

## Attenuated virulence of chitin-deficient mutants of *Candida albicans*

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**ABSTRACT** We have analyzed the role of chitin, a cell-wall polysaccharide, in the virulence of *Candida albicans*. Mutants with a 5-fold reduction in chitin were obtained in two ways: (i) by selecting mutants resistant to Calcofluor, a fluorescent dye that binds to chitin and inhibits growth, and (ii) by disrupting *CHS3*, the *C. albicans* homolog of *CSD2/CAL1/DIT101/KT12*, a *Saccharomyces cerevisiae* gene required for synthesis of ≈90% of the cell-wall chitin. Chitin-deficient mutants have no obvious alterations in growth rate, sugar assimilation, chlamyospore formation, or germ-tube formation in various media. When growing vegetatively in liquid media, the mutants tend to clump and display minor changes in morphology. Staining of cells with the fluorescent dye Calcofluor indicates that *CHS3* is required for synthesis of the chitin rings found on the surface of yeast cells but not formation of septa in either yeast cells or germ tubes. Despite their relatively normal growth, the mutants are significantly less virulent than the parental strain in both immunocompetent and immunosuppressed mice; at 13 days after infection, survival was 95% in immunocompetent mice that received *chs3/chs3* cells and 10% in immunocompetent mice that received an equal dose of *chs3/CHS3* cells. Chitin-deficient strains can colonize the organs of infected mice, suggesting that the reduced virulence of the mutants is not due to accelerated clearing.

A key step in the development of an antifungal drug is the determination of its efficacy. For practical reasons, much of the initial testing is done in culture (*in vitro*), where the test conditions differ markedly from the conditions encountered by a fungal pathogen during infection (*in vivo*). The limited ability of *in vitro* testing to accurately predict *in vivo* efficacy is illustrated by the azoles, which are highly effective *in vivo* despite modest fungicidal activity *in vitro* (1). Thus, it is possible that some fungal components are required for pathogenicity *in vivo* but not for growth *in vitro*. Possible virulence determinants include factors required for recognition and invasion of the host and for protection against host defense systems. Because the cell wall is involved in these processes—the wall protects the fungal cell from external injury and cell-wall components mediate adherence (2–4) and immune response (5–8)—changes in cell-wall structure and/or composition may affect virulence.

We have begun to investigate the role of the cell-wall polysaccharide chitin in the virulence of *Candida albicans*. Chitin is found in all true fungi; therefore agents that inhibit chitin synthesis are potential broad-spectrum antifungal drugs. In the past decade, many of the genes involved in chitin synthesis have been isolated in *Saccharomyces cerevisiae*, and, more recently, the homologs of these genes have been identified in *C. albicans* (9–11).

In *S. cerevisiae*, three chitin synthases have been detected (for review, see refs. 12 and 13). One of them, chitin synthase III, makes 90% of the cell-wall chitin. At least three genes, *CSD2/CAL1/DIT101/KT12*, *CSD4/CAL2*, and *CAL3*, are required for this activity. Mutants lacking this enzyme are chitin-deficient and, consequently, are resistant to Calcofluor, a fluorescent dye that binds to chitin and inhibits growth by disrupting microfibril assembly (14, 15).

Like *S. cerevisiae*, *C. albicans* has at least three chitin synthases (9–11). Recently, the *C. albicans* homolog of *CSD2*, designated *CHS3*, was cloned and sequenced (11). By analogy to *S. cerevisiae*, *C. albicans CHS3* mutants should be chitin-deficient and Calcofluor-resistant. In the present report, we show that chitin-deficient mutants of *C. albicans* obtained by two methods are significantly less virulent than the wild-type strain.

### MATERIALS AND METHODS

**Strains, Media, and Mutagenesis.** The *C. albicans* strains used in this study are listed in Table 1. The following media were used: YPD [1% yeast extract (Difco)/2% peptone (Difco)/2% glucose], YPDC (YPD containing Calcofluor at 0.5 mg/ml), Sabouraud-dextrose [2% neopeptone (Difco)/1% glucose], and SD [0.7% yeast nitrogen base (Difco)/2% glucose]. Media were supplemented with uridine at 0.1 mg/ml as required. Agar (2%, Difco) was added for solid media. Urd<sup>-</sup> auxotrophs were selected on medium containing 5-fluoroorotic acid (5-FOA) as described (16). Germ-tube formation was induced in 20% fetal bovine serum (GIBCO/BRL), RPMI 1640 medium (Sigma preparation supplemented with 0.2% Na<sub>2</sub>CO<sub>3</sub>, 165 mM 3-[N-morpholino]propanesulfonic acid, pH 7), or N-acetylglucosamine-containing medium (19) at 37°C at an inoculation density of 10<sup>6</sup> cells per ml. Mutagenesis with ethyl methanesulfonate was done as described for *S. cerevisiae* (20).

**Virulence Studies.** Strain ICR 4-week-old male mice (Harlan–Sprague–Dawley) were housed five per cage; food and water were given *ad libitum*, according to National Institutes of Health guidelines for the ethical treatment of animals. Strains of *C. albicans* were grown in Sabouraud-dextrose at 26°C to a density of 10<sup>7</sup> cells per ml. Cells were harvested, washed, and resuspended in sterile water. Mice were infected via the lateral tail veins. Cages were checked three times daily for mice dead or moribund (exhibiting severe lethargy, vertigo, and ruffled fur) mice. Moribund mice were euthenized by cervical dislocation and necropsied. The left and right kidneys were removed and patched onto YPD agar, and the plates were incubated overnight at 37°C. The recovered *C. albicans* were tested for their ability to grow on YPDC plates. In experiments using neutropenic mice, cyclophosphamide was administered (150 mg/kg) by i.p. injection 96 and 24 hr before infection. Injections were repeated every 3 days for the duration of the experiment. Neutropenia was verified by comparing the per-

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Abbreviation: 5-FOA, 5-fluoroorotic acid.

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Table 1. *C. albicans* strains used in this study

Strain	Genotype	Source or ref.
<i>CHS3/CHS3</i>		
ATCC 10261	Clinical isolate	ATCC
CAI4	$\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> , congenic to SC5314	16
SC5314	Clinical isolate	17
SGY243	<i>ade2/ade2</i> $\Delta$ <i>ura3::ADE2</i> / $\Delta$ <i>ura3::ADE2</i>	18
<i>CHS3/<math>\Delta</math><i>chs3</i></i>		
CACB3, CACB8, and CACB10	Independent transformants of CAI4, $\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> <i>CHS3/<math>\Delta</math><i>chs3::hisG_URA3_hisG*</i></i>	This work
CACB3A, CACB8A, and CACB10A	5-FOA-resistant derivatives of CACB3, -8, -10, $\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> <i>CHS3/<math>\Delta</math><i>chs3-2::hisG</i></i>	This work
CACB8B-6	$\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> <i>CHS3/<math>\Delta</math><i>chs3-3::hisG_URA3_hisG_URA3_hisG</i></i> , derived from CACB8A, sister transformant of CACB8B-5	This work
CACB10B-8	$\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> <i>CHS3/<math>\Delta</math><i>chs3-1::hisG_URA3_hisG</i></i> , derived from CACB10A, sister transformant of CACB10B-6 and CACB10B-10	This work
<i><math>\Delta</math><i>chs3</i>/<math>\Delta</math><i>chs3</i></i>		
CACB3B-5	$\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> $\Delta$ <i>chs3-2::hisG</i> / $\Delta$ <i>chs3-3::hisG_URA3_hisG_URA3_hisG</i> , derived from CACB3A	This work
CACB8B-5	Same genotype as CACB3B-5, derived from CACB8A	This work
CACB10B-6	Same genotype as CACB3B-5, derived from CACB10A	This work
CACB10B-10	$\Delta$ <i>ura3::imm434</i> / $\Delta$ <i>ura3::imm434</i> $\Delta$ <i>chs3-2::hisG</i> / $\Delta$ <i>chs3-1::hisG_URA3_hisG</i> , derived from CACB10A	This work

\*The DNA from these strains were not examined for amplification of *hisG* and *URA3*; consequently, specific allele designations cannot be assigned.

centage of neutrophils to total number of leukocytes before and after injection with cyclophosphamide.

**Recovery of *C. albicans* from Mouse Kidneys.** For quantitation of colony-forming units of *C. albicans* in kidney tissue, mice were anesthetized using methoxyflurane and killed by cervical dislocation on days 3, 7, and 14. Kidneys were removed, suspended in 10 ml of sterile water, homogenized using a Waring blender, serially diluted in sterile water, and plated on the appropriate medium.

## RESULTS

**Identification of Chitin-Deficient Mutants of *C. albicans* by Selection of Calcofluor Resistance.** Two strains of *C. albicans*, ATCC 10261 and SGY243, were treated with ethyl methane-sulfonate, and aliquots of each were plated on YPD agar containing Calcofluor at 100  $\mu$ g/ml. From strain ATCC 10261, Calcofluor-resistant colonies were obtained at a frequency of  $\approx 10^{-5}$ . Six mutants were isolated and designated pcd 1, 2, 3, 4, 5, and 9 (putative chitin deficiency). From SGY243, no Calcofluor-resistant mutants were obtained. It has been shown that SGY243 is triploid at certain loci (21, 22), and this may explain the inability to recover Calcofluor-resistant mutants from this strain.

To determine whether Calcofluor resistance of the pcd mutants is caused by a reduction in cell-wall chitin, the amount of chitin in yeast cells was measured as described (23). The results show that four of the mutants, pcd 3, 4, 5, and 9, have a 5- to 10-fold reduction in cell-wall chitin, containing 0.36, 0.33, 0.34, and 0.23  $\mu$ g of chitin per mg of wet cells, respectively, compared to 1.73  $\mu$ g of chitin per mg of wet cells in the parental strain ATCC 10261.

**The pcd Mutants Display Reduced Virulence in Immunocompetent Mice.** We tested the virulence of the chitin-deficient mutants by infecting outbred ICR mice. Mice were injected via the tail vein with  $1 \times 10^6$  cells of each of the *C. albicans* strains (pcd 3, 4, 5, 9, and ATCC 10261), and the mice were monitored three times a day for symptoms of morbidity. Twenty-four of 25 pcd-infected mice survived for 30 days after infection, whereas none of the mice infected with ATCC strain 10261 survived >12 days. Autopsies indicated the presence of kidney lesions in mice infected with pcd mutants or ATCC 10261. Organisms were recovered from the infected organs and tested

for their ability to grow with Calcofluor. Organisms recovered from ATCC 10261-infected mice were Calcofluor-sensitive, whereas those recovered from pcd-infected mice were Calcofluor-resistant. Thus, the pcd mutants retain an ability to colonize organs.

**Isolation of Chitin-Deficient Strains by Sequential Disruption of *CHS3*.** Because they were obtained by chemical mutagenesis, the pcd strains may have other defects in addition to chitin loss. To create a mutant specifically defective in chitin biosynthesis, we disrupted *CHS3*, the *C. albicans* homolog of *S. cerevisiae* *CSD2*. *CSD2* has been proposed to encode the catalytic component of chitin synthase III, the enzyme that makes 90% of the cell-wall chitin. Using exact match primers to *CHS3*, *C. albicans* chromosomal DNA, and PCR, we synthesized a 4.6-kb DNA fragment containing *CHS3*. To disrupt the *CHS3* allele, 1.8 kb, which represents  $\approx 50\%$  of the open reading frame, was replaced by the "URA blaster" cassette, a 4-kb molecular construct consisting of a functional *C. albicans* *URA3* gene flanked by direct repeats of *hisG* from *Salmonella typhimurium* (16). Restriction maps of the wild-type and disrupted ( $\Delta$ *chs3-1::hisG\_URA3\_hisG*) alleles of *CHS3* are shown in Fig. 1, and the legend provides a detailed description of the constructions.

The parental strain for the disruption, CAI4, is a homozygous  $\Delta$ *ura3* strain congenic to the clinical isolate SC5314 (16). Strain CAI4 was transformed with pCHC2-11 that had been digested with *Pst* I and *Not* I. Ten Urd<sup>+</sup> transformants were analyzed by Southern hybridization (see Fig. 1 legend), and nine gave the results expected for integration of the disrupted allele at the *CHS3* locus. Urd<sup>-</sup> derivatives were obtained by growing the transformants to saturation in nonselective medium (YPD) and then plating a portion of the culture on medium containing 5-FOA. Southern analysis indicated that two classes of 5-FOA-resistant strains were recovered with approximately equal frequency: (i) strains containing *chs3::hisG*, which probably arose by cis recombination between the repeated *hisG* sequences, and (ii) strains containing only the wild-type *CHS3* allele, which may have arisen by mitotic recombination between the chromosome containing *chs3::hisG\_URA3\_hisG* and its wild-type homolog or by gene conversion. Our recovery of two classes of 5-FOA-resistant strains is consistent with the results of Gow *et al.* (22).

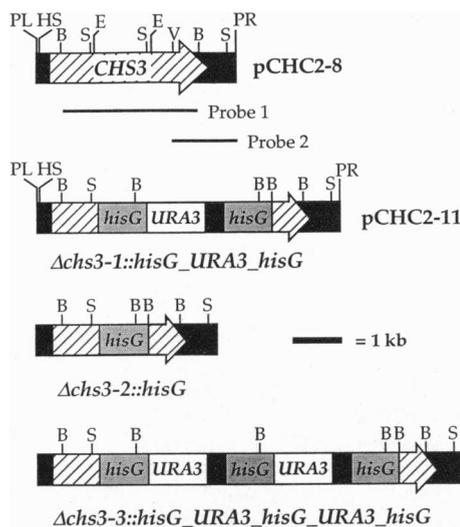


FIG. 1. Restriction enzyme maps of wild-type and disrupted alleles of *CHS3*. The *C. albicans CHS3* gene (GenBank accession no. D13454) was synthesized in two pieces from *C. albicans* chromosomal DNA using two pairs of oligonucleotide primers and the PCR. **Primer pair A:** Primer CaCSD2-1 (5'-CCCAGGCTCACACAGATCATTCGC; nt 27–43 of GenBank accession no. D13454 are shown in boldface type; a *Stu* I site was added at the 5' end) and primer CaCSD2-2 (5'-GTGAATCACGCT-TACCTC; the complement of nt 2648–2665 of GenBank accession no. D13454) yielded a fragment of 2.6 kb (fragment A). Reaction conditions were 10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM MgCl<sub>2</sub>/0.2 mM of each dNTP/≈1 μM of each primer/SGY243 chromosomal DNA at 5–10 ng/μl and *Taq* polymerase at 0.05 unit/μl. After incubation at 92°C for 3 min, the mixture was subjected to 31 amplification cycles (1 min at 92°C, 30 sec at 62°C, 3 min at 72°C), followed by a 7-min incubation at 72°C. **Primer pair B:** Primer CaCSD2-3 (5'-CGATGAAACTGTGCCACCAG, nt 2547–2566 of GenBank accession no. D13454) and primer CaCSD2-4 (5'-CCCTCTAGAGGGACCCTTGAGTATTAGC; the complement of nt 4551–4570 of GenBank accession no. D13454 are shown in boldface type; an *Xba* I site was added at the 5' end) yielded a fragment of 2.0 kb (fragment B). Reaction conditions were the same as for fragment A, except that MgCl<sub>2</sub> concentration was 6 mM. Fragments A and B overlap by 119 nt; a single *Asp* 718 site (the only one in GenBank accession no. D13454) is located in the overlap region, and fragments A and B were joined at this site to give the intact *CHS3* gene. Fragment A was digested with *Stu* I and *Asp* 718, and fragment B was digested with *Xba* I and *Asp* 718. These fragments were ligated into the *Hinc*II and *Xba* I sites of pSKΔEcoRIEcoRV, a derivative of pSK lacking the *Eco*RI and *Eco*RV sites in the polylinker, to give pCHC2-8. To make a disrupted allele of *CHS3*, pCHC2-8 was digested with *Eco*RI, the ends were filled in with Klenow fragment, and *Bgl* II linkers [5'-d(pCAGATCTG)] were added (which regenerated the *Eco*RI site) to give pCHC2-9. The “URA blaster” cassette was inserted into this plasmid. To obtain the cassette, pMB-7 (16) was cut with *Sal* I, the ends were made blunt with Klenow, and then a second digestion was done with *Bgl* II. The 4-kb fragment was ligated into the *Eco*RV and *Bgl* II sites of pCHC2-9 to give pCHC2-11. The latter was digested with *Pst* I and *Not* I, producing a 7-kb linear fragment containing the disrupted gene. Sequential transformation of strain CA14 was done as described (16). Two Southern analyses were done. In the first analysis, chromosomal DNA was digested with *Bam*HI and probed with the 3.2-kb *Bam*HI fragment from pCHC2-8 (probe 1). Sizes of the hybridizing bands were 3.2 kb for *CHS3* and 1.75 and 0.75 kb for *Δchs3-1::hisG\_URA3\_hisG*, *Δchs3-2::hisG*, and *Δchs3-3::hisG\_URA3\_hisG\_URA3\_hisG*, respectively. In the second analysis, chromosomal DNA was digested with *Spe* I and probed with the 1.5-kb *Eco*RV-*Xba* I fragment from pCHC2-8 (probe 2). Sizes of the hybridizing bands were 1.7 kb for *CHS3*, 5.5 kb for *Δchs3-1::hisG\_URA3\_hisG*, 2.6 kb for *Δchs3-2::hisG*, and 8.6 kb for *Δchs3-3::hisG\_URA3\_hisG\_URA3\_hisG*. PL, polylinker left—*Kpn* I-*Apa* I-*Xho* I-*Sal* I-*Cl*a I-*Hind*III-*Pst* I-*Hind*III-*Cl*a I; PR, polylinker right—*Xba* I-*Not* I-*Eag* I-*Sac* II-*Bst*XI-*Sac* I; HS, *Hinc*II-*Stu* I junction; B, *Bam*HI; E, *Eco*RI; S, *Spe* I; V, *Eco*RV.

To derive a homozygous disruptant, three *CHS3/chs3::hisG* heterozygotes (CACB3A, CACB8A, and CACB10A) were subjected to a second round of transformation. Homologous

integration of the disrupted *chs3* gene could occur at either *CHS3* or *chs3::hisG* to give the desired disruptant (*chs3::hisG/chs3::hisG\_URA3\_hisG*) or the heterozygote (*CHS3/chs3::hisG\_URA3\_hisG*), respectively. Because we expected the disruptants to be chitin-deficient, and consequently Calcofluor-resistant, ≈10 transformants from each parental heterozygote were streaked onto plates containing a gradient of Calcofluor ranging from 0 to 1 mg/ml. Out of 29 transformants, most were inhibited by a relatively low Calcofluor concentration; only six transformants exhibited uniform growth across the entire gradient. These six strains and four Calcofluor-sensitive transformants were analyzed by Southern hybridization. An intact *CHS3* gene was present in all four Calcofluor-sensitive strains but was absent from all six Calcofluor-resistant strains. Further characterization of the transformants revealed the presence of two forms of the disrupted allele; *Δchs3-1::hisG\_URA3\_hisG* was recovered, as was a larger allele that contains an additional copy of *hisG* and *URA3*, as shown in Fig. 1 (*Δchs3-3::hisG\_URA3\_hisG\_URA3\_hisG*). The formation of multiple arrays of *hisG* and *URA3* has been reported (22). The genotypes of these transformants and their relationships to one another are given in Table 1. For the remainder of this paper, all disrupted alleles will be referred to as *Δchs3*.

We next determined the amount of chitin in *Δchs3/Δchs3* disruptants and in the related *CHS3/Δchs3* heterozygotes. In the yeast form, the disruptants are chitin-deficient, as expected from the Calcofluor-resistant phenotype. A small amount of chitin, ≈10–20% of the wild-type level, which is probably synthesized by the other chitin synthases, was detected (Table 2). To assess the role of *CHS3* in germ-tube formation, strains CACB8B-5 (*Δchs3/Δchs3*) and CACB8B-6 (*CHS3/Δchs3*) were induced to form germ tubes in three different media: 20% serum, a synthetic medium with *N*-acetylglucosamine (19), and RPMI 1640 medium. The frequencies of germ-tube formation were similar for the *Δchs3/Δchs3* and *CHS3/Δchs3* strains in the various media (data not shown) indicating that *CHS3* is not required for germ-tube formation.

Chitin synthesis was localized by staining *CHS3/Δchs3* (CACB8B-6) and *Δchs3/Δchs3* (CACB8B-5) cells with Calcofluor (24). Yeast cells (Fig. 2 A and B) and hyphae (Fig. 2 C and D) were examined. Bud scars, clearly visible on *CHS3/Δchs3* yeast cells (Fig. 2A), were absent from *Δchs3/Δchs3* yeast cells (Fig. 2B). Septa were observed in the yeast and mycelial forms of both strains (Fig. 2 C and D). Thus, *CHS3* is required for chitin-ring formation but not for septation. The most striking differences between the strains were seen when Calcofluor was added to the growth medium. Elorza *et al.* (14) showed that adding Calcofluor to cultures of *C. albicans* produces delocalized deposition of chitin in both yeast cells and germ tubes, and we obtained similar results with the *CHS3/Δchs3* strain. In contrast, the *Δchs3/Δchs3* was resistant to the action of Calcofluor during both yeast (data not shown)

Table 2. Determination of cell-wall chitin in mutants of *C. albicans* homozygous or heterozygous for a disrupted allele of *CHS3*

Strain	Relevant genotype*	Chitin, μg/ml
CACB3B-5	<i>Δchs3/Δchs3</i>	0.286
CACB8B-5	<i>Δchs3/Δchs3</i>	0.305
CACB10B-6	<i>Δchs3/Δchs3</i>	0.291
CACB10B-10	<i>Δchs3/Δchs3</i>	0.316
CACB3A	<i>Δchs3/CHS3</i>	1.34
CACB8B-6	<i>Δchs3/CHS3</i>	1.85
CACB10B-8	<i>Δchs3/CHS3</i>	1.80
CA14	<i>CHS3/CHS3</i>	1.98

Yeast cells were harvested, and chitin was quantitated as described (23). Chitin is expressed as μg of chitin per mg of wet cells.

\*Specific alleles of *chs3* are given in Table 1.

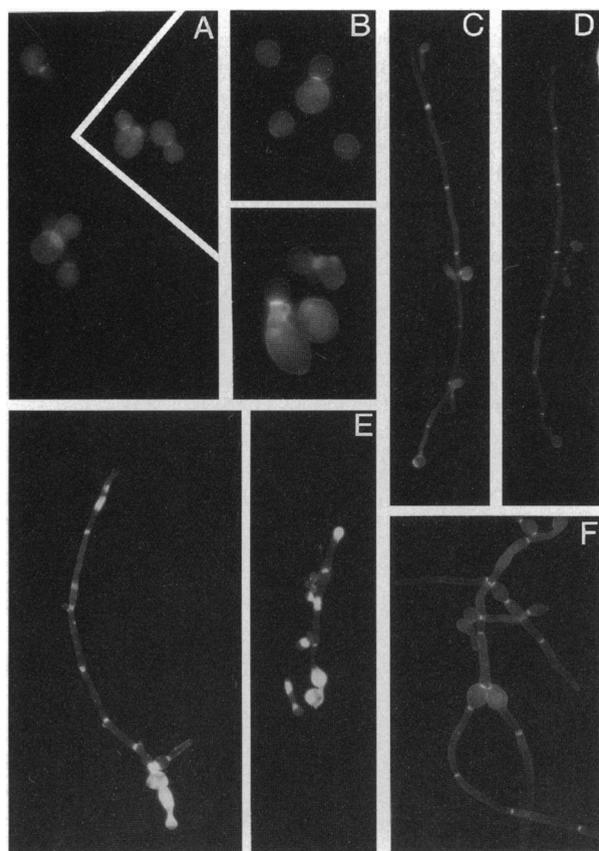


FIG. 2. Visualization of chitin in  $\Delta chs3/CHS3$  and  $\Delta chs3/\Delta chs3$  cells of *C. albicans* with Calcofluor. In *A–D*, cells were grown overnight in YPD at 26°C (*A* and *B*) or RPMI 1640 medium at 37°C (*C* and *D*) and then harvested and resuspended in water containing Calcofluor at 100  $\mu\text{g}/\text{ml}$ . Photomicroscopy was done with an Olympus B $\times$ 50 microscope as described (23). (*A*) Yeast cells of CACB8B-6 ( $\Delta chs3/CHS3$ ). (*B*) Yeast cells of CACB8B-5 ( $\Delta chs3/\Delta chs3$ ). (*C*) Germ tubes of CACB8B-6 ( $\Delta chs3/CHS3$ ). (*D*) Germ tubes of CACB8B-5 ( $\Delta chs3/\Delta chs3$ ). (*E* and *F*) Same as *C* and *D*, respectively, except that the RPMI 1640 medium contained Calcofluor at 100  $\mu\text{g}/\text{ml}$ ; aliquots were removed from each culture and examined directly. [Magnification:  $\times 640$  (*A* and *B*);  $\times 260$  (*C–F*).]

and hyphal growth. The effect of adding Calcofluor to cells undergoing germ-tube formation is illustrated in Fig. 2 (*E* and *F*). In  $CHS3/\Delta chs3$  cultures, patches of intense fluorescence (Fig. 2*E*) were observed on most cells, and the frequency of germ-tube formation was low. In contrast, the rate of frequency of germ-tube formation of  $\Delta chs3/\Delta chs3$  cells was not significantly altered by incubation with Calcofluor. Septa were clearly visible, but no abnormal deposition of chitin was observed (Fig. 2*F*). Thus, *CHS3* is required for the Calcofluor-induced synthesis of chitin.

Additional characterization indicated that the chitin-deficient disruptants have no obvious alterations in growth rate, sugar assimilation, or chlamydo-spore formation (data not shown). When growing vegetatively in liquid medium, the disruptants tend to clump, and in a fraction of the cells, the neck region is distended (Fig. 2*B* Lower). The disruptants are hypersensitive to  $\beta$ -glucuronidase, as judged by the accelerated formation of spheroplasts relative to wild-type strains.

**The *chs3* Disruptants Display Reduced Virulence in Both Immunocompetent and Immunocompromised Mice.** The virulence of the disruptants and the heterozygotes was determined in male ICR mice as described above. The results (see Fig. 3) are similar to those obtained with the *pcd* mutants—the chitin-deficient disruptants are less virulent but, nonetheless, retain the ability to grow *in vivo*. Because virulence is atten-

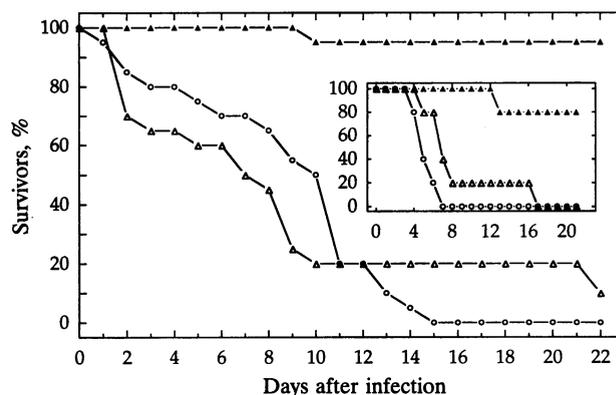


FIG. 3. Virulence of *CHS3* disruptants and heterozygotes against immunocompetent and immunocompromised mice. Mice were infected with  $1 \times 10^6$  cells of *C. albicans* as described. Seven congenic diploids, four  $\Delta chs3/\Delta chs3$  strains, and three  $\Delta chs3/CHS3$  strains, were tested; each strain was injected into five mice. Symbols denote infection with  $\Delta chs3/\Delta chs3$  (20 mice total) ( $\blacktriangle$ ),  $\Delta chs3/CHS3$  (15 mice total) ( $\triangle$ ), and wild-type (20 mice total) ( $\circ$ ). (Inset) Neutropenia was induced with cyclophosphamide as described. Mice were neutropenic for four days before infection; each strain was injected into five mice, and  $5 \times 10^3$  cells were administered per mouse. Symbols denote infection with CACB8B-5 ( $\Delta chs3/\Delta chs3$ ) ( $\blacktriangle$ ), CACB8B-6 ( $\Delta chs3/CHS3$ ) ( $\triangle$ ), and SC5314 (wild-type) ( $\circ$ ).

uated by the *ura3* mutation (25), we compared stability of the  $Urd^+$  phenotype in  $\Delta chs3/CHS3$  and  $\Delta chs3/\Delta chs3$  strains *in vivo*. Seven days after infection, kidneys were recovered and homogenized, and samples were spread onto plates with and without 5-FOA. Recovery of uridine auxotrophs was similar, 0.05% and 0.07%, for the  $\Delta chs3/CHS3$  and  $\Delta chs3/\Delta chs3$  strains, respectively, showing that the attenuated virulence of the chitin-deficient strain is not due to an enhanced generation of  $Urd^-$  segregants. The total number of colony-forming units recovered from the two strains was also similar (data not shown), indicating that the chitin-deficient strain colonizes this organ and proliferates. Thus, the reduced virulence of the  $\Delta chs3/\Delta chs3$  strain is not due to increased clearing or to an inability to grow *in vivo*.

To quantitate the difference in virulence between the  $\Delta chs3/CHS3$  and  $\Delta chs3/\Delta chs3$  strains, various numbers of cells were injected into mice, and morbidity was monitored. At 13 days after infection, morbidity reached 80% with  $1 \times 10^5$  cells of CACB8B-6 ( $\Delta chs3/CHS3$ ) versus  $1 \times 10^7$  cells of CACB8B-5 ( $\Delta chs3/\Delta chs3$ ). Thus, under these experimental conditions, the chitin-deficient mutants were  $\approx 100$ -fold less virulent than the  $\Delta chs3/CHS3$  strain.

Strains CACB8B-6 ( $\Delta chs3/CHS3$ ), CACB8B-5 ( $\Delta chs3/\Delta chs3$ ), and SC5314 ( $CHS3/CHS3$ , parental clinical isolate) were assayed for virulence in immunocompromised mice. Neutropenia was induced by i.p. injections of cyclophosphamide, and groups of five mice were infected with  $5 \times 10^3$  cells of each strain of *C. albicans*. As shown in Fig. 3 Inset, the virulence of the  $\Delta chs3/\Delta chs3$  is significantly attenuated.

## DISCUSSION

Using two strategies, chemical mutagenesis and disruption of *CHS3*, we have isolated chitin-deficient mutants of *C. albicans* and have shown that these mutants are less virulent than wild-type strains, as judged by the increased survival of infected mice. Not all chitin biosynthetic genes are important for virulence; *C. albicans* mutants lacking *CHS2*, the structural gene for a chitin synthase zymogen, have a modest reduction in cell-wall chitin and show no alteration in virulence (22).

In *S. cerevisiae*, *CSD2* (the homolog of *C. albicans CHS3*) is required for chitin synthase III activity. This enzyme, in

contrast to chitin synthases I and II, does not require proteolytic activation (12, 13, 26). [Under the appropriate experimental conditions, partial proteolysis stimulates chitin synthase III activity  $\approx$ 3-fold relative to untreated samples (26).] In *C. albicans*, a similar protease-independent activity has been identified (9). Contrary to expectations, the protease-independent activity in *C. albicans* does not require *CHS3*; *chs3* disruptants have no reduction in this activity. Our preliminary studies suggest that the protease-independent activity is encoded by *CHS2*; it appears that a small proportion of the *CHS2* gene product is detectable in membrane preparations without trypsin treatment. The elucidation of the biochemical defect in the *CHS3* mutant has been hampered by the existence of multiple chitin synthase isozymes.

Although the genetic defect(s) in the *pcd* mutants have not yet been identified, the chitin deficiency and Calcofluor resistance of these mutants suggest that the enzyme encoded by *CHS3* is impaired. Mutations in the other chitin synthases do not significantly reduce cell-wall chitin and do not confer resistance to Calcofluor.

The attenuated virulence of chitin-deficient mutants is consistent with earlier studies on the treatment of candidiasis with nikkomycins, competitive inhibitors of chitin synthases. Becker *et al.* (27) showed that the survival of *C. albicans*-infected mice is increased by nikkomycin therapy. Chapman *et al.* (28) demonstrated that nikkomycin Z inhibits chitin synthesis during the infection. When nikkomycin therapy was discontinued, the mice died of candidiasis. Consistent with the results of the present study, these findings can be explained as follows. Nikkomycin-treated mice harbor chitin-deficient organisms; when nikkomycin therapy is discontinued, chitin synthesis resumes and full virulence is regained.

In addition to chitin, other cell-wall components have been implicated in virulence. In *Paracoccidioides brasiliensis* (29), *Histoplasma capsulatum* (30), and *Blastomyces dermatitidis* (31), mutants deficient in  $\alpha$ -1,3-glucan are avirulent. In cerulenin-resistant mutants of *C. albicans*, alteration of mannan structure is correlated with reduced adherence (32) and virulence (33). In *Wangiella dermatitidis*, mutants lacking the cell-wall pigment melanin cause little or no mortality in mice. Interestingly, the latter mutants, like the chitin-deficient *C. albicans* mutants, remain able to proliferate in tissues; melanin-deficient mutants grow exponentially in mouse brains and cause neurological disease (34).

*C. albicans* treated with polyoxin D, leading to decreased cellular chitin content, have been shown to exhibit decreased adherence to buccal epithelial cells, suggesting a role for chitin in the attachment process (35). However, our results clearly indicate that chitin-deficient mutants readily colonize tissues of infected mice and adhere to either endothelial cells (S. Filler and J. Edwards, personal communication) or epithelial cells (P. Fidel and J. Sobel, personal communication). We conclude that adherence plays little or no role in the reduced virulence of the mutants investigated in this study.

Why are chitin-deficient strains less virulent? It is clear that loss of *CHS3* alters the polysaccharide composition of the cell wall. A concomitant alteration of protein composition is also possible because there is some evidence that certain cell-wall proteins are covalently attached to chitin (36). Furthermore, good evidence exists that glucan and chitin are covalently attached in the cell wall (37–39), raising the possibility that chitin deficiency also results in loss or damage of cell-wall glucan. Because cell-wall components have immunomodulatory properties, the structural changes caused by chitin deficiency may, in turn, alter host response. Recent studies suggest that *C. albicans* infection triggers a nonprotective host response (helper T cell type 2 response), in which the action of inhibitory cytokines leads to down-regulation of host-cell-mediated immunity (40). Thus, a mutant lacking a chitin or a chitin-associated component that triggers a helper T cell type

2 response deleterious to the host might be less virulent than a wild-type strain. Such a mutant might readily establish infection in the host tissue and yet be unable to elicit the host response that is also important for progression of the pathogenic process.

This study demonstrates that the role of various cellular components in the infection process of fungal pathogens is manifold and difficult to predict. Not only are virulence factors putatively produced by the pathogen, but also the host responses determine the outcome of the infection. Searches for antifungal therapies must take into account all of these factors.

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