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 *cgknh1Δ* 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 kre9Δ* 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 *cgknh1Δ* background rendered cell growth tetracycline sensitive on media containing glucose or galactose. *cgkre9Δ* 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and toxins, are required for fungal viability and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to *C. 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 a broad spectrum. Developing new antifungal drugs with novel modes of action and function have been demonstrated to

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TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<td>S. Emr</td>
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<tr>
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<td>pRS416</td>
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<td></td>
</tr>
<tr>
<td>pCgACT-14</td>
<td></td>
<td></td>
</tr>
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<tr>
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</tr>
<tr>
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**Construction of tetracycline-sensitive mutants of *S. cerevisiae KRE9* and *C. glabrata KNH1**

The construction of tetracycline-sensitive mutants of *S. cerevisiae* and *C. glabrata* was performed using the one-step gene replacement method. A PCR fragment harboring the entire coding sequence for the *C. glabrata* HIS3 gene (39) was generated by PCR with *C. glabrata* HIS3 (CGTRP1) sequence (28) to generate pCGK9

**Construction of tetracycline-sensitive mutants of *S. cerevisiae KRE9* (Tot*)**

Replacement of the cognate *KRE9* promoter with the tetracycline-responsive promoter, pCgACT-1 (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), cgknh1Δ deletion strain SNBG1-7-7 (D), and cgknh1Δ deletion strain SNBG2-26 (E) were cultured on galactose medium in the presence of tetracycline. Cells precultured on galactose medium were cultured on glucose medium.

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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 EcoRI 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+ CgKRE9). Disruption of CgKRE9 in strain 2001HTU was achieved by using a DNA fragment amplified by PCR using pCGK9tetAB as a template and a pair of primers (5’-CCATCGATGAATTCATGCTGCTGCGCTATCTGCTATCG-3’, 5’-CAACTGGACAAATATCTAAC-3’) (Fig. 1). The correct integration was confirmed by PCR, and the strain was designated SNBG1-7-7. A KpnI-SacI fragment of pCGK1tetAB 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 KpnI-ClaI 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 KpnI-SacI fragment of pCGK1tetAB 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 analysis.
analysis by using anti-β-1,3-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<sup>s</sup> 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<sup>s</sup> 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* kre9Δ 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<sup>s</sup> KRE9 mutant was complemented by introduction of an extragenic copy of KRE9 on pRS316 (6) (data not shown). Disruption of KNH1 in a Tet<sup>s</sup> 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<sup>D</sup> and knh1<sup>D</sup> mutations in *S. cerevisiae* (15). This Tet<sup>s</sup> KRE9 knh1Δ 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 EcoRI 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 EcoRI fragments ranging from 4 to 7 kbp to assist in their cloning by functional complementation.

After screening Tet<sup>s</sup> KRE9 knh1Δ 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 EcoRI 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 EcoRI 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 EcoRI fragment was isolated, as well as pSB2-1. As shown in Fig. 4, while...
the tetracycline sensitivity of Tet<sup>+</sup> KRE9 knh1Δ cells was complemented by pSBG9-1 partially on glucose medium but completely on galactose medium, pSB2-1 completely complemented the tetracycline sensitivity of Tet<sup>+</sup> KRE9 knh1Δ cells on both media.

Deletional analysis of the inserts of the two plasmids demonstrated that a 1.4-kbp BamHI-PstI fragment of pSB2-1 and a 3.0-kbp PstI-EcoRI 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<sup>+</sup> KRE9 knh1Δ 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 kre9Δ 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 kre9Δ 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 kre9Δ 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<sup>+</sup> CgKRE9).** To explore the physiological essentialness of CgKRE9 and...
Knh1p proteins. introduced to improve alignment. (B) Sequence identities between Kre9p and signals for secretion are underlined in each protein. Gaps (shown as dashes) were all proteins are underlined in the consensus sequence. The putative N-terminal containing either glucose or galactose as a carbon source.

formation for disruption of CgKRE9 (KRE9 and C. glabrata evisiae 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. cgkre9Δ mutants were obtained from only galactose plates. Although cgknh1Δ cells on all plates and cgkre9Δ cells on the galactose containing plate grew as well as wild-type cells, the growth of cgkre9Δ 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 cgkre9Δ mutant. As shown in Fig. 1D, microscopic examination of cgkre9Δ 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 kre9Δ null mutant (6). However, cgknh1Δ 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" CgKRE9) was generated by replacing the cognate CgKRE9 promoter region with the tetracycline-responsive promoter in C. glabrata (CgHIS3) genes, respectively (Fig. 1). Trans-locus Plasmid KRE9 C. glabrata TRP1 D::HIS3 pRS416 ND ND 1.10 2.00 301.51 0.03 1.02 \\
HAB813 kre9::HIS3 pRS424 1.54 1.53 1.20 0.10 1.02 \\
HAB813 kre9::HIS3 pSBG9-1 (CgKRE9::HIS3) 10.53 0.70 0.00 0.31 0.10 \\
HAB813 kre9::HIS3 pSBG9-1 (CgKNH1::HIS3) 10.53 0.00 0.00 0.21 0.10 \\
HAB813 kre9::HIS3 pRS424 1.54 1.53 1.20 0.10 1.02 \\
HAB813 kre9::HIS3 pSBG9-1 10.53 0.00 0.00 0.21 0.10 \\
HAB813 kre9::HIS3 pRS416 1.54 1.53 1.20 0.10 1.02 \\
HAB813 kre9::HIS3 pSBG9-1 10.53 0.00 0.00 0.21 0.10 \\

b Killer zone size (diameter) was determined by seeded-plate assays as previously described (8).

TABLE 2. Killer phenotypes of alkali-insoluble β-glucan levels of the S. cerevisiae kre9Δ cells harboring either CgKRE9 or CgKNH1Δ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele at KRE9 locus</th>
<th>Plasmid</th>
<th>Alkali-insoluble glucan(s)</th>
<th>Killer zone size (cm) on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-1,6-Glucan</td>
<td>β-1,3- and β-1,6-Glucan</td>
</tr>
<tr>
<td>SEY6210 KRE9</td>
<td>pRS424</td>
<td>138.13 ± 5.15</td>
<td>354.55 ± 1.54</td>
<td>1.53 ± 0.06</td>
</tr>
<tr>
<td>HAB813 kre9::HIS3</td>
<td>pRS424</td>
<td>32.33 ± 2.00</td>
<td>301.51 ± 17.20</td>
<td>No zone</td>
</tr>
<tr>
<td>HAB813 kre9::HIS3</td>
<td>pSBG9-1 (CgKRE9::HIS3)</td>
<td>85.29 ± 2.24</td>
<td>315.28 ± 19.66</td>
<td>1.20 ± 0.09</td>
</tr>
</tbody>
</table>

These values are the means of at least three determinations ± 1 standard deviation.

4 β-Glucan levels are expressed as micrograms of glucan per milligram (dry weight) of cell wall.

5 Killer zone size was determined by seeded-plate assays as previously described (8).

6 ND, not determined.

7 All values are the means of at least three determinations ± 1 standard deviation.

8 Killer phenotypes and β-1,6-glucan levels of cgkre9Δ and cgknh1Δ mutants.

Although cgkre9Δ 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 cgkre9Δ mutants for further analysis as described below.

To address the killer phenotypes of cgkre9Δ and cgknh1Δ
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 cgkre9Δ cells clearly formed smaller killer zones than those of wild-type cells, whereas cgknh1Δ cells formed slightly larger killer zones than those of wild-type cells (Table 3). We also examined the killer sensitivity of cgkre9Δ 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 cgkre9Δ cells (data not shown).

To establish that the killer toxin resistance seen in the growth-suppressed cgkre9Δ 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 cgkre9Δ 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 cgkre9Δ 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 cgkre9Δ and cgknh1Δ mutants, CFW sensitivities of both growth-suppressed cgkre9Δ and cgknh1Δ cells were determined by a spotting assay (31) on plates containing glucose or galactose as a carbon source. Although cgknh1Δ cells grew as well as wild-type cells even in the presence of 25-μg/ml CFW, growth-suppressed cgkre9Δ failed to grow at this concentration of CFW when glucose was used as a carbon source (Table 3).

In *S. cerevisiae*, kre9Δ mutant cells gave strong fluorescence when stained by CFW (6). This evidence and glucose-specific CFW sensitivity of growth-suppressed cgkre9Δ 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 cgkre9Δ cells than in wild-type cells, while cgknh1Δ 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 cgkre9Δ cells without such suppressor mutations. For this purpose, two different strategies were taken. In one, a Tet<sup>CgKRE9</sup> mutant was used. In the other, cgkre9Δ 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 tetrazycline 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<sup>CgKRE9</sup> mutant cells while there was no obvious change in cells of the parent strain, ACG22. When cgkre9Δ 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 cgkre9Δ 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 cgkre9Δ cells.** We asked if multiple copies of either CgKNH1 or *S. cerevisiae* KRE9 could complement the phenotypes of a cgkre9Δ mutant. CgKNH1 was cloned into pRS316 (56), which is known to be a multicycop plasmid for *C. glabrata* (60). CgKNH1-pRS316 and KRE9-pRS316 (6) were transformed into growth-suppressed cgkre9Δ 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 cgkre9Δ 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<sup>KRE9</sup> knh1Δ 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
The kreu9Δ 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 kreu9Δ null mutant (6): a severe growth defect on glucose medium, resistance to the K1 killer toxin, a reduction of β-1,6-glucan synthesis in C. albicans, 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 biosynthesis 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.

cgknh1Δ 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 cgkre9Δ cells grown on glucose medium (Table 4). However, in addition to the synthetic lethality suggested by the tetracycline sensitivity of Tet+ CgKRE9 cgknh1Δ mutant (Fig. 6B), its ability to complement a range of kreu9 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 kreu9 mutant phenotypes (Fig. 4 and Table 2), CFW sensitivity, and chitin increase of growth-suppressed cgkre9Δ cells (Table 4).

We have demonstrated that cellular chitin levels were significantly increased in cgkre9Δ 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 gas1Δ, fks1Δ, and knr4Δ mutants (22, 27, 45, 47). Based on genetic interaction between gas1Δ and chs3Δ mutations and the sensitivity to nikkomycin Z (a competitive inhibitor of chitin syntheses) of a gas1Δ 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

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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Strain</th>
<th>Genotype</th>
<th>Killer zone size (cm)</th>
<th>Alkali-insoluble glucan(s)</th>
<th>CFW sensitivity</th>
<th>Chitin (μg GlcNAc/mg dry cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-1,6-Glucan</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-1,3- and β-1,6-glucan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YPD</td>
<td>2001HTU</td>
<td>WT</td>
<td>1.35 ± 0.00</td>
<td>52.48 ± 0.54</td>
<td>R</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>SNBG5</td>
<td>cgkre9Δ::CgTRP1</td>
<td>0.73 ± 0.08</td>
<td>20.14 ± 1.34</td>
<td>S</td>
<td>3.87 ± 1.10</td>
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<tr>
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<td>SNBG26</td>
<td>cgknh1Δ::CgHIS3</td>
<td>1.55 ± 0.00</td>
<td>52.57 ± 1.40</td>
<td>R</td>
<td>0.90 ± 0.04</td>
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<tr>
<td>YPGal</td>
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<td>WT</td>
<td>1.17 ± 0.02</td>
<td>76.14 ± 1.07</td>
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<td>1.02 ± 0.02</td>
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<td>0.63 ± 0.03</td>
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<td>1.12 ± 0.05</td>
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<td>cgknh1Δ::CgHIS3</td>
<td>1.53 ± 0.02</td>
<td>84.11 ± 2.20</td>
<td>R</td>
<td>1.08 ± 0.03</td>
</tr>
</tbody>
</table>

*β-Glucan levels are expressed as micrograms of glucan per milligram (dry weight) of cell wall.
*Killer zone size (diameter) was determined by seeded-plate assays as previously described (8).
*CFW sensitivity was scored by growth of 10⁶ cells on plates containing CFW (25 μg/ml). R, resistant; S, sensitive.
*Chitin levels are expressed as micrograms of N-acetylglucosamine per milligram of dry cells.
*All values are the means of at least three determinations ± 1 standard deviation.

**FIG. 7.** Cellular chitin levels in cgkre9Δ mutants of C. glabrata. (A) Effects of addition of tetracycline on cellular chitin levels in Tet+ CgKRE9 mutants. About 10⁶ 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 cgkre9Δ 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.
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 \(\beta-1,6\)-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\(^+\) CgKRE9 cells grown on glucose medium (Fig. 7A) and cgkre9\(\Delta\) cells transferred from galactose to glucose medium (Fig. 7B). Second, there was no obvious increase in chitin levels in cgkre9\(\Delta\) 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 \(\beta-1,6\)-glucan (Tables 3 and 4). This emphasizes the increased chitin in cgkre9 mutants and its apparent glucose dependence. In S. cerevisiae, at least five genes have been shown to be involved in the chitin synthase activity (11, 14). Cloning of these homologs and an enzymatic analysis of chitin synthetase 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 \(\beta-1,6\)-glucan levels (4, 8, 34, 48).

Glucose-specific cross-linking changes in the cell wall of cgkre9\(\Delta\) 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 SS (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 CgKRE9 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 suggests that 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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