ISOLATION OF PLEIOTROPIC YEAST MUTANTS REQUIRING ERGOSTEROL FOR GROWTH

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Summary : Mutant strains of *Saccharomyces cerevisiae* which require ergosterol for growth have been isolated. These mutants are all petite and require a fatty acid. Several mutants require methionine in addition. These mutants have been classified into 6 complementation groups. For one of the mutants the enzymatic block has been localized after lanosterol. These mutants do not show a stringent requirement for ergosterol, as sitosterol, stigmasterol or cholesterol also support growth. Mutants of this type will be of value not only in studies of sterol biosynthesis, but also in assessing the biological role of sterols in the cytoplasmic yeast membrane. Similar mutants but without a stringent requirement for a sterol have been previously isolated by Resnick and Mortimer (8).

The precise role of ergosterol in yeast metabolism has not yet been determined. The requirement of sterols for growth by these organisms has been well documented (3, 4, 9, 11 and 12). Anaerobic growth prevents *Saccharomyces cerevisiae* from producing ergosterol ; in which case, sterols must be supplied to the culture. In the absence of sterols, growth stops after 5 to 7 generations and the cells begin to die 24 h afterwards (6). An ultrastructure analysis reported by Mopurgo et al. (6) shows that yeast cells grown in these conditions usually do not have any internal structure, i.e., cytoplasmic membrane, nuclear membrane, vacuoles and mitochondria.

Since it is well known that all the membraneous systems of the yeast cell contains ergosterol it seems reasonable to conclude that ergosterol is required for the formation of the membranes. In spite of this fact and the numerous enzymes involved in the ergosterol biosynthetic pathway, mutants which absolutely require ergosterol for growth have not yet been obtained. Strains which are unable to synthesize ergosterol have been obtained by selecting for strains resistant to nystatin, but these mutants display normal growth characteristics (14). Mutants that require either oleic acid or ergosterol have been isolated and genetically analyzed by Resnick and Mortimer (10). A recent study by Bard (5) shows that these strains are unable to synthesize ergosterol.

We have obtained by conventional mutagenesis and selection (by diagnostic replica plating) 11 yeast mutants which simultaneously require ergosterol, and a fatty acid for growth.

MATERIAL AND METHODS : *Saccharomyces cerevisiae* wild type, *FL100*, was mutagenized with nitrous acid to 1% survival. The cells were incubated on complete medium supplemented with Tergitol np40 (1 mg/ml), ergosterol (100 µg/ml) and oleic acid (200 µg/ml). After growth the cells were replicated to complete medium without supplements. Among approximatively 40,000 colonies tested, 11 mutants were found to be auxotrophic for ergosterol and oleic acid.

The genetic analyses were performed by the methods of Mortimer and Hawthorne (7). Difco Yeast nitrogen base without amino acids was used as minimal medium. The complete medium (YPG) contained yeast extract (1%), bacto peptone (1%) and glucose (2%).

Sterol extraction : Yeast cultures were centrifuged, washed once with distilled water before being acid saponified for 1 h at 30° in 0.1 N HCl (12). After additional washing the pellets were vacuum dried. The total lipids were extracted with chloroform-methanol (2:1, V/V). The lipid extract was vacuum dried with a rotary evaporator and saponified for 1 h in methanolic KOH (10%, W/V) under nitrogen. The non-saponifiable fraction was extracted with petroleum ether. The non-saponifiable components were separated by thin layer chromatography using silica gel G plates activated at 105° for 30 mn. The plates were developed in a cyclohexane-ethyl acetate (9:1, V/V) solvent. To visualize the components, the plates were sprayed with an ethanol solution saturated with berberine hydrochloride and then examined under UV light. For further analysis the spots were scraped off and eluted with diethyl ether. Squalen was identified by a second chromatography on silica gel G using a cyclohexane-benzene (95:5, V/V) solvent. Lanosterol was identified after acetylation with acetic anhydride and epoxidation (8) with p-nitroperbenzoic acid on silica gel G using a cyclohexane-ethyl acetate (9:1, V/V) solvent. The radioactivity was measured in a Beckman Scintillation counter (LS 150).

RESULTS : All eleven mutants studied, generally require an unsaturated fatty acid (i.e. oleic acid) in addition to ergosterol. For the mutant *olerg 6-1* (table 1) a saturated fatty acid, such as stearic acid, was sufficient for growth. Eight mutants also required methionine or its biosynthetic precursor homocysteine. All these mutants are petite.

Hybrids produced from crosses between the wild type and the mutants show that the mutations are recessive, and segragate 2/2. These matings established that the lipid requirement, the methionine requirement and the petite

TABLE 1

COMPLEMENTATION GROUPS AND REQUIREMENTS

OF STEROL DEPENDANT MUTANTS

Complementation Group	1	2	3	4	5	6
Mutants	olerg 1-1 olerg 1-2	olerg 2-1 olerg 2-2 olerg 2-3 olerg 2-4	olerg 3-1 olerg 3-2	olerg 4-1	olerg 5-1	olerg 6-1
Requirements	unsaturated fatty acid	unsaturated fatty acid methionine	unsaturate fatty acid methionine	unsaturated fatty acid methionine	unsaturated fatty acid	saturated or unsaturated fatty acid methionine

phenotype segregated together. The phenotypic complementation tests allow one to classify the mutants into 6 complementation groups (table 1).
On complete solid medium (YPG) the mutants require 10 µg/ml ergosterol to obtain their optimal growth. In liquid YPG medium the amount of sterol can be reduced to 4 µg/ml to obtain the maximal cell yield in stationary phase. The sterol requirement is not stringent, i.e. stigmasterol, sitosterol, or cholesterol can replace ergosterol as a growth supplement, but cholesterol is less efficient than the other two (9, 13). This means that the degree of unsaturation within the ring structure is not as important as the side chain characteristics in the cells physiology. Lanosterol does not permit growth of the mutants strains. This suggests that they are all blocked at a step after lanosterol.

It has been shown that the absence or a severe reduction in the amount of ergosterol in the cell, or its replacement by sterols which bind nystatin less efficiently, results in nystatin resistance (5, 13, 14). For this reason, we have examined the nystatin resistance of the mutant strains and of the wild type growing with different sterol supplements. On ergosterol, the mutant strains are more sensitive to nystatin than the wild type *FL100* ; the mutants are inhibited by 8 µg/ml nystatin, whereas the wild type resists to 10 µg/ml. On cholesterol, sitosterol or stigmasterol, the wild type is always inhibited by 10 µg/ml of nystatin ; whereas, the mutants are resistant up to 15 µg/ml. This strongly suggests that the three sterols are incorporated into the membrane and bind nystatin less efficiently than ergosterol.

We have tried to localize the enzymatic block in the ergosterol pathway for one of the mutants by studying the amount of radioactive ^{14}C-acetate incorporated into the sterol precursors of the wild type, *FL100*, and mutant *olerg2-1*. Table 2 shows that in the wild type, 80% of the radioactivity of the non-saponifiable lipids is recovered in the sterols fraction. For the mutant 61% is in products migrating with squalen, 30% in the 4,4-dimethyl sterols fraction (that includes lanosterol), 6% in the 4-α-methyl sterols fraction, and only 1% in the sterols fraction. The squalen fraction was rechromatographed in a second solvent system and it appears that the ratio of the amount of radioactivity incorporated into squalen by the mutant and by the wild type is 80:1, respectively. The 4,4-dimethyl sterols fraction has been identified as lanosterol by acetylation and epoxidation. The ratio of radioactivity in this fraction between the mutant and wild type is 6 to 1, respectively.
It appears clear from these data that either the *olerg 2-1* mutant is blocked after lanosterol or 4-α-methyl sterols, or that all of the oxydation processes leading to ergosterol are drastically reduced. This could explain the

TABLE 2

STEROL SYNTHESIS IN *Saccharomyces cerevisiae* STRAINS *FL100* AND *FL100 olerg*$_{2-1}$

Strains	Sterols	4-α-Methyl sterols	4,4 dimethyl sterols	squalen oxide	fraction migrating with squalen
FL100	1030	23	75	34	114
FL100 olerg$_{2-1}$	22	102	540	36	1090

Cells growing in logarithmic phase are harvested, washed and suspended in 0.1 M phosphate buffer containing 2% glucose, Tergitol np 40 0.05%,oleic acid 50 µg/ml, Methionine 100 µg/ml and 1 µ Ci/ml of ^{14}C acetate. The suspension is vigorously aerated during 2 hours. The non-saponifiable lipids are extracted, chromatographied as described in Material and Methods. Results are expressed in c.p.m. for 100 Klett units/ml.

progressive lowering of the radioactivity in precursor fractions going from squalen to ergosterol. The first explanation seems more likely because the chromatograms of the non-saponifiable lipids from the mutant had always a spot at position of the 4-α-methyl sterols which was never seen with the wild type.

DISCUSSION : Strict sterol requiring mutants were isolated. These mutations have been divided into six complementation groups. Preliminary ascus dissections show that these complementation groups are not linked. These six classes of mutants are not necessarily lesions in ergosterol biosynthesis *per se*, but could be blocks in the electron transport system that requires molecular oxygen and that is associated with both ergosterol biosynthesis and desaturation of fatty acids. Mutants of this kind have been described by Resnick and Mortimer (10) and Bard (5). These mutants are very similar to ours as they also have a lipid requirement (either unsaturated fatty acid or sterol), a methionine requirement, and are all petite. They differ from our mutants in that they do not have a stringent requirement for ergosterol.

Isolation of mutants that are strictly sterol requiring has many interesting possibilities. These mutants may enable one to study the relationship between the structure and the physiological function of sterols in the yeast cell membrane. This can be done by studying the permeability of the cell membrane (by single diffusion or by specific permeases) in cells starved for ergosterol or in which ergosterol has been replaced by another sterol. Such mutants will also provide information on the steps of ergosterol biosynthesis in yeast.

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