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**WdChs5p of *Wangiella (Exophiala) dermatitidis*, a class V chitin
synthase, is essential for sustained cell growth at temperature of
infection**

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Dedication

To my parents and my wife for their love and encouragement

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WdChs5p of *Wangiella (Exophiala) dermatitidis*, a class V chitin synthase, is essential for sustained cell growth at temperature of infection

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The chitin synthase structural gene, *WdCHS5* was isolated from *Wangiella dermatitidis*. Sequence analysis revealed that this gene has a myosin motor-like-encoding region at its 5'-end and a chitin synthase (class V)-encoding region at its 3'-end. The sequence analysis also revealed that the 5,655-bp open reading frame of *WdCHS5* encoded 1,885 amino acids, and had a 53-bp intron near one end and a 57-bp intron near the other. Northern blotting showed that *WdCHS5* was highly expressed under conditions of stress, such as temperature shift from 25°C to 37°C, or conditions that induce morphology changes. Analysis of the 5' upstream region of *WdCHS5* indicated that one or more of the potential regulatory elements detected might have contributed to the high expression. Disruption of *WdCHS5* by

different methods produced mutant strains with temperature-sensitive phenotypes, which grew normally at 25°C, but had severe growth and cellular abnormalities at 37°C. The observation that supplementing osmotic stabilizers, such as sorbitol and sucrose could rescue the phenotype indicated that the loss of WdChs5p caused the cell wall integrity defects. Animal survival tests using a mouse model of acute-infection showed that all *wdchs5Δ* strains were less virulent than the wild-type parental strain. Fungal organ burden assays demonstrated that the *wdchs5Δ11* mutant could not survive long enough in mice to cause any infection. Reintroduction of the *WdCHS5* gene into *wdchs5Δ* mutants rescued the ts-phenotype and reestablished their virulence. Four double chitin synthase gene disruption mutants involving *WdCHS5* were constructed. Three of them showed similar phenotype as that of the *wdchs5Δ* mutant. However, the double disruption of *WdCHS4* and *WdCHS5* resulted in a mutant which had the combined phenotype of *WdCHS4* and *WdCHS5* single gene disruption mutants and grew very poorly in a less rich medium. This report documents that the product of *WdCHS5* is required for the sustained growth of *W. dermatitidis* at 37°C, and this is of critical importance to its virulence.

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INTRODUCTION

Fungal infection and fungal cell wall

Fungi are eukaryotic organisms, which exist virtually everywhere in the world. Despite approximately 200,000 known species of fungi, only a relatively small number of them have the ability to cause disease in humans (Perfect, 1996). During the past two decades, the number of cases of fungal infections (mycoses) has been increasing concomitantly with the number of immunocompromised hosts and advances in medical technology. Three major factors are believed to contribute to this rise: (1) more severely ill and/or immunocompromised patients with either AIDS or having cancer chemotherapy or immunosuppressive therapy for organ transplants; (2) more aggressive medical procedures, such as extensive surgery; (3) broad-spectrum antibiotics or glucocorticosteroids treatment (Georgopapadakou, 1995). The symptoms of fungal diseases range from minor or superficial infections to invasive and often life-threatening mycoses. Among the infections, many are caused by dematiaceous (phaeoid) fungal pathogens, which represent a large group of organisms that are darkly pigmented because of the polymerization of melanin in their cell walls. The diseases produced by dematiaceous fungi include phaeohyphomycosis, chromoblastomycosis and black grain mycetoma. In general, each species causes only relatively few infections, but collectively they give rise to a large number of primary and opportunistic diseases.

Dematiaceous pathogens are attracting more attention, because more cases of infection by the same species are being identified and more species have been documented as dematiaceous pathogens (Matsumoto et al., 1991; Merz, 1991; Kwon-Chung et al., 1992; Walsh et al., 1994; Matsumoto et al., 1994). Treatment of dematiaceous mycoses is extremely difficult, often involves surgery and frequently is associated with failed antifungal therapy because of the insensitivity of the fungus involved to available therapeutic agents (Paul et al., 1991; Kwon-Chung et al., 1992; Matsumoto et al., 1994; Walsh et al., 1988). A major obstacle of developing effective antifungal agents is that fungi are complex organisms that share many potential biochemical targets with other eukaryotic cells. However, the fungal cell wall, which is usually the outermost boundary between host and fungus in tissue, and is absent from cells of the mammalian host, represents an attractive potential target for antifungal drug development. The successful use of penicillins and cephalosporins to inhibit bacterial cell wall biosynthesis lends credibility to the hypothesis that antibiotics targeting the cell wall of fungi can also be developed. The fungal cell wall is not only a protective shell but also a dynamic organelle essential to the viability of the organism. Its complex structure serves many functions including osmotic protection, transport of macromolecules, growth, conjugation, and spore formation (Perfect, 1996). The integrity of the cell wall is essential for cell survival in hostile environments and because fungal cells have a very high turgor pressure, even a minor defect in the cell wall can lead to

bursting and death (Cabib et al., 1989). From the basic research point of view, the fungal cell wall together with the septum make up about one-third of the cell dry weight. Consequently, cells devote a great deal of energy and synthetic apparatus to generate these structures (Cabib et al., 2001). Moreover, the formation of cell wall is part of cell growth and division, therefore, it must be strictly regulated to accompany other processes that are part of the cell cycle. Because the fungal cell wall imparts shape to the cell in a constantly changing pattern and because of its relatively simple composition, it serves as an excellent model for studying cell development and morphogenesis at the molecular level (Cabib et al., 2001).

Structure and composition of the fungal cell wall

In general, the fungal cell wall is composed of a complex of proteins and polycarbohydrates such as glucan, mannan, and chitin. Alkali solubilization analysis showed that the polysaccharides can be divided into two general categories: alkali soluble polymers, including mannoproteins and some α - and β -(1, 3)-D-glucan; alkali insoluble polymers, including β -(1, 3)-D-glucan, β -(1, 6)-D-glucan, chitin, chitosan, and cellulose. Basically, the insoluble polysaccharides provide mechanical strength to the cell, whereas the soluble polysaccharides act as interstitial components that link and restrict the skeletal polymers (Debono et al., 1994). Although each fungus has a somewhat different biomolecular composition, the overall cell wall structure of fungi is quite similar and the cell

wall components are attached to each other directly or indirectly. The first characterized linkage between cell wall components was the β - (1, 4) bond between the reducing terminal GlcNAc of a chitin chain and the nonreducing terminal glucose of β - (1, 3) glucan. Subsequently, the linkages between mannoproteins, β - (1, 6) glucan and β - (1, 3) glucan were connected. Finally, the linkages among all the major components were established. In this complex, β - (1, 6) glucan holds a central position, to which chitin and β - (1, 3) glucan are directly attached by glycosidic linkages, and the mannoprotein is connected to the polysaccharide through the lipidless remnant of a glycosylphosphatidylinositol anchor. The cross-linkings are considered to contribute to the mechanical strength of the cell wall. They are also important in the loosening and remodeling of the cell wall, such as during development and morphogenesis, when some linkages could be disconnected temporarily by specific enzymes (Cabib et al., 2001).

Mannoproteins, one of the major cell wall components, can be classified into two groups: structural mannoproteins that form integral components of the cell wall and enzymes located in the cell wall or in the periplasmic space. Although mannoproteins are considered to be a filling material which is enmeshed in the glucan structural network, the actual protein part of mannoproteins also plays an important role and functions as an acceptor for crosslinks with other sugar chains (Cabib et al., 1988).

β -Glucan usually is the most abundant component of the fungal cell wall and is uniformly distributed around the cell. β - (1, 3)-D-glucan is the major structural component of the cell wall and is biosynthesized from UDP glucose by a membrane-bound enzyme, β - (1, 3)-D-glucan synthase. Another β -glucan, β - (1, 6) glucan is relatively minor but very important for cross-linking. As to the synthesis of β - (1, 3)-glucan, studies from *Saccharomyces cerevisiae* and other fungi led to the discovery of two factors that are required simultaneously for glucan synthesis: one is a small (26 kD) GTP-binding protein; the other is the catalytic component of the synthase. The GTP-binding protein was later identified as Rho1p, whereas the catalytic component was identified as Fks1p (Douglas et al., 1994; Drgonova et al., 1996; Qadota et al., 1996). The discovery of the GTP-binding proteins sheds light on how the glucan synthesis can be started and shut off (Cabib et al., 1998). GTP-binding proteins can function as molecular switches because when bound to GTP, they are kept in an active state, whereas when bound to GDP, they are kept in an inactive state. GTPase-activating proteins control the conversion from an active to inactive enzyme form, whereas GTP-GDP exchange factors control the opposite shift. Little is known about the synthesis of the β - (1, 6)-glucan. However, it is speculated that the synthesis of β - (1, 6)-glucan probably starts in the endoplasmic reticulum, then continues in the Golgi, and finally is completed at the cell surface (Shahinian et al., 2000).

Chitin and chitin synthesis

Chitin, the linear polymer of β -1, 4-N-acetylglucosamine, is an essential structural component of fungal cell walls and plays an important role in fungal morphogenesis (Cid et al., 1995; Ruiz-Herrera et al., 2001). The linear chains form hydrogen bonds between residues of adjacent chains, which are in antiparallel directions resulting in the formation of microfibrils. The chitin microfibrils are crystalline and extraordinarily strong (Munro et al., 2001). Chitin appears to be widely distributed in the fungal kingdom and the same fungus at different developmental stages may have different amounts of chitin. Three methods are commonly used for detecting chitin, (i) staining with chitin-binding dyes, such as Calcofluor and Congo red; (ii) labeling with wheat germ agglutinin, which is a lectin that recognizes and binds GlcNAc; (iii) chemical assay, which quantitates chitin; e.g. the amount of GlcNAc released by chitinase is measured with a colorimetric assay (Bulawa, 1993). In *S. cerevisiae*, chitin only accounts for 1-2% of the dry weight of the cell wall, whereas, in filamentous fungi chitin constitutes as high as 40% of the cell wall (Bartnicki-Garcia et al., 1969). In *S. cerevisiae*, chitin is found mostly in the bud scar, which is a craterlike structure formed on the surface of the mother cell after bud cell separation (Cabib et al., 1982). A small amount of chitin is also localized at the lateral cell wall (Molano et al., 1980). However, newborn daughter cells do not have detectable amounts of chitin (Roncero et al., 1988; Shaw et al., 1991). Chitin is also synthesized during

the process of polar elongation and in response to sexual pheromone stimulation, as well as during sporulation (Molano et al., 1980; Lipke et al., 1976; Briza et al., 1988). In filamentous fungi, chitin is mostly concentrated in the septum and apex of the growing hyphae (Gooday, 1990).

Chitin synthases [UDP-N-acetyl-D-glucosamine: chitin 4- β -N-acetylglucosamine transferase, EC (2.4.1.16)], which are membrane bound proteins, are responsible for the synthesis and deposition of the chitin (Muzzarelli et al., 1986; Munro et al., 2001). Chitin synthase was first described in the late 1950s in *Neurospora crassa* (Glazer et al., 1957). However, the first chitin synthase gene was not described until 1986 in *S. cerevisiae* (Cabib, 1996). Since then, more and more *CHS* genes have been identified from various fungi. It is predicted that the chitin synthase protein has a number of transmembrane spanning regions at the C-terminus and a catalytic domain at the cytoplasmic side based on hydrophobicity plots of the derived protein sequence. This model is consistent with previous biochemical analyses, which demonstrated that the catalytic domain was located at the inner face of the cytoplasmic membrane, so as to provide the access to the substrate for the enzyme (Cabib et al., 1983). All chitin synthases are assumed to perform the same reaction, which results in additions of N-acetylglucosamine to the elongating oligosaccharide chain. The monomer N-acetylglucosamine acts as an activator of the enzyme, which is inhibited by the reaction product UDP

(Gooday, 1990). Because the substrate for chitin synthase, UDPGlcNAc (UDP-N-acetylglucosamine), is in the cytoplasm, but the chitin synthase is an integral membrane protein and the chitin product is extracellular, the process of chitin biosynthesis is believed to be a transmembrane event.

Chitin and chitin synthesis in *Saccharomyces cerevisiae*

The extensive study of the chitin synthases of *S. cerevisiae* (ScChsp) documented that a specific function can be assigned to each of its three chitin synthases (Cid et al., 1995; Orlean, 1997). ScChs1p (class I) was the first chitin synthase to be discovered in yeast. It is the major chitin synthase detected in *S. cerevisiae* and represents about 90% of the *in vitro* activity measurable in a wild-type strain. Because ScChs1p is 10 to 20 times more active than the other two chitin synthases, the detection of the other two was difficult and for 15 years, ScChs1p was thought to be the only chitin synthase in yeast. The contribution of ScChs1p to chitin synthesis is negligible and its activity normally does not participate in the synthesis of cellular chitin *in vivo*, (Bulawa et al., 1986). For activity, ScChs1p shows an absolute requirement for a divalent cation (Mg^{2+} or Mn^{2+}), and because it is uniformly distributed on the yeast plasma membrane in the zymogenic form, it requires activation, at least *in vitro* (Duran et al., 1975; Duran et al., 1979). Interestingly, although the ScChs1p activity is described as zymogenic, no direct evidence documents that proteolytic processing of this activity occurs *in vivo*.

The isolation of the mutant, *ScChs1*, revealed that it apparently lacked chitin synthase activity *in vitro*, but could grow at a normal growth rate with normal chitin content. The *ScCHS1* gene was then cloned by complementation of the *ScChs1* mutant. Disruption of *ScCHS1* neither is lethal nor affects chitin synthesis *in vivo* (Bulawa et al., 1986). However, the defect in this enzyme has a subtle phenotype when grown in a medium with poor buffering capacity: many daughter cells lyse during cell separation, and have small holes in the center of the birth scars. The defects can be prevented by buffering the growth medium or by adding sorbitol as an osmotic stabilizer (Cabib et al., 1987). Adding chitinase inhibitors, such as allosaminin or demethylallosamidin- α , or by disrupting the chitinase structural gene, can also prevent the defects in the mutant. All these facts led to the conclusion that ScChs1p is responsible for the synthesis of chitin after cell separation and counterbalances the chitinase activity: it is thus considered to be a repair enzyme (Bulawa et al., 1986; Cabib et al., 1992).

The finding that the absence of ScChs1p does not produce any apparent phenotype indicated that other chitin synthase(s) must be presented in *S. cerevisiae*. Two different approaches were performed independently in the search for the postulated additional chitin synthases. This effort led to the identification of ScChs2p. Study of ScChs2p (class II) revealed that it is also located in the plasma membrane and can be activated *in vitro* by treatment with proteases.

Subsequently, *ScCHS2*, the gene that encodes ScChs2p was cloned by detection of its overexpression *in vitro*, in an *ScChs1* mutant background (Silverman et al., 1988). The major difference between ScChs1p and ScChs2p is that they have different preferences for divalent cations. Co^{2+} is the best metal stimulator for ScChs2p, however, it inhibits ScChs1p. The other major difference is that the optimum pH for ScChs2p activity is pH 7.5 to 8, which is more alkaline than that for ScChs1p (pH 6.5). ScChs2p only contributes about 5% of the chitin synthase activity of ScChs1p (Sburlati et al., 1986). However, ScChs2p has significant enzyme activity without trypsin treatment *in vitro*, which suggests that ScChs2p may function in an unprocessed form *in vivo*. Nevertheless, trypsin treatment of the intact enzyme or truncated enzyme produces a common 35 kD fragment that is still fully capable of synthesizing chitin. Disruption of *ScCHS2* produces mutants with thickened amorphous septa that have no defined primary septa. Furthermore, later studies revealed that disruption of *ScCHS1* and *ScCHS3* in the same background gave a mutant that has a primary septum and the mutant cells are abnormally large and aggregate in clumps (Shaw et al., 1991). Taken together, these results demonstrate that ScChs2p is responsible for the chitin deposition in the primary septum (Silverman et al., 1988; Shaw et al., 1991).

Because the double disruption of *ScCHS1* and *ScCHS2* had no *in vivo* defect on chitin synthesis, and produced mutant cells with 2-fold more chitin under certain

conditions, it became apparent that at least one other chitin synthase exists in *S. cerevisiae* (Bulawa et al., 1990). This led to the discovery of another chitin synthase, ScChs3p (class IV). ScChs3p was originally found to be the major activity in the cell membrane without trypsin treatment (Orlean, 1987; Bulawa et al., 1990; Valdivieso et al., 1991; Bulawa et al., 1992), to contribute to the synthesis of most of the cell wall chitin during vegetative growth and to be responsible for the chitin deposition in the ring and lateral cell wall (Roncero et al., 1988; Shaw et al., 1991). Also, ScChs3p has an optimal activity at pH 8.0 in the presence of Mg^{2+} . Ni^{2+} , a powerful inhibitor of ScChs1p and ScChs2p, has very little effect on ScChs3p, especially in the presence of Co^{2+} (Choi et al., 1994). ScChs3p was first thought to be nonzymogenic, because it was found to be inactivated by trypsin (Orlean 1987; Bulawa et al., 1990; Valdivieso et al 1991). However, subsequent studies showed that in the presence of the substrate UDPGlcNAc and after incubation with a protease, a 10-fold increase of ScChs3p activity occurred when cell extracts were treated with detergents, which normally leads to a significant reduction in ScChs3p activity (Choi et al., 1994). Therefore, ScChs3p also has, at least *in vitro*, a zymogenic property.

The *ScCHS3* gene was subsequently cloned by complementing a mutant with a Calcofluor white resistance phenotype (Valdivieso et al., 1991). Although the mutants with double disruption of *ScCHS1* and *ScCHS2*, or *ScCHS1* and *ScCHS3*

are viable, double disruption of *ScCHS2* and *ScCHS3* is lethal (Shaw et al., 1991). The reason for this synthetic lethality is unknown, but the result suggests that chitin is essential for cell survival. Another important discovery during characterization of *ScCHS3* is that overexpression of *ScCHS3* does not result in overproduction of ScChs3p activity and transformation of *Schizosaccharomyces pombe* with *ScCHS3* does not increase ScChs3p activity (Valdivieso et al., 1991). Together, these results indicate that other factors are required for ScChs3p activity or its activation *in vivo*.

Analysis of Calcofluor white resistant and chitin-deficient mutants led to the identification of numerous key regulators of the ScChs3p activity. However, the same screening method was not used to find the regulators of ScChs1p and ScChs2p, because these proteins only contribute to a minor amount of cell-wall chitin and, therefore, are largely unaffected by the presence of Calcofluor. It was found that the products of the *ScCHS4* (also called *ScSKT5*, *ScCSD*, *ScCAL2*), *ScCHS5*, *ScCHS6* and *ScCHS7* genes are required for functional ScChs3p activity (Valdivieso et al., 1999), although they do not have any similarity to true chitin synthase at the sequence level (Bulawa et al., 1992; Kawamoto et al., 1992; Santos et al., 1997; Trilla et al., 1999; Ziman et al., 1998). The current model suggests that ScChs3p activity is controlled inside cells by a specialized mechanism of vesicle sorting (Ziman et al., 1996), coupled with a pathway

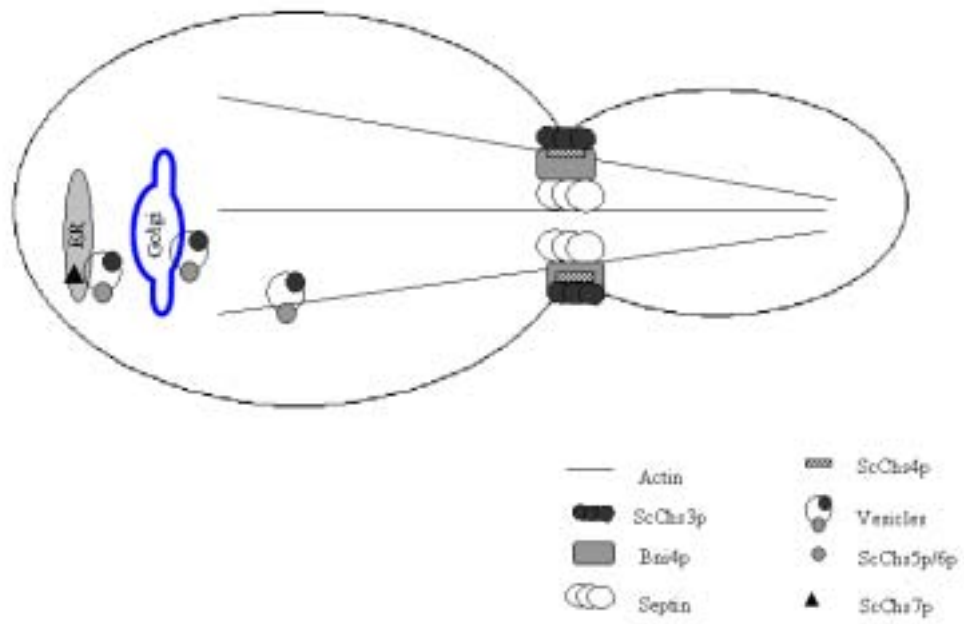
through endocytic recycling (Chuang et al., 1996; Holthuis et al., 1998). According to this model, ScChs3p is maintained inside specialized vesicles called chitosomes (TGN/early endosome vesicles) and is transported to the specific sites of function (Valdivia et al., 2002), where it becomes activated. On the other hand, inactivation occurs by endocytosis. Under this scenario (Fig. 1), ScChs4p, ScChs5p, ScChs6p and ScChs7p are believed to be post-translational regulators of ScChs3p, which are important for the correct targeting of this chitin synthase to cellular sites. Demarni and his colleagues found that ScChs3p interacts with ScChs4p, and ScChs4p interacts with ScBni4p (for bud-neck involved), which in turn binds to ScCdc10p, a septin protein that forms a ring at the site of bud emergence (Demarini et al., 1995). Disruption of *ScCHS4* or *ScBNI4* causes mislocalization of ScChs3p. GFP-tagging of ScChs4p also demonstrated that this protein localizes in the same region of the septin ring as ScChs3p does, and that its localization depends on functional ScBni4p. ScChs4p is also involved directly in the activation of ScChs3p through protein-protein interaction (Ono et al., 2000). *In vitro*, 3 to 10-fold ScChs3p activity is observed when *ScCHS4* is overexpressed. However, overexpression of *ScCHS3* does not increase activity further (Bulawa et al., 1993). It was suggested that the *ScCHS4* gene product functions as a posttranslational activator of ScChs3p and acts as a limiting subunit of a complex containing ScChs3p. This protein has a putative E-F hand loop, a calcium-binding domain and a cluster of basic amino acids followed by a C-

terminal CAAX motif (Bulawa et al., 1993; Trilla et al., 1997; DeMarini et al., 1997). ScChs4p was then shown to interact with ScCdc10p septin through an anchor protein, ScBni4p. Cells devoid of *ScBNI4* have normal amounts of chitin and ScChs3p activity. However, the chitin is mislocalized in the mutant (Sanz et al., 2002). The model indicates that a complex containing ScChs3p/ScChs4p is positioned at the septum site through its interaction with the ScBni4p/Septin complex (DeMarini et al., 1997). Complementation of a Calcofluor-resistant mutant also led to the cloning of *ScCHS5* and ScChs5p was shown to be required for ScChs3p activity and also for chitin synthesis *in vivo* (Santos et al., 1997). Mutants lacking ScChs5p have a 75% reduction in vegetative cell-wall chitin. It was suggested that ScChs5p is a Golgi protein required for targeting ScChs3p to polarized growth sites during vegetative growth, because *ScCHS5* mutants accumulate ScChs3p in Golgi vesicles. Its function seems to be specifically related to ScChs3p transport during vegetative growth, although it may participate in the transport of other membrane proteins. This protein has a characteristic fibronectin type III domain (Bateman et al., 1996), which is probably related to its function in vesicle trafficking, and resembles animal neurofilaments. ScChs6p was shown to be required for chitin synthesis *in vivo* but not for ScChs3p activity *in vitro* (Bulawa et al., 1993). This observation indicates that ScChs6p participates in a late step in the control of ScChs3p activity and it is a Golgi protein required for the anterograde transport of ScChs3p to the membrane (Ziman et al., 1996).

This hypothesis was further supported by the evidence that ScChs6p is involved in the correct transport of chitosomes, which contain ScChs3p, to the plasma membrane (Ziman et al., 1998). Its function and localization seem to be similar to those of ScChs5p and together with ScChs5p, ScChs6p is required in the recycling of endocytic vesicles. The *ScCHS6* mutant also accumulates ScChs3p in cytoplasmic chitosomes, but the localization of ScChs1p is not affected. *ScCHS7* encodes a membrane-bound protein that is located in the ER (Trilla et al., 1999). ScChs7p functions as a specific chaperone for ScChs3p, directing its sorting from the ER (Trilla et al., 1999). In the absence of this protein, ScChs3p accumulates in the ER, producing an inactive protein both *in vivo* and *in vitro*. ScChs7p seems to be present in a limited amount in the cell, because overexpression of ScChs3p leads to the accumulation of this protein in the ER. Increasing the intracellular ScChs7p level can relieve this accumulation. The fact that overexpression of *ScCHS3* does not increase chitin levels unless *ScCHS4* and *ScCHS7* are overexpressed simultaneously, suggests that the extent of ScChs3p activity *in vivo* depends on a delicate balance between the levels of ScChs3p, ScChs4p and ScChs7p (Trilla et al., 1999).

Each of the chitin synthases of *S. cerevisiae* has its specific function, but little is known about the relationships among them. Comparison of their deduced amino acid sequences revealed that *ScCHS1* and *ScCHS2* are closely related to each

Figure 1. Schematic representation of ScChs3p and related proteins. ScChs7p is an integral ER protein and responsible for ScChs3p exiting from ER. ScChs5 and ScChs6p are Golgi proteins and required for ScChs3p transport into plasma membrane. ScChs4p is a plasma membrane-associated protein and the potential activator of ScChs3p. ScBni4p is a septin-associated protein involved in anchoring the ScChs3p complex to the septin structure through direct interaction with ScChs4p (adapted from Valdivia et al., 2002).



other and that the last 750 amino acids have 40% identity. They also share a significant homology with *ScCHS3*, especially in the last third of the sequence at the C-terminal end (22% identity). This region contains the QRRRW motif, which is considered to be the signature motif for chitin synthases (Valdivieso et al., 1999), because it is essential for their catalytic activity (Cos et al., 1998). The hydrophobicity profiles of the *ScCHS1* and *ScCHS2* gene products are very similar, but differ considerably from that of *ScCHS3*. No significant homology exists among them at the N-terminal region, and a large portion of the protein is considered to be nonessential. However, the C-terminal region seems to be essential and the deletion of this region usually leads to loss of activity and function (Shaw et al., 1991).

Classification of chitin synthases

The rapid finding of multiple chitin synthase genes from other fungi was promoted by aligning the deduced amino acid sequences of two chitin synthase genes from *S. cerevisiae* (*ScCHS1*, *ScCHS2*) and one from *Candida albicans* (*CaCHS1*), which identified short, but conserved sequences that were then used to design PCR primers (Bowen et al., 1992; Miyazaki et al., 1993)). Using the degenerated primers, numerous *CHS* gene fragments from different fungi were amplified (Bowen et al., 1992) and led to the isolation of chitin synthase genes from *C. albicans* (Chen-Wu et al., 1992), *N. crassa* (Yarden et al., 1991; Yarden

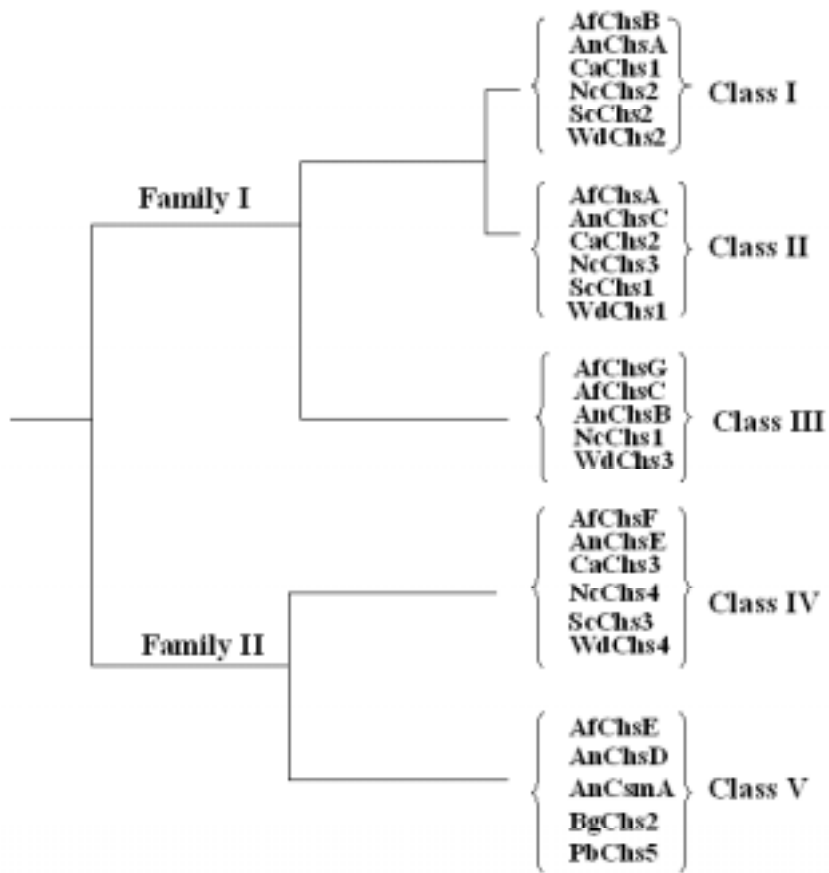
et al., 1993), and *Ustilago maydis* (Gold et al., 1994). The accumulation of more *CHS* genes in turn led to the design of more specific primers and more chitin synthase genes since have been found from more fungi (Miyazaki et al., 1993; Chow et al., 1994; Peng et al., 1995; Karappayil et al., 1996). To date, more than 150 fungal *CHS* genes have been identified or partially sequenced, although only a few of them have been subject to functional characterization (Roncero, 2002). Interestingly, except in *S. pombe*, which has only one chitin synthase gene, most species studied so far have multiple chitin synthase genes. The Zygomycete fungus, *Phycomyces blakesleeanus* has 10 *CHS* genes, the highest number of *CHS* genes reported so far (Ruiz-Herrera, 2001).

As more and more *CHS* genes were identified, the classification of chitin synthases became important. The sequence alignment work from the original PCR amplifications of *CHS* gene fragments from 14 different fungi laid the foundation for their initial classification (Bowen et al., 1992). At first, three distinct classes, class I to III, were suggested. However, the initial primers were only able to amplify sequences of *CHS* genes that encode orthologs of ScChs1p (Class I), ScChs2p (Class II) and a third type (Class III) with no ortholog in yeast. Subsequent investigations showed that new primers were required to amplify genes that encode orthologs of ScChs3p (Class IV) (Peng et al., 1995; Karuppayil et al., 1996). The PCR primers derived from *ScCHS3* led to the discovery of other

classes: Class V and VI (Mellado et al., 1995; Peng et al., 1995; Karuppayil et al., 1996; Specht et al., 1996; Nino-Vega et al., 2000; Wang et al., 1999). However, the existence of the sixth class of chitin synthases is still controversial. Nonetheless, there are two well-defined families of chitin synthases (Fig. 2). The first (family I) includes orthologs of the isozymes encoded by *ScCHS1/2*. This family can be further divided into three classes: I, II and III. Classes I and II are present in yeast and filamentous fungi, while class III is exclusive to filamentous fungi and absolutely absent from ascomycete yeast species. The second (family II) includes the orthologs of the isozyme encoded by *ScCHS3*. Proteins in this family share a high degree of similarity, but are clearly distinct from those in family I. This family can also be further divided into two classes: Class IV and class V. To date, Class IV isozymes are present in both yeast and filamentous fungi, while class V isozymes are exclusive to filamentous fungi. Surprisingly, most if not all of the class V isozymes have been found to encode proteins that contain a myosin motor-like domain in their N-terminal regions. In *Aspergillus fumigatus* (Mellado et al., 1996) and *Aspergillus. oryzae* (Chigira et al., 2002), a sixth class of isozymes has been described, but there is still some controversy about the existence of this class and caution should be exercised about its classification.

Interestingly, *CHS*-like genes have also been found in bacteria (Bulawa et al.,

Figure 2. Classification of chitin synthases (adapted from Munro, et al., 2001). *Af*: *Aspergillus fumigatus*. *An*: *Aspergillus nidulans*. *Ca*: *Candida albicans*. *Nc*: *Neurospora crassa*. *Pb*: *Paracoccidioides brasiliensis*. *Sc*: *S. cerevisiae*. *Wd*: *Wangiella dermatitidis*.



1991), insects (Gagou et al., 2002) and nematodes (Veronico et al., 2001). The *CHS*-like genes from insects and nematodes cluster with class IV and V, suggesting a common evolutionary origin. Based on current knowledge, a tentative hypothesis was proposed (Roncer, 2002) to explain the main evolutionary trends in chitin synthases. Their origin probably was very old, and a very ancient gene duplicated at the base of the fungal kingdom gave rise to the two families. The unique gene of family I also duplicated very early and most fungi contain class I and II isozymes. Some time later, further duplications led to the third isozyme class, although this class seems to have emerged in filamentous fungi exclusively. In certain specific cases, such as in *A. fumigatus*, further duplications produced several additional isozymes in the same class, and this probably occurred in the more recent past. Similar events might have happened in family II, in which an initial gene (class IV) present in most fungi was duplicated, leading to the emergence of class V isozymes. Because class III isozymes have only been identified in filamentous fungi and some dimorphic/polymorphic fungi that are capable of forming true hyphae, these enzymes may have important roles in filamentous fungi and affect a variety of processes, but their function in dimorphic/polymorphic fungi remains to be elucidated. In general, the class IV enzymes make the greatest contribution to chitin synthesis. Class IV and class V enzymes can be differentiated by the presence or absence of tryptophan residues at two positions within a conserved region (Miyazaki et al., 1997).

Chitin and chitin synthesis in other fungi

Of the medically important fungi, most chitin synthase work has been focused on *C. albicans* and *A. fumigatus*. In *C. albicans*, a dimorphic fungus, three chitin synthase structural genes have been cloned and characterized. *CaCHS2* (Class I) was originally identified by using the conserved primers designed by Bowen (Bowen et al., 1992; Chen-Wu et al., 1992). *CaChs2p* is zymogenic and contributes some 80% of the total chitin synthase activity *in vitro* (Munro et al., 1998), which is consistent with the characteristics of *ScChs1p*, another class I chitin synthase (Chen-Wu et al., 1992; Munro et al., 1998). *CaCHS2* is not essential and disruption of this gene produces a mutant with normal growth, morphogenesis and virulence characteristics (Gow et al., 1994; Mio et al., 1996). *CaCHS3* (class IV) was initially cloned by screening the genomic DNA library of *C. albicans* using the *ScCHS3* gene of *S. cerevisiae* as a probe. An 80-90% reduction in the cell wall chitin content was observed in mutants without *CaCHS3*. However, these mutants again had a normal cell shape and growth rate (Mio et al., 1996; Bulawa et al., 1995). Controversial results have been reported from different groups concerning the virulence of the *CaChs3* mutants (Mio et al., 1996; Bulawa et al., 1995). The difference may be related to the different strains and conditions used in respective studies. However, an exciting result was obtained from the study of *CaCHS1* (class II), which represents the first chitin synthase gene reported as an essential gene (Munro et al., 2001). Because of the

lethal phenotype of this mutant, null mutants could not be recovered by conventional gene disruption approaches. However, a conditional mutant was constructed in which a single functional copy of *CaCHS1* was placed under the control of the MRP1 promoter, which was then induced by maltose and repressed by glucose. Similarly to ScChs2p, which is another class II chitin synthase, this chitin synthase is required for chitin deposition in the primary septum. Under repressing conditions, in *C. albicans* yeast form, the mutant cells form chains of inflated compartments with multiple nuclei and are septum-less. In its hyphal form, cells initially form normal parallel-sided hyphae in serum, but the lateral wall then develops balloon-like swellings and lyses at locations that include sites unrelated to the predicted sites of septa. This result showed that besides its function in chitin synthesis in the primary septum, it is also required for maintaining the integrity of the lateral cell wall (Munro et al., 1998). An *ScCHS4* homologue was found in *C. albicans*, which has the ability to complement the chitin synthesis defect of a *S. cerevisiae* *ScCHS4* mutant by restoring chitin synthase activity (Sudoh et al., 1999). However, its function in chitin localization in *C. albicans* has not been clarified. Recently, an *ScCHS7* homologue was found in *C. albicans* by genome database searching, but *CaCHS7* cannot complement the *S. cerevisiae* *ScCHS7* mutant (Roncero, 2002). Although a close homologue of the *ScCHS5* gene has been found and sequence similarity is sufficiently high, its function remains to be tested. No close homologue of *ScCHS6* was found in *C.*

albicans (Roncero, 2002).

In another medically important fungus, *A. fumigatus*, seven chitin synthase genes have been identified. The seven chitin synthase genes of *A. fumigatus* belong to five classes: *AfCHSA* (class I), *AfCHSB* (class II), *AfCHSC* (class III), *AfCHSD* (class IV), *AfCHSE* (class V), *AfCHSF* (class IV) and *AfCHSG* (class III). Disruption of each individual gene was successfully achieved (Munro et al., 2001), however, no single gene is essential. In addition, none of the mutants has obvious abnormality in septum formation. Disruption of *AfCHSA*, *AfCHSB*, *AfCHSD* or *AfCHSF* individually, has little effect on morphology and growth (Mellado et al., 1996). Disruption of *AfCHSG* results in a mutant with an obvious phenotype, such as low levels of conidiation, inhibited radial growth rates of colonies and hyphae in these colonies have about twice the normal number of branches (Mellado et al., 1996). At the same time, reduced chitin synthase activity is detected. Although, disruption of *AfCHSC* produces mutants with abnormal phenotypes, a double disruption of *AfCHSC* and *AfCHSG* in the same background has a phenotype comparable to the *AfCHSG* gene disruption mutant. The double gene disruption mutants produce a decreased mortality rate in mice and the symptoms of disease are delayed. Disruption of *AfCHSE* produces a mutant with a profound phenotype. This mutant has reduced chitin content, periodic swelling along its hyphae, and abnormal and greatly reduced ability to conidiate

(Aufauvre-Brown et al., 1997). Despite the severe phenotype of the *AfCHSE* gene disruption mutants, they still have unaffected virulence in terms of their ability to establish disease in a neutropenic mouse model of pulmonary aspergillosis (Aufauvre-Brown et al., 1997).

In the better studied nonpathogenic model filamentous fungus, *Aspergillus nidulans*, chitin constitutes up to 40% of the cell dry weight (Specht et al., 1996), and five chitin synthase genes have been identified: *AnCHSA* (class II), *AnCHSB* (class III), *AnCHSC* (class I), *AnCHSD* (class V), and *AnCHSE* (class IV). Similar to the situation with *A. fumigatus*, no single chitin synthase is essential and no chitin synthase has been found to contribute to septum formation, indicating that another isozyme may be responsible for this, or perhaps more than one chitin synthase is responsible for the chitin synthesis at the septum. In *A. nidulans*, disruption of *AnCHSA* produces no obvious phenotype, but disruption of *AnCHSB* gives rise to mutants that stop growing and have swollen hyphal tips shortly after spore germination and this defect is not rescued by adding osmotic stabilizer (Yanai et al., 1994). Although, disruption of *AnCHSC* produces no defect in morphology and growth rates in the asexual life cycle, disruption of *AnCHSD* generates mutants with abnormal conidiation and hyphal swelling indicating that *AnCHSD* is involved in synthesizing chitin in the lateral cell wall (Specht et al., 1996). The *AnChsE* disruptant is indistinguishable from the wild type in term of

morphology and development, although the chitin content of hyphae is reduced by about 30 to 40% (Specht et al., 1996). Interestingly, another class V chitin synthase (AnCsmA) was found recently. The two class V proteins are identical except that AnCsmA has an extended N-terminus that has homology to a myosin motor-like domain (Fujiwara et al., 1997). This kind of chitin synthase also exists in numerous other fungi.

One fundamental question that remains to be answered is why most fungi have more than one chitin synthase? During evolution, selection favors the retention of beneficial traits, and elimination of redundancy. The current hypothesis for the existence of multiple chitin synthases is that each chitin synthase plays a different role (Ruiz-Herrera, 2001). On the other hand, the multiplicity of chitin synthase genes in a fungus may provide an important safety mechanism for its survival. The existence of several chitin synthases may guarantee survival by allowing the adaptation of the fungus to the different growth conditions it may encounter.

As previously discussed, functions of some of the chitin synthases have been identified, but still for most of them, their functions remain to be elucidated. The biggest challenge has been to obtain double, triple or multiple gene disruption mutants, especially in filamentous fungi, for which genetic manipulation tools are not developed as well as for yeasts. Nonetheless, considerable information has

been gathered through the diligent work of many groups. The gene disruption mutants at hand can be classified into five groups based on the severity of phenotype alterations (Table 1). Interestingly, there is little correlation between the severity of the phenotypes and the class of enzyme affected in the mutants.

Regulation of chitin synthesis

It has become clear that different chitin synthases perform different functions at specific times and specific locations, and that the synthesis and deposition of chitin are under temporal and spatial control (Choi et al., 1994; Ruiz-Herrera et al. 2001). This realization raised another fundamental question: how is the regulation of a chitin synthase achieved? In *S. cerevisiae*, the regulation of chitin synthase has been attributed mainly to the changes in chitin contents and chitin synthase activities. With advances of molecular techniques, more and more attention is being directed to elucidating this regulation at molecular levels. However, caution is required in the matter of interpreting any observation because most measuring methods may be subject to quantitative errors, which are always accompanied by the enzyme multiplicity and assay conditions. For example, most chitin synthases are activated by proteolysis, however, no strong evidence exists to support that this process actually occurs *in vivo*, and little is known about the mechanism of this activation process. Nevertheless, several conclusions have been reached from previous studies concerning the regulation of chitin synthases: (i) it seems that

Table 1. Phenotypic alterations in *Chs* mutants

1. No apparent defects:

AfChsC (Class III), *AfChsA* (Class I), *AfChsB* (Class II), *NcChsI* (Class III), *WdChs2* (Class I), *WdChs3* (Class III), *ScChsI* (Class I).

2. Minor changes (lower chs activity, lower chitin content, or some growth defects):

AfChsG (Class III), *AnCsmA* (class V), *CaChs2* (Class I), *NcChs2* (Class II), *ScChs2* (class II), *WdChs1* (Class II), *WdChs4* (Class IV).

3. Significant changes (very low level of chs activity and/or chitin contents, reduced growth, morphological changes, reduced virulence, etc):

AfChsE (Class V), *AnChsD* (Class V), *CaChs3* (Class IV), *CaChs2/3*, *ScChs3* (Class IV).

4. Severe phenotypes: (poor growth, aberrant morphology, loss of virulence)

AnChsB (Class III), *WdChs1/2* (Class II/I), *WdChs2/3* (Class I/III).

5. Lethality:

ScChs1/2/3, *CaChs1* (Class I).

Abbreviations: *Af*: *Aspergillus fumigatus*. *An*: *Aspergillus nidulans*. *Ca*: *Candida albicans*. *Nc*: *Neurospora crassa*. *Sc*: *S. cerevisiae*. *Wd*: *Wangiella dermatitidis*.

(adapted from Ruiz-Herrera et al., 2001).

most chitin synthases are under some kind of regulation; (ii) no universal regulatory mechanism exists; (iii) the regulation depends on various environmental conditions and internal effectors; (iv) no general agreement as to whether regulation happens at the transcriptional level or post-transcriptional levels, but most likely that chitin synthase regulation is the combined result of transcriptional and post-transcriptional regulatory mechanisms (Ruiz-Herrera, 2001). In *S. cerevisiae*, fluctuations of *ScCHS1* mRNA are observed throughout the cell cycle (Pammer et al., 1992), although ScChs1p activity remains quite constant (Choi et al., 1994). This suggests that the regulation of ScChs1p may occur through activation of the zymogen form. The mRNA of *ScCHS2* and enzyme activity of ScChs2p both reach maximal levels in synchronized cells before cell division when the primary septum is formed. ScChs2p activity decreases rapidly after transcription of this gene stops (Pammer et al., 1992; Choi et al., 1994; Spellman et al., 1998). It was suggested that ScChs2p expression is regulated by temporal synthesis and rapid turnover during the cell cycle. However, the possibility that the regulation is through the activation of the zymogen cannot be excluded. A fluctuation of *ScCHS3* mRNA is also observed during the cell cycle and peaks late in the cell cycle after septum formation (Pammer et al., 1992). However, it was also reported that ScChs3p is stable throughout the cell cycle, which indicates that the regulation of ScChs3p is mainly post-transcriptional (Choi et al., 1994). In *C. albicans*, the *CaCHS* genes

are regulated differentially during the switch from yeast to hyphal growth. *CaCHS2* and *CaCHS3* mRNA are present at high levels compared with the relatively low level of *CaCHS1* mRNA, and they reach their maximal levels two hours after the switch to hyphal growth (Munro et al., 1998). In *P. brasiliensis*, the mRNA levels of four chitin synthase genes, *PbrCHS1*, *PbrCHS2*, *PbrCHS4*, and *PbrCHS5* are higher in hyphal form cells than in yeast form cells. This is not consistent with the chitin levels, which are higher in yeast cells. However, this may also indicate that post-transcriptional regulation is involved in the control of the chitin synthesis (Munro et al., 2001).

Another important aspect of chitin synthase is its localization and deposition. ScChs1p and ScChs3p are synthesized constitutively and located at the plasma membrane and in endocytotically-derived chitosomes, and may be subject to cell-cycle specific recycling (Ziman et al., 1996; Chuang et al., 1997). Studies of the targeting and localization of ScChs3p suggest that it is targeted to the site where the new bud occurs and to the mother-bud neck. Targeting of ScChs3p requires ScChs5p, ScChs4p, ScMyo2p and actin (Santos et al., 1997). In contrast, ScChs2p is believed to be processed through the constitutive secretory pathway via the ER and the Golgi where it is packed into vesicles and transported to the plasma site where a new septum forms. After cytokinesis, ScChs2p is internalized and transported through an endosome to the vacuole where it is degraded by Pep4p

(Chuang et al., 1996). It was suggested that ScChs1p and ScChs3p follow a similar pathway as ScChs2p, but once in the endosome (endocytotically derived chitosome), they are not subject to degradation but remain in the cytoplasm as a reservoir of these enzymes.

The cell-wall salvage pathway also regulates chitin synthase and alterations in the cell wall affects chitin synthesis. Disruption of *ScGGPI* and *ScGAS1*, which are involved in GPI-glycoproteins synthesis and glucan synthesis in *S. cerevisiae*, respectively, leads to a dramatic reduction in the levels of β -glucan, and ultimately cause an increase in chitin by a compensatory mechanism. ScChs3p produces this newly synthesized chitin, since *ScCHS3/ScGGPI* double mutants are severely affected in growth and viability. Similar results were obtained by disruption of *ScFKSI*, which is responsible for glucan synthesis in *S. cerevisiae* (Popolo et al., 1997).

Chitin synthase as a drug target

Chitin and glucan are both considered good drug design targets because they are unique and essential to fungi (Georgopapadakou et al., 1995). Echinocandins and papulocandins are specific and noncompetitive inhibitors of fungal β (1,3)-glucan synthase. Inhibition leads to depletion of cell wall glucan in growing cells, osmotic instability, and, ultimately, lysis of the fungal cells (Gopal et al., 1984).

Because of the narrow *in vitro* spectrum of the papulocandins, this group of compounds is no longer pursued for pharmacological development (Georgopapadakou, 1995). However, echinocandins have demonstrated useful fungicidal activity both *in vitro* and in animal models, and semisynthetic derivatives with broader antifungal spectra and improved pharmacologic properties have been selected for clinical development (Groll et al., 1998).

Polyoxins and nikkomycins, which are structurally closely related peptide nucleoside antibiotics, are well-studied inhibitors of chitin synthase (Debono 1994). They were first found to be produced by *Streptomyces cacaoi* and *Streptomyces tendae*, respectively (Suzuki et al., 1965; Dahn et al., 1976) and numerous naturally derived congeners have been described since then (Hector et al., 1993). These compounds are substrate analogs of UDP-N-GlcNAc (Endo et al., 1969; Endo et al., 1970). Polyoxin D was shown to be effective *in vitro* against a wide range of fungi including *C. albicans* (Becker et al., 1983). It inhibits cell septation and separation of yeast cell buds, which leads to chaining, osmotic swelling and cell death. However, this compound is not useful in therapy because of its toxicity. The effectiveness of polyoxins and nikkomycins also depends on their ability to enter an intact cell, as well as their susceptibility to degradation by cellular proteases and the affinity of the specificity for each chitin synthase enzyme (Yadan et al., 1984; Payne et al., 1985; Decker et al., 1991). The

three chitin synthases of *S. cerevisiae* have different sensitivities to polyoxin D, nikkomycin X and nikkomycin Z. ScChs1p is more sensitive than ScChs3p, which in turn is more sensitive than ScChs2p. The recent discovery that CaChs1p of *C. albicans* is essential for its growth led to an antifungal screen targeted to find inhibitors of this enzyme. The screen resulted in the finding of the CaChs1p-specific inhibitor RO-09-3143 (Masubuchi et al., 2000; Sudoh et al., 2000). Growth experiments showed this compound only had a fungistatic effect against the wild-type cells but was fungicidal in an *ScCHS2* null mutant background (Masubuchi et al., 2000; Sudoh et al., 2000). Further study of the fungal cell wall may be expected to continue to furnish both exciting new findings and clinical applications. To date, most antifungal drug screenings are focused on selecting drugs against Chsp itself. However, the finding that most chitin synthase genes are under transcriptional and/or translational regulation suggests additional potential drug targets.

Melanin of dematiaceous fungi

Dematiaceous fungi represent a group of fungi that are dark walled and the dark pigmentation is caused by melanin synthesis and deposition. Melanin, which is the main characteristic component of the dematiaceous fungal cell wall, has been proven to be a virulence factor in numerous dematiaceous fungi (Langfelder et al., 2003). Recent studies have seen a huge increase in our knowledge of fungal

melanins, both regarding their biosynthesis and their importance for fungal pathogenicity. In general, melanins are macromolecules formed by oxidative polymerization of phenolic or indolic compounds. Often the resulting pigments are brown or black in color (Langfelder et al., 2003). Several different types of melanin have been reported in fungi, and the two most important types are dihydroxyphenylalanine (DOPA)-melanin and dihydroxynaphthalene (DHN)-melanin. Although neither type of melanin is essential for fungal growth, both have been implicated in pathogenesis (Langfelder et al., 2003).

***Wangiella dermatitidis* as a model for study of dematiaceous fungi and the fungal cell wall**

Wangiella (Exophiala) dermatitidis is an asexual, polymorphic, dematiaceous (melanized) fungal pathogen of humans, which predominately exists as a yeast form *in vitro*, but can be easily manipulated to undergo morphological transitions to produce isotropically enlarged yeast, multicellular form and hyphae (Szaniszlo et al., 1983; Cooper et al., 1993; Karuppayil et al., 1997; Szaniszlo, 2002). *In vivo*, this fungus also produces various morphological forms, such as budding yeast, pseudohyphae, true hyphae, isotropically enlarged yeast cells and sclerotic bodies (Kwon-Chung et al., 1992; Matsumoto et al., 1993; de Hoog et al., 1994; Matsumoto et al., 1994; Nachman et al., 1996). Although considered a paradigm for phaeohyphomycosis, because of its increasing detection in cutaneous,

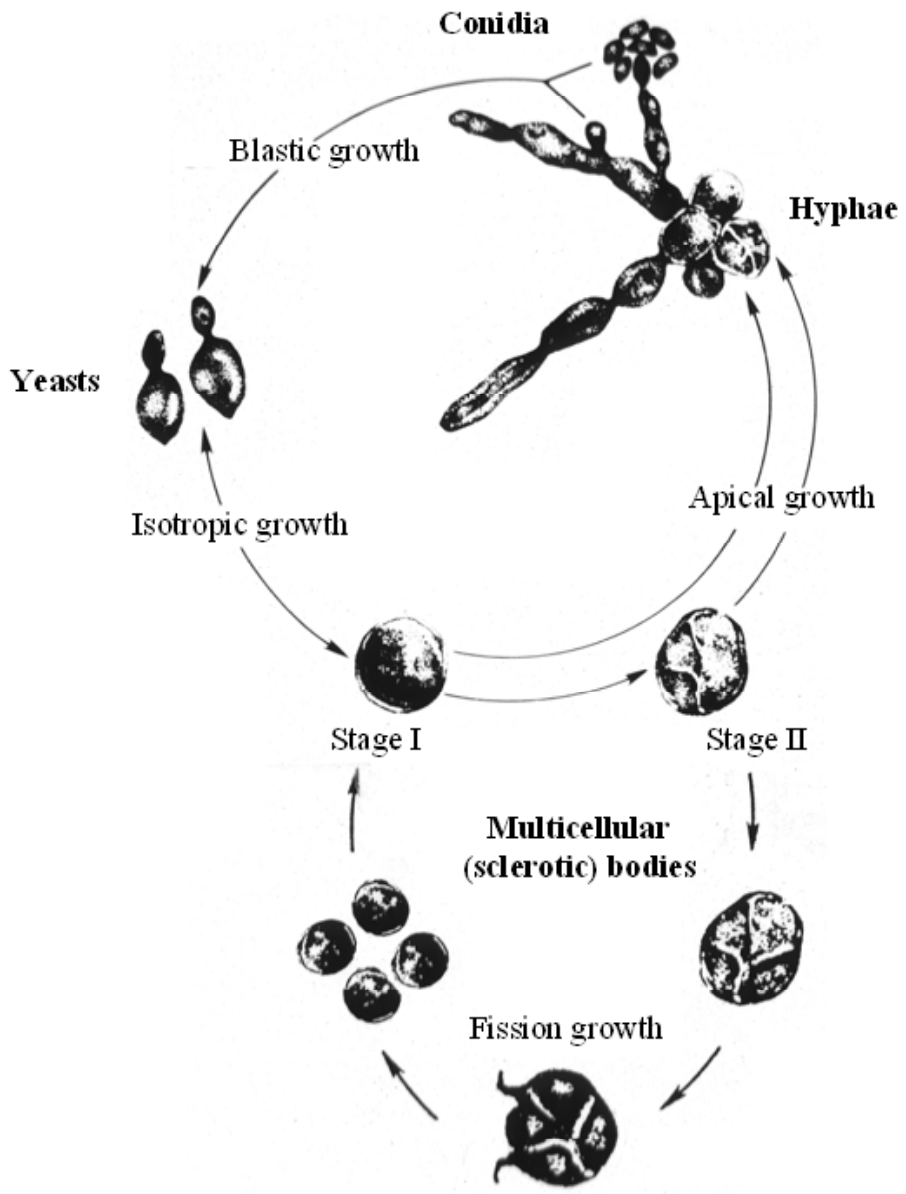
subcutaneous and the central nervous infections (Kwon-Chung et al., 1992; Matsumoto et al., 1993; Matsumoto et al., 1994), it is better known as a model for studies of more than 100 other dematiaceous agents of mycoses (Szanişzlo et al., 1993, deHoog et al., 1994).

The usefulness of *W. dermatitidis* as a model for studies of many morphologically diverse pathogenic dematiaceous fungi has been established by the results from a variety of past investigations (Szanişzlo et al., 1993; de Hoog et al., 1994; Szanişzlo, 2002). First, it was documented in this fungus for the first time among dematiaceous human pathogens that its melanin represents the polymerization product of DHN-melanin, which is chemically distinct from DOPA-melanin (Geis et al., 1985). The close relationship between this fungus and some other dematiaceous fungi was further strengthened after other species were shown to melanize their cells in the same manner (Taylor et al., 1991; Cooper, 1997). Sequence analysis of rDNA subsequently confirmed that the majority of dematiaceous pathogens are closely related (Haase et al., 1999). Second, another important finding that validated the use of *W. dermatitidis* as a model dematiaceous fungus is the well-established relationship between growth conditions and cell morphological transitions (Szanişzlo, 2002). Particular interest has been directed toward the nature and development of its unique multicellular form. For example, in extreme acidic condition (pH 2.5), *W. dermatitidis* forms

isotropically enlarged, multinucleate cells, which with prolonged incubation, may become internally partitioned with one or more septa (Szaniszlo et al., 1976). This cell type strongly resembles the sclerotic body, which is the tissue-phase of all chromoblastomycosis agents produced in human subcutaneous lesions. This early finding suggested that *W. dermatitidis* is a good model to study agents of chromoblastomycosis, as well as other phaeohyphomycosis agents (Szaniszlo et al., 1983; Cooper et al., 1985).

The single fungus, *W. dermatitidis* exhibits all of the vegetative phenotypes of any other dematiaceous pathogen observed *in vivo* (Fig. 3), and can be induced to produce *in vitro* homogeneous populations of different forms at 25°C and 37°C for the study of the relevance of cell wall components, such as chitin and melanin, to pathogenicity and virulence. In addition, it has been proven that this fungus is an excellent choice for experimentation and as a dematiaceous fungal model (Szaniszlo, 2002) for the following reasons: (i) its yeast phase allows exact quantification, which is more difficult with molds; (ii) it is amenable to mutagenesis because of the uninucleate haploid feature of *W. dermatitidis*. Numerous auxotrophic mutants (*wdarg*, *wdade*, *wdura5*, etc), melanin mutants (e.g. *mel1*, *mel2*, *mel3*, *mel4*, etc.), temperature-sensitive cell-division-cycle (*cdc*) and other morphological mutants (e.g. *wdchs1*, *wcdc2*, Hf1, Hf2 etc.) have been derived, which permit studies that would be impossible with only a wild-type

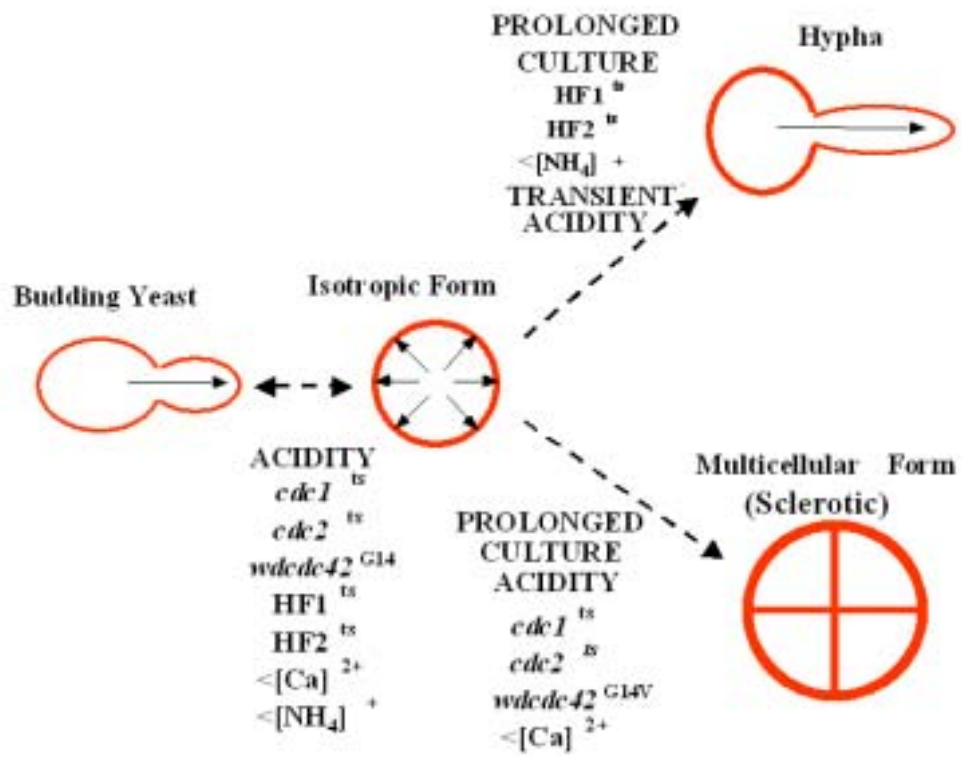
Figure 3. The life cycle of and vegetative growth models of *Wangiella dermatitidis* (adapted from Geis and Jacobs, 1985; Mendoza and Szaniszlo, unpublished data).



strain (Szaniszlo, 2002); (iii) a protoplast fusion system for parasexual genetic analyses has been established, which allows complementation studies and has provided useful synthetic diploids that can be used when investigating essential genes (Cooper, et al., 1993; Szaniszlo, 2002); (iv) molecular-based transformation, gene disruption, and gene expression systems have been developed and used extensively; (v) a variety of genes have been cloned and are being characterized and some of them have been proven to be relevant to its viability, morphology and virulence (Szaniszlo, 2002).

The fact that *W. dermatitidis* is easily manipulated to undergo morphological transition *in vitro* provides a useful model for study of dematiaceous fungal morphogenesis as well as pathogenesis (Fig. 4). The yeast form of this fungus originates by budding from lateral or terminal hyphal blastospores or their yeast-like progeny (Oujezdsky et al., 1973). Prolonged culture of the yeast-like cells on a carbon rich solid medium, such as Sabouraud dextrose agar, gives rise to thick-walled and spore-like unicellular forms (Oujezdsky et al., 1973). Subculture of these forms in fresh minimal medium induces hyphal outgrowth (Oujezdsky et al., 1973). Similar morphological transitions can be obtained in culture medium with a limited nitrogen source (McIntosh, 1996). Also, a temperature-sensitive mutant, Hf1, which was derived from the wild-type *W. dermatitidis* 8656 forms hyphae at the restrictive temperature of 37°C (McIntosh, 1996). In this mutant, yeast cells

Figure 4. Diagram of *W. dermatitidis in vitro* morphological conversions. The conditions are marked for transitions of the yeast form to multicellular (sclerotic) bodies or hyphae. The arrows inside the cell show the changes in cell polarity during morphogenic processes. Cell-division-cycle (*cdc*) and hyphal form temperature-sensitive (*ts*) mutants could switch, under the nonpermissive temperature, from isotropic to multicellular forms (*cdc1* and *cdc2* mutants) or to hyphal form (Hf1 and Hf2 mutants) (modified from McIntosh and Szaniszlo, unpublished data).



seem to be able to pass through an intermediate stage of isotropic growth prior to the formation of hyphae by polarized apical extension. The first condition found to induce morphological transition from yeast forms to sclerotic body-like multicellular forms involved incubating yeast cells in acidic media of pH 2.5 (Szaniszlo et al., 1976; Karuppayil et al., 1997). Transfer of the acid-induced multicellular forms back to a medium of neutral pH leads to hyphal growth instead of a simple reverse of the process with the production of yeast form. An important role for Ca^{2+} in *W. dermatitidis* to this morphological transition has also been established (Karuppayil et al., 1997). In synthetic media under various pH and temperatures, two thresholds of Ca^{2+} have been identified. At lower concentration (0.1 mM), cells tend to favor non-polarized growth, which leads to the multicellular form, whereas at higher concentrations (1-5 mM), cells tend to favor polarized growth characterized by yeast budding or pseudohyphal or true hyphal forms. Other temperature-sensitive mutants, Mc1, Mc2 (*wdcdc1*) and Mc3 (*wdcdc2*), which were also derived from the wild-type *W. dermatitidis* 8656 form the multicellular form at the restrictive temperature of 37° (Roberts et al., 1978).

In terms of virulence, two cell wall components, melanin and chitin have been shown to have relevance to the full virulence of *W. dermatitidis* (Feng et al., 2001; Liu et al., 2001; Wang et al., 2001; Szaniszlo, 2002). All DHN-melanin biosynthesis mutants (*mel*) derived so far by traditional mutagenesis or that arise

spontaneously during plating are less virulent than their wild-type parent when tested in an acute mouse model of infection (Dixon et al., 1991). Disruption of *WdPKS1* (polyketide synthase), which is essential for melanin synthesis, also produces albino mutants with less virulence (Feng et al., 2001). Because this particular gene is non-essential for cell growth and dramatic color changes are caused by disrupting this gene, a color-selectable and site-specific integrative transformation system has been established and used to study the effects of truncations of the 5' URS of *WdCHS3* fused to *lacZ* on the expression of β -galactosidase (Wang et al., 2000). Also, this system was used to study the effects of overexpression of the wild-type and a number of dominant positive and dominant negative mutant alleles for the previously cloned *WdCdC42* gene (Ye et al., 1999; Ye et al., 2000).

In *W. dermatitidis*, vegetative morphological transitions are usually accompanied by changes of cell-wall chitin. Chitin is found throughout the cell wall in hyphae and isotropic forms, but the primary site of chitin localization in yeast cells is in septal regions (Cooper et al., 1985; Harris et al, 1986; McIntosh, 1996). Chitin synthase activities are detected from membranes of *W. dermatitidis*, and original analysis indicated the possibility of multiple chitin synthases in this fungus (Szanişzlo and Momany, 1993). The direct evidence that multiple chitin synthases present in this fungus came to light when 600-bp PCR products were successfully

amplified from genomic DNA of *W. dermatitidis* using the degenerate primers designed by Bowen et al (1992). After cloning and sequencing of the PCR products, three chitin synthase homologous gene fragments (*WdCHS1*, *WdCHS2*, and *WdCHS3*) were identified (Szanişzlo et al., 1993). Another PCR product was later amplified and identified as the *WdCHS4* gene fragment (Karuppayil et al., 1996). The full-length gene of each chitin synthase was then isolated either by library screening using the PCR products as probes or by marker rescue after a site-specific gene disruption was obtained. Sequence comparisons put the derived proteins of each gene into a different chitin synthase class (WdChsp): *WdCHS2*, class I; *WdCHS1*, class II; *WdCHS3*, class III; *WdCHS4*, class IV. Disruption of *WdCHS1* produced mutants that tended to form short polarized chains of relatively normal-appearing yeast cells and that grew at rates comparable to the wild type at both 25°C and 37°C. However, cytological and ultrastructural studies showed that mutant cells were defective in cell separation and sometimes were enlarged, had multiple nuclei, and had chitin enrichments at septal regions. Nonetheless, the mutants did not have reduced virulence in an acute mouse infection model (Zheng, et al., unpublished data).

Disruption of *WdCHS2* produces mutants with no obvious morphological defects in yeast vegetative growth or their ability to carry out polymorphic transitions. However, chitin synthase activity assays show that mutants have drastically

reduced chitin synthase activity. No loss of virulence with these mutants is detected in a mouse model of acute infection (Wang et al., 2001). Disruption of *WdCHS3* results in significantly reduced chitin synthase activity, but does not obviously affect morphology, growth rate, chitin content, or virulence (Wang et al., 2000). Disruption of *WdCHS4* results in mutants with reduced chitin content, abnormal yeast clumpiness, and increased melanin secretion. No significant loss of virulence is apparent in any single chitin synthase gene disruption mutant (Wang et al., 1999). However, to study the function of each chitin synthase and to determine systematically its contribution to the biology of *W. dermatitidis*, it requires different chitin synthase genes to be disrupted in various combinations. Through years of work, a large collection of such mutants with double, and triple gene disruptions has been collected. Disruption and characterization of each *WdCHS* individually or in different combinations have revealed some insights into the individual and collective relevance of each WdChsp isozyme to the biology and virulence of *W. dermatitidis*. Two general conclusions can be drawn from characterizations of these mutants. First, some WdChsp must have partially overlapping functions, but they are not completely redundant. Second, no single WdChsp is essential for the viability of *W. dermatitidis* at 25°C, even though some *wdchsΔ* mutants have significantly reduced chitin synthase activities or chitin contents (Szanišslo, 2002). However, double mutants with both *WdCHS1* and *WdCHS2* being disrupted are incapable of growth at 37°C. In addition, they

have abnormal phenotypes when grown at 25°C, such as grow weakly and have defective septal regions. This suggests that WdChs1p and WdChs2p have overlapping functions for normal cell growth. The fact that *wdchs1Δ* mutants have a slightly altered morphology as compared to that of *wdchs2Δ* mutants indicates that WdChs1p and WdChs2p do not have completely redundant functions. Not surprisingly, because of their temperature sensitivity, *wdchs1Δwdchs2Δ* double mutants have reduced virulence when tested in mouse models of acute infections, most likely because they cannot grow at infection temperatures. On the other hand, the disruption of both *WdCHS2* and *WdCHS3* genes results in mutants that have reduced virulence too, but grow normally at both 25°C and 37°C (Wang et al., 2001). Although the basis for the reduced virulence in the *wdchs2Δwdchs3Δ* double gene disruption background is unknown, it suggests that WdChs2p and WdChs3p are functionally overlapping for cell survival in mice, and that they provide protection against a factor other than the higher temperature involved in mice. Therefore, three out of the four previously identified WdChsp isozymes have been proven to contribute to the full virulence of *W. dermatitidis* directly or indirectly.

The complicated relationships among the expression of the four *WdCHS* genes at the transcriptional level have been addressed preliminarily by using semi-quantitative RT-PCR (Wang et al., 2002). The expression of *WdCHS1* and

WdCHS2 are very constant at both 25°C and 37°C, suggesting that *WdCHS1* and *WdCHS2* play basic roles in cell proliferation. On the other hand, both genes appear to compensate for each other. *WdCHS1* disruption is compensated only by higher expression of *WdCHS2*, whereas *WdCHS2* disruption appears to be mainly compensated by increased expression of *WdCHS1*. This indicates that *WdCHS1* and *WdCHS2* are functionally overlapping *in vivo*, and is consistent with the previous observation that *WdCHS1* and *WdCHS2* cannot both be defective in the same background for cell viability at 37°C, in spite of the fact that they can grow weakly, albeit abnormally, at 25°C (Wang et al., 2001). RT-PCR and RNA dot-blots results also demonstrate that *WdCHS3* transcript is the most abundant transcript among all the chitin synthase genes. Its transcription level increases quickly after temperature shift from 25°C to 37°C for 3 h and its expression continues to increase to a level of 3.5 times higher than those observed at 25°C. This finding is consistent with a previous result from Northern analysis showing that *WdCHS3* is highly expressed at elevated temperature and under other stress conditions that induce cellular morphogenesis. Western blotting also indicates that the production of WdChs3p is temperature dependent and temporally regulated (Wang et al., 2000).

Objectives

My project was initiated by my finding of a fifth chitin synthase structural gene (*WdCHS5*) in *W. dermatitidis*, which represents a class V chitin synthase that has

a myosin motor-like-encoding domain at its 5'-end. This finding is important because this unique type of chitin synthase has been identified in only a relatively few other filamentous fungi. In addition, it is the most poorly understood chitin synthase of the isozymes. Considerable attention has been drawn to the unique structure of this kind of chitin synthase because of the presence of the myosin motor-like domain. Motors are molecular machines that move their cargo along F-actin or microtubules. Most motors hydrolyze ATP to move their “cargo” unidirectionally along the microtubules or the F-actin (Gero, 2000). Furthermore, some myosin tail domains contain structural and functional motifs, such as SH3 domains, GAP domains, FERM domains, and pleck-strin homology (PH) domains (Sellers et al. 2000). However, the function for myosin tail domain is largely unknown. A common assumption is that the tail directs the interaction of a given myosin with its cargo (Mermall, 1998). In *S. cerevisiae*, genome sequencing has revealed genes encoding five myosins, six kinesins and one dynein (Steinberg, 1998), of which, a class V myosin, Myo2p appears to support polar growth during budding. It was suggested that Myo2p binds secretory vesicles via its carboxy-terminal tail and moves its cargo along F-actin filaments to sites of growth (Schott et al., 1999; Pruyne et al., 1998). At present, Myo2p appears to be the main membrane-bound motor in yeast and, therefore, is likely to participate in the transport of many components, and one such component is Chs3p (Santos et al., 1997).

The first gene found to encode a chitin synthase with a myosin motor-like domain was identified in *A. nidulans* (Fujiwara et al., 1997). The gene was *AnCsmA* with a long open reading frame of 5.5 kb. The N-terminal domain of about 700 residues of *AnCsmA* shows significant similarity to myosins and the positions of the P-loop, the switch I and the switch II, and the essential residues in these motifs are conserved. Classification puts it in the myosin family class XVII (Hodges et al., 2000) and this is also the first example of a myosin fused to a metabolic enzyme. The C-terminal half of the *AnCsmA* product can be grouped among the class V chitin synthases, and its sequence is almost identical to that of *AnChsD*, a class V chitin synthase from *A. nidulans* strain FGSC4. *AnChsD* lacks the myosin motor-like domain and might be a partial sequence of *AnCsmA* or its homologue. The co-existence of a chitin synthase and a myosin motor-like domain in the same molecule of *AnCsmA* provides a new potential insight into the transport and localization of this enzyme. Speculation was made that the N-terminal domain could be active as a myosin motor, and that it would be necessary for proper localization of the C-terminal chitin synthase domain. In filamentous fungi, actin is concentrated in the form of fibers or patches at hyphal tips, septa, and branching sites where cell wall or septal synthesis is active (Harris et al., 1993). In yeast, spatial organization of actin cytoskeleton is apparently linked to the distribution of chitin (Cid et al., 1995) and Myo2p is essential for proper localization of ScChs3p (Santos et al., 1997). Thus, it is possible that some

chitin synthases are incorporated into vesicles, which are transported to the cell surface by myosins along actin cables, whereas AnCsmA is transported by its N-terminal myosin motor-like domain. Disruption of *AnCsmA* produces mutants that show aberrant growth on solid medium and form balloons in their hyphae, indicating involvement of AnCsmA in hyphal tip growth. However, abnormalities are predominantly seen in older parts of hyphae, suggesting AnCsmA may function in chitin metabolism in those old regions.

Reintroduction of the chitin synthase domain-encoding part of *AnCsmA* did not rescue the phenotype caused by disruption of the whole gene, suggesting that the myosin motor-like domain of AnCsmA plays an important role in the proper localization of AnCsmA. Later work showed that the entire coding region of *AnCsmA* is translated as a single polypeptide with an approximate molecular mass of 210 kD. AnCsmA-HA is detected through day five of cultivation and its levels are significantly reduced under high osmotic conditions. Because of the detection of a band of 140 kD corresponding to the size of AnCsmA-HA without the myosin motor-like domain, it is speculated that AnCsmA is cleaved between the myosin motor-like domain and the chitin synthase domain after 2 days cultivation and post-translational processing of AnCsmA may occur as a means of activating the chitin synthase domain. Studies of *AnCsmA* expression also revealed that *AnCsmA* transcript levels are regulated in response to an alteration in external

osmolarity. The *AnCsmA* null mutants are sensitive to low osmotic conditions and levels of AnCsmA-HA protein and *AnCsmA* transcript are significantly reduced under high osmotic conditions suggesting that AnCsmA plays an important role in maintenance of cell wall integrity under low osmotic conditions (Takeshita et al., 2002). Sequence analysis of the *AnCsmA* promoter region revealed that potential functional promoter elements are present. Interestingly, a potential Rlm1p binding site was found among them. Rlm1p is a transcription factor and a downstream target of MAP kinase cascade. Most of the genes known, or suspected to encode cell wall protein or proteins that are involved in cell wall biogenesis are regulated by Rlm1p in response to the activation of the PKC pathway (Jung et al., 1999). The finding of this sequence in the promoter region of *AnCsmA* suggests that an orthologous gene of RLM1 may be present in *A. nidulans*, and that its product may regulate *AnCsmA* expression in response to alterations in external osmolarity. Among the other potential functional promoter elements present in the promoter region of *AnCsmA* are a number of STREs, which are well-conserved elements in stress-induced genes of fungi. In *S. cerevisiae*, the expression of genes that contain STREs in their promoter regions is induced under high osmolarity, heat shock, or oxidative stress conditions (Estruch et al., 2000). Thus, the transcription of *AnCsmA* may similarly be induced under these stress conditions.

Genes similar to *AnCsmA* have been isolated from other filamentous fungi, including *MgCsm1* of *Pyricularia oryzae* (perfect stage, *Magnaporthe grisea*), *BgChs2* of *Blumeria graminis* (Zhang et al., 2000), and *GgChsA* of *Glomerella graminicola* (Amnuaykanjanasin et al., 2003). It was suggested that *GgChsA* of *G. graminicola* is responsible for the synthesis of one-fourth to one-third of the chitin in conidial walls and it appears to have a non-redundant function in chitin synthesis. Characterizations of *GgChsA* of *G. graminicola* demonstrated that it is essential for conidial wall strength in media with low osmotic pressure and contributes to strength of hyphal tips. *GgChsA* disruption in *G. graminicola* results in phenotypes that are different from *AnCsmA* disruption in *A. nidulans*, which may indicate a different role for *GgChsA* of *G. graminicola*. For example, *AnCsmA* disruption in *A. nidulans* causes spherical swellings along the length of its hyphae, but swellings also occur at hyphal apices. In contrast, *GgChsA* disruption in *G. graminicola* causes hyphal swellings, occurs primarily at the apices and less commonly in the intercalary regions (Amnuaykanjanasin et al., 2003). It was suggested that the chitin produced by the *GgChsA*-encoded isozyme contributes to the strength of the hyphal tip. Since no orthologue exists in the genome of *S. cerevisiae* and *S. pombe*, it was suggested that Csm-type proteins have unique functions that are peculiar to filamentous growth. However, this kind of gene has also been identified in the dimorphic fungus, *Paracoccidioides*

brasiliensis (Nino-Vega et al., 2000), and the polymorphic fungus, *W. dermatitidis* (Liu et al., 2001).

The suggestion that the chitin synthase domain of a class V type of isozyme might be translocated to its proper localization site by the help of the myosin domain, a motor protein, by moving along actin cables adds intrigue to its study (Fujiwara et al., 1997; Horiuchi et al., 1999; San-Blas et al., 2002). However, little evidence exists to support the hypothesis that chitin transportation has a close relationship with the cytoskeletal structure. My discovery of *WdCHS5* in *W. dermatitidis* broadens the range of this kind of gene to a polymorphic fungus, which we think will eventually allow a more definitive functional characterization of a class V isozyme. My report represents the first step toward that goal, and also documents the unique contribution that this isozyme makes to the biology and virulence of *W. dermatitidis*.

MATERIALS AND METHODS

Strains

The *W. dermatitidis* strains used in this work are listed in Table 2. The wild-type strain of *W. dermatitidis* 8656 (ATCC34100 [*E. dermatitidis* CBS525.76]) and the temperature-sensitive mutants Mc3 (*wcdc2*; ATCC38716) and Hf1 have been described in detail previously (Cooper et al., 1993; Roberts et al., 1978; Ye et al., 1999; Wang et al., 2000). Strains XL1-blue, DH5 α , JM109, and SOLRTM (Stratagene, La Jolla, CA) of *Escherichia coli* were used for the construction of genomic and cDNA libraries, subcloning and plasmid preparation.

Media and growth conditions

Propagation of the *W. dermatitidis* wild type 8656, Hf1 and Mc3 strains was normally either in the nutritionally rich medium YPD (2% peptone, 1% yeast extract, 2% dextrose) or the nutritionally poor semisynthetic medium MCD [Bacto Czapek Dox broth (Difco) plus 0.1% yeast extract], when required, agar (1.5% w/v) was added to produce solid media for strain maintenance, mutant selection, etc. For temperature induced morphological transitions, the log-phase yeast cells of the wild-type, Mc3, and Hf1 grown in YPD broth at 25°C were used to inoculate pre-warmed YPD broth at a density of 10⁶ cells/ml and grown for 24 h at 37°C, which is considered the restrictive temperature for the temperature-

Table 2. Strains^a used in this study.

Strains	Parent strains	Genotype	Reference or source
Wd8656		wild type	ATCC34100
Mc3	Wd8656	<i>wcdc2</i>	ATCC38716
Hfl	Wd8656	ts mutant	Wang et al 2000
<i>wdchs1Δ-LA</i>	Wd8656	<i>wdchs1::ble</i>	Unpublished
<i>wdchs2Δ-1</i>	Wd8656	<i>wdchs2::hph</i>	Wang et al 2001
<i>wdchs3Δ-1</i>	Wd8656	<i>wdchs3::hph</i>	ATCC MYA-886
<i>wdchs4Δ-1</i>	Wd8656	<i>wdchs4::hph</i>	Wang et al 1999
<i>wdchs5Δ11</i>	Wd8656	<i>wdchs5::hph</i>	This work
<i>wdchs5Δ236</i>	Wd8656	<i>wdchs5::hph</i>	This work
<i>wdchs5Δ316</i>	Wd8656	<i>wdchs5::hph</i>	This work
<i>wdchs5Δ11-1</i>	<i>wdchs5Δ11</i>	<i>wdchs5::hph-WdCHS5-sur</i>	This work
<i>wdchs5Δ236-1</i>	<i>wdchs5Δ236</i>	<i>wdchs5::hph-WdCHS5-sur</i>	This work
<i>wdchs5Δ316-1</i>	<i>wdchs5Δ316</i>	<i>wdchs5::hph-WdCHS5-sur</i>	This work
<i>wdchs1Δ5Δ11-1</i>	<i>wdchs5Δ11</i>	<i>wdchs1::hphwdchs5::sur</i>	This work
<i>wdchs2Δ5Δ11-1</i>	<i>wdchs5Δ11</i>	<i>wdchs2::hphwdchs5::sur</i>	This work
<i>wdchs3Δ5Δ11-1</i>	<i>wdchs5Δ11</i>	<i>wdchs3::hphwdchs5::sur</i>	This work
<i>wdchs4Δ5Δ11-1</i>	<i>wdchs5Δ11</i>	<i>wdchs4::surwdchs5::hph</i>	This work
<i>wdchs4Δ5Δ11-2</i>	<i>wdchs5Δ11</i>	<i>wdchs4::surwdchs5::hph</i>	This work
<i>wdchs4Δ5Δ11-3</i>	<i>wdchs5Δ11</i>	<i>wdchs4::surwdchs5::hph</i>	This work

^a All mutants used in this study were derived from the wild-type parental strain 8656.

sensitive mutants Mc3 and Hf1 to form isotropic and hyphal forms, respectively. For other morphological transition-inducing conditions, log-phase wild-type yeast cells grown at pH 6.5 in MCD broth were used to inoculate pH 2.5 MCD broth at a density of 10^6 cells/ml and then cultured with shaking at 25°C. By 72 h, most of the yeast inocula had converted to isotropically enlarged cells and multicellular forms. The log-phase wild-type yeast cells were also used to inoculate pH 6.5 CDN broth, which was prepared by adding the following components to 0.05 M sodium succinate buffer (pH 6.5): dextrose, 3%; NaNO₃, 0.3%; K₂HPO₄, 0.1%; MgSO₄ · 7H₂O, 0.05%; FeSO₄ · 7H₂O, 0.001%; NH₄Cl, 0.53%; filter-sterilized thiamine (3µg/liter) was added after autoclaving. The CDN medium contained different concentration of EGTA or was devoid of nitrogen: by 24 h, most yeast cells cultured in the presence of 5 or 30 mM EGTA were arrested in their cell cycles either as normal-sized yeast cells without buds or with tiny buds, and those cultured without nitrogen had initiated yeast-to-hypal transitions. Methods for transformation by electroporation of intact yeast cells were also described previously (Wang et al., 1999; Zheng et al., 1999). Drug selection plates for isolating *W. dermatitidis* transformants were made by adding agar (1.5% w/v) and hygromycin B (HmB; Sigma, St. Louis, Mo) to YPD at a final concentration of 50 µg/ml for selection to the resistance conferred by the hygromycin B phosphotransferase gene (*hph*) or the chlorimuron ethyl (provided by J. Sweigard, Dupont Co., Wilmington, Del) to SD at a final concentration of 20µg/ml for

detection of the resistance conferred by the sulfonyleurea resistance (*sur*) gene. Growth rates of the wild-type, and the *wdchs5Δ* and *wdchs4Δ5Δ* mutants of *W. dermatitidis* were determined by three different methods, and all started with 10^6 cells/ml initial inoculation in 50 ml YPD media. Subcultures grown at 25C° and 37C° were collected at each 4 h or 8 h and subjected to spectrophotometric, hemocytometric and plate viable counting measurements, respectively. Three independent measurements were performed and the average numbers were used for growth curve plotting.

Escherichia coli XL-1 Blue (Stratagene, La Jolla, CA), which was used for the subcloning and plasmid preparation, was grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml). *E. coli* cells used for cDNA library screening were grown in LB broth supplemented with 0.2% maltose and 10 mM MgSO₄. For ZIPII cDNA library screening, the following media were prepared: NZY broth (0.5% NaCl, 0.2% MgSO₄ · 7H₂O, 0.5% yeast extract, 1% NZ amine casein hydrolysate, pH 7.5); top agar NZY broth (0.7% (w/v) agarose added into NZY broth); and NZY plates (1.5% Difco agar was added to the NZY broth).

Chemical reagents, enzymes and molecular biology kits

Most inorganic and general organic chemicals were purchased from Sigma

Chemical Company (St. Louis, MO) and EM Science (Gibbstown, NJ). Sulfonyleurea was kindly provided by Dr. J. Sweigard (Dupont, Wilmington, DE). Hygromycin B was purchased from Sigma Chemical Company (St. Louis, MO). Restriction enzymes were purchased from Promega (Madison, WI), New England BioLabs (Beverly, MA), and Invitrogen (Carlsbad, CA). T4 DNA ligase was purchased from Promega (Madison, WI) or Invitrogen (Carlsbad, CA). DNA markers were from GIBCO BRL (Gaithersburg, MD). Taq polymerase, large fragment of DNA polymerase I (Klenow fragment), calf intestine phosphatase and RQ1 RNase-free DNase were purchased from Promega (Madison, WI). RNase and the Expand long template PCR system were purchased from Roche (Germany). The DECA prime II DNA labeling kit and MicroPoly(A)PureTM were purchased from Ambion (Austin, TX). Unincorporated nucleotides were removed by chromatography through Sephadex G-50 column (Boehringer Mannheim Biochemicals, Indianapolis, IN). The PERFECTHYBTMPLUS was purchased from Sigma (Saint Louis, MO). The SuperScript® plasmid system for cDNA synthesis and for plasmid cloning was purchased from Life Technologies Inc. (Gaithersburg, MD). A pGEM-T easy vector system (Promega, Madison, WI) was used for cloning PCR products. The QIAprep spin Miniplasmid kit (QIAGEN Inc. Chatsworth, CA) was used to isolate plasmid DNA and the QIAquick Gel Extraction kit (QIAGEN Inc. Chatsworth, CA) was used to purify PCR products or DNA fragments from agarose gels.

Radioisotopes, membranes, autoradiography and photography

α -³²P-dATP and α -³²P-dCTP (Ci/mM) were purchased from DuPont NEN Research Products (Boston, MA). For chitin synthase activity assays, ¹⁴C-UDP-N-acetylglucosamine (Amersham Life Science, Arlington Heights, IL; Specific activity: 14.87 mCi/ml) was used. NYTRAN nylon transfer membranes (Schleicher & Schuell, Keene, NH) were used for Southern and Northern blotting. Nytran super charge membranes from Schleicher & Schuell (Keene, NH) were used for plaque lifts and colony hybridizations. X-OMAT film (Kodak) was used for Southern and Northern blots. DNA was photographed with UV light using a Fotoprep I system transilluminator and camera (Fotodyne, New Berlin, WI) and with Polaroid Type 667 instant film (Cambridge, MA).

Miscellaneous equipment

Membrane proteins were separated from cell-wall-free extracts using a Beckman L5-75B ultracentrifuge (Beckman Inc., Irvine, CA). Concentrations of nucleic acids, proteins and cell suspensions were determined by using a Beckman Model 25 Spectrophotometer and radioactivity of labeled samples was evaluated in a Beckman LS 6800 liquid scintillation counter. PCR reactions were performed in Perkin Elmer Thermocyclers (Norwalk, CT). The hybridizations of Southern and Northern blots were performed in PersonalHyb hybridiser (Stratagene, La Jolla,

CA). Electroporation was done with a Bio-Rad Gene Pulser and 0.2 mm cuvettes (BioRad Lab, Hercules, CA).

Nucleic acid manipulations

Methods for the isolation of genomic DNA of *W. dermatitidis* were carried out either by the method previously described (Wang et al., 1999; Zheng et al., 1999) by spheroplasting with zymolyase-20T (ICB Biomedicals, Inc., Aurora, Ohio), followed by detergent lysis or by using a glass bead method adapted from Charles S. Huffman (Ausubel et al., 1989). Briefly, after the cells were collected and washed by centrifugation with dH₂O, breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0), glass beads (400-520 μm diameter, Thomas Scientific, Swedesboro, NJ) and phenol/chloroform were added. This mixture was then vortexed vigorously using Multi-Tube Vortexer (West Chester, PA) for 3 min, then TE (pH 8.0) was added and the mixture was vortexed briefly and then centrifuged. Genomic DNA was finally isolated by precipitation using 100% ethanol, washed with 75% ethanol, and treated with RNase (Roche, Germany) to eliminate any RNA coprecipitated with DNA. For RNA isolation, total RNA was prepared by extraction with hot acidic phenol (pH 4.7) (Ambion, Austin, TX). RNA samples were then treated with RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 1 h, followed by phenol/chloroform extraction, ethanol precipitation and washing. The DNase-

treated RNA pellets were finally dissolved in RNase-free water.

Southern blot analysis

For Southern blot analyses, fungal genomic DNA (~ 5 µg) was first digested with appropriate restriction enzymes, next separated by electrophoresis in an 0.8% agarose gel, after which the gel was denatured with 0.25 M HCl, neutralized with a solution of 0.5 M NaOH and 1.5 M NaCl and then transferred to a Nytran membrane by capillary blotting in 20 x SSC buffer (3 M NaCl and 0.3 M Sodium citrate). After the DNA was cross-linked to the Nytran membranes in a UVP CL-1000 ultraviolet crosslinker for 3 min, the DNA-bound membranes were first prehybridized in PERFECTHYB™PLUS hybridization buffer at 68°C for 30 min and then probes labeled with α -³²P-dATP or α -³²P-dCTP prepared by using the DECA prime II DNA labeling kit (Ambion, Austin, TX), were added and hybridized for 3 h. Following the hybridization, the membranes were washed first in low stringency wash buffer (2 x SSC and 0.1% SDS) for 5 min at room temperature, then they were washed in high stringency wash buffer (0.5% x SSC and 0.1% SDS) twice at the hybridization temperature (68°C). When necessary, ultra-high stringency wash buffer (0.1 x SSC and 0.1% SDS) was also used for further washing. The membranes were finally exposed to the X-ray film at -70°C for 8 to 24 h.

Northern blot analysis

Total RNA (~ 20 µg) extracted from yeast, hyphal or multicellular form cells was suspended in 20 µl loading buffer containing 1 µl 400 µg/ml ethidium bromide, 2.5 µl 10 x MOPS (0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 10 mM EDTA), 1.5 µl 37% formaldehyde and 12.5 µl formamide and incubated at 65°C for 10 min followed by quenching on ice. The samples were loaded into 1.2% agarose/formaldehyde gels and electrophoresed in 1 x MOPS buffer at 8 volts/cm for about 3 h. Then, the gels were transferred to the NYTRAN membranes by capillary blotting. Procedures for prehybridization, hybridization and washing were the same as for the Southern blotting analysis. The membranes were hybridized first using part of *WdCHS5* as the gene probe, then stripped using ultrastringent washing buffer and finally rehybridized with the *WdACT* gene probe.

Primers and PCR amplification

Primers for PCR were synthesized by IDT (Integrated DNA Technologies, Inc. Coralville, IA) or Invitrogen (Carlsbad, CA) and had the following sequences:

Chs51: 5'-TGGGGATCCCARGTNTAYGARTAYTA-3'

Chs53: 5'-ATAGAATTCTTIATCCAICKICKICKYTG-3'

pATG: 5'-ATGGCCACTCGAGGGAACGTC-3'

pATG2: 5'-ATGCTCGACACGAAAGGCGCTCGCG-3'

pIntron1: 5'-GCTTTGGAAGCAGTTGGGTCG-3'

pTGA: 5'-TCACAGTTGCCAGACAAAAT-3'

pIntron2: 5'-CTTCCCCTGGCTCTGTCTAT-3'

Primers designed to amplify 5' upstream sequences were as follows:

psmaI: 5'-TGTCCCGGGGTGAACTTCAATGGC-3' (*Sma*I site was underlined).

p1.2: 5'-TCAGGGCCCATCAGAAGGAGCGGTA-3' (*Apa*I site was underlined).

p1.0: 5'-TCAGGGCCCAACTTGACCTCGACTT-3' (*Apa*I site was underlined).

p0.88: 5'-TCAGGGCCCAGAGGTAGGTTGGAAT-3' (*Apa*I site was underlined).

p0.68: 5'-TCAGGGCCCTATTCTAGAGGGTCTA-3' (*Apa*I site was underlined).

p0.2: 5'-TCAGGGCCCTTGAT TACGACTTGA-3' (*Apa*I site was underlined).

Primers designed to introduce the point mutation into the putative REPCAR1 site were as follows:

prepr: 5'-TGGAGGTCGCTCCTTGGCCGA-3' (the mutated sites were underlined).

prepf: 5'-TCGGCCAAGGAGCGACCTCCA-3' (the mutated sites were underlined).

Primers used for RT-PCR detection of *WdPKS1* expression:

WDPKS-F: 5'- CCGCCACTTCAGTCACCATTAGCG-3'

WDPKS-R: 5'- CCAGGTGGAGTGGTCTTCCAGCG-3'

Primers designed for P-loop mutation:

Pmut1: 5'- GGAGTCTGGGTCGGGGGCAACGACAGTCAGGTCGA-3'

Ala

Pmut2: 5'- GCGACCTGACTGTCGTTGCCCCGACCCAGACTCC-3'

Ala

PCR amplification using Taq polymerase (Promega, Madison, WI) was carried out in a Perkin Elmer Thermocycler (Norwalk, CT). The PCR reaction mixture contained 2.5 mM MgCl₂, 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 10 mM dNTPs, 0.1 µg genomic DNA and 1 U Taq polymerase. The following program was used when the PCR amplification of DNA fragments were less than 1 kb: one cycle of 4 min at 94°C for denaturation; 30 to 35 cycles of amplification with 1 min at 94°C, 1 min at 50 or 55°C, and 1 min at 72°; followed by one extension cycle for 10 min at 72°C. PCR amplifications of the whole length *WdCHS5* gene and the chitin-synthase domain encoding region with the Expand long template PCR system (Roche, Germany) were performed in a GeneAmp PCR system 9799 (Applied Biosystems, Foster City, CA). The reaction contained 10 mM dNTPs, PCR buffer system 1 (10 x), 0.1 µg *W. dermatitidis* genomic DNA, corresponding primers and enzyme mix. The following program was used to amplify the PCR product, 1 cycle at 94°C for 2 min for denaturation, 10 cycles of 10 sec at 94°C (denaturation), 30 sec at 65°C (annealing) and 6 min at 68°C (elongation), followed by 10 cycles of 10 sec at 94°C, 30 sec at 65°C and 7 min at 68°C, and 10 sec at 94°C, 30 sec at 65°C and 8 min at 68°C.

RT-PCR

The RT-PCR was carried out using the Access RT-PCR system (Promega). The reaction of 25 µl contained 1 µl MgSO₄ (25 mM), 0.5 µl dNTP (10 mM), 2.5 µl buffer (10 x), 1 µl forward and reverse primer (25 µM), 0.5 µl avian myeloblastosis virus (AMV) reverse transcriptase, 0.5 µl *Tfl* DNA polymerase, 2 µl of 1 µg mRNA isolated from wild-type cells grown at 25 or 37°C, and finally, RNase-free water supplemented to 25 µl. The RT-PCR amplifications were run in a GeneAmp 2400 PCR System (Perkin-Elmer, Wellesley, MA) using the following cycling conditions: 48°C for 1 h, 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 68°C for 1 min, then an extra step of elongation at 68°C for 5 min. The RT-PCR products were examined using 1.2% ethidium bromide/agarose gel electrophoresis. The differential expression patterns of the *WdPKS1* gene were investigated by a semi-quantitative RT-PCR method previously described (Wang, 2002). Primers 18S for the 18S rRNA gene and the 18S PCR Competimer (Ambion) were used to provide an internal control to ensure that an equal amount of total RNA was present in each of the different samples. The RT-PCR amplification was performed as described above.

Screening of the cosmid library

For attempts to isolate the *WdCHS5*, a genomic cosmid library of *W. dermatitidis* constructed as described previously (Feng, et al., 2001) was screened. For the

screening, the cosmid library was first spread on LB plates containing 25 µg/ml chloramphenicol and incubated at 37°C overnight. Nylon membranes were then placed for 2 min over colonies that had developed on the agar, and then lifted and transferred to 1.5 M NaCl, 0.5 M NaOH solution for 2 min, 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) solution for 5 min and 0.2 M Tris-HCl (pH 7.5), 2 x SSC solution for 30 sec. After the membranes were cross-linked in a UVP CL-1000 ultraviolet crosslinker for 3 min, followed by rinsing with 3 x SSC, 0.1% SDS solution, Southern blot analysis was performed using the PCR product generated by primer Chs51 and Chs53 as a probe.

Construction and screening of the partial genomic library

The partial genomic library for *WdCHS5* isolation was constructed using genomic DNA of the wild-type *W. dermatitidis* completely digested with *EcoRI*. Following electrophoresis, the resulting 2.5 to 3.5-kb fragments were excised from the gel and ligated into the pBS-KS(+) vector, which was cut by *EcoRI* and dephosphorylated with CIAP (calf intestine alkaline phosphatase, Promega, Madison, WI). The ligation product was then transformed into XL-1 Blue competent cells and the resulting subgenomic library of about 6,000 independent clones was screened by colony hybridization using the PCR product generated by primer Chs51 and Chs53 as a probe. The screening method was the same as that described for screening the cosmid library.

Screening of the cDNA library

A ZAPII cDNA library of *W. dermatitidis* was constructed in our laboratory as described previously (Wang et al., 2000). For cDNA library screening, 1 μ l of the appropriately tittered ZAPII cDNA library (phage) was mixed with 600 μ l of SURE cells (OD₆₀₀ 0.6), incubated at 37°C for 10 min, and then mixed with 6.5 ml warm top agar for 1 min. Each sample was next poured on a NZY agar plate. After incubation at 37°C for 8 to 10 h, approximately 10³ to 10⁴ plaques were formed on each plate. To prepare a plaque replica membrane, the plates were chilled at 4°C for at least 2 h before making nylon membrane lifts from the top agar. The remaining steps were the same as described for the cosmid library screening.

Site-specific mutagenesis

Site-specific mutation of the lysine (Lys) residue to alanine (Ala) in the P-loop was performed using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, two primers Pmut1 and Pmut2, which had the mutated nucleotides, were synthesized (Invitrogen, Carlsbad, CA). The reaction contained 5 μ l 10 x reaction buffer, 2 μ l (50 ng) of dsDNA template, 2.5 μ l (125 ng) of each primer, 1 μ l dNTP mix and 1 μ l *PfuTurbo* DNA polymerase (2.5 U/ μ l) in a total volume of 50 μ l. The cycling parameters were as following: 95°C for 30 sec, followed by 16 cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 10 min.

After the PCR, the reaction mixture was placed on ice for 2 min, and 1 μ l of the *DpnI* restriction enzyme (10 U/ μ l) was added to the reaction, which was then incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The *DpnI* treated sample (3 μ l) was transformed into XL1-blue supercompetent cells, after which transformants were selected, and next plasmids were isolated and subject to sequencing to confirm the site-specific mutation.

Sequencing and computer analysis

The nucleotide sequence of the *WdCHS5* gene was assigned GenBank accession number AF469116. Sequencing of the *WdCHS5* gene was done by the Institute of Cellular and Molecular Biology of the University of Texas at Austin. The deduced amino acid sequence of *WdCHS5* and the sequence comparisons were determined by using the BLAST software system from NCBI. The promoter sequence analysis was performed using MacInspect software and the TRANSFAC 4.0 program (BCM Search Launcher). The sequence alignment and cluster phylum analysis were done using the ClustalW software from European Bioinformatics Institute.

Cell-wall-free extracts and membrane protein preparation

For chitin synthase activity assays, log-phase cultures (30 ml) were collected by centrifugation and washed with cold water and TM buffer (50 mM Tris-HCl, 2.5

mM MgCl₂, pH 7.0). The harvested cells were resuspended in 1.5 ml TM buffer, and then an equal volume of glass beads (0.45 – 0.55 mm) (Thomas Scientific, Swedesboro, NJ) was added. This mixture was subsequently vortexed vigorously using Multi-Tube Vortexer (West Chester, PA) for 30 sec six times. Between each agitation, the tubes were cooled on ice. The final cell slurry from each tube was then recovered by washing with 1 ml TM buffer for six times. The pooled washings were centrifuged at 3,500 x g for 5 min, and the supernatant (cell wall-free extract) was collected. The wall-free extracts were finally ultracentrifuged at 60,000 x g for 45 min and homogenized in TM buffer (1 ml) containing 33% glycerol for immediate use or stored at -70°C.

Transformations of *W. dermatitidis* and *E. coli*

For transformation of *W. dermatitidis*, yeast cells were prepared from a mid-log phase culture grown at 25°C, which was washed once with cold ddH₂O at 4°C, and then washed with cold 10% glycerol. The final competent cells were resuspended in 10% glycerol to approximately 4 x 10⁸ cells/ml. Prior to transformation, plasmids were usually linearized and repurified to remove salts or DNA fragments were purified from gels using the QIAquick gel extraction kit (QIAGEN Inc, Chatsworth, CA). Competent cells (0.2 ml) were mixed with DNA at a ratio of about 1 µg DNA per 1 x 10⁸ cells. Electroporation of the cell suspensions was carried out with a Gene Pulser[®] electroporation system with 0.2

mm cuvettes (Bio-Rad, Richmond, CA) at a setting of 1.45 kV field strength, 25 μ F capacitance, and 200 Ω resistance. After a 2-4 h regeneration period in 0.8 ml YPD or SD broth, the cell suspensions were spread on YPD agar plates containing 50 μ g/ml hygromycin B or SD agar plates containing 30 μ g/ml of chlorimuron ethyl and incubated at 25°C for about four days.

For general cloning, *E. coli* competent cells were made as follows: 1 ml of an overnight culture was inoculated into 50 ml fresh LB broth and allowed to grow for additional 3-4 h until the OD₆₀₀ reached 0.6; cells next were chilled on ice for at least 15 min and then harvested by centrifugation at 4°C. Subsequently, these cells were washed with 25 ml cold TFBI buffer (30 mM KoAC, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, and 15% glycerol, pH 5.8, Filter sterilize), and allowed to stand on ice for at least 15 min before they were collected again by centrifugation at 4°C. Finally, the cells were resuspended in 1 ml filter sterilized TFBII buffer (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl and 15% glycerol, pH 6.5). For each transformation, 100 μ l of competent cells were mixed with plasmid DNA, incubated on ice for 45 min. This was followed by a 90 sec heat shock at 42°C and a 3 min chill on ice. Transformed cells then were incubated with 1 ml LB at 37°C for 1 h with shaking before plating on LB agar containing corresponding antibiotics. For construction of the partial genomic library, the harvested cells (OD₆₀₀ = 0.6) were washed three times with ice-cold water

followed by one wash with 10% glycerol. Cells were finally resuspended in 10% glycerol and aliquoted to 100 μ l for each transformation. Electroporation was performed at 12.5 kV/cm field strength, 200 Ω resistance and 25 mF capacitance, corresponding to a time range of 4 – 4.5 ms.

Chitin content assays

Chitin contents were measured by a modification of the procedure described by Yabe (Yabe et al., 1996). Yeast cells were harvested from 20-ml cultures at required times, suspended in 4 M HCl (1 ml) and boiled for 4 h. After the hydrolysates were diluted with water (19 ml), the amount of hexosamine in a diluent (1 ml) was determined by the Elson-Morgan method (Boas et al., 1953), using N-acetyl-D-glucosamine (Sigma, St. Louis, MO) as a standard. Cell dry weight for calculation of chitin contents per milligram of cells was determined by collecting cells from 20 ml cultures on 0.45 μ m membrane filters (Type HA, Millipore Corp., Bedford, MA), which were subsequently dried at 65°C to a constant weight.

Chitin synthase activity assays

Cell membrane proteins were prepared as described above (see Cell-wall-free extracts and membrane protein preparation) and the activities of the chitin synthases were determined by the method described by Orlean (Orlean et al.,

1987). Concentrations of membrane proteins were measured using the Commaissie Protein Assay Reagent (PIERCE, Rocdkford, IL). Each chitin synthase assay was carried out in 40 μ l reaction mixture consisting of 30 μ g of membrane protein, 3 μ l of 0.5 M Tris-HCl (pH 7.3), 3 μ l of 40 mM MgAc, 2 μ l of 0.8 M N-acetylglucosamine (Sigma, St. Louis, MI), 10 mM UDP-N-acetylglucosaminae (Sigma), and 5 μ l of 10 mM UDP-N-acetyl-D-[U-14C] glucosamine (Amersham, Arlington Heights, IL, specific activity: 271 mCi/mmol). For trypsin-activated chitin synthase activity assays, 2 μ l of 1 mg/ml trypsin from bovine pancreas (type III; Sigma) was added to the membrane preparations that were subsequently incubated at 30°C for 15 min. To terminate the trypsin activation, 2 μ l of 1.5 mg/ml soybean trypsin inhibitor (Sigma) was added. The mixture was then incubated at 30°C for 30 min and reactions were stopped by adding 1 ml of 10% TCA. After the chitin precipitate was collected by filtration on 25 mm glass fiber filters (type A/E) (Gelman Science, Ann Arbor, MI), the filters were washed with 5 ml of 95% ethanol and their radioactivities were counted using a Beckman LS6800 liquid scintillation counter (Beckman Inc., Irvine, CA). The chitin synthase activities and chitin contents of each sample were measured at least three times. Differences among groups were evaluated for statistical significance by the parametric one-way analysis of variance using the Newman-Keuls method for paired data. The analysis was performed by using PRISM version 2.0 software

(GraphPad Software, Inc., San Diego, CA). Probability values of <0.05 were considered significant.

β -galactosidase activity assays

For β -galactosidase assay, specific activities of β -galactosidase were determined according to the method used with *S. cerevisiae* (Ausubel et al., 1989). Mid-log phase yeast cells (5 ml) grown at 25°C or 37°C were collected and resuspended in an equal volume of Z buffer (60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄·H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 50 mM β -mercaptoethanol, pH 7.0). In each assay, 100 μ l cells mixed with 900 μ l Z buffer were permeabilized by adding 1 drop of 0.1% SDS and 2 drops of chloroform using a Pasteur pipette. After the mixture was allowed to equilibrate at 30°C for 15 min, 0.2 ml of 4 mg/ml of the chromogenic substrate *o*-nitrophenyl- β -D-galactoside (ONPG) was added and allowed to react. The reaction was then stopped by adding 0.5 ml of 1 M Na₂CO₃ after incubating at 30°C for 60 min. The OD₄₂₀ and OD₅₅₀ were determined and the following equation was used to calculate the β -galactosidase activity: $U = 1000 \times [(OD_{420}) - (1.75 \times OD_{550})] / 6 \times OD_{600}$.

Alkaline phosphatase activity assays

The relative activities of alkaline phosphatase were determined by the method modified from Nombela (Nombela et al., 1998). The initial wild-type *W.*

dermatitidis and *wdchs5Δ11* cultures were grown at 25°C for 2 days, and then approximately 5×10^7 cells were added to 50 ml YPD media and YPD plus 1 M sorbitol. After 48 h and 72 h incubation, cells were harvested by low-speed centrifugation. The resulting supernatants were collected and used for alkaline phosphatase activity assays. In each assay, 0.5 ml of 20 mM Na₂PNPP (*p*-nitrophenylphosphate disodium, Sigma, St. Louis, MO) (dissolved in 0.1 M glycine/NaOH buffer at pH 9.8) was added to 0.5 ml of sample. The reaction mixtures were then incubated at 37°C for 30 min. The OD₄₂₀ and OD₆₀₀ of the samples after incubation were determined and the following equation was used to calculate the relative alkaline phosphatase activity (arbitrary units) = $1000 \times (\Delta \text{OD}_{420}) / \Delta \text{OD}_{600} \times 30 \times (\text{sample volume}/\text{total volume})$.

Photomicroscopy

Photomicroscopy of *W. dermatitidis* wild-type and the mutant cells was performed by using an Olympus BX-60 microscope. For fluorescence photomicroscopy, cells were first fixed in 5% formaldehyde for 3 h and washed twice with 75% ethanol at room temperature. Staining of cell walls was carried out using 0.5 mg/ml Calcofluor (Sigma) at room temperature for 2 to 5 min, followed by thorough washing with PBS saline buffer. Nuclear staining with DAPI (4',6'-diamidino-2-phenylindole) (Accurate Chemical, Westbury, N.Y.) was carried out by staining the fixed cells with 1 μg/ml DAPI at room temperature for

2 to 5 min, which were then washed 2-3 times by centrifugation with PBS saline buffer.

Electron microscopy

The procedure for preparing specimens for scanning electron microscopy was as following: wild type and mutant cells were harvested from YPD cultures at specific time points and fixed overnight in sodium cacodylate-buffered 2.5% glutaraldehyde at 4°C. The fixed cells were washed twice with the same buffer and dehydrated in an ethanol series (0, 25, 50, 75, 100, 100% ethanol) and followed by ethanol-acetone (10, 25, 40, 50, 100% acetone) series. The dehydrated specimens were then submerged in acetone for 30 min, critical-point dried (Tousimis Samdri-790), and sputter coated (Ladd model 30800 LaddResearch83HollyCourtWilliston, VT 05495) with gold for 60 sec at 2.5 kV and 20 mA. Specimens were examined in a Philips 515 scanning electron microscope.

Virulence tests

For determination of virulence, test strains (Wd8656, *wdchs5Δ11*, *wdchs5Δ236*, *wdchs5Δ316*, *wdchs5Δ11-1*, *wdchs5Δ236-1*, *wdchs5Δ316-1*) of *W. dermatitidis* were sent to the laboratory of Dr. Jeffrey M. Becker (University of Tennessee) for evaluations. For these evaluations, test strains were cultured in YPD (5 ml) overnight at 30°C with shaking, and then an aliquot of the overnight culture was

used to inoculate 50 ml YPD cultures, which were then again grown overnight to mid-log phase. The resulting yeast cells were harvested, washed three times with sterile water, counted on a hemacytometer, and diluted to a final concentration of 9×10^7 cells/ml. Virulence of the strains was then determined in an immunocompetent (normal) acute infection mouse model system. Male ICR mice (22 to 25 g; Harlan Sprague-Dawley) were housed five per cage; food and water were supplied ad libitum, according to National Institute of Health guidelines for the ethical treatment of animals. Mice (10 per yeast strain) were inoculated via the lateral tail vein with 100 μ l of cell suspension (9×10^7 cells/ml), such that each mouse received a final dose of 9×10^6 cells. To determine the number of viable yeast cells injected into each mouse, an aliquot of the suspension used for injection was diluted and plated in top agar (0.1% Noble agar) onto YPD plates. The plates were incubated at 30°C for 48 to 72 h, and percent viability was determined. Mice were checked three times a day for survival or signs of infection. Visible signs of infection were torticollis, ataxia, or lethargy. Infected mice were considered moribund when they were unable to access food and water. Moribund mice were humanely sacrificed by cervical dislocation under anesthesia. Survival fractions in virulence tests were calculated by the Kaplan-Meier method, and survival curves were tested for significant difference ($P < 0.01$) by the Mantel-Haenszel test using GraphPad Prism software (version 3.0 for windows). A tissue burden analysis, also carried out at the University of

Tennessee, involved injecting *wdchs5Δ11* into mice and sacrificing them at day 1, 3, 5, and 10 post-infection to determine the fungal organ burden of the brain, kidney, liver, and spleen.

Plasmid construction

The *WdCHS5* integrative gene disruption plasmids pHB0320 and pHB0510 (see Fig. 15A, B) were constructed by cloning 1.8-kb *SacI* and 1.2-kb *SalI* fragments from the 5'- and 3'-end of *WdCHS5* into corresponding sites of vectors pCB1636 and pCB1004, which contained the hygromycin B phosphotransferase (*hph*) gene from *E. coli* and the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter from *A. nidulans* (Wang et al., 2001), respectively. After the resulting vectors were linearized with *BclI* or with *BstEII* to target integrations into genomic sites of *WdCHS5*, respectively, they were used to transform *W. dermatitidis* competent cells by electroporation as described previously (Wang et al., 1999; Ye et al., 2000). In order to construct the one-step replacement disruption vector pHB0280 (see Fig. 16A), the 1-kb *EcoRI* and *BamHI* fragment of the 3'-end and 0.5-kb *KpnI* and *PstI* (fill-in to blunt end using Klenow enzyme) fragment of the 5'-end of *WdCHS5* were cloned into the *EcoRI* and *BamHI*, *KpnI* and *ApaI* (fill-in to blunt end) sites of vector pCB1636, which positioned the *hph* gene to the middle of the construct. The 4.5-kb fragment obtained by digestion of pHB0280 with *KpnI* and *BamHI* was then used for the transformation. Another one-step

replacement disruption vector pHB0901 (see Fig. 28A) was constructed by cloning 0.5-kb *Xho*I and *Kpn*I fragment of the 5'-end and 1-kb *Eco*RI and *Bam*HI fragment of the 3'-end of *WdCHS5* into the corresponding sites of vector pCB1551, which contained a 3-kb sulfonyleurea-resistance allele (*sur*) of the *M. grisea* (Sweigard et al., 1997). This construct was mainly used to derive double chitin synthase gene disruption mutants. Another disruption vector pHY was constructed by Heather Yarbrough (Heather et al., unpublished data) and used for disruption of *WdCHS4* in the *wdchs5Δ11* background. The complementation vector pHB2080, which contained the full-length *WdCHS5* gene was constructed by cloning a 5-kb *Bam*HI fragment from the cosmid library clone and 3-kb *Eco*RI fragment from a subgenomic library clone into the pBS-KS(+) vector, after which the combined fragment was released with *Bss*HIII digestion, filled in with Klenow enzyme and blunt ligation into the *Sma*I site of pCB1551 (provided by J. Sweigard). Putative randomly reconstituted strains were selected by prescreening for temperature-insensitive revertants, which were subsequently confirmed to contain *WdCHS5* by Southern analysis. All the plasmids for analysis of the 5' upstream sequence of *WdCHS5* were derived from pYEX303-gal (Ye et al., 1999). Briefly, the different length 5' upstream sequences before the ATG start codon were amplified with primer psmall (which has a *Sma*I restriction enzyme site) from one end and primers p1.2, p1.0, p0.88, p0.68, p0.2 (which have *Apa*I restriction enzyme sites) from the other end, respectively. All the PCR products

were subject to *Sma*I digestion first and then *Apa*I (partially digested). The corresponding 1.2-kb, 1.0-kb, 0.88-kb, 0.68-kb, 0.2-kb and 0.45-kb fragments obtained from complete digestion of the 1.2-kb PCR product with *Sma*I and *Apa*I were used to replace the *gla*A promoter in plasmid pYEX303-gal, to generate pHB8040, pHB9000, pHB 9010, pHB9020, pHB9030, and pHB8050, respectively. Prior to transformation, the plasmids were linearized with *Fse*I. The *WdPKS1* fragment incorporated into these plasmids facilitated the identification of strains with site-specific integrations among hygromycin B (HmB)-resistant transformants as indicated by the production of white colonies (Ye et al, 1999). The plasmids used to introduce two point mutations in the REPCAR1 site of the promoter region were constructed using recombinant-PCR amplification. Two pairs of primers p1.0-prepr and psmal-prepf were used to amplify 240 bp and 800 bp of the promoter region, respectively. These two PCR products had a 20-bp overlap and were used as templates for the second round PCR using primer set p1.0 and psmal. The final 1-kb product was cloned into pGEM-T easy vector (Promega). After sequencing confirmed the expected mutation, which also showed there is no other mutation introduced during PCR amplification, the 1-kb fragment released with *Sma*I and *Apa*I (partial digestion) was cloned into pYEX303-gal. The final plasmid pH9101 was used to transform wild-type *W. dermatitidis* following the procedures described above.

To construct the plasmid used for expression of the chitin synthase domain, the 4.5-kb chitin synthase domain-encoding region was first amplified using primers pATG2 and pTGA, and then subsequently cloned into pHB7010, which had the 1.2-kb *WdCHS5* promoter and a 2-kb *TglaA* terminator. The resulting plasmid, pHB3510, was transformed into the *wdchs5Δ11* mutant. In order to mutate the conserved Lys residue in the P-loop, two primers Pmut1 and Pmut2 were designed to mutate the Lys into Ala. The 2-kb *BstEII-AgeI* fragment from pHB2080 was first cloned into the corresponding sites of pCDNA3.1 (kindly provided by Dr. X. Ye) vector and site-specific mutagenesis was performed following the instructions of the QuikChange Site-Directed Mutagenesis Kit. After sequencing confirmed the expected mutations and that there was no other mutation introduced during PCR amplification, then the 2-kb *BstEII-AgeI* fragment was cloned back into corresponding sites of pHB2080. Plasmids derived from this study are all listed in Table 3.

Table 3. Plasmids derived in this study.

plasmid	Description	Purpose
pHB0041	a cosmid clone containing 5-kb <i>WdCHS5</i>	cloning of
pHB0051	5-kb <i>Bam</i> HI fragment from pHB0041 in pBluescript KS (+)	<i>WdCHS5</i>
pHB0061	4-kb <i>Sal</i> I fragment from pHB0041 in pBluescript KS (+)	as above
pHB0091	1.2-kb <i>Bam</i> HI- <i>Eco</i> RI fragment from pHB0051 in pBluescript KS (+)	as above
pHB0101	2-kb <i>Pst</i> I fragment from pHB0051 in pBluescript KS (+)	sequencing of
pHB0111	1.2-kb <i>Sal</i> I fragment from pHB0051 in pBluescript KS (+)	<i>WdCHS5</i>
pHB0121	1.7-kb <i>Xho</i> I fragment from pHB0051 in pBluescript KS (+)	as above
pHB0131	2.5-kb <i>Pst</i> I fragment from pHB0051 in pBluescript KS (+)	as above
pHB0230	1.8-kb <i>Sac</i> I fragment from pHB0051 in pBluescript KS (+)	as above
pHB0240	2.3-kb <i>Sac</i> I fragment from pHB0051 in pBluescript KS (+)	as above
pHB0250	2-kb <i>Xho</i> I fragment from pHB0051 in pBluescript KS (+)	as above
pHB0270	1-kb <i>Bam</i> HI- <i>Eco</i> RI from pHB0051 in pCB1636	disrupt <i>WdCHS5</i>
pHB0280	0.5-kb <i>Kpn</i> I- <i>Pst</i> I from pHB0051 in pHB0270	disrupt <i>WdCHS5</i>
pHB0290	2.5-kb <i>Pst</i> I from pHB0051 in pCB1532	as above
pHB0300	3-kb <i>Pst</i> I from pHB0051 in pCB1532	as above
pHB0320	1.8-kb <i>Sac</i> I from pHB0051 in pCB1636	as above
pHB0510	1.2-kb <i>Sal</i> I from pHB0051 in pCB1004	as above
pHB0801	0.5-kb <i>Xho</i> I- <i>Kpn</i> I from pHB0051 in pCB1551	as above
pHB0901	1-kb <i>Bam</i> HI- <i>Eco</i> RI from pHB0051 in pHB0801	as above
pHB1070	500-bp RT-PCR product from pATG-pIntron1 in pGEM-T easy vector	determine intron 1
pHB1210	500-bp RT-PCR product from pTGA-pIntron2 in pGEM-T easy vector	determine intron 2
pHB1174	3-kb <i>Eco</i> RI fragment of <i>WdCHS5</i> in pBluescript KS(+)	cloning of
pHB1180	0.6-kb <i>Bam</i> HI- <i>Kpn</i> I fragment from pHB1174 in pBluescript KS (+)	<i>WdCHS5</i>
pHB1190	1.2-kb <i>Bam</i> HI- <i>Kpn</i> I fragment from pHB1174 in pBluescript KS (+)	as above
pHB2060	3-kb <i>Eco</i> RI- <i>Pvu</i> II from pHB1174 in pHB0051	as above

pHB2070	8-kb <i>ApaI</i> from pHB2060 in pCB1551	as above
pHB2080	8-kb <i>BstHII</i> from pHB2060 in pCB1551	as above
pHB2100	2-kb cDNA clone on the <i>EcoRI-XhoI</i> site of pBulescript SK (+)	cloning of cDNA of <i>WdCHS5</i>
pHB8040	1.2-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	promoter
pHB9000	1.0-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	analysis
pHB9010	0.88-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	as above
pHB9020	0.68-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	as above
pHB9030	0.2-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	as above
pHB8050	0.45-kb <i>SmaI-ApaI</i> promoter in pYEX303-gal	as above
pHB9101	1-kb <i>SmaI-ApaI</i> promoter (with two point mutation) in pYEX303-gal	as above as above
pHB2200	1.2-kb <i>EcoRI WdCHS5</i> promoter in pCB1532	complementation
pHB7010	2-kb <i>XbaI-EcoRI A. niger gla4</i> gene terminator in pHB2200	study
pHB3500	4.5-kb PCR from pATG2-pTGA in pGEM-T easy vector	as above
pHB3510	4.5-kb <i>NotI</i> fragment from pHB3500 in pHB7010	as above
pHB3610	2-kb <i>BstEII-AgeI</i> fragment from pHB2080 into pCDNA3.1	P-loop mutation
pHB3620	105K to A mutation in pHB3610	study
pHB3630	2-kb <i>BstEII-AgeI</i> fragment from pHB3620 into pHB2080	as above

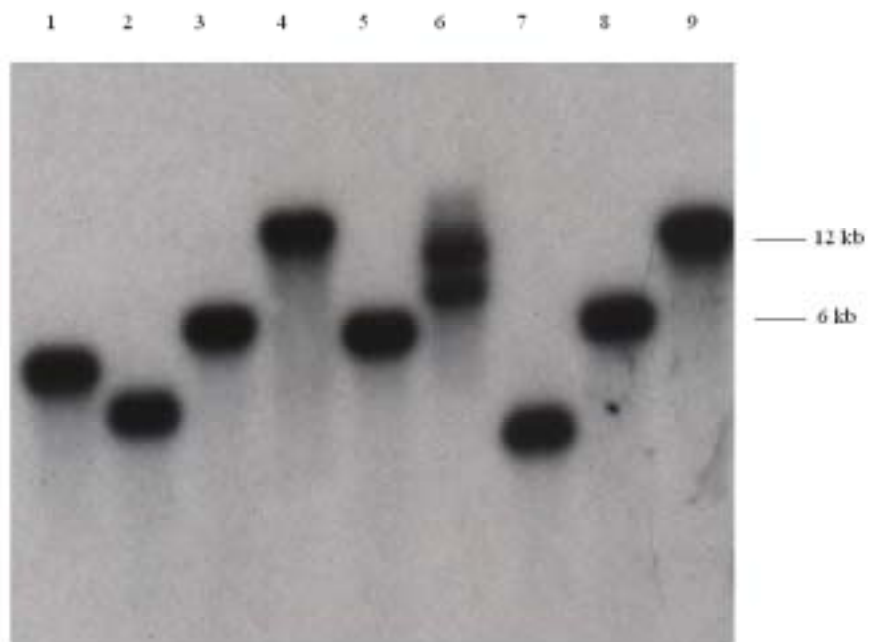
RESULTS

Cloning of the *WdCHS5* gene

A 362-bp PCR product was amplified by using the degenerate primers Chs51 and Chs53. The PCR product was ligated into pGEM-T easy vector (Promega), and subsequent sequence analysis of the PCR product showed that the derived amino acid sequence had high homology to equivalent portions of AnCsmAp (83% identity) of *A. nidulans*, BgChs2p (82% identity) of *B. graminis* and MgCsm1p (85% identity) of *Magnaporthe grisea*, all of which are considered to be class V chitin synthases. Southern analysis (Fig. 5) with genomic DNA of *W. dermatitidis* digested with different restriction enzymes and probed with the 362- bp *WdCHS5* PCR fragment indicated that this gene was present as a single copy, and had a hybridization pattern different from that obtained with any of the other four previously cloned *WdCHS* genes (data not shown). Therefore, this gene was named *WdCHS5*.

To isolate the whole gene, a cosmid library was first screened by colony hybridization using the *WdCHS5* PCR-product as a probe. Unfortunately, only a 5-kb 5'-end portion of the gene, which had the 1.2-kb promoter region of *WdCHS5*, was present in this particular cosmid clone. Further screening of this cosmid library gave two other positive clones that were identical to the previously

Figure 5. Southern analysis of genomic DNA of *W. dermatitidis* probed with the *WdCHS5* PCR product. Total DNA was digested with *Xho*I (lane 1), *Xba*I (lane 2), *Sal*I (lane 3), *Pst*I (lane 4), *Kpn*I (lane 5), *Hind*III (lane 6), *Eco*RI (lane 7), *Bgl*II (lane 8), and *Bam*HI (lane 9).



identified one, indicating that the large size of the gene prevented direct cloning or that the cosmid library did not evenly cover the whole genome of *W. dermatitidis*. Based on the restriction map and Southern analysis result, a 3-kb *EcoRI* subgenomic library was constructed and screened to get the remaining portion of the gene. Two positive clones were identified from this subgenomic library and direct sequencing confirmed that they contained the remaining 3'-end portion of *WdCHS5*.

Characterization of the *WdCHS5* gene sequence

The restriction map of *WdCHS5* and schematic structure of the derived WdChs5p are shown in Figure 6. The nucleotide sequence of the cloned *WdCHS5* contained a single open reading frame of 5655 bp interrupted by two introns of 53 and 57 bp at its 5'- and 3'-ends, respectively. Each intron showed a consensus GT/AG splicing site, and the invariant CTG in the middle. Subsequently, the existence of the two introns was confirmed by RT-PCR (Fig. 7) and by sequence analysis of the RT-PCR products (data not shown). The *WdCHS5* gene encoded a putative protein (WdChs5p) of 1885 amino acids with a calculated mass of 208.9 kD, and a pI of 7.76 (Fig. 8). Two putative unconventional TATA boxes (positions -605 and -648) and a putative CCAAT box in reverse orientation (ATTGG; position -191) were found at the upstream of the putative ATG start codon. The cloning of the 3'-end of the cDNA of the *WdCHS5* gene by cDNA library screening and

Figure 6. Schematic representations of WdChs5p (A) and *WdCHS5* (B). A. Schematic structure of WdChs5p. The putative P-loop (99-106: GESGSGKT), switch I and Switch II (148-153: TASKAG; 407-411: DFPGF) are represented by different red boxes. B. Restriction map of *WdCHS5*. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; Xa, *Xba*I; X, *Xho*I. The hatched box over the open reading frame corresponds to a 362-bp probing position in the gene. The grey box indicates the region has no significant homology to others. Two introns, 1 and 2, are drawn as blue boxes.

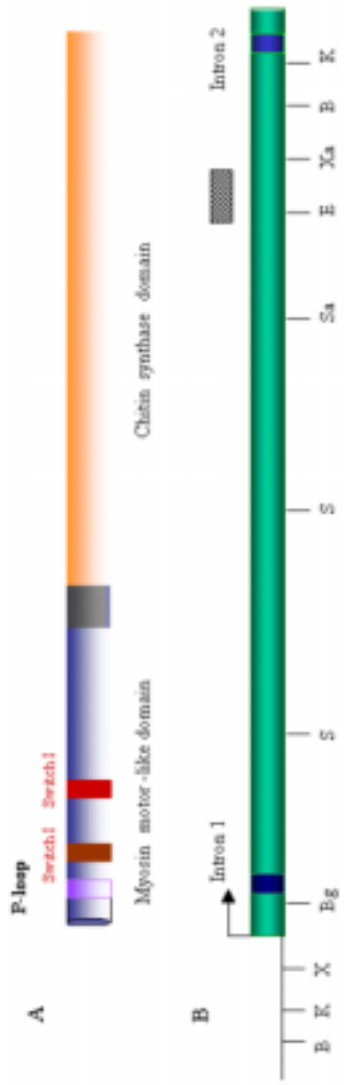


Figure 7. RT-PCR confirmation of the introns in *WdCHS5*. Primers pATG and pIntron1 were used for amplification of the intron 1 (53-bp) from cDNA (lane 4) and genomic DNA (lane 3). Primers pTGA and pIntron2 were used for amplification of the intron 2 (57-bp) from cDNA (lane 2) and genomic DNA (lane 1). The introns were also confirmed by direct sequencing of the RT-PCR products (data not shown).

1

2

3

4

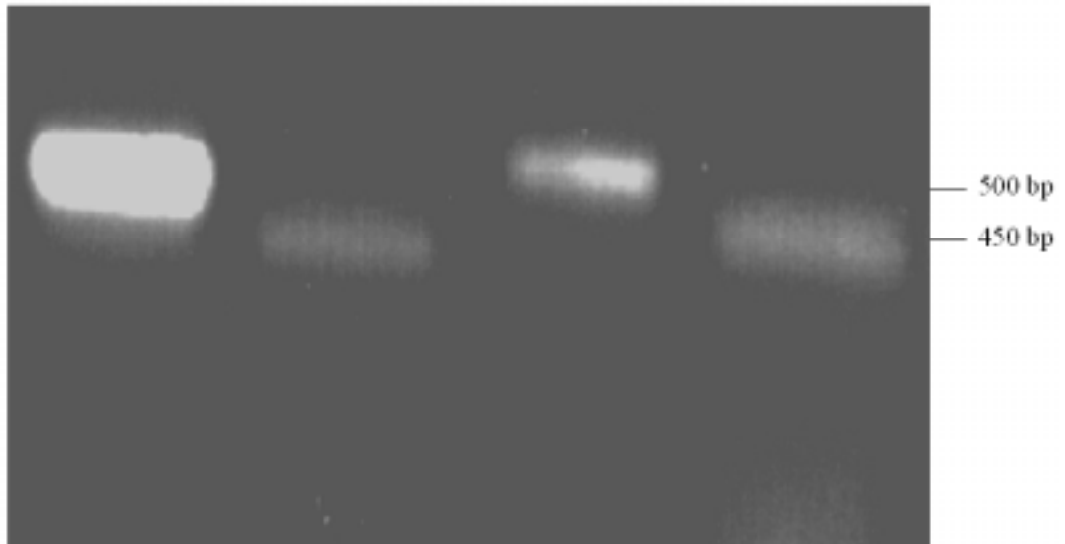


Figure 8. Nucleotide and derived amino acid sequences of *WdCHS5*. The putative intron sequences are represented by lower case letters. The positions of the primers Chs51 and Chs53 for PCR amplification are underlined. The putative P-loop is indicated in bold amino acid letters. The putative unconventional TATA boxes are boxed. A putative CCAAT box in reverse orientation (ATTGG) is indicated by bold italic letters.

-1254 ATCAGAAGGAGCGGTAGGGTCTTTTCTGCTACTTTGGACGGTTTTTCGGAAGTTCATGGCCATGACGCCTTTGAGAATGTTTTCTCCCTCG
-1164 GATTTGTCAGTCTCAAGGCCTCCGTTCTCCTCGGGCTTTATCACTTACAAGCCTAATTGCTCGAAAAGAAGTAGCCGAGTACAAGGGAT
-1074 TGTTCCGGTCTGCTTGCATGTCCGCAACTTGACCTCGACTTCAAACCTCCGGCTCTTGCAACTCCGATCCCAAAAAGGTGCGGTGGCCG
-984 CATCGACCTGAGAGACCGACTCGGACGCAAGGGCTCTGGCTTTGACTCTTTGGGCTTCTTCTCGTCCACCTTTGTAACCCGACAGGGGT
-894 TTTCTGTCCGCGCAAGCGCCTCATCTGCCACGATGTGCCAGAGGTAGGTTGGAATGACTCGGCCTGCGCCTCATTGTGTCCGGTT
-804 TGGAGATACGGTCCGCAAGGAGCGCCTCCAGAAGTAGGAGCGGACATTTGCCCTGCGACGCTGGGGTGGATTGCGCGCAAGCGGGA
-714 TCAACAAGGGAGGATCGAAAAGACTTGGTGGGGATCTATAAGTTAGTGTACCTATTCTAGAGGGTCTATAAGTATTGTTTAAAGTTTTTC
-624 ATAGAAGTTGAGCTTCTGATAAGTAATCAAGTTCTTGTAGGACGGTTTTTAGGGAATAAACCCGCAATTATTTTTGACACCAAGC
-534 CCTAGGCCGACTGGGAGGGCCACGCTTGGGGAGCAAACACAGGGCAGTCCCCGGCAGCAGCGGTACCCGTAACAGCGTTACAATA
-444 CGAGTGTGCAAGCTCACGCTCTTGTCCATACGTGCAAGCGCCCAAAACACCTAAGGAGCCTGCCCCGACCAAAAACACTCCTTGA
-354 ATATTGTCCAAAGATCAGACTCCTGGCACATCATTTCGCTCGCTCGCTCGATATTGTTGTTGTTGTTTTCACATTGCTGCAACT
-264 CTTGATTACGACTTGACGATTCTTGTTCGCTTCTTTCGCTTGTATGATTAAATCCAGCGCCTTTGCATTGCAATTGCTCCTCGCCGT
-174 TCGCTCTGCAGCCTGCGGGCTGTCTCGCACTCGTTGACTTCAATTCAAGCACTACTTCATCTTAAACCGAACTCGTCCGATGAGGAGAG
-84 GCGCCAGGTTGTAGAGCATCGGAGCCCGCAAGATCTCTGGTTGCAACACCATTGACGCGCTGGCCATTGAAGTTCCACCATGGCC
M A
7 ACTCGAGGGAACGTCCTGCACATATGCAAGCGTCTTGCCTGCTCTTCCGGCTCATTTGCAATCAGATACCCACATAACCGCCCATCTT
T R G N V P A H M Q A S L P A L P A H L Q S D T H I T A H L
97 GCGAGCCGATTCCATGTTTCTTGGCGACAGCGAGTTATCGTGCAGGACTCATATGCCTCAACACCTTCACTCTTCGACGCGAGGA
A S R P H V S L P T A R L S S Q G L I C L N T P T S S T R G
187 CCGAATGGAGACAAGGAAGGAGTCCATGGGGAGCCGAGGACCTGGCACGCCGGCCTGGCCAGGCTGGGTAATAGAGCGGAAGAC
P N G D K E G S A M G E P E D L A R R A W A R L G N R A E D
277 CAAGCATTTGGTTTCTTgtaaatgctgctgaaagtgcggattaaatgggtcaacactgactgtgctacagCGGGGAGTCTGGGTCCGGGA
Q A F G F F G E S G S G K
367 AGACGACAGTCAAGTCCGACCTTCTTTCTCGGTCTCTGTTTCTGTCACACCTTATCATCAAACTATCCCTCGCTGCCTTCGCT
T T V R S H L L P S V L S P S S T P L S S K L S L A A F V F
457 TTGACACCTGACCACTACCAAGACAACACGACCCAACTGCTCCAAAGCAGGTTTATTTTACGAGCTACAGTACGATGTTTCGTCAA
D T L T T T K T T T T Q T A S K A G L F Y E L Q Y D V S S I
547 TAACCTTACCTTATCGAAGCAAGTTGTTGGACCATCGCTTGAAGAAGCGGATTTTACATGTCGCCAAGCGGTGAACGAGGTTTC
T L T L I E G K L L D H R L E R S R I S H V P T G E R S P H
637 ATGTATTACTACCTTCTCGCCGTAACAAGCGCCGAGAAAGTCCCATCTCGCCCTGGATGGCCATGTCAACATCACAAACCCCGGAA
V L Y Y L L A G T S A A E K S H L G L D G H V N I T T A G T
727 CCGCCTAAGCAGGTCGCTTCTGTATCGCACAAAGATGGAGATATTTTTTGGCCACCCACGCAATGAAAGTTGGCATCAATGACG
G L S R S A S V S H K R W R Y F L G H P T Q M K V G I N D A
817 CAGAAGCTTCCAGCATTTCAAAAACGCGCTGCGGAAATGGAGTTCCCGAGGACGAAATCGCCGAAATCGCCAGGTAACGCAACAA
E G F Q H F K N A L R K L E F P P R T E I A E I C Q V L A A I
907 TTCTTATATCGCCCAATGGAGTTCCGTCACAGGACAGGCAACTCTTACAGCAGCCGAGGAAAGCGGGTATTCTCATGAGGGCGGT
L H I G Q L E F G T G Q A T L T A A E E S G G Y G E
997 AGACCGTACCCGTTCAAGAATAGGACACACTTGTATAGTGGCTGCTTCTTGGGCTTGGTGTTCAGGACCTGGAGGAGAGCCTGA
T V T V V K N R D T L A I V A A P L G L G V Q D L E E S L R
1087 GGTACAAGACGAGGACCATCCACCGAGCGTGTCCCGTATGCTCGACAGGAAAGCGGCTCGGAAAACGCTGATGAGCTGACGACGCA
Y K T R T I H R E R V T V M L D T K G A R E N A D E L A T T
1177 CTCTGTATTGCTTGGTTACCTACATATCGAGAGTATCAACCAGAGGTTTGCAGCGCCGAGGACTCGGTGGCTAACACTATCTCCA
L Y S L L V T Y I I E S I N Q R V C A A E D S V A N T I S I
1267 TTGTGGACTTTCCTGGCTTGTGACCACTCGTCCACCGGCTGTCCTAGACCAACTGCTGAACAATGCAGCAATGAGTCGCTCTACA
V D F P G F A D H S S T G S V L D Q L L N N A A N E S L Y N
1357 ACAGCTTGCACAGCATTTTCGAGAAGACTGCTGAGATGCTGAGAGCGAAGAGGTCAGTGTCCGGCAACCAAGCTATTTGATAACT
T C L H S I F E K T A E M L E S E E V S V P A T S Y F D N S
1447 CAGATGCTTGCCTGGCTTTTGAAGCACGGTAACGGATTACTGGCTATTCTCGACGATCAAAACCGGCGGTCGACAGATGTACAGT
D A V R G L L K H G N G L L A I L D D Q T R R G R T D V Q P
1537 TCCTGGAAAAGCTTGGGAAAGAGATCGAAAACAAGAACAGGCCATCACCGTGGGCGAGCCACATCAACAATGCCGGGTAGCAATTTG
L E S L R K R F E N K N K A I T V G S A T S T M P G S N F A
1627 CAACCACCAACTTGGCCCTTCACTCCCGTGGCCACTATGCTGGAGAAGTGCATTACCCAGTGCATAGTCTGGTGGAAAGAGAAATGGGG
T T N L A A S F T V R H Y A G E V D Y P V H S L V E E N G D
1717 ACGTTGTTTCTGGCAGCTTGTGAAATATGATCAAAGCCACCAAAAGCGATTTCGTCGCAACTGTTTGGTCAAGAAGCCTTGAACACCG
V V S G D L M N M I K A T K S D F V A N L F G Q E A L N T V
1807 TCAGCCACCCAGCCAAAAGACAGCATCGTCCAGGCCAGGTCAGCTCGAAAACCACTCGGGATGCCAGCGTCTCGAGGAAAGAACATG
S H P A E K T A I V Q A V S S K P L R M P S V S R K K H D
1897 ATCAGTTGCGAGAATGGCAAGCCGAAGGCGAGTCTCTCCAGCCCTCAGGAAGAAGAACCCCTGCCAGGTACAGAAGAACGCAAG
Q L R R M A S R R A D R S P A P Q E E E P L P G T E E A K V
1987 TCAGGCGGACAAAGCCACTGCGACCGGATTGACTCAGGGTGTGACGACAGTTCTTATCGGCTCTGGCAACATTACCAAAATCGCTCA
R R T K P T A T G L T Q G A A A Q F L S A L D N I T K S L T
2077 CCGCACCAATGTGAACAATTACTTTTGTCTCAAGCCCAACGACAGCGGATAGCCAAACAGTTTCGACCAAGTGTGTACGTC
A P N V N N Y F V F C L K P N D R R I A N Q F D S K C V R Q

2167 AACAAAGTTCAGATGTTCCGGCATTGCCGAAATCAGCCAAAGACTACGCACTGCGGATTTTACTATTTTCCTGCCTTTTGGAGAATTTCTCG
Q V Q M F G I A E I S Q R L R T A D F T I F L P F G E F L G
2257 GGCTGACAAACGCTGACGGCGGTTCGTCGCGGAGCGATCGTAAAAGGCGCAGCTTGTCTGACAGTAAACACTGGCCCCAAACGAGGC
L T N A D G G V V G S D R E K A Q L V L T V N T G P Q T R H
2347 ACATCGAAATACTGSTGTCTTCTGAGTGAACGTTGCTGGGCCAGCATTGCGCTTACAGGGTCTCAAGCAGCAGCTTACTTCGGAGGCG
I G N T G V F L S E R C W A S I A L T G S Q A A A Y F G G D
2437 ATATCGGCTCGCCGCTAGACCTGATACCCCGGTGATAATCCGTTCACTGATTCCAAGGCCGACTAGTTGGTTCAGCTGATGGAACTC
I G S P S R P D T P G H N P P S D S K A R L V G S A D G T P
2527 CCGGGTCGTTTTATGGCGATGAAGCCAAAGGCGGTGGCTATTTGGCAGTCGAGAACTGACGCCAAAGTCAGACGCTGGAGCATCCGCTT
G S F Y G D E A K G G G Y F G S R E L D A K S D A G A S A F
2617 TTCATTGAGGGGATATGTTCCGCAACCTCGAAACCAAGGAGGAACCTCGCCGAGAAGGGCAACAAGAAGAAGGTTGAAAGAAGTCGATGTTG
H S G D M F R N L E T K E E L A E K G N K K K V E E V D V V
2707 TTCCGGTTTCGCTAGCCGGAAGCGATGGCTTGTCTACTCTACTTCTGACCTGGTACTTGCCTGACTTCGCCATCAAATGGATTGGTG
P V S S S R K R W L A I V Y P L T W Y L P D F A I K W I G G
2797 GTATGAAACGAAAGGATGTCGCAACGCTGGAGAGAAAAGTTCCGCCATCAATCTCCGATCTGGCTCAGCTGTGCTGTGGTGGTCTTCT
M K R K D V R T A W R E K F A I N L L I W L S C G L V V P F
2887 TCATCATCGTGTCCCTGAGTTGATTTGTCCAAACAGAATGTATACTCGGCCGCTGAGCTGTGAGCCATGATGAAAAGGCAACACA
I I V F P E L I C P K Q N V Y S A A E L S A H D G K G K H S
2977 GCGCCTATGTGGCTATTCGAGGACAGTCTTCGACTTGGGGGCTTTCATGCCGAATCATTATCCAAAAATCATCCCGCAGAGCTCGCTCA
A Y V A I R G Q V F D L G A F M P N H Y P K I I P Q A S L K
3067 AGAAGTACGCGGGCGTCGATGCCACCGGGTGTCCAGTTCAAGTCTCGGCCCTCTGCCAAGGTAAGACGGACGGGTTGATCCTACCG
K Y A G V D A T G L F P V Q V S A L C Q G K D G R V D P T V
3157 TCCAGCTTGACTATACGCAACCAACATCAGTGGCACTCGGCCGCTCATCAGCTCAACAGACGCCAACCGGAAATACCATGATTTCCGCT
Q L D Y T A T N I S G T A A V I S S T D A N R K Y H D F R Y
3247 ACTTACCAATGATTTCCGACCAGATTGGTCTACGAGCAAATGATCATGCTCAAGGCCAATTATCGCAAGGGCAGCATCGGTTATACCC
P T N D S R P D W F Y E Q M I M L K A N Y R K G S I G Y T P
3337 CTCAGTATGTGAAGACGCTGGCTAAAAAGTCGAAGTCGATAGCCATTCTGAATGATAGGTCCTACGACTTACCACCTTACAATGAAGGTG
Q Y V K T L A K K S K S I A I L N D R V Y D F T T Y N E G G
3427 GCGCGAGGTACGGGCTCCTCCTGGTGAAGAAAGTGCATCAGGGGTCGATACTGATTTATGGACTCTTTGGTGGTCGACCTCTTACCC
R S V R A P P G E E V P S G V D T D F M D S L V V D L F T Q
3517 AGCGAGCAGGCCACGCTCACCAAGTATTGGAATCGCTACCCCTTACCCGGGCTTGGAGGTGCAAGTGCAGCTTTGTCTCGCAACT
R A G H D V T K Y W N A L P L D P G L R S R M Q L N L
3607 TGTTCTTCGTTGGTGTAAACGATACTCGGAATTCACCACGTTGCTTTTCGCCCCGATACATTCTGCTCGCAGTATCGATTCTGCTGTGCA
F F V G V T D T R N S P R C L F A R Y I L L A V S I L L C S
3697 GTGTCAATGGCTTCAAATTTCTTCCCGCTTCAAATTTGGAGGCAAAAATGTGCTGAGAACCCTGGCAAGTTTGTCACTTCCAGGTTG
V I G P K P F A A L Q P G G K N V P E N L D K F V I C Q V P
3787 CTGCTACACAGAAGATGAGGACTCACTTCCCGCTGCCATTGACTCTGCGGCTCGCATGCGATGCAAAACGCAAAATGCTCATTG
T T E D E D S L R R A I D S A A R M R Y D D K R K L L I V
3877 TGGTGTGCGATGGTATGATTATTGGCCAGGGCAATGACCACCGACCCCTCGTATCGTGTGGATATTCTGGGGGTTCTGAAACCGTGG
V C D G M I I G Q G N D R P T P R I V L D I L G V S E T V D
3967 ATCCAGAACCCTCAGCTTCGAATCGCTTGGTGAAGGTATGAAACAGCACACATGGGCAAGTCTATTGCGTCTCTATGAAGTGCAGG
P E P L S F E S L G E G M K Q H N M G K V Y S G L Y E V Q G
4057 GGCATATTGTCCTTTCATGGTGGTTGTCAAGTTCGGTAAACCCGCAAGTATCTCGGCCGGGCAACCGAGGCAAGCGGATTTCTCAGA
H I V P F M V V V K V G K P S E V S R P G N R G K R D S Q M
4147 TGGTATCATGCGCTTCTCCACAGATTCACTATAACCTGCCAATGAGTCCCTTGGAACTTGAGATGCACCACCAGATTGCAACATCA
V I M R P L H R V H Y N L P M S P L E L E M H H Q I R N I I
4237 TCGGGTCAATCCGACTTTCTACGAGTTTATGCTTCAAATCGATGCCGACCGGTCGTCGCCCCGATTTCGGCCACGCGCATGGTTTCGG
G V N P T F Y E F M L Q I D A D T V V A P D S A T R M V S A
4327 CATTCTGCGTATACCCGCTGATGGTGTGTTGGCGAAAACGTCACCTGTCATGCAATGCAAGTCTGCTTCAATCAGGATGATGAGGTTT
F L R D T R L I G V C G E T S L S N A K S S F I T M M Q V Y
4417 ACGAGTACTACATTTTCGCAACCTCACCAAGGCGTTTGGTTCGTTGTTGCGCTCGGTGACATGTGTGCCAGGTTGTTTACCATTGATC
E Y Y I S H N L T K A F E S L P G S V T C V P G C F T M Y R
4507 GCATTCGGGCGGCCGAGACAGGCAAGCCGCTGTTGCTCAGCAAGGAGATCATCCAAGACTATTTCGAAATCCGAGTTGACACCCTGCACA
I R A A E T G K P L F V S K E I I Q D Y S E I R V D T L H M
4597 TGAAGAATCTGCTCCATCTCGGTGAAGACAGGTATCTGACCCAGCTGCTATTGAAGTACCATTCCAAGTACAAGCAAGTACATTTTCC
K N L L H L G E D R Y L T T C T L L K Y H S K Y K T K Y I F H
4687 ACGTCAACGATGGACCATTTGCTCCGACAGCTGGAAGTCTTTCATGTCGCAACCGCGGATGGATCAACATGACCTACACAACCTGA
A H A W T I A P D S W K V F M S Q R R R W I N S T V H N L I
4777 TTGAAGTATCCCGCTACAACAGCTATGTGGTTCGCTGTTTCAGCATGCGGTTGCTGCTGTTCTAGATTTGTTGCTACGGTGTG
E L I P L Q Q L C G F C F S M R F V V F L D L S T V V A
4867 CCCCAGTACAGTGCATACATTTGATTTGCTCCTCGCAACAGAAATCCGATGTGGTACCATTGACCGCATTCATCCTTCTGG
P V T V A Y I A Y L I V L L A T E S D V V P L T A F I L L G

4957 GCGCAATCTATGGTCTACAAGCCATCATCTTCATTCTTCGTCGGAATGGGAAATGATTGGCTGGATGATTGTCTACATCCTGGCGATGC
A I Y G L Q A I I F I L R R K W E M I G W M I V Y I L A M P
5047 CGGTGTCTCCCTGGGACTTCCTTTGTACGCCCTTCTGGCATATGGATGACTTCAGTTGGGGCAACACCCGTCCTGGTCAGGGGCGAACATG
V F S L G L P L Y A F W H M D D F S W G N T R L V R G E H G
5137 GGAAGCAGATCTTGCTGTGATGAAGGCAAGTTCGGCCCTGACTCCATTCCAAGAAGAAATGGGAGGAGTATCAAGCGGAACTTTGGG
K Q I L L S D E G K F G P D S I P K K K W E E Y Q A E L W D
5227 ACGCGCAAACCTCAACGAGACGATGCGCCGCTCCGAACTGTGAGTTACAGTTATGGCACAAAGTCTTATCTTCCCACTGGCTCTGTCTATG
A Q T Q R D D A R S E L S G Y S Y G T K S Y L P T G S V Y G
5317 GTGGAGGGTACAATGACACACAGCACCTGATGATGGCGCCCTCGCGGTCCGCATCGCAGCTCGACATGCACCCTACGCCAATGTACGGCG
G G Y N D T Q H L M M A P S R S A S Q L D M H P T P M Y G G
5407 GTGGAGCGGGCACAATCAATCCCGGATGTCACTGGCCCATCGGAGATGCTGGGCAGCCAGAGTAACCTGATGATGCCAAGCGGAAAGGT
G G G H N Q S R M S L A P S E M L G S Q S N L M M P S G R S
5497 CCGTCGCGGATATGGAGATGTCCGACCTGACCGGATTACCTACGACGACATGCTCCTCAACGAGATCCGAGACATTTTGGAGCGGCCG
V A D M E M S D L T G L P T D D M L L N E I R D I L R T A D
5587 ACCTAATGACAGTACGAAGAAAGGTATCAAGCAAGAATTGGAACGGCGGTTCAATGTCAACCTGGATATGAAGCGGGCGTATATTGGAA
L M T V T K K G I K Q E L E R R F N V N L D M K R A Y I G S
5677 GTGgtatgtttgaaatccccgataccctgtacccttttactctactgactcttttactacagCCACCGAAGCCATTTTGTCTGGGCAACTGT
A T E A I L S G Q L -
5767 GACGGATCTGTTTGATTAGAATGCTCGCCATGTCTATTTAGCAAGTCATGATCTTGTATATACTGCTTAATATAGGACCGGAGTCTGTGG
5867 GGTGACCAAAAAGACGGCTCAGTATGTGGCGGCATGCAGGTGGTAAATTTCTATCTATACATATCATGAAAGTGTCTGCCATGGTCCGA
5957 TGCAACATTTAGCTGCGATGAAGATGAACATTTGTTTTCAACCGCGCGGATGCGAGACCCAGTACCCTGTATCGCTATGGGCGGTATGC
6047 CGTTTACAAGTTTGTTCATGAGGGTAGAACTGCTCCGACAGGTTTCTCATCGTA

subsequent analysis revealed that the poly(A) began at a position 225-bp downstream of the stop codon. Hydropathicity and transmembrane domain analysis indicated that WdChs5p is a six-transmembrane protein with the six-transmembrane part located at the carboxyl terminus (Fig. 9).

Comparison of the deduced protein sequence of WdChs5p with those of other class V chitin synthases having myosin motor-like domains (data not shown) indicated that it has 67%, 67%, 66%, 66%, 65%, 64%, and 41% identity (based on the pairwise blast results) to ChsZ of *A. oryzae* (Chigira et al., 2002), CsmA of *A. nidulans* (Fujiwara et al., 1997), Chs2 of *B. graminis* (Zhang et al., 2000), ChsA of *G. graminicola* (Amnuaykanjanasin et al., 2002), ChsV of *Fusarium oxysporum* (Madrid et al., unpublished data), Csm1 of *M. grisea* (Park et al., 1999), PbrChs5p of *Paracoccidioides brasiliensis* (Nino-Vega et al., 2000), respectively (Table 4). Recently, analysis of *N. crassa* genome revealed that it has a hypothetical protein that has high identity to this kind of chitin synthase (Galagan, 2003). Interestingly, in *A. fumigatus*, another similar chitin synthase (ChsE) with very close identity to this kind of chitin synthase was identified, but its N-terminal was 350 amino acids shorter than the others (Aufauvre-Brown et al. 1997) (Table 4). The affiliation of WdChs5p with the class V family of chitin synthases was further supported by the identification in its N-terminal domain (first 800 residues) of a myosin motor-like region, which to date is only associated

Figure 9. Hydropathic analysis and transmembrane prediction of the deduced amino acid sequence of *WdCHS5*.

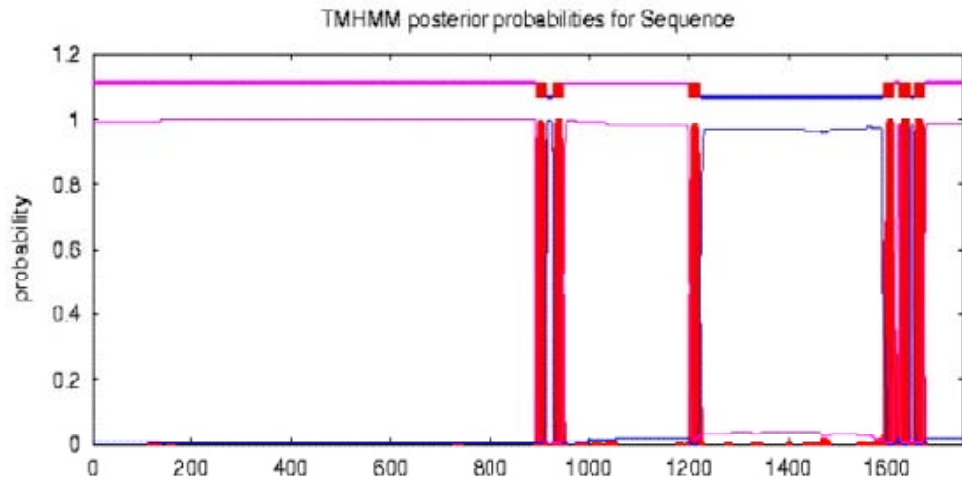
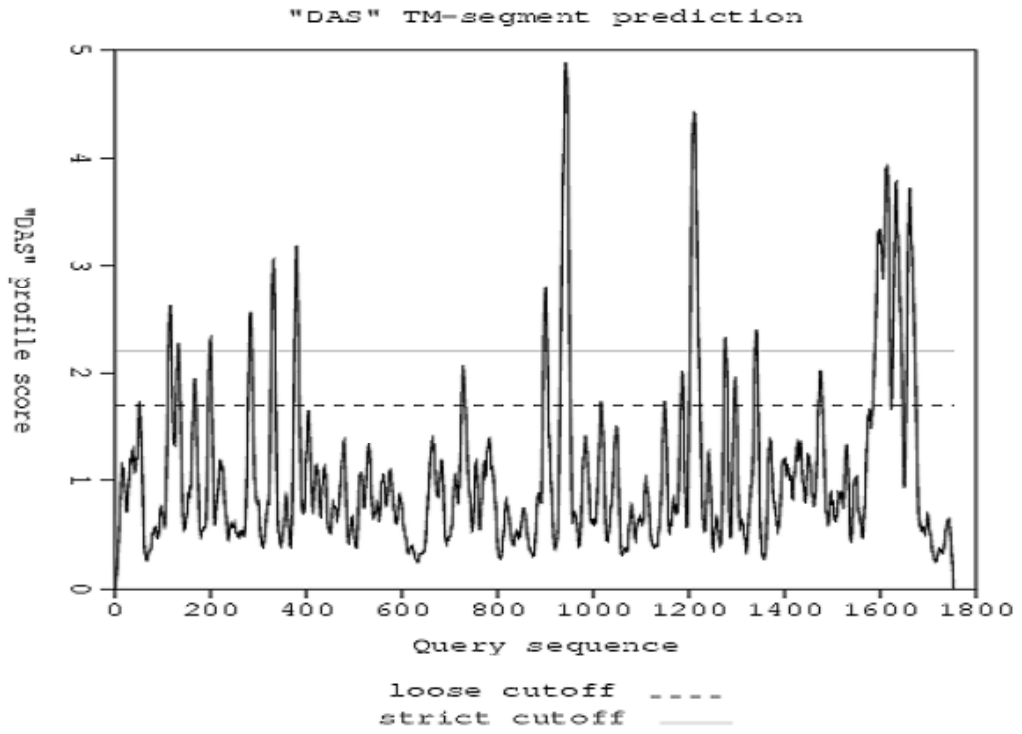


Table 4. Percent identity of the deduced amino acids of other class V chitin synthases to WdChs5p

Chitin synthases	Percentage identity to WdChs5p
AoChsZ	67%
AnCsmA	67%
AfChsE	67%
BgChs2p	66%
GgChsA	66%
FoChsV	65%
MgCsm1	64%
PbrChs5p	41%
Nchp	41%

with members of this isozyme class (Munro et al., 2001). Further support for a class V affiliation for WdChs5p was provided by identification of a characteristic ATP/GTP-binding site motif (P-loop: GESGSGKT), located in the myosin motor-like domain from amino acid residues 99 to 106 and the presence of switch I (TASKAG) and switch II (DFPGF) motifs at residues 148 to 153 and 407 to 411, respectively. However, none of the class V chitin synthases with a myosin motor-like domain has an actin-binding site or the IQ motif, which is a helical sequence in the neck of traditional myosins that binds light chains and calmodulin (Cheney, et al., 1992; Sellers, 2000). WdChs5p also has the “QRRRW” (1548-1552) signature motif for all chitin synthases in the C-terminal chitin synthase domain. Phylogram analysis and neighbor-joining tree analysis of class V chitin synthases from ten different fungi (Fig. 10) revealed that eight of them were closely related, whereas, PbrChs5p of *P. brasiliensis* and the hypothetical protein of *N. crassa* were not closely related to the others. The significance of this subdivision is currently unknown.

Stress conditions resulted in increased levels of cellular *WdCHS5* mRNA

The regulation of *WdCHS5* expression was investigated by Northern analysis using a *WdCHS5*-specific PCR probe amplified with primers chs5F and chs5R. Total RNA was from cells grown under different stress conditions, which included elevated temperature (37°C) for the wild type and the two temperature-

Figure 10. ClustalW phylogram analysis of amino acid sequences of the class V chitin synthases from various fungi. AoChsZ: ChsZ of *Aspergillus oryzae*; AfChsE: ChsE of *Aspergillus fumigatus*; AnCsmA: CsmA of *Aspergillus nidulans*; WdChs5p: WdChs5p of *Wangiella dermatitidis*; GgChsA: ChsA of *Glomerella graminicola*; FoChsV: ChsV of *Fusarium oxysporum*; MgCsm1: Csm1 of *Magnaporthe grisea*; BgChs2p: Chs2p of *Blumeria graminis*; PbrChs5p: Chs5p of *Paracoccidioides brasiliensis*; Nchp: hypothetical protein of *Neurospora crassa*.

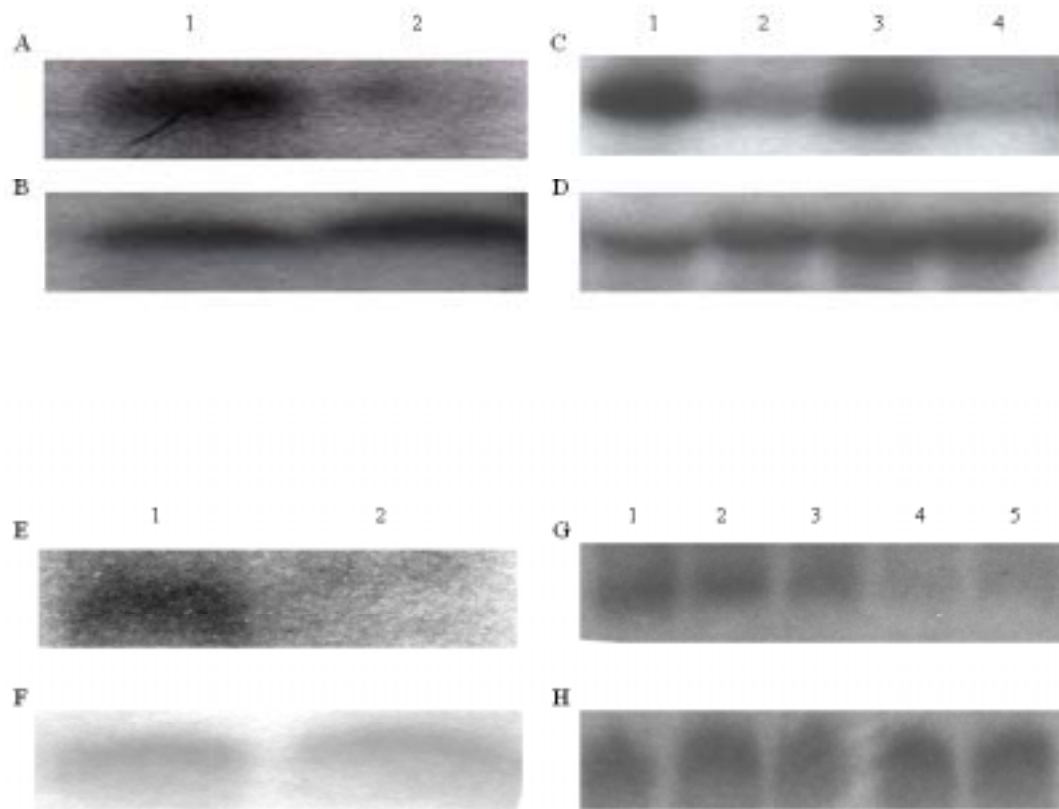


sensitive morphological mutants Mc3 and Hf1, which form multicellular forms and hyphal forms at elevated temperature, respectively, and also conditions that initiate the development of the isotropic sclerotic morphology (acidic condition or Ca²⁺ limitation induced by increasing EGTA concentrations) or hyphae in the wild type (by nitrogen limitation). In response to all these conditions, *WdCHS5* transcripts were detected at higher levels in the stressed cells than were found in control cells (Fig. 11). The dramatic differential expression of *WdCHS5* associated with cells shifted from 25°C to 37°C was confirmed by the semi-quantitative RT-PCR (Wang et al., 2002). Based on these observations, we speculated that *WdCHS5* is a stress response gene. Because a similar differential expression pattern was previously found for *WdCHS3* (Wang et al., 2000), it was not surprising to find two identical regulatory elements, REPCAR1 and STUAP, and several similar regulatory elements, such as STRE, ABAA and HAP in upstream regions of both *WdCHS3* (data not shown) and *WdCHS5* (see Fig. 12C), suggesting that these two genes may have similar mechanisms for regulating their transcriptions.

Study of the *WdCHS5* promoter

Because *WdCHS5* was found to be differentially expressed and there are numerous potential regulatory elements present in its promoter region, it was of

Figure 11. Northern analysis of *WdCHS5* expression. A. Total RNA prepared from wild-type *W. dermatitidis* (grown at 37°C, lane 1 and 25 °C, lane 2); B. The same membrane from A was then probed with *WdACT* as a control to show the approximately equal amount of RNA loading; C. Total RNA prepared from Mc3 (grown at 37 °C, lane 1 and 25 °C, lane 2) and Hf1 (grown at 37 °C, lane 3 and 25 °C, lane 4); D. The same membrane from B probed with *WdACT* as a control. E. Total RNA prepared from the wild-type strain grown in MCD [pH2.5] (lane 1), or in MCD [pH6.5] (lane 2). G. Total RNA prepared from wild type grown in SM [pH6.5], without nitrogen (lane 1), or with addition of 20 mM EGTA (lane 2), 5 mM EGTA (lane 3), 0.5 mM EGTA (lane 4), or 0 mM EGTA (lane5). F and H are the actin loading controls.



interest and importance to study the promoter region of *WdCHS5* to see if any actual *cis*-acting elements could be identified. In order to do this, a series of 5'-deletion fragments in the *WdCHS5* URS (upstream regulatory sequence) was fused in frame with the *LacZ* gene (Fig 12B) and then used to replace the original *glaA* promoter of plasmid pYEX303-lacZ, which also contains a *WdPKS1* targeting sequence and the *glaA* terminator of *A. niger*. The use of these constructs ensured that all alleles were integrated into the same nonessential *WdPKS1* genomic locus, which is required for melanin biosynthesis (Ye et al., 1999; Wang et al., 2000). After the constructs were linearized in the *WdPKS1* sequence with *FseI* and transformed into yeast cells, transformants were selected for resistance to HmB and mutants with a site-specific integration were identified by production of albino colonies. The albino transformants derived from each construct were first streaked on X-gal-containing YPD plates and incubated at 25°C and 37°C for 2 days. Different intensities of blue color were formed from the colonies grown on plates incubated at 37°C, indicating the *LacZ* gene was mainly expressed at this temperature and different length promoters had different abilities to drive *LacZ* expression (data not shown). Subsequently, the *LacZ* expression in cells grown at 25°C and 37°C was quantitatively assessed by β -galactosidase activity assay and by use of o-nitrophenyl- β -galactosidase as a chromogenic substrate. The resulting data (Fig. 12A) led to two conclusions. First, all samples from 37°C cells, except that from cells transformed with the plasmid control

without any *WdCHS5* URS, had significantly higher amounts (4 to 6-fold) of β -galactosidase activity than those of the corresponding samples from 25°C, which was consistent with the Northern analyses (Fig. 11A). Second, the most dramatic changes of β -galactosidase activities were observed among samples from cells with constructs having truncations between position -880 and -450 (Fig. 11A: compare -880 with -680 and -680 with -450), which is the region that includes most of the potential *cis*-acting elements identified by sequence analysis using MacInspector software and the TRANSFAC 4.0 program (Fig. 12C). Truncations between bp -880 to bp -680 significantly ($p < 0.05$) increased β -galactosidase activity, about 2.5 fold, which indicated that at least one negative regulator binding sequence exists at this region. The data also showed that other regulatory binding site(s) or transcription start machinery binding sites were localized between bp -680 to bp -450, because truncation in this region caused a 2-fold β -galactosidase activity decrease. Therefore, besides the temperature regulation of its expression, which led to its high expression at 37°C, *WdCHS5* also appeared to be under a more complicated type of transcriptional regulation.

Interestingly, a REPCAR1 site was found at position -789, which is a well-studied negative transcriptional regulatory element in *S. cerevisiae* (Luche et al., 1990). In *S. cerevisiae*, the product of the arginase (*CARI*) gene responds to both induction and nitrogen catabolite repression (Middlehoven 1964; Middlehoven

1970). In the presence of inducer (arginine or its analog, homoarginine), arginase is produced at high levels. In the absence of inducer, only low, basal levels of arginase production occur. It was found that the regulation is mediated through a *cis*-dominantly regulated upstream repression sequence (URS, or REPCAR1). Deletion of this element resulted in the high-level expression of the *CAR1* gene without an inducer. A two-point mutation (CG-to-GA) was made in the potential REPCAR1 site of the *WdCHS5* promoter region and recombinant PCR was performed to introduce this mutation. The resulting 1-kb promoter region with the site-specific mutation was site-specifically integrated into the *WdPKS1* locus, as was done for all the other constructs. The resulting albino transformants were then subject to β -galactosidase activity assay. The strain with the REPCAR1 site mutated showed significantly ($p < 0.05$) higher amount of β -galactosidase activity than that of the corresponding strain, which has the original 1-kb promoter region (Fig. 12A, compare -1000 with -1000*). However, the β -galactosidase activity of this construct did not reach as high as that of the strain with the 680-bp length of promoter region. In another words, the site-specific mutation of REPCAR1 only partially abolished the repression. Nonetheless, this result suggested that REPCAR1 element was at least partially responsible for the repression. The reason that the mutation did not totally relieve the repression could be either that there are other factors involved or that the mutation did not cause a complete loss of the regulatory function of this site.

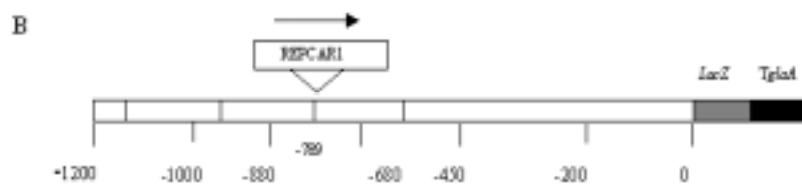
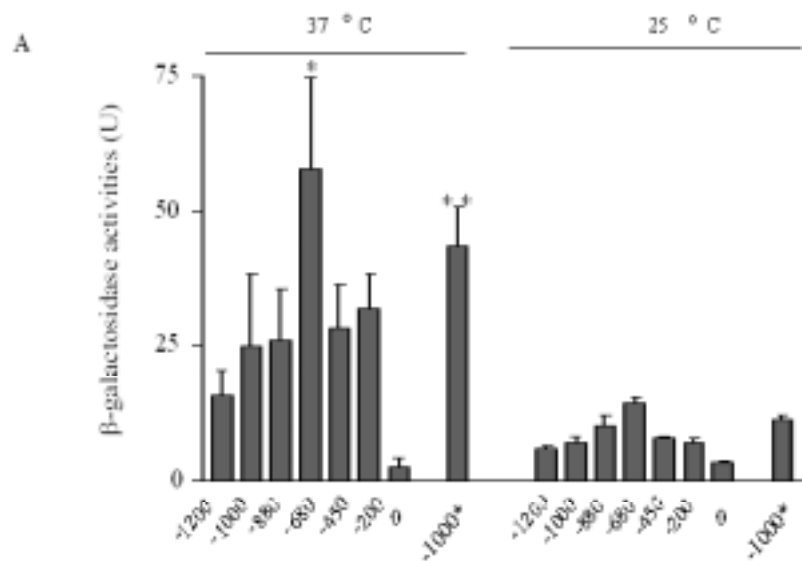
Figure 12. Analysis of the *WdCHS5* promoter. (A) Analysis of the 5' -upstream region of *WdCHS5* by expressing *WdCHS5:LacZ* reporter fusions in the wild-type strain. Two independent white strains from each transformation were grown in YPD broth at 25°C or 37°C for 24 h and then assayed for β -galactosidase activity. Results shown here were derived from at least three independent experiments. Standard deviations are shown. (B) Scheme of the upstream region of *WdCHS5* showing the putative binding position for REPCAR1. (C) Other putative binding sites for transcription factors in the 5' -USR of *WdCHS5*.

Detection of a significantly different ($p < 0.05$) β -galactosidase activity at 37°C between the full length promoter and the truncation at position – 680 is indicated by a single asterisk, whereas the significantly different ($p < 0.05$) activity between the original 1-kb promoter and the promoter with mutations is indicated by two asterisks.

REPCAR1 element in yeast: 5'-AGCCGCCGA-3'

REPCAR1 in *W. dermatitidis*: 5'-AGCCGCCTC-3'

Base substitutions: 5'-GA-3'



Disruption of *WdCHS5*

To elucidate the functions of *WdChs5p*, as well as the possible function of each of its domains, three different disruption vectors were constructed. Two were then used for site-specific integrative gene disruptions that targeted the encoding region for the myosin motor-like domain and the chitin synthase domain, respectively (Fig. 13 A, B). The *WdCHS5* integrative gene disruption plasmids pHB0320 and pHB0510 were first linearized with *Bcl*I and *Bst*EII, respectively. The linearized DNA was then used to transform wild-type *W. dermatitidis* yeast cells by electroporation. Hygromycin-resistant transformants were obtained from each transformation. Site-specific integrations of the linearized pHB0320 and pHB0510 were predicted to result in two truncated fragments of *WdCHS5* separated by the vector sequence and the *hph* gene (Fig. 13A, B). Southern analysis identified several transformants that had the site-specific integrations. Total DNA from one of each putative *wdchs5* Δ mutant was then digested with *Bgl*III and subject to Southern analysis using the PCR product as a probe. The expected band shifts from 5.5-kb to 11.7-kb and 11.5-kb, respectively (Fig. 13C, D; data shown only for *wdchs5* Δ 236 and *wdchs5* Δ 316), which verified these transformants were *WdCHS5* disruptants. Another gene disruption vector (pHB0280) was also constructed and used for a one-step gene replacement of a part of *WdCHS5* with the *hph* selective marker (Fig. 14A). The 4.5-kb fragment released by digestion of pHB0280 with *Kpn*I and *Bam*HI was used for the one-

step replacement gene disruption of *WdCHS5*. Southern analysis then identified numerous mutants with most of *WdCHS5* replaced by the *hph* marker (Fig. 14B; data shown only for *wdchs5Δ11*).

Characterization of *wdchs5Δ* mutants

1. Disruption of *WdCHS5* produced mutants that were hyperpigmented and died at 37°C, but not at 25°C

To determine the consequences of the *WdCHS5* disruption in the wild-type background, the *wdchs5Δ* mutants were first grown on YPD agar or in YPD broth at different temperatures. Characterization of the mutants demonstrated that in general, all three types of *wdchs5Δ* mutants had identical phenotypes, which differed significantly from that of the wild type when grown at 37°C, but not at 25°C. At 25°C, all *wdchs5Δ* strains grew normally in the manner of the wild-type strain on YPD agar medium (Fig. 15A). However, at 37°C, differences between the mutants and the wild type became apparent by 72 h on the agar medium (Fig. 15B) and became even more obvious as time passed. On the agar medium, the colonies of the mutants incubated at 37°C became much darker and smaller than those of the wild type (Fig. 15: compare sector 15B1 with sectors 15B2, 4, and 6). The darkening of mutants occurred similarly in liquid media (Fig. 15E). Subsequent quantitative growth studies of *wdchs5Δ* mutants and the wild-type strain again showed that the disruption of *WdCHS5* did not seem to affect the

Figure 13. Disruption of *WdCHS5* by site-specific integrative gene disruption and complementation of *wdch5Δ* mutants with *WdCHS5*. (A) Construction of pHB0320 to disrupt the myosin motor-like domain encoding region of *WdCHS5*. (B) Construction of pHB0510 to disrupt the chitin synthase domain encoding region of *WdCHS5*. (C, D) Southern blots of genomic DNA digested with *Bgl*III (Bg) from *W. dermatitidis* wild-type 8656 (lane 1 and 4), *wdch5Δ236* (lane 2), *wdch5Δ236-1* (lane 3, complementation of *wdch5Δ236* with *WdCHS5*), *wdch5Δ316* (lane 5) and *wdch5Δ316-1* (lane 6, complementation of *wdch5Δ316* with *WdCHS5*) and hybridized with a 362-bp *WdCHS5* PCR product. Probes are indicated by broken arrows.

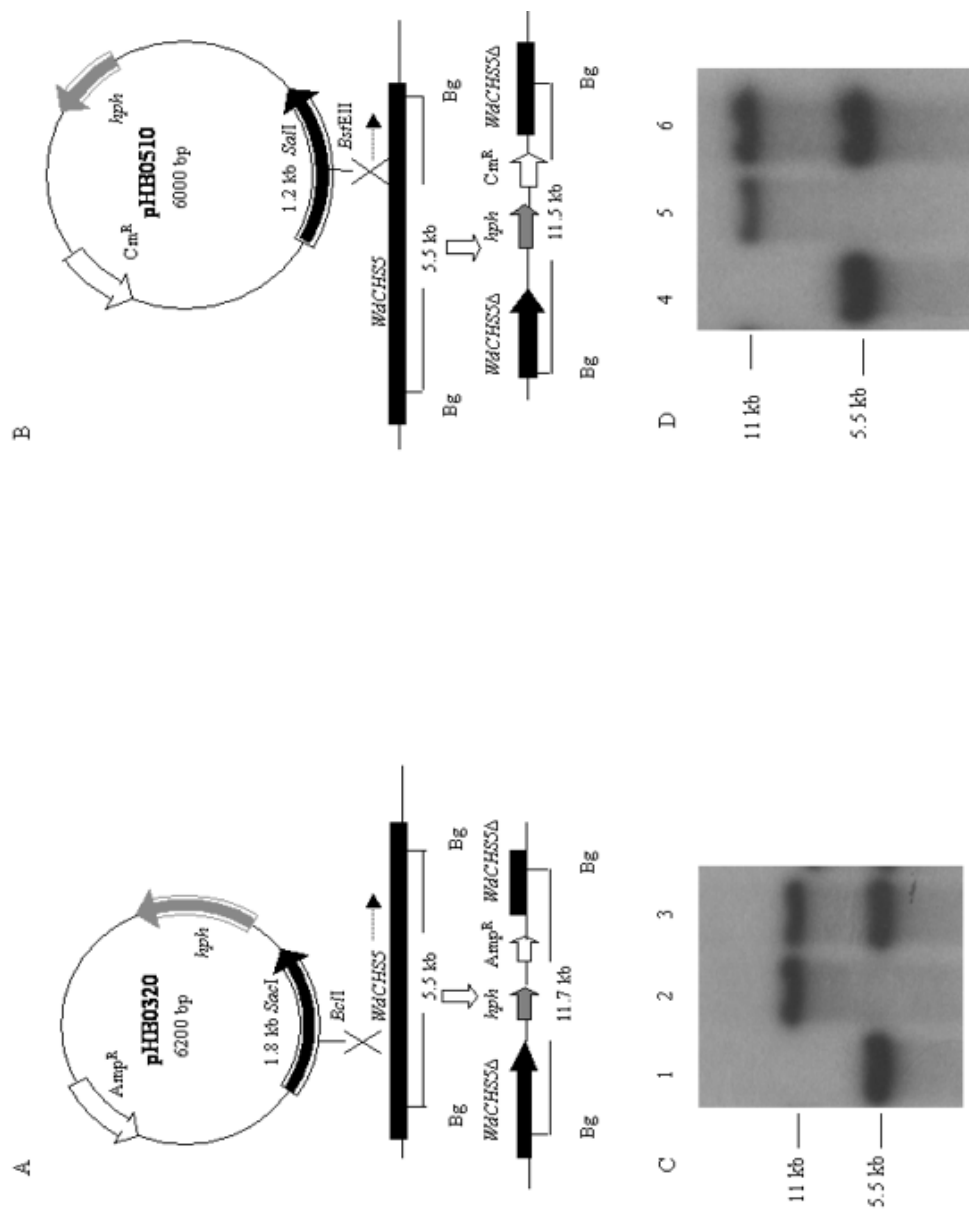
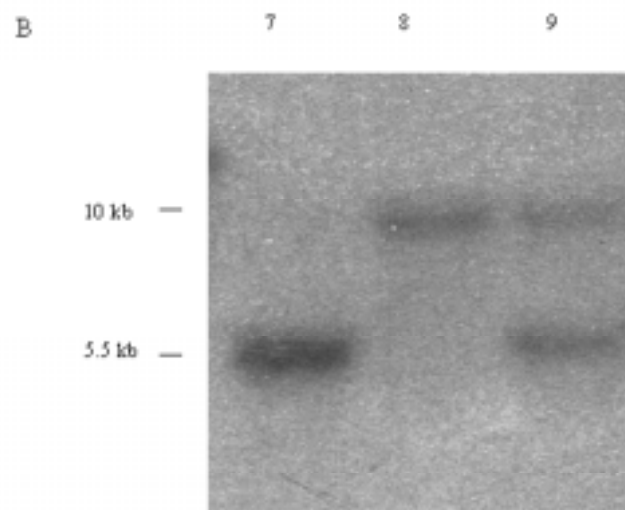
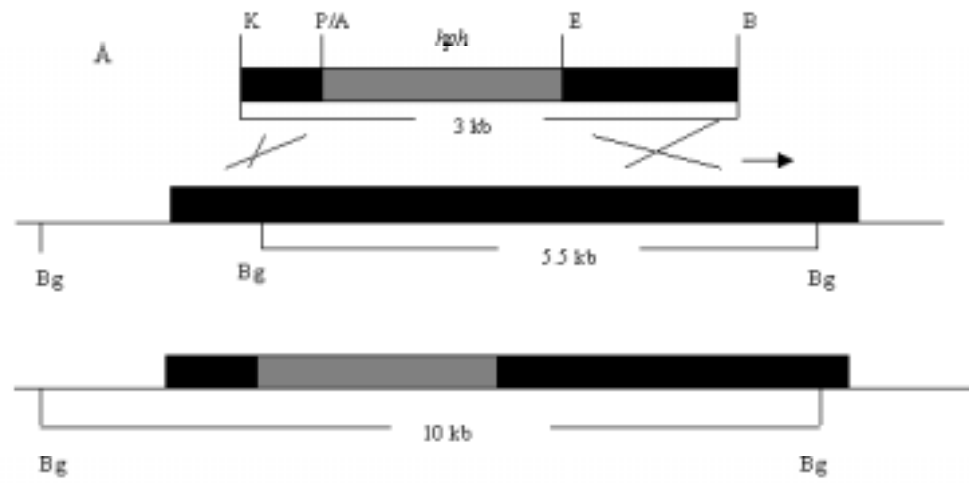


Figure 14. Disruption of *WdCHS5* by a one-step gene replacement strategy and complementation of the *wdchs5Δ11* mutant with *WdCHS5*. (A) The strategy for the construction of the vector, pHB0280 for the one-step gene replacement of *WdCHS5*. (B) Southern analysis of the wild type (lane 1), representative mutant *wdchs5Δ11* (lane 2), and the complementation strain *wdchs5Δ11-1* (lane 3). Abbreviations: *hph*, hygromycin B phosphotransferase gene; K, *KpnI*; P, *PstI*; A, *ApaI*; E, *EcoRI*; B, *BamHI*; Bg, *BglII*. The probe used for the Southern analysis is indicated by an arrow.



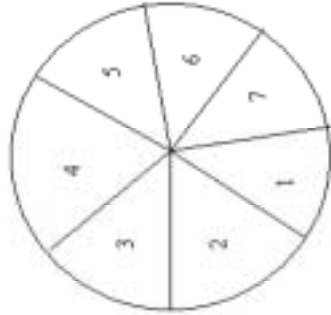
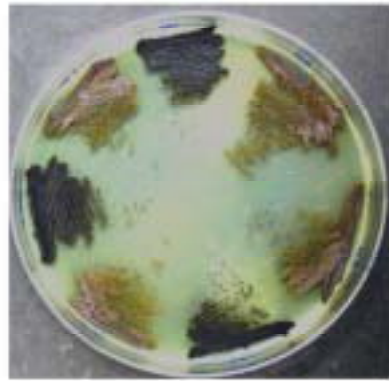
growth of *wdchs5Δ* mutants in the liquid medium at 25°C when measured by spectrophotometric, hemocytometric and plate viable counting methods (Figure 16A, B, C). However, each of these same methods provided different growth kinetic patterns when the mutants and the wild type were grown at 37°C (Fig. 17). Although subtle, the discrepancies detected were easily explained by correlating the microscopic observation and the color change of cultures previously noticed. For example, the OD 600 of the *wdchs5Δ* cultures continued to increase even after 40 h (Fig. 17A). However, this apparent increase was due to the culture becoming much darker than that of the wild type and not to an increase in the cell number, as shown by both hemocytometry counting and the viable counting procedures (Fig. 17B, C). More importantly, as shown by plate counting, the number of *wdchs5Δ* viable cells rapidly decreased after 40 h. Taken together, these results suggested that the *wdchs5Δ* mutants grew normally at 25°C, but lost viability gradually after entering late-log or early stationary phase when grown at 37°C. This strongly indicated that WdChs5p is important for the normal cell growth and the cell maturation at 37°C. This hypothesis was strengthened by the demonstration that reintroduction of the *WdCHS5* wild-type gene into the *wdchs5Δ* mutant backgrounds restored their wild-type colony and growth characteristics (Fig. 15, and 17). Similar growth patterns were observed when the

wild type and the *wdchs5Δ11* mutant were grown in MCD liquid medium, which is less rich than the YPD medium (Fig. 18). Generally, both strains grew slower in this medium. However, the OD600 of the *wdchs5Δ11* mutant kept increasing and the viable cell numbers dropped after the cells entered late-log or early stationary phase, which is consistent with their growth patterns in YPD broth.

2. The *wdchs5Δ11* mutant had abnormal yeast morphology at 37°C but not at 25°C

Because different *wdchs5Δ* mutants all had the same apparent phenotype, the mutant derived by the replacement disruption strategy (*wdchs5Δ11*) was used for additional more extensive microscopic investigations. Once again, at 25°C, no significant difference was observed between the wild type and the *wdchs5Δ11* mutant even after 72 h growth in the YPD liquid medium (Fig. 19). However, at 37°C, the phenotype of this mutant could be distinguished from that of the wild type by as early as 48 h of growth (Fig. 20A, D), and by 72 h, aberrant cellular morphologies became considerably more obvious (Fig. 20G, J). By this time, the mutant cells had clearly begun to clump and to form cell aggregation, whereas the wild-type cells did not. Also, individual cell of the mutant often had swelled, with some swelling to about double the size of normal cells as time increased. Eventually most of these particular cells lost their round shape and smooth surface, to the extent that their surfaces became crinkled and their cell shapes

Figure 15. Temperature-sensitive phenotype of *wdchs5Δ* mutants. (A, B) Colony characteristics of the wild type, representative *wdchs5Δ* mutants and the *wdchs5Δ* complemented strains grown on YPD agar media and incubated at 25°C (A) or 37°C (B) for 3 days. Sector 1, wt; 2, *wdchs5Δ11*; 3, *wdchs5Δ11-1*; 4, *wdchs5Δ236*; 5, *wdchs5Δ236-1*; 6, *wdchs5Δ316*; 7, *wdchs5Δ316-1*. (C, D) Colony characteristics of the wild type, the *wdchs5Δ11* mutant and the *wdchs5Δ11-1* strain grown on YPD agar media containing 1 M sorbitol at 37°C for 3 days. (E) The color characteristics of the wild type and the *wdchs5Δ11* mutant grown in liquid YPD media with or without adding 1 M sorbitol at 37°C for 3 days.



D wt wdr5Δ11 wdr5Δ11-1



Figure 16. Comparison of the growth rate and the viability of different strains grown at 25°C. The wild-type *W. dermatitidis*, the representative *wdchs5Δ* mutants (*wdchs5Δ11*, *wdchs5Δ236* and *wdchs5Δ316*), and the representative complementation strains (*wdchs5Δ11-1*, *wdchs5Δ236-1*, *wdchs5Δ316-1*) grown in YPD liquid media at 25°C and assayed by optical density (A), hemacytometer counting (B) and viable counting (C) methods. The initial inoculation level was 10^6 cells/ml.

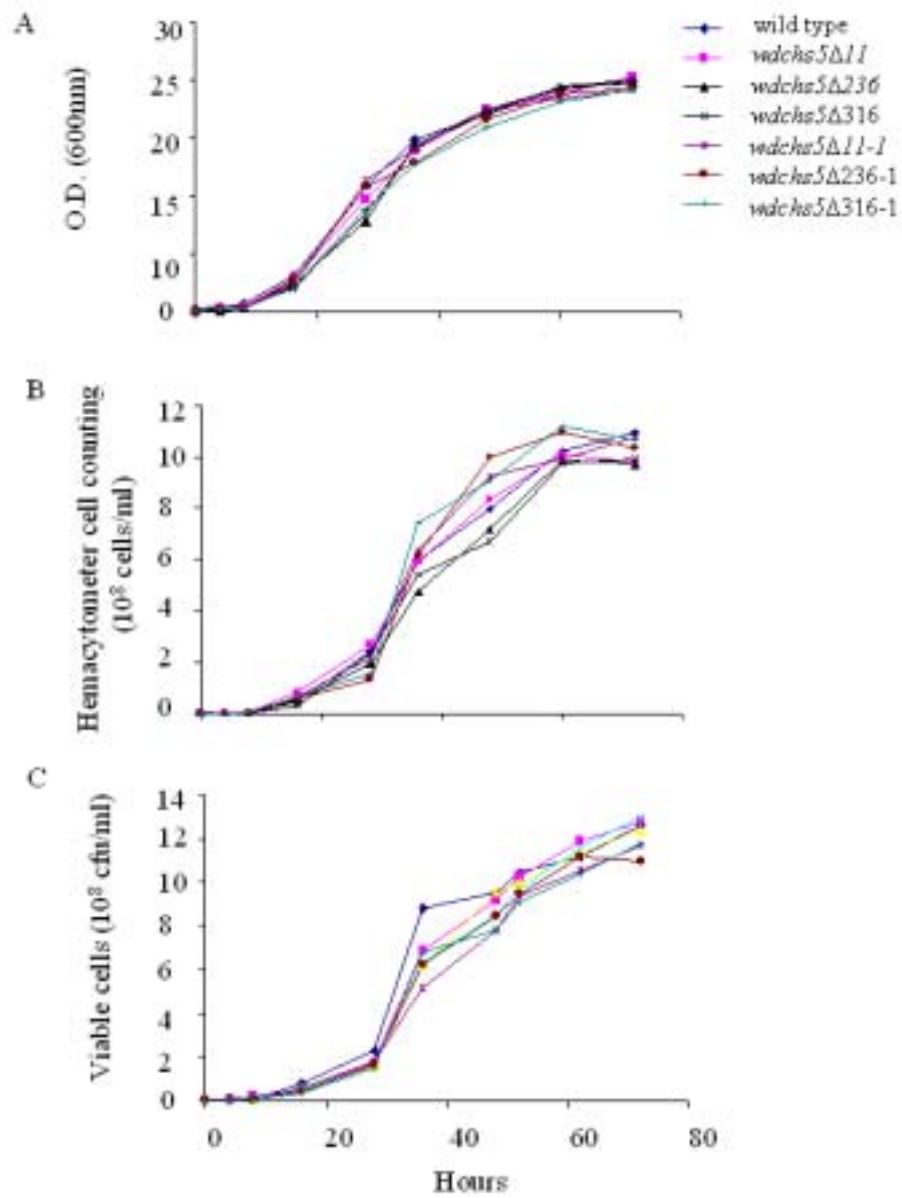


Figure 17. Comparison of the growth rate and the viability of different strains grown at 37°C. The wild-type *W. dermatitidis*, the representative *wdchs5Δ* mutants (*wdchs5Δ11*, *wdchs5Δ236* and *wdchs5Δ316*), and the representative complementation strains (*wdchs5Δ11-1*, *wdchs5Δ236-1*, *wdchs5Δ316-1*) grown in YPD liquid media at 37°C and assayed by optical density (A), hemacytometer counting (B) and viable counting (C) methods. The initial inoculation level was 10⁶ cells/ml.

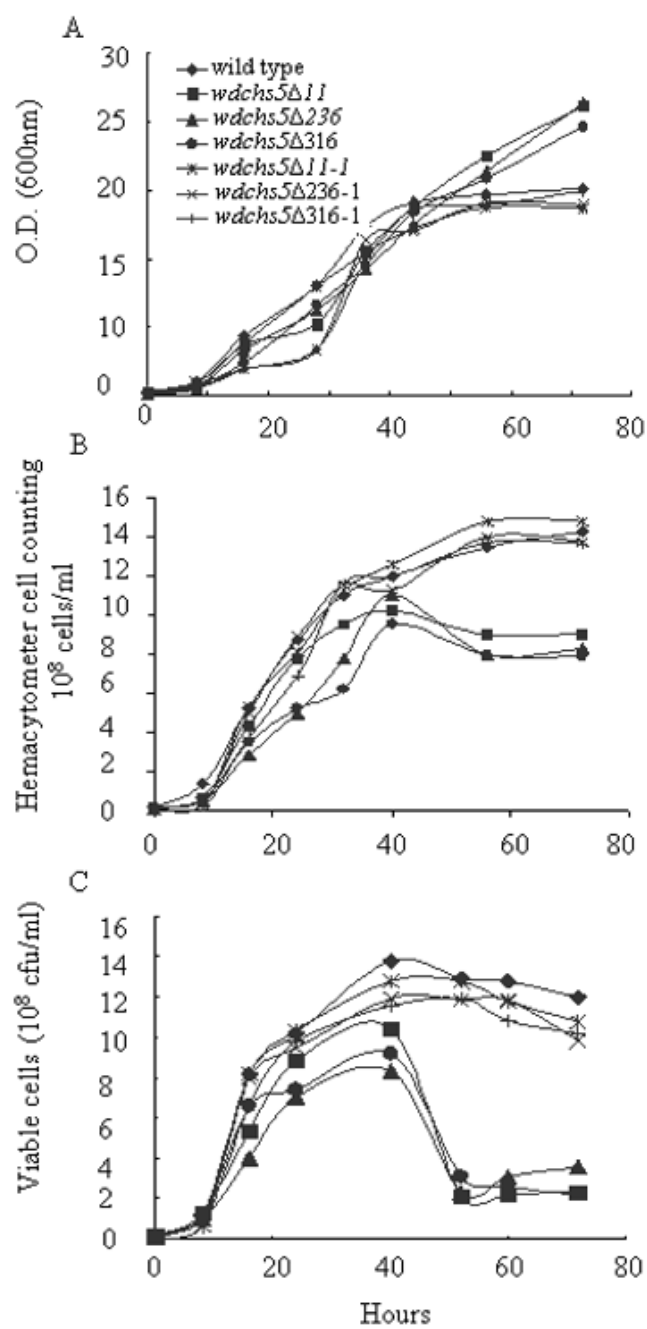
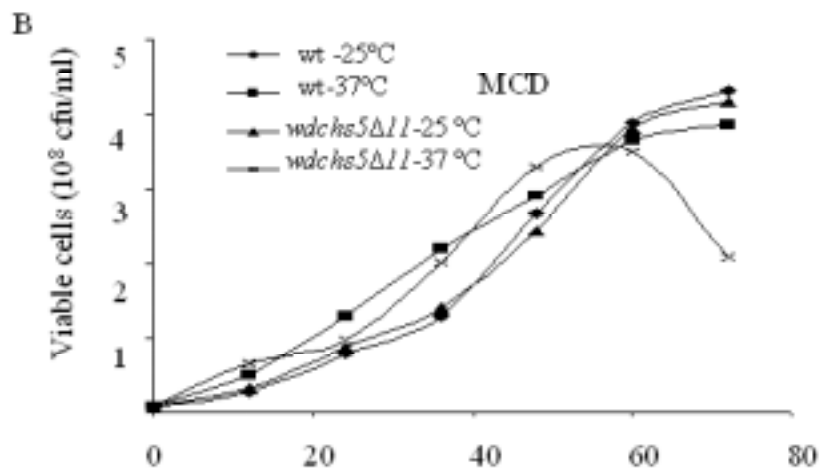
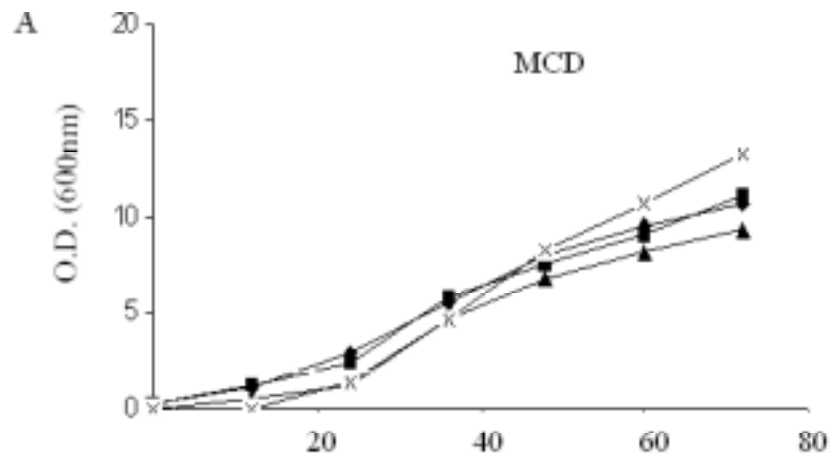


Figure 18. Comparison of the growth rate and the viability of wild-type *W. dermatitidis* and the *wdchs5Δ11* mutant grown in MCD liquid media at 25°C and 37°C and assayed by optical density (A), and viable counting (B) methods. The initial inoculation levels for all cultures were 10⁶ cells/ml.



became flat, irregular, indicating these cells lost their cell wall integrity and underwent lysis. Calcofluor staining showed that the mutant cells tended to have more cell wall chitin at 48 h (Fig. 20E), which was not as uniformly localized as that in the wild-type cells (Fig. 20B). However, by 72 h the Calcofluor staining patterns between the strains became less apparent (Fig. 20K, H). DAPI staining showed that some of the mutant cells had even lost nuclei by 72 h, indicating that their nuclei had probably leaked from cytoplasm due to the cell wall damage (Fig. 20L). Scanning electron microscopy confirmed that the *wdchs5Δ11* mutant cells had severe cellular defects when grown at 37°C for 72 h (Fig. 21A, B, and C) when compared with the wild-type strain (Fig. 21D, E, F). Most of the mutant cells showed irregular cell shapes and crinkled surfaces. In contrast, the wild-type cells were uniformly oval in shape and had smoother surfaces. Some of the mutant cells were enlarged abnormally and occasionally, cellular content leakage or wall debris was observed on some cells. Infrequently, holes on the surface of the mutant cells were seen, indicating that the cell wall perforated at those places, suggesting that these might be sites where the cellular contents possibly sometimes leaked. Interestingly, the phenotype of this mutant could be reversed by adding an osmotic stabilizer, such as 1.2 M sorbitol or 1 M sucrose to the growth medium (Fig. 20M). Under these conditions the mutants did not swell and lyse, which suggested that the lysis and death of the mutant was, in fact, due to the cell wall damage and loss of cell wall integrity. Similarly, at 37°, adding 1M

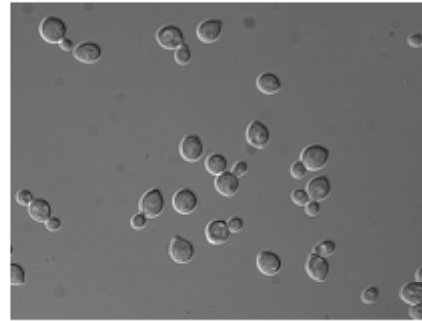
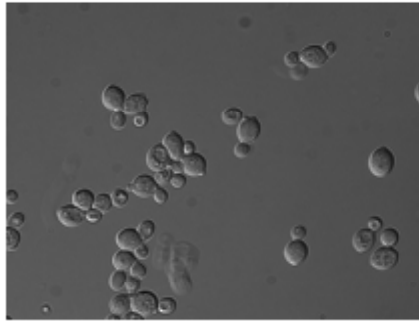
Figure 19. Cellular morphologies and staining properties of *W. dermatitidis* wild-type strain and the *wdchs5Δ11* mutant grown at 25°C. The cells of the wild-type strain and the *wdchs5Δ11* mutant were grown in YPD medium at 25°C for 72 h then fixed with 5% formaldehyde and stained with Calcofluor or DAPI. All cells are shown at the same magnification. Nomarski, Nomarski phase contrast.

72h

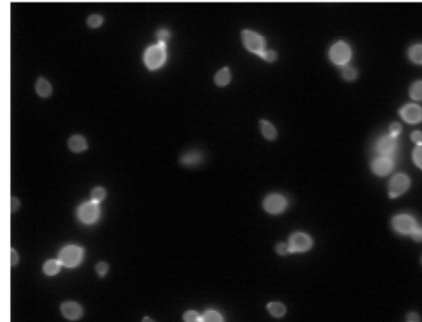
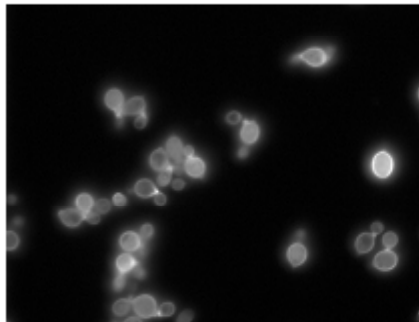
wt

wdchs5Δ11

Nomarski



Calcofluor



DAPI

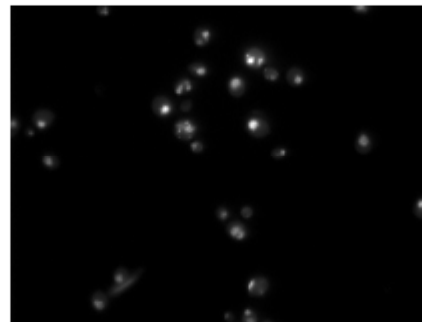
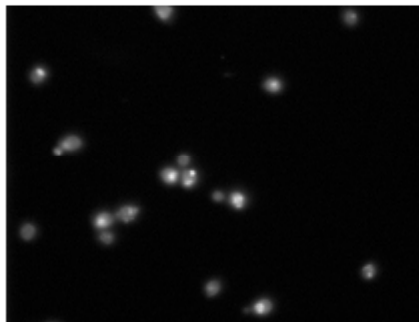


Figure 20. Cellular morphologies and staining properties of *W. dermatitidis* wild-type strain and the *wdchs5Δ11* mutant grown at 37°C. The cells of the wild-type strain (A, B, C, G, H, I) and the mutant, *wdchs5Δ11* (D, E, F, J, K, L) were grown in YPD broth at 37°C for 48 h (A, B, C, D, E, F) and 72 h (G, H, I, J, K, L). The *wdchs5Δ11* mutant (M, N, O) was grown in YPD broth with 1 M sorbitol at 37°C for 72 h. All the cells were fixed with 5% formaldehyde, and stained with Calcofluor or DAPI. All cells are shown at the same magnification. Arrows point to mutant cells that were swelled or enlarged and lost their nuclei. Nomarski, Nomarski phase contrast.

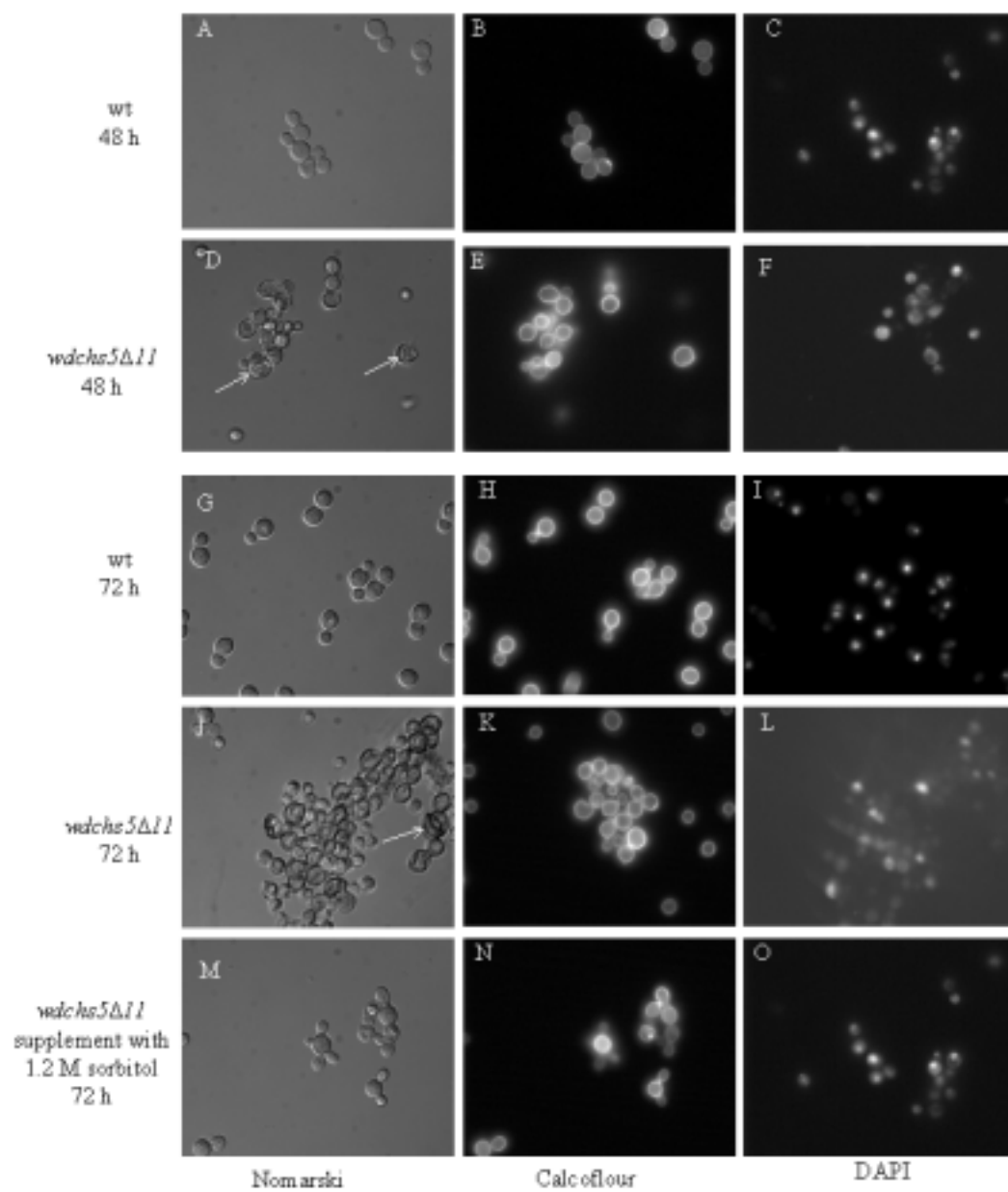
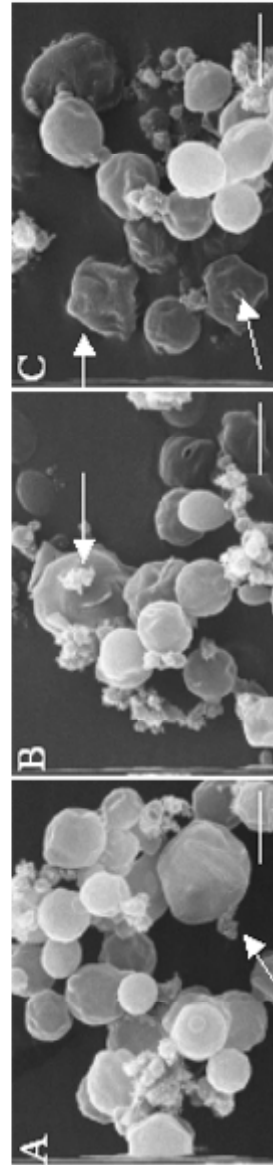
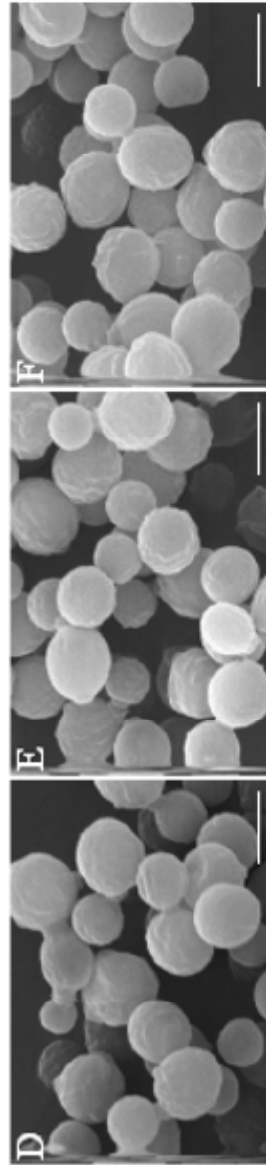


Figure 21. Scanning electron micrographs of the wild-type (wt) (A, B, C) and the *wdchs5Δ11* mutant (D, E, F) cells grown in YPD broth at 37°C for 72 h. Arrows point to the enlarged, irregular mutant cells, and cells with a hole on their surface or cells appearing to leak intracellular materials. Bars, 5μm.

wdchs5Δ1



wt



sorbitol in YPD agar also reversed the defect in the phenotype (Fig. 15C).

3. Disruption of *WdCHS5* did not reduce overall chitin synthase activity

Because the *wdchs5*Δ mutant had severe growth defects at 37°C, it was speculated that the chitin synthesized by WdChs5p has an important function in maintaining cell wall integrity. However, no significant differences were detected between the total chitin synthase activity of the wild-type strain and that of the *wdchs5*Δ mutant strains at both 25°C and 37°C, when evaluated under zymogenic or nonzymogenic assay conditions (Fig. 22). This result indicated that either the chitin synthase assay itself was not sensitive enough to detect any difference or other chitin synthase(s) was induced to compensate for the loss of WdChs5p.

4. Disruption of *WdCHS5* affected the chitin content at 37°C

Although, no significant difference of chitin synthase activity was detected between the wild-type and *wdchs5*Δ mutants, significantly different ($p < 0.05$) and increased chitin contents (about 50%) were detected when the *wdchs5*Δ11 mutant was compared to the wild-type, but only in cells cultured at 37°C for at least 48 h (Fig. 23B). This supported the hypothesis that the cell wall damage caused by the disruption of *WdCHS5* induced a compensatory pathway, which resulted in the other chitin synthases being activated or stimulated to produce more chitin. Nonetheless, this newly synthesized chitin was unable to rescue the damage

caused by the loss of WdChs5p, most likely because the specific function of WdChs5p was not repaired by any of the other four WdChsp isozymes. Conversely, this difference may simply be due to the fact that the cell wall of the mutant was damaged or degraded to such an extent that it may have affected the dry weight measurements required for the final chitin content determinations. As expected, complementation of the *wdchs5Δ11* mutant with *WdCHS5* (*wdchs5Δ11-1*) lowered the chitin content to the level of the wild-type strain in cells cultured at 37°C for 48 h.

5. Disruption of *WdCHS5* changed melanin deposition and expression patterns

The observation that *wdchs5Δ* mutants were hyperpigmented when grown on YPD agar and in YPD broth suggested that more melanin was being incorporated into their cell walls. Interestingly, the media of *wdchs5Δ* cultures were much darker than that of the wild-type strain when grown at 37°C for 2 days (data not shown), indicating that some melanin or melanin precursors had leaked out from the mutant cells. A semi-quantitative RT-PCR result (Fig. 24) confirmed that in the *wdchs5Δ11* mutant background, the transcription of the polyketide synthase gene (*WdPKS1*) increased by about 3.5 fold, indicating more *WdPKS1* was transcribed and more melanin was deposited in the cell wall, in an attempt to

Figure 22. Chitin synthase activities of the wild-type strain, different *wdchs5Δ* mutants and complementation strains grown at 37°C or 25°C for 24 h and assayed after trypsin treatment or without trypsin treatment. Results were derived from at least three independent experiments. Standard deviations are shown. No significant differences ($P>0.05$) were found among the samples with the same treatment.

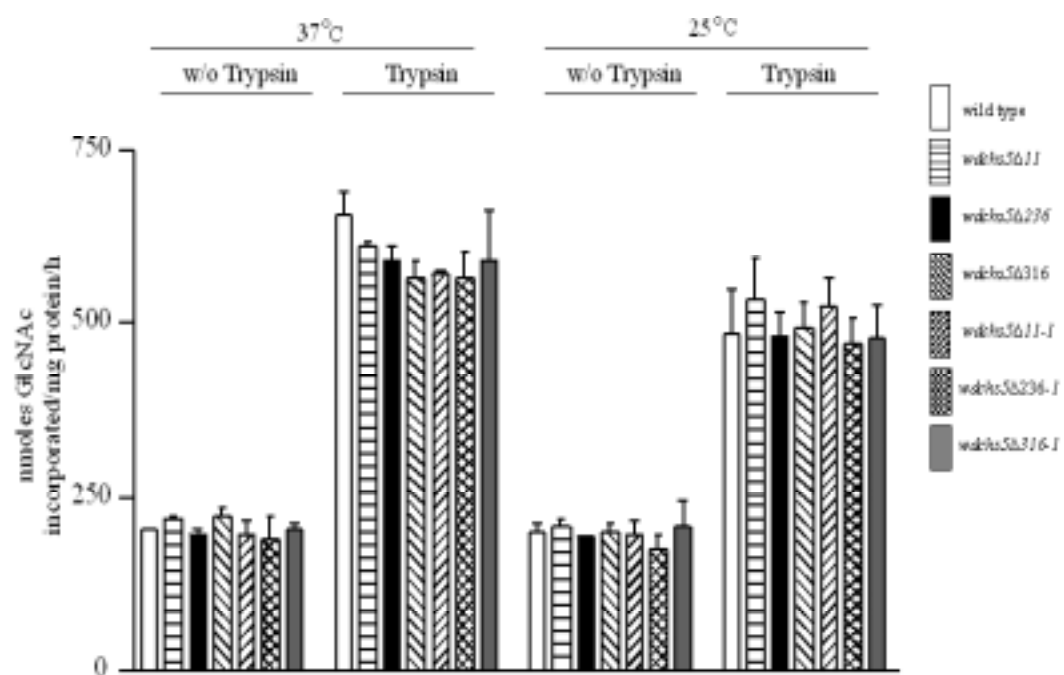
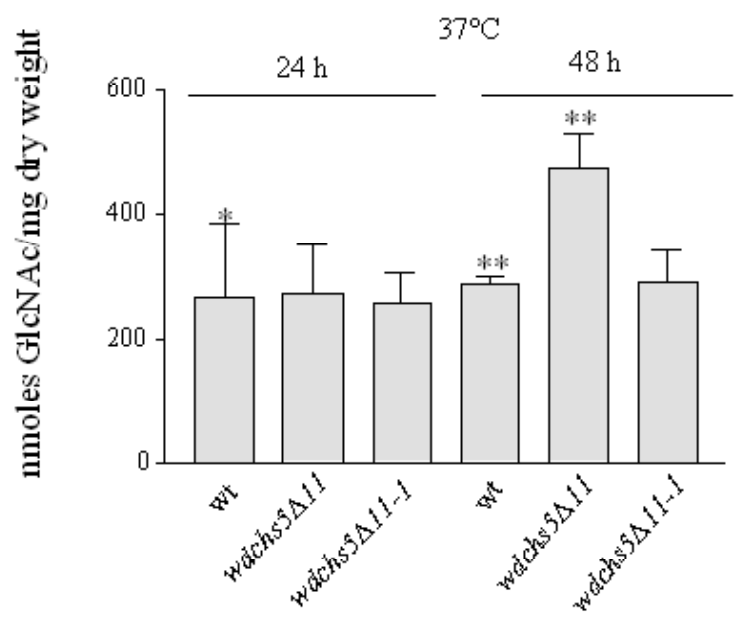
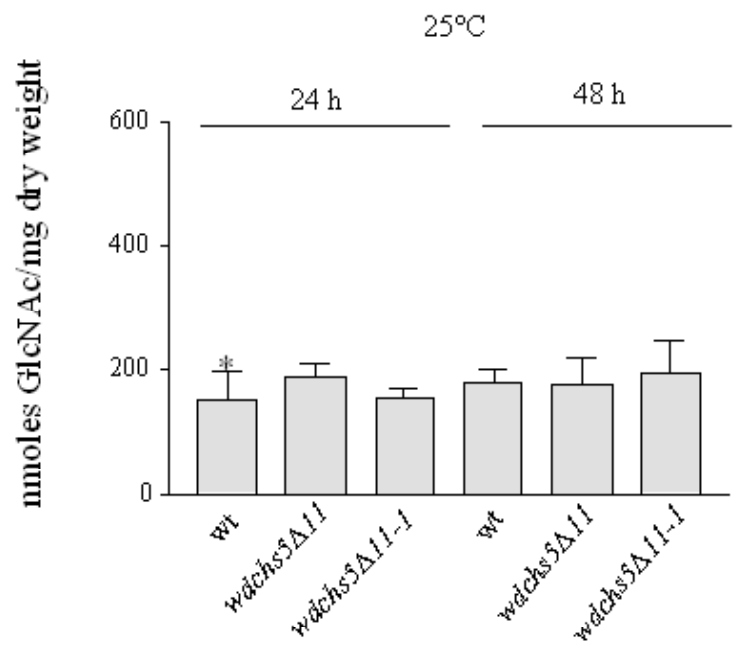


Figure 23. Comparison of chitin content of the wild-type strain, *wdchs5Δ11* and *wdchs5Δ11-1*. Strains were grown at 25°C (A) and 37°C (B) for 24 h and 48 h. The significantly different ($p < 0.05$) chitin content of the wild-type strain grown at 25°C and 37°C is indicated by a single asterisk, whereas the significantly different ($p < 0.05$) content of the wild-type and the *wdchs5Δ11* mutant grown at 37 °C for 48 h is indicated by two asterisks.



compensate for the cell wall damage caused by *WdCHS5* disruption. However, it was obvious that the increased melanin could not compensate for the loss of *WdChs5p*, which eventually led to the extra melanin or melanin precursors being leaked into the media.

6. Disruption of *WdCHS5* damaged the cell wall

A loss of cell wall integrity usually results in cell lysis and is characterized by increased cell permeability and release of intracellular contents (such as alkaline phosphatase) to the external medium (Molina et al., 1998). Therefore, an alkaline phosphatase assay is commonly used to detect the release of intracellular contents. Because such a cellular defect is restricted to the cell wall structure and the plasma membrane remains largely intact, the lytic phenotype can be rescued by providing an osmotic stabilizer, such as 1 M sorbitol, to the growth medium. For this reason, the alkaline phosphatase assay is usually performed both in the absence and presence of an osmotic stabilizer. To measure the release of alkaline phosphatase by *wdchs5Δ11* to the external medium, PNPP (*p*-nitrophenylphosphate) hydrolysis was quantified in the supernatant of a liquid culture. At 37°C, without supplementing with osmotic stabilizer, the relative alkaline phosphatase activities of the *wdchs5Δ11* mutant were about 8 times higher than that of the corresponding wild-type samples at 48 h and 72 h (Fig. 25A and B). This suggested that the mutant had a lytic defect in its cell wall and had

released higher amounts of alkaline phosphatase from the cell than did the wild-type strain. It was not surprising to see that after adding 1 M sorbitol into the medium, the alkaline phosphatase activities returned to the wild-type level, which indicated that osmotic stabilizer could reverse the lytic phenotype and further confirmed that the cell lytic defects were due to the loss of cell wall integrity.

7. The *wdchs5*Δ mutants had reduced virulence in mice

As might be expected of mutants with a temperature-lethal phenotype, all three types of *wdchs5*Δ mutants showed a significant reduction in virulence compared to that of the wild-type strain when tested in an acute murine infection model (Fig. 26). Reintroduction of the *WdCHS5* gene into the *wdchs5*Δ background fully reconstituted the virulence of the mutants. To determine fungal organ burdens of the brain, kidney, and liver/spleen, mice injected with *wdchs5*Δ11 were sacrificed at day 1, 3, 5, and 10 post-infection. Starting from day 3, the viable counts began to decrease in all the organs examined, suggesting the cells were being cleared from those organs (Table 5). Furthermore, the infection was totally cleared from the liver/spleen by day 5 and 98% of the infection was cleared from the brain and the kidney by day 10. This result indicated that the mutant cells were incapable of sustained growth and survival in the mice, probably because of the elevated temperatures associated with the mice.

Figure 24. RT-PCR analysis of *WdPKS1* expression at 37°C. Total RNA samples from wild-type strain (lanes 1, 2) and *wdchs5Δ11* (lanes 3, 4) were reverse transcribed and further amplified by PCR with primers WdPKS-F and WdPKS-R, which were standardized against internal controls (lane B1 to B4) represented by RT-PCR products from the same RNA samples used in lanes A1 to A4, but generated using primers exclusive for the amplification of the 18S rRNA gene. (C) Densitometry analysis of the RT-PCR products depicted in (A) and (B), to estimate the relative transcriptional level of the *WdPKS1* gene. Results were derived from two independent experiments. Standard deviation is shown.

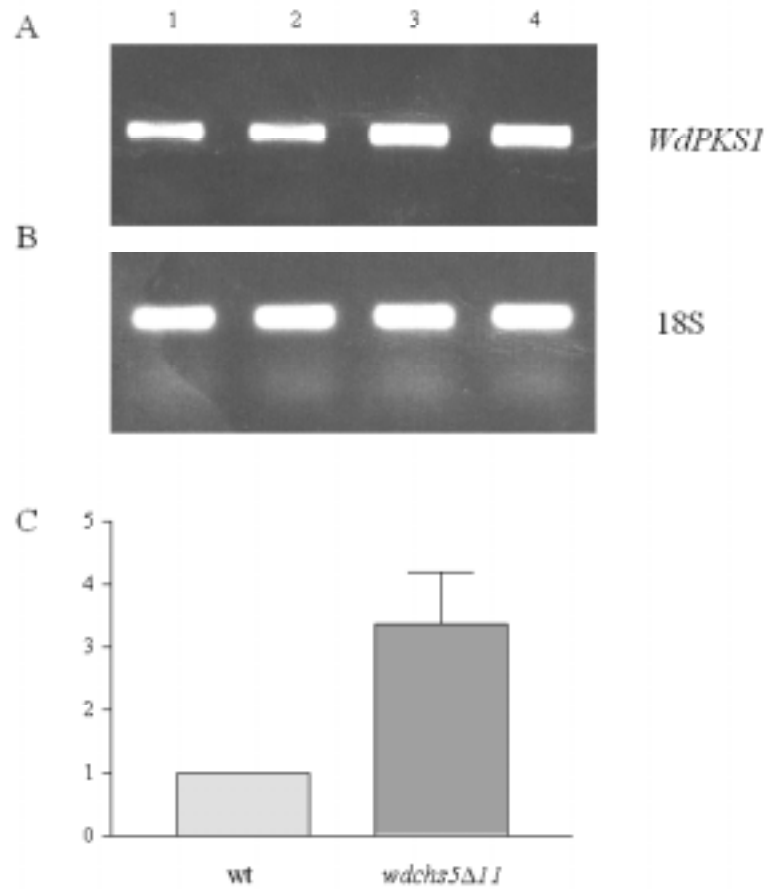


Figure 25. Alkaline phosphatase activities of the wild-type strain and *wdchs5Δ11*. The wild type and the *wdchs5Δ11* mutant were grown in YPD broth with or without addition of 1 M sorbitol at 37°C for 48 h (A) and 72 h (B). Results were derived from at least three independent experiments. Standard deviations are shown.

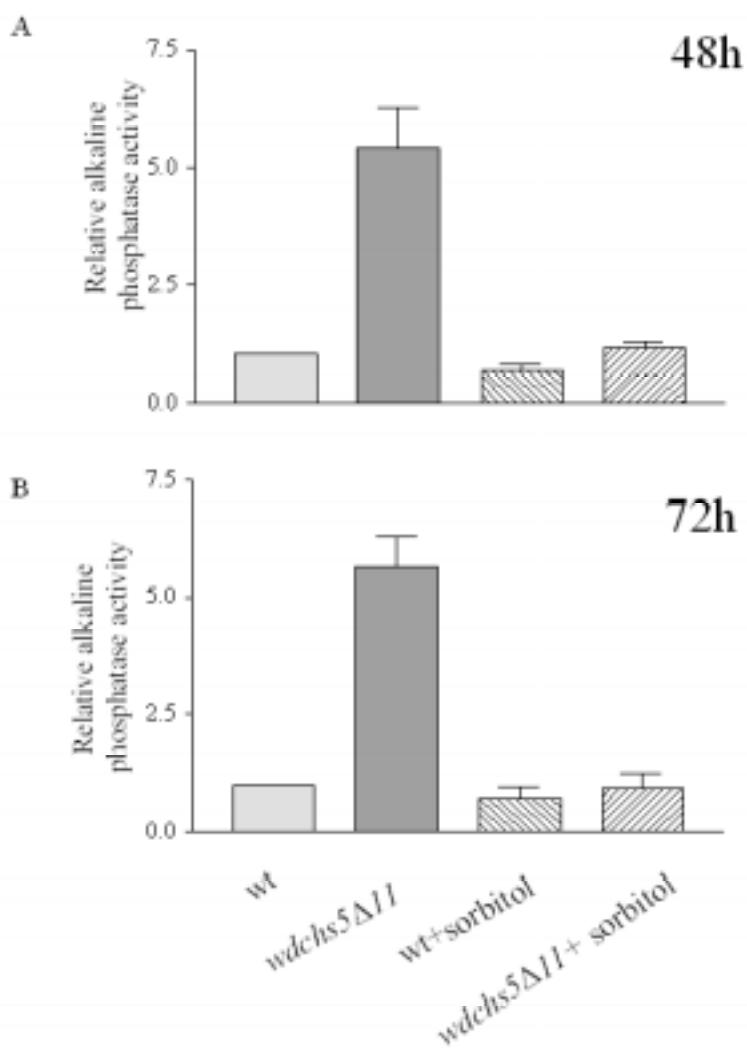


Figure 26. Virulence test of *W. dermatitidis* wild-type strain, *wdchs5*Δ mutants and complementation strains. Groups of 10 mice received injections of log-phase yeast cells. The injections contained 9×10^9 cells per mouse, and the mice were monitored for 14 days to determine the survival rate. Survival fractions were calculated by the Kaplan-Meier method, and survival curves were tested for significant difference ($P < 0.01$) by the Mantel-Haenszel test using GraphPad Prism software (version 3.00 for Windows).

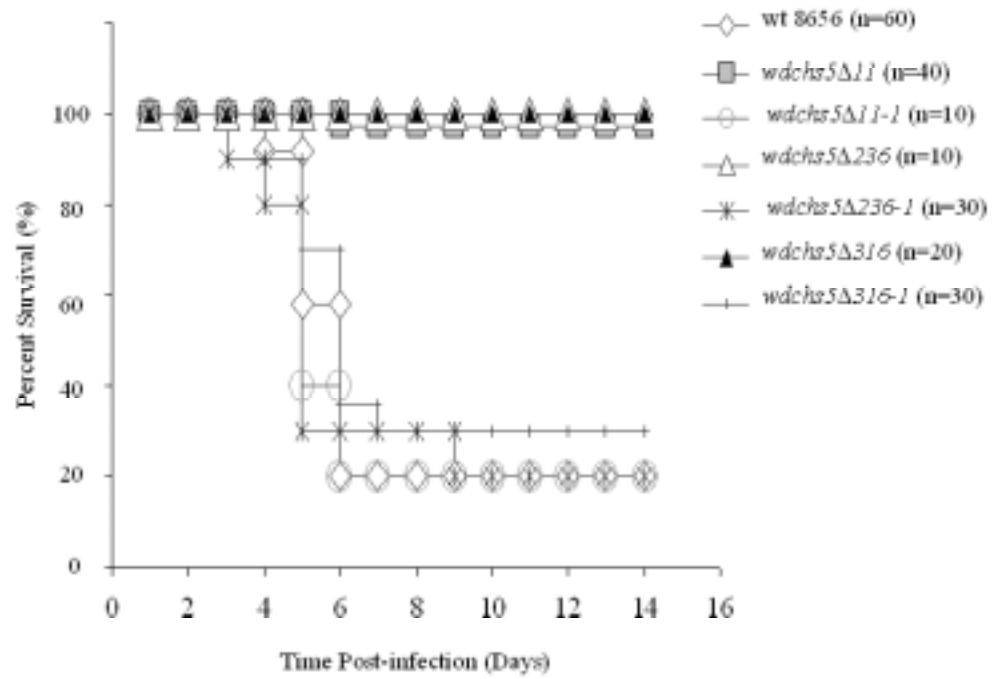


Table 5. Fungal organ burden analysis of the *wdchs5Δ11* mutant. The fungal organ burden analysis involved injecting *wdchs5Δ11* into mice and sacrificing them at day 1, 3, 5, and 10 post-infection to determine the fungal organ burden of the brain, kidney, liver, and spleen. *** represents no colonies were formed.

Table 5. Fungal organ burden of *wdchs5Δ11*-infected mice

Organ \ cfu/g	Day 1	Day 3	Day 5	Day 10
Brain	3.6×10^5	1.4×10^6	7.0×10^5	6.7×10^3
Kidney	1.5×10^5	7.9×10^4	4.8×10^4	4.3×10^3
Liver/Spleen	2.2×10^5	5.4×10^4	***	***

Construction of double gene disruption mutants with each of other four *CHS* gene disrupted together with *WdCHS5*

Although, the loss of WdChs5p function resulted in yeast cell wall weakening at elevated temperature, which in turn brought about a loss of cell viability, the observation that *wdchs5* Δ mutant cells were identical to the wild-type cells at 25°C suggested that either WdChs5p has little or no function at this temperature or one or more of the other chitin synthases compensated for the function loss of WdChs5p in *wdchs5* Δ mutants at lower temperatures. In either case, it was obvious that none of the other four WdChsp isozymes could compensate for the loss of WdChs5p at the higher temperature. Under the scenario that WdChsp5p has little or no function at 25°C, together with conclusions from our previous studies that none of the four other chitin synthases is essential for cell viability at 25°C, we should be able to disrupt each other single *WdCHS* gene in *wdchs5* Δ background without effect at 25°C. On the other hand, if one or more than one other WdChsp compensates for the function of WdChs5p at 25°C, then the disruption of *WdCHS5* in certain *wdchs* Δ backgrounds would produce strains incapable of growth at both 25°C and 37°C. To distinguish these two possibilities and to study the relationships between WdChs5p and other chitin synthases in *W. dermatitidis*, double mutants with either *WdCHS1*, *WdCHS2*, *WdCHS3*, or *WdCHS4* disrupted together with *WdCHS5* were required to be derived. In order to do this, first, another one-step replacement gene disruption vector, pHB0901,

was constructed by cloning a 0.5-kb *Xho*I and *Kpn*I fragment of the 5'-end of *WdCHS5* and a 1-kb *Eco*RI and *Bam*HI fragment of the 3'-end of *WdCHS5* into the corresponding sites of vector pCB1551, which contained the *sur* gene marker. The 4.5-kb fragment released by digestion of pHB0280 with *Kpn*I and *Bam*HI was used for the one-step replacement gene disruption of *WdCHS5* in *wdchs1*Δ, *wdchs2*Δ, *wdchs3*Δ mutant backgrounds (Fig. 27A). Because of the unique temperature-sensitive phenotype of *WdCHS5* gene disruption, transformants were first screened by comparing their growth at 25°C and 37°C: transformants that showed hyperpigmentation at 37°C were potential *WdCHS5* gene disruption mutants. Southern analysis was then performed to verify the site-specific *WdCHS5* gene disruption, which identified numerous mutants with part of *WdCHS* replaced by *sur* marker (Fig. 27B; data shown only for *wdchs1*Δ*wdchs5*Δ11-1, *wdchs2*Δ*wdchs5*Δ11-1, and *wdchs3*Δ*wdchs5*Δ11-1). However, this methodology was not successful when used to screen the *WdCHS5* disruption in the *wdchs4*Δ mutant background, mainly because *wdchs4*Δ mutant itself has a hyperpigmentation phenotype when grown at 37°C. Therefore, another gene disruption vector pHY (Fig. 28A), which has 2-kb *Eco*RI fragment of the *WdCHS4* gene and the *sur* marker was used to disrupt *WdCHS4* in the *wdchs5*Δ11 background. After pHY was linearized with *Bam*HI, it was transformed into the *wdchs5*Δ11 mutant background. The DNA isolated from 18 transformants was subjected to Southern analysis, and three of them were identified as site-specific

gene disruption mutants. The expected band shift indicated that the *WdCHS4* gene had been successfully disrupted in *wdchs5Δ11* background (Fig. 28B).

Characterization of double chitin synthase gene disruption mutants

Successful derivation of all the double mutants involving *WdCHS5* enabled me to study the possible relationships between WdChs5p and other chitin synthases in *W. dermatitidis*. At 25°C, all double mutant strains grew as normally as the wild-type strain did on YPD agar (Fig. 29A). Although, at 37°C, the double mutants, *wdchs1Δ5Δ11-1*, *wdchs2Δ5Δ11-1*, and *wdchs3Δ5Δ11-1* showed essentially the same phenotype as that of *wdchs5Δ* (Fig. 29B, sector 6, 7, 8), a more severe phenotype was observed for the *wdchs4Δ5Δ11-1* mutant. This particular double mutant showed even more hyperpigmentation than *wdchs4Δ* or *wdchs5Δ* alone (Fig. 29B: compare sectors 29B5 and 29B6 with sectors 29B10). The colonies it formed were also much smaller and their surfaces were much rougher. Basically, it showed the combined or synergistic phenotype of *wdchs4Δ* and *wdchs5Δ* single disruption mutant. The growth rate of the *wdchs4Δwdchs5Δ11-1* mutant was similar to those of *wdchs5Δ* mutants when grown in YPD broth (Fig. 30A and B). However, when grown in less rich MCD medium, the *wdchs4Δwdchs5Δ11-1* double mutant grew very poorly at 37°C as shown by spectrophotometric and plate viable counting measurements (Fig. 30A and B).

Figure 27. Disruption of *WdCHS5* in *wdchs1Δ*, *wdchs2Δ*, and *wdchs3Δ* backgrounds by a one-step gene replacement strategy. (A) Strategy for the construction and use of the one-step replacement vector. (B) Southern analysis of the wild type (lane 1), representative mutant, *wdchs1Δ5Δ11-1* (lane 2), *wdchs2Δ5Δ11-1* (lane 3), and *wdchs3Δ5Δ11-1* (lane 4). The genomic DNA was digested with *ApaI*, Abbreviations: *sur*, surfonylurea resistance gene; K, *KpnI*; X, *XbaI* A, *ApaI*; E, *EcoRI*; B, *BamHI*. The probe used to detect *WdCHS5* in the Southern analysis is indicated by an arrow.

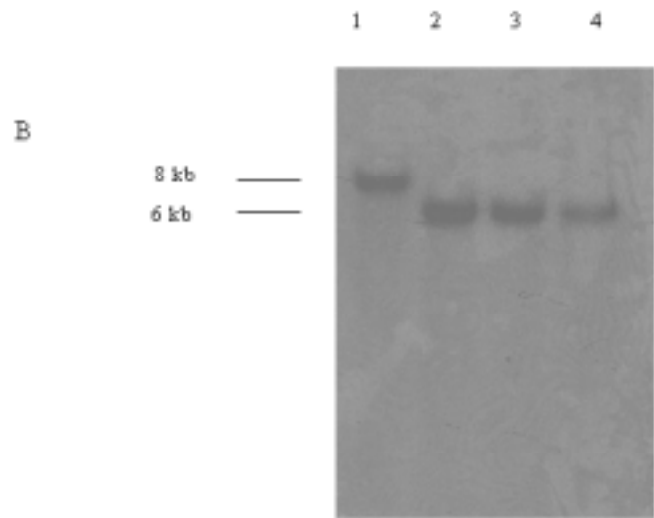
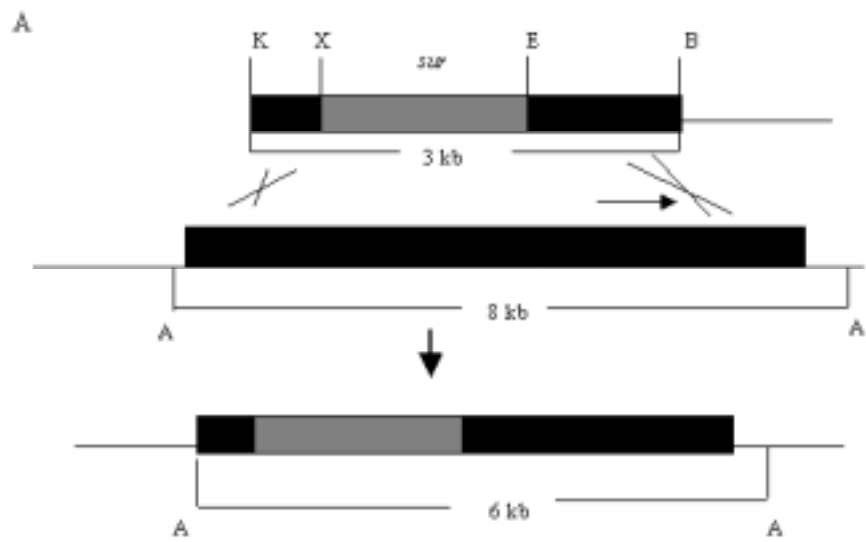


Figure 28. Disruption of *WdCHS4* in the *wdchs5Δ11* background by site-specific integrative gene disruption strategy. (A) Construction of the site-specific integrative vector. (B) Southern analysis of the wild-type (lane 4), representative mutant, *wdchs4Δ5Δ11-1* (lane 1), *wdchs4Δ5Δ11-2* (lane 2), and *wdchs4Δ5Δ11-3* (lane 3). Genomic DNA was digested with *Bgl*III. Abbreviations: B, *Bam*HI; Bg, *Bgl*III. The probe used to detect *WdCHS4* in the Southern analysis is indicated by a broken arrow.

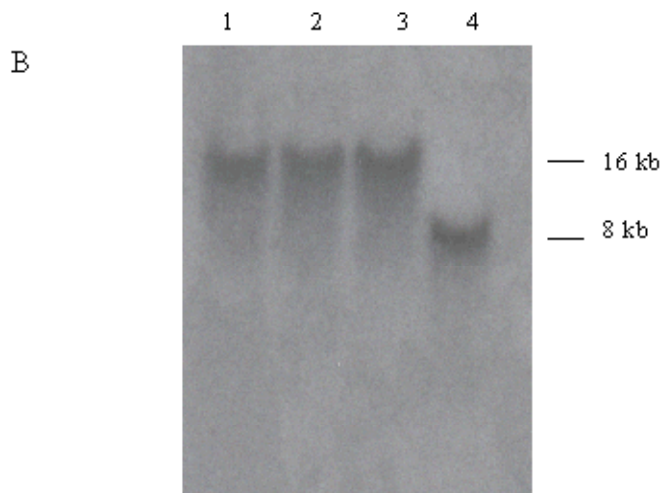
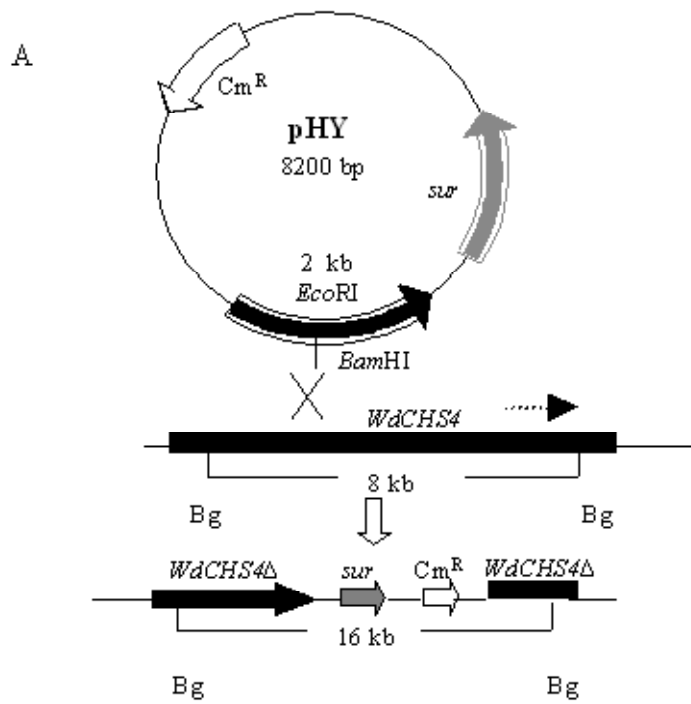
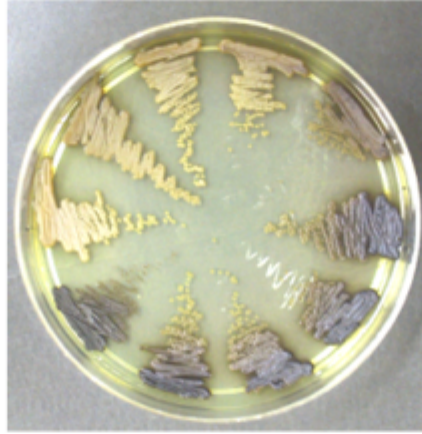
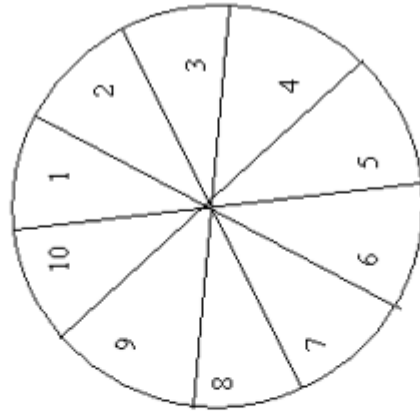
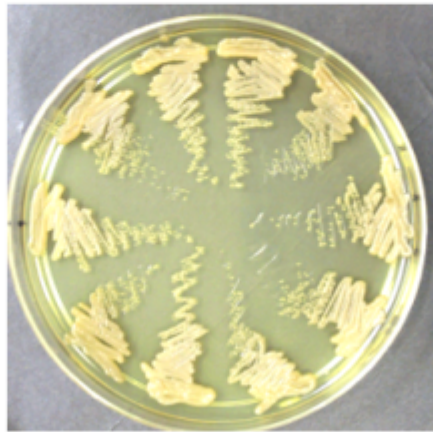


Figure 29. Colony characteristics of all the double mutants involving *WdCHS5* grown on YPD agar and incubated at 25°C (A) and 37°C (B) for 3 days. Sector 1, wt; 2, *wdchs1Δ-1A*; 3, *wdchs2Δ-1*; 4, *wdchs3Δ-1*; 5, *wdchs4Δ-1*; 6, *wdchs5Δ11*; 7, *wdchs1Δ5Δ11*; 8, *wdchs2Δ5Δ11*; 9, *wdchs3Δ5Δ11*; 10, *wdchs4Δ5Δ11-1*.



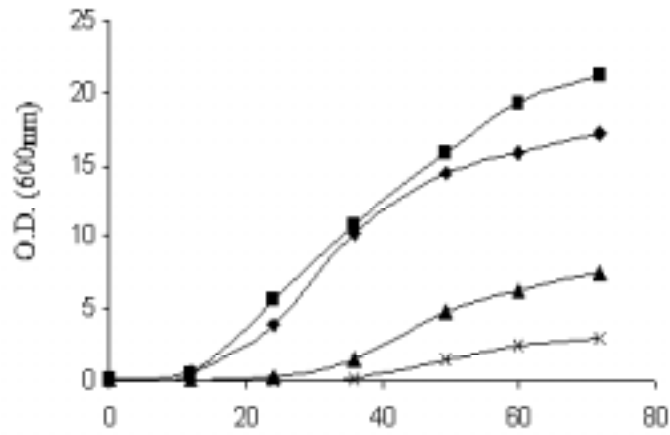
B



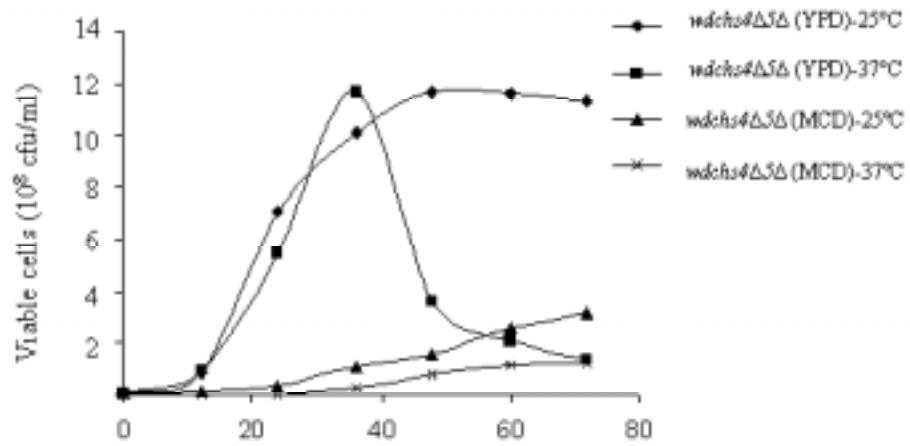
A

Figure 30. Growth and viability of the *wdchs4Δ5Δ11-1* mutant. The *wdchs4Δ5Δ11-1* mutant was grown in different media and at different temperatures. Its growth rate and viability were assayed by optical density (A) and viable counting (B) methods. Log-phase cultures were transferred to YPD or MCD media to the final concentration of 10^6 cell/ml. Cells were grown at 25°C or 37°C.

A



B



DISCUSSION

In this report, I demonstrated that WdChs5p was essential for the sustained growth of *W. dermatitidis* at 37°C both *in vitro* and *in vivo*. Cloning of the *WdCHS5* gene and analysis of its deduced amino acid sequence showed that a myosin motor-like domain was fused to a chitin synthase domain, a condition only found among some class V chitin synthases (Munro et al., 2001). Even though the significance of this putative gene fusion remains unknown, it is becoming clear that genes encoding class V isozymes are not uncommon (Munro et al., 2001). So far, at least nine similar chitin synthases, which have this unique structure, were identified from various fungi. Furthermore, class V chitin synthases are apparently only present in obligately filamentous fungi and some species of dimorphic and polymorphic fungi, including *P. brasiliensis* and *W. dermatitidis*. However, prior to the present investigation, only AnCsmA of *A. nidulans* and GgChsA of *G. graminicola* had received extensive study (Horiuchi et al., 1999; Amnuaykanjanasin et al., 2003). Particularly, studies from AnCsmA have established the important role of this chitin synthase in the maintenance of hyphal wall integrity and polarized hyphal wall synthesis, and especially its importance to this mold in growth and morphogenesis under low osmotic conditions. These studies also suggest that the post-translational processing of AnCsmA might be necessary for activating the chitin synthase domain. In *G. graminicola*, it is also found that GgChsA is essential for conidial wall strength in

media with high water potential and contributes to the strength of hyphal tips. Also, it has been previously reported from gene disruption studies in *A. nidulans* and *A. fumigatus* that class V chitin synthases have important functions (Specht et al., 1996; Aufauvre-Brown 1997). These studies showed that the disruptants had reduced mycelial chitin contents and growth rates, lysis of germinating conidia and swelling of hyphae. Taken together, the class V chitin synthases and the subclass of class V chitin synthase that have a myosin motor-like domain, play an important role in fungal growth and maintenance of cell wall integrity. The results of this study consistently demonstrated that WdChs5p functions in maintaining cell wall integrity in *W. dermatitidis*, but only at 37°C. Studies of AnCsmA also suggest that its expression might be subject to transcriptional regulation and that post-translational processing might be necessary for activating the chitin synthase domain. However, no evidence as yet supports the latter hypothesis or suggests that the putative myosin motor even associates with the actin. Nonetheless, because myosins with different functions have been identified in different cells, and some myosin tail domains contain a structural motif that may be used to direct the interaction of a given myosin with its cargo (Mermall et al., 1998; Steinberg et al., 2000), it remains tempting to speculate that the fusion of a myosin motor-like domain to a chitin synthase domain helps localize the chitin synthase in association with cytoskeletal structures. Support for this idea is provided by studies from *S. cerevisiae*, where Myo2p, a class V myosin, acts as a

transport motor required for the delivery of chitin synthase 3 (Chs3p; class IV) to the growing buds (Santos et al., 1997). Thus, it is speculated that the N-terminal domain of WdChs5p is a myosin motor that is necessary for the proper localization of the C-terminal chitin synthase domain by interacting with the actin. The localization of WdChs5p and its interaction with actin are currently under our investigation.

The study of the regulation of the *WdCHS5* expression strongly suggests that *WdCHS5* is a stress response gene (this study and Liu et al., 2001). The mRNA of *WdCHS5* was found at significantly higher levels in cells grown at 37°C, and under other stress conditions that promoted morphology changes, such as Ca²⁺ limitation, nitrogen starvation and low pH. The importance of this gene to the viability and virulence of *W. dermatitidis*, and the interesting expression pattern of its transcription prompted me to further characterize the promoter region of this gene. My analyses suggested that at least one negative regulatory element exists in the 5'-upstream regulatory region of *WdCHS5*. A similar conclusion has been made with *WdCHS3* (Wang et al., 2000). Taken together, it indicates that *W. dermatitidis* has at least two chitin synthase genes that are differentially expressed in response to a variety of adverse conditions. However, unlike the situation with the disruption of *WdCHS5*, the disruption of *WdCHS3* does not produce a temperature-sensitive phenotype. Nevertheless, we speculate that these two genes

share a similar regulatory mechanism or global regulation based on their similar differential expression patterns and the presence of common potential *cis*-acting elements in their promoter regions.

The original work from *S. cerevisiae* indicated the repression effect of the REPCAR1 site. Subsequently, the REPCAR1 sequences were found in several other eukaryotic organisms. Sequence alignment revealed that the symmetrical core sequence $C^{-2}C^{-1}G^0C^{+1}C^{+2}$ (CCGCC) possesses the greatest homology, and the potential REPCAR1 site in the *WdCHS5* promoter region also had this core sequence. Saturation mutagenesis of the REPCAR1 site in *S. cerevisiae* demonstrated that a C^{-1} -to-G change almost fully abolished the repression and a G^0 -to-A change also partially abolished the repression (Sumrada et al., 1987; Luche et al., 1990). However, changing other sites had no or little effect on the repression. A preliminary site-specific mutation study in *W. dermatitidis* demonstrated that when the REPCAR1 site of *WdCHS5* was mutated (C^{-1} -to-G and G^0 -to-A), the repression effect was partially relieved. This indicates that this unique site is actually a negative regulatory element and at least partially responsible for the repression. That the site-specific mutation did not totally abolish the repression may suggest either that there are other factors involved or the mutations did not fully abolish the function of this site.

Although, the mechanisms responsible for the increased mRNA levels of *WdCHS5* in stressed cells have not been determined, it is possible that either or both transcription initiation and posttranscriptional regulation are contributing to the increased mRNA levels of *WdCHS5*. The steady-state mRNA levels detected by Northern analyses are the cumulative result of mRNA synthesis and decay, as well as increased transcriptional initiation. Nonetheless, I favor the hypothesis that the increased transcriptional initiation is the major factor that contributes to the increased mRNA levels of *WdCHS5* in stressed conditions. Support for this possibility is provided by the previous semi-quantitative RT-PCR studies that detected comparable amounts of *WdCHS5* mRNA in cells grown continuously at 37°C for 24 h and cells grown at 25°C for 21 h and then shifted to 37°C and grown for additional 3 h (Wang et al., 2002). This strongly argues that the increased level of *WdCHS5* mRNA at 37°C is not due to the increased mRNA half-life, because if this were so, a much higher amount of *WdCHS5* mRNA from the cells grown continuously at 37°C compared to that of cells subjected to the temperature shift for just 3 h would have been seen. Experiments designed to distinguish which mechanism is more important are in progress.

The notion that *CHS* gene expression is under transcriptional regulation is not totally new. Previous studies of *S. cerevisiae*, *C. albicans* and other fungi have already indicated that the transcription of some *CHS* genes is under specific

regulation (Pammer et al., 1992; Munro et al., 1998). A computer analysis has identified many possible targets for transcriptional regulators in the 5' upstream regions (USR) of numerous *CHS* genes (Ruiz-Herrera, 2001). However, the presence of these transcriptional regulator targets does not mean that they are actually functional. Instead, such information can only be used to support the possibility and point one toward finding and investigating potential specific regulators. Based on this reasoning, our lab performed a similar *cis*-acting elements search in all *WdCHS* promoter regions. Both common regulatory elements and gene specific regulatory elements were found (data not shown), indicating that they may share general transcription regulation as well as individual specific regulation to perform their unique function. Another interesting observation was that most of the putative regulatory elements were not isolated; rather, they appeared to be clustered in short regions with some even overlapping. This further indicated that they might be parts of higher order regulatory units, which in turn supports the notion of coordinated regulation of given genes by different effectors.

Three different methods were used to disrupt *WdCHS5*. In each case, the disruption of *WdCHS5* resulted in mutants with temperature-sensitive phenotypes. At 25°C, the mutant yeast cells grew normally both on agar and in liquid medium, but at 37°C, dramatic changes of the cell morphology were observed after

prolonged incubation. This resulted in mutant cells that became hyperpigmented, swollen, and then often lysed and died. These observations lead to the conclusion that the loss of WdChs5p function results in yeast cell wall weakening at elevated temperature, which in turn brought about the loss of cell viability. This scenario is consistent with the hypothesis that WdChs5p has an essential function at the temperature of infection. Reintroduction of the *WdCHS5* gene successfully complemented the temperature-sensitive phenotype, which confirmed that the phenotype was the result of the loss of *WdCHS5* itself. The fact that *wdchs5Δ* mutant cells are identical to the wild-type cells at 25°C suggests that either WdChs5p has little or no function at this temperature or one or more of the other chitin synthases compensate for the loss of function of WdChs5p at lower temperatures. Nonetheless, it is obvious that none of the other four WdChsp isozymes can compensate for the loss of WdChs5p at the higher temperature. Successful derivation of all the possible double mutants involving *wdchs5Δ* disruption reinforced the above hypothesis.

To address the question of whether the expression of only the chitin synthase domain would complement the phenotype of *wdchs5Δ11* mutant, a vector, pHB3510, which contains the 3'-end chitin synthase domain-encoding region of *WdCHS5*, was constructed. This vector also contains the 1.2-kb *WdCHS5* promoter and a 2-kb *A. niger glaA* gene terminator. About one hundred

transformants were screened for their temperature sensitivity, but none of them showed the reversed phenotype. This indicated that the expression of only the chitin synthase domain was not able to complement the loss of *WdCHS5* and that the myosin motor-like domain was indispensable for the function of *WdCHS5*. This result is consistent with the study of *AnCsmA* in *A. nidulans*, which proved that expression of the chitin synthase domain cannot remedy the defects of an *AnCsmA* null mutant (Horiuchi et al., 1999). To investigate this possibility further, the myosin motor-domain's Lys residue in the conserved P-loop of *WdChs5p* was mutated to Ala in the vector previously used for complementation of *wdchs5Δ* mutants. Among 80 transformants, 20 of them were confirmed by Southern blots to have integrations with the mutated construct (data not shown), but none of them had the ability to complement the *wdchs5Δ11* mutant. This suggested that site-specific mutation of this Lys to Ala abolished this construct's ability to complement the phenotype of the *wdchs5Δ11* mutant. This further argued that the myosin motor-like domain is important for the proper function of *WdChs5p*. The P-loop is a highly conserved motif found in ATP- and GTP-binding proteins. The lysine residue is absolutely conserved in this motif and lysine side chain has been implicated in hydrogen bonding to the γ -phosphate as demonstrated by x-ray analysis of skeletal myosin bound to an ATP analog (Rayment et al., 1993; Fisher et al., 1995). In such a structure, alanine would clearly be unable to compensate for the charged lysine residues. The observed

detrimental effect of the lysine substitutions is well supported in the literature (Deyrup et al., 1998).

Cell wall weakening and abnormal cell shapes resulting from the disruption of a particular chitin synthase gene have been documented by a number of groups in different fungi, including *CaCHS1* of *C. albicans* (Munro et al., 2001) and *CsmA* of *A. nidulans* (Horiuchi et al., 1999). Interestingly, in *A. nidulans*, the growth defects of a *CsmA* null mutant were predominantly observed in old hyphal regions and the defects could be suppressed with an osmotic stabilizer (Takeshita et al., 2002). In *W. dermatitidis*, the weakening of the yeast cell wall and loss of cell viability at 37°C caused by the disruption of *WdCHS5* were only observed when mutant cells similarly entered mature stages of growth. Thus, it appears that this temperature-sensitive phenotype is different from that of a *wdchs1Δwdchs2Δ* double mutant, which is not able to grow at 37°C at all (Szaniszlo, 2002). This strongly suggests that *WdChs5p*, or the chitin synthesized by *WdChs5p* is essential for sustained or progressive yeast cell growth, but only at elevated temperature. Possibly either *WdChs1p* or *WdChs2p* must be functional at 37°C for the initial cell growth, but then *WdChs5p* is required for maintaining that growth at the high temperature. An additional possibility is that without *WdChs5p*, the mutant cells can grow for several generations, but when those cells become mature and older, especially while their intracellular contents are

increasing, they require their cell wall to have extra strength to sustain increased internal pressures. The observation that the temperature-sensitive phenotype can be rescued by supplementing media with osmotic stabilizers, such as sorbitol and sucrose, supported this idea and confirmed that the resulting cell lysis and death is largely due to the loss of cell wall integrity.

In yeast, several MAPK-mediated pathways have been identified that control different cellular processes (Molina et al., 1998). Among them is the so-called cell integrity pathway, which responds to high temperature, exposure to pheromones and hypotonic shock. In this particular pathway, signal transduction requires the action of protein kinase C (PKC), which is regulated by a small GTPase, Rho1. Downstream of the MAPK cascade is usually transcription factors, whose activation is controlled by the MAPKs through phosphorylation. Previously, in *S. cerevisiae*, the cell integrity pathway has been shown to play an important role in the transcriptional control of *ScCHS1*, *ScCHS2* and *ScCHS3* (Iguar et al., 1996). For the cell integrity pathway, alterations in the signaling pathway that controls the cell integrity lead to defects in cell wall assembly or maintenance and result in cell lysis. While inactivation of the initial common components of the pathway results in cell lysis at any temperature, loss of function of any of the components of the MAPK downstream pathway results in cell lysis only at high temperature. Because this cellular defect is confined to the cell wall structure and does not

affect the plasma membrane directly, the lytic phenotype can be remediated by providing osmotic support to the growth medium (e.g. 1 M sorbitol). Also, the lytic defect is usually characterized by the increase of cell permeability and causes release of intracellular contents to the external medium. Results from this study clearly demonstrated that the disruption of *WdCHS5* resulted in lysis of mutant cells at 37°C and supplementing osmotic stabilizer such as sorbitol and sucrose would prevent this cell lysis. Also, significantly higher amounts of alkaline phosphatase activity were detected from the mutant grown at 37°C for 48 h or 72 h and supplementing sorbitol to the medium completely reversed the increased alkaline phosphatase activity detected to the level of the wild type. Therefore, I propose that *WdChs5p* is an important factor in cell wall integrity pathway and the chitin synthesized by this specific enzyme is essential for sustained cell growth at 37°C in *W. dermatitidis*.

Any unfavorable circumstance that adversely affects cell growth can be designated a stress. Such circumstances differ widely, including not only environmental challenges such as heat or cold shock, osmotic dehydration, salt stress, extremes of pH, or oxidative stress, but also conditions of nutrient limitation. The molecular responses evoked to deal with such a diverse range of stress conditions are often not identical and are characteristic of a specific stress response (Mager et al., 1998). It is well-documented that STRE elements (general

Stress Responsive Elements) are a group of regulatory factors that are responsible for gene induction under stress conditions, including heat shock, osmotic stress, oxidative stress, and nitrogen starvation. Different stresses appear to induce the transcription of many genes by activating (generally multiple) STREs in the promoters of these genes. Based on the detection of high levels of *WdCHS5* mRNA when the wild-type *W. dermatitidis* cells were exposed to various stresses, including elevated temperature, low pH, calcium limitation and nitrogen starvation, it is proposed that *WdCHS5* is a stress response gene. Interestingly, at least three putative STRE elements were found in the promoter region of *WdCHS5*, which further supported this speculation. Whether or not the STRE elements or other potential *cis*-acting elements detected in the 5'-URS of *WdCHS5* are functional factors that contribute to the increased level of *WdCHS5* mRNA requires further studies.

My inability to show an absence of significant reductions of chitin synthase activity in the *wdchs5* Δ mutants compared to that of the wild-type strain, indicates that either the assay itself is not sensitive enough to detect the difference or that other chitin synthases are induced or activated to compensate for the loss of WdChs5p activity. However, the possibility that the increased chitin content of the *wdchs5* Δ mutants grown at 37°C is due to the addition of misplaced chitin contributed by one or more of the other WdChsp is favored. This notion was

supported by the semi-quantitative RT-PCR results, because at 37°C in *wdchs5Δ* background, the transcriptions of all other *WdCHS* genes were increased in various amounts (Wang et al., 2002). This hypothesis was further reinforced by the observation that the *wdchs5Δ* mutant cells tended to have considerably more chitin deposited in their cell walls than the wild-type strain when grown at 37°C, especially, for 48 h, as detected by Calcofluor staining. Additional supporting evidence that the disruption of *WdCHS5* triggered a compensatory pathway to protect cell wall integrity was provided by the finding that both the melanin expression and deposition were increased in the *wdchs5Δ11* mutant at 37°C based on the observation that the mutants were hyperpigmented and *WdPKS1* transcription increased by about 3.5 folds. However, the hyperpigmented *wdchs5Δ11* mutant could not cross-feed the albino melanin-deficient *wdpksΔ* mutant (data not shown) in which the polyketide synthase was disrupted (Feng et al., 2001), suggesting that the dark pigmentation and black secretions of the *wdchs5Δ11* mutant were not precursors of melanin but were probably polymerized melanin itself.

The fungal cell wall is a complicated and dynamic structure that performs many important functions that are essential for the cell survival in hostile environment. It has been reported that even a minor crack or fissure in the cell wall can lead to cell lysis (Cabib et al., 1989). The disruption of *WdCHS5* obviously voided the *W.*

dermatitidis yeast cell of a certain amount of chitin that was specifically synthesized by WdChs5p, and further led to a cell wall weakening and ultimately to cell lysis and death. Because the cell wall normally maintains a relatively intact structure and all its components are directly or indirectly connected to each other, when a specific essential factor is missing from it, either other factors are triggered to compensate for the loss or the loss itself triggers a cell wall integrity compensatory pathway that induces other factors to be overexpressed or activated to try to compensate for the loss.

Finally, it is proposed in this study that the loss of virulence in the *wdchs5Δ* mutants when tested in a mouse model of acute-infection is likely due to their inability to grow progressively at temperatures of infection. This hypothesis is supported by necropsy data that suggest the number of viable cells of the *wdchs5Δ11* mutant in mice decreased significantly with time: the infection was totally cleared from the liver/spleen by day 5 and 98% the infection was cleared from brains and kidneys by day 10. This result indicated that the mutant cells were incapable of sustained growth and survival under the elevated temperature associated with the mice. I thus suspect that the unique temperature-sensitive phenotype of the *wdchs5Δ11* mutant is a major contributing factor to this clearance and that the chitin contributed by WdChs5p, together with melanin, contributes greatly to its virulence.

To date, in *W. dermatitidis*, five chitin synthase structure genes have been identified (Table. 6) and characterized to different degrees. Successful derivation of all the double mutants involving disruption of *WdCHS5* made the collection of all the possible double mutants complete. Preliminary characterization of the double mutants involving *WdCHS5* demonstrated that three double mutants: *wdchs1Δwdchs5Δ11*, *wdchs2Δwdchs5Δ11* and *wdchs3Δwdchs5Δ11* had a phenotype similar to that of the *wdchs5Δ* single gene disruption mutant. However, the double mutant *wdchs4Δwdchs5Δ-11* had the combined phenotype of the *wdchs4Δ* and the *wdchs5Δ* mutants when grown on YPD agar (Table 7). The observation that the *wdchs4Δwdchs5Δ-11* mutant grew very poorly in MCD medium at 37°C indicated that both genes are required for this particular mutant's growth under certain conditions. That the *wdchs5Δ11* mutant can grow several generations in MCD medium at 37°C indicates that *WdCHS5* itself is only essential for cell maturation or progressive cell growth at this condition. However, *WdCHS4* is another important gene for normal cell growth in MCD medium at 37°C, as shown previously by disruption of *WdCHS4*. At 25°C, the *wdchs4Δ* mutant has similar growth rates to those of the wild-type strain in both YPD and MCD media. However, at 37°C, the *wdchs4Δ* mutant has significant lower growth rate in MCD medium, but not in YPD medium (Wang et al., 1999). Taken together, the disruption of *WdCHS4* and *WdCHS5* at the same time caused more severe growth defects when the double mutant was grown in MCD medium at

37°C. More interestingly, sequence alignment and phylogram analysis of all five chitin synthases of *W. dermatitidis* using the ClustalW program demonstrated that WdChs5p is most closely related to WdChs4p (Fig. 31). The further relationship between these two proteins is under our current investigation.

The absence of chitin from human and other animals makes it an attractive target for designing antifungal drugs. However, the presence of multiple chitin synthases and the potential different sensitivities of each chitin synthase to different drugs or inhibitors have mitigated some efforts. This study demonstrates that WdChs5p is an essential enzyme for the viability of *W. dermatitidis* both *in vitro* and *in vivo* at 37°C and that the loss of WdChs5p alone can result in a mutant's inability to survive long enough to produce a lethal infection in mice. This in turn suggests that WdChs5p, because of its essential role in the maintenance of *W. dermatitidis* at the elevated temperatures associated with infections, is a particularly suitable target for the design of an antifungal drug with therapeutic value in the treatment of the various mycoses caused by this fungus. Should additional class V chitin synthases with similar attributes be present in other dematiaceous pathogens, possibly such an antichitin drug would have wide usefulness in the treatment of the diseases caused by this large group of fungi that are so difficult to eradicate during human infections.

Table 6. Summary of chitin synthase genes in wild-type *W. dermatitidis*

Gene/Protein	Base pairs/Amino acids	Introns	Isozyme class	GenBank #
<i>WdCHS1</i> / WdChs1p	3011/988	1(47)	II	AF054503
<i>WdCHS2</i> / WdChs2p	2843/928	1(59)	I	AF052606
<i>WdCHS3</i> / WdChs3p	2756/885	2(54&48)	III	AF053314
<i>WdCHS4</i> / WdChs4p	3714/1238	0	IV	AF126146
<i>WdCHS5</i>/ WdChs5p	5765/1885	2(53&57)	V	AF469116

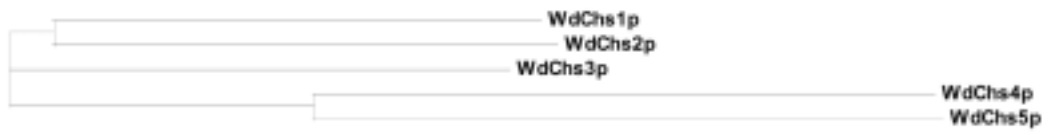
Used with the author's permission (Szamiszlo, 2002)

Table 7. Summary of chitin synthases gene disruption in wild-type *W. dermatitidis*

Disruption strain	Isozyme classes affected	Gene markers used	Phenotypic characteristics produced
<i>wächs1</i> Δ	II	<i>hph/ble</i>	Short yeasts chains
<i>wächs2</i> Δ	I	<i>hph/WdURA5</i>	No morphological change, but drastically reduced Chsp activity at 25 and 37°C
<i>wächs3</i> Δ	III	<i>hph</i>	No morphological change, but reduced Chsp activity at 37°C
<i>wächs4</i> Δ	IV	<i>hph</i>	Clumped yeasts, multiple buds, lowered chitin, hyperpigmentation at 37°C
<i>wächs5</i> Δ	V	<i>hph</i>	No morphological change at 25°C, but conditionally lethal and hyperpigmented at 37°C
<i>wächs1</i> Δ <i>wächs2</i> Δ	I, II	<i>ble & hph</i>	Abnormal hyphal growth with defective septa at 25°C
<i>wächs1</i> Δ <i>wächs3</i> Δ	II, III	<i>ble & hph/hph & ben</i>	Like <i>wächs1</i> Δ
<i>wächs1</i> Δ <i>wächs4</i> Δ	II, IV	<i>hph & sur</i>	Combined phenotype of <i>wächs1</i> Δ and <i>wächs4</i> Δ
<i>wächs1</i> Δ <i>wächs5</i> Δ	II, V	<i>hph & sur</i>	Like <i>wächs5</i> Δ
<i>wächs2</i> Δ <i>wächs3</i> Δ	I, III	<i>sur & ble</i>	Normal morphology except for greatly reduced Chsp activity at 37°C; less virulent in mouse models
<i>wächs2</i> Δ <i>wächs4</i> Δ	I, IV	<i>hph & sur</i>	Like <i>wächs4</i> Δ
<i>wächs2</i> Δ <i>wächs5</i> Δ	I, V	<i>hph & sur</i>	Like <i>wächs5</i> Δ
<i>wächs3</i> Δ <i>wächs4</i> Δ	III, IV	<i>hph & sur</i>	Like <i>wächs4</i> Δ
<i>wächs3</i> Δ <i>wächs5</i> Δ	III, V	<i>hph & sur</i>	Like <i>wächs5</i> Δ
<i>wächs4</i> Δ <i>wächs5</i> Δ	IV, V	<i>sur & hph</i>	Combined phenotype of <i>wächs4</i> Δ and <i>wächs5</i> Δ
<i>wächs1</i> Δ <i>wächs2</i> Δ <i>wächs3</i> Δ	II, I, III	<i>ble & sur & hph</i>	Like <i>wächs1</i> Δ <i>wächs2</i> Δ except morphology even more abnormal at 25°C
<i>wächs1</i> Δ <i>wächs3</i> Δ <i>wächs4</i> Δ	II, III, IV	<i>ble & sur & hph</i>	Combined phenotype of <i>wächs1</i> Δ and <i>wächs4</i> Δ

(modification of Szaniszló, 2002)

Figure 31. ClustalW phylogram analysis of the amino acid sequences of five chitin synthases of *W. dermatitidis*



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