# Distinct chitinases are expressed during various growth phases of the human pathogen *Paracoccidioides brasiliensis*

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*The aim of this work was the partial purification and subsequent evaluation of chitinase expression during the various growth phases of* Paracoccidioides brasiliensis. *Initially,* Pb*CTS1r was expressed as a recombinant protein and displayed enzymatic activity against 4-MU-[N-acetylglucosamine (GlcNAc)]$_3$ and 4-MU-(GlcNAc)$_2$. Two proteins, 45 kDa and 39 kDa in size, were partially purified from* P. brasiliensis *yeast crude extract using cation-exchange chromatography coupled with HPLC and were characterised as* Pb*CTS1 and* Pb*CTS2, respectively. Anti-*Pb*CTS1r antibody recognised two proteins in the crude extracts of yeast and the transitional stage between mycelial and yeast phases. In crude extracts of mycelium, only the 45 kDa protein was detected. However, quantitative real-time polymerase chain reaction led to the detection of small quantities of* Pbcts2 *transcript in the mycelial phase. In the yeast cell wall extract, only the 39 kDa protein was detected. Moreover, both proteins were secreted by the yeast parasitic phase, suggesting that these proteins participate in the modulation of the fungal environment. Phylogenetic analysis of the predicted* Pb*CTS1 and* Pb*CTS2 proteins indicated that they code for distinct chitinases in* P. brasiliensis. *During evolution,* P. brasiliensis *could have acquired the paralogues* Pbcts1 *and* Pbcts2 *for growth and survival in diverse environments in both saprophytic and parasitic phases.*

Key words: *Paracoccidioides* - chitinase - cell wall - chromatography

*Paracoccidioides brasiliensis* is a dimorphic fungus pathogenic to humans, growing as yeast during infection and as mycelium in the environment. The cell wall of *P. brasiliensis* consists of chitin and glucan and is involved in pathogenesis (de Agostino Biella et al. 2006). The percentage of chitin in *P. brasiliensis* ranges from 37-48% in the yeast phase and from 7-18% in the mycelial phase, indicating the importance of this carbohydrate and the enzymes involved in cell wall metabolism in dimorphism and the fungal infection process (Kanetsuna et al. 1969).

Chitin is a polymer of β-1.4-linked N-acetylglucosamine (GlcNAc) and is the major structural component of crustacean exoskeletons, insect cuticles, diatoms and fungal cell walls, but it is not present in humans (Barrett 2002). Due to its absence in humans, chitinases have been examined as interesting targets in the exploratory design of specific antifungal agents (Rush et al. 2010).

Chitinases (EC 3.2.1.14) hydrolyse chitin and can be classified into two families of glycoside hydrolases, families 18 and 19, based on amino acid sequence, structural homology and mechanism of action (Henrissat & Bairoch 1996). Furthermore, chitinolytic enzymes can be divided into two major categories based on their mode of action: endochitinases and exochitinases. Endochitinases (EC 3.2.1.14) cleave chitin and chito-oligomers and release multimers of GlcNAc. Exochitinases can be divided into two subcategories: chitin 1.4-β-chitobiosidases and β-N-acetylhexosaminidases. Chitin 1.4-β-chitobiosidases are not officially recognised with an EC number. They cleave chitin and chito-oligomers and release only (GlcNAc)$_2$. β-N-acetylhexosaminidases (EC 3.2.1.52) release only GlcNAc monomers (Lorito 1998).

Fungal chitinases are important for nutrition, developmental processes, morphogenesis and pathogenesis (Dahiya et al. 2006, Binod et al. 2007). Many fungal chitinases have been purified and characterised and most fungi express more than one chitinolytic enzyme (Seidl et al. 2005, Duo-Chuan 2006).

In this work, our aim was to partially purify chitinases from *P. brasiliensis* and to evaluate their expression during different growth phases, including mycelium, yeast and the transition from mycelium to yeast, in addition to the cell wall of yeast and the supernatant of the yeast culture medium.

## MATERIALS AND METHODS

*Fungus and growth conditions* - *P. brasiliensis*, *Pb*01 isolate (ATCC-MYA-826), was cultivated in modified Sabouraud dextrose solid medium (4% glucose, 1% peptone, 0.5% yeast extract, 0.1% brain-heart infusion, 1.2% agar) at 22ºC for 15 days for the mycelial phase and at 36ºC for seven days for the yeast phase. For the transition from mycelium to yeast, the fungus growing at 22ºC

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was transferred to liquid medium at 36ºC and grown for 24 h under shaking at 150 rpm.

*Recombinant DNA preparation and transformation* - The *Pbcts1* cDNA (accession AQ75798) (Bonfim et al. 2006) was amplified by polymerase chain reaction (PCR) using forward (5'-CAGATCCACCAGAATTCATGACG-3') and reverse (5'-CACCTCGAGCTACTCCCCAGG-3') primers containing *Eco*RI and *Xho*I sites (underlined), respectively, for cloning into the expression vector pET-32a(+) (Novagen). PCR conditions were as follows: 94ºC (3 min), 25 cycles at 94ºC (1 min and 30 s), 58ºC (2 min) and 72ºC (1 min and 30 s) and a final elongation at 72ºC (10 min). The PCR product was digested with *Eco*RI and *Xho*I and cloned into the respective sites of pET-32a(+). The resulting plasmid was used to transform *Escherichia coli* BL21(DE3)/pLysS (Certificate of Quality in Biosafety 0037-97).

*Production and purification of the recombinant protein* - The transformed *E. coli* cells were grown at 37ºC in LB-medium with 34 µg $mL^{-1}$ chloramphenicol, 100 µg $mL^{-1}$ ampicillin and 200 mM glucose at 200 rpm until the $A_{600}$ reached 0.6-0.8. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The cells were incubated for 3 h at 37ºC with shaking at 200 rpm, followed by centrifugation at 10,000 *g* for 5 min at 4ºC. The cell pellet containing insoluble inclusion bodies was suspended in phosphate buffered saline (PBS), incubated for 1 h with lysozyme (1 mg $mL^{-1}$) and sonicated, and 1% sarkosyl was added. The sample was centrifuged at 10,000 *g* for 20 min at 4ºC, following which 2% Triton X-100 was added to the supernatant. The recombinant chitinase (*Pb*CTS1r) was purified using Ni-NTA resin (Invitrogen). Briefly, the supernatant was applied on a Ni-NTA resin column and *Pb*CTS1r was recovered using elution buffer [250 mM imidazole, 0.5 M sodium chloride (NaCl), 50 mM sodium phosphate, pH 8.0]. For later assays, the affinity-purified fusion protein was pooled, dialysed in sodium acetate buffer at pH 6.0 and hydrolysed with enterokinase.

*Antibody production* - *Pb*CTS1r was used to produce anti-*Pb*CTS1r antibody in New Zealand rabbits. The immunisation consisted of three injections of 200 µg of *Pb*CTS1r, with an interval of two weeks between injections. The serum containing anti-*Pb*CTS1r antibody was collected and ELISA and Western blotting were used to test antibody reactivity. The protocol concerning animal immunisation was approved by the local Ethical Committee on Animal Care of Federal University of Minas Gerais (protocol CETEA-UFMG 043/2008).

*Protein extraction* - Protein extracts from mycelium, yeast cells, the transition from mycelium to yeast and the cell wall of the yeast were obtained by disruption of frozen *P. brasiliensis* cells. The cells were resuspended in Tris-Ca buffer [2 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.8; protease inhibitor (GE-Healthcare)], harvested and shaken for 20 min at 4ºC with glass beads. Complete cell disruption was verified by the absence of fungal growth on Sabouraud medium. Lysed cells were separated into two fractions, the cell wall (pellet) and a crude protein extract (supernatant), using centrifugation at 5,000 *g* and 4ºC for 15 min. The pellet was washed five times with distilled water, rinsed with varying concentrations of NaCl (85 mM; 34 mM; 17 mM) and boiled in sodium dodecyl sulfate (SDS)-extraction buffer (50 mM Tris-HCl, pH 8.0, 2% SDS, 100 mM EDTA, 10 mM DTT) for 10 min. This treatment was repeated twice to ensure complete liberation of the SDS-solubilised proteins. Finally, the pellet was washed six times with water, resuspended in Tris-Ca buffer and stored as an extract composed of cell wall proteins (Pitarch et al. 2002).

Yeast-secreted proteins were obtained by filtration (0.22 µm filter) of Sabouraud dextrose medium containing *P. brasiliensis* yeast grown for 24 h. The protein extract was dialysed against distilled water and precipitated with 20% trichloroacetic acid and acetone.

*Purification of native chitinases from P. brasiliensis* - The soluble protein crude extract of yeast cells was consecutively dialysed against distilled water and 50 mM sodium acetate buffer, pH 5.4 (buffer A), filtered through a 0.22 µm filter and applied to a CM Sepharose™ Fast Flow column pre-equilibrated with buffer A (HPLC Äkta purifier GE-Healthcare). The proteins were eluted with a linear gradient of buffer A containing 0-1 M NaCl with a flow rate of 1 mL $min^{-1}$. Fractions of 1 mL and 0.5 mL fractions were collected before and after the application of the NaCl gradient, respectively. The fractions were dialysed against distilled water and their chitinase activity was determined.

*Electrophoresis and protein quantification* - SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions, as described by Laemmli (1970). Proteins were separated by 10% SDS-PAGE followed by staining with Coomassie Brilliant Blue. The protein concentration was measured according to the Bradford method (1976) using bovine serum albumin as a standard.

*Western blot analysis* - Western blot analysis was performed as described by da Silva Neto et al. (2009). The *P. brasiliensis* chitinases were detected with the anti-*Pb*CTS1r antibody (diluted 1:500 in PBS). The secondary antibody was anti-rabbit immunoglobulin G coupled to alkaline phosphatase. The reactions were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (BCIP/NBT). Negative controls were obtained with rabbit preimmune serum.

*Enzymatic activity assay* - The chitinase activity was determined as described by Selvaggini et al. (2004) with some modifications, using 4-methylumbelliferyl-N-N'-diacetylchitobiose [trimer; 4-MU-$(GlcNAc)_2$] or 4- methylumbelliferyl-N-N'-N''-triacetylchitotriose [tetramer; 4-MU-$(GlcNAc)_3$] (Sigma-Aldrich) as the substrate. These substrates were employed based on their ability to detect and distinguish exochitinases (chitobiosidases) and endochitinases. Exochitinases release a fluorescent product from only the trimeric substrates and endochitinases can be identified by digestion from the tetramer (Tronsmo & Harman 1993, Duo-Chuan 2006).

In a standard assay, 20 µL of diluted enzyme solution was incubated with 5 µL of 0.4 mM substrate solution and 80 µL of 100 mM citrate-phosphate buffer, pH 5.0, at 37ºC for 30 min. The reaction was terminated with 120 µL glycine/NaOH buffer, pH 10.6. After 5 min of incubation, a reading was taken at 355 nm excitation and 460 nm emissions in a spectrofluorometer (SpectraMax M2$^e$, Molecular Devices). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 pmol of 4-MU $mL^{-1}$ $min^{-1}$.

*Quantitative real-time PCR (qRT-PCR) analysis* - Total RNAs from *P. brasiliensis* (isolate *Pb*01) mycelium, the transition from mycelium to yeast and the yeast cells were extracted with Trizol (Invitrogen). Single-stranded cDNAs were constructed using a reverse transcription system (Promega). As a control for genomic contamination, the reactions were performed in the absence or presence of reverse transcriptase. qRT-PCR was performed in duplicate with samples from two independent experiments using the StepOnePlus™ Real-Time PCR system (Applied Biosystems). The PCR thermal cycling program was 40 cycles at 95ºC (15 s) and at 60ºC (1 min). The reaction mixture consisted of SYBR green PCR master mix (Applied Biosystems), 10 pmol of each primer and the template cDNA (40 ng) in a final volume of 25 µL. A melting curve analysis was performed to confirm the amplification of a single PCR product. The data were normalised using tubulin cDNA for each set of qRT-PCR experiments. A non-template control was included. A relative standard curve was generated by pooling an aliquot of cDNA from each sample. The cDNA standard was serially diluted 1:5 and a standard curve was generated using four samples from the pooled cDNA. The relative expression levels of genes of interest were calculated using the standard curve method for relative quantification (Bookout et al. 2006). The qRT-PCR primers for each gene were as follows: CTS1 ss 5'CTACTTGTCCGATACTTGGTCC3', CTS1 as 5'GGAGAGGAGCACATTCAGATTC3'; CTS2 ss 5'GTCCCAAAACGCCAGAATG3', CTS2 as 5' TGAGAACGCAACCGATTGAC3'; tubulin: Tub ss 5'ACAGTGCTTGGGAACTATACC3', Tub as 5'GGGACATATTTGCCACTGCC3'.

*Comparison of the sequences and inferred phylogeny* - The predicted proteins from the *Pb*CTS1 and 39 kDa chitinase (*Pb*CTS2) gene sequences, obtained from a search in the *P. brasiliensis* isolate *Pb*01 genome database (BROAD Institute, MIT and Harvard, Cambridge, MA; broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis.1/MultiHome.html), were aligned with chitinases from the National Center for Biotechnology Information (NCBI) server (ncbi.nlm.nih.gov) (Altschul et al. 1990). The phylogenetic analysis of the identified chitinases was generated using sequences from 23 complete ascomycete chitinases. A chitinase from *Cryptococcus neoformans*, a basidiomycete, was used as an outgroup. A phylogenetic tree was constructed by multiple sequence alignments using CLUSTALX (Thompson et al. 1997), generated by the neighbour-joining method (Saitou & Nei 1987) and visualised using TreeView software (taxonomy.zoology.gla.ac.uk/rod/treeview.html). The robustness of the branches was estimated using 100 bootstrap replicates and indicates the percentage of times that all species appeared as a monophyletic cluster. The alignments of the sequences are shown in Supplementary data.

## RESULTS

*Expression and purification of PbCTS1r protein* - To express the recombinant protein, the cDNA coding for *Pb*CTS1 was subcloned into the expression vector pET-32a(+). After induction with IPTG, a 58 kDa recombinant protein was detected in bacterial lysates (Fig. 1A, Lane 2). The fusion protein was purified from bacterial lysates using Ni-NTA and was cleaved with enterokinase (Fig. 1A, Lane 3) to remove the His-Tag, Trx-Tag and S-Tag. The molecular mass of *Pb*CTS1r agreed with the predicted mass of 45 kDa. An aliquot of *Pb*CTS1r was used to raise the anti-*Pb*CTS1r antibody. The purified recombinant protein was blotted on a nylon membrane and reacted with the antibody (Fig. 1B, Lane 1). No cross-reactivity to the rabbit preimmune serum was detected (Fig. 1B, Lane 2). *Pb*CTS1r showed higher specific activity in the hydrolysis of 4-MU-$(GlcNAc)_3$ (142.92 U/mg) compared to 4-MU-$(GlcNAc)_2$ (52.32 U/mg), indicating the release of multimers of GlcNAc; in this case, specifically a tetramer of GlcNAc.

*Characterisation of the P. brasiliensis PbCTS2 novel chitinase* - To confirm our hypothesis of a second chitinase encoded by *P. brasiliensis* (*Pb*CTS2), the protein was partially purified from crude extracts of yeast cells using cation-exchange chromatography. Two protein peaks were detected possessing chitinase activity (Fig. 2A). The presence of chitinases in these peaks was further confirmed by immunoblotting analysis. Fractions

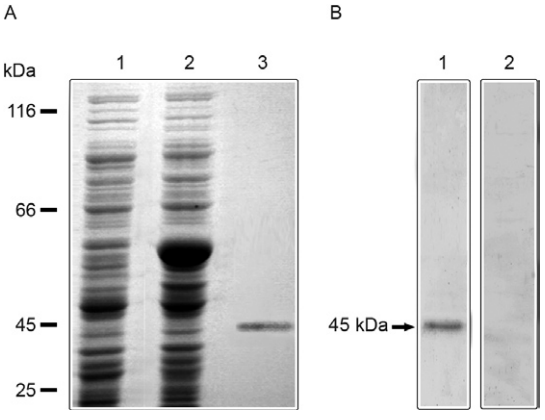


Fig. 1: analysis of the *Pb*CTS1r. A: the proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue R-250 [Lane 1: total protein from non induced *Escherichia coli* BL21(DE3)/pLysS containing pET-32a(+); 2: proteins prepared from *E. coli* BL21(DE3)/pLysS after induction with isopropyl-β-D-thiogalactopyranoside; 3: *Pb*CTS1r purified]. Molecular masses are indicated at the side; B: Western blot of purified chitinase after incubation with anti-*Pb*CTS1r antibody (Lane 1) and with rabbit preimmune serum (Lane 2). The arrow indicates the overexpressed chitinase protein.

from the first peak displayed higher activity towards the 4-MU-$(GlcNAc)_3$ substrate; fractions from the second peak displayed higher activity towards the 4-MU-$(GlcNAc)_2$ substrate. Three fractions were investigated (Fig. 2B). Two of the analysed fractions (fractions 3 and 5) were collected when the column was washed with buffer A; the other (fraction 34) was collected during the NaCl linear gradient. In fraction 3, both proteins were detected, with masses of 45 kDa and 39 kDa, corresponding to *Pb*CTS1 and *Pb*CTS2, respectively (Fig. 2B, Lane F3). In addition, fraction 3 displayed weaker activity towards the 4-MU-$(GlcNAc)_2$ substrate and higher enzymatic activity towards 4-MU-$(GlcNAc)_3$ (Table). Fraction 5, in which only *Pb*CTS1 was detected (Fig. 2B, Lane 3), displayed high levels of enzymatic activity when 4-MU-$(GlcNAc)_3$ was the substrate (Fig. 2A, Table). Both *Pb*CTS1 and *Pb*CTS2 were detected in fraction 34 (Fig. 2B, Lane 4). A higher level of enzymatic activity towards 4-MU-$(GlcNAc)_2$ compared to 4-MU-$(GlcNAc)_3$ was attributed to *Pb*CTS2 because *Pb*CTS1 showed higher levels of enzymatic activity towards 4-MU-$(GlcNAc)_3$ (Table). In addition, fraction 34 showed a strong reaction to *Pb*CTS2, as observed by Western blotting.

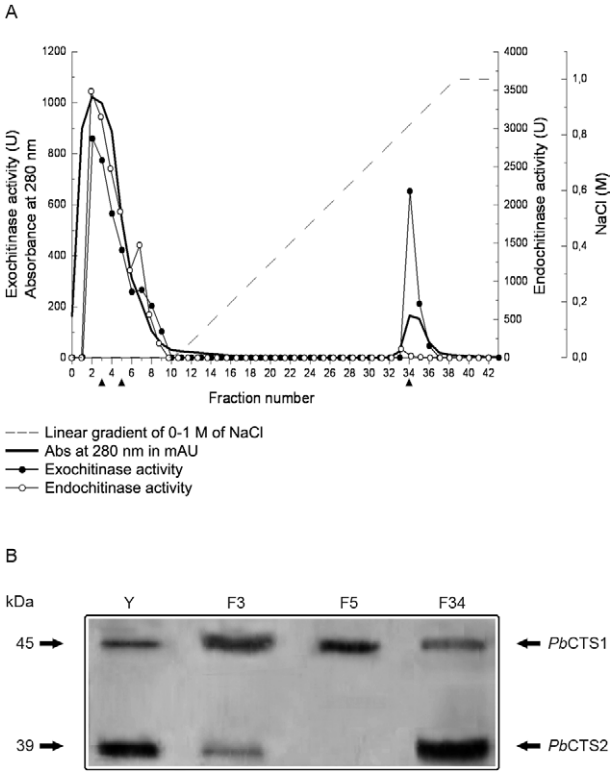


Fig. 2: purification analysis of *Paracoccidioides brasiliensis* chitinases. A: cationic exchange chromatography profile of yeast crude extract on HPLC and profile of chitinase activity were plotted. Arrowheads indicate fractions 3, 5 and 34; B: three fractions of this purification were analyzed by Western blot using anti-*Pb*CTS1r (Lane Y: crude extract from yeast cells; F3, F5 and F34: fractions 3, 5 and 34 of the cationic exchange chromatography, respectively). The left numbers indicate the size of proteins. In each lane was used 20 µg of total protein. NaCl: sodium chloride.

*Detection of P. brasiliensis chitinases* - The anti-*Pb*CTS1r antibody was able to detect the presence of *Pb*CTS1 in yeast cell fractions and in different *P. brasiliensis* phases (Fig. 2B, 3A). *Pb*CTS1 was found in the crude extracts of mycelium, the transition from mycelium to yeast and yeast cells (Fig. 3A, Lanes 1-3, respectively). *Pb*CTS1 was not detected in the purified yeast cell wall protein fraction (Fig. 3, Lane 4), but it was present in the secreted protein fraction from the yeast phase (Fig. 3, Lane 5). Serendipitously, the anti-*Pb*CTS1r antibody cross-reacted with the novel *P. brasiliensis* chitinase protein in the immunoblotting (Fig. 3A, Lanes 2-5). *Pb*CTS2 was not detected in the mycelium phase (Fig. 3A, Lane 1). It was detected during the transition from mycelium to yeast (Fig. 3A, Lane 2) and during the yeast phase (Fig. 3A, Lanes 3-5). No cross-reactivity to the rabbit preimmune serum was detected (Fig. 3B). qRT-PCR results examining the transition from mycelium to yeast and the yeast phase corroborate the Western blot results. However, a low *Pbcts2* transcript level was detected in the mycelium phase (Fig. 3C).

*Phylogenetic analysis* - A search of the *P. brasiliensis* isolate *Pb*01 genome (BROAD Institute) using BlastP (Altschul et al. 1990) was performed to find the putative gene encoding *Pb*CTS2. In the search, we used

TABLE

Total and specific activity in different protein fractions of the cationic exchange chromatography

| Fraction number[a] | Total protein (mg/mL) | Total activity (pmol/mL/min) | | Specific activity ($U^b$/mg) | | Ratio |
|---|---|---|---|---|---|---|
| | | 4-UM-$(GlcNAC)_2$ | 4-UM-$(GlcNAC)_3$ | 4-UM-$(GlcNAC)_2$ | 4-UM-$(GlcNAC)_3$ | 4-UM-$(GlcNAC)_2$: 4-UM-$(GlcNAC)_3$ |
| Yeast crude extract | 0.852 | 864.17 | 3.170.11 | 1.014 | 3.721 | 0.27 |
| 3 | 0.443 | 773.14 | 3.145.77 | 1.745 | 7.101 | 0.25 |
| 5 | 0.197 | 421.50 | 1.910.57 | 2.140 | 9.698 | 0.22 |
| 34 | 0.0156 | 163.14 | 29.12 | 10.457 | 1.867 | 5.60 |

*a*: these fractions refers to Fig. 2; *b*: one unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 pmol of 4-MU per mL $min^{-1}$; GlcNAC: N-acetylglucosamine.

*Pb*CTS1 and 18 *Hypocrea jecorina* chitinase protein sequences (Seidl et al. 2005). We identified five putative *P. brasiliensis* chitinases. Based on the sequence similarity (e-value $\leq 10^{-5}$), the size of the open reading frame (ORF) and the size of the protein, a putative gene was identified corresponding to *Pb*CTS2 (broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/GeneDetails.html?sp=S7000001961010990).

A phylogenetic tree was constructed with *Pb*CTS1, *Pb*CTS2 and other chitinase genes from ascomycetes available on the NCBI database (Altschul et al. 1990) (Fig. 4). The predicted *Pb*CTS1 and *Pb*CTS2 shared 40% similarity. Phylogenetic analysis indicated that the paralogues *Pbcts1* and *Pbcts2* encode two different chitinases in *P. brasiliensis*.

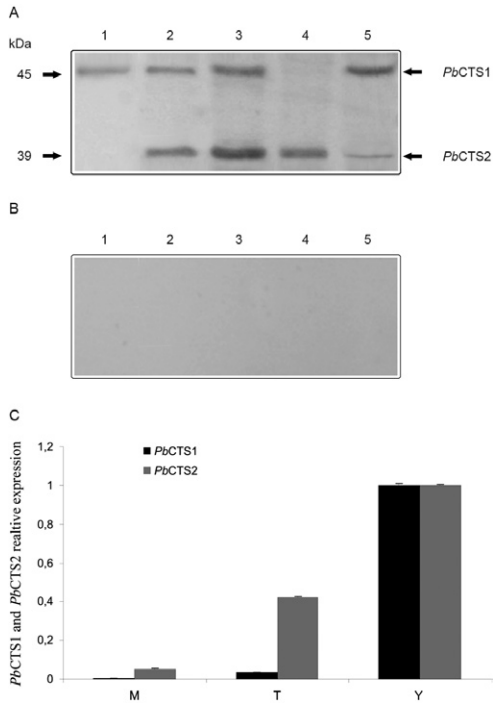


Fig. 3: Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analyses of *Paracoccidioides brasiliensis* chitinases. A: the proteins were transferred from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel to a nylon membrane and incubated with *anti-Pb*CTS1r antibody; B: the cross-reactivity of *P. brasiliensis* proteins was analyzed by Western blotting with rabbit preimmune serum (Lane 1: crude extract from mycelium; 2: crude extract from mycelium in transition to yeast cells after 24 h of temperature shift; 3: crude extract from yeast; 4: yeast cell wall extract; 5: yeast secreted proteins). The numbers on the left indicate the size of proteins. In each lane it was used 20 µg of total protein; C: qRT-PCR plot of *Pbcts1* and *Pbcts2* expression levels in mycelium (M), transition from mycelium to yeast after 24 h (T) and yeast (Y) cells of *P. brasiliensis*. The primers were as following: CTS1 ss 5'CTACTTGTCCGATACTTGGTCC3', CTS1 as 5'GGAGAGGAGCACATTCAGATTC3', CTS2 ss 5'GTCCCAAAACGCCAGAATG3', CTS2 as 5'TGAGAACGCAACCGATTGAC3'; tubulin: Tub ss 5'ACAGTGCTTGGGAACTATACC3', Tub as 5'GGGACATATTTGCCACTGCC3'. The values of expression of the *Pbcts1* and *Pbcts2* were standardized using the values of expression of the constitutive gene encoding to the protein tubulin. The expression level was calculated by relative standard curve method. The standard deviations are presented from two independent experiments.

## DISCUSSION

We have previously characterised the cDNA encoding the *Pbcts1* chitinase (Bonfim et al. 2006). To further characterise this chitinase, we expressed the recombinant protein in *E. coli* and raised anti-*Pb*CTS1r antibodies to study the chitinase expression pattern. *Pb*CTS1 was detected in the crude extracts of *P. brasiliensis* mycelium, the transition from mycelium to yeast and yeast cells. This pattern of expression is in accordance with our previous analysis of *Pbcts1* transcripts in the tested fungal phases (Bonfim et al. 2006). The high expression of *Pb*CTS1 in yeast and the parasitic form of *P. brasiliensis* as well as in the medium of yeast cell culture and in experimental infection conditions (Bonfim et al. 2006) reinforces a role for this protein in the maintenance of the fungal environment.

One study on *P. brasiliensis* strain IVIC *Pb*9 found chitinase activity in the culture medium, but not in the crude protein extracts from yeast cells (Flores-Carreón et al. 1979). In this study, *Pb*CTS1 was detected both in the culture medium and in the crude extracts of yeast cells from isolate *Pb*01, which can be explained by the differences between the isolates and by the use of different substrates to measure chitinase activity.

Bonfim et al. (2006) described the presence of both a glycosaminoglycan attachment site and a laminin G heparin-binding domain in *Pb*CTS1. These domains mediate mycobacterial adhesion to lung epithelial cells and macrophages (Jeffrey 1999) and contribute to cell invasion and systemic dissemination in the host cells (Adams 2004). We found *Pb*CTS1 in the culture medium of yeast, indicating that this protein is secreted and therefore could participate in the maintenance of the fungal environment.

Many chitinases identified to date are secreted, but some are found on the cell wall, as occurs in *Candida albicans* (Iranzo et al. 2002), *Aspergillus fumigatus* (Hearn et al. 1998) and *Aspergillus nidulans* (Yamazaki et al. 2008). *Pb*CTS1 was not detected in the cell wall fraction, suggesting that this chitinase is not involved in yeast cell wall biosynthesis.

Fungi such as *C. albicans*, *A. fumigatus* and *Saccharomyces cerevisiae* encode many chitinases, each possessing diverse chitinolytic activities (Adams 2004). Here, we describe a second chitinase of *P. brasiliensis*, *Pb*CTS2. The five chitinases found in the *P. brasiliensis* isolate *Pb*01 genome database had lengths varying from 26-58 kDa; *in silico* prediction indicated that one of the chitinase genes would generate a 39 kDa protein. Therefore, based on the size of the ORF, the gene sequence and the predicted protein size, we assumed that the 39 kDa gene encoded *Pb*CTS2, which was partially purified in the same fraction as *Pb*CTS1.

The cross-recognition of *Pb*CTS2 with our anti-*Pb*CTS1r antibody can be explained by the similarity between the two proteins, which are likely to share antigenic determinants. This cross-reactivity allowed us to characterise the activity of a second chitinase. Chitinases of diverse molecular weights have been described in the literature (Seidl et al. 2005). Similar to *Pb*CTS1 and *Pb*CTS2, two other endochitinases of 42 kDa and 37 kDa from *Clonostachys rosea* (Mamarabadi et al.

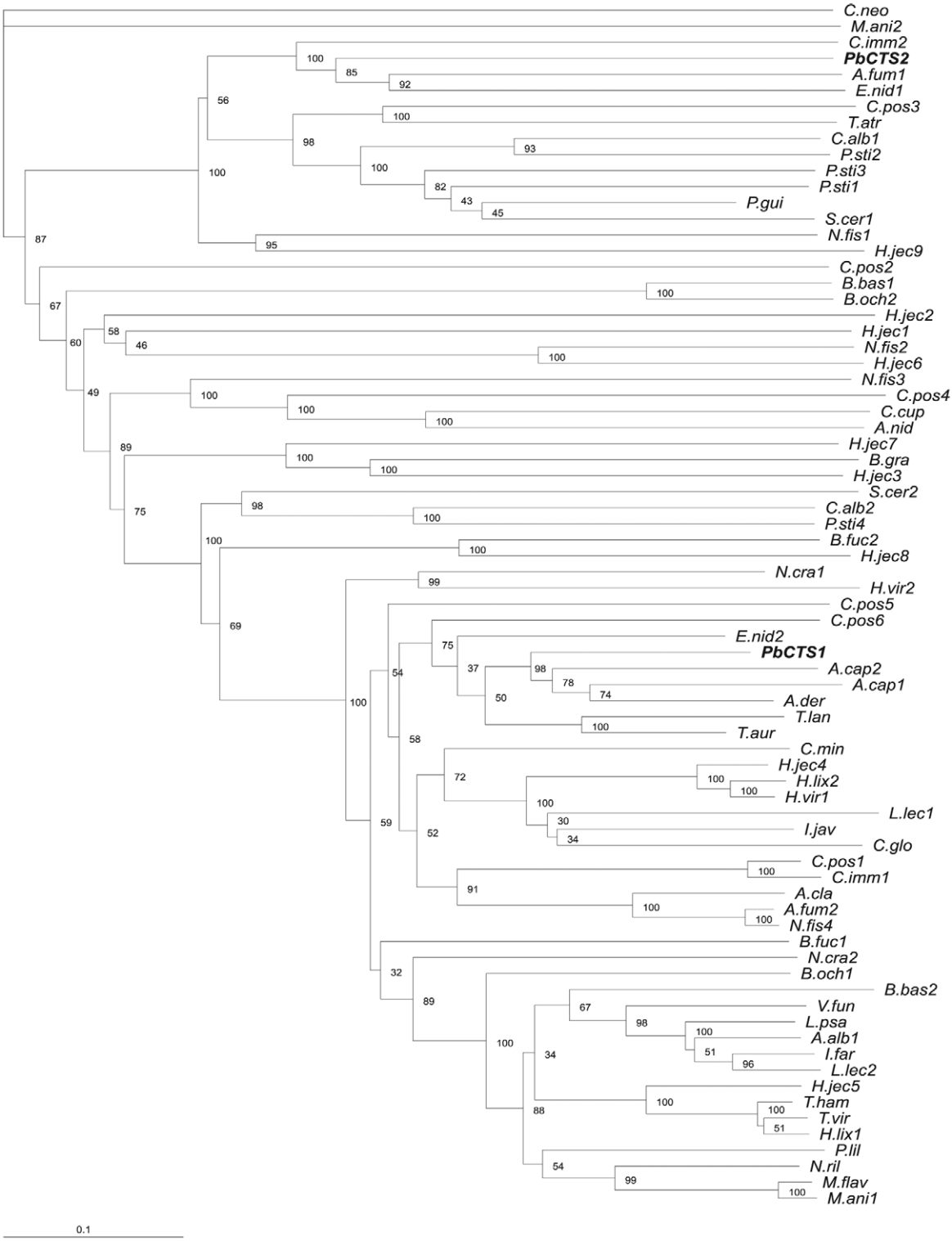


Fig. 4: phylogenetic tree illustrating the relationship among *Pb*CTS1, *Pb*CTS2 and others chitinases. Sequences were aligned and subjected to phylogenetic analysis using minimum evolution (neighbour-joining). The numbers on branches indicate bootstrap values obtained for 100 replications. The species (named with binomial name) and the respective GenBank accessions are shown following: *Ajellomyces capsulatus 1* (*A. cap1*) (AAF80370), *A. capsulatus 2* (*A. cap2*) (AAG41982), *Aspergillus fumigatus 1* (*A. fum1*) (AAO61685), *A. fumigatus 2* (*A. fum2*) (AAO61686), *Candida albicans 1* (*C. alb1*) (AAG35112), *C. albicans 2* (*C. alb2*) (AAA68015), *Coccidioides immitis 1* (*C. imm1*) (2204242A), *C. immitis 2* (*C. imm2*) (Q1EAR5), *Coccidioides posadasii 1* (*C. pos1*) (P54196), *C. posadasii 2* (*C. pos2*) (AAO88269), *Cryptococcus neformans* (*C. neo*) (XP_572898), *Emericella nidulans 1* (*E. nid1*) (BAA36223), *E. nidulans 2* (*E. nid2*) (BAA35140), *Hypocrea jecorina 1* (*H. jec1*) (DAA05858), *H. jecorina 2* (*H. jec2*) (DAA05857), *Metarhizium anisopliae 1* (*M. ani1*) (AAY32603), *M. anisopliae 2* (*M. ani2*) (AAC33265), *Neorospora crassa 1* (*N. cra1*) (XP_965309), *N. crassa 2* (*N. cra2*) (XP_957924), *Paracoccidioides brasiliensis 1* (*Pb*CTS1) (AAQ75798), *P. brasiliensis 2* (*Pb*CTS2) (PAAG_03848.1), *Sacharomyces cerevisae 1* (*S. cer1*) (P29029), *S. cerevisae 2* (*S. cer2*) (Q06350).

2008) have been identified. During the partial chitinase purification, fraction 34 showed a large concentration of *Pb*CTS2, according to the Western blot assays, and a high activity when using the 4-MU-$(GlcNAc)_2$ substrate, which can be attributed to *Pb*CTS2 because only a trace quantity of *Pb*CTS1 was detected in that fraction. In addition, *Pb*CTS1 has a higher enzymatic activity towards 4-MU-$(GlcNAc)_3$ than towards 4-MU-$(GlcNAc)_2$. Nevertheless, the activity towards 4-MU-$(GlcNAc)_3$ in fraction 5 and the lack of a visible signal for *Pb*CTS2 in the Western blot could be attributed to other *P. brasiliensis* chitinases present in those fractions.

Although *Pb*CTS2 was not detected in mycelium by Western blot analysis, a low expression level was detected by qRT-PCR, presumably due to the high sensitivity of the technique or post-transcriptional regulation of the protein. *Pb*CTS2 was detected in all of the analysed protein extracts from yeast cells. Because *Pb*CTS2 is expressed in the cell wall, unlike *Pb*CTS1, it may have a role in yeast cell wall biosynthesis and structural maintenance. In addition, the presence of the 39 kDa chitinase during the transition from mycelium to yeast and in the extracellular medium of yeast cells suggests a role in the maintenance of the fungal environment.

The phylogenetic analysis of *Pb*CTS1 and *Pb*CTS2 indicated that they clustered separately and could have different functions. This is similar to chitinases of *Emericella nidulans* (*E. nid* 1 and *E. nid 2*) and *Coccidioides immitis* (*C. imm* 1 and *C. imm 2*). Fungal chitinases can play multiple and diverse roles (Duo-Chuan 2006). During evolution, *P. brasiliensis* could have acquired the paralogues *Pbcts1* and *Pbcts2* to grow and survive in different environments in both saprophytic and parasitic phases.

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Multiple alignment of the sequences deduced protein chitinases

| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | ---------- |
| *H. jec2* | ---------- |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | ---------- |
| *H. jec2* | ---------- |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | ----------MAWRLSPRVLIRLTTAVGLAHLAQSEAMHEVGNPQC |
| *H. jec2* | ----------MPWISPGGIACWLASATAIVSFHGVSATANEVDAFQSQLNLC |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |

↳

| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ----------MKQLPRC |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ----------MLL |
| *H. jec1* | PSLCVGNGRHEWTAYSSMESLSLCDQPMLFDFAIHVRVDDPESTLRFRACTAANMTERQV |
| *H. jec2* | PLACSDKSPEQWSVYSSFERLSQCDQPVLFDLAIHTPINDKTNSVLIRACTTSPAAGGSQ |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | MGPFFLYIGALLFGAVGAVNHGPPEFGSG----YRQGVDFL----------PGRDTA---- |
| *A. cap1* | RSLAWCFVALLLFTALSSVSAAPGCRPSVNVLPFKDDVDDY----------TKIDCV---- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ----------MVGHSAQH---- |
| *C. neo* | STPAFLSFALLLVSQSSAQGHTAPPGRHRHPRIFK----------DLQERGQL---- |
| *H. jec1* | LNSTHHGSQHSETHMPGDAHLSSADIARWSPGEAESGLNAVVAFDS-----LRHHL---- |
| *H. jec2* | PMASTVKREAEACGGSFVESKVPLLISQNPGQATAPFGNGLVTILEGLKEYRQKAV---- |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ----------MPSLFAQSLAIIATLQATLGLATPVSAP---------- |
| *N. cra2* | ----------MFSIKAAALAVLSFSSFLVDAAPAPMCP---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ----TAWR-------TVTETVTVPAAATEGACSSPPEPSQSPSS---------- |
| *A. cap1* | ----SVWRSTVWVTSTTTITLPFPSASSSASASSFPAATSTPAADYPVISQASESTPPPP |
| *E. nid2* | ---------- |
| *C. pos1* | ----------MRFLIG-----ALLTLQTLVQAS---------- |
| *C. imm1* | ----------MRFLIG-----ALLTLQTLRWLS---------- |
| *A. fum2* | ----------MRFATSTIVKVALLLSSLCVDAA---------- |
| *N. cra1* | ----------MLSSIVTCVIPLLVISTPAAAATS---------- |
| *C. alb2* | ----------MCHKMMDKFQQRLHHTTS---------- |
| *A. fum1* | ----------MVSSKLSFVATAVAALAPLASAFDASSRSNLA---------- |
| *E. nid1* | ----------MAPKLFTFVS-ALSGLASLASAFHAEAKSNIA---------- |
| *PbCTS2* | ----------MAFEN---------- |
| *C. imm2* | ----------MGLTNILAAFIAVSSLFIQSLALNPYAKSNLA---------- |
| *C. alb1* | ----------MLSFKSLLAAAVVASSALAS--ASNQVA---------- |
| *S. cer1* | ----------MSLLYIILLFTQFLLLPTDAFDRSANTNIA---------- |
| *S. cer2* | ----RSKSSLVSHLLILLIFITIIIEMCLYNKIFKNQRSDDIRDN---------- |
| *C. neo* | ----DERLLPITATTTVVVPMTATRTIWVDSTQLVLGDGGNDGDNDASG---------- |
| *H. jec1* | ----GEATDSGENIILFASVHNTVLGLYVGASVDAASAVSALAPGLVDSPYTTGASR--- |
| *H. jec2* | ----ATSGCTGDNNIMLGYFDGAVVGTYAGKALSSATIPSIVDYLLEEDKEQGSAPFG-- |
| *C. pos2* | ----------MPPIPESHG---------- |
| *M. ani2* | ----------MLFSMVMFTERWWVGSKDCPR---------- |
| *M. ani1* | ----------DTVIG----------KHA |
| *N. cra2* | ----------GTNVTSTSADTNVTVPDR---------ADS |
| *Pb*CTS1 | ----------MTNP---------- |
| *A. cap2* | ----------SATEPPSPSPTGNM-------PHV |
| *A. cap1* | YEGSCRVRPTPKPPSPSSPIPS--------PPSVYPTRPSPPSPSPSHNM-------SSP |
| *E. nid2* | ----------M |
| *C. pos1* | ----------SMSSMPNSYPVPEA-------PAE |
| *C. imm1* | ----------LRCLFCELGRFMFTLS-------TVT |
| *A. fum2* | ----------VMWNRDTSSTDLEA-------RAS |
| *N. cra1* | ----------QFQVQSSGN---------TTV |
| *C. alb2* | ----------T |
| *A. fum1* | ----------IYWGQG--PNQLRLSHFCQETS |
| *E. nid1* | ----------VYYGQG--VNQPRLAEFCAETS |
| *PbCTS2* | ----------HIGQA--PNQPRLRHICANDR |
| *C. imm2* | ----------VYWGQG--AGQNRLSYFCEKTS |
| *C. alb1* | ----------LYWGQNGAGGQERLAQYCQETD |
| *S. cer1* | ----------VYWGQNSAGTQESLATYCESSD |
| *S. cer2* | ----------FNNGGHRVPSNVQNHGTHIRD |
| *C. neo* | ----------DVSSVWSSQASQSTEGASTSS |
| *H. jec1* | ----------SLAQICGQGRNSEHTVGVAISFENDLAAVQSCVASWSRAECAPTDD |
| *H. jec2* | ----------MLAQICDHDSDMDHTAGVAVALKADIGSVQSALLSWNQGECAKAQS |
| *C. pos2* | ----------NST |
| *M. ani2* | ----------VPALENSNNP |

↳

| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | GGYVNAVYFTNWG--------IYGRNYQPADLPASQISH---------- |
| *N. cra2* | SGYKNVVYFTNWG--------IYGRNYQAADLPADKITH---------- |
| *Pb*CTS1 | -GFRSVVYFVNWA--------IYSRNYHPQDLPVEKLTH---------- |
| *A. cap2* | DGFKSVVYFVNWA--------IYGRNYQPQDLPAAKLTH---------- |
| *A. cap1* | DGYKSIVYYVNWA--------IYARNYNPQDLPVKKLTH---------- |
| *E. nid2* | SGYKTVGYFVNWA--------IYGRNYNPQDLPAEKLTH---------- |
| *C. pos1* | GGFRSVVYFVNWA--------IYGRGHNPQDLKADQFTH---------- |
| *C. imm1* | A--VTVVTDDIQA--------IYGRGHNPQDLKADQFTH---------- |
| *A. fum2* | SGYRSVVYFVNWA--------IYGRNHNPQDLPVERLTH---------- |
| *N. cra1* | SSYRNAVYFTNWG--------IYGANFQPQQLPASQITH---------- |
| *C. alb2* | PLFKTCVYFSNWS--------VYQKKHFPQDIPIEYFTH---------- |
| *A. fum1* | LD-IINIGFIN----------YFPDMSPGH-WPGSNFGN---------- |
| *E. nid1* | YD-IINIGFIN----------SFPEQNPLTGLPGSDFGN---------- |
| *PbCTS2* | FTNVIVLGFVN----------VFPERGKGG-YPGTNFGN---------- |
| *C. imm2* | FD-IIVVGFIN----------VFPDQGPAG-WPGSNFGN---------- |
| *C. alb1* | VD-IVLLSFLN----------LFPDP------LNVNFAN---------- |
| *S. cer1* | AD-IFLLSFLN----------QFPT-------LGLNFAN---------- |
| *S. cer2* | EAFISGVYYSNWSP-------YKPRFHFPHDINLKQVSH---------- |
| *C. neo* | EAMLPTASASSSSVS-------DLTSSSYDTDAQSSSFVT---------- |
| *H. jec1* | SSEISTLTDLNI---------WTRSDLPGRPLTRHSHTHLHPRGECKAIKVVPGDSCASL |
| *H. jec2* | GTTLRELVISQVPLPSINAKASNISLAEAPGRKAQALDSKVDGKYCKMRTIAAGDSCASI |
| *C. pos2* | LTNPRVICYYQT---------YYPNNGTDYISTLPLLTN---------- |
| *M. ani2* | WRLERTATAAELS--------QYGNPTTCEIDNGGVIVA---------- |
| *M. ani1* | ----------VLYSFLNLSNN----------GTVYSGD |
| *N. cra2* | ----------VLYSFANLKED----------GTVFSSD |
| *Pb*CTS1 | ----------VLYAFANVRPES----------GEVYLSD |
| *A. cap2* | ----------VLYAFANVHGDT----------GEVYLTD |
| *A. cap1* | ----------VLYAFANVRAES----------GEVFLTD |
| *E. nid2* | ----------ILYAFANVRPET----------GEVYLSD |
| *C. pos1* | ----------ILYAFANIRPS----------GEVYLSD |
| *C. imm1* | ----------ILYAFANIRPS----------GEVYLSD |
| *A. fum2* | ----------VLYAFANVRPET----------GEVYMTD |
| *N. cra1* | ----------ALTPSR----------VSSD |
| *C. alb2* | ----------IFYAFILIDEQT----------GKLKFSD |
| *A. fum1* | ----------QCDGSVYVTN----------DGVVTKLLS |
| *E. nid1* | ----------QCWADTFVV----------DGIASQLYS |
| *PbCTS2* | ----------QCSAEVFKNK----------DGVETQLLS |
| *C. imm2* | ----------QCADSYYYTK----------NGTKTKLLD |
| *C. alb1* | ----------QCGNTFESG----------LL |
| *S. cer1* | ----------ACSDTFSDG----------LL |
| *S. cer2* | ----------IYYAFFKINSRT----------GGIENTD |
| *C. neo* | ----------GSYIEDVTATS----------SLLSDSLP |
| *H. jec1* | ASKCGVSGANFERYNSYDKRLCSKLAVGKPVCCSPGKLPDLRPKPKPSGECATYTVKSSD |
| *H. jec2* | AKACKVSVADFFKYNGVKGNGNDWCRKLQAGRNICCSSGSSKPLPEANGDCFSYTIKAGD |
| *C. pos2* | ----------DCGVSHIIL----------AAIHIND |
| *M. ani2* | ----------DGFQASKGYTG----------DSIVDYND |
| *M. ani1* | SWADIDKHY---------- |
| *N. cra2* | TWSDTDKRY---------- |
| *Pb*CTS1 | TWSDIEKHY---------- |
| *A. cap2* | NYADTDKHY---------- |
| *A. cap1* | TWADTDKHF---------- |
| *E. nid2* | TWSDIEKHY---------- |
| *C. pos1* | TWADTDKHY---------- |
| *C. imm1* | TWADTDKHY---------- |
| *A. fum2* | SWADIEKHY---------- |
| *N. cra1* | TYADLEKRY---------- |
| *C. alb2* | EWCDLQMPQ---------- |
| *A. fum1* | GCHQIMEDI---------- |
| *E. nid1* | HCPNIAEDI---------- |
| *PbCTS2* | GCQNLIEDI---------- |
| *C. imm2* | GCYQIKEDL---------- |
| *C. alb1* | HCSQIGADI---------- |
| *S. cer1* | HCTQIAEDI---------- |
| *S. cer2* | SWSDLEMNL---------- |
| *C. neo* | TSSSISSDY---------- |
| *H. jec1* | TCADIAASNGLETTDVVEFNRK-TWGWNGCKSLLVGQSICLSKGTPPLPAPVPNAVCGPT |
| *H. jec2* | DCSSIGMPWNLTPKDIEGFNQKVTWGWRGCPNLTVGLKICLSKGSPPMPAPVSNAVCGPQ |
| *C. pos2* | EPGNITLNDHS---------- |
| *M. ani2* | AHYKTSVDQ---------- |
| *M. ani1* | -PNDSWN------DVGNN----------VYGCVKQLYLLKK---------- |
| *N. cra2* | -PTDSWN------DNGTN----------VYGCVKQLYLLKK---------- |
| *Pb*CTS1 | -PTDSWN------DVGTN----------VYGCIKQLFLLKK---------- |
| *A. cap2* | -PTDSWE------DVGNN----------VYGCVKQLFLLKK---------- |
| *A. cap1* | -PTDSWS------ETGNN----------VYGCVKQLFLLKK---------- |
| *E. nid2* | -PTDSWN------DTGNN----------VYGCVKQLGLLKR---------- |
| *C. pos1* | -PGDKWD------EPGNN----------VYGCIKQMYLLKK---------- |
| *C. imm1* | -PGDKWD------EPGNN----------VYGCIKQMYLLKK---------- |
| *A. fum2* | -PGDSWS------DTGNN----------VYGCIKQLYLLKK---------- |
| *N. cra1* | -PGDSWS------EPGEN----------AYGCVKQMYLLKK---------- |
| *C. alb2* | -PSPNQS------ITGN----------LQQFYEMKK---------- |
| *A. fum1* | -PICQAAGKKVLLSIGG----------AYPPDQSILSEDS---------- |
| *E. nid1* | -PKCQAAGKKVFLSLGG----------ATP-TYWFDTIDA---------- |
| *PbCTS2* | -PVCQEIGIKVLLSLGG----------GVG-NYTVTNKRA---------- |
| *C. imm2* | -PKCKALGKTILLSLGGG----------AVHDFYEVKSEES---------- |
| *C. alb1* | -KTCQSLGKTVLLSLGG----------GVGDYGFSDVAS---------- |
| *S. cer1* | -ETCQSLGKKVLLSLGG----------ASGSYLFSDDSQ---------- |
| *S. cer2* | -YKSLAIKNSELIKESSNN------SVQ-NILPLGCIGELFYLKNT---------- |
| *C. neo* | -PSLSVSQSEASSSTTGVA------TSS----ATVTDSSFSTFAEEPAS---------- |
| *H. jec1* | KPGTKKPPAGVALATLNPC---PLNACCNIWGSCGTTKEFCTISRSGTGNPGTSRPGENG |
| *H. jec2* | KPGTVRPESVKDAFELAKLNPCPLNSCCNVWGQCGIDALFCTKADGPTGNPGTAPAGSNG |
| *C. pos2* | -PDDPRY----------VPLWAEMRVMQA---------- |
| *M. ani2* | ---DAWGFVRAAYNRGRN----------TNRQSSGPHPLCTR---------- |

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| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | CISNCGMGIVN-NKEAPDSFKKVTYYESWNLDRSCMHMDVRTVGDLGQGYTHLHFAFVNI |
| *H. jec2* | CISNCGTGIVNNAKPPSGGFRRIGYYEAFNWERPCLHMESKLSNTDKYTHMHWAFG--DI |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ----------ANRNMKTMLSIGGWTWSTN----------FPAAASTAA |
| *N. cra2* | ----------ANRNVRVLLSIGGWTYSQTSPS---------RFALTASTAE |
| *Pb*CTS1 | ----------KNRNLNVLLSIGGWTYSSN----------FALPASTPA |
| *A. cap2* | ----------QNRNLKILLSIGGWTYSSN----------FPGAASTAA |
| *A. cap1* | ----------QNRHLKVLLSIGGWTYSPH----------FGAAVSTPA |
| *E. nid2* | ----------QHRQLKVLLSIGGWTYSPN----------FTNGAGTPE |
| *C. pos1* | ----------NNRNLKTLLSIGGWTYSPN----------FKTPASTEE |
| *C. imm1* | ----------NNRNLKTLLSIGGWTYSPN----------FKTPASTEE |
| *A. fum2* | ----------QNRNLKVLLSIGGWTYSPN----------FAPAASTDA |
| *N. cra1* | ----------RNRNMKVLLSIGGWTYSPK----------FPPVAATEE |
| *C. alb2* | ----------KNRHLKLIMSIGGWGICHL----------FESVVSNDT |
| *A. fum1* | ----------AVAFATFLWGAFGPVAEGW----------EGPRPFGD |
| *E. nid1* | ----------STKLADFLWGAFGPVTDAWTV----------ADKPRPFGN |
| *PbCTS2* | ----------GEKFADFLWGAFGPKTPEWG----------NGPRPFGD |
| *C. imm2* | ----------ALNFADFLWGAFGPLTPDWT----------GPRPFGE |
| *C. alb1* | ----------ATKFADTLWNKFGAGE-DPE----------RPFDD |
| *S. cer1* | ----------AETFAQTLWDTFGEGTGASE----------RPFDS |
| *S. cer2* | ----------CSDKKFKVIMSIGGWSDSEN----------FKIIIKDDK |
| *C. neo* | ----------TGSSSSQSIAADSAPSSSASTSASTGSSSSRPTSLPQTESAT |
| *H. jec1* | TKDLKVSVQDPRGQFEHFKGLSGIKRIAAFGGWSFSVGVDT--------YATLRQAIKPE |
| *H. jec2* | KSDFSVYINDTHHQWDGFMGLKNVKRIVSFGGWGFSTGVAS--------YDVLRKAMTPE |
| *C. pos2* | ----------SGTKVMGMVGGAAKGSY----------QRLDGSVA |
| *M. ani2* | ----------KCIAWLLMGRLMNAVLTQSD----------NPALVPNQN |
| *M. ani1* | TRSNFAKSAVTIMKDWGFDG-----IDVDWEYP----------ADDVQATN |
| *N. cra2* | SRTKFATSALALVKDWGFDG-----IDIDWEYP----------ASETEAQN |
| *Pb*CTS1 | GRAKFAETAVKLLLDLGLDG-----LDVDWEYP----------MNDGEAKD |
| *A. cap2* | NRAHFADTATKLMLDMGFDG-----LDIDWEYP----------NDDEEAKN |
| *A. cap1* | ARTKFADSATQLLLNLGFDG-----LDIDWEYP----------KDDEEAKS |
| *E. nid2* | NRARFAQTATKLITDLGFDG-----IDIDWEYP----------QNDQQAQN |
| *C. pos1* | GRKKFADTSLKLMKDLGFDG-----IDIDWEYP----------EDEKQAND |
| *C. imm1* | GRKKFADTSLKLMKDLGFDG-----IDIDWEYP----------EDEKQAND |
| *A. fum2* | GRKNFAKTAVKLLQDLGFDG-----LDIDWEYP----------ENDQQAND |
| *N. cra1* | GRRRFASSAVKLVQDWGFDG-----LDIDWEYP----------TNAREAQD |
| *C. alb2* | KFDNFVNSTIEFVEKYGFDG-----VDIDWEYP----------KNSTQAAK |
| *A. fum1* | VVVDGFDFDIEHNGGFGYAT---MVNTFRQYF----------NQVPERKF |
| *E. nid1* | AVVDGFDFDIEFFGSKGYAN---MIKRFRRRF----------GEVPDQTF |
| *PbCTS2* | VVVDGFDFDIEHNESFVYHPYIFMVNRLRSHF----------SRFPNKKF |
| *C. imm2* | ASVDGFDFDIEKGSNFGYSI---MVRRLRELF----------LQDPLNRY |
| *C. alb1* | AVVDGFDFDIEHGGATGYPE---LATALRGKF----------AKDTSKNY |
| *S. cer1* | AVVDGFDFDIENNNEVGYSA---LATKLRTLF----------AEGT-KQY |
| *S. cer2* | LLQNFVDSSVETMFRLGFDG-----IDLDWEFPG----------NNESEPRG |
| *C. neo* | KSEDLGAASTNIASNATVSDN-VSVSESQWASVTSTKSSSISE-------TGSASLSGSE |
| *H. jec1* | HREFFATQVVDFLKQHKLDG-----VDFDWEYPG----------ATDLPIPAG |
| *H. jec2* | NRGHFVSNVVAFAKKMGIDG-----IDLDWEYPG----------APDIPGIPKG |
| *C. pos2* | DFEAFYCPLRDMIRTRNLNG-----LDLDVEED----------MSLDG |
| *M. ani2* | ATGSNSRPWKPLGKAQSYSNE---ELNNAPQFN----------PETLYASD |
| *M. ani1* | MVLLLQAVRDELDAYAAKFA-------QGYHFQLSIAAPAGPANYNKLH------LGDLG |
| *N. cra2* | FLLLLKEIRSQMDKYAAAHA-------DGYHFLLTMAASAGPSKYGVLES----SMKEIG |
| *Pb*CTS1 | FVSLLKATREELDRVGGGG----------RKFLLTIACPAGPENYRKLR------LQEMT |
| *A. cap2* | FVELLKVTREKLDELSKN----------RKFYLTVACPAGPKNFQKLR------LKEMT |
| *A. cap1* | LVELLKTTREVLDLAGGKD----------RRFLLTVACPAGRQNFEKLR------LREMT |
| *E. nid2* | YVDLLRRCREALNAAQGQ----------RRFQLTVAVPAGPDNYNKLR------LQEMT |
| *C. pos1* | FVLLLKACREALDAYSAKHP-------NGKKFLLTIASPAGPQNYNKLK------LAEMD |
| *C. imm1* | FVLLLKACREALDAYSAKHP-------NGKKFLLTIASPAGPQNYNKLK------LAEMD |
| *A. fum2* | FVLLLKEVRTALDSYSAANA-------GGQHFLLTVASPAGPDKIKVLH------LKDMD |
| *N. cra1* | FVLLLRACRQALDDYARQYA-------PGYHFLITIAAPAGPQHYGVMD------LPGMN |
| *C. alb2* | LVELLARLRNKLN----------SKYIITVAAPGGSDNIEILK------IQEMD |
| *A. fum1* | YLSAAPQCIIPDAQLSDAIF----NAAFDFIWIQYYNTAACSAKSFIDT-----SLGTFN |
| *E. nid1* | YISAAPQCSIPDEQLSVAIK----NAVIDFVWVQFYNTPGCSARDFVLG-----TKNGFN |
| *PbCTS2* | FISAAPECLITERALDLVIK----YAKFDWISVQFYNAYQCSARSWVIDG----DKSGFT |
| *C. imm2* | YISAAPQCIMPDKYLSHAIS----NSAFDFIFIQFYNNPSCSAKRWVTNP----KSVTYT |
| *C. alb1* | FLSAAPQCPYPDASLGDLLS----KVPLDFAFIQFYNNYCSIN----------GQFN |
| *S. cer1* | YLSAAPQCPYPDASVGDLLE----NADIDFAFIQFYNNYCSVS----------GQFN |
| *S. cer2* | YLKLVRMLRLKLNSLESQIFG----KRTEDHFQLSIAAPAFKDKLFYLP------ITEID |
| *C. neo* | SVSLSDSFSISATSTDSAAVIG---TASSSYSRFESSATASETASASANE----TGSKTK |
| *H. jec1* | SPEDPANYLRFLQLLRKKLP---------AEMSLSIAAPASFWYLKAFP------IADIA |
| *H. jec2* | LPSDGPNYLETLRALRKALP---------SKYSLSIAVPASYWYLRPFP------IREMS |
| *C. pos2* | IIRLIDRLKSDFG----------DEFIITLAPVATAMVRGLR---------- |
| *M. ani2* | TLIRFNGVNYISQSKEQKVS-----PSDSNPWRVFVDWTGTKERVGTPKKAWP--KHVYA |

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| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | KVLDYINLMAYDFSGS---------- |
| *N. cra2* | ETLDFMNLMAYDYAGA---------- |
| *Pb*CTS1 | PYLDFYNLMAYDYSGS---------- |
| *A. cap2* | PYLDFYNLMAYDYAGS---------- |
| *A. cap1* | PYLDFYNLMAYDYSGS---------- |
| *E. nid2* | PYLDFYNLMAYDYAGS---------- |
| *C. pos1* | KYLDFWNLMAYDFSGS---------- |
| *C. imm1* | KYLDFWNLMAYDFSGS---------- |
| *A. fum2* | QQLDFWNLMAYDYAGS---------- |
| *N. cra1* | PYIDSWHLMAYDYAGS---------- |
| *C. alb2* | KYLTFWNLMCYDFAGEG---------- |
| *A. fum1* | --FDAWVTVLKAS---------- |
| *E. nid1* | --YDSWVEVIKAG---------- |
| *PbCTS2* | YMFEKWMKLIDAS---------- |
| *C. imm2* | --VDDWVKYIRKS---------- |
| *C. alb1* | --YDTWSKFADSA---------- |
| *S. cer1* | --WDTWLTYAQTVS---------- |
| *S. cer2* | QYVDYWNMMTYDYYGS---------- |
| *C. neo* | SHGNWWETSTSSSAWAS---------- |
| *H. jec1* | KTVDYIVYMTYDLHGQW---------- |
| *H. jec2* | ETVDYIVYMAYDLHGNGNCLRSHVNQTEVVLALSMITKAGVSASKVVVGESSYGRSFKMA |
| *C. pos2* | -HLSGFDYRAL---------- |
| *M. ani2* | PYVDFTLNTIPDLRALA---------- |
| *M. ani1* | ----------WSNSSAHNANLYANPGNLNA |
| *N. cra2* | ----------WDKKAGHQANLYPDEKNPDT |
| *Pb*CTS1 | ----------WDTIAGHQSNIKPSRSNPKS |
| *A. cap2* | ----------WDTVAGHQANLEVSKSDPKS |
| *A. cap1* | ----------WDTIAGHQSNIEISKSNRNS |
| *E. nid2* | ----------WDQTAGHQANLYPSTSNPTS |
| *C. pos1* | ----------WDKVSGHMSNVFPSTTKPES |
| *C. imm1* | ----------WDKVSGHMSNVFPSTTKPES |
| *A. fum2* | ----------FSSLSGHQANVYNDTSNPLS |
| *N. cra1* | ----------WDSTTGHQANLLPSPKNLLT |
| *C. alb2* | ----------WSSKTAFHSNLFGNNGD--- |
| *A. fum1* | ----------ASKDAKLYVGLPASETAANQ |
| *E. nid1* | ----------ANPNAKLYVGLPASGAAANL |
| *PbCTS2* | ----------VNPSAKLLIGLPAAPKAALP |
| *C. imm2* | ----------GNPLAKLFIGLPASKSAAAK |
| *C. alb1* | ----------PNKNIKLFVGVPATSNIAG- |
| *S. cer1* | ----------PNKNIKLFLGLPGSASAAGS |
| *S. cer2* | ----------WSETTGYHSNLFS------E |
| *C. neo* | ----------SSLSASESASESASLTASGT |
| *H. jec1* | ----------DFNSRWASDGCPKGSCLRSH |
| *H. jec2* | KAGCTGPLCKFTGANGKSEAAAGRCTNARGYLANAEINEIISKSKGHPKTWYDKDTASDY |
| *C. pos2* | ----------ETARGSKISWYNVQFYNGR |
| *M. ani2* | ----------KNHNVNHFTLAFVVSKDAN |
| *M. ani1* | TPFNTDDAVNDYIKGG-VPASKIVLGMPIYGKSFQKTNGI----------GKPFSGVG- |
| *N. cra2* | TPFSTDRAVTDYIKFG-IPSNKIVLGMPLYGRAFASTDGP----------GTAYSGVG- |
| *Pb*CTS1 | TPVSTEAALNHYIGVGGVPASKIVLGMPLYGRTFANTDGP----------GTPFQGNGG |
| *A. cap2* | TPYSTEAALDYYIGVGEVPASKMILGMPLYGREFADTDGP----------GTPFTGTGG |
| *A. cap1* | TPFSTKEAVDYYVGVGKVPPSKLILGMPLYGRTFADTDGP----------GTPFHGDGG |
| *E. nid2* | TPFNTVQAVNHYIDAGGVPSNKIILGMPIYGRAFQNTDGP----------GRPYSGIG- |
| *C. pos1* | TPFSSDKAVKDYIKAG-VPANKIVLGMPLYGRAFASTDGI----------GTSFNGVG- |
| *C. imm1* | TPFSSDKAVKDYIKAG-VPANKIVLGMPLYGRAFASTDGI----------GTSFNGVG- |
| *A. fum2* | TPFNTQTALDLYRAGG-VPANKIVLGMPLYGRSFANTDGP----------GKPYNGVG- |
| *N. cra1* | TRFNTDQAVRDFVRRG-IPANKIVLGLPLYGRSFEGTDGL----------GKPYSGIG- |
| *C. alb2* | NSLNASDVVQTYINKG-VHPTKLILGMPMYGRIFHGVDRPEI---------GIPFTKERK |
| *A. fum1* | GYYLTPDEVESLVSTYMDRYPDTFGGIMLWEATASENN-QID---------GAPYADHMK |
| *E. nid1* | GYYLTPEEVKPLVKKYMDKYPETFGGVMLWEATQARNN-QID---------GVGYNEKIR |
| *PbCTS2* | NYYLDIKEN--YINCFINRHITINKHITINKHITINKH-FYQ---------CINY----- |
| *C. imm2* | EDYLTPGEATKIVSTYMAKYPSTFGGMMVWEATASENN-KLG---------GLPYADIMK |
| *C. alb1* | --YVDTSKLSSAIEEIKC--DSHFAGVSLWDASGAWLNTDEK---------GENFVVQVK |
| *S. cer1* | GYISDTSLLESTIADIAS--SSSFGGIALWDASQAFSN-ELN---------GEPYVEILK |
| *S. cer2* | TELNGNFAMHYMIDRFGVNSRKLVLGMAAYGRSFHIKDNKFEPFNQNTVLINKIFKGVGK |
| *C. neo* | VSVSDSSTWPESASVSSAATATTFESSTSISATSTSTSSS----------SSPTSTLAS |
| *H. jec1* | VNMTETLNALAMITKAGVEARQVVVGVASYGRSFEMNDPKCKG-------PMCTFTGPQS |
| *H. jec2* | LVYNDVEWVAYMSDKTKQSRREKWKGLNFLGTVDWAVDLQEFN----VTDHTEPPQGGPG |
| *C. pos2* | GHMLHPSVYDTIIHQG-WEAERIVIGLLTNPANGSQGYVP----------METISS |
| *M. ani2* | TTCGTAYGMQNYAQYSKIKALREAGGDVMLSIGGANNAPLAAS--------CKNVDDLMQ |
| *M. ani1* | ------DGSWENGIWDYKVLPKA---------- |
| *N. cra2* | ------EGSWEKGIWDYKVLPKS---------- |
| *Pb*CTS1 | ------SGSWETGVWDYKVLPKP---------- |
| *A. cap2* | ------SGSFEPGIWDYKALPKE---------- |
| *A. cap1* | ------QGSFERGIWDYKSLPKV---------- |
| *E. nid2* | ------QGTWEQGVYDYKALPRP---------- |
| *C. pos1* | ------GGSWENGVWDYKDMPQQ---------- |
| *C. imm1* | ------GGSWENGVWDYKDMPQQ---------- |
| *A. fum2* | ------QGSWENGVWDYKALPQA---------- |
| *N. cra1* | ------AGTLEPGTWVYRDLPRP---------- |
| *C. alb2* | ------SGCIEADVVDYNKFGDT---------- |
| *A. fum1* | ------DILLHCDP--SPPVTSS--SAVP---------- |
| *E. nid1* | ------EILYDLDPNHPPPTTSP--TPTPTPSTTTSTTSTTSTTSATSTTSTTSTTSTT |
| *PbCTS2* | ---------- |
| *C. imm2* | ------EVLLRCDPDPPTSTVTSTISASTSTQTSSQSTTMETKTLSASTTPSSPSTVSPS |
| *C. alb1* | ------NVLNQNACVAPSSSATT---------- |
| *S. cer1* | ------NLLTS-----ASQTATT---------- |
| *S. cer2* | PTKEIDKADGKEGIWPYKNLPKI---------- |
| *C. neo* | TLMLGYYPDWSAYYLSPESVDWDR---------- |
| *H. jec1* | -------LAAPGGCTNSSGYISN---------- |
| *H. jec2* | ANETGCINVFDNMIWDWVNPSIEAAIGCTN---------- |
| *C. pos2* | --------VLANVLTKYPSFGGVS---------- |
| *M. ani2* | H----YYDIVDNLNLKVLDFDIEG---------- |

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| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | ----------GVTVIYDDV----AKGYYSYDNRTQE---------- |
| *N. cra2* | ----------GAKVFLDEK----VGASWSYDETNKV---------- |
| *Pb*CTS1 | ----------GAGEQMLSIRSGECGATWSYDRPSRT---------- |
| *A. cap2* | ----------GAEEHLESGSKGGCGASWSYDKSSRS---------- |
| *A. cap1* | ----------GAVEHIDSLEKGGCGASWSYDASSRT---------- |
| *E. nid2* | ----------GATEQLDTN----IGASWSYDPSSRE---------- |
| *C. pos1* | ----------GAQVTELED----IAASYSYDKNKRY---------- |
| *C. imm1* | ----------GAQVTELED----IAASYSYDKNKRY---------- |
| *A. fum2* | ----------GATEHVLPD----IMASYSYDATNKF---------- |
| *N. cra1* | ----------GAKEEYDNL----AKATYSYDALSRE---------- |
| *C. alb2* | ----------FDYEDFDPR----KVGALKYDSHSKQ---------- |
| *A. fum1* | ---------SSTPVTTPSPS---SSAVPSSTPAVSETPSPS---------SSAVPS-STP |
| *E. nid1* | STTPTTSTTSTTSTTTPTPSPSPSTASSSTTETVTPSPKPSPSESSTTSETSSLPSTSTP |
| *PbCTS2* | ---------- |
| *C. imm2* | STMQTTSTGSTSTGTGTTSSQVTSSTTISTRSASTETVTTRSQEPPSTTISTRPASTETV |
| *C. alb1* | ----------QSTTTTSSAVTQSTTTTSAAITQSAT---------- |
| *S. cer1* | ----------TVATSKTSAASTSSASTSSASTSQKK---------- |
| *S. cer2* | ----------GTIEQYDPK----YVSAYCFDEKNSI---------- |
| *C. neo* | ----------FDILDFAFAIPNSDGSLYFTDDSSTDSLQRLVTTGHAAGKRVKLSIGGW |
| *H. jec1* | ----------AEILDIMEDRKSYPVQAWYDEKTDSDYLIYN---------- |
| *H. jec2* | ----------ILQPSPLSTTVTLTAYTTLTLQSGTKLSTSVVSAPFSISEVSYQPFIIDY |
| *C. pos2* | ----------GWEYFNAMPGG---------- |
| *M. ani2* | ----------TWVAVQASIERRNLAVKKVQDKWKS---------- |
| *M. ani1* | ----------LISYDTPDITKEKVTYLKSKGL------GGSMFWEASADR------Q |
| *N. cra2* | ----------MVSYDTPEMVKQKVSYIKEKGL------GGAMYWEASGDR------T |
| *Pb*CTS1 | ----------MITYDTVPMVEEKTRYLIERGL------GGGMWWEASGDRDPKTGNK |
| *A. cap2* | ----------MISYDTVPMVEKKTKYIIDKGL------GGGMWWEASGGRDPRTAEK |
| *A. cap1* | ----------MISYDNVAMVEEKTKYIIQKGL------GGGMWWESSGDRDPRNGDK |
| *E. nid2* | ----------MVSYDTVAAADLKAAYIQSRRL------GGAMWWETSADKGGKTANK |
| *C. pos1* | ----------LISYDTVKIAGKKAEYITKNGM------GGGMWWESSSDK------T |
| *C. imm1* | ----------LISYDTVKIAGKKAEYITKNGM------GGGMWWESSSDK------T |
| *A. fum2* | ----------LISYDNPQVANLKSGYIKSLGL------GGAMWWDSSSDK------T |
| *N. cra1* | ----------LITYDNVLSALVKTKYIFLRGL------GGAVFWEASGDK------T |
| *C. alb2* | ----------LITFDNPQCARIKASFVQSRQL------GGGMWWDSAGDVS----VT |
| *A. fum1* | VAS----------STP--VVPGTSASSSPVSSSSAIAP---STPVVPG-TSTPSSTPVAS |
| *E. nid1* | VVS----------ETPSETKTPTSSSAPPLSSSSPVGG---SSSTASSSTSTPSETPSAS |
| *PbCTS2* | ---------- |
| *C. imm2* | TTRSQ--------EPPSSTISTRSASTETVTTRSQEPP---SSTISTRSASTETSTSSQD |
| *C. alb1* | ----------TTSAAVTTKSNQIVTSSSSS----SSSIFYGNSTTESSTGIAT |
| *S. cer1* | ----------TTQSTTSTQSKSKVTLSPTA----SSAIKTSITQTTKTLTSST |
| *S. cer2* | ----------FISYDNTKSVKTKAEYVTHNNL------GGGFWWESCGEAY----AN |
| *C. neo* | TGSAYFS-----TIVADDSLRATFVSNIYDIYNQYN---LDGIDIDWEYPGTAGADGNAV |
| *H. jec1* | ----------QTQWVAYMSKSVKEARIKKYQALN---FGGISNWAIDLEEWWVDMYD |
| *H. jec2* | PDLLSKS-----DGQMITYNPTPQITPDPITIRIPDGWTVTGGHLASPGSGPGSSSGPSS |
| *C. pos2* | ----------ISRPWEWAASISLIVGMKTVLAC------ATSALYSKLATA------- |
| *M. ani2* | ----------EGKDIAIWYTLPILPTGLTPEGMN----VLSDAKAKGVELAGVNVMTM |
| *M. ani1* | GPDSLIGTS---------- |
| *N. cra2* | DKDSLMTVV---------- |
| *Pb*CTS1 | ANGSLIGTF---------- |
| *A. cap2* | AKGSLIGTF---------- |
| *A. cap1* | ANGSLIGTF---------- |
| *E. nid2* | ADGSLIGTF---------- |
| *C. pos1* | GNESLVGTV---------- |
| *C. imm1* | GNESLVGTV---------- |
| *A. fum2* | GSDSLITTV---------- |
| *N. cra1* | GAESLIGTL---------- |
| *C. alb2* | NDGCLVKNF---------- |
| *A. fum1* | STPVVPGTS----------ASSSPVSSSSAVASSTPVVP----GTSV |
| *E. nid1* | STRAVSETSTHISTSTSSGPETSLTGSSTSVPATSSSVPSSAISPSSTPVIS----ETPR |
| *PbCTS2* | ---------- |
| *C. imm2* | SPSTTISTKSAPTG----------TVTTRSQDLPSTTISTRSPETETETVTT----KSQD |
| *C. alb1* | GTVLPTGSN---------- |
| *S. cer1* | KTKSSLGTT---------- |
| *S. cer2* | ESRSLINAFNEG---------- |
| *C. neo* | SSDDSANFLIFLQD---------- |
| *H. jec1* | LDGAEKEDTDGLDELFDTCEG---------- |
| *H. jec2* | KPAKATSTTSDDGAG---------- |
| *C. pos2* | ---------- |
| *M. ani2* | DYGNAICQS---------- |
| *M. ani1* | ----------SNKLGGP---DATENLL |
| *N. cra2* | ----------KDGLGTL---DSEKNLL |
| *Pb*CTS1 | ----------VEEIGGTSKLQKVQNVL |
| *A. cap2* | ----------VEGIGGG--LEKHENAL |
| *A. cap1* | ----------VVGVGGK--LETSENVL |
| *E. nid2* | ----------VEDVGGVNNLDRTQNAI |
| *C. pos1* | ----------VNGLGGTGKLEQRENEL |
| *C. imm1* | ----------VNGLGGTGKLEQRENEL |
| *A. fum2* | ----------VNALGGTGVFEQSQNEL |
| *N. cra1* | ----------ATQMKRL---DQTKNLL |
| *C. alb2* | ----------VDQLGGVEVLEKSANNL |
| *A. fum1* | P----------SSTPAIPGGSSS----------SSEAVASSTPLVTLTLT |
| *E. nid1* | PPVTSSSSSTFVSSTSTSTDCSESSTAIGTHSSSSISETPSASTPAASPSTSPETTKTLT |
| *PbCTS2* | ---------- |
| *C. imm2* | SPSITLSTRSSSAETVSTRSQHSSSTTISTKSAPTETGTTSEHSTSMPVSTRSASTETVI |
| *C. alb1* | ----------ENAATTGSGSNT----KLAIS |
| *S. cer1* | ----------TTESTLNS-----VAIT |
| *S. cer2* | ----------LHFNVSSKPSIFQDVRVK |
| *C. neo* | ----------LRAALPSEAIITSATQVW |
| *H. jec1* | ----------TYETLEDIPQDLDSRCASFYILTVLSSQL |
| *H. jec2* | ----------YLLPITFYPTLSYRIPSILTPKPPAPTKLPDNDEHPI |
| *C. pos2* | ---------- |
| *M. ani2* | ----------ANTEGQNIHGKCATSAI |

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| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | NYPDSKYDNMRKQMA---------- |
| *N. cra2* | EYPDSQYDNMKKGMSS---------- |
| *Pb*CTS1 | SFPESKYDNLKNQFPGE---------- |
| *A. cap2* | SFPESQYDNLKAGFAEK---------- |
| *A. cap1* | RYPESKYDNLRAGFPNGTTQH---------- |
| *E. nid2* | SYPDSQYDNLKAGFPSS---------- |
| *C. pos1* | SYPESVYDNLKNGMPS---------- |
| *C. imm1* | SYPESVYDNLKNGMPS---------- |
| *A. fum2* | DYPVSQYDNLRNGMQT---------- |
| *N. cra1* | AYPASRYANIRAGVPES---------- |
| *C. alb2* | HGC---------- |
| *A. fum1* | VSPTPAPSSSE--SSSTDLSSSTQTDVG----------TAPSQPAGPSTTATATTSSSSS |
| *E. nid1* | VFPTPGSSVSTGTTSASTLSSSVPATSGGHTETSTVSTSSANQTPSASTSKPLIPTNSAS |
| *PbCTS2* | ---------- |
| *C. imm2* | TRSQNSDSQSMTVSTRSPSTESITTRSQG-----SPSETFSTKSVPVDTISTELPSQTHS |
| *C. alb1* | TVTDVQKTVITITSCSEHKCVATPVTTG----------VVVVTDIDTVYTTYCP |
| *S. cer1* | SMKTTLSSQITSAALVTPQTTTTSIVSS----------APIQTAIT---STLSP |
| *S. cer2* | KYYLNKYGDGGFLSPYLKHLDSRKQ---------- |
| *C. neo* | PFADSNGNPMTDVSEFAKVLDWILIMNYDVWGSSSTPGPNAPLSDGCGNSTQPLANAYAA |
| *H. jec1* | SLAVEAYKEVSEGYDKKFKWYVEWVKDGINDDLLDFMTGSGEGNKYMDCKWESEVSEGSG |
| *H. jec2* | NTPSSSTSSGRPGDGQQSSGTPRPPSNGQNPSSTPKPPGDGPDPINTQKPPGDGPDPINT |
| *C. pos2* | ---------- |
| *M. ani2* | AFLHSQLKGLHPNKSD----------A |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | STDE---SSTTVGS-GNGNGSGSTTTTAATDSITAAPTATSS--ATATGATSEPVTITTI |
| *E. nid1* | STSTGSVTSTPSAP-GVPSSSAGSDETATTSTTDSEPTSTSSGSVTAKPTTTEPATTTTI |
| *PbCTS2* | ---------- |
| *C. imm2* | TTDSTPVSSSPTIPSGSTTIIPGTASDPVSAPTTTVPPNPTLTLAPSSSTTEDRTTITTI |
| *C. alb1* | LTNSQVYVPVQTVVCTEETCVPSPTSTAQKPKASTTIKGVEKGQTTSYPVVGTTEGVKKI |
| *S. cer1* | ATKSSSVVSLQTATTSTLSPTTTSTSSGSTSSGSTSSDSTAR---TLAKELNAQYAAGKL |
| *S. cer2* | ---------- |
| *C. neo* | VSSWTSAGMPANQITLGVPAYGYIQVSSASSLIQRRSLPLLPHKRSKHAKKASYVTVQNE |
| *H. jec1* | PCSEMKLRVDPSNPGARYIEYTLRDEDGFYKALQASHNIERDWVTFEDYAVRDPCTCPPG |
| *H. jec2* | QKPPGDGPDPSNAHNTPASTKRPGDNQHPTPTPAPIRDIDCKDDSCTRGRDCESDDCLRG |
| *C. pos2* | ---------- |
| *M. ani2* | EIDAMMGTTPMVGVNDVQGEVFYLSDARLVMQDAQKRNLGMVGIWSIARDLPAALTCLRN |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | IVTSYIDICPTGFTTVTTTYTTTYCPGTNTATATATVTN---PPSGPGGAGSQ-TTAPTV |
| *E. nid1* | IVTSYTSICPTGFTTITTTITSTYCPGTASATATAIAPTTDVPGSGSGSSPAQPTITADI |
| *PbCTS2* | ---------- |
| *C. imm2* | ITTSYVTVCPTGFTTVTITYTTTYCPETASLTPTQAPIP----------GAPAPP |
| *C. alb1* | VTTSAQTVGSS-TKYVTIELTSTITPVTYPTSVASNGTN----------TTVP |
| *S. cer1* | NGKSTCTEG-----EIACSADGKFAVCDHSAWVYMECAS----------GTT- |
| *S. cer2* | ---------- |
| *C. neo* | SGGTTDGQVMWYGLLNQGALTLSDGEYVATGGFTRHWDDCSSTPWLKSSESGQIVTYDDP |
| *H. jec1* | GGGTFCRCMNYFVMYKNFPRRIKDASKIDVPDPKELVDEALPRMDELAQLLAWTIPLVRM |
| *H. jec2* | GDCEGENCVEGGKCRGKRCISGGNCKGPKCKTGGPCEGENCEKGGGCAKTLFGDCGSGGC |
| *C. pos2* | ---------- |
| *M. ani2* | STA---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | PEGWTTTVTVCTQCAAKPTTVTLTLPVTETGSTSTDAVPAPPAATGEGSNPTQPSGASPT |
| *E. nid1* | PEGWTTTVTVCTVCAATPTTVTLTLPPATTTEESTSAQPTGEVPSSDGSGSGEVSTTTVV |
| *PbCTS2* | ---------- |
| *C. imm2* | PDGWTTIVTVCPQCAPTPTTVTLTVPTRSAFLP----------APTETRPV |
| *C. alb1* | VFTFEGGAAVANSLNSVWFPVPFLLAAFAF---------- |
| *S. cer1* | CYAYDSGDSVYTQCNFSYLESNYF---------- |
| *S. cer2* | ---------- |
| *C. neo* | QSMNLKAQFAAQAGLRGCNVFSVDGDWTGSSWPLTDAVRSGLGLPAV---------- |
| *H. jec1* | GSLEVSYEDPAVAFSMPVFMLEDAIESIKKIKEIGEKEEEKKQHERVLQILEIVFAILPL |
| *H. jec2* | QGARCFSKRDCFGAQCERLTIKPLPKPDPKSTPATPPKPTCLVDCPKLPECPDWDPLCHD |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |

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| Microorganism[a] | Multiple alignment |
|---|---|
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | GGNGSFSEEPVPPPAVTQVSTSTEIVTLVRPTSSRPLILG------TGTVHPSSTLAVKP |
| *E. nid1* | VVPAPTGNAGDGVPAPGANVGEEYTAAPGSATTSKPLIGGGASGAHTAYPYASSTFHIIP |
| *PbCTS2* | ---------- |
| *C. imm2* | VTVVPVPENPIKNVKPSESGDFVTVTTVAPATVTKTLEYN---------NPVDSDVNVQP |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | VAEGAAAAFGAASLIARGLALAAELGNGALTVVEIVDDPLSAPFAILGLLIGPLGVRAKG |
| *H. jec2* | PCPFSACPVYRRPTGKACTTLQTARDCTEFVSSTRVKTKPTTSWSTTTRTLCETMVDCEA |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | SAKPSGQNSGSSSHVPIPPSYTQEAVSPLSTGAASRVTGLGHGLVLTVLTLSAFFVL--- |
| *E. nid1* | SASAHVPVPSGSGSS---PSGTQGGASPTFTGAGSRYDVVKGVPALVALALSLLAVL--- |
| *PbCTS2* | ---------- |
| *C. imm2* | TGGSS----------PVEFEGGAMTVRSMDVVAKALITAGAAVLGLFLGL------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | SRSGFRSAADARRALDEGKLKLFSEAFRRKDSLVHMIMKQSKSCKP---------- |
| *H. jec2* | ADITATTTITTTHTPDPIESVGPAVPYYGVSWSFGEKEKSSMLADEEAYFSALETPMTTT |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | ---------- |
| *H. jec2* | TTTSAEPSTTDDTPTETTADGPTSTVGPNDLVCGFALYAAFYRFDIVKMVGDWVWDDEGH |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |
| *M. ani1* | ---------- |
| *N. cra2* | ---------- |
| *Pb*CTS1 | ---------- |
| *A. cap2* | ---------- |
| *A. cap1* | ---------- |
| *E. nid2* | ---------- |
| *C. pos1* | ---------- |
| *C. imm1* | ---------- |
| *A. fum2* | ---------- |
| *N. cra1* | ---------- |
| *C. alb2* | ---------- |
| *A. fum1* | ---------- |
| *E. nid1* | ---------- |
| *PbCTS2* | ---------- |
| *C. imm2* | ---------- |
| *C. alb1* | ---------- |
| *S. cer1* | ---------- |
| *S. cer2* | ---------- |
| *C. neo* | ---------- |
| *H. jec1* | ---------- |
| *H. jec2* | KLKKELKGCGALTGWKWYRRDDGSREARFNLPVFLTAGCVESAIKSAGGPGLSCIFAT-- |
| *C. pos2* | ---------- |
| *M. ani2* | ---------- |

↳

*a*: the species (named with binomial name) and the respective GenBank accessions: *Ajellomyces capsulatus 1* (*A. cap1*) (AAF80370), *A. capsulatus 2* (*A. cap2*) (AAG41982), *Aspergillus fumigatus 1* (*A. fum1*) (AAO61685), *A. fumigatus 2* (*A. fum2*) (AAO61686), *Candida albicans 1* (*C. alb1*) (AAG35112), *C. albicans 2* (*C. alb2*) (AAA68015), *Coccidioides immitis 1* (*C. imm1*) (2204242A), *C. immitis 2* (*C. imm2*) (Q1EAR5), *Coccidioides posadasii 1* (*C. pos1*) (P54196), *C. posadasii 2* (*C. pos2*) (AAO88269), *Cryptococcus neformans* (*C. neo*) (XP_572898), *Emericella nidulans 1* (*E. nid1*) (BAA36223), *E. nidulans 2* (*E. nid2*) (BAA35140), *Hypocrea jecorina 1* (*H. jec1*) (DAA05858), *H. jecorina 2* (*H. jec2*) (DAA05857), *Metarhizium anisopliae 1* (*M. ani1*) (AAY32603), *M. anisopliae 2* (*M. ani2*) (AAC33265), *Neorospora crassa 1* (*N. cra1*) (XP_965309), *N. crassa 2* (*N. cra2*) (XP_957924), *Paracoccidioides brasiliensis 1* (*Pb*CTS1) (AAQ75798), *P. brasiliensis 2* (*PbCTS2*) (PAAG_03848.1), *Saccharomyces cerevisae 1* (*S. cer1*) (P29029), *S. cerevisae 2* (*S. cer2*) (Q06350).