Wax ester fermentation and fatty acid biosynthesis in the facultatively anaerobic flagellate *Euglena gracilis*

Inaugural-Dissertation

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Abbreviations

General abbreviations, chemicals and enzymes

A	absorbance
Aa	amino acid
ACCase	acetyl-CoA carboxylase
ACP	Acyl-Carrier-Protein
Amp	ampicillin
APS	ammoniumperoxidisulfate
ATP	adenosine triphosphate
BLAST	basic Local Alignment Search Tool
BSA	bovine-serume albumine
CCR	crotonoyl-CoA reductase
CHAPS	3-[(3-cholamidopropyl)- dimethylammonio]-1-propane-sulfonate
$CHCl_3$	chlorophorm
CoA	coenzym A
DE	diethylether
DMSO	dimethyl sulfoxide
DNase	desoxyribonuclease
DTT	dithiotreitol
EDTA	ethylenediamintetraacetate
ECL	enhanced chemoluminescence
ER	Endoplasmic Reticulum
FAD	Flavin Adenine Dinucleotide reduced
FADH	Flavin Adenine Dinucleotide oxidized
FAlcs	Fatty Alcohols
FAMEs	Fatty Acid Methyl Esters
FAS	Fatty Acid Synthase
FPLC	Fast-Protein-Liquid-Chromatography
Fig	Figure
Ga	billion of years
GC	gas chromatography
HEPES	2-[4-(2-hydroxyethyl)1-1-piperazinyl)-ethansulfonic acid
HPLC	High Performance Liquid Chromatography
IPTG	isopropyl-D-thiogalactoside
KAS	ketoacyl-ACP synthase
Km	kanamycin

LB	Luria-Bertani medium
Luminol	3-aminophtalhydrazide
Me_2CO	acetone
MeOH	methanol
MS	mass spectroscopy
NAD	nicotinamide adenine dinucleotide reduced
NADH	nicotinamide adenine dinucleotide oxidized
NADP	nicotinamide adenine dinucleotide phosphate reduced
NADPH	nicotinamide adenine dinucleotide phosphate oxidized
NCBI	National Center for Biotechnology Information
NiNTA	Nickel Nitriloacetic Acid
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Polymethyl Sulfonic Acid
PE	petrol ether
PKS	polyketide synthase
Pre-	precursor
RNAse	ribonuclease
RT	Room Temperature
S	substrate
SDS	Sodium Dodecylsulfate
SiO_2	Silica gel
Tab.	table
TCA	Trichloroacetic Acid
TE	Tris-EDTA
TEMED	N, N, N',N'-Tetramethylethylendiamin
TER	*trans*-2-enoyl-CoA reductase
TLC	Thin Layer Chromatography
Tris	2-amino-2(hydroxymethyl) 1,3-propandione
Triton X-100	octylphenoxy poly-(8-10)-ethyleneglycol
Tween 20	polyoxyethylene sorbitan monolaureate
X-Gal	5-Bromo-4-Chlor-3-Indol-β-D-galactopyranoside

Amino acids

A,	Ala	alanine
C,	Cys	cysteine
D,	Asp	aspartic acid
E,	Glu	glutamic acid
F,	Phe	phenylalanine
G,	Gly	glycine
H,	His	histidine
I,	Ile	isoleucine
K,	Lys	lysine
L,	Leu	leucine
M,	Met	methionine
N,	Asn	asparagine
P,	Pro	proline
Q,	Gln	glutamine
R,	Arg	arginine
S,	Ser	serine
T,	Thr	threonine
V,	Val	valine
W,	Trp	triptophane
Y,	Tyr	tyrosine

Nucleic acids

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
dNTP	desoxynucleoside-5'-triphosphate

Units

bp	base pair
°C	degree Celsius
g	gram
g	gravity
h	hour

kb	kilo base pair
kDa	kilo Dalton
l	litre
m	meter
M	molar
mg	milligram
mm	minute
µg	microgram
µl	microlitre
mM	millimolar
µM	micromolar
nm	nanometer
rpm	revolution per minute
s	second
U	Unit: µmol mg^{-1} min^{-1}
v/v	volume per volume
w/v	weight per volume

1. Introduction

1.1 Mitochondria: function and origin

Mitochondria are double-membrane-bounded organelles present in most eukaryotic cells. They are considered as essential functional component of the cell because of their capability to generate energy by aerobic respiration and oxidative phosphorylation (Henze and Martin 2003; McBride et al. 2006). The electrons, generated by the oxidation of nutrients such as glucose, undergo a series of transfers along the respiratory enzyme complexes. The electrons release energy by each transfer among the transport chain towards oxygen which serves as terminal electron acceptor. The energy released by the transfer among the transport chain, localized on the mitochondrial inner membrane, is stored in form of a proton gradient and then used for the synthesis of ATP (Saraste 1999). Beside the synthesis of ATP, mitochondria possess other biosynthetic pathways. They perform □-oxidation of fatty acids, Fe-S cluster and Fe-S protein synthesis, amino acid biosynthesis and are yet involved in apoptosis and cell death (Lill and Kispal 2000; Giulivi et al. 2008).

Mitochondria originated as result of an endosymbiotic event (Lang et al. 1999) by association of a facultatively anaerobic α-proteobacterium with a strictly anaerobically functioning archaebacterial host. (Martin and Müller 1998; Gray et al. 2001). Their origin is estimated at least 1.5 Ga together with the discovery of the unicellular eukaryotes architarchs and multicellular red algae which must have possessed mitochondria (Javaux et al. 2001; Butterfield 2000). At the time deep ocean water was still anoxic and the sulfide level was very high. The advent of oxygen took place during the time from 2.5 to ca 0.6 Ga (Canfield 1998; Anbar and Knoll 2001; Canfield et al. 2007) and the eukaryotes must have arisen and diversified by that time at very low oxygen tension and high sulfide concentration as anaerobic organisms (Martin and Müller 1998). Many recent eukaryotes still live in anaerobic environments and perform alternative pathways by oxygen lack, leading to ATP production (Fenchel and Finlay 1995; Martin et al. 2003; Mentel and Martin 2008). Depending on the pathway they use to synthesize ATP, they can be divided into three groups: those that perform cytosolic fermentation (type I), eukaryotes which possess anaerobic organelles called hydrogenosomes (type II) and anaerobic mitochondria (Tielens et al. 2002; Müller 1993; Martin and Müller 1998).

1.1.1 Type I and type II eukaryotes

Some eukaryotes don't need oxygen for ATP production. Instead, they use endogenously produced electron acceptors. These organisms perform substrate level phosphorylation and are classified into two groups, depending on the presence or absence of highly specialized organelles called hydrogenosomes (Müller 1998). Both groups share a key enzyme responsible for the oxidative decarboxyilation of pyruvate, pyruvate:ferredoxin oxidoreductase (PFO) (Hrdy and Müller 1995; Müller 1998), while in the mitochondria this reaction is usually catalyzed by pyruvate dehydrogenase (PDH).

The first group (type I) consists of amitochondriate protists like *Giardia intestinalis* and *Entamoeba histolytica* which are dependent on substrate level phosphorylation and do not possess ATP producing organelles.

Anaerobic organisms with specialized organelles belong to the second group (type II). These organelles are related to mitochondria and are called hydrogenosomes (Tielens et al. 2002; Henze and Martin 2003; Martin 2005; Tielens and van Hellemond 2006). Hydrogenosomes in contrast to mitochondria lack oxidative phosphorylation and rely on substrate level phosphorylation resulting in acetate, formate and H_2 as end products (Müller 1988; Boxma et al. 2004).

1.1.2 Anaerobic mitochondria

Not all mitochondria require oxygen for ATP production. Organisms that live temporarily or for prolonged time in the absence of oxygen have to maintain, in some way, their redox balance and need a different terminal electron acceptor than oxygen.

There exist eukaryotes that possess specialized facultatively anaerobic mitochondria that produce ATP through oxidative phosphorylation even under anaerobiosis. Instead of oxygen, anaerobic mitochondria require a proton-pumping electron transporter and excrete end products of electron transport other than H_2O. Organisms that possess anaerobically functioning mitochondria can be divided in two groups.

Unicellular protists belong to the first group including several fungi (Kobayashi et al. 1996; Takaya et al. 2003) and ciliates (Finlay et al. 1983) which use environmental electron acceptors, nitrate or nitrite, to produce nitrous oxide using electrons from the cytochrome *c* pool (Rotte et al. 2000; Tielens et al. 2002).

The other group consists of multicellular organisms as flatworms (platyhelminths) (Tielens 1994), parasitic nematodes and invertebrates (mussels and snails) (Grieshaber and Völkel 1998; Doeller et al. 2001) that inhabit hypoxic or anoxic environments, such as marine and fresh water sediments or the digestive tracts of animals. These organisms survive prolonged hypoxic phases of their life or alternating periods in the presence or absence of oxygen (De Zwaan 1991; Grieshaber et al. 1994) and possess anaerobic

mitochondria performing fermentative processes using endogenously produced substrates (i.e. fumarate) as terminal electron acceptors (Tielens et al. 2002).

In the absence of oxygen the carbohydrates are degraded in the cytosol via phosphoenolpyruvate (PEP) to pyruvate by the glycolytic pathway and to malate by the fermentative malate dismutation pathway. Malate and pyruvate are then transported into the mitochondria to be further metabolized (**Fig. 1.1**). A portion of this malate is oxidized via pyruvate and acetyl-CoA to acetate and the other portion is converted to succinate and propionate via fumarate reduction (Tielens 1994). The reaction is catalyzed by two typical components of anaerobically functioning mitochondria. Fumarate reductase (FRD) reduces fumarate to succinate and rhodoquinone shuttles the electrons necessary for the reduction of fumarate from NADH dehydrogenase complex I towards FRD.

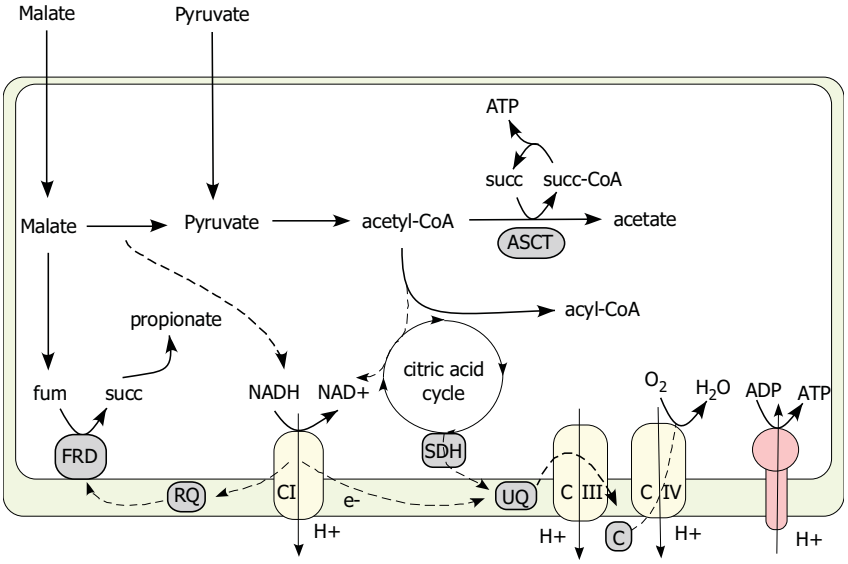


Fig. 1.1: Possible metabolic pathways in facultatively anaerobic mitochondria. Under aerobic conditions acetyl-CoA undergoes the citric acid cycle. In absence of oxygen, acetate, succinate, propionate and acyl-CoA are end products of fermentative processes. ASCT: acetate:succinate–CoA transferase; FRD: fumarate reductase; RQ: rhodoquinone; SDH: succinate dehydrogenase; UQ: ubiquinone; C: cytochrome; CI, CIII, CIV: complexes I, III and IV of the respiratory chain; fum: fumarate; succ: succinate (modified from Tielens et al. 2002).

Two other processes are known to be used as an electron sink to reoxidize the NADH produced by the oxidative catabolic pathways and therefore to maintain the redox balance. The first is the unusual mitochondrial lipid biosynthesis of *Ascaris suum*.

This parasitic nematode produces in addition to succinate, acetate and propionate, methyl-branched-chain fatty acids. 2-methylbutyrate and 2-methylvalerate are the predominant end products of carbohydrate metabolism and are synthesized using acetyl-CoA and propionyl-CoA as primer (Suarez de Mata et al. 1977; Komuniecki et al. 1989;

Kita 1992). The reactions involved in this pathway are comparable with those of mammalian β-oxidation.

The second process is the mitochondrial wax ester fermentation of the photosynthetic flagellate *Euglena gracilis* (Tielens et al. 2002).

1.1.3 The mitochondrion of *Euglena gracilis*

E. gracilis mitochondria have the basic architecture common to all mitochondria but are quite plastic in size and shape and have an irregular outer membrane (Pringsheim and Hovasse 1948; Buetow 1968). However the structure of the mitochondrial reticulum varies with the conditions of growth, temperature or exogenously supplied carbon source (Pellegrini 1980a).

The mitochondrion of *E. gracilis* is a facultatively anaerobic organelle able to produce ATP under aerobic and anaerobic conditions. Several *Euglena* species can adapt to a wide range of O_2 tension and can tolerate even very low oxygen concentrations (Buetow 1989). *E. gracilis* can survive anoxia up to six months when grown in the dark with cultivation on lactate (Carre et al. 1988).

In the presence of oxygen the pyruvate stemming from glycolysis is transported into the mitochondrion and undergoes oxidative phosphorylation. The synthesized acetyl-CoA enters a modified Krebs cycle with a succinate-semialdehyde shunt. Oxygen is the final acceptor for the electrons resulting from the glucose breakdown and the most of ATP is produced via oxidative phosphorylation (Buetow 1989; Kitaoka et al. 1989).

Under anaerobic conditions the mitochondrion of *E. gracilis* is able to perform a unique wax ester fermentation, in which the fatty acid synthesis serves as electron sink when the wax esters are synthesized from its polysaccharide paramylon (□-1-3-glucan) (Inui et al. 1984a). In the absence of oxygen the pyruvate stemming from glycolysis is reduced to acetyl-CoA by pyruvate:$NADP^+$ oxidoreductase (PNO), an oxygen sensitive enzyme (Inui et al. 1987; Rotte et al. 2001). The resulting acetyl-CoA is used as both primer and C2 donor, with NADH as electron donor for the mitochondrial malonyl-CoA-independent fatty acid biosynthesis (**Fig. 1.2**).

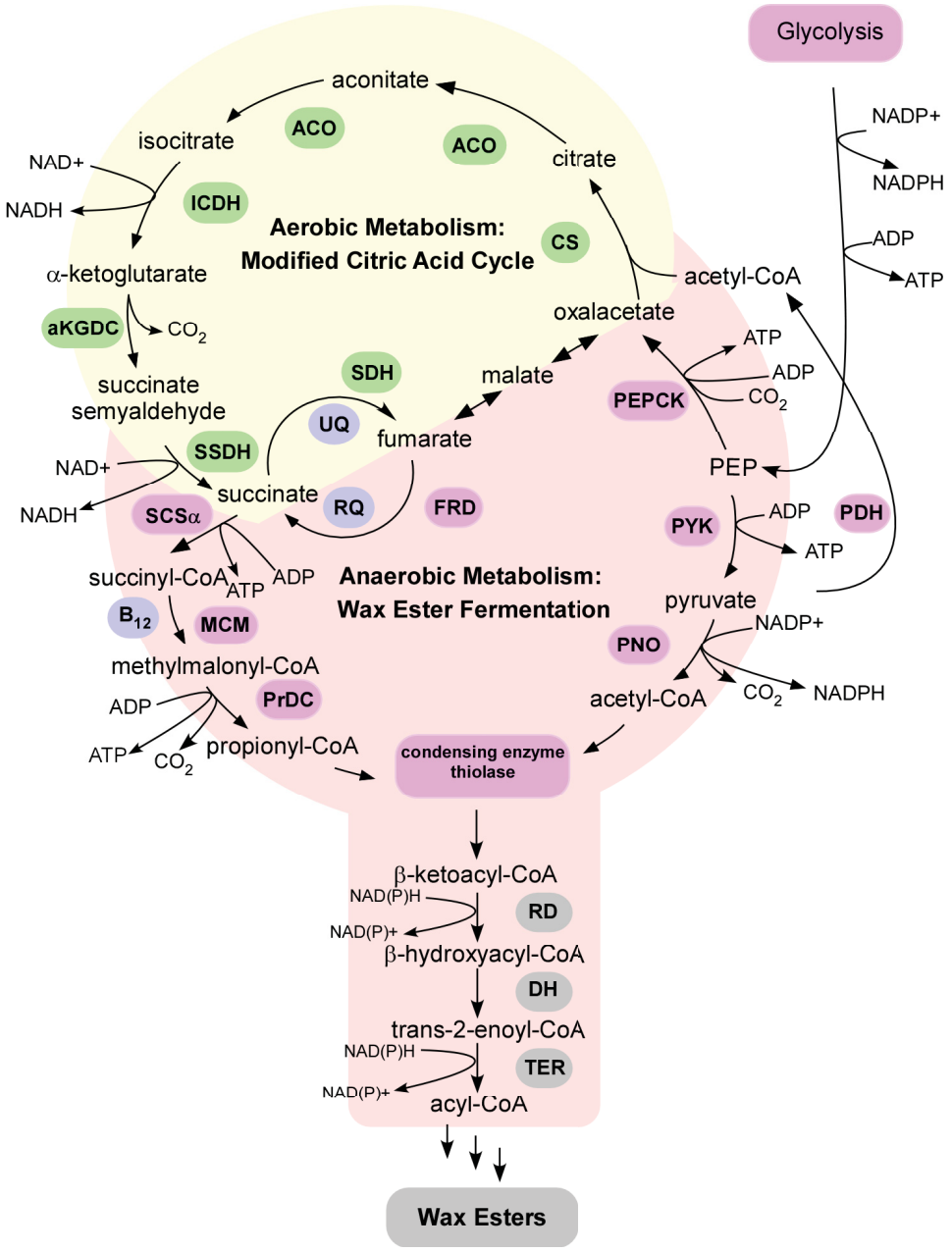


Fig. 1.2: Working hypothesis for the pathway of wax ester fermentation in *E. gracilis*. CS: citrate synthase; ACO: aconitase; ICDH: isocitrate dehydrogenase; α-KGDC: α-ketoglutarate decarboxylase; SSDH: succinate semyaldehyde dehydrogenase; SDH: succinate dehydrogenase; UQ: ubiquinone; SCS α: succinyl-CoA α-synthetase; MCM: methylmalonyl-mutase; PrDC: pyruvate decarboxylase; PNO: pyruvate:NADP$^+$ oxidoreductase; PYK: pyruvate kinase; PDH: pyruvate dehydrogenase; PEPCK; phosphoenylpyruvate carboxykinase; RD: β-ketoreductase; DH: β-hydroxyacyl-dehydrogenase; TER: *trans*-2-enoyl-CoA reductase (modified from Schneider and Betz 1985).

This pathway proceeds via CoA esters instead of acyl-carrier-protein (ACP) intermediates. The synthesized fatty acids are transported in the cytosol by an acyl-carnitine transferase (Kolattukudi 1970; Kahn and Kolattukudi 1973b). A portion of the synthesized fatty acids is reduced to alcohol, esterified with the fatty acids and

accumulated in the cytosol as waxes. On return to aerobiosis the wax esters are rapidly degraded and paramylon granules are resynthesized (Inui et al. 1982). The wax esters are synthesized up to 40 µg/10^6 cell in the first 24 h of anaerobic incubation and about 60 % of them consist of chains with an even number of carbons. The chains with an odd number of carbons are produced via the methyl-malonyl-CoA pathway using propionyl-CoA as primer (Nagai et al. 1971; Inui et al. 1983; Pönsgen-Schmidt et al. 1988). In this pathway phosphoenolpyruvate (PEP) is transformed to oxaloacetate by PEP carboxykinase (PEPCK) and enters a reverse Krebs cycle in which it is subsequently reduced to succinyl-CoA by two reductive steps catalyzed by malate dehydrogenase and fumarate reductase in presence of rhodoquinone (Takamiya et al. 1993; Van Hellemond et al. 1996; Miyadera et al. 1997; Hoffmeister et al. 2004). Succinyl-CoA is converted to methyl-malonyl-CoA by methyl-malonyl-CoA mutase (MCM), a vitamin B_{12} dependent enzyme, and finally decarboxylated to propionyl-CoA by methylmalonyl-CoA decarboxylase/propionyl-CoA carboxylase (Schöttler 1977; Schöttler and Wienhausen 1981; Schultz et al. 1983;).

1.2 The importance of fatty acids

Fatty acid biosynthesis is a primary metabolic pathway and it is essential for growth. In the cell fatty acids are never found as free acids. Their carboxyl group is mostly esterified or otherwise modified to form a class of compound known as lipids. Lipids assume different functions in the cell. Polar lipids such as phospholipids and glycerolipids, are the major compounds of cell membranes necessary for cellular compartmentalization (Harwood 1996). Lipids, as storage compounds in form of triacylglycerols, represent an important reserve of free energy while cuticular lipids such as wax esters are involved in waterproofing and surface protection of plant leaves (von Wettstein-Knowles 1993). Furthermore, many lipids or their metabolic derivates play a crucial role in biological activity as signal transductors, hormones and vitamins (Wang and Quinn 1999; Zmijewski 2005; Paschold 2008).

Fatty acids are carboxylic acids of highly reduced carbon chains. They can be divided in saturated chains which do not have double bonds and unsaturated fatty acids harbouring one or more (poly unsaturated fatty acids, PUFAS) double bonds in the chain. Usually the most widely distributed fatty acids in nature are even numbered saturated chains with 12-24 carbons. Fatty acids with a length of C20-C26 are called very long chain fatty acids (VLCFAs). Beside usual straight chains, it is possible to find, mostly in plants and bacteria, compounds with different structures, i.e. odd-numbered chains, methyl- and

epoxy-acids. Short hydroxy-branched fatty acids synthesized by many genera of eubacteria and some representatives of the archeabacteria are also very important (Doy 1990). The short-branched acids, used as primer in the biosynthesis of polyesters polymers, are accumulated as granules in the cell and are described as poly-β-hydroxyalkanoate (PHA) (Anderson and Daves 1990; Satoh et al. 2003).

1.3 Fatty acid biosynthesis in plants

The fatty acid biosynthesis in plants occurs in the plastids and the carbon source for this pathway derives from the pool of acetyl-CoA present in the organelle. It has been estimated that chloroplasts isolated from spinach leaves contain about 75 % of the total cellular acetyl-CoA (Post-Beittenmiller 1992; Tumaney et al. 2004). However, the exact biochemical origin of the plastidial acetyl-CoA is still unknown and many theories have been proposed in the past 30 years. Chloroplasts possess an extremely active acetyl-CoA synthetase (Behal et al. 2002) that covers a part of the plastidial acetyl-CoA requirement for TCA cycle, certain amino acids and isoprenoid biosynthesis (Ke et al. 2000). Another pathway could involve the activity of plastidial pyruvate dehydrogenase (PDH) acting on pyruvate deriving from glycolysis. Furthermore, the production of acetyl-CoA by a mitochondrial PDH where the synthesized acetyl-CoA is then transferred as free acetate or acetylcarnitine into the plastid. The fourth route involved in the synthesis of acetyl-CoA is the ATP-citrate lyase reaction (ACL), whereas the proposed role is somewhat controversial. In leaves of *Brassica napus* L. the ACL activity has been reported to be predominantly plastidial, while in tobacco and pea cytosolic (Rangasamy and Ratledge 2000). Furthermore, in *Arabidopsis* no plastidial ACL activity or protein could be demonstrated to date (Lernmark and Gardeström 1994; Ohlrogge and Browse 1995; Ohlrogge et al. 2000; Rawsthorne 2002; Tumaney et al. 2004). Acetyl-CoA plays a crucial role in many different metabolic pathways. It is, therefore, conceivable that more than one pathway may contribute to maintaining the acetyl-CoA pool.

Fatty acid biosynthesis starts with the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACCase). This reaction takes place in two steps. The first step is catalyzed by the biotin carboxylase portion of ACCase and is ATP-dependent. Biotin carboxylase activates the HCO_3^- by transferring it to a lysin residue of the biotin-carboxyl-carrier protein. In the second reaction, catalyzed by carbonyltransferase, the CO_2 is transferred from biotin to acetyl-CoA to form malony-CoA.

Two different forms of ACCase have been identified in plants (Alban et al. 1993; Konishi and Sasaki 1994; Sasaki and Nagano 2004; Belkebir et al. 2006). The prokaryotic

type consists of a multisubunit ACCase present in the plastids of all plants, except Geraniaceae and Poaceae, and is composed of four different subunits encoded by nuclear genes with the exception of the carbonyltransferase which is encoded by the plastidial genome (Konishi et al. 1996). The second type, known as eukaryotic ACCase, is a multifunctional enzyme assigned to the cytosolic compartment consisting of a single polypeptide encoded by nuclear genes. The eukaryotic ACCase is involved in fatty acid elongation and flavonoid biosynthesis while the prokaryotic type plays a key role for *de novo* fatty acid synthesis (Belkebir et al. 2006).

While in animals, fungi and some bacteria fatty acid biosynthesis is catalyzed by a multifunctional polypeptide complex situated in the cytosol (FAS I), in plants individual enzymes are organized into a multienzymatic complex (FAS II) which is localized in the stroma of plastids. The synthesis starts with the transfer of malonyl-CoA from CoA to ACP, a protein cofactor containing phosphopantethein as prosthetic group, on which the growing fatty acid chain is attached as thioester, and the condensation with an acetyl-CoA moiety (**Fig. 1.3**).

During this first condensation a C-C bond is generated and a CO_2 molecule is released. To synthesize an 18-carbon fatty acid at least three different condensing enzymes are required, distinguished on their substrate specificity. β-ketoacyl synthase III (KAS III) acts only on the first condensation between malonyl-ACP and acetyl-CoA to form β-ketobutyryl-ACP, a four carbon product (Jaworski et al. 1989). The elongation of the fatty acid chain up to C12-C16 carbons is catalyzed by KAS I and the final condensation to give stearoyl-ACP with 18 carbons requires KAS II. After the initial condensation, whose product is β-ketoacyl-ACP, three additional steps are necessary to synthesize a saturated fatty acid. The first reaction is the reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP catalyzed by the β-ketoreductase, which uses NAD(P)H as electron donor. The next reaction is dehydratation by β -hydroxyacyldehydratase to form *trans*-2-enoyl-ACP. The last step of one round of fatty acid biosynthesis cycle is again a reduction step. *Trans*-2-enoyl-reductase converts 2-*trans*-enoyl-ACP to the corresponding saturated chain. Acyl-ACP can be removed from ACP in two different ways.

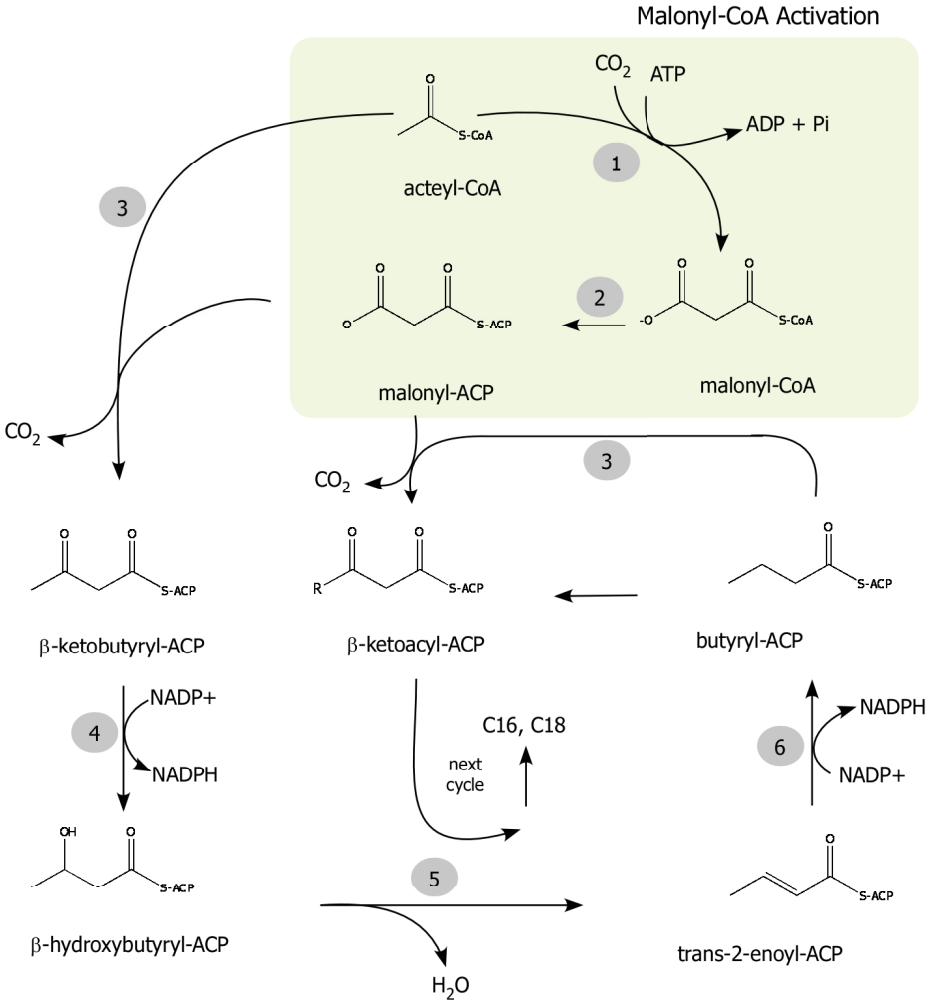


Fig. 1.3: **Reaction and enzymes involved in the fatty acid biosynthesis**. 1: acetyl-CoA carboxylase; 2: malonyl-CoA:ACP transacylase; 3: β-ketoacylsynthase; 4: β-ketoreductase; 5: β-hydroxyacyldehydratase; 6: *trans*-2-enoyl-reductase (modified from Ohlrogge 1995).

Mostly an acyl-ACP thioesterase hydrolyzes the acyl-ACP to free fatty acid which is transferred to the outer envelope of the plastid where it is reactivated to acyl-CoA to form glycerolipids. Alternatively, an acyltransferase transfers the fatty acid to glycerol-3-phosphate for the biosynthesis of plastidial membrane lipids (Ohlrogge 1995; Koo et al. 2004).

Beside the plastidial fatty acid biosynthesis plants possess a fatty acid synthesis system localized in the mitochondria (Chuman and Brody 1989; Shintani and Ohlrogge 1994). In contrast to the plastidial one, the rate of the mitochondrial fatty acid synthesis is marginal and might involve repair processes of membrane phospholipids as previously suggested for yeast (Schneider et al. 1997; Hiltunen 2005). Additionally, as demonstrated in pea leaves mitochondria, this pathway is responsible for the synthesis of lipoic acid, an essential cofactor of glycine decarboxylase (Wada et al. 1997). Similarly to FAS type I and type II, the mitochondrial pathway uses malonyl-CoA as C2 donor. However,

dicotyledonous plant mitochondria lack ACCase and require for activation of malonate into malonyl-ACP a malonyl-CoA synthetase and a malonyl-CoA:ACP transacylase (Baldet et al. 1993; Gueguen et al. 2000). Mitochondria of grasses and rice, on the other hand, have been demonstrated to possess a mitochondrial ACCase (Focke et al. 2003).

When the synthesis of saturated fatty acids in the chloroplasts is concluded, double bonds, usually stereospecific "*cis",* have to be introduced to enhance membrane fluidity and to lower the melting point. The plastidial stearoyl-ACP desaturase is a membran-bound enzyme that catalyzes the introduction of the *cis*-double bond in the Δ9 position in several fatty acids (Hongsthong et al. 2003). This enzyme requires molecular oxygen and ferredoxin as electron donor for its activity as shown in **Fig. 1.4**. (Sperling et al. 2003; Hongsthong et al. 2006).

Generally, fatty acid biosynthesis results in C16 and C18 chains, but plants possess VLCFAs with chain length of 20 or more carbons. VLCFAs result from the elongation of plastidial generated fatty acids by a membrane-bound enzymatic complex localized in the endoplasmatic reticulum (Joubes et al. 2008). The fatty acid elongation proceeds with the same sequence of reactions involved in *de novo* fatty acid biosynthesis but with CoA-esters instead of ACP-esters. *Trypanosoma brucei*, the sleeping sickness parasite, is the first and only organism known so far, in which an unusual elongase system is involved in *de novo* fatty acid biosynthesis (Lee et al. 2006; Lee et al. 2007).

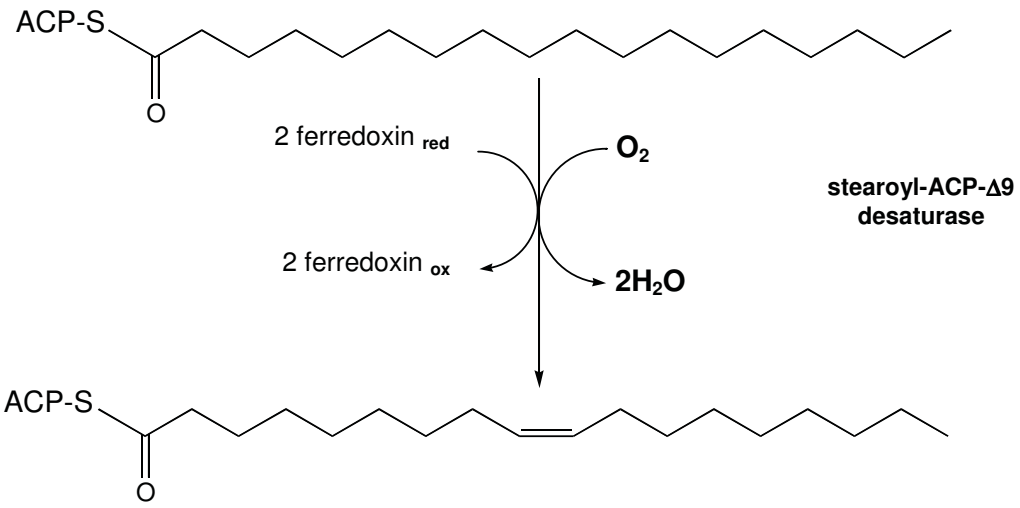


Fig. 1.4: Desaturation of stearic acid catalyzed by stearoyl-ACP-Δ9 desaturase.

1.4 Fatty acid biosynthesis in *E. gracilis*

Of particular interest, from an evolutionary and biochemical point of view, is the coexistence of different types of fatty acid synthesis (FAS) systems in *E. gracilis*. The first is a multifunctional FAS type I, typical for yeast and mammalian cells, located in the cytosol which produces mainly C16 acids as major products followed by C14 and C18 chains (Goldberg and Bloch 1972; Walker et al. 1981). The FAS type II is a highly conserved group of enzymes characteristic for plants and bacteria, where all the reactions are carried out at active sites located on the same polypeptide chain (Mc Carthy and Hardy 1984). *E. gracilis* possesses two ACP-dependent FAS type II localized in the chloroplasts, whereas one of those is an elongase system (Ernst-Fonberg and Bloch, 1971; Hendren and Bloch, 1980). The fourth fatty acid synthetic system has been reported to be present in the microsomes and seems to be involved in *de novo* wax ester synthesis under aerobic conditions. The last one is the ACP- and malonyl-CoA-independent mitochondrial fatty acid synthesis system involved in the anaerobic wax ester fermentation (Inui et al. 1982, 1984a). In contrast to the other fatty acid synthases, the mitochondrial one occurs with a net gain of ATP since the activation from acetyl-CoA to malonyl-CoA is not required (Buetow 1989). This mitochondrial synthase system has been long described as a reversal of β-oxidation in which the acyl-CoA dehydrogenase was substituted by *trans*-2-enoyl-CoA reductase (TER)(Inui et al. 1984a). However, a key difference occurs since the acyl-CoA dehydrogenation steps of the mitochondrial β-oxidation are linked to O_2 reduction and are irreversible (Graham and Eastmond 2002). Recently, Hoffmeister et al. (2005) characterized a novel *trans*-2-enoyl-CoA reductase (EC 1.3.1.44) from *E. gracilis* that catalyzes the reduction of enoyl-CoA to acyl-CoA in the wax ester fermentation pathway of the facultatively anaerobic *E. gracilis* mitochondrion. *Euglena* TER defines a new family of enzymes that is widely distributed among prokaryotic genomes, but without functionally characterized prokaryotic homologues. This enzyme showed sequence similarity to the *Treponema denticola* open reading frame TDE0597 and the *Streptomyces avermitilis* open reading frame SAV2368, annotated, like all other prokaryotic members of the family, with unknown function. Members of this protein family in prokaryotes are often organized in gene clusters that suggest involvement in fatty acid or related synthesis operating with CoA esters instead of acyl-carrier-protein (ACP)-esters (Hoffmeister et al. 2005).

1.5 Aims of this work

The facultatively anaerobic mitochondrion of *E. gracilis* performs an unusual wax ester fermentation. Despite much work reported in the literature over the past 25 years the biochemical pathway is not yet completely described. In light of the foregoing, the aims of this work were to characterize the mitochondrial fatty acid synthesis system of *E. gracilis*. Since this pathway shares high similarity between the usual fatty acid synthesis and the polyketide biosynthesis, the prokaryotic TER homologoues TDE0597 from *T. denticola* and SAV2368 from *S. avermitilis* have to be heterologously expressed and biochemically characterized.

In order to understand how the presence of oxygen influences the content and the composition of the wax esters, *E. gracilis* 1224-5/25 strain Z has to be cultivated under aerobic and anaerobic conditions and in the presence of the herbicide flufenacet. The wax esters of the different cultures have to be isolated, transesterified and analysed via gas chromatography as fatty acid methyl esters and fatty alcohols.

To compare the ability to survive anoxia and to perform mitochondrial anaerobic fermentation within the same genus, growth curves of eleven different *E. gracilis* strains have to be generated. The wax ester fraction of the cultures has to be isolated and analyzed. In order to test the expression of mitochondrial protein involved in the wax ester fermentation Northern blot analysis has to be performed.

The amino acid sequence of the thiolase/condensing enzyme, one of the key enzymes involved in the wax esters fermentation, has to be identified and the protein heterologously expressed and biochemically characterized.

2. Materials and Methods

2.1 Chemicals

All chemicals used in this work were purchased in p.a. quality from J.T. Backer, Biomol, Biorad, Biozym, Calbiochem, Fluka, ICN, Merck, Riedel de Haën, Roche, Roth, Sigma and Serva.

2.2 Kits

ECL Western Blotting Analys System	Amersham Bioscience
Nucleospin Plasmid Kit (Mini, Midi, Maxi)	Macherey-Nagel
ThermoSequenase DYEnamic direct cycle sequencing kit	Amersham Bioscience
QIAquick Gel Extraction Kit	Qiagen
Quick Ligation Kit	Qiagen

2.3 Chromatographic material

In this work the following chromatographic materials were used:

acetone	VWR
acetonitrile	Riedel de Haën
chloroform	Fluka
$[Ce(SO_4)_2 \times 4H_2O]$	Sigma
TLC_{F254} Plate (5 x 10 cm)	Merck
TLC_{F254} Plate (10 x 20 cm)	Merck
TLC_{F254} Plate (20 x 20 cm)	Merck
2'-7'-dichlorfluorescein	Sigma
diethyl ether	Riedel de Haën
silica gel 60	Merck
methanol	Riedel de Haën
methylpalmitate	Sigma
molybdenum (VI)-oxid	Riedel de Haën

molybdenum	VWR
α-naphthol	Riedel de Haën
n-hexane	Normapur
Ni-NTA agarose	Qiagen
petrol ether	Roth
1-tetradecanol	Fluka

2.4 Enzymes

alkaline phosphatase (CIP)	New England Biolabs
DNAse I	Roche
Klenow-fragment	MBI Fermentas
Pfu DNA polymerase	Promega
restriction endonucleases	MBI Fermentas, New England Biolabs
RNAse A	Serva
Taq DNA polymerase	Eppendorf
Triple Master DNA polymerase	Eppendorf
T4 DNA ligase	Fermentas

2.5 Strains

2.5.1 *Euglena gracilis* strains

The *Euglena gracilis* strains used in this work were obtained from **SAG** (Sammlung von Algenkulturen), Göttingen.

E. gracilis SAG **1224-5/3**	*E. gracilis* SAG **1224-5/4**
E. gracilis SAG **1224-5/10**	*E. gracilis* SAG **1224-5/13** var. Saccharophyla
E. gracilis SAG **1224-5/15** var. Bacillaris	*E. gracilis* SAG **1224-5/18**
E. gracilis SAG **1224-5/19**	*E. gracilis* SAG **1224-5/23**
E. gracilis SAG **1224-5/25 strain Z**	*E. gracilis* SAG **1224-5/27**

2.5.2 *Treponema denticola*

Treponema denticola ATCC 35405 DNA was a kind gift of Prof. Howard F. Jenkinson University of Bristol, U.K.

2.5.3 *Streptomyces avermitilis*

Streptomyces avermitilis MA-4680 DNA was obtained from **DSMZ** (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

2.5.4 *Escherichia coli* strains

The following *E. coli* strains were used in this work:

XL1-Blue MRF' (Stratagene)	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]
BL21 (DE3) (Invitrogen)	F- ompT hsdSB (rB-mB-) gal dcm(DE3)
C41 (DE3) (Avidis)	BFdcm ompT hsdS(rBmB) Gal λ (DE3) + 1 not characterized mutation
C43 (DE3) (Avidis)	BFdcm ompT hsdS(rBmB) Gal λ (DE3) + 2 not characterized mutation
Origami (DE3) (Novagen)	Δara-leu 7697 ΔlacX74 ΔphoAPvull phoR araD139 galE galK rpsLF`[lac+ (lacq)pro] gor522::Tn10(TcR) trxB::kan
Rosetta (DE3) (Novagen)	F-ompT hsdSB(rB –mB -) gal dcm (DE3) pRA RA (argU, argW, ileX, glyT, leuW, proL

2.5.5 *Saccharomyces cerevisie*

The heterologous expression in yeast was perfomed with the following strain:

INVSc1 (Invitrogen)	his3Δ1/his3Δ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3- 52

2.6 Plasmids

For the heterologous expression in *E. coli* and *S. cerevisie* the followings plasmids were used:

pBluescript SK+ (Stratagene)	*E. coli* plasmid with ampicillin resistance and blue-white selection
pGEM®-T Easy (Promega)	*E. coli* plasmid with ampicillin resistance and blue-white selection
pET28a (Novagen)	*E. coli* expression vector with kanamycin-resistance and N- and C-terminal His-Tag
pET32a (Novagen)	*E. coli* expression vector with ampicillin resistance und N- und C-terminal His-Tag as well as N-terminal thioredoxin-fusion
pQE30 (Qiagen)	*E. coli* expression vector with ampicillin resistance and N-terminal His-Tag
pYES2/CT (Invitrogen)	*S. cerevisiae* expression vector with C-terminal His-Tag
pYCT/2CT (Invitrogen)	*S. cerevisiae* expression vector with C-terminal His-Tag

2.7 Oligonucleotides

Oligonucleotides for this work were synthesized by Metabion GmbH (Martinsried, Germany) and stored in aqueous solution at -20 °C.

The following fluorescence marked primer were used for sequencing the pBluescript SK^+ plasmid:

M13(24)for	5'-IRD700-CGC CAG GGT TTT CCC AGT CAC GAC-3'
M13(24)rev	5'-IRD800-AGC GGA TAA CAA TTT CAC ACA GGA-3'

The following fluorescence marked primer were used for sequencing pET28a and pET32a plasmids:

T7proext	5'-IRD700-CGC GAA ATT AAT ACG ACT CAC TAT AGG-3'
T7term	5'-IRD800-ATG CTA GTT ATT GCT CAG CGG-3'

The following oligonucleotides were synthesized for cloning *T. denticola* and *S. avermitilis* TER homologues and the *E. gracilis* propionyl-CoA carboxylase (PCC). The restriction sites are underlined.

TdenNdeI_F	5'-TAT ACA TAT GAT TGT AAA ACC AAT GGT TAG-3' *Nde*I
TdenBglII_F	5'-TAT AGA TCT TAT GAT TGT AAA ACC AAT GGT TAG-3' *Bgl*II
TdenXhoI_R	5'-TAT CTC GAG TTA AAT CCT GTC GAA CCT TTC TAC-3' *Xho*I
SaveNdeI_F	5'-TAT ACA TAT GTG CGT CTG GAC ACC GCG TCG-3' *Nde*I
SaveBglII_F	5'-TAT AGA TCT TAT GTG CGT CTG GAC ACC GCG TCG-3' *Bgl*II
SaveXhoI_R	5'-TAT CTC GAG CTA CGA CTC CGG CCA GGG CAG-3' *Xho*I
SaveHindIII_HF	5'-TAT AAG CTT GTT ATG TGC GTC TGG ACA CCG CGT-3' *Hind*III
SaveXhoI_HR	5'-TAT CTC GAG CGA CTC CGG CCA GGG CAG-3' *Xho*I
Eg2493BamHI_F	5'- TAT GGA TCC ATG AAG GGA ATG CGG AAG GTT-3' *Bam*HI
Eg2493SacI_R	5'- TAT GAG CTC CTA GCA CAG CGT CAA CTC GGT-3' *Sac*I
Eg2493BamHI_HF	5'- TAT GGA TCC AATG AAG GGA ATG CGG AAG GTT-3' *Bam*HI
Eg2493XbaI_HR	5'- TAT TCT AGA GCA CAG CGT CAA CTC GGT CGC-3' *Xba*I

Eg_PCCFor 5'-TAT AAG CTT GAG GCG CTT GCT TCT TTT GGT GAT-3'
*Hind*III

Eg_PCCRev 5'- TAT AAG CTT GGA ACT CGA GCT CGA TCA ATT TTG-3'
*Hind*III

2.8 Working with *E. gracilis*

Cultures of *E. gracilis* were maintained in 50 ml TYS medium [0.1 % (w/v) sodium acetate; 0.1 % (w/v) meat extract; 0.2 % (w/v) trypton; 0.2 % (w/v) yeast extract; 0.02 % (w/v) KNO_3; 0.001 % (w/v) $MgSO_4x\ 7H_2O$; 0.002 % (w/v) $(NH_4)_2HPO_4$; 0.004 % (w/v) $CaSO_4$] at 28 °C with constant light at 5000 lux in a plant growth chamber (Sanyo MLR-350). About 5 ml of this permanent culture were used to inoculate 200 ml of preculture. After 6 days the preculture was used to inoculate larger cultures in a 10 liter flask or in a fermenter (BIOSTAT B 10L, Braun Biotech).

The cultivation in the 10 liter flask was carried in 7 liters TYS medium for 6 days under the same conditions of the precultures. Aerobic cultures were treated with 2 l/min CO_2 in air and anaerobic cultures with 2 l/min CO_2 in N_2.

The cultivation in the fermenter was performed in a 7 liters defined Koren-Hutner medium [composition for 1 liter medium: 12 g glucose; 0.8 g KH_2PO_4; 1.5 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4x\ 7H_2O$; 0.2 g $CaCO_3$; 0.0144 g H_3BO_3; 2.5 mg vitamin B_1; 20 µg vitamin B_{12}; 1 ml trace elements solution; 1 ml Fe-solution. The trace elements solution was made up of 4.4 g $ZnSO_4x\ 7H_2O$; 1.16 g $MnSO_4x\ H_2O$; 0.3 g $Na_2MoO_4x\ 2H_2O$; 0.32 g $CuSO_4x\ 5H_2O$; 0.38 g $CoSO_4x\ 5H_2O$ in 100 ml destilled water; the Fe solution was made up of 1.14 g $(NH_4)_2\ SO_4Fe(SO_4)_2x\ 6H_2O$ and 1 g EDTA in 100 ml destilled water]. The cultures were grown under stirring at 200 rpm at constant temperature of 28 °C and pH 2.8. The vitamins and the glucose were added to the medium after sterilization. Aerobic cultures were treated with 2 l/min CO_2 in air and anaerobic cultures with 2 l/min CO_2 in N_2. The cells were harvested six days after the inoculation and centrifuged at 2800 *g* for 5 min. The harvested cells were frozen at -20 °C or immediately used for lipids extraction.

E. gracilis bleached cells were grown in 50 ml modified Hutner's medium [19.8 g glucose; 0.04 mM $FeSO_4x\ 7H_2O$; 0.01 mM $ZnSO_4x\ 7H_2O$; 0.009 mM $MnSO_4x\ 5H_2O$; 0.1 mM $Ca(NO_3)_2x\ 4H_2O$; 0.6 mM $MgSO_4x\ 7H_2O$, 2 mM KCl; 2 mM D_2-malic acid; 0.1 % $(NH_4)_2HPO_4$] at constant temperature of 28 °C and pH 3.5.

2.9 *E. gracilis* bleaching

Three Erlenmeyer flasks with 20 ml defined Koren-Hutner medium were inoculated with 50,000 cells/ml (final concentration) *E. gracilis* 1224-5/25 strain Z cultures. One flask was used as a control while the other two flasks were supplemented with 200 µg/ml ofloxacin and 500 µg/ml streptomycin, respectively. The cultures were incubated overnight at 28 °C. The next day between 150 and 300 cells were plated on defined Koren-Hutner medium plates with 7.5 % agar and incubated at 28 °C over 10 days. White colonies of ofloxacin and streptomycin mutants were picked and grown in modified Hutner's medium at 28 °C.

2.10 Working with nucleic acids

The following protocols of basic methods of molecular biology were conducted as described by Sambrook et al. (1989):

- Phenolic extraction of nucleic acids and its concentration by ethanol or isopropanol extraction
- Photometric method to determine nucleic acids concentration
- Separation of nucleic acids or DNA fragments by agarose gel electrophoresis
- DNA digestion with restriction enzymes
- Ligation in expression vectors
- Vectors dephosphorylation by alkaline phosphatase

2.10.1 Isolation of total RNA from *E. gracilis*

For total RNA isolation, about 500 mg fresh weight *Euglena* cells were used. The frozen cells were trasnfered to a mortar and were ground in mortar and pestle in the presence of liquid N_2. To isolate RNA 0.5 ml Trizol® reagent (Invitrogen) was added. The powder was transfered in a test tube with 0.1 ml chloroform, it was vigorously shaken and was left on ice for 10 minutes. Afterwards, it was centrifuged at 10000 *g* for 10 min and 4 °C. One volume isopropanol was added to the supernatant and was incubated on ice for 30 min. The mixture was centrifuged at 10000 *g* for 10 min and 4 °C. The supernatant was discarded and the pellet washed 2 x with 70 % ethanol. The supernatant was discarded again and the dried pellet was solubilized in 0.01 % DEPC water and stored at -80 °C.

2.10.2 cDNA synthesis

E. gracilis cDNA was synthesized from 4 µg total RNA with the First strand Synthesis Kit for RT-PCR (Invitrogen) as recommended by the manufacturer, and was stored at -20 °C.

2.10.3 Plasmid DNA isolation

Plasmid DNA isolation was performed with the Nucleospin Plasmid Kit (Mini, Midi, Maxi; Macherey-Nagel) as recommended by the manufacturer. The isolated plasmid DNA was digested with restriction enzymes and the size of the insert, as well as its concentration, was tested on a 1 % agarose gel electophoresis.

2.10.4 Sequencing

All fragments were sequenced by AGOWA (Berlin), GATC Biotech (Konstanz) or by the method of Sanger et al. (1977) with didesoxynucleotides modified as follows. The fragments were sequenced using the laserfluorescence method performed by a DNA Sequencer Long ReadIR 4200 (Licor). 2-3 µg plasmid were dissolved in 20 µl water. The sequence reactions were carried out using the ThermoSequenase DYEnamic direct cycle sequencing Kit (GE Healthcare Biosciences) as recommended by the manufacturer and the PCR reaction performed in a Primus 96 Plus Thermocycler (MWG Biotech). Initial denaturation at 98 °C for 150 s with a PCR programm over 25 cycles including a short denaturation at 98 °C for 60 s, annealing at 68.5 °C for 35 s and elongation at 71 °C for 120 s was employed. To the PCR samples 7 µl loading buffer [200 µl 0.5 M EDTA pH: 8.0; 100 ml Paraosanilin (100 mg/ml); 9.5 ml formamide] were added. The samples were denaturated at 70 °C for 3 min and loaded onto a sequencing gel prepared as recommended by the manufacturer.

2.10.5 Polymerase chain reaction (PCR)

For amplification of specific *E. gracilis* DNA sequences between 70 and 150 ng cDNA were used as template. For amplification of specific *T. denticola* and *S. avermitilis* sequences between 100 and 250 ng genomic DNA were used as template. A typical PCR reaction was performed in 25-50 µl end volume and included 1x *Triple Master* or *Pfu*-buffer, 0.25 mM each dNTP, 2 µM each primer and 1 U *Triple Master* or *Pfu*-polymerase. The reaction was carried out in an Eppendorf thermocycler with a denaturation at 98 °C for 2 min, 30 cycles of 15 s at 94 °C, 30 s at 65 ± 5 °C for annealing, 90 s at 72 °C for elongation and a final elongation step of 5 min at 72 °C. In some cases an initial denaturation for 15 min and a Hot Start reaction were necessary for a successful PCR.

2.10.6 Cloning of PCR fragments

The PCR products were separated on a 1 % agarose gel, the specific fragments were excized from the gel and eluted by QIAquick Gel Extraction Kits (Qiagen) as recommended by the manufacturer. The phosphorylation was performed with 20 U T4-DNA kinase and 0.01 M ATP for 30 min at 37 °C. Afterwards the PCR fragments were filled in with 3 U Klenow polymers and 50 □M of each dNTP for 30 min at 37 °C. The reaction was stopped by addition of 50 mM EDTA pH 8.0. The PCR fragments were separated with the QIAquick Gel Extraction Kit (Qiagen) and ligated into the pBluescript SK+ vector (Stratagene).

2.10.7 Preparation of DNA radioactively labeled probes

The preparation of radioactively labeled probes for the Northern blot analysis was performed as described by Sambrook et al. (1989). The DNA fragment was denaturated and incubated over 3 hours at room temperature with 1x Oligo-Mix [5 OD_{260} $p(dN)_6$ (GE Healthcare Biosciences); 10 mM $MgCl_2$; 50 mM Tris-HCl, pH 7.0; je 50 µM dATP, dGTP, dTTP; 7 mM β-mercaptoethanol] 5 □g BSA, 2.5 U Klenow polymers und 3 µl (1.1 MBq) α^{32}PdCTP. Surplus nucleotides were removed on a Sephadex G50 column and the purified hybridization probe was used for the homologous or heterologous hybridization.

2.10.8 Northern blot analysis

The electrophoretic separation of RNA was performed under denaturing conditions on a 1.2 % agarose gel [1x MOPS-Puffer (20 mM MOPS pH 7.0; 5 mM sodium acetate pH 6.4; 1 mM EDTA pH 8.0); 6.6 % (v/v) formaldehyde and 3 µl ethidiumbromide]. Each solution and buffer were prepared with 0.01 % DEPC water and proteinase K (10 µg/ml) to prevent RNA degradation. All bowls and flasks were washed with 0.65 % sodium hypochloride. 5 mg RNA were dissolved in 15 µl 0.01 % DEPC water and 5 µl RNA loading buffer [57 % (v/v) formamide; 17.5 mM MOPS pH 7.0; 4.4 mM sodium acetate pH 6.4; 0.8 mM EDTA; 8 % (v/v) formaldehyde; 1.4 % (w/v) Ficoll 400; 0.09 mg/ml bromphenol blue]. The samples were loaded onto the gel and separated at 5 V/cm over 2 hours in a 1x MOPS buffer (0.04 M MOPS pH 7.0; 0.01 M sodium acetate; 0.001 M EDTA). The agarose gel was washed 3x 20 min in 0.01 % DEPC water with proteinase K (10 µg/ml). The transfer was performed using the capillary transfer method onto a nylon membrane (N, Amersham Biosciences) as described by Sambrook et al. (1989). The transfer occured overnight in SSC buffer (3 M NaCl and 0.3 sodium citrate pH 7.0). The membrane was dried and the RNA samples fixed by UV light (λ=312 nm) for 3 min. The prehybridisation of the membrane was performed in a buffer containing 6x SSPE (3 M NaCl; 0.2 M NaH_2PO_4;

20 mM EDTA pH 7.4), 0.1 % SDS, 0.02 % (w/v) PVP 90, 0.02 % (w/v) Ficoll 400 at 55 °C for 2 hours. The hybridization occured at 55 °C for heterologous hybridization and at 68 °C for homologous hybridization overnight in 3x SSPE, 0.1% (w/v) SDS, 0.02 % (w/v) PVP 90, 0.02 % (w/v) Ficoll 400, RNA samples and 10 ng/ml radioactively labeled probe. Hybridization probes were prepared by the Feinberg and Vogelstein method (1984) and radioactively marked with α-^{32}P-dCTP as described in **2.10.7**. The membrane was washed in 2x SSPE, 0.1 % SDS, dried and was exposed overnight onto a Fuji Medical X-Ray Film (Fujifilm) at -80 °C. The mRNA expression level detected on SR imaging plates with a FLA3000 Fluorescence scanner version 1.8E and quantified with the Image Gauge software (version 3.0) (Fuji Photo film Co., Düsseldorf, Germany).

2.11 Working with *E. coli*

The following protocols of basic methods of molecular biology were conducted as described by Sambrook et al. (1989):

- Preparation of culture media: FB and LB
- Cultivation of different bacterial strains on solid and fluid media
- Preparation and transformation of $CaCl_2$ competent and ultracompetent *E. coli* cells with plasmid-DNA
- *E. coli* cells transformed with recombinant pBluescript-plasmid-DNA were use for blue-white selection on LB-agar plates supplemented with 0.1 mg/ml ampicillin, 0.047 mg/ml IPTG and 0.04 mg/ml XGal.

2.11.1 Preparation of competent *E. coli* cells

XL1-Blue cells were grown overnight at 37 °C on LB agar plates supplied with tetracyclin (30 µg/ml). About 10-12 large colonies were picked to inoculate 250 ml SOB medium (0.5 % yeast extract; 2 % tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$) in a 1 liter flask and grown at 16 °C under vigorous shaking to OD_{600}= 0.5. The culture was left on ice for 10 min and was then pelleted by spinning at 3400 *g* for 10 min and 4 °C. The supernatant was descarded and the pellet was gently resuspended in 80 ml ice-cold TB medium (10 mM PIPES; 15 mM $CaCl_2$; 250 mM KCl; 55 mM $MnCl_2$; pH 6.7). The resuspended cells were spun at 3400 *g* for 10 min and 4 °C, the supernatant was discarded and the pellet was gently resuspended in 20 ml ice-cold TB and 1.4 ml DMSO. The cells were aliquoted and stored at -80 °C.

2.11.2 Expression cultures

The plasmids (pQE30, pET-28a und pET-32a) carrying the recombinant genes for protein expression were transformed freshly into different *E. coli* strains. The colonies selected from LB plates supplemented with antibiotics were inoculated in 5 ml of fresh LB medium (with antibiotics) and were incubated overnight. 2 ml of these culture were used to inoculate 200 ml expression cultures and were incubated at 37 °C until they reached an OD_{600} 0.6-0.8. The induction was performed by addition of 0.01 mM-1 mM IPTG and incubation at different temperature (16 °C, 30 °C und 37 °C) between 1 h and overnight.

2.11.3 Isolation of recombinant proteins from *E. coli* expression cultures

E. coli cultures were harvested by spinning at 3400 *g* for 10 min and 4 °C. The pellet was resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8.0; 300 mM NaCl; 10 mM imidazol) with lysozyme (1 mg/ml) and incubated on ice for 20 min. The cells were sonicated 6x at 80 W for 10 s. RNase A (10 µg/ml) and DNase I (5 µg/ml) were added to the homogenate and the mixture was incubated 10 min at 4 °C. The cell debris was centrifuged at 16000 *g* for 30 min and 4 °C. The supernatant was then used for enzymatic activity assays and for SDS-PAGE analysis.

2.11.4 Isolation and disruption of inclusion bodies

Fifty milliliter *E. coli* cultures were harvested by spinning at 3400 *g* for 10 min and 4 °C. The pellet was resuspended in lysis buffer 1 (50 mM Tris-HCl, pH 8.0; 25 % saccharose; 1 mM EDTA), 120 µl lysozyme (10 mg/ml) were added and the suspension was incubated for 30 min and 4 °C. 25 µl DNAse I (100 µg/ml) and 83 µl buffer 2 (100 mM $MgCl_2$; 10 mM $MnCl_2$) were added to the lysate and the mixture was again incubated for 30 min and 4 °C. Afterwards, 1.5 ml buffer 3 (20 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1 % Triton X-100; 1 % sodium cholate) were added and the solution was centrifuged at 16000 *g* for 10 min at room temperature. The supernatant containing the cytosolic proteins was taken off and the pellet was redissolved in 1 ml buffer 4 (0.5 % Triton X-100; 1 mM EDTA). This step was repeated several times. The final pellet was resuspended in 600 µl buffer 5 (10 mM Tris-HCl, pH 8.0; 8 M urea; 100 mM NaH_2PO_4; 20 mM β-mercaptoethanol) containing a strong denaturant and reducing agents necessary to reduce the cysteine residues and to cleave the disulfide bonds of the proteins enclosed in the inclusion bodies.

2.11.5 Dialysis and refolding of the recombinant proteins

To allow an optimal refolding of the denaturated protein it has been necessary dialyze the suspension containing the recombinant protein enclosed in the inclusion bodies. The suspension was transferred in a Slide-A-Lyzer® 7 K dialysis cassette (Pierce) and dialyzed

for 24 hours and 4 °C in 1 L dialysis buffer (10 mM Tris-HCl, pH 8.0; 100 mM ; 20 mM β-mercaptoethanol). The next day the protein suspension was pipetted off, was concentrated with a polypropylene column and the refolded proteins were used for further analysis.

2.12 Working with *S. cerevisiae*

2.12.1 Yeast media

A. YPD (Yeast Extract Peptone Dextrose-medium):
1 % (w/v) yeast extract
2 % (w/v) peptone
2 % (w/v) D-glucose

B. SC medium:
0.67 % (w/v) Yeast Nitrogen Base w/o amino acids (Sigma)
2 % (w/v) glucose *or* for induction
1 % (w/v) raffinose
2 % (w/v) galactose

Yeast Synthetic Dropout mix w/o uracil (Sigma):
0.01 % (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophane)
0.005 % (asparagine, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine)

2.12.2 Preparation of competent *S. cerevisiae* cells

Five milliliters of *S. cerevisiae* preculture were incubated in YPD medium overnight. The preculture was used to inoculate 40 ml YPD culture and incubated at 30 °C under stirring at 200 rpm until it reached an OD_{600} of 0.4. The culture was harvested by spinning at 1800 *g* for 5 min. The pellet was resuspended in 2 ml buffer 1 [0.6 M KCl; 10 mM bicin, pH 8.35; 3 % (w/v) ethylene-glycol], it was aliquoted in 200 ml and was stored at -80 °C.

2.12.3 Transformation of *S. cerevisiae* cells

Fifty micrograms of DNA carrier (DNA from fish sperm, Roche) were denaturated at 95 °C for 10 min. These, together with 1-4 µg plasmid-DNA were given to frozen yeast cells and were incubated under stirring at 300 rpm in a thermomixer compact (Eppendorf) at 37 °C for 60 s. 15 µl 100 mM $CaCl_2$ were added and were incubated for more 3 min. 75 µl

100 mM $CaCl_2$ and 1 ml buffer 2 [40 % (w/v) PEG 1000; 200 mM bicin, pH 8.35] were then added and the mixture was gently mixed by inverting the tube and incubated for one hour at 30 °C. The yeast cells were centrifuged for 1 min at 1800 *g*, the pellet was washed with 500 µl buffer 3 (150 mM NaCl; 10 mM bicin, pH 8.35) and was resuspended in 200 µl buffer 3. The resuspended cells were plated on SC plates and incubated for 2 days at 30 °C.

2.12.4 *S. cerevisiae* expression cultures

Fifty milliliters SC medium with glucose were inoculated with one colony and were incubated under stirring at 200 rpm for 2 days at 30 °C. Afterwards, 200 ml SC medium with 0.1 % raffinose and 0.2 % galactose were inoculated with the preculture to OD_{600}= 0.4. For this purpose the corresponding volume of preculture was centrifuged at 1500 *g* and the resulting pellet was resuspended in SC medium with 0.1 % raffinose 0.2 % glucose for induction. The cells were incubated under stirring at 200 rpm overnight at 30 °C and were used to inoculate 2 liter of SC medium with 0.1 % raffinose and 0.2 % galactose to OD_{600}= 0.1. The culture was incubated under stirring at 200 rpm overnight at 30 °C.

2.12.5 Isolation of *S. cerevisiae* mitochondria

Yeast cells from 2 liter cultures were centrifuged at 1800 *g* for 5 min at room temperature. The pellet was washed with 100 ml water and was centrifuged. The pellet was then resuspended in 100 ml washing buffer (20 mM Tris-HCl, pH 7.4; 50 mM NaCl; 0.6 M sorbitol) and was centrifuged as already described. The pellet was resupended in 60 ml washing buffer and 1 µl/ml Yeast/Fungal Protease Inhibitor Cocktail (Sigma) was added. The cell suspension was portionated in Falcon flasks and ½ volume glas beads (Ø 0.4 – 0.6 mm) was added. After 5 min incubation on ice the cells were disrupted by 6x vortexing (Reax Top, Heidolph) at 4 °C for 1 min. The disrupted cells were centrifuged at 2800 *g* and 4 °C for 10 min to separate cell debris and glas beads. The supernatant was transfered in Nalgene flasks and was centrifuged at 10000 *g* and 4 °C for 20 min. The supernatant (PMS = Post mitochondrial supernatant) was discarded and the pellet (mitochondria) was resuspended in 30 ml washing buffer supplemented with 1 µl/ml Protease Inhibitor Cocktail.

2.12.6 Isolation of *S. cerevisiae* mitochondrial membrane

Isolated mitochondria were disrupted by sonification (Sonoplus HD 60, Bandelin) with 5x 10 s impulse at 50 % intensity. Between each sonification the tube was kept 1 min on ice.

The disrupted mitochondria were centrifuged (T-865, Sorvall Ultra Pro 80) at 30000 rpm (ca. 105,000 *g*) and 4 °C for 1 h. The supernatant (soluble mitochondrial proteins) was discarded and the mitochondrial membranes were resuspended in 5 ml resuspending buffer [50 mM sodium phosphat, pH 7.2; 5 % (v/v) glycerin; 320 mM NaCl; 0.5 % (v/v) Triton X-100; 20 mM imidazol] supplemented with 1 µl/ml Protease Inhibitor Cocktail (Sigma).

2.12.7 Isolation of recombinant protein from mitochondrial membranes

Mitochondrial membrane proteins were solubilized by stirring at 4 °C for 1 h and were separated from the membrane residues by ultracentrifugation (T-865, Sorvall Ultra Pro 80) at 30000 rpm (ca. 105,000 *g*) and 4 °C for 1 h. The recombinant protein present in the supernatant was purified on Ni-NTA matrix (Qiagen). 3 ml of supernatant were added to 0.75 ml Ni-NTA matrix and were incubated at 4 °C for 1 h. The solution was loaded on a polypropylene column and washed 2 times with washing buffer [50 mM sodium phosphat, pH 7.2; 5 % (v/v) glycerin; 320 mM NaCl; 0.5 % (v/v) Triton X-100; 20 mM imidazol]. The elution was performed using elution buffer [50 mM sodium phosphat, pH 7.2; 5 % (v/v) glycerin; 320 mM NaCl; 0.5 % (v/v) Triton X-100; 250 mM imidazol] and the purified proteins were stored in 25 % glycerin at -20 °C.

2.13 Working with proteins

2.13.1 Determination of protein concentration

Concentration of recombinant protein was determinated by Bradford's method (1976). 0.5-25 µl of sample were mixed with 200 µl Bradford reagent (Bio Rad Protein Assay) and diluted to 1 ml with H_2O. After 10 min incubation time the protein concentration was measured at λ= 595 nm. The standard curve was prepared with bovine serum albumin.

2.13.2 SDS-PAGE

Separation of purified proteins under denaturing conditions was performed by discontinuous SDS polyacrylamide gel electrophoresis by the method of Laemmli (1970). The solutions, the gels and the electrophoretic separation were performed as described in Sambrock et al. (1989). The 12 % SDS-PAGE resolving gel was prepared with the PerfectBlue Twin S or Twin M system (Peqlab) as recommended by the manufacturer. The samples were dissolved in 0.2 volume loading buffer [50 mM Tris-HCl pH 6.8; 5 mM

EDTA; 5 % (w/v) SDS; 25 % (v/v) β-mercaptoethanol; 50 % (v/v) glycerin; 0.25 % (w/v) bromophenol blue] and were denaturated at 95 °C for 5 min.

2.13.3 Coomassie staining

SDS-PAGE coomassie staining was performed with a method from Heukeshofen and Dernick (1988) modified as follows. One tablet Phast Gel Blue R-350 (GE-Healthcare Biosciences) was dissolved in 80 ml H_2O under stirring. 120 ml methanol were added, the mixture was then filtrated and stored at 4 °C. To stain the gel, the stock solution was diluted with 10 % acetic acid (v/v) and heated at 90 °C. The gels were left 30-60 min in the solution and bleached with 10 % acetic acid.

2.13.4 Westernblot

For immunological analysis, proteins from the SDS-PAGE were transfered onto a nitrocellulose membrane as described in Gershoni und Palade (1983).

Transfer was performed in a Semi-Dry Blot System (Nova Blot Multiphor II; Amersham Biosciences). The nitrocellulose membrane (Hybond C Extra, Amersham Biosciences) was equilibrated for 5 min in H_2O. The anode of the Semi-Dry Blot System was moisturized with H_2O and coated with 3 Whatman papers impregnated with transfer buffer [39 mM glycin; 48 mM Tris; 0.037 % (w/v) SDS; 20 % (v/v) methanol]. The nitrocellulose membrane was placed onto the Whatman paper and on it the SDS-PAGE gel followed from 3 Whatman papers impregnated with transferbuffer. The transfer was performed at 0.8 mA/cm^2 for 1 h. After the transfer onto the membrane the proteins were reversibly stained with Ponceau S solution [0.2 % (w/v) Ponceau S; 3 % (v/v) acetic acid].

2.13.5 Immunodetection

For detection of recombinant protein with His-Tag a monoclonal mouse IgG His-Tag antibody (Novagen) was used. The Ponceau S stained membrane was incubated for 1 h in blocking solution [3 % (w/v) BSA in TBS (10 mM Tris-HCl, pH 7.5; 150 mM NaCl)] followed by 2 washing steps for 10 min in TBS-T [20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05 % (v/v) Tween 20; 0.2 % (v/v) Triton X-100] and one step for 10 min in TBS. Incubation with the first antibody (0.1 µg/ml in blocking solution) was performed at room temperature for 1 h or at 4 °C overnight. The membrane was washed 2 x 20 min with TBS-T, 1 x 10 min with TBS and incubated at room temperature for 1 h with the secondary antibody [ImmunoPure Goat Anti-Mouse IgG, (H + L) Peroxidase Conjugated, Pierce]. The secondary antibody was diluted 1:30000 in TBS and 1.5 % (w/v) skimmed

milk. After incubation with the secondary antibody the membrane was washed 3 x in TBST and the signals detected by horseradish reaction in the ECL Western Blotting Analysis Systems (Amersham Biosciences) carried out as recommended by the manufacturer. The signals were documented using Lumi-Chemoluminescent Detection Film.

2.13.6 *trans*-enoyl-CoA reductase enzymatic assay

Trans-enoyl-CoA reductase activity was measured spectrophotometrically with a method from Inui et al. (1984a) and Seubert et al. (1968) modified as follows. The reaction was performed in 200 µl (GENios microplate reader, Tecan Instruments) contained 100 mM phosphate buffer pH 6.2; 0.5 mM crotonoyl-CoA (Sigma); 0.4 mM NADH; 2 µM FAD and the recombinant protein. The reaction mixture was incubated at 30 °C for 10 min and the reaction was started by addition of substrate. The activity was followed by decreasing absorbance at 340 nm for 10 min. For the enzymatic characterization, different concentration of crotonoyl-CoA, NADH, FAD and protein were used.

2.13.7 Thiolase assay

The condensing activity of the recombinant thiolase was measured spectrophotometrically following the fluorescence at 460 nm after excitation at 355 nm. The reaction was performed in 40 mM NaH_2PO_4, 50 µM acetyl-CoA, 50 µM propionyl-CoA and between 10-200 ng/µl recombinant protein. The reaction mixture was incubated at 30 °C for 30 min and stopped with 25 µl 100 □M CPM solution [7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarine]. The condensation of CPM with CoA residues was carried out at 30 °C for 30 min.

The second method for the tiolase assay was performed by HPLC. The reaction mixture contained 50 mM Tris-HCl buffer pH 8.2, 10 mM acetyl-CoA and 15 mM propionyl-CoA. The reaction, started by addition of recombinant protein, was incubated at 30 °C for 20 min or over night. The reaction was then stopped with 30 % perchloric acid and centrifuged at 13000 rpm and 4 °C for 5 min. The supernatant was neutralized with 0.5 M Na_2CO_3 and analysed in a L7100 Hitachi Merck HPLC equipped with a L7420 UV Vis detector and a LiChrospher® 100 RP-18 (5 µm) column. The analysis was performed in 50 mM pH 5 NaH_2PO_4/acetonitrile 98:2 (v/v) to 65:35 (v/v) in 20 min and then to 30:70 (v/v) in 30 min. The elution of the reaction products were followed at λ=254 nm.

2.14 Wax ester analysis

2.14.1 Lipid extraction from *E. gracilis* cells

Extraction of the total lipid fraction from *E. gracilis* cells was performed with a method from Folch et al. (1957) modified as follows. At the end of the growth period, the cells were collected by centrifugation at 4500 *g* for 5 min. The collected cells were boiled in hot methanol and sonified 2x for 5 min. The cell residue was covered with $CHCl_3$/MeOH 2:1 (v/v; 25 ml/g cells) and removed by filtration in a separatory funnel. The organic phase was extracted with ¼ in volume of a 0.88 % KCl solution. After phase separation the organic phase was extracted with MeOH/H_2O 1:1 (v/v). The total lipid extract was then evaporated under reduced pressure (Heidolph) at 40 °C.

2.14.2 Purification of the wax ester fraction

The lipid extract was fractionated on a chromatografic column filled with Silica gel 60 (F_{254} Merck Kieselgel). The wax ester fraction was eluted with a PE/DE 99:1 (v/v) solution. Furthermore triglycerides, glycolipids and phospholipids were eluted with the following mixtures of solvents: PE/DE 9:1 (v/v); $CHCl_3$ 100 % (v); Me_2CO/MeOH 9:1 (v/v); MeOH 100 % (v); $CHCl_3$/MeOH/H_2O 65:25:4 (v/v). The lipid composition of the collected fractions was analysed on TLC plates (Kieselgel 60, 0.25 mm, Merck) and the resulting bands compared with commercially available standards. Wax esters were identified by developing TLCs plates with $Ce(SO_4)_2$, glycolipids by developing TLCs plates with α-naphothol/sulfuric acid reagent and phospholipids with the molybdenum blue reaction according to Dittmer and Lester (1964).

2.14.3 Preparation of fatty acid methyl esters (FAMEs) and fatty alcohols (FAlcs)

For a complete analysis of *E. gracilis* wax esters it has been necessary to transesterify the isolated fractions and to analyze the reaction products as fatty acid methyl esters (FAMEs) and fatty alcohols (Falcs). The dried wax esters were dissolved in 2 ml n-hexane, 2 ml of 3 % concentrated H_2SO_4 in dry methanol was added and the mixture heated overnight at 80 °C. After cooling, the reaction mixture was diluted with 6 ml of double-distilled water and the FAMEs and Falcs mixture was extracted three times with n-hexane by centrifugation at 2800 *g* for 2 min.

2.14.4 GC analysis of FAMEs and FAlcs

FAMEs and FAlcs reaction products were separated by thin-layer chromatography on Silica gel plates (20 x 20 cm, 0.25 mm, Kieselgel 60 F254, Merck) with PE/DE 8:2 (v/v) according to Kattner and Fricke (1986). Bands corresponding to FAMEs and FAlcs were identified with commercially available standards, scraped off after visualizing with 0.2 % 2',7'-dichlorofluorescein in ethanol and submitted to GC analysis. The analysis was performed with a Agilent 6850 series GC coated with a HP-5 % Phenyl-Methyl Siloxane Capillar column (30.0m x 250 µm x 0.25 mm Supelco). Temperature programming from 120 °C for 8 min then 4 °C/min to 220 °C and a final hold for 8 min at 220 °C was employed. Nitrogen was used as carrier gas.

2.15 Bioinformatics methods

2.15.1 Sequence analysis

To analyze and edit the sequences the GCG software (version 10.3) of the Genetic Computer Groups, University of Wisconsin was used (Devereux et al. 1984).

2.15.2 Data bank comparison

To search homologous sequences in GenBank the programs BLASTn, BLASTx and BLASTp of the BLAST (basic local alignment tool search) package in NCBI was used (Altschul et al. 1990).

2.15.3 Calculation of molecular weight and isoelectric point

The molecular weight and the isoelectric point were calculated with the online tool „Compute pI/Mw" (http://www.expasy.ch/cgi-bin/pi-tool).

2.15.4 Prediction of hypothetical transmembrane peptides

Putative transmembrane peptides of amino acids sequences were identified with the TMAP software from EMBOSS (http://bioinfo.hku.hk/EMBOSS/).

2.15.5 Prediction of hypothetical transitpeptides

Putative transit peptides of amino acid sequences were identified with the software MitoProt II (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter; Claros and Vincens 1996) and the iPSORT software (http://psort.nibb.ac.jp/; Bannai et al. 2001, Bannai et al. 2002).

3. Results

3.1 Wax ester fermentation in *Euglena gracilis*

E. gracilis cells synthesize promptly wax esters when they are brought to anaerobiosis. In absence of oxygen the mitochondria of *Euglena* perform a malonyl-CoA independent synthesis of fatty acids leading to accumulation of wax esters (Inui et al. 1982). In order to characterize this unusual fatty acid biosynthesis, *E. gracilis* cells were cultivated under different conditions on glucose as carbon source. The lipid fraction was extracted, the wax esters were purified on a silica gel column and were transesterified. The reaction products were separated by thin layer chromatography (TLC) and were analyzed by gas chromatography as fatty acid methyl esters (FAMEs) and fatty alcohols (FAlcs). FAMEs and FAlcs were identified by comparison with commercial standards.

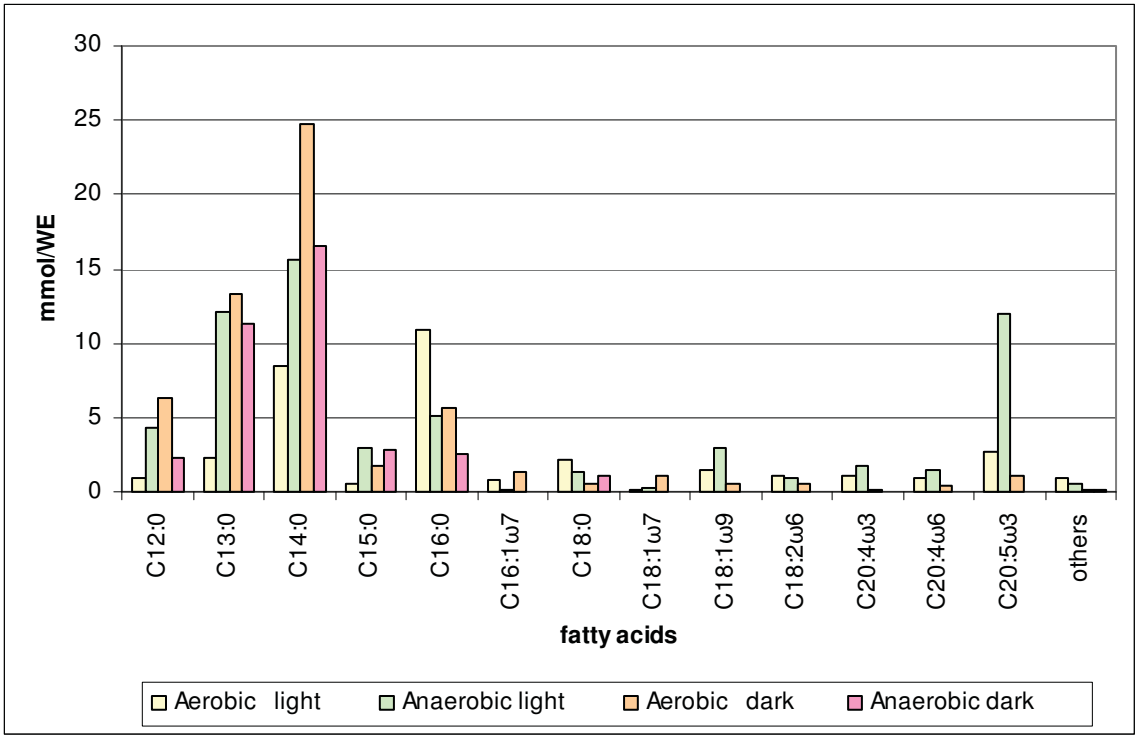
3.1.1 The effect of oxygen on the wax ester content and composition of *E. gracilis* 1224-5/25 strain Z

To investigate how oxygen affects the mitochondrial fatty acid synthesis and the wax ester fermentation, cultures of *E. gracilis* 1224-5/25 strain Z were grown in the presence and absence of oxygen, in the light and in the dark. As shown in **Tab. 3.1**, wax esters were synthesized under aerobic conditions and represented about 6 % of the total lipid fraction, corresponding to 2.3 mg/g fresh weight.

Tab. 3.1: Wax ester content in *E. gracilis* 1224-5/25 strain Z cultures. The wax ester content is expressed in mg/g fresh weight (FW) and in percent of the total lipid fraction (WE %). The cultures were grown under different conditions.

	Aerobic		Anaerobic	
	light	dark	light	dark
WE mg/FW	2.3	2.8	1.3	25.4
WE/lip	6.0	8.0	3.5	48.5

The FAMEs profile of **Fig. 3.1** showed a pattern of compounds, including longer and unsaturated fatty acids, and consisted mainly of myristic (C14:0) and palmitic acid (C16:0)(Fig. 3.1). On the other hand, in the FAlcs fraction the main products were



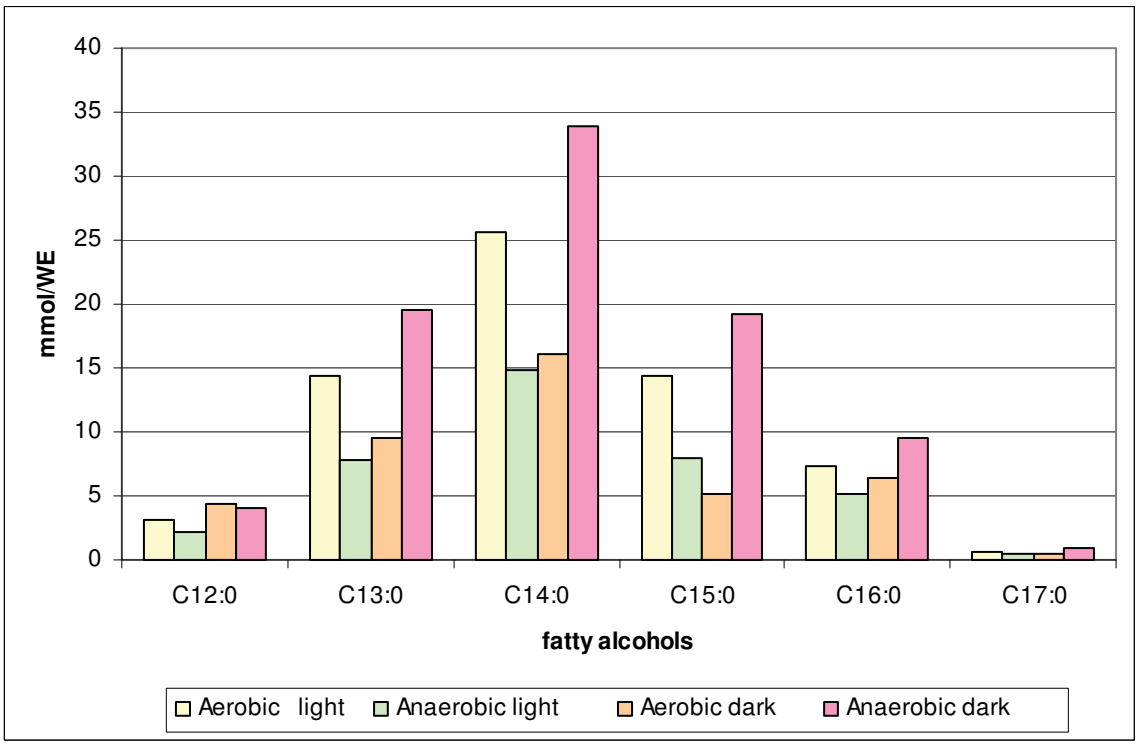


Fig. 3.1: FAMEs and FAlcs composition of *E. gracilis* 1224-5/25 strain Z. The cultures were grown aerobically and anaerobically, in the light and in the dark. The bars represent the concentration of FAMEs and FAlcs expressed in mmol/WE. *Others*, represents longer and unsaturated fatty acid with a concentration ≤0.3 mmol/WE. C16:1ω7 represent, i.e., chain length, number of double bonds and position of the last methyl group.

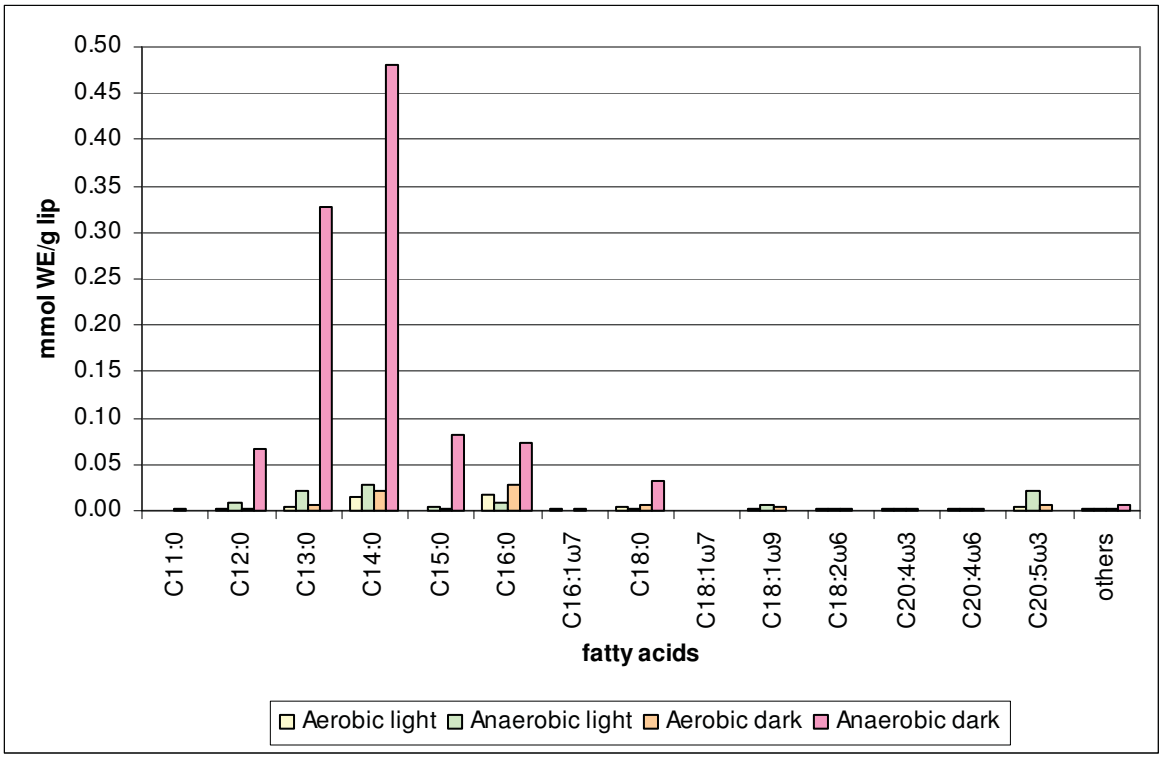
myristyl alcohol (C14:0) followed by C13:0 and C15:0. In contrast to the FAMEs fraction, FAlcs consisted only of saturated chains (**Fig. 3.1**). Growth of aerobic cultures in

the dark had no significant effect on the wax ester accumulation which was about 8 % of the total lipid fraction corresponding to 2.8 mg/g fresh weight, but on the FAMEs and FAlcs profiles. The unsaturated fatty acids were strongly reduced with the exception of C16:1 □7. In contrast, a prevalence of saturated acids were detected, in particular C12:0, C13:0 and C14:0. The FAlcs profile shifted to shorter chains, in proportion with a higher concentration of C12:0 and C13:0.

The absence of oxygen had not a great influence on the wax ester accumulation in cultures grown in a lower oxygen environment (N_2/CO_2) in the light, since the amount of waxes was only 3.5 % of the total lipid fraction. However, the FAMEs and FAlcs composition was clearly shifted towards shorter and saturated odd-chain like C13:0 and C15:0, as shown in **Fig. 3.1**. Of particular note was the four fold higher accumulation of eicosapentaenoic acid (20:5 ω3; EPA) in comparison to cells grown aerobically in the light.

On the other hand, growth at strictly anaerobic conditions led to a ca. ten fold higher accumulation of wax ester, up to 48.5 % of total lipids, corresponding to 25.4 mg/g fresh weight. Anaerobiosis showed a very strong effect on the saturation degree of the fatty acids, as the insaturated fatty acids were absent. In the FAMEs and FAlcs fractions the main products were odd-number chain C13:0 and C15:0 acids and alcohols, while the proportion of C16 and C18 fatty acids and alcohols in dark-synthesized wax esters decreased substantially (**Fig. 3.1**).

The bar graphs of **Fig. 3.2** shows, the fatty acid and fatty alcohol concentrations of the wax esters were normalized per gram of the total lipid fraction (mmol/WE/g lip) isolated from the cells. Aerobic cultures grown in the light and in the dark accumulated as main products C14:0 and C16:0 and the fatty acids showed a wide distribution among long unsaturated chains. In contrast, under anaerobic condition was detected a impressive accumulation of odd-number fatty acids and alcohols, in particular C13:0 and C15:0. Futhermore, the synthesis of unsaturated fatty acids was inhibited (**Fig. 3.2**).



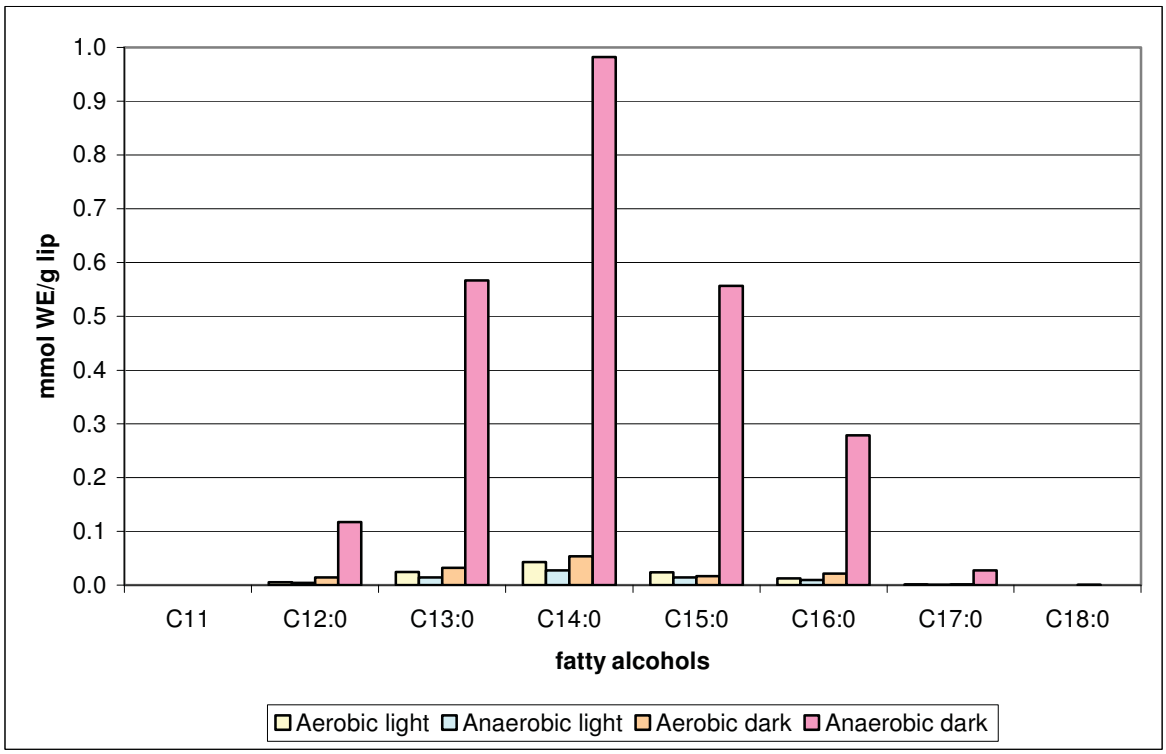


Fig. 3.2: FAMEs and FAlcs composition of *E. gracilis* 1224-5/25 strain Z. The cultures were grown aerobically, anaerobically, in the light, in the dark. The bars represent the concentration of FAMEs and FAlcs normalized per gram lipids (mmol/WE/g lip). Others, represents longer and unsaturated fatty acid with a concentration ≤0.001 mmolWE/g lip.

3.1.2 The effect of flufenacet on the wax ester content and composition of *E. gracilis* 1224-5/25 strain Z

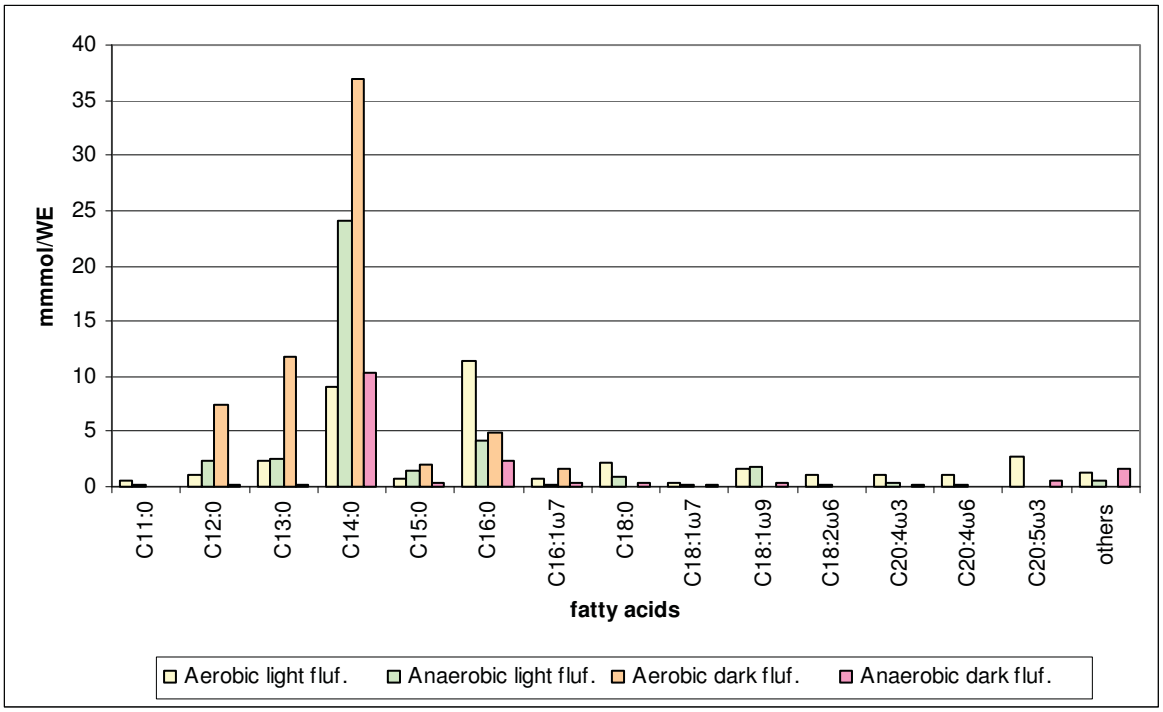
Flufenacet belongs to the class of K_3 herbicides and has demonstrated to inhibit higher plant elongases but not FAS complexes in either *Arabidopsis* (in the chloroplast) or yeast (Trenkamp et al. 2004). *E. gracilis* mitochondrial fatty acid synthesis occurs, as well as the elongation system, via CoA esters instead of ACP-proteins (Hoffmeister et al. 2005). To characterize this biochemical pathway, *E. gracilis* 1224-5/25 strain Z cells were cultivated in the presence of 100 µM flufenacet.

Tab. 3.2: Wax ester content in *E. gracilis* 1224-5/25 strain Z cultures. The wax ester content is expressed in mg/g fresh weight (FW) and in percent of the total lipid fraction (WE %). The cultures were grown at the indicated conditions.

	100 µM flufenacet			
	Aerobic		Anaerobic	
	light	dark	light	dark
WE/mg FW	2.5	4.4	23.2	42.9
WE/lip	9.6	21.6	33.3	59.6

In all treated cultures, flufenacet seemed to influence the synthesis of waxes in *Euglena* cells. Aerobic cultures supplemented with 100 µM flufenacet showed an accumulation of wax esters up to 9.6 % of the lipid fraction corresponding to 3.5 mg/g fresh weight, without any substantial change in the fatty acids profile, in comparison with untreated aerobic cultures. In contrast, as shown in **Fig. 3.3**, in the fatty alcohol fraction a higher accumulation of palmityl alcohol at the expense of C15:0 was observed.

In dark grown aerobic cultures the treatment with the herbicide caused a significant increase in the wax ester synthesis up to ca. 22 % of the lipid fraction, as reported in **Tab. 3.2**, but in contrast to flufenacet treated cultures grown in the light, the fatty acid profile showed important differences. Longer and unsaturated fatty acids, with the exception of C16:1 ω7, were below detection (**Fig. 3.3**). In the FAlcs fraction an inhibition of the synthesis of odd-number chains was observed, in particular of C13:0, resulting in a higher concentration of C14:0 and C12:0. Moreover, the synthesis of C16:0 and C18:0 was inhibited.



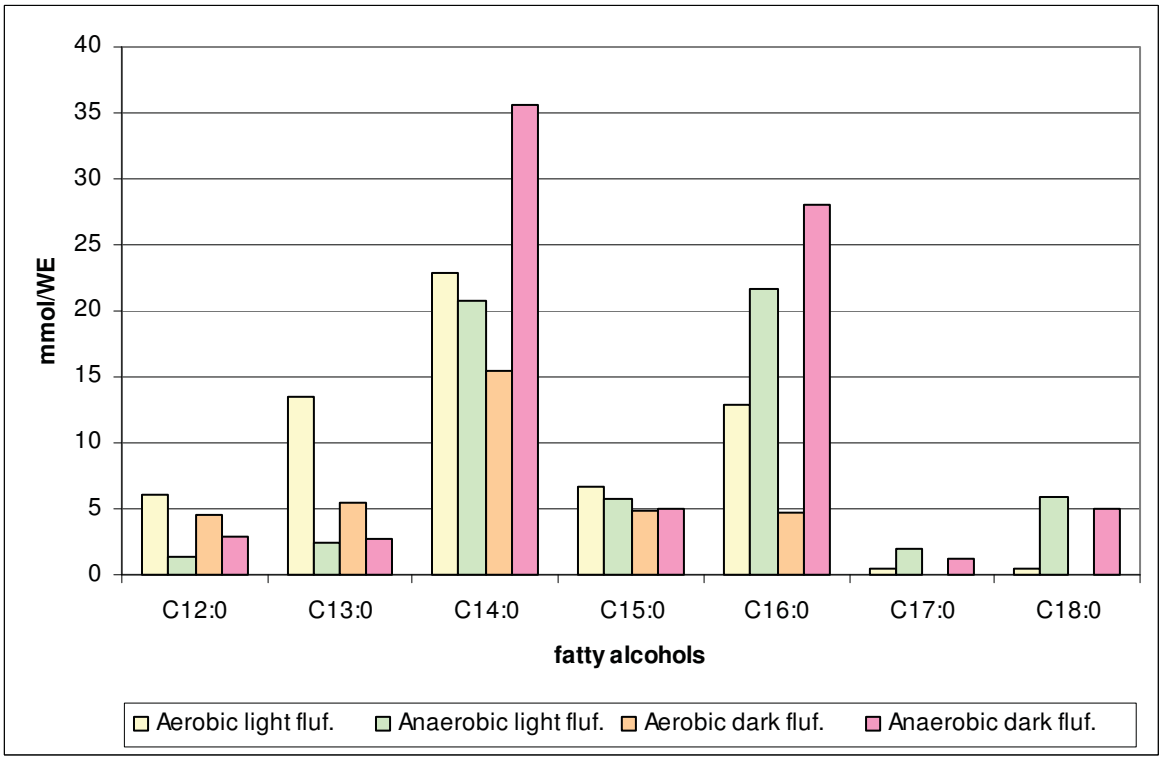


Fig. 3.3: FAMEs and FAlcs composition of *E. gracilis* 1224-5/25 strain Z. The cultures were grown aerobically and anaerobically, in the light and in the darkand in the presence of 100 µM flufenacet. The bars represent the concentration of FAMEs and FAlcs expressed in mmol/WE. *Others*, represents longer and unsaturated fatty acids with a concentration ≤0.3 mmol/WE. C16:1ω7 represent, i.e., chain length, number of double bonds and position of the last methyl group.

The combination of the two factors, anaerobiosis and the herbicide flufenacet, resulted in a surprisingly high amount of wax esters in *E. gracilis* cells. Cultures grown in a lower oxygen environment (N_2/CO_2) in the light possessed 3.5 fold more wax esters, 33.3 % of the lipid fraction, than treated aerobic light grown cultures. The odd-chain fatty acid concentration in the wax ester fraction was strongly reduced, compared to those grown without flufenacet, as shown in **Fig. 3.1**, although the total wax ester production was not inhibited (**Tab. 3.2**). In the FAlcs fraction the shift towards even-number chains with C14:0 and C16:0 as major compounds, was particularly evident.

In strictly anaerobic grown cultures an increase up to ca. 60 % wax esters was detected after treatment with flufenacet. All fatty acids and alcohols >C18 were below detection, but, despite anaerobiosis, the proportion of odd-chain constituents (C13:0 and C15:0) in the wax esters was dramatically reduced and resulted very low. Unexpected, in contrast to untreated anaerobic cultures as shown in the bar graph of **Fig. 3.1**, unsaturated and longer chains were incorporate into the wax esters.

Fig. 3.4 represents the fatty acid and fatty alcohol concentrations of the wax esters normalized per gram of the total lipid fraction (mmol/WE/g Lip) isolated from aerobically and anaerobically grown cells grown in the presence of flufenacet.

The FAME and FAlc profiles isolated from all cultures, with the exception of anaerobically grown cells supplemented with flufenacet, showed a very similar pattern of compounds. Both, even- and odd- number chain acids and alcohols were present in the same proportion. In contrast, in strictly anaerobic culture supplemented with flufenacet the synthesis of odd-number chain constituents (C13:0 and C15:0) was dramatically reduced, while the accumulation of compounds with a chain length > C18 was not inhibited.

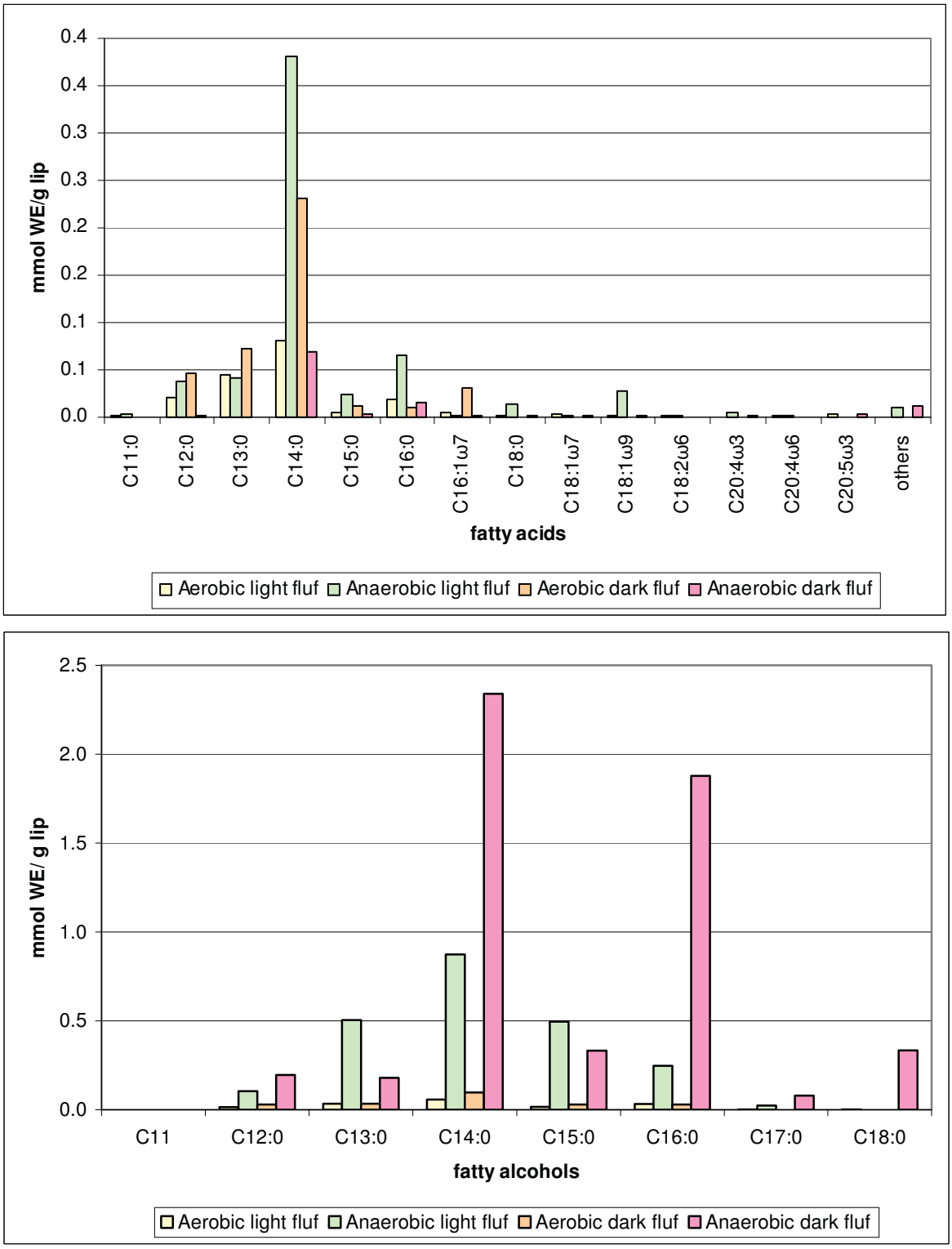


Fig. 3.4: FAMEs and FAlcs composition of *E. gracilis* 1224-5/25 strain Z. The cultures were grown aerobically and anaerobically, in the light, in the dark. All cultures were supplemented with 100 µM flufenacet. The bars represent the concentration of FAMEs and FAlcs normalized per gram lipids (mmol/WE/g lip). *Others*, represents longer and unsaturated fatty acid with a concentration ≤0.001 mmolWE/g lip.

3.2 Preparation of *E. gracilis* bleached mutants

E. gracilis possesses very active FAS type II localized in the chloroplasts (Buetow 1989). To characterize the mitochondrial fatty synthesis and to avoid the presence in the wax esters of fatty acids stemming from the plastidial FAS system, *E. gracilis* bleached mutants were generated. Two *E. gracilis* 1224-5/25 strain Z cultures were incubated overnight in the presence of 200 µg/ml ofloxacin and 500 µg/ml streptomycin, respectively. The cells were plated and after 10 days incubation at 28 °C about 60 % ofloxacin and 40 % streptomycin bleached colonies were detectable on the respective plates. Six white colonies were picked for each antibiotic treated culture and were incubated in fluid medium. Three of the ofloxacin and one of the streptomycin mutants revealed to be not a stable substrain and turned to green after few days of cultivation. After successive six days of incubation one of the ofloxacin mutants and two of the streptomycin bleached cells showed bacterial contamination and were discarded. The five stable substrains were called as following: Ofl1, Ofl2, Strep1, Strep2 and Strep3. To establish the growth rate of the bleached cells, growth curves were performed. As shown in **Fig. 3.5 (C)**, marginal differences occurred between the bleached strains. The exponential phase was reached between the fourth and the fifth day of growth and a cell division rate of eight hours was observed. For the wax ester analysis and anaerobic fermentation the strains Ofl1 and Strep1 were chosen.

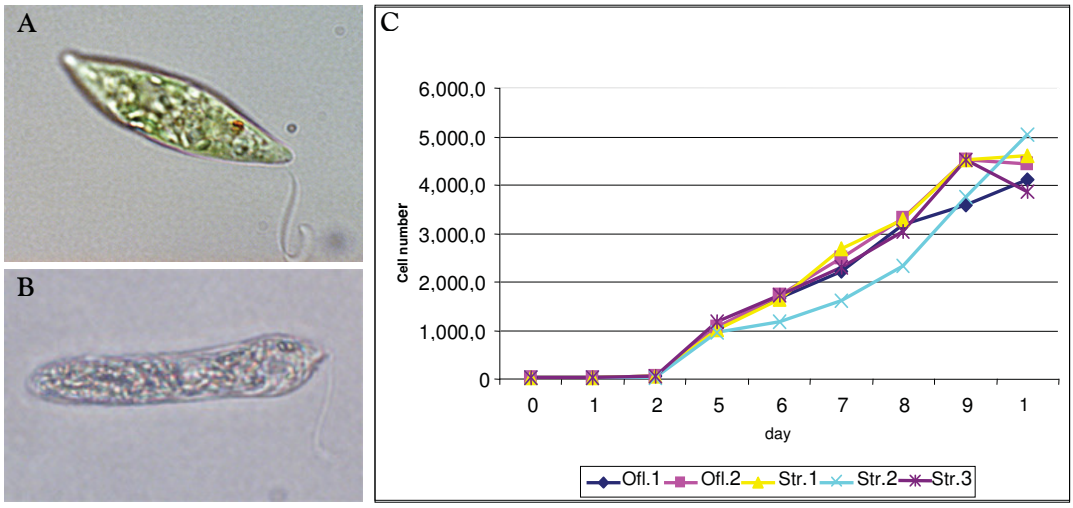


Fig. 3.5: *E. gracilis* 1224-5/25 before (A) and after (B) bleaching treatment with ofloxacin. (C) Growth curves of *E. gracilis* bleached mutants. The cultures were grown under aerobic conditions on a modified Hutner medium over 12 days (**see section 2.8**).

3.3 *E. gracilis* growth curves

All studies on the wax ester fermentation were carried out on *E. gracilis* strain Z and on the streptomycin bleached mutant *E. gracilis* SM-ZK (Inui et al. 1982; Inui et al. 1984; Schneider and Betz 1985). To test the ability to survive lower oxygen tension growth curves for the *E. gracilis* strains 1224-5/3, 1224-5/4, 1224-5/10, 1224-5/13, 1224-5/15, 1224-5/19, 1224-5/23, 1224-5/27 and for the *Euglena* bleached mutants 1224-5/25 Ofl1 and 1224-5/25 Strep1 were performed. The cultures were grown by strictly anaerobic conditions under N_2 flow in the dark at 28 °C for 8 days.

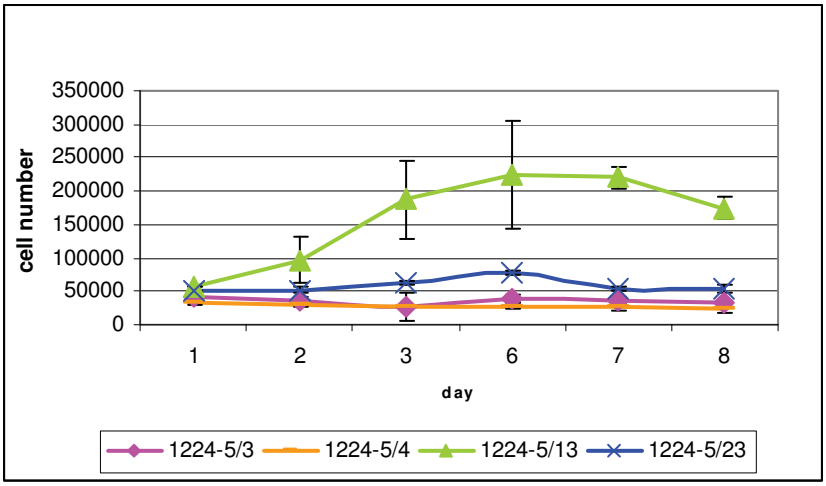


Fig. 3.6: Growth curves of *E. gracilis* 1224-5/3, 1224-5/4, 1224-5/13 and 1224-5/23 strains over 8 days. The cells were grown under constant N_2 flow, in the dark and at 28 °C (n=3).

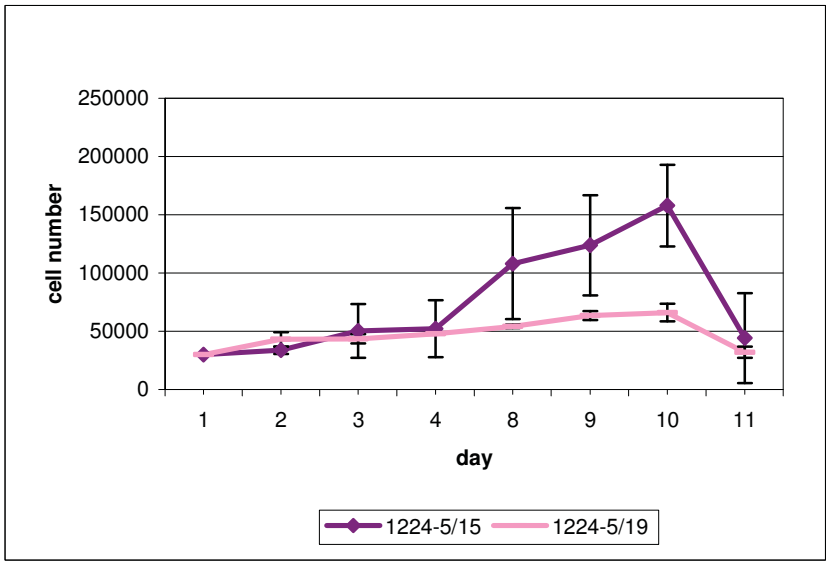


Fig. 3.7: Growth curves of *E. gracilis* strains 1224-5/15 and 1224-5/19 over 12 days. The cells were grown under constant N_2 flow, in the dark and at 28 °C (n=3).

As shown in **Fig. 3.6** and **Fig. 3.7**, the strains 1224-5/3, 1224-5/4 and 1224-5/19 survived by the time of cultivation but did not proliferate. In contrast, the strains 1224-5/13, 1224-5/15 and 1224-5/23 showed an increase in the cell number. *E. gracilis* 1224-5/13 was the fastest and best growing strain, reaching in six days the maximum of growth with about 200,000 cells/ml. The strain 1224-5/15 reached about 150,000 cells/ml in ten days, whereas this result was not reproducible since the cells died when they were cultivated anaerobically in larger volumes. All other *E. gracilis* 1224-5/10, 1224-5/18, 1224-5/27 1224-5/25 Ofl1 and 1224-5/25 Strep1 strains died on the second day after inoculation and the cultivation was stopped.

3.4 Wax ester fermentation of different *E. gracilis* strains

The characterization of the wax esters was performed only in the *E. gracilis* 1224-5/25 strain Z and no additional information are reported for other strains, to date. To verify whether the wax ester synthesis occurs not only in *E. gracilis* 1224-5/25 strain Z, other *E. gracilis* species were cultivated under different conditions on glucose as carbon source. To characterize the fatty acid and fatty alcohol profiles of the wax ester fraction and their differences occurring by the diverse conditions of growth, the lipid fraction of different *Euglena* strains was extracted. The wax esters were purified on a silica gel column and were transesterified. The reaction products were separated by thin layer chromatography (TLC) and were analyzed by gas chromatography as fatty acid methyl esters (FAMEs) and fatty alcohols (FAlcs). FAMEs and FAlcs were identified by comparison with commercial standards.

3.4.1 The effect of oxygen on the wax ester content of the *E. gracilis* bleached mutants and of different green *E. gracilis* species

All strains accumulated wax esters in different amounts and different proportion to fresh weight under aerobic conditions, as shown in **Tab. 3.3**. The highest concentration of waxes, was detected in the *E. gracilis* bleached mutants 1224-5/25 Ofl1 and Strep1 which accumulated 55.2 % and 59.6 %, respectively, wax esters of the total lipid fraction with 17.8 mg/g FW and 15.7 mg/g FW. In *E. gracilis* 1224-5/25 Ofl1 and Strep1 waxes represented respectively 8.9 % and 7.8 % of the dry weight (DW) of the cells. The strain *E. gracilis* 1224-5/15 showed wax esters up to 27.6 % of the total lipid fraction corresponding to 7.1 mg/g FW and 3.5 % DW. The fastest growing *E. gracilis* strain 1224-5/13 accumulated 12.4 % wax esters and 3.5 mg/g FW. In all other *E. gracilis* strains no significant differences were detected.

As already reported for *E. gracilis* 1224-5/25 strain Z (**s.s. 3.1.3**) oxygen had a strong influence on the cultures and on their wax ester content. Under strictly anaerobic conditions some of the cultures died (**s.s. 3.3**), but those strains which survived anaerobiosis accumulated about ten fold more wax esters than in the presence of oxygen. In contrast to the corresponding aerobic cells, anaerobically grown *E. gracilis* 1224-5/13 was the most enriched strain with 62.1 % wax esters of the lipid fraction. Waxes with 78.4 mg/g FW represented in this culture more than 31 % of the dry weight. By anaerobiosis *E. gracilis* strains 1224-5/3, 1224-5/4, 1224-5/19 and 1224-5/23 accumulated wax ester up to 33.3 % (14.3 mg/g FW), 28.8 % (10.5 mg/g FW), 20.8 % (8.4 mg/g FW) and 34.4 % (20.8 mg/g FW), respectively, of the total lipid fraction.

Tab. 3.3: Wax ester content in different *E. gracilis* strains. The cultures were grown under aerobic and anaerobic conditions. The wax ester content is expressed in mg/g fresh weight (FW), in percent of the total lipid fraction (WE/lip) and in percent of dry weight (DW).

Strain	Aerobic			Anaerobic		
E. gracilis	mg/g FW	WE/DW	WE/lip	mg/g FW	WE/DW	WE/lip
1224-5/3	1.1	0.5	4.3	14.3	5.7	33.3
1224-5/4	0.6	0.3	3.2	10.5	4.2	28.8
1224-5/10	5.5	2.7	3.2	-	-	-
1224-5/13	3.5	1.6	12.4	78.4	31.4	62.1
1224-5/15	7.1	3.5	27.6	-	-	-
1224-5/19	1.0	1.3	2.9	8.4	3.4	20.8
1224-5/23	4.1	1.9	3.8	20.8	8.3	34.4
1224-5/27	2.6	1.3	4.0	-	-	-
1224-5/Ofl1	17.8	8.9	55.2	-	-	-
1224-5/Strep1	15.7	7.8	59.6	-	-	-
1224-5/-25	2.3	0.9	6.0	25.4	10.2	48.5

3.4.2 The effect of flufenacet on the wax ester content of different *Euglena* strains

Addition of 100 µM flufenacet to anaerobic cultures affected the synthesis of wax esters which were accumulated in the different strains in higher concentrations (**Tab. 3.4**). Only the strain *E. gracilis* 1224-5/4 died by addition of flufenacet. *E. gracilis* strains 1224-5/3 and 1224-5/19 yielded two fold higher amounts of waxes with 70.1 % and 44 % of the lipid fraction, corresponding to 26.1 % and 6.3 % of dry weight, respectively. In *E. gracilis* 1224-5/13 and *E. gracilis* 1224-5/19 the increase of wax esters in the lipid fraction was not so striking 77.4 % and 53.8 % of the lipid fraction, respectively. However, in *E. gracilis*

1224-5/13 the proportion of waxes in the dry weight grew up to 57 %, comparable to the data reported in the literature (Buetow 1989).

Tab. 3.4: Wax ester content in different *E. gracilis* strains. The cultures were grown under anaerobic conditions in the presence of 100 µM flufenacet. The wax ester content is expressed in mg/g fresh weight (FW), in percent of the total lipid fraction (WE/lip) and in percent of dry weight (DW).

Strain	Anaerobic fluf		
E. gracilis	mg/g FW	WE/DW	WE/lip
1224-5/3	59.4	26.1	70.1
1224-5/4	-	-	-
1224-5/10	-	-	-
1224-5/13	114.0	57.0	77.4
1224-5/15	-	-	-
1224-5/19	15.1	6.3	44.0
1224-5/23	31.3	14.0	53.8
1224-5/27	-	-	-
1224-5/Ofl1	-	-	-
1224-5/Strep1	-	-	-
1224-5/-25	42.9	19.7	59.6

3.4.3 FAMEs and FAlcs composition of strictly aerobic *E. gracilis* strains

The basic fatty acid and fatty alcohol profiles of *E. gracilis* strains 1224-5/10, 1224-5/15, 1224-5/27 grown aerobically showed a very similar distribution (**Fig. 3.8**). The FAMEs consisted mostly of the even number chains C14:0 and C16:0, whereas in *E. gracilis* 1224-5/15 a discrete amount of C13:0 was detected. Unsaturated fatty acids were present in the wax esters fraction at very low concentration. The FAlcs profile showed the same distribution of the FAMEs profile. Only the *E. gracilis* strain 1224-5/27, beside C14:0 and C16:0, accumulated a very high amount of C15:0.

In the *E. gracilis* bleached mutants 1224-5/25 Ofl1 and 1224-5/25 Strep1, which showed nearly identical profiles, the unsaturated fatty acids were present in a very low concentration and the synthesis of fatty acids and alcohols was clearly shifted towards shorter, saturated chain. FAMEs consisted mostly of C12:0, C13:0 and C14:0, while the FAlcs fraction accumulated also C15:0 as major compound.

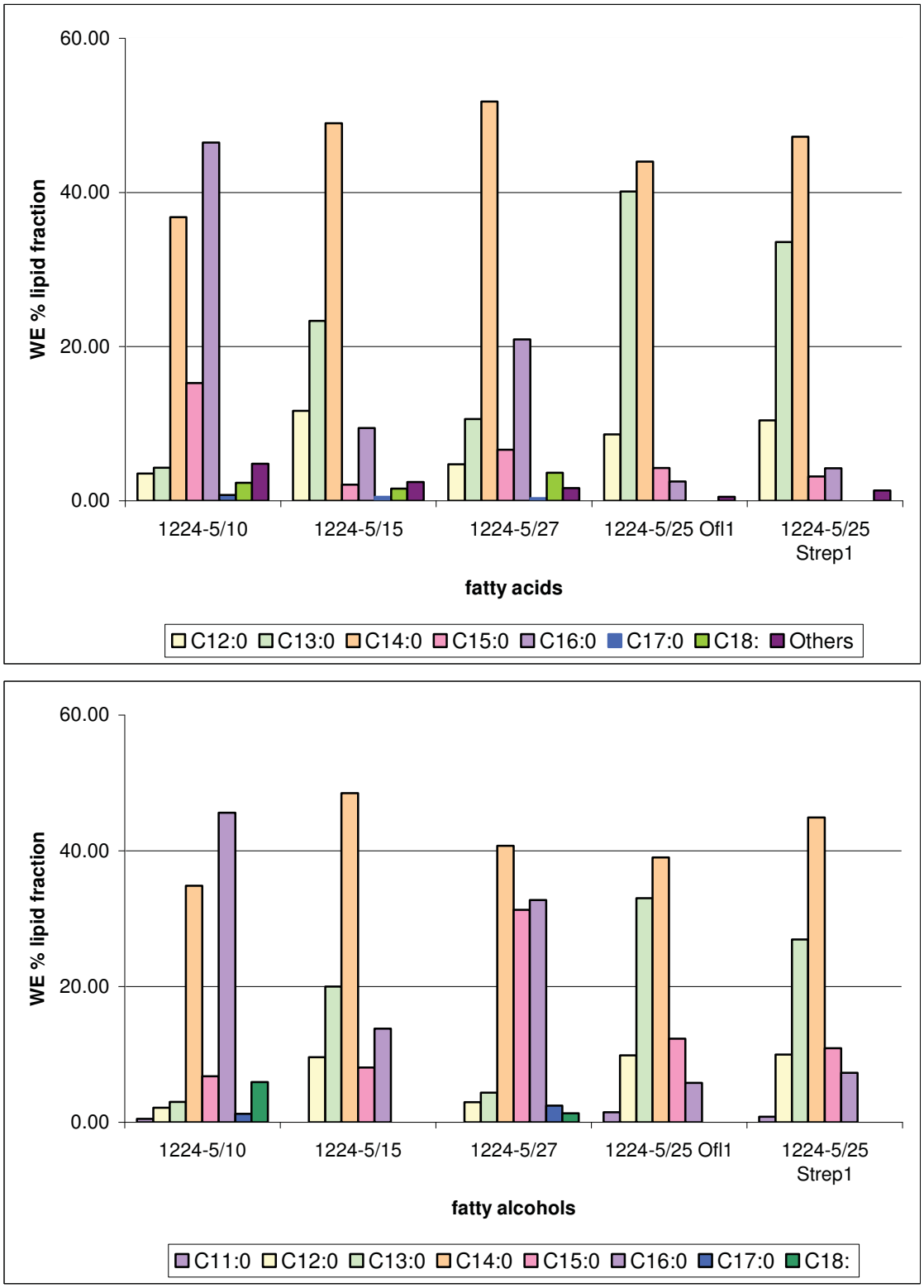


Fig. 3.8: FAMEs and FAlcs composition of the wax ester fraction in *E. gracilis* strains 1224-5/10, 1224-5/15, 1224-5/27, 1224-5/25 Ofl1 and 1224-5/25 Strep1. The bars represent the amount of the fatty acids and alcohols in the wax ester fraction of *E. gracilis* strains grown under aerobic conditions. In the FAMEs graph the bar for *others* includes all unsaturated fatty acids present in the wax ester fraction.

3.4.4 FAMEs and FAlcs composition of *E. gracilis* 1224-5/3

To characterize the wax ester composition, *E. gracilis* 1224-5/3 cultures were grown under aerobic conditions in the light, anaerobic conditions in the dark and anaerobic conditions in the presence of 100 µM flufenacet.

As shown in **Fig. 3.9**, FAMEs and Falcs isolated from the wax ester fraction of aerobically grown cultures yielded as major products even-number chains C14:0 and C16:0.

The absence of oxygen induced a reduction of C14:0 in the FAMEs profile. An increment of odd-number chains was not evident. Unexpected, the concentration of C16:0 increased. On the other hand, in the FAlcs fraction possessed two fold higher concentration of C13:0 and C15:0.

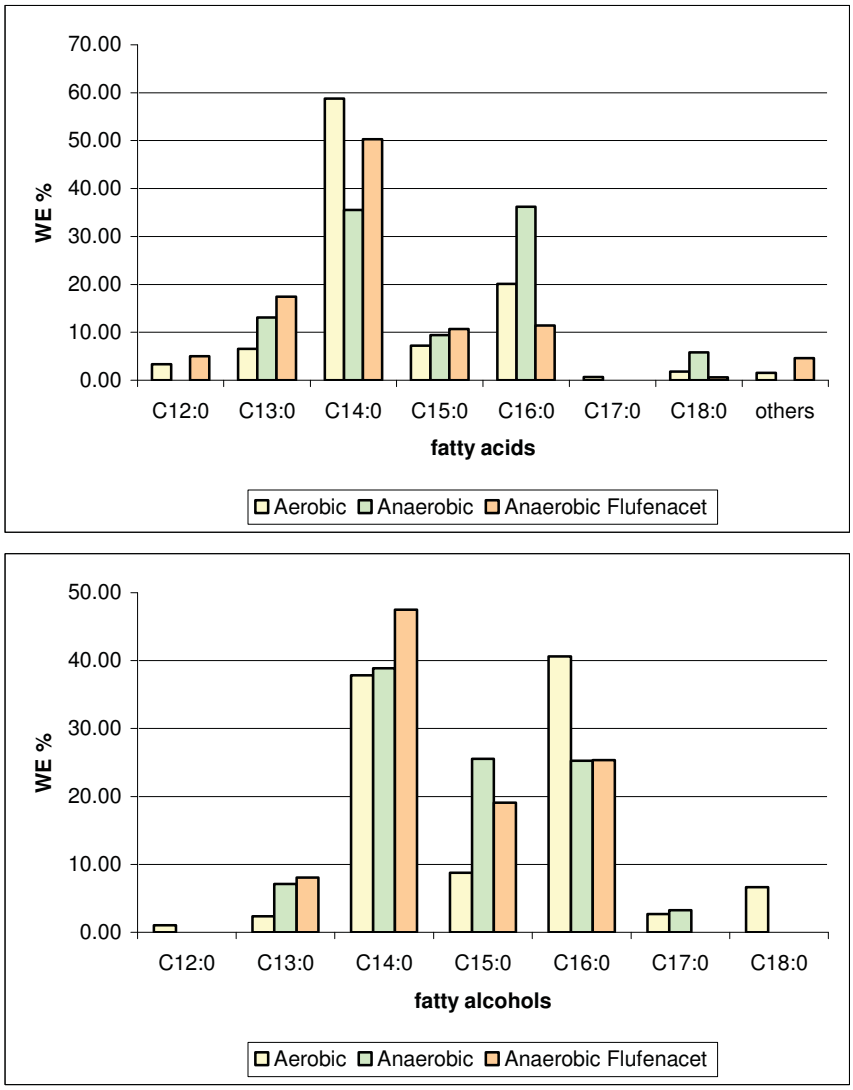


Fig. 3.9: FAMEs and FAlcs composition of *E. gracilis* 1224-5/3. The cultures were grown aerobically, anaerobically and in the presence of 100 µM flufenacet. The bars represent the proportion of FAMEs and FAlcs in the wax ester fraction. *Others*, represents all unsaturated fatty acids detected in the wax ester fraction.

Addition of flufenacet to the cultures caused the shift of fatty acid and alcohol profiles to shorter chains (C12:0, C13:0 and C14:0) and inhibited the incorporation of >C16 chains in the wax esters.

3.4.5 FAMEs and FAlcs composition of *E. gracilis* 1224-5/4

To characterize the wax ester composition, *E. gracilis* 1224-5/4 cultures were grown under aerobic conditions in the light, anaerobic conditions in the dark and anaerobic conditions in the presence of 100 µM flufenacet.

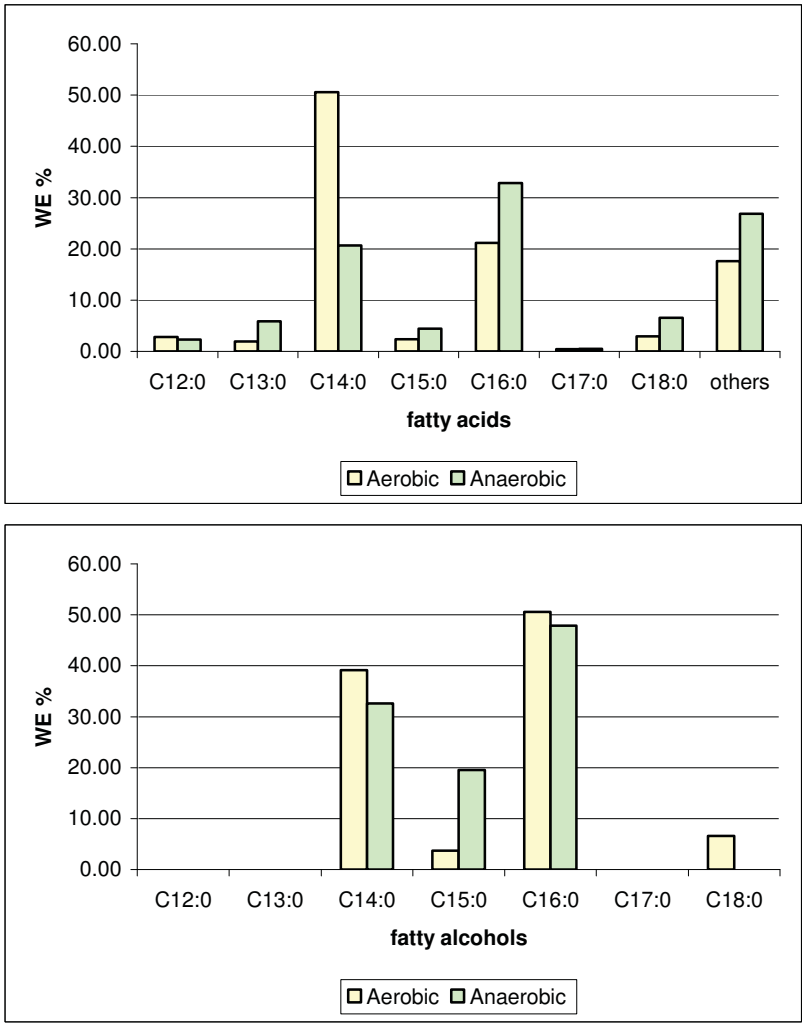


Fig. 3.10: FAMEs and FAlcs composition of *E. gracilis* 1224-5/4. The cultures were grown aerobically and anaerobically. The bars represent the proportion of FAMEs and FAlcs in the wax ester fraction. *Others*, represents all unsaturated fatty acids detected in the wax ester fraction.

Aerobically grown *E. gracilis* 1224-5/4 accumulated mostly even-number chains (C14:0 and C16:0) in the FAMEs and FAlcs isolated from the wax ester fraction. Shorter, odd-number chains were present in the FAMEs fraction in a very low concentration and in the FAlcs fraction were absent, with the exception of C15:0. Unsaturated fatty acids were incorporated in the wax ester in a discrete amount, ca. 20 % of the wax ester fraction as shown in **Fig. 3.10**.

During anaerobiosis the proportion of myristic acid (C14:0) was strongly reduced and the concentration of unsaturated fatty acids increased. In the FAlcs fraction the amount of C15:0 grew up to ca. 20 %, as shown in **Fig. 3.10**, about five fold more than under aerobic conditions. Unexpected, the cells died by addition of flufenacet to the culture.

3.4.6 FAMEs and FAlcs composition of *E. gracilis* 1224-5/13

To characterize the wax ester composition, *E. gracilis* 1224-5/13 cultures were grown under aerobic conditions in the light, anaerobic conditions in the dark and anaerobic conditions in the presence of 100 µM flufenacet.

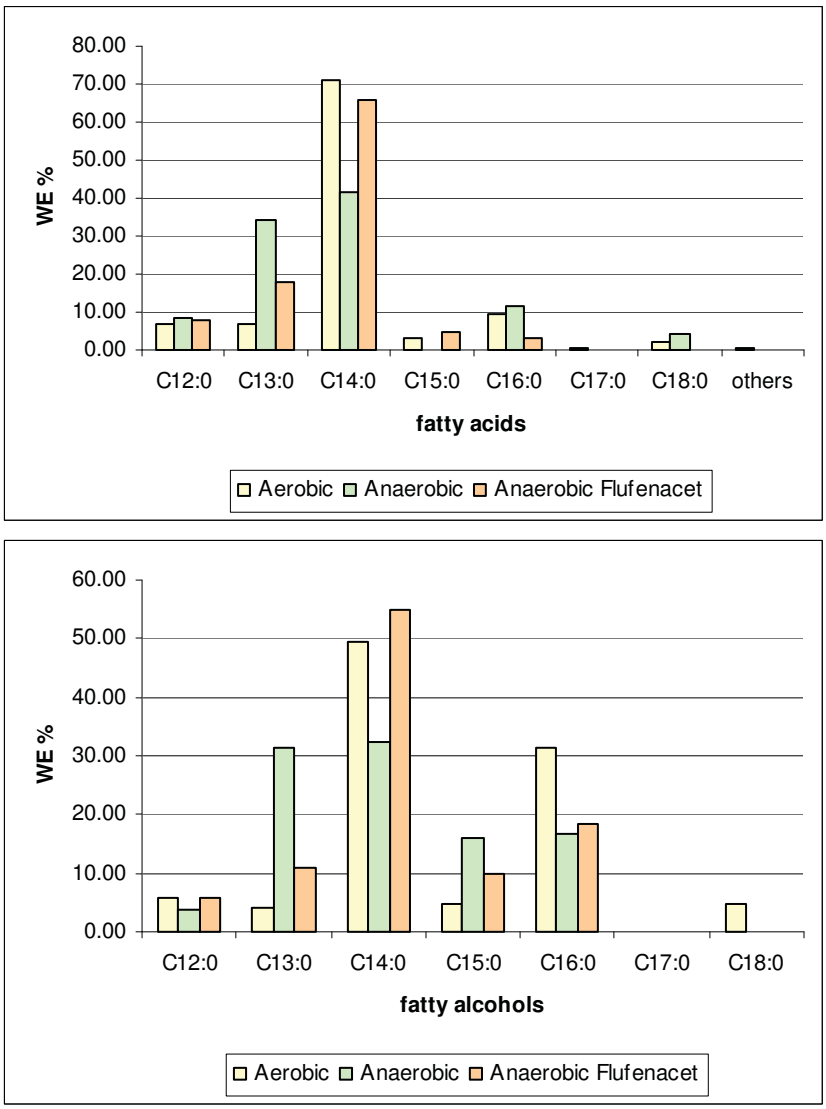


Fig. 3.11: FAMEs and FAlcs composition of *E. gracilis* 1224-5/13. The cultures were grown aerobically, anaerobically and in the presence of 100 µM flufenacet. The bars represent the proportion of FAMEs and FAlcs in the wax ester fraction. *Others*, represents all unsaturated fatty acids detected in the wax ester fraction.

In the presence of oxygen *E. gracilis* 1224-5/13 cultures accumulated mostly C14:0 and C16:0 in the FAMEs in the FAlcs fraction and the concentration of unsaturated fatty acids was extremely low.

In, contrast, when the cultures were brought to anaerobiosis the fatty acid and alcohol profiles were clearly shifted to odd-number chains. In particular, C13:0 was accumulated in the FAMEs and FAlcs fraction about six fold more than under aerobic conditions and the synthesis of C14:0 and C16:0 was inhibited.

By addition of flufenacet to the culture a strong reduction of odd-number constituents was observed. Fatty acids and alcohols accumulated again even-number chain compounds and >C18 chains, as well as unsaturated fatty acids, were below detection.

3.4.7 FAMEs and FAlcs composition of *E. gracilis* 1224-5/19

To characterize the wax ester composition, *E. gracilis* 1224-5/19 cultures were grown under aerobic conditions in the light, anaerobic conditions in the dark and anaerobic conditions in the presence of 100 □M flufenacet.

Growth by aerobic conditions induced an accumulation of mainly myristic (C14:0) and palmitic acid (C16:0) in the FAMEs profile (**Fig. 3.12**). Additionally, a pattern of compounds, including longer and unsaturated fatty acids, were detected. On the other hand in the FAlcs fraction the main products were myristyl (C14:0) and pamityl alcohol (C16:0) followed by a very high concentration of C15:0.

The absence of oxygen had a very strong effect on the wax esters composition. While in the FAMEs profile no significant change was detected, the only two constituents of the FAlcs fraction were myristyl and pamityl alcohols.

When flufenacet was added to the culture, no change was detected in the FAMEs profile. On the other hand, the FAlcs fraction was enriched in alcohols with different chain length and the incorporation of odd-number chain fatty alcohols was enhanced, in particular of C15:0.

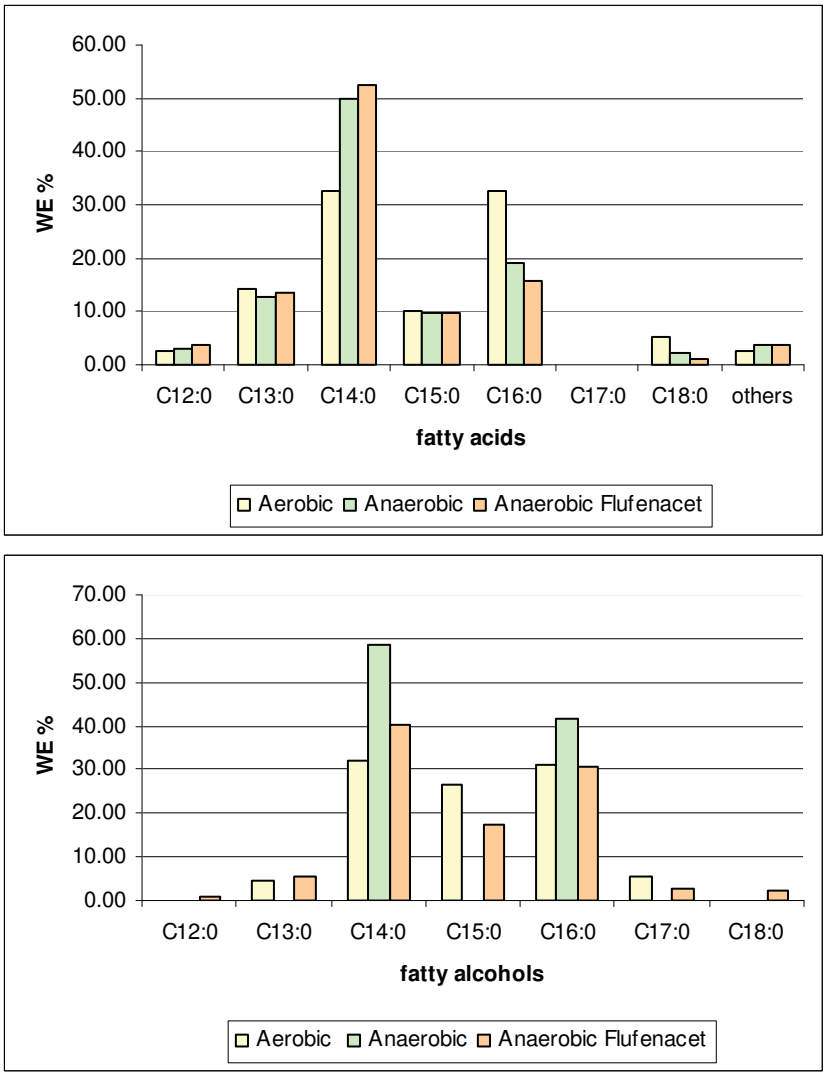


Fig. 3.12: FAMEs and FAlcs composition of *E. gracilis* 1224-5/19. The cultures were grown aerobically, anaerobically and in the presence of 100 µM flufenacet. The bars represent the proportion of FAMEs and FAlcs in the wax ester fraction. *Others*, represents all unsaturated fatty acids detected in the wax ester fraction.

3.4.8 FAMEs and FAlcs composition of *E. gracilis* 1224-5/23

To characterize the wax ester composition, *E. gracilis* 1224-5/23 cultures were grown under aerobic conditions in the light, anaerobic conditions in the dark and anaerobic conditions in the presence of 100 µM flufenacet.

In anaerobically grown *E. gracilis* 1224-5/23 cultures the unsaturated fatty acids were present in a very low concentration. The synthesis of fatty acids and alcohols was clearly shifted towards shorter saturated chains and consisted mainly of C12:0, C13:0 and C14:0.

Anaerobiosis did not show a very strong effect on the fatty acid profile. The proportion of fatty acids and alcohols did not change significantly in comparison to the FAMEs and FAlcs fraction isolated from aerobic cultures.

In anaerobic cultures supplemented with flufenacet the proportion of C13:0 and C15:0 fatty acids decreased substantially (**Fig. 3.13**) while the synthesis of C14:0 and C16:0 increased. In the fatty alcohol fraction, the effect of flufenacet was also remarkable, since the incorporation of all other alcohols, with the exception of C14:0 and C16:0, was inhibited.

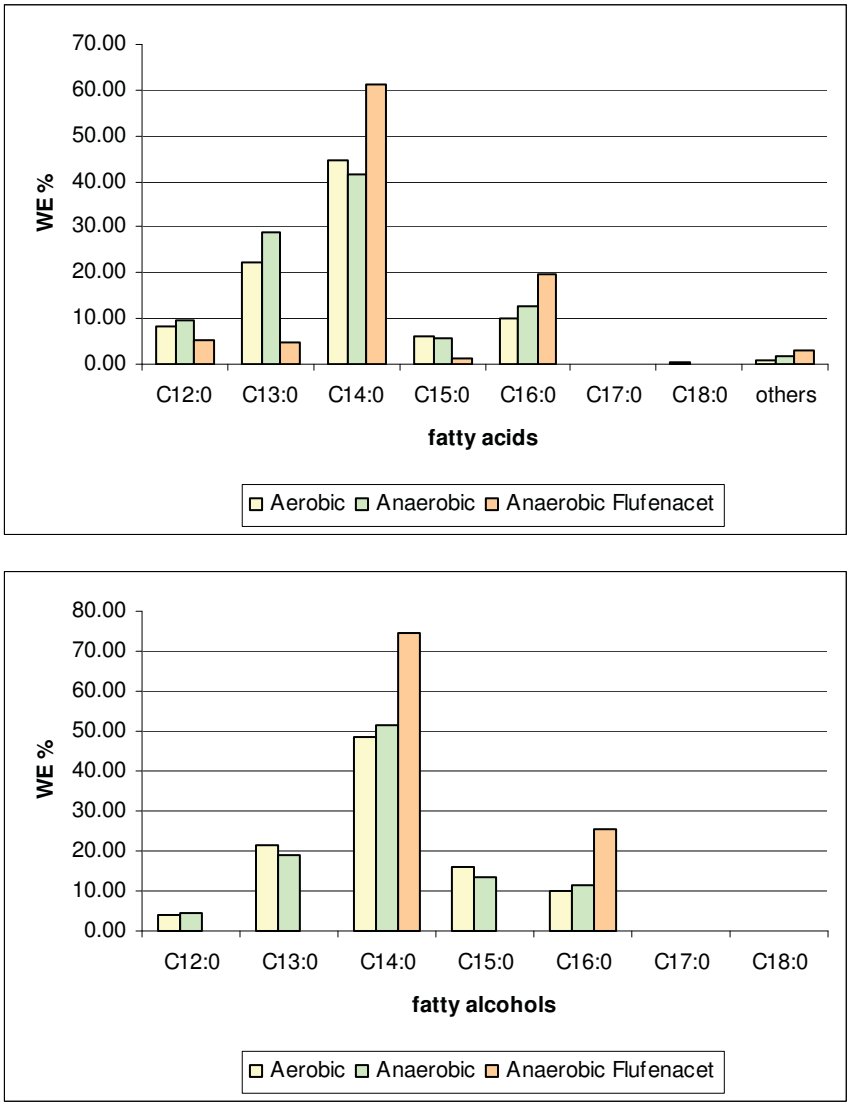


Fig. 3.13: FAMEs and FAlcs composition of *E. gracilis* 1224-5/23. The cultures were grown aerobically, anaerobically and in the presence of 100 µM flufenacet. The bars represent the proportion of FAMEs and FAlcs in the wax ester fraction. *Others*, represents all unsaturated fatty acids detected in the wax ester fraction.

3.5 Northern blot analysis of different Euglena strains

The Northern blot analysis was performed as described in **2.10.8** with RNA isolated from the different *E. gracilis* strains cultivated under aerobic conditions. The electrophoretic

separation was carried out on a 1 % agarose gel. The RNA samples were transferred onto a nylon membrane (N, Amersham Biosciences) and hybridized with the 1157 bp *trans*-2-enoyl-CoA reductase (TER) fragment or the 5841 bp pyruvat:NADP$^+$ oxidoreductase fragment from the *E. gracilis* 1225-5/25 strain Z. The fragments used for the radioactively labelled hybridization were isolated by double digestion from the plasmids pEgTER2 (Hoffmeister et al. 2005) and pEgPNO3 (Rotte et al. 2001).

For the hybridization with the TER probe 8 µg RNA of each *E. gracilis* strain were used. The film was exposed seven days at -80 °C. Bands corresponding to about 1600 bp were detected in the samples *E. gracilis* 1224-5/3, 1224-5/4, 1224-5/13, 1224-5/15, 1224-5/19, 1224-5/23 and 1224-5/27, although in apparently different intensity at expression level as shown in **Fig. 3.14**. In the slots corresponding to the samples 1224-5/10, 1224-5/25-Ofl1 and 1224-5/25-Strep1 signals for TER homologous sequences could not be detected.

The hybridization with the PNO probe was performed using 5 µg RNA isolated from each *E. gracilis* strain. After 24 hours exposition at -80 °C bands of about 6000 bp were detected in each slot. The expression level of the PNO in the lane corresponding to the *E. gracilis* 1224-5/13 and *E. gracilis* 1224-5/15 RNA samples appeared to be higher than in the RNA samples isolated from the other strains.

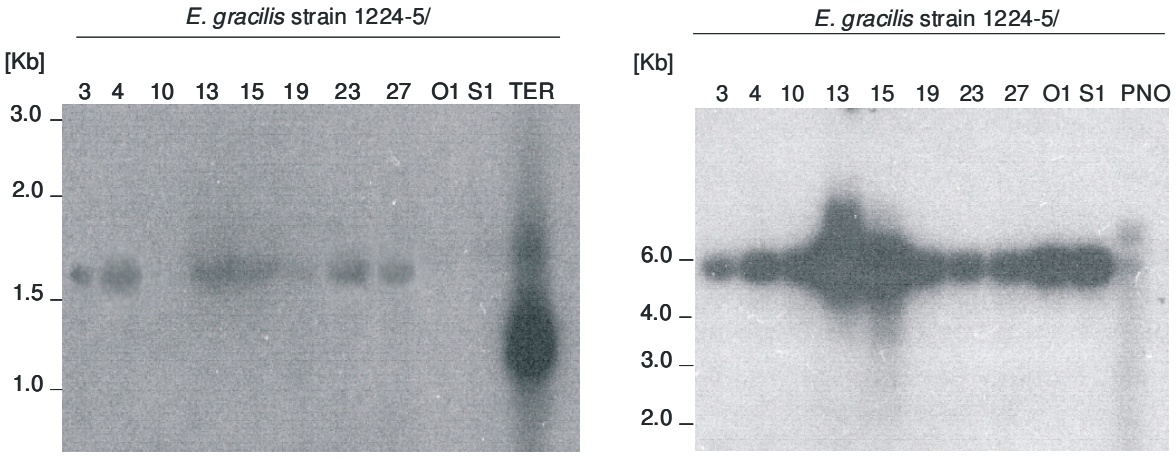


Fig. 3.14: Northern blot analysis with RNA isolated from different *E. gracilis* strains using the TER sequence and, the PNO sequence. *3*, *E. gracilis* 1224-5/3; *4*, *E. gracilis* 1224-5/4; *10*, *E. gracilis* 1224-5/10; *13; E. gracilis* 1224-5/13; *15*, *E. gracilis* 1224-5/15; *19*, *E. gracilis* 1224-5/19; *23*, *E. gracilis* 1224-5/23; *27*, *E. gracilis* 1224-5/27; *O1*, *E. gracilis* 1224-5/25-Ofl.1; *S1*, *E. gracilis* 1224-5/25-Strep1; TER, 1157 bp *trans*-2-enoyl-CoA reductase fragment; PNO, 5841 bp pyruvate:NADP$^+$ oxidoreductase fragment.

To quantify the RNA expression level for the TER and PNO sequences the Northern blot analysis was repeated with RNA samples isolated from the *E. gracilis* strains 1224-5/3, 1224-5/4, 1224-5/13, 1224-5/19 and 1224-5/23. As positive control the actin sequence was used, isolated by double digestion from the *Euglena* EST clone eg_003051049r prepared in-house (Ahmadinejad et al. 2007). The RNA expression was quantified with the Image Gauge software (3.0).

The hybridization for the TER sequence was carried out with the 1617 bp fragment *ter1* (Hoffmeister 2004). In all RNA samples bands ~ 2000 bp and ~1600 were detectable (**Fig. 3.15**). The RNA expression level was calculated for the bands at ~ 2000 bp since the characterized TER fragment involved in wax ester fermentation has a length of 1912 bp (Hoffmeister et al. 2005). The *E. gracilis* strains 1224-5/3 and 1224-5/13 showed 2.4 and 2 fold, respectively, higher expression at RNA level than the *Euglena* strains 1224-5/4 and 1224-5/23, while in *E. gracilis* 1224-5/19 they showed an increase of 1.4 fold.

The PNO was expressed mostly in *E. gracilis* strains 1224-5/13 and 1224-5/3, with 3.6 and 2.4 fold more, respectively, than in the other strains (**Fig. 3.15**). Only in the *E. gracilis* 1224-5/23 the RNA expression level was extremely low, about 0.2 fold than the reference.

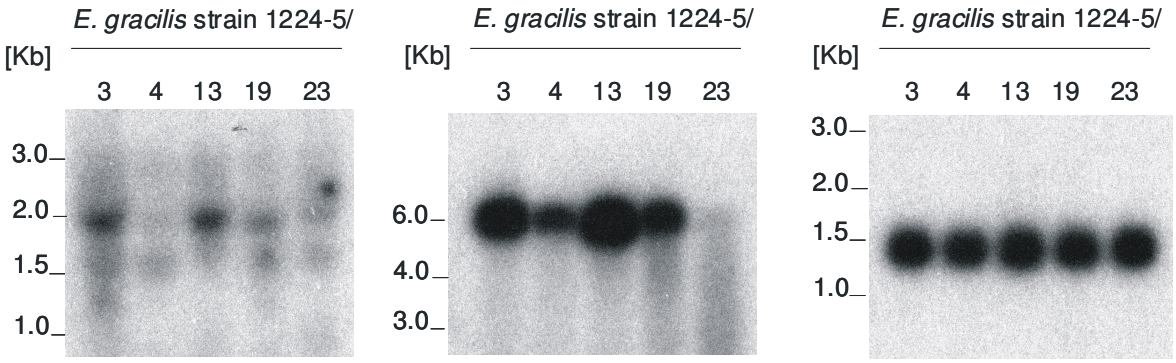


Fig. 3.15: Northern blot analysis with RNA isolated from different *E. gracilis* strains using in A, the TER sequence in B, the PNO sequence and in C the actin sequence as radioactively labelled hybridization probes. *3*, *E. gracilis* 1224-5/3; *4*, *E. gracilis* 1224-5/4; *13, E. gracilis 1224-5/13; 19, E. gracilis 1224-5/19; 23, E. gracilis 1224-5/23; Kb, kilobase.* 5 µg RNA of each *Euglena* strain were loaded onto the gel.

3.6 Identification of a putative mitochondrial 3-ketoacyl-CoA thiolase from *E. gracilis*

A tBLASTn search with the mitochondrial 3-oxoacyl-CoA thiolase gene (D16294; EC 2.3.1.16) from *Homo sapiens* in the Euglena EST database at NCBI, showed a hit with an e-value of $6e^{-70}$ and an amino acids identity of 39 % which was annotated as a putative 3-ketoacyl-CoA thiolase mitochondrial related cluster. The protein sequence ELL00002493 had a length of 420 amino acid, a molecular weight of 44.5 kDa and a light alkaline isoelectric point of 8.43. The software MitoProtII predicted a probability of 90,05 % of export to mitochondria and the presence of a transit peptide sequence of 30 amino acids. With the software TMAP from the EMBOSS-package two membrane spanning regions were detected at the amino acids 20-48 and 107-124.

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 TATTTTTTTTCGACCAGAATGAAGGGAATGCGGAAGGTTGCTATTGTAGCAGCATGCAGG  60
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ACTCCAATTGGTGCCTTCGGTGGGTCCTTGAAATCCGTGATTGGTGCTCAACTTGCAGCA  120
T  P  I  G  A  F  G  G  S  L  K  S  V  I  G  A  Q  L  A  A

ACTGTCATCCGAGAAATTCTGAACCGAACAAAAATTGATCCGTCAATTATCGGAGATGTT  180
T  V  I  R  E  I  L  N  R  T  K  I  D  P  S  I  I  G  D  V

CGATTCGGGTGCTGCATGGATCATTACGATGCGATTAATGTTGCCCGCGTCGCTGGTTTG  240
R  F  G  C  C  M  D  H  Y  D  A  I  N  V  A  R  V  A  G  L

CTGGCTGGTGTTCCGGATACGGTTCCTGGAGTGACAATGAACAGAGTCTGTGTCAGTGCC  300
L  A  G  V  P  D  T  V  P  G  V  T  M  N  R  V  C  V  S  A

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M  G  L  T  A  E  F  L  A  H  K  H  R  I  S  R  E  E  Q  D

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E  C  G  E  I  V  P  V  E  V  K  Q  K  K  K  V  T  I  I  D

AAAGATGAGCACTTCCGCCCTGGTCTCACCATGGCACAGCTTGAAAAACTCCCACCTGCC  780
K  D  E  H  F  R  P  G  L  T  M  A  Q  L  E  K  L  P  P  A
```

```
TTCATCCCTAATGTTGGAACAGTCACTGCTGGGAATTCTTCTGGAATCAACGATGGAGCC  840
F  I  P  N  V  G  T  V  T  A  G  N  S  S  G  I  N  D  G  A

GCTGCTTTATTGCTGGTGGAACTGGAGAAAGCGAAGGCTTTGGGTTTGAAGCCTCTTGCA  900
A  A  L  L  L  V  E  L  E  K  A  K  A  L  G  L  K  P  L  A

ATTATTTCTGGAATGGGCAAGGGTGGGTGTGCTCCTGAATTGATGGGTGAAAGTCCAGTG  960
I  I  S  G  M  G  K  G  G  C  A  P  E  L  M  G  E  S  P  V

CCAGCAGTCAACGACCTTTGCGAGAAGACAGGGCACAAGGTGTCTGATTATCAACGAGTG  1020
P  A  V  N  D  L  C  E  K  T  G  H  K  V  S  D  Y  Q  R  V

GAAATGAATGAGGCATTTGCATCCCAGTACATCGCTTGTGAACGTCTTCTGAAAATGAAC  1080
E  M  N  E  A  F  A  S  Q  Y  I  A  C  E  R  L  L  K  M  N

CGTGATGTCACCAATGTGAATGGCAGTGGTATTGGCCTTGGACATCCTGTGGGCTGCACT  1140
R  D  V  T  N  V  N  G  S  G  I  G  L  G  H  P  V  G  C  T

GGGGCACGTATCGTGGTTTCTCTCTTGCATGAAATGATCCGCTCTGGTTCACAGCTCGGC  1200
G  A  R  I  V  V  S  L  L  H  E  M  I  R  S  G  S  Q  L  G

ATGGCTTCCATGTGTGGTGGTGGAGGTGTGTCTCTTGCGACCGAGTTGACGCTGTGCTAG  1260
M  A  S  M  C  G  G  G  G  V  S  L  A  T  E  L  T  L  C  *
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Fig 3.16. Nucleotides and amino acid sequence of the putative mitochondrial 3-ketoacyl-CoA thiolase from *E. gracilis*. The light grey marked amino acids represent the mitochondrial transit peptide. The dark grey marked amino acids represent the two membrane spanning regions present in the sequence.

Multiple alignments of the putative 3-ketoacyl-CoA thiolase revealed homologous sequences sharing a high similarity among bacterial genomes. The 3-ketoacyl-CoA thiolases of *Desulfococcus oleovorans* and *Bacillus cereus*, for example, showed sequence identity of 58 % and 46 % with e-values of $3e^{-127}$ and $3e^{-83}$, respectively. After CLUSTALW alignment the amino acids responsible for the enzyme activity were identified. Cysteine 98 and 405 (the position are based on the *E. gracilis* thiolase sequence) responsible for the activation of acyl-CoA are present on the sequence, as well as asparagine Asn 343, histidine His 384 and glycine Gly 407 as reported in Haapalainen et al. (2006).

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Burkholderia: --------MQREVVVVSGVRTAIGDFGGSLKDFAPTELGARVVREVLSRAQVSGDEVGHVVFGNVVH :  59
Ralstonia so: --------MAREVVVVSGVRTAVGTFGGSLKDVAPCELGALVVREALARAQVGGEEVGHVVFGHVIN :  59
Clostridium : ---------MKEVVIVSAVRTAMGKFGGTLKDVPAVELGATVIKEAINRAGIKPEIIDEVIMGNVIQ :  58
Bacillus cer: ---------MHNVVITAAVRSPIGTFGGALKNVTPVELAVPVLQEAVKRGGVEPHEVDEVILGHCIQ :  58
Desulfococ  : ---------MKDVVIVSACRTAIGAFGGTLKNMHASRIASIAMKEAIRRAGIDAGIIDDIRFGCCLD :  58
Euglena grac: YFFSTRMKGMRKVAIVAACRTPIGAFGGSLKSVIGAQLAATVIREILNRTKIDPSIIGDVRFGCCMD :  67

Burkholderia: TEPKDMYLARVAAINGGIAQHAPALTVNRLCGSGLQAIVSATQNVLLGDADIAVAGGAENMSRAPYS : 126
Ralstonia so: TEPRDMYLSRVAALEGGVPAETPAFNVNRLCGSGLQAVVSAAQTILLGDVDVAIGGGAESMSRAPYL : 126
Clostridium : AG-LGQSPGRQAAVKAGIPVEVPAFTLNKVCGSGLRAVGLAAQMIKAGDADVVIAGGMENMSAAPYV : 124
Bacillus cer: RT-DEANTARTAALAAGFPDTVTGYTIQRQCSSGMQAIMSAAMQIQLGVSDVVVAGGVEAMSSSPYA : 124
Desulfococ  : PT-DSMNVTRTAALLAGIPDSVPAVTINRVCISGMEATLSGMAMIQAGMADVLLTGGVEHMSGVPYV : 124
Euglena grac: HY-DAINVARVAGLLAGVPDTVPGVTMNRVCVSAMEAIQSGYLNIATGFADVIVAGGVESMSNAPYM : 133
                                            *
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Burkholderia: VPAARFGQRMGDAKLVDMMIG-ALNDP---------------------FQSIHMGVTAENVAAKYGI : 171
Ralstonia eu: APAARWGSRMGDAGLVDMMLG-ALHDP---------------------FQAIHMGVTAENVAREYGI : 171
Clostridium : LPNARWGQRMFDGKMVDTMVKDGLWES---------------------FNDYHMGMTAENIAEKWEL : 170
Bacillus cer: LKQHRWGQRLQHGEIRDTVWE-VLEDP---------------------IHHIMMGETAENLVEQYEI : 169
Desulfococ  : VQDARWGCRLQDTTFVDALIRGLHCGSHIIPHPEDGPLKTGELIERLKGKPYIMGHTAEMVAELYNI : 191
Euglena grac: VPNARWGKRLQDDSLLDAMIHGLMVGSTVIPYPKDGPIKM------MRGQPYIMGLTAEFLAHKHRI : 194

Burkholderia: TRDAQDALALESHRRASHATKSGYFKD--QILPIEIASR--KGTVVFDADEHVRHDASLDDFTKLKP : 234
Ralstonia so: GRELQDQTAVESHRRASRAIQAGYFKG--QIVPVTIKSR--KGDVQFDTDEHVRHDATLEDMAKLRP : 234
Clostridium : TREMQDEFACASQNKAEKAIKEGKFKD--EIVPVVIKTR--KGEVVFDTDEFPRFGATVESLAKLKP : 233
Bacillus cer: TREEQDEVALRSHTLALKAIESGYFDD--QIVPITIKER--RKEVIFSKDEHPRADITAEKLAGLKP : 232
Desulfococ  : SREEMDEVALRSHNNVERATKEGDFAE--EIVPVEIPQKKGKPPVIFDKDEHFRPGLTMEQLTKLPP : 256
Euglena grac: SREEQDEIALRSHNNVERVTKSGILRECGEIVPVEVKQK--KKVTIIDKDEHFRPGLTMAQLEKLPP : 259

Burkholderia: VFAKENGTVTAGNASGINDAAAAVVLMERGVADKRGAKPLARLVSYAHAGVDPAYMGIGPVPATKKA : 301
Ralstonia so: VFAKENGTVTAGNASGLNDAAAALVLMERSLAERRGLKPLARLVSYGHAGVDPKVMGIGPVPATRKA : 301
Clostridium : AFKKD-GTVTAGNASGINDGAAALVIMSAEKAEELGLKPLAKIASYGSKGLDPAYMGYGPVGATKVA : 299
Bacillus cer: AFRKD-GSVTAGNASGLNDGSAVLVLMSEEKAKEKGLQPLARIVGYSVAGVDPKIMGIGPAPAIRKG : 298
Desulfococ  : AFVPGIGKVTAGNSSGLNDGAAAMVIMSADKAKELGLKPLAKIVASGRGACHPSVMGISPVPAVKNM : 323
Euglena grac: AFIPNVGTVTAGNSSGINDGAAALLLVELEKAKALGLKPLAIISGMGKGGCAPELMGESPVPAVNDL : 326

Burkholderia: LERA-GITVADLDVIEANEAFAAQACAVSNALGLDPAKVNPNGSGISLGHPIGATGALITVKALYEL : 367
Ralstonia so: LERA-GLTVKDLDVVEANEAFAAQACAVTQQLGLDPARVNPNGSGISLGHPIGATGALITVKALHEL : 367
Clostridium : LEKA-GWKVEDLDLIEANEAFASQSLAVAKDLGFDMEKVNVNGGAIALGHPVGCSGARILVTLLHEM : 365
Bacillus cer: LEKV-DWSLEDADLLEINEAFAAQYLAVEKELGLDREKVNVNGSGVGLGHPIGCTGARITVSLIHEL : 364
Desulfococ  : FDKHPDLSLDAFELIELNEAFAAQYLGCEKELGLNREITNVNGSGIGLGHPVGCTGARIIVSLIHAM : 390
Euglena grac: CEKT-GHKVSDYQRVEMNEAFASQYIACERLLKMNRDVTNVNGSGIGLGHPVGCTGARIVVSLLHEM : 392
                              *                                   *

Burkholderia: QRIGGRYALVTMCIGGGQGIAAVFERL : 394
Ralstonia so: QRIGGRYALVTMCIGGGQGIAAVFERI : 394
Clostridium : QRRDSKKGLATLCIGGGMGTALLVER- : 391
Bacillus cer: KRRGLEKGIASLCVGGGIGVALFIEAL : 391
Desulfococ  : KKRGKSVGMATLCGGGGVSMATAVEML : 417
Euglena grac: IRSGSQLGMASMCGGGGVSLATELTLC : 419
                          * *
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Fig. 3.17: Sequence alignment of bacterial 3-ketothiolases with the *E. gracilis* putative mitochondrial thiolase. The binding domains are indicate in bold and the position of the amino acids is based on the *E. gracilis* sequence. *Burkholderia pseudomallei*, SAVP1 YP_108160.1; *Ralstonia solanacearum*, GMI1000, NP_519758.1; *Clostridium tetani*, NP_781017.1; *Bacillus cereus* AH1134, ZP_ 02523830,1; *Desulfococcus oleovorans* Hx, YP_001530041.1; *E. gracilis* ELL00002493.

3.7 Cloning and over expression of the putative *E. gracilis* thiolase T_2493

To PCR amplified the putative 3-ketoacyl-CoA thiolase, cDNA from *E. gracilis* cells was prepared. Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel) and cDNA was synthesized with the SuperScript Synthesis System (Invitrogen) as recommended by the manufacturer. The 3-ketoacyl-CoA thiolase was amplified with the primers Eg2493BamHI and Eg2493SacI to introduce the restriction sites 5'-*Bam*HI and 3'-*Sac*I. The PCR fragment was cloned into pQE30 expression vector which provides N-terminal His-Tag fusion proteins. The construct T_2493 in pQE30 was expressed in the *E. coli* strain M15[pREP4] after induction with 0.4 mM IPTG and it was subsequently

incubated at 37 °C for three hours. After Ni-NTA purification on Coomassie-stained 12 % SDS-PAGE and Western blot analysis one band corresponding to 44.5 kDa was detectable in the sample carrying the construct T_2493 in pQE30, while in the sample carrying the control vector no additional bands were present. However, the recombinant protein was expressed as insoluble inclusion bodies in the pellet fraction.

Different IPTG concentrations, temperature and time of induction were tested unsuccessfully. The inclusion bodies were isolated and disrupted in presence of β-mercaptoethanol as denaturing and reducing agent. The denaturated protein mixture was dialyzed overnight to allow the complete refolding of the proteins. The dialyzed protein was purified on Ni-NTA agarose but it did not bind onto the matrix and it was eluted completely in the flow through (data not shown).

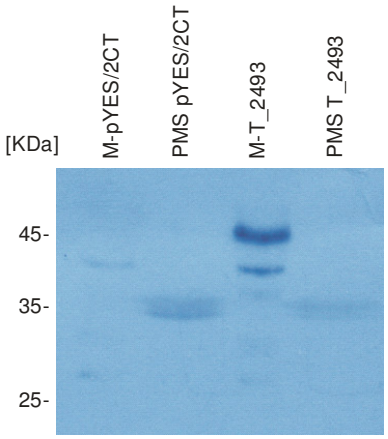

Fig. 3.18: Western blot with immunodetection of the His-Tag carrying recombinant protein after mitochondrial isolation. *M-pYES/2CT*, Mitochondrial fraction of INVSc cells carrying the pYES/2CT vector as negative control; *PMS-pYES/2CT*, Post Mitochondrial Surnatant (PMS) of INVSc cells carrying the pYES/2CT vector; *M-T_2493*, Mitochondrial fraction of INVSc cells carrying the construct T_2493 in pYES/2CT; *PMS-T_2493*, PMS of INVSc cells carrying the construct T_2493 in pYES/2CT.

For the expression in yeast cells the 3-ketoacyl-CoA thiolase was amplified with the primers Eg2493BamHI_HF and Eg2493XbaI_HR to introduce the restriction sites 5'-*Bam*HI and 3'-*Xba*I. The PCR fragment was cloned into pYES/2CT expression vector which provides C-terminal His-Tag fusion proteins. The construct T_2493 in pYES/2CT was expressed in INVSc yeast strain by induction with 1 % of raffinose and 2 % galactose and was incubated at 30 °C overnight. The recombinant protein was expressed in the mitochondrial fraction. After Western blot analysis with immunodetection of the His-Tag a clear band corresponding to 44.5 kDa was detectable in the mitochondria of yeast cells carrying the construct (**Fig. 3.18**). The mitochondria were isolated, disrupted by sonification and tested for condensing activity as described in **s. 2.12.5**. The recombinant protein revealed inactive since no condensing activity was detectable with all three different assays (data not shown).

3.8 Identification and amplification of a putative mitochondrial propionyl-CoA carboxylase (PCC) α-subunit from *E. gracilis*

A tBLASTn search with the mitochondrial PCC gene from *Homo sapiens* (X14608; EC 6.4.1.3) in the *Euglen*a EST database at the NCBI showed two hits that exhibit high similarity with the query sequence. The first was the sequence EC683153.1 (823 bp; ELE00005336 Low light non-normalized long fraction *E. gracilis*) with an e-value of $1e^{-104}$ and an amino acid identity of 63 %, and the second sequence EL581823.1 (518 bp; s_eg003053054r Euglena gracilis strain SAG 1224-5/25) with an e-value of $6e^{-42}$ and an amino acid identity of 47 %. The 823 bp fragment was amplified by PCR with the primers Eg_PCCFor and Eg_PCCRev to introduce 5'-*Hind*III and 3'-*Hind*III restriction sites. cDNA synthesized from the isolated total RNA was used as template. The PCR product was cloned into pGEM®-T Easy vector and after sequencing was used to prepare the radioactively labelled hybridization probes for the Northern blot analysis.

3.8.1 Northern blot analysis of *E. gracilis* strain Z

The Northern blot analysis was performed as described in **s. 2.10.8** with total RNA isolated from *E. gracilis* cultures grown under aerobic and anaerobic conditions. Of each RNA sample 12 □g were separated electrophoretically under denaturing conditions on a 1 % agarose gel. The RNA samples were transferred onto a nylon membrane (N, Amersham Biosciences) and hybridized at 68 °C with the 823 bp PCC □-subunit fragment amplified from the *E. gracilis* cDNA. After hybridization and three days film exposition at -80 °C a clear band of about 3000 bp corresponding to the complete PCC gene sequence was detectable in the RNA sample isolated from aerobic cultures (lane 1; **Fig. 3.19**). In the RNA sample isolated from an anaerobic culture beside a band at about 3000 bp many degradation products were present (lane 2; **Fig. 3.19**).

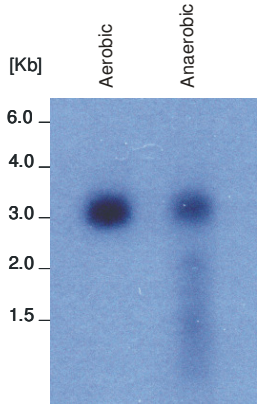

Fig. 3.19: Northern blot analysis with RNA isolated from *E. gracilis* cultures cultivated under aerobic and anaerobic conditions. In each lane 12 □g RNA sample were loaded onto the gel. *Lane 1*, RNA isolated from aerobic cultures. *Lane 2*, RNA isolated from anaerobic cultures. The amplified 823 bp fragment corresponding to the PCC α-subunit was used as hybridization probe.

To quantify the RNA expression level for the PCC sequence the Northern blot analysis was repeated. As positive control the actin sequence isolated by double digestion from the *Euglena* expressed sequence tags (EST) contig eg_003051049r generated in-house was used (Ahmadinejad et al. 2007). The RNA expression was quantified with the Image Gauge software (3.0). As shown in **Fig. 3.20** the PCC was expressed under both, aerobic and anaerobic conditions and either additional bands nor degradation products were detectable. However, the expression of PCC during anaerobiosis was about 15 % higher than in aerobic cells.

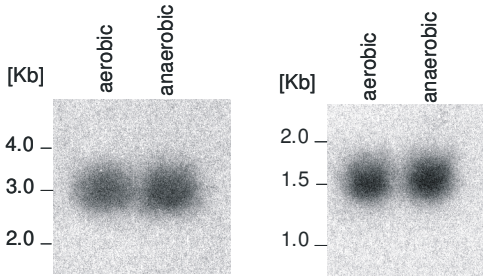

Fig. 3.20: Northern blot analysis with RNA isolated from *E. gracilis* cultures cultivated under aerobic and anaerobic conditions. In **A,** the amplified 823 bp fragment corresponding to the PCC α-subunit was used as hybridization probe. In **B**, the ca. 1500 bp actin fragment was used. 5 µg RNA sample were loaded onto the gel. *Lane 1*, RNA isolated from aerobic cultures. *Lane 2*, RNA isolated from anaerobic cultures.

3.9 Cloning and overexpression of the *T. denticola* TER (TdenTER) in *E. coli*

A freeze dried genomic DNA sample of *T. denticola* ATCC 35405, kindly provided by Prof. H.F. Jenkinson (University of Bristol, UK), was used as template to perform PCR amplification. The 1194-bp fragment from the gene sequence TDE0597 of *T. denticola* ATCC 35405 was amplified by PCR with the primers Tden1Ndefor and Tden1Xhorev to introduce 5'-*Nde*I and 3'-*Xho*I restriction sites. The amplification products were cloned into pET28a vector, which provides N-terminal His-Tag fusion proteins and were expressed in *E. coli* strain BL21(DE3) by addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 37 °C for four hours. The TdenTER ORF expressed in pET28a with BL21(DE3) resulted in soluble protein after purification by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose. As shown in **Fig. 3.21**, in the Coomassie-stained 12 % SDS-PAGE and in the Western blot analysis with immunodetection a band of about 45 kDa was visible in the samples carrying the recombinant protein (lanes 1, 3, 5, 6, 8-11), while it was not present in the samples carrying the control vector (lanes 2, 4 and 7).

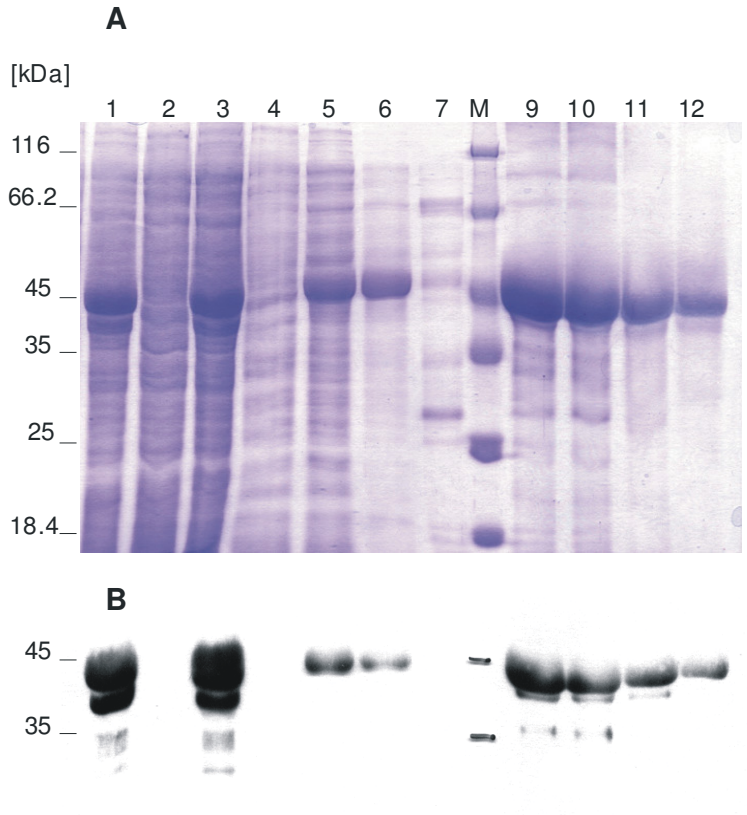

Fig. 3.21: *A*. Coomassie-stained 12 % SDS-PAGE of the fractions after Ni-NTA purification and *B*. Western blot analysis with immunodetection of the TdenTER ORF in pET28a expressed in BL21(DE3). *Lane 1*, soluble bacterial fraction. *Lane 2-11,* different fractions after Ni-NTA purification. *Lane 2*: flow-through pET28a; *lane 3*: flow-through pET28a-TdenTER; *lane 4*: first wash pET28a; *lane 5-6*: first and second wash pET28a-TdenTER; *lane7*, first elution pET28a; *lane 8-11*: respectively, first, second, third and fourth elution pET28a-TdenTER.

3.9.1 Catalytic properties of TdenTER

The kinetic parameters were determined using the TdenTER construct in *E. coli*. The recombinant protein showed NADH-dependent activity, but was not active when NADPH was used as cofactor. The specific activity of TdenTER was determined as 43.4 ± 4.8 U/mg (µmol mg^{-1} min^{-1}) using crotonoyl-CoA as substrate, while *trans*-hexenoyl-CoA as a substrate was not reduced. TdenTER also exhibited a much higher affinity for crotonoyl-CoA with K*m* values of 2.7 µM and a lower substrate inhibition with K*i* = 0.198 µM for crotonoyl-CoA using NADH as cofactor (**Fig. 3.22**). Oxidizing activity of TdenTER was not detectable in the reverse reaction when butyryl-CoA was used as substrate. The K*m* value and the specific activity were defined using the “enzyme kinetics module” of the Sigma Plot software.

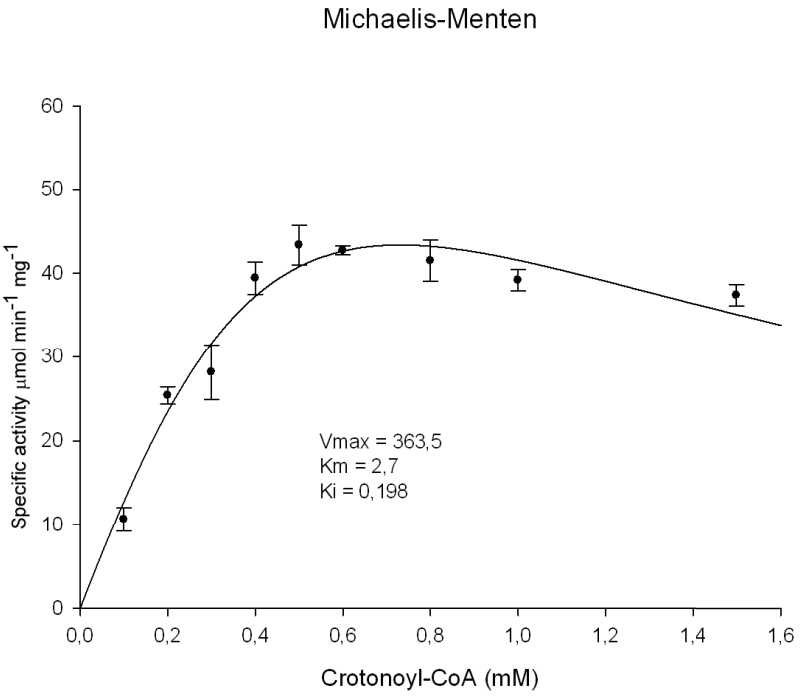

Fig. 3.22: Specific activity of TdenTER with crotonoyl-CoA as substrate. The specific activity is reported in □mol min^{-1} mg^{-1} and the K*m* value is calculated with the "enzyme kinetics modules" of the Sigma Plot software based on Michaelis and Mentel reaction. (n=4).

The *E. gracilis* TER is a NAD(P)H dependent enzyme (Hoffmeister et al. 2005). In this work it has been demonstrated that the presence of NADH as cofactor is indispensable for the activity of TdenTER. Consequently, the k*m* value has also been calculated for NADH. The calculated K*m* showed a value of 0.83 □M and a maximum activity of 114.3 □M. Additionally, the TdenTER activity was also measured in the presence of the inhibitors Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether, Irgasan Sigma) and Isoniazid. A stock solution of Triclosan was prepared in 98 % ethanol. Appropriate amounts of ethanol were tested in control assays to check possible inhibitory effects on the enzymatic activity. Whereas added to the assay mixture in a wide range of concentrations, between 5-1000 µM, both inhibitors had no effect on the TdenTER activity (data not shown). The K*m* value and the specific activity were defined using the "enzyme kinetic modules" of the Sigma Plot software.

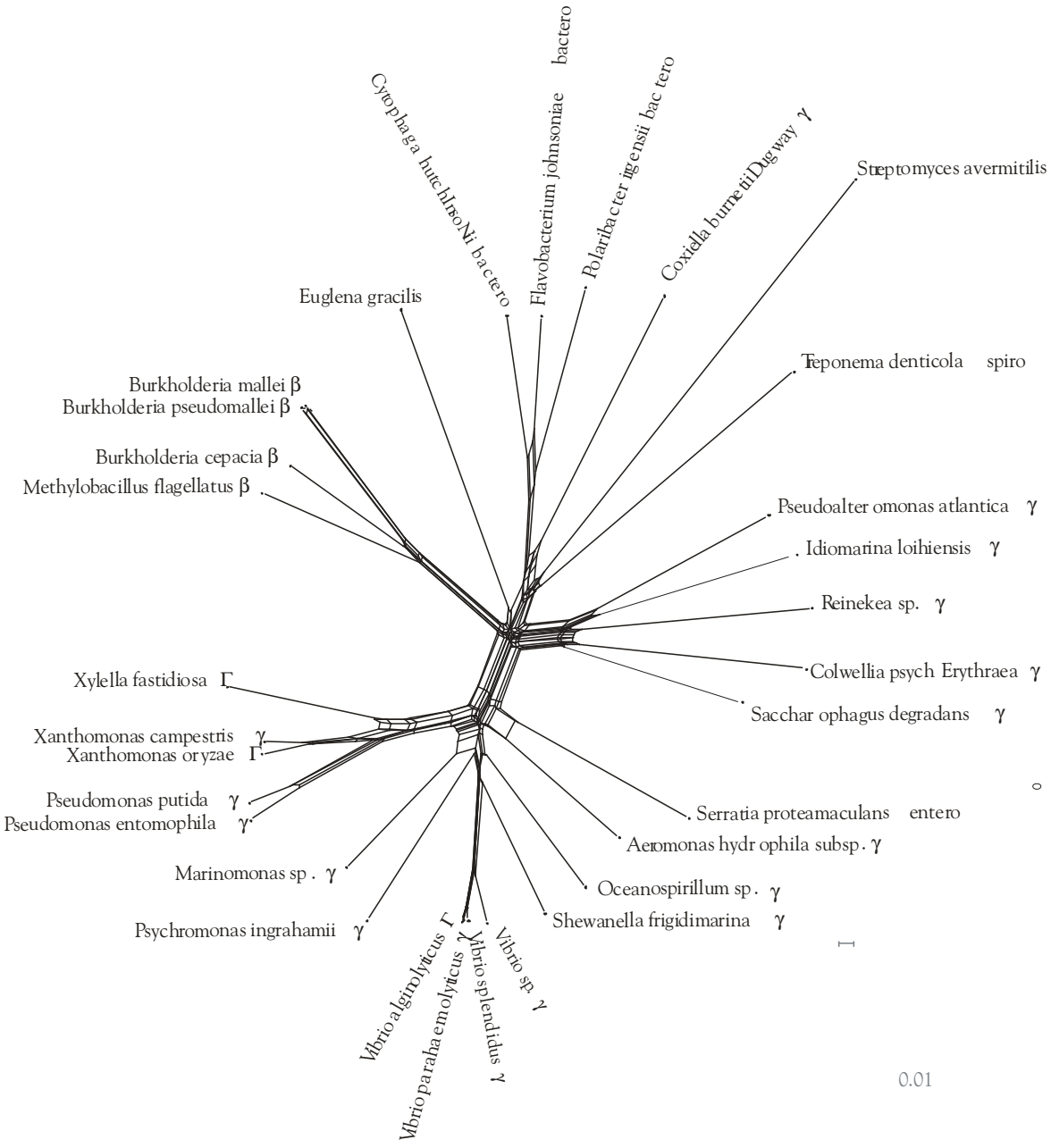

Fig. 3.23: NeighborNet graph of TdenTER homologous sequences. The scale bar at the lower right side indicates estimated substitutions per site. Abbreviations are as follows: *β*- and *γ*-, proteobacteria; *bactero*, bacteroides; *entero*, enterobacteria, *spiro*, spirochete.

```
C.burn :    ------------------------------------------------------------------------ :   -
E.grac :    MSCPASPSAAVVSAGALCLCVATVLLATGSNPTALSTASTRSPTSLVRGVDRGLMRPTTAAALTTMREVPQM :  72
F.john :    ------------------------------------------------------------------------ :   -
C.hutc :    ------------------------------------------------------------------------ :   -
P.irge :    ------------------------------------------------------------------------ :   -
T.dent :    ------------------------------------------------------------------------ :   -

C.burn :    ------------------------------------------------------------------------MI :   2
E.grac :    AEGFSGEATSAWAAAGPQWAAPLVAAASSALALWWWAARRSVRRPLAALAELPTAVTHLAPPMAMFTTTAKV : 144
F.john :    ------------------------------------------------------------------------MI :   2
C.hutc :    ----------------------------------------MCWIELKRISTLFYQTKKIS---------IMI :  23
P.irge :    ------------------------------------------------------------------------MI :   2
T.dent :    ------------------------------------------------------------------------MI :   2
```

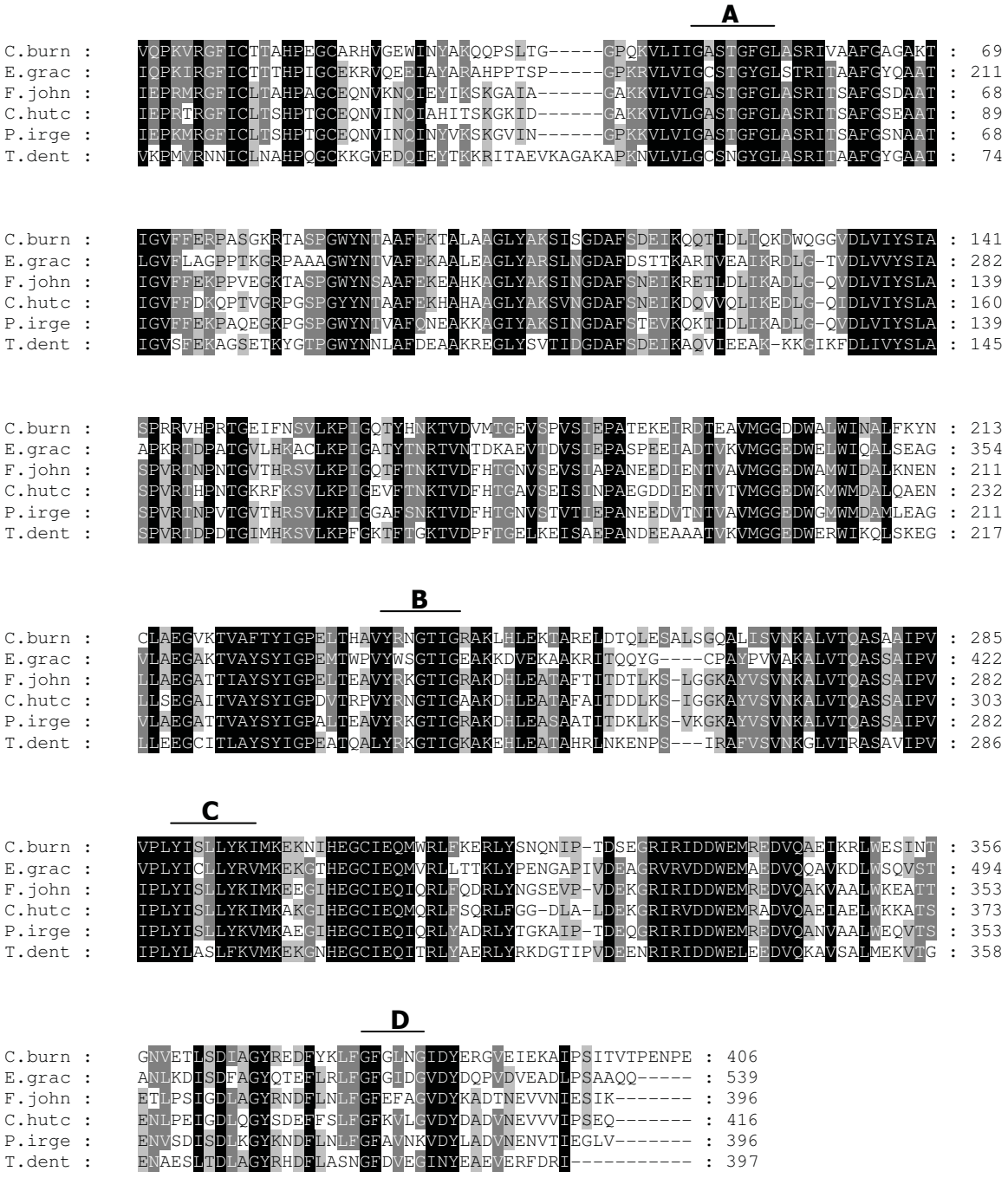

Fig. 3.24: Multiple sequence alignment of TdenTER with hypothetical *trans*-2-enoyl-CoA reductases. *A*: represents the putative binding site for NAD(P)H; *B and C*: for the substrate and D: for FAD. Organisms and accession numbers are given as follows: *Treponema denticola* ***AE017248***; *Euglena gracilis* ***Q5EU90***; *Flavobacterium johnsoniae* ***ZP01243065***; *Cytophaga hutchinsonii* ***YP677688***; *Polaribacter irgensii* ***ZP01118954***; *Coxiella burnetii* ***ZP01298067***. The protein sequences retrieved from GenBank were aligned with CLUSTALW (Thompson et al. 1994).

3.10 Cloning and over expression of the *S. avemitilis* TER (SaveTER)

A genomic DNA sample of *S. avermitilis* was used as template to perform PCR amplification. The 1320-bp fragment from the gene sequence SAV2368 (GenBank accession number ***AB070934***) from the *S. avermitilis* polyketide biosynthetic cluster 5 (*pks5*) was amplified by PCR with the primers SaveNdefor, SaveBglII_F and SaveXho_R to introduce 5'-*Nde*I, *Bgl*II and 3'-*Xho*I restriction sites. The amplification products were cloned into pET28a and pET32 vectors, which provide both N-terminal His-tag fusion proteins and the pET32 additionally a thioredoxin-fusion protein. The expression was carried out in *E. coli* strain BL21(DE3) by addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for four hours. For both constructs clear bands corresponding to the recombinant protein were visible on the Coomassie-stained 12 % SDS-PAGE and after Western blot analysis with immunodetection. However the protein was expressed as insoluble inclusion bodies in the pellet fraction (data not shown). To solubilize the expressed protein from the inclusion bodies the pellet fraction was treated with 1.2 % sodium-cholate. A positive result was obtained when the construct SaveTER in pET28a was expressed in *E. coli* strain Rosetta(DE3) by induction with 0.1 mM IPTG at 37 °C for three hours. As shown in **Fig. 3.25** a band corresponding to ~ 48 kDa was detected in the lane 3 of the Coomassie-stained 12 % SDS-PAGE and Western blot analysis, whereas the proteins expressed in the inclusion bodies remained for the most part in the pellet fraction (lane 1) and the purified solubilized protein was not active.

Different IPTG concentrations, temperatures and times of induction as well as different *E. coli* strains [Origami(DE3), Rosetta(DE3), C43(DE3) and C41(DE3)] were tested unsuccessfully. Only in the case of the construct SaveTER in pET28a expressed in C41 (0.5 mM IPTG, 30 °C over 24 hours) bands corresponding to ~ 48 kDa were visible in the clear lysate fraction as soluble protein (**Fig. 3.26**). However, after Ni-NTA purification the recombinant protein revealed inactive with either crotonoyl-CoA and hexenoyl-CoA as substrate.

The SaveTER fragment was then cloned in pYES/2CT and pCT/2CT vectors carrying C-terminal His-Tag fusion proteins for the expression in the yeast strain INVSc. The recombinant protein was expressed in the mitochondrial fraction as demonstrated after Western blot analysis with immunodetection. But, after mitochondria isolation and Ni-NTA purification the recombinant protein was completely degraded and inactive (data not shown).

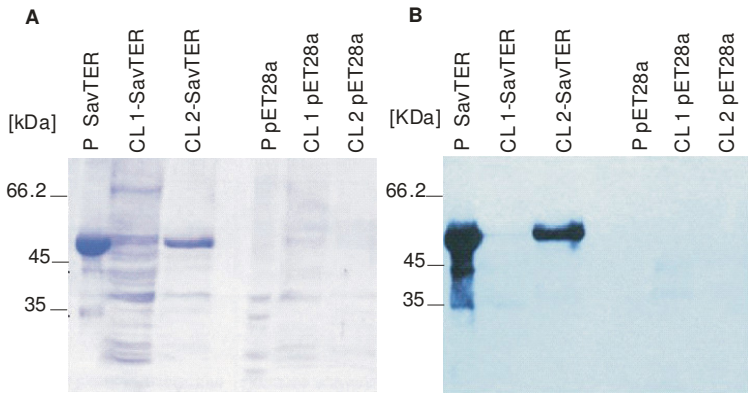

Fig. 3.25: Coomassie-stained 12% SDS-PAGE of the fractions after Ni-NTA purification and Western blot analysis with immunodetection of the SavTER expressed in Rosetta(DE3). *P SavTER*, pellet fraction SavTER in pET28a; *CL1 SavTER,* 1° clear lysate SavTER in pET28a; *CL2- SavTER,* 2° clear lysate SavTER in pET28a*; P pET28a*, pellet fraction pET28a; *CL1 pET28a,* 1° clear lysate pET28a; *CL2 pET28a*, 2° clear lysate pET28a.

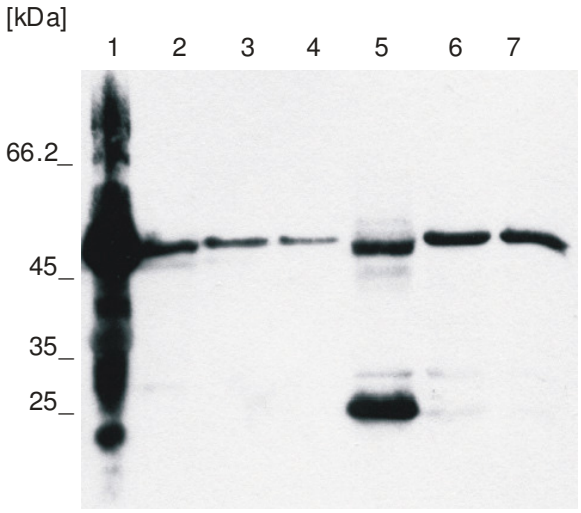

Fig. 3.26: Western blot analysis with immunodetection of the SaveTER ORF in pET28a expressed in C41(DE3). Induction was carried out with 0.1 mM IPTG at 30 °C over 24 hours. *Lane 1*, bacterial pellet fraction. *Lane 2-7,* different fractions after Ni-NTA purification. *Lane 2*: flow-through; *lane 3*: wash; *lane 5-7*: first, second and third elution pET28a-SaveTER.

4. Discussion

The mitochondrion of the flagellate *Euglena gracilis* is a facultatively anaerobic organelle able to produce ATP in the presence and absence of oxygen. The shift of *Euglena* cells from aerobic to anaerobic conditions induces the malonyl-CoA independent synthesis of wax esters from the reserve polysaccharide paramylon (Inui et al. 1982). Mitochondrial fatty acid synthesis uses propionyl-CoA and acetyl-CoA as C2- and C3-donors, for *de novo* synthesis of even- and odd-numbered fatty acids, respectively.
Aims of this work were to characterize the mitochondrial fatty acid synthesis of *E. gracilis* by means of analysis of the wax ester fraction and by comparison of enzymatic activity of the *Euglena* TER (Hoffmeister et al. 2005) with two prokaryotic homologues.

4.1 The effect of oxygen on the wax ester content and composition of *E. gracilis* 1224-5/25 strain Z

To analyze how the oxygen influences the wax ester content and the fatty acid and fatty alcohol profiles different cultures of *E. gracilis* 1224-5/25 strain Z were performed. Aerobic cultures grown in the light accumulated wax esters up to ca. 6 % of the fresh weight, which yielded the even-number myristic (C14:0) and palmitic (C16:0) acid as major compounds, as already reported in earlier work (Inui et al. 1982), beside unsaturated chains. The analysis of the fatty acid composition of *E. gracilis* 1224-5/25 strain Z cultures grown aerobically in the dark revealed a correlation between the growth in the light and the presence of unsaturated fatty acids in the wax ester fraction. Although the wax ester content was not significantly effected by aerobically cultivation in the dark, the concentration of usaturated fatty acids in *Euglena* cultures was strongly reduced. A similar result was described by Barsanti et al. (2000). They showed that the content of unsaturated fatty acids in an *E. gracilis* spontaneous white mutant grown in the light is as low as those of green cells in the dark, suggesting a correlation of unsaturated fatty acids to the presence of chloroplasts. In plants the introduction of double bonds on the fatty acid chain is a reaction that occurs in the plastids and is catalyzed by soluble and membrane-bound desaturases responsible for the insaturation on the fatty acid chain (Somerville and Browse 1996; Lopez Alonso et al. 2003). The absence of unsaturated fatty acids in the wax ester fraction of dark grown *E. gracilis* strain Z cultures could be therefore attributed to a lost of desaturase activity. Furthermore, heterotrophical cultivation in the dark leads in *E. gracilis* to a loss of chloroplasts and galactolipids and a marked increase in

mitochondrial structure (Vannini 1983; Hulanicka et al. 1964). Dark grown *Euglena* cells produce a different fatty acid pattern with a gain in longer polyunsaturated chains, but these are used for the synthesis of phospholipids (Korn 1964; Rosenberg 1963; Hulanicka et al. 1964). Hence, they are possibly not incorporated in the wax esters.

When the cultures were brought to anaerobiosis, the absence of oxygen caused a striking effect on the accumulation and composition of the wax esters. Lower oxygen tension (N_2/CO_2) in the light did not influence the wax ester content but the fatty acid and alcohol profiles. In fact, the proportion of even-number chain compounds was reduced and the fatty acid and alcohol pattern were shifted towards shorter and odd-number chains, suggesting an activation of the mitochondrial fatty acid synthesis. In agreement with earlier data (Schneider and Betz 1985; Inui et al. 1982) the presence of odd-numbered fatty acids and fatty alcohols such as C13:0 and C15:0 implicates the participation of the fumarate reductase-rhodoquinone [FRD(RQ)]-propionyl-CoA route (Schneider and Betz 1985; Tielens et al. 2002) since the formation of odd-chains by α-oxidation can be excluded in fermenting *Euglena* cells (Schneider et al. 1984a). Additionally, α-oxidation is an energy consuming reaction leading to the synthesis of fatty acids hydroperoxydes which serve as important intermediates in the oxylipins pathway in plants, fungi and diatoms as response to wounding and as defence mechanism in host-pathogen interaction (Hamberg and Gardner 1992; Blee 1998; Pohnert and Boland 2002; Hamberg 2003; Ianora et al. 2004). *E. gracilis* strain Z has been reported to synthesize the oxylipin jasmonic acid (Ueda et al. 1991). However the biosynthesis of jasmonate is a reaction proceeding neither through α-oxidation nor in absence of oxygen. It can be excluded, therefore, that in *Euglena* cells grown by lower oxygen tension the biosynthesis of odd-number chain fatty acids and fatty alcohols occurs via α-oxidation rather than by anaerobic mitochondrial fermentation.

By lower oxygen tension (N_2/CO_2) in the light anaerobic wax esters yielded a high concentration of eicosapentenoic acid (C20:5 ω3, EPA). Several species of marine bacteria including *Shewanella* and marine microalgae have been reported to possess polyunsaturated fatty acids such as EPA and docosahexaenoic acid (22:6 ω3, DHA)(Barclay et al. 1994; Nichols et al. 1999). These organisms are known to synthesize EPA *inter alia* via specialized polyketide synthases that produce highly unsaturated linear products (Metz et al. 2001), performing *cis-trans* isomerization and enoyl reduction reaction in selected cycles.

Under strictly anaerobic conditions (in the dark) *E. gracilis* strain Z cells accumulated wax esters up to ca. 50 % of the total lipid fraction, as expected (Buetow 1989). The ability of *E. gracilis* strain Z to survive anaerobiosis up to 6 months at 5 °C on a non-fermentable

carbon source was described by Carre et al. (1988). During these conditions ca. 70 % of *E. gracilis* dry weight was represented by wax esters (Buetow 1989). However, in this work it has been demonstrated that *E. gracilis* strain Z accumulated only about 10 % of the dry weight as waxes under anaerobiosis **Tab. 3.3**. As reported for cultures grown by lower oxygen tension (N_2/CO_2) in the light, the fatty acid and fatty alcohol composition was shifted towards shorter and odd-number chains. The presence of these components represents a strong evidence for the involvement of the [FRD(RQ)]-propionyl-CoA route. In *Euglena* the synthesis of propionyl-CoA occurs via the methyl-malonyl-CoA pathway (Nagai et al. 1971; Schneider and Betz 1985) and one of the key enzymes is the fumarate reductase (FRD) that catalyzes the reduction of fumarate to succinate and requires the participation of rhodoquinone (RQ)(Takamiya et al. 1993; Van Hellemond und Tielens 1994; Tielens 2002; Hoffmeister et al. 2004). The reduction of fumarate is a NADH-consuming reaction, essential to maintain the the redox balance in anaerobically functioning mitochondria (Van Hellemond und Tielens 1994; Tielens and Van Hellemond 1998; Tielens 2002; Van Hellemond et al. 2003). The increment of odd-numbered fatty acids and alcohols in the wax ester fraction of anaerobically grown cultures confirms, therefore, the involvement of the anaerobic energy metabolism and the activation of the mitochondrial fatty acid biosynthesis. Moreover, the results of the measurements with dark-grown anaerobic cultures indicate that EPA is lacking entirely while odd-chain fatty acids accumulate, suggesting that EPA synthesis occurs by enzymes that are wholly independent of the short chain saturated wax ester route (**Fig. 3.1**).

4.2 The effect of flufenacet on the wax ester content and composition of *E. gracilis* 1224-5/25 strain Z

Flufenacet belongs to the class of K_3 herbicides and has demonstrated to inhibit higher plant elongases but not FAS complexes in either *Arabidopsis* (in the chloroplast) or yeast (Trenkamp et al. 2004). *E. gracilis* mitochondrial fatty acid synthesis occurs, as well as elongation system, via CoA esters instead of ACP-proteins (Hoffmeister et al. 2005). To characterize this biochemical pathway, *E. gracilis* 1224-5/25 strain Z cells were cultivated in the presence of 100 µM flufenacet.

In this work it has been demonstrated that the combination of two factors, the growth in the light and the addition of the herbicide flufenacet, plays an important role for the synthesis of the wax esters and their fatty acid and alcohol pattern. In aerobic cultures, flufenacet influenced only the accumulation of wax esters up to ca. 10 % of the lipid

fraction, while the fatty acid and fatty alcohol profiles were not not affected if compared with those of untreated cultures (**Fig. 3.1** and **Fig. 3.3**). On the other hand, dark grown *Euglena* cells supplemented with flufenacet yielded wax esters up to 22 % of the lipid fraction and the synthesis of shorter and saturated chains was enhanced. By cultivation in the prsence of flufenacet all fatty acids and alcohols longer than C18 in the wax ester fraction were below detection limit. The proportion of odd-chain constituents in the wax esters was increased, in particular of C13:0 (**Fig. 3.3**). Furthermore, the proportion of C16 and C18 fatty acids and alcohols in dark-synthesized wax esters decreases substantially. This, together with the finding that unsaturated fatty acids, with the exception of C16:1 ω7, were absent, suggests that they may indeed stem from plastidial or cytosolic synthesis in ambient or by low oxygen conditions.

The shift to anaerobiosis and the treatment with flufenacet resulted in an unexpected high concentration of wax esters in cultures grown anaerobically in the light. In fact, *E. gracilis* strain Z cells accumulated wax esters up to 33.3 % of the lipid fraction, a ca. three fold higher amount than the corresponding aerobically grown cultures. However, the odd chain fatty acids and alcohols are reduced in the wax ester fraction compared to growth without flufenacet (**Fig. 3.1**), although the total wax ester production is not inhibited (**Tab. 3.1**) and the accumulation of even-number chain fatty acids and alcohols with C14:0 and C16:0 as major compounds, was particularly evident. This suggests that redox balance is maintained by diversion of reduction equivalents to cytosolic and/or plastidial fatty acid synthesis (in the light), as evidenced by the synthesis of longer and unsaturated fatty acids. The suggestion that flufenacet diverts reduction equivalents to fatty acid synthesis in other compartments is consistent with the finding that this herbicide does not inhibit the fatty acid synthesis but only the elongation in *Arabidopsis* or yeast (Trenkamp et al. 2004).

The presence of flufenacet in anaerobic dark cultures caused an increase of waxes up to about 60 % of the dry weight. Moreover, the paucity of organics with a chain length longer than C18 suggested a very broad inhibition of very long chain fatty acid elongases in *Euglena* by flufenacet. Most important, however, is the finding that the low O_2-specific synthesis of odd-chain fatty acids and EPA is inhibited by flufenacet, whereas fatty acid synthesis in general is not. Eckermann et al. (2003) already described an inhibitory activity due to the covalent binding of the chloroacetanilides, metazachlor and metolachlor, to the active site cysteine of plant type III polyketide synthases. Similarly to this class of enzymes, the 3-ketoacyl synthase, which catalyze the first step in the elongation process, possesses a catalytic amino acid triad of Cys258, His425 and Asn458 acting on CoA esters subtrates (Lassner et al. 1996; Joubès et al. 2008). This finding which is correlated with the inhibition of elongases by flufenacet (Trenkamp et al. 2004) suggests that the

enzymatic mechanism of mitochondrial fatty acid synthesis in *Euglena* differs from a FAS-type mechanism and probably involves elongase- or PKS-like activities.

4.3 Wax ester content and composition in different *E. gracilis* strains

The ability to survive lower oxygen tension has been reported for at least several *Euglena* species (Buetow 1989), however all studies on the wax ester fermentation were carried out on *E. gracilis* strain Z and on the streptomycin bleached mutant *E. gracilis* SM-ZK (Inui et al. 1982; Inui et al. 1984).

To compare the resistance to anoxia and the capability to perform wax ester fermentation, nine different *Euglena* species were cultivated and growth curves under anaerobic conditions were prepared. In this work it has been demonstrated that oxygen plays a crucial role. Not all green *Euglena* strains survived anaerobiosis confirming that the mitochondrial biochemical aptitude to synthesize fatty acids in the mitochondria leading to ATP production is not evenly spread among *E. gracilis* species and must have been lost in some strains (**s.s 3.3**). Anyway, all *Euglena* species accumulate wax esters in their lipid fraction by aerobic cultivation, as already demostrated for *E. gracilis* 1224-5/25 strain Z.

4.3.1 Wax ester content and composition of strictly aerobic *E. gracilis* strains

E. gracilis species accumulate wax esters in their lipid fraction by aerobic cultivation in different concentrations. Unexpected, *Euglena* 1224-5/15 var. *Bacillaris* yielded four fold more wax esters, up to 27.6 % of the lipid fraction corresponding to 3.5% of the dry weight, than other *Euglena* species. On the other hand, in the *Euglena* strains 1224-5/10 and 1224-5/27 the waxes concentrations were comparable with that of *Euglena* 1224-5/25 strain Z. All three *Euglena* strains presented fatty acid and alcohol profiles with clear prevalence of C14:0 and C16:0 acids. Of particular note was the accumulation in the fatty alcohol fraction of C15:0 in Euglena cells 1224-5/27. Moreover, the very high concentration of C16:0 together with the discrete concentration of C18:0 and unsaturated fatty acids in *Euglena* 1224-5/10 cells indicates a clear plastidial origin under aerobic conditions.

4.3.2 Wax ester content and composition of facultatively anaerobic *E. gracilis* strains

All analyzed *Euglena* species accumulated wax esters in very similar concentration and proportion to dry weight with the exception of the strain *E. gracilis* 1224-5/13 in which the

wax esters corresponded to ca. 12 % of the lipid fraction (**Tab. 3.3**). The fatty acid and fatty alcohol profiles did not show significant differences in comparison to those of *E. gracilis* 1224-5/25 strain Z. Only in the strain *E. gracilis* 1224-5/23 the composition was shifted towards shorter chains, in particular C12:0 C13:0 and C14:0.

As expected, growth under strictly anaerobic conditions led to a ten fold higher accumulation of waxes in comparison to growth under aerobiosis (**Tab. 3.3**). While no significant differences were detected between the *Euglena* strains 1224-5/3, 1224-5/4, 1224-5/19 and 1224-5/23, in *E. gracilis* 1224-5/13 the wax ester fraction represented ca. 31 % of the dry weight. Surprisingly, the analysis of the fatty acids and alcohols showed a clear increment of shorter, saturated odd-number chains only in *E. gracilis* 1224-5/13 and *E. gracilis* 1224-5/23. This suggests that the synthesis of fatty acids in the *Euglena* strains 1224-5/3, 1224-5/4 and 1224-5/19 probably does not require the participation of the fumarate reductase-rhodoquinone [FRD(RQ)]-propionyl-CoA route as already demonstrated for the *E. gracilis* 1225-5/25 strain Z during this work.

Addition of flufenacet to anaerobically grown cultures in the dark led to a further increment of the wax ester concentration of all *Euglena* species. In *E. gracilis* strain 1224-5/13 the increase was particularly evident with a wax ester concentration reaching ca. 60 % of the dry weight, a value comparable with those already reported in the literature (Carre et al. 1988; Buetow 1989). In contrast, the herbicide did not effect significantly the fatty acid and alcohol profiles, with the exception of *Euglena* strains 1224-5/13 and 1224-5/23 resulting in a strong reduction of C13:0 and C15:0 chains probably due to the inhibition of the condensing step in the mitochondrial fatty acid synthesis, as already observed for *E. gracilis* 1224-5/25 strain Z (**Fig. 3.3**).

4.4 Wax ester content and composition of *E. gracilis* 1224-5/25 bleached mutants

E. gracilis possesses both cytoplasmic type I and a very active plastidial type II fatty acid synthase (FAS)(Delo et al. 1971) whose fatty acids when synthesized or transported in the cytosol could be incorporate the waxes stored in the cell. To avoid the assimilation of non-mitochondrial synthesized fatty acids in the wax ester fraction, bleached *E. gracilis* cells strain 1224-5/25 were generated by treatment with streptomycin and ofloxacin. The effect of streptomycin as bleaching agent on *Euglena* chloroplasts was already described by Privasoli et al. (1948). Aminoglycosidic antibiotics including streptomycin inhibit the bacterial protein- and DNA-synthesis by blocking the 30 S ribosomal subunit (Chamber

and Sande, 1997). Eukaryotic non-histone protected DNA is also affected, resulting i.e. in *E. gracilis* in a permanent elimination of functional chloroplasts and in a loss of photosynthetic activity. Ofloxacin treatment also causes loss of photosynthetic activity in *E. gracilis* as well as in plants (Pyke et al. 1989). However the mode of action differs from aminoglycosides inhibiting the bacterial gyrase activity and in higher concentration the eukaryotic topoisomerase II, required for DNA replication, transcription, repair and recombination (Mandell and Petri 1997).

Within this work five different *E. gracilis* 1224-5/25 bleached mutants were generated: two by treatment with ofloxacin and three with streptomycin. Since just marginal differences were observed by aerobic cultivation, for the analysis of the wax ester content and composition the substrains *E. gracilis* 1224-5/25 Ofl1 and *E. gracilis* 1224-5/25 Strep1 were chosen. Both substrains showed a very high amount of waxes in the lipid fraction up to 60 % corresponding to 7.8 % and 8.9 % of the dry weight for *E. gracilis* 1224-5/25 Ofl1 and *E. gracilis* 1224-5/25 Strep1, respectively. The wax ester concentration was in the bleached cells even higher than in all other green *Euglena* strains with the exception of *E. gracilis* 1224-5/13. In the fatty acid and alcohol profile any significant difference between the both substrains was detected. In agreement with earlier data (Inui et al. 1984), the cells accumulated more shorter and saturated chains with a clear prevalence of C13:0 and C14:0 acids and alcohols. The paucity of unsaturated acids in the wax ester fraction confirms the chloroplasts damage and the loss in enzymatic activity including that of the FAS type II. Siebenlist et al. (1991) described in an *E. gracilis* strain Z streptomycin mutant a cytoplasmic FAS type I complex, similar to the FAS multienzyme of lower fungi, whose expression and repression was directly correlated to the plastidial development. It is, therefore, conceivable that in *E. gracilis* 1224-5/25 Ofl1 and *E. gracilis* 1224-5/25 Strep1 a relevant portion of the fatty acids accumulated in the wax esters were synthesized by the cytoplasmic FAS type I complex.

In contrast to all data reported to date (Inui et al. 1982, 1983, 1984), the bleached mutants were unable to survive the absence of oxygen when they were brought to anaerobiosis. Moreover, the extremly high wax ester content in the bleached cells together with the finding that they are incapable to survive anaerobiosis and, therefore, to perform wax ester fermentation suggests a damage also at mitochondrial level. Injuring effects on mitochondria by inhibitors of the DNA gyrase were already described (Polónyi et al. 1990). However, any mitochondrial toxic effect has been reported for streptomycin and most of the wax ester fermentation experiments carried out to date were performed on streptomycin bleached *E. gracilis* cells (Inui et al. 1982, 1984, 1986).

4.5 TER-PNO Northern blot hybridization

In this work it has been demonstrated that all analyzed strains accumulate wax esters under aerobic conditions and some of them are even able to survive anoxia performing wax ester fermentation. To test if also in these *Euglena* strains the *trans*-2-enoyl-CoA reductase (TER) and the pyruvat:NAD^+ oxidoreductase (PNO) which play an important role in the anaerobic metabolism of *E. gracilis* 1224-5/25 strain Z are present (Rotte et al 2001; Hoffmeister et al. 2005), a Northern blot analysis was performed. The PNO is an oxygen sensitive enzyme expressed in *E. gracilis* strain Z under aerobic and anaerobic conditions. It catalyzes the reduction of pyruvate to acetyl-CoA in the mitochondria under anaerobiosis (Inui et al. 1985; Rotte et al. 2001). On the other hand, TER catalyzes the reduction of crotonoyl-CoA to butyryl-CoA during the mitochondrial wax ester fermentation (Inui et al. 1984, 1985; Hoffmeister et al. 2005).

The results showed an expected signal at about 6000 bp corresponding to the PNO ORF as reported in the literature (Rotte et al. 2001) suggesting that the PNO was expressed in all analyzed strains under aerobic conditions. In contrast the TER was not detectable in all strains. Surprisingly in both bleached mutants no TER expression at RNA level was detectable, indicating a repression of the mitochondrial proteins due to the antibiotics effects. Moreover, *E. gracilis* strains 1224-5/15 and 1224-5/27, although they died in the absence of oxygen, apparently expressed the TER sequence at RNA level.

The expression of TER and PNO at RNA level was then quantified in the strains which survive anaerobiosis and differences were actually observed probably reflecting the diverse aptitude to anoxia and wax ester fermentation. Interestingly, in *E. gracilis* 1224-5/23 the PNO expression was very low, while in *E. gracilis* 1224-5/13 was four fold higher compared to the other strains. Northern blot hybridization using the 1617 bp TER fragment resulted in two bands, one at ~ 1600 bp and the second at ~ 2000 bp. The TER ORF consists of 1912 bp of which 276 bp at the 3'-terminus represent a non-coding region (Hoffmeister 2004). However, three TER species with different chain-length specificities have been reported to coexist in the *Euglena* mitochondria (Inui et al. 1986). Hoffmeister et al. (2005) purified one of these enzymes. Since the highly conserved protein sequence was used in this work as hybridization probe it is possible that the obtained signals corresponded to a different TER hit. The Northern blot analysis was performed with total RNA isolated from *Euglena* cells, therefore the two dectected bands could represent both forms, pre-mRNA and maturated mRNA. On the other hand, the detection of two ORFs with similar nucleotide sequence expressing different TER proteins can not be excluded.

4.6 *E. gracilis* mitochondrial T_2493 thiolase gene: identification and heterologous expression

The condensation of acetyl-CoA and propionyl-CoA units catalyzed by the thiolase/condensing enzyme is one of the most important steps in the mitochondrial wax ester fermentation of *E. gracilis*. The gene encoding for a putative mitochondrial thiolase was identified by a BLASTp search and consisted of 1260 bp. The molecular weight of 44.5 kDa agreed with the literature data for bacterial catabolic thiolases which possess a molecular weight between 40-46 kDa (Kunau et al. 1995). The T_2493 thiolase is possibly organized in dimers or tetramers since this class of enzymes has never been observed as monomers. Most of them are active as dimers like peroxisomal degradative thiolases (Middelton 1973). Homotetramers were found only in the biosynthetic thiolase subfamily as result of dimerization of two dimers (Arakawa et al. 1987).

All thiolases characterized so far possess an N- and C-terminal region carrying the same βαβαβαββ structure (Mathieu et al. 1994). At the C-terminal half are localized most of the catalytic residues whereas, the essential cysteine is found on the N terminus (Modis and Wierenga 1999; Jez and Noel 2000; Olsen et al. 2001). On the T_2493 gene the amino acids Cys98 and Cys405 could be identified (*E. gracilis* numbering) as the ones on which the substrate is bound during the acetyl-transfer reaction and the Claisen condensation (Modis and Wierenga 2000). The formation of two oxyanion holes is a necessary event for the reaction kinetics. The amino acid pair His384-Asn343 forms the first oxyanion hole together with a water molecule as one of the hydrogen bonding donors. The first oxyanion hole contributes to the nucleophylic attack to the carbonyl-carbon atom, while the second oxyanion provided by Cys98-Gly407 is responsible for the activation and stabilization of the substrate (Kursula et al. 2002; Haapalainen et al. 2006).

The presence of these amino acids on the gene sequence suggested that T_2493 belongs to the family of the biosynthetic thiolases which catalyze the condensation of acetyl-CoA and/or propionyl-CoA moietys in the PHA biosynthesis (Yabutani et al. 1995; Modis and Wierenga 2000). However, when the T_2493 was expressed in *E. coli* the protein was accumulated in the insoluble fraction as inclusion bodies and showed no activity. Furthermore, the recombinant protein heterologously expressed in yeast was accumulated in the mitochondrial fraction and was inactive as well (**see 3.7**).

As reported for other eukaryotes (Rotte et al. 2001) the T_2493 sequence showed a transit peptide of 30 amino acids and the presence of two membrane spanning regions suggested an association with the mitochondrial inner membrane.

Recently, the ability of *Trypanosoma brucei* to synthesize fatty acids via elongases and acting on CoA esters rather than ACP intermediates has been demonstrated (Lee et

al. 2007). The presence of two membrane spanning regions on the T_2493 sequence and the deep evolutionary relationship between *Trypanosoma* and *Euglena* (Kivic and Walne 1984; Simpson and Roger 2004) could suggest an involvement of the T_2493 gene in a fatty acid biosynthesis performed by elongase like enzymes. However, this theory has not been supported as the recombinant protein expressed in *E. coli* and yeast was inactive (**s.s. 3.7**).

4.6.1 The thiolase superfamily

Thiolases catalyze the carbon-carbon bond formation in many important pathways such as fatty acid, steroid and polyketide synthesis. Although thiolases share significant sequence similarity (Igual et al. 1992) and the same reaction mechanism based on acetylated cysteine as covalent intermediate (Modis and Wirenga 2000), they can be functionally divided in three subfamilies. Two subfamilies include the KAS leading to the synthesis of fatty acids (Smith et al. 2003) and the PKS synthase (Austin and Noel 2003). The third subfamily consists of thiolases which perform biosynthetical and degradative reactions. The enzymes belonging to this last subfamily act on CoA esters instead of ACP esters and work mostly in synthetic pathways with the exception of β-oxidation thiolases (Haapalainen et al. 2006). Among the subfamilies thiolases are divided in acyl-CoA:acetyl-CoA acyl-transferase (thiolase I, EC 2.3.1.16) and acetyl-CoA:acetyl-CoA acetyl-transferase (thiolase II, EC 2.3.1.9).

The thiolase protein family has been well biochemically characterized and seems to share a common ancestor (Arakawa et al. 1987). In the cell compartments of eukaryotes different thiolases can be detected. Animal mitochondria possess thiolase I and II, while peroxisomes show only the thiolase I. In contrast in peroxisomes and glyoxysomes of plants and yeast only thiolase II has been found. After a phylogenetic analysis Pereto et al. (2005) could demonstrate that all thiolases show an ancient origin in a proteobacterial ancestor. The animal cytosolic thiolase II showed its closest relative sequences among □- and β-proteobacterial genomes, while the mitochondrial and peroxisomal thiolase I share high similarity with sequences of δ-proteobacteria. Although, any relationship could be found between the ancestor sequence, enzymatic function and cell localization (Pereto et al. 2005).

It could not be excluded that the T_2493 gene encodes for a thiolase involved in the β-oxidation pathway. In *E. gracilis* this multifunctional protein constitutes of four non-identical subunits organized in a complex by non-covalent interactions (Winkler et al. 2003). But since neither condensing nor thiolytic activity could be detected it has been impossible to define the metabolic function of the T_2493 protein sequence.

4.7 The propionyl-CoA (PCC) carboxylase of *E. gracilis* strain Z

The analysis of the wax ester fraction isolated from *Euglena* cells demonstrated that the biosynthesis of odd-number chain fatty acids and alcohols was particular evident under anaerobic conditions. The propionyl-CoA used as starter unit for the odd-number chains is synthesized by decarboxylation of methylmalonyl-CoA and the reaction is catalyzed by methylmalonyl-CoA decarboxylase (MMD)/propionyl-CoA carboxylase (PCC)(Schöttler 1977). The PCC involved in the wax ester fermentation of *E. gracilis* was identified by EST analysis (Ahmadinejad et al. 2007) but it is still uncharacterized. Whereas it has already been demonstrated that proteins involved in the anaerobic fermentation are also expressed in aerobically grown cells (Hoffmeister et al. 2004), a Northern blot analysis was performed. The bands at ~ 3000 bp obtained after hybridization in both samples under aerobic and anaerobic conditions corresponded to the ORF length reported in the literature for PCC/MMD (Lamhonwah et al. 1986; Huder and Dimroth 1993). Furthermore, the PCC/MMD expression at RNA level was in absence of oxygen marginally higher, about 15 %, than under aerobic conditions suggesting an upregulation also at protein level due to the fermentation and ATP production exigency.

The decarboxylation of methylmalonyl-CoA to propionyl-CoA leading to ATP production within fermentative processes is a very important reaction for strictly anaerobic bacteria. In fact, some rumen bacteria (Hilpert and Dimroth 1982) and some anaerobic gram-negative bacteria, isolated from anoxic marine and freshwater mud samples (Denger and Schink 1990) use the decarboxylation of methylmalonyl-CoA to propionyl-CoA as the only energy-conserving step in lactate or succinate fermentation (Schink and Pfennig 1982b; Hilpert and Dimroth 1983). During this process the anaerobic bacteria convert decarboxylation energy into an Na^+ ion gradient across the membrane generating a motive force used for ATP synthesis (Hilpert and Dimroth 1982).

The methylmalonyl-CoA decarboxylase (MMD; EC 4.1.1.41) is a biotin dependent enzyme organized in four non-identical subunits of which the α-chain carries the carboxyltransferase. The β-chain represents the carboxybiotin carrier protein decarboxylase, the γ-chain expresses the biotin carrier protein and the δ-chain is presumably essential for the assembly of the complex (Bott et al. 1997).

In contrast, the eukaryotic PCC (EC 6.4.1.3) is a mitochondrial enzyme which plays a key role in the catabolism of branched-chain amino acids and β-oxidation of odd-number chain fatty acids (Lamhonwah et al. 1986). The enzyme catalyzes the carboxylation of propionyl-CoA to methylmalonyl-CoA and consists of two non-identical subunits. One is the α-chain which shares the same role as in the MMD, carrying the carboxyltransferase covalently bound with the biotin prosthetic group and the second is the β-chain. In

humans, deficiency in PCC results in metabolic disorders leading to death (Jiang et al. 2005). Both enzymes share a high similarity, especially at the α-subunit level and together belong to the biotin dependent carboxylase family which includes also oxaloacetate decarboxylase (EC 4.1.1.3) acetyl-CoA carboxylase (ACC; EC 6.4.1.2), and pyruvate carboxylase (PC; EC 6.4.1.1) (Wolf and Feldman 1982; Huder and Dimroth 1995).

A Network analysis with the *Euglena* contig sequence obtained by several *E. gracilis* EST clones shows the wide distribution of the PPC/MMD proteins among genomes (**Fig. 4.1**). A difference can be observed for homologous proteins isolated from eukaryotes and proteobacteria. All proteobacterial sequences cluster in one group. Interestingly, the contig sequence of *E. gracilis* splits together with protein sequences isolated in eukaryotes usually living under aerobic conditions but which have to tolerate in some phases of their life short periods of anoxia. The nematode *Caenorhabditis elegans* stops its development when environmental conditions do not allow reproduction. In this “dauer larvae” stage *C. elegans* can survive anoxia performing malate dismutation and excreting acetate, propionate and succinate from its anaerobic functionally mitochondria (Burnell et al. 2005). Even the sea urchin *Strongylocentrotus droebachiensis*, although an aerobic animal, relies during the reproductive cycle exclusively on anaerobic metabolism in its internal organs (Bookbinder and Shick 1986).

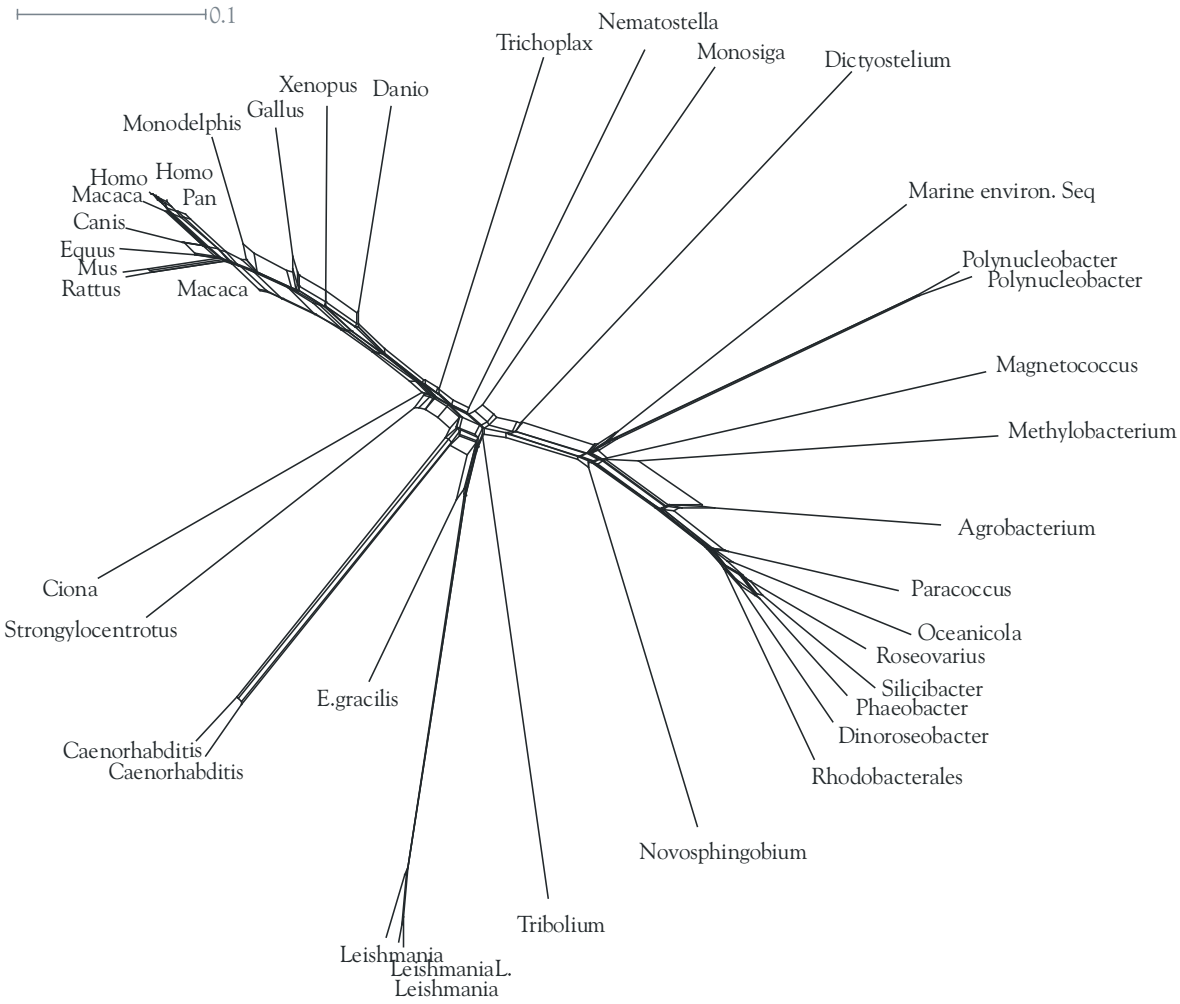

Fig. 4.1: NeighborNet graph of the PCC/MMD homologous sequences. The scale bar at the upper left side indicates estimated substitutions per site. A*grobacterium tumefaciens* str.C58, NP_357031; *Caenorhabditis elegans*, NP_509293; *C. briggsae* AF16, XP_001677115; *Canis familiaris*, XP_534175.2; *Ciona intestinalis*, XP_002131306; *Danio rerio*, CAQ15408; *Dictostelium discoideum* AX4, XP_643676; *Dinoroseobacter shibae* DFL12, YP_001532069; *Euglena gracilis* contig sequence; *Equus caballus*, XP_001092670; *Gallus gallus*, XP_416970.2; *Homo sapiens*, BAG59350; *H. sapiens*, NP_000273.2; *Leishmania braziliensis* MHOM/BR/75/M2904, XP_001561449; *L. infantum*, XP_001462618; *L. major* strain Friedlin, XP_001687397; *Macaca mulatta*, XP_001493941.2; *M. fascicularis*, BAE88139; *Magnetococcus* sp.MC-1, YP_864317; marine gamma proteobacterium HTCC2207, ZP_01225350; *Methylobacterium nodulans* ORS 2060, ZP_02123879; *Monodelphis domestica*, XP_001376722; *Monosiga brevicollis*, XP_001743619; *Mus musculus*, NP_689093; *Nematostella vectensis*, XP_001627485; *Novosphingobium aromaticivorans* DSM 12444, YP_496094; *Oceanicola granulosus* HTCC2516, ZP_01155059; *Phaeobacter gallaeciensis*, ZP_02148282; *Pan troglodytes*, XP_001148157; *P. troglodytes*, EAX09033; *Paracoccus denitrificans* PD1222, XP_860279; *Polynucleobacter* sp. QLW-P1DMWA-1, YP_001155693; *P. necessarius* STIR1, YP_001797741; *Rattus norvegicus*, EDM02587; *Roseovarius* sp TM103, ZP_01879190; *Rhodobacterales bacterium* HTCC2150, ZP_01743293; *Silicibacter sp.* TM1040, YP_613864; *Strongylocentrotus purpuratus* XP_797409.2; *Triboleum castaneum*, XP_974679; *Trichlopax adhaerens*, XP_002108817; *Xenopuis laevis*, NP_001089298.

4.8 Prokaryotic *trans*-2-enoyl-CoA reductase (TER)

4.8.1 Treponema denticola TdenTER

T. denticola is a spirochete that is commonly found in the human oral cavity and is responsible for the pathogenesis of human periodontal diseases and acute necrotizing ulcerative gingivitis (Loesche 1988; Loesche and Grossman 2001). This results in the inflammatory response of the host to the presence of subgingival plaque bacteria (Sokransky and Haffajee 1992). Whereas *T. denticola* is closely related to the syphilis-causing obligate human pathogen *Treponema pallidum* sub. *pallidum,* their genomes show distinct metabolic differences due to the greater biosynthetic capabilities of *T. denticola*. The *T. denticola* genome encodes 2786 proteins, of which 352 are currently annotated as unidentified open reading frames (Seshadri et al. 2004). One of these proteins belongs to a family of enzymes widely distributed among prokaryotic genomes, but without functionally characterized prokaryotic homologues. The *T. denticola* open reading frame TDE0597, annotated, like all other prokaryotic members of the family with unknown function, shows sequence similarity to the recent characterized *trans*-2-enoyl-CoA reductase (TER, EC 1.3.1.44) from *E. gracilis* (Hoffmeister et al. 2005). Members of this protein family in prokaryotes are often organized in gene clusters that suggest involvement in fatty acid or related synthesis operating with CoA esters instead of acyl-carrier-protein (ACP)-esters (Hoffmeister et al. 2005).

4.8.1.1 Expression and catalytic properties of TdenTER

Since the function of prokaryotic TER has not been investigated so far, the *T. denticola* TER was heterologous expressed in *E. coli*. The 1194-bp fragment from the gene sequence TDE0597, hitherto annotated as a hypothetical reading frame, was cloned into the expression vector pET28a and transformed into *E. coli* BL21(DE3). The presence of N-terminal His-Tag fusion proteins allowed a complete purification by Ni-NTA affinity chromatography of the recombinant protein expressed in the soluble fraction. As already reported for the *E. gracilis* TER the presence of a cofactor is essential for the enzyme activity (Hoffmeister et al. 2005). However, many differences occurred between the TER homologues. In contrast to the *E. gracilis* TER, which accepts both cofactors NADH and NADPH, the TdenTER showed an exclusive NADH-dependent activity. Additionally, the specific activity with a value of 43.4 ± 4.8 U/mg (μmol mg^{-1} min^{-1}) using crotonoyl-CoA as substrate was about ten-fold higher than *E. gracilis* TER activity (3.9 U/mg) and in contrast did not accept *trans*-hexenoyl-CoA as substrate. The TdenTER K*m* value of 2.7 μM for crotonoyl-CoA as substrate indicates a much higher affinity for the substrate than the *E. gracilis* TER (68 μM). The lower substrate inhibition with K*i* value of 0,198 μM for crotonoyl-CoA using NADH as cofactor, not reported for the *E. gracilis* TER (Hoffmeister

et al. 2005), suggested a regulation mechanism similar to that described for the *fabI* gene which encodes for enoyl-ACP reductase in the bacterial FAS type II (Heath and Rock 1996a). The acyl-ACP chains synthesized by *fabI* inhibits the *fabI* itself and the potency of inhibition increases with the acyl-ACP chain-lengths (Heath and Rock 1996b).

Enoyl-CoA/ACP reductases in *E. coli* and other organisms are an ideal target for antimicrobial compounds (Hoang and Schweizer 1999). Triclosan, in particular, is a very effective antibacterial agent due to its capability to inactivate the enzyme by a slow binding formation of a fabI-NAD^+-triclosan ternary complex (Heath et al. 1999; Heath et al. 2002). However, essential amino acids such as Leu100, Tyr146, Tyr156, Met159, Ala196, Ala197 (fabI, *E. coli* numbering) required for the formation of the ternary complex (Steart et al. 1999) were missing on the gene sequence of TdenTER and, as expected, the addition of Triclosan to the assay mixture had no effect on the enzyme activity (Tucci and Martin 2007). A decrease of 5 fold TdenTER activity was measured only with 1000 μM Triclosan, but the loss in activity was the result of ethanol present in the mixture required to solubilize the drug. The TdenTER activity was also tested with Isoniazid, a clinically used antituberculosis drug, but without any inhibiting effect, as well.

Multiple alignment of the TdenTER amino acid sequence (**Fig. 3.24**) showed homologous sequences among prokaryotic genomes with more than 50 % amino acid identities and an interesting similarity to the short-chain dehydrogenase/reductase (SDR) enzyme family (Jörnvall et al. 1995). Whereas highly divergent, enzymes which belong to this protein family show characteristic motifs for nucleotide binding and specific residues for the active site (Jörnvall et al. 1995; Persson et al. 1995; Kallberg et al. 2002). The TdenTER displayed the motif $G^{53}XXXGXXG^{59}$ typical for NAD(P)H binding Rossmann fold (G*XXX*G*XX*G) in the N-terminal part of the consensus sequence (Wierenga et al. 1985; Das et al. 2000). Similarly the highly conserved triad of serine, tyrosine and lysine with the typical motif YX_6K usually present in the catalytic centre and responsible for the enzyme activity was also identified on the TdenTER sequence at residues 241-248. As reported for the *E. gracilis* TER (Hoffmeister et al. 2005), a second hypothetical substrate binding domain could be identified on the TdenTER at residues 290-296 with the motif YX_5K (Das et al. 2000). As shown in **Fig. 3.24** in contrast to the *E. gracilis* TER and to the protein sequence of *Cytophaga hutchinsonii* (YP677688) a putative FAD binding site with the motif G*X*G*XX*G localized at the C terminusl of the amino acid sequence (Chang and Hammes 1989), was missing in TdenTER. Accordingly, addition of FAD in the enzyme assay (0-10 μM) had no effects on the enzyme activity (Tucci and Martin 2007).

4.8.1.2 Metabolism of *T. denticola*

T. denticola is a facultative anaerobe, growing in partially aerobic or anaerobic environments (Syed et al. 1993). *T. denticola* possesses a genome considerably larger in size than the genomes of other spirochetes, possibly reflecting niche adaptation in the oral biofilm environment (Seshadri et al. 2004, Kuramitzu et al. 2005).

In contrast to other spirochetes in which their reduced genomes do not allow great biosynthetic abilities, the genome analysis of *T. denticola* revealed the existence of genes encoding enzymes for important metabolic and biosynthetic pathways. Genes involved in pathways for *de novo* biosynthesis of cofactors, nucleotides and fatty acids are apparent on the genome, while, i.e., *T. pallidum* and *Borrelia burgdorferi* are completely dependent on the fatty acids present as substrates in the culture medium (Seshadri et al. 2004, Fraser et al. 1997; Fraser et al. 1998). *T. denticola* is able to perform glycolysis and gluconeogenesis, and possesses, additionally, a pentose phosphate pathway. However, the absence of tricarboxylic acid cycle (TCA) and an electron transport chain (ETC) suggests that ATP is generated mainly through sugar fermentation and substrate level phosphorylation (Seshadri et al. 2004). Because of their anaerobic lifestyle spirochetes are known to synthesize short chain fatty acids (SCFA) as result of fermentative processes (Miller et al. 1991). *T. hyodysenteriae*, *T. innocens* and *T. vincentii* produce acetate and butyrate as terminal electron acceptors and end products of the pyruvate fermentation which is directly associated with ATP production. In the butyrate pathway, the reduction from crotonoyl-CoA to butyryl-CoA is catalyzed by an enoyl-reductase (EC1.3.1.44)(Miller and Wolin 1996; Boynton et al. 1996; Bennet and Rudolph 1995), that is, however clearly distinct from TdenTER.

Unlike other spirochetes *T. denticola* ferments preferentially the amino acids cysteine, serine, alanine, and glycine to acetate as major compound with smaller amounts of lactate, succinate, formate and pyruvate. Glucose fermentation has also been reported for *T. denticola* cells, but far less efficient than amino acid fermentation, and butyrate is present only in trace amounts (Miller and Wolin 1996; Hespel and Canale–Parola 1971).

4.8.1.3 TdenTER: a novel fatty acid biosynthesis route?

The phylogenetic analysis of TER homologues showed a prevalence among □- and □-proteobacteria while archaebacterial homologues were missing (**Fig. 3.23**). Many □- and □-proteobacteria such as *Burkholderia mallei* ATCC 23344, *Burkholderia pseudomallei* K96243, *Colwellia psychrerythraea* 34H, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas putida* KT2440 which possess the TER homologous sequence perform polyhydroxyalcanoate (PHA) biosynthesis. PHAs are a class of biodegradable polyesters produced mostly under oxygen limitation as carbon and energy reserve (Anderson and

Dawes 1990; Nierman et al. 2004; Methe et al. 2005). The most well-studied type of PHA is poly-hydroxybutyrate (PHB), synthesized from acetyl-CoA by a sequence of three reactions catalyzed by β-ketothiolase (EC 2.3.1.9), acetoacetyl-CoA reductase (EC1.1.1.36) and poly(β-hydroxybutyrate) synthase (no EC number)(Haywood et al 1989; Anderson and Daves 1990). However, in this pathway the presence of TdenTER is not necessary since only the product of the first reduction step, β–hydroxybutyryl-CoA, is required for the synthesis of PHB.

Treponema, in general, has not been reported to synthesize PHAs and lacks the fatty acid biosynthesis pathway. In contrast, *T. denticola* showed on its genome genes encoding proteins involved in the fatty acid biosynthesis (Seshadri et al. 2004). The position of TdenTER on the downstream of fatty acid biosynthesis as shown in **Fig. 4.2** could indicate an involvement of this enzyme in a novel fatty acid synthesis pathway possibly similar to PHA biosynthesis. The activity of TdenTER operating with CoA esters also suggested a possibly malonyl-CoA independent pathway circumventing ATP consumption at the malonyl-CoA activation step catalyzed by the acetyl-CoA carboxylase (Ohlrogge and Browse 1995). Based upon their distribution among various gene clusters (**Fig. 4.2**), the family of prokaryotic TER enzymes would appear to belong to the more versatile and flexible components of metabolism, rather than to dedicated core pathways of energy metabolism in these organisms, in contrast to the situation in *E. gracilis* (Tucci and Martin 2007).

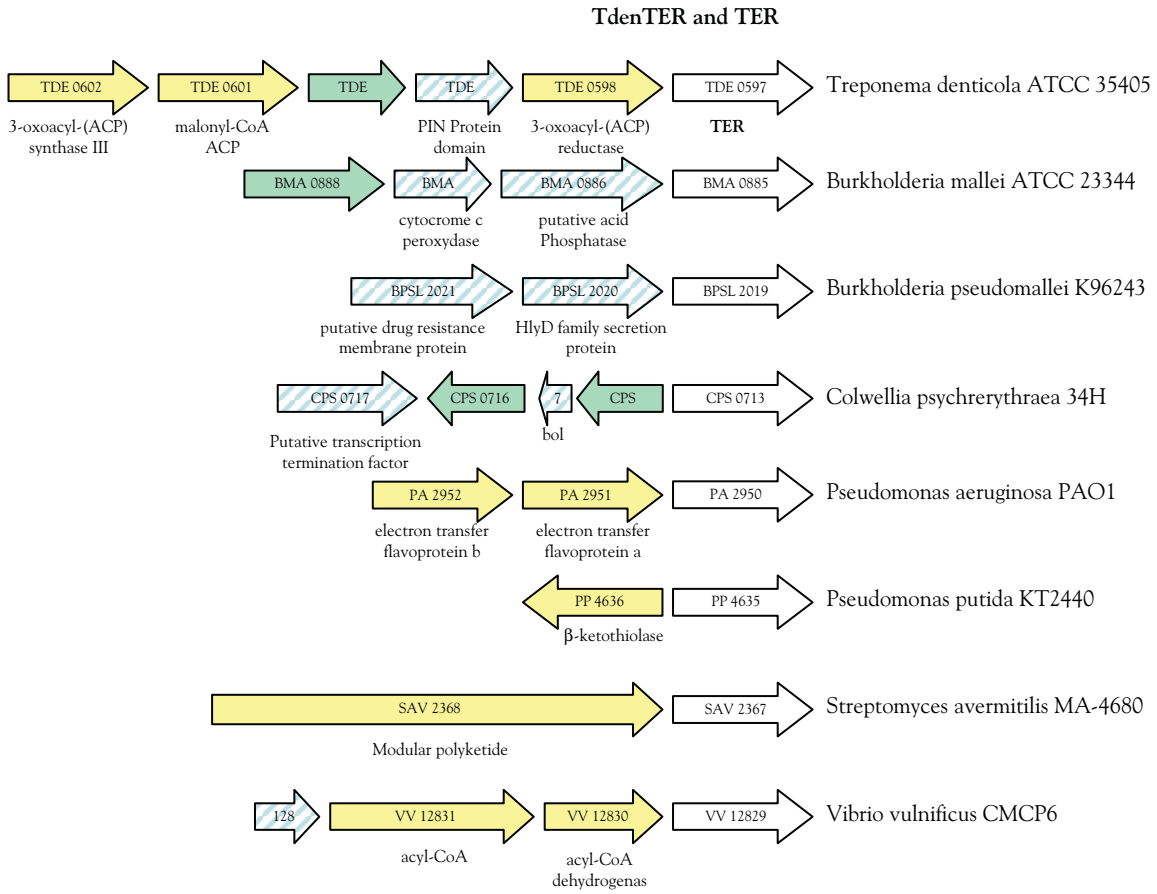

Fig. 4.2: Operon analysis of TdenTER and TER homologues through the CMR resource at TIGR. White arrows show TER homologues of TdenTER, grey arrows indicate hypothetical reading frames and hatched arrows proteins with distinct functions.

4.8.2 Expression and catalytic properties of SaveTER

The SAV2367 gene of *Streptomyces avermitilis* is the first sequence that shares a high similarity among enoyl-reductase of polyketide synthase (PKS) modules (Hoffmeister et al. 2005). To determine the enoyl-reductase activity of SAV2367 the protein was heterologously expressed and purified to further biochemical characterization. After overexpression in *E. coli*, the recombinant SaveTER was accumulated in the pellet fraction as insoluble inclusion bodies and each test to get a soluble and active protein failed (**s.s. 3.10**). Additionally, in yeast the recombinant SaveTER was expressed in the mitochondrial fraction, but in this case the purified protein was completely degraded (**s.s. 3.10**).

The gene SAV2367 encoding for the SaveTER is localized on the modular polyketide synthase complex *pks5,* which is one of the smaller PKS clusters present on the *S. avermitilis* genome. This Gram positive bacterium possesses other seven PKS clusters and they all contain PKS type I genes. Three of these clusters, which represent more than 80 % of the PKS modules of the *S. avermitilis* genome, are well studied and are involved

in the biosynthesis of oligomycin, avermectin and polyene macrolide (Ikeda et al. 1994; Omura et al. 2001). Within the remaining clusters only the *pks5* cluster showes high amino acid similarity with those three largest (Omura et al. 2001; Jenke-Kodama et al. 2006). Despite the high similarity with the *E. gracilis* TER and the position on the downstream of the *pks5* cluster (**Fig**. **4.2**), the SaveTER showed no activity with crotonoyl-CoA or hexenoyl-CoA as substrates.

Modular PKS type I shows biochemical steps to chain extension and correspondingly enzymatic activities closely related to those involved in fatty acid biosynthesis (Hopwood and Sherman 1990). The active sites of PKS type I are organized linearly into modules in which different catalytic domains are present. Each module provides an elongation cycle and contains KAS, acyltransferase (AT), ACP protein and optionally KR, DH and ER domains. The combination onto the modules of the single domains, which are used only once during the assembly of the products, reflects the structures of the generated polyketides. Due to the complex molecular organization it is moreover conceivable a specific enoyl-reductase activity on different substrate than crotonoyl-CoA and hexenoyl-CoA, as reported for other PKS genes (Shen 2000; Moore and Hertweck 2002).
On the other hand, *S. avermitilis* displays the largest number of PKSs clusters on the genome of all other bacteria sequenced yet. Hence, a loss in domain functionality due to recombination events could not be excluded (Jenke-Kodama et al. 2006).

4.9 Outlook

In this work the direct correlation between anaerobiosis and mitochondrial biosynthesis of odd-number fatty acids and fatty alcohols during the wax ester fermentation in *Euglena gracilis* 1224-5/25 strain Z has been demonstrated. However, further analyses are necessary. The expression conditions for the putative thiolase could be improved and the condensing activity should be tested with more sensitive assays using radioactively labelled substrates.

Furthemore, the hetrologous expression and following characterization of the methylmalonyl-CoA decarboxylase could explain which role exactly this enzyme plays during anaerobic metabolism in *E. gracilis* cells.

5. Summary

The mitochondrion of the flagellate *Euglena gracilis* is a facultatively anaerobic organelle that is able to produce ATP in the presence and in the absence of oxygen. The shift of *Euglena* cells from aerobic to anaerobic conditions induces a mitochondrial wax ester fermentation coupled to a net synthesis of ATP. The mitochondrial fatty acid synthesis system uses acetyl-CoA and propionyl-CoA as primer and as C2- and C3-donor, respectively. A portion of fatty acids is reduced to alcohols, esterified with another fatty acid and deposited in the cytosol as waxes.

E. gracilis strain Z cultures were grown under different conditions. The wax esters were isolated, transesterified and analysed by gaschromatography (GC) as fatty acid methyl esters and fatty alcohols. In this work it has been demonstrated that oxygen play a key role in the accumulation of wax esters which are mostly synthesized under anaerobic conditions. The synthesis of odd-number chain fatty acids and alcohols during anoxia and their lack by addition of the herbicide flufenacet, which inhibits the elongation but not the synthesis of fatty acids, confirm the involvement of the methylmalonyl-CoA pathway. Bleached mutants were generated by treatment with ofloxacin and streptomycin and their wax ester fraction was isolated and analyzed. In contrast to the data reported in the literature to date, the bleached mutants did not survive anaerobiosis.

Growth curves of eleven different *E. gracilis* strains demonstrated that in absence of oxygen not all *Euglena* strain were able to survive anoxia and to perform wax ester fermentation. Their wax esters were isolated and analyzed. Northern blot analysis with RNA samples of the different *Euglena* strains showed that pyruvate:NAPD$^+$ oxidoreductase homologues were present in all analyzed strains, while the *trans*-2-enoyl-CoA reductase was apparently not expressed in all cultures.

The amino acid sequence of a putative mitochondrial thiolase/condensing enzyme of *E. gracilis* strain Z was identified and the protein was heterologously expressed in *Saccharomyces cerevisiae* and *Escherichia coli*. However, the recombinant protein was not active and it could not be characterized.

The *trans*-2-enoyl-CoA reductase of *E. gracilis* strain Z which catalyzes the reduction of crotonoyl-CoA and hexenoyl-CoA during the mitochondrial fatty acid synthesis shares sequence similarity only with prokaryotic homologues. Two of these sequences, TDE0597 from *Treponema denticola* and SAV2368 from *Streptomyces avermitilis,* were chosen for further characterization. SAV2368 was heterologously expressed in *E. coli* and *S. cerevisiae*. However, the recombinant protein was not active and it could not be

characterized. The TDE0597 was successfully expressed in *E. coli.* The protein was purified by affinity chromatography and biochemically characterized.

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