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CLONING AND CHARACTERIZATION OF THE CHITIN SYNTHASE GENE FROM THE OOMYCETE PATHOGEN PYTHIUM INSIDIOSUM

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ABSTRACT

CLONING AND CHARACTERIZATION OF THE CHITIN SYNTHASE GENE FROM THE OOMYCETE PATHOGEN *Pythium insidiosum*

By

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Pythium insidiosum is an organism in the kingdom Stramenopila class Oomycetes that causes disease in mammals including humans. Current therapeutic strategies, including anti-fungal drug treatment, have been largely ineffective. Chitin synthase genes (*CHS*) have been found in the true Fungi and in the Oomycetes and may be a potential target for drug development. The objective of this study was to clone and characterize the *CHS2* gene from *P. insidiosum* (*PiCHS2*). Degenerate PCR primers (Bowen 1992) were used to amplify a conserved 600 bp region of *PiCHS2*. The deduced amino acid sequence of *PiCHS2* and homologous sequences from true Fungi and other Oomycetes were used to create a phylogenetic tree. The Oomycetes CHS grouped together away from the true Fungi, which suggests that *CHS* from the Oomycetes represent a new family of *CHS* genes. A biased genomic DNA library was screened using the 600 bp fragment of *PiCHS2* as a probe but failed to yield a clone containing *PiCHS2*. A second product co-amplified with the 600 bp fragment of *PiCHS2* did not share any significant sequence homology with any known *CHS* gene or any sequences in the GenBank database. However, the presence of the *PiCHS2* gene in the genome was supported by Southern Blot analysis.

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1.1 History of Infections Caused by *Pythium insidiosum*

Pythium insidiosum is a microorganism classified in the kingdom Stramenopila, phylum Pseudofungi, class Oomycetes, order Pythiales and family Pythiaceae. *P. insidiosum* is the causative agent of a disease termed pythiosis insidiosi, which is characterized by the formation of cutaneous granulomas in infected animals. The history of pythiosis is reviewed by Mendoza (1998). The first documented cases of equine cutaneous granulomas linked to pythiosis occurred during the 19th century in India. Smith in 1884, Fish in 1895 and Drouin in 1896, however, first suggested its fungal-like etiology. But they did not isolate the etiologic organism. The organism was first isolated by de Haan and Hoogkamer in 1901 who were working with equines with cutaneous granulomas in Indonesia. They named the disease hyphomycosis destruens equi. The identity of the organism remained obscure and this uncertainty was compounded by the fact that pythiosis insidiosi was clinically confused with other equine cutaneous diseases, specifically, a skin condition caused by species of the genus *Habronema*. During the 20th century Bridges and Emmons isolated an organism from Texan horses with cutaneous granulomas. They believed it was a fungus similar to *Mortierella*. Austwick and Copland induced the formation of biflagellate zoospores upon transfer to an aqueous medium containing rotten maize silage (Austwick 1974). Based on their findings they suggested the genus *Pythium* was a more appropriate classification for this organism. Ichitani and Amemiya induced oogonia with aplerotic spores and suggested the species

Pythium gracile. Later deCock *et al.*, working with several isolates from different geographic locations, concluded that this agent was a single species and *Pythium insidiosum* was introduced (de Cock 1987). Today, the disease remains an important cause of economic loss for horse farmers, a significant killer of humans in tropical and subtropical third world countries like Thailand and an important cause of death in dogs and cats.

1.2 Life Cycle of *Pythium insidiosum*

Organisms of the genus *Pythium* are important plant pathogens and some species are also pathogens of fishes. However, *P. insidiosum* is the only species of the genus that causes disease in mammals. *Pythium insidiosum* has a worldwide distribution in temperate, sub tropical and tropical areas.

The life cycle of *P. insidiosum* in nature was originally studied by Miller (1983) and involves the growth of hyphal elements on plant tissue, the formation of sporangia followed by the release of motile biflagellate zoospores. Miller was able to show that the zoospores of *P. insidiosum* swam toward the hairs of all mammals tested and even suggested that they swam toward the follicle of the hair (1983). In this experiment the zoospores changed from a random path to a continuous line to the hairs. The same experiment was performed using leaves, stems and roots of the Australian water lily (*Nymphaea gigantean*), the water hyacinth (*Eichhornia crassipes*) and salvinia weed (*Salvinia molesta*). Of all plants tested, the zoospores showed affinity for the Australian water lilies and displayed a similar change of direction as previously described. Miller proposed

that *P. insidiosum* grows as hyphae on water lilies where it forms sporangia and zoospores. The zoospores are released and swim in the swamp until encountering either another plant host, in which case the cycle is repeated, or the tissue of an injured animal where they germinate and causes pythiosis.

The model proposed by Miller, although plausible, does not address some important issues such as the mechanisms for the colonization and germination of the zoospores in an animal host. Mendoza *et al.* studied the phenomenon of chemotaxis in *P. insidiosum* and proposed a more detailed model of its life cycle (1993). Using a scanning electron microscope, they not only confirmed the chemotactic behavior of the zoospores, but they also showed that, upon reaching the tissue, the zoospores lost their flagella, encysted and then secreted an amorphous substance over their surface. The encysted zoospores, using the amorphous substance, attached themselves to the new host and then emitted a germ tube. Mendoza *et al.* also found that the germ tubes were always oriented toward the infected tissue. This observation was in agreement with similar findings in other *Pythium* species and in other Protoctistans, which also secreted a similar amorphous material (Mendoza 1993). Mendoza *et al.* proposed that chemotactic signals, produced by the plant and the animal tissues, direct the zoospores to swim toward the new host, secrete the amorphous substance and orient their germ tubes toward the tissue. The zoospores in their control experiment, which contained no plant or animal tissue, swam in a random manner, finally encysted, produced a germ tube but did not secrete the amorphous substance. Mendoza *et al.* proposed that secretion of the

amorphous substance aids the zoospores in attachment to the host's tissue and, therefore, could be an important virulence factor. Finally, they asserted that the zoospores are more likely the infectious propagule. Based on their findings they proposed a model similar to the one proposed previously by Miller. They suggested that *P. insidiosum* first colonizes a plant, grows in hyphal form and then differentiates into sporangia with zoospores. The zoospores, attracted by chemotactic signals, swim toward a new plant or an injured animal, encyst, secrete an amorphous substance, attach to the host tissue and produce a germ tube that penetrates the host's tissue. If the new infected host is a plant, the life cycle repeats, however, if the new host is an animal, the zoospores germinate causing pythiosis.

The models proposed by these two groups of investigators are both essentially the same on the most basic level, however, the Mendoza *et al.* model illustrates the life cycle at higher resolution. Their model shows an association between the production of the amorphous substance and the presence of animal tissue. This association suggests that the amorphous substance is involved in the virulence of *P. insidiosum*, however, the study by Mendoza *et al.* fails to show the molecular events involved in the attachment and penetration of the infected tissues. Furthermore, to prove that this substance is truly involved in the virulence of *P. insidiosum*, chemical identification of the amorphous substance followed by mutation of the genes involved in its synthesis would be required. To achieve this goal, an animal or plant model of the infection would be of great

value to assess the virulence of *P. insidiosum* in isolates that express or lack this substance.

1.3 Pythiosis insidiosi in Humans and Animals

Infections caused by *P. insidiosum* occur in temperate, subtropical and tropical areas of the world with cases being reported in Argentina, Australia, Brazil, Colombia, Costa Rica, Haiti, India, Indonesia, Japan, New Guinea, New Zealand, Thailand and the United States (Mendoza 1998). Horses, dogs, cats and humans are all susceptible to the infection, which usually occurs after exposure to stagnant water containing zoospores of *P. insidiosum*. However, some reported cases of animal and human pythiosis without exposure to stagnant water suggest that infection can be induced by other propagules of *P. insidiosum* (Mendoza 1998).

Three main forms of human pythiosis have been described: 1) ophthalmic which includes keratitis and corneal ulcer, 2) subcutaneous /cutaneous and 3) systmeic/vascular (Thianprasit 1996). Predisposing factors such as α-thalassemia, β-thalassemia, hemoglobin E and paroxysmal nocturnal hemoglobinuria have all been incriminated in reported cases of systemic and cutaneous human pythiosis in Thailand (Thianprasit 1996). Patients infected with *P. insidiosum* did not receive deferoxamine therapy, so some Thai clinicians believed that iron overload might contribute to the mechanism of infection (Wanachiwanawin 1993). However, based on data from experimental rabbit pythiosis and reports of human pythiosis in Australia, Haiti, New Zealand and the

USA, the infection does not appear to be associated with impaired immune function (Miller 1983). Thus, the high incidence of Thalassemia in Thailand could better explain why human pythiosis is always associated with this disorder. Based on reports of pythiosis in animals and humans, it has been shown that pythiosis occurs in apparently healthy hosts (Triscott 1993, Shenep 1998, Thitithanyanont 1998).

Clinical Presentation

The cutaneous form of the disease is commonly observed in the lower parts of the limbs but can occur in virtually any anatomical area of body that has been exposed to contaminated swamp water in the endemic areas. This form of the disease is common in horses, however, there have been at least two cases of human subcutaneous pythiosis in Thailand affecting the extremities (Thianprasit 1996). The clinical manifestation of the disease in horses is characterized by tumor like lesions with necrotic tissue and yellow-white coral like masses known as "kunkers" (Mendoza 1986). These granulomatous cutaneous lesions are 5mm to 500mm in diameter and have a characteristic serosanguineous discharge. Intense pruritus of the infected areas is also characteristic of the disease in horses (Mendoza 1998).

Patients with systemic arterial pythiosis usually develop progressive gangrene and pain in the extremities. Clinically, progressive ascending arteritis with thrombosis and aneurysms of the main arteries is observed (Thianprasit 1996). After exposure and infection due to *P. insidiosum*, the duration of the disease varies from 1-6 months before diagnosis or death (Thianprasit 1996).

The ophthalmic form of the disease rarely involves patients with hemoglobinopathies. This form occurs in otherwise healthy people usually after handling infected plants. Corneal ulcers and keratitis are typical clinical findings and both are indistinguishable from eye infections caused by fungi. Patients often end up with keratoplasty, evisceration or enucleation (Imwidthaya 1994).

Diagnosis

Pythiosis is difficult to diagnose based on clinical features alone. Cutaneous forms of the disease are often confused with infections caused by zygomycetes such as *Basidiobolus ranarum* and *Conidiobolus coronatus.* Microscopic examination of clinical material from the infected areas is the most common means of diagnosing pythiosis. The coral-like kunkers in equine pythiosis are the ideal clinical material for laboratory testing. The masses should be cut into small pieces and placed in 10% KOH and then examined using light microscopy (Mendoza 1998). Humans with pythiosis rarely develop kunkers, so tissue biopsies, pus or tissue debris should be used for microscopic examination. The presence of coenocytic hyphae from infected areas 4.0-9.0μm in diameter with branches at about 90° angles is indicative of pythiosis. The production of biflagellate zoospores is an important diagnostic tool and can be performed within 24 hours. Once biflagellate zoospores are induced, one can rule out the confusing possibility of an infection caused by a zygomycete.

Histological examination of tissue sections from infected areas can also be used to diagnose infections caused by *P. insidiosum.* Coenocytic hyphae with branches at 90-degree angles surrounded by eosinophils is a typical

histopathological feature of pythiosis. However, these tissue sections can easily be mistaken for those found during infections caused by organisms in the fungal phylum Zygomycota, specifically *B. ranarum* and *C. coronatus*, which also cause cutaneous granulomas in horses. Thus, other methodologies have been developed to differentiate these etiologic agents.

Serological tests have proven to be the most reliable way to diagnose infections caused by *P. insidiosum*. The enzyme-linked immunosorbent assay (ELISA) has been used to diagnose pythiosis in horses. The test proved to be specific and sensitive in dogs, humans and horses with active pythiosis (Mendoza 1998). This test employs dilute culture filtrate antigen (CFA) and anti-horse IgG or IgM. Some horses in endemic regions without any other signs of disease were found to be positive in this test, suggesting that sub-clinical infections may occur (Mendoza 1998).

Immunohistochemical assays including immunofluorescence (Mendoza 1987) and immunoperoxidase (Brown 1988, Triscott 1993) tests have been used to identify *P. insidiosum* hyphae in tissue sections. The immunofluorescence test is specific for *P. insidiosum* in infected cats, dogs and humans, but uniform fluorescence among tissue sections of the "kunkers" was detected in equine pythiosis (Mendoza 1998). The immunoperoxidase test was able to distinguish *P. insidiosum* in tissue sections from the troublesome zygomycetes *B. ranarum* and *C. coronatus* (Triscott 1993).

The complement fixation test was also used in the past; however, this test detects only 82% of horses infected with *P. insidiosum*. This test is also prone to

false positives, specifically, in sera from cured patients or from apparently healthy horses (Miller 1982). These facts combined with the difficulty in performing the test have precluded its use as a diagnostic tool.

Finally, several laboratories have used the immunodiffusion test (ID) with success. The ID test is by far the best serological test for diagnosis. Its success is due to its economical features and also its simplicity to perform and interpret. Although it is insensitive, its specificity is remarkable. The test uses concentrated CFAs from *P. insidiosum* (Mendoza 1986). This test did not show precipitin bands using sera from horses recovering from infection after two months of successful treatment. Further, the test showed no cross-reaction using sera from patients with conidiobolomycosis, habronemiasis or horses with excessive granulomatous tissue (Mendoza 1986). This test is also useful in monitoring response to treatment and may be applied to cats, cattle, dogs, horses and humans with pythiosis (Mendoza 1998).

In summary, the diagnosis of pythiosis can be accomplished using direct microscopic examination of clinical samples from infected sites followed by a confirmatory ID test as well as other serological tools. In well-equipped laboratories the histochemical assays can provide a reliable alternative to the immunodiffusion and ELISA tests. In all cases care should be taken to avoid misdiagnosis of cutaneous zygomycosis.

Treatment

Surgical removal of lesions caused by *P. insidiosum* has been the main treatment in horses and humans. This procedure is not practical since most

lesions occur on the limbs and this area, at least in horses, possesses a delicate anatomical structure. Furthermore, it is very difficult to remove all of the infected tissues and reoccurrence of the disease is common (McMullan 1977, Alfaro 1990). Amputation to treat pythiosis has been attempted in humans with the vascular form (Thianprasit 1996). Although some patients responded to this form of treatment, many did not. Patients that do not respond to amputation often die of disseminated abdominal pythiosis (Mendoza 1998). Nonetheless, surgery is one of the options available to treat pythiosis in horses and humans. A combination of surgery with other forms of treatment has been attempted. For example, two horses with subcutaneous pythiosis were treated by surgical removal of the granulomatous masses on their legs. The sites were then treated with a neodymium:yttrium-aluminum-garnet laser and the horses were given trimethoprim-sulfa-methoxazole and phenylbutazone for 2 weeks after surgury (Sedrish 1997). One year later both horses were disease free and had minimal scarring.

In summary, surgery has limited use in cases of systemic pythiosis and amputation is often required. Reoccurrence of the disease is common following surgical treatment of cutaneous forms due to incomplete removal of all of the infected tissue. However, surgery followed by drug therapy or laser cauterization, has been used successfully to treat cutaneous forms of the disease.

The most significant advance in the treatment of pythiosis was the development of a curative vaccine in 1981. Miller first used an

immunotherapuetic vaccine consisting of sonicated hyphal antigens to cure horses with pythiosis (1981). Miller achieved a 53% cure rate after vaccination alone and a 33% cure rate using the vaccine after surgery (n=40: 30 horses vaccine only, 10 horses surgery followed by vaccine). Miller suggested that the failure of the vaccine might be due to the age of some of the lesions or the age of the vaccine preparation. Later, Mendoza developed a new version of the vaccine made of precipitated proteins from CFAs (1986). This vaccine gave results similar to one Miller tested in 1982 but it had a longer shelf life and fewer side effects. Mendoza *et al.* noted that horses with chronic pythiosis (lesions >2 months old) did not respond to the vaccine. Overall, horses with chronic pythiosis all died in spite of receiving the vaccine, whereas, horses with acute pythiosis were all cured after vaccination (Mendoza 1992). Horses that were cured by immunotherapy all developed a local inflammatory response at the site of the injection. Horses that were not cured by the vaccine did not respond at the site of the injection. These findings strongly suggest that the success of immunotherapy could be directly related to the immune status of the horse (Mendoza 1998).

In addition to its usefulness in horses, there is one documented case where the vaccine was successfully used to cure human pythiosis and at least seven new human cases from Thailand (Thitithanyanont 1998, Mendoza unpublished data, personal communication). The first case treated with the vaccine was a boy from Thailand with chronic disseminated arterial pythiosis. The vaccine was given as a last resort. He progressed over a 4-week period

until all symptoms of the disease virtually disappeared. Two years after the vaccination, the boy was still in good condition and free of pythiosis. The other seven cases also responded in the same fashion. The vaccine does not appear to provide any lasting immunity to the disease.

Based on histopathological and serological findings in cases of vaccinated and cured humans and animals, a potential mechanism of action for the curative properties of the vaccine has been proposed (Thitithanyanont 1998). It was found that, in cured cases, the classic eosinophilic inflammatory reaction gradually changed to a mononuclear response with macrophages and possibly cytotoxic T lymphocytes (CTLs). This switching of the response indicated this is an example of a possible immunomodulation from a Th2 (eosinophils and IgE) to a Th1 response (mononuclear cells). While the immunotherapuetic vaccine remains an exciting new treatment option, further investigation on the mechanism of its curative properties should be pursued before its commercialization. Although the vaccine is of value in acute cases, it is of little use in chronic equine pythiosis presumably because horses become anergic and unable to respond to the stimulation provided by the immunogens in the vacccine.

Drug therapy is another therapeutic option that has been used over the years to treat pythiosis in animals and humans. Many traditional anti-fungal compounds target ergosterols, however, this compound is not present in the cell membrane of *P. insidiosum*. Interestingly, some investigators have reported success rates up to 80% using amphotericin B, a drug that targets ergosterols, to

treat pythiosis in horses (McMullan 1977). Strikingly, since that report, other reports have indicated that Amphotericin B is ineffective for treating pythiosis (Imwidthaya 1994, Thianprasit 1996). In addition, amphotericin B has no effect on *P. insidiosum in vitro* and it is highly toxic to the host. Potassium iodide is another compound that has been used to treat equine pythiosis with contradictory results. Although some reports indicate that potassium iodide or sodium iodide is effective against the pathogen (Gonzales 1979, Hutchins 1972), other reports indicate these drugs are not effective (Mendoza 1998, Murray 1978). In addition to their low cure rate these drugs are toxic the host.

In human pythiosis, amphotericin B was ineffective in curing systemic pythiosis, the most common type (Wanachiwanawin 1993, Thianprasit 1996, Sathapatayavongs 1989). A combination therapy using terbinafine and itraconazole was effective in treating one case of human ophthalmic pythiosis (Shenep 1998). In two cases of subcutaneous pythiosis a combination of amphotericin B and 5-flurocytosine was effective (Triscott 1993). Additionally, some studies indicate that a saturated solution of potassium iodide can cure subcutaneous human pythiosis (Thianprasit 1996, Mendoza 1998). However, other reports have suggested that potassium iodide is ineffective (Mendoza 1998). A combination of amphotericin B, flucytosine and hydrocortisone was successful in treating two cases of subcutaneous human pythiosis in Australia (Triscott 1993). Although drug therapy has proven to be feasable in some cases of subcutaneous pythiosis, it is ineffective in cases of systemic human pythiosis. Unfortunately, many clinicians resort to amputation above the infected site for

the treatment of systemic pythiosis (Thianprasit 1996). In summary, human subcutaneous and ophthalmic pythiosis have been treated successfully with drugs in a few cases, however, drug therapy is not effective in treating systemic or vascular pythiosis.

Drug therapy for pythiosis applies anti-fungal compounds to a disease that is not caused by a fungus, consequently, the drugs give contradictory results. This is most likely because the targeted compounds of the drugs are not present in *P. insidiosum*. Therefore, new drugs with a broader spectrum of action should be developed in order to treat non-fungal diseases like pythiosis. New targets common to fungal and fungal-like organisms should be explored. Some potential targets for this type of therapeutic strategy are the cell wall components of *P. insidiosum* including the glucan and chitin synthase enzyme systems.

1.4 Chitin and Chitin Synthase

Chitin: Poly N-acetylglucosamine

Chitin is an unbranched polysaccharide widely distributed in the Kingdom Fungi. It has also been found in animals (insects and crustacea), plants (algae) and protoctista. In fungi and fungal-like microorganisms chitin is part of the cell wall and gives strength and rigidity to the cell. Chitin is composed of repeating units of N-acetyl-D-glucosamine (GlcNAc). A single strand of chitin is estimated to be between 1800 and 2300 residues long (Ruiz-Herrera 1992). Individual chitin strands associate with other strands by hydrogen bonding and non-bonded interactions between pyranose rings in arrays of sheets or chains (Ruiz-Herrera

1992). Chitin is classified as alpha, beta or gamma according to how the strands interact with each other. Like DNA strands, chitin strands have polarity, which is based on their reducing and non-reducing ends. Alpha chitin is the only form identified to date in true Fungi.

Chitin Synthase Genes (*CHS*)

The cell wall of most fungi is composed, in part, of the polysaccharides chitin and glucan, which provide rigidity and strength to the cell. It has been known for quite some time that Oomycetes possess cellulose and glucan in their cell walls. More recently, however, it was reported that some Oomycetes also contain chitin. There is evidence that chitin and glucan are structurally linked in the cell wall, a fact that may be important in planning therapeutic strategies (El-Sherbeini 1995, Kollar 1995). More importantly, the enzymes responsible for the synthesis of these two polymers, glucan and chitin synthases, are inhibited by the echinocandins and nikkomycins, respectively. This finding suggests that the cell wall constituents might be good targets for the development of new anti-fungal drugs.

Several unique genes or *CHS* genes encode the chitin synthases. Much of what is known about *CHS* comes from studies of organisms in the Kingdom Fungi. True Fungi typically have multiple isoforms of chitin synthase, each with a different role in the cell. In yeast, such as *Saccharomyces cerevisiae,* at least three isoforms of CHS have been found (Bulawa 1993). *CHS1* and *CHS2* are both activated by protease treatment, whereas, *CHS3* is not (Bulawa 1993). *CHS1* and *CHS2* together produce only 10% of cellular chitin while *CHS3* is

believed to make up the remaining 90%. Using mutagenic studies, it was found that *CHS1* is required for normal budding while *CHS2* is required for normal morphology, septation and cell separation (Bulawa 1993). *CHS3* mutants are resistant to Calcofluor and *Kluyveromyces lactis* killer toxin. They lack alkali-insoluable glucan, and are, in some cases, temperature sensitive for growth (Bulawa 1993). In filamentous fungi, like *Aspergillus nidulans*, four *CHS* genes have been described so far. These *CHS* genes are involved in conidia formation and growth at the apical portion of the hyphae (Borgia 1996, Motoyama 1997). In general, these isozymes have redundancy and multiple *CHS* genes must be disrupted in order to perturb the growth of the fungus.

Inhibition of CHS Enzymes

The problems associated with current anti-fungal drugs including limited effectiveness, toxicity and cost have prompted interest in finding new drugs with targets more specific to the pathogens. The cell wall has proven to be a very effective target in prokaryotes and is now being investigated as a potential target in fungal and para-fungal pathogens.

The echinocandins, inhibitors of glucan synthase, are currently in clinical trials. They proved to be effective in murine models infected with *Candida* sp., *Aspergillus fumigatus* and *Histoplasma capsulatum* (Powles 1998). An echinocandin class anti-fungal drug was fungicidal to *Candida albicans* in an *in vitro* study (Gordee 1988). This compound inhibited glucose incorporation into β-glucan in about 75-95% of treated fungal cells. A semi-synthetic pneumocandin was also effective *in vivo* against the fungus *Pneumocystis carinii* in a rat model

of the infection (Powles 1998). Echinocandins are limited by their low oral absorption, so they need to be applied parenterally until perfected for oral use. The anti-fungal drugs that target glucan synthase could also be applied to patients infected with *P. insidiosum*, which also contains glucan.

Another class of compounds, the nikkomycins, have been explored as potential chemotherapeutic agents. Although the nikkomycins showed effectiveness *in vitro* against some of the pathogenic dimorphic fungi (Hector 1990), they are no longer in clinical trials (Georgopapadakou 2001). Efficiency of transport into fungal cells and susceptibility to degradation may account for their limited clinical use (El-Sherbeini 1995). In spite of this, the current nikkomycin anti-microbials may find application in dual therapy with echinocandins. A combination of a nikkomycin and an echinocandin was effective *in vitro* against *Aspergillus fumigatus*, *Coccidioides immitus* (Stevens 2000) and *Candida albicans* (Hector 1986). One study indicates that fungi may compensate for inhibition of synthesis of one polymer by up regulation of another polymer (Stevens 2000). In another study, it was found that the mycelial phase of *Paracoccidioides brasiliensis* synthesized more α-glucan in response to treatment with papulacandin B, an inhibitor of β-glucan synthesis (Hector 1990). These studies suggest that dual inhibition of glucan and chitin synthase may enhance the therapeutic value of drugs that target the cell wall components.

Although current chitin synthase inhibitors have not proven effective in treating fungal infections, it seems that the short half-life and delivery of the drug to the fungal target might explain the problems. If these pharmacological

problems could be solved, chitin synthase inhibitors may find application alone or in concert with other cell wall inhibitors. Therefore, chitin synthase remains an unexploited target. Characterization and comparison of the cell wall components of fungal-like organisms to true fungi may be important for the evaluation of new anti-fungal drugs for the treatments of infections caused by fungal-like organisms such as *P. insidiosum*.

Study of CHS from the Oomycetes

The Oomycetes, including *P. insidiosum*, are a group of organisms in the kingdom Stramenopila that are morphologically, but not phylogenetically, related to organisms in the kingdom Fungi. These organisms are protists near the plants and the green algae (Kwon-Chung 1994). Due to their morphological features, the Oomycetes were at one time considered to be members of the kingdom Fungi. Before phylogenetic analysis, the Oomycetes and the true fungi were separated into two groups based on their cell wall polymers. The Oomycetes were placed into the cellulose-glucan group and the fungi into the chitin-glucan group (Bartnicki-garcia 1968).

During the middle of the 20th century, several investigators explored the possibility of chitin in some Oomycetes (Thomas 1942, Frey 1950, Cooper 1967, Lin 1970, Cherif 1992, Bulone 1992, Mort-Bontemps 1997). Thomas in 1942 first proposed the idea of chitin and cellulose in the Oomycete *Pythium debaryanum*. However, Frey (Frey 1950), who found only cellulose in this Oomycete, could not replicate his work. Using X-ray diffraction and working with *Apodachlya* sp., Lin and Aronson revealed the presence of chitin in this

Oomycete (1970). Using a wheat germ agglutinin-ovomucoid-gold complex (WGA), a lectin that binds to GlcNAc, Cherif *et al.* found chitin in *Pythium ultimum* (1992). However, it is well known that the WGA assay is not entirely specific for chitin and that it has weak affinity for N-acetylneuraminic acid, *N*-acetylgalactosamine and Man-β-(1-4)-GlcNAc-β-(1-4)-GlcNAc-β-*N*-Asn (Bulawa 1993). Later, Bulone *et al.* were able to demonstrate the presence of chitin in the Oomycete *Saprolegnia monoica* using several assays. They demonstrated chitin in the cell wall using X-ray diffraction, electron diffraction and infrared spectroscopy. They obtained a residue hydrolysable by chitinase, an enzyme that degrades chitin. They also detected a 'chitin synthase activity' from membrane fractions of the mycelium of this oomycete and showed that the activity was enhanced after treatment with trypsin, a protease that activates chitin synthase *in vitro*. In addition, the activity was decreased by addition of polyoxin D, a competitive inhibitor of chitin synthase. They also detected chitin synthase activity in regenerated protoplasts. These investigators noted that although chitin synthase was present in *S. monoica*, the levels of chitin were low relative to most fungi.

Later, Mort-Bontemps *et al.* (1997) demonstrated the existence of chitin synthase genes in the Oomycete *Saprolegnia monoica* for the first time. This was the first chitin synthase gene ever cloned from an Oomycete. This group used a pair of degenerate primers, designed by Bowen *et al.* (1992) to amplify a conserved 600 bp region from fungal chitin synthase genes, to amplify homologous regions in three different oomycetes: *S. monoica*, *Phytophthora*

capsici and *Achlya ambisexualis*. They cloned and characterized the entire *CHS2* gene from *S. monoica*. In addition, they also detected a chitin synthase activity from the mycelium of *S. monoica* and demonstrated the expression of chitin synthase transcripts from mycelium and regenerated protoplasts using RT-PCR. As part of this work, Mort-Bontemps *et al.* constructed a phylogenetic tree using the deduced amino acid sequences of CHS2 from *S. monoica*, and the fungi *Rhizopus oligosporus*, *Candida albicans*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Neurospora crassa*. They suggested that the fungi and Oomycetes, "despite having a divergent evolution, have evolved with conserved chitin synthase systems" (Mort-Bontemps 1997). However, their analysis was limited by the fact that only one Oomycete was used for comparison. In addition, their results clearly show that *CHS2* from *S. monoica* segregates away from fungal *CHS* genes, which formed a compact group.

CHS Phylogeny

Later, Perpich *et al.* (1999), using the same set of degenerate primers, amplified a 600 bp fragment of the *CHS2* gene from the Oomycete *P. insidiosum*. They used the deduced amino acid sequences of the conserved 600 bp region from four Oomycetes and several fungi, rather than the entire *CHS* molecule, to construct a phylogenetic tree. Their phylogenetic analysis (figure 1) showed that the Oomycetes grouped together away from the fungal *CHS*. In addition, the Oomycetes, like the fungi, appear to be further divided into clades composed of *CHS1* and *CHS2* classes. These results suggested that *CHS* from the Oomycetes represents a new family of *CHS* genes distinct from

the fungal *CHS*. This finding also agrees with the current taxonomical placement of the Oomycetes in a separate kingdom from the true fungi.

1.5 Objectives

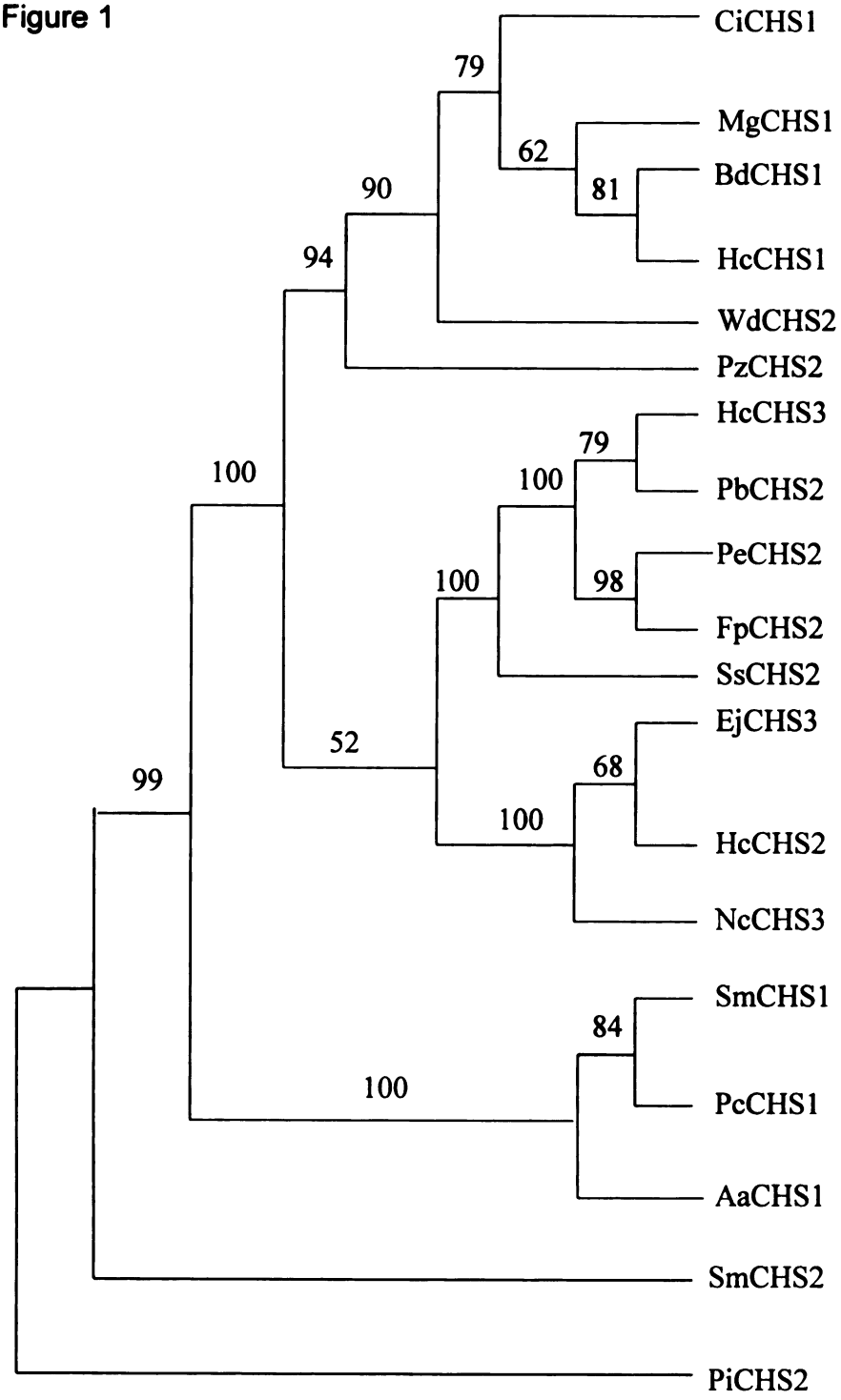
The general objective of the current study was to establish the presence of chitin synthase genes in *P. insidiosum*. The original specific goals of this study were:

- Cloning and sequence characterization of *CHS2* from *P. insidiosum* (*PiCHS2*).
- Determine the number of CHS isozymes present in *P. insidiosum*.
- Phylogenetic analysis using the deduced amino acid sequence of *PiCHS2*.

Figure 1

CHS Phylogenetic Tree. The tree was constructed using deduced amino acid sequences from several fungal chitin synthase genes and known Oomycete sequences: Aa=*Achlya ambisexualis*, Pc=*Phytophthora capsici*, Sm=*Saprolegnia monoica* and Pi=*Pythium insidiosum*. The fungi grouped into clades as *CHS1*, *CHS2*, *CHS3* or other isoforms of *CHS*. The Oomcyetes (bottom of the tree) grouped together away from the true fungi, which suggests that the Oomycetes *CHS* might be a new family of *CHS* genes. The phylogram was generated using PAUP (Phylogenetic Analysis Using Parsimony) with 500 bootstrap replicates.

Figure 1


CiCHS1
MgCHS1
BdCHS1
HcCHS1
WdCHS2
PzCHS2
HcCHS3
PbCHS2
PeCHS2
FpCHS2
SsCHS2
EjCHS3
HcCHS2
NcCHS3
SmCHS1
PcCHS1
AaCHS1
SmCHS2
PiCHS2
79
62
81
90
94
79
100
100
98
100
52
68
100
99
84
100

2. Materials and Methods

2.1 DNA Extraction

Pythium insidiosum (ATCC #200269) was subcultured in Sabouraud broth for 5 days at 37°C while shaking at approximately 150 rpm. The culture was killed by the addition of merthiolate (0.05% final concentration). The hyphae were concentrated using a vacuum pump and filter flask system. The hyphal mat was washed with sterile water and placed into a pre-cooled mortar. The mat was then ground under liquid nitrogen using a pestle and reduced to a fine powder. Microfuge tubes were filled half way up the conical portion with the powder and 600μL of lysis buffer (5 mM Tris-HCl (pH 7.2), 5 mM EDTA (pH 8.0), 0.9% SDS, 0.01% 2-mercaptoethanol) was added. The mixture was homogenized using a syringe and a 20 G 1 ½ needle and then centrifuged at maximum speed in a microcentrifuge. After centrifugation, 250μL of the supernatant was placed into a fresh tube and 250μL of lysis buffer was added. Proteinase K (200μg/mL final concentration) was added and the mixture was incubated at 55°C for 1 hour. Following incubation, 500μL of Phenol: Chloroform: isoamyl alcohol (25:24:1) was added and the tube mixed until an emulsion formed. The mixture was centrifuged for 15 minutes at maximum speed at room temperature. Following centrifugation, 400μL of the supernatant was placed into a fresh tube and 10μL of 3M sodium acetate followed by 2 volumes of ice cold 100% ethanol were added. The tube was inverted gently several times. After an overnight incubation at –20°C, the tube was centrifuged at maximum speed at room temperature for 5 minutes. One mL of 70% ethanol

was added to the resulting nucleic acid pellet. The tube was centrifuged at maximum speed at room temperature for 2 minutes. The 70% ethanol was removed from the tube and the pellet was air dried for 10 minutes and then resuspended in 50-100μL of sterile water depending on the downstream application of the DNA.

The RNA was removed by the addition of 30 units of DNase free RNase1 (Promega, Madison, WI) and buffer to a 1X working concentration and the mixture was incubated for 1 hour at 37°C. Following this incubation, the mixture was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), precipitated and washed following the technique above for the nucleic acid isolation.

2.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction was prepared on ice in a total reaction volume of 25μL containing the following (final concentration): 12.25μL H_2O, 1X Geneamp® PCR Buffer II (50mM KCl and 10mM Tris-HCl (pH 8.3, Applied Biosystems, Foster City, CA), 2 mM $MgCl_2$, 1.25 mM each dNTP, 20pmol each primer, 500ng DNA template (*P. insidiosum* genomic DNA) and 1.25 U Taq polymerase (Applied Biosystems, Foster City, CA). Negative control reactions containing the same reagents, except the DNA template, were included in all experiments. The PCR thermal cycling conditions were as follows: hot start for 10 minutes at 95°C, 40 cycles as follows: 1 minute at 94°C, 2 minutes at 50°C

and 3 minutes at 72°C and an extension file: 1 minute at 94°C, 2 minutes at 50°C and 10 minutes at 72°C.

Amplification products were analyzed by running portions of each reaction on a 1.4% agarose gel at 135 volts for 45 minutes to 1 hour. Ethidium bromide (0.5μg/mL) was added to all agarose gels. DNA was isolated from the agarose by using a protocol from the S.N.A.P.™ mini-prep kit (Invitrogen®, Carlsbad, CA). This purified PCR product was cloned into the TOPO-TA vector (Invitrogen ®, Carlsbad, CA) and used for sequencing or labeled radioactively and used as a probe for Southern hybridization experiments.

2.3 Southern Hybridization

Genomic DNA from *P. insidiosum* was digested with *Bam* H I (Promega, Madison, WI) as follows: 40μg DNA, 6 mM Tris-HCl (pH 7.5), 100mM NaCl, 6mM $MgCl_2$ and 1mM DTT and 40 units of *Bam* H I (buffer and enzyme from Promega, Madison, WI) in a total reaction volume of 60μL. The mixture was incubated at 37°C for 3 hours and heat inactivated by incubating at 65 °C for 15 minutes.

The reaction was loaded into 2 lanes of a 0.8% agarose gel along with undigested DNA, unlabeled probe and a DNA size marker as controls. The gel was run at 75 volts for approximately 4 hours.

The DNA was transferred to a nylon membrane using the capillary method. For this, the gel was first treated with 0.25 N HCl in a shaking bath at room temperature for 30 minutes. The gel was then placed into a shaking bath

containing 1.5 M NaCl and 0.5 N NaOH for 30 minutes and then transferred to a fresh bath of the same solution and the incubation was repeated. Finally, the gel was placed in a bath containing 0.5M Tris-HCl/1.5M NaCl (pH 8.0) and incubated for 15 minutes. Once the gel treatment was complete, a capillary transfer was set up using 3MM paper filters, 20X SSC (3M NaCl and 0.3M sodium citrate pH 7.0) as the transfer buffer and a positively charged nylon membrane (BioRad, Hercules, CA). After overnight transfer, the membrane containing the DNA was rinsed briefly in 10X SSC, to remove debris, and then briefly blotted. The DNA was cross-linked to the membrane by exposure to 50mJoules of UV energy in the Gene linker UV chamber (BioRad, Hercules, CA).

Pre-hybridization of the membrane was carried out at 42°C for 2 hours in 50% Formamide, 5X Denhardt's solution (0.1% ficoll Type 400, 0.1% polyvinylpyrrolidone and 0.1% BSA), 0.25% SDS, 3X SSPE (3M NaCl, 0.02 M EDTA and 0.2M NaH_2PO_4 pH 7.4) and salmon sperm DNA (0.1mg/mL).

The 600 bp PCR amplicon was radioactively labeled with ^{32}P using random priming with hexamers (Gibco, Rockville, MD). Unincorporated nucleotides were removed from the labeling reaction using a push column (Stratagene, La Jolla, CA). The probe was denatured by heating in a boiling water bath for 5 minutes and immediately transferred to ice. The labeled denatured probe was added to the pre-hybridization mixture, containing the membrane, and incubated at 42°C for 24 hours. The hybridization buffer was decanted from the hybridization bottle and 100mL of low stringency wash buffer (1X SSC and 0.5% SDS) was added. Washing at low stringency proceeded for

30 minutes at room temperature with one change of solution. After the second low stringency wash solution, 100mL of high stringency wash buffer (0.5X SSC and 0.5% SDS) was added and washing proceeded at 68°C for 30 minutes with one change of solution. The membrane was removed from the hybridization bottle, wrapped in plastic wrap and placed on an X-ray film (Eastman Kodak, New Haven, CT) at –70°C. The film was developed a various times depending upon the signal strength using an automated film processor (Eastman Kodak, New Haven, CT).

2.4 Construction of a Biased Genomic DNA Library

The same conditions as previously described in section 2.3 (Southern Hybridization) were repeated and a small portion of the agarose containing DNA fragments between 6-12 kb was physically excised from the agarose gel. The DNA was purified using glassmilk (Bio101 Carlsbad, CA).

A portion of the purified DNAs was run on an agarose gel, blotted and hybridized with the radioactively labeled *CHS* PCR product as described in section 2.3 (Southern Hybridization). The purified 6-12 kb mixture of DNA fragments was cloned into the Lambda FIX II vector (Stratagene La Jolla, CA) according to the manufacturer's protocol. The cloning reaction was scaled down proportionally to accommodate 10μg of DNA rather than the suggested 50μg. The resulting biased library was amplified and purified according to the manufacturer's protocol.

2.5 Screening of the *P. insidiosum* Biased Genomic DNA Library (Plaque Lifts)

Primary screening of the library was performed by plating 50,000 plaques on a 150mm plate. Five microliters of a 1/100 dilution (50,000 pfu) of the amplified library were plated by mixing with *E. coli* cells as described in the Lambda FIX II protocol. Plates were incubated at 37°C for 8 hours until the plaques were each about 1mm in diameter. The plates were then chilled for 2 hours at 4°C. Plaque lifts were done by placing circular nylon membrane over the plates for 2 minutes for the first membrane and 4 minutes for the second replica. The membranes were transferred to a solution of 1.5 M NaCl and 0.5 N NaOH for 2 minutes at room temperature. They were then placed into a solution containing 1.5M NaCl and 0.5M Tris-HCl (pH 8.0) for 5 minutes and finally a solution containing 0.2M Tris-HCl (pH 7.5) and 2X SSC for 1 minute. The membranes were briefly blotted and cross-linked by exposing to 120mJoules of UV energy using a Gene Linker (BioRad, Hercules, CA). The membranes were washed in 5X SSC, 0.5% SDS and 1mM EDTA for 1 hour at 42°C with shaking.

Pre-hybridization and hybridization steps were performed as described above for Southern Hybridization (section 2.3). Washing was done in the same manner as the Southern Hybridization except for the low stringency and high stringency wash buffers, which contained 1X SSC and 0.1% SDS and 0.2X SSC and 0.1% SDS, respectively. Low stringency washes were performed at room temperature and high stringency washes were performed at 42°C. Membranes were placed on an X-ray film and incubated at -70°C. Putative positive clones were picked up from the original phage/*E. coli* plates and placed in 1 mL SM

buffer (100 mM NaCl, 8mM $MgSO_4$, 50mM Tris-HCl (pH 7.5), 0.01% gelatin) and 20μL of chloroform.

For secondary screening, a portion of eluted phage was used to produce well-separated plaques on a 150mm agar plate. This plate was screened in the same manner as for the primary screening except high stringency washing steps were performed at 52°C. Single putative positive clones were picked up from the plates and placed in 0.5 mL of SM buffer with 10 μL of chloroform. A tertiary screening was performed using the phage eluent from the secondary screening. This was done in the same manner as the secondary screening, except the high stringency washing, which was performed at 62°C.

2.6 PCR Screening of the Putative Positive Clones Isolated by Plaque Lifts

Five microliter portions of SM buffer containing putative positive clones were used as template for PCR as described in section 2.2 above. A single clone that was positive by both PCR and plaque lifting was selected for DNA isolation and further analysis.

2.7 DNA Isolation from a Putative Positive Lambda Phage Clone

A putative positive clone was plated to 50,000 plaques on a 150mm plate and incubated for 8 hours. The plate was overlaid with 10mL SM buffer and incubated at 4 °C overnight with shaking. The SM buffer containing the lambda phage was collected from the plate and 500 μL of chloroform was added. The mixture was incubated for 15 minutes at room temperature and then centrifuged

at 500 X g for 10 minutes. The supernatant, containing the phage, was transferred to a fresh tube and 30 μL of chloroform was added. DNase (3 μg, Boehringer Manheim, Indianapolis, IN) and Rnase (3.6 μg, Promega, Madison, WI) were added to the tubes which contained about 10 mL of SM with the phage. The tubes were incubated at 37 °C for 15 minutes. An equal volume of a solution containing 20% polyethylene glycol (PEG 8000) and 2M NaCl in lambda diluent (10mM Tris-HCL (pH 7.5) and 10mM $MgSO_4$) was added and this mixture was vortexed gently and incubated in ice water for 1hour to precipitate the lambda phage. The phage were recovered by centrifugation at 4°C and 10,000 X g for 10 minutes. The supernatant was removed and discarded. The pellet containing the phage was resuspended in 0.5 mL of TE buffer (pH 8.0) by vortexing and 5μL of 10% SDS was added. The mixture was incubated for 5 minutes at 68°C. Ten microliters of 5M NaCl was added and the lambda DNA was extracted once by mixing with an equal volume (500μL) of phenol:chloroform and once by mixing with an equal volume of chloroform alone. The aqueous phase was transferred to a fresh tube between extractions. Following the last organic extraction, an equal volume of isopropanol was added and the tube was mixed and stored at -80°C for 15 minutes. The precipitated DNA was recovered by centrifugation at 12,000 X g at 4°C for 15 minutes. The pellet was washed with 70% ethanol and resuspended in water. The results of the DNA isolation were assessed by running a small portion of the DNA sample on a 0.8% agarose gel.

2.8 Isolation of the Insert DNA from the Lambda Phage DNA

Purified DNA from a putative positive clone was treated with *Not* I (Promega, Madison, WI). Four identical *Not* I digests were set up as follows: 1μg DNA from the positive clone, 6mM Tris-HCl (pH 7.9), 0.15M NaCl, 6mM $MgCl_2$ 1mM DTT, 0.1mg/mL BSA and 5 units of *Not* I in a total volume of 20 μL. The digestions were incubated at 37°C for 1, 2, 3 and 4 hours. Each digestion was heat inactivated by incubating at 65°C for 15 minutes.

Following inactivation, the reactions were loaded into a 0.7% agarose gel and run at 75 volts for 4 hours and 30 minutes. The gel was blotted and the 600 bp *CHS* PCR product was used as a probe in a Southern hybridization experiment as described in section 2.3. The restriction enzyme digestion and electrophoresis were repeated and bands were excised from the gel and purified using a capture column from the S.N.A.P. mini-prep kit for purifying DNA from agarose gels (Invitrogen®, Carlsbad, CA).

2.9 Sub-cloning the Insert DNA into the pZErO™ Plasmid Vector

The purified insert DNA from the lambda phage screening was sub-cloned into the pZErO™-2 vector (Invitrogen® Carlsbad, CA), which uses Kanamycin selection. The insert DNA from the lambda phage clone was recombined with pZErO™-2 vector according to the manufacturer's protocol.

2.10 Isolation of Plasmid DNA from Bacterial Clones Harboring pZErO™-2

Bacterial clones were grown for 24 hours in LB broth supplemented with

Kanamycin. Plasmid DNA was isolated using the S.N.A.P. mini-prep kit (Invitrogen® Carlsbad, CA) and quantified by absorbance at 260 nm before sequencing.

2.11 Nucleotide Sequencing

Sequencing was performed using the Sanger dideoxy chain termination method, big dye terminator chemistry (Applied Biosystems, Foster City, CA) and the Applied Biosystems ABI Prism 310 automated DNA sequence analyzer. Dye terminator sequencing reactions were set up as follows: 500ng of plasmid DNA, 4μL TRRM (Template Ready Reaction Mixture, Applied Biosystems, Foster City, CA), 3.2 pmol of primer (Invitrogen, Carlsbad, CA) and water to a final volume of 10μL. The Reactions were cycled 25 times as follows: 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. The reactions were incubated at 4°C until the cleanup step.

Before loading on to the ABI Prism 310 Genetic Analyzer, a clean-up procedure was used to eliminate unincorporated Dye Terminators from the reaction mixture. This was performed by mixing the entire 10μL sequencing reaction with 32μL of 95% ethanol and 6μL of sterile water. This mixture was vortexed briefly and incubated at room temperature for 15 minutes. The mixture was then centrifuged at maximum speed for 30 minutes at room temperature in a microcentrifuge. The supernatant was removed and discarded and the DNA pellet was dried for 5 minutes in a vacuum centrifuge. The pellet was resuspended in 18μL of TSR (Template Suppression Reagent, Applied

Biosystems, Foster City, CA). The tube was vortexed, briefly centrifuged, incubated at 95°C for 2 minutes and immediately chilled on ice for 10 minutes. The tube was vortexed and briefly centrifuged before being loaded on to the ABI Prism 310 Genetic Analyzer. Primer walking was employed for sequencing the insert by successive rounds of gathering sequence data and generating new primers.

2.12 Engineering a Specific *PiCHS2* Probe

The following primer set (hereafter referred to as *CHS* nested) was used in a PCR reaction as described in section 2.2 to generate a 500 bp amplicon of *PiCHS2*: 5'-AGG AAC ACT CAC AGG CAT CGC AAG-3' and 5'-TGG GTT GAA GTA GTT GGG CTG CTC-3'. The PCR products were cloned and sequenced as described in sections 2.2 and 2.11, respectively.

2.13 Southern Blot Using the Purified 500 bp *PiCHS2* Probe

A Southern hybridization experiment was performed as described in section 2.3 using the following three probes: 1) the 600 bp PCR product of *PiCHS2* obtained using the degenerate primers (Bowen 1992), 2) the purified 600 bp unknown PCR product and 3) the purified *CHS2* 500 bp PCR product obtained using the *CHS2* nested primers. The following control samples were loaded into a single agarose gel in triplicate: all three unlabeled probes, *Not* I digestion (prepared as described in section 2.8) of a pZErO™-2 plasmid vector containing a 7.5 kb fragment of the insert DNA from the putative positive lambda

phage clone, *Bam* H I digestion of *P. insidiosum* genomic DNA (prepared as described in section 2.3) and a DNA marker (Promega, Madison, WI). Following electrophoresis and capillary transfer, the nylon membrane was divided into 3 equal parts each containing a copy of all the above samples. Each membrane was reacted with one of the three probes. The methods for this Southern hybridization experiment were the same as those described in section 2.3.

2.14 Screening Putative Positive Clones from the Biased Library by Dot Blot Hybridization

Aliquots of nine positive phage clones from the primary screening, dissolved in SM buffer, were spotted on a nylon membrane in triplicate. The membrane was allowed to dry, divided into three and treated as described in section 2.5. The membranes were briefly blotted and the DNA was cross-linked using 120mJoules of UV energy (Biorad, Carlsbad, CA). Each of the membranes was screened either with the 600 bp mixture probe, the 500 bp *CHS2* probe or the 600 bp unknown probe in a Southern hybridization experiment. The pre-hybridization, hybridization and washing steps were performed as described in section 2.3.

2.15 Screening a Putative Positive Clone Identified by Dot Blot Hybridization

PCR reactions were prepared using phage clones in SM buffer as template. A positive control reaction was prepared using genomic DNA from *P. insidiosum* as template. The reactions were set up using the same formulation

as described in section 2.2 but with 5 µl of each phage eluent as template. The reactions were cycled as described in section 2.2. The results were assessed by running 10 µl of each reaction on a 2% agarose gel for 45 minutes at 135 volts.

3. Results

3.1 Amplification of a 600 bp Fragment of *PiCHS2*

PCR amplification of *P. insidiosum* genomic DNA using the primers designed by Bowen *et al.* (1992) yields *CHS* amplicons of approximately 600 bp. Figure 2 shows a single band at approximately 600 bp in all reactions containing genomic DNA from *P. insidiosum* and no reaction in a negative control containing no DNA template.

3.2 Southern Hybridization Using Genomic DNA from *P. insidiosum*

Figure 3 shows hybridization to undigested genomic DNA and unlabeled 600 bp probe. A strong band at approximately 8 to 9 kb is present in each of the two lanes containing the *Bam* H I digestion. A faint band at approximately 11 kb was also observed in the *Bam* H I digestion in some blots exposed to film overnight. A slight cross-reaction is observed with the DNA used as a marker.

3.3 Constructing a Biased Genomic DNA Library

The biased library of DNA fragments was screened for *PiCHS2* by Southern blot using the 600 bp *PiCHS2* PCR amplicon as a probe prior to cloning. A single band is observed in the lane containing the library of fragments that matches the 8 kb band in the *Bam* H I digestion (Figure 4). The probe hybridized to the undigested DNA control and the unlabeled 600 bp probe control.

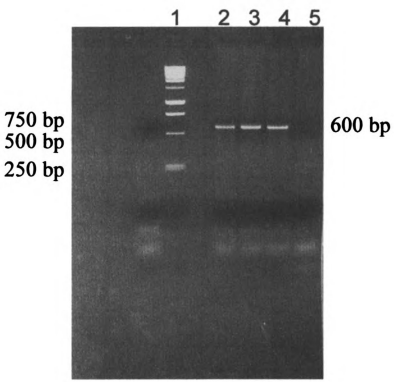


Figure 2

Chitin Synthase PCR. Amplicons obtained using genomic DNA from *P. insidiosum* as template and Bowen *et al.* (1992) *CHS* primers. The figure shows a single 600 bp product. Lane 1- DNA marker, lanes 2, 3 and 4- identical PCR reactions with *P. insidiosum* genomic DNA as template and lane 5- Negative control reaction (no DNA-reagent blank).

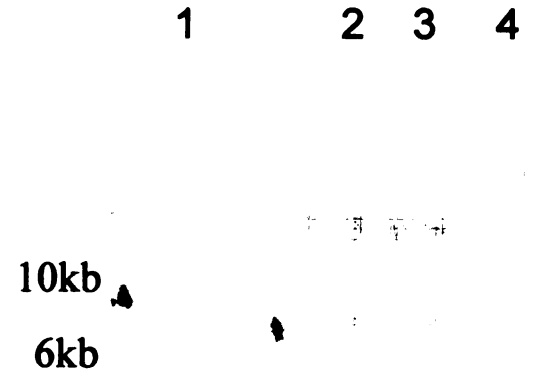


Figure 3

Southern blot analysis. The figure shows a single distinct band at approximately 8 kb. Lane 1- 1 kb DNA marker (Promega Madison, WI), lanes 2 and 3- Identical *Bam* H I digestions of *P. insidiosum* genomic DNA and lane 4- undigested genomic DNA from *P. insidiosum*.

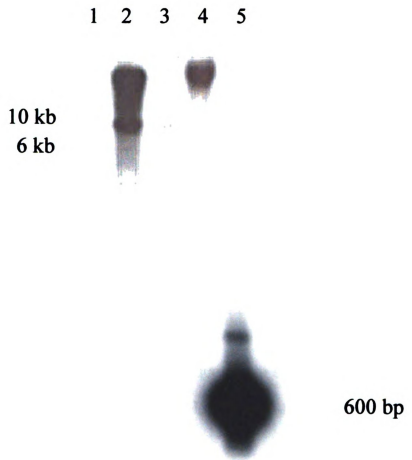


Figure 4

Screening the Mixture of Genomic DNA Fragments Prior to Cloning.

Screening the biased library of fragments by Southern hybridization using the 600 bp fragment of *PiCHS2* as a probe prior to cloning into Lambda FIX II. Lane 1- 1 kb DNA marker (Promega, Madison, WI) lane 2- *Bam* H I digestion of *P. insidiosum* genomic DNA, lane 3- biased library of fragments used for cloning into Lambda FIX II, lane 4- undigested genomic DNA from *P. insidiosum* and lane 5- unlabeled 600 bp *PiCHS2* probe.

3.4 Screening the Library

Primary screening was done by making five plates with 50,000 plaques each. Two replicas of each plate were made using nylon membranes. The membranes were used in hybridization reactions with the 600 bp PCR amplicon as a probe. The resulting X-ray films were aligned with the plates and plaques that were positive on both membranes were selected. Figure 5 shows an X-ray film from one of the primary screening plates. Plaques were excised from the agar plates and the phage were eluted into buffer. The phage eluent was used to make two additional plates for secondary screening. Each of these plates was screened with two membranes and the 600 bp *PiCHS2* probe. Figure 6 shows the results of the secondary screening. Several plaques were positive on both membranes and two well-separated plaques were selected for further screening. These plaques were excised from the plates and the phage were eluted into SM buffer.

A tertiary screening was done to select phage clone. Two separate clones were selected from the secondary screening and each was grown on an agar plate with approximately 50 plaques on each plate, which gave adequate separation of the plaques. The results of the tertiary screening can be seen in Figure 7. All of the plaques on one of the plates were positive except one. Several plaques were selected and placed individually into buffer.
PCR was performed using the intact phage clones from the tertiary screening as template to ensure that the clones contained *PiCHS2* as an insert. Several clones that were positive by plaque screening were selected. Additionally,

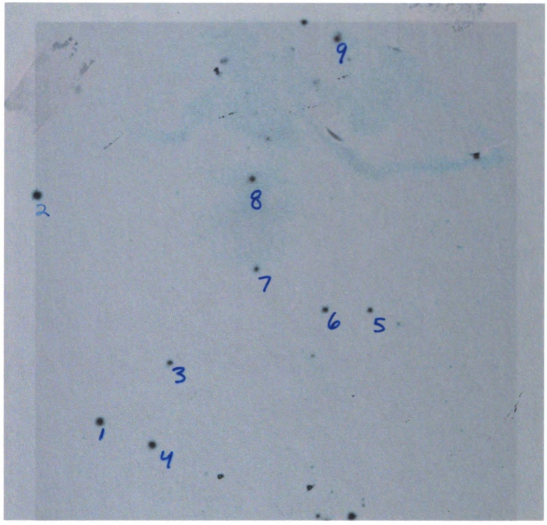


Figure 5

Primary screening of the biased library. Numbered spots represent putative positive plaques that reacted with the 600 bp *PiCHS2* probe on both membranes.

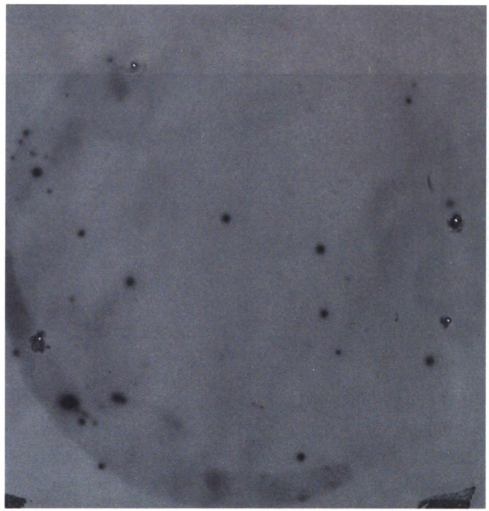


Figure 6

Secondary screening of the biased library. Plaques on these plates were larger and better separated than the plaques observed during the primary screening. Seven of the plaques hybridized to the probe on both membranes in the same relative position.

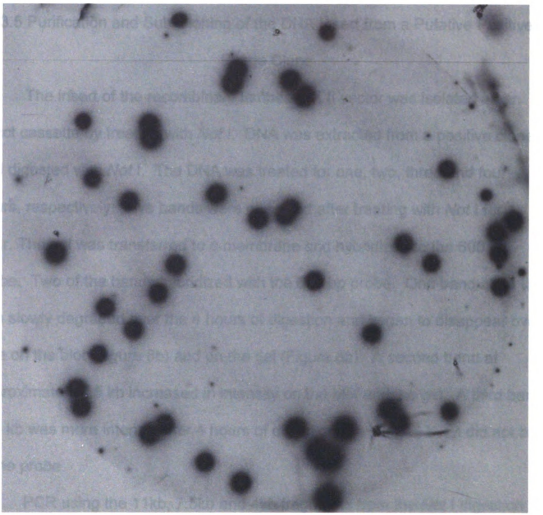


Figure 7

Tertiary screening of the biased library. The plaques were well separated on the agar plates and 44 of 45 plaques hybridized to the 600 bp probe and were deemed putative positive clones.

several clones that were negative by plaque screening were also selected as negative controls. Agreement between the PCR screening and plaque screening methods was noted for all clones tested (data not shown).

3.5 Purification and Sub-Cloning of the DNA Insert from a Putative Positive Phage Clone

The insert of the recombinant lambda FIX II vector was isolated as an intact cassette by treating with *Not* I. DNA was extracted from a positive clone and digested with *Not* I. The DNA was treated for one, two, three and four hours, respectively. Five bands were observed after treating with *Not* I for 1 hour. The gel was transferred to a membrane and hybridized to the 600 bp probe. Two of the bands hybridized with the 600 bp probe. One band at 11 kb was slowly degraded over the 4 hours of digestion and began to disappear over time on the blot (Figure 8b) and on the gel (Figure 8a). A second band at approximately 7.5 kb increased in intensity on the blot and the gel. A third band at 4 kb was more intense after 4 hours of digestion (on the gel) but it did not bind to the probe.

PCR using the 11kb, 7.5kb and 4kb fragments from the *Not* I digestion, cloned separately into plasmids, as templates with the *CHS* primers revealed 600 bp amplicons in reactions containing the 11kb and 7.5kb *Not* I fragments (Figure 9). No amplification of a 600 bp product was observed in the reaction containing the 4kb fragment as template. A positive control reaction prepared using genomic DNA from *P. insidiosum* as template revealed an expected

Figure 8

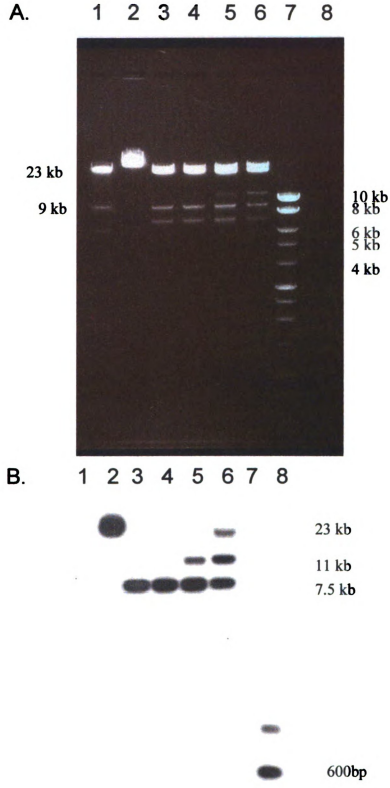
Not I Digestion and Blot of the DNA insert from a Putative Positive Clone.

Not I digestion of DNA from the lambda phage clone after 1, 2, 3 and 4 hours and the corresponding blot using the 600 bp PCR amplicon as a probe.

A) The gel before transfer. Lane 8- unlabeled 600 bp probe (Note that the amount of DNA loaded is lower than the limit of detection using ethidium bromide), lane 7- 1kb DNA marker (Promega, Madison, WI), lane 6- 1 hour, lane 5- 2 hours, lane 4- 3 hours, lane 3- 4 hours and lane 2- undigested DNA from the lambda phage clone, lane 1- 23 kb DNA marker (BioRad, Carlsbad, CA). The relevant MW of selected bands in the lanes containing DNA markers is indicated.

B) The corresponding blot using the 600 bp PCR amplicon as a probe. Lanes are labeled the same as in figure 9B. The unlabeled 600 bp *PiCHS2* probe is visible at the bottom.

Figure 8


A.
1 2 3 4 5 6 7 8
23 kb
9 kb
10 kb
8 kb
6 kb
5 kb
4 kb
B.
1 2 3 4 5 6 7 8
23 kb
11 kb
7.5 kb
600bp

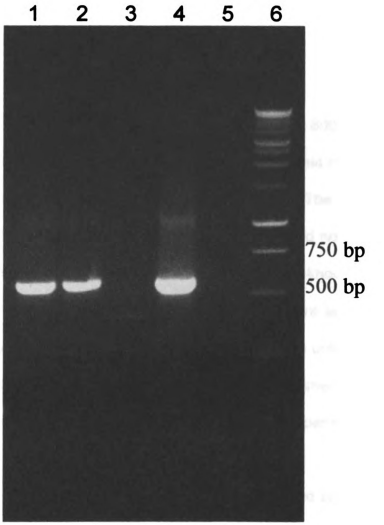


Figure 9

PCR Screening of Plasmid Clones carrying either the 4 kb, 7.5 kb or 11 kb insert from the *Not* I digestion (see figure 8) of the lambda phage clone. Bowen *et al.* (1992) *CHS* primers were used. Lane 1- 11 kb fragment, lane 2- 7.5 kb fragment, lane3- 4 kb fragment, lane 4- positive control (*P. insidiosum* genomic DNA used as template), lane 5- negative control (no DNA template) and lane 6- 1kb DNA marker.

product of approximately 600 bp. A negative control reaction containing no DNA template was also prepared and showed no amplification.

3.6 Sequencing the Putative *PiCHS2* Molecule and the 600 bp Probe

PCR was used to amplify a 600 bp product from plasmid clones of the 7.5 kb fragment of the original lambda phage clone insert DNA. The 600 bp product was cloned and sequenced. The sequence of this product did not match the *PiCHS2* sequence obtained by Perpich *et al.* (1999). This unknown sequence (Figure 10) was one bp shorter than *PiCHS2* and did not share significant homology with any sequence in the GenBank database. The unknown sequence matched the sequence of the Bowen *CHS* PCR primers, however, it had different residues at the degenerate positions when compared to *PiCHS2* (Figure 11).

The 600 bp probe was cloned into a plasmid vector and sequenced. The probe was found to be a mixture of the 600 bp *PiCHS2* sequence obtained by Perpich *et al.* (1999) and a 600 bp fragment of the unknown sequence obtained from the biased lambda phage library.

3.7 Southern Blot Using Each Probe Separately

A Southern blot experiment was performed using each product, obtained by PCR with genomic DNA from *P. insidiosum*, as a probe. Three probes were prepared. First, the same probe used to screen the library was selected. This probe is a

Figure 10

Sequence of Both Products Obtained by PCR with the CHS Primers.

Sequence comparison of the two products obtained by PCR using the degenerate *CHS* primers designed by Bowen *et al* (1992). Primer binding sites are from residues 1 to 27 and from 553 to 579 (on *PiCHS2*). *PiCHS2* is the 600 bp conserved region of the chitin synthase class 2 gene from *P. insidiosum*. The unknown fragment is the lower sequence (Unknown), which was co-amplified with *PiCHS2* using the primers designed by Bowen *et al* (1992). Sequence dissimilarities are shown with an asterisk.

Figure 10

```
                    10         20         30         40         50         60         70         80
8 PiCHS2     CTGAAGCTTA CGATGTACAA CGAAGATGGC TCCGAGCTGA AAGGAACACT CACAGGCATC GCAAGAAACT TGGCGTACAT
10           ---------- -*-----*-- *--*---*-* -**-****** *--**-*-** -***-***** -**-*-*-** **-****-**
9 Unknown    CTGAAGCTTA CTATGTATAA TGAGGATTGG TGTGCAGCTG CAGTCAGAGC CTTGGTTCCA GTTAAACAAA GAGGAGGCGC

                    90        100        110        120        130        140        150        160
8 PiCHS2     GGCGGAAGTG TGGGGCgAgC gTGCGTGGGA GAATGTTGCG GTGGCGATTG TATCCGATGG TCGCACCAAA GCGAGTGCTT
10           ******--** *****-*-** -**-**-*-* ********-* *********- ***--****- *******-** *-*-*-**-*
9 Unknown    CAGCACAGAA AACAACAAAG GGACAGGTGG ATCATGGTCA TATCGTCGAG ATACCACAAG AAAAGGTaCT TCCACTTTTG

                   170        180        190        200        210        220        230        240
8 PiCHS2     CCTGCCTCGA CTATCTTACC AGTCTTGGAG CCTTTGACGA AGAGATTATG ACAGTGACCA GCGCGGGTGT CGACGTGCAG
10           ****-***-* ---******* --***-**** *******-** *-******** -**-****-* **-**-**** *******-**
9 Unknown    ATACCAATGG CTACAGCGGA AGCAATCATT AGAAGCTCAG CGGTTGATGA AATGATCGCG TTGGTGACAC GCTTTCCCGT

                   250        260        270        280        290        300        310        320
8 PiCHS2     ATGCACCTTT TCGAATCCAC GGTGCAGTTC ATGCGAGACG ATAACTTTGA GGCGTACTAT CCGCCAATCC AGATCATTTA
10           ****-***-* *******--- *****-*-** **-******- ******-*** ********** *--*---**- -*********
9 Unknown    TACGAATCTC GATGCGGCAC TTGCAATTCA TAGTCGTCTG GACGAGTGCG TTTTCGGCTA TCGGCAAGTC ATGAAGGCAC

                   330        340        350        360        370        380        390        400
8 PiCHS2     TGCGTTGAAA GAGCACAACG GTGGCAAGCT GAACTCCCAT CTATGGTTTT TCAACGCGTT TTCCGAGCAG CTTCTACCGA
10           -****-**** -****-*-*- *-*---***- -********* **-*-****- *****-*--* *******-** ****-***-*
9 Unknown    TCTCGTTCTT GTCTGCGATG TTAGCATCAT GCTTCGGGCC GCAAGCAGCT CTCCAGAGTC GAGAAGACCT GAATTCGTGG

                   410        420        430        440        450        460        470        480
8 PiCHS2     CGTATACGGT ACTCGTCGAC GTAGGAaCGA TTCCAgGACC CGATTCAATT TTTCGACTTG TCCGAAGCAT GGATCGTAAC
10           *-****-*** *-****-**- --*-****** **-****-*- **---***-* *-******** ****-*-*-* **--****--
9 Unknown    GGCCATCTTC TCGTTACTTC GTTGAGTGAC CACTGCAATC GCATTTGTTC GTCGACGCCC GTGAAGGAAG ATATATGCAC

                   490        500        510        520        530        540        550        560
8 PiCHS2     CCTCAGATTG GAGGCGTGGC TGGCGAGATT GCAGTTGAGC AGCCCAACTA CTTCAACCCA GTCATCGCTG CCCAAAACTT
10           **--****-- *****-**** -*-*****-* -***-***** ****---**- **-******* -**-****-- --*---**--
9 Unknown    TATCGTTATG CGCATGATCG TAGGAGCGTC GTCCTCTGTG CCAGCAATGA TATTCTTTGC GGAAATCATG CCAAAACTTT

                   570        580        590        600        610        620        630        640
8 PiCHS2     CGAATACAAA CTCGAGAAC
10           *******-** ******-*
9 Unknown    GAGTACAAGC TCGAGAAC
```

```
                        A
                        C
                        G          C   C   A   C
1)    5' CTG AAG CTT ACT ATG TAT AAT GAG GAT  3'

2)    5' CTG AAG CTT ACG ATG TAC AAC GAA GAT  3'

3)    5' CTG AAG CTT ACT ATG TAT AAT GAG GAT  3'
```

Figure 11

Sequences of one of the Bowen primers and primer binding regions of *PiCHS2* and the unknown sequence. 1) Bowen *et al.* degenerate *CHS* primer, 2) Primer binding region of 600 bp unknown sequence and 3) Primer binding region of 600 bp *PiCHS2*. Positions with multiple residues are degenerate areas. Differences between *PiCHS2* and the unknown sequence at the degenerate positions are shown in bold.

mixture of the unknown sequence and *PiCHS2*. Second, the 600 bp unknown sequence alone. This probe was obtained by PCR using the cloned 7.5 kb fragment of genomic DNA on a plasmid as template. Third, a 500 bp portion of *PiCHS2* was used. This probe was obtained by PCR using a set of nested primers designed to amplify only *PiCHS2* but not the unknown sequence from genomic DNA of *P. insidiosum*. Purity of all of the probes was confirmed by sequencing.

The mixture probe reacted with all three unlabeled probes and the 7.5 kb insert DNA from the lambda phage clone. In addition, this probe hybridized to the same 8 kb band observed in the *Bam* H I digestion of previous Southern blot experiments. It also hybridized weakly to an 11 kb fragment in the *Bam* H I digestion. The probe composed only of the unknown sequence recognizes itself, the unlabeled mixture probe and the 7.5 kb insert DNA from the lambda phage clone. It bound to a band larger than 8 kb in the *Bam* H I digestion. Finally, the 500 bp *PiCHS2* probe bound to the mixture probe and to itself. It did not react with the 7.5 kb insert from the biased library. This probe hybridized to a band at the 8 kb position but did not display a band at the 11 kb position in the *Bam* H I digestion.

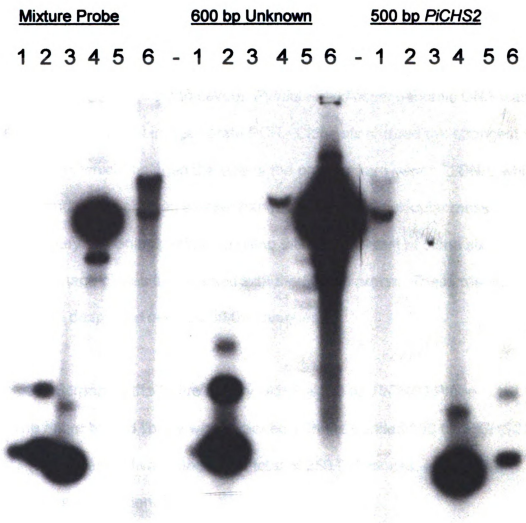
3.8 Dot Blot of Nine Clones from the Primary Screening of the Library

Nine of the strongest positive clones from the primary screening of the biased library were selected for this experiment. Each clone was spotted once

Figure 12

Southern blot using 3 different probes and 3 identical blots. All samples were loaded in triplicate and reacted with a different probe. The samples for the mixture probe: lane 1- unlabeled mixture probe, lane 2- unlabeled unknown probe, lane 3- unlabeled 500 bp *PiCHS2* probe, lane 4- 7.5 kb insert from lambda phage clone (digested from a plasmid), lane 5- 1 kb marker (Promega, Madison, WI) and lane 6- *Bam* H I digest of *P. insidiosum* genomic DNA. The samples for the unknown probe: lane 1- unlabeled mixture probe, lane 2- unlabeled unknown probe, lane 3- unlabeled 500 bp *PiCHS2*, lane 4- *Bam* H I digestion, lane 5- 1 kb marker and lane 6- 7.5 kb insert. The samples for the 500 bp *PiCHS2* probe: lane 1- *Bam* H I digest, lane 2- 1 kb marker, lane 3- 7.5 kb insert DNA, lane 4- unlabeled 500 bp *PiCHS2* probe, lane 5- unlabeled unknown probe and lane 6- unlabeled mixture probe.

Figure 12


Mixture Probe
600 bp Unknown
500 bp PiCHS2
1 2 3 4 5 6 - 1 2 3 4 5 6 - 1 2 3 4 5 6

each on three small pieces of nylon membrane. The membranes were reacted with all three probes used in the experiment in section 3.8 (Figure 13). The probe composed of the unknown sequence reacted with all nine clones. The mixture probe also reacted with all nine clones. The 500 bp *PiCHS2* probe reacted with clone six only.

PCR screening of clone six and several other clones that were negative in the dot blot using the *CHS* nested primers revealed an expected 500 bp amplicon for clones five, six and seven. *Pythium insidiosum* genomic DNA was used as a positive control in a separate PCR. Clone six showed the strongest band at 500 bp, which matched the size of the product from genomic DNA, while clones five and seven showed weaker bands at the same molecular mass.

A primary screening performed using the phage eluent of clone six revealed no phage clones that reacted with the 500 bp probe. These results were obtained despite screening 250,000 plaques.

3.9 Screening the Entire Library with the 500 bp *PiCHS2* Probe

The entire biased library was screened with the purified 500 bp *PiCHS2* probe. After screening five plates and a total of 250,000 clones, no putative positive plaques were identified.

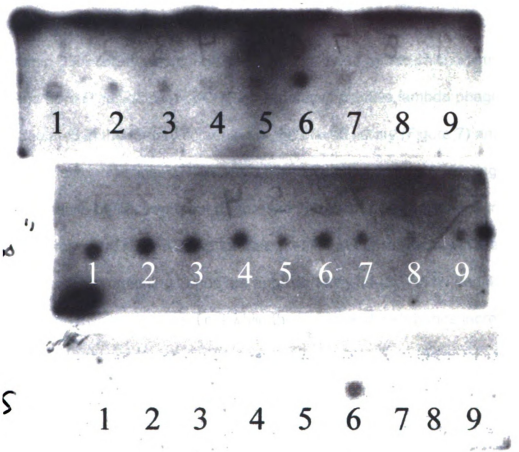


Figure 13

Dot blot of nine putative positive clones from the primary screening of the biased library. Samples were spotted in triplicate on three different membranes. Each membrane was reacted with one of the three probes used for the Southern blot experiment (Figure 12): 600 bp mixture probe (top membrane), 600 bp unknown probe (middle membrane) and 500 bp *PiCHS2* (bottom membrane).

4. Discussion

The primary goal of this study was to establish the presence of *CHS* genes in *P. insidiosum* and to isolate and sequence one of the genes. A biased genomic DNA library was constructed and screened for the chitin synthase class 2 gene from *P. insidiosum* (*PiCHS2*). A putative positive lambda phage clone was isolated in the tertiary screening of the biased library (Figure 7) and DNA was extracted. The insert DNA was excised from the vector by treating with *Not* I. The results of this treatment (Figure 8 a and b) show five distinct bands. The vector arms are 9 kb and 20 kb and are present at approximately those positions. The remaining bands are approximately 11 kb, 7.5 kb and 4 kb. The 11 kb band disappears over time while the 7.5 kb and 4 kb bands increase in intensity over time. This suggests that the insert DNA from the putative positive lambda phage clone is 11 kb in size and contains a *Not* I recognition site which produces bands that are 7.5 kb and 4 kb in size (Figure 8b). The 11 kb and 7.5 kb bands was recognized by the probe while the 4 kb band was not. Therefore, the probe recognition site is present within the 7.5 kb portion of the 11 kb insert DNA. These conclusions are supported by PCR (Figure 9) where the expected 600 bp amplicon was observed using the 7.5 kb and 11 kb fragments as template, while the 4 kb fragment did not yield the 600 bp band.

The nucleotide sequencing of the 600 bp PCR product showed that the 11 kb fragment did not carry the *PiCHS2* gene. Instead, the 600 bp product was found to contain primer recognition sites for the Bowen *et al. CHS* primers but was not homologous to any known *CHS* (Figures 10 and 11).

A Southern blot experiment was prepared using a purified 500 bp fragment of *PiCHS2*, the unknown probe and the mixture of the two (Figure 12). The 500 bp *PiCHS2* probe reacted with a band at approximately 8 kb that appears to be at the same MW as observed in the original Southern blot. The 600 bp unknown probe, however, reacted with a band at a higher molecular mass, nearly 11 kb. This finding suggests that the strong 8 kb band observed in the original Southern blot (figure 3) is a fragment that contains the *PiCHS2* gene and that the 11 kb band is a fragment that contains the unknown sequence. Therefore, since the 11 kb fragment was isolated from the lambda phage library, it was possible that the 8 kb fragment could be contained in another putative positive clone from the library. Since the primary screening of the library (Figure 4) revealed several putative positive clones, one of them could contain the *PiCHS2* gene.

The results of the dot blot experiment (figure 12) show that clone six could contain *PiCHS2*. PCR screening, using the primers known to amplify a pure 500 bp product of *PiCHS2*, was done using this clone and several clones negative in the dot blot (data not shown). Clone six reacted strongly, two other putative negative clones were weakly positive and the clone containing the 11 kb fragment was negative. These results, while seemingly inconclusive, could be explained by diffusion across the plate of clone six to clones in other areas. The phage eluent containing clone six was plated and plaque lift screening was done. This method failed to isolate a putative positive phage clone.

The purified 500 bp *PiCHS2* probe was used to screen the entire library.

No putative positive clones were obtained despite screening 250,000 plaques. Thus, this library seems to contain several copies of clones with the 11 kb insert but none containing the 8 kb *PiCHS2* gene.

An unexpected result of this research was the co-amplification by PCR, using the Bowen *et al. CHS* primers, of a segment of DNA approximately 600 bp in length that shows no homology to any known chitin synthase genes. The primers used for this PCR experiment are well characterized and have been widely used (Bowen 1992, Motoyama 1994, Mort-Bontemps 1997, Nino-Vega 1998). Bowen *et al.* (1992) designed the primers to amplify conserved 600 bp regions of chitin synthase genes from fungi. Since their design in 1992, several investigators have used them to amplify fungal and Oomycete *CHS*. None of these investigators reported anomalous amplification of non-*CHS* DNA segments. Perpich *et al.* (1999) used these primers to amplify a 600 bp fragment of *CHS2* from *P. insidiosum* and did not report any non-*CHS* amplicons. The PCR conditions in this study were not different from those reported in that study. Yet, these data show two PCR products using the degenerate *CHS* primers. Due to the fact that the PCR artifact was only 1 base pair smaller than the intended product it was not detected after agarose gel electrophoresis and, consequently, was co-purified with the 600 bp fragment of *PiCHS2*. The artifact was detected later as a minor contaminant of the *CHS* probe preparation.

Artifacts during PCR amplification do occur. Some investigators have reported the formation of hybrid molecules during PCR amplification. This was

usually reported when heterogeneous mixtures of rDNA templates were used for PCR. Hybrid products composed of a stretch of one sequence fused to a different but homologous sequence were found (Amann 1995). This example, however, does not explain why Perpich *et al.* (1999) did not obtain contaminated amplicons under conditions similar to those used in this study. Despite sequencing over 30 clones of the 600 bp PCR product, those investigators obtained only the 600 bp fragment of the *PiCHS2* gene. This discrepancy could be explained by a manufacturer's error in the synthesis of the primers used for this research. Both experiments were done in the same laboratory with similar reagents and conditions. The primary difference was the lot of primers used for amplification. This study used new primers synthesized by a different company. Therefore, aberrant products from the synthesis of the primers may account for the differential results. However, examination of Figure 11, which shows a comparison of the sequences from the Bowen *et al.* primers and the two PCR products obtained in this study, suggests that the primers were synthesized correctly and the anomalous amplification of the artifact may be due to the degeneracies in the primers. This raises the possibility that the original primers used by Perpich *et al.* (1999) were somehow biased for amplification of *PiCHS2*. This idea is supported by the fact that the authors obtained only one *CHS* isozyme, which is in contrast to all other reports were mulitiple isozymes of *CHS* were identified using the same primer set. For example, Mort-Bontemps *et al.* (1997) reported two PCR products, homologous to *CHS1* and *CHS2* from *Saccharomyces cerevisiae,* using the Bowen *et al.* primers to amplify genomic

DNA from the Oomycete *Saprolegnia monoica*. In addition, most fungal chitin synthase systems studied to date contain two or more isozymes. Finally, it is possible that the artifact obtained in this study is actually an ancient *CHS* isozyme that has become a pseudogene. However, no other reports of such pseudogenes have been discovered so far using the Bowen *et al.* primers.

The problems isolating *PiCHS2* could be due to the vector used for cloning in phage lambda. Lambda FIX II preferentially packages lambda DNAs with inserts ranging from 9 to 23 kb. The size of the *Bam* H I fragment of *PiCHS2* in this study is approximately 8 to 9 kb, which is at the low end of this range. Although an 8 kb insert would ligate into FIX II more efficiently than an 11 kb insert, such a construct would not package with nearly the efficiency of a lambda DNA carrying an 11 kb insert since packaging efficiency is largely dependent upon the size of the DNA in the head of the phage. This is consistent with the fact that all of the putative positive clones obtained in the primary screening hybridized to the purified unknown probe. Presumably all of these are equivalent clones containing the 11 kb *Bam* H I fragment. However, this was an unexpected finding since other investigators have cloned fragments as low as 6 kb using the same vector and cloning kit (Mendoza, personal communication).

In conclusion, the problems encountered during this research seem to be technical rather than biological in nature. Chitin synthase genes have been identified in several fungi and Oomycetes (Mort-Bontemps 1997, Bowen 1992, Motoyama 1994, Au-Young 1990). These genes were identified by PCR using the same primers that were used in this research. The entire *CHS2* from *S.*

monoica was isolated and characterized (Mort-Bontemps 1997). This gene is expressed during hyphal growth and in regenerated protoplasts. Furthermore, the amplification of conserved regions of *CHS* genes containing open reading frames in a total of four Oomycetes including *P. insidiosum* suggests that these genes are not pseudogenes. Rather, they are likely important to the Oomycetes at some stage in their life cycles. In addition, the taxonomic classification proposed by Bartnicki-Garcia which placed the Oomycetes into the cellulose-glucan group and the true fungi into the chitin-glucan group is no longer appropriate in light of the fact that the Oomycetes clearly contain chitin and chitin synthase enzymes in their cell walls. Further, it appears that the presence of *CHS* in the Oomycetes is widespread.

This research confirmed the existence of a *CHS2* gene in *P. insidiosum* and established that *CHS* genes from the Oomycetes are phylogenetically distinct from fungal *CHS* genes. Further work is needed in order to characterize the entire *PiCHS2* gene. The results of this study will undoubtedly be important for future studies on the cloning and characterization of *CHS* genes in *P. insidiosum*.

5. Future Direction

As soon as *PiCHS2* has been isolated from *P. insidiosum*, expression experiments should be performed to determine the role of chitin in this organism. If *P. insidiosum* utilizes chitin during hyphal growth as *S. monoica* does, *CHS* could be an excellent target for drug design. Although development of drugs that target chitin synthase is in its infancy, chitin synthase remains an attractive target for drug design in this group of pathogens. In addition, the recent development of glucan synthase inhibitors could also be investigated as a chemotherapeutic option in the infections caused by *P. insidiosum*.

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