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Effects of Culture Conditions on Ergosterol Biosynthesis by *Saccharomyces cerevisiae*

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Ergosterol is an essential component of yeast cells that maintains the integrity of the membrane. It was investigated as an important factor in the ethanol tolerance of yeast cells. We investigated the effects of brewing conditions on the ergosterol contents of *S. cerevisiae* K-9, *sake* yeast, several kinds of *Saccharomyces cerevisiae* that produce more than 20% ethanol, and X2180-1A, laboratory yeast. K-9 had a higher total ergosterol contents under all the conditions we examined than X2180-1A. Ethanol and hypoxia were found to have negative and synergistic effects on the total ergosterol contents of both strains, and significantly reduced the free ergosterol contents of X2180-1A but only slightly reduced those of K-9. The maintenance of free ergosterol contents under brewing conditions might be an important character of *sake* yeast strains. DNA microarray analysis also showed higher expression of ergosterol biosynthesis genes in K-9 than in X2180-1A.

Key words: *Saccharomyces cerevisiae*; ergosterol; ethanol tolerance; *sake* yeast

Sterols are essential components of all eukaryotic cells. The predominant sterol in *Saccharomyces cerevisiae* is ergosterol, which is the counterpart of cholesterol in mammalian cells. In *S. cerevisiae* cells, as in other eukaryotes, sterols are present in two forms, as free sterols and sterol esters. Free sterols are mainly located on the plasma membrane where they are very important on the fluidity and permeability of the membrane and have various effects on the activities of membrane-bound proteins.[1] The steryl esters are sequestered in cytosolic lipid particles, where they function in sterol homeostasis.[2,3] Recently, there has been growing interest in the role of ergosterol in the formation of rafts, microdomains of the membrane that are involved in membrane traffic and cell signaling.[4,5] Since blocking of any of the steps of ergosterol biosynthesis pathways, from acetyl-CoA to zymosterol, is lethal, sterols must be essential for yeast cell functions.

Sake yeast strains, used in *sake* making, show higher ethanol tolerance than any other industrial yeast strains,[6] and produce more than 20% ethanol. We reported previously that an ethanol-sensitive mutant of *sake* yeast complemented with *ERG6*, which encodes Delta(24)-sterol C-methyltransferase.[7] This enzyme methylates position C-24 of zymosterol to convert it to fecosterol in the ergosterol biosynthetic pathway. Moreover, the ability to synthesize ergosterol is regarded as one of the important factors in the ethanol tolerance of yeast cells.[7–9] Although ergosterol has a positive effect on ethanol tolerance, the ergosterol content of yeast cells appears to be low during the *sake*-making procedure, which is hypoxic and shows high ethanol concentration, because hypoxity[10,11] and high concentrations of ethanol[12,13] have been reported to affect the ergosterol biosynthesis of yeast cells.

To investigate the behavior of intracellular ergosterol of a *sake* yeast strain and a laboratory yeast strain under *sake*-making conditions, we analyzed ergosterol biosynthesis in those yeast strains under several conditions related to the *sake*-making procedure. Until now, the ergosterol contents of sake yeast cells have been measured as total ergosterol, which includes free sterol and sterol esters.[7,8] In this study, we first measured the total ergosterol contents of yeast cells (*S. cerevisiae* K-9, a sake yeast, and X2180-1A, a laboratory yeast) cultured under various conditions to verify the factors affecting ergosterol biosynthesis. We investigated not only total ergosterol but also free ergosterol of yeast cells under hypoxic and high ethanol conditions, which are characteristics of sake-making procedures. Furthermore, we analyzed the expression of the ergosterol biosynthetic genes of K-9 and X2180-1A with a DNA microarray to investigate the relation between these differences of the behavior in intracellular ergosterol in these strains and the gene expression profiles.

Materials and Methods

Yeast strains and culture media. The yeast strains used in this study were *Saccharomyces cerevisiae* K-9 (Kyokai 9) as a sake yeast, and X2180-1A as a laboratory yeast. SD medium (0.67% Difco yeast

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nitrogen base without amino acids, 1.0% glucose) was used for normal yeast cultivation, and 1.8% agar was added for plate cultivation.

Ethanol tolerance of yeast. We compared the ethanol tolerances of *Saccharomyces cerevisiae* K-9 and X2180-1A using plate cultures and the growth profiles in a liquid medium containing ethanol. Yeast cells that had been aerobically precultured in SD medium for 24 h were washed twice with sterile distilled water and suspended in sterile distilled water to a cell concentration corresponding to $OD_{660\,nm}$ to 1.0. The cell suspensions were diluted 10, 10^2, 10^3, and 10^4 fold, and 3 µl of each cell suspension was spotted on plates containing 0, 5, or 7% ethanol and then incubated for 2 d at 30 °C.

The growth profiles of *S. cerevisiae* K-9 and X2180-1A were measured as follows: Yeast cells precultured in SD medium for 24 h at 30 °C were inoculated in SD medium containing 0, 5, or 7% ethanol. The amount of cells added was adjusted to bring $OD_{660\,nm}$ to 0.1. The cultures were incubated at 30 °C aerobically. The increase in absorbance of the cultures was measured automatically using Biophotorecorder model TN1506 (ADVANTEC, Tokyo, Japan).

Determination of total and free ergosterol contents of yeast cells. Total ergosterol was extracted and determined following the procedure described by Inoue *et al.*[7] Yeast cells, which were cultivated under the conditions described for each experiment, were collected and washed twice with sterilized distilled water. Washed cells were freeze-dried, weighed dry cell weight, and suspended in 5 ml of ethanol before 30 ml of methanol and 2.0 g of KOH were added. Saponification of the mixtures was carried out by heating at 75 °C for 30 min under N_2 gas. After the mixture was cooled to room temperature, 10 ml of distilled water was added. Sterols were extracted twice from the mixture with 10 ml of petroleum ether. The fractions of petroleum ether were collected and evaporated to dryness. Sterol fractions were dissolved in 2 ml of CH_2Cl_2·methanol (1:1 v/v), and subjected to HPLC (TSK gel ODS-80Ts column, Tosoh, Tokyo, Japan) to determine ergosterol concentrations.

Free ergosterol of yeast cells were extracted and determined by the method of Bailey and Parks,[14] with some modifications. Yeast cells, which were cultivated under the conditions described for each experiment, were collected and washed twice with sterilized distilled water, freeze-dried, weighed, suspended in 400 µl of dimethyl sulfoxide (DMSO), and heated for 1 h at 100 °C. After cooling to room temperature, the mixture was supplemented with 3 ml of H_2O and then extracted twice with 2 ml of petroleum ether. The combined extracts were evaporated to dryness, dissolved in 500 µl of chloroform, and then analyzed by HPLC, as described above.

DNA microarray analysis. Yeast cells were cultivated in SD liquid medium at 30 °C aerobically, and harvested when $OD_{660\,nm}$ reached 5.0. Cultivated cells were collected by centrifugation, and washed twice with ice chilled-sterilized distilled water. Total RNA was extracted from the yeast cells by the hot phenol method.[15] The isolated total RNA was dissolved in an appropriate volume of DEPC-treated water.

All RNA labeling procedures were performed on ice. The total RNA (25 µg) was mixed with 8 µl of 5× First-Strand Buffer (Invitrogen-Life Technologies, Carlsbad, CA), 1 µl of yeast-specific primer mix (Eurogentech, Seraing, Belgium), 3 µl of 20 mM dNTP without dCTP (6.67 mM each of dATP, dGTP, dTTP), 1 µl of 1 mM dCTP, 1.5 µl of 1 mM Cy3- or Cy5-conjugated dCTP (Amersham Pharmacia Bioscience, Piscataway, NJ), 4 µl of 0.1 M DTT, 1 ml of RNasin (Promega, Madison, WI), and DEPC-treated H_2O to adjust the final volume to 40 µl. The mixture was incubated at 65 °C for 5 min and then at 42 °C for 5 min, mixed with 1 µl of RNasin and 1 µl of Superscript II RT (Invitrogen-Life Technologies) and incubated at 42 °C for 2 h for reverse transcription. An additional 1 µl of Superscript II RT was added after 1 h of incubation.

After incubation, 5 µl of 50 mM EDTA and 2 µl of 10 N NaOH were added to the mixture, and the mixture was incubated at 65 °C for 20 min to stop the reverse transcriptional reaction. To neutralize the mixture, 4 µl of 5 M acetic acid was added to the mixture.

The probes were cleaned using a QIA-quick PCR purification kit (Qiagen, Hilden, Germany), and a mixture of each Cy3- and Cy5-labeled cDNA probe was concentrated to approximately 24.5 µl using the Microcon-30 ultrafiltration device (Millipore, Bedford, MA).

Yeast chip ver. 2.0 (DNA Chip Research, Yokohama, Japan) was used for a DNA microarray. Hybridization of the microarray was carried out according to the instructions of the manufacturer. The microarray was dried and scanned using an FLA-8000 Fluorescent Image Analyzer (Fuji Film, Tokyo), and the data were analyzed by GeneSpring 6.0 software produced by Silicon Genetics 6.1 (Redwood City, CA).

Results

Effect of ethanol on the growth of S. cerevisiae K-9 and X2180-1A

Ethanol tolerance is one of the most important characteristics of brewing yeast. As ethanol concentration in sake mash approaches 20% at the end of fermentation, sake yeast strains are thought to have higher ethanol tolerances than other yeast strains.[6] In this study, we measured the cell growth of *S. cerevisiae* K-9, a sake yeast strain, and *S. cerevisiae* X2180-1A, a laboratory yeast strain, in media containing 5% and 7% ethanol (Fig. 1).

The growth of X2180-1A was poorer than the growth

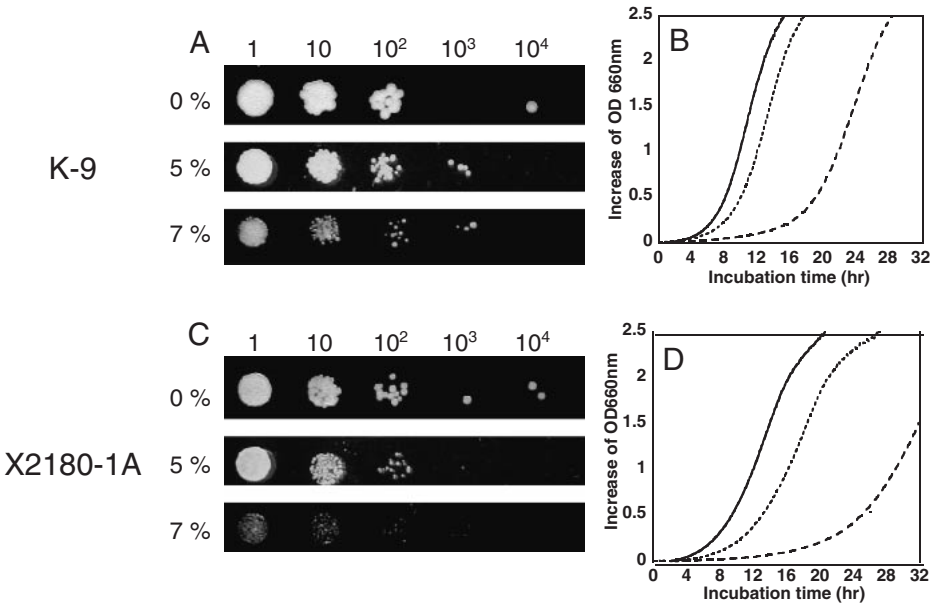

Fig. 1. Ethanol Tolerances of *S. cerevisiae* K-9 and X2180-1A.

The growth of K-9 (A) and X2180-1A (C) on solid SD medium containing 0, 5, or 7% ethanol. The series of spots was made by dilution of the yeast cell suspensions of their cell density at $OD_{660\,nm}$ at 1.0 by 10 times. Yeast cells spotted on the medium were cultured at 30 °C for 48 h. The growth of K-9 (B) and X2180-1A (D) in liquid SD medium containing ethanol at a concentration of 0%, solid lines (—); 5%, dotted lines (···); 7%, broken lines (– –). The yeast cells were inoculated at $OD_{660\,nm}$ 0.1. The growth of the two yeast strains was measured by $OD_{660\,nm}$ every 30 min at 30 °C aerobically.

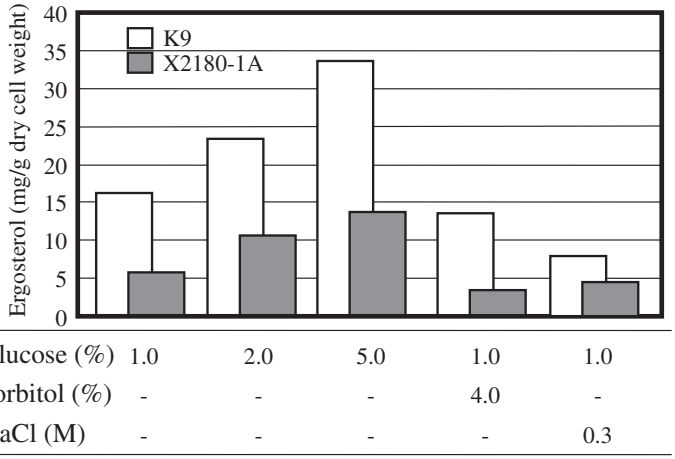

Fig. 2. Ergosterol Contents of *S. cerevisiae* K-9 and X2180-1A.

Each type of yeast cells was cultured at 25 °C for 96 h aerobically in SD medium containing glucose and an osmotic adjuster respectively. Ergosterol of yeast cells was extracted and saponified as described in "Materials and Methods".

of K-9 on the SD plate containing ethanol. In liquid media, the growth rates of both strains decreased with the addition of ethanol, but ethanol inhibition on growth was significantly greater for X2180-1A than for K-9. These results clearly show that *S. cerevisiae* K-9 has higher growth ability under high ethanol concentrations.

In previous studies, we reported that ergosterol biosynthesis is an important factor in ethanol tolerance. Mutation of the *erg6* gene leads to a reduction in the ergosterol content of cells and an ethanol-sensitive phenotype.[7] However, we focused on differences in the behavior of the ergosterol contents of K-9 and X2180-1A under various conditions.

Total ergosterol contents of K-9 and X2180-1A under various conditions

The total ergosterol contents of K-9 and X2180-1A were 16.5 and 5.8 mg/g dry cell weight respectively (Fig. 2). The total ergosterol content of K-9 was about 3-fold higher than that of X2180-1A.

At the beginning of the sake-making procedure, the osmotic pressure of sake mash is high because of high

glucose concentrations and this might affect the ergosterol biosynthesis of yeast cell. Hence, we analyzed the effects of glucose concentrations and osmotic pressure on the total ergosterol contents of yeast cells. The results of the glucose additive experiment are shown in Fig. 2. In both the K-9 and the X2180-1A strains, the total ergosterol concentration of cells increased when the glucose concentration of medium increased. The amount of total ergosterol of K-9 was higher than that of X2180-1A in all conditions. D-Sorbitol and NaCl instead of glucose were used to adjust the osmotic pressure of the medium. The total ergosterol concentration of yeast cells cultivated in 1% glucose and 4% D-sorbitol or 0.3 M NaCl medium, — the osmolalities of these media are same as that of 5% glucose medium — were rather lower than the cells cultivated in 1% glucose medium (Fig. 2). These results indicate that glucose has a positive effect on the total ergosterol concentration of yeast cells, but not through its effect on osmotic pressure.

Influence of O_2 and ethanol on total ergosterol contents of the yeasts

Sake mash is hypoxic, its ethanol concentration can reach 20% at the end of fermentation. Although molecular oxygen is required for ergosterol biosynthesis, sake yeasts have to synthesize ergosterol to maintain plasma membrane integrity through the sake-making procedure. Hence it is important to analyze the effects of oxygen on the ergosterol biosynthesis of sake yeast.

Yeast cells of K-9 and X2180-1A were cultured in liquid SD medium under aerobic and hypoxic conditions at 25 °C for 96 h, and the total ergosterol contents of the yeast cells were measured. Under hypoxic conditions, the total ergosterol contents of the yeast cells decreased to one-fourth in K-9 and one-half of those of yeast cells cultured under aerobic conditions (Fig. 3). Even though the total ergosterol concentration of K-9 strain was greatly reduced under hypoxic conditions, it was still higher than that of X2180-1A. Thus the ergosterol biosynthesis ability of the K-9 strain is higher than that of X2180-1A under both aerobic and hypoxic conditions.

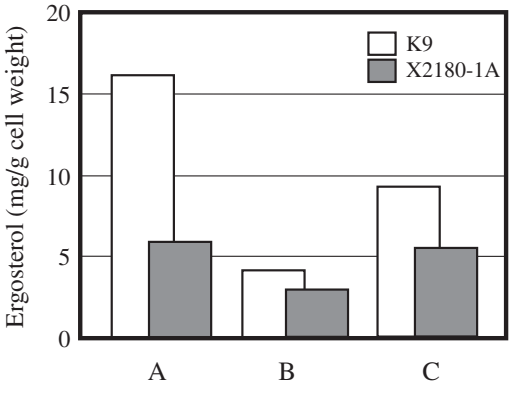

Fig. 3. Influences of Oxygen and Ethanol on Ergosterol Contents of Yeast Cells of *S. cerevisiae* K-9 and X2180-1A.

Yeast cells were cultured at 25 °C for 96 h in SD medium containing 0% (A and B) or 5% ethanol (C) with shaking (aerobic, A and C) and without shaking (hypoxic, B). Ergosterol of yeast cells was extracted and saponified as described in "Materials and Methods".

Sake yeasts produce up to 20% ethanol at the end of the sake-making procedure, and ethanol affects the membrane fluidity and ergosterol biosynthesis of yeast cells. Hence we investigated the effects of ethanol on the ergosterol contents of sake yeast cells. Ethanol reduced the total ergosterol content of K-9 cells but not that of X2180-1A cells (Fig. 3C), but the amount was still higher in K-9 cells than in X2180-1A cells.

Influence of O_2 and ethanol on free and total ergosterol contents of yeast cells

As mentioned above, hypoxia and ethanol had negative effects on the ergosterol biosynthesis of yeast cells. These two factors are common in all kinds of brewing procedures, and they might affect the biosynthesis of ergosterol during the fermentation period. Hence we investigated the synergistic effects of hypoxia and ethanol the ergosterol biosynthesis in detail. As described above, ergosterol presents as two forms in yeast cells, a free form, which fulfills various functions in the membrane, and an ester form, which is stored in lipid particles. Especially, the amount of free ergosterol is important for ethanol tolerance and fermentation. Free ergosterol is mainly present in the plasma-membrane of yeast cells and affects the activity of several plasma-membrane proteins such as Pma1p, which is essential for ethanol fermentation, Gas1p, Tat2p, and uracil permease *etc.* though raft formation and membrane fluidity.[4,5] Hence we measured the free ergosterol and total ergosterol contents of yeast cells separately.

Time courses of free and total ergosterol contents of yeast cells are shown in Fig. 4. Under aerobic conditions, the total ergosterol concentration of K-9 cells increased with increasing cultivation time, reaching 15 mg/g dry cells, while under hypoxic conditions it decreased slightly (Fig. 4). Total ergosterol concentrations of X2180-1A cells grown under both aerobic and hypoxic conditions did not change significantly with cultivation time. In both yeast strains and culture conditions, free ergosterol increased transiently at 48 h. In K-9 cells grown under aerobic and hypoxic conditions, the free ergosterol concentration remained almost constant, even though the total ergosterol concentration decreased significantly under hypoxic conditions. In X2180-1A cells, however, the free ergosterol concentration was lower under hypoxic conditions than under aerobic conditions (about one half as much as at 48 h).

Because the presence of ethanol also affected total ergosterol concentration, we investigated the synergistic effects of hypoxia and ethanol on the amount of free and total ergosterol concentrations in yeast cells. Ethanol

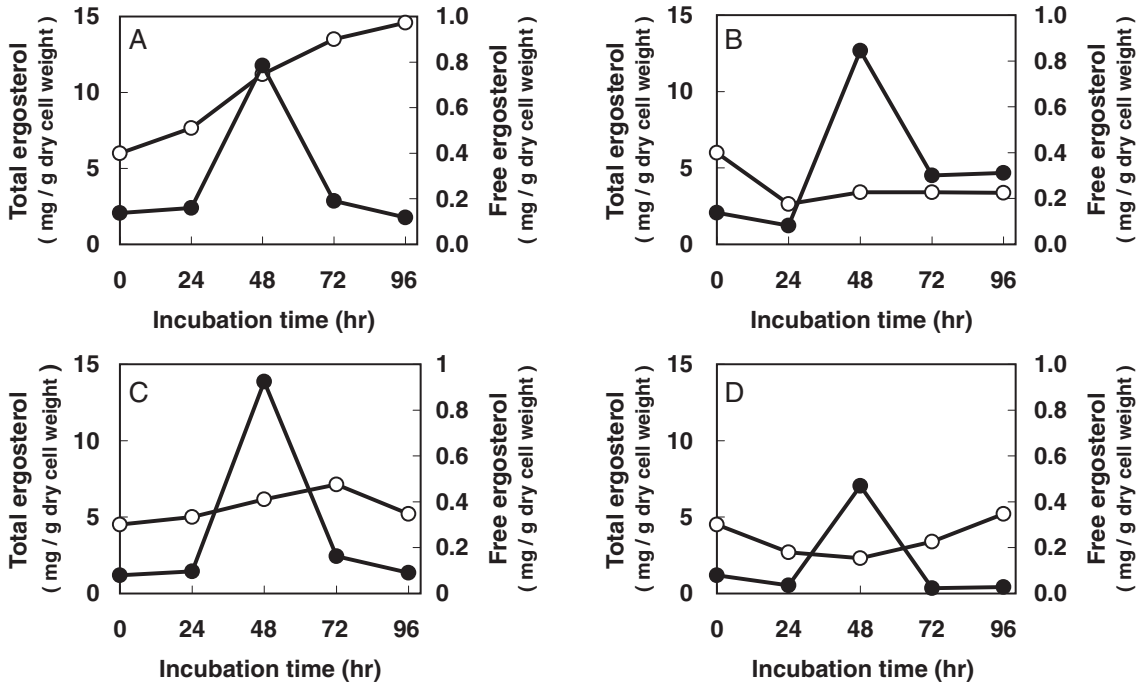

Fig. 4. Time Courses of Free and Total Ergosterol Contents of Yeast Cells under Aerobic and Hypoxic Conditions without Ethanol.
A, K-9 cultured under aerobic conditions; B, K-9 cultured under hypoxic conditions; C, X2180-1A under aerobic conditions; D, X2180-1A under hypoxic conditions. Yeast cells cultured in liquid SD medium without ethanol at 25 °C. Open circles, total ergosterol contents; closed circles, free ergosterol contents.

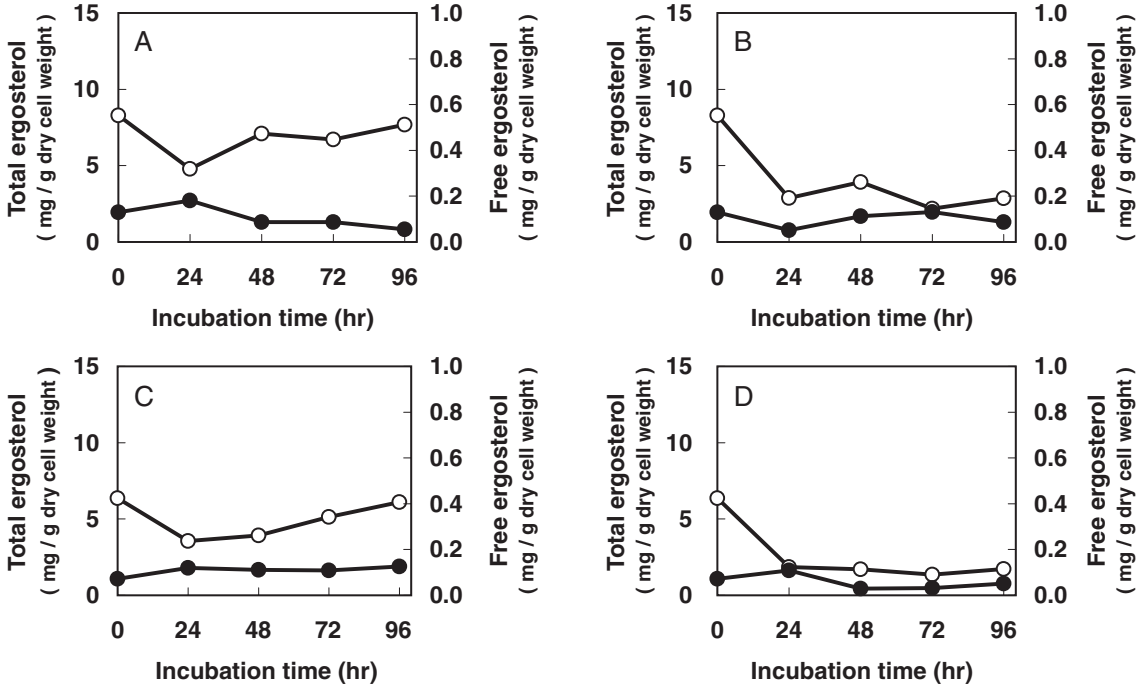

Fig. 5. Time Courses of Free and Total Ergosterol Contents of Yeast Cells under Aerobic and Hypoxic Conditions with Ethanol.
A, K-9 cultured under aerobic conditions; B, K-9 cultured under hypoxic conditions; C, X2180-1A under aerobic conditions; D, X2180-1A under hypoxic conditions. Yeast cells were cultured in liquid SD medium containing 5% ethanol under aerobic and hypoxic conditions at 25 °C. Open circles, total ergosterol contents; closed circles, free ergosterol contents.

affected both free and total ergosterol concentrations in K-9 and X2180-1A cells. The total ergosterol concentrations in both types of yeast cells were reduced by the addition of ethanol (Fig. 5). The effects were most significant in aerobic K-9 cells and hypoxic X2180-1A cells. But, under all conditions, the amounts of ergo-

sterol in K-9 cells were still higher than those in X2180-1A cells.

Ethanol had its strongest effect on the concentration of free ergosterol. With the addition of 5% ethanol, the transient peaks at 48 h disappeared in K-9 and X2180-1A cells under both aerobic and hypoxic conditions. The free ergosterol concentration of K-9 cells remained constant under both aerobic and hypoxic conditions, while the free ergosterol concentration of X2180-1A cells decreased under the hypoxic condition.

Expression analysis of ergosterol biosynthesis genes in sake yeast

K-9 cells had a greater ability to synthesize and accumulate ergosterol than X2180-1A cells. It is important to determine whether this ergosterol has to do with a difference in ergosterol synthetic gene expression. The expressions of these genes were analyzed using a DNA microarray. Both strains were cultivated in SD medium, and total RNAs were extracted from two independent cultures. The extracted total RNAs were used for probe preparation, and were analyzed by two independent microarray hybridizations by swapping of dyes.

Among 19 ergosterol biosynthesis genes examined in the microarray, 9 genes were expressed 2-fold more in K-9 than in X2180-1A (Fig. 6 and Table 1), and none of genes was expressed more strongly in X2180-1A than in K-9. Upregulation of ergosterol biosynthesis genes in K-9 cells is partly responsible for the higher accumulation of ergosterol in K-9 cells than in X2180-1A cells.

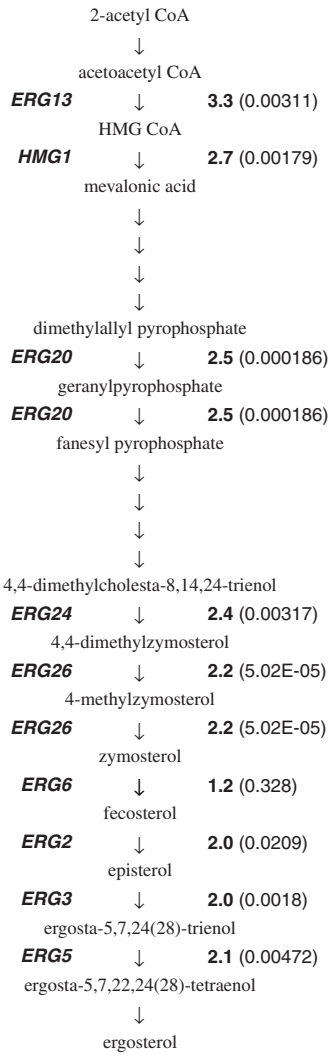

Fig. 6. Overexpressed Genes Involved in Ergosterol Biosynthesis in K-9.

From DNA microarray analysis (n = 4), overexpressed genes involved in ergosterol biosynthesis in K-9, its expression ratio compared with that of X2180-1A, and p-value of t-test.

Table 1. Overexpressed Ergosterol Biosynthesis Genes in K-9

Gene	Gene product	Ratio	p-value
ERG13	CoA synthetase	3.3	0.00311
HMG1	HMG CoA reductase	2.7	0.00179
ERG20	farnesyldiphosphate synthetase	2.5	0.000186
ERG24	C-14 sterol reductase	2.4	0.00317
ERG26	C-3 sterol dehydrogenase	2.2	5.02E-05
*ERG6**	C24 sterol methyltransferase	1.2	0.328
ERG2	C-8 sterol isomerase	2.0	0.0209
ERG3	C-5 sterol desaturase	2.0	0.0018
ERG5	C-22 sterol desaturase	2.1	0.00472
ERG28	ER transmembrane protein	2.1	0.0251

**ERG6* was not overexpressed in K-9, shown as control gene.

Discussion

Sake-making conditions, which are characterized by hypoxia, high ethanol concentrations, high osmotic pressure, low temperature, and high glucose concentrations, are unfavorable for normal yeast cell functions. Sake yeast strains are known to have a higher ethanol tolerance than other industrial yeast strains.[6] *S. cerevisiae* K-9, a sake yeast strain, has higher growth ability under higher ethanol conditions than X2180-1A, a laboratory yeast strain, according to the results of both plate and liquid medium with 5% ethanol (Fig. 1). Ergosterol has been found to be one of the components which play an important role in maintaining cell-membrane integrity[1] and the ethanol tolerance of yeast.[7–9] We found that the total ergosterol concentration of K-9 was 3-fold higher than that of X2180-1A (Fig. 2, 3A), and this result agrees well with previous studies.[7] Sake yeasts can produce more than 20% ethanol concentration during the sake-making procedure and show higher ethanol tolerance than other yeast strains. To investigate the behavior of intracellular ergosterol of the sake yeast strain, we analyzed the effects of factors relating sake-making procedure, such as glucose concentration, osmotic pressure, hypoxia and ethanol concentration, on the ergosterol contents of yeast cells of K-9, a sake yeast, and X2180-1A, a laboratory yeast.

We found that a high concentration of glucose increased ergosterol biosynthesis in yeast cells. On the

contrary osmotic pressure did not have any effect on the total ergosterol contents of yeast cells (Fig. 2). Therefore, the positive effect of glucose is not due to its effect on osmotic pressure. Ergosterol biosynthesis is a metabolically expensive pathway,[16] and consumes a large amount of ATP, which is supplied by the glycolysis pathway. Furthermore, the ergosterol biosynthesis pathway starts from acetyl-CoA, which is also supplied by the glycolysis pathway. This relationship between ergosterol biosynthesis and the glycolytic pathway has to do with high ergosterol biosynthesis under the high glucose condition, but further studies are needed to determine why ergosterol biosynthesis increases with increasing amounts of glucose in a media.

Since molecular oxygen is required for ergosterol biosynthesis,[16,17] hypoxic conditions should also have a negative effect on total ergosterol content in sake yeast. Furthermore, ethanol concentration affects the elasticity of the plasma-membrane[18] and the ergosterol biosynthesis in yeast cells.[12,13] The total ergosterol contents of both strains were reduced by both hypoxia and high concentrations of ethanol in the medium. But the amount of ergosterol in K-9 cells was still higher than that of the laboratory strain of X2180-1A cells under all conditions examined (Fig. 2, 3). These results suggest that K-9 cells have higher ergosterol biosynthesis ability than X2180-1A cells under these conditions related to sake making.

Ergosterol is present in two forms in yeast cells, free ergosterol and ergosterol ester. Free ergosterol is more important for cell integrity because it is present mainly in the plasma-membrane, where it forms micro-domains that decrease membrane fluidity and maintain the activity of plasma-membrane proteins.

As mentioned above, the hypoxic condition did not have any significant effect on the amount of free ergosterol in K-9 yeast cells, although it had a significant negative effect on total ergosterol contents. On the other hand, the hypoxic condition had a negative effect on the amount of free ergosterol in X2180-1A yeast cells, while it did not have a significant effect on total ergosterol contents. These results indicate that sake yeast K-9 is better able to maintain homeostasis of free ergosterol than laboratory yeast of X2180-1A, even under hypoxic conditions.

In addition to the effect of the hypoxic condition of the medium, ethanol concentration had a considerable effect to the biosynthesis and homeostasis of ergosterol. The addition of ethanol on the medium decreased the total amount of ergosterol in K-9 yeast cells but did not in X2180-1A cells, and the transient peaks disappeared at 48 h in both K-9 and X2180-1A. Although the total ergosterol contents of yeast cells in X2180-1A did not decrease with the addition of ethanol to the medium, the transient peaks at 48 h disappeared in X2180-1A cells. This result indicates that the disappearance of transient peaks of free ergosterol was not due to down regulation of ergosterol biosynthesis. Even though the increase in free ergosterol was transient, the basal level of free ergosterol remained stable in both types of cells. This indicates that the homeostasis of free ergosterol was not affected by the biosynthesis of ergosterol and that the system that maintains free ergosterol homeostasis is essential for the viability of yeast cells under hypoxic and high ethanol conditions.

The free ergosterol contents of K-9 and X2180-1A yeast cells should both be regulated to maintain normal cell function. Steryl esters accounted for an estimated 65% to 99% of the total ergosterol of most yeast cells, and the total ergosterol contents of K-9 cells was more than that of X2180-1A cells under all conditions.

These results were supported by a DNA microarray analysis of K-9 and X2180-1A (Table 1, Fig. 6). Nine genes involved in ergosterol biosynthesis were expressed more strongly in *S. cerevisiae* K-9 than in X2180-1A. Especially, overexpression of *HMG1*, which encodes HMG CoA reductase and is involved in the main bottleneck of the early ergosterol pathway,[19] and *ERG28*, which encodes a key protein in the yeast sterol biosynthetic enzyme complex,[20] appear to be related to the higher total ergosterol contents of K-9 than of X2180-1. This might result in the greater ethanol tolerance of K-9 than of X2180-1A. Because ergosterol biosynthesis during sake-making is difficult due to the high ethanol concentration and hypoxic conditions, ergosterol biosynthesis genes in sake yeasts might be overexpressed to maintain their ethanol tolerance and cell activities. The differences in the expressions of the genes in ergosterol biosynthesis might come from the natural mutation of *HAP1* in X2180-1A, which is derived from S288c.[21] The *HAP1* gene encodes a complex transcriptional regulator of the ergosterol-related gene *HMG1* and many other genes that are involved in electron transfer reactions. In addition, a strain constructed from X2180 that contained a wild *HAP1* gene originating from sake yeast showed higher expression levels of ergosterol-related genes and ergosterol content than X2180.[22] Further research is required to make clear the alternations of regulations in ergosterol biosinthesis in K-9 cells.

Until now, the ethanol tolerance of yeast cells has been considered to depend on total ergosterol contents. *S. cerevisiae* K-9, a sake yeast, maintained both total and free ergosterol contents of yeast cells even under hypoxic and high ethanol conditions. This ability of sake yeast strain might be related to higher ethanol tolerance.

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