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# Nystatin-Resistant Mutants of Yeast: Alterations in Sterol Content

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Mutants of the genes *nys1* and *nys3* differ from sensitive strains (*nys*$^+$) in their sterol content. Ultraviolet absorption spectra of the nonsaponifiable material extracted from cells of *nys*$^+$ demonstrated the presence of ergosterol and 24(28)-dehydroergosterol. In *nys1* mutants, the spectrum suggests the presence of a new sterol. The absorption spectrum of extracts from *nys3* mutants indicates absence of both ergosterol and 24(28)-dehydroergosterol and presence of another new sterol. Conversion of *nys*$^+$ and *nys3* to petite results in loss of 24(28)-dehydroergosterol in the former and the new sterol in the latter, whereas the new sterol in *nys1* is only reduced. The sterols in ethanol-grown cells of all genotypes are essentially the same as is found for growth on glucose. With the exception of *nys3* grown on ethanol, the mutants do not appear to be at a disadvantage compared to wild type.

The implication of sterols in the mode of action of polyene antibiotics was first suggested by the observation that exogenous sterols protected fungi against their fungicidal effects (5). Subsequently it was shown that both ergosterol and cholesterol complex with polyenes in solution and alleviate the inhibition of growth and metabolic activity caused by the antibiotics (10). Experiments with fractionated yeast and labeled nystatin have shown that sterols in the cell membrane are primarily responsible for binding this polyene. In particular, ergosterol, the major membrane sterol in yeast, has been identified as the binding site for nystatin (9). These observations led Lampen (8) to propose a general scheme for polyene action in which the binding of antibiotic to sterol leads to irreversible changes in membrane permeability. Such a scheme is in accord with the data obtained from work with artificial membrane systems and polyenes (7).

A genetic analysis of nystatin-resistant mutants of yeast in this laboratory led to the identification of three genes for nystatin resistance, *nys1*, *nys2*, and *nys3* (2), and it was suggested that resistance might result from changes in the composition of the cell membrane.

Two of the sterols most abundant in yeast, ergosterol and 24(28)-dehydroergosterol, can be readily identified by their ultraviolet absorption spectra (3). The other sterols found in yeast do not have distinctive absorption spectra and are not detected by this assay method. This communication describes the results of analyses for ergosterol and 24(28)-dehydroergosterol in *nys*$^+$, *nys1*, and *nys3* under a variety of different cultural conditions.

A previous report describing some of these findings (R. A. Woods, J. Hogg, and L. Miller, Heredity **24**:516, 1969) included studies of the gene *nys2*. It has since been found that the strain used carried *nys3*, and we have not been able to recover *nys2* from our laboratory stocks.

## MATERIALS AND METHODS

**Yeast strains.** The sensitive strain *nys*$^+$ and mutants of *nys1* and *nys3* were derived from stocks described previously (2, 14). None of the strains used carried any of the known modifiers of nystatin resistance (2, 14). Petite derivatives of these strains were induced by treatment with acriflavine (4).

**Media.** The yeast complete medium (YCM) contained 0.5% yeast extract, 0.5% casein hydrolysate, and 0.3% peptone plus glucose (4%) or ethanol 2% as carbon source. The medium was adjusted to *p*H 5.8, solidified with 1.5% Oxoid No. 3 agar when required, and sterilized by autoclaving. Nystatin, dissolved in propylene glycol, was added to give final concentrations of 5 or 10 units/ml.

**Growth conditions.** Cultures for sterol analyses were grown for 48 or 72 hr (stationary phase) in 50 ml of YCM in 250-ml Erlenmeyer flasks on a rotary shaker at 200 rev/min. The inoculum was taken from an overnight culture on YCM and adjusted to give ca. $10^6$ cells/ml. All cultures were incubated at 28 C.

**Genetic techniques.** Matings and ascus dissections were carried out as described previously (2). In these experiments, nystatin resistance was determined by testing inocula for their ability to grow on YCM plus 5 or 10 units of nystatin per ml with 48 hr of incubation.

Under these conditions, strains carrying *nys1* and *nys3* were able to grow on 10 and 5 units per ml, respectively, whereas *nys*$^+$ strains failed to grow on 5 units per ml. The absolute resistance of particular strains was not determined.

**Sterol analyses.** Nonsaponifiable sterols were extracted by the method of Breivik and Owades (3). Cells from 25- or 40-ml samples of the cultures were saponified with 10 ml of 40% alcoholic KOH at 85 to 90 C for 3 hr. The saponification mixtures were extracted with 10 ml of *n*-heptane. These extracts were diluted in absolute ethanol, and absorption spectra were recorded between 310 and 200 nm with a Beckman DB spectrophotometer coupled to a 10-inch chart recorder. The relative percentages of ergosterol and 24(28)-dehydroergosterol were calculated by the method of Breivik and Owades (3).

The dry weight yields of cultures were determined by filtering 5-ml samples through tared membrane filters (Millipore type HAWPO45). The filters were washed twice with 10 ml of distilled water and reweighed after 48 hr at 85 C.

**Materials.** Ergosterol and squalene were purchased from the Sigma Chemical Co., U.K. Stock solutions in absolute ethanol were stored in light-proof containers at 4 C. Spectroscopic grade *n*-heptane (Fluka A. G.) was purchased from E. R. Emmanuel & Co. Ltd., London. Nystatin was purchased from E. R. Squibb & Sons Ltd., U.K. All other reagents were Analar grade; the absolute ethanol was benzene-free.

## RESULTS

The absorption spectra of the sterols extracted from *nys*$^+$ show that this strain contains both ergosterol and 24(28)-dehydroergosterol (*see* Fig. 1). Both of these sterols have peaks at 293, 281, and 271 nm, typical of a $\Delta 5,7$-diene system; in addition, 24(28)-dehydroergosterol has a flat peak between 230 and 220 nm which is not present in ergosterol. Also shown in Fig. 1 are the spectra of ergosterol and squalene; the latter has a peak at 205 nm which is also found in *nys*$^+$ and the mutant strains. The spectra of mutants *nys1a*, *nys1b*, *nys1c*, and *nys3a*, *nys3b* are shown in Fig. 2. All of the mutants of *nys1* have a spectrum similar to that given by the mixture of ergosterol and 24(28)-dehydroergosterol found in *nys*$^+$; however, the flat peak in the lower end of the spectrum is shifted to lie between 228 and 236 nm.

This change in the absorption spectrum indicates that the mutants contain a new sterol. The retention of the peaks at 293, 281, and 271 nm suggests either that this new sterol has a $\Delta 5,7$-diene system or that the cells still contain an appreciable amount of ergosterol or 24(28)-dehydroergosterol, or both. In contrast extracts from the mutants of *nys3* show little or no absorption between 310 and 250 nm but have a peak at 205 nm with marked shoulders at 228 and 220 nm. These mutants are deficient in both ergosterol and 24(28)-dehydroergosterol.

Thus, for both *nys1* and *nys3*, resistance to nystatin is associated with altered sterols. Surprisingly, the growth yields of the resistant mutants after 48 hr in YCM are very close to that found for *nys*$^+$ (see Table 1). The association between resistance and altered sterols has been confirmed by sterol analyses on cultures obtained from tetrads segregating sensitivity versus resistance for all mutant alleles.

The petite condition in yeast is associated with the loss of a range of mitochondrial functions (4). When *nys*$^+$ strains are converted to petite, they no longer accumulate 24(28)-dehydroergosterol (L. Miller, *personal communication*). The mutants of *nys1* and *nys3*, together with *nys*$^+$, were converted to petite with acriflavine and tested for nystatin resistance and sterol content. The mutants were still resistant after conversion, and *nys*$^+$ remained sensitive. The results of the sterol analyses are shown in Fig. 3 and Table 1. The extract of *nys*$^+$ petite shows only the absorption spectrum of ergosterol whereas, in *nys3* petite, the shoulders at 228 and 220 nm are no longer visible. In contrast, the spectrum of *nys1* petite is little changed apart from a significant reduction in the 235/281 nm absorbance ratio.

Since the petite state is associated with loss of the ability to grow on nonfermentable carbon sources (4), it was of interest to test the nonpetite (grande) nystatin-sensitive and -resistant strains for sterol content and resistance when grown on ethanol. The mutants retained resistance and *nys*$^+$ remained sensitive. The sterol spectra obtained were essentially the same as when the strains were grown on 4% glucose: *nys*$^+$ contained both ergosterol and 24(28)-dehydroergosterol, *nys1* contained the new sterol, and the spectra of *nys3* showed the shoulders at 228 and 220 nm. The growth yields and sterol data for *nys*$^+$, *nys1c*, and *nys3a* are listed in Table 1.

## DISCUSSION

These experiments have shown that nystatin resistance in yeast is associated with changes in cell sterols. In mutants of *nys1*, the ultraviolet absorption spectra suggest the presence of a sterol which retains the $\Delta 5,7$-diene system associated with the three peaks at 293, 281 and 272 nm; the shift in the peak in the lower range of the spectrum (from 220/225 to 230/235 nm) suggests that there is a new sterol present. The mutant strains have growth yields comparable with *nys*$^+$ under all of the conditions tested, indicating that this new sterol does not interfere with growth. The *nys3* mutants are deficient in ergosterol and 24(28)-dehydroergosterol, although the spectrum of *nys3a* suggests that this mutant may contain a little ergosterol. Heptane extracts from

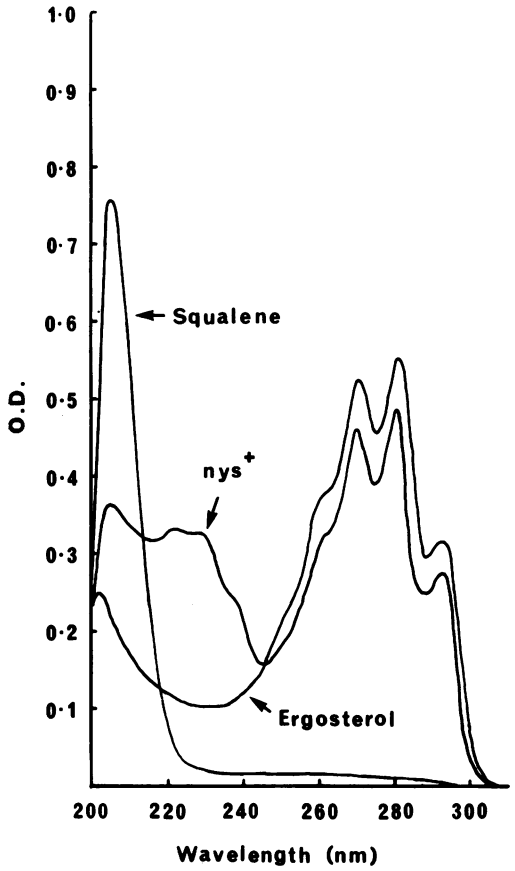


FIG. 1. *Absorption spectra of ergosterol, squalene, and sterols extracted from nys+. Ergosterol (20 μg/ml) and squalene (10 μg/ml) in heptane/ethanol (1:9); nys+ heptane extract diluted 1:19 in ethanol.*

*nys3* mutants do contain substantial amounts of material, giving a positive reaction in the Lieberman-Burchard assay for sterols (13; S. Molzahn and R. A. Woods, *unpublished data*). Strains of this genotype also grow as well as *nys+* on glucose but give lower yields on ethanol, suggesting that whatever sterol replaces ergosterol in these mutants is not as effective for growth on a nonfermentable substrate.

Despite the demonstration by Breivik and Owades (3) that many strains of yeast contain as much, if not more, 24(28)-dehydroergosterol as ergosterol, there have been few subsequent references to this sterol. Longley et al. (11) found it to be the major membrane sterol in the strain NCYC366; Lampen et al. (9) referred to its detection in log-phase cells; and Katsuki and Bloch (6) suggested that it was an intermediate in ergosterol biosynthesis. The majority of sterol determinations have quantified "ergosterol" gravimetrically as the digitonide, colorimetrically by the Lieberman-Burchard reaction, or spectrophotometrically by absorbance at 281 nm. All of these methods will estimate ergosterol plus 24(28)-dehydroergosterol. The results obtained with *nys+* suggest that the levels of 24(28)-dehydroergosterol are considerably more dependent on cultural conditions than those of ergosterol. The petite derivative of *nys+* contains no 24(28)-dehydroergosterol, and cells grown in 4% glucose contain twice as much as those grown in ethanol.

The sterols in *nys1* and *nys3* are also affected by cultural conditions. The 235/281-nm absorbance ratio in grande *nys1* strains ranged from 1.62 to 1.73, whereas, in the petite derivatives, it was only about 1.1. This suggests that the absorption spectrum of these mutants is made up of two components and that conversion to petite reduces the level of the one which is responsible for the peak at 230 to 235 nm. It is thus possible that the sterol giving the "ergosterol peaks" at 293, 281, and 271 nm is in fact ergosterol. Experiments to separate the sterols in these various strains are in progress. The sterol component in

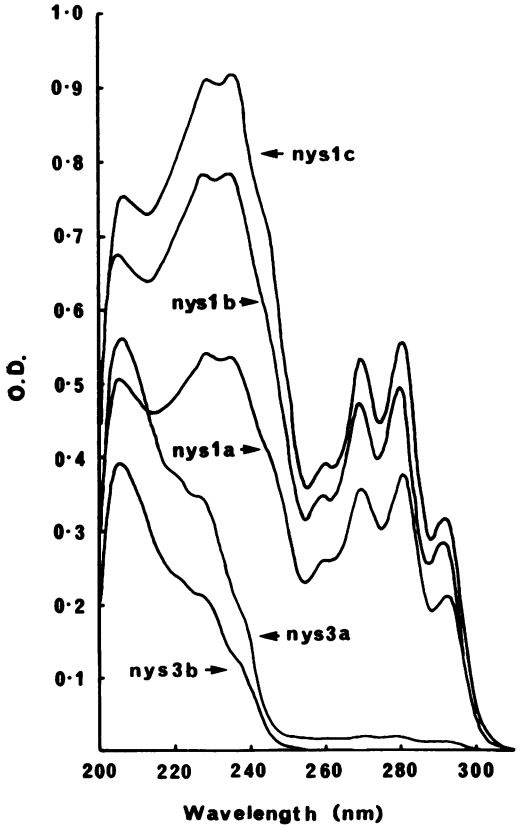


FIG. 2. *Absorption spectra of sterols extracted from mutants of nys1 and nys3. Heptane extracts diluted 1:19 in ethanol.*

*nys3* which causes the shoulders on the absorption spectra at 228 and 220 nm is lost as a result of conversion to petite, but it is retained when the grande strains are grown in ethanol.

The sterol patterns of normal and petite derivatives of *nys*$^+$ suggest that synthesis or accumulation of 24(28)-dehydroergosterol can be added to the list of metabolic defects associated with the petite condition. The changes in the spectra of *nys1* and *nys3* petites indicate that these mutant strains also contain sterols whose synthesis is at least in part dependent on functional mitochondria.

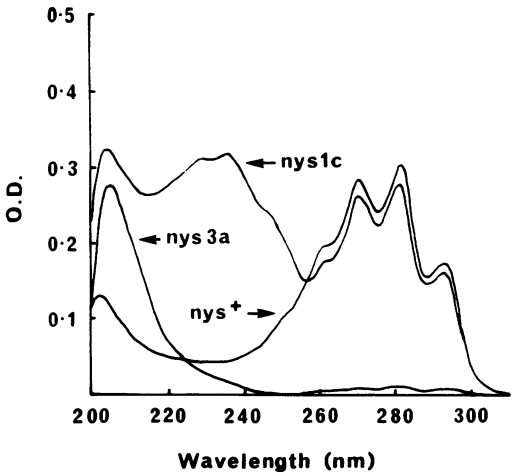


FIG. 3. *Absorption spectra of sterols extracted from petite derivatives of nys*$^+$, *nys1c, and nys3a. Heptane extracts diluted 1:19 in ethanol.*

The levels of ergosterol in *nys*$^+$ appear to be relatively independent of carbon source and respiratory capacity, suggesting that this sterol may not necessarily be associated with mitochondrial function. Preliminary experiments indicate that accumulation of 24(28)-dehydroergosterol by *nys*$^+$ is repressed by high glucose concentrations, whereas the new sterol in *nys1* is relatively unaffected (R. A. Woods, *unpublished data*). It thus seems possible that a proportion of the reported variations in yeast ergosterol associated with changes in cultural conditions have in fact been variations in 24(28)-dehydroergosterol.

The correlation between altered sterols and nystatin resistance is in accord with the model for polyene action proposed by Lampen (8). Ergosterol is the major membrane sterol in yeast (9) and binds nystatin and other polyenes effectively (15). Absence or severe reduction in the amount of ergosterol in the membrane and its replacement with sterols which bind nystatin less effectively is a predictable mechanism for the origin of resistance. There is little or no ergosterol in *nys3*, so that the resistance of this genotype is probably due to a reduction in the number of binding sites for the polyene. However, these mutants show only low levels of resistance (2), thus indicating that the remaining sterols can bind the antibiotic. The resistance of *nys1* is not so readily explicable, since the mutants may contain ergosterol; certainly resistance is associated with the presence of a new sterol, so that it seems likely that it binds nystatin less effectively than those which it replaces in the membrane. Studies on mutants selected for re-

TABLE 1. *Growth yields and sterol contents*

| Strain | Carbon source | Yield[a] (mg/5 ml) | Wt of sample analyzed (mg) | 230/281 or 235/281-nm ratio[b] | Ergosterol (%) | 24(28)-Dehydroergosterol (%) |
|---|---|---|---|---|---|---|
| nys$^+$ | Glucose | 43.6 | 218 | 0.68 | 0.98 | 0.54 |
| nys1a | Glucose | 40.6 | 203 | 1.73 | | |
| nys1b | Glucose | 46.2 | 231 | 1.62 | | |
| nys1c | Glucose | 45.2 | 226 | 1.65 | | |
| nys3a | Glucose | 46.6 | 233 | | | |
| nys3b | Glucose | 45.6 | 229 | | | |
| nys$^+$ (petite) | Glucose | 19.6 | 156 | 0.13 | 1.33 | 0.00 |
| nys1a (petite) | Glucose | 18.1 | 145 | 1.00 | | |
| nys1b (petite) | Glucose | 18.4 | 146 | 1.12 | | |
| nys1c (petite) | Glucose | 15.8 | 126 | 1.14 | | |
| nys3a (petite) | Glucose | 18.4 | 148 | | | |
| nys3b (petite) | Glucose | 16.2 | 130 | | | |
| nys$^+$ | Ethanol | 25.6 | 128 | 0.30 | 1.05 | 0.27 |
| nys1c | Ethanol | 36.6 | 183 | 1.58 | | |
| nys3a | Ethanol | 9.2 | 56 | | | |

[a] Cultures grown for 48 hr with glucose and 72 hr with ethanol.
[b] Ratios were 230/281 nm for *nys*$^+$ and 235/281 nm for *nys1*.

sistance to other polyenes have shown that a change in sterol pattern is involved in all mutants investigated in this laboratory (S. Molzahn and R. A. Woods, *manuscript in preparation*).

At present we have no knowledge of the biochemical lesions in *nys1* and *nys3*. Ergosterol is probably the "end product" of sterol biosynthesis in yeast and so it is possible that *nys1* and *nys3* represent blocks in this pathway. Attempts to isolate ergosterol-deficient mutants of yeast have been based on the assumption that such mutants would require ergosterol for growth on a defined medium (1, 12). A mutant of this kind, responding to ergosterol or oleic acid, has been isolated and analyzed genetically by Resnick and Mortimer (12), but its sterol composition was not determined. Neither *nys1* nor *nys3* mutants require ergosterol for growth in a defined medium, though little or no ergosterol is present in *nys3*. This suggests that ergosterol deficiency may not necessarily result in ergosterol requirement.

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