Disruption of the *SNF1* Gene Abolishes Trehalose Utilization in the Pathogenic Yeast *Candida glabrata*

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The *SNF1* gene product, a serine/threonine protein kinase, is a global regulatory protein which has been isolated from several organisms. In *Saccharomyces cerevisiae* the *SNF1* gene product is essential for the derepression of glucose repression since *snf1* strains are unable to utilize sucrose, galactose, maltose, melibiose, or nonfermentable carbohydrates. Moreover, the *SNF1* gene product was suggested to interact with additional regulatory pathways and to affect the expression of multiple target genes as reflected by the pleiotropic nature of the *snf1* mutation. Here we report the characterization of the *SNF1* homolog of *Candida glabrata*, a pathogenic yeast phylogenetically related to *S. cerevisiae*. The carbon utilization spectrum of *C. glabrata* is considerably narrower than that of other pathogenic yeasts, and the majority of the strains utilize solely glucose and trehalose from among 20 of the most commonly tested carbohydrates. Disruption of the *C. glabrata* *SNF1* homolog resulted in the loss of the ability to utilize trehalose, indicating that even in an organism with such a limited carbon utilization spectrum, the regulatory mechanism governing catabolic repression is preserved.

Candida glabrata (*Torulopsis glabrata*) is a haploid, asexual pathogenic yeast increasingly recovered from clinical specimens (18). The yeast can cause superficial mucosal infection as well as fungemia in immunocompromised or severely ill immunocompetent patients (18, 32). Frequently, *C. glabrata* coinfects with the more virulent yeast *Candida albicans* from which it can be readily discriminated by its inability to produce germ tubes in serum and its distinct pattern of carbon source utilization (18). Recently, with the increasing number of infections caused by drug-resistant isolates and the emergence of fungal nosocomial infections (14, 24), considerable attention has been directed to the study of *C. glabrata* and other less prominent opportunistic yeasts. In addition to its importance as a pathogen, *C. glabrata* may also be a model for the study of other closely related pathogenic yeasts, such as *C. albicans* and *Candida tropicalis*. The advantages presented by *C. glabrata* include haploidy and its ability to express *Saccharomyces cerevisiae* genes (20).

Although the knowledge regarding the molecular biology of *C. glabrata* is rather limited, notable progress has been made in recent years. Winge and coworkers developed a transformation system by cloning an ARS-like sequence and the *S. cerevisiae* *LEU2* gene into an episomal shuttle vector which was further improved to confer stability (19, 20). Another transformation system using a *C. glabrata* *ura3* mutant has also been described (34). Several structural sequences including the *MT-II* gene cluster (20), *ERG11* (8), *ERG3* (8), *HIS3*, *TRP1* (16), and the DNA binding domain of the *AMT1* transcription factor (19) have been characterized and provide the basis for further molecular studies.

The *SNF1* (sucrose nonfermenting) gene was first identified by a screening of *S. cerevisiae* for mutants unable to grow on sucrose as the sole carbon source (5). Additional *snf1* mutants were independently isolated based on their inability to utilize

ethanol (*ccr1*) or glycerol and maltose (*cat1*) (6). Genetic and molecular analysis of these mutants led to the recognition of the *SNF1* gene as an indispensable component of the catabolic repression pathway. The *SNF1* gene encodes a serine/threonine protein kinase (72 kDa) which is essential for the removal of catabolic repression (5). It was shown that the Snf1p acts antagonistically to protein phosphatase type I by interacting with the Mig1p repressing complex, thereby relieving the transcriptional inhibition of the targeted genes (28). The *snf1* mutation exerts pleiotropic effects on multiple cellular processes such as glycogen accumulation (12), hypersensitivity to heat shock and starvation (27), peroxisome proliferation (25), and sporulation (15). Homologous sequences obtained from humans (1), rats (4), plants (2, 10, 11, 21), and protozoan parasites (22) are suggested to play a crucial role in various stress response mechanisms and in the regulation of metabolic pathways such as the biosynthesis of cholesterol and other isoprenoid compounds (4). Recently it was suggested that in *S. cerevisiae* the Snf1p interacts directly or indirectly with another distinct signal transduction pathway, RAS-cAMP, in addition to its primary role in the catabolic repression mechanism (29).

Unlike *S. cerevisiae* or *C. albicans*, *C. glabrata* does not ferment or assimilate glucose-repressible sugars such as sucrose, maltose, or galactose. Trehalose, a glucose dimer, is the only sugar other than glucose that is assimilated by a majority of the *C. glabrata* strains (17). Is the *SNF1* gene, which controls the complex mechanism of glucose repression, being maintained in *C. glabrata*? Do the major components of this regulatory pathway have additional roles in this organism?

In this study, we describe the isolation and characterization of the *CgSNF1* gene and demonstrate that its disruption results in the loss of trehalose utilization in *C. glabrata*.

MATERIALS AND METHODS

Strains. The sources and genotypes of the strains used in this study are listed in Table 1. *C. glabrata* L5SD contains an insertion of the linearized pGDV1 plasmid (see below) into the genomic *SNF1* sequence, resulting in the disruption of the *SNF1* gene while complementing the leucine auxotrophy of L5.

Media and growth conditions. *C. glabrata* and *S. cerevisiae* strains were maintained on YEPD agar (1% yeast extract, 2% peptone, 2% glucose) slants.

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TABLE 1. Strains used in this study.

Strain	Genotype	Source
<i>S. cerevisiae</i>		
MCY2916	<i>MATα snf1Δ10 his3Δ200 ura3-52 lys2-801 leu2-3,112</i>	M. Carlson
MCY829	<i>MATα his3Δ200 ura3-52 lys2-801</i>	M. Carlson
<i>C. glabrata</i>		
NCSSL84	Wild-type reference strain	D. Winge
L5	<i>leu2</i>	D. Winge
L5SD	<i>snf1::LEU2</i>	This study

Transformants were selected on yeast nitrogen base (YNB) agar (6.7 g/liter, Difco) supplemented with 2% of either glucose (YNBG), sucrose (YNBS), or trehalose (YNBT) and any other compound required for the growth of each mutant. *Escherichia coli* cells (DH10B; GibcoBRL, Gaithersburg, Md.) were grown on Luria-Bertani broth or agar supplemented with 150 µg of ampicillin (Amersco, Solon, Ohio) per ml. Bacterial and yeast cells were grown at 37 and 30°C, respectively.

PCR. PCRs were performed in a thermo cycler (MJ Research, Watertown, Mass.) using *Taq* DNA polymerase and nucleotides obtained from Boehringer-Mannheim Corp. (Indianapolis, Ind.). Two degenerative oligonucleotide primers compatible with the conserved regions of *SNF1* genes were used to amplify a 300-bp fragment of *CgSNF1* (sense, 5'-CGAAGCTT[CT]TC[AG]TC[AG]TC[AG]AA[ACGT]GG-3'; antisense, 5'-CGCAAGCTT[CT]CA[GA]CA[GA]AT[CAT]AT[CAT]AG-3'). Twenty-nine amplification cycles (melting, 94°C, 1 min; annealing, 50°C, 1 min; polymerization, 72°C, 1 min) were followed by an additional cycle of an extended polymerization step (10 min). A 300-bp product which hybridized with the *ScSNF1* sequence probe under low-stringency conditions (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], 50°C) was cloned into the *HindIII* site of the Bluescript SK⁺ vector (Stratagene, La Jolla, Calif.), and its nucleotide sequence was determined.

DNA transformation. *S. cerevisiae* was transformed by the modified LiAc method (9, 13). *C. glabrata* was transformed by electroporation as described by Varma et al. for *Cryptococcus neoformans* (31). Electrocompetent *E. coli* cells were transformed by electroporation following the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Cloning of the *CgSNF1* genomic sequence. On the basis of the results of Southern blot analysis of genomic DNA with the 300-bp PCR-generated probe, *EcoRI*-*Sall* fragments (2.5 to 4.5 kb) were cloned into the Bluescript SK⁺ vector. The size-selected genomic library was introduced into *E. coli* DH10B cells by electroporation, and the clones were screened with the same PCR-generated probe.

Construction of a complementation vector. The 3.3-kb genomic fragment encoding the *CgSNF1* sequence and flanking regions was cloned into the *Sall*-*Bam*HI sites of the plasmid YEp24 (NEB, Beverly, Mass.). The resulting plasmid, pGLY1, was used to transform the *S. cerevisiae snf1* deletion mutant MCY2916 (Table 1). Transformants were selected for sucrose utilization and/or uracil prototrophy. Genomic DNA was obtained from the complemented *S. cerevisiae* mutants by a previously described method (7) and used to transform electrocompetent *E. coli* cells. Plasmids were rescued from these transformants and subjected to further characterization.

Construction of a *SNF1* disruption vector. A 1-kb *EcoRV* fragment of the

FIG. 1. Partial restriction map of the cloned 3.3-kb genomic fragment containing the *CgSNF1* gene (gray box) and its flanking regions (dashed boxes). In order to construct the *SNF1* disruption vector the 1.15-kb *EcoRV* fragment was cloned into a Bluescript SK⁺ vector. The fragment carrying the *S. cerevisiae LEU2* gene, obtained from pRS405, was cloned into the *HpaI* site within the *CgSNF1* coding region to yield the vector pGDV1. The vector was linearized and used to transform the *leu2* auxotroph L5.

CgSNF1 coding region (Fig. 1) was cloned into the pBluescript SK⁺ vector. A 2.78-kb *NaeI*-*AatII* DNA fragment containing the *S. cerevisiae LEU2* gene was obtained from the pRS405 vector (Stratagene) and was treated with T4 DNA polymerase to generate blunt ends. The *LEU2* gene was inserted into the *HpaI* site of the cloned *CgSNF1* fragment (Fig. 1). The resulting disruption vector, pGDV1, was digested with *Sall*-*PstI* to release the *CgSNF1*-*LEU2* chimeric sequence, which was used for the transformations of the *leu2* mutant of *C. glabrata*, L5 (Table 1).

Susceptibility to antifungal agents. The E-test kit (AB Biodisk, Solna, Sweden) was used to compare the in vitro susceptibilities of the wild type, *leu2* mutant, and the *snf1*-disrupted mutants to amphotericin B and fluconazole, according to the instructions of the manufacturer.

Utilization pattern of carbon sources. The diagnostic kit api20C (BioMerieux Vitek Inc., Hazelwood, Mo.) was used to determine the carbon utilization spectrum of the reference strain and mutants. Cells were grown at 30°C up to 96 h and were monitored at 24-h intervals.

Nucleotide sequence analysis and accession numbers. DNA sequencing was performed by the dideoxy-mediated chain-termination method with the Sequenase version 2.0 sequencing kit as instructed by the manufacturer (U.S. Biochemicals, Cleveland, Ohio). Sequence analysis, comparison, and database searches were performed with MacVector 3.0 (Oxford Molecular Group, Campbell, Calif.) and various programs of the Genetics Computer Group (Madison, Wis.). The nucleotide sequence data reported in this paper were submitted to the National Center for Genome Research with the accession numbers L78129 (*CaSNF1*) and L78130 (*CgSNF1*).

RESULTS

Isolation of the *C. glabrata SNF1* gene. Two degenerative oligonucleotide primers were derived from conserved regions of the *SNF1* gene sequence of *S. cerevisiae* and its plant and mammalian homologs (Fig. 2) (4, 11, 21). Amplification by PCR, with *C. glabrata* genomic DNA as template, yielded a single 300-bp DNA fragment. The PCR product hybridized strongly with the *ScSNF1* probe, and analysis of its nucleotide sequence revealed a high degree of similarity with the *ScSNF1* gene. Southern analysis of genomic DNA digested with various restriction enzymes indicated the existence of a single *SNF1* gene (data not shown). A size-selected genomic library of *C. glabrata* (2.5 to 4.5 kb) was constructed in the pBluescript SK⁺ vector, and a total of 750 clones were screened. Two independent clones, each carrying an identical 3.3-kb insert containing the *CgSNF1* gene and its flanking regions, were isolated and subjected to further studies.

Characterization of the *CgSNF1* gene. A partial restriction map of the *EcoRI*-*Sall* 3.3-kb DNA fragment encoding the *CgSNF1* gene is shown in Fig. 1. Nucleotide sequence analysis of the *CgSNF1* gene revealed an open reading frame of 1,839 bp encoding a putative 613-amino-acid polypeptide with no introns. Comparisons of the deduced amino acid sequences revealed an overall 91% similarity and 84% identity with the putative *ScSnf1p* sequence while only a 77% similarity and 65% identity were observed with the putative *CaSnf1p* sequence (Fig. 2). Similar to *ScSNF1* and *CaSNF1* (23), a stretch of histidine residues was found at the amino terminus of the putative protein (Fig. 2).

Complementation of the *SNF1* deletion mutation in *S. cerevisiae* with the *CgSNF1* gene. A *Sall*-*Bam*HI 3.3-kb fragment consisting of the *CgSNF1* coding regions and flanking sequences was cloned into the YEp24 vector to yield the plasmid pGLY1. This vector was used to transform an *S. cerevisiae snf1Δ* mutant (MCY2916, Table 1) which cannot assimilate sucrose in order to test its ability to complement this phenotype. Transformants were selected for their uracil prototrophy on YNBG agar and then tested for sucrose utilization on YNBS agar plates. The randomly chosen 196 transformants tested were all able to utilize sucrose as a sole carbon source, demonstrating that both the *ura3* and the *snf1* mutations were complemented by pGLY1 (Fig. 3). Crude total DNA extracts, obtained from 10 independent transformants, were used to transform electrocompetent *E. coli* cells. The plasmid DNA

FIG. 2. Alignment (PileUp) of the putative Snf1p amino acid sequences deduced from the *C. glabrata* (*CgSNF1*), *S. cerevisiae* (*ScSNF1*), and *C. albicans* (*CaSNF1*) nucleotide sequences. The locations of the primers used for PCR in vitro synthesis are marked by arrows. The histidine residue stretches at the amino-terminal end of the proteins are marked with asterisks.

obtained from the transformed *E. coli* clones exhibited restriction patterns identical to those of the pGLY1 vector (data not shown). Simultaneous screening of *S. cerevisiae* cells for uracil prototrophy and sucrose utilization on YNBS agar resulted in a greater than 100-fold decrease in the total number of transformants obtained.

Disruption of the *CgSNF1* gene. An integrative vector, termed pGDV1 (Fig. 1), was designed to disrupt the *CgSNF1* sequence with the *S. cerevisiae* *LEU2* gene via homologous recombination. A *C. glabrata* *leu2* mutant, L5 (Table 1), was transformed with the linearized pGDV1 vector by electroporation, and the resulting transformants were selected on YNBG agar for leucine prototrophy. Southern blot analysis of *SalI*-digested transformant DNAs with a radiolabeled *CgSNF1* probe demonstrated the predicted structural modification of the *SNF1* locus due to gene replacement (Fig. 4). The shift observed in the molecular sizes of the bands hybridizing with the *CgSNF1* probe (4.7 kb for the reference strain and 7.5 kb for the mutant) in about 50% of the tested clones indicated that an insertion had occurred at the targeted *SNF1* sequence thereby disrupting its coding region. The rest of the clones exhibited insertions into ectopic sites in the genome (data not shown). Six of the *snf1* mutants obtained were subjected to further characterization.

Analysis of the *snf1* disruption mutants. The *snf1* disruption mutants were tested to detect possible phenotypic modifications. The *S. cerevisiae* *SNF1* gene and its mammalian homolog are known to play a central role in the regulation of fatty acid and cholesterol metabolism. Alteration of membrane structure and permeability therefore may result from a *snf1* mutation (25, 33). Since most commonly used antifungal agents either target the enzymes in the sterol biosynthetic pathway or bind to membrane sterol, we were interested in the susceptibility of *snf1* mutants to an azole or polyene antifungal agent. None of the mutants tested with the E-test kit exhibited any change in its susceptibility to either fluconazole or amphotericin B com-

FIG. 3. (I) Complementation of *S. cerevisiae* *snf1* deletion mutant with the *SNF1* homolog obtained from *C. glabrata*. A, *S. cerevisiae* *snf1* deletion mutant complemented with pGLY1; B, *S. cerevisiae* MCY2916 (*snf1* deletion mutant), and C, *S. cerevisiae* MCY829 (reference strain). Cells were grown at 30°C on either YNBG agar (upper panel) or YNBS agar (lower panel). (II) Disruption of the *CgSNF1* gene of *C. glabrata*. D, *snf1* disruption mutant L5SD; E, the *leu2* mutant L5; and F, *C. glabrata* reference strain NCSSL84. Cells were grown at 30°C on either YNBG agar (upper panel) or YNBS agar (lower panel).

FIG. 4. Southern blot hybridization of *C. glabrata* disruption mutants. Lane M, molecular weight marker; lanes A and B, independent disruption mutants; lane C, wild-type strain NCSSL84. Genomic DNA was digested with *Sal*I and electrophoretically resolved on an 0.8% TAE-agarose gel. The DNA was transferred to a nylon membrane and hybridized with the 1-kb *Eco*RV *CgSNF1* probe at 65°C. The filter was washed under high-stringency conditions (0.1× SSC–0.1% SDS, 65°C) and exposed for 12 h at –70°C with an intensifying screen.

pared with the wild-type reference strain or its parental *leu2* mutant.

Growth rates of the *snf1* disruption mutant L5SD, the *leu2* mutant L5, and the wild-type culture NCSSL84 were determined in YEPD media at 30 and 37°C. The generation time of L5 and L5SD was about 40% longer (88 min at 30°C) than that of the wild-type reference strain (63 min at 30°C). Since the L5-complemented mutant, *LEU2*, did not regain the wild-type generation time (8), the slower growth rate of L5SD is probably not related to the *snf1* phenotype but rather to an as yet unidentified mutation(s) acquired during the chemical mutagenesis of the *leu2* mutant (20).

The carbon utilization profile of the *snf1* disruption mutants was determined with the api20C kit, which includes 20 different carbon sources. The assay indicated that both the wild type and the *leu2* mutant, L5, utilize glucose and trehalose while the *snf1* mutation hampered the utilization of trehalose. Further analysis on YNB agar confirmed that *snf1* mutants grow only on glucose media (YNBG, YEPD) at 30 and 37°C (Fig. 3).

DISCUSSION

This report describes the isolation and characterization of the *SNF1* gene homolog from the pathogenic yeast *C. glabrata*. An open reading frame encoding a putative 613-amino-acid protein with high similarity to those obtained from *C. albicans* (23) and *S. cerevisiae* was detected (5). Although *C. glabrata* is classified in the genus *Candida*, a higher degree of homology was observed with the *SNF1* gene of *S. cerevisiae* than with the *SNF1* homolog of *C. albicans*. This observation supports the relationship of the three yeasts depicted in the phylogenetic tree constructed on the basis of the small subunit rRNA se-

quence (3). These results also emphasize the heterogeneity among members of the genus *Candida*.

The similarity of *CgSNF1* with *ScSNF1* was demonstrated at the functional level since *CgSNF1* complemented a *snf1* mutant of *S. cerevisiae*. This result suggests that the *CgSNF1* product plays similar regulatory roles in *C. glabrata* and that *CgSnf1p* can associate with the components of *S. cerevisiae* regulatory mechanisms. The observation that the *snf1* disruption mutants lost their ability to utilize trehalose further supports this assumption.

Direct selection of *S. cerevisiae* mutants complemented for both the uracil auxotrophy and sucrose utilization on YNBS agar was significantly less efficient. A similar phenomenon was observed when the mutant was complemented with the *C. albicans SNF1* gene homolog (23a). This phenomenon may be the result of the double selection. It may reflect, however, the requirement for basal levels of *Snf1p* to derepress the transcription of genes necessary for sucrose utilization.

In *S. cerevisiae*, *Snf1p* was suggested to play a role in other cellular processes in addition to the catabolic repression pathway (29). Such a hypothesis may be supported by the presence of a histidine residue stretch at the amino-terminal end of the protein in which mutations had no effect on glucose catabolic repression (6). A similar structure was observed in *C. glabrata* and *C. albicans* (13-mer in *ScSNF1* and 12-mer in *CaSNF1* and *CgSNF1*), indicating that this unique nickel binding site is well conserved and suggesting a yet unknown functional significance.

The carbon utilization spectrum of *C. glabrata* is extremely narrow and most of the glucose-repressible carbon sources are not utilized. It is interesting that the complex mechanism for the catabolic repression was preserved in such an organism. Trehalose, a nonreducing disaccharide of glucose, was first thought to serve as a storage molecule. Recent studies, however, have shown that this disaccharide plays a role in protection mechanisms against various environmental stresses such as heat shock, frost, and high osmolarity in yeasts, filamentous fungi, and bacteria (26, 30). It may well be that the involvement of trehalose in the various cellular processes requires strict regulation of its catabolism and accounts for the maintenance of this regulatory pathway.

Since the carbon utilization pattern of *C. glabrata* is much simpler than that of *S. cerevisiae* or *C. albicans*, *C. glabrata snf1* mutants may prove to be a useful model for the study of carbon catabolic regulation.

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