JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2416–2419 Vol. 178, No. 8
0021-9193/96/$04.00+0

NOTES

Role of Three Chitin Synthase Genes in the Growth of *Candida albicans*

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Received 21 November 1995/Accepted 1 February 1996

The *CHS2* and *CHS3* genes of *Candida albicans* were disrupted. The double disruptant was still viable. Assessment of chitin and of calcofluor white resistance shows that *CHS1* is responsible for septum formation and *CHS3* is responsible for overall chitin synthesis otherwise. There were only small differences in virulence to immunocompromised mice of homozygous *chs2*Δ and homozygous *chs3*Δ null mutants.

Like *Saccharomyces cerevisiae*, *Candida albicans* harbors three chitin synthase genes, designated *CHS1*, *CHS2*, and *CHS3* (2, 6, 13). In *S. cerevisiae*, it was demonstrated by gene disruption experiments that chitin synthase 1 (Chs1p) is involved in the repair of damaged chitin, Chs2p is required for primary septum formation, and Chs3p is responsible for all other chitin syntheses (5, 12, 14). More recently, Kollar et al. reported that *CHS3* also contributes to the formation of linkage between chitin and β-1,3-glucan in *S. cerevisiae* (10). In order to gain more insights into the physiological roles of the chitin synthases of *C. albicans*, we have disrupted both *CHS2* and *CHS3* in *C. albicans* by means of the URA blaster protocol (1).

The homozygous *chs2*Δ null mutant and the homozygous *chs3*Δ null mutant strains of *C. albicans* were obtained by transforming CAI-4 cells (*ura3*Δ::*imm34*/*ura3*Δ::*imm34*) with DNA fragments containing either *CHS2* in which the *hisG-URA3-hisG* cassette was inserted at the unique *Xho*I site or *CHS3* in which the 0.8-kb *Nco*I-*Cla*I region was replaced by the *hisG-URA3-hisG* cassette by the lithium acetate method (9). These DNA fragments were successfully integrated into one of the diploid *CHS2* or *CHS3* alleles, respectively, and the *URA3* gene was efficiently eliminated by 5-fluoroorotic acid (5-FOA) selection (11) (Fig. 1). Then these DNA fragments were again transfected into cells in which one of the diploid *CHS2* or *CHS3* alleles was already flanked by the *hisG* sequence. Although the second allele of the *CHS2* locus was efficiently targeted by the same DNA fragment used to disrupt the first allele, the remaining *CHS3* allele was not easily disrupted by transfection of the same DNA fragment. Therefore, we constructed another plasmid in which the *hisG-URA3-hisG* cassette was inserted at the *Nco*I site of *CHS3*. We assumed that use of this DNA for the second round of transfection would increase the efficiency of homologous recombination between the transfected DNA and the remaining intact *CHS3* allele because the 0.8-kb *Nco*I-*Cla*I region of *CHS3* was missing in the already targeted *CHS3* locus. As expected, in 3 of 24 uracil auxotrophs, both of the *CHS3* alleles were found to be flanked by the *hisG* sequence after 5-FOA selection, resulting in the homozygous *chs3*Δ null mutation (Fig. 1).

Cells lacking functional *CHS3* grew in a rich medium such as YPD (1% peptone, 2% yeast extract, and 2% dextrose), but their growth was somewhat slower than that of cells missing *CHS2* or the parental strain CAI-4 (the doubling times for CAI-4, the homozygous *chs2*Δ null mutant, and the homozygous *chs3*Δ null mutant were about 70, 72, and 90 min, respectively). In addition, the homozygous *chs3*Δ null mutant became somewhat swollen, whereas the *chs2*Δ null mutant cells were morphologically indistinguishable from the parental CAI-4 cells. This slight increase in size of the homozygous *chs3*Δ null mutant cells was not obvious in the presence of sorbitol, suggesting that the homozygous *chs3*Δ null mutation increased susceptibility to osmotic shock.

It has been reported that the disruption of *CHS2* decreased the cellular chitin content in a hyphal-cell-specific manner, while that of *CHS3* led to about 80% reduction in cellular chitin levels in both yeast and hyphal cells (4, 8). We also addressed how cellular chitin synthesis was affected by the disruption of *CHS3*. The amount of cell wall chitin was estimated by measuring the *N*-acetylglucosamine content in alkali-insoluble fractions prepared from cells in either the yeast or hyphal form (3, 8, 15). As shown in Fig. 2, when the cells were grown as hyphae, the cellular chitin content in the homozygous *chs3*Δ null mutant cells was less than one-third of that in the parental CAI-4 cells, while those of the homozygous *chs2*Δ null mutant and the heterozygous *chs3*Δ null mutant were almost the same as that of CAI-4. Essentially similar results were obtained with cells in the yeast form. The amount of chitin in yeast form cells was about half of that in hyphal form cells in CAI-4. Neither the homozygous *chs2*Δ null mutation nor the heterozygous *chs3*Δ null mutation changed the chitin content, while deletion of both *CHS3* alleles led to a greater than 60% reduction in chitin content (Fig. 2). These results indicate that the cell wall chitin is synthesized largely by Chs3p in both yeast and hyphal cells and that *CHS2* contributes little to cellular chitin synthesis.

Calcofluor white staining of mutant cells that were growing as hyphae revealed that the entire cell surface was strongly stained by calcofluor white in the parental CAI-4 cells as well as the homozygous *chs2*Δ null mutant cells, although fluores-

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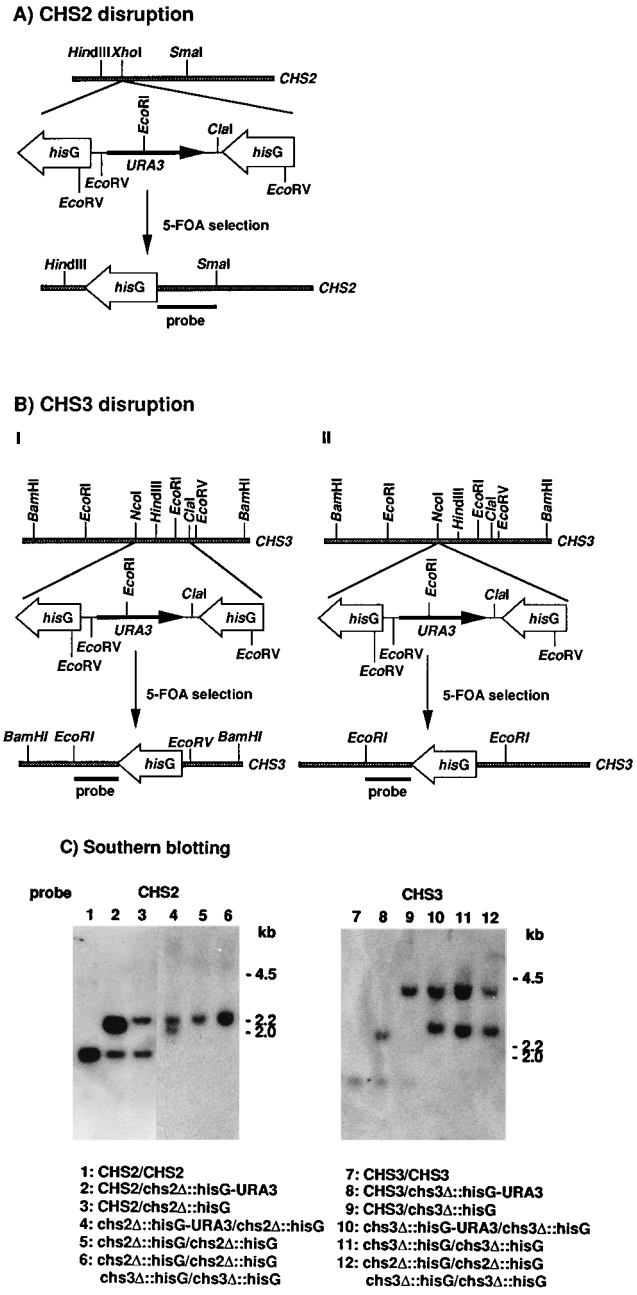

FIG. 1. Disruption of *CHS2* and *CHS3*. The schemes for the disruption of *CHS2* and *CHS3* in *C. albicans* are illustrated in panels A and B, respectively. After each transformation, the *URA3* gene that had been integrated into the genome was excised by treating the cells with 5-FOA. The probes used for Southern blotting are also indicated as bold bars. (C) Genomic Southern blotting of the mutant strains. Genomic DNA (25 μg) extracted from the indicated strains was digested with *Hin*dIII and *Sma*I (for the *CHS2* probe) or *Eco*RI (for the *CHS3* probe), separated by agarose gel electrophoresis, and hybridized with the probes. Bands of 1.4, 2.0, and 2.3 kb detected by the *CHS2* probe derived from the endogenous intact *CHS2* allele, the *chs2*Δ::*hisG-URA3* allele, and the *chs2*Δ::*hisG* allele, respectively. Bands of 1.4, 2.8, and 4.0 kb detected by the *CHS3* probe derived from the endogenous intact *CHS3* allele, the *chs3*Δ::*hisG-URA3* allele, and the *chs3*Δ::*hisG* allele, respectively. The positions of size markers are indicated (in kilobases).

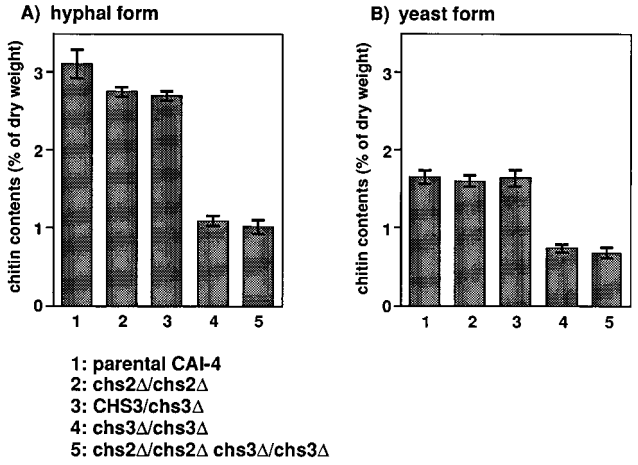

FIG. 2. Changes in the chitin content of *C. albicans* cells caused by the disruption of *CHS2* and *CHS3*. *C. albicans* cells of the parental strain CAI-4 (lane 1) and *chs2*Δ::*hisG*/*chs2*Δ::*hisG* (lane 2), *CHS3*/*chs3*Δ::*hisG* (lane 3), *chs3*Δ::*hisG*/*chs3*Δ::*hisG* (lane 4), and *chs2*Δ::*hisG*/*chs2*Δ::*hisG chs3*Δ::*hisG*/*chs3*Δ::*hisG* (lane 5) mutants were induced (B) or uninduced (A) to form hyphae for 12 h, and the chitin content of each strain was determined.

cence was somewhat weaker in the homozygous *chs2*Δ null mutant cells (Fig. 3A and B). In the homozygous *chs3*Δ null mutant cells, lateral fluorescence was significantly decreased compared with the homozygous *chs2*Δ null mutant and CAI-4 cells, yet the septum was still strongly stained with calcofluor white (Fig. 3C). Essentially the same results were obtained with cells in the yeast form.

The above results indicate that *CHS3* is required for chitin synthesis and strongly suggest that *CHS1* is involved in chitin synthesis at the septum in *C. albicans*. The involvement of *CHS1* in septum formation was further confirmed by generating a *C. albicans* strain in which both *CHS2* and *CHS3* were disrupted. The homozygous *chs2*Δ *chs3*Δ null mutant was obtained in the same way as the homozygous *chs3*Δ null mutant in a *chs2*Δ::*hisG*/*chs2*Δ::*hisG* background. After confirming that the one of the *CHS3* alleles was flanked by the *hisG* sequence, cells harboring the homozygous *chs2*Δ *chs3*Δ null mutation were screened in the presence of 1 mg of calcofluor white per ml, because the homozygous *chs3*Δ null mutation conferred resistance to calcofluor white. Of 104 Ura^+ clones, 14 clones were resistant to calcofluor white at 1 mg/ml. By genomic Southern blotting, all 14 clones were found to harbor the homozygous *chs2*Δ *chs3*Δ null mutations (Fig. 1), demonstrating that *C. albicans* cells were viable even without functional *CHS2* and *CHS3*. The morphology of the homozygous *chs2*Δ *chs3*Δ null mutant cells was quite similar to that of homozygous *chs3*Δ null mutant cells; they were somewhat swollen in the regular medium but restored to normal size in the presence of 1 M sorbitol. In addition, they underwent hyphal formation upon addition of horse serum and a temperature shift from 30 to 37°C. When both yeast and hyphal forms of the homozygous *chs2*Δ *chs3*Δ null mutant cells were stained with calcofluor white, the septum was still well stained, as shown for the homozygous *chs3*Δ null mutant (Fig. 3D and F), demonstrating that *CHS1* is involved in septum formation. Surprisingly, weak fluorescence was still visible in the lateral cell wall of the homozygous *chs2*Δ *chs3*Δ null mutant cells (Fig. 3D and F). This implies that a part of the chitin in the lateral cell wall is also synthesized by *CHS1*. However, we cannot rule out the possibility that more than three chitin synthases are present in *C. albicans*. We also tried to generate a homozygous *chs1*Δ null mutant of *C. albicans* to define the function of *CHS1* in more details but did not succeed in disrupting both alleles of *CHS1*. As already pointed out by Gow (7), one possibility is that *CHS1* is an essential gene in *C. albicans.*

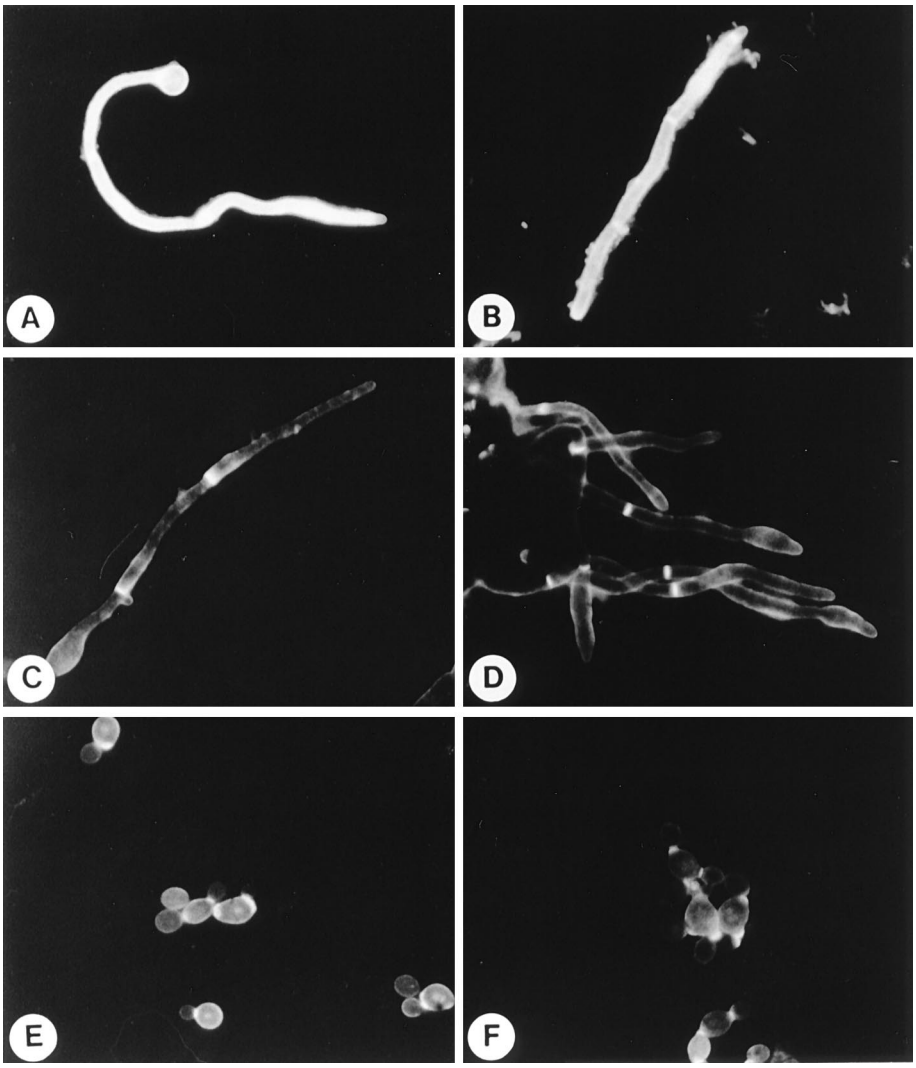

FIG. 3. Changes in the pattern of calcofluor staining in *C. albicans* cells after disruption of *CHS2* and *CHS3*. Cells of the parental strain CAI-4 (A and E) and *chs2*Δ::*hisG*/*chs2*Δ::*hisG* (B), *chs3*Δ::*hisG*/*chs3*Δ::*hisG* (C), and *chs2*Δ::*hisG*/*chs2*Δ::*hisG chs3*Δ::*hisG*/*chs3*Δ::*hisG* (D and F) mutants were induced (A, B, C, and D) or uninduced (E and F) to form hyphae for 12 h and stained with calcofluor white (1 mg/ml).

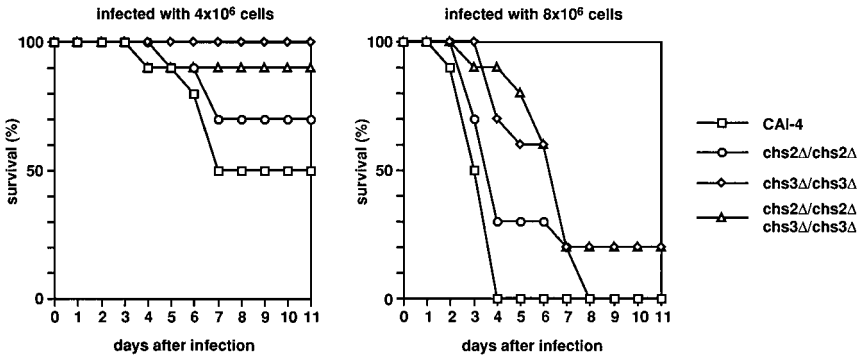

FIG. 4. Effects of disruption of *CHS2* and *CHS3* on the virulence of *C. albicans* cells. Male CD-1 mice that had been treated with 200 mg of cyclophosphamide per kg three times at 3 days before infection, on the day of infection, and 3 days after infection were infected with 6×10^6 cells (left panel) or 8×10^6 cells (right panel) of the parental strain CAI-4 or the *chs2*Δ::*hisG*/*chs2*Δ::*hisG*, *chs3*Δ::*hisG*/*chs3*Δ::*hisG*, or *chs2*Δ::*hisG*/*chs2*Δ::*hisG chs3*Δ::*hisG*/*chs3*Δ::*hisG* mutant. In each experiment, 10 mice were used for each strain, and the number of surviving mice after infection was scored.

According to the report of Bulawa et al. (4), disruption of *CHS3* drastically reduced the virulence of *C. albicans*. We also examined the virulence of the homozygous *chs2*Δ null mutant as well as the homozygous *chs3*Δ null mutant in immunocompromised CD-1 mice. Consistent with the earlier report by Gow et al. (8), the homozygous *chs2*Δ null mutant was as virulent as the parental CAI-4 (Fig. 4). However, the homozygous *chs3*Δ null mutation and the homozygous *chs2*Δ *chs3*Δ null mutation only slightly attenuated the virulence when 6×10^6 or 8×10^6 cells were injected intravenously (Fig. 4). When the mice were infected with 10^7 cells of CAI-4, the homozygous *chs2*Δ null mutant, or the homozygous *chs3*Δ null mutant, all of them died within 3 days (data not shown).

These results demonstrate that essentially neither *CHS2* nor *CHS3* is significantly involved in virulence and that the virulence of *C. albicans* is not tightly associated with chitin content or cellular chitin synthesis. However, it is possible that *CHS1* is critical for both vegetative growth and virulence. Our results for the virulence of the homozygous *chs3*Δ null mutant are inconsistent with the previous report by Bulawa et al. (4). We do not know the reason for this contradiction, but the involvement of *CHS3* in virulence might differ in different strains and conditions.

We thank N. Kleckner (Harvard University) and A. Shinohara (Osaka University) for plasmid pNKY51 and W. Fonzi (Georgetown University) for *C. albicans* CAI-4.

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