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Construction of squalene-accumulating *Saccharomyces cerevisiae* mutants by gene disruption through homologous recombination

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Abstract *Saccharomyces cerevisiae* synthesizes ergosterol via squalene, but squalene is hardly detected in aerobically grown cells. To obtain a stable squalene-accumulating yeast strain, we attempted to disrupt a gene required in the conversion of squalene to ergosterol, by homologous recombination with a short piece of the gene fragment conjugated with an integration plasmid vector carrying the *LEU2* gene. Two mutants that required ergosterol at least for fast growth were isolated. In an aerobic cultivation and with ergosterol supplementation, the two mutants accumulated squalene up to 5 mg/g dry cells. Southern hybridization analysis indicated that both mutants had acquired the vector DNA integrated in the same gene, or nearby genes, on chromosome 12.

Introduction

Squalene has some medical effects on hypertension, liver diseases and various skin diseases (Ikekawa et al. 1986; Kaiya 1990). The livers of deep sea sharks have been used as raw material for squalene production. However, in the near future, these natural resources may be limited.

Squalene is an intermediate of ergosterol biosynthesis in *Saccharomyces cerevisiae*. Although aerobically growing yeast does not accumulate squalene, semiaerobically growing cells take up exogeneous sterols including cholesterol and accumulate squalene because of the loss of the formation or activation of squalene epoxidase (Jahnke and Klein 1983). Karst and Lacroute (1977) isolated some temperature-sensitive mutants defective in ergosterol biosynthesis by screening for sterol requirement and nystatin resistance, after ultraviolet irradiation. These mutants were defective in one of the following enzymatic activities: squalene epoxidase (gene erg1), 2,3-oxidosqualene-lanosterol cyclase (*erg7*), phosphomevalonic kinase (*erg8*), mevalonic kinase (*erg12*) and squalene synthetase (*erg 9, 10, 11*). Among them, only the *erg1* gene mutants accumulated squalene under the aerobic condition, although to a small extent. Nonetheless, this result has indicated the possibility of obtaining a yeast mutant accumulating squalene by manipulating a gene involved in the conversion of squalene to ergosterol.

Homologous recombination occurs frequently in yeast cells and this property helps the establishment of the target gene disruption technique (Shortle et al. 1982). When sufficiently small pieces of yeast chromosomal DNA are conjugated with a foreign DNA into a circular form and introduced into the original yeast strain, any chromosomal gene might be disrupted by the single-point homologous recombination between the introduced incomplete gene and the corresponding chromosomal gene. In this paper, we report the construction of squalene-accumulating *S. cerevisiae* mutants by random gene disruption through homologous recombination with random short pieces of chromosomal DNA.

Materials and methods

Strains and plasmids

A haploid *S. cerevisiae* strain, SHY3 (*a, ste-VC9, leu2-3, leu2-112, trp-289, his-D1, ura3-52, ade1-101, can1-100*) (Botstein et al. 1979), was used as the source of chromosomal DNA and as the host of the transformation. The integration vector, named pIA02, was constructed as follows. An episomal plasmid, YEp13 (Broach et al. 1979), was separated into two fragments by *Sal*I digestion. One of them, which

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contains the 6.8-kb fragment carrying the yeast *LEU2* gene and the larger half of pBR322 carrying the ampicillin-resistance gene and the replication origin (Bolivar et al. 1977), was self-ligated and propagated in *Escherichia coli* K12 HB101 (Hanahan 1983). This plasmid was used as the integration vector for the yeast transformation.

Media

For the cultivation of *S. cerevisiae* SHY3, YPD medium (1% yeast extract, 2% peptone, 2% glucose) was routinely used. A minimal medium (0.67% Difco Bacto Yeast Nitrogen Base without amino acid, 2% glucose) was used as the basic medium for the selection of the leucine-autotrophic transformants. Ergosterol or cholesterol was added at the final concentration of 4 mg/l in liquid medium and 40 mg/l agar plates. YPDE and YPDC were YPD media supplemented with ergosterol and cholesterol, respectively.

Preparation of a bank of yeast chromosomal DNA fragments

The chromosomal DNA of *S. cerevisiae* SHY3 was isolated according to the method of Cryer et al. (1975). The DNA was digested with DNaseI until the length of most of the generated fragments became less than 1 kb. The fragments were ligated with *Pvu*I-digested pIA02 after blunting their ends with T4 DNA polymerase, and introduced into *E. coli* HB101. Approximately 25,000 ampicillin-resistant transformants were collected and divided into 60 groups. Plasmid DNAs isolated from each group were separately used to transform *S. cerevisiae* SHY3.

Transformation of yeast

The plasmid DNAs were introduced into yeast cells according to the method of Ito et al. (1983), except that the heat shock treatment was performed at 45C for 10 min. This severe condition increased the transformation efficiency approximately four times as much as original condition (data not shown).

Analysis of the squalene content

The yeast cells grown in 100 ml media were harvested, resuspended in 30 ml methanol and broken by shaking with glass beads in a Braun homogenizer. After repetition of the extraction of total lipids from the homogenate with 30 ml chloroform–methanol (1:2) three times, the organic phases were combined and evaporated under a stream of nitrogen gas, and dissolved in chloroform. To analyze for the contents of sterols and squalene, the solution was directly applied to silica-gel thin-layer chromatography (TLC) plates (Merck 60 F_{254}) or to gas-liquid chromatography (GLC) after saponifying the lipids by incubation with 20% KOH at 85C for 1 h. The thin-layer plate was developed with petroleum ether-diethyl-ether-acetic acid (95:4:1) and scanned by a Shimazu TLC Scanner CS-90 with irradiation of 200 nm ultraviolet light. For detection of sterols and squalene by GLC, the saponified lipids were loaded on the silica-gel column, which was activated by heating at 150C for several hours, and eluted with chloroform. The eluted peak was then applied to GLC using HP-1 (Hewlett Packard). As an internal standard for the quantitative measurement, *n*-heptacosane was used. The Rf values and the retention times were calibrated with authentic squalene, ergosterol and cholesterol purchased from Sigma.

Results

Transformation of yeast and selection of ergosterol-requiring mutants

We constructed a bank of 25,000 *E. coli* transformants containing short fragments of *S. cerevisiae* chromosomal DNA by using a plasmid lacking the replication origin of YEp13. These transformant colonies were divided into 60 groups, each of them containing about 420 colonies. The 60 groups of recombinant plasmids were amplified in *E. coli* and introduced into the *S. cerevisiae* SHY3 cells independently. For enrichment of ergosterol-requiring transformants, the cells in the transformation mixture were cultivated in minimal medium broth supplemented with the required amino acids except for leucine, cholesterol and nystatin at 30°C for 1 day with vigorous shaking. The concentration of nystatin, 10 mg/l, is sufficient to kill the wild-type yeasts capable of ergosterol biosynthesis (Woods 1971).

Cells were washed with distilled water, and plated onto minimal medium supplemented with the required amino acids except for leucine and ergosterol. After incubating at 30°C for 5 days, about 15,000 leucine-autotrophic transformants were obtained. All the colonies were then transferred onto YPD and YPDE plate to test for growth. Two transformants, E13 and E16 hardly grew on YPD plates and they were used in the subsequent analyses. As shown in Fig. 1, the growth of the two mutants considerably depends on the presence of ergosterol. The growth rate of E16 in the sterol-free medium was slower than that of E13, suggesting that these two clones are mutually independent mutants. Different growth rates between E13 and E16 were also observed on the YPD plates (data not shown).

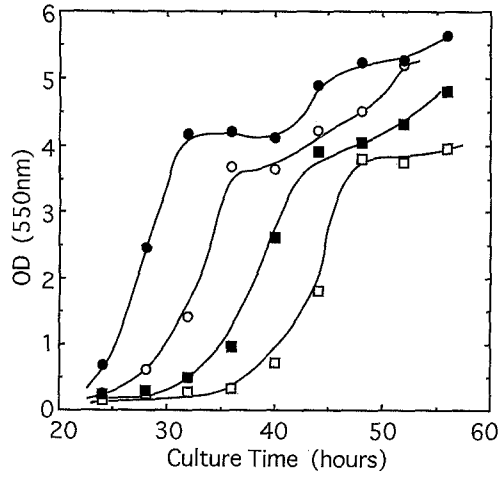

Fig. 1 The growth of *Saccharomyces cerevisiae* E13 (○, ●) and E16 (□, ■) mutants in aerobic culture conditions in medium with (YPDE, ●, ■) or without ergosterol (YPD, ○, □) (*OD* optical density)

Accumulation of squalene in E13 and E16 cells

To examine the ergosterol synthesis activity, E13 and E16 as well as their parental strain, SHY3, were cultivated in YPDC broth with shaking, and the total lipids were extracted from the cells for analysis of the sterol and squalene contents by GLC. In SHY3, as shown in Fig. 2, a peak for ergosterol (retention time = 25.6 min) was observed, but neither squalene nor cholesterol was detected. In contrast, a large peak for squalene was detected in each mutant, in addition to the smaller peaks for ergosterol and cholesterol. The contents of ergosterol in E13 and E16 were one-half and one-fifth of that in SHY3, respectively (see Table 1), indicating that these two mutants were not completely defective in ergosterol biosynthesis. Nonetheless, the mutants took up cholesterol from the media, as in the case of the temperature-sensitive mutants for ergosterol biosynthesis under the non-permissive conditions (Taylor and Parks 1980), suggesting that the synthesis rate of ergosterol in each mutant is actually reduced. Both mutants accumulated squalene in the cells. The amount of squalene in E13 increased with cultivation and its accumulation was enhanced by cultivation in YPDE in comparison with that in YPDC (Fig. 3). The final amount of squalene in E13 reached approximately 5 mg/g dry cell weight (d.c.w.). The properties of E16 were quite similar to that of E13 and it also accumulated 5 mg/g d.c.w (data not shown).

Southern hybridization analyses of the recombination site on the chromosome of each mutant

Mutants E13 and E16 resemble each other and they are quite different from SHY3 concerning their squalene-accumulation ability. To know whether the insertion of a recombinant plasmid to a specific chromosomal site caused these changes, Southern hybridization analyses (Southern 1975) were performed against *Bgl*II, *Eco*RI, and *Xba*I-digested chromosomal DNAs and chromosomal DNAs separated by pulsed-field electrophoresis of the parent and mutant strains (see Fig. 4 and Fig. 5). Among these restriction enzymes, *Xba*I does not cut pIA02, which should be integrated into the chromosomes.

When SHY3 DNA was probed with the *Bgl*II-*Sal*I fragment of pIA02, only the original *leu2* gene on the chromosome was detected in each digestion sample (Fig. 4A). In case of the mutants, some more bands were detected in addition to those in SHY3. The revealed band pattern was the same between E13 and E16; the new bands corresponded to the size of 8 kb (*Bgl*II), 18 kb (*Xba*I) and, 5 and 9 kb (*Eco*RI). The intensity of each extra band was quite similar to that of the *leu2* gene band. By hybridization using the total fragments of pBR322 digested with *Alu*I as a probe, which did not include *LEU2* gene and constituted the main portion of pIA02 vector, the 8-kb *Bgl*II fragment, the 18-kb *Xba*I fragment and the 5-kb *Eco*RI fragment showed positive signals in the mutants DNAs (Fig. 4B). For *Eco*RI or

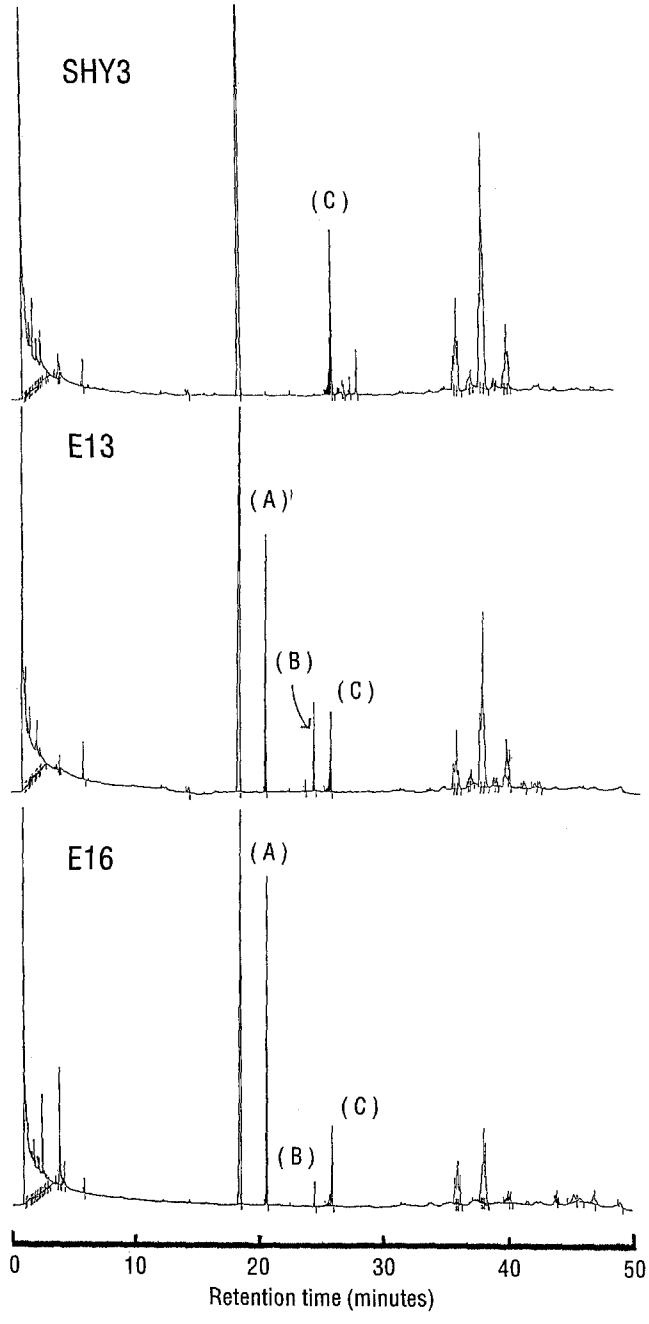

Fig. 2 Gas-liquid chromatograms (GLC) of the lipids extracted from *S. cerevisiae* SHY3 and mutants E13 and E16. GLC analyses were performed using a Shimazu GC-14A gas chromatograph equipped with an HP-1[25 m × 0.32 mm i.d. × 0.17 μm fused silica capillary column coated with 100% dimethylpolysiloxane (Gum)] (Hewlett Packard) and flame ionization detection. The column oven was programmed from 200C to 330C at the rate of 4C/min, with the injector at 330C. Helium gas was used as a carrier gas at 90 ml/min. The peaks for squalene, cholesterol and ergosterol are marked (*A*), (*B*) and (*C*), respectively. The large peak detected beside the squalene peak is that of *n*-heptacosane added as the internal standard

Table 1 Squalene and sterol contents of yeast cells in the logarithmic growth phase

Compounds	Concentration (mg/g Dry cell weight)		
	SHY3	E13	E16
Squalene	0.00	1.33	1.92
Ergosterol	1.16	0.68	0.23
Cholesterol	0.00	0.34	0.10

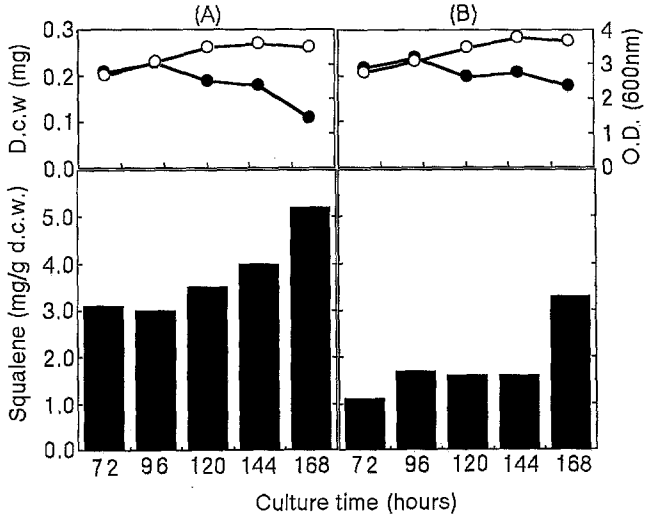

Fig. 3 The amount of accumulated squalene in E13 by cultivation with YPDE (**A**) and YPDC (**B**). *Histograms* show the squalene contents: ○ OD at 600 nm; ● dry cell weight (D.C.W.) per 100 ml culture broth

*Bgl*II digestion, we should observe two hybridizing bands because the vector pIA02 has one cutting site each for these enzymes. However, only one band was observed in both cases for unknown reason. When the 1.4-kb *Sal*I-*Pvu*II fragment of pBR322 was used as the probe, the same results were obtained (data not shown). In the hybridization analysis of the chromosomal DNA separated by pulsed-field electrophoresis, the pBR322 gave rise to a positive signal to chromosome 12 in both mutants (Fig. 5). Although we detected concomitant shortening of chromosome 4 in the mutant E16, we do not think this is related to squalene accumulation.

Discussion

We applied the integrative gene disruption technique to mutate yeast cells. So far, incubation with chemical mutagens and/or irradiation of ultraviolet were used for the yeast mutagenesis. However, we expected that the high frequency of the homologous recombination would help the random integrative disruption of the yeast genome, and we introduced the short fragment of the yeast chromosomal DNA into yeast cells using an integration vector. By this method, we obtained two stable squalene-accumulating mutants, E13 and E16, after the enrichment of nystatin-resistant transformants. They are leaky mutants of ergosterol biosynthesis. The remaining ergosterol synthesis activity of E13 is higher than that of E16, and in the absence of sterols E13 grows faster than E16. In spite of these differences, both mutants accumulate squalene at the concentration of 5 mg/g d.c.w.

Southern hybridization analyses of digested chromosomal DNAs indicate that only one copy of a pIA02 recombinant had been integrated into a specific chromosomal site, leading to the generation of the two mutants, E13 and E16. This integration event had not occurred through the homologous recombination at the *leu2* locus of SHY3, but most probably through the homologous recombination between a short piece of DNA inserted in pIA02 and the corresponding portion of the chromosome. The length of the fragment

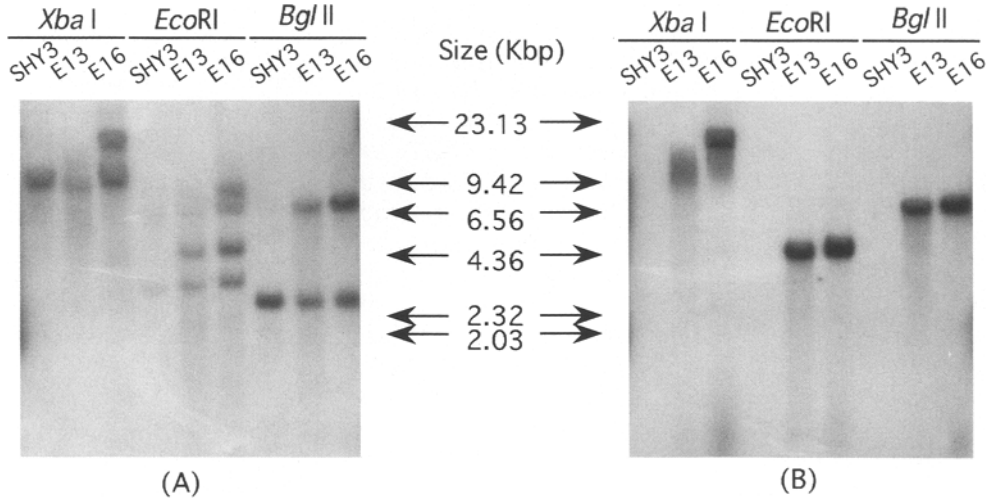

Fig. 4A,B Southern hybridization analysis of the chromosomal DNA of the mutant and wild-type strains. The *LEU2* gene probe was the *Bgl*II-*Sal*I fragment of pIA02 (**A**), and the vector probe was the total fragments of pBR322 digested by *Alu*I (**B**). The probes were labeled with ^{32}P by the random-primed labeling method. The restriction enzymes used to digest the chromosomal DNAs isolated from SHY3, E13 and E16 are indicated in the *top row*

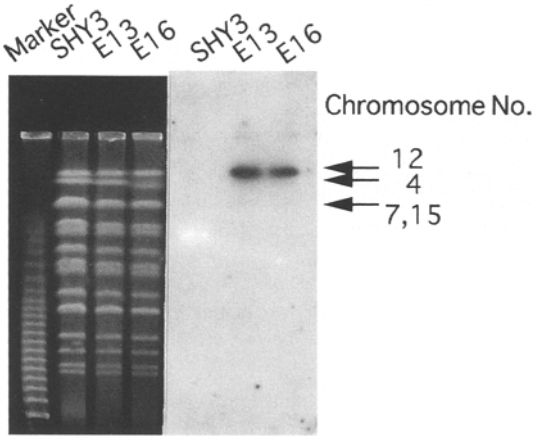

Fig. 5 Southern hybridization analysis of the SHY3, E13 and E16 chromosomes separated by pulsed-field electrophoresis. The probe was all fragments of pBR322 digested by *Alu*I labeled with ^{32}P by the random-labeling method. Lambda DNA Ladder was used as a molecular mass marker

detected by hybridization coincided between E13 and E16, suggesting that the homologous recombination occurred within the same or nearby loci in the two mutants. The pulsed-field electrophoresis experiment indicates the integration of the recombinant plasmid on chromosome 12. This suggests that the integration site is not the *ERG1* gene because it is localized on chromosome 15 (Jandrositz et al. 1991).

In conclusion, these results indicated that the points of integration were quite close on chromosome 12 in the two mutants; the points are clearly different from the *ERG1* gene located on chromosome 15. Possibly, the same gene was disrupted both in E13 and E16 by the homologous recombination between the introduced short fragment and the chromosome. The enzyme, 2,3-oxidosqualene-lanosterol cyclase, converts squalene epoxide, which is produced from squalene by the reaction catalyzed with the squalene epoxidase, to lanosterol. A temperature-sensitive mutant (*erg7*) lacking this enzymatic activity accumulated squalene epoxide in the non-permissive condition but no squalene was detected in the cells (Karst and Lacroute 1977). Taking this into account, we assume we have detected a new gene, or new genes, involved in squalene metabolism; it may be a structural or controlling gene for enzyme involved in squalene metabolism. Recovering the integrated plasmid with the flanking chromosomal fragments and analyzing its sequence will elucidate which gene was disrupted.

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