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A Novel Sterol Desaturase-Like Protein Promoting Dealkylation of Phytosterols in *Tetrahymena thermophila*[▽]

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The gene TTHERM_00438800 (*DES24*) from the ciliate *Tetrahymena thermophila* encodes a protein with three conserved histidine clusters, typical of the fatty acid hydroxylase superfamily. Despite its high similarity to sterol desaturase-like enzymes, the phylogenetic analysis groups Des24p in a separate cluster more related to bacterial than to eukaryotic proteins, suggesting a possible horizontal gene transfer event. A somatic knockout of *DES24* revealed that the gene encodes a protein, Des24p, which is involved in the dealkylation of phytosterols. Knocked-out mutants were unable to eliminate the C-24 ethyl group from C_{29} sterols, whereas the ability to introduce other modifications, such as desaturations at positions C-5(6), C-7(8), and C-22(23), were not altered. Although C-24 dealkylations have been described in other organisms, such as insects, neither the enzymes nor the corresponding genes have been identified to date. Therefore, this is the first identification of a gene involved in sterol dealkylation. Moreover, the knockout mutant and wild-type strain differed significantly in growth and morphology only when cultivated with C_{29} sterols; under this culture condition, a change from the typical pear-like shape to a round shape and an alteration in the regulation of tetrahymanol biosynthesis were observed. Sterol analysis upon culture with various substrates and inhibitors indicate that the removal of the C-24 ethyl group in *Tetrahymena* may proceed by a mechanism different from the one currently known.

Sterols are lipophilic membrane components essential for the structural integrity of most eukaryotic cells. Together with phospholipids, they regulate the fluidity of the lipid bilayers and permeability barrier properties (4); they also serve as precursors of bile salts and a number of different steroid hormones in mammals, brassinosteroids in plants and fungi (2), and ecdysteroids in arthropods (14). Sterols are also actively involved in the modulation of cell signaling, in the transport and distribution of lipophilic molecules, and in the formation of lipid rafts (22).

For most organisms in which sterols are *de novo* synthesized, such as vertebrates (cholesterol), plants (stigmasterol, β-sitosterol, and campesterol), and fungi (ergosterol), the enzymes involved have been well identified and characterized. Most of them can be grouped into four families of proteins: (i) the cytochrome b_5 (Cytb_5)-dependent fatty acid hydroxylase superfamily, composed of C-5 sterol desaturases (erg3), C-4 sterol methyl oxidases (erg25), and cholesterol 25-hydroxylases; (ii) the *S*-adenosyl-L-methionine sterol methyltransferase (SMT) family, composed of the SMT1 and SMT2 types, which are typical in plants, and C-24 sterol methyltransferase, which has been described for fungi (erg6); (iii) the highly hydrophobic reductases, which include C-7, C-14, and C-24 sterol reductases; and (iv) the cytochrome P450 family, with C-22 sterol desaturases (erg5) and C-14 sterol demethylases (erg11) as its main representatives. Other eukaryotic organisms from diverse orders cannot synthesize sterols. This is the case for invertebrates such as nematodes and arthropods (36), the alveolates *Tetrahymena* spp. (6), *Paramecium tetraurelia* (37), and *Plasmodium falciparum* (10), and the flagellated parasites *Giardia intestinalis* and *Trichomonas vaginalis* (18), among others. Most of these organisms are strictly auxotrophic for sterols but are still able to perform some structural modifications on the sterol moiety, such as dehydration, reduction, oxidation, dealkylation, or desaturation, at specific positions. For instance, the nematode *Caenorhabditis elegans* and the insects *Drosophila melanogaster* and *Bombyx mori* take sterols from their diet and convert phytosterols, like campesterol, sitosterol, and stigmasterol, into cholesterol (19); a C-24-dealkylating activity for these conversions is required. In insects, cholesterol is metabolized to ecdysteroids, 20-hydroxyecdysone (20E), and ecdysone (E), hormones involved in both molting and metamorphosis (14). In contrast, the nematode *C. elegans* metabolizes cholesterol into the 4-methyl sterols required for its development (12).

Among organisms with this metabolic diversity, *Tetrahymena* spp. are peculiar: the ciliates neither are auxotrophic nor have the machinery to synthesize sterols *de novo*. Therefore, when sterols are absent, *Tetrahymena* satisfies its vegetative growth with self-made triterpenoid alcohols—mainly tetrahymanol (a surrogate sterol similar to the hopanoids found in bacteria) (39). On the other hand, if a sterol is present, the synthesis of tetrahymanol is repressed; the sterol is incorporated and, in

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FIG. 1. Conversion of sterols by *Tetrahymena* sp. Final product and some of the intermediates formed in cultures supplemented with sitosterol. Tetrahymanol is synthesized in the absence of exogenous sterols. Abbreviations: C5-DES, C-5(6) sterol desaturase; C7-DES, C-7(8) sterol desaturase; C22-DES, C-22(23) sterol desaturase; C-24-DET, C-24 deethylation.

some cases, also modified. Four sterol modifications have been confirmed in *Tetrahymena*: desaturation on the sterol moiety at positions C-5(6), C-7(8), and C-22(23) and the removal of the ethyl group at C-24 of the lateral chain from C_{29} sterols (Fig. 1) (8). To date, it is not clear why these ciliates incorporate and modify the exogenous sterols when they are able to synthesize tetrahymanol.

Previous characterizations of sterol desaturases in *T. thermophila* have revealed some typical features, such as their oxygen, cytochrome b_5, and NAD(P)H dependence (28), which groups these enzymes within the superfamily of fatty acid hydroxylases (FAH). A bioinformatic analysis of the genome of this ciliate shows the presence of at least 8 putative sterol desaturase genes. One sequence (TTHERM_00487050) that encodes a hypothetical 2,049-amino-acid protein was excluded from the analysis, as it appears to be a fusion protein not containing the full canonical sequence of a desaturase. Four other sequences (TTHERM_01194720, TTHERM_00446080, TTHERM_00438800, and TTHERM_00758950) were assigned to a C-5 sterol desaturase cluster on the basis of our phylogenetic analysis; one of them (TTHERM_01194720) was conclusively characterized as such in our laboratory. Three other hypothetical proteins (TTHERM_00077800, TTHERM_00876970, and TTHERM_00348230) were assigned to the C-4 methyl oxidase cluster; however, it is not clear if the last activity really exists in *T. thermophila* (29).

In this work, another member of this superfamily was identified and characterized by a gene knockout (KO) approach. The encoded protein of *T. thermophila* is active in the C-24 deethylation of phytosterols. Although C-24 dealkylation has been reported for other organisms, the enzymes involved and their structural genes have not been identified to date. A distinctive feature of the mutant was its morphology and the altered regulation of tetrahymanol biosynthesis. Furthermore, phylogenetic analysis groups the enzyme in a well-defined branch in a separated cluster of the FAH superfamily, indicating higher similarities with bacterial sequences than with eukaryotic sequences, suggesting that the acquisition of the gene by the ciliate could be the result of a horizontal gene transfer event.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *T. thermophila* strain CU428 *mpr1-1/mpr1-1* (mp-s, VII), designated the wild type (WT) in this work, and plasmid pBS-MnB-3 were a gift from M. A. Gorovsky (University of Rochester, NY) (30). Cells were grown in 250-ml Erlenmeyer flasks containing 100 ml SPP medium of the following composition (wt/vol): 1% proteose-peptone (Oxoid, United Kingdom), 0.1% yeast extract (Merck, Germany), 0.2% glucose (Merck,

TABLE 1. Sequences and oligonucleotides used for PCR amplifications[a]

Primer target	Forward primer	Sequence of primer	Reverse primer	Sequence of primer
UP 607	1	CCGAGCTCAGAAGGACGGTCAATTAAAG	2	CGGGATCCATAAACACCCTTTAAACAACC
DW 607	3	GCGTCGACTTTGAGAAGATTTTCTGACA	4	CCCTCGAGTCAATCAAAGTCAACAAACAAAG
neo3	5	TGAATAACTCCTTTAATTTAAATACAC	6	AGAGCTAACATGTATGTGAAGAGG
WT 607 allele	7	GTGAAGATTTAGTCGATATAATTTG	8	TGCAGAGCTTAAATTGATTGGC
KO 607 allele	7	GTGAAGATTTAGTCGATATAATTTG	9	AATGAGCTATGTTTTGAGCAGC
TTHERM_438800	10	TCTATAGTCTTTGGAGGTTTCG	8	TGCAGAGCTTAAATTGATTGGC
α-Tubulin	11	TGTCGTCCCCAAGGAT	12	GTTCTCTTGGTCTTGATGGT
DES24g	13	ATGTCATTTATTTGTTTATGACAG	14	TCATAAAAATAATTGCTAAAGTAAC
DES24c	10	TCTATAGTCTTTGGAGGTTTCG	15	CTAATTTTTATTAAAATTCTTGTTTG

[a] UP 607 and DW 607 are the upstream and downstream regions from the TTHERM_00438800 putative gene. *neo3* is the cassette from the plasmid pBS-MnB-3 (38). WT 607 allele and KO 607 allele are the sequences amplified to check the replacement of the endogenous gene in the KO607 mutant; TTHERM_438800 and α-tubulin sequences were amplified in the RNA expression analysis. DES24g and DES24c are the fully amplified sequences of the *DES24* gene from the genomic DNA and cDNA, respectively, in the CU428 WT strain.

Germany), and 0.003% iron citrate (Sigma-Aldrich). In sterol desaturase activity assays, medium was supplemented with lathosterol (5α-cholest-7-en-3β-ol), cholesterol (cholest-5-en-3β-ol), stigmasterol (stigmasta-5,22-dien-3β-ol), β-sitosterol (stigmast-5-en-3β-ol), or sitostanol (stigmastan-3β-ol) at a final concentration of 20 μg/ml, which was added from 5-mg/ml stock solutions in ethanol (28). When indicated, paromomycin (Sigma-Aldrich) was added from a 100-mg/ml stock solution in water, together with 1 μg/ml of $CdCl_2$, which was prepared as a 1-mg/ml stock solution in water.

Cultures were inoculated daily with a 1:10 dilution of a 24-h culture. Cultivation was carried out in a rotary shaker (180 rpm) at 30°C. Plasmid pBS-MnB-3, containing the neomycin resistance gene under a cadmium-inducible metallothionein (MTT) promoter (the *neo3* cassette) expressing paromomycin resistance, was used throughout this study (30).

Standard DNA and RNA manipulation procedures. Genomic DNA of *T. thermophila* CU428 was prepared as previously described (13). The isolation of plasmid DNA was performed with a Wizard Plus SV miniprep DNA purification system kit (Promega, Madison, WI). Total RNA was prepared from *T. thermophila* cultures grown for 24 h using TRIzol reagent (Invitrogen, Carlsbad, CA). Nucleic acid fragments were amplified by PCR using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). When high-fidelity PCR was required, high-fidelity DNA polymerase from Finnzymes was the choice. Amplifications involved an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing from 50 to 60°C for 40 s, and extension at 72°C for 2 min. The products were separated on 1% agarose gels, isolated, and recovered using a Wizard PCR Preps kit. Sequencing reactions were performed in an automatic capillary analyzer using BigDye Terminator reagents (Applied Biosystems) by following the protocols supplied by the manufacturer. For RNA analysis, reverse transcription (RT) reactions were carried out using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI) according to the manufacture's instructions. All RNA samples were treated with DNase I prior to amplification. The amplification was done for 35 cycles (94°C for 1 min, 55°C for 40 s, and 72°C for 1 min). cDNA synthesis was monitored by PCR with α-tubulin primers. The primers used for all amplification reactions are listed in Table 1.

Construction of the transformation sequence C607 and the KO607 mutant. For *DES24* gene disruption in *T. thermophila*, we constructed the transformation sequence C607 for somatic knockout by overlapping PCR. The 0.88-kb upstream (UP) and 1.17-kb downstream (DW) flanking regions of the putative sequence TTHERM__00438800 (from the J. Craig Venter Institute database) were amplified separately using *T. thermophila* CU428 genomic DNA as the template with primers 1 and 2 (for fragment UP) or 3 and 4 (for fragment DW). The *neo3* cassette (1.9 kb) was amplified separately with primers 5 and 6 (Table 1) from plasmid pBS-MnB-3, expressing paromomycin resistance under the control of a cadmium-inducible promoter (MTT1) (30). The PCR products were purified from agarose gel with the Wizard SV gel and PCR clean-up system (Promega). The entire final C607 construct of 3.95 kb was PCR amplified, and the purified product was used for the transformation of *Tetrahymena* cells. For the transformation of the recipient, *T. thermophila* CU428, cells were grown in 50-ml cultures in SPP medium at 30°C to reach a density of 2×10^5 to 3×10^5 cells/ml. Cultures were starved overnight in 10 mM Tris buffer, pH 7.5, and transformed with 2.5 mg of the purified C607 DNA fragment delivered with gold particles according to a biolistic-gun protocol (3). Bombardment was performed with a Dupont biolistic PDS-1000/He particle delivery system (Bio-Rad). Transformants were recovered in 50 ml SPP medium containing 1.0 μg/ml $CdCl_2$. After 4 h, 80 μg/ml paromomycin was added, and the entire mixture was distributed in seven microtiter plates of 96 wells each.

Phenotypic assortment and gene replacement assays. The selection procedure was carried out as previously described by Nusblat et al. (29). Proper integration of the construct was checked by PCR, and the level of gene segregation was assayed by RT-PCR. In the first case, the WT gene and the corresponding fragment from the deletion mutant (KO607) were amplified using primers 7 and 8 for the WT copy of the *DES24* gene and primers 7 and 9 for the KO locus. For transcript-level assays, the WT and KO607 mutant were grown in SPP medium. After RNA extraction and purification, cDNAs were obtained and analyzed by PCR amplification with primers 10 and 8, corresponding to part of the exon of the *DES24* gene. The products were separated on 1% agarose gels. α-Tubulin transcript detection with primers 11 and 12 was used as a control. For primer sequences, see Table 1.

Growth curves. Mid-log-phase cultures of *T. thermophila* strain CU428 and the KO607 mutant were used to inoculate 50 ml of fresh SPP medium at an initial cell density of 0.4×10^5 to 1.0×10^5 cells/ml. Cell numbers were determined after 0, 6, 16, 24, 30, and 48 h. Cells were treated with 1% trichloroacetic acid (TCA) and then were counted in a Neubauer chamber.

Microscopy. The *Tetrahymena thermophila* WT and KO607 strains were grown in SPP medium (5 ml) with 20 μg/ml stigmasterol or cholesterol for up to 72 h. Untreated cells served as a control. Cells were collected by centrifugation, washed twice with PHEM buffer {60 mM PIPES [piperazine *N*,*N*′-bis(2-ethanesulfonic acid)], 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9} and fixed for 30 min with 3% paraformaldehyde in PHEM buffer. Fixed cells were rinsed twice with phosphate-buffered saline (PBS) buffer and stained with 1 mM DAPI (4′,6-diamidino-2-phenylindole, diluted in PBS) for 1 h at room temperature. After being rinsed with PBS, cells were mounted onto poly-L-lysine-coated coverslips, allowed to settle for 20 min, and placed onto slides with a drop of Vectashield mounting medium (Vector Laboratories, Inc.). Cells were examined by fluorescence and Nomarski differential-contrast microscopy with a Nikon Eclipse TE-2000-E2 microscope. Images were analyzed with EZ-C1 FreeViewer Gold version 3.90 (Nikon Corporation) and edited with SPOT Advanced 4.0.5 software (Diagnostic Instruments, Inc.) and Adobe Photoshop CS3 version 10.0 (Adobe Systems Inc.).

Detection of C-5(6), C-7(8), and C-22(23) sterol desaturase and C-24 dealkylase activities. Sterol desaturation and deethylation activities were analyzed upon culture in medium with selected sterols added. Cells were grown for up to 72 h in SPP medium containing 20 μg/ml of lathosterol, cholesterol, stigmasterol, β-sitosterol, or sitostanol. Two milliliters of grown cultures was withdrawn and submitted to lipid saponification using 2 ml of 2 M NaOH in methanol-water (1:1, vol/vol) at 60°C for 1 h. After the cultures cooled, sterols were extracted twice with 5 ml of hexane and dried under a nitrogen flow. Cholesterol or ergosterol were used as the internal standard and added prior to extraction at a final concentration of 20 μg/ml. The sterols were resuspended in ethanol and separated by high-performance liquid chromatography (HPLC) on a C_{18} Ultrasphere column, using methanol-water (95:5, vol/vol) as the mobile phase. Sterol identification was performed by using standards and by gas chromatography-mass spectrometry (GC-MS) analysis (see below).

Identification and quantification of sterols by gas chromatography-mass spectrometry analysis. Cells from cultures with added sterols were collected by centrifugation at 3,000 × *g* for 5 min at 4°C and extracted with 5 ml hexane, after

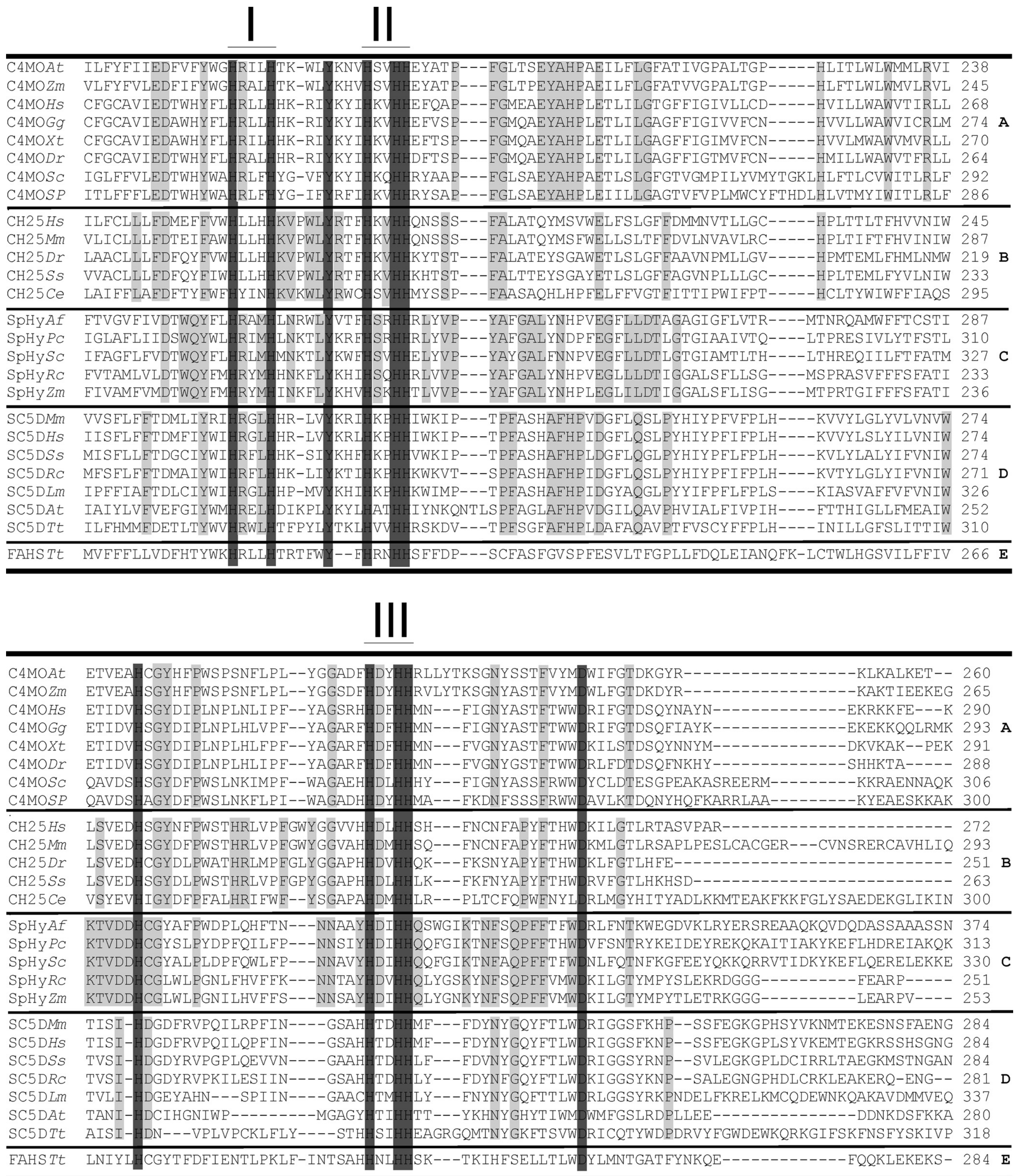

FIG. 2. Multiple sequence alignment of fatty acid hydroxylase superfamily members (FAHS). Alignment of C-4 methyl oxidase (A), cholesterol 25-hydroxylase (B), C-4 sphingolipid hydroxylase (C), C-5 sterol desaturase (D), and the putative protein of the TTHERM_00438800 *T. thermophila* sequence (E). I, II, and III are the three conserved histidine clusters of these members of the FAHS. Dark gray indicates conserved amino acids in all enzymatic groups. Light gray indicates conserved amino acids within each group. Note that the putative *T. thermophila* protein (FAHS*Tt*) has a conserved motif that is different from those of the other enzymatic groups. The organisms and the accession numbers of the amino acid sequences used for the analysis are as follows: C4MO*At*, *Arabidopsis thaliana* Q8W5A2; C4MO*Zm*, *Zea mays* B6TCQ7; C4MO*Hs*, *Homo sapiens* Q15800; C4MO*Gg*, *Gallus gallus* Q5ZLL6; C4MO*Xt*, *Xenopus tropicalis* Q0V9N6; C4MO*Dr*, *Danio rerio* Q7ZW77; C4MO*Sc*, *Saccharo-*

which they were subjected to saponification with 2 ml of 2 M NaOH in methanol-water (1:1, vol/vol) at 60°C for 1 h. Cholesterol or ergosterol was used as the internal standard and was added prior to extraction with hexane at a final concentration of 20 μg/ml. The organic phase was evaporated under nitrogen, and the residue was resuspended in 100 μl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and then incubated for 30 min at 70°C. The composition of the steryl trimethylsilyl ether derivatives was analyzed by running samples through an HP-5MS (30 m by 0.25 mm by 0.25 μm; 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 an HP mass selective detector (model MSD 5973) operated at an ionization voltage of 70 eV with a scan range of 50 to 600 atomic mass units (amu). The retention times and mass spectra of all new peaks obtained were compared to those of standards (Steraloids) and those available in the National Institute of Standards and Technology (NIST) mass spectral library.

Phylogenetic and sequence analyses. Available C-5(6) sterol desaturase, C-4 sterol methyl oxidase, 25-cholesterol hydroxylase, C-4 sphingolipid hydroxylase, fatty acid hydroxylase, and β-carotene hydroxylase protein sequences were aligned using Clustal W (34). Phylogenetic analyses were carried out by the neighbor-joining method using the program MEGA4, version 4.0.2 (32), with 10,000 bootstrap samplings or by minimum evolution with 5,000 bootstrap replicates. Both methods gave very similar tree topologies. All the sequences were retrieved from the UniProtKB database except XP_001017777.1, which was retrieved from the RefSeq database (NCBI).

Nucleotide sequence accession number. The nucleotide sequence for the *DES24* gene has been deposited in GenBank under accession number HM448899.

RESULTS

Sequence analysis. Sterol desaturases in *T. thermophila* are members of the fatty acid hydroxylase superfamily (24, 33). The enzymes of this superfamily are integral membrane proteins, use similar electron transport systems, and display conserved histidine motifs, which are presumably involved as ligands for an iron atom(s) and are the active sites of the enzyme (31).

Previous bioinformatic analysis of putative proteins of the ciliate belonging to cytochrome b_5-dependent sterol desaturases retrieved 8 putative sequences, with the E value being below 1.0e−10 (29).

The sequence TTHERM_00438800, which has no potential ortholog definition, as noted in the OrthoMCL database, corresponds to a putative gene with a single exon of 855 bp, according to the *Tetrahymena* Genome Database (TGD). This sequence shares the three typical histidine clusters (HX_3H, HX_2HH, and H/XHX_2HH) and some conserved amino acids with C-4 sphingolipid hydroxylases, C-4 methyl oxidases, C-25 cholesterol hydroxylase, and C-5 sterol desaturases, as shown in Fig. 2. It is also noted that, while each of the members of each enzymatic group has its own conserved motif, the TTHERM_00438800 sequence does not share any of these and does not group with them (Fig. 2).

Another distinctive feature of this sequence is the number of hydrophobic transmembrane regions in the protein. The prediction in the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) shows that the putative protein of *Tetrahymena* has five transmembrane regions but that the other enzymes have three or four.

Interestingly, searching for similar amino acidic sequences in the NCBI database retrieved putative proteins from bacteria as the most similar homologs, with an E value of <1.1e−8. The most similar sequence was ZP_01908611.1 from the deltaproteobacterium *Plesiocystis pacifica*, displaying 31.9% identity and 45.4% similarity.

A consensus phylogenetic tree generated by the neighbor-joining and minimum-evolution methods with 53 amino acid sequences belonging to the fatty acid hydroxylase superfamily clearly shows that the TTHERM_00438800 sequence grouped in a different branch within bacterial sequences of unknown function. The resulting tree is composed of eight clusters, with six clusters belonging to well-characterized protein families (C-4 methyl oxidase, C-25 cholesterol hydroxylase, C-4 sphingolipid hydroxylase, C-5 sterol desaturase, fatty acid hydroxylase, and β-carotene hydroxylase) and the remaining two clusters belonging to uncharacterized proteins (unknown 1, choanoflagellate and ciliate sequences; unknown 2, bacterial and TTHERM_00438800 sequences). The two clusters belonging to the fatty acid hydroxylase and β-carotene hydroxylase families result in a good out-group for the phylogenetic analysis. It has to be noted that for some sequences of the six well-characterized branches, information about biological activity is still pending. This is the case for the ciliate sequences belonging to the C-4 methyl oxidase group (Fig. 3).

Although the activity of the TTHERM_00438800 sequence cannot be predicted by bioinformatic analysis, the presence of the conserved histidine clusters, typically found in enzymes that metabolize sterols and sphingolipids, are indicative that the activity could be related to lipid metabolism.

Knockout of the TTHERM_ 00438800 sequence and characterization of the KO607 mutant. To assign a function to the protein encoded by the TTHERM_00438800 sequence, an analysis of different enzymatic activities involved in sterol metabolism was employed with a knockout mutant cell line. *T. thermophila* has two nuclei, the somatic macronucleus, which is transcriptionally active, and the germinative micronucleus. Aiming to knock out the TTHERM_00438800 gene, we introduced the construct C607, which provides paromomycin resistance (see Materials and Methods), into the macronucleus by somatic transformation using a biolistic bombardment protocol (see Fig. 4A for a schematic representation of the construction) (3). From 7 plates with 96 wells each, 35 wells showed cell growth with 80 μg/ml paromomycin. Transformants were subsequently transferred to fresh SPP medium with increasing concentrations of paromomycin (35). The selected clones were

myces cerevisiae P53045; C4MO*Sp*, *Schizosaccharomyces pombe* Q9UUH4; CH25*Hs*, *Homo sapiens* O95992; CH25*Mm*, *Mus musculus* Q9Z0F5; CH25*Dr*, *Danio rerio* Q5PRC0; CH25*Ss*, *Salmo salar* B5X8D7; CH25*Ce*, *Caenorhabditis elegans* B6TCB9; SpHy*Af*, *Aspergillus fumigatus* Q4WRB7; SpHy*Pc*, *Pichia ciferrii* Q22AK3; SpHy*Sc*, *Saccharomyces cerevisiae* Q6YGT5; SpHy*Rc*, *Ricinus communis* Q5PRC0; SpHy*Zm*, *Zea mays* Q9UUH4; SC5D*Mm*, *Mus musculus* P38992; SC5D*Hs*, *Homo sapiens* O75845; SC5D*Ss*, *Saccharomyces cerevisiae* Q5ZLL6; SC5D*Rc*, *Rana catesbeiana* Q39208; SC5D*Lm*, *Leptosphaeria maculans* P32353; SC5D*At*, *Arabidopsis thaliana* A0CQM6; SC5D*Tt*, *Tetrahymena thermophila* Q8W5A2; FAHS*Tt*, *Tetrahymena thermophila* XP_001017777.1. All the sequences were retrieved from the UniProtKB database except XP_001017777.1, which was retrieved from the RefSeq database (NCBI).

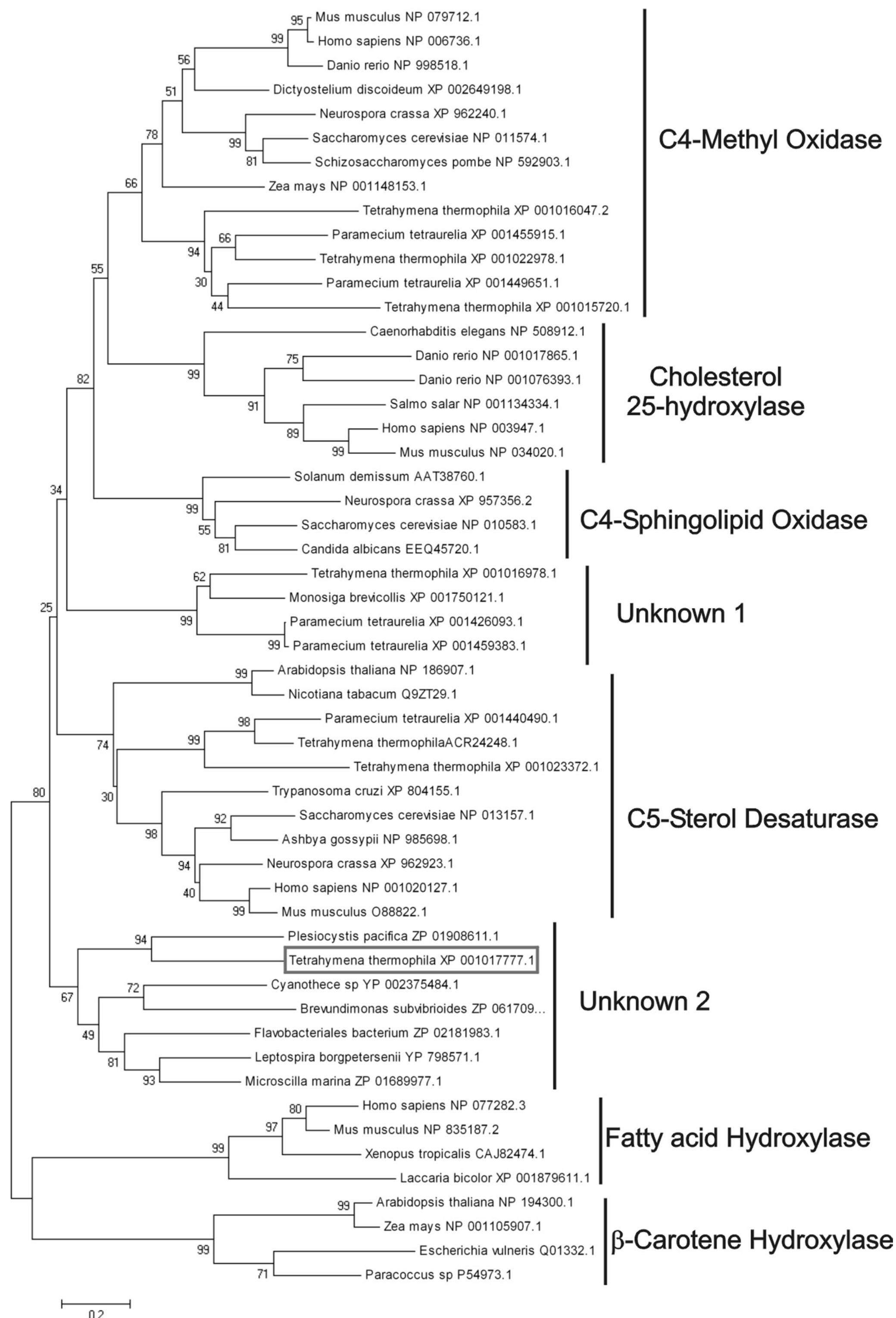

FIG. 3. Phylogenetic analysis of the fatty acid hydroxylase superfamily. The phylogenetic tree of 56 amino acid sequences (NCBI) was created using the neighbor-joining method with MEGA4 software, as described in Materials and Methods. The groups include C-4 methyl oxidase, cholesterol 25-hydroxylase, C-4 sphingolipid oxidase, C-5 sterol desaturase, fatty acid hydroxylase, β-carotene hydroxylase, and two of unknown functions (Unknown 1 and 2). The *Tetrahymena* putative genes (GenBank accession numbers) used in this analysis are TTHERM_01194720 (XP_001029976.1) (in a gray box), TTHERM_00446080 (XP_001023372.1), TTHERM_00438800 (XP_001017777.1), TTHERM_00758950 (XP_001016978.1), TTHERM_00077800 (XP_001015720.1), TTHERM_00876970 (XP_001016047.2), and TTHERM_00348230 (XP_001022978.1). The bars indicate percentages of substitution.

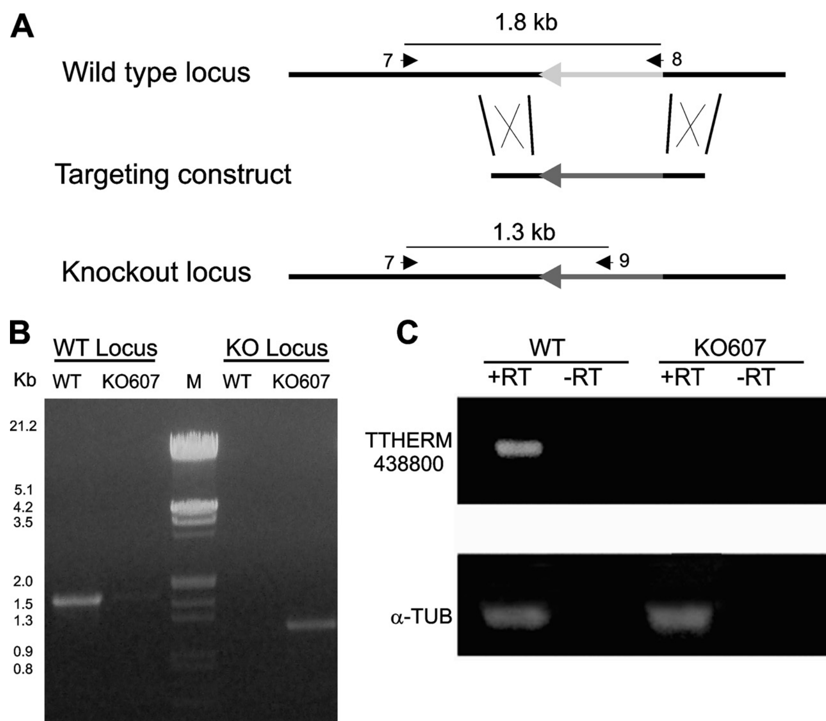

FIG. 4. Generation of the knockout mutant KO607. (A) Schematic representation of the gene replacement in the sequence TTHERM_00438800, in which a target construction was inserted by homologous recombination, and the knockout locus generated in the KO607 strain. The numbered arrows indicate primers used for PCR amplifications, and the sizes of the corresponding fragments are indicated above them. (B) DNA amplification of the WT locus and KO locus from WT and KO607 cells, respectively, using the same forward primer (primer 7) and two specific reverse primers (primers 8 and 9) for each locus. The 1.81-kb DNA fragment from the WT locus is clearly seen in the WT strain and faintly in the KO607 mutant, whereas the 1.58-kb DNA fragment from the KO locus is present only in the latter. Lane M, DNA markers for fragment length. (C) RNA transcripts from the TTHERM_00438800 open reading frame measured in the WT and KO607 mutant by RT-PCR. The fragment was amplified with primers 10 and 8. α-Tubulin (α-TUB) was used as a control. See Table 1 for primer descriptions.

those where the gene construct, C607, was integrated into the proper locus and grew in 3 mg/ml of paromomycin. PCR amplification of genomic DNA from the knockout mutant KO607 showed copies of both the WT and mutated alleles (Fig. 4B). Nevertheless, an expression analysis by RT-PCR showed that the gene is expressed only in the wild type and not in the knockout cell line (Fig. 4C). These results indicate that the small amount of the wild-type allele amplified from the mutated strain proceeds from the nontranscriptional micronucleus of the ciliate.

The gene for sequence analysis was amplified from CU428 genomic DNA and cDNA, using the primer pairs 13 and 14 and 10 and 15 (Table 1). The gene sequence obtained from strain CU428 (GenBank accession number HM448899) was 100% identical to the one from SB210, the strain used by the J. Craig Venter Institute for the genome project (11). The sequence analysis shows that the gene is composed of only one exon, as previously reported from an *in silico* analysis.

C-24 sterol deethylation is abolished in the KO607 mutant. All sterol modifications reported for *T. thermophila* can be detected by analysis of the conversion products formed when it is cultured with specific substrates. To illustrate the approach, we summarized in Table 2 the products expected in cultures of wild-type and hypothetical-knockout strains supplemented with different sterols.

Comparative analysis of the conversion products formed by the WT and KO607 mutant with medium supplemented with C_{27} sterols, such as cholesterol or lathosterol, showed that both strains were able to produce the tri-unsaturated derivative cholesta-5,7,22-trien-3β-ol, with similar yields. The amounts recovered were 12.1 ± 1.3 μg/ml and 9.8 ± 1.1 μg/ml for the WT and KO607 mutant, respectively, in cholesterol-containing medium and 9.2 ± 0.8 and 8.6 ± 0.9 μg/ml for the WT and KO607 mutant, respectively, in lathosterol-containing medium. These results suggest that C-5, C-7, and C-22 sterol desaturases were not affected. However, when cultures of KO607 were supplemented with C_{29} sterols, such as stigmasterol, β-sitosterol, or sitostanol, the final product was stigmasta-5,7,22-trien-3β-ol, indicating that the C-24 ethyl group was not removed in any of the three substrates and that the C-5, C-7, and C-22 desaturase activities remained unaffected. We therefore concluded that the KO607 mutant cell line was a sterol desaturase-like mutant involved in deethylation activity on C_{29} sterols (Table 3). Also, HPLC and GC-MS results, displayed in Fig. 5, confirmed that the C-24 deethylated products (the C_{27} diene $\Delta^{5,22}$ and triene $\Delta^{5,7,22}$) are formed in the wild-type strain but missing in KO607 cultures containing stigmasterol.

The levels of growth and cellular morphologies of the WT and KO607 mutant were compared in cultures with and without sterols. As shown in Fig. 6, both in sterol-free and in the C_{27} sterol cholesterol-containing medium, growth and mor-

TABLE 2. Expected products of sterol metabolism in supplemented cultures of the *T. thermophila* WT and putative knockout mutants[a]

Sterol added to cultures	Activities measurable	Final product accumulated in hypothetical strain:	
		WT	KO C5Des
Sitostanol A	C-5, C-7, and C-22 sterol desaturases and C-24 sterol deethylation	Cholesta-5,7,22-trien-3β-ol F	Cholesta-7,22-dien-3β-ol E
β-Sitosterol	C-7 and C-22 sterol desaturases and C-24 sterol deethylation	Cholesta-5,7,22-trien-3β-ol F	Cholesta-5,7,22-trien-3β-ol F
Stigmasterol	C-7 sterol desaturase and C-24 sterol deethylation	Cholesta-5,7,22-trien-3β-ol F	Cholesta-5,7,22-trien-3β-ol F
Cholesterol	C-7 and C-22 sterol desaturases	Cholesta-5,7,22-trien-3β-ol F	Cholesta-5,7,22-trien-3β-ol F
Lathosterol	C-5 and C-22 sterol desaturases	Cholesta-5,7,22-trien-3β-ol F	Cholesta-7,22-dien-3β-ol E
Campesterol (cholest-5-en-24α-methyl-3β-ol)	C-7 and C-22 sterol desaturases	Cholesta-5,7,22-trien-24α-methyl-3β-ol	Cholesta-5,7,22-trien-24α-methyl-3β-ol
Ergosterol (cholesta-5,7,22-trien-24β-methyl-3β-ol)	SWM	Cholesta-5,7,22-trien-24β-methyl-3β-ol	Cholesta-5,7,22-trien-24β-methyl-3β-ol

[a] List of products expected in cultures supplemented with sterols in the WT and putative knockout mutants. KO C5Des, C-5 sterol desaturase knockout mutant; KO C7Des, C-7 sterol desaturase knockout mutant; KO C22Des, C-22 sterol desaturase knockout mutant; KO C24Det, C-24 sterol deethylation knockout mutant; SWM, sterol recovered from the ciliate without modifications.

phology were indistinguishable between the WT and mutant, whereas in the mutant growth was severely impaired in the presence of the C_{29} sterol stigmasterol. The same decrease in growth parameters (i.e., growth rate and biomass yield) were observed with all C_{29} sterols assayed, namely, stigmasterol, β-sitosterol, and sitostanol (data not shown). The KO607 mutant also revealed a change in morphology in stationary-phase cultures, characterized by the loss of its typical pear-like shape and adoption of a spherical shape (Fig. 6F).

Another significant difference in the mutant cell line refers to the regulation of the synthesis of its own hopanoid (tetrahymanol). As has been stated in previous studies, *Tetrahymena* suppresses the synthesis of tetrahymanol in the presence of an exogenous C_{27}, C_{28}, or C_{29} sterol, such as cholesterol, ergosterol, or stigmasterol, respectively, without a noticeable change in growth parameters (7). In cultures of the KO607 mutant with C_{29} sterols, the relative percentage of tetrahymanol recovered was higher than with C_{27} sterols, such as cholesterol, indicating that the tetrahymanol inhibition was lower only in the presence of C_{29} sterols (Fig. 7). Although there are no previous studies of the effects of C_{29} sterols on tetrahymanol synthesis, together, these results suggest that the ethyl group (at position C-24) impairs significantly the normal growth of the ciliate, affecting tetrahymanol regulation.

DISCUSSION

In this study, we report the first identification of a gene encoding a C-24 sterol dealkylation activity, named *DES24*. Although C-24 dealkylation of sterols has been reported in the ciliate *Tetrahymena*, green algae, metazoan-like sponges, nematodes, arthropods, mollusks, cnidarians, and ctenophores, neither the enzymes involved in this metabolic conversion nor the corresponding genes have been identified so far (26, 16, 20, 5, 15, 19).

The mutation introduced into the *DES24* gene through reverse genetics completely abolished the transcriptional activity of the gene where no mRNA production could be detected (Fig. 4C). The mutant was unable to carry out the deethylation of C_{29} sterol substrates, namely, stigmasterol, β-sitosterol, and sitostanol, whereas all three sterol desaturase activities (at C-5, C-7, and C-22) were not affected, as indicated by the formation of the final product, stigmasta-5,7,22-trien-3β-ol.

A C-24 dealkylation mechanism of phytosterols has been described in arthropods, nematodes, and sponges. The reaction is carried out in three successive steps consisting of dehydrogenation, epoxidation, and epoxide fragmentation of the alkyl group (19), as depicted in Fig. 8. As a consequence of the epoxide fragmentation, a compound with a $\Delta^{24(25)}$ double

TABLE 3. Sterol derivatives recovered from cultures of the *T. thermophila* WT and KO607 mutant[a]

Compound(s) measured	Amt of sterol (μg/ml) in indicated culture with:					
	Stigmasterol		β-Sitosterol		Sitostanol	
	WT	KO607	WT	KO607	WT	KO607
Cholesta-5,7,22-trien-3β-ol	3.62 ± 0.07	–	1.93 ± 0.31	–	2.22 ± 0.37	–
Stigmasta-5,7,22-trien-3β-ol	2.23 ± 0.22	2.01 ± 0.46	0.61 ± 0.09	0.88 ± 0.12	2.20 ± 0.35	2.35 ± 0.25
Cholest derivatives[b]	4.42 ± 0.15	–	2.22 ± 0.38	–	2.22 ± 0.37	–
Stigmast derivatives[c]	2.23 ± 0.22	2.01 ± 0.46	1.52 ± 0.22	2.78 ± 0.36	8.56 ± 1.20	7.02 ± 1.12

Strains were grown in SPP medium with 20 μg/ml of the indicated sterol. At 48 h of incubation, sterols were extracted and quantified by GC-MS, as described in Materials and Methods. The results are the mean values of two determinations ± standard deviations (combined from three independent experiments). –, not detected.
[b] Cholest derivatives are all bioconversion products without a C_{24} ethyl group formed in cultures.
[c] "Stigmast derivatives" refers to all the bioconversion products formed preserving the C_{24} ethyl group. For details, see Fig. 1.

TABLE 2—*Continued*

Final product accumulated in hypothetical strain:		
KO C7Des	KO C22Des	KO C24Des
Cholesta-5,22-dien-3β-ol D	Cholesta-5,7-dien-3β-ol C	Stigmasta-5,7,22-trien-3β-ol B
Cholesta-5,22-dien-3β-ol D	Cholesta-5,7-dien-3β-ol C	Stigmasta-5,7,22-trien-3β-ol B
Cholesta-5,22-dien-3β-ol D	Cholesta-5,7,22-trien-3β-ol F	Stigmasta-5,7,22-trien-3β-ol B
Cholesta-5,22-dien-3β-ol D Cholesta-5,7,22-trien-3β-ol F Cholesta-5,22-dien-24α-methyl-3β-ol	Cholesta-5,7-dien-3β-ol C Cholesta-5,7-dien-3β-ol C Cholesta-5,7-dien-24α-methyl-3β-ol	Cholesta-5,7,22-trien-3β-ol F Cholesta-5,7,22-trien-3β-ol F Cholesta-5,7,22-trien-24α-methyl-3β-ol
Cholesta-5,7,22-trien-24β-methyl-3β-ol	Cholesta-5,7,22-trien-24β-methyl-3β-ol	Cholesta-5,7,22-trien-24β-methyl-3β-ol

bond is formed and is subsequently removed by a Δ^{24} sterol reductase.

Several pieces of evidence suggest that this mechanism is unlikely to occur in *T. thermophila.* In principle, none of the suspected intermediates, i.e., epoxy, methyl, or methylene derivatives, have been detected in *Tetrahymena* so far (26). Moreover, we did not detect them upon culture with stigmasterol plus 0.5 to 3.0 ppm of *N,N*-dimethyldodecanamine (data not

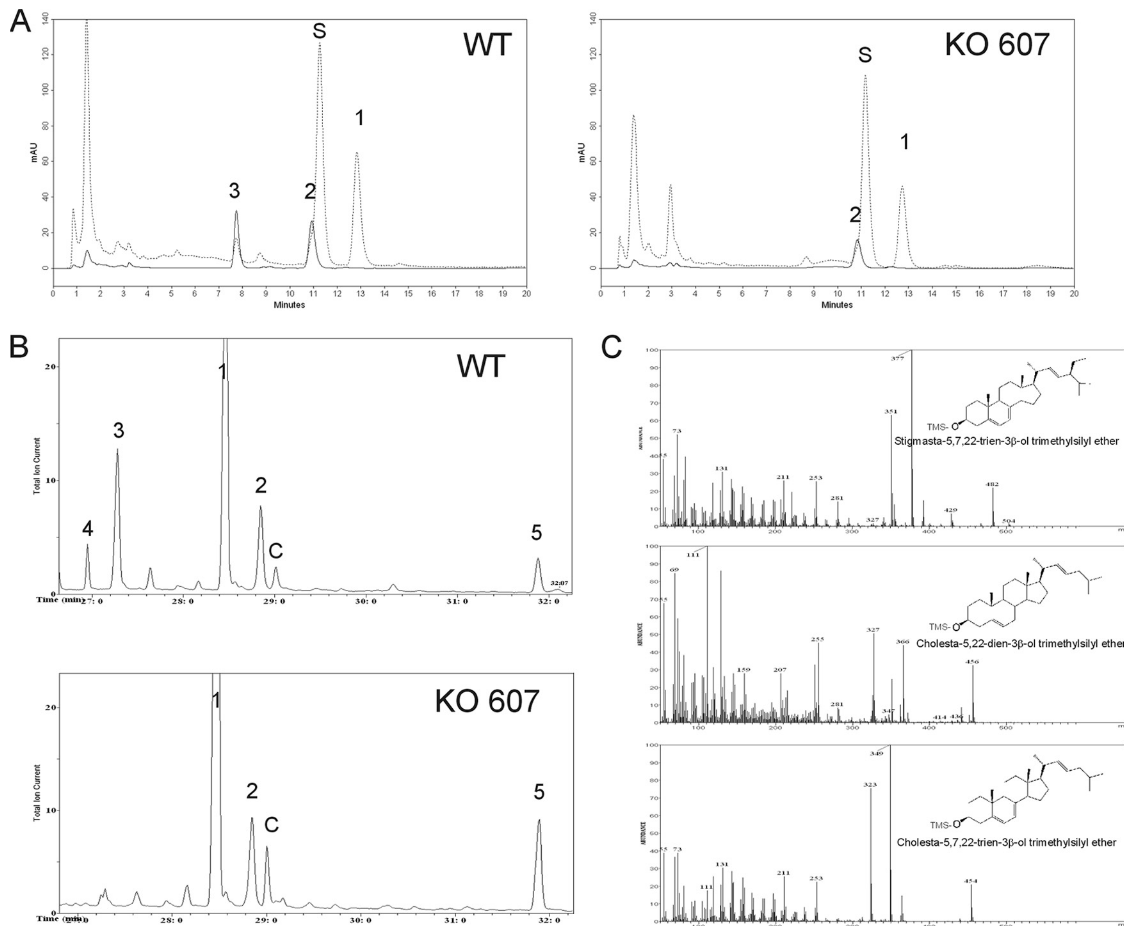

FIG. 5. Sterol profile of the *T. thermophila* WT and KO607 mutant cultivated with stigmasterol. (A) HPLC analysis of sterols from the WT (left) and KO607 mutant (right) grown with stigmasterol for 24 h. Total sterols were extracted with hexane as described in Materials and Methods. Absorbance was monitored at 210 nm (dotted line) and 285 nm (solid line), the latter specific for sterols displaying conjugate double bonds (5,7 diene derivatives) (peaks 2 and 3). Sterol identification was done with available standards. Time in minutes is noted on the *x* axis. (B) Gas chromatography (GC) analysis of trimethylsilyl ether derivatives of total sterols extracted from the WT and KO607 upon cultivation with stigmasterol for 48 h. The *y* axis indicates the total ion current. (C) MS results for peaks 2, 3, and 4 are not available in the NIST library. The *y* axis indicates relative abundance, and the *x* axis indicates *m*/*z*. Peaks: 1, stigmasterol; 2, stigmasta-5,7,22-trien-3β-ol; 3, cholesta-5,7,22-trien-3 β-ol; 4, cholesta-5,22-dien-3 β-ol; 5, tetrahymanol; S, cholesterol used as internal standard; C, contaminant.

FIG. 6. Growth and cellular morphology of the *T. thermophila* WT and KO607 mutant cultivated with different sterols. (A to F) Stationary-phase cultures of the WT and KO607, grown in SPP medium (A and D) with cholesterol (B and E) or stigmasterol (C and F). Under the last condition, mutant cells lost their typical pear-like shape. (G) Growth curves of the *T. thermophila* WT (●) and mutant strain KO607 (○) in SPP medium without added sterols, of the WT (▼) and KO607 mutant (Δ) in cholesterol-containing medium, and of the WT (■) and KO607 mutant (□) in stigmasterol-supplemented medium. All cultures were inoculated at an initial cell density of 0.4×10^5 to 1.0×10^5 cells/ml.

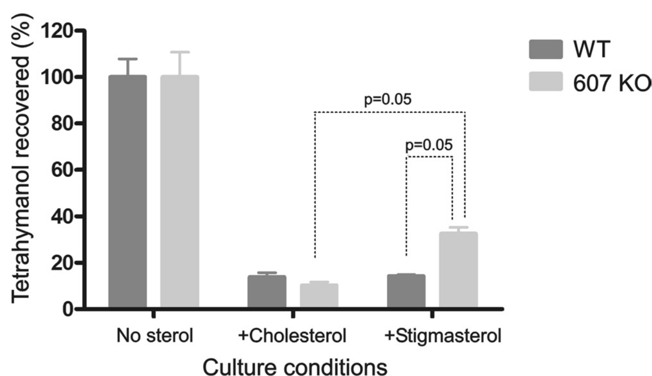

FIG. 7. Tetrahymanol recovered from the WT and KO607 mutant cultivated with stigmasterol. Relative percentages of tetrahymanol recovered from the WT (dark-gray bars) and KO607 mutant (light-gray bars) grown in SPP medium without added sterols (No sterol) or in SPP with cholesterol or stigmasterol for 48 h, normalized to that recovered from 10^6 cells. Tetrahymanol was measured by GC-MS, and ergosterol, added prior extraction, was used as an internal standard for all calculations. The data are the mean values of three determinations ± standard deviations and are representative of three independent experiments.

shown), a known inhibitor of Δ^{24} sterol reductase (23). Concomitantly, the ciliate was unable to remove the C-24 methyl group of C_{28} sterols, such as ergosterol and campesterol, or the C-24 methylene group, considered one of the intermediates of the dealkylation reaction, when either 24-methylenecholesterol, fucosterol, or isofucosterol was used as a substrate (26). This behavior is clearly different from that of insects (19) and sponges (20). Moreover, no Δ^{24} sterol reductase activity has been described in *Tetrahymena*; thus, when sterols such as desmosterol were added to the culture, no products carrying a reduction of the $\Delta^{24(25)}$ double bond were detected (27). Consistently with our results, we were also unable to identify the presence of sterols with a double bond at $\Delta^{24(25)}$ in wild-type cells when the ciliate was grown in cultures supplemented with C_{29} sterols. Additionally, the absence of a clear *DES24* ortholog in the genomes of arthropods, nematodes, and sponges reinforces the idea that Des24p has significant differences from the enzymes responsible for the sterol dealkylation (not yet identified) in these organisms.

An analysis of the amino acid sequence revealed that, besides the histidine clusters typical of the fatty acid hydroxylase superfamily, the protein does not have any other conserved motif and no remarkable features. It must be noted that the

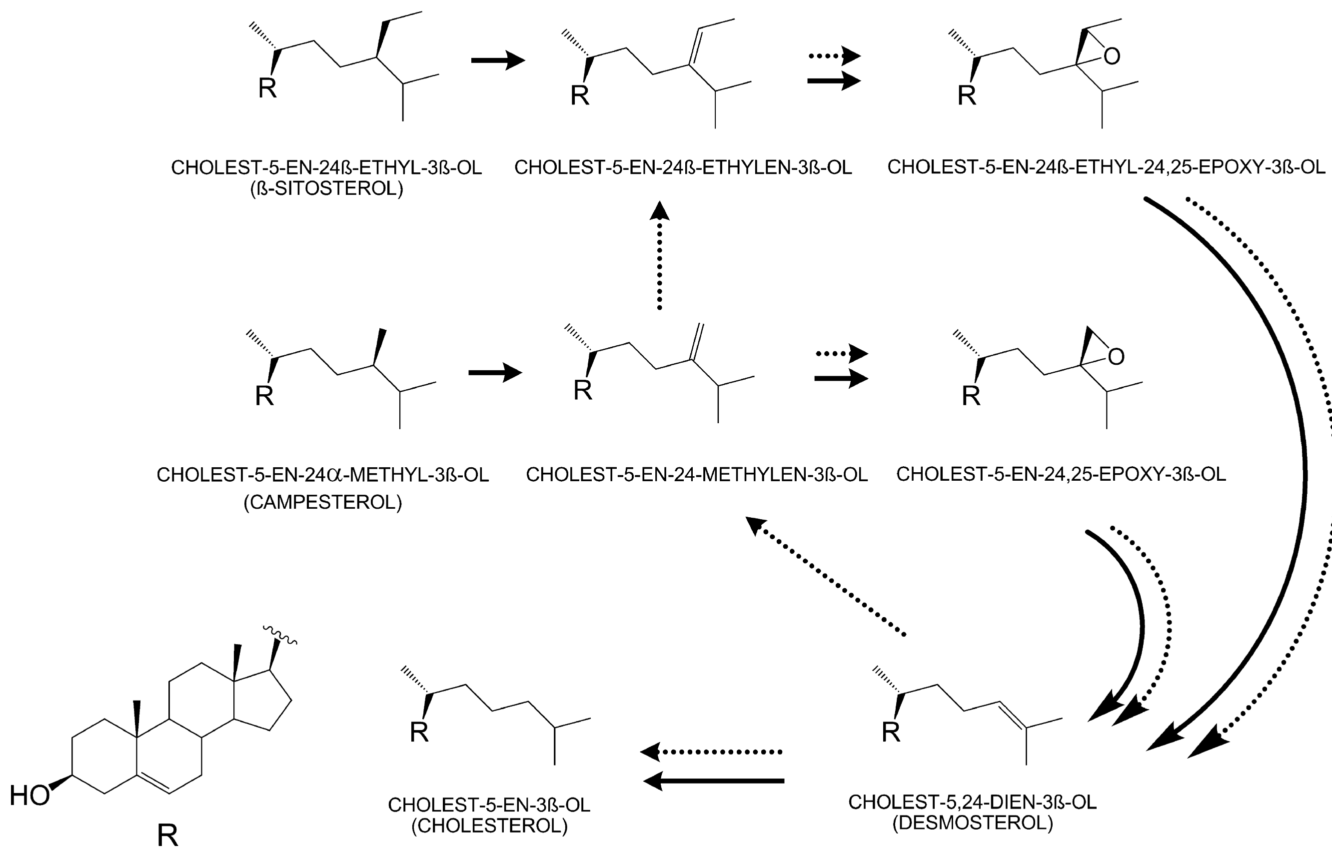

FIG. 8. Dealkylating pathways. Dealkylation in arthropods/nematodes (solid arrows) and sponges (dotted arrows).

presence of nonhomologous enzymes involved in dealkylations in the sterol pathways in other organisms has already been reported. This is the case for Δ^8,Δ^7 isomerase and Δ^{24} reductase (10). Together, these facts suggest that Des24p is a novel class of protein probably involved in the first step of the C-24 dealkylation process in *Tetrahymena.*

Dealkylation activity is important for plant predators that are faced with the metabolization of phytosterols, such as some arthropods and nematodes, although we could not find Des24p orthologs in these and other eukaryotes. Also, it was not found in the ciliate *Paramecium*, which belongs, together with *Tetrahymena*, to the single major evolutionary lineage Oligohymenophorea (37), suggesting that this activity might be unique in *Tetrahymena* and was probably transferred horizontally from evolutionarily distant organisms. Furthermore, the fact that Des24p does not branch with its typical eukaryote counterparts while it does with bacterial putative proteins supports this hypothesis.

A few bacteria harbor putative enzymes for sterol biosynthesis, i.e., *Methylococcus capsulatus*, *Gemmata obscuriglobus*, and *Plesiocystis pacifica*, and the first two are also able to synthesize them (10). There have been reports on bacterium-like genes transferred among distantly related organisms, particularly protists with phagotrophic lifestyles, including *T. thermophila* (1). Therefore, the acquisition of a bacterial gene was proposed for this ciliate (25). The tetrahymanol cyclase gene of *Tetrahymena* (TTHERM_00382150) has, like Des24p, only bacterial orthologs and might have been acquired from a nonrelated organism.

Nevertheless, the hypothesis of a horizontal gene transfer from prokaryotes to *Tetrahymena* must be carefully taken into account, due to the lack of documentation on sterol dealkylation in these organisms.

The *DES24* gene is not essential, but its disruption in the KO607 mutant gives rise to some detectable phenotypic changes, such as a round shape and a decrease in growth rate and biomass yield, when cultured with C_{29} substrates. Another interesting feature of the mutant is that tetrahymanol inhibition typically observed in WT cells upon the addition of sterol substrates is affected, suggesting that C_{29} sterols impair the normal growth of the ciliate.

However, the ratios between tetrahymanol and sterols and between saturated and unsaturated fatty acids, as a consequence of this mutation, must be further investigated.

If we assume that the acquisition of a gene has been beneficial to the organism which acquires it, a possible explanation inferred from our results could be that the presence of C_{29} sterols in the membrane of the ciliate might be harmful to the normal growth and development of the organism. Therefore, considering that *Tetrahymena* lives in aquatic environments in the presence of exogenous sterols from different sources, the ciliate is able to obtain all sort of sterols, for instance, from phytoplankton, higher plants, and algae, and thus forego tetrahymanol biosynthesis.

It is interesting to highlight that, according to the expression profile of this gene (http://tged.ihb.ac.cn/search.aspx?keyword=TTHERM_00438800), it is expressed not only under growth conditions but also during starvation.

The presence of sterols also influences the swimming velocity of the cell (17), and it has been postulated that steroid hormones strongly influence the chemotaxis and chemotactic selection of *Tetrahymena* cells and that these hormones also have a moderate effect on phagocytosis, cell shape, and cell growth (21). However, it is not clear why the ciliate incorporates and modifies exogenous sterols when it is able to synthesize tetrahymanol. Another possible explanation, as for arthropods (14), may be that *Tetrahymena* uses sterols to synthesize steroid hormones. Although the presence of vertebrate hormone-like molecules has been demonstrated for *Tetrahymena*, it is obscure whether the ciliate can store or produce steroid hormones (9). In summary, although the demonstration of this activity in bacteria and the gene identification in arthropods are still lacking, this work contributes new insight into phytosterol metabolism.

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