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Pishko, Elizabeth J Kirkland, Theo N Cole, Garry T

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# Isolation and characterization of two chitinase-encoding genes (cts1, cts2) from the fungus Coccidioides immitis

(Fungal respiratory pathogen; genomic/cDNA sequences; protein homology; complement fixation antigen)

## Elizabeth J. Pishko<sup>a</sup>, Theo N. Kirkland<sup>b</sup> and Garry T. Cole<sup>a</sup>

\*Department of Botany, University of Texas, Austin, TX 78713, USA; and <sup>b</sup>Veterans Affairs Medical Center, Departments of Pathology and Medicine, University of California, San Diego, CA 92161, USA. Tel. (1-619) 552-8565

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#### SUMMARY

Two chitinase(CTS)-encoding genes (cts) from Coccidioides immitis (Ci), a respiratory fungal pathogen of humans, were cloned and sequenced. Both the genomic and cDNA sequences are presented. The transcription start points and poly(A)-addition sites were confirmed. The cts1 gene contains five introns and a 1281-bp ORF which translates a 427-amino-acid (aa) protein of 47.4 kDa. The cts2 gene contains two introns and a 2580-bp ORF which translates a 860-aa protein of 91.4 kDa. The deduced CTS1 protein showed highest homology to the Aphanocladium album and Trichoderma harzianum CTS (74% and 76%, respectively), while CTS2 showed highest homology to the CTS of Saccharomyces cerevisiae (Sc) and Candida albicans (47% and 51%, respectively). The putative N-terminal sequence of the mature CTS1 protein also showed 89% homology to the reported N-terminal sequence of a 48-kDa complement fixation antigen (CF-Ag) of Ci which has demonstrated chitinase activity. The CF-Ag is a clinically important antigen used in serodiagnosis of this fungal disease. CTS2 showed several of the conserved features of the Sc CTS, including putative catalytic and Ser/Thr-rich domains, and a C-terminal Cys-rich region. We propose that CTS1 and CTS2 of Ci are members of two distinct classes of fungal chitinases, an observation not previously reported for a single fungus.

## INTRODUCTION

Coccidioides immitis (Ci) grows as a saprobe in alkaline soil of the desert Southwest (Paggagianis, 1988). If airborne, infectious propagules (arthroconidia) of the saprobic phase are inhaled by a susceptible host, the fungal cells typically convert into large, multinucleate spherules ( $\geq 20 \,\mu$ m diameter). The parasitic phase cells subsequently undergo a process of segmentation which results in differentiation of a multitude of uninucleate endospores (approx. 2  $\mu$ m diameter), still contained within the maternal spherule. The spherule wall eventually ruptures and the pathogen disseminates in the host by hematogenous and lymphatic spread of the endospores (Cole and Sun, 1985). Chitin is a major structural component of *Ci* parasitic cell walls (Hector and Pappagianis, 1982). A 48-kDa protein with CTS activity has been isolated from the culture filtrate of the parasitic phase of *Ci* and

Correspondence to: Dr. G.T. Cole, at his present address: Department of Microbiology, Medical College of Ohio, Toledo, OH 43699-0008, USA, Tel. (1-419) 381-5423; Fax (1-419) 381-3002; e-mail: gtoole@opus.mco.edu

Abbreviations: aa, amino acid(s); Aa, Aphanocladium alhum; Bc, Bacillus circulans; bp, base pair(s); C., Coccidioides; Ci, C. inmitis; CF-Ag, complement fixation antigen; CTS, chitinase(s); cts, gene(s) encoding CTS;

kb. kilobase(s) or 1000 bp; NCBI, National Center for Biotechnology Information; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; PCR, polymerase chain reaction; Rn, Rhizopus niveus; Ro, Rhizopus oligosporus; RT-PCR, reverse transcription-polymerase chain reaction; Sc, Saccharomyces cerevisiae; Sm, Serratia marcescens; Th, Trichoderma harzianum; T<sub>m</sub>, melting temperature; tsp, transcription start point(s); UTR, untranslated region(s); X, any aa.

suggested to play a role in chitin remodeling and endospore release (Johnson and Pappagianis, 1992). The N-terminal sequence of this CTS has been determined and shown to be identical to that of the serodiagnostic CF-Ag (Johnson et al., 1993). In addition, alignment of the N-terminal sequence of this initially described Ci chitinase (CTS1) with sequences of reported CTS has suggested that the hydrolase is a member of a class of fungal enzymes with homology to bacterial CTS (Blaiseau and Lafay, 1992; Hayes et al., 1994). A second distinct class of fungal CTS has been described, represented by Sc (Kuranda and Robbins, 1991) and Ca (McCreath et al., 1995), which shows protein sequence homology to certain plant CTS (e.g., Cucumis sativis; Lawton et al., 1994). The aims of this study were to isolate and characterize the cts1 gene which encodes the CF-Ag (CTS 1), and probe the genomic library of Ci for presence of a second cts gene (cts2) with homology to the plant-like class of chitin hydrolases.

### EXPERIMENTAL AND DISCUSSION

## (a) Cloning and nt sequence analysis of cts1 and cts2

Degenerate sense and antisense oligo PCR primers were designed on the basis of the reported N-terminal sequence of the Ci CTS/CF-Ag (MPNYYPV; Johnson et al., 1993), and a consensus sequence (DIDWEYP) derived from alignment of Bc CTSA1 (GenBank accession no. S46770), Sm CTSB (X15208), and Aa CHI1 (M57601). The PCR of Ci genomic DNA (strain C634) yielded a prominent band of 515 bp, visible in the agarose gel. The sequenced PCR product translated into a 151-aa ORF and a 62-bp intron in the 5' region (Fig. 1A). A 73-bp intron at the 3' end of the genomic sequence was looped out of the PCR product as a result of hybridization between the antisense primer and template DNA. After alignment of the translated sequence of the PCR product with the Aa CH11, the ORF showed 54.7% identity. We concluded that the PCR product was a fragment of the *Ci cts1* gene.

The PCR product was random hexamer primer-labeled (Pan and Cole, 1992) and used to screen a Ci genomic library. A positive clone was isolated and digested with EcoRI+BamHI to yield DNA fragments in the range 0.8-5.0 kb. A 2.4-kb fragment which hybridized with the labeled PCR product in a Southern blot (Pan and Cole, 1992) was isolated, subcloned into pBluescript II KS, and subjected to sequence analysis. The nt sequence shown in Fig. 1A includes the 515-bp PCR product. A cDNA clone corresponding to the entire predicted coding region of the gene was obtained by RT-PCR as described (Pan et al., 1995). The cDNA was entirely sequenced and confirmed that the genomic DNA contained five introns ranging in size from 59 bp to 73 bp. Each intron contained conserved 5' GT, 3' AG, and lariat sequences characteristic of fungal introns (Ballance, 1986). The cDNA also confirmed the location of the stop codon and identified the site for addition of the poly(A) tail. The primer extension assay (Pan et al., 1995) resolved the position of the terminal Met and putative tsp. The latter corresponds to nt - 100 from the AUG start codon (Fig. 1A). The tsp is located 50 nt downstream from a transcriptional promoter consensus sequence, TATATTA, in the 5' UTR.

Fig. 1. The nt sequence of the Ci cts1 and deduced aa sequence. A: CTS1. The solid line below the nt sequence indicates the 515-bp PCR product used to screen the genomic library. The dotted line near the 3' end of the nt sequence of the PCR product indicates a DNA loop-out site which resulted from hybridization between the antisense primer and template genomic DNA. The double lines and parentheses indicate the conserved 5'/3' and lariat sequences of the introns, respectively. A conserved block of aa (164-DGXDXDXE-171) reported to occur in other CTS are indicated by boldface. The upward arrow indicates the predicted cleavage site of the signal peptide. The square brackets locate a potential N-glycosylation site. The open circle above nt -100 indicates the putative tsp, the solid circle above nt 1871 locates the poly(A) tail addition site, and the asterisk identifies the stop codon. The putative transcriptional promoter sequence (Tata box) is boxed. Methods: The genomic library of Ci (strain C735) was constructed in  $\lambda$ FIXII (Stratagene, La Jolla, CA, USA) as previously described (Wyckoff et al., 1995). The nt sequences of the degenerate sense and antisense oligo PCR primers were as follows: 5'-ATGCCMAAYTAYTAYCCHG (48-fold degeneracy) and GGRTAYTCCCAMTCDATRTC-3' (64-fold degeneracy) (D, A or G or T; H, A or C or T; M, A or C; R, A or G. Y, C or T). The T<sub>m</sub> ranges for the two degenerate primers were 50-60°C and 54-64°C. respectively. Isolation of DNA, Southern hybridization, and DNA sequencing were performed as described (Pan et al., 1995). Sequence analysis was performed using the program DNA Strider (Marck, 1988). Isolation of total RNA for RT-PCR was performed by the method previously reported (Wyckoff et al., 1995). The signal peptide cleavage site was predicted using the method of von Heijne (1986). B: CTS2. The solid line below the nt sequence indicates the 664-bp PCR product used to screen the genomic library. The dotted line near the 5' end of the nt sequence of the PCR product indicates a loop out which occurred by hybridization as described in A, except that in this case it involved the sense primer and template genomic DNA. The conserved sequences of the introns are indicated by double lines and parentheses as explained in A. Conserved blocks of aa in the N-terminal region (34-YWGQ-37; 188-LLSLG-122; 168-DGXDXDXE-175) reported to occur in other CTS are indicated by boldface. The upward arrow indicates the predicted signal peptide cleavage site. The square brackets locate potential N-glycosylation sites. The dashed line below the aa sequence indicates the conserved Ser/Thr-rich domain. Seven conserved Cys residues in the C-terminal region are indicated in boldface enlarged type. The open circle above nt - 123 indicates the putative tsp, and the solid circle above nt - 2807 locates the poly(A) tail addition site. The putative transcriptional promoter sequence (Tata box) is boxed, and the stop codon is indicated by an asterisk. Methods: Same as described in A. The nt sequences of the degenerate sense and antisense oligo PCR primers were as follows: 5'-GTNTAYTGGGGNCARAAY (128-fold degeneracy; Tm = 58-68°C), and CTGCAGTARTTRTTRTARAAYTG (32-fold degeneracy;  $T_m = 56-66^{\circ}C$ ) (N, A or C or G or T, and see A).

	atacgcgcgcgtctactctttgccaaacgggtccactccggactcttgcgggtcaccttgacttctcaggcgggggatcgtcaccatctgacagatgcacccgtcatcgagggcaaAga ccatttgcattcgattctctctattgcattaacctgcttacactgccatccgcgagatctatccattgctccggctaatccctgctgaaaccttctatgaggcctcattctac
-500	
-186	tgcgtgatgtettagtggteaggtgtattg <mark>tatetta</mark> eetggtttgatatttaeeetaagatgtgtteetettetgtattettgtgaatatatat
-66 1	tggggctatatcetettteetggtcaettttteattatcaagatcgacgacagattceatetcaaca ATGAGGTTCETTATTGGCGCTTTACTTACTCACCAAACTETTGTCCAGGCATE M R F L I G A L L T L Q T L V Q A + S
54	TTCGATGTCAAGTATGCCAATTCTTATCCAGTCCCCGAGGCTCCGGCGGAAGGTGGCTTTCGCTCCGTTGTTTATTTTGTGAACTGG_gtaggtLtatgtttacttttccaccgtagac
19	S M S S M P N S Y P V P E A P A E G G F R S V Y F V N W
173	ggccgtgscgttgittaittact)ggracatecag_GCGATCTACGGACGTCGTCATAACCCCGCAAGATTTGAAGGCTGATCACTCAC
48	
	S G E V Y L S D T W A D T D K H Y P G D K W D E P G N N V Y G C I K Q M Y L L K
	<u>AGAACAACCGGAACIITGAAGACGTTGCTTTCGATTGGAGGATGGACCTATTCCCCCAACTTCAAAACCCCCGGCTAGGAGGAAGGA</u>
117	K N N R N L K T L L S I G G W T Y S P N F K T P A S T E E G R K K F A D T S L K
532	TGATGAAGGACCTTGGCTTTGATGGGATTGACATCGATTGGGAG c: aagettggqgtttetececeatettaaaggaatgetattaattettgqqqtegatt $($ ctae $)$ tbggageag T
650	ACCCCGAAGATGAGAAGCAGGCGAATGATTTTGTGTTGTTAGTCAAAGCATGTAGAGAA gtatgatcaaattccaaacgggaaaacaaagacatatacattgttga(ctaan)agtatag
172	Y PEDEKOANDFVLLLKACRE
769	GCACTCGACGCGTACTCCCCAAAACACCCCGAATGGCAAGAAATTCTTGCTCACTATTGCTTCACCGGCAG qtaaqtctagcaagcccaccqtgtgtttttgggaaatctgtc(ctat)a
192	
	ADDOTIVITION AND ADDOTIVITION AND ADDOTIVITION AND ADDOTIVITAD AND ADDOTIVITAD AND ADDOTIVITAD AND ADDOTIVITAD ADDOTIV
215	
	DORTAREDTDTCTCTARACTALDORADDTRACTARDACTARDACTARDACADECODERACABITACADACTARDACADECODERACA
252	
	ACTORNAGE ACTIVISCIES ACTORNAGE ACTIVISCIES ACTIVISCIE
292	
	GTTACGAACTTGAGGATATCGCTGCGTCGGTATGCCTACGAGAACAACCCTACCTGATCAGCTATCAGTTAAGATCGCGGCCAAGAAGCCCGAGTACATTACGAAGAATGG
332	
	ANTOGONOTIGANTOTOGNAGAGAGCAGCAGCAGAAGAAGAAATUGGATICTCOTTGGAACC gtaagtgetttgeattggeggettaggatatgacatactaatag(teat)gea
372	
394	ccasccag GTCGTCAACCGATGGGAGGTACCGCGAAATTICAGCACGCGGAACGACGCTCAGTATCATAACTTCAAGAATGGGAGGAAGAAGTAACTTCA
	aaaacccgctgctgctgcctgcatcactgctatccccgtctttcgtttcaacttgtacatcttagttaactagtcacctttcccgttgtalcttcaacttggtttcacgttggggccca
	actitggetttteatgaatatggettagttatgtgtettaegagaaatatggeegaeetttgttgtattggetttgtttetegatteatgaaegttetetaaacagttaaetagttagte
1844	aggcagttaactatagetetttteeeeaaacetgaaactecatetggatagttaaaaacteeeaeteegaeeteegteeetateeeaaacaetae

## В

-281 aaattgtccgtcgagggagtetgatagccettgtttagtatgggtetetetggettgageeteataatetettegteteetgteeettaaatetttt<mark>tataga</mark>gtgatateteea -161 tttcctctccttgtccatctgcattgcagcagtcatacactgcaaacctctggtttgcgtcctttccttgagtgcactaccagectcacgtcctgttggggccggcttgcttacctgtt 1155 ASTACTSTTACAASTACCACATCTSCCTCGACTTCAACCCAGACTTCAACCCAGACTTCAAASCACTACTATGGAAACCAAGACTTTGTCGGCTTCTACGACCCCGAGCAGTCCGAGTACTGTGTGCG STVTSTTSASTSTQTSSQSTTMETKTLSASTTPSSPSTVS 387 PSSTMQTTSTGSTSIETVTTRSQEPPSTTISTRSASTEP 1395 ACAACGAGAAGTCAAGAGCCACCATCAACCACAATCTCCACAAGGTCAGCTTCCACTGAGACTGTGAGGACAAGAAGTCAAGAGCCACCATCAACCACAATCTCCACATGGTCGGCCTCC TTRSQEPPSTTISTRSASTETVTTRSQEPPSTTISTWSAS 427 1515 ACTGARACTAGTACAAGCAGCCAGGATTCACCATCACCATCACGACAATTTCGACGAAGTCGGCCCCACTGGCACTGCACAACTAGGAGTCAGGATTTACCCTCAACGACCATCTCTACGAGA 467 T E T S T S S Q D S P S T T I S T K S A P T G T V T T R S Q D L P S T T I S T P. 507 <u>SPETETETATTKSQCSPSITLSTRSSSAETVSTRSQHSSS</u> 1755 ACAACAATTTCAACGAAGTCTGCACCAACTGGAACTGGTACGACAAGTGAACATCAACATCAACGCCTGTCTCTACGACGACTGCCCCCCCAGACTGTAATAACGAGAAGTCAGAAT 547 TT I STKSAPTETGTTSEHSTSMPVSTRSASTETVITRSQN S D S Q S M T V S T R S P S T E S I T T R S Q G S P S E T F S T K S V P V D T I 587 1995 TCAACTGAATTGCCTTCTCAAACGCCAACAACGATTATAACGGGAACACCTTCTGATCCTGTATCAGCCCCGACCACCACGGTTCCTCCCCAATCCTGCCCCATGACGCCCCATCTTCC 627 STELPSQTPTTIITGTPSDPVSAPTTTVPP[NPT]LTLAFSS S T T E D R T T I T T I I T T S Y V T V C P T G F T T V T I T Y T T T Y C P E T 667 2235 GOTTOCTCACCCACAGOTCACTACTCCCACCCACAGOTCACACACAGOTCACCCACGOTCACGOTCACCCACGOTCAC  $2714 \ \texttt{attttcttcggaggggtgtgtacataatgtcacgggtgctgtacttgtacatacttgacacttacctacatatacagtacatatatacttcatatccctcgaggtgctgtacatatactgacacttgacacttgacacttgacactatacagtacatatatacttcatatacctcgaggtgctgtacatatacttgacacttgacacttgacacttgacacttgacacttgacactatacacgtacatatacatgtacatatacttcatatacctcgaggtgctgtacacgggtgctgtacttgtacatgtacatgtacatgtacatgacactgacacttgacactgacacttgacacttgacacttgacacttgacacttgacacttgacacttgacacttgacactgacacttgac$ 

The cts2 gene was cloned using the same strategy as described above. Degenerate primers were designed on the basis of aa consensus sequences derived from alignment of Sc CTS1 (M74070), Ro CHI1 and 2 (D10157 and D10158, respectively) and Rn CTS (D10154). The consensus sequences used were VYWGQN and QFYNNYCS. In this case, the PCR product was approx. 660 bp which, after sequence analysis, translated into a 203-aa ORF and included a 55-bp intron (Fig. 1B). As in cts1, the PCR product of cts2 excluded a 61-bp intron as a result of hybridization between the sense primer and genomic template DNA. The translated sequence showed 35.3% identity to the aa sequence of the Sc CTS. The ORF of the PCR product included a conserved aa sequence (DGFDFDIE), which was identical to part of the putative catalytic domain of the Sc CTS (Kuranda and Robbins, 1991). We concluded that the PCR product is a fragment of the Ci cts2 gene. As above, the PCR product was used as a probe to isolate the full length cts2 gene. A clone was isolated and digested with EcoRI+BamHI to yield fragments in the range of 1.5-5 kb. A 2.8-kb fragment which hybridized with the labeled PCR product in a Southern blot was subcloned and sequenced (Fig. 1B). The cDNA clone obtained by RT-PCR was entirely sequenced and proved to be identical to the genomic sequence except that the latter included two introns of 61 and 55 bp. The introns demonstrated the same conserved sequences as described for cts1. The location of the stop codon, site of the poly(A) tail addition, position of the terminal Met, and putative tsp (nt -123 from the AUG start codon; Fig. 1B) were confirmed. The tsp was located 51 nt downstream from a transcription promoter consensus sequence, TATAGA, in the 5' UTR.

# (b) Analysis of the deduced aa sequences of CTS1 and CTS2

The cts1 cDNA contained a single ORF encoding a predicted 427-aa protein of 47397 Da and pI of 5.80. A single potential N-glycosylation site (N-X-S/T) was identified (Fig. 1A). Comparison of the Ci aa sequence with proteins in the GenBank database revealed highest similarity among certain fungal and bacterial CTS, including Aa (Blaiseau and Lafay, 1992), Th (Hayes et al., 1994), and Bc (Watanabe et al., 1990). Percent similarities (based on conservative aa substitutions) and percent identities between Ci and Aa are 74.4% and 49.9%, respectively; for Ci and Th are 75.5% and 49.1%; and for Ci and Bc are 48.9% and 28.6%. A conserved block of aa was identified that contained invariant Asp and Glu residues (Fig. 1A) implicated in the catalytic mechanism of fungal and bacterial CTS (Watanabe et al., 1990, McCreath et al., 1995). The ORF of the Ci protein also

contained a 35-aa sequence in the N-terminus region (aa 18-52; Fig. 1A) which . nowed 8...7% identity to the N-terminal sequence of the Ci (strain Silveira) CTS/CF-Ag reported by Johnson et al. (1993). The predicted cleavage site of the Ci protein in Fig. 1A based on the Von Heijne (1986) rule was between residues 17 and 18, which corresponded to the N-terminus of the CF-Ag. The minor differences between the two aa sequences may reflect Ci strain variation (i.e., strain C735 vs. strain Silveira). Results of these sequence comparisons support our conclusion that cts1 encodes the serodiagnostic CF-Ag, which has been reported to be a CTS and is related to the bacterial class of chitin hydrolases. The genomic and deduced aa sequences of the Ci cts1 gene and CTS1 were deposited in GenBank (accession No. L41663).

The cts2 cDNA contained a single ORF encoding a predicted 860-aa protein of 91390 Da and pI of 4.98. Two potential N-glycosylation sites were identified (Fig. 1B). Comparison of the Ci aa sequence in the region of the putative catalytic domain (aa 22-239; Fig. 1B) with proteins in the GenBank revealed a similarity of 43-51% with fungal and plant CTS (Table 1). Several features of this Ci protein showed striking homology to the Sc and Ca CTS. The Ci protein has several conserved blocks of aa throughout the N-terminal region, including the invariant Asp and Glu residues (DGXDXDXE) identified in Ci CTS1 as part of the putative CTS catalytic domain (Fig. 1B; Milewski et al., 1992). The Ci protein also contains six invariant Cys residues which have been suggested to be involved in correct folding of the catalytic domain (McCreath et al., 1995). The putative catalytic domain of the Ci CTS (aa 22-239) is followed by a variable region (i.e., no evident homology to fungal/plant CTS; aa 240-345), and then by a conserved Ser/Thr-rich domain (aa J46-682; Fig. 1B). This region shows approx. 55% of the aa encoded by either Ser or Thr, which is comparable to the Sc CTS (Kuranda and Robbins, 1991).

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Sequence similarities and identities between C. immitis CTS2 and CTS of other organisms

Sequence source <sup>*</sup>	Similarity <sup>b</sup>	Identity
_	(%)	(%)
Saccharomyces cerevisiae (Sc)	46.9	28.4
Candida albicans CHT2 (Ca)	46.7	28.7
Candida albicans CHT3 (Ca)	51.4	31.1
Cucumis sativis (Cs)	43.0	20.9

<sup>a</sup> CTS sequences of Sc, Ca and Cs (cucumber) obtained from Kuranda and Robbins (1992), McCreath et al. (1995) and Lawton et al. (1994), respectively.

<sup>b</sup> Based on alignment of conservative aa substitutions.

The Ser/Thr-rich domain has been suggested to serve as a potential site for O-mannosylation. The native Sc homolog of this Ci protein has been shown to be highly glycosylated. In the Sc CTS, the Ser/Thr-rich domain is followed by a Cys-rich, high-affinity chitin-binding region (Kuranda and Robbins, 1991). This same putative region of the Ci protein contains seven Cys residues (Fig. 1B) but no sequence homology to the equivalent region of the Sc CTS. These data support our conclusion that cts2encodes a chitinase (CTS2) with homology to the fungal/plant class of chitin hydrolases. The genomic and deduced aa sequences of the Ci cts2 gene and CTS2 were deposited in GenBank (accession No. L41662).

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