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WdChs1p, a class II chitin synthase, is more responsible than WdChs2p (Class I) for normal yeast reproductive growth in the polymorphic, pathogenic fungus *Wangiella (Exophiala) dermatitidis*

Li Zheng, Leonel Mendoza, Zheng Wang, Hongbo Liu, Changwon Park, Sarah Kauffman, Jeffrey M. Becker, and Paul J. Szanislo

Section of Molecular Genetics and Microbiology, School of Biological Science and Institute for Cellular and Molecular Biology, University of Texas at Austin, 1 University Station A5000, Austin, TX 78712-0162, USA

Microbiology Department, University of Tennessee, Knoxville, TN 37919, USA

Abstract

The chitin synthase gene *WdCHS1* was isolated from a partial genomic DNA library of the pathogenic polymorphic fungus *Wangiella dermatitidis*. Sequencing showed that *WdCHS1* encoded a class II chitin synthase composed of 988 amino acids. Disruption of *WdCHS1* produced strains that were hyperpigmented in rich media, grew as yeast at wild-type rates at both 25 and 37°C and were as virulent as the wild type in a mouse model. However, detailed morphological and cytological studies of the *wdchs1Δ* mutants showed that yeast cells often failed to separate, tended to be enriched with chitin in septal regions and, sometimes, were enlarged with multiple nuclei, had broader mother cell–daughter bud regions and had other cell wall defects seen considerably less often than in the wild type or *wdchs2Δ* strains. Disruption of *WdCHS1* and *WdCHS2* in the same background revealed that WdChs1p had functions synergistic to those of WdChs2p, because mutants devoid of both isozymes produced growth that was very abnormal at 25°C and was not viable at 37°C unless osmotically stabilized. These results suggested that WdChs1p was more responsible than WdChs2p for normal yeast cell reproductive growth because strains with defects in the latter exhibited no morphological abnormalities, whereas those with defects in WdChs1p were frequently impaired in one or more yeast developmental processes.

Introduction

Wangiella dermatitidis (Kano) McGinnis [*Exophiala dermatitidis* (Kano) de Hoog] is one among more than 100 other dematiaceous (melanized) fungi known to cause human disease (McGinnis 1977; Hoog de and Hermanides-Nijof 1977; Matsumoto et al. 1994). Although traditionally most associated with primary dermatotrophic forms of cutaneous and subcutaneous phaeohyphomycosis, this asexual fungus has recently become better known as a paradigm for this emerging mycosis because of its increasing detection as a systemic pathogen in both immunocompetent and immunocompromised patients (Matsumoto et al. 1993, 1994; Chang et al. 2000). Moreover, because *W. dermatitidis* has a well-defined and easily

E-mail: pjszanislo@mail.utexas.edu, Tel.: +1-512-4713384, Fax: +1-512-4717088.

U. S. Patent and Trademark Office, Remsen Building, Room 2D18, 400 Dunlany Street, Alexandria, VA 22313, USA

Medical Technology Program, Michigan State University, East Lansing, MI 48824, USA

The Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, 4555 Overlook Ave. Bldg. 30/Code 6910, Washington, DC 20375, USA

Wyeth Research, Chemical and Screening/Natural Products Research, Pearl River, NY 10965, USA

manipulated polymorphic vegetative nature, a well-characterized cell wall chemistry and a rapidly improving molecular tractability, it currently serves as the model of choice for discovering cell wall-related virulence determinants among the dematiaceous fungi, irrespective of the mycoses they cause and the morphotypes of the agents involved (Szaniszlo et al. 1983; Montijn et al. 1997; Kwon-Chung et al. 1998; Szaniszlo 2002). The model studies have confirmed by molecular methods that both 1,8-dihydroxynaphthlene (1,8-DHN) melanin and chitin, a linear homopolymer of $\beta(1 \rightarrow 4)$ *N*-acetylglucosamine, have relevance to the virulence of *W. dermatitidis*, and therefore presumably the virulence of other dematiaceous pathogens of humans (Feng et al. 2001; Wang et al. 2001; Szaniszlo 2002; Liu et al. 2004). They have also shown that chitin and $\beta(1 \rightarrow 3)$ glucan serve as the main structural constituents of the cell walls of *W. dermatitidis* and that chitin content varies widely with morphotype (Szaniszlo et al. 1983; Montijn et al. 1997). For example, chitin is a relatively minor component in yeast cells where it is localized primarily in septal regions (Cooper et al. 1984; Szaniszlo and Momany 1993). In contrast, hyphae and sclerotic bodies, which are the two main alternative vegetative morphotypes of *W. dermatitidis*, not only have chitin in their septal regions, but also have considerable additional chitin in other cell wall areas (Szaniszlo et al. 1983; Cooper et al. 1984; Jacobs et al. 1985; Harris and Szaniszlo 1986; McIntosh 1996).

In previous reports, we described in detail results about the cloning and effects of disruption in *W. dermatitidis* of the four chitin synthase structural genes *WdCHS2*, *WdCHS3*, *WdCHS4*, and *WdCHS5*, but only preliminarily described results related to *WdCHS1* (Wang et al. 1999, 2001, 2002; Wang and Szaniszlo 2000, 2002; Szaniszlo 2002; Liu et al. 2004). Our studies showed that each *WdCHS* gene encodes an isozyme (WdChsp) of a different class, according to the system first established by Bowen et al. (1992) and later extended by others (Din et al. 1996; Specht et al. 1996). They further suggested that isozymes WdChs1p (class II), WdChs2p (class I) and WdChs4p (class IV) are orthologs of Chs2p, Chs1p and Chs3p, respectively, in *Saccharomyces cerevisiae*, whereas isozymes WdChs3p (class III) and WdChs5p (class V) have no equivalents in that fungus (Szaniszlo 2002). In addition, our analyses of a number of chitin synthase mutants (*wdchsΔ*) with each gene disrupted singly have revealed that no single WdChsp is essential for viability and good growth at 25 and 37°C, with the important exception that mutants with only *WdCHS5* disrupted cannot sustain wild-type growth rates and viability for more than a few generations at 37°C (Wang et al. 1999, 2001; Wang and Szaniszlo 2000; Szaniszlo 2002; Liu et al. 2004; this study and unpublished data). Consequently, the *wdchs5Δ* mutants show little virulence in a mouse model of acute infection, unlike the *wdchs2Δ*, *wdchs3Δ* and *wdchs4Δ* single disruption mutants tested previously in the same model (Szaniszlo 2002). Among the double *wdchsΔ* mutants tested for virulence prior to this report, only those with both *WdCHS2* and *WdCHS3* disrupted in the same strain have similarly reduced virulence (Wang et al. 2001; Szaniszlo 2002). However, in this case, the loss of virulence is not attributable to the acquisition of a temperaturelethal phenotype or to any other readily apparent abnormalities.

In this report, we describe how *WdCHS1* of *W. dermatitidis* was cloned and new details about the effects of its disruption in the wild-type strain and in strains with *WdCHS2* disrupted. Our new results document that mutants with *WdCHS1* disrupted are as virulent as the wild type when tested in a mouse model, even though they are sometimes defective in normal yeast reproductive development. We further document that in contrast to *wdchs1Δ* and *wdchs2Δ* single disruption strains, mutants with both *WdCHS1* and *WdCHS2* disrupted in the same background are without virulence, because in the manner of the *wdchs5Δ* mutants this double disruption combination again produces strains with a temperaturelethal phenotype, which can be partially rescued by growth in the presence of osmotic stabilizers. Cytological and ultrastructural examinations of the *wdchs1Δwdchs2Δ* mutants grown at 25°C show that they are much more defective in cell shape, septum formation and cell separation ability than are the yeasts of the *wdchs1Δ* single mutants, and very different from the wild-type yeast and the

normal-appearing yeast of the *wdchs2Δ* mutants as described here and previously (Wang et al. 2001). We suggest that these particular results with the *wdchs1Δwdchs2Δ* mutants mean that WdChs1p has functions that are synergistic to those of WdChs2p, such that at least one of these two isozymes must be functional for good growth at 25 and 37°C. However, our finding that *wdchs1Δ* mutants, but not *wdchs2Δ* mutants, frequently exhibit defects in normal yeast cell development and cell division also implies that the functions of WdChs1p are not completely redundant with those of WdChs2p, and instead suggest that of these two isozymes WdChs1p, the class II chitin synthase of *W. dermatitidis*, is the primary isozyme responsible for normal yeast reproductive growth and that WdChs2p (class I) serves as an efficient, but less than perfect backup chitin synthase isozyme when WdChs1p fails to function or is not produced.

Materials and methods

Strains and media

The *W. dermatitidis* strains used in this work are listed in Table 1. The laboratory wild-type strain 8656 (ATCC 34100 [*E. dermatitidis*, CBS 525.76]), used as the original stock for the derivation of all the *wdchsΔ* mutants, has been extensively described previously (see, for example, Karuppayil and Szaniszlo 1997). Mutant *wdchs2Δ-1* and the methods used for its derivation have also been described in detail, whereas the derivation and nature of mutant *wdchs1Δwdchs2Δ-A* have only been described preliminarily (Wang et al. 1999,2001;Wang and Szaniszlo 2002). Strains INVαF' (Invitrogen), JM019 (Promega) and XL1-Blue (Stratagene) were used as *Escherichia coli* plasmid hosts. Culture of *W. dermatitidis* was in the rich broth medium YPD or in the synthetic media CDN and SD (Wang et al. 1999;Zheng and Szaniszlo 1999). Drug selection plates for isolating *W. dermatitidis* transformants, resistant to hygromycin B (HmB) or to bleomycin/phleomycin/chlorimuron ethyl, were prepared as described previously (Wang et al. 1999,2001). *E. coli* strains were grown in Luria-Bertani medium supplemented with 100 μg of ampicillin or 20 μg of chloramphenicol/ml.

Preparation and analysis of DNA and construction of a genomic library

Genomic DNA was isolated from *W. dermatitidis* by spheroplasting with zymolyase (ICN Biochemicals, Inc.), followed by detergent lysis, phenol–chloroform extraction and ethanol precipitation as described previously (Zheng and Szaniszlo 1999). Southern blotting was performed by standard methods (Ausubel et al. 1989). DNA fragments (~25 ng) used for probes in Southern analysis and colony hybridizations were labeled with [α -³²P]dATP using the Prime-a-Gene kit (Promega). To clone *WdCHS1*, a partial genomic library was constructed by digesting DNA with *Bam*HI and then excising and eluting DNA fragments of 5–10 kb from a gel after electrophoresis. After ligation of the DNA fragments into the Lambda FIXII vector (Stratagene), the ligation products were transformed into XL1-Blue *E. coli* cells for amplification according to the manufacturer's instructions. Portions (~2.0–5.0×10⁴ pfu/plate) of the resulting *Bam*HI library were then cultured on NZY medium, prior to transfer to Nytran membranes (Schleicher and Schull) for screening by colony hybridization with the mixture of PCR-generated fragments of *WdCHS1*, *WdCHS2* and *WdCHS3* described previously (Bowen et al. 1992) for an initial probe. Southern hybridization experiments were then used to identify clones that contained the *WdCHS1* gene. Methods for nucleic acid analysis have been described previously (Wang et al. 1999; Zheng and Szaniszlo 1999). Chromosome preparation and CHEF electrophoretic methods have also been described (Zheng and Szaniszlo 1999).

Plasmids and transformations

Two methods were used to disrupt *WdCHS1* in the wild-type strain background. For the first, the integrative disruption vector pLZ44 was made by ligating a 1.4 kb *Bgl*III/*Hind*III fragment from *WdCHS1* into the *Bam*HI/*Hind*III sites of PAN7-1 (Peng et al. 1995; Wang et al. 2001), which contains the *gpd* promoter and the *hph* gene for selection of HmB transformants. Prior

to use in transformations, this integrative disruption vector was linearized in the *WdCHS1* targeting sequence with *KpnI*. For the second, the replacement vector pLZ56, briefly described previously (Zheng and Szaniszlo 1999; Wang et al. 2001), was constructed to ensure the removal of the complete *WdCHS1* sequence and to produce mutants with the same phenotype as those derived by the integrative disruption method, but with the *ble* gene for selection of phleomycin-resistant transformants: this strategy was used to provide a *wdchs1Δ* strain (*wdchs1Δ-2*) in which *WdCHS2* could subsequently be disrupted using a vector with the *hph* gene that produces transformants with better efficiency. Specifically, pLZ56 was constructed by inserting a 3.3 kb *BglIII* fragment from pUT737 (from CAYLA Co.), which has the *TRI* promoter from *Trichoderma reesei* that allows strong expression of the bacterial *Streptoalloteichus hindustanus ble* gene in filamentous fungi, into the *BglIII* site located in the middle of pWdCHS1.KS (Mendoza 1995; Wang et al. 2001). In this case, the actual gene replacement of *WdCHS1* was accomplished with a 7.8 kb *StuI* linear fragment. The *wdchs1Δwdchs2Δ* double mutants were then generated using pLZ41 (Wang et al. 2001) to disrupt *WdCHS2* in the *wdchs1Δ-2* mutant (*wdchs1::ble*) by selection for HmB resistance. All transformations of *W. dermatitidis* were carried out by electroporation of intact cells as described previously (Zheng and Szaniszlo 1999).

Primers and RT-PCR amplifications

The primers used to detect *WdCHS1* expression and confirm the presence and size of the putative intron were *WdCHS1*-RT1 (5' TCGCATCGCAGAACTTTGA 3') and *WdCHS1*-RT2 (5' TAAGCCATCGTCGTCGCTCCGCT 3'). The methods for the synthesis of the first strand of cDNA and subsequent PCR and RT-PCR amplifications were as described previously (Wang et al. 2001).

Microscopy and photomicroscopy

Cellular morphology was documented as described previously (Karuppayil and Szaniszlo 1997; Wang et al. 1999) using a Zeiss ICM 405 inverted microscope and a Zeiss Axioimager (Carl Zeiss, Inc.). Procedures for staining of the cell wall with Calcofluor (Sigma) and for the staining of nuclei with DAPI (4',6'-diamide-2-phenylendole; Accurate Chemical), as well as those for transmission electron microscopy, have also been described (Cooper et al. 1984; Ye and Szaniszlo 2000).

Chitin content and chitin synthase activity assays

Chitin contents and chitin synthase activity levels were measured by the method described previously (Wang et al. 1999; Zheng and Szaniszlo 1999).

Virulence studies in mice

Virulence test of the *wdchsΔ* mutants in an immunocompetent mouse model of acute infection were mostly done as described previously (Wang et al. 1999, 2001). However, because the *wdchs1Δwdchs2Δ* double mutants grew more slowly than the other strains at 25°C and in an aberrant, more hyphal and pseudohyphal morphology that was difficult to quantify by hemacytometer counting, inocula for these mutants were adjusted by OD 600 to the proper concentrations by comparison with the standard inocula of the other strains.

Nucleotide sequence accession number

The nucleotide sequence of the *WdCHS1* gene was assigned the GenBank accession number AF054503.

Results

***WdCHS1* encodes a class II chitin synthase**

A probe consisting of a labeled mixture of 600 bp PCR products amplified with degenerate primers designed from conserved chitin synthase sequences identified ten putative clones with *WdCHS* genes in a Lambda FIXII genomic library, one of which contained the full-length *WdCHS1* gene. After release of *WdCHS1* from the Lambda FIXII vector with *NotI*, the resulting fragment was mapped by PCR (data not shown) and restriction analysis (Fig. 1a), which showed that the gene was about 5.8 kb in length, and that the 600 bp sequence that was amplified by PCR was located about 1.0 kb from its 5' end and about 4.2 kb from its 3' end. Subsequent chromosome mapping showed that *WdCHS1* was located in chromosome 2 of strain 8656 (Fig. 1b). Sequence analysis revealed that *WdCHS1* consisted of a 2,964 bp open reading frame, which was interrupted by a 47 bp intron (position bp 1,694–1,740) and encoded a class II chitin synthase consisting of 988 amino acids (data not shown). Confirmation of the intron length and position was accomplished by RT-PCR amplifications that produced bands from mRNA with the expected size of about 350 bp. The exact nature of the 47 bp intron was then confirmed by comparing the sequence of the 350 bp RT-PCR product amplified with mRNA from cells grown at 25 and 37°C with that of a 390 bp product amplified with genomic DNA (data not shown). A TATA-like element was also identified at –40 bp upstream of the start codon. Comparisons of the deduced WdChs1p amino acid sequence with those of other chitin synthases, minus 121 amino acids at their N-termini where there are divergences too great to produce meaningful comparisons, indicated highest amino acid identity with CiChs2p (78%) of *Coccidioides immitis* (GenBank submission no. AF329357). Progressively lower amino acid identities were found with AnChsAp (73%) of *Aspergillus nidulans* (Yanai et al. 1994), NcChs2p (73%) of *Neurospora crassa* (Din and Yarden 1994), CaChs1p (53%) of *Candida albicans* (Au-Young and Robbins 1990) and Chs2p (52%) of *S. cerevisiae* (Silverman 1989).

WdChs1p is not essential for viability

As a prerequisite for the functional analyses of WdChs1p, *WdCHS1* was disrupted by two methods that produced viable strains, and thus documented that this gene was not essential for the viability of *W. dermatitidis*. In the first, *WdCHS1* disruption was accomplished by an integrative method with pLZ44, which carried the *hph* selection marker (Fig. 2a). This methodology produced two transformants that were shown by Southern analysis to be *wdchs1Δ* mutants (*wdchs1Δ-1A*, *wdchs1Δ-1B*) with the *WdCHS1* gene suitably enlarged from 5.8 to 13.1 kb, as predicted (Fig. 2b). In the second method, disruption was accomplished by replacement with pLZ56 (Wang et al. 1999,2001), which carried the *ble* selection marker (Fig. 2c). Although the transformation efficiency in this case was extremely high (100–500 transformants/μg DNA), the frequency of site-specific integration was very low and produced only one *wdchs1Δ* mutant (*wdchs1Δ-2*) out of the 76 transformants initially screened microscopically for the frequent chaining yeast phenotype observed for *wdchs1::hph* mutants (see Fig. 3b). Nonetheless, Southern analysis showed that *WdCHS1* had been deleted in *wdchs1Δ-2* (Fig. 2d), which further confirmed that *WdCHS1* was not an essential gene for *W. dermatitidis*.

Loss of WdChs1p affects pigmentation and sometimes alters yeast cell shape, septation and cell separation, but has little or no effect on chitin synthase level, chitin content, growth rate or virulence

Light microscopic observations of the three *wdchs1Δ* strains grown at 25°C (Fig. 3b) or at 37°C (data not shown) showed that all were essentially identical and often formed short yeast chains, whereas the wild-type strain and the *wdchs2Δ* mutants grown identically showed this tendency much less frequently (Fig. 3a, c; Table 2; Wang et al. 2001). In addition, cells of the

wdchs1Δ mutants more often than the wild type (Fig. 4a) or the *wdchs2Δ* mutants (Fig. 4g) were aberrantly enlarged, had broader mother cell–daughter bud necks and had more than one nucleus (Fig. 4d, e; Table 2), although the vast majority of *wdchs1Δ* cells had only one nucleus per cell in the manner of the wild type and the *wdchs2Δ* mutants (Fig. 4b, h; Wang et al. 2001). Calcofluor staining patterns reflective of sites of major chitin deposition in the *wdchs1Δ* mutants, the wild type and *wdchs2Δ* mutants were also generally similar from the point of view that the most intense staining tended to be restricted to septal regions (Fig. 4c, f, i). However, septal regions and certain side wall regions of the *wdchs1Δ* mutants (Fig. 4f), but not those of the wild type or the *wdchs2Δ* mutants (Fig. 4c, i), were occasionally stained more intensely. Transmission electron micrographs of cells of the *wdchs1Δ-A* mutant confirmed that whereas most cells indeed had normal cytoplasm, many had septal regions and septa that were, in fact, abnormal when compared to those of the wild type. They also confirmed that whereas the wild-type strain usually showed a narrow mother cell–daughter bud neck region and both primary and secondary septa (Fig. 5a; see also Figs. 4a, b, 5, 6, 7 of references Cooper et al. 1984; Grove et al. 1973, respectively), the septal regions of budding *wdchs1Δ-A* cells were not only broader sometimes, but also had cell walls that were thicker and septa devoid of clearly defined primary and secondary layers (Fig. 5b–d) or that were sometimes even absent, which produced cells that were multinucleate (Fig. 5e). Although these microscopic data suggested that the disruption of *WdCHS1* frequently affected septal regions, septa and cell separations to some degree, this perturbation was not reflected by major reductions in total chitin synthase activity (Fig. 6a), chitin content (Fig. 6b), or growth rates at 25°C (Fig. 7a) or 37°C (Fig. 7b). Interestingly, the *wdchs1Δ* mutants also formed colonial growth at 25°C that was more black than brown on YPD agar medium, compared to that of the wild-type strain and the *wdchs2Δ* strains (Fig. 8a). However, this hyperpigmentation difference was not apparent at 25°C (Fig. 8b) when 1 M sorbitol was added to the medium. Lastly, as with the *wdchs2Δ*, *wdchs3Δ* and *wdchs4Δ* single chitin synthase disruption mutants (Wang et al. 1999, 2001; Wang and Szaniszlo 2000), the *wdchs1Δ* mutants showed no loss of virulence when tested in a mouse model of acute infection (Fig. 9).

Mutants with both *WdCHS1* and *WdCHS2* disrupted are severely abnormal in morphology and cytology when grown at 25°C

Although only one *wdchs1Δ* disruption strain (*wdchs1Δ-2*) was initially obtained using pLZ56 and selection for bleomycin/phleomycin resistance, its derivation was important because it provided a strain background that allowed additional *WdCHS* genes to be disrupted by the use of more efficient vectors containing either *hph* or *sur* selection markers (Table 1; Wang et al. 1999). For example, the three *wdchs1Δwdchs2Δ* double mutants were generated for this study using the disruption vector pLZ41 (Wang et al. 2001) to disrupt *WdCHS2* in the *wdchs1::ble* mutant background. Among the eight transformants obtained with pLZ41, three were shown by Southern analysis to be *wdchs1Δwdchs2Δ* mutants (data not shown), and one (*wdchs1Δwdchs2Δ-A*) has been described previously (Wang et al. 1999). Microscopic observations of the three *wdchs1Δwdchs2Δ* double mutants cultured at 25°C showed that they all had dramatically altered cell morphologies (Figs. 3d, 4j), many cells with increased nuclear numbers (Fig. 4k) and considerably more intense cell wall and septal region chitin staining patterns (Fig. 4l), when compared to the wild type (Figs. 3a, 4a–c) or either the *wdchs1Δ* (Figs. 3b, 4d–f) or *wdchs2Δ* mutants (Figs. 3c, 4g–i; Wang et al. 2001). Furthermore, at this temperature, the *wdchs1Δwdchs2Δ* mutants grew at slower rates (Fig. 7a) and were hyperpigmented (black vs. brown) in the manner of the *wdchs1Δ* mutants (Fig. 8a). Transmission electron microscopic images confirmed that the septal region profiles in the *wdchs1Δwdchs2Δ-A* double mutant cultured at 25°C were again sometimes devoid of septa and when septa were present they were consistently even more aberrant than those of the *wdchs1Δ-1* mutant (Fig. 10a–c).

Mutants with both *WdCHS1* and *WdCHS2* disrupted have lower chitin synthase activities, wild-type levels of chitin, cannot grow at 37°C unless protected with osmotic stabilizers and do not cause lethal infections in mice

Because the majority of chitin synthase activity found in *W. dermatitidis* is contributed by WdChs2p (Wang et al. 2001), the finding that the *wdchs1Δwdchs2Δ* mutants grown at 25°C had chitin synthase levels reduced approximately to low levels of *wdchs2Δ* mutants was not unexpected (Fig. 6a). However, these same mutants showed no equally dramatic decreases in chitin content (Fig. 6b). These results suggested that the chitin synthesized by one or more of the remaining WdChsp isozymes compensated for the loss of chitin contributed by WdChs1p and WdChs2p at 25°C in the *wdchs1Δwdchs2Δ* mutants and allowed for their growth, albeit in an abnormal morphology: supporting this hypothesis was the intensified and more generalized Calcoflour fluorescent staining indicative of chitin deposition throughout the entire cell surfaces of the *wdchs1Δwdchs2Δ* double mutants (Fig. 4l) and presence in some cells of the abnormal cell wall structures that potentially may be enriched with chitin (Fig. 10d).

In contrast to the growth at 25°C, the *wdchs1Δ-wdchs2Δ* mutants cultured at 37°C grew very little in liquid or on plate media. This lack of growth at 37°C was observed with cells grown on either nutrient-rich YPD medium (Figs. 7b, 8c) or nutrient-poor SD and CDY media (data not shown). However, when the YPD agar medium was amended with 1 M sorbitol (Fig. 8d), considerable growth at 37°C was observed, suggesting that the cell wall integrity of the double mutant had been partially restored. Light microscopic examination of the *wdchs1Δwdchs2Δ* mutant grown at 37°C in sorbitol-amended YPD liquid medium (Fig. 8e) also showed it was obviously protected from the lysis and the death that occurred at 37°C in the same medium in the absence of sorbitol (Fig. 8f) and had reverted to the multinucleate (data not shown), more filamentous morphology of the double mutant that develops under those conditions at 25°C. Although the *wdchs1Δwdchs2Δ* mutants with this temperaturelethal sensitivity were expected to be avirulent in our mouse model of acute infection, three *wdchs1Δwdchs2Δ* strains were nonetheless tested to substantiate this supposition (Fig. 9). In all cases, the *wdchs1Δwdchs2Δ* mutants were found to have lost virulence in comparison to the wild type and the *wdchs1Δ* and *wdchs2Δ* mutants.

Discussion

In a recent publication, Ichinomya et al. (2005) reported that the class I (ChsCp) and class II (ChsAp) chitin synthases of the filamentous, conidiogenous fungus *A. nidulans* are both involved in septum formation. They further state that prior to their report no evidence documented that a class II chitin synthase was involved in septum synthesis in such a filamentous fungus. Our findings with *W. dermatitidis* in the present report provide similar evidence with a second conidiogenous, filamentous fungus and extend the involvement of the two isozymes types to the processes that occur during normal yeast cell development and cell division in one capable of both hyphal and yeast growth. However, our results indicate that this pathogen's class I and class II isozymes function synergistically and also that the class II chitin synthase of *W. dermatitidis* is more important than its class I isozyme and thus is not completely redundant functionally with it during yeast reproductive growth. The evidence for our first conclusion that the class I and class II chitin synthases of *W. dermatitidis* have synergistic functions is based on our findings that whereas neither isozyme is essential for viability, and strains with either a functional WdChs1p (class II) or a functional WdChs2p (class I) grow at wild-type rates at 25 or 37°C, strains with both isozymes inactivated in the same background grow poorly, have very abnormal morphologies with aberrant septal regions and septa when cultured at 25°C and are not viable when cultured at 37°C unless osmotically protected. The evidence for our contention that WdChs1p is more important for normal yeast reproductive growth than WdChs2p is as follows: (1) disruption of *WdCHS1* causes mutants

to produce inordinate numbers of short polarized chains of yeast, whereas disruption of *WdCHS2* does not, even though total chitin synthase activities are greatly reduced in the latter; (2) cells of *wdchs1Δ* mutants, but not *wdchs2Δ* mutants, are sometimes abnormally enlarged and have more than one nucleus; (3) some cells and the septal regions between cells of *wdchs1Δ* mutants, but not of *wdchs2Δ* mutants, are enriched with chitin and frequently appear defective when observed ultrastructurally; (4) cells of *wdchs1Δ* mutants, but not of *wdchs2Δ* mutants, are hyperpigmented, a condition that has been suggested previously to be correlated with the loss of cell wall integrity in *W. dermatitidis* (Szanişzlo 2002; Liu et al. 2004); (5) the hyperpigmentation phenotype of the *wdchs1Δ* mutants can be reversed by sorbitol additions to their growth media, which supports the notion that the hyperpigmentation is associated with loss of cell wall integrity. Taken together, we suggest these results mean that WdChs1p is more responsible than WdChs2p for normal cell wall integrity, septation and error-free cytokinesis and yeast cell separation, even though the latter can substitute for the former with adequate, but not perfect, efficiency.

Additional support for our contention that WdChs1p (class II) is more intimately involved with normal yeast reproductive growth than WdChs2p (class I) is indirect and based largely on the analogy between the functional roles played by its nonessential ortholog (Chs2p) in *S. cerevisiae* and its essential ortholog (Chs1p) in *C. albicans* (Cabib 2004; Munro et al. 2001). For example, our identification of WdChs1p as a class II chitin synthase means that our suggested functions for it in *W. dermatitidis* are very similar to those played by their equivalents in both these yeasts: in these well-studied fungi, the class II isozymes are similarly responsible for primary septum formation, whereas at least in *S. cerevisiae* the class I isozyme (Chs1p) acts primarily in an auxiliary capacity to repair any defects that occur in septal regions during cell separations (Cabib 2004; Cabib et al. 1992). In addition, disruption of *WdCHS1*, as in the manner of disruption of *CHS2* in *S. cerevisiae* or lack of expression of *CaCHS1* in *C. albicans*, produces mutants having little reduction in total chitin synthase activity. In contrast, total chitin synthase activity is drastically reduced in strains of the same three species devoid of their functional class I isozymes (Cabib et al. 1989; Gow et al. 1994; Wang et al. 2001). Furthermore, and also in the manner of *chs2* disruption mutants of *S. cerevisiae* and the lack of expression of *CaChs1p* in conditional mutants of *C. albicans* (Munro et al. 2001; Shaw et al. 1991; Schmidt et al. 2002), *wdchs1Δ* cells are occasionally larger and frequently show septal region abnormalities that are not found in mutants with a defective class I isozyme or the wild type, such as failing to separate, showing septa that lack clearly defined primary and secondary septa that are reminiscent of those observed in TEM profiles of *W. dermatitidis* treated with the chitin synthase inhibitor polyoxin (Cooper et al. 1984) and sometimes having abnormally broad mother cell–daughter bud necks and not forming even a defective septum between daughter nuclei. Finally, similar to the *wdchs1Δwdchs2Δ* double mutants of *W. dermatitidis*, *chs1chs2* double mutants of *S. cerevisiae* also have more abnormal and exaggerated morphologies than their single parental *chs* mutant strains, although in the case of the latter the loss of function of both its class I and class II isozymes in the same background is much less severe and, to our knowledge, does not lead to lethality at any temperature (Shaw et al. 1991). We suggest that these functional similarities among the class II orthologous chitin synthases of *S. cerevisiae*, *C. albicans* and *W. dermatitidis*, and the morphological similarities among mutants of these three species with defects in these particular isozymes, indicate that the primary function of the protein encoded by *WdCHS1* is to facilitate faithful primary septum formation, cytokinesis and, in the case of a filamentous fungus capable of yeast reproductive growth, cell separation, whereas its class I chitin synthase WdChs2p functions more in the manner of ChsCp in *A. nidulans* to provide a less-than-perfect backup when the function of the class II chitin synthase WdChs1p is impaired.

It is presently unclear why the *wdchs1Δwdchs2Δ* double mutants retain an ability to grow, albeit poorly, at 25°C but not at 37°C unless osmotically stabilized. However, we suspect that

of the remaining WdChsp, at least one that is maintaining the cell wall integrity of *W. dermatitidis* at the lower temperature is differentially produced or does not function at the higher temperature. Support for this hypothesis is provided by at least two observations. The first is that mutants with both *WdCHS1* and *WdCHS2* disrupted, while capable of growth at 25°C, nonetheless have severely distorted cell shapes that tend to be elongated, ballooned and multinucleate, in addition to having even more abnormal septal regions and septal structures than the *wdchs1Δ* single mutants. Since the same mutants die at 37°C, it also seems logical to conclude that one or more of the other WdChsp of *W. dermatitidis* must be able to synthesize enough chitin at the lower temperature to maintain general cell wall integrity, but not at the higher temperature, even though normal septation is not carried out. The second is from results published previously that document *wdchs5Δ* mutants have the ability to grow like the wild type at 25°C, but die after a few generations when shifted to 37°C (Liu et al. 2004). We take these particular results to also mean that one or more of the remaining WdChsp isozymes are not produced or do not carry out chitin synthesis properly in this mutant at the higher temperature. However, neither of these possibilities presently can be substantiated completely for a number of reasons. Paramount among these is the absence of a complete genome sequence for *W. dermatitidis*, which is required to exclude the possibility that this species has more than the five WdChsp already discovered. Another is our inability to produce the needed quadruple disruption strains that would allow mutants with only one WdChsp to be tested for abilities to grow at one temperature but not another. We suspect this is because the disruption of so many *WdCHS* genes in the same background leads to synthetic lethality and thus precludes our ability to recover transformants.

Our past and present results, however, do document that neither individually nor collectively can the four other known WdChsp isozymes of *W. dermatitidis* correct for the loss of WdChs5p function at 37°C, even though one or more allows normal growth at 25°C (Liu et al. 2004). This seems to mean that WdChs5p, an isozyme with no ortholog in *S. cerevisiae* or *C. albicans*, carries out a specific function that is not compensated for by any one or all of the other WdChsp isozymes combined at 37°C. Similarly, our present and past results further document that even the combined functions of WdChs3p, WdChs4p and WdChs5p cannot perfectly substitute for the combined loss of WdChs1p and WdChs2p in strains grown at 25°C or sustain the viability of those same strains grown at 37°C without osmotic stabilization. However, among these three isozymes, WdChs3p (class III), another isozyme with no ortholog in *S. cerevisiae* or *C. albicans*, can be formally excluded as being responsible for the maintenance of the cell wall integrity of the *wdchs1Δwdchs2Δ* mutant at 25°C because a *wdchs1Δwdchs2Δwdchs3Δ* triple mutant with only WdChsp4p and WdChs5p isozymes has been produced, the phenotype of which is only slightly more severe than that of a *wdchs1Δwdchs2Δ* double mutant (Zheng 1997; Wang et al. 1999; Szaniszlo 2002). This result indicates that either WdChsp4p, the ortholog of Chs3 of *S. cerevisiae*, or WdChs5p together or alone must function well enough at 25°C to sustain some growth of *W. dermatitidis*, albeit in about the same abnormal phenotype of the *wdchs1Δwdchs2Δ* double mutants, in the absence of WdChs1p, WdChs2p and WdChs3p (Zheng 1997; Wang et al. 1999; Szaniszlo 2002).

Although WdChs4p might seem likely to be more responsible than WdChs5p for maintaining the viability of the *wdchs1Δwdchs2Δ* double and *wdchs1Δwdchs2Δwdchs3Δ* triple mutants of *W. dermatitidis* at 25°C, because mutants of *S. cerevisiae* without functional class II (Chs2p) and class IV (Chs3p) isozymes are not viable and Chs3p is now reported to be responsible not only for chitin ring formation, but also for cell wall rigidity, bud neck integrity and cytokinesis in the absence of a primary septum (Cabib and Schmidt 2003), it appears not to be the case. Instead we suggest that WdChs5 might be a better possibility. Support for this idea is based on the observations addressing the hypothesis that triple mutants with only WdChs5p (class V) and either WdChs1p (class II) or WdChs2p (class I) would grow at wild-type rates at both 25 and 37°C and only have the phenotypes of *wdchs4Δ* mutants or of *wdchs1Δwdchs4Δ*

mutants, respectively. In this respect, we have previously derived a triple mutant of *W. dermatitidis* having only WdChs2p (class I) and WdChs5p (class V), which, in fact, indeed grows at wild-type rates at both 25 and 37°C, has the combined morphology of a *wdchs1Δwdchs4Δ* mutant and is as virulent as the wild-type strain (Wang et al. 2001; Szaniszlo 2002; H. Liu and P.J. Szaniszlo, unpublished data). Unfortunately, we have so far been unable to derive a triple chitin synthase mutant of *W. dermatitidis* with only WdChs1p (class II) and WdChs5p (class V). We suspect this result is because such strains are synthetically lethal, but we are nonetheless continuing our attempts to derive this particular mutant in order to explain more exactly how all the chitin synthases of this fungus interact and together functionally contribute to its biology, pathogenicity and virulence.

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Fig. 1. Mapping of *WdCHS1*. **a** Restriction enzyme map of the *Not1* fragment showing positions of the *WdCHS1* sequence (longer arrow) and the *WdCHS1* PCR sequence (shorter arrow) amplified to provide the 600 bp product used initially as a probe for library screening and chromosome mapping. Restriction site abbreviations depicted: *N* *NotI*, *S* *StuI*, *H* *HindIII*, *E* *EcoRI*, *K* *KpnI*, *B* *BglII*, *X* *XbaI*, *Sa* *SalI*. **b** Southern blot hybridization of the chromosomes of strain 8656 hybridized with a PCR-generated mixture of 600 bp fragments of *WdCHS1*, *WdCHS2* and *WdCHS3* (lane 2) or with cloned *WdCHS1* (lane 3), *WdCHS2* (lane 4) or a 591 bp PCR product specific for *WdCHS3* (lane 5). The karyotype at the left (lane 1) shows the four known chromosomes of *W. dermatitidis* 8656, as reported previously (Zheng and Szaniszlo 1999)

Fig. 2.

Disruption of *WdCHS1* with pLZ44 or pLZ56. **a** Strategy for the integrative disruption of *WdCHS1* with pLZ44, which contains the *hph* selection marker and was linearized by digestion with *KpnI* in its 1.4 kb *BglIII/HindIII* *WdCHS1* targeting sequence prior to transformations by electroporation. Restriction site abbreviations depicted: *B* *BglIII*, *Ba* *BamHI*, *H* *HindIII*, *S* *StuI*, *Sa* *SalI*, *K* *KpnI*. **b** Southern analysis of genomic DNA of wild-type strain 8656 (lane 1) and two representative HmB-resistant *wdchs1Δ* mutants (*wdchs1Δ-1A* and *wdchs1Δ-1B*, lanes 2 and 3, respectively) digested with *BamHI* and hybridized with a *WdCHS1* DNA fragment. **c** Strategy for the replacement of *WdCHS1* with pLZ56, which contains the *ble* selection marker and was used in a linear form by digestion with *BamHI*. Restriction site abbreviations depicted: *B* *BglIII*, *Ba* *BamHI*, *S* *StuI*. **d** Southern analysis of genomic DNA digested with *BamHI* of the wild-type strain 8656 (lane 1) and the phleomycin-resistant mutant *wdchs1Δ-2* (lane 2) and probed with 800 bp PCR-generated amplicons of *WdCHS1*.

Fig. 3. Cell morphologies of *W. dermatitidis* wild-type strain 8656 (a), *wdchs1Δ-1A* (b), *wdchs2Δ-1* (c) and *wdchs1Δwdchs2Δ-A* (d). Cells were first grown to mid-log phase in liquid YPD and then photographed at 63× using Nomarski differential interference contrast (DIC) optics and a Zeiss ICM 405 Inverted Photomicroscope

Fig. 4.

Cellular characteristics of *W. dermatitidis* wild-type strain 8656 (a–c), *wdchs1Δ-1A* (d–f), *wdchs2Δ-1* (g–i) and *wdchs1Δwdchs2Δ-A* (j–l), photographed at 100× with Nomarski differential interference (DIC) optics (a, d, g, j) or with epifluorescence optics after staining the cells depicted as DIC images with DAPI (b, e, h, k) and other cells with Calcofluor white (c, f, i, l) and using a Zeiss Axioimager A1 microscope. All cells depicted were grown for 24 h in liquid YPD at 25°C. Arrows indicate cells with obvious abnormal morphology (d), clearly evident multiple nuclei (k) and unusually intense chitin staining of wall and septal regions (f, l). Note the insets in d and e showing what appears to be an abnormally shaped, septumless cell with three nuclei

Fig. 5. Transmission electron micrographs of *W. dermatitidis* wild-type strain 8656 and mutant strain *wdchs1Δ-1A*. **a** A wild-type yeast cell with a typical cell wall (*W*), a narrow mother cell–daughter bud neck and a clearly differentiated primary septum (*PS*) separating two secondary septa (*SS*). **b–e** Various septal regions of the *wdchs1Δ-1A* mutant, which show mother cell–daughter bud areas that tend to be broader than normal and have septa that are abnormal (*AS*) because they do not have clearly differentiated primary and secondary layers (**b–d**) or appear as septumless septal regions (*SSR*) in multinucleate cells (**e**). All cells depicted were grown for 24 h in liquid YPD at 25°C. Other structures that are clearly evident include mitochondria (*M*) with well-defined cristae, vacuoles (*V*) and nuclei (*N*). Note the presence of three nuclei in the cellular region of the septumless profile depicted in **e**. Bar=1 μM

Fig. 6.

Chitin synthase activities (**a**) and chitin contents (**b**) of *W. dermatitidis* wild-type strain (lane 1), *wdchs1Δ-1A* (lane 2), *wdchs2Δ-1* (lane 3) and *wdchs1Δwdchs2Δ-A* (lane 4) grown at 25°C and assayed as described in detail previously (Wang et al. 1999; Zheng and Szaniszlo 1999). The chitin synthase activities were derived after activation of the chitin synthases with trypsin. The data represent the averages derived from at least four independent experiments, each of which involved triplicate assays of membrane preparations having known protein concentrations. The chitin contents are averages derived from two independent experiments. The *error bars* represent standard deviations and all data were calculated as relative percentages compared to those of the wild-type strain

Fig. 7.

Comparisons of the rates of growth at 25°C (a) and 37°C (b) of the wild-type strain 8656 (open diamond) and selected *wdchs1Δ* (filled square) and *wdchs2Δ* (filled triangle) single and *wdchs1Δwdchs2Δ* (filled circle) double mutants. Mid-log phase cells from 25°C cultures were used to inoculate liquid YPD medium at an initial concentration of about 10^5 cells/ml. Cultures were incubated with shaking at 37°C in a Psychrotherm Incubator Shaker (New Brunswick Scientific Co.) or at 25°C in a New Brunswick G-75 Waterbath Shaker at speed settings of 200 rpm. Optical densities were measured with a DU spectrophotometer (Beckman Instruments, Inc.)

Fig. 8.

Colony and cellular morphologies of wild-type strain 8656 and the *wdchs1Δ-1A*, *wdchs2Δ-1* and *wdchs1Δwdchs2Δ-A* mutants grown at two temperatures in YPD plate medium with or without osmotic stabilizer. Colony growth at 25°C without (a) and with (b) supplemental 1 M sorbitol and at 37°C without (c) and with (d) supplemental 1 M sorbitol. Cellular morphologies of the *wdchs1Δwdchs2Δ-A* mutant grown at 37°C in liquid YPD medium with (e) or without supplemental 1 M sorbitol (f)

Fig. 9.

Mouse survival analysis after injection with the wild-type strain 8656 (wt), *wdchs1Δ-1A*, *wdchs2Δ-1* or the *wdchs1Δwdchs2Δ* double mutants A, B and C. Groups of ten mice were injected with log phase yeast cells of each strain. Mice were generally injected with 9×10^6 cells per mouse and all were then monitored for 10–15 days to determine survival rate. However, because it was difficult to quantify the *wdchs1Δwdchs2Δ* double mutants by hemacytometer counting, the inocula for those strains were adjusted by OD 600 to the proper concentrations by comparison with the standard inocula of the other strains. The data presented are from two experiments that showed very similar results. Survival fractions were calculated by the Kaplan–Meir method and survival curves tested for significant difference ($P < 0.01$) by the Mantel–Haenszel Test using Graphpad Prism 3.00 for Windows

Fig. 10. Transmission electron micrographs of the *W. dermatitidis* double mutant strain *wdchs1Δwdchs2Δ-A* showing **a** a septumless septal region (*SSR*), **b, c** abnormal septa (*AS*) in septal regions (*SR*) and **d** sites of putative abnormal side wall chitin deposition (*CP*)

Table 1

Strains used in this study

Strain	Parent strain	Genotype	Reference or source
Wd8656		Wild type	ATCC 34100
<i>wdchs1</i> Δ-1A	Wd8656	<i>wdchs1::hph</i>	This work
<i>wdchs1</i> Δ-1B	Wd8656	<i>wdchs1::hph</i>	This work
<i>wdchs1</i> Δ-2	Wd8656	<i>wdchs1::ble</i>	This work
<i>wdchs2</i> Δ-1	Wd8656	<i>wdchs2::hph</i>	Wang et al. (2001)
<i>wdchs1</i> Δ <i>wdchs2</i> Δ-A	<i>wdchs1</i> Δ-2	<i>wdchs1::blewdchs2::hph</i>	ATCC MYA-887; Wang et al. (1999)
<i>wdchs1</i> Δ <i>wdchs2</i> Δ-B	<i>wdchs1</i> Δ-2	<i>wdchs1::blewdchs2::hph</i>	This work
<i>wdchs1</i> Δ <i>wdchs2</i> Δ-C	<i>wdchs1</i> Δ-2	<i>wdchs1::blewdchs2::hph</i>	This work

All strains used in this study are isogenic

Morphological differences between the wild type (WT) and the disruption mutants *wdchs1Δ-1A* (*chs1*) and *wdchs2Δ-1* (*chs2*) cultured at 25 or 37°C

Table 2

Strain	Time (h)	Temperature (°C)	Yeast morphology (%)					Chain with more than three cells	Chain with more than three cells
			With no bud	With small bud	With large bud	With wide bud neck	Chain with three cells		
WT	0	25	31.1	28.8	40.1	0.0	0.0	0.0	0.0
<i>chs1</i>	37	37	31.1	28.8	40.1	0.0	0.0	0.0	0.0
	25	25	29.4	28.5	40.8	1.3	0.0	0.0	0.0
<i>chs2</i>	37	37	29.4	28.5	40.8	1.3	0.0	0.0	0.0
	25	25	32.7	26.6	40.7	0.0	0.0	0.0	0.0
WT	24	37	32.7	26.6	40.7	0.0	0.0	0.0	0.0
	25	25	48.2	18.3	26.7	1.5	5.4	0.0	0.0
<i>chs1</i>	37	37	67.6	10.8	16.5	2.5	2.6	0.0	0.0
	25	25	44.9	10.5	17.8	8.0	7.3	11.5	14.9
<i>chs2</i>	37	37	36.0	10.2	16.0	10.0	13.0	3.6	0.0
	25	25	58.1	12.5	23.9	1.9	3.6	0.0	0.0
		37	67.2	10.0	17.8	1.9	3.2	0.0	0.0

Values are the averages of three different counts of 100 cellular units