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A Squalene Epoxidase Is Involved in Biosynthesis of Both the Antitumor Compound Clavaric Acid and Sterols in the Basidiomycete *H. sublateritium*

Ramiro P. Godio,[1] Roberto Fouces,[1] and Juan F. Martín[1,2,*]
[1]Instituto de Biotecnología de León (INBIOTEC), Avda. Real 1., 24006 León, Spain
[2]Área de Microbiología, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, Campus de Vegazana, s/n. 24071 León, Spain
*Correspondence: jf.martin@unileon.es
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SUMMARY

The basidiomycete *Hypholoma sublateritium* produces the triterpenoid antitumor clavaric acid, an inhibitor of the human Ras-farnesyl transferase. The *H. sublateritium* squalene epoxidase gene (*erg1*) has been cloned and shown to encode a flavoprotein monooxygenase that requires FAD, NADPH, and P450 cofactors. Silencing of the *erg1* gene in *H. sublateritium* using constructions expressed from the *gdh* promoter of *Agaricus bisporus* showed that the squalene epoxidase is involved in clavaric acid formation and in ergosterol biosynthesis; silenced expression of *erg1* resulted in an ergosterol-dependent phenotype for full growth. Overexpression of *erg1* gene resulted in up to 32% to 97% increment of clavaric acid production confirming its involvement in the biosynthesis of this antitumor product. Oxidosqualene (or dioxidosqualene) appears to be the branching point for primary metabolism (sterols) and secondary metabolites in basidiomycetes.

INTRODUCTION

Mushrooms have been widely used in traditional medicine [1]. They produce hundreds of secondary metabolites, many of them having antitumor activity [2, 3]. Fungi, particularly basidiomycetes, are excellent sources for the screening of new antitumor agents [2, 4–6]. However, progress on the elucidation of the biosynthesis of these metabolites in basidiomycetes has been slow due to the lack of adequate molecular genetics tools for gene manipulation in those fungi.

Mutant *ras* genes (oncogenes) are associated with a variety of solid tumors in humans [7]. The Ras proteins RasH, RasN, RasK-4A, and RasK-4B act as biological switches that are regulated by GDP and GTP binding. They are modified posttranslationally by prenylation (introduction of a lipophilic 15-carbon farnesyl moiety) by the action of the RAS farnesyl transferase (FPTase) [8–10]. The prenylation of the RAS protein plays an essential role in the uncontrolled cell proliferation in tumor cells as compared to normal cells. Inhibitors of the FPTase have been searched as candidates for antitumor agents [6, 11].

In 1998, researchers at Merck isolated from a mushroom a potent inhibitor of the RAS farnesyltransferase that was named clavaric acid [12, 13]. Although the producer fungi was initially thought to be *Clavariadelphus truncatus*, detailed taxonomic studies showed that it corresponds to *Hypholoma sublateritium* (colloquially named "brick cap") [6]. Clavaric acid is a triterpenoid compound, structurally related to fasciculic acid, a calmodulin inhibitor produced by *Naematoloma* (Syn. *Hypholoma*) *fasciculare* [14, 15]. Clavaric acid appears to be synthesized from the triterpene squalene by a pathway related to ergosterol biosynthesis involving the enzymes squalene epoxidase and dioxidosqualene cyclase converting 2,3:22,23-dioxidosqualene to clavarinone, the putative last intermediate in clavaric acid biosynthesis (Figure 1).

The enzyme squalene epoxidase (SE) (EC. 1.14.99.7) catalyzes the conversion of squalene to (3*S*)2,3-oxidosqualene (Figure 1). Genes encoding SE involved in sterol biosynthesis have been cloned from vertebrates [16, 17], yeasts, and other ascomycetes [18–22], but very little information on clavaric acid biosynthesis is available. In the clavaric acid biosynthesis the SE may also catalyze a second step converting 2,3-oxidosqualene to 2,3:22,23-dioxidosqualene.

An important question is whether the SE involved in biosynthesis of clavaric acid in *H. sublateritium* (and other structural analogs in other basidiomycetes) is different from the Erg1 protein or not, i.e., if there are separate SEs for primary and secondary metabolism or if the biosynthesis of clavaric acid diverges from that of ergosterol after the oxidosqualene biosynthetic step. On the other hand, the last steps of clavaric acid biosynthesis are clearly different from those involved in lanosterol and ergosterol biosynthesis. It is therefore of great interest to clone the genes involved in the last steps of ergosterol and clavaric acid biosynthesis in *H. sublateritium* to clarify these questions. Knowledge of the genetics of clavaric acid biosynthesis will allow us to understand the biosynthesis of this interesting isoprenoid molecule and to obtain

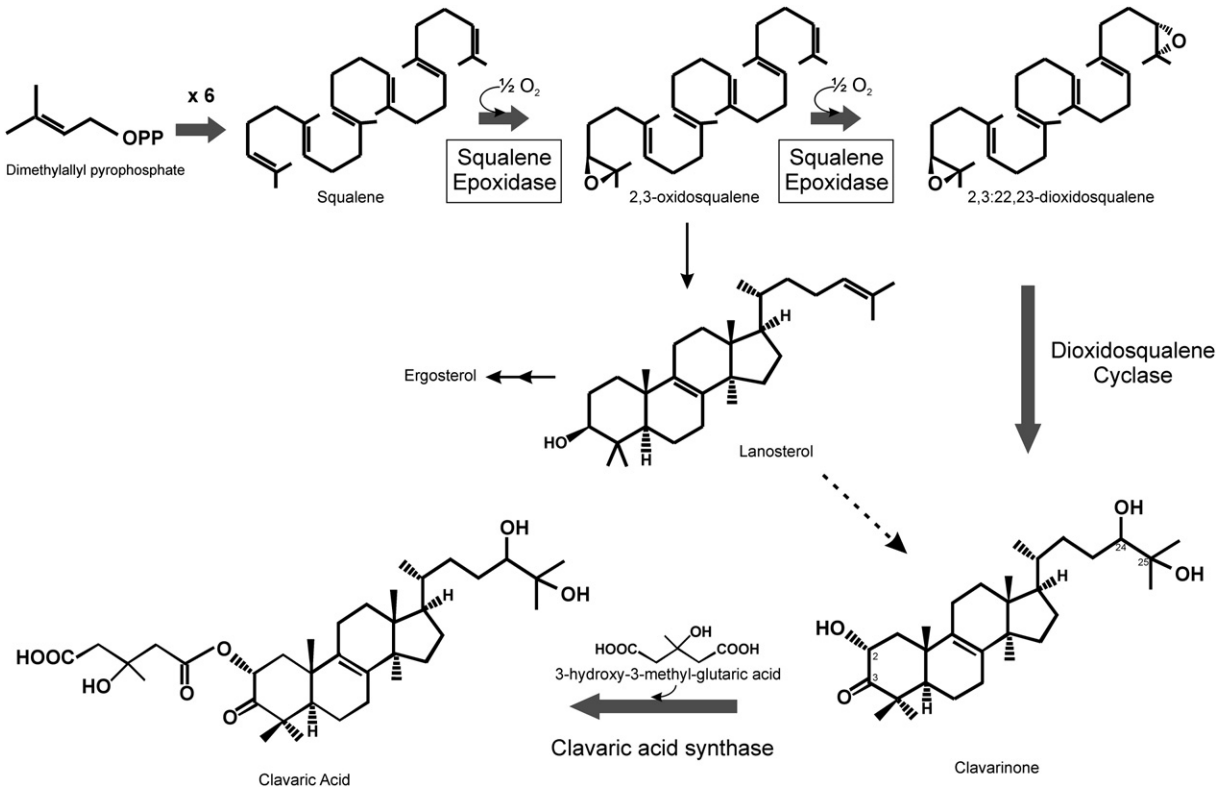


Figure 1. Proposed Clavaric Acid Biosynthetic Pathway

Clavaric acid derives from the triterpenoid squalene. The squalene epoxidase (monooxygenase) reactions converting squalene to 2,3-oxidosqualene and 2,3:22,23-dioxidosqualene are boxed. The pathway to ergosterol branches out from 2,3-oxidosqualene. The conversion of 2,3:22,23-dioxidosqualene to clavarinone and to clavaric acid are hypothetical reactions (see text for details).

higher producing *H. sublateritium* strains since clavaric acid is barely available for animal clinical studies.

In this article, we report the cloning, characterization of the *erg1* gene of *H. sublateritium*, and the effect of its overexpression and silencing by antisense RNA on clavaric acid biosynthesis. Our results indicate that the *erg1* gene encodes a squalene epoxidase that is involved in both ergosterol biosynthesis and clavaric acid production.

RESULTS

Cloning and Sequencing of the *erg1* Gene Encoding the Squalene Epoxidase of *H. sublateritium*

A fragment of the *erg1* gene of *H. sublateritium* was cloned by PCR with degenerated oligonucleotides SE1 and SE2 (Table 1), based on known squalene epoxidase sequences, as primers and a range of temperatures from 50°C to 70°C. A band of about 0.8 kb was obtained that was cloned in pGEM-T-Easy and sequenced. The nucleotide sequence revealed that it contained a fragment of an ORF corresponding to a putative squalene epoxidase (the plasmid was designated pGEM-SE). The cloned fragment lacked *Eco*RV restriction sites, and therefore, this enzyme was used for a complete digestion of total *H. sublateritium* DNA to clone a larger DNA fragment containing the SE gene.

Since an *H. sublateritium* DNA library was not available at that time, the DNA regions adjacent to the cloned 0.8 kb DNA fragment were amplified by inverse PCR with oligonucleotides EPO1 and EPO2 (Table 1) as primers and total *H. sublateritium* DNA, digested with *Eco*RV and self-ligated, as template. As the initially cloned DNA fragment contained a unique *Nco*I site, the pool of self-ligated (circularized) *Eco*RV fragments was digested with *Nco*I thus allowing linearization of those plasmids. The primers EPO1 and EPO2 annealed at the ends of the linearized fragments and allowed amplification of the *Eco*RV fragment containing the EPO1 and EPO2 sequences.

This strategy resulted in amplification of a DNA region of about 3 kb that included the regions adjacent to the 0.8 fragment cloned initially. The entire 3 kb *Eco*RV fragment was mapped with restriction endonucleases and sequenced. It contained an ORF of 1659 bp with five putative introns.

To confirm the presence of the introns, cDNA was obtained from RNA of *H. sublateritium* (18 d static liquid cultures in MEA broth) as template by using oligonucleotides EX1F, EX1R, EX2F, EX2R, EX5F, and EX5R (Table 1) flanking each of the putative introns as primers. Sequencing of

Table 1. Oligonucleotides Used as Primers

Primers	
Name	Sequence (5′→3′)
EMBLF5	TTATGCCCGAGAAGATGTT
EPO1	CCTCAAGACAGCCCTCCATTCC
EPO2	AGGGGAAGGACGGGGAAGAGG
EX1F	GAGCTCGTCGAGGCGACT
EX1R	GAGCTGCGGTACGATGTT
EX2F	TACCCATCCAGCGCGCGC
EX2R	CCCCCACGCTCGAAGTAC
EX5F	GGAAGTGCAAAGCAAGTG
EX5R	CCCGAACACGACGCACGC
HPH4	ACTTCTCGACAGACGTCGCGG
PGDHA-F	GGAGCTCTTGGAATGGAATTA
PGDHA-R	GGGCATGTTGTATATTGGCTG
RTSEF	ATGTCCAAATCCCGCTCAA
RTSER	CAGATCGCAGGGCGTGAAA
SE1	CCSGACCGSATCGTSGGCGAG
SE2	GTSAGSGGGTGSCGCATGTT
SE4	TACAGGTTTAGGGCGAGTT
SE6	GGAACTCCAGCCGTCCATC
SE14	GGTGGGTGCACAAAATGGAAG
TGDHA-F	GGGTAGGCGATTAGTGGAGG
TGDHA-R	GGGATCCAGAGAAACTCTGAT

the obtained DNA confirmed that the cloned *erg1* gene contained five introns of 57, 57, 53, 33, and 73 nucleotides, at positions 751–807, 1138–1194, 1281–1333, 1403–1435, and 1530–1602 numbered from the ATG translation initiation codon. The cloned gene showed an ORF of 1659 bp and encoded a protein of 461 amino acids with a deduced molecular weight of 49,079 Da.

The Cloned ORF1 Encodes a Squalene Epoxidase

Searches of the ORF1-encoded protein in the databases revealed a high similarity with proteins annotated as squalene epoxidases and with proteins of unknown function deduced from the genomes of different fungi. The highest similarity was with an unknown protein of *Ustilago maydis* (E value = 1×10^{-101}) and a protein annotated as squalene epoxidase of *Cryptococcus neoformans* (E value = 1×10^{-97}), both of which are basidiomycetes. When the protein encoded by ORF1 was aligned with the squalene epoxidases of ascomycetes [22], the identity ranged from 33% to 49% (Figure 2B), indicating that the protein encoded by ORF1 is a squalene epoxidase-like protein, but the low percentage of identity when compared with the ascomycetes SEs suggests that it might have a slightly different substrate specificity.

The squalene epoxidases are FAD-dependent monooxygenases. An analysis of the conserved domains of the Erg1 protein with the Pfam bioinformatic tool (protein families database of alignments, http://www.sanger.ac.uk/Pfam/) revealed the conservation in Erg1 and other squalene epoxidases of three strictly conserved motifs (two in the first exon and one in the second exon) (Figure 2A) known to be involved in FAD binding and squalene recognition (see Discussion).

Genes Flanking the *erg1* Gene

In fungi, genes for secondary metabolism are sometimes scattered in the genome, but frequently they are arranged in clusters [23, 24]. To study the genes adjacent to *erg1*, a phage containing the region around *erg1* was isolated by screening a genomic library of *H. sublateritium* HS898 in the EMBL3 phage vector (see Experimental Procedures) by the PCR procedure of Vaiman [25] by using oligonucleotides SE1 and SE2 (Table 1) as primers. Three phages were positives giving an amplification of 0.8 kb corresponding to an internal fragment of *erg1*. Phage F36-2 was selected because the *erg1* gene was located in the center of the insert and sequenced in both DNA strands. As shown in Figure 3A, the *erg1* gene was flanked upstream by a truncated ORF2 that encodes a putative epoxide hydrolase and downstream by a putative α-D-glucosidase.

The putative epoxide hydrolase contains a domain (α/β fold) characteristic of fungal microsomal epoxide hydrolases (pfam00561.13). It is unclear at this time if any of these proteins may be involved in clavaric acid biosynthesis.

Overexpression of *erg1* from the *Agaricus bisporus gdhA* Promoter Leads to Increased Clavaric Acid Production

The *erg1* gene was overexpressed by using a construction pGDHA-SEover in which the *erg1* gene was coupled (at the ATG translation initiation codon) to the *A. bisporus gdhA* promoter (cloned for this purpose; see Experimental Procedures), a regulated promoter (repressed by ammonium ions and derepressed in asparagine/glutamate-based medium). The GDHA-SEover cassette for overexpression of *erg1* was then subcloned in the *Agrobacterium-Hypholoma* conjugative plasmid pBGgHg, giving the construction pHgSEover (Figure 3B) that was used to transform *H. sublateritium* HS898 by conjugation.

The presence of the correct expression cassette in the transformants was confirmed by PCR with primers SE14 (internal to the *erg1* gene) and HPH4 (internal to the hygromycin resistance *hph* gene in the cassette). A 1.1 kb amplification band (Figure 3C) indicated that the cassette had been integrated without rearrangement. The wild-type *H. sublateritium* strain showed a single copy of the *erg1* gene. The presence of at least one additional copy of the *erg1* gene in the transformants was confirmed by Southern hybridization with a 340 bp probe internal to the *erg1* gene (Figure 3D).

Expression of *erg1* in two of the transformants, HS898-SEover-43 and SEover-21, was tested by northern analyses with total RNA from 15 day static liquid cultures in minimal medium (MM, Experimental Procedures)

containing either ammonium acetate (10 or 30 mM) or asparagine/glutamate (15 mM) as nitrogen sources. Results of the northern analyses (Figure 4A) showed that both transformants formed higher levels of *erg1* transcript as compared to the untransformed strain in the asparagine/glutamate but not in cultures in 30 mM ammonium medium where expression occurs primarily from the endogenous *erg1* gene (unregulated by ammonium) and not from the ammonium-repressed *gdhA* cassette.

The effect of *erg1* overexpression on clavaric acid production was quantified in liquid cultures of both transformants and the control untransformed strain in defined medium containing either asparagine/glutamate or ammonium acetate (10 or 30 mM) as nitrogen sources. As observed in Figure 4B, the yield of clavaric acid of the transformants increased to 132% and 197% of the production of the control strain in the derepressing nitrogen source, whereas there was only a small increase (to 104% and 119%) in medium with 10 mM ammonium and no increase at all in medium with 30 mM ammonium (concentrations that repress expression from the *A. bisporus gdh* promoter).

Overexpression of *erg1* Reduces the Sensitivity to Terbinafine

A comparative analysis of the resistance to terbinafine [21, 22] showed that transformants SEover-43 and SEover-21 were less sensitive to terbinafine (in the range of 2.5 to 12.5 μg/ml) (not shown) probably because of the increased levels of squalene epoxidase in these two strains. Transformant SEover-43 showed always 30%–50% higher growth than transformant SEover-21, at the different levels of terbinafine tested, which correlates well with the level of expression of the *erg1* gene in these two transformants (see Discussion).

Silencing of *erg1* Gene Expression by Antisense RNA Reduces Clavaric Acid Production

Constructions for *erg1* gene disruption by gene replacement were made by using plasmid pBS-SKSEI in which the internal region of *erg1* was replaced by a cassette containing the hygromycin resistance (*hph*) gene coupled to the *A. bisporus gpd* promoter. After testing 315 transformants by PCR and Southern hybridization, none of the tested transformants showed the desired gene replacement (not shown) even when the expected recombinants were grown in presence of ergosterol.

Therefore, silencing of *erg1* gene expression by the antisense RNA was used as an alternative strategy [26]; the constructions for antisense RNA expression (pHg-SEa1 and pBG-SEa2) contained, respectively, a 800 or 1600 bp fragment of the *erg1* gene in the 3′-5′ orientation expressed from the nitrogen source-regulated *gdhA* promoter of *A. bisporus* and flanked downstream by the *TgdhA* transcriptional terminator (Figure 5A). Integration of the intact antisense expression cassette in the *H. sublateritium* transformant was tested by PCR with primers PGDHA-F and TGDHA-R (Table 1) corresponding, respectively, to the *A. bisporus gdhA* promoter and the *gdhA* terminator, as shown in the construction in Figure 5A.

These primers amplified a DNA fragment of 1.8 kb in the HS898 SEa1 transformant (67 transformants tested) or of 2.5 kb in the HS898 SEa1 transformants (31 transformants tested). The correct integration of the antisense expression cassette was confirmed by Southern hybridization with, as probe, a 340 bp fragment of the *erg1* gene that was present in the antisense construction. As shown in Figure 5B, four of these transformants (two with the SEa1 and two with SEa2 constructions) showed the expected hybridization bands.

Northern analysis of the expression of *erg1* in two of the transformants HS898 SEa1-23 (with the SEa1 antisense construction) and HS898 SEa2-4 (with the antisense SEa2 construction) showed (Figure 6A) that there was a strong reduction (50%) of the expression of *erg1* in the transformants with the antisense constructions in the asparagine/glutamate-based medium and a small reduction of *erg1* gene expression in the transformants grown in medium with 10 mM NH_4^+ and no significant reduction in medium containing 30 mM NH_4^+ as nitrogen source (repressing conditions for the *gdhA* promoter that drives the antisense RNA expression).

The reduced expression of *erg1* in the transformants containing the antisense cassette correlated with a decrease in clavaric acid production in minimal medium containing asparagine/glutamate as nitrogen source as compared to the untransformed strain (Figure 6) and no significant reduction in medium with 30 mM ammonium as nitrogen source. These results support the conclusion that the squalene epoxidase encoded by *erg1* is involved in the conversion of squalene to epoxide-squalene during clavaric acid biosynthesis.

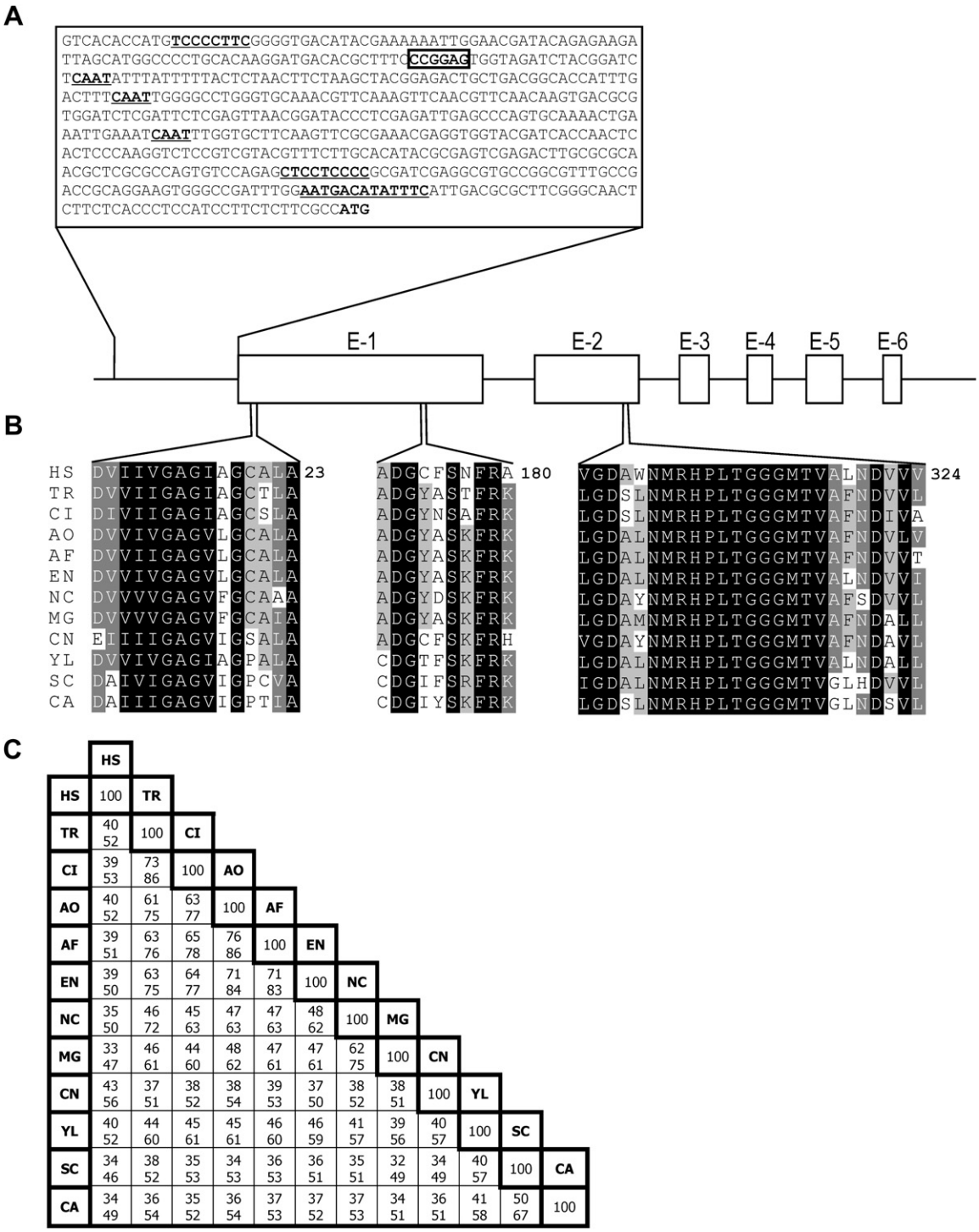
Silencing of *erg1* Results in a Partial Requirement of Ergosterol

During the fermentation with the transformants carrying the antisense RNA constructions, a clear reduction of growth (measured as dry weight) was observed in the transformants grown in asparagine/glutamate medium (allowing expression of the antisense RNA), but not in the medium with 30 mM ammonium (Figure 6C).

Growth of the transformants was only obtained if the cultures of the transformants were supplemented with 12 μg/ml ergosterol (Figure 6D). In the static liquid cultures of transformants in MM supplemented with ergosterol, the growth was lower than that of the parental strain grown under the same conditions (Figure 6D, right). In conclusion, all evidence suggests that the squalene epoxidase encoded by *erg1* is required for clavaric acid production and also for ergosterol biosynthesis.

Lanosterol Does Not Stimulate Clavaric Acid Biosynthesis

Since lanosterol might serve as an intermediate in an alternative pathway for conversion of 2,3-oxidosqualene to clavaric acid (Figure 1) the effect of lanosterol on clavaric acid biosynthesis was studied both in the HS898SEa1-23



	HS	TR	CI	AO	AF	EN	NC	MG	CN	YL	SC	CA
HS	100											
TR	40 52	100										
CI	39 53	73 86	100									
AO	40 52	61 75	63 77	100								
AF	39 51	63 76	65 78	76 86	100							
EN	39 50	63 75	64 77	71 84	71 83	100						
NC	35 50	46 72	45 63	47 63	47 63	48 62	100					
MG	33 47	46 61	44 60	48 62	47 61	47 61	62 75	100				
CN	43 56	37 51	38 52	38 54	39 53	37 50	38 52	38 51	100			
YL	40 52	44 60	45 61	45 61	46 60	46 59	41 57	39 56	40 57	100		
SC	34 46	38 52	35 53	34 53	36 53	36 51	35 51	32 49	34 49	40 57	100	
CA	34 49	36 54	35 52	36 54	37 53	37 52	37 53	34 51	36 51	41 58	50 67	100

Figure 2. Conserved Motifs in the Squalene Epoxidases

(A) Promoter region upstream of the *erg1* gene (starting at the ATG, in bold face). Relevant regulatory sequences recognized by different transcriptional factors are shown in bold face (see text). The exons E-1 to E-6 are shown in boxes.

(B) Three conserved motifs in the squalene epoxidase encoded by the *erg1* gene of *H. sublateritium* and the homologous genes of other fungi (see text for details on the role of each one of the motifs). The numbers at the right of the HS sequence correspond to the position of these amino acids in the *H. sublateritium* SE.

(C) Percentage of amino acid identity between pairs of fungal squalene epoxidases. HS, *Hypholoma sublateritium*; TR, *Trichophyton rubrum*; CI, *Coccidioides immitis*; AO, *Aspergillus oryzae*; AF, *Aspergillus fumigatus*; EN, *Emericella nidulans*; NC, *Neurospora crassa*; MG, *Magnaporthe grisea*; CN,

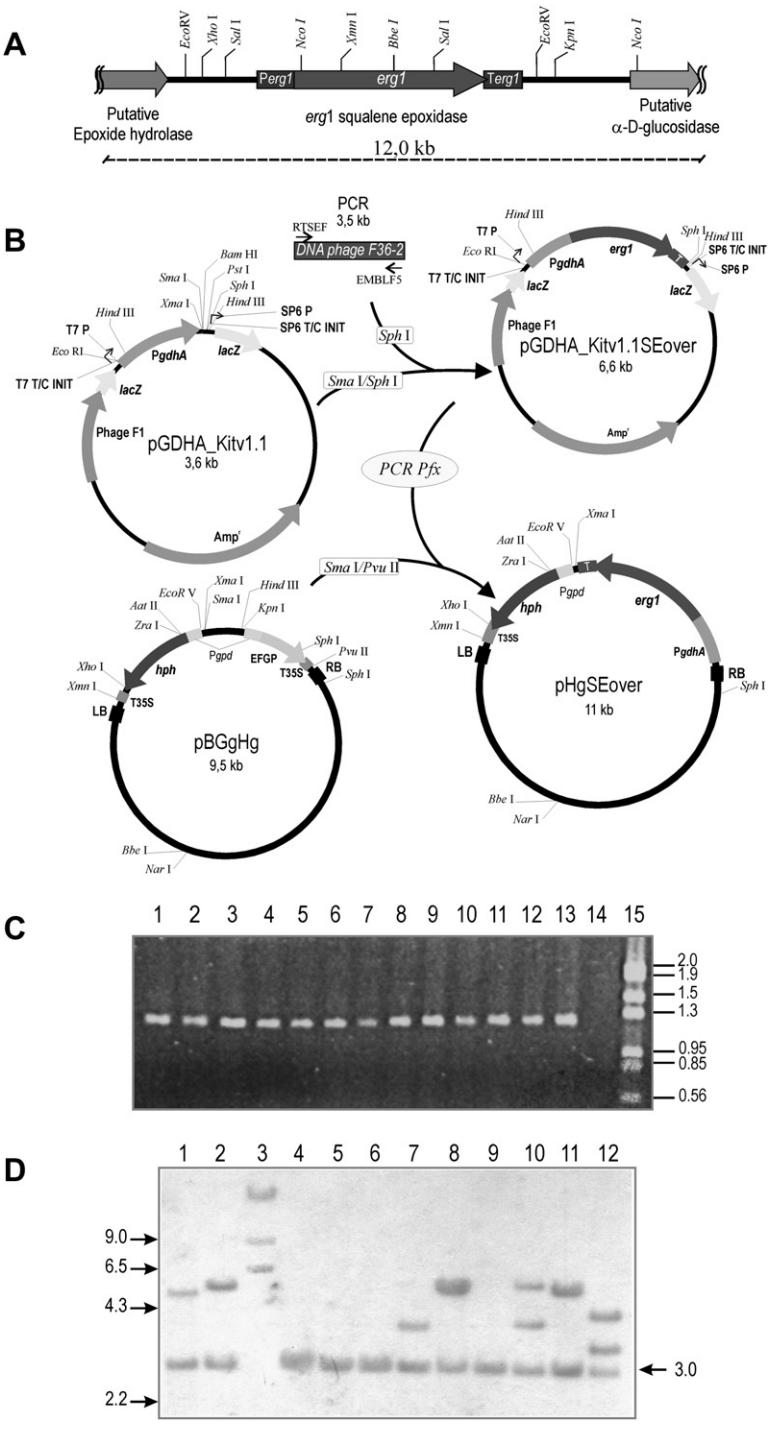


Figure 3. Organization of the DNA Region around *erg1* and Overexpression of the Gene

(A) Organization of the three genes existing in the 12.0 kb sequenced region of *H. sublateritium*. The *erg1* promoter and terminator regions are shown in black.

(B) Plasmids used to construct the expression vector pHgSEover; *erg1* is expressed from the *A. bisporus gdh* promoter. The hygromycin resistance gene (*hph*) is expressed from the *A. bisporus gpd* promoter. T35S, 35S cauliflower mosaic virus transcriptional terminator. LB and RB, left border and right border sequences of the *Agrobacterium tumefaciens* T-DNA region.

(C) PCR of different transformants (lanes 1 to 13) with primers SE14 (internal to *erg1*) and HPH4 (internal to the *hph* gene) to confirm the integration of the intact *erg1* expression cassette. Lane 14, untransformed *H. sublateritium*. Lane 15, size markers (in kb on the right).

(D) Southern hybridization using a 340 bp probe internal to *erg1* confirming the presence of at least one additional copy of the *erg1* gene (different band sizes depending upon the integration site). The endogenous *erg1* copy (3.0 kb) is indicated by an arrow on the right. Size markers (lane 3) are indicated by arrows on the left. Lanes 4, 5, and 6 show the profile of the untransformed *H. sublateritium* strain.

and HS898SEa2-4 transformants expressing the antisense *erg1* gene and in the wild-type strain.

Lanosterol (20 μg/ml) added to defined minimal medium did not stimulate clavaric acid biosynthesis but clearly reduced its production in all strains. The reduction in the wild-type was of 76%, whereas in both transformants, it almost suppressed clavaric acid production (Table S1, see the Supplemental Data available with this article online). These results do not support an involvement of lanosterol as a direct precursor of clavaric acid and instead

Cryptococcus neoformans; YL, *Yarrowia lipolytica*; SC, *Saccharomyces cerevisiae*; CA, *Candida albicans*. The first number in each square box corresponds to the percentage of identical amino acids, and the second includes identical and functionally conserved ones.

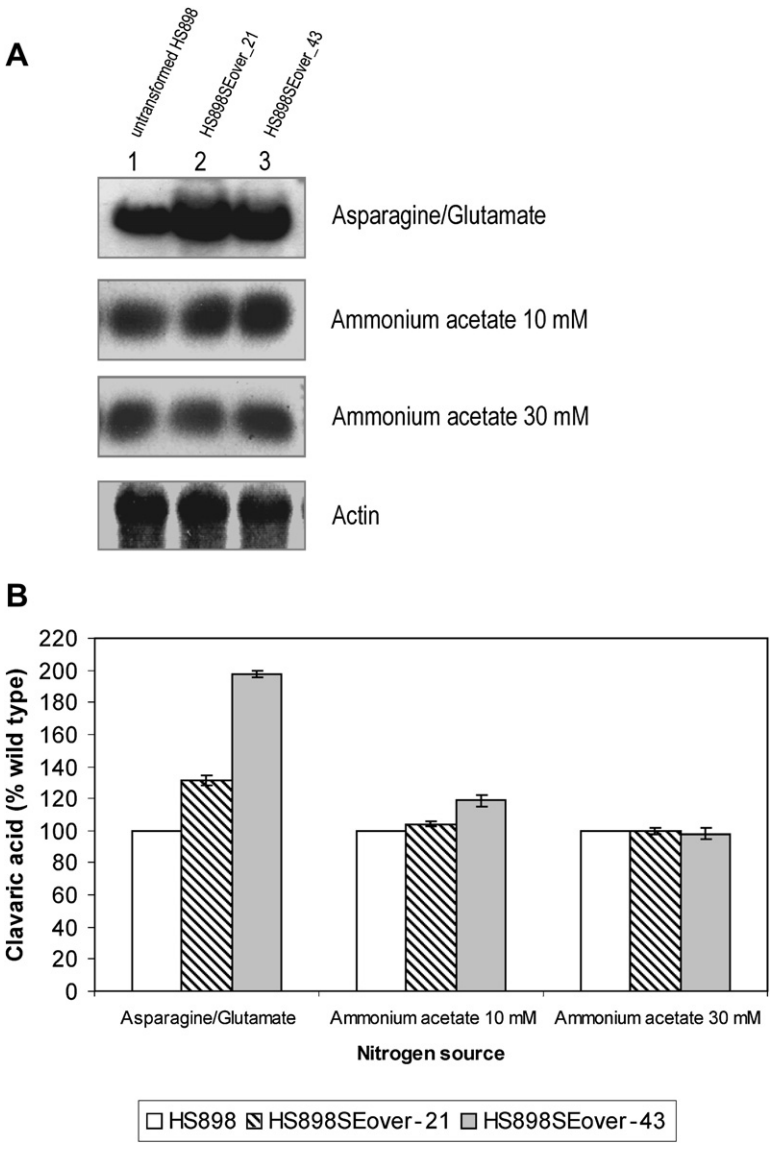


Figure 4. Overexpression of *erg1* and Clavaric Acid Production in *H. sublateritium*

(A) Northern hybridizations showing increased expression of *erg1* in transformants HS898SE over-21 and HS898SEover-43 in MM with asparagine/glutamate, differences were smaller in MM with 10 mM ammonium acetate and 30 mM ammonium acetate, respectively. Hybridizations were performed with a 340 bp probe internal to *erg1* as indicated in Experimental Procedures.

(B) Comparative levels of clavaric acid production in cultures of *H. sublateritium* HS898 and the transformants with the *erg1* expression cassette in MM with asparagine/glutamate, 10 mM ammonium acetate, or 30 mM ammonium acetate, respectively. Static cultures were grown for 15 days in triplicate. Vertical lines on top of each bar indicate standard deviation values of the mean of the three flasks. Note the increased clavaric acid production in the SEover-21 and SEover-43 strains in asparagine/glutamate medium. Clavaric acid producton is referred to that of the wild-type (160 μg/ml = 100%).

suggest that high levels of lanosterol exert a feedback effect on some of the steps involved in biosynthesis of the antitumor compound.

DISCUSSION

The cloned *erg1* gene encodes a protein very similar to other fungal SEs, particularly to those of basidiomycetes *Cryptococcus neoformans* and *Ustilago maydis* and mammalian cells, but they are more distantly related to the SEs of ascomycetes [22]. The cloned *H. sublateritium erg1* gene contained five introns. The presence of relatively large numbers of introns is frequent in other genes of basidiomycetes [27]. In contrast, the *erg1* gene of the ascomycete *T. rubrum* contains a single intron [22, 28].

Squalene epoxidases (EC. 1.14.99.7) are flavoprotein mono-oxygenases that catalyze the conversion of squalene to (3*S*)2,3-oxidosqualene, which in mammals, is rate limiting in the biosynthesis of cholesterol and in yeast in the biosynthesis of ergosterol [29]. In addition to oxygen, the enzyme requires FAD, NADPH [29–31], NADPH-cytochrome P450 reductase (EC. 1.6.2.4) [32], and a still unknown protein factor existing in the cell supernatants [33, 34]. The molecular mechanism of oxidation of squalene has been studied by Abe and coworkers [29, 35]. The reduced form of the flavoprotein appears to react with a molecular oxygen to produce a superoxide anion that leads to the formation of flavin C(4a)-hydroperoxide that may act then as an electrophile [35]. Finally, the oxygen is transferred to the C-C-terminal double bond of squalene.

Recent photoaffinity labeling [36, 37] and site-directed mutagenesis studies [35] led to the identification of conserved residues that play important roles in enzyme activity. These motifs are conserved in the *H. sublaterium* SE. These include a Rossman fold consisting of alternating

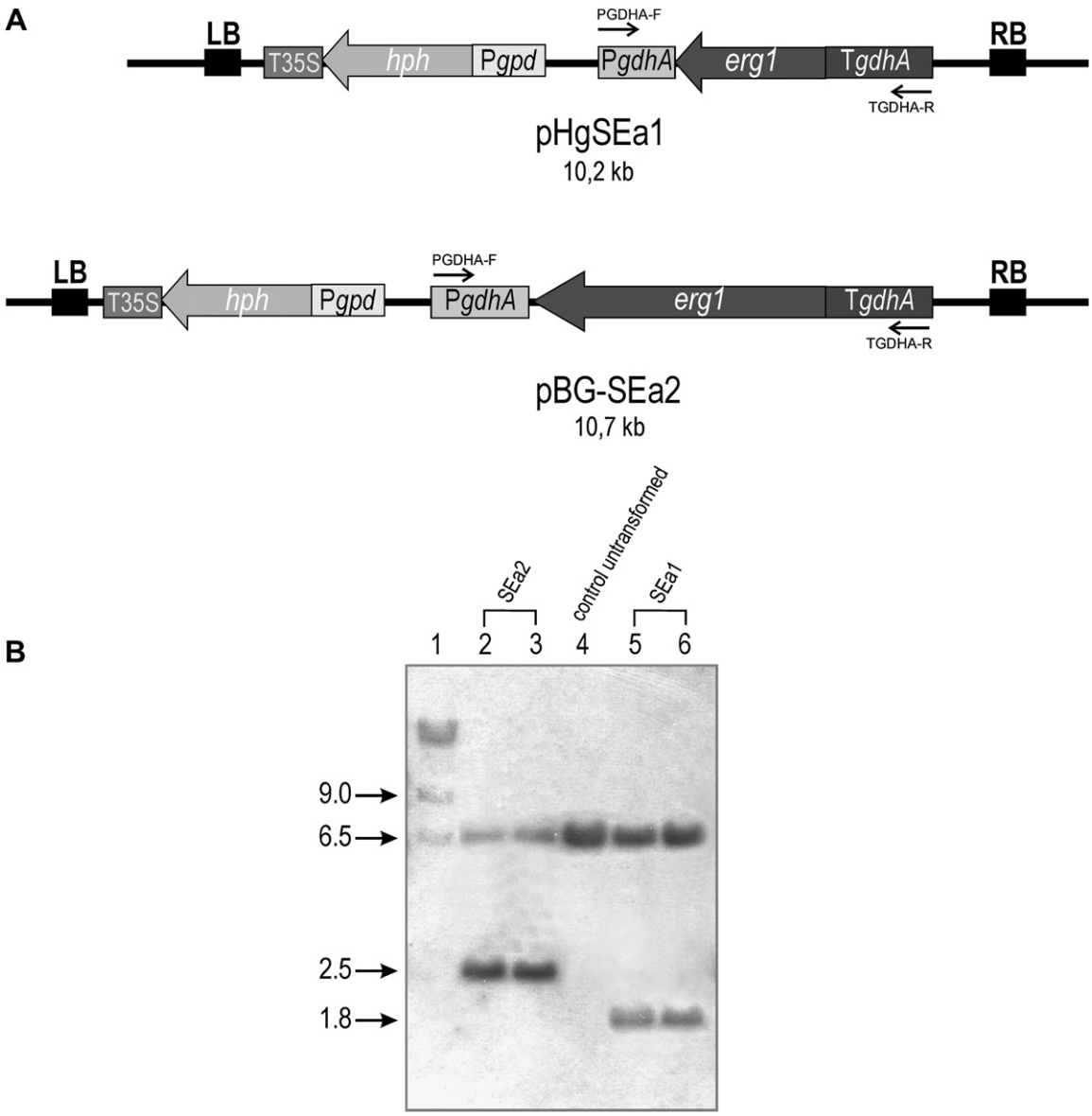


Figure 5. Silencing of the *erg1* Gene by Antisense RNA

(A) Constructions pHgSEa1 and pBGSEa2 containing fragments of different size of *erg1* in the opposite (3′ to 5′) orientation (for antisense RNA formation). In both constructions, the *erg1* antisense strand is expressed from the promoter of the *A. bisporus gdhA* promoter and is flanked by the *gdhA* transcriptional terminator. The hygromycin resistance cassette and the LB and RB border sequences are as in the legend of Figure 3.

(B) Southern hybridizations showing the correct integration of the antisense cassettes in two transformants with each of the antisense constructions. Lane 1, size markers (indicated on the left, in kb). Lanes 2 and 3, transformants with pBGSEa2. Lane 4, control, untransformed *H. sublateritium*. Lanes 5 and 6, transformants with pHgSEa1. Hybridizations were performed with a 340 bp fragment internal to *erg1*. The endogenous *erg1* gene corresponds to the 6.5 kb hybridyzing band. Note the different size of the additional copy or copies of the antisense constructions in transformants SEa2 as compared to transformants SEa1.

α helices and β sheets that contains the Gly-X-Gly-X-X-Gly sequence [38, 39] located in the C-terminal region of the first β sheet (amino acids 14–19 in the *H. sublateritium* Erg1 protein). A second motif Asp-Gly-X-X-Ser-X-Phe-Arg that appears to interact with the pyrophosphate group of FAD [40] is located at positions 172–179 of the *H. sublateritium* Erg1. The third motif Gly-Asp-X-X-Asn-Met-Arg-His-Pro-Leu-Thr-X-X-Gly-Met located in the *H. sublateritium* Erg1 at positions 301–315 is found in a variety of flavoproteins and appears to interact with the ribityl moiety of FAD [41]. These enzymes catalyze the stereospecific conversion of squalene to (3*S*)2,3-epoxidosqualene.

The SE gene (*erg1*) of *S. cerevisiae* is known to be involved in ergosterol biosynthesis and its overexpression confers resistance to allylamines (terbinafine) [42]. In *Candida albicans*, disruption of the *erg1* gene caused

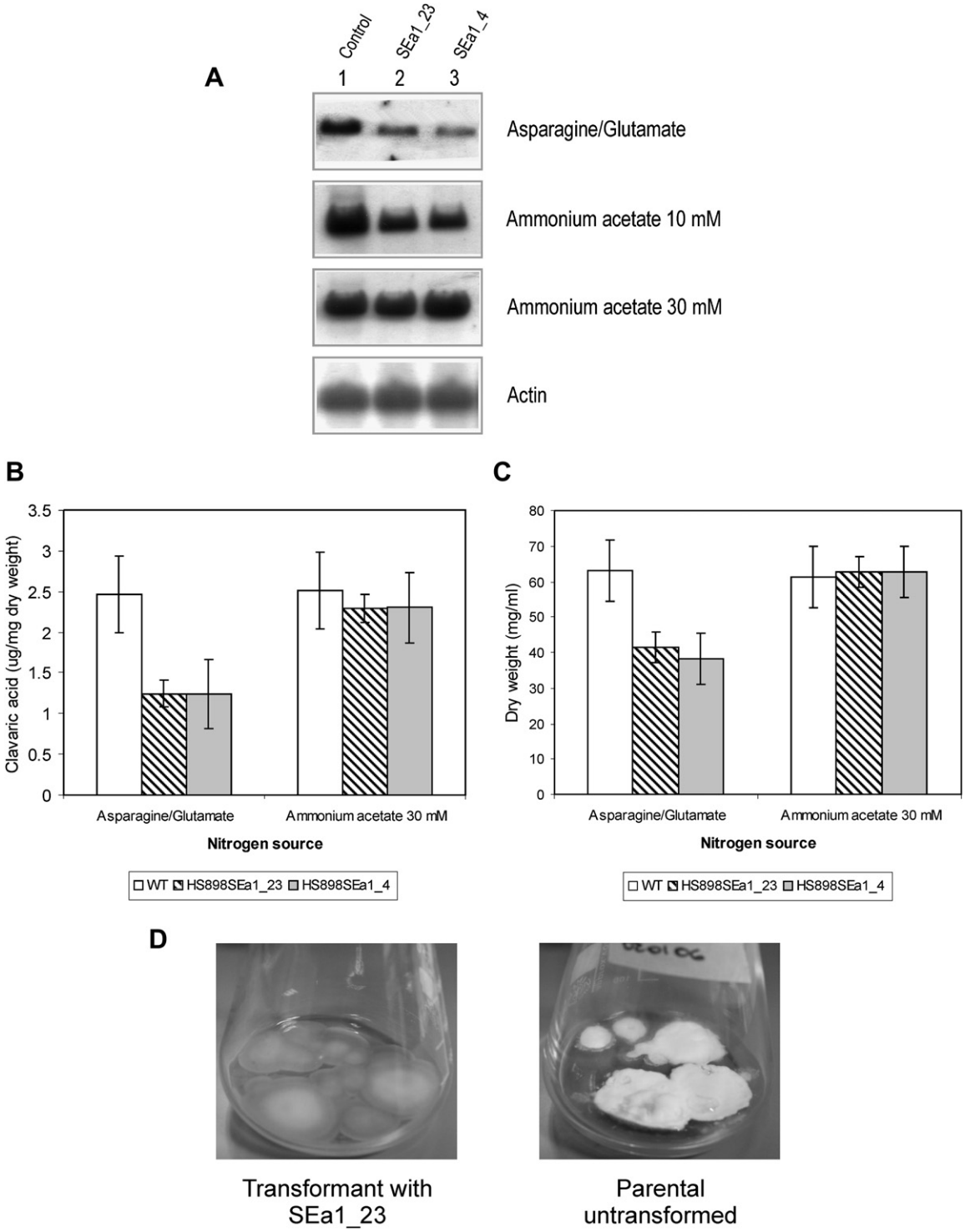


Figure 6. Expression of *erg1*, Growth, and Clavaric Acid Production

(A) Northern hybridizations showing expression of *erg1* in two different transformants containing the antisense constructions SEa1 in MM containing either asparagine/glutamate, 10 mM ammonium acetate, or 30 mM ammonium acetate, respectively. Hybridizations were performed with a 340 bp probe internal to *erg1*. RNA samples were taken from cultures at 15 days.

(B and C) Clavaric acid production (specific yield) and dry weight in static cultures of the same transformants and the control strain grown under the conditions of (A). Cultures were performed in triplicate. Vertical bars on top of each column indicate the standard deviation values of the mean of the three cultures.

(D) Aspect of growth in static cultures in MM supplemented with 20 μg/ml ergosterol of a transformant SEa1-23 with the antisense construction pHgSEa1 (left) as compared to the untransformed parental strain (right). The growth of the transformant with the antisense construction is dependent upon ergosterol supplementation.

alteration of the morphology and increased sensibility to several antifungal drugs including terbinafine [43]. Similarly, overexpression of the *erg1* gene in *H. sublateritium* increases the resistence to terbinafine.

The cloned *H. sublateritium* SE seems to be involved in the biosynthesis of ergosterol and clavaric acid. Similarly, the SE has been shown to be involved both in phytosterols and bioactive triterpene saponins in *Panax ginseng* [44]. The similarity of the early steps of the ergosterol and clavaric acid biosynthesis explains that the Erg1 protein is common for both the clavaric acid and ergosterol biosynthesis. The isoprenoid pathway for both compounds appears to be identical up to the (3*S*)2,3-oxidosqualene (Figure 1). However, whereas the (3*S*)2,3-oxidosqualene is used directly for lanosterol biosynthesis (transformed later into ergosterol), the clavaric acid intermediate clavarinone requires another oxidation step at carbons 22, 23 to form (3*S*,22*S*)2,3:22,23-dioxidosqualene. Dioxidosqualene is a known precursor of onocerin, a plant secondary metabolite. There is also a report suggesting that dioxidosqualene may be a precursor of lanosterol in the ergosterol biosynthetic pathway [45]. The hydroxyl groups at carbons 2,3 are retained as hydroxyl and keto groups respectively, in the molecule of clavarinone, and the C-3 hydroxyl group is finally esterified with a 3-hydroxy-3-methylglutaryl moiety in the clavaric acid. The two rounds of epoxidation may be catalyzed by the Erg1 SE since the same occurs in other organisms; e.g., partially purified SE from pig liver converts 2,3-oxidosqualene to 2,3:22,23-squalene dioxide [46]. The existence of a related enzyme catalyzing the second oxidation steps in *H. sublateritium* seems unlikely since the hybridization studies argue against the presence of more than one allele of *erg1* in this basidiomycete. An alternative pathway for the conversion of oxidosqualene to clavarinone involving lanosterol as an intermediate (dashed lines in Figure 1) does not seem likely since supplementation of the *Hypholoma* cultures with lanosterol did not increase clavaric acid production. The observed inhibitory effect of lanosterol indicates a feedback regulation of clavaric acid biosynthesis by this sterol.

The nitrogen source-regulated *gdh* promoter from *A. bisporus* (cloned in this work; see Experimental Procedures) proved to be an excellent promoter for overexpressing in *Hypholoma* either the antisense RNA or the *erg1* gene itself. Previously, we have reported that commonly used ascomycete promoters are not efficient for gene expression in the basidiomycetes [47]. Unfortunately the number of constitutive or regulable basidiomycete promoters available for molecular genetics studies is very low, and this is a serious limitation for progress in our understanding of the biosynthesis of pharmacologically active secondary metabolites in mushrooms. The good expression of the antisense RNA from the nitrogen source-regulated *A. bisporus gdh* promoter allowed us to silence partially the *erg1* gene. The results of these experiments proved that *erg1* is involved in both ergosterol and clavaric acid biosynthesis.

Overexpression of *erg1* from the *gdh* promoter in asparagine/glutamate medium led to a clear increase of clavaric acid specific production; only minor increments of production were observed in the ammonium-based (repressing) medium, thus confirming that the effect of *erg1* amplification on clavaric acid production is directly correlated to the level of expression of *erg1*.

Clavaric acid is a valuable natural product synthesized in minute amounts by the wild-type *H. sublateritium* strain. The constructions overexpressing the *erg1* gene are very useful to obtain increased amounts of this antitumor drug.

SIGNIFICANCE

This study provides clear evidence for the involvement of the squalene epoxidase encoded by the cloned *erg1* gene in the biosynthesis of the antitumor compound clavaric acid in the producer basidiomycete *Hypholoma sublateritium*. Basidiomycetes are very difficult to manipulate at the molecular level. Genetic modification of *Hypholoma* has been achieved by *Agrobacterium tumefaciens* mediated transformation. Silencing of the *erg1* gene by antisense expression of *erg1* DNA fragments resulted in a reduction of the specific yield of clavaric acid and also in an ergosterol-dependent growth of the transformants. These results support the conclussion that the *erg1*-encoded squalene epoxidase is involved in both clavaric acid and ergosterol biosynthesis. Natural yields of clavaric acid are very low. Overexpression of the *erg1* gene in *H. sublateritium* resulted in a 32% to 97% increment of clavaric acid production.

EXPERIMENTAL PROCEDURES

Microbial Strains and Culture Media

H. sublateritium ATCC74314 [6], a dikaryotic strain, was routinely grown on solid MEA (20 g/l malt extract, 5 g/l peptone, 20 g/l glucose, 15 g/l agar) medium at 20°C. To obtain arthrospores, the cultures were grown on solid PDA medium (Difco, Detroit, Michigan) at 20°C for 3 weeks and collected by scraping the surface with glass beads in sterile water. Liquid *H. sublateritium* cultures were grown in 100 ml of MEA broth at 20°C and 200 rpm. For clavaric acid production studies or transcriptional analysis, *H. sublateritium* wild-type strain and the transformants were grown in minimal medium (MM) (glucose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; H_2KPO_4, 0.5 g; HK_2PO_4, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.4 mg [1.6 μM]; $MnCl_2 \cdot 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamin hydrochloride, 0.1 mg; biotin, 5 μg; distilled water, up to 1 l) with different nitrogen sources (asparagine-glutamate, 15 mM each or ammonium acetate 10 mM and 30 mM). Erlenmeyer flasks (250 ml) containing 50 ml medium were inoculated with two agar plugs (each 0.25 cm^2) cut out from a colony grown on solid MEA medium. Incubation was carried out at 20°C under static conditions since previous studies revealed that static incubations were optimal for clavaric acid production.

Escherichia coli LE392 was used as a host strain for propagation of bacteriophages. All plasmid subcloning experiments were performed in *E. coli* DH5α that was transformed with competent cells by standard protocols [48]. *Agrobacterium tumefaciens* AGL1 was used as a host strain for transformation in conjugation experiments.

Preparation of Ergosterol and Terbinafine

Ergosterol was prepared at a stock concentration of 12 mg/ml in ethanol and Tween 80 (1:1, v/v). The solution was heated at 65°C during 5 min before using it. Terbinafine (TRB) (Terbinafina 250 mg, Teva

Genéricos Madrid, Spain) was dissolved in 100% DMSO at a stock concentration of 25 mg/ml and used at the final concentrations indicated in the Results section.

DNA Procedures and Plasmids

All basic DNA methods were performed as described by Sambrook et al. [48]. Genomic DNA from both wild-type and hygromycin-resistant *H. sublateritium* transformants was obtained by freezing cells from a 20 d liquid culture in liquid nitrogen and grounding the frozen pellet to a fine powder in a mortar, as described previously [47].

A binary plasmid vector (8.5 kb), designated pHg3Zf, consisting of a pBGgHg backbone [49] containing the *hph* gen, the CaMV 35S transcriptional terminator, and the *gpd* promoter from *A. bisporus*, was constructed. The polylinker (*Pvu*II fragment) of the plasmid pGEM 3Zf(+) (Promega, Madison, WI) was used to replace a *Pvu*II-SmaI (*egfp* gene) fragment of plasmid pBGgHg (novel construction pHg3Zf).

In order to construct the expression vector pGDHA-SEover, intermediate plasmid pGDHA was made by inserting the *A. bisporus gdhA* promoter sequence (obtained by PCR amplification with primers PGDHA-F and PGDHA-R containing *Sac*I and partial SmaI restriction sites, respectively) in the vector pGEM 3Zf(+) (Promega, Madison, WI) previously digested with *Sac*I and SmaI restriction endonucleases. The intermediate plasmid pGDHA-SE was made by inserting the *erg1* gene sequence obtained by PCR amplification (3.5 kb) with primers RTSEF and EMBLF5, in the SmaI restriction site of the plasmid pGDHA. Finally, pGDHA-SEover was constructed by inserting the cassette containing the *erg1* gene with the *gdhA* promoter from pGDHA-SE (obtained by PCR amplification with primers PGDHA-F and EMBLF5) by blunt-end ligation at the *Pvu*II-*Sma*I site in the plasmid pBGgHg [49] (Figure 3).

Antisense RNA Constructions

For the *erg1* gene attenuation studies, plasmids pHg-SEa1 and pBG-SEa2 contained a fragment of the *erg1* gene of *H. sublateritium* in the antisense position under control of the *A. bisporus* glutamate dehydrogenase promoter (P*gdhA*) and the transcriptional terminator of the same gene. The hygromycin resistance gene was used as a selectable marker.

Intermediate plasmid pGDHA-2 was made by inserting the *A. bisporus gdhA* transcriptional terminator region (obtained by PCR amplification with primers TGDHA-F and TGDHA-R containing BamHI and partial SmaI restriction sites, respectively) in the vector pGDHA previously digested with SmaI and BamHI restriction endonucleases. The 800 bp fragment of the *erg1* gene used in the antisense construction pHg-SEa1 was amplified by PCR with oligonucleotides SE4 and SE6. This PCR product was cloned in the SmaI site of the plasmid pGDHA-2 to create the pGDHA-2SEa. Finally, pHg-SEa was constructed by inserting the *Sac*I-BamHI fragment containing the antisense cassette of plasmid pGDHA-2SEa in the vector pHg3Zf, previously digested with the same restriction endonucleases (Figure 3).

In order to construct vector pBG-SEa2, intermediate plasmid pGDHA-4 was made by inserting the polylinker (*Pvu*II fragment) of the plasmid pGEM 5Zf(+) (Promega, Madison, WI) in the SmaI site of the plasmid pGDHA-2. The 1600 bp fragment of *erg1* gene used in the antisense construction pBG-SEa2 was amplified by PCR with oligonucleotides RTSEF and RTSER and cloned in the plasmid pGEM-T Easy (Promega, Madison, WI). The plasmid obtained, designated pGEM-ERG1, was digested with the *Not*I restriction enzyme. This fragment was subcloned in vector pGDHA-4 to obtain plasmid pGDHA-4SEa. The *Hind*III fragment of this vector was refilled (Klenow fragment of the DNA polymerase) and cloned in plasmid *Pvu*II-digested pBGgHg digested *Pvu*II, to obtain plasmid pBG-SEa2 (Figure 5). Artrospores of *H. sublateritium* were transformed by the *A. tumefaciens*-mediated method [47].

Genomic Library Construction and Screening

Genomic DNA from *H. sublateritium* ATCC74314 was partially digested with *Sau*3A. DNA fragments (10 to 22 kb) were ligated into the BamHI site of the lambda phage vector EMBL3, followed by in vitro packaging. Recombinant bacteriophage plaques of the genomic library were screened by the PCR procedure of Vaiman [25] with oligonucleotides SE1 and SE2 as primers.

Southern Blot Analysis

Southern blotting was performed by standard procedures [48]. Digoxigenin labeling, hybridization, and detection were performed with the Genius Kit (Roche Diagnostics). Hybridizations were performed at 42°C with a 5× SSC solution containing 0.1% lauryl-sarcosine, 0.02% SDS, 40% formamide, and 2% blocking reagent. The hybridization signals were visualized with a chemiluminescent substrate for alkaline phosphatase (CDP-Star), according to the manufacturer's (Roche) protocol. For Southern experiments, a 340-bp *Nco*I-*Xmn*I DNA fragment, internal to the *erg1* gene, were used as probe.

Primers Used for Cloning and Sequencing the *erg1* Gene of *H. sublateritium*

Three conserved flavin-binding sequence motifs were used to design the primer pairs SE1 and SE2 according to the codon usage in filamentous fungi. PCR amplifications were performed with *Taq* DNA polymerase (Promega, Madison, WI), using genomic DNA of *H. sublateritium* as template. The resulting 0.8 kb DNA fragment was sequenced to confirm the presence of the gene of interest and used as a probe to screen the *H. sublateritium* genomic library.

PCR Analysis

The presence of the *erg1* expression cassette (construction pGDHA-Seover) in the transformants was confirmed by PCR with primers SE14 (internal to the *erg1* gene) and HPH4 (internal to the hygromycin resistance *hph* gene in the cassette).

Attenuation of *erg1* gene expression was studied by the antisense RNA strategy. The presence of the intact attenuation cassettes (pHg-SEa1 and pBG-SEa2) in the transformants was confirmed by PCR with primers PGDHA-F and TGDHA-R; these primers correspond, respectively, to the *A. bisporus gdhA* promoter and the *gdhA* terminator.

Inverse PCR (IPCR) was used for the rapid in vitro amplification of DNA regions that flank a region of known *erg1* sequence. The template for the reverse primers was a mixture of *Eco*RV restriction fragments that were circularized by self-ligation. For circularization, 50 ng of the appropriate restriction fragment (2.8–3.5 kb) was diluted to a concentration of 50 ng/ml in ligation buffer. The ligation reaction was allowed to proceed for 10 hr at 16°C. The PCR was performed in reactions containing 0.1 ng of *Nco*I digested circularized DNA, using one cycle of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1.5 min, primer annealing at 55°C for 1 min, and extension by *Taq* polymerase (Promega, Madison, WI) at 70°C for 4 min.

RNA Isolation and Northern Analysis

Total *H. sublateritium* RNA was isolated with the RNeasy kit (QIAGEN). Northern analyses were performed by standard methods with 10 μg of total RNA per lane. A 340 pb *Nco*I-*Xmn*I fragment internal to the *H. sublateritium erg1* gene was labeled and used as the probe. Hybridizations were carried out as described by Sambrook et al. [48]. The intensity of the hybridizing bands was determined with the Quantity one 1D Analysis software (BioRad). A 666 bp *Sac*I probe internal to the *H. sublateritium* γ-actin gene was used to estimate the total amounts of RNA in gels.

Reverse Transcriptase-Polymerase Chain Reaction

cDNA was obtained from total RNA extracted from mycelia grown for 15–18 days in MEA medium. To verify the presence of a putative intron in the genomic DNA sequence, the region containing the expected intron splice sites was amplified from RNA with platinum Taq polymerase (SuperScript One-Step RT-PCR kit, Invitrogen, San Diego, CA), by using the oligonucleotides EX1F and EX1R, EX2F and EX2R, EX5F and EX5R, or EX2F and EX5R as primers. The PCR products obtained were directly cloned in the pGEM-T Easy vector (Promega,

Madison, WI) and sequenced to confirm the presence of the intron. All RNA samples were treated with RQ1 Rnase-Free Dnase (Promega, Madison, WI).

Computer Analysis

Computer analysis of nucleotide and amino acid sequences was carried out with the Lasergene DNASTAR programs, BLASTX2 and BLASTN software packages (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were constructed with CLUSTALW (http://www.ebi.ac.uk/tools/clustalw/). Putative conserved domains were detected with PFAM (http://www.sanger.ac.uk/Software/Pfam/). The nucleotide sequence of *H. sublateritium erg1* gene was deposited in the GenBank database under accession number EF581376.

Clavaric Acid Analysis and Quantification

Samples of 15 ml taken from the cultures of *H. sublateritium* wild-type and transformants were extracted twice with chloroform (60 ml) and sulphuric acid 5% (50 μl) for 5 hr with shaking (220 rpm). The organic extracts were pooled, concentrated to dryness in a vacuum evaporator, and dissolved in methanol (1 ml). The organic solvent solutions (200 μl) were derivatized according to the method of García et al. [50] and quantified by HPLC with pure clavaric acid as standard. The chromatography was performed in a Shimadzu 10 AD-VP chromatograph connected to a photodiode detector with a LiChrospher 100RP-18 (250 × 4 mm) 5 μm pore column. A mobile phase (flow of 0.7 ml/min) containing water:acetonitrile 95:5 (v/v) (solvent A) and methanol:acetonitrile 95:5 (v/v) (solvent B) was used with a gradient as follows: time zero 53% B, 13–18 min 89% B, 18–20 min 100% B, and 25–30 min 53% B. Under these conditions, the retention time of the clavaric acid was 21.4 min quantified at 254 nm. Standards of the clavaric acid were provided by F. Pelaez (CIBE, Merck Sharp & Dohme de España).

Terbinafine Susceptibility Test

The analysis of the resistance to terbinafine was performed with a petri dish growth assay where the growth of *H. sublateritium* and the *erg1* overexpression transformants SEover-43 and SEover-21 was examined. Fresh 0.25 cm^2 agar plug of a 10 day old culture grown on solid MEA medium were plated on MM plates with 0, 2.5, 6.25, or 12.5 μg/ml of terbinafine. The plates were incubated at 20°C, and the growth was estimated as previously described by Panagou et al. [51]. Images were captured with a high-sensitivity Canon EOS 10D (DS6031) (Canon USA, Inc., New York, USA) digital camera and processed with Image Pro Express version 6.0 (Media Cybernetics) image-analysis software. All plates were photographed every day. Each plate was corrected for background by subtracting the image of an uninoculated (blank) plate incubated together with the test plates. Images were 512 by 512 pixels. Visible growth is expressed in integrated optical density (IOD) units.

Supplemental Data

Supplemental Data include one table on the effect of lanosterol on clavaric acid production and are available at http://www.chembiol.com/cgi/content/full/14/12/1334/DC1/.

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Accession Numbers

The nucleotide sequence of *H. sublateritium erg1* gene was deposited in the GenBank database under accession number EF581376.