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# Nikkomycin Z Supersensitivity of an Echinocandin-Resistant Mutant of *Saccharomyces cerevisiae*

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**Echinocandins and nikkomycins are antibiotics that inhibit the synthesis of the essential cell wall polysaccharide polymers 1,3-β-glucan and chitin, respectively. Some 40 echinocandin-resistant *Saccharomyces cerevisiae* mutants were isolated and assigned to five complementation groups. Four complementation groups contained mutants with 38 recessive mutations. The fifth complementation group comprised mutants with one dominant mutation, *etg1-3* (strain MS10), and one semidominant mutation, *etg1-4* (strain MS14). MS10 and MS14 are resistant to the semisynthetic pneumocandin B, L-733,560, and to aculeacin A but not to papulacandin. In addition, microsomal membranes of both mutant strains contain 1,3-β-glucan synthase activity that is resistant to L-733,560 but not to papulacandin. Furthermore, MS14 is also supersensitive to nikkomycin Z. The echinocandin resistance and the nikkomycin Z supersensitivity of MS14 cosegregated in genetic crosses. The wild-type gene (designated *ETG1* [C. Douglas, J. A. Marrinan, and M. B. Kurtz, J. Bacteriol. 176:5686–5696, 1994, and C. Douglas, F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. Dahl, P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. Mandala, B. R. Frommer, and M. B. Kurtz, Proc. Natl. Acad. Sci. USA, in press]) was isolated from a genomic library in the plasmid YCp50 by functional complementation of the nikkomycin Z supersensitivity phenotype. The cloned DNA also partially complements the echinocandin resistance phenotype, indicating that the two phenotypes are due to single mutations. The existence of a single mutation, in MS14, simultaneously affecting sensitivity to a glucan synthase inhibitor and a chitin synthase inhibitor implies a possible interaction between the two polymers at the cell surface.**

Cell walls are vital for the existence of fungi and are absent from mammalian cells. Two polysaccharide polymers, chitin and 1,3-β-glucan, are essential components of fungal cell walls. Therefore, antibiotics that interfere with the synthesis of these polymers are potentially useful in mycosis therapy. Polysaccharides have been estimated to account for as much as 80 to 90% of the *Saccharomyces cerevisiae* cell wall. The major cell wall polymers are glucan and mannan, and small amounts of chitin are also present (21). Glucans account for 30 to 60% of the cell wall (20, 22). These have been divided into three types: the alkali-soluble and the alkali- and acid-insoluble glucans that are rich in 1,3-β linkages and a less abundant form of glucan that is alkali insoluble and acid soluble and contains mainly 1,6-β linkages.

Glucan synthases are likely targets for several antifungal drugs including echinocandin B (24, 52), pneumocandin B (4, 46), aculeacin A (36, 58), and papulacandin (1, 54). The first three antibiotics belong to a class of antifungal lipopeptides collectively known as echinocandins. The echinocandins are noncompetitive inhibitors of 1,3-β-glucan synthase of fungi (4, 15, 53).

The nikkomycins are antibiotics which competitively inhibit chitin synthase of fungi and insects (8, 13, 16, 19). Only very high concentrations of nikkomycins inhibit cell growth of *Candida albicans* and *S. cerevisiae* (34). This may explain, at least in part, the lack of genetic analysis of nikkomycin resistance in yeasts. Efficiency of transport through the peptide transport systems and susceptibility to degradation are two important factors that influence the antifungal activities of the nikkomycins (16, 28, 59).

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There are several reports of mutants with defects in cell wall and 1,3-β-glucan synthesis (3, 41, 42), but in none of these mutants is the 1,3-β-glucan synthase resistant to glucan synthase inhibitors. A new semisynthetic pneumocandin B compound, L-733,560, is a potent antifungal agent (7) and, compared with other echinocandins, has enhanced activity against *S. cerevisiae*. The availability of this compound afforded the opportunity to isolate and characterize mutants resistant to the drug (17, 18). Importantly, the glucan synthase activity in extracts of these mutants is also resistant to the drug, like the previously described *etg1-1* (for echinocandin target gene) mutant (17, 18). Unexpectedly, one of the mutants is also concomitantly supersensitive to the chitin synthase inhibitor nikkomycin Z. This finding supports earlier evidence (6, 55–57) of a possible interaction between chitin and 1,3-β-glucan.

## MATERIALS AND METHODS

**Strains, media, antibiotics, and genetic methods.** The yeast strains used in this study were derivatives of strain X2180-1A and GG100-14D and are listed in Table 1. YPAD solid medium contained 1% Bacto Yeast Extract, 2% Bacto Peptone, 2% dextrose, and 0.003% adenine sulfate. Synthetic dextrose (SD) medium contained 0.67% Bacto Yeast Nitrogen Base without amino acids (Difco), 2% dextrose, and 2% Bacto Agar (Difco). Synthetic complete (SC) medium is SD medium supplemented (per liter) with 20 mg each of adenine, histidine, tryptophan, and uracil; 60 mg of leucine; and 30 mg of lysine. Sporulation medium contained 2% Bacto Agar (Difco) and 0.3% potassium acetate. Standard methods were used for genetic analysis (50).

**Chemicals.** Nikkomycin Z and polyoxin D were purchased from CalBiochem. Amphotericin B, cerulenin, calcofluor white, cycloheximide, filipin, nystatin, tunicamycin, and trifluoperazine were purchased from Sigma. Aculeacin was from Toyo Jozo, and fluconazole and ketoconazole were from Janssen. L-733,560 and L-731,373 (semisynthetic pneumocandins [4]) and L-687,781 (dihydropapulacandin [54]) were from R. Schartz and J. Balkovec, Merck & Co.

**Isolation of spontaneous mutants resistant to L-733,560.** The wild-type yeast strain X2180-1A was grown to stationary phase in SD minimal medium. Approximately $1 \times 10^6$ to $3 \times 10^6$ cells were spread on SD plates containing 7.5, 15, or 45 μM L-733,560. Following incubation at 28°C for 4 days, echinocandin-resistant colonies appeared at a frequency of $1 \times 10^{-6}$ to $3 \times 10^{-6}$.

**DNA manipulation and transformation.** Standard techniques for DNA ma-

TABLE 1. Strains used in this study

| Strain | Relevant properties[a] | Source |
|---|---|---|
| X2180-1A | *MAT***a***ETG1* Ech$^S$ NZ$^R$ | C. Ballou |
| GG100-14D | *MAT*α *ura3-52 his3 trp1 ETG1* Ech$^S$ NZ$^R$ | K. Bostian |
| MS10 | *MAT***a** *etg1-3* Ech$^R$ NZ$^R$ | This work |
| MS14 | *MAT***a** *etg1-4* Ech$^R$ NZ$^S$ | This work |
| D1.22C | *MAT***a** *etg1-4 ura3-52* Ech$^R$ NZ$^S$ | This work |
| D11.8D | *MAT***a** *etg1-3 his3 trp1* | This work |
| D11.12B | *MAT*α *etg1-3 ura3-52* | This work |
| D11.3A | *MAT***a** *etg1-3 ura3-52* | This work |
| D1.6D | *MAT*α *etg1-4 ura3-52 his3* | This work |
| D1.17A | *MAT*α *etg1-4 ura3-52* | This work |
| D1.17B | *MAT***a** *etg1-4 his3 trp1* | This work |
| D1 | *MAT***a**/*MAT*α *ETG1*/*etg1-4* (GG100-14D × MS14) | This work |
| D11 | *MAT***a**/*MAT*α *ETG1-3*/*etg1-3* (GG100-14D × MS10) | This work |
| D151 | *MAT***a**/*MAT*α*etg1-4*/*etg1-4* (D1.17A × D1.17B) | This work |
| D152 | *MAT***a**/*MAT*α*etg1-3*/*etg1-3* (D11.8D × D11.12B) | This work |
| D153 | *MAT***a**/*MAT*α*etg1-4*/*etg1-3* (D1.6D × D11.3A) | This work |
| D154 | *MAT***a**/*MAT*α*ETG1*/*ETG1* (GG100-14D × X2180-1A) | This work |

[a] Abbreviations: Ech, echinocandin; NZ, nikkomycin Z; S, sensitive; R, resistant.

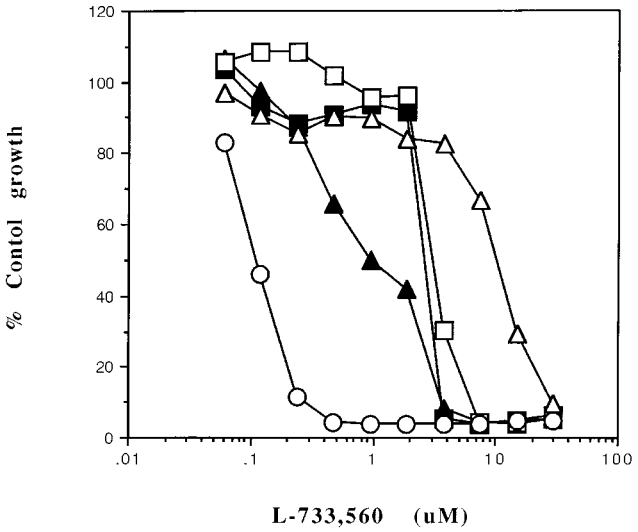



FIG. 1. Relative echinocandin resistance of the mutant haploids and the heterozygous diploids. Haploid cells from the MS10 and MS14 strains and diploid cells from the heterozygous diploids obtained by mating of each mutant to the wild-type strain GG100-14D were tested for resistance to L-733,560 by the broth microdilution method. ○, X2180-1A; △, D1.22C *etg1-4*; □, D11.3A *etg1-3*; ■, D11 *ETG1-3*/*etg1-3*; ▲, D1 *ETG1-4*/*etg1-4*.

nipulation were utilized (45). *Escherichia coli* DH5α (25) was used as the host in bacterial transformation. Yeast transformation with the DNA libraries was performed by electroporation (2). All other yeast transformations with different plasmid subclones were by the alkali cation method (31). Plasmid DNA was prepared from *E. coli* by the alkaline lysis method (45). Plasmids were isolated from yeast cells for transformation into *E. coli* as previously described (29). The wild-type gene complementing the mutation from MS14 was isolated from a yeast genomic library constructed in the centromeric shuttle vector YCp50 (44).

**Diffusion assay.** Approximately $3 \times 10^6$ cells of cultures grown to logarithmic phase were added to 5 ml of molten SC medium that was precooled to 50°C and poured into petri dishes (60 by 50 mm). Following solidification of the medium, sterile 5-mm disks containing known amounts of antibiotics were placed onto the plates, which were then incubated at 30°C for 24 h.

**Liquid broth microdilution assay.** To quantify drug resistance, 75 μl of a suspension containing $6 \times 10^5$ cells per ml was inoculated into an equal volume of SC broth containing 1:2 serial dilutions of L-733,560 or nikkomycin Z in columns 3 to 12 of 96-well microtiter plates. As a control, column 2 contained cells in SC medium without drug. The plates were then incubated at 28°C for 24 to 48 h, and growth in the presence or absence of the drug was measured by reading the turbidity at 600 nm.

**Glucan synthase assay.** Membrane extracts were prepared from mutant and wild-type cells grown to logarithmic phase as previously described (32). After homogenization with glass beads, unbroken cells and debris were removed by low-speed centrifugation (1,000 × *g* for 5 min). The supernatant fluids were centrifuged at 100,000 × *g* for 60 min, and the pellets were washed with 2.5 ml (per g of wet cells) of buffer containing 0.05 M potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride. The washed pellet was resuspended in the same buffer containing 5% glycerol. This particulate protein extract served as the source for both 1,3-β-glucan synthase and the chitin synthases utilized in the enzymatic assays. Protein concentrations were determined by using a bicinchoninic acid protein assay reagent kit, available commercially (Pierce Corp.), and utilizing bovine serum albumin (BSA) (Pierce Corp.) as a standard according to the manufacturer's protocols. The 1,3-β-glucan synthase reactions were performed as previously described (10, 47). Briefly, an 80-μl reaction volume contained 125 mM Tris · HCl (pH 7), 0.25 mM dithiothreitol, 30 mM KF, 0.3 M glycerol, 0.23% BSA, 0.125 mM phenylmethylsulfonyl fluoride, 2 mM UDP-glucose, 10 μM GTPγS, 0.1 nmol of UDP-[$^3$H]glucose (4.5 Ci/mmol; Amersham), plus 25 μg of membrane protein extract. The reactions were performed in the presence of 0.0, 0.1, 0.5, 5, 25, and 50 μM L-733,560 for dose titration of the drug. Following incubation at 25°C for 150 min, the [$^3$H]glucose incorporated into trichloroacetic acid-insoluble material was collected onto glass fiber filters (102 by 258 mm) and measured by using a betaplate liquid scintillation counter (series 2800 harvester; Cambridge Technologies Inc.) at 25% efficiency. The product of such reactions was verified by solubilization by laminarinase (Sigma catalog no. L9259) but not by α-amylase (Sigma catalog no. A2643).

**Chitin synthase assay.** A previously described chitin synthase assay (33) was used. Approximately 125 μg of the membrane protein extracts described above was trypsin activated and used to catalyze chitin synthase reactions. A reaction volume of 100 μl contained 50 mM Tris HCl (pH 7.5), 40 mM MgCl, 32 mM *N*-acetylglucosamine (GlcNAc), 1 mM UDP-*N*-acetyl-[$^{14}$C]glucosamine ($4 \times 10^5$ cpm/μmol), and 0.8 mg of digitonin per ml. The reactions were performed in the presence of 0.0, 0.0625, 0.25, 1, and 4 μg of nikkomycin Z per ml. After 30 min of incubation at 30°C, the reaction products were precipitated with 10% trichloroacetic acid and collected onto Whatman glass microfiber GF/A discs, after which counting of the incorporated [$^{14}$C]GlcNAc was performed.

## RESULTS

**Isolation of echinocandin-resistant mutants.** To isolate mutants with mutations in genes involved in biosynthesis of 1,3-β-glucan, we utilized the 1,3-β-glucan synthesis inhibitor L-733,560 to identify resistant mutants of the wild-type sensitive strain, X2180-1A. Some 40 spontaneous mutants capable of colony formation in the presence of 7.5 μM L-733,560 were isolated as described in Materials and Methods. The mutants exhibited different levels of resistance, ranging from a 5- to a 50-fold increase over the wild-type level.

**Genetic analysis of the echinocandin-resistant mutants.** L-733,560-resistant mutants were crossed nonselectively to the wild-type sensitive strain GG100-14D, and isolates were tested for their ability to sporulate. The diploids D1 and D11 (Table 1) were tested for drug resistance by replication on plates containing SC medium supplemented with 7.5 μM L-733,560. Following growth at 28°C for 2 days, sensitivity to the drug was scored. By this analysis, the mutations in MS10 and MS14 were dominant, while the mutations in the 38 remaining mutants were recessive. The two mutant strains MS10 and MS14 were chosen for further analysis because they exhibited a dominant phenotype and showed the highest level of drug resistance.

To determine the levels of resistance of MS10 and MS14, a drug titration assay was performed by using mutant haploids and heterozygous diploids. The results depicted in Fig. 1 show a 40- to 50-fold increase in resistance of the haploid mutants over the haploid parental wild-type level. While the drug resistance phenotype of MS10 is dominant, the mutation in MS14 is semidominant (Fig. 1).

Genetic analyses, summarized below, indicate that the mu-

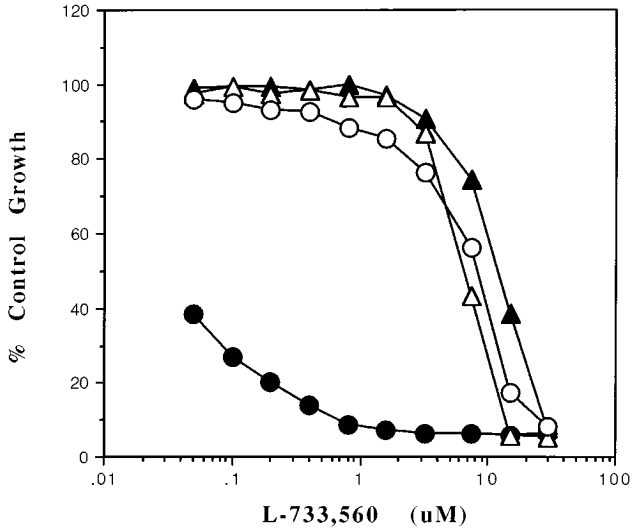



FIG. 2. Relative L-733,560 resistance of homozygous and heterozygous diploids. Diploid cells were tested for resistance by the broth microdilution assay. ▲, D151 *etg1-4/etg1-4*; △, D152 *etg1-3/etg1-3*; ○, D153 *etg1-3/etg1-4*; ●, D154 *ETG1/ETG1*.

tations in MS10 and MS14 are alleles of a single gene which we have designated *ETG1-3* and *ETG1-4*, respectively. Analysis of 23 tetrads of D1 (*ETG1-4/etg1-4*) and 17 tetrads of D11 (*ETG1-3/etg1-3*) showed a 2:2 segregation of echinocandin resistance versus sensitivity. The other four heterozygous markers present in these diploids (*ura3-52*, *his3*, *trp1*, and the mating type marker) also segregated 2:2. These results indicate that the drug resistance phenotypes of MS10 and MS14 are due to a single mutation in each. Two drug-resistant meiotic segregants of D1 (strain D1.6D) and D11 (strain D11.3A) were crossed to yield a heterozygous diploid, D153 (*etg1-3/etg1-4*). Two homozygous diploids, D151 (*etg1-4/etg1-4*) and D152 (*etg1-3/etg1-3*), were also constructed by mating drug-resistant meiotic segregants of the same parent. The D153 heterozygous diploid exhibited a level of L-733,560 resistance similar to that of the D151 and D152 homozygous diploids (Fig. 2).

The heterozygous diploid, D153, was allowed to sporulate. Among the nine tetrads which were dissected, seven gave four viable spores. For each of these, all spore colonies were resistant to L-733,560. The other markers (*ura3-52*, *his3*, and the mating type marker) segregated 2:2. These results indicated that the drug resistance mutation derived from MS10 and that from MS14 are tightly linked and probably allelic. This gene was designated *ETG1* (17, 18), and the mutations derived from MS10 and MS14 are referred to as *etg1-3* and *etg1-4*, respectively.

**Characterization of the *etg1-3* and the *etg1-4* mutants.** Strains MS10 and MS14 exhibited no multiple drug resistance when tested against several antifungal inhibitors affecting cell wall, membrane, sterol, and protein synthesis (Table 2). A characteristic feature of the MS14 strain is its supersensitivity to nikkomycin Z in addition to its resistance to L-733,560 (Table 2). In contrast to the semidominant L-733,560 resistance of the MS14 strain, the supersensitivity to nikkomycin Z is recessive (Fig. 3). The mutant strains, MS10 and MS14, did not show any defect in growth rate or in mating phenotype when tested in YPAD medium under standard conditions.

**Cosegregation of echinocandin resistance with nikkomycin supersensitivity.** To determine whether the nikkomycin Z supersensitivity was due to a single gene mutation, we analyzed the drug resistance phenotypes of the four meiotic segregants of 12 tetrads of strain D1 (*ETG1-4/etg1-4*). The results of this analysis demonstrated a 2:2 segregation pattern for the nikkomycin supersensitivity phenotype. In addition, echinocandin resistance cosegregated with nikkomycin Z supersensitivity in all 12 tetrads tested, suggesting that a single mutation may lead to both phenotypes (Fig. 4).

TABLE 2. Effects of antifungal antibiotics on MS10 and MS14

| Target | Compound | Amt of drug (μg/disc) | Zone of inhibition (mm) for: | | |
|---|---|---|---|---|---|
| | | | X2180-1A | MS10 | MS14 |
| 1,3-β-Glucan synthesis | L-733,560 | 2 | 20 | 8 | 8 |
| | L-731,373 | 50 | 20 | 0 | 0 |
| | L-687,781 | 20 | 10 | 10 | 17 |
| | Aculeacin A | 50 | 15 | 0 | 0 |
| Chitin synthesis | Nikkomycin Z | 15 | 0 | 0 | 34 |
| | Polyoxin D | 20 | 0 | 0 | 0 |
| Chitin assembly | Calcofluor white | 50 | 0 | 0 | 0 |
| Glycosylation | Tunicamycin | 20 | 21 | 21 | 22 |
| Membrane | Amphotericin B | 20 | 18 | 16 | 18 |
| | Filipin | 20 | 24 | 21 | 23 |
| | Nystatin | 20 | 30 | 28 | 31 |
| | Monensin | 40 | 0 | 0 | 0 |
| | Valinomycin | 20 | 0 | 0 | 0 |
| Sterol synthesis | Cerulenin | 10 | 43 | 42 | 41 |
| | Fluconazole | 5 | 18 | 19 | 21 |
| | Ketoconazole | 20 | 24 | 23 | 25 |
| Protein synthesis | Cycloheximide | 2.5 | 45 | 45 | 43 |
| Secretion | Chloroquine | 100 | 0 | 0 | 0 |
| | Trifluoperazine | 100 | 8 | 8 | 8 |

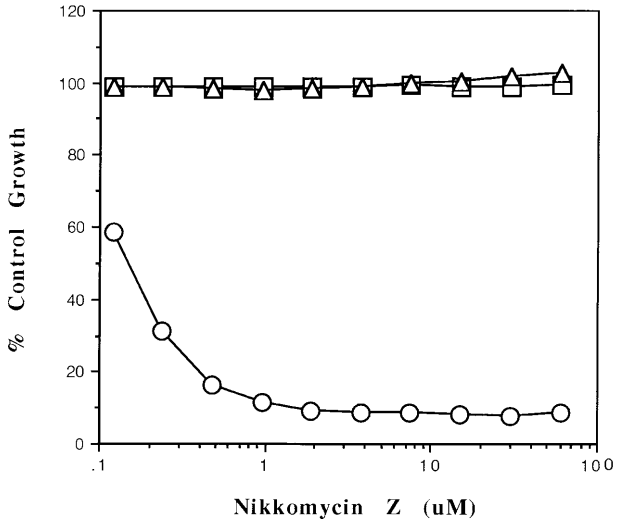



FIG. 3. Supersensitivity of MS14 mutant cells to nikkomycin Z. Cells of the MS14 strain grown to log phase in SC medium were tested for sensitivity to nikkomycin Z by the broth microdilution method. ○, MS14 *etg1-4*; △, X2180-1A *ETG1*; □, D1 *ETG1-4/etg1-4*.

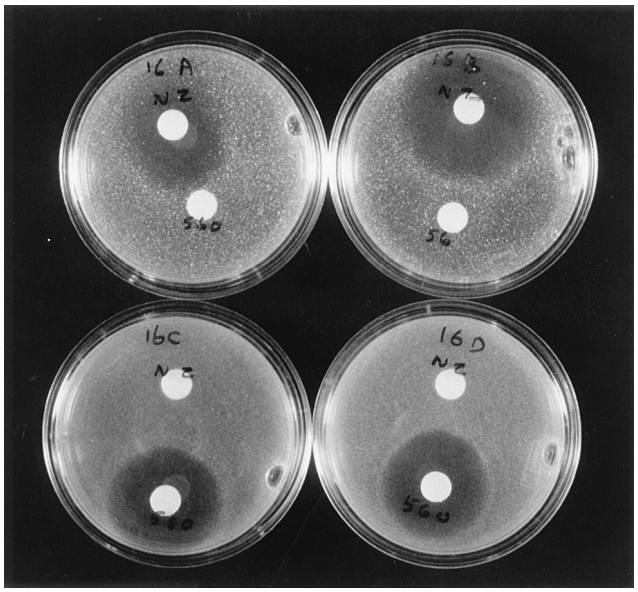



FIG. 4. Cosegregation of the L-733,560 resistance and the nikkomycin Z supersensitivity phenotypes. Approximately $2 \times 10^6$ cells from four meiotic segregants of a tetrad resulting from the diploid D1 (*ETG1-4*/*etg1-4*) were added to 10 ml of SC medium and poured into petri dishes. Filter disks containing 10 μg of either nikkomycin Z (top disk) or L-733-560 (bottom disk) were added to the plates, which were then incubated at 30°C for 2 days.

**1,3-β-Glucan synthase and chitin synthase activities.** Membrane extracts of the wild-type and the mutant strains were tested for their associated 1,3-β-glucan synthase and chitin synthase 1 (Chs1) activities. The sensitivity of the 1,3-β-glucan synthases to L-733,560 and to L-687,781 were determined (Fig. 5A and B). The effect of nikkomycin Z on Chs1 activity was also tested (Fig. 5C). Both the MS10 and MS14 membrane extracts catalyzed synthesis of wild-type levels of 1,3-β-glucan. While the mutant 1,3-β-glucan synthases exhibited resistance to L-733,560, no significant cross-resistance to papulacandin was observed (Table 3). Chs1 activity catalyzed by the mutant membranes is not affected in its sensitivity to nikkomycin Z. The mutant membrane extracts also exhibit a wild-type level of Chs2 and Chs3 activities (data not shown). Table 3 shows the 50% inhibitory concentration of L-733,560 for glucan synthase and that of nikkomycin Z for Chs1.

An important aspect of the 1,3-β-glucan synthases is their activation by GTPγS (32). To study whether this activation is affected in the mutant enzymes, reactions were performed in the presence and absence of GTPγS. Approximately 20-fold stimulation by GTPγS was observed for the wild-type and the mutant enzymes (Fig. 6). Thus, the mutant 1,3-β-glucan synthases are not impaired in stimulation by GTPγS. The glucan synthase specific activity in the mutant membranes approximated that of the wild type.

**Complementation of nikkomycin Z supersensitivity by a plasmid carrying *ETG1*.** The nikkomycin Z supersensitivity was exploited to isolate a plasmid (YCp-ETG1) carrying the

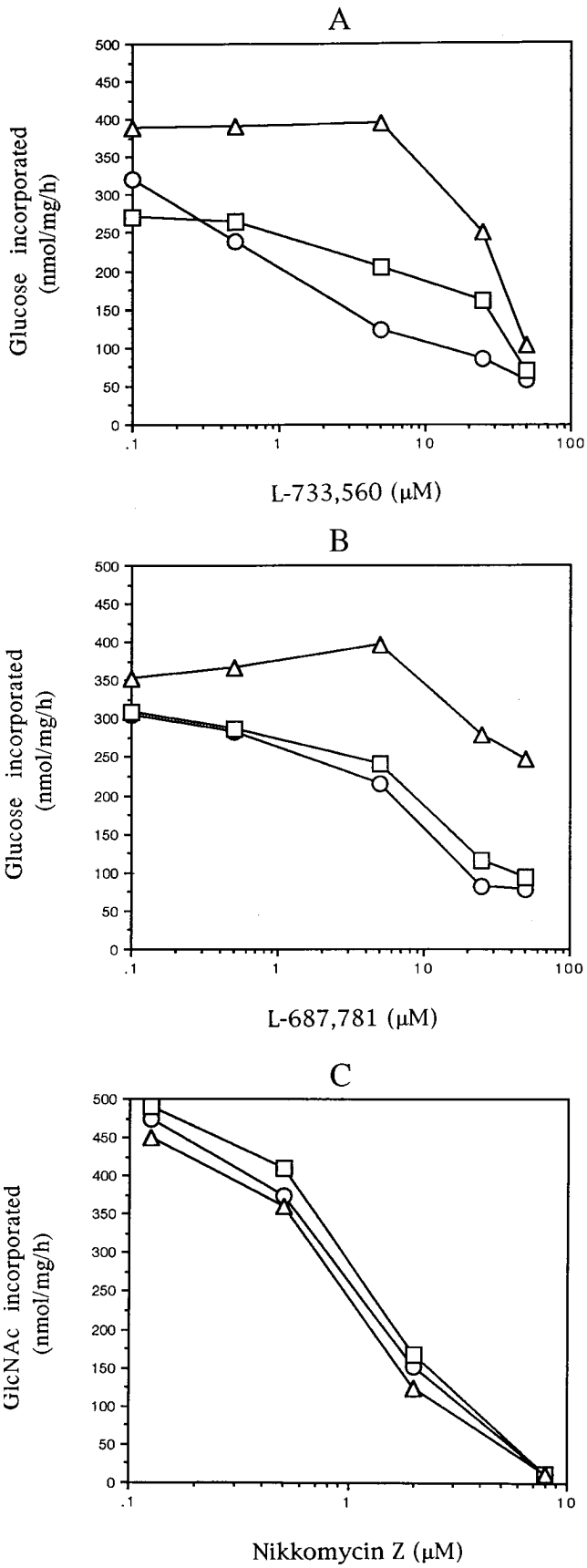



FIG. 5. Effect of L-733,560, L-687,781, and nikkomycin Z on 1,3-β-glucan synthesis and chitin synthesis. Membrane extracts prepared from cells of the wild-type parental strain X2180-1A and of the mutant strains MS10 and MS14 were used to catalyze 1,3-β-glucan synthase reactions (A and B) and chitin synthase reactions (C) utilizing UDP-glucose and UDP-*N*-acetylglucosamine as respective substrates. ○, X2180-1A; □, MS10 mutant; △, MS14 mutant.

TABLE 3. Drug sensitivities of 1,3-β-glucan synthase and chitin synthase

| Microsomal membrane source (strain) | 1,3-β-glucan synthase $IC_{50}$[a] (μM) for | | Nikkomycin Z $IC_{50}$[a] (μM) for chitin synthase |
|---|---|---|---|
| | L-733,560 | L-687,781 | |
| X2180-1A | 2.77 | 8.30 | 1.57 |
| MS10 | 20.23 | 6.80 | 1.60 |
| MS14 | 23.46 | 5.14 | 1.47 |

[a] $IC_{50}$, 50% inhibitory concentration.

*ETG1* gene. The molecular genetic analysis and sequencing of the cloned DNA, a detailed description of which is detailed elsewhere (17), show that the cloned DNA is a single gene. As discussed above, the L-733,560 resistance and the nikkomycin supersensitivity phenotypes cosegregated in a fashion suggesting a single mutation leading to both phenotypes in the MS14 strain. Yeast cells containing the *etg1-4* mutation and the *ura3-52* selectable marker (strain D1.22C) were transformed with YCp-ETG1. The transformants exhibited L-733,560 sensitivity and partial resistance to nikkomycin Z (Fig. 7). Transformants of strain D11.3A (*etg1-3 ura3-52*) containing YCp-ETG1 exhibited partial sensitivity to L-733,560 (Fig. 7).

**Synergism between L-733,560 and nikkomycin Z.** A synergistic interaction between echinocandins and nikkomycins against *S. cerevisiae* is demonstrated in Fig. 8. A heterozygous diploid, strain D1, resulting from a genetic cross between cells of the MS14 strain and the wild-type strain GG100-14D is resistant to both echinocandins and nikkomycin Z. This phenotype was expected since the MS14 cells exhibit a recessive nikkomycin Z supersensitivity and a semidominant L-733,560 resistance (Fig. 1 and 3). The reduced resistance level of the diploid cells to L-733,560 has allowed us to detect a synergistic interaction between L-733,560 and nikkomycin Z in *S. cerevisiae*. In this case, while nikkomycin Z was inactive by itself, it promoted the activity of L-733,560 applied at a subinhibitory concentration.

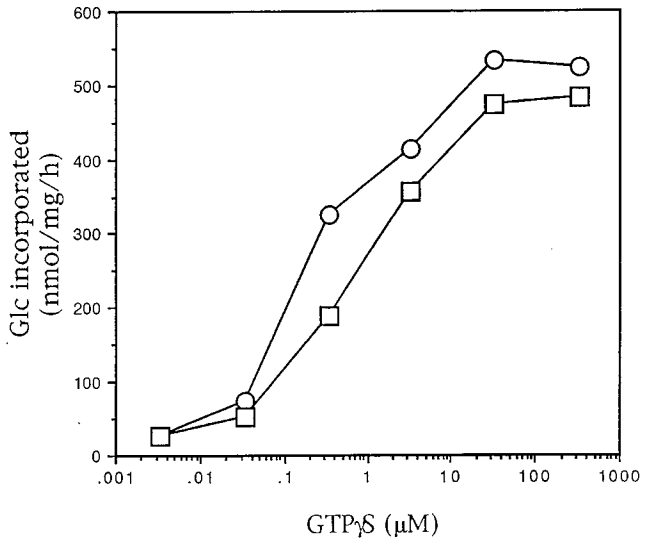



FIG. 6. Effect of GTPγS on 1,3-β-glucan synthase activity. Membrane extracts from wild-type and mutant strains were used to prime 1,3-β-glucan synthase reactions in the presence of increasing concentrations of GTPγS. ○, X2180-1A; □, MS10.

## DISCUSSION

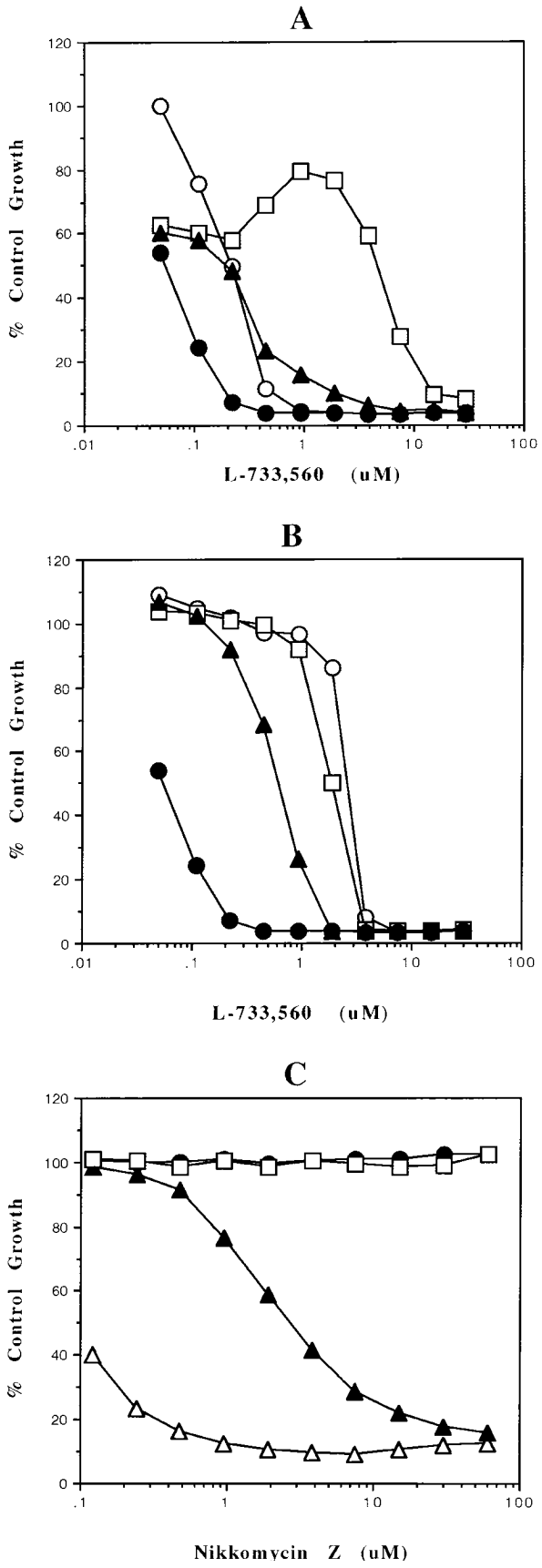
Echinocandins and nikkomycins exert their antifungal effects by interfering with cell wall biosynthesis. While nikkomycins are competitive inhibitors of chitin synthases (8), echinocandins are noncompetitive inhibitors of 1,3-β-glucan synthase (4, 15, 24). The two drugs bring about similar effects, culminating in cessation of cell growth and swelling and, in some cases, bursting of hyphal tips and buds (8, 9, 41, 59). Both chitin and 1,3-β-glucan have potential as chemotherapeutic targets in fungi (23, 28, 59).

The *etg1-3* and *etg1-4* mutations appear to affect 1,3-β-glucan synthase. The mutant cells are specifically resistant to echinocandins. Membrane extracts prepared from the MS10 and MS14 strains show a wild-type level of 1,3-β-glucan synthase. This activity is resistant to L-733,560 but not to papulacandin (Fig. 5; Table 2). These in vitro properties parallel those of the whole cells. An independently isolated yeast mutant, designated *etg1-1*, with an echinocandin-resistant 1,3-β-glucan synthase has recently been described (18), and the complementing gene, *ETG1*, was cloned (17). Genetic analysis indicates that the locus of the cloned DNA is the same as that of *etg1-1* and *etg1-3* (17).

Three chitin synthases (Chs1, Chs2, and Chs3) were described for *S. cerevisiae* cell extracts (7, 8, 48). Chs1 and Chs2 (39, 46), but not Chs3 (7, 39), are zymogens and require partial proteolysis for activation. Different functions were assigned to the three Chs enzymes as follows: Chs2, septum formation and cell division; Chs1, cell wall repair; and Chs3, formation of chitin in the bud ring and at bud emergence and in the cell wall (11, 48, 51). Most of the trypsin-activated in vitro chitin synthesis is due to Chs1 (12). While polyoxin D, nikkomycin X, and nikkomycin Z are competitive inhibitors of Chs1 and Chs2, Chs1 is much more sensitive than Chs2 (8). Because of the nikkomycin Z supersensitivity phenotype of strain MS14, we examined its in vitro chitin synthase activity. Membrane extracts of MS14 stimulated a wild-type level of GlcNAc incorporation into chitin. In addition, the chitin synthesis reactions catalyzed by the mutant and the wild-type membranes show similar sensitivities to nikkomycin Z (Table 3). Therefore, the Chs1 in vitro activity is not affected in the MS14 strain. Neither the Chs2 nor the Chs3 activity is altered in this mutant.

A positive or synergistic interaction is an alternative approach to increasing the therapeutic index of anti-infective drugs. Such positive interactions of antifungal drugs are well documented (5, 14, 30, 38, 40). Several recent reports have described synergy between nikkomycins and azoles (27, 35) and between azoles and papulacandin or echinocandin (26). In the above-mentioned studies, several *Candida* species were utilized and in no case was *S. cerevisiae* used as the test organism. The data presented here demonstrate a synergistic interaction between L-733,560 and nikkomycin Z against *S. cerevisiae*. Nikkomycin Z promoted the activity of L-733,560. This interaction is similar to that reported between rifampin and amphotericin B. Rifampin, which is inactive by itself, potentiated the activity of amphotericin B both in vitro and in vivo (30, 40).

The data presented here lend support to previous observations related to possible interactions between chitin and 1,3-β-glucan. Several reports pointed out that the 1,6-β-glucan that is highly branched with 1,3-branch points is found extensively linked to the wall chitin and is alkali insoluble (37, 55). An analysis of the β-glucan of a yeast mutant defective in Chs3, responsible for synthesis of the cell wall chitin (48), demonstrated that all the β-glucan present was alkali soluble (43). An interpretation of these data is that the alkali-soluble and the



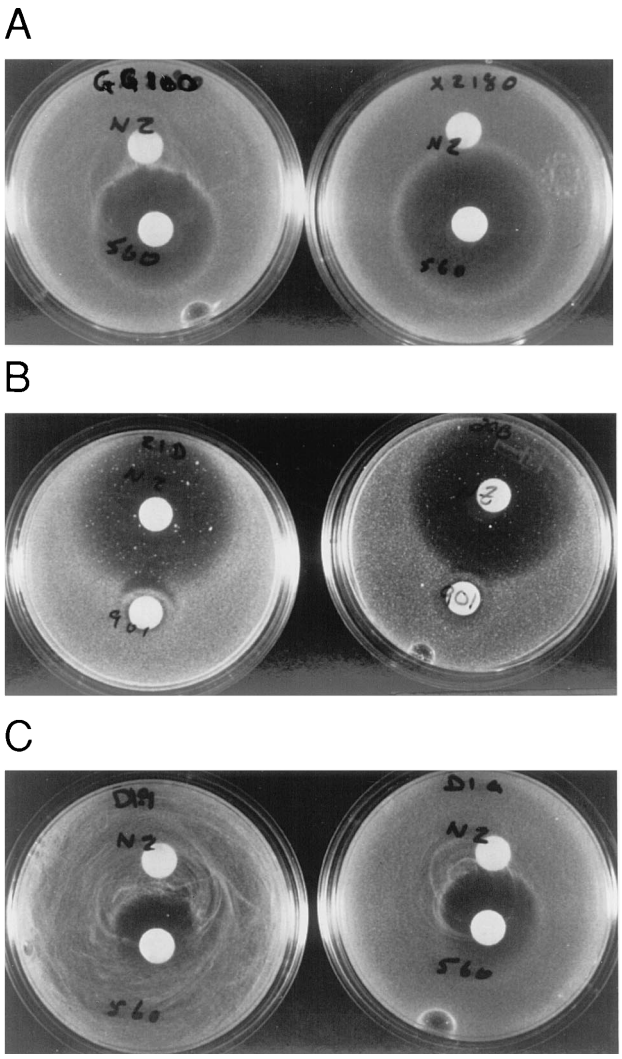




FIG. 8. Synergistic interaction of echinocandin and nikkomycin Z. Approximately $2 \times 10^6$ cells were mixed with 10 ml of SC medium and poured into petri plates as described in the legend to Fig. 3. The disks in the upper part of each plate contained 10 μg of nikkomycin Z. The disks in the lower part of each plate contained 15 μg of L-733-560. (A) Two wild-type strains, GG100-14D (left) and X2180-1A (right). (B) Two mutant strains, MS14 (left) and a meiotic segregant, D1-22C (right). (C) Two isolates of the diploid strain D1 *ETG1-4/etg1-4.*

alkali-insoluble fractions of the yeast β-glucan may differ only in the extent of their cross-linkage to the cell wall chitin (6). Other studies have demonstrated that inhibition of 1,3-β-glucan synthase activity by aculeacin led to a 1.5-fold increase of chitin synthase activity (56, 57). In those studies, treatment of cells with high levels of antibiotic had no effect on mannan synthesis.

Since both 1,3-β-glucan synthase and chitin synthase are

FIG. 7. Complementation of L-733,560 resistance and nikkomycin Z supersensitivity. Mutant haploids transformed with YCp-ETG1 or with the control plasmid YCp50 were tested for complementation of the drug-resistance–sensitivity phenotypes by the broth microdilution assay. (A) ○, D1 *ETG1-4/etg1-4* diploid; □, D1.22C *etg1-4* containing YCp50; ▲, D1.22C containing YCp-ETG1; ●, X2180-1A *ETG1*. (B) ○, D11 *ETG1-3/etg1-3*; □, D11.3A *etg1-3* containing YCp50; ▲, D11.3A containing YCp-ETG1; ●, X2180-1A. (C) ●, D1; △, D1.22C containing YCp50; ▲, D1.22C containing YCp-ETG1; □, X2180-1A.

membrane enzymes, it is possible that specific perturbation of the membrane environment by altering one enzyme may affect the activity of the other. The *ETG1* gene is likely to represent a 1,3-β-glucan synthase subunit. Mutations in this gene, such as those present in MS10 and MS14, may lead to a reduced level of 1,3-β-glucan. As a way to compensate for the reduction in the wall β-glucan, the cells may need to synthesize more chitin, which under these conditions may be more critical for growth. Alternatively, an altered 1,3-β-glucan synthase that has acquired resistance to echinocandins may interfere with the cross-linking of 1,3-β-glucan to the wall chitin by an unidentified mechanism, offering an explanation for the nikkomycin Z supersensitivity phenotype.

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