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ORIGINAL ARTICLE

Multifunctional Acyltransferases from *Tetrahymena thermophila*

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Abstract Multifunctional acyltransferases are able to catalyze the esterification of various acyl-acceptors with activated fatty acids. Here we describe the identification of four proteins from *Tetrahymena thermophila* that share certain properties with mammalian acyltransferases regarding their predicted transmembrane structure, their molecular mass and the typical acyltransferase motif. Expression of the *Tetrahymena* sequences results in production of triacylglycerols and wax esters in recombinant yeast when appropriate substrates are provided. The in vitro characterization shows, that these enzymes are capable of esterifying different acyl-acceptors including fatty alcohols, diols, diacylglycerols and isoprenols with acyl-CoA thioesters. Based on these catalytic activities and the sequence similarities of the *Tetrahymena* proteins with acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) family members, we conclude that we identified a new group of DGAT2-related multifunctional acyltransferases from protozoan organisms.

Keywords Multifunctional acyltransferase · *Tetrahymena* · Wax ester · DGAT · Prenyl ester · Diester

Abbreviations

ASAT	Acyl-CoA:sterol acyltransferase, steryl ester synthase
CoA	Coenzyme A
DGAT	Acyl-CoA:diacylglycerol acyltransferase
DES	Diester synthase
PES	Prenylester synthase
TAG	Triacylglycerol(s)
WE	Wax ester(s)
WS	Wax synthase

Introduction

Acyltransferases belong to the large group of enzymes, transferring groups other than amino-acyl groups (EC 2.3.1) onto acyl-acceptors like fatty alcohols (EC 2.3.1.75) or diacylglycerols (EC 2.3.1.20). Genes encoding multifunctional acyltransferases catalyzing the esterification of a variety of acyl-acceptor moieties with acyl-CoA thioesters have been characterized from prokaryotic and eukaryotic organisms [1–5].

More recently, genes essential for wax ester synthesis became important tools for developing renewable resources for the technical industry [6–8]. The increasing prices of fossil material make up the market for tailor-made wax esters (WE). These lipids in general are excellent lubricants with good stability under high temperature and pressure and they are very resistant to hydrolysis [6], which makes them attractive as lubricants. While saturated long-chain monoesters have good lubricity but poor low temperature fluidity, unsaturated monoesters or diesters overcome this lack and combine it with good thermal and oxidative stability and high viscosity indices [9]. To achieve the renewable production of WE, experiments are in progress to identify new enzymes catalyzing respective esterification

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reactions [10–13]. Production of high levels of WE in oil crops [6, 7] or microorganisms [8, 12] might be able to replace fossil materials in future.

Tetrahymena is a unicellular protozoan and belongs to the phylum of Ciliophora [14]. It is common in temperate freshwater [15] and can be cultivated in the laboratory. *Tetrahymena* is a model system for eukaryotic cells that provides features of higher eukaryotic organisms which cannot be found in other unicellular model systems like *Saccharomyces cerevisiae* [16]. Studies on lipid components in *Tetrahymena thermophila* (before 1976 *T. thermophila* was referred to as *T. pyriformis* variety or syngen 1 [17]) showed that the cells are able to accumulate large amounts of lipids. During the logarithmic phase, 24% of the cell dry weight is made up by lipids [18], 28% of which are neutral lipids located in lipid droplets [19], mainly triacylglycerols (TAG) esterified with saturated fatty acids of 14–18 carbon atoms [20]. The WE make up a small portion of the total lipid content of only about 0.15% [21]. Unlike TAG they contain branched-chain acyl-groups of 17–21 carbon atoms (76%) and branched-chain alcohols of 17–19 carbon atoms (45%) [21]. In addition to long-chain WE, fatty acid methyl and ethyl esters have also been found in *Tetrahymena*, of which methyl linoleate, ethyl myristate and ethyl linoleate are the most abundant [22]. The physiological function of these esters and WE is not clear because the low amount excludes storage functions [21]. They might be communication scents or simply a way to control the level of free alcohols to prevent toxic effects for the cells [22].

Like other ciliates, *Tetrahymena* has two specialized nuclei: the micronucleus (MIC) is the diploid germline while the haploid macronucleus (MAC), which differentiates from the MIC, is the somatic gene expression machine [15, 23]. The recent whole genome shotgun sequencing and assembly of the *Tetrahymena* MAC genome by the Institute for Genomic Research (TIGR) in 2006 revealed that the genome contains 104 Mb which cluster on 250–300 chromosomes [16, 24]. Computational analyses revealed about 25,000 protein coding reading frames, about 17,000 of which have strong matches to genes in other organisms [16]. Similarity based analyses of the *Tetrahymena* genome database (TGD) [24] with known mammalian wax synthase sequences [1, 3, 13] revealed several proteins, some of which were analyzed in this work.

In this study we identified four sequences related to DGAT2 family members. Heterologous expression studies in a suitable yeast mutant and enzyme assays with transgenic yeast membranes revealed that these sequences encode multifunctional acyltransferases which accept various acyl-acceptors.

Materials and Methods

Identification and Cloning of Putative Wax Synthases

BLASTp [25] studies were undertaken to investigate the predicted *Tetrahymena thermophila* proteome for putative wax synthases using the NCBI server (http://blast.ncbi.nlm.nih.gov/). Human wax synthase sequences AWAT1 (NCBI: NP_001013597) and AWAT2 (NCBI: NP_001002254) were used as query sequences. The derived protein sequences (for NCBI accession numbers and nomenclature see below) were used to generate nucleotide sequences adapted to expression in *Brassica* and synthesized by GeneArt. The respective sequences were cloned from GATEWAY® entry vectors (pDONR221) into pYES-DEST52 expression vectors (Invitrogen) and expressed in *S. cerevisiae* BY4741 Δlro1 Δdga1 [26], a yeast mutant lacking TAG synthesis (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lro1-Δ::kanMX4 dga1-Δ::natMX4). In the following the *Tetrahymena* proteins are termed as follows: XP_001027910: TtWS1; XP_001026090: TtWS2; XP_001008104: TtWS3; XP_001017939: TtWS4.

Functional Analysis in Yeast

Transgenic yeast strains expressing a *Tetrahymena* sequence in pYES-DEST52 vectors (Invitrogen) were cultivated in SD minimal medium containing 0.17% (w/v) yeast nitrogen base (MP Biomedicals), 0.068% (w/v) complete supplement medium without uracil and leucine (MP Biomedicals), 0.5% NH_4SO_4 (w/v), 0.01% (w/v) leucine and 2% glucose (Roth) for 24 h at 28 °C. The cells of 50 ml cultures were induced with 2% galactose for 48 h and were supplemented with 125 μM decanol, dodecanol, tetradecanol, hexadecanol and octadecanol (Roth). Cells were harvested, washed and stored at −20 °C until extraction.

Extraction of yeast cells was performed according to the protocol of Bligh and Dyer [27]. The lipid extracts were separated by TLC on preparative TLC plates (Silica Gel 60 plates 0.5 mm thickness, Merck) in heptane/diethyl ether/acetic acid (90/30/1, v/v/v) and visualized under UV light after spraying with dichlorofluorescein (0.3% (w/v) dissolved in isopropanol) [28]. Myristoyl-dodecanoate (Sigma Aldrich) and TAG isolated from sunflower oil were used as standards.

GC Analysis of WE and TAG

Bands co-chromatographing with the WE and TAG standards were transmethylated in 0.5 M sulfuric acid and 3% dimethoxypropane in methanol for 1 h at 80 °C. 250 nmol docosanoic acid was added as an internal standard before

transmethylation. Fatty acid methyl esters (FAME) and fatty alcohols were extracted with heptane, concentrated and analyzed via gas chromatography (GC) with flame ionization detection (FID). For quantification of WE the total amount of fatty alcohols in the fractions was summarized, for quantification of TAG the sum of FAME was divided by 3.

GC-FID analysis was carried out using the HP6890 gas chromatograph equipped with an OPTIMA225 column (Macherey & Nagel) (25 m length, 0.25 mm diameter, 0.25 µm film thickness). 1 µl of the extract was analyzed in splitless injection with N_2 as carrier gas (constant flow, 0.9 bar pressure, total column flow 1 ml/min) and inlet and detector temperatures of 260 °C. A temperature program was carried out starting at 120 °C, 8 °C/min to 144 °C, 4 °C/min to 240 °C. Peaks were identified via comparison of the respective retention times with standard substances of different fatty alcohols and FAME (Sigma Aldrich).

Phylogenetic Analysis and Structure Prediction

Sequence analyses were carried out using ClustalX2 [29] and GeneDoc [30] software. Phylograms were computed with MEGA5 [31] and neighbor-joining method [32] with 1,000 bootstrap replicates using *p*-distance method. All gaps were deleted for computation of evolutionary distances.

Molecular mass and isoelectric points were calculated using ProtParam [33] on the ExPASy Server (http://web.expasy.org/protparam/). Transmembrane structures of *Tetrahymena* proteins and mammalian wax synthases were predicted using TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) [34–36]. Predictions were compared to Kyte Doolittle plots [37] with window parameters of 19, which revealed similar results. Acyltransferase superfamily motifs and putative acyl-acceptor binding pockets were discovered by NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/) [38].

Preparation of Yeast Membranes and In Vitro Wax Synthase Assay

Transgenic yeast cells of 200-ml cultures were harvested after 16 h of induction, washed in Tris–H_2SO_4 (50 mM, pH 7.4), frozen for 15 min at −20 °C and disrupted using glass beads (0.75–1 mm diameter, Roth) by vortexing for 5 min in 2 ml Tris–H_2SO_4 buffer. Cell disruption was repeated three times and the supernatants were collected by centrifugation and combined. After sonication (two times for 30 s) the cell debris was sedimented (2,500×*g*, 15 min and 4 °C) and the membranes were isolated from the supernatant by high speed centrifugation (1 h, 140,000×*g*, 4 °C). The sedimented membranes were resuspended in an appropriate volume of Tris–H_2SO_4 buffer and stored in aliquots at −80 °C. The protein concentration was determined [39].

WS activity was measured in 10 mM BIS–Tris-propane pH 9, 13 µM [1-^{14}C]-labeled acyl-CoA ([1-^{14}C]-myristoyl-CoA and [1-^{14}C]-stearoyl-CoA purchased from Biotrend, specific activity 2.03 Bq/pmol, [1-^{14}C]-palmitoyl-CoA purchased from Perkin Elmer, specific activity 2.22 Bq/pmol, [1-^{14}C]-oleyl-CoA purchased from Perkin Elmer, specific activity 2.03 Bq/pmol, [1-^{14}C]-decanoyl-CoA specific activity 0.09 Bq/pmol and [1-^{14}C]-dodecanoyl-CoA, specific activity 0.7 Bq/pmol and 2-methyl-acyl-CoA (14:0, 16:0 and 18:0) specific activity 0.62 Bq/pmol were prepared by Prof. Sten Stymne and members of his laboratory, SLU Alnarp, Sweden) and 300 µM acyl-acceptors (Sigma Aldrich) using 1–2 µg protein from total membrane fractions. Acyl-acceptors were dissolved in heptane and evaporated to dryness in the reaction tubes before addition of further assay components, DGAT activity was measured under standard WS assay conditions with endogenous DAG present in the yeast membranes. Incubation was carried out at 35 °C for 20 min. Lipids were extracted with 250 µl chloroform:methanol (1:1) and 100 µl 0.45% NaCl-solution. 80 µl of chloroform phase was applied to TLC silica gel plates (Merck) and chromatographed in heptane/diethyl ether/acetic acid (90/20/1 v/v/v). The bands were visualized with the FLA-3000 bioimager system (Fujifilm). For quantification, single bands were analyzed in a liquid scintillation counter LS 6500 (Beckman Coulter).

Results

Identification of Putative Wax Synthases from *Tetrahymena thermophila*

Sequence similarity based searches conducted with human AWAT1 and AWAT2 wax synthase sequences as query against the annotated *Tetrahymena thermophila* proteome resulted in several protein sequences with similarities to the human wax synthases. Four sequences (for NCBI accession number see experimental procedures) with the highest identities to human sequences (15–21%) were analyzed. Sequence identity between the *Tetrahymena* proteins varied between 34 and 44%. No sequence similarities could be detected with jojoba type wax synthases from plants [11, 40] or *Euglena* [10] or the bifunctional proteins from petunia [41] or *Acinetobacter* [4, 42]. The *Tetrahymena* sequences share certain identities with DGAT2 related proteins from plants and fungi (13–20%) [43–47]. As shown in Fig. 1, the *Tetrahymena* proteins build a new branch of proteins related to the DGAT2 protein family.

The relation to the mammalian DGAT2 family is supported by some distinct features that can be found in both families. All proteins contain 306–333 amino acids and have a predicted molecular mass between 35 and 38 kDa. The isoelectric point lies between 9.3 and 9.4, which is a typical feature of DGAT2 family members [48]. In addition TtWS1, TtWS2 and TtWS4 contain a predicted transmembrane helix at the N-terminus like AWAT1 (Fig. 2a), while the C-terminal part of the protein is predicted to be exposed to the cytoplasmic phase. TtWS3, like AWAT2, shows a slightly different topology in the models with two to three transmembrane domains at the N-terminal part of the protein, but the C-terminus is also reaching out into the cytosol. Conserved sequence search displayed an acyltransferase superfamily motif (NCBI database cl00357) in all sequences in which several conserved amino acids can be found that are predicted to belong to an acyl-acceptor binding pocket (cd07986) (Fig. 2a).

Sequence alignments with human AWAT showed the highest variety in the N-terminal part of the protein, where the transmembrane domains are located. Furthermore the HPHG (amino acids 103–106 in HsAWAT1) motif which is characteristic for DGAT2 family members [48–50] was found in TtWS1 and TtWS4, while TtWS2 and TtWS3 showed a modified version in which glycine is substituted with asparagine or tyrosine, respectively (Fig. 2b, Supplemental data 1).

Functional Expression in Yeast

To determine the identities of the four *Tetrahymena* proteins, lipid extracts of transgenic yeasts expressing a *Tetrahymena* sequence were analyzed. Thin layer chromatography suggested that TtWS2, TtWS3 and TtWS4 were able to restore triacylglycerol synthesis in yeast, while TtWS1, TtWS2 and TtWS4 appear to cause wax production as well, if they are provided with appropriate alcohols. The identities of the products were verified and the amounts were quantified by GC analysis after transmethylation of the WE and TAG bands scraped off from the TLC plates (Fig. 3).

By GC analysis decanol (10:0-OH), dodecanol (12:0-OH), tetradecanol (14:0-OH) and hexadecanol (16:0-OH) and the FAME corresponding to palmitic (16:0),

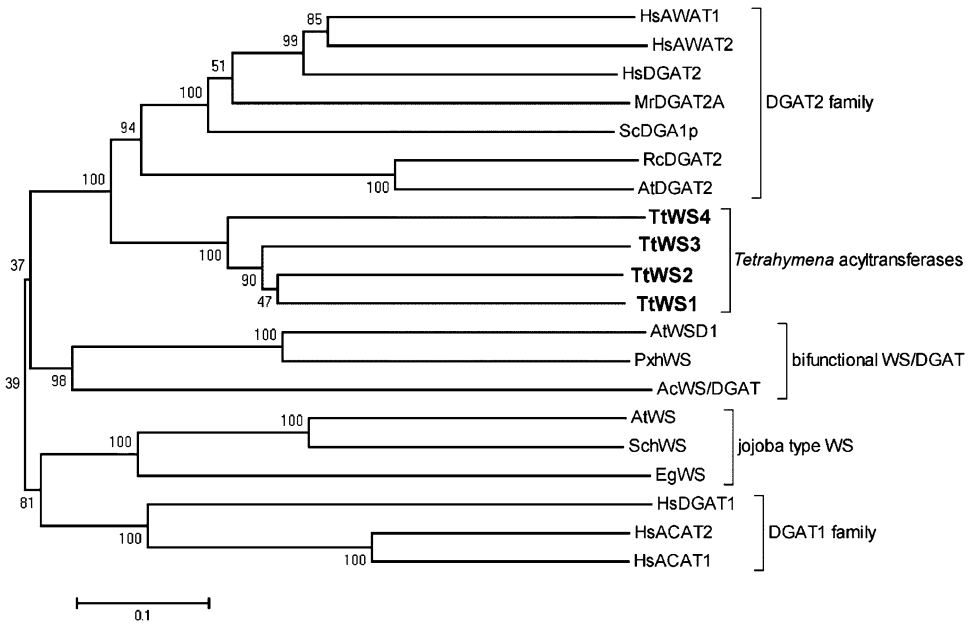

Fig. 1 Phylogram showing the relation of four *Tetrahymena* acyltransferases with selected acyltransferases from human, plant, fungi and bacteria. HsAWAT2 (*Homo sapiens*, NCBI: NP_001013597.1), HsAWAT2 (*H. sapiens*, NCBI: NP_001002254.1), HsDGAT2 (*H. sapiens*, NCBI: NP_115953.2), MrDGAT2A (*Mortierella ramanniana*, NCBI: AF391089_1), ScDGAT1p (*Saccharomyces cerevisiae*, NCBI: NP_014888.1), RcDGAT2 (*Ricinus communis*, NCBI: ABI83668.1), AtDGAT2 (*Arabidopsis thaliana*, NCBI: Q9ASU1.1), TtWS1 (*Tetrahymena thermophila*, NCBI: XP_001027910), TtWS2 (*T. thermophila*, NCBI: XP_001026090), TtWS3 (*T. thermophila*, NCBI: XP_001008104), TtWS4 (*T. thermophila*, NCBI: XP_001017939), AtWSD1 (*A. thaliana*, NCBI: NP_568547.1), PxhWS (*Petunia x hybrida*, NCBI: AAZ08051.1), AcWS/DGAT (*Acinetobacter calcoaceticus*, NCBI: YP_045555.1), AtWS (*A. thaliana*, NCBI: NP_200345.1), SchWS (*Simmondsia chinensis*, NCBI: AF149919.1), EgWS (*Euglena gracilis*, NCBI: ADI60058.1), HsDGAT1 (*H. sapiens*, NCBI: NP_036211.2), HsACAT2 (*H. sapiens*, NCBI: NP_003569.1), HsACAT1 (*H. sapiens*, NCBI: NP_003092.4). The *scale* corresponds to amino acid substitutions per site in the given alignment with 237 positions and 20 sequences. Numbers next to the branches are bootstrap values indicating the probability of this relationship in %. The phylogram was created with ClustalX2 and MEGA5 software

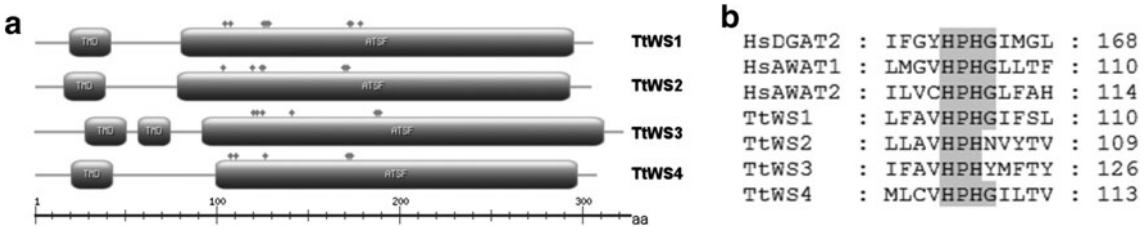

Fig. 2 Predicted domains and motifs of *Tetrahymena* WS proteins. The putative transmembrane domain (TMD) and the acyltransferase superfamily motif (ATSF) are shown by squares, amino acids of the putative acyl-acceptor binding pocket are labeled with pins (**a**). The conserved HPHG motif of DGAT2 family members and the substitutions of the glycine residues in TtWS2 and TtWS3 are depicted (**b**). Figure **a** was created using ExPASy Prosite Mydomains and the alignment (**b**) was created with ClustalX2 and GeneDoc

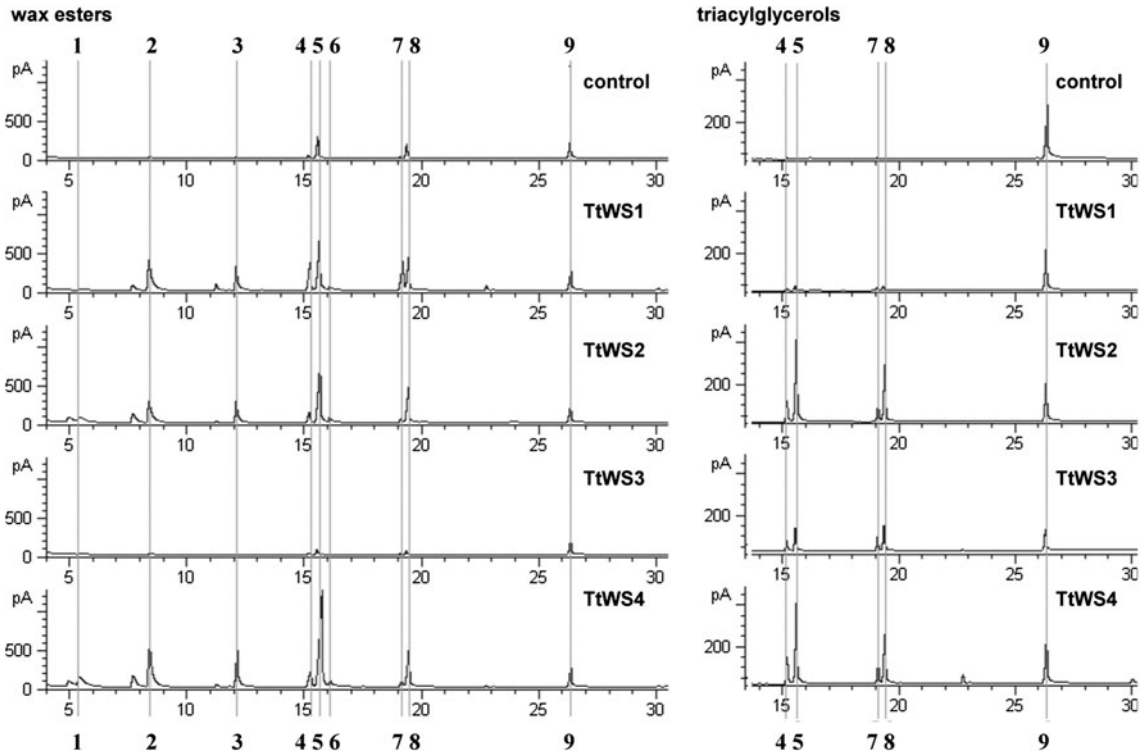

Fig. 3 Functional expression of TtWS sequences in yeast cells. Lipids were extracted from the yeast cells after 48 h cultivation in medium supplemented with 125 μM 10:0-, 12:0-, 14:0-, 16:- and 18:0-OH, separated by TLC and wax esters and triacylglycerols were quantified by GC using 250 nmol methyl docosanoate as internal standard. The respective GC chromatograms of transmethylated WE and TAG are depicted, the *x-axis* displays the retention time [min] and the *y-axis* corresponds to the FID signal strength [pA]. *1* 10:0-OH, *2* 12:0-OH, *3* 14:0-OH, *4* methyl-16:0, *5* methyl-16:1, *6* 16:0-OH, *7* methyl-18:0, *8* methyl-18:1, *9* methyl-22:0 (internal standard)

palmitoleic (16:1), stearic (18:0) and oleic acid (18:1) were identified. The empty vector control and TtWS3 expressing cells showed very small amounts of alcohols in the WE fractions only, while TtWS1, TtWS2 and TtWS4 expressing cells contained distinctly higher amounts (Fig. 4). 12:0-OH was the most abundant alcohol (50% of alcohol in WE) followed by 14:0-OH (5–30%) then 10:0-OH (10–15%) and 16:0-OH (<10%). Regarding the fatty acids in the WE fraction the biggest portion was made up by mono-unsaturated fatty acids with 16 or 18 carbon atoms (Fig. 3). The fatty acid pattern of the TAG fractions (Fig. 3) of TtWS2, TtWS3 and TtWS4 expressing cells resembled the one found in the WE fractions, where the 16:1 and 18:1 were most abundant and comprised 80% of the total fatty acid mixture of WE and TAG. GC-analysis of WS and TAG of TtWS3 expressing cells showed almost equal amounts of C_{16} and C_{18} fatty acids.

Quantification of the WE and TAG content showed that TtWS4 expressing cells had the highest content of waxes of about 5 μmol WE per gram cell fresh weight; the TAG production was about 1 μmol/g. TtWS2 expressing yeasts produced about 4 μmol/g WE and 1 μmol/g TAG. In TtWS1 expressing yeast cells, about 2 μmol/g WE could be detected but no significant amounts of TAG. TtWS3

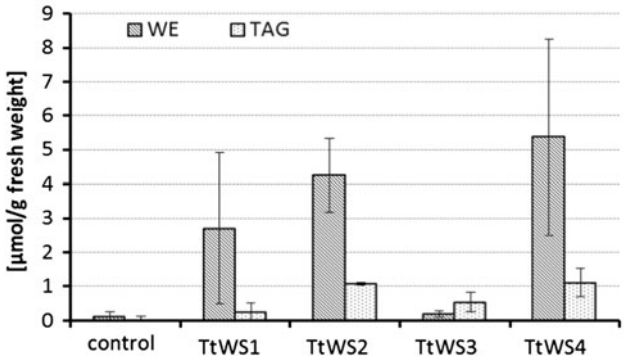

Fig. 4 Production of wax esters (WE) and triacylglycerols (TAG) in transgenic yeast cultures expressing the empty vector (control) or one of the *Tetrahymena* sequences (TtWS1-4). The amount of wax esters and TAG was quantified by GC as shown in Fig. 3. Mean values and standard deviations of three preparations are given

showed low amounts of both WE and TAG, but merely the TAG level was significantly above the background level (Fig. 4).

In summary it can be concluded that all four proteins were functionally expressed in yeast and caused wax ester or TAG production.

Optimizing an In Vitro WS Assay

To analyze the properties of the four *Tetrahymena* enzymes, an in vitro wax synthase assay was optimized, using membranes of TtWS1 expressing yeast cells as the enzyme source. Enzymatic activity was only detectable in the membrane fraction and not in the soluble fraction indicating that the protein is associated with membranes. These results are in line with the predicted protein structure. The highest wax synthase activities were obtained using approximately 13 µM palmitoyl-CoA (Supplemental data 2a), 300 µM decanol (Supplemental data 2b) and 10 mM BIS–Tris-propane buffer at pH 9.0 (Supplemental data 2c). Under these conditions the WS activities were linear with at least 2 µg membrane protein and an incubation time of 20 min at 35 °C.

Substrate Specificities

As wax synthesis was already observed in yeast when cultures were provided with straight-chain alcohols, they were tested as acyl-acceptors in the optimized enzyme assay first. It could be shown that all enzymes had the highest wax synthase activities with medium-chained saturated alcohols of 10–12 carbon atoms (Fig. 5), while all except TtWS3 were inactive with alcohols of <9 carbon atoms. In contrast to TtWS1, which showed a narrow chain-length preference between 10:0-OH and 12:0-OH, TtWS2 and TtWS4 also accepted alcohols with chain-lengths of 14 or 16 carbon atoms and TtWS3 esterified 16:0-CoA with 8:0-OH (Fig. 5).

In addition to WE, one further product was detected on TLC that co-migrated with TAG. It was clearly detected in all WS assays using membranes from yeasts expressing TtWS2, TtWS3 and TtWS4, but it was barely detected in assays with TtWS1 expressing membranes. These results were in line with the TAG level determined in the yeast cells expressing one of the *Tetrahymena* sequences (Fig. 4). The amount of TAG produced inversely depended on the amount of WE synthesized. When less attractive alcohols were used as acyl-acceptors, more TAG were produced and vice versa, which led to the assumption, that there was a competition reaction for the acyl-acceptor binding site of the enzyme. Since these *Tetrahymena* proteins were at least bifunctional and the fact that several acyltransferases show quite broad substrate acceptances [1, 2, 5], we had a closer look at further substrates.

In addition to saturated 1-alcohols, we ran assays with decan-2-ol and mono-unsaturated fatty alcohols. All enzymes had distinctly lower activities with 2-alcohols than with 1-alcohols of the same chain length. With the mono-unsaturated fatty alcohols 16:1-OH and 18:1-OH, TtWS2, TtWS3 and TtWS4 performed higher activities than with the corresponding saturated alcohols, while TtWS1 was inactive with unsaturated alcohols (Fig. 5).

As branched-chain WE are of high interest for the chemical industry, we assayed different kinds of branched-chain alcohols. TtWS4 was found to be active with 3,7-dimethyl-octanol (3,7-diMe-8:0-OH), but transferred the branched alcohols to palmitoyl-CoA at nearly the same rate as 10:0-OH. As some *Tetrahymena* species contain 2-methyl-branched alcohols in their waxes [21], it was interesting to see whether the *Tetrahymena* enzymes were active with a mixture of 2-methyl-branched alcohols of 16–20 carbon atoms. As given in Fig. 5, TtWS2 and TtWS4 performed WS activities with iso-alcohols, while TtWS1 and TtWS3 did not.

Another class of branched alcohols are isoprenols which can be esterified with acyl-CoAs to form prenyl esters. We investigated phytol, farnesol and geranylgeraniol as substrates for prenyl ester synthase (PES) activity. Especially TtWS4 displayed high activities with farnesol and geranylgeraniol, but a lower one with phytol. Unlike TtWS4, TtWS2 and TtWS3 were active with geranylgeraniol (Fig. 5) only.

As *Tetrahymena* also contains sterol esters, we investigated whether these acyltransferases possess sterol ester synthase (ASAT) activity. *Tetrahymena* species contain a special sterol termed tetrahymanol [51], but they are able to utilize both exogenously added cholesterol and ergosterol for ester synthesis [52]. WS assays with those sterols revealed that none of the *Tetrahymena* enzymes was active

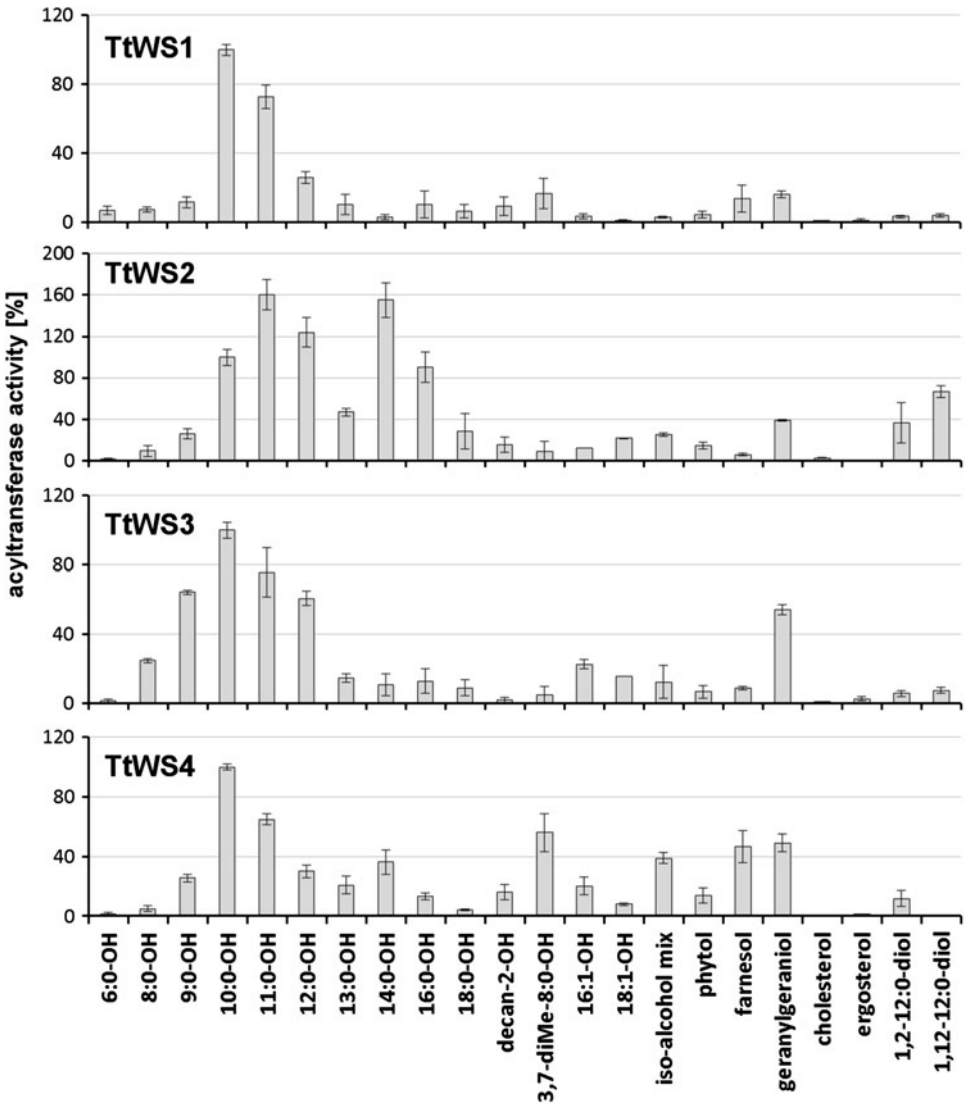

Fig. 5 Acyl-acceptor specificities of TtWS enzymes expressed in yeast. Relative formation rates of WE by yeast membranes harboring a TtWS enzyme from 16:0-CoA and the given acyl-acceptors under otherwise standard assay conditions are depicted as mean values of at least four enzyme assays. Before calculating the mean values, the activities of the control membranes (empty vector) of about 18 pmol mg^{-1} min^{-1} were subtracted from the activity of the yeast membranes with the *Tetrahymena* enzymes. 100% correspond to the activity of the respective TtWS enzyme with 10:0-OH and 16:0-CoA, namely TtWS1: 330 pmol mg^{-1} min^{-1}, TtWS2: 270 pmol mg^{-1} min^{-1}, TtWS3: 114 pmol mg^{-1} min^{-1}, TtWS4: 558 pmol mg^{-1} min^{-1}

with cholesterol and ergosterol, suggesting that they possess no ASAT activity (Fig. 5).

A last group of interesting alcohols are diols. These can be esterified with two acyl-CoAs to form wax diesters, or they are esterified at only one hydroxyl-group to form a hydroxyl-wax monoester. Diester synthase (DES) activity was obtained with TtWS2 and 1,2-dodecanediol (1,2-12:0-diol) and the respective 1,12-isomer (1,12-12:0-diol), while the other enzymes were hardly active with these substrates (Fig. 5).

Analyses of the acyl-CoA specificities of the TtWS enzymes gave the following results (Fig. 6): the highest WE formation rates were obtained with saturated long-chain acyl-CoA thioesters of 14–18 carbon atoms, while activities with 18:1-CoA were detectable with TtWS3 only. Acyl-CoAs with chain-lengths of 10–12 carbon atoms were hardly incorporated into WE or TAG. The thioester specificity of TtWS3 and TtWS4 differed between WS and acyl-CoA:diacylglycerol acyltransferase (DGAT) activities. While TtWS3 produced high levels of WE with 18:1-CoA, TAG were hardly detectable. TtWS4 performed the highest WS activity with 18:0-CoA and the highest DGAT activity with 14:0-CoA (Fig. 6). In addition to straight-chain acyl-CoAs, we also investigated 2-methyl-branched acyl-CoAs with chain-lengths of 14–18 carbon atoms. The activities with these substrates were distinctly lower than with the non-branched acyl-CoAs and could only be detected when incubation time and protein amount were increased (data not shown).

In summary, we were able to characterize four new acyltransferases with different properties (Fig. 7). TtWS1 synthesized WE as the main product especially with 10:0-OH and 14:0-CoA. DGAT and PES activities of TtWS1 were distinctly lower than WS activities, while DES and ASAT activity were hardly detectable. TtWS2 displayed high WS and DGAT activities with a broad range of

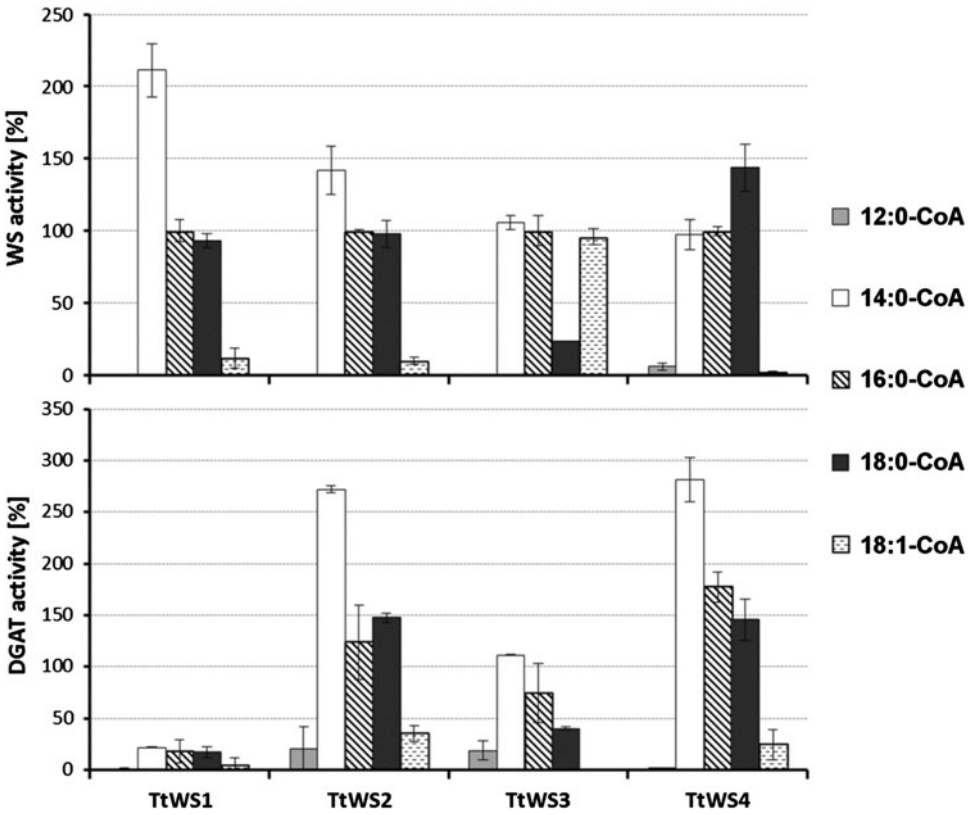

Fig. 6 Acyl-CoA specificities of TtWS enzymes expressed in yeast. Relative formation rates of WE and TAG by yeast membranes harboring a TtWS enzyme from 10:0-OH and the given acyl-CoA thioester under otherwise standard assay conditions are depicted as mean values of at least four enzyme assays. 100% correspond to the activity of the respective TtWS enzyme with 10:0-OH and 16:0-CoA as given in Fig. 5

saturated and mono-unsaturated alcohols ranging from 10 to 18 carbon atoms and saturated acyl-CoAs of 14–18 carbon atoms. In addition, it readily accepted diols as substrates. TtWS3 performed similar DGAT and WS activity, while PES activity was twofold lower. It had the highest activities not only with 10:0-OH and 14:0-CoA, but also with 10:0-OH and 18:1-CoA. In comparison to TtWS2, TtWS4 had a broad but different substrate specificity. WS activity of TtWS4 was high with straight-chain alcohols from 9 to 14 carbon atoms as well as with methyl-branched alcohols or isoprenols. TtWS4 formed more TAG than WE, but the proportion of the two products varied depending on the acyl-group of the thioester as observed with TtWS3 (Fig. 6).

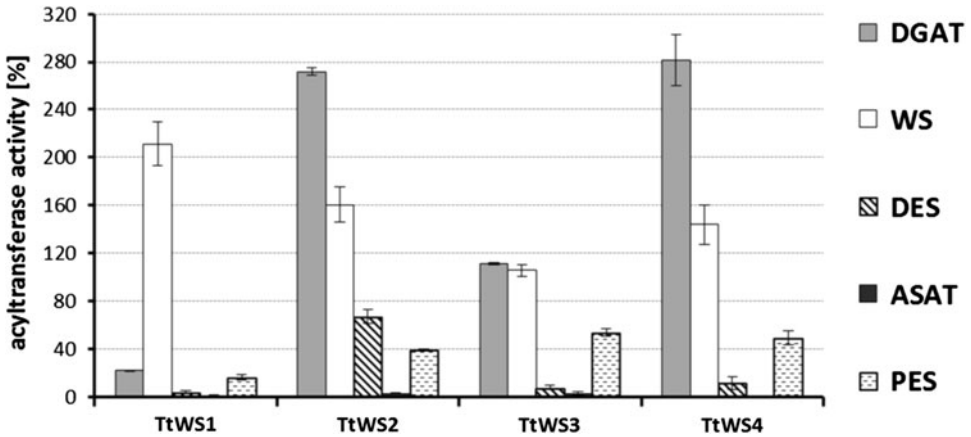

Fig. 7 Comparison of the maximal acyltransferase activities of yeast membranes harboring *Tetrahymena* enzymes with various acyl-acceptors. Data of the highest activities with different substrate groups were taken from Figs. 5 and 6. (DGAT: diacylglycerol acyltransferase activity determined with 10:0-OH and 14:0-CoA, WS: wax synthase activity with 10:0 or 11:0-OH and 16:0-CoA, DES: diester synthase activity with 12:0-diol and 16:0-CoA, PES: prenyl-ester synthase activity with geranylgeraniol and 16:0-CoA, ASAT: sterol-ester synthase activity with ergosterol and 16:0-CoA)

Discussion

In this study we identified four proteins from *Tetrahymena thermophila* which possess distinct properties of DGAT2 family members including the HPHG motif [49]. This motif is found in vertebrate DGAT2 acyltransferases [48–50] and the histidine and proline residues have been shown to be essential for acyltransferase activity [49]. Mutagenesis experiments will show whether these amino acids conserved in the four *Tetrahymena* proteins are critical for the enzymatic activities as well and whether the glycine group in the motif, which is substituted by arginine or tyrosine in two of the four *Tetrahymena* proteins (Fig. 2b), affects the activity or the properties of the enzymes. Sequence analyses suggest that these TtWS proteins build up a new branch of DGAT2 related multifunctional acyltransferases (Fig. 1), which are perhaps typical for protozoal organisms, as protein sequences similar to those of *Tetrahymena* were identified in other protozoa like *Paramecium tetraurelia* (NCBI: XP_001425482, XP_001451326, XP_001433749).

The identity of the TtWS proteins was further supported by functional expression studies in yeast in combination with in vitro enzyme assays. These experiments clearly demonstrated that the four *Tetrahymena* proteins possess acyltransferase activities but differ in their acyl-acceptor and -donor specificities, as they caused the formation of different patterns of lipophilic reaction products when provided with the respective substrates. TtWS1 was found to have the most pronounced and TtWS2 and TtWS4 the most relaxed acyl-acceptor specificity (Fig. 5). Unlike TtWS1, which largely produces WE when expressed in yeast membranes, expression of the other TtWS proteins caused biosynthesis of TAG, WE, prenyl esters and diesters but in different proportions (Fig. 6).

Multifunctional acyltransferases like the ones described here have been identified in human. AWAT2, for instance, shows equal WS and diacylglycerol synthase activities and lower DGAT and retinyl ester synthase activities [1, 3]. DGAT1, which belongs to a protein family different from that of AWAT2 (Fig. 1) efficiently catalyzes the production of TAG, while production of WE, diesters and retinyl esters was eightfold lower [2]. Hence both mammalian enzymes utilize an acyl-acceptor spectrum different from that of the *Tetrahymena* enzymes. An acyltransferase from *Acinetobacter*, which does not possess sequence similarity to the enzymes from *Tetrahymena* or mammals, was found to be a multifunctional acyltransferase as well [5]. Unlike *Tetrahymena* enzymes it performs not only WS, DGAT and diester, but also steryl ester synthase activity.

The broad acyl-acceptor specificities of most *Tetrahymena* acyltransferases and the observation that the amount of TAG production depended inversely on that of WE, suggests that *Tetrahymena* acyltransferases have flexible hydrophobic binding pockets in which various acyl-acceptors compete for binding. However, we cannot exclude other protein models like different interacting binding sites. Protein structure analyses will show in which way the acyltransferases mediate and regulate the esterification of acyl-acceptors.

In contrast to the broad acyl-acceptor specificities of the *Tetrahymena* proteins, the acyl-donor specificities were more pronounced and higher activities were obtained with saturated than with unsaturated acyl-CoA thioesters (Fig. 6). In that way, the *Tetrahymena* enzymes differ from that of *Acinetobacter*, which was shown to readily accept acyl-CoA thioesters of 8–20 carbon atoms [42]. They also have an acyl-donor specificity different from that of AWAT2, that was highly active with unsaturated long-chain acyl-CoA thioesters [3]. Interestingly, yeast cells expressing a *Tetrahymena* sequence formed WE and TAG mainly esterified with unsaturated acyl-groups (Fig. 3). These results are likely caused by the high level of unsaturated acyl groups in yeast cells [53] so that the introduced acyltransferases are predominantly provided with 16:1- and 18:1-CoA. Such a substrate channeling might allow incorporation of branched-chain acyl-moieties into WE as well.

The application of heterologous expression of genes encoding wax synthesizing enzymes in host systems is well under way. For instance, expression of genes from jojoba in transgenic *Arabidopsis thaliana* seeds led to the accumulation of WE [11, 40]. The protozoan enzymes described here were found to differ not only in their acyl-CoA but also in their acyl-acceptor specificities. Expression of sequences like TtWS2 or TtWS4 in plant systems in combination with suitable fatty acyl-CoA reductases will reveal their suitability for field application and industrial uses.

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