Cloning of the Late Genes in the Ergosterol Biosynthetic Pathway of *Saccharomyces cerevisiae*—A Review[1]

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ABSTRACT: Research on the ergosterol biosynthetic pathway in fungi has focused on the identification of the specific sterol structure required for normal membrane structure and function and for completion of the cell cycle. The pathway and its end product are also the targets for a number of antifungal drugs. Identification of essential steps in ergo-sterol biosynthesis could provide new targets for the development of novel therapeutic agents. Nine of the eleven genes in the portion of the pathway committed exclusively to ergosterol biosynthesis have been cloned, and their essentiality for aerobic growth has been determined. The first three genes, *ERG9* (squalene synthase), *ERG1* (squalene epoxidase), and *ERG7* (lanosterol synthase), have been cloned and found to be essential for aerobic viability since their absence would result in the cell being unable to synthesize a sterol molecule. The remaining eight genes encode enzymes which metabolize the first sterol, lanosterol, to ultimately form ergosterol. The two earliest genes, *ERG11* (lanosterol demethylase) and *ERG24* (C-14 reductase), have been cloned and found to be essential for aerobic growth but are suppressed by mutations in the C-5 desaturase (*ERG3*) gene and *fen1* and *fen2* mutations, respectively. The remaining cloned genes, *ERG6* (C-24 methylase), *ERG2* (D8Æ7 isomerase), *ERG3* (C-5 desaturase), and *ERG4* (C-24(28) reductase), have been found to be nonessential. The remaining genes not yet cloned are the C-4 demethylase and the C-22 desaturase *(ERG5).*
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Sterol biosynthesis in eucaryotic cells has been intensely studied for decades. The biosynthetic pathways of animal, plant, and fungal sterols are now well-defined, and the regulation of cholesterol biosynthesis in animal cells has been well documented (1). Much of the interest in the cholesterol pathway has resulted from the association of poorly regulated cholesterol synthesis and cardiovascular disease.

Research on the fungal sterol pathway which produces the C_{28} sterol, ergosterol, as its end product has followed two major lines of investigation. The first of these is directed to answer fundamental questions concerning the role or roles of sterol in membrane structure and function. Using a variety of systems, sterols have been shown to play critical roles in maintaining appropriate membrane fluidity (2,3), regulating membrane permeability (4,5), influencing the activity of membrane-bound enzymes (6), and altering cell growth rate (7). Beyond these membrane-related functions, sterols have been implicated in providing an essential function in the cell cycle. In this capacity, a sterol of specific structural features is required at hormonal levels (10 nM) to allow cell cycle completion. This phenomenon has been demonstrated in yeast (8) and animal cells (9,10). The elucidation of this sterol function as well as the sterol molecule responsible have been subjects of recent research. The second focus of fungal sterol research is driven by the need to develop new antifungal agents for use in medicine and agriculture. There has been an alarming recent rise in the number of human fungal infections (11) resulting from several factors that include both increases in the incidence of immunosuppressive disease and increases in immunosuppression resulting from technological advances in areas of science and medicine that have modified clinical practices.

As eucaryotes, fungal pathogens possess few potential therapeutic targets that would leave the host unaffected. The rise in microbial resistance to currently available therapeutic agents has been cited in publications varying from basic scientific journals to the lay press (12,13). Although the major emphasis of many of these articles is bacterial resistance, all officially approved antifungal agents have major deficiencies that limit their effective use. Of additional consequence here is the fact that some of the best fungal antibiotics are fungistatic rather than fungicidal. Coupled with immunosuppressive disease and medical advances in chemotherapy and transplantation, all of which result in or require immunosuppression, a fungistatic compound merely retards the pathogen which must then be controlled by a nonfunctional immune response. Human immunodeficiency virus infection is a particularly important area in which currently available antifungals have

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Abbreviations: PCR, polymerase chain reaction; SBI, sterol biosynthesis inhibitor.

been of limited efficacy (14,15). Thus, the identification of essential or required steps in sterol synthesis might provide ideal targets for new drugs that could have improved antifungal activity.

The major targets for antifungal compounds include ergosterol and its biosynthesis. There are a number of drugs that specifically block ergosterol biosynthesis with little effect on host cholesterol synthesis. Specificity is based upon differential enzyme susceptibility or differences in cell permeability. In many cases, adverse side effects result from inhibition of host enzymes. The allylamines inhibit squalene epoxidase (16), an enzyme appearing in the pathway just before the synthesis of the first sterol molecule (Fig. 1). These compounds are restricted to primarily topical applications and show little activity against pathogens that produce life-threatening systemic infections. The morpholines (17) are a group of sterol biosynthesis inhibitors (SBI) which block two separate steps in sterol synthesis, C-14 sterol reduction (*ERG24*) and sterol Δ8→7 isomerization (*ERG2*). This group is used primarily in agricultural applications although amorolfine is under investigation for human topical use (18). The azoles (imidazole and triazoles) are the most widely employed SBI (19) and are used in both topical and systemic situations. These compounds inhibit the C-14 demethylation of lanosterol (*ERG11* gene product) resulting in the formation of C-14 methylsterols. As a substitute for ergosterol, these sterols produce membrane effects leading to reduced growth (20), decreased membrane fluidity (3), and reduced ability to form invasive mycelia (20), an important feature in fungal pathogenesis. The polyene group (21) of antifungals interacts selectively with membrane ergosterol and introduce channels through which essential cell constituents escape, resulting in cell death. Due to their hydrophobicity and metabolism, some polyenes, such as nystatin, are restricted to topical application. Others, such as amphotericin B, can be used systemically but produce serious side effects.

Although not recognized as a major pathogen (22), the yeast *Saccharomyces cerevisiae* has been used as a model system for the elucidation of the ergosterol pathway, and it has been possible in this organism to determine the essentiality of the genes responsible for some of the enzymatic steps. Yeast has the advantage of a defined life cycle and sexual reproduction, making haploid existence and genetic crosses routine. Progress using pathogenic fungi, such as *Candida albicans*, has been considerably slower and largely dependent on procedures and information derived from work with *S. cerevisiae*.

The review will focus on the latter part of the pathway, beginning with the synthesis of squalene, the precursor of lanosterol, which is the first sterol intermediate in the pathway (Fig. 1). Steps prior to squalene formation are important for pathway regulation, and early pathway intermediates are me-

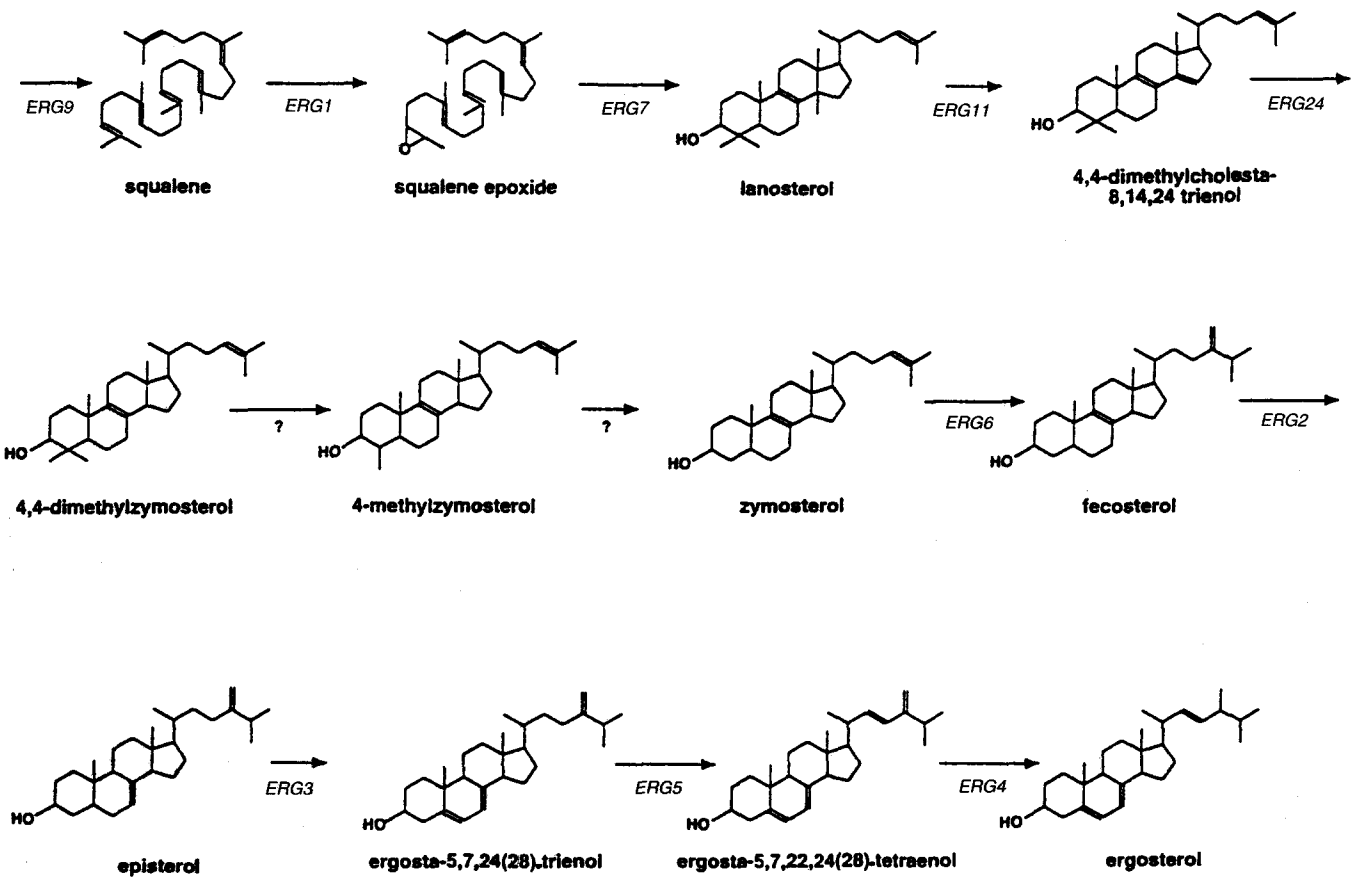

FIG. 1. The sterol biosynthetic pathway in *Saccharomyces cerevisiae* (adapted from Ref. 57).

tabolized to produce other essential cellular components (1). The genes responsible for coding nine of the eleven enzymes in the squalene to ergosterol part of the pathway have been cloned, and the essentiality of each for aerobic growth has been determined. For biosynthetic steps from lanosterol to ergosterol, essentiality was determined by gene disruption, followed by allelic replacement and growth under aerobic conditions. Blocks in the steps from squalene formation to lanosterol synthesis are essential because they do not allow the formation of any sterol molecule. Table 1 lists the eleven reactions from lanosterol formation to ergosterol and, where known, the genes involved, their essentiality for aerobic growth, their chromosomal location and drugs that inhibit the gene products. The genes specifying the enzymes responsible for two of the eleven steps have not been cloned. These include the genes encoding the enzymes responsible for the removal of the two bulky C-4 methyl groups and for desaturation of the side chain at the C-22 position (*ERG5*).

ERG9

Squalene, the first sterol precursor in ergosterol biosynthesis, is formed from two farnesyl diphosphate molecules by the enzyme squalene synthetase (farnesyl diphosphate farnesyl transferase). The substrate of this reaction, farnesyl diphosphate, serves as a beginning compound in the synthesis of several other important molecules that have isoprenoid components. Thus, the action of the *ERG9* gene product represents the first reaction in the branch committed to sterol synthesis. Two laboratories have reported the cloning of the *ERG9* gene (23,24). In both studies, *erg9* sterol auxotrophs containing a marker allowing the aerobic uptake of sterol were transformed with a wild-type yeast genomic library. Transformants capable of aerobic growth without ergosterol supplementation were selected. Plasmids containing the *ERG9* gene resulted in squalene synthase activity in *erg9* cells as well as in *Escherichia coli*. Characterization of the insert DNA indicated an open reading frame coding for a 444 amino acid protein with a molecular mass of approximately 51,700. This enzyme is highly conserved between yeast and humans (25). The *ERG9* gene has been mapped to chromosome 8.

ERG1

The product of the *ERG1* gene is an oxygen-requiring enzyme that converts squalene to 2,3-oxidosqualene, the immediate precursor of lanosterol. This enzyme is sensitive to allylamine inhibition, and this characteristic has been exploited to clone *ERG1* (26). An allylamine-resistant mutant (based on enzyme resistance) of *S. cerevisiae* was used to construct a library that was employed to transform the wild-type parental strain. Transformants were then isolated based on resistance to the drug. The plasmid containing the *erg1* allele was analyzed and found to contain an open reading frame encoding a 496 amino acid protein. The *ERG1* gene has been localized to chromosome 15.

ERG7

The *ERG7* gene encoding 2,3-oxidosqualene cyclase or lanosterol synthase has been cloned from both *C. albicans* (27) and, more recently, *S. cerevisiae* (28). A similar gene encoding the plant enzyme, cycloartenol synthase, which performs a parallel step in plant sterol synthesis, has also been cloned from *Arabidopsis thaliana* (29). In *S. cerevisiae*, polymerase chain reaction (PCR) amplification of yeast genomic DNA was performed using oligonucleotide primers derived from the conserved amino acid sequences from the *Candida* and *Arabidopsis ERG7* gene products. The PCR product was then employed to probe both cDNA and genomic libraries, but no full-length sequences were identified, possibly because the sequence is unstable in *E. coli*. Ultimately, two overlapping clones were utilized to reconstruct the entire coding sequence. The gene comprises a 2196bp open reading frame corresponding to a 732 amino acid protein. As is the case with the other genes preceding the formation of a sterol molecule, *ERG7* is required for aerobic viability. The *Candida ERG7* gene (27) was cloned by using a wild-type *C. albicans* library

TABLE 1
Cloned Genes in the Ergosterol Biosynthetic Pathway

Gene	Enzyme encoded	Essential for viability	Known enzyme inhibitors	Chromosomal location
ERG9	Squalene synthase	Yes	Squalestatin	8
ERG1	Squalene epoxidase	Yes	Allylamines	15
ERG7	Squalene cyclase	Yes	U1866A	8
ERG11	Lanosterol demethylase	Yes[a]	Azoles	8
ERG24	Δ14 Reductase	Yes[b]	Morpholines	?
ERG6	C-24 Methyltransferase	No		13
ERG2	C-8 Isomerase	No	Morpholines	13
ERG3	C-5 Desaturase	No		12
ERG4	Δ24 Reductase	No		7

[a]Suppressed by *erg3*.
[b]Suppressed by *fen1* and *fen2*.

to complement an *erg7* mutant of *S. cerevisiae*. *ERG7* has been mapped to chromosome 8 in *S. cerevisiae*.

ERG11

The product of the *ERG11* gene is a cytochrome P-450 demethylase which is responsible for the removal of the methyl group from the C-14 position. Several aerobically viable *erg11* mutants have been reported (30,31), but all have been found to be leaky and were accompanied by a second mutation in the *ERG3* gene whose product introduces a double bond at the C-5 position (C-5 desaturase) later in the pathway. The essentiality of the *ERG11* gene was in question until it was cloned and characterized by Kalb *et al.* (32,33) and found to be essential for aerobic growth. The cloning was accomplished by transforming yeast with a yeast genomic library in a high copy number plasmid and screening transformants for resistance to the azole, ketoconazole, a specific inhibitor of the C-14 demethylase. Subsequent analysis indicated a protein of 530 amino acids. The gene encoding the demethylase has been mapped to chromosome 8.

The suppression of *ERG11* essentiality by *erg3* mutants remained a question. Recently, Bard *et al.* (34) have shown that double disruptions of *ERG11* and *ERG3* are aerobically viable, indicating that the removal of the C-14 methyl group is not an essential step. The suppression of the *erg11* phenotype by *erg3* is explained by the hypothesis of Watson *et al.* (35). Since the enzymes of the sterol pathway show a high level of substrate infidelity, enzymes subsequent to a blocked step will continue to catalyze the available sterol intermediates. In this case, C-14 methyl sterols continue down the pathway to the C-5 desaturase which attempts to desaturate the C-5 position but is unable to complete the process. The result of this aborted effort is the formation of an intermediate, 14α-methyl-ergosta-8,24(28)-diene-3β,6α-diol, a sterol proposed to disrupt membrane structure and function resulting in cell death. This diol has been identified in *erg11* mutants of *C. albicans* and is tolerated by this organism (36,37). This difference may be the result of subtle variabilities in membrane structure or may reflect alternative mechanisms for esterification of toxic sterols in the two species.

ERG24

The product of the C-14 demethylase reaction, 4,4-dimethylcholesta-8,14,24-trienol, is reduced at the C-14 position to form 4,4-dimethylcholesta-8,24-dienol by the action of the product of the *ERG24* gene. The C-14 reductase is one of the two sites inhibited by the morpholine antibiotics, and this characteristic was employed by one group to clone *ERG24* (38). Using a strategy similar to that employed in the cloning of *ERG11*, *S. cerevisiae* was transformed with a yeast genomic library in a high copy number plasmid, and resulting transformants were screened for resistance to fenpropidin, a morpholine. Resistance was confirmed to be due to the overproduction of the C-14 reductase. Subsequent gene disruption and allele replacement resulted in strains that were unable to grow in the presence of oxygen. To address the issue of whether nonviability under these conditions is due to the accumulation of toxic sterol intermediate (primarily ignosterol, ergosta-8,14-dienol) or the lack of a sterol possessing an essential structural feature, aerobic growth was attempted in a genetic background allowing for uptake of exogenous sterols on a medium containing ergosterol. Growth did occur, arguing that ignosterol did not block aerobic growth.

Simultaneously, a second research group cloned the C-14 reductase using a clever alternative strategy (39). This group selected for a fenproprimorph-resistant mutant that carried two recessive mutations, *fen1* and *fen2*. Both the wild-type and the resistant mutant produce ergosterol when the drug is absent and ignosterol when it is present. The presence of the *fen1* and *fen2* mutations allow survival of cells producing only ignosterol. Nystatin-resistant isolates of a strain carrying *fen1* and *fen2* were generated by mutagenesis and screened for the accumulation of ignosterol. One such isolate was obtained. The *ERG24* gene was cloned by transforming the nystatin-resistant, ignosterol-producing, *fen1, fen2* strain with a wild-type yeast genomic library and by selecting an ergosterol-producing, nystatin-sensitive isolate. The plasmid conferring the desired phenotype was characterized, and a 1314bp open reading frame encoding a 438 amino acid protein of a molecular mass of 50,612 was deduced. This gene has yet to be mapped.

A third research group has recently reported the cloning and characterization of the *ERG24* gene (40). The approach used was similar to that employed by Marcireau *et al.* (38). Sequence analysis confirmed the conclusions of the previous reports. In addition, this study reported sequence similarities among *ERG24*, the *sts1*$^+$ gene of *Schizosaccharomyces pombe* (41) and the *YGL022* gene of *S. cerevisiae* (42). The latter two genes and their relationship to the ergosterol pathway are discussed in the *ERG4* section of this review.

ERG6

The product of the *ERG6* gene methylates the C-24 position of zymosterol to produce the 28 carbon sterol structure common to most fungi. C-24 methylation was considered to be an essential step in the synthesis of functional sterol (43,44). However, since only hormonal amounts of "essential" sterol were required, absolute proof of this hypothesis was difficult. The *ERG6* gene was ultimately cloned by complementation using an *erg6* mutant (45). Cloning using the *erg6* strain presented technical difficulties since cell membrane function was significantly affected by the presence of unmethylated sterol, resulting in low transformation rates and diminished mating efficiencies. Significant protocol alterations were required to successfully transform *erg6* with a yeast genomic library and select for clones that were insensitive to cycloheximide (a permeability defect in *erg6* results in increased sensitivity to this drug). Restoration of wild-type cycloheximide resistance was an effective initial screen for *erg6* complementation.

Transformants were then assayed for ergosterol production and nystatin sensitivity. Two deletion-substitutions of the *ERG6* gene were constructed on a plasmid used to replace the wild-type *ERG6* gene by transformation in which the *LEU2* gene inserted into the *ERG6* gene provided a selectable marker. These disrupted strains were viable. A subsequent report (46) has indicated that gene *SED6*, which suppressed a mutant form of *ERD2*, a gene involved in protein retrieval from endoplasmic reticulum, is identical to the *ERG6* gene. The *ERG6* gene has been localized on chromosome 13.

ERG2

The Δ8→7 isomerase, the product of the *ERG2* gene, is one of the targets of the morpholine antibiotics (47). Based on the efficacy of these drugs, it was suggested that this step may be essential. Using the same protocol as was used with *ERG6*, *erg2* mutants were transformed with a yeast genomic library and screened for cycloheximide resistance (48). Sequence analysis of functional plasmid inserts conferring ergosterol synthesis indicated an open reading frame encoding a 222 amino acid protein. Disruption of the coding sequence did not affect viability, indicating the gene is not essential (49). The *ERG2* gene has also been mapped to chromosome 13.

ERG3

The C-5 desaturase, the product of the *ERG3* gene, converts episterol [ergosta-7,24(28)-dienol] to ergosta-5,7,24(8)-trienol. Based on feeding experiments, Parks and co-workers (50–52) concluded that only sterols containing a C-5 unsaturation or the capability of undergoing this alteration could provide the essential sterol function. The *ERG3* gene was cloned (53) using a protocol for transformation, selection and verification identical to that employed for *ERG6* (45) and *ERG2* (48). Sequencing of the *ERG3* gene indicated a 365 amino acid protein. The essentiality of *ERG3* was determined by deletion and substitution, and strains carrying the allele containing the deletion and disruption are viable. The *ERG3* gene is located on yeast chromosome 12.

ERG4

The *ERG4* gene provides the enzyme which reduces the double bond at C-24(28), the final step in ergosterol biosynthesis. Direct cloning of this gene has not been possible using the protocols employed in cloning the genes operating earlier in the pathway. The sterols accumulated in *erg4* mutants are similar in structure to ergosterol, and the effects of sterol substitution are insufficient to distinguish mutant and wild-type, based on permeability or antibiotic sensitivity differences. Recently, the sequence for *ERG24* was shown (40) to have similarity to the sequences reported for the *sts1*$^+$ gene of *S. pombe* (41), the *YGL022* gene of *S. cerevisiae* (42) and the chicken nuclear lamin B receptor gene (54). Based on the accumulation of C-24(28) sterols, both *sts1*$^+$ and *YGL022* were determined to contain defects in the C-24(28) reductase (*ERG4*). In addition, a disruption of *YGL022* was shown to be allelic to an *erg4* mutant. The authors thus concluded that *YGL022* is *ERG4* and that the reductase is not essential for viability. Figure 2 shows the amino acid sequences of the *ERG24* and *ERG4* gene products. *ERG4* has been localized to chromosome 7.

THE REMAINING GENES

The remaining two steps not yet cloned in the ergosterol biosynthetic include the *ERG5* gene and the C-4 demethylase gene. The latter gene is interesting since no mutants have been detected using the standard polyene resistance selection procedure. This might mean that the inability to demethylate may result in the accumulation of sterol intermediates that are unacceptable for functional inclusion in the membrane or that the resulting sterols may not possess the essential structural features to provide the essential cell cycle function. The process of C-4 demethylation is complex and involves three separate enzymes—an oxidase, a decarboxylase, and a 3-keto reductase. Little work has been done on the yeast enzymes although the counterpart reactions in the cholesterol pathway have been described (55,56).

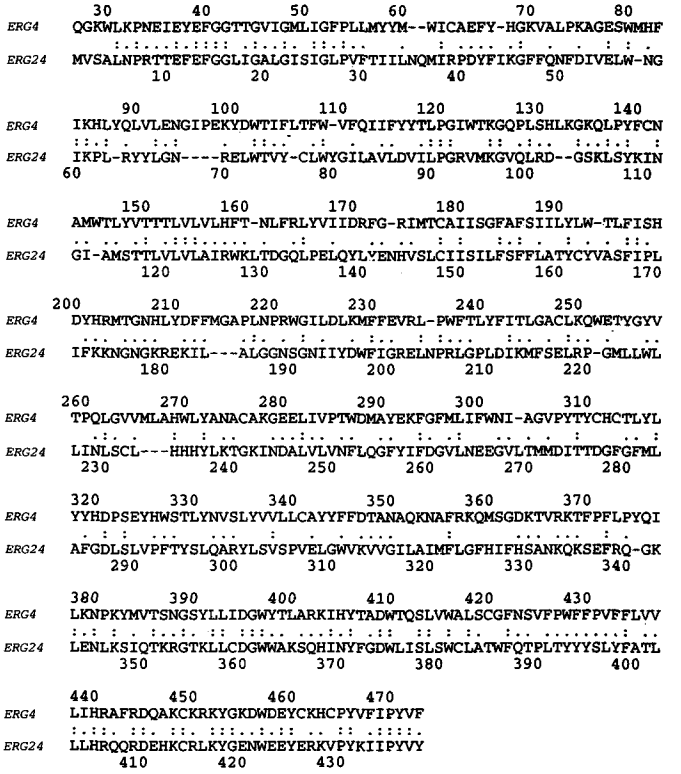

FIG. 2. The amino acid sequences of the *ERG4* and *ERG24* genes of *Saccharomyces cerevisiae*. Single dots represent amino acid similarity and double dots represent amino acid identity.

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