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13. ABSTRACT *(Maximum 200 Words)*

Fungal infections continue to be a growing problem in present day health care. Part of this trend is due to advanced medical treatments that either allow entry of opportunistic pathogens or suppress the normal immune response. Disease states which diminish the immune response are also contributors. This situation is exacerbated by the increased incidence of resistance to the current arsenal of antifungal drugs. Thus, there is a pressing need for the discovery and development of new antifungal compounds. The research supported by this award seeks to identify new fungal sites against which novel classes of antifungals would be effective. Targeted in this work are unexplored steps in ergosterol biosynthesis in the human pathogen, *Candida albicans*. Initial identification of essential sterol biosynthetic steps were performed in *Saccharomyces cerevisiae*. Three genes, *ERG25*, *ERG26*, and *ERG27*, encoding the enzymes for sterol demethylation at C-4, have been characterized in these two organisms and been found to be excellent targets for antifungal development. A fourth gene, *ERG6*, encoding the C-24 transmethylase, has also been investigated and found to be potentially effective site for inhibition based on impaired membrane function of cells unable to perform this reaction.

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Martin Bard 10/12/99
N Douglas Lees 10/12/99

PI - Signature Date

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INTRODUCTION

The research described in this report was designed to address the problem of increased fungal infection due to a number of factors including the rise is resistance to the currently available antifungal compounds (1). The approach used in these studies was to identify novel targets in the ergosterol biosynthetic pathway of the human pathogen, *Candida albicans* to which new antifunagl compounds might be isolated and employed This work will follow preliminary discovery in the common yeast, *Saccharomyces cerevisiae* where genetic and molecular manipulations are more easily accomplished. Two ergosterol biosynthetic steps have been be investigated as potential antifungal sites. The first was the C-24 sterol transmethylase, a step not found in host cholesterol biosynthesis and one that has been shown to have serious, negative effects on membrane function in *S. cerevisiae.* The second step was the complex C-4 demethylation step where the three *Saccharomyces* enzymes responsible have been isolated and characterized in our lab over the past four years. Finally, the process of genetic suppression was analyzed in *C. albicans* because it has been described in several *S. cerevisiae* steps where a lethal mutation can be overcome by secondary mutations.

BODY

Experimental Methods and Results:

CLONING AND DISRUPTION OF THE *C. ALBICANS ERG6* GENE

The *C. albicans ERG6* gene has been cloned, sequenced, and homozygously disrupted. This work has recently been published (2) and a reprint of the manuscript can be found in the Appendix. The *Candida* gene is comprised of 377 amino acids and is 66% identical to the *Saccharomyces ERG6* gene. It also shows 40% and 49% identity to the *ERG6* genes from *Arabidopsis* and *Triticum,* respectively. Amino acid positions 129-137 represent the highly conserved S-adenosyl methionine binding site characteristic of sterol methyl transferases.

Disruption of the two copies of the *C. albicans ERG6* gene was accomplished in two ways. In the first the "ura3 blaster" (3) was inserted into an isolated copy of the *Candida ERG6* gene. The "ura3 blaster" is comprised of about a 1.2 kb repeat of the *hisG* elements derived from *Salmonella* flanking the *Candida URA3* gene. This construct was used to transform a wild type strain to obtain the *ERG6* heterozygote. Following loss of one of the *hisG* repeats and the *URA3* gene in the "ura3 blaster" by intrachromosomal recombination, the "ura3 blaster" was again employed in transformation to disrupt the second copy of the *ERG6* gene. The second method used the *ERG6* heterozygote to select for mitotic recombination by plating on nystatin which selects for cells producing no ergosterol. Both approaches yielded double disruptants which were confirmed by PCR and GC/MS. The doubly disrupted *erg6* strain accumulated only cholesta-(27 carbon)-type sterols rather than ergosta-(28 carbon)-type sterols.

The characterization of the *Candida erg6* strains was undertaken to ascertain whether inhibitors of ERG6p might serve as effective antifungal agents. Fig. 1 shows the growth patterns of the wild type strain (CAI4) and two homozygous *erg6* strains, 5AB-15 and HO11-A3, grown in the presence of sterol biosynthesis and metabolic inhibitors. Both mutants were resistant to nystatin as would be expected; both also show no change in sensitivity to the azoles. The *erg6* mutants were shown to be hypersensitive to a number of sterol synthesis and metabolic inhibitors including terbinafine, tridemorph, fenpropiomorph, fluphenazine, cycloheximide, cerulenin, and brefeldin A. Table 1 expresses the susceptibilities of these strains in quantitative terms. These altered susceptibilities are likely due to increased permeability characteristics similar to those reported for *erg6* mutants of *S. cerevisiae* . Azoles, which inhibit the ERG11p show no increase in inhibition implying that their entry into cells is not enhanced by the altered sterol composition of the plasma membrane present in the *erg6* mutants. The other compounds, however, show increased efficiency of inhibition. Terbinafine, an inhibitor of squalene epoxidase, the product of the *ERG1* gene, is 50 times more effective against the *erg6* mutant than the wild type. The morpholines, tridemorph and fenpropimorph, inhibitors of sterol C-14 reductase (ERG24p) and C8-C7 sterol isomerase (ERG2p), were 3,000 and 1,000 times more effective, respectively, against the *erg6* strain. Currently, the morpholines are restricted to agricultural applications but reformulations for human use would be particularly effective in combination with an ERG6p inhibitor. Brefeldin A, an inhibitor of Golgi function, cerulenin, an inhibitor of fatty acid synthesis, cycloheximide, a protein synthesis inhibitor, and fluphenazine, a calmodulin antagonist, all show increases in effectiveness of 2 to 50 fold in the *erg6* mutant. In terms of speculating about human applications, an inhibitor of ERG6p would also enhance the antifungal efficacy of these compounds as well as other compounds that otherwise could not enter the cell.

CLONING AND DISRUPTION OF THE *C. ALBICANS ERG25* GENE

The *C. albicans ERG25* gene was cloned and sequenced and the DNA and amino acid sequences have been submitted to the GenBank data base under accession number AF051914 (see Appendix). The base and amino acid sequences of the *C. albicans ERG25* gene are presented in Fig. 2. The open reading frame of the gene is comprised of 927 bases which encode a 308 amino acid protein. Fig. 3 shows the multiple alignment sequence of the *C. albicans ERG25* gene along with those from *S. cerevisiae* and *Homo sapiens*. Shaded regions indicate sequences common to all three organisms. In *C. albicans*, the CTG codon is translated as serine (4). At the level of the amino acid sequence, the *C. albicans* gene is 65% homologous to the *S. cerevisiae* gene (5) and 38% homologous to the human gene (6). The *C. albicans ERG25* gene also has several other features in common with the *ERG25* genes from other organisms. It contains three histidine-rich clusters comprising the eight histidine motif HX_{3-4}, HX_{2-3}, and $HX_{2-3}HH$ starting at amino acid positions 156, 173, and 258, respectively. This motif is found in many iron binding, non-heme integral membrane desaturases, hydroxylases, and oxidases (7) including the *S. cerevisiae* C-

5 sterol desaturase (8). It also contains a C-terminal endoplasmic retrieval signal as do other *ERG25* genes (5, 6).

Since the *ERG25* gene in *S. cerevisiae* has been shown to be essential and since *C. albicans* has not been shown to be able to take up exogenous sterol under any conditions, the disruption of the *C. albicans ERG25* gene was problematical because there would be no obvious way to keep a potential homozygous *erg25* mutant viable. This obstacle has been circumvented by the construction of a plasmid containing a conditional lethal version of the *ERG25* gene that can produce the ERG25p under specific conditions.

The disruption of the *C. albicans ERG25* gene was accomplished using the "ura3 blaster" system (3). A 4 kb *Cla*I-*Xho*I DNA fragment was isolated from an *ERG25* containing plasmid, pCERG25-7, and inserted into the *Cla*I-*Xho*I site in the bluescript vector to generate plasmid pIU879A (Fig. 4; the *Bam*HI site in pIU879A present in the multiple cloning site was destroyed by filling in this site with Klenow enzyme). pIU879A was restricted at the *Nco*I site (within the *ERG25* gene), filled in with Klenow, and *Bam*HI linkers were added to this vector to create a unique *Bam*HI site. The 4 kb "ura blaster" obtained from plasmid p5921 as a *Bam*HI-*Bgl*II fragment was then ligated into the newly created *Bam*HI site to yield plasmid pIU1300. The *ERG25* heterozygote was made by transforming *C. albicans* CAI8 with a 6.3 kb *Cla*I-*Bgl*II fragment obtained from pIU1300 and selecting for uracil prototrophy. The disruption of the first *ERG25* allele was confirmed by PCR using the primers indicated in Fig. 5. In order to repeat this procedure to disrupt the second *ERG25* allele, we selected for loss of the *URA3 hisG* region by plating colonies (strain TJC1) on medium containing 5-fluroorotic acid which allows growth of uridine requiring strains only. Colonies capable of growing on this medium had eliminated the *URA3* gene and were designated as strain TJC2.

To circumvent the problem associated with keeping a cell with a double disruption of a likely essential sterol gene viable in view of the inability of the cell to take up exogenous sterol, we embarked on constructing conditional lethals mutants of *ERG25*, which would be introduced into TJC2 (as an integrant at the *ADE2* locus or on an independent plasmid) before attempting the disruption of the second *ERG25* genomic allele. A conditional lethal such as a temperature-sensitive would result in a functional Erg25p at the permissive temperature but a non-functional Erg25p at the non-permissive temperature. Introducing the *ERG25* conditional lethal mutant allele into TJC2 followed by transformation with the "ura3 blaster" should result in a strain which has both genomic *ERG25* alleles disrupted and is viable at low temperature (permissive) but not at higher temperature (non-permissive). Integrations and disruptions at the correct genetic loci would be checked by PCR. This construct will allow also us to determine whether suppressors accumulate at the non-permissive temperatures.

In order to validate the efficacy of this approach we first tried the procedure in a *S. cerevisiae erg25* mutant strain and attempted rescue using conditional lethal

ERG25 mutants of *C. albicans*. Using pIU870 (Fig. 6), we have undertaken the construction of two different kinds of conditional lethals including a temperature-sensitive (ts) mutant and the other a conditional lethal requiring high concentrations of iron in the growth medium. The *E. coli* mutator strain XL-1 Red (Stratagene) is deficient in three of the primary DNA repair pathways making its mutation rate approximately 5,000 fold higher than that of its wild type parent. This strain was grown carrying a plasmid containing the *Candida* wild type *ERG25* gene. Plasmid DNA was isolated and transformed into a *Saccharomyces erg25* auxotroph (*erg25*-25C). Replicas of the transformed colonies were made and one set incubated at 30°C and the second at 38.5°C. Out of 3,500 transformants one, pIU908 (pIU936 is a *C. albicans* plasmid with the ts *ERG25* allele) , showed temperature sensitivity of the *ERG25* gene.

Sterol analysis shown in Table 2 of the transformant with the ts *erg25* showed significantly elevated levels of 4,4-dimethylzymosterol and decreased amounts of ergosterol at the non-permissive temperature. This transformant also showed slightly increased 4,4-dimethylzymosterol and decreased ergosterol at the permissive temperature indicated diminished enzyme activity even at the normal growth temperature. In contrast, the same strain carrying a plasmid with the *ERG25* allele showed nearly identical profiles at both temperatures. Cells grown at the non-permissive temperature were pre-grown at 30°C and shifted to 38.5°C for 24 hr. prior to sterol extraction. Growth at 38.5°C proceeded for about 1-1.5 generations after the shift. This indicates that the ergosterol present was made prior to the shift and suggests that the cessation of growth is due to the accumulation of C-4 sterol species.

Passage of the *ERG25*-containing plasmid through XL-1 Red could result in a plasmid that picks up a mutation that might confer a ts phenotype. In order to eliminate this possibility, the ts *erg25* insert was removed from pIU980 and reinserted into an identical, non-mutagenized plasmid backbone. When transformed into *S. cerevisiae erg25*-25C, the re-constructed plasmid yielded the same sterol profiles as shown in Table 2 demonstrating that the ts lesion is in the *ERG25* gene. The location of the *erg25* ts mutation was confirmed by sequencing the ts allele. The base sequences from positions 837 to 857 of the wild type and ts alleles are shown in Fig. 7. At position 846, A is replaced by G in the ts allele resulting in an amino acid substitution of aspartic acid for asparagine at amino acid 247. This location is between histidine clusters 2 and 3 and might result in altered iron binding of the ts gene product.

These preliminary results indicate that the *C. albicans ERG25* can be expressed in *S. cerevisiae* and that plasmid-borne *erg25* temperature sensitive mutants can potentially be employed to rescue double disruptions of essential genes in *C. albicans*. These results have recently been accepted for publication (9) and a preprint can be found in the Appendix.

The temperature sensitive *erg25* insert was then removed from pIU980 and inserted into a *C. albicans* vector (pIU936) containing the *ADE2* gene. Strain TJC2,

which carries an *ade2* marker, was transformed with this vector and *ADE2* prototrophs were obtained. An isolate of TJC2 carrying the integrated *C. albicans* ts *erg25* gene was subjected to a second round of transformation using the "ura3 blaster" yielding 4,100 *URA3* transformants of which only 2 were reliably temperature sensitive. However, GC analysis and PCR indicated that the temperature sensitivity was unrelated to the change in the *ERG25* allele. In addition, TJC2/pIU936 was plated onto various concentrations of nystatin in hopes of inducing mitotic recombination. The idea was that colonies which showed resistance to nystatin at higher temperatures may have become nystatin resistant at intermediate temperatures such as 37°C. Again, out of 955 colonies scored none were found to be nystatin resistant. It was concluded that the ts phenotype of the ts *erg25* was not expressed in *C. albicans* under the same conditions described in *S. cerevisiae*.

We then decided to generate new temperature sensitive alleles this time starting with a plasmid pIU908 which already has single amino acid change (described above) in the hopes of obtaining a ts allele based upon multiple amino acid changes . This plasmid was cycled through XL-1 Red *E. coli* cells for 8 or 30 hours and transformed into *S. cerevisiae erg25*-25C cells to determine temperature sensitivity. Two ts colonies were obtained that failed to grow at the even lower non-permissive temperature of 37°C. The subcloning of the 2.5 kb *Bam*HI-*Blg*lII *erg25* fragments confirmed that the inability to grow at the lower temperature was due to the *erg25* containing fragments. The new ts alleles were then inserted into *Candida* vectors and used to transform TJC2. This heterozygous *C. albicans* strain now carrying one of the these two vectors (containing the new ts *erg25* plasmid) was transformed with the "ura3 blaster" in hopes of obtaining homozygous *erg25* disruptants covered by a *erg25* ts allele. We have analyzed 16 out of 525 transformants for temperature sensitivity, *erg25* homozygosity and accumulation of 4,4-dimethylsterols. One of the transformants is homozygous for *erg25* and we are currently testing whether the ts phenotype disappears when the wild type *ERG25* allele is reintroduced.

Another type of conditional lethal specific to *ERG25* has been demonstrated by Kaplan (6). Kaplan isolated *erg25* mutants by screening *Saccharomyces* mutagenized cells able to grow on yeast complete medium (high iron) but unable to grow on the same medium after the iron had been drastically reduced by chelation (low iron). Since the sterol C-4 methyloxidase is a non-heme iron containing protein, Kaplan was able to isolate *erg25* mutants as *fet6* (mutants demonstrate a decreased ability to bind or transport iron). Again, the *E. coli* mutator strain XL-1 Red was employed as above except that the screen was on high and low iron media. Out of approximately 1,000 transformants, 6 that could not grow on low iron medium (non-permissive) were isolated.

One of the high iron-requiring isolates, plasmid pIU912, was chosen to test for the ability to rescue a *S. cerevisiae erg25* mutant. Table 3 shows the sterol composition of the wild type (MKC8) and high iron requiring *ERG25* alleles (MKC5)

in *S. cerevisiae erg25*-25C grown on high (50μM) and low (6μM) $FeSO_4$. The low iron concentration was above the 5μM selection concentration and allowed minimal growth to demonstrate the sterols that accumulate. Sterol profiles at high and low iron for the strain carrying the wild type *ERG25* allele were normal except that ergosterol levels were somewhat higher than typically seen. The strain carrying the high iron-requiring *ERG25* allele showed diminished ergosterol and increased levels of C-4 sterols at low iron concentration. This alteration was evident, but much less so, at the high iron concentration. When parallel experiments were attempted in *C. albicans* with the high iron-requiring alleles, no differences were noted. In fact, after as many as ten transfers on media containing no iron supplementation, the *C. albicans* strains continued to grow indicating that this organism, unlike *S. cerevisiae*, cannot be depleted of iron.

Simulataneous to the work using the high-iron-requiring mutants in *C. albicans* , plasmid backbone replacement was also done as in the case of the ts *erg25* and the reconstructed plasmid behaved in the same way. Sequencing of the high iron-requiring *erg25* allele, however, did not yield any differences from the wild type *ERG25* coding sequence. In returning to the other five high iron-requiring *ERG25* alleles isolated in the original screen, it was noted that four behaved exactly like pIU912 in terms of sterol profiles at high and low iron, in the verification that the lesion is not in the plasmid, and in that the *ERG25* base sequences did not vary from that of the wild type allele. The original plasmid and one of the other high iron-requiring mutants (the only ones tested) both were found to have a base insertion in the *ERG25* promoter. Where this might explain the phenotype and prove quite interesting in its own right, we had no obvious explanation regarding the molecular nature of the mutation and, thus, this line of investigation was put aside.

CLONING AND DISRUPTION OF THE C. *ALBICANS ERG26* GENE

The *Saccharomyces ERG26* gene has been isolated, cloned, and disrupted gene in our laboratory (10). The *Saccharomyces* gene, YGL001c, is approx. 30% similar to a cholesterol dehydrogenase from *Nocardia* sp.(11) The *Nocardia* cholesterol dehydrogenase converts cholesterol to the 3-keto derivative, exactly what is expected of the C-3 sterol dehydrogenase (decarboxylase) involved in ergosterol biosynthesis. The surprising result was that the *Saccharomyces erg26* null mutant growing with ergosterol supplementation was still not viable unless a *hem3* mutation was present. Thus, the *erg26 hem3* double mutant is viable if supplemented with ergosterol but the *erg26 HEM3* strain is not. The *hem3* mutation can be replaced by mutations in other heme genes. The *ERG26* gene is, therefore, an attractive target for antifungals because the absence of the gene product is lethal.

The *C. albicans ERG26* gene was recently cloned in our lab. A *Saccharomyces erg26 hem1* strain was transformed with a *C. albicans* genomic library and transformants were plated onto minimal medium containing ergosterol. Out of 4,500 total transformants, three were able to grow on δ-aminolevulinic acid (ALA)

media without ergosterol. Characterization of these plasmids revealed that two were identical and contained a 5 kb insert of *Candida* genomic DNA while the other plasmid contained a 20 kb insert. Fig. 8 shows subclones of the *Candida* genomic clones in which the *ERG26* gene could be localized to a 1.9 kb *Bam*HI-*Cla*I fragment. DNA sequencing of this fragment demonstrated a sequence which encodes a 347 amino acid open reading frame which is 70% identical to the *ERG26* amino acid sequence of *Saccharomyces cerevisiae* (Fig. 9). The complete DNA and corresponding amino acid sequence of the *Candida albicans ERG26* gene are shown in Fig. 10.

The *Candida albicans ERG26* gene is in the process of undergoing the disruption process. Previously we have used the "ura3 blaster" to disrupt *ERG25* but our strategy for disrupting *ERG26* is based on one-step disruption using PCR primers as demonstrated by Mitchell (12). Mitchell uses oligomers containing *ERG26* short homology regions (~55 bp) flanking the *HIS1, ARG4,* and *URA3* genes which are available from him on plasmids. The PCR products are then used to transform the *C albicans* strain BWP17 containing deletions in all three of these genes. Transformants which are simultaneously prototrophic for *HIS1* and *URA3* would be good candidates for having disruptions at both *ERG26* loci. The problem of lethality when both copies of an essential gene are eliminated will be circumvented by introducing a plasmid containing the *ERG26* gene under the control of an inducible promoter. Dr. Carol Kumamoto has provided us with a plasmid containing the pMAL promoter which is active only when cells are grown on maltose containing media and not glucose containing media (13). We have now constructed a fusion between the pMAL promoter and the *ERG26* open reading frame (on a 1.2 kb *BamHI-HindIII* fragment) and are in the process of sequencing this construct to determine that the *ERG26* gene is correct as well as functional.

The specific strategy to disrupt the *C. albicans ERG26* gene is shown in Fig. 11. Stain BWP17 was transformed with a PCR fragment comprised of the *HIS1* gene flanked on each side by 50-60 base pairs of the *ERG26* sequence. *HIS1* transformants will represent *ERG26* heterozygotes in which a significant portion of the *ERG26* gene is deleted and replaced by the complementary *erg26* flanking sequences and the *HIS1* gene. The next step is a repeat of the procedure using the *URA3* marker with flanking *erg26* sequences that are inside those used in the formation of the heterozygote and, thus, are present only in the non-disrupted gene. The resulting disrupted chromosomes will be different sizes allowing for PCR confirmation of the double disruption.

CLONING AND DISRUPTION OF THE C. *ALBICANS ERG27* GENE

The last unidentified gene encoding an enzyme involved in ergosterol biosynthesis in *S. cerevisiae,* the 3-keto sterol reductase, which in concert with the C-4 sterol methyloxidase (*ERG25*) and the C-3 sterol dehydrogenase (*ERG26*) removes the two methyl groups from the C-4 position, was recently cloned in our lab (14). We developed a strategy to isolate a *Saccharomyces* mutant deficient in converting

3-keto to 3-hydroxy-sterols. An ergosterol auxotroph (disrupted at squalene epoxidase, *erg1*) unable to synthesize sterol or grow without sterol supplementation was mutagenized. Colonies were then selected that were nystatin resistant in the presence of 3-ketoergostadiene and cholesterol. The cholesterol was added to keep the cells viable while the nystatin resistance was expected to result in mutants that are deficient in converting the 3-ketoergostadiene to ergosterol. One of eight resulting resistant colonies was then crossed to a wild type strain to separate the *erg1* and putative *erg27* mutations. A resulting segregant, SDG100, was transformed with a *S. cerevisiae* library and gene YRL100w was identified as the complementing gene. Disruptions of YLR100w failed to grow on various types of 3-keto sterol substrates. Surprisingly when *erg27* was grown on cholesterol or ergosterol supplemented media, the endogenous compounds that accumulated were the non-cyclic sterol intermediates squalene, squalene epoxide and squalene dioxide, with little or no accumulation of lanosterol or 3-ketosterols. Feeding experiments in which *erg27* strains were supplemented with lanosterol (an upstream intermediate of the C-4 demethylation process) and cholesterol (an end-product sterol) demonstrated accumulation of four types of 3-keto sterols identified by GC/MS and chromatographic properties: 4-methyl-zymosterone, zymosterone, 4-methyl-fecosterone, and ergosta-7,24 (28)-dien-3-one. In addition, a fifth intermediate was isolated and identified by ^{1}H NMR as a 4-methyl-24,25-epoxy-cholesta-7-en-3-one.

We are now in a position using *S. cerevisiae erg27* auxotrophs to isolate by complementation the *C. albicans ERG27* gene. Twenty-two thousand transformants were obtained after transforming a *Candida* genomic library into the *S. cerevisiae erg27* strain. Of these, 400 colonies were able to grow on a minimal medium without ergosterol. These colonies are either transformants containing the *Candida ERG27* gene or revertants of the *Saccharomyces erg27* mutation. Screening of these colonies is now in progress.

SUPPRESSION OF *ERG* MUTATIONS IN *C. ALBICANS*

The third objective of the project was to determine the likelihood and mechanisms by which lethal mutations in the ergosterol pathway of *C. albicans* can be suppressed by other mutations. This phenomenon is noted in *S. cerevisiae* in some of the steps from lanosterol to zymosterol and in each case the mechanism is unique. Mutations in *ERG11* are suppressed by downstream mutations in the *ERG3* gene (15, 16). Mutations in the *ERG24* gene are suppressed by mutations in *FEN1* (17), a gene whose end product is involved in the synthesis of very long chain fatty acids used in ceramide and sphingolipid synthesis (18). Mutations in the *ERG25* gene are suppressed by a pair a of mutations (19). The first is in the upstream *ERG11* gene while the second is a leaky mutation in the heme biosynthetic pathway. More recently, mutations in *ERG26* have been shown not to undergo suppression although mutations in the heme pathway allow sterol uptake under aerobic conditions and growth can occur if exogenous sterol is provided (10). Mutations in *ERG27*, are not subject to suppression since these mutants accumulate only sterol precursors (14).

UNPUBLISHED DATA

The presence of suppression in *C. albicans* can be evaluated only when double disruptions of the target genes are available. Sterol biosynthesis and its relationship with fungal physiology are very similar but not identical in *S. cerevisiae* and *C. albicans*. One point of departure of interest here is the fact that *ERG11* mutants in *C. albicans* are viable presumably because this organism can utilize the types of sterol intermediates that accumulate with this pathway block (20). However, the situation with the *S. cerevisiae ERG26* and *ERG27* genes predicts that suppression will not occur and that these will remain excellent targets for antifungal drug development.

KEY RESEARCH ACCOMPLISHMENTS

- cloning and sequencing of the *ERG6* gene from *C. albicans*
- determination of the enhanced permeability characteristics of *C. albicans erg6* mutants
- verification that the *ERG6* gene would make a suitable target for antifungal development
- availability of the *ERG6* gene as screen for the isolation of sterol C-24 transmethylase inhibitors
- cloning and sequencing of the *ERG25* gene from *C. albicans*
- the functional expression of *C. albicans* sterol genes in *S. cerevisiae*
- development of techniques using conditional lethals for the rescue of sterol mutations in *C. albicans* that are lethal
- cloning and sequencing of the *C. albicans ERG26* gene
- cloning of the *C. albicans ERG27* gene
- identification of essential sterol biosynthetic steps in *S. cerevisiae* that are not subject to suppression indicating that such steps would be effective drug targets in *C. albicans*

REPORTABLE OUTCOMES

Manuscripts

Jensen-Pergakes, K.L., Kennedy, M.A., Lees, N.D., Barbuch, R., Koegel, C., and Bard, M. (1998). Sequencing, Disruption, and Characterization of the *Candida albicans* Sterol Methyltransferase (*ERG6*) Gene: Drug Susceptibility Studies in *erg6* Mutants. Antimicrob. Agents Chemother. **42**: 1160-1167

UNPUBLISHED DATA

Kennedy, M A., Johnson, T.A., Lees, N.D., Barbuch, R., Eckstein, J.A., and Bard, M. (1999). 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, *in press*.

Abstracts and Presentations

Johnson, T.A.*, Kennedy, M.A., Lees, N.D., and Bard, M. Cloning of the *Candida albicans ERG25* Gene. Indiana Acad. Sci. 112th Annual Meeting, DePauw University, Proc. In. Acad. Sci. **112**: 86. 1996.

Kennedy, M.A., Pergakes, K.J.*, Lees, N.D., and Bard, M. Cloning of the *Candida albicans ERG6* Gene. Indiana Acad. Sci. 112th Annual Meeting, DePauw University, Proc. In. Acad. Sci. **112**: 86-87. 1996.

Aaron, K.*, Lees, N., and Bard, M. Cloning and disruption of the *ERG26* Gene in the Pathogenic Yeast *Candida albicans*. Abstract submitted for Indiana Acad. Sci. 115th Annual Meeting, Evansville, IN, Nov. 5, 1999.

Kennedy, M.A.*, Johnson, T.A., Lees, N.D., Barbuch, R., Eckstein, J.A., and Bard, M. Identification of the C-4 Sterol Methyloxidase Gene (*ERG25*) from *Candida albicans* and Expression of *ERG25* conditional lethal mutations in *Saccharomyces cerevisiae*. Abstract submitted for Indiana Acad. Sci. 115th Annual Meeting, Evansville, IN, Nov. 5, 1999

* Presenter

Patents applied for

DNA Encoding Sterol Methyltransferase SLW# 00740.003US1

Degrees obtained that are supported by this award

Theresa Johnson, M.S. degree, December, 1998
Kristen Jensen-Pergakes, M.S. degree, May,1999
Matthew Kennedy and Kora Aaron will receive their Ph.D. and M.S. degrees, respectively, in May 2000 and were supported in part by this grant.

Submission to GenBank database

Jensen-Pergakes, K.L., Kennedy, M.A., Lees, N.D., Barbuch, R., Koeger, C., and Bard, M. Sequencing, Disruption, and Characterization of the *Candida albicans* Sterol Methyltransferase (*ERG6*) Gene: Drug Susceptibility Studies in *erg6* Mutants. Accession number: AF031941

Funding applied for on work supported by this award

Burroughs Wellcome Fund Scholar Award in Pathogenic Mycology to Martin Bard. Characterization of New Target Sites for Antifungal Intervention in the *Candida albicans* Ergosterol Pathway, $425,000, July, 1999-June, 2004

Employment opportunities received as result of training

Kristen Jensen-Pergakes employed by Selective Genetics Inc., San Diego CA

CONCLUSIONS

The research sponsored in this project is comprised of the three aims listed in the Statement of Work (see Appendix). This first of these is to explore the characteristics of *erg6* mutants of *C. albicans* to determine the suitability of the ERG6p as an antifungal target site. This aspect of the project has been completed and published (2). The *erg6* mutant of *Candida* has been shown to have permeability characteristics similar to those reported for *S. cerevisiae erg6* mutants. The susceptibility of the *erg6* mutant to a number of antifungal agents has been tested and has been shown to be greatly enhanced for most. Thus, inhibitors of ERG6p may prove to be effective antifungal agents. The availability of the *ERG6* gene resulting from this work provides a mechanism with which to screen for ERG6p inhibitors. A patent for the *ERG6* DNA sequenced has been sought.

A second aim of the project calls for the isolation, cloning, and disruption of the *C. albicans* genes involved in sterol C-4 demethylation. Once accomplished, we will know whether the genes are essential in *C. albicans*, a determination which will tell us whether the gene products they produce represent good targets for the development of new antifungals. Preliminary to these studies, the genes from *S. cerevisiae* have been characterized in the same way and the information obtained has been employed in the *Candida* system. Our progress in *S. cerevisiae* to date has resulted in the isolation, sequencing, and disruption of all three genes.

The *S. cerevisiae ERG25* gene encoding the sterol C-4 methyloxidase has been characterized and found to be essential (5). In addition, a suppression system allowing the survival of *erg25* mutants has been defined (19). The availability of the *S. cerevisiae* mutant has allowed us to isolate the *Candida ERG25* gene from a library by complementation. The *C. albicans* gene has been sequenced and characterized (9). The only remaining component of this work is to create a strain in which the *erg25* mutant can be kept viable for direct determination of its essentiality. We have explored two conditional lethal systems in which to accomplish this. In both, a plasmid containing a conditional lethal mutation of the gene to be disrupted is introduced into a *C. albicans* strain in which one copy of the gene has been disrupted. The second chromosomal copy is then disrupted under permissive conditions as confirmed by a marker in the disrupting construct. Essentiality of the gene is demonstrated by switch to the non-permissive condition.

We explored a high iron-requiring conditional lethal but abandoned that approach in favor of a temperature sensitive (ts) system. Preliminary experiments where a plasmid-borne *C. albicans* ts *ERG25* rescues a *S. cerevisiae erg25* strain has demonstrated the efficacy of this approach and the creation of a ts mutant that is expressed in *C. albicans* is now well underway. This preliminary work should pave the way for the utilization of this tool to investigate the essentiality of other probable lethal genes in *C. albicans*.

The *S. cerevisiae ERG26* gene encoding the sterol C-3 dehydrogenase (C-4 sterol decarboxylase) has recently been sequenced and disrupted and found to be essential (10). This essential gene has also been isolated by complementation and cloned from *C. albicans*. The sequence of the *C. albicans ERG26* gene has been determined. We have a new protocol for the sequential disruption on the two *C. albicans ERG26* alleles and a new plasmid mediated rescue using a maltose promoter fused to the coding sequence of the *ERG26* gene. This approach defines another innovative way to determine the essentiality of required genes in *C. albicans*.

The final gene encoding the enzyme employed in the sterol biosynthetic step, *ERG27*, has recently been identified and characterized (14). The sterol 3-keto reductase restores the critical 3-OH and represents the final component of the complex reaction which removes the two methyl groups from the C-4 position. Initial screenings for the isolation of clones containing the *C. albicans ERG27* gene are currently taking place.

The final aim of our proposal was to evaluate the occurrence of and mechanisms by which suppressors to mutations in essential sterol genes in *C. albicans* arise. This is an important phenomenon in *S. cerevisiae* and might be a critical factor in attempts to develop new antifungal drugs. While we cannot provide an absolute answer to whether suppression of *erg25*, *erg26*, and *erg27*, mutations of *C. albicans* occurs until we have the double disruptions, emerging information from the cognate mutants of *S. cerevisiae* should be a predictor. A suppression system involving two mutations, an *erg11* and a leaky heme, has been described in *S. cerevisiae* (19). Although growth is restored, it is very poor growth and indicates that if such a suppression system occurs in *C. albicans* the resulting cell would be severely compromised in its ability to proliferate and induce the typical pathogenic responses in the host. In addition, as a diploid, the occurrence of simultaneous double mutations is highly unlikely. We have detected no evidence of true suppression of *erg26* and *erg27* mutants in *S. cerevisiae*. This organism allows survival of lethal sterol mutations by being able to take up exogenous sterol under certain conditions such as anaerobiosis or in the presence a mutations that allow sterol uptake. Since *C. albicans* is an obligate aerobe and no known mutations allow uptake, this does not seem to be a viable alternative to escape the effects of removing the ERG26p and ERG27p from the cell. Thus, we conclude that the ERG25p, ERG26p and ERG27p are excellent targets for new classes of antifungal drugs.

REFERENCES

1. White, T.C., Marr, K. A., and Bowden, R. A. Clinical, cellular, and molecular factors that contribute to antifungal resistance, Clin. Microbiol. Rev. **11**: 382-402. 1998.

2. Jensen-Pergakes, K. L., Kennedy, M. A., Lees, N. D., Barbuch, R., Koegel, C., and Bard, M. Sequencing, disruption, and Characterization of the *Candida albicans* sterol methyltransferase (*ERG6*) gene: Drug susceptibility studies in *erg6* mutants. Antimicrob. Agents Chemother. **42**: 1160-1167. 1998.

3. Fonzi, W. A., and Irwin, M. Y. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134**: 717-728. 1993.

4. Santos, M. A. S., Perreau, V. M., and Tuite, M. F. Transfer RNA structural change is a key element in the reassignment of the CUG codon in *Candida albicans*, EMBO J. **15**: 5060-5068. 1996.

5. Bard, M., Bruner, D. A., Pierson, C. A., Lees, N. D., Biermann, B., Frye, L., Koegel, C., and Barbuch, R. Cloning and characterization of *ERG25*, the *Saccharomyces cerevisiae* gene encoding the C-4 sterol methyl transferase, Proc. Nat. Acad. Sci. USA **93**: 186-190. 1996

6. Li, L., and Kaplan, J. Characterization of yeast methyl sterol oxidase (*ERG25*) and identification of a human homologue, J. Biol. Chem. **271**: 16927-16933. 1996.

7. Shanklin, J., Achim, C., Schmidt, H., Fox, B. G., and Munck, E. Mossbauer studies of alkane ω-hydroxylase: Evidence for a diiron cluster in an integral-membrane protein, Proc. Nat. Acad. Sci. USA **94**: 2981-2986. 1997.

8. Arthington, B. A., Bennett, L. G., Skatrud, P.L., Guynn, C. J., Barbuch, R. J., Ulbright, C. E., and Bard, M. Cloning, disruption and sequence of the gene encoding yeast C-5 desaturase, Gene **102**: 39-44. 1991.

9. Kennedy, M. A., Johnson, T. A., Lees, N. D., Barbuch, R., Eckstein, J. A., and Bard, M. Cloning and sequencing of the *Candida albicans* C-4 sterol methyl oxidase gene (*ERG25*) and expression of an *ERG25* conditional mutation in *Saccharomyces cerevisiae*. Lipids, in press.

10. Gachotte, D., Barbuch, R., Gaylor, J., Nickel, E., and Bard, M. Characterization of the *Saccharomyces cerevisiae ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 sterol decarboxylase) involved in sterol biosynthesis. Proc. Nat. Acad. Sci. USA **95**: 13794-13799. 1998.

11. Horinouchi, S., Ishizuka, H., and Beppu, T. Cloning, nucleotide sequence, and transcriptional analysis of the NAD(P)-dependent cholesterol dehydrogenase gene

from a *Nocardia* sp. and its hyperexpression in *Streptomyces* spp. Applied Environ. Microbiol. **57**: 1386-1393. 1991.

12. Wilson, R. B., Davis, D., and Mitchell, A. P. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. **181**: 1868-1874. 1999.

13. Brown, D. H., Slobodkin, I. V., and Kumamoto, C. A. Stable transformation and regulated expression of an inducible reporter construct in *Candida albicans* using restriction enzyme-mediated integration. Mol. Gen. Genet. **251**: 75-80. 1996.

14. Gachotte, D., Sen, S., Eckstein, J., Barbuch, R., Krieger, M., Ray, B. D., and Bard, M. Characterization of the *Saccharomyces cerevisiae ERG27* gene encoding the 3-keto reductase involved in C-4 demethylation. Proc. Nat. Acad. Sci. USA **96**: in press.

15. Watson, P. F., Rose, M. E., Ellis, S. W., England, H., and Kelly, S. L. Defective sterol C-5,6 desaturation and azole resistance: A new hypothesis for the mode of action of azole antifungals. Biochem. Biophys. Res. Commun. **164**: 1170-1175. 1989.

16. Kelly, S. L., Lamb D. C., Corran, A. J., Baldwin, B. C., and Kelly, D. E. Mode of Action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3-beta,6-alpha-diol. Biochem. Biophys. Res. Commun. **207**: 910-915. 1995.

17. Lorenz, R. T., and Parks, L. W. Cloning, sequencing, and disruption of the gene encoding the sterol C-14 reductase in *Saccharomyces cerevisiae*. DNA Cell Biol. **11**: 685-692. 1992.

18. Oh, C.-S., Toke, D. A., Mandala, S., and Martin, C. E. *ELO2* and *ELO3*, homologues of the *Saccharomyces cerevisiae ELO1* gene, function in fatty acid elongation and are required for sphingolipid formation. J. Biol. Chem. **270**: 29836-29842. 1997.

19. Gachotte, D., Pierson, C. A., Lees, N. D., Barbuch, R., Koegle, C., and Bard, M. A yeast sterol auxotroph is rescued by addition of azole antifungals and reduced levels of heme. Proc. Nat. Acad. Sci. **94**: 11173-11178. 1997.

20. Bard, M., Lees, N. D., Turi, T., Craft, D., Cofrin, L., Barbuch, R., Koegle, C., and Loper, J. C. Sterol synthesis and viability of *erg11* (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. Lipids **28**: 963-967. 1993.

BIBLIOGRAPHY AND INDIVIDUALS SUPPORTED

A complete bibliography of publications and abstracts to this point are listed under REPORTABLE OUTCOMES. We anticipate individual publications on the C, albicans ERG25, ERG26, and ERG27 genes in the future.

The following individuals received support from this award:

Dr. Martin Bard (PI)
Matthew Kennedy (Ph.D. student
Teresa Johnson (M. S. student)
Kristen Jensen-Pergakes (M. S. student)
Kora Aaron (M. S. student)

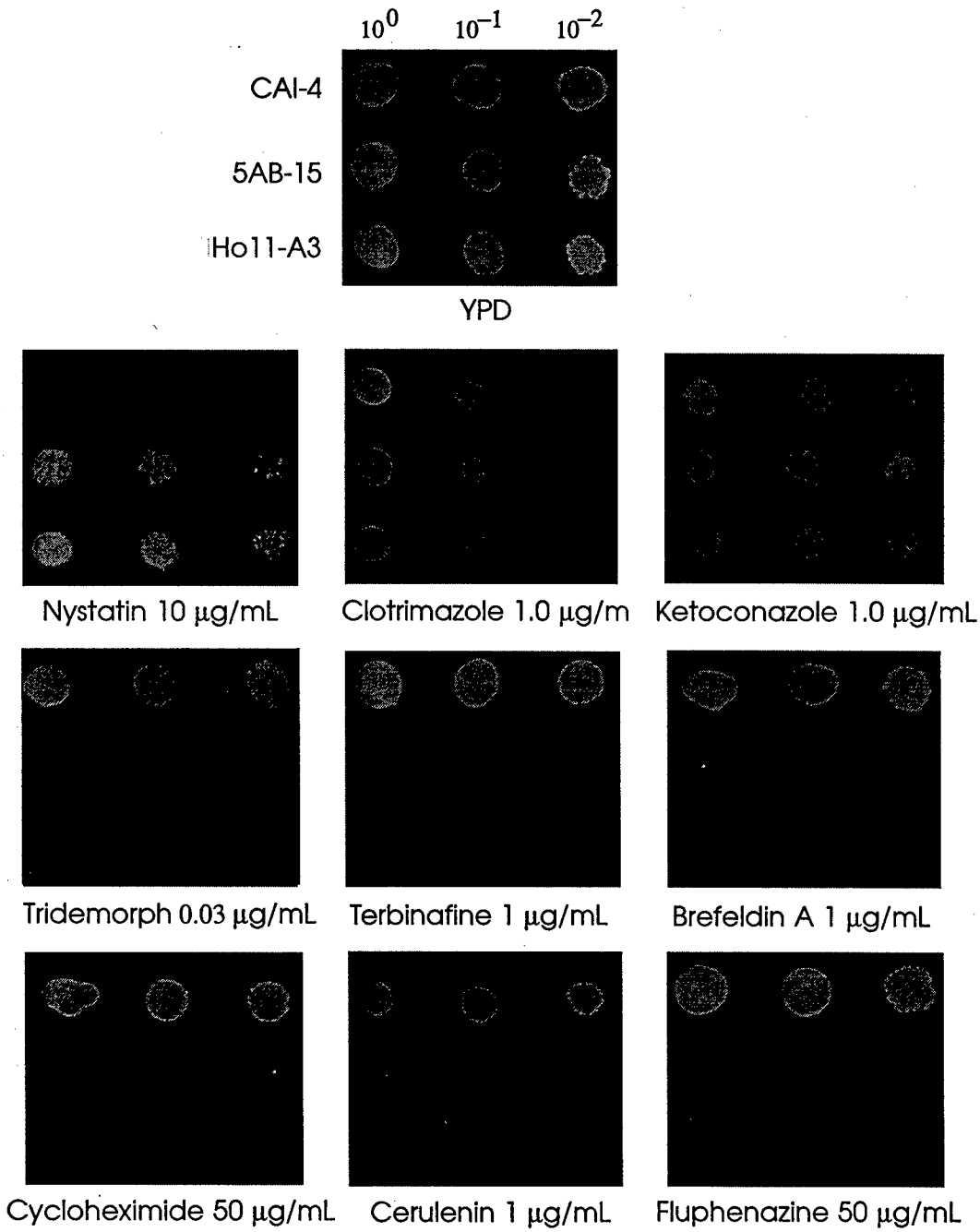

Figure 1. Growth responses of wild type (CAI4), homozygous *erg6* derived from "ura3 blaster" transformation (5AB-15), and homozygous *erg6* derived from mitotic recombination (HO11-A3) in the presence of sterol biosynthesis inhibitors and metabolic inhibitors. Cells were grown at 37°C to a density of 1 X 10^7 cells/ml and 5µl inoculated at 10^0, 10^{-1}, and 10^{-2} dilutions.

Table 1. Drug susceptibilities[a] of *ERG6* and *erg6* strains of *C. albicans* to antifungal agents and metabolic inhibitors.

DRUG	*ERG6*	*erg6*
Nystatin	2.5	15
Clotrimazole	1	1
Ketoconazole	5	5
Terbinafine	>50	1
Fenpropiomorph	0.5	0.005
Tridemorph	>90	0.03
Brefeldin A	50	1
Cerulenin	2	1
Cycloheximide	>600	50
Fluphenazine	100	50

[a] denotes inhibitor concentration (μg/ml) at which no growth appeared after 48 hours; cells were grown at 37C to a density of $1x\ 10^7$ cells/ml and 5μl inoculated at 10^0, 10^{-1}, and 10^{-2} dilutions.

```
   1  ttttgattcattaattgttatatttcaacatatacatattcctttattccttgatccttttttaaagtattcaatttat
  80  tatttatttgtttgtttgaagtttata ATG TCT TCC ATT AGT AAT GTT TAT CAT GAC TAT TCG AGT
                                  M   S   S   I   S   N   V   Y   H   D   Y   S   S     13

 147  TTT CTG AAT GCA ACT ACT TTT TCC CAA GTT TAT CAA AAT TTC AAT CAA TTA GAT AAT TTA
      F   S   N   A   T   T   F   S   Q   V   Y   Q   N   F   N   Q   L   D   N   L     33

 207  AAT GTT TTT GAA AAA TTA TGG GGG TCA TAT TAT TAT TAT ATG GCC AAT GAT TTA TTT GCT
      N   V   F   E   K   L   W   G   S   Y   Y   Y   Y   M   A   N   D   L   F   A     53

 267  ACT GGA TTA TTA TTT TTT TTA ACT CAT GAA ATT TTT TAT TTT GGT AGA TGT TTA CCA TGG
      T   G   L   L   F   F   L   T   H   E   I   F   Y   F   G   R   C   L   P   W     73

 327  GCT ATA ATT GAT AGA ATT CCT TAT TTT AGA AAA TGG AAA ATT CAA GAT GAA AAA ATC CCT
      A   I   I   D   R   I   P   Y   F   R   K   W   K   I   Q   D   E   K   I   P     93

 387  AGT GAT AAA GAA CAA TGG GAA TGT CTT AAA TCA GTT TTA ACA TCT CAT TTC TTA GTT GAA
      S   D   K   E   Q   W   E   C   L   K   S   V   L   T   S   H   F   L   V   E    113

 447  GCT TTC CCA ATT TGG TTT TTC CAT CCA TTA TGT CAA AAA ATT GGT ATT AGT TAT CAA GTA
      A   F   P   I   W   F   F   H   P   L   C   Q   K   I   G   I   S   Y   Q   V    133

 507  CCA TTC CCT AAA ATT ACT GAT ATG TTG ATT CAA TGG GCA GTA TTT TTT GTT TTG GAA GAT
      P   F   P   K   I   T   D   M   L   I   Q   W   A   V   F   F   V   L   E   D    153

 567  ACT TGG CAT TAT TGG TTT CAT AGA GGA TTA CAT TAT GGG GTT TTC TAT AAA TAT ATT CAT
      T   W   H   Y   W   F   H   R   G   L   H   Y   G   V   F   Y   K   Y   I   H    173

 627  AAA CAA CAT CAT AGA TAT GCT GCT CCA TTT GGA TTG GCA GCA GAA TAT GCT CAT CCA GTT
      K   Q   H   H   R   Y   A   A   P   F   G   L   A   A   E   Y   A   H   P   V    193

 687  GAA GTT GCC TTA TTA GGA TTG GGT ACG GTT GGT ATT CCG ATT GTT TGG TGT CTT ATC ACT
      E   V   A   L   L   G   L   G   T   V   G   I   P   I   V   W   C   L   I   T    213

 747  GGT AAC TTG CAT CTT TTC ACA GTT TCC ATT TGG ATC ATT TTA AGA TTA TTC CAA GCC GTT
      G   N   L   H   L   F   T   V   S   I   W   I   I   L   R   L   F   Q   A   V    233

 807  GAT GCT CAT TCC GGT TAT GAA TTC CCT TGG TCT TTA CAT AAT TTC TTG CCA TTT TGG GCT
      D   A   H   S   G   Y   E   F   P   W   S   L   H   N   F   L   P   F   W   A    253

 867  GGT GCT GAT CAT CAT GAT GAA CAT CAT CAT TAT TTC ATT GGT GGA TAC TCT TCA TCT TTT
      G   A   D   H   H   D   E   H   H   H   Y   F   I   G   G   Y   S   S   S   F    273

 927  AGA TGG TGG GAT TTC ATT TTG GAT ACC GAA GCT GGT CCA AAA GCT AAA AAG GGT AGA GAA
      R   W   W   D   F   I   L   D   T   E   A   G   P   K   A   K   K   G   R   E    293

 987  GAC AAA GTC AAA CAA AAT GTT GAA AAA TTA CAA AAG AAG AAC TTA TAG agagagaaagagtat
      D   K   V   K   Q   N   V   E   K   L   Q   K   K   N   L                       308

1049  atgtgtacaacttctcaatgtttgtaccactttcaatattaatactgtttatttttggttttatttaatatatatatc
1126  atatctattcatagtgctacat
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Figure 2. The base and amino acid sequences of the *C. albicans ERG25* gene.

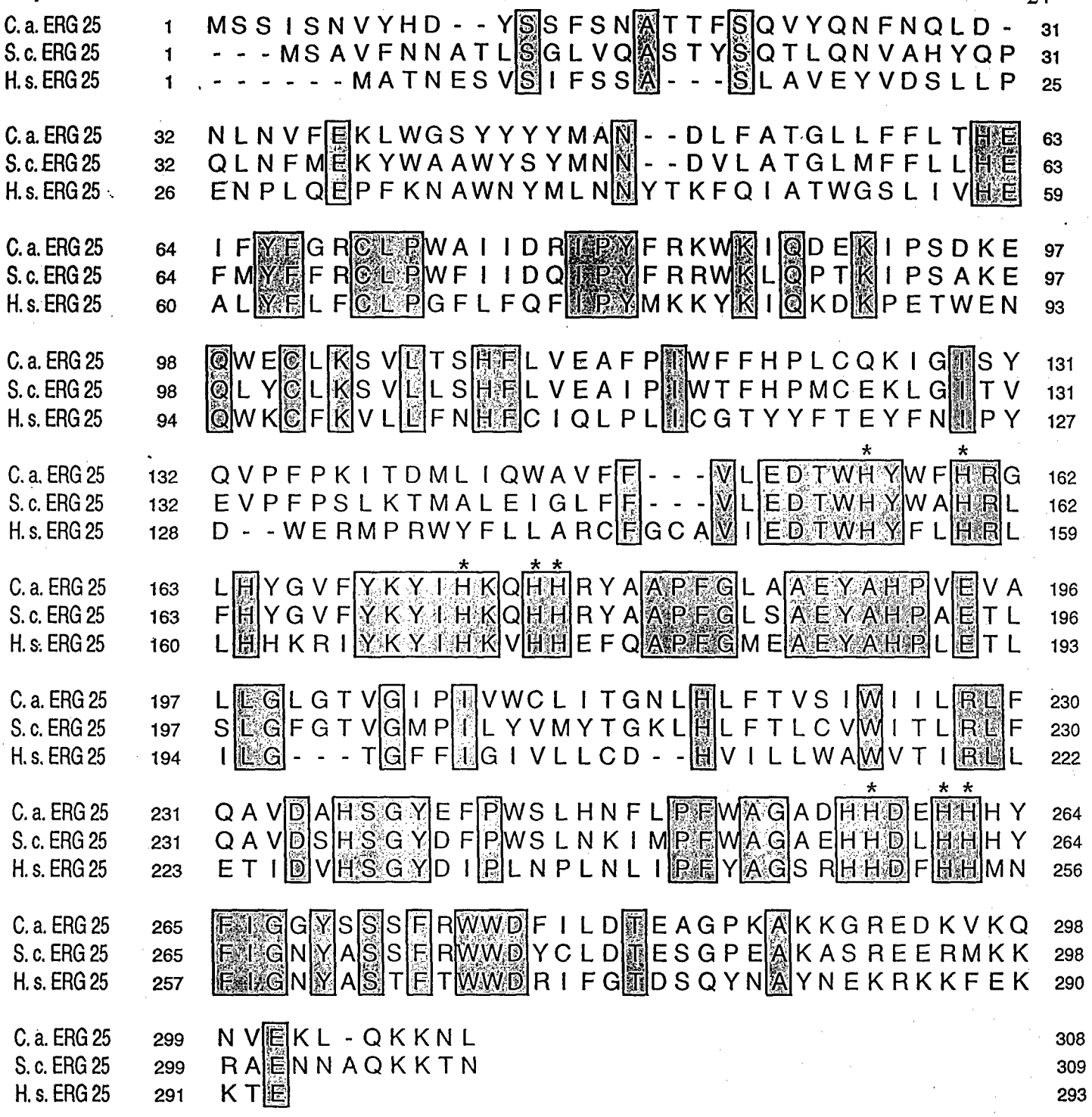


Figure 3. The multiple sequence alignment for the *ERG25* genes from *C. albicans*, (C. a.), *S. cerevisiae* (S. c.), and *H. sapiens* (H. s.). Shaded boxes indicate regions of amino acid homology among all three species. Histidines in the three histidine clusters are designated by * at each position.

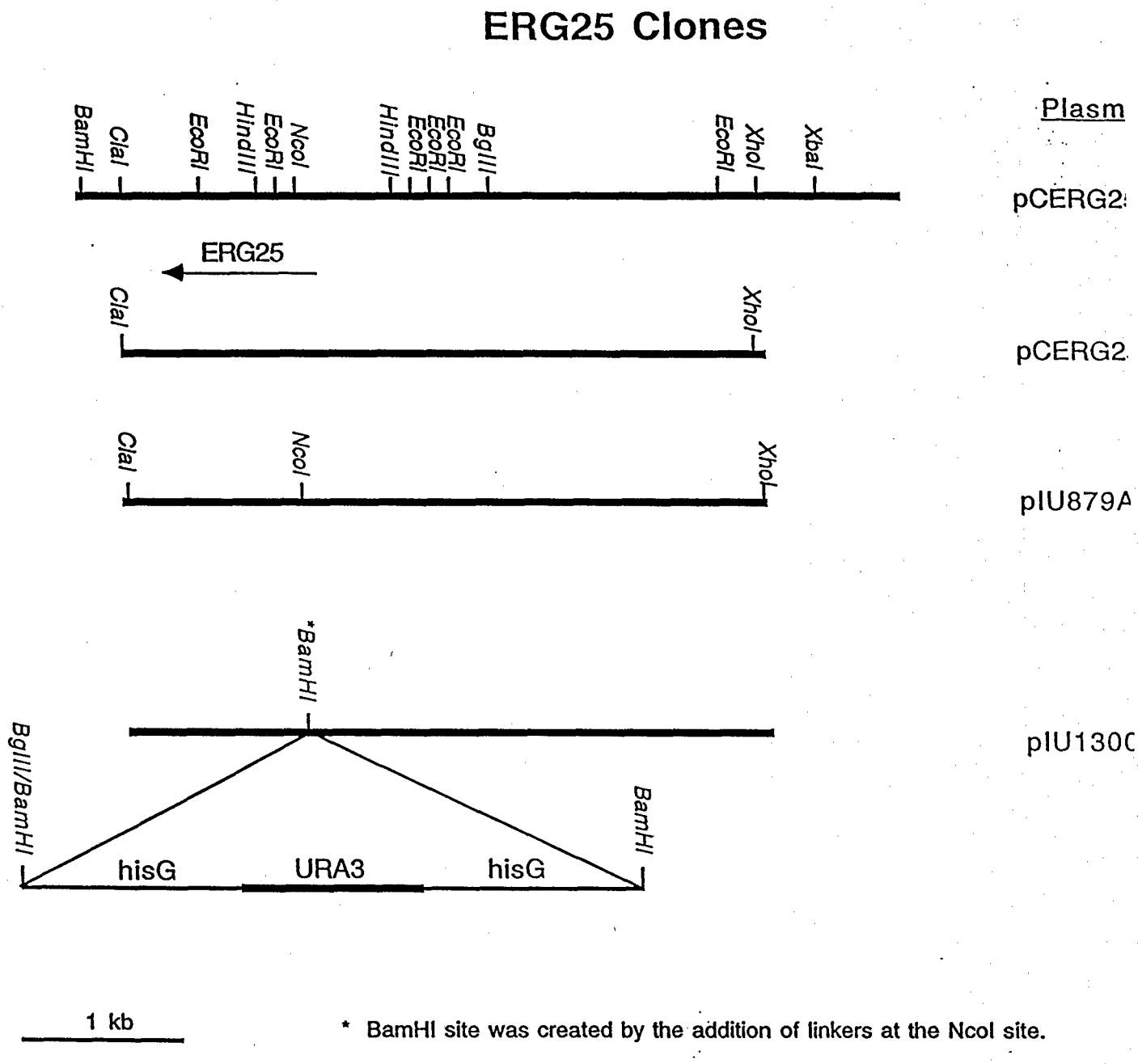


Figure 4. *C. albicans* genomic clones pCERG25-7 and pCERG25-9 and two subclones pIU879A and pIU1300. The "ura3 blaster" was inserted into the newly created *Bam*H1 site using linkers.

Confirmation of ERG25 heterozygote

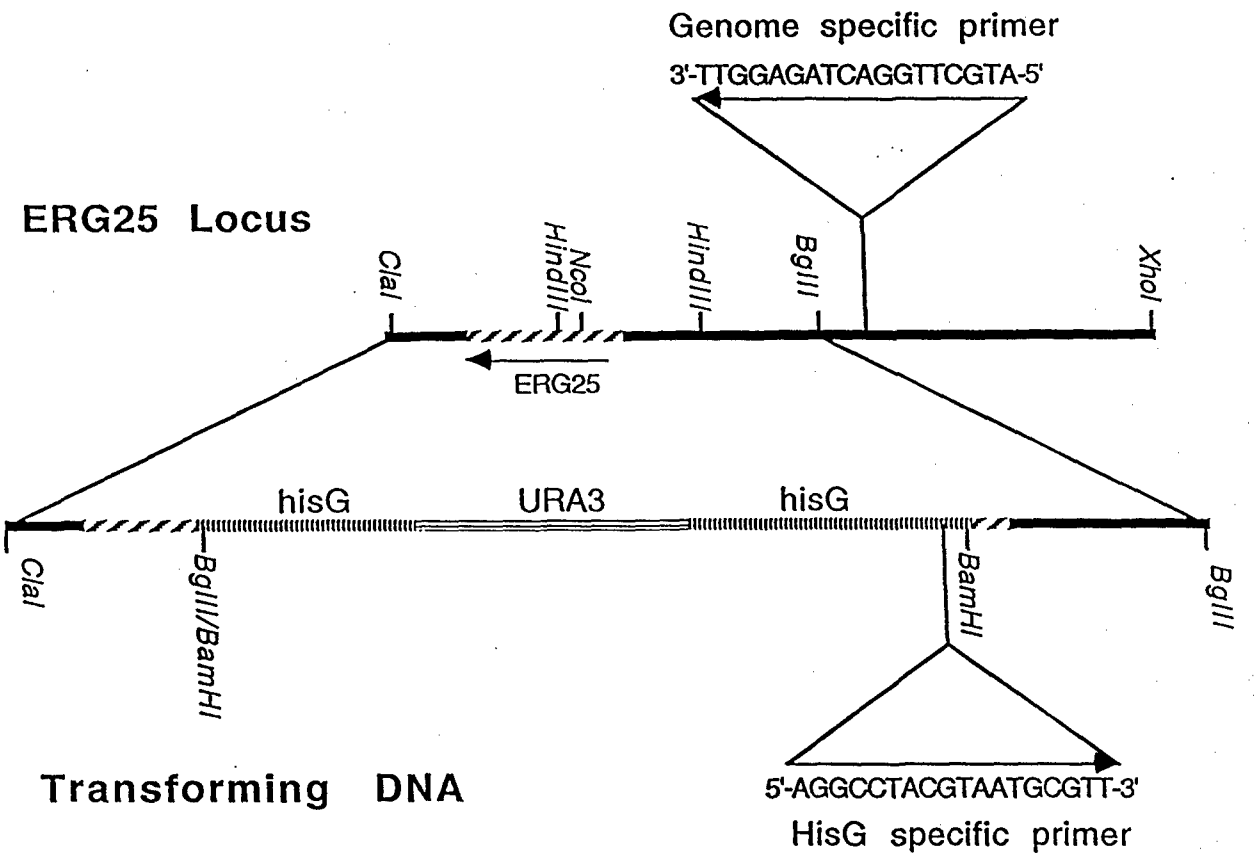


Figure 5. A genomic specific primer and *hisG* specific primer were used as indicated to confirm the location of the "ura3 blaster".

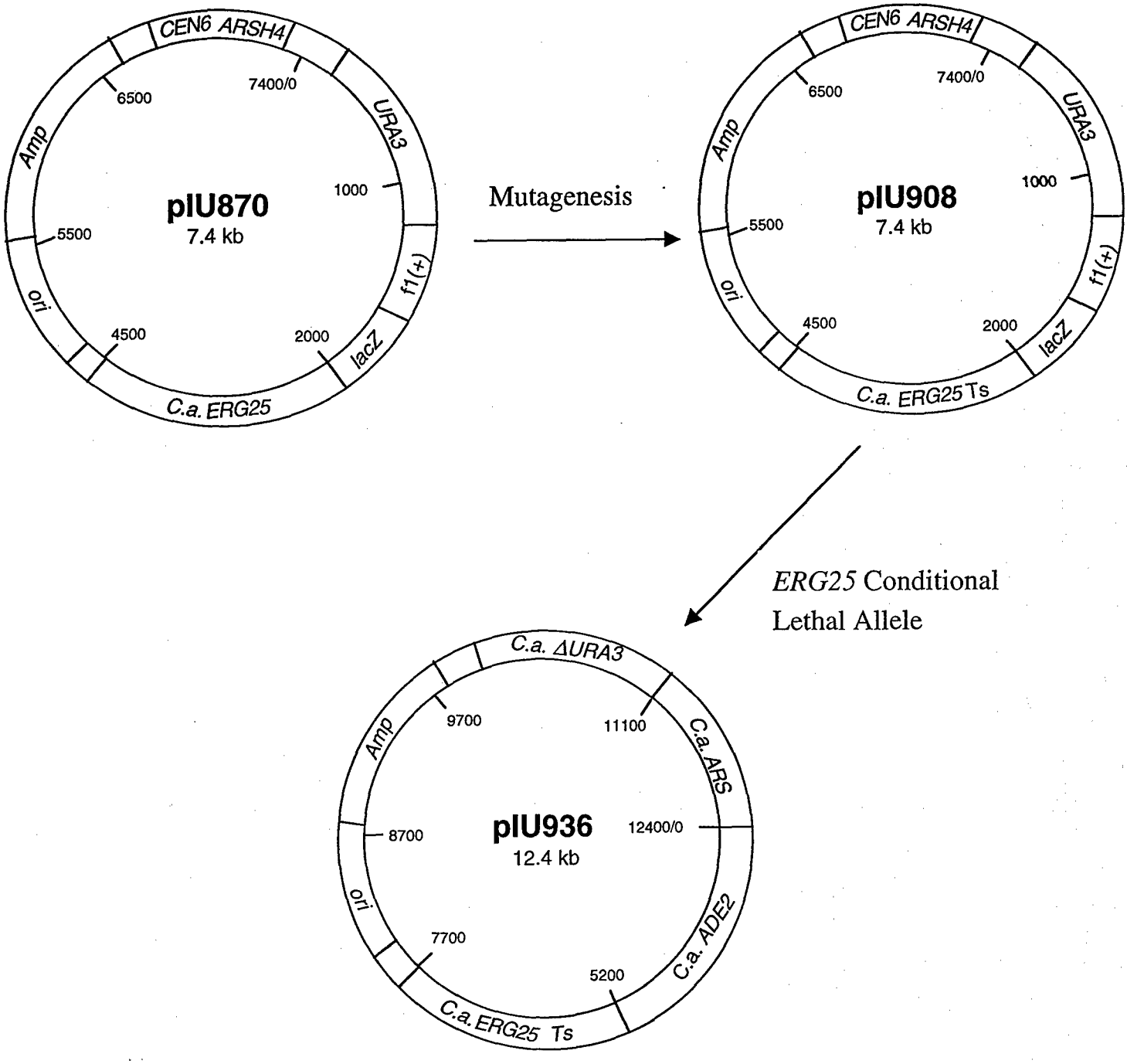


Figure 6. Creation of the *C. albicans ERG25* ts allele and its transfer into a C. *albicans* plasmid vector.

Table 2. Accumulated sterols at permissive and non-permissive growth temperatures of *S. cerevisiae erg25* mutants carrying wild type (pIU870) or temperature sensitive (pIU908)[b] *C. albicans ERG25* alleles.

STEROL[a]	*erg25*-25C/pIU870		*erg25*-25C/pIU908	
	30°C	38.5°C	30°C	38.5°C
squalene	5.9	2.4	31.9	15.2
zymosterol	4.0	5.7	<1	<1
ergosterol	61.9	63.6	32.6	16.2
fecosterol	2.7	6.4	<1	<1
4-methyl fecosterol	3.6	6.7	20.6	29.4
lanosterol	1.7	2.2	<1	<1
4,4-dimethyl zymosterol	6.6	8.0	14.9	34.8
4,4-dimethyl fecosterol	-	-	-	4.4

[a] Sterols represented as percent of total sterol.

[b] cells containing the temperature sensitive *ERG25* allele were pre-grown at the permissive temperature (30°C) before being shifted to the non-permissive temperature (38.5°C) and grown for 24 h.

WT	837	TCT	TTA	CAT	AAT	TTC	TTG	CCA	857
	244	S	L	H	N	F	L	P	250
T^S	837	TCT	TTA	CAT	GAT	TTC	TTG	CCA	857
	244	S	L	H	D	F	L	P	250

Figure 7. Base and amino acid sequences surrounding the ts mutation in the *C. albicans ERG25* gene.

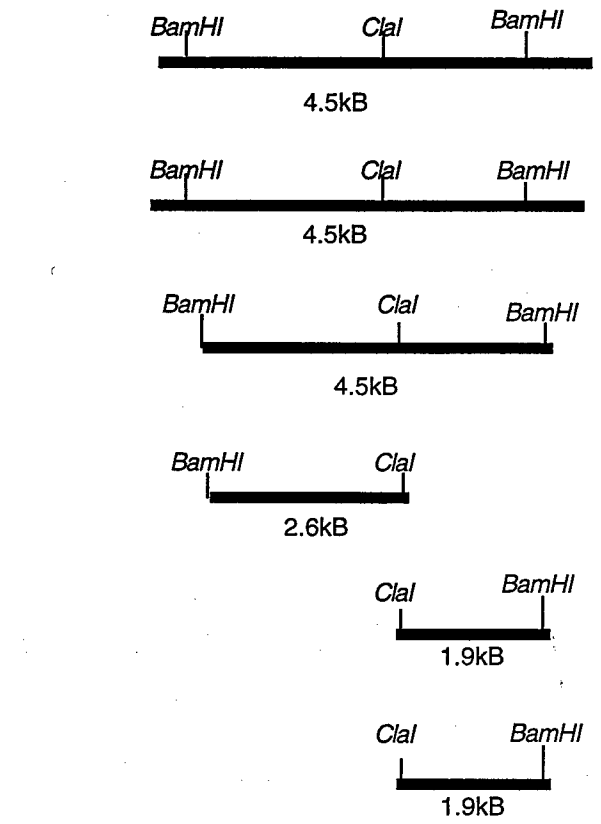
Table 3. Accumulated sterols[a] at high and low iron concentrations of *S. cerevisiae erg25* mutants carrying wild type (MKC8) or high iron-requiring (MKC5)[b] *C. albicans ERG25* alleles.

STEROL	MKC8		MKC5	
	50 μM $FeSO_4$	6 μM $FeSO_4$	50 μM $FeSO_4$	6 μM $FeSO_4$
squalene	2.6	7.5	7.0	7.9
zymosterol	2.1	2.9	2.0	<1
ergosterol	83.1	65	55.2	31.9
fecosterol	1.5	4.3	4.1	3.7
4-methyl fecosterol	3.4	9.5	15.9	29.4
lanosterol	<1	2.1	<1	4.3
4.4-dimethyl zymosterol	4.4	8.3	13.9	22.4
4,4-dimethyl fecosterol	-	-	-	-

[a]sterol extraction was performed as previously described [5] and GC/MS analyses were carried out as described in Fig 1. Sterols represented as percent of total sterol.

[b]low iron medium (6 μM $FeSO_4$) was just above the non-permissive concentration (5 μM $FeSO_4$) for MKC5 in order to permit minimal growth.

ERG26 Clones



Insert	Plasmid	Ability to Compliment erg26 mutants	Vector
	pIU1401 (CaERG26-2)	+	YPB1
	pIU1403 (CaERG26-3)	+	YPB1
	pIU1404	+	pRS316
	pIU1407	-	pRS316
	pIU1408	+	pRS316
	pIU1413	N/A	Bluescript SK

Figure 8. Subclones of the of the *C. albicans ERG26* clones.

```
ATGAGTGAATCTTTACAGTCAGTATTAATCATTGGAGGATCAGGATTTTTGGGA
 M  S  E  S  L  Q  S  V  L  I  I  G  G  S  G  F  L  G
CTTCATTTAATAGAACAATTTTATAGACATTGTCCTAATGTGGCAATCACAGTT
 L  H  L  I  E  Q  F  Y  R  H  C  P  N  V  A  I  T  V
TTCGATGTTCGTCCTTTACCTGAGAAGTTATCGAAATATTTCACATTTGATCCT
 F  D  V  R  P  L  P  E  K  L  S  K  Y  F  T  F  D  P
TCAAAAATACAATTTTTCAAAGGTGATTTAACTTCCGATAAAGATGTCTCTGAT
 S  K  I  Q  F  F  K  G  D  L  T  S  D  K  D  V  S  D
GCTATAAATCAATCTAAATGTGATGTAATTGTTCATTCAGCTTCACCGATGCAT
 A  I  N  Q  S  K  C  D  V  I  V  H  S  A  S  P  M  H
GGATTACCTCAAGAAATTTATGAAAAAGTTAATGTTCAAGGAACCAAAAATTTG
 G  L  P  Q  E  I  Y  E  K  V  N  V  Q  G  T  K  N  L
CTTTCAGTAGCACAGAAATTACATGTCAAGGCTTTAGTCTACACCTCCTCAGCT
 L  S  V  A  Q  K  L  H  V  K  A  L  V  Y  T  S  S  A
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 G  V  I  F  N  G  Q  D  V  I  N  A  D  E  T  W  P  Y
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 P  E  V  H  M  D  G  Y  N  E  T  K  A  A  A  E  E  A
GTCATGAAAGCCAACGATAATGATCAATTGCGTACTGTTTGTTTACGTCCTGCG
 V  M  K  A  N  D  N  D  Q  L  R  T  V  C  L  R  P  A
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 G  I  F  G  P  G  D  R  Q  L  V  P  G  L  R  A  S  A
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 K  L  G  Q  S  K  Y  Q  L  G  D  N  N  N  L  F  D  W
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 T  Y  V  G  N  V  A  D  A  H  V  L  A  A  Q  K  I  L
GATAAATCTACAAGAGACGACATTAGTGGTCAAACATTTTTTATAACTAATGAC
 D  K  S  T  R  D  D  I  S  G  Q  T  F  F  I  T  N  D
TCACCAACATATTTTTGGACATTGGCAAGAACTGTTTGGAAAAATGATGGTTAC
 S  P  T  Y  F  W  T  L  A  R  T  V  W  K  N  D  G  Y
ATTGATAAATATTACATTAAATTGCCATATCCAGTAGCATTGACTTTAGGTTAT
 I  D  K  Y  Y  I  K  L  P  Y  P  V  A  L  T  L  G  Y
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 I  S  E  F  V  A  K  N  I  L  K  K  E  P  G  I  T  P
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 F  R  V  K  V  V  C  A  I  R  Y  L  E  A  K  K  L  L
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 G  Y  K  P  E  V  D  L  E  T  G  I  N  Y  T  L  D  W
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 M  N  E  D  L  *
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Figure 9. The base and amino acid sequences of the *C. albicans* *ERG26* gene.

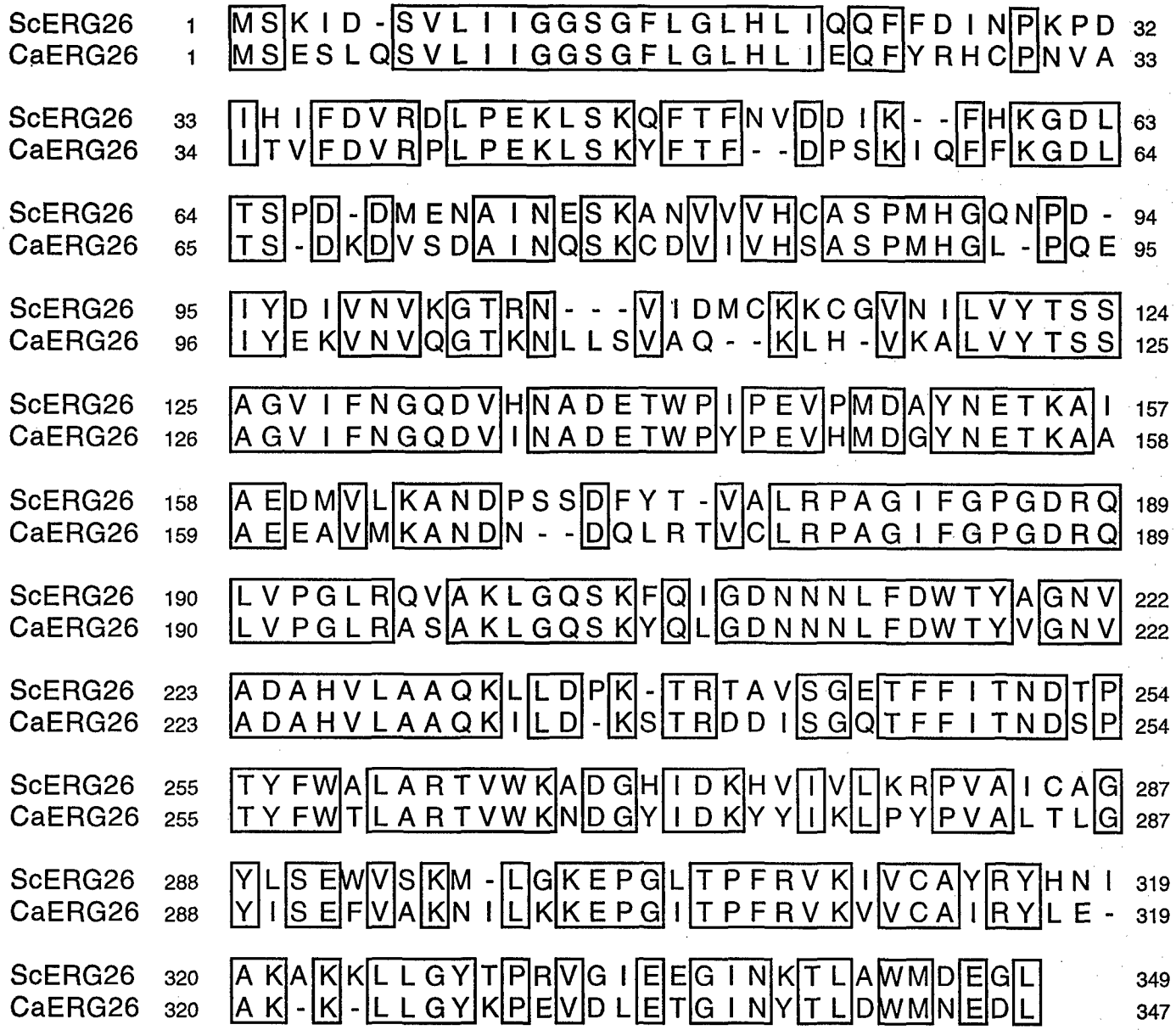

Figure 10. The multiple sequence alignment for the *ERG26* genes from *C. albicans*, (Ca), and *S. cerevisiae* (Sc).

ERG26 Short Homology Region Disruption Strategy

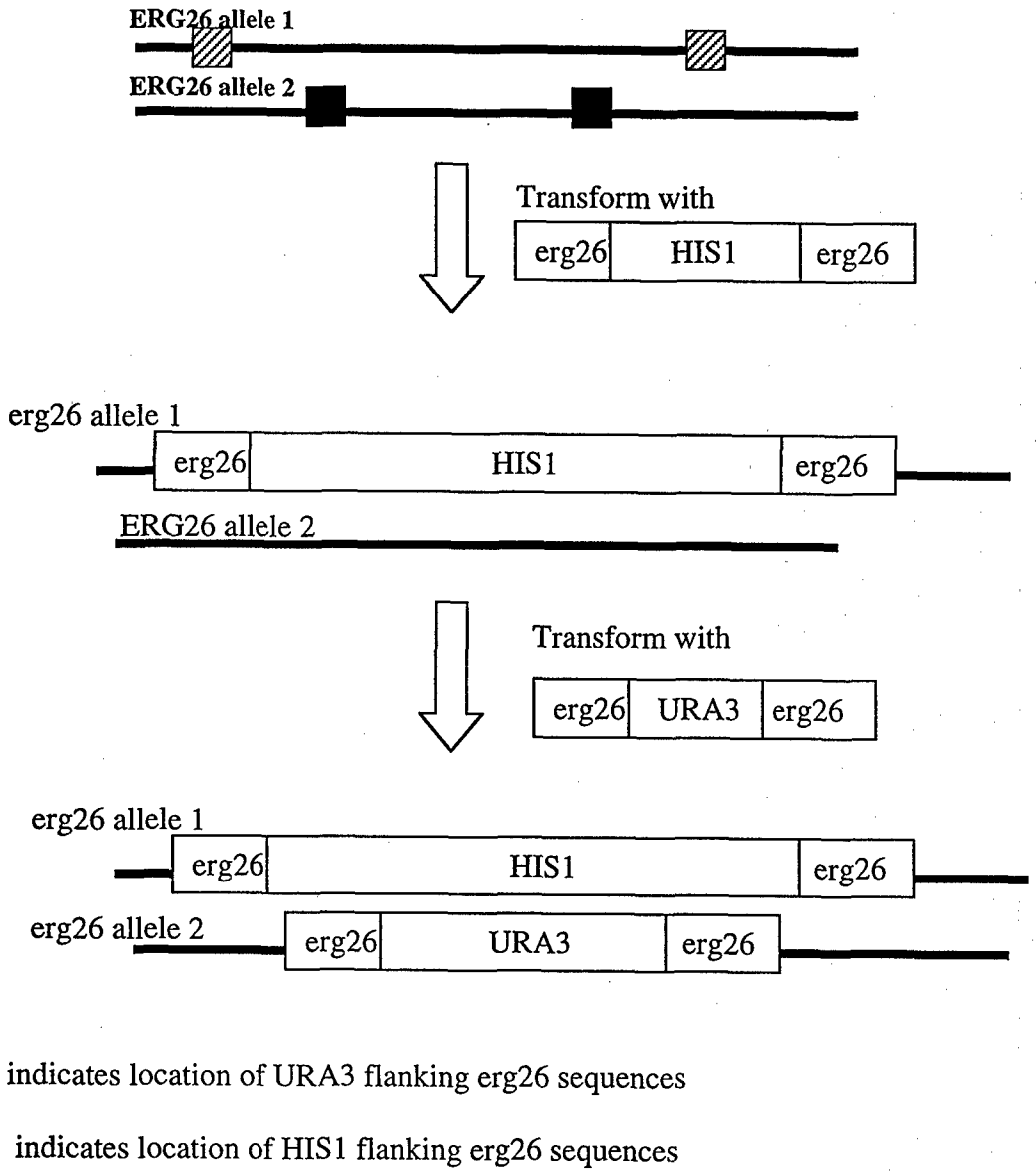

Figure 11. Sequential disruption strategy for the disruption of the *C. albicans ERG26* gene.

STATEMENT OF WORK

Aim 2 Cloning and disruption of the C-24 Transmethylase gene (*ERG6*) of *Candida albicans*

-cloning by complementation of a *C. albicans* genomic library with a *Saccharomyces cerevisiae erg6* mutant
-confirmation of plasmid-borne phenotype (FOA,) GC/MS analysis
-characterization by restriction mapping and subcloning
-determination of essentiality by sequential disruption
-physiological characterization of *C. albicans ERG6* disruptions including susceptibility testing
Months 0-24

Aim 1 Isolation of C-4 demethylase mutants of *C. albicans*

Following isolation of the three genes for C-4 demethylation from *S. cerevisiae*:

Isolation of C-4 demethylase genes from *C. albicans*
-complementation of *S. cerevisiae* C-4 demethylase mutants with a genomic library from *C. albicans*
-confirmation of plasmid-borne phenotype (FOA), GC/MS analysis
-characterization by restriction mapping and subcloning
-gene disruption and allele replacement (sequential)
-analysis of essentiality
- sequencing of the *C. albicans* C-4 demethylase genes
Months 12-36

Aim 3 Suppressor analysis

isolation of suppressors of C4 demethylase mutants
characterization of suppressors
GC/MS analysis
sensitivity to inhibitors
Months 30-48

```
LOCUS       AF031941     1221 bp    DNA             PLN       17-JUL-1998
DEFINITION  Candida albicans sterol transmethylase (ERG6) gene, complete cds.
ACCESSION   AF031941
NID         g3323499
KEYWORDS    .
SOURCE      Candida albicans.
  ORGANISM  Candida albicans
            Eukaryotae; Fungi; Ascomycota; Hemiascomycetes; Saccharomycetales;
            mitosporic Saccharomycetales; Candida.
REFERENCE   1  (bases 1 to 1221)
  AUTHORS   Jensen-Pergakes,K.L., Kennedy,M.A., Lees,N.D., Barbuch,R.,
            Koegel,C. and Bard,M.
  TITLE     Sequencing, disruption, and characterization of the Candida
            albicans sterol methyltransferase (ERG6) gene: drug susceptibility
            studies in erg6 mutants
  JOURNAL   Antimicrob. Agents Chemother. 42 (5), 1160-1167 (1998)
  MEDLINE   98253976
REFERENCE   2  (bases 1 to 1221)
  AUTHORS   Jensen-Pergakes,K.L., Kennedy,M.A., Lees,N.D. and Bard,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-OCT-1997) Biology, IUPUI, 723 W. Michigan St.,
            Indianapolis, IN 46202
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                     FRQATARHEHFLAHKMNLNENMKVLDVGCGVGGPGREITRFTDCEIVGLNNNDYQIER
                     ANHYAKKYHLDHKLSYVKGDFMQMDFEPESFDAVYAIEATVHAPVLEGVYSEIYKVLK
                     PGGIFGVYEWVMTDKYDETNEEHRKIAYGIEVGDGIPKMYSRKVAEQALKNVGFEIEY
                     QKDLADVDDEIPWYYPLSGDLKFCQTFGDYLTVFRTSRIGRFITTESVGLMEKIGLAP
                     KGSKQVTHALEDAAVNLVEGGRQKLFTPMMLYVVRKPLEKKD"
BASE COUNT      413 a    145 c    242 g    421 t
ORIGIN
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       61 aattacgaaa gagatgaaca attcactaaa gctttacatg gtgaatctta taaaaaaact
      121 gggttatcag ctttaatagc taaatctaaa gatgctgctt ctgttgctgc tgagggttat
      181 ttcaaacatt gggatggtgg tatttctaaa gatgatgaag agaaaagatt gaatgattat
      241 tcccaattga ctcatcatta ttataattta gtcactgact tttatgaata tggttggggt
      301 tcttcattcc atttttcaag atattataaa ggtgaagctt ttagacaagc tactgctaga
      361 catgaacatt tcttggccca taaaatgaat cttaatgaaa acatgaaagt tttagatgtt
      421 ggttgtggtg taggtggtcc tggtagagaa atcacaagat ttactgattg tgaaattgtt
      481 ggattaaata ataatgatta tcaaattgaa agagctaatc attatgctaa aaaataccat
      541 ttagatcata aattatctta tgttaaaggt gattttatgc aaatggattt tgaaccagaa
      601 tcattcgatg ctgtttatgc cattgaagct accgttcatg ctccagtttt agaaggagtt
      661 tattcagaaa tttataaagt tttgaaacca ggtggtattt tcggtgttta tgaatgggtc
      721 atgactgata aatacgatga aactaatgaa gaacatcgta aaattgctta tggtattgaa
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     1141 aaaccattag aaaagaaaga ttaatggggc ttgacaaaca acaagtaaac agggtgagtt
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//
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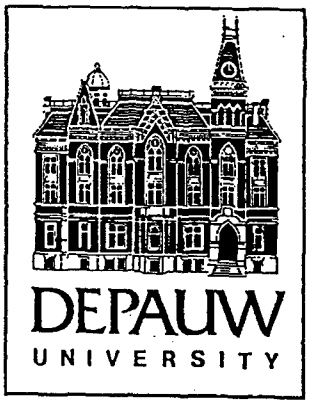
INDIANA ACADEMY *of* SCIENCE

112th Annual Meeting

November 7-8, 1996

PROGRAMS & ABSTRACTS



antisense coat protein gene with DNase I and are ready to be cloned into the vector. Transformants will be selected for with herbicide and and allowed to grow. Periodic challenges with the virus will be performed to assay for resistance.

10:30 a.m. Cloning of the *Candida albicans ERG25* Gene T.A. Johnson*, M.A. Kennedy, N.D. Lees and M. Bard, Department of Biology, IUPUI, Indianapolis, IN 46202

In *Candida albicans*, like other fungi, ergosterol is the primary sterol molecule and functions to regulate the permeability and fluidity of the plasma membrane. *Candida albicans* is a pathogenic yeast and can have a devastating effect on immunocompromised individuals.

Our lab isolated *erg25*, a C-4 sterol methyl oxidase mutant that accumulates 4,4-dimethyl zymosterol. ERG25 is the first of three gene products required for the process of C-4 demethylation in both yeast and mammals. Recently, our lab has cloned and characterized ERG25 from *Saccharomyces cerevisae* by gene disruption and found it to be an essential gene. We hypothesize that *ERG25 C. albicans* will be essential and therefore a good target candidate for new antifungal drug development.

A *S. cerevisae erg25* mutant was used to screen a *C. albicans* library for complementing clones. Two such clones were isolated. The complementing region was localized to a 2.7 kb fragment subcloned into the centromeric shuttle vector pRS316. Sequencing of the 2.7 kb fragment and disruption of the *C. albicans erg25* are in progress. Cloning, sequencing and disruption strategies and results of these experiments will be presented.

10:45 a.m. Cloning of the *Candida alicans ERG6* Gene. M.A. Kennedy, K.J. Pergakes*, N.D. Lees and M. Bard. Department of Biology, IUPUI, Indianapolis, IN 46202

In the pathogenic fungi *Candida albicans*, ergosterol is the primary sterol and is critical for regulating the permeability and fluidity of membranes. This research focuses on one of the late sterol pathway

genes, *ERG6*. *ERG6* codes for S-adenosylmethionine:Δ^{24}-sterol-C-methyltransferase (SMT), which methylates zymosterol at the C-24 position. This produces a C-28 methyl group unique to ergosterol. C-24 methylation in not required in cholesterol synthesis, so this is a possible route for producing safe antifungal drugs.

ERG6 was first cloned in *Saccharomyces cervisiae*. The *erg6* mutants accumulate zymosterol and other 27 carbon sterols. ERG6 was determined to be a non-essential gene in *S. cervisiae* by gene disruption. Although *ERG6* mutants are viable, the cells are permeable to many drugs that wild type cells are resistant to. It is unknown whether *ERG6* in C. *albicans* is essential. It is being explored as an antifungal target due to the ability of mutants in *S. cervisiae* to alter the permeability of the plasma membrane.

The C. *albicans ERG6* gene was cloned by complementation of a C. *albicans* library with a S. *cervisiae erg6* mutant strain. The smallest complementary fragment was 2.3kb inserted in the centromeric plasmid pRS316. Restriction enzyme analysis and further complementation studies indicated that the 2.3kb fragment contains ERG6. Sequencing of the entire gene, along with a disruption *of ERG6* are in progress. Strategies and results of these experiments will be discussed.

11:00 a.m. DNA Fingerprinting of Trout Lilies - A High School Research Project. S. Elliades, T. Brblic, S. Lambert, J. Shaw, R. Smith, M. Inman, H. Saxon, and C. Vann. Ball State University, Burris High School, and the Indiana Academy for Science, Mathematics, and Humanities, Muncie, IN 47306

In May 1996 six high students participated in a threeweek course on applications of DNA fingerprinting, the purpose of which was for students to experience the entire scientific research process. DNA was isolated from 14 trout lily leaves collected from 5 populations. RAPD/PCR with 2 primers yielded 24 polymorphic bands. Genetic diversity within the species was higher than expected (~60%), suggesting sexual reproduction was occurring frequently. The diversity was apportioned such that 94% occurred within and only 6% between populations, precluding separation of Indiana populations into discrete genetic units.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1998, p. 1160–1167
0066-4804/98/$04.00+0

Vol. 42, No. 5

Sequencing, Disruption, and Characterization of the *Candida albicans* Sterol Methyltransferase (*ERG6*) Gene: Drug Susceptibility Studies in *erg6* Mutants

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The rise in the frequency of fungal infections and the increased resistance noted to the widely employed azole antifungals make the development of new antifungals imperative for human health. The sterol biosynthetic pathway has been exploited for the development of several antifungal agents (allylamines, morpholines, azoles), but additional potential sites for antifungal agent development are yet to be fully investigated. The sterol methyltransferase gene (*ERG6*) catalyzes a biosynthetic step not found in humans and has been shown to result in several compromised phenotypes, most notably markedly increased permeability, when disrupted in *Saccharomyces cerevisiae*. The *Candida albicans ERG6* gene was isolated by complementation of a *S. cerevisiae erg6* mutant by using a *C. albicans* genomic library. Sequencing of the *Candida ERG6* gene revealed high homology with the *Saccharomyces* version of *ERG6*. The first copy of the *Candida ERG6* gene was disrupted by transforming with the *URA3* blaster system, and the second copy was disrupted by both *URA3* blaster transformation and mitotic recombination. The resulting *erg6* strains were shown to be hypersusceptible to a number of sterol synthesis and metabolic inhibitors, including terbinafine, tridemorph, fenpropiomorph, fluphenazine, cycloheximide, cerulenin, and brefeldin A. No increase in susceptibility to azoles was noted. Inhibitors of the *ERG6* gene product would make the cell increasingly susceptible to antifungal agents as well as to new agents which normally would be excluded and would allow for clinical treatment at lower dosages. In addition, the availability of *ERG6* would allow for its use as a screen for new antifungals targeted specifically to the sterol methyltransferase.

The frequency of occurrence of human fungal infections has been increasing over the past decade in response to a combination of factors (12) which include advances in invasive surgical techniques which allow for opportunistic pathogen access, immunosuppression employed in transplantation or resulting from chemotherapy, and disease states such as AIDS. The threat to human health is further compounded by the increased frequency with which resistance to the commonly employed antifungal agents is appearing.

The most prevalently utilized antifungal agents include the polyenes and the azoles. The polyenes are effective by binding to ergosterol, the fungal membrane sterol, and inducing lethal cell leakage (7). Polyenes often have negative side effects, and resistance has been reported (15, 28). The azoles function by inhibition of the cytochrome P-450-mediated removal of the C-14 methyl group from the ergosterol precursor, lanosterol (32). The azoles are fungistatic drugs and are thus subject to the accumulation of resistant phenotypes due, in part, to the need to continuously administer the drug to patients who are immunocompromised. Resistance has been reported in *Candida albicans* (8, 30, 31, 37, 38) as well as in other species of *Candida* (24, 26). In addition, other fungal pathogens, including species of *Histoplasma* (36), *Cryptococcus* (19, 33), and *Aspergillus* (9), have been the subjects of recent reports on azole resistance. The increase in infections coupled with the reduced efficacy of the currently available drugs makes the discovery and development of new antifungals an urgent matter.

The pathway for fungal sterol biosynthesis has provided an excellent target for antifungal development, but there remain additional sites in the pathway that have not been thoroughly investigated. The sterol methyltransferase gene (*ERG6*) represents a particularly good example because this step is not found in cholesterol biosynthesis, thus avoiding some elements of possible side effects. *Saccharomyces cerevisiae erg6* mutants have been available for some time (23), and the *ERG6* gene was isolated and disrupted several years ago (11). Although the absence of the *ERG6* gene product was not lethal, it did result in several severely compromised phenotypes.

erg6 mutants have been shown to have diminished growth rates as well as limitations on utilizable energy sources (21), reduced mating frequency (11), altered membrane structural features (18, 20), and low transformation rates (11). In addition, several lines of evidence have indicated that *erg6* mutants have severely altered permeability characteristics. This has been demonstrated by using dyes (3), cations (3), and spin labels used in electron paramagnetic resonance studies (18). These early observations have been corroborated recently by the cloning of the *LIS1* gene (35), mutants of which were selected on the basis of hypersensitivity to sodium and lithium; sequencing of *LIS1* has indicated identity to *ERG6*. This study demonstrated that while the rate of cation uptake was increased three- to fourfold in the mutant strain, the rate of cation efflux was indistinguishable from that of the wild type. In addition, studies using the Golgi inhibitor brefeldin A have routinely employed *erg6* mutant strains because of their permeability by this compound (34). Since the absence of a func-

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tional sterol methyltransferase would make the cell hypersensitive to exogenous compounds, blocks in *ERG6* gene product function could increase the effectiveness of new or existing antifungals. Thus, we have utilized an *S. cerevisiae erg6* mutant to isolate the *C. albicans ERG6* gene, disrupted both copies in the latter organism, and characterized the resulting phenotype of the *C. albicans erg6* mutant.

MATERIALS AND METHODS

Strains and plasmids. *C. albicans* CAI4 (*Δura3::imm434/Δura3::imm434*), received from W. Fonzi (10), was used for disruption of both copies of *ERG6*. The *S. cerevisiae erg6* deletion strain BKY48-5C (α *leu2-3 ura3-52 erg6Δ::LEU2*) was used as the recipient strain for transformation with the *Candida* genomic library (13). *Escherichia coli* DH5α was used as the host strain for all plasmid constructions. Plasmid pRS316 was obtained from P. Heiter, and Bluescript plasmid was obtained from Stratagene, La Jolla, Calif.

Media. CAI4 was grown on YPD complete medium containing 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and 2% glucose. Complete synthetic medium (CSM) was used for transformation experiments and contained 0.67% yeast nitrogen base (Difco), 2% glucose, and 0.8 g of a mixture of amino acids plus adenine and uracil (Bio 101) per liter. CSM dropout medium contained the same ingredients as CSM, but without uracil. Uridine was added at 80 mg per liter to ensure growth of CAI4. CSM containing uridine and 5-fluoroorotic acid (5-FOA) at 1 g/liter was used to regenerate the *ura3* genetic marker as outlined by Fonzi and Irwin (10). All experiments were carried out at 30°C unless otherwise indicated.

Cloning of *ERG6*. Transformation of *S. cerevisiae* BKY48-5C by using the *Candida* gene library was carried out by a lithium acetate-modified protocol developed by Gaber et al. (11) for *erg6* transformations. The *C. albicans ERG6* gene was cloned by transforming a *S. cerevisiae erg6* deletion strain (BKY48-5C) with a *Candida* genomic DNA library obtained from S. Scherer at the University of Minnesota (13). Transformants containing putative *Candida ERG6* DNA were subcloned into the *Saccharomyces* vector pRS316 for complementation analyses and DNA sequencing. All *Candida* transformations for disruption experiments were carried out essentially in accordance with the procedures of Sanglard et al. (30). Plasmid p5921, obtained from Fonzi (10), was the source of the *URA3* blaster for *Candida ERG6* disruption experiments.

Approximately 1,250 transformants were obtained by plating on a uracil dropout medium that ensured the presence of the plasmid. These transformants were then screened on medium containing 0.06 μg of cycloheximide per ml. *S. cerevisiae erg6* strains are nystatin resistant and cycloheximide sensitive. Transformants that were resistant to this level of cycloheximide (Cyh^r) were further tested for the presence of intracellular ergosterol. Sterols extracted from the *S. cerevisae erg6* strains and the transformants were analyzed by UV spectrophotometry and gas chromatography-mass spectrometry (GC-MS) to confirm the sterol profile.

DNA sequencing of the *Candida ERG6* gene. Both strands of the plasmid insert containing the *ERG6* gene were sequenced by the Sanger dideoxy chain termination method. Initially, T3 and T7 primers were used, and as DNA sequence became available, primers were generated from sequenced DNA.

PCR. PCR analyses were used to verify disruptions of both *Candida ERG6* genes. Primers P1, P2, and P3 were used to distinguish disrupted *ERG6* genes on the basis of size and are in the *ERG6* gene itself. Primer 4 is in the *hisG* region of the URA3 blaster. P1 was 5′-CACATGGGTGAAATTAG-3′ and could be used with all other primers. P2 was 5′-CTCCAGTTCAATTAGCAG-3′, P3 was 5′-TGTGCGTGTACAAAGCAC-3′, and P4 was 5′ GATAATACCGAGATC GAC-3′. PCR buffers and *Taq* polymerase were obtained from Promega. The buffer composition was 10 mM Tris-HCl (pH 9) and 2 mM $MgCl_2$, and reactions mixtures contained 0.2 mM deoxynucleoside triphosphates and 0.5 U of polymerase. Conditions for amplification were as follows: the first cycle was denaturation at 94°C for 5 min; this was followed by 40 cycles of annealing at 50°C for 2 min, elongation at 72°C for 3 min, and denaturation at 94°C for 1 min. A final elongation step at 72°C for 20 min completed the reaction. The protocols used for preparation of the *Candida* template DNA described by Ausubel et al. (1).

Sterol analyses. Nonsaponifiable sterols were isolated as described previously (23). UV analysis of sterols in extracts was accomplished by scanning wavelengths from 200 to 300 nm with a Beckman DU 640 spectrophotometer. GC analyses of nonsaponifiable sterols were conducted on a HP5890 series II equipped with the Hewlett-Packard Chemstation software package. The capillary column (HP-5) was 15 m by 0.25 mm by 0.25 mm (film thickness) and was programmed to increase from 195 to 300°C (3 min at 195°C and then increased at 5.5°C/min until the final temperature of 300°C was reached and held for 4 min). The linear velocity was 30 cm/s with nitrogen as the carrier gas, and all injections were run in the splitless mode. GC-MS analyses were done with a Varian 3400 GC interfaced to a Finnigan MAT SSQ 7000 MS. The GC separations were done on a DB-5 fused-silica column (15 m by 0.32 mm by 0.25 mm [film thickness]) programmed to increase from 50 to 250°C at 20°C/min after a 1-min hold at 50°C. The oven temperature was then held at 250°C for 10 min before the temperature was increased to 300°C at 20°C/min. Helium was the carrier gas, with a linear velocity of 50 cm/s in the splitless mode. The MS was in the electron impact ionization mode at an electron energy of 70 eV, an ion source temperature of 150°C, and scanning from 40 to 650 atomic mass units at 0.5-s intervals.

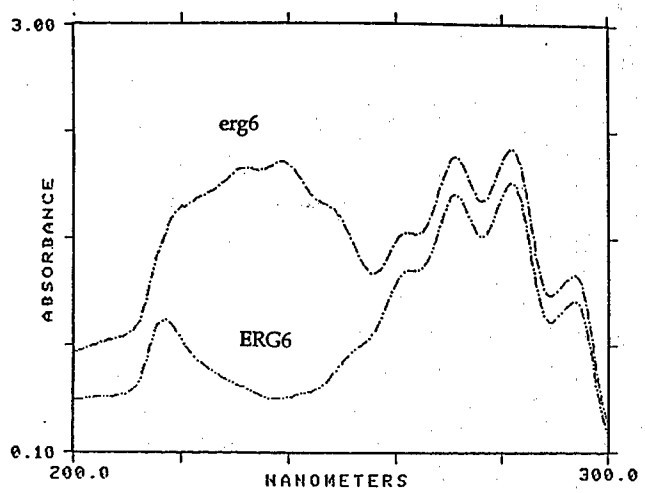

FIG. 1. UV scan of nonsaponifiable sterols in which *erg6* sterols containing a conjugated double bond in the sterol side chain show absorption maxima at 230 and 238 nm. Wild-type *erg6* transformants containing the *Candida ERG6* gene do not have the conjugated double-bond system in the sterol side chain.

Drug susceptibility testing in *C. albicans*. Drug susceptibilities of *C. albicans* wild-type and *erg6* strains were conducted by using cells harvested from overnight YPD plates grown at 37°C. Cells were suspended in YPD medium to a concentration of 10^7 (optical density at 660 nm of 0.5) cells per ml. Cells were plated by transferring 5 μl of the original suspension (10^0) plus 10^{-1} and 10^{-2} dilutions onto YPD plates containing the drug to be tested. The plates were incubated for 48 h at 37°C and observed for growth. Clotrimazole, brefeldin A, cerulenin, cycloheximide, nystatin, and fluphenazine were obtained from Sigma, St. Louis, Mo. Fenpropiomorph and tridemorph were obtained from Crescent Chemical Co., Hauppage, N.Y. Ketoconazole was obtained from ICN, Costa Mesa, Calif. Terbinafine was a gift from D. Kirsch (American Cyanamid, Princeton, N.J.). Stock solutions of terbinafine, tridemorph, brefeldin A, and cerulenin were prepared in ethanol. Clotrimazole, ketoconazole, and fenpropiomorph stocks were prepared in dimethyl sulfoxide, and fluphenazine and cycloheximide stocks were prepared in water. Nystatin was dissolved in *N,N*-dimethyl formamide (Sigma).

Nucleotide sequence accession numbers. The GenBank accession number for the *C. albicans ERG6* gene is AF031941. GenBank accession numbers for the previously determined nucleotide sequences of *ERG6* from *S. cerevisiae*, *Arabidopsis thaliana*, and *Triticum ativum* are X74249, U71400, and U60755, respectively.

RESULTS

Cloning of the *C. albicans ERG6* gene. Four *Saccharomyces erg6* transformants which grew on cycloheximide were analyzed for sterol content. *erg6* mutants which fail to synthesize ergosterol due to defects in the C-24 transmethylase gene accumulate principally zymosterol, cholesta-5,7,24-trien-3β-ol, and cholesta-5,7,22,24-tetraen-3β-ol (23). UV scans of the sterols obtained from a *Saccharomyces erg6* strain as well as an *erg6* transformant containing the *Candida ERG6* gene are shown in Fig. 1. Sterols giving the *erg6* spectrum contain absorption maxima at 262, 271, 282, and 293 nm as well as maxima at 230 and 238 nm. The latter two absorption maxima are due to conjugated double bonds which occur in the sterol side chain (cholesta-5,7,22,24-tetra-en-3β-ol). The *ERG6* transformed strain does not have a conjugated double bond in the side chain and gives absorption maxima only at 262, 271, 282, and 293 nm. The remaining three transformants yielded similar profiles. Additionally, GC analysis of the *erg6* mutant and the *ERG6* transformants confirmed the presence of ergosterol in the latter strains (data not shown). These results were confirmed by MS.

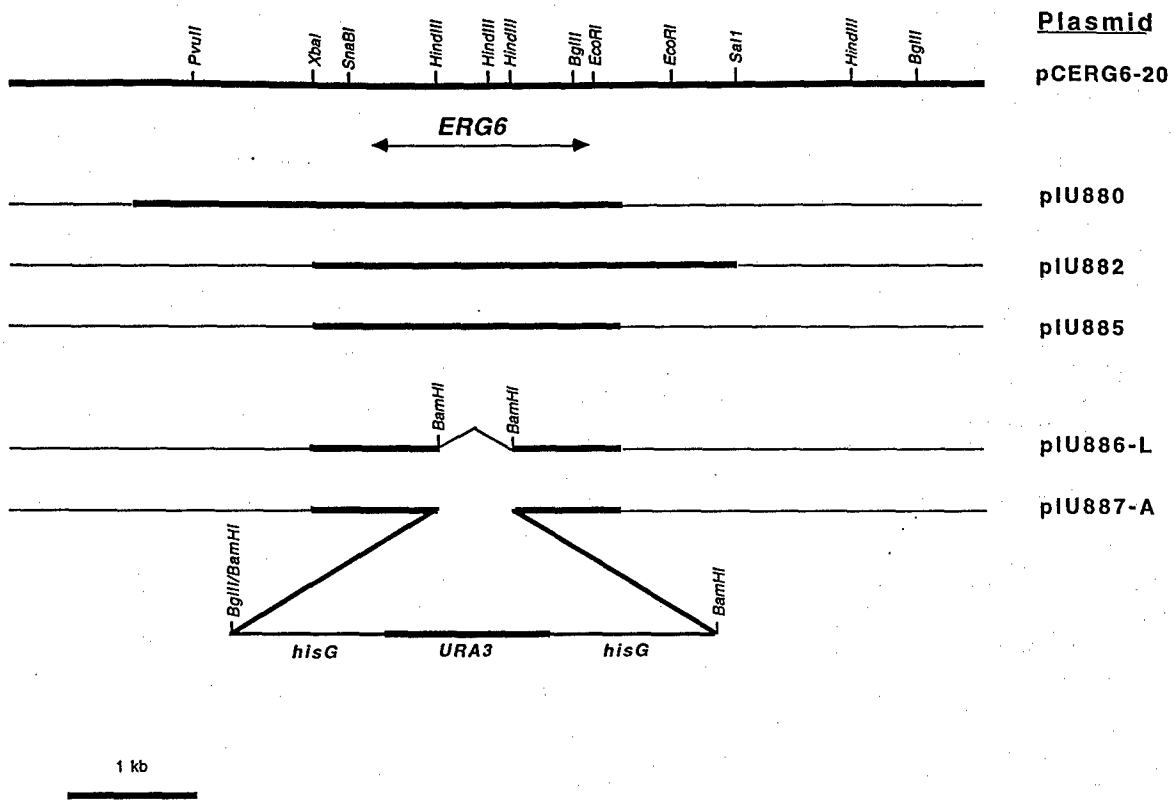

FIG. 2. A *C. albicans ERG6* genomic clone (pCERG6-20) with restriction sites and three complementing subclones, pIU880, pIU882, and pIU885. Deletion of a 0.7-kb *Hin*dIII fragment within pIU885, filling in of cohesive ends, addition of *Bam*HI linkers (pIU886-L), and subsequent insertion of the *URA3* blaster into this site as shown (pIU887-A) are represented.

Of the four transformants restoring the ability of the *erg6* mutant to synthesize ergosterol, there were two different types, designated pCERG6-20 and pCERG6-9, with insert sizes of 8 and 14 kb, respectively (Fig. 2). The pCERG6-9 insert contained the entire 8-kb DNA fragment of pCERG6-20, suggesting that the *ERG6* gene resided within the 8-kb fragment. Growth of ergosterol-producing transformants on media containing 5-FOA resulted in the loss of the transforming plasmid, which restored the BKY48-5c strain back to the *erg6* phenotype; this indicated that ergosterol production of the pCERG6-20 and -9 transformants was plasmid mediated. To locate the *ERG6* gene within the plasmid insert, an approximately 4-kb subclone of the left arm of pCERG6-20 was inserted into the *Saccharomyces* vector pRS316, yielding plasmid pIU880, which was able to complement *erg6* (Fig. 2). Plasmid pIU882, which contains a 2.4-kb overlap with pIU880, also complemented *erg6*, suggesting that the *Candida ERG6* gene lies within this 2.4-kb fragment. A 2.4-kb *Xba*I-*Eco*RI subclone of pIU880 inserted into pRS316 resulted in pIU885 containing the entire *ERG6* gene.

DNA sequencing of the *Candida ERG6* gene. The 2.4-kb *Xba*I-*Eco*RI DNA insert of pIU885 (Fig. 2) was selected for sequencing. The DNA and amino acid sequences are presented in Fig. 3. The *Candida ERG6* gene encodes the sterol methyltransferase, which contains 377 amino acids and is 66% identical to the *Saccharomyces* enzyme. Figure 4 shows the sequence alignment between the *Candida*, *Saccharomyces*, *Arabidopsis*, and *Triticum* sterol methyltransferases, and the levels of identity of *Candida* to the latter two are 40 and 49%, respectively. A 9-amino-acid region (Fig. 4; amino acids 127 to 135 in the *C. albicans* sequence) represents the highly conserved *S*-adenosylmethionine binding site (6).

Creation of a *C. albicans ERG6* heterozygote. Disruption of the *Candida ERG6* gene to derive a sterol methyltransferase-deficient strain was made more difficult since *Candida*, unlike *Saccharomyces*, is diploid and, thus, both copies of the *ERG6* gene must be disrupted. To accomplish this, the *URA3* blaster system developed by Fonzi (10) was used. The *URA3* blaster contains ~3.8 kb comprised of repeat elements of *hisG* (derived from *Salmonella*) flanking the *Candida URA3* gene. The plasmid pIU887-A containing the *URA3* blaster inserted into the *ERG6* gene is shown in Fig. 2. The 2.4-kb *Xba*I-*Eco*RI *ERG6* DNA fragment was cloned into the pBluescript vector KS(+) in which a *Hin*dIII site was filled in with the Klenow fragment of DNA polymerase I (pIU886). pIU886-L was subsequently derived by deleting a 0.7-kb *Hin*dIII fragment within the *ERG6* coding sequence, filling in this site with Klenow fragment, followed by the addition of *Bam*HI linkers. Plasmid 5921, containing the *URA3* blaster, was digested with *Sna*BI and *Stu*I, both blunt-cutting enzymes, followed by religation. This resulted in a deletion of 6 bp in one of the *hisG* regions and destruction of these two sites. The modified 5921 plasmid was then digested with *Bam*HI and *Bgl*II to release the 3.8-kb *URA3* blaster, which was then ligated into pIU886-L that had been digested with *Bam*HI to generate pIU887-A.

C. albicans CAI4 was transformed by using the 5.3-kb *Bgl*II-*Sna*BI fragment containing the *URA3* blaster and *ERG6* flanking recombinogenic ends of 0.8 and 0.9 kb. Transformants containing the single disrupted *ERG6* allele resulting in heterozygosity for *ERG6* were confirmed by using PCR after se-

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1     ATACAAAATTTCTTTCTTTCCTTTTTACTTTACAATTATTTAAATCAAGTTAAAGAACCTTTCATTTTCTTATTTTAATTTAATTTAGAAATTTTCAA

101   CCTAATTAATTCATAATCTAAGATTCAACTCATTAACAATGTCTCCAGTTCAATTAGCAGAAAAAAATTACGAAAGAGATGAACAATTCACTAAAGCTTT
                                  M  S  P  V  Q  L  E  A  K  N  Y  R  E  D  E  Q  F  T  K  A  L                21

201   ACATGGTGAATCTTATAAAAAAACTGGGTTATCAGCTTTAATAGCTAAATCTAAAGATGCTGCTTCTGTTGCTGCTGAGGGTTATTTCAAACATTGGGAT
      H  G  E  S  Y  K  K  T  G  L  S  A  L  I  A  K  S  K  D  A  A  S  V  A  A  E  G  Y  F  K  H  W  D        54

301   GGTGGTATTTCTAAAGATGATGAAGAGAAAAGATTGAATGATTATTCCCAATTGACTCATCATTATTATAATTTAGTCACTGACTTTTATGAATATGGTT
      G  G  I  S  K  D  D  E  E  K  R  L  N  D  Y  S  Q  L  T  H  H  Y  Y  N  L  V  T  D  F  Y  E  Y  G  W        88

401   GGGGTTCTTCATTCCATTTTTCAAGATATTATAAAGGTGAAGCTTTTAGACAAGCTACTGCTAGACATGAACATTTCTTGGCCCATAAAATGAATCTTAA
         G  S  S  F  H  F  S  R  Y  Y  K  G  E  A  F  R  Q  A  T  A  R  H  E  H  F  L  A  H  K  M  N  L  N     121

501   TGAAAACATGAAAGTTTTAGATGTTGGTTGTGGTGTAGGTGGTCCTGGTAGAGAAATCACAAGATTTACTGATTGTGAAATTGTTGGATTAAATAATAAT
      E  N  M  K  V  L  D  V  G  C  G  V  G  G  P  G  R  E  I  T  R  F  T  D  C  E  I  V  G  L  N  N  N        154

601   GATTATCAAATTGAAAGAGCTAATCATTATGCTAAAAAATACCATTTAGATCATAAATTATCTTATGTTAAAGGTGATTTTATGCAAATGGATTTTGAAC
      D  Y  Q  I  E  R  A  N  H  Y  A  K  K  Y  H  L  D  H  K  L  S  Y  V  K  G  D  F  M  Q  M  D  F  E  P     188

701   CAGAATCATTCGATGCTGTTTATGCCATTGAAGCTACCGTTCATGCTCCAGTTTTAGAAGGAGTTTATTCAGAAATTTATAAAGTTTTGAAACCAGGTGG
      E  S  F  D  A  V  Y  A  I  E  A  T  V  H  A  P  V  L  E  G  V  Y  S  E  I  Y  K  V  L  K  P  G  G        221

801   TATTTTCGGTGTTTATGAATGGGTCATGACTGATAAATACGATGAAACTAATGAAGAACATCGTAAAATTGCTTATGGTATTGAAGTCGGTGATGGTATT
      I  F  G  V  Y  E  W  V  M  T  D  K  Y  D  E  T  N  E  E  H  R  K  I  A  Y  G  I  E  V  G  D  G  I        254

901   CCAAAAATGTATTCTCGTAAAGTTGCTGAACAAGCTTTGAAAAATGTTGGATTTGAAATTGAATATCAAAAAGATTTGGCTGATGTTGATGATGAAATTC
      P  K  M  Y  S  R  K  V  A  E  Q  A  L  K  N  V  G  F  E  I  E  Y  Q  K  D  L  A  D  V  D  D  E  I  P     288

1001  CTTGGTATTATCCATTAAGTGGTGATTTGAAATTTTGTCAAACTTTTGGTGATTATTTGACTGTTTTCAGAACTTCAAGAATTGGTAGATTCATTACTAC
      W  Y  Y  P  L  S  G  D  L  K  F  C  Q  T  F  G  D  Y  L  T  V  F  R  T  S  R  I  G  R  F  I  T  T        321

1101  TGAATCAGTTGGTTTAATGGAAAAAATTGGTTTAGCTCCAAAAGGTTCTAAACAAGTTACTCATGCTTTAGAAGATGCTGCTGTTAATTTAGTTGAAGGT
      E  S  V  G  L  M  E  K  I  G  L  A  P  K  G  S  K  Q  V  T  H  A  L  E  D  A  A  V  N  L  V  E  G        354

1201  GGTAGACAAAAATTGTTTACTCCAATGATGTTGTACGTTGTTAGAAAACCATTAGAAAAGAAAGATTAATGGGGCTTGACAAACAACAAGTAAGGTGAGT
      G  R  Q  K  L  F  T  P  M  M  L  Y  V  V  R  K  P  L  E  K  K  D                                        376

1301  TTATGTTGGGGGTGTTCAATTCGTGTATCTATTCATAGAGGTATTGATTTGCAATTTGTTTTTTGTTTATTCTATTTATTATTTTAAATACTGTTTATAT
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FIG. 3. The DNA and amino acid sequences of the *C. albicans ERG6* gene. The *S*-adenosylmethionine binding site is indicated by underlining.

lection for loss of the *URA3-hisG* region. Intrachromosomal recombination between the linear *hisG* sequences resulted in the loss of one of these *hisG* repeats and the *URA3*, thus permitting reuse of the *URA3* blaster for the subsequent disruption of the *ERG6* gene on the homologous chromosome. Selection for colonies on medium containing 5-FOA resulted in growth of only uridine-requiring strains (5).

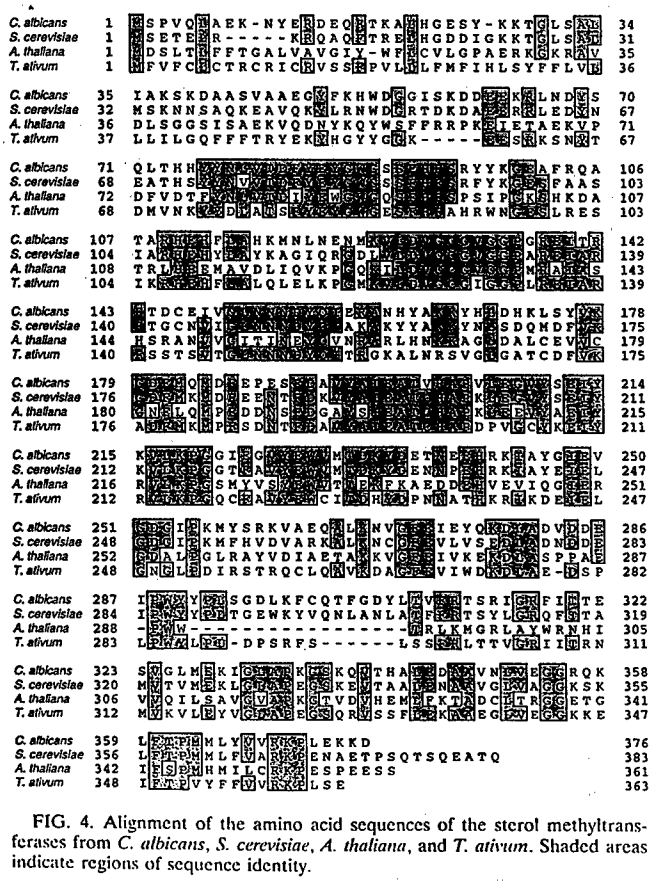

FIG. 4. Alignment of the amino acid sequences of the sterol methyltransferases from *C. albicans*, *S. cerevisiae*, *A. thaliana*, and *T. ativum*. Shaded areas indicate regions of sequence identity.

Creation of *C. albicans erg6* strains. The creation of a *Candida erg6* mutant strain in which both alleles were disrupted was accomplished in two different ways. The *ERG6* heterozygote was placed onto plates containing high concentrations of nystatin (15 μg/ml), and nystatin-resistant colonies appeared after 3 days. We surmised that mitotic recombination resulted in homozygous *ERG6* and *erg6* segregants and that these nystatin-resistant colonies might be the *erg6* homozygotes. When colony purified, these resistant colonies indeed turned out to be *erg6* homozygotes (see below). The second method used to generate *erg6* homozygotes was to transform the *ERG6* heterozygote with the *URA3* blaster. Two kinds of transformants were obtained, wild-type and slow-growing colonies. Both types of colonies were tested for resistance to nystatin, and only the slower-growing colonies were nystatin resistant.

Confirmation of *erg6* homozygosity by sterol analyses. The sterols isolated from wild-type and putative *erg6* homozygotes were analyzed by UV spectrophotometry and GC-MS. All of our putative *erg6* homozygotes contained *erg6*-like UV scans similar to the *S. cerevisiae erg6* scan shown in Fig. 1. Additionally, GC-MS of *erg6* mutant sterols confirmed that only cholesterol-like (C-27) sterols accumulate since the side chain cannot be methylated. Figure 5 shows a GC profile demonstrating that the putative *erg6* mutants accumulate C-27 sterols and are deficient in side chain transmethylation. Whereas the predominant sterol in the CAI4 wild type is ergosterol (peak B, 76%), the principal sterols in *erg6* mutants are zymosterol (peak A, 43%), cholesta-5,7,24-trien-3β-ol (peak D, 6%), cholesta-7,24-dien-3β-ol (peak E, 9%), and cholesta-5,7,22,24-tetraen-3β-ol (peak F, 29%).

PCR confirmation of homozygous disruptions. Confirmation of the disruption of both copies of the *C. albicans ERG6* gene by mitotic recombination of the heterozygote and by a second transformation using the *URA3* blaster was performed by using four PCR primers. The *URA3* blaster containing a 3.8-kb region of *hisG-URA3-hisG* replaced 0.7 kb of *ERG6*

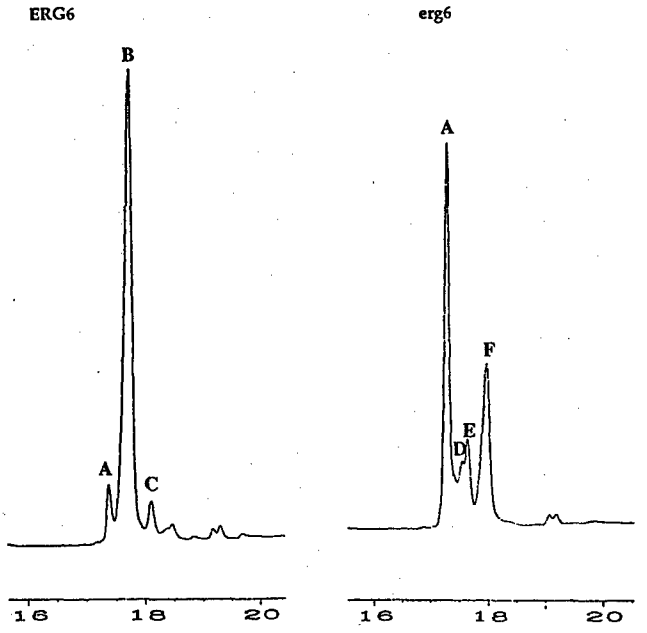

FIG. 5. GC of the sterols of the wild type and an *erg6* strain of *C. albicans*. Peak A, zymosterol; peak B, ergosterol; peak C, fecosterol; peak D, cholesta-5,7,24-trien-3β-ol; peak E, cholesta-7,24-dien-3β-ol; peak F, cholesta-5,7,22,24-tetraen-3β-ol.

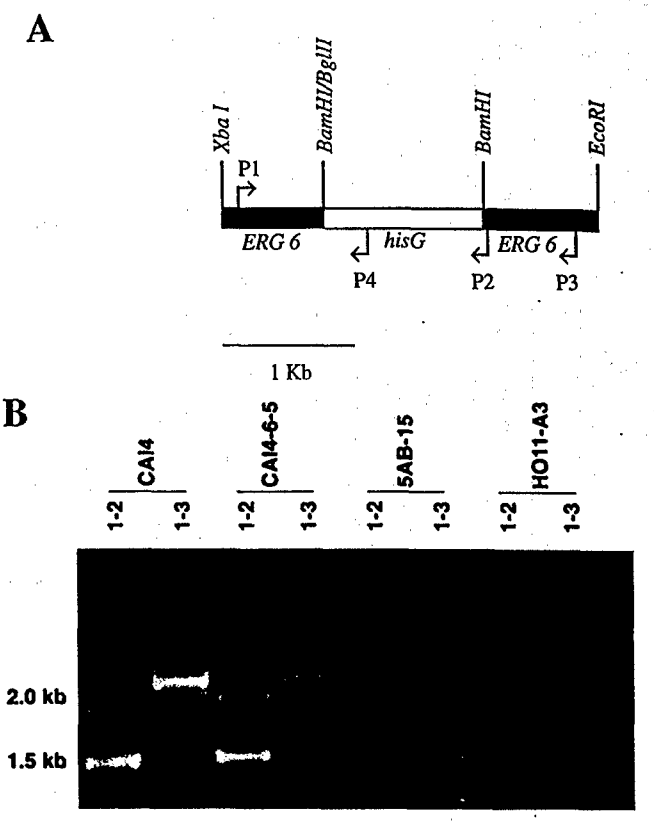

FIG. 6. (A) *URA3* blaster disruption of the *ERG* gene showing location of PCR primers; (B) agarose gel electrophoresis confirmation of heterozygote and homozygote disruptants of the *ERG6* gene. Lanes (left to right): 1 and 2, CAI4 (wild type); 3 and 4, CA14-6-5 (heterozygote); 5 and 6, 5AB-15 (homozygote derived from *URA3* blaster transformation followed by mitotic recombination); 7 and 8, HO11-A3 (homozygote derived from two rounds of *URA3* blaster transformation). The PCR primer pairs used are indicated at the tops of the lanes (e.g., 1-2 is P1-P2). The image was captured on disc and the photograph was generated by using Photoshop on Macintosh.

DNA (Fig. 6A). This was followed by deletion of the *hisG-URA3* sequence such that, in effect, the remaining 1.2-kb *hisG* sequence replaces a 0.7-kb *ERG6* deletion. The expected PCR amplifications of CAI4 using primer pair P1-P2 or P1-P3 are 1.5 and 2.15 kb, respectively (Fig. 6B, lanes 1 and 2). The expected products from P1-P2 amplification of the heterozygote CAI-4-6-5 are 1.5 kb (wild-type allele) and 2.01 kb (disrupted *ERG6* allele), and the expected products from amplification using the P1-P3 primers are 2.15 kb (wild type) and 2.65 kb (disrupted *ERG6*); these products are visible in Fig. 6B, lanes 3 and 4. Primer pair P1-P4 gives a 1.1-kb band, demonstrating the presence of *hisG* within the *ERG6* sequence (data not shown). The *erg6* homozygotes 5AB-15, obtained by mitotic recombination, and HO11-A3, obtained by *URA3* blaster disruption, yield identical amplification products with primer pairs P1-P2 (2.01 kb) and P1-P3 (2.65 kb), as shown in Fig. 6B, lanes 5 to 8.

Drug susceptibilities of *C. albicans erg6* strains. The susceptibilities of the *erg6* strains as compared to that of wild-type *C. albicans* were determined by using a number of antifungal compounds and general cellular inhibitors (Fig. 7). The *erg6* strains were shown to be more resistant to nystatin while showing nearly identical sensitivities to the azole antifungals clotrimazole and ketoconazole. Significantly increased susceptibilities of the *erg6* strains were noted for tridemorph and fenpropiomorph, inhibitors of sterol Δ14-reductase and Δ8-Δ7 isomerase (2); terbinafine, an allylamine antifungal inhibiting squalene epoxidase (16); brefeldin A, an inhibitor of Golgi function (33); cycloheximide, a common protein synthesis inhibitor; cerulenin, an inhibitor of fatty acid synthesis (25); and fluphenazine, a compound which interferes with the function of calmodulin (14).

The determination of drug concentrations sufficient to completely inhibit growth on plates yielded the data shown in Table 1. The concentration of nystatin required for complete inhibition of the wild type (2.5 μg/ml) is within the normal range for a wild-type strain (23), while the *erg6* mutants show a resistance level similar to that noted for *erg6* mutants of *S. cerevisiae* (23). As demonstrated by growth on plates (Fig. 7), the azoles show equal efficacies against both wild-type and *erg6* mutant strains. In contrast, the *erg6* mutants show significantly increased susceptibilities to other antifungals and metabolic inhibitors. *erg6* susceptibilities to cerulenin and fluphenazine were twofold greater, while those for terbinafine and brefeldin A were about 50 times greater, than those of the wild type. Cycloheximide susceptibility was increased about 11-fold in the *erg6* mutants, while the greatest increases in susceptibility were shown for the morpholines fenpropiomorph (100-fold) and tridemorph (several thousandfold). The *erg6* heterozygote showed essentially the same drug sensitivities as those of the wild type, CAI4, for all inhibitors tested.

DISCUSSION

Strains with mutations in the *erg6* gene of *S. cerevisiae* have been available for many years (23). Since the biosynthetic step that adds the C-24 methyl group is found in fungal but not in human sterol biosynthesis, it was proposed (27) that this step might be essential and that inhibition at this point in the pathway would be lethal. This hypothesis could not be tested until

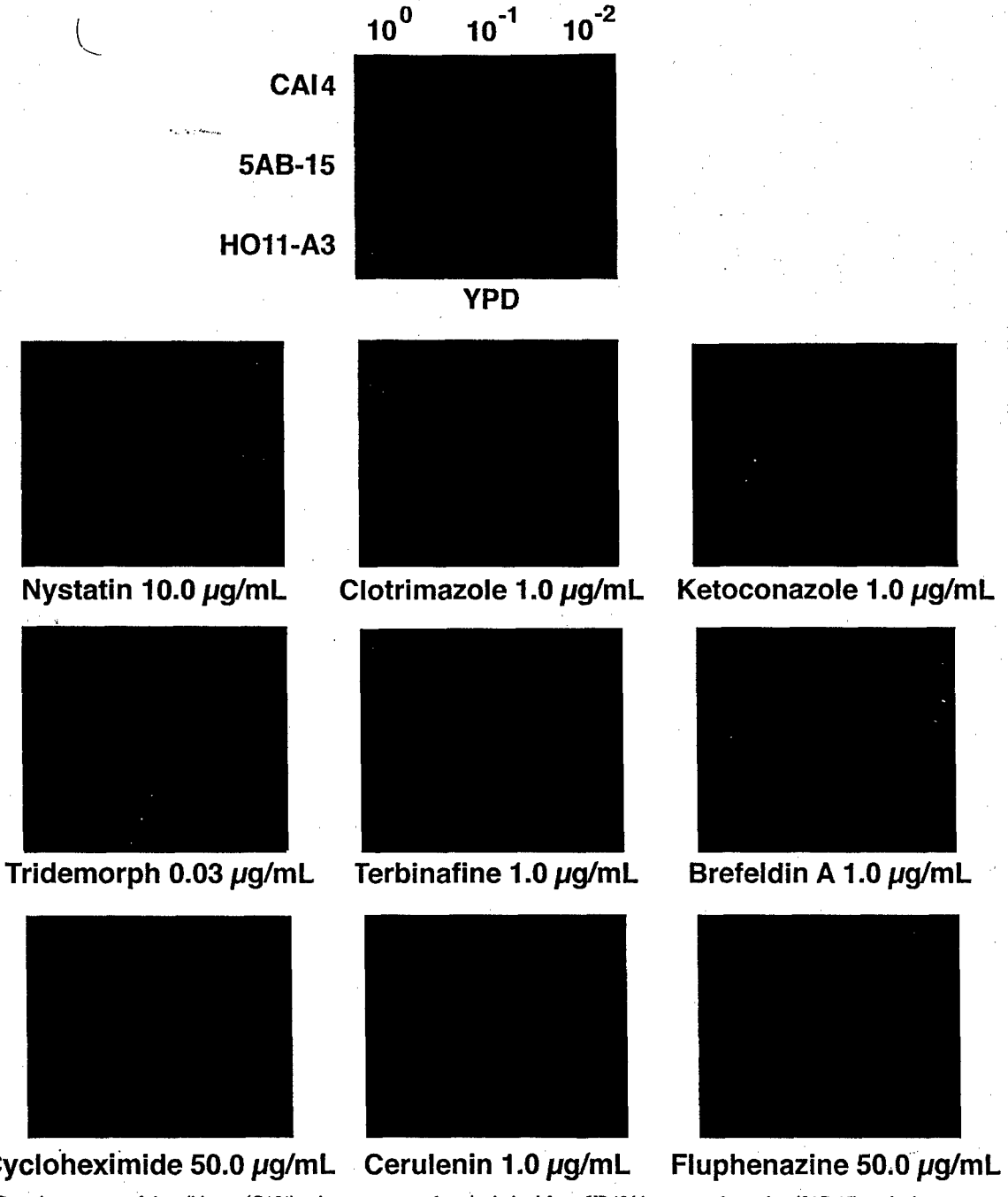

FIG. 7. Growth responses of the wild type (CAI4), a homozygous *erg6* strain derived from *URA3* blaster transformation (5AB-15), and a homozygous *erg6* strain derived from mitotic recombination (HO11-A3) in the presence of sterol biosynthesis inhibitors and metabolic inhibitors. Cells were grown at 37°C to a density of 10^7 cells/ml, and 5 μl was inoculated at 10^0, 10^{-1}, and 10^{-2} dilutions. The image was captured on disc and the photograph was generated by using Photoshop on Macintosh.

the *ERG6* gene could be shown to be completely inactivated, since low levels of leakiness could allow viability. The cloning and disruption of the *ERG6* gene (11) provided definitive evidence that the gene is not essential in *S. cerevisiae*. However, the same study reinforced previous work done with *erg6* point mutations that had demonstrated that *erg6* mutants have several altered phenotypes (3, 18, 20, 21). Our particular interest is in the alteration of permeability characteristics.

The essential nature of the *ERG6* gene in *C. albicans* has not been reported prior to the work described here. It was possible that this gene could be essential since the *ERG11* gene has been shown to be essential in *S. cerevisiae* but not in *C. albicans*, indicating that these two species are not identical in their abilities to survive and grow on various sterol intermediates. In addition, it would be of particular interest to assess the permeability of *Candida erg6* mutant cells since this characteristic might make them more sensitive to known and new antifungals or might even make them sensitive to compounds previously found not to be effective when ergosterol is present in the cell.

Using a *Candida* genomic library, we have isolated the *Can-*

TABLE 1. Susceptibilities of *ERG6* and *erg6* strains of *C. albicans* to antifungal agents and metabolic inhibitors

Drug	Inhibitory concn (μg/ml)[a]	
	ERG6	*erg6*
Nystatin	2.5	15
Clotrimazole	4	4
Ketoconazole	5	5
Terbinafine	>50	1
Fenpropiomorph	0.5	0.005
Tridemorph	>90	0.03
Brefeldin A	50	1
Cerulenin	2	1
Cycloheximide	>600	50
Fluphenazine	100	50

[a] Concentration at which no growth appeared after 48 h under the conditions described in the legend to Fig. 7.

dida ERG6 gene by complementing an *erg6* mutant of *Saccharomyces*. As part of our screen for complementation, sensitivity to nystatin and resistance to cycloheximide were employed. Nystatin functions by binding to membrane ergosterol and causing cell leakage, which leads to cell death (7). Mutants such as *erg6* do not produce ergosterol and utilize sterol intermediates in place of membrane ergosterol. Nystatin has lower affinity for sterol intermediates, thus leading to resistance in non-ergosterol-containing strains. Restoration of the *ERG6* gene from *Candida* in *Saccharomyces erg6* mutants would restore the nystatin-sensitive phenotype. The wild-type *ERG6* gene also reconstitutes the cell permeability barrier to normal levels, thus conferring cycloheximide resistance at low drug concentrations. Cloning of the *Candida ERG6* gene was also confirmed by UV analysis of sterol composition and GC-MS analysis of accumulated sterols in *Saccharomyces erg6* and transformed strains containing the *Candida ERG6* gene. Final confirmation that we had cloned *ERG6* was provided by sequencing the *Candida ERG6* gene. The *Candida* sequence showed high identity to the *S. cerevisiae ERG6* gene sequence and good agreement with the same gene from *Arabidopsis* and *Triticum*. The high homology of the *Candida* and *Saccharomyces* sequences accounts for the successful complementation noted in this study.

To determine the essentiality of the *ERG6* gene in *Candida*, the two copies were disrupted by first creating the heterozygote by using the *URA3* blaster disruption protocol. The second copy of the *ERG6* gene was disrupted either by allowing for mitotic recombination or by a second disruption with the *URA3* blaster. In both cases, the resulting *erg6* homozygotes were viable, indicating that the *ERG6* gene in *C. albicans* is not essential for viability. Both types of *erg6* mutants were confirmed by sterol and PCR analyses of the disruptions.

With the continued increase in resistance to the azole antifungals, new approaches to antifungal chemotherapy are strongly indicated. One approach is to disarm the resistance mechanism. A primary mechanism in *C. albicans* for azole resistance is the increase in expression of efflux systems which utilize the azoles as substrates. Both the ABC (ATP-binding cassette) transporter gene *CDR1* and a gene (*BEN*^{r}) belonging to a major facilitator multidrug efflux transporter have been implicated in this process (31). A report by Sanglard et al. (30) has shown that disruption of the *CDR1* gene results in a cell that shows increased susceptibilities to the azole, allylamine, and morpholine antifungals as well as other metabolic inhibitors, including cycloheximide, brefeldin A, and fluphenazine. Although not effective alone, disruptions of *BEN*^{r} were shown to work synergistically with *CDR1* with two metabolic inhibitors. The *CDR1* system could provide for an assay for drugs not subject to efflux by these transporters or could also be used to select for compounds which could block the action of the transporters directly. Such approaches would avoid or disarm resistance mechanisms, respectively.

In this report, the testing of *Candida erg6* mutants for their susceptibility to antifungal and metabolic inhibitors indicated that these mutants had increased sensitivity to a wide variety of compounds. Azoles were an exception in that they showed no difference in efficacy for wild-type and mutant strains. Apparently, the permeability changes are unrelated to the entry mechanism for these compounds. The remainder of the compounds tested, including two other antifungal compounds with different mechanisms of action, are significantly more inhibitory toward the *erg6* strain.

These findings have important applicability from several perspectives. First, the results predict that an inhibitor of the *ERG6* gene product would result in a fungal organism that is hypersensitive to known compounds or new compounds to which the cell is normally impermeable. Treatment of a cell with both inhibitors would thus produce a synergistic effect. Synergism has been shown (4) by using the experimental sterol methyltransferase inhibitor ZM59620 added simultaneously with allylamine and morpholine antifungals. In these studies, the concentrations of the drugs in the combined treatment were significantly below the individual concentrations necessary for both the inhibition of ergosterol biosynthesis and growth inhibition. Thus, because of the increased drug access produced by inhibitors of the sterol methyltransferase, other inhibitors can be clinically employed at reduced dosages. Second, the availability of the *C. albicans ERG6* gene allows it to be used as a screen for the identification of inhibitory compounds that specifically target the *ERG6* gene product. This approach has been successfully utilized in cloning of one of the 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase genes (29) as well as the *ERG11* (17) and *ERG24* (22) genes. In applying this strategy for the purpose of identifying *ERG6* gene product inhibitors, the sensitivity of a wild-type strain would be compared to that of a strain carrying additional copies of *ERG6* on a high-copy-number plasmid. Inhibition of the wild type but not the multiple-copy strain would identify inhibition specific to the sterol methyltransferase. Treatment of a fungal pathogen with such an inhibitor would result in a metabolically compromised cell that, as in the first application, would be more susceptible to existing antifungals and metabolic inhibitors. Finally, the *erg6* system allows for the replacement of in vitro testing of inhibitors by utilizing the increased permeability characteristics inherent in the in vivo mutant system. This will allow characterization of potential inhibitors that normally fail to reach intracellular targets due to a lack of permeability.

Since the *erg6* system results in a compromised cell which is highly permeable to a variety of compounds and since selection of new inhibitors using high-copy-number *ERG6* plasmids allows for easy identification, we believe that this system has superior potential for the development of new antifungal treatment protocols.

ACKNOWLEDGMENTS

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REFERENCES

1. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Short protocols in molecular biology, 3rd ed. John Wiley & Sons, Inc., New York, N.Y.
2. **Baloch, R. I., E. I. Mercer, T. E. Wiggins, and B. C. Baldwin.** 1984. Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropiomorph, and fenpropidin. Phytochemistry **23**:2219–2226.
3. **Bard, M., N. D. Lees, L. A. Burrows, and F. W. Kleinhans.** 1978. Differences in crystal violet dye uptake and cation-induced death among yeast sterol mutants. J. Bacteriol. **135**:1146–1148.
4. **Barrett-Bee, K., and G. Dixon.** 1995. Ergosterol biosynthesis inhibition: a target for antifungal agents. Acta Biochim. Pol. **42**:465–480.
5. **Boeke, J. D., J. Truehart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoro-orotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154**:164–175.
6. **Bouvier-Navé, P., T. Husselstein, T. Desprez, and P. Benveniste.** 1997. Identification of cDNAs encoding sterol methyl transferases involved in the second methylation step of plant sterol biosynthesis. Eur. J. Biochem. **246**: 518–529.
7. **Brajtburg, J., W. G. Powderley, G. S. Kobayashi, and G. Medoff.** 1990. Amphotericin B: current understanding of mechanism of action. Antimicrob. Agents Chemother. **34**:183–188.
8. **Clark, F. S., T. Parkinson, C. A. Hitchcock, and N. A. R. Gow.** 1996. Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species: possible role for drug efflux in drug resistance. Antimicrob. Agents Chemother. **40**:419–425.
9. **Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly.** 1997. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **41**:1364–1368.
10. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134**:717–728.
11. **Gaber, R. F., D. M. Copple, B. K. Kennedy, M. Vidal, and M. Bard.** 1989. The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle sparking sterol. Mol. Cell. Biol. **9**:3447–3456.
12. **Georgopapadakou, N. H., and T. J. Walsh.** 1994. Human mycoses: drugs and targets for emerging pathogens. Science **264**:371–373.
13. **Goshorn, A. K., S. M. Grindle, and S. Scherer.** 1992. Gene isolation by complementation in *Candida albicans* and applications to physical and genetic mapping. Infect. Immun. **60**:876–884.
14. **Hait, W. N., J. F. Gesmonde, and J. S. Lazo.** 1993. Effect of anti-calmodulin drugs on the growth and sensitivity of C6 rat glioma cells to bleomycin. Anticancer Res. **14**:1711–1721.
15. **Hebeka, E. K., and M. Solotorovsky.** 1965. Development of resistance to polyene antibiotics in *Candida albicans*. J. Bacteriol. **89**:1533–1539.
16. **Jandrositz, A., F. Turnowski, and G. Hogenaur.** 1991. The gene encoding squalene epoxidase from Saccharomyces cerevisiae: cloning and characterization. Gene **107**:155–160.
17. **Kalb, V. F., J. C. Loper, C. R. Dey, C. W. Woods, and T. R. Sutter.** 1986. Isolation of a cytochrome P-450 gene from *Saccharomyces cerevisiae*. Gene **45**:237–245.
18. **Kleinhans, F. W., N. D. Lees, M. Bard, R. A. Haak, and R. A. Woods.** 1979. ESR determination of membrane permeability in a yeast sterol mutant. Chem. Phys. Lipids **23**:143–154.
19. **Lamb, D. C., B. C. Baldwin, K. J. Kwon-Chung, and S. L. Kelly.** 1997. Stereoselective interaction of the azole antifungal agent SCH39304 with the cytochrome P-450 monooxygenase system isolated from *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **41**:1465–1467.
20. **Lees, N. D., M. Bard, M. D. Kemple, R. A. Haak, and F. W. Kleinhans.** 1979. ESR determination of membrane order parameter in yeast sterol mutants. Biochim. Biophys. Acta **553**:469–475.
21. **Lees, N. D., S. L. Lofton, R. A. Woods, and M. Bard.** 1980. The effects of varied energy source and detergent on the growth of sterol mutants of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **118**:209–214.
22. **Marcireaux, D., D. Guyonnet, and F. Karst.** 1992. Construction and growth properties of a yeast strain defective in sterol 14-reductase. Curr. Genet. **22**:267–272.
23. **Molzhan, S. W., and R. A. Woods.** 1972. Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **72**:339–348.
24. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. **41**:617–623.
25. **Morisaki, N., H. Funabashi, R. Shimazawa, J. Furukawa, A. Kawaguchi, S. Okuda, and S. Iwasaki.** 1993. Effect of side-chain structure on inhibition of yeast fatty-acid synthase by cerulenin analogues. Eur. J. Biochem. **211**:111–115.
26. **Parkinson, T., D. J. Falconer, and C. A. Hitchcock.** 1995. Fluconazole resistance due to energy-dependent drug efflux in *Candida glabrata*. Antimicrob. Agents Chemother. **39**:1696–1699.
27. **Pinto, W. J., and W. D. Nes.** 1983. Stereochemical specificity for sterols in *Saccharomyces cerevisiae*. J. Biol. Chem. **258**:4472–4476.
28. **Powderley, W. G., G. S. Kobayashi, G. P. Herzig, and G. Medoff.** 1988. Amphotericin B-resistant yeast infection in severely immunocompromised patients. Am. J. Med. **84**:826–832.
29. **Rine, J., W. Hansen, E. Hardeman, and R. W. Davis.** 1983. Targeted selection of recombinant clones through gene dosage effects. Proc. Natl. Acad. Sci. USA **80**:6750–6754.
30. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1996. Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob. Agents Chemother. **40**:2300–2305.
31. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39**:2378–2386.
32. **Vanden Bossche, H., G. Willemsens, and P. Marichal.** 1987. Anti-*Candida* drugs—the biochemical basis for their activity. Crit. Rev. Microbiol. **15**:57–72.
33. **Venkateswarlu, K., M. Taylor, N. J. Manning, M. G. Rinaldi, and S. L. Kelly.** 1997. Fluconazole tolerance in clinical isolates of *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **41**:748–751.
34. **Vogel, J. P., J. N. Lee, D. R. Kirsch, M. D. Rose, and E. S. Sztul.** 1993. Brefeldin A causes a defect in secretion in *Saccharomyces cerevisiae*. J. Biol. Chem. **268**:3040–3043.
35. **Welihinda, A. A., A. D. Beavis, and R. J. Trumbly.** 1994. Mutations in *LIS1* (*ERG6*) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1193**:107–117.
36. **Wheat, J., P. Marichal, H. Vanden Bossche, A. Le Monte, and P. Connolly.** 1997. Hypothesis on the mechanism of resistance to fluconazole in *Histoplasma capsulatum*. Antimicrob. Agents Chemother. **41**:410–414.
37. **White, T.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41**:1482–1487.
38. **White, T.** 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14α demethylase in *Candida albicans*. Antimicrob. Agents Chemother. **41**:1488–1494.

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*

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Abstract

The *ERG25* gene encoding the *Candida albicans* C-4 sterol methyl oxidase was cloned and sequenced by complementing a *Saccharomyces cerevisiae erg25* mutant with a *C. albicans* genomic library. The Erg25p is comprised of a 308 amino acids and shows 65% and 38% homology to the enzymes from *S. cerevisiae* and *Homo sapiens*, respectively. The protein contains three histidine clusters common to non-heme iron binding enzymes and an endoplasmic reticulum retrieval signal as do the proteins from *S. cerevisiae* and human. A temperature-sensitive (ts) conditional lethal mutation of the *C. albicans ERG25* was isolated and expressed in *S. cerevisiae*. Sequence analysis of the ts mutant indicated an amino acid substitution within the region of the protein encompassed by the histidine clusters involved in iron binding. Results indicate that plasmid-borne conditional lethal mutants of target genes have potential use in the rescue of *Candida* mutations in genes that are essential for viability.

The ergosterol biosynthetic pathway in fungi remains the target of the major classes of antifungal compounds currently employed to treat human infections. The recent, dramatic increase in reports of resistance (1) to these compounds has mandated a continued search for new antifungal target sites against which novel compounds can be developed and employed. Of particular concern is the rapidly emerging resistance to the azole antifungals which are now the most widely employed antifungals in systemic, life-threatening infections. Most of the basic research in defining the ergosterol biosynthetic pathway and in determining the types of sterol molecules that support fungal growth has been conducted in *Saccharomyces cerevisiae* (2,3). This organism is readily amenable to genetic analysis and the availability of haploid strains has made the isolation and characterization of ergosterol biosynthetic genes possible. At this time all the genes encoding sterol biosynthetic enzymes in this organism have been cloned and disrupted with the exception of one of the genes involved in the complex series of reactions responsible for removing the two methyl groups from the C-4 position of the sterol molecule. All genes prior to the sterol methyl transferase (*ERG6*) gene have been shown to be essential for viability and thus represent possible new targets for the discovery of novel antifungal compounds.

The use of S. *cerevisiae* as a model system for gene isolation and the determination of essential pathway steps is a useful tool in making similar determinations in pathogenic fungi such as *Candida albicans* where haploid strains and sexual reproduction are not found. The complementation of *S. cerevisiae* sterol mutants with a *C. albicans* genomic library to isolate sterol genes in *C. albicans* is a first step in verifying gene essentiality in this organism and the suitability of that step for seeking inhibitors that might serve as antifungal agents. This approach has recently been used to clone and disrupt the *ERG6* gene of *C. albicans* (4). Although the *ERG6* gene is not essential in *S. cerevisiae*, its disruption results in a cell that is

severely compromised in several functions associated with membrane permeability (5,6). The disruption of both copies of the *ERG6* gene in *C. albicans* likewise does not render the cell inviable but does make it hyper-susceptible to a number of antifungals compounds and cellular inhibitors (4).

The final step in fungal sterol biosynthesis to be explored at the molecular level in *S. cerevisiae* is the multi-component reaction which sequentially removes the two methyl groups from the C-4 position. Three enzymes are required and the genes of two of these, the C-4 methyl oxidase gene (*ERG25*) and the C-3 sterol dehydrogenase (C-4 decarboxylase) gene (*ERG26*), have been isolated and characterized (7,8). Disruptions of *ERG25* and *ERG26* have been found to be lethal (7,8). Here we report the cloning and sequencing of the C-4 sterol methyl oxidase gene from *C. albicans* achieved by the complementation of a *S. cerevisiae erg25* mutant with a *C. albicans* library. Disruption of both copies of an essential sterol gene in *C. albicans*, however, is problematic since this organism will not take up exogenous sterol to allow viability in order to verify that the double disruption has taken place. As a possible means to circumvent this situation, the *C. albicans ERG25* gene has been used to create a conditional lethal *ERG25* mutation which can be expressed on autonomous plasmids in *S. cerevisiae* to rescue *erg25* mutations under permissive conditions but not under non-permissive conditions.

MATERIALS AND METHODS

Strains and plasmids: The *S. cerevisiae erg25* strain used in this study has been previously described (7). The *C. albicans* strain CAI8 (*ade2::hisG/ ade2::hisG, Δura3::imm434/Δura3::imm434*) was a gift from W. Fonzi and has been previously described (9). Plasmid pCERG25, the original complementing clone of 4 kb in the *C. albicans* shuttle vector YPB1, was isolated from a *C. albicans* genomic library

obtained from Stew Scherer, University of Minnesota. pIU870 contains the 2.5 kb *BglII-BamHI C. albicans ERG25* gene subcloned from pCERG25 into pRS316 at the *BamHI* site. pIU908 is identiccal to pIU870 but with a temperature-sensitive conditional lethal mutation in *ERG25*. pIU873 contains the 3.7 kb *XbaI-SalI C. albicans ERG25* gene subcloned from pCERG25 into pRS316 at the *XbaI-SalI* sites. *Escherichia coli* strain DH5α was used to manipulate and maintain all the plasmids used in this study.

Growth conditions: Sterol auxotrophs were grown anaerobically on complete medium supplemented with 1% ergosterol at 30°C. *Candida* strains were grown in complete or SD (synthetic dropout) media supplemented with 100 μg/ml uridine. Temperature shift growth conditions were as follows: duplicate cultures were grown to early log phase (OD_{660}=0.4) at the permissive temperature in complete or SD media at which time one set of the cells was shifted to the non-permissive temperature (38.5°C) and the second set remained at 30°C. Both sets were incubated for 24 h prior to sterol analysis.

Transformation: *S. cerevisiae* transformations were carried out with the high efficiency lithium acetate procedure previously described (10). *E. coli* transformations were carried out by standard methods.

Mutagenesis: Conditional lethal mutations were isolated following mutagenesis of plasmid pIU870 carrying the *ERG25* gene. pIU870 was transformed into *E. coli* strain XL1-Red (11), a strain which is deficient in three genes employed in DNA repair and has a mutation rate about 5,000 times higher than that in wild type strains. Following 8 h of growth in XL1-Red the plasmid DNA was isolated and transformed into *S. cerevisiae erg25*-25c. Transformants were plated at 30°C followed by replica

plating on the same medium but incubated at 38.5°C. Colonies not growing at 38.5°C were screened to make certain that the ts mutation was in the *ERG25* gene and not in the selectable *URA3* gene. The mutant strain carrying the plasmid with the ts *ERG25* (pIU908) was designated *erg25*-25C/pIU908 or ts. A control strain carrying a plasmid with the wild type *ERG25* gene (pIU870) was designated *erg25*-25C/pIU870 or wild type.

Sequencing: Sequencing was performed with the Sequenase 2.0 dideoxy-sequencing kit and [^{35}S-dATP] purchased from Amersham.

Sterol Analysis: Sterol extraction and preliminary gas chromatographic (GC) sterol analyses were carried out as described previously (7). GC separation and mass spectral (MS) analyses were done with a 5890 Hewlett Packard GC coupled to a Hewlett Packard 5972 mass selective detector. All data analyses were performed with the Hewlett Packard Chemstation software. GC separations of sterols were obtained on a J and W Scientific DB-5MS column (20 m X 0.18 mm) with a film thickness of 0.18 microns. Injections were programmed in the splitless mode with an inlet temperature of 280°C. Helium was used as the carrier gas with a linear velocity of 30 cm/sec. The oven was programmed to hold the initial temperature of 100°C for 1 min and then increased to 300°C at 10°C per min. The oven temperature was held at 300°C for an additional 15 min. The interface temperature of 280°C resulted in a detector temperature of 180°C. Mass spectra were generated in the electron impact mode at an electron energy of 70eV. The instrument was programmed to scan from 40 to 700 amu at 1 sec intervals.

RESULTS AND DISCUSSION

The *ERG25* gene from *S. cerevisiae* has been cloned and characterized in our lab (7). The Erg25p is an essential enzyme that is responsible for the first of three reactions which sequentially remove the two methyl groups from the C-4 position of the sterol intermediate, 4,4-dimethylzymosterol. In this study *S. cerevisiae* strain *erg25*-25C was transformed with a *C. albicans* genomic library (12) and two clones (pCERG25) capable of growing without ergosterol supplementation were isolated. From these two clones several subclones containing the *ERG25* gene were created. The two smallest complementing subclones, pIU870 (2.5 kb insert) and pIU873 (3.7 kb insert), were employed in this study. Restoration of ergosterol biosynthesis was used to confirm the cloning of the *ERG25* gene. Fig. 1 shows the sterol profiles, as determined by GC, of the *S. cerevisiae erg25* mutant, grown on cholesterol to maintain viability, and a *C. albicans ERG25*-complemented transformant (*erg25*/pCERG25). The *erg25* strain accumulated 4,4-dimethylzymosterol, and no ergosterol. The complemented strain accumulated both ergosterol and 4,4-dimethylzymosterol indicating only partial complementation by the *C. albicans* gene.

DNA from several *ERG25* subclones was used to sequence the *C. albicans ERG25* gene. The nucleotide base and amino acid sequences (GenBank Accession Number AF051914) of the *C. albicans ERG25* are shown in Fig. 2. The *ERG25* open reading frame is comprised of 927 bases which encode a 308 amino acid protein. Fig. 3 shows the multiple sequence alignment of the *ERG25* genes from *C. albicans*, *S. cerevisiae*, and *H. sapiens*. The shaded regions indicate where the sequences are identical among all three organisms. The CTG leucine codon at amino acid position 15 is translated as a serine in *C. albicans* (13). The *C. albicans* gene is 65% homologous at the amino acid level to the *S. cerevisiae* gene (7) and 38%

homologous to the human gene (14). As in the case of the *S. cerevisiae* and human Erg25p, the *C. albicans* Erg25p contains three histidine-rich clusters comprising the eight histidine motif $HX_{3-4}H$, $HX_{2-3}HH$, and $HX_{2-3}HH$ starting at amino acid positions 156, 173 and 258, respectively. This motif is common to over 60 iron binding, non-heme integral membrane desaturases, hydroxylases and oxidases (15), including the *S. cerevisiae* C-5 sterol desaturase (16). The *C. albicans ERG25* gene also contained a C-terminal endoplasmic reticulum retrieval signal that is also present in the *S. cerevisiae* (7) and human (14) versions of the gene.

In the process of selecting new target sites in the ergosterol pathway for the identification of new antifungals, it would be advantageous to identify reactions that are essential for viability. This has been done extensively in *S. cerevisiae* and several genes (7,8,17,18) have been identified by disruption techniques as providing sterol structural characteristics that are essential for cell viability. Repeating these determinations in *C. albicans* is more problematic due to its diploid nature, which requires two separate disruption events, and because of the inability of the organism to take-up exogenous sterol (19). Thus, the successful disruption of an essential gene would create an inviable cell with no obvious mechanism for rescue.

The isolation of conditional lethal mutations of the gene in question would provide a potential solution to this problem. The conditional lethal could be introduced into the cell on a complementing plasmid before the second allele is disrupted and the absence of the wild type gene product would occur only under non-permissive conditions. This allows for the survival of the double disruptant and the opportunity for molecular confirmation of the disruption.

A temperature-sensitive (ts) conditional lethal of the *C. albicans ERG25* was isolated following mutagenesis in *E. coli* XL1-Red (11). Table 1 shows the sterol compositions of *S cerevisiae* strains *erg25*-25C/pIU870 (wild type *ERG25* allele) and *erg25*-25C/pIU908 (ts *ERG25* allele) grown under permissive (30°C) and non-

permissive (38.5°C) growth conditions on SD medium. Cells containing the wild type allele show nearly identical sterol profiles at both temperatures. On the other hand, cells containing the ts allele produce significantly elevated levels of 4,4-dimethylzymosterol at the non-permissive growth temperature. Growth of this strain at the permissive temperature results in some elevation of C-4 methyl sterol content indicating that the temperature sensitive mutation results in diminished enzyme efficiency even at 30°C. Identical results were obtained with cells grown on complete medium. The 38.5°C grown cells were pre-grown at 30°C and shifted to 38.5°C for 24 h prior to sterol extraction. Thus, the ergosterol detected was produced during pre-growth at 30°C. The cells grew for 1-1.5 generations after the shift to 38.5°C. Cessation of growth is likely due to the increased ratio of C-4 methyl sterol to ergosterol that results from the absence of a functional *ERG25* allele.

In order to conclusively demonstrate that the ts phenotype of *erg25*-25C/pIU908 is due to a mutation in the *ERG25* gene and not defects in the plasmid created by passage through XL1-Red, the *ERG25* insert was removed from the plasmid and reinserted into an identical, but non-mutagenized, plasmid backbone. The re-constructed plasmid was then transformed into the *S. cerevisiae erg25*-25c and analyzed for sterol composition following growth at 30°C and 38.5°C. The profiles that emerged were identical to those shown for *erg25*-25C/pIU908 shown in Table 1 indicating that a mutation in the *ERG25* gene is responsible for the ts phenotype of *erg25*-25C/pIU908.

Further evidence for the nature of the ts mutation was derived from sequencing of the open reading frame of the ts allele. Fig. 4 shows the *C. albicans* wild type *ERG25* allele and the ts *ERG25* allele from base positions 837 to 857. The base at position 846 in the wild type allele has been changed from an A to a G resulting in a change from asparagine to aspartic acid at amino acid position 247.

The location of the change is between histidine clusters 2 and 3 (Fig. 3) and could involve the inability of the altered gene product to bind iron.

The data presented report the cloning and sequencing of the *C. albicans ERG25* gene encoding the C-4 sterol methyl oxidase, a non-heme, iron-binding enzyme, is similar in sequence and other characteristics to the enzymes found in *S. cerevisiae* and human. A temperature-sensitive conditional lethal mutation of the *C. albicans ERG25* can be expressed in *S. cerevisiae* indicating that plasmid-borne *ERG25* conditional lethals can be employed to rescue double disruptions of essential genes in C. *albicans*.

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References

1. White, T.C., Marr, K. A., and Bowden, R. A. (1998) Clinical, Cellular, and Molecular Factors that Contribute to Antifungal Resistance, *Clin. Microbiol. Rev. 11*, 382-402.

2. Daum, G., Lees, N.D., Bard, M., and Dickson, R. (1998) Biochemistry, Cell Biology and Molecular Biology of Lipids of *Saccharomyces cerevisiae, Yeast 14*, 1471-1510.

3. Lees, N.D., Bard, M., and Kirsch, D.R. (1997) Biochemistry and Molecular Biology of Sterol Synthesis in *Saccharomyces cerevisiae*, in *Biochemistry and Function of Sterols* (Parish, E.J., and Nes W.D. Eds.) pp. 85-99, CRC Press, Boca Raton.

4. Jensen-Perkages, K.L., Kennedy, M.A., Lees, N.D., Barbuch, R., Koegel, C., and Bard, M. (1998) Sequencing, Disruption, and Characterization of the *Candida albicans* Sterol Methyltransferase (*ERG6*) Gene: Drug Susceptibility Studies in *erg6* Mutants, *Antimicrob. Agents Chemother. 42*, 1160-1167.

5. Gaber, R.F., Copple, D.M., Kennedy, B.K., Vidal, M., and Bard, M. (1989) The Yeast Gene *ERG6* is Required for Normal Membrane Function but is not Essential for Biosynthesis of the Cell-cycle Sparking Sterol, *Mol. Cell Biol. 9*, 3447-3456.

6. Lees, N.D., Lofton, S.L., Woods, R.A., and Bard, M. (1980) The Effects of Varied Energy Source and Detergent on the Growth of Sterol Mutants of *Saccharomyces cerevisiae*, *J. Gen. Microbiol. 118*, 209-214.

7. Bard, M., Bruner, D.A., Pierson, C.A., Lees, N.D., Biermann, B., Frye, L., Koegel, C., and Barbuch, R. (1996) Cloning and Characterization of *ERG25*, the *Saccharomyces cerevisiae* Gene Encoding the C-4 Sterol Methyl Transferase, *Proc. Nat. Acad. Sci. USA* 93, 186-190.

8. Gachotte, D., Barbuch, R., Gaylor, J., Nickel, E., and Bard, M. (1998) Characterization of the *Saccharomyces cerevisiae ERG26* Gene Encoding the C-3 Sterol Dehydrogenase (C-4 Decarboxylase) Involved in Sterol Biosynthesis, *Proc. Nat. Acad. Sci. USA 95*, 13794-13799.

9. Fonzi, W.A., and Irwin, M.Y. (1993) Isogenic Strain Construction and Gene Mapping in *Candida albicans*, *Genetics 134*, 717-728.

10. Gietz, R.D., Schiestl, R.H., Willems, A. R., and Woods, R.A. (1995) Studies on the Transformation of Intact Yeast Cells by the LiAc/SS-DNA/PEG Procedure, *Yeast 11*, 355-360.

11. Greener, A., Callahan, M., and Jerpseth, B. (1996) An Efficient Random Mutagenesis Technique Using an *E. coli* Mutator Strain, *Meth. Mol. Biol. 57*, 375-385.

12. Goshorn, A.K., Grindle, S.M., and Scherer, S. (1992) Gene Isolation by Complementation in *Candida albicans* and Applications to Physical and Genetic Mapping, *Infect. Immun. 60*, 876-884.

13. Santos, M.A.S., Perreau, V.M., and Tuite, M.F. (1996) Transfer RNA Structural Change is a Key Element in the Reassignment of the CUG Codon in *Candida albicans*, *EMBO J. 15*, 5060-5068.

14. Li, L., and Kaplan, J. (1996) Characterization of Yeast Methyl Sterol Oxidase (*ERG25*) and Identification of a Human Homologue, *J. Biol. Chem. 271*, 16927-16933.

15. Shanklin, J., Achim, C., Schmidt, H., Fox, B.G., and Munck, E. (1997) Mossbauer Studies of Alkane ω-hydroxylase: Evidence for a Diiron Cluster in an Integral-membrane Protein, *Proc. Nat. Acad. Sci. USA 94*, 2981-2986.

16. Arthington, B.A., Bennett, L.G., Skatrud, P.L., Guynn, C.J., Barbuch, R.J., Ulbright, C.E., and Bard, M. (1991) Cloning, Disruption and Sequence of the Gene Encoding Yeast C-5 Desaturase, *Gene 102*, 39-44.

17. Bard, M., Lees, N.D., Turi, T., Craft, D., Coffrin, L., Barbuch, R., Koegel, C., and Loper, J.C. (1993) Sterol Synthesis and Viability of *erg11* (Cytochrome P-450 Lanosterol Demethylase) Mutations in *Saccharomyces cerevisiae* and *Candida albicans*, *Lipids 28*, 963-967.

18. Lorenz, R.T., and Parks, L.W. (1992) Cloning, Sequencing and Disruption of the Gene Encoding the C-14 Reductase in *Saccharomyces cerevisiae*, *DNA Cell Biol. 11*, 685-692.

19. Kurtz, M.B., and Marrinan, J. (1989) Isolation of *hem3* Mutants from *Candida albicans* by Sequential Gene Disruptions, *Mol. Gen. Genet. 217*, 47-52.

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   1  ttttgattcattaattgttatatttcaacatatacatattcctttattccttgatccttttttaaagtattcaatttat
  80  tatttatttgtttgtttgaagtttata ATG TCT TCC ATT AGT AAT GTT TAT CAT GAC TAT TCG AGT
                                  M   S   S   I   S   N   V   Y   H   D   Y   S   S     13

 147  TTT CTG AAT GCA ACT ACT TTT TCC CAA GTT TAT CAA AAT TTC AAT CAA TTA GAT AAT TTA
      F   S   N   A   T   T   F   S   Q   V   Y   Q   N   F   N   Q   L   D   N   L     33

 207  AAT GTT TTT GAA AAA TTA TGG GGG TCA TAT TAT TAT TAT ATG GCC AAT GAT TTA TTT GCT
      N   V   F   E   K   L   W   G   S   Y   Y   Y   Y   M   A   N   D   L   F   A     53

 267  ACT GGA TTA TTA TTT TTT TTA ACT CAT GAA ATT TTT TAT TTT GGT AGA TGT TTA CCA TGG
      T   G   L   L   F   F   L   T   H   E   I   F   Y   F   G   R   C   L   P   W     73

 327  GCT ATA ATT GAT AGA ATT CCT TAT TTT AGA AAA TGG AAA ATT CAA GAT GAA AAA ATC CCT
      A   I   I   D   R   I   P   Y   F   R   K   W   K   I   Q   D   E   K   I   P     93

 387  AGT GAT AAA GAA CAA TGG GAA TGT CTT AAA TCA GTT TTA ACA TCT CAT TTC TTA GTT GAA
      S   D   K   E   Q   W   E   C   L   K   S   V   L   T   S   H   F   L   V   E    113

 447  GCT TTC CCA ATT TGG TTT TTC CAT CCA TTA TGT CAA AAA ATT GGT ATT AGT TAT CAA GTA
      A   F   P   I   W   F   F   H   P   L   C   Q   K   I   G   I   S   Y   Q   V    133

 507  CCA TTC CCT AAA ATT ACT GAT ATG TTG ATT CAA TGG GCA GTA TTT TTT GTT TTG GAA GAT
      P   F   P   K   I   T   D   M   L   I   Q   W   A   V   F   F   V   L   E   D    153

 567  ACT TGG CAT TAT TGG TTT CAT AGA GGA TTA CAT TAT GGG GTT TTC TAT AAA TAT ATT CAT
      T   W   H   Y   W   F   H   R   G   L   H   Y   G   V   F   Y   K   Y   I   H    173

 627  AAA CAA CAT CAT AGA TAT GCT GCT CCA TTT GGA TTG GCA GCA GAA TAT GCT CAT CCA GTT
      K   Q   H   H   R   Y   A   A   P   F   G   L   A   A   E   Y   A   H   P   V    193

 687  GAA GTT GCC TTA TTA GGA TTG GGT ACG GTT GGT ATT CCG ATT GTT TGG TGT CTT ATC ACT
      E   V   A   L   L   G   L   G   T   V   G   I   P   I   V   W   C   L   I   T    213

 747  GGT AAC TTG CAT CTT TTC ACA GTT TCC ATT TGG ATC ATT TTA AGA TTA TTC CAA GCC GTT
      G   N   L   H   L   F   T   V   S   I   W   I   I   L   R   L   F   Q   A   V    233

 807  GAT GCT CAT TCC GGT TAT GAA TTC CCT TGG TCT TTA CAT AAT TTC TTG CCA TTT TGG GCT
      D   A   H   S   G   Y   E   F   P   W   S   L   H   N   F   L   P   F   W   A    253

 867  GGT GCT GAT CAT CAT GAT GAA CAT CAT CAT TAT TTC ATT GGT GGA TAC TCT TCA TCT TTT
      G   A   D   H   H   D   E   H   H   H   Y   F   I   G   G   Y   S   S   S   F    273

 927  AGA TGG TGG GAT TTC ATT TTG GAT ACC GAA GCT GGT CCA AAA GCT AAA AAG GGT AGA GAA
      R   W   W   D   F   I   L   D   T   E   A   G   P   K   A   K   K   G   R   E    293

 987  GAC AAA GTC AAA CAA AAT GTT GAA AAA TTA CAA AAG AAG AAC TTA TAG agagagaaagagtat
      D   K   V   K   Q   N   V   E   K   L   Q   K   K   N   L                      308

1049  atgtgtacaacttctcaatgtttgtaccactttcaatattaatactgtttatttttggttttatttaatatatatatc
1126  atatctattcatagtgctacat
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C. a. ERG 25    1  MSSISNVYHD--YSSFSNATTFSQVYQNFNQLD-  31
S. c. ERG 25    1  ---MSAVFNNATLSGLVQASTYSQTLQNVAHYQP  31
H. s. ERG 25    1  ------MATNESVSIFSSA---SLAVEYVDSLLP  25

C. a. ERG 25   32  NLNVFEKLWGSYYYYMAN--DLFATGLLFFLTHE  63
S. c. ERG 25   32  QLNFMEKYWAAWYSYMNN--DVLATGLMFFLLHE  63
H. s. ERG 25   26  ENPLQEPFKNAWNYMLNNYTKFQIATWGSLIVHE  59

C. a. ERG 25   64  IFYFGRCLPWAIIDRIPYFRKWKIQDEKIPSDKE  97
S. c. ERG 25   64  FMYFFRCLPWFIIDQIPYFRRWKLQPTKIPSAKE  97
H. s. ERG 25   60  ALYFLFCLPGFLFQFIPYMKKYKIQKDKPETWEN  93

C. a. ERG 25   98  QWECLKSVLTSHFLVEAFPIWFFHPLCQKIGISY 131
S. c. ERG 25   98  QLYCLKSVLLSHFLVEAIPIWTFHPMCEKLGITV 131
H. s. ERG 25   94  QWKCFKVLLFNHFCIQLPLICGTYYFTEYFNIPY 127

                                        *       *
C. a. ERG 25  132  QVPFPKITDMLIQWAVFF---VLEDTWHYWFHRG 162
S. c. ERG 25  132  EVPFPSLKTMALEIGLFF---VLEDTWHYWAHRL 162
H. s. ERG 25  128  D--WERMPRWYFLLARCFGCAVIEDTWHYFLHRL 159

                    *   **
C. a. ERG 25  163  LHYGVFYKYIHKQHHRYAAPFGLAAEYAHPVEVA 196
S. c. ERG 25  163  FHYGVFYKYIHKQHHRYAAPFGLSAEYAHPAETL 196
H. s. ERG 25  160  LHHKRIYKYIHKVHHEFQAPFGMEAEYAHPLETL 193

C. a. ERG 25  197  LLGLGTVGIPIVWCLITGNLHLFTVSIWIILRLF 230
S. c. ERG 25  197  SLGFGTVGMPILYVMYTGKLHLFTLCVWITLRLF 230
H. s. ERG 25  194  ILG---TGFFIGIVLLCD--HVILLWAWVTIRLL 222

                          *     **
C. a. ERG 25  231  QAVDAHSGYEFPWSLHNFLPFWAGADHHDEHHHY 264
S. c. ERG 25  231  QAVDSHSGYDFPWSLNKIMPFWAGAEHHDLHHHY 264
H. s. ERG 25  223  ETIDVHSGYDIPLNPLNLIPFYAGSRHHDFHHMN 256

C. a. ERG 25  265  FIGGYSSSFRWWDFILDTEAGPKAKKGREDKVKQ 298
S. c. ERG 25  265  FIGNYASSFRWWDYCLDTESGPEAKASREERMKK 298
H. s. ERG 25  257  FIGNYASTFTWWDRIFGTDSQYNAYNEKRKKFEK 290

C. a. ERG 25  299  NVEKL-QKKNL                       308
S. c. ERG 25  299  RAENNAQKKTN                       309
H. s. ERG 25  291  KTE                              293
```

Table 1. Accumulated sterols at permissive and non-permissive growth temperatures of *S. cerevisiae erg25* mutants carrying wild type (pIU870) or temperature sensitive (pIU908)[b] *C. albicans ERG25* alleles.

STEROL[a]	*erg25*-25C/pIU870		*erg25*-25C/pIU908	
	30°C	38.5°C	30°C	38.5°C
squalene	5.9	2.4	31.9	15.2
zymosterol	4.0	5.7	<1	<1
ergosterol	61.9	63.6	32.6	16.2
fecosterol	2.7	6.4	<1	<1
4-methyl fecosterol	3.6	6.7	20.6	29.4
lanosterol	1.7	2.2	<1	<1
4,4-dimethyl zymosterol	6.6	8.0	14.9	34.8
4,4-dimethyl fecosterol	-	-	-	4.4

[a] Sterols represented as percent of total sterol.

[b] cells containing the temperature sensitive *ERG25* allele were pre-grown at the permissive temperature (30°C) before being shifted to the non-permissive temperature (38.5°C) and grown for 24 h.

WT	837	TCT	TTA	CAT	AAT	TTC	TTG	CCA	857
	244	S	L	H	N	F	L	P	250
T^S	837	TCT	TTA	CAT	**G**AT	TTC	TTG	CCA	857
	244	S	L	H	**D**	F	L	P	250

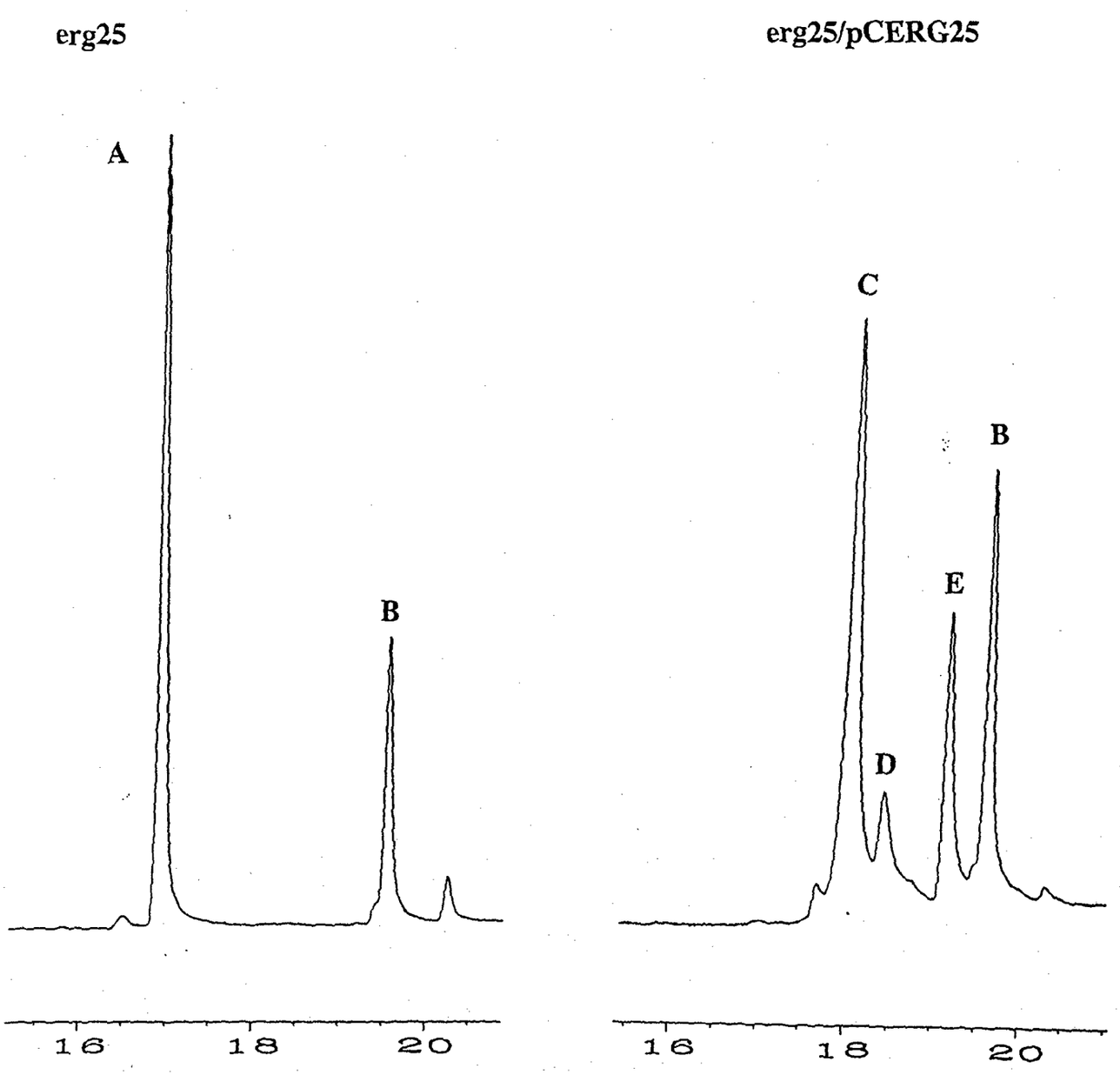
Figure Legends:

Figure 1. Sterol profiles of *Saccharomyces cerevisiae* strain *erg25-25C* (left panel) and the same strain containing the *Candida albicans ERG25* on plasmid pIU873 (*erg25*/pCERG25). Sterol extraction was carried out as described previously [5]. Peak A: cholesterol; peak B: 4,4-dimethyl zymosterol; peak C: ergosterol; peak D: fecosterol; peak E: 4-methyl fecosterol.

Figure 2. The base and amino acid sequences of the *ERG25* gene from *C. albicans*.

Figure 3. The multiple sequence alignment for the *ERG25* genes from *C. albicans* (C. a.), *S. cerevisiae* (S. c.), and *H. sapiens* (H. s.). Shaded boxes indicate regions of amino acid homology among all three species. Histidines in the three histidine clusters are designated by an * at each position.

Figure 4. Base and amino acid sequences of the region surrounding the ts mutation in the *C. albicans ERG25* gene.


erg25
erg25/pCERG25
A
B
C
D
E
B
16
18
20
16
18
20
Retention Times

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March 3, 1998

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VIA FEDERAL EXPRESS

Re: SLW # 00740.003US1
DNA ENCODING STEROL METHYLTRANSFERASE

Dear Martin:

Enclosed please find a copy of the revised draft of the above-mentioned specification for your review.

Please contact Janet Embretson at (612) 373-6959 at your earliest convenience to discuss this draft.

With kind regards,

Gregory J. Hanson
Paralegal

Enclosure

DNA ENCODING STEROL METHYLTRANSFERASE

Statement of Government Rights

This invention was made with a grant from the Government of the United States of America (Grant No. DAMD17-95-1-5067). The Government may have certain rights in the invention.

Background of the Invention

The frequency of occurrence of human fungal infections has been increasing over the past decade in response to a combination of factors (Georgopapadakou et al., 1994). These factors include advances in invasive surgical techniques which allow for opportunistic pathogen access, the administration of immunosuppressive agents employed in transplantation, and an increase in the number of immunosuppressed patients resulting from chemotherapy and disease states such as AIDS. The threat to human health is further compounded by the increased frequency with which resistance to the commonly employed antifungal agents is occurring.

Currently, the most common antifungals include the polyenes and the azoles. The polyenes bind to ergosterol, the fungal membrane sterol, and induce lethal cell leakage (Brajtburg et al., 1990). However, polyenes often have negative side effects and resistance to polyenes has been reported (Hebeka et al., 1965; Powderley et al., 1988). The azoles are fungistatic agents that inhibit the cytochrome P450-mediated removal of the C-14 methyl group from the ergosterol precursor, lanosterol (Vanden Bossche et al., 1987). Resistance to azoles has been reported in *Candida albicans* (Clark et al., 1996; Sanglard et al., 1996; Sanglard et al., 1995; White, 1997a; White, 1997b) as well as in other species of *Candida* (Moran et al., 1997; Parkinson et al., 1995), and in other fungal pathogens, including species of *Histoplasma* (Wheat et al., 1997), *Cryptococcus* (Lamb et al., 1997; Venkateswarlu al., 1997), and *Aspergillus* (Denning et al., 1997).

The pathway for fungal sterol biosynthesis is one target for antifungal development. In particular, fungal genes that catalyze a step in sterol biosynthesis that is not found in cholesterol biosynthesis (Pinto et al., 1983) are of interest in this regard. One such fungal gene is the sterol methyltransferase gene *(ERG6)*. Non-recombinant *Saccharomyces cerevisiae erg6* mutants have been available for some time (Molzhan et al., 1972). The *S. cerevisiae ERG6* gene has been isolated, and recombinant strains prepared (i.e., via genetic engineering) in which the gene has been disrupted (Gaber et al., 1989). Although the absence of the *ERG6* gene product in *S. cerevisiae* was not lethal, it did result in several severely compromised phenotypes (Bard et al., 1978; Kleinhans et al., 1979; Lees et al., 1979; Lees et al., 1980).

S. cerevisiae erg6 mutants have been shown to have diminished growth rates as well as limitations on utilizable energy sources (Lees et al., 1980), reduced mating frequency (Gaber et al., 1989), altered membrane structural features (Kleinhans et al., 1979; Lees et al., 1979), and low transformation rates (Gaber et al., 1989). In addition, several lines of evidence have indicated that *S. cerevisiae erg6* mutants have severely altered permeability characteristics. This has been demonstrated using dyes (Bard et al., 1978), cations (Bard et al., 1978), and spin labels used in electron paramagnetic resonance studies (Kleinhans et al., 1979). These early observations have been corroborated recently by the cloning of the *S. cerevisiae LIS1* gene (Welihinda et al., 1994), mutants of which were selected on the basis of hypersensitivity to sodium and lithium. Sequencing of *LIS1* has indicated identity to *ERG6*. In addition, studies using the Golgi inhibitor, brefeldin A, have routinely employed *erg6* mutants because of their remarkably increased permeability to the compound (Vogel et al., 1993). However, as *S. cerevisiae* and *Candida albicans* differ in their ability to survive and grow on various sterol intermediates, and as *S. cerevisiae* is rarely the cause of a human disease, it was unknown whether the *ERG6* gene in the common fungal pathogen, *C. albicans*, effected similar properties.

Thus, a continuing need exists for fungal genes and strains that can aid in the identification of agents that increase the susceptibility of pathogenic fungi to conventional anti-fungal or anti-metabolic agents.

Summary of the Invention

The present invention provides an isolated nucleic acid segment comprising a nucleic acid sequence encoding a *Candida albicans* sterol methyltransferase (ERG6), a biologically active variant or subunit thereof. As described hereinbelow, the *Candida albicans ERG6* gene was isolated by complementation of a *Saccharomyces cerevisiae erg6* mutant using a *Candida albicans* genomic library. Preferably, the sterol methyltransferase of the invention comprises SEQ ID NO:2, which is encoded by a DNA having SEQ ID NO:1. Thus, the invention further provides isolated, purified recombinant *Candida albicans* sterol methyltransferase, a biologically active variant or subunit thereof, e.g., a polypeptide having SEQ ID NO:2. Methods to isolate and purify sterol methyltransferase are known to the art (see, for example, Ator et al., 1989).

ERG6 can be used in a method to identify antifungals targeted specifically to sterol methyltransferase. Therefore, the invention also provides a method to identify inhibitors of fungal sterol methyltransferase. The method comprises contacting an amount of isolated, purified recombinant *Candida albicans* sterol methyltransferase, or a biologically active variant or subunit thereof, with an amount of an agent. The activity of the sterol methyltransferase in the presence of the agent is then determined or detected relative to an amount of sterol methyltransferase not contacted with the agent.

The isolation and characterization of a *Candida albicans* ERG6 gene also permits the preparation of recombinant *Candida albicans* isolates that lack a functional sterol methyltransferase, e.g., isolates which have decreased or reduced amounts of sterol methyltransferase, or lack sterol methyltransferase activity. Inhibiting the functional *ERG6* gene product may make the cell hypersensitive to exogenous compounds, and thus could increase the effectiveness of new or existing

antifungals. Thus, as described hereinbelow, the first copy of the *Candida ERG6* gene was disrupted by transforming a wild type isolate with the ura blaster system. The second copy of the *Candida ERG6* gene was disrupted by ura blaster transformation or mitotic recombination. The resulting *erg6* strains were shown to be more susceptible to a number of sterol synthesis and metabolic inhibitors including terbinafine, tridemorph, fenpropiomorph, fluphenazine, cycloheximide, cerulenin and brefeldin A, relative to the corresponding isolate of *Candida albicans* which encodes a functional sterol methyltransferase. No increase in susceptibility to azoles was noted.

Therefore, recombinant *Candida albicans* isolates lacking, or having reduced, sterol methyltransferase activity are useful in a method to identify antifungal agents that would otherwise have low or no ability to permeate the fungal cell membrane and thus may be overlooked as therapeutic agents. Moreover, the administration of inhibitors of the *ERG6* gene product to a host organism having a fungal infection may make the fungal cell increasingly susceptible to antifungals or other agents which normally would be excluded, e.g., due to their lack of ability to permeate the cell, and may permit clinical treatment at lower dosages. Hence, the invention provides a method to enhance the efficacy of an agent such as an antifungal agent, comprising: administering to a mammal having, or at risk of having, a fungal infection, an amount of agent that inhibits *Candida albicans* sterol methyltransferase and an amount of an anti-fungal agent effective to inhibit or treat the infection. Preferably, the sterol methyltransferase inhibitor is administered in an amount that reduces or decreases the effective amount of the anti-fungal agent administered relative to the effective amount of the anti-fungal agent administered in the absence of the inhibitor.

Also provided is a method to identify inhibitors of fungal sterol methyltransferase. The method comprises contacting an isolate of a fungus, e.g., *Candida albicans*, with an amount of an agent, wherein the genome of the isolate has two functional sterol methyltransferase genes which are expressed so as to yield wild type levels of sterol methyltransferase. Then it is determined whether the agent

inhibits the growth of the isolate or reduces the amount or activity of the sterol methyltransferase of the isolate, relative to a corresponding isolate having and expressing more than two copies of a functional sterol methyltransferase.

Brief Description of the Figures

Figure 1. Codons for specified amino acids.

Figure 2. Exemplary and preferred substitutions for variant *C. albicans* sterol methyltransferase (*ERG6*) polypeptide.

Figure 3. UV scan of non-saponifiable sterols in which *erg6* sterols containing a conjugated double bond in the sterol side chain show absorption maxima at 230 and 238 nm. Wild type strains containing the *Candida ERG6* gene do not have the conjugated double bond system in the sterol side chain.

Figure 4. A schematic representation of a *Candida albicans ERG6* genomic clone (pCERG6-20) and three complementing subclones, pIU880, pIU882, and pIU885. Selected restriction endonuclease sites are shown. A subclone in which a 0.7 kb HindIII fragment within pIU885 was deleted, the cohesive ends filled in, and BamHI linkers (pIU886-L) added is shown. The insertion of the ura blaster into the BamHI site of pIU886-L resulted in pIU887-A.

Figure 5. The DNA sequence (SEQ ID NO:1) and corresponding inferred amino acid sequence (SEQ ID NO:2) of a *Candida albicans ERG6* gene. The underlined region indicates the S-adenosyl-methionine binding site.

Figure 6. Alignment of the amino acid sequences of the sterol methyltransferases from *Candida albicans* (C. a.; SEQ ID NO:2), *Saccharomyces cerevisiae* (S. c.; SEQ ID NO:3), *Arabidopsis thaliana* (A. t.; SEQ ID NO:4), and *Triticum ativum* (T a.; SEQ ID NO:5).

Figure 7. Gas chromatography of the sterols of the wild type and an *erg6* strain of *Candida albicans*. Peak A; zymosterol, Peak B; ergosterol, Peak C; fecosterol, Peak D; cholesta-5,7,24-trien-3β-ol, Peak E; cholesta-7,24-dien-3β-ol, Peak F; cholesta-5,7,22,24tetraen-3β-ol.

Figure 8. (A). A schematic representation of the ura blaster disruption of the *ERG6* gene. The location and orientation of PCR primers is shown ("P2", "P3", and "P4"). (B). Agarose gel electrophoretic confirmation of heterozygote and homozygote disruptants of the *ERG6* gene. CAI4 (wild type), lanes 1 and 2; CAI4-6-5 (heterozygote), lanes 3 and 4; 5AB-15 (homozygote derived from ura blaster transformation followed by mitotic recombination), lanes 5 and 6; HO11-A3 (homozygote derived from two rounds of ura blaster transformation), lanes 7 and 8. The image was captured on disc and the photograph generated using Photoshop on MacIntosh.

Figure 9. Growth responses of wild type (CAI4), homozygous *erg6* derived from ura blaster transformation (5AB-15) and homozygous *erg6* derived from mitotic recombination (HO11-A3) in the presence of sterol biosynthesis inhibitors and metabolic inhibitors. Cells were grown at 37°C to a density of 1 x 10^7 cells/ml and 5 µl inoculated at 10^0, 10^{-1}, and 10^{-2} dilutions. The image was captured on disc and the photograph generated using Photoshop on MacIntosh.

Detailed Description of the Invention

Definitions

As used herein, a "variant" of a *C. albicans* sterol methyltransferase is a polypeptide that has at least about 70%, preferably at least about 80%, and more preferably at least about 90%, but less than 100%, contiguous amino acid sequence homology or identity to the amino acid sequence of the corresponding wild type *C. albicans* sterol methyltransferase polypeptide, e.g., SEQ ID NO:2. Thus, a variant sterol methyltransferase polypeptide of the invention may include amino acid residues not present in the corresponding wild type sterol methyltransferase polypeptide, and may include amino and/or carboxy-terminal or internal deletions or insertions relative to the corresponding wild type polypeptide. Variants of the invention include polypeptides having at least one D-amino acid. Preferably, the variant polypeptides of the invention are biologically active. A "biologically active" sterol methyltransferase of the invention has at least about 1%, more preferably at

least about 10%, and more preferably at least about 50%, of the activity of the sterol methyltransferase having SEQ ID NO:2. Methods to determine the biological activity of sterol methyltransferase are well known to the art (see, for example, Ator et al., 1989).

Sterol methyltransferase polypeptides, variants or subunits thereof which are subjected to chemical modifications, such as esterification, amidation, reduction, protection and the like, are referred to as "derivatives."

As used herein, a "susceptible" isolate means that the isolate has decreased growth in the presence of a particular agent relative to the growth of the isolate in the absence of the agent.

A "recombinant" isolate of the invention is a strain or isolate of *C. albicans* that has been manipulated *in vitro* so as to alter, e.g., decrease or disrupt, the function or activity of the endogenous sterol methyltransferase. A "recombinant" isolate of the invention also includes a strain or isolate of *C. albicans* that has been manipulated *in vitro* so as to increase the amount or activity of sterol methyltransferase present in that isolate or strain.

As used herein, the terms "isolated and/or purified" refer to *in vitro* preparation, isolation and/or purification of a nucleic acid molecule or polypeptide of the invention, so that it is not associated with *in vivo* substances.

A "variant" nucleic acid molecule of the invention is a molecule that has at least about 70%, preferably about 80%, and more preferably at least about 90%, but less than 100%, contiguous nucleotide sequence homology or identity to the nucleotide sequence corresponding to a wild type *C. albicans* sterol methyltransferase gene, e.g., SEQ ID NO:1. A variant sterol methyltransferase gene of the invention may include nucleotide bases not present in the corresponding wild type gene, e.g., 5', 3' or internal deletions or insertions such as the insertion of restriction endonuclease recognition sites, relative to the wild type gene.

A. Nucleic Acid Molecules of the Invention

1. Sources of the Nucleic Acid Molecules of the Invention

Sources of nucleotide sequences from which the present nucleic acid molecules encoding a *C. albicans* sterol methyltransferase, a subunit or a variant thereof, or the nucleic acid complement thereof, include total or polyA$^+$ RNA from any isolate of *C. albicans* from which cDNAs can be derived by methods known in the art. Other sources of the nucleic acid molecules of the invention include genomic libraries derived from any *C. albicans* strain or isolate. Moreover, the present nucleic acid molecules may be prepared *in vitro*, or by subcloning at least a portion of a DNA segment that encodes a particular *C. albicans* sterol methyltransferase polypeptide.

2. Isolation of a Gene Encoding *C. albicans* Sterol Methyltransferase

A nucleic acid molecule encoding a *C. albicans* sterol methyltransferase polypeptide can be identified and isolated using standard methods, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). For example, reverse-transcriptase PCR (RT-PCR) can be employed to isolate and clone *C. albicans* sterol methyltransferase cDNAs. Oligo-dT can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from isolated RNA which contains RNA sequences of interest, e.g., total RNA isolated from human tissue. RNA can be isolated by methods known to the art, e.g., using TRIZOL™ reagent (GIBCO-BRL/Life Technologies, Gaithersburg, MD). Resultant first-strand cDNAs are then amplified in PCR reactions.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers comprising at least 7-8 nucleotides. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences, specific DNA sequences from

total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, and the like. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51, 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). Thus, PCR-based cloning approaches rely upon conserved sequences deduced from alignments of related gene or polypeptide sequences.

Primers are made to correspond to relatively highly conserved regions of polypeptides or nucleotide sequences which were identified and compared to generate the primers, e.g., by a sequence comparison of non-*C. albicans* sterol methyltransferase genes. One primer is prepared which is predicted to anneal to the antisense strand, and another primer prepared which is predicted to anneal to the sense strand, of a nucleic acid molecule which encodes a *C. albicans* sterol methyltransferase.

The products of each PCR reaction are separated via an agarose gel and all consistently amplified products are gel-purified and cloned directly into a suitable vector, such as a known plasmid vector. The resultant plasmids are subjected to restriction endonuclease and dideoxy sequencing of double-stranded plasmid DNAs.

Another approach to identify, isolate and clone DNAs which encode *C. albicans* sterol methyltransferase is to screen a cDNA or genomic *C. albicans* DNA library. Screening for DNA fragments that encode all or a portion of a DNA encoding *C. albicans* sterol methyltransferase can be accomplished by screening the library with a probe which has sequences that are highly conserved between genes believed to be related to *C. albicans* sterol methyltransferase, e.g., the homolog of *C. albicans* sterol methyltransferase from a different species, or by screening of plaques for binding to antibodies that recognize sterol methyltransferase. DNA fragments that bind to a probe having sequences which are related to *C. albicans* sterol methyltransferase, or which are immunoreactive with antibodies to *C. albicans* sterol methyltransferase, can be subcloned into a suitable vector and sequenced and/or used as probes to identify other DNAs encoding all or a portion of *C. albicans* sterol methyltransferase.

Yet another method to identify and isolate a DNA encoding a *C. albicans* sterol methyltransferase is to employ a complementation assay. Thus, *erg6* mutants are transformed with either genomic DNA or cDNA of *C. albicans* and clones are identified that complement the *erg6* mutation and/or are cycloheximide resistant, as described below.

As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid molecule or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide, so that it can be sequenced, replicated, and/or expressed. For example, "isolated *C. albicans* sterol methyltransferase nucleic acid" is RNA or DNA containing greater than 7, preferably 15, and more preferably 25 or more, sequential nucleotide bases that encode at least a portion of *C. albicans* sterol methyltransferase, or a variant thereof, or a RNA or DNA complementary thereto, or that is complementary or hybridizes, respectively, to RNA or DNA encoding *C. albicans* sterol methyltransferase or a variant thereof and remains stably bound under stringent conditions, as defined by methods well known in the art, e.g., in Sambrook et al., *supra*. Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell.

An example of isolated *C. albicans* sterol methyltransferase nucleic acid is RNA or DNA that encodes *C. albicans* sterol methyltransferase and wherein the methyltransferase shares at least about 70%, preferably at least about 80%, and more preferably at least about 90%, sequence identity with at least a portion of the C. *albicans* sterol methyltransferase polypeptide having SEQ ID NO:2, e.g., a DNA molecule corresponding to SEQ ID NO:1.

As used herein, the term "recombinant nucleic acid" or "preselected nucleic acid," e.g., "recombinant DNA sequence or segment" or "preselected DNA sequence or segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate cellular source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been transformed with exogenous DNA. An example of preselected DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

Thus, recovery or isolation of a given fragment of DNA from a restriction digest can employ separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn et al., Nucleic Acids Res., 9, 6103 (1981), and Goeddel et al., Nucleic Acids Res., 8, 4057 (1980). Therefore, "preselected DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

As used herein, the term "derived" with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular nucleic acid molecule.

3. Variants of the Nucleic Acid Molecules of the Invention

Nucleic acid molecules encoding amino acid sequence variants of *C. albicans* sterol methyltransferase are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of *C. albicans* sterol methyltransferase.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing truncated forms and amino acid substitution variants of *C. albicans* sterol methyltransferase polypeptide, including variants that are truncated. This technique is well known in the art as described by Adelman et al., DNA, 2, 183 (1983). Briefly, *C. albicans* sterol methyltransferase DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of *C. albicans* sterol methyltransferase. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the *C. albicans* sterol methyltransferase DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al., Proc. Natl. Acad. Sci. U.S.A., 75, 5765 (1978).

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage

origin of replication as described by Viera et al., Meth. Enzymol., 153, 3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, N.Y. 1989). Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the *C. albicans* sterol methyltransferase, and the other strand (the original template) encodes the native, unaltered sequence of *C. albicans* sterol methyltransferase. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for polypeptide production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified so that a homoduplex molecule is created wherein both strands of the plasmid contain the mutations(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine triphosphate (dATP), deoxyriboguanosine triphosphate (dGTP), and deoxyribothymidine triphosphate (dTTP), is combined with a modified thiodeoxyribocytosine triphosphate called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture

is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(αS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101.

For example, a preferred embodiment of the invention is an isolated and purified DNA molecule comprising a preselected DNA segment encoding *C. albicans* sterol methyltransferase having SEQ ID NO:2, wherein the DNA segment comprises SEQ ID NO:1, or variants of SEQ ID NO:1, having nucleotide substitutions which are "silent" (see Figure 1). That is, when silent nucleotide substitutions are present in a codon, the same amino acid is encoded by the codon with the nucleotide substitution as is encoded by the codon without the substitution. For example, valine is encoded by the codon GTT, GTC, GTA and GTG. A variant of SEQ ID NO:2 at the fourth codon in the polypeptide (GTT in SEQ ID NO:1) includes the substitution of GTC, GTA or GTG for GTT. Other "silent" nucleotide substitutions in SEQ ID NO:1 which can encode *C. albicans* sterol methyltransferase having SEQ ID NO:2 can be ascertained by reference to Figure 1 and page D1 in Appendix D in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989). Nucleotide substitutions can be introduced into DNA segments by methods well known to the art, see, for example, Sambrook et al., *supra*. Moreover, the nucleic acid molecules of the invention may be modified in a similar manner so as to result in *C. albicans* sterol methyltransferase polypeptides that have deletions,

for example, the polypeptides are truncated at the C-terminus of *C. albicans* sterol methyltransferase. Such deletions can be accomplished by introducing a stop codon, i.e., UAA, UAG, or UGA, in place of a codon for an amino acid.

4. Chimeric Expression Cassettes and Host Cells Transformed Therewith

To prepare expression cassettes for transformation herein, the recombinant or preselected DNA sequence or segment may be circular or linear, double-stranded or single-stranded. A preselected DNA sequence which encodes an RNA sequence that is substantially complementary to a mRNA sequence encoding a sterol methyltransferase is typically a "sense" DNA sequence cloned into a cassette in the opposite orientation (i.e., 3′ to 5′ rather than 5′ to 3′). Generally, the preselected DNA sequence or segment is in the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by control sequences which promote the expression of the preselected DNA present in the resultant cell line.

As used herein, "chimeric" means that a vector comprises DNA from at least two different species, or comprises DNA from the same species, which is linked or associated in a manner which does not occur in the "native" or wild type of the species.

Aside from preselected DNA sequences that serve as transcription units for a sterol methyltransferase, or portions thereof, a portion of the preselected DNA may be untranscribed, serving a regulatory or a structural function. For example, the preselected DNA may itself comprise a promoter that is active in mammalian cells, or may utilize a promoter already present in the genome that is the transformation target. For mammalian cells, such promoters include the CMV promoter, as well as the SV40 late promoter and retroviral LTRs (long terminal repeat elements), although many other promoter elements well known to the art may be employed in the practice of the invention.

Other elements functional in the host cells, such as introns, enhancers, polyadenylation sequences and the like, may also be a part of the preselected DNA. Such elements may or may not be necessary for the function of the DNA, but may

provide improved expression of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the cell.

"Control sequences" is defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" is defined to mean that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a peptide or polypeptide if it is expressed as a preprotein that participates in the secretion of the peptide or polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

The preselected DNA to be introduced into the cells further will generally contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of transformed cells from the population of cells sought to be transformed. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide-resistance genes, such as *neo, hpt,*

dhfr, bar, aroA, dapA and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Preferred genes include the chloramphenicol acetyl transferase gene (cat) from Tn9 of *E. coli*, the beta-glucuronidase gene (gus) of the *uidA* locus of *E. coli*, and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

The general methods for constructing recombinant DNA which can transform target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein. For example, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2d ed., 1989), provides suitable methods of construction.

The recombinant DNA can be readily introduced into the host cells, e.g., mammalian, bacterial or insect cells, by transfection with an expression vector comprising DNA encoding a sterol methyltransferase or its complement, by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a transformed cell having the recombinant DNA stably integrated into its genome or having the recombinant DNA stably maintained as an extrachromosomal element (e.g., a plasmid), so that the DNA molecules, sequences, or segments, of the present invention are expressed by the host cell.

Physical methods to introduce a preselected DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. The main advantage

of physical methods is that they are not associated with pathological or oncogenic processes of viruses. However, they are less precise, often resulting in multiple copy insertions, random integration, disruption of foreign and endogenous gene sequences, and unpredictable expression. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. For insect cells, baculovirus vectors are generally employed to introduce foreign genes to those cells.

As used herein, the term "cell line" or "host cell" is intended to refer to well-characterized homogenous, biologically pure populations of cells. These cells may be eukaryotic cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art, as well as primary cells, or prokaryotic cells. The cell line or host cell is preferably of mammalian, bacterial or insect origin, but other cell lines or host cells may be employed, including plant, yeast or fungal cell sources.

"Transfected" or "transformed" is used herein to include any host cell or cell line, the genome of which has been altered or augmented by the presence of at least one preselected DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered," "non-native," or "foreign DNA," wherein said DNA was isolated and introduced into the genome of the host cell or cell line by the process of genetic engineering. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence. Preferably, the transfected DNA is a chromosomally integrated recombinant DNA sequence, which comprises a gene encoding the sterol methyltransferase or its complement, which host cell may or may not express significant levels of autologous or "native" sterol methyltransferase.

To confirm the presence of the preselected DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular

biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; and/or "biochemical" assays, such as detecting the presence or absence of sterol methyltransferase, e.g., by immunological means (ELISAs and Western blots) or enzymatic assays.

To detect and quantitate RNA produced from introduced preselected DNA segments, RT-PCR may be employed. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique demonstrates the presence of an RNA species and gives information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the preselected DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the protein product of the introduced preselected DNA sequence or evaluating the phenotypic changes brought about by the expression of the introduced preselected DNA segment in the host cell.

B. Polypeptides, Variants, and Derivatives Thereof of the Invention

The present isolated, purified *C. albicans* sterol methyltransferase polypeptides, variants or derivatives thereof, can be synthesized *in vitro*, e.g., by the solid phase peptide synthetic method or by recombinant DNA approaches which are well known to the art. The solid phase peptide synthetic method is an established and widely used method, which is described in the following references: Stewart et al., Solid Phase Peptide Synthesis, W. H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., 85 2149 (1963); Meienhofer in "Hormonal Proteins

and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; and Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer, Vol. 2 (Academic Press, 1980) pp. 3-285. These peptides or polypeptides can be further purified by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; or ligand affinity chromatography.

Purification of recombinant *C. albicans* sterol methyltransferase from the culture medium or the intact cells, if desired, may be achieved by conventional purification means such as ammonium sulfate precipitation, column chromatography, and the like, and fractions containing the *C. albicans* sterol methyltransferase polypeptide can be identified by, for example, enzymatic activity or Western blot analysis. Purification of a *C. albicans* sterol methyltransferase may be accomplished by methods such as those disclosed in Ator et al., 1989 and Ator et al., 1992.

Once isolated and characterized, derivatives, e.g., chemically derived derivatives, of a given *C. albicans* sterol methyltransferase polypeptide can be readily prepared. For example, amides of *C. albicans* sterol methyltransferase polypeptide or variants thereof may be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the polypeptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

Salts of carboxyl groups of a polypeptide or polypeptide variant of the invention may be prepared in the usual manner by contacting the polypeptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

N-acyl derivatives of an amino group of the *C. albicans* sterol methyltransferase polypeptide or polypeptide variants may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected polypeptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy polypeptide or polypeptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired.

Formyl-methionine, pyroglutamine and trimethyl-alanine may be substituted at the N-terminal residue of the polypeptide or polypeptide variant. Other amino-terminal modifications include aminooxypentane modifications (see Simmons et al., Science, 276, 276 (1997)).

In addition, the amino acid sequence of *C. albicans* sterol methyltransferase can be modified so as to result in a *C. albicans* sterol methyltransferase variant. The modification includes the substitution of at least one amino acid residue in the polypeptide for another amino acid residue, including substitutions which utilize the D rather than L form, as well as other well known amino acid analogs. These analogs include phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, gamma-carboxyglutamate; hippuric acid, octahydroindole-2-carboxylic acid, statine, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, penicillamine, ornithine, citrulline, α-methyl-alanine, para-benzoyl-phenylalanine, phenylglycine, propargylglycine, sarcosine, and tert-butylglycine.

One or more of the residues of the polypeptide can be altered, preferably so long as the polypeptide variant is biologically active. For example, for *C. albicans* sterol methyltransferase variants, it is preferred that the variant has a similar, if not greater, biological activity than that of the corresponding non-variant wild type polypeptide, e.g., a polypeptide having SEQ ID NO:2. Conservative amino acid substitutions are preferred--that is, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine,

methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

Conservative substitutions are shown in Figure 2 under the heading of exemplary substitutions. More preferred substitutions are under the heading of preferred substitutions. After the substitutions are introduced, the variants are screened for biological activity.

Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic; trp, tyr, phe.

The invention also envisions polypeptide variants with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes described above for another.

Acid addition salts of the polypeptide or variant polypeptide or of amino residues of the polypeptide or variant polypeptide may be prepared by contacting the polypeptide or amine with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the polypeptides may also be prepared by any of the usual methods known in the art.

The invention will be further described by the following example.

Example I

Materials and Methods

Strains and plasmids. *C. albicans* CAI4 *(Δura3::imm434/Δura3::imm434)* (Fonzi et al., 1993) was used for disruption of both copies of *ERG6*. *S. cerevisiae erg6* deletion strain BKY48-5C (α *leu2-3 ura3-52 erg6Δ::LEU2)* was used as the recipient strain for transformation with the *Candida* genomic library (Goshorn et al., 1992). *E. coli* DH5α was used as the host strain for all plasmid constructions. Plasmid pRS316 was obtained from P. Heiter (Sikorski et al., 1989) and Bluescript plasmid was obtained from Stratagene (La Jolla, California).

Media. CAI4 was grown on YPD complete medium containing 1% yeast extract (Difco), 2% Bacto-peptone (Difco) and 2% glucose. Complete synthetic medium (CSM) was used for transformation experiments and contained 0.67% yeast nitrogen base (Difco), 2% glucose and 0.8 g/l of a mixture of amino acids plus adenine and uracil (Bio101). CSM "drop out" media contained the above without uracil. Uridine was added at 80 mg/l. CSM media containing uridine and 5-fluoroorotic acid (5-FOA) at 1 g/l was used to regenerate the *ura3* genetic marker as outlined by Fonzi et al. (1993). All experiments were carried out at 30°C unless otherwise indicated.

Cloning of *ERG6*. Transformation of *S. cerevisiae* strain BKY48-5C using the *Candida* gene library was carried out according to a lithium acetate modified protocol developed by Gaber et al. (1989) for *erg6* transformations. The *C. albicans ERG6* gene was cloned by transforming a *S. cerevisiae erg6* deletion strain (BKY485C) with a *Candida* genomic DNA library obtained from Dr. Stew Scherer (University of Minnesota) (Goshorn et al., 1992). The library contained Sau3A fragments of *Candida* genomic DNA ligated into the BamHI site of the 9.6 kb *Candida-Saccharomyces* shuttle vector, p1041. p1041 contains the *C. albicans URA3* gene as a selectable marker and two origins of replication. One origin of replication is a portion of 2 μ DNA that is required for plasmid replication in *Saccharomyces* and the other origin is a CARS sequence *(Candida* autonomously replicating sequence) that is required for replication in *Candida.* Additionally, this vector contains DNA sequences so as to permit plasmid replication in *E. coli.* Transformants containing *Candida ERG6* DNA were subcloned into the

Saccharomyces vector, pRS316, for complementation analyses and DNA sequencing. All *Candida* transformations for disruption experiments were carried out essentially according to procedures of Sanglard et al. (1996). Plasmid p5921, obtained from Fonzi et al. (1993), was the source of the ura blaster for *Candida* *ERG6* disruption experiments.

Approximately 1250 transformants were obtained after plating potential transformants on a uracil dropout medium which ensures the presence of the plasmid. These transformants were then screened on 0.06 μg/ml cycloheximide. *S. cerevisiae erg6* strains are nystatin resistant and cycloheximide sensitive. Transformants that were resistant to this level of cycloheximide (cyh^r) were further tested for the presence of intracellular ergosterol. Sterols extracted from *S. cerevisiae erg6* and the transformants were analyzed by UV and GC/MS to confirm the sterol phenotypes.

DNA sequencing of the *Candida ERG6* gene. The plasmid insert containing the *ERG6* gene was sequenced in both directions using the Sanger dideoxy chain termination method. Initially T3 and T7 primers were used and as DNA sequence became available, primers were generated from sequenced DNA.

PCR. PCR analyses were used to verify disruptions of both *Candida ERG6* genes. Primers P1, P2, and P3 were used to distinguish *ERG6* disrupted genes on the basis of size. The primers correspond to *ERG6* sequences. Primer 4 corresponds to sequences in the *hisG* region of the ura blaster. P1 has the sequence 5′-CACATGGGTGAAATTAG-3′ (SEQ ID NO:6); P2 corresponds to 5′-CTCCAGTTCAATTAGCAG-3′ (SEQ ID NO:7); P3 is 5′-TGTGCGTGTACAAAGCAC-3′ (SEQ ID NO:8); and P4 is 5′-GATAATACCGAGATCGAC-3′ (SEQ ID NO:9). PCR buffers and Taq polymerase were obtained from Promega. Buffer composition was 10 mM Tris-HCl (pH 9) and 2 mM $MgCl_2$ and reactions mixtures contained 0.2 mM dNTPs and 0.5 U of polymerase. Conditions for amplification were as follows: the first cycle of denaturation at 94°C was for 5 minutes followed by 40 cycles of annealing at 50°C for 2 minutes, elongation at 72°C for 3 minutes, and denaturation at 94°C for 1

minute. A final elongation step was performed at 72°C for 20 minutes. Protocols for preparation of the *Candida* template DNA is described in Ausubel et al. (1995).

Sterol analyses. Sterols were isolated as non-saponifiables as described previously (Molzhan et al., 1972). UV analysis of sterols in extracts was accomplished by scanning from 200 to 300 nm using a Beckman DU 640 spectrophotometer. GC analyses of non-saponifiables were conducted on a HP5890 series II equipped with the HP chemstation software package. The capillary column (HP-5) was 15 m x 0.25 mm x 0.25 mm film thickness and was programmed from 195°C to 300°C (three minutes at 195°C, then an increase at 5.5°C/minute until the final temperature of 300°C was reached and held for 4 minutes). The linear velocity was 30 cm/sec using nitrogen as the carrier gas and all injections were run in the splitless mode. GC/MS analyses were done using a Varian 3400 gas chromatograph interfaced to a Finnigan MAT SSQ 7000 mass spectrometer. The GC separations were done on a fused silica column, DB-5, 15 m x 0.32 mm x 0.25 mm film thickness programmed from 50°C to 250°C at 20°C/minute after a 1 minute hold at 50°C. The oven temperature was then held at 250°C for 10 minutes before programming the temperature to 300°C at an increase of 20°C/minute. Helium was the carrier gas with a linear velocity of 50 cm/second in the splitless mode. The mass spectrometer was in the electron impact ionization mode at an electron energy of 70 eV, an ion source temperature of 150°C, and scanning from 40 to 650 atomic mass units at 0.5 second intervals.

Drug susceptibility testing in *C. albicans*. Drug susceptibilities of *C. albicans* wild type and *erg6* strains were conducted using cells harvested from overnight YPD plates grown at 37°C. Cells were suspended in YPD to a concentration of 1×10^7 (OD_{660} of 0.5) cells per ml. Cells were plated by transferring 5 µl of the original suspension (10^0) plus 10^{-1} and 10^{-2} dilutions to YPD plates containing the drug to be tested. The plates were incubated for 48 hours at 37°C and observed for growth.

Clotrimazole, ketoconazole, brefeldin A, cerulenin, cycloheximide, nystatin, and fluphenazine were obtained from ICN (Costa Mesa, CA). Fenpropiomorph and tridemorph were obtained from Crescent Chemical Co., Hauppage, New York.

Terbinafine was a gift from Dr. D. Kirsch. **(ALSO AVAILABLE FROM SANDOZ?)**

Stock solutions of terbinafine, tridemorph, brefeldin A, and cerulenin were prepared in ethanol. Clotrimazole, ketoconazole, and fenpropiomorph stocks were prepared in dimethyl sulfoxide and fluphenazine and cycloheximide stocks were prepared in water. Nystatin was dissolved in *N,N*-dimethyl formamide (Sigma).

Nucleotide sequence accession numbers. GenBank accession numbers for the nucleotide sequences of *ERG6* from *S. cerevisiae, A. thaliana, and T. ativum* are X74249 (SEQ ID NO:3), U71400 (SEQ ID NO:4), and U60755 (SEQ ID NO:5), respectively.

Results

Cloning of the *C. albicans ERG6* gene. Four *S. cerevisiae erg6* transformants which grew on cycloheximide were analyzed for sterol content. *erg6* mutants which fail to synthesize ergosterol due to defects in the C-24 transmethylase gene accumulate principally zymosterol, cholesta-5,7,24-trien-3β-ol and cholesta-5,7,22,24-tetraen-3β-ol (Molzhan et al., 1972). UV scans of the sterols obtained from a *Saccharomyces erg6* strain as well as an *erg6* transformant containing the *Candida ERG6* gene are shown in Figure 3. Sterols giving the *erg6* spectrum contain absorption maxima at 262, 271, 282, and 293 nm as well as maxima at 230 and 238 nm. The latter two absorption maxima are due to conjugated double bonds which occur in the sterol side chain (cholesta-5,7,22,24-tetra-en-3β-ol). The *ERG6* transformed strain does not have a conjugated double bond in the side-chain and gives absorption maxima only at 262, 271, 282, and 293 nm. The remaining three transformants yielded similar profiles. Gas chromatographic analysis of the *erg6* mutant and the *ERG6* transformants confirmed the presence of ergosterol in the latter strains. These results were also confirmed by mass spectrometry.

Of the four transformants that restored the ability of the *erg6* mutant to synthesize ergosterol, two of the transformants had an insert size of 8 kb (e.g., pCERG6-20; Figure 4) and the other two had an insert of 14 kb (e.g., pCERG6-9).

The pCERG6-9 insert contained the entire 8 kb DNA fragment of pCERG6-20, suggesting that the *ERG6* gene resided within the 8 kb fragment. Growth of ergosterol producing transformants on media containing 5-FOA resulted in loss of the transforming plasmid. The loss of the plasmid restored the *erg6* phenotype, which indicated that ergosterol production of the pCERG6-20 and pCERG6-9 transformants was plasmid mediated.

In order to locate the *ERG6* gene within the plasmid insert, an approximately 4 kb subclone of the left arm of pCERG6-20 was inserted into the *Saccharomyces* vector pRS316 yielding plasmid pIU880 (Figure 4). pIU880 was able to complement *erg6*. Plasmid pIU882, which contains a 2.4 kb overlap with pIU880, also complemented *erg6* suggesting that the *Candida ERG6* gene lies within this 2.4 kb fragment. This 2.4 kb fragment was subcloned into pRS316 by digesting pIU880 with XbaI-EcoRI to yield pIU885.

DNA sequencing of the *Candida ERG6* gene. The 2.4 kb XbaI-EcoRI fragment was sequenced. The DNA sequence of this fragment and its corresponding amino acid sequence are shown in Figure 5 (SEQ ID NO:1 and SEQ ID NO:2, respectively). The *Candida ERG6* gene encodes a sterol methyltransferase which contains 377 amino acids and is 66% identical to the *Saccharomyces* counterpart. Figure 6 shows an alignment between the *Candida*, *Saccharomyces*, *Arabidopsis*, and *Triticum* sterol methyltransferases. The percent identity of the *Candida* sterol methyltransferase to the *Arabidopsis* and *Triticum* sterol methyltransferase are 40% and 49%, respectively. A nine amino acid region (Figure 6; amino acids 127-135 in the *C. albicans* sequence) represents the highly conserved S-adenosyl methionine binding site (Bouvier-Nave et al., 1997).

Creation of a *C. albicans ERG6* heterozygote. Disruption of the *Candida ERG6* gene to derive a sterol methyltransferase deficient strain was made more difficult since *Candida*, unlike *Saccharomyces*, is an obligate diploid and, thus, both copies of the *ERG6* gene must be disrupted. To accomplish this, the "ura blaster" system developed by Fonzi et al. (1993) was employed. The ura blaster contains about 3.8 kb of repeat elements of *hisG* (derived from *Salmonella)* which flank the

Candida URA3 gene. The plasmid pIU887-A containing the ura blaster inserted into the *ERG6* gene is shown in Figure 4. The 2.4 kb XbaI-EcoRI *ERG6* DNA fragment was cloned into the pBluescript vector-KS(+) in which a HindIII site was filled in with the Klenow fragment of DNA polymerase I, yielding pIU886. pIU886-L was subsequently derived by deleting a 0.7 kb HindIII fragment within the *ERG6* coding sequence, filling in this site with Klenow followed by the addition of BamHI linkers. p5921, containing the ura blaster, was digested with SnaBI and StuI, followed by religation, which resulted in a deletion of 6 bp in one of the *hisG* regions and destruction of these two sites. The modified p5921 was then digested with BamHI and BglII to release the 3.8 kb ura blaster which was then ligated into pIU886-L that had been digested with BamHI to generate pIU887-A.

Candida strain CAI4 was transformed with the 5.3 kb BglII-SnaBI fragment containing the ura blaster and *ERG6* flanking recombinogenic ends of 0.8 and 0.9 kb. Transformants containing the single disrupted *ERG6* allele resulting in heterozygosity for *ERG6* were confirmed using PCR after selection for loss of the *URA3-hisG* region. Intrachromosomal recombination between the linear *hisG* sequences resulted in loss of one of the *hisG* repeats and the *URA3* thus permitting reuse of the ura blaster for the subsequent disruption of the *ERG6* gene on the homologous chromosome. Selection for colonies on 5-FOA resulted in growth of only uridine requiring strains (Boeke et al., 1987).

Creation of *C. albicans erg6* strains. The creation of a *Candida erg6* mutant strain in which both alleles were disrupted was accomplished in two different ways. The *ERG6* heterozygote was placed onto plates containing high concentrations of nystatin (15 μg/ml) and after 3 days nystatin resistant colonies appeared. When colony purified, these resistant colonies were found to be *erg6* homozygotes that resulted from mitotic recombination. A second method used to generate *erg6* homozygotes was to transform the *ERG6* heterozygote with the ura blaster. Two kinds of transformants were obtained: wild type and slow growing colonies. Both types of colonies were tested for resistance to nystatin and only the slower growing colonies were nystatin resistant.

Confirmation of *erg6* homozygosity by sterol analyses. The sterols isolated from wild type and putative *erg6* homozygotes were analyzed by UV spectrophotometry and gas-chromatography/mass spectroscopy. All of the putative *erg6* homozygotes contained *erg6*-like UV scans similar to the *S. cerevisiae erg6* scan shown in Figure 3. Additionally, GC/MS of *erg6* mutant sterols confirmed that only cholesterol-like (C27) sterols accumulate since the side-chain cannot be methylated. Figure 7 represents a GC profile demonstrating that the putative *erg6* mutants accumulate C-27 sterols and are deficient in side-chain transmethylation. Whereas the predominant sterol in the CAI4 wild type is ergosterol (peak B, 76%), the principal sterols in *erg6* mutants are: zymosterol (peak A, 43%), cholesta-5,7,24-trien-3β-ol (peak D, 6%) cholesta-7,24-dien-3β-ol (peak E, 9%), and cholesta-5,7,22,24-tetraen-3β-ol (peak F, 29%).

PCR confirmation of homozygous disruptions. Confirmation of the disruption of both copies of the *C. albicans ERG6* gene by mitotic recombination of the heterozygote and by a second transformation using the ura blaster was performed by employing four PCR primers. The ura blaster containing a 3.8 kb region of *hisG-URA3-hisG* replaced the 0.7 kb of *ERG6* DNA (Figure 8A). This was followed by deletion of the *hisG-URA3* sequence such that in effect the remaining 1.2 kb *hisG* sequence replaced a 0.7 kb *ERG6* deletion. The expected PCR amplification product of CAI4 using P1-P2 or Pl-P3 was observed, i.e., a product of 1.5 kb and 2.15 kb, respectively (Figure 8B, lanes 1 and 2). The product from amplification of the heterozygote CAI-4-6-5 with Pl-P2 was 1.5 kb (wild type allele) and 2.01 kb (disrupted *ERG6* allele). The product from amplification of CAI-4-6-5 with P1-P3 primers was 2.15 kb (wild type) and 2.65 kb (disrupted *ERG6*) (Figure 8B, lanes 3 and 4). Primer pair P1-P4 gives a 1.1 kb band demonstrating the presence of *hisG* within the *ERG6* sequence. The *erg6* homozygotes, 5AB15, obtained by mitotic recombination, and HO11-A3, obtained by ura blaster transformation, yielded identical amplification products using primers P1-P2 (2.01 kb) and primers P1-P3 (2.65 kb), as shown in Figure 8B, lanes 5-8.

Drug susceptibilities of *C. albicans erg6* strains. The susceptibilities of the *erg6* strains as compared to wild type *C. albicans* were determined using a number of antifungal compounds and general cellular inhibitors (Figure 9). The *erg6* strains were shown to be more resistant to nystatin while showing near identical sensitivity to the azole antifungals, clotrimazole and ketoconazole. Significantly increased susceptibilities of the *erg6* strains were noted for tridemorph and fenpropiomorph, inhibitors of sterol Δ14-reductase and Δ8-Δ7 isomerase (Baloch et al., 1984); terbinafine, an allylamine antifungal inhibiting squalene epoxidase (Jandrositz et al., 1991); brefeldin A, an inhibitor of Golgi function (Venkateswarlu et al., 1997); cycloheximide, a common protein synthesis inhibitor; cerulenin, an inhibitor of fatty acid synthesis (Monsaki et al., 1993); and fluphenazine, a compound which interferes with the function of calmodulin (Hait et al., 1993).

Table 1. Drug susceptibilities[a] of *ERG6* and *erg6* strains of *C. albicans* to antifungal agents and metabolic inhibitors.

DRUG	*ERG6*	*erg6*
Nystatin	2.5	15
Clotrimazole	1	1
Ketoconazole	5	5
Terbinafine	>50	1
Fenpropiomorph	0.5	0.005
Tridemorph	>90	0.03
Brefeldin A	50	1
Cerulenin	2	1
Cycloheximide	>600	50
Fluphenazine	100	50

[a] denotes inhibitor concentration (μg/ml) at which no growth appeared after 48 hours under the growth conditions described in the brief description of Figure 9.

The determination of drug concentrations sufficient to completely inhibit growth on plates yielded the data shown in Table 1. The concentration (2.5 μg/ml) of nystatin required for complete inhibition of wild type is within the normal range for a wild type strain (Molzhan et al., 1972) while the *erg6* mutants showed a resistance level similar to that noted for *erg6* mutants of *S. cerevisiae* (Molzhan et al., 1972). As demonstrated on plates (Figure 9), the azoles show equal efficacy against both wild type and *erg6* strains. In contrast, the *erg6* mutants show significantly increased susceptibilities to other antifungals and metabolic inhibitors. *Erg6* susceptibilities to cerulenin and fluphenazine showed two-fold increases while those for terbinafine and brefeldin A were about 50 times greater than for wild type. Cycloheximide susceptibility increased about eleven-fold while the greatest increases in susceptibility increase was shown for the morpholines, fenpropiomorph (100-fold) and tridemorph (several thousand-fold).

Discussion

Candida erg6 mutants are of interest as the permeability of these mutants might make them more sensitive to known and new antifungals or even make them sensitive to compounds previously found not to be effective when ergosterol is present in the cell. Using a *Candida* genomic library, *Candida ERG6* was isolated by complementing an *erg6* mutant of *Saccharomyces.* To further identify the *ERG6* gene for sensitivity to nystatin and resistance to cycloheximide were also employed in conjunction with the complementation assay. Nystatin functions by binding to membrane ergosterol and causing cell leakage which leads to cell death (Brajtburg et al., 1990). Mutants such as *erg6* do not produce ergosterol and utilize sterol intermediates in place of membrane ergosterol. Nystatin has lower affinity for sterol intermediates thus leading to resistance in non-ergosterol containing strains. Expression of the *Candida ERG6* gene in *Saccharomyces erg6* mutants restores the nystatin-sensitive phenotype. The *ERG6* gene also elevates the cell permeability barrier to normal levels, thus conferring cycloheximide resistance at low drug concentrations. Cloning of the *Candida ERG6* gene was also confirmed by UV

analysis of sterol composition and GC/MS analysis of accumulated sterols in *Saccharomyces erg6* and transformed strains containing the *Candida ERG6* gene. Final confirmation that the *ERG6* had been obtained was provided by sequencing of the *Candida ERG6*.

To determine whether the *ERG6* gene in *Candida* was essential for viability, the two copies of the gene were disrupted by first creating a heterozygote using the ura blaster disruption protocol. The second copy of the *ERG6* gene was disrupted by either allowing for mitotic recombination or by a second disruption with the ura blaster. In both cases the resulting *erg6* homozygotes were viable, indicating that the *ERG6* gene in *C. albicans* is not essential for viability. Both types of *erg6* mutants were confirmed by both sterol analysis and PCR analysis of the disruptions.

With the continued increase in resistance to the azole antifungals, new approaches to antifungal chemotherapy are strongly indicated. One approach is to disarm the resistance mechanism. A primary mechanism in *C. albicans* for azole resistance is the increase in expression of efflux systems which utilize the azoles as substrates. Both the ABC (ATP-binding cassette) transporter gene *CDR1* and a gene (*BEN*^{r}) belonging to a major facilitator multidrug transporter have been implicated in this process (Sanglard et al., 1995). A report by Sanglard et al. (1996) has shown that disruption of the *CDR1* gene results in a cell that showed increased susceptibilities to the azole, allylamine, and morpholine antifungals as well as other metabolic inhibitors including cycloheximide, brefeldin A, and fluphenazine. Although not effective alone, disruptions of *BEN*^{r} were shown to work synergistically with *CDR1* with two metabolic inhibitors. The *CDR1* system can provide for an assay for drugs not subject to efflux by these transporters and could also be used to select for compounds which could block the action of the transporters directly. Such approaches would avoid or disarm resistance mechanisms, respectively.

The testing of *Candida erg6* mutants for their susceptibility to antifungal and metabolic inhibitors indicated that these mutants had increased sensitivity to a wide variety of compounds. Azoles were an exception in that they show no difference in

efficacy between wild type and mutant strains. Apparently, the permeability changes are unrelated to the entry mechanism for these compounds. The remainder of the compounds tested, including two other antifungal compounds with different mechanisms of action, showed significantly increased efficacy in the *erg6* strain.

These findings have important applicability from several perspectives. First, the availability of the *C. albicans ERG6* gene allows it to be used as a screen for the identification of inhibitory compounds that specifically target the *ERG6* gene product. This approach has been successfully utilized in cloning of one of the HMGCoA reductase genes (Rine et al., 1983) as well as the *ERG11* (Kalb et al., 1986) and *ERG24* (Marcireaux et al., 1992) genes. In applying this strategy for the purpose of identifying *ERG6* gene product inhibitors, the sensitivity of a wild type strain would be compared to that of a strain carrying additional copies of *ERG6* on a high copy number plasmid. Inhibition of the wild type but not the multiple copy strain would identify inhibition specific to the sterol methyltransferase. Treatment of a fungal pathogen with such an inhibitor would result in a metabolically compromised cell that would be more susceptible to existing antifungals and metabolic inhibitors.

Second, the results predict that an inhibitor of the *ERG6* gene product would result in a fungal organism that is hypersensitive to known compounds or new compounds to which the cell is normally impermeable. Treatment of a cell with both inhibitors would thus produce a synergistic effect. Synergism has been shown (Barrett-Bee et al., 1995) using the experimental sterol methyltransferase inhibitor, ZM59620, in tandem with allylamine and morpholine antifungals. In these studies, the concentrations of the drugs in the combined treatment were significantly below the individual concentrations necessary for both the inhibition of ergosterol biosynthesis and growth inhibition. Thus, because of the increased drug access produced by inhibitors of the sterol methyltransferase, other inhibitors can be clinically employed at reduced dosages.

The *erg6* system also allows for the replacement of *in vitro* testing of inhibitors by utilizing the increased permeability characteristics inherent in the *in*

vivo mutant system. This allows characterization of potential inhibitors that normally fail to reach intracellular targets due to a lack of permeability.

Since the *erg6* system results in a compromised cell which is highly permeable to a variety of compounds and since selection of new inhibitors using high copy number *ERG6* plasmids allows for easy identification, this system has superior potential for the development of new antifungal treatment protocols.

References

Ator, M. A., S. J. Schmidt, J. L. Adams and R. E. Dolle. 1989. Mechanism and inhibition of Δ^{24} - sterol methyltransferase from *Candida albicans* and *Candida tropicalis*. Biochemistry, 28, 9633-9640.

Ator, M. A., S. J. Schmidt, J. L. Adams, R. E. Dolle, L. I. Kruse, C. C. Frey and J .M. Barone. 1992. Synthesis, specificity and antifungal activity of inhibitors of the *Candida albicans* Δ^{24} - sterol methyltransferase. J. Med. Chem., 35, 100-106.

Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.) 1995. Short Protocols in Molecular Biology, Third Ed.

Baloch, R. I., E. L. Mercer, T. E. Wiggins, and B. C. Baldwin. 1984. Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae and Ustilago maydis* by tridemorph, fenpropiomorph, and fenpropidin. Phytochemistry 23:2219-2226.

Bard, M., N. D. Lees, L. A. Burrows, and F. W. Kleinhans. 1978. Differences in crystal violet dye uptake and cation-induced death among yeast sterol mutants. J. Bacteriol. 135:1146-1148.

Barrett-Bee, K., and G. Dixon. 1995. Ergosterol biosynthesis inhibition: a target for antifungal agents. Acta Biochim. Polon. 42:465-480.

Boeke, J. D., J. Truehart, G. Natsoulis, and G. R. Fink. 1987. 5-Fluoro-orotic acid as a selective agent in yeast molecular genetics. Meth. Enzymol. 154:164-175.

Bouvier-Navé, P., T. Husselstein, T. Desprez, and P. Benveniste. 1997. Identification of cDNAs encoding sterol methyl transferases involved in the second methylation step of plant sterol biosynthesis. Eur. J. Biochem. 246:518-529.

Brajtburg, J., W. G. Powderley, G. S. Kobayashi, and G. Medoff. 1990. Amphotericin B: current understanding of mechanism of action. Antimicrob. Agents Chemother. 34:183-188.

Clark, F. S., T. Parkinson, C. A. Hitchcock, and N. A. R. Gow. 1996. Correlation between rhodamine 123 accumulation and azole sensitivity in *Candida* species: possible role for drug efflux in drug resistance. Antimicrob. Agents Chemother. 40:419-425.

Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly. 1997. Itraconazole resistance in *Aspergillits fumigatus*. Antimicrob. Agents Chemother. 41:1364-1368.

Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134:717-728.

Gaber, R. F., D. M. Copple, B. K. Kennedy, M. Vidal, and M. Bard. 1989. The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle sparking sterol. Mol. Cell Biol. 9:3447-3456.

Georgopapadakou, N. H., and T. J. Walsh. 1994. Human mycoses: drugs and targets for emerging pathogens. Science 264:371-373.

Goshorn, A. K., S. M. Grindle, and S. Scherer. 1992. Gene isolation by complementation in *Candida albicans* and applications to physical and genetic mapping. Infect. Immun. 60:876-884.

Hait, W. N., J. F. Gesmonde, J. S. Lazo. 1993. Effect of anti-calmodulin drugs on the growth and sensitivity of C6 rat glioma cells bleomycin. Anticancer Res. 14:1711-1721.

Hebeka, E. K., and M. Solotorovsky. 1965. Development of resistance to polyene antibiotics in *Candida albicans*. J. Bacteriol. 89:1533-1539.

Jandrositz, A., F. Turnowski, and G Hogenaur. 1991. The gene encoding squalene epoxidase from *Saccharomyces cerevisiae*: cloning and characterization. Gene 107:155-160.

Kalb, V. F., J. C. Loper, C. R. Dey, C. W. Woods, and T. R. Sutter. 1986. Isolation of a cytochrome P-450 gene from *Saccharomyces cerevisiae.* Gene 45:237-245.

Kleinhans, F. W., N. D. Lees, M. Bard, R. A. Haak, and R. A. Woods. 1979. ESR determination of membrane permeability in a yeast sterol mutant. Chem. Phys. Lipids 23:143-154.

Lamb, D. C., B. C. Baldwin, K. J. Kwon-Chung, and S. L. Kelly. 1997. Stereoselective interaction of the azole antifungal agent SCH39304 with the cytochrome P-450 monooxygenase system isolated from *Cryptococcus neoformans.* Antimicrob. Agents Chemother. 41:1465-1467.

Lees, N. D., M. Bard, M. D. Kemple, R. A. Haak, R. and F. W. Kleinhans. 1979. ESR determination of membrane order parameter in yeast sterol mutants. Biochim. Biophys. Acta 553:469-475.

Lees, N. D., S. L. Lofton, R. A. Woods, and M. Bard. 1980. The effects of varies energy source and detergent on the growth of sterol mutants of *Saccharomyces cerevisiae.* J. Gen. Microbiol. 118:209-214.

Marcireaux, D., D. Guyonnet, and F. Karst. 1992. Construction and growth properties of a yeast strain defective in sterol 14-reductase. Curr. Genet. 22:267-272.

Mercer, E. I. 1993. Inhibitors of sterol biosynthesis and their applications. In: Progress in Lipid Research, Sprecher H. and J. L. Harwood (eds.), Pergamon Press, NY, pp. 357-416.

Molzhan, S. W., and R. A. Woods 1972. Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae.* J. Gen. Microbiol. 72:339-348.

Monsaki, N., H. Funabashi, R. Shimazawa, J. Furukawa, A. Kawaguchi, S. Okuda, and S. Iwasaki. 1993. Effect of side-chain structure on inhibition of yeast fatty acid synthase by cerulenin analogues. Eur. J. Biochem. 211:111-115.

Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman. 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency

virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives *in vitro*. Antimicrob. Agents Chemother. 41:617-623.

Parkinson, T., D. J. Falconer, and C. A. Hitchcock. 1995. Fluconazole resistance due to energy-dependent drug efflux in *Candida glabrata*. Antimicrob. Agents Chemother. 39:1696-1699.

Pinto, W. J., and W. D. Nes. 1983. Stereochemical specificity for sterols in *Saccharomyces cerevisiae*. J. Biol. Chem. 258:4472-4476.

Powderley, W. G., G. S. Kobayashi, G. P. Herzig, and G. Medoff. 1988. Amphotericin B-resistant yeast infection in severely immunocompromised patients. Amer. J. Med. 84:826-832.

Rine, J., W. Hansen, E. Hardeman, and R. W. Davis. 1983. Targeted selection of recombinant clones through gene dosage effects. Proc. Natl. Acad. Sci. USA 80:6750-6754.

Sanglard, D., F. Ischer, M. Monod, and J. Bille. 1996. Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob. Agents Chemother. 40:2300-2305.

Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille. 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. 39:2378-2386.

Sikorski, R. S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19-27.

Vanden Bossche, H., G. Willemsens, and P. Marichal. 1987. Anti-*Candida* drugs the biochemical basis for their activity. Crit. Rev. Microbiol. 15:57-72.

Venkateswarlu, K., M. Taylor, N. J. Manning, M. G. Rinaldi, and S. L. Kelly. 1997. Fluconazole tolerance in clinical isolates of *Cryptococcus neoformans*. Antimicrob. Agents Chemother. 41:748-751.

Vogel, J. P., J. N. Lee, D. R. Kirsch, M. D. Rose, and E. S. Sztul. 1993. Brefeldin A causes a defect in secretion in *Saccharomyces cerevisiae.* J. Biol. Chem. 268:3040-3043.

Welihinda, A. A., A. D. Beavis, and R. J. Trumbly. 1994. Mutations in *LIS1* (*ERG6*) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae.* Biochim. Biophys. Acta 1193:107-117.

Wheat, J., P. Marichal, H. Vanden Bossche, A. Le Monte, and P. Connolly. 1997. Hypothesis on the mechanism of resistance to fluconazole in *Histoplasma capsulatum.* Antimicrob. Agents Chemother. 41:410-414.

White, T. 1997a. Increased MRNA levels of *ERG16, CDR, and* MDRI correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 41:14821487.

White, T. 1997b. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14a demethylase in *Candida albicans.* Antimicrob. Agents Chemother. 41:1488-1494.

All publications and patents are incorporated by reference herein, as though individually incorporated by reference, as long as they are not inconsistent with the present disclosure. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid segment comprising a nucleic acid sequence encoding a *Candida albicans* sterol methyltransferase having SEQ ID NO:2, a biologically active variant or subunit thereof.

2. The isolated nucleic acid segment of claim 1 which comprises a DNA sequence comprising SEQ ID NO:1.

3. The isolated nucleic acid segment of claim 1 which encodes a polypeptide having SEQ ID NO:2.

4. An isolated polypeptide comprising SEQ ID NO:2, or a biologically active variant or subunit thereof.

5. An isolated, purified *Candida albicans* sterol methyltransferase, a biologically active variant or subunit thereof.

6. The sterol methyltransferase of claim 5 having SEQ ID NO:2.

7. A recombinant isolate of *Candida albicans*, the genome of which does not encode a functional sterol methyltransferase.

8. The isolate of claim 7 in which at least one sterol methyltransferase gene has been disrupted by an insertion of DNA.

9. A recombinant isolate of *Candida albicans*, in which one genomic copy of the sterol methyltransferase gene does not encode a functional sterol methyltransferase.

10. The isolate of claim 7 or 9 which is susceptible to terbinafine, tridemorph, fenpropriomorph, fluphenazine, cycloheximide, cerulenin or brefeldin A relative to the susceptibility of the corresponding isolate of *Candida albicans* that has two copies of a sterol methyl transferase gene, each of which encodes a functional sterol methyltransferase.

11. The isolate of claim 7 or 9 which has increased permeability to antifungal agents or metabolic inhibitors.

12. The isolate of claim 7 or 9 in which the amount or activity of sterol methyltransferase is reduced or decreased relative to the amount or activity of sterol methyltransferase in an isolate that has two copies of a sterol methyl transferase gene, each of which encodes a functional sterol methyltransferase.

13. A method to identify inhibitors of fungal sterol methyltransferase, comprising:
    (a) contacting an isolate of *Candida albicans*, the genome of which has two copies of a sterol methyltransferase gene, with an amount of an agent, wherein both copies of the gene are expressed at wild type levels; and
    (b) determining or detecting whether the agent inhibits the growth of the isolate relative to the inhibition of the growth of a corresponding recombinant *Candida albicans* isolate which has more than two copies of a functional sterol methyltransferase gene and has increased activity or amounts of functional sterol methyltransferase relative to the isolate of step (a).

14. The method of claim 13 wherein the recombinant isolate is stably transformed with an expression cassette which encodes a sterol methyltransferase.

15. The method of claim 14 wherein the expression cassette comprises SEQ ID NO:1.

16. A method to identify inhibitors of fungal sterol methyltransferase, comprising:
    (a) contacting an amount of the isolated sterol methyltransferase of claim 5 with an amount of an agent; and
    (b) determining or detecting whether the agent inhibits the activity of the sterol methyltransferase relative to the activity of an amount of sterol methyltransferase not contacted with the agent.

17. A method to identify an anti-fungal agent which has reduced permeability to a wild type isolate of *Candida albicans*, comprising:
    (a) contacting the isolate of claim 7 or 9 with an amount of the agent; and
    (b) determining or detecting whether the agent inhibits the growth of the isolate relative to the corresponding wild type *Candida albicans* isolate.

18. The method of claim 17 wherein the agent is a metabolic inhibitor.

19. The method of claim 17 wherein the agent is a polyene, allylamine or morpholine.

20. A therapeutic method, comprising:

    administering to a mammal having, or at risk of having, a fungal infection an amount of an inhibitor of the sterol methyltransferase of claim 5 and an amount of an agent effective to inhibit or treat the fungal infection, wherein the sterol methyltransferase inhibitor is administered in an amount that reduces or decreases the effective amount of the agent administered relative to the effective amount of the agent administered in the absence of the

sterol methyltransferase inhibitor, and wherein the sterol methyltransferase inhibitor increases the ability of the agent to permeate the fungus.

21. The method of claim 20 wherein the agent is an antifungal agent.

22. The method of claim 20 wherein the agent is a metabolic inhibitor.

23. The method of claim 20 wherein the agent is normally impermeable to fungi.

24. The method of claim 20 wherein a *Candida albicans* infection is inhibited or treated.

25. The method of claim 20 wherein the sterol methyltransferase of claim 5 comprises SEQ ID NO:2.

26. An isolated DNA segment comprising a DNA sequence having SEQ ID NO:1 or its complement, a subunit or a variant thereof.

27. The isolated DNA segment of claim 26 wherein the variant DNA segment encodes a polypeptide having SEQ ID NO:2.

Abstract of the Invention

The invention provides an isolated and purified nucleic acid molecule encoding a *Candida albicans* sterol methyltransferase (*ERG6*). Also provided is a *C. albicans* strain or isolate that has reduced levels of sterol methyltransferase as a result of the disruption of at least one sterol methyltransferase gene. Preferred isolates are more susceptible to a number of sterol synthesis and metabolic inhibitors relative to wild type isolates. Further provided are methods to identify sterol methyltransferase inhibitors and methods to screen for antifungals or metabolic inhibitors which are not normally permeable to the fungal cell.

FIGURE 1

Amino Acid	Codon
Phe	UUU, UUC
Ser	UCU, UCC, UCA, UCG, AGU, AGC
Tyr	UAU, UAC
Cys	UGU, UGC
Leu	UUA, UUG, CUU, CUC, CUA, CUG
Trp	UGG
Pro	CCU, CCC, CCA, CCG
His	CAU, CAC
Arg	CGU, CGC, CGA, CGG, AGA, AGG
Gln	CAA, CAG
Ile	AUU, AUC, AUA
Thr	ACU, ACC, ACA, ACG
Asn	AAU, AAC
Lys	AAA, AAG
Met	AUG
Val	GUU, GUC, GUA, GUG
Ala	GCU, GCC, GCA, GCG
Asp	GAU, GAC
Gly	GGU, GGC, GGA, GGG
Glu	GAA, GAG

FIGURE 2

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

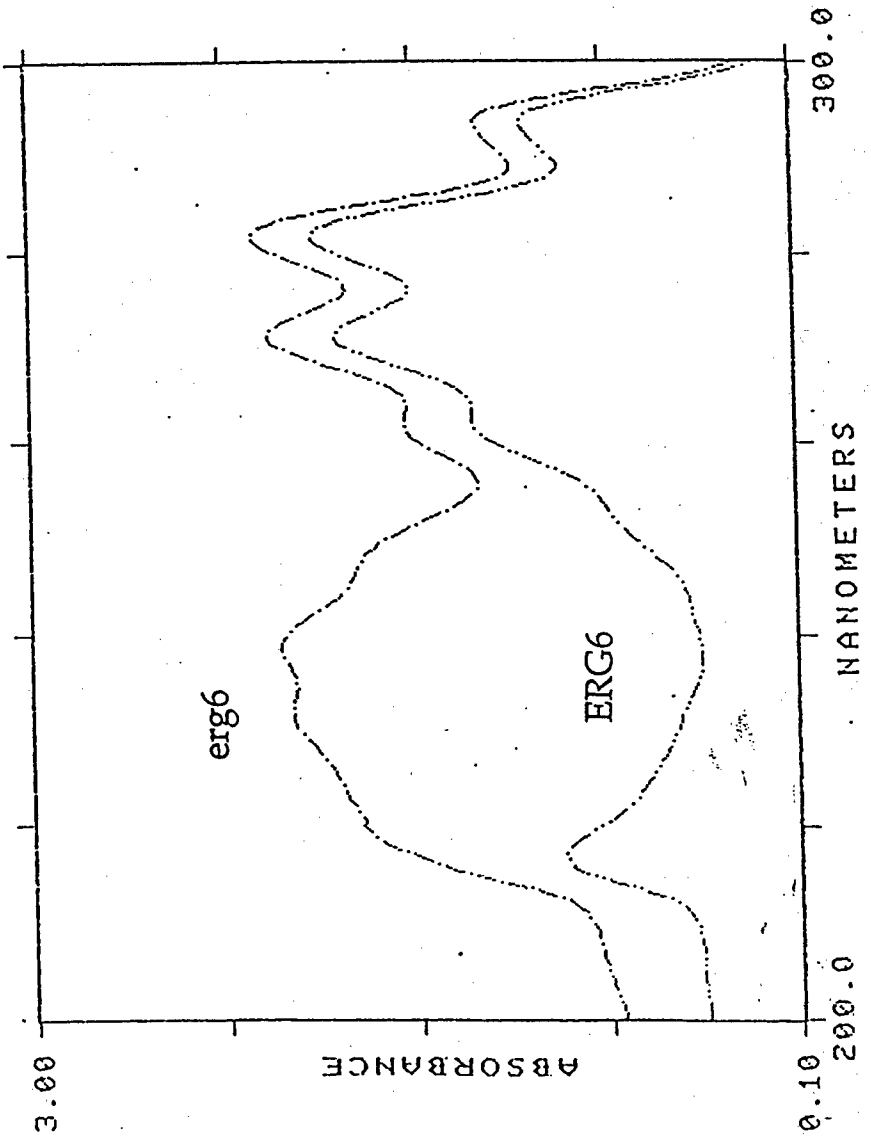

Figure 3

ERG6 Clones

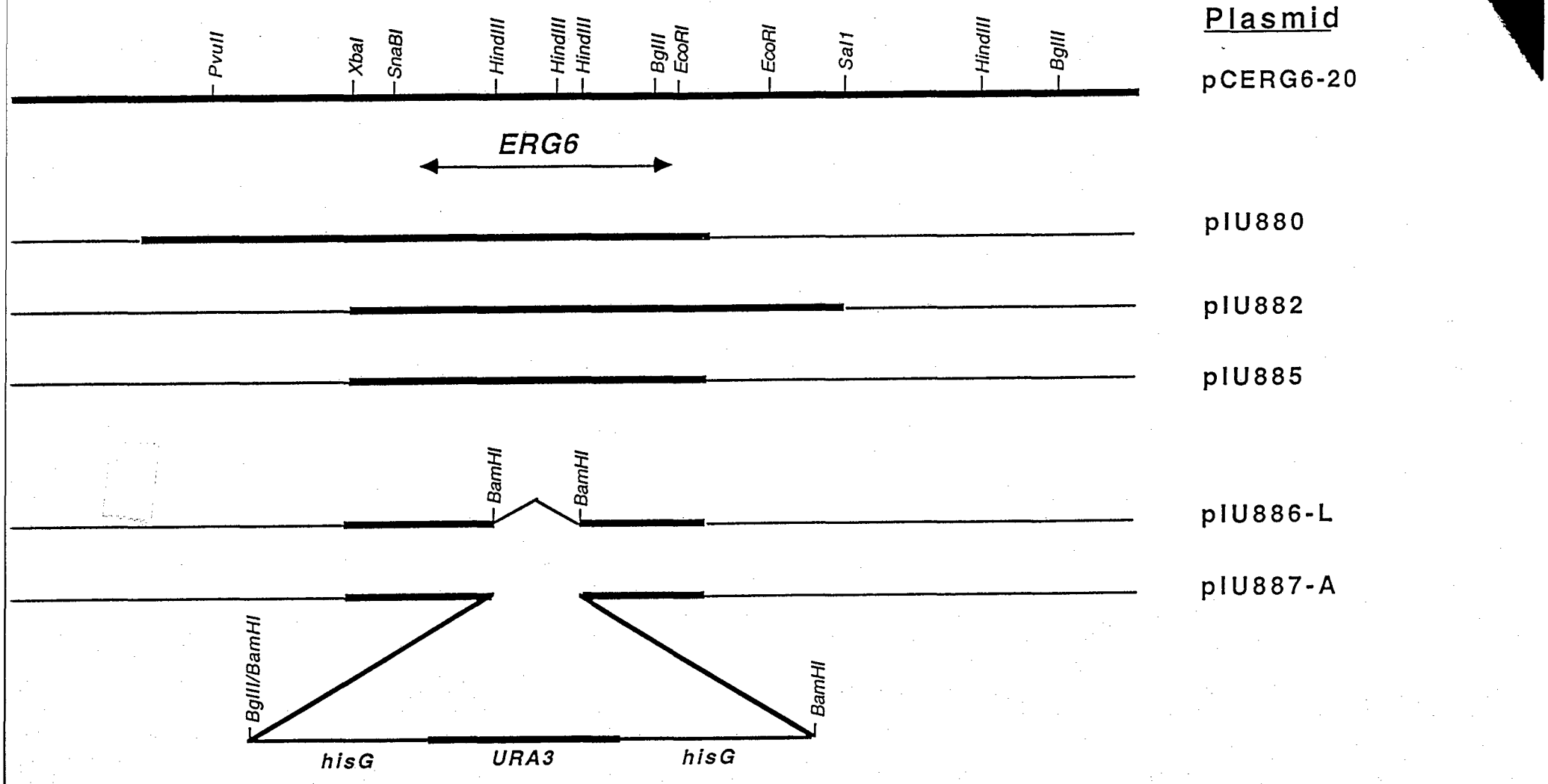

Figure 4

```
    NTACAAAATTTCTTTCTTTCCTTTTTTACTTTTACAATTATTTAAATCAAGTTAAAGAACCTTTCATTTTCTTATTTTAATTTAATTTAGAAATTTTCAA

101 CCTAATTAATTCATAATCTAAGATTCAACTCATTAACAATGTCTCCAGTTCAATTAGCAGAAAAAAATTACGAAAGAGATGAACAATTCACTAAAGCTTT
                                M  S  P  V  Q  L  E  A  K  N  Y  R  E  D  E  Q  F  T  K  A  L    21

201 ACATGGTGAATCTTATAAAAAAAACTGGGTTATCAGCTTTAATAGCTAAATCTAAAGATGCTGCTTCTGTTGCTGCTGAGGGTTATTTCAAACATTGGGAT
     H  G  E  S  Y  K  K  T  G  L  S  A  L  I  A  K  S  K  D  A  A  S  V  A  A  E  G  Y  F  K  H  W  D    54

301 GGTGGTATTTCTAAAGATGATGAAGAGAAAAGATTGAATGATTATTCCCAATTGACTCATCATTATTATAATTTAGTCACTGACTTTTATGAATATGGTT
     G  G  I  S  K  D  D  E  E  K  R  L  N  D  Y  S  Q  L  T  H  H  Y  Y  N  L  V  T  D  F  Y  E  Y  G  W    88

401 GGGGTTCTTCATTCCATTTTTCAAGATATTATAAAGGTGAAGCTTTTAGACAAGCTACTGCTAGACATGAACATTTCTTGGCCCATAAAATGAATCTTAA
       G  S  S  F  H  F  S  R  Y  Y  K  G  E  A  F  R  Q  A  T  A  R  H  E  H  F  L  A  H  K  M  N  L  N   121

501 TGAAAACATGAAAGTTTTAGATGTTGGTTGTGGTGTAGGTGGTCCTGGTAGAGAAATCACAAGATTTACTGATTGTGAAATTGTTGGATTAAATAATAAT
     E  N  M  K  V  L  D  V  G  C  G  V  G  G  P  G  R  E  I  T  R  F  T  D  C  E  I  V  G  L  N  N  N   154

601 GATTATCAAATTGAAAGAGCTAATCATTATGCTAAAAAATACCATTTAGATCATAAATTATCTTATGTTAAAGGTGATTTTATGCAAATGGATTTTGAAC
     D  Y  Q  I  E  R  A  N  H  Y  A  K  K  Y  H  L  D  H  K  L  S  Y  V  K  G  D  F  M  Q  M  D  F  E  P   188

701 CAGAATCATTCGATGCTGTTTATGCCATTGAAGCTACCGTTCATGCTCCAGTTTTAGAAGGAGTTTATTCAGAAATTTATAAAGTTTTGAAACCAGGTGG
       E  S  F  D  A  V  Y  A  I  E  A  T  V  H  A  P  V  L  E  G  V  Y  S  E  I  Y  K  V  L  K  P  G  G   221

801 TATTTTCGGTGTTTATGAATGGGTCATGACTGATAAATACGATGAAACTAATGAAGAACATCGTAAAATTGCTTATGGTATTGAAGTCGGTGATGGTATT
     I  F  G  V  Y  E  W  V  M  T  D  K  Y  D  E  T  N  E  E  H  R  K  I  A  Y  G  I  E  V  G  D  G  I   254

901 CCAAAAATGTATTCTCGTAAAGTTGCTGAACAAGCTTTGAAAAATGTTGGATTTGAAATTGAATATCAAAAAGATTTGGCTGATGTTGATGATGAAATTC
     P  K  M  Y  S  R  K  V  A  E  Q  A  L  K  N  V  G  F  E  I  E  Y  Q  K  D  L  A  D  V  D  D  E  I  P   288

1001 CTTGGTATTATCCATTAAGTGGTGATTTGAAATTTTGTCAAACTTTTGGTGATTATTTGACTGTTTTCAGAACTTCAAGAATTGGTAGATTCATTACTAC
       W  Y  Y  P  L  S  G  D  L  K  F  C  Q  T  F  G  D  Y  L  T  V  F  R  T  S  R  I  G  R  F  I  T  T   321

1101 TGAATCAGTTGGTTTAATGGAAAAAATTGGTTTAGCTCCAAAAGGTTCTAAACAAGTTACTCATGCTTTAGAAGATGCTGCTGTTAATTTAGTTGAAGGT
       E  S  V  G  L  M  E  K  I  G  L  A  P  K  G  S  K  Q  V  T  H  A  L  E  D  A  A  V  N  L  V  E  G   354

1201 GGTAGACAAAAATTGTTTACTCCAATGATGTTGTACGTTGTTAGAAAACCATTAGAAAAGAAAGATTAATGGGGCTTGACAAACAACAAGTAAGGTGAGT
     G  R  Q  K  L  F  T  P  M  M  L  Y  V  V  R  K  P  L  E  K  K  D                                   376

1301 TTATGTTGGGGGTGTTCAATTCGTGTATCTATTCATAGAGGTATTGATTTGCAATTTGTTTTTTGTTTATTCTATTTATTATTTTAAATACTGTTTATAT
```

Figure 5

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[redacted]RG 6    1  MSPVQLAEK-NYERDEQFTKATHGESY-KKTGLSALL 34
[redacted]ERG 6   1  MSETELR-----KRQAQFTREIHGDDIGKKTGLSALL 31
[redacted]t. ERG 6 1  MDSLTLFFTGALVAVGIY-WFICVLGPAERKGKRAV 35
[redacted]T. a. ERG 6 1  MFVFCLCTRCRICRVSSEPVLILFMFIHLSYFFLVT 36

C. a. ERG 6   35  IAKSKDAASVAAEGYFKHWDGGISKDDEEKRLNDYS 70
S. c. ERG 6   32  MSKNNSAQKEAVQKYLRNWDGRTDKDAEERRLEDYN 67
A. t. ERG 6   36  DLSGGSISAEKVQDNYKQYWSFFRRPKEIETAEKVP 71
T. a. ERG 6   37  LLILGQFFFTRYEKYHGYYGGK-----EESRKSNYT 67

C. a. ERG 6   71  QLTHHYYNLVTDFYEYGWGSSFHFSRYYKGEAFRQA 106
S. c. ERG 6   68  EATHSYYNVVTDFYEYGWGSSFHFSRFYKGESFAAS 103
A. t. ERG 6   72  DFVDTFYNLVTDIYEWGWGQSFHFSPSIPGKSHKDA 107
T. a. ERG 6   68  DMVNKYYDLATSFYEYGWGESFHFAHRWNGESLRES 103

C. a. ERG 6   107  TARHEHFLAHKMNLNENMKVLDVGCGVGGPGREITR 142
S. c. ERG 6   104  IARHEHYLAYKAGIQRGDLVLDVGCGVGGPAREIAR 139
A. t. ERG 6   108  TRLHEEMAVDLIQVKPGQKILDVGCGVGGPMRAIAS 143
T. a. ERG 6   104  IKRHEHFLALQLELKPGMKVLDVGCGIGGPLREIAR 139

C. a. ERG 6   143  ETDCEIVGLNNNDYQIERANHYAKKYHLDHKLSYVK 178
S. c. ERG 6   140  ETGCNVIGLNNNDYQIAKAKYYAKKYNLSDQMDFVK 175
A. t. ERG 6   144  HSRANVVGITINEYQVNRARLHNKKAGLDALCEVVC 179
T. a. ERG 6   140  ESSTSVTGLNNNDYQITRGKALNRSVGLGATCDFVK 175

C. a. ERG 6   179  GDFMQMDFEPESFDAVYAIEATVHAPVLEGVYSEIY 214
S. c. ERG 6   176  GDFMKMDEEENTFDKVYAIEATCHAPKLEGVYSEIY 211
A. t. ERG 6   180  GNFLQMPFDDNSFDGAYSIEATCHAPKLEEVYAEIY 215
T. a. ERG 6   176  ADFMKMPFSDNTFDAVYAIEATCHAPDPVGCYKEIY 211

C. a. ERG 6   215  KVLKPGGIFGVYEWVMTDKYDETNEEHRKIAYGIEV 250
S. c. ERG 6   212  KVLKPGGTFAVYEWVMTDKYDENNPEHRKIAYEIEL 247
A. t. ERG 6   216  RVLKPGSMYVSYEWVTTEKFKAEDDEHVEVIQGIER 251
T. a. ERG 6   212  RVLKPGQCFAVYEWCITDHYDPNNATHKRIKDEIEL 247

C. a. ERG 6   251  GDGIPKMYSRKVAEQALKNVGFEIEYQKDLADVDDE 286
S. c. ERG 6   248  GDGIPKMFHVDVARKALKNCGFEVLVSEDLADNDDE 283
A. t. ERG 6   252  GDALPGLRAYVDIAETAKKVGFEIVKEKDLASPPAE 287
T. a. ERG 6   248  GNGLPDIRSTRQCLQAVKDAGFEVIWDKDLAE-DSP 282

C. a. ERG 6   287  IPWYYPLSGDLKFCQTFGDYLIVERTSRIGRFIITE 322
S. c. ERG 6   284  IPWYYPLTGEWKYVQNLANLAIFFRTSYLGRQFITA 319
A. t. ERG 6   288  -PWW----------------TRLKMGRLAYWRNHI 305
T. a. ERG 6   283  LPWYLPL-DPSRFS------LSSERLTTVGRIITRN 311

C. a. ERG 6   323  SVGLMEKIGLAPKGSKQVTHALEDAAVNDVEGGRQK 358
S. c. ERG 6   320  MVTVMEKLGLAPEGSKEVTAALENAAVGLVAGGKSK 355
A. t. ERG 6   306  VVQILSAVGVAPKGTVDVHEMLFKTADCLTRGGETG 341
T. a. ERG 6   312  MVKVLEYVGLAPEGSQRVSSFLEKAAEGLVEGGKKE 347

C. a. ERG 6   359  LFTPMMLYVVRKPLEK                    374
S. c. ERG 6   356  LFTPMMLFVARKPENAETPSQTSQEATQ        383
A. t. ERG 6   342  IFSPMHMILCRKPESPEESS                361
T. a. ERG 6   348  IFTPVYFFVVRKPLSE                    363
```

Figure 6

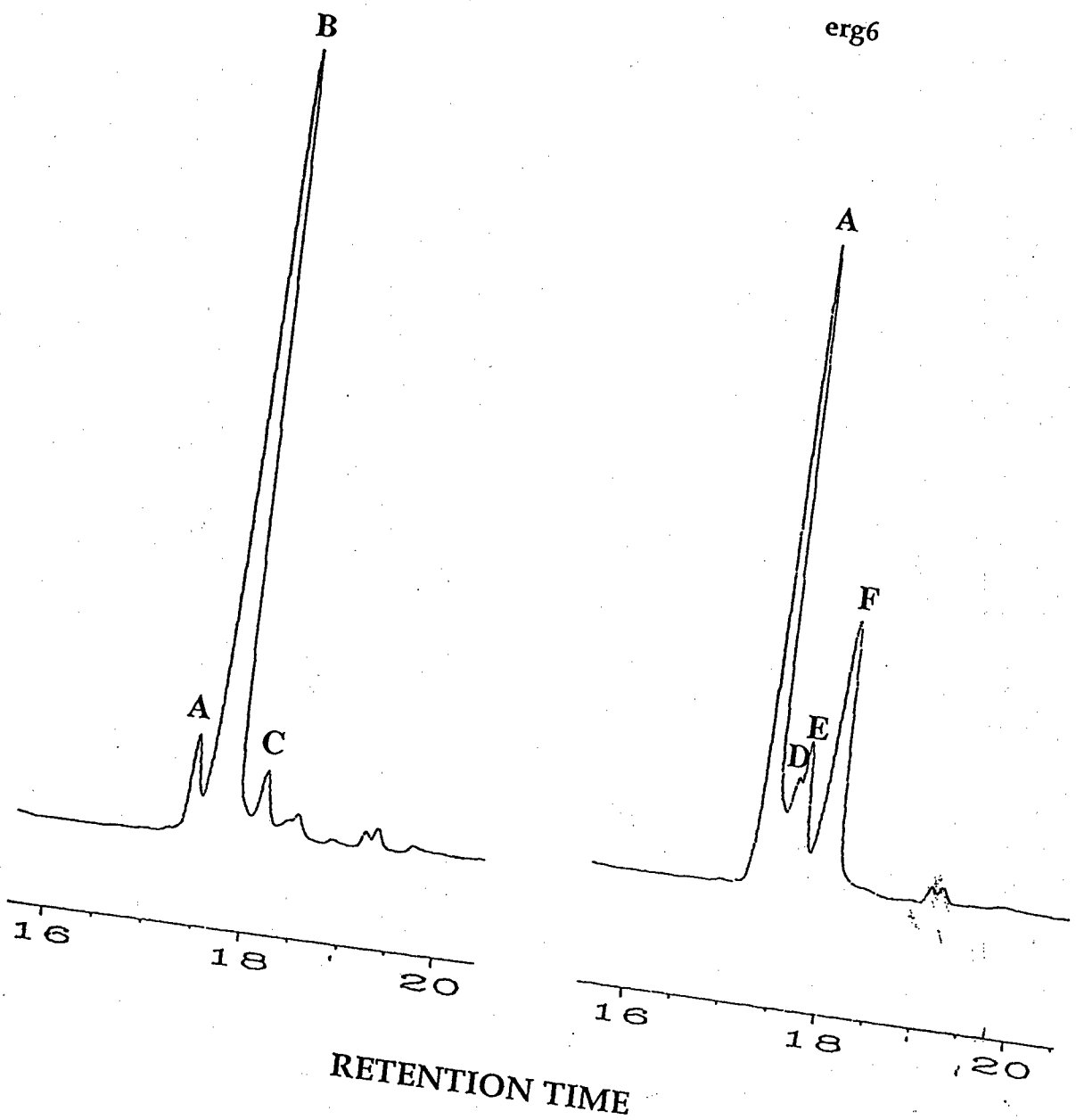

Figure 7

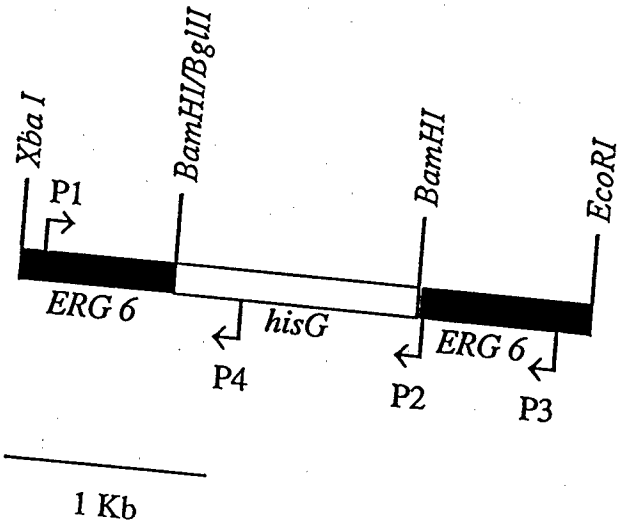

Figure 8A

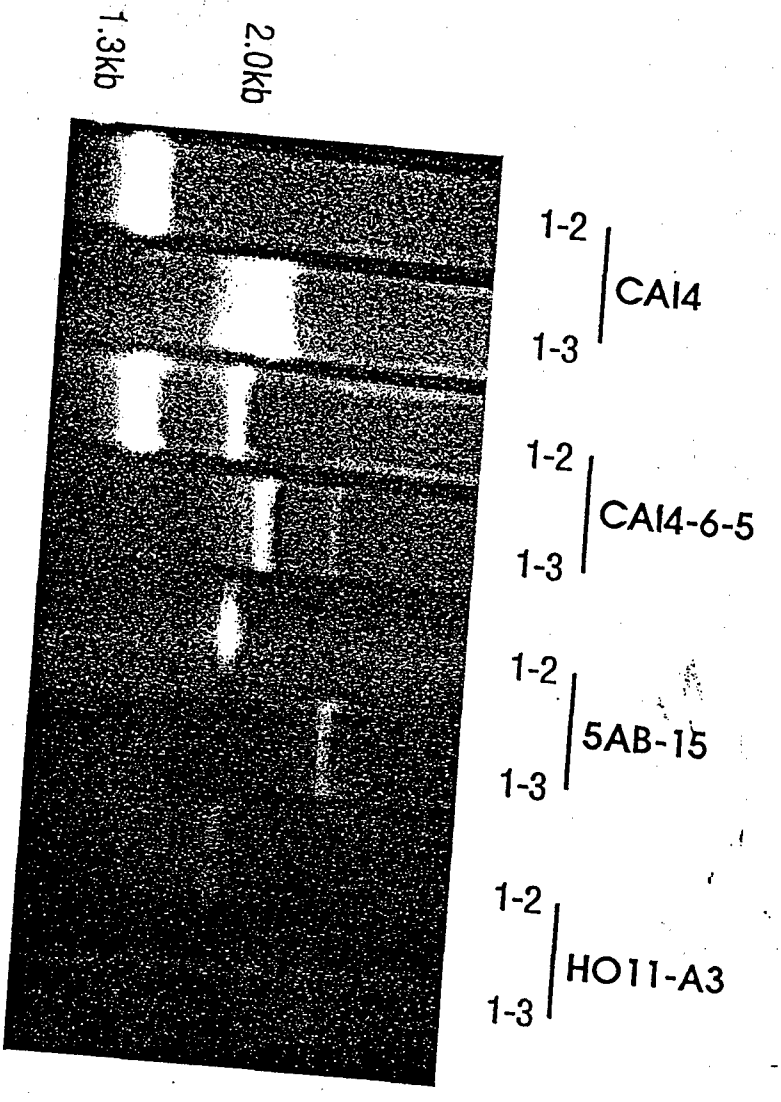

Figure 8B

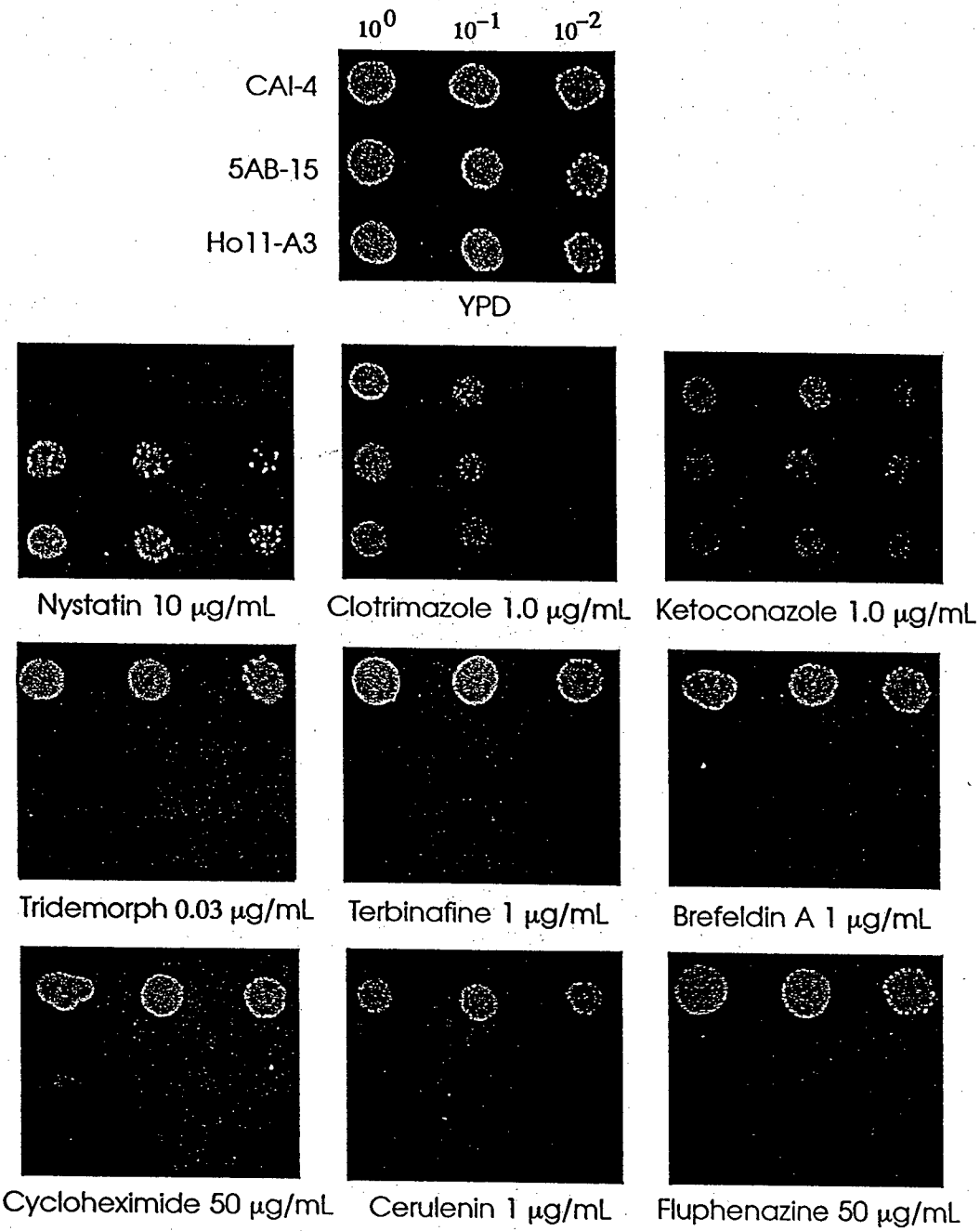
10^0
10^{-1}
10^{-2}
CAI-4
5AB-15
Ho11-A3
YPD
Nystatin 10 μg/mL
Clotrimazole 1.0 μg/mL
Ketoconazole 1.0 μg/mL
Tridemorph 0.03 μg/mL
Terbinafine 1 μg/mL
Brefeldin A 1 μg/mL
Cycloheximide 50 μg/mL
Cerulenin 1 μg/mL
Fluphenazine 50 μg/mL

Figure 9

Rec'd
7/19/2000

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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PHYLLIS M. RINEHART
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