VOL. 55 NO. 11, NOV. 2002 THE JOURNAL OF ANTIBIOTICS pp. 962 — 968

Isolation of Temperature-sensitive *Saccharomyces cerevisiae* with a Mutation in *erg25* for C-4 Sterol Methyl Oxidase

HIROSHI NOSE*, TAKAKO MIYARA, NOBUAKI KUSHIDA and SHIGERU HOSHIKO

Drug Discovery, Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd.,
760 Morooka-cho, Kohoku-ku, Yokohama 222-0002, Japan

(Received for publication May 20, 2002)

C-4 sterol methyl oxidase encoded by the ERG25 gene is a key enzyme in the ergosterol biosynthetic pathway in fungi. ERG25p contains three histidine clusters common to nonheme iron binding enzymes and endoplasmic reticulum retrieval signal. In order to characterize ERG25p, we generated a series of temperature-sensitive(ts) erg25 mutants by random mutagenesis. One of the resulting mutants, the mERG25 strain, accumulated 4,4-dimethlzymosterol at the nonpermissive temperature. Sequence analysis of the mERG25 mutant indicated three amino acid substitutions in ERG25p, namely N48D, V133A, and F135S. These results indicate that the ERG25 gene product is a new antifungal target.

The ergosterol biosynthetic pathway is the target of the major classes of antifungal drugs currently used to treat human systemic fungal infections. Among these antifungals, fluconazole is widely used because it dose not cause serious side effects. Recently, however, the emergence of fluconazole-resistant organisms is compromising the reliability of this agent. Thus, the pharmaceutical industry now faces an urgent need to develop novel compounds and new antifungal target sites. In *Saccharomyces cerevisiae*, all of the genes encoding ergosterol biosynthetic enzymes have been cloned and characterized[1]. Earlier studies have demonstrated that all of the genes prior to the sterol C-24 methyltransferase (*ERG6*) gene are essential for viability, hence these genes have the potential to be singled out as new targets for the discovery of novel antifungal drugs. For example, C-14 reductase (ERG24p) is inhibited by a series of morpholines now employed as agricultural fungicides[2,3]. Squalene epoxidase (ERG1p) is inhibited by terbinafine, an agent used as a topical medication for mycoses of the skin[4,5]. Lanosterol 14α-demethylase (ERG11p) is inhibited by azoles[6].

Inhibitors of the C-4 demethylation step have not been previously reported. C-4 demethylation is separated into three reactions: (I) a C-4 methyloxidase reaction in which the 4α-methyl group is converted to an alcohol, then an aldehyde, and finally to a carboxylic acid, (II) a C-3 sterol dehydrogenation, which removes the 3α-hydrogen leading to the decarboxylation of a 3-ketocarboxylic acid sterol intermediate, (III) a 3-keto reduction, which converts the 3-keto to the β-hydroxy sterol. The *ERG25*, *ERG26* and *ERG27* genes were cloned as the genes concerned with each step[7~9]. The *ERG25* gene encodes a protein containing three histidine clusters and a KKXX golgi-to-endoplasmic reticulum retrieval signal[7]. Histidine clusters are common among non-heme diiron enzymes such as hydroxylases and fatty acid desaturases[10]. In order to demonstrate that the C-4 methyloxidase ERG25 gene product is a new antifungal target, we constructed and characterized a temperature-sensitive *erg25* mutant.

Materials and Methods

Strains and Culture Conditions

The wild type *S. cerevisiae* strain used in this work was YPH499 (Stratagene). The other strains were derivatives of YPH499 (Table 1). Yeast strains were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) or SD (0.67% Yeast Nitrogen Base without amino acids, 2% glucose) supplemented with appropriate amino acids and 0.004% adenine. For solid media, 2% Bacto-agar was added. 5-Fluoroorotic acid (5-FOA) medium for selection of yeast

* Corresponding author: hiroshi_nose@meiji.co.jp

Table 1. Genotypes and origin of yeast strains used in these studies.

Strain	Genotype	Ref.
YPH499	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1*	12
PDR5	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1*	this work
sERG25	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 pRD416-ERG25*	this work
dERG25	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 erg25::HIS3 pRD416-ERG25*	this work
ssERG25	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 erg25::HIS3 pRD415-ERG25*	this work
mERG25	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 erg25::HIS3 pRD415-mERG25*	this work

ura3 cells was prepared as described previously[11].

DNA Sequencing

DNA was sequenced directly with synthetic oligonucleotide primers using a Thermo Sequenase II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc.) on a model 377 DNA autosequencer (Applied Biosystems Inc.).

Cloning and Construction of Plasmids Containing the ERG25 Gene

The plasmids pRD415 and pRD416 were constructed in our laboratory from pRS415 and pRS416, respectively[12]. pRD415 is a yeast centromere vector that consists of the *S. cerevisiae GPD1* promoter region[13], a multiple cloning site, the *S. cerevisiae CYC1* transcription termination signal, and the *LEU2* gene as a selectable marker. The only difference between pRD416 and pRD415 is that the former includes the *URA3* gene as a selectable marker. A 1.0-kb fragment containing the coding sequence for *ERG25* was amplified by PCR using strain FL100 genomic DNA as a template and the primers ERG25-3 and ERG25-4 shown in Table 2. The ERG25-3 and ERG25-4 primers were designed to contain the recognition sites for *Hin*dIII and *Xho*I site, respectively (italic letters in Table 2). The PCR product was digested with *Hin*dIII and *Xho*I and ligated into plasmids pRD415 and pRD416, resulting in the formation of pRD415-*ERG25* and pRD416-*ERG25*, respectively.

Construction of the Yeast Strain dERG25

The mutant with *erg25* gene disruption was constructed by the method of LORENZ *et al.*[14] with some modification, and the yeast transformations were performed according to the method of ITO *et al.*[15]. The plasmid pRD416-*ERG25* was transformed into the haploid strain PDR5 (Table 1). The resulting Ura$^+$ colonies, termed sERG25, were isolated and used to disrupt the chromosomal *ERG25* gene (Figure 1). The chimera erg25::HIS3 gene was amplified by PCR using the primers erg25::HIS-3 and erg25::HIS-4. The underlined residues in Table 2 indicate nucleotides homologous to *HIS3* selectable marker. The amplified 1,110 bp fragment was introduced to the sERG25 strain. The His$^+$ transformants were isolated and examined by PCR to determine whether the interrupted erg25 gene was integrated into the chromosome by homologous recombination, thereby satisfying the precondition for obtaining the strain dERG25 (*erg25::HIS3 pRD416-ERG25*).

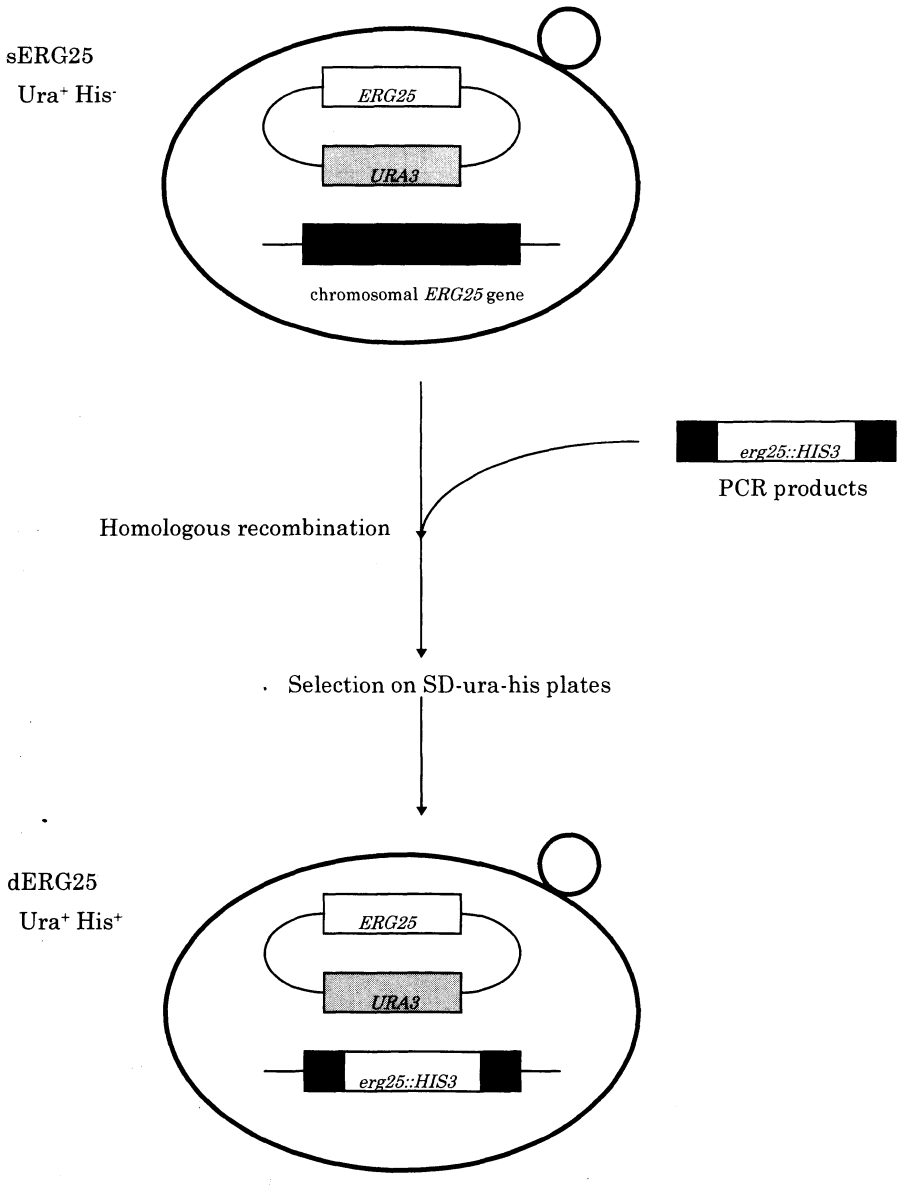
Isolation of the Temperature-sensitive mERG25 Strain

To construct pRD415-*mERG25*, mutagenic PCR was performed as previously described[16]. The *ERG25* gene was amplified using pRD416-*ERG25* as a template and primers ERG25-3 and ERG25-4. The PCR product was digested with *Hin*dIII and *Xho*I and ligated into plasmid pRS415. Mutagenized plasmids were transformed into the dERG25

Table 2. PCR primers used in this work.

Gene	Oligonucleotide designation and sequence
ERG25	ERG25-3; 5'-CATC*AAGCTT*GTAGTACAGCCATAAAAAAA-3'
	ERG25-4; 5'-GTAC*CTCGAG*TTTGAAGTATGTTTCTTCTC-3'
erg25::HIS3	erg25::HIS-3; 5'-CCTTCCAGTAAATCTTTATATTAGTTGTAACTTTTT CTCTTTAGATAGTACTCTTGGCCTCCTCTAG-3'
	erg25::HIS-4; 5'-TAAACAATTGTGAAGGTAAAAAGAAAGAGTTAGGG AGGTCAAACAAAGTTCGTTCAGAATGACACG-3'

Fig. 1. Construction of the yeast strain dERG25.



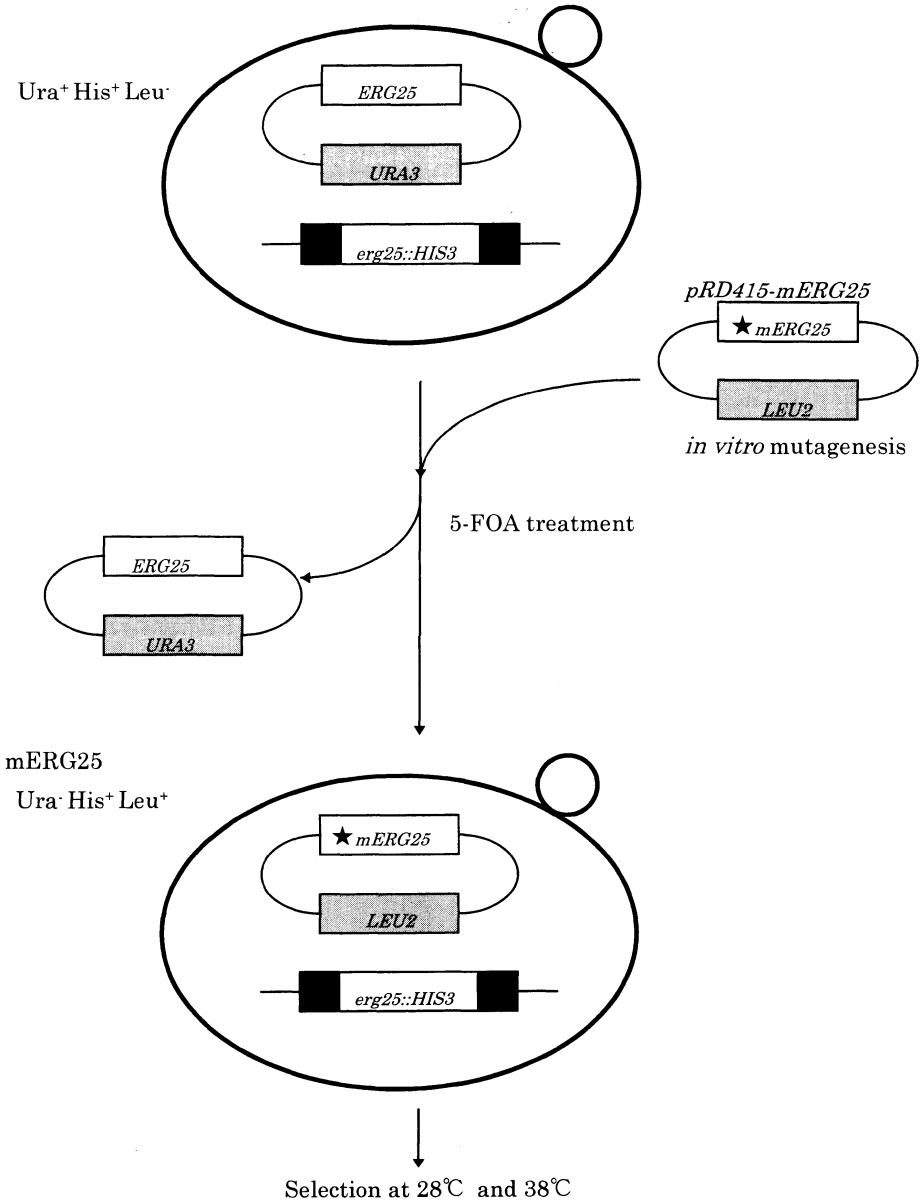
A yeast strain was transformed with a single copy, *URA3*-based plasmid bearing the yeast *ERG25* gene. Then, the chromosomal *ERG25* gene was deleted and replaced by the yeast *HIS3* gene.

strain. Leu$^+$ transformants were isolated and inoculated on SD plates containing 5-FOA to remove pRD416-*ERG25*. Each colony was then streaked onto an SD plate and incubated at 28°C or 38°C. Sterol extraction and analyses were carried out with colonies thatgrew at 28°C and not at 38°C.

Sterol Analysis

S. cerevisiae strains were inoculated at 1×10^7 cells/ml in 100 ml of YPD medium. After incubation at 28°C (permissive) or 38°C (nonpermissive) for 9 hours with shaking, the yeast cells were collected. Sterols were isolated as nonsaponifiables as described previously[17]. Individual sterols were identified using LC/MS. The LC/MS analyses were performed using a 1090L Hewlett-Packard LC connected to a Hewlett-Packard 5989A mass selective detector equipped with a Capcell Pak CN column (4.6× 250 mm). The solvent system consisted of 21% methanol with 2.5 mM ammonium acetate in water at a flow rate of 1.0 ml/minute. A mass range of *m/z* 300~600 was scanned

Fig. 2. Strategy for isolating temperature-sensitive mutants.



The expression plasmid pRD415-*mERG25* constructed by the error-prone PCR method was transformed into the dERG25 strain. The Leu$^+$ transformants were isolated and each strain was plated on an SD plate containing 5-FOA. The Ura$^-$ Leu$^+$ colonies were streaked on SD plates and incubated at 28°C and 38°C to select ts mutants.

in 1.0 second.

Results

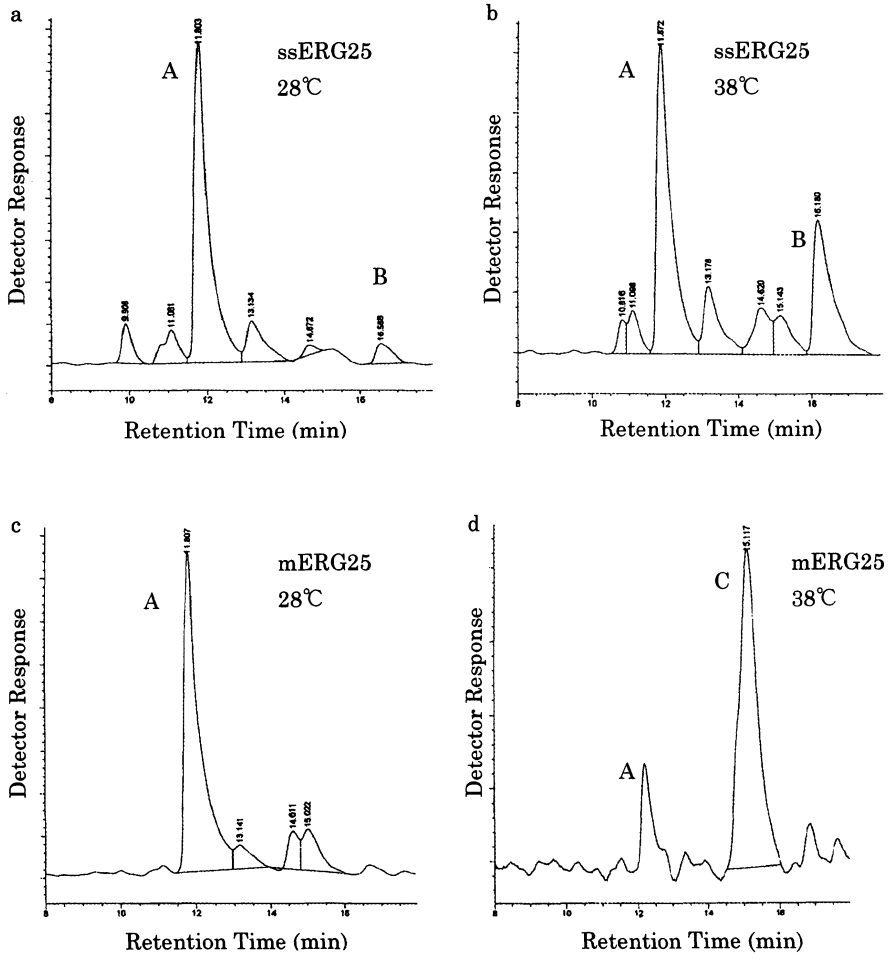
Isolation of the Temperature-sensitive mERG25 Strain

To construct the ts *erg25* mutants, we prepared dERG25, a strain, in which the chromosome *ERG25* gene locus was completely disrupted by the *HIS3* gene, but the cell was rescued by the plasmid pRD416-*ERG25* containing a single yeast *ERG25* gene (Fig. 1). This dERG25 strain failed to grow on media containing 5-FOA, a reagent that selectively kills the *URA3* cells. Thus, the growth of the dERG25 strain depended on the plasmid pRD416-*ERG25*, and this was confirmed by PCR analyses (data not shown). Using dERG25 strain, we isolated the ts *erg25* strains. The method is summarized in Figure 2. The dERG25 strain was transformed with either pRD415-*ERG25* or pRD415-*mERG25* and inoculated on SD-leu plates. The Leu^+ cells were isolated and plated on SD-leu plates containing 5-FOA in order to select the *ura3* cells. After incubation at 28°C for 3 days, each of the resulting Ura^- Leu^+ colonies was streaked on SD-leu plates and incubated at 28°C and 38°C to select the temperature-sensitive mutants. Only one strain, mERG25, grew normally at 28°C, but this strain failed to grow at 38°C.

Characterization of the Temperature-sensitive mERG25 Strain

In order to demonstrate that the ts phenotype of the mERG25 strain was due to a mutation in the *ERG25* gene, we analyzed the sterol compositions of the ssERG25 and mERG25 strains grown at 28°C and 38°C on SD-leu

Fig. 3. LC/MS analyses of sterols extracted from ssERG25 and mERG25 strains.



The strains were grown in YPD medium. Sterols were identified by LC/MS analysis. Peak A, ergosterol; peak B, lanosterol; peak C, 4,4-dimethylzymosterol.

medium. The wild-type ssERG25 strain showed an identical sterol profile at both temperatures (Fig. 3 a, b). The sterol profile of the mutant mERG25 strain grown at 28°C was identical to that of the wild-type strain, but notably the mERG25 strain produced another sterol different from ergosterol and lanosterol at the nonpermissive temperature (Fig. 3 c, d). The another sterol (retention time, 15.117 minutes) was confirmed to be 4,4-dimethylzymosterol by its mass spectrum and ^{13}C NMR. The molecular ion was observed at 412 Da and the fragmentation pattern was identical to the spectrum of 4,4-dimethylzymosterol[18].

Sequence analysis of the mERG25 mutant indicated that the bases at position 142, 390, and 404 were changed from an A to a G, a T to a G, and a T to a C, respectively, resulting in three amino acid substitutions, *i.e.*, N48D, V133A, and F135S, in the ERG25p (Fig. 4).

Discussion

Sterols are known to be important in membrane fluidity, membrane permeability, cell morphology, enzyme activity, and cell cycle progression. Therefore, the enzymes of the ergosterol biosynthetic pathway are likely to offer unique targets for antifungal drugs, such as lanosterol 14α-demethylase (ERG11p).

The *ERG25* gene from *S. cerevisiae* has been cloned and characterized previously[7]. An amino acid sequence analysis showed that the ERG25p was a membrane protein localized in the endoplasmic reticulum. ERG25p is an important enzyme that catalyzes the first reaction that remove the two methyl groups from the C-4 position of 4,4-dimethylzymosterol. The ERG25p contains three histidine motiefs, H^{159}RLFH, H^{172}KQHH, and H^{256}HDLHHH. These motifs are common to iron-binding, nonheme integral membrane desaturases, hydroxylases, and oxidases. It was surmised that three histidine motifs in these enzymes act as ligands for the iron atom(s).

In this report, we constructed the ts mutant of *S. cerevisiae* and identified the ts mutations of *S. cerevisiae ERG25* gene. Three amino acid substitutions occurred in our ts mutant strain of *S. cerevisiae*, N48D, V133A, and F135S. MATTHEW *et al.* isolated a ts mutation of the *C. albicans ERG25* gene and identified the amino acid substitution N247D within the region of histidine clusters[19]. They reported that the amino acid change at this position was between histidine clusters and could involve the inability of the altered ERG25p to bind iron. The mERG25 strain of *S. cerevisiae* had another mutation of the *ERG25* gene. The amino acid changes were located

Fig. 4. Amino acid sequences of the temperature-sensitive mutation (mERG25) in the *S. cerevisiae* ERG25 gene.

```
ERG25      1 MSAVFNNATLSGLVQASTYSQTLQNVAHYQPQLNFMEKYWAAWYSYMNNDVLATGLMFFL   60
mERG25     1 .............................................D.............   60
             ********************************************* ************

ERG25     61 LHEFMYFFRCLPWFIIDQIPYFRRWKLQPTKIPSAKEQLYCLKSVLLSHFLVEAIPIWTF  120
mERG25    61 ............................................................  120
             ************************************************************

ERG25    121 HPMCEKLGITVEVPFPSLKTMALEIGLFFVLEDTWHYWAHRLFHYGVFYKYIHKQHHRYA  180
mERG25   121 ............A.S.......................................... ...  180
             ************ * ********************************************

ERG25    181 APFGLSAEYAHPAETLSLGFGTVGMPILYVMYTGKLHLFTLCVWITLRLFQAVDSHSGYD  240
mERG25   181 ............................................................  240
             ************************************************************

ERG25    241 FPWSLNKIMPFWAGAEHHDLHHHYFIGNYASSFRWWDYCLDTESGPEAKASREERMKKRA  300
mERG25   241 ............................................................  300
             ************************************************************

ERG25    301 ENNAQKKTN   309
mERG25   301 .........   309
             *********
```

Histidine motifs are underlined.

between the histidine clusters. Hydropathy analysis using the algorithm of DOOLITTLE and KYTE indicated that ERG25p had as many as four transmembrane domains[20]. Three amino acid substitutions, *i.e.*, N48D, V133A, and F135S, were involved in these transmembrane regions. Therefore, these amino acids could be important for binding iron and maintaining the enzyme activity.

More analysis of the *ERG25* gene will demonstrate that ERG25p is an attractive target for the development of antifungal drugs.

References

1) LEES, N. D.; M. BARD & D. R. KIRSCH: Biochemistry and molecular biology of sterol synthesis in *Saccharomyces cerevisiae*. Crit. Rev. Biochem. Mol. Biol. 34: 33~47, 1999
2) BALOCH, R. I. & E. I. MERCER: Inhibition of sterol Δ^8-Δ^7-isomerase and 14-reductase by fenpropimorph tridemorph and fenpropidin in cell-free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry 26: 663~668, 1987
3) BALOCH, R. I.; E. I. MERCER, T. E. WIGGINS & B. C. BALDWIN: Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. Phytochemistry 23: 2219~2226, 1984
4) RYDER, N. S.: Squalene epoxidase as a target for the allylamines. Biochem. Soc. Trans. 19: 774~777, 1991
5) RYDER, N. S.: Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br. J. Dermatol. 126: 2~7, 1992
6) ISAACSON, D. M.; E. L. TOLMAN, A. J. TOBIA, M. E. ROSENTHALE, J. L. MCGUIRE, H. VANDEN BOSSCHE & P. A. JANSSEN: Selective inhibition of 14 alpha-desmethyl sterol synthesis in *Candida albicans* by terconazole, a new triazole antimycotic. J. Antimicrob. Chemother. 21: 333~343, 1988
7) BARD, M.; D. A. BRUNER, C. A. PIERSON, N. D. LEES, B. BIERMANN, L. FRYE, C. KOEGEL & R. BARBUCH: Cloning and characterization of *ERG25*, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. Proc. Natl. Acad. Sci. USA 93: 186~190, 1996
8) GACHOTTE, D.; R. BARBUCH, J. GAYLOR, E. NICKEL & M. BARD: Characterization of the *Saccharomyces cerevisiae ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. Proc. Natl. Acad. Sci. USA 95: 13794~13799, 1999
9) GACHOTTE, D.; S. E. SEN, J. ECKSTEIN, R. BARBUCH, M. KRIEGER, B. D. RAY & M. BARD: Characterization of the *Saccharomyces cerevisiae ERG27* gene encoding the 3-keto reductase involved in C-4 sterol demethylation. Proc. Natl. Acad. Sci. USA 96: 12655~12660, 1999
10) SHANKLIN, J.; E. WHITTLE & B. G. FOX: Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33: 12784~12794, 1994
11) BOEKE, J. D.; F. LACROTE & G. R. FINK: A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197: 345~347, 1984
12) SIKORSKI, R. S. & P. HIETER: A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19~27, 1989
13) BITTER, G. A. & K. M. EGAN: Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilizing the glyceraldehyde-3-phosphate dehydrogenase gene promoter. Gene 32: 263~274, 1984
14) LORENZ, M. C.; R. S. MUIR, E. LIM, J. MCELVER, S. C. WEBER & J. HEITMAN: Gene disruption with PCR products in *Saccharomyces cerevisiae*. Gene 158: 113~117, 1995
15) ITO, H.; Y. FUKUDA, K. MURATA & A. KIMURA: Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163~168, 1983
16) IKEDA, M.; K. HAMANO & T. SHIBATA: Epitope mapping of anti-recA protein IgGs by region specified polymerase chain reaction mutagenesis. J. Biol. Chem. 267: 6291~6296, 1992
17) MOLZAHN, S. W. & R. A. WOODS: Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 72: 339~348, 1972
18) BYSKOV, A. G.; C. Y. ANDERSON, L. NORDOHOLM, H. THOGERSEN, X. GUOLIANG, Q. WASSERMAN, J. V. ANDERSEN, E. GUDDAL & T. ROED: Chemical structure of sterols that activate oocyte meiosis. Nature (London), 374: 559~562, 1995
19) KENNEDY, M. A.; T. A. JOHNSON, N. D. LEES, R. BARBUCH, A. JAMES, J. A. ECKSTEIN & M. BARD: Cloning and sequencing of the *Candida albicans* C-4 sterol methyl oxidase gene (*ERG25*) and expression of an *ERG25* conditional lethal mutation in *Saccharomyces cerevisiae*. Lipids 35: 257~262, 2000
20) KYTE, J. & R. F. DOOLITTLE: A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105~132, 1982