

# Biochemical and Genetic Aspects of Nystatin Resistance in *Saccharomyces cerevisiae*

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Two phenotypically distinct sets of nystatin-resistant mutants were investigated. One set is resistant, respiratory competent, and requires no lipid for growth. The other set is more resistant, respiratory deficient, and lipid requiring (unsaturated fatty acid or sterol). Both sets show altered sterol composition as demonstrated by the Liebermann-Burchard colorimetric reaction, ultraviolet spectrophotometry, and gas-liquid chromatography. Genetic analysis indicates that all nystatin-resistant mutants can be placed into one of six distinct genetic groups. The phenotype's nystatin resistance, lipid requirement, and respiratory deficiency are recessive. There was one case of allelism for mutants from different sets. Revertants of mutants which have the tripartite phenotype retain a residual level of nystatin resistance, but they are no longer lipid requiring or respiratory deficient. Growth studies in mutants which have the tripartite phenotype reveal that the addition of ergosterol to the growth medium results in decreased resistance to nystatin.

A relationship between the polyene antibiotic, nystatin, and sterols in the cell membrane of sensitive organisms has been demonstrated by a number of investigators. For two excellent reviews on permeability alterations and resulting cell death due to the administration of this antibiotic see Lampen (15) and Kinsky (13).

The ready availability of nystatin-resistant mutants in the yeast *Saccharomyces cerevisiae* afforded an opportunity to correlate genetic changes with alterations in sterol composition. In addition, a combined biochemical genetic approach offered opportunities to observe phenomena closely related to sterol production such as mitochondrial formation and lipid metabolism.

Ahmed and Woods (2) identified three nystatin-resistant genes. These are recessive and subject to enhanced resistance by three dominant modifier genes. Some of these mutants do not synthesize ergosterol and/or an additional sterol, 24(28)-dehydroergosterol (24). Resnick and Mortimer (20) reported a class of yeast lipid mutants which require the addition of either an unsaturated fatty acid or ergosterol to yeast extract-peptone-dextrose (YEPD) medium. These mutants, now designated *ole2*, *ole3*, and *ole4*, were also petite (respiratory deficient), and preliminary genetic analysis

demonstrated that both the lipid requirement and petite phenotype segregated together (12). The findings that the *ole2*, 3, and 4 mutants are also highly resistant to nystatin and that all three phenotypes segregate together is an important aspect of this investigation and strengthens the evidence for the role of lipids in the function and biogenesis of mitochondria.

Several investigators have observed relationships between lipogenesis and the production of functional mitochondria in yeast. Sarachek (21), using a pantothenate-deficient strain, demonstrated that during the period of transition from the logarithmic to the stationary phase, cells grown in the absence of pantothenate, a metabolite essential in lipogenesis, become respiratory deficient. The addition of exogenous acetate or unsaturated fatty acids prevented the occurrence of respiratory deficiency, whereas the addition of saturated fatty acids and sterols did not. Parks and Starr (18) demonstrated that raising the temperature of growing cells to 42 C, a condition conducive to a sharp reduction in sterol production, coincided with petite induction. Supplementation of the growth medium with ergosterol at elevated temperatures resulted in the induction of only one-half as many petites.

This report is concerned with some of the relationships discussed above. In particular,

correlations are made between nystatin resistance and sterol patterns, and between sterol composition, lipid requirement, and respiratory deficiency in mutant strains of yeast.

### MATERIALS AND METHODS

**Organisms.** A wild-type strain of *S. cerevisiae*, S288C, obtained from the Berkeley collection was the parent strain of all mutants used in this investigation. Nystatin-resistant mutants *nyr1*, 2, 5, 6, and 15, as well as Resnick and Mortimer's (20) *ole1*, 2, 3, and 4 mutants, were employed. In view of the existence of mutants which are phenotypically similar to the *nyr* set (2), no attempt is made to appropriate genetic symbols to this set. *nyr* mutants are referred to by their isolation numbers. This is not the case for the *ole* set, wherein the numeral after the *ole* symbol does refer to a genetic locus.

Because of the time lapse between the isolation of the *nyr* and *ole* sets (3 years), another wild-type strain, X2180, derived as an "a" mating type segregant from a diploidized S288C culture was used as an internal control. This was necessary because different wild-type *S. cerevisiae* strains differ substantially in amounts of ergosterol synthesized. All growth was at 30 C.

**Media and genetic analysis.** A YEPD culture medium of 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar (solid) was the standard culture medium with 1% Tween 80 (a polyoxyethylene derivative of sorbitan monoleate) added as the source of unsaturated fatty acid for *ole1*, 2, 3, 4 mutants. Ergosterol (Nutritional Biochemicals Corp., Cleveland, Ohio) was solubilized by adding 1.5% Tergitol (v/v, Union Carbide Institute, W. Va.) a nonionic detergent free of lipid. Various concentrations of this sterol from  $10^{-4}$  M to  $10^{-5}$  M (solid) and  $5 \times 10^{-5}$  M to  $7.5 \times 10^{-6}$  M (liquid) were employed. An aqueous nystatin suspension (Grand Island Biological Co., Long Island, N.Y.) was added to solid medium before pouring at 49 C or less.

Minimal medium contained 0.67% yeast nitrogen base (Difco), 2% dextrose, 1% Tween 80, and 2% agar. For the testing of petites, 0.5% ethanol replaced dextrose as the carbon source.

Hybridization was accomplished by mixing haploids of opposite mating types on YEPD plus Tween 80 medium, and after 2 to 3 hr zygotes were isolated by micromanipulation. After 24 hr of growth on YEPD plus Tween 80, diploids were sporulated by transfer to a medium composed of 2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 2% agar, and the following additions per liter: L-leucine, 6 mg; L-threonine, 300 mg; L-tryptophan, 2 mg; L-methionine, L-histidine, L-arginine, adenine, and uracil, 1 mg each; and L-lysine, 20 mg. After 3 to 5 days of incubation, the cultures were examined for asci, and if well formed asci were abundant the cell walls were digested with a 1:25 dilution of Glusulase (a digestive enzyme from the snail crop, Endo Laboratories, Garden City, N.Y.). Dissected asci were tested for levels of resistance, respiratory competency, and lipid requirements by replica plating.

**Nystatin resistance determinations.** Plates containing 25 to 800 units (U) of nystatin per ml were scored 24 hr after replica plating from YEPD plus 1% Tween 80 medium lacking the antibiotic. Plates were again scored after 36 hr, particularly for the slower growing *ole2*, 3, and 4 mutants. Scoring was as follows. The designation "+" signifies confluent growth on nystatin plates for at least 50% of the determinations. The latter qualification is especially important for plates containing 25 U of nystatin per ml, the minimum amount of antibiotic used. Higher concentrations of nystatin gave more consistent results. The notation "±" refers to nonconfluent growth such that only single colonies are seen rather than a uniform layer of cells. This same notation is also used for a uniform layer of cells growing more slowly than the same strain on a plate having a lower concentration of nystatin. The designation "-" means that the strain is nystatin sensitive and fails to grow. Segregants from dissected asci were routinely tested on several plates with varying concentrations of nystatin to establish the resistance level of each of the four ascospore colonies.

**Extraction of nonsaponifiables and sterols.** Yeast cultures grown for 48 to 96 hr were pelleted and washed once with distilled water before being acid-saponified for 1 hr at 30 C in 0.1 N HCl (1). After an additional washing, pellets were subjected to base-saponification at reflux temperatures for 90 min in 15% methanolic KOH. The nonsaponifiable fraction was extracted with petroleum ether (bp, 30–60 C) and analyzed for ergosterol by either the Liebermann-Burchard colorimetric reaction or ultraviolet (UV) spectrophotometry. For gas-liquid chromatographic (GLC) analysis of sterols, batch cultures of yeast were grown, pelleted, washed, and subjected to total lipid extraction by overnight stirring in chloroform-methanol (2:1). The lipid extract, approximately 250 ml for each sample, was vacuum dried on a rotary evaporator and saponified for 3 hr at reflux temperatures in Florence flasks containing 72 ml of absolute ethanol, 24 ml of ethyl ether, and 4 ml of 10 N KOH. The nonsaponifiable fraction was extracted with petroleum ether.

**Colorimetry.** The Liebermann-Burchard colorimetric reaction to detect  $\Delta^5$ -sterols was carried out essentially according to the procedure of Stadtman (22). Upon the addition of Liebermann-Burchard reagent, lanosterol a  $\Delta^8$ -sterol, gives a clear yellow color (10).

**UV spectrophotometry.** UV spectrophotometry of ergosterol in absolute ethanol gives four absorption bands at 262, 271, 282, and 293.5 nm (8). The presence of ergosterol in each sample was determined by dissolving the nonsaponifiable fractions in absolute ethanol and scanning from 300 to 200 nm, with a Beckman DB spectrophotometer.

**Chromatography.** Preparative thin-layer chromatography for GLC analysis was accomplished by applying nonsaponifiable samples to Silica Gel-G plates activated at 110 C for 1 hr before use. Ergosterol and lanosterol (Mann Research, New York, N.Y.) were applied to each plate as standards. Plates were developed in a solvent system of hexane-di-

ethyl ether-acetic acid (80:20:1), and sterols were made visible with an iodine (1% in methanol) spray. Sterols were eluted with hexane.

GLC was performed on a Varian Aerograph model 1200. Two kinds of columns were employed. (i) A stainless-steel column (1/8 inch by 6 ft) packed with 3% neopentyl glycolsuccinate (NPGS) on 100/20 mesh Chromosorb W was operated at a column temperature of 220 C and N<sub>2</sub> gas flow of 19.6 ml/min. (ii) A stainless-steel column (1/8 inch by 5 ft) packed with the less polar 5% silicone gum rubber (SE-30) on 60/80 mesh Chromosorb W was operated at 225 C and N<sub>2</sub> gas flow of 32.4 ml/min. For both columns the detector temperature was 265 C, and the injector temperature was 255 C.

Silylation of sterols was accomplished by reacting bis(trimethylsilyl)trifluoroacetamide (Regisil, Regis Chemical, Chicago, Ill.) with sterol samples for 30 min at 60 C in screw-cap test tubes in the dark.

Columns were coated with silylating agent prior to packing. Ergosterol purchased from Nutritional Biochemicals Corp. and Sigma Chemical Co. (St. Louis, Mo.) was used to determine a standard relative retention time. Both purchased products were isolated from yeast. The retention times are relative to cholestane.

**Growth determination.** Growth studies were carried out with a Klett-Summerson photoelectric colorimeter (red filter) in side-arm Erlenmeyer flasks. Doubling times were computed from areas of the growth curve of 30 to 100 Klett units, known to be linear with cell concentration (23).

## RESULTS

**Isolation and description of mutants.** A wild-type strain of *S. cerevisiae*, S288C, was subjected to 10 and 60 U of nystatin per ml on minimal medium plus 1% Tween 80 plates. Fifteen spontaneously produced nystatin-resistant mutants were isolated, five of which will be discussed in the present investigation (*nyr1*, 2, 5, 6 isolated as single colonies from nystatin plates at 10 U/ml, and *nyr15* isolated as a single colony from a 60 U/ml plate).

Resnick and Mortimer (20) used a YEPD plus Tween 80 medium to isolate the more phenotypically complex *ole* mutants. The *ole* mutants employed throughout this investigation are *ole2-1* (KD46), *ole2-2* (KD91), and the double mutant *ole3ole4* (KD18). In addition to being nystatin resistant, these mutants are petite and require a lipid supplement, unlike the *nyr* mutants which have resistance as their sole phenotype.

Revertants at the *ole2* locus were easily detected by their morphological appearance on solid medium. *ole2* colonies are always small and retain a white liquidy appearance even on aged plates; in contrast, grande revertant colonies are larger and after several days of growth

on solid medium appear dry and amber-colored.

Table 1 records the range of resistance levels of the *ole* and *nyr* mutants, two grande non-lipid-requiring revertants of *ole2* (*ole2-1R* and *ole2-2R*), and two genetically related wild-type strains, S288C and X2180. The resistance levels of *ole1* (KD20 and KD115) are also listed. *ole1-1* and *ole1-2* are two grande alleles of a fatty acid desaturase mutant (12).

The higher resistance values reported in this investigation are largely due to the presence of Tween 80 in the medium (2). Tween 80 is a source of oleic acid for the lipid-requiring mutants, and, as a detergent, it solubilizes nystatin. Concomitantly, Tween 80 decreases the interaction of the antibiotic with the cell surface (13). Therefore, with detergent effects taken into consideration, Table 1 reveals the relative levels of resistance of the mutants. The most resistant mutants are *ole2-1*, *ole2-2*, and the double mutant *ole3ole4* which can grow on plates of 800 U of nystatin per ml. The remaining mutants in order of decreasing resistance are: *nyr1* and *nyr15* greater than *nyr2*, *nyr5*, *nyr6*, *ole2-1R*, and *ole2-2R*. Wild types S288C and X2180 and the fatty acid desaturase mutants, *ole1-1* and *ole1-2*, are sensitive.

TABLE 1. Range of nystatin resistance levels of mutant and wild-type *Saccharomyces cerevisiae*

Strain <sup>a</sup>	Resistance <sup>b</sup>						
	25 <sup>c</sup>	50	75	100	400	600	800
Wild-type strains							
S288C	+	-	-				
X2180	+	-	-				
<i>nyr</i> mutants							
<i>nyr1</i>	+	+	+	+	+	±	±
<i>nyr2</i>	+	+	+	±	-		
<i>nyr5</i>	+	+	+	±	-		
<i>nyr6</i>	+	+	+	±	-		
<i>nyr15</i>	+	+	+	+	+	±	±
<i>ole</i> mutants							
<i>ole2-1</i>	+	+	+	+	+	+	+
<i>ole2-2</i>	+	+	+	+	+	+	+
<i>ole3ole4</i>	+	+	+	+	+	+	+
<i>ole2-2R</i>	+	+	+	-			
<i>ole2-1R</i>	+	+	±	-			
<i>ole1-1</i>	+	-	-				
<i>ole1-2</i>	+	-	-				

<sup>a</sup> Recorded 24 hr after replica plating to YEPD plus 1% Tween 80 plates containing nystatin.

<sup>b</sup> +, Confluent growth for at least 50% of the determinations; ±, spotted colonies without confluence, or appreciable thinning out of the cell plaque; -, no growth.

<sup>c</sup> Units of nystatin per milliliter.

**Liebermann-Burchard colorimetric reaction.** Ergosterol has long been known as the primary sterol in yeast, although several other sterols have been reported in lesser amounts (7, 8). The Liebermann-Burchard colorimetric reaction was used to demonstrate qualitative differences in sterols among the two sets of nystatin-resistant mutants. Preliminary investigations of mutants *ole2-1*, *ole2-2*, and *ole3ole4* established that these strains do not contain ergosterol or a closely related  $\Delta^5$ -sterol. These mutants gave a Liebermann-Burchard colorimetric response of clear yellow ( $\lambda_{max}$ , 460 nm), identical to the response given by lanosterol (10). Only the wild-type strains S288C and X2180 and nystatin-resistant mutants *nyr5*, *nyr6*, and *nyr15* produced the green or blue-green color demonstrating a  $\Delta^5(6)$ -sterol. Mutants *nyr1*, *nyr2*, *ole2-2R*, and *ole2-1R* gave colors of blue, blue-black, or green-black. Although this colorimetric reaction gave the green color for *nyr5* and *nyr6*, a yellowish background was always present in these two mutant samples that was not present in S288C, X2180, or purchased ergosterol. The hybrids *nyr1*  $\times$  X2180, *nyr5*  $\times$  X2180, and *ole2-2*  $\times$  X2180 gave reactions which appeared colorimetrically identical to the wild-type strains.

**UV spectrophotometry.** Figure 1 illustrates UV absorption spectra of nonsaponifiable extracts from the wild-type strain S288C and several representative nystatin-resistant mutants. In addition to S288C, both mutants *nyr5* and *nyr6* gave the typical ergosterol UV spectrum when grown on YEPD plus Tween 80 medium. Mutants *nyr1*, *nyr2*, and *ole2-2R* did not show absorbance in the 240 to 310 nm range when grown on YEPD plus Tween 80 medium or YEPD plus  $5 \times 10^{-5}$  M ergosterol medium, indicating that these mutants neither produce ergosterol nor incorporate this sterol from the medium.

Mutant *nyr15*, in addition to the normal ergosterol absorption maxima, had an additional band at 235 nm. Woods (24) detected this sterol in one of his nystatin-resistant strains and observed that this new sterol appears with concomitant loss of the wild-type sterol, 24(28)-dehydroergosterol.

Mutant *ole2-2* showed incorporation of a low level of ergosterol when grown in YEPD plus  $5 \times 10^{-5}$  M ergosterol, whereas no ergosterol could be demonstrated for this mutant when it was grown on YEPD plus Tween 80 medium (Fig. 2).

**GLC.** Trimethylsilylated ethers of the sterol fractions of nystatin mutants *nyr1*, 2, 5, 15,

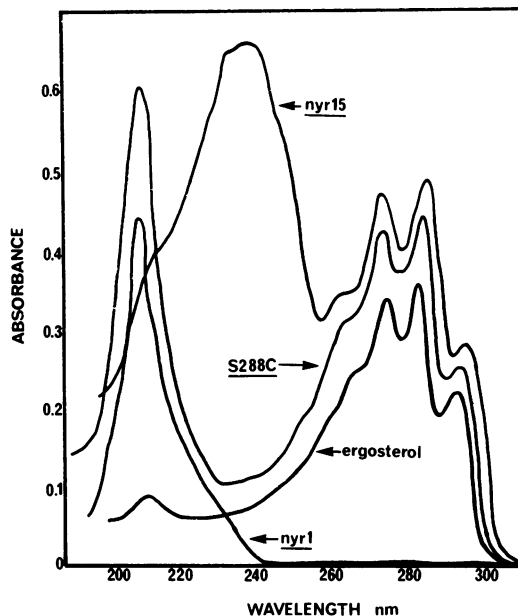


FIG. 1. Ultraviolet absorption spectra of the nonsaponifiable fractions from *Saccharomyces cerevisiae* strains, S288C and nystatin-resistant mutants *nyr1* and *nyr15*. Ergosterol absorbs maximally at 262, 271, 282, and 293.5 nm. Growth was in liquid YEPD medium plus Tween 80.

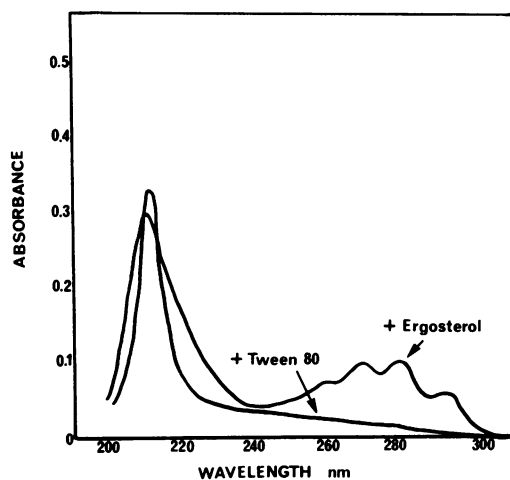


FIG. 2. Ultraviolet absorption spectrum of nonsaponifiable fractions from mutant strain *ole2-2* grown in liquid YEPD medium plus 1% Tween 80 or plus  $5 \times 10^{-5}$  M ergosterol.

*ole2-2*, *ole2-2R*, and wild type, S288C, revealed on GLC analysis that the spectrum of mutant yeast sterols was grossly affected (Fig. 3). A comparison of the different sterol patterns showed that only S288C, *nyr5*, *nyr1*, and *ole2-2* had peaks with the approximate relative retention time of ergosterol (RRT = 2.9, see Fig.

3A, B, C, and G). UV spectrophotometry of the latter two mutants, however, suggested an inability to synthesize ergosterol. S288C and *nyr5*, although differing in the number of peaks, had as their major sterol fractions peaks with an RRT of 2.9. Although mutant *nyr1* had a minor peak with the approximate RRT of ergosterol, the major sterol fraction in this chromatogram had an RRT of 2.4. It is not unlikely that a sterol intermediate synthesized by *nyr1* would give the same RRT as ergosterol on a 3% NPGS column. The *ole2-2* chromatogram also revealed a sterol fraction with an RRT at or too close to that of ergosterol to distinguish it unequivocally. Analysis of this mutant on the less polar 5% SE-30 column confirmed the above evidence that *ole2-2* does

not synthesize ergosterol. The latter column gives peak broadening and tends to merge sterol fractions that are more sharply delineated on the NPGS column. Thus the more complex sterol pattern of *nyr1* made it unsuitable for further resolution on the SE-30 column.

Mutants *nyr2*, *ole2-2R*, and *nyr15* clearly did not show an ergosterol fraction. The chromatograms of *nyr2* and *ole2-2R* had approximately the same sterol fractions present and in the same relative proportions (Fig. 3 D, E).

**Genetic analysis.** The types of questions amenable to genetic analysis of these two seemingly different sets of nystatin-resistant mutants, the *ole2*, 3, 4 and *nyr* sets are: (i) the dominance-recessivity relationships, (ii) the association and possible dissociation of the tripartite phenotype, (iii) the possible allelic relationships within and between mutant sets, and (iv) the minimum number of genetic loci involved in nystatin-resistant mutants.

Hybrids produced from crosses between the wild type nystatin-sensitive strain, X2180, and mutants *nyr2*, 5, 6, and 15 were all clearly sensitive to nystatin (defined as no growth on 50 U/ml nystatin plates). The hybrid from *nyr1* mated to X2180 exhibited nonconfluent growth ( $\pm$ ) on 50 U/ml plates, finally becoming sensitive on 75 U/ml plates. The nonconfluent growth manifested by all *nyr1* hybrids made scoring of these diploids difficult, and in some cases low levels of resistance had to be attributed to such diploids.

The hybrids *nyr1*  $\times$  X2180 (15 asci), *nyr5*  $\times$  X2180 (13 asci), and *nyr15*  $\times$  X2180 (8 asci) were analyzed and gave two sensitive: two resistant ascospore colonies, indicating that nystatin-resistant mutants *nyr1*, *nyr5* and *nyr15*, were single mutations (scoring was on nystatin plates containing 100 U/ml for *nyr1*  $\times$  X2180, and 75 U/ml for *nyr5*  $\times$  X2180 and *nyr15*  $\times$  X2180).

Hybrids of *ole2-2* and *ole3ole4* each mated to X2180 were nystatin sensitive. These matings also demonstrated that the lipid requirement and petite phenotype were recessive. The following data establish that these three phenotypes segregate together. Eleven asci dissected from the hybrid *ole2-2*  $\times$  X2180 gave 2:2 segregation for the lipid requirement, petite phenotype, and nystatin resistance (100 U/ml plates). Thirty-one asci dissected from the hybrid *ole3ole4*  $\times$  X2180 also demonstrated that the lipid requirement, petite phenotype, and nystatin resistance always segregated together. In addition to ascospores showing the tripartite phenotype, wild-type ascospore colonies were also observed. The ratio of the

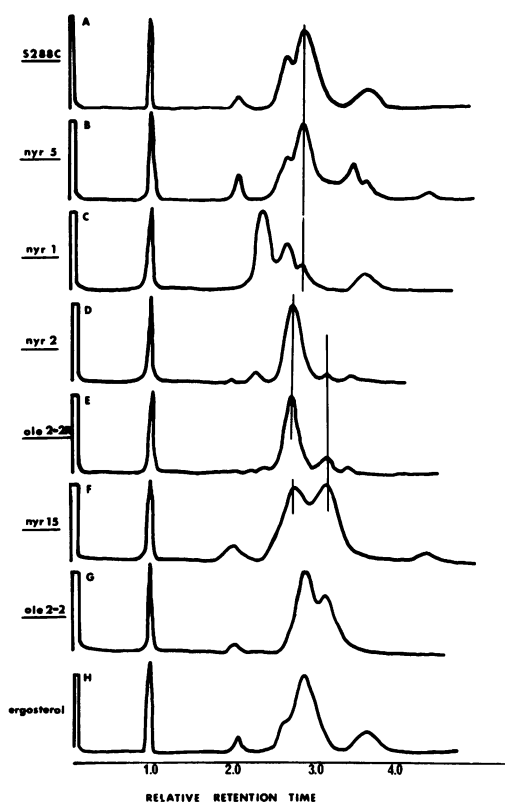


FIG. 3. Gas-liquid chromatography of trimethylsilyl ethers of sterols from *Saccharomyces cerevisiae* wild-type and nystatin-resistant strains. Operating conditions:  $\frac{1}{8}$  inch by 6 ft stainless-steel columns packed with 3% neopentyl glycolsuccinate on  $100/20$  mesh Chromosorb W, operated at 220 C and  $N_2$  flow rate of 19.6 ml/min. Retention time is relative to cholestane, with ergosterol (Nutritional Biochemicals Corp.) giving a relative retention time of 2.9. The largest peak in each chromatogram was reduced to 1.5 inches, with all other peaks reduced proportionately.

tetrad classes PD:NPD:T (parental ditype: non-parental ditype:tetratype) was 7:3:21 which corresponds to 2:2, 0:4, and 1:3 (scored as grande:petite, sensitive:resistant; the petite phenotype was scored with and without 1% Tween 80 supplementation). The above data fit the 1PD:1NPD:4T distribution of tetrad classes for two unlinked genes, with at least one of the genes showing no centromere linkage (19), and demonstrate that the separable single genetic lesions *ole3* and *ole4* display the tripartite phenotype. In the combined 42 asci analyzed, no segregant showed dissociation of the tripartite phenotype. This accords with observations by Resnick and Mortimer (20) and Keith et al. (12), who also failed to detect dissociation of the lipid requirement and petite phenotype.

Grande revertants of *ole2-1* and *ole2-2* were isolated and found to be non-lipid-requiring and less nystatin resistant than the parental strains (Table 1). Phenotypically, the reverted *ole* mutants resemble the *nyr* set. Their sole phenotype is a level of resistance that is more comparable to several less resistant *nyr* mutants (*nyr2*, 5, 6) than the highly resistant *ole2*, 3, 4 set.

The hybrid *ole2-2R* (revertant) × X2180 is nystatin sensitive, demonstrating that the resistance phenotype of the revertant is recessive. Twenty-three asci analyzed from this cross yielded 2:2 segregation for sensitivity: resistance when scored on 100 U/ml nystatin plates. All segregants were grande and, in addition, sensitive on plates containing 150 and 300 U of nystatin per ml. The original *ole2-2* mutant was not recovered, indicating that the revertant phenotype results from a reverse mutation at or within a few map units of the original locus rather than an unlinked modifier or suppressor gene.

Resistance levels of pairwise combinations of nystatin-resistant mutants are listed in Table 2. These crosses assess the allelic relationships among mutants and provide the basis for estimating the number of genetic loci involved. Diploids *nyr2* × *ole2-2*, *nyr2* × *ole2-2R*, *ole2-2* × *ole2-2R*, and *nyr5* × *nyr6* demonstrate levels of resistance indicating the absence of complementation. Segregation analysis of these diploids confirm that mutants *ole2*, *ole2-2R*, and *nyr2* are allelic as well as mutants *nyr5* and *nyr6*. The diploids *nyr2* × *ole2-2*, *nyr2* × *ole2-2R*, and *ole2-2* × *ole2-2R* are neither petite, lipid requiring or highly nystatin resistant; the lower resistance levels of *nyr2* and *ole2-2R* are dominant in crosses to *ole2-2*. The phenotypic relationships within this latter

group illustrate that not only is the entire tripartite phenotype recessive but also, phenotypically, a low level of resistance is dominant to a higher level.

The ambiguous results given by *nyr1* hybrids obscured the complementation data. Whereas the hybrid *nyr2* × *nyr5* was clearly nystatin sensitive on 50 U/ml plates, indicating two independent loci, the hybrid *nyr1* × *nyr2* was resistant on 50 U/ml plates, nonconfluent at slightly higher concentrations, and clearly sensitive above 100 U/ml. Dissected asci of *nyr1* crossed to *nyr2*, 5, and 15 in each case revealed a 1:1:4 ratio indicative of two independent loci with at least one of the genes not linked to its centromere (Table 3).

*ole3* and *ole4* complement *nyr1*, *nyr5*, and *nyr15* and therefore represent two additional loci. Thus all nystatin-resistant mutants in this investigation can be placed into the following six separate genetic groups: (A) *ole2-1*, *ole2-2*, *ole2-2R*, *nyr2*; (B) *ole3*; (C) *ole4*; (D) *nyr1*; (E) *nyr5*, *nyr6*; (F) *nyr15*.

**Growth characteristics of mutant *ole2-2*.** Experiments were carried out in an attempt to elucidate the relationship between nystatin resistance and growth rate of *ole2-2* in YEPD plus lipid supplemented medium (Table 4). Several important observations can be made from this data. (i) Mutant *ole2-2* supplemented with two lipids, an unsaturated fatty acid and a sterol, has the doubling time of the wild-type strain S288C (1.5 hr). (ii) *ole2-2* is resistant to 100 U of nystatin per ml in liquid

TABLE 2. Nystatin resistance determinations of hybrids

Hybrid <sup>a</sup>	Resistance <sup>b</sup>			
	25 <sup>c</sup>	50	75	100
<i>nyr1</i> × <i>nyr2</i>	+	+	±	
<i>nyr1</i> × <i>nyr5</i>	+	±	-	
<i>nyr1</i> × <i>nyr15</i>	+	±	±	
<i>nyr1</i> × <i>ole2-2R</i>	+	-	-	
<i>nyr1</i> × <i>ole2-2</i>	+	±	±	
<i>nyr2</i> × <i>nyr5</i>	+	-	-	
<i>nyr2</i> × <i>nyr15</i>	+	-	-	
<i>nyr2</i> × <i>ole2-2</i>	+	+	+	-
<i>nyr2</i> × <i>ole2-2R</i>	+	+	+	-
<i>ole2-2</i> × <i>ole2-2R</i>	+	+	+	±
<i>nyr5</i> × <i>nyr6</i>	+	+	+	±
<i>nyr5</i> × <i>nyr15</i>	+	-	-	

<sup>a</sup> Recorded 24 hr after replica plating to YEPD plus Tween 80 plates containing nystatin.

<sup>b</sup> +, Confluent growth for at least 50% of the determinations; ±, spotted colonies without confluence, or appreciable thinning out of the cell plaque; -, no growth.

<sup>c</sup> Units of nystatin per milliliter.

YEPD plus Tween 80 medium with no growth inhibition. In comparison, S288C is sensitive to 10 U of nystatin per ml with an approximate 12-hr delay in appearance of turbidity compared to a nystatin-free control. (iii) The low growth level of *ole2-2* in YEPD medium with no lipid supplement is probably due to trace amounts of lipid in Difco yeast extract (11). Figure 4 illustrates growth of *ole2-2* in YEPD plus  $5 \times 10^{-6}$  M ergosterol medium (plus a lipid-free detergent, Tergitol) with varying concentrations of nystatin. There was little difference in growth curves in the presence of 0, 10, or 25 U of nystatin per ml, but at 50 U of the antibiotic per ml there was a long lag phase, and 100 U/ml completely inhibited growth. Nystatin is sensitive to both light and oxygen with the potency of the antibiotic decreasing rapidly during the course of the growth experiments.

The data illustrate that nystatin sensitivity of *ole2* in the presence of exogenous ergosterol and nystatin (even when ergosterol is not required as a growth supplement) is a result of incorporation of the sterol by *ole2* into its cell membrane (also see Fig. 2).

DISCUSSION

A biochemical analysis of the sterol composition of nystatin-resistant mutants was undertaken in an attempt to elucidate the possible mechanism(s) of resistance in yeast. The wealth of research on polyene toxicity suggests that polyene antibiotics complex with sterols in the cell membrane of sensitive organisms resulting in leakage of essential cellular metabolites (16). Kinsky et al. (14) demonstrated that erythrocyte membranes lysed by various polyene antibiotics possessed nearly circular pits approximately 12.5 nm in diameter under electron microscope examination.

Nystatin resistance may arise in a yeast strain as a result of a mutation leading to qualitative or quantitative changes in the sterols in

the cell membrane, and, depending upon the nature of such changes, different mutations would lead to varying levels of resistance. The finding that different sterols afford yeast cultures unequal degrees of protection against polyene antibiotics suggests that certain sterols have a greater affinity for polyenes than

TABLE 4. Doubling times of *ole2-2* in YEPD plus lipid-supplemented medium<sup>a</sup>

YEPD plus:	Doubling time (hr)
1% Tween 80 + $7.5 \times 10^{-6}$ M ergosterol	1.5-1.75
1% Tween 80	2.0
1% Tween 80 + 100 U of nystatin/ml	2.0
1% Tween 80 + $7.5 \times 10^{-6}$ M ergosterol + 100 U of nystatin/ml	3.0
1.5% Tergitol + $7.5 \times 10^{-6}$ M ergosterol	3.5-4.0
1.5% Tergitol + 0 U of nystatin/ml	5.0 <sup>+</sup>
1.5% Tergitol + 100 U of nystatin/ml	5.0

<sup>a</sup> Growth was monitored on side-arm Erlenmeyer flasks measured on a Klett colorimeter. Detergent Tween 80 is a source of unsaturated fatty acid and Tergitol NP-40 is not. Doubling times were calculated over the range of 30 to 100 Klett units, known to be linear with cell concentration.

TABLE 3. Tetrad analysis of several *nyr1* hybrids<sup>a</sup>

Hybrid	Type asci			Total asci
	PD (0:4)	NPD (2:2)	T (1:3)	
<i>nyr1</i> × <i>nyr2</i>	2	2	8	12
<i>nyr1</i> × <i>nyr5</i>	0	2	9	11
<i>nyr1</i> × <i>nyr15</i>	3	2	15	20

<sup>a</sup> Scored as sensitive:resistant colonies on YEPD plus 1% Tween 80 plates containing 75, 150, and 400 U of nystatin per ml. Abbreviation: PD, parental ditype; NPD, non-parental ditype; T, tetratype.

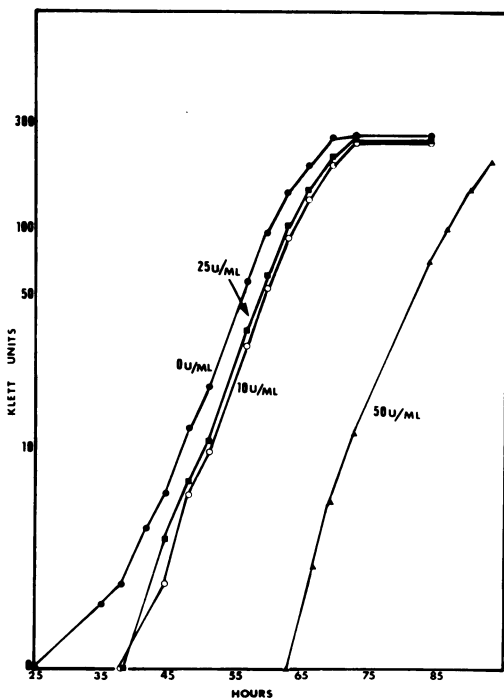


FIG. 4. Effect of adding various concentrations of nystatin (0-100 U/ml) to cultures of nystatin-resistant mutant *ole2-2* growing in liquid YEPD plus  $5 \times 10^{-6}$  M ergosterol. The addition of 100 U of nystatin per ml resulted in no growth.

others. For example, Gottlieb et al. (9) demonstrated that a cholesterol-filipin ratio of 1:4 was more effective in reversing the *in vitro* inhibition of the antibiotic than was a lanosterol-filipin ratio of 4:1.

Mutations in which (i) ergosterol is synthesized in reduced amounts, (ii) ergosterol is synthesized but must compete for sterol sites in the cell membrane with other more predominant sterols having less affinity for nystatin, or (iii) ergosterol is not synthesized but is replaced in the cell membrane by one or more sterol intermediates, could lead to resistance. The above mechanisms assume that mutant strains which do not synthesize ergosterol do make a sterol which meets the structural and functional requirements of the cell. A second required assumption is that nystatin has evolved as an antibiotic with a greater chemical affinity for sterols terminal in the metabolic pathway such as ergosterol and cholesterol than for sterols earlier in the pathway.

The biochemical evidence relating nystatin resistance in yeast to sterol synthesis reveals that the above mechanisms may be operative. The strains under consideration here such as S288C, *nyr5*, and *nyr6* most closely resemble one another in synthesizing ergosterol as the primary sterol, although GLC chromatograms of S288C and *nyr5* vary in total number of peaks. *nyr1* may or may not synthesize ergosterol as a minor sterol component. UV spectrophotometry suggests that *nyr15* synthesizes a sterol having, in addition to the normal ergosterol absorption spectrum, a band at 235 nm. This unidentified sterol differs from ergosterol most probably by the formation of a conjugated double bond system (8). Mutants *nyr2* and *ole2-2R* are allelic, and analysis of their sterols indicates the absence of ergosterol. *ole2-2* reveals a different sterol spectrum compared to the revertant *ole2-2R* or wild-type strain.

Although six separate genes were identified, it cannot be assumed that each represents a lesion in the ergosterol biosynthetic pathway. For example, at the *ole2-nyr2* locus, the *ole2* allele may be responsible for the production of a sterol early in the biosynthetic pathway resulting in a cell that is lipid requiring and petite. The alternative *nyr2* allele may produce a sterol late in the pathway wherein the cell is non-lipid-requiring and grande. If these alternative alleles produce vastly different sterols, this locus may have regulatory rather than biosynthetic functions.

It is interesting to note that wild-type, anaerobically grown yeast deprived of lipid sup-

plement show a number of similarities to the lipid-requiring *ole2*, 3, and 4 mutants. (i) Andreason and Stier (4, 5) demonstrated that the former require an unsaturated fatty acid and ergosterol for growth. (ii) Whereas the *ole2*, 3, 4 mutants are petite and, therefore, lack functional mitochondria, mitochondrial function in anaerobically grown cells is repressed. (iii) Bulder (6) has demonstrated that in *Schizosaccharomyces japonicus* anaerobically grown cells are hardly affected by the polyene antibiotic, pimarinin, compared to aerobically grown cells. (Anaerobically grown cells have one-twenty-fourth as much ergosterol as aerobically grown cells.) His observation that resistance is concomitant with an inability to synthesize ergosterol helps to explain the high levels of nystatin resistance manifested by the *ole2*, 3, and 4 mutants. The Liebermann-Burchard colorimetric reaction suggests that these mutants are blocked at or near lanosterol formation.

Keith et al. (12) established the inability of mutants *ole2-1*, *ole2-2*, and *ole3ole4* to desaturate palmitic and stearic acids given labeled acetate as precursor. They also found that these mutants required an unknown amino acid or base in synthetic complete medium. I have now established that methionine is required for growth. Parks (17) showed that the methyl group at the C-24 position of ergosterol is donated by *S*-adenosylmethionine, and in view of this it is suggested that mutations occurring just before or just after this methylation step are related to the primary genetic lesions in the *ole2*, 3, and 4 mutants. Although there is no clear correlation between the inability to synthesize ergosterol and desaturate fatty acids, Akhtar et al. (3) suggest that the mechanism by which certain double bonds in sterols are formed may be analogous to the way in which some long-chain fatty acids are desaturated. If regulation of lipid synthesis were dependent upon key desaturation steps, a lesion preventing desaturation in the sterol pathway (whether in the ring or side chain) may lead to a concomitant loss of desaturation of fatty acids.

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