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Heterologous Expression of Sterol-O-acyltransferases in *S. cerevisiae*

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KURZFASSUNG

Saccharomyces cerevisiae, Sterolbiosynthese, Lipidpartikel, Sterol-O-acyltransfersen

Die Biosynthese von Ergosterol in der Hefe *S. cerevisiae* ist weitgehend erforscht. Dies erleichtert die genetische Manipulation der einzelnen Reaktionsschritte und rückt die Sterolbiosynthese in den Blickpunkt zahlreicher wissenschaftlicher und industrieller Interessen.

Sterole sind wesentlicher Bestandteil eukaryotischer Zellmembranen, doch können sie bei zu starker Präsenz die Membranhomöostase stören. Um toxische Effekte zu meiden und gleichzeitig Sterole intrazellulär zu speichern, werden diese verestert und in *S. cerevisiae* in sogenannten Lipidpartikeln akkumuliert.

Die für die Veresterung verantwortlichen Enzyme sind Sterol-O-acyltransferasen, die im endoplasmatischen Retikulum zu finden sind. Es konnte bereits gezeigt werden, dass über die Aktivität der Sterol-O-acyltransferasen in *S. cerevisiae* die Gesamtsterolmenge sowie auch die Sterolmuster beeinflusst werden können.

Inwieweit sich die heterologe Expression von Sterol-O-acyltransferasen auf den Sterolmetabolismus in *S. cerevisiae* auswirkt, ist Gegenstand dieser Forschungsarbeit.

ABSTRACT

Saccharomyces cerevisiae, sterol biosynthesis, lipid particles, sterol-O-acyltransferases

The biosynthesis of ergosterol in the yeast *S. cerevisiae* has been extensively investigated. This facilitates the genetic manipulation of individual reaction steps and puts sterol biosynthesis into the focus of numerous scientific and industrial interests.

Sterols are an essential component of eukaryotic cell membranes, but presence of high amounts can disturb membrane homeostasis. In order to avoid toxic effects and simultaneously facilitate their intracellular storage for periods of depletion, they are esterified and accumulate in *S. cerevisiae* in so-called lipid particles.

The enzymes responsible for esterification, namely sterol-O-acyltransferases, are to be found in the endoplasmic reticulum. It has already been shown that overall sterol content as well as sterol pattern can be influenced by the activity of sterol-0-acyltransferases in *S. cerevisiae*.

To which extent heterologous expression of sterol-0-acyltransferases affects sterol metabolism in *S. cerevisiae* is subject of this Master thesis.

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ABBREVIATIONS

aa	amino acid
ab	antibody
ACAT	acylCoA:cholesteroltransferase
amp	ampicillin
bidest	bidestilled
bp	base pairs
BP	Birgit Ploier
Ca.are2	*ARE2* of *Candida albicans*
CoA	coenzyme A
DC	disruption cassette
DG	diglycerides
DMSO	dimethyl sulfoxid
dNTP	dideoxynucleotid triphosphate
DTT	dithiothreitol
ER	endoplasmic reticulum
ERG	ergosterol related gene
EtOH	ethanol
Fig	figure
FS	free sterols
G418	geneticin 418 sulfate
GC/MS	gas chromatography/ mass spectroscopy
GST	glutathion-S-transferase
HAP	heme activator protein
HAT	heterologes steryl-O-acyltransferase
his	histidin
HMG-CoA	(*3S*)-3-hydroxy-3-methylglutaryl-CoA
Hs.acat1	*ACAT1* of *Homo sapiens*
Incub	incubation
kan	kanamycin
LB	Luria-Bertani Broth (Lennox)
leu	leucin
LiAc	lithium acetate
LV	Leervektor (reference vector without insert but with selective marker)
Mat	mating type
MeOH	methanol
Mut	mutant
No	number
OD	optical density
ON	over night
ONC	over night culture
p	page
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
Pfu	*Pyrococcus furiosus*
PL	phospholipids
Prep gel	preparative agarose gel
Rn.acat1	*ACAT1* of *Rattus norvegicus*

Rn.acat2	*ACAT2* of *Rattus norvegicus*
rpm	revolutions per minute
RT	room temperature
Sc.are2	*ARE2* of *Saccharomyces cerevisiae*
SD	synthetic defined
SDS- PAGE	sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SE	sterylester
T	temperature
TG	triglycerides
Tg.acat1	*ACAT1* of *Toxoplasma gondii*
TLC	thin layer chromatography
Trafo	transformation
Tris	tris(hydroxymethyl)-aminomethan
trp	tryptophan
ura	uracil
WB	Western Blot
WT	wild-type
YPD	yeast extract peptone dextrose
Zym	zymolyase

1 Introduction

1.1 Sterol biosynthesis in *S. cerevisiae*

Sterols are essential components in the eukaryotic cell membrane. Viability of cells is dependent on their presence as they are important for a variety of cell physiologies (Daum *et al.,* 1998).

The main part of sterols is apolar. Just the polarity of the hydroxyl group at the C3-atom gives them the necessary amphiphilic character for their proper incorporation into the membrane. As polarity gives them characteristics of a tenside and excessive sterols therefore may cause toxicity to the membrane, they are esterified for storage as neutral lipids in separated organelles (lipid particles). The end product of sterol biosynthesis in mammalian cells is cholesterol, whereas fungi and yeast produce ergosterol, which differs in structure by two additional double bonds at the C7- and C22-atom and by a methyl group at the C24-atom.

Enzymes of the ergosterol biosynthesis pathway are located either to the endoplasmic reticulum (ER) or to lipid particles (Figure 1).

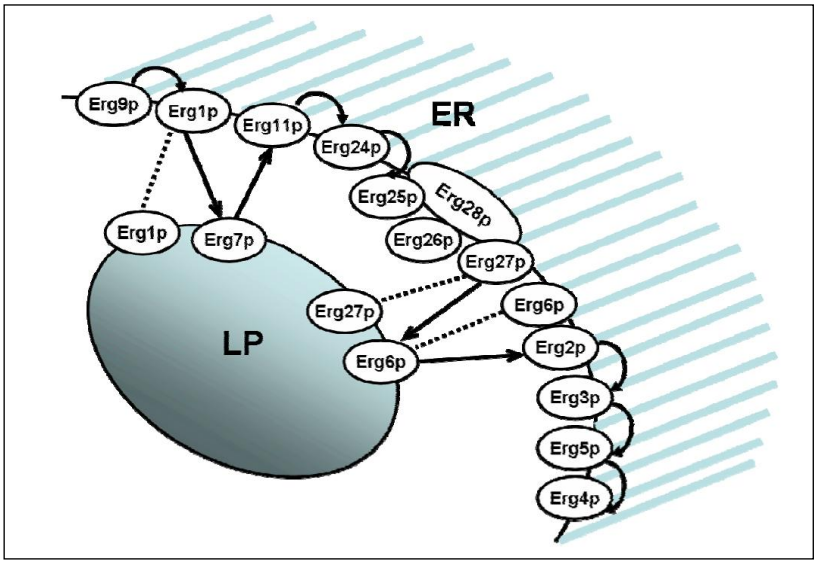


Figure 1: Enzymes of the ergosterol pathway located to the endoplasmic reticulum (ER) or to lipid particles (ER) (Maczek, 2009)

Sterol biosynthesis in eukaryotic cells is complex. Therefore regulation mechanisms are still not completely elucidated. Ergosterol formation can be divided into the pre- and post-squalene pathway.

The pre-squalene pathway is largely independent of oxygen supply. It includes all reactions from glucose to the end product squalene, which is a triterpene consisting of 30 C-atoms (Figure 1).
The reaction of hydroxymethylglutaryl-CoA (HMG-CoA) to mevalonate by the respective HMG-CoA reductases is of high interest, as it is a main regulatory point of ergosterol biosynthesis. The two responsible isoenzymes, namely Hmg1p and Hmg2p, are regulated differently under various conditions. Activity is influenced by sterol end products as well as by starting materials, by oxygen and heme concentrations and by various transcription factors, like the Hap1 (heme activator protein) (Maczek, 2009). *HMG1* is stimulated by heme, whereas expression of *HMG2* is repressed by heme. Moreover, it was shown that heme stimulated transcription of *HMG1* through the action of the transcriptional factor Hap1 (Thorsness *et al.*, 1989). Yeast strains derived from the S288C strain carry a Ty1 insertion mutation in the *HAP1* gene resulting in a decrease of its transcription level. Consequently *HMG1* expression is decreased among other enzymes related to ergosterol biosynthesis pathway. Ergosterol levels were found to be lower in yeast strains with this defect in the *HAP1* gene (Tamura *et al.*, 2004). Hmg1p is a membrane bound protein and over-expression of the whole encoding gene did not significantly change sterol metabolism. However, over-expression of a truncated version of *HMG1*, that lacks its membrane-binding region and is therefore cytosolic, from a constitutive promoter to prevent down-regulation, resulted mainly in the accumulation of squalene beside slight elevation of ergosterol and other sterol components (Polakowski *et al.*, 1997).

In contrast to the pre-squalene pathway, some later reaction steps are dependent on oxygen so that aerobic conditions are obligatory to complete ergosterol biosynthesis (Figure 2).
More than 20 reaction steps are governed by Erg7 to convert squalene epoxide into lanosterol. Reactions to obtain the first compound that shows the typical tetracyclic ring system of sterols are complex and their elucidation was the aim of many investigations.
Sterol biosynthesis leading to zymosterol formation is quite conserved among eukaryotic cells. Mutations of genes responsible for metabolizing zymosterol further are not immediately lethal whereas mutations earlier in ergosterol biosynthesis cause sterol auxotrophy. By mutations of genes responsible for lanosterol formation the cell's capability for sterol uptake is abolished.
Also the deletion of *ERG6*, responsible for the methylation of zymosterol at the C24-atom, is problematic as for example membrane permeability is disturbed, cross-breeding competence diminished and tryptophan uptake is diminished dramatically (Maczek, 2009).

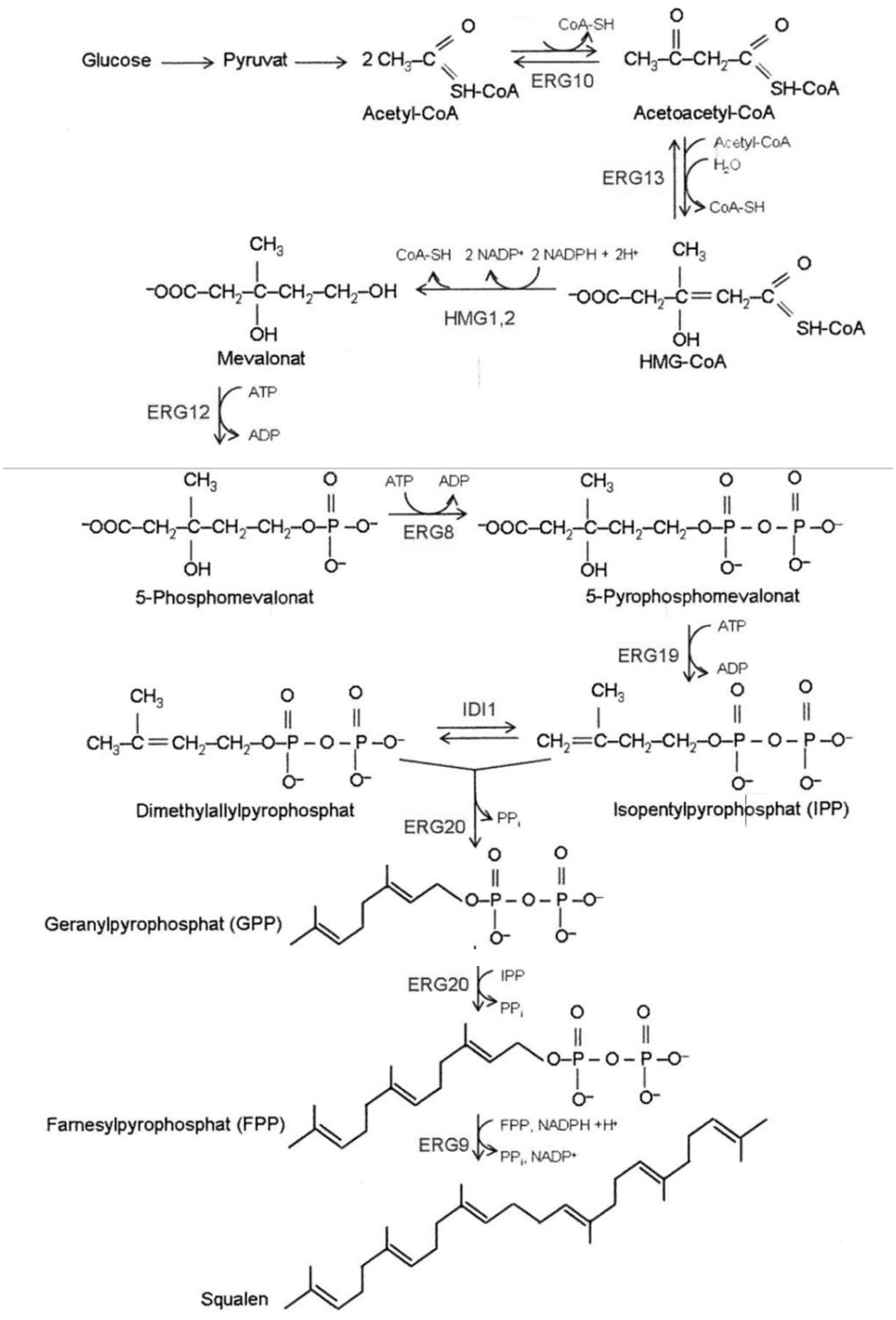


Figure 2: Pre-squalene pathway of ergosterol biosynthesis in yeast S. cerevisiae (Maczek, 2009)

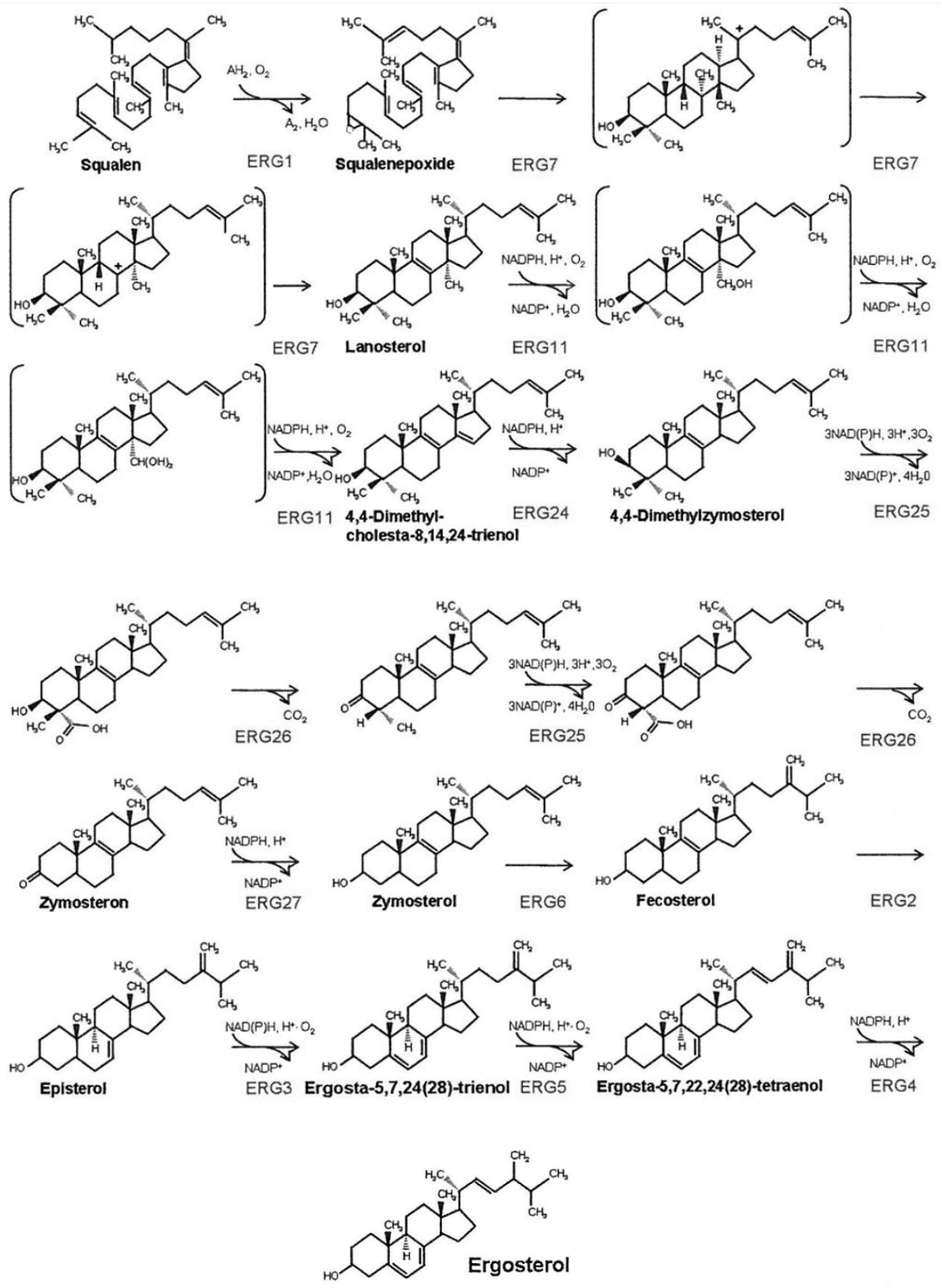


Figure 3: Post-squalene pathway of ergosterol biosynthesis in yeast S. cerevisiae (Maczek, 2009)

1.2 Sterol esterification *S. cerevisiae*

The major subcellular location of free sterols is the plasma membrane (Zinser *et al.,* 1991) whereas steryl esters are extramembranous lipids and stored together with triacylglycerols in so-called lipid particles (Zinser *et al.,* 1993). Esterification occurs at the OH group of the C3-atom with CoenzymA activated fatty acids (Figure 4). The major fatty acid used for steryl ester synthesis in *S. cerevisiae* is C16:1 followed by C18:1 (Zweytick *et al.,* 2000).

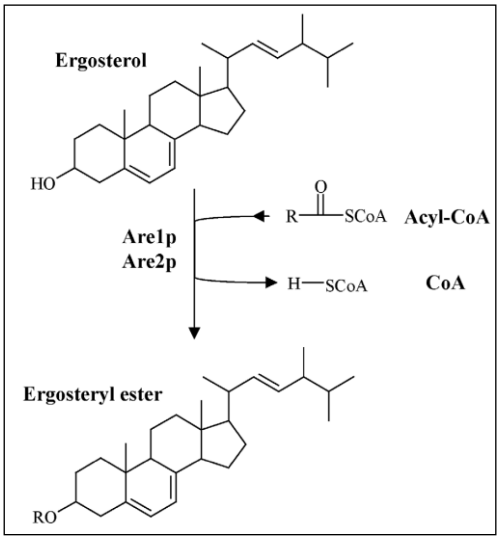


Figure 4: Steryl ester formation in yeast (Müllner and Daum, 2004)

Sterol esters are synthesized under a variety of conditions like in stationary growth phase or during sporulation (Taylor *et al.*, 1978). These phases account for an excess of free sterols as they are devoid of any membrane proliferation. Esterification under sterol excess and the reversible hydrolysis of sterol esters provide a tool to maintain membrane homeostasis and guarantee for sterol availability in times of depletion (Taylor *et al.*, 1978, Leber *et al.*, 1995). Steryl esters are sequestered from lipid particles, if demanded, so that free sterol levels in the plasma membrane stay equal (Leber *et al.*, 1995). Interconversion between free sterols and steryl esters is mediated by steryl ester synthases and sterol ester hydrolases (Zinser *et al.*, 1993).

Steryl esters need to be hydrolized if required for quick response to environmental conditions (Czabany *et al.,* 2007). Three genes were identified to be responsible for this event, namely *YEH2*, *YEH1* and *TGL1* (Köffel, 2006).

Steryl esters and triacylglycerols form the hydrophobic core of lipid particles. They are surrounded by a phospholipid monolayer with a small amount of proteins embedded (Wagner and Daum, 2005). At the moment there is no universally valid model to explain the biogenesis of lipid particles. For some time a budding model was thought to be likely as it was supported by experimental results. The

model proposed the accumulation of neutral lipids, steryl esters and triacylglycerides between the two leaflets of the phospholipid bilayer in certain regions in the ER until reaching a critical size and budding off to form lipid particles (Czabany *et al.*, 2007). Work by Jaquier *et al.* (2011) showed that the transport of integral membrane proteins from the ER to lipid particles occurred fast, in an energy and temperature independent manner, was temporally controlled and possible in both directions. These results support (i) a model where ER and lipid particles are permanently connected or (ii) that their association occurs frequently enough for the uniform transfer of proteins. However, the precise nature and structure of the association between the ER membrane and lipid particles still needs to be defined.

Steryl ester synthesis in yeast is carried out by two acyl-CoA:cholesterol acyltransferase (**A**CAT) **r**elated **e**nzymes (**ARE**) (Yang *et al.,* 1996) also designated sterol-O-acyltransferese or acyl-CoA:sterol acyltransferases. Those are located to the endoplasmic reticulum as significant activities of both enzymes were found exclusively in microsomal fractions (Zinser *et al.,* 1993; Zweytick *et al.,* 2000) and GFP tagged ARE proteins were detected in the endoplasmic reticulum (Zweytick *et al.,* 2000). Two genes responsible for steryl ester formation were identified in *S. cerevisiae* by sequence search for yeast homologs to the human *ACAT1* gene exhibiting 23% identity. The two isoenzymes, namely *ARE1* and *ARE2*, are 61% identical at the DNA and 49% identical at the predicted protein levels (Yang *et al.,* 1996). The high level of sequence conservation counts for their common evolution arising from a gene duplication event (Oelkers *et al.,* 1998). The requirement for multiple enzymes for the same reaction would be advantageous to a cell, if one enzyme was either differentially regulated, alternatively localized, or specific for a substrate (Yang *et al.,* 1997). Also Jensen-Perkages *et al.* (2001) raised the question why yeast contains two sterol esterification genes, yet neither is essential for survival.

Yeast cells deleted of both *ARE1* and *ARE2* do not contain detectable amounts of steryl esters (Zweytick *et al.,* 2000). *ARE1* encodes the minor isoform in terms of its contribution to esterification, whereas Are2p is the main esterification enzyme conferring about 80% of the activity in the wild type, *in vitro* and *in vivo*. Deficiencies in sterol esterification were only apparent in the *are2* and *are1are2* mutant, but normal sterol ester biosynthesis was observed for an *are1* knockout strain (Yang *et al.*, 1996). By the knockout of *ARE2* the capacity to esterify ergosterol *in vivo* was shown to decrease by 20%, whereas in the WT and *are1* knockout strain ergosterol constitutes the major esterified sterol with 50 to 60% in the sterol ester fraction (Zweytick *et al.,* 2000). Polakowsky *et al.* (1999) confirmed that Are1p contributes much less to the esterification reaction than Are2p. Moreover, overexpression of *ARE2* showed that it predominantly influences the quantity and

enrichment of late sterol intermediates. All enriched sterols are characterized by the demethylation at the C4-atom (zymosterol, fecosterol, episterol, ergosterol).

By overexpression of *ARE1* in an *are1are2* mutant, esterification of lanosterol was significantly enhanced over the esterification of zymosterol compared to the *ARE2* overexpressing strain. *ARE1* might have the function to limit conversion of lanosterol to zymosterol to interrupt the ergosterol pathway and store ergosterol precursors in lipid particles. Moreover, *ARE1* expression could be shown to increase when sterol precursors accumulate as in the case of *erg2*, *erg3* and *erg6* knockout strains (Jensen-Perkages *et al.,* 2001).

Indeed, yeast sterol-O-acyltransferases also seem to be differentially regulated regarding esterification under anaerobic conditions. Under anaerobiosis, *S. cerevisiae* is not able to produce ergosterol and its addition to the growth medium is required to maintain cell growth. Contrarily to aerobic cells, disruption of *ARE1* and active Are2p reduced the esterification level to 25% compared to the WT without oxygen supply. Intriguingly, sterol ester levels did not change upon the disruption of *ARE2* (Valachovic *et al.,* 2001). Furthermore, *ARE1* was up-regulated under heme-deficient growth conditions while *ARE2* was depressed. Such a contra-regulation like for *ARE1* and *ARE2* is also observed for *HMG1* and *HMG2*, which are also positively and negatively regulated by heme. In accordance with this observation, *ARE2* expression also decreased with the knockout of *HAP1,* the heme activator protein (Jensen-Perkages *et al.*, 2001). *HAP1* is responsible for oxygen sensing and heme signalling in *S. cerevisiae* (Hach *et al.,* 1999).

End product regulation could be excluded as a regulatory mechanism for both enzymes. Neither deletion nor overexpression of one *ARE* gene altered its own expression level or the one of its isoform (Jensen-Perkages *et al.,* 2001).

1.3 Heterologous sterol-0-acyltransferases (HATs)

In this study, five different sterol-O-acyltransferases from four organisms were investigated for their substrate specificities towards cholesta-5,7,24-trienol. Three mammalian acyl-CoA:cholesterol acyltransferase (*ACAT*) genes, human *ACAT1*, rat *ACAT1* and *ACAT2*, the *ARE2* gene from *Candida albicans*, a yeast often associated with opportunistic infections and the *ACAT1* gene from *Toxoplasma gondii* an intracellular protozoan were chosen.

Rat liver cells were the first cells for which ACAT activity was identified in the 1940s and 1950s (Buhman *et al.,* 2000). Cholesterol was shown to be the preferred substrate for mammalian ACAT enzymes (Yang *et al.,* 1997, Tavani *et al.,* 1982). An investigation of rat liver microsomes showed that alkylation at the C24-atom, as in the case of ergosterol, significantly reduces esterification (Tavani *et al.,* 1982). Existence of a second ACAT isoform was established for the first time by a mouse model deficient in *ACAT1* activity, as mutant mouse liver still showed normal contents of cholesterol esters (Meiner *et al.,* 1996). Acat2p was the second enzyme identified in humans to esterify cholesterol, but to a less extent than Acat1p. Both human ACAT isoforms were not able to esterify ergosterol (Oelkers *et al.,* 1998) and lanosterol (Nishikawa *et al.,* 2005). Human *ACAT1* shares 17% amino acid identity with Are2p and 16% with Are1p with the most homologous regions near the C-terminus (Yu *et al.,* 1996). Eight transmembrane domains were found for Acat1p with the PredictProtein algorithm (Oelkers *et al.,* 1998). Human *ACAT1* expressed in yeast was used a wide range of various Acyl-CoAs as substrates (Yang *et al.,* 1997).

Interest on sterol-O-acyltransferases in *C. albicans* is mainly based on the probability to use conversion of free sterols to steryl esters as a target for antifungal agents, as the organism is known to induce serious fungal infections (Kim *et al.,* 2004). The gene was found by PCR on mRNA with primers designed based on a consensus sequence of ACAT or ACAT related enzymes and was designated *ARE2*. It shows seven putative transmembrane domains. *ARE2* of *C. albicans* was expressed in a *S. cerevisiae are1are2* double knockout and was shown to esterify yeast wild type sterols. *In vitro* experiments showed its preference for cholesterol over ergosterol and for oleoyl-CoA as substrates.

Toxoplasma gondii is an obligate intracellular parasite capable of infecting nearly all types of nucleated cells and is responsible for fatal diseases in neonates and immunocompromised individuals. *T. gondii* is able to incorporate exogenous cholesterol and fatty acid into its ester fraction (Nishikawa *et al.,* 2005). *T. gondii* does not synthesize sterols from precursors of the mevalonate pathway and parasite growth is dependent on cholesterol acquisition from the host cell (Coppens *et al.,* 2000).

The parasite expresses two ACAT isoforms, *ACAT1α* and *ACAT1β*, both located to the ER. Concerning substrate specificities, *T. gondii* showed a broad use of fatty acids *in vivo* with preferential uptake for palmitate over oleate, arachidonate, stearate and linoleate what does not correlate to the preferences of sterol-O-acyltransferases in yeast. As mammalian enzymes, *T. gondii* ACATs did not esterify lanosterol *in vitro* but readily esterified ergosterol. Only one isoform *ACAT1α* reconstituted lipid droplet formation in a yeast strain devoid of endogenous diglyceride-O-acyltransferase and sterol-O-acyltransferase activities, suggesting the use of yeast wild type sterols including ergosterol. *ACAT1β* could not be expressed in the mutant yeast (Nishikawa *et al.*, 2005).

1.4 Vitamin D_3

Vitamin D_3 is the only vitamin which can be produced by the human body on his own. Skin exposure to sun light causes the photolysis of 7-dehydrocholesterol, provitamin D_3, by the action of ultraviolet B rays. The formed previtamin D_3 is spontaneously converted to vitamin D_3 by rearrangement of its double bonds. After this isomerization reaction the vitamin needs to be further metabolized for its biological activity by the hydroxylation at the C25- and C1-atoms.

Although Vitamin D_3 uptake through our diet is not necessarily required, a variety of factors can limit its production in the skin. Limiting factors include that ultraviolet B photons might be not sufficient for the conversion of 7-dehydrocholesterol because of high skin pigmentation or alteration of the zenith angle of the sun. Additionally, the ability for enzymatic production of provitamin D_3 from cholesterol decreases with aging. A lack of vitamin D_3 is known to cause rachitis in children and is associated with osteoporosis as well as with an increased risk of colon cancer (Holick, 1999). For those reasons Vitamin D_3 deficiencies are a severe health concern and, therefore, of therapeutic and economic interest.

2 Project specification

This Master thesis was carried out in cooperation with DSM Nutritional Products Ltd during a long-term project. At the time of this Master thesis the main goal of this project was to increase the production of cholesta-5,7,24-trienol in a genetically engineered yeast strain. Cholesta-5,7,24-trienol is a precursor of provitamin D_3 (7-dehydrocholesterol) which further can be photochemically converted to vitamin D_3 (cholecalciferol).

To obtain cholesta-5,7,24-trienol in *S. cerevisiae* the ergosterol pathway needs to be manipulated by the knockout of *ERG6* and *ERG5*. To push sterol biosynthesis, a truncated version of *HMG1* is over-expressed. The use of a strain with an intact *HAP1*, i.e. a wild type *HAP1* background, is a precondition for high level production of sterols. A defect in the transcription factor Hap1 in some yeast strains is caused by an insertion mutation of transposon Ty1 and results in decreased levels of expression of ergosterol related genes and decreased ergosterol content (Tamura *et al.,* 2004).

This genetically modified yeast strain produces cholesta-5,7,24-trienol but also high amounts of zymosterol. Conversion of zymosterol to cholesta-5,7,24-trienol seems to be hampered. Different approaches to improve cholesta-5,7,24-trienol to zymosterol ratio had already been disproved before project start (Martin Lehmann, DSM, personal communication). One hypothesis has been the limited activity of Erg2p and Erg3p due to their preference for ergosta sterols. However, over-expression of *ERG2* or *ERG3* respectively, neither from yeast nor from vertebrate origin, gave satisfying improvements.

Erg6p is part of a complex where seven Erg proteins, including the enzymes to convert zymosterol to cholesta-5,7,24-trienol, Erg2p and Erg3p, closely interact (Mo *et al.,* 2004). Inactivation of Erg6p instead of its deletion was suggested to avoid the probable decomposition of this protein complex which may causes limited activities of Erg2p and Erg3p. Introduction of point mutations into the *ERG6* gene resulted in the desired sterol pattern, but so far none of the modifications did change the cholesta-5,7,24-trienol to zymosterol ratio (Ploier, 2010).

Nearly all zymosterol and 90% of cholesta-5,7,24-trienol are present as steryl esters in lipid particles (Martin Lehmann, DSM, personal communication). Preference of endogenous sterol-O-acyltransferases, Are1p and Are2p, for zymosterol would make the compound unavailable for further processing enzymes by its efficient esterification. To make esterified zymosterol available, the three steryl esterhydrolases Yeh1p, Yeh2p, and Tgl1p were over-expressed but this measure did not give any change in sterol composition. The knockout of *ARE1* neither changed the situation and the knockout of *ARE2* was not yet possible at the start of this Master thesis. The idea was to prevent esterification of zymosterol to allow for its conversion to cholesta-5,7,24-trienol, which was to be specifically acylated. Moreover, withdrawing the end product from the reaction equilibrium and high

amounts of free zymosterol should push cholesta-5,7,24-trienol formation. Specific esterification of cholesta-5,7,24-trienol may be efficiently carried out by sterol-O-acyltransferases of other organisms with differing substrate preferences. Five sterol-O-acyltransferases of four organisms, further referred to as **h**eterologous sterol-O-**a**cyl**t**ransferases (**HAT**s), were selected by Regina Leber, based on sequence similarity and literature search, for their heterologous expression in *S. cerevisiae* mutant strains.

2.1 Objectives of this Master thesis

Objective of this work was to assess if and how expression of selected HATs in an *erg5erg6are1are2* mutant influences cholesta-5,7,24-trienol to zymosterol ratio.

To confirm that they show esterification activity and are correctly expressed in *S. cerevisiae*, a strain devoid of endogenous sterol-O-acyltransferases was required. The additional knockout of *ERG6* and *ERG5* enables to assess for substrate preferences of HATs for cholesta-5,7,24-trienol and zymosterol.

Protein expression should be observed by endowing HATs with an adequate tag for immunological detection. Plasmids harboring respective tagged HATs should be transformed, correct expression and substrate specificities assessed and genomic integration should be carried out for promising sterol-O-acyltransferases. During this Master thesis, the respective quadruple knockout was never available, wherefore other strategies were developed.

Another point was the elucidation of free sterol and steryl ester composition. At the start of this Master thesis only analysis of total sterol content, including free sterol as well as steryl ester fraction, by GC/MS was possible. To conclude on substrate specificities of HATs by the change of total sterol composition is an indirect method, and requires confirmation by analyzing steryl ester composition. Therefore the establishment of a respective analysis method was required.

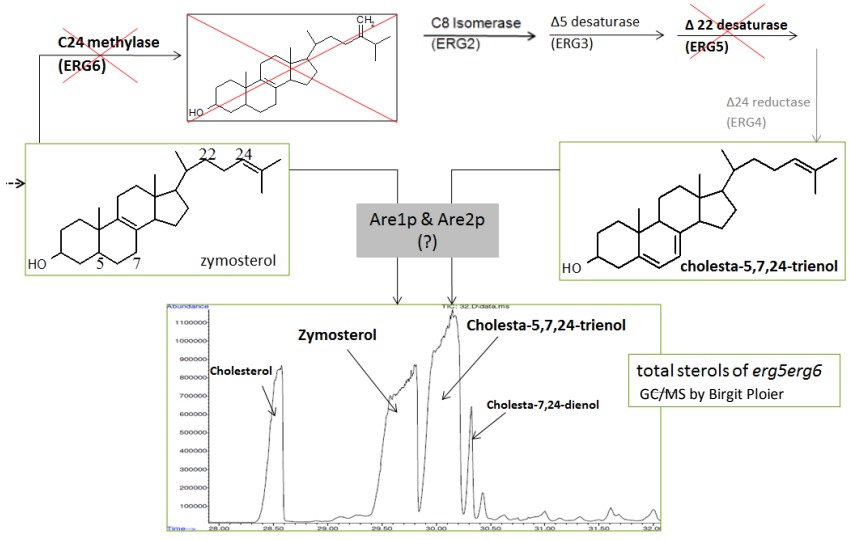


Figure 5: Manipulation of the ergosterol synthesis pathway to obtain cholesta-5,7,24-trienol

3 Material

3.1 Strains

Table 1: *S. cerevisiae* strains in this work

Strain background	**Name**	**Genotype [Plasmid** (backbone + insert)**]**	**Source**
FY834	FY-*are1are2*	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*	this work
FY834	FY-YEX	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1]	this work
FY834	FY-YEX-120	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Hs.acat1]	this work
FY834	FY-YEX-121	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Rn.acat2]	this work
FY834	FY-YEX-122	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Rn.acat1]	this work
FY834	FY-YEX-123	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Ca.are2]	this work
FY834	FY-YEX-124	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Tg.acat1]	this work
FY834	FY-YEX-*Sc.are2*	*MATa, ura3-52, leu2-Δ1, lys2-Δ202, are1::HIS3, are2::TRP1*, [pYEX4T-1+Sc.are2]	this work
CEN.PK2-1C	CEN.PK2-1C	*MATa, ura3-52, trp1-289, leu2-3_112, his3Δ1, MAL2-8^C, SUC2*	EUROSCARF
CEN.PK2	BP2_*erg5erg6*	*MATα, ura3-52, his3Δ1, MAL2-8^C, SUC2, erg5::LEU2, erg6::TRP1*	Birgit Ploier
CEN.PK2	*erg6are1are2*	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*	Birgit Ploier
CEN.PK2	612-YEX	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1]	this work
CEN.PK2	612-YEX-120	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Hs.acat1]	this work
CEN.PK2	612-YEX-121	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Rn.acat2]	this work
CEN.PK2	612-YEX-122	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Rn.acat1]	this work
CEN.PK2	612-YEX-123	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Ca.are2]	this work
CEN.PK2	612-YEX-124	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Tg.acat1]	this work
CEN.PK2	612-YEX- *Sc.are2*	*MAT?, ura3-52, leu2-3_112, MAL2-8^C, SUC2, erg6::TRP1, are1::HIS3, are2::loxP-kanMX-loxP*, [pYEX4T-1+Sc.are2]	this work

Table 2: *P. pastoris* strains in this work

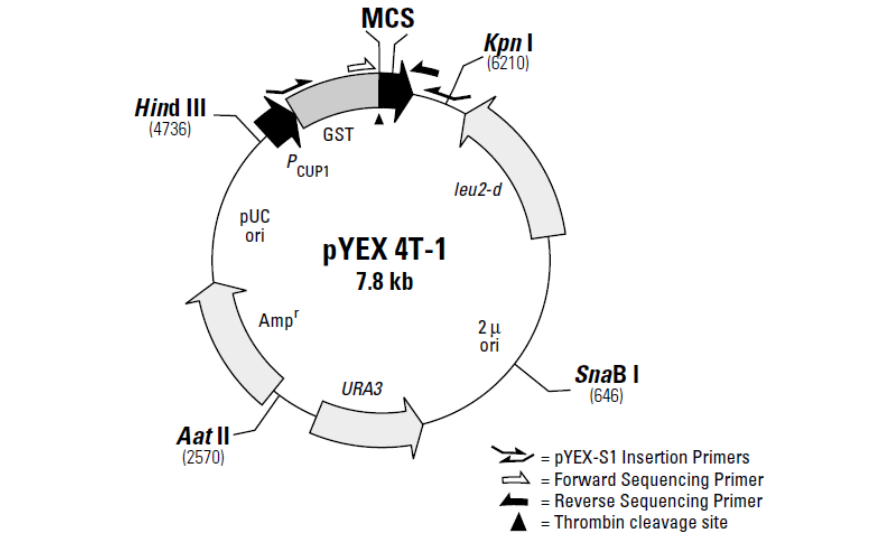
Strain background	**Name**	**Genotype / Plasmid** (backbone + insert)	**Source**
CBS57435	WT	*ΔKu70, his4*	Tamara Wriessnegger
CBS57435	*erg6*	*ΔKu70, his4, Δerg6::KanMX6*	Tamara Wriessnegger
CBS57435	*DHCR7*	*ΔKu70, his4, Δaox1::pPpGAPZ-A[DHCR7]*	Tamara Wriessnegger
CBS57435	*erg6DHCR7*	*ΔKu70, his4, Δerg6::KanMX6, Δaox1::pPpGAPZ-A[DHCR7]*	Tamara Wriessnegger

Cloning work was performed in *E. coli* TOP10F' (Invitrogen) with the following genotype: F´{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG

3.2 Plasmids

Table 3: Plasmids used in this work

Plasmid	Source
pYEX 4T-1	Regina Leber
pJ201	DSM NP Ldt.
pJet1.2	Fermentas



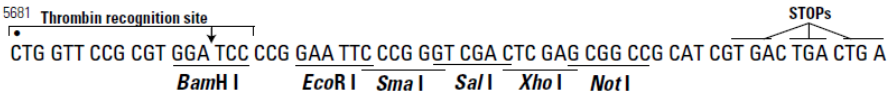


Figure 6: Vector card of pYEX 4T-1

3.2.1 Heterologous sterol-O-acyltransferase genes

Five different acyltransferases of four organisms (Table 4) were selected and ordered as synthetic genes (DSM, In-Kind contribution). Genes have also been provided as part of an integration cassette for homologous recombination at the *ARE2* locus with an integral *URA3* marker for selection. All gene constructs were available in a pJ201 vector and optimized for the expression in *S. cerevisiae* (detailed list see Table 5).

Table 4: HAT genes

Gene	Host organism
ACAT1	*Homo sapiens*
ACAT2	*Rattus norvegicus*
ACAT1	*Rattus norvegicus*
ARE2	*Candida albicans*
ACAT1	*Toxoplasma gondii*

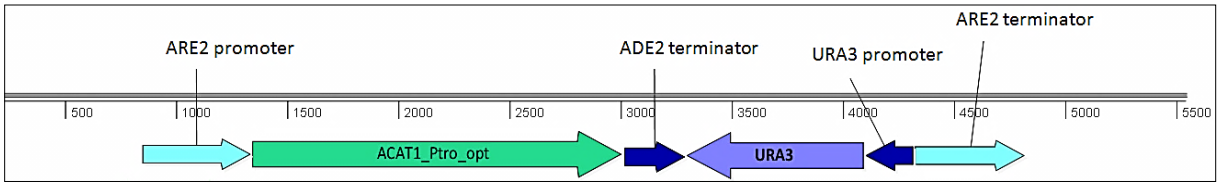


Figure 7: Integration cassette for genomic integration at the ARE2 locus

Table 5: HAT genes and respective substitution cassettes in the pJ201 vector

Name of vector (DSM)	Description by DSM
pHYD-0120	Ptro ACAT1_optSc1 (DNA 2.0 plasmid 41919)
pHYD-0121	Rnor ACAT2_optSc1 (DNA 2.0 plasmid 41920)
pHYD-0122	Rnor ACAT1_optSc1 (DNA 2.0 plasmid 41921)
pHYD-0123	Calb ARE2_optSc1 (DNA 2.0 plasmid 41922)
pHYD-0124	Tgon ACAT1_optSc1 (DNA 2.0 plasmid 41923)
pHYD-0125	*are2::URA3* substitution construct (DNA 2.0 plasmid 41924)
pHYD-0126	*are2::Ptro ACAT1_optSc1 URA3* substitution construct
pHYD-0127	*are2::Rnor ACAT2_optSc1 URA3* substitution construct
pHYD-0128	*are2::Rnor ACAT1_optSc1 URA3* substitution construct
pHYD-0129	*are2::Calb ARE2_optSc1 URA3* substitution construct
pHYD-0130	*are2::Tgon ACAT1_optSc1 URA3* substitution construct

Table 6: xBlast search of HAT gene sequences sent by DSM

Name of vector harbouring HAT gene	xBlast search of HAT gene	Code
pHYD-0126 & pHYD-0120	sterol O-acyltransferase 1 [*Homo sapiens*]	NP_003092.4
pHYD-0127 & pHYD-0121	sterol O-acyltransferase 2 [*Rattus norvegicus*]	EDL86862.1
pHYD-0128 & pHYD-0122	sterol O-acyltransferase 1, isoform CRA_a [*Rattus norvegicus*]	EDM09484.1
pHYD-0129 & pHYD-0123	hypothetical protein CaO19.2248 [*Candida albicans* SC5314]	XP_714838
pHYD-0123 & pHYD-0124	sterol O-acyltransferase, putative [*Toxoplasma gondii* VEG]	EEE27951.1

3.3 Primers

Table 7: Primers for the amplification of *ARE2* of *Saccharomyces cerevisiae* CEN.PK2

Primer name	Sequence (from 5' to 3'end)	Tm [°C]
are2_fw	CTATATGGATCCAAAATGGACAAGAAGAAGGATCTACTGG	61.6
are2_rv	GACTGCGAATTCTTAGAATGTCAAGTACAACGTACACATG	62.1

Table 8: Primers for sequencing of *ARE2* of *Saccharomyces cerevisiae* CEN.PK2 in pJET1.2

Primer name	Sequence (from 5' to 3'end)	Tm [°C]
pJETfw	see manual	-
pJETrv	see manual	-
are2-fw.bp638	CCAATTTCTCCGGGTTGTACG	56.0

Table 9: Primers for the verification of integration of HATs at *ARE2* locus

Primer name	Sequence (from 5' to 3'end)	Tm [°C]
upstr1_fw	GGTTTGAGCATGTACGAGAATAAGTTAC	55.8
upstr2_fw	GTCAACGAAATGCGCCG	55.1
ura1_fw	CCTAATGCTTCAACTAACTCCAGTAATTCC	57.2
ura2_fw	GGCAGCAACAGGACTAGGATGAG	59.4
downstr1_rv	CGATTACTGTCCCGACTGGAAGG	59.0
downstr2_rv	GAAGACGACTCTAAAGCTCGAGAAC	56.8
120_rv	GGTTGGTTACAAAGTCATCGAAATG	54.7
121_rv	GAGGTCTGTCCTGCTTAGGGTATGC	60.6
122_rv	CGGACTTGTCTATTAGATTTGTTACAAAATC	54.7
123_rv	CTAAGTCTAAGTTCATTTTGTGGAGCG	55.9
124_rv	GATGAAAATGGTGATGATGAAGATGG	54.4
KA2up	GCGGAGTTAACATTTAACGGC	54.6
KA2rev	CTCTGTATTGCCTTCGGTAGC	55.3
KA2down	CTTCGCCGATACGAGACCG	57.8

Table 10: Primers for the verification of *ERG5* and *ERG6* disruption cassettes

Primer name	Sequence (from 5' to 3'end)	Tm [°C]
K1E6	CTGCTCCACTTCGTCTCAATGG	57.8
K2E6	CTTGCCACGACTCATCTCCATG	57.9
K2exE6	GCCTGCTAGCAATGAACGTGCTA	59.8
K1E5	GGTTTCCCTCGTTTAAGTCTGCG	58.1
K2E5	ATCAGCCTTCTTGGAGGCTTCC	59.3
K2exE5	CAAAACGCCAACCCTTAATGAAG	55.0

3.4 Instruments and Devices

Table 11: List of instruments and devices used in this work

Instrument/ Device/Enzyme	Supplier
Automatic TLC sampler	CAMAG
Avanti J-20XP Centrifuge	Beckman Coulter Inc.
Centrifuge tubes, 50 ml and 500 ml	Nalgene Thermo Fisher Scientific Inc.
Centrifuges	Centrifuge 5810R, Eppendorf, Germany Centrifuge 5415R, Eppendorf, Germany
Daunce homogenizer	Sartorius AG
Dispensette	Brand GmbH, Germany
Electrophoresis gel chambers	PowerPac™ Basic + Sub-Cell GT, Biorad, USA
Electrotransformation	MicroPulser™, BIO-RAD, USA Electroporation Cuvettes (2mm 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 gripper	VWR International, GmbH
GC vials	VWR International, GmbH
GC/MS	Agilent Technologies, Austria
Glass beads	Carl Roth GmbH + Co KG
Glass bottles	Schott/ Duran, Ilmabor TGI
Incubator (30°C and 37°C)	Binder GmbH
Kolben	Simax
Laminar flow chamber BSB4A	Gelaire Flow Laboratories
Merckenschlager, MSK homogenizer	B. Braun, Biotech International GmbH, Germany
Merckenschlager glass vessels, GL25	Schott AG, Germany
MF Membrane filters, 0.025 µm VSWP	Millipore, USA
Mixing of small volumes	Vortex-Genie 2, Scientific Industries Inc, USA
NuPAGE®SDS Gels: 4-12% Bis-Tris Gel 1 mm x 15 wells	Invitrogen Life Technologies Corp.
Optima LE-80 Ultracentrifuge	Beckman Coulter Inc.
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 scanner	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
Ultracentrifuge tubes	Sorvall Instruments, USA
UV-cuvettes	Greiner bio-one International AG
Vibrax	Vibrax VXR basic, IKA GmbH& Co KG, Germany
Western blot membrane	Sartorius AG, nitrocellulose blotting membrane

3.5 Reagents

Table 12: 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
Cholesta-5,7,24-trienol	DSM (in-kind contribution)
Cholesterol	Sigma- Aldrich Corp.
Cholesteryl oleate	Stigma- Aldrich Corp.
CloneJet™PCR Cloning Kit, #K1231 #K1232	Fermentas Thermo Fisher Scientific Inc.
$CuSO_4$	TU Graz, Austria
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
Ethyl acetate	Carl Roth GmbH + Co KG
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
GoTaq DNA Polymerase	Promega Corp.
Green GoTaq reaction buffer (5x)	Promega Corp.
GST antibody produced in rabbit, G7781	Sigma-Aldrich Corp.
HCl	Carl Roth GmbH + Co KG
L-Adenine	Carl Roth GmbH + Co KG
L-Histidine	Carl Roth GmbH + Co KG
Lithium acetate	Fluka/ Sigma- Aldrich Corp.
L-Leucine	Carl Roth GmbH + Co KG
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.
Lyticase (from *A. luteus*)	Sigma- Aldrich Corp.
Magnesium chloride	Carl Roth GmbH + Co KG
Manganese chloride	Carl Roth GmbH + Co KG
Mass Ruler DNA Ladder Mix	Fermentas Thermo Fisher Scientific Inc.
Maxima Hot Start Green PCR Mastermix	Fermentas Thermo Fisher Scientific Inc.
Methanol	Carl Roth GmbH + Co KG
N'O'-bis(trimethylsilyl)-trifluoracetamid	Sigma- Aldrich Corp.

n-Heptane	Carl Roth GmbH + Co KG
Nitrogen base without 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
Pyridine	Carl Roth GmbH + Co KG
Pyrogallol	Carl Roth GmbH + Co KG
Restriction enzymes	Fermentas Thermo Fisher Scientific Inc.
RNaseA	Fermentas Thermo Fisher Scientific Inc.
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
Sodium chloride	Carl Roth GmbH + Co KG
Sodium citrate	Carl Roth GmbH + Co KG
Sodium hydroxide	Carl Roth GmbH + Co KG
Sorbitol	Carl Roth GmbH + Co KG
Spectra Broad Range Protein Standard	Invitrogen Life Technologies Corp.
Sulfuric acid	Carl Roth GmbH + Co KG
SuperSignal West Pico Chemiluminescent Substrate Kit	Pierce
T4 DNA Ligase	Promega Corp.
T4 DNA ligase buffer 10x	Promega Corp.
Trichloroacetic acid	Carl Roth GmbH + Co KG
Triolein	Stigma- Aldrich Corp.
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 Corp.
Yeast extract	Carl Roth GmbH + Co KG
Zymolyase (from *A. luteus*)	Seikagaku Biobusiness, Corp.
β-Mercaptoethanol	Carl Roth GmbH + Co KG

3.6 Media and Buffers

Table 13: Cultivation media used in this work; the agar is omitted for liquid media

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, final concentration 40 mg/ L
SD all	6.7 g/L Bacto- 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 tryptophane in the dropout powder mix
SD-leu	Like SD all but without leucine in the dropout powder mix
SD-trp	Like SD all but without tryptophane in the dropout powder mix
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
YPD	10 g/L Yeast extract (1%), 20 g/L Peptone (2%) , 20 g/L Glucose (2%), 20 g/L Agar (2%)

4 Methods

4.1 Electrocompetent *E. coli* cells

30 mL of LB media were inoculated with the chosen *E. coli* strain and incubated over night at 37°C and 220 rpm. The following day, the main culture of 500 mL of LB media was inoculated with 5 mL of the overnight culture and incubated at 37°C until reaching an OD between 0.7 and 0.9, which usually took between two and four h. After transferring the culture to chilled 500 mL centrifuge bottles, it was cooled on ice for 30 min before harvesting the cells at 2,000 x g and 4°C for 15 min. The supernatant was discarded and the pellet was carefully resuspended in pre-chilled 500 mL ddH_2O. The suspension was centrifuged as before and the supernatant was discarded. Then, the cell pellet was resuspended in 35 mL of pre-chilled, sterile 10% glycerol and centrifuged at 4,000 x g and 4°C for 15 min. After discarding the supernatant, the pellet was resuspended in 1 mL ice-cold, sterile 10% glycerol before aliquoting the electrocompetent cells to 80 µL into sterile Eppendorf tubes. The cells were frozen in liquid N_2 and stored at -80°C until needed.

4.2 Electroporation of *E. coli* cells

The transformation of plasmids into electrocompetent *E. coli* cells TOP10F' followed a standard procedure. 50 µL of electrocompetent cells, prepared as described above, were thawed on ice. After adding 2 µL of a plasmid preparation and transferring the mixture to chilled transformation cuvettes, they were incubated on ice for ten min before pulsing them in the electroporator. Immediately after the electro-pulse, 1 mL of SOC medium was added and the cells were regenerated at 37°C for one h at 650 rpm before plating them on selective media.

4.3 Transformation of yeast

The transformation of plasmids was carried out according to a quick and easy protocol described by Elble (1992). Twenty five mL of YPD were inoculated with cells from a fresh plate and incubated for 5 h at 30°C and 140 rpm. For every transformation event, 1 mL of culture was spun at 3000 x g for 10 s. After discarding the supernatant, the pellet was carefully resuspended in 10 µL of carrier DNA (10 mg/mL), 1 µg transforming DNA and 500 µL of transformation-mix (consisting of 90 mL sterile 45% PEG 4000, 10 mL of 1 M lithium acetate, 1 mL of 1 M Tris-HCl, pH 7.5, and 0.2 mL of 0.5 M EDTA, pH 8). The transformation mixture was incubated at room temperature overnight on the benchtop. After collecting the cells by centrifugation at 3000 x g for 10 s and discarding the supernatant, the pellet was resuspended in 200 µL of YPD and incubated for 2 h at 30°C. The whole volume was plated onto selective media.

For the transformation of substitution cassettes a roundup of the protocol described by Adams *et al.* (1997) was used. Fifty mL of YPD were inoculated to an OD of 0.1 with a fresh ONC and incubated at 30°C between three and five hours. The culture was then harvested in a sterile 50 mL centrifuge tube at 2500 rpm for 5 min. The pellet was washed with 25 mL of sterile, ice-cold H_2O, centrifuged as before and, after discarding the water, resuspended in 1 mL of 100 mM LiAc and transferred to a sterile 1.5 mL microfuge tube. The cells were harvested at top speed for 5 s and the LiAc was removed with micropipette. Then the pellet was resuspended in 400 µL of 100 mM LiAc, which yielded sufficient material for 10 transformations. The transformation mix was added in the following order to 50 µL of this LiAc competent cell suspension:

240 µL PEG 50% w/v

36 µL 1 M LiAc

5 µL single stranded carrier DNA (10 mg/mL)

500- 1000 ng of disruption cassette DNA

After snapping each tube until the pellet was completely resuspended, the mixture was incubated for 30 min at 30°C before being heat shocked at 42°C for 25 min. The cells were collected by centrifugation at 3000 rpm for 15 s, before the transformation mix was removed with a micropipette. Subsequently, the cells were resuspended in 1 mL of YPD and incubated for 1 h at 30°C. Regeneration was followed by centrifugation at top speed for 10 s and the supernatant was discarded. Regenerated cells were resuspended in 100 µL of sterile H_2O and plated onto selective media which were then incubated for three to seven days at 30°C. Single colonies of transformants were tested by colony PCR.

4.4 PCR to test functionality of primers

Some primers designed for cPCRs were tested for their functionality with the respective plasmid as template with the "Maxima^{TM} Hot Start Green PCR Master Mix" under following conditions:

0.5 µL	Plasmid DNA template
19.5 µL	deion. H_2O
25 µL	Maxima Mastermix
2.5 µL	Primer fw of 10 µM
2.5 µL	Primer rv of 10 µM
50 µL	Total volume

Cycling conditions:

95°C/4 min-{95°C/30s-($T_m$primer-5°C/30 s)-(72°C/1 min per kbp)} x40 -72°C/10 min -4°C/∞

4.5 Colony PCR

To verify disruption of respective genes in knockout strains and site-specific integration of HAT integration cassettes to substitute the endogenous *ARE2,* different methods of cell disruption were used. Cell disruption by heat and enzymatic digestion of cell wall by zymolyase turned out to work best. In every case, cells should be fresh proceeding from a single colony grown over night preferably on YPD media.

To lyse cells by heat, cell material was resuspended in 25 ml of dd$H_2$0 and incubated for 5 min at 95°C followed by cooling for 5 min on ice. After centrifugation at top speed for 3 min, 3 µl of supernatant were used for PCR.

For enzymatic cell wall digestion minimal quantity of cell material taken by a pipette tip was resuspended in 50 µL of zymolyase (2.5 mg/mL ~ 50 U) in a PCR reaction tube and incubated for 15 min at 37°C. After harvesting the cells at 5000 rpm for 1 min the supernatant was discarded and the pellet heated for 5 min at 92°C to inactivate DNases. The pellet was resuspended in a prepared PCR Master-Mix before starting the PCR under the following conditions:

When using "DreamTaq™ DNA polymerase":

~ 3 µL	Pellet of *S. cerevisiae* / supernatant
31.75 µL	deion. H_2O
5 µL	Dream Taq Buffer (10x)
5 µL	dNTP mix of 2 mM each
2.5 µL	Primer fw of 10 µM
2.5 µL	Primer rv of 10 µM
0.25 µL	DreamTaq DNA polymerase of 5 U/µL
50 µL	Total volume

Cycling conditions:

95°C/3 min-{95°C/30 s-($T_m$primer-5°C/30 s)-(72°C/1 min per kbp)} x40 -72°C/10 min -4°C/∞

When using "Maxima™ Hot Start Green PCR Master Mix":

~ 3 µL	Pellet of *S. cerevisiae* / supernatant
19 µL	deion. H_2O
25 µL	Maxima Mastermix
2.5 µL	Primer fw of 10 µM
2.5 µL	Primer rv of 10 µM
52 µL	Total volume

Cycling conditions:

95°C/4 min-{95°C/30 s-($T_m$primer-5°C/30 s)-(72°C/1 min per kbp)} x40 -72°C/10 min -4°C/∞

When using "GoTaq® DNA polymerase"

1,5 µL	Pellet of *S. cerevisiae*
32.25 µL	deion. H_2O
10 µL	Green GoTaq® Reaction Buffer (5x)
1 µL	dNTP mix of 10 mM each
2.5 µL	Primer fw of 10 µM
2.5 µL	Primer rv of 10 µM
0.25 µL	GoTaq DNA polymerase of 5 U/µL
50 µL	Total volume

Cycling conditions:

95°C/2,5 min-{95°C/30 s-($T_m$primer-5°C/30 s)-(72°C/1 min per kbp)} x30 -72°C/10 min -4°C/∞

When using "DreamTaq™ Green PCR Master Mix":

~ 3 µL	Pellet of *S. cerevisiae* / supernatant
19 µL	deion. H_2O
25 µL	DreamTaq Mastermix
2.5 µL	Primer fw of 10 µM
2.5 µL	Primer rv of 10 µM
52 µL	Total volume

Cycling conditions:

95°C/3 min-{95°C/30 s-($T_m$primer-5°C/30 s)-(72°C/1 min per kbp)} x40 -72°C/10 min -4°C/∞

4.6 Gel electrophoresis

The separation and purification of DNA fragments by agarose gel-electrophoresis was performed according to standard protocols. 1% agarose gels in TAE buffer were run at 90 V for about 80 min for preparative gels and 120 V for 45 min for analytical gels. The sizes of DNA fragments were assessed by comparison to the standard "GeneRuler DNA Ladder Mix", and the concentrations were estimated based on the "MassRuler DNA Ladder Mix" standard, both by Fermentas.

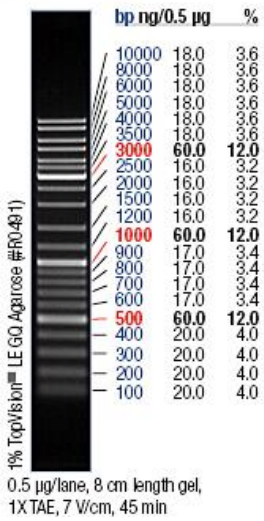


Figure 8: GeneRuler™ DNA Ladder Mix

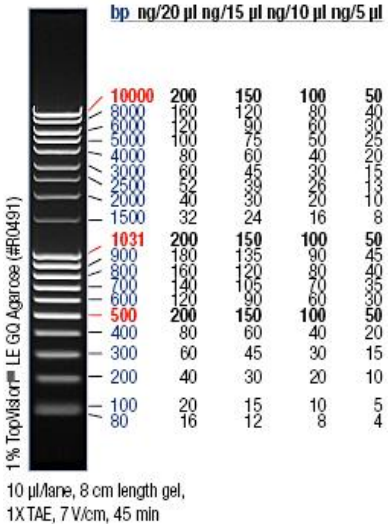


Figure 9: MassRuler ™ DNA Ladder Mix

4.7 Isolation of genomic DNA

Genomic DNA isolation was carried OUT according to the so-called “Bust n’ Grab” method (Harju, 2004). For this purpose, 1.5 ml of an overnight culture were transferred into a microfuge tube and pelleted at 13,000 x g for five min. The pellet was resuspended in 200 µL of lysis buffer (2% Triton X-100,1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and then placed in a -80°C freezer until completely frozen. The mixture was quickly thawed in a thermomixer at 95°C. This procedure was repeated twice before vortexing the sample for 30 s. After adding 200 µL of chloroform, the tubes were vortexed for two min and centrifuged for three min at room temperature and maximum speed. For DNA precipitation, the upper aqueous phase was transferred to a microcentrifuge tube containing 400 µl of ice-cold 100% ethanol which was then mixed by inversion. To increase the yield, samples were incubated at -20°C for five to ten min and centrifuged for five min at maximum speed. The supernatant was removed with a micropipette and the pellet was dried in an incubator at 37°C before dissolving it in 20 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

4.8 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 µl to 50 µl ddH_20. Cell material was abraded with a toothpick from a quarter of an agar-plate.

DNA fragments and PCR products were purified over standard DNA agarose gels and extracted with “Wizard SV Gel Slice and PCR Product Preparation” by Promega, as described in the manual. Elution volume was adjusted to estimated concentration in the agarose gel and varied from 20 µl to 50 µl.

Herein this procedure is described as “purification by a preparative gel”.

4.9 Restriction Enzymes

Restriction was performed from two h to 20 h/overnight in 1x respective buffer with standard restriction enzymes (U) at 37 °C.

Definition of 1 U: 1 unit (U) is defined as the amount of enzyme required to digest 1 µg of λ-DNA in 1 hour at 37°C in 50 µl of recommended reaction buffer.

4.10 Ligation

To ligate DNA fragments which had been purified before over standard DNA preparative agarose gels, a molar ratio of vector to insert of 1:2 was used with about 100 to 150 ng of total DNA per ligation mixture, as follows:

17 - x- y µL	deion. H_2O
2 µL	T4 DNA ligase buffer (10x)
x µL	insert
y µL	vector backbone
1 µL	T4 DNA ligase
20 µL	total volume

Mixture was incubated for two hours at room temperature and afterwards for two hours at 16°C. Before transformation into electrocompetent *E. coli* Top10F' cells, ligation mixture was desalted for 30 min with MF Membrane filters of 0,025 µm pore size.

4.11 Amplification and molecular cloning of *ARE2* from *S. cerevisiae*

To amplify *ARE2*, genomic DNA of CEN.PK2-ID was isolated and a two step PCR was carried out. Annealing temperature of first cycles was lower, designed according to base pairs of the primer homologous to *ARE2*. Additionally, the primer provides overhangs with restrictions sites for the molecular cloning into the pYEX4T-1. To facilitate restriction, additional bases were added downstream and upstream of restriction sites. First PCR cycles should provide sufficient template for further cycles with an annealing temperature according to the whole length of the primer to support specific amplifications (Figure 10).

are2_fw	5'-CTATAT***GGATCC***AAA**ATG**GACAAGAAGAAGGATCTACTGG-3'
are2_rv	5'-GACTGC***GAATTC***T**TA**GAATGTCAAGTACAACGTACACATG-3'
underlined…	overhang
bold…	start/stop codon
italic & bold…	restriction sites

Figure 10: Primer design for the amplification and molecular cloning of *ARE2* from *S. cerevisiae*

PCR was carried out under following conditions:

23.5 µL	deion. H_2O
5 µL	Phusion HF Buffer (5x)
1 µL	dNTP mix of 10 mM each
2.5 µL	are2_fw of 10 µM
2.5 µL	are2_rv of 10 µM
15 µL	Template DNA
0.5 µL	Phusion Hot Start DNA polymerase of 2 U/µL
50 µL	Total volume

Cycling conditions:

98°C/1 min

-{98°C/10 s-(67.8°C/50 s)-(72°C/58 s)} x5

-{98°C/10 s-(78°C/50 s)-(72°C/59 s)} x30

-72°C/10 min -4°C/∞

Amplified DNA was purified by a preparative gel and molecular cloning was carried out with the Clone Jet™ PCR Cloning Kit according to the producer's manual. The whole amplicon was cloned blunt end into the pJet1.2 and transformed into *E. coli* TOP10F'. After plasmid isolation the vector was treated with restriction enzymes to clone the *ARE2* coding sequence into the pYEX4T-1.

4.12 Sequencing

Amplified *ARE2* of CEN.PK2 cloned into pJET1.2 was sequenced by LGC Genomics GmbH (Berlin, Germany), in very high quality. 10 µL of a plasmid preparation were mixed with 4 µL of a 5 µM sequencing primer.

4.13 Cultivation of FY834 *are1are2* expressing GST-HAT fusion proteins from pYEX4T-1

Strains were grown over night in SD-ura media at 30°C and moderate shaking. Main cultures were inoculated to an OD_{600} of one. After two h of growth expression of GST-HAT fusion proteins was induced by adding sterile filtered 0,5 M $CuSO_4$ to a final concentration of 0.5 or 0.75 mM, respectively. As culture media contained 40 µg/l, i.e. 0,25 µM, of $CuSO_4$ expression might have already been induced before actual induction with the $CuSO_4$ solution. Samples for immunodetection of GST-HAT fusion proteins were taken from uninduced culture (t_0) and for lipid pattern analysis as well as for immunodetection, samples were taken after six or 20 hours of induction, respectively.

4.14 Microsome Preparation

Strains were grown over night in SD-ura or YPD media, respectively, at 30°C and moderate shaking. Main cultures of 500 ml were inoculated to an OD_{600} of one. After two h of growth expression of GST-HAT fusion proteins was induced by adding sterile filtered 0.5 M $CuSO_4$ to a final concentration of 0.5 mM. After a total incubation time of 20 h cultures were harvested at 5000 rpm for five minutes in the Avanti J-20XP Centrifuge in the rotor JA-10. Supernatant was discarded and the cell pellet was resuspended in about 100 ml of 10 mM Tris/Cl buffer, pH 7.4. Cells were once more pelleted at 5000 rpm for five min. For cell disruption in a Merckenschlager MSK homogenizer, cells were resuspended in about 30 ml of 10 mM Tris/Cl buffer, pH 7.4, and transferred into a Merckenschlager glass vessel filled with glass beads in a ratio of 1:2, v/v to cell suspension. Cell disruption was carried out for three to four min with cooling every 45 to 60 s. Supernatant was transferred to 50 ml centrifuge tubes and cell nuclei and cell debris were removed by centrifugation at 3500 rpm for five min in the rotor JA-25,50. Then, supernatant was centrifuged for 10 min at 20,000 rpm to remove mitochondria. Remaining microsomal fraction was collected from the supernatant by ultracentrifugation at 45,000 rpm for one h in Optima LE-80K Ultracentrifuge in the rotor SW-41. The pellet was resuspended in 1.2 ml of 10 mM Tris/Cl buffer, pH 7.4, and transferred into a Dounce homogenizer. Aliquots of 200 µl of homogenate were transferred into Eppendorf tubes and stored at -20°C.

4.15 Protein Analysis: Cell disruption, SDS-PAGE and Western Blot

Expression of HATs as GST fusions was verified by immunological detection of the GST tag. 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, 7 OD_{600} units of an overnight culture of each sample were harvested. The pellets were resuspended in 300 µL of 1.85 M NaOH/7.5% (v/w) β-mercaptoethanol and incubated for ten min on ice. Upon addition of 300 µL of 50% TCA, the samples were mixed thoroughly and incubated for additional 30 min on ice. Protein was pelleted at 10,000 rpm for five min and the supernatant was removed with a micropipette. For neutralization of the TCA, the pellet was washed once with 1 mL of ice cold water and then resuspended in a mixture of

66 µL of NuPage sample buffer 1x,

33 µL of 1M Tris Base and

2 µL of β- Mercaptoethanol

per pellet.

The proteins were denatured for five min at 95°C prior to loading 5 to 10 µL on a NuPAGE SDS Gel (4-12% Bis-Tris Gel), following standard protocols.

For Western Blot analysis the blotting sandwich was built up as shown in Figure 11. Transfer was performed for up to one h with a current of 400 mA as the limiting variable.

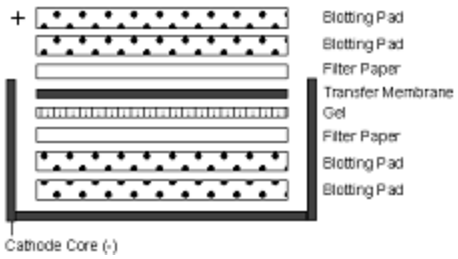


Figure 11: Scheme for Western Blot assembly, (copyright by Invitrogen)

The membranes were stained with Ponceau S solution, a sodium salt of a diazo dye that reversibly stains protein bands to check if protein transfer to the membrane has 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 or for one h at room temperature. This step was followed by rinsing the membrane with 1xTBST before applying a 1:1000 working solution of the primary antibody, i.e. polyclonal anti-GST produced in rabbit, on the membrane and incubating over night at room

temperature and moderate shaking. After washing the membrane three times for five min with 1xTBST, the secondary antibody, i.e. goat anti-rabbit IgG, horseradish peroxidase conjugated, was applied in a 1:5000 dilution for one h at room temperature and moderate shaking. After repeating the washing steps three times for five 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. This working solution was protected from sunlight and used immediately after preparation. For one membrane, 5 mL of the working solution were applied with a micropipette directly onto the membrane and incubated for one to eight min. During incubation, chemiluminescence signals were detected with the G:Box Bioimager.

4.15.1 Coomassie blue staining of SDS-PAGE

To visualize protein bands on SDS-PAGE by Coomassie blue the Roti-Blue Colloidal Coomassie staining solution was used. Gels were incubated overnight in the staining solution and washed with 10% aqueous acetic acid solution for half an hour to remove the background and an image was taken immediately.

4.16 Lipid extraction

Lipids were extracted by the method of Folch *et al.* (1957). To get equal amounts of each strain, 100 to 200 OD_{600} units of early stationary cultures were harvested in Pyrex glass tubes. The cell pellets were resuspended in 4 mL $CHCl_3$:MeOH (2:1, v/v) and agitated in the presence of glass beads for one h at room temperature on a Vibrax at full speed to extract the total lipids. After sedimenting the glass beads at 1500 rpm for 3 min the crude lipid extract was transferred to a new Pyrex tube. Proteins and non-polar substances were removed by sequentially washing the organic phase with 2 mL of 0.034% $MgCl_2$, 2 mL of 2 N KCl:MeOH (4:1, v/v) and 1.5 mL of $CHCl_3$:MeOH:H_2O (3:48:47, per vol.). Each washing step consisted of 2 min of vortexing and subsequent centrifugation to separate the phases. The upper, aqueous phases were always discarded. In cases when lipid quantification or purity was irrelevant washing with KCl: MeOH and $CHCl_3$:MeOH:H_2O was omitted.

The lipid extracts were dried under a stream of nitrogen and finally dissolved in 50 µL of $CHCl_3$:MeOH (2:1, v/v). These lipid extracts were used for TLC analysis or stored at -20°C immediately.

4.17 Thin layer chromatography

To analyse the ratio between free sterols and steryl esters, a two step separation on Silica TLC plates was carried out. Therefore, lipid extracts were automatically spotted onto 20 x 10 cm silica plates. In parallel, the first solvent mixture, petroleum ether/diethyl ether/acetic acid (49:49:2 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, the same plates were developed in a second, pre-equilibrated chamber using petrol ether/diethyl ether (49:1, v/v) as a solvent mixture until the solvent front reached the top of the plate.

For a better separation of triglycerides and steryl ester one eluent, petrol ether/diethyl ether/acetic acid (90:10:1 per vol.), was applied (Assmann *et al*., 1975).

Bands of free sterols, steryl esters, triglycerides and squalene were visualized by charring or iodine vapour if further processed. TLC plates were 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 seconds and then heated at 105°C for 40 min.

4.18 Analysis of sterols and steryl esters subsequently to Silica TLC

To evaluate composition of sterol and steryl ester fractions, respective bands on TLC were analysed. Lipid extract of 200 to 360 OD_{600} units or 7.5 mL of 10 mg/mL stock solutions of sterol and steryl ester standards were separated on TLC. Reversible visualization was achieved by incubation of the TLC plate in iodine vapour chamber until bands were clearly visible, which took about 3 min. After relevant bands were labelled, iodine was volatilized by moderate heat from a blow dryer. The TLC plate was sufficiently moistened with dH_20 to scrape off relevant bands and transfer them into Pyrex tubes.

The following protocols as preparations for GC/MS analysis were examined for evaluation of free sterol and steryl ester composition, respectively.

4.18.1 Saponification of steryl esters directly on silica gel

The scraped off silica gel was prepared for GC/MS as described in "4.19 GC/MS". Silica gel was processed like cell pellets.

4.18.2 Extraction of steryl esters before saponification

For this purpose, 7.5 µL of 10 mg/mL cholesterol, cholesteryl oleate and cholesta-5,7,24-trienol instead of cell lipid extracts were processed like described above. Sterols were extracted from silica gel with 1.5 mL of n-heptane or $CHCl_3$:MeOH (2:1, v/v) respectively, for one h under agitation on a Vibrax. Silica gel was sedimented by spinning for 10 min at 4000 rpm and supernatant was transferred to a new Pyrex tube. The extract was dried under a stream of nitrogen before GC/MS preparation as described in "4.19 GC/MS".

4.18.3 Purification through cotton wool after extraction of steryl esters from silica gel

(Recommendation by Tanja Wrodnigg, Institute of Organic Chemistry, Graz University of Technology, obtained by personal communication)

Cholesteryl oleate standard and cholesta-5,7,24-trienol were processed like described above (0 to 4.18.2) with the difference that a purification step was incorporated after extraction from silica gel. Extracts were filtered through cotton wool filled into a Pasteur pipette. The extract was dried under a stream of nitrogen before GC/MS preparation as described in "4.19 GC/MS". The filtered extracts were dried under a stream of nitrogen before GC/MS preparation as described in "4.19 GC/MS".

4.19 GC/MS

For GC/MS, samples were prepared by harvesting 15 OD_{600} units of ONC in Pyrex glass tubes. The culture medium was discarded, and the pellets were suspended in 0.6 mL methanol, 0.4 mL of 0.5% pyrogallol dissolved in methanol and 0.4 mL of 60% KOH each. After addition of 5 µL of 2 mg/mL internal cholesterol standard, the pellets were vigorously vortexed and heated for two h in a sandbath at 90°C. After cooling to room temperature, saponified lipids were extracted three times with 1 mL of n-heptane. For good extraction efficiency, the samples were vortexed for one min. The phases were separated by centrifugation at 1500 rpm for 3 min. The upper phases were collected in a new Pyrex tube, while the lower phases were re-extracted. The combined upper phases of each sample were dried under a stream of nitrogen. The extracted sterols were dissolved in 10 µL of pyridine and derivatized with 10 µL of N'O'-bis(trimethylsilyl)-trifluoracetamid to improve volatility. Then, the samples were diluted in 50 µL ethyl acetate and, subsequently, analysed 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 14.

Table 14: 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), range of 10°C/min to 250°C (0 min) and range 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

5 Results

5.1 Expression of HATs as GST-fusion proteins from pYEX4T-1

The high copy number plasmid pYEX4T-1 was chosen for the over-expression of HATs. A high expression level should ensure for sufficient enzyme activity to detect differences in steryl ester formation within the cell and to reveal acyltransferase specificities. In terms of toxicity high level expression seemed unproblematic as human ACAT1 was already shown to be harmless when over-expressed in *S. cerevisiae* (Yang, 1996). By providing a GST-tag the pYEX4T-1 simplifies immunological detection as expression of all HAT fusion proteins can be detected at the same time by the same antibody. Furthermore, GST-tagging could provide a tool for purification, thus enabling detailed analysis of HATs.

The original strategy was the transformation into the CEN.PK2 *erg5erg6are1are2* quadruple knockout. As the strain was not available at the time of this Master thesis, pYEX4T-1 plasmids carrying HAT encoding genes were transformed into a FY834 *MATa are1are2* double knockout and later on into a CEN.PK2 *erg6are1are2* triple knockout strain.

The strain FY834 *MATa are1are2* was kindly provided by Prof. Dr. Günther Daum, Institute of Biochemistry, Graz University of Technology. The knockout of the two sterol-O-acyltransferase encoding genes leads to the total lack of steryl esters in this strain (Figure 15).

Once detection of GST by Western blotting was established, immunodetection revealed proteins of variable sizes in several experiments and bands corresponding to complete protein mass were not observed. Nevertheless, analysis of lipid composition by TLC and subsequent charring revealed the presence of steryl esters, which implies the activity of the heterologous acyltransferases (Figure 15). Protein expression of the strain to express GST-Are2p from *C. albicans* which showed highest activity in sterol ester formation was also examined by SDS-PAGE and subsequent Coomassie blue staining (Figure 12).

5.1.1.1 SDS-PAGE, Coomassie blue staining and Western blot

Table 15: Lane description of Figure 12, Figure 13 and Figure 14

Sample	Protein	Source organism	Mass of GST fusion protein [kDa]
EV	empty vector control		27,5
120	GST-Acat1p	*Homo sapiens*	89
121	GST-Acat2p	*Rattus norvegicus*	85
122	GST-Acat1p	*Rattus norvegicus*	89
123	GST-Are2 p	*Candida albicans*	96
124	GST-Acat1p	*Toxoplasma gondii*	97
Sc.are2	GST-Are2p	*Saccharomyces cerevisiae*	99
GST-F	Positive control, a GST-fusion protein of unknown function and size obtained by Dr. Regina Leber		
Std.	Page Ruler Prestained Protein Ladder (Fermentas)		
t_0	Time point before $CuSO_4$ induction		
t_x	Time point x h after $CuSO_4$ induction		
R- t_x	Samples from a fermentation carried out by Dr. Regina Leber		

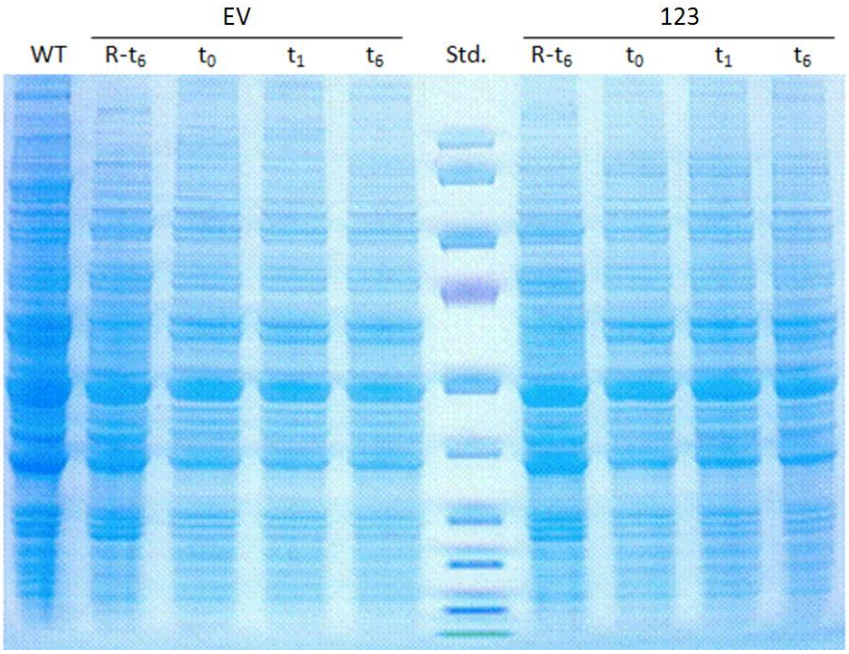


Figure 12: Coomassie blue staining of SDS-PAGE

Coomassie blue staining did not show any apparent change in protein expression within 6 hours after induction (Figure 12).

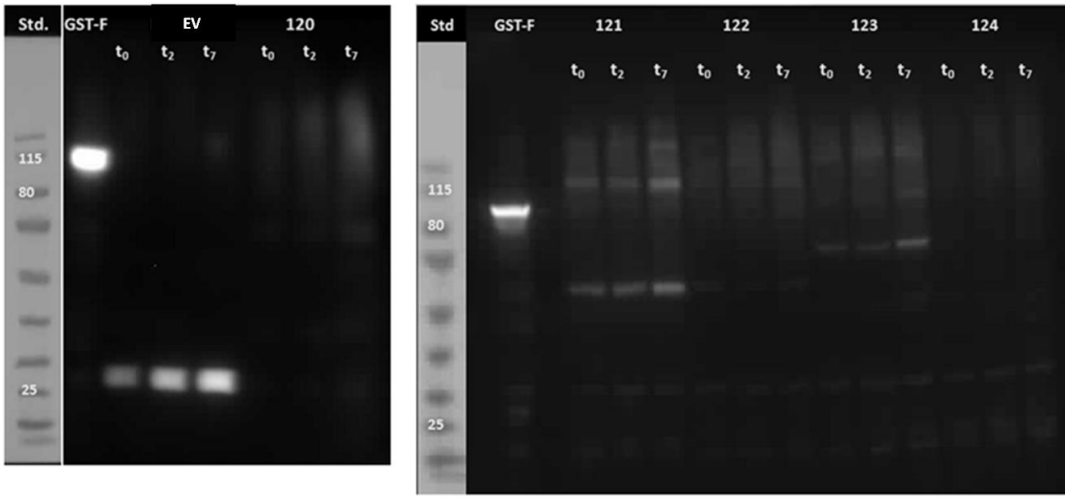


Figure 13: Western blot of GST-HAT fusion proteins after 2 and 7 hours of induction

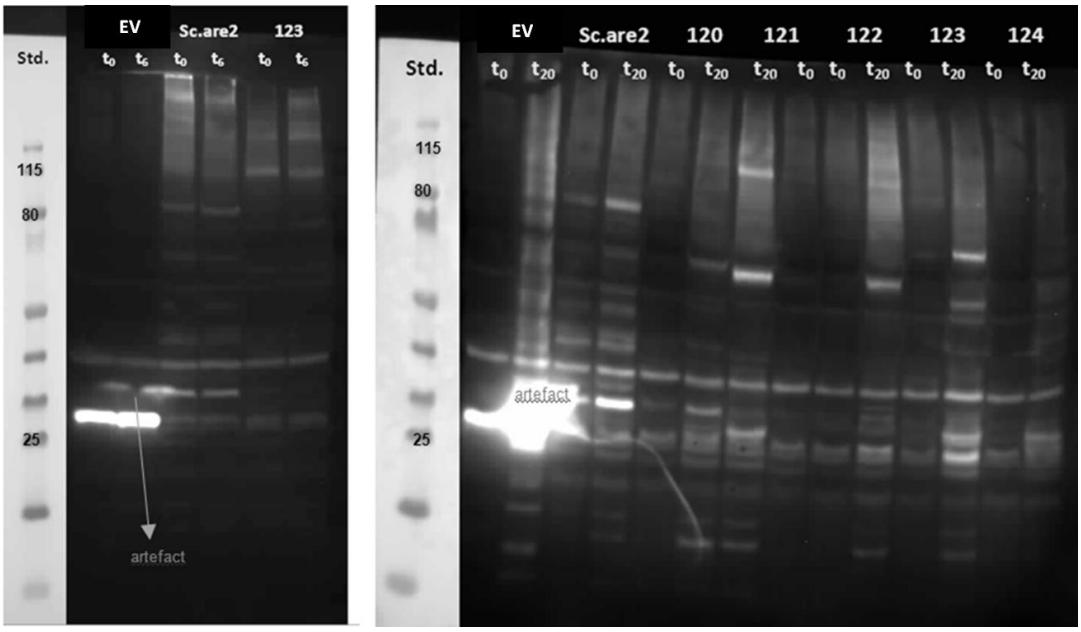


Figure 14: Western blot of GST-HAT fusion proteins after 20 hours of induction

As revealed by immunodetection, full-length expression of GST-HAT fusion proteins was not found for any strain. To elucidate if expression of HATs is negatively influenced by GST-tagging, *ARE2* was amplified from CEN.PK2-1C genome by PCR and arranged within the same vector construction. Sequencing confirmed correct gene amplification. Moreover, the pYEX4T-1 vector harboring the wild type *ARE2* was used as a reference for further analysis procedures. Results of the subsequent Western blot supported the assumption that GST-tagging leads to detected fragments of variable size as the GST-Are2p fusion protein from *S. cerevisiae* showed a similar fragmentation pattern as the likewise tagged heterologous acyltransferases (Figure 14).

5.1.1.2 Analysis of lipid composition by TLC

To estimate activities of GST-HAT fusion proteins, steryl ester formation was evaluated. For this purpose, 150 OD_{600} units were harvested after 20 h of induction with $CuSO_4$ to a final concentration of 0.75 mM and their lipid extracts were separated on TLC.

Table 16: Lane description of Figure 15

Lane	Sample		OD_{600}
1	empty vector in FY834 *MATa are1are2*		150
2	GST-Are2p	*Saccharomyces cerevisiae*	
3	GST-Acat1p	*Homo sapiens*	
4	GST-Acat2p	*Rattus norvegicus*	
5	GST-Acat1p	*Rattus norvegicus*	
6	GST-Are2p	*Candida albicans*	
7	GST-Acat1p	*Toxoplasma gondii*	
Std.	Cholesteryl oleate		15 µg
	Triolein		
	Cholesterol		10 µg

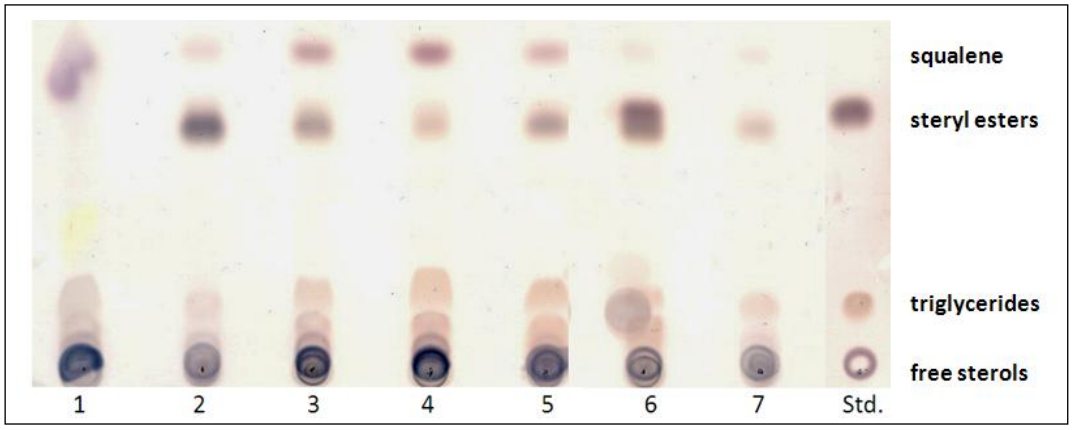


Figure 15: TLC showing esterification by heterologous acyltransferases, TLC was developed in petrol ether/diethyl ether/acetic acid (90:10:1 per vol.)

All strains harbouring HAT-GST fusion proteins showed steryl ester formation, however to a different extent. For the strains that produced less steryl esters an increased amount of squalene was observed except for the strain expressing *T. gondi ACAT1*. As expected the over-expression of endogenous GST-Are2p from *S. cerevisiae* yielded the highest level of steryl esters, which was matched by the amount of steryl esters formed by the strain habouring *C. albicans* GST-Are2p. Efficiency in steryl ester formation by yeast enzymes is followed by human GST-Acat1p and GST-Acat2p from *R. norvegicus*. GST-Acat1p from *T. gondii* and GST-Acat2p from *R. norvegicus* showed

lowest esterification rate. Interestingly, the strain expressing GST-Are2p from *C. albicans* was the only one to show two bands for the steryl ester fraction. The same phenomenon was also observed when developing the TLC using a two solvent system. It is assumed that the two bands correspond to different types of steryl esters. However, as it is not possible to determine the molecular species composition of the steryl ester fraction so far, this assumption waits to be proven.

It has to be taken into account that a high efficiency of esterification of main wild type sterols does not necessarily reflect the situation for the sterol of interest, cholesta-5,7,24-trienol. Therefore, a strain to express cholesta-5,7,24-trienol or an *in vitro* assay is necessary to reveal esterification efficacy and specificity of HATs towards the target sterol.

5.1.2 Transformation into CEN.PK2 *erg6are1are2*

An *erg6are1are2* knockout strain was obtained from tetrad dissection and was used for the transformation with the pYEX4T-1 vector constructs. The knockout of *erg6* was observed to show a similar if not identical sterol pattern as the *erg5erg6* double knockout (Figure 17) as long as sterol production is not pushed by the insertion of the truncated version of HMG (Martin Lehmann, DSM, personal communication).

During this project strain constructing a strain devoid of endogenous sterol-O-acyltransferase genes did not work out. Although the knockout of *are1* and *are2* was confirmed by cPCR for several CEN.PK2 knockout strains, i.e. an *erg6are1are2* triple knockout, these strains still showed esterification of sterols as revealed by TLC of its lipid extracts (Figure 16).

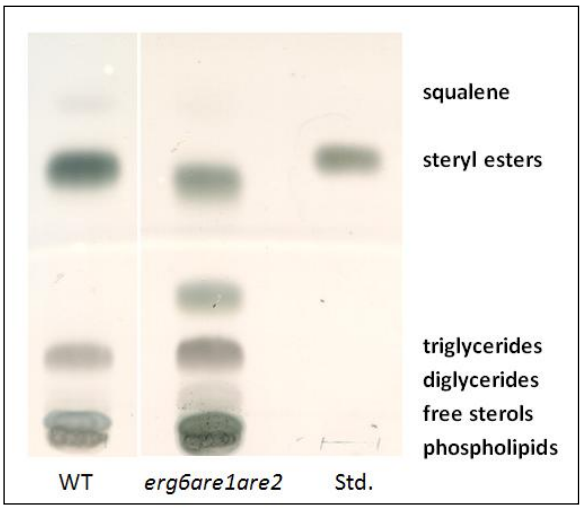


Figure 16: TLC of *erg6are1are2* showing presence of steryl esters (data provided by Birgit Ploier)

However, total sterol composition significantly changed by the supposed knockout of *are1* and *are2* genes leading to a clear shift in total sterol composition from zymosterol to cholesta-5,7,24-trienol. As shown in Figure 17 and Figure 18 deleting *are1* and *are2* in an *erg6* background lowered zymosterol levels and concomitantly increased the concentration of cholesta-5,7,24-trienol.

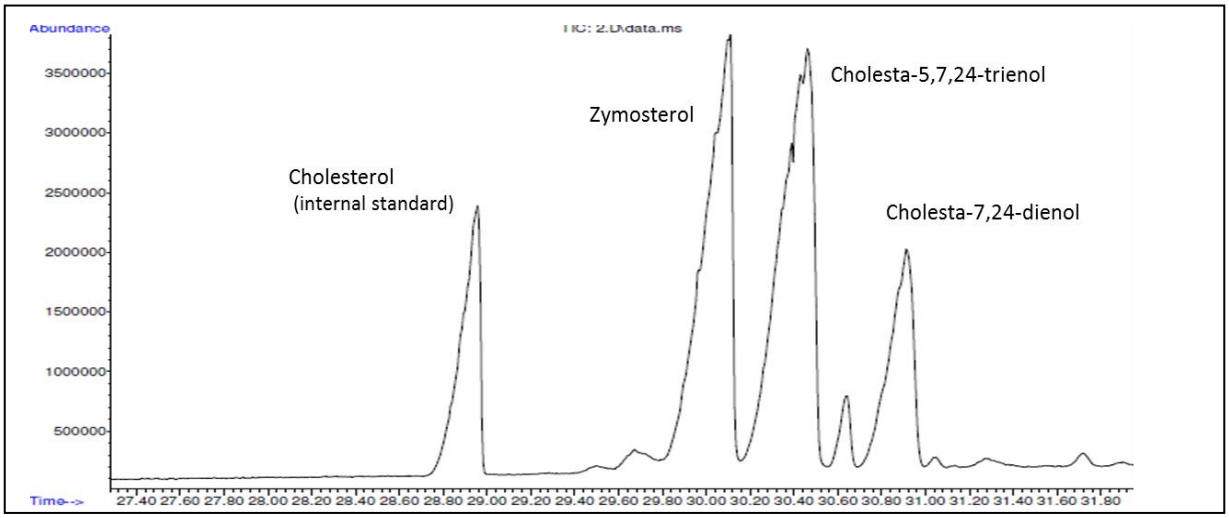


Figure 17: GC/MS chromatogram of *erg6* (data provided by Birgit Ploier)

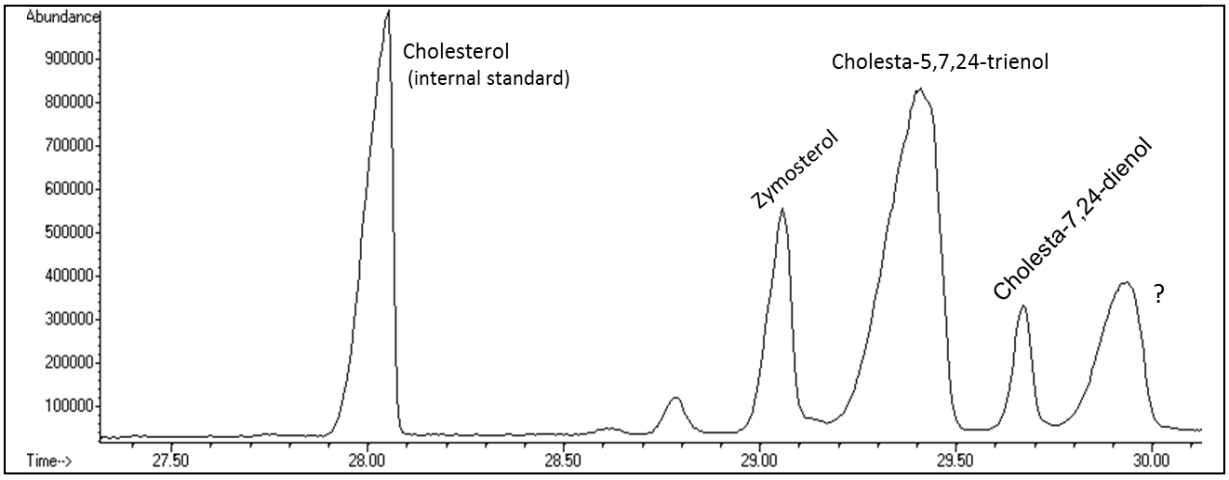


Figure 18: GC/MS of *erg6are1are2* with empty vector (pYEX4T-1)

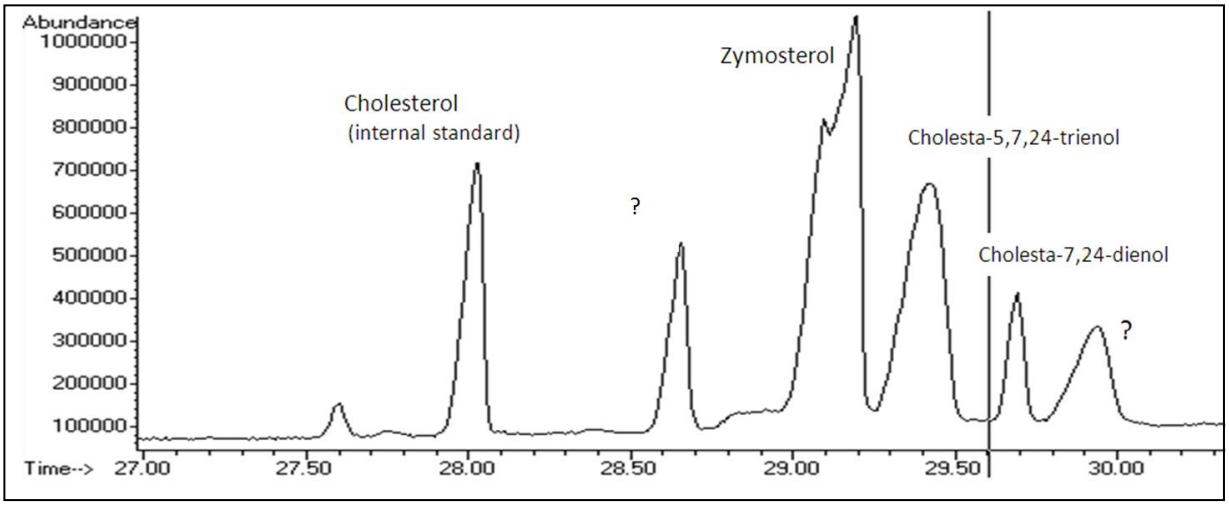


Figure 19: GC/MS of *erg6are1are2* with vector expressing GST-Are2p of *S. cerevisiae*

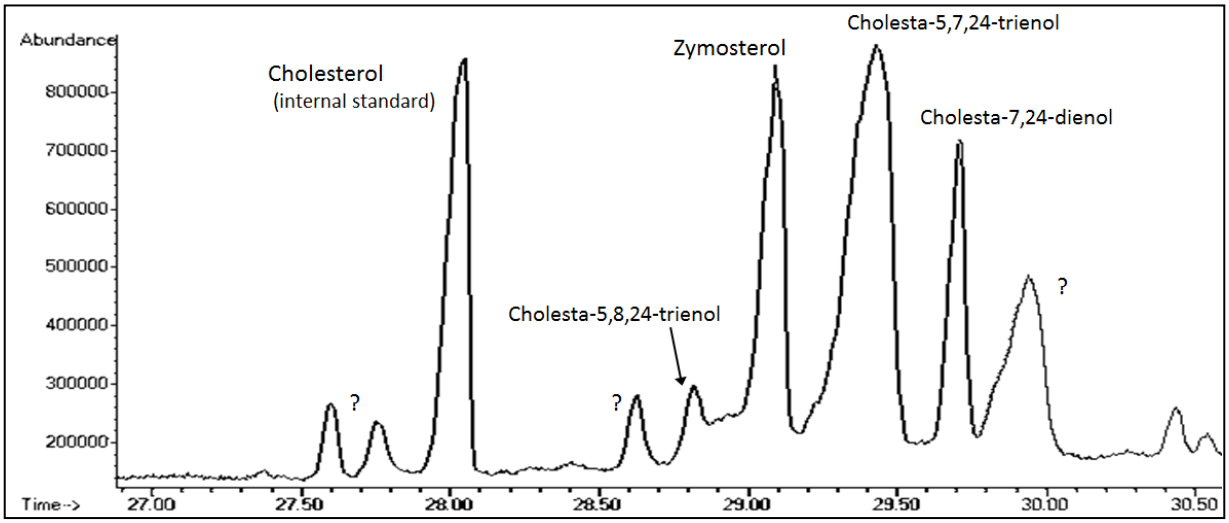


Figure 20: GC/MS of *erg6are1are2* with vector expressing GST-Acat1p of *H. sapiens*

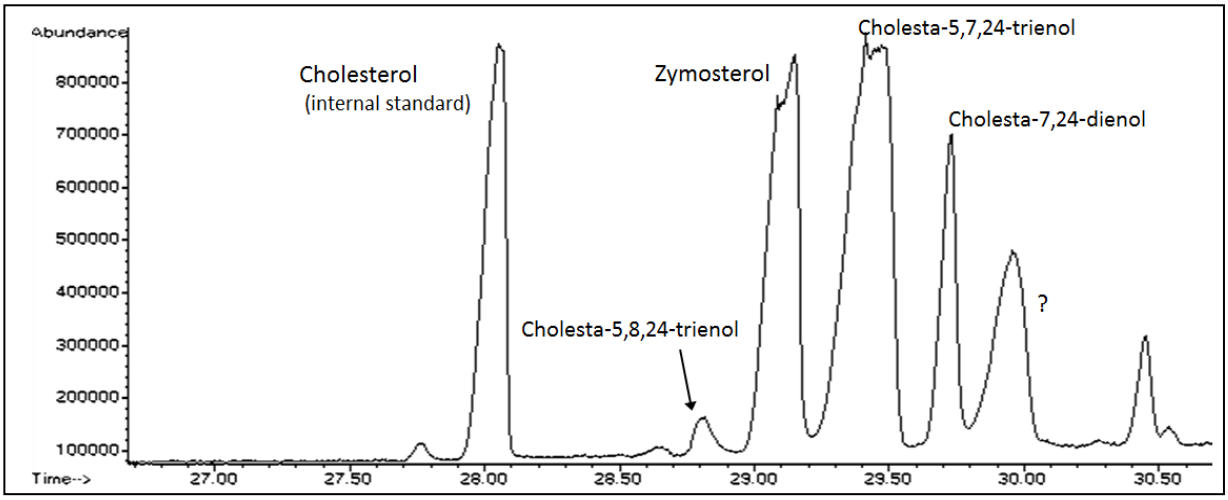


Figure 21: GC/MS of erg6are1are2 with vector expressing GST-Acat2p of *R. novegicus*

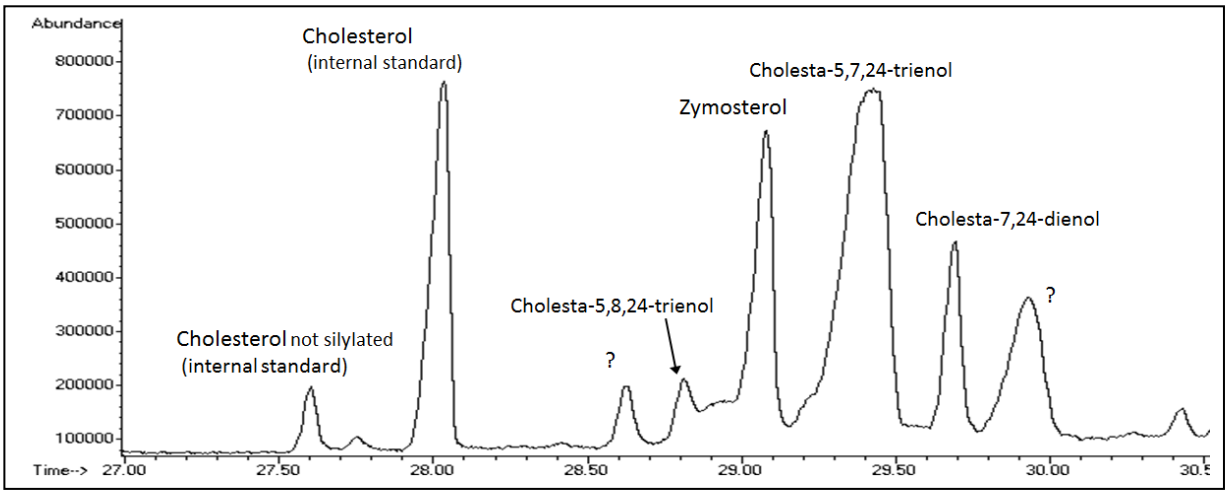


Figure 22: GC/MS of *erg6are1are2* with vector expressing GST-Acat1p of *R. novegicus*

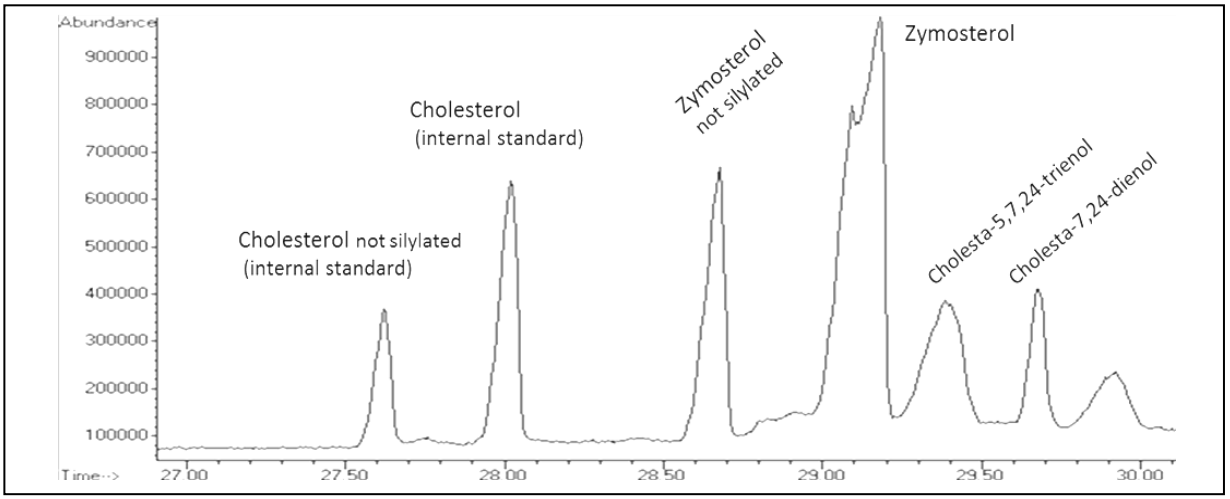


Figure 23: GC/MS of *erg6are1are2* with vector expressing GST-Are2p of *C. albicans*

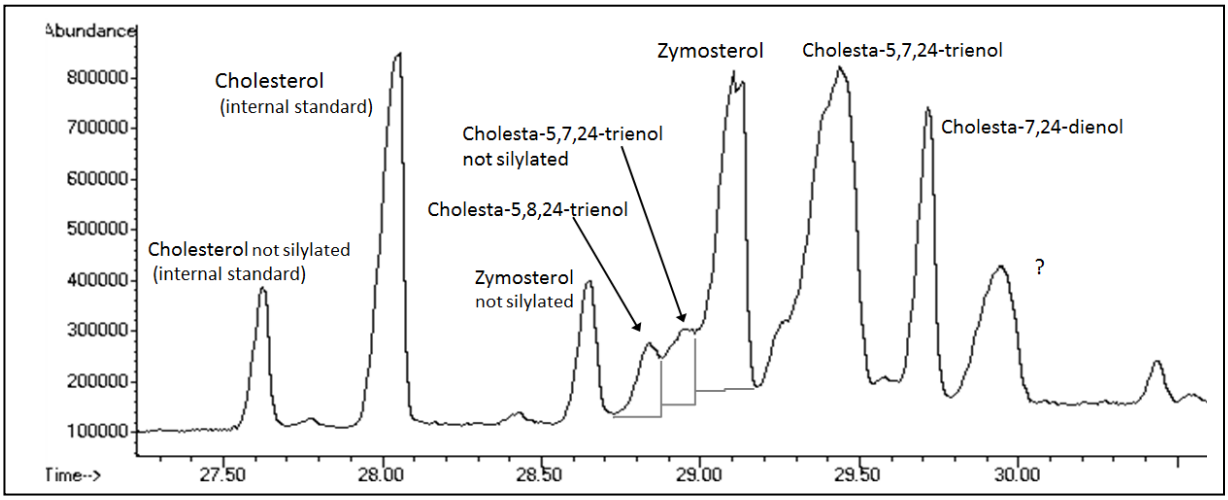


Figure 24: GC/MS of erg6are1are2 with vector expressing GST-Acat1p of *T. gondii*

To quantitatively evaluate sterol contents, peak areas were calculated by integration and compared to the internal standard cholesterol.

Table 17: Sterol content

		m [µg]/ 15 OD units		Ratio [%]	
Protein in GST-fusion	**Source organism**	**trienol**	**zymosterol**	**trienol**	**zymosterol**
empty vector		**15**	**4**	**78**	**22**
Are2p	*S. cerevisiae*	16	27	37	63
Acat1p	*H. sapiens*	17	7	70	30
Acat2p	*R. norvegicus*	20	13	60	40
Acat1p	*R. norvegicus*	19	7	74	26
Are2 p	*C. albicans*	6	28	18	82
Acat1p	*T. gondii*	15	10	59	41

Table 18: Change of sterol pattern in comparison

		Ratio		Change of sterol content*		
Protein in GST-fusion	**Source organism**	**trienol**	**zymosterol**	**trienol**	**zymosterol**	**overall**
empty vector		**3.6**	**1**	**1**	**1**	**1**
Are2p	*S. cerevisiae*	0.6	1	1.1	6.5	3.8
Acat1p	*H. sapiens*	2.3	1	1.1	1.8	1.4
Acat2p	*R. norvegicus*	1.5	1	1.3	3.1	2.2
Acat1p	*R. norvegicus*	2.8	1	1.3	1.6	1.4
Are2 p	*C. albicans*	0.2	1	0.4	6.8	3.6
Acat1p	*T. gondii*	1.4	1	1.0	2.5	1.7

*normalized to empty vector strain

By comparison of sterol contents the influence of heterologous acyltransferase fusion proteins was evaluated (Table 17 and

Table 18). Expression of any HAT resulted in an decreased ratio of cholesta-5,7,24-trienol to zymosterol compared to the empty vector control. For some transformants total amount of cholesta-5,7,24-trienol was increased up to 1.3 fold compared to the empty vector control. In all cases the overall sterol content was increased by HAT activity, for some, however only insignificantly. The increase in total sterol content correlated with the decrease in the cholesta-5,7,24-trienol to zymosterol ratio suggesting that it was particularly the surplus of zymosterol that was esterified. The strongest effects were observed in the case of GST-Are2p from *S. cerevisiae* and for GST-Are2p of *C. albicans* showing highest augmentation in zymosterol content with a raise of 6.5-fold and 6.8-fold,

respectively. By the expression of GST-Are2p from *S. cerevisiae* no significant change of the cholesta-5,7,24-trienol content was observed. For the strain expressing GST-Are2p of *C. albicans* even a decrease of cholesta-5,7,24-trienol content of 60% was calculated suggesting a substrate preference for zymosterol.

Sterol-O-acyltransferases with supposedly less preference for zymosterol are the three enzymes of the higher eukaryotes *R. norvegicus* and *H. sapiens*. Thereof, expression of GST-Acat1p of *R. norvegicus* yielded highest amounts of cholesta-5,7,24-trienol with a raise of 1.3-fold, concomitantly showing the lowest level of zymosterol compared to the empty vector control. Although yielding the highest ratio of cholesta-5,7,24-trienol to zymosterol within transformants, the expression of rat GST-Acat1p decreased the ratio from 3.6:1 to 2.8:1.

Overall conclusions on the sterol specificity of sterol-O-acyltransferases are hampered by the mysteriously remaining esterification of sterols in the *erg6are1are2* host strain.

5.2 Microsome preparation and Western blot

With the intent to establish a microsomal assay to determine substrate specificities, microsomes of the FY834 *MATa are1are2* double knockout strain and of a transformant, harboring the pYEX4T-1 vector to express the Are2p of *C. albicans* in as N-terminal GST fusion, were prepared.

Table 19: Lane description of Figure 25

Lane	Sample	m [µg]	Protein size [kDa]
Std.	PageRuler Prestained Protein Ladder (Fermentas)		
1	*are1are2*	10	-
2		20	-
3		60	-
4	GST-Are2p of *C. albicans*	10	96
5		20	96
6		60	96

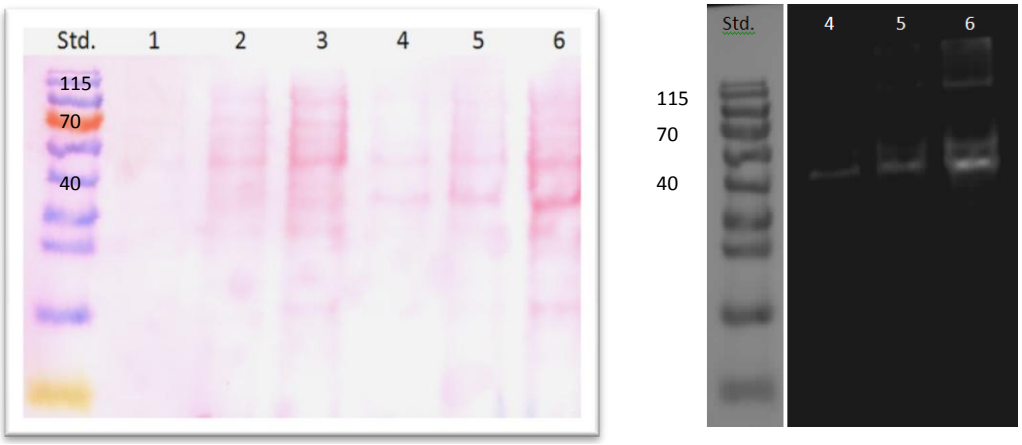


Figure 25: Microsome preparation: Ponceau staining (left) and chemiluminescent detection of GST-fusion protein (right)

Immunodetection of GST-Are2p from *C. albicans* in microsome preparation revealed that visible band variability was clearly minimized compared to the Western blot of total protein. That suggests that only protein that still carries the required transmembrane domains is held back in the microsomal fraction (Figure 25).

5.3 Genomic integration of HAT genes at the *ARE2* locus

Since the construction of the quadruple knockout was delayed by the unsuccessful deletion of *ARE2*, a CEN.PK2 *MATα erg5erg6are1* triple knockout strain was transformed with cassettes carrying HAT genes for the integration into the *ARE2* locus. The knockout of both sterol-O-acyltransferases had caused problems in the past, among others, because of the low transformation rate (Birgit Ploier, personal communication).

Therefore the idea arose to substitute the endogenous *ARE2* directly with the HAT genes. In this way also the probably critical situation of strains to lack sterol-O-acyltransferase activity completely would be circumvented.

5.3.1 Colony PCRs to verify integration of HAT genes

For the verification of positive transformants several primers had been designed upstream and downstream the *ARE2* substitution cassette (upstr1_fw & upstr2_fw), beside a specific primer for each of the corresponding heterologous acyltransferases (120_rv to 124_rv) and primers in the *URA3* marker region (ura1_fw & ura2_fw). Also primers inside the substitution cassettes (KA2up & KA2down) and for wild type *ARE2* (KA2rev) were used to verify integration and/or presence of the endogenous *ARE2* (Figure 26).

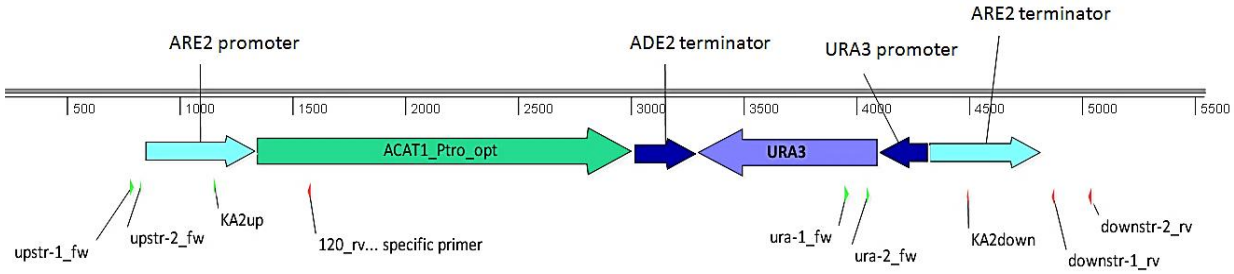


Figure 26: Primers inside and outside the substitution cassettes (coloured arrows) to verify integration of heterologous acyltransferases at the *ARE2* locus

As results of colony PCRs were unsteady and did not allow for a straight-forward selection of transformants, several procedures for cell disruption as well as different polymerases were tested. Zymolyase treatment of cells for cPCR turned out to be most adequate. In some cases, cPCRs neither showed positive results for the integration of a heterologous acyltransferase nor for the presence of the endogenous *ARE2* coding region. Here only results are shown that allowed sound conclusions.

The DNA polymerase used is indicated in the respective figure description. 20 µl of the PCR mix and 5 µl of standard were seperated by agarose gel electrophoresis. PCR cycling conditions can be found in the section Methods.

Table 20: General description of abbreviations for templates used in cPCRs

Template	**Description**		
erg5erg6are1	positive control for *ARE2* of *S. cerevisiae*, CEN.PK2 triple knockout strain		
are1are2	negative control for *ARE2* of *S. cerevisiae*, FY834 double knockout strain		
T-126-x	*ACAT1*	*Homo sapiens*	Transformants of substitution cassettes harboring indicated HAT genes
T-127-x	*ACAT2*	*Rattus norvegicus*	
T-128-x	*ACAT1*	*Rattus norvegicus*	
T-129-x	*ARE2*	*Candida albicans*	
T-130-x	*ACAT1*	*Toxoplasma gondii*	
x…	number given to transformant		

5.3.1.1 PCRs to test primer functionality

Primers were tested for their functionality with a plasmid as template, apart from the primers downstr1_rv and downstr2_rv as there was no adequate plasmid template available.

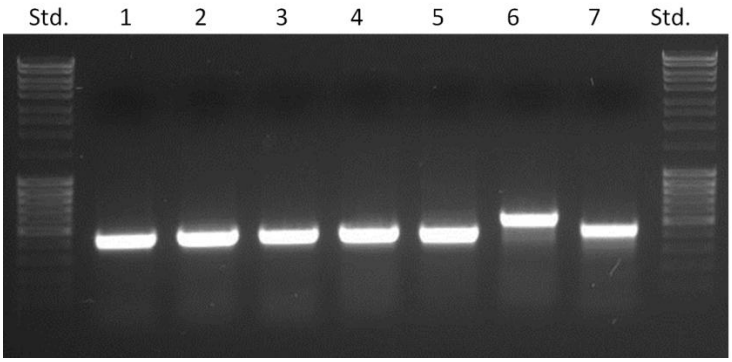


Figure 27: PCRs on plasmid DNA template to prove functionality of primers

Table 21: Lane description of Figure 27

PCR Nr.	Plasmid	Description: *ARE2* substitution cassette with indicated HAT gene		Primers	Expected fragment size [bp]
1	pHYD-0126	*ACAT1*	*H. sapiens*	KA2up + 120_rev	441
2	pHYD-0127	*ACAT2*	*R. norvegicus*	KA2up + 121_rev	441
3	pHYD-0128	*ACAT1*	*R. norvegicus*	KA2up + 122_rev	441
4	pHYD-0129	*ARE2*	*C. albicans*	KA2up + 123_rev	442
5	pHYD-0130	*ACAT1*	*T. gondii*	KA2up + 124_rev	442
6	pHYD-0125	*ARE2* substitution cassette without HAT gene		ura1_fw + KA2_down	560
7	pHYD-0125			ura2_fw + KA2_down	462

All tested primers showed fragments of expected sizes. Thus primer design was excluded as being responsible for faulty fragment amplification.

5.3.1.2 Colony PCRs to verify integration of the HAT cassettes using upstream primers

For this purpose two forward primes upstream the substitution cassettes were designed (upstr1_fw and upstr2_fw) and one primer inside the respective HAT coding sequence was obtained. Primers specific for HAT genes were designed after sequence alignments to *ARE2* of *S. cerevisiae* choosing regions of little sequence homology to achieve specific fragment amplification (see Appendix). Also a forward primer inside the *ARE2* promoter sequence was used to amplify HAT genes as well as the endogenous *ARE2* independently of their localization (KA2up).

For almost all cPCRs and all methods to disrupt cells as for different DNA polymerases, positive and negative controlls worked out perfectly showing defined bands at the expected sizes.

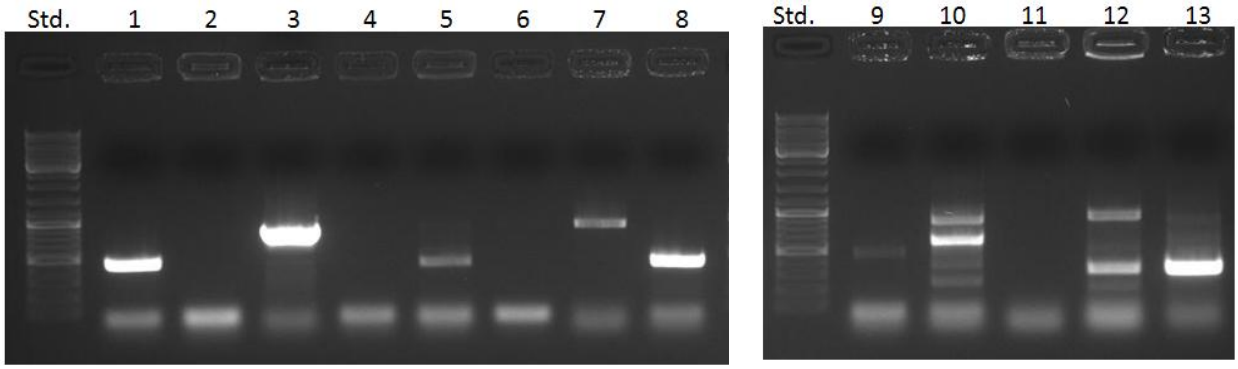


Figure 28: cPCRs with DreamTaq Green PCR Master Mix using primers upstream of *ARE2*

Table 22: Lane description of Figure 28

Lane	Template	Primers		Expected fragment size [bp]
Std.	GeneRuler™ DNA Ladder Mix (Fermentas)			
1	*erg5erg6are1*	KA2up	KA2rev	449
2	*are1are2*			x
3	*erg5erg6are1*	upstr1_fw	KA2rev	823
4	*are1are2*			x
5	T-129-4	upstr1_fw	KA2rev	823
6	T-129-4	KA2up	KA2rev	449
7	T-129-4	upstr1_fw	123_rv	813
8	T-129-4	KA2up	123_rv	442
9	T-126-5	upstr1_fw	120_rv	812
10	T-127-5	upstr1_fw	121_rv	812
11	T-128-5	upstr1_fw	122_rv	812
12	T-129-5	upstr1_fw	123_rv	813
13	T-130-1	upstr1_fw	124_rv	813

cPCR with the DreamTaq Green PCR Master Mix (Figure 28) resulted in one or several unspecific bands for all transformants. In case of T-129-4 the fragment suggesting a crossover event within the *ARE2* promoter site as well as the intrinsic fragment of the integration cassette could be amplified (Lane 7 and 8). The endogenous *ARE2* could not be amplified for this transformant. This seems to confirm a positive substitution event of the endogenous *ARE2*. Also T-129-5 showed the locus specific fragment, but also an unspecific amplicon which is suspicious as T-129-4 did not show that unspecific band. In general cPCRs with the DreamTaq DNA Polymerase apparently resulted in unspecific fragment amplification.

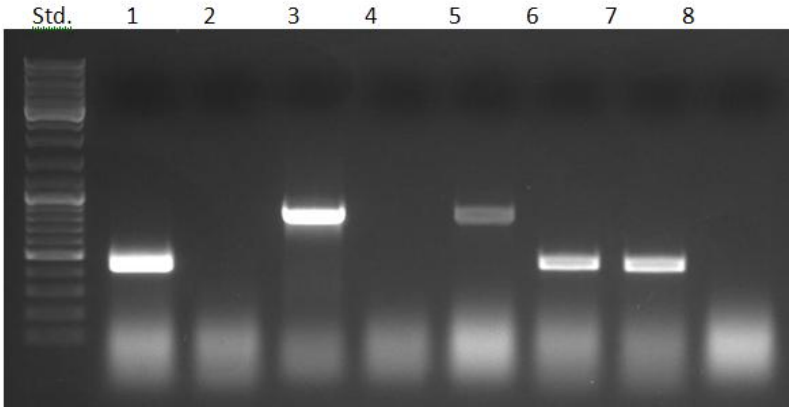


Figure 29: cPCRs of T-126-4 with DreamTaq DNA polymerase using primers upstream of *ARE2*

Table 23: Lane description of Figure 29

Lane	Template	Primers		Expected fragment size [bp]
Std.	GeneRuler™ DNA Ladder Mix (Fermentas)			
1	*erg5erg6are1*	KA2up	KA2rev	449
2	*are1are2*			x
3	*erg5erg6are1*	upstr1_fw	KA2rev	823
4	*are1are2*			x
5	T-126-4	upstr1_fw	KA2rev	823
6	T-126-4	KA2up	KA2rev	449
7	T-126-4	KA2up	120_rv	441
8	T-126-4	upstr1_fw	120_rv	812

For the transformant T-126-4 cPCRs revealed the integration of the respective substitution cassette by a single cross over event (Figure 29). Locus specific and unspecific fragments for the endogenous *ARE2* could be amplified while for the HAT cassette only intrinsic fragment was present. That means that the substitution of the endogenous *ARE2* had not been successful and both, endogenous as well as heterologous, sterol-O-acyltransferases were present in the genome.

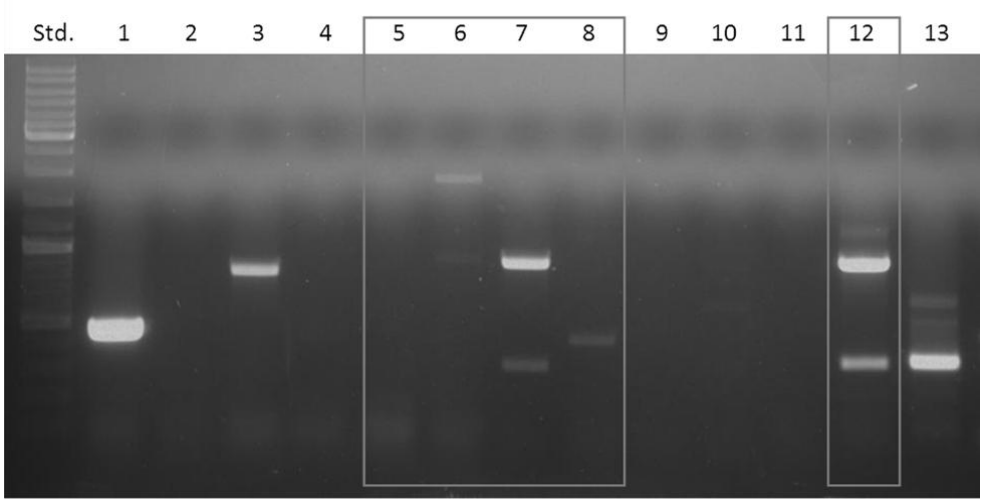


Figure 30: cPCRs with GoTaq polymerase using primers upstream of *ARE2*

Table 24: Lane description of Figure 30

Lane	Template	Primers		Expected fragment size [bp]
Std.	GeneRuler^{TM} DNA Ladder Mix (Fermentas)			
1	*erg5erg6are1*	KA2up	KA2rev	449
2	*are1are2*			x
3	*erg5erg6are1*	upstr1_fw	KA2rev	823
4	*are1are2*			x
5	T-129-4	upstr1_fw	KA2rev	823
6	T-129-4	KA2up	KA2rev	449
7	T-129-4	upstr1_fw	123_rv	813
8	T-129-4	KA2up	123_rv	442
9	T-126-5	upstr1_fw	120_rv	812
10	T-127-5		121_rv	812
11	T-128-5		122_rv	812
12	T-129-5		123_rv	813
13	T-130-1		124_rv	813

As in former cPCRs (Figure 28) locus specific and unspecific fragments were amplified for T-129-4 (Figure 30). The unspecific band for T-129-5 (Lane 12) was found again and now also for T-129-4 (Lane 7) and might refer to unspecific binding of a primer. For cPCRs on transformants T-126-5, T-127-5, T-128-5 site-specific primer combinations did not show any amplicon and T-130-1 showed an unspecific band of the same size as in the previous cPCR (Figure 28).

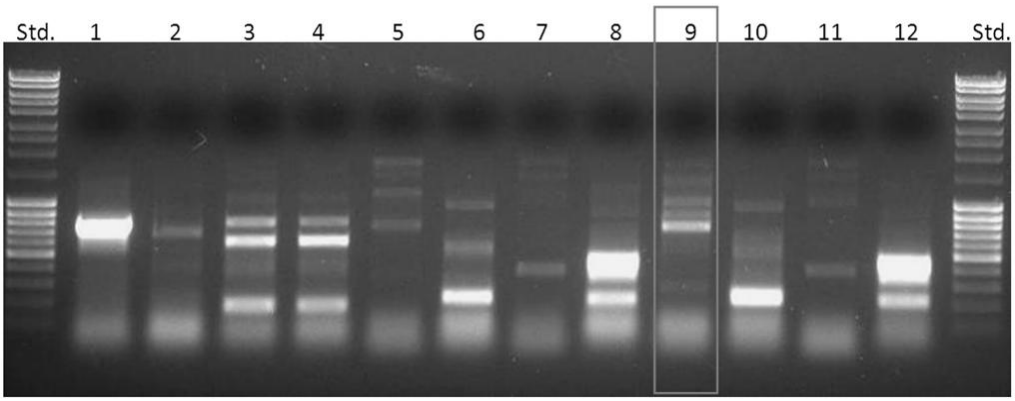


Figure 31: cPCR with Maxima Hot Start Green PCR Mix using primers upstream of *ARE2*

Table 25: Lane description of Figure Figure 31

Lane	Template	Primers		Expected fragment size [bp]
Std.	MassRuler™ DNA Ladder Mix (Fermentas)			
1	*erg5erg6are1*	upstr2_fw.	KA2rev	775
2	*are1are2*		KA2rev	x
3	T-129-4		123_rv	767
4	T-129-5		123_rv	767
5	T-126-6		120_rv	767
6	T-127-6		121_rv	767
7	T-128-6		122_rv	768
8	T-130-1		124_rv	768
9	T-126-7		120_rv	767
10	T-127-7		121_rv	767
11	T-128-7		122_rv	768
12	T-130-2		124_rv	768

As so far cPCRs were too unsteady to test as many transformants as would have been necessary, a second primer in the *ARE2* promoter region was ordered (upstr2_fw). cPCRs with this primer turned out to result in the amplification of unspecific fragments for all tested transformants (Figure 31). Substitution could not even be proven for T-129-4 and T-129-5 whose fragment patterns of former cPCRs already had suggested a positive integration event. Also the negative control, an *are1are2* double knockout, showed a slight band referring to a functional *ARE2* gene. Amplified fragments of transformants carrying the same HAT gene are consistent, e.g. T-127-6 and T-127-7 etc. The only one to show slight differences to his counterpart and a band of expected size is T-126-7, for which reason it was chosen for further analysis.

cPCRs with the upstr2_fw were only carried out with conditions and for the transformants shown above. At the same time GC/MS analysis of strains made further verification of integration events and therefore optimization of cPCR conditions redundant.

5.3.1.3 Colony PCRs to verify integration of the HAT cassettes using downstream primers

As some results to verify integration of the substitution cassettes indicated positive integration by a single crossover event upstream, two forward primers inside the *URA3* marker gene and two reverse primers downstream of endogenous *ARE2* were ordered (Figure 26). Four primer combinations were available to verify site-specific integrations.

As for the primer upstr2_fw, all conditions for cPCRs to verify crossover events in the *ARE2* terminator region were not further optimized. All cPCRs that were carried out are shown here.

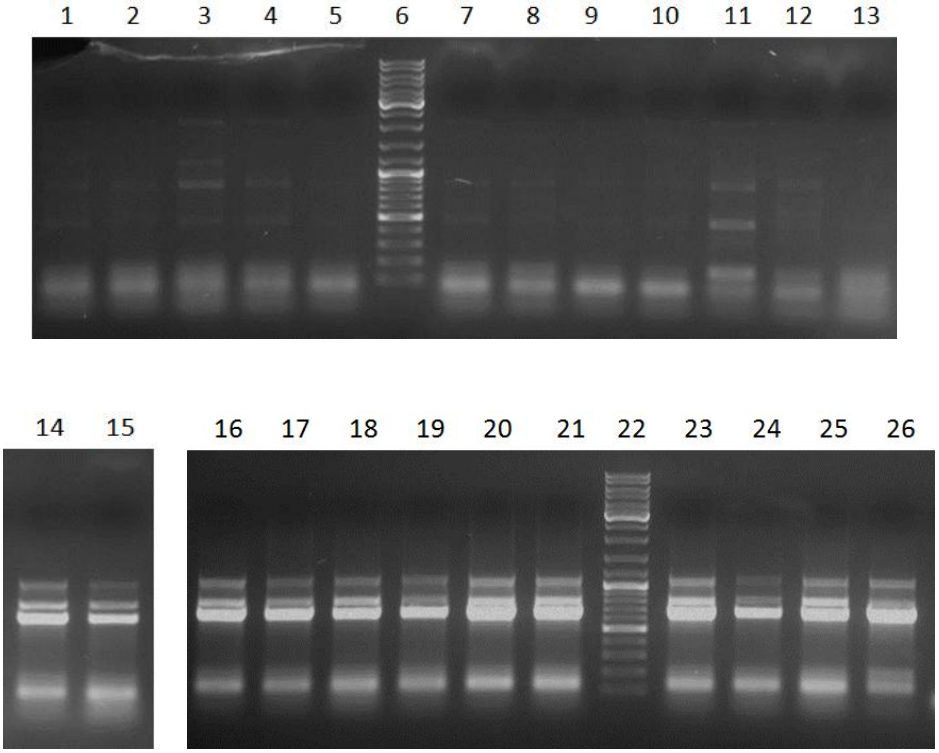


Figure 32: cPCR with Maxima Hot Start Green PCR Mix using primers downstream of *ARE2*

Table 26: Lane description of Figure 32

Lane	Description	Primers	Expected fragment size [bp]
1	T-129-4	ura1_fw + downstr-1_rv	941 bp
2	T-129-5		
3	T-126-6		
4	T-127-6		
5	T-128-6		
6	Gene Ruler™ DNA Ladder Mix		
7	T-126-7	ura1_fw + downstr-1_rv	941 bp
8	T-127-7		
9	T-128-7		
10	T-126-8		
11	T-127-8		
12	T-128-8		
13	T-130-2		
14	T-129-4	ura2_fw + downstr-2_rv	1007 bp
15	T-129-5		
16	T-126-6		
17	T-127-6		
18	T-128-6		
19	T-126-7		
20	T-127-7		
21	T-128-7		
22	Gene Ruler™ DNA Ladder Mix		
23	T-126-8	ura2_fw + downstr-2_rv	1007 bp
24	T-127-8		
25	T-128-8		
26	T-130-2		

Both primer combinations, i.e. ura1_fw + downstr-1_rv and ura2_fw + downstr-2_rv, did not result in the expected fragment amplification. For the primer combination ura1_fw + downstr-1_rv the visible bands in the expected range of fragment size is about 100 bp too small except for transformant T-127-8 which shows a slight band of correct size. In the case of primer combination ura2_fw + downstr-2_rv all tested transformant show same band pattern with variable intensities of unspecific fragment amplification.

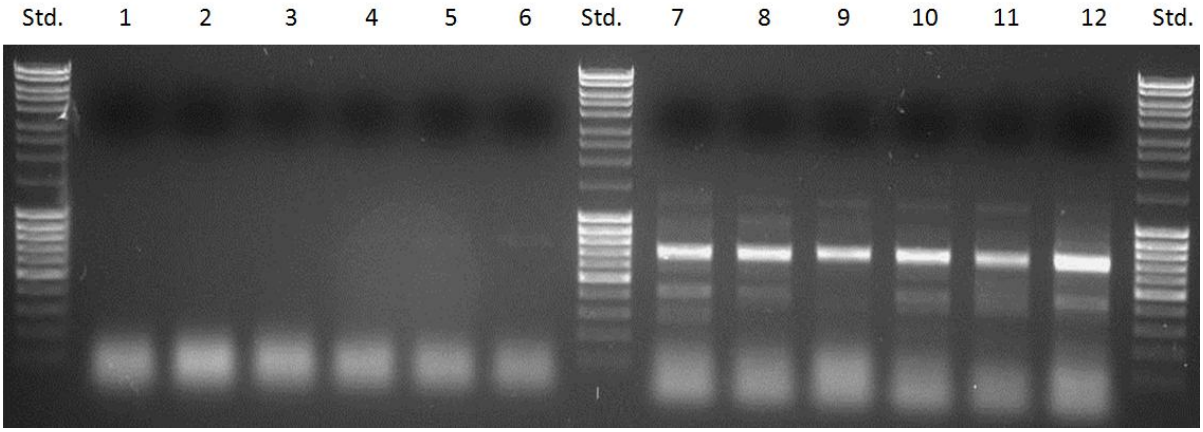


Figure 33: cPCR with Maxima Hot Start Green PCR Mix using primers downstream of *ARE2*

Table 27: Lane description of Figure 33

Lane	Template	Primers	Expected fragment size [bp]
Std.	MassRuler^{TM} DNA Ladder Mix (Fermentas)		
1	T-129-4	ura1_fw + downstr-2_rv	1106
2	T-129-5		
3	T-126-6		
4	T-127-6		
5	T-128-6		
6	T-130-2		
7	T-129-4	ura2_fw + downstr-1_rv	843
8	T-129-5		
9	T-126-6		
10	T-127-6		
11	T-128-6		
12	T-130-2		

Primer combination ura1_fw + downstr-2_rv did not result in any fragment amplification, whereas primer combination ura2_fw + downstr-1_rv produced a fragment to give high intensity which is about 100 bp too small to correspond to correct gene integration.

5.3.2 PCRs to elucidate integration events

Although cPCR results were not satisfactory, three transformants were chosen for more precise examination of possible integration events. Transformants T-126-7, T-129-4 and T-129-5 were chosen due to positive results (marked with grey frames in Figure 30 and Figure 31) in cPCRs that suggested crossover events upstream of *ARE2*, i.e. in the *ARE2* promoter site, for two transformants of *ARE2* of *Candida albicans* (T-129-4 and T-129-5, Figure 30) and for one of human *ACAT1* (T-126-7, Figure 31).

PCRs with Phusion polymerase on genomic DNA of mentioned strains should confirm correct upstream as well as downstream integration of substitution cassettes.

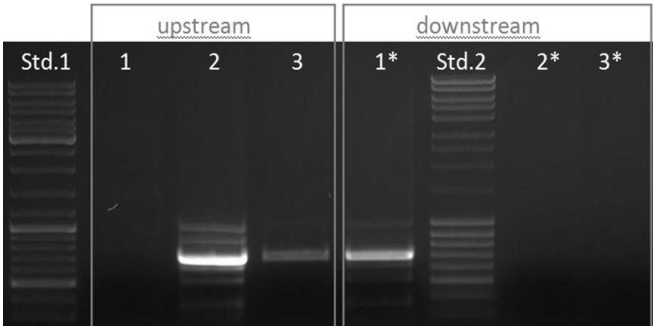


Figure 34: PCR on gDNA to verify integration of HATs

Table 28: Lane description of Figure 34

PCR	Template		Primer		Expected fragment size [bp]
1	T-126-7	*ACAT1 H. sapiens*	upstr-1_fw	120_rv	767
2	T-129-4	*ARE2 C. albicans*		123_rv	768
3	T-129-5	*ARE2 C. albicans*		123_rv	768
1*	T-126-7	*ACAT1 H. sapiens*	ura2_fw	downst-1_rv	843
2*	T-129-4	*ARE2 C. albicans*			
3*	T-129-5	*ARE2 C. albicans*			

In no case both fragments to verify upstream and downstream crossover events could be amplified. cPCR of human *ACAT1* using downstream primer resulted in a fragment that is about 100 bp smaller than calculated like it was seen as well in the cPCR shown in Figure 33. Although the band seems to indicate unspecific fragment amplification independently of the supposed integrated HAT gene (see Figure 33) it was not amplified for transformant T-129-4 and T-129-5. According to the amplified bands, crossover of substitution constructs at the *ARE2* promoter site occurred for transformants of *C. albicans ARE2* but cPCRs monitoring downstream crossover did not yield any bands. Obviously, rather insertion than substitution had occurred.

The whole fragment between *ARE2* promoter and termination site could not be successfully amplified which was probably due to its expected size of approximately 7 kbp.

5.3.3 GC/MS of probable positive transformants

In spite of the non-promising PCR results three transformants, T-129-5, T-129-5, T-126-7, were also characterized by GC/MS analysis as at least integration of HAT genes had been verified.

As the substitution cassettes of heterologous acyltransferases were transformed into an *erg5erg6are1* triple knockout strain, the main sterol components should only be zymosterol and cholesta-5,7,24-trienol (Figure 35). Anyway by GC/MS only one main compound was detected for all measured transformants, which was ergosterol (Figure 36). Only the GC/MS chromatogram of T-129-4 is shown as other transformants showed the same results.

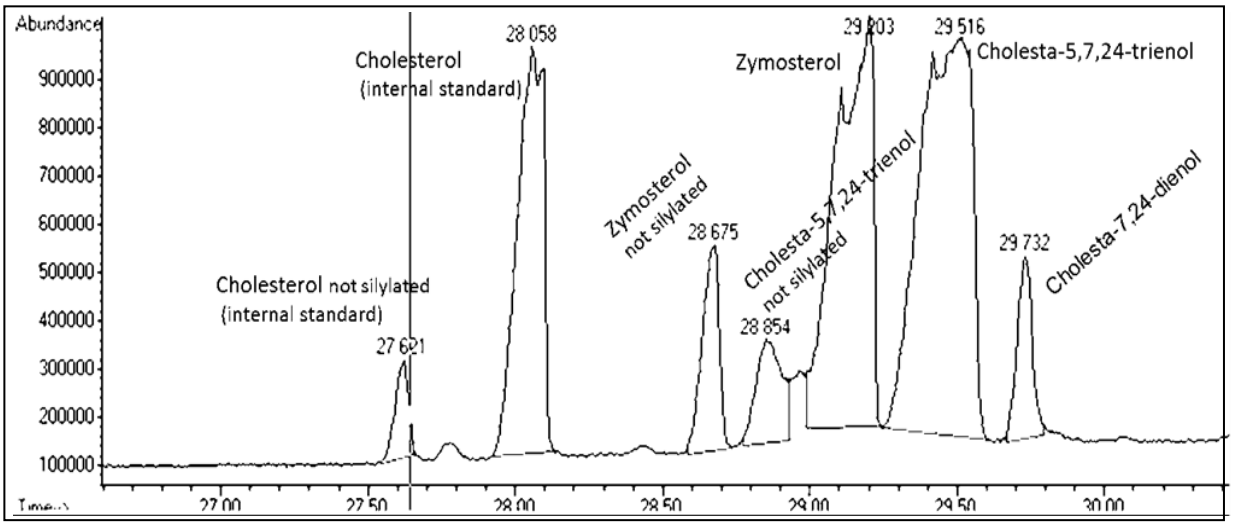


Figure 35: GCMS of CEN-PK2 *erg6erg5are1*

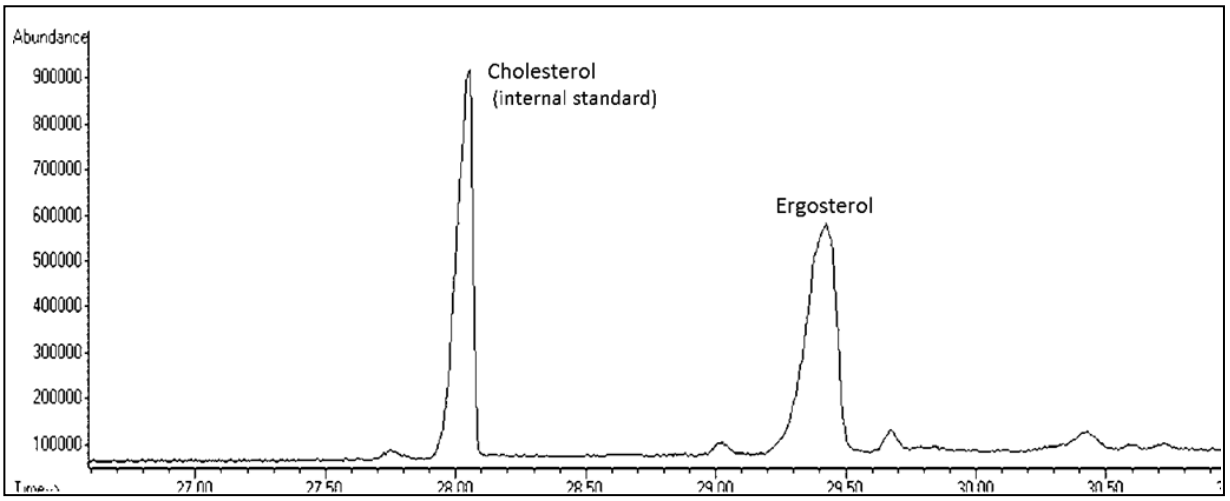


Figure 36: GC/MS chromatogram of T-129-4

As these results were not explainable, analysis was repeated with the transformants streaked out several times on different growth media. On the one hand, analysis was made for cells proceeding from YPD plates and on the other hand for cells proceeding from triple selective media (SD –trp –leu –his) and with its ONC for GC/MS grown in SD-ura. Growing on selective media should remove any possible impurities as only positive transformants should be able grow.

Selective growth conditions did not have any effect on sterol composition as former results, with ergosterol as the main sterol, were reproducible for cultivation on YPD as well as on SD –trp –leu –his.

5.3.3.1 Control of *ERG5* and *ERG6* disruption

Because of the unexplainable GC/MS results *ERG5* and *ERG6* disruption was controlled by cPCR. cPCRs were made for each of the transformants proceeding from full media on the one and selective media on the other hand as described in 0. Primers were provided by Birgit Ploier. Forward primers, K1E5 and K1E6, bind upstream the respective locus carrying the disruption cassettes. Reverse primers, K2E5 and K2E6, bind inside the respective marker gene and K2E5ex and K2E6ex bind downstream of disruption cassettes.

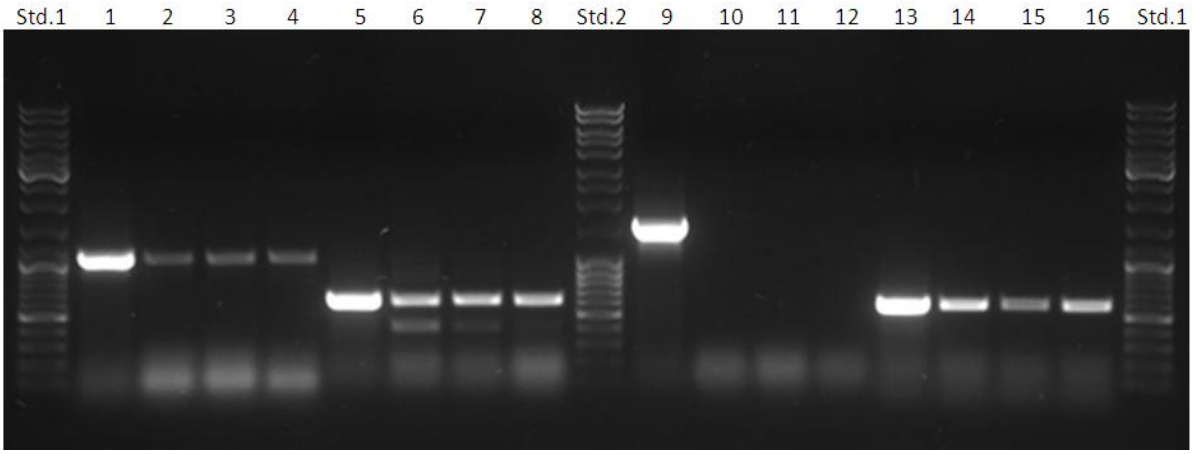


Figure 37: cPCR with Maxima Hot Start Green PCR Mix to control *ERG5* and *ERG6* disruption cassettes

Table 29: Lane description of Figure 37

PCR Nr.	Template	Primer combination	Fragment size [bp]
1	*erg5erg6are1*	K1E6 + K2E6ex	1075
2	T-129-4		
3	T-129-5		
4	T-127-6		
5	*erg5erg6are1*	K1E6 + K2E6	590
6	T-129-4		
7	T-129-5		
8	T-127-6		
9	*erg5erg6are1*	K1E5 + K2E5ex	1543
10	T-129-4		
11	T-129-5		
12	T-127-6		
13	*erg5erg6are1*	K1E5 + K2E5	642
14	T-129-4		
15	T-129-5		
16	T-127-6		
Std.1	GeneRuler™ DNA Ladder Mix, Fermentas		
Std.2	MassRuler™ DNA Ladder Mix, Fermentas		

Results for both growth conditions, selective as well as full media, verified the presence of disruption cassettes and looked completely the same as shown in Figure 37. cPCR with the primer combination K1E5 + K2E5ex to amplify the whole *ERG5* disruption cassette did only work out for the control (Lane 9) but its presence was proven for all transformants with primer combination K1E5 + K2E5.

Anyway, GC/MS results are only explainable by the presence of a functional *ERG5* and *ERG6* gene. Obviously, the substitution of *ARE2* by heterologous acyltransferases did not result in any reliably verified transformant.

5.4 Analysis of ester fraction from Silica TLC plate

As a precondition for the evaluation of substrate specificity and specific activity of heterologous acyltransferases, an analysis method which allows for distinction between free sterol and sterol ester fraction and identification of sterols in these fractions had to be established. An analysis method was essential for the assignation of sterol esterification pattern of whole cell lipid extracts as well as for microsomal assay experiments.

For this purpose, the separation of free and esterified sterols was achieved by TLC after Folch lipid extraction. Visualization by iodine staining was followed by scraping off the relevant bands from the TLC plate. Saponification, as part of the sample preparation for GC-MS analysis, was carried out directly on the scraped off silica gel. Results from GC-MS analysis suggested that sterols are modified during sample preparation. Therefore, sterols were extracted from silica gel with n-heptane or $CHCl_3$:MeOH (2:1, v/v), respectively, and brought to dryness by a N_2 stream before saponification. The approach was not successful as in the end impurities, which might derive from the extraction, did not allow for GC-MS analysis. Therefore, a purification step was incorporated after extraction, in which a Pasteur pipette filled with cotton wool was used to remove impurities.

5.4.1 First trials

Cultures of FY834 *MATa are1are2* double knockout strains harboring the pYEX4T-1 vector to express the respective GST-HAT fusion proteins were used to identify their sterol pattern. For this purpose, strains were cultivated like described in the section Methods and 200 OD_{600} units were harvested after 6 h of induction for lipid extraction. After separation by TLC and iodine staining (Figure 38) bands representing free sterol and steryl ester fraction were scraped off the Silica TLC plate and silica gel was directly used for GC/MS sample preparation which started with the saponification step to hydrolyze esters at 90°C in the presence of KOH.

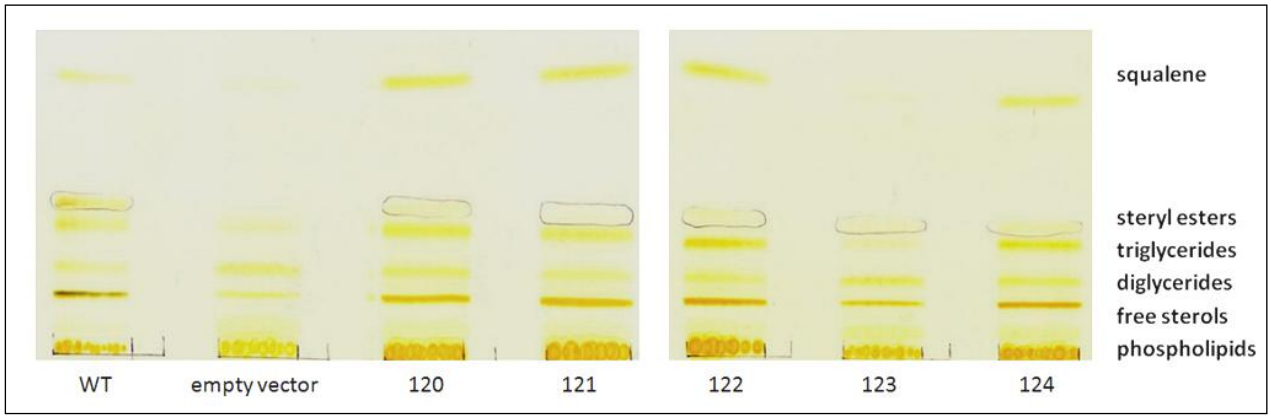


Figure 38: TLC plate with separated lipid extracts of FY834 *MATa are1are2* transformed pYEX4T-1 HAT vector constructs stained with iodine vapor

Table 30: Lane description of Figure 38

Sample	Protein	Source organism
WT (CEN.PK2)	-	-
empty vector	GST	-
120	GST-Acat1p	*Homo sapiens*
121	GST-Acat2p	*Rattus norvegicus*
122	GST-Acat1p	*Rattus norvegicus*
123	GST-Are2 p	*Candida albicans*
124	GST-Acat1p	*Toxoplasma gondii*

Against expectations no sterol component apart from the internal standard cholesterol was found by GC/MS analysis. As for direct GC/MS analysis of total sterols 15 OD units are sufficient, the processed 200 OD units should be enough for evaluable signal strength. The compounds seemed to be lost or modified during sample preparation.

5.4.2 Evaluation of lipid loss & substrate specificity of sterol-O-acyltransferase of *P. pastoris*

To evaluate if sterols and sterol esters were lost or modified during sample preparation *P. pastoris* wild type and three mutant strains were chosen for analysis as their sterol patterns suggested for an enhanced substrate preference of its sterol-O-acyltransferase towards cholesta-5,7,24-trienol. Mutants were an *erg6* knockout strain, a strain expressing dehydrocholesterol reductase 7 from *Danio rerio* (*DHCR7*) and a strain with both mentioned alterations (*erg6DHCR7*). By the knockout of *ERG6* the strain produces mainly cholesta-5,7,24-trienol which is only partly converted to cholesta-5,24-dienol by *DHCR7*, whilst the WT harboring the *DHCR7* gene mainly produces ergosta-5,22-dienol (Figure 39).

Table 31: *P. pastoris* mutants and their main sterols

***P. pastoris* strain**	**Main sterols**
WT	ergosterol
erg6	cholesta-5,7,24-trienol
DHCR7	ergosta-5,22-dienol
erg6DHCR7	cholesta-5,24-dienol & cholesta-5,7,24-trienol

The assumption of substrate preference of sterol-O-acyltransferase towards cholesta-5,7,24-trienol was based on the fact that the introduction of *DHCR7* into a WT strain led to the quite efficient conversion of ergosterol to ergosta-5,22-dienol whilst *DHCR7* expressed in an *erg6* knockout resulted only in a moderate conversion of cholesta-5,7,24-trienol to cholesta-5,24-dienol. The Dhcr7p substrate in the *erg6* knockout, cholesta-5,7,24-trienol, might be not available for the enzyme because of its storage in esterified form. This is just one among other possible explanations for the apparently higher activity of Dhcr7p in the *erg6* knockout. As the possible modification or loss, respectively, of cholesta-5,7,24-trienol was anyway of interest, clarification of sterol-O-acyltransferase substrate preference at the same time was reasonable.

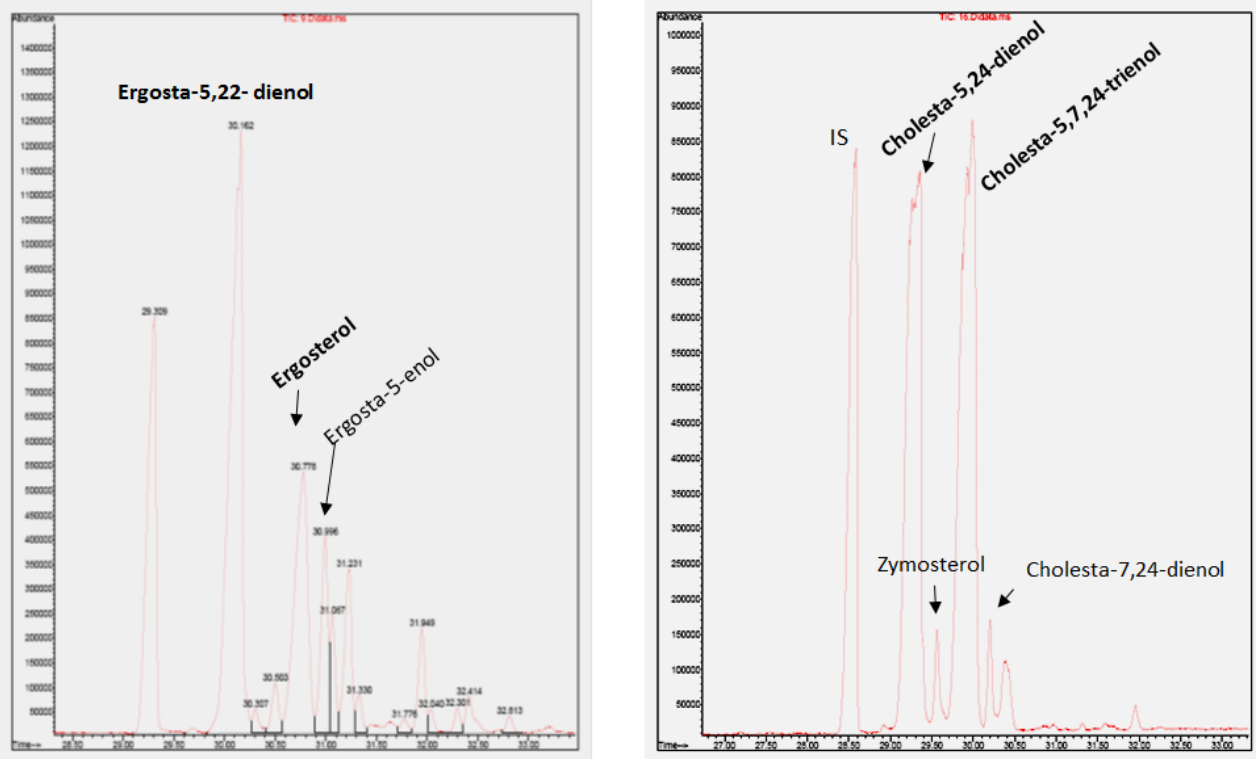


Figure 39: GC/MS chromatograms of *P. pastoris* mutants: *erg6* on the left and *erg6*+DHCR7 on the right, with main sterol components indicated (data provided by Tamara Wriessnegger)

5.4.2.1 Lipid loss by Folch lipid extraction

First to exclude was a probable loss of sterols and steryl esters in Folch lipid extraction. Therefore 10 µl of 25 µl lipid extract of 120 OD_{600} units, so equivalent to 48 OD_{600} units, were separated by TLC and sterol and steryl ester concentrations were estimated by comparison with cholesterol standard of which 4 µg were applied (Figure 40). A steryl ester standard to estimate concentrations was not available at this time.

Concomitantly differences in the amounts of steryl esters in mutant strains were evaluated to support the assumption that the efficient esterification of cholesta-5,7,24-trienol by *P. pastoris* sterol-O-acyltransferase is responsible for less substrate conversion to cholesta-5,24-trienol in the *erg6DHCR7* mutant.

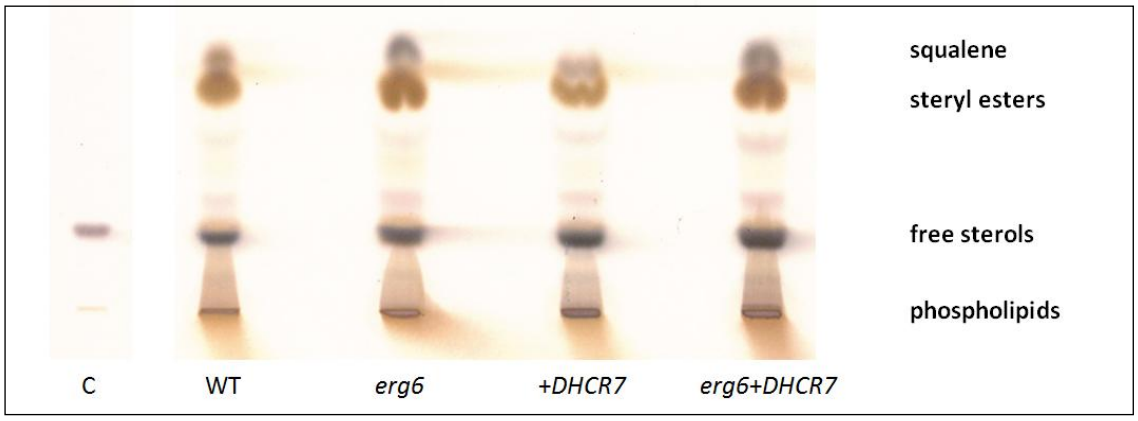


Figure 40: Charred TLC plate with lipid extracts of *P. pastoris* mutants after lipid extraction

Estimated masses were recalculated to the equivalents of OD_{600} units and resulted in a content of about 200 ng/OD_{600} in steryl esters and a content of about 400 ng/OD_{600} in free sterols.

So in 15 OD units about 10 µg of total sterols would be recovered after Folch lipid extraction whereas by normal GC/MS analysis about 25 µg of total sterols are detected in a WT strain (information obained by personal comunication with Tamara Wriessnegger). That means that compared to GC/MS sample preparation Folch lipid extraction might recover less sterols but this loss should not be severe enough to result in non evaluable signal strengh.

Concerning *P. pastoris* sterol-O-acyltransferase specificity the assumption that cholesta-5,7,24-trienol is extraordinarily highly esterified in the *erg6* mutant could not be confirmed as WT and *erg6* showed more or less the same intensity of the bands representing steryl ester fraction. Ergosta-5,22-dienol seems to be a less preferred substrate as steryl ester content in this strain is clearly lower compared to all other strains. Another explanation would be a generally lower sterol content in the *DHCR7* strain resulting in less need for esterification.

5.4.2.2 Lipid loss by GC/MS sample preparation

For the evaluation of lipid loss during sample preparation for GC/MS analysis, lipid extracts of 120 OD_{600} units were separated on TLC, bands representing free sterols and steryl esters were scraped off and GC/MS sample preparation was carried out until the derivatisation step after n-heptane extraction. After drying the extract by a nitrogen stream, the residues were dissolved in 30 µl $CHCl_3$:MeOH (2:1, v/v) and the total volume was applied on a Silica TLC plate to estimate sterol content.

For samples of mutant strains, 5 µL of 2 mg/mL internal cholesterol standard were added before application of lipid extracts on the Silica TLC plate. In the case of WT, the same amount of cholesterol standard was added to both respective samples, free sterols and steryl esters, but right before saponification according to the GC/MS sample preparation protocol. 12.5 µg of cholesterol standard were applied on the TLC for quantitative comparison.

The strain expressing Dhcr7p was not further analysed as it was of interest to compare intensity of steryl ester bands after Folch lipid extraction. However, its main sterol components and their possible modification or loss respectively in GC/MS analysis were not of major interest in this project.

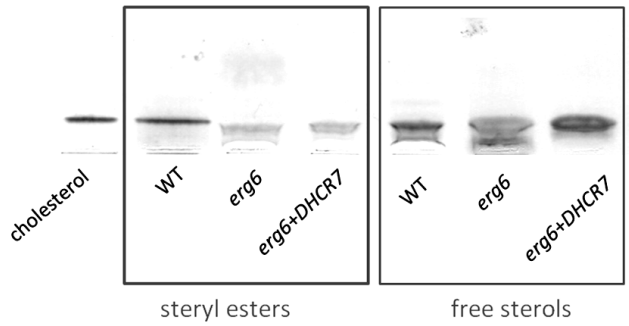


Figure 41: Charred TLC plate with lipid extracts of *P. pastoris* mutants before the derivatisation step in GC/MS sample preparation, steryl ester fraction of WT and all free sterol fractions contained internal standard (see text for details)

Although the already applied internal cholesterol standard made estimations of concentrations difficult, recalculations to the processed OD_{600} units in lipid extraction were done, but have to be seen as vague indications. At about 80 ng/OD_{600} steryl esters and 150 ng/OD_{600} free sterols the loss in GC/MS preparation, with direct saponification on silica gel could be estimated to be at least around 50%. However, this loss still would not be sufficient for non-evaluable signal strength in GC/MS analysis.

5.4.2.3 Evaluation of free sterol and steryl ester pattern by GC/MS

For the identification of sterols by GC/MS analysis, lipid extracts derivedd from 360 OD_{600} units were prepared like described in the section Methods.

The comparison of the two GC/MS chromatograms in

Figure 42 revealed that the main compound, cholesta-5,7,24-trienol, of the *P. pastoris erg6* knockout strain could not be found again neither in the free sterol nor in the steryl ester fraction after the described procedure.

Also in the case of the *erg6DHCR7* strain its main sterols cholesta-5,24-dienol and cholesta-5,7,24-trienol were not detected in neither of the two fractions. Free sterol fraction of WT could not be measured for unexplainable impurities, but analysis of its sterol ester fraction did not show any ergosterol.

It seems that the conjugated double bond at the C5- and C7-position is destroyed. It is assumed that the combination of high temperature and reagent contact to the silica gel in the saponification procedure might be responsible for the destructive process. This makes sense as the same sterol analysis procedure applied on *S. cerevisiae* FY834 *are1are2* strains expressing GST-HAT fusion proteins did not show an evaluable ergosterol peak. Ergosterol is the main sterol to be produced by this strains and also possesses a conjugated double bond at the C5- and C7-position.

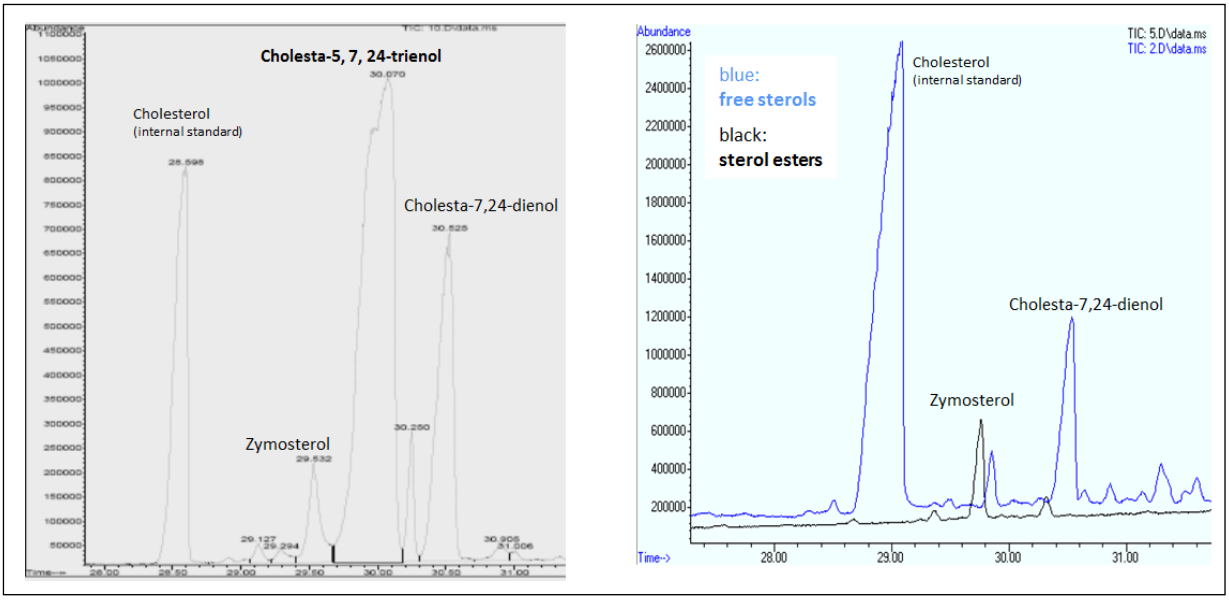


Figure 42: GC/MS chromatograms *P. pastoris erg6*: Total sterol content after "normal" GC/MS preparation (on the left) and of free sterol (blue) and sterol ester (black) fractions processed from lipid extracts, TLC separation and GC/MS preparation with the saponification step on silica gel, are shown

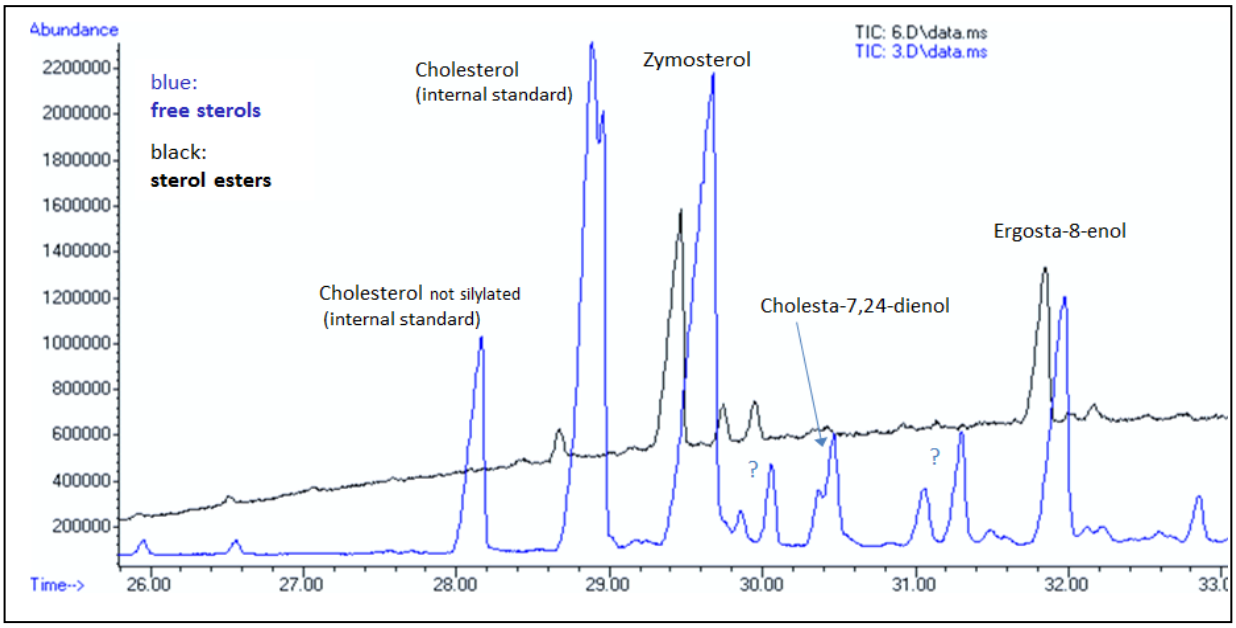


Figure 43: GC/MS chromatograms *P. pastoris erg6+DHCR7* of free sterol (blue) and sterol ester (black) fraction proceeded from lipid extracts, TLC separation and GC/MS preparation with the saponification step on silica gel

5.4.3 Extraction from silica gel and incorporation of an purification step before saponification

As the presence of silica gel during saponification in GC/MS sample preparation was suspected to catalyze the modification of certain sterols, these were extracted from silica gel by n-heptane or $CHCl_3$:MeOH (2:1, v/v), respectively. For this purpose a cholesteryl oleate standard and cholesta-5,7,24-trienol provided by DSM was used.

GC/MS sample preparation was carried out on dried extracts of silica gel scraped off a TLC plate. Samples extracted with n-heptane could not be brought to dryness after the n-heptane extraction steps in GC/MS sample preparation. Because of the formation of a metastable phase, which impeded the total removal of solvent and therefore its derivatisation, analysis by GC/MS could not be carried out. Samples extracted from silica gel with $CHCl_3$:MeOH (2:1, v/v) neither could be measured as they showed white impurities after the derivatisation steps and dilution with ethyl acetate.

After these results the recommendation (obtained by personal communication with Tanja Wrodnigg, Institute of Organic Chemistry, Technical University of Graz) to purify extracts with cotton wool was pursued. Extraction from silica gel was carried out like before, but before GC/MS sample preparation extracts were purified through cotton wool in a Pasteur pipette.

Indeed, sample preparation was possible and cholesteryl oleate was recovered in its saponified form as cholesterol (Figure 44 and Figure 45) by GC/MS analysis. However, cholesta-5,7,24-trienol was not detected anymore in GC/MS analysis as it seems to be modified during sample preparation. An amount of peaks occurred in the GC/MS chromatogram which could not be attributed to any known

compound. The used cholesta-5,7,24-trienol material still needs to be analyzed to exclude its decomposition or impurities to be responsible for GC/MS results.

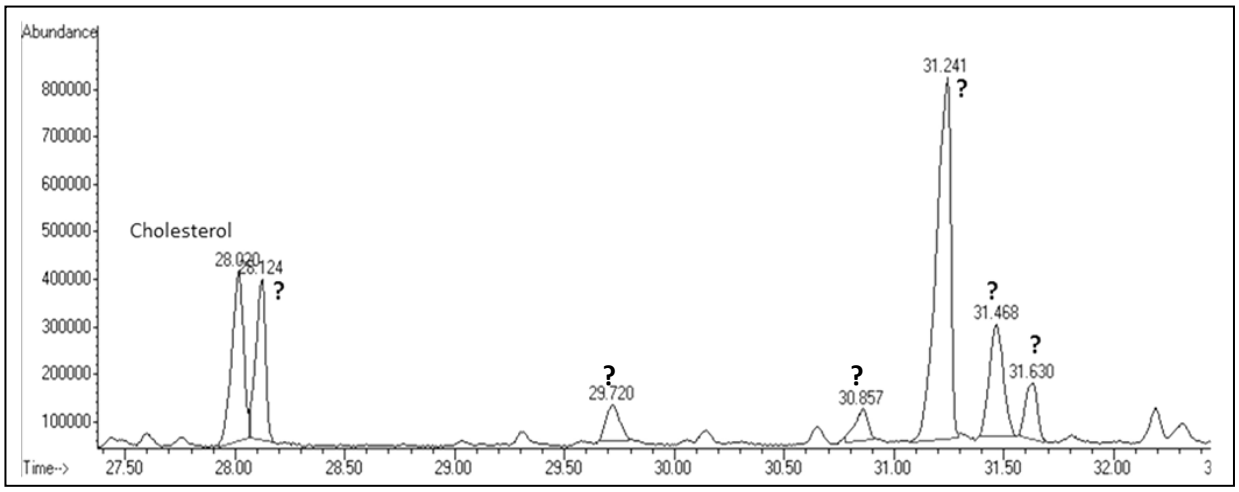


Figure 44: GC/MS chromatogram after n-heptane extraction of sterols from silica gel

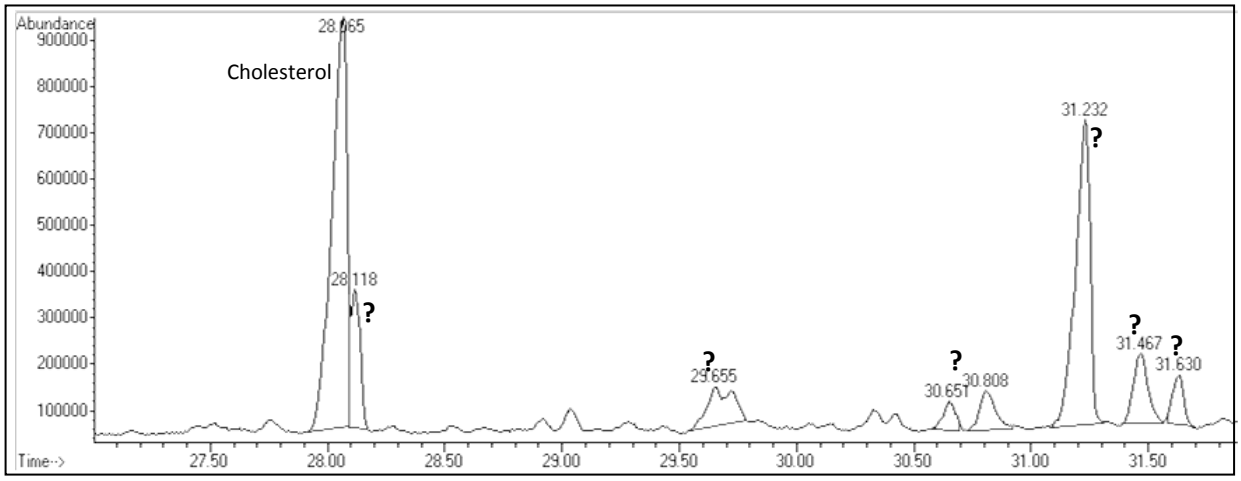


Figure 45: GC/MS chromatogram after $CHCl_3$:MeOH (2:1, v/v) extraction of sterols from silica gel

6 Discussion

6.1 Expression of HATs as GST-fusion proteins from pYEX4T-1

pYEX4T-1 is a high copy number plasmid and should ensure for sufficient protein expression to detect differences in activities and specificities of HATs. Tagging with GST at the N-terminus was supposed to facilitate the immunodetection of all GST-HAT fusion proteins at the same time.

First, [pYEX4T+HAT] vector constructs were transformed into a FY834 *are1are2* double knockout strain. For this host strain no sterol-O-acyltransferase activity was detectable as the strain did not show any steryl ester formation. HATs expressed as N-terminal GST fusion in this strain were all able to esterify yeast wild type sterols, but to a different extent. Conclusions concerning their activities were derived from the band intensity of the steryl ester fraction after TLC of their lipid extracts and charring. GST-Are2p of *C. albicans* showed the most intense steryl ester band almost equal to GST-Are2p from *S. cerevisiae*. It was followed in activity by human GST-Acat1p and GST-Acat1p from *R. norvegicus*. GST-Acat1p of *T. gondii* and GST-Acat2p from *R. norvegicus* were the proteins to esterify fewest sterols. Activities correspond to the esterification of wild type sterols and not necessarily reflect the situation in a mutant strain producing mainly zymosterol and cholesta-5,7,24-trienol. As the sterol-O-acyltransferase of *C. albicans* is a yeast enzyme, it was most likely to esterify yeast wild type sterols of *S. cerevisiae*. Also its expression and corresponding activity in *S. cerevisiae* might be facilitated in contrast to other HATs by its yeast origin. Human Acat1p is known to show low activity in yeast cells when expressed as the only sterol-O-acyltransferase with about 4% activity compared to wild type yeast cells (Yang *et al.*, 1996).
Low steryl ester concentrations triggered high squalene concentrations like it was already reported for the *are1are2* double mutant to give a threefold rise in squalene content (Zweytick *et al.*, 2000). It seems that squalene concentrations are indirectly proportional to the cell´s ability to esterify sterols.

Expression of GST-HAT fusion proteins was documented by immunodetection of GST. Western blotting revealed fragmentation of all GST-HAT fusions. As also the GST-Are2p of *S. cerevisiae* was not detected with its complete size, the GST tag was assumed to be responsible for fragmentation and not the heterologous expression in *S. cerevisiae*. The size of GST-HAT fusion proteins of about 90 kDa is a point of concern. With the size the probability of intracellular proteolytic degradation increases although literature says that GST tagging provides protection from intracellular protease cleavage and stabilizes the recombinant protein (Terpe, 2003).
Endogenous Are2p was successfully overexpressed before with a HA tag of a total size of about 80 kDa (Polakowski *et al.*, 1999). Degradation of human Acat1p heterologously expressed in *S. cerevisiae*

as a fusion protein was already observed by Yang *et al.*, 1996. Degradation was assumed to be responsible for the observed lower molecular weight. Are2p of *C. albicans* was expressed in *S. cerevisiae* with a myc tag and the full size of the fusion protein was detected (Kim *et al.*, 2004).
Conclusively, the observed steryl ester forming activity of HATs either refers to an active fragment or to the fact that fragmentation is due to sample preparation for Western blotting and does not necessarily reflect the *in vivo* state inside the cell.

To elucidate if chosen HATs show the desired substrate specificity for cholesta-5,7,24-trienol, [pYEX4T+HAT] vector constructs were transformed into a CEN.PK2 *erg6are1are2* triple knockout strain. Changes in total sterol composition of transformants compared to the empty vector control were evaluated by GC/MS. Although the CEN.PK2 *erg6are1are2* triple knockout strain still showed unexplainable steryl ester formation, the cholesta-5,7,24-trienol to zymosterol ratio clearly changed when compared to a *erg6* knockout strain. By the alleged knockout of sterol-O-acyltransferases the cholesta-5,7,24-trienol to zymosterol ratio changed from an 1:1 (estimated by Figure 18) to an 3.6:1 distribution. This suggests that cholesta-5,7,24-trienol content is strongly influenced by endogenous sterol-O-acyltransferases activity level.
As expected zymosterol content increased with the expression of GST-Are2p from *S. cerevisiae* and cholesta-5,7,24-trienol content stayed more or less equal. This might indicate that there is a substrate preference of endogenous Are2p of *S. cerevisiae* for zymosterol compared to cholesta-5,7,24-trienol *in vivo* and suggests that total sterol composition is very much dependent on endogenous Are2p activity.
Like in the transformed FY834 *are1are2* double knockout strains, GST-Are2p of *C. albicans* was the HAT to show highest sterol esterification levels, but with substrate specificities undermining the project aims. With 6.8-fold increase in zymosterol and a decrease to 40% in cholesta-5,7,24-trienol content, cholesta-5,7,24-trienol seems to be even less preferred as substrate for GST-Are2p of *C. albicans* than for GST-Are2p of *S. cerevisiae*. Alternatively, one might blame the high activity of *C. albicans* GST-Are2p for the over-proportional esterification of zymosterol, which therefore can not be further metabolized to cholesta-5,7,24-trienol.
Apparently, the highest affinity towards cholesta-5,7,24-trienol was shown for GST-Acat1p from *R. norvegicus* with an augmentation of about 1.3-fold upon expression. As zymosterol content increased only 1.6-fold, GST-Acat1p expression showed the lowest gain for this sterol compared to other HATs. Anyway, only low substrate affinity toward zymosterol was assessed and not the highly sought for specific esterification of cholesta-5,7,24-trienol. In general, transformation of HATs resulted in higher sterol contents and always went along with a higher increase of total zymosterol than of total cholesta-5,7,24-trienol levels. Only in strains that showed a generally low increase of

total sterol content a slight rise in cholesta-5,7,24-trienol content was detected. It might be that we only observe an esterification activity dependent change of sterol composition that has nothing to do with substrate specificities of sterol-0-acyltransferases. Moreover, evaluation of substrate preference *in vivo* can not be seen as completely trustful as the transformed CEN.PK2 *erg6are1are2* triple knockout strain still showed unexplainable esterification of sterols. Whether this remaining endogenous sterol-O-acyltransferase activity does influence results when HATs are co-expressed can not be excluded.

For the above mentioned reasons, and to be more independent of strain construction and its difficulties, the establishment of an *in vitro* microsomal assay was reasonable. In order to do so, microsomes of the FY834 *are1are2* double knockout strain harboring the vector to express GST-Are2p from *C. albicans* were prepared. Immunoblotting of microsome preparation resulted in a detectable fragment of about 50 kDa. It seems that only protein which still carries the necessary transmembrane domains is located in the ER and therefore held back in microsomal preparations.
In vitro assay experiments were pursued by other group members.

6.2 Genomic integration of HAT genes at the *ARE2* locus

The substitution of the *ARE2* gene by HATs seemed to be a good strategy to circumvent the need for an *erg5erg6are1are2* quadruple knockout strain the construction of which was impaired in part by the unsuccessful deletion of *ARE2*. Transformation rate should be enhanced as substitution by a HAT circumvented the critical situation of a strain lacking steryl ester formation capability. Thus a CEN.PK2 *erg5erg6are1* triple knockout strain was transformed.
cPCRs should verify integration events by site-specific and unspecific amplifications. Site-specific cPCRs applied one primer upstream or downstream, respectively, the substitution cassette immediately bordering the supposed locus of integration and one primer inside the substitution cassette. Also primers to reveal if the endogenous *ARE2* was still present were applied. Despite of several cell disruption protocols, several primers and different polymerases used, cPCRs did not work out in most cases. cPCR often gave no result apart of the positive controls or unspecific fragment amplification was observed. Correct primer design was confirmed for all primers that anneal inside the substitution cassette by PCRs with plasmids as templates. cPCRs with the forward primers upstream the *ARE2* locus, upstr1_fw and upstr2_fw, and a reverse primer to bind inside the endogenous *ARE2*, KA2rev, worked out perfectly on the control strain *erg5erg6are1* for almost every protocol applied. To check primers that bind inside the substitution cassettes on the control strains *are1are2* and *are1erg5erg6* would have additionally excluded wrong positives and might have explained unspecific fragment amplification.

Two transformants of *ARE2* of *C. albicans*, T-129-4 and T-129-5, were the only ones which gave positive results for a cross-event in the *ARE2* promoter region in several cPCRs. This was confirmed by PCRs on genomic DNA. Also for one transformant of human *ACAT1,* T-126-7, a fragment of correct size indicating insertion at the *ARE2* promoter site could be obtained in a cPCR. But PCR on genomic DNA applying a different forward primer as in the cPCR did not confirm this result.

As cPCRs hinted at insertions by single crossover events as in the case of a T-126-4, i.e. the human *ACAT1* gene, *ARE2* replacement had to be confirmed as well with downstream primers. Four different primer combinations, two different forward and two different reverse primers, were tested to do so. None of these resulted in the amplification of the expected fragments.

Primer design was always based on the genomic sequence of *S. cerevisiae* S288c which might be not completely identical to the CEN.PK2 strain background. Thus, incorrect fragment amplification due to a primer design or calculation of fragment sizes based on a wrong sequence information can not be excluded, although it appears unlikely.

Three transformants showing positive results for integration in cPCR were subjected to analysis of steryl ester content by GC/MS measurement. The only sterol detected was ergosterol, an unexplainable result as the recipient strain of HAT substitution cassettes, the *erg5erg6are1* triple knockout, showed the typical sterol distribution with zymosterol and cholesta-5,7,24-trienol as the main sterols. The only way to reconstitute the strain's capability to produce ergosterol are functional *ERG5* and *ERG6* genes. Disruption of these in the triple knockout was controlled by cPCRs and verified. To exclude "impurities" to be responsible for the unexplainable ergosterol formation, strains were streaked out several times on triple selective media, SD –trp –leu –his and grown overnight in SD-ura media. In this way, growth should be only permitted to strains that carry all four auxotrophic marker genes. This measure was not successful, as further GC/MS analysis revealed the same result as before, namely ergosterol as the only sterol to be found.

For genotypic verification transformants were checked for *ERG5* and *ERG6* disruption by cPCRs. Strains were cultivated on selective media as described above. Additionally, strains originating from cultivation on full media YPD were checked to see if disruption is invariable even without selective pressure. Strains from both growth conditions gave same results verifying disruption of the *ERG6* as well as of the *ERG5* gene. An explanation why transformants do not show inherent genotypic and phenotypic characteristics might be that they are not haploid. Crossing of the *erg5erg6are1* triple knockout with a wild type yeast strain would yield a heterozygous strain to express *ERG5* and *ERG6* for ergosterol production and at the same time show positive results in cPCRs for their disruption. Yet in this case some of the cPCRs should have yielded in bands corresponding to native *ERG5* and *ERG6* loci, which was never observed.

6.3 Analysis of ester fraction from Silica TLC plate

To reveal to which extent certain sterols are esterified inside the cell an analysis method which allows for distinction between free sterols and esterified ones is necessary.

Whole lipid extracts were separated by TLC and respective bands scraped off to be analyzed by GC/MS. Fatty acids of triacylglycerides had already been analyzed by a similar procedure (Athenstaedt *et al.*, 2005).

First strains to be tested for their steryl ester composition were the FY834 *are1are2* double knockouts harboring vectors to express GST-HAT fusion proteins. Respective bands originating from lipid extract of 200 OD_{600} units were analyzed. Neither free sterol nor steryl ester fractions gave any signal apart from the internal standard cholesterol. This was surprising as normally only 15 OD units are used for GC/MS analysis of total sterols. Hence, sterols must be lost at some point of sample preparation.

To evaluate at which point sample preparation needed to be improved, sterol contents were estimated after Folch lipid extraction and during GC/MS sample preparation before the derivatisation step. For this purpose a WT and three *P. pastoris* sterol-modified strains were used to evaluate if its sterol-O-acyltransferase shows substrate specificities for cholesta-5,7,24-trienol.

An *erg6* knockout strain, a strain expressing dehydrocholesterol reductase 7 from *Danio rerio* (*DHCR7*) and a strain with both mentioned modifications (*erg6DHCR7*) were used. The main sterols produced in these strains were ergosta-5,22-dienol by *DHCR7*, cholesta-5,7,24-trienol by *erg6* and cholesta-5,24-dienol and cholesta-5,7,24-trienol by *erg6DHCR7.* Whilst *DHCR7* was capable to convert ergosterol to ergosta-5,22-dienol almost completely, in the *erg6DHCR7* strain only about half of cholesta-5,7,24-trienol was converted to cholesta-5,24-dienol (data obtained by Tamara Wriessnegger). One explanation of this observation could be the storage of cholesta-5,7,24-trienol as steryl esters, which would make the substrate less accessible for Dhcr7p.

Lipid separation by TLC and subsequent charring revealed that the *erg6* strain showed a similar free sterol to steryl ester distribution as the wild type. It seems that steryl ester content is more or less equal in both strains and, therefore, bad substrate accessibility is unlikely to be responsible for the bad conversion of cholesta-5,7,24-trienol to cholesta-5,24-dienol in *erg6DHCR7*. Lowest content of steryl esters was found in the *DHCR7* mutant indicating that (i) ergosta-5,22-dienol might be a less preferred substrate of the sterol-O-acyltransferase in *P. pastoris* compared to ergosterol and cholesta-5,7,24-trienol or that (ii) overall sterol levels are lower in this particular strain.

Recovered masses of free and esterified sterols after Folch lipid extraction were roughly estimated on TLCs to be 400 ng/OD and 200 ng/OD, respectively, and determined to be sufficient for further GC/MS analysis of respective bands. Total sterol contents after Folch lipid extraction were recalculated to be about 10 µg/ 15 OD units and therefore less than the amount to be detected after

GC/MS sample preparation which is about 25 µg/ 15 OD units for a WT strain (data for WT obtained by Tamara Wriessnegger). Folch lipid extraction seems to recover less sterols than GC/MS sample preparation, but this loss should not be severe enough to result in no evaluable signal strength as a 13-fold higher amount of cell material or OD units, respectively, were applied. Estimation of concentrations after GC/MS sample preparation before the derivatisation step indicated a loss of about 50%. However, sufficient amounts should be present for detection in GC/MS.

For final analysis of free sterol and steryl ester composition, respective bands derived from 360 OD units were scraped off the Silica TLC plate and processed for GC/MS. The *DHCR7* mutant was not measured as its main sterols and their possible modification was of minor interest.

GC/MS data of mutants, *erg6* and *erg6DHCR7*, revealed that the main compound cholesta-5,7,24-trienol could not be detected anymore, neither in the steryl ester nor in the free sterol fraction. Although only the steryl ester fraction of WT was analyzed in detail, it was suspicious that no trace of ergosterol could be detected there. It seems that compounds with a C5-C7 conjugated double bond are modified during sample preparation as no respective compounds were found after processing TLC-separated sterol and steryl ester fractions for GC/MS. The saponification step, including high temperature and basic conditions, was directly carried out on scraped off silica gel and suspected to be responsible for the modification of samples. It might be that the silica gel is catalyzing a reaction, that destroys the C5-C7 conjugated double bond and/or oxidizes the sterols.

To underscore this assumption an extraction step before saponification was incorporated. For this purpose cholesteryl oleate standard and purified cholesta-5,7,24-trienol provided by DSM were separated on TLC. $CHCl_3$:MeOH (2:1, v/v) or n-heptane, respectively, was used to extract sterol compounds from silica gel. In the end, the attempt failed as impurities did not allow completing sample preparation. To remove impurities, extracts were purified through cotton wool which made sample preparation possible. However, in the end cholesta-5,7,24-trienol was again not found in GC/MS. Cholesterol, as the saponified form of cholesteryl oleate, was the only compound which could be identified in the GC/MS chromatograms. Moreover, the chromatograms showed a range of peaks which could not be attributed to any compound and might be modified products or impurities. Obviously, cholesta-5,7,24-trienol needs to be analyzed directly by GC/MS to confirm its presence and quantify its amount.

6.4 Prospects

Further work is necessary to completely elucidate HATs specificities.

Strain construction to obtain an *erg5erg6are1are2* mutant with no esterification activity is a precondition to reveal substrate preferences of HATs *in vivo.* Furthermore, transformation into a strain expressing the truncated version of *HMG1* would be interesting to elucidate influence of heterologous sterol-0-acyltransferase activities under conditions of elevated sterol production.

For further work, a shorter tag to detect protein expression immunologically is reasonable. HATs should also be expressed without tag to exclude influence of the protein tag by comparing activities of the tagged and untagged versions. For promising HATs immunodetection by specific antibodies is worth considering.

Genomic integration of HATs by substitution of the *ARE2* gene caused problems. Further transformations should be carried out in a strain deficient of endogenous *ARE2* as in this case single cross over events will not be a hindrance, even though screening for double crossover events would also be facilitated by the marker of the *ARE2* disruption cassette.

Substrate specificities should also be evaluated *in vitro*. Therefore, a microsomal assay has to be established and moreover an adequate analysis method is required. For the *in vitro* assay as well as for *in vivo* experiments parallel analysis of free sterol and steryl ester fractions might be a prerequisite, unless radioactive compounds are applied.

It should be examined if there is an optimal esterification level or highly specific sterol-O-acyltransferase activity, respectively, supporting the efficient conversion of zymosterol to cholesta-5,7,24-trienol *in vivo*.

7 Summary

It was shown that by heterologous expression of sterol-O-acyltransferases of human, rat, yeast and protozoan origin esterification of sterols can be reconstituted to a *S. cerevisiae are1are2* mutant, which itself is devoid of esterification activity.

In the same strains evidence for correct expression of HATs on the protein level failed as only fragmentation patterns were observed by immunodetection of the attached GST-tag. Not even expression of endogenous Are2p of *S. cerevisiae* resulted in one detectable band of correct size when expressed as GST-fusion. Therefore, the GST-tag was thought to be responsible for fragmentation. Its influence on HAT activities or on the observed fragmentation, respectively, can only be speculated about.

Expression of the GST-tagged HATs in an *erg6are1are2* mutant strain, that still showed unexplainable esterification activity, clearly influenced sterol composition and content. By the expression of any HAT total sterol content increased, but to different extents. By the expression of GST-Are2p of *C. albicans* only zymosterol content was elevated whereas cholesta-5,7,24-trienol was found in even lower levels than in the empty vector control or in the strain expressing endogenous Are2p in a GST-fusion. Most elevated content of cholesta-5,7,24-trienol and lowest increase of the zymosterol level was found in the strain expressing GST-Acat1p of *R. norvegicus*. In general, the increase of zymosterol content by HAT expression was up 6.8-fold, whereas cholesta-5,7,24-trienol level could only be elevated 1.3-fold compared to the empty vector control.

Intentions of homologous integration of HATs at the *ARE2* locus of an *erg6erg5are1* triple knockout did not result in any verified transformant. No or unspecific fragment amplification in cPCRs were observed in most cases. GC/MS analysis of three selected transformants showed ergosterol as the only sterol to be produced by these strains, which is virtually impossible. The *erg6* and *erg5* knockout was confirmed by cPCR and GC/MS results were reproducible when strains were cultured on multiple selective media before analysis. No conclusive explanation for the contradicting phenotypic and genotypic characteristics could be found so far.

Analysis of sterol and steryl ester fractions from Silica TLC plates by GC/MS sample preparation directly on scraped off silica gel seems to destroy sterol compounds with a C5-C7 conjugated double bonds. Intentions to avoid the probably catalytic effect of silica gel causing the loss of mentioned compounds during GC/MS sample preparation steps failed. The intended analysis method seems to be not appropriate for the project needs.

8 Indices

8.1 References

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8.2 List of figures

8.3 List of tables

9 Appendix

9.1 The standard genetic code

First base in codon	Second base in codon: U	Second base in codon: C	Second base in codon: A	Second base in codon: G	Third base in codon
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

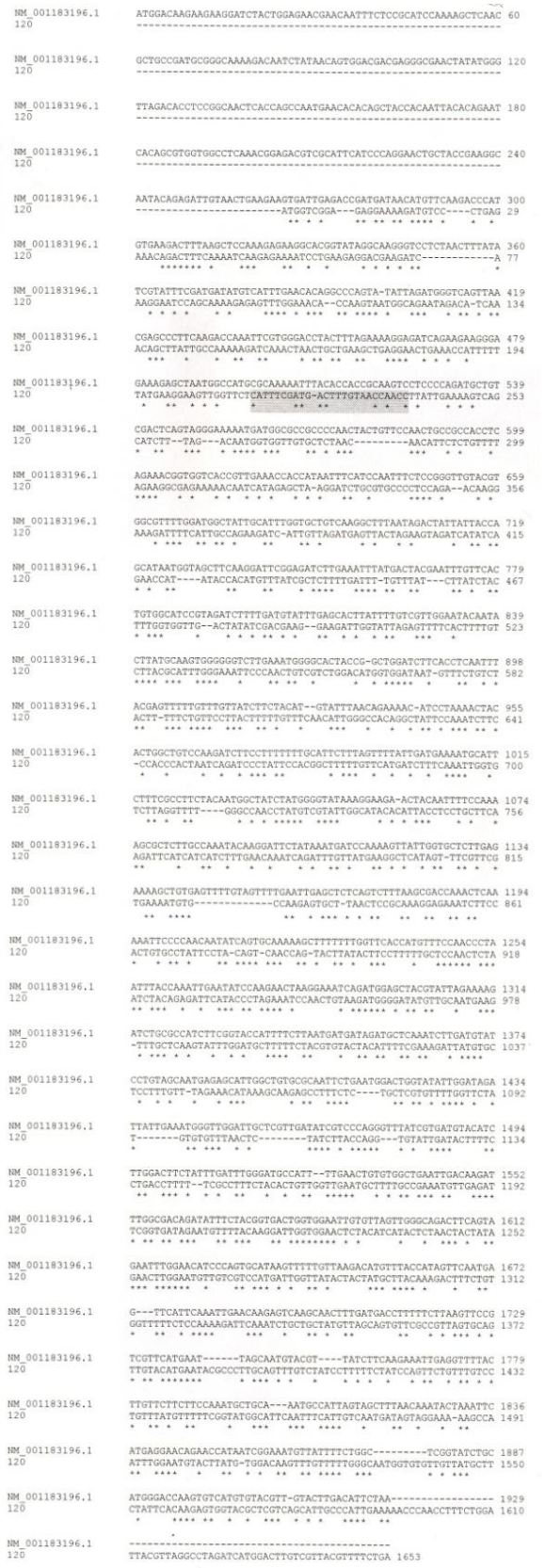
9.2 Single-letter and three-letter amino acid codes

Amino acid	Three letter code	One letter code
Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamic acid	glu	E
Glutamine	gln	Q
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	try	W
Tyrosine	tyr	Y
Valine	val	V

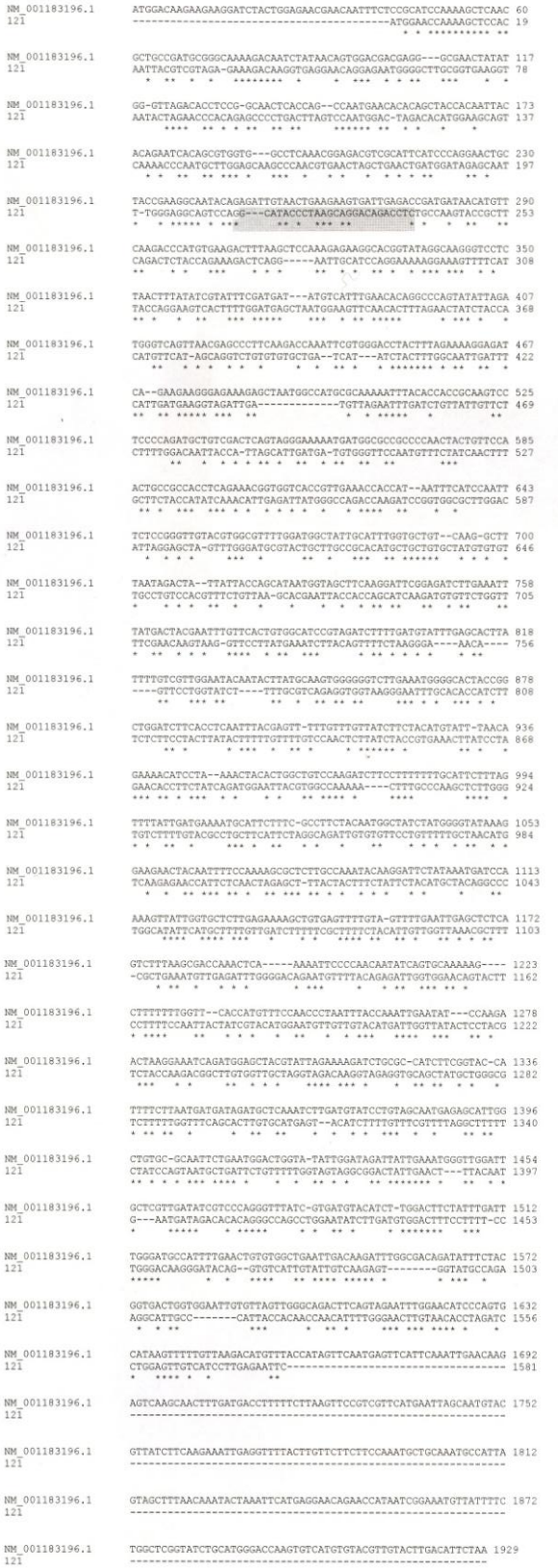
9.3 Alignment of *S. cerevisiae ARE2* with HATs

Primers are shown in grey.

H. sapiens ACAT1, Primer 120_rv



R. norvegicus ACAT2, Primer 121_rv



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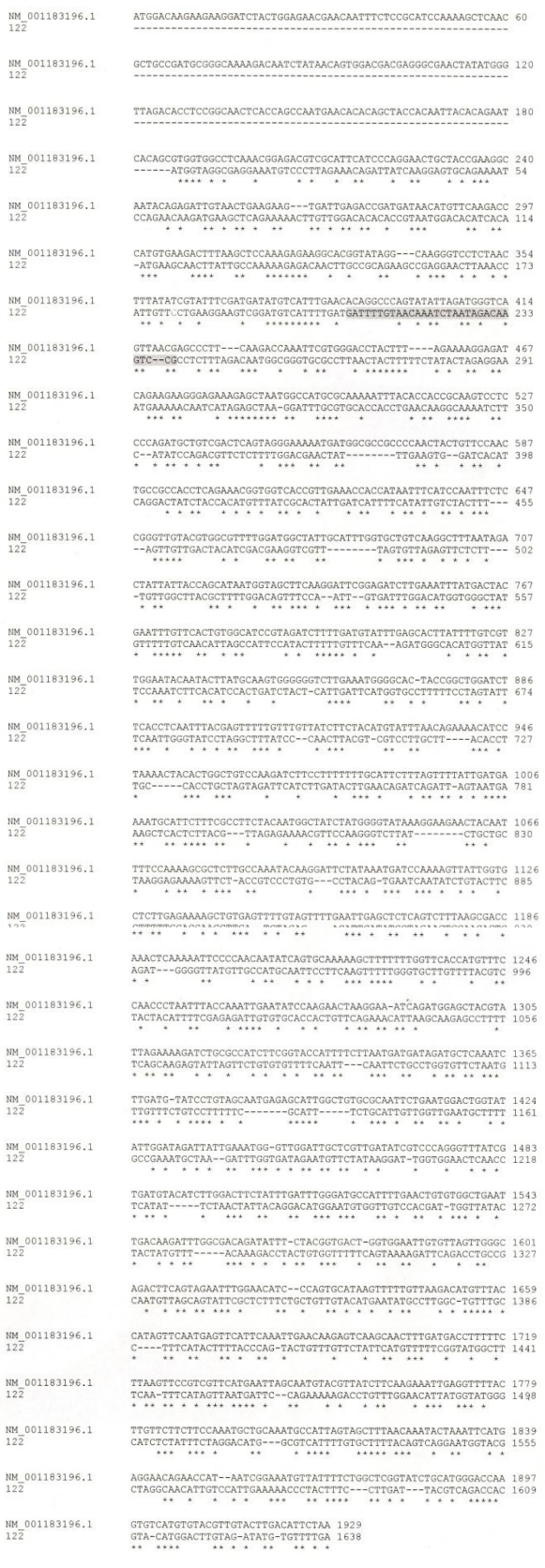
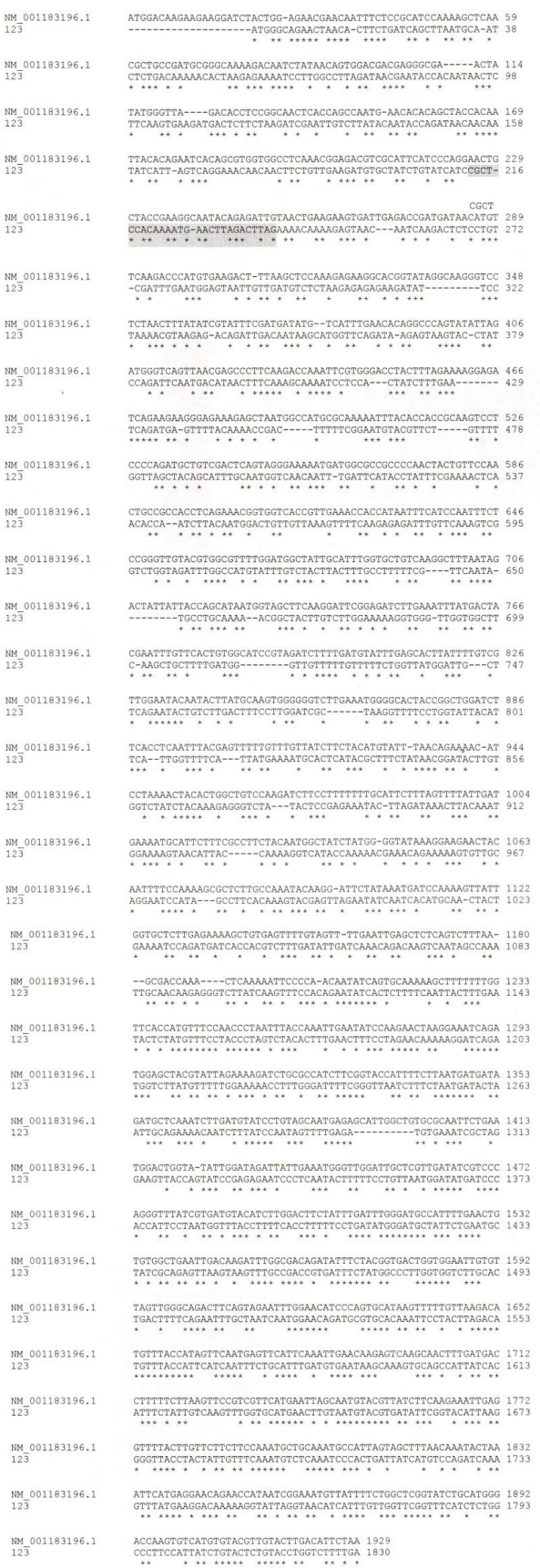


C. albicans ARE2, Primer 123_rv



T. gondii ACAT1, Primer 124_rv