

Differential expression of chitin synthase (*CHS*) and glucan synthase (*FKS*) genes correlates with the formation of a modified, thinner cell wall in *in vivo*-produced *Beauveria bassiana* cells

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Abstract

During infection (*in vivo*), the entomopathogenic fungus *Beauveria bassiana* produces yeast-like cells that are surrounded by modified cell walls. These modifications have been related to the fungus ability to limit recognition by the host defense system. The composition of the *in vivo* cell wall was analyzed using a combination of cytochemical and molecular techniques. The *in vivo* cell walls still contained both chitin and 1,3- β -glucan, but they were significantly thinner than *in vitro* cell walls (50–60 nm versus 100–160 nm, respectively). The difference in cell wall thickness was correlated with transcriptional regulation of cell wall-related genes: quantitative RT-PCR reactions demonstrated that *B. bassiana* chitin synthase (*CHS*) and glucan synthase (*FKS*) genes are down regulated *in vivo*. These analyses indicate that *in vivo*-triggered phenotypic modifications, including cell wall adjustments, are controlled by molecular mechanisms that include regulation of gene expression at the transcriptional level.

Key words: AY743592, AY743593, CF350282, dimorphism, fungal cell wall, host-pathogen interactions

Introduction

The fungal wall is a complex structure that protects cells from the environment and provides them with their inherent shape. The fungal cell wall is dynamic; the arrangement of particular components and their relative amounts on or within the wall vary depending upon the life cycle stage and environmental conditions, including temperature or nutrient availability. In insect pathogenic fungi, the cell wall generally forms an interface between the pathogen and the host. Several studies have suggested that components of cell wall serve to elicit the host encapsulation response [1, 2]. Carbohydrate surface epitopes have been implicated both in the activation of the phagocytic hemocytes and as receptors for

opsonin-mediated phagocytosis [3]. Fungal cells that gain ingress through the transcuticular route normally undergo replication in the hemocoel, where they diminish -and ultimately overcome- the host defense reactions. The outer matrix of *in vivo* cells might lack some of the chemical cues responsible for nonself recognition. Certain species of *Entomophaga* (Zygomycetes) produce wall-less protoplasts in the insect hemocoel that escape immune recognition and replicate in the osmotically stable nutrient-rich hemolymph [4, 5]. The production of unsaturated fatty acids by these protoplasts has been reported to inhibit 1,3- β -glucan synthase activity [6]. Studies on the insect pathogen *Beauveria bassiana* have demonstrated significant differences in the wall composition between *in vitro* and *in vivo* cell phenotypes [7, 8].

In vitro-produced cells possessed a well-developed cell wall and were readily phagocytosed, whereas *in vivo* hyphal bodies found free floating in the insect hemolymph possessed a modified wall that lacks galactose residues. However, unlike the *Entomophaga* protoplasts, the *in vivo* *B. bassiana* hyphal bodies retained their cylindrical shape suggesting the presence of structural cell wall components.

Structurally, the fungal wall may be considered analogous to the extracellular matrix (ECM) of animal cells which consists of fibrous proteins such as collagen embedded in a gel-like polysaccharide substrate. The beta-linked polysaccharide, 1,3- β -D-glucan, is the major structural component of the cell wall, giving yeasts and other fungi their shape and providing osmotic support [9]. The multi-subunit enzyme 1,3- β -D-glucan synthase (UDP-glucose: 1,3- β -D-glucan 3- β -D-glycosyltransferase; EC 2.4.1.34) catalyzes the formation of 1,3- β -D-glucan. Biochemical studies with *Saccharomyces cerevisiae* revealed that this enzyme utilizes UDP-glucose as the substrate and consists of a regulatory subunit and a catalytic subunit [10]. The regulatory GTP-binding component, encoded for by *RHO1* gene, is copurified with membrane-bound glucan synthase activity [11, 12]. The two closely related genes *FKS1* and *FKS2* coding for the catalytic subunit of the 1,3- β -D-glucan synthase were first isolated from *S. cerevisiae* [13]. The finding of an *Aspergillus nidulans* gene, *FKSA*, which is similar to *S. cerevisiae* *FKS*, suggested that the *FKS* genes are conserved in fungi [14]. Homologous *FKS* genes have also been reported for *Candida albicans*, *Neurospora crassa*, *Cryptococcus neoformans* and *Paracoccidiodes brasiliensis* [10, 15, 16]. The chitin synthase (*CHS*) synthesizes chitin through transfer of UDP-N-acetylglucosamine to a chitin chain. Chitin synthesis has been most extensively studied in the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, where three different genes have been cloned (*CHS1*, *CHS2* and *CSD*). Since then, *CHS* genes or gene fragments have been characterized in numerous fungal species either by amplification using nucleotides based on peptide sequences strictly conserved between *S. cerevisiae* and *C. albicans*, or by hybridization with the yeast genes [17].

Herein, the presence and distribution of chitin and 1,3- β -glucan have been examined on *in vitro*

and *in vivo* produced *B. bassiana* cells using a combination of cytochemical probes. In addition, synthase genes responsible for the formation of these polymers have been investigated. Using a combination of molecular methods the partial sequence of a *B. bassiana* 1,3- β -D-glucan synthase (*BbFKS*) and complete sequence of chitin synthase (*BbCHS*) homologues have been generated and analyzed. The transcript levels of both chitin and glucan synthase as well as the regulatory *RHO* gene have been measured in the *in vivo* and *in vitro* cells to examine if their activities are being regulated at a transcriptional level.

Methods and materials

Maintenance of fungal culture

Beauveria bassiana UFL 5477 was maintained on Sabouraud maltose agar plus 2% yeast extract (SMY) at 25 °C. Conidia produced on these plates were inoculated into flasks containing Sabouraud dextrose broth plus 2% yeast extract and shaken at room temperature for 3–4 days. Blastospores produced in broth cultures were separated from mycelial fragments by filtering through Miracloth (Calbiochem Co., San Diego, CA), and were collected by low speed centrifugation in deionized H₂O. The concentration of washed blastospores was enumerated with a Brite-line hemacytometer and either used immediately or stored at 4 °C.

Production of *in vivo* fungal cells

The beet armyworm *Spodoptera exigua*, the cabbage looper *Trichoplusia ni*, and the tobacco hornworm *Manduca sexta*, accessed from laboratory colonies, were used as hosts to propagate the *in vivo* cell phenotype of *B. bassiana*. In all cases, larvae were reared on artificial diet and at 25 °C until they reached the fifth larval instar. At this time the insects were challenged by injection of blastospores into the hemocoel. A total of 5×10^4 cells were injected into *S. exigua* and *T. ni* whereas 1×10^6 cells were injected into the larger *M. sexta* larvae. Injected larvae were incubated for 68–72 h at 25 °C. Quantities of *in vivo* produced fungal cells were harvested from *M. sexta* larvae as out-

lined by Tartar and Boucias [18], and were processed for RNA extraction.

Fluorescence microscopy

In vitro and *in vivo* fungal cells were harvested from either broth or hemolymph, washed in PBS (Phosphate Buffer Saline), and fixed for 2 h at room temperature in 2% paraformaldehyde in PBS. After fixation, the fungal cells were washed in PBS. To detect mannose and galactosidic residues, cell preparations were stained with either concanavalin-A (Con-A) fluorescein isothiocyanate (FITC) or peanut lectin-FITC conjugates, respectively, with and without competing sugars (200 mM) as outlined by Pendland et al. [8]. Fixed cells were probed with aniline blue fluorochrome (Biosupplies Australia, Parkville, Victoria Australia) for the detection of 1,3- β -glucans. Unfixed cell preparations were incubated in an aqueous solution of aniline blue (330 μ g/ml) for 30 min at room temperature, and washed in H₂O. Cell preparations were examined with a Leitz Laborlux S microscope fitted with epifluorescence optics using either FITC or DAPI filter cubes. Images were captured with a Spot-RT monochrome digital camera and processed with Photoshop 7.0 software (Adobe).

Electron microscopy

Fungal cell preparations were collected from *in vivo* hemolymph 48 h post injection and *in vitro* sources by low speed centrifugation. Pellets were resuspended in 2.5% (v/v) glutaraldehyde (EM grade) in 0.1 M cacodylate buffer containing 0.1% CaCl₂ and fixed for 3 h. Following fixation, samples were washed three times in 0.1 M cacodylate buffer (pH 7.2) and post-fixed in 1% (w/v) aqueous osmium tetroxide for 1 h at room temperature. Samples were washed three times with water and pellets were embedded in 3% aqueous agar for easier handling. Samples were dehydrated through a standard ethanol series (30–100% ethanol, 15 min per step) and cleared with two washes in acetone. Tissues were infiltrated with Epon-Araldite resin (Epon 812 2 gm, Araldite 502 1 gm, DDSA 4.5 gm, DMP-30 4 drops; Ernest F. Fullam Inc., Schenectady, NY) in a stepwise fashion with ratios of acetone to resin of 3:1, 1:1 and 1:3 and finally several changes of pure resin. Resin blocks

were cured overnight in a 65 °C oven. Thin gold interference sections (85–100 nm thick) cut with a diamond knife on a UltraCut-E microtome (Reichert-Jung, Wein, Austria) were collected on 200-mesh nickel grids.

To localize 1,3- β -glucans, sections were first blocked against nonspecific labeling with 1% cold water fish gelatin in PBS (pH 7.2) for 30 min. Sections were then incubated with the primary antibody 1:100 solution in PBS containing 0.5% gelatin for 1 h. Three washes in PBS followed, and the sections were incubated with the secondary antibody (1:20 solution in PBS) for 1 h. Sections were washed twice in PBS, twice in deionized, post-stained with 2.5% aqueous uranyl acetate for 10 min, followed by standard Reynolds lead citrate stain for 5 min and were examined with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV. The primary antibody used for labeling was a monoclonal antibody raised in mouse against a laminarin-haemocyanin conjugate (purchased from Biosupplies Australia Pty Ltd, Parkville Victoria, Australia). The secondary antibody, 18 nm colloidal gold-affinipure goat anti-mouse IgG, H+L, was obtained from Jackson ImmunoResearch Labs (West Grove, Pennsylvania). Cytochemical controls included: (i) replacement of the primary antibody with non-specific antibody raised in mouse against neurofilaments, obtained from the Hybridoma Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida; (ii) omission of the primary antibody; (iii) incubation of the primary antibody with laminarin before the labeling. The above-mentioned staining protocols produced no detectable labeling in all of the cytochemical controls.

To localize chitin, sections were blocked against nonspecific labeling with 1% bovine serum albumin in PBS (pH 7.2) for 30 min. Then they were floated on the undiluted gold-conjugated lectin solution for 30 min followed with three washes in PBS and two washes in water. The sections were counterstained with 2.5% aqueous uranyl acetate for 10 min, followed by standard Reynolds lead citrate stain for 5 min and were examined with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV. Colloidal gold-conjugated (10 nm) tomato lectin was purchased from EY Laboratories, Inc. (San Mateo, California). Cytochemical controls inclu-

ded: (i) pre-incubation of the labeling solution with re-acetylated chitosan before labeling; and (ii) labeling of the mosquito larva cross-section.

Nucleic acids extraction

Beauveria bassiana genomic DNA was isolated from lyophilized mycelium (25 mg) that was homogenized in liquid nitrogen, and subjected to modified cetyltrimethylammonium (CTAB) DNA extraction protocol. Total RNA was isolated using TriReagent (Sigma) following the manufacturer's protocol [18]. Two total RNA pools were extracted: the *in vitro* RNA was obtained from *B. bassiana* blastospores and mycelium isolated following a 48h incubation period. *In vivo* RNA was extracted from cells harvested from infected *Manduca sexta* prior to insect death. Both RNA pools were resuspended in formamide and stored at -70°C . RNA concentration was estimated using a spectrophotometer.

PCR reactions

Aliquots of *Beauveria bassiana* genomic DNA were amplified with a mixture of *Taq* DNA polymerase (Promega) and *PFU* polymerase (Stratagene). Two sets of primers (FKS2up/low and CHSup/2low, Table 1) were designed to amplify both chitin synthase and glucan synthase genes. Amplification conditions were 30 cycles 94°C , 30s, 50°C , 30s, and 68°C , 3 min., and 30 cycles 94°C , 30s, 55°C , 30s, and 68°C , 3 min, for the *FKS* and the *CHS* homologues, respectively. Both amplified fragments were purified with the QIAEX II gel extraction kit (QIAGEN), following the manufacturer's protocol, and sequenced by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

RACE PCR

To obtain the remainder of the gene sequences, PCR amplifications of cDNA ends were performed using the *in vitro* extracted total RNA and the SMART RACE cDNA amplification kit (Clontech). The oligonucleotide used included the Clontech primers combined with either the 3' or 5' RACE *CHS* homologues, and the 3' RACE *FKS* homologue (Table 1).

Sequence analyses

The deduced *B. bassiana* CHS protein was aligned with selected fungal CHS protein sequence downloaded from GenBank. The alignment was performed using ClustalX with default parameters [19]. The BbCHS class was determined by sequence comparison, using the unweighted pair group method using arithmetic average (UPGMA), as described previously by Bowen et al. [17]. The UPGMA-based calculations were performed in PAUP* version 4.0 [20].

The *B. bassiana* FKS and RHO protein sequences were aligned with fungal homologues in ClustalX [19]. The C-terminal sequences of both proteins were found to be highly divergent and therefore were not considered in phylogenetic analyses. Amino-acid alignments that were used for phylogenetic analyses were 949- and 191-character long for FKS and RHO, respectively, and are available upon request. Relationships were reconstructed by using the Neighbor Joining distance model as implemented in PAUP* (with default parameters), and node support was assessed by bootstrap analyses (100 replicates). Additional reconstructions were performed using maximum parsimony analyses and bootstrapping (100 replicates).

Quantitative real-time RT-PCR

The cDNA templates for quantitative PCR were synthesized separately from DNase-treated total RNA from both *in vivo* and *in vitro* *B. bassiana* cell preparations [18]. First strand cDNA syntheses and RT-PCR reactions were performed using the ThermoScript RT-PCR system from Life Technologies, following the manufacturer's directions. Primers for use in quantitative PCR were designed from cDNA sequences by MIT Primer 3 program (Table 1). Quantitative PCR primers were designed to contain 45–55% GC content, possess $T_m = 60\text{--}62^{\circ}\text{C}$, and to provide products of 100–200 base-pairs (bp) within the putative ORFs and no secondary structure. Quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Five replicates were conducted for each gene. Quantitative PCR conditions were optimized for

Table 1. Primers used in the initial amplification of glucan and chitin synthase sequences from genomic DNA, as well as in the 3' and 5' RACE reactions and Real-time PCR conducted on the *in vivo* and *in vitro* cDNA preparations

<i>Degenerate primers</i>	
Glucan synthase	FKS2 up, 5'-GTTAAGGAYACBAAGATTYTGGC-3' FKS2 low, 5'-CATYTGYTWCWCATACCAG-3'
Chitin synthase	CHS up, 5'-CARATGMTTTTCTGTCTCAAGGA-3' CHS2 low, 5'-GAKGTRAACATRTGCCARGG-3'
<i>RACE primers</i>	
FKS 3' homologue	CGGTGACGTCGCTGCTGGAAAGGAA
CHS 5' homologue	TTTGCAGAGCGTGGTATCGGTAGGC
CHS 3' homologue	ACGCCAGCTGACGGACAAGACGAAC
<i>Real-time PCR primers</i>	
Beta tubulin	TubF: 5'-TCCTTCGTACGGTGACCTGA-3' TubR: 5'-CGAGCTTGCGAAGATCAGAG-3'
Glucan synthase	GluF: 5'-TTACCTCGGCACTCAGCTTC-3' GluR: 5'-AGCGTGAGCATGAATGACTG-3'
Chitin synthase II	ChitIIF: 5'-AACGTGGATGGTAGCCAACG -3' ChitIIR: 5'-AATGTGGTGGAGCCGATAGC-3'
RHO	RHOF: 5'-ACAACGTCCAGGAGAAGTGG-3' RHOR: 5'-GCACCAATCTTCTTGCGAAT-3'

where Y = C/T, B = C/G/T, W = A/T, R = A/G, M = A/C and K = G/T.

cDNA template dilution 1:25 and annealing temperature $T_m = 60$ °C. The thermal cycling conditions were 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s performed for 40 cycles. The absence of 'primer-dimers' was confirmed by examining products following agarose electrophoresis and ethidium-bromide staining. Determination of relative expression ratio for specific gene *in vivo* and *in vitro* was determined from quantitative PCR results in relation to the control gene β -tubulin using REST software [21].

Results

Light and electron microscopy

The test insects, *T. ni*, *S. exigua* and *M. sexta*, injected with *in vitro* produced blastospores displayed symptoms of mycosis within two to three days post injection. Larvae became sluggish and ceased to feed within 2–3 days, and by 3–4 days post-challenge all treated larva had succumbed to fungal disease. Examination of the hemolymph at 48 h post-challenge revealed that all three host insects contained numerous hyphal body cells and degenerate insect hemocytes (Figure 1b). The *in vivo* cells produced in the three different hosts produced identical staining patterns when probed with the different light-

and electron-microscope cytochemical probes. In all cases, the *in vivo* hyphal cells were morphologically similar to *in vitro* produced blastospores. Staining the *in vitro* and *in vivo* cells with Con-A FITC conjugate produced similar patterns of fluorescence, the general cell wall was moderately stained whereas, the apical and septal regions displayed strong fluorescence demonstrating the presence of surface mannose and/or α -D glucose residues. The *in vitro* blastospores and affiliated hyphal elements possessed peanut lectin receptors evenly distributed over their cell surfaces (Figure 1a). FITC-labeled peanut lectin also bound to discrete patches on the insect hemocytes. As reported previously [8], *in vivo* cells lacked the peanut lectin binding sites on their surface (Figure 1b–c). Control staining reactions, amending probes with either 250 mM mannose or D-galactose, suppressed fluorescent labeling of these cells. Staining *in vivo* and *in vitro* cells with aniline blue probe produced intense fluorescence over the majority of the cell surfaces (Figure 1d–f). Positive labeling demonstrated that *in vivo* and *in vitro* cells contain 1,3- β -glucan residues on their cell wall surface and in septal regions. However, the apical bud regions of both cell phenotypes lacked 1,3- β -glucan surface residues.

Electron microscopy of *in vitro* cells revealed the presence of hyphal filaments with a well-

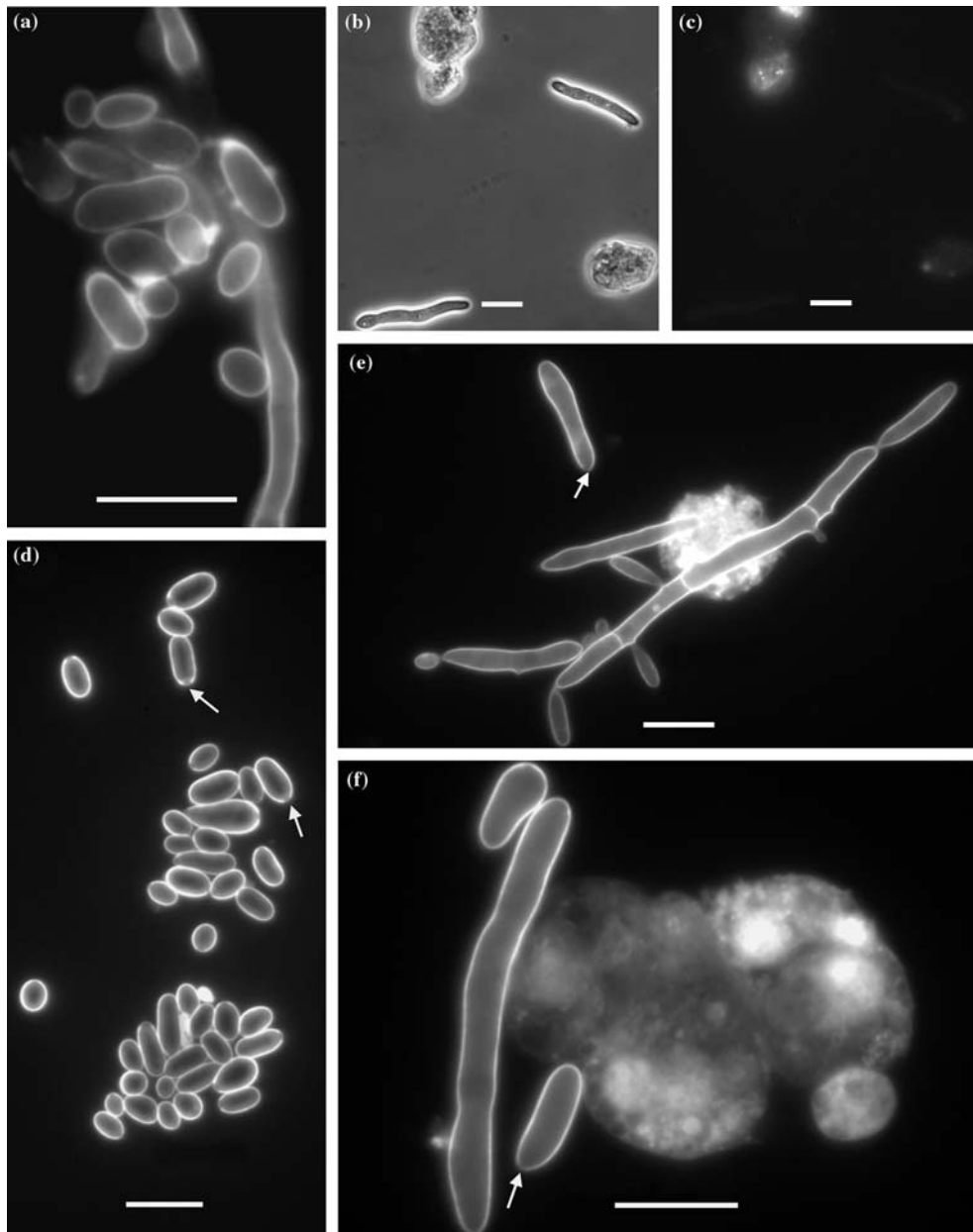


Figure 1. Light micrographs of *in vitro* and *in vivo* *B. bassiana* cells stained with either peanut lectin (a–c) or aniline blue (d–f). Different cell phenotypes of the *in vitro* cells all possessed external galactose residues as reflected by the binding of the peanut lectin in Figure 1a. The *in vivo* cells observable under phase optics (Figure 1b), did not display any peanut lectin binding sites (Figure 1c). However the hemocytes harvested with the *in vivo* cells did contain localized regions that bound to the peanut lectin (Figure 1c). Both *in vitro* (Figure 1d) and *in vivo* cells (Figure 1e–f) readily bound the aniline blue stain demonstrating the presence of 1,3- β -glucan residues on their surfaces. In both cases the bud zone appeared to lack these surface residues (see arrows Figure 1d–f). Scale bar equals 10 micrometers.

defined cell wall measuring 100–160 nm in thickness. As shown in Figure 2a–c, the *in vitro* cells anti-laminarin monoclonal antibody localized 1,3- β -glucan in the cell wall exclusively. Gold

particles were detected throughout the outer and septal wall regions. The distribution of the gold particles in the wall was even, with a very low level of nonspecific labeling. Probing *in vitro* cells with

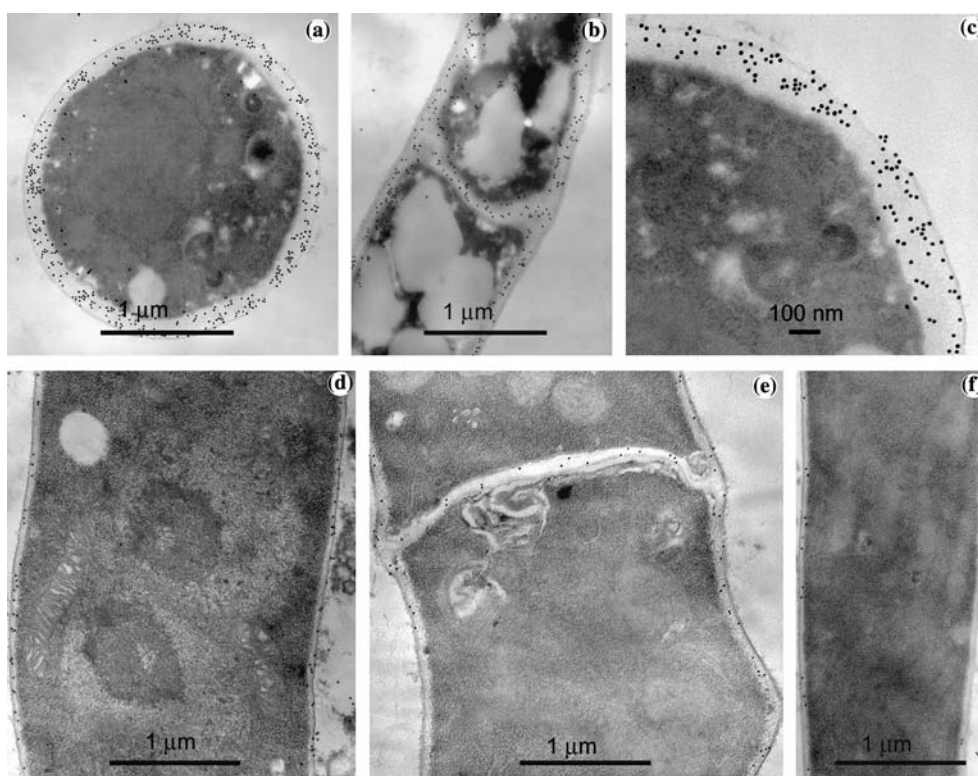


Figure 2. Transmission electron micrographs of *Beauveria bassiana* showing the localization of 1,3- β -glucan using a monoclonal antibody raised in mouse against a laminarin-haemocyanin conjugate. (a–c) *in vitro* cell; (d) extracted from *M. sexta*; (e) extracted from *S. exigua*; (f) extracted from *T. ni*.

the gold-conjugated tomato lectin resulted also in the specific labeling of the cell wall regions (Figure 3a–c). However, the tomato lectin did not produce the uniform intense label observed with the anti-1,3- β -glucan IgG probe. Examination of thin sections of fungal cells extracted from infected *M. sexta*, *T. ni*, and from *S. exigua* (Figures 2d–f, 3d–f) demonstrated the presence of hyphal filaments enclosed by a 50–60 nm cell wall, which was significantly thinner than that found in *in vitro* cells (Figure 2a–c; 3a–c). Cytochemical analysis of the *in vivo* thin-walled cells demonstrated the presence of both 1,3- β -glucan and chitin. Binding sites for the anti-glucan antibody were detected throughout the thin cell wall and the thicker septal wall of the *in vivo* cells extracted from the three insect hosts. Very few gold particles were observed outside of the cell wall. The tomato lectin gold conjugate sparsely labeled the cell wall region. Gold particles did bind well to the septal regions and to sites associated with the membranes and vesicles affiliated with the cell walls. All cyto-

chemical controls supported the specificity of the labeling for 1,3- β -glucan and chitin.

Sequencing and sequence analyses of Beauveria bassiana CHS, FKS and RHO homologues

Fragments homologous to both chitin synthase (*CHS*) and glucan synthase (*FKS*) genes were successfully amplified from *B. bassiana* genomic DNA using the degenerate primer pairs. RACE PCR reactions subsequently produced a 3119 bp sequence for *BbCHS*, which included the entire coding sequence, and a 3096 bp sequence for *BbFKS*, which corresponded to the 3' end of the gene, as 5' end reactions failed to produce amplification. Both *BbCHS* and *BbFKS* sequences are publicly available in GenBank, with the accession numbers AY743592 and AY743593, respectively. The gene coding for the regulatory subunit of the glucan synthase (*BbRHO*) has been sequenced previously during our pilot scale EST analysis of

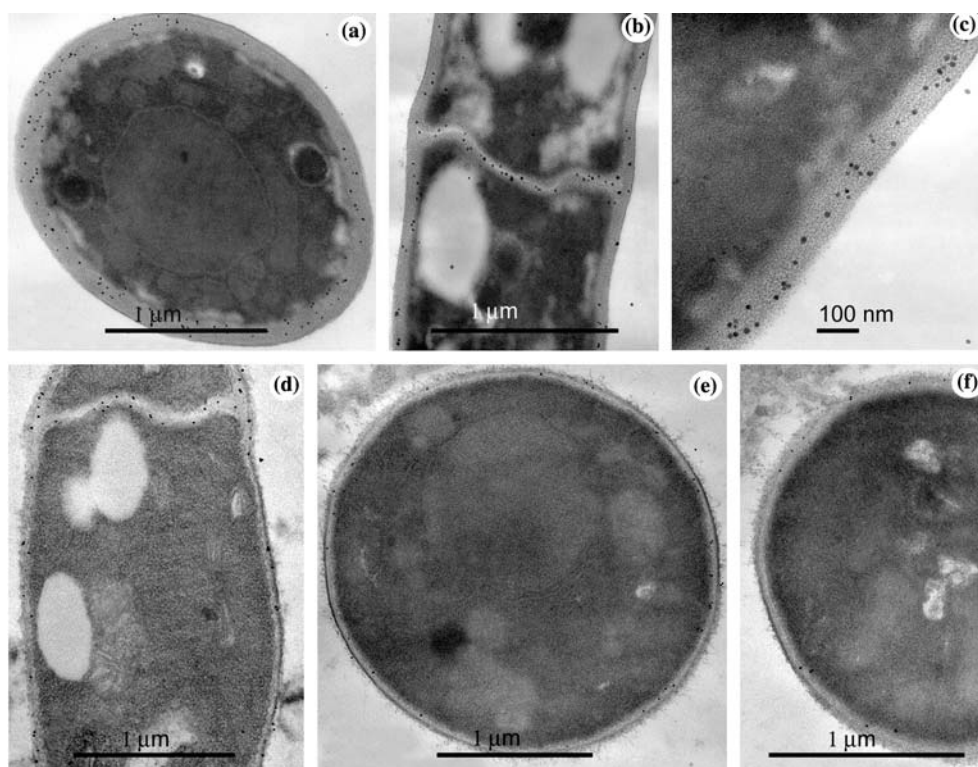


Figure 3. Transmission electron micrographs of *Beauveria bassiana* showing the localization of chitin using a tomato lectin-gold conjugate. (a–c) *in vitro* cell; (d) extracted from *M. sexta*; (e) extracted from *S. exigua*; (f) extracted from *T. ni*.

B. bassiana [18], and is also available in GenBank with the accession number CF350282.

The BbCHS-deduced amino acid sequence is 362 AA long and has been used in a UPGMA analysis in order to determine the class of the chitin synthase encoded by *BbCHS* [17]. UPGMA dendrograms usually cluster in the appropriate manner but should not be used as phylogenetic trees. The inferred dendrogram is represented in Figure 4, and revealed that the *BbCHS* gene likely encodes a class II chitin synthase. This result is consistent with BLAST analysis [22] of the protein sequence that showed that class II chitin synthases produce the best homology scores.

The BbFKS and BbRHO proteins were compared to homologous sequences from various filamentous fungi. The partial BbFKS sequence was 362 amino acids long, and exhibited 90% and 88% identity with the homologous amino acid fragment of the *P. brasiliensis* and *A. nidulans* FKS protein, respectively. The BbRHO protein is 240 AA long. The two protein sequences were also used in phylogenetic analyses (Figure 5). Phylogenetic

trees include fungal species for which both genes encoding the glucan complex (FKS and RHO) have been sequenced. They are rooted with *Cryptococcus neoformans* and exhibit a very similar pattern (Figure 5). Both trees depict *B. bassiana* at the same overall position within the Ascomycetes.

The three cloned *B. bassiana* genes are similar to homologous genes previously sequenced from other fungal species. This confirms that the molecular determinants responsible for cell wall biosynthesis and plasticity are well conserved in fungi, and suggests that *B. bassiana* may prove to be an excellent model for fungal cell wall research in relation to both dimorphism and host-pathogen interactions.

Real time-PCR

The gene expression levels for *BbCHS*, *BbFKS*, and *BbRHO* in the *in vitro* and *in vivo* cells were determined using quantitative PCR. Randomization test results normalized by the reference gene (β -tubulin) expression demonstrated that the

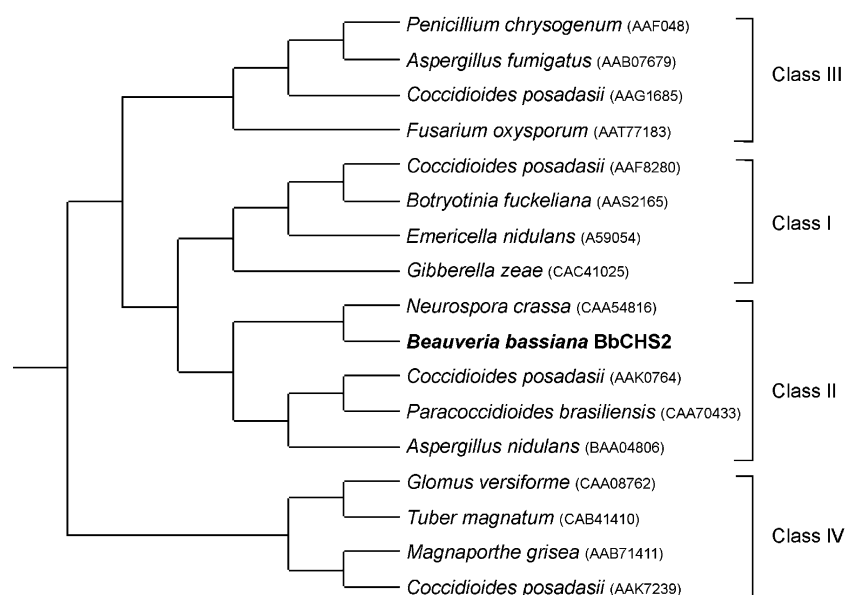


Figure 4. Unweighted pair group method using arithmetic averages (UPGMA) dendrogram showing the four distinct chitin synthase classes. The tree was calculated from amino acid sequence alignment. It shows that the amplified fragment *BbCHS* is homologous to class II chitin synthase genes.

target genes *BbCHS* and *BbFKS* in the *in vivo* cell preparation were down-regulated by factors of 3.002 and 2.882 respectively in comparison to the *in vitro* group (Figure 6). Pairwise fixed reallocation randomization analysis demonstrated that the rates of down-regulation were significantly different (P-values of 0.008 and 0.001, obtained for *BbCHS* and *BbFKS*, respectively, are lower than the 0.05 cut-off value. See Figure 6). The relative transcript levels of the *BbRHO* gene in the *in vivo* cells were did not differ significantly (P-value 0.117) from that calculated for *in vitro* cells: the calculated expression ratio was 0.8.

Discussion

In the case of *Beauveria bassiana*, understanding the regulation of the cell wall synthesis may provide insight into the pathogenic process of this entomopathogenic fungus. Indeed, whereas *B. bassiana* cells exhibit a regular cell wall when grown *in vitro*, fungal cells that are found freely circulating in the host hemolymph possess a modified wall [8]. The cell surface properties of *in vivo* cells examined herein have similar properties to those reported by Pendland et al. [8]. In both preparations, the *in vivo* cell wall is significantly

thinner than the *in vitro* cell wall. Secondly the *in vivo* cells lack detectable galactose residues, epitopes that are used in non-self recognition by the circulating insect opsonin [3, 7, 8]. This difference in both the cell wall thickness and carbohydrate composition is believed to lead to the decrease of possible surface epitopes recognized by the host defense system as non-self, allowing fungal cells to escape defense reactions. However, our study demonstrated that the *B. bassiana in vivo* cells maintain cell shape via the presence of glucan and chitin, and that cell walls, albeit thinner, still contain both major constituents at a detectable level (Figure 1–3).

In addition to these microscopic observations, we initiated investigations on the molecular regulation of the fungal cell wall *in vivo*. Several genes responsible for the presence of chitin and glucan in the cell wall were cloned and sequenced. These genes included *BbCHS*, which encodes for a class II chitin synthase (Figure 4) and the glucan synthase complex of *BbFKS* and *BbRHO* (Figure 5, [23]). Sequence analyses showed that the three *B. bassiana* genes were homologous to other genes obtained from ascomycetes, providing us with a basis to compare the molecular regulation of cell wall in several fungal species. Importantly, differential expression analyses performed by

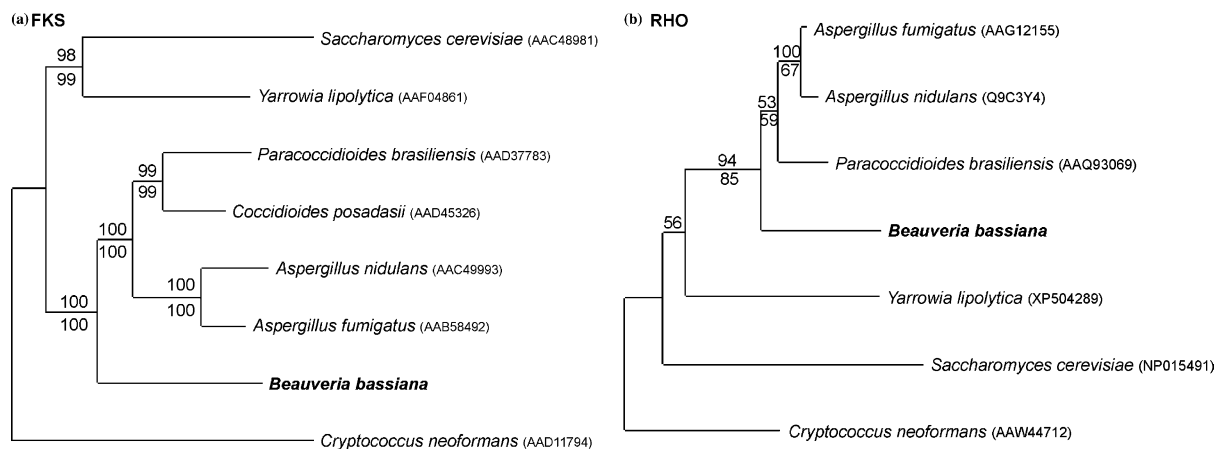


Figure 5. Neighbor Joining trees inferred from (a) FKS and (b) RHO protein sequence alignments. Numbers above and below the nodes represent the results of bootstrap analyses (100 replicates), using Neighbor Joining and Maximum Parsimony method, respectively. Both trees are rooted with the Basidiomycete *Cryptococcus neoformans*, and show that the *Beauveria bassiana* cloned genes are closely related to homologous sequences obtained from Ascomycetes.

quantitative PCR (Figure 6) were remarkably concordant with our microscopy observations. First, all three transcripts were detected in *in vivo* *B. bassiana* cells, suggesting that both glucan and chitin are present in the cell wall of these cells. Second, the expression of both *BbFKS* and *BbCHS* was shown to be 3 times weaker *in vivo*, and this differential expression may explain, in part, the difference observed in cell wall thickness.

Our study is a first step toward understanding the molecular mechanisms that control the synthesis and regulation of cell wall during the *B. bassiana* infection. It reveals that *B. bassiana* *in vivo*-produced cells do not form protoplasts as previously suspected [8], but retain thin, modified cell walls. These unique modifications are probably essential for pathogenicity because they function to maintain cell integrity but limit the amount of epitopes that may trigger the host defense system. To our knowledge, such a strategy has never been reported for any other pathogenic fungus. Furthermore, it differs from the total absence of cell wall and the formation of *in vivo* protoplasts known for species of *Entomophaga* [5]. In these entomophthorean entomopathogens, glucan synthases were shown to be inhibited by unsaturated fatty acids, at the post-translational level [6]. In the case of *B. bassiana*, although post-translational regulations cannot be ruled out, our analysis demonstrated that *in vivo* cell wall adjustments involve transcriptional regulations, as both *BbFKS* and *BbCHS* genes are

down-regulated *in vivo*. In addition, the similarity in expression patterns (3-fold decrease) suggests that both genes may be controlled by the same regulatory motif. This motif may resemble the negative regulatory element that has recently been reported for the class III chitin synthase in the dimorphic fungus *Wangiella dermatitis* [24].

Although our analysis shows that *BbCHS* is transcriptionally regulated, the molecular control of chitin content in *B. bassiana* cell wall is likely to be more complicated and to involve a larger number of genes. The characterization of a class II *CHS* gene in *B. bassiana* suggests that this fungus may also possess genes that encode for other types of chitin synthases (class I, III and maybe IV), as this is the case for most fungus [17], including *Metarhizium anisopliae*, another entomopathogen [25]. Previous studies [24, 26, 27] indicate that chitin synthase genes are differentially expressed in response to environmental conditions, or during defined stages of the life cycle. However, attempts to correlate this differential expression with phenotypic observations failed to produce a clear and uniform picture, thus suggesting that post-transcriptional regulations play an important role during cell wall morphogenesis. Cloning and sequencing of all chitin synthase genes from *B. bassiana* will refine our understanding of *in vivo* cell wall regulation.

In contrast to chitin synthases, glucan synthases are encoded by a single copy of the *FKS* gene in various fungi [14, 15, 28]. Therefore, glucan

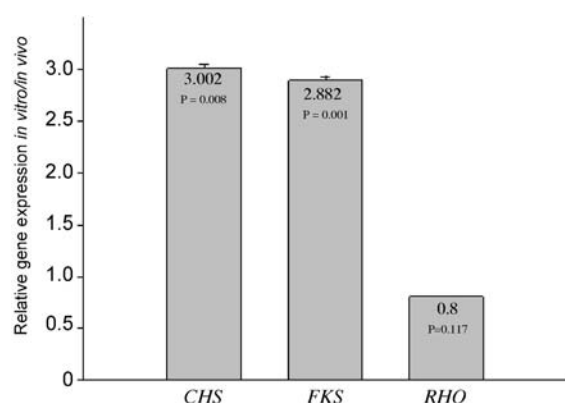


Figure 6. Ratios of the relative expression levels of *BbCHS*, *BbFKS*, and *BbRHO* gene transcripts in *in vitro* versus *in vivo* cell preparations. Relative expression levels for each gene ($n = 5$) was calculated using the Pair-wise Fixed Reallocation Randomization test normalized to the control gene β -tubulin. P values lower than 0.05 indicate significant differences between *in vivo* versus *in vitro* transcript levels.

synthesis may be controlled by a more straightforward regulation mechanism. To date, these mechanisms have only been studied in the model organism *Saccharomyces cerevisiae* [29, 30]. In fact, our study represents the first investigation of differential expression of glucan synthase genes (*FKS* and *RHO*) in a filamentous fungus. In that regard, it is interesting to note that molecular control of glucan synthesis in *B. bassiana* involves transcriptional regulation of the glucan synthase catalytic subunit (*FKS*), and not the regulatory subunit (*RHO*). This is concordant with various studies that demonstrated that *RHO* is required for several essential functions, including cell signaling pathways [30].

This study demonstrates that the *B. bassiana* cell wall is modified both quantitatively and qualitatively *in vivo*, and that these phenotypic changes are the reflect of the differential gene expression of cell-wall related genes. The understanding of the molecular mechanisms responsible for cell wall adjustments will be improved by similar expression studies involving a larger sample of genes. For example, the genes and regulation mechanisms responsible for the loss of terminal galactose residues in *in vivo* cells ([8]; this study) have yet to be characterized. On a broader level, this study echoes our previous EST analysis of the *B. bassiana in vivo* transcriptome [18]. It confirms our hypothesis that the *in vivo* gene expression is highly specific to the growth conditions provided by the host hemolymph.

Whereas some genes are up-regulated during the infection [18], others, such as *BbFKS* and *BbCHS*, and potentially other genes involved in cell wall synthesis are down-regulated in response to the host conditions, thereby facilitating the infection and the evasion of the host defense system. It is interesting to note that an association between morphogenesis and virulence has long been presumed for dimorphic fungi, and pathways that co-regulate pathogenesis and dimorphism have also been detailed in the human pathogen *Candida albicans* [31]. Similar pathways may be revealed by further studies on regulation mechanisms underlying the formation of the pathogenic, *in vivo* phenotype in *B. bassiana*.

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References

1. Boucias DG, Latgé JP. Invertebrate fungal elicitors. In: Drouhet E, Cole GT, DeRepentigny L, Latgé JP, Dupont B, eds. Fungal Antigen Isolation, Purification, and Detection, Plenum PressNY, 1988: 121–137.
2. Gillespie JP, Bailey AM, Cobb B, Vilcinskas A. Fungi as elicitors of insect immune responses. Arch Insect Biochem Physiol 2000; 44: 49–68.
3. Pendland JC, Boucias DG. Phagocytosis of lectin-opsonized fungal cells and endocytosis of the ligand by insect *Spodoptera exigua* granular hemocytes: An ultrastructural and immunocytochemical study. Cell Tissue Res 1996; 285: 57–67.
4. Beauvais A, Latgé JP. Chitin and (1–3) glucan synthases in the protoplasmic entomophthorales. Arch Microbiol 1989; 152: 229–236.
5. Beauvais A, Latgé JP, Vey A, Prevost MC. The role of surface components of the entomopathogenic fungus *Entomophaga aulicae* in the cellular immune response of *Galleria mellonella* (Lepidoptera). J Gen Microbiol 1989; 135: 489–498.
6. Mackichan J, Thomsen L, Kerwin J, Latgé JP, Beauvais A. Unsaturated fatty acids are the active molecules of a glucan-

- synthase-inhibitory fraction isolated from entomophthorean protoplasts. *Microbiology* 1995; 141: 2757–62.
7. Pendland JC, Heath MA, Boucias DG. Function of a galactose-binding lectin from *Spodoptera exigua* larval hemolymph: opsonization of blastospores from entomogenous hyphomycetes. *J Insect Physiol* 1988; 34: 533–540.
 8. Pendland JC, Hung SY, Boucias DG. Evasion of host defense by *in vivo* produced protoplast like cells of the insect mycopathogen *Beauveria bassiana*. *J Bacteriol* 1993; 175: 5962–5969.
 9. Farkas V. Fungal cell walls: their structure, biosynthesis and biotechnological aspects. *Acta Biotechnol* 1990; 10: 225–238.
 10. Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, Yamada-Okabe T, Arisawa M, Watanabe T, Yamada-Okabe H. Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae* *GSC1/FKS1* and its involvement in β -1,3-glucan synthesis. *J Bacteriol* 1997; 179: 4096–4105.
 11. Kang MS, Cabib E. Regulation of fungal cell wall growth: a guanine nucleotide-binding, proteinaceous component required for activity of 1,3- β -D-glucan synthase. *Proc Natl Acad Sci USA* 1986; 83: 5808–5812.
 12. Mazur P, Baginsky W. In vitro activity of 1,3- β -D-glucan synthase requires the GTP-binding protein Rho1. *J. Biol. Chem.* 1996; 271: 14604–14609.
 13. Douglas CM, Foor F, Marrinan JA, Morin N, Nielsen JB, Dahl AM, Mazur P, Baginsky W, Li W, El-Sherbeini M, Clemas JA, Mandala SM, Frommer BR, Kurtz MB. The *Saccharomyces cerevisiae* *FKS1* (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3- β -D-glucan synthase. *Proc Natl Acad Sci USA* 1994; 91: 12907–12911.
 14. Kelly R, Register E, Hsu M, Kurtz M, Nielsen J. Isolation of a gene involved in 1,3- β -glucan synthesis in *Aspergillus nidulans* and purification of the corresponding protein. *J Bacteriol* 1996; 178: 4381–4391.
 15. Thompson JR, Douglas CM, Li W, Jue CK, Pramanik B, Yuan X, Rude TH, Toffaletti DL, Perfect JR, Kurtz M. A glucan synthase *FKS1* homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. *J Bacteriol* 1999; 181: 444–453.
 16. Pereira M, Felipe MSS, Brigido MM, Soares CMA, Azevedo MO. Molecular cloning and characterization of a glucan synthase gene from the human pathogenic fungus *Paracoccidioides brasiliensis*. *Yeast* 2000; 16: 451–462.
 17. Bowen AR, Chen-Wu JL, Momany M, Young R, Szanislo PJ, Robbins PW. Classification of fungal chitin synthases. *Proc Natl Acad Sci USA* 1992; 89: 519–523.
 18. Tartar A, Boucias DG. A pilot-scale expressed sequence tag analysis of *Beauveria bassiana* gene expression reveals a tripeptidyl peptidase that is differentially expressed *in vivo*. *Mycopathologia* 2004; 158: 201–209.
 19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acid Res* 1997; 24: 4876–4882.
 20. Swofford DL. PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0. Sunderland MA: Sinauer Associates, 2000.
 21. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (rest®) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nuc Acids Res* 2002; 30: e36.
 22. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nuc Acids Res* 1997; 25: 3389–3402.
 23. Beauvais A, Bruneau JM, Mol PC, Buitrago MJ, Legrand R, Latgé JP. Glucan synthase complex of *Aspergillus fumigatus*. *J Bacteriol* 2001; 183: 2273–2279.
 24. Wang Z, Szanislo PJ. *WdCHS3*, a gene that encodes a class III chitin synthase in *Wangiella (Exophiala) dermatitis*, is expressed differentially under stress conditions. *J Bacteriol* 2000; 182: 874–881.
 25. Nam JS, Lee DH, Lee KH, Park HM, Bae KS. Cloning and phylogenetic analysis of chitin synthase genes from the insect pathogenic fungus, *Metarhizium anisopliae* var *anisopliae*. *FEMS Microbiol Lett* 1998; 159: 77–84.
 26. Nino-Vega GA, Munro CA, San-Blas G, Gooday GW, Gow NAR. Differential expression of chitin synthase genes during temperature-induced dimorphic transitions in *Paracoccidioides brasiliensis*. *Med Mycol* 2000; 38: 31–39.
 27. Lee JI, Choi JH, Park BC, Park YH, Lee MY, Park HM, Maeng PJ. Differential expression of the chitin synthase genes of *Aspergillus nidulans*, *chsA*, *chsB*, and *chsC*, in response to developmental status and environmental factors. *Fungal Genet Biol* 2004; 41: 635–646.
 28. Kellner EM, Orsborn KI, Siegel EM, Mandel MA, Orbach MJ, Galgiani JN. *Coccidioides posadasii* contains a single 1,3- β -glucan synthase gene that appears to be essential for growth. *Eukaryot Cell* 2005; 4: 111–120.
 29. Mazur P, Morin N, Baginsky W, El-Sherbeini M, Clemas JA, Nielsen JB, Foor F. Differential expression and function of two homologous subunits of yeast 1,3- β -D-glucan synthase. *Mol Cell Biol* 1995; 15: 5671–5681.
 30. Zhao C, Jung US, Garrett-Engle P, Roe T, Cyert MS, Levin DE. Temperature-induced expression of yeast *FKS2* is under the dual control of protein kinase C and calcineurin. *Mol Cell Biol* 1998; 18: 1013–1022.
 31. Rooney PJ, Klein BS. Linking fungal morphogenesis with virulence. *Cell Microbiol* 2002; 4: 127–137.

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