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A hyphal-specific chitin synthase gene (*CHS2*) is not essential for growth, dimorphism, or virulence of *Candida albicans*

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ABSTRACT **In the dimorphic fungus *Candida albicans*, the *CHS2* gene encodes a chitin synthase that is expressed preferentially in the hyphal form. Gene disruption of *CHS2* in this diploid asexual fungus was achieved by the "ura-blaster" protocol described for *Saccharomyces* [Alani, E., Cao, L. & Kleckner, N. (1987) *Genetics* 116, 541–545]. This involves the sequential disruption of multiple alleles by integrative transformation with *URA3* as a single selectable marker. After disrupting the first *CHS2* allele, the Ura⁻ phenotype was recovered through cis recombination between repeated *hisG* sequences that flanked the *URA3* marker in the disruption cassette, which was then used again to disrupt further *CHS2* alleles. This method of gene disruption is well suited to the mutational analysis of this genetically recalcitrant human pathogen. Three rounds of disruption were required, suggesting that the strain SGY243 is triploid for the *CHS2* locus. The resulting homozygous Δ*chs2*::*hisG* null mutants were viable and made germ tubes with a normal morphology. The germ tubes were formed more slowly than parental strains in serum-containing medium and the germinating cells had a 40% reduction in their chitin content compared to germ tubes of the parent strain. The chitin content of the yeast form was not affected. A prototrophic strain of the *chs2* null mutant was not attenuated significantly in its virulence when tested in normal and immunosuppressed mice.**

Chitin is a fibrous polymer of β-1,4-*N*-acetylglucosamine that constitutes a major structural component of the cell wall of many species of fungi, including those species that are pathogenic in humans. Because chitin is not found in mammals and its biosynthesis is normally essential for the shape and viability of the fungal cell (1, 2), chitin synthesis is an attractive target for the design of antifungal drugs.

The most common medical mycosis is caused by *Candida albicans*, which is a diploid organism with no sexual cycle. This fungus is capable of dimorphic growth where growth can occur by unicellular budding or filamentous hyphal extension and branch formation (3). The hyphal cell wall has three to five times the chitin content of the yeast cell wall (4, 5) and the cells have up to 10 times the *in vivo* chitin synthase activity (6). Three genes encoding chitin synthases have been cloned and sequenced in *C. albicans* (7, 8, 37). Two encode chitin synthase zymogens (*CHS1* and *CHS2*), homologous to the *CHS* genes of *Saccharomyces cerevisiae*. The third chitin synthase gene, *CHS3*, is homologous to the *S. cerevisiae* *CSD2* (9) gene (also called *CAL1*) and is apparently the structural gene for an enzyme, which in *S. cerevisiae*, does not require proteolysis for its activity. Northern blot analysis of the *C. albicans* *CHS* genes showed that CHS2 mRNA levels were elevated in cells undergoing hyphal development, whereas the CHS1 mRNA was expressed only at low levels early in germ-tube formation (8).

The role of each of the chitin synthase activities described in *S. cerevisiae* has been investigated by disrupting each of the relevant genes (for a review, see ref. 10). Gene disruptions are more difficult in *C. albicans* because the organism is constitutively diploid and because stable multiply marked strains are required for sequential gene disruptions. Sequential gene disruption of *HEM3* of *C. albicans* has been achieved with two genetic markers (11), but host strains with sufficient markers for the disruption of more than one structural gene are not yet available. In this study, we employed the "ura-blaster" protocol originally described by Alani *et al.* (12) for use with *S. cerevisiae*, to disrupt all alleles of the hyphal-specific *C. albicans* *CHS2* gene. This method allows the sequential disruption of target alleles by using Ura3⁻ auxotrophy as a single selectable marker. Because the selectable marker can be regenerated after the disruption of each allele, this method overcomes the need for multiply marked host strains and is, therefore, ideally suited for the analysis of families of genes such as the *CHS* genes in *C. albicans*. We show that the *chs2* null mutant was still able to form germ tubes, albeit with a reduced chitin content compared with the isogenic wild-type parent. A prototrophic *chs2*⁻ null mutant was still able to cause disease in normal and immunosuppressed mice and had a similar virulence to the parental *CHS2* strain. A preliminary report of the construction of the Δ*chs2*::*hisG* null mutant has appeared (13).

MATERIALS AND METHODS

Strains and Plasmids. *C. albicans* Robin Berkhout SGY 243 (*ade2* Δ*ura3*::*ADE2*/*ade2* Δ*ura3*::*ADE2*) was a gift from Rosemarie Kelly (14) and was the parental strain for all strains constructed in this study (Table 1). The plasmid pCUB6 (15) was derived from pNKY50 (11), which was modified by replacement of the *Saccharomyces URA3* gene with the *C. albicans URA3* gene obtained from Squibb. The *Bam*HI–*Bgl* II fragment containing the *C. albicans URA3* gene flanked by 1.1-kb direct repeats of the *Salmonella typhimurium hisG* gene was subsequently transferred into pUC18 to give p5921. The *Bgl* II site was created at the *Sma* I site of the pUC18 polylinker using T4 ligase to add *Bgl* II linkers to *Sma* I-digested phosphatase-treated vector.

Media and Growth Conditions. The yeast form of *C. albicans* was grown at 30°C in YPD or the defined medium SD (16). Germ-tube growth was induced using 20% (vol/vol) newborn calf serum (17), *N*-acetylglucosamine-containing medium (18), or the regimen of pH- and temperature-

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Abbreviation: FOA, 5-fluoroorotic acid.

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Table 1. *C. albicans* strains used

Strain	Genotype	Ref.
SGY243	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, CHS2/CHS2/CHS2*	12
NGY1	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, CHS2/CHS2/Δchs2::hisG::URA3::hisG*	This work
NGY2	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, CHS2/CHS2/Δchs2::hisG*	This work
NGY5	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, CHS2/Δchs2::hisG/Δchs2::hisG*	This work
NGY7	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, Δchs2::hisG/Δchs2::hisG/Δchs2::hisG::URA3::hisG*	This work
NGY10	*ade2/ade2, Δura3::ADE2/Δura3::ADE2, Δchs2::hisG/Δchs2::hisG/Δchs2::hisG*	This work

Strain SGY243 appears to be triploid for the *CHS2* locus. This conclusion is based on the following three observations: (*i*) three rounds of transformation were required to create a *chs2* homozygous null mutant, (*ii*) at each stage of the disruption, the only new *Xho* I band observed upon Southern blot analysis was 8.5 kb (diagnostic for *chs2::hisG*), and (*iii*) the relative intensities of the wild-type (4.7 kb) and mutant (4.0 kb) *Pst* I bands and the wild-type (3.6 and 5.6 kb) and mutant (8.5 kb) *Xho* I bands (Fig. 1). Tandem duplications of the *CHS2* gene occurred in some transformants, but not in the above strains. These duplications, which were recognized through the generation of large diagnostic *Xho* I bands, were always resolved to a single *chs2::hisG* in FOA-resistant progeny (data not shown). Strain NGY1 was created by insertion of the complete (*chs2::hisG::URA::hisG*) cassette into *CHS2* that, after selection on FOA medium, resulted in the generation of NGY2, which carries a resolved *chs2::hisG* locus. After transformation of NGY2 and selection with FOA, a second heterozygous mutant (NGY5) was obtained that had a disruption in the second wild-type *CHS2* allele. Southern blot analysis showed that the relative intensities of the wild-type and mutant alleles of *CHS2* were reversed in NGY2 and NGY5, which suggested a 2:1 and 1:2 ratio for the wild-type and disrupted *CHS2* alleles (see Fig. 1). Transformation of NGY5 with the disruption cassette yielded the prototrophic null strain NGY7, which was used in pathogenicity experiments. Selection of auxotrophic recombinants of NGY7 on FOA agar yielded the homozygous null NGY10.

stimulated dimorphism (19). For growth of *C. albicans ura3* strains, medium was supplemented with uridine (50 μg/ml). Growth rates were calculated from spectrophotometric measurements, and cell biomass yields were measured from dry weight of tared samples after 38 h of growth in liquid medium at 30°C. The medium for regenerating *C. albicans* spheroplasts was supplemented with 1 M sorbitol.

Disruption of *CHS2*. The method used was essentially that described by Alani *et al.* (12). The *C. albicans CHS2* gene was obtained originally from a λGEM-11 genomic library from J. Zwicker (Center for Cancer Research, Massachusetts Institute of Technology). A 4.7-kb *Pst* I fragment containing the *CHS2* gene was cloned into pBluescript from which the *Hin*dIII site had first been deleted. A *Bam*HI–*Bgl* II fragment of pCUB6 containing the *hisG::URA3::hisG* cassette was then cloned between the two internal *Hin*dIII sites of the *CHS2* gene to give p1043. Spheroplasts from mid-exponential-phase yeast cells of *C. albicans* SGY243 (Table 1) were prepared using β-glucuronidase (Sigma; type H-2S) according to Kurtz *et al.* (20). Competent spheroplasts were not necessarily osmotically fragile, even in the presence of SDS. Spheroplasts were transformed with 10–15 μg of *Pst* I-digested p1043 and transformants were plated on SD without uridine at 30°C. The transformation frequency varied between 5 and 15 transformants per μg of DNA in various experiments. Transformants were streaked out to obtain single colonies, which were then grown in YPD for genomic DNA preparations. Southern blot analysis was performed to confirm the constructions of primary transformants and strains generated at all the subsequent steps of the disruption. Ura^- segregants were selected from the Ura^+ transformants by plating on SD agar containing 5-fluoroorotic acid (FOA, 1 g/liter) with added uridine (21). Regeneration of the Ura^- auxotrophy enabled the same *Δchs2*::hisG::*URA3*::*hisG* cassette to be used to disrupt the second allele of the *CHS2* gene.

Measurement of Chitin Content. Chitin contents for the yeast and hyphal forms of the parental SGY243 and homozygous *chs2* null mutants were determined. Yeast cells were grown overnight in YPD plus uridine (50 μg/ml) at 30°C, and hyphal cells were harvested after 6 h of growth at 37°C in 20% newborn calf serum with uridine (50 μg/ml). Cells were washed, dried to constant weight, and extracted in 5% (wt/vol) KOH at 100°C for 12 h in the absence of air. The KOH was exchanged three times during the extraction. The chitin content of the alkali-insoluble material was determined colorimetrically after deacetylation and deamination by the method of Ride and Drysdale (22). Chitin standards were subjected to all extractions to assess the efficiency of recovery of chitin.

Calcofluor Staining. The morphology and chitin distribution of parental and *chs2* null mutants were also examined by fluorescence microscopy after staining with Calcofuor white (American Cyanamid) at 25 μg/ml.

Pathogenicity *in Vivo*. Female mice (Charles River Harefield strain, Glaxo) of 18–22 g were used to assess the virulence of *C. albicans* strains in systemic disease. Saline-washed yeast cells, grown in YPD plus uridine (50 μg/ml), were used as inocula. Inoculation was by intravenous injection in normal and immunosuppressed mice. Immunosuppression was achieved by intraperitoneal administration of cyclophosphamide (100 mg/kg) every 3 or 4 days, with an initial dose 4 days prior to infection. Mouse survival was recorded and LD_{50} values were estimated after 21 days. Fungal cultures from postmortem kidney homogenates suggested that deaths were due to *C. albicans*. Histological examination of mouse kidney sections stained with Alcian blue periodic acid Schiff's stain was by the method of Mowry (23).

RESULTS

Construction of *Δchs2::hisG* Null Mutant. Disruption of the *C. albicans CHS2* gene was achieved by replacement of the 1.8-kb *Hin*dIII fragment in the 3.0-kb open reading frame with a 1.1-kb fragment encoding a bacterial *hisG* gene using the ura-blaster protocol (12). The homozygous *Δchs2::hisG* null mutant was generated after three rounds of transformation with the *Δchs2::hisG::URA3::hisG* ura-blaster cassette using *Pst* I-digested p1043 DNA. After each transformation, the Ura^+ prototrophs were purified and plated on FOA-containing agar to select for Ura^- auxotrophs that had undergone cis recombination via the *hisG* repeats to excise the *URA3* marker. Genomic DNA was isolated from transformants and FOA-resistant progeny and analyzed on a Southern blot by using a 4.7-kb probe consisting of most of the *CHS2* open reading frame (Fig. 1). Three rounds of transformation were necessary to disrupt all copies of the *CHS2* gene, and the intensities of the mutant *Pst* I (4.0 kb) and *Xho* I (8.5 kb) bands increased at each stage relative to the corresponding wild-type bands. This suggests that the original SGY243 strain was triploid for the *CHS2* locus (Table 1).

Complications were encountered during the *CHS2* gene disruption. All transformants had insertions of the *hisG::URA3::hisG* at the *CHS2* locus prior to FOA selection, but some heterozygotes reverted to a wild-type restriction

pattern after FOA treatment or after subsequent transformation. Only 25% of the strains recovered after FOA selection in the first round of transformation were heterozygous and 75% of second-round transformants had a wild-type pattern in *Pst* I and *Xho* I digests on Southern blots (data not shown). Recreation of the wild-type allele could have occurred by gene conversion with a wild-type *CHS2* allele.

Southern blot analysis of transformants and FOA-resistant progeny that were generated during the construction of the *chs2* null mutant revealed additional integration and recombination events. Genomic DNA from transformants arising from the third round of gene disruption (prior to FOA selection) revealed four bands when digested with *Xho* I. The largest predicted band in this Southern blot (11.3 kb) corresponded to an allele incorporating the full *hisG*::*URA3*::*hisG* cassette at the *CHS2* locus. While most transformants showed a band at this position, a few transformants had higher molecular weight bands, suggesting that multiple copies of the cassette were present in the cell, either integrated within the *CHS2* locus or possibly free, as a replicating plasmid. The existence of multiple tandem arrays implies that a circularized intermediate form of the linear cassette was generated *in vivo* during the transformation in some cells. Since the *URA3* gene contains an *Eco*RI site the presence of multiple arrays was further tested by digesting *Xho* I-cut DNA with *Eco*RI. This reduced the size of all of these high molecular weight bands to a smaller fragment corresponding to the monomeric size, suggesting that the high molecular weight bands were due to multimers of the ura-blaster (data not shown). Tandem Δ*chs2*::*hisG*::*URA3*::*hisG* copies were always resolved to a single Δ*chs2*::*hisG* in FOA-resistant progeny. Tandem duplication events have also been observed during ura-blaster-mediated gene disruption of another *Candida* gene (R. Swoboda, N.A.R.G., G. W. Gooday, and A.J.P.B., unpublished data) and tandem integrations have been observed in *S. cerevisiae* (24).

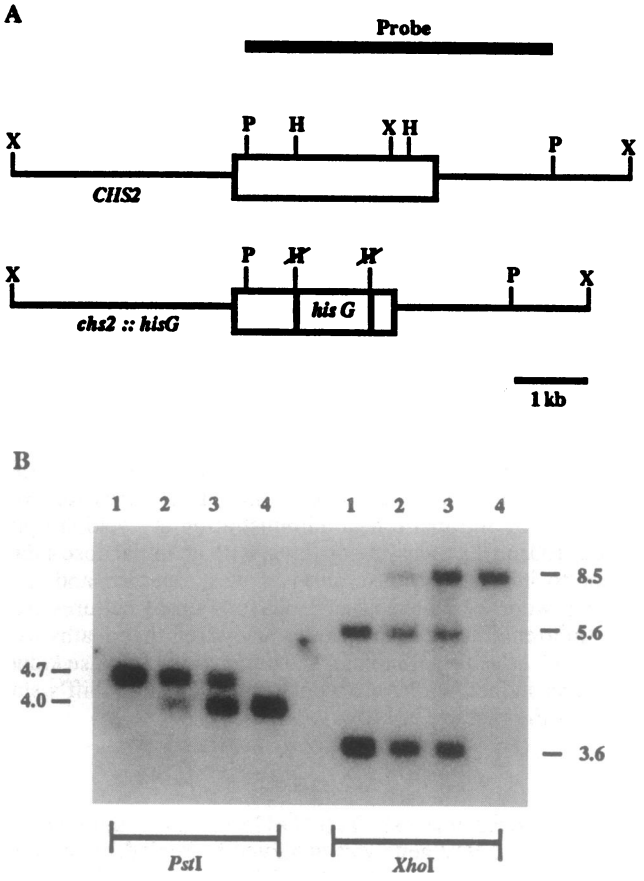

FIG. 1. Sequential disruption of the *CHS2* gene by using the ura-blaster technique. (*A*) Structure of the *CHS2* and Δ*chs2*::*hisG* genes and the probe used to analyze transformants. (*B*) Southern blot analysis. The *CHS2* open reading frame is represented by the open box that contains a 1.1-kb bacterial *hisG* gene replacing the 1.8-kb *Hin*dIII–*Hin*dIII fragment of the *C. albicans CHS2* gene. The single line represents chromosomal DNA. H, *Hin*dIII; Ḥ, destroyed *Hin*dIII sites; P, *Pst* I; X, *Xho* I. The probe is a 4.7-kb *Pst* I–*Pst* I fragment used in the Southern blot analysis. Lanes: 1, parental strain SGY243; 2 and 3, NGY2 and NGY5, respectively, from the first and second rounds of transformation, and are heterozygous mutants; 4, NGY10 from the third round of transformation and is the homozygous null mutant. DNA is from strains after transformation, strain purification, and subsequent treatment with FOA. For *Pst* I digestions, the disruption results in the loss of a band at 4.7 kb and generation of a new fragment of 4.0 kb. *Xho* I digestions generate two bands of 3.6 and 5.6 kb for wild-type alleles and a single 8.5-kb fragment in a disrupted allele. The changes in the relative band intensities reflect the numbers of alleles present and are reversed after the first and second rounds of transformations. NGY2, isolated after the first transformation, is interpreted as having two wild-type and one disrupted alleles, and NGY5, the second-round transformant, has two mutant and one wild-type alleles.

To summarize, unsuspected integration and recombination events can occur after transformation of *C. albicans* with the ura-blaster cassette and careful checking of individual steps in the disruption protocol by Southern blot analysis is, therefore, required. However, despite these complications the ura-blaster strategy was able to generate homozygous *chs2* null mutants.

Phenotype and Chitin Content of *chs2* Null Mutant. The specific growth rates of the yeast form of the parental SGY243 strain and the null strain NGY10 were similar in YPD (0.23 h^{-1}) and SD (0.20 h^{-1}) media at 30°C. The yield of cells after overnight growth of the yeast form in these media was again similar (Table 2). In certain conditions, buds of *S. cerevisiae chs1* mutants lyse and appear refractile under phase-contrast optics (25). Bud lysis has been shown to be due to the action of a chitinase with an acidic pH optimum and it was prevented by buffering of the medium to pH >5.0 (26, 27). However, buds of logarithmic-phase or stationary-phase cells of the *C. albicans* NGY10 *chs2* null mutant were not refractile when grown in either rich or minimal medium at either pH 4.5 or at pH 6.8, the pH optimum for *C. albicans* chitinase (R. O'Donnell and G. W. Gooday, unpublished data).

The percentage of yeast cells of the parental and null strains that formed germ tubes was tested under three regimens for stimulation of the dimorphic switch. In serum-containing medium, the *chs2* null strain initially formed germ tubes more slowly than the parental strain, but ultimately the extent of germ-tube formation was similar in each (Table 2). Germ-tube formation was similar for both strains using either pH and temperature (19) or *N*-acetylglucosamine-containing medium (18) to stimulate dimorphism and was reduced compared to clinical isolates and nongenetically marked strains under identical conditions (data not shown). Thus NGY10 was fully competent to form germ tubes, albeit at a reduced rate in some media.

Calcofluor white-stained yeast and hyphal cells of SGY243 and the NGY10 null strain were indistinguishable, suggesting that there were no dramatic aberrations in the arrangement of chitin in the cell walls. The chitin contents of yeast and hyphal cells of the null mutant and parent strains were determined after alkali extraction of whole cells. The chitin content of the yeast cells of the NGY10 null and the SGY243 parental strains were not significantly different (Fig. 2). However, the chitin content of the hyphal cells of SGY243 was five times that in the yeast form. In comparison, the chitin content of the hyphal form of the null mutant NGY10 was only 155% of that in the parental yeast form of SGY243

Table 2. Growth of parental strain SGY243 and *chs2* null mutant NGY10

Growth	Medium	SGY243	NGY10
Germ-tube formation, %	Serum (1 h)	84 ± 3	40 ± 5
	Serum (3 h)	95 ± 2	95 ± 2
	Temperature/pH shift (2 h)	15 ± 1	18 ± 1
	Temperature/pH shift (6 h)	66 ± 4	68 ± 4
	N-Acetylglucosamine (24 h)	7 ± 2	1 ± 1
Yield (yeast form), mg/ml	YPD	4.9 ± 0.1	5.9 ± 0.3
	SD	2.5 ± 0.1	1.9 ± 0.1

Germ-tube formation was in prewarmed medium at 37°C at an inoculation density of 5×10^6 yeast cells per ml in 20% newborn calf serum (17), in Soll's medium for pH/temperature shift (19), or *N*-acetylglucosamine-containing medium (18). Yield measurements are for cells in overnight cultures at 30°C in nondefined (YPD) and defined (SD) media (16). Values are the mean ± SD (n = 3).

(Fig. 2). Therefore, the *CHS2* gene does contribute to chitin synthesis in hyphal cells.

The Homozygous Δ*chs2*::*hisG* Null Mutant Is Virulent. Ura3⁻ auxotrophic and prototrophic strains of *C. albicans*, with and without the Δ*chs2*::*hisG* double disruption, were tested for virulence in systemic infections after intravenous injection of yeast cells into normal and immunosuppressed mice. As reported (28–31), auxotrophic strains including Ura⁻ auxotrophs had greatly attenuated virulence in animals (Table 3). Thus Ura⁺ strains of the heterozygous and homozygous Δ*chs2*::*hisG* null mutants, isolated prior to treatment with FOA, were also used in virulence tests. In a Ura⁺ background, the apparent LD_{50} for the NGY7 null mutant was increased marginally compared to NGY1, which retained a functional copy of *CHS2* (Table 3). This was reflected in a slight increase in the mean time of mouse survival when normal or immunosuppressed mice were injected with NGY1 compared to NGY7 at three inoculation doses (data not shown). Histological examination of the kidneys of mice infected with the heterozygous and homozygous Δ*chs2*::*hisG* prototrophic strains revealed an infiltration of invasive hyphae in the cortex (Fig. 3). The kidneys were also positive for the growth of *C. albicans*, which, upon isolation and culture, was identical phenotypically to the inoculum.

DISCUSSION

The ura-blaster technique described here is well suited to genetic analysis of *C. albicans*, which lacks a sexual cycle and is constitutively diploid. This technique also circumvents the need for multiple genetic markers that are needed for the disruption of pairs of alleles from one or several target genes.

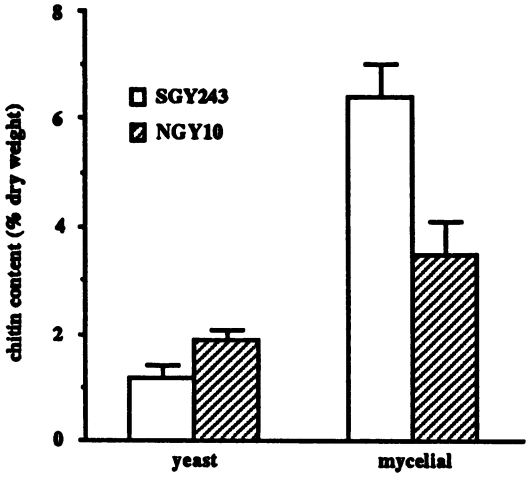

FIG. 2. Chitin contents of the yeast and hyphal cells of the parental SGY243 strain and NGY10, a homozygous Δ*chs2*::*hisG* null mutant. The error bars are SDs of five replicate samples.

Because auxotrophic strains of *C. albicans* have greatly attenuated virulence (28–31), a further advantage of the technique is that virulence studies can be performed on Ura⁺ isolates of the null strains by utilizing the prototrophic strains recovered after initial transformation and prior to FOA treatment in animal models. The strong negative selection against spontaneous auxotrophic revertants in pathogenicity tests presumably helps maintain the stability of the inoculum in animals.

This study shows that the *CHS2* gene is not essential for growth of the yeast or hyphal form of *C. albicans*. Northern blot analysis has shown that *CHS2* is expressed preferentially in the hyphal form of *C. albicans* (8), and this finding is consistent with our analysis of the homozygous Δ*chs2*::*hisG* null mutant, which had a reduced chitin content in hyphal cells but a normal chitin content in yeast cells. However, the morphology of the null mutant hypha was normal despite the reduced chitin content of the cells.

In *S. cerevisiae*, *chs1* mutants have a normal chitin content but have an increased tendency to undergo bud lysis in some media (10, 25–27). In this yeast, *chs2* mutants often have aberrant morphologies and have a normal or increased chitin content but apparently lack the chitinous primary septum upon which the bulk of the chitin containing bud and birth scar material is assembled (10, 32, 33). The *C. albicans CHS2* gene may be more homologous to *CHS1* of *S. cerevisiae* (34). However, our results suggest that the *C. albicans CHS2* is not functionally homologous to either *CHS* gene of *S. cerevisiae* since the null mutant did not form fragile buds, had a normal morphology in both yeast and hyphal forms, and had a reduced chitin content in the hyphal cell. In hyphae of *Neurospora crassa*, *chs1*^{RIP} mutants were morphologically abnormal and had a greatly reduced chitin content but still produced some chitin in the cell wall (2). These data suggest that the *CHS* genes may play an active role in chitin synthesis in filamentous fungi, including the hyphal form of *C. albicans*. In *S. cerevisiae*, the *CHS3 (CSD2)* gene product is responsible for the synthesis of most of the chitin in the cell wall (9, 10). Northern blot analysis of all three chitin synthase

Table 3. LD_{50} values of normal and immunosuppressed mice after intravenous administration of Ura⁺ and Ura⁻ strains of *C. albicans* with heterozygous or homozygous null mutations in *CHS2*

Strain	Phenotype	LD_{50} Normal mice	LD_{50} Immunosuppressed mice
SGY243	Ura⁻ Chs2⁺	>7.02*	6.71
NGY1	Ura⁺ Chs2⁺	6.60	5.69
NGY7	Ura⁺ Chs2⁻	6.84	5.78
NGY10	Ura⁻ Chs2⁻	>6.86*	6.80

LD_{50} values are expressed as the logarithm of the colony-forming units.
*No death occurred even at the highest doses with these mice.

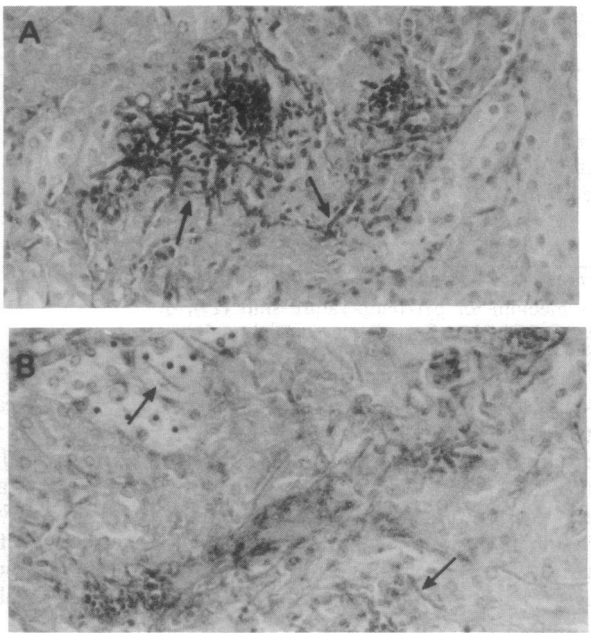

FIG. 3. Effect of disruption of the *CHS2* gene on virulence of *C. albicans*. Sections of mouse kidneys 2 days after intravenous inoculation of normal mice with NGY1 (*A*) and NGY7 (*B*), stained with Alcian blue periodic acid Schiff's stain. These strains are *URA3*, are prototrophic for uridine, and contain heterozygous (*A*) or homozygous (*B*) disruptions in *CHS2*. Some hyphal cells are indicated with arrows.

genes in *C. albicans* grown under a wide range of conditions again suggests that *CSD2* is the most abundant transcript in both yeast and hyphal cells (D. Schofield, B. Hube, G. W. Gooday, and N.A.R.G., unpublished data). Gene disruption of the *Candida CSD2* gene is, therefore, likely to have a more profound affect on the chitin content of both growth forms of the fungus than disruption of the *CHS2* gene.

In support of previous findings, the virulence of auxotrophic mutants was severely attenuated compared to prototrophic strains in an otherwise isogenic background (25, 26, 35). However, the virulence of the homozygous Δ*chs2*::*hisG* mutant was only marginally attenuated compared to the heterozygote, which is phenotypically Chs2$^+$. Recent progress in establishing methodologies such as the ura-blaster technique and other systems for reverse genetics in *C. albicans* (36) should significantly increase progress in the analysis of virulence and dimorphism in this human pathogen.

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