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Expression and functional characterization of a C-7 cholesterol desaturase from *Tetrahymena thermophila* in an insect cell line

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ABSTRACT

Tetrahymena thermophila transforms exogenous cholesterol into pro-vitamin D_3 (7-dehydrocholesterol) with remarkable efficiency in a one-step reaction carried out by a C-7 cholesterol desaturase. The enzyme *DES7* is encoded by the gene TTHERM_00310640, identified with RNAi and gene knock-out experiments, but has not yet been heterologously expressed actively in any organism. A model derived from its amino acid sequence classified *DES7*p as a Rieske-type oxygenase with transmembrane localization. The protein has catalytic activity, sequence and topological similarity to DAF-36/Neverland proteins involved in the synthesis of steroid hormones in insects and nematodes. Due to their structural and functional similarity, we analyzed the expression of a codon optimized *DES7* gene from *Tetrahymena* in the insect Sf9 cell line, identified and measured the steroid metabolites formed, and extended the actual knowledge on its localization. We found that the accumulation of 7-dehydrocholesterol could be increased 16–40-fold in *Spodoptera frugiperda*, depending on physiological conditions, by overexpression of *T. thermophila DES7*. The protein was detected in the microsomal fraction, in accordance with previous reports. Although the electron transfer chain for Des7p/DAF-36/Neverland Rieske-type oxygenases is presently unknown, we identified possible donors in the ciliate and insect genomes by bioinformatic analysis. In spite of the large evolutionary distance between *S. frugiperda* and *T. thermophila*, the results indicate that there is significant functional conservation of the electron donors, since the ciliate's sterol desaturase can function in the context of the insect electron transport system. The results achieved demonstrate that *DES7* is the first gene from a ciliate, coding for a microsomal enzyme, expressed in active form in an insect cell line.

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1. Introduction

The ciliate *Tetrahymena thermophila* can transform exogenous cholesterol into pro-vitamin D_3 (7-dehydrocholesterol) with remarkable efficiency in a one-step reaction, carried out by a C-7 cholesterol desaturase. This conversion process can be applied to reduce the amount of cholesterol in foodstuffs, with simultaneous enrichment in pro-vitamin D_3 [1,2], and/or can become an alternative in the industrial production of vitamin D_3, replacing complex and highly polluting chemical methods that are still in use [3–5]. Development of industrial applications, such as the cholesterol conversion in foodstuffs or the production of bulk chemicals, requires large scale production of the relevant enzymes in robust hosts, and in a metabolic environment that is optimal for their activity.

C-7 cholesterol desaturase is encoded in the gene *DES7* (TTHERM_00310640), identified with RNAi and gene knock-out experiments [6]. The corresponding enzyme localizes in the perinuclear membranous fraction in the protozoa, and can be recovered in active form in microsomes [6,7]. A model derived from its amino acid sequence classified Des7p as a Rieske-type oxygenase, with transmembrane localization. Detailed information on its catalytic site and its physiological electron donors are presently unknown.

Among eukaryotes only few Rieske-type oxygenases have been functionally characterized beyond the fact that they contain a typical iron-sulfur center. These enzymes include the so-called DAF-36/Neverland protein (Nvd), involved in the synthesis of ecdysteroides and dafachronic acids in animals, and a group of four Rieske-type proteins involved in the synthesis of chlorophyll in plants. Nvd is a C-7 cholesterol desaturase, present both in ecdysozoan and in deuterostome organisms, and plays an important role

Abbreviations: RO, Rieske oxygenases; KSH, 3-Ketosteroid-9a-hydroxylase; ETC, electron transfer chain.

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in the regulation of development, reproduction, and longevity [8,9]. In insects, C-7 sterol desaturation mediated by the Nvd protein is the initial step in the synthesis of 20E ecdysteroids from dietary cholesterol or from phytosterols [10,11]. The same requirement for C-7 cholesterol desaturation was demonstrated in *Caenorhabditis elegans daf-36* mutants, via the addition of 7-dehydrocholesterol to cultures of *daf-36* mutants [12]. Moreover, for Opistokonts it was postulated that DAF-36/Nvd could be a node of regulation of the partitioning of cholesterol to steroidogenic and bile acid pathways, thereby influencing development and longevity [13].

The ciliate *Tetrahymena* neither requires nor synthesizes ecdysteroids nor dafachronic acids; therefore a similar function for its C-7 cholesterol desaturase as in Opistokonts seems unlikely. Noteworthy, *Tetrahymena* does respond to hormones of different nature, including steroids [14], and the expression profile of *DES7* shows a differential response in starvation conditions than during conjugation. Altogether, these evidences suggest that the physiological role of the *DES7* gene product is presently unknown [15].

Spodoptera littoralis carries a putative *nvd* gene, whose expression has been confirmed in the testis of its larva's (along with five cytochrome P450 enzymes encoded by the so-called Halloween genes) [16]. Des7p of *Tetrahymena* has sequence and topological similarity to DAF36/Nvd, and the coding gene has been identified by knockout and RNAi, but has not yet been expressed heterologously in any organism. This observation prompted us to analyze the expression of *DES7* from *Tetrahymena* in a *Spodoptera* host. For this purpose, we selected the commercially available baculovirus Sf9 cell line, a well-known expression system for eukaryotic proteins [17], which has already successfully been used for heterologous expression of proteins from *C. elegans*, as mentioned above. Furthermore, C-7 cholesterol desaturase activity has not been investigated in the species *Spodoptera frugiperda* under any physiological condition, making it necessary to test endogenous levels and responses to addition of sterols in the cell line itself too. The results achieved demonstrate that *DES7* is the first gene from a ciliate, coding for a microsomal enzyme, expressed in active form in an insect cell line.

2. Materials and methods

2.1. Recombinant baculovirus construction

The DNA sequence of Des7p (TTHERM_00310640) was codon-optimized for baculovirus expression, synthesized and cloned in the pVL1392 vector (BD Bioscience, San Diego, CA, USA) by GenScript (Piscataway, NJ, USA) to obtain DES7i (i.e., a codon-optimized insect version of Des7p) and DES7i-His-Tag. The latter was synthesized by adding a 6xHis-tag coding sequence at the C-terminal end of the gene, prior to the stop codon. The DES7i and DES7i-His-Tag genes were cloned using the added Pst1 and BamH1 cloning sites, which rendered the constructs pVL1392-DES7i and pVL1392-DES7i-His-Tag, respectively.

Each construct was co-transfected with the Baculogold Bright AcMNPV genome, according to BD Biosciences protocols. For control purposes, a recombinant baculovirus was constructed using solely the pVL1392 transfer vector (C). The constructs were checked with primers flanking the recombination site. (Forward: 5′ TCCGGATTATTCATACCGTCCCACCATC 3′ and Reverse: 5′ GCTTCATCGTGTCGGGTTTAACATTACGG 3′).

2.2. DES7i baculovirus expression

Sf9 suspension cultures (2×10^6 cells/ml) grown in Sf900 II medium (Invitrogen, Gaithersburg, MD, USA) supplemented with 1% of fetal bovine calf serum (Internegocios S.A., Mercedes, Buenos Aires, Argentina), plus 0.5 μg/ml hemin chloride, 100 μM δ-Aminolevulinic acid and 100 μM ferric citrate, were infected either with DES7i, DES7i-His-Tag or Control baculoviruses (C), using a multiplicity of infection of 2. Infected cultures were incubated in an orbital shaker (100 rpm) at 27 °C. Three days after infection (3 dpi) the culture medium was separated by centrifugation at 10,000×*g* for 10 min and cell pellets were then re-suspended in Sf900 II medium, containing a mixture of methyl-β-cyclodextrin and cholesterol (80 μg/ml or 80 μg/ml plus [1,2-^{3}H] cholesterol, 4 μCi/ml), prepared according to Christian et al. [18] after which the cells were incubated at 27 °C and 100 rpm for 1 extra day (4 dpi). Infected cells were analyzed for sterol content and composition, prior to the addition of the methyl-β-cyclodextrin:cholesterol mixture and after 24 h of incubation (i.e., 3 and 4 dpi, respectively). Cells were incubated with [1,2-^{3}H] cholesterol for 5 h at 27 °C in a shaking incubator rotating at 100 rpm.

2.3. Preparation of homogenates and microsomes

Culture supernatants of Sf9 suspension cultures were separated after centrifugation at 5000×*g* for 10 min. The cell pellets were recovered, washed twice with buffer (100 mM sodium phosphate buffer, pH = 7,2 plus 1 mM EDTA and 20% (v/v) glycerol) and re-suspended in the same buffer with the addition of a cocktail of protease inhibitors (50 μM PMSF, 50 μM Leupeptin and 0,5 mM AEBSF). Homogenates were obtained by sonication of the re-suspended cells (3 cycles of 10 s at 30 W). For preparation of microsomal fraction, homogenates were centrifuged at 9000×*g* for 30 min at 4 °C, supernatants were collected and subsequently centrifuged at 100,000×*g* for 70 min at 4 °C. The pellet fractions were then recovered, re-suspended in the same buffer and kept at −80 °C for further analysis.

2.4. Western blot analyses

Proteins from cell homogenates and from microsomal fractions were separated on 15% (w/v) SDS–PAGE. Proteins were blotted onto PVDF membrane (Amersham) for Western blot analyses. The DES7i-His-Tag protein were detected using a mouse anti-His-Tag (BD Biosciences) primary antibody (after 1/5000 dilution) and a peroxidase conjugated anti-mouse (Sigma) secondary antibody (in a 1/10,000 dilution). The presence of the DES7i-His-Tag protein was revealed by chemiluminescence (Pierce, ECL, Thermo).

2.5. Sterol extraction and HPLC analysis

To analyze sterol content in 3 dpi- and 4 dpi-grown cells, cultures were harvested by centrifugation at 5000 rpm for 10 min at 4 °C, re-suspended in 1.5 ml distilled water and then saponified in 1.5 ml 2 M NaOH in methanol–water (1:1, v/v) at 80 °C for 1 h. Sterols were extracted according to Bligh and Dyer and dried under a flow of nitrogen. The dried fractions were re-suspended in ethanol and centrifuged. Supernatants were separated by high-performance liquid chromatography (HPLC) on a C18 Ultrasphere column, using methanol–water (97:3, vol/vol) as the mobile phase. Stigmasterol (Sigma–Aldrich) was added as an internal standard for HPLC quantification.

To analyze biotransformation of radioactive cholesterol, HPLC coupled to a Flo-one Beta Radio 105 chromatography detector was used, following the manufacturer's instructions (Radiomatic, Canberra Company).

2.6. GC–MS analysis

To identify specific sterols in the samples, ethanol extracts were dried under a gentle flow of nitrogen and re-suspended in 100 μl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), followed by 30 min incubation at 70 °C. The composition of the steryltrimethylsilyl ester derivatives was analyzed by running samples through a 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 50–600 amu. The retention time and mass spectrum of all newly identified peaks obtained were compared to those of standards (Steraloids, USA) and/or available in the NIST library.

3. Results

3.1. Expression of T. thermophila C-7 sterol desaturase in S. frugiperda Sf9 cells

T. thermophila and other *Tetrahymena* species, like *T. piriformis*, harbor three sterol desaturases, with specificity towards C-5(6), C-7(8) or C-22(23) desaturation activity, as shown in Fig. 1 [7]. The enzymes react with different sterol substrates randomly, that is, in no strict reaction order. In the case of 5-cholesten-3β-ol (cholesterol) a C-7 sterol desaturase converts it to 5,7-cholestadien-3β-ol (provitamin D_3).

In spite of the apparent similarity with respect to the reaction that they catalyze, the three enzymes share no phylogenetic links, functional domains, nor topological motifs. Regarding the C-7 desaturase protein, Des7p, the model derived from its amino acid sequence classified this enzyme as a Rieske-type oxygenase, with a trans-membrane localization. This sequence suggests that the following architectural elements are present: (i) at least one N-terminal hydrophobic segment; (ii) a Rieske domain, harboring an [2Fe–2S] cluster coordinated by two His- and two Cys-(C–X–H–X16–17–C–X2–H) residues and that functions as an electron acceptor; (iii) a C-terminal catalytic domain harboring the active-site Fe(II) motif, in which the iron atom is coordinated by a His-His-carboxylate facial triad [6].

It has been proposed that Rieske-type oxygenases, though very diverse in sequence, share significant topological similarity [19]. The putative *nvd* gene identified in *S. littoralis* (GenBank: GU391576.1) is homologous to *Tetrahymena*'s Des7p, with an *E*-value of $2e^{-38}$ in a Tblastn (NCBI) analysis and shares the same topological distribution of functional groups, according to the classification scheme proposed by Capyk and Eltis [19]. Because of this similarity between both proteins, we selected *S. frugiperda* Sf9 as the host for the expression of a codon-optimized insect version of Des7p from *T. thermophila* (DES7*i*). As described in Section 2, three recombinant polyhedron-negative baculoviruses were obtained, following a standard protocol: (i) Control baculovirus used as control; (ii) DES7*i*, baculovirus with the DES7*i* sequence inserted into its genome, and DES7*i*-His-Tag, a baculovirus with a DES7*i* gene with an additional sequence encoding a 6×His-*Tag* attached to the C-terminus of the protein, for expression- and localization studies.

Protein expression was analyzed by Western blot of cell homogenates and isolated microsomal fractions. As shown in Fig. 2, a 62 kDa band in agreement with the predicted MW of the DES7*i* protein, was visible in such fractions from lysates of Sf9 cells infected with DES7*i*-His-Tag baculovirus, and absent in Sf9 fractions obtained from cells infected with non-tagged DES7*i* baculovirus, which served as controls. This microsomal localization of DES7*i* in insect cells confirms results of previous studies in *Tetrahymena*, where it was shown that a Des7p::eGFP fusion localized in defined compartments compatible with microsomes, such as the membranes surrounding both micro and macronucleus [6].

3.2. Sterol profile and cholesterol conversion in Sf9 cells

The profile of sterols isolated from cell homogenates at 3 dpi is shown in Fig. 3. In the HPLC chromatogram (A), it can be seen that the control strain contains only cholesterol and a small amount of

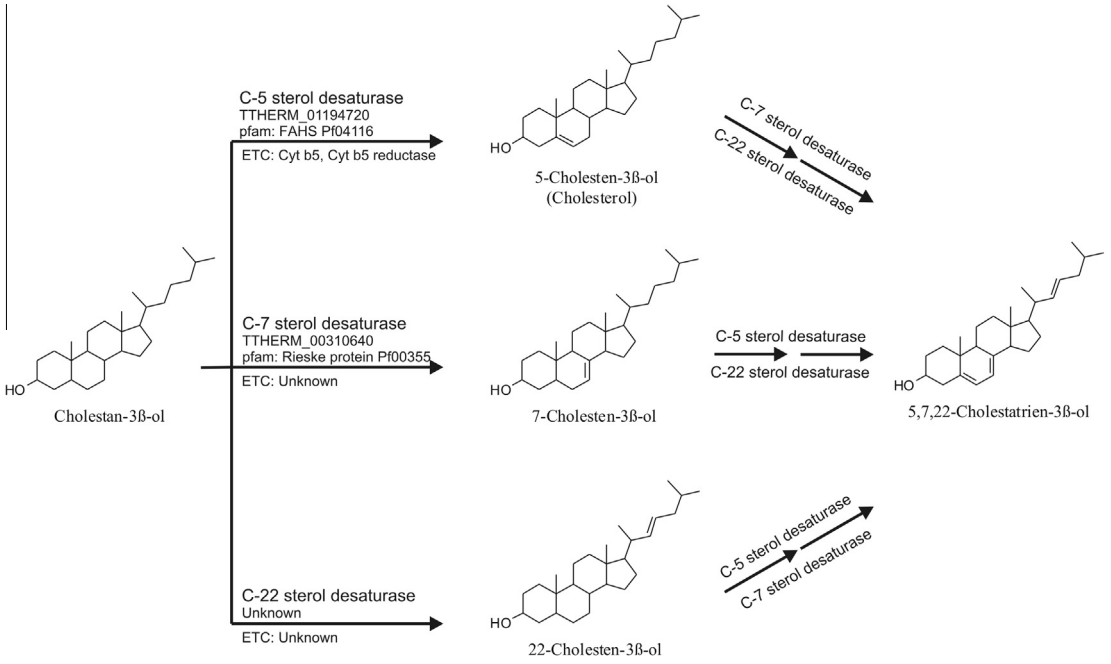

Fig. 1. Substrates and products of sterol desaturases present in *T. thermophila*. Sterol desaturase gene ID, motif involved and relevant electron transfer chain (ETC) are indicated.

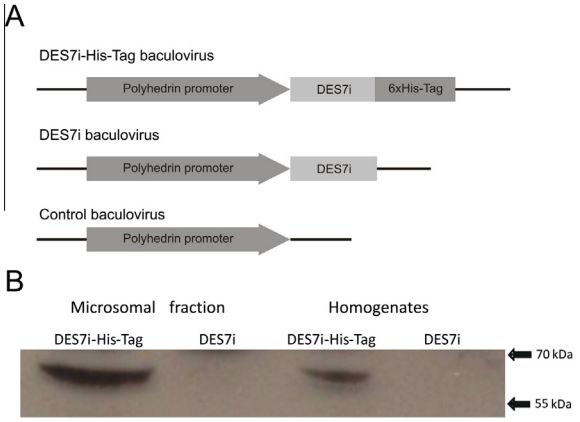

Fig. 2. (A) Schematic representation of constructs synthesized for recombinant baculovirus infection in Sf9 cells. (B) Expression of C-7 sterol desaturase from *Tetrahymena* in Sf9 cells. Samples from Sf9 cells infected with DES7*i*-His-Tag baculovirus (lanes 1 and 3) and DES7*i* baculovirus (lanes 2 and 4), which was used as a control. The proteins were detected using a mouse anti-His-Tag antibody.

7-dehydrocholesterol, whereas DES7*i*-expressing cells show the same profile, except that the level of 7-dehydrocholesterol was significantly increased. Noteworthy, with detection at 285 nm no other sterols appear in any of the chromatograms. To further corroborate the identity of 7-dehydrocholesterol in the samples, the UV absorption spectrum of peak (a) was measured using the HPLC variable wavelength detector. The same profile of DES7i sample and 7-dehydrocholesterol standard confirm the identity of the compound. Supplementary Fig. 1. Moreover, the identification of sterols performed by GC–MS (B–C), revealed the same predominant sterols in a similar quantitative relation (see below).

As the Sf9 cells infected with a control baculovirus accumulated low but significant amounts of 7-dehydrocholesterol (presumably from endogenous cholesterol), the cultures were supplemented with extra cholesterol, added at 3 dpi, to prevent possible sterol-substrate limitations. Measurements of the concentration of 7-dehydrocholesterol in homogenates prepared from cultures infected with the control baculovirus or with DES7i-expressing baculovirus were then compared at 3 dpi, with and without addition of exogenous cholesterol. As can be seen in Fig. 4, the control cells accumulates 0.092 ± 0.004 μg/ml and 0.210 ± 0.010 μg/ml of 7-dehydrocholesterol, with and without exogenous cholesterol added, respectively. This is indicative for the endogenous production of the oxidized sterol by this cell line (Fig. 4A). Cells expressing the DES7*i* gene produced higher levels of 7-dehydrocholesterol, which were not further significantly affected by the addition of exogenous cholesterol (1.5 ± 0.1 μg/ml and 1.25 ± 0.01 μg/ml, with and without added cholesterol, respectively).

The transformation of cholesterol to 7-dehydrocholesterol due to the expression of DES7*i* was also confirmed using radiolabeled cholesterol. After 5 h incubation with [1,2-^{3}H]cholesterol dispersed in methyl-β-cyclodextrin and added to 3 dpi cultures, the control

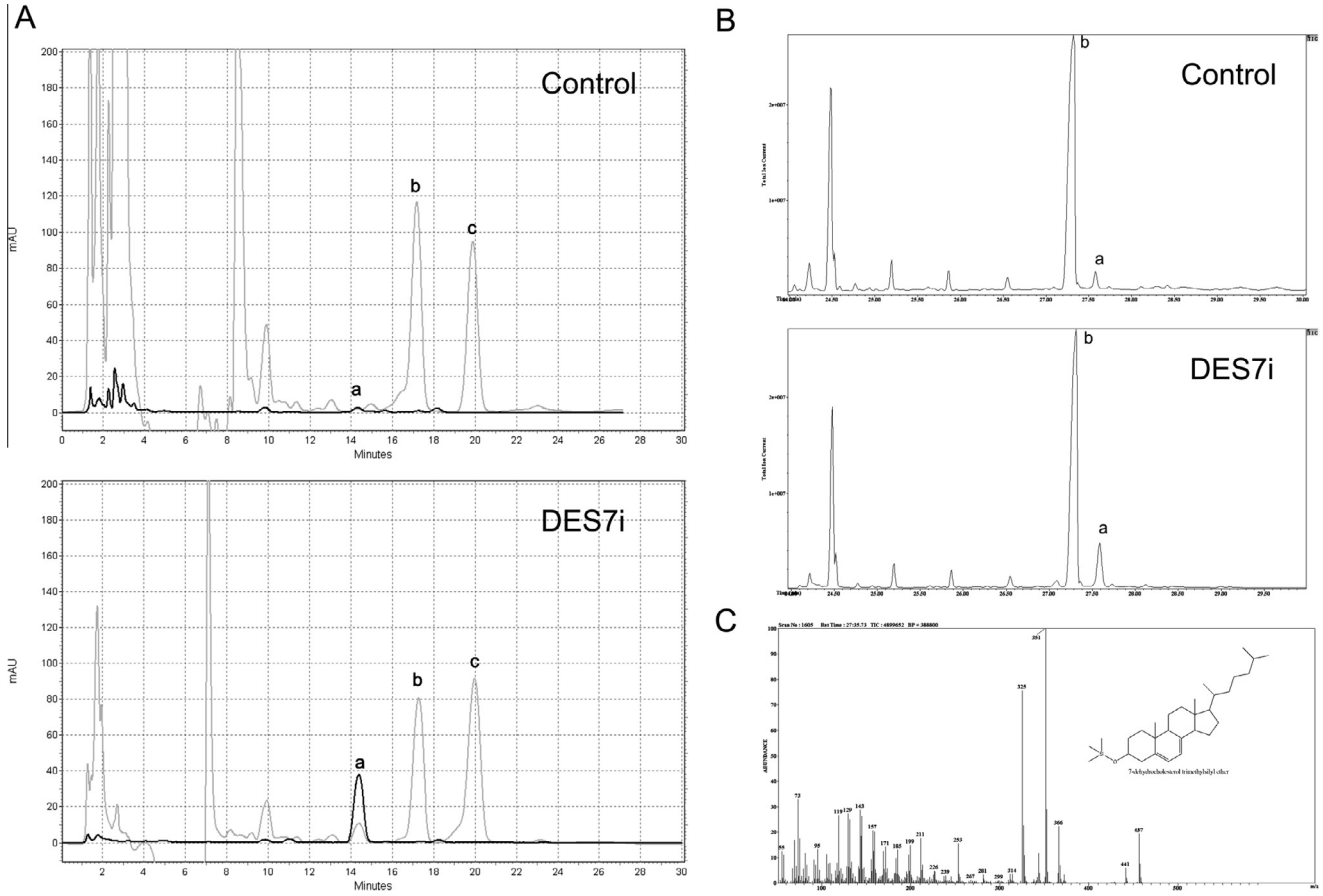

Fig. 3. Sterol profile of Sf9 cells infected with recombinant baculovirus. (A) HPLC analysis of cells infected with control (up) and DES7*i* (down) baculovirus. Absorbance was monitored at 210 nm (light gray line) and 285 nm (dark gray line), the latter is specific for sterols containing conjugate double bonds (here: 5,7diene derivatives). Sterol identification was done with available standards. (B) Gas chromatography (GC) analysis of trimethylsilyl ether derivatives of the total sterol fraction extracted from cells infected with control (upper panel) and DES7*i* (lower panel) baculovirus. The *y* ordinate indicates the total ion current. (C) MS results from peak a from the lower panel of part A. Peaks: a, 7-dehydrocholesterol; b, cholesterol; c, stigmasterol used as the internal standard.

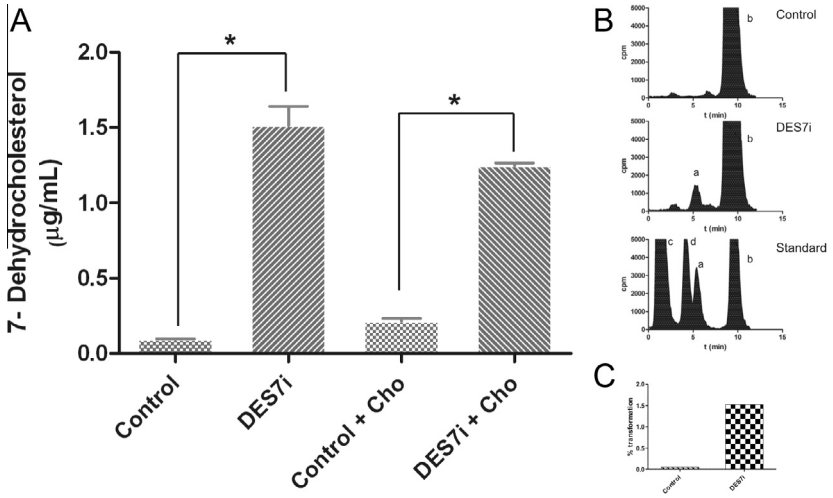

Fig. 4. (A) HPLC quantification of 7-dehydrocholesterol produced in cell cultures infected with: control baculovirus (Control), DES7*i* baculovirus (DES7*i*), control cells with added methyl-β-cyclodextrin:cholesterol (Control + Cho) and DES7*i* baculovirus incubated with added methyl-β-cyclodextrin:cholesterol (DES7*i* + Cho). Data are shown as mean ± SE ($n = 3$), $^{*}P < 0.001$, *t*-test. (B) HPLC coupled to radioactive Beta detection. Cells infected with control baculovirus or DES7*i* baculovirus at 3 dpi, and then incubated for 5 h with a mixture of methyl-β-cyclodextrin:[1,2-^{3}H]cholesterol (80 µg/ml, 4 µCi/ml). Standard: product formation during the incubation of live *Tetrahymena* with radioactive cholesterol. Peaks: a, 7-dehydrocholesterol; b, cholesterol; c, 7,22-bisdehydrocholesterol; d, 22-dehydrocholesterol. (C) Biotransformation of radioactive cholesterol to 7-dehydrocholesterol in Sf9 cells infected with control and DES7*i* baculovirus. The total amount of radiolabeled sterols present (i.e., cholesterol + 7-dehydrocholesterol) is set as 100%.

cell accumulated 0.04% 7-dehydrocholesterol, considering the total amount of radiolabeled sterols present (i.e., cholesterol + 7-dehydrocholesterol) as 100%, whereas cells infected with DES7*i* baculovirus accumulated 1.52% 7-dehydrocholesterol, in the same period (Fig. 4B). This almost 40-fold increase in the conversion of radiolabeled cholesterol to 7-dehydrocholesterol observed in cells infected with DES7*i* baculovirus, may only be attributed to the expression of active Des7 protein (Fig. 4C).

3.3. Phylogenetics analysis of C-7 sterol desaturase from T. thermophila

Analysis of Rieske oxygenases (RO) based on topological similarity grouped the Nvd protein (C-7 cholesterol desaturase) from *Drosophilia melanogaster* with bacterial RO's, close to the KshA protein from *Mycobacterium tuberculosis* which has 3-ketosteroid-9a-hydroxylase (KSH) activity [19]. As expected, Des7p from *Tetrahymena* also groups in this clan (Fig. 5). Moreover, the relevant phylogenetic tree shows two distinct groups of RO sequences, with different structural features of their Rieske domain; together with KshA, *Tetrahymena*'s Des7p belongs to the so-called Group II.

3.4. Electron transfer donors for eukaryotic Rieske oxygenases

Based on studies on the cholesterol degradation pathway in *Rhodococcus* species and *M. tuberculosis*, it was proposed that KSH may function as a two-component system composed of a ferredoxin reductase, i.e., KshB, and a terminal oxygenase, i.e., KshA [20]. We hypothesize that if Des7p is part of a similar system, another Rieske-type protein – currently unknown – must be part of an electron transfer chain (ETC) for Des7p. To test this, a bioinformatics search on possible electron donors for the C-7 desaturase in *Tetrahymena* was performed. This search retrieved a number of putative reductases and ferredoxins that can be part of such an ETC in the ciliate. Table 1 shows all iron-sulfur proteins of *T. thermophila*, including three putative ferredoxins, five putative reductases and two putative oxidases, plus the already known Des7p oxygenase.

Among the reductases there are: a (mitochondrial, membrane-bound) NADH-Ubiquinone oxidoreductase (Q23KA9), and an Ubiquinol-cytochrome c reductase (I7MIC7), both likely involved in respiratory electron transfer; an NAD(FAD)-dehydrogenase (Q241B3) and a ferredoxin-NAD(+) reductase (Q24D68), involved in cell redox homeostasis in metazoans, and an NADH dehydrogenase (I7MDW5) involved in Ubiquinone reduction. In the oxidase/oxygenase group are included a (mitochondrial) succinate dehydrogenase (I7M403) and a dihydro-pyrimidine dehydrogenase (Q23YG3) involved in uridine monophosphate biosynthesis, plus the oxygenase Des7p (I7ML19), involved in sterol metabolism.

From motifs-comparison with the bacterial KshB reductase, only two putative reductases from the ciliate (Q24D68 and Q241B3) share the three motifs identified in the bacterial KshB reductase (i.e., the flavin-binding motif, the NADH-binding motif and the Rieske-binding motif). Hence we assume that these latter two proteins could be involved in the electron transfer chain from NADH to the final oxygenase Des7p. Additionally, a STRING analysis (database of known and predicted protein interactions) [21] selected NAD(FAD)-dehydrogenase Q241B3 as the most likely electron donor, but this hypothesis has yet to be tested, because prediction was made based on gene neighborhood in members of the bacterial phyla Actinobacteria and Proteobacteria, and co-expression of homologs in other taxonomic groups, but none in ciliates.

On the other hand, to test the presence of orthologs in Lepidoptera, a BLAST search was performed using the ciliate NAD (FAD)-dehydrogenase as query. Interestingly, the search retrieved putative proteins in *Bombyx mori* and *S. frugiperda* with the three motifs identified in the bacterial KshB reductase, that can explain the functionality of *Tetrahymena*'s 7-cholesterol desaturase in these hosts.

4. Discussion

Tetrahymena harbors at least three microsomal sterol desaturases with C-5(6), C-7(8) and C-22(23) activity on C27 and C29 sterols. All three enzymes require molecular oxygen, a reduced NAD(P)H cofactor, and respond similarly to various oxygenase

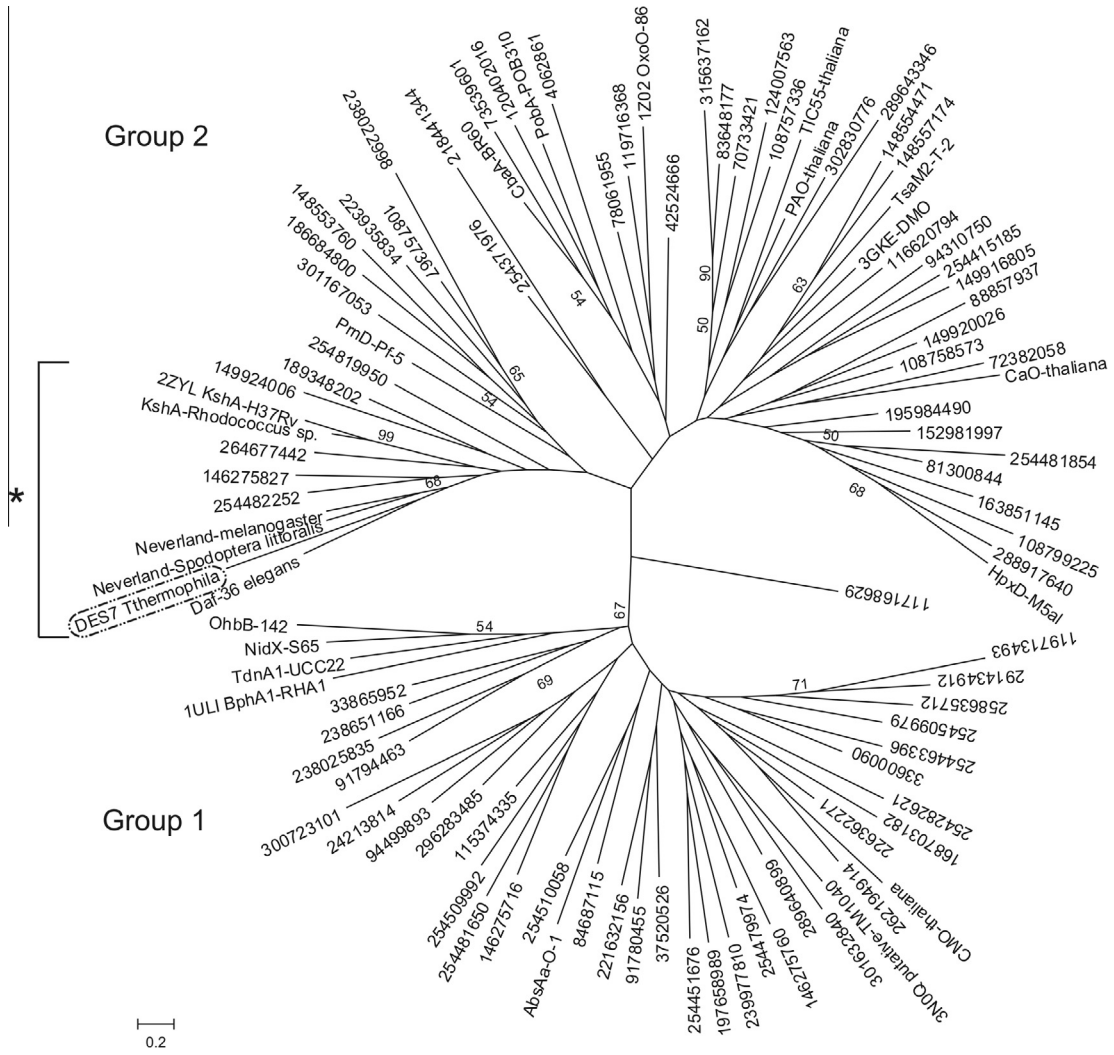

Fig. 5. Consensus tree of representative sequences of Rieske oxygenases [9]. The 100 sequences were aligned using the MUSCLE algorithm and the phylogenetic tree was created using the maximum-likelihood method with 100 bootstrap replicates, using MEGA5.2 software [34]. C-7 sterol desaturase from *T. thermophila* (DES7 *T. thermophila*, in circle), *C. elegans* (Daf-36 elegans), *D. melanogaster* (Neverland-melanogaster) and *S. littoralis* (Neverland *Spodoptera littoralis*) as well as KshA proteins from *R. rhodochrous* (KshARhodococcus) were included in the analysis. These proteins form a more robust clan, denoted with an asterisk, in group II from the bilobar tree. The bar indicates percentage substitution.

inhibitors [7]. The so-far identified proteins display different structural motifs, electron donor specificity and phylogenetic background. The C-5 desaturase belongs to the fatty acid hydroxylase superfamily (pfam: PF04116), is dependent on a Cytb_5/Cytb_5 reductase combination and can complement an *erg3* null mutant in yeasts [22], whereas the C-7 sterol desaturase is a Rieske-type oxygenase of the Nvd type, that is mostly present in animals [6], and the C-22 sterol desaturase has not been identified yet in any canonical group, excluding also the cytochrome P450 (CYP) enzymes, typical of all currently known C-22 desaturases [23]. This diversity in motifs and specificities of sterol modifying enzymes of the post-squalene route may reflect a relatively fast evolution of *Tetrahymena*, a phenomenon that has been noted for other ciliate genes as well [24]. This then raises the question whether fast-evolving *Tetrahymena* sterol desaturases are still compatible with the microsomal electron transport machinery in distantly-related organisms like the insects. To address this question, we expressed a codon-optimized variant of *Des7p of Tetrahymena* in *Spodoptera*, and tested its functionality in the insect cell line.

Among the eukaryotes, the Rieske-like oxygenases are conserved between plants, worms, insects, sea urchins, amphibians, fish and birds. Recently they were also described in choanoflagellates and in the more distant ciliates *Tetrahymena* and *Paramecium* [6]. Functional studies in worms and flies of DAF-36/Nvd proteins are amongst the first to assign a role of such enzymes to steroidogenesis.

Transcription and expression of the Nvd protein/s in the insect *S. littoralis* have been reported in the larva's prothoracic gland and testis, but so far the process has not been studied in cells suspensions [16]. Additionally, it was reported that the ratio of cholesterol over phospholipids was significantly lower in membranes of the insect than in the vertebrates [25], and that this ratio may further decrease upon infection with baculovirus, which is known to interfere with the developmental process in insects. For this reason, and to prevent possible substrate limitation, we decided to test the supplementation of cultures with additional cholesterol.

During this work we observed that the C-7 cholesterol desaturase activity in *S. frugiperda* could be increased by the overexpression of *T. thermopohila* DES7*i*.The protein was detected in the microsomal fraction, in agreement with previous reports on its localization [6,10]. In spite of the large evolutionary distance between *S. frugiperda* and *T. thermophila*, our results indicate that

Table 1
Proteins harboring an iron-sulfur cluster in the *T. thermophila*'s genome, including three putative ferredoxins, five putative reductases, and two putative oxidases, plus the already known Des7p oxygenase (*). Pfam denotes domains present in each protein.

Protein class	Gene ID	Uniprot ID	pfam code	pfam	Length (aa)
Ferredoxins	TTHERM_00161210	Q22W11	PF00111	Fer2	172
	TTHERM_00161840	Q22VV0	PF00111	Fer2	165
	TTHERM_00256970	Q23QI1	PF00111	Fer2	130
Oxygenases/oxidases	TTHERM_00310640*	I7ML19	PF00355	Rieske	514
	TTHERM_00241700	I7M403	PF13085	Fer2 3	312
			PF13534	Fer4 17	
	TTHERM_01250100	Q23YG3	PF14691	Fer4_20	1023
			PF13450	NAD_binding_8	
			PF01180	DHO_dh	
			PF14697	Fer4_21	
Reductases	TTHERM_00194260	Q23KA9	PF13510	Fer2.4	718
			PF10588	NADH-G 4Fe-4S 3	
			PF00384	Molybdopterin	
			PF09326	DUF1982	
	TTHERM_00622710	Q241B3	PF00355	Rieske	631
			PF07992	Pyr_redox_2	
			PF00070	Pyr_redox	
			PF14759	Reductase_C	
	TTHERM_00295080	I7MIC7	PF00355	Rieske	269
	TTHERM_01104910	Q24D68	PF00355	Rieske	686
			PF07992	Pyr redox 2	
	TTHERM_00294640	I7MDW5	PF12838	Fer4_7	236

there is significant conservation of physiological electron donors, since the ciliate's sterol desaturase can function in the context of the insect's electron transport system.

Our results are in agreement with the proposition that the last eukaryotic common ancestor already harbored enzymes for sterol biosynthesis, and that subsequent evolution in the eukaryotic domain occurred by gene modification, gains and/or losses [26]. In this respect, the C-7 cholesterol desaturase seems to be another example of a horizontal gene-transfer event, just as it has been suggested forthe HMG-CoA reductase, the squalene–hopene-cyclase, the squalene synthase and the C-24 sterol desaturase-like [27,28].

Rieske-type oxygenases (ROs) are known to catalyze a range of chemical reactions such as mono- and di-oxygenation, ring desaturations, O-dealkylation, sulfoxidation, among others [29,30]. Additionally, several ROs are known to catalyze more than one reaction simultaneously, like e.g. carbazole 1,9a-dioxygenase. This range of reactions performed by ROs demonstrates the broad catalytic capacity of the active site of these non-heme iron proteins. In fact the 3-ketosteroid-9α-hydroxylase activity from *Rhodococcus rhodochrous* is based on the presence of five homologous enzymes (encoded by *kshA1–kshA5*), each with a preference for suitable substrates [31].

Previous reports on *nvd*/*daf-36* emphasized the high degree of conservation of this family of genes across the animal phyla, including insects and nematodes, and several deuterostome species, with the exception of mammals [10,11]. In accordance, Des7p from *T. thermophila* groups with Nvd from *D. melanogaster*, but surprisingly, both also share structural and topological motifs with the bacterial Rieske oxygenase KshA from *R. rhodochrous* and *M. tuberculosis*.

Among the animal phyla, the C-7 sterol desaturase is the only Rieske-type oxygenase identified so far, although no physiological electron transport chain has yet been linked to its activity. In contrast, the ETC from the bacterial homologous, KshA, has been well characterized [32,33]. It is expected that the structural similarity between Des7p and KshA may provide a template for the analysis of unexplored eukaryotic ETC members. In this respect it is relevant to note that the two putative reductases from the ciliate, Q24D68 and Q241B3, do have the three motifs identified in the bacterial KshB reductase (i.e., a flavin-binding site, an NADH-binding site and the rieske-binding motifs) and could be involved in the electron transfer chain from NADH to the final oxygenase Des7p. Nevertheless, as no other information is yet available on the mechanism of the desaturation reaction performed by Des7p or of any other eukaryotic Nvd protein, as for now none of the predicted genes can be discarded as the potential electron donor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2015.02.001.

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