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# Cloning and Characterization of *GNS1*: a *Saccharomyces cerevisiae* Gene Involved in Synthesis of 1,3-β-Glucan In Vitro

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**The *GNS1* gene product is required for the synthesis of 1,3-β-glucan in vitro, since mutations in this gene result in exhibit an 80 to 90% reduction in 1,3-β-glucan synthase specific activity. *gns1* mutant strains display a pleiotropic phenotype including resistance to a pneumocandin $B_0$ analog (L-733,560), slow growth, and mating and sporulation defects. The *gns1-1* mutation was genetically mapped to within 1.35 centimorgans from the *MAT* locus on chromosome III. The wild-type *GNS1* gene was isolated by complementing the pneumocandin resistance phenotype of the *gns1-1* mutation and by hybridization with a chromosome III-derived sequence being used as a probe. The nucleotide sequence of *GNS1* was determined and compared with the homologous region of the chromosome. The genetic and nucleotide sequence analyses revealed that *GNS1* and the open reading frame, *YCR34* [S. Oliver, Q. van der Aart, M. Agostoni-Carbone, and the Chromosome III Sequencing Group, Nature (London) 357:38–46, 1992], represent identical loci in the genome. Cells deleted for *GNS1* are viable but exhibit slow growth as well as the pleiotropic phenotype of the *gns1* mutants. The putative protein product is predicted to be an integral membrane protein with five transmembrane helices displaying an exoplasmic orientation for the N terminus and a cytoplasmic orientation for the C terminus. This protein may be a subunit of 1,3-β-glucan synthase.**

The main function of the cell wall is to provide a skeletal support and a mechanical barrier for the protection of the cell from its surroundings. The cell wall also plays roles in many other functions, such as the selective uptake of macromolecules, osmoregulation, and cell growth and division (35). Furthermore, several enzymatic activities related to hydrolysis of extracellular nutrients (1) and turnover of the cell wall macromolecules during morphogenesis (36) are associated with the extracellular matrix.

Polysaccharides account for as much as 80 to 90% of the *Saccharomyces cerevisiae* cell wall. The major cell wall polysaccharide polymers are glucan, a polymer of glucose, and mannan, which is present as a mannoprotein. In addition, small amounts of chitin, a polymer of *N*-acetylglucosamine, are also present at the cell surface (9). The glucans support and maintain the rigidity of the cell wall, while the mannoproteins determine its permeability (12, 51). Three types of glucan account for 30 to 60% of the *S. cerevisiae* cell wall (19, 47). A major form of glucan (60% of the total) is insoluble in alkali or acetic acid and exists as a branched 1,3-β polymer with fibrillar structures containing 3% 1,6-β lateral chains. A second major form of glucan (32% of the total) is soluble in dilute alkali, has an amorphous structure, and contains mainly 1,3-β linkages with some 1,6-β linkages. A minor form of glucan (8% of the total) is acid soluble and highly branched and contains mainly 1,6-β linkages with some glucose residues connected through 1,3-β linkages.

Several antifungal antibiotics target the biosynthesis of specific cell wall polymers. Tunicamycin inhibits the enzyme *N*-acetylglucosylaminyl transferase, which catalyzes *N*-acetylglucosamine transfer to the lipid carrier dolichol phosphate (28, 34). Nikkomycins and polyoxins are competitive inhibitors of chitin synthases (7, 10, 11, 18). Papulacandin B (2, 30, 43, 48), aculeacin A (29, 49), echinocandin B (46), and pneumocandin $B_0$ (6) inhibit synthesis of 1,3-β-glucan, most likely by inhibiting activity of 1,3-β-glucan synthase. The killer toxin of *Hansenula mrakii* kills sensitive strains of *S. cerevisiae* by inhibiting 1,3-β-glucan synthase activity (25, 50). Imidazole and triazole may indirectly inhibit a cell wall function (3, 32) by their interference with sterol biosynthesis (5).

The fact that a 1,3-β-glucan synthase enzyme is absent in mammalian cells makes it an attractive target for antifungal antibiotics. This membrane-associated enzyme utilizes UDP-glucose as a substrate and is stimulated by a detergent-soluble GTP-binding protein (23). Recent analysis of mutations conferring resistance to some of the 1,3-β glucan synthase inhibitors has led to the cloning of several genes affecting 1,3-β-glucan synthesis (13, 14, 16, 25, 37). In addition, the *KRE6* gene affects synthesis of 1,6-β-glucan (38). The water-soluble pneumocandin $B_0$ derivative L-733,560 was used to isolate several pneumocandin-resistant mutants in *S. cerevisiae* (14, 16). Three of these mutants contain single mutations in the *FKS1*/*ETG1* gene and harbor 1,3-β-glucan synthases resistant to L-733,560 (13, 14, 16). The *FKS1*/*ETG1* gene is a candidate for a 1,3-β-glucan synthase subunit.

In this paper, we describe a complementation group composed of three mutants that map to the *GNS1* locus. In addition to having the whole-cell echinocandin resistance phenotype, this complementation group is characterized by a dramatic decrease in 1,3-β-glucan synthase specific activity in vitro. We also report on the cloning and disruption of the wild-type gene, *GNS1*, that complements the three *gns1* mutations.

## MATERIALS AND METHODS

**Strains, plasmids, and media.** YCp50 and a yeast genomic DNA library constructed by M. Rose were provided by F. Foor. The yeast strains used in this study are listed in Table 1. The mutant strains are derived from strain X2180-1A. The YPAD solid medium contained 1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose, and 0.003% adenine sulfate. Synthetic Dextrose (SD) media contained 0.67% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose,

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TABLE 1. Yeast strains

| Strain | Relevant properties[a] |
|---|---|
| X2180-1A[b] | *MAT***a** *GNS1* (wt, Ech$^s$) |
| MS1 | *MAT***a** *gns1-1* (Ech$^r$) |
| MS41 | *MAT***a** *gns1-2* (Ech$^r$) |
| MS7-43 | *MAT***a** *gns1-3* (Ech$^r$) |
| GG100-14D[b] | *MAT*α *GNS1 ura3-52 his3 trp1* (wt, Ech$^s$) |
| D2-1B | *MAT*α *ura3-52 gns1-2* |
| D2-1D | *MAT***a** *ura3-52 gns1-2* |
| D2-2D | *MAT***a** *his3 gns1-2* |
| D2-5A | *MAT*α *his3 ura3-52 GNS1* |
| D2-8B | *MAT***a** *ura3-52 gns1-1* |
| D12-7D | *MAT***a** *his3 gns1-1* |
| D12-11D | *MAT***a** *ura3-52 gns1-3* |
| D28-3B | *MAT***a** *his3 gns1-3* |
| D28-9D | *MAT***a** *his3 gns1-3* |
| D28-18C | *MAT***a** *ura3-52 his3 gns1-3* |
| MS100 | *MAT*α *his3 trp1 gns1*::*URA3* |
| MS101 | *MAT*α *his3 gns1*::*URA3* |
| DJ211-5-3 | *MAT***a** *bar1-1 ura3-52 his4-580 trp1* |
| D2 | *MAT***a**/*MAT*α (MS41 × GG100-14D) |
| D12 | *MAT***a**/*MAT*α (MS1 × GG100-14D) |
| D28 | *MAT***a**/*MAT*α (MS7-43 × GG100-14D) |
| D132 | *MAT***a**/*MAT*α (D28-18C × D28-3B) |
| D136 | *MAT***a**/*MAT*α (D28-18C × D2-1D) |
| D137 | *MAT***a**/*MAT*α (D28-18C × D12-11D) |
| D140 | *MAT***a**/*MAT*α (D28-9D × D2-1B) |
| D141 | *MAT***a**/*MAT*α (D12-7D × D2-1B) |
| D142 | *MAT***a**/*MAT*α (D2-2D × D2-1B) |

[a] wt, wild type; Ech, echinocandin.

[b] Strains GG100-14D and X2180-1A were obtained from K. Bostian and C. Ballou, respectively. The mutants were generated in X2180-1A and outcrossed to GG100-14D.

and 2% Bacto agar (Difco). Synthetic Complete (SC) medium is SD medium supplemented with 20 mg (each) of adenine, histidine, tryptophan, and uracil; 60 mg of leucine; and 30 mg of lysine per liter of medium. Sporulation medium is 2% Bacto agar (Difco) and 0.3% potassium acetate.

**DNA manipulation, transformation, and genetic analysis techniques.** The standard techniques of DNA manipulation and growing bacterial cultures were performed as described elsewhere (41). *Escherichia coli* DH5α (20) was used in bacterial transformation. Yeast transformations with the DNA libraries were performed by electroporation (4). All other yeast transformations with different plasmid subclones were performed by the alkali cation method (22). Plasmid DNA from *E. coli* was prepared by the alkaline lysis method (41). Plasmids were isolated from *S. cerevisiae* for transformation into *E. coli* as previously described (21). A yeast genomic library constructed in the centromeric shuttle vector YCp50 (39) was used to clone the *GNS1* gene by complementation of the *gns1-1* mutation. The techniques of yeast genetic crosses, tetrad dissection, and other procedures for working with *S. cerevisiae* are described elsewhere (44). Genetic crosses of the drug-resistant mutants and the wild-type sensitive strain GG100-14D were performed nonselectively on SC-uracil plates. Crosses of drug-resistant meiotic segregants containing either *ura3-52* or *his3* markers were carried out under selective conditions on SC plates lacking both uracil and histidine. Diploid isolates were tested for their ability to form asci on sporulation plates as previously described (44).

**DNA sequencing.** The nucleotide sequence of *GNS1* was determined by the dideoxy chain termination method (42) with synthetic oligonucleotide primers complementary to specific regions of *GNS1* and with a Sequenase reagent kit (U.S. Biochemical Corp.).

**Ethyl methanesulfonate mutagenesis.** YPAD broth (5 ml) was inoculated with an overnight culture of strain X2180-1A to give an initial cell density of $10^6$ cells per ml, and this was followed by incubation at 30°C overnight. From that saturated culture, a 2.5-ml aliquot was washed twice in 50 mM $KPO_4$ buffer, pH 7.0, and resuspended in 10 ml of this buffer. To a 5-ml aliquot, 150 μl of ethyl methanesulfonate was added, and the mixture was vortexed and incubated at 30°C for 1 h. The remainder of the culture was kept on ice untreated. To the 5 ml of treated cells, an equal volume of a freshly made 10% (wt/vol) filter-sterilized solution of sodium thiosulfate was added and mixed. Cells were collected, washed twice with sterile water, resuspended in 5 ml of YPAD broth, and incubated at 24°C for 4 to 6 h. Appropriate cell dilutions were plated on minimal medium (SD). The untreated culture was diluted and plated in the same way. After 3 days of growth at 24°C, the surviving colonies were replicated onto YPAD medium with and without 7.5 μM L-733,560 and incubated at 30 and 37°C.

**PCR amplification.** PCR was performed according to a previously published procedure (31). Approximately 5 ng of genomic DNA from strain X2180-1A was used as the template. Synthetic oligonucleotide primers were designed to amplify a 1.5-kb fragment of a region 6 kb to the left of *MAT*. The sequences of the two primers were 5′ TGACAGTAGTTTCACAAGTACTTAATATTGGAAATG 3′ and 5′ TCAGATAATTTTATCGGTACCTTTTATATGTTAAAT 3′. Amplified DNA fragments were gel purified, radiolabeled by the random priming method (17), and used as probes to screen bacterial colonies containing a yeast genomic library in the centromeric vector YCp50 (39), all according to published protocols (41).

**Gene disruption.** Chromosomal disruption of the *GNS1* gene was performed by one-step gene disruption (40). The plasmid pJAC4 was constructed by cloning a 1.6-kb *Dpn*I fragment from the library clone pJAC2-1, which contains a 17-kb DNA insert, into the *Hin*cII site of pUC18. A 1.5-kb DNA fragment carrying the *S. cerevisiae URA3* gene was isolated from the YCp50 plasmid as an *Nru*I-*Sma*I fragment and inserted between the *Nru*I-*Eco*RV sites of pJAC4, resulting in plasmid pJAC9 in which 1.2 kb of the *GNS1* sequence has been replaced with *URA3*. The *gns1*::*URA3* disruption fragment was purified from pJAC9 as a 2.7-kb *Xba*I-*Hin*dIII fragment that was used to transform two *ura3-52* yeast strains (GG100-14D and D2-5A) containing a wild-type *GNS1*. Two *gns1* disruptants designated MS100/GG100Δ*gns1* and MS101/D2-5AΔ*gns1* were subjected to further analysis. Disruption of *GNS1* in these strains was confirmed by Southern hybridization. Total genomic DNA was isolated from stationary cultures of the disruptants and the parent strains. Approximately 5 μg of each DNA was digested with *Dra*I, *Kpn*I, or *Hpa*I-*Cla*I, and the digestion products were resolved on 1% agarose gels, transferred to Zeta probe GT nylon membranes, and hybridized according to the manufacturer's protocols (Bio-Rad Laboratories). $^{32}$P-radiolabeled probes of the DNA fragment internal to *GNS1* were prepared by the random primer method (17).

**Drug resistance assays.** The sources of the antibiotics, the liquid broth microdilution assay, and the disk diffusion assay were described elsewhere (16). The structures of the pneumocandin compounds L-733,560 and L-731,373 (6) and the dihydropapulacandin compound L-687,781 (48) were previously described.

**Glucan synthase and chitin synthase assays.** Cell extracts were prepared from mutant and wild-type cells grown to logarithmic phase as previously described (23). Assays of 1,3-β-glucan synthase (8, 43) and chitin synthase (24) were performed as previously described (16). A published procedure (23) for the preparation of soluble and insoluble membrane fractions was used. To particulate membrane extracts, tergitol Nonidet P-40 (NP-40) was added to a final concentration of 2% (vol/vol). The suspension was mixed by vigorous vortexing and fractionated into soluble and insoluble fractions as previously described (23).

**β-Glucan analysis.** Total alkali-insoluble β-glucan was isolated from stationary-phase cultures grown in YPAD medium as previously described (38). Carbohydrate was measured as hexose according to a published protocol (15).

## RESULTS

**Isolation of mutants resistant to L-733,560.** To identify genes required for 1,3-β-glucan synthesis in *S. cerevisiae*, we utilized the semisynthetic pneumocandin $B_0$ derivative L-733,560 to isolate drug-resistant mutants. Some 40 spontaneous L-733,560-resistant mutants were isolated from strain X2180-1A as previously described (16). In addition, 10 mutagen-induced L-733,560-resistant mutants were also isolated. Strain X2180-1A was mutagenized with ethyl methanesulfonate, grown for 16 h to eliminate dying cells, and plated for single colonies (see Materials and Methods). Approximately 2,000 colonies were tested for L-733,560 resistance by replica plating onto YNBD medium supplemented with 7.5 μM L-733,560. One of the ethyl methanesulfonate-induced mutants (MS43) and two of the spontaneously isolated mutants (MS1 and MS41) constitute a single complementation group as described below. The mutations in these three strains were tested for dominance or recessiveness by mating the *MAT***a** L-733,560-resistant mutants to the *MAT*α L-733,560-sensitive strain GG100-14D. All three *MAT***a**/*MAT*α diploid strains, D2, D12, and D28, exhibited sensitivity to L-733,560, indicating that the drug resistance mutations were recessive. Tetrad analysis revealed that L-733,560 resistance segregated as a single trait in each of the three mutant strains (Fig. 1). Complementation tests were performed with drug-resistant segregants from D2, D12, and D28. The diploids formed between these resistant isolates, D132, D136, D137, D140, D141, and D142 (Table 2), exhibited resistance to L-733,560. Thus, the mutations in the three resistant mutant isolates failed to complement each other. On

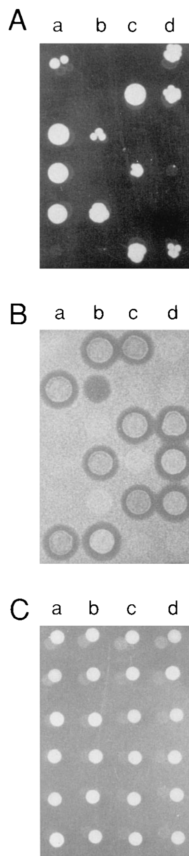


FIG. 1. Segregation pattern (2:2) of the L-733,560 resistance phenotype of the *gns1-1* mutant. Cells ($10^5$) of each of four meiotic products (lanes a to d) from the diploid strain D2 (*GNS1/gns1 URA3/ura3-52 TRP1/trp1 HIS3/his3*) were dropped on SC plates containing 7.5 μM L-733,560 (A) or $10^7$ cells of the *bar1* strain DJ211-5-3 (B). Panel C shows control SC plates without additions. The plates were incubated for 2 to 3 days at 30°C.

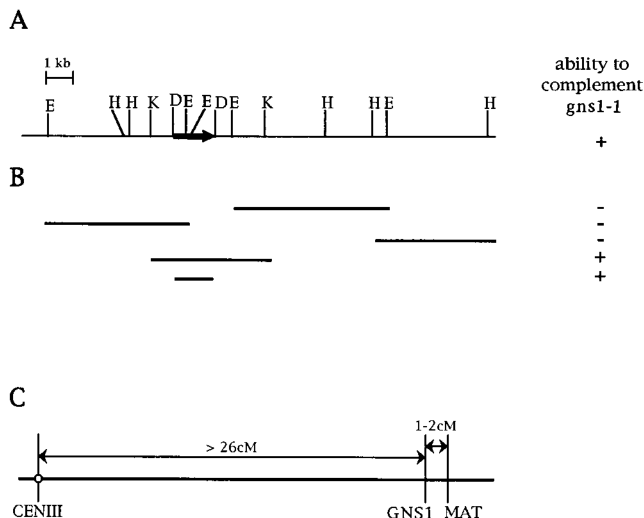


FIG. 2. Localization of the *gns1-1* minimum complementing fragment. A partial restriction map of the 17-kb clone containing *gns1-1* complementing activity is depicted (A). The direction of the transcription of *GNS1* is indicated by the arrow. The yeast genomic DNA fragments represented by the lines below the restriction map (B) were inserted into the centromeric plasmid YCp50, and the recombinant plasmids were transformed into strain D2-8B. Yeast transformants that received plasmids carrying one of these fragments were tested for complementation (indicated by the plus sign) of the echinocandin resistance phenotype of the *gns1-1* mutation. Genetic mapping of *gns1-1* to the left arm of chromosome III (C) was performed by standard procedures (44). The plasmids as represented from top to bottom of panels A and B are pJAC2-2, pEF1, pEF2, pHF1, pJAC1, and pJAC4. D, *Dpn*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

the basis of this result and on their linkage to the *MAT* locus (discussed below), the three mutants are placed in one complementation group. We refer to these alleles as *gns1-1* (strain MS1), *gns1-2* (strain MS41), and *gns1-3* (strain MS43).

**Genetic mapping of *gns1-1*.** Genetic analysis of the meiotic segregants of 37 tetrads resulting from the outcrossing of MS1 to GG100-14D led to the mapping of the *gns1-1* mutation to within 1 to 2 centimorgans (cM) of the *MAT* locus on chromosome III (Table 2; Fig. 2C). A similar analysis revealed that both *gns1-2* and *gns1-3* are linked to *MAT* (Table 2). Parental ditype tetrads (2 *MAT***a** echinocandin resistant:2 *MAT*α echinocandin sensitive) were the only class of tetrads obtained from crosses between GG100-14D and MS41 (12 tetrads) and between GG100-14D and MS43 (19 tetrads). The distance from *GNS1* to *CEN3*, measured by the frequency of second-division segregation (44), is approximately 26 cM (data not shown). The drug-resistant meiotic segregants capable of growth in the presence of L-733,560 are *MAT***a**, while the sensitive progeny unable to grow in the presence of the drug are *MAT*α (Fig. 1).

TABLE 2. Genetic mapping of the *gns1* mutations

| Interval | No. by ascus type: Parental ditype | Nonparental ditype | Tetratype | Map distance (cM) |
|---|---|---|---|---|
| *gns1-1–MAT* | 36 | 0 | 1 | >1.35 |
| *gns1-2–MAT* | 12 | 0 | 0 | >3.8 |
| *gns1-3–MAT* | 19 | 0 | 0 | >2.5 |

**Phenotypes of the *gns1* mutants.** The mutant cells exhibit a bilateral mating defect, i.e., mating between two mutant cells for production of homozygous diploids containing two copies of the mutant gene is very inefficient. In this case, the resultant homozygous diploids fail to form spores upon subculturing on sporulation media. These diploids are osmotically unstable and often burst when resuspended in water. In contrast, matings of wild-type cells with cells containing any of the three *gns1* mutations produced heterozygous diploids which were not affected. Morphologically, MS1 shows some aggregated cells and occasional flocculated growth. The growth of the MS1 mutant is significantly slower than that of its wild-type parental strain. This slow growth is characterized by a long lag period before the cells enter the division cycle (data not shown). The generation time of MS1 cells grown in YPAD medium is 4 h compared with the 2-h generation time of its parent strain grown under the same conditions.

The MS1 cells exhibited no overall multiple drug resistance when tested against antifungal inhibitors with different modes of action. This strain was not resistant to papulacandin L-687,781, another glucan synthase inhibitor. However, it exhibited resistance to ketoconazole and hypersensitivity to monensin and valinomycin (Table 3). The MS41 and MS43 mutant strains exhibited slow growth and drug resistance and sensitivity profiles similar to that of MS1 cells.

**Isolation of the *GNS1* gene by functional complementation and by hybridization.** The *GNS1* gene was cloned by complementation of the L-733,560 resistance phenotype. Yeast strain D2-8B (*MAT***a** *ura3-52 gns1-1*) was transformed with a yeast

TABLE 3. Effect of antifungal antibiotics on MS1 and X2180-1A

| Target | Compound | Amt of compound (μg/disc) | Zone of inhibition (mm) | |
|---|---|---|---|---|
| | | | X2180-1A | MS1 |
| β-Glucan synthesis | L-733,560 | 2 | 20 | 12 |
| | L-731,373 | 50 | 20 | 15 |
| | L-687,781 | 20 | 10 | 19 |
| | Aculeacin A | 50 | 15 | 9 |
| Chitin synthesis | Nikkomycin Z | 15 | 0 | 0 |
| | Polyoxin D | 20 | 0 | 0 |
| Chitin assembly | Calcofluor white | 50 | 0 | 0 |
| Glycosylation | Tunicamycin | 20 | 21 | 26 |
| Membrane | Amphotericin B | 20 | 18 | 22 |
| | Filipin | 20 | 24 | 24 |
| | Nystatin | 20 | 30 | 29 |
| | Monensin | 40 | 0 | 15 |
| | Valinomycin | 20 | 0 | 10 |
| | Cerulenin | 10 | 43 | 40 |
| Sterol synthesis | Fluconazole | 5 | 18 | 18 |
| | Ketoconazole | 20 | 24 | 18 |
| Protein synthesis | Cycloheximide | 2.5 | 45 | 43 |
| Secretion | Chloroquine | 100 | 0 | 0 |

genomic DNA library in the centromeric vector YCp50 (39), and this was followed by selection of transformants on SC-uracil medium. Some 2,400 transformants were picked onto master plates and replicated onto plates containing SC-uracil medium supplemented or not with 7.5 μM L-733,560. Following incubation at 30°C for 2 to 3 days, four sensitive colonies were isolated. One of these four colonies, designated D2-8B (pJAC2-1), was shown to contain a complementing plasmid, pJAC2-1, capable of reversing the L-733,560 resistance conferred by the *gns1-1* mutation (strain D2-8B). The size of the DNA insert in pJAC2-1 is about 17 kb. As described above, the *GNS1* gene is closely linked to the *MAT* locus on chromosome III. Since the nucleotide sequence of this chromosome was previously determined (33), we attempted to clone *GNS1* simultaneously by DNA hybridization. In a separate experiment, a 1.5-kb DNA fragment representing a sequence located at about 6 kb to the left of *MAT* was amplified by PCR, radiolabeled, and used as a probe to screen a YCp50-based yeast genomic library by colony hybridization (41). Screening of about 4,800 bacterial colonies resulted in a hybridizing bacterial clone containing a plasmid designated pJAC2-2. Both pJAC2-1 and pJAC2-2 contained DNA fragments of identical restriction maps. A partial restriction map for pJAC2-2 is shown in Fig. 2A. pJAC2-2 was introduced into strain D2-8B containing the *gns1-1* mutation. Three yeast transformants were tested and found to acquire a wild-type level of sensitivity to L-733,560. A transformant designated D2-8B(pJAC2-2) was subjected to curing of the transforming plasmid, pJAC2-2, by three successive rounds of overnight growth in YPAD followed by plating on YPAD for single colonies. Cured clones that have lost the plasmid, as judged by their inability to grow on uracil-deficient medium, exhibited resistance to L-733,560. These results showed that the *gns1-1* complementing activity was plasmid mediated. The L-733,560 sensitivity conferred by the transforming plasmids (pJAC2-1 and pJAC2-2) coincided with

TABLE 4. Specific activity of 1,3-β-glucan synthase[a]

| Yeast strain | Allele | Plasmid | Sp act of 1,3-β-glucan synthase (nmol/mg/h) |
|---|---|---|---|
| X2180-1A | *GNS1* | | 405.6 |
| GG100-14D | *GNS1* | | 378.3 |
| MS1 | *gns1* | | 41.2 |
| MS41 | *gns1* | | 200.1 |
| MS43 | *gns1* | | 58.6 |
| MS100 | *gns1*::*URA3* | | 48.1 |
| MS101 | *gns1*::*URA3* | | 20.3 |
| MS1 | *gns1* | YCp50 | 32.3 |
| MS1 | *gns1* | pJAC2-1 | 397.3 |

[a] 1,3-β-Glucan synthase assays were performed with 25 μg of membrane proteins as described in Materials and Methods.

a reversal of the low glucan synthase activity associated with the mutant microsomal membranes (Table 4).

**Determination of the *gns1-1* minimum complementing fragment.** To define the *gns1-1* complementing region of pJAC2-2, several fragments obtained by digestion with restriction enzymes that cut within the cloned insert DNA were generated (Fig. 2B). Plasmids containing restriction fragments subcloned in YCp50 were propagated in *E. coli* DH5α, characterized with regard to restriction patterns, and then reintroduced into strain D2-8B. A 4-kb *Kpn*I fragment internal to the original 17-kb library clone was subcloned in the centromere-based vector YCpLac33 to yield pJAC2-1. This plasmid reversed the L-733,560 resistance and the low 1,3-β-glucan synthase activity of the MS1 mutant cells (Table 4). By a similar analysis, a 1.6-kb *Dpn*I fragment derived from pJAC2-2 was cloned into the *Hin*cII site of the bacterial vector pUC18 to produce the plasmid pJAC4. A *Bam*HI-*Sph*I 1.6-kb fragment containing *GNS1* was purified from pJAC4 and subcloned between the *Bam*HI and *Sph*I restriction sites of both YCp50 and YEp24 plasmid vectors to yield pJAC3 and pJAC5, respectively. Both recombinant plasmids were able to complement the drug resistance and the low glucan synthase specific activity of the mutant cells (data not shown).

**Sequence analysis.** The dideoxy chain termination method was used to determine the nucleotide sequence of the gene. The sequence was compared with that of the homologous region of chromosome III. This analysis confirmed the identity of *GNS1* with the open reading frame *YCR34* (33) on the right arm of the chromosome, within 1 to 2 cM from *MAT* and 26 cM from *CEN3* (Fig. 2C). The putative protein product of *GNS1*/*YCR34* was compared with those in protein banks, and no significant homology with any of the known proteins was found. The Gns1p product is a 40-kDa protein that is basic (pI = 10.3) and hydrophobic but lacks a cleavable N-terminal signal sequence. The protein contains an N-linked glycosylation site (Asn-X-Ser, amino acid positions 32 to 35) and at least two leucine zipper motifs (amino acid positions 12 to 33 and 68 to 89). A computer-assisted hydropathy analysis was used to predict the membrane topology of Gns1p (45). By this analysis, Gns1p is predicted to be a membrane protein with five transmembrane helices (Fig. 3). The likely topology of the protein is such that the hydrophilic N terminus, consisting of 61 residues, is exoplasmically oriented, while the C terminus, with 51 residues, is cytoplasmically oriented, as predicted by the "positive inside" rule (45).

***GNS1* gene disruption and characterization of resistance to L-733,560.** To obtain a stable *gns1* mutant and to test whether *GNS1* is essential, a chromosomal deletion of the *GNS1* gene

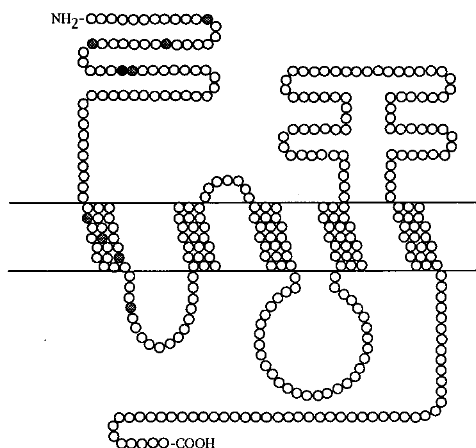


FIG. 3. Topology prediction for Gns1p. A full window of 21 residues and a core window of 11 residues were used as described by Sipos and von Heijne (45). Each residue was then drawn as a circle. Positions of glycosylation (●) and dimerization (○) are indicated.

was generated by one-step gene disruption (40). A 1-kb region of plasmid pJAC4 containing most of the *GNS1* N-terminal coding sequence in addition to part of the promoter region was deleted and replaced with the *URA3* gene. The disrupted copy of *GNS1* was excised as a 2.7-kbp *Hin*dIII-*Xba*I fragment, which was then used to transform the two *GNS1*-containing strains GG100-14D and D2-5A. Two disrupted mutants, GGΔ*gns1* (strain MS100) and D2-5AΔ*gns1* (strain MS101), acquired resistance to L-733,560 and were subjected to further analysis. The alteration of the *GNS1* locus in the disrupted strains was confirmed by Southern hybridization (data not shown). The *gns1* null mutant (strain MS100) exhibited resistance to L-733,560 (Fig. 4) in addition to the pleiotropic phenotype of the *gns1-1* cells described above. The results depicted in Fig. 4 show that the *gns1* null mutant is 10-fold more resistant to L-733,560 than its wild-type parental strain (GG100-14D). In liquid cultures, 3 μM and 0.3 μM L-733,560 inhibited growth of the *gns1* null mutant and its wild-type parent strain, respectively (Fig. 4). The *gns1* mutants were capable of growth on SC solid medium supplemented with 7.5 μM L-733,560.

**1,3-β-Glucan synthase and chitin synthase activities.** Since the *gns1* mutants were isolated on the basis of their resistance to an inhibitor of 1,3-β-glucan synthase, L-733,560, we decided to evaluate 1,3-β-glucan synthase activities of the mutant membranes. While the MS41 mutant membranes exhibited an about 50% decrease in specific activity, the MS1 and MS43 membranes showed an up to 90% reduction in activity (Table 4). The *gns1* null mutant membranes stimulated only 5 to 10% total activity. Membrane extracts of the wild-type and mutant strains were tested for their associated 1,3-β-glucan synthase and chitin synthase activities in the presence of serially diluted L-733,560 and nikkomycin Z, respectively. While the 50% inhibitory concentration ($IC_{50}$) of the mutant 1,3-β-glucan synthase was similar to that of the wild type, the specific activity was dramatically reduced by comparison with that of the wild-type parent strain (Table 4). In contrast to the reduced level of 1,3-β-glucan synthesis, the trypsin-activated chitin-synthesizing activity of the mutant extracts was equivalent to wild-type levels (Fig. 5B). The $IC_{50}$ of nikkomycin Z for the mutant chitin-synthesizing activity was equivalent to that for the wild type (Table 5). Table 5 shows a comparison of the $IC_{50}$s and MICs of L-733,560. Growth inhibition of the wild-type parental strain by 0.3 μM L-733,560 contrasts with the relatively high concentration (7.58 μM) required for 50% inhibition of glucan synthase activity in vitro (Table 5). In contrast to the reduced specific activity of 1,3-β-glucan synthase in the *gns1* mutants, they had normal or slightly elevated levels of β-glucan content (Table 6).

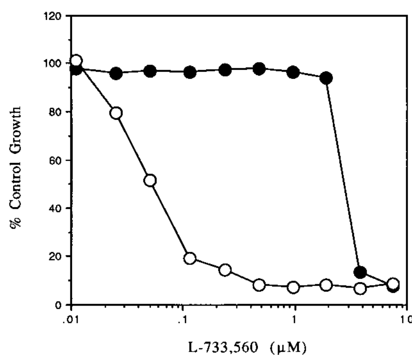


FIG. 4. Resistance of *gns1*Δ cells to L-733,560. Yeast cells (strain MS100) were inoculated from an overnight culture into fresh SC medium and grown to $1 \times 10^7$ to $2 \times 10^7$ cells per ml. The cells were harvested by centrifugation and resuspended in the same growth medium to a density of $6 \times 10^5$ cells per ml. The liquid broth microdilution assay was performed in order to quantitate the level of pneumocandin resistance as described in Materials and Methods. Wild-type GG100-14D, open circles; MS100 *gns1*Δ mutant, solid circles.

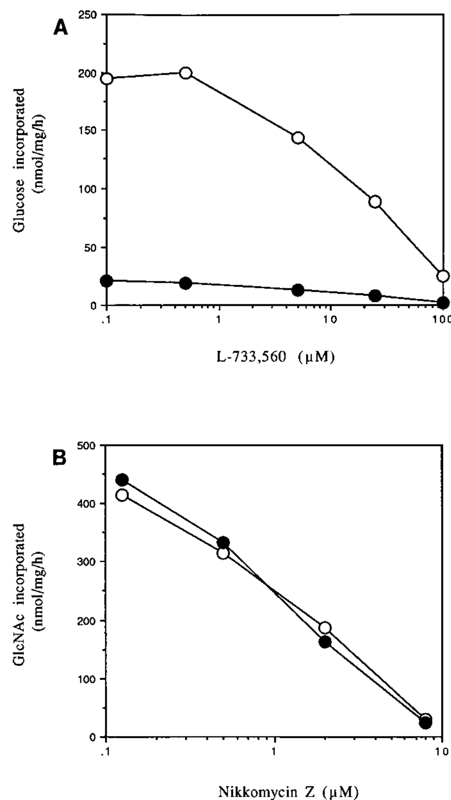


FIG. 5. Drug titration and specific activity of the 1,3-β-glucan synthase and the chitin synthase. Microsomal membranes prepared from wild-type yeast cells (strain GG100-14D, open circles) and *gns1* null mutant cells (strain MS100, solid circles) were used to catalyze 1,3-β-glucan synthase (A) and chitin synthase (B) reactions utilizing UDP [$^3$H]glucose and [$^{14}$C]*N*-acetylglucosamine as the respective substrates. The reactions of 1,3-β-glucan synthase and chitin synthase were performed in the presence of increasing concentrations of L-733,560 and nikkomycin Z, respectively.

TABLE 5. Whole-cell resistance to L-733,560 and drug sensitivities of 1,3-β-glucan synthase and chitin synthase

| Strain | MIC of L-733,560 (μM) | $IC_{50}$ of L-733,560 (μM) | $IC_{50}$ of nikkomycin Z (μM) |
|---|---|---|---|
| GG100-14D | 0.3 | 7.58 | 0.94 |
| MS100 | 3.0 | 5.48 | 1.1 |

The yeast 1,3-β-glucan synthase activity can be fractionated into a soluble and insoluble fraction by treating yeast membranes with salt and detergent. While neither fraction is active by itself, 1,3-β-glucan synthase activity can be reconstituted by mixing the two fractions in the presence of GTP (23). Experiments to test whether the *gns1* mutant is defective in the soluble or insoluble fraction of the enzyme were performed. The otherwise low 1,3-β-glucan synthase activity of the mutant membranes is significantly stimulated by the wild-type insoluble membrane fraction but not by the wild-type solubilized fraction (Table 7). These data indicate that the mutant enzyme is defective in its insoluble component. To demonstrate that the wild-type soluble and insoluble fractions were functional, the two fractions were mixed together and used in the assay. 1,3-β-Glucan synthase activity was reconstituted by mixing these fractions in the presence of GTPγS (Table 7) as previously described (23).

## DISCUSSION

The gene *GNS1* is required for normal synthesis of 1,3-β-glucan in vitro. Mutations in this gene lead to a dramatic reduction (up to 90%) in 1,3-β-glucan synthase specific activity. This reduction in activity is reversed when membranes from the mutant cells carrying *GNS1* on plasmids are used in the assay. The slow growth, mating defects, and echinocandin resistance phenotypes of the mutant cells are all complemented by *GNS1*, indicating that this gene mediates the pleiotropic phenotype mentioned above.

The genetic analysis and sequence of the cloned *GNS1* gene showed that it is encoded by the *YCR34* open reading frame on chromosome III (33). Hydropathy analysis of the deduced amino acid sequence revealed an integral membrane protein with five or six transmembrane domains. This is consistent with the finding that the 1,3-β-glucan synthase catalytic activity fractionates with the yeast membranes. The fact that the *gns1* mutants are associated with a sharp decrease in 1,3-β-glucan synthase activity and are apparently defective in the detergent-insoluble component of glucan synthase lends support to the idea that Gns1p may be a subunit of the enzyme.

The predicted topology of Gns1p suggests a product with an exoplasmic orientation for the N terminus and a cytoplasmic orientation for the C terminus. This pattern of topological

TABLE 6. Alkali-insoluble β-glucan content

| Strain | β-Glucan content (μg/mg [dry wt])[a] |
|---|---|
| X2180-1A | 164 |
| MS1 | 171 |
| MS41 | 108 |
| MS43 | 173 |
| GG100-14D | 145 |
| MS100 | 187 |

[a] Both 1,3-β-glucan and 1,6-β-glucan.

TABLE 7. Reconstitution of 1,3-β-glucan synthase activity[a]

| Fraction or crude extract | Glucose incorporated (nmol/mg/h) |
|---|---|
| Wild-type crude membranes | 374.1 |
| Wild-type crude membranes + NP-40 | 672.6 |
| Mutant crude membranes | 39.9 |
| Mutant crude membranes + NP-40 | 45.1 |
| Wild-type soluble | 3.4 |
| Wild-type insoluble | 25.6 |
| Mutant soluble | 0.4 |
| Mutant insoluble | 1.0 |
| Wild-type insoluble + wild-type soluble | 405.7 |
| Wild-type insoluble + mutant soluble | 400.3 |
| Mutant insoluble + mutant soluble | 3.5 |
| Mutant insoluble + wild-type soluble | 19.4 |

[a] Membrane extracts were prepared from GG100-14D (wild-type) and MS100 (mutant) cells. 1,3-β-Glucan synthase assays were performed at 22°C with 72 μg of soluble fractions and 25 μg of either crude unfractionated proteins or insoluble fractions as described in Materials and Methods. The insoluble fractions were pellets of membrane fractions adjusted to 2% NP-40–2 M NaCl, while the soluble fractions are desalted supernatants of similar membrane fractions adjusted to 2% NP-40–2 M NaCl–10 μM GTPγS.

orientation is characteristic of such membrane receptors as β-adrenergic receptors and the yeast **a** and α pheromone receptors. The presence of a glycosylation site near the N terminus is conserved in this class of transmembrane receptors. There is an N-linked glycosylation site (Asn-X-Ser [or Thr], amino acid positions 32 to 34) at the N-terminal exoplasmic domain of Gns1p.

The abundance of leucine zippers in the sequence (amino acid positions 12 to 33 and 68 to 89) suggest that Gns1p is capable of protein-protein interaction. Leucine zippers are present not only in DNA-binding proteins but also in some membrane glycoproteins (26). The idea that the Gns1p may dimerize is intriguing. There is evidence to indicate that the fungal 1,3-β-glucan synthase is a multimeric enzyme and that heteromeric 1,3-β-glucan synthases can be formed between subunits from unrelated fungal species (23). The leucine zipper motifs function in oligomerization of peptides before their export to the cell surface as well as in protein-protein interactions (26).

The viability of the haploid strains with *gns1* disruptions indicates that the gene is not essential for growth. Since the loss of 1,3-β-glucan synthase activity is expected to be lethal, a functionally redundant homolog of *GNS1* may be present in the yeast genome. Null mutations in the *FKS1/ETG1* gene were previously reported to be associated with supersensitivity to L-733,560 (16). This contrasts with the L-733,560 resistance of the *gns1* null mutant. Resistance to L-733,560 was also reported in several strains carrying different mutant alleles of *FKS1* (14, 16). The resistance phenotype of these mutants was either semidominant or dominant (14, 16). In contrast to the L-733,560 resistance of the 1,3-β-glucan synthase of the *fks1-2 fks1-3 fks1-4* mutants, the glucan synthase present in the *gns1-1* membranes exhibits a wild-type level of sensitivity to L-733,560. Both the *gns1* null and the *fks1* null mutants exhibit reduced levels of 1,3-β-glucan synthase activity in vitro. The simultaneous disruption of *FKS1* and *GNS1* was not lethal, but the doubly disrupted haploid mutant (*gns1*::*URA3 fks1*::*HIS3*) grew poorly. In addition, the doubly disrupted mutant exhibited 1,3-β-glucan synthase activity lower than that of either of the single disruptants (16a), suggesting that the two genes may function in a common pathway. The resistance of *gns1*Δ mutants and the supersensitivity of *fks1*Δ mutants to L-733,560 suggest that Gns1p may be the primary target for the drug,

functioning in an earlier step in that pathway. If proven, that would be consistent with a role for Gns1p as a receptor for the drug, as discussed below. A possible interaction between Gns1p and Fks1p is presently under investigation.

Mutations in the *KRE6* gene confer resistance to the yeast K1-type killer toxin because of a reduction in its receptor (1,6-β-glucan) content (38). A *kre6* null mutant is also associated with a reduction in 1,3-β-glucan synthase activity. Unlike the *kre* mutants, the *gns1* mutants are not affected in their resistance to the yeast killer toxin and therefore are likely to contain unaltered 1,6-β-glucan. Mutations in *KRE6*, *SKN1* (38), or *GNS1* reduce the specific activity of 1,3-β-glucan synthase without adverse effects on the alkali-insoluble β-glucan content in the cell wall.

The recessive resistance phenotype of the *gns1* mutant cells to L-733,560 indicates that Gns1p may mediate sensitivity to L-733,560. Whether or not Gns1p actually binds L-733,560 awaits further studies. If such binding occurs, this would strengthen the idea that Gns1p may be a receptor. A ligand that may bind to such a presumed receptor could be the drug itself. Alternatively, the drug may modulate this receptor in an indirect fashion. Some cell wall degradation products resulting from treatment of the cells with the drug may function as a ligand.

Despite the fungicidal activity of L-733,560, the *S. cerevisiae* β-glucan synthase is much more resistant to the drug by comparison with the *Candida albicans* or *Aspergillus fumigatus* enzymes (27). The drug also achieves a 50% inhibition of the *S. cerevisiae* enzyme at relatively higher concentrations than its MIC against whole cells (14, 16, 27). This may be attributed to the crude nature of the enzyme preparations. It is also possible that the drug acts at some other point in addition to acting on glucan synthase.

The actual number of the enzyme subunits present in the soluble and insoluble components of 1,3-β-glucan synthase is unknown. Fks1p (13, 14, 16) and Gns1p are candidates for insoluble subunits. Reconstituting a wild-type level of 1,3-β-glucan synthase activity by mixing a *gns1* mutant soluble fraction with a wild-type insoluble fraction indicates that the mutant soluble component of the enzyme is functional. This is supported by the lack of stimulation of 1,3-β-glucan synthase activity upon mixing the mutant insoluble and wild-type soluble fractions. Similarly, 1,3-β-glucan synthase activity of the mutant unfractionated crude membrane extract was stimulated by mixing with the wild-type insoluble fraction but not by mixing with the wild-type soluble fraction. The fact that activity can be reconstituted by adding a wild-type insoluble fraction to mutant unfractionated extract indicates that the mutant soluble component of the enzyme was fully competent and readily available for interaction with the wild-type insoluble component. Either the soluble component is present in excess over the insoluble component in the mutant extract or a mutant defective insoluble component had lost its ability to bind to the soluble component, which becomes available for interaction with the wild-type insoluble fraction upon mixing.

In summary, *GNS1* encodes an integral membrane protein that fractionates with the detergent-insoluble component of the membrane. It is required for the activity of 1,3-β-glucan synthase and appears to mediate the sensitivity of *S. cerevisiae* to L-733,560, an inhibitor of 1,3-β-glucan synthase. A conclusion from this work is that Gns1p may be a subunit of 1,3-β-glucan synthase. Alternatively, this protein may be required for the activation of 1,3-β-glucan synthase by an unidentified regulatory mechanism.

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