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Use of Sterol Mutants as Probes for Sterol Functions in the Yeast, *Saccharomyces cerevisiae*

Leo W. Parks, James H. Crowley, Frank W. Leak, Steven J. Smith, and Michele E. Tomeo

CONTENTS

I. INTRODUCTION

This is the centenary of the isolation of ergosterol from yeast.[1] While there was much interest in this compound as a substitute for vitamin D_3, little attention was paid to the possible physiological significance of ergosterol in the metabolism of yeast. Numerous *in vitro* studies on the interactions of sterols, particularly cholesterol, with various other membrane lipid components led to the conclusion that sterols merely served structural roles to membranes, providing rigidity, stability, and resistance to physical stresses. This is discussed in an earlier review.[2] Generally ignored in those studies were the observations that, when ergosterol was included in the experiments, it often did not behave like cholesterol. Reading those results led to the notion that ergosterol, although it must have a structural function, might have additional functions. The possibility that ergosterol had at least dual physiological roles was first proposed in 1967.[3]

Although the chemistry of sterols had been thoroughly studied, it was obvious that yeast mutants, defective in one or more steps in ergosterol biosynthesis, would be critical for a study of physiological functions of sterols. The first reported attempt at isolating stable sterol mutants in yeast was unsuccessful, and the paper clearly established the frustration in such an undertaking.[4] The seminal observation for obtaining a variety of sterol mutants was by Lynn Miller, who noticed that polyene resistant organisms had altered sterol composition. Subsequently expanded,[5] this finding has been exploited in virtually every laboratory working on sterol synthesis or metabolism in yeast. All of the polyene resistant organisms continue to produce sterols, albeit of structural features modified from ergosterol.[2] Obtaining sterol auxotrophs was frustrated by the inability of yeast to take sterols from the medium under aerobic conditions. This has been called "aerobic sterol exclusion." Although this phenomenon has been reviewed recently,[6] it is important to mention in a discussion of the physiological roles of sterols.

Substantial confusion exists on whether or not genes mediating certain ergosterol functions are essential to yeast. A gene is considered essential if, when that gene is inactivated, the organisms cannot grow. There is no doubt that many genes fall into that category. Conditional lethality has facilitated defining the roles of many essential genes. For example, varying the temperature or osmolarity of media has been used to remediate otherwise lethal defects. Mutations that cause auxotrophy for amino acids, purines, pyrimidines, vitamins, etc. are generally considered essential, even though those substituents can be added to media and the yeast can then grow. Few would classify *TRP1* as nonessential. Although *trp1* mutants cannot grow on defined media without added tryptophan, they grow abundantly on the same medium that is supplemented with the amino acid. It is interesting, therefore, that the ability of certain sterol mutants to grow on a routine glucose-yeast extract-tryptone medium has been used as the metric for nonessentiality of that mutant's altered gene.[7–9]

Here, we discuss the effects of three structural genes in ergosterol biosynthesis on the growth and physiology of yeast. Genes *ERG24*, the C-14 sterol reductase, *ERG6*, the C-24 sterol methyltransferase, and *ERG3*, the C-5 desaturase, will be explored with regard to their possible functions and essentiality.

II. *ERG24*

The *ERG24* gene encodes the C-14 sterol reductase,[10–12] which is the primary target for the antimycotic 15-azasterol,[13] and the morpholine class of antifungal agents.[14] Treatment of yeast cells with the morpholine antifungal fenpropimorph leads to the accumulation of ignosterol ($\Delta^{8,14}$-ergostadien-3ß-ol) as the primary sterol.[14] The C-14 sterol reductase (Erg24p) and C-8 sterol isomerase (Erg2p) are inhibited *in vitro* by fenpropimorph, with ERG24p being affected at higher concentrations of the drug.[15] Accumulation of Δ^8-sterols by yeast in low concentrations of fenpropimorph does not appear to cause growth inhibition,[14] and yeast strains containing the *erg2* mutation (C-8 sterol isomerase) are viable despite the presence of only Δ^8-sterols, further indicating that Δ^8-sterols are not toxic to the cell.[16] In addition, multiple copies of the *ERG2* gene do not confer resistance to fenpropimorph.[17] However, the accumulation of $\Delta^{8,14}$-sterols in fenpropimorph-treated cells does appear to be correlated to growth inhibition.

The *ERG24* gene was cloned and sequenced in our laboratory and determined to encode a predicted polypeptide of 438 amino acids (M_r 50,612).[10] This was accomplished by first isolating a strain with an *erg24* mutation in a genetic background containing the *fen1* and *fen2* mutations.[10] Strains having the *fen1* and *fen2* mutations are highly resistant to fenpropimorph and are believed to tolerate ignosterol as the major sterol.[18] The *ERG24* gene was then isolated by complementation of the *erg24-1* mutation in the *fen1, fen2* background. Upon inactivation of the *ERG24* gene, it was found that *erg24*::*LEU2, fen1, fen2* strains were viable and produced ignosterol as the major sterol. In addition, this sterol mutant, like most other sterol mutants, is resistant to nystatin and supersensitive to cycloheximide. The ERG24 gene has since been cloned by other researchers,[11,12] thus confirming the identity of *ERG24* as the gene encoding the yeast C-14 sterol reductase.

The inactivation of the *ERG24* gene in an otherwise wild-type background has been shown to result in cell inviability in an aerobic environment.[11,12] However, *erg24* null mutants are viable anaerobically where *de novo* sterol synthesis is precluded in the absence of oxygen, and the cells are able to grow on ergosterol supplemented to the medium. These results indicate that ignosterol is not able to substitute for ergosterol in performing some critical function in the cell. Recent results in our laboratory (unpublished observations) have confirmed that *erg24* null mutants are inviable when grown aerobically on rich media (2% dextrose, 1% peptone, and 0.5% yeast extract) without the presence of either the *fen1* or *fen2* mutation to suppress the growth inhibition resulting from ignosterol synthesis. However, we have consistently observed that *erg24* null mutants are able to grow aerobically on synthetic complete media (unpublished observations) despite the presence of ignosterol as the principal sterol. The ability of the *erg24* mutant to grow would seem to question the notion that the *ERG24* is an essential gene as has been reported.[11,12]

It is clear that yeast strains defective in Erg24p function produce ignosterol as the major sterol, which is detrimental to some aspect of normal cellular physiology. This indicates that the growth inhibitory effects mediated by fenpropimorph may indeed be a result of interference with the C-14 sterol reductase. However, fenpropimorph may affect other aspects of cellular metabolism, such as the uptake of pyrimidine bases, uracil and cytosine, as we have reported.[19] Nevertheless, it seems that ignosterol is not suitable for growth of yeast only under certain growth conditions. Incorporation of 24-methyleneignosterol into membranes increases membrane fluidity and permeability to glucose as a result of changes in the normal membrane composition;[20] it is not known if these changes are sufficient to completely inhibit cell growth and division. Future studies with the *ERG24* gene may provide an answer to the question of why ignosterol is not a suitable sterol under certain conditions of growth.

III. *ERG6*

In yeast zymosterol ($\Delta^{8,24}$-cholestadien-3ß-ol) is methylated by S-adenosylmethionine to yield fecosterol ($\Delta^{8,24(28)}$-ergostadien-3ß-ol). This is catalyzed by the sterol C-24 methyltransferase, the structural gene for which is *ERG6*. Insertional inactivation of this gene does not prevent the growth of mutants containing this lesion on routine laboratory media, but those strains do show interesting defects.

The *erg6* mutants have enhanced permeability to Ni^+,[21] Na^+, and Li^+.[22] The hypersensitivity to these monovalent cations was determined to be a result of increased uptake rather than decreased efflux. These mutants are also much more sensitive to the protein synthetic inhibitor, cycloheximide,[8], and Brefeldin A,[23,24] which leads to the disassembly of the Golgi apparatus. By contrast, despite the ability of wild-type yeast to transport tryptophan through two different mechanisms,[25,26] *erg6* mutants have a sixfold reduction in tryptophan uptake.[8] These alterations in permeability and the diversity of compounds that

are affected may reflect the participation of ergosterol in a component central to the transport of all of the affected components.

Another consequence of the *erg6* mutation is a reduction in mating efficiency especially between two *erg6* null mutants.[8] This finding is in agreement with a report that sterol auxotrophic yeast grown on sterols other than ergosterol demonstrate perturbations in conjugation.[27] The zymosterol which accumulates in the *erg6* mutant may not fully compensate for ergosterol in the membrane fusion events which occur during mating.

Early experiments with *ERG6* showed that the cellular fraction most enriched with the sterol methyltransferase was a lipid-enriched fraction that was called the floating lipid layer.[7] Recently, working with highly purified lipid microdroplets prepared from the floating lipid layer, it was established that this is the principal repository of the methyltransferase.[28] As a surface protein on those particles, the sterol methyltransferase may have some role in sterol distribution in yeast.

The secretory pathway mediates the localization of most nascent proteins to the vacuole, plasma membrane, or for excretion.[29] Resident luminal proteins of the endoplasmic reticulum (ER) are transferred to the Golgi apparatus, presumably adventitiously. The *ERD2* structural gene encodes a receptor that is responsible for the retrieval of these proteins.[30] The *ERG6* gene can act as a multicopy suppressor of *erd2Δ* strains.[31] Whether it is the methyltransferase *per se*, its transmethylated sterol products, or some unknown other role of *ERG6* that mediates this suppression is not known.

Defects in sterol transmethylation appear to cause a multitude of physiological effects in cells. However, mutants defective in *ERG6* are viable on routine laboratory media.[7,8]

IV. *ERG3*

The final step in the transformation of the sterol nucleus in ergosterol synthesis is the desaturation of episterol ($\Delta^{7,24(28)}$-ergostadien-3ß-ol) to $\Delta^{5,7,24(28)}$-ergostatrien-3ß-ol. The *ERG3* gene, which encodes the C-5 desaturase, was thought to be essential under all growth conditions. This resulted from the inability to isolate a non-leaky *erg3* lesion, and from data defining the physiological value of specific sterol structural features.[32] A microamount of Δ^5-sterol is necessary to satisfy the "sparking" function,[32,33] one of four essential functions of sterols.[34] These studies employed yeast strains having an *erg7* or *erg1*::*URA3* lesion in combination with a *hem1* defect, thus allowing sterol uptake by the sterol auxotrophic strains.

The insertional inactivation of *ERG3*[9] proved that yeast could survive and grow comparably to wild type without any Δ^5-sterols, making predominantly $\Delta^{7,22}$-ergostadien-3ß-ol, when maintained on media containing a fermentable carbon source. However, a possible role for the Δ^5-sterols in respiration was suggested in early experiments in which *erg3* mutants were selected.[35] The *erg3* mutants that were isolated were found to be conditionally temperature defective in respiration. The effect was due to the co-selection of a second mutation that conferred the respiratory defect. The co-selection of mutations for enhanced survival of mutants defective in sterol biosynthesis has also been seen in the *erg11* mutants.[36] *ERG11* is the gene for the cytochrome P450 C-14 demethylase. The co-selected mutation in that instance is *erg3*. Without the associated *erg3*, the *erg11* mutant is viable only under anaerobic conditions. Strains that contain an insertionally inactivated *ERG3* allele are unable to grow on nonfermentable (respiratory) carbon sources.[37] Reevaluation of previous work revealed that the sparking sterol could be replaced by the addition of δ-amino-levulinic acid to the media, allowing heme competency. Thus, the presence of Δ^5-sterols is required only when heme biosynthesis is blocked, as with *hem1* strains, or during times of special heme requirements, such as the utilization of nonfermentable carbon sources.

As with the isolation of mutations in most other genes in the yeast sterol biosynthetic pathway, *erg3* mutants have been selected by resistance to polyene antifungal agents.[5] If a Δ^5-sterol is required for utilization of respiratory carbon sources, such as glycerol or ethanol, mutants lacking Δ^5-sterols should not be selected on respiratory carbon and energy sources. We selected polyene-resistant (ergosterol) mutants on media containing either dextrose or glycerol as the carbon source. To avoid strain-dependent prejudice in mutant selection, three different wild-type strains were used. Mutants were selected on rich media containing either dextrose or glycerol, and each medium was supplemented with either nystatin or filipin. While dextrose supports growth of strains containing many different ergosterol mutations, all mutants isolated on glycerol were found to produce some amount of Δ^5-sterol.[38]

The *ERG3* gene has also been cloned by selecting for mutants resistant to the phytotoxin syringomycin, and sensitive to high Ca^{2+} concentrations.[39] The treatment of wild-type yeast with syringomycin alters K^+ efflux, Ca^{2+} influx, and protein phosphorylation.[39] That *erg3* mutants are resistant to syringo-

mycin while being more sensitive to high levels of exogenous Ca^{2+} is indicative of an alteration in membrane composition and integrity associated with the structural change by the mutant sterol.

These findings reveal no obvious selective advantage for yeast cells maintaining the *ERG3* gene, when grown on fermentable substrates. However, as described below, the native environment for yeast is vastly different from artificial laboratory conditions. The utilization of ethanol, produced in the fermentation of sugars found in the natural habitat of yeast, apparently requires sterols containing an unsaturation in the C5-6 position. While the role of Δ^5-sterols in respiration is currently being studied actively, it is clear that sterols lacking the *ERG3* are viable on glucose, either aerobically or anaerobically. This has been interpreted also to reflect the nonessentiality of *ERG3*.[9]

V. CONCLUSIONS

We have shown here that some genes, *ERG3* and *ERG6*, that have been designated as nonessential, are in fact essential under some growth conditions. The *ERG24* gene, that has been reported to be essential, may not be required in some media. Likewise, the *ERG11* gene has been reported to be essential, yet it is not required if there is a concomitant defect in *ERG3*. A physiological basis for these results has not been established, but is under investigation. It seems reasonable to assume, however, that these genes and their accompanying proteins have been important to the survival of the species, and consequently offered a selective advantage in evolution.

Ecologically, yeast cells "show a marked preference for sugary micro-habitats, usually of vegetable origin. Their ability to develop anaerobically, together with the inability of most other fungi to tolerate the alcohol produced, enables yeasts to compete successfully for those habitats."[40] Representatives of *Saccharomyces* have been isolated from sugar cane, sorghum, honey, maple syrup, soft Italian cheeses, extracts of peppermint, exudations of oaks and elms, and fruits, such as grapes, figs, apples, holly berries, etc.[41] *Saccharomyces* is the most common yeast found on grapes.[42] Many yeasts, although generally not *Saccharomyces*, are found in the nectar of flowers. Some yeasts tolerate up to 24% salt in brine solutions.[43] The picture that emerges, then, is that of a group of organisms that are tolerant of and proliferate in highly osmotic environments.

It is interesting to speculate on the role of ergosterol in the osmotolerance of yeast. The alterations in transport processes or sensitivities to solutes that we have described in the sterol mutants suggests an intimate relationship in the native ergosterol being involved in the maintenance of cellular integrity, even in media containing high concentrations of sugar and salts.

Certainly, there are other roles for ergosterol.[34] Taken together, the multiple functions of ergosterol suggest that the yeast sterol is a consensus sterol, being able to function in a variety of physiological activities. The uniqueness and importance of ergosterol *per se* is supported by the phenomenon of aerobic sterol exclusion.

External sterols are taken up only very poorly by wild-type yeast cells, when grown aerobically.[6] This exclusion of external sterols requires that the cells be competent for the synthesis of heme compounds.[44,45] In wild-type yeast that means that aerobic conditions must be maintained. Circumstantially, this strongly suggests a relationship between ergosterol and the respiratory process in this organism. Anaerobically, yeast cells are incapable of heme and sterol synthesis, but sterols are required for growth. They must be supplied in the medium and a wide variety of sterols can satisfy this requirement. This suggests that, although mechanisms exist for getting sterols into the cells, there have evolved mechanisms to exclude exogenous sterols from being taken into the cells. Aerobic sterol exclusion thus may assure that only ergosterol is available for its various physiological functions, even if the endogenous synthesis of ergosterol is very costly to the cell. The potential metabolic disruptions of exogenous sterols must more than be offset by the expense of synthesizing the 28-carbon molecule that is ergosterol. Aerobic sterol exclusion and the distinctive features of ergosterol must be important to the organism. Otherwise, they would have been eliminated by competitive selection in the evolution of the organism.

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