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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)

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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 (≥ 20 μm diameter). The parasitic phase cells subsequently undergo a process of segmentation which results in differentiation of a multitude of uninucleate endospores (approx. 2 μm 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

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Abbreviations: aa, amino acid(s); *Aa*, *Aphanocladium album*; *Bc*, *Bacillus circulans*; bp, base pair(s); *C.*, *Coccidioides*; *Ci*, *C. immitis*; 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_m, melting temperature; *tsp*, transcription start point(s); *UTR*, untranslated region(s); X, any aa.

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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.

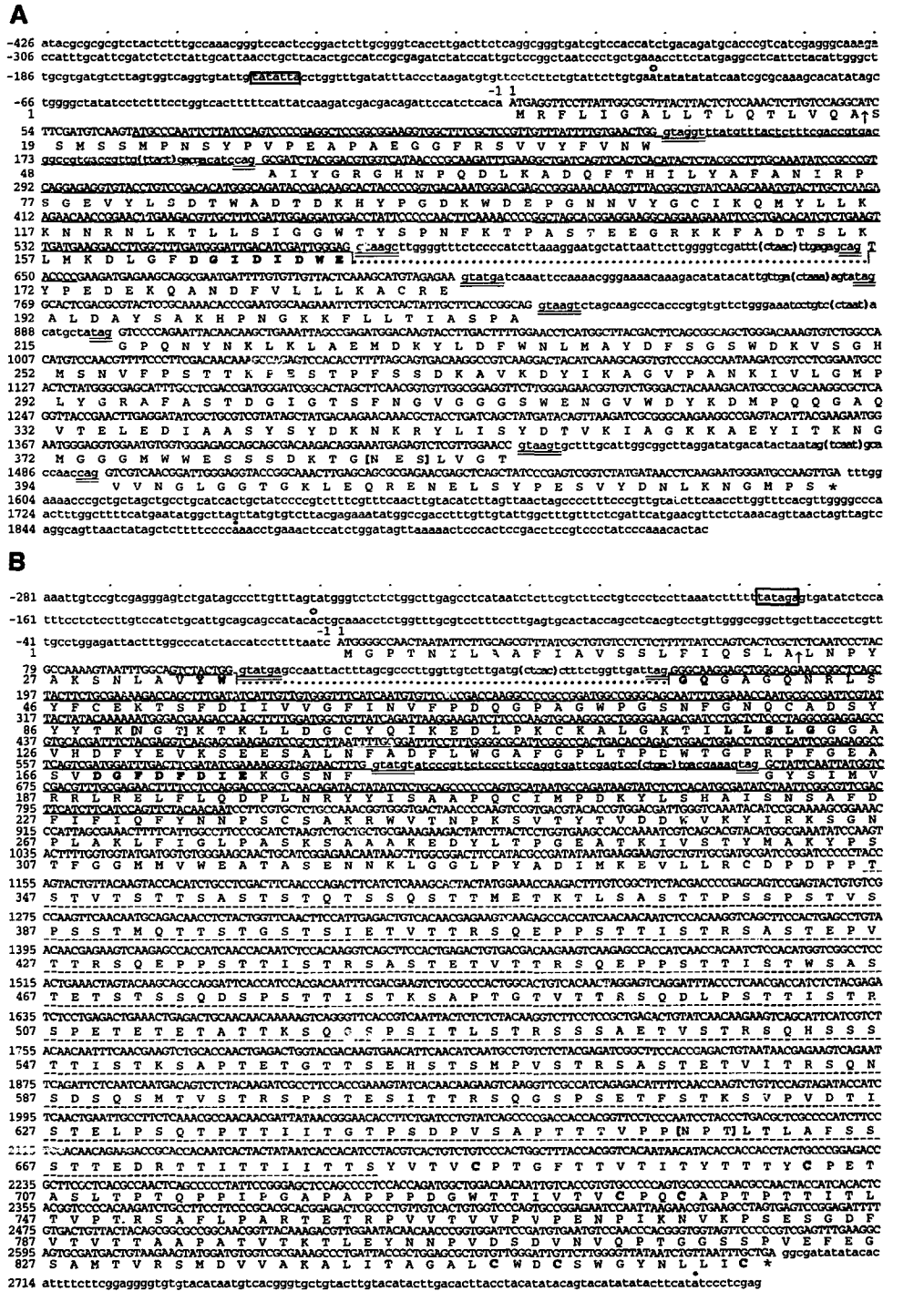
EXPERIMENTAL AND DISCUSSION

(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* CHI1, 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 *Eco*RI + *Bam*HI 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 λFIXII (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_m 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; T_m = 58–68°C), and CTGCAGTARTTRTTRTARAAYTG (32-fold degeneracy; T_m = 56–66°C) (N, A or C or G or T, and see A).


A
B

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 *Eco*RI + *Bam*HI 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 showed 85.7% 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 I). 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 346–682; 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).

TABLE I

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

Sequence source[a]	Similarity[b] (%)	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

[a] CTS sequences of *Sc*, *Ca* and *Cs* (cucumber) obtained from Kuranda and Robbins (1992), McCreath et al. (1995) and Lawton et al. (1994), respectively.

[b] 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 *cts2* encodes 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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REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J.: Basic local alignment search tool. J. Mol. Biol. 215 (1990) 404–410.

Ballance, D.J.: Sequences important for gene expression in filamentous fungi. Yeast 2 (1986) 229–236.

Blaiseau, P.-L. and Lafay, J.-F.: Primary structure of a chitinase-encoding gene (*chi1*) from the filamentous fungus, *Aphanocladium album*: similarity to bacterial chitinases. Gene 120 (1992) 243–248.

Cole, G.T. and Sun, S.H.: Arthroconidium-spherule-endospore transformation in *Coccidioides immitis*. In: Szaniszlo, P.J. (Ed.), Fungal Dimorphism. Plenum Press, New York, NY, 1985, pp. 281–333.

Hayes, C.K., Klemsdal, S., Lorito, M., Di Pietro, A., Peterbauer, C., Nakas, J.P., Tronsmo, A. and Harman, G.E.: Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum*. Gene 138 (1994) 143–148.

Hector, R.F. and Pappagianis, D.: Enzymatic degradation of the wall of spherules of *Coccidioides immitis*. Exp. Mycol. 6 (1982) 136–148.

von Heijne, G.: A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14 (1986) 4683–4690.

Horiuchi, H., Yanai, K., Odazaki, T., Takagi, M. and Yano, K.: Isolation and sequencing of a genomic clone encoding an aspartic proteinase of *Rhizopus niveus*. J. Bacteriol. 170 (1988) 272–278.

Johnson, S.M. and Pappagianis, D.: The coccidioidal complement fixation and immunodiffusion-complement fixation antigen is a chitinase. Infect. Immun. 60 (1992) 2588–2592.

Johnson, S.M., Zimmerman, C.S. and Pappagianis, D.: N-terminal sequence analysis of the *Coccidioides immitis* chitinase/immunodiffusion-complement fixation protein. Infect. Immun. 61 (1993) 3090–3092.

Kuranda, M.J. and Robbins, P.W.: Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J. Biol. Chem. 266 (1992) 19758–19767.

Lawton, K.A., Beck, J., Potter, S., Ward, E. and Ryals, J.: Regulation of cucumber class III chitinase gene expression. Mol. Plant-microbe Interact. 7 (1994) 48–57.

Marck, C.: 'DNA strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acid. Res. 16 (1988) 1829–1836.

McCreath, K.J., Specht, C.A. and Robbins, P.W.: Molecular cloning and characterization of chitinase genes from *Candida albicans*. Proc. Natl. Acad. Sci. USA 92 (1995) 2544–2548.

Milewski, S., O'Donnell, R.W. and Gooday, G.W.: Chemical modification studies of the active center of *Candida albicans* chitinase and its inhibition by allosamidin. J. Gen. Microbiol. 138 (1992) 2545–2550.

Pan, S. and Cole, G.T.: Electrophoretic karyotypes of clinical isolates of *Coccidioides immitis*. Infect. Immun. 60 (1992) 4872–4880.

Pan, S., Zhang, M. and Cole, G.T.: Isolation and characterization of the arginase-encoding gene (*arg*) from *Coccidioides immitis*. Gene 154 (1995) 115–118.

Pappagianis, D.: Epidemiology of coccidioidomycosis. Curr. Top. Med. Mycol. 2 (1988) 199–228.

Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H.: Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. J. Bacteriol. 172 (1990) 4017–4022.

Wyckoff, E.E., Pishko, E.J., Kirkland, T.N. and Cole, G.T.: Cloning and expression of a gene encoding a T-cell reactive protein from *Coccidioides immitis*: homology to 4-hydroxyphenylpyruvate dioxygenase and the mammalian F antigen. Gene 161 (1995) 107–111.

Yanai, K., Takaya, N., Kojima, N., Horiuchi, H., Ohta, A. and Takagi, M.: Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. J. Bacteriol. 174 (1992) 7398–7406.