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Isolation of *Candida glabrata* Homologs of the *Saccharomyces cerevisiae KRE9* and *KNH1* Genes and Their Involvement in Cell Wall β-1,6-Glucan Synthesis

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The *Candida glabrata KRE9* (*CgKRE9*) and *KNH1* (*CgKNH1*) genes have been isolated as multicopy suppressors of the tetracycline-sensitive growth of a *Saccharomyces cerevisiae* mutant with the disrupted *KNH1* locus and the *KRE9* gene placed under the control of a tetracycline-responsive promoter. Although a *cgknh1*Δ mutant showed no phenotype beyond slightly increased sensitivity to the K1 killer toxin, disruption of *CgKRE9* resulted in several phenotypes similar to those of the *S. cerevisiae kre9*Δ null mutant: a severe growth defect on glucose medium, resistance to the K1 killer toxin, a 50% reduction of β-1,6-glucan, and the presence of aggregates of cells with abnormal morphology on glucose medium. Replacement in *C. glabrata* of the cognate *CgKRE9* promoter with the tetracycline-responsive promoter in a *cgknh1*Δ background rendered cell growth tetracycline sensitive on media containing glucose or galactose. *cgkre9*Δ cells were shown to be sensitive to calcofluor white specifically on glucose medium. In *cgkre9* mutants grown on glucose medium, cellular chitin levels were massively increased.

Candida (*Torulopsis*) *glabrata*, an imperfect fungus, is a haploid yeast of the genus *Candida* and has been demonstrated to be a pathogen of opportunistic yeast infections (1). There are increasing concerns over *C. glabrata*, because it causes not only mucocutaneous but also systemic infections in transplant and immunosuppressed patients (21, 58, 59). Moreover, the extensive use of topical and systemic antifungal drugs has resulted in the appearance of azole-resistant infections with *Candida* species, including *C. glabrata* (41, 59). Thus, there is a need to develop new antifungal drugs with novel modes of action and broad spectra.

Fungal cell wall biosynthesis is one possible target for new antifungal drugs, since it is essential for fungal viability and does not occur in mammals (18, 19). Fungal cell wall biosynthesis has been studied quite extensively in *Saccharomyces cerevisiae* (11, 14, 30) and *Candida albicans* (5, 11, 19, 36, 37) but not in *C. glabrata*. However, in addition to the advantage of its haploidy in genetic manipulation, recent progress on the molecular biology of *C. glabrata*, including development of host-vector systems (28, 29, 60), a controllable gene expression system (40), and the isolation of several structural sequences (17, 28, 35, 44), provides us with an opportunity to study cell wall biosynthesis in this organism.

β-1,6-Glucan is a component of fungal cell walls, where it occurs as a polymer covalently attached to glycoproteins (26, 38) and to other cell wall structural polymers such as β-1,3-glucan and chitin (14, 30). In *S. cerevisiae*, many genes involved in β-1,6-glucan synthesis were isolated through mutations (*kre* [killer resistant] mutations) that confer resistance to the K1 killer toxin, which kills sensitive yeast cells following binding to this β-1,6-glucan polymer (4, 6, 8, 15, 34, 48, 49). While other genetic studies have identified additional genes affecting cellular levels of β-1,6-glucan (24, 25, 46, 55), it still remains unclear how these genes, including the *KRE* genes, are concerned in β-1,6-glucan biosynthesis. Among them, *KRE9* and its homolog *KNH1*, genes encoding cell surface O glycoproteins, are required for β-1,6-glucan synthesis in *S. cerevisiae* (6, 8, 15). The *S. cerevisiae kre9*Δ null mutant shows several phenotypes: resistance to K1 killer toxin; slow growth, especially on glucose media; an 80% reduction of alkali-insoluble β-1,6-glucan; and defects in cell separation. Overexpression of *KNH1* can partially suppress these phenotypes of a *kre9*Δ null mutant (15). Although a *knh1*Δ null mutant showed no obvious phenotype, disruption of both *KRE9* and *KNH1* was synthetically lethal (15). Further, the *SKN7* gene encoding a yeast homolog of bacterial two-component regulators has also been isolated as a multicopy suppressor of the slow-growth phenotype of the *kre9*Δ null mutant (7, 9). Recently, a homolog of the *KRE9* gene has been isolated from *C. albicans* (33).

Here we report isolation of the *KRE9* and *KNH1* homologs in *C. glabrata* and several lines of evidence, including the first analysis of cell wall components in *C. glabrata*, suggesting evolutionary conservation of these molecules as essential components of β-1,6-glucan synthesis.

MATERIALS AND METHODS

Strains, growth media, and procedures. The *S. cerevisiae* and *C. glabrata* strains used in this study are listed in Table 1. YPD and YPGal are complex yeast media with 2% glucose and 2% galactose, respectively, and YNB is a synthetic medium with either 2% glucose or 2% galactose and supplemented for auxotrophic requirements. Yeast transformations were carried out by the modified lithium acetate method (20, 23) and the one-step transformation method (12). Tetracycline assays were carried out as previously described (39). Seeded-plate assays for killer toxin sensitivity were performed as previously described (8). Spotting assays were performed as previously described (31). 5-Fluoro-orotic acid, G418 (Geneticin), and calcofluor white (CFW) were purchased from PCR Inc. (Gainesville, Fla.), GIBCO BRL (Grand Island, N.Y.), and Polysciences Inc. (Warrington, Pa.), respectively. Plasmid DNA was propagated in *Escherichia coli* XL-1-blue cells (Stratagene, La Jolla, Calif.).

Manipulation of DNA. Techniques for manipulation of DNA were performed as previously described (52). Yeast genomic DNA was prepared as previously described (51). Southern blots were performed by using nylon membranes (Hybond N; Amersham Canada Limited, Oakville, Ontario, Canada) and following

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TABLE 1. Yeast strains used in this study

Strain	Genotype or description	Source or reference
S. cerevisiae		
SEY6210	*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 lys2-801 trp1-*Δ*901 suc2-*Δ*9*	S. D. Emr
HAB813	*MAT*α *kre9*Δ::*HIS3* in SEY6210	6
FAHAP4	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63*::*TRP1-tetRHAP4AD ura3-52*	39
SNB50-1	*MAT***a** *Kan*^{r}*-97t-KRE9* in FAHAP4	This work
SNB54-5	*MAT***a** *knh1*Δ::*his*G in SNB50-1	This work
C. glabrata		
2001HTU	*cgura3*Δ *cgtrp1*Δ *cghis3*Δ	28
ACG22	*cgtrp1*Δ::*CgTRP1-TAGAL4* in 2001HTU	40
SNBG1-7-7	*cgkre9*Δ::*CgTRP1* in 2001HTU	This work
SNBG2-26	*cgknh1*Δ::*CgHIS3* in 2001HTU	This work
SNBG3-10	*URA3-97t-CgKRE9* in ACG22	This work
SNBG4-49	*cgknh1*Δ::*CgHIS3* in SNBG3-10	This work
SNBG5	*cgkre9*Δ::*CgTRP1*; suppressor from SNBG1-7-7	This work

the instructions of the manufacturer. A PCR fragment harboring the entire coding sequence for *S. cerevisiae KRE9* was used as a probe. DNA sequencing was performed by the dideoxy method (53) on an ABI 373A sequencer with Bluescript universal and reverse primers and synthetic oligonucleotides complementary to specific regions of *CgKRE9* and *CgKNH1*.

Plasmids. A 0.7-kbp *Hin*dIII fragment harboring the *tetO-HOP1* chimeric promoter and a 1.4-kbp *Not*I fragment harboring the kanamycin resistance gene (*Kan*^{r}) were excised from p97t (39) and pKanMX2 (57), respectively, and systematically cloned into Bluescript SKII+ (Stratagene) to generate p97tKan. A 0.4-kbp *Spe*I-*Sac*II fragment of pMPY-ZAP (54), harboring the *hisG* sequence, was blunted with T4 DNA polymerase (GIBCO BRL) and cloned into the *Eco*RV site of Bluescript SKII+ to construct phisG+ and phisG−. The latter plasmids have their *hisG* sequences in opposite orientations. A 0.4-kbp *Sma*I-*Eco*RV fragment of phisG+, a 1.1-kbp *Sma*I-*Hin*dIII fragment of pMPY-ZAP (harboring the *S. cerevisiae URA3* gene), and a 0.4-kbp *Hin*dIII-*Sma*I fragment of p*his*G− were systematically cloned into Bluescript SKII+ to generate pSNZAP3, harboring a modified *hisG-URA3-hisG* module.

A 1.0-kbp *Eco*RI-*Hin*dIII fragment harboring the entire *CgKRE9* sequence was generated by PCR from *C. glabrata* genomic DNA with a pair of primers (5′-AAAGAATTCGGATCCAACACGCCTGTTGTG-3′, 5′-TTTCTCAAGCT TTTGGAAGATGGGAGGAC-3′), cloned into pUC118, and subjected to replacement of the region between *Kpn*I and *Sal*I sites with the *C. glabrata TRP1* (*CgTRP1*) sequence (28) to generate pCGK9ΔT (Fig. 1A). The 5′ portion of the *CgKNH1* sequence was generated by PCR with a pair of primers (5′-ATATGG TACCAATCAAATGCTCTCG-3′, 5′-CGTTGGGCCCGACACTCTGCGAC ACTTC-3′) as a 0.3-kbp *Kpn*I-*Sma*I fragment. The 3′ portion of the *CgKNH1* sequence was generated by PCR with a pair of primers (5′-ATATGGATCCTT ACGGGGAACAGAACGG-3′, 5′-AAGAGAGCTCAGTAAGTAGAGTGAA TATAC-3′) as a 0.4-kbp *Bam*HI-*Sac*I fragment. These two fragments and a 1.0-kbp *Xho*I fragment harboring the *C. glabrata HIS3* (*CgHIS3*) gene (28) were cloned into Bluescript SKII+ to generate pCGK1ΔH (Fig. 1B). A portion of the *CgKRE9* sequence including the start codon was generated by PCR with pSB2-1 as a template and a pair of primers (5′-CCATCGATGAATTCATGCTGCTG CTGGCTATACTGCTATC-3′, 5′-TTTCTCAAGCTTTTGGAAGATGGGAG GAC-3′) as a 0.3-kbp *Eco*RI-*Kpn*I fragment. This fragment and a 1.4-kbp *Sac*I-*Bam*HI fragment of pSB2-1 were cloned into p97t (39) to generate pCGK9tetAB (Fig. 2A).

pRS424 (13) was used to clone fragments for deletional analysis of the inserts of pSB2-1 and pSBG9-1. A 4.4-kbp *Pst*I fragment of pSB2-1 (Fig. 3A) and a 3.2-kbp *Sac*I-*Eco*RI fragment of *Pst*I fragment-deleted pSBG9-1 (Fig. 3B) were used for construction of plasmids derived from pRS316, pRS416 (56), pCgACT-14, and pCgACH-3 (29).

Construction of tetracycline-sensitive mutants of *S. cerevisiae KRE9* (Tet^s *KRE9*). Replacement of the cognate *KRE9* promoter with the tetracycline-responsive promoter, 97t (39), was achieved by the one-step gene replacement method (3, 54) with slight modifications. A DNA fragment was amplified by PCR using p97tKan as a template and a pair of primers (5′-GAATAGAACAGGAG TCTCAAAGCATTCTTGAAGCCAGATTGCAACAGCTATGACCATG-3′, 5′-AAAGCACATATGATGGAATTTCTTTGTAAACGCATTATGAATTCT TTTCTGAGATAAAG-3′) and subsequently was used for transformation of the *S. cerevisiae* strain FAHAP4, which harbors the tetR-HAP4AD fusion activator gene (39). After selection on G418-containing plates, the correct integration was confirmed by PCR and the strain was designated SNB50-1. Disruption of the *KNH1* gene in SNB50-1 was achieved by using a DNA fragment amplified by PCR using pSNZAP3 as a template and a pair of primers (5′-CTGATAGTAT TATTCTTAACATTATTTTGTTCGGTAGTGTTCCGTAAAACGACGGCC

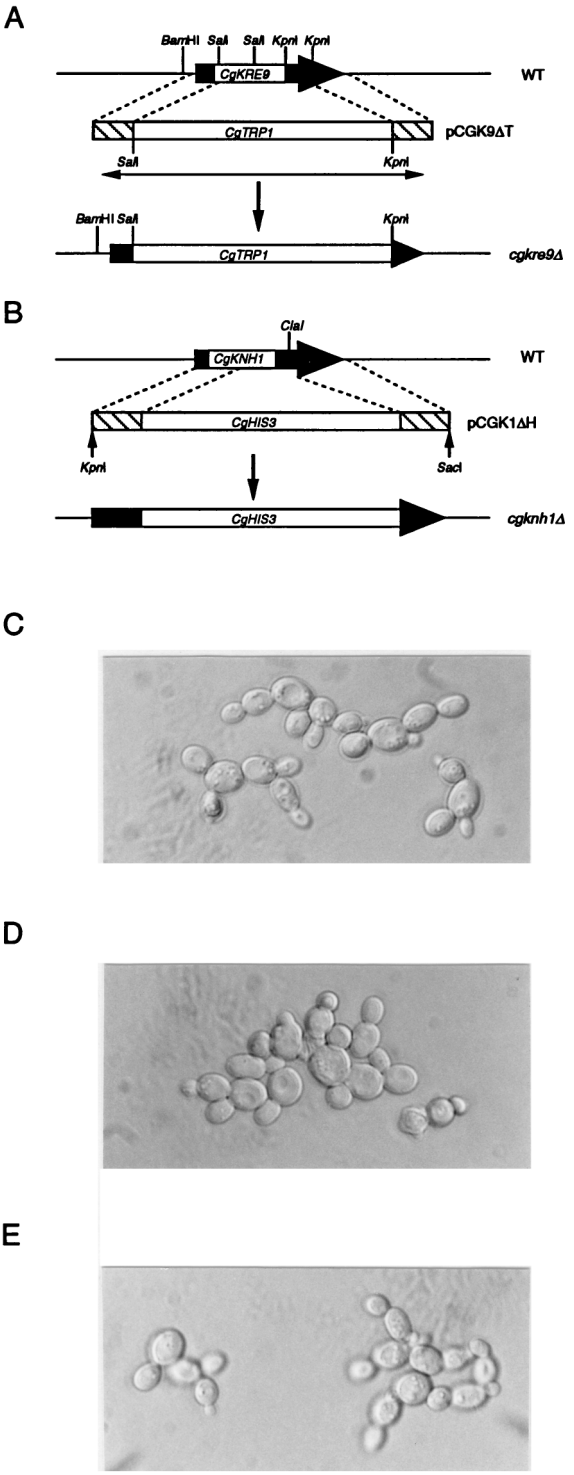

FIG. 1. Disruption of *CgKRE9* and *CgKNH1* and morphological effects of the deletions. (A) Disruption of *CgKRE9*. A PCR-amplified fragment (double-headed arrow) from pCGK9ΔT (Materials and Methods) was used for the one-step gene replacement. (B) Disruption of *CgKNH1*. A *Kpn*I-*Sac*I fragment of pCGK1ΔH (Materials and Methods) was used for the one-step gene replacement. Homologous recombination between the two regions (hatched boxes) resulted in disruption of the chromosomal copy. The wild-type strain, 2001HTU (C), *cgkre9*Δ deletion strain SNBG1-7-7 (D), and *cgknh1*Δ deletion strain SNBG2-26 (E) as viewed by Normarski optics are shown. Cells precultured on galactose medium were cultured on glucose medium.

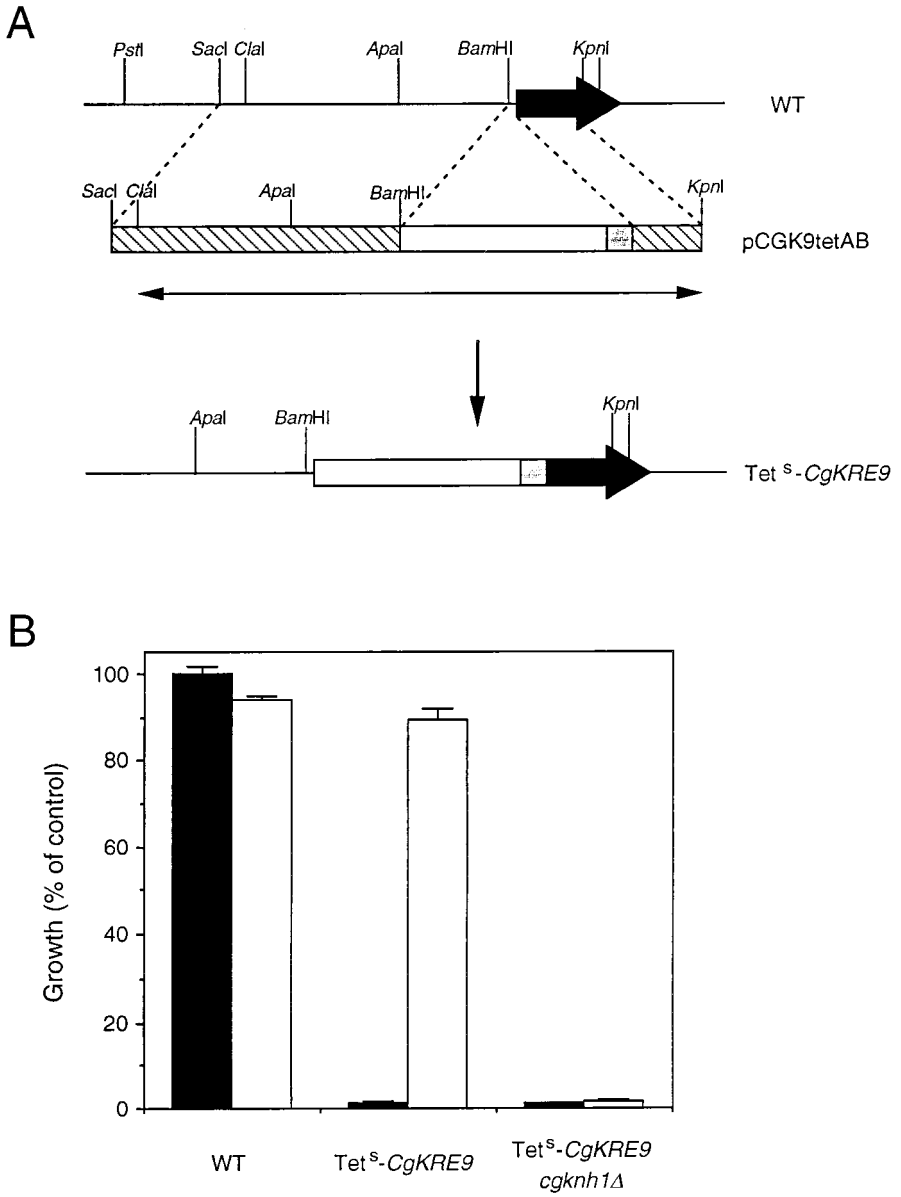

FIG. 2. Construction of a tetracycline-sensitive mutant of *CgKRE9* (Tet^s *CgKRE9*). (A) Scheme for replacement of the cognate *CgKRE9* promoter region with the tetracycline-responsive promoter. A PCR-amplified fragment (double-headed arrow) from pCGK9tetAB (Materials and Methods) was used for the one-step gene replacement. The solid arrow indicates the ORF of *CgKRE9*. Open and shaded boxes indicate the *S. cerevisiae URA3* gene and the tetracycline-responsive promoter, 97t, respectively. Homologous recombination between the two regions (hatched boxes) resulted in generation of the Tet^s *CgKRE9* mutant. (B) Growth inhibition by tetracycline on the Tet^s *CgKRE9* mutants. A total of 10^4 cells were inoculated and were cultured on YPD (solid bars) or on YPGal (open bars) for 20 h at 30°C. Growth of cells with tetracycline (50 μg/ml) is expressed as percent of optical density at 600 nm of cells without tetracycline. As the wild type (WT), strain ACG22 (Table 1) was used. Error bars, standard deviations.

AGT-3′, 5′-CATTATCTGTGCCTCAAAGCATTAACTTTTCTTGCAGTCA GAGAAACAGCTATGACCATG-3′). The correct integration was confirmed by PCR. The strain was subjected to 5-fluoro-orotic acid selection and finally designated SNB54-5 after the elimination of the *URA3* gene was confirmed by PCR.

Cloning of *C. glabrata KRE9* and *KNH1* genes. SNB54-5 cells were transformed with a pRS424-based *C. glabrata* subgenomic bank, harboring *Eco*RI 4- to 7-kbp fragments of *C. glabrata* genomic DNA, and spread onto both YNB-glucose and YNB-galactose plates containing tetracycline (50 μg/ml). After incubation at 30°C for 3 days, colonies appeared on the plates, cells were collected, and plasmid DNA was recovered from them.

Disruption of *CgKRE9* and *CgKNH1* and construction of tetracycline-sensitive mutants of *CgKRE9* (Tet^s *CgKRE9*). Disruption of *CgKRE9* in strain 2001HTU was achieved by using a DNA fragment amplified by PCR using pCGK9ΔT as a template and a pair of primers (5′-CCATCGATGAATTCATGCTGCTGCT GGCTATACTGCTATC-3′, 5′-CAACTGGACAAATATCTAAC-3′) (Fig. 1). The correct integration was confirmed by PCR, and the strain was designated SNBG1-7-7. A *Kpn*I-*Sac*I fragment of pCGK1ΔH was used to disrupt *CgKNH1* (Fig. 1) in strain 2001HTU. The correct integration was confirmed by PCR, and the strain was designated SNBG2-26.

A *Kpn*I-*Cla*I fragment harboring target sequences for *CgKRE9* and *S. cerevisiae URA3* was excised from pCGK9tetAB and used for replacement of the *CgKRE9* promoter region with the tetracycline-responsive promoter, 97t (39, 40), in the *C. glabrata* strain ACG22 (40) (Fig. 2A). After the correct integration was confirmed by PCR, the strain was designated SNBG3-10. To construct SNBG4-49, a *Kpn*I-*Sac*I fragment of pCGK1ΔH was used to disrupt *CgKNH1* in SNB3-10. The correct integration was confirmed by PCR.

Cell wall component analysis. The levels of cell wall alkali-insoluble β-glucan were determined as previously described (15). The alkali-soluble and alkali-insoluble Zymolyase-resistant cell wall fractions were subjected to a dot blot

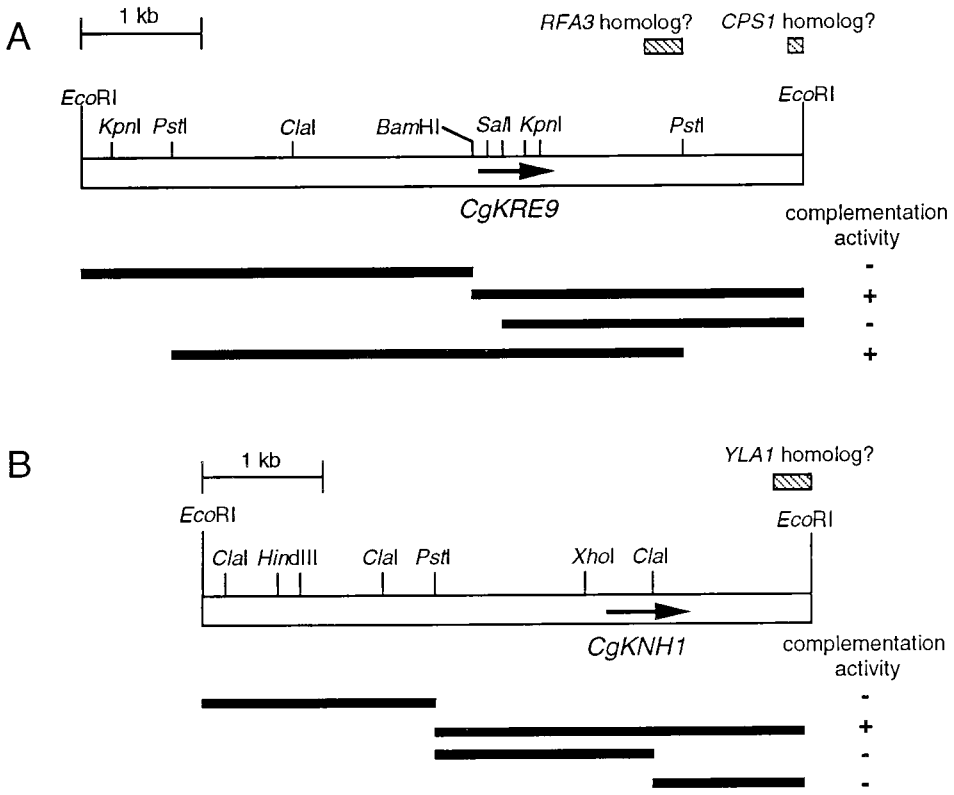

FIG. 3. Restriction maps and deletional analysis of inserts of *C. glabrata* genomic DNA on pSB2-1 and pSBG9-1. Open bars indicate the inserts on pSB2-1 (A) and pSBG9-1 (B). Fragments used for deletional analysis are represented by solid bars. The presence and absence of complementation activity in Tet^s *KRE9 knh1*Δ cells are indicated as + and −, respectively. Arrows indicate ORFs of *CgKRE9* (A) and *CgKNH1* (B). Hatched bars indicate regions with homology to the syntenic *S. cerevisiae* genes.

analysis by using anti-β-1,6-glucan antibody as previously described (33) with standardization by cell wall dry weight. The content of cellular chitin was determined as previously described (10) with *Streptomyces griseus* chitinase (Sigma, St. Louis, Mo.) and standardization by cell dry weight.

Sequence analysis and homology search. Sequence analysis was performed by using GeneWorks (Intelligenetics, Mountain View, Calif.) and GeneJockey (Biosoft, Cambridge, United Kingdom) software. A homology search for *C. glabrata* sequences against *S. cerevisiae* sequences was performed by using the WU-BLAST2 program in the *Saccharomyces* Genome Database (Stanford University).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to the GenBank database. The accession numbers of the *C. glabrata KRE9* (*CgKRE9*) and *KNH1* (*CgKNH1*) genes are AF064251 and AF064252, respectively.

RESULTS

Construction of tetracycline-sensitive mutants of the *S. cerevisiae KRE9* gene. To isolate the *S. cerevisiae KRE9* homolog from *C. glabrata*, we performed complementation screening. As convenient hosts for the screening, tetracycline-sensitive mutants of the *S. cerevisiae KRE9* gene (Tet^s *KRE9*) were constructed. The *KRE9* promoter region was replaced with a tetracycline-responsive promoter in a strain, FAHAP4, harboring the *tetR*-HAP4AD fusion activator gene for tetracycline-controllable gene expression (39). As shown in Fig. 4, addition of tetracycline (50 μg/ml) inhibited growth of cells of Tet^s *KRE9* mutant strain SNB50-1 on glucose medium but not on galactose medium. These observations resemble and are consistent with the finding that an *S. cerevisiae kre9*Δ mutant grows extremely slowly on glucose medium while growing somewhat better on galactose medium (15) and suggest that the concentration of tetracycline used in the present study is sufficient to repress the expression of *KRE9* driven by the tetracycline-responsive promoter. The tetracycline sensitivity of the Tet^s *KRE9* mutant was complemented by introduction of an extragenic copy of *KRE9* on pRS316 (6) (data not shown). Disruption of *KNH1* in a Tet^s *KRE9* mutant rendered cell growth tetracycline sensitive on glucose or galactose media (Fig. 4). This result is consistent with the known synthetic lethality between *kre9*Δ and *knh1*Δ mutations in *S. cerevisiae* (15). This Tet^s *KRE9 knh1*Δ mutant strain, SNB54-5, was used for complementation cloning of a *C. glabrata* homolog(s).

Cloning of *C. glabrata KRE9* and *KNH1* genes. By genomic Southern hybridization using the *S. cerevisiae KRE9* sequence as a probe, 5- and 6-kbp *Eco*RI fragments of *C. glabrata* genomic DNA were shown to contain sequences hybridizing to *S. cerevisiae KRE9* (data not shown). This result allowed us to make a subgenomic *C. glabrata* bank harboring *Eco*RI fragments ranging from 4 to 7 kbp to assist in their cloning by functional complementation.

After screening Tet^s *KRE9 knh1*Δ cells transformed with the subgenomic bank on plates containing glucose as a carbon source and tetracycline (50 μg/ml), pSB2-1 harboring the 6-kbp *Eco*RI fragment was isolated as a plasmid which allowed the mutant cells to grow as well as wild-type cells. However, plasmids harboring the 5-kbp *Eco*RI fragment, which also gave a hybridization signal in Southern analysis, were not isolated. Since the expression of *KNH1* is induced by galactose in *S. cerevisiae* (15), we screened a population of transformed cells for growth on plates containing galactose as a carbon source. In this way, pSBG9-1, a plasmid harboring the 5-kbp *Eco*RI fragment was isolated, as well as pSB2-1. As shown in Fig. 4, while

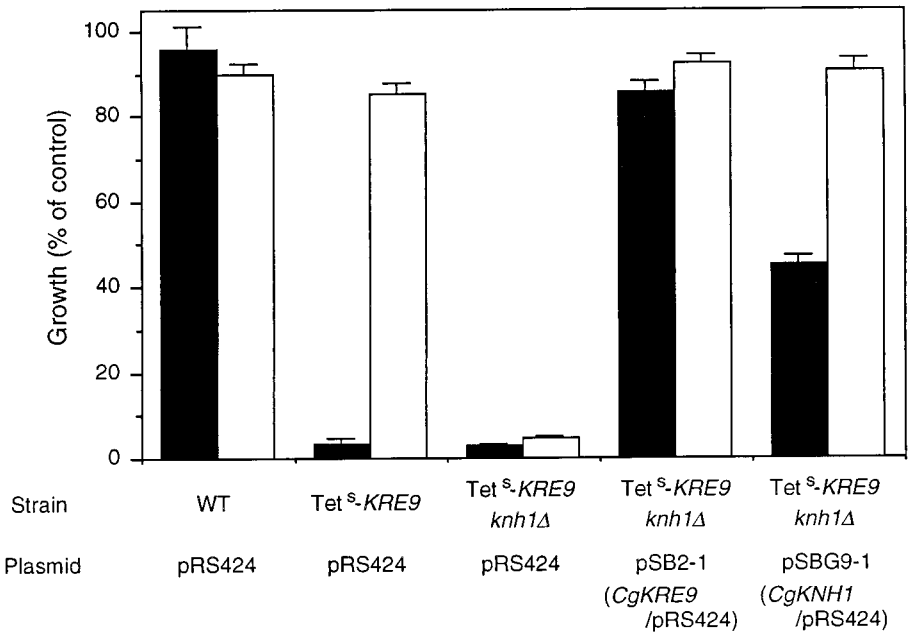

FIG. 4. Growth of *S. cerevisiae* Tet^s *KRE9 knh1*Δ cells harboring either pSB2-1 or pSBG9-1. About 10^4 cells were inoculated and cultured on YNB-glucose for 20 h (solid bars) or on YNB-galactose for 40 h (open bars) at 30°C. Cells were grown with or without tetracycline (50 μg/ml), and growth on tetracycline is expressed as the percentage of optical density at 600 nm of cells grown without tetracycline. The strain FAHAP4 (Table 1) was used as the wild type (WT). Error bars, standard deviations.

the tetracycline sensitivity of Tet^s *KRE9 knh1*Δ cells was complemented by pSBG9-1 partially on glucose medium but completely on galactose medium, pSB2-1 completely complemented the tetracycline sensitivity of Tet^s *KRE9 knh1*Δ cells on both media.

Deletional analysis of the inserts of the two plasmids demonstrated that a 1.4-kbp *Bam*HI-*Pst*I fragment of pSB2-1 and a 3.0-kbp *Pst*I-*Eco*RI fragment of pSBG9-1 were sufficient for the complementation activity (Fig. 3). DNA sequencing determined that the two plasmids harbored distinct open reading frames (ORFs). The ORF on pSB2-1 was predicted to encode a protein (276 amino acids) similar to *S. cerevisiae* Kre9p with 53% overall identity, and the protein (265 amino acids) deduced from the ORF on pSBG9-1 revealed 48% overall identity with *S. cerevisiae* Knh1p (Fig. 5). We designated the genes on pSB2-1 and pSBG9-1 *CgKRE9* and *CgKNH1*, respectively. Both predicted gene products showed features characteristic of their *S. cerevisiae* counterparts: putative N-terminal signals for secretion, a high proportion of serine/threonine residues (22% in both proteins) that could be potential sites for O glycosylation, and C termini rich in basic amino acid residues (Fig. 5).

Extensive sequencing on 3′ flanking regions of both *CgKRE9* and *CgKNH1* identified additional regions similar to the genes flanking the *KRE9* and *KNH1* genes in the *S. cerevisiae* genome. On pSB2-1, two sequences homologous to the *RFA3* and *CPS1* genes, respectively, which are located in the 3′ region of the *KRE9* locus on chromosome X of *S. cerevisiae*, were found (Fig. 3A). A sequence homologous to the *YLA1* gene, located in the 3′ region of the *KNH1* locus on chromosome IV, was found on pSBG9-1 (Fig. 3B).

Complementation activity of either *CgKRE9* or *CgKNH1* on a yeast centromeric plasmid, pRS416 (56), was also examined in the Tet^s *KRE9 knh1*Δ mutant. The tetracycline sensitivity of the mutant cells on glucose or galactose medium was complemented by introducing a plasmid, CgKRE9-pRS416, whereas CgKNH1-pRS416 complemented the sensitivity only on galactose medium (Fig. 6), suggesting that expression of *CgKNH1* is induced by galactose in *S. cerevisiae*.

Complementation of the killer phenotype of the *S. cerevisiae kre9* mutant by *CgKRE9* and *CgKNH1*. Mutations in *KRE9* confer resistance to the K1 killer toxin in *S. cerevisiae* (6, 8). In order to test whether multiple copies of *CgKRE9* and *CgKNH1* could complement this phenotype, pSB2-1 and pSBG9-1 were transformed into the *S. cerevisiae kre9*Δ null mutant strain HAB813 (Table 1) and the killer sensitivities of the transformants were examined by measuring zones of killing in a seeded-plate assay (8). The *kre9*Δ mutant cells are known to show no killer zone in the assay, since the mutant has an 80% reduction of β-1,6-glucan, which is necessary for the toxin binding. As shown in Table 2, cells harboring pSB2-1 formed killer zones when grown on glucose or galactose plates while cells harboring pSBG9-1 did so only when grown on galactose plates. The killer zone sizes, however, were smaller than those of wild-type strain SEY6210 cells, suggesting that the complementation was partial. We also examined complementation activity of either *CgKRE9* or *CgKNH1* on a single-copy plasmid as assayed via the killer resistance. Cells harboring CgKRE9-pRS416 formed killer zones in the seeding assay on glucose or galactose plates to the same extent as those harboring multiple copies of *CgKRE9* (Table 2), whereas cells harboring CgKNH1-pRS416 failed to form killer zones (data not shown). To show that the partial complementation of the killer phenotype of *kre9*Δ mutant was due to restoration of β-1,6-glucan levels, alkali-insoluble β-1,6-glucan levels in the mutant cells harboring either pSB2-1 or pSBG9-1 were determined. As shown in Table 2, although cells harboring pSBG9-1 showed no restoration, in cells harboring pSB2-1, the alkali-insoluble β-1,6-glucan level was partially elevated over that of the mutant when the cells were grown on glucose medium.

Disruption of *CgKRE9* and *CgKNH1* genes and construction of tetracycline-sensitive mutants of *CgKRE9* (Tet^s *CgKRE9*). To explore the physiological essentialness of *CgKRE9* and

A

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CgKre9p      M-L----LLA TLLSLFALIR A--DVNIVEP GPGDTFSGAD GT-VSINLKW   42
  Kre9p      MRLQRNSIIC ALVFLVSFVL G--DVNIVSP SSKATFSPSG GT-VSVPVEW   47
CgKnh1p      M-LSIILLLM QLVAA----- ---DIAVVGP SLDQSFDASG GT-AKIPIQW   40
  Knh1p      M-L--IVLFL TLFCSVVFRT AYCDVAIVAP EPNSVYDLSG TSQAVVKVKW   47

Consensus    M-L....L.. .L..... .--DV.IV.P .....F..SG GT-......W     50

CgKre9p      VDNGAYPPLD KVAYYLFSLC YGPNTKIQCV YKPDTKITPD DLDKDDAGTY   92
  Kre9p      MDNGAYPSLS KISTFTFSLC TGPNNNIDCV AVLASKITPS ELTQDDK-VY   96
CgKnh1p      LFTPNTPSQD DFTSLTFSLC SGPNVKIEAF KVIG-KL--S DIGTTD----   83
  Knh1p      MHTDNTPQEK DFVRYTFTLC SGTNAMIEAM ATLQ-TLSAS DLTDNE----   92

Consensus    ......P... .....TFSLC .GPN..I... .....K...S DL...D.--   100

CgKre9p      SYKFDIQSSV VGSGQPYVQV FAWVDGQGYT LHYTPRIELT NMGGPTTFEL   142
  Kre9p      SVTAEFASTL TGNGQYYIQV FAQVDGQGYT IHYTPRFQLT SMGGVTAYTY   146
CgKnh1p      -FEAEVSQSV GANGYYYVQI YA-ATTDGYT IHYSPRFKLT GMTGSKLPDT   131
  Knh1p      -FNAIIENTV GTDGVYFIQV FA-QTAIGYT IHYTNRFKLK GMIGTKAANP   140

Consensus    ...A.....V ...G.YY.QV FA.....GYT IHYTPRF.LT .M.G......   150

CgKre9p      LTYTDTIQPV PQTSIRTGTD TNTQSFD-AS SVYSLFYTEQ TLKTRVAPIQ   191
  Kre9p      ---SATTEPT PQTSIQTTTT NNAQATTIDS RSFTVPYTKQ TGTSRFAPMQ   193
CgKnh1p      L--LITAPPT PETRVTTG-- --DLGATIDS KSFDIPYGEQ NGKAKFAPMQ   175
  Knh1p      S--MITIAPE AQTRITTG-- --DVGATIDS KSFTVPYNLQ TGVVKYAPMQ   184

Consensus    .---.T..P. PQT.I.TG.. ......TIDS .SF..PY..Q TG....APMQ   200

CgKre9p      QQPGTKVTAT TWSRRYPTSA VTFYSTFRSS LDQLSTLTPA WNYTVSSAVN   241
  Kre9p      MQPNTKVTAT TWTRKFATSA VTYYSTFGSL PEQATTITPG WSYTISSGVN   243
CgKnh1p      TQPGTKITAT TWSRRYATSA VSFFTSLTAT PVQHTTLTPG WSYYISSDYN   225
  Knh1p      LQPATKVTAK TWKRKYATSE VTYYYTLRNS VDQHTTVTPG WSYIITADSN   234

Consensus    .QP.TKVTAT TW.R.YATSA VT.Y.T.... ..Q.TT.TPG WSY.ISS..N   250

CgKre9p      YATPAAMPSD NGGWYNPIKR QSLSTRKLNV RYLSS-----              276
  Kre9p      YATPASMPSD NGGWYKPSKR LSLSARKINM R--KV-----              276
CgKnh1p      YAPPAPFPSD NGGWYDPKKR QSFTTRKLNM DKLRKRRQTS              265
  Knh1p      YATPAPMPAD NGGWYNPRKR LSLTARKVNA LRHR------              268

Consensus    YATPA.MPSD NGGWY.P.KR .SL..RK.N. .....-----              290
```

B

	CgKnh1p	Kre9p	Knh1p
CgKre9p	43%	53%	38%
CgKnh1p		44%	48%
kre9p			44%

FIG. 5. Sequence comparisons of CgKre9p and CgKnh1p with their *S. cerevisiae* counterparts. (A) Alignment of the putative Kre9p and Knh1p amino acid sequences deduced from the *C. glabrata* (*CgKRE9* and *CgKNH1*) and *S. cerevisiae* (*KRE9* and *KNH1*) nucleotide sequences. The residues with conserved identity in all proteins are underlined in the consensus sequence. The putative N-terminal signals for secretion are underlined in each protein. Gaps (shown as dashes) were introduced to improve alignment. (B) Sequence identities between Kre9p and Knh1p proteins.

CgKNH1, each gene was disrupted with the *C. glabrata TRP1* (*CgTRP1*) and *HIS3* (*CgHIS3*) genes, respectively (Fig. 1). Transformation for disruption of *CgKRE9* was performed on plates containing either glucose or galactose as a carbon source. *cgkre9*Δ mutants were obtained from only galactose plates, whereas *cgknh1*Δ mutants were obtained from glucose plates. This carbon source dependency on the growth of *cgkre9*Δ mutant was confirmed by spotting cells precultured on galactose medium onto plates containing either 2% galactose, 2% glucose, or 2% glucose and galactose. Although *cgknh1*Δ cells on all plates and *cgkre9*Δ cells on the galactose containing plate grew as well as wild-type cells, the growth of *cgkre9*Δ cells was severely impaired on plates containing glucose as a carbon source (data not shown). These results suggest that the presence of glucose is involved in the slow-growth phenotype of the *cgkre9*Δ mutant. As shown in Fig. 1D, microscopic examination of *cgkre9*Δ cells transferred from galactose to glucose medium revealed the presence of aggregates of cells with abnormal morphology, which are also observed in the *S. cerevisiae kre9*Δ null mutant (6). However, *cgknh1*Δ cells showed no morphological change compared to the wild type (Fig. 1C and E).

To test for a possible synthetic lethality between *cgkre9* and *cgknh1* mutations, a *C. glabrata* tetracycline-controllable gene expression system (40) was applied to control the expression of *CgKRE9*. This system uses the same tetracycline-responsive promoters and *tetR* fusion activator as the system for *S. cerevisiae*. As shown in Fig. 2A, a tetracycline-sensitive mutant (Tet^s *CgKRE9*) was generated by replacing the cognate *CgKRE9* promoter region with the tetracycline-responsive promoter in *C. glabrata* ACG22, harboring the *tetR*-GAL4AD fusion activator gene (40). Consistent with the growth phenotype of a *cgkre9*Δ mutant, tetracycline (50 μg/ml) inhibited the growth of Tet^s *CgKRE9* cells specifically on glucose medium (Fig. 2B). This glucose-specific tetracycline sensitivity was complemented by introducing an extragenic copy of *CgKRE9* on pCgACH-3 (29), a centromeric plasmid for *C. glabrata* (data not shown). When *CgKNH1* was disrupted in a Tet^s *CgKRE9* mutant, cells failed to grow on glucose or galactose media in the presence of tetracycline (Fig. 2B). This result indicates that the disruption of both *CgKRE9* and *CgKNH1* is synthetically lethal in *C. glabrata*.

Killer phenotypes and β-1,6-glucan levels of *cgkre9*Δ and *cgknh1*Δ mutants. Although *cgkre9*Δ cells showed severe growth defects on glucose medium, spontaneous second-site suppressor mutations partially restoring growth arose when the cells were cultured by serial passage on glucose medium. Since it is known in *S. cerevisiae* that those second-site suppressors have no effects on the killer phenotypes except for enhanced growth of the original mutants (4, 8, 34, 48), we used such growth-suppressed *cgkre9*Δ mutants for further analysis as described below.

To address the killer phenotypes of *cgkre9*Δ and *cgknh1*Δ

TABLE 2. Killer phenotypes of alkali-insoluble β-glucan levels of the *S. cerevisiae kre9* cells harboring either *CgKRE9* or *CgKNH1*[d]

Strain	Allele at *KRE9* locus	Plasmid	Alkali-insoluble glucan(s)[a]		Killer zone size[b] (cm) on:	
			β-1,6-Glucan	β-1,3- and β-1,6-glucan	Glucose	Galactose
SEY6210	*KRE9*	pRS424	138.13 ± 5.15	354.55 ± 1.54	1.53 ± 0.06	1.25 ± 0.05
HAB813	*kre9*Δ::*HIS3*	pRS424	32.33 ± 2.00	301.51 ± 17.20	No zone	No zone
HAB813	*kre9*Δ::*HIS3*	pSB2-1 (*CgKRE9*/pRS424)	85.29 ± 2.24	315.28 ± 19.66	1.20 ± 0.09	1.02 ± 0.03
HAB813	*kre9*Δ::*HIS3*	pSBG9-1 (*CgKNH1*/pRS424)	32.86 ± 1.31	267.45 ± 10.53	No zone	0.70 ± 0.00
SEY6210	*KRE9*	pRS416	ND[c]	ND	1.48 ± 0.08	1.23 ± 0.08
HAB813	*kre9*Δ::*HIS3*	pRS416	ND	ND	No zone	No zone
HAB813	*kre9*Δ::*HIS3*	CgKRE9-pRS416	ND	ND	1.10 ± 0.05	1.02 ± 0.10

[a] β-Glucan levels are expressed as micrograms of glucan per milligram (dry weight) of cell wall.
[b] Killer zone size (diameter) was determined by seeded-plate assays as previously described (8).
[c] ND, not determined.
[d] All values are the means of at least three determinations ± 1 standard deviation.

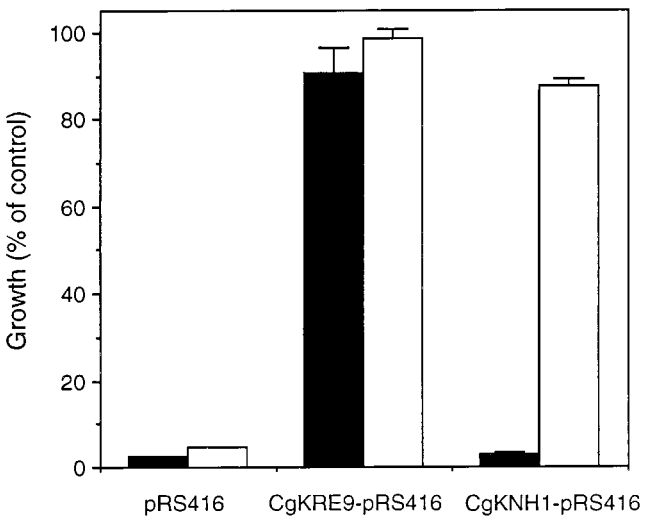

FIG. 6. Growth of *S. cerevisiae* Tet^s *KRE9 knh1*Δ cells harboring a single copy of either *CgKRE9* or *CgKNH1*. About 10^4 cells were inoculated and cultured on YNB-glucose for 20 h (solid bars) or on YNB-galactose for 40 h (open bars) at 30°C. Growth of cells with tetracycline (50 μg/ml) is expressed as percent of optical density at 600 nm of cells without tetracycline. FAHAP4 (Table 1) was used as the wild-type. Error bars, standard deviations.

mutants, we asked whether *C. glabrata* was sensitive to the K1 killer toxin. *C. glabrata* wild-type strain 2001HTU (Table 1) was found to be sensitive to the toxin on plates containing glucose or galactose as carbon sources, as measured by killer zones formed in a seeded-plate assay (Table 3). When mutant cells were assayed, growth-suppressed *cgkre9*Δ cells clearly formed smaller killer zones than those of wild-type cells, whereas *cgknh1*Δ cells formed slightly larger killer zones than those of wild-type cells (Table 3). We also examined the killer sensitivity of *cgkre9*Δ cells which had been stored on galactose medium to prevent second-site suppressor mutations. Although such mutant cells grew extremely slowly on glucose plates, sizes of killer zones of the cells were the same as those of growth-suppressed *cgkre9*Δ cells (data not shown).

To establish that the killer toxin resistance seen in the growth-suppressed *cgkre9*Δ cells was directly due to decreased levels in β-1,6-glucan, we attempted to determine β-1,6-glucan levels in *C. glabrata* cells. Following the method used in *S. cerevisiae*, alkali-insoluble cell wall fractions were digested with Zymolyase, a commercial β-1,3-glucanase preparation, and residual polymers were measured as hexose. As shown in Table 3, in growth-suppressed *cgkre9*Δ cells, hexose levels in the alkali-insoluble Zymolyase-resistant fraction were reduced to 40 and 50% of wild-type levels in cells grown on glucose and galactose medium, respectively. To verify the presence of β-1,6-linkage in these fractions, alkali-soluble and alkali-insoluble Zymolyase-resistant fractions from all three strains grown on glucose medium were subjected to a dot blot analysis using affinity-purified anti-β-1,6-glucan polyclonal antibody (33). In *cgkre9*Δ cells, the amount of material recognized by the antibody in both fractions was estimated at less than 50% of those of wild-type by comparing signals from serially diluted spotted samples (data not shown). These results strongly suggest that disruption of *CgKRE9* results in a more than 50% reduction of cell wall β-1,6-glucan independent of the carbon source used for growth.

Sensitivity to CFW and cellular chitin levels in *cgkre9* and *cgknh1* mutants. CFW, a negatively charged fluorescent dye that preferentially binds to nascent chains of chitin and interferes with cell wall assembly (16, 50), is a useful compound for surveying a broad range of cell wall defects in *S. cerevisiae* (32, 46). To test for cell wall defects in *cgkre9*Δ and *cgknh1*Δ mutants, CFW sensitivities of both growth-suppressed *cgkre9*Δ and *cgknh1*Δ cells were determined by a spotting assay (31) on plates containing glucose or galactose as a carbon source. Although *cgknh1*Δ cells grew as well as wild-type cells even in the presence of 25-μg/ml CFW, growth-suppressed *cgkre9*Δ failed to grow at this concentration of CFW when glucose was used as a carbon source (Table 3).

In *S. cerevisiae*, *kre9*Δ mutant cells gave strong fluorescence when stained by CFW (6). This evidence and glucose-specific CFW sensitivity of growth-suppressed *cgkre9*Δ cells led us to determine cellular chitin levels in *C. glabrata* cells. As shown in Table 3, on glucose medium, more than fourfold more cellular chitin was detected in growth-suppressed *cgkre9*Δ cells than in wild-type cells, while *cgknh1*Δ cells had almost the same amount of chitin as wild-type cells. On galactose medium, no significant difference was seen in chitin levels among these three strains.

To assess a possible correlation between this chitin increase and the second-site mutations suppressing the growth defect on glucose medium, we measured cellular chitin levels in *cgkre9*Δ cells without such suppressor mutations. For this purpose, two different strategies were taken. In one, a Tet^s *CgKRE9* mutant was used. In the other, *cgkre9*Δ cells, which had been stored on galactose medium, were switched from galactose to glucose medium. As shown in Fig. 7A, although the repression of *CgKRE9* expression is expected to be partial since the inoculum for the tetracycline assay was increased to permit sufficient cells to be obtained for the chitin measurement, addition of tetracycline resulted in an ~17-fold increase of chitin levels in the Tet^s *CgKRE9* mutant cells while there was no obvious change in cells of the parent strain, ACG22. When *cgkre9*Δ cells were transferred from galactose to glucose medium, cellular chitin levels increased by >15-fold (Fig. 7B). These results suggest that a considerable amount of chitin is present in *cgkre9*Δ cells grown in the presence of glucose and that such levels are unrelated to second-site mutations leading to growth suppression.

Overexpression of *CgKNH1* and *S. cerevisiae KRE9* in *cgkre9*Δ cells. We asked if multiple copies of either *CgKNH1* or *S. cerevisiae KRE9* could complement the phenotypes of a *cgkre9*Δ mutant. *CgKNH1* was cloned into pRS316 (56), which is known to be a multicopy plasmid for *C. glabrata* (60). CgKNH1-pRS316 and KRE9-pRS316 (6) were transformed into growth-suppressed *cgkre9*Δ cells. As summarized in Table 4, the killer sensitivities and β-1,6-glucan levels of the mutant cells were partially restored by multiple copies of *S. cerevisiae KRE9* whereas multiple copies of *CgKNH1* showed no effect. Further, multiple copies of either *CgKNH1* or *S. cerevisiae KRE9* allowed growth-suppressed *cgkre9*Δ cells to grow as well as wild-type cells on plates containing glucose and CFW (25 μg/ml). In the cells harboring CgKNH1-pRS316, the chitin increase was slightly suppressed (Table 4).

DISCUSSION

The *CgKRE9* and *CgKNH1* genes have been identified by functional screening using an *S. cerevisiae* Tet^s *KRE9 knh1*Δ mutant. Both *C. glabrata* gene products have significant overall identity with their *S. cerevisiae* counterparts (Fig. 5B). Partial restoration of the killer sensitivity and β-1,6-glucan levels of *kre9*Δ mutant cells harboring multiple copies of *CgKRE9* (Table 2) clearly indicates that CgKRE9 is an ortholog of *S. cerevisiae KRE9*. Furthermore, a single copy of *CgKRE9* was sufficient to partially complement the killer phenotype of the

TABLE 3. Alkali-insoluble glucan and cellular chitin levels in *C. glabrata* cells grown on either glucose or galactose[e]

Medium	Strain	Genotype	Killer zone size[b] (cm)	Alkali-insoluble glucan(s)[a]		CFW sensitivity[c]	Chitin[d]
				β-1,6-Glucan	β-1,3- and β-1,6-glucan		
YPD	2001HTU	WT	1.35 ± 0.00	52.48 ± 0.54	178.44 ± 4.54	R	0.88 ± 0.07
	SNBG5	*cgkre9*Δ::*CgTRP1*	0.73 ± 0.08	20.14 ± 1.34	233.56 ± 5.75	S	3.87 ± 1.10
	SNBG2-26	*cgknh1*Δ::*CgHIS3*	1.55 ± 0.00	52.57 ± 1.40	179.46 ± 4.29	R	0.90 ± 0.04
YPGal	2001HTU	WT	1.17 ± 0.02	76.14 ± 1.07	243.82 ± 9.06	R	1.02 ± 0.02
	SNBG5	*cgkre9*Δ::*CgTRP1*	0.63 ± 0.03	38.66 ± 1.62	260.74 ± 1.92	R	1.12 ± 0.05
	SNBG2-26	*cgknh1*Δ::*CgHIS3*	1.53 ± 0.02	84.11 ± 2.20	242.65 ± 5.46	R	1.08 ± 0.03

[a] β-Glucan levels are expressed as micrograms of glucan per milligram (dry weight) of cell wall.
[b] Killer zone size (diameter) was determined by seeded-plate assays as previously described (8).
[c] CFW sensitivity was scored by growth of 10^4 cells on plates containing CFW (25 μg/ml). R, resistant; S, sensitive.
[d] Chitin levels are expressed as micrograms of *N*-acetylglucosamine per milligram of dry cells.
[e] All values are the means of at least three determinations ± 1 standard deviation.

*kre9*Δ mutant (Table 2). This result also supports the argument for the functional similarity between Kre9p and CgKre9p and implies that the promoter activity of *CgKRE9* and the N-terminal signal for secretion of CgKre9p are active in *S. cerevisiae*.

Disruption of *CgKRE9* resulted in cells with phenotypes similar to that of the *S. cerevisiae kre9*Δ null mutant (6): a severe growth defect on glucose medium, resistance to the K1 killer toxin, a reduction of β-1,6-glucan, and the presence of aggregates of cells with abnormal morphology on glucose medium (Table 3; Fig. 1D). Some of these phenotypes were partially complemented by multiple copies of *S. cerevisiae KRE9* (Table 4). Recent cloning of the *C. albicans KRE9* (*CaKRE9*) gene has demonstrated that CaKre9p is also required for β-1,6-glucan synthesis in *C. albicans* (33). These lines of evidence indicate that the function of Kre9p as an essential component for β-1,6-glucan biosynthesis is conserved at least among *S. cerevisiae*, *C. albicans,* and *C. glabrata.*

*cgknh1*Δ mutants, however, had no phenotype beyond a slightly increased sensitivity to the K1 killer toxin. Further, multiple copies of *CgKNH1* failed to restore the killer sensitivity and alkali-insoluble β-1,6-glucan levels in *cgkre9*Δ cells grown on glucose medium (Table 4). However, in addition to the synthetic lethality suggested by the tetracycline sensitivity of Tet[s] *CgKRE9 cgknh1*Δ mutant (Fig. 6B), its ability to complement a range of *kre9* defects in *S. cerevisiae* and *C. glabrata* implies that CgKnh1p is related to Kre9p/CgKre9p and is an ortholog of *S. cerevisiae* Knh1p. These complementation abilities include *S. cerevisiae kre9* mutant phenotypes (Fig. 4 and Table 2), CFW sensitivity, and chitin increase of growth-suppressed *cgkre9*Δ cells (Table 4).

We have demonstrated that cellular chitin levels were significantly increased in *cgkre9* mutants on glucose medium (Table 3 and Fig. 7). It is known that chitin levels are also increased in several cell wall mutants of *S. cerevisiae* such as *gas1*Δ, *fks1*Δ, and *knr4*Δ mutants (22, 27, 45, 47). Based on genetic interaction between *gas1*Δ and *chs3*Δ mutations and the sensitivity to nikkomycin Z (a competitive inhibitor of chitin synthases) of a *gas1*Δ mutant, it has been hypothesized that such a chitin increase is essential for growth as a compensation mechanism to support the impaired cell wall integrity of

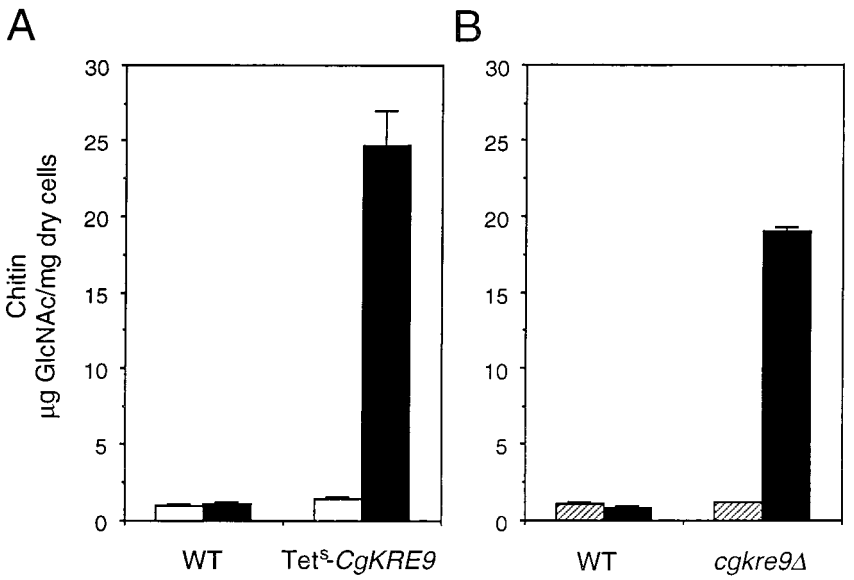

FIG. 7. Cellular chitin levels in *cgkre9* mutants of *C. glabrata*. (A) Effects of addition of tetracycline on cellular chitin levels in Tet[s] *CgKRE9* mutants. About 10^6 cells were cultured on YPD with (solid bars) or without (open bars) tetracycline (50 μg/ml) at 30°C for 20 h, and the cellular chitin levels were measured. As the wild type (WT), strain ACG22 (Table 1) was used. (B) Effect of switching the carbon source on cellular chitin levels in *cgkre9*Δ mutant. Cells precultured on YPGal were inoculated onto either YPD (solid bars) or YPGal (hatched bars) and cultured at 30°C for 20 h, and the cellular chitin levels were measured. As the wild type (WT), strain 2001HTU (Table 1) was used. Error bars, standard deviations.

TABLE 4. Effects of multiple copies of either *CgKNH1* or *S. cerevisiae KRE9* on the phenotypes of growth-suppressed *cgkre9*Δ cells[f]

Strain	Allele at *CgKRE9* locus	Plasmid	Alkali-insoluble glucan(s)[a]		Killer zone size[b] (cm)	CFW sensitivity[c]	Chitin[d]
			β-1,6-Glucan	β-1,3- and β-1,6-glucan			
2001HTU	*CgKRE9*	pRS316	76.05 ± 3.40	228.22 ± 6.11	1.46 ± 0.02	R	0.94 ± 0.02
SNBG5	*cgkre9*Δ::*CgTRP1*	pRS316	35.14 ± 1.42	246.14 ± 3.79	1.10 ± 0.02	S	3.71 ± 0.38
SNBG5	*cgkre9*Δ::*CgTRP1*	CgKNH1-pRS316	32.72 ± 0.78	218.78 ± 10.09	0.89 ± 0.01	R	2.95 ± 0.52
SNBG5	*cgkre9*Δ::*CgTRP1*	KRE9-pRS316	44.97 ± 3.01	204.59 ± 4.96	1.56 ± 0.02	R	ND[e]

[a] β-Glucan levels are expressed as micrograms of glucan per milligram (dry weight) of cell wall.
[b] Killer zone size was determined by seeded-plate assays as previously described (8).
[c] CFW sensitivity was scored by growth of 10^3 cells on plates containing CFW (25 μg/ml). R, resistant; S, sensitive.
[d] Chitin levels were expressed as micrograms of *N*-acetylglucosamine per milligram of dry cells.
[e] ND, not determined.
[f] All values are the means of at least three determinations ± 1 standard deviation.

these mutants (27, 45, 47). However, the increase of chitin in *cgkre9* cannot simply be concluded to be the result of such a compensation mechanism, since it is correlated with a severe growth defect on glucose medium and is independent of the reduction of β-1,6-glucan. This idea that increased chitin levels slow the growth of *cgkre9* mutants is supported by several observations in the present study. First, considerable amounts of cellular chitin were detected in both tetracycline-treated Tet[s] *CgKRE9* cells grown on glucose medium (Fig. 7A) and *cgkre9*Δ cells transferred from galactose to glucose medium (Fig. 7B). Second, there was no obvious increase in chitin levels in *cgkre9*Δ cells grown on galactose medium (Table 3 and Fig. 7B), on which they grew as well as the wild type did, in spite of a 50% reduction of alkali-insoluble β-1,6-glucan (Tables 3 and 4).

The mechanism and physiological relevance of the chitin increase in *cgkre9* mutants and its apparent glucose dependence remain to be elucidated. In *S. cerevisiae*, at least five genes have been known to be involved in the chitin synthase activity (11, 14). Cloning of these homologs and an enzymatic analysis of chitin synthesis in *C. glabrata* will be helpful in addressing this question. It will be useful to see if a chitin increase is common to *S. cerevisiae kre9* and other *kre* mutants, since second-site mutations suppressing growth defects have been isolated in many *kre* mutants and act without restoration of killer sensitivity or β-1,6-glucan levels (4, 8, 34, 48). Glucose-specific cross-linking changes in the cell wall of *cgkre9*Δ cells may result in elevated chitin levels and a severe growth defect on glucose medium.

Extensive sequencing of regions around both the *CgKRE9* and *CgKNH1* loci show that genomic organization in the 3′ regions of both homologs is conserved between *C. glabrata* and *S. cerevisiae* (Fig. 3). This synteny in regions of two chromosomes further indicates a close evolutionary relationship between *C. glabrata* and *S. cerevisiae*, consistent with the phylogenetic trees deduced from comparison of 5S (2) and 18S (43) rRNA genes. Further, CgKre9p and CgKnh1p have lower overall identity between themselves than to their orthologous *S. cerevisiae* counterparts (Fig. 5B). This observation implies that the duplication of the *KRE9* and *KNH1* genes took place before the divergence of these two fungi from a common ancestor. In contrast, no chromosomal conservation between *S. cerevisiae* and *C. albicans* was found in the 8-kbp fragment containing the *CaKRE9* locus (data not shown). This result supports the idea of a more distant relationship of *C. albicans* and *S. cerevisiae* based on phylogenetic trees deduced from the distribution of the serine-tRNA gene (42, 43) and comparison of rRNA genes (2, 43). Although the presence of a *KNH1* homolog in *C. albicans* still remains a possibility, this result suggests that extensive genomic reorganization around the *CaKRE9* locus has occurred since its divergence from a common ancestor with *S. cerevisiae*. For example, it is possible that the duplication event leading to the *KRE9* and *KNH1* pair in *S. cerevisiae* and *C. glabrata* occurred after the divergence of these yeast lineages from that of *C. albicans*.

In summary, although the molecular functions of the Kre9p/Knh1p proteins still remain to be characterized, the evolutionary conservation of the essentiality of these proteins supports the idea that compounds that interfere with their functions would be new antifungal drugs affecting a broad spectrum of pathogenic fungi. Our data also indicate that *C. glabrata* is a useful model pathogenic fungus for understanding biological processes, including cell wall biosynthesis.

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