Extracellular Fibrils of Pathogenic Yeast Cryptococcus gattii Are Important for Ecological Niche, Murine Virulence and Human Neutrophil Interactions

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Abstract

Cryptococcus gattii, an emerging fungal pathogen of humans and animals, is found on a variety of trees in tropical and temperate regions. The ecological niche and virulence of this yeast remain poorly defined. We used Arabidopsis thaliana plants and plant-derived substrates to model C. gattii in its natural habitat. Yeast cells readily colonized scratch-wounded plant leaves and formed distinctive extracellular fibrils (40–100 nm diameter × 500–3000 nm length). Extracellular fibrils were observed on live plants and plant-derived substrates by scanning electron microscopy (SEM) and by high-voltage electron microscopy (HREM). Only encapsulated yeast cells formed extracellular fibrils as a capsule-deficient C. gattii mutant completely lacked fibrils. Cells deficient in environmental sensing only formed disorganized extracellular fibrils as apparent from experiments with a C. gattii STE12α mutant. C. gattii cells with extracellular fibrils were more virulent in murine model of pulmonary and systemic cryptococcosis than cells lacking fibrils. C. gattii cells with extracellular fibrils were also significantly more resistant to killing by human polymorphonuclear neutrophils (PMN) in vitro even though these PMN produced elaborate neutrophil extracellular traps (NETs). These observations suggest that extracellular fibril formation could be a structural adaptation of C. gattii for cell-to-cell, cell-to-substrate and/or cell-to-phagocyte communications. Such ecological adaptation of C. gattii could play roles in enhanced virulence in mammalian hosts at least initially via inhibition of host PMN-mediated killing.

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Introduction

‘Primary’ pathogenic fungi that cause serious diseases in healthy humans occur in relatively specialized niches in nature. This trait differentiates them from ‘opportunist’ fungal pathogens that afflict immunodeficient individuals, since ‘opportunist’ pathogenic fungi are more widely distributed in soil and plant vegetable matter. During the last decade, more than 400 species of ‘opportunist’ fungi have been recognized as pathogens for humans and animals [1]. The broad framework for understanding relationships between natural occurrence of the fungal pathogens and their human infectivity currently rests on the idea of ‘dual use’ factors. It suggests that human pathogenic fungi have attributes (‘virulence factors’) that allow them to survive in soil in competition with other life forms, to cycle through invertebrate hosts such as amoebae and to renew their ability to infect human cells [2,3]. Not specifically addressed by this idea is whether growth on plants and vegetable matter plays a role in the virulence of pathogenic fungi. Such a scenario needs systematic investigation considering that many bacteria, especially enteric pathogens, acquire ecological fitness through growth on plants [4]. Indeed, the model plant Arabidopsis thaliana has proven to be a tractable host for the study of bacterial pathogenesis, especially in efforts to unravel virulence factors that are shared between plant pathogens and animal pathogens [5,6].

C. gattii is an encapsulated yeast that causes pulmonary and cerebromeningeal cryptococcosis. C. gattii is an emerging pathogen that has triggered serious public health concerns due to (i) its appearance in previously unknown geographic areas, (ii) its outbreaks among healthy humans, pets, and wildlife, (iii) the intractable nature of cryptococcal disease, and (iv) the difficulty of diagnosis in clinical laboratories. Reliable estimates of cryptococcosis due to C. gattii are currently lacking, but one estimate suggests that one-third to one-tenth of cryptococcosis cases worldwide are caused by C. gattii [7]. Diagnostic laboratories routinely do not distinguish C. gattii from the closely related pathogen C. neoformans, since the reagents required are not readily available. Current information on C. gattii comes either from environmental surveys, which are patchy and far between or from retrospective evaluation of C. neoformans/C. gattii clinical isolates.

C. gattii is readily distinguished from closely related pathogen C. neoformans by occurrence on trees, rather than the pigeon...
drippings colonized by the latter. Other diagnostic characteristics of *C. gattii* include the presence of distinctive cigar-shaped yeast morphology in the host cerebrospinal fluid, agglutinating serotypes, creatinine assimilation and smooth, elongate, rod shaped basidiospores of the teleomorphic form *Pleospora helianthi* [8]. Any of these characteristics could serve as the starting point for systematic studies into virulence of *C. gattii*, but other questions have arisen. Why do rural to semi-urban forested areas pose higher risks for *C. gattii* infection? How do trees figure in the infectious cycle? What is the nature of the infectious propagules, and how are they spread in the environment? Why are apparently healthy humans and animals so prone to infection by this pathogen?

To date, *C. gattii* has been associated with decayed hollows, in the trunks and/or branches of over 50 species of angiosperms and gymnosperms. The growing body of sampling data supports the idea that *C. gattii* is long established among the fungal flora of native trees in many parts of the world [7]. Some experimental studies also provide evidence for *C. gattii*-plant associations. Huerfano et al. [9] demonstrated that *C. gattii* can survive on and be recovered from experimentally infected stems of almond seedlings 100 days post-inoculation, demonstrative of this pathogen’s ability to colonize and thrive on live plants. Previously, we reported that *C. gattii* will grow profusely on wood and wood extracts from a variety of trees [10]. This fungal growth was heavily melanized, which was significant as melanin is a known virulence factor. A mutation in transcription factor, *Ste12*, caused impaired growth and loss of pigmentation on plant-based media, and a concomitant loss of virulence in a murine model of infection [11].

Fungal Strains

*C. gattii* NIH444 (ATCC32609; serotype B, *MATα*), the wild-type strain isolated from cerebrospinal fluid (CSF), was a gift from Dr. K.J. Kwon-Chung (National Institutes of Health, Bethesda, MD). We have previously described this strain as optimum for *C. gattii* molecular pathogenesis studies [12]. *C. gattii* ste12ΔA, a mutant strain with targeted knockout of the transcription factor STE12α, was included as it exhibits reduced colonization of woody substrates, and reduced pathogenicity in mice as a result of defective environmental sensing [10]. We also included a capsular mutant of *C. gattii* (cap59ΔC), because the capsule of *C. neoformans* has critical roles in fungal biology and virulence [3,13]. The *C. gattii* cap59ΔA mutant was constructed by homologous recombination of the cap59::NAT disruption cassette based on sequences from *C. neoformans* CAP59 [14]. The PCR amplifications, biolistic transformation and confirmation of acapsular morphology was according to standard methods [10,15,16].

Growth Media

*C. gattii* cells were cultured in YPD broth at 30°C with shaking (180 rpm), and were maintained in YPD agar and preserved at -70°C in 15% sterile glycerol or in liquid N2. Un-treated black cherry (*Prunus serotina*) wood chips were obtained from Roger Dziengelski, Finch, Pruyln & Co., Inc. (Glens Falls, NY). Leaf agar was prepared by mincing of 10 g of *A. thaliana* leaves into small pieces with scissors, and addition of 2% agar with 0.1% glucose followed by autoclaving for 15 min at 121°C [10].

Arabidopsis thaliana

*A. thaliana* ecotype Columbia (Col-0) and Landsberg erecta (Ler-0), and various mutant lines *eds1* (enhanced disease susceptibility 1; lipase/signal transducer/triacylglycerol lipase), *nahG* (transgenic lipase lesioning salicylic acid; SA); *nip1* (nonexpressor of PR genes 1; pathogenesis-related 1), *sid1* (SA-induction deficient), *rpm1* (resistance to *Pseudomonas syringae* pv maculicola 1), were grown in a greenhouse at the Department of Biological Sciences, Syracuse University, Syracuse, NY [17,18,19,20]. Four- to six-week old plants were transferred to the Mycology Laboratory of the Wadsworth Center where they were maintained at 20–23°C with a 12 hr light/dark cycle under 50–70% humidity, in a modified incubator with HEPA filtration.

Plant inoculation, harvest and light microscopy

*C. gattii* cells were subcultured twice in YPD broth at 30°C with 180 rpm shaking and were then collected by centrifugation (3 min at 3,800 rpm), washed twice in deionized sterile water (DSW) and re-suspended to a final concentration of 1.0×10⁶ cells/ml, by counting in a hemacytometer. *A. thaliana* plants from the growth chamber were placed in a sealed carrier case and moved to a BSL-2 safety cabinet. Four to six leaves on each *A. thaliana* plant were marked with a glass marker. Leaves were lightly wounded on the adaxial leaf surface with a 2–3 mm shallow scratch (without complete puncture) on either side of the mid-vein with a 27-gauge syringe needle [21,22,23]. Two 5-μl drops of 10⁶ *C. gattii* cells/ml were placed at the wound site, and allowed to air dry (5–10 min). Plants were replaced in modified growth incubator maintained at 20–23°C, 12 hr light/dark cycle, and 50–70% humidity. Macrophotographic images of *A. thaliana* plants and leaves were obtained with a consumer digital camera.

Infected *A. thaliana* leaves were chosen at random and cut from the plant 7 days post-inoculation and homogenized with a tissue grinder in 1 ml of DSW, and the homogenate was serially diluted. 100-μl of each dilution was plated onto YPD agar and incubated
for 3–5 days at 25°C, and colonies were counted for determination of fungal viability.

For light microscopy, leaves were cut from plants 7 or 14 days post-inoculation, fixed in 2% glutaraldehyde (EM grade) in phosphate-buffered saline (PBS) or in 0.2 M sodium cacodylate buffer, pH 7.4, alcohol dehydrated by graded series (25%, 50%, 75%, 95%, 100%), and stored for further processing in 100% ethanol at 4°C [24]. Staining of whole leaves was accomplished by soaking of leaves in an aqueous solution of equal parts of Trypan blue (1 mg/ml), lactic acid, deionized water, and glycerolfor 4–min [25]. Leaves were destained in chloral hydrate (2.5 g/ml), slide-mounted, and viewed and imaged with a table top scanner and light microscope.

Growth on plant-based and artificial substrates

A number of natural and artificial substrates were used to study growth characteristics of C. gattii. The natural substrates were intended to recapitulate growth of C. gattii on plants in nature, and for comparisons to growth on organic or synthetic substrates. Wild-type and C. gattii cap59A mutant strains were grown to mid-logarithmic phase in YPD broth. Cells were collected by centrifugation, washed two times in minimal asparagine medium, with 1% glucose, and re-suspended in the same medium to a final concentration of 1×10⁶ cells/ml. Wild-type C. gattii cap59A mutant cells were treated with sodium azide (2 mM), followed by incubation at 65°C for 30 minutes to render them non-viable; these cells were used in experiments that compared effects of viable and non-viable cells. The plant-based and artificial substrates used were: autoclaved black cherry wood shavings (Finch, Pryn & Co., Inc., Glens Falls, NY), consumer brown paper bag, Whatman #1 qualitative filter paper with 98% cellulose (Whatman Limited, Kent, England), Therma flock® plastic coverslips surface treated for optimal cell adhesion (Nalge Nunc International, Rochester, NY), and glass coverslip (Ted Pella, Inc., Redding, CA) and dialysis membrane. The substrate was placed at the bottom of 18-mm round wells of a 24-well flat-bottom polystyrene cell culture plate. One milliliter of 1×10⁶ cells in minimal asparagine medium, was added to each well in one row of the 24 wells in duplicate. The culture plates were covered and incubated for 24- or 48-hr at 25°C. The cell suspension was removed, and wells were washed 3 times with 0.05% Tween-80 in Tris-buffered saline (TBS), to remove non-adherent cells. Substrates were fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4 for 24 hr at 4°C, and dehydrated by exposure to a graded ethanol-water series, and stored in 100% ethanol in plates. Average numbers of attached cells were determined by counting cells in four SEM fields per sample.

Scanning electron microscopy (SEM)

A. thaliana leaves inoculated with C. gattii were harvested at indicated intervals, fixed in 2% glutaraldehyde (EM grade) in phosphate-buffered saline (PBS) or 0.2 M sodium cacodylate buffer, pH 7.4, alcohol dehydrated by graded series (25%, 50%, 75%, 95%, 100%), and stored in 100% ethanol. The dehydrated specimens were critical point dried with liquid CO2 at 70°C, and dehydrated by exposure to a graded ethanol-water series, and stored in 100% ethanol in plates. Average numbers of attached cells were determined by counting cells in four SEM fields per sample. The plant-based and artificial substrates were used to study growth characteristics of C. gattii. The natural substrates were intended to recapitulate growth of C. gattii on plants in nature, and for comparisons to growth on organic or synthetic substrates. Wild-type and C. gattii cap59A mutant strains were grown to mid-logarithmic phase in YPD broth. Cells were collected by centrifugation, washed two times in minimal asparagine medium, with 1% glucose, and re-suspended in the same medium to a final concentration of 1×10⁶ cells/ml. Wild-type C. gattii cap59A mutant cells were treated with sodium azide (2 mM), followed by incubation at 65°C for 30 minutes to render them non-viable; these cells were used in experiments that compared effects of viable and non-viable cells. The plant-based and artificial substrates used were: autoclaved black cherry wood shavings (Finch, Pryn & Co., Inc., Glens Falls, NY), consumer brown paper bag, Whatman #1 qualitative filter paper with 98% cellulose (Whatman Limited, Kent, England), Therma flock® plastic coverslips surface treated for optimal cell adhesion (Nalge Nunc International, Rochester, NY), and glass coverslip (Ted Pella, Inc., Redding, CA) and dialysis membrane. The substrate was placed at the bottom of 18-mm round wells of a 24-well flat-bottom polystyrene cell culture plate. One milliliter of 1×10⁶ cells in minimal asparagine medium, was added to each well in one row of the 24 wells in duplicate. The culture plates were covered and incubated for 24- or 48-hr at 25°C. The cell suspension was removed, and wells were washed 3 times with 0.05% Tween-80 in Tris-buffered saline (TBS), to remove non-adherent cells. Substrates were fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4 for 24 hr at 4°C, and dehydrated by exposure to a graded ethanol-water series, and stored in 100% ethanol in plates. Average numbers of attached cells were determined by counting cells in four SEM fields per sample.

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High voltage electron microscopy (HVEM)

A. thaliana leaves that have been scratch-wounded and inoculated with C. gattii cells were harvested and fixed in 2% glutaraldehyde (EM grade) in 0.2 M sodium cacodylate buffer, pH 7.4 overnight. Leaves were washed three times with 0.2 M sodium cacodylate buffer and stained with 1% osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate for 1-hr at 4°C, rinsed two times with DSW, and dehydrated through a graded ethanol series [26]. Samples were then embedded in Spurr low-viscosity embedding medium (Polysciences, Inc.; Warrington, PA, USA), which was allowed to polymerize at 70°C for 48-hr. Blocks with embedded A. thaliana leaves were cut and trimmed with a hot razor blade, and mounted on pre-formed TEM pegs. Sections were cut approximately 1 μm thick, on a microtome and were mounted on formvar-coated grids. Grid-mounted sections were then stained with 2% uranyl acetate for 60 min, followed by Reynold’s lead stain for 20 min; they were then allowed to air dry, and were stored in an EM grid box. Sections were observed and imaged with the 1.2 MeV AEI EM7 MK II high voltage electron microscope (HVEM) at the Wadsworth Center.

Effects of inhibitors of cytoskeletal proteins

We wanted to examine the roles of actin, tubulins and other cytoskeletal proteins on C. gattii microtubes, since these proteins are important determinants of cell shape [27,28]. C. gattii wild-type and cap59A mutant strain were grown in YPD broth, 30°C, 180 rpm for 12–16 hr and treated with thiabendazole, cytochalasin B, mebendazole, or latrunculin B (details in Suppl. information).

Murine model of cryptococcosis

Virulence of C. gattii was tested in a murine model of cryptococcosis that mimics either progressive pulmonary cryptococcosis (intranasal inoculation, IN) or rapid onset of cerebrone-ngeal cryptococcosis (intravenous inoculation, IV) as described previously [10,29,30]. C. gattii cells were grown with two successive passages on YPD agar and A. thaliana leaf-agar at 25°C for 4 days.

For six-week-old male BALB/c mice were obtained from Charles River Laboratories, Inc. Groups of 8 BALB/c mice were inoculated either via IN or IV route with 30 μl or 100 μl DSW containing 10⁵ cells or 10³ cells, respectively. Mice were given food and water ad libitum, and were observed daily according to a welfare score sheet that addresses appearance of disease symptoms, general malaise, and any apparent pain and discomfort in the infected animals. After signs of progressive poor health or discomfort were noted, the infected animals were euthanized by CO₂ inhalation followed by cervical dislocation. Data from five infected mice (IN or IV) were used to determine Kaplan-Meyer survival curve using SAS software (SAS Institute, Inc.; Cary, N.C). An assessment of colonization and multiplication within lungs and brain of infected mice was obtained by using three mice from each group, which were euthanized at day 7 (IV) or day 14 (IN) post infection. None of the animals displayed any signs of overt illness at these two time periods. One-half of lung or brain harvested from infected animals was preserved for histopathological analyses, in Bouin’s fixative (brain section) or buffered formalin (lungs). The other halves of target organs were used to calculate relative fungal loads. Tissues were weighed and homogenized in 1 ml DSW, serial dilutions were plated on YPD agar, and incubated at 30°C for determination of CFU. Histopathological examinations were done as described previously [10]. Briefly, after 24 hr of fixation,
tissues were sectioned, and brain sections were washed in distilled water for several hours. Processing was done in a vacuum infiltration processor, the Tissue-Tek VIP 5 (Sakura Finetech), starting with 70% ethanol and proceeding through a series of dehydrating alcohols and xylene into paraffin, for 15 min per station. Tissues were then embedded in paraffin blocks and sectioned at a 7-µm (brain tissues) and 3-4 µm (lung tissues) thickness. Sections were stained with hematoxylin & eosin and mucicarmine (Richard Allen Scientific) and examined by light microscopy.

C. gattii–PMN interactions

We have previously shown that in vitro interactions between human PMN and C. gattii can be used for a complimentary evaluation of virulence outcome in mouse models, and to assess the likely outcome of initial interactions between C. gattii and phagocyte cells [10,29,31]. We compared C. gattii cells grown on leaf agar to C. gattii cells grown on YPD agar; a clinical isolate of Candida glabrata was used as control as was C. gattii cap59 D mutant.

Blood was obtained from human volunteers per protocol approved by the New York State Department of Health Institutional Review Board. PMN were isolated by Ficol-Paque (Pharmacia LKB Biotechnology) centrifugation [10,29,30]. PMN were washed twice and resuspended in RPMI-1640. PMN and yeast cells were incubated at a 10:1 ratio for 4 hr at 37°C under 5% CO2, followed by plating on YPD agar for CFU determination [31]. Percent fungicidal activity was calculated as: \(1 - \frac{\text{CFU experiment}}{\text{CFU controls}}\) \(\times\) 100. Results were expressed as mean±standard error (SE) from the values from at least four individual blood donors [10,29]. SEM analysis of C. gattii-PMN interactions were carried using Thermanox® plastic cover slips in 24-well flat-bottom tissue culture plates. PMNs and yeast cells were mixed at a 1:10 ratio, added to each well, and incubated for 4 hr at 37°C under 5% CO2. Following incubation, cells were fixed with 2% glutaraldehyde and processed as described earlier.

Results

C. gattii proliferates on A. thaliana leaves

Gross examination of A. thaliana ecotype Col-0 leaves inoculated with C. gattii wild-type strain showed noticeable chlorotic lesions around the initial wound site. Leaves inoculated with C. gattii cap59 D mutant strain had smaller chlorotic lesions (Figs. 1D, 1G). However, neither of two C. gattii strains caused systemic plant disease in contrast to what has been reported for a number of plant pathogenic fungi grown on A. thaliana plants [32,33]. Microscopic examinations of trypan blue-stained leaves for the presence of yeast cells showed C. gattii colonization along wound sites, as well as punctuated colonization across the leaf, even distant from the initial inoculum site and wound (Fig. 1E,1F). In contrast, A. thaliana leaves inoculated with C. gattii cap59 D mutant had much lower number of yeast cells that were confined to the initial inoculation site.

Figure 1. C. gattii colonized experimentally inoculated A. thaliana leaves. A. thaliana leaves on live plants were inoculated with sterile deionized water control (A), C. gattii wild type cells (D), or C. gattii cap59 D mutant (G). Gross examination of the control leaves with mock inoculations showed no scars while large chlorotic lesions were seen in leaves inoculated with C. gattii wild type cells; much smaller lesions were visible in leaves inoculated with C. gattii mutant cells. Further examination of inoculated leaves after fixation and staining showed no significant fungal colonization around inoculation sites in the mock-inoculated and cap59 D mutant-inoculated leaves (B, H), but leaves inoculated with C. gattii wild-type cells showed fungal cells around inoculation sites (E). Microscopic examinations of leaves revealed numerous C. gattii wild-type cells at the inoculation site and also cells that had dispersed away from the original inoculation (F); in contrast, cap59 D mutant cells were localized at few spots around the inoculation site (I) (100× magnification). Panels A, B and C are from different leaves to illustrate salient features of these observations. doi:10.1371/journal.pone.0010978.g001
sites (Figs. 1H and 1I). Control leaves with mock-inoculation did not display any specific staining (Fig. 1B–1C). Yeast cells were stained most heavily at the wound edges in the leaves consistent with observed chlorotic regions (Fig. 1E). Overall, there was ample evidence for *C. gattii* wild-type strain colonization and dispersal from the wound sites on *A. thaliana* Col-0 ecotype leaves. Preliminary examination of another ecotype *A. thaliana* Ler-0 indicated almost two-times more colonization visually as seen on Col-0 ecotype (Fig. S1). Additionally, a number of mutant plant lines derived from *A. thaliana* Col-0 ecotype and deficient in plant defense against fungal pathogens, showed higher colonization of inoculated leaves by *C. gattii* wild-type strain (*eds1*/*nahG*/*sid2*/*npr1*/*rpm1*; Fig. S2).

**C. gattii** extracellular fibrils on plant leaves

Further examinations by SEM revealed that *C. gattii* profusely colonized wounded *A. thaliana* leaves, with the highest numbers of yeast cells present at the wound sites, but significant numbers of cells seen away from the wounds, a pattern consistent with the light microscopic observations (Fig. 2A, B). Although *C. gattii* cells on leaves were intact, and showed buds of various sizes, we could not tell whether colonization away from wound sites resulted by active

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**Figure 2.** *C. gattii* formed extracellular fibrils on *A. thaliana* leaves. SEM images of infected leaves showed that *C. gattii* colonized wound site and crevices along leaf surfaces (A, B), extracellular fibrils were visible projecting from *C. gattii* cells, connecting yeast cells to each other and to the leaf surface (C, D). Note a yeast cell at higher magnification with prominent extracellular fibrils extending to other yeast cells and to the leaf tissue (D). Higher magnification of *C. gattii*- inoculated leaf surface revealed formation of 'leaf halo' and 'pocket' in *A. thaliana* in some instances (E, F). Scale bar equals 100 μm (A), 5.0 μm (B, C), and 1.0 μm (D, E, F).

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spread or by contact with other inoculated leaves or possibly by deposits of yeast aerosols in the custom growth chamber. *C. gattii* colonization demonstrated a number of unique features that have not been reported in literature: a) extracellular fibrils of variable lengths on the cells (Fig. 2C, D); b) extensive inter-connected individual yeast cells via extracellular fibrils (Fig. 2B, C), and c) formation of pockets or holes on leaf surfaces around *C. gattii* cells (Fig. 2E, F). The dimensions of the fibrils were measured from SEM images: 40–100 nm length ×500–3000 nm diameter. Further experiments revealed that the incorporation of leaves and other plant based substrate in sterile agar also induced the production of extracellular fibrils similar to those seen on intact leaves (Fig. S3).

Further examination of *C. gattii* extracellular fibrils was carried out in HVEM using thick-section microscopy of inoculated leaves (Fig. 3). The sections revealed that extracellular fibrils extended uniformly from cell surfaces in association with leaf tissue (Fig. 3A, D). Both mature and budding cells were covered with fibrils while young buds displayed relatively less projections.

*C. gattii* mutants have vestigial or no fibrils

Although *C. gattii* cap59Δ mutant showed attachment to the inoculation sites, no extracellular fibrils were seen on these mutant cells (Fig. 4A–E). The growth of *C. gattii* cap59Δ mutant was restricted, and cells appeared in clumps (Fig. 4A, B). The data were consistent with the mutant’s reduced production of chlorotic lesions and colonization on *A. thaliana* leaves, as described in the previous section. Other features seen on *A. thaliana* inoculated with *C. gattii* wild-type cells such as pockets and holes in the leaf surfaces were not observed in leaves inoculated with *C. gattii* cap59Δ mutant. The sparse appearance of a digestion-like halo surrounding a few *C. gattii* cap59Δ mutant cells suggested either absence of these structures or their rare occurrence (Fig. 4D).

We also tested *C. gattii* ste12Δ mutant, to see whether defect in environmental signaling has any role in fungus–plant interactions. As seen in SEM micrographs, the inoculated *C. gattii* ste12Δ mutant colonized *A. thaliana* leaves and displayed extracellular fibrils (Fig. 4F, G). However, these fibrils were not as well-formed as those seen in *C. gattii* wild-type strain, and colonized leaves did not show pockets or holes.

*C. gattii* cells exposed to cytoskeletal protein-inhibitors namely, thiabendazole, cytochalasin B, mebendazol or latrunculin B demonstrated variable disorganization of extracellular fibrils on live *A. thaliana* leaves (Fig. S4).

*C. gattii* extracellular fibrils can be induced in vitro

*C. gattii* cells were further tested to determine whether extracellular fibrils formation was an exclusive response to a live plant host or these features could also be induced on other

Figure 3. *C. gattii* extracellular fibril organization visualized by high-voltage EM. HVEM examination of infected leaves showed extracellular fibrils covering *C. gattii* cells in close proximity to the leaf tissues (A–D). Both mature and budding *C. gattii* cells produced extracellular fibrils although young buds displayed relatively less projections. The fibrils were uniformly distributed along the entire cell surface.

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Substrates. Extracellular fibrils were observed to form on a variety of substrates such as cherry wood chips, brown-paper-bag, Thermoplastic coverslips, cellulose filter paper, and glass coverslips (Fig. 5A–F). Extracellular fibrils were more numerous and better formed on plant-based substrates than on synthetic substrates and less frequent on artificial substrates. The rates and pattern of colonization of the different substrates between 24–48 hours was as follows: cherry wood chip > brown paper bag > dialysis tubing > filter paper = Thermoplastic coverslip > glass coverslip (Fig. 5, histogram). Cytoskeletal protein-inhibitors thiabendazole, cytochalasin B, mebendazole or latrunculin B caused disorganization of extracellular fibrils formation in *C. gattii* cells grown in vitro assay (Fig. S5).

*C. gattii* cells with extracellular fibrils are hypervirulent

Kaplan-Meyer survival curves revealed that mice infected IV with *C. gattii* cells grown on leaf agar had significantly lower survival ($p<0.001$) than did mice infected with *C. gattii* cells grown on traditional YPD agar (Fig. 6). For IN route of infection, the difference in virulence of *C. gattii* cells grown on leaf agar and cells grown on YPD agar was even more significant ($p<0.0001$).

Lungs and brains of mice infected with *C. gattii* cells grown on leaf agar had higher total fungal counts than lungs and brains of mice infected with *C. gattii* cells grown on YPD agar (Fig. S6). This raised the possibility that fungal cells with extracellular fibrils proliferated more rapidly in the infected tissues. Post-mortem...
examination of mice IN-infected with C. gattii cells from leaf agar revealed extensive tissue damage in form of enlarged, mottled lung tissue consistent with extensive pneumonia, while lung and brains of mice infected with YPD grown C. gattii cells appeared less damaged (Fig. 7). Notably, more PMNs infiltrated the lungs of mice infected with C. gattii cells grown on YPD agar (Fig. 7B) than was observed for PMN infiltration in lungs of mice infected with C. gattii cells grown on leaf agar (Fig. 7D). C. gattii cells from leaf agar were also numerous in extracellular spaces without the presence of any neutrophils. This raised a possibility that lack of or inhibition of PMN infiltration was related to more tissue damage in mice infected with C. gattii cells grown on leaf agar. A comparison of brain sections of IV-infected animals revealed larger numbers and more prominent brain lesions in mice infected with C. gattii cells with extracellular fibrils; these lesions also contained relatively more yeast cells than did lesions seen in mice infected with C. gattii cells grown on YPD agar (details not shown). This observation was also consistent with more proliferation of C. gattii cells with extracellular fibrils. Unfortunately, it was not practicable to examine these infected tissues by electron microscopy. Therefore, we are unable to provide any evidence of the presence or absence of the C. gattii extracellular fibrils in the infected murine tissues.

C. gattii with extracellular fibrils are more resistant to PMN killing

C. gattii cells grown on leaf agar were significantly more resistant to in vitro killing by human PMN than were C. gattii cells grown on YPD agar (Fig. 8; p<0.05). Controls cells in this assay including Candida glabrata and C. gattii cap59 Δ mutant were highly susceptibility to PMN killings as expected. PMN assay results were consistent with earlier findings in this study from mouse survival experiments, and these findings raised the possibility that at least one mechanism behind the increased virulence of C. gattii cells with extracellular fibrils is their greater ability to escape from PMN-mediated host defenses.

By SEM, we further examined roles of C. gattii extracellular fibrils in PMN interactions. Neutrophils extracellular traps (NETs) were prominent against control organism Candida glabrata. NETs from one neutrophil entrapped many Candida glabrata cells (Fig. 9A). This observation was similar to an earlier report on involvement of NETs in PMN- Candida albicans interactions [34]. C. gattii cap59 Δ mutant–neutrophils interactions showed many yeast cells trapped by single neutrophils with NETs (Fig. 9C, D). C. gattii cells grown in standard YPD broth did not form fibrils, and multiple yeast-cells were seen in interactions with one activated neutrophil (Fig. 9E–G). Interaction of C. gattii cells grown on YPD agar showed 1–5 yeast...
cells with an activated neutrophil (Fig. 9H, I). The PMN in these interactions had rudimentary NETs while  
*C. gattii* cells from YPD agar formed a few extracellular fibrils. Leaf agar grown *C. gattii* cells made extensive extracellular fibrils; these fibrils made yeast cell-yeast cell connections and also connections to many activated PMN (Fig. 9J, K). Notably, *C. gattii* extracellular fibrils appeared to lie atop or extend over PMN.

**Discussion**

The present study revealed several unreported aspects of *C. gattii* that have implications for its ecology and virulence. *C. gattii* readily colonized scratch-wounded *A. thaliana* leaves on live plants forming organized layers of fungal cells with unique extracellular fibrils emanating from and between them. Extracellular fibrils were also formed when *C. gattii* cells were grown on plant-based substrates, and on materials promoting cell adhesion. Formation of *C. gattii* extracellular fibrils was associated with hypervirulence in mice infected via IN route, and with an increased resistance to killing by human PMN in vitro. Paradoxically, human PMN NETs were observed in the presence of extracellular fibrils-bearing *C. gattii* cells, but the fibrils apparently conferred enhanced fungal resistance to PMN killing. These observations suggest that *C. gattii* extracellular fibrils formation could be a structural adaptation for cell-to-cell, cell-to-substrate and cell-to-host communication. Pathogenic fungi might possess features enabling them to adapt to their ecological niche; such features could also serve to enhance fungal virulence in the mammalian hosts at least initially via inhibition of host PMN-mediated killing.

Discernment and characterization of extracellular fibrils in *C. gattii* prompted us to search for precedents for such structures, in the literature. Bacteria, of course, have pili and flagella for locomotion and virulence [35]. Other extra-cellular extensions have been extensively recorded from both prokaryotic and eukaryotic cells. In eubacteria, they are variously termed adhesion threads, extracellular appendages, fibrils, and filaments [36,37,38,39] while in mammalian cells they include blebs, filopodia, lamellipodia, podosomes, and nanotubes [40,41,42,43,44]. *Cryptococcus gattii* extracellular fibrils did not fully share features such as their induction and size with previously recorded descriptions. Among

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**Figure 7. *C. gattii* cells with extracellular fibrils elicited less robust tissue responses.** The histopathological results are from the post-mortem examination of lungs of mice infected IN with *C. gattii* cells were taken either from YPD agar (A, B) or leaf agar (C, D). (A) a focus of fungal replication is bordered by intense neutrophil and pulmonary macrophage inflammatory response in alveoli and perivascular connective tissues, Mucicarmine, 100X; (B) Higher magnification showing intense neutrophilic inflammation borders mucicarmine positive *C. gattii* cells, Mucicarmine, 400X; (C) Severe diffuse lung invasion by *C. gattii* from leaf agar in the mouse model of intranasal inoculation. All alveoli are either colonized by the fungus or otherwise compromised by a predominantly suppurative inflammatory response. Mucicarmine, 100X; and (D) Higher magnification of showing numerous mucicarmine positive *C. gattii* cells fill alveoli; there is a weaker neutrophilic response than see in (B). Notably, there are many extracellular organisms with no inflammation, the hallmark of cryptococcosis caused by hypervirulent *C. gattii* strains.

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Further studies are needed to confirm the extent and nature of approximation of the sizes of tubular protuberances emanating from the capsule [46,47,48].

We preferred our A. thaliana scratch wound model over other inoculation methods, because it is more consistent with natural wounding of plant tissues as a result of herbivory, insect attack, animal damage or abiotic damaging events [57]. Extensive experimental evidence for ecological specialization is available for C. neoformans. For example, in landmark studies, Staib and colleagues established that pigeon dropping could provide adequate nutrition for C. neoformans asexual and sexual growth, notable because these droppings are a relatively nutrient-poor source for microbial growth [58]. More recently, it was shown that C. neoformans undergoes filamentation and sexual mating on pigeon manure agar [59]. Our present studies raise the possibility for ecological specialization in C. gattii. Extracellular fibrils formation could be an adaptation for colonization on plant leaves, which offer an inhospitable surface for microbial colonization due to limited nutrients, extreme exposure to temperature changes, dehydration and radiation, and unique inter-specific competition [4,60]. The inference for leaf colonization is supported by the fact that leaf colonization is a wide-spread trait in basidiomycetous yeasts especially many Cryptococcus species from the Tremellales lineage that includes C. gattii [61].

C. gattii infections are most likely acquired from host exposure to environments where the fungus grows on or in association with leaves and trees especially tree hollows [62]. We sought to model this situation in the laboratory, by growing C. gattii on plant substrates, and then examining its virulence in a murine model of cryptococcosis. We utilized growth on A. thaliana leaf agar, which induced C. gattii to produce extracellular fibrils. Mice infected using C. gattii cells with extracellular fibrils had higher rates of lung and brain colonization by the fungus and significantly lower survival. Histopathological examination of diseased tissues also showed extensive fungal proliferation and destruction of pulmonary tissue in infected mice; the host response appeared rather limited, in terms of PMN infiltration. The ease with which we were able to produce a hypervirulent variant of the parent C. gattii strain, most notably for IN infection, by simply changing the growth substrate, underscored the crucial effect that natural habitat could exert as a modulator of innate virulence of a pathogen. Alternately, C. gattii grown on YPD agar did not express all putative virulence factors. Further experiments are warranted to clarify the roles that growth substrates exert on the virulence of C. gattii.

To examine cause-effect relationship between extracellular fibrils formation and hypervirulence by a complementary approach, we utilized in vitro fungus–PMN assay since this assay is rapid and results correlate well with virulence properties seen in animal model [31,63]. A significant reduction in PMN-mediated killing of C. gattii cells grown on plant-based substrates showed expected correlation

![Image](58x24 to 76x41)

Figure 8. C. gattii with extracellular fibrils were more resistant to human PMN killing. C. gattii cells grown on leaf agar showed reduced susceptibility to in vitro killing by human PMN in phagocyte interaction assay. The data showed that only 24% of initial 10⁷ yeast cells used in the inoculum were susceptible to PMN-induced killing. In contrast, higher PMN fungicidal activity was seen with C. gattii cells grown on YPD agar (41% killing). The two control used in these experiments showed expected high susceptibility to PMN-mediated fungicidal activity: C. gattii cap59::mutant (64% killing), and Candida glabrata (65% killing). Results are mean±SE of at least four independent experiments.

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neutrophils are also likely critical in allowing eventual survival and actin, tubulin and extra-cellular transport for characterization of design of antifungal drugs and vaccines.

distinguishes these pathogens from myriad of non-pathogenic specialized niches would be a fruitful approach to discern what examination of pathogenic fungi in conditions that simulate their fibrils interact with these cell types? In summary, we believe that C. gattii also be important to examine in the lungs, and then cause systemic disease [68].

Plants were transferred to the Mycology Laboratory of the Wadsworth Center where they were maintained at 20–23°C with a 12 hr light/dark cycle under 50–70% humidity, in a modified incubator with HEPA filtration. C. gattii cells were subcultured twice in YPD broth at 30°C with 180 rpm shaking and were then collected by centrifugation, washed twice in deionized sterile water (DSW) and re-suspended to a concentration of 1.0 × 10⁶ cells/mL. Four to six leaves on each A. thaliana plant were lightly wounded on the adaxial surface on either side of the mid-vein with a 27-gauge syringe needle (4, 6, 7). Two 5-μL drops of 10⁷ C. gattii cells/mL were placed at the wound site, and allowed to air dry (5–10 min). Plants were replaced in modified growth incubator maintained at 20–25°C, 12 hr light/dark cycle, and 50–70% humidity. After 7 days, inoculated plants were transferred to a BSL 2 cabinet, leaves excised and photographed with a digital camera. Whole plants and close up of inoculated leaves showed varying levels of scars. [References [1.Cao, H., S. A. Bowling, A. S. Gordon, and X. Dong. 1994. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6:1503–1592]; [2.Delaney, T. P., S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessmann, E. Ward, and J. Ryals. 1994. A central role of salicylic acid in plant disease resistance. Science 266:1247–1250]; [3.Falk, A., B. J. Feys, L. N. Frost, J. D. Jones, M. J. Daniels, and J. E. Parker. 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc Natl Acad Sci U S A 96:3292–7]; [4.Fullner, J. K. 1999. Role of Agrobacterium vizB genes in transfer of T complex and RSF1010. J Bacteriol 181:430–4]; [5.Grant, M. R., L. Godiard, E. Straube, T. Ashfield, J. Lewald, A. Satther, R. W. Innes, and J. L. Dangl. 1995. Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. Science 269:3436; [6. Mithofer, A., G. Wanner, and W. Boland. 2005. Effects of Feeding Spodoptera litoralis on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. Plant Physiol. 137:1160–1168]; [7.Polizzi, G., and A. Vitale. 2006. Dasylirion serratifolium as a new host of Brotynia cennanis, the causal agent of leaf spots and blight in Italy. Plant Disease 90:114–114]. Found at: doi:10.1371/journal.pone.0010978.s001 (3.56 MB DOC)

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**Supporting Information**

**Figure S1** C. gattii colonized A. thaliana mutant plant leaves. A. thaliana ecotype Columbia (Col-0) and various mutant ecotypes such as eds1 (enhanced disease susceptibility 1; lipidase/signal transducer/triacylglycerol lipase), nahG (transgenic line degrading salicylic acid; SA), npr1 (nonexpressor of PR genes 1; pathogenesis-related 1), sid2 (SA-induction deficient), rpm1 (resistance to Pseudomonas syringae pv maculicola 1), and pad4 (phytoalexin deficient 4) were grown in a greenhouse at the Biology Department, Syracuse University, Syracuse, NY (1–3, 5). Four-to six-week old

**Figure S2** C. gattii showed enhanced colonization of A. thaliana mutant plant leaves. A number of mutant plants with genotypes derived from A. thaliana Col-0 ecotype were inoculated as described in supplementary figure S1. Inoculated leaves were homogenized in glass tissue grinders, homogenate suspended in sterile deionized water and a series of dilutions plated on YPD agar for recovery of fungal colony forming units (CFU). The mutant plants showed higher susceptibility to colonization with C. gattii wild-type cells. The colonization was highest in eds1 and nahG mutants. Overall, colonization was eds1>nahG>sid2>npr1>rpm1. The experiment was repeated once.

**Figure S3** C. gattii extracellular projections were formed on agar with plant substrates. Dialysis tubing was cut to small squares

**Figure 9. C. gattii fibrils interfered with entrapment by neutrophil extracellular traps (NETs).** SEM analysis revealed that activated PMN made profuse NETs against control organism Candida albicans (A, scale bar 10 μm; B, scale bar 5 μm). Rudimentary to no NETs were visible against C. gattii caps9A mutant cells (C, scale bar 10 μm; D, 5 μm). C. gattii cells grown either in YPD broth (E, F) or on YPD agar (G, H) had no or rudimentary fibrils, and group of 1–5 yeasts were seen around an activated neutrophil, which produced only rudimentary NETs. C. gattii grown on leaf agar made extensive fibrils, which extended between yeast cells, and yeast cells and multiple activated PMN (I and K scale bar 10 μm; J, scale bar 5 μm). Symbols: (*) fungal cells, (+ or N) PMN. doi:10.1371/journal.pone.0010978.g009
approximately 1 cm × 1 cm and sterilized by boiling in water for 20–30 min. Three squares of sterilized tubing were then laid flat over YPD, *A. thaliana* leaf, black cherry wood chip or Nger seed agar plates. Each square was inoculated with 100 μl of 10^7 cells/ml *C. gattii* wild-type cells. Plates were allowed to dry and incubated at 25°C for 4 days. Small blocks of agar (~1 cm × 1 cm) were removed and fixed in 2% glutaraldehyde. 0.2 M sodium cacodylate buffer, and dehydrated by graded alcohol series, critical point dried, gold sputter coated and imaged by. (A) YPD agar, (B) *A. thaliana* leaf agar, (C) Nger seed agar, and (D) Black cherry wood chip agar. SEM micrographs of one representative *C. gattii* cells obtained from each treatment described above. Controls seed agar, and (H) Black cherry wood chip agar. DOC micrographs of one representative leaf agar, (C) Niger seed agar, and (D) Black cherry wood chip agar. SEM micrographs of one representative

Notably, *C. gattii* extracellular fibrils were absent on YPD agar in contrast to abundant formation on agar supplemented with plant substrates.

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**Figure S4** *C. gattii* extracellular fibrils are altered by cytoskeletal protein inhibitors. We examined the roles of actin, tubulins and other cytoskeletal proteins on the formation of *C. gattii* extracellular fibrils since these proteins are important determinants of cell shape. (1, 3). *C. gattii* wild-type and cap59A mutant strain were grown in YPD broth for 12–16 hr. Thiabendazole (25–150 μg/ml), cytochalasin B (25–150 μg/ml), mebandazole (20–80 μg/ml), or latrunculin B (100 μM/100 μM/ml), were added to individual cultures and incubated for an additional 6 hr (2). An aliquot of yeast cells were also treated with 2 M sodium azide at 65°C for 30 min to render them non-viable. Cells were collected by centrifugation, washed twice with SDW, and re-suspended to a concentration of 10^6 cells/ml. Cells were serially diluted in SDW and plated on YPD agar to determine any loss in viability. None of the drug treatments caused significant loss of viability. Six to eight leaves each from at least three different *A. thaliana* plants were scratch wounded and inoculated with two 5–μl drops of 1×10^6 *C. gattii* cells obtained from each treatment described above. Controls included leaves from wounded plants inoculated with SDW, and non-wounded, non-inoculated *A. thaliana* plants. Leaves were harvested 7-days post inoculation and prepared for light microscopy and SEM as described in the previous section. Microfilament (Actin) and microtubule (Tubulin) inhibitors cytochalasin B (Top left), latrunculin B (Top right), thiabendazole (Bottom left) or mebandazole (Bottom right) alter *C. gattii* cell attachment and extracellular fibril formation in a concentration dependent manner. Lower magnification (left panel) and higher magnification images (right panel) for each treatment are shown with red borders demarcating original inoculation sites. The concentrations used were cytochalasin B (Top left panels [A-B 25 μg/mL], [C-D 50 μg/mL], [E-F 100 μg/mL], and [G-H 150 μg/mL]); latrunculin B (Top right panels [A–B 100 μM], [C–D 200 μM] and [E–F 400 μM]); thiabendazole (Bottom left panels [A–B 25 μg/mL], [C–D 50 μg/mL], [E–F 100 μg/mL], and [G–H 150 μg/mL]); mebandazole (Bottom right panels [A–B 20 μg/mL], [C–D 40 μg/mL], [E–F 60 μg/mL], and [G–H 80 μg/mL]). A concentration dependent effect on extracellular fibril formation is most pronounced with latrunculin and mebandazole.

*References*

1. de Hoog GS, Guarro J, Gené J, Figueras (2009) Atlas of Clinical Fungi. Utrecht: CBS/Universitat Rovira i Virgili.
2. Mylonakis E, Casadevall A, Ausubel FM (2007) Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog 3: e101.
3. Casadevall A, Steenbergen JN, Nosanchuk JD (2003) ‘Ready made’ virulence and ‘dual use’ virulence factors in pathogenic environmental fungi—the *Cryptococcus neoformans* paradigm. Curr Opin Microbiol 6: 332–337.
4. Brandl MT (2006) Fitness of human enteric pathogens on plants and implications for food safety. Annu Rev Phytopathol 44: 367–392.

**Author Contributions**

Conceived and designed the experiments: DJS RR SC VC. Performed the experiments: DJS PR YD MBJ SSB WAS SC VC. Analyzed the data: DJS PR YD MBJ BFM SSB WAS SC VC. Contributed reagents/materials/analysis tools: RR MBJ BFM SSB WAS SC VC. Wrote the paper: DJS SC VC.

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5. van Baardwijk P, van Belkum A, Thomma BP (2007) Disease induction by human microbial pathogens in plant-model systems: potential, problems and prospects. Drug Discov Today 12: 167–173.

6. Rahme LG, Ausubel FM, Cao H, Drenkard E, Goumnerov BC, et al. (2000) Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci USA 97: 4813–4821.

7. Springer D, Chaturvedi V (2010) Projecting global occurrence of Cryptococcus gattii Tazner Infect Dis 16: 14–20.

8. Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B (2001) (1557) Proposal to conserve the name Cryptococcus gattii against C. hondurianus and C. baileyi (Basidiomycota, Hymenomycetes, Tremellomycotina). Taxon 51: 804–806.

9. Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M (2002) (1557) Characterization of Cryptococcus neoformans var. gattii, serotype C. Rev Iberoam Microl 18: 111–132.

10. Wright LC, Chen SC, Wilson CF, Simpanya MF, Blackstock RD, et al. (2002) Characterization of adhesion threads of Dimorphodacnum galactinum. Haldenborough is dependent upon protein filaments. Environ Microbiol 4: 2844–2854.

11. Walker JR, Gnanam A, Schnickova I, Harmison J, Karymov MA, et al. (2007) Cryptococcus neoformans var. grubii-like capsule formation and epigenetic changes of the primary pathogenic yeast Cryptococcus gattii. Eukaryot Cell 5: 1065–1080.

12. Xue C, Tada Y, Dong X, Heitman J (2007) Microreview: capsule- associated genes of Cryptococcus neoformans. Mycopathologia 168: 207–218.

13. Narasipura SD, Ault JG, Behr MJ, Chaturvedi V, Chaturvedi S (2003) Selection of optimal media for the generation of cell polarity. Mol Biol Cell 14: 4912–4919.

14. Okabayashi K, Hasegawa A, Watanabe T (2007) Microreview: capsule-associated genes of Cryptococcus neoformans. Mycopathologia 163: 1–8.

15. Chang YC, Kwon-Chung KJ (1994) Characterization of the capsule-deficient mutation of Cryptococcus neoformans restores its virulence. Mol Cell Biol 14: 952–9421.

16. Hu G, Kneudest JD (2006) Gene disruption in Cryptococcus neoformans and Cryptococcus gattii by in vitro transposition. Curr Genet 49: 341–350.

17. Delaney TP, Uknes S, Verhoog J, Friedrich L, Weyman K, et al. (1994) A central role of saccharic acid in plant disease resistance. Science 266: 1217–1250.

18. Cao H, Bowling AS, Gordon AS, Dong X (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1533–1552.

19. Saarimaa C, Peltola M, Raulio M, Neu TR, Salkinoja-Salonen MS, et al. (2006) Characterization of adhesion threads of Myxococcus xanthus. Bioessays 28: 590–595.

20. Mithofer A, Wanner G, Boland W (2005) Identification of functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J 28: 293–305.

21. Fullner KJ (1998) Role of Arabidopsis thaliana ligament fibroblasts to non-demineralized dentin surface in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 97: 393–397.

22. Springer DJ, Chaturvedi V (2010) Functional characterization of the human fungal pathogen Cryptococcus neoformans var. grubii. FEMS Microbiol Lett 52: 843–846.

23. Jarrell K, Gruber H, Vassallo J, Tamashiro WM, Goncalves EM, et al. (2005) Morphological characterization of a human gloma cell line. Cancer Cell Int 5: 1–263–273.

24. Frases S, Pontes B, Nimrichter L, Viana NB, Rodrigues ML, et al. (2009) Phylloplane Yeasts. In: Péter G, Rosa C, eds. Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci USA 97: 4813–4821.
63. Narasipura SD, Chaturvedi V, Chaturvedi S (2005) Characterization of Cryptococcus neoformans variety gattii SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. Mol Microbiol 55: 1762–1800.

64. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532–1535.

65. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, et al. (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr Biol 16: 396–400.

66. Urban CF, Leurido S, Zychlinsky A (2006) How do microbes evade neutrophil killing? Cell Microbiol 8: 1687–1696.

67. Dong ZM, Murphy JW (1995) Effects of the two varieties of Cryptococcus neoformans cells and culture filtrate antigens on neutrophil locomotion. Infect Immun 63: 2632–2644.

68. Wartha F, Bäier K, Abiger B, Fernebro J, Zychlinsky A, et al. (2007) Capsule and D-alanylated peptidoglycan protect Streptococcus pneumoniae against neutrophil extracellular traps. Cell Microbiol 9: 1162–1171.