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DIFFERENCES IN MEMBRANE ORDER PARAMETER AND ANTIBIOTIC SENSITIVITY IN ERGOSTEROL-PRODUCING STRAINS OF *SACCHAROMYCES CEREVISIAE*

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Gas chromatography of free sterols derived from exponential cultures of *Saccharomyces cerevisiae* strains which produce varying amounts of ergosterol indicated the presence of a previously unreported sterol peak. Gas chromatographic/mass spectral analyses of the composition of this peak have identified ergosta-5,7-dien-3β-ol, ergosta-7,22-dien-3β-ol and an unidentified sterol, molecular weight *m/z* 400, as peak components in wild-type strains. Analysis of strain *nyr5*, an ergosterol-producing slightly nystatin-resistant variant, showed this strain to be a leaky mutant of the 22(23) desaturase in the ergosterol biosynthetic pathway. Electron paramagnetic resonance (EPR) studies of membrane fluidity indicated that increased ergosterol levels resulted in higher order parameters when 5-doxyl stearic acid was employed as a membrane probe. There was no order parameter change with 7-doxyl stearic acid which seems to indicate a specificity of location in sterol packaging into the membrane. Yeast strains can be distinguished from each other based on the level of ergosterol produced by their sensitivity to the polyene antibiotic, nystatin.

Introduction

Sterols have been shown to be essential components of eukaryotic membranes, although their specific function has not been defined despite extensive study [1,2]. However, sterols have been demonstrated to alter membrane fluidity [1,3–5], membrane phase-transition temperatures [1,3,6,7], membrane permeability [8,9], the activity of membrane-bound enzymes [1,3,10] and cellular growth characteristics [11–13]. Several reports [14–17] have indicated additional roles for sterols although mechanisms for these functions remain undetermined. For example, Dahl et al. [14] have described the sparing effect of sterols where pairs of sterols have a synergistic effect on cell growth. These authors reported that the sterol-requiring procaryote, *Mycoplasma capricolum*, grew at an elevated rate in a medium supplemented with a high concentration of lanosterol and a low concentration of cholesterol. The growth rate observed was greater than the combined rates obtained when the medium was supplemented with high lanosterol or low cholesterol concentrations. Since the bulk physical state of the membranes of cells grown on cholesterol plus lanosterol versus lanosterol alone was shown to be identical, a more specific function for the sparing effect of low levels of cholesterol is implied. Subsequent to this observation, the same authors [15] reported that low cholesterol levels also synergistically affect the rates of lipid and protein synthesis and fatty acid uptake.

Rodriguez et al. [16,17] have recently reported a sterol-sparking effect on the growth of sterol auxotrophs of *Saccharomyces cerevisiae* where a small

amount of normal end-product sterol (ergosterol) was required for the initiation and maintenance of growth. The specific amount of ergosterol necessary to elicit this response was much less than the minimum amount required to support growth. Therefore, a second sterol (cholestanol, in this case) was required to satisfy the bulk membrane requirements of the cell. If present in greater amounts, ergosterol alone could support growth, satisfying all sterol requirements of the cell, while cholestanol could satisfy only the bulk membrane requirements.

Most eukaryotic organisms rigorously regulate sterol synthesis [7], making quantitative and qualitative changes in sterol content possible in only a few systems such as erythrocytes [3] and species of *Mycoplasma* [4]. *S. cerevisiae* has provided an ideal system in which to study the role of sterols in membranes, since sterol variability can be achieved. Studies utilizing mutants blocked at various stages of ergosterol synthesis [18–21] have been used to demonstrate the effects of sterol substitution, since these mutants incorporate sterol intermediates into their membranes in place of ergosterol. EPR and crystal violet dye uptake studies [9,8] of these mutants have shown significant alterations in membrane permeability. In addition, EPR studies have indicated changes in membrane fluidity in these cells [5]. Sterol substitution has also been shown to alter growth characteristics on a variety of energy sources [13].

Yeast strains which have quantitative differences in ergosterol levels have also been described. Downing et al. [22] and Bard and Downing [23] have isolated and characterized several yeast strains in which 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in sterol biosynthesis, is deregulated. As a result, these strains produce increased levels of squalene and total sterols. A low ergosterol-producing strain originally reported by Bard [24] will be shown in this communication to be a leaky sterol biosynthetic mutant accumulating a high level of squalene and significantly reduced sterol levels. This strain, *nyr5*, was isolated based upon a weak resistance to the polyene antibiotic, nystatin.

In this report, sterol content, as determined by gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS), in strains producing altered levels of sterol will be correlated with membrane-structural alterations and antibiotic sensitivity. EPR techniques using spin-labeled fatty acids have been widely applied to a number of membrane systems, including yeast [5]. Here, 5-doxyl stearic acid (5DS) and 7-doxyl stearic acid (7DS) will be employed to show a significant change in membrane fluidity at one position in the membrane, while a nearby position remains unchanged. Since sterol biosynthetic mutants lacking ergosterol are typically selected on the basis of polyene resistance [21,24,25], the level of antibiotic sensitivity of cells producing varying amounts of ergosterol should be related to ergosterol content. Data will be presented to indicate such a correlation.

Materials and Methods

Strains. Strain *ole3*R is derived from the heme mutant strain *ole3*. *ole3* cannot complete ergosterol biosynthesis. Downing et al. [22] have isolated an *ole3* variant, designated R6K6, which shows increased HMG-CoA reductase activity and increased sterol levels but still cannot synthesize ergosterol. *ole3* and R6K6 ergosterol-producing revertants were selected spontaneously by plating on yeast minimal medium (0.67% yeast nitrogen base and 2% dextrose) and designated *ole3*R and R6K6R, respectively [23]. *ole3* was originally derived from the wild-type strain, S288C. *nyr5* was selected as a nystatin-resistant mutant of strain S288C [24].

Growth. Cells were grown aerobically at 30 °C in a medium containing 1% Difco yeast extract, 2% Bacto-peptone and 2% dextrose (medium 1, YEPD. All growth was monitored using a Klett-Summerson colorimeter with a No. 66 red filter. Exponential phase cells were harvested at Klett readings ranging from 90 to 150, while stationary phase cells were harvested at readings of 360 Klett units or greater.

Cells for sterol analysis using GC and GC-MS were grown in medium 1 containing 5% dextrose. For exponential phase cells, 300-ml cultures were grown to Klett readings of 100. A 50-ml fraction to be used for dry weight determination and a 200-ml fraction which was saponified for sterol

analysis were collected by centrifugation. 50-ml cultures divided into a 5-ml fraction for dry weight analysis and a 20-ml fraction for saponification were prepared for stationary phase cells.

Cells for nystatin-sensitivity studies were grown overnight in medium 1. Cell samples of appropriate volume were collected by centrifugation ($5000 \times g$) and suspended in 20 ml fresh medium 1 in 250 ml nephelometer flasks to yield starting concentrations of 8–20 Klett units. Nystatin stocks (4 mg/ml) were prepared in dimethylformamide and stored frozen. Appropriate amounts of thawed nystatin stock were added to each nephelometer. All flasks including the control were adjusted to the same dimethylformamide concentration. Growth was calculated as the number of generations per 24 h. For those samples reaching stationary phase before 24 h, mean generation times were determined and the number of generations per 24 h period was calculated by extrapolation.

GC Analysis and GC-MS. Nonsaponifiable sterols were extracted into *n*-heptane (10 ml) according to the method of Breivik and Owades [26]. The extracts were evaporated to dryness under a stream of nitrogen and after redissolving in 0.5 ml *n*-heptane were analyzed on a Hewlett-Packard 5710 gas chromatograph. The following columns were used: (a) SE-30, 1% on Gas Chrom Q (80–100 mesh), 6 ft glass column at 240 °C, (b) OV-17, 1% on Gas Chrom Q (100–120 mesh), 6 ft glass column at 270 °C. The carrier gas was nitrogen at 60 ml/min for both columns and retention times of sterols were calculated relative to cholestane, cholestanol, or cholesterol. To ascertain the sterols not resolvable by the above analyses, GC-MS analysis was utilized.

The GC separations and mass analysis were carried out on a Finnigan MAT Model 4500 GC/MS/DS instrument. The GC separation was carried out on a 15 m × 0.323 mm Durabond DB-5 column with 0.25 μm film thickness. The column oven temperature was programmed from 200–300 °C at 10 K per min and the carrier gas was helium at a linear velocity of 60 cm/s. The capillary column was interfaced directly into the mass spectrometer ion source. The indicated ion source temperature was 150 °C. The mass spectrometer was operated in the electron impact mode at 70 eV ionizing potential. The mass spectrometer was scanned from m/z 50 to m/z 600 with a 1 s scan-time. Mass spectra were enhanced by manual subtraction when necessary.

EPR sample preparation. Spin label dissolved in ethanol was evaporated by a stream of nitrogen to a thin film on the bottom of a 15-ml conical centrifuge tube. A 100-μl cell sample suspended in a small volume of medium (approx. 20 μl) was transferred to the centrifuge tube containing the spin label. The mixture was vortexed and transferred into a 110-mm microcap (outside diameter 1.2 mm, inside diameter 0.9 mm, SF type; Drummond Scientific, Bromall, PA). Following flame-sealing of one end, the mixture was centrifuged ($800 \times g$ at 25 °C, 1 min). The tip containing the sample of packed, labeled cells was broken off and excess liquid above the sample was removed.

EPR data acquisition and analysis. EPR spectra of the spin-labeled cells were obtained with an X-band spectrometer comparable to a Varian V4502. A cylindrical, large sample-access cavity (Varian V4535) fitted with a fused silica variable temperature dewar was used. Spectrometer parameters for observation of the spin-label signals were: microwave frequency, 9.1 GHZ; microwave power, 33 mW; central magnetic field 3240 G; magnetic field modulation, 2.3 G at 50 kHZ; and magnetic field sweep, 100 G in 5 min with a time constant of 0.6 s. The nitroxyl spin labels used, 5-doxyl stearic acid (Syva, Palo Alto, CA, now available from Aldrich, Milwaukee, WI) and 7-doxyl stearic acid (Molecular Probes, Junction City, OR) are hydrophobic and locate themselves preferentially in membranes. The spectra produced from the EPR signal were typical of those previously reported [5]. The EPR signal from the spin label allows for determination of an order parameter, S, from the expression,

$$S = \frac{T_{\parallel} - T_{\perp} - C}{T_{\parallel} - 2T_{\perp} + 2C}(1.66), \quad C = 1.4 - 0.053\,(T_{\parallel} - T_{\perp})$$

where $T_{\parallel}$ and $T_{\perp}$ are the apparent parallel and perpendicular ^{14}N hyperfine interaction parameters of the spectrum measured in gauss [27]. S may vary from zero to 1 with typical values from membranes in the range 0.5–0.7. Lower values of S correspond to more freedom of motion of the spin label and thus a more fluid membrane. For

individual samples, the temperature was constant to within $\pm 0.1°C$. The temperature values for all the spectra were within a 2°C range of 25°C. All order parameters were corrected to 25°C using $dS/dT = -0.0007°C^{-1}$ (where T is the temperature). In addition, the isotropic hyperfine coupling constant, a, was (computed from Ref. 27) $a = 1/3(T_{\|} + 2T_{\perp} + 2C)$. The value of a depends on the polarity of the solvent (or environment). Values of a obtained in these experiments (15.3 G) were characteristic of the nitroxyl being in a non-ionic environment, indicating that the label molecules were located in lipid regions.

Results and Discussion

This investigation describes the sterol composition, membrane structural properties, and antibiotic sensitivity of four strains of *S. cerevisiae*. Strains S288C, *ole3*R, and R6K6R are wild-type ergosterol-producing strains. *ole3*R and its variant, R6K6R, are low and high ergosterol-producing strains, respectively [22,23]. Strain *nyr5* is the lowest ergosterol producer; it is slightly nystatin-resistant and was derived from strain S288C, which itself is a low ergosterol producer [24]. We will show that *nyr5* is a leaky mutant in the ergosterol biosynthetic pathway.

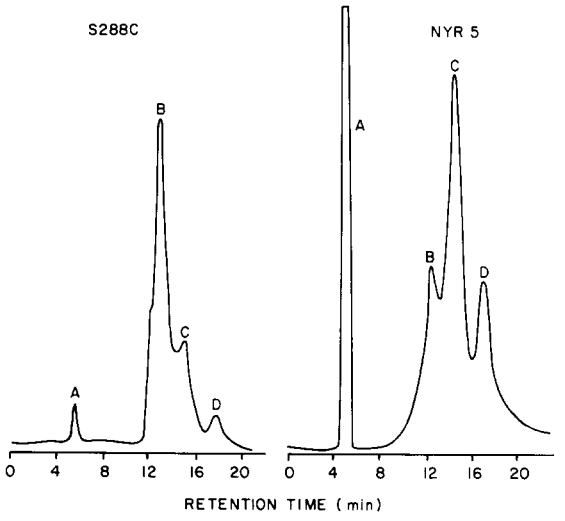

Fig. 1. Gas-liquid chromatogram of free sterols from strains S288C and *nyr5*. A, squalene, B, ergosterol, C, previously unreported sterol peak, D, lanosterol.

Sterol analysis of wild-type and mutant strains

Gas chromatography of free sterols on 1% SE-30 and 1% OV-17 columns was utilized to determine the types of sterols and the relative amounts of sterols accumulated by mutant and wild-type strains. Fig. 1 shows the spectra obtained from exponential cultures of the wild-type, S288C, and the weakly nystatin-resistant strain, *nyr5*. For S288C, the sterol precursor, squalene, was evident (peak A) along with ergosterol (peak B) and lanosterol (peak D). The identification of these compounds was authenticated by using squalene, ergosterol and lanosterol standards. In older cultures of wild-type strains, zymosterol has been shown to be present as a discrete peak [18,28]. In the early exponential cultures used here, zymosterol accumulation is represented as a shoulder on the left side of the ergosterol peak. Similarly, 24(28)-dehydroergosterol has been reported as an end-product of wild-type yeast sterol synthesis [29]. This sterol does not accumulate in these early cultures. The presence of peaks A, B, and D is in agreement with previous reports (23,29). Peak C, however, represents a previously unidentified sterol in wild-type *S. cerevisiae*. Using an authentic standard, peak C was shown not to be 24(28)-dehydroergosterol. The chromatogram of *nyr5* shows a large increase in the squalene component, decreased levels of ergosterol and lanosterol and a significantly increased amount of peak C sterol.

TABLE I

SQUALENE AND TOTAL STEROL ACCUMULATION IN YEAST STRAINS

Cells were harvested in mid log phase designated as (e) for exponential or stationary phase designated as (s). Sterols were quantified by weight determination of peak areas from original chromatograms. The values obtained were normalized using cell dry weight determinations from each culture. All values relative to amounts of squalene and total sterols accumulated in S288C. Values in parentheses indicate total numbers of independent determinations for squalene and sterol values.

Strain	Squalene	Total sterols
S288C (e)	1.00	1.00 (6)
*ole3*R (e)	0.61	1.52 (6)
R6K6R (e)	3.08	2.77 (3)
nyr5 (e)	38.92	0.44 (3)
nyr5 (s)	1.75	0.65 (e)

TABLE II

GAS CHROMATOGRAPHIC ANALYSIS OF PEAK C STEROLS ACCUMULATED BY WILD-TYPE AND MUTANT YEAST STRAINS

Values in parentheses are normalized by peak area for the largest peak in each strain.

Peak	Retention time (s)	*m/z* (normalized)		
	Strain:	S288C	*nyr5*	*pol5*
C_2	249	398 (0.25)		398 (0.1)
C_1	255	398 (0.25)	398 (1.0)	398 (0.8)
C_3	257	400 (0.1)		400 (0.25)

The chromatograms obtained for *ole3*R and R6K6R, strains that were previously demonstrated to be low and high producers of ergosterol, respectively [22,23], were typical of wild-type yeast spectra with the exception that they also showed small peaks corresponding to peak C. The relative amounts of squalene and total sterols for all four strains are shown in Table I.

The identification of the peak C sterol could not be resolved using the SE-30 and OV-17 columns. Gas chromatography using a capillary column and mass spectrometry analysis of this sterol peak in S288C and *nyr5* were performed. A sterol biosynthetic mutant, *pol5*, which has been characterized [19–21,29,30] as unable to accomplish 22(23) desaturation in ergosterol biosynthesis and which accumulates ergosta-5,7-dien-3β-ol, zymosterol and ergosta-7,24(28)-dien-3β-ol as principal sterols, was also analyzed to provide standards for these sterol intermediates. When prepared in the same manner as the other strains, *pol5* was also found to contain a peak analogous to peak C.

Peak C in strains S288C and *pol5* was resolved into three components (C_1, C_2 and C_3) with molecular ions at *m/z* 398, 398, and 400, respectively. Peak C in *nyr5* yielded a single peak with a molecular weight of 398. Peak C_1 was found as a relatively minor component (25% the amount of the major sterol, ergosterol) in S288C, a major component (80% of the major sterol peak, zymosterol) in *pol5*, and the only peak C sterol in *nyr5* (Table II). This sterol component was identified as ergosta-5,7-dien-3β-ol by comparing the spectrum with that of an authentic sample [30]. The molecular ion *m/z* 398 and fragment ions *m/z* 383, 380, 365, 339, 271 and 253 (see Table III) were indicative of this structure and the ion *m/z* 253 confirmed that both double-bonds were in the sterol nucleus. On this basis, we conclude that *nyr5* is a leaky mutant of the 22(23) desaturase.

The second peak C component with a molecular ion at *m/z* 398 (C_2) was found as a minor component in S288C and *pol5* (25 and 10% of the major sterol, respectively, Table II). This sterol yielded a spectrum very similar to the reported spectrum for an authentic sample of ergosta-7,22-dien-3β-ol [31]. The major high mass fragment ions and ion intensities are shown in Table III.

Component C_3 was found as a minor sterol in S288C and *pol5* (10 and 25% of the major sterol, respectively, Table II). This sterol could not be identified due to interference from ergosta-5,7-dien-3β-ol. The *m/z* 255 ion (Table III) indicates a double-bond in the sterol nucleus. This ion was probably the result of the loss of water and the alkyl side-chain as a neutral fragment.

The appearance of peak C sterols in our wild-type strains results from the fact that we are examining our cultures during early exponential growth. Previous GC analyses of yeast sterols have

TABLE III

MAJOR HIGH MASS FRAGMENT IONS AND RELATIVE IONS INTENSITIES (R.I.) OF ERGOSTEROL AND PEAK C STEROLS

	Molecular weight: *m/z* (R.I.)	Fragment ions: *m/z* (R.I.)
Ergosterol	396(40)	378(2), 363(45), 337(18), 271(10), 253(21)
Ergosta-7,22-dien-3β-ol	398(31)	383(21), 365(7), 271(22)
Ergosta-5,7-dien-3β-ol	398(49)	383(6), 380(3), 365(68), 339(30), 271(40), 353(13)
Unidentified	400(7)	385(2), 273(4), 255(16)

used much older cultures, most typically 18-h and 48-h cultures [18,32]. It has been reported that yeast increase sterol synthesis during the stationary growth phase [33]. Table I shows an analysis of squalene and sterol content from a stationary phase culture of *nyr5*. Under these conditions, the overall profile approaches that of the wild-type strain with most of the squalene converted to sterol and an increase in ergosterol level at the expense of peak C sterol. Evidently, peak C sterols are also converted to ergosterol during stationary phase in wild-type cultures.

EPR analysis

EPR was employed to ascertain the effects of sterol concentration (primarily ergosterol, except for *nyr5*) on the physical state of the cytoplasmic membrane. Different levels of the membrane were examined using the spin labels 5-doxyl stearic acid and 7-doxyl stearic acid. Table IV shows membrane order parameter data for all four strains in both exponential and stationary phases of growth. The results using 7-doxyl stearic acid show no significant difference in order parameter for *nyr5* and its wild-type parent, S288C. Likewise, *ole3*R and R6K6R do not vary from one another. However, when 5DS is used, *nyr5* varies significantly from S288C as does R6K6R from *ole3*R. *nyr5* shows increased fluidity, while R6K6R shows decreased fluidity. This correlates with previous observations, indicating that increased sterol content (Table II) results in increased rigidity (decreased fluidity) of membranes in the liquid-crystalline state [1,34]. The amount of sterol present in the membrane has a dramatic effect on membrane structure at one level, while just two carbons deeper into the membrane, the fluidity is unaltered. In all cases, stationary phase cultures show more rigid membranes than do exponential cultures due to elevated sterol synthesis during stationary phase. This increased packing of sterol into the membrane reduces the order parameter differences noted in exponential cultures. This phenomenon has also been reported for ergosterol-deficient yeast strains using 5-doxyl stearic acid [5].

TABLE V

PERCENT GROWTH INHIBITION OF EXPONENTIAL CULTURES OF *S. CEREVISIAE* DUE TO NYSTATIN

Data given as mean of duplicate experiments. Values of each experiment are given in parentheses.

Strain	1 mg/l Nystatin	2 mg/l Nystatin	4 mg/l Nystatin
S288C	58 (57,59)	65.5(66,65)	92.5(95,90)
nyr5	36.5(32,41)	51.5(43,48)	74 (87,61)
*ole3*R	63 (60,66)	78 (83,73)	91.5(95,86)
R6K6R	71.5(72,71)	90 (94,86)	93.5(97,90)

Antibiotic sensitivity

Since sensitivity to the antibiotic, nystatin, is based on its interaction with ergosterol in the cell membrane [35], it was of interest to know whether nystatin sensitivity could be used to distinguish among strains producing varying amounts of this sterol. The inhibition of growth by varying concentrations of nystatin for exponential cultures of the four strains is shown in Table V. Based upon the relative sterol content of the four strains (Table I), an order of sensitivity (most sensitive to

TABLE IV

MEMBRANE ORDER PARAMETER, *S*, OF WILD TYPES AND STEROL MUTANTS OF *S. CEREVISIAE* IN EXPONENTIAL AND STATIONARY GROWTH PHASES

Data given as mean ± S.D. Value in parentheses are numbers of replicas.

Strain	5-Doxyl stearic acid		7-Doxyl stearic acid	
	exponential phase	stationary phase	exponential phase	stationary phase
S288C	0.580 ± 0.007 (24)	0.595 ± 0.012(11)	0.582 ± 0.007 (6)	0.611 ± 0.007(26)
nyr5	0.566 ± 0.005 (14)	0.579 ± 0.006 (9)	0.586 ± 0.009 (6)	0.609 ± 0.007(24)
*ole3*R	0.571 ± 0.010 (24)	0.600 ± 0.008(18)	0.592 ± 0.006(20)	0.614 ± 0.006(12)
R6K6R	0.585 ± 0.007 (14)	0.607 ± 0.012(21)	0.595 ± 0.007(16)	0.620 ± 0.006 (9)

least sensitive) of R6K6R > *ole3*R > S288C > *nyr5* would be expected. This sequence is clearly evident at concentrations of 1 mg/l and 2 mg/l of nystatin. This order becomes obscured at 4 mg/l of nystatin where the inhibition for three of the strains approaches 100% and only *nyr5* is distinguishable as being somewhat resistant.

In this investigation, we have noted the appearance in wild-type yeast of three previously unreported sterol intermediates during the early phases of growth. One of these intermediates, ergosta-5,7-dien-3β-ol, was identified as the major sterol component in *nyr5* during the exponential growth phase. Since this sterol is also a major sterol accumulated by the previously characterized sterol mutant, *pol5, nyr5* was concluded to be a leaky mutant of *pol5*. EPR analysis of the ergosterol-producing strains indicated that increased and decreased sterol synthesis does affect the physical state of the cytoplasmic membrane. Specifically for a sterol overproducing strain, R6K6, excess sterol, rather than being stored in lipid vacuoles, was packed into the membrane resulting in an elevated order parameter. The increased nystatin sensitivity of strain R6K6 also indicates the presence of elevated levels of ergosterol in the cytoplasmic membrane. The overall effect of sterol content on membrane fluidity was noted using the spin label 5-doxyl stearic acid but was not seen when 7-doxyl stearic acid was employed. This observation may mean that the cell has a location specificity for sterol packed into the membrane. Finally, we have conclusively shown that nystatin sensitivity can be used to distinguish among yeast strains, including wild types, that produce varying amounts of ergosterol.

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