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Sterol specificity of acyltransferases in yeast

MASTER'S THESIS

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AFFIDAVIT

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28.12.2014
Date

Signature

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2. Abstract

The pathway of ergosterol biosynthesis in yeast is highly conserved. Twenty-eight enzymes, located in the endoplasmic reticulum, are involved in ergosterol production. Cellular sterols are present in two forms, as free sterols and as sterol esters. Sterol esters are stored in organelle-like structures, so-called lipid droplets or lipid particles. The acylation in *S. cerevisiae* is carried out via the two enzymes Are1p and Are2p. These enzymes and five heterologous acyltransferases were expressed in *S. cerevisiae*. Expression was confirmed via western blot analysis and the enzymes were tested for their substrate specificity towards different sterols via an enzymatic *in vitro* assay with radioactively labeled oleoyl-CoA. This radioassay was optimized concerning protein amount, sterol amount, oleoyl-CoA amount and reaction time to make it a feasible tool for testing sterol specificity of the different acyltransferases.

Der Biosyntheseweg von Ergosterol in Hefe ist besonders konserviert. Im endoplasmatischen Retikulum sind 28 Enzyme an der Produktion von Ergosterol beteiligt. Sterole kommen in der Zelle auf zwei Arten vor: als freie Sterole und als Sterolester-Verbindungen. Diese Sterolester werden in organell-ähnlichen Strukturen, den sogenannten „Lipid-Tröpfchen“ oder „Lipid-Partikeln“ gespeichert. Die Acylierung wird in der Bäckerhefe (*Saccharomyces cerevisiae*) von den beiden Enzymen Are1p und Are2p durchgeführt. Diese beiden Enzyme wurden neben fünf heterologen Acyltransferasen in Hefe exprimiert. Die Expression wurde durch Western Blot Analyse bestätigt und die Substratspezifität der Enzyme in einem enzymatischen *in vitro*-Assay mit radioaktiv-markiertem oleoyl-CoA als Substrat gemessen. Der „Radioassay“ wurde hinsichtlich Protein- Sterol- und oleoyl-CoA-Menge sowie hinsichtlich Reaktionsdauer optimiert, um eine praktikable Methode für weitere Experimente zu entwickeln.

3. Table of contents

4. Introduction

Membranes of eukaryotic cells are composed of many different lipids. The major ones are glycerophospholipids, sphingolipids and sterols. Among these lipids, the abundance and structure of sterols is a decisive factor for the properties of yeast membranes. (Pichler, 2005)

Sterols (for cholesterol see Figure 1) are produced in the cell mainly in the endoplasmic reticulum (ER) through a highly conserved pathway and then transported to other membranes. They play an important role for the regulation of the membrane permeability and fluidity. Although other membrane lipids are also important for these properties, eukaryotic cells are not viable without sterols. (Daum et al., 1998)

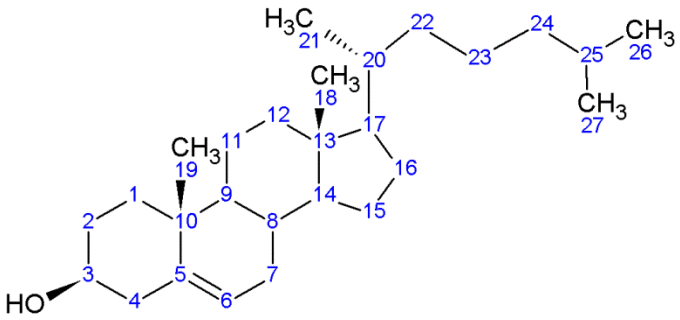

Figure 1: Structure of cholesterol

Sterols produced by mammals, plants and fungi have only small structural differences in a few double bonds and methyl/ethyl side chains, but even these minimal differences have an influence on the properties of the membranes these lipids are embedded in. (Xu et al., 2001)

Baker's yeast cells usually produce more sterols than can be embedded into membranes. Moreover there are two counteracting mechanisms to maintain sterol homeostasis: esterification of free sterols and ester hydrolysis (Chang et al., 2006).

The enzymes in yeast, responsible for sterol esterification are called **A**cyl Coenzyme A: **S**terol **A**cyl**t**ransferases (**ASATs).** Their main function is the transfer of an acyl-group from Acyl Coenzyme A to the OH-group of the sterol (Figure 2). ASATs have certain substrate specificity depending on the side chain of the sterol (Taketani et al. 1979). In organisms mainly producing cholesterol, ASATs are called **A**cyl Coenzyme A: **C**holesterol **A**cyl**t**ransferases (**ACATs**). This name can easily lead to confusion due to the fact, that the Acetyl Coenzyme A: Cholesterol Acetyltransferase is also abbreviated as ACAT (http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&term=Acetyl-CoA+C-Acetyltransferase).

ASATs belong to the class of acyltransferases, which are part of the enzyme family of transferases. They are described to carry out a direct transfer of the fatty acid group from a fatty acyl-Coenzyme A derivative to cholesterol (http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&term=Sterol+O-Acyltransferase). This is not very precise due to the fact, that this enzyme group esterifies a wider range of sterols, not only cholesterol, as shown below.

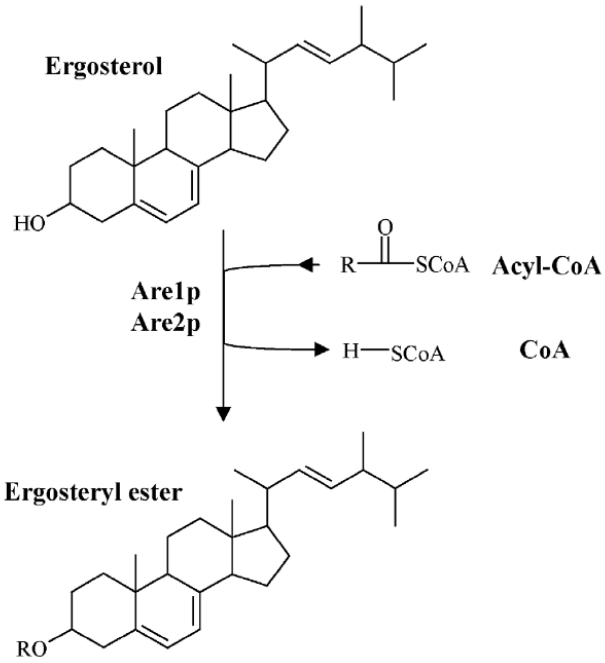

Figure 2: Sterol ester formation in yeast (Müllner et al., 2004)

Sterol esters of *S. cerevisiae* are synthesized by the two ASAT enzymes Are1p and Are2p. These enzymes are 49 percent identical to each other and are located in the endoplasmic reticulum (ER) (Zweytick et al., 2000; Yang et al., 1996). Are2p is suggested to be the more active enzyme, because in Δ*are*2 mutants the esterification rate was reduced to less than 26 percent of the wild type. (Yang et al., 1996)

There seems to be an increased specificity of Are2p towards ergosterol, whereas Are1p is more specific for esterifying earlier compounds of sterol biosynthesis. It is suggested that Are1p serves preventing the incorporation of these ergosterol precursors into the plasma membrane. (Zweytick et al., 2000)

Δare1Δare2 double knockout strains are viable but contain less than one percent sterol esters. The lower level of sterol esters is compensated by an increased amount of free sterols. (Zweytick et al., 2000)

For the utilization of CoA-derivatives in yeast, it was demonstrated that ASATs present in membrane preparations from wild-type cells preferentially utilized CoA-derivatives of C18:1 and C18:2 as

substrates in vitro; C16:0, C18:0 and C20:4 acyl CoAs were shown to be poorer substrates. (Yang et al., 1997)

The fatty acids C18:2 and C20:4 are not found in *S. cerevisiae* under normal growth conditions whereas utilization of C16:1, a dominant fatty acid in steryl esters of yeast, was not tested by Yang et al. (1996). Results of Zweytick et al. (2000) indicated that in vivo C16:1 and C18:1 are the major fatty acid species incorporated into steryl esters. No significant difference was observed between Are1p and Are2p regarding the efficiency of fatty acid utilization. (Zweytick et al., 2000)

Sterol esters are stored together with triacylglycerols, as so-called neutral lipids, in lipid droplets, aka lipid particles, lipid bodies, or oil bodies with a diameter of approximately 400 nm. Their composition regarding the neutral lipids depends on the activity of the different acyltransferases. (Czabany et al., 2008)

Lipid droplets are cellular organelles that consist of a neutral lipid core covered by a monolayer of phospholipids and many proteins. They are thought to function in the storage, transport, and metabolism of lipids, in signaling, and as a specialized microenvironment for metabolism in most types of cells from prokaryotic to eukaryotic organisms. (Yang et al., 2012)

As only free sterols are used as substrates in the ergosterol biosynthesis pathway and as membrane building blocks, this creation of an inert sterol pool is considered to be a protective mechanism, storing components needed for membrane formation (Leber, 1994).

There is still an ongoing discussion, how these lipid droplets are formed and how enzymes can reach them. Regarding formation, there are some models proposed, “the most widely cited model posits, that lipid droplets form in the ER membrane as a result of an accumulation of neutral lipids within the bilayer that drives a “budding” of a nascent droplet from the cytosolic leaflet of the membrane.” (Walther et al., 2009). Other explanations are that those particles “are excised from both leaflets of the ER membrane bilayer as a bicelle” or a third model proposes a mechanism called “vesicular budding”, where “droplets are initially formed within small bilayer vesicles, utilizing the machinery of vesicle formation of the secretory pathway” (Walther et al., 2009).

There are also some models proposed, how enzymes can be present in lipid droplets. They can either be embedded during formation or transported to lipid droplets after the droplets are formed. (Athenstaedt et al., 1999)

The reverse reaction of esterification, i.e. hydrolysis, is carried out in *S. cerevisiae* by three sterol ester hydrolases Yeh1p, Yeh2p and Tgl1p with Yeh2p showing the highest activity. (Müllner et al., 2005)

Yeh2p is located in the plasma membrane, whereas the other two enzymes are located in lipid droplets. The three hydrolases are most likely specific for hydrolysis of sterol esters from lipid droplets. It is still unclear, how sterol esters and Yeh2p in the plasma membrane meet. (Daum et al., 2007)

The mobilization of steryl esters by the above hydrolases recycles sterol intermediates to the sterol biosynthetic pathway. These intermediates are mainly zymosterol, fecosterol, lanosterol and episterol, which are the four most abundant esterified sterols. (Wagner et al., 2008)

Besides other regulatory mechanisms, esterification of ergosterol and sterol intermediates and hydrolysis of sterol esters play an important role in cellular sterol homeostasis. (Daum et al., 2007)

4.1.Probing specificity of heterologous acyltransferases

The heterologous acyltransferases, to be examined in this work, had been heterologously expressed in several studies. For some of them esterification for different sterols had been documented. But none of them was tested for their acylation specificity towards 7-dehydrocholesterol (7-DHC) as compared to zymosterol.

Research on the human ACAT1 indicates also that there could be a domain in the enzyme that is mainly activated by cholesterol (Liu et al., 2005). It was shown that certain other sterols as dehydroergosterol, sitosterol, enantiomeric cholesterol (*ent*-cholesterol) or epicholesterol did not activate ACAT1 (structures see Figure 3). The human ACAT1 was also reported to have specificity for cholesterol and cholestanol, when compared to other cholesta and ergosta compounds. (Liu et al., 2005)

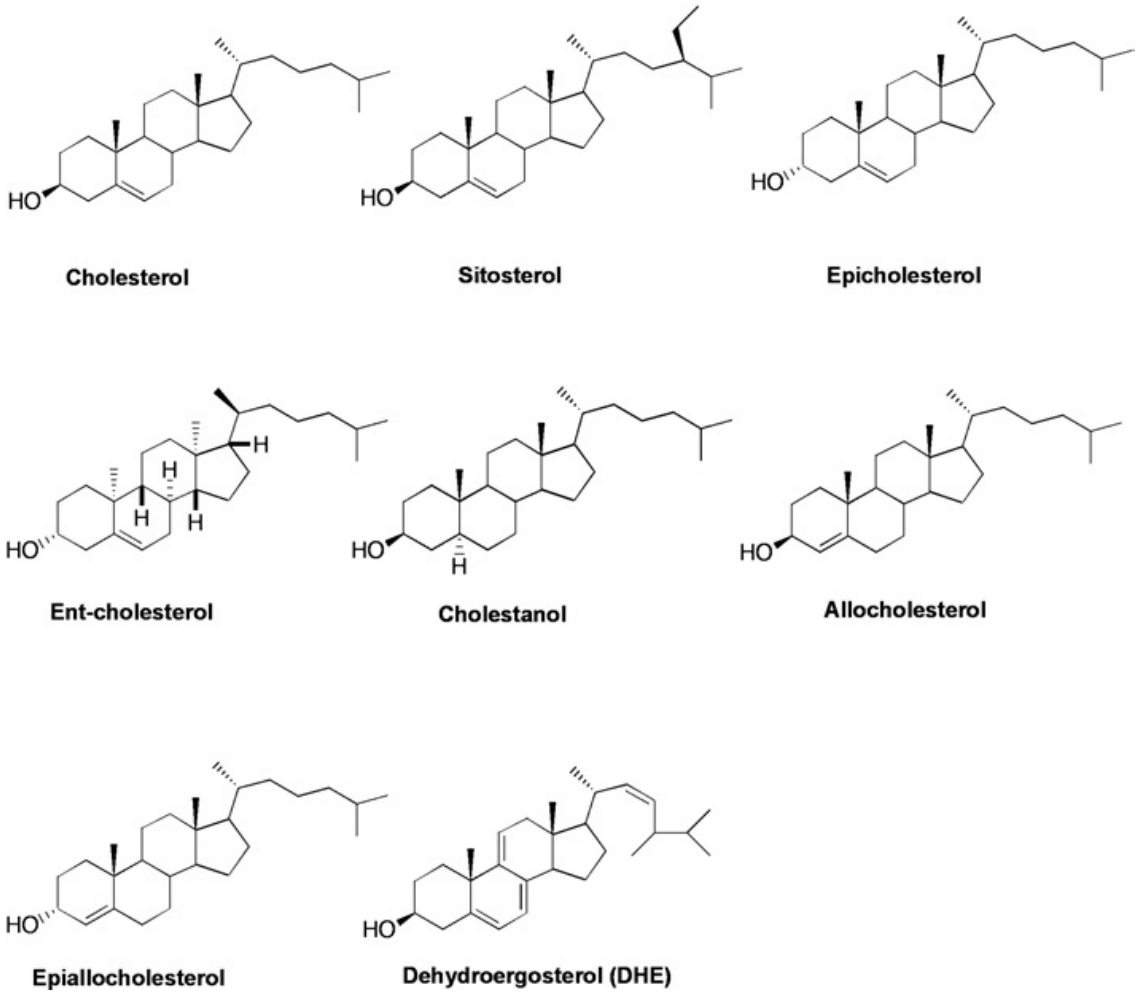

Figure 3: Cholesta and ergosta compounds tested for specificity of the human ACAT1: Ent-cholesterol: enantiomeric cholesterol; (Liu et al., 2005)

The human ACAT was also tested for the esterification rate of cholesterol compared to ergosterol and 7-DHC. The human ACAT esterified cholesterol at the highest rate, whereas 7-DHC and ergosterol were not esterified. (Yang et al., 1997)

For the rat ACATs there is a single paper that focuses on the substrate specificity but it does not distinguish between ACAT 1 and ACAT 2 (Tavani et al., 1982). It is reported that some sterols (campesterol, 4α-methyl-cholest-7-en-3β-ol, cholestanol, desmosterol, lathosterol and cholest-4-en-3β-ol) were esterified by these enzymes, but less efficiently than cholesterol. Some other sterols were not esterified, i.e. 3-epicholesterol, sitosterol, stigmasterol, ergosterol, lanosterol, and 4,4-dimethyl-cholest-7-en-3β-ol. It is pointed out, that “a 3β-hydroxyl group is required for the sterol to be a substrate”. Furthermore, it is suggested, “that the side chain is very important in the binding of the substrate in the active site of the ACAT[s].” (Tavani et al., 1982)

The *Candida albicans* ASAT (Are2) was functionally expressed in *S. cerevisiae* but substrate specificity towards certain sterols was not tested. (Kim et al., 2004)

Toxoplasma gondii ACAT 1 had been heterologously expressed in yeast cells, but was only tested for substrate specificity when expressed in mammalian cells. It was capable of esterifying cholesterol, ergosterol, and 25-hydroxycholesterol at a higher rate and 7-DHC as well as β-sitosterol at a lower rate of approximately 50-65 % of cholesterol esterification activity. Lanosterol was not esterified by the *T. gondii* ACAT 1. (Nishikawa et al., 2005)

5. Targets of this Master thesis

The goal of this work is to characterize the substrate specificity of heterologous acyltransferases and compare them to the homologous Are1p and Are2p of *S. cerevisiae* for the two sterols zymosterol and 7-DHC (Figure 4). Therefore, a *S. cerevisiae Δare1Δare2* double knockout strain was used that produced no steryl esters. Eight acyltransferases were cloned into a vector and were transformed into *S. cerevisiae* followed by protein expression. From these induced transformants, microsomal preparations were carried out for an enrichment of the respective acyltransferases. Sterol specificity of ASATs was then tested via an enzymatic assay using radioactively labeled oleoyl-CoA substrate.

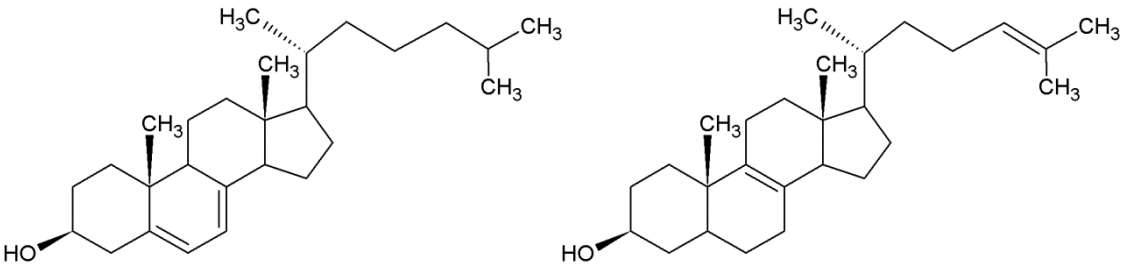

Figure 4: structures of 7-dehydrocholesterol (left) and zymosterol (right)

6. Project aims

This Master thesis was part of an ACIB project named “Enzyme engineering and substrate channelling for vitamin production” which aims at engineering acyltransferases to enhance the production of 7-DHC.

The major aim of this project is to increase the production of 7-DHC in yeast. Upon deletion of the two genes, ERG5 and ERG6, *S. cerevisiae* accumulates mainly two sterol intermediates, namely cholesta-5,7,24-trienol and zymosterol. These sterols are largely esterified by the two acyltransferases Are1p and Are2p and stored in organelle-like structures called lipid particles, where they become inaccessible to enzymes modifying the sterol structure. Cholesta-5,7,24-trienol is converted to 7-DHC by the enzyme dehydrocholesterol-24-reductase (DHCR24). Both substances serve as precursor for vitamin D3 production and are, therefore, point of interest for DSM Nutritional Products (DNP). The yield limiting factor to get high amounts of this molecule is the conversion of zymosterol to cholesta-5,7,24-trienol by the two enzymes Erg2p and Erg3p which can only work on zymosterol as long as it is not yet esterified.

In this project, two different strategies to convert accumulating zymosterol to 7-dehydrocholesterol were pursued. The first was to create Are2p muteins with substrate specificity for 7-dehydrocholesterol which were tested in the *Δerg5Δerg6Δare1Δare2* quadruple knockout strain. Additionally, acyltransferases from different organisms were selected from literature to be expressed in a *Δare1Δare2* double knockout strain to be tested for sterol selectivities, which comprises the work presented in this Master thesis.

7. Materials

7.1.Strains

Table 1: Yeast strains used in this work

Strain	Genotype	Source
FY 834	*MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-Δ202 Δare1::HIS3 Δare2::TRP1*	Günther Daum
CEN.PK2 *erg5erg6are1are2**	*MATα his3Δ1 ura3-52 trp1-289 leu2-3_112 MAL2-8^{C} SUC2 erg6::TRP1 erg5::LEU2 are1::HIS3 are2*::loxP-kanMX-loxP	Birgit Ploier

*an additional copy of *ARE*1 was found

Table 2: E. coli strain used in this work

Strain	Genotype	Source
Top10F'	F´{lacIq Tn10 (TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	Invitrogen

7.2.Primers

Table 3: Primers used in this work; restriction sites are underlined: ecoRI: GAATTC, bamHI: GGATCC, short Kozak sequence marked in yellow, FLAG tag coding sequence marked in grey

Primername	Sequence	Tm [C°]
Are1FLAGfwd	GACTAGTCAAAATGGACTACAAAGACGATGACGATAAAACGGAG ACTAAGGATTTGTTGC	77.5°C
Are1FLAGrev	CCCAAGCTTGGGTCATAAGGTCAGGTACAACGTCATAATGATACT GGG	77.1°C
Are2FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAGACAAGAA GAAGGATCTACTGGAG	81.4°C
Are2FLAGrev	GCGGAATTCTTAGAATGTCAAGTACAACGTACACATGACACTTGG TCCCATGCAGATACC	80.9°C
120FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAGTCGGAGA GGAAAAGATGTCCCTGAC	82.1°C

Primername	Sequence	Tm [C°]
120FLAGrev	GCGGAATTCTCAGAAAACGTAACGACAAGTCCATGATCTAGGCCTAACGTAATCCAGAAAGG	79.0°C
121FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAAGAACCAAAAGCTCCACAATTACGTCG	82.3°C
121FLAGrev	GCGGAATTCTCAAGGATGACAACTCCAGGATCTAGGTGTTACAAGTTCCCAAAATGTTGGTTGTGGTAATG	79.6°C
122FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAGGATCCAAAATGGTAGGCGAGGAAATG	83.6°C
122FLAGrev	GCGGAATTCTCAAAACACATATCTACAAGTCCATGTACGTGGTCTGACGTAATCAAGGAAAGTAGGGTTTTTCAATGG	81.1°C
123FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAGGCAGAACTAACACTTCTGATCAGC	80.4°C
123FLAGrev	GCGGAATTCTCAAAAGACCAGGTACAGAGTACAGATAATGGAAGGGCCAGAGATGAAACCGAACCAACAAATGATGTTACC	83.6°C
124FLAGfw	GCGGGATCCATGGACTACAAAGACGATGACGATAAATTAGATGATCCTTTGTCTAAAACTAG	78.8°C
124FLAGrev	GCGGAATTCTCTCAAAACAATCTTAAAGGTTGATGATCGGCATGAGCATTGTAAAAGTGTATTTGTGCCC	80.2°C
Kozak-FLAG	CGCGGATCCGCGAAAATGGACTACAAAGACGATGACGATA	78.1°C
p426Seqfwd	TTAGTTTTAAAACACCAGAACTTAGTTTCG	55.7 °C
p426Seqrev	TTACATGACTCGAGGTCGACGG	56.5 °C
pYEX4T-1 fwd	CTTTGCAGGGCTGGCAAGCCAC	64.5°C
pYEX4T-1 rev	GCACTCATGACCTTCATTTTGGAA	56.9°C
Are1-inside-fw	CAGTACATGACAACGGACTTGTTC	55.7°C
123-inside-fw	AGAGAGATTTGTTCAAAGTCGGTC	54.0°C
124-inside-fw	TGGTGATCGTAGACCAGCCAGTGG	60.8°C

7.3.Instruments and Devices

Table 4: List of instruments and devices used in this work

Instrument/ Device/Enzyme	Supplier
Centrifuges	Centrifuge 5810R, Eppendorf Centrifuge 5415R, Eppendorf Avanti J-20XP Centrifuge, Beckman Coulter Inc.
Dispensette	Brand GmbH, Germany
Electrophoresis gel chambers	PowerPac™ Basic + Sub-Cell GT, Biorad, USA
Electrotransformation	MicroPulser, BIO-RAD, USA Electroporation Cuvettes (2 mm gap) Molecular BioProducts Inc., USA
Eppendorf tubes	Greiner bio-one International AG
Falcon tubes	Greiner bio-one International AG
G:Box HR	Syngene, UK
GC caps	VWR International, GmbH
GC vials	VWR International, GmbH
GC pliers	VWR International, GmbH
GCMS	Agilent Technologies, Austria
Glas bottles	Schott/ Duran, Ilmabor TGI
Glass beads	Carl Roth GmbH + Co KG
Incubator (30°C and 37°C)	Binder GmbH
Flasks	Simax
Laminar flow chamber BSB4A	Gelaire Flow Laboratories,
Mixing of small volumes	Vortex-Genie 2, Scientific Industries Inc, USA
NuPAGE SDS Gels: 4-12 % Bis-Tris Gel 1mm x 15 wells	Invitrogen- Life Technologies Corp.
PCR machines	GeneAmp®PCR System 2700, Applied Biosystems, USA
PCR tubes	Greiner bio-one International AG
Petridishes	Greiner bio-one International AG
Photometer	BioPhotometer, Eppendorf, Germany
Pipette tips	Greiner bio-one International AG
Pipettes	Pipetman P20N Gilson Inc., USA Pipetman P200N Gilson Inc., USA Pipetman P1000N Gilson Inc., USA Eppendorf research 0.5-10 µL, Eppendorf, Germany
Pyrex tubes	Pyrex, Incorp.
Scanner	HP scanjet 4370
Shaker	HT MiltronII, Infors AG, Swiss Certomat® BS-1, Sartorius, Germany
Thermomixer	Thermomixer comfort, Eppendorf, Germany
TLC chambers	CAMAG
TLC silica plates: aluminium sheets 20 x 20 cm, silica gel 60	Merck GmbH.
Transferpettor (200-1000 µL; 10-50 µL)	Brand GmbH, Germany
UV-cuvettes	Greiner bio-one International AG

Instrument/ Device/Enzyme	Supplier
Vibrax	Vibrax VXR basic, IKA GmbH& Co KG. Germany
Western Blot membrane: nitrocellulose blotting membrane	Sartorius AG

7.4.Reagents

Table 5: List of reagents used in this work

Reagent	Supplier
Acetic acid	Carl Roth GmbH + Co KG
Agar	BD Bacto- Becton, Dickinson and Company
Agarose	Biozym Scientific GmbH
Chloroform	Carl Roth GmbH + Co KG
Cholesterol	Sigma- Aldrich Corp.
dATP	Fermentas- Thermo Fisher Scientific Inc.
dCTP	Fermentas- Thermo Fisher Scientific Inc.
Deionised water	Fresenius Kabi Austria GmbH
dGTP	Fermentas- Thermo Fisher Scientific Inc.
Diethylether	Carl Roth GmbH + Co KG
DreamTaq buffer	Fermentas- Thermo Fisher Scientific Inc.
DreamTaq DNA polymerase	Fermentas- Thermo Fisher Scientific Inc.
DTT	Carl Roth GmbH + Co KG
dTTP	Fermentas- Thermo Fisher Scientific Inc.
EDTA	Carl Roth GmbH + Co KG
Ethanol	Australco Handels GmbH
Gene Jet Plasmid Miniprep Kit	Fermentas- Thermo Fisher Scientific Inc.
Gene Ruler DNA Ladder Mix	Fermentas- Thermo Fisher Scientific Inc.
Glucose Monohydrate	Carl Roth GmbH + Co KG
Glycerol	Carl Roth GmbH + Co KG
HCl	Carl Roth GmbH + Co KG
L-Adenine	Carl Roth GmbH + Co KG
L-Histidine	Carl Roth GmbH + Co KG
Lithiumacetate	Fluka/ Sigma- Aldrich Corp.
L-Leucine	Carl Roth GmbH + Co KG

Reagent	Supplier
L-Lysine	Carl Roth GmbH + Co KG
Loading Dye (6x)	Fermentas- Thermo Fisher Scientific Inc.
L-Tyrosine	Carl Roth GmbH + Co KG
L-Uracil	Fluka/ Sigma- Aldrich Corp.
Maxima Hot Start Green PCR Mastermix	Fermentas- Thermo Fisher Scientific Inc.
Methanol	Carl Roth GmbH + Co KG
n-heptane	Carl Roth GmbH + Co KG
N'O'-bis(trimethylsilyl)-trifluoracetamid	Sigma- Aldrich Corp.
Yeast Nitrogen Base (w.o. amino acids)	Difco- Becton, Dickinson and Company
NuPAGE Antioxidant	Invitrogen- Life Technologies Corporation
PageRuler Prestained Protein Ladder	Fermentas- Thermo Fisher Scientific Inc.
PEG 4000	Sigma- Aldrich Corp.
Petrolether	Carl Roth GmbH + Co KG
PfuUltra buffer 10x	Promega Corp.
PfuUltra DNA polymerase	Promega Corp.
Phusion DNA polymerase	Finnzymes- Thermo Fisher Scientific Inc.
Phusion HF buffer	Finnzymes- Thermo Fisher Scientific Inc.
Ponceau S	Amersham Life Science
Potassiumacetate	Carl Roth GmbH + Co KG
Potassiumchloride	Carl Roth GmbH + Co KG
Potassiumhydroxide	Carl Roth GmbH + Co KG
Primary antibody- Rabbit anti Erg6p	Ao.Univ-Prof. Dr. Günther Daum
Pyridine	Carl Roth GmbH + Co KG
Pyrogallol	Carl Roth GmbH + Co KG
Quik Change Mutagenesis Kit	Invitrogen- Life Technologies Corp.
RNaseA	Fermentas- Thermo Fisher Scientific Inc.
Secondary antibody- Goat anti rabbit IgG, horseradish peroxidase conjugated	Sigma- Aldrich Corp.
Single stranded carrier DNA (fish sperm)	Roche Diagnostics, GmbH
Skim milk powder (Eiweiß 90)	DM Drogerie Markt GmbH
Sodiumchloride	Carl Roth GmbH + Co KG
Sodiumcitrate	Carl Roth GmbH + Co KG
Sodiumhydroxide	Carl Roth GmbH + Co KG
Sorbitol	Carl Roth GmbH + Co KG

Reagent	Supplier
Spectra Broad Range Protein Standard	Invitrogen- Life Technologies Corp.
Sulfuric acid	Carl Roth GmbH + Co KG
SuperSignal West Pico Chemiluminescent Substrate Kit	Pierce
Trichloroacetic acid	Carl Roth GmbH + Co KG
Tris	Carl Roth GmbH + Co KG
Tryptophan	Carl Roth GmbH + Co KG
Tween20	Carl Roth GmbH + Co KG
Wizard- Gel Slice and PCR Product Preparation	Promega
Yeast extract	Carl Roth GmbH + Co KG
β-Mercaptoethanol	Carl Roth GmbH + Co KG

7.5.Media and Buffers

Table 6: List of media used in this work

Medium	Compostion
LB	10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 20 g/L agar
LB- amp	LB + 1 ml ampicillin stock solution (1000x)/1L
SD all dropout mix	3 g Adenine, 3 g Lysin, 3 g Tyrosin, 3 g Histidin, 3 g Leucin, 3 g Uracil Tryptophan was added after autoclaving of the media: 4 ml of 250x stock (10 g/L) sterile filtered 0.2 µm f.c. 40 mg/ L
SD all	6.7 g/L Yeast Nitrogen Base without amino acids (0.67 %), 20 g/L Glucose (2 %), 20 g/L Agar (2 %), 2 g/L SD all dropout powder mix (0.2 %)
SD-his	Like SD all but without histidine in the dropout powder mix
SD-his-leu-trp	Like SD all but without histidine, leucine and tryptophan in the dropout powder mix
SD-leu	Like SD all but without leucine in the dropout powder mix

Medium	Compostion
SD-trp	Like SD all but without tryptophan
SD-ura	Like SD all but without uracil in the dropout powder mix
SOC	20 g/L bacto tryptone, 0.58 g/L NaCl, 5 g/L bacto yeast extract, 2 g/L $MgCl_2$, 0.16 g/L KCl, 2.46 g/L $MgSO_4$, 3.46 g/L dextrose
Sporulation medium	1 g potassium acetate, 0.25 g Yeast extract, 0.1 g glucose in 100 ml distilled H_2O
YPD	10 g/L Yeast extract (1 %), 20 g/L Peptone (2 %) , 20 g/L Glucose (2 %), 20 g/L agar (2 %)

Table 7: List of buffers used in this work

Buffer/ solution	Composition
Protein sample buffer (2x)	100 mM Tris/HCl pH 8.8, 4 mM EDTA, 4 % SDS, 20 % glycerol, 0.002 % bromophenol blue
Ponceau S	0.1 % Ponceau S in 5 % acetic acid
MOPS NuPAGe Running buffer (1x)	50 ml MOPS NuPAGe Running buffer (20x), 950 ml bidest H_2O
EDTA stock solution (0.5 M)	93.05 g EDTA disodium salt, dissolved in 400 ml bidest H_2O ; pH adjusted to about 8, solution topped up to a final volume of 500 ml
TAE buffer (50x)- stock solution for electrophoresis	242 g Tris base dissolved in 750 ml deionized water; addition of 57.1 ml glacial acid acid and 100 ml of 0.5 M EDTA (pH 8.0) solution adjusted to a final volume of 1 L
TAE Buffer (1x) for electrophoresis	200 ml of TAE (50x), 9.8 L bidest H_2O
TBS (10x)	30.3 g Tris (0.25 M), 87.6 g NaCl (1.5 M), pH adjusted with 1 M HCl to pH 7.5, to 1 L with ddH_2O
TBST	999.5 ml 1x TBS, 0.5 ml Tween 20
TBST milk	5 g dry milk powder dissolved in 100 ml TBST
Transfer buffer (20x)	14.5 g Tris (24 M), 72 g Glycin (1920 mM), to 500 ml ddH_2O

Buffer/ solution	**Composition**
Transfer buffer (1x)	50 ml 20x transfer buffer, 100 ml methanol to 1 L with ddH_2O
Transformation mix	90 ml 45 % PEG4000, 10 ml of 1 M LiAc, 1 ml of 1 M TrisHCl, pH 7.5, 0.2 ml of 0.5 M EDTA subjected to vortex mixing
Tris-HCl (100 mM, pH 7)	12.1 g Tris diluted in ddH_2O pH adjusted with 1 M HCl
Ampicillin stock (1000x)	100 mg/ml dissolved in ddH_2O; sterile filtered 0.2 µm

8. Methods

8.1. Enzymatic restriction

Inserts (see Table 8) were cut out of vector pJET201 (provided by Corinna Odar) with the restriction enzymes *Eco*RI and *Bam*HI according to standard protocols (Current Protocols in Molecular Biology, 2011). Vector backbone p426GPD and pYEX-4T were also cut with these restriction enzymes and were re-ligated with the different inserts according to standard protocols (Lohman et al., 2011).

Table 8: Names of acyltransferases, organisms they derive from, short term and DNA base pair length

Acyltransferases	Organism	Short term	Base pairs
Sc ARE1mut	*Saccharomyces cerevisiae*	Are1	**1833**
Sc ARE2	*Saccharomyces cerevisiae*	Are2	**1939**
***Pt* ACAT*1*_opt*Sc*1**	*Pan troglodytes*	120	**1659**
***Rn* ACAT*2*_opt*Sc*1**	*Rattus norvegicus*	121	**1581**
***Rn* ACAT*1*_opt*Sc*1**	*Rattus norvegicus*	122	**1644**
***Ca ARE2*_opt*Sc*1**	*Candida albicans*	123	**1836**
***Tg* ACAT*1*_opt*Sc*1**	*Toxoplasma gondii*	124	**1955**

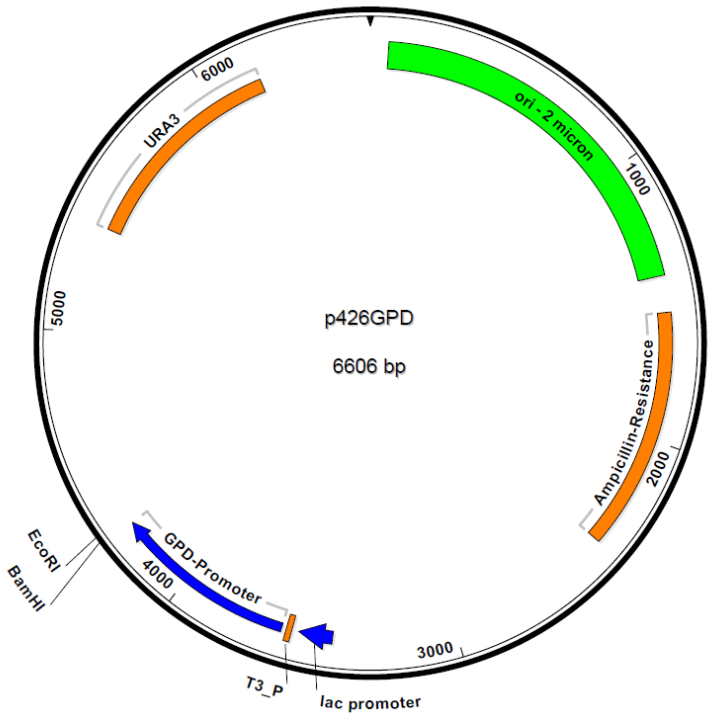

Figure 5: Vector p426GPD: A 2µ plasmid with ampicillin resistance cassette, *URA3*-Marker and constitutive *GPD* promoter

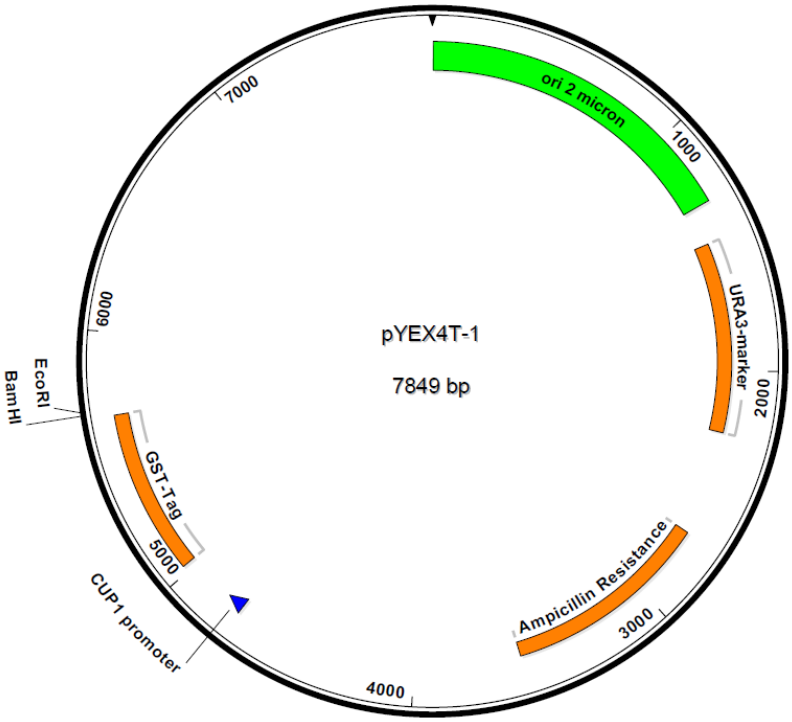

Figure 6: Vector pYEX-4T-1: A 2µ plasmid with ampicillin resistance cassette, *URA3*-Marker, inducible *CUP1* promoter and *GST*-Tag sequence

8.2.Gel electrophoresis

DNA fragments and vector backbone were separated by agarose gel-electrophoresis, performed according to standard protocols (Current Protocols in Molecular Biology, 2011). One percent agarose gels in TAE buffer were run at 90 V for 90 to 100 min for preparative gels and 120 V for 45 min for analytical gels. The sizes of DNA fragments were compared to the "GeneRuler DNA Ladder Mix" standard by Fermentas.

8.3.Preparation of plasmids, PCR products, and DNA fragments

All plasmids were isolated with Fermentas "GeneJet Plasmid Miniprep Kit" according to the supplier's manual and finally eluted with 20-30 µl of ddH_20. DNA fragments and PCR products were partly purified over standard DNA agarose gels and/or extracted with "Wizard SV Gel Slice and PCR Product Preparation" by Promega, as described in the manual.

8.4.Electroporation of *E. coli* cells

The transformation of plasmids into electrocompetent *E. coli* cells TOP10F' followed a standard procedure. Fifty µL of electrocompetent cells, kindly provided by Thorsten Bachler, were thawed on ice. After adding 2-3 µL of a plasmid preparation (80-100 ng) and transferring the mixture to cooled transformation cuvettes, they were incubated on ice for approximately ten min before pulsing them in the electroporator with program Ec_1. After the electro-pulse, 1 ml of SOC medium was added and the cells were regenerated at 37°C for one h at 500-600 rpm before plating them on selective media.

8.5.Sequencing

All plasmids were sequenced by LGC Genomics GmbH (Berlin, Germany). Ten µL of plasmid preparation (approx. 1 µg DNA) were mixed with 4 µL of a 5 µM sequencing primer, as demanded on the homepage of LGC genomics.

8.6.LiOAc-Transformation

An overnight pre-culture of the yeast strain was grown and OD was measured. The main culture was inoculated to an OD of 0.1 and grown in a shaker at 200 rpm and 30°C until the OD reached a value between 0.2 and 0.5. (3 to 5 h). Fifty ml cell suspension were harvested via centrifugation at 2500 rpm (3000 x g) for 5 min. Medium was discarded and the pellet was washed once with 25 ml sterile H_2O and pelleted again as described above. The supernatant was discarded and the pellet was resuspended in 1 ml of 100 mM lithium acetate and transferred into a sterile 1.5 ml Eppendorf tube. It was then centrifuged at 13,500 rpm (16,000 x g) for 5 s and most of the lithium acetate was removed with a micropipette. The pellet was resuspended in sterile water to a final volume of 500 µl. Single-stranded carrier DNA (2 mg/mL) was boiled at 95°C in a thermomixer and quickly chilled in ice water. Cell suspensions were vortexed and 50 µl were pipetted in a sterile Eppendorf tube. The tube was centrifuged at 13,500 rpm (16,000 x g) for 5 s and LiOAc was removed with a micropipette.

The transformation was carried out with the following amounts added in the following order:

240	µL	PEG 50 % w/v
36	µL	1.0 M LiOAc
5	µL	single stranded carrier DNA 10 mg/ml
50	µL	H_2O and plasmid DNA (1000 ng)

The cell suspension was vortexed for approximately 1 min until the pellet was completely resuspended. The Eppendorf tube was then incubated at 30°C for 30 min and heat-shocked at 42°C for 20-25 min in a thermomixer. The mix was centrifuged at 7,000 rpm (4,547 x g) for 15 s and the transformation mix was removed with a micropipette. Cells were resuspended in YPD medium and cells were grown at 30°C for 1 h with slight agitation. Cells were then pelleted at 13,500 rpm (16,000 x g) for 10 s, the pellet was resuspended in 200 µl sterile H_2O and plated in two equal aliquots onto selective medium (SD-ura). Cells were grown for approximately 24 h until colonies were visible. Colonies were re-streaked on selective medium.

8.7.Preparation for GC-MS measurement

The yeast strains were grown over night in 10 ml SD-ura medium at 30°C and 160 rpm. 15 OD units, e.g. 3 ml of an OD=5 culture were centrifuged in a Pyrex tube at 2,500 rpm (1125 x g) for 5 min and the supernatants were discarded. One ml solvent mixture (0.6 mL Methanol, 0.4 ml of 0.5 % Pyrogallol in methanol, 0.4 ml of 60 % KOH and 5 µL of 2 mg/mL cholesterol serving as an internal standard were added. Pyrex tubes were vortexed until the pellet was resuspended. Then, tubes were heated in a sand bath at 90°C for 2 h and, after cooling, were extracted with 1 mL n-heptane. Therefore, tubes were vortexed for 5 min and centrifuged at 1,500 rpm (405 x g) for 3 min. The organic phases were transferred into new Pyrex tubes and the aqueous phases were re-extracted twice. Then, the organic phases were pooled and were brought to dryness in a nitrogen stream. The extract was dissolved in 10 µl pyridine. Ten µl of N'O'-bis (trimethylsilyl) - trifluoracetamide were added for derivatization and were diluted with 50 µl ethyl acetate.

The samples were analyzed by Prof. Dr. Erich Leitner at the Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology. The technical data of GC-MS analysis are described in Table 9.

Table 9: Technical data of GC-MS analysis

GLC	HP 5890 Series II Plus with Electronic Pressure Control and 6890 automated liquid sampler (ALS)
Injector	Split/splitless 270°C, mode: splitless, purge on: 2 min
Injection volume	1 µl

Column	HP 5-MS (Crosslinked 5 % Phenyl Methyl Siloxane), 30 m x 0.25 mM i.d. x 0.25 µm film thickness
Carrier	Helium, 5.0
Flow	0.9 ml, linear velocity 35.4 cm/s, constant flow
Oven	100°C (1 min), ramp of 10°C/min to 250°C (0 min) and ramp of 3°C/min to 300°C (0 min)
Detector	selective Detector HP 5972 MSD
Ionization	EI, 70 eV
Mode	Scan, scan range: 100-550 amu, 2.58 scans/s
EM Voltage	Tune Voltage
Tune	Auto Tune

8.8.Determination of total protein concentration

Protein concentrations in the microsomal fractions were determined with the Pierce BCA Protein Assay Kit. The microsomal samples were diluted in 100 mM Tris/Cl buffer, pH 7.4, like the self-prepared BSA standard. Standards and samples were measured in duplicates in different dilutions, the measurements were carried out according to the Kit protocol.

8.9.Western Blot Analysis

The double knockout strains *Δare1Δare2*, harboring the recombinant acyltransferases were analysed by Western Blot to determine whether the enzymes were expressed or not. The first step of protein analysis was to break the yeast cells and precipitate the proteins by a method described first by Volland et al. (1993), modified by Schimmoeller et al. (1995), and finally adapted to own specific needs.

For this purpose, three OD units of an overnight culture of each sample were harvested. The pellets were resuspended in 50 µL of 1.85 M NaOH/7.5 % (v/w) β-mercaptoethanol and incubated for ten min on ice. Upon addition of 50 µL of 50 % TCA, the samples were mixed thoroughly and incubated for additional 30 min to 1 h on ice. Protein was pelleted at 10,000 rpm (9279 x g) for 5 min and the supernatants were removed with a pipette. For neutralization of the TCA, the pellets were washed once with 1 ml of ice cold water and then resuspended in a mixture of 33 µL of sample buffer 1x, 15

µL of 1 M Tris base and 2 µL of β-mercaptoethanol per pellet. The proteins were denatured for 10 min at 37°C prior to loading 5 - 10 µL on a NuPAGE SDS Gel (4-12 % Bis-Tris Gel), following NuPAGE protocol.

For Western Blot analyses the blotting sandwich was built up as shown in Figure 7:

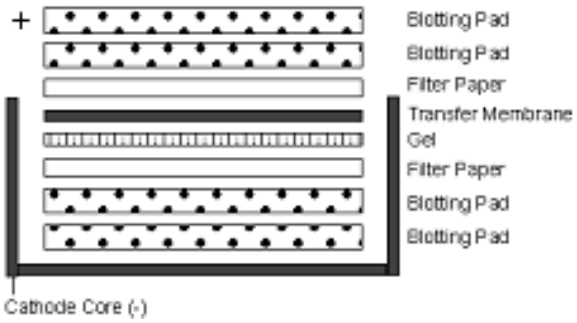

Figure 7: Scheme for Western Blot assembly, ®Invitrogen

Transfer was performed for up to 1 h with a current of 400 mA as the limiting variable.

The membranes were stained with Ponceau S solution to check if protein transfer from the gel to the membrane had worked out. After taking an image, the Ponceau S stain was washed away with water before blocking the membrane with TBST-milk over night at 4°C. This step was followed by rinsing the membrane with 1xTBST before applying a 1:30,000 working solution of the primary antibody (Anti-FLAG or Anti-GST) and incubating over night at room temperature and moderate shaking. After washing the membrane three times for ten min with 1xTBST, the secondary antibody (Goat anti mouse IgG for FLAG/Goat anti rabbit IgG for GST, both horseradish peroxidase conjugated) was applied at 1:5000 dilution for one h at room temperature and moderate shaking. After repeating the washing steps three times for ten min with 1xTBST, the detection was done with the SuperSignal West Pico Chemiluminescent Substrate Kit from Pierce. The two substrate components, peroxide solution and enhancer solution, were mixed in a 1:1 ratio. For one membrane, 5 ml of the working solution were applied with a micropipette directly onto the membrane and incubated for one to five min. During incubation, chemiluminescence signals were detected with the G:Box Bioimager.

8.10. Microsomal Preparation (light microsomes)

The preparation was carried out according to the following description for the microsomal assays and for the first radioassays. For the later radioassays it was slightly changed to achieve a more active microsomal fraction, i.e. the heavy microsomes.

Fifty ml of SD-ura medium were inoculated with the different strains from a freshly grown SD-ura plate. The pre-culture was grown in an incubator at 30°C and 160 rpm for 24 to 36 h to an OD of at least 2.

Thus, the main culture in 400 ml of SD-ura medium was inoculated to an OD of 0.1 and incubated at 30°C and 110 rpm for 20 h to an OD of approximately 3. Then, cells were harvested via centrifugation at 5,000 rpm (2,744 x g) for 5 min at 4°C. Subsequently, the pellet was washed with cold water and re-centrifuged at 5,000 rpm (2,744 x g) for 5 min at 4°C. The pellet was dispersed in 30 ml of 100 mM Tris/HCl buffer, pH 7.4. The cell suspension and approx. 20 ml of glass beads were transfered into the homogenization glasses of a Merckenschlager desintegrator. The cells were disrupted for 4 min with CO_2 cooling every 30 s. Unbroken cells and cell debris were spun out at 4,000 rpm (JA 25.50, 1,313 x g) for 5 min at 4°C. The supernatant was centrifuged for removal of mitochondria at 20,000 rpm (JA 25.50, 32,816 x g) for 30 min at 4°C. Then, the supernatant was ultracentrifuged at 45,000 rpm (100,000 x g) for 1 h at 4°C to pellet the light microsomes. The pellet was transferred using a pipette tip into a Dounce homogeniser and homogenized in 200 to 1000 µl of 1 M Tris/HCl buffer, pH 7.4. Aliquots were frozen down and stored at -20°C.

8.11. Adaptions: Microsomal Preparation (heavy microsomes)

To achieve a more active enzyme fraction, heavy microsomes were prepared for the radioassay. Therefore, mitochondria removal was carried out at 12,859 rpm (JA 25.50, 20,000 x g) for 30 min at 4°C and another centrifugation step was done at 18,185 rpm (JA 25.50, 40,000 x g) for 30 min at 4°C to isolate the heavy microsomes. The pellet was homogenized via pipetting in 100 mM Tris/HCl buffer, pH 7.4. Aliquots of 50 µl were taken, frozen down and stored at -20°C.

8.12. Microsomal Assay

The microsomal assay was prepared as the radioassay (see below) but with non-radioactive substrate oleoyl-CoA only.

8.12.a. Preparation procedure

307 nmol of sterol substrate, solved in acetone or $CHCl_3$:MeOH (2:1, v/v) were taken from stock and dispersed in 77 mg of tyloxapol by vortexing. The dispersion was brought to dryness in a nitrogen stream. The white residue was then resuspended in 307 µl of 50 mM KH_2PO_4, pH 7, to get a sterol concentration of 1 µmol/ml. The suspension was mixed on a Vibrax for approximately 1 h until it was only slightly cloudy and stored at -20 °C.

77 nmol of oleoyl-CoA, dissolved in 10 mM NaOAc, pH 6, were freshly prepared for each assay.

0.1 to 1 mg of protein were diluted in KH_2PO_4 (50 mM, pH 7) for each assay.

8.12.b. Carrying out the assay

The sterol-tyloxapol mixture was combined with the protein dilution in a Pyrex tube and was pre-incubated for 3 min in a water bath at 30°C. At timepoint t=0 of the assays, the first aliquot was taken right after the pre-incubation without addition of oleoyl-CoA. For the further aliquots or those assays lacking the t=0 timepoints, oleoyl-CoA was added and aliquots were withdrawn at defined timepoints. Therefore, the aliquots were directly transferred into Pyrex tubes filled with $CHCl_3$:MeOH (2:1, v/v) to stop the esterification reaction. For the last aliquot, $CHCl_3$: MeOH (2:1, v/v) was directly added to the Pyrex tube.
Then, lipids were extracted similarly as in Folch et al. (1957) for one h on a Vibrax. The phases were separated through centrifugation at 1500 rpm for 3 min (453 x g) and the lower phase was transferred into another Pyrex tube. The organic phase was washed with 1 ml of 0,034 % $MgCl_2$ solution for 2 min on the Vibrax and centrifuged for 3 min at 2500 rpm (1258 x g). Then, the supernatant was discarded and the organic phase was washed with 1 ml of artificial upper phase ($MeOH/H_20/ CHCl_3$ = 48:47:3, v/v/v) for 2 min on the Vibrax. The Pyrex tubes were centrifuged again for 3 min at 2500 rpm, the supernatants were discarded and the solutions were brought to dryness in a nitrogen stream. The residue was dissolved in 20 µl $CHCl_3$:MeOH (2:1, v/v), vortexed and 5 µl were put on TLC silica plates for lipid separation.

8.13. Radioassay

The radioassay was set up according to the enzymatic assay described in the diploma thesis of Harald Pichler (Pichler, 1996, according to Taketani, 1979 and Zinser, 1993). Sterols were prepared with a 25-fold excess of tyloxapol (w/w), also named Triton WR 1339, as a detergent, and were resuspended in 50 mM KH_2PO_4, pH 7. This treatment with tyloxapol was reported to lead to an increase of ACAT activity by 250 percent when compared to adding cholesterol without any detergent. This is achieved by making the exogenous sterol more accessible for the enzyme (Billheimer, 1981). The sterol was added in a 10-fold excess over oleoyl-CoA as the test assays pointed out that there was a plateau reached at that concentration. (see results section)

The [Oleoyl-1-^{14}C]- CoA (0.02 µCi/µL, 0.3412 nmol/µL) (Figure 8) was prepared with non-radioactive oleoyl-CoA in 10 mM Na-acetate, pH 6, and was diluted to the concentration needed.

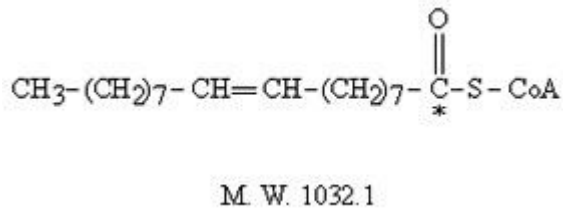

Figure 8: [oleoyl-1-^{14}C]- Coenzyme A

8.13.a. Initial assays

For testing the method, different amounts of sterol, oleoyl-CoA and protein were applied and the assay was carried out as described below (see results section).

8.13.b. Final assay

102 nmol of sterol substrate, dissolved in acetone or MeOH: $CHCl_3$ (2:1, v/v) were taken from stock and mixed with 984 mg tyloxapol as a detergent. The liquids were vortexed and brought to dryness in a nitrogen stream. The white residue was resuspended in 300 µl of 50 mM KH_2PO_4, pH 7. The suspension was mixed on a Vibrax for approximately 1 h until it was only slightly cloudy and then was stored at -20 °C.

10.2 nmol of mixed *oleoyl-CoA, dissolved in 100 µl of 10 mM NaOAc, pH 6, were freshly prepared for each assay. Therefore, 1.5 nmol of radioactively labeled oleoyl-CoA and 8.5 nmol of non-labeled oleoyl-CoA were mixed.

0.5 mg of protein were dissolved in 50 mM KH_2PO_4, pH 7, for each assay.

The sterol-tyloxapol mixture (300 µl) was mixed with 100 µl protein dilution in a Pyrex tube and pre-incubated for 3 min in a water bath at 30°C. The oleoyl-CoA (100 µl) was added and the reaction mixture was incubated at 30°C. To stop the esterification reaction, 4 ml of $CHCl_3$:MeOH (2:1, v/v) was added at defined timepoints.

8.14. Folch extraction

After the assays, the lipids were extracted on a Vibrax for one h at approx. 1000 rpm. One ml of 0.034 % $MgCl_2$ solution was added and the solution was shaken for 2 min on the Vibrax and centrifuged for 3 min at 2500 rpm (1258 x g) for phase separation. The supernatant was discarded and the organic phase was washed with 1 ml of artificial upper phase (MeOH/H_20/ $CHCl_3$ = 48:47:3, v/v/v) for 2 min on the

Vibrax. Upon centrifuging again for 3 min at 2500 rpm, the supernatant was discarded and the solution was brought to dryness in a nitrogen stream. The residue was dissolved in 100 µl of MeOH: $CHCl_3$ (2:1, v/v), vortexed for 3 s and 10 µl were applied on TLC silica plates for lipid separation. As a standard, a lipid extract of a WT CEN.PK2 strain that was compared to lipid standards was also applied on the plate. Respective bands were stained with iodine (approx. 2 min) and were marked with a pencil. Then, the iodine was removed under aeration. The plate was wetted and selected bands were scraped off the plate with a sharp piece of metal and put into scintillation vials filled with 8 ml of scintillation cocktail. The vials were placed in a scintillation counter and were repeatedly counted for 10 min until count numbers were stable and the quenching parameter SIS showed an acceptable value according to user's manual.

8.15. Thin layer chromatography

8.15.a. For microsomal assay

To analyze the amount of sterol esters formed by the acyltransferases during the microsomal assay, a two-step separation on Silica TLC plates was carried out. Therefore, lipid extracts were spotted with a Hamilton syringe onto 20 x 10 cm silica plates. In parallel, the first solvent mixture, petrol ether/diethyl ether/acetic acid (20:20:0.8 per vol.) was prepared. After equilibration of the TLC chamber for about 30 min, TLC plates were developed until reaching one third of the plate's height. After drying the TLC plates completely - until the plate did not smell of acetic acid - the same plates were developed in a second, pre-equilibrated chamber using petrol ether/diethyl ether (40:0.8, v/v) as a solvent mixture until the solvent front reached a line 1 cm beneath the top of the plate. TLC plates were then dried and dipped into a solution of 0.4 g $MnCl_2$, 60 ml H_2O, 60 ml methanol and 4 ml H_2SO_4 conc. for 10 s and then heated at 105°C for 40 min.

8.15.b. For radioassay

To separate the radioactively labeled sterol esters from other lipid compounds after the enzymatic reaction, a single separation on Silica TLC plates was carried out. Therefore, 10 µl lipid extracts were spotted with a Hamilton syringe onto 20 x 10 cm silica plates. In parallel, the solvent mixture, petrol ether/diethyl ether/acetic acid (35:15:1 per vol.) was prepared. After equilibration of the TLC chamber for about 30 min, TLC plates were developed until reaching a line 1 cm beneath the top of the plate. After drying the TLC plates completely - until the plate did not smell of acetic acid - they were stained with iodine for approximately 3 min. Spots were marked with a pencil and iodine was removed inside the vent at room temperature.

9. Results

9.1. Constructing the plasmids: p426GPD

Six acyltransferases (Table 10), chosen by Regina Leber, available in the vector pJ201, were cloned into the 2µ-plasmid p426GPD (Figure 9), which should provide high expression rates by a GPD-promoter. The sequences were codon optimized for expression in *S. cerevisiae*.

Table 10: Acyltransferases used for first cloning strategy; optSc: codon optimization for expression in *S. cerevisiae*

Acyltransferases	Organism	Short term	Base pairs
Sc ARE2	*Saccharomyces cerevisiae*	Are2	**1939**
***Pt* ACAT1_opt*Sc*1**	*Pan troglodytes*	120	**1659**
***Rn* ACAT2_opt*Sc*1**	*Rattus norvegicus*	121	**1581**
***Rn* ACAT1_opt*Sc*1**	*Rattus norvegicus*	122	**1644**
***Ca ARE*2_opt*Sc*1**	*Candida albicans*	123	**1836**
***Tg* ACAT1_opt*Sc*1**	*Toxoplasma gondii*	124	**1955**

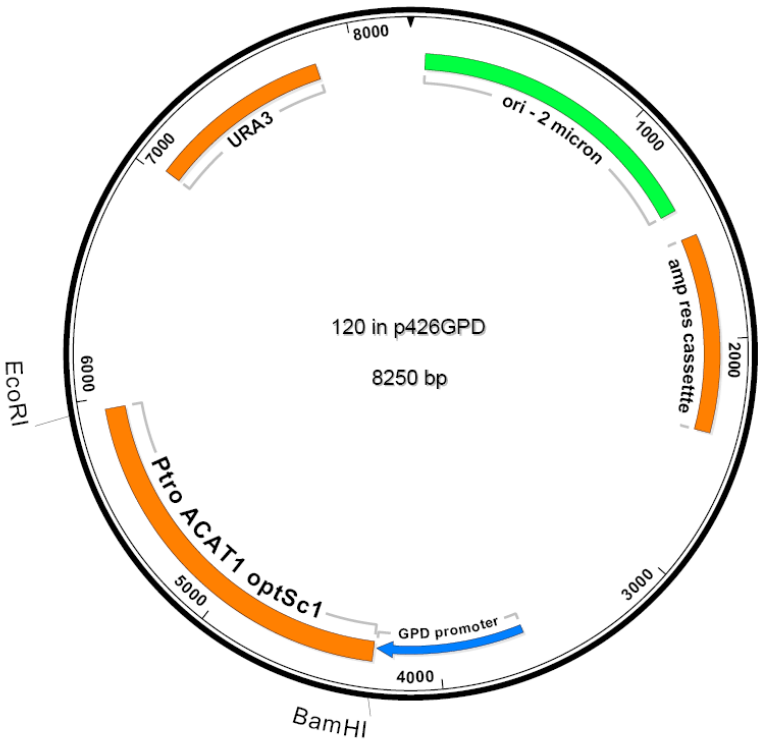

Figure 9: Insert 120 in vector p426GPD: A 2µ plasmid with ampicillin resistance cassette, *URA3*-Marker and constitutive *GPD* promoter

The plasmids were then transformed into *E.coli*-TOP10F'-cells via electro-transformation. Positive transformants were confirmed via restriction analysis (Figure 10).

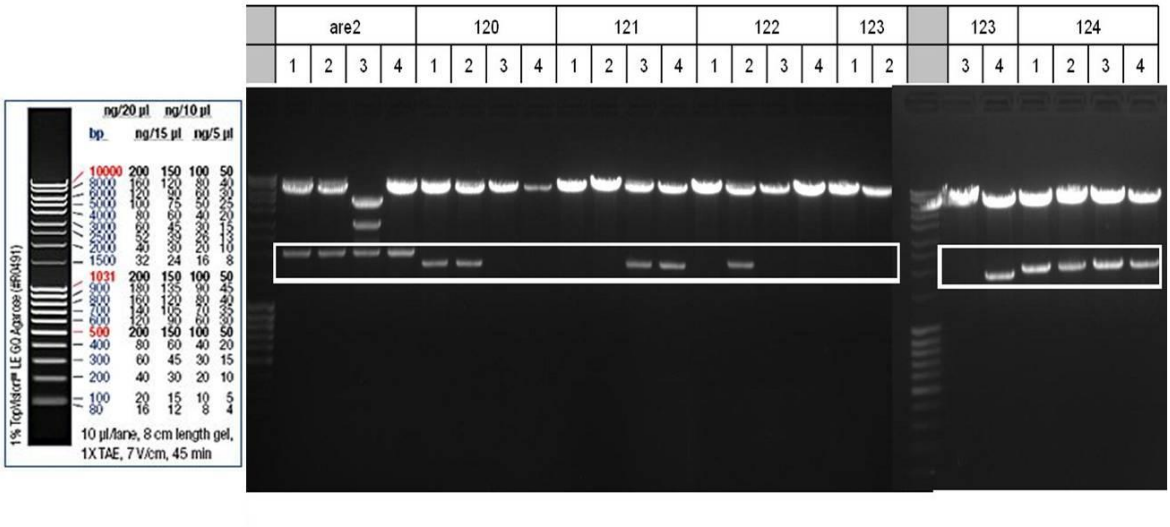

Figure 10: Agarose gel electrophoresis of restriction control for cloning of ACAT genes into p426GPD: For each ACAT (index see **Table 10**) at least one tested transformant carried the expected vector construcht as indicated by the fragment lengths of the restriction cut (compare expected insert lengths, see **Table 10**)

In addition, specific primers for every insert were designed to add a FLAG-tag by PCR (Figure 11). After restriction with enzymes *Bam*HI and *Eco*RI, the PCR-products were ligated into vector p426GPD and transformed into TOP10F'-cells. Since the Kozak-sequence (AAA) was missing for the first series of constructs, another PCR was carried out to add a Kozak-sequence at the 5' end before FLAG-tag and the start codon (ATG). The sequences were then confirmed via Sanger-sequencing. The results showed that all plasmids carried the FLAG-tagged insert.

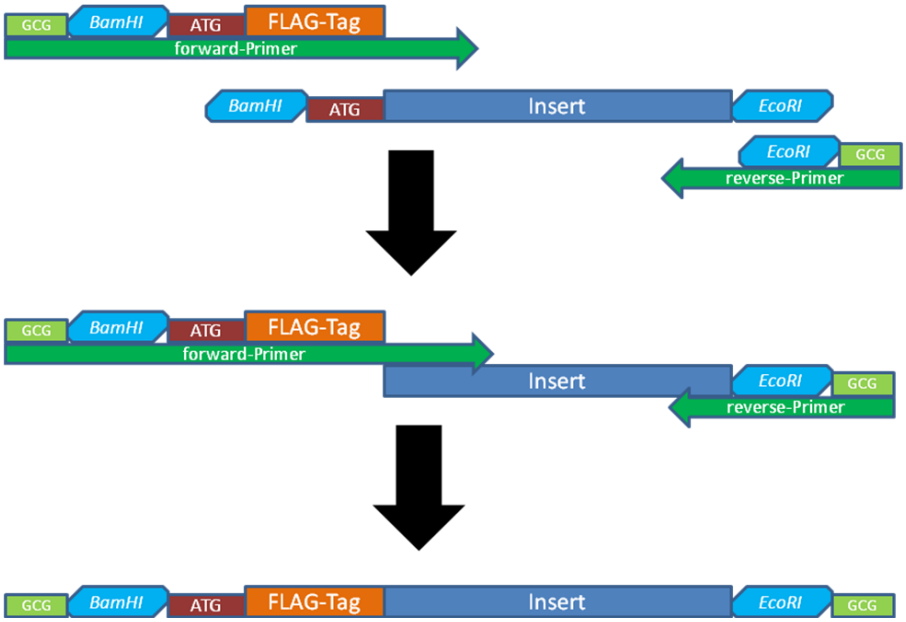

Figure 11: schematic view of PCR reaction

The plasmids with the heterologous acyltransferases - with and without the FLAG-tag - were transformed into two *S. cerevisiae* strains: double knockout *are1are2* (Δ12, strain-background: FY834, G.Daum) and triple knock out *erg5erg6are1are2* (Δ5612, strain-background: CEN.PK2, B.Ploier). The triple knockout strain had first been constructed by Birgit Ploier as a quadruple knockout *erg5erg6are1are2* but colony PCR analysis showed that the *ARE1* gene was still present after the substitution cassette was inserted. We hypothesise that the *ARE1* cassette re-integrated somewhere else in the genome.

In these two strain backgrounds (Δ12 and Δ5612), sterol composition analysis and microsomal assays were carried out.

9.2.Sterol composition analysis

The *S. cerevisiae* strains with acyltransferases were grown on SD-ura medium. Then, 15 OD units, i.e. 15 ml of OD=1 culture, were harvested through centrifugation and cells were broken via treatment with KOH. Sterol esters were hydrolysed, cholesterol was added as an internal standard and lipid-extraction was carried out. Total lipids were analysed via GC-MS by Prof. Dr. Erich Leitner at the Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology. (Figure 12 to Figure 15).

As indicated in Figure 13, the Δ12 double knockout strain accumulated zymosterol only if acyltransferases Are2 (*S. cerevisiae*) or 123 (*C. albicans*) were present. An increased level of lanosterol was observed for insert 123 (*C. albicans*). The most abundant compound was ergosterol as the strain background has no mutation/deletion in the *ERG* genes. *ERG6* is coding for the C24-methyltransferase

which adds a methyl group at C24 and converts zymosterol to fecosterol. *ERG6* is therefore essential for the conversion of cholesta- to ergosta-compounds. As Birgit Ploier had shown in her Diploma thesis, ergosta-sterols are not detectable in significant amounts, when Erg6p is non-functional. (Ploier, 2010)

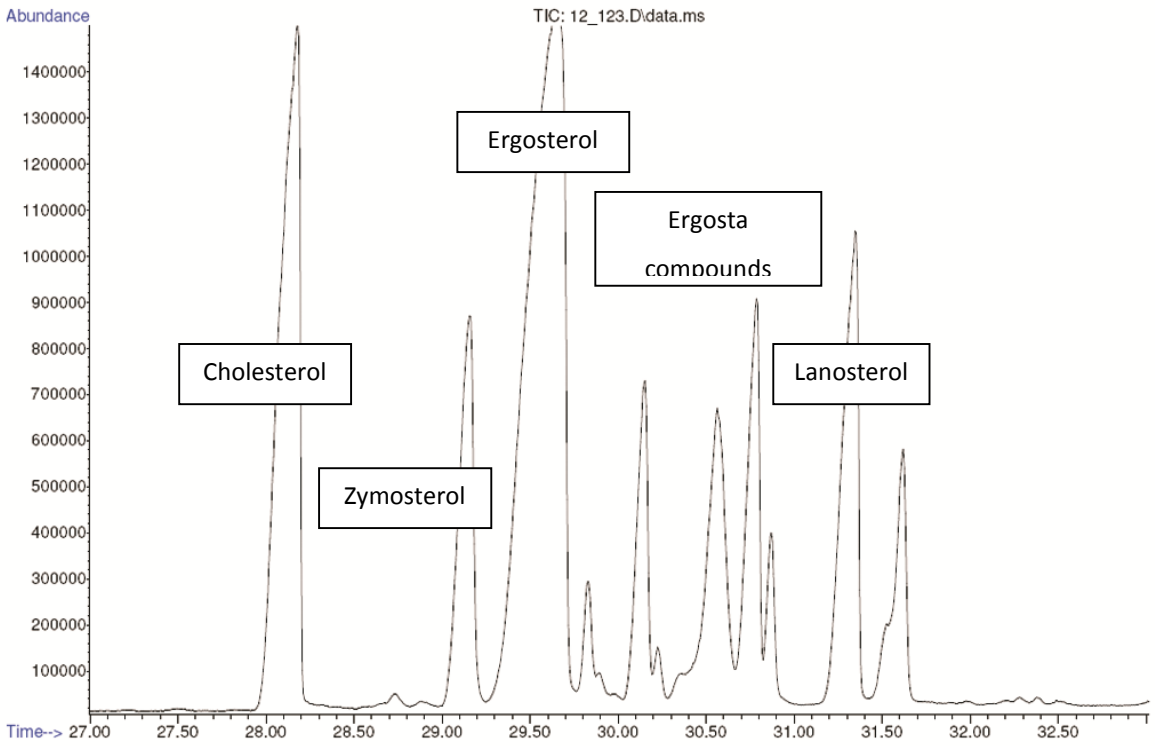

Figure 12: GC-MS chromatogram of 123 (*C.a.*) in Δ12; the x-axis gives the retention time, the y-axis gives the abundance; cholesterol was used as an internal standard

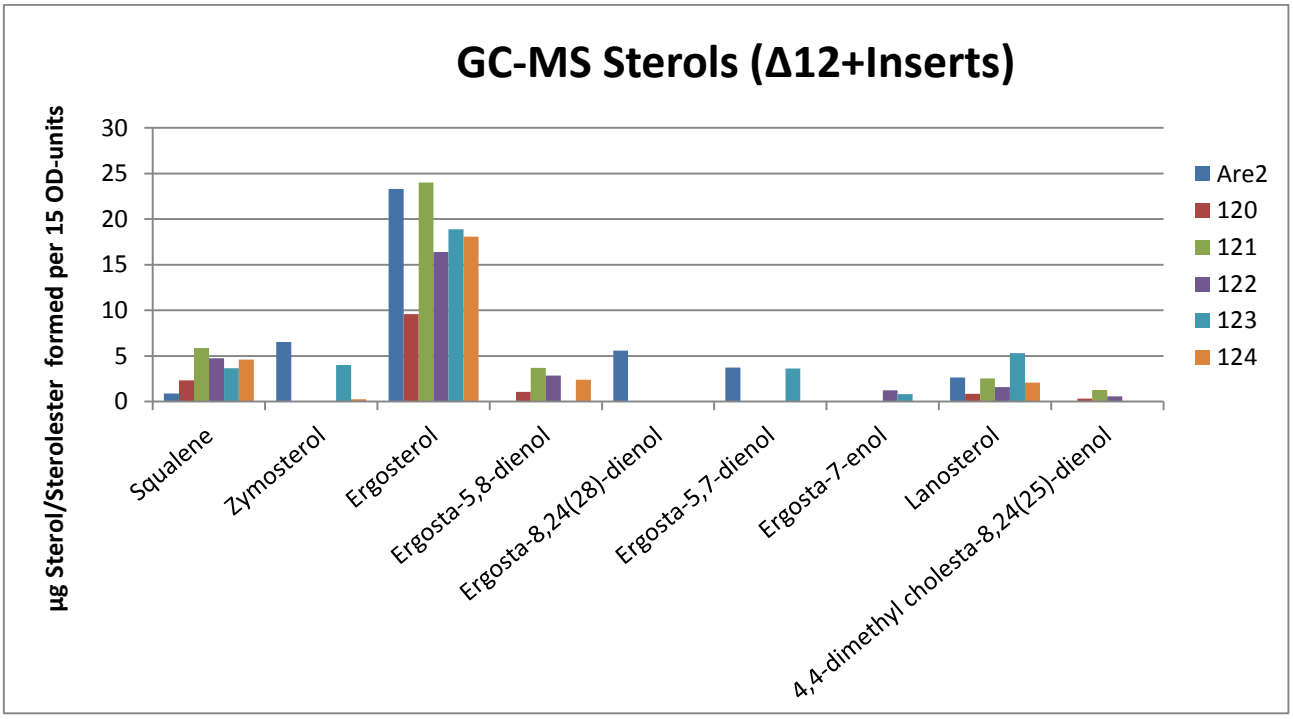

Figure 13: Sterol analysis of Δ12 expressing different acyltransferases; the y-axis gives the amount of sterols & sterol esters formed in 15 OD-units; Are2: *S. cerevisiae*, 120: *P. troglodytes*, 121: *R. norvegicus* (ACAT2), 122: *R. norvegicus* (ACAT1), 123: *C. albicans* (Are2), 124: *T. gondii* (ACAT1);

Through deletion of the genes *ERG5* and *ERG6*, the strain Δ5612 showed a different sterol pattern, as there was no production of ergosta-compounds but of cholesta-compounds (Figure 15). Upon the expression of the acyltransferases Are2 (S.c.) and 123 (C.a.) in Δ*5612*, there was a clear increase in zymosterol when compared to the other acyltransferases. As the Are1p enzyme was still active in this strain, these results represented the sum of sterol esters esterified by Are1p and/or the heterologously expressed acyltransferases and of free sterols. This could also explain, why the empty vector control (EVC) had a higher amount of cholesta-5,7,24-trienol than 120, 122 and 124.

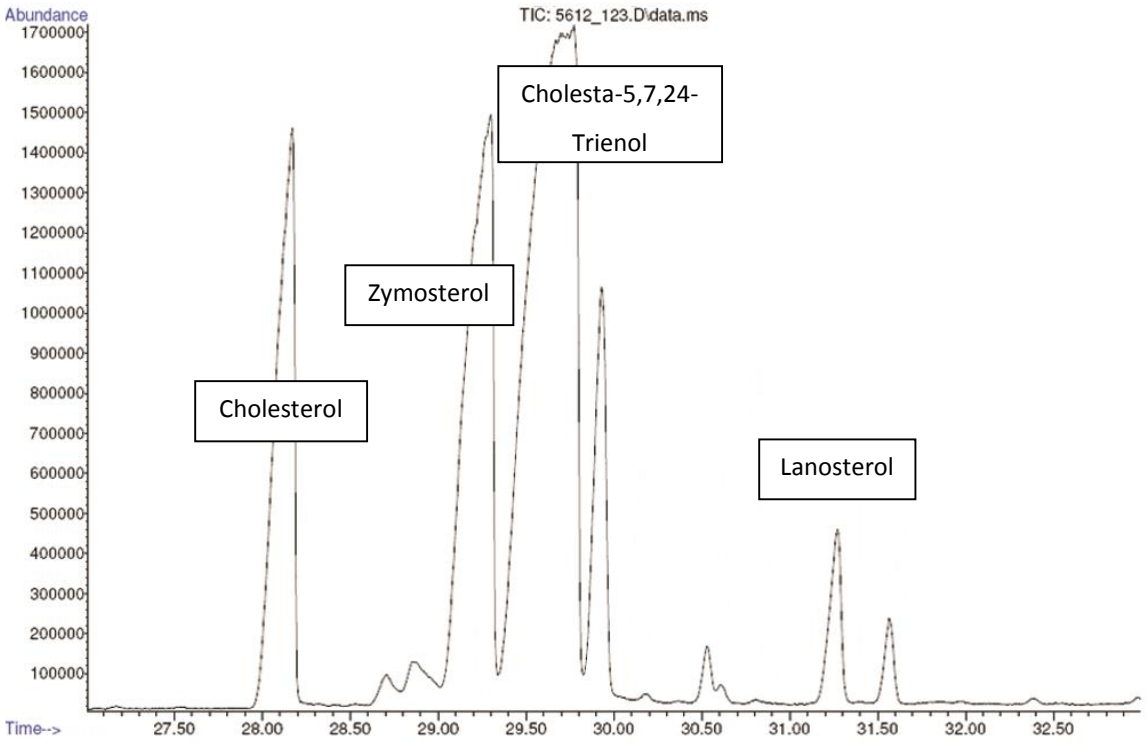

Figure 14: GC-MS chromatogram of 123 (*C.a.*) in Δ5612; the x-axis gives the retention time, the y-axis gives the abundance; cholesterol was used as an internal standard

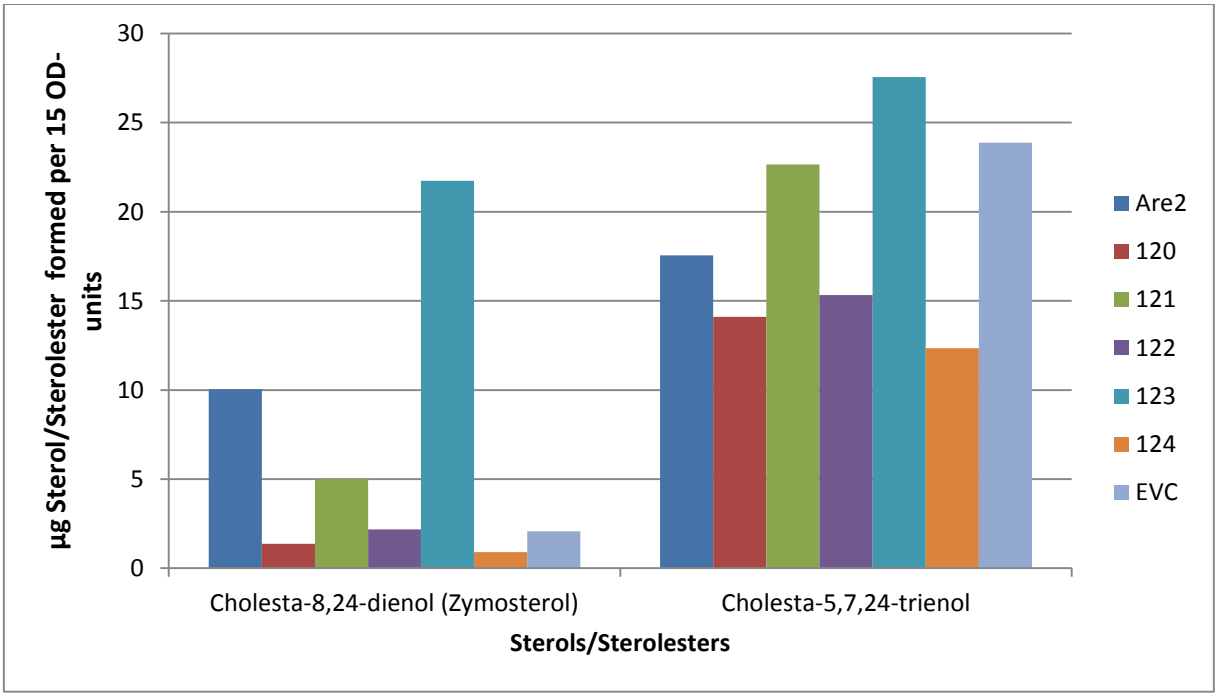

Figure 15: Sterol analysis of Δ5612 expressing different acyltransferases; the y-axis gives the amount of sterols & sterol esters formed in 15 OD-units; Are2: *S. cerevisiae* , 120: *P. troglodytes*, 121: *R. norvegicus* (ACAT2), 122: *R. norvegicus* (ACAT1), 123: *C. albicans* (Are2), 124: *T. gondii* (ACAT1);

The transformation of the p426GPD FLAG-tagged constructs was later on checked via Western blot analysis (Figure 16) which showed, that only 3 acyltransferases (namely Are2p from *S. cerevisiae*, Are2p form *C. albicans* and ACAT1 form *T. gondii*) were well expressed in the *S. cerevisiae* strains. So for further tests new constructs were made. (see chapter 9.2.b).

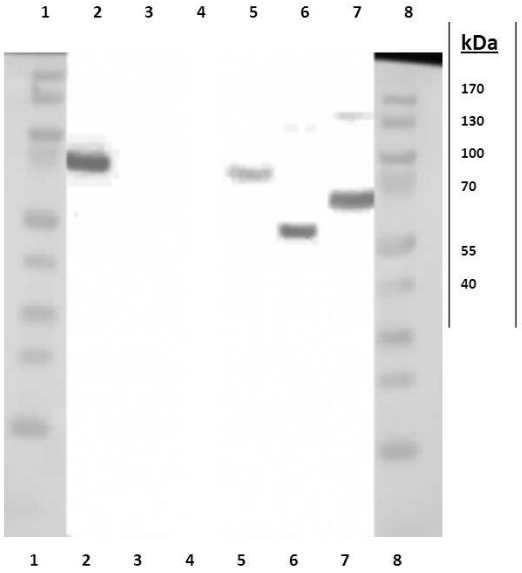

Figure 16: Western Blot of FLAG-constructs; Lane 1: Page Ruler Protein Ladder, Lane 2: *S.c.* Are2 (expected band: 100.1 kDa), Lane 3: *Pt* ACAT1_opt*Sc*1 (expected band: 90.2 kDa), Lane 4: *Rn* ACAT2_opt*Sc*1 (expected band: 86.0 kDa), Lane 5: *Tg* ACAT1_opt*Sc*1 (expected band: 98.2 kDa), Lane 6: positive control: *E. coli* HPOTWopt (6+3) cytP450 (expected band: 54 kDa), Lane 7: *Ca ARE2*_opt*Sc*1 (expected band: 96.8 kDa), Lane 8: Page Ruler Protein Ladder

9.2.a. Microsomal Assay

For the double KO strain (Δ12) carrying the different plasmids, a microsomal assay was carried out to determine the sterol specificity of esterification. Therefore, microsomal preparations were obtained. The pre-cultures were inoculated and grown for approximately 24 h in SD-ura medium. Then, the main cultures were incubated and grown in SD-ura medium for approx. 40 h at 30°C until an OD of at least 4 was reached. Cells were harvested, broken and the microsomes were separated via several centrifugation steps as described in the methods section (chapter 8.10). The protein concentration was measured via a BCA assay and then a microsomal assay was carried out with the following 4 sterols: cholesterol, cholesta-5,7,24-trienol, zymosterol (i.e. cholesta-8,24-dienol) and 7-dehydrocholesterol. The microsomes were mixed with an excess of the different sterols as the first substrate. For the assays with cholesta-5,7,24-trienol, a lower sterol-concentration was used. Then, oleoyl-CoA was added as the limiting substrate in the esterification reaction. The mix was incubated at 30°C and aliquots were pipetted into $CHCl_3$/MeOH (2:1, v/v) to stop the reaction. In the first assays, three aliquots were taken (after 5, 10 and 15 min) to determine when the oleoyl-CoA was exhausted. For the further

experiments, the time points were changed (0 min, 2 min, 60 min) to determine, whether there were any steryl esters in the microsomes and whether there was any further turnover after a longer period of incubation.

The turnover was detected after lipid extraction via TLC (examples with cholesta-5,7,24-trienol as substrate are shown in Figure 17 & Figure 18). Results indicated that there were already well detectable levels of sterol esters in some of the microsomes - especially from strains expressing *S. cerevisiae* Are2p *and C. albicans* Are2 (=123) - while there was no additional esterification detectable after adding the oleoyl-CoA. This indicated that the ergosterol, still produced in the *Δare1Δare2* double knockout strains, was present in a large quantity in the form of sterol esters and that further esterification was not detectable by this method. So the method was changed to a radioassay that should provide a specific signal for the esterification of sterols of interest.

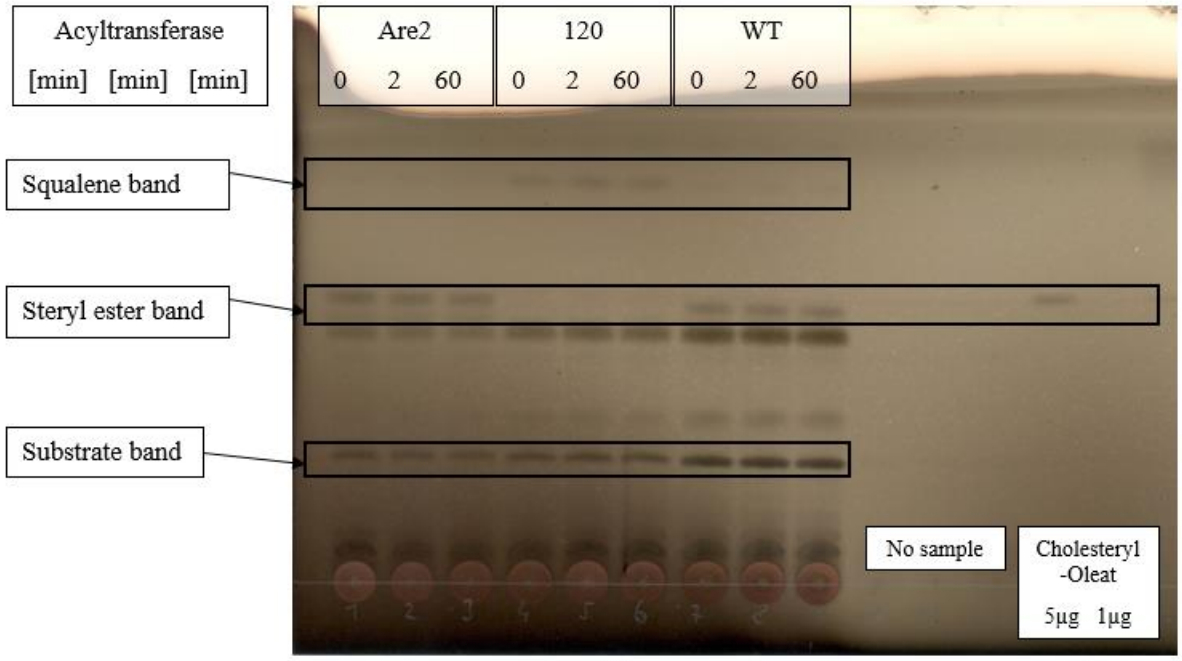

Figure 17: TLC after enzymatic assay; lipids were separated in two steps: 1st step to one third of the TLC plate: petrol ether:diethyl ether:acetic acid 20:20:0.8, v/v/v ; 2nd step to 1 cm below the top: petrol ether:diethyl ether 40:0.8, v/v; the TLC showed no further turnover after 60 min; shown on the TLC are two constructs, Are2 and 120, compared to Wild type (WT) and cholesteryl-oleate as a sterol ester standard.

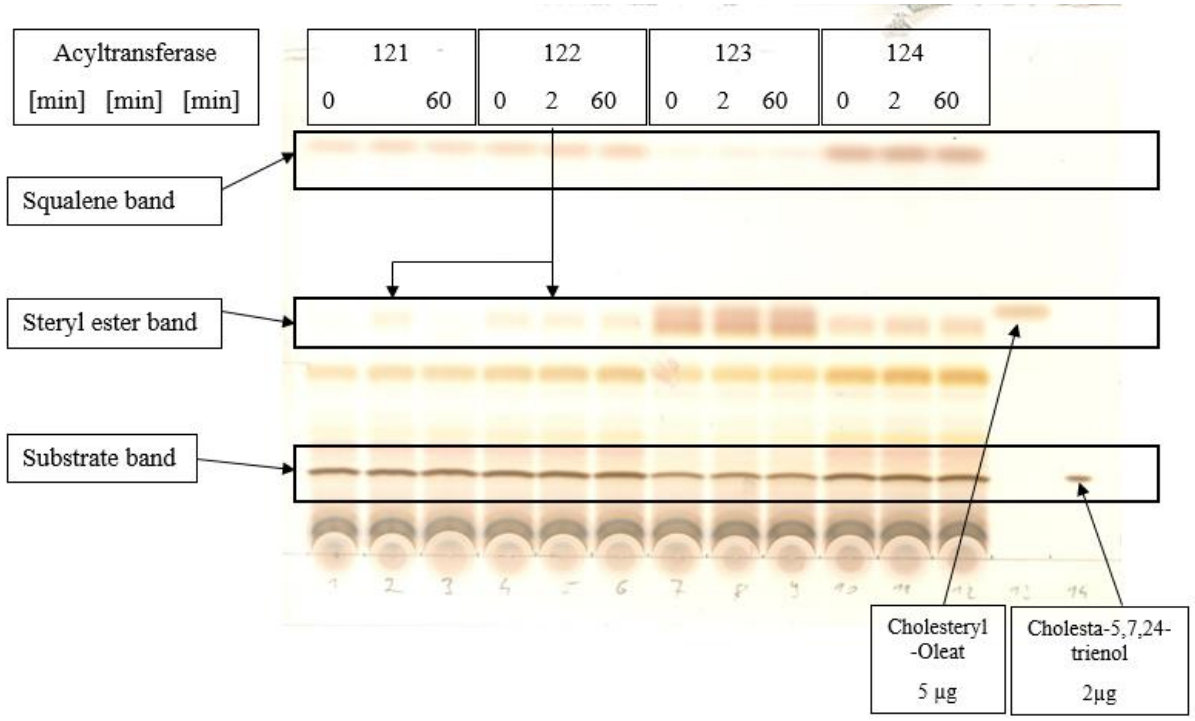

Figure 18: TLC after enzymatic assay; lipids were separated in two steps: 1st step to one third of the TLC plate: petrol ether:diethyl ether:acetic acid 20:20:0.8, per volume ; 2nd step to 1 cm below the top: petrol ether:diethyl ether 40:0.8, v/v; the TLC showed no further turnover after 60 min; shown on the TLC are four constructs, 121, 122, 123 and 124 compared to cholesteryl-oleate as a sterol ester standard and cholesta-5,7,24-trienol as a substrate standard.

9.2.b. Changing to other constructs

Due to the situation that even after repeated transformation with the FLAG-constructs, only three acyltransferases (Are2p from *S. cerevisiae*, Are2p from *C. albicans* and ACAT1 form *T. gondii*) could be expressed in the *S. cerevisiae Δare1Δare2* double knockout strain the work was continued with constructs in the vector pYEX4T-1 (Figure 19). This vector is similar to p426GPD, it is as well a 2µ plasmid with ampicillin resistance cassette, *URA3* marker but instead of a constitutive, it carries an inducible *CUP1* promoter.

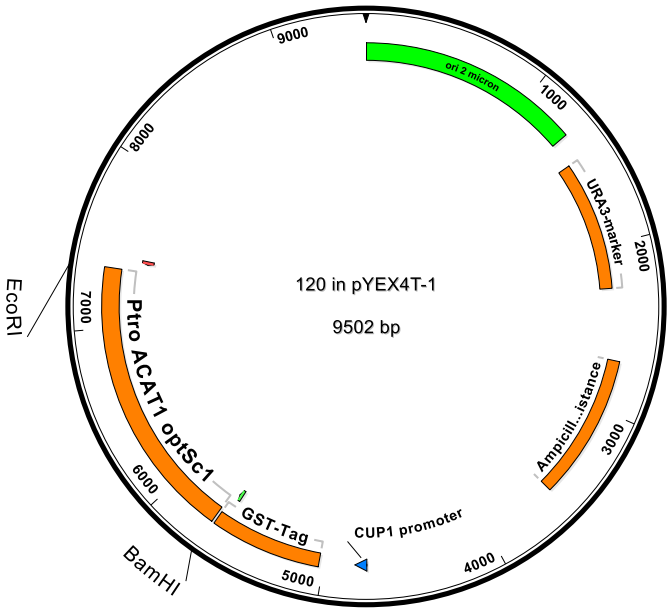

Figure 19: Insert 120 (*P. troglodytes*) in vector pYEX4T-1: A 2µ plasmid with ampicillin resistance cassette, *URA3* marker and inducible *CUP1* promoter

A Western blot by Corinna Odar showed that all acyltransferases were expressed with approximately the right sizes (Figure 20 & Table 11).

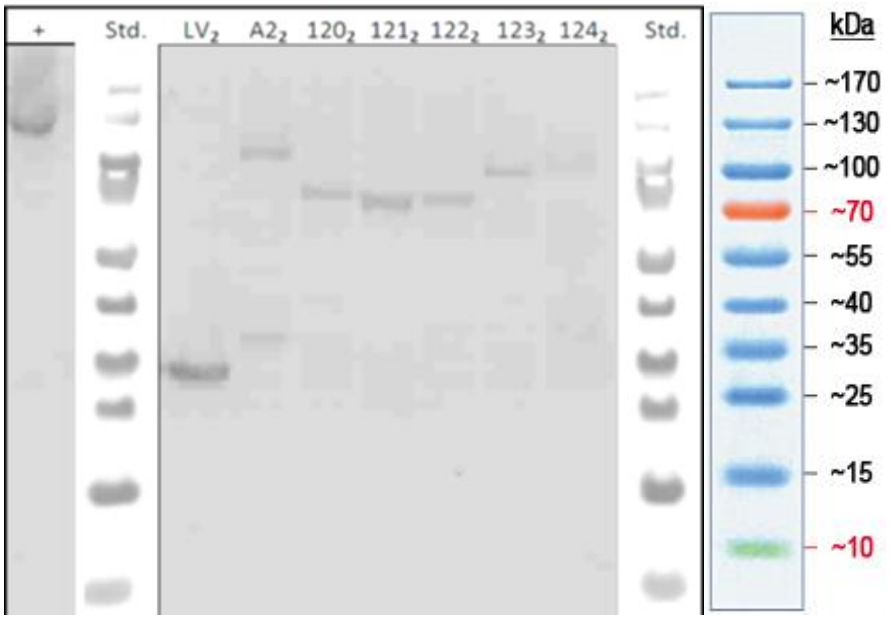

Figure 20: Western Blot kindly provided by Dipl.-Ing[in] Corinna Odar; LV_2: empty vector (GST-Tag only), $A2_2$: *S. cerevisiae* (*ARE2*), 120_2: *P. troglodytes* (ACAT1), 121_2: *R. norvegicus* (ACAT2), 122_2: *R. norvegicus* (ACAT*1*), 123_2: *C. albicans* (*ARE2*), 124_2: *T. gondii* (ACAT1); GST-tagged acyltransferase visualized by chemiluminescence

The new plasmids had been constructed by Corinna Odar. Additionally to the acyltransferase constructs in vector p426GPD, Are1 was also cloned into vector pYEX4T-1. (Table 11)

Table 11: Acyltransferases used for second cloning strategy; optSc: codon optimization for expression in *S. cerevisiae*

Acyltransferases	Organism	Short term	Base pairs	kDa (GST-Tag included)
***Sc ARE1*mut**	*Saccharomyces cerevisiae*	Are1	1833	**97.1**
Sc ARE2	*Saccharomyces cerevisiae*	Are2	1939	**100.1**
***Pt* ACAT1_opt*Sc*1**	*Pan troglodytes*	120	1659	**90.2**
***Rn* ACAT2_opt*Sc*1**	*Rattus norvegicus*	121	1581	**86.0**
***Rn* ACAT1_opt*Sc*1**	*Rattus norvegicus*	122	1644	**89.6**
***Ca* ARE2_opt*Sc*1**	*Candida albicans*	123	1836	**96.8**
***Tg* ACAT1_opt*Sc*1**	*Toxoplasma gondii*	124	1955	**98.2**

The constructs had not been confirmed via sequencing by Corinna Odar, so this was carried out. The sequencing showed that sequences for the inserts Are2, 120 -122 and 124 were identical to the sequences designed by Regina Leber for the (heterologous) expression in S. cerevisiae. (Figure 21 to Figure 26)

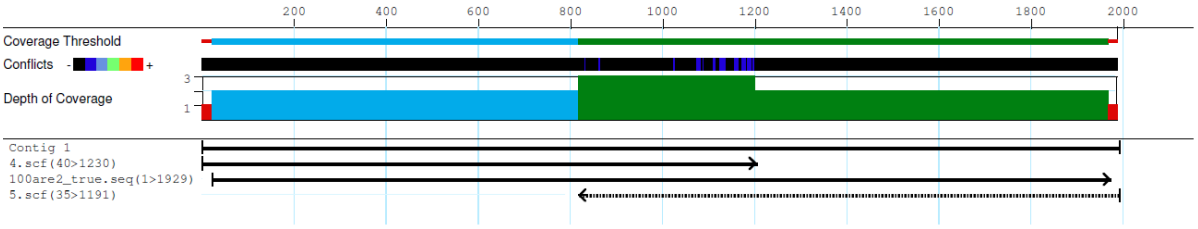

Figure 21: Sequencing result for Are2 cloned into vector pYEX4T-1; the blue lines indicate regions, where sequencing showed a divergence to the original sequence but only for one sequencing strand (details see Figure 22).

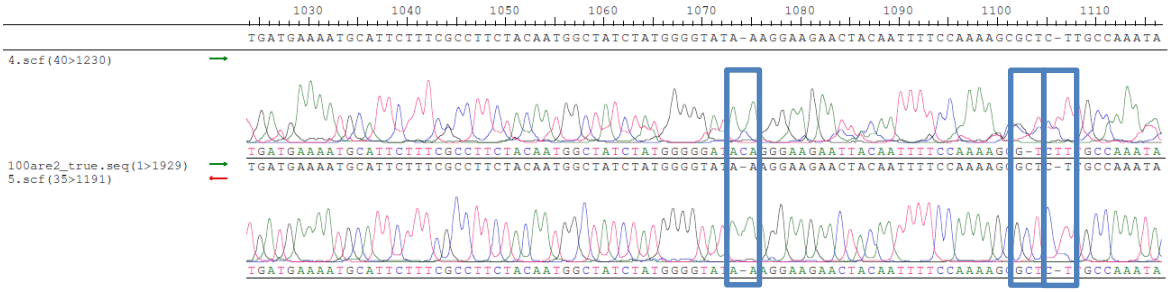

Figure 22: Details of sequencing results for the insert Are2 in vector pYEX4T-1; errors occur only on one sequencing strand in the end region of the sequence for the forward and reverse primer.

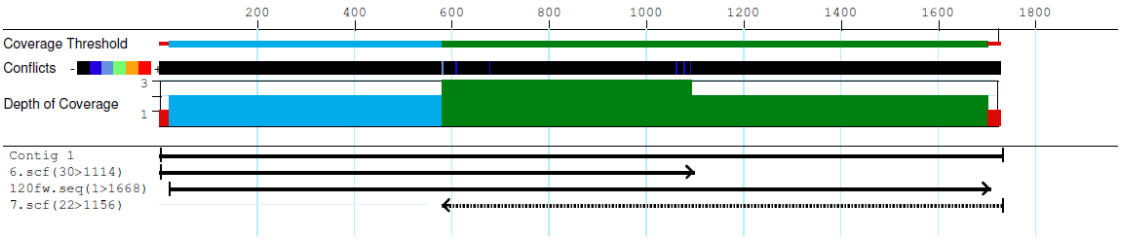

Figure 23: Sequencing result for 120 (*P. troglodytes*) cloned into vector pYEX4T-1; the blue lines indicate regions, where sequencing showed a divergence to the original sequence but only for one sequencing strand

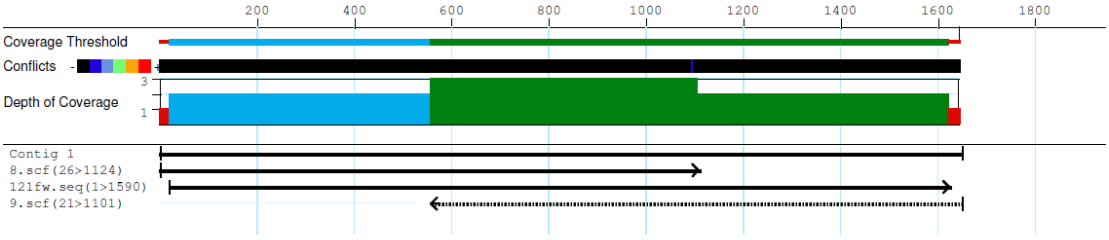

Figure 24: Sequencing result for 121 (ACAT 2, *R. norvegicus*) cloned into vector pYEX4T-1; the blue lines indicate regions, where sequencing showed a divergence to the original sequence but only for one sequencing strand

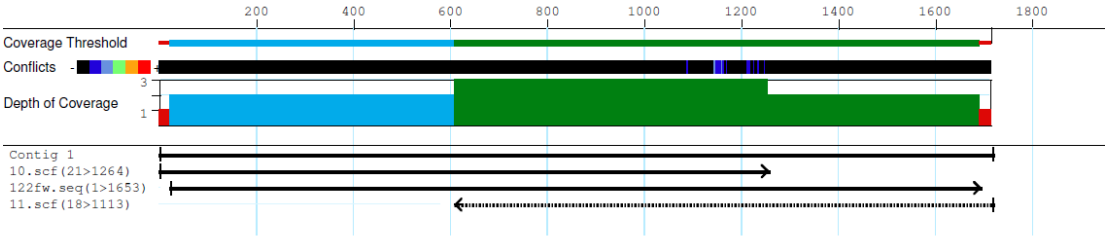

Figure 25: Sequencing result for 122 (ACAT 1, *R. norvegicus*) cloned into vector pYEX4T-1; the blue lines indicate regions, where sequencing showed a divergence to the original sequence but only for one sequencing strand

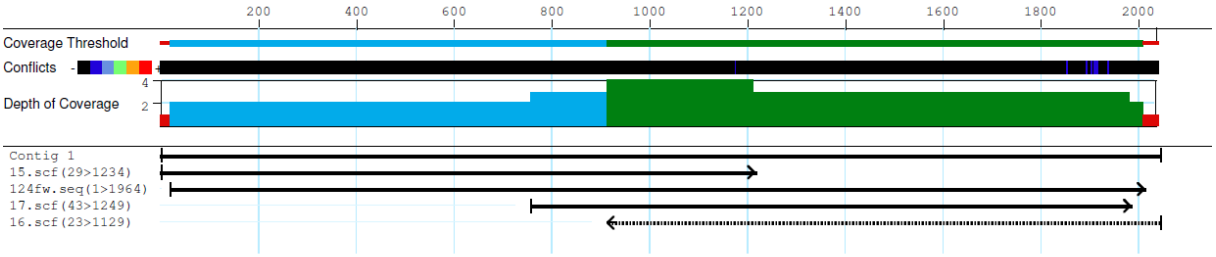

Figure 26: Sequencing result for 124 (*T. gondii*) cloned into vector pYEX4T-1; the blue lines indicate regions, where sequencing showed a divergence to the original sequence but only for one sequencing strand

For the Are1 insert, Corinna Odar exchanged some sequences that were identical to the recognition sequence of *Bam*HI. Additionally, the Are1 insert showed some irregular sequences around base pair 200 (Figure 27). Nevertheless, further experiments were carried out, because Are1p seemed to be expressed (as shown in Western Blot Analysis) and showed acyltransferase activity.

For the 123 insert (*C. albicans*) two mutations occurred: The first at base 46 and the second at base 1515 from the *Bam*HI cutting sequence (see Figure 28), both times a thymine was changed to a cytosine. This led to an exchange in the amino acid sequence, for the first mutation I13T (Isoleucine to Threonine) and for the second mutation F503L (Phenylalanine to Leucine). Unfortunately, this change was detected at the end of the assay experiments, so cloning was therefore not repeated. This change could have also led to changes in expression or activity of the acyltransferase from *C. albicans*.

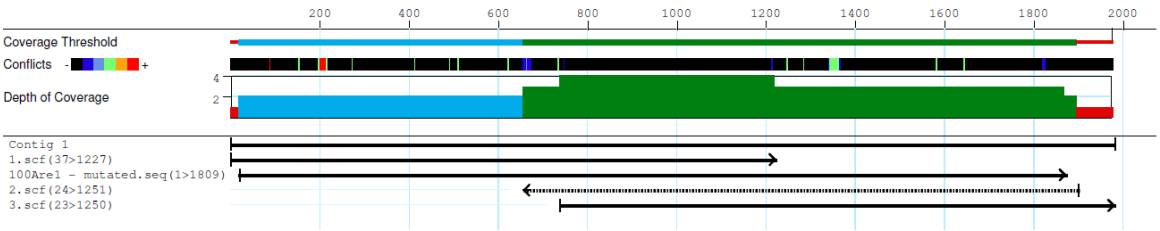

Figure 27: Sequencing result for Are1 (*S. cerevisiae*) cloned into vector pYEX4T-1; the conflicts show site-directed mutation and a deletion at bp 200 that occurred during cloning by Corinna Odar.

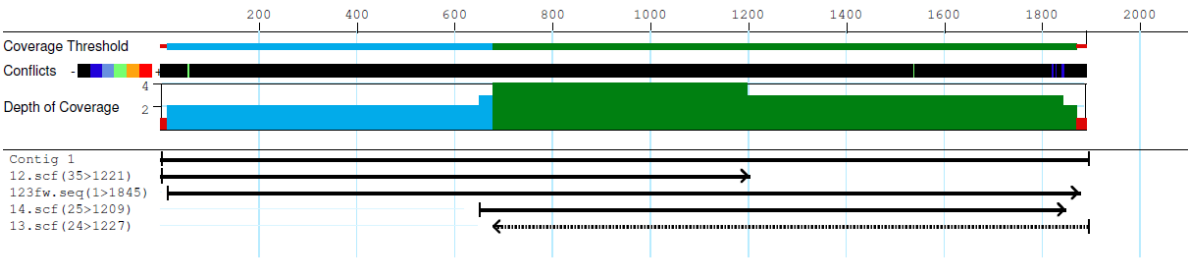

Figure 28: Sequencing result for 123 (*C. albicans*) cloned into vector pYEX4T-1; the green lines at the left end and at approximately base number 1530 show two mutations where thymine was changed to cytosine both times

The pYEX4T-1 constructs have an inducible *CUP1* promoter, so the microsomal preparation also had to be modified, to respond to these new constructs. During cultivation, 0.4 mL of 0.5 M $CuSO_4$ was added after 4 h of growth to a concentration of 0.5 mM $CuSO_4$ to express the acyltransferases. Then, the cells were induced for further 16 h and the microsomal preparation was carried out as described in the methods section (chapter 8.10).

A Western Blot was also carried out to confirm expression of the acyltransferases. (Figure 29)

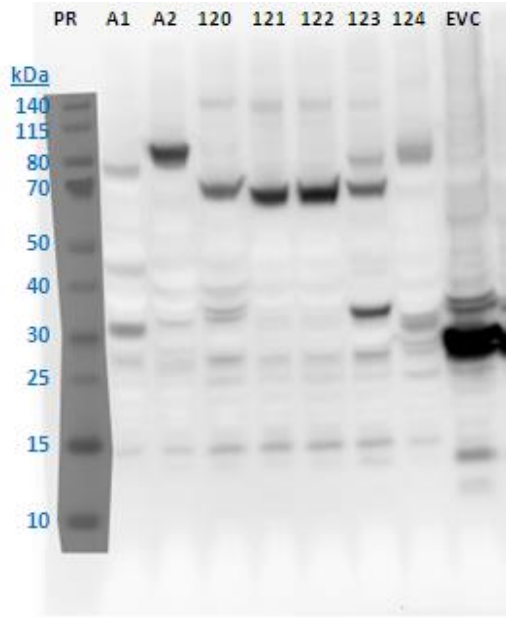

Figure 29: Western Blot kindly provided by BSc Holly Stolterfoht; A1: *S. cerevisiae* (*ARE*1), A2: *S. cerevisiae* (*ARE*2), 120: *P. troglodytes* (ACAT1), 121: *R. norvegicus* (ACAT2), 122: *R. norvegicus* (ACAT1), 123: *C. albicans* (*ARE2*), 124: *T. gondii* (ACAT1) EVC: empty vector (GST-Tag only); GST-tagged acyltransferase visualized by chemiluminescence; expected protein sizes see Table 11

9.3.Radioassay

9.3.a. Overview

The radioassay was set up with nearly the same components as the microsomal assay. Microsomal fractions of the *S. cerevisiae Δare1Δare2* double knockout strain background were mixed with a mixture of the sterol of interest and tyloxapol as a detergent. The reaction mix was then pre-incubated at 30°C for approximately 3 min and the assay was started by the addition of a mixture of non-labelled oleoyl-CoA with ^{14}C-labelled oleoyl-CoA (Figure 30).

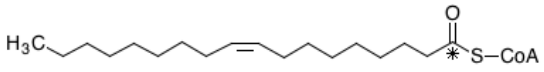

Figure 30: Radiolabelled 1-^{14}C-cis-9-Octadecenoyl coenzyme A (oleoyl-CoA);

After a certain incubation time – for the final assays 2 min - a lipid extraction modification of the method by Folch et al. (1957), was carried out. The lipids were dissolved in $CHCl_3$: MeOH (2:1, v/v) and separated on a TLC-plate. Respective bands were stained with iodine (Figure 31), scraped off the TLC, put into a scintillation cocktail and radioactivity was quantified in a scintillation counter. (overview see Figure 32)

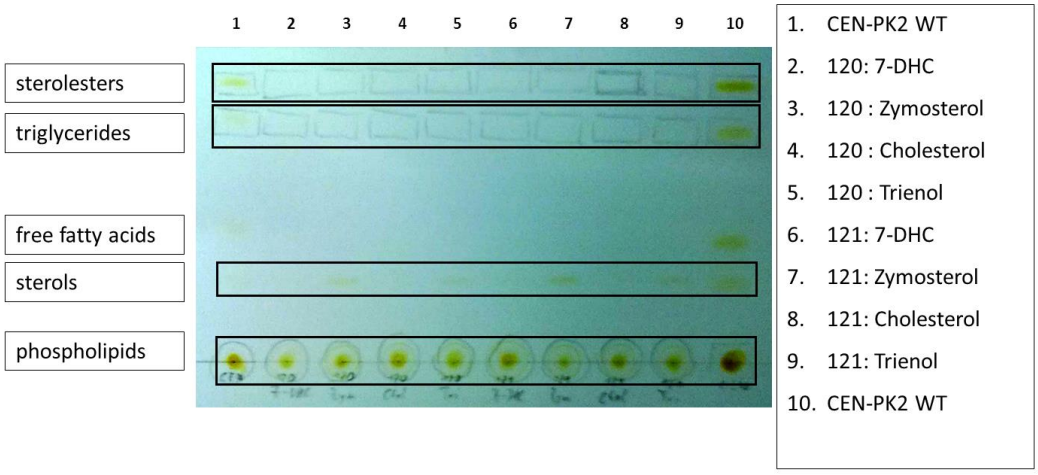

Figure 31: TLC of lipids from radioassay and lipid extraction; bands were stained with iodine (approximately 8 min); 7-DHC: 7-dehydrocholesterol, Trienol: Cholesta-5,7,24-trienol

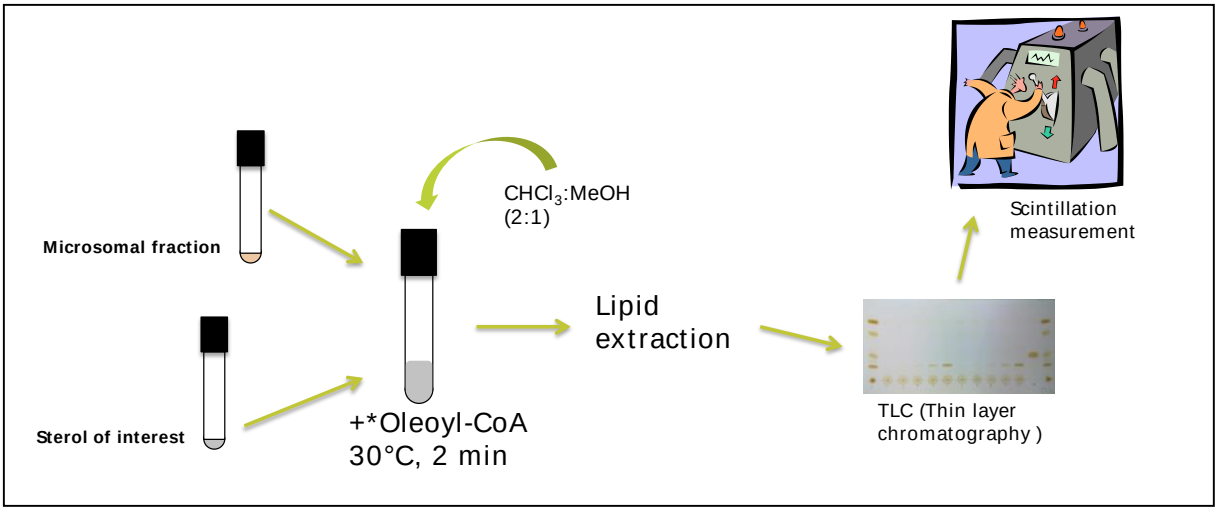

Figure 32: Overview of the radioassay steps

Since the counting results with the same acyltransferase fluctuated between measurements and the sum of all counts also differed, a strategy to make the measurements more comparable was developed. So for the later assays, the same amount of the lipid extractions was counted without TLC-separation and the measured counts were taken as 100 percent.

9.3.b. Pre-tests

First assay results (Figure 33) showed that the only acyltransferases that showed any ester formation were the Are2 proteins of *S. cerevisiae, C. albicans* and *T. gondii*. Therefore, it was decided for the microsomes of the non-active acyltransferases, to be tested in a smaller scale (with only one substrate) before testing them with more substrates.

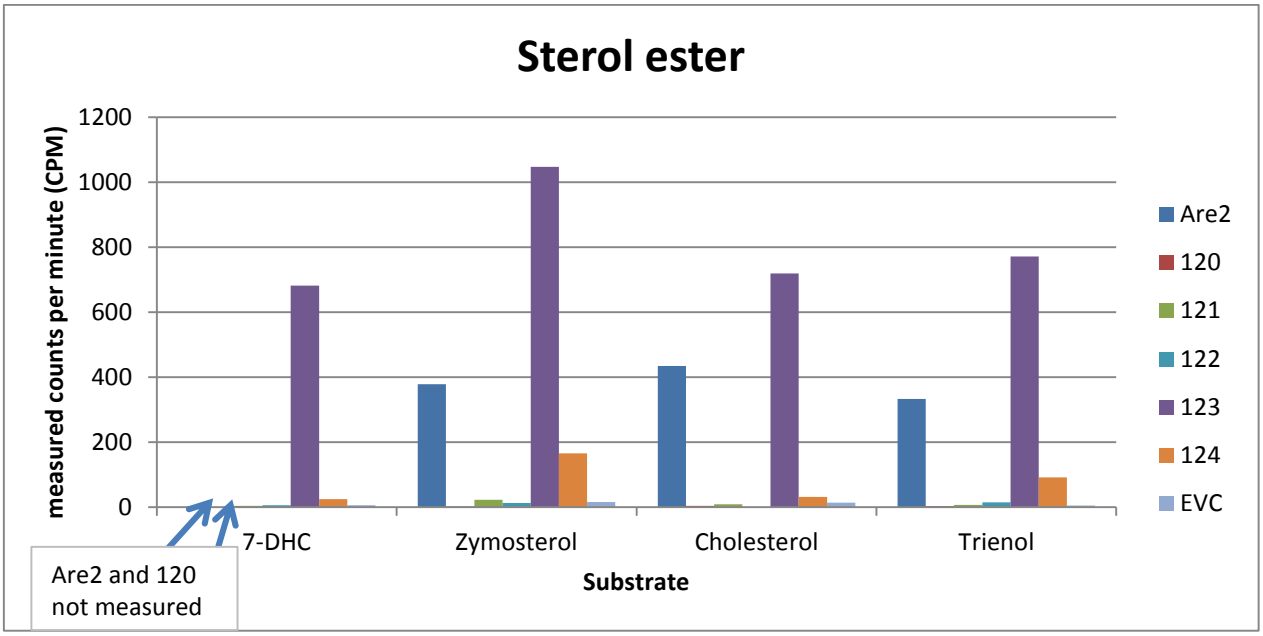

Figure 33: First measurements with the radioassay: 7-DHC: 7-dehydrocholesterol; Are2: *S. cerevisiae* (*ARE2*), 120: *P. troglodytes* (ACAT1), 121: *R. norvegicus* (ACAT2), 122: *R. norvegicus* (ACAT1), 123: *C. albicans* (*ARE2*), 124: *T. gondii* (ACAT1); for 7-dehydrocholesterol as a substrate Are 2 and 120 were not measured

In these first measurements, only the lipids scraped from the TLC were measured in the scintillation counter. It was observed, that the sum of the counts (i.e. sterol esters, triglycerides and phospholipids together) differed between assays. To describe this effect, a triple measurement was carried out and results were compared (Figure 34). The measured counts showed only moderate reproducibility, whereas when they were related to counts of total lipid extracts reproducible results were obtained (Figure 35). A reason for the difference in measured counts could be the different lipid extraction efficiency.

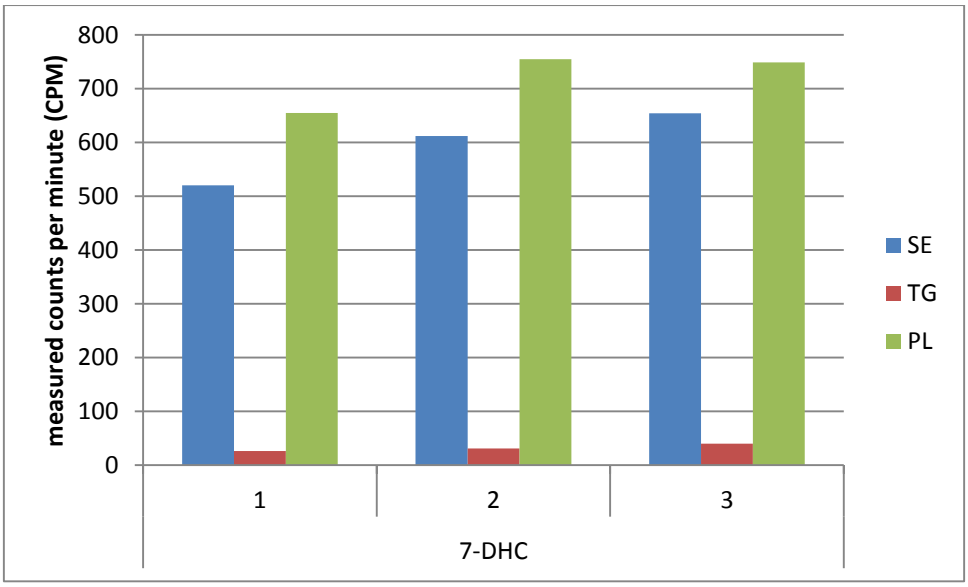

Figure 34: Triplicate of single measurements with 123 (*C. albicans*) and 7-dehydrocholesterol as a substrate. The experiments showed only moderate reproducibility; SE: sterol esters, TG: triglycerides, PL: phospholipids

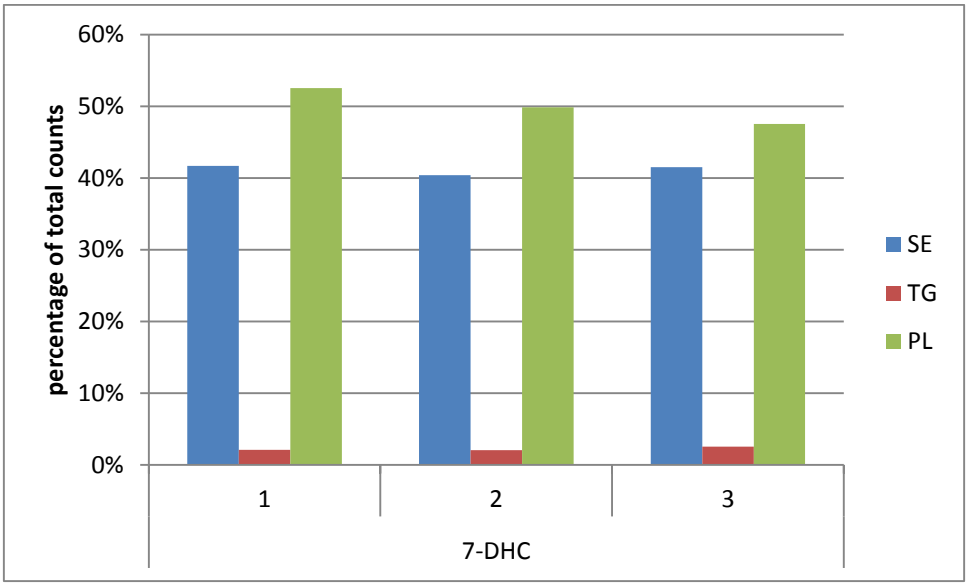

Figure 35: Triplicate of single measurements with 123 (*C. albicans*) and 7-dehydrocholesterol as a substrate. Measured counts are shown in relation to the measured total counts as 100 %; SE: sterol esters, TG: triglycerides, PL: phospholipids

Measuring 'total counts', i.e. counts of total lipid extracts, and relating the counts of specific lipid bands to these total counts, also led to other observations when comparing test assay results with different protein amounts.

Figure 36 shows the measured counts without any relation to total counts. It shows that some radioactivity was found in the supposed phospholipid spot, even without any microsomal material added! This means that at least some part of the *oleoyl-CoA was extracted and applied on the TLC plate and measured in the phospholipid fraction. The figure also shows that the measured counts for all lipids increase with increasing protein amount, but counts for sterol esters increased at a much higher rate. This indicates, that the microsomal material has some influence on how much radioactivity is found in the different lipid spots, and that there is a different correlation to amount of the microsomal material concerning the different lipid classes.

This can be seen better, when the counts are related to the total counts (Figure 37). It is shown that the percentage of counts for phospholipids, triglycerids and oleate stay nearly on the same level or even decrease slightly with increasing protein amount.

The relative counts of the sterol esters on the other hand increase with protein amount at a much higher rate than the sum of the relative counts of the other lipids decrease.

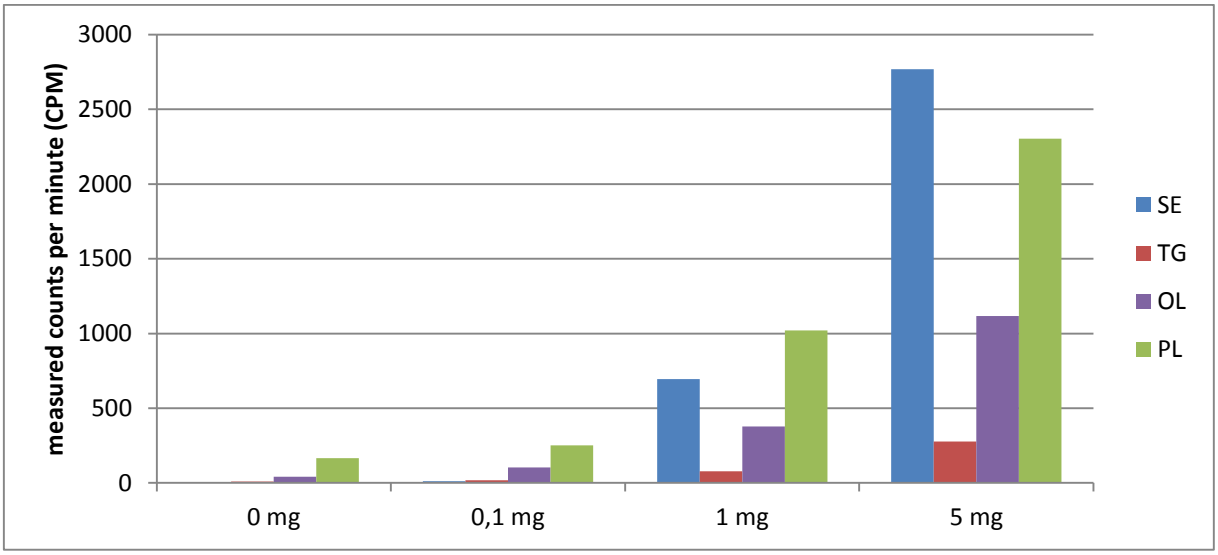

Figure 36: Test assay with 7-DHC as a substrate and the acyltransferase 123 (*C. albicans*) for different protein amounts; single measurements; SE: sterol esters, TG: triglycerides, OL: oleate, PL: phospholipids.

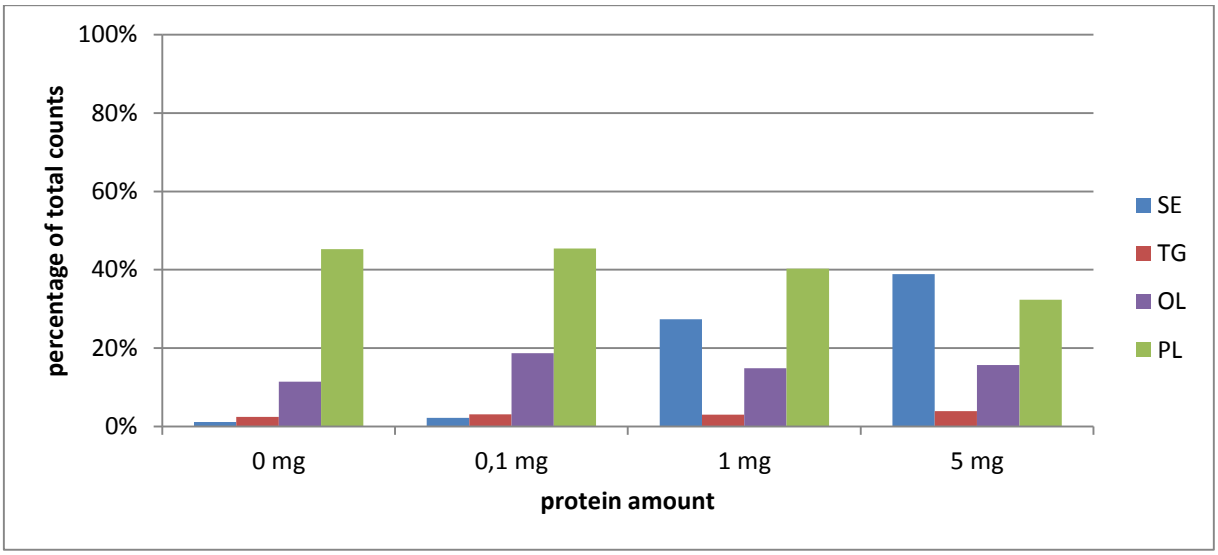

Figure 37: Test assay with 7-DHC as a substrate and the acyltransferase 123 (*C. albicans*) for different protein amounts; single measurements; counts are given as a percentage to total counts in lipid extracts; SE: sterol esters, TG: triglycerides, OL: oleate, PL: phospholipids.

9.3.c. Setting the various steps of the assay

The first test assays showed that the assay had to be adjusted regarding sterol amount, protein amount and assay time to a reasonable experimental window. All the variations were carried out with acyltransferase 123 (*C. albicans*) and 7-DHC as a substrate.

The amount of *oleoyl-CoA was fixed at 10.2 nmol per assay, because that had led to a rather well detectable signal in the first experiments. The *oleoyl-CoA was also measured directly, which led to approximately 197,000 counts per minute. The total counts in the different experiments ranged from

3,000 to 7,000 counts per minute for 10-fold diluted extracts. This means that there were 30,000 – 70,000 counts per minute in the lipid extracts, so 70-85 % of the *oleoyl-CoA not ending up in the lipid fraction. This could have happened due to side reactions, e.g. hydrolysis of the thioester bond, that could eliminate the radioactively labeled molecule from the lipid phase or due to losses during the lipid extraction steps.

- ***Sterol amount:***

To determine the amount of exogenously added sterol to achieve saturation with the sterol of interest, and to determine how much other sterol was present in the microsomal preparation, measurements with different exogenously added sterol concentrations were carried out: Test assays with 0-, 0.2-, 1-, 5- and 10-fold excess of exogenous sterol over the amount of total *oleoyl-CoA were carried out (Figure 38 and Figure 39).

The results for the 0- and 0.2-fold excess indicated that – as to be expected - there were some sterols present in the microsomal fraction which were employed for the esterification reaction. First estimates suggested around 10-20 µg sterols per mg protein in the microsomal fraction. GC-MS measurements, later performed by Barbara Petschacher, showed that there was only 1.4 µg sterol, mainly ergosterol, per mg protein in the microsomal preparation.

The results for the higher sterol concentrations indicate that the saturation was nearly reached at 10-fold excess, because doubling the sterol amount from 5- to 10-fold excess increased production of sterol esters by only 10 %. So a 10-fold excess was used in the later assays.

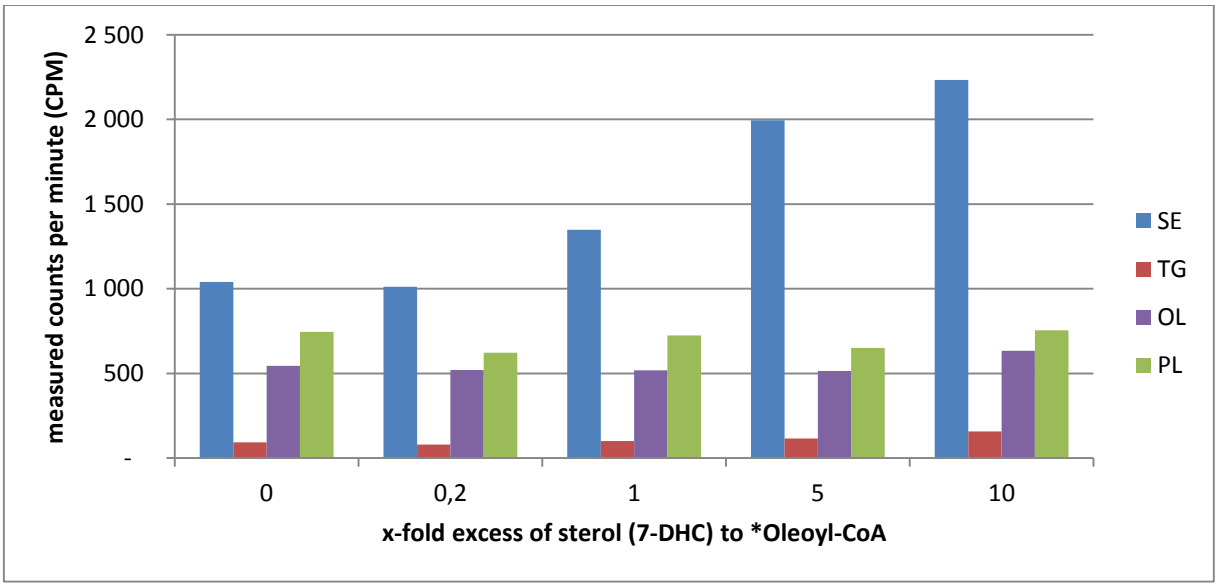

Figure 38: Test assay with 123 (*C. albicans)* and 7-dehydrocholesterol for different sterol amounts; results show the mean value of two single measurements; SE: sterol esters, TG: triglycerides, OL: oleate, PL: phospholipids

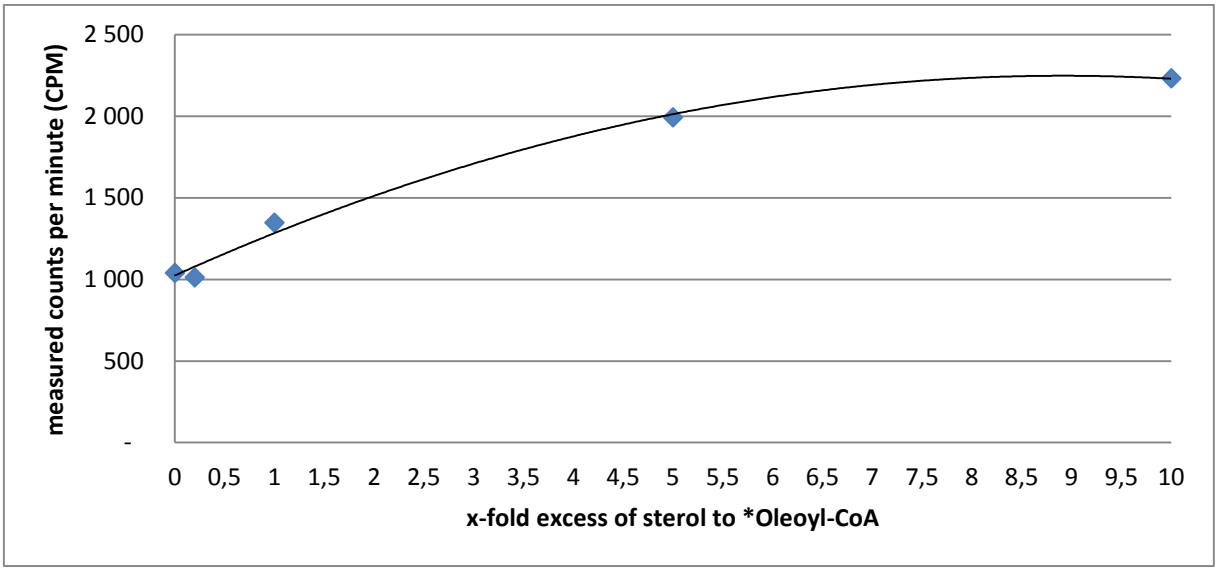

Figure 39: Test assay with 123 (*C. albicans)* for different sterol concentrations of 7-DHC to show, when saturation is reached; results show the mean value of two single measurements; only counts of the sterol esters are shown;

- ***Protein amount***

The assay was tested for different protein amounts: 0, 0.1, 1 and 5 mg total microsomal protein were taken and radioassays with 7-DHC at 10-fold excess were carried out (Figure 40).

Results indicate that for increasing protein amounts the relative counts of phospholipids decrease and sterol esters increase, while relative counts of triglycerides and oleate remain nearly constant.

For the final assays, only 0.5 mg of microsomal protein was used, because microsomal material was limited. The microsomal preparations had total protein concentrations ranging from 1.5 to 65.9 µg/µL. Most preparations had a concentration between 8 and 20 µg/µL.

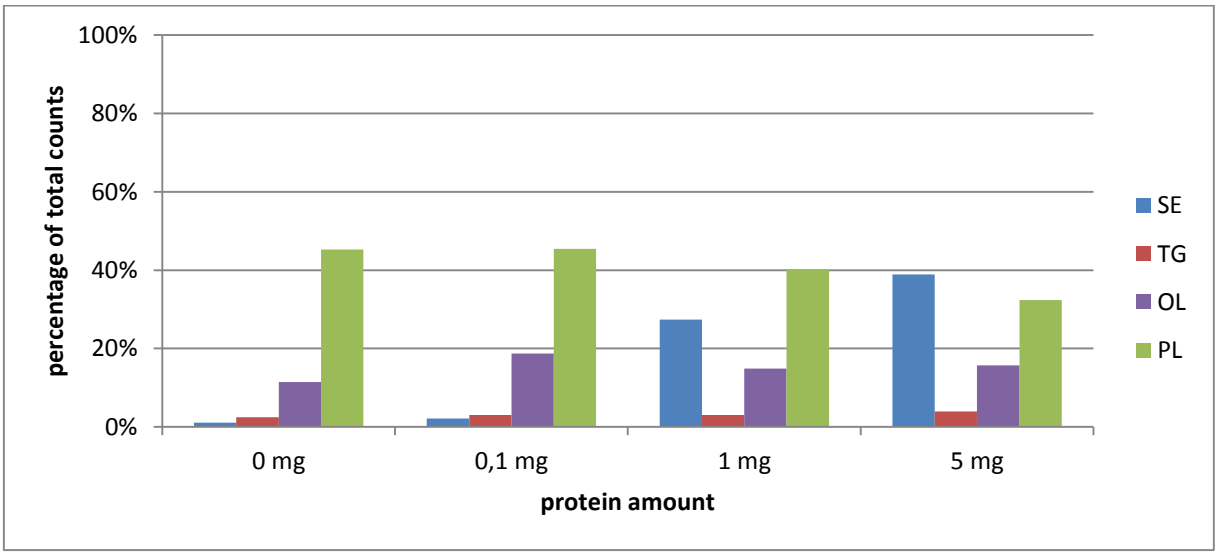

Figure 40: results of the test assay with 123 (*C. albicans*) for different protein amounts; SE: sterol esters, TG: triglycerides, OL: oleate, PL: phospholipids

- ***Assay time***

In the microsomal assay there was no visible change for the ester formation after 4, 15 or 60 min, so the test assays were stopped at time points below 15 min. The first radioassays optimised for sterol amount and protein amount were carried out with 4 min incubation time and delivered significant labeling of sterol esters. Nevertheless, several time points were tested to determine a time point where *oleoyl-CoA was not depleted (Figure 41). The graph suggests that the reaction was near-linear between 0 and 2 min. It also indicates that the reaction might reach a plateau after 10 min.

For the final radioassays, the reaction was stopped after 2 min, because it was estimated that at this timepoint there was still *oleoyl-CoA available for the reaction. On the other hand, turnover, even for the lesser active acyltransferases should be detectable.

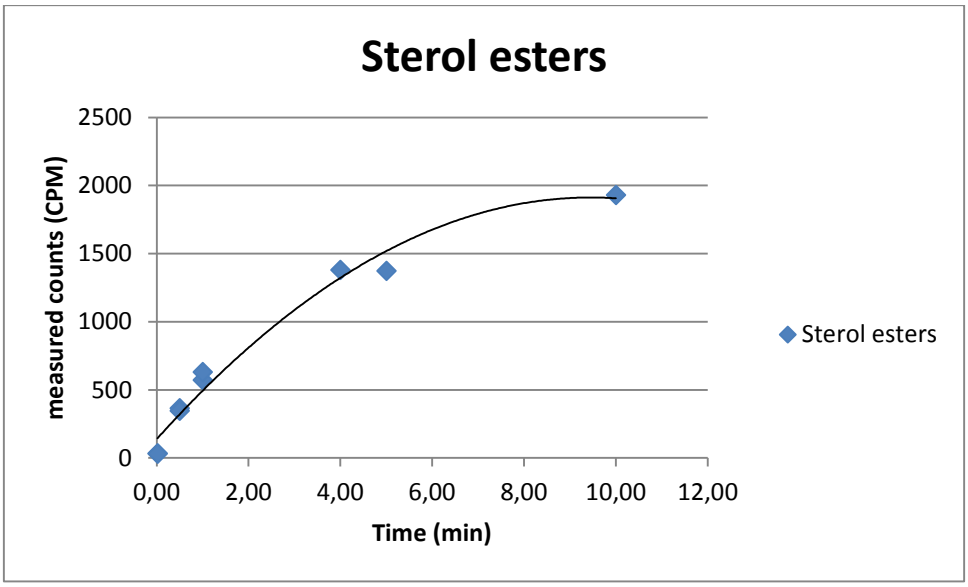

Figure 41: Time-resolved sterol ester formation; two single measurements of 123 (*C. albicans*) with 7-DHC as a substrate.

9.3.d. The final radioassay

Using the different homogenates harboring heterologous acyltransferases, an enzymatic assay with *oleoyl-CoA was carried out, to find out whether there was substrate-specificity for 7-dehydrocholesterol over zymosterol. 102.4 nmol of each sterol was prepared as a mixture with tyloxapol in a 25-fold excess as detergent and dissolved in 50 mM KH_2PO_4, pH 7.4.

8.5 nmol oleoyl-CoA - as the second substrate - was mixed with 1.7 nmol ^{14}C-labelled *oleoyl-CoA. At first, the sterol-tyloxapol-mixture with a 10-fold excess in sterol molarity compared to oleoyl-CoA was mixed with 500 µg of heavy microsomal fraction protein. The mixture was pre-incubated for 3 min at 30°C and the assay was started by adding *oleoyl-CoA. The reaction was stopped after 2 min by adding

4 ml of $CHCl_3$:MeOH (2:1, v/v). Then, a lipid extraction with a modified Folch extraction method was carried out. The lipids were dissolved in 100 µl $CHCl_3$:MeOH (2:1, v/v) and 10 µl thereof were separated on a TLC-plate, which was stained with iodine. Respective bands were scraped off the TLC and radioactivity was measured by scintillation counting. Another 10 µl of dissolved lipids were counted without TLC-separation.

The results indicate, that there was no enhanced specificity for 7-dehydrocholesterol for none of the heterologous acyltransferases, most of them prefered zymosterol over 7-dehydrocholesterol (Figure 42 and Table 12). The degree of zymosterol preference varied, for instance Are1p (*S. cerevisiae*) esterified zymosterol 15 % more effectively than 7-DHC, whereas ACAT1 from *T. gondii* esterified zymosterol 7-fold more effectively than 7-DHC.

Furthermore the results clearly showed, that the heterologously expressed Are2p from *C. albicans* was the most active enzyme.

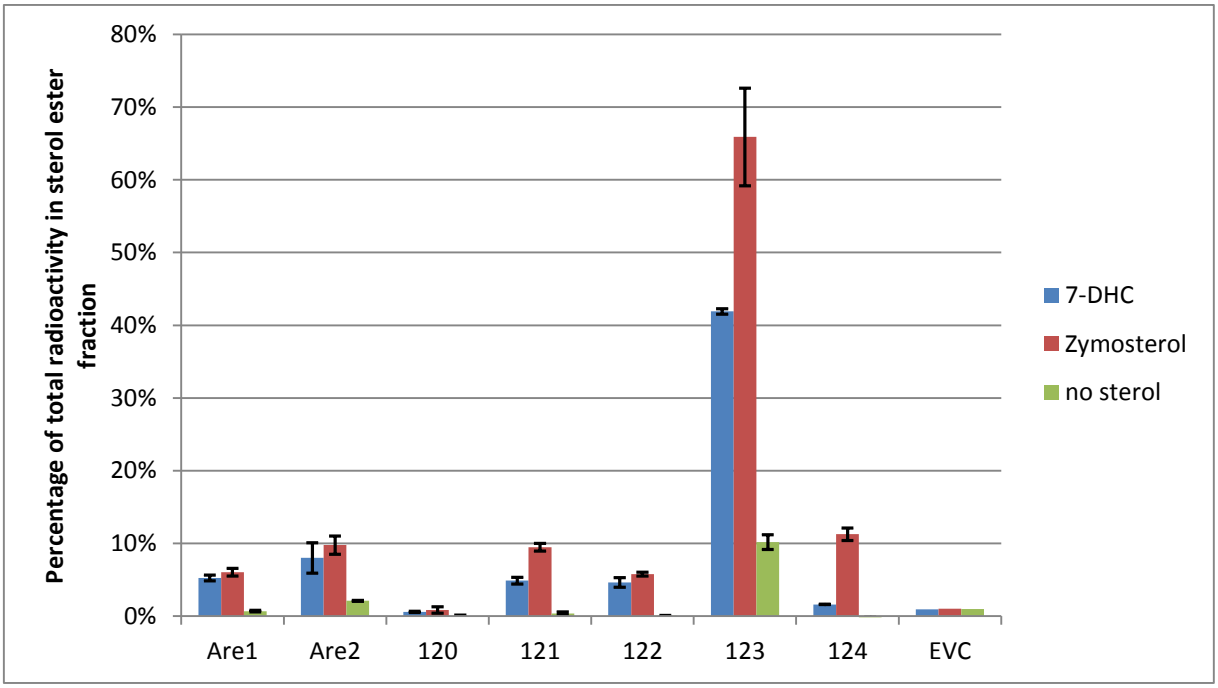

Figure 42: Final results of the radioassay: the y-axis gives the percentage of total radioactive compounds in a lane found in the ester band; two independent measurements were made in parallel; 7-DHC: 7-dehydrocholesterol, no sterol: assay without added sterol; Are1: *S. cerevisiae*, Are2: *S. cerevisiae*, 120: *P. troglodytes* (ACAT1), *121*: *R. norvegicus* (ACAT2), *122*: *R. norvegicus* (ACAT1), *123*: *C. albicans* (*ARE2*), *124*: *T. gondii* (ACAT1);

Table 12: Final results of the radioassay; percent radioactivity in sterol ester fraction of lipid extract; n.d.: not detectable (<0.1 %)

Organism	Acyltransferase	Abbreviations	Sterol	Sterolester formation (mean)
Saccharomyces cerevisiae	*Sc ARE*1	Are1	7-dehydrocholesterol	5.3 %
			zymosterol	6.0 %
			no sterol added	0.7 %
Saccharomyces cerevisiae	*Sc ARE2*	Are 2	7-dehydrocholesterol	8.0 %
			zymosterol	9.8 %
			no sterol added	2.1 %
Pan troglodytes	*Pt* ACAT1_opt*Sc*1	120	7-dehydrocholesterol	0.6 %
			zymosterol	0.8 %
			no sterol added	n.d.
Rattus norvegicus	*Rn* ACAT2_opt*Sc*1	121	7-dehydrocholesterol	4.9 %
			zymosterol	9.5 %
			no sterol added	0.4 %
Rattus norvegicus	*Rn* ACAT1_opt*Sc*1	122	7-dehydrocholesterol	4.6 %
			zymosterol	5.8 %
			no sterol added	0.0 %
Candida albicans	*Ca ARE*2_opt*Sc*1	123	7-dehydrocholesterol	41.9 %
			zymosterol	65.9 %
			no sterol added	10.2 %
Toxoplasma gondii	*Tg* ACAT1_opt*Sc*1	124	7-dehydrocholesterol	1.6 %
			zymosterol	11.3 %
			no sterol added	n.d.
	empty vector control	EVC	7-dehydrocholesterol	0.9 %
			zymosterol	1.0 %
			no sterol added	1.0 %

10. Discussion

10.1. Radioassay

The implemented radioassay was established as a feasible tool for the determination of acyltransferase activity and substrate specificity concerning the two sterols 7-dehydrocholesterol and zymosterol.

The test assays for setting up the radioassay had not given enough reproducible and coherent results for the detection of sterol specificity. The procedure of determining the different assay conditions was very time consuming. It was shown, that tuning various parameters as substrate amount, protein amount and assay time were essential to get results that allowed conclusions on sterol specificity.

Since it is suggested that the *in vitro* assay works as an assay with mixed micelles, which are formed right before the assay, there are some parameters that cannot be excluded by the assay setup. The different sterols could for instance have an influence on transfer rates, the microsomal preparations could differ in their composition and that could lead to differences in structure and/or size of those micelles. This could also have an influence on accessibility and distribution of different sterols, as sterols must be integrated into the microsomal membranes/micelles.

Compared to the situation *in vivo*, where lipid droplets/particles are formed and transformed permanently, mixed micelles in the *in vitro* assay are formed at once and should not change a lot during the assay. The detergent tyloxapol should enhance the accessibility of all different sterols and therefore should lead to equal assay conditions. Nonetheless, it cannot be excluded, that different sterols could have an effect on the formation of the mixed micelles and that the activity of the homologous and heterologous acyltransferases could also be affected by that.

10.2. ASAT/ACAT activity

From all heterologous acyltransferases, Are2p from *C. albicans* (123) was the most active enzyme, even for different microsomal preparations. An explanation could lie in the amino acid sequence. The FYxDWWN motif that is supposed to be the binding site for the oleoyl-CoA (Guo et al., 2001) is different in the *Candida* Are2p, as it is a FYxPWWS motif (Figure 45). On the one hand this contradicts the statement in the paper of Guo et al. (2001), that all yeast acyltransferases have this common motif. On the other hand it indicates, that this change from aspartic acid to proline (D to P) and from asparagine to serine (N to S) could influence the oleoyl-CoA binding of the enzyme. This change in a motif that is conserved for many other acyltransferases might make a difference in enzyme folding and particularly in the activity of this site. Thus, since the oleoyl-CoA was added in an aqueous solution to start the assay, it must have been taken up into the artificial membrane, built up from tyloxapol, sterols

and the ER components from the microsomal preparation. The higher turnover could be explained by an advantage for the *Candida* Are2p in the uptake of oleoyl-CoA. This could for instance be shown via carrying out some radioassays with different point mutations for the *C. albicans* Are2p.

Other explanations for the high activity of the *C. albicans* Are2p could be small mutations at two sites (both times thymine -> cytosine) as described in chapter 9.2.b. This could have also had an influence on the activity by altering the enzyme folding which could have led to higher affinity for oleoyl-CoA or the different sterols.

Another explanation could be that the expression rate of *C. albicans* Are2p was higher due to codon optimization, when compared to the *S. cerevisiae* Are1p/Are2p.

10.3. Sterol specificity

When comparing the esterification rate of the two substrates, results of the radioassay indicate, that ACAT1 from *T. gondii* showed a huge preference for zymosterol over 7-dehydrocholesterol which was nearly not esterified. Nishikawa et al. (2005) showed that ACAT1 from *T. gondii* esterified 7-dehydrocholesterol at approximately 65 percent compared to cholesterol when expressed in mammalian cells. So the question was, why 7-dehydrocholesterol was not esterified at a higher rate in our experiments. As Nishikawa et al. (2005) compared 7-dehydrocholesterol to cholesterol and we compared 7-dehydrocholesterol to zymosterol, further testing would be essential to find out, if those two results actually contradict each other.

ACAT2 from *R. norvegicus* and Are2p from *C. albicans* also showed a clear preference for zymosterol, whereas Are1p and Are2p from *S. cerevisiae* and ACAT1 from *R. norvegicus* only showed a slight preference for zymosterol. The *P. troglodytes* ACAT1 was nearly inactive, thus preventing any speculations on sterol specificity of this enzyme.

The chemical structure of 7-DHC differs from zymosterol only in 2 positions: the lack of the C24 double bond and the shift of the conjugated double bond from C8 to C7 (Figure 43).

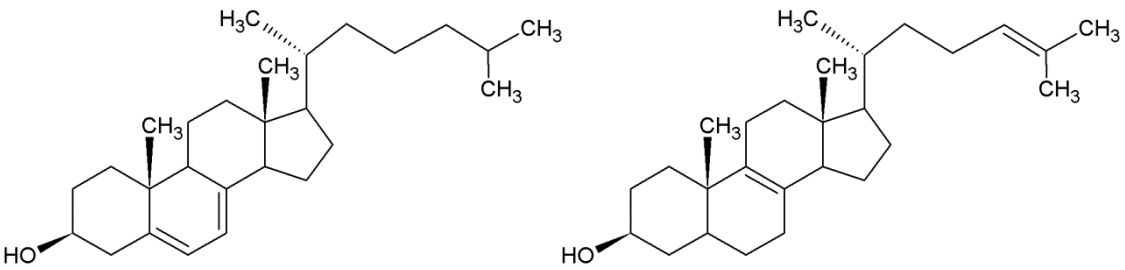

Figure 43: structures of 7-dehydrocholesterol (left) and zymosterol (right)

This structural difference is most likely responsible for the observed substrate specificity of some of the sterol acyltransferases. Further research, to pinpoint the enzymes' active sites, should make this point clearer. Concerning the side chain, Tavani et al. (1982) described an effect for ACAT from rat as follows: "The enzyme [ACAT] is very sensitive toward even minor changes of the structure of the side chain of a sterol […]." The electric field induced by the C24 double bond for zymosterol should have an influence on the bending of the side chain or could interact in a different way with the surrounding of the molecule (Tavani et al., 1982).

A 3D structure of the sterols also shows a difference for the polycyclic structure where the double bond at C8 seems to make a difference: the cyclopentane ring is directing out of plane for 7-dehydrocholesterol whereas for zymosterol it lies nearly in plane with the cylcohexane ring aside. Therefore, also the side chains point in different direction when comparing the two sterols (Figure 44).

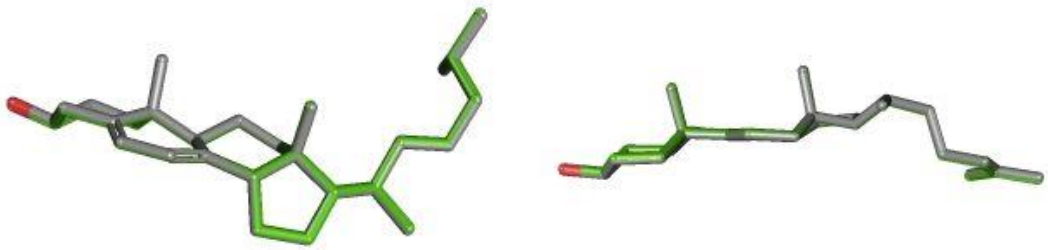

Figure 44: 3D structure of 7-dehydrocholesterol (left) and zymosterol (right)

The results showed that there was none of the tested homologous and heterologous acyltransferases that clearly prefered 7-dehydrocholesterol over zymosterol in vitro. Most of the acyltransferases prefered zymosterol as a substrate.

11. Indices

11.1. References

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11.2. Internet ressources:

http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&term=Acetyl-CoA+C-Acetyltransferase

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http://coli.usal.es/web/abydl/biblioteca/bibelectro.alu/documentos/protocolos/Yeast_protocols_Clontech.pdf

http://www.ebi.ac.uk/Tools/msa/clustalw2/

http://coli.usal.es/web/abydl/biblioteca/bibelectro.alu/documentos/protocolos/Yeast_protocols_Clontech.pdf

11.3. List of figures

11.4. List of tables

12. Appendix

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Are1            --------------------------------MTETKDLLQDEEFLKIRRLNSAEANKRHS 29
Are2            --------------------------------MDKNKDLLENEQFLRIQKLNASDAGKRQS 29
120             -------------------------------------MVGEEKMSLRNRLSKSRENPEED 23
121             -------------------------------------MEPKAPQLRRRERQGEEQENGA 22
122             -------------------------------------MVG-EETSLRNRLSRSAENPEQD 22
123             -------------------------------------MGRTNTSDQLNAISDKNTKRKS 22
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Are2            ILVDNEDELYGLTSSNNSCASEHEGEGEGEDERPATTSSAPTQNHSAGDVAFIPGKTAEE 89
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122             EAQKN----------------------LLDTHRNGHITMKQLIAKKRQLAAEAEELKPLF 60
123             LALDN--------------------EYHNNSSSEDDSSKIELSYTIPDNNNIISQETTTS 62
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Are2            DTETVTKVVESDDQVFRTHVQTLSSKGKSRY-RKGSSNFISFFDDMAFENRPSILDGSVN 148
120             MKEVGSHFDDFVTNLIEKSAS-------------------LDNGGCALTTFSVLEGEKNN 106
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122             LKEVGCHFDDFVTNLIDKSAS-------------------LDNGGCALTTFSILEEMKNN 101
123             VEDVLSLSSAPQNELRLRKQK-------------------SNNQDSPVDLNGVIVDVSKR 103
124             LPHQQGAEPRVSLEATMCSPSP-EKTGPPCSPCPESCGKQEGNNTDPCTVSSPCESRGSR 179
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Are2            DPFKTKFVGPTLEKEIRKREKE--------LMAMRKNLHHGKPAPDADAADAPALTTTTT 200
120             HRAKDLRAPPEQGKIFIA------------------------------------------- 124
121             STRKTQELHPGKRKVFIT------------------------------------------- 104
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120             ---------RRSLLDELLEVDHIRTIYHMFIALLILFILSTLVVDYIDEGRLVLEFSLLS 175
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122             ---------RRSLLDELFEVDHIRTIYHMFIALLIIFILSTLVVDYIDEGRLVLEFSLLA 170
123             NDITFKAKSSTIFESDEFYKTDFFGMYVLFWLATAFAMVNNLIHTYFENSTPILQWTVVK 189
124             DGKTFPSKLDFFDANSDLARSDFRGVAVLLFIAAIFYLVANPILRWYD-SKEFVDPSLAR 298
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Are2            FMTTNLSTVALIDLIMYLSTFFVVGIQYLCKWGVLNWSSTGWAFTSIYELLFVGFYMYLT 320
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Are1            -VYYFDFNWVTRIFLFLHSVVFVMKSHSFAFYNGYLWDIKQELEYSSKQLQKYK------ 336
Are2            -ENILKLHWLSKIFLFLHSLVLLMKMHSFAFYNGYLWGIKQELQFSKSALAKYK------ 373
120             GFGPTYVVLAYTLPPASRFIIIFEQIRFVMKAHSFVRENVPRVLNSAKEKSSTVPIPTVN 295
121             CVLPVHVSVKHELPPASRCVLVFEQVRFLMKSYSFLRETVPGIFCVRGGKG--ICTPSFS 271
122             GFIPTYVVLAYTLPPASRFILILEQIRLVMKAHSYVRENVPRVLSAAKEKSSTVPVPTVN 290
123             SEYCLDFPWIAKVFLVLHSLVFIMKMHSYAFYNGYLWSIYKEGLYSEKYLDKLTNGKVTL 309
124             SCLYNAWPIIPAAFVQMIAVVQFMKMHSYSSTNMNFCDDMRQGKQTLGYPENVTLR---- 411
                                  :: . : :        .
```

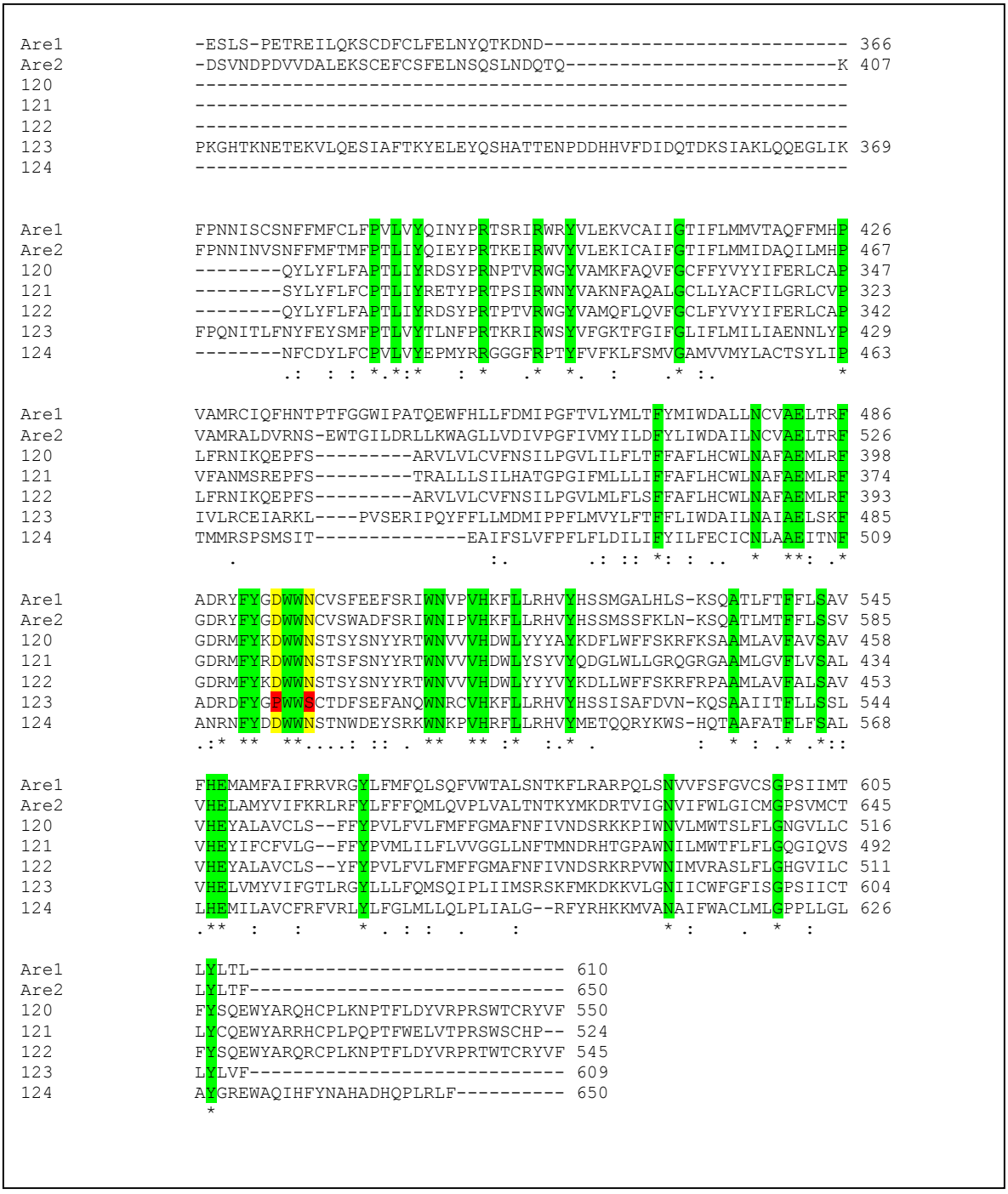

Figure 45: Sequence alignment made with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/); Are1: *S. cerevisiae* , Are2: *S. cerevisiae* , 120: *P. troglodytes*, 121: *R. norvegicus* (ACAT2), 122: *R. norvegicus* (ACAT1), 123: *C. albicans* (Are2), 124: *T. gondii* (ACAT1); amino acids in green devote consensus sequences; the yellow amino acids of the putative oleoyl-CoA binding motif show consensus sequence for all acyltransferases except *Candida albicans* (123) that are marked with red