JOURNAL OF BACTERIOLOGY, Sept. 1994, p. 5686–5696
0021-9193/94/$04.00+0

Vol. 176, No. 18

A *Saccharomyces cerevisiae* Mutant with Echinocandin-Resistant 1,3-β-D-Glucan Synthase

C. M. DOUGLAS,* J. A. MARRINAN, W. LI, AND M. B. KURTZ

Merck Research Laboratories, Rahway, New Jersey 07065

Received 2 May 1994/Accepted 15 July 1994

A novel, potent, semisynthetic pneumocandin, L-733,560, was used to isolate a resistant mutant in *Saccharomyces cerevisiae*. This compound, like other pneumocandins and echinocandins, inhibits 1,3-β-D-glucan synthase from *Candida albicans* (F. A. Bouffard, R. A. Zambias, J. F. Dropinski, J. M. Balkovec, M. L. Hammond, G. K. Abruzzo, K. F. Bartizal, J. A. Marrinan, M. B. Kurtz, D. C. McFadden, K. H. Nollstadt, M. A. Powles, and D. M. Schmatz, J. Med. Chem. 37:222–225, 1994). Glucan synthesis catalyzed by a crude membrane fraction prepared from the *S. cerevisiae* mutant R560-1C was resistant to inhibition by L-733,560. The nearly 50-fold increase in the 50% inhibitory concentration against glucan synthase was commensurate with the increase in whole-cell resistance. R560-1C was cross-resistant to other inhibitors of *C. albicans* 1,3-β-D-glucan synthase (aculeacin A, dihydropapulacandin, and others) but not to compounds with different modes of action. Genetic analysis revealed that enzyme and whole-cell pneumocandin resistance was due to a single mutant gene, designated *etg1-1* (echinocandin target gene 1), which was semidominant in heterozygous diploids. The *etg1-1* mutation did not confer enhanced ability to metabolize L-733,560 and had no effect on the membrane-bound enzymes chitin synthase I and squalene synthase. Alkali-soluble β-glucan synthesized by crude microsomes from R560-1C was indistinguishable from the wild-type product. 1,3-β-D-Glucan synthase activity from R560-1C was fractionated with NaCl and Tergitol NP-40; reconstitution with fractions from wild-type membranes revealed that drug resistance is associated with the insoluble membrane fraction. We propose that the *etg1-1* mutant gene encodes a subunit of the 1,3-β-D-glucan synthase complex.

The fungal wall is a complex, dynamic structure that provides rigidity and shape to the cell. A fibrillar network of polysaccharides, including 1,3-β-D-glucan, 1,6-β-D-glucan, and chitin, creates a meshlike structure that protects the cell from osmotic shock while remaining sufficiently plastic to allow cells to change shape as they progress through the cell cycle (15). Producing this complex array of polymers is a highly regulated process involving numerous synthetic enzymes, the delivery of enzymes, substrates, and primers to the site of assembly, and integration of new polysaccharides into the overall wall ultrastructure. One of the key enzymes of cell wall biosynthesis is 1,3-β-D-glucan synthase (EC 2.4.1.34 [UDP-glucose:1,3-β-D-glucan 3-β-glucose transferase]), which transfers glucose from UDP-glucose to an acid-insoluble, alkali-soluble, exo-β-1,3 glucanase-sensitive polysaccharide (16, 52). Crude microsomal fractions from a number of organisms, including *Candida albicans* (40), *Aspergillus fumigatus* (57), *Neurospora crassa* (42), and *Saccharomyces cerevisiae* (52), can catalyze this reaction. The membrane-associated enzymatic activity has a millimolar K_m for UDP-glucose, requires guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) for maximal synthesis, and produces a polymer reported to be 60 to 80 glucose units in length (52). Activity from several organisms can be fractionated with salt and detergent into soluble and insoluble components, which are inactive by themselves but produce an active complex when reconstituted (29). Partial purification of the enzyme from *N. crassa* (2) and solubilization of the activity from *C. albicans* (58) and *A. fumigatus* (6, 57) have been reported, but to our knowledge, the genes encoding bona fide subunits of 1,3-β-D-glucan synthase have not been cloned.

* Corresponding author. Mailing address: Department of Infectious Disease Research, Merck Research Laboratories—RY80Y-230, P.O. Box 2000, Rahway, NJ 07065-0900.

Yeast K_1 killer toxin and the chitin binding dye Calcofluor White have been used to identify genes important for the biosynthesis of 1,6-β-D-glucan (27) and chitin (45), respectively. Binding to the cell surface is the first step in the lethal action of *S. cerevisiae* K_1 toxin (14). By selecting for toxin-resistant mutants, Bussey and coworkers (10) have isolated several mutants with altered cell walls. The killer resistance (*KRE*) genes identified by these mutations encode a variety of proteins, including Kre1p, a serine- and threonine-rich secretory pathway protein with a C-terminal hydrophobic tail (10); Kre6p, a membrane-associated enzyme with a UDP-glucose binding motif (44); and Kre5p, a glycoprotein which is retained within the endoplasmic reticulum (35). These results suggest that synthesis of the 1,6-β-D-glucan to which killer toxin binds is a complex process involving a rich array of gene products. Our understanding of chitin synthesis has been aided by isolation of *S. cerevisiae* mutants resistant to the antifungal dye Calcofluor White (46). Four separate complementation groups have been identified, including one (*CAL1*) that is required for the activity of at least one of the chitin synthase isozymes (*CHS3*) (60). The various roles for chitin in the yeast cell cycle, including repair of the bud scar and formation of filaments in the bud neck, may reflect the need for several different isoforms of this enzyme (13).

Echinocandins and pneumocandins are fungicidal, acyl-substituted cyclic hexapeptides (lipopeptides) that inhibit in vitro synthesis of 1,3-β-D-glucan noncompetitively (47) and lead to lysis of growing cells of *C. albicans* (19). Recent results with cilofungin, an echinocandin B derivative, and pneumocandin B_0 suggest that β-glucan synthesis in *A. fumigatus* is also a target of the lipopeptides (5, 21, 32, 57). In an effort to identify genes specifically involved in 1,3-β-D-glucan synthesis, de Mora et al. (20) isolated *S. cerevisiae* mutants resistant to the echinocandin aculeacin A. Glucan synthesis in these mutants, as measured by whole-cell labeling, was aculeacin A

resistant, but the mutations did not affect aculeacin-sensitive polysaccharide synthesis in a regenerating spheroplast system or an in vitro 1,3-β-D-glucan synthesis assay. While limited inhibition of *S. cerevisiae* in vitro β-glucan synthesis by aculeacin has been described (62), no other inhibitor of the *C. albicans* enzyme (echinocandin B, cilofungin, papulacandin) has been shown to inhibit *S. cerevisiae* activity, and none of the mutants isolated to date has drug-resistant enzyme activity.

We describe here the use of the novel semisynthetic pneumocandin L-733,560 to isolate an *S. cerevisiae* mutant with resistant β-glucan synthase activity. This mutant, R560-1C, is specifically resistant to inhibitors of 1,3-β-D-glucan synthesis such as the pneumocandins, echinocandins, and papulacandins. Resistance to L-733,560 as measured in the in vitro reaction is commensurate with whole-cell resistance, and both phenotypes cosegregate in genetic crosses. The mutation defines a new locus, which we have named *ETG1* for echinocandin target gene 1. Other properties of the enzyme (substrate binding and specific activity) and characteristics of the product synthesized in vitro are unaffected by the mutation. Fractionation experiments with crude membranes from R560-1C and its wild-type parent show that the *etg1-1* mutation affects the insoluble fraction believed to contain the catalytic subunit (29). Our results suggest that *ETG1* encodes a subunit of 1,3-β-D-glucan synthase.

MATERIALS AND METHODS

Strains and libraries. *S. cerevisiae* W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and W303-1B (*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) were provided by R. Rothstein (59). X2180-1A (*MAT***a**) and X2180-1B (*MAT*α) were obtained from the American Type Culture Collection. *Escherichia coli* DH5α from Bethesda Research Laboratories was used as a host for recombinant plasmids. An *S. cerevisiae* genomic library constructed from DNA partially digested with *Sau*3AI and ligated into YEp24 (18) was obtained from S. Parent (Merck & Co., Inc).

Antifungal compounds and assays. Pneumocandins L-733, 560 (12), L-731,373 (12), and pneumocandin B_0 (49), as well as L-687,781 (61), sphingofungin B (26), mevinolin (1), and the zaragosic acids A and B (8), were synthesized or isolated at Merck Research Laboratories, Rahway, N.J. Fluconazole (39) and ketoconazole were obtained from Janssen, and terbinafine (SF86-327) (41) was obtained from Sandoz. Aculeacin A (37) was from Toyo Jozo, and tetrahydroechinocandin B (31) was from J. Balkovec, Merck & Co, Inc. Tunicamycin, cerulenin, amphotericin B, nystatin, trifluoroperazine, and cycloheximide were purchased from Sigma Chemical Co., St. Louis, Mo. Nikkomycin Z was purchased from CalBiochem, San Diego, Calif. Susceptibility to these compounds was measured with either a broth microdilution (MIC) assay (4) or an agar diffusion assay. For the MIC assay, saturated cultures grown at 30°C in minimal (SD) medium (0.7% yeast nitrogen base without amino acids, 2% glucose, and amino acid supplements with or without uracil) were subcultured 1:100 and grown for 4 to 6 h. The cell density was adjusted to 2×10^4 cells per ml, and 75 μl of the suspension was added to an equal volume of SD medium in microtiter dishes containing twofold serial dilutions of the test compound. Growth after 24 h was determined either visually or spectrophotometrically at 600 nm in a microtiter plate reader (SLT model 340ATCC). For the agar diffusion assay, logarithmic-phase cells were inoculated into molten SD medium or rich (YPAD [2% peptone, 1% yeast extract, 2% glucose, 0.08 mg of adenine per ml]) medium with 2% agar at a final cell density of 2×10^5 cells per ml before pouring. Filter disks were placed on the agar surface, and compounds were applied. Zones of inhibition were measured after 24 h at 30°C.

In vitro glucan synthesis assay. Crude membrane fractions were prepared essentially as described previously (55). Briefly, cells grown in 1.0 liter of YPAD medium to 7.5×10^5 cells per ml were harvested by centrifugation and washed with breakage buffer (0.1 M phosphate [pH 7.0], 1 mM EDTA, 1 mM dithiothreitol). The cells were resuspended in 50 ml of ice-cold breakage buffer, placed in a Bead Beater (BioSpec Products, Bartlesville, Okla.) with 50 g of 0.45-μm-diameter acid-washed glass beads (Braun), packed in ice, and subjected to four 1-min pulses alternated with 2 min of cooling. Breakage was typically >80% as judged by microscopy. Cell debris and unbroken cells were removed by low-speed centrifugation (2,000 × *g* for 10 min at 4°C). Crude microsomes were harvested from the supernatant by centrifugation at 100,000 × *g* for 1 h at 4°C; the pellet was resuspended in 5 ml of breakage buffer with 25% glycerol and homogenized with a Dounce homogenizer. Aliquots of the crude membrane fraction were stored at −80°C. To determine the protein concentration, a modification of the bicinchoninic acid assay (54) was used. Sodium deoxycholate was added to samples at a final concentration of 0.03%, followed by precipitation with trichloroacetic acid (TCA) (7). Precipitates were harvested by centrifugation at 1,200 × *g* for 10 min at 4°C and resuspended in 0.01 ml of 0.1 N NaOH, and the protein assay was performed with reagents from Pierce Chemicals. Typical protein concentrations ranged from 10 mg/ml to 20 mg/ml.

1,3-β-D-Glucan synthesis was measured by a modification of the procedure detailed by Schmatz et al. (48). Two separate cocktails were prepared and mixed together to initiate the reaction. Cocktail I contained TEK buffer (0.45 M Tris-HCl [pH 7.5], 3.3 mM EDTA, 0.11 M potassium fluoride), buffer B (Dulbecco's phosphate-buffered saline [PBS], pH 7.0, containing 1.2 M glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol), 2.5% (wt/vol) bovine serum albumin (BSA) in buffer B, GTP-γ-S, and 25 μg of crude 1,3-β-D-glucan synthase. Cocktail II contained α-amylase [Sigma; 18,000 U/ml in 3.2 M $(NH_4)_2SO_4$ diluted 1:20 in Dulbecco's PBS (pH 7.0) containing 1.2 M glycerol], UDP–D-glucose (Sigma), and either UDP–D-[6-^{3}H]glucose (4.5 Ci/mmol; Amersham) or UDP–D-[1-^{3}H]glucose (2 to 12 Ci/mmol; NEN). The final reaction mixture contained 69 μl of cocktail I, 7 μl of cocktail II, and 4 μl of water or drug (described below). Final concentrations for all components were 500 mM glycerol, 100 mM Tris-HCl (pH 7.5), 60 mM NaCl, 25 mM potassium fluoride, 3.5 mM $Na_2HPO_4 \cdot 7H_2O$, 3.2 mM $(NH_4)_2SO_4$, 1.2 mM KCl, 0.9 mM KH_2PO_4, 0.8 mM EDTA, 0.4 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.2% BSA, 3 μM GTP-γ-S, 40 U of α-amylase, and 0.4 mM UDP–D-glucose at 80,000 dpm/nmol (UDP–D-[6-^{3}H]glucose) or 3,750 dpm/nmol (UDP–D-[1-^{3}H]glucose). For 50% inhibitory concentration (IC_{50}) determinations, L-733,560 was prepared as a 2-mg/ml stock in water and added to individual reaction mixtures in place of water (4 μl per reaction mixture). Stocks of pneumocandin B_0 and L-687,781 were prepared in dimethyl sulfoxide, which had no effect on the assay at the final concentration used (5%). Reactions (80 μl final volume) were run in duplicate in microtiter dishes agitated gently at 22°C for 2.5 h. After incubation, an equal volume of ice-cold 20% TCA was added and the precipitates were harvested onto glass-fiber filter mats (Pharmacia) and washed with water in a 96-channel cell harvester (Cambridge Scientific). The mats were air dried, sealed in bags with Betaplate Scint scintillation cocktail (LKB/Wallac), and counted with a Betaplate scintillation counter (LKB/Wallac). The counting efficiency was estimated at 40%.

Specific activity was expressed as nanomoles of radiolabeled product per hour per milligram of protein.

Screening for drug-resistant clones. *S. cerevisiae* W303-1A was transformed with three independent pools of the multicopy yeast genomic library by the standard spheroplast transformation procedure (53). L-733,560 was added to molten SD medium at 0.5 or 1.25 μg/ml. Aliquots of each library were spread on the surface at 5×10^3 CFU per plate. Colonies which appeared after 5 days at 30°C were clonally purified and retested for auxotrophic markers and drug resistance by replica plating. Antibiotic resistance was quantitated by the broth microdilution method.

Transformants were cured of plasmid DNA by growth in uracil-supplemented minimal medium followed by selection with 5-fluoroorotic acid (9). Uracil auxotrophs were identified and tested for drug sensitivity. Plasmid DNA was isolated from clones by standard methods (53), transformed into *E. coli* DH5α, and analyzed for inserts by digestion with restriction endonucleases (34). To test for plasmid-dependent phenotypes, *S. cerevisiae* W303-1A was transformed as described with plasmids isolated from *E. coli* and assayed for drug resistance by the broth microdilution method.

Chitin and squalene synthase assays. Crude membrane fractions from W303-1A and R560-1C were assayed for chitin synthase I activity. Crude protein (750 μg) was pretreated with trypsin (Sigma) and incubated with 10 nCi of UDP-*N*-acetyl-D-[glucosamine-^{14}C(U)] (>200 mCi/mmol; NEN), and then the product was harvested as described previously (17). The IC_{50} for nikkomycin Z was measured by titrating the drug from 0.02 μg/ml to 5 μg/ml and calculating the percentage of inhibition of synthesis.

Squalene synthase activity was measured according to the method of Bergstrom et al. (8). [1-^{3}H(*N*)]farnesyl pyrophosphate (15 to 30 Ci/mmol; NEN) was added to 2 μg of crude microsomal protein from W303-1A or R560-1C in 50 mM *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) buffer (pH 7.5), with 1 mM NADPH, 5.5 mM $MgCl_2$, 11 mM potassium fluoride, 3 mM dithiothreitol, 1 μg of terbinafine per ml, and 5 μM farnesyl pyrophosphate (final specific activity, 1.1×10^6 dpm/nmol). After a 12-min incubation at 30°C, 1 volume of ethanol was added and squalene was extracted from the reaction mixture with 2 volumes of *n*-heptane containing 1 μl of squalene per ml. Radioactivity in the organic phase of the extract was determined by liquid scintillation counting. Titration of zaragosic acid A from 0.02 nM to 1.8 nM against the enzyme from mutant and wild-type strains was used to determine the IC_{50}.

Genetic analysis. Standard methods were used for mating, sporulation, tetrad dissection, and analysis of auxotrophic markers (53).

High-performance liquid chromatography (HPLC) analysis of L-733,560. Standard in vitro glucan synthesis reaction mixtures with crude membrane fractions from R560-1C and W303-1A were prepared with L-733,560 added at a final concentration of 2 μM. After 150 min at 22°C, the samples were centrifuged at 100,000 × *g* for 1 h at 4°C and L-733,560 in the supernatants was quantitated essentially by the method of Hadju et al. (24). Samples (25 μl) were injected onto a Zorbax RXC_8 column equilibrated at 37°C and eluted at 1.2 ml/min with 60% methanol–32.4 mM acetic acid–25 mM triethylammonium phosphate (pH 2.9). The drug was detected by fluorescence (excitation, 224 nm; emission, 302 nm) and quantitated against a standard curve of L-733,560.

Fractionation of crude microsomes. Crude membranes from R560-1C and W303-1A were prepared and fractionated by the method of Kang et al. (29). Cells grown to 7×10^5 cells per ml were harvested by centrifugation and broken with glass beads, and the crude membranes were extracted with Tergitol NP-40 and 2 M NaCl with (soluble fraction) and without (insoluble fraction) 20 μM GTP-γ-S. For reconstitution experiments, 25 μg of particulate fraction and 7.5 μg of soluble fraction were added to reaction mixtures with or without added L-733,560.

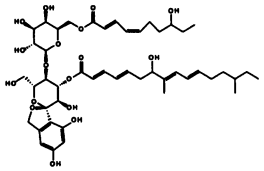
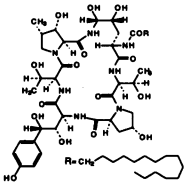
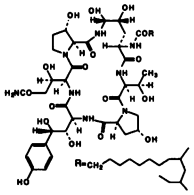
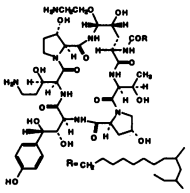
Gel filtration of product. Radiolabeled product was partially purified from glucan synthesis reaction mixtures by gel filtration. After synthesis, duplicate samples were combined, made alkaline by the addition of 0.25 volumes of 50% NaOH, and centrifuged for 1 min at 10,000 × *g* at 25°C. The supernatant (200 μl) was chromatographed at 0.3 ml/min on a Superose 12 column (Pharmacia) with 150 mM NaOH as eluant. Fractions were collected, neutralized with 150 mM HCl, and counted. To calibrate the column, linear dextrans (Sigma) ranging in size from 9.3 kDa to 515 kDa were chromatographed under the same conditions and detected by the phenol-sulfuric acid method (22). Curdlan, a linear 1,3-β-D-glucan with a size of ca. 450 glucose units isolated from *Alcaligenes faecalis* (Accurate Chemical & Scientific Corp., Westbury, N.Y.), was also used as a standard.

Product analysis. Peak fractions containing radiolabeled product from repeat runs on the Superose 12 column were pooled, dialyzed overnight at 4°C against water, and dried under a vacuum. Over 90% of the radioactivity was recovered after dialysis. Samples were resuspended in 0.1 M acetate buffer (pH 5.0). One aliquot was treated with 2 U of laminarinase (Sigma) for 18 h at 37°C (42); a duplicate aliquot without enzyme served as a control. A solution of NaOH (50%; 0.1 sample volume) was added, and the samples were sonicated for 15 min at 25°C before separation by gel filtration and scintillation counting as described. The fractions from the gel filtration column containing the radiolabeled digestion product were pooled, and an aliquot (50 μl) was subjected to anion-exchange chromatography on a CarboPac PA1 column (Dionex Corporation, Sunnyvale, Calif.) equilibrated and eluted at ambient temperature with 8 mM NaOH at a flow rate of 1 ml/min. Carbohydrate in the effluent was detected by pulsed amperometry (sampling time [*t*] = 200 ms) with the following settings: $E_1 = 0.10$ V ($t_1 = 500$), $E_2 = 0.60$ V ($t_2 = 100$), and $E_3 = -0.60$ V ($t_3 = 50$ ms) (28). A standard mixture of neutral and amino sugars was chromatographed under the same conditions. The profile of ^{3}H-labeled material was determined by collecting fractions and counting with Scintiverse (Fisher Scientific), with a counting efficiency of 40%.

RESULTS

Activity of L-733,560 against *S. cerevisiae*. Naturally occurring pneumocandins, echinocandins, and papulacandins have relatively weak antifungal activity against *S. cerevisiae*. Recent chemical efforts to improve upon the antifungal properties of the pneumocandins led to the synthesis of L-733,560, a dibasic water-soluble derivative of pneumocandin B_0 (12). Table 1 gives the MICs of L-733,560 and the natural products pneumocandin B_0, dihydropapulacandin (L-687,781), and aculeacin A against *S. cerevisiae* W303-1A and X2180-1A. L-733,560 is at least 16-fold more active against both strains than the natural products. After 48 h, samples from the MIC assay were spotted on drug-free agar plates to determine the minimum fungicidal concentration; wells with no visible growth failed to produce colonies. Against *C. albicans*, the minimum fungicidal concentration for L-733,560 (0.15 μg/ml) was sevenfold lower than the value for pneumocandin B_0 (1.06 μg/ml). The significant potency of L-733,560 made this compound especially attractive as a selective agent.

TABLE 1. Activity of glucan synthesis inhibitors against *S. cerevisiae*

Compound	Structure	MIC (μg/ml) X2180-1A	MIC (μg/ml) W303-1A
L-687,781 (Dihydropapulacandin)		16	16
Aculeacin A		4	4
Pneumocandin B_0		32	32
L-733,560		0.25	0.25

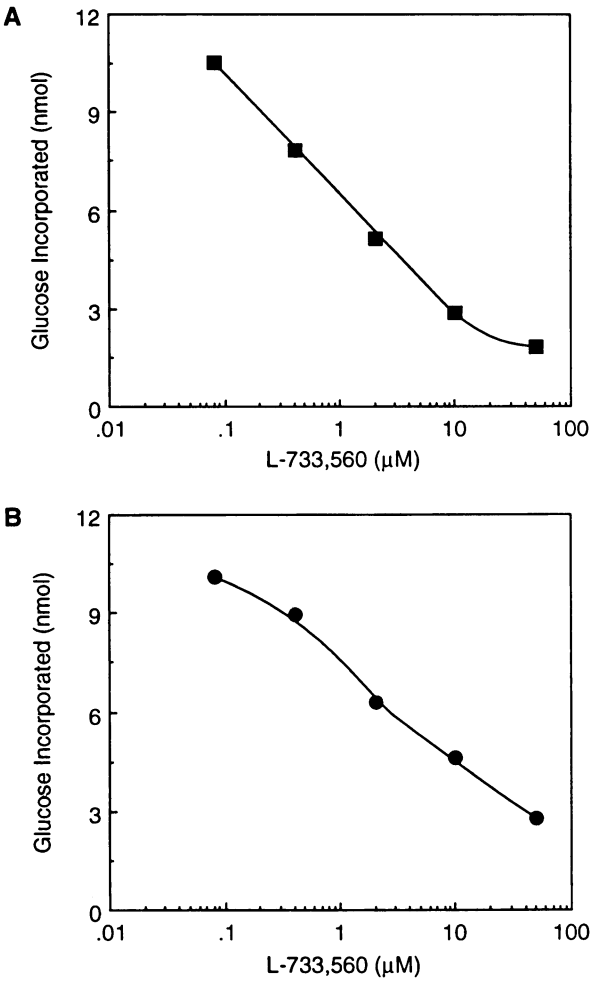


FIG. 1. 1,3-β-D-Glucan synthesis assay of crude membrane fractions from *S. cerevisiae* W303-1A (A) and X2180-1A (B). Reactions were performed as described in Materials and Methods with UDP-D-[1-^{3}H]glucose. For W303-1A, uninhibited synthesis was 12.9 nmol of glucose incorporated, and the specific activity of the enzyme was 205 nmol · h^{-1} · mg of protein^{-1}. For X2180-1A, uninhibited synthesis was 12.6 nmol of glucose incorporated, and the specific activity of the enzyme was 200 nmol · h^{-1} · mg of protein^{-1}. Final concentrations of L-733,560 in the reactions ranged from 0.08 μM to 50 μM.

Inhibition of in vitro glucan synthesis. In *C. albicans*, the improved antifungal activity of L-733,560 was correlated with its increased potency against 1,3-β-D-glucan synthase (33). We evaluated the activity of L-733,560 against *S. cerevisiae* glucan synthase in crude membranes from W303-1A and X2180-1A. Inhibition curves of the logarithm of the L-733,560 concentration versus the product formed (Fig. 1) show that synthesis was inhibited in a dose-dependent fashion, with a maximum of 90% inhibition at 50 μM L-733,560, an IC_{50} of 1 μM, and a linear portion spanning a nearly 100-fold range of drug concentrations, from 0.08 μM to nearly 10 μM. The reaction is also inhibited by pneumocandin B_0 and L-687,781, but the estimated IC_{50} values were greater than 10 μM for both compounds, with virtually no inhibition at concentrations below 1 μM (data not shown). We examined the kinetics of L-733,560 inhibition (Fig. 2). Typically, noncompetitive inhibitors give rise to lines which converge at the x axis, whereas the lines for a competitive inhibitor are parallel and those of a mixed inhibitor converge at a point where $[S]/v$ is <0. The plot for L-733,560 inhibition is consistent with noncompetitive inhibition.

Isolation of R560-1C. The improved potency of L-733,560 compared with that of other β-glucan synthesis inhibitors suggested that it might be useful as a selective agent for cloning *S. cerevisiae* target genes that confer antibiotic resistance when they are overexpressed. Three independent libraries of *S. cerevisiae* total genomic DNA were transformed into W303-1A, and 27 drug-resistant colonies were isolated. These clones were challenged with drug in a liquid MIC assay to estimate the level of resistance. One clone (R560-1) was at least 10-fold more resistant than the parent strain; the others were 2- to 4-fold more resistant. We examined the effect of plasmid loss on the drug resistance phenotype by selecting for spontaneous plasmid-free, uracil-requiring derivatives with 5-fluoroorotic acid and assaying them for sensitivity to L-733,560. Surprisingly, the phenotype of R560-1 was unchanged by the loss of plasmid (Fig. 3). The twofold difference in resistance between strain R560-1 and the cured derivative R560-1C is within the experimental error. Plasmid DNA isolated from R560-1 failed to confer L-733,560 resistance when retransformed into W303-1A.

Specificity of resistance. A panel of known antifungal antibiotics with various modes of action were tested against R560-1C and W303-1A to determine the specificity of the resistance phenotype (Table 2). R560-1C was resistant to inhibitors of *C. albicans* 1,3-β-D-glucan synthase, such as pneumocandin B_0, L-731,373, tetrahydroechinocandin B, aculeacin, and L-687-781 (dihydropapulacandin). For all other

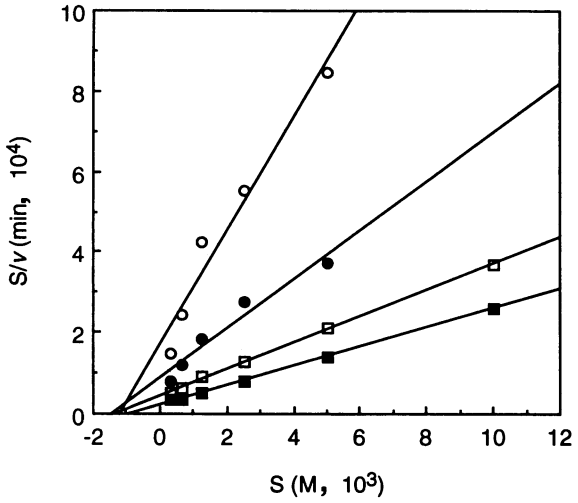


FIG. 2. Hanes-Wolff plot of L-733,560 inhibition of 1,3-β-D-glucan synthesis in crude membranes from W303-1A. Assays were performed with UDP-D-[6-^{3}H]glucose as described in Materials and Methods. The final concentration of UDP-glucose ranged from 0.31 mM to 10 mM in twofold dilutions at a specific activity of 15,000 dpm/nmol. The concentrations of L-733,560 were 10 μM (○), 2 μM (●), 0.4 μM (□), and 0.08 μM (■).

compounds tested, the sensitivities of R560-1C and W303-1A were equivalent. There was no change in the mutant's sensitivity to the chitin synthesis inhibitor nikkomycin Z or the glycosylation inhibitor tunicamycin. Sterol and fatty acid synthesis inhibitors such as the zaragosic acids, cerulenin, fluconazole, ketoconazole, mevinolin, and terbinafine produced similar zones of growth inhibition on both strains. We also observed no difference in sensitivity to antifungal agents which inhibit protein synthesis (cycloheximide), secretion (trifluoroperazine), sphingolipid biosynthesis (sphingofungin B), or

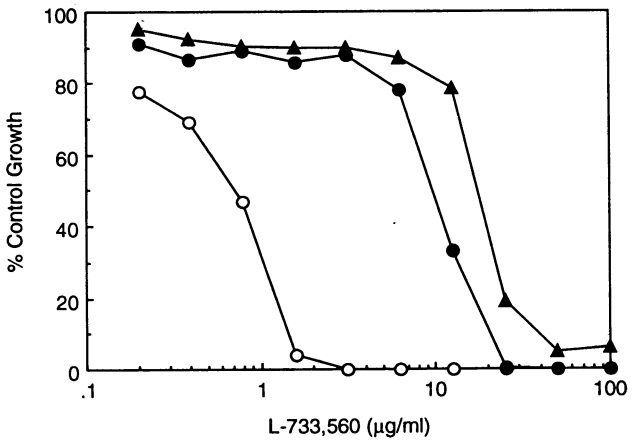


FIG. 3. L-733,560 inhibition of growth. W303-1A (○) and R560-1C (cured [▲]) were grown overnight in SD medium with uracil; R560-1 (●) was grown without uracil. Cell suspensions were seeded in microtiter plates containing twofold dilutions of L-733,560 (M_r 1,163) from 100 μg/ml to 0.20 μg/ml (final concentration) in SD medium with or without uracil. After 24 h at 30°C, the optical density at 600 nm was measured for each well. Growth relative to that of a drug-free control well was used to calculate the dose-response curves.

TABLE 2. Sensitivity of R560-1C and W303-1A to antifungal antibiotics

Compound (μg/disc)	Zone of inhibition (mm) with:	
	W303-1A	R560-1C
L-733,560 (20)	23	9
L-731,373 (100)	20.5	8.5
Pneumocandin B_0 (150)	13	NZ[a]
Aculeacin A (15)	14	NZ
L-687,781 (dihydropapulacandin)[b] (50)	16	9
Tetrahydroechinocandin B (60)	11.5	8
Nikkomycin Z[c] (50)	NZ	NZ
Tunicamycin (20)	17	17
Zaragosic acid A (70)	20.5	21
Zaragosic acid B (20)	13	14
Cerulenin (10)	34	35
Fluconazole (5)	18	18
Ketoconazole (20)	NZ	NZ
Mevinolin (30)	16 (H)[d]	16 (H)
Terbinafine (30)	13.5 (H)	12 (H)
Amphotericin B (20)	13.5	14
Nystatin (600)	13	13.5
Cycloheximide (50)	41	43
Trifluoroperazine[b] (100)	14	13.5
Sphingofungin B (20)	28 (H)	29 (H)

[a] NZ, no zone.
[b] Assay was run in YPAD medium rather than SD medium.
[c] Nikkomycin Z sensitivity was tested in yeast carbon base medium (Difco) with 20 mM L-proline.
[d] H, hazy zone.

the integrity of the cell membrane (amphotericin B and nystatin).

Enzyme resistance in R560-1C. We tested the sensitivity of R560-1C glucan synthase to inhibition by L-733,560. Crude microsomal fractions from mutant and wild-type cells grown to early logarithmic, mid-logarithmic, or stationary phase were assayed for β-glucan synthesis activity. The specific activity of the mutant enzyme was commensurate with that of the wild type at each phase of growth (data not shown). Therefore, all remaining experiments were done with membranes prepared from logarithmic-phase cells. Enzymatic activities from W303-1A and R560-1C were inhibited by L-733,560 in a dose-dependent fashion (Fig. 4), but the IC_{50} was much higher for the mutant (50 versus 1.1 μM). The nearly 50-fold differential in IC_{50} is commensurate with the difference in MIC (4 to 8 μg/ml for R560-1C versus 0.125 to 0.25 μg/ml for W303-1A).

We determined the effect of the mutation on other membrane-associated enzyme activities. Two independent reactions, synthesis of chitin from UDP-GlcNAc and synthesis of squalene from farnesyl pyrophosphate, were measured. Trypsin-activated chitin synthase activity was unaffected by the mutation, and the IC_{50} for nikkomycin Z was also unchanged (1.6 μg/ml for W303-1A; 2.1 μg/ml for R560-1C). The specific activity of squalene synthetase in mutant and wild-type membranes was 2 to 3 nmol · min^{-1} · mg of protein^{-1}, and the IC_{50}s of zaragosic acid A were 0.2 and 0.3 nM, respectively. Of the three membrane-associated activities assayed in membranes from R560-1C, only the IC_{50} of L-733,560 was affected.

Genetics of echinocandin resistance. We performed a genetic analysis to determine if the pneumocandin resistance phenotype was due to a single gene mutation segregating in standard Mendelian fashion. R560-1C was mated to the pneumocandin-sensitive strains W303-1B and X2180-1B. Results for the whole-cell and in vitro enzyme sensitivities to L-733,560

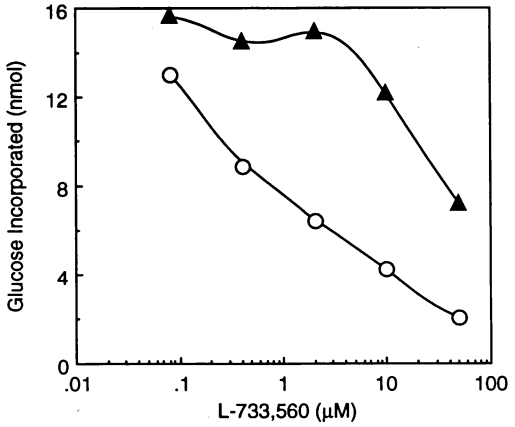


FIG. 4. Resistance of R560-1C glucan synthesis activity. Conditions were as described in the legend to Fig. 1. L-733,560 was titrated against crude membrane fractions from W303-1A (○) and R560-1C (▲). For W303-1A, uninhibited synthesis was 19.0 nmol of glucose incorporated and the specific activity of the enzyme was 313 nmol · h^{-1} · mg of $protein^{-1}$. For R560-1C, 100% synthesis was 16.4 nmol of glucose incorporated and the specific activity of the enzyme was 260 nmol · h^{-1} · mg of $protein^{-1}$.

for haploid progeny from two tetrads (DYLIP3-7 and DYLIP4-6), the parents, and both heterozygous diploids are reported in Tables 3 and 4. Whole-cell resistance segregated 2:2 in the tetrads, and the sensitivity of both diploids (YLIPD3 and YLIPD4) was intermediate between that of R560-1C and the sensitive parents. Whole-cell resistance data from 40 four-spore tetrads of R560-1C crossed with W303-1B confirmed these results (data not shown). Crude membrane fractions prepared from the haploid progeny of DYLIP3-7 and DYLIP4-6, as well as parental diploids, were challenged with fivefold dilutions of L-733,560 (Tables 3 and 4). The IC_{50}s from these experiments illustrate that each resistant spore had resistant glucan synthase activity, while each sensitive spore had sensitive glucan synthase activity. Intermediate drug resistance in the diploid membrane preparations also reflects the whole-cell phenotype and demonstrates that the mutation is semidominant. The mutation that confers resistance to glucan synthase inhibitors is a single allele, which we have called *etg1-1* for echinocandin target gene 1.

Drug stability in crude membrane fractions. Whole-cell and in vitro resistance to pneumocandins, particularly resistance resulting from a dominant or semidominant mutation, could be due to enhanced ability to degrade the inhibitor. The stability of L-733,560 in typical glucan synthase reaction mixtures from R560-1C and W303-1A was measured. In these experiments, the concentration of drug added (2 μM) was sufficient to inhibit wild-type synthesis by ca. 60% but inhibited the mutant

TABLE 3. Segregation of whole-cell and enzyme resistance to L-733,560 in tetrad *DYLIP3-7* progeny

Strain	IC_{50} (μM)	MIC (μg/ml)
R560-1C (parent 1)	49	3.13
W303-1B (parent 2)	1.1	0.125
YLIPD3 (diploid)	10	0.78
YLIP3-7A (spore A)	1.2	0.125
YLIP3-7B (spore B)	>50	3.13
YLIP3-7C (spore C)	>50	3.13
YLIP3-7D (spore D)	3.6	0.125

TABLE 4. Segregation of whole-cell and enzyme resistance to L-733,560 in tetrad *DYLIP4-6* progeny

Strain	IC_{50} (μM)	MIC (μg/ml)
R560-1C (parent 1)	49	6.25
X2180-1B (parent 2)	2.5	0.13
YLIPD4 (diploid)	5.3	0.78
YLIP4-6A (spore A)	27	6.25
YLIP4-6B (spore B)	17	6.25
YLIP4-6C (spore C)	0.9	0.13
YLIP4-6D (spore D)	0.1	0.13

enzyme by less than 10% (Fig. 4). After 2.5 h of incubation, quantitation of L-733,560 by HPLC revealed no difference in the amount of compound recovered (data not shown) and no novel peaks which might indicate metabolism of the compound.

Properties of the mutant enzyme. Crude membrane fractions from W303-1A and R560-1C were assayed for GTP-γ-S stimulation of glucan synthase activity and affinity for substrate. If the *etg1-1* mutation is in a subunit of 1,3-β-D-glucan synthase, there may be some alteration in these properties of the enzyme. Titration of GTP-γ-S from 400 pM to 40 μM in reaction mixtures showed that glucan synthase activity from both W303-1A and R560-1C was stimulated ca. 10-fold at concentrations of ≥4 μM (data not shown). We also measured the K_m for UDP-glucose for both enzymes (Fig. 5). The crude enzyme from R560-1C had a K_m of 1.4 mM, while wild-type glucan synthase had a K_m of 1.1 mM. The values for V_{max} were similar but not identical (2.2 μM · min^{-1} for R560-1C and 1.4 μM · min^{-1} for W303-1A).

Glucan synthase activity in crude microsomes can be separated into two fractions by extraction with NaCl and Tergitol NP-40 (29). Each is inactive by itself, but reconstitution of the soluble and insoluble fractions produces an active complex. Table 5 shows that over 95% of the activity for crude microsomes from W303-1A and R560-1C is lost upon fractionation of the enzyme but that activity can be partially restored by recombining the fractions. Only the combination of a soluble fraction with an insoluble fraction reconstitutes activity (data not shown). Mixing fractions from the mutant and wild-type strains also restored activity; either insoluble fraction was functional with either soluble fraction. By titrating L-733,560 against the four different reconstituted enzymes, we determined the IC_{50}s reported in Table 5. Both complexes containing the mutant insoluble fraction were partially resistant, while those containing the mutant soluble fraction were fully sensitive to L-733,560.

Product analysis. We analyzed the radiolabeled material synthesized in glucan synthase reaction mixtures to determine if the *etg1-1* mutation altered the properties of the product. Typical chromatograms from W303-1A and R560-1C alkali-solubilized reaction mixtures separated on Superose 12 are shown in Fig. 6A. Two peaks of radioactivity, one in the void volume and one near the included volume, eluted from the column, and the profiles for the mutant and the wild type were equivalent. The late eluting peak was determined to be unincorporated UDP-[^{3}H]glucose by thin-layer chromatography (data not shown). Several experiments suggest that the peak in the void volume is a product of the enzyme. First, chromatograms from reaction mixtures quenched before incubation by the addition of NaOH do not contain this peak. Second, the relative area of the void volume peak is consistent with our estimates, by TCA precipitation, of ca. 10% incorporation of glucose from UDP-glucose into product. Third, inhibition of

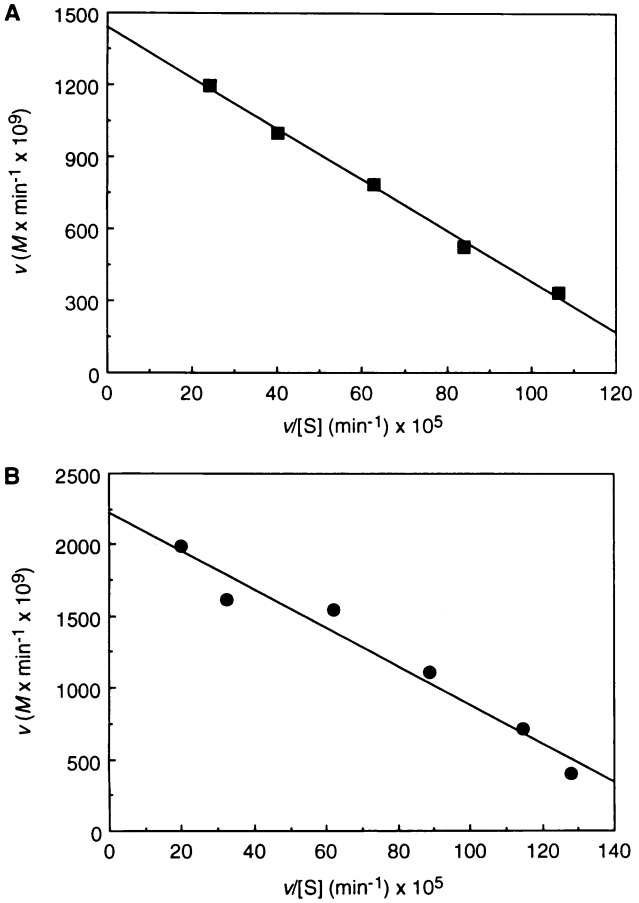


FIG. 5. Kinetics of 1,3-β-D-glucan synthase from W303-1A (A) and R560-1C (B). UDP-D-[6-^{3}H] glucose was used, and the final specific activity was 5,600 dpm/nmol. The UDP-glucose concentration was titrated in twofold dilutions from 0.3 mM to 10 mM. The reaction velocity ($M \cdot min^{-1} \cdot 10^9$) was calculated and used to generate Woolf-Augustinsson-Hofstee plots (50). The K_m for W303-1A was estimated at 1.1 mM, and the V_{max} was 1.4 $\mu M \cdot min^{-1}$. For R560-1C, the K_m was 1.4 mM and the V_{max} was 2.2 $\mu M \cdot min^{-1}$.

synthesis by L-733,560 reduces the amount of TCA insoluble radioactivity in the reaction and the area of the void volume peak to an equivalent extent. Figure 6 also shows the radioactivity profile for reactions catalyzed in the presence or absence of 20 μM L-733,560. The percentage of inhibition for duplicate reactions was measured by TCA precipitation. Weak inhibition of mutant activity (18%) and strong inhibition of wild-type activity (70%) are reflected in the reduction of the sizes of the respective product peaks.

The properties of the partially purified product from the R560-1C and W303-1A reaction mixtures were compared. Roughly 65% of the labeled product from both mutant and wild-type reaction mixtures was soluble at pH 2.4, while over 95% was soluble at pH 14. Authentic 1,3-β-D-glucan (curdlan) had the same properties. The products from gel filtration were treated with laminarinase, a crude exo-1,3-β-D-glucanase. Over 95% of the material from both reaction mixtures was susceptible to digestion, as measured by TCA precipitation or chromatography on Superose 12. A single radiolabeled peak that eluted near the included volume was generated by digestion. This material was separated by HPLC anion-exchange chromatography as described in Materials and Methods, and

TABLE 5. L-733,560 inhibition of fractionated and reconstituted glucan synthesis activity from R560-1C and W303-1A

Fraction[a]	Protein (mg/ml)	Sp act (nmol of product · h^{-1} · mg of protein^{-1})	IC_{50} (μM) of L-733,560
W303-1A			
I	2.8	10.2	
S	0.3	<0.3	
Unfractionated	3.2	300.0	0.8
Reconstituted		72.6	0.9
R560-1C			
I	4.4	8.4	
S	0.3	0.5	
Unfractionated	5.6	263.4	50
Reconstituted		111.0	6
Mixed			
W303-1A (I) + R560-1C (S)		106.8	0.8
R560-1C (I) + W303-1A (S)		82.2	7

[a] I, insoluble; S, soluble.

fractions were assayed for radioactivity. The digestion product from both R560-1C and W303-1A eluted as a single peak that comigrated with authentic D-glucose (data not shown).

Other phenotypes of R560-1C. If glucan synthesis were impaired in R560-1C, the mutant cells might show phenotypic changes consistent with a defect in cell wall biosynthesis. Temperature sensitivity (43), osmotic fragility (51), and hypersensitivity to exo-1,3-β-D-glucanase (44) are a few of the phenotypes described previously for cell wall mutants. Stationary-phase cultures of R560-1C and W303-1A were subcultured in rich medium, incubated at 25, 30, 37, and 42°C, and plated for CFU. Both strains grew well at every temperature except 42°C, where neither W303-1A nor R560-1C grew. Addition of 1 M sorbitol, an osmoticum which stabilizes strains with a weakened cell wall, did not enhance the growth rate of R560-1C; in fact, the mutant grew as well as W303-1A in all media tested, including minimal and rich broths. To test for exo-1,3-β-D-glucanase sensitivity, fresh log-phase cultures of each strain were incubated with zymolyase and plated in medium with and without osmotic support and colonies were counted to determine the percentage of osmotically fragile cells. The concentrations of crude enzyme that reduced the number of viable cells by 2 logs were equivalent for both R560-1C and W303-1A. In these assays measuring cell wall-related phenotypes, R560-1C was indistinguishable from W303-1A.

DISCUSSION

The *S. cerevisiae* mutant strain R560-1C has several properties that suggest that the *etg1-1* mutation directly affects 1,3-β-D-glucan synthase activity. First, unlike those of any other mutants described to date, the in vitro enzyme activity of R560-1C is resistant to echinocandins. We have shown that the reaction has the properties previously reported for 1,3-β-D-glucan synthase (52). The K_m for UDP-glucose (1.1 mM for R560-1C, 3.8 mM for *S. cerevisiae* GS-1-36 [55]) is within the range of values (0.7 to 7.1 mM) given for glucan synthases from several different fungi (16), and the acid insolubility, base solubility, and laminarinase sensitivity of the product are comparable for W303-1A, R560-1C, and GS-1-36. Second, the phenotypes of enzyme and whole-cell resistance cosegregated in genetic crosses. Third, the level of enzyme resistance, as

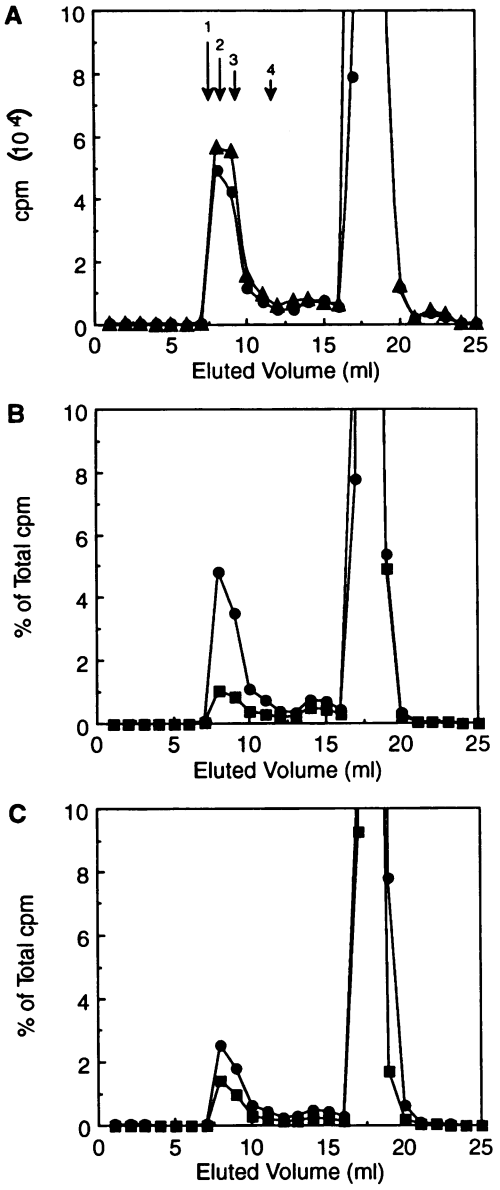


FIG. 6. Gel filtration profile of radiolabeled product from wild-type and mutant glucan synthesis reaction mixtures. Radioactivity profiles for W303-1A (●) and R560-1C (▲) reaction mixtures chromatographed as described in Materials and Methods are shown in panel A. The substrate was UDP-D-[6-^{3}H] glucose diluted with unlabeled UDP-glucose to a final specific activity of 80,000 dpm/nmol. Arrows indicate the peak position of linear 1,6-α-glucans. The sizes in kilodaltons and glucose units (DP [degree of polymerization]) of these standards are as follows: 1, 515 (DP = 2,860); 2, 162 (DP = 900); 3, 40 (DP = 222); 4, 9.3 (DP = 52). (B) W303-1A synthesis in the presence (■) or absence (●) of 20 μM L-733,560. (C) R560-1C synthesis in the presence (■) or absence (●) of 20 μM L-733,560.

judged by the elevated IC_{50}, was sufficient to account for the resistance of the whole cells. Fourth, the mutant was cross-resistant to dihydropapulacandin (L-687,781), a structurally distinct glucan synthase inhibitor with a fatty acyl side chain attached to a disaccharide core (Table 1), but not to antifungal agents with other modes of action. Unlike the pleiotropic drug resistance mutations described by others (3), which confer resistance to several classes of inhibitors, the *etg1-1* mutation is specific to inhibitors of 1,3-β-D-glucan synthase.

Two alternative mechanisms that might explain in vitro resistance to L-733,560 were addressed. The *etg1-1* mutation could alter the lipid composition of the R560-1C plasma membrane and cause a general perturbation of membrane-associated proteins. Aculeacin A-resistant *S. cerevisiae* mutants (20) have changes in cell surface hydrophobicity that could reflect changes in lipid composition similar to those described for *C. albicans* mutants resistant to aculeacin A (36). We analyzed two other membrane-associated activities (chitin synthesis and squalene synthesis) and found no difference in either specific activity or sensitivity to inhibitors. Another explanation for R560-1C resistance could be metabolism of L-733,560 through such mechanisms as proteolysis or cleavage of the acyl tail. HPLC analysis of L-733,560 from mutant and wild-type reaction mixtures confirmed that the compound was recovered intact.

Most efforts to isolate fungal mutants specifically affected in the synthesis of 1,3-β-D-glucan have followed two approaches. In the first, cells or spores have been mutagenized and screened for phenotypes associated with defective cell walls. Ribas et al. isolated *Schizosaccharomyces pombe* thermosensitive mutants requiring the presence of an osmotic stabilizer to survive (43). While the incorporation of radiolabeled glucose into β-glucan was reduced when these mutants were grown at the nonpermissive temperature and the specific activity of 1,3-β-D-glucan synthase was reduced in extracts prepared from cells grown at 37°C, the activity itself was not thermolabile. Likewise, *Aspergillus nidulans* mutants identified by Borgia and Dodge have not been shown to have a specific defect in 1,3-β-D-glucan synthase (11). The second approach has involved the use of inhibitors to select for drug-resistant mutants. The aculeacin A-resistant mutants (*acr-1*, *acr-2*, *acr-3*, and *acr-4*) isolated by de Mora et al. (20) are affected in cell wall-related phenotypes, but these strains are not cross-resistant to other 1,3-β-D-glucan synthesis inhibitors such as papulacandin, and the IC_{50} of the in vitro enzyme activity is unchanged. There is one report of papulacandin B-resistant mutants of both *S. cerevisiae* and *S. pombe* (23). The *S. cerevisiae pap1* mutant shows no apparent defect in β-glucan synthesis, and the gene which complements the mutation (*PAP1*) is nonessential but reduces in vitro 1,3-β-D-glucan synthase activity ≥50% when disrupted.

We believe the *etg1-1* mutation affects the catalytic subunit of 1,3-β-D-glucan synthase. The model proposed by Kang et al. (29) for glucan synthase predicts a minimum of two subunits. When crude microsomes are fractionated, the catalytic center, with the binding site for UDP-glucose, remains membrane associated, while the regulatory subunit, with GTP binding activity, is soluble. The requirement for GTP-γ-S for maximal activity, a hallmark of fungal glucan synthases from *S. cerevisiae*, *C. albicans*, *A. fumigatus*, *S. pombe*, and others (6, 32, 55), was not altered by the *etg1-1* mutation. Our evidence from fractionation and reconstitution of the L-733,560-resistant enzyme activity from R560-1C suggests that resistance is associated with the particulate fraction, which is thought to contain the catalytic subunit. We considered that the mutation might change the conformation of the binding site for UDP-glucose. However, inhibition of in vitro glucan synthesis activity by echinocandins and pneumocandins in *C. albicans* (38, 47) and *A. fumigatus* (32, 57) is noncompetitive, and the Hanes-Woolf plots for L-733,560 inhibition of *S. cerevisiae* glucan synthesis are typical of noncompetitive inhibition (50). The absence of a change in the K_m for UDP-glucose also supports the idea that the mutation does not distort the catalytic center.

If there is direct interaction between the compound and the enzyme, it occurs in a manner that does not interfere with UDP-glucose binding. L-733,560 and related compounds may interact with the catalytic subunit at a site other than the substrate binding pocket. Moreover, if direct interaction between the pneumocandins and glucan synthase is required for inhibition and the enzyme is a multisubunit complex, overexpression of a single subunit is unlikely to lead to gene dosage-based resistance to these inhibitors. Our original screen for the echinocandin target gene used a multicopy library transformed into W303-1A and may have been unsuccessful for this reason.

Pneumocandin-resistant glucan synthase activity from R560-1C was unaffected in other properties we measured. The product synthesized by the mutant enzyme was indistinguishable from that of the wild type. Both polymers were alkali-soluble, partially acid-soluble polysaccharides with a high apparent molecular weight. The *kre1* mutant described by Boone et al. (10) affected not only the level of cell wall 1,6-β-glucan but the structure as well. Analysis of the 1,6-β-glucan fraction isolated from a strain with a disruption of *KRE1* showed that it had an altered structure with a smaller average polymer size. Our results illustrate that the mutant and wild-type polysaccharide products were equally sensitive to digestion with exo-1,3-β-D-glucanase, suggesting that the linkage between glucose units is exclusively β-1,3. The mutation did not alter either the degree of polymerization or the overall structure of the polymer.

A surprising feature of the glucan synthesized in vitro is the apparent size of the polymer. We anticipated, on the basis of reports from Shematek et al. (52), that the reaction product would be 60 to 80 glucose units in length. The product from W303-1A and R560-1C crude membranes elutes in the void volume of Superose 12, which suggests that the polymer length could be 3,000 glucose units or longer. Under the same elution conditions, curdlan, an authentic 1,3-β-D-glucan with a degree of polymerization estimated at 450 glucose units (56), migrates as expected. The unexpected mobility of the in vitro product could be due to association with high-molecular-weight species (other polysaccharides, lipids, proteins, etc.) present in the crude membrane fraction. Experiments to define the degree of polymerization of the synthetic polymer are in progress.

Two recent reports describe the use of the HM-1 killer toxin from *Hansenula mrakii* to identify genes involved in 1,3-β-D-glucan synthesis. This *Hansenula* toxin inhibits glucan synthesis in whole yeast cells but inhibits the in vitro enzyme reaction at concentrations 10 to 100 times higher than the MIC. Kasahara et al. (30) reported on the cloning of a single-copy essential gene (*HKR1*) that confers resistance to HM-1 toxin by its overexpression, and Hong et al. (25) cloned *KNR4* by complementing a killer toxin-resistant mutant. Disruption of *KNR4* is not lethal but results in several cell wall-associated phenotypes: osmotic-sensitive lysis, resistance to 1,3-β-glucanase, threefold reduction of in vitro glucan synthase activity, and a twofold decrease in cell wall glucan. The cloned gene that complements all of these defects turned out to be identical to *SMI1*, which was discovered in a screen for proteins that bind matrix-associated DNA regions. *KRN4/SMI1* may be a regulator affecting cell wall synthesis directly or indirectly. In contrast, the *HKR1* gene encodes a large (189-kDa) serine- and threonine-rich protein with an EF hand motif for calcium binding and limited homology to *CDC24*, a gene encoding a protein implicated in GDP-GTP exchange required for bud emergence. The relationship of the *HKR1* gene to glucan synthesis is unclear. While the sequence does not have obvious regulatory gene motifs, overexpression of the C-terminus-encoding half of the cloned gene made cells more resistant to toxin than overexpression of the intact gene. In addition, overexpression of *HKR1* increases the β-glucan content in the cell wall without affecting in vitro β-glucan synthase activity. Understanding the relationship between *ETG1*, *KNR4*, and *HKR1* will further our understanding of β-glucan synthesis, and direct demonstration of the role of the *etg1-1*-encoded protein in glucan synthesis awaits cloning and disruption of *ETG1*.

ACKNOWLEDGMENTS

We thank J. Balkovec, M. Hammond, J. Williamson, and S. Parent for critical reading of the manuscript. We also thank F. A. Bouffard and J. Dropinski for providing L-731,373 and L-733,560 and R. Schwartz for pneumocandin B_0. We are indebted to R. Thompson and R. Hadju for HPLC analysis of L-733,560, J. Milligan for assistance with chitin and squalene synthesis assays, and J. Williamson for thin-layer chromatography analysis of UDP-glucose.

REFERENCES

1. **Alberts, A., J. Chen, G. Kuron, V. Hunt, J. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, E. Harris, A. Patchett, R. Monaghan, S. Currie, E. Stapley, G. Albers-Schonberg, O. Hensens, J. Hirschfield, K. Hoogsteen, J. Liesch, and J. Springer.** 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. USA **77**:3957–3961.
2. **Awald, P., M. Zugel, C. Monks, D. Frost, and C. P. Selitrennikoff.** 1993. Purification of 1,3-β-glucan synthase from *Neurospora crassa* by product entrapment. Exp. Mycol. **17**:130–141.
3. **Balzi, E., W. Chen, S. Ulaszewski, E. Capieaux, and A. Goffeau.** 1987. The multidrug resistance gene *PDR1* from *Saccharomyces cerevisiae*. J. Biol. Chem. **262**:16871–16879.
4. **Bartizal, K., G. Abruzzo, C. Trainor, D. Krupa, K. Nollstadt, D. Schmatz, R. Schwartz, M. Hammond, J. Balkovec, and F. Vanmiddlesworth.** 1992. In vitro antifungal activities and in vivo efficacies of 1,3-β-D-glucan synthesis inhibitors L-671,329, L-646,991, tetrahydroechinocandin B, and L-687,781, a papulacandin. Antimicrob. Agents Chemother. **36**:1648–1657.
5. **Beaulieu, D., J. Tang, D. J. Zeckner, and T. R. Parr.** 1993. Correlation of cilofungin *in vivo* efficacy with its activity against *Aspergillus fumigatus* (1,3)-β-D-glucan synthase. FEMS Microbiol. Lett. **108**:133–138.
6. **Beauvais, A., R. Drake, K. Ng, M. Diaquin, and J. P. Latgé.** 1993. Characterization of the 1,3-β-glucan synthase of *Aspergillus fumigatus*. J. Gen. Microbiol. **139**:3071–3078.
7. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**:241–250.
8. **Bergstrom, J. D., M. M. Kurtz, D. J. Rew, A. M. Amend, J. D. Karkas, R. G. Bostedor, V. S. Bansal, C. Dufresne, F. L. Vanmiddlesworth, O. D. Hensens, J. M. Liesch, D. L. Zink, K. E. Wilson, J. Onishi, J. A. Milligan, G. Bills, L. Kaplan, M. N. Omstead, R. G. Jenkins, L. Huang, M. S. Meinz, L. Quinn, R. W. Burg, Y. L. Kong, S. Mochales, M. M. S. Mochales, I. Martin, F. Pelaez, M. T. Diez, and A. W. Alberts.** 1993. Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc. Natl. Acad. Sci. USA **90**:80–84.
9. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
10. **Boone, C., S. S. Sommer, A. Hensel, and H. Bussey.** 1990. Yeast *KRE* genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. **110**:1833–1843.
11. **Borgia, P. T., and C. L. Dodge.** 1992. Characterization of *Aspergillus nidulans* mutants deficient in cell wall chitin or glucan. J. Bacteriol. **174**:377–383.
12. **Bouffard, F. A., R. A. Zambias, J. F. Dropinski, J. M. Balkovec, M. L. Hammond, G. K. Abruzzo, K. F. Bartizal, J. A. Marrinan, M. B. Kurtz, D. C. McFadden, K. H. Nollstadt, M. A. Powles, and D. M. Schmatz.** 1994. Synthesis and antifungal activity of novel

cationic pneumocandin B_0 derivatives. J. Med. Chem. **37:**222–225.
13. **Bulawa, C. E.** 1993. Genetics and molecular biology of chitin synthesis in fungi. Annu. Rev. Microbiol. **47:**505–534.
14. **Bussey, H., D. Saville, K. Hutchins, and R. G. E. Palfree.** 1979. Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. J. Bacteriol. **140:**888–892.
15. **Cabib, E., B. Bowers, A. Sburlati, and S. J. Silverman.** 1988. Fungal cell wall synthesis: the construction of a biological structure. Microbiol. Sci. **5:**370–375.
16. **Cabib, E., and M. S. Kang.** 1987. Fungal 1,3-β-glucan synthase. Methods Enzymol. **138:**637–642.
17. **Cabib, E., M. S. Kang, and J. Au-Young.** 1987. Chitin synthase from *Saccharomyces cerevisiae*. Methods Enzymol. **138:**643–649.
18. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28:**145–154.
19. **Cassone, A., R. E. Mason, and D. Kerridge.** 1981. Lysis of growing yeast-form cells of *Candida albicans* by echinocandin: a cytological study. Sabouraudia **19:**97–110.
20. **DeMora, J. F., R. Gil, R. Sentandreu, and E. Herrero.** 1991. Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to aculeacin A. Antimicrob. Agents Chemother. **35:**2596–2601.
21. **Douglas, C., J. Marrinan, J. Curotto, J. Onishi, and M. Kurtz.** 1992. Activity of a new echinocandin, L-688,786, against filamentous fungi, abstr. A-41, p. 7. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
22. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
23. **Durán, A., C. Castro, T. deCos, M. Díaz, C. Godoy, and B. Santos.** 1992. Characterization of genes involved in yeast cell wall synthesis: an attempt to find novel targets for antifungal agents, p. 221–232. *In* T. G. Villa and J. Abalde (ed.), Profiles in biotechnology. Servicio de Publicacions de la, Universidad de Salamanca e Intercambio Ciontifico, Santiago de Compostela, Salamanca, Spain.
24. **Hadju, R., R. Thompson, K. White, B. Stark Murphy, and H. Kropp.** 1993. Comparative pharmacokinetics of three water-soluble analogues of the lipopeptide antifungal compound L-688,786 in mice and rhesus monkeys, abstr. 357, p. 185. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C.
25. **Hong, Z., P. Mann, N. H. Brown, L. E. Tran, K. J. Shaw, R. S. Hare, and B. DiDomenico.** 1994. Cloning and characterization of *KNR4*, a yeast gene involved in (1,3)-β-glucan synthesis. Mol. Cell. Biol. **14:**1017–1025.
26. **Horn, W. S., J. L. Smith, G. F. Bills, S. L. Raghoobar, G. L. Helms, M. B. Kurtz, J. A. Marrinan, B. R. Frommer, R. A. Thornton, and S. M. Mandala.** 1992. Sphingofungins E and F: novel serine palmitoyltransferase inhibitors from *Paecilomyces variotii*. J. Antibiot. **45:**1692–1696.
27. **Hutchins, K., and H. Bussey.** 1983. Cell wall receptor for yeast killer toxin: involvement of (1→6)-β-D glucan. J. Bacteriol. **154:**161–169.
28. **Johnson, D. C., and W. R. LaCourse.** 1990. Liquid chromatography with pulsed electrochemical detection at gold and platinum electrodes. Anal. Chem. **62:**589A–597A.
29. **Kang, M. S., and E. Cabib.** 1986. Regulation of fungal cell wall growth: a guanine nucleotide-binding, proteinaceous component required for activity of (1-3)-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **83:**5808–5812.
30. **Kasahara, S., H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi.** 1994. Cloning of the *Saccharomyces cerevisiae* gene whose overexpression overcomes the effects of HM-1 killer toxin, which inhibits β-glucan synthesis. J. Bacteriol. **176:**1488–1499.
31. **Keller-Scheirlein, W., and J. Widmer.** 1976. Stoffwechselprodukte von Mikroorganisem. Uber die aromatische Aminosaure des Echinocandin B: 3,4-Dihydroxyhomotyrosin. Helv. Chim. Acta **59:**2021–2031.
32. **Kurtz, M. B., I. B. Heath, J. Marrinan, S. Dreikorn, J. Onishi, and C. Douglas.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activity against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38:**1480–1489.
33. **Kurtz, M. B., et al.** Submitted for publication.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Sommer, and H. Bussey.** 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1→6)-β-D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10:**3013–3019.
36. **Mehta, R. J., J. M. Boyer, and C. H. Nash.** 1984. Aculeacin resistant mutants of *Candida albicans*: alterations in cellular lipids. Microbios Lett. **27:**25–29.
37. **Mizuno, K., A. Yagi, S. Satoi, M. Takada, M. Hayashi, K. Asano, and T. Matsuda.** 1977. Studies on aculeacin. I. Isolation and characterization of aculeacin A. J. Antibiot. **30:**297–302.
38. **Nollstadt, K., and D. Schmatz.** Unpublished results.
39. **Odds, F. C., S. L. Cheesman, and A. B. Abbott.** 1986. Antifungal effects of fluconazole (UK49858), a new triazole antifungal, *in vitro*. J. Antimicrob. Chemother. **18:**473–478.
40. **Orlean, P. A. B.** 1982. (1,3)-β-D-Glucan synthase from budding and filamentous cultures of the dimorphic fungus *Candida albicans*. Eur. J. Biochem. **127:**397–403.
41. **Petranyi, G., N. S. Ryder, and A. Stutz.** 1984. Allylamine derivatives: a new class of synthetic antifungal agents inhibiting squalene epoxidase. Science **224:**1239–1241.
42. **Quigley, D. R., and C. P. Selitrennikoff.** 1984. β(1-3) Glucan synthase activity of *Neurospora crassa*: stabilization and partial characterization. Exp. Mycol. **8:**202–214.
43. **Ribas, J. C., M. Diaz, A. Duran, and P. Perez.** 1991. Isolation and characterization of *Schizosaccharomyces pombe* mutants defective in cell wall (1-3)β-D-glucan. J. Bacteriol. **173:**3456–3462.
44. **Roemer, T., and H. Bussey.** 1991. Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. Proc. Natl. Acad. Sci. USA **88:**11295–11299.
45. **Roncero, C., M. H. Valdivieso, J. C. Ribas, and A. Durán.** 1988. Effect of Calcofluor White on chitin synthases from *Saccharomyces cerevisiae*. J. Bacteriol. **170:**1945–1949.
46. **Roncero, C., M. H. Valdivieso, J. C. Ribas, and A. Durán.** 1988. Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to Calcofluor White. J. Bacteriol. **170:**1950–1954.
47. **Sawistowska-Schroder, E. T., D. Kerridge, and H. Perry.** 1984. Echinocandin inhibition of 1,3-β-D-glucan synthase from *Candida albicans*. FEBS Lett. **173:**134–138.
48. **Schmatz, D. M., M. A. Romancheck, L. A. Pittarelli, R. E. Schwartz, R. A. Fromtling, K. H. Nollstadt, F. L. Vanmiddlesworth, K. E. Wilson, and M. J. Turner.** 1990. Treatment of *Pneumocystis carinii* pneumonia with 1,3-β-glucan synthesis inhibitors. Proc. Natl. Acad. Sci. USA **87:**5950–5954.
49. **Schwartz, R. E., D. F. Sesin, H. Joshua, K. E. Wilson, A. Kempf, K. E. Golken, D. Kuehner, P. Gaillot, C. Gleason, R. White, E. Inamine, G. Bills, P. Salmon, and L. Zitano.** 1992. Pneumocandins from *Zalerion arboricola*. I. Discovery and isolation. J. Antibiot. **45:**1853–1866.
50. **Segel, I. H.** 1976. Biochemical calculations. John Wiley & Sons, New York.
51. **Selitrennikoff, C. P.** 1983. Use of a temperature-sensitive, protoplast-forming *Neurospora crassa* strain for the detection of antifungal antibiotics. Antimicrob. Agents Chemother. **23:**757–765.
52. **Shematek, E. M., J. A. Braatz, and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. I. Preparation and properties of β-(1,3) glucan synthetase. J. Biol. Chem. **255:**888–894.
53. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1983. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
55. **Szaniszlo, P. J., M. S. Kang, and E. Cabib.** 1985. Stimulation of β(1→3)glucan synthetase of various fungi by nucleoside triphos-

phates: generalized regulatory mechanism for cell wall biosynthesis. J. Bacteriol. **161**:1188–1194.

56. **Takahashi, F., and T. Harada.** 1986. Structure of curdlan that is resistant to (1-3)β-D-glucanase. Carbohydr. Polym. **6**:407–421.
57. **Tang, J., D. Beaulieu, and T. R. Parr, Jr.** 1992. The solubilized and membrane bound *Aspergillus fumigatus* (1,3)-β-D-glucan synthase: cilofungin inhibition, kinetics, and comparison to the *Candida albicans* (1,3)-β-D-glucan synthase, abstr. 112, p. 129. Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C.
58. **Tang, J., and T. Parr, Jr.** 1991. W-1 solubilization and kinetics of inhibition by cilofungin of *Candida albicans* (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **35**:99–103.
59. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. Cell **56**:619–630.
60. **Valdivieso, M. H., P. C. Mol, J. A. Shaw, E. Cabib, and A. Duran.** 1991. *CAL1*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. J. Cell Biol. **114**:101–109.
61. **Vanmiddlesworth, F., M. N. Omstead, D. Schmatz, K. Bartizal, R. Fromtling, and G. Bills.** 1991. L-687,781, a new member of the papulacandin family of β-1,3-D-glucan synthesis inhibitors. I. Fermentation, isolation, and biological activity. J. Antibiot. **44**:45–51.
62. **Yamaguchi, H., T. Hiratani, K. Iwata, and Y. Yamamoto.** 1982. Studies on the mechanism of antifungal action of aculeacin A. J. Antibiot. **35**:210–219.