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Generation of *Sporothrix schenckii* mutants expressing the green fluorescent protein suitable for the study of host-fungus interactions

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Abbreviations: GFP, green fluorescent protein; PBMCs, peripheral blood mononuclear cells; CFU, colony forming units

Abstract (198/200 words)

Sporotrichosis is an infection caused by members of the *Sporothrix* genus, and among them, *Sporothrix schenckii* is one of the etiological agents. Both, the disease and the causative agent have gained interest in the recent year, because of the report of epidemic outbreaks, and the description of the disease transmission from animals to human beings. Despite the relevance of *S. schenckii* in the clinical field, there are basic aspects of its biology poorly explored. So far, *Agrobacterium tumefaciens*-mediated transformation has been reported as an alternative for genetic manipulation of this fungal pathogen. Here, we report the optimization of the transformation method and used this to generate insertional mutants that express the green fluorescent protein in *S. schenckii*. We obtained five mutant strains that showed mitotic stability and expression of the reporter gene. The strains displayed normal cell wall composition, and a similar ability to interact *ex vivo* with human monocytes and monocyte-derived macrophages. Moreover, the virulence in larvae of *Galleria mellonella* was similar to that obtained with the wild-type control strains. These data indicate that these fluorescent mutants with normal ability to interact with the host could be used in bioimaging to track the host-*Sporothrix* interaction *in vivo*.

Keywords: *Sporothrix schenckii*; *Agrobacterium tumefaciens,* cell wall; immune sensing; virulence; green fluorescent protein

1. Introduction

Sporotrichosis is the term used to describe the subacute or chronic mycosis caused by *Sporothrix* spp. (Chakrabarti et al., 2015; Lopes-Bezerra et al., 2006; Lopez-Romero et al., 2011). Among the 51 species composing the *Sporothrix* genus, only *Sporothrix schenckii*, *Sporothrix brasiliensis*, *Sporothrix globosa*, and *Sporothrix luriei* have been described as pathogenic species (de Beer et al., 2016), affecting humans and other mammals (Rodrigues et al., 2016). In the environment, these organisms usually grow like molds, generating hyphae and conidia, but have the ability to undergo dimorphism when invading the host tissues, generating yeast-like cells (Lopez-Romero et al., 2011). Even though sporotrichosis is regarded as a responsive infection to the standardized antifungal strategies, it is considered an emerging health problem in immunocompromised patients, and outbreaks have been reported in North and South America, Africa, Asia, and Oceania, some of them with high mortality and morbidity rates (Chakrabarti et al., 2015; Govender et al., 2015; Gremião et al., 2017; Lopez-Romero et al., 2011; Rodrigues et al., 2016; Rodrigues et al., 2013; Sanchotene et al., 2015). Despite the unquestionable relevance of sporotrichosis, the progress in understanding the biology of *Sporothrix* species and several aspects of the clinical forms, epidemiology, natural reservoirs, alternatives for the diagnosis and treatment is still limited (Mora-Montes et al., 2015). In 2015, we reported that the scientific literature dealing with *Sporothrix* was extremely limited, with only 1124 published papers, since the first report of the disease by Schenk more than a century ago (Mora-Montes et al., 2015). This figure has not been significantly modified in 2017, as

only 1519 papers are listed in the PubMed from the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/pubmed/?term=Sporothrix). Although this is a world-wide distributed disease, the list of countries where a high frequency of sporotrichosis cases have been reported is limited (Chakrabarti et al., 2015), offering a possible explanation to the modest scientific interest on this disease and the causative agent. In addition, the limited repertoire of molecular tools to facilitate the study of *Sporothrix* spp. can also partially explain the limited interest in this organism (Mora-Montes et al., 2015). This is relevant when the scientific interest in *Candida* spp. and *Aspergillus* spp. is analyzed in terms of publications: there was an exponential production of scientific literature after the development of tools for genetic manipulation and the release of the genome sequences (Mora-Montes et al., 2015).

The first approach to assess the relevance of genes and molecular pathways in *S. schenckii* physiology and virulence was the use of mutants generated by exposure to UV light (Romero-Martinez et al., 2000; Torres-Guerrero and Arenas-Lopez, 1998). Later, the study of the calcium/calmodulin kinase I allowed the adaptation of the RNA interference methodology to *S. schenckii* (Rodriguez-Caban et al., 2011); the *Agrobacterium tumefaciens*-mediated transformation was reported for this organism (Zhang et al., 2011), the genome sequencing of *S. schenckii*, *S. brasiliensis*, *S. globosa* and *S. pallida* were reported (D'Alessandro et al., 2016; Huang et al., 2016; Teixeira et al., 2014), and *S. schenckii* genes of stable and constitutive expression were identified and used

for data normalization during analysis of gene expression (Trujillo-Esquivel et al., 2017). To assess the fitness and virulence, the models of systemic and subcutaneous sporotrichosis in mice and the systemic infection in larvae of *Galleria mellonella* are currently available (Castro et al., 2013; Clavijo-Giraldo et al., 2016; Hachisuka and Sasai, 1981).

The study of the host-fungus interaction is relevant to understand the mechanisms responsible for the damage to the host, the ability of the immune system to control the pathogen, and the strategies used to avoid the immunosurveillance (Hernández-Chávez et al., 2017; Mendes-Giannini et al., 2005; Rizzetto and Cavalieri, 2011). Among the alternatives to study the host-fungus interaction, the expression of fluorescent molecules within the fungal cell has been used to study fungal pathogenesis (Bobard et al., 2011; Hoppe et al., 2009). Here, we used the *Agrobacterium tumefaciens*-mediated transformation to generate *S. schenckii* mutants that stably expressed the green fluorescent protein (GFP). The mutant strains showed similar phenotypical parameters, including cell wall composition, virulence, and ability to interact with human peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages, indicating they are suitable for the study of the host-*Sporothrix* interaction.

2. Materials and methods

2.1 Microorganisms and culture media

S. schenckii 1099-18 ATCC MYA 4821 (Castro et al., 2013) was used as genetic background to generate strains expressing GFP and is referred in the text

as wild-type (WT) control strain. Cells were maintained and propagated in YPD medium (1% [w/v] yeast extract, 2% [w/v] gelatin peptone, and 3% [w/v] dextrose). When solid medium was required, 2% agar (w/v) was included. Conidia were obtained in solid YPD medium, pH 4.5, at 28°C for 6-9 days, and harvested by mechanical means as reported (Martinez-Alvarez et al., 2017). Hyphae were obtained by incubating conidia in YPD broth, pH 4.5, at 28°C for 48 h and reciprocal shaking (120 rpm), and harvested by filtering, using a vacuum system and a 5-µm nylon membrane (Monodur®). Cells were washed 6 times with sterile cold water and kept at -20ºC until used. Yeast cells were obtained by growing conidia in YPD broth, pH 7.8, for 7 days at 37°C and shaking at 120 rpm (Martinez-Alvarez et al., 2017). Yeast-like cells were harvested by centrifuging at 5000 x *g* for 5 min at 4°C, washed three times with deionized water, and kept at -20°C until used. Cell inactivation by heat was performed at 60°C for 2 h (Martinez-Alvarez et al., 2017), and the loss of cell viability was confirmed on YPD plates, pH 4.5, incubated at 28°C for 5 days. Bacteria were kept and maintained in LB medium (0.5 [w/v] yeast extract, 1% [w/v] gelatin peptone, and 1% [w/v] NaCl).

2.2 *Agrobacterium tumefaciens*-mediated transformation

We based our transformation strategy in a previously published protocol (Zhang et al., 2011) with some modifications. *A. tumefaciens* AGL-1 harboring the pBGgHg vector (Chen et al., 2000) was grown overnight at 28°C in LB broth supplemented with 100 µg mL^{-1} ampicillin and 100 µg mL^{-1} kanamycin, cell density adjusted to 0.2, and cell induction was achieved by incubating bacteria in minimal medium [0.34 M K_2HPO_4, 0.16 M NaH_2PO_4, 0.37M NH_4Cl, 0.24 $MgSO_4$, 0.04M

KCl, 1.8 mM $CaCl_2$, $FeSO_4$ 0.18 mM, pH 7.0 adjusted with 1 N HCl) supplemented with 200 µM acetosyringone (Sigma) for 4.5 h at at 28°C and reciprocal shaking (250 rpm). Then, an aliquot containing 100 µL was used for co-incubation with 100 µL of freshly harvested conidia at $1x10^6$ conidia mL^{-1} and were placed on a cellophane disk on top of a plate containing solid minimal medium and incubated 3 days at 28°C. Then, the cellophane disk was placed on YPD, pH 4.5, supplemented with 400 mg mL^{-1} hygromycin B and 200 µM cefotaxime, and incubated for 3 days at 28°C. Interactions, where no acetosyringone was added to induce *A. tumefaciens* cells, were included as a control.

Hygromycin B-resistant colonies were isolated, grown in solid medium to stimulate conidia production and selected again in YPD plates, pH 4.5, containing 400 mg mL^{-1} hygromycin B. This selection was performed for four times and then cells were stimulated three times to undergo dimorphism in YPD, pH 7.8.

2.3 Isolation of *S. schenckii* genomic DNA

Genomic DNA was isolated as reported (Robledo-Ortiz et al., 2012). Briefly, hyphae grown in YPD broth overnight were frozen in liquid nitrogen, mechanically ground with mortar, resuspended in 200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, 40 µg mL^{-1} RNase (Sigma), and incubated 1 h at 37 ºC. Then, one volume of a phenol:chloroform (1:1) solution was added, thoroughly mixed with vortex, and the sample was centrifuged for 10 min at 8,000 x *g* and 4ºC. The liquid phase was saved, mixed with 0.5 volumes of neat isopropanol, and

161 incubated 4 h at -20ºC. The nucleic acid was washed with 70% (v/v) ethanol and
162 kept at -20ºC until used.
163
164 **2.4 Molecular characterization of transformant strains**
165 Confirmation of the presence of pBGgHg within the genomic DNA of
166 *S. schenckii* was performed by PCR, using the primer pair 5'-
167 GGCGACCTCGTATTGGGAATC-3' and 5'-CTATTCCTTTGCCCTCGGACGAG-3',
168 which align in the *pdg* promoter and inside the *hph* marker, respectively.
169 Quantitative PCR was used to determine the number of integrative events within
170 the *S. schenckii* genome. Nucleic acids were quantified in a NanoDrop 2000
171 (Thermo Scientific). The quantitative PCR reactions were performed in a
172 thermocycler StepOne Plus (Life Technologies) and the SYBR Green PCR Master
173 Mix (Life Technologies). Reactions were prepared with 800 ng of genomic DNA,
174 following the instructions provided by the manufacturer, and the copy number
175 estimated with the StepOne software V 2.2 (Life Technologies) by calculating $2^{-\Delta\Delta Ct}$
176 (Livak and Schmittgen, 2001). The encoding gene for the ribosomal protein L6 was
177 used as an endogenous control; while the WT strain was defined as the reference
178 condition (Trujillo-Esquivel et al., 2017). The primer pairs used were 5'-
179 CCTGAAGTTCATCTGCACCA-3' and 5'-GAAGTCGTGCTGCTTCATGT-3' for the
180 encoding gene for GFP; and 5'-ATTGCGACATCAGAGAAGG-3' and 5'-
181 TCGACCTTCTTGATGTTGG-3' for the encoding gene for the ribosomal protein L6.
182
183 **2.5 Fluorescent microscopy**

Fungal cells were examined by fluorescence microscopy using a Zeiss Axioscope-40 microscope and an Axiocam MRc camera. All images were captured using 395nm/509nm for excitation and emission, respectively, and exposing the sample for 900 milliseconds to the laser beam.

2.6 Analysis of cell wall composition

Yeast-like cells were disrupted in a Braun homogenizer as previously described (Mora-Montes et al., 2010). Cell walls were recovered by centrifuging the homogenate at 18 000 x *g* for 10 min, were extensively washed with deionized water, cleansed and acid-hydrolyzed as described (Mora-Montes et al., 2007). Acid-hydrolyzed samples were analyzed by HPAEC-PAD in a carbohydrate analyzer system from Dionex, using a CarboPac PA10 column and the separation conditions previously reported (Estrada-Mata et al., 2015).

2.7 Isolation and stimulation of human PBMCs with *S. schenckii* cells

Human EDTA-treated whole blood was isolated from healthy adult volunteers after information of the study was disclosed and a written informed consent was signed. This study was approved by the Ethics Committee from Universidad de Guanajuato (permission number 17082011). The PBMC population was isolated by density centrifugation using Histopaque-1077 (Sigma) as reported (Martinez-Alvarez et al., 2017). Cells were suspended in RPMI 1640 Dutch modification (added with 2 mM glutamine, 0.1 mM pyruvate and 0.05 mg mL^{-1} gentamycin; all reagents from Sigma), and aliquots of 100 µL containing $5x10^5$

PBMCs were plated onto round-bottom 96-well microplates, and 100 µL with $1x10^5$ fungal cells were added to each well. Plates were incubated for 24 h at 37°C with 5% (v/v) CO_2, centrifuged for 10 min at 3000 x *g* at 4ºC, and the supernatants saved and used for cytokine quantification. TNFα and IL-6 were measured with a commercial ELISA kit from Peprotech.

2.8 Differentiation of human PBMC-derived macrophages and phagocytosis assays

Human PBMCs cells were differentiated into macrophages as reported (Perez-Garcia et al., 2016). Briefly, aliquots of 1 mL containing $5x10^6$ cells in RPMI supplemented with 1% (v/v) penicillin-streptomycin solution (PS, Sigma) were seeded in flat bottom 24-well plates and incubated for 2 h at 37°C and 5% (v/v) CO_2. Non-adherent cells and cell debris were removed by washing twice with PBS at 37°C. Next, X-VIVO 15 serum-free medium (Lonza) supplemented with 1% (v/v) PS and 10 ng mL^{-1} recombinant human granulocyte-macrophage colony stimulating factor (Sigma) were added to each well, and plates were incubated for 7 days at 37°C and 5% (v/v) CO_2. Fresh medium was exchanged every 3 days. Yeast-like cells were washed twice with PBS, labeled with 1 mg mL^{-1} Acridine Orange (Sigma), and cell concentration adjusted at $1x10^7$ yeast cells mL^{-1}, as described (Gonzalez-Hernandez et al., 2017). Interactions were carried out in 6-well plates containing 1 mL of DMEM medium (Sigma) per well, and a macrophage-yeast ratio 1:6. Plates were incubated for 2.5 h at 37°C and 5% (v/v) CO_2, macrophages were washed twice with cold PBS and suspended in PBS containing 1.25 mg mL^{-1} Trypan Blue as an external fluorescence quencher

(Gonzalez-Hernandez et al., 2017). A MoFlo XDP system (Beckman Coulter) was used to analyze samples by flow cytometry. A total of 50,000 events gated for macrophage cells were collected per sample. Fluorescence was acquired from the compensated FL1 (green) and FL3 (red) channels using macrophage cells without any labeling. Phagocytosis of fungal cells was assessed from acquired events in the green (recently phagocytosed cells) and red (cells within acidified phagolysosomes) fluorescence channels.

2.9 *Galleria mellonella* survival assays

The virulence of *S. schenckii* cells was assessed in larvae of *G. mellonella* as described (Clavijo-Giraldo et al., 2016). Briefly, the last left pro-leg was disinfected with 70% (v/v) ethanol and used to inject $1x10^5$ yeast-like cells contained in 10 µL of PBS, using a Hamilton syringe and a 26-gauge needle. Insects were kept at 37°C and survival monitored daily for two weeks. Body melanization and lack of response to stimuli were taken as signs of animal death. To determine the fungal burden, animals were decapitated, and serial dilutions of the hemolymph were incubated on YPD plates, pH 4.5, at 28°C for 72 h. Each experimental group contained 30 larvae, including a control group injected only with PBS.

2.10 Statistical analysis

Statistical analyses were conducted with the GraphPad Prism 6 software. Cytokine stimulation and phagocytosis were performed in duplicate with eight healthy donors, whereas the *in vitro* experiments were performed at least three

254 times in duplicates. Data represent the cumulative results of all experiments
255 performed. The Mann-Whitney *U* test was used to establish statistical significance.
256 Survival experiments with *G. mellonella* larvae were carried out three times, with a
257 total of 30 larvae per strain tested. Data were analyzed using the Log-rank test and
258 are reported in Kaplan-Meier survival curves. For all cases, the statistical
259 significance was set at $P < 0.05$.
260

261 **3. Results**

262 **3.1 Generation of *S. schenckii* strains expressing GFP**

263 The *A. tumefaciens*-mediated transformation of *S. schenckii* (Zhang et al.,
264 2011) was used here with some modifications: we found the activation of
265 *A. tumefaciens* for 4.5 h instead 8 h generated similar amount of transformant
266 colonies using pBGgHg (722 ± 48 vs. 708 ± 55 transformants per 10^6 conidia^{-1}, for
267 4.5 h and 8 h incubation in presence of 200 µM acetosyringone, respectively; P =
268 0.768). We also observed a high frequency of false transformant cells immediately
269 recovered from the co-incubation plates, forcing to include in the selection stage
270 five monoconidial passages, before considering the colonies as transformants.
271 Since the *S. schenckii* hyphae are multinuclear, but yeasts are considered
272 mononuclear cells (Torres-Guerrero, 1999), three steps of yeast-like cell formation
273 were included to eliminate non-transformed nuclei contained within hyphae. After
274 following this strategy, using *A. tumefaciens* ALG-1 containing pBGgHg, a binary
275 vector that contains the encoding gene for the enhanced GFP from *Aequorea*
276 *victoria* (Chen et al., 2000), we randomly selected five transformant colonies to
277 assess expression of GFP by fluorescent microscopy. The WT strain showed

green autofluorescence when observed under fluorescent microscopy, however, the fluorescent signals obtained from the mutants transformed with pBGgHg were brighter (Fig. 1), indicating expression of the recombinant protein. The presence of pBGgHg within the *S. schenckii* genome was confirmed by PCR, amplifying part of the selection cassette *hph* that confers resistance to hygromycin B (data not shown). The mutant strains showed mitotic stability after sequential passages for eight months in YPD medium with no selective agent included (data not shown). Next, we determined the number of insertional events within the genome of the selected mutants by qPCR. Since the sequencing of *S. schenckii* indicated that this organism is haploid (Teixeira et al., 2014), the comparative amplification of any gene present as a sole copy within the *S. schenckii* genome is likely to be useful in the quantification of insertional events of pBGgHg within the *S. schenckii* genome. Since the gene encoding for the ribosomal protein L6 has been recently characterized by our group (Trujillo-Esquivel et al., 2017), we used this gene for data normalization in qPCR assays. Results indicated that strains HSS2, HSS3, and HSS4 had one integrative event in the genome; whereas HSS5 and HSS6 had three integrative events into the genome (Fig. 2). Collectively, these data indicate that we obtained five *S. schenckii* mutant strains expressing GFP.

3.2 The *S. schenckii* strains expressing GFP have normal cell wall composition

The cell wall is the outermost fungal structure, and therefore, along with soluble components, the first element in contact with host cells and humoral factors, including those from the immune response (Díaz-Jiménez et al., 2012;

Martinez-Alvarez et al., 2014; Martinez-Alvarez et al., 2017). Therefore, we analyzed the cell wall of yeast-like cells, as this cell morphology is associated with the infection and is demonstrated to interact with immune cells (Lopez-Romero et al., 2011; Mora-Montes et al., 2015). Cell dimorphism was stimulated in YPD, pH 7.8, and more than 97% cells were in the yeast-like morphology, as previously reported (Martinez-Alvarez et al., 2017; Trujillo-Esquivel et al., 2017). Cells were mechanically disrupted and wall purified as described in Materials and methods, and then acid-hydrolyzed to break down sugar polysaccharides, releasing the corresponding monosaccharide units (Martinez-Alvarez et al., 2017). Sugars were separated by HPAEC-PAD and the proportion of glucosamine, glucose, mannose, and rhamnose, the basic units of chitin, glucans, and oligosaccharides from glycoproteins, were determined. In the WT strain, chitin (the polysaccharide composed of N-acetylglucosamine) was the less abundant in the cell wall of yeast-like cells (15.6 ± 1.6% of total sugar content, Table 1), while glucans (glucose-based polysaccharides) were the most abundant components (40.2 ± 2.6 %, Table 1). Altogether, rhamnose and mannose levels were as abundant as the glucose content (Table 1). These data are similar to those previously reported by our group (Martinez-Alvarez et al., 2017). When the cell wall composition of the five mutant strains expressing GFP was analyzed, the sugar content was indistinguishable from that obtained from the WT strain, suggesting no significant changes in the cell wall composition upon cell transformation (Table 1).

3.3 The *S. schenckii* strains expressing GFP have normal interaction with components of the innate immune system

Next, we assessed whether the transformation of the fungal cells had an impact on their ability to interact with cellular elements from the human innate immune system. The fungal cells were co-incubated with human PBMCs and then, the level of the proinflammatory cytokines TNFα and IL-6 were measured. The WT yeast-like cells showed the ability to stimulate similar levels of both cytokines (Fig. 3). Conidia and germlings stimulated slightly higher cytokine levels, although they were not statistically significant (Fig. 3). The three cell morphologies of the five mutants expressing GFP stimulated TNFα and IL-6 production like WT cells (Fig. 3). Next, we used yeast-like cells to evaluate the ability of human monocyte-derived macrophages to phagocyte these fungal cells. Results in figure 4 indicate that the WT control cells and the five mutant strains expressing GFP were similarly phagocytosed by the immune cells: total fungal cells interacting with the human monocyte-derived macrophages were similar for all the strains analyzed. From the total cells interacting with macrophages (100% was around 2500 cells), about 70% of the population was in the late stages of the phagocytic process, indicated as the red signal of acidified phagolysosomes was prominent (Gonzalez-Hernandez et al., 2017) (Fig. 4). The rest of fungal cells was in an earlier stage of the interaction, as macrophages displayed mainly the green signal from fungal cells and minimal red fluorescence of acidified phagocytic vesicles (Gonzalez-Hernandez et al., 2017) (Fig. 4). Overall, these data indicate the mutant cells have a normal ability to interact with both PBMCs and monocyte-derived macrophages.

3.4 The *S. schenckii* strains expressing GFP have no changes in the virulence in the model *G. mellonella*

Next, to analyze the virulence of the mutant strains, we used the model of systemic sporotrichosis in larvae of *G. mellonella* (Clavijo-Giraldo et al., 2016). Larvae were inoculated with yeast-like cells and monitored daily for two weeks. We observed an animal mortality of about 60% in animals infected with the WT strain, and similar results were obtained with any of the five mutant strains that express GFP (Fig. 5). The control group inoculated only with PBS show no mortality during the observation period (Fig. 5). To assess whether the *in vivo* cell fitness of the mutant strains was similar to that shown by the WT strain, the fungal load in animals that succumbed to the infection was determined by quantifying the colony forming units (CFU) for each strain. No significant differences in this parameter were observed when results from the WT and mutant strains were compared (P = 0.243). Therefore, these data indicate the WT and mutant strains generated in this study displayed a similar behavior *in vivo*.

4. Discussion

The current repertoire of tools to understand the gene function and relevance for *S. schenckii* is currently limited, and heterologous complementation using other fungal systems is the strategy most frequently reported (Hernandez-Cervantes et al., 2012; Lopes-Bezerra et al., 2015; Lopez-Esparza et al., 2013; Pérez-Sánchez et al., 2010; Robledo-Ortiz et al., 2012; Sánchez-López et al., 2015). Thus far, no gene disruption has been reported in *S. schenckii* cells, and gene silencing has emerged as an alternative to generating mutant strains (Rodriguez-Caban et al., 2011). This strategy was achieved using protoplast transformation, but thus far, no other group has been capable to reproduce this

strategy. As an alternative, the *A. tumefaciens*-mediated transformation has been standardized for *S. schenckii* (Zhang et al., 2011). Even though the current method for *S. schenckii* is reproducible and has been used for the generation of a collection of insertional mutants (Zhang et al., 2011), we aimed to the optimization of the method. The activation of the *vir* genes is critical for the transference of DNA from the prokaryotic to the eukaryotic cell, and this event is enhanced by using acetosyringone (Singh and Prasad, 2016). The bacterial activation via acetosyringone is not required for DNA transference, but positively influences cell transformation (Singh and Prasad, 2016). For the transformation of other fungal species, such as *Lasiodiplodia theobromae* (Muniz et al., 2014), *Valsa mali*: (Wang et al., 2013), *Fusarium oxysporum* (Mullins et al., 2001) and *Magnaporthe grisea* (Hee-Sool et al., 2001), the activation time of *A. tumefaciens* is lower than 8 h, as reported for *S. schenckii* (Zhang et al., 2011). Our protocol reduced this parameter to almost half of the one originally reported, and we consider this modification significant to reduce the transformation time. Another improvement included in our method for cell transformation was the number of passages to select transformed nuclei. *S. schenckii* does not sporulate, even though has all the machinery to undergo mating (Teixeira et al., 2014); therefore, monosporic cultures to obtain cells with a homogeneous genome (Balcázar-López et al., 2016; Walker et al., 2016) are not currently available in *S. schenckii*. As an alternative, we selected mutant strains undergoing dimorphism, as yeast-like cells are mononuclear and thus a true monoconidial culture was established. The strain used here showed increased resistance to hygromycin B when compared with that were the *A. tumefaciens* transformation assay was firstly established (400 µg mL^{-1} vs. 100

398 µg mL^{-1} (Zhang et al., 2011)). This is likely to be a strain-specific trait, as
399 *S. schenckii* cells are reported to display a heterogeneous phenotype when drug
400 sensitivity is tested (Han et al., 2017; Oliveira et al., 2015).The mutants generated
401 here showed mitotic stability and a notorious expression of GFP. We identified two
402 groups of strains: those with one integrative event after transference of DNA from
403 bacteria to fungal cells, and a group with three integrations. We could not see a
404 significant difference in the fluorescence associated with strains from both groups,
405 i.e., all the analyzed strains displayed similar fluorescent intensity. It is possible
406 that the brightness associated with the expression of one copy of GFP was enough
407 to saturate the acquisition system of the microscope and thus, strains with more
408 than one copy did not show increased fluorescence. Alternatively, it is possible to
409 speculate that the additional copies of GFP were subjected to gene silencing via
410 small RNAs, which is commonly found in eukaryotes when more than one copy of
411 foreign DNA is introduced (Carthew and Sontheimer, 2009). In addition, the
412 plasmid integration in telomeric or subtelomeric regions could account for gene
413 silencing (Smith et al., 2008). Additional experiments are required to address this
414 subject. Although we did not determine the sites of insertion, and therefore is likely
415 this occurred within a gene, we demonstrated that if there is a loss-of-function
416 mutation, it occurred in a gene dispensable for dimorphism, cell wall composition,
417 interaction with immune cells, normal growth *in vivo* and virulence. The random
418 insertional mutagenesis using *A. tumefaciens* cells is a common strategy to
419 generate mutant libraries to identify genes associated with specific phenotypical
420 traits (Blaise et al., 2007; Zhang et al., 2011). Therefore, the phenotypical

characterization of the mutants reported in this study is not a futile task, but a requirement to demonstrate their usefulness in further studies.

One limitation we must acknowledge in our present study is that we did not perform a thorough phenotypical characterization and any other phenotype has to be confirmed to be similar to that shown by WT control cells. Nevertheless, we do think these strains could be a useful first step to develop bioimaging *in vivo* with *S. schenckii*, to track cellular dissemination, homing, and tissue specificity during the *Sporothrix*-host interaction, as previously reported for *C. albicans* and *Aspergillus fumigatus* (Doyle et al., 2006; Mosci et al., 2013; Vecchiarelli and d'Enfert, 2012).

In conclusion, here we report the optimization of the *A. tumefaciens*-mediated transformation in *S. schenckii* and the generation of strains expressing GFP with normal cell wall composition, virulence, and ability to interact with human PBMCs and macrophages.

5. Acknowledgments

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6. References

444 Balcázar-López, E., Méndez-Lorenzo, L.H., Batista-García, R.A., Esquivel-Naranjo, U., Ayala, M.,
445 Kumar, V.V., Savary, O., Cabana, H., Herrera-Estrella, A., Folch-Mallol, J.L., 2016. Xenobiotic
446 compounds degradation by heterologous expression of a *Trametes sanguineus* laccase in
447 *Trichoderma atroviride*. PLoS ONE 11, e0147997.
448 Blaise, F., Rémy, E., Meyer, M., Zhou, L., Narcy, J.-P., Roux, J., Balesdent, M.-H., Rouxel, T., 2007. A
449 critical assessment of *Agrobacterium tumefaciens*-mediated transformation as a tool for
450 pathogenicity gene discovery in the phytopathogenic fungus *Leptosphaeria maculans*. Fungal
451 Genet Biol 44, 123-138.
452 Bobard, A., Mellouk, N., Enninga, J., 2011. Spotting the right location— imaging approaches to
453 resolve the intracellular localization of invasive pathogens. Biochim Biophys Acta 1810, 297-307.
454 Carthew, R.W., Sontheimer, E.J., 2009. Origins and Mechanisms of miRNAs and siRNAs. Cell 136,
455 642-655.
456 Castro, R.A., Kubitschek-Barreira, P.H., Teixeira, P.A.C., Sanches, G.F., Teixeira, M.M., Quintella,
457 L.P., Almeida, S.R., Costa, R.O., Camargo, Z.P., Felipe, M.S.S., de Souza, W., Lopes-Bezerra, L.M.,
458 2013. Differences in cell morphometry, cell wall topography and Gp70 expression correlate with
459 the virulence of *Sporothrix brasiliensis* clinical isolates. PLoS ONE 8, e75656.
460 Chakrabarti, A., Bonifaz, A., Gutierrez-Galhardo, M.C., Mochizuki, T., Li, S., 2015. Global
461 epidemiology of sporotrichosis. Med Mycol 53, 3-14.
462 Chen, X., Stone, M., Schlagnhaufer, C., Romaine, C.P., 2000. A fruiting body tissue method for
463 efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Appl Environ Microbiol 66,
464 4510-4513.
465 Clavijo-Giraldo, D.M., Matinez-Alvarez, J.A., Lopes-Bezerra, L.M., Ponce-Noyola, P., Franco, B.,
466 Almeida, R.S., Mora-Montes, H.M., 2016. Analysis of *Sporothrix schenckii sensu stricto* and
467 *Sporothrix brasiliensis* virulence in *Galleria mellonella*. J Microbiol Methods 122, 73-77.
468 D'Alessandro, E., Giosa, D., Huang, L., Zhang, J., Gao, W., Brankovics, B., Oliveira, M.M.E., Scordino,
469 F., Lo Passo, C., Criseo, G., van Diepeningen, A.D., Huang, H., de Hoog, G.S., Romeo, O., 2016. Draft
470 genome sequence of the dimorphic fungus *Sporothrix pallida*, a nonpathogenic species belonging
471 to *Sporothrix*, a genus containing agents of human and feline sporotrichosis. Genome Announc 4,
472 e00184-00116.
473 de Beer, Z.W., Duong, T.A., Wingfield, M.J., 2016. The divorce of *Sporothrix* and *Ophiostoma*:
474 solution to a problematic relationship. Stud Mycol 83, 165-191.
475 Díaz-Jiménez, D.F., Pérez-García, L.A., Martínez-Álvarez, J.A., Mora-Montes, H.M., 2012. Role of
476 the fungal cell wall in pathogenesis and antifungal resistance. Curr Fungal Infect Rep 6, 275-282.
477 Doyle, T.C., Nawotka, K.A., Kawahara, C.B., Francis, K.P., Contag, P.R., 2006. Visualizing fungal
478 infections in living mice using bioluminescent pathogenic *Candida albicans* strains transformed
479 with the firefly luciferase gene. Microb Pathog 40, 82-90.
480 Estrada-Mata, E., Navarro-Arias, M.J., Perez-Garcia, L.A., Mellado-Mojica, E., Lopez, M.G., Csonka,
481 K., Gacser, A., Mora-Montes, H.M., 2015. Members of the *Candida parapsilosis* complex and
482 *Candida albicans* are differentially recognized by human peripheral blood mononuclear cells. Front
483 Microbiol 6, 1527.
484 Gonzalez-Hernandez, R.J., Jin, K., Hernandez-Chavez, M.J., Diaz-Jimenez, D.F., Trujillo-Esquivel, E.,
485 Clavijo-Giraldo, D.M., Tamez-Castrellon, A.K., Franco, B., Gow, N.A.R., Mora-Montes, H.M., 2017.
486 Phosphomannosylation and the functional analysis of the extended *Candida albicans MNN4*-like
487 gene family. Front Microbiol 8, 2156.
488 Govender, N.P., Maphanga, T.G., Zulu, T.G., Patel, J., Walaza, S., Jacobs, C., Ebonwu, J.I., Ntuli, S.,
489 Naicker, S.D., Thomas, J., 2015. An outbreak of lymphocutaneous sporotrichosis among mine-
490 workers in South Africa. PLoS Negl Trop Dis 9, e0004096.

491 Gremião, I.D.F., Miranda, L.H.M., Reis, E.G., Rodrigues, A.M., Pereira, S.A., 2017. Zoonotic
492 epidemic of sporotrichosis: cat to human transmission. PLoS Pathog 13, e1006077.
493 Hachisuka, H., Sasai, Y., 1981. Development of experimental sporotrichosis in normal and modified
494 animals. Mycopathologia 76, 79-82.
495 Han, H.S., Kano, R., Chen, C., Noli, C., 2017. Comparison of two in vitro antifungal sensitivity tests
496 and monitoring during therapy of *Sporothrix schenckii sensu stricto* in Malaysian cats. Vet
497 Dermatol 28, 156-e132.
498 Hee-Sool, R., Seogchan, K., Yong-Hwan, L., 2001. *Agrobacterium tumefaciens*-mediated
499 transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Mol Cells 12, 407-411.
500 Hernandez-Cervantes, A., Mora-Montes, H.M., Alvarez-Vargas, A., Jimenez, D.F., Robledo-Ortiz,
501 C.I., Flores-Carreon, A., 2012. Isolation of *Sporothrix schenckii MNT1* and the biochemical and
502 functional characterization of the encoded alpha1,2-mannosyltransferase activity. Microbiology
503 158, 2419-2427.
504 Hernández-Chávez, M., Pérez-García, L., Niño-Vega, G., Mora-Montes, H., 2017. Fungal strategies
505 to evade the host immune recognition. J Fungi 3, 51.
506 Hoppe, A.D., Seveau, S., Swanson, J.A., 2009. Live cell fluorescence microscopy to study microbial
507 pathogenesis. Cell Microbiol 11, 540-550.
508 Huang, L., Gao, W., Giosa, D., Criseo, G., Zhang, J., He, T., Huang, X., Sun, J., Sun, Y., Huang, J.,
509 Zhang, Y., Brankovics, B., Scordino, F., D'Alessandro, E., van Diepeningen, A., de Hoog, S., Huang,
510 H., Romeo, O., 2016. Whole-genome sequencing and *in silico* analysis of two strains of *Sporothrix*
511 *globosa*. Genome Biol Evol 8, 3292-3296.
512 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
513 quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
514 Lopes-Bezerra, L.M., Lozoya-Perez, N.E., Lopez-Ramirez, L.A., Martinez-Alvarez, J.A., Teixeira,
515 M.M., Felipe, M.S., Flores-Carreon, A., Mora-Montes, H.M., 2015. Functional characterization of
516 *Sporothrix schenckii* glycosidases involved in the *N*-linked glycosylation pathway. Med Mycol 53,
517 60-68.
518 Lopes-Bezerra, L.M., Schubach, A., Costa, R.O., 2006. Sporothrix schenckii and sporotrichosis. An
519 Acad Bras Cienc 78, 293-308.
520 Lopez-Esparza, A., Alvarez-Vargas, A., Mora-Montes, H.M., Hernandez-Cervantes, A., Del Carmen
521 Cano-Canchola, M., Flores-Carreon, A., 2013. Isolation of *Sporothrix schenckii GDA1* and functional
522 characterization of the encoded guanosine diphosphatase activity. Arch Microbiol 195, 499-506.
523 Lopez-Romero, E., Reyes-Montes M del, R., Perez-Torres, A., Ruiz-Baca, E., Villagomez-Castro, J.C.,
524 Mora-Montes, H.M., Flores-Carreon, A., Toriello, C., 2011. *Sporothrix schenckii* complex and
525 sporotrichosis, an emerging health problem. Future Microbiol 6, 85-102.
526 Martinez-Alvarez, J.A., Perez-Garcia, L.A., Flores-Carreon, A., Mora-Montes, H.M., 2014. The
527 immune response against *Candida* spp. and *Sporothrix schenckii*. Rev Iberoam Micol 31, 62-66.
528 Martinez-Alvarez, J.A., Perez-Garcia, L.A., Mellado-Mojica, E., Lopez, M.G., Martinez-Duncker, I.,
529 Lopes-Bezerra, L.M., Mora-Montes, H.M., 2017. *Sporothrix schenckii sensu stricto* and *Sporothrix*
530 *brasiliensis* are differentially recognized by human peripheral blood mononuclear cells. Front
531 Microbiol 8, 843.
532 Mendes-Giannini, M.J.S., Soares, C.P., da Silva, J.L.M., Andreotti, P.F., 2005. Interaction of
533 pathogenic fungi with host cells: molecular and cellular approaches. FEMS Immunol Med Microbiol
534 45, 383-394.
535 Mora-Montes, H.M., Bates, S., Netea, M.G., Diaz-Jimenez, D.F., Lopez-Romero, E., Zinker, S.,
536 Ponce-Noyola, P., Kullberg, B.J., Brown, A.J., Odds, F.C., Flores-Carreon, A., Gow, N.A., 2007.
537 Endoplasmic reticulum alpha-glycosidases of *Candida albicans* are required for N glycosylation, cell
538 wall integrity, and normal host-fungus interaction. Eukaryotic cell 6, 2184-2193.

539 Mora-Montes, H.M., Dantas Ada, S., Trujillo-Esquivel, E., de Souza Baptista, A.R., Lopes-Bezerra,
540 L.M., 2015. Current progress in the biology of members of the *Sporothrix schenckii* complex
541 following the genomic era. FEMS Yeast Res 15.
542 Mora-Montes, H.M., Robledo-Ortiz, C.I., Gonzalez-Sanchez, L.C., Lopez-Esparza, A., Lopez-Romero,
543 E., Flores-Carreon, A., 2010. Purification and biochemical characterisation of endoplasmic
544 reticulum alpha1,2-mannosidase from *Sporothrix schenckii*. Mem Inst Oswaldo Cruz 105, 79-85.
545 Mosci, P., Pericolini, E., Gabrielli, E., Kenno, S., Perito, S., Bistoni, F., d'Enfert, C., Vecchiarelli, A.,
546 2013. A novel bioluminescence mouse model for monitoring oropharyngeal candidiasis in mice.
547 Virulence 4, 250-254.
548 Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M., Kang, S., 2001. *Agrobacterium*-
549 mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and
550 gene transfer. Phytopathology 91, 173-180.
551 Muniz, C.R., da Silva, G.F., Souza, M.T., Jr., Freire, F.C., Kema, G.H., Guedes, M.I., 2014.
552 *Agrobacterium tumefaciens*-mediated transformation of *Lasiodiplodia theobromae*, the causal
553 agent of gummosis in cashew nut plants. Genet Mol Res 13, 2906-2913.
554 Oliveira, D.C., de Loreto, É.S., Mario, D.A.N., Lopes, P.G.M., Neves, L.V., da Rocha, M.P., Santurio,
555 J.M., Alves, S.H., 2015. *Sporothrix schenckii* complex: susceptibilities to combined antifungal agents
556 and characterization of enzymatic profiles. Rev Inst Med Trop Sao Paulo 57, 289-294.
557 Perez-Garcia, L.A., Csonka, K., Flores-Carreon, A., Estrada-Mata, E., Mellado-Mojica, E., Nemeth, T.,
558 Lopez-Ramirez, L.A., Toth, R., Lopez, M.G., Vizler, C., Marton, A., Toth, A., Nosanchuk, J.D., Gacser,
559 A., Mora-Montes, H.M., 2016. Role of protein glycosylation in *Candida parapsilosis* cell wall
560 integrity and host interaction. Front Microbiol 7, 306.
561 Pérez-Sánchez, L., González, E., Colón-Lorenzo, E.E., González-Velázquez, W., González-Méndez,
562 R., Rodríguez-del Valle, N., 2010. Interaction of the heterotrimeric G protein alpha subunit SSG-1
563 of *Sporothrix schenckii* with proteins related to stress response and fungal pathogenicity using a
564 yeast two-hybrid assay. BMC Microbiology 10, 317-317.
565 Rizzetto, L., Cavalieri, D., 2011. Friend or foe: using systems biology to elucidate interactions
566 between fungi and their hosts. Trends Microbiol 19, 509-515.
567 Robledo-Ortiz, C.I., Flores-Carreon, A., Hernandez-Cervantes, A., Alvarez-Vargas, A., Lee, K.K., Diaz-
568 Jimenez, D.F., Munro, C.A., Cano-Canchola, C., Mora-Montes, H.M., 2012. Isolation and functional
569 characterization of *Sporothrix schenckii ROT2*, the encoding gene for the endoplasmic reticulum
570 glucosidase II. Fungal Biol 116, 910-918.
571 Rodrigues, A.M., de Hoog, G.S., de Camargo, Z.P., 2016. *Sporothrix* species causing outbreaks in
572 animals and humans driven by animal–animal transmission. PLoS Pathog 12, e1005638.
573 Rodrigues, A.M., de Melo Teixeira, M., de Hoog, G.S., Schubach, T.M.P., Pereira, S.A., Fernandes,
574 G.F., Bezerra, L.M.L., Felipe, M.S., de Camargo, Z.P., 2013. Phylogenetic analysis reveals a high
575 prevalence of *Sporothrix brasiliensis* in feline sporotrichosis outbreaks. PLoS Negl Trop Dis 7,
576 e2281.
577 Rodriguez-Caban, J., Gonzalez-Velazquez, W., Perez-Sanchez, L., Gonzalez-Mendez, R., Valle, N.,
578 2011. Calcium/calmodulin kinase1 and its relation to thermotolerance and HSP90 in *Sporothrix*
579 *schenckii*: an RNAi and yeast two-hybrid study. BMC Microbiology 11, 162.
580 Romero-Martinez, R., Wheeler, M., Guerrero-Plata, A., Rico, G., Torres-Guerrero, H., 2000.
581 Biosynthesis and functions of melanin in *Sporothrix schenckii*. Infect Immun 68, 3696-3703.
582 Sánchez-López, J.F., González-Ibarra, J., Álvarez-Vargas, A., Milewski, S., Villagómez-Castro, J.C.,
583 Cano-Canchola, C., López-Romero, E., 2015. Isolation of the *GFA1* gene encoding glucosamine-6-
584 phosphate synthase of *Sporothrix schenckii* and its expression in *Saccharomyces cerevisiae*. Protein
585 Expression and Purification 110, 57-64.

586 Sanchotene, K.O., Madrid, I.M., Klafke, G.B., Bergamashi, M., Terra, P.P.D., Rodrigues, A.M., de
587 Camargo, Z.P., Xavier, M.O., 2015. *Sporothrix brasiliensis* outbreaks and the rapid emergence of
588 feline sporotrichosis. Mycoses 58, 652-658.
589 Singh, R.K., Prasad, M., 2016. Advances in *Agrobacterium tumefaciens*-mediated genetic
590 transformation of graminaceous crops. Protoplasma 253, 691-707.
591 Smith, K.M., Kothe, G.O., Matsen, C.B., Khlafallah, T.K., Adhvaryu, K.K., Hemphill, M., Freitag, M.,
592 Motamedi, M.R., Selker, E.U., 2008. The fungus *Neurospora crassa* displays telomeric silencing
593 mediated by multiple sirtuins and by methylation of histone H3 lysine 9. Epigenetics Chromatin 1,
594 5-5.
595 Teixeira, M.M., de Almeida, L.G., Kubitschek-Barreira, P., Alves, F.L., Kioshima, E.S., Abadio, A.K.,
596 Fernandes, L., Derengowski, L.S., Ferreira, K.S., Souza, R.C., Ruiz, J.C., de Andrade, N.C., Paes, H.C.,
597 Nicola, A.M., Albuquerque, P., Gerber, A.L., Martins, V.P., Peconick, L.D., Neto, A.V., Chaucanez,
598 C.B., Silva, P.A., Cunha, O.L., de Oliveira, F.F., dos Santos, T.C., Barros, A.L., Soares, M.A., de
599 Oliveira, L.M., Marini, M.M., Villalobos-Duno, H., Cunha, M.M., de Hoog, S., da Silveira, J.F.,
600 Henrissat, B., Nino-Vega, G.A., Cisalpino, P.S., Mora-Montes, H.M., Almeida, S.R., Stajich, J.E.,
601 Lopes-Bezerra, L.M., Vasconcelos, A.T., Felipe, M.S., 2014. Comparative genomics of the major
602 fungal agents of human and animal Sporotrichosis: *Sporothrix schenckii* and *Sporothrix brasiliensis*.
603 BMC Genomics 15, 943.
604 Torres-Guerrero, H., 1999. Ploidy study in *Sporothrix schenkii*. Fungal Genet Biol 27, 49-54.
605 Torres-Guerrero, H., Arenas-Lopez, G., 1998. UV irradiation induced high frequency of colonial
606 variants with altered morphology in *Sporothrix schenckii*. Med Mycol 36, 81-87.
607 Trujillo-Esquivel, E., Martinez-Alvarez, J.A., Clavijo-Giraldo, D.M., Hernandez, N.V., Flores-Martinez,
608 A., Ponce-Noyola, P., Mora-Montes, H.M., 2017. The *Sporothrix schenckii* gene encoding for the
609 ribosomal protein L6 has constitutive and stable expression and works as an endogenous control
610 in gene expression analysis. Front Microbiol 8, 1676.
611 Vecchiarelli, A., d'Enfert, C., 2012. Shedding natural light on fungal infections. Virulence 3, 15-17.
612 Walker, C., Muniz, M.F., Rolim, J.M., Martins, R.R., Rosenthal, V.C., Maciel, C.G., Mezzomo, R.,
613 Reiniger, L.R., 2016. Morphological and molecular characterization of *Cladosporium*
614 *cladosporioides* species complex causing pecan tree leaf spot. Genet Mol Res 15.
615 Wang, C., Guan, X., Wang, H., Li, G., Dong, X., Wang, G., Li, B., 2013. *Agrobacterium tumefaciens*-
616 mediated transformation of *Valsa mali*: an efficient tool for random insertion mutagenesis.
617 Scientific World Journal 2013, 968432.
618 Zhang, Y., Li, G., He, D., Yu, B., Yokoyama, K., Wang, L., 2011. Efficient insertional mutagenesis
619 system for the dimorphic pathogenic fungus *Sporothrix schenckii* using *Agrobacterium*
620 *tumefaciens*. J Microbiol Methods 84, 418-422.

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Figure Legends

Figure 1. Expression of the enhanced green fluorescent protein in *S. schenckii*. Conidia were transformed with pBGgHg and selected by five monoconidial passages and three rounds of induction of the yeast morphology. Then, cells were inspected by either bright-field (upper panel) or fluorescent microscopy (lower panels). The WT strain, 1099-18 ATCC MYA 4821, showed low levels of autofluorescence; while the transformed strains (HSS2-HSS6), expressing the enhanced green fluorescent protein showed a sharp and bright fluorescent signal. Scale bars = 10 μm.

Figure 2. Estimation of the number of insertional events of pBGgHg within the genome of *S. schenckii*. Genomic DNA was isolated from the WT strain, 1099-18 ATCC MYA 4821, and the mutant strains, HSS2-HSS6, and used to perform qPCR reactions to amplify the encoding gene for the enhanced green fluorescent protein. Data were normalized using the amplification of the gene encoding for the ribosomal protein L6 as control and the WT strain as reference condition.

Figure 3. Stimulation of TNFα and IL-6 by *Sporothrix schenckii* mutant cells expressing GFP. Live conidia, yeast-like cells or germlings were co-incubated 24 h with human PBMCs, the supernatant saved and used to quantify the cytokine levels by ELISA. WT, strain 1099-18 ATCC MYA 4821; HSS2-HSS6, mutant

strains expressing GFP. The data represent means ± SD of data collected from eight healthy volunteers assayed by duplicate.

Figure 4. Phagocytosis of *Sporothrix schenckii* yeast-like cells by human monocyte-derived macrophages. Yeast-like cells were labeled with Acridine Orange and incubated with the human cells at a MOI 1:6 for 2.5 h at 37◦C under a CO_2 atmosphere. Then, macrophages were gated by FACS system and 50,000 cells were counted/sample. Results represent macrophages interacting with at least one fluorescent yeast-like cell (Total cells). Cells were only green fluorescence was detected were regarded as recently phagocyted, while those emitting both green and red fluorescence were classified as macrophages with yeast-like cells within acidified phagolysosomes. WT, strain 1099-18 ATCC MYA 4821; HSS2-HSS6, mutant strains expressing GFP. The data represent means ± SD of three independent biological replicates performed by duplicate.

Figure 5. Mortality of *Galleria mellonella* larvae infected with different strains of *Sporothrix schenckii* expressing GFP. Upper panel, Groups containing 10 larvae were inoculated with 1×10^5 yeast-like cells and mortality was recorded daily during a two-week period. As a control, one animal group was injected with 10 µL of PBS. The Kaplan–Meier plots were generated with the cumulative data of three independent experiments, and each survival curve contains 30 animals. The statistical analysis showed no differences across the analyzed strains ($P = 0.523$). **Lower panel**, Colony forming units isolated from larvae infected with *S. schenckii*

668 cells. After registration of the animal death, the hemolymph was isolated and used
669 to calculate colony forming units by serial dilution in plates containing YPD, pH 7.8,
670 and incubated at 37°C. No significant differences were observed (P = 0.243).
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Table 1. Cell wall composition of *Sporothrix schenckii* wild-type and mutant strains expressing GFP

Strain	Glucosamine (%)	Glucose (%)	Rhamnose (%)	Mannose (%)
WT (ATCC MYA-4821)	15.6 ± 1.6	40.2 ± 2.6	21.1 ± 1.9	23.1 ± 3.4
HSS2	14.0 ± 1.2	40.5 ± 1.5	22.2 ± 1.3	23.3 ± 1.6
HSS3	17.1 ± 2.6	40.3 ± 1.4	17.7 ± 2.0	24.8 ± 1.6
HSS4	15.3 ± 1.7	40.4 ± 1.4	20.9 ± 1.7	23.3 ± 1.3
HSS5	15.9 ± 2.1	38.8 ± 2.9	21.1 ± 1.2	23.7 ± 2.4
HSS6	15.6 ± 1.8	40.4 ± 1.9	20.9 ± 2.2	23.0 ± 1.5

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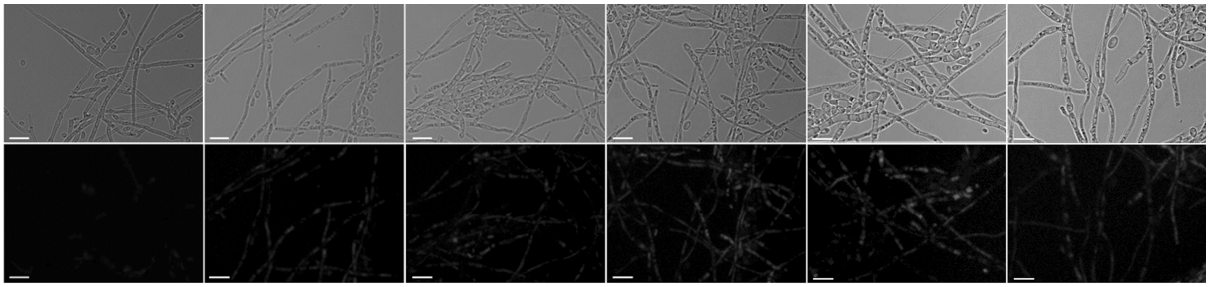

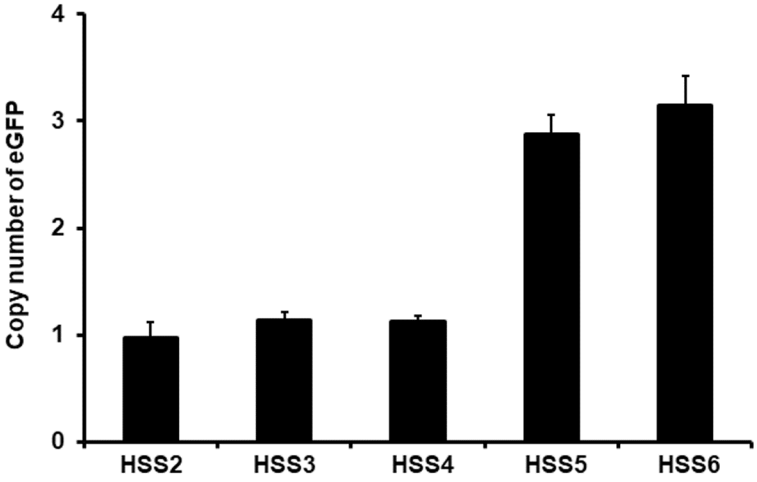

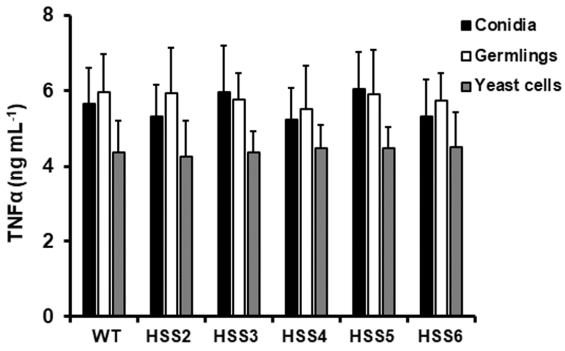

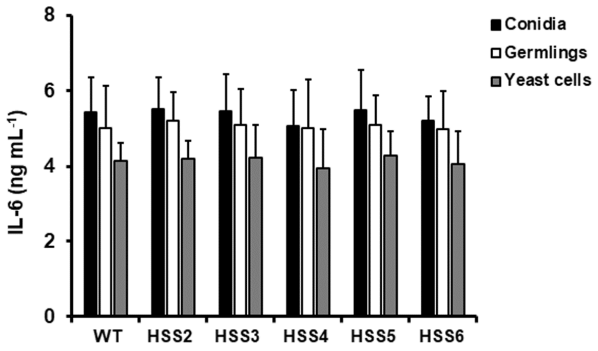

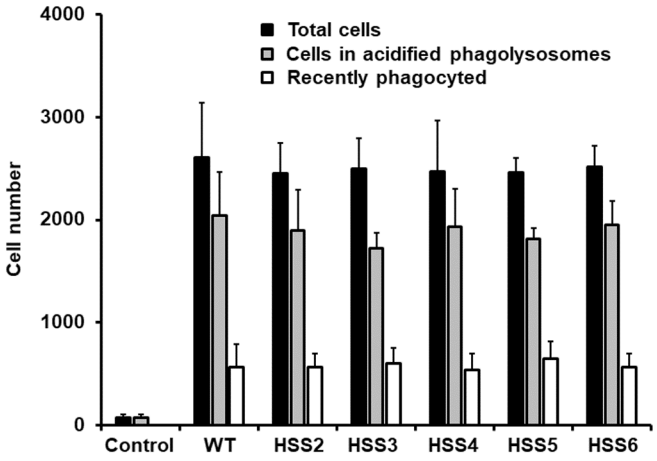

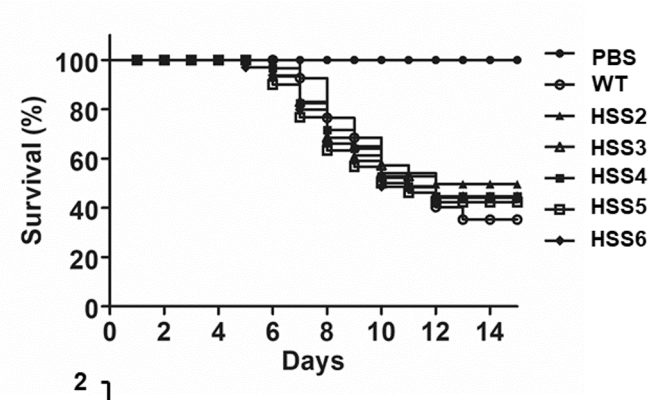

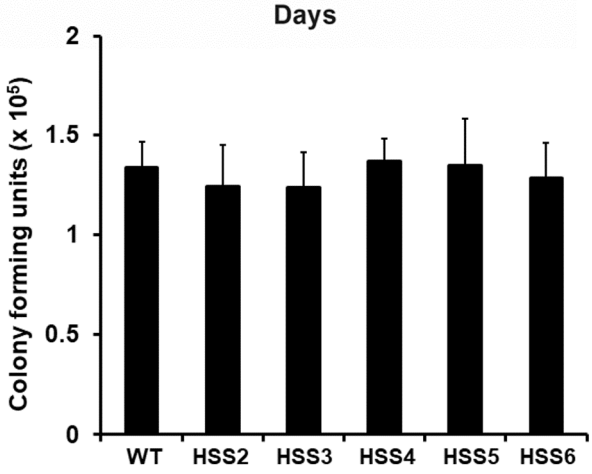

Research Highlights

- *Agrobacterium*-mediated transformation of *Sopothrix schenckii* was optimized
- Insertional mutants expressing GFP were generated
- The mutant strains showed normal cell wall composition and interaction with the host
- The GFP-expressing mutants are suitable for the study of the *Sporothrix*-host interaction