Cloning, sequencing and characterization of a chitin synthase gene fragment from the fungus *Phascolomyces articulosus*

by

George D. Zouganelis, B.Sc.

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Abstract

Phascolomyces articulosus genomic DNA was isolated from 48 h old hyphae and was used for amplification of a chitin synthase fragment by the polymerase chain reaction method. The primers used in the amplification corresponded to two widely conserved amino acid regions found in chitin synthases of many fungi. Amplification resulted in four bands (820, 900, 1000 and 1500 bp, approximately) as visualized in a 1.2% agarose gel. The lowest band (820 bp) was selected as a candidate for chitin synthase because most amplified regions from other fungi so far exhibited similar sizes (600-750 bp). The selected fragment was extracted from the gel and cloned in the *Hinc* II site of pUC19. The derived plasmid and insert were designated pUC19-*PaCHS* and *PaCHS* respectively. The plasmid pUC19-*PaCHS* was digested by several restriction enzymes and was found to contain *BamH*I and *Hinc*II sites. Sequencing of *PaCHS* revealed two intron sequences and a total open reading frame of 200 amino acids. The derived polypeptide was compared with other related sequences from the EMBL database (Heidelberg, Germany) and was matched to 36 other fully or partially sequenced fungal chitin synthase genes. The closest resemblance was with two genes (74.5% and 73.1% identity) from *Rhizopus oligosporus*. Southern hybridization with the cloned fragment as a probe to the PCR reaction showed a strong signal at the fragment selected for cloning and weaker signals at the other two fragments. Southern hybridization with partially digested *Phascolomyces articulosus* genomic DNA showed a single band. The amino acid sequence was compared with sequences from other chitin synthase gene classes using the CLUSTALW program. The chitin synthase fragment from *Phascolomyces articulosus* was initially grouped in class II along with chitin synthase fragments from *Rhizopus oligosporus* and *Phycomyces blakesleeanus* which also belong to the same class, Zygomycetes. Bootstrap analysis using the neighbor-joining method

available by CLUSTALW verified such classification. Comparison of *PaCHS* revealed conservation of intron positions that are characteristic of chitin synthase gene fragments of zygomycetous fungi.

Table of contents

List of illustrations

List of tables

Introduction

Chitin synthase is the enzyme that catalyses the last step in chitin synthesis where N-acetyl glucosamine units are linked in a β (1-4) fashion to synthesize chitin fibrils. Chitin synthase was first isolated by Glaser and Brown in 1957. Since then chitin synthases have been isolated from many other fungi. Chitin synthase has been implicated in the growth, branching (Gooday, 1971), septum and lateral wall formation in *Saccharomyces cerevisiae* (Bulawa, 1993) and in transition from yeast to hyphal forms in *Candida albicans* (Masyiki *et al.*, 1993). The isolation of mutants lacking particular chitin synthase activities was invaluable for cloning, sequencing and assigning functions in several genes from species such as *Neurospora crassa* (Yarden and Yanofsky, 1991; Din and Yarden, 1994) and *C. albicans* (Chen-Wu *et al.*, 1992; Au-Young and Robbins, 1990; Mio *et al.*, 1996). For example, the dimorphic fungus *C. albicans* contains three genes for chitin synthase: *CanCHS1*, *CanCHS2* and *CanCHS3*. *CanCHS1* is responsible for the synthesis of septal chitin, because when the other two genes were disrupted, calcofluor white (a stain that binds to chitin) was primarily visible at the septum. Disruption of *CanCHS2* affected more the levels of chitin content in the yeast form than in the hyphal form, suggesting that *CanCHS2* plays an important role in dimorphism. *CanCHS3* disruption reduced the levels of chitin in both hyphal and cell forms and the mutants appeared swollen, suggesting that *CanCHS3* may play an important role in morphogenesis (Mio *et al.*, 1996).

Phascolomyces articulosus is a resistant host to the biotrophic mycoparasite *Piptocephalis virginiana*, whereas the related fungus *Choanephora cucurbitarum* is a susceptible host. Manocha and Graham (1982) implicated chitin synthase activity at the infection site to host resistance. In an *in vivo* study using autoradiographic techniques, they demonstrated that the activity of chitin synthase as measured by

the degree of incorporation of N-[H^3]acetylglucosamine ([^{3}H]GlcNAc) into chitin at the penetration sites varied as a function of host-parasite compatibility. At 18 and 24 h after inoculation, the label incorporation was higher at the penetration sites in *P. articulosus* than in *C. cucurbitarum*, coinciding with the formation of papilla and sheath in the former species.

Manocha and McCullough (1985) carried this work further and observed similar patterns of localized incorporation of [^{3}H]GlcNAc in the mechanically wounded hyphae of susceptible and resistant hosts. Label incorporation at the penetration as well as the wounding sites was inhibited by polyoxin D (chitin synthesis inhibitor). Cycloheximide (protein synthesis inhibitor) treatment greatly increased the label in subapical regions and reduced that at the hyphal tips in both hosts, whereas this treatment did not prevent localized incorporation at the penetration sites on the resistant host. Suppression of localized incorporation of [^{3}H]GlcNAc at penetration sites in *C. cucurbitarum* was not due to the lack of mobility of chitin synthase to the penetration site or its activating factor. Consequently, absence or negligible incorporation of N-[H^3] acetylglucosamine at the penetration sites on the susceptible host 18 h after inoculation could not be due to the inability of the susceptible host to mobilize chitin synthase to the penetration site or to activate the zymogen form of chitin already present at the penetration site.

Manocha and Begum (1985) compared the biochemical properties of chitin synthases from the resistant and susceptible hosts in an attempt to explain the different host responses at the penetration sites. Crude chitin synthase was extracted from young (24 hours) hyphae of *C. cucurbitarum* and *P. articulosus* and its activity was characterized by measuring the incorporation of the substrate [^{14}C]UDP-N-acetylglycosamine (UDP-GlcNAc) into chitin. The enzyme activity was mainly associated with the mixed membrane fraction. Properties of the enzyme

preparation such as activation by proteases, UDP-GlcNAc, and response to pH were examined. Enzyme activity from both fungi displayed basically the same features as the corresponding enzymes reported from other mucoraceous fungi. However, the two enzyme preparations differed from each other in their response to various commercial proteases and storage at 4°C. All proteases activated the *P. articulosus* enzyme but only acidic proteases activated the enzyme from *C. cucurbitarum*. Consequently, Manocha and Balasubramanian (1988) studied the effect of a neutral protease from *P. viriginiana* on the chitin synthases from the two hosts and concluded that such an activity was suppressed in both hosts alike.

Differences in structure, activation and expression of the chitin synthase genes may account for distinct responses of the two hosts to the mycoparasite invasion. To elucidate such details chitin synthase genes from the two hosts must be cloned and characterized. In this study the chitin synthase from the resistant host is dealt with. The easiest route to the cloning of chitin synthase genes from the two hosts should have been by cross-hybridization to chitin synthase genes from other fungi. This method was used to clone two chitin synthase genes from *Rhizopus oligosporus* using *CHS2* from *S. cerevisiae* as a probe (Motoyama *et al.*, 1994). However, low stringency Southern blotting experiments between most chitin synthase genes showed no cross-hybridization (Bulawa *et al.*, 1986; Au-Young, *et al.*, 1990; Valdivieso *et al.*, 1991; Szaniszlo and Momany, 1993). Despite this apparent lack of DNA level conservation, the derived amino acid sequences of chitin synthase genes share a region of high homology (Au-Young and Robbins, 1990). Based on this region Bowen and coworkers (1992) designed degenerate polymerase chain reaction (PCR) primers to amplify chitin synthase related fragments from 15 different fungi. A PCR fragment was employed as a probe for the isolation of *CHS2* from *S. cerevisiae* (Bulawa and Osmond, 1990). Similarly, the

PCR strategy employed previously could be used for cloning and characterization of chitin synthase genes from the mucoraceous fungus, *P. articulosus*, which is resistant to the mycoparasite.

The objectives of the current research were to:

1. Amplify a chitin synthase gene fragment from *Phascolomyces articulosus*.
2. Clone this fragment and characterize it by restriction fragment analysis and by nucleotide sequencing.
3. Test the homology of the fragment with *Phascolomyces articulosus* genomic DNA and secondary amplification products
4. Employ sequence alignment techniques such as CLUSTALW to compare and classify the fragment with other chitin synthase fragments

Literature review

Fungal chitin

Chitin, a linear polysaccharide composed of β (1-4)-linked *N*-acetyl glucosamine units is one of the most abundant polymers found in nature. It is a component of the exoskeleton of insects and crustaceans and it is present in the cell wall of some fungi and algae. Structural studies of chitin revealed that the chain length varies in different organisms. The exoskeleton of crabs such as *Scylla seratia* contain polymers as large as 5000 to 8000 units, whereas yeasts such as *Saccharomyces cerevisiae* contain in their cell walls chains of about 100 units (Cabib, 1987). The chains are associated with one another by very strong hydrogen bonding between the >N-H groups of one chain and the >C=O groups of the adjacent chain. Such bonding accounts for the high insolubility of crustacean and fungal fibrils. Fibril chitin is found in three forms: α-chitin which is prevalent in crustaceans and fungi and has an antiparallel arrangement of adjacent chains, β-chitin chains in which are parallel with each other and γ-chitin in which two of these chains are parallel and the third is antiparallel (Gooday, 1971).

The coexistence of chitin with other fungal polysaccharides was an essential criterion for fungal taxonomy (Bartnicki-Garcia, 1968). Basidiomycetes and Ascomycetes have glucan and chitin as predominant building blocks of their cell walls, whereas Zygomycetes cell walls contain chitin and chitosan. Chitin is also present in lower fungi such as two classes of Mastigomycotina, Chitridiomycetes and Hypochitridiomycetes. The vast majority of the Oomycetes on the other hand contain a combination of glucan and cellulose in their cell walls.

Chitin occurs primarily at the apex. Gooday (1971) reported, using microscopic autoradiographic techniques, that 50 times more chitin was deposited at the apex than 50 to 75 μm behind the apex. Non-apical deposition of chitin,

occurs during septum formation, during formation of side branches and during the rare intercalary elongation of cells in such specialized structures as the stipes of Agaric fruit bodies. Chitin was also shown to increase three times in the walls of invasive mycelia of *C. albicans* as in the walls of the yeast form (Mio *et al.*, 1996)

Biochemistry of chitin synthesis

The enzyme responsible for chitin biosynthesis in many fungi and invertebrates is chitin synthase and it catalyses the following reaction:

2n UDPGlcNAc ------> 2n UDP + [GlcNAc-β-(1-4)-GlcNAc]n

From the biochemical point of view, chitin biosynthesis can be considered as a transglycosylation reaction, where acetylglucosaminyl residues are transferred from a donor to an acceptor. The universal donor for the reaction is the nucleotide uridine-diphosphate-N-acetyl-glucosamine, while the acceptor is the growing chitin chain (Sentardeu *et al.*, 1994).

Table 1: General properties of fungal chitin synthases (Ruiz-Herrera,1991)

Parameter	Properties
Substrate	UDPGlcNAc
Products	Chitin, UDP
Acceptor	Lipids?, protein?
Kinetics	Cooperative
Km	0.5-3.0 mM
Hill's number	1.0-4.0
T_{opt}	15-40 °C
pH_{opt}	5.8-8.0
Activating metals	Mg^{+2}, Co^{+2}, Mn^{+2}
Activators	Several proteases
Stimulators	UDPGlcNAc, GlcNAc
Inhibitors	Polyoxins, nikkomycins

Chitin synthesis was first observed *in vitro* by Glaser and Brown (1957), who isolated a mixed membrane fraction obtained by high speed centrifugation of cell-free extracts of *Neurospora crassa* and was able to synthesize chitin. Glaser and

Brown, showed the utilization of UDPGlcNAc as a substrate and further studies supported that view (Porter and Jargowski, 1966), (Camargo *et al.*, 1967). Additionally, incubation with *Mucor rouxii* cell-free extracts with different nucleotides, demonstrated that, only the couple dUTP and GlcNac gave rise to a sugar nucleotide derivative (McMurrough *et al.*, 1971).

The requirement for divalent cations was not demonstrated by Glaser and Brown although they used 10 mM $MgCl_2$ for the incubations. Most further studies have revealed a stimulatory effect of divalent metals, although their efficiency varies according to the metal added to the reaction mixtures. Ruiz-Herrera (1982) pointed out that removal of divalent cations from the membrane preparations of *M. rouxii* by exhaustive dialysis against the chelating compound EDTA (later removed by the same method against water) resulted in Mg^{2+} dependency. Manocha and Begum (1985) noted an equally significant stimulatory effect of Co^{2+} to chitin synthases from *Choanephora cucurbitarum* and *Phascolomyces articulosus*. Sburlati and Cabib (1986) reported that the order of efficiency of divalent kations for chitin synthase 2 is $Co^{2+} > Mn^{2+} > Mg^{2+}$. Optimum pH reported in *N. crassa* was 7.5 but further reports show a ranging optimum pH, from 5.8 in *Mortierella vinacea* to 8.0 in *Coprinus cinereus*. Temperature optima exhibited a more drastic range: the minimal value was reported to be 15 °C in an *Aspergillus flavus* enzyme (Lopez-Romero and Ruiz-Herrera, 1976) and the maximal value of 40 °C was shown by chitin synthase I from *Saccharomyces cerevisiae* (Orlean, 1987).

The kinetics of chitin synthases appear to be non-Michaelian, in other words plots are clearly sigmoidal suggesting cooperative effects. Because of this abnormal behavior values of apparent Km reported, and which oscillate between 0.5-3.0 mM, are only approximate (McMurough and Bartnicki-Garcia, 1971; Adjimani and

Manocha, 1985). Most of them have been calculated with very high substrate concentrations, at which the kinetics look more or less normal.

The observation that chitin synthase from both *Saccharomyces carlsbergensis* and *S. cerevisiae* was inhibited by a heat-stable soluble compound which was susceptible to trypsin treatment opened the way to the finding of proteolytic activation of fungal chitin synthases. The compound, in fact, did not inhibit the enzyme itself, but instead a protease responsible for the endogenous activation of chitin synthase (Cabib and Keller, 1971). The role of trypsin was not to destroy the putative inhibitor, but to activate chitin synthase. Most fungal chitin synthases display proteolytic activation (Manocha and Begum, 1985; Cabib, 1987; Manocha and Balasubramanian, 1988). Some chitin synthases such as *CAL1* and *CanCHS3* are proteolysis independent (Valdiveaso *et al.*, 1991; Masyuki *et al.*, 1993). Proteases exhibit a degree of specificity; for example chitin synthase from *M.rouxii* is stimulated by acid proteases whereas the enzyme from *Phycomyces blakesleeanus* (Gamow *et al.*, 1987) is stimulated by trypsin. Not all proteases are activating; for example proteinase A and carboxypeptidase C are unable to activate the *S. cerevisiae* enzyme which otherwise activated by chymotrypsin and papain (Orlean, 1987). Ruiz-Herrera (1991) purified a soluble acid protease from *M.rouxii* which inactivated chitin synthase. The most accepted hypothesis for the protease activation of chitin synthesis is that the chitin synthase is initially in an zymogenic (inactive) form and then is later activated at the required time and site, triggering localized chitin synthesis (Cabib, 1987). This view can be supported by the fact that inactive chitin synthase from a slime variant of *N. crassa* is 38 kDa polypeptide, where in the case of tripsin activation this polypeptide was missing and was substituted by two other polypeptides of 18 and 21 kDa (Ruiz-Herrera, 1991) .

Two groups of compounds with similar structures have been established as competitive inhibitors of fungal chitin synthesis. These are the polyoxins and nikkomycins. When these compounds are applied to growing cells they give rise to the appearance of abnormally shaped cells, cell bursting and partial lysis (Bowers *et al.*, 1974). The most comprehensive analysis of the interaction between polyoxins and the active center of chitin synthase was performed by Hori and coworkers (1974). They concluded that the UDPGlcNAc pyrimidine binding site of the enzyme interacts with the 2,4-dioxypyrimidine nucleoside moiety of the inhibitor protein containing different substituents. The amino group at C-2 was most important for the inhibitor binding to the enzyme. The methylation of polyoxin D brought about a drastic loss in its inhibitory activity. Hydrogen bondings which stabilized the attachment of polyoxin were established by the carboxyl oxygen atom at C-1, the hydroxyl groups at C-3 and C-4, and the carbamoyloxy group (-O-$CONH_2$) of the carbamoylpolyoxamic moiety. Finally, an ionized amino group (pKa=6.3) and an unionized imidazole group (pKa=7.7) were important for catalysis. In fact, site directed mutagenesis with chitin synthase 2 of *S.cerevisiae*, recently implicated similar residues to the active site (Nagahashi *et al.*, 1995). Considering the structural similarities between polyoxins and nikkomycins similar mechanisms of inhibition should apply.

Chitin synthase is isolated in the form of chitosomes. These are microvesicles measuring 40-100 nm in diameter. Electron microscopy has been used to study the role of chitosomes in the mechanism of fibrillogenesis *in vitro*. Bracker and coworkers (1976) incubated chitosomes with an activating protease, UDP-GlcNAc and GlcNAc (allosteric activator). In the first step, delicate microfibrils appear inside the chitosomes giving the inside a fibroid appearance. The crystallized chitin

gives breakage to the chitosomes which remains adherent to coils and is lost in advanced stages. When the membrane is open straight or coiled fibrils are formed.

In vivo the origin and the fate of chitosomes are speculative. They may originate either from the endoplasmatic reticulum or a multivesicular body (Bartnicki-Garcia, 1987). Leal-Morales and coworkers (1988) showed that chitin synthase 1 from *S. cerevisiae* is both present in chitosomes and plasma membrane. More recently the same group (Leal-Morales *et al.*, 1994) demonstrated that both chitin synthase 1 and 2 are located in the same subcellular fractions despite their functional and genetic differences.

There is no general model describing the catalytic reaction carried out by chitin synthase. Several details about how the reaction is initiated to build up the chain or how many catalytic sites it has remain unresolved. The reaction is thought by some to be initiated by a certain polypeptide (Bartnicki-Garcia, 1979) or a glycolipid (Mills and Cantino, 1978). Data of Hill's (nH) coefficients from *Coprinus cinereus* (Rouset-Hall and Gooday, 1975) which may be indicative of the number of active sites, oscillate from 1.0 to 4.0; but significantly, nH numbers were higher at low concentrations and close to 1.0 at high substrate concentrations. Since UDPGlcNAc is an allosteric activator of the enzyme, it is not likely that most binding sites are allosteric and that the enzyme has a single catalytic site. Therefore not one but tow catalytic polypeptides would have to interact during synthetic reaction, plus another one or two for binding the growing chain (Ruiz-Herrera, 1991).

Fungal Chitin Synthase Genes

The first chitin synthase gene to be cloned was *CHS1* from *S. cerevisae*, which encodes for a plasma membrane-bound zymogen that exhibits the highest activity in yeast cell-free extracts. Bulawa and coworkers (1986) isolated mutants,

lacking chitin synthase activity and used these to clone *CHS1*. The gene had an open reading frame of 3400bp and encoded a protein of 130 Kd. From the hydrophobicity plot, it appeared that the chitin synthase protein contained a hydrophilic region at the amino terminus. The presence of four or five membrane

Table 2: Genes involved in chitin synthesis in *S.cerevisiae* (Bulawa, 1993).

Gene	Essential	Chromosome	Proposed function
CAL1	No	2R	Catalyic component of chitin III. Required for synthesis of spore wall-chitosan.
CSD4	No	2L	Postranslational activator of chitin synthetase III
CAL3	No	12	Required for chitin synthetase III activity
CSD3	No	10L	Unknown
CHS1	No	-	Structural gene for chitin synthase I. Cell wall repair.
CHS2	No	5R	Structural gene for chitin synthase II. Synthesis of septal chitin.
CSD1	No	-	May be required for UDPGlcNAc biosynthesis

spanning domains was consistent with the known membrane localization of the enzyme (Duran *et al.*, 1975). When a plasmid encoding *CHS1-lacZ* fusion protein was transformed into *Schizosaccharomyces pombe* which lacked chitin synthase and chitin, both enzymatic activities were expressed in the same ratio as in *S. cerevisiae* chitin synthase. Furthermore, *CHS1* was disrupted with *URA3* in three different sites. All mutants lacked measurable chitin synthase activities but were viable, containing normal levels of chitin and mated and sporulated efficiently. The authors then concluded that *CHS1* gene is a dispensable form of chitin synthase which displays a high level of activity under the artificial conditions *in vitro*, but which does not play an essential role *in vivo*.

Such contradictory results led to the search for a second gene responsible for chitin synthesis in *S. cerevisiae* (Silverman *et al.*, 1988). Since no mutant for *CHS2* was available, the gene could not be cloned by complementation. Therefore researchers relied on the overproduction of the enzyme conferred by the presence of *CHS2* on a high copy plasmid. Accordingly, a strain containing a *leu2* mutation and a disrupted *CHS1* gene was transformed with a Yep13 plasmid (which contains a *LEU2* gene), genomic library. Chitin synthase activity was measured directly on the colonies of transformants. The transformants that overproduced chitin synthase were selected. Transformants regained chitin synthase activity *in vitro* and this was activated by proteolysis. The evidence that the *CHS2* gene was indeed the structural gene of a different chitin synthase, was also obtained by transformation of *S. pombe*. The activity of the enzyme was inhibited by polyoxin D and the reaction product was hydrolyzed by a chitinase from *S. seratia* as was found for *CHS2* from wild type cells. The conclusion that *CHS1* and *CHS2* are structural genes for chitin synthases has been challenged by Sietsma and Wessels (1990) who observed that *S. pombe* does contain chitin in the cell wall and chitin synthase activity measurable *in vivo*.

The detection of a third form of chitin synthase in the *S. cerevisiae* cell by Bullawa and Osmond (1991) in strains defective in chitin synthase 1 and 2 lead to an investigation for a third chitin synthase gene in *S. cerevisiae*. A gene that was linked with chitin synthase 3 activity was cloned by two independent groups (Bulawa *et al.*, 1992; Valdivieso *et al.*, 1991) and was named *CSD2* or *CAL1* respectively. *CAL1* (*CSD2*) was cloned by complementation of the defect in calcofluor-resistant cal^R1 mutants of *S. cerevisiae*. Calcofluor white is a substance that binds specifically with chitin *in vivo* and exhibits anti fungal activity (Roncero and Duran, 1985; Roncero *et al.*, 1988; Valdivieso *et al.*, 1991). Transformation of

the mutants with a plasmid carrying the appropriate insert restored calcofluor sensitivity, wild type chitin levels and normal spore maturation. Southern blotting using the DNA fragment as a probe showed hybridization to a single locus. Allelic tests indicated that the cloned gene corresponded to the cal^R1 locus. The DNA insert contained a single open reading frame encoding a protein of 1099 amino acids with a MW of 124 kD. Several regions of homology were apparent with those of chitin synthase 1 and 2 from *S. cerevisiae*. cal^R1 mutants were defective in chitin synthase 3, a trypsin independent synthase. Transformation of *CAL1* to *S. pombe* did not result in expression of chitin synthase 3 or the alteration of the natural three other mutants (*cal^R2, cal^R3, cal^R5*) deficient in chitin *in vivo* and in chitin synthase 3 *in vitro*. These facts led to the assumption that the corresponding gene products are required for expression of chitin synthase 3, possibly as subunits of the enzyme or as activators. *CAL2* (*CSD4*) and *CAL3* genes have been cloned and they are thought to be regulated, by transcription, translation or postranslational modification, the synthesis of the active *CAL1* (CSD2) gene product if the chitin synthase is monomeric. Otherwise *CAL2* (CSD4) and *CAL3* may be coding for subunits of the active enzyme (Bulawa *et al.*, 1991).

Chitin synthase genes have been also cloned from a variety of other fungal species, such as the dimorphic fungus *C. albicans*, the filamentous Ascomycetes *N. crassa* and *A. nidulans* and the zygomycetous fungus *Rhizopus oligosporus*. In *C. albicans*, chitin synthase profiles showed that yeast enzyme had an optimum at pH 6.8 and an activity independent of proteolysis that showed a peak at pH 8.0 (Au-Young and Robbins, 1990). These observations lead to the cloning of the first *C. albicans* chitin synthase (*CanCHS*1) by heterologous expression in a S. *cerevisiae chs1* mutant. *CanCHS1* complemented the *S. cerevisiae CHS1* mutation and encoded enzymatic activity which was stimulated by partial proteolysis. The

enzyme present in transformants catalyzed incorporation of [C^{14}]-GlcNAc from the substrate, UDP[U-C^{14}]-GlcNAc. Southern analysis showed hybridization of *CanCHS1* probe only with *C. albicans* DNA and not with *S. cerevisiae*. The pH profile showed an optimum at pH 6.8, suggesting the presence of other forms of chitin synthase in *C. albicans*. DNA sequencing revealed an open reading frame of 2328 bp which predicts a polypeptide of MW 88281 Da with 776 amino acids. *CanCHS1* was homologous (37% amino acid identity) to the *CHS1* gene from *S.cerevisiae*. Hydropathic analysis suggested that it encodes an integral membrane protein.

Table3: Genes involved in chitin synthesis in *N. crassa* (Yarden and Yanofsky, 1991; Din and Yarden,1994) and *C. albicans* (Au-Young and Robins, 1990; Chen-wu *et al.*, 1992; Valvidieso *et al.*, 1991).

Species	Gene	Proposed function
Neurospora crassa	*NcCHS1*	Cell wall biogenesis
	NcCHS2	Structural gene for *NcHS2*
Candida albicans	*CanCHS1*	Structural gene for *CanCHS1*. Synthesis of septal chitin.
	CanCHS2	Structural gene for *CanCHS2*. Cell wall repair
	CanCHS3	Catalytic component of *CanCHS3*. May be involved in morphogenesis

Chen-Wu and coworkers (1991) focused on a second gene that encodes a chitin synthase that requires proteolytic activation. Comparison of derived amino acid sequences from *CHS1*, *CHS2* and *CanCHS1* revealed a region of high homology. Primers corresponding to that region were synthesized and used to amplify a chitin synthase related fragment by the PCR (polymerase chain reaction). A single band of 600 bp was detected and was cloned in M13 vector. Analysis of these clones revealed two different DNA sequences, one corresponding to *CanCHS1* and one to the second gene designated as *CanCHS2*. The PCR fragment

was used as a probe to isolate *CanCHS2* from a lambda genomic library. *CanCHS2* had an open reading frame (ORF) of 3860 bp and encoded 1067 amino acids. The hydropathy plots showed similarity with *CHS1*, *CHS2*, *CanCHS1* genes. Northern Blot analysis from yeast and hyphal forms with the two PCR products showed that *CanCHS2* transcripts strongly present in the yeast and hyphal growth phase, where the response for *CanCHS2* was much lower and was only present in the yeast phase.

A chitin synthase 3 gene (*CanCHS3*), encoding for a non-zymogenic chitin synthase was also cloned from *C. albicans*, using the *CAL1* gene that encodes for chitin synthase 3 in *S. cerevisiae* as a probe (Sudoh *et al.*, 1993). *CanCHS3* had an ORF of 3552 bp encoding a protein of 1175 amino acids and MW of 132 kD. Southern blot analysis indicated that *CanCHS3* is present in one copy in the genome and maps to chromosome I. Northern blot analysis showed that *CanCHS3* was highly expressed in the transition to hyphal growth. This suggested that *CanCHS3* may play an important role in fungal morphogenesis.

Degenerate oligonucleotide primers were used in the amplification of several *CHS*-related sequences from *N.crassa* genomic DNA by PCR. These sequences appear as nonclustered single copies in the genome and were used as probes to clone two chitin synthase genes. *NcCHS1* (Yarden and Yanofsky, 1991) and *NcCHS2* (Din and Yarden, 1994) genes were mapped to linkage group V and IV, respectively. Repeat-induced point mutation (RIP) has been used for the *NcCHS1* gene for inactivation experiments, producing multiple effects. The chs-1 ^{RIP} mutants grow slowly, form abnormal, swollen hyphae and have a 10-fold reduction in chitin synthase activity. No defect was observed in the formation or abundance of hyphal swollen conidia. Inactivation of *NcCHS2* produced progeny which under standard

growth conditions were indistinguishable from the wild type. An increase in sensitivity to the antifungal compound Epidenphos was observed.

In *A. nidulans*, a filamentous ascomycete, five chitin synthase genes have been isolated and characterized (Yanai *et al.*, 1994; Motoyama *et al.*, 1994; Specht *et al.*, 1996). Yanai and coworkers (1994), used *CHS2* of *S. cerevisiae* as a probe to isolate two zymogenic chitin synthases genes, designated as *CHSA* and *CHSB*, from a genomic library. Nucleotide sequencing showed that *CHSA* and *CHSB* encoded polypeptides consisting of 1013 and 916 amino acid residues respectively. Northern analysis indicated that both genes were transcribed, suggesting that cellular chitin in *A. nidulans* is synthesized by at least two chitin synthases. PCR was employed by Motoyama and coworkers (1994) to construct a probe for a third zymogenic chitin synthase gene (designated as *CHSC*) from *A. nidulans*. Nucleotide sequencing and Northern analysis showed that *CHSC* encodes a polypeptide of 914 amino acids and has four introns. Comparison with other chitin synthases reveals high homology with *CanCHS2*. Recently, Specht and coworkers (1996) cloned two more genes (*CHSD* and *CHSC*) from *A. nidulans* that code for non-zymogenic chitin synthases. PCR was employed to amplify fragments of the genes using genomic DNA as a template and two sets of degenerate primers. These fragments were cloned and sequenced. Translation of the first fragment showed homology to amino acids 879-996 of *CHS3* polypeptide of *S. cerevisiae*, whereas translation of the second fragment showed homology to amino acids 833-1112 of the *CHS3* polypeptide. These fragments were presumed to derive from genes *CHSD* and *CHSE*, respectively. Clones from a chromosome-specific cosmid library were probed with the PCR fragments. *CHSD* was found to be localized in chromosome I, whereas *CHSE* was found to be localized in chromosome IV. Restriction fragments containing the genes were subcloned into pUC18, sequenced and the open reading frames (ORF) of the genes

were deduced. *CHSD* encodes a ORF of 1491 amino acids, whereas *CHSE* codes for 898 amino acids.

Two chitin synthase genes (*RhiolCHS1*, *RhiolCHS2*) were isolated from *Rhizopus oligosporus* by plaque hybridization probed with the chitin synthase 2 gene from *S. cerevisiae*. From their deduced amino acid sequences, they were both class II chitin synthases according to the classification proposed by Bowen and coworkers (1992). The expression of these genes was controlled differently in each stage of differentiation. It was suggested that the gene products of *RhiolCHS1* and *RhiolCHS2* function mainly in the hyphae growing stage but not in the late stage of spore formation. When each of these genes was expressed in *S. cerevisiae*, elevation of chitin synthase activity was observed in both cases.

Chitin synthase gene fragments and gene classification

The observation that *CanCHS1* and *CanCHS2* regions can be amplified using PCR enabled several groups to clone and sequence partially (Bowen *et al.*, 1992; Miyazaki *et al.*, 1993; Causier *et al.*, 1994) or fully (Yarden and Yanofski, 1991; Din and Yarden, 1994) other fungal chitin synthases. Bowen and coworkers (1992) amplified approximately 600 bp fragments of chitin synthase DNA from 15 different fungal species. These fragments were sequenced and their deduced amino acid sequences were aligned and grouped by the CLUSTAL program. All sequences with the exception of *CHS1* fell into three distinct classes, which could represent three separate functional groups. Within each CLUSTAL class some close relationships were seen. Similarities were apparent between *A. niger* and *Aspergillus nidulans*, which are known or suspected Ascomycetes of the genus Emericella, between *Ajellomyces* (*Histoplasma*) *capsulatum* and *Ajellomyces* (*Blastomyces*) *dermatitis*, which are both Ascomycetes of the genus Ajellomyces and among the opportunistic pathogens *Emericella jeanselmei*, *Wengiella*

dermatitis, *Phaeococcomyces exophialale* and *Xylophyla bantiana* which are all members of the family Dematiaciae of Fungi imperfecti. Further analysis of class I and class II fragments with a phylogeny inference package (PHYLIP) grouped two Basidiomycetes *Schizophyllum commune* and *Ustilago maydis* and separated *Schizosaccharomyces pombe* into a class by itself. Large evolutionary separation was also observed between *S. cerevisiae*, *S. pombe* and *C. albicans* and other fungi. Should *C. albicans* be an ascomycete like *S. cerevisiae* and *S. pombe*, then it might not be surprising that these three fungi exhibit such large separations from the remaining fungi investigated. Bootstrap analysis was also performed on each chitin synthase class. Bootstrapping is a statistical method used to evaluate the confidence level of the phylogenetic estimate by random resampling of the data. One hundred bootstrap replicates were performed in each class. *E. jeanselmei and P. exophillae* belong in the family Dematiaciae were group together in 95 out of 100 trials, giving a 95% bootstrap confidence limit as a phylogenetic group. *H. capsulatum* and *B. dermatitis* appeared with >99% confidence limit as a group. *C. albicans* and *S. cerevisiae* appeared as outgroups from the other fungal species in 100% of the bootstrap replicates.

In conclusion, Bowen et al. (1992) classified chitin synthases in three "zymogenic type" classes of chitin synthase and a "non-zymogenic type" class (genes similar to *CAL1* or *CanCHS3*, designated as IV). Although this classification was limited because only conserved regions were examined, cloning, sequencing and comparison of full sequences revealed similar relationships between chitin synthase genes (Din and Yarden, 1994). Consequently, all chitin synthase genes cloned are assigned to a class. For example *CHSC* from *Aspergillus nidulans* and the two genes from *Rhizopus oligosporus* belong to class I and II, respectively. With the addition of more sequences new classes were proposed. Mehman *et al.*

(1994) in a study on chitin synthase gene fragments from ectomycorrhizal fungi indicated that certain sequences although similar to class II demonstrate enough difference to constitute a fourth zymogenic class. Recently, Specht *et al.* (1996) isolated two non-zymogenic chitin synthase genes from *A. nidulans* that exhibit similarity with *CAL1* from *S. cerevisiae* but exhibit enough differences among each other to constitute separate classes. In summary chitin synthases can be classified in six classes, four zymogenic classes and two non zymogenic.

Partial chitin synthase gene sequences generated by PCR have also been derived from *P. blakesleeanus* (Miyazaki *et al.*, 1993), *Botrytis cinerea* (Causier *et al.*, 1994), *Phialophora verrucosa*, a large group of ectomycorrhizal fungi (Mehmann *et al.*, 1994), *Aspergillus fumigatus* (Mellado *et al.*, 1995) and *Entomophaga aulicae* (Thomsen and Beauvais, 1995). The *P. blakesleeanus* fragment was 750 bp long and best matched the motifs found in class II zymogen. The fragment included an approximately 160 bp region that was attributed to an intron. Southern hybridization of restriction enzyme digested genomic DNA, using the PCR fragment as a probe suggested that *P. blakesleeanus* contained additional *CHS* genes. Two fragments were cloned from the zygomycetous fungus *E. aulicae*. They were shorter (561 bp) than the fragment from the related fungus *P. blakesleeanus* and they were lacking intron sequences. The deduced amino acid sequences were homologous to class II chitin synthases. The *B. cinerea* fragments were approximately 600 bp and had high homology with the fragments of *Neurospora crassa* (74-85.7 %). When the predicted amino acid sequences retrieved from the database were aligned and used to generate a phylogenetic tree the *CHS* fragments from *B. cinerea* were grouped with the fragments of *N. crassa* (Causier *et al.*, 1994). Two products were amplified by two different primer sets from *P. verrucosa* DNA. The first product (615 bp) contained two distinct chitin

synthase fragments as determined by Southern hybridization. These two fragments were classified among classes I and II according to the Bowen and coworkers (1992) classification. The second product (316 bp) corresponded to a single chitin synthase homologous to *CAL1* from *S.cerevisiae*. In ectomycorrhizal fungi (Mehmann *et al.*, 1994) the PCR products varied from one to four fragments of lengths from 600 to 900 bp. The fragments amplified from the ascomycetous species were approximately of the same size as *S. cerevisiae* (600 bp), whereas the basidiomycetous species gave larger fragments. Cloning and sequencing of the most prominent DNA fragments revealed that these differences were due to various introns at conserved positions. The presence of introns in basidiomycetous fungi therefore has a potential use in identification of genera by analyzing PCR-generated fragments. By comparison of the deduced amino acid sequences, the majority of chitin synthase fragments from ectomycorrhizal fungi belonged to class II according to Bowen and coworkers (1992) classification. In addition CLUSTAL analysis revealed a fourth class of putative zymogenic chitin synthases characterized by a gap and their own set of conserved amino acid residues.

Two approaches were used to isolate fragment of chitin synthase genes from the opportunistic human pathogen *Aspergillus fumigatus*. First, regions of amino acid conservation in chitin synthases of *S. cerevisiae* were used to design degenerate primers for amplification of portions of related genes, and second, a segment of the *S.cerevisiae CAL1* gene was used to screen an *A. fumigatus* λ genomic DNA library. The PCR approach led to the identification of five different genes designated *ChsA*, *ChsB*, *ChsC*, *ChsD* and *ChsF*. *ChsA*, *ChsB* and *ChsC* fall into Classes I, II and III of the zymogen type respectively. The *ChsD* fragment had approximately 35% amino acid sequence identity to both zymogen type genes and the non-zymogen type *CAL1* gene. *ChsF* appears to be a homologue of *CAL1* gene,

being 80% identical to *CAL1* over 100 amino acids. An unexpected finding was the isolation by heterologous hybridization of another gene (*ChsE*), which also had strong sequence similarity (54%) to *CAL1*. Reverse transcriptase PCR was used to show that each gene was expressed during hyphal growth in submerged cultures.

Fungal chitin synthase mutants

Chitin synthase mutants have been identified in *S. cerevisiae*, *C. albicans*, *N. crassa* and *A. nidulans*. In *S. cerevisiae* mutants have been identified for all genes thought to be involved in chitin synthesis (see Table 3). Mutants for *CHS1*, the structural gene for chitin synthase 1 exhibit hypersensitivity to the chitin synthase inhibitor polyoxin D and under certain culture conditions form small abnormal buds that are susceptible to lysis (Bulawa, 1986). This budding effect has been well characterized by Cabib and coworkers (1989). Lysis occurs late in the cell cycle, after the separation of nuclei, and appears to be caused by a defect in cell-wall structure; a hole is visible in the birth scar of the daughter cell, and addition of sorbitol to the medium prevents lysis. The frequency of lysed buds increases in low pH and by inhibition of the chitinase. The studies on the lysis of *chs1* buds led to the proposal that chitin synthase 1, acts as a repair enzyme by replenishing chitin during cytokinesis (Cabib *et al.*, 1989; Cabib *et al.*, 1992).

Mutants of *CHS2*, the structural gene for chitin synthase 2 show a 0-90% range in spore viability, depending on the strains and media used. In certain strains, spores containing *chs2* disruptions cannot form colonies on rich medium with glucose as the carbon source (Silverman *et al.*, 1988). In some strains, this phenotype is partially suppressed, but the recovery of the mutant is low and the colonies are small. In all strains that have been tested, the recovery of the *chs2* mutant is improved when spores are germinated on synthetic medium or on rich medium with glycerol as the carbon source. Other effects in *chs2* disruptants include

abnormal cell separation, multinucleus phenotypes and the formation of large cell clumps. Surprisingly the cellular chitin is elevated almost twofold (Cabib *et al.*, 1989). These effects are less severe in synthetic medium than a glucose rich medium. The most striking effect in *chs2* disruptants is the abnormal formation of primary septa. A comparison between wild type and *chs2* disruptant cells shows that the septa in the former cells are thicker, amorphous and as a result their cell separation is impaired. This fact led Cabib and coworkers (1989) to propose that *CHS2* in *S. cerevisiae* is responsible for the formation of the primary septum.

Table 4: Characteristics of *S. cerevisiae* mutants defective in chitin synthesis (Bulawa, 1993)

Mutant	Enzyme defect	Chitin	Phenotypes
csd2	*CHS3*	<10%	calcofluor resistant, temperature sensitive.
csd4	*CHS3*	< 5%	calcofluor resistant.
cal3	*CHS3*	~20%	calcofluor resistant.
csd3	None	~10%	calcofluor resistant, temperature sensitive.
chs1	*CHS1*	>90%	Forms refractive buds. Hypersensitive to polyoxin D.
chs2	*CHS2*	>90%	Defects in septation and nuclear separation.
sch1	-	>90%	Sporulates poorly
csd1	-	-	Temperature sensitive.

Mutants that lack chitin synthase 3 activity in *S. cerevisiae* exhibit abnormalities that are associated with *CSD2* (*CAL1*) the catalytic component for this enzyme and two other genes; *CSD4*, the postranslational activator and *CAL3* a gene required for chitin synthase 3 activity (Bulawa, 1992). The *csd2* mutants have been studied in most detail. They contain a small amount of residual chitin, 5-10 % of the wild type level, indicating that another chitin synthase is active during vegetative growth. The residual chitin has been localized by staining thin sections of

disrupted cells with gold-labeled wheat germ agglutinin. A thin line of chitin is detected in the division septum; no staining is observed elsewhere in the cell (Shaw *et al.*, 1991). Thus, chitin synthase makes the chitin of the bud scars and the lateral wall, and a different synthase makes the chitin of the primary septum. The absence of chitin in the bud scars of *csd2* cells has also been demonstrated with calcofluor staining (Roncero *et al.*, 1988). The analysis of chitin deposition in certain cell-division (*cdc*) mutants led to the conclusion that chitin synthase 3 makes the chitin in the lateral wall. Several *cdc* mutants, when shifted to nonpermissive conditions, are defective in polarized growth and synthesize considerable chitin in the wall, producing cells that are uniformly and brightly stained by calcofluor (Roberts et al., 1983; Sloat *et al.*, 1989). In contrast, *cdc3/csd2* and *cdc24/csd2* double mutants shifted to nonpermissive conditions stained poorly (Shaw *et al.*, 1991), indicating that *CSD2* (*CAL1*) is required for the accumulation of lateral wall chitin in the *cdc3* and *cdc24* mutants. Several mutants of *CSD4* have been isolated (Roncero *et al.*, 1988), and most are phenotypically identical to *csd2* mutants during vegetative growth. One mutant, assigned *csd4-1*, exhibits a different phenotype; the amount of cellular chitin is reduced only twofold, suggesting that the mutant protein is partially active (Bulawa, 1992). All *csd4* mutants are calcofluor sensitive but *csd4-1* is calcofluor resistant when grown in synthetic buffered medium (Bulawa, 1993).

Santos and coworkers (1992) studied disruptants of *CAL3* and found a four to five-fold reduction in cell wall chitin, a specific loss of chitin synthase activity and calcofluor resistance. Although much is not known about the role of *CAL3* it is there to regulate in conjunction with *CSD4*, by transcription, translation or postranslational modification, the synthesis of the active *CSD2* gene product (Bulawa, 1993).

Certain strains that are deficient in chitin synthase 3 activity exhibit temperature sensitivity on media of low osmolarity. Inclusion of sorbitol and salts in the media or the presence of a suppresser gene cancel temperature effects (Bulawa,1992). Sutton and corworkers (1991) demonstrated that the transformation of a plasmid containing a polymorphic gene that suppresses *csd* mutations to a double mutant with *csd* and temperature sensitive phenotype corrected temperature sensitivity but not chitin synthase activity.

Like *Saccharomyces cerevisiae*, *Candida albicans*, a dimorphic fungus, harbors three chitin synthase genes, designated *CanCHS1*, *CanCHS2* and *CanCHS3*. In order to gain more insights into the physiological functions of the chitin synthases of *C. albicans*. Mio and coworkers (1996) disrupted both *CanCHS2* and *CanCHS3* genes. Four types of mutants were examined in this study: homozygous disruptants of *CaCHS2* (*chs2Δ*); homozygous disruptants of *CanCHS3* (*chs3Δ*); heterozygous disruptants of *CanCHS3* (*CHS3/chs3Δ*) and double homozygous disruptants in both *CanCHS2* and *CanCHS3* (*chs2Δ/chs3Δ*). The *chs2Δ* null mutants were indistinguishable from the parental wild type cells, cellular chitin was reduced to about 80 % in both hyphal and yeast forms and were as well stained by calcofluor as wild type cells. The *chs3Δ* null mutants appear swollen in the absence of sorbitol, suggesting that the homozygous *chs3Δ* mutation increased susceptibility to osmotic shock, cellular chitin was reduced to 25 % and they only showed calcofluor fluorescence at the septum. The heterozygous disruptants of *CanCHS3* (*CHS3/chs3Δ*) showed small difference from the wild type in chitin content in both hyphal and yeast form. The above results indicated that the cell wall chitin is synthesized largely by *CanCHS3* in both yeast and hyphal forms and is required for chitin synthesis. Additionally, these results demonstrated that *CanCHS2* contributes little to cellular chitin and strongly suggested that *CanCHS1*

is involved in chitin synthesis at the septum in *C. albicans*. In order to confirm the involvement of *CanCHS1* in septum formation a double mutant (chs2Δ/chs3Δ) for both *CanCHS2* and *CanCHS3* was constructed. The morphology of this mutant was similar to the homozygous *chs3Δ* mutant: cells were swollen in both hyphal and yeast forms in normal medium but the morphology was restored in the presence of sorbitol. When hyphal and yeast forms were stained with calcofluor, strong fluorescence was detected in the septum and weak fluorescence was detected in the lateral wall. These results show involvement of *CanCHS1* in septum formation and partial involvement in lateral wall formation. Mio and coworkers (1996) attempted to disrupt *CanCHS1* but such a mutant was not viable. As already pointed out by Gow (1994), one possibility is that *CanCHS1* is an essential gene in *C. albicans*.

Table 5: Characteristics of *C. albicans* mutants defective in chitin synthase activity. (Mio *et al.*,1996)

Mutant	Defect	Hyphal chitin	Yeast chitin	Phenotypes
chs2Δ	*CanCHS2*	80%	80%	entire cells stained by calcofluor
chs3Δ	*CanCHS3*	35%	25%	swollen cells, calcofluor resistant
CHS3/chs2Δ	*CanCHS2* and *CanCH3*	80%	60%	entire cells stained by calcofluor
chs2Δ/chs3Δ	*CanCHS2* and *CanCHS3*	35%	25%	swollen cells, calcofluor resistant

Mutant analysis was also used for the characterization of chitin synthase genes in *Neurospora crassa*. Yarden and Yanofsky (1991) inactivated *NcCHS1* with a repeat-induced point mutation process. The resulting mutants (designated as $chs1^{RIP}$) were slow in growth and exhibited hyphal swelling in the presence and absence of sorbitol. However, cross-wall formation and conidiation were not

affected. The *chs1*^{RIP} phenotype was correlated with a 7 to 20-fold reduction in chitin synthase activity and a hypersensitivity to Nikkomycin Z, a competitive inhibitor of chitin synthase. These observations suggested that *N. crassa* produces an additional chitin synthase that can participate in cell wall formation. This view was supported by Din and Yarden (1994), who cloned and sequenced *NcCHS2*. The RIP phenomenon was used to study the role of *NcCHS2 in vivo*. In contrast to the consequence of *NcCHS1* disruption (Yarden and Yanofsky, 1991), progeny from crosses in which *NcCHS2* had undergone RIP did not exhibit any abnormal morphology. Though a significant reduction (approximately 75 %) in *in vitro* chitin synthase activity was observed in the *chs2*^{RIP} strain, no striking decrease of cell-wall chitin accompanied the reduction of in enzyme activity when compared to that measured in the wild type. Even though there was no apparent morphological effect when *NcCHS2* was disrupted, it is possible that loss of chitin synthase 2 activity may result in reduced fitness of the fungus. This was demonstrated by its increased sensitivity to the lipid-biosynthesis inhibitor epidephnos. However, *chs2*^{RIP} mutants did not show an increased sensitivity to nikkomycin Z. Thus in contrast to *S. cerevisiae* chitin synthases (Bulawa, 1993), it seems that not all *N. crassa* chitin synthases may be equally sensitive to the nikkomycin/polyoxin drug family.

In *Aspergillus nidulans* five chitin synthase genes have been isolated and characterized (Motoyama *et al.*, 1994; Yanai *et al.*, 1994; Specht *et al.*, 1996). Yanai and coworkers (1994) isolated *CHSA* and *CHSB* and disrupted them by recombination depending on sequence similarity, to produce in each disruptant tandemly arranged genes with 5'- and 3'- truncation. The 5'-truncated gene could not be expressed because of the lack of its promoter. The 3'-truncated gene, which had its promoter, may be transcribed and translated into a polypeptide lacking the usual carboxy-terminal region. Examination of disruptant phenotypes showed that

chsA mutant grew as well as the wild type but the *chsB* mutant did not form colonies. The *chsB* mutant did not grow on a medium with 1.2 M sorbitol as an osmotic stabilizer. Microscopic observations of the *chsB* disruptant revealed that hyphal growth stopped immediately after germination of conidia with hyphae swollen at their tips. These results suggest that *chsB* but not *chsA* is an essential gene for hyphal growth. Motoyama *et al.* (1994) isolated and disrupted *CHSC*. The resulted mutants showed no difference of morphology in the asexual cycle and no difference in growth rate compared to a wild type strain. In conclusion *CHSC* may or may not have some redundant functions with other chitin synthases of *A. nidulans* or it may be required under special circumstances. Specht and coworkers (1996) identified and disrupted *CHSD* and *CHSE*. Each of the wild-type *A. nidulans* genes was replaced by a copy that had a substantial fraction of its coding region replaced by the *A. nidulans argB* gene. Hyphae from both *chsE* and *chsD* disruptants contain about 60-70% of the chitin content of wild-type hyphae. The morphology and development of *chsE* disruptants are indistinguishable from those of wild type. Nearly all of the conidia of *chsD* disruption strains swell excessively and lyse when germinated in low osmotic strength medium. Conidia that do not lyse produce hyphae that initially have normal morphology but subsequently lyse at subapical locations and show ballooned walls along their length. The lysis of germinating conidia and hyphae of *chsD* disruptants is prevented by the presence of osmotic stabilizers in the medium. Conidiophore vesicles from *chsD* disruption strains frequently swell excessively and lyse, resulting in colonies that show reduced conidiation. These properties indicate that chitin synthesized by the *CHSD*-encoded isozyme contributes to the rigidity of the walls of germinating conidia, of the subapical region of hyphae and of conidiophore vesicles, but is not necessary for

normal morphology of cells. The phenotypes of *chsD* and *chsE* disruptants indicate that the chitin synthesized by each isozyme serves a distinct function.

Chitin synthase and mycoparasitism

The role of chitin synthase in a mycoparasitic system is the interest of this study. The term mycoparasitism refers to one fungus parasitic on another fungus. *Piptocephalis virginiana* is a biotrophic, haustorial mycoparasite that infects successfully mucoraceous hosts such as *Choanephora cucurbitarum*, whereas the related fungus *Phascolomyces articulosus* exhibits resistance. In an interaction between the mycoparasite and its host the first step is directed growth and physical contact with the host hypha. At the site of contact, the germ tube of the mycoparasite attaches to the hyphal wall and develops an appressorium. From the appressorium an infection peg is formed to attempt host penetration. In the susceptible host, a haustorium is formed after successful penetration. Through the haustorium the mycoparasite draws nutrition from the host cytoplasm. In the case of the resistant host, penetration is impeded by thickening of the host cell wall. The thickening of the cell wall is apparently a defense mechanism mounted by the host in response to the attempted penetration. In some instances, the penetration of the mycoparasite is successful. This results in the formation of a haustorium. The resistant host responds by forming a thick sheath around the haustorium preventing the mycoparasite from establishing a nutritional relationship with the host (Manocha and Golesorkhi, 1979). Manocha and Graham (1982) in an *in vivo* study using autoradiographic techniques, demonstrated that the activity of chitin synthase as measured by the degree of incorporation of N-[H^3] acetylglucosamine into chitin at the penetration sites, varied as a function of host-parasite compatibility. Autoradiographs of the resistant host prepared 18, 24 and 36 h after being challenged by the mycoparasite revealed label incorporation over the penetration

sites. This pattern of label accumulation coincides with the formation of papilla (18 h) and rapid development of a sheath (22-24 h) to wall-off the haustorium completely. In contrast, negligible levels of label accumulated at the penetration sites 18 h after inoculation on the susceptible host, *C. cucurbitarum*. Although incorporation of label increased significantly after 24 h, the level of accumulation comparable to that observed in the resistant host was not detected until 36 h following inoculation. This coincided with the development of extrahaustorial matrix around the haustorium in the susceptible host. Obviously, the two hosts, *C. cucurbitarum* and *P. articulosus* differ in their response, at least during earlier stages to the challenge by mycoparasite.

Absence or negligible incorporation of N-[H^3] acetylglucosamine at the penetration sites on the susceptible host 18 h after inoculation could be due to the inability of the susceptible host to mobilize chitin synthase to the penetration site or to activate the zymogen form of chitin already present at the penetration site. This view was not supported by experimental evidence presented by Manocha and McCullough (1985). Autoradiography of mechanically wounded hyphae of the susceptible and resistant host showed similar patterns of localized incorporation of N-[H^3] acetylglucosamine within 5 min after a short pulse of sonication. The label incorporation was inhibited by polyoxin D (a chitin synthase inhibitor). Cycloheximide treatment reduced the label incorporation at the hyphal tip, but increased the label in subapical region. However, this treatment did not prevent localized incorporation of the label at wounding sites on both hosts or at the penetration sites on the resistant host.

Manocha and Begum (1985) reported the biochemical differences between the crude chitin synthase extracts from the susceptible and resistant, *C. cucurbitarum* and *P. articulosus,* respectively, and characterized their activity by

measuring the incorporation of the substrate [^{14}C]UDP-N-acetylglycosamine (UDP-GlcNAc) into chitin. The enzyme activity was mainly associated with the mixed membrane fraction. Properties of the enzyme preparation such as activation by proteases, UDP-GlcNAc, and response to pH were examined. Enzyme activity from both fungi displayed basically the same features as the corresponding enzymes reported from other mucoraceous fungi. However, the two enzyme preparations differed from each other in their response to various proteases and storage at 4°C. Enzyme preparation from *P. articulosus* was activated by all proteases, whereas the *C. cucurbitarum* preparation was activated by acid protease, slightly activated by trypsin over narrow concentration range, and was inhibited by neutral protease. Enzyme from *C. cucurbitarum* showed a rapid decrease in activity within the first 5 hours of storage at 4°C and also exhibited relatively higher activity of endogenous proteases than that from *P. articulosus*.

Presence or absence of [^{3}H]GlcNAc accumulation at the penetration sites may be attributed to differential activation of chitin synthase and chitinase by proteinases in response to mycoparasite invasion. The latter enzyme is known to degrade effectively the nascent chitin synthesized by chitin synthase (Romero *et al.*, 1982). Chitinase and chitin synthase have common properties of being zymogenic, membrane bound, and activated by partial proteolysis as reported by Humphreys and Gooday (1984) in a mucoraceous fungus, *Mucor mucedo*. Both enzymes are involved in the regulation of growth and metabolic activities in fungi (Cabib *et al.*, 1982). The possibility that the lack of chitin synthesis at the penetration site on a susceptible host may be due to the differential activation of chitin synthase and (or) chitinase by proteinases in response to the challenge by the mycoparasite was examined by Manocha and Balasubramanian (1988). Effects of partially purified preparations of proteinases extracted from *C. cucurbitarum* and *P. articulosus* were

studied *in vitro* on the activities of chitinase and chitin synthase of the same hosts. Both chitinase and chitin synthase are membrane bound, zymogenic and activated by partial proteolysis. Host proteases of acid and neutral type stimulated chitinase activity, but only the acid protease stimulated chitin synthase activity of the two hosts. Neutral proteinase of *C. cucurbitarum* inhibited its chitin synthase, whereas that of *P. articulosus* increased its chitin synthase activity. Partially purified preparations of neutral proteinase from *Piptocephalis virginiana*, however, enhanced chitinase and suppressed chitin synthase activity of both the hosts alike. In light of these results, Manocha and Balasubramanian (1988) suggested that host proteinases regulate the activity of chitinase and chitin synthase of the two host species.

In order to further characterize chitin synthases in these species the genes from both hosts must be isolated and characterized. This study focused on chitin synthase gene from *P. articulosus*. Since low stringency Southern hybridizations show that most chitin synthase genes do not cross-hybridize (Bulawa *et al.*, 1986; Au-Young, *et al.*, 1990; Valdivieso *et al.*, 1991; Szaniszlo and Momany, 1993), traditional techniques involving probes from genes of other species may not be applicable. Based on the fact that most chitin synthases conserve highly two amino acid sequences we decided to synthesize primers analogous to the ones that Bowen and coworkers (1992) used to amplify genomic DNA regions that correspond to chitin synthase genes. In this work an amplification product that corresponds to chitin synthase gene fragment from *P. articulosus* was cloned, sequenced and characterized.

Materials and Methods

Stock Cultures and Growth Conditions

A culture of *Phascolomyces articulosus* was maintained at 22°C±1 °C on a medium consisting of the following (Manocha *et al.*, 1988):

Malt extract	20g
Yeast extract	2g
Agar	20g
Distilled water	1L

Fungal DNA Isolation

The method used was described by Tonuchi and coworkers (1986). A culture was grown in 1L medium for 48 h. The hyphae were collected using a funnel and a cheese cloth. The culture was washed with 100 mL of 250 mM EDTA and was frozen in liquid nitrogen. The culture was lyophilized overnight. A portion of this preparation (0.5 g) was used for the extraction of genomic DNA. The lyophilized hyphae were incubated at 51°C for 30 min in a buffer containing 5 mg of Proteinase K (Sigma), 100 mM Tris-Cl pH 8, 0.5 % SDS and 0.5 M EDTA pH 8, with occasional shaking. After centrifugation the supernatant was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) twice. To this 0.25 volume of 7.5 M ammoniun acetate and 2.5 volume of 95 % ethanol were added. After incubation at -20°C for 15 min a pellet was recovered by centrifugation. The DNA pellet was washed with 70 % ethanol, dried and dissolved in TE (10 mM Tris-Cl, pH 8 and 1 mM EDTA pH 8). The genomic DNA was treated with 50 μg of RNAase A and reextracted with phenol:chloroform and reprecipitated. The final pellet was dissolved in TE.

Restriction enzyme digestion

Restriction enzymes (New England Biolabs) were used at a concentration range of 0.5 U/μL (units/μL). The amount of DNA varied with the size of molecule being digested; 200-400 ng of plasmid DNA and 15 μg of genomic DNA. The sample and the enzyme were suspended in a total volume of 20 μL with the buffer specified by the manufacturer for the enzyme. The samples were incubated at 37 °C. The digestion times were 30 min for genomic DNA and 1 h for plasmid DNA.

Agarose gel electrophoresis

DNA was analyzed by electrophoresis in 0.7% to 1.2 % agarose gels (w/v). Electrophoresis buffer (TAE) contained 40 mM Tris-Cl, 20 mM acetic acid, 1 mM EDTA, pH 8.0. To the DNA samples 1/10 volume of loading buffer (0.25 % bromophenol blue and 20 % glycerol in the electrophoresis buffer) was added. The samples were loaded on the gel and a constant voltage of 65 volts was applied. Gels were stained in ethidium bromide (3 μg/mL) for 7 minutes. The gels were then photographed under UV light to visualize the DNA.

Miniprep plasmid isolation

The method used for miniprep plasmid isolation was as reviewed by Sambrook *et al.* (1989). Cells of an overnight Luria-Bertrani broth (LB) (10g tryptone, 5g yeast extract and 10g NaCl for 500 mL medium per 1 L) culture (1.5 mL) were spun down at high speed for 30 seconds. To the bacterial pellet, 100 μL of lysozyme solution (2mg/mL lysozyme, 50 mM glucose, 25 mM Tris-Cl, pH 8 and 10 mM EDTA) was added. Next, 200 μL of alkaline SDS (0.5% SDS and 0.2 N NaOH) was added. This solution was incubated at room temperature for 15 min. The solution was neutralized by adding 150 μL of 3 M sodium acetate and was incubated in ice for 40 min. After centrifugation the supernatant was collected into a sterile tube and two volumes of ethanol were added and the tubes were placed in -

70 °C for 15 min. The pellet resulting from a high speed 5 min centrifugation was twice washed with 70 % ethanol and was resuspended in 50 to 100 µL of water.

Large scale plasmid extraction

Large scale plasmid extraction was performed as reviewed by Sambrook *et al.* (1989). Transformed cells were grown in 250 mL LB-Amp (LB + 50 µg/mL Ampicillin). The culture was resuspended in 6 mL extraction buffer (25 mM Tris-Cl, 10 mM EDTA, 15% sucrose and 12 mg lysozyme, pH 7.5). The mixture was incubated on ice for 20 min, followed by 10 min with addition of 12 mL of 0.2 N NaOH and 1% SDS and 20 min with addition of 7.5 mL of 3 M sodium acetate (pH 4.5). The mixture was transferred into 30 mL Corex centrifuge tubes and centrifuged at 10000 x g for 15 min. The resulting pellet was suspended in CsCl in TE to a final density of 1.55 g/mL with 25 µg ethidium bromide. The suspension was centrifuged at 60000 rpm for 20 h in a Beckman TL-100 microultracentrifuge. Plasmid DNA was visualized under ultraviolet (UV) illumination and drawn off with an 18 gauge syringe. The samples were divided to 400 µL aliquots and 1 mL of 95 % ethanol was added. These mixtures were centrifuged at 4000 x g at room temperature for 10 min. The resulting pellets were diluted in 50 µL of water.

Polymerase chain reaction

Two primer sets synthesized by Vetrogen (London, ON) were employed for PCR according to Miyazaki and coworkers (1993). The first primer set included: 5'- CTT AAG CTT ACH ATG TAY AAY GAR GAT-3' (primer 1) and 5'-GTT CTC GAG YTT RTA YTC RAA RTT YTG-3' (primer 2). The second primer set included: 5'- CTT AAG CTT ACH ATG TAY AAY GAR GA[T/C/GA]-3' (primer 1) and 5'-GTT CTC GAG YTT RTA YTC RAA RTT YTG-3' (primer 2), where H= A or C or T; R= A or G; Y=C or T. Primers in both sets contained a *Hin*dIII site or a *Xho*I site. Both primers in the second set were phosphorylated.

PCR reactions were carried out in 50 or 100-μL tubes. Amount of DNA (3 ng/μL-10 ng/μL) and $MgCl_2$ (2-14.5 mM) were varied for optimization. The thermophylic enzymes *Taq* and *Pwo* (Boehringer Manheim) were of 2.5 units per reaction mixture. A mixture of the two enzymes (2.5 units *Taq* and 0.5 units *Pwo* per reaction mixture) was invaluable for high specificity and yield. Two PCR programs were employed. The first program was constituted by: (1) 5 min denaturation step at 94 °C, 2 min annealing at 47 °C or 46 °C and 3 min extension 72 °C for the first cycle; (2) 3 min denaturation step at 94 °C, 2 min annealing at 47 °C or 46 °C and 3 min extension 72 °C, for 40 cycles. The last cycle included an extension step of 10 min 72 °C. The second program was constituted by 3 min denaturation at 94 °C, 1 min annealing at 47 °C or 50 °C and 3 min at 72 °C. The last cycle included an extension step of 7 min 72 °C. Both programs ran for 40 cycles. Detected fragments were isolated from gel using the GENECLEAN kit (Promega).

Dephosphorylation of pUC19

CsCl purified plasmid (10μg) was fully digested by *Hinc*II (20 units) endonuclease for at least two hours. The digestion was confirmed on agarose gel. The digest extracted by phenol:chloroform, was precipitated with a final concentration of 3 M NaAc, pH 8 and two volumes of ethanol and was washed with ethanol and resuspended in 10 μL of TE. The plasmid was incubated with 10 units of Calf Intestine Phosphatase (CIP) (Promega) and 1X CIP buffer (1mM $ZnCl_2$, 1mM $MgCl_2$, and 10 mM Tris-Cl pH 8.3) for 15 min at 37°C (according to the instructions of the manufacturer). Another aliquot of enzyme was added and the mixture was incubated for 45 min. The reaction was stopped using 10 mM Tris-Cl pH 8, 1 mM EDTA, 200 mM NaCl and 0.5 % SDS. The plasmid was extracted with phenol:chloroform and was precipitated to a final concentration of 3 M NaAc with

two volumes of ethanol, was washed twice with 70 % ethanol and resuspended in water.

Cloning of the PCR fragment in pUC 19

The agarose free PCR fragment was ligated with pUC19 in the following ligation mixes: 1 μL 10X ligation buffer, 1μL T4 ligase (200u/μL) (New England Biolabs) and 2 μL insert (~ 350 ng) and 0.5 μL plasmid (335 ng) and was made up to 10 μL with water or 1 μL 10X ligation buffer 1 μL ligase, 4 μL insert and was made up to 10 μL with water. Ligations were performed overnight at 4 °C. Meanwhile competent cells were made by growing *E.coli* strain DH5α overnight in 2 mL LB cultures. Half mL aliquots were transferred in 50 mL LB medium. These were allowed to grow for 2-3 h (OD_{600}=0.5-0.6). Cells were collected by centrifugation and were mixed in 25 mL transformation buffer that contained 5 mM Tris-Cl and 75 mM $CaCl_2$ pH 7.5. Cells were left over night on ice and were collected by centrifugation and were resuspended in 1 mL transformation buffer. The cell not used for transformation were stored at -70 °C in 15 % glycerol in small aliquots.

For transformation, 100 μL of competent cells was added to up to 5 μL ligation mix and placed on ice for 30 min. The cells then were heat-shocked for 45 sec at 42 °C and immediately placed on ice for two minutes. 900 μL of SOC (2 % bactotryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ and 20 mM glucose) medium was added. This mixture was incubated for 45 min at 37 °C. 100 μL samples were plated on LB-Amp-Xgal plates (LB plates with 40 μg/mL ampicillin and 800 μg 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)). The plates were incubated at 37°C overnight. Colonies with possible insert were detected by non-complementation of β-galactosidase activity (colonies appeared white) according to Ullman *et al.* (1967) and were transferred to 2 mL LB medium to grow overnight.

Alkaline Southern Blotting

The agarose gels containing the PCR products and the partially digested genomic DNA were placed in 0.25 N HCl for 15 min at room temperature in order to nick the DNA. The gels were placed on a filter paper wick with ends immersed in NaOH. A nylon membrane (ICN Biotrans) was placed directly on top of the gel and several layers of absorbent paper were stacked on top. DNA was eluted from the gel to the membrane for a minimum of 4h. The nylon membrane was neutralized for less than a minute in a small volume of SSC (150 mM NaCl, 15 mM $Na_3C_6H_5O_7.2H_2O$ pH 7.0). DNA was cross-linked to the membrane with UV light in a Stratagene crosslinker. The membrane was then allowed to dry on filter paper.

Labeling reaction

A radioactive probe was generated by using the Pharmacia Oligolabeling kit by the random priming method of Feinberg and Vogelstein (1983). Up to 75 ng insert DNA, isolated previously by GENECLEAN, was boiled for 10 min. To this was added 5 µL of the random hexamer mix and immediately placed on ice. Label 30 µCi α-^{32}P dCTP (ICN), and 5 units of Klenow fragment of DNA polymerase I were added and the mixture was incubated at 37°C for 2 to 5 h.

Hybridization of insert DNA with PCR products and digested genomic DNA

Hybridization was done as reviewed by Sambrook *et al.* (1989). Blots were incubated in a prehybridization solution (2XSSC, 1 % SDS and 5X Deinhart's solution) for 30 min at 65 °C. Radiolabelled DNA was denatured for 5 min at 100 °C with 100 µg/mL herring sperm DNA. The denatured DNA was added to the prehybridization mixture and was incubated at 65 °C overnight. Following hybridization the membrane was rinsed first with 2X SSC and 0.5 % SDS for 5 min at room temperature, 2X SSC and 0.1 % SDS for 30 min at 64 °C and finally with 0.1X SSC and 0.5% SDS at room temperature. The washed membrane was placed

on moist filter paper, sealed with Saran Wrap and exposed to X-ray film (KODAK X-OMAT) with a Cronex intensifying screen overnight.

DNA sequence determination

DNA was sequenced with the dideoxy nucleotide chain termination method of Sanger *et al.* (1977). The reactions were done using the Applied Biosystems Cycle DNA sequencing kit and the analysis of reaction products was done using DNA sequencer 373A (Applied Biosystems). Two sequences resulted corresponding to the forward and reverse primers for pUC19. The sequencing was done by the automatic sequencing facility at McMaster University.

DNA Sequence analysis and alignment

The DNA sequences for the left and right hand primers were compared and the overlapping region was determined. With the entire fragment sequence in hand we were able to determine short sequences characteristic to the beginning, middle and end of introns. The resulting open reading frame was translated by the GeneJockey program. The resulting amino acid sequence was compared with sequences deposited in EMBL database (Heidelberg, Germany) by the Pearson and Lipman FastA program (1988) and was aligned with existing chitin synthase fragment sequences by the CLUSTALW Version 1.6 program (Thomson *et al.*, 1994). CLUSTALW is a program that creates a multiple sequence alignment from a group of sequences using progressive pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. The alignments in CLUSTALW are carried out in three stages: (1) all pairwise similarity scores between all the sequences are calculated using the slow/full dynamic programming method that uses two gap penalties (for opening and extending gaps) and a full amino acid matrix; (2) the similarity scores are used to construct a dendrogram using the Neighbor-Joining method of Saitou and Nei (1987) and (3) the final

multiple alignment is performed by gradually aligning groups of sequences, according to the branching order in the dendrogram. A series of alignments of larger and larger groups of sequences is performed with the most similar sequences aligned first. Any gaps that are introduced in the early alignment are fixed. When the two groups of sequences are aligned against each other, a full protein weight matrix (BLOSUM 30) is used. The two gap penalties, - a "fixed" penalty for opening up a gap, and a "floating" penalty for extending a gap, are used.

The CLUSTALW program offers also a phylogenetic tree menu that includes a bootstrapping option. Bootstrapping is a method for deriving confidence values for the groupings in a tree (Felsenstein, 1985). It involves making N random samples of sites from the alignment (N should be large eg. 500-1000), drawing N trees (1 from each sample) and counting how many times each grouping from the original tree occurs in the sample trees. In this study after the initial alignment of *PaCHS* and related fragments the bootstrapping method was used to assign confidence values to apparent groupings. For example, in Figure 8 (Results) *PaCHS* was found to be grouped with *RhiolCHS1* in 820 out of 1000 trials as compared with the initial tree, giving them an 82% bootstrap confidence limit as a phylogenetic group. CLUSTALW does not offer a visual representation of the trees drawn. Consequently, the program TREEVIEW Version 1.2 (available on the Internet, courtesy of R. D. M. Page, 1996) was used for visual representation of the CLUSTALW trees.

Results

Extraction of DNA from *Phascolomyces articulosus*

Genomic DNA was extracted from *Phascolomyces articulosus* using as a source lyophilized hyphae, incubated in a high EDTA extraction buffer (0.5 M) at 51°C. This preparation was extracted with phenol:chloroform twice and precipitated in salt and ethanol. RNA was digested with RNAase A. High molecular DNA was observed after agarose electrophoresis in a TAE 0.7% gel (Figure 1).

PCR optimization

The optimization of PCR for the amplification of a chitin synthase gene fragment from *P. articulosus* included variables such as annealing temperature, template concentration, thermocycler programs, thermophilic enzymes and $MgCl_2$ concentration (Table 6). The two programs tested differed in time length of melting and annealing steps. Program 1 was longer than program 2. It appeared that program 2 gave more specific products. Primer sets were similar in nucleotide sequence with the exception of an extra degenerate site in the second set. Primer set 2 gives more specific amplification than Primer set 1. Increase in annealing temperature showed a more specific amplification pattern. Template DNA concentrations of 3-10 ng/mL were tested. Specific amplification occurs with 7 ng/mL of DNA. $MgCl_2$ concentration was a very important variable, because amplification did not occur in concentrations below 5 mM and specific amplifications occurred at 14.5 mM concentration. The addition of *Pwo* thermophilic enzyme was invaluable because it produces fragments with blunt ends and has proof reading properties. The optimum reaction resulted in four distinct bands (820, 900, 1000 and 1500 bp approximately) (Figure 2).

Figure1: Agarose gel electrophoresis of *P. articulosus* genomic DNA

Figure 1: 500 ng of genomic DNA is electrophoresed in a 0.7% agarose gel in TAE at 60V for 2 h and stained with ethidium bromide. The marker used is λ DNA digested by *Hind* III (Boehringer-Manheim). The sizes of the marker fragment are: 23 Kbp, 9.4 Kbp, 6.6 Kbp, 4.4 Kbp, 2.3 Kbp, 2 Kbp and 564 bp.

Table 6: Summary of PCR optimization for the amplification of chitin synthase gene fragments from *Phascolomyces articulosus*.

Program	Primer set	Enzyme	T_{an} in ^0C	[DNA] in ng/μL	[$MgCl_2$] in mM	Results	Band appearance
1	1	*Taq*	47	3	2	no amplification	-
					5	no amplification	-
					10	nonspecific amplification	streak
					14.5	nonspecific amplification	streak
				7	2	no amplification	-
					5	no amplification	-
					10	no amplification	-
					14.5	non specific amplification	streak
1	1	*Taq*	46	7	5	non specific amplifications	streaks
					10		
					14.5		
2	1	*Taq*	47	7	2	no amplification	-
					5	no amplification	-
					10	non specific amplification	ladder
					14.5	non specific amplification	ladder
				10	2	noamplification	-
					5	no amplification	-
					10	non specific amplification	ladder
					14.5	non specific amplification	ladder
2	1	*Taq*	50	7	10	no amplification	-
					14.5	no amplification	-
2	1	*Pwo*	50	7	10	no amplification	-
					14.5	no amplification	-
2	1	*Taq* + *Pwo*	50	7	10	no amplification	-
					14.5	amplification	faint band + streek
				10	10		
					14.5	no amplification	-
						amplification	faint band + streak
2	2	*Taq* + *Pwo*	50	7	10	amplification	distinct bands
				10	14.5	no amplification	-
					10	no amplification	-
					14.5	amplification	faint bands

DNA cloning of the amplified fragment

A band corresponding to 820 bp was selected to be cloned because chitin synthase fragments isolated from a variety of fungi were of similar size (600-750 bp). The band was extracted from the gel using the GENECLEAN kit and was cloned in the *Hin*cII site of pUC19. The process was facilitated by using phosphorylated primers, coamplifying with *Pwo*, another thermophilic enzyme that gives PCR products with blunt ends, and by dephosphorylating the plasmid to limit religation. Ten white colonies were randomly selected after transformation and incubation of transformants in LB-X-Gal-Agar, for miniprep plasmid isolation. Two transformants showed an insert of 820 bp after digestion with *Hin*dIII and *Xho*I, sites present in the primer sequence (Figure 2).

Restriction enzyme mapping of puc19-*PaCHS*

One plasmid (puc19-*PaCHS*) was digested with several enzymes (*Hin*dIII, *Bam*HI, *Pst*I, *Hin*cII, *Eco*RI and *Xho*I) (Figure 3a). All digestions linearized the plasmid and *Bam*HI produced a small fragment (~ 300 b). Double digestions with *Eco*RI and *Hin*dIII, *Hin*cII and *Pst*I, and *Bam*HI and *Hin*dIII, released the insert, one small fragment and two small fragments respectively (Figure 3b). The resulting map is shown in Figure 4.

Southern Hybridization

To show that the two clones contained the same insert and that the insert was derived from the PCR products, the 820 bp insert band was extracted from the gel, was labeled by the random priming method and was hybridized to the PCR products and the two clones by Southern hybridization. The probe hybridized to the PCR products and the two inserts. Hybridization also occurred at uncut portions of the plasmids (Figure 5a). The probe did not hybridize with the lambda marker that served as negative control.

Figure 2: Agarose electrophoresis of PCR products and clones of the 820 bp fragment

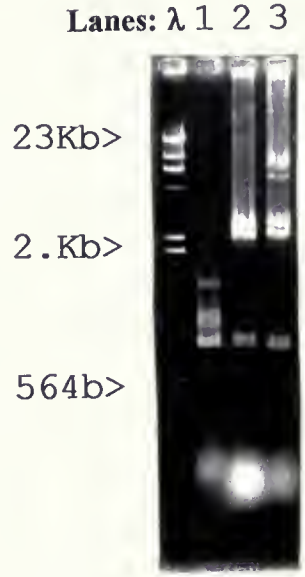

Figure 2: Lane 1 contains 15 mL of PCR from *P. articulosus* genomic DNA. Lanes 2 and 3 contain 400 ng of double digested minipreparation plasmid containing an insert (two independent clones). The fragment is released after 1 h digestion with 0.5U/μL *Xho*I and *Hin*dIII. DNA is separated by agarose electrophoresis at 60 V for 2 h in a 1.2% agarose gel. The bands below 564 b correspond to excess primer in the case of PCR and RNA from the minipreparation in the case of the two plasmids. The marker used is λ DNA digested by *Hin*d III (Boehringer-Manheim). The sizes of the marker fragment are: 23 Kbp, 9.4 Kbp, 6.6 Kbp, 4.4 Kbp, 2.3 Kbp, 2 Kbp and 564 bp.

Figure 3: Restriction fragment characterization of pUC19-*PaCHS*

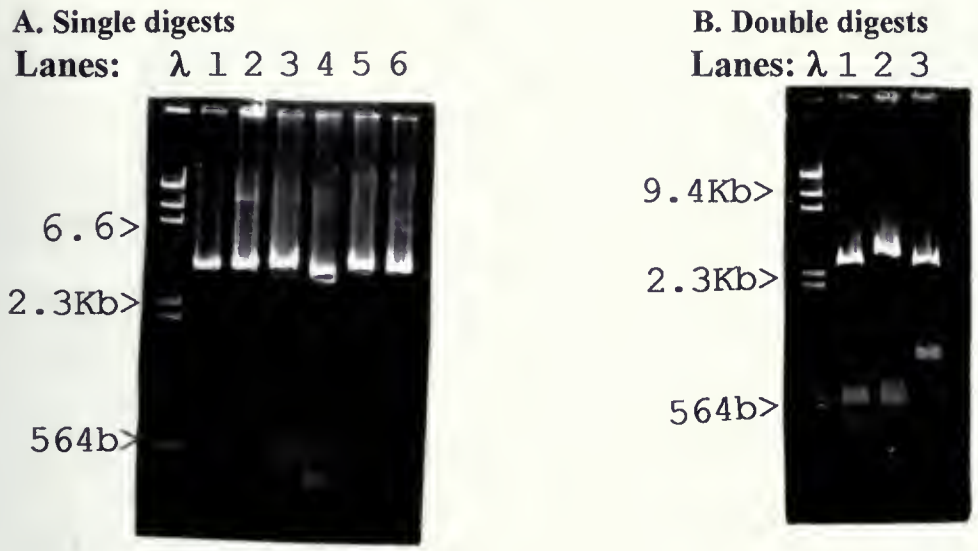

Figure 3: A. 400ng of CsCl purified insert-containing plasmid were digested with 0.5U/μL *Hind* III (lane 1), *Xho*I (lane 2), *Hinc*II (lane 3), *Bam*HI (lane 4), *Eco*RI (lane 5), *PstI* (lane 6) for 1 h and separated on a 1.2% agarose gel. B. 400ng of the insert containing plasmid were digested with 0.5u/μL *Hind* III and *Bam*HI (lane 1), *Hinc*II and *Pst*I (lane 2) and *Eco*RI and *Hind*III (lane 3) for 1 h and separated on a 1.2 % agarose gel at 60V and 2 h. The marker used is λ DNA digested by *Hind* III (Boehringer-Manheim). The sizes of the marker fragment are: 23 Kbp, 9.4 Kbp, 6.6 Kbp, 4.4 Kbp, 2.3 Kbp, 2 Kbp and 564 bp.

Figure 5. Southern hybridizations of PaCHS with the two clones, PCR product and *P. articulosus* genomic DNA.

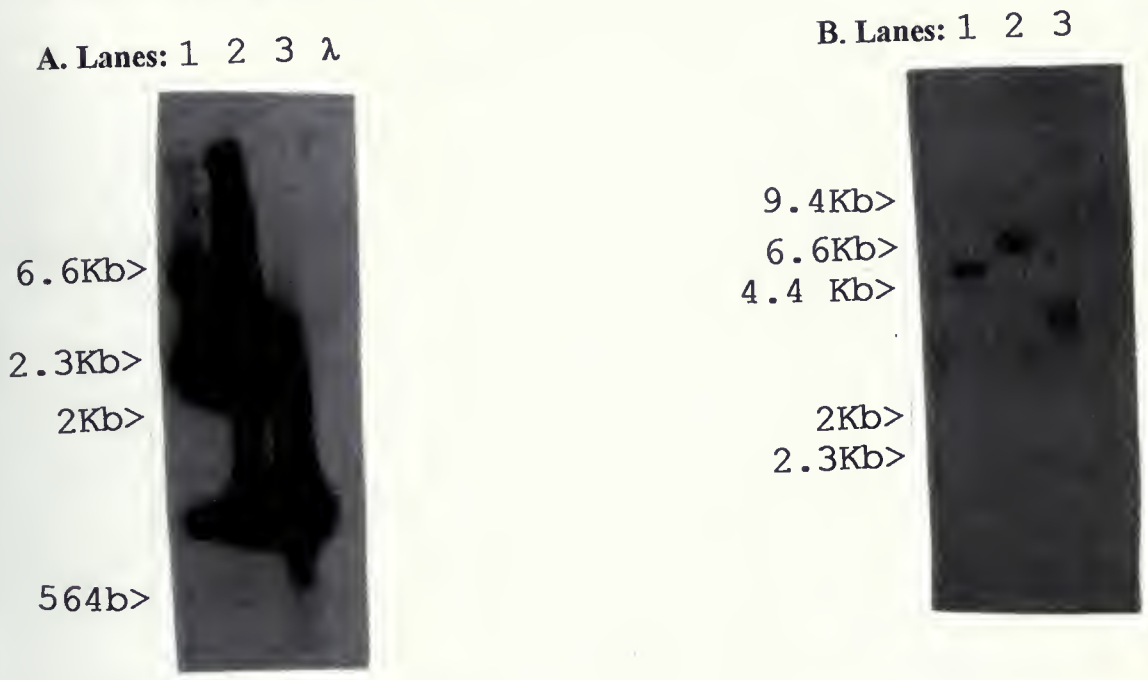

Figure 5: A. The contents from the agarose electrophoresis (Figure 2) were transfered to a nitrocellulose membrane and were probed with *PaCHS*. Lanes 1 and 2 contain the two selected clones digested with *Hin*dIII and *Xho* I, lane 3 contains the total PCR product. λ marker (λ digested with *Hin*dIII) served as a negative control.
B. Genomic DNA (20 μg) from *P. articulosus* was partially digested with 0.5U/μL *Hin*dIII (lane 1) *BamH*I (lane 2) and *Eco*RI (lane 3) for 30 min, transfered to a nitrocellulose membrane and probed with *PaCHS*.

Figure 4: Restriction map of pUC19-PaCHS

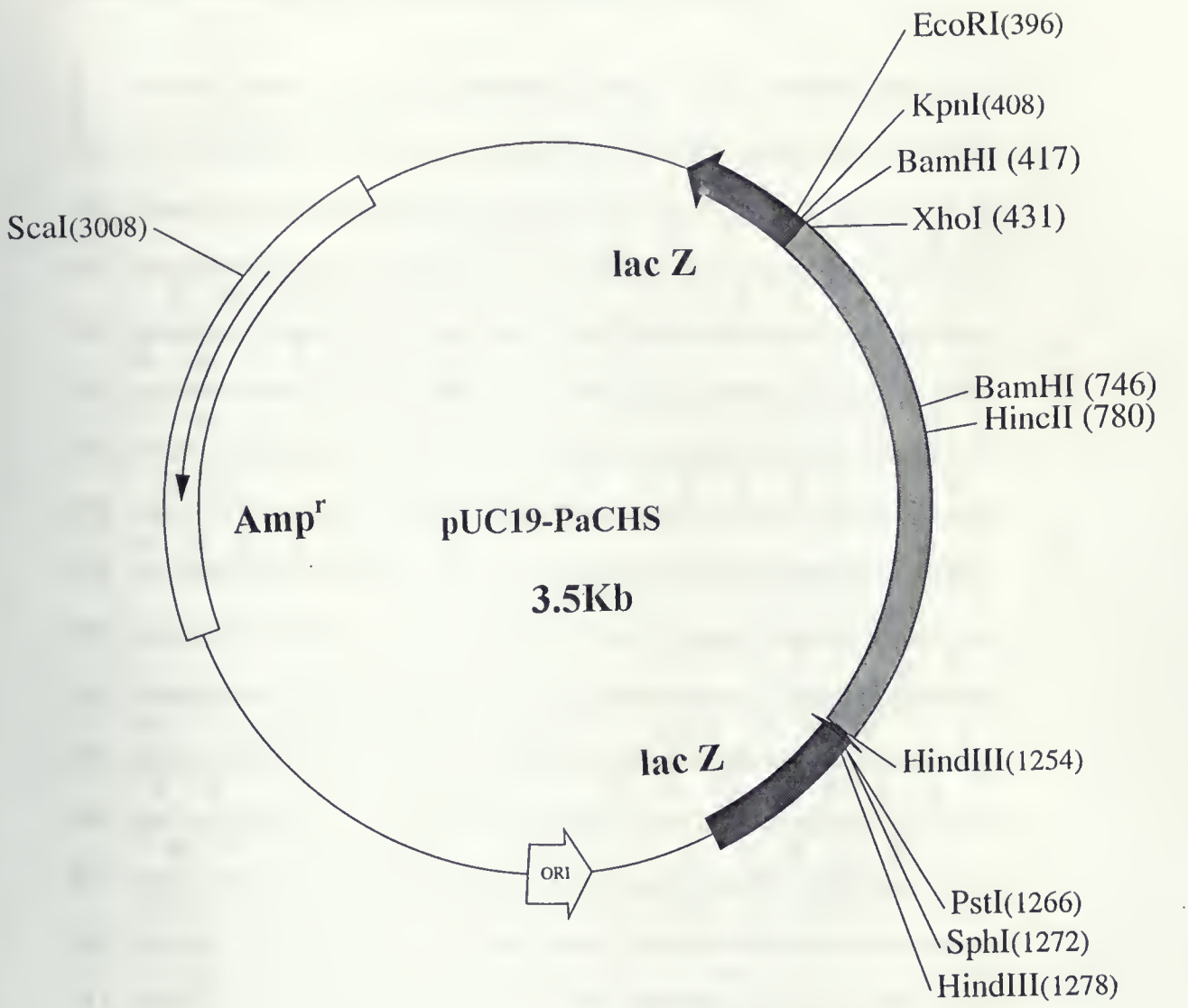

Figure 4: The restriction sites of the cloned fragment in pUC19 are demonstrated above, according to previous analysis (Figure 3). Amp^r corresponds to the ampicilin resistance gene. ORI corresponds to the origin of replication. The lac-Z gene is disrupted by the inserted fragment. *Xho*I (431) and *Hin*dIII (1278) are as predicted by the primer sequences, and *Bam*HI (764) and *Hin*cII (780) are within the cloned fragment. *Eco*RI (396), *Kpn*I (408), *Bam*HI (417), *Pst*I (1266), *Sph*I (1272) and *Hin*dIII (1278) are sites within the multicloning site of puC19.

Figure 6: Nucleotide and aminoacid sequences of PaCHS

```
1   cttaccatgtacaacgtaaaataatatagttcctgctataaatattatat
1    L  T  M  Y  N
51  aacctataatatattatttttagcacttaaatgctgatctttacccaat

101 ttaatttattattaggaggatgaagttttgttttgtcgtacgatgcatgg
                    E  D  E  V  L  F  C  R  T  M  H  G
151 cgtaatgaagaatattgcccacctttgttcgcgccacaggtcctccacgt
     V  M  K  N  I  A  H  L  C  S  R  H  R  S  S  T
201 ggggggttggaggatacgaaaaagttgttgtttgcattatctctgatggt
    W  G  V  G  G  Y  E  K  V  V  V  C  I  I  S  D  G
251 cgtaataagattaatccaagaacactgtcagttttaacaactatgggtgt
     R  N  K  I  N  P  R  T  L  S  V  L  T  T  M  G  V
301 ttatcaagaaggtgttgctaagaatatcgtcgaaggtaaaccagtgacag
      Y  Q  E  G  V  A  K  N  I  V  E  G  K  P  V  T
351 cgcatatttatgatgtaatggcactatttttatttaatttccttctatc
    A  H  I  Y  E
401 ttgggattaattacatataaaatttgcatgctcataactaaggaagtaac

451 atacttatttttgctatttgttacgcgatttagtatacaacacaaatat
                                      Y  T  T  Q  I
501 cagtggatcccgacatgacacttaagggttcggaaaaaggtatggttccc
    S  V  D  P  D  M  T  L  K  G  S  E  K  G  M  V  P
551 gtccaaattctattctgtctcaaagagaaaaaccaaaaaagatcaactcg
     V  Q  I  L  F  C  L  K  E  K  N  Q  K  K  I  N  S
601 catcgctggtttttcaagcatttggtccactcttaaagcccaacatatg
      H  R  W  F  F  Q  A  F  G  P  L  L  K  P  N  I
651 tgtccttcttgatgtcggtactcgtccaggacatacatccatctaccacc
    C  V  L  L  D  V  G  T  R  P  G  H  T  S  I  Y  H
701 tgtggaaatcttttagtattcaccgtaatgtggctggagcatgcggtgag
     L  W  K  S  F  S  I  H  R  N  V  A  G  A  C  G  E
751 attcgcgccatggctggatgtcatttatggaatccgctagtcgcctgccc
      I  R  A  M  A  G  C  H  L  W  N  P  L  V  A  C
801 aaaacttcgaatacaaactc
    P  K  L  R  I  Q     200
```

Figure 6: The deduced amino acid sequence for *PaCHS* was derived from the nucleotide sequence. The amino acids are listed below the corresponding codon. The nucleotide positions are listed in the left margin. The abbreviations to represent amino acids are: A (alanine), C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (aspsaragine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), W (tryptophan) and Y (tyrosine). The abbreviations used to represent nucleotides are adenine (a), guanine (g), cytosine (c), and thymine (t). The underlined sequences represent intron specific sequences and the primer sequences are represented with italics.

Table 7: Comparison of PaCHS with other chitin synthase sequences.

Species	Gene Fragment	Affinity	Class	Match %
Rhizopus oligosporus	RHIOL_CHS1	zygomycetes	II	74.7
	RHIOL_CHS2			71.6
Schizosaccharomyces pombe	SCHCO_CHS1	ascomycetes	II	63.5
Schizophylum commune	SCHPO_CHS2	basidiomycetes	II	63.4
Aspergillus niger	ASPNG_CHS1	ascomycetes	I	50.9
	ASPNG_CHS2		II	58
Ustillago maydis	CHS1_USTMA	basidiomycetes	III	47.9
	CHS2_USTMA		VI	46.5
Xylophylla bantiana	CHS1_XYLBA	fungi imperfectii	I	51.2
	CHS2_XYLBA		II	55.7
Exophialla jeanselmei	CHS1_EXOJE	fungi imperfectii	I	52
	CHS2_EXOJE		II	56.9
	CHS3_EXOJE		III	36.6
Saccharomyces cerevisiae	CHS1	ascomycetes	nonclass	46.2
	CHS2		II	46.8
Ajellomyces capsulatas	CHS1_AJECA	ascomycetes	I	52.7
	CHS2_AJECA		III	40.4
	CHS3_AJECA		II	58.7
Ajellomyces dermatitis	CHS1_AJEDE	ascomycetes	I	52.7
	CHS2_AJEDE		II	58.7
Candida albicans	CHS1_CANAL	fungi imperfectii	II	53.5
	CHS2_CANAL		I	43.9
Wengellia dermatitis	CHS1_WD	fungi imperfectii	I	61
	CHS2_WD		II	55.4
	CHS3_WD		III	42.3
Botritis cinerea	CHS1_BOTCI	fungi imperfectii	I	54.1
Neurospora crassa	CHS1_NEUCR	ascomycetes	I	47.4
	CHS2_NEUCR		II	63.1
	CHS3_NEUCR		III	42.4
Emericella nidulans	CHS1_EMENI	ascomycetes	I	52.9
	CHS2_EMENI		II	58.1
Rhinocladiella atrovirens	CHS1_RHIAT	fungi imperfectii	I	51.3
	CHS2_RHIAT		III	38.4
Phaecoccomyces exophialae	CHS1_PHAEX	fungi imperfectii	I	51.5
	CHS2_PHAEX		II	56.1

Table 7. 36 chitin synthase gene fragments are found to be related to *PaCHS*. The fungal classes and the gene fragment classifications are as in Bowen *et al.*, 1992. The percent matches and the gene designations are from the EMBL database (Heidelberg, Germany). The comparison was done with the Pearson and Lipman FastA program (1988)

Figure 7: CLUSTAL analysis for PaCHS and 40 other fungal chitin synthase genes

Sequences are represented as alinged by the CLUSTAL program. The names of the species are listed on the left hand side in abreviated forms. These abbreviations are: CHS1_Yeast, CHS2_Yeast (*Saccharomyces cerevisiae*), CHS_1CANAL, CHS_2CANAL, (*Candida albicans*), CHS1_NEUCR, CHS_2NEUCR, CHS_3NEUCR (*Neurospora crassa*), CHS1_RHIOL, CHS2_RHIOL (*Rhizopus oligosporus*), CHS_PB (*Phycomyces blakesleeanus*), CHS_PA (*Phascolomyces articulosus*) CHS1_EXOJE, CHS2_EXOJE, CHS3_EXOJE, (*Exophylla jeanselmei*), CHS1_XYLBA, CHS2_XYLBA (*Xylophylla bantiana*), CHS1_ETG, CHS2_ETG (*Entomophaga aulicae*), CHS1_BOTCI (*Botritis cinerea*), CHS1_USTMA, CHS2_USTMA, CHS3_USTMA (*Ustillago maydis*), CHS1_WD, CHS2_WD, CHS3_WD (*Wengellia dermatitis*), CHS1_LAL, CHS2_LAL, (*Laccaria laccata*), CHS1_SCHCO (*Schizophyllum commune*), CHS1_1CAC (*Cantharelus cubarius*), CHS1_SCPO (*Scizosaccharomyces pombe*), CHS1_AJEDE, CHS2_AJEDE (*Ajellomyces dermatitis*), CHS1_AJECA, CHS2_AJECA, (*Ajellomyces capsulatas*), CHS1_EMENI, CHS2_EMENI (*Emericella niger*), CHS1_PHAEX, CHS2_PHAEX (*Phaecoccomyces exophialae*). The abbreviations to represent amino acids are: A (alanine), C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (aspsaragine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), W (tryptophan) and Y (tyrosine). The abbreviations used to represent nucleotides are adenine (A), guanine (G), cytosine (C),and thymine (T). Dots (.) represent homology and stars (*) represent matching.

Figure 7. CLUSTALW analysis for PaCHS and 40 other chitin synthase genes

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Class_I
CHS1_YEAST HILLGRTLKGIMDNVKVMYKKNSSTW------GPDAMKKIVVCIISDGRSKINERSLALLSSLGCYQDGFAKDEINEKGVAMHVYEHTTMINITN---ISESEVSLECNQGTVPIQLLF
CHS2_CANAL DILLGRTLKGVFKNIKYLESKARSSTW------GKDSWKKIVVCIVSDGRTKINERAQALLAGLGVYQEGLAKSRVDDKKVQAHMFEYTTRVGIS---KVTDDVVKLT-TEKVVFVQMLF
CHS1_NEUCR EILFARTMIGVFKNIEYMCKRTESKTW------GKDAMKKIVVCVVSDGRAKINPRTRALLAGMGVYQEGIAKQQVNGKDVTAHIYEYTTQVGMT----IKNDVVQLI-PKQQ-FVQMLF
CHS1_EXOJE EFLFARTMIGVFKNIEYMCKRTRSSKTW------GKEAMKKIVVCIVSDGRAKINPRTRAVLAGLGVYQEGVAKNIVEGKPVTAHIYDYTTQIGLE----LKGTQGLKS-PRSATPVQILF
CHS1_PHAEX EFLFARTMIGVFKNIEYMCNRTSSKTW------GKEAMKKIVVCIVSDGRAKINTRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGTQVSLK-PRSATPVQLLF
CHS2_WD    EFLFARTMIGVFKNIEFMCNRSSSKTW------GKEAMKKIVVCIVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGTQVLSK-PRSATPVQLLF
CHS1_BOTCI DVLFARTMHGVFKNIEFMCTRKDSKTW------GKDAMKKIVVCVVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGIS----LKKDIVTLT-PKQQ-FVQLLF
CHS1_EMENI DFLFARTMAVGVFKNIEHMCSRTRRSTW------GKDAMKKIVVCVISDGRAKINPRTRAVLAGLGCYVDGIAKQQVNGKDVTAHIYEYTTQVGME----LKGNQVHLK-PRSGVFVQMIF
CHS1_AJECA DFLFARTMIGVFKNIEYMCSRTNSKTW------GKEAMKKIVVCVVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQIGME----LKGNQVHLK-PRSGVFVQMIF
CHS1_AJEDE DFLFARTMIGVFKNIEYMCSRTNSKTW------GKEAMKKIVVCVVSDGRAKINQRTKAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQIGME----LKGTQVHLK-PRSGVFVQMIF
CHS1_XYLRA DFLFARTMIGVFKNIEIMCNKGSSQTW------GKEAMKKIVVCIVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGID----LKGTQVSLR-PKGATPVQLLF
CHS1_ASPNG DFLFARTMIGVFKNIEIMCGRTSSKTW------GKDAMKKIVVCVISDGRAKINPRTRAVLAGLGCYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGGQVSLK-PRTGCFVQMIF
ClasII
CHS2_YEAST KYSLARTTHSIMKNVAHLCKREKSHVW------GPNGWKKVSVLISDGRAKVNQGSLDYLAALGVYQEDMAKASVNGDPVKAHIFELTTQVSINADLDY--VSKDIV------PVQLVF
CHS1_CANAL EVAFARTMHGVMKNIAHLCSRHKSKIW------GKDSWKKVQVLIVADGRNKVQQSVLELLTATGCYQENLARPYVNNSKVNAHLFEYTTQISIDENLKFKGDEKNLA------PVQVLF
CHS_PA     EVLFCRTMHGVMKNIAHLCSRHRSSTW------GVGGYEKVVVCIISDGRNKINPRTLSVLTTMGVYQEGVAKNIVEGKPVTAHIYEYTTQISVDPDMTLKGSEKGMV------PVQILF
CHS1_RHIOL EVLFCRTNGVMKNVSHLCSRNRSSTW------GPEGWKKVVVCIVSDGRTKIHPRTLSVLAAMGVYQEGMAKNIVEGKPVTAHIYEHTTQLSIDADMNFRGDEKGVV------PVQILF
CHS2_RHIOL EVLFARTMHGVMKNIAHLCTRDRSRTW------GPNGWEKVVVCIVSDGRNKINQRTLSVLALLGVYQDGIAKNVVHGKPVTAHIYEYTTQVSVDPNMEVKKAGSKNVV------PCQILF
CHS_PB     EILFARTMHGVMKNIAHLCSHIRSNVW------EGPKAWEKVVVCIVSDGRKKIHPRTLSLLATLGVYQDGVAKNVVGDKPVTAHIYEYTTQLSVDPEMKFKGADKGMP------PCQILF
CHS1_ETG   ENLFTKTMTSVMKNVAHLCRRNRSRTW------GDNGWQKVVVCIVSDGRLKINKRVLTVLAAMGVYQDGIAQYAVNGKPVTGHIYEYTTQIVVDTDLNIRGADAGLV------PVQTIF
CHS2_ETG   EHLFVKTMRAVMKNVTHLCSRNRSRIW------GDQGWEKVVVCIVADGRTKIHPLTLKVLAAMGIYQDNVSQTSVNGNPVTAHIYEYTTSQVTMDSDLKVRASQGETV------PIQTIF
CHS2_PHAEX EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWPKIVVCVIADGRQKVHPRTINALAALGVYQDGIAKNVVNKKEVTAHVYEYTTQVSLDEGMKFKGAEKGIV------PCQMIF
CHS2_NEUCR EFGFTRTMHAVMKNISHFCSRNKSRTW------GADGWQKIVVCVVSDGREIIHPRTLDALAAMGVYQHGIAKNFVNQKAVQAHVYEYTTQVSLDSDLMFKGAEKGIV------PCQMIF
CHS2_EXOJE EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWQKIVVCVIADGRQKVHPRTINALAALGVYQDGIAKNIVNQKEVTAHVYEYTTQVSLDEGMKFKGAEKGIV------PCQMIF
CHS1_WD    EIEFTRTMHGIMRNIAHFCSRTRSRTW------GKDGWQKIVVCVIADGRQKVHPRTINALAAMGVYQDGIAKNVVNQKEVTAHVYEYTTQVSLDETLKFKGAEKGIV------PCQMIF
CHS1_SCHPO EVLFARTMHSVMKNISHLCTRKNSQVW------GKDAMKKVVVCIISDGRTKIHPRTLAYIAAIGVYQDGIAKNQVNDKEVKAHIYEYTTQLSIDPNLKFKGSDRGIV------PVQMIF
CHS1_SCHCO EELFCRTMHGVIKNIAHLCKRDRSKTW------GKEGWKKVVVCIVSDGRQKINSRTLSVIAAMGRHQDGIAKNVVNKKPVTAHIYEYTTQITVTPSMKIEGAERGTM------PVQLIF
CHS3_AJECA EIHFTRTTHGIMANITHFCSRTKSRTW------GKDGWQKIVVCIADGRQKVHPRTLNALAAMGVYQDGIAKNIVNQKPVNAHVYEYTTQVSLDPDLKFKGAEKGIM------PCQIIF
CHS2_AJEDE EIHFTRTMHGIMRNITHFCSRTKSRTW------GKDGWQKIVVCIIADGRQKVHPRTINALAAMGVYQDGIAKNIVNQKPVNAHVYEYTTQVSLDPDLKFKGAEKGYK------PCQIIF
CHS2_EMENI ETHFTRTMHGVMQNISHFCSRSKSRTW------GKDGWKKIVVCIISDGRKKVHPRTINALAALGVYQEGIAKNVVNQKQVNAHVYEYTTQVSLDSDLKFKGAEKGIV------PCQVLF
CHS2_ASPNG EIGFTRTLHGVMQNITHLCSRSKSRTW------GKDGWKKIVVCIIADGRKKVHPRTINALAALGVYQEGIAKNVVNQKQVNAHVYEYTTQVSLDPDLKFKGAEKGIM------PCQVLF
CHS2_XYLBA EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWQKIVVCVIADGRQNVHPRTLNALAAMGVYQDGIAKNEVNSKEVTAHVYEYTTQVSLDETLKFKGAEKGTV------PCQDVF
CHS2_IAL   EDLFCRTMHGVIKNIAHLCKRDRSKTW------GKDGWKKVVVCIVSDGRGKINSRTLSVIAAMGAYGEGIAKNKYGTKDVTAHIYEYTTQISYTPSMKIEGAERGTV------PVQLIF
Class_III
CHS1_USTMA RILLARTLHGVMLNIRDICKSKSSKFWRRSAEEGRPGWQRIVVSLIFDGIDPCDEEVLDIAATVGVYQDGVMKRKVDGKDTVAHLFEYTTQLSVDPTPALIQPHADD--ASNLVFVQMIF
CHS3_NEUCR KVLLSRTLHSVMTNIRDIVNLKKSSFWNRGG----PAWQKIVVCLVFDGLDKTDKNVLDVLATIGVYQDGVIKKDVDGKETVAHIFEYTSQLSVTPNQALIRPVDDG--PQTLPFVQFIF
CHS3_EXOJE KVLTARTLHGVMQNIRDIVNLKKSEFWNKGG----PAWQKIVVCLVFDGIDPCDKRTLDLIATVGIYQDGVMKKDVDGKDTVWHIFEYTTQLSVTPNQLIRPNDND--STSLPFVQMIF
CHS3_WD    KVLTARTLMGVMQNIRDIVNLKKSEFWNKGG----PAWQKIVVCLVFDGIDPCDKRTLDVLATIGVYQDGVMKKDVDGKETTAHIFEYTTQLSVTANQQLIRPNDND--PSTLPFVQMIF
CHS2_AJECA KMLTSRTLHGVMQNIRDIVNLKKSEFWNKGG----PAWQKIAVCLVFDGIDPCDKRTLDVLATIGIYQDGVMKKDVDGKETTAHIFEYTTQLSVTANQQLIRPEDDG--PSTLPFVQMIF
CHS1_IAL   KTLYARTLHGVMLNIRDICKTKQSKFWRRHAEEGNPGWKKITVALIVDGIEPMDKSAVLDILATVGVYQDGIMKKEVDGKETAHIFEYTTQLSYDATPQLVLPQPNN--PNNLMPVQIIF
Class_IV
CHS1_CAC   DELFCKTMNAYIKNIAHLCGRSRSKTW------GADGWGKVVVCVVSDGRSEINQRTLKVLTLMGVYGDGYAKDTYNGKDVQAHIFEFTTGYMTL----NDESTQAPC------PIQVIF
CHS2_USTMA DVLFARTMGGVIKNIAHLCSRTRSKTW------GPDAMKKVVVIIVADGRKKANERMLKALGLMGCYNEGVMKDHVLKKPVEAHIFEYTTRVQITE----KGEVKVTPC------PIQVVF
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Figure 7. CLUSTALW analysis for PaCHS and 40 other chitin synthase genes

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Class_I
CHS1_YEAST HILLGRTLKGIMDNVKYMVKKKNSSTW------GPDAWKKIVVCISDGRSKINERSLALLSSLGCYQDGFAKDEINEKKVAAMAYYEHTMINITN---ISESEVSLECNQGTVPIQLLF
CHS2_CANAL DILLGRTLKGVFKNIKYLESKARSSTW------GKDSWKGIVVCISVGSRTKINERAQALLAGLGVYQEGLAKSRVDDKKVQAMMFEYTTRVGIS---KVTDDVVKLT-TEKVVPFVQMLF
CHS1_NEUCR EILFARTMIGVFKNIEYMCKRTESKTW------GKDAWKGIVVCVVSDGRAKINPRTRALLAGMGVYQEGIAKQQVNGKDVTAHIYEYTTQVGMT-----IKNDVVQLI-PKQQ-PVQMLF
CHS1_EKOJE EFLFARTMIGVFKNIEYMCNRTSSKTW------GKEAWKGIVVCISVGSRAKINPRTRAVLAGLGVYQEGVARNIVEGKPVTAHIYDYTTQIGLE----LKGTQGLKS-PRSATPVQILF
CHS1_PHAEX EFLFARTMIGVFKNIEYMCNRTSSKTW------GKEAWKGIVVCISVGSRAKINTRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGTQVSLK-PRSATPVQLLF
CHS2_WD    EFLFARTMIGVFKNIEFMCNRSSSKTW------GKEAWKGIVVCISVGSRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGTQVLSK-PRSATPVQLLF
CHS1_BOTCI DVLFARTMHGVFKNIEFMCTRKDSKTW------GKDAWKGIVVCVVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGIS----LKKDIVTLT-PKQQ-PVQLLF
CHS1_EMENI DFLFARTMVGVFKNIEHMCSRTRRSTW------GKDAWKGIVVCVISDGRAKINPRTRAVLAGLGCVYDGIAKQQVNGKDVTAHIYEYTTQVGME----LKGNQVHLK-PRSGVPVQMIF
CHS1_AJECA DFLFARTMIGVFKNIEYMCSRTNSKTW------GKEAWKGIVVCVVSDGRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQIGME----LKGNQVHLK-PRSGVPVQMIF
CHS1_AJEDE DFLFARTMIGVFKNIEYMCSRTNSKTW------GKEAWKGIVVCVVSDGRAKINQRTKAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQIGME----LKGTQVHLK-PRSGVPVQMIF
CHS1_XYLBA DFLFARTMIGVFKNIEIMCNKGSSQTW------GKEAWKGIVVCIVSDRRAKINPRTRAVLAGLGVYQDGIAKQQVNGKDVTAHIYEYTTQVGID----LKGTQVSLR-PKGATPVQLLF
CHS1_ASPNG DFLFARTMIGVFKNIEYMCSRTSSKTW------GKDAWKGIVVCVISDGRAKINPRTRAVLAGLGCYQDGIAKQQVNGKDVTAHIYEYTTQVGLE----LKGGQVSLK-PRTGCPVQMIF
ClasII
CHS2_YEAST KYSLARTIHSIMKNVAHLCKREKSHTW------GPNGWKKVSVILISDGRAKVNQGSLDYLAALGVYQEDMAKASVNGDPVKAHIFELTTQVSINADLDY--VSKDIV------PVQLVF
CHS1_CANAL EVAFARTMHGVMKNIAHLCSRHKSKIW------GKDSWKGKVQVIIVADGRNKVQQSVLELLTATGCYQENLARPVNNSKVNAHLFEYTTQISIDENLKFKGDEKNLA-----PVQVLF
CHS_PA     EVLFCRTMHGVMKNIAHLCSRHRSSTW------GVGGYEKVVVCIISDGRNKINPRTLSVLTTMGVYQEGVARNIVEGKPVTAHIYEYTTQISVDPDMTLKGSEKGMV------PVQILF
CHS1_RHIOL EVLFCRTMNGVMKNVSHLCSRNRSSTW------GPEGWKKVVVCIVSDGRTKIHPRTLSVLAAMGVYQEGMARNIVEGKPVTAHIYEHTTQLSIDADMNFRGDEKGVV------PVQILF
CHS2_RHIOL EVLFARTMHGVMKNIAHLCTRDRSRTW------GPNGWEKVVVCIVSDGRNKINQRTLSVLALLGVYQDGIAKNVVHGKPVTAHIYEYTTQVSVDPNMEVKKAGSKNVV-----PCQILF
CHS_PB     EILFARTMHGVMKNIAHLCSHIRSNVW------EGPKAWEKVVVCIVSDGRKKIHPRTLSLLATLGVYQDGVAKNVVGDKPVTAHIYEYTTQLSVDPEMKFKGADKGMP-----PCQILF
CHS1_ETG   ENLFTKTMTSVMKNVAHLCRRNRSRTW------GDNGWQKVVVCIVSDGRLKINKRVLTVLAAMGVYQDGIAQYAVNGKPVTGHIYEYTTQIVVDTDINIRGADAGLV------PVQTIF
CHS2_ETG   EHLFVKTMRAVMKNVTHLCSRNRSRIW------GDQGWEKVVVCIVADGRTKIHPLTLKVLAAMGIYQDNVSQTSVNGNPVTAHIYEYTSQVMMDSDLKVRASQGETV------PIQTIF
CHS2_PHAEX EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWQPKIVVCVIADGRQKVHPRTLNALAALGVYQEGMARNIVEGKPVTAHIYEYTTQVSLDEGMKFKGAEKGIV------PCQMIF
CHS2_NEUCR EFGFTRTMHAVMKNISHFCSRNKSRTW------GADGWQKIVVCVVSDGREIIHPRTLDALAAMGVYQHGIAKNFVNQKAVQAHVYEYTTQVSLDSDLMFKGAEKGIV------PCQMIF
CHS2_EXOJE EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWQKIVVCVIADGRQKVHPRTLNALAALGVYQDGIAKNIVNQKEVTAHVYEYTTQVSLDEGMKFKGAEKGIV------PCQMIF
CHS1_WD    EIEFTRTMHGIMRNIAHFCSRTRSRTW------GKDGWQKIVVCVIADGRQKVHPRTLNALAAMGVYQDGIAKNVVNQKEVTAHVYEYTTQISIDFNLKFKGSDRGIV------PVQMIF
CHS1_SCHCO EELFCRTMHGVIKNIAHLCKRDRSKTW------GKEGWKKVVVCIVSDGRQKINSRTLSVIAAMGRHQDGIAKNVVNKKPVTAHIYEYTTQITVTPSMKIEGAERGTM-----PVQLIF
CHS3_AJECA EIHFTRTTHGIMRNITHFCSRTKSRTW------GKDGWQKIVVCIADGRQKVHPRTLNALAAMGVYQDGIAKNIVNQKPVNAHVYEYTTQVSLDPDLKFKGAEKGIM-----PCQIIF
CHS2_AJEDE EIHFTRTMHGIMRNITHFCSRTKSRTW------GKDGWQKIVVCIADGRQKVHPRTLNALAAMGVYQDGIAKNIVNQKPVNAHVYEYTTQVSLDPDLKFKGAEKGYK-----PCQIIF
CHS2_EMENI ETHFTRTMHGVMQNISHFCSRSKSRTW------GKDGWKKIVVCIISDGRKKVHPRTLNALAALGVYQEGIAKNVVNQKQVNAHVYEYTTQVSLDSDLKFKGAEKGIV------PCQVIF
CHS2_ASPNG EIGFTRTLHGVMQNITHLCSRSKSRTW------GKDGWKKIVVCIADGRKKVHPRTLNALAALGVYQEGIAKNVVNQKQVNAHVYEYTTQVSLDPDLKFKGAEKGIM-----PCQVLF
CHS2_XYLBA EIEFTRTMHGIMRNISHFCSRTKSRTW------GKDGWQKIVVCVIADGRQNVHPRTLNALAAMGVYQDGIAKNEVNSKEVTAHVYEYTTQVSLDETLKFKGAEKGTV------PCQDVF
CHS2_LAL   EDLFCRTMHGVIKNIAHLCKRDRSKTW------GKDGWKKVVVCIVSDGRGKINSRTLSVIAAMGAYGEGIAKNKYGTKDVTAHIYEYTTQISYTPSMKIEGAERGTV------PVQLIF
Class_III
CHS1_USTMA RILLARTLHGVMLNIRDICKSKSSKFWRRSAEEGRPGWQRIVVSLIFDGIDPCDEEVLDLLATVGVYQDGVMKGKVDGKDTVAHLFEYTTQLSVDPTPALIQPHADD--ASNLVPVQMIF
CHS3_NEUCR KVLLSRTLHSVMTNIRDIVNLKKSSFWNRGG---PAWQKIVVCLVFDGIDLDKTDKNVLDVLATIGVYQDGVIKKDVDGKETVAHIFEYTTQLSVTPNQALIRPVDDG--PQTLPFVQFIF
CHS3_EXOJE KVLTARTLHGVMQNIRDIVNLKKSEFWNKGG---PAWQKIVVCLVFDGIDPCDKNTLDLLATVGIYQDGVMKGKVDGKDTVVHIFEYTTQLSVTPNQQLIRPNDND--STSLPFVQMIF
CHS3_WD    KVLTARTLMGVMQNIRDIVNLKKSEFWNKGG---PAWQKIVVCLVFDGIDPCDKNTLDVLATIGVYQDGVMKKDVDGKETVAHIFEYTTQLSVTANQQLIRPNDND--ATSLPPAQMIF
CHS2_AJECA KMLTSRTLHGVMQNIRDIVNLKKSEFWNKGG---PAWQKIAVCLVFDGIDPCDKDTLDVLATIGIYQDGVMKGKVDGKETIAHIFEYTTQLSVTANQQLIRPHDDG--PSTLPFVQMMF
CHS1_LAL   KTLYARTLHGVMLNIRDICKTKQSKFWRRHAEEGNPGWKKITVALIVDGIEPMDKSVLDIIATVGVYGDGIMKKEYDGKETVAHIFEYTTQLSIDATFQLVLPQPNN--PNNLMPVQIIF
Class_IV
CHS1_CAC   DELFCKTMNAYIKGIAHLCGRSRSKTW------GADGWGKVVVCVVSDGRSEINQRTLKVLTLMGVYGDGYAKDTYNGKDVQAHIFEFTTGYMYTL----NDESTQAPC-----PIQVIF
CHS2_USTMA DVLFARTMGGVIKGIAHLCSRTRSKTW------GPDAWKGVVVVIIVADGRKKANERMLKALGLMGCYNEGVMKDHVLKKPVEAHIFEYTTRVQITE----KGEVKVTPC-----PIQVVF
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class_I
CHS1_YEAST CLKEQNQKKINSHRWAFEGFAELLRPNIVTLLDAGTMPGKDSIYQLWREF-RNPNVGGACGEIRTDLGKREV------KLINPLVAS
CHS2_CANAL CLKETNAKKINSHRWCFQAIGQVLDFKIVVLLDCGTQPSGRSLYELWKEFDRHRVAGACGEITTSLKKR-Q------MITNPLVAS
CHS1_NEUCR CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTEPGGSSIYHLWKAFDLD-----------------------------
CHS1_EXOJE CLKEKNQKKINSHRWFFQAFGRVLDPNICVLFDAGTKPGKDSIYQLWKAFDLEPMCGGACGEIKVML---DHG-K---KLYNPLIAT
CHS1_PHAEX CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTKPGKDSIYQLWKAFDLEPMCGGACGEIKVML---DHG-K---KLYNPLIAT
CHS2_WD    CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTKPGNDSIYQLWKAFDLEPMCGGACGEIKVML---DHG-----KLINPLVAT
CHS1_BOTCI CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTKPGKDSIYHLWKAFDLEPHCAGACGEIKAMLGMLGPGGK--NPLVNPLVAT
CHS1_EMENI CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTQPGKDSIYRLWKAFDVEPMCGGACGEIKVML---DHG-K---KLYNPLVAT
CHS1_AJECA CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTKPGRDSIYHLWRAFDLQPMCGGACGEIKTML---SHG-K---KLINPLVAG
CHS1_AJEDE CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTKPGRDSIYEHLWRAFDLHPMCGGACGEIKAML---SHG-K---KLYNPLIA-
CHS1_XYLBA CLKEKIRRKINSHRWFFQAFGRVLDPNICVLIDAGTKPGKDSIYHLWKAFDLEPMIGGACGEIKVML---EHG-K---KLYNPLVAT
CHS1_ASPNG CLKEKNQKKINSHRWFFQAFGRVLDPNICVLIDAGTRXGKDSIYHLWKXFDVDPMCGGACGEIKVM----SHG-K---KLINPLVAG
CHS2_YEAST CLKEENKKKINSHRWLFNAFCPVLQPTVVTLVDVGTRLNNTAIYRLWKVFDMDSNVAGAAGQIKTMKGKWGL------KLFNPLVAS
Class_II
CHS1_CANAL CLKESNQKKINSHRWLFNAFCPVLDPNVIVLIDVGTKPDNHAIYNLWKAFDRDSNVAGAAGEIKAMKGKGWI------INLTPLVAS
CHS_PA     CLKEKNQKKINSHRWFFQAFGPLLKPNICVLLDVGTRPGHTSIYHLWKSFSIHRNVAGACGEIRAMA--GC--------HLWNPLVAC
CHS1_RHIOL CLKEKNQKKINSHRWFFQAFGPLLKPNVCVLIDVGTKPGGRSIYHLWKCFDINSNVAGACGEIKAMLGPGGV--------HLLNPLVAS
CHS2_RHIOL CLKEKNQKKINSHRWFFQAFGPVIDPHICVLIDVGTKPGGTSIYHLWKAFDINSNIAGACGEIRAMAGTRGV--------ALLVPLVAA
CHS_PB     CLKENNQKKINSHRWFFQAFGPVINPNVCVLIDVGTRPGKTSIYHLWKAFDISSNIAGACGEIRGMSGTAGV--------ALLNPLVAS
CHS1_ETG   CLKEKNAKKLNSRWFFNAFGPLLNPLKEKLIDVGTRPSGTSIYHLWKAFDRNPNLGGACGEIYAELGKGWN--------KLINPLVAA
CHS2_ETG   CLKEKNAKKLNSRWFFNAFGPVLSPNVCVLIDVGTKPTPTSIYHLWKAFDRNPDLGGACGEIYAELGKGGV--------KLINPLVAA
CHS2_PHAEX CLKEQNKRKLNSRWFFNAFGRALTPNVCILLDVGTKPDSKALYHLWKAFDQDSNVAGAAGEIKADKGKGWL--------GLLNPLVAS
CHS2_NEUCR CLKEKNQKKLNSRWFFNAFGKALNPNVCILLDVGTRPGGTSLYHLWKAFDTDSNVAGACGEIKAMKGRFGG--------NLLNPLVAS
CHS2_EXOJE CLKEQNKKKLNSRWFFNAFGRALTPNVCILLDVGTKPDSKALYHLWKAFDQDSNVAGAAGEIKADKGKGWL--------GLFNPLVAT
CHS1_WD    CLKEKNKKKLNSRWFFNAFGRALIPNVCILLDVGTKPDSKALYHLWKAFDQNSNVAGAAGEIKADKGKGWL--------GLLNPLVAS
CHS1_SCHPO CLKEKNQKKLNSHLWFFQAFCPILKPEVCILLDAGTRPGDQSIYHLWKSTDLNPQVAGACGEIVVMKGKLGS--------GLINPLVAT
CHS1_SCHCO CLKEKNQKKINSHRWFFNAFGPILQPNVCVLLDVGTMPGPTSIYHLWKAFDINSNVGGACGEIVALKGKWGL--------NLLNPLVAS
CHS3_AJECA CLKERNEKKLNSRWFFNAFGRALTPNVCILLDVGTKPGPTALYHLWKAFDQDSNVAGAAGEIKAGKGKGWL--------GLFNPLVAS
CHS2_AJEDE CLKERNEKKLNSRWFFNAFGRALTPNVCILLDVGTKPAPTALYHLWKAFDQDSNVAGAAGEIKAGKGKGWL--------GLFNPLVAS
CHS2_EMENI CLKEHNQKKLNSRWFFNAFGRALQPNICILLDVGTRPEPTALYHLWKAFDQDSNVAGAAGEIKASKGKNML--------GLINPLVAS
CHS2_ASPNG CLKEHNKKKLNSRWFFNAFGRALQPNICILLDVGTKPAPTALYHLWKAFDQNSNVAGAAGEIKAGKGKGML--------GLINPLVAS
CHS2_XYLBA CLKEKNKKKLNSRWFFNAFGRALTPNVCILLDVGTKPDSKALYHLWKAFDQDSNVAGAAGEIKADKGKGWM--------GLINPLVAS
CHS2_LAL   CLKEKNQKKINSHRWFFNAFGAILQPNYCYLIDVGTMPGPTSIYHLWKAFDINSNVGGACGEIVALKGKYGQ--------NLINPLVAA
Class_III
CHS1_USTMA CLKQKNSKKINSHRWFFNALGRHLQPELCVLIDAGTKPGHKSLYYLWEAFTNNANLGGACGEIHAMIKN---------GRKLFNPLVAA
CHS3_NEUCR CLKQKNTKKINSHRWLFNAFGRINPEVCILLDAGTKPSPRSLLALWEGFTNDKDLGGACGEIHAMLGKG---------GKKLINPLVAV
CHS3_EXOJE CLKQKNSKKINSHRWLFNGFGRINPEVCILLDAGTKPGPKSLAALWEAFTNDKDLGGACGEIHAMLG-RGGV--FGRKLINPLVAA
CHS3_WD    CLKQKNSKKINSHRWLFNAFGRINPEVCILLDAGTKPCSKSLMALWQAFTNDKDLGGACGEIHAMLCGPGGV--FGRL---PLVA-
CHS2_AJECA CLKQKNSKKINSHRWLFNAFGRINPEICILLDAGTKPGHKSLLALWEAFTNDKDLGGSCGEIHAMLG-KGWK---------NLINPLVAS
CHS1_LAL   VLKAKNQNNINSHRWLFNAIGKQLNPEICYLIDAGTKPGHKSIFYLWEAFTNDAHLGGCCGEIHAMIQGGRK---------LINPLVAA
Class_IV
CHS1_CAC   CYKEQNKKKLNSHRWFFNLGP-LINPNVCYLIDVGRSPTGTSIYELWKCFDKHPNVGGACGEIAYDTGRGCSL---------LLTSPLAAS
CHS2_USTMA CLKEQNKKKLNSHRWYFNAFCQMLKPNVCILLDVGTKPTGTSIYELWKSFDKHHRVGGACGEICVDTGRGCT---------ALFNPLVAS
```

Figure 8: Phylogenetic tree showing the relationships between chitin synthase fragments

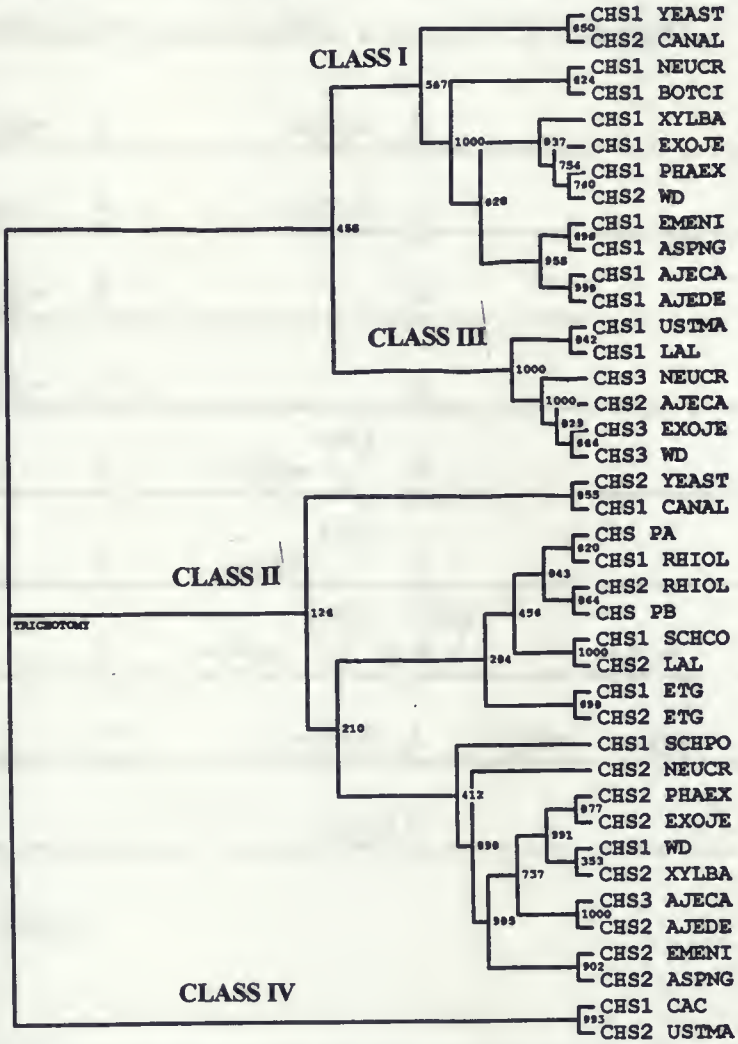
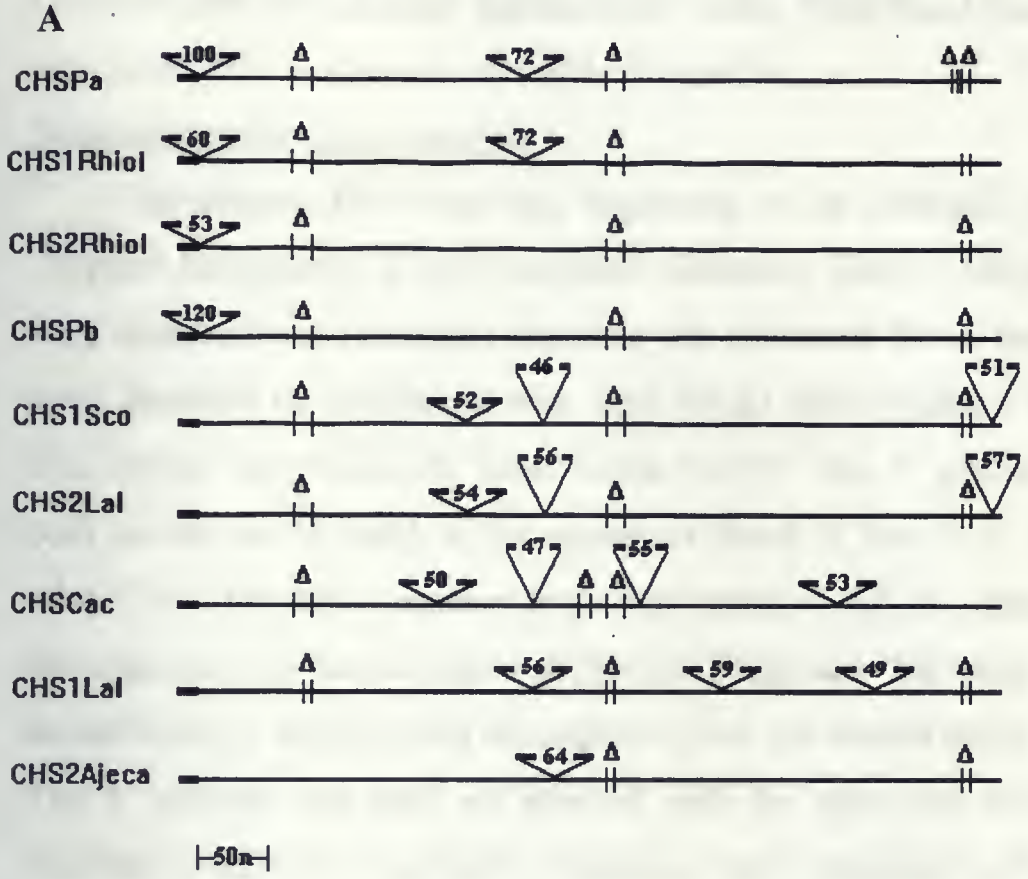


Figure 8: Sequences are alinged by the CLUSTALW program and bootsraping option (1000 trials) was selected.The resulting tree is viewed by the TREEVIEW program The names of the species are listed in abreviated forms: CHS1_YEAST, CHS2_YEAST (*Saccharomyces cerevisiae*), CHS1_CANAL, CHS2_CANAL (*Candida albicans*), CHS1_NEUCR, CHS_2NEUCR, CHS_3NEUCR (*Neurospora crassa*), CHS1_ASPNG, CHS2_ASPNG (*Aspergillus niger*), CHS1_RHIOL, CHS2_RHIOL (*Rhizopus oligosporus*), CHS_PB (*Phycomyces blakesleeanus*), CHS_PA (*Phascolomyces articulosus*), CHS1_EXOJE, CHS2_EXOJE, CHS3_EXOJE, (*Exophylla jeanselmei*), CHS1_XYLBA, CHS2_XYLBA (*Xylophylla bantiana*), CHS1_ETG, CHS2_ETG (*Entomophaga aulicae*), CHS1_BOTCI (*Botrytis cinerea*), CHS1_USTMA, CHS2_USTMA, CHS3_USTMA (*Ustilago maydis*), CHS1_WD, CHS2_WD, CHS3_WD (*Wengellia dermatitis*), CHS1_LAL, CHS2_LAL, (*Laccaria laccata*), CHS1_CAC (*Cantharellus cibarius*), CHS1_SCHCO (*Schizophyllum commune*), CHS1_SCPO (*Schizosaccharomyces pombe*), CHS1_AJEDE, CHS2_AJEDE (*Ajellomyces dermatitis*), CHS1_AJECA, CHS2_AJECA (*Ajellomyces capsulatas*), CHS1_EMENI, CHS2_EMENI (*Emericella nidulans*), CHS1_PHAEX, CHS2_PHAEX (*Phaecoccomyces exophialae*). Internal labels show the number of trials where particular sequences are drawn together.

Figure 9: Intron conservasion in chitin synthase PCR fragments

A



B

Species	Genes	Affinity	# Introns	CHS Class
Ajellomyces capsulatum	*AjecaCHS2*	Ascomycetes	1	III
Laccaria laccata	*LalCHS1*	Basidiomycetes	3	III
	LalCHS2		3	II
Schizophylum commune	*ScoCHS1*	Basidiomycetes	3	II
Cantharellus cibarius	*CacCHS1*	Basidiomycetes	3	IV
Rhizopus oligosporus	*RhiolCHS1*	Zygomycetes	2	II
	RhiolCHS2		1	II
Phycomyces blakesleeanus	*PbCHS*	Zygomycetes	1	II
Phascolomyces articulosus	*PaCHS*	Zygomycetes	2	II

Figure 9: A. This scheme represents chitin synthase conserved regions from three fungal classes. The sequences were aligned by CLUSTALW and the relative positions of introns were determined. The sequence information was down loaded from the Genbank database. Triangles represent introns. The numbers within the triangles represent number of nucleotides. The vertical lines with (Δ) represent gaps introduces due to alignment. The thickened lines represent the left hand primer sequences. B. The table summarizes the species, the fungal class and the putative zymogenic class.

Southern hybridization was used to show homology with the genomic DNA. Genomic DNA was partially digested with *Eco*RI, *Hin*dIII and *Bam*HI, hybridized with insert DNA and exposed overnight (Figure 5b).

Sequencing and sequence analysis

The plasmid DNA was fully sequenced by the automatic sequencer 373A (Applied Biosystems) at the McMaster University facility. Overlapping regions were identified and a complete sequence was generated. Intron specific sequences were identified by comparison with other fungal chitin synthase gene sequences. Two introns are presumably found within *PaCHS*. The 5' splicing sites (gtaa) in both introns are identical to the sequences found in introns of *RhiolCHS1* and *RhiolCHS2*. The lariat sequence in the first intron of *PaCHS* (gctgat) is similar to the sequence from the first intron of *RhiolCHS2* (gctaat). The lariat sequence in the second intron is identical with the sequence from the second intron of *RhiolCHS1*. The 3' splicing sites (tag) are identical with the splice site from *PbCHS*. The resulting continuous nucleotide sequence was translated with the help of Genejockey program. The resulting polypeptide was 200 amino acids long (Figure 6). Comparison of *PaCHS* amino acid sequence with sequences deposited in the EMBL databank in Heidelberg, Germany, revealed similarity with 36 chitin synthase fragment. The closest matching was with gene fragments that belong to zygomycetous fungus Rhizopus oligosporus (74.7% for *RhiolCHS1* and 71.6% for *RhiolCHS2*). *PaCHS* was also closely matched with all class II fragments listed in the database. The results are listed in Table 7.

Classification and of *PaCHS*

The deduced amino acid sequence was aligned with 40 other partial sequences (down loaded from the GenBank database) from other fungi from all three zymogenic classes proposed by Bowen et al. (1992) and a fourth class

proposed by Mehman and coworkers (1994), by the CLUSTALW method (Figure 7). The classification was done according to the initial tree given by the program. *PaCHS* is grouped with chitin synthases that are classified as class II by Bowen and coworkers (1992). Bootstraping analysis done by the CLUSTALW program groups all zygomycetous chitin synthase genes in class II group (Figure 8). Mucorales fungi are grouped together at 93.4% (934 out of 1000 trials) bootstrap support whereas the fragments of Entomophthorales (*Entomophaga aulicae*) are grouped together at a 99.9% (999 out of 1000 trials) bootstrap support. Fragments from two Basidiomycetes (*Schizophyllum commune* and *Laccaria laccata*) are grouped with higher level of bootstrap support 43.3 % (433 out of 1000 trials) with the fragments from Mucorales than the level of bootstrap confidence 29.5% (295 out of 1000 trials) between the fragments of Entomophthorales and the group consisting of fragments from Mucorales and the two Basidiomycetes. Fragments from the family Dematiaciae are grouped in classes I, II and III with high level of bootstrap confidence (82.8%, 92.5% and 73.7% respectively) and are also grouped with fragments from ascomycetous fungi such as *Aspergillus niger* and *Neurospora crassa*. Fragments of Candida albicans and Saccharomyces cerevisiae constitute outgroups in classes I and II.

Comparison of introns of *PaCHS* with introns from other chitin synthase fragments

Sequences of chitin synthase gene fragments containing introns were aligned with *PaCHS* and the position of introns and breaks due to alignment were identified. The fragments considered here are: *RhiolCHS1* and *RhiolCHS2* (*Rhizopus oligosporus*), *PbCHS* (*Phycomyces blakesleeanus*), *ScoCHS1* (*Schizophyllum commune*), *LalCHS1* and *LalCHS2* (*Laccaria laccata*), *CacCHS1* (*Cantharellus cibarius*) and *AjecaCHS2* (*Ajellomyces capsulatas*). The fragments from *C. cibarius* and *L. laccata* are included as representatives of a large group of

ectomycorrhizal fungi that contain intron sequences within PCR generated chitin synthase fragments. *PaCHS* contains an intron conserved in all Mucorales investigated to date. A second intron is conserved in *RhiolCHS1*. *PaCHS* showed no conservation in intron position with the rest of fungi considered here. Results shown in Figure 9.

Discussion

Chitin synthase has been extracted in the past from *Choaneophora cucurbitarum* and *Phascolomyces articulosus*, the susceptible and resistant hosts of the mycoparasite *Piptocephalis virginiana*, respectively. The chitin synthases of the two species are both proteolytically activated, membrane bound and are both zymogen forms (Manocha and Begum, 1985; Manocha and Balasubramanian, 1988). Manocha and Graham (1982) implicated chitin synthase activity at infection site to host resistance. In an *in vivo* study using autoradiographic techniques, they demonstrated that the activity of chitin synthase as measured by the degree of incorporation of N-[H^3]acetylglucosamine ([^{3}H]GlcNAc) into chitin at the penetration sites varied as a function of host-parasite compatibility. At 18 and 24 h after inoculation, the label incorporation was higher at the penetration sites in *P. articulosus* than in *C. cucurbitarum*, coinciding with the formation of papilla and sheath in the former species.

Differences in structure, activation and expression of the chitin synthase genes may account for distinct responses of the two hosts to the mycoparasite invasion. To elucidate such details cloning and characterization of chitin synthase genes from both hosts is necessary. This study focused on the isolation of a chitin synthase gene fragment from *P. articulosus*. PCR was employed to amplify genomic DNA regions homologous to chitin synthase. PCR detected four bands (820, 900, 1000 and 1500 bp, approximately) that are candidates for chitin synthase gene fragments. The 820 bp fragment was chosen as a primary candidate because it is similar in size with the existent chitin synthase fragments (600-750 bp), previously amplified with similar primers (Bowen *et al.*,1992; Miyazaki *et al.*, 1993). This fragment was cloned in pUC19 vector and was recovered after double digestion with *Hin*dIII and *Xho*I restriction enzymes. The recombinant plasmid was

designated pUC19-*PaCHS*. Restriction enzyme analysis revealed two internal restriction sites; *Bam*HI (746) and *Hin*cII (780), that can be useful for further cloning experiments. Sequencing of our fragment (designated *PaCHS*) revealed two nucleotide regions that are most likely introns since they contain intron characteristic sequences. Translation of the remaining nucleotide sequence resulted in a polypeptide sequence of 200 amino acids in length.

Comparison with the EMBL database sequences revealed a high percentage in matching ranging from 74.5 to 36% between *PaCHS* and other partial or full chitin synthase amino acid sequences (Table 6). It was concluded that the cloned fragment corresponded to a chitin synthase. *PaCHS* is mostly related at the amino acid level with two chitin synthase genes from *Rhizopus oligosporus* (*RhiolCHS1* and *RhiolCHS2*), another zygomycetes fungus (Motoyama *et al.*,1994). Comparison of *PaCHS* with other genes shows that it is mostly related with class II chitin synthases and less related with class III synthases. These facts indirectly suggest that *PaCHS* is a class II chitin synthase and most likely codes for a zymogenic chitin synthase (Bowen *et al.*, 1992).

To substantiate such a claim, *PaCHS* was aligned with 40 other chitin synthase genes by the CLUSTALW program (Figure 7). *PaCHS* was best aligned with class II chitin synthases according to the classification proposed by Bowen and coworkers (1992) and expanded by Mehman *et al.*(1994). Class II includes the genes from *Rhizopus oligosporus* and *Phycomyces blakeleeanus* and *Entomophaga aulicae* that belong to the zygomycetes. Bootstrap analysis of the same alignment reveals that the chitin synthases examined belong to four distinct classes. The chitin synthases from zygomycetous fungi are grouped in class II and appear closely related. Chitin synthase genes from mucoraceous fungi are closely grouped together with 93.4% (943 out of 1000 bootstrap trials) and they appear less related with the

genes of *Entomophage aulicae*, a fungus in Entomophthorales. This observation is consistent with the taxonomic classification of Zygomycetes (Moore-Landecker, 1990). On the other hand genes from *Schizophyllum commune* and *Laccaria laccata*, two Basidiomycetes, are also closely related to genes from Zygomycetes. This association was also, observed by Miyazaki and coworkers (1993). Chitin synthase gene fragments from Zygomycetes are not closely associated with Ascomycetes such as *S. cerevisiae*, *S. pombe*, *N. crassa* and *A. niger* within class II. This observation is consistent with the classification of 18S ribosomal RNA genes, where Mucorales and Entomophthorales are grouped together but classified separately from Ascomycetes. The Fungi imperfecti included in this study are grouped as in Bowen et al (1992). The dimorphic fungus *C. albicans* constitutes an outgroup with *S. cerevisiae* and the members of the family Dematiaciae such as *X. bantiana* *E.jeanselmei*, *P. exophylla* and *E. jeanselmei*, constitute a closely related group with 99.1% bootstrap confidence (991 out of 1000 bootstrap trials). Class I exhibits similar grouping as class II. The fragments from *S. cerevisiae* and *C. albicans* constitute an outgroup within class I. The fragments of *N. crassa* and *B. cinerea* are closely related as reported by Causier and coworkers (1994). Fragments from Fungi imperfecti of the family Dematiaciae are grouped together. In a similar fashion fragments from Ascomycetes such as *E. nidulans*, *A. niger*, *A. dermatitis* and *A. capsulatas* are closely associated. In Class III fragments from *L. laccata* and *U. maydis*, two Basidiomycetes, constitute an outgroup. Fragments from the ascomycetes *N. crassa* and *A. capsulatas* are grouped with fragments from *W. dermatitis* and *E. jeanselmei* two fungi imperfecti. Similar groupings are evident in three classes and can be explained by the fact that members of the family Dematiaciae are most likely loculomycetous ascomycetes (deHoog and McInnis,

1987). The emergence of a fourth class is consistent with the classification of Mehmann *et al.* (1994).

As pointed out by Valencia *et al.* (1991) in their study about *ras* proteins, relatedness in sequences such as those considered by Bowen *et al.* (1992) and the present study can be interpreted in terms of similarity of function and/or in terms of similarity of species. The classification of Bowen *et al.* (1992) and by extension this classification suggest both types of similarity for the chitin synthase genes fragments chosen for analysis. *PaCHS* has been classified as class II in the present study. The class II chitin synthases from *S. cerevisiae* and *C. albicans* are responsible for septal chitin synthesis (Bulawa, 1993; Chen-Wu *et al.*, 1992). In the zygomycetous fungus *Rhizopus oligosporus* both chitin synthase genes isolated so far belong to class II (Motoyama and coworkers 1994). Expression studies suggested that the gene products for *RhiolCHS1* and *RhiolCHS2* function mainly in the hyphae growing stage but not in the late stage of spore formation. The possibility that *PaCHS* plays a similar role in cell wall biogenesis of *Phascolomyces articulosus* must be explored by similar expression studies.

The phylogenetic data in the present study and Bowen *et al.* (1992) exhibit certain limitations. In the first place, there is an important difference between the data presented here and data that include whole protein sequences. Xoconostle-Cazares *et al.* (1996) demonstrated that *CHS* genes exhibit high homology at the central part of the molecules (that includes the PCR fragments studied) and no homology at the amino and carboxy termini. This led to the suggestion that the highly conserved regions are in fact regulatory regions. Multiple alignment and bootstrapping demonstrated the disappearance of the three *CHS* classes originally described (Bowen, 1992) and the early divergence of two branches. In addition, homology of many *CHS* gene products did not appear to coincide with phylogenetic

relationships of the fungi analysed. This inconsistency was difficult to explain unless ancient evolution of *CHS* genes and convergence is invoked.

A second limitation of the present analysis is in the interpretation that can be made of evolutionary relationships (Bowen *et al.*, 1992). Because probably for functional reasons, the sequences are very similar, the apparent evolutionary relationships may be different from those derived by other methods. On the other hand, all chitin synthases may catalyse the same reaction using the same residues key residues. The variation seen in the segment examined may, in fact reflect the accumulation of neutral amino acid changes; therefore , the greater the evolutionary separation, the greater the number of such changes.

Sequence characterization of *PaCHS* revealed two introns. *PaCHS* was compared with seven other homologous regions from a variety of fungi. The fragments considered here are representatives of all published chitin synthase fragments that contain introns to date. An intron was conserved among all Mucorales examined and a second intron was conserved in *RhiolCHS1* of *R. oligosporus*. In contrast, fragments from the Entomophthorales fungus *Entomophaga aulicae* do not contain introns. In Basidiomycetes, introns are completely conserved between *ScoCHS1* of *Schizophyllum commune* and *LalCHS2* of *Laccaria laccata* that belong to class II and are related in our CLUSTALW analysis with zygomycetous chitin synthase fragments. This conservation is extended to all basidiomycetous class II chitin synthase fragments. One intron is conserved between a representative of class III (*LalCHS2* from *Laccaria laccata*) and a representative of class IV (*CacCHS1* from *Cantharellus cibarius*). The intron found in *AjecaCHS2* of *Ajellomyces capsulatum* (class III), the only ascomycetes containing an intron in the conserved region, is not matched with any introns of the examined chitin synthase fragments. In summary, intron positions in fragments from

Zygomycetes are different from the intron positions of Basidiomycetes within class II and the intron positions in class III fragments from ascomycetes are not conserved in class III fragments from basidiomycetes. Such observations are consistent with the assumption of Mehmann *et al.* (1994) that none of the events giving rise to introns observed in chitin synthase gene fragments examined here occurred in a putative ancestral gene and before the divergence of Basidiomycetes, Zygomycetes and Ascomycetes, irrespective of whether that event involved a net loss or gain of introns (Doolittle, 1978).

Southern hybridization of the insert as a probe with the PCR product and the two clones showed strong signals at the PCR portion of the gel and at the two inserts. It also hybridized in a weaker fashion to the plasmid portion and the other two PCR bands (Figure 5a).This observations can be attributed to incomplete digestion of the minipreparation plasmid DNA and to lower homology between the cloned fragment and the secondary PCR bands. In contrast, the insert hybridizes at a single locus with *P. articulosus* partially digested DNA (Figure 5b). The failure of the probe to hybridize to other loci can be attributed to high stringency conditions, low copy number of *PaCHS* and/or low homology between the insert and other chitin synthase gene related. Nevertheless, Southern hybridization shows that the cloned fragment can be used to isolate the entire *PaCHS* sequence from a genomic library.

In conclusion, a chitin synthase gene fragment designated as *PaCHS* was cloned, sequenced and characterized. *PaCHS* was 820 bp long, contained two intron sequences and coded for 200 amino acids. Comparison of the polypeptide sequence with other sequences from the EMBL database shows identity ranging from 74.1%-33% with 36 other chitin synthase fragments. Alignment of *PaCHS* polypeptide sequence with 40 other chitin synthase fragments revealed that it

belonged to class II zymogen-type chitin synthases. Bootsrapping analysis groups *PaCHS* among all the chitin synthase fragments available to date. Zygomycetes appear to be closely related. Comparison of introns in zygomycetous with basidiomycetous and ascomycetous chitin synthase fragments reveal different patterns of intron conservation in each class. Southern hybrdizations show homology between the cloned fragment, the PCR products and genomic DNA.

Future prospects

We have cloned and characterized a chitin synthase gene fragment from *P. articulosus*, the resistant host of a mycoparasite. The full sequence of the gene could be isolated by probing a genomic library with *PaCHS*. Cloning of the other PCR fragments could result in a full arsenal of probes for other *P.articulosus* chitin synthase genes. Alternatively, additional PCR primers could be used for amplification and cloning of other chitin isoforms in *P. articulosus*. This work could be extended to *Choanephora cucurbitarum*, the susceptible host to the mycoparasite, *Piptocephalis virginiana*. With the entire genes cloned from both hosts further experiments could be conducted. For example, disruption mutants can be generated from both hosts and the infection from the mycoparasite can be studied. On the other hand one or more isoforms of chitin synthase could be transformed from the resistant host to the susceptible host and the interaction with the mycoparasite parasite could be investigated.

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