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Structural Discrimination in the Sparking Function of Sterols in the Yeast *Saccharomyces cerevisiae*†

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A *Saccharomyces cerevisiae* sterol auxotroph, SPK14 (a *hem1 erg6 erg7 ura*), was constructed to test the ability of selected C-5,6 unsaturated sterols at growth-limiting concentrations to spark growth on bulk cholestanol. The native sterol, ergosterol, initiated growth faster and allowed a greater cell yield than did other sterols selectively altered in one or more features of the sterol. Although the C-5,6 unsaturation is required for the sparking function, the presence of the C-22 unsaturation was found to facilitate sparking far better than did the C-7 unsaturation, whereas the C-24 methyl was the least important group. The addition of δ-aminolevulinic acid to the medium allowed the sparking of FY3 (*hem1 erg7 ura*) on bulk cholestanol due to the derepression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the production of endogenous ergosterol. The optimal concentration of δ-aminolevulinic acid to spark growth was 800 ng/ml, whereas higher concentrations caused a growth inhibition. The growth yield of FY3 reached a plateau maximum at about 5 μg/ml when the bulk cholestanol was varied in the presence of 10 ng of sparking ergosterol per ml.

Evidence for differential utilization of sterols has been described in insects (3) and bacteria (5, 6, 17). Because of its ease of growth and facile genetic manipulations, *Saccharomyces cerevisiae* has been particularly valuable in studying the diverse functions of sterols. Using the sterol auxotrophs RD5-R and FY3, we demonstrated that the sterol requirement for growth could be fulfilled by cholestanol only when a very small concentration of ergosterol (10 ng/ml) was also present in the medium (23, 25, 26). We have systematically varied the combinations of sterols that were provided to the auxotrophs, and we proposed multiple roles for sterols in this organism under aerobic conditions. We have designated the cellular requirement that could be satisfied by cholestanol as the bulk function, and the high specificity function fulfilled by microamounts of ergosterol has been termed the sparking function. By feeding different sterols and stanols to RD5-R and FY3, we showed that most sterols and stanols could satisfy the bulk requirement. However, only those sterol derivatives that have a C-5,6 unsaturation or are capable of being desaturated at C-5 fulfill the sparking function (25).

Other studies with *S. cerevisiae* have shown growth preferences for different sterols, depending on the cultural conditions. The sterol auxotroph GL7, grown on limiting amounts of ergosterol, has higher growth rates and yields in media additionally supplemented with cholesterol when compared with growth of the organism on either sterol alone (21). The necessity of the 24 β-methyl group on the sterol was proposed for *S. cerevisiae* when the wild-type organism is grown under anaerobic conditions in the presence of the synthetic sterol inhibitor 2,3-iminosqualene (19, 20).

Numerous sterols at nonlimiting concentrations (5 μg/ml or greater) appear to satisfy the sterol requirement in the presence (22, 26) or absence (2, 10) of cholestanol. Although it requires only 10 ng of ergosterol per ml to satisfy the microrequirement, it is necessary to use other highly purified C-5,6 unsaturated sterols at growth-limiting concentrations (less than 200 ng/ml), in conjunction with a bulk sterol such as cholestanol, to define the precise structural features involved in sparking. By feeding sterols systematically altered for specific groups of ergosterol, it should be possible to determine preference and priority for structural features that facilitate the sparking function of C-5,6 unsaturated sterols (Fig. 1). In this paper we assess the ability of selected sterols and the heme intermediate, δ-aminolevulinic acid (ALA), to fulfill the microrequirement of *S. cerevisiae* in the presence of bulk cholestanol.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* FY3 (a *hem1 erg7 ura*) (28) was crossed to JR5 (α *erg6*) (15) and sporulated to construct strain SPK14 (a *hem1 erg6 erg7 ura*). Both heme mutants require a C-5,6 unsaturated sterol, unsaturated fatty acids, methionine, and uracil for growth.

Medium and growth conditions. The medium used for the growth curves and cholestanol cycling consisted of 2% glucose–0.67% yeast nitrogen base (Difco Laboratories)–1% Casamino Acids (Difco) and was supplemented with 20 μg each of methionine and uracil per ml. The medium was buffered with 0.05 M succinic acid and adjusted to pH 5.5 with KOH pellets. All sterols and stanols were purified by high-performance liquid chromatography before use (24). Unsaturated fatty acid supplementation consisted of a mixture of oleic and palmitoleic acids (4:1, vol/vol) at 0.01% final concentration from a 10% (vol/vol) stock solution in Tergitol-ethanol (1:1, vol/vol). ALA was dissolved in distilled water and filter sterilized (0.45-μm Millipore HA filter) before addition to the medium. Cultures were grown aerobically at 28°C in 25-ml test tubes with constant shaking. Growth was monitored with a Klett-Summerson photoelectric colorimeter equipped with a green filter. Klett units were found to be proportional to cell number by the following formula: log cells per ml = (log Klett units + 1.86)/0.6486 over the range of 5 to 500 Klett units, as verified by direct cell counts with an electronic cell counter.

Construction of a new sterol auxotroph for sparking studies. Since it was necessary to use a sterol auxotroph that would not interconvert the test sparking sterols in vivo to other

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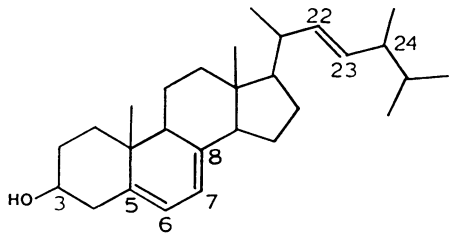

FIG. 1. Structure of ergosterol. The numbers designate points of modification for sterols used in this study. See Table 1.

forms, a new strain was constructed to conduct the studies. Strain FY3 (**a** *heml erg7 ura*) was mated with JR5 (α *erg6*) and allowed to sporulate. Spores were dissected from the tetrads by micromanipulation and screened after colony growth. Heme incompetence was assayed by the inability to grow on YPD medium (1% yeast extract, 1% peptone, 2% glucose) without sterol and unsaturated fatty acids. Production of 2,3-oxidosqualene was determined by lipid extraction and gas chromatography (13). The presence of the sterol methyltransferase defect (*erg6*) was assayed by adding 0.1 μCi of [^{14}C]methionine per ml to medium containing cholesta-5,7,22,24-tetraen-3β-ol, unsaturated fatty acids, and 20 μg each of methionine and uracil per ml. Strains that could not convert the cholesta-5,7,22,24-tetraen-3β-ol to ergosterol were considered to contain the *erg6* gene (data not shown). The strain used in this study was termed SPK14 (*heml erg6 erg7 ura*). The *erg6* mutation ensures that the side chain of C-24 unsaturated sterols fed to SPK14 are not methylated. Under heme-incompetent conditions (*heml*), the C-22 unsaturation is not introduced (27, 27a). The unsaturation at the C-7 position cannot occur with the test sterols used.

Cholestanol cycling. FY3 or SPK14 was grown on 1.0 μg of cholesterol per ml to the midlog phase, harvested, and washed twice with a 0.5% aqueous solution of Tergitol-ethanol (1:1, vol/vol). The cells were then transferred to fresh medium that contained 10 μg of cholestanol per ml, unsaturated fatty acids, methionine, and uracil. The cells were suspended at a concentration of 75 to 100 Klett units and incubated for 24 h. A 200-μl volume was then used from this culture to inoculate 1 liter of medium containing 5 μg of bulk cholestanol per ml in addition to unsaturated fatty acids, methionine, and uracil. Samples from this mixture were pipetted in 10-ml volumes to test tubes, and the appropriate concentration of the test sparking sterol was added from a solution of 20 μg/ml in ethanol, diluted from an original stock of 2 mg/ml.

Chemicals. Ergosterol, cholesterol, 7-dehydrocholesterol, Tergitol Nonidex P-40, ALA, and amino acids were purchased from Sigma Chemical Co. Stigmasterol, sitosterol, cholestanol, and cholesta-5,22(*E*)-dien-3β-ol were from Research Plus. Ergosta-5-en-3β-ol, ergostanol, and cholesta-5,22(*Z*)-dien-3β-ol were a gift from the late Henry Kircher. Cholesta-5,7,22,24-tetraen-3β-ol was isolated from strain JR5 (16), and ergosta-5,7-dien-3β-ol was isolated from strain G204 (27a).

RESULTS

Determination of the maximum yield on differing amounts of cholestanol. To determine the minimal concentration of bulking cholestanol necessary for the maximal cell yield on sparking levels of ergosterol, the following experiment was performed. Strain FY3 was cycled on cholestanol as described in Materials and Methods and then suspended into fresh medium containing 10 ng of ergosterol per ml and various concentrations of cholestanol. Figure 2 illustrates the results of the maximal amount of growth attained in the different cholestanol concentrations at stationary phase as determined by optical density. Cell yield increased the most between 0 and 1 μg of bulking cholestanol per ml and then began to plateau at about 5 to 6 μg of cholestanol per ml. In subsequent experiments, bulking cholestanol was added at 5 μg/ml to obtain an optimal yield.

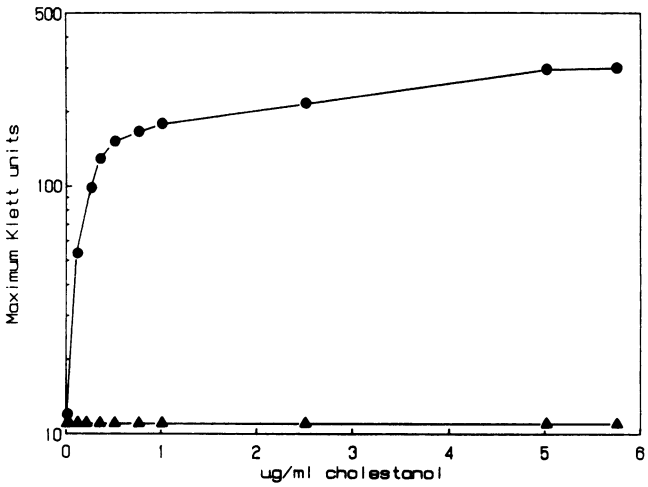

FIG. 2. Determination of the maximum yield on the minimal concentration of cholestanol sparked by 10 ng of ergosterol per ml. FY3 was grown to the stationary phase in different concentrations of cholestanol in the presence of 10 ng of ergosterol per ml to spark growth. At 5 μg of cholestanol per ml, a maximum yield was obtained. Symbols: ●, 10 ng of ergosterol added per ml; ▲, control culture with no added ergosterol.

Sparking of FY3 by ALA on bulk cholestanol. The *heml* mutation in FY3 can be bypassed by the addition of ALA to the medium to allow heme competency. The attainment of heme competency in this strain has been shown to stimulate the activity of the sterol regulatory enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase at least fivefold. This derepression results in the biosynthesis and accumulation of 2,3-oxidosqualene, some of which can leak through the *erg7* mutation to form ergosterol (13). Thus, we tested the ability of FY3 to spark on bulk cholestanol by utilizing ALA to biosynthesize endogenous ergosterol.

The optimal concentration on ALA found to spark FY3 was 0.8 μg/ml, whereas lower concentrations took longer to initiate growth. At higher ALA concentrations of 10 to 20 μg/ml, growth inhibition was observed during the midlog phase after a longer lag period (Fig. 3). The growth inhibition at high ALA concentrations has been reported before but is not fully understood (12).

Ability of different sterols at limiting concentrations to spark growth on cholestanol. Since it is known that C-5,6 unsaturated sterols in nonlimiting concentrations such as 7-dehydrocholesterol, stigmasterol, sitosterol, and cholesterol allow growth of sterol auxotrophs (2, 10, 22, 25), we tested different sterols for their ability to spark at growth-limiting concentrations with 5 μg of cholestanol per ml as the bulking sterol.

Sterol-depleted SPK14 was cycled on cholestanol for 24 h and transferred to fresh medium containing 5 μg of cholestanol per ml and 25 ng of the different test sterols per ml

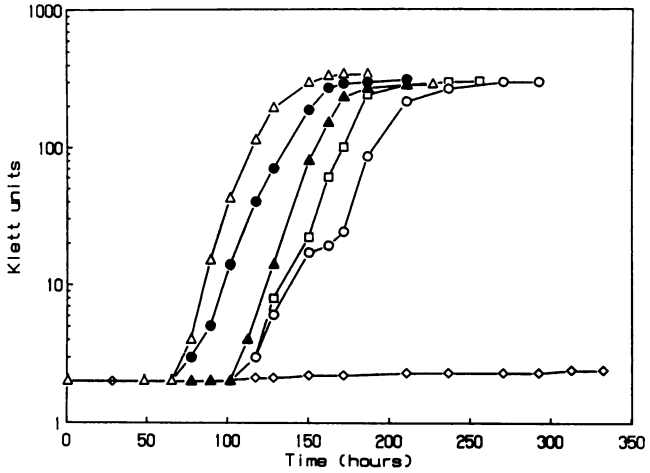

FIG. 3. Sparking ability of FY3 on different concentrations of ALA. FY3 was cycled on cholestanol and transferred to medium containing 5 μg of cholestanol per ml and ALA at the following concentrations: ▲, 0.1 μg/ml; ●, 0.4 μg/ml; △, 0.8 μg/ml; □, 10 μg/ml; ○, 20 μg/ml.

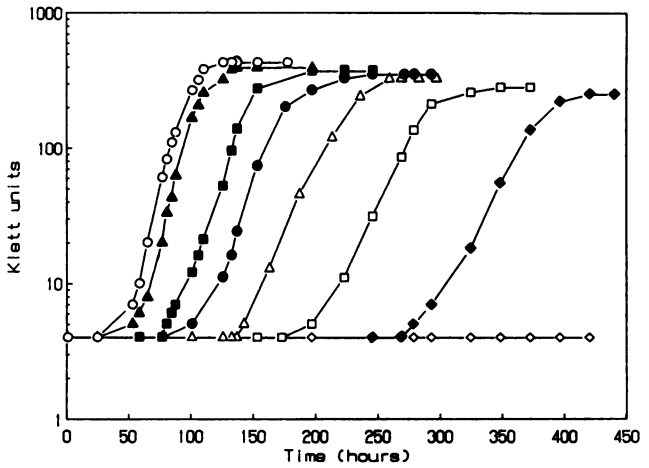

FIG. 4. Sparking ability of different sterols at 100 ng/ml in the presence of 5 μg of bulking cholestanol per ml. Symbols: ○, ergosterol; ▲, cholesta-5,7,22,24-tetraen,3β-ol; ■, cholesta-5,22-dien-3β-ol; ●, ergosta-5,7-dien-3β-ol; △, cholesta-5,7-dien-3β-ol; □, ergosta-5-en-3β-ol; ◇, ergostanol or cholestanol only; ◆, cholesterol.

(Table 1 and Fig. 1). Ergosterol is known to spark cell growth at this concentration (26). The culture containing ergosterol initiated growth after 85 h and was followed by the sparking on cholesta-5,7,22,24-tetraen-3β-ol at about 100 h. The other cultures containing cholesta-5,22-dien-3β-ol, ergosta-5,7-dien-3β-ol, and cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), respectively, came up after additional lag periods. Ergosta-5-en-3β-ol displayed a lag of about 250 h before initiating growth. However, the cells exposed to cholesterol did not grow after a period of 450 h. The control culture, which received 5 μg of bulking cholestanol per ml only, did not grow. Additionally, ergostanol, which does not have a C-5,6 unsaturation to allow sparking, did not allow growth as expected (25). From optical density measurements taken throughout growth, it was determined that as the lag period increased for the different sterols, the final cell yield was decreased (data not shown).

When the concentration of sparking sterols was increased to 50 ng/ml in the presence of 5 μg of bulking cholestanol per ml, the lag times of all cultures decreased and the growth curve with cholesta-5,7,22,24-tetraen-3β-ol approached that of ergosterol. However, the culture containing cholesterol still was not able to grow after 450 h. The final growth yield of the cultures was again lower as the lag times increased but was greater than their respective yields on 25 μg of cholestanol per ml. Again, the slope of the growth curves decreased as the lag times for the different sterols increased, revealing slower growth rates (data not shown).

When the concentration of the sparking sterols was increased to 100 ng/ml, cholesterol was able to spark growth after a lag period of about 275 h. The lag times of the other test sterols were decreased compared with those of the previous experiment, and the yields were again less as the lag time was greater. The exponential growth rate was slower as the lag time increased (Fig. 4).

SPK14 was able to grow equally well when provided with pure sterols at 5 μg/ml, except with cholestanol and ergostanol, which lack the C-5,6 unsaturation. Conversely, no growth was detected when SPK14 was fed 100 ng of the test sterols per ml in the absence of bulk cholestanol (data not shown). However, it was found that after a period of over 500 h in the sparking experiments, SPK14 was able to grow on cholestanol or ergostanol because of endogenous desaturation at the C-5,6 position (25). Cells taken from the end of the experiment and recycled on cholestanol underwent the same lag periods when retested, demonstrating that adaptation had not occured (data not shown).

Comparison of other C-22 unsaturated sterols for sparking ability. From the previous experiments it was clear that C-5,6 unsaturated sterols also possessing the C-22 unsaturation had a superior sparking ability compared with those possessing the C-7 unsaturation or the C-24 methyl group. Thus, it appears that the C-22 unsaturation plays a major role facilitating the sparking process. We then compared the sparking ability of stigmasterol, which contains unsaturations at C-5 and C-22, with that of sitosterol, which only has the C-5 unsaturation. Each of these sterols has a C-24 ethyl group in the α configuration opposite that of ergosterol. Also, the conformation at C-22 was tested by comparing cholesta-5,22(*E*)-dien-3β-ol with cholesta-5,22(*Z*)-dien-3β-ol. The additional C-22 unsaturation of stigmasterol elicited a much greater sparking response than did sitosterol (Fig. 5). The poor sparking ability of cholesta-5,22(*Z*)-3β-ol, demonstrates that the chirality of the C-22 unsaturation is also critical for the optimal facilitation of sparking.

TABLE 1. Description of sterols[a]

Sterol	Features
Cholestanol	
Cholesterol	Δ5
Ergostanol	C-24 β-methyl
Ergosta-5-en-3β-ol	Δ5, C-24 β-methyl
Cholesta-5,7-dien-3β-ol	Δ5, 7
Cholesta-5,22-dien-3β-ol	Δ5, 22
Ergosta-5,7-dien-3β-ol	Δ5, 7, C-24 β-methyl
Cholesta-5,7,22,24-tetraen-3β-ol	Δ5, 7, 22, 24
Ergosterol	Δ5, 7, 22, C-24 β-methyl
Sitosterol	Δ5, C-24 α-ethyl
Stigmasterol	Δ5, 22, C-24 α-ethyl

[a] The numbering system for sterols is illustrated for ergosterol, which is a Δ5,7, 22 triene with a C-24 β-methyl group. The C-22 unsaturation is in the *E* conformation.

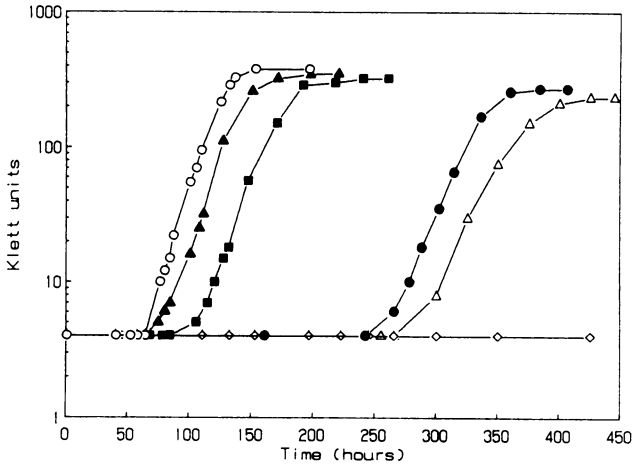

FIG. 5. Sparking ability of test sterols at 50 ng/ml in the presence of 5 μg of bulking cholestanol per ml. Symbols: ○, ergosterol; ■, cholesta-5,22(*E*)-dien-3β-ol; ▲, stigmasterol; ●, sitosterol; △, cholesta-5,22(*Z*)-dien-3β-ol; ◇, cholestanol only. The *E* conformation for the C-22 unsaturation is the normal form of ergosterol.

DISCUSSION

The *S. cerevisiae* sterol auxotroph FY3 requires exogenous sterol in addition to unsaturated fatty acids and methionine due to the *hem1* and *erg7* mutations (8, 28). FY3 does not grow on cholestanol alone and must be supplied with a sterol having a C-5,6 unsaturation or capable of being desaturated at the C-5 position to fulfill the sparking function (25). Others have found a requirement for a C-24 β-methyl sterol for growth under anaerobic conditions in the presence of the inhibitor 2,3-iminosqualene (19, 20). However, it appears that either the anaerobic system does not have sterol requirements that are as strict as those of the aerobic system using heme mutants or the two systems represent two different phenomena. For example, the heme mutant GL7 (*hem3 erg7*) does not grow on lanosterol (which lacks the C-5,6 unsaturation) aerobically but does grow on lanosterol anaerobically (1, 8). It has also been demonstrated that the unsaturated fatty acid source used to supplement sterol auxotrophs is a critical factor in the resulting phospholipid composition and growth response to different sterols (2). In all sparking studies to date and in this report, the unsaturated fatty acid supplementation has been a mixture of oleic and palmitoleic acids (4:1), which simulates the ratio found in wild-type *S. cerevisiae* grown aerobically (15).

Studies have been conducted demonstrating that growth of sterol auxotrophs can be sustained on various C-5,6 unsaturated sterols at nonlimiting concentrations (5 μg/ml or greater) with or without cholestanol (2, 22, 25). It has been determined that as little as 10 ng of ergosterol per ml will spark growth of FY3 on bulking concentrations of cholestanol (23, 26). However, we wished to test the sparking ability of other C-5,6 unsaturated sterols supplied at growth-limiting concentrations (0.2 μg/ml or less) to ascertain whether a specific feature of the ergosterol molecule facilitates the sparking process or whether all C-5,6 sterols have the same sparking capacity.

By systematically feeding sterols differing from ergosterol by the absence of specific groups, we found that the ability of C-5,6 unsaturated sterols to spark growth varied greatly depending on certain structural features. The most striking observation was the different sparking abilities of ergosterol and cholesterol. Although they each have the C-5,6 unsaturation required for sparking, cholesterol does not possess a C-24 methyl group or unsaturations at C-7 and C-22. SPK14 could not spark growth until 100 ng of cholesterol per ml was supplied, and a lag period of about 275 h was observed before initiation, whereas at this same concentration ergosterol sparked growth after only 60 h (Fig. 4). Thus, one or more of the three additional features of ergosterol were critical for the facilitation of the sparking process.

To determine which feature(s) was the most important, other sterols altered in just one or two of the functional groups were tested for their sparking ability. The sterols possessing the C-22 unsaturation had the greater sparking ability (cholesta-5,7,22,24-tetraen-3β-ol and cholesta-5,22-dien-3β-ol), whereas those missing the C-22 unsaturation had the poorer sparking ability (7-dehydrocholesterol, ergosta-5-en-3β-ol). Interestingly, cholesta-5,22-dien-3β-ol, which lacks the C-7 unsaturation and the C-24 methyl group, was able to spark growth before ergosta-5,7-dien-3β-ol, which lacks only the C-22 unsaturation. Thus, it appears that the C-22 unsaturation is the most important feature for the facilitation of sparking and outweighs the presence of the C-7 unsaturation and methyl group together.

By comparing the sparking ability of 7-dehydrocholesterol, which lacks C-22 unsaturation and the C-24 methyl group, with that of ergosta-5-en-3β-ol, which lacks the C-22 and C-7 unsaturations, it can be ascertained whether the other features promote sparking. 7-Dehydrocholesterol sparked growth far better, indicating that the C-7 unsaturation plays the secondary role in the facilitation of sparking, whereas the C-24 methyl is the least important feature. The importance of the C-22 unsaturation compared with the C-24 methyl group can be illustrated further by the superior sparking ability of cholesta-5,7,22,24-tetraen-3β-ol compared with that of ergosta-5,7-dien-3β-ol (Fig. 4).

Although the C-24 methyl group appears to be the least important feature, its presence is clearly facilitative. Cholesterol was not capable of sparking growth until a concentration of 100 ng/ml was reached, whereas ergosta-5-en-3β-ol, which has a single C-24 methyl addition, allowed sparking at 25 ng/ml. The single addition of a C-7 unsaturation to cholesterol (7-dehydrocholesterol) also allowed sparking at the lower concentrations, demonstrating the facilitative capability of this feature.

To test further the importance of the C-22 unsaturation, we compared the sparking abilities of stigmasterol (C-5 and C-22 unsaturations, C-24 α-ethyl) and sitosterol (C-5 unsaturation, C-24 α-ethyl). The superior sparking ability of stigmasterol again demonstrates the facilitative nature of this feature and indicates that the C-24 position can be filled equally well by a β-methyl or α-ethyl group. We also compared the effect of an altered conformation at the C-22 position. When the sparking ability of cholesta-5,22(*E*)-dien-3-β-ol (the native conformation) was compared with that of cholesta-5,22(*Z*)-dien-3β-ol, the chirality at the C-22 position was also found to be a critical factor for optimum sparking (Fig. 5). The C-22 unsaturation has also been shown to increase ethanol tolerance and reduce membrane stretching in a anaerobic system (9, 30).

The different sparking abilities of these C-5,6 unsaturated sterols demonstrates that the conformation and chirality of a given sterol are crucial for optimal sparking. We believe that these results reflect a much higher specificity for the sparking site(s) than previously thought and strongly suggest the involvement of protein binding in the sparking process. Competition between the bulking cholestanol and the C-5,6

unsaturated sterol for the sparking site appears to favor greatly sterols with C-22 unsaturation and to a lesser degree, those with C-7 unsaturation and a C-24 methyl group. Although there are some differences in the uptake efficiencies of the sparking sterols, this factor cannot account for their different sparking abilities (14, 29). Others have recently implicated sterol specificity by a greater stimulation from ergosterol than from cholesterol for a protein kinase (4), a methyltransferase (11), and polyphosphoinositide metabolism (7).

The addition of ALA to FY3 has been shown to derepress 3-hydroxy-3-methylglutaryl-coenzyme A reductase and cause the accumulation of 2,3-oxidosqualene. This high concentration of sterol intermediate allows some leakage through the *erg7* mutation to form ergosterol (13). We found that the low levels of ergosterol biosynthesized endogenously by induction from ALA would allow optimal sparking on bulk cholestanol at a concentration of 800 ng/ml. Lower concentrations of ALA increased the lag period and reduced the yield, whereas high concentrations induced a longer lag period and a growth inhibition during the log phase (Fig. 3). This growth inhibition has been reported before (12) and is currently being investigated. A previous report described a water-soluble compound isolated from wild-type *S. cerevisiae* that allowed sparking on cholestanol (18). This sparking ergosterol replacement factor has the same properties of the heme intermediate ALA, which was shown to spark growth at very low concentrations. We believe that the sparking ergosterol replacement factor may actually be ALA and causes sparking on cholestanol by the induction of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the production of microamounts of ergosterol formed through leakage of the *erg7* mutation.

ACKNOWLEDGMENTS

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