YEAST MUTANTS REQUIRING ERGOSTEROL AS ONLY LIPID SUPPLEMENT

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Summary: Mutants of Saccharomyces cerevisiae were isolated which required ergosterol or cholesterol as the only lipid supplement. They also required methionine, were petite, and showed complete absence of respiratory cytochromes. Revertants of these strains grew without ergosterol and methionine, were grande, and had respiratory cytochromes. Most revertants did not make ergosterol and were nystatin resistant. Sterol analysis and enzyme assays suggested a block in sterol formation after lanosterol.

Owing to interest in regulation of sterol biosynthesis in mammalian cells and yeast, and to the generally accepted view of the importance of sterols in membrane structure, it was thought desirable to study regulation of sterol biosynthesis with yeast mutants which require ergosterol for growth. Nystatin resistant mutants have been described in which little or no ergosterol was formed (1,2), but they do not require added sterol for growth. In this report are described a group of mutants which require ergosterol for growth. They also require methionine, and, with the exception of one strain, they are petite. Similar mutants were recently described by Karst and Lacroute (3) which required additional supplementation with oleic acid.

Materials and Methods: The parent strain used in this study was Saccharomyces cerevisiae D587-4B (α his 1-1). Minimal medium was 0.67% Difco yeast nitrogen base (without amino acids), 2% glucose, and 20 mg per liter of L-histidine. Complete medium was 1% yeast extract, 2% Bactopeptone and 2% glucose (YPD). Plates were prepared with 2% Bacto agar. A 0.2% solution of ergosterol in ethanol-Tween 20 or Tween 80 (1:1), or in ethanol-Tergitol (2:1) was added to a final concentration of 20 mg/liter. Petites were identified by inability to grow on YPD in which glucose was replaced by 3% glycerol, and by the tetrazolium overlay technique (4).

Erg$^-$ mutants were obtained from the parent strain or from nystatin resistant derivatives. Strain D587 was plated on YPD containing ergosterol in Tween 20, and irradiated by a 15W germicidal lamp for 20-60 seconds to 10-20% survival. After 48 hr growth at 30° colonies were replicated to YPD plates. Ergosterol was required for growth of 6 strains out of 20,000 examined. Nystatin resistant mutants were isolated by plating strain D587 on varying concentrations of nystatin in synthetic medium. Alternatively, wild type cells were mutagenized with ethyl methanesulfonate or N-methyl-N'-nitro-N nitrosoguanidine and then spread on nystatin plates. Resistant strains which showed low levels of ergosterol were mutagenized again and mutants selected for ergosterol requirement as described

above. We obtained 10 Erg^- strains in this manner and 1 Erg^- strain which arose spontaneously from a nystatin resistant strain. Petite strains were obtained from strain D587 treatment with ethidium bromide (5). Erg^+ revertants were isolated on YPD plates in the absence of ergosterol, and Met^+ revertants on minimal agar containing ergosterol. (Mutagen was present in most procedures.)

Extracts were prepared by breaking washed cells (grown to late log phase on 1% peptone, 0.5% yeast extract and 0.5% glucose) in an Eaton press (6), and centrifuging the suspension at 800 x g for 5 min at 2°. Published procedures were used to determine acetoacetyl CoA thiolase (7), HMGCoA synthase (7), HMGCoA reductase (8), citrate synthase (9), formation of squalene and lanosterol from ^{14}C-mevalonate (10), and ubiquinone (11). α-Ketoglutarate dehydrogenase was determined (12) on mitochondria prepared according to Schatz and Kovac (13).

Non-saponifiable lipid fractions were prepared from whole cells or cell extracts by the method of Molzahn and Woods (14). UV absorption was recorded in spectroscopic grade n-heptane with a Cary 15 spectrophotometer. Sterol fractions were separated with benzene-ethyl acetate (5:1) on silica gel plates impregnated with 3% $AgNO_3$, and located with Liebermann-Burchard reagent (15). Gas liquid chromatography (glc) was carried out on trimethylsilylated mixtures of sterols with a Hewlett-Packard 7300 gas chromatograph and a 6 x 180 cm glass column of 1% neopentylglycolsuccinate on Chromosorb W (80-100 mesh) at 220° and a flow rate of 40-50 ml per min.

Results: Isolation and Properties of Ergosterol Requiring Mutants. It was considered that nystatin resistant strains, already deficient in ergosterol, would be more effective as a source of ergosterol requiring mutants. However, isolation of Erg^- strains of identical phenotype proved possible not only from nystatin resistant mutants, but also from wild type cells. Nystatin resistant strains chosen for study showed very low UV absorption in the non-saponifiable lipid fraction at 250-300 nm indicating low levels of ergosterol. They grew on YPD with a generation time of approximately 3 hr as compared to 1.5 hr for wild type cells and 4 hr for petite strains. Erg^- mutants were isolated from nystatin resistant strains on YPD medium plus ergosterol in Tween 20 in order to avoid recovery of oleic acid mutants which appear when Tween 80 is used. Erg^- mutants obtained directly from wild type by UV irradiation showed similar properties. All Erg^- strains were petite, required methionine or homocysteine for growth, and had a generation time of approximately 3 hr. Although cholesterol was able to replace ergosterol in all strains there was no growth in the absence of sterol.

Sterol Analysis of Mutants. Surprisingly ergosterol was not the predominant sterol in our strain of wild type yeast but a sterol (or sterols) which was not further identified and showed in glc a relative retention time (rrt) compared to squalene of 3.9 (Fig. 1A). Ergosterol constituted only 10% of total sterols,

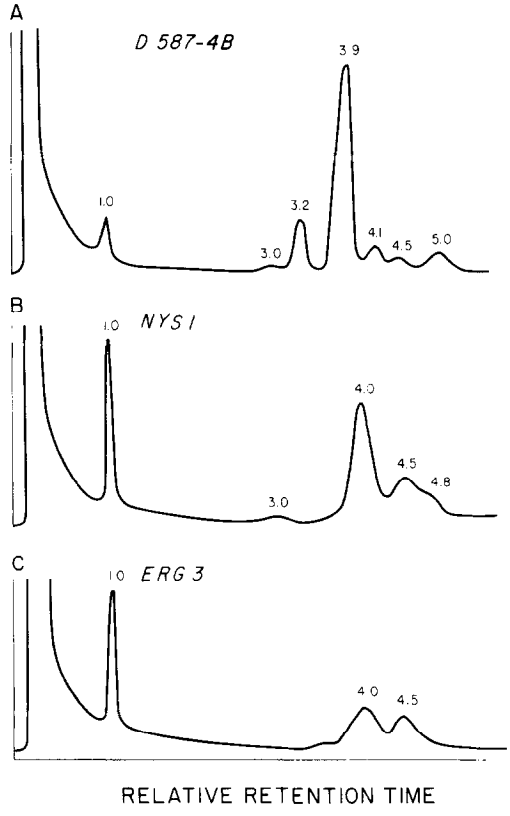

Fig. 1. Gas-liquid chromatography of sterol trimethylsilyl ethers. Curves were normalized for constant weight and sensitivity. Retention time is relative to squalene; ergosterol and cholesterol had rrt 4.1 and 2.8, respectively.

and squalene and lanosterol were present in trace amounts (<1%). An identical pattern was found in a petite mutant of strain D587. Ergosterol was absent in a nystatin resistant strain (Nys3), but lanosterol and squalene were present in considerably higher amounts than in wild type cells, and the major fraction was a sterol (or sterols) with rrt 4.0 (Fig. 1B). In Erg$^-$ mutants grown in limiting amounts (0.1 mg/liter) of ergosterol squalene was the predominant intermediate, and the unknown sterol fraction with rrt 4.0 was present in relatively lower quantities than in nystatin resistant strains (Fig. 1C). Erg$^-$ strains grown on optimal amounts (20 mg/liter) of ergosterol or cholesterol incorporated these sterols, but showed lower levels of endogenous sterols.

Preliminary attempts to analyze the sterol fraction from wild type cells by thin layer chromatography afforded separations into a weak lanosterol band (R_F

Table 1. Enzyme Activities in Yeast Mutants Relative to Wild Type Levels

	Yeast Strains					
Enzymic activities	Petite	Nys1	Nys3	Erg3[a]	Erg2[b]	Erg11[b]
Acetoacetyl-CoA thiolase	0.81	0.75	0.75	0.25	0.38	0.39
HMGCoA synthase	0.77	1.0	1.0	0.33	0.10	0.41
HMGCoA reductase	1.2	0.67	0.82	0.45	0.25	0.46
Conversion of ^{14}C-mevalonate to squalene	1.2	0.61	0.76	0.22	0.10	0.31
Conversion of ^{14}C-mevalonate to lanosterol	1.2	1.0	0.38	0.22	0.04	0.01
Citrate synthase	0.13	1.0	0.70	0.16	0.15	0.11
Glucose-6-phosphate-dehydrogenase	1.0	1.0	1.0	0.9	1.0	1.1

[a] Grown on limiting sterol; optimal sterol gave similar values.
[b] Grown on optimal sterol concentrations.

0.63), a small ergosterol band (R_F 0.23), and a large band with intermediate R_F (0.45) which gave a blue color changing to blue-green in the Liebermann-Burchard reaction. In nystatin resistant mutants a band was obtained corresponding to lanosterol and a larger band (R_F 0.45). Both bands gave a yellow color in the Liebermann-Burchard reaction which changed to yellow-green. Similar results were obtained with Erg$^-$ mutants.

Enzyme Activities of Ergosterol Deficient Mutants. An effort was made to associate the observed alterations in sterol metabolism with an enzyme deficiency (Table 1). A petite strain had essentially wild type activity of ergosterol biosynthetic enzymes, but it was quite deficient in citrate synthase, a mitochondrial enzyme. On the other hand, glucose-6-phosphate dehydrogenase, a cytosol marker, was normal in all strains. Nystatin resistant mutants had essentially normal acetoacetyl CoA thiolase, HMGCoA synthase, and HMGCoA reductase. Nys1, which had low levels of ergosterol, was closer to normal than Nys3 in conversion of mevalonate to lanosterol. In accord with their grande phenotype these strains

had normal levels of citrate synthase. However, ergosterol requiring mutants (Erg2, Erg3, and Erg11) had approximately 1/4 to 1/3 wild type activity of sterol biosynthetic enzymes, and like the petite strain, were also deficient in citrate synthase. Enzyme activities in cell extracts of Erg3 remained unchanged whether grown in limiting or optimal sterol, but Erg1 and Erg11 (grown on optimal sterol) showed very low conversions of ^{14}C-mevalonate to lanosterol. α-Ketoglutarate dehydrogenase was 20-30% of wild type levels in mitochondria of one petite and one Erg$^-$ strain (Erg13). Incorporation of label into non-saponifiable fraction of cells grown in presence of methyl-^{14}C-methionine was normal in a petite strain, 60% of wild type in Nys1 and Nys4, 28% in Erg5 and Erg13, and 44% in a revertant of Erg5. Ubiquinone levels were normal in a petite strain, and 70% of wild type levels in Erg2 and Erg5. Low temperature spectroscopy (16) on suspensions of whole cells (500 mg/ml) in collaboration with Dr. M. Erecinska and Dr. W. D. Bonner indicated complete absence of respiratory cytochromes (a,b,and c) in Erg$^-$ mutants, and an essentially wild type pattern in the revertants.

Isolation of Revertants. Revertants of Erg$^-$ mutants (originating in wild type or nystatin resistant strains) were obtained by selecting separately for Met$^+$ and Erg$^+$ in order to determine whether the ergosterol and methionine requirements resulted from a single mutation. All revertants selected for Met$^+$ were also Erg$^+$ and grande, and those selected as Erg$^+$ were also Met$^+$ and grande. Selection on complete glycerol-ergosterol media did not yield grande revertants. Only one revertant, Erg5R, selected as Met$^+$, still required ergosterol and was grande. Most revertants were resistant to nystatin, and showed sterol patterns very similar to those found in nystatin resistant mutants. Only 2 revertants had wild type phenotype.

Discussion: Ergosterol is the only lipid required for growth by the Erg$^-$ mutants described in this report. The phenotype of these strains also includes respiratory deficiency and requirement for methionine. Owing to complex formation between ergosterol and nystatin (17) the mutants can not be tested unequivocally for nystatin resistance. However, since nearly all of the revertants were nysta-

tin resistant it seems likely that the parent Erg$^-$ strains were also resistant to this antibiotic. The ole mutants of Resnick and Mortimer (1,18) have a similar phenotype (petite, Met$^-$, nystatin resistant), except that they require either oleic acid or ergosterol for growth. The mutants recently described by Karst and Lacroute (3) are also petite, Met$^-$ and Erg$^-$, but require supplementation with oleic acid. Homocysteine was able to replace methionine in our mutants as in those of Karst and Lacroute (3). Although ergosterol is the only lipid requirement for growth of the present mutants, it is apparent that they are similar to the two types previously reported.

Although yeast strains are known to differ considerably in their sterol content, the wild type strain used in this investigation differed extremely from others tested in this laboratory and elsewhere. Ergosterol was a minor component, and a sterol fraction as yet unidentified was the main constituent of the non-saponifiable fraction. Nystatin resistant strains and Erg$^-$ mutants had a different main sterol fraction with rrt 4.0, and 10-fold higher concentrations of squalene and lanosterol, suggesting a defect in the conversion of lanosterol to other sterols.

A single mutation appears to be responsible for the complex phenotype of the Erg$^-$ mutants, since nearly all revertants had the Erg$^+$, Met$^+$, and grande phenotype; respiratory cytochromes, entirely absent in the mutants, were present in revertants. (Genetic analysis is in progress.) Since petite mutants (ρ^-) showed normal sterol patterns and sterol biosynthetic enzymes, the ergosterol requirement of Erg$^-$ mutants was not a consequence of a petite mutation. Supplementation with ergosterol on complete medium did not result in normal growth rate or relieve respiratory deficiency. Hence, it is suggested that the respiratory deficiency was due to a mutation in sterol biosynthesis or its regulation, and that biosynthesis of sterol intermediates is simultaneous with and essential to formation of the respiratory apparatus.

Addendum. Further work has shown that hemin or protoporphyrin IX satisfied the growth requirement for ergosterol and methionine in all Erg$^-$

mutants. δ-Aminolevulinic acid replaced ergosterol in two mutants. Representative strains, grown with hemin, had sterol patterns similar to those of nystatin resistant strains, but were still respiratory deficient.

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