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The Cytochrome b_5 dependent C-5(6) sterol desaturase *DES5A* from the endoplasmic reticulum of *Tetrahymena thermophila* complements ergosterol biosynthesis mutants in *Saccharomyces cerevisiae*

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Abstract

Tetrahymena thermophila is a free-living ciliate with no exogenous sterol requirement. However, it can perform several modifications on externally added sterols including desaturation at C5(6), C7(8), and C22(23). Sterol desaturases in *Tetrahymena* are microsomal enzymes that require Cyt b_5, Cyt b_5 reductase, oxygen, and reduced NAD(P)H for their activity, and some of the genes encoding these functions have recently been identified. The *DES5A* gene encodes a C-5(6) sterol desaturase, as shown by gene knockout in *Tetrahymena*. To confirm and extend that result, and to develop new approaches to gene characterization in *Tetrahymena*, we have now, expressed *DES5A* in *Saccharomyces cerevisiae*. The *DES5A* gene was codon optimized and expressed in a yeast mutant, *erg3*Δ, which is disrupted for the gene encoding the *S. cerevisiae* C-5(6) sterol desaturase *ERG3*. The complemented strain was able to accumulate 74% of the wild type level of ergosterol, and also lost the hypersensitivity to cycloheximide associated with the lack of *ERG3* function. C-5(6) sterol desaturases are expected to function at the endoplasmic reticulum. Consistent with this, a GFP-tagged copy of Des5Ap was localized to the endoplasmic reticulum in both *Tetrahymena* and yeast. This work shows for the first time that both function and localization are conserved for a microsomal enzyme between ciliates and fungi, notwithstanding the enormous evolutionary distance between these lineages. The results suggest that heterologous expression of ciliate genes in *S. cerevisiae* provides a useful tool for the characterization of genes in *Tetrahymena*, including genes encoding membrane protein complexes.

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Keywords

C-5(6) sterol desaturase; *Tetrahymena*; endoplasmic reticulum; complementation; *Saccharomyces cerevisiae*; ergosterol

INTRODUCTION

Sterol composition in eukaryotic organisms is very diverse; while cholesterol is the predominant sterol in vertebrates and ergosterol in fungi, stigmasterol, β-sitosterol and campesterol are the most abundant in plants. More complex and diverse sterol profiles can be found in protozoans and microalgae, with additional sterol ring and side chain modifications such as (de)methylation, (de)saturation and oxidation [1]. In addition, some invertebrates, alveolates (a group to which ciliates belong) and a few flagellated parasites cannot synthesize sterols at all, and are therefore strictly auxotrophic [2]. Interestingly, many of them are nonetheless able to perform structural modifications or rearrangements on the sterol moiety [3, 4].

The ciliate *Tetrahymena* is an interesting case. This fresh-water protozoan has no sterol requirement and no detectable sterols in its membranes. Instead, it makes and uses tetrahymanol, a compound closely related to hopanoids which function as sterol surrogates in prokaryotes [5]. However, in response to the addition of selected sterols to the growth medium, *Tetrahymena* suppresses the formation of tetrahymanol and replaces this compound by the added sterol, either with or without prior modification(s). Four sterol-modifying activities have been detected in the ciliate: desaturation in positions C-5(6), C-7(8) and C-22(23) and the removal of the ethyl group on position C24 in C29 sterols [6, 7]. As a result of these transformations, the ciliate accumulates a Δ5,7,22 trien (C27) sterol in its membrane, as the major product.

We have recently identified *DES5A* (GenBank ID: FJ940725.1) (TTHERM_01194720), the ene encoding a C-5(6) sterol desaturase in the ciliate, by a knockout approach [8]. The gene is divergent in sequence from known C-5(6) sterol desaturases,, raising the question of whether it has also diverged in fundamental mechanistic aspects. One way to explore this question is to ask whether the Tetrahymena gene can rescue sterol desaturation mutants in other organisms. In *S. cerevisiae*, the C-5(6) sterol desaturase is an endoplasmic reticulum enzyme encoded by the *ERG3* gene [9,10], whose disruption blocks ergosterol biosynthesis. In previous studies the yeast *erg3* null mutant has been successfully complemented with C-5(6)sterol desaturase homologs from plants, mammals and algae[11, 12], providing a powerful approach to confirming functional homologs. Erg3p, as well as the corresponding enzyme in plants and vertebrates, is a membrane-bound enzyme that requires cytochrome b_5 and cytochrome b_5 reductase for its activity [13, 14]. Cyt b_5 is a small tail anchored protein of the endoplastic reticulum membrane with an N-terminal globular cytosolic haem-binding domain, and a hydrophobic transmembrane domain followed by a carboxyl ER-targeting luminal domain [15]. Interestingly, structural studies of the *T. thermophila* Cyt b_5 suggest that the ciliate protein may differ slightly, both in molecular mass and spectrophotometric absorption peaks from the ones found in mammals and yeast [16]. This apparent divergence may reflect relatively fast evolution of Tetrahymena Cytb5, a phenomenon that has been noted for other ciliate genes, and raises the question of whether fast-evolving *Tetrahymena* sterol desaturases are still compatible with the microsomal electron transport machinery in distantly-related organisms like *S. cerevisiae*. This question is underscored by the unusual sterol metabolism in *Tetrahymena*. To address these questions, we expressed a codon-optimized variant of *DES5A* in *S. cerevisiae*, and tested its function as well as its

localization. We also expressed the GFP-tagged protein in *Tetrahymena*, in order to compare localizations in the two distantly-related lineages.

MATERIAL AND METHODS

Strains, growth conditions and plasmids

The following yeast strains were used in this study: Saccharomyces cerevisiae W303 (MATa/MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} [phi$^+$]) denoted as wild-type strain and the erg3 mutant strain (MATα ade2-1 his3-11,15 leu2-3,112, trp1-1, ura3-1, can1, erg3::TRP1 upc2::HIS3) [17]. These strains were grown aerobically at 30° C either in YPD containing 2% glucose (Merck), 1% Yeast Extract (Oxoid) and 2% peptone (Oxoid) or in synthetic minimal media containing 0.67% Yeast Nitrogen Base (US Biological) supplemented with the appropriate amino acids and 2% glucose as carbon source. The DES5A gene (TTHERM_01194720) was synthesized with codons optimized for expression in S. cerevisiae (Genscript, USA). The ORF was re-amplified with primers containing HindIII/XhoI restriction sites and subcloned into p425GPD, the 2-micron yeast expression vector containing a glyceraldehyde-3-phosphate dehydrogenase promoter [18]. The plasmid was designed pC-5T. For expression of GFP, the enhanced green fluorescent protein was cloned into HindIII/XhoI sites of p425GPD, obtaining the pGFP plasmid, used as a control. For the GFP fused to DES5A construct, the enhanced green fluorescent protein was fused at the C-terminal of DES5A gene by overlapping PCR and cloned into the HindIII/XhoI sites of p425GP, obtaining the pC-5T. GFP plasmid. S. cerevisiae strain were transformed by electroporation and selected on minimal agar plates without leucine [19].

T. thermophila strain CU428 (mpr1- 1/mpr1-1; mp-s, VII), is designated as wild type (WT) in this work. Cells were grown at 30°C in 125 ml Erlenmeyer flasks containing 20 ml SPP medium with the following composition (wt/vol): 1% proteose-peptone (Oxoid, U.K), 0.1% yeast extract (Merck, Germany), 0.2% glucose (Merck, Germany), and 0.003% iron citrate (Sigma-Aldrich).

Plasmid pmEGFP [20] contained a monomeric variant (A206K) of GFP together with the neomycin/paromomycin resistance gene under a cadmium-inducible metallothionein (MTT) promoter (*neo* 4 cassette) [21].

Construction of *DES5A*-GFP fusion in *Tetrahymena*

A 791 base pair C-terminal fragment of the *DES5A* gene, lacking the stop codon, and a downstream flanking region of 910 base pairs were amplified by PCR and cloned into the BamHI and HindIII sites respectively of pmEGFP using directional In-Fusion Cloning techniques (Clontech). The *DES5A*-GFP construct was then liberated with NheI and XhoI restriction enzymes from the vector and was introduced into the endogenous *DES5A* locus by homologous integration as diagrammed in Fig. 4A. After biolistic treatment [22], the somatic transformants were selected with 120 μg/ml of paromomycin in the presence of 1.0 ug/ml $CdCl_2$, which induces the *MTT1* promoter of the *neo* 4 expression cassette, and were transferred daily in increasing concentrations of paromomycin to allow phenotypic assortment. Single cells were then isolated and the clones expanded with daily transfers for 2 weeks in 40 mg/ml paromomycin.

Sterol extraction and GC-MS analysis

To analyze sterols in whole cells, 30 ml of a 48h (stationary) culture were harvested, resuspended in 1.5 ml distilled water and then saponified in 1.5ml 2M NaOH in methanol-water (1:1 v/v) at 80°C for 1 h. Sterols were extracted 3 times with 3ml hexane each and dried under nitrogen flow. The residue was resuspended in 100 μl N-methyl-N-

(trimethylsilyl) trifluoroacetamide (MSTFA), and then incubated for 30 min at 70°C. The composition of the steryl trimethylsilyl ester derivatives was analyzed by running samples through an HP-5MS (30 m. × 0.25 mm × 0.25 um, Agilent Technologies) in a Hewlett Packard HP 6890 gas chromatograph. The column was temperature programmed at 10°C/min from 100 to 310°C and subsequently held for 10 min at 310°C. MS was carried out using a HP mass selective detector (model MSD 5973) operated at an ionization voltage of 70 eV with a scan range of 50 to 600 amu. The retention time and mass spectrum of all new peaks obtained were compared to those of standards (Steraloids, USA) and those available in the NIST library.

The sterol content in samples are expressed as percentage of the total amount of sterols present in each sample (% w/w of total sterols).

Localization of GFP tagged proteins

Tetrahymena expressing *DES5A*-GFP were fixed with 4.2 % paraformaldehyde in 50 mM HEPES pH 7.0 for 10 min at room temperature, and permeabilized with ice-cold 0.1% Triton X-100 in 50 mM HEPES pH 7.0 for 8 min. After being washed with ice-cold HEPES three times, the fixed cells were treated with blocking solution (1% bovine serum albumin in TBS buffer) for 15 min at room temperature, and incubated for 30 min in blocking solution with anti-GFP primary antibody (A-11122; Invitrogen) at a 1:400 dilution. The cells were washed three times for 5 min each with TBS buffer containing 0.1% BSA and subsequently incubated for 30 min at room temperature with donkey anti-rabbit–Alexa Fluor 488 secondary antibody (A21206; Invitrogen) at a 1:200 dilution in blocking solution. After one wash in TBS buffer containing 0.1% BSA and two washes in 50 mM HEPES pH 7.0 the cells were mounted with Trolox antibleaching solution. Digital images were collected using a Carl Zeiss Axio imager M2 fluorescence microscope.

To localize *Des5Ap*-GFP in yeast, cells were collected by centrifugation for 5 minutes at 600 g and resuspended in 3 ml of freshly prepared 4% paraformaldehyde. After incubation for 20 minutes at room temperature, the suspension was centrifuged for 5 minutes at 350 *g* and cells were washed 3 times with 2 ml 0.1 M potassium phosphate buffer, pH 7.4 (KPB). Cells were resuspended in 2 ml KPB supplemented with 1.2 M sorbitol and incubated at 4°C overnight. Then cells were collected by centrifugation for 5 minutes at 600 *g*, resuspended in 0.1% Triton X-100 in PBS and incubated 10 min at room temperature. After washing three times with 1 ml PBS, cells were incubated for 10 minutes with 5 μM Hoechst 33258 stain (prepared in PBS also containing 3% BSA). The cells were washed three times with PBS and resuspended in DakoCytomation Fluorescent Mounting Medium (Dako). Cells were visualized using a Olympus FV300 confocal microscope (model BX61) with acquisition software FluoView version 3.3.

RESULTS

Divergence in *Tetrahymena thermophila* Cyt b_5 enzymes

Our previous analysis indicated that the *T. thermophila DES5A* gene is a member of the fatty acid hydroxylase superfamily (FAHS) [8]. This enzyme requires the cytochrome b_5 microsomal electron transfer for its activity. The family of cytochrome b_5-like proteins includes, besides cytochrome b_5 itself which is denominated as free Cyt b5, hemoprotein domains covalently associated with other redox domains in diverse fusion proteins.

In querying the *Tetrahymena thermophila* genome, we identified a large number of putative Cyt b5 genes. By searching for domains corresponding to Cyt b_5 (PFAM: PF00173) we identified 13 sequences, six of which possess a sequence length of 109-227 aa and include a hydrophobic transmembrane domain, and which therefore resemble free Cyt b5 proteins in

other organisms. The seven remaining sequences are similar but longer and possess other domains such as dopamine beta-monooxygenase (DOMON), catecholamine-binding domain (DOH) and fatty acid desaturase, and which therefore are not free Cyt b5 genes.

A phylogenetic tree generated by the Maximum likelihood method, based on 43 amino acid sequences of cytochrome b_5 proteins including the six putative homologs in Tetrahymena, clearly shows that animal, fungal, and plant proteins cluster according to lineage (Fig. 1). The Tetrahymena sequences do not group in a specific cluster, and thus appear to have undergone the extensive divergence noted previously for protist cytochrome b_5 proteins [23].

Complementation by heterologous expression of *DES5A* in the *Saccharomyces cerevisiae erg3* mutant strain

Given the extensive sequence divergence, it is possible that the Tetrahymena Des5Ap enzyme can only function with the Tetrahymena cytochrome b_5 providing microsomal electron transfer. Alternatively, the Tetrahymena desaturase may be able to use budding yeast (*S. cerevisiae*) Cyt $b_{5,}$, that only shares between 12.1% and 30.1% identify, or 17.6% and 53.4% similarity, with the Tetrahymena homologs. If the latter is true, it would allow us to characterize ciliate genes involved in sterol metabolism by taking advantage of the many tools available in *S. cerevisiae.* To explore this, we expressed *DES5A* in a haploid yeast strain deficient in *ERG3*. Since *Tetrahymena* utilizes an alternative nuclear genetic code [24], we first optimized codon assignment and frequency bias for expression in yeast. The optimized gene was cloned into a GPD expression shuttle vector (p425-GPD, with LEU2 as selectable marker), obtaining the pC-5T plasmid that was electroporated into the *erg3* mutant strain. The Leu+ isolates were checked by PCR for the presence of the *DES5A* gene and then screened for resistance to low levels of cycloheximide. The *erg3* mutant displays a marked hypersensitivity to cycloheximide, compared to wild type *Saccharomyces* strains [25]. As shown in Fig. 2, the *erg3* mutant expressing *DES5A* (*erg3* + pC-5T) shows increased resistance to cycloheximide.

Sterol profile in *S. cerevisae* WT, *erg3* mutant and *erg3* + pC-5T-complemented strain

We performed GC-MS analysis to determine the sterol composition of WT, the *erg3* mutant and *erg3+*pC-5T transformed cells (Fig 3 and Table 1). Major differences in sterol composition were noted between WT and *erg3*Δ strains in ergosterol and 5α-ergosta-7,22-dien-3β-ol content. In particular, *erg3*Δ cells accumulated less than 1% the level of ergosterol found in WT cells. Importantly, expression of the pC-5T plasmid in *erg3*Δ cells restored ergosterol synthesis in the mutant to 74% of the WT level. This result suggests that *DES5A* could significantly but incompletely complement the *ERG3* deletion. The conclusion that complementation was incomplete was also supported by analysis of intermediates in ergosterol synthesis. The *erg3* mutant accumulated an ergosterol precursor, 5α-ergosta-7,22-dien-3β-ol (47%) as the major sterol. This precursor makes up only 12% of the sterol pool in WT cells, but accumulates to high levels (41%) in the *erg3*Δ strain expressing *DES5A*. The profile of other intermediates in the ergosterol biosynthesis pathway, such as fecosterol (5α-ergosta-8-en-3β-ol) and lanosterol were also slightly different between the three strains (Table 1).

Sub-cellular localization of Des5Ap

Based on work in other systems, we predicted that Des5Ap would function at the level of the endoplasmic reticulum. The desaturase contains the C-terminal di-lysine motif, QIKQKKN, which have been demonstrated to be necessary for the retention of ER integral membrane proteins in different organisms[26]. To localize the enzyme in *Tetrahymena*, and to ask whether it localizes similarly when expressed in yeast, we C-terminally tagged both Des5Ap

and the variant optimized for yeast expression, with eGFP. To avoid potential misexpression artifacts in *Tetrahymena*, we targeted the tagged gene to its endogenous locus [21]. *T. thermophila* wild type cells (CU428 strain) were transformed with a *DES5A*-GFP construct that integrated in the somatic (expressed) macronucleus, so that the tagged gene was under the control of the endogenous promoter, as depicted in Fig 4A. Due to high autofluorescent background even in wild type cells, we increased the selectivity of the signal using anti-GFP antibody and indirect immunofluorescence microscopy. As shown in Fig. 4B, Des5Ap-GFP showed a strong signal around the nucleus, in a pattern that is consistent with nuclear envelope localization, and a more diffuse one in the cytoplasm. This pattern is consistent with ER localization in these cells[27, 28].

To assay localization of the C-5(6) sterol desaturase from *Tetrahymena thermophila* in yeast, the *DES5A* gene fused to eGFP was cloned into a GPD expression shuttle vector obtaining the pC-5T. GFP plasmid, which was introduced into the *S. cerevisiae erg3*Δ strain. A perinuclear signal, typical of ER anchored proteins [10], was observed in the complemented strain (Fig 5.). Expression of eGFP by itself under the same promoter (pGFP), produced only diffuse cytoplasmic fluorescence (Fig. 5). These results are consistent with those reported for other ER localized proteins in the ciliate [27] and with studies of the microsomal C-5 sterol desaturase in *Saccharomyces cerevisiae* [10].

DISCUSSION

We found that the *S. cerevisiae erg3* null mutant could be partially complemented by expression of the *T. thermophila DES5A* gene, confirming that Des5Ap has C-5(6) sterol desaturase activity. This enzyme is likely to require the activity of a cytochrome b_5 -dependent microsomal electron transport system, as previously shown in *Tetrahymena* for a stearoyl-CoA desaturase [29]. Although the cytochrome b_5 sequences are highly divergent between *S. cerevisiae* and *T. thermophila*, our results indicate that there is significant functional conservation since the ciliate sterol desaturase can function in the context of fungal cytochrome b_5. These results are in concordance with the work of Desmond and Gribaldo [2] which proposed that the last eukaryotic common ancestor already harbored many enzymes for sterol biosynthesis, and that subsequent evolution over the eukaryotic tree occurred by modifications or gene losses, as reported for other enzymes involved in sterol biosynthesis. Overall, our current analysis, based on expression of a ciliate gene in an evolutionarily-distant species, complements our previous studies based on disruption of the gene in *T. thermophila* via homologous recombination [8].

Sterol C-5(6) desaturases belong to the fatty acid hydroxylase superfamily (pfam: PF04116). These enzymes are integral membrane proteins that localize in the endoplasmic reticulum. An ER retention signal for similar proteins that has been characterized in other systems, consists of a C-terminal di-lysine or di-arginine motif [26]. Examples of sterol C-5(6) desaturasas containing this motif are found in different organisms, such us *Saccharomyces cerevisiae* (P32353 UniProtKB accession number), *Mus musculus* (O88822) and *Arabidopsis thaliana* (Q39208). Tetrahymena Des5Ap contains the QIKQKKN sequence at the C-terminal predicted cytosolic domain, which may be functioning as an ER retention signal. This is in accordance with ER localization in both organisms (Fig. 4 and Fig. 5). Other ciliates proteins with C-terminal di-lysine motif have been also localized in ER like the *Paramecium tetraurelia* Ca^{2+}-ATPase [30], implying that this type of mechanisms of ER retention for integral proteins are conserved in ciliates.

The ability to express ciliate proteins in heterologous systems with strong experimental tools, like *S. cerevisiae*, may be strongly advantageous for characterizing their activities. A handful of *T. thermophila* proteins and one enzymatic RNA have previously been expressed

in yeast, namely Histone H2A[31], the translation release factor eRF1[32], the cytosolic methionine salvage pathway protein mtnBD [33] and the ribozyme Group I intron [34]. Our report adds to this list, and moreover shows that a membrane-associated *Tetrahymena* protein that requires a compatible Cyt b_5/Cyt $b_5$reductase electron transport system, can function in a heterologous context. Our results are similar to a recent report in which a Δ5 fatty acid desaturase gene from another ciliate, *Paramecium tetraurelia,* was functionally expressed in *S. cerevisiae* [35], but with the significant difference that the *Paramecium* enzyme, a so-called "front-end" desaturase, contains a fused N-terminal Cyt b_5-like domain and therefore does not require the activity of the yeast Cyt b_5.

The rescue of erg3Δ by *DES5A* expression was incomplete, as evidenced by the only partial restoration of ergosterol levels in the complemented strain. Similar results have been reported when mammalian or plant C-5(6) sterol desaturases were expressed in *S. cerevisiae*. For example, the enzyme from *Arabidopsis* restored 46% of ergosterol accumulation when expressed in the erg3Δ mutant [36]. The incomplete complementation may be a consequence of aberrant transcriptional regulation of the transgenes, but downstream steps (e.g., translation, or trafficking) may also be non-optimal for the transgenes. In summary, our results suggest that functional complementation of *Saccharomyces cerevisiae* mutants can be used as an efficient approach for the analysis of genes in *Tetrahymena*. This includes both the study of membrane protein complexes, and the characterization of genes whose functions have not yet been established.

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Abbreviation key

Cyt	Cytochrome
ER	endoplasmic reticulum

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HIGHLIGHTS

- Ciliate *DES5A* gene restores ergosterol biosynthesis in *erg3 S. cerevisiae* strain.
- *DES5A* gene reverses the hypersensitivity to cycloheximide in *erg3* strain.
- Des5Ap is localized in endoplasmic reticulum of *T. thermophila* and *S. cerevisiae*.

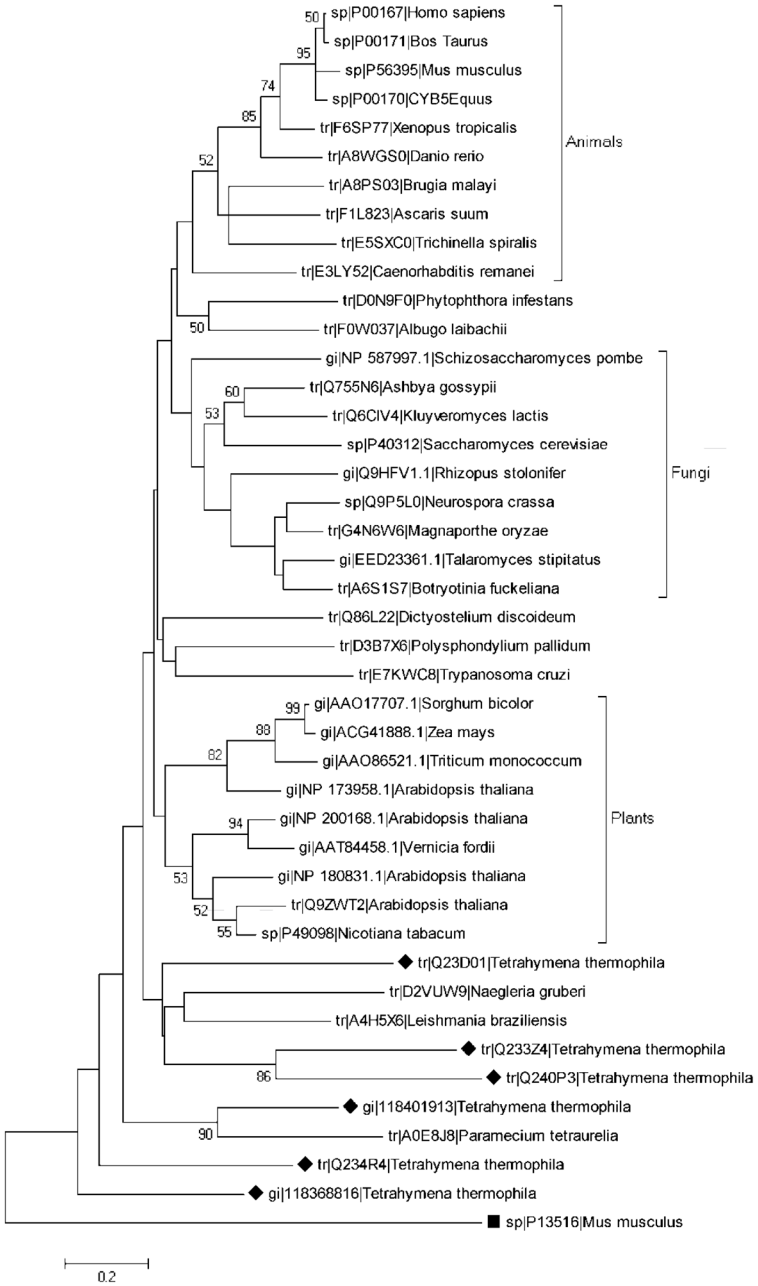

Fig. 1.

Phylogenetic analysis of cytochrome b5 proteins. The phylogenetic tree of 43 amino acid sequences (UniProtKB) was generated using a Maximum likelihood method with 500 bootstrap replicates with MEGA5 software, as described in Materials and Methods. The black rhombus indicate *Tetrahymena thermophila* putative cytb5 enzymes. The black box indicates a *Mus musculus* Acyl-CoA desaturase protein, containing a Cytb_5 domain, which is used as an outgroup. The bar indicates percentages of substitution.

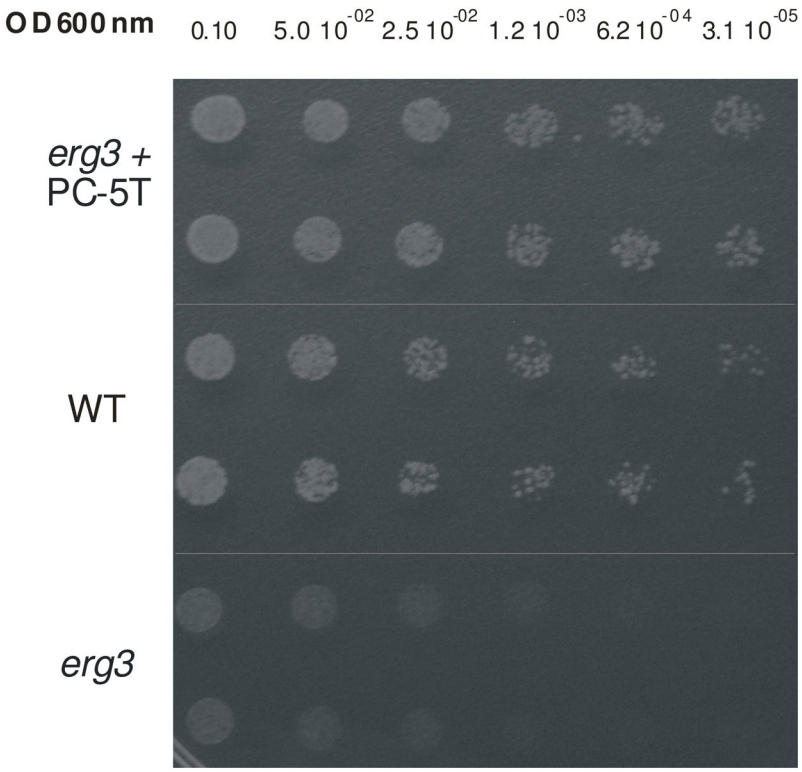

Fig. 2.

Hypersensitivity to cycloheximide in the *erg3* null strain is resuced by expression of the *DES5A* gene. Serial dilutions of the wild type, *erg3* null and *erg3*+pC-5T complemented strains were spotted onto plates containing minimum medium plus 0.01 μg/ml of cycloheximide and grown for 4 days at 28 °C.

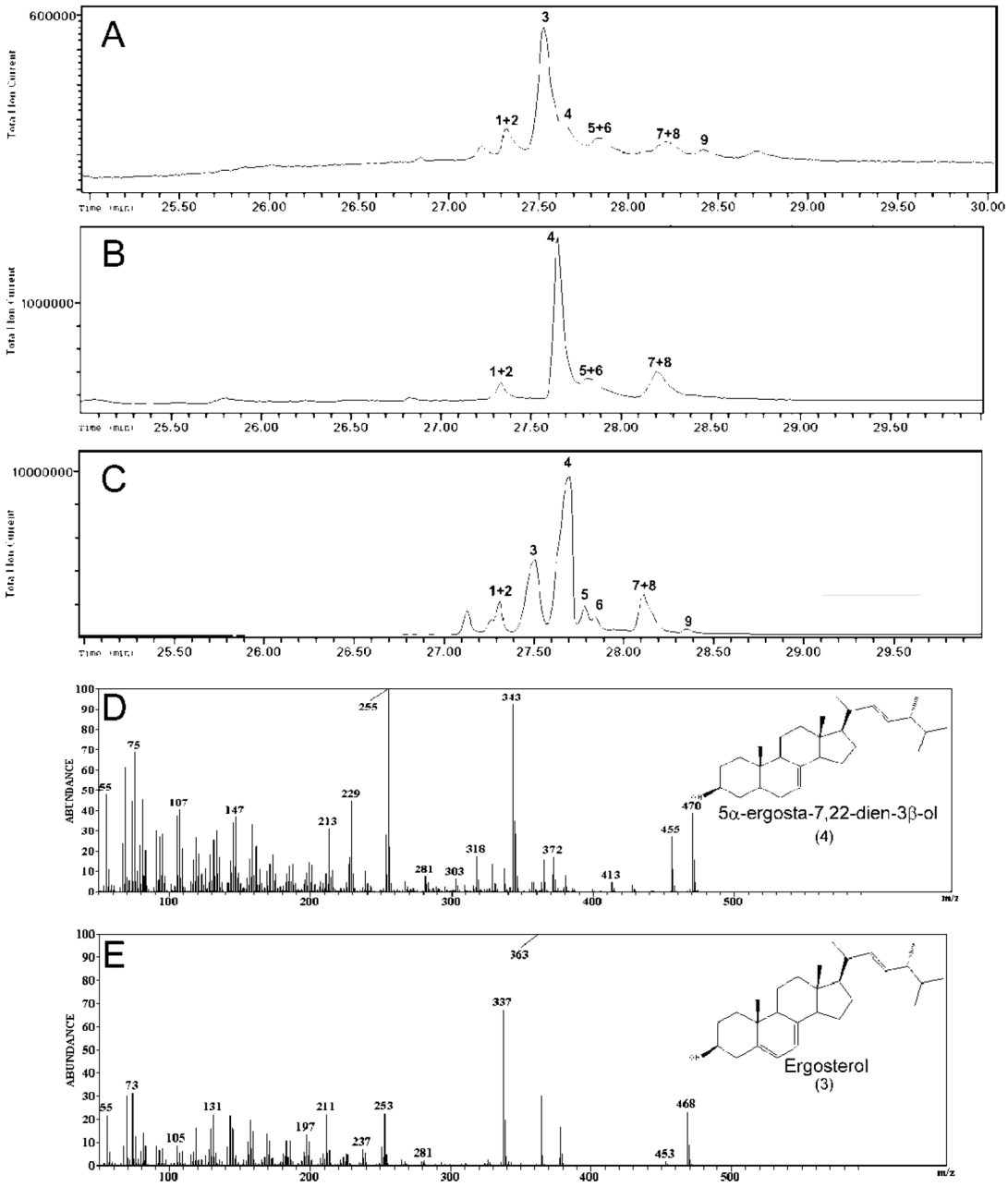

Fig. 3.

GC-MS analysis of sterols. A: *S. cerevisiae* WT 303. B: *erg3* null. C: *erg3+*p C-5T strain. D and E: mass spectra of 5α-ergosta-7,22-dien-3β-ol and ergosterol trimethylsilyl derivatives respectively. The compounds were identified with the NIST library. Peaks: 1 - zymosterol, 2 - 5α-ergosta-8,22-dien-3β-ol, 3 - ergosterol, 4 - 5α-ergosta-7,22-dien-3β-ol, 5 - fecosterol, 6 - 5α-ergosta-8-en-3β-ol, 7 - 5α-ergosta-7,24-dien-3β-ol, 8 - 5α-ergosta-7-en-3β-ol, 9 - lanosterol.

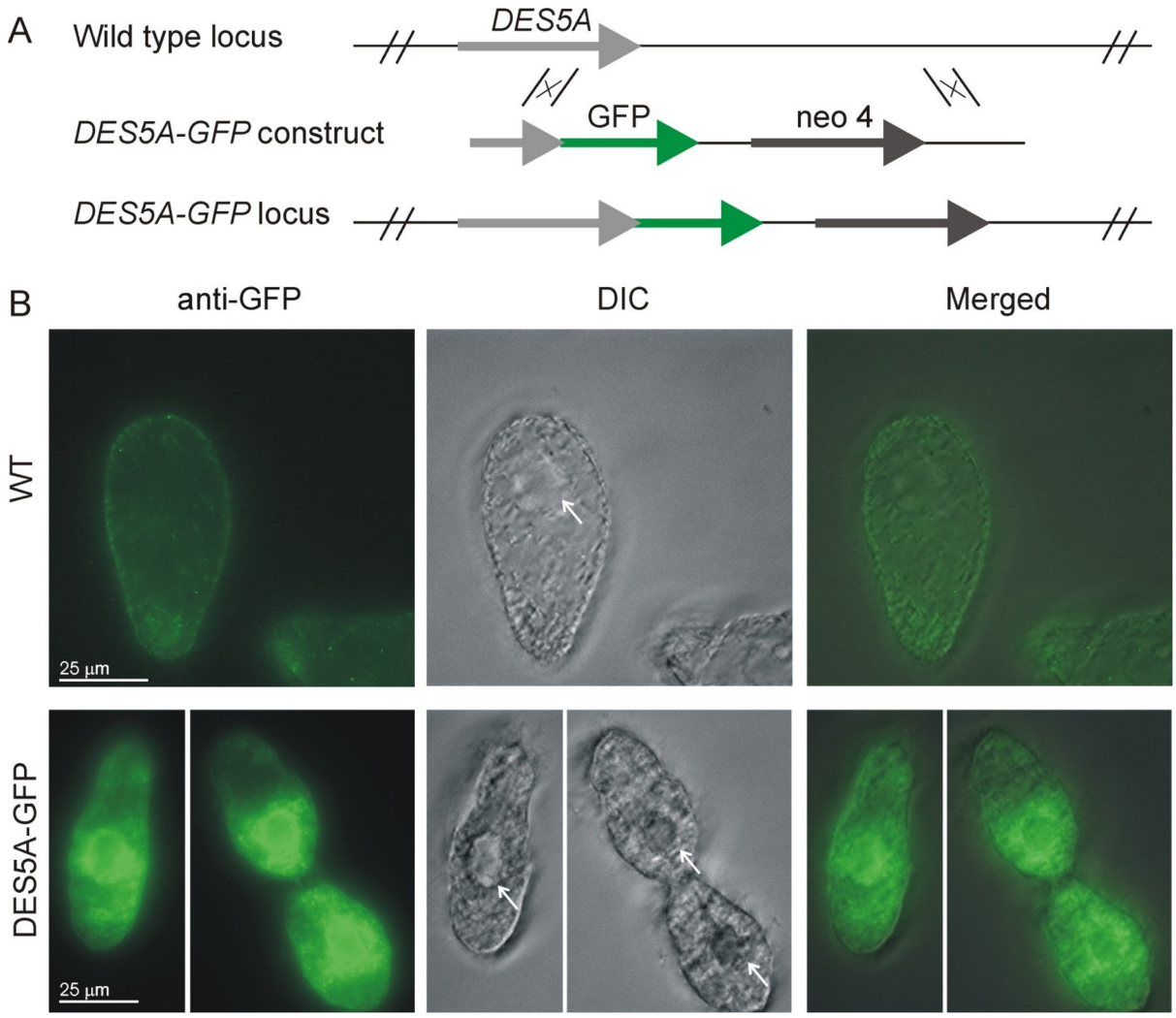

Fig. 4.

Localization of GFP tagged Des5Ap in *Tetrahymena thermophila* (A) Schematic representation of gene replacement in the WT locus of the *DES5A* gene by targeting the DES5A-GFP construct by homologous recombination, using a cassette in which neo4 confers paramomycin resistance. (B) Immunofluorescence localization of GFP-tagged *DES5A.* Wild-type (WT) and DES5A-GFP strains were grown to Log phase and fixed for indirect immunofluorescence staining. The GFP was localized by anti-GFP primary antibody followed by labeling with anti-rabbit–Alexa Fluor 488 secondary antibody. DIC: Differential interference contrast microscopy. White arrows indicate the nucleus of the ciliate.

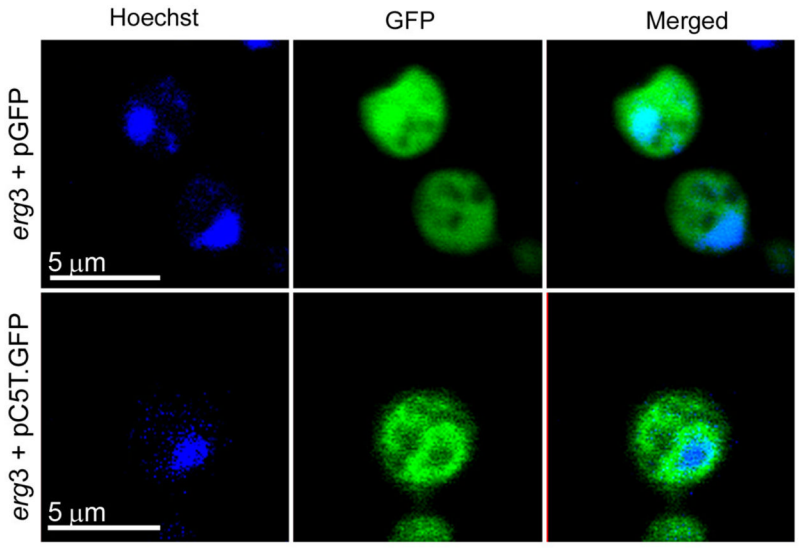

Fig. 5.

Localization of GFP-tagged Des5Ap in *Saccharomyces cerevisiae.* The *erg3* strain expressing GFP by itself (top) and GFP-tagged *DES5A* (bottom) were grown in minimum medium and analyzed at early log phase by direct fluorescence microscopy. DNA was stained with Hoechst.

Table 1

Sterol profile in *S. cerevisae* WT W303, *erg3* null mutant and *erg3*+ pC-5T complemented strains

Peak	m/z TMS	Sterol	Sterol content in strains (% w/w)*		
			Wild type	***erg3***	***erg3* + pC-5T**
1	456	Zymosterol	11	6	7
2	470	5α-ergostan-8,22-dien-3β-ol			
3	468	Ergosterol	46	<1	34
4	470	5α-ergostan-7,22-dien-3β-ol	12	47	41
5	470	Fecosterol	14	15	1
6	472	5α-ergostan-8-en-3β-ol			3
7	470	5α-ergostan-7,24-dien-3β-ol	11	16	12
8	472	5α-ergostan-7-en-3β-ol			
9	498	Lanosterol	6	16	2

*Sterol content is expressed as percentage of total sterols isolated in each strain (% w/w)