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Authors

Yu, J-J
Kirkland, TN
Hall, LK
et al.

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Characterization of a Serodiagnostic Complement Fixation Antigen of *Coccidioides posadasii* Expressed in the Nonpathogenic Fungus *Uncinocarpus reesii*

J.-J. Yu,[1] T. N. Kirkland,[2]* L. K. Hall,[2] J. Wopschall,[2] R. C. Smith,[2] C.-Y. Hung,[1] X. Chen,[1]† E. Tarcha,[1] P. W. Thomas,[1]‡ and G. T. Cole[1]

Department of Medical Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio,[1] *and Departments of Pathology and Medicine, Veterans Administration San Diego Healthcare System and University of California San Diego, San Diego, California*[2]

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***Coccidioides* spp. (*immitis* and *posadasii*) are the causative agents of human coccidioidomycosis. In this study, we developed a novel system to overexpress coccidioidal proteins in a nonpathogenic fungus, *Uncinocarpus reesii*, which is closely related to *Coccidioides*. A promoter derived from the heat shock protein gene (*HSP60*) of *Coccidioides posadasii* was used to control the transcription of the inserted gene in the constructed coccidioidal protein expression vector (pCE). The chitinase gene (*CTS1*) of *C. posadasii*, which encodes the complement fixation antigen, was expressed using this system. The recombinant Cts1 protein ($rCts1_{Ur}$) was induced in pCE-CTS1-transformed *U. reesii* by elevating the cultivation temperature. The isolated $rCts1_{Ur}$ showed chitinolytic activity that was identical to that of the native protein and had serodiagnostic efficacy comparable to those of the commercially available antigens in immunodiffusion-complement fixation tests. Using the purified $rCts1_{Ur}$, 74 out of the 77 coccidioidomycosis patients examined (96.1%) were positively identified by enzyme-linked immunosorbent assay. The $rCts1_{Ur}$ protein showed higher chitinolytic activity and slightly greater seroreactivity than the bacterially expressed recombinant Cts1. These data suggest that this novel expression system is a useful tool to produce coccidioidal antigens for use as diagnostic antigens.**

Coccidioides posadasii is a fungal pathogen that grows as a saprobe in the alkaline desert soil of the southwestern United States, as well as in parts of Mexico and Central and South America (14). Coccidioidomycosis (San Joaquin Valley fever) occurs in susceptible individuals by inhalation of airborne infectious arthroconidia of the saprobic phase. Vaccine development against coccidioidal infection is in progress, and new diagnostic agents are being evaluated. Immunogenic proteins necessary for successful vaccine and serodiagnosis development have been difficult to isolate from culture filtrates of the organism. Furthermore, posttranslational modification and protein conformation have been shown to be important for immunogenicity (6). Ideally, native proteins isolated from *Coccidioides* would be the best antigen source for evaluation of their protective properties against coccidioidal infection and/or use as diagnostic antigens. However, using current technology, most of the antigens are produced in small amounts in *Coccidioides* and are difficult to isolate. In order to produce large amounts of coccidioidal antigens with proper protein folding to retain their immunogenicity, we developed a eukaryotic expression system to overexpress coccidioidal proteins in *Uncinocarpus reesii*, a nonpathogenic fungus closely related to *Coccidioides*. Expression of coccidioidal proteins in a nonpathogenic organism is desirable, since growth of *Coccidioides* spp. requires a biosafety level 3 facility. Although *U. reesii* has been collected from the lungs of wild rodents, it seems to be only a transient and apparently harmless inhabitant of the animals, and its life cycle does not include the production of spherules or endospores, stages that are presumed to be adaptations for the infective process (20). In a murine model, arthroconidia of *U. reesii* failed to cause organ-specific or systemic infection (unpublished observations). Phylogenetic relatedness between *C. posadasii* and *U. reesii* has been well documented (1, 7, 13). *U. reesii* is the closest relative of *C. posadasii* among the *Onygenaceae* so far examined by comparative biochemical, immunological, and molecular studies.

MATERIALS AND METHODS

Cultivation. *Uncinocarpus reesii* UAMH 3881 (ATCC 34534; American Type Culture Collection, Manassas, Va.) was grown on GYE agar (1% glucose, 0.5% yeast extract, 1.5% agar) at 30°C for 1 week to produce arthroconidia for transformation.

Construction of the pCE-CTS1 plasmid used for expressing the *C. posadasii* chitinase protein. A coccidioidal protein expression vector (pCE) (Fig. 1A) was constructed using standard molecular cloning methods (10). The pCE vector contains the promoter and terminator of the heat shock protein gene (*HSP60*) of *C. posadasii* and the hygromycin resistance gene, *HPH*, derived from pAN7-1 (17). The 0.54-kb promoter and 0.41-kb terminator were amplified from the DNA template isolated from an *HSP60* genomic clone (22) by PCR using primer pairs A-B and C-D (Table 1), respectively. To facilitate cloning, restriction sites were added to the 5′ ends of the upstream and downstream primers (primers A to D in Table 1). A 3.9-kb fragment harboring the hygromycin resistance gene (*HPH*) was obtained by digestion of the pAN7-1 plasmid with BglII and XbaI restriction enzymes. The 8,112-bp pCE plasmid was constructed by subsequently cloning the digested *HSP60* promoter (HindIII and SpeI) and terminator (SpeI and BglII) and the *HPH* gene (BglII and XbaI) into the pZErO-2.1 plasmid (Invitrogen, Carlsbad, Calif.). To construct the *CTS1* expression vector, pCE-

* Corresponding author. Mailing address: VASDHCS, 111F, 3350 La Jolla Village Dr., San Diego, CA 92161. Phone: (858) 552-7446. Fax: (858) 642-4398. E-mail: tkirkland@ucsd.edu.

† Present address: Department of Medicine, University of Wisconsin—Madison, Madison, WI 53705.

‡ Present address: College of Pharmacy, The University of Texas at Austin, Austin, TX 78758.

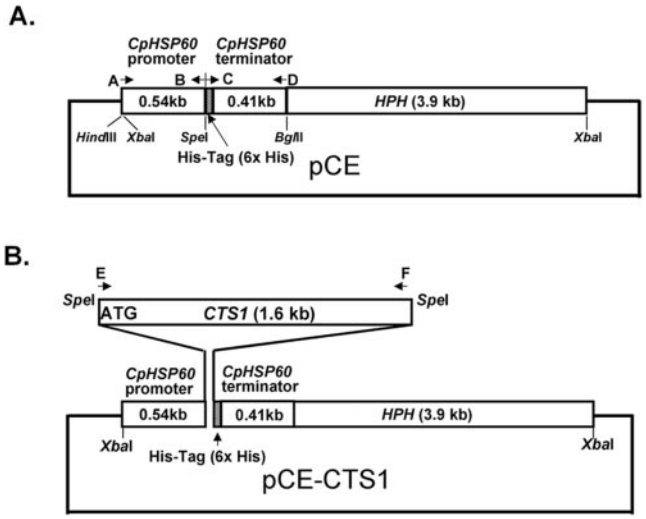

FIG. 1. Representations of pCE vector (A) and pCE-CTS1 plasmid (B). The hatched box represents an 18-nucleotide fragment that encodes the His tag. Primer pairs used to amplify *CpHSP60* (A-B and C-D), as well as *CpCTS1* (E-F), genes are positioned, and their sequences are presented in Table 1. Two XbaI sites used to release the Cts1 expression cassette are indicated.

CTS1 (Fig. 1B), one pair of primers with an engineered SpeI site (primers E and F) (Table 1) was used to amplify a 1.6-kb PCR product using the *CpCTS1*genomic clone as a template (16). A SpeI-restricted *CTS1* fragment was inserted into pCE using the same restriction site to yield the pCE-CTS1 plasmid. This plasmid was then used to transform an *Escherichia coli* strain, TAM-1 (Activemotif, Carlsbad, Calif.). The pCE-CTS1 plasmid was amplified from the transformed bacteria, isolated, and used for subsequent transformation of *U. reesii*.

Transformation procedure. Transformation of *U. reesii* was performed using a method that has been employed successfully for *C. posadasii* (18). Prior to transformation, the pCE-CTS1 plasmid was linearized by XbaI digestion and purified. DNA was taken up by the protoplasts of *U. reesii* in the presence of polyethylene glycol and calcium ion. Transformants were selected on GYE agar supplemented with 75 μg/ml hygromycin B (HmB) and subsequently maintained on 100 μg/ml HmB-GYE agar.

Screening of pCE-CTS1 transformants. To obtain DNA for PCR screening, approximately two inoculating loops of fungal mycelia were isolated from plate cultures of each of the putative transformants or the parental strain and transferred separately to 2-ml microcentrifuge tubes containing 100 mg of glass beads (0.45 to 0.55 mm in diameter) and 0.5 ml of CTAB buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA, 0.2% β-mercaptoethanol). The fungal cells were homogenized using a Mini-Beadbeater (Biospec, Bartlesville, OK) at 3,000 rpm for 60 s, incubated at 60°C for 30 min, extracted with 0.5 ml chloroform-isoamyl alcohol (24:1), and centrifuged (16,500 relative centrifugal force; 10 min). The DNA present in the aqueous phase was precipitated with ethanol in accordance with standard protocols. The transformants were screened for the presence of *CpCTS1* by PCR using primers E and F (Table 1). Expression of His-tagged Cts1 protein from pCE-CTS1-transformed *U. reesii* was examined by Western blot analyses. Putative transformants or the parental strain was grown in 2 ml GYE plus 50 μg/ml HmB or in GYE alone for 5 days at 30°C, followed by 24 h of growth at 37°C. Proteins were prepared from 0.3 ml culture (hyphae plus media) by sonication, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15 μl of the preparation), transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc. Hercules, Calif.), and probed with an anti-His-tag monoclonal antibody (Sigma Chemical Co., St. Louis, Mo.) using standard protocols (2).

Heat shock-induced expression of the *U. reesii*-expressed recombinant Cts1 protein ($rCts1_{Ur}$). The pCE-CTS1-transformed *U. reesii* (no. 8) bacteria were grown in GYE medium on a gyratory shaker at room temperature (25°C) for 4 days. Fungal cultures were allowed to continue to grow at either room temperature or elevated temperature (37°C) for an additional 24 h. The proteins were then isolated from each culture filtrate by ammonium sulfate precipitation (90% saturation) and subjected to SDS-PAGE analyses.

Isolation of $rCts1_{Ur}$. The pCE-CTS1-transformed *U. reesii* (no. 8) was grown in GYE medium at 30°C for 3 to 4 days, followed by overnight growth at an elevated temperature (37°C) for isolation of the expressed $rCts1_{Ur}$ protein. Culture filtrates were collected and subjected to ammonium sulfate precipitation (90% saturation). The protein precipitate was solubilized in water, exhaustively dialyzed against sterile distilled water, and subjected to nickel column chromatography according to the manufacturer's recommendation (Novagen, Madison, Wis). The purified $rCts1_{Ur}$ was analyzed using surface-enhanced laser desorption-ionization time-of-flight (SELDI-TOF) mass spectrometry to determine its molecular mass and homogeneity as described previously (3). The purified $rCts1_{Ur}$ was also subjected to trypsin digestion, followed by peptide fingerprinting analysis using matrix-assisted laser desorption-ionization (MALDI) (12). This procedure was performed to confirm that the isolated recombinant protein was the product of the expression of the pCE-CTS1 construct.

Partial purification of Cts1 protein from *Coccidioides posadasii*. Total protein containing Cts1 was precipitated from 10-day-old mycelial culture filtrate using ammonium sulfate (90% saturation), and the Cts1 protein was further purified by hydrophobic interaction and ion-exchange chromatography. The $(NH_4)_2SO_4$ precipitates were resuspended in 20 mM Tris-HCl, pH 8.0 (buffer A), and 0.4 M $(NH_4)_2SO_4$ and loaded onto a 1- by 10-cm phenyl-Sepharose hydrophobic interaction column. Proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ from 0.4 to 0 M in buffer A. Fractions containing Cts1 were identified by the enzymatic assays described below, dialyzed against 20 mM Tris-HCl, pH 9.0 (buffer B), and loaded onto a Q-hyperD high-performance liquid chromatography column (Beckman, Fullerton, Calif.). The Cts1 protein was eluted with a linear gradient of NaCl from 1 to 0.1 M in buffer B.

Isolation of the bacterium-expressed recombinant Cts1 protein ($rCts1_{Ec}$). The full-length open reading frame (1,281 bp) plus stop codon of the *CpCTS1* gene was amplified by PCR using sense and antisense primers engineered to include restriction sites for ease of subcloning into an expression vector. The nucleotide sequences of these primers were as follows: 5′-ACCATGGGGTTCCTTATTG GCGC-3′ and 5′-TCTCGAGTCAACTTGGCATCCCATTC-3′ (the underlined sequences represent the NcoI and XhoI restriction sites, respectively). The PCR-amplified 1.3-kb product was digested with NcoI and XhoI and ligated into

TABLE 1. PCR primers used to construct pCE-CTS1 plasmid

Primer[a]	Nucleotide sequence[b]	Sequence derivation[c]
A	5′-GAAGCTTTCTAGAGGTTCATGAAACTCGTAGC-3′	*HSP60*
B	5′-GACTAGTCTTGAATGAGTAACGACTAAG-3′	*HSP60*
C	5′-GACTAGTCATCACCATCACCATCACTAAGAGGCTCACAGATTTAC-3′	*HSP60*
D	5′-GAGATCTATAAAACAAATCCCCACACTAAA-3′	*HSP60*
E	5′-CCATACTAGTATGAGGTTCCTTATTGGCGCT-3′	*CTS1*
F	5′-TTCCACTAGTACTTGGCATCCATTCTTGAG-3′	*CTS1*

[a] Primers A to F were used to amplify fragments of *HSP60* or *CTS1*, as indicated in Fig. 1 and described in the text.
[b] Engineered restriction sites (underlined) included in primers A to F (HindIII, SpeI, SpeI, BglII, SpeI, and SpeI, respectively) were used to facilitate cloning. The double-underlined sequence in primer A is an engineered XbaI site used to release the expression cassette (from the 5′ end of *HSP60* promoter to the 3′ end of the *HPH* terminator). The His6 coding sequence is double-underlined in primer C and followed by a stop codon (TAA).
[c] Nucleotide sequences of primers are based on reported DNA sequences of *HSP60* (GenBank accession no. U81786) (22) and *CTS1* (GenBank accession no. L41663) (16).

the same enzyme restriction sites of the pET32b vector (Novagen). The sequence of the plasmid insert was confirmed by DNA sequencing. The pET32-CTS1 construct was used to transform the *E. coli* strain BLR (DE3). The N terminus of the pET32-CTS1 construct contained a 109-amino-acid thioredoxin sequence for enhancing the solubility of the target proteins (9), a polyhistidine (His_6) sequence for facilitating protein purification, and a thrombin site sequence for removing thioredoxin from the fusion proteins. Growth of the transformed cells, induction of expression, and purification of the recombinant protein were conducted according to the manufacturer's protocol. The nickel affinity-purified $rCts1_{Ec}$ was subjected to thrombin digestion using a biotinylated thrombin kit (Novagen) for 2 h at room temperature with the enzyme diluted 1:50 in the reaction solution according the manufacturer's recommendations (Novagen). The biotinylated enzyme was removed at the end of the digestion reaction by streptavidin agarose (Novagen), and the thrombin-released 109-amino-acid thioredoxin with a His tag was removed by nickel affinity chromatography. The mass of the purified $rCts1_{Ec}$ was determined by SELDI-TOF mass spectrometry as described above.

Deglycosylation. Deglycosylation was performed by incubation of the boiled $rCts1_{Ur}$ protein (1 μg) with 500 units of peptide–*N*-glycosidase F (PNGase F) (New England BioLabs, Beverly, Mass.) in a total 13-μl solution for 90 min at 37°C according to the manufacturer's protocol.

Chitinase assay. 4-Methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNac)_2$; Sigma] and 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotrioside [4-MU-$(GlcNac)_3$; Sigma] were used as substrates to determine exochitinase and endochitinase activities, respectively. Chitinase activity was assayed by incubating 20 μl of 100 μM 4-MU-$(GlcNac)_2$ or 4-MU-$(GlcNac)_3$ dissolved in 50 mM sodium phosphate buffer, pH 6.2, and 20 μl of the test sample in wells of a 96-well, flat-bottom, untreated black microtiter plate (Corning Inc., Acton, Mass.) at 37°C for 10 min (11). The reactions were terminated by the addition of 100 μl 1 M glycine-NaOH, pH 10.6. The product of the chitinolytic reaction, 4-MU, is fluorescent under alkaline conditions. Fluorescence was measured using an HTS 7000 microtiter plate fluorometer (Perkin-Elmer, Boston, Mass.) with excitation at 360 nm and emission at 465 nm. One unit of enzyme was defined as the amount of enzyme able to liberate 1 μmol of 4-methylumbelliferone per min under the described assay conditions. Samples were assayed in triplicate.

ID-CF assay. The immunodiffusion (ID) assay was performed by a method previously described (8) for examining complement fixation (CF) antigenicity of the purified $rCts1_{Ur}$ and $rCts1_{Ec}$. The reference *Coccidioides immitis* CF antigen (Ag) and goat anti-CF reference serum were obtained from Meridian Diagnostics (Cincinnati, Ohio). The volume of antigen or serum added to each well of the ID-CF plates was 10 μl unless otherwise specified. Precipitin lines were visible within 48 h and were documented by photography. Comparisons of the protein contents of the reference CF Ag (10 μl) and $rCts1_{Ur}$ (0.25 μg), which were used in the ID-CF assays, were conducted by SDS-PAGE separation and silver staining using a SilverQuest kit (Invitrogen).

Complement fixation assay. The complement fixation assay was performed in the Clinical Microbiology Laboratory at the VA San Diego Healthcare System using standard techniques.

ELISA. Human sera obtained from 77 coccidioidomycosis patients and 31 control individuals were tested for seroreactivity to the recombinant Cts1 by standard enzyme-linked immunosorbent assay (ELISA) methodology. All sera were anonymous except for the CF titer, and the protocol for this study was approved by the University of California San Diego Institutional Review Board. ELISA plate wells (Immulon 2HB Flat Bottom Microtiter plates; ThermoLab Systems, Franklin, Mass.) were coated with 50 ng of $rCts1_{Ur}$ or $rCts1_{Ec}$ in 50 μl of phosphate-buffered saline buffer (pH 7.4) overnight at 4°C. Wells without protein coating were used as a blank control for each serum sample examined. The plates were blocked with 1% casein in phosphate-buffered saline plus 0.1% Tween 20 for 1 hour, and the coated wells were reacted with 50 μl of diluted human sera for 2 h, followed by 45 min of incubation with 50 μl of 1:8,000-diluted HRP-rec-Protein G peroxidase conjugate (Zymed Laboratories, San Francisco, Calif.). The peroxidase substrate (100 μl), tetramethyl benzidine (Sigma), 10 mg/ml in 0.1 M sodium acetate, pH 6.0, with 0.1% H_2O_2, was then added to each well and allowed to develop for 30 min. The reaction was terminated by the addition of 50 μl of stop solution (2.4 N sulfuric acid), and the ELISA plates were read within 30 min at 450 nm in an Emax precision microplate reader (Molecular Devices, Sunnyvale, Calif.).

Statistical analyses. Statistical analyses were performed using the SPSS program (SPSS Inc., Chicago, Ill.). Spearman's rank correlation test was used for analysis of the correlation between the ELISA optical density (OD) and CF titers. The difference in the reactivities of $rCts1_{Ur}$ and $rCts1_{Ec}$ was analyzed by a paired *t* test. A probability value of ≤0.05 was considered to be significant.

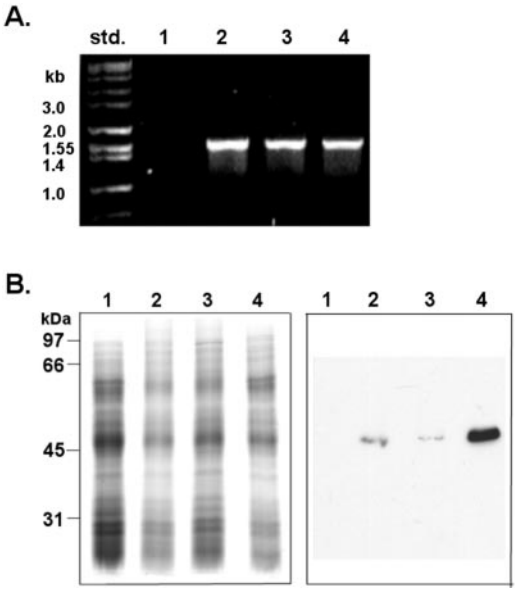

FIG. 2. Confirmation of pCE-CTS1 transformants 2, 3, and 8 (lanes 2 to 4, respectively) by PCR amplification of the inserted *CpCTS1* gene (A) and detection of the expressed $rCts1_{Ur}$ protein (B). (A) Ethidium bromide-stained electrophoresis gel of PCR products amplified from a parental strain of *U. reesii* (lane 1) and the pCE-CTS1 transformants. std., standards. (B) Proteins prepared from cytosol plus culture filtrate of the parental strain (lanes 1) or transformants were separated in a 10% SDS-PAGE and stained with Coomassie blue (left) or detected by anti-His-tag antibodies in a Western blot (right).

RESULTS

Construction of pCE and pCE-CTS1. The 8.1-kb coccidioidal protein expression plasmid (pCE) (Fig. 1A) was constructed as a vector to overexpress proteins of interest. The promoter of the *CpHSP60* gene in the pCE vector was used to control the transcription of the inserted gene, and the terminator was used to provide a poly(A) addition site. His_6-encoding oligonucleotides were introduced for tagging the expressed proteins at their C termini. A stop codon (TAA) (Table 1, primer C) immediately following the His_6 coding sequence was used to terminate the translation of the tagged protein. The hygromycin resistance gene (*HPH*) was included in the pCE vector to allow positive selection of transformants (Fig. 1B). The *CTS1* insert (1.6 kb) is a genomic amplicon containing the full-length Cts1 coding region but without its own stop codon.

Transformation of *U. reesii* with pCE-CTS1. Approximately 5×10^5 protoplasts of *U. reesii* were transformed with 3 μg of pCE-CTS1 DNA. A total of 23 HmB-resistant transformants were obtained. Three out of eight randomly selected HmB-resistant transformants were shown to contain the heterologous *CTS1* gene and to express His-tagged recombinant Cts1 protein by PCR screening and Western analyses (Fig. 2). The 1.6-kb PCR amplicon of the introduced *CpCTS1* gene was evident among the *U. reesii* transformants (Fig. 2A, lanes 2 to 4), but not the parental strain (lane 1). Various amounts of His-tagged $rCts1_{Ur}$ were detectable in the crude preparation of cytosol plus culture filtrate from pCE-CTS1 transformants by Western analysis using the anti-His-tag monoclonal antibodies (Fig. 2B, right, lanes 2 to 4). Clone number 8 of the pCE-CTS1

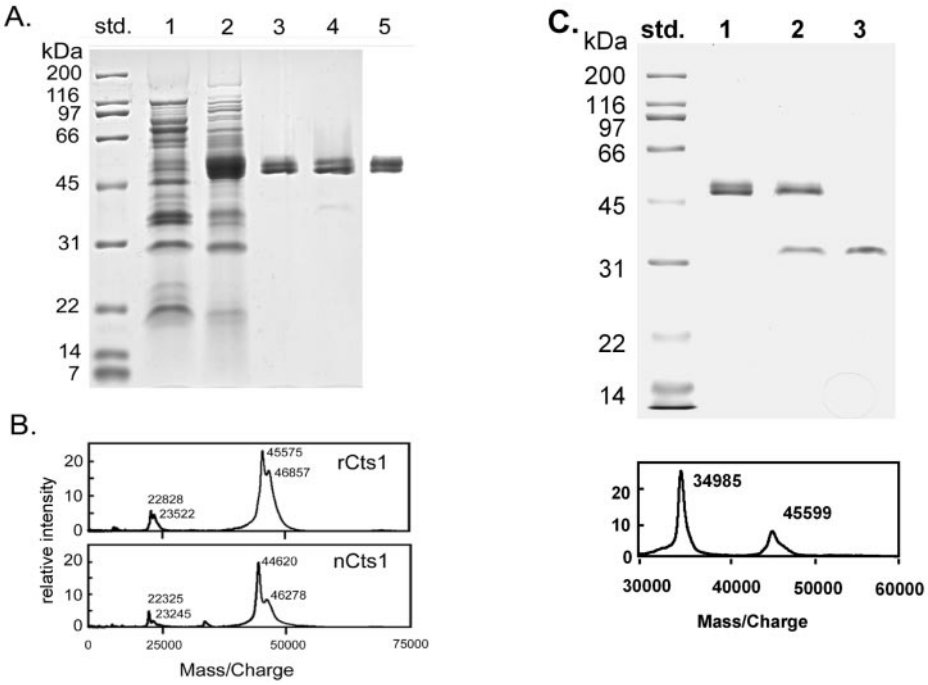

FIG. 3. (A) SDS-PAGE separation of *U. reesii*-expressed recombinant Cts1 protein (lanes 1 and 2) and partially purified native CpCts1 (lanes 3 and 4). Total proteins were prepared from non-heat-shocked (lane 1) or heat-shocked (lane 2) culture filtrate by ammonia sulfate precipitation. $rCts1_{Ur}$ was isolated by Ni affinity chromatography (lane 3) and reacted with an anti-His-tag antibody (lane 5; Western blot). (B) Molecular sizes of $rCts1_{Ur}$ and native CpCts1 (nCts1) were determined by SELDI-TOF mass spectrometry. (C) Deglycosylation of $rCts1_{Ur}$ using PNGase F. Coomassie blue-stained SDS-PAGE revealed untreated $rCts1_{Ur}$ (lane 1), enzyme-treated $rCts1_{Ur}$ (lane 2), and PNGase F alone (lane 3) as a reference. SELDI-TOF mass spectrometry of the enzyme-treated $rCts1_{Ur}$ is also presented. std., standards.

transformants expressed the largest amount of detectable $rCts1_{Ur}$ and was used as the source for scale-up production of $rCts1_{Ur}$.

Induction of $rCts1_{Ur}$ expression by heat shock. To demonstrate that expression of the $rCts1_{Ur}$ protein in the pCE-CTS1-transformed *U. reesii* can be induced by elevated temperature, total secreted proteins were isolated from fungal cultures with or without heat shock treatment and analyzed by SDS-PAGE. Large amounts of $rCts1_{Ur}$ were evident in the sample prepared from the heat-shocked fungal culture (Fig. 3A, lane 2) compared to the sample from the culture grown at room temperature (Fig. 3A, lane 1).

Isolation of $rCts1_{Ur}$ from the pCE-CTS1-transformed *U. reesii*. The secreted $rCts1_{Ur}$, which contained a C-terminal His tag, was isolated from heat shock-treated culture filtrate by ammonium sulfate precipitation and nickel affinity chromatography. One to 5 mg of $rCts1_{Ur}$ can be isolated routinely from 1 liter of culture filtrate. The isolated $rCts1_{Ur}$ contains two species, which was evident in a Coomassie blue-stained (Fig. 3A, lane 3) or a silver-stained (Fig. 4D) SDS-PAGE gel. Two species of native Cts1 protein isolated from *C. posadasii* also can be detected in an SDS-PAGE gel (Fig. 3A, lane 4). Both species of the isolated $rCts1_{Ur}$ contained a C-terminal His tag, which can be detected by an anti-His-tag monoclonal antibody in a Western blot (Fig. 3A, lane 5). The presence of the higher-molecular-weight species of $rCts1_{Ur}$ was due to glycosylation. Glycosylation of Cts1 protein was predicted by the presence of a putative N glycosylation site in the *CpCTS1* gene (16) and confirmed by deglycosylation of the $rCts1_{Ur}$ with PNGase F (Fig. 3C). SELDI-TOF mass spectrometry was used to determine the molecular size of the isolated $rCts1_{Ur}$ (Fig. 3B, top), and the results indicated a mass smaller (45,575 Da) than the predicted molecular mass (46,539 Da) based on the translated gene sequence, with a suggested signal peptide cleavage site between amino acid residues 17 and 18 (16). Results of SELDI-TOF analysis of partially purified secreted Cts1 from *C. posadasii* also showed a molecular mass smaller (44,620 Da) than the predicted mass (45,528 Da) (Fig. 3B, bottom). Peaks at the 22- to 24-kDa range (both $rCts1_{Ur}$ and native CpCts1) (Fig. 3B) represented the doubly-charged Cts1 protein ion. In order to confirm that $rCts1_{Ur}$ is the product of expression of the *CTS1* gene, we performed MALDI-TOF analyses of trypsin digests of the purified recombinant protein (a mixture of the two $rCts1_{Ur}$ species). In Table 2, we report the molecular masses of nine separate peptides derived from the MALDI-TOF analysis of $rCts1_{Ur}$, each of which matched the molecular mass of a predicted trypsin-digested peptide of the translated *CTS1* gene. These nine peptides constitute 40% of the full-length $rCts1_{Ur}$ sequence. The sequence of the first reported peptide in Table 3 was confirmed by tandem mass spectrum analysis and could be the N terminus of the isolated $rCts1_{Ur}$ with a predicted molecular weight of 45,717. A secreted chitinase with an almost identical N-terminal sequence, YYPVPE APAEGGFRAVVYFVNRAIYGR, has been isolated from the 60-h endospore filtrate of *C. posadasii* strain Silveira (19).

Enzyme activity. Results of enzyme assays using either 4-MU-$(GlcNac)_3$ or 4-MU-$(GlcNac)_2$ as a substrate demonstrated that the purified $rCts1_{Ur}$ is a functional enzyme with

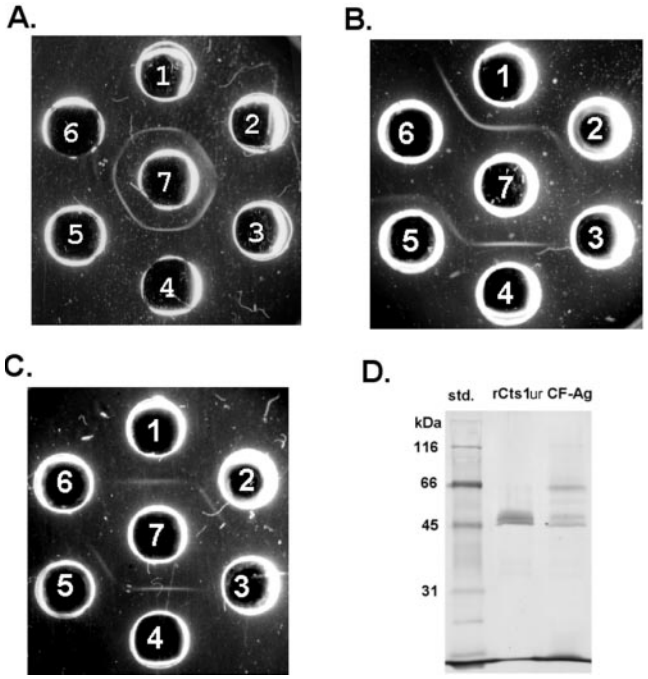

FIG. 4. ID-CF assays (A to C) using the isolated $rCts1_{Ur}$ as an antigen. (A) Samples in wells are reference CF Ag (wells 1 and 4); 1 μg, 0.5 μg, 0.25 μg, or 0.125 μg of $rCts1_{Ur}$ (wells 2, 3, 5, and 6, respectively); and reference CF antibody (Ab) (well 7). (B) Samples in wells are reference CF Ab (wells 1 and 4), sera from coccidioidomycosis patients (wells 2 and 5), $rCts1_{Ur}$ (well 6), heat-treated $rCts1_{Ur}$ (well 3), and reference CF Ag (well 7). (C) Samples in wells are reference CF Ab (wells 1 and 4), sera from patient (wells 2 and 5), sera from healthy individuals (wells 3 and 6), and $rCts1_{Ur}$ (well 7). (D) Protein samples of reference CF Ag or $rCts1_{Ur}$ used in the ID-CF assays were separated in an SDS-PAGE (12%) and stained with silver reagents. std., standards.

both endo- and exochitinase activities (Table 3). The chitinolytic activities of $rCts1_{Ur}$ and native CpCts1 were almost identical when the synthetic 4-MU-$(GlcNac)_2$ or 4-MU-$(GlcNac)_3$ was used as a substrate. Heat treatment (65°C for 10 min or 95°C for 3 min) inactivated the chitinase activities of both $rCts1_{Ur}$ and native CpCts1.

Serologic activity. ID-CF assay and ELISA were used to confirm the seroreactivity of the purified $rCts1_{Ur}$. The results

TABLE 2. Molecular masses of peptides derived from trypsin digestion of the purified $rCts1_{Ur}$ protein measured by MALDI mass spectrometry compared to the predicted molecular masses of translated peptides of CTS1

Translated *CTS1* sequence (start-end)	Molecular mass (Da)	
	Predicted	Measured
SYPVPEAPAEGGFR (26–39)	1,476.71	1,476.42
SVVYFVNWAIYGR (40–52)	1,573.81	1,573.56
HYPGDKWDEPGK (92–112)	1,428.65	1,428.58
TLLSIGGWTYSPNFK (124–138)	1,683.87	1,683.66
KFLLTIASPAGPQNYNK (205–221)	1,862.02	1,861.84
VSGHMSNVFPSTTKPESTPFSSDK (248–271)	2,583.20	2,583.80
IVLGMPLYGR (286–295)	1,134.63	1,134.42
YLISYDTVK (350–358)	1,101.56	1,101.54
TGNESLVGTVVNGLGGTGK (385–403)	1,759.91	1,760.04

TABLE 3. Chitinolytic activities of native CpCts1 and the isolated recombinant Cts1

Cts1	Endochitinase sp act (units/mg)[a]	Exochitinase sp act (units/mg)[a]
CpCts1	1.83 ± 0.09	1.40 ± 0.03
$rCts1_{Ur}$	1.85 ± 0.07	1.50 ± 0.09
$rCts1_{Ec}$	0.37 ± 0.02	0.31 ± 0.01

[a] Definition of unit is described in Materials and Methods.

of a titration of $rCts1_{Ur}$ in the ID-CF assay suggested that 25 μg/ml $rCts1_{Ur}$ has seroreactivity comparable to that of the reference CF antigen provided by the manufacturer (Fig. 4A). Immunodiffusion resulted in a single precipitin line yielding a line of identity between the purified $rCts1_{Ur}$ and the ID-CF reference antigen (Fig. 4B). This suggested that antigenic epitopes are shared by the $rCts1_{Ur}$ and the coccidioidal CF antigen. The CF antigen of *Coccidioides* has been reported to be heat labile (4). Heat treatment (65°C; 10 min) of $rCts1_{Ur}$ abolished its ability to form precipitin lines with either the reference antibody or sera from coccidioidomycosis patients (Fig. 4B). Precipitin lines were visible between wells of $rCts1_{Ur}$ and patient serum in the ID-CF assay (Fig. 4B and C), but not sera from healthy individuals, suggesting that $rCts1_{Ur}$ could be used for the serodiagnosis of coccidioidomycosis. Protein profiles of $rCts1_{Ur}$ and reference CF antigen used in ID-CF assays are shown in a silver-stained SDS-PAGE gel (Fig. 4D). It is evident that the commercial reference CF antigen contains proteins other than Cts1.

A total of 108 serum samples (1:640 dilution) were tested for reactivity to the isolated $rCts1_{Ur}$ by ELISA. The range of adjusted absorbances (absorbance of antigen-coated wells minus absorbance of buffer-coated wells) of 31 CF-negative sera was from 0.000 to 0.226, with a mean of 0.07 and standard deviation of 0.06. A cutoff value of 0.19 (mean plus 2 standard deviations) was imposed to define positive reactivity of sera with $rCts1_{Ur}$. Seventy-four out of the 77 coccidioidomycosis patients examined (96.1%) were positively identified by ELISA (range, 0.271 to 2.571 at a 1:640 dilution) using the 0.19 cutoff value, and one of the 31 control sera (3.2%) was false positive. All three false-negative sera (range from 0.122 to 0.183) had low CF titers; two of them were 1:2, and the third was 1:4. A direct correlation was also observed between the ELISA OD readings and the CF titers ($r = 0.429$; $P < 0.01$) (Fig. 5) of the patient sera.

Comparison of the endpoint titers between the CF-positive and -negative sera showed the same trend as the single OD values, with the median in the CF^+ sera being 51,200 and the median value in the CF^- sera being 1,000 (Fig. 6). This understates the difference, since the sera were only diluted to 51,200. The difference between the two groups is highly significant ($P < 0.0001$; Mann-Whitney U test). If a titer of >64,000 was used to define positive reactions, 5/62 (8.0%) CF-positive sera had false-negative results by ELISA and 1/44 (2.2%) CF-negative sera had false-positive results. The endpoint titers did not correlate with the CF titers. $rCts1_{Ur}$ was tested in the CF assay using six CF-positive sera with a range of titers. The CF titers using the recombinant and the native antigens were the same. Presumably, there is something about the nature of the

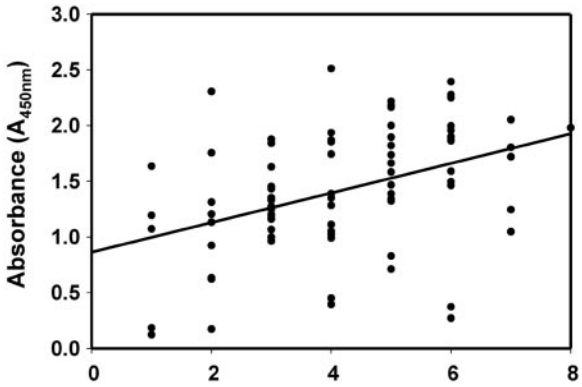

FIG. 5. Correlation of CF titer with reactivity at a single dilution in the $rCts1_{Ur}$ ELISA.

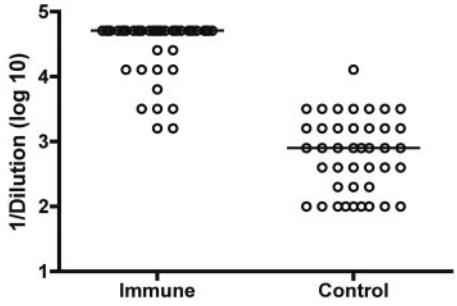

FIG. 6. Endpoint titers of CF-positive and control sera. The endpoint was defined as an OD of 0.20, which is more than twice the value obtained with human serum binding to blank wells. The lines represent the medians of the two groups.

CF test that make titers in this assay not correlate well with ELISA titers rather than a difference between the recombinant and native antigens.

Comparison of $rCts1_{Ur}$ and $rCts1_{Ec}$. $rCts1_{Ec}$ was isolated from the IPTG (isopropyl-β-D-thiogalactopyranoside)-induced pET32b-CTS1-transformed *E. coli* by nickel affinity chromatography, followed by thrombin digestion to remove the fused thioredoxin (Fig. 6A). The digested and purified $rCts1_{Ec}$ contains a 30-amino-acid fusion peptide derived from the pET32b vector at its N terminus and has a predicted molecular weight of 50,416, which agrees with the mass (50,605 Da) determined by SELDI-TOF mass spectrometry. The specific chitinolytic activity of $rCts1_{Ur}$ was fivefold higher than that of $rCts1_{Ec}$ (Table 3). Seroreactivity analyzed using ID-CF (Fig. 7B) and ELISA (Fig. 7C) indicated that $rCts1_{Ur}$ has greater sensitivity than $rCts1_{Ec}$. In an ID-CF test, a minimum of 2 μg $rCts1_{Ec}$ per 20-μl well was required to produced a visible precipitation line to the reference antibody. However, as little as 0.25 μg of $rCts1_{Ur}$ was able to form a visible line under the same reaction conditions. Results of ELISA using serial dilutions of three randomly selected sera and the same amount of antigen, either $rCts1_{Ur}$ or $rCts1_{Ec}$, also showed that $rCts1_{Ur}$ had slightly stronger reactivity with patient sera than $rCts1_{Ec}$ in this assay as well ($P < 0.01$) (Fig. 7C).

DISCUSSION

In this paper, we describe an expression system for the production of *Coccidioides* proteins in the closely related nonpathogenic fungus *U. reesii*. This is the first reported genetic manipulation of *U. reesii*. The construct coding for $Cts1_{Ur}$ contains an upstream *HSP60* promoter, and the temperature shift of *U. reesii* from 25°C to 37°C did induce $rCts1_{Ur}$ production significantly. We chose to produce the CF antigen in this system because it is the major antigen in the diagnostic CF test and the ID-CF tests (4, 16). It has the added advantage of having chitinolytic activity, so we had enzymatic, as well as immunologic, reactivity to evaluate correct folding.

We produced and purified the $rCts1_{Ur}$ protein and studied its enzymatic activity and antigenicity in several types of assays. The conditions for inducing $rCts1_{Ur}$ production and the protocol for protein purification reported here might not be optimal; however, milligrams of $rCts1_{Ur}$ can be isolated from 1 liter of growth medium. The fate of the introduced foreign DNA has not been determined in the pCE-CTS1 transformants. However, based on the larger amounts of $rCts1_{Ur}$ produced in transformant no. 8 compared to others, we believe that multiple-copy random integration might have occurred in this transformant.

The $rCts1_{Ur}$ was glycosylated, and the amino acid sequence matched the predicted amino acid sequence. A significant drawback for the use of a commercially available eukaryotic system to express recombinant proteins is hyperglycosylation, as has been demonstrated for recombinant *Trichoderma* chitinase in transformed *Pichia pastoris* (23). $rCts1_{Ur}$ expressed in *U. reesii* had electrophoretic mobility similar to that of the native Cts1 protein (Fig. 3A), suggesting that hyperglycosylation did not occur in this system. The production of enzymatically active and seroactive rCts1 from *C. posadasii* strain Silveira in bacteria has been reported (5, 24). A minimum of 2.5 μg of protein in 10 μl well was required to produce a visible precipitation line between the chitin affinity-purified rCts1 and the pooled patient sera (5). This result is comparable to our results with $rCts1_{Ec}$ in the ID-CF assays (2 μg per 20-μl well). We compared bacterially and fungally expressed rCts1 of *C. posadasii* strain C735 and showed that $rCts1_{Ur}$ had superior chitinolytic activity and higher seroreactivity than the bacterially expressed $rCts1_{Ec}$ in the ID-CF assay and slightly higher reactivity in the ELISA. These results may be due to better folding of $rCts1_{Ur}$ in the fungal host. We have also demonstrated that heat inactivated chitinolytic activity and abolished seroreactivity, suggesting that conformation is critical for $rCts1_{Ur}$ to be functional.

The CF test is particularly useful because it has prognostic as well as diagnostic value (15, 21). However, it is technically difficult, relatively slow, and labor-intensive, so a convenient assay, such as an ELISA, would be an important step forward. We tested $rCts1_{Ur}$ in an ELISA. We found that 92 to 96% of CF^+ sera were reactive in the ELISA. The lower limit of detection was set high enough that very few of the sera from people who were CF negative had a positive reaction. The OD reading of ELISA also correlated to some degree with the CF titer of the examined sera. However, the endpoint titers of the sera did not correlate with the CF titers. These results suggest caution in replacing the CF assay with the ELISA against $rCts1_{Ur}$. Although the sensitivity of diagnosis of coccidioidomycosis using the CF antigen is high, cross-reactivity of native

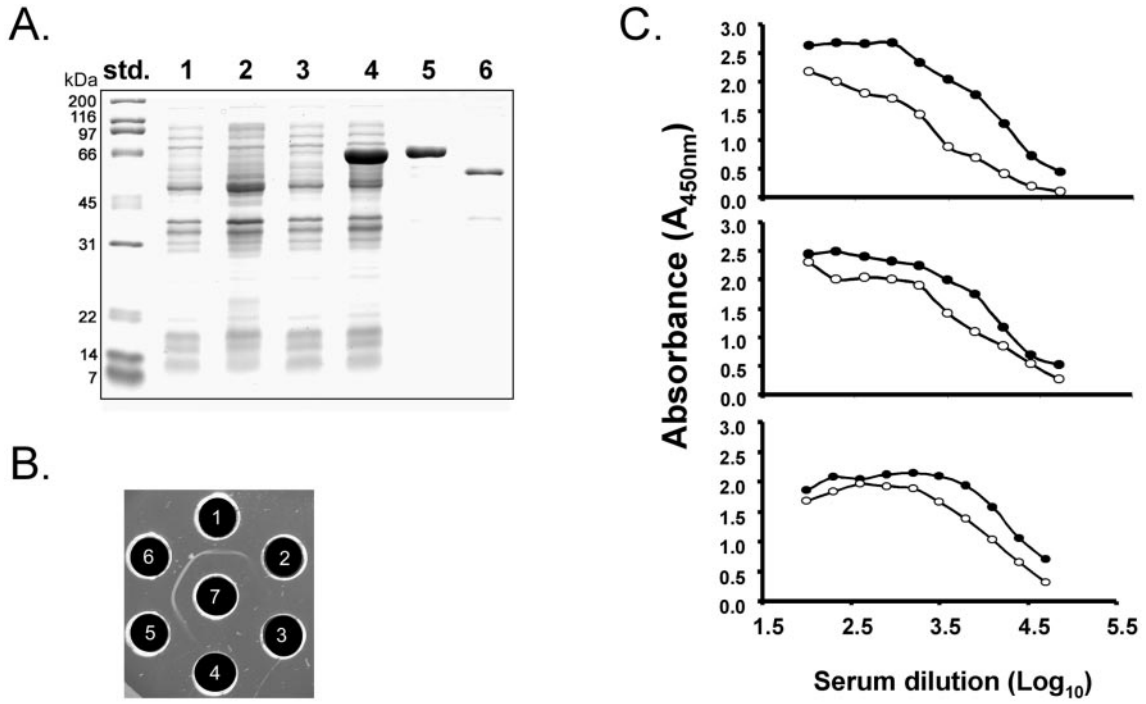

FIG. 7. Expression and seroreactivity of $rCts1_{Ec}$. (A) SDS-PAGE analysis of expression of $rCts1_{Ec}$. Lanes 1 to 4 show electrophoretic separation of cytosolic proteins from bacteria transformed with either pET32b alone (lanes 1 and 2) or pET32b-CTS1 (lanes 3 and 4) without IPTG induction (lanes 1 and 3) and with IPTG induction (lanes 2 and 4). Lanes 5 and 6 show the nickel affinity-purified $rCts1_{Ec}$ before and after thrombin cleavage, respectively. std., standards. (B) ID-CF assays. Samples in wells are 4 μg, 2 μg, 1 μg, or 0.5 μg of $rCts1_{Ec}$ (wells 1 to 4, respectively); 0.25 μg (well 5) or 1 μg (well 6) of $rCts1_{ur}$; and reference antibody (well 7). (C) Comparison of reactivity of $rCts1_{Ur}$ (closed circles) and $rCts1_{Ec}$ (open circles) with sera (1/100 to 1/512,000; twofold dilution) from three coccidioidomycosis patients, using ELISA.

CF antigen and the bacterium-expressed rCts1 with sera from patients with histoplasmosis and blastomycosis has been documented (25, 26). The highest percentage of cross-reactivity has been from the native CF antigen with sera from patients with histoplasmosis. We also found that sera from patients with histoplasmosis reacted in the $rCts1_{Ur}$ ELISA (data not shown). Among the limited numbers of sera we have tested, $rCts1_{Ur}$ reacted positively with more sera from patients with histoplasmosis than sera from patients with blastomycosis. More studies are needed to evaluate serospecificity using $rCts1_{Ur}$ by our ELISA method. However, it is clear that expression of $rCts1_{Ur}$ using *U. reesii* provides proper folding of the expressed protein, which is important for retaining its chitinolytic activity and functional CF antigenicity. This is a major advantage of *U. reeseii* over prokaryotic expression systems. Expression of *C. immitis* proteins in *U. reeseii* may provide a substantial step forward in the expression of *C. immitis* proteins for design of immunologic tests for coccidioidomycosis.

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