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DISSERTATION

Functional expression of the *C. elegans* fat-1 gene in wild-type yeast

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Abbreviations:

AA	arachidonic acid
ADH	alcohol dehydrogenase I
ALA	alpha-linolenic acid
Amp	ampicillin
ARS	autonomous replication sequence
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
CEN	centromeric
CIP	calf intestinal alkaline phosphatase
COX	cyclooxygenase
CYC1	cytochrome-c oxidase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EPA	eicosapentaenoic acid
FAD2	fatty acid desaturase 2
FAD3	fatty acid desaturase 3
fat-1 / FAT-1	fatty acid metabolism 1
fat-1t / FAT-1t	truncated fat-1 / FAT-1
GAL1	galactokinase
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GLA	gamma-linolenic acid
GPD	glyceraldehyde-3-phos-phate dehydrogenase
GRAS	generally regarded as safe
KanMX4	kanamycin-resistance module
kb	kilo bases
LA	linoleic acid
LB	Lysogeny broth
MCS	multiple cloning site
MGH	Massachusetts General Hospital
n-3	omega-3
n-6	omega-6
ori	origin of replication
PCR	polymerase chain reaction
PEG	polyethylene glycol
PUFA	polyunsaturated fatty acid(s)
rpm	rounds per minute
S. boulardii	*Saccharomyces boulardii*
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
S.O.C.	super-optimal catabolite repression

TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)-aminomethane
U	unit(s)
URA3	orotidine 5-phosphate decarboxylase
Vol	volume
w/v	weight per volume
YCp	yeast centromeric plasmid(s)
YEp	yeast episomal plasmid(s)
YIp	yeast integrating plasmid(s)
YM	yeast mold
YPD	yeast peptone dextrose

1 INTRODUCTION

1.1 Polyunsaturated fatty acids

Fat is an important dietary source of energy in human beings. Dietary fat comprises saturated and unsaturated fatty acids. Unsaturated fatty acids can be further differentiated into monounsaturated and polyunsaturated fatty acids (PUFA).
Polyunsaturated fatty acids are important structural components of membrane lipids and serve as precursors of families of signaling molecules[1].
PUFA can be further divided into two classes, which differ in the position of their first double bond from the methyl end – the omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids.

1.1.1 Metabolism and biological effects of polyunsaturated fatty acids

Of all fatty acids, there are two – linoleic acid (LA, 18:2 n-6) and alpha-linolenic acid (ALA, 18:3 n-3) – that are essential for humans since they cannot be synthesized by the human body but need to be supplied by nutrition[2]. These essential fatty acids can be converted to long-chain PUFA in the liver, retaining the omega type (n-3 or n-6) of the parent essential fatty acid.

1.1.2 Metabolic pathways of omega-3 and omega-6 fatty acids

Omega-3 and omega-6 fatty acids go through the same oxidation and metabolization pathways (Figure 1)[3]. Once ingested, ALA and LA can be elongated and desaturated into long-chain PUFA[4]. LA is converted into gamma-linolenic acid (GLA, 18:3 n-6) and GLA, in turn, can be converted to arachidonic acid (AA, 20:4 n-6). ALA, the precursor of omega-3 fatty acids, can be converted to the long-chain omega-3 PUFA eicosapentaenoic acid (EPA, 20:5 n-3) and subsequently docosahexaenoic acid (DHA, 22:6 n-3)[5]. However, the conversion from parent fatty acids into long-chain polyunsaturated fatty acids occurs only slowly in humans[3] and there is competition among the enzymes involved in the process of elongation and desaturation of LA and ALA[6]. This is why humans have to take up AA, EPA, and DHA with their diet.

Both EPA and AA are 20-carbon fatty acids and are precursors for the formation of eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes. Eicosanoids derived from omega-6 fatty acids often have metabolic properties opposite to those derived from omega-3 fatty acids[6]. Dietary amounts of LA as well as the ratio of LA to ALA appear to be important for the metabolism of ALA to long-chain omega-3 PUFA[7]. A balanced intake of both, omega-6 and omega-3 fatty acids, is probably important for good health[7, 8].

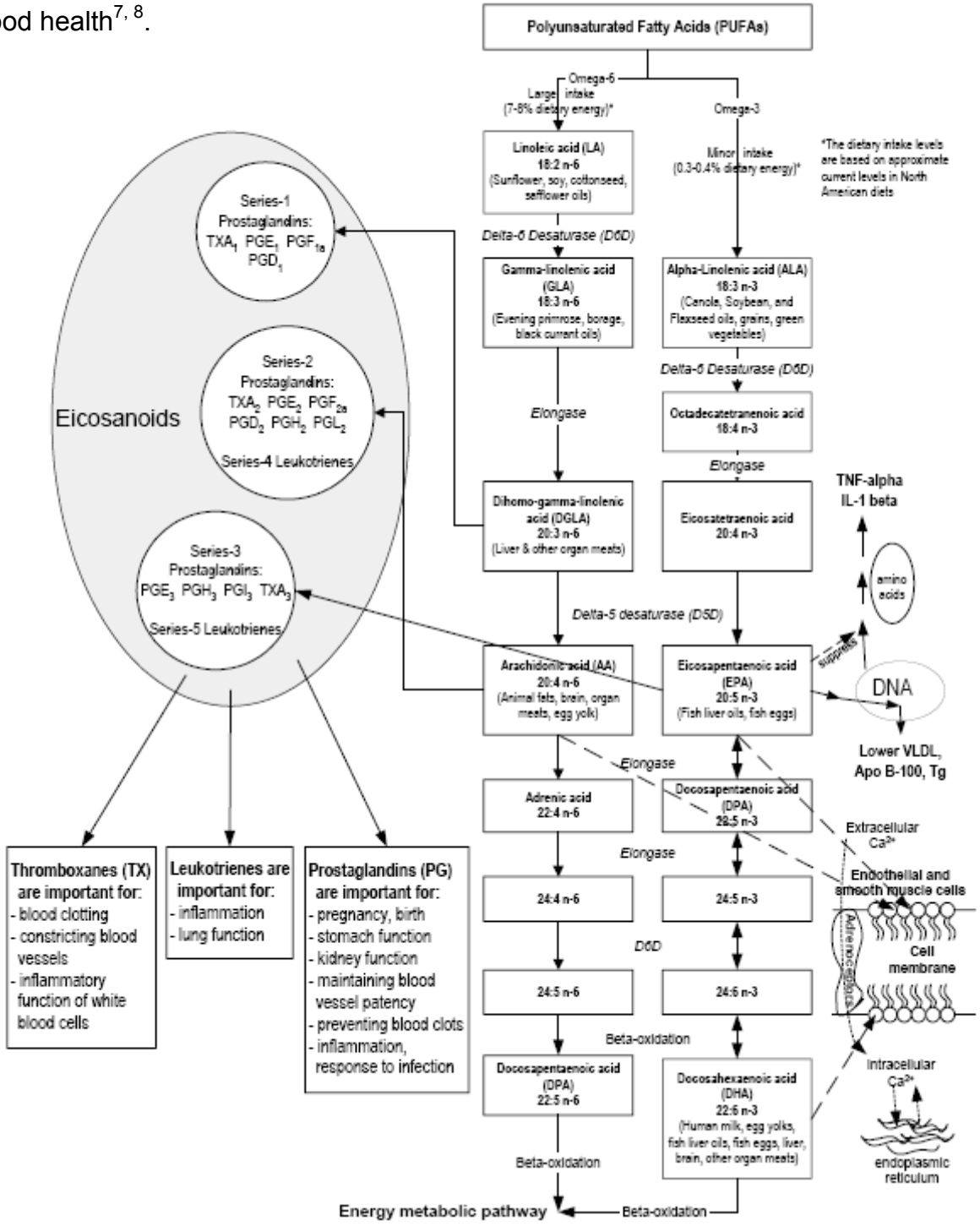

Figure 1: Classical omega-3 and omega-6 fatty acid synthesis pathways and the role of omega-3 fatty acid in regulating health/disease markers[3].

1.1.3 Health effects of omega-3 and omega-6 fatty acids

The role of omega-6 PUFA, especially of arachidonic acid, has been investigated in detail during the last decades[9, 10]. The cyclooxygenase (COX) enzymes catalyze the first step in the conversion of AA into prostaglandins and thromboxanes. There are at least two different COX isoforms: The expression of COX-1 is constitutive in most cell types, whereas COX-2 is expressed under physiological conditions only in some tissues but is particularly induced in response to acute stress such as that caused by e.g. inflammation[11]. In turn, the resulting high levels of substances such as prostaglandin E_2, $F_{2\alpha}$, and I_2 can contribute to tumor, increased perfusion, pain, and fever – typical signs of an inflammatory process.
The leukotrienes are another series of lipid mediators formed by lipoxygenase from arachidonic acid. These are highly potent proinflammatory mediators that cause bronchial constriction, mucus production, and submucosal oedema[11].

Omega-3 fatty acids and their role in inflammation were more precisely characterized in recent years. These substances can act as substrates for the production of signaling molecules or functioning mediators as well as modulators in the regulation of gene expression[8] and are associated with an anti-inflammatory effect. Besides the inhibiting impact on the metabolism of omega-6 PUFA to proinflammatory mediators[11], lipid mediators generated from omega-3 PUFA such as prostaglandin E3, resolvins, and protectins are of anti-inflammatory and antiproliferative significance. Resolvins and protectins stop further recruitment of leukocytes in the event of inflammation hence dampening the inflammatory process and promoting resolution[12].

Many human studies have shown that the lack of omega-3 PUFA correlates with an increased risk of numerous major diseases and that omega-3 PUFA supplementation exhibits cardioprotective, anti-inflammatory, anticancer, and neuroprotective effects[8]. Beneficial effects of omega-3 PUFA have been shown to be involved in the secondary prevention of coronary heart disease, hypertension, type 2 diabetes, and in some patients with renal disease, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and chronic obstructive pulmonary disease[7].

1.2 Situation in Western countries

Dietary habits of Western societies underwent a major change over the past 100 to 150 years[7]. Human beings evolved consuming a diet that contained small and about equal amounts of omega-6 and omega-3 PUFA (ratio of 1 to 2:1). The introduction of agriculture with its dependence on grains led to an increase in total saturated fatty acids and in omega-6 PUFA. In addition, the emergence of agribusiness with processed foods, grain fattened livestock, and hydrogenation of vegetable fats have all further reduced the content of omega-3 fatty acids[7].
As a result, modern Western diets are deficient in omega-3 fatty acids but have too much omega-6 fatty acids[8], with a current estimated ratio of omega-6 to omega-3 fatty acids of 10 to 25:1[6] instead of 1 to 2:1[7].

On account of the increased intake of omega-6 fatty acids in the Western diet, eicosanoid products from AA, specifically prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids, and lipoxins, are formed in larger quantities than those formed from omega-3 fatty acids, particularly EPA[6]. The dietary habits adopted by Western societies over the past 100 to 150 years, especially the shift in omega-6 to omega-3 fatty acid ratio with a deficiency of omega-3 fatty acids, could thus make an important etiologic contribution to coronary heart disease, hypertension, diabetes, and some types of cancer[7, 13].

1.2.1 Dietary sources of omega-3 fatty acids

Unlike other fatty acids, which are widely available in foods, the major source of omega-3 fatty acids is fatty fish, certain vegetable oils, and nuts[8]. Table 1[3] lists the amount of omega-3 fatty acids in some commonly consumed fish and shellfish species, as well as nuts and edible oils, that contain at least 5 percent omega-3 fatty acids.

Food item	EPA	DHA	ALA	Food item	EPA	DHA	ALA
Fish (Raw[a])				**Fish, continued**			
Anchovy, European	0.6	0.9	-	Tuna, Fresh, Yellowfin	trace	0.2	trace
Bass, Freshwater, Mixed Sp.	0.2	0.4	0.1	Tuna, Light, Canned in Oil[e]	trace	0.1	trace
Bass, Striped	0.2	0.6	trace	Tuna, Light, Canned in Water[e]	trace	0.2	trace
Bluefish	0.2	0.5	-	Tuna, White, Canned in Oil[e]	trace	0.2	0.2
Carp	0.2	0.1	0.3	Tuna, White, Canned in Water[e]	0.2	0.6	trace
Catfish, Channel	trace	0.2	0.1	Whitefish, Mixed Sp.	0.3	0.9	0.2
Cod, Atlantic	trace	0.1	trace	Whitefish, Mixed Sp., Smoked	trace	0.2	-
Cod, Pacific	trace	0.1	trace	Wolffish, Atlantic	0.4	0.3	trace
Eel, Mixed Sp.	trace	trace	0.4				
Flounder & Sole Sp.	trace	0.1	trace				
Grouper, Mixed Sp.	trace	0.2	trace	**Shellfish (Raw)**			
Haddock	trace	0.1	trace	Abalone, Mixed Sp.	trace	-	-
Halibut, Atlantic and Pacific	trace	0.3	trace	Clam, Mixed Sp.	trace	trace	trace
Halibut, Greenland	0.5	0.4	trace	Crab, Blue	0.2	0.2	-
Herring, Atlantic	0.7	0.9	0.1	Crayfish, Mixed Sp., Farmed	trace	0.1	trace
Herring, Pacific	1.0	0.7	trace	Lobster, Northern	-	-	-
Mackerel, Atlantic	0.9	1.4	0.2	Mussel, Blue	0.2	0.3	trace
Mackerel, Pacific and Jack	0.6	0.9	trace	Oyster, Eastern, Farmed	0.2	0.2	trace
Mullet, Striped	0.2	0.1	trace	Oyster, Eastern, Wild	0.3	0.3	trace
Ocean Perch, Atlantic	trace	0.2	trace	Oyster, Pacific	0.4	0.3	trace
Pike, Northern	trace	trace	trace	Scallop, Mixed Sp.	trace	0.1	-
Pike, Walleye	trace	0.2	trace	Shrimp, Mixed Sp.	0.3	0.2	trace
Pollock, Atlantic	trace	0.4	-	Squid, Mixed Sp.	0.1	0.3	trace
Pompano, Florida	0.2	0.4	-				
Roughy, Orange	trace	-	trace				
Salmon, Atlantic, Farmed	0.6	1.3	trace	**Fish Oils**			
Salmon, Atlantic, Wild	0.3	1.1	0.3	Cod Liver Oil	6.9	11.0	0.9
Salmon, Chinook	1.0	0.9	trace	Herring Oil	6.3	4.2	0.8
Salmon, Chinook, Smoked[b]	0.2	0.3	-	Menhaden Oil	13.2	8.6	1.5
Salmon, Chum	0.2	0.4	trace	Salmon Oil	13.0	18.2	1.1
Salmon, Coho, Farmed	0.4	0.8	trace	Sardine Oil	10.1	10.7	1.3
Salmon, Coho, Wild	0.4	0.7	0.2				
Salmon, Pink	0.4	0.6	trace				
Salmon, Pink, Canned[c]	0.9	0.8	trace	**Nuts and Seeds**			
Salmon, Sockeye	0.6	0.7	trace	Butternuts, Dried	-	-	8.7
Sardine, Atlantic, Canned in Oil[d]	0.5	0.5	0.5	Flaxseed			18.1
Seabass, Mixed Sp.	0.2	0.4	-	Walnuts, English	-	-	9.1
Seatrout, Mixed Sp.	0.2	0.2	trace				
Shad, American	1.1	1.3	0.2				
Shark, Mixed Sp.	0.3	0.5	trace	**Plant Oils**			
Snapper, Mixed Sp.	trace	0.3	trace	Canola (Rapeseed)	-	-	9.3
Swordfish	0.1	0.5	0.2	Flaxseed Oil	-	-	53.3
Trout, Mixed Sp.	0.2	0.5	0.2	Soybean Lecithin Oil	-	-	5.1
Trout, Rainbow, Farmed	0.3	0.7	trace	Soybean Oil	-	-	6.8
Trout, Rainbow, Wild	0.2	0.4	0.1	Walnut Oil	-	-	10.4
Tuna, Fresh, Bluefin	0.3	0.9	-	Wheatgerm Oil	-	-	6.9
Tuna, Fresh, Skipjack	trace	0.2	-				

Trace = <0.1; - = 0 or no data; Sp. = species; [a]Except as indicated; [b]Lox.; [c]Solids with bone and liquid; [d]Drained solids with bone; [e]Drained solids.

Table 1: Omega-3 fatty acid content, in grams per 100 g food serving, of a representative sample of commonly consumed fish, shellfish, and fish oils, nuts and seeds, and plant oils that contain at least 5 g omega-3 fatty acids per 100 g [3].

1.2.2 Association of omega-3 fatty acid intake and health

Early epidemiological studies noted a very low cardiovascular mortality in populations with high fish consumption[7, 14, 15]. This benefit of dietary fish can probably be explained by the intake of long chain omega-3 PUFA[16].

Studies have shown that populations, e.g. Eskimos, whose diets are rich in fish, which is a natural source of omega-3 fatty acids, have relatively high proportions of omega-3 fatty acids and low concentrations of AA in their plasma phospholipids[17].
Further studies demonstrated that plasma phospholipids, platelet and erythrocyte membrane fatty acids, as well as cholesteryl ester and free fatty acid compositions, are responsive to total dietary fat content and that the omega-3 fatty acid composition of adipose tissue is associated primarily with the amount of the dietary omega-3 fatty acid intake[18, 19].

In April 1999, an international working group of scientists met at the National Institute of Health to discuss the scientific evidence relative to dietary recommendations of omega-6 and omega-3 fatty acids. They agreed on the importance of reducing the omega-6 PUFA in the diets, with an increase in the uptake of omega-3 PUFA. The increase of ALA, together with EPA and DHA, and a reduction of vegetable oils with high LA content are thought to be necessary to achieve this healthier diet in Western countries[6]. Such dietary recommendations have been implemented by the American Heart Association in 2002[20].

1.3 *Caenorhabditis elegans* fat-1 gene

The nematode *Caenorhabditis elegans* is one of the few animals able to produce the essential fatty acids LA and ALA. These are generated by the action of desaturases that successively direct the conversion of monounsaturated fatty acids to PUFA[2].
One of the enzymes involved in the biosynthesis of polyunsaturated fatty acids in *C. elegans* is the FAT-1 protein encoded by the *C. elegans* fat-1 (fatty acid metabolism 1) gene. The FAT-1 protein was found to be a membrane-bound omega-3 fatty acid desaturase that catalyzes the introduction of an omega-3 double bond into a range of omega-6 substrates (illustrated in Figure 2)[1, 21, 22].

In contrast to mammals, expression of the fat-1 gene thus enables the nematode to endogenously convert omega-6 into omega-3 PUFA.

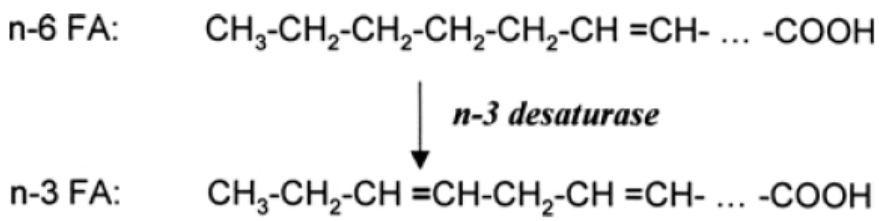

Figure 2: Conversion of omega-6 fatty acids to omega-3 fatty acids by an omega-3 desaturase that does not exist in mammalian cells. The omega-3 desaturase catalyzes the introduction of a double bond into omega-6 fatty acids at the omega-3 position of their hydrocarbon chains to form omega-3 fatty acids[8].

By virus-mediated gene transfer, the *C. elegans* fat-1 gene has been successfully introduced into various mammalian cells, including heart cells[23], neurons[24], endothelial cells[25], and human cancer cell lines[26, 27]. These in vitro studies performed by Kang et al., Ge et al., Meiler et al., and Xia, Wang & Kang in recent years indicate that the expression of the fat-1 gene in mammalian cells can alter their lipid profile and balance their omega-6 to omega-3 fatty acid ratio without the requirement of exogenous omega-3 PUFA supplementation[8]. This genetic approach offers a possibility to change the omega-6 to omega-3 fatty acid ratio but does not alter the total amount of cellular fatty acids.

1.4 How to meet the demand of omega-3 fatty acids in the future?

The demand for omega-3 fatty acids is rising since their health benefits are increasingly recognized, but their source is limited as overfishing leads to depletion of edible fish in the oceans[8]. So new approaches will be necessary in order to meet the growing demand of dietary omega-3 fatty acids in the future.

Although omega-6 fatty acids are highly abundant in the Western diet, elevating tissue concentrations of omega-3 PUFA relies on the continuous dietary intake of fats rich in omega-3 fatty acids since omega-6 PUFA cannot be converted into omega-3 PUFA in mammals[2, 8, 28].

Increasing the amount of omega-3 PUFA in beef and dairy products offers a way to improve the nutritional content of omega-3 fatty acids and can help to balance the omega-6 to omega-3 ratio[2]. Currently, this is achieved by feeding animals with exogenous omega-3 fatty acids (e.g., fish or other marine products), thereby enriching animal tissues with omega-3 PUFA[8].

Based on the in vitro results of the expression of the fat-1 gene in mammalian cells, fat-1 transgenic mice[29] and pigs[30] have been successfully generated. Due to the expression of the omega-3 fatty acid desaturase, these transgenic animals convert omega-6 to omega-3 PUFA, thus leading to an omega-6 to omega-3 fatty acid ratio in the tissues that approaches 1:1.
The development of a variety of omega-3-rich foodstuff such as the fat-1 pig would allow increased dietary intake with only little change in dietary habits[6], and these experiments provide a basis for producing omega-3 PUFA-rich food in a cost-effective and sustainable way for the increasing demand in the future[8].

An alternative approach of supplying humans with omega-3 PUFA could be the development of transgenic omega-3 PUFA producing organisms as dietary probiotic additives. These organisms could be supplied by nutrition and could convert the omega-6 PUFA contained in normal diet into omega-3 PUFA in the intestinal tract. In this way an increased omega-3 PUFA as well as decreased omega-6 PUFA intake could be realized without any change in habitual diet.

1.5 *Saccharomyces cerevisiae* as host for the expression of proteins involved in fatty acid metabolism

Saccharomyces cerevisiae (baker's yeast) is a eukaryotic organism. It is easy to grow and has been genetically well-characterized. Despite its small genome size of 12,068 kilobases[31], yeasts display most of the features of higher eukaryotes[32]. This fact and the availability of powerful genetic and molecular tools for *S. cerevisiae* have made yeast a common host for studies in molecular biology.

1.5.1 Heterologous protein expression

Heterologous expression is a technique to express genes of a certain species in a different organism. For heterologous protein expression, DNA of a particular gene is cloned into an expression plasmid which is then inserted into the host by transformation.

The yeast *S. cerevisiae* is an often used host for the expression of proteins – not only for the production of recombinant protein[33] but also as a model eukaryote to understand the function and properties of many mammalian proteins[34]. Many yeast cloning vectors are available for heterologous protein expression in *S. cerevisiae*. Most of them contain regions for autonomous replication and thereby allow extrachromosomal amplification of the gene of interest; others permit integration of foreign DNA directly into the yeast genome.

Yeasts have several properties that make them a preferred host for the production of heterologous proteins: They are able to perform posttranslational modifications and are considered as GRAS (generally regarded as safe) organisms[34] since there are no known yeast viruses or toxins and yeasts have been safely used in the baking and brewing industries for centuries. They can be cultivated at high-yield and low cost, and secrete only few proteins into the medium which makes it easy to recover secreted heterologous proteins[35]. Furthermore, the yeast genome can be easily manipulated, suitable host strains exist or can be easily generated, and a variety of plasmids, promoters etc. have been described and are widely available[34].
Examples of heterologous proteins expressed in yeast are interferons[36, 37], hepatitis surface antigene[38-40], and human growth hormone[33].

1.5.2 Fatty acid metabolism in yeast

Fatty acids are essential compounds in the cell. As in other eukaryotes, fatty acids in the yeast *Saccharomyces cerevisiae* serve important roles as a source of metabolic energy and as components of membrane lipids[41]. The narrow spectrum of fatty acids in yeast consists mostly of 16 and 18 carbon fatty acids[41]. Very-long-chain fatty acids with more than 18 carbons comprise only 1 to 2 percent of the total fatty acids[42].
It is well known that *S. cerevisiae* possesses only a very limited range of endogenous desaturation activities[1]. It is only able to synthesize monounsaturated fatty acids containing a delta-9 double bond[41] which – besides saturated fatty acids – account for 80 percent of yeast fatty acids[43].
Since yeast does not typically feed on fatty acids, cellular function and growth relies on de novo biosynthesis of fatty acids[43]. However, exogenous fatty acids provided to *S. cerevisiae* in the medium can be readily taken up and incorporated into complex lipids[41].

1.5.3 Synthesis of omega-3 fatty acids in yeast

Previous work on *C. elegans* fat-1 gene expression has been performed by Spychalla et al.[1] and Meesapyodsuk et al.[22, 44].

Spychalla et al. identified the DNA sequence of a *C. elegans* fatty acid desaturase through its homology with the FAD2 and FAD3 genes (encoding a delta-12 desaturase and an omega-3 desaturase) of the plant *Arabidopsis thaliana* and designated this gene fat-1. For a first attempt to determine the function of the gene product the fat-1 gene was expressed in *Arabidopsis* and the overall fatty acid composition of root tissues from wild-type and fat-1 transgenic plants was analyzed. The expression of fat-1 resulted in a 90 percent increase in the proportion of alpha-linolenic acid (18:3 n-3) accompanied by decreases in the proportion of linoleic acid (18:2 n-6) in root lipids of transgenic plants. Further experiments showed that when wild-type and transgenic *Arabidopsis* plants were sprayed with solutions of arachidonic acid (20:4 n-6) or homogamma-linolenic acid (20:3 n-6) as sodium salts, the exogenously supplied fatty acids were incorporated into the leaves. Analysis of total leaf lipids from plants expressing the fat-1 gene revealed that the supplied omega-6 fatty acids were substantially replaced by peaks corresponding to the omega-3 desaturated products eicosatetraenoic acid *(*20:4 n-3) and eicosapentaenoic acid *(*20:5 n-3) which could not be detected in wild-type leaves. These results suggested that the *C. elegans* FAT-1 protein is an omega-3 fatty acid desaturase that recognizes a range of 18- and 20-carbon omega-6 substrates.

Further experiments to characterize the fatty acid desaturase produced by the fat-1 gene were performed by Meesapyodsuk et al.[22, 44]. In these experiments the cDNA sequence of the *C. elegans* fat-1 gene was cloned into the yeast episomal plasmid pYES2.1, containing the galactose-inducible GAL1 promoter as well as the URA3 gene, and the *S. cerevisiae* strain INVSc2, carrying an ura3-mutation, was transformed with the constructed expression plasmid. Transformants were selected on minimal agar plates lacking uracil. Yeast cultures were grown in uracil-deficient media containing galactose and incubated with a range of possible fatty acid substrates. Gas chromatography (GC) analyses of fatty acid methyl esters from yeast cultures were performed and the positions of the newly formed double bond in the desaturation products were determined by gas chromatography-mass spectrometry (GC-MS). The results indicated that the FAT-1 protein catalyzes the introduction of a cis double bond

at the omega-3 position of a wide range of mono- and polyunsaturated fatty acid derivatives. From their experiments Meesapyodsuk et al. concluded that the enzyme encoded by the *C. elegans* fat-1 gene has an omega-3 regioselectivity, the ability to desaturate unsaturated substrates in the 16-20 carbon range, and a preference for substrates with omega-6 double bonds.

2 PROBLEM

In mammalian cells the endogenous synthesis of omega-3 PUFA is not possible. Omega-3 as well as omega-6 PUFA are essential fatty acids and thus need to be supplied by nutrition. Various health benefits of omega-3 fatty acids have been shown in recent years leading to an increase of their demand, but the sources of omega-3 PUFA are limited. There is thus an imbalance in the intake of omega-3 and omega-6 fatty acids in modern Western diets with a deficiency of omega-3 and abundance of omega-6 PUFA, but the human body is not able to convert these into omega-3 fatty acids.

The *Caenorhabditis elegans* fat-1 gene encodes the FAT-1 protein which is an omega-3 fatty acid desaturase that catalyzes the introduction of an omega-3 double bond into a range of omega-6 substrates[1, 21, 22].

The objective of the research presented here was the development of a transgenic industrial yeast strain of *Saccharomyces cerevisiae* as another model organism[8, 29, 30] which functionally expresses the *C. elegans* fat-1 gene. For this, different yeast expression plasmids had to be constructed. In a next step, the plasmids needed to be introduced into a wild-type *S. cerevisiae* strain industrially used, e.g. in baking. Finally, functionality of the protein had to be confirmed by demonstration of omega-3 desaturation of exogenously applied fatty acids.

The approach introduced here could lead to an alternative way of supplying humans with omega-3 PUFA, thereby meeting the increasing demand in the future.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Strains

Escherichia coli DH10B (Invitrogen Corporation, CA)

Escherichia coli TOP10 (Invitrogen Corporation, CA*)*

Saccharomyces cerevisiae Meyen ex E.C. Hansen (ATCC, VA)

3.1.2 Plasmids

HO-poly-KanMX4-HO (ATCC, VA)

p416 ADH (kindly supplied by Tiago Fleming Outeiro, PhD, MassGeneral Institute for Neurodegenerative Disease, Harvard Medical School, Charlestown, USA)

p426 GPD (kindly supplied by Tiago Fleming Outeiro, PhD, MassGeneral Institute for Neurodegenerative Disease, Harvard Medical School, Charlestown, USA)

pCE8 (available in the laboratory, formerly kindly supplied by John Browse's laboratory, Institute of Biological Chemistry, Washington State University, Pullman, USA)

pCR®-Blunt II-TOPO (Invitrogen Corporation, CA)

pRS306 GPD (kindly supplied by Tiago Fleming Outeiro, PhD, MassGeneral Institute for Neurodegenerative Disease, Harvard Medical School, Charlestown, USA)

3.1.3 Oligonucleotides

<u>Primers used to produce blunt-ended fat-1 fragments by PCR from pCE8:</u>

EcoRI + fat-1 start: 5' CAGGAATTCATGGTCGCTCATTCCTCAGAAGGG 3'

SacI + fat-1 end: 5' TCCGAGCTCTTACTTGGCCTTTGCCTTCTCCTC 3'

<u>Primers for PCR amplification of parts of fat-1 cDNA in expression plasmids:</u>

fat1for: 5' GCACCACGCCTTCACCAACCACAT 3'

fat1rev: 5' CCAAGCCGAGGCCATAGTAACGAT 3'

Primers used for sequencing the fat-1 PCR product:

fat-1 seq low 1: 5' GGTGAGAAGGCGATATGTCCAATG 3'

fat-1 seq up 1: 5' GTTTTTGGATTCGCGTTGTTCGTC 3'

fat-1 seq up 2: 5' CGCTGAGGTGTACGAGGCTGATG 3'

Primers for sequencing p416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4:

fw1 p416pRS306 w fat-1+KanMX: 5' CTTGTCTGTAAGCGGATGCC 3'

fw2 p416pRS306 w fat-1+KanMX: 5' CTGGAGTTAGTTGAAGCATTAGGT 3'

fw3 p416pRS306 w fat-1+KanMX: 5' TCAAAGAGACATGGGTGGAAGAGA 3'

fw4 p416pRS306 w fat-1+KanMX: 5' CCGAGATAGGGTTGAGTGTTGT 3'

fw5 p416pRS306 w fat-1+KanMX: 5' CGGGCCTCTTCGCTATTACG 3'

ADH1 forward primer 1: 5' AATAGAGCGACCATGACCTTGAA 3'

ADH1 reverse primer 1: 5' ATAATAAAGTGCACACCCAAATGA 3'

ADH1 forward primer 2: 5' CAAGACATAATGGGCTAAACAAGA 3'

ADH1 rv 2: 5' CATGCTCCTTGATTTCCTATTTC 3'

GPDp rv 1: 5' GAGATAGATACATGCGTGGGTCAA 3'

GPDp fw 1: 5' CGGGCAAAGAAATCGTAGTT 3'

fat-1 seq low 1: 5' GGTGAGAAGGCGATATGTCCAATG 3'

fat-1 seq up 1: 5' GTTTTTGGATTCGCGTTGTTCGTC 3'

fat-1 seq up 2: 5' CGCTGAGGTGTACGAGGCTGATG 3'

KanMX forward primer 1: 5' TCTCACATCACATCCGAACATAAA 3'

KanMX reverse primer 1: 5' AATTCCGTCAGCCAGTTTAGTCT 3'

KanMX forward primer 2: 5' TGATGCGAGTGATTTTGATGACGA 3'

rv1 p416pRS306 w fat-1+KanMX: 5' TCATTAGGCACCCCAGGCTTTACA 3'

rv2 p416pRS306 w fat-1+KanMX: 5' CGAAGGGAGAAAGGCGGACAG 3'

rv3 p416pRS306 w fat-1+KanMX: 5' TGAATTGAAAAGCTGTGGTATGGT 3'

fwMCS p416pRS306 w fat-1+Kan: 5' GATATCAAGCTTATCGATACCGTC 3'

fw6 p416pRS306 w fat-1+KanMX: 5' CATCACAAAAATCGACGCTCAA 3'

fw7 p416pRS306 w fat-1+KanMX: 5' ATCCTTTGATCTTTTCTACGGG 3'

fw8 p416pRS306 w fat-1+KanMX: 5' ATTGCTACAGGCATCGTGGTG 3'

fw9 p416pRS306 w fat-1+KanMX: 5' GGGTGAGCAAAAACAGGAAG 3'

fw10 p416pRS306 w fat-1+KanMX: 5' TAAATTCCCAAATTATTCCATCAT 3'

Primers for sequencing parts of HO-poly-KanMX4-HO + GPD prom + fat-1t + term:
forward primer in HO-L: 5' ATAGTTAATCGGGCAATGTCC 3'
reverse primer in KanMX PstI: 5' CAGGTCTGCAGCGAGGAGC 3'
fat-1 seq low 1: 5' GGTGAGAAGGCGATATGTCCAATG 3'
fat-1 seq up 1: 5' GTTTTTGGATTCGCGTTGTTCGTC 3'
fat-1 seq up 2: 5' CGCTGAGGTGTACGAGGCTGATG 3'

Customized primers, designed using the DNAStar PrimerSelect software, were ordered from the Invitrogen Corporation, CA and TIB MOLBIO GmbH, Germany.

3.1.4 Media

LB:
2% tryptone, 1% yeast extract, 1% NaCl.
For LB plates, 1.5% agar was added.
For selection, ampicillin at a concentration of 50 µg/ml or kanamycin at 70 µg/ml was added to the autoclaved and cooled media.

YM:
0.3% yeast extract, 0.3% malt extract, 0.5% veggie peptone, 1% dextrose.
For YM plates, 2% agar was added.
Liquid medium was acidified to pH 3.0-4.0 using 2 M HCl.
For G418 selection, G418 was added to the autoclaved and cooled media at a concentration of 200 µg/ml for agar plates and 80 µg/ml for broth.

YPD:
1% yeast extract, 2% veggie peptone, 2% dextrose.
For YPD plates, 2% agar was added.
When necessary for selection, G418 was added at a concentration of 200 µg/ml for agar plates and 80 µg/ml for broth.

3.1.5 Buffers, solutions, chemicals

Agar (Sigma-Aldrich Corporation, MO)

Agarose (Sigma-Aldrich Corporation, MO)

Ampicillin (Sigma-Aldrich Corporation, MO)

Boron trifluoride (Sigma-Aldrich Corporation, MO)

BSA (New England BioLabs, MA)

Buffered lithium solution: 1 vol 10X TE buffer, 1 vol 10X lithium acetate stock solution, 8 vol sterile water

Cloned Pfu DNA polymerase reaction buffer (10X) (Stratagene, CA): 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton® X-100, 1 mg/ml nuclease-free BSA

Dextrose (Sigma-Aldrich Corporation, MO)

DNA Ladder 1 kb Plus exACTGene (Fisher Scientific, PA)

DNA Ladder 100 bp (Promega Biosciences, CA)

DNA, single-stranded from salmon testes (Sigma-Aldrich Corporation, MO)

DTT (Sigma-Aldrich Corporation, MO)

Ethanol (Sigma-Aldrich Corporation, MO)

Ethidium bromide (Sigma-Aldrich Corporation, MO)

Fatty acid standards (Nu-Chek Prep, Inc., MN)

G418 (Sigma-Aldrich Corporation, MO)

Glycerol (Sigma-Aldrich Corporation, MO)

Hexane (Sigma-Aldrich Corporation, MO)

Igepal CA-630 (Sigma-Aldrich Corporation, MO)

Isopropanol (Sigma-Aldrich Corporation, MO)

Kanamycin (Sigma-Aldrich Corporation, MO)

Lithium acetate (Sigma-Aldrich Corporation, MO)

Lithium acetate stock solution (10X): 1 M lithium acetate, pH 7.5, filter sterilized

Loading Dye (6X) Blue/Orange (Promega Biosciences, CA)

Malt extract (BD, NJ)

NaCl (Sigma-Aldrich Corporation, MO)

NEBuffer 1 (10X) (New England BioLabs, MA): 0.1 M Bis-Tris-Propane-HCl, 0.1 M $MgCl_2$, 10 mM DTT, pH 7.0

NEBuffer 2 (10X) (New England BioLabs, MA): 0.1 M Tris-HCl, 0.5 M NaCl, 0.1 M $MgCl_2$, 10 mM DTT, pH 7.9

NEBuffer 3 (10X) (New England BioLabs, MA): 1 M NaCl, 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 10 mM DTT, pH 7.9

NEBuffer 4 (10X) (New England BioLabs, MA): 0.5 M potassium acetate, 0.2 M Tris-acetate, 0.1 M magnesium acetate, 10 mM DTT, pH 7.9

NEBuffer EcoRI (10X) (New England BioLabs, MA): 0.5 M NaCl, 1 M Tris-HCl, 0.1 M $MgCl_2$, 0.25 % Triton X-100, pH 7.5

Omega-6 PUFA (Nu-Chek Prep, Inc., MN)

PCR Master Mix 2X (Promega Biosciences, CA): 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400μM dATP, 400μM dGTP, 400μM dCTP, 400μM dTTP, 3mM $MgCl_2$

PEG 3350 (Sigma-Aldrich Corporation, MO)

PEG solution: 8 vol 50% PEG, 1 vol 10X TE buffer, 1 vol 10X lithium acetate stock solution

Phenol-chloroform-isoamyl alcohol (25:24:1), pH 8.0 (Sigma-Aldrich Corporation, MO)

Salt solution (Invitrogen Corporation, CA): 1.2 M NaCl, 0.06 M $MgCl_2$

SDS (Sigma-Aldrich Corporation, MO)

S.O.C. medium (Invitrogen Corporation, CA): 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

dNTP Mix (New England BioLabs, MA): 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP

T4 ligase reaction buffer (10X) (New England BioLabs, MA): 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 0.1 M DTT, 10 mM ATP, pH 7.5

TBE buffer (10X): 890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA, pH 8.3

TE buffer (10X): 100 mM Tris HCl, 10 mM EDTA, pH 7.5

Tryptone (Fisher Scientific, PA)

Veggie Peptone (Novagen, NJ)

Yeast extract (Fisher Scientific, PA)

3.1.6 Enzymes

Alkaline Phosphatase (CIP) (New England BioLabs, MA)

DNA polymerase I, large Klenow fragment (New England BioLabs, MA)

PfuTurbo DNA polymerase (Stratagene, CA)
Restriction endonucleases (New England BioLabs, MA, Promega Biosciences, CA, and Boehringer Mannheim GmbH, Germany)
T4 DNA ligase (New England BioLabs, MA)
Taq DNA polymerase in PCR Master Mix 2X (Promega Biosciences, CA)

3.2 Programs and data bases

Chromas 2 Technelysium Pty Ltd, Version 2.31
DNAStar DNASTAR Inc., Version 5.00
GenBank (Benson et al. 2008) access via www.ncbi.nlm.nih.gov/
SaccharomycesGenomeDatabase (SGD) (Cherry et al. 1997)
WormBase (Chen et al. 2005)

3.3 DNA techniques

3.3.1 Preparation of plasmid DNA from *E. coli*

Small amounts (<30 µg) of plasmid DNA from different colonies were isolated from *E. coli* using the PureLink Quick Plasmid Miniprep Kit (Invitrogen Corporation, CA – catalog number K2100-10) and QIAprep Spin Miniprep Kit (Qiagen Inc., CA – catalog number 27104), following the manufacturer's protocol.
Greater amounts of plasmid DNA were extracted using the HiSpeed Plasmid Midi Kit (Qiagen Inc., CA – catalog number 12643) according to the manufacturer's protocol.

3.3.2 Purification and concentration of DNA

The purification of DNA fragments from aqueous solutions was performed in two different ways.
One method to purify DNA after PCR was to apply isopropanol and precipitate the desired DNA fragment. 100 µl Tris EDTA buffer, 40 µl of 3M NaCl and 200 µl isopropanol were added to the PCR reaction mix and incubated at -20°C.

After 20 minutes of incubation, the reaction mix was centrifuged for 25 minutes at maximum speed at 4°C. The resulting pellet was washed with 70% ethanol and dried before dissolving it in nuclease-free water. Alternatively, the QIAquick Gel Extraction Kit (Qiagen Inc., CA – catalog number 28706) was used following the protocol for PCR product purification.

In case of low DNA yield, DNA was concentrated using Microcon® centrifugal filter devices (Millipore Corporation, MA).

3.3.3 PCR amplification of parts of fat-1 to verify cloning and transformation

In order to search for *E. coli* colonies containing plasmid DNA with fat-1 insert and thereby proving successful cloning, polymerase chain reaction amplifying parts of fat-1 was performed. Plasmid DNA extracted by Mini- or MidiPrep was used as template DNA with "fat1for" and "fat1rev" primers as well as PCR Master Mix (Promega Biosciences, CA – catalog number M7502). PCR was performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., MA) and reaction parameters were set as follows:

Step 1) initial denaturation: 95°C for 5 minutes
Step 2) denaturation: 94°C for 30 seconds
Step 3) annealing: 62°C for 30 seconds
Step 4) extension: 72°C for 1 minute
Step 5) steps 2 to 4 repeated 30 times
Step 6) final extension: 72°C for 8 minutes

For PCR from yeast cells, DNA was obtained by harvesting cells from 100 µl liquid culture. The supernatant was removed and 30 µl 0.2% SDS were added. The cells were then vortexed for 15 seconds and incubated for 4 minutes at 90°C. After spin down, 1 µl of the supernatant was used for PCR. The reaction was set up in the same way as described above.

3.3.4 PCR producing blunt-ended fat-1 fragments

The PfuTurbo DNA polymerase (Stratagene, CA – catalog number 600250) was used to produce blunt-ended fat-1 fragments with desired restriction sites by amplification via polymerase chain reaction from pCE8. The primers "EcoRI + fat-1 start" and "SacI + fat-1 end" (0.5 µM each) were added to a 50 µl reaction mix containing 10 ng template DNA, 2.5 U PfuTurbo DNA polymerase, dNTPs, and Cloned Pfu DNA polymerase reaction buffer. PCR parameters were set as follows:

Step 1) initial denaturation: 98°C for 30 seconds
Step 2) denaturation: 98°C for 10 seconds
Step 3) annealing: 72°C for 1 minute
Step 4) extension: 72°C for 30 seconds
Step 5) → steps 2 to 4 repeated 33 times
Step 6) final extension: 72°C for 10 minutes

3.3.5 Digestion of DNA with restriction endonucleases

Restriction sites within the plasmid DNA of the expected construct were located using the DNAStar MapDraw program. Restriction endonucleases suitable for verification of the plasmid construct were selected, and isolated plasmid DNA of different colonies was digested according to the reaction conditions of the particular enzyme stated by the manufacturer. The resulting fragments were analyzed by agarose gel electrophoresis.

3.3.6 Agarose gel electrophoresis

Agarose gels at concentrations between 0.7 and 1.2 % (w/v) agarose, depending on the expected fragment sizes, were prepared with TBE buffer and boiled. After boiling, 10 µl ethidium bromide per 100 ml TBE were added to the heated gel. The gel was poured into the plastic tray and cooled until it solidified. The solid gel on its tray was then placed into the electrophoresis chamber which was filled with TBE buffer. The samples (10 µl each) mixed with loading dye and a DNA ladder used to subsequently determine the fragments' length were applied to the slots in the gel. Electrophoresis was operated with constant voltage at 75 to 100 V. The separated DNA fragments were then visualized as fluorescent bands under ultraviolet light.

3.3.7 Isolation and purification of DNA fragments from agarose gel

Bands of DNA fragments of interest were cut off from agarose gel under UV light and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen Inc., CA) following the manufacturer's protocol.

3.3.8 Blunting

For blunt-end ligation, blunted fragments were produced using the DNA polymerase I, large (Klenow) fragment at 1 U/µg DNA, NEBuffer 2, and 40 µM of each dNTP. The reaction was performed for 30 minutes at room temperature. The enzyme was afterwards heat inactivated at 75°C for 10 minutes and the remaining dNTP were removed from the reaction mix using the Invitrogen PureLink PCR Purification Kit (Invitrogen Corporation, CA – catalog number K3100-01).

3.3.9 Dephosphorylation of DNA fragments

In order to prevent larger DNA fragments from self-ligation prior to further ligation steps, CIP was used for dephosphorylation of these fragments. CIP was added at a concentration of 0.5 U/µg DNA. The mix was then incubated for 30 minutes at 37°C and 30 minutes at 50°C.

For further ligation, CIP was inactivated by adding an equal amount of phenol-chloroform-isoamyl alcohol to the solution and incubating for 30 to 60 minutes at room temperature in a shaking incubator. Afterwards, the reaction mix was centrifuged at maximum speed and 4°C for 20 minutes. The upper phase was taken and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., CA – catalog number 28706) following the protocol for PCR product purification.

3.3.10 DNA ligation

Amounts of DNA of the different fragments to be ligated for vector construction were calculated according to the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio (insert / vector)} = \text{ng of insert}$$

A ratio of 1:3 and 500 ng of vector were used for calculations.
Between 200 and 800 U of T4 DNA ligase and 10X ligase buffer were added to the insert and vector DNA. Ligation was performed at 16°C overnight.

3.3.11 DNA sequencing

Sequencing of the plasmid DNA was performed by the MGH DNA Core Facility of the Department of Molecular Biology at Massachusetts General Hospital.
Each sample was submitted in a labelled 1.5 ml tube containing 2 µg of DNA template and 100 ng of primer. Samples were submitted in duplicates to the MGH sequencing core. The DNA samples were there sequenced using Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing kits which utilize a fluorescently-labelled dideoxy-nucleotide chain termination method. After cycle sequencing and clean up, the DNA samples were resolved by capillary electrophoresis on an ABI 3730XL DNA Analyzer which translates the fluorescent signals into their corresponding base pair sequence.

Unknown base pairs in DNA sequences obtained from the sequencing core were compared to the fluorescent signals using Chromas 2 software and manually translated. Plasmid sequences were arranged and merged using DNAStar EditSeq and MagAlign software.

3.4 Introduction of plasmid DNA into *E. coli*

3.4.1 Transformation of *E. coli* by electroporation

In order to amplify plasmid DNA, plasmids were introduced into *E. coli*. For electroporation 1 µl plasmid DNA was added to a chilled microcentrifuge tube. Electrocompetent ElectroMAX DH10B cells (Invitrogen Corporation, CA – catalog number 18290-015) were thawed on ice and 20 µl were added to the microcentrifuge tube containing plasmid DNA. The mixture was then carefully transferred to a chilled 0.1 cm electroporation cuvette (Fisher Scientific, PA – catalog number FB101). Electroporation was performed using a MicroPulser Electroporator (Bio-Rad Laboratories, CA) and Ec1 setting at 1.8 to 2.0 kV for 4.3 to 6.10 ms.

After electroporation, 0.5 ml of S.O.C. medium were added to the cuvette and the solution was transferred to a sterile 1.5 ml microcentrifuge tube and incubated for 1 hour in a shaking incubator at 37°C and 225 rpm.
Different amounts (10 to 100 µl) of the solution were spread on selective LB plates containing 50 µg/ml ampicillin and incubated overnight at 37°C.

3.4.2 Transformation of *E. coli* by heat shock

A 50 µl vial of One Shot TOP10 chemically competent *E. coli* cells (Invitrogen Corporation, CA) was thawed on ice. 5 µl of the cloning reaction mix were pipetted into the vial of competent cells and mixed by gentle tapping. The vial was incubated on ice for 30 minutes and afterwards heat shocked by incubation for 45 seconds in a 42°C water bath. The vial was immediately put on ice for 2 more minutes before 250 µl of S.O.C medium (Invitrogen) were added to the vial. The vial was further incubated at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. 30 and 50 µl of the solution were spread on selective kanamycin LB plates (70 µg/ml). The plates were incubated at 37°C overnight.

3.5 Introduction of DNA into *S. cerevisiae*

3.5.1 Yeast transformation using lithium acetate

Yeast transformation using lithium acetate provides transformation efficiencies of 10^5 to 10^6 transformations/µg[45].
The night before transformation, a flask containing 50 ml YPD or YM medium was inoculated and grown overnight at 30°C to a concentration of 1 x 10^7 cells/ml. For higher efficiency, the yeast was diluted to 2 x 10^6 cells/ml in fresh YPD or YM medium and grown for another 2 to 4 hours.
Yeast cells were harvested by centrifuging 5 minutes at 4000 x g at room temperature and then resuspended in 5 ml highest-quality sterile water. Cells were pelleted by centrifuging 5 minutes at 5000 to 6000 x g at room temperature and resuspended in 0.75 ml buffered lithium solution.

200 µg single-strand carrier DNA were transferred to a sterile 1.5-ml microcentrifuge tube, boiled for 15 min and frozen afterwards. Less than 5 µg transforming DNA, 200 µl yeast suspension, and 1.2 ml PEG solution were added to the 1.5-ml microcentrifuge tube and incubated for 30 minutes at 30°C in a shaking incubator. Then the yeast cells were heat shocked for exactly 15 minutes at 42°C.
Afterwards the cells were pelleted and resuspended in 500 µl to 1 ml of 1x TE buffer and incubated for a few hours at 30°C and 200 rpm before spreading aliquots of up to 200 µl onto YPD or YM plates containing 200 µg/ml G418 and incubation for 2 to 5 days at 30°C.

3.5.2 Yeast transformation by electroporation

Yeast cells were grown in 50 ml YPD or YM medium at 30°C to a concentration of 10^8 cells/ml. The culture was harvested by centrifuging at 4000 x g and 4°C and re-suspended in 8 ml sterile water. The cells were then treated with lithium acetate in order to increase electrocompetence of the cells. 1 ml 10x TE (pH 7.5) and 1 ml 10x lithium acetate stock solution (1 M) were added. After gentle shaking for 45 minutes at 30°C, 250 µl of 1 M DTT were added, followed by 15 minutes at 30°C with gentle shaking. The yeast suspension was then diluted with water to 50 ml and washed and concentrated by three cycles of centrifuging at 4°C and 4000 to 6000 x g and resuspension of the pellets as follows: First pellet in 25 ml ice-cold water, second pellet in 2.5 ml ice-cold water, third pellet in 50 µl ice-cold water.
For electroporation, 40 µl concentrated yeast cells were mixed with less than 100 ng transforming DNA contained in less than 5 µl volume in a sterile, ice-cold 1.5 ml tube and then transferred to an ice-cold 0.2-cm gap electroporation cuvette (Fisher Scientific, PA – catalog number FB102). Electroporation was performed using a MicroPulser Electroporator (Bio-Rad Laboratories, CA) and Sc1 setting at 1.5 kV for 4.2 to 5.6 ms. After electroporation, 1 ml of YPD or YM medium was added to the cuvette, and the solution was transferred to a sterile 1.5 ml microcentrifuge tube and incubated for 1 hour in a shaking incubator at 37°C and 225 rpm.
Different amounts (10 to 100 µl) of the solution were spread on selective YPD or YM plates containing 200 µg/ml G418 and incubated for 2 to 5 days until colonies appeared.

3.6 Fatty acid incubation

Yeast colonies were grown to saturation in selective YPD medium. Exogenous omega-6 PUFA (arachidonic and linoleic acid, Nu-Check Prep) were then added to a final concentration of 100 mg/l with Igepal CA-630 (Sigma-Aldrich Corporation, MO – product number I8896) 0.1% (v/v). Yeast colonies were incubated for three days at 30°C in a shaking incubator.

3.7 Fatty acid analysis

3.7.1 Lipid extraction from yeast cells for gas chromatographic analysis

Yeast cells were harvested from liquid culture by centrifuging for 20 minutes at 4000 rpm. 50 µl of yeast cells were transferred to clean glass screw-cap tubes. Boron trifluoride and hexane were added at 1.5 ml each. The tubes were then sealed under nitrogen to prevent fatty acid oxidation. The samples were incubated for 60 minutes at 100°C in a dry bath incubator. After incubation samples were cooled to room temperature.

3.7.2 Sample preparation for gas chromatography

1 ml double distilled water was added to each tube after cooling. After vortexing, phases were separated by centrifuging for 5 min at 3000 rpm. The upper hexane phase of each sample was removed with a Pasteur pipette and transferred to a clean culture tube. The samples were dried under nitrogen, re-dissolved in 70 µl hexane, and transferred to gas chromatography (GC) tubes.

3.7.3 Fatty acid analysis by gas chromatography

Fatty acid methyl esters were analyzed by gas chromatography using a fully automated 6890N Network GC System (Agilent Technologies, CA) equipped with a flame-ionization detector. Peaks of resolved fatty acids were identified by comparison with fatty acid standards (Nu-Chek Prep, Inc., MN), and area percentage for all resolved peaks was analyzed using GC ChemStation Software (Agilent Technologies, MN).

4 RESULTS

4.1 Fat-1 containing yeast expression plasmids

4.1.1 Plasmids and principles for expression in yeast

Plasmids are extrachromosomal DNA molecules capable of autonomous replication naturally occurring in bacteria and some eukaryotic organisms, e.g. *S. cerevisiae*. Plasmids are mostly double-stranded and circular in structure but vary widely in size and copy number in a cell. Plasmids used in genetic engineering are called vectors. Each vector consists of a replicator, a multiple cloning site (MCS), and a selectable marker. The replicator contains a specific DNA sequence, the origin of replication (ori), allowing independent replication in the host. The MCS is a short region containing several commonly used restriction sites allowing the insertion of DNA fragments at this location. A selectable marker is necessary in order to maintain presence of the plasmids in a cell. This typically is a gene encoding resistance to a certain antibiotic or, as in many yeast expression plasmids, cloned yeast genes. Laboratory yeast strains usually carry mutant alleles of genes necessary for the biosynthesis of certain amino acids. Cloned yeast genes on plasmids are able to complement an auxotrophic mutation in the yeast genome and thereby allow selection of transformants.

There are different classes of yeast expression vectors varying in their mode of replication. Yeast centromeric plasmids (YCp) and yeast episomal plasmids (YEp) belong to the class of yeast vectors capable of extrachromosomal replication in yeast. YCp vectors contain an autonomous replication sequence (ARS) as well as a centromeric (CEN) element for maintenance of the plasmids in dividing cells[46, 47]. These plasmids are present at very low copy numbers (1 to 2 copies per cell) and replicate once during the cell cycle. With a loss rate of approximately 1 percent per generation they are relatively stable during mitosis and meiosis[46]. YEp vectors contain a segment of the yeast 2 µm plasmid that serves as an origin of replication and is responsible for high copy numbers (20 to 50 copies) and high frequency of transformation (10^4 to 10^5 transformants per µg DNA) of these plasmids. However, most of these plasmids are relatively unstable[48-51].

Yeast integrating plasmids (YIp) do not contain sequences for autonomous replication, but instead transformation results from integration into the yeast genome by

recombination between yeast sequences carried on the plasmid and homologous sequences in the yeast genome. Although transformation frequency is only 1 to 10 transformants per µg DNA, integration is very stable. Also, transformation frequency can be increased 10- to 1000-fold by linearization of the plasmid[52-54].

Besides the copy number and hence gene dosage determined by the plasmid's replicator sequence, promoters influence transcription of the gene of interest. Inducible promoters, such as the GAL1 (galactokinase) promoter, allow conditional expression of the plasmid-encoded protein. Transcription only occurs if a certain substance – galactose in the case of GAL1 – is added to the yeast medium that then activates the promoter. In contrast, there are constitutive promoters which permit constant transcription of the gene.

Since first of all galactose is expensive and cannot always be supplied in addition to the yeast when used as a dietary supplement and secondly a constant expression of the gene is necessary for the purpose of this study, constitutive promoters were chosen for the design of the expression plasmids.

4.1.2 Original fat-1 gene and plasmids available for this study

The original fat-1 gene was available in the laboratory in the plasmid pCE8[1], a pBluescript SK- vector[55], into which the cDNA sequence of the *C. elegans* fat-1 gene had been cloned as an EcoRI and XhoI fragment, as shown in Figure 3 (personal communication with Jennifer Watts of John Browse's laboratory, Institute of Biological Chemistry, Washington State University, Pullman, USA).

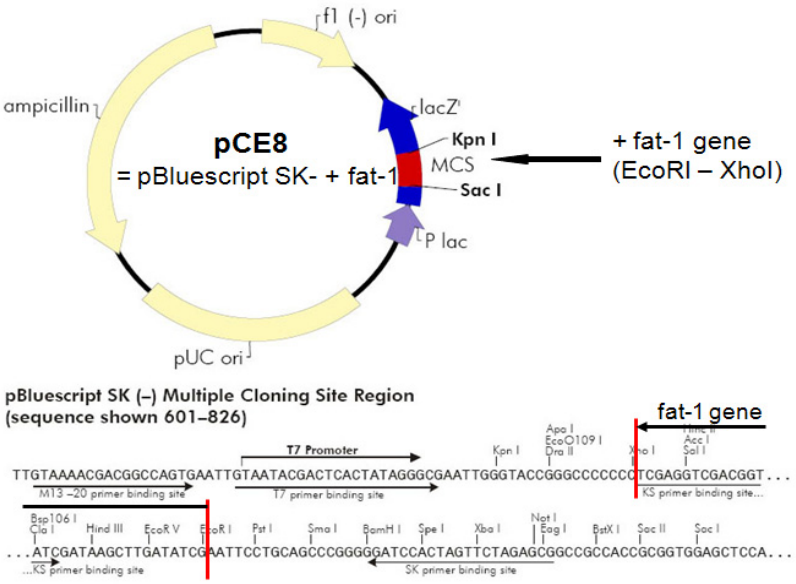

Figure 3: Map of pCE8, modified from that of the Stratagene Instruction Manual[55].

In order to generate suitable restriction sites for sticky-end cloning of fat-1 into different yeast expression plasmids, a blunt-ended fat-1 fragment was produced by PCR using the PfuTurbo DNA polymerase (Stratagene) with “EcoRI + fat-1 start” and “SacI + fat-1 end” primers and the plasmid pCE8 as template for the reaction. The resulting PCR fragment was ligated into pCR-Blunt II-TOPO (Invitrogen)[56] and the resulting plasmid termed pCR-Blunt II-TOPO + fat-1 (Figure 4) was amplified in TOP10 *E. coli* bacteria.

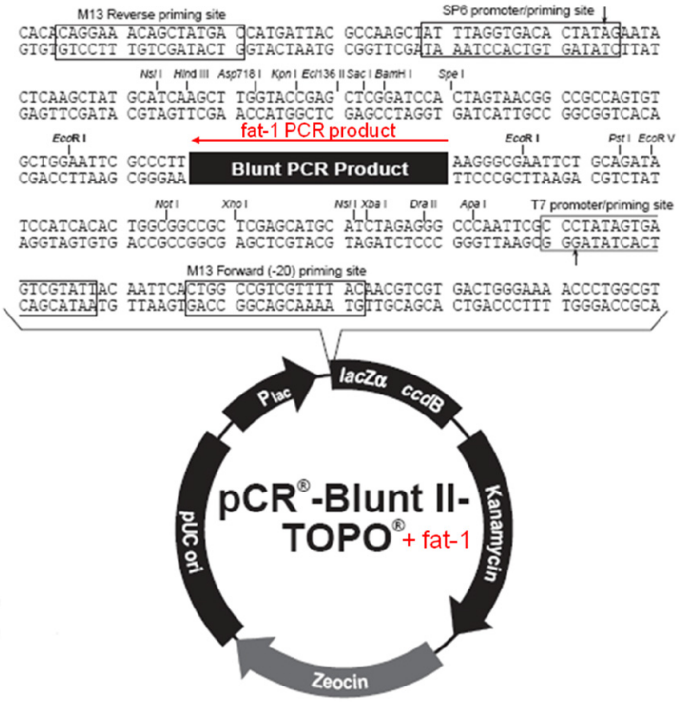

Figure 4: Map of pCR-Blunt II-TOPO + fat-1, modified from that of Invitrogen[56].

Although the Pfu DNA Polymerase is a high fidelity polymerase and exhibits the lowest error rate of any thermostable DNA polymerases studied[57], plasmid DNA isolated from different colonies grown under kanamycin selection was sequenced using the primers "fat-1 seq low 1", "fat-1 seq up 1", and "fat-1 seq up 2" to exclude errors in the fat-1 gene sequence.

Sequencing results are illustrated in Figure 5.

NNNNNTNNNNNNNTNNTNNNNNCNCCNNNTTNNNNCCCNGATNNNCNCCNCNGNGNAAANTTCANNNGNN
ACTTTNTNNNNCAGCAGACGTNCNCNNNNNAGNGNGATCACCATNNGTNNCCCNGGNGNGTCAATAATAT
CACTCTGTACATCCACAANCAGANGATAANGGCTCTCTCTTTTATAGGNGTAAACCTTAAACTGCCGTAC
GTATAGGCTGCGCAACTGTTGGGAAGNGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAA
GGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGA
CGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCG
CCAGTGTGATGGATATCTGCAGAATTCGCCCTTCAGGAATTCATGGTCGCTCATTCCTCAGAAGGGTTAT
CCGCCACGGCTCCGGTCACCGGCGGAGATGTTCTGGTTGATGCTCGTGCATCTCTTGAAGAAAAGGAGGC
TCCACGTGATGTGAATGCAAACACTAAACAGGCCACCACTGAAGAGCCACGCATCCAATTACCAACTGTG
GATGCTTTCCGTCGTGCAATTCCAGCACACTGTTTCGAAAGAGATCTCGTTAAATCAATCAGATATTTGG
TGCAAGACTTTGCGGCACTCACAATTCTCTACTTTGCTCTTCCAGCTTTTGAGTACTTTGGATTGTTTGG
TTACTTGGTTTGGAACATTTTTATGGGAGTTTTTGGATTCGCGTTGTTCGTCGTTGGACACGATTGTCTT
CATGGATCATTCTCTGATAATCAGAATCTCAATGATTTCATTGGACATATCGCCTTCTCACCACTCTTCT
CTCCATACTTCCCATGGCAGAAAAGTCACAAGCTTCACCATGCTTTCACCAACCACATTGACAAAGATCA
TGGACACGTGTGGATTCAGGATAAGGATTGGGAAGCAATGCCATCATGGAAAAGATGGTTCAATCCAATT
CCATTCTCTGGATGGCTTAAATGGTTCCCAGTGTACACTTTATTCGGTTTCTGTGATGGATCTCACTTCT
GGCCATACTCTTCACTTTTTGTTCGTAACTCTGAACGTGTTCAATGTGTAATCTCTGGAATCTGTTGCTG
TGTGTGTGCATATATTGCTCTAACAATTGCTGGATCATATTCCAATTGGTTCTGGTACTATTGGGTTCCA
CTTTCTTTCTTCGGATTGATGCTCGTCATTGTTACCTATTTGCAACATGTCGATGATGTCGCTGAGGTGT
ACGAGGCTGATGAATGGAGCTTCGTCCGTGGACAAACCCAAACCATCGATCGTTACTATGGACTCGGATT
GGACACAACGATGCACCATATCACAGACGGACACGTTGCCCATCACTTCTTCAACAAAATCCCACATTAC
CATCTCATCGAAGCAACCGAAGGTGTCAAAAAGGTCTTGGAGCCGTTGTCCGACACCCAATACGGGTACA
AATCTCAAGTGAACTACGATTTCTTTGCCCGTTTCCTGTGGTTCAACTACAAGCTCGACTATCTCGTTCA
CAAGACCGCCGGAATCATGCAATTCCGAACAACTCTCGAGGAGAAGGCAAAGGCCAAGTAAGAGCTCGAA
GGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCT
TGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTG
TTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT
CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCNGCTCNCTCNAAGGCNGTAATACNG
NTATCCNCNGAATCNNNGGATAACNNNNNAAAGAANNTGNNANCNAAAGGNNNNNCNAAAGNNNNNGAAN
NNNNAAAAANGGNNNNNNTNNNNNN

Figure 5: Sequencing results of the pCR-Blunt II-TOPO plasmid containing the blunt-ended fat-1 fragment produced from pCE8 using the PfuTurbo DNA polymerase (Stratagene) with "EcoRI + fat-1 start" and "SacI + fat-1 end" primers. Printed in red is the PCR product insert, the start and stop codon sequences are underlined.

Alignment with the original *C. elegans* fat-1 sequence using the DNAStar MegAlign program and a blast search of the sequence obtained from the sequencing core using BLASTN 2.2.17 at http://www.wormbase.org/db/searches/blast_blat demonstrated that the PCR-amplification of fat-1 did not introduce errors (see Figure 6). Sequencing of the plasmid DNA furthermore revealed information regarding the orientation of the insert, as shown in Figure 4.

```
BLASTN 2.2.17 [Aug-26-2007]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= Query Sequence
         (2195 letters)

Database: c_elegans nucleotide release [WS192]
           7 sequences; 100,281,426 total letters

Searching.................................................done

                                                                  Score    E
Sequences producing significant alignments:                      (bits) Value

IV                                                                975    0.0
[Alignment] [Genome View]
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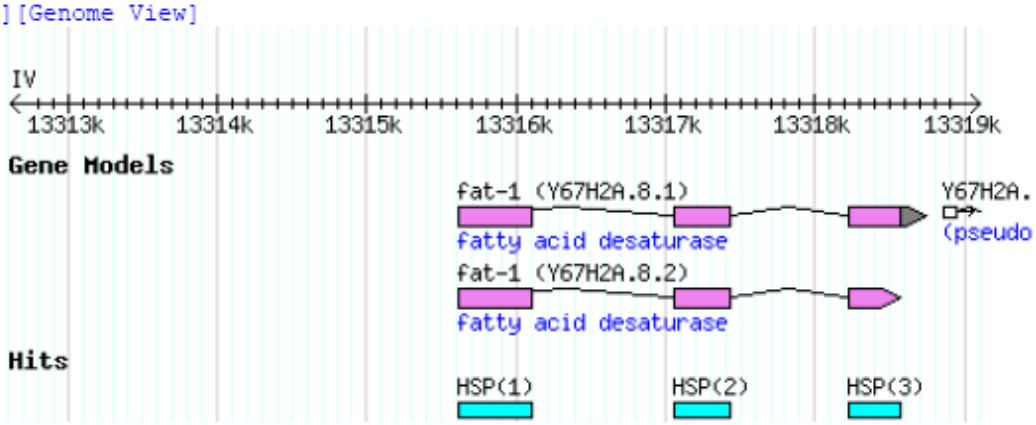


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 Strand = Plus / Plus


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Query: 523      ctggttgatgctcgtgcatctcttgaagaaaaggaggctccacgtgatgtgaatgcaaac 582
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Figure 6: Results from the blast search performed using BLASTN 2.2.17 at http://www.wormbase.org/db/searches/blast_blat. An identity of 100 percent between the fat-1 sequence in pCR-Blunt II-TOPO + fat-1 obtained from the sequencing core and the *C. elegans* genome was verified.

In the study presented here the following four different yeast cloning vectors were used to construct different fat-1 expression plasmids: Three of these were kindly supplied by Tiago Fleming Outeiro, PhD, MassGeneral Institute for Neurodegenerative Disease, Harvard Medical School, Charlestown, USA. P416 ADH is a YCp vector carrying, in addition to the ARS and CEN element, an URA3 auxotrophic marker, an ampicillin resistance gene, and a constitutive promoter of the alcohol dehydrogenase I (ADH from ADH1 gene) as well as a CYC1 (cytochrome-c oxidase) terminator sequence.

P426 GPD is similar to p416 ADH but carries a different, strong constitutive GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter and, instead of the CEN/ARS element, contains a 2µm origin for extrachromosomal replication and high copy number. The p416 ADH and p426 GPD expression plasmids (Figure 7) were constructed by Mumberg et al.[58].

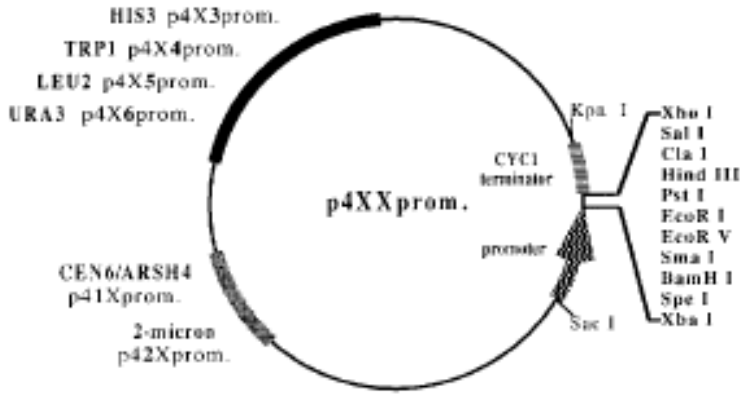

Figure 7: Schematic map and nomenclature of expression vectors[58].
Plasmid p416 ADH carries an ARS and CEN element, an URA3 auxotrophic marker, and ADH promoter as well as CYC1 terminator sequence. In contrast, p426 GPD contains a 2µm origin and carries a GPD promoter.

The pRS306 GPD shuttle vector is a derivative of pRS306 (Figure 8)[59]. It is similar in design to p426 GPD, also comprising an URA3 auxotrophic marker, an ampicillin resistance gene, GPD promoter, and CYC1 terminator but lacks a sequence for autonomous replication, resulting in transformation by integration of the plasmid into the yeast genome.

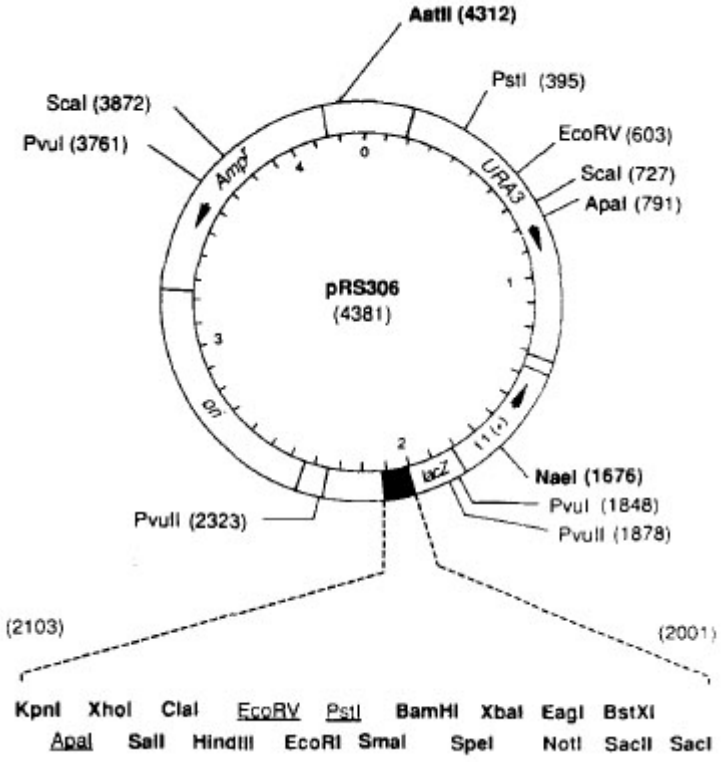

Figure 8: Map of pRS306[59].

The HO-poly-KanMX4-HO plasmid (ATCC number 87804)[60] is a yeast integration plasmid containing two fragments from the HO gene for integration at the HO locus[61]. The HO gene is expressed in yeast haploids, leading to mating-type switching and formation of diploid strains but is not expressed in diploids[62]. Since the HO locus is not required for growth, there is no consequence of disrupting HO in the industrial *S. cerevisiae* stain selected and described in 4.2.1 (personal communication with David Stillman, Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, USA). Furthermore, the HO-poly-KanMX4-HO plasmid obtained from ATCC contains the KanMX4 selectable marker which confers resistance to the antibiotic G418[61], thereby allowing selection of yeast transformants, and an ampicillin resistance gene for selective growth in *E. coli* (Figure 9).

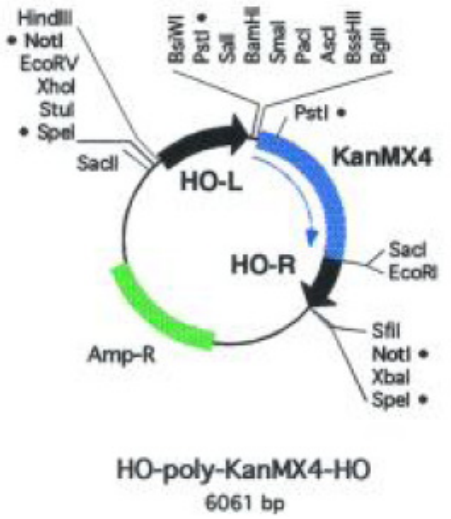

Figure 9: Map of HO-poly-KanMX4-HO[61].

4.1.3 Design and cloning of fat-1 containing yeast expression vectors

In order to achieve the objective to functionally express the fat-1 gene in wild-type yeast, which could be used industrially or as a dietary supplement in the future, several factors important for the design of the fat-1 containing yeast expression vectors had to be considered.

First, a selectable marker necessary to maintain the presence of plasmids in a cell and allowing selection of successfully transformed yeast cells had to be part of the expression plasmids. An auxotrophic marker available from most yeast expression vectors could not be employed since wild-type yeast naturally does not carry any auxotrophic mutation. Therefore, an antibiotic resistance gene, the KanMX4 selectable marker, which confers resistance to G418, was chosen for this purpose.

In addition, different expression plasmids were designed, yielding low (YIp) and high (YCp) transformation efficiencies. This guaranteed that in case transformation by integration of the desired gene into the yeast genome was not sufficient, higher transformation efficiencies could be achieved with plasmids carrying the ARS element. Furthermore, constitutive ADH and GPD promoters were chosen for the construction of different yeast expression vectors.

4.1.3.1 Cloning of p416 ADH + fat-1 + KanMX4 and pRS306 GDP + fat-1 + KanMX4

Cloning strategy

Two vectors (p416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4) were constructed as described below using p416 ADH and pRS306 GPD plasmids.
In order to allow future selection of *S. cerevisiae* transformants, the kanamycin resistance gene from the plasmid HO-poly-KanMX4-HO was cloned into p416 ADH and pRS306 GPD respectively, as illustrated in Figure 10. The fragment containing the KanMX4 cassette was cut out from HO-poly-KanMX4-HO using an EcoRI and BamHI restriction endonuclease digest and was separated from the rest of the plasmid vector by agarose gel electrophoresis. After extraction of the KanMX4 fragment from the agarose gel it was ligated into p416 ADH and pRS306 GPD pre-digested with EcoRI and BamHI, resulting in the two new constructs termed p416 ADH + KanMX4 and pRS306 GPD + KanMX4. The two new plasmids were introduced into *E. coli* by electroporation. Transformants were selected on LB plates containing ampicillin. Plasmid DNA was extracted from *E. coli* and analyzed by restriction digestion with EcoRI and BamHI endonucleases. Colonies showing a band at approximately 1,500 bp in agarose gel electrophoresis (Figure 12) after restriction digestion contained the expected KanMX4 fragment and were used for further experiments.

In a next step, the fat-1 fragment from pCR-Blunt II-TOPO + fat-1 was cloned into p416 ADH + KanMX4 and pRS306 GPD + KanMX4 (demonstrated in Figure 11). For this, each of the three plasmids was digested with XbaI and SpeI restriction endonucleases. After digestion of pCR-Blunt II-TOPO + fat-1, the fragment containing fat-1 was separated from the other digestion products by agarose gel electrophoresis and

extracted from the gel. The fat-1 fragment was then ligated into the XbaI, SpeI restriction sites of p416 ADH + KanMX4 and pRS306 GPD + KanMX4 giving rise to the two fat-1 yeast expression plasmids named p416 ADH + fat-1+ KanMX4 and pRS306 GPD + fat-1 + KanMX4. *E. coli* were transformed with the new constructs and transformants were again selected on LB plates containing ampicillin.

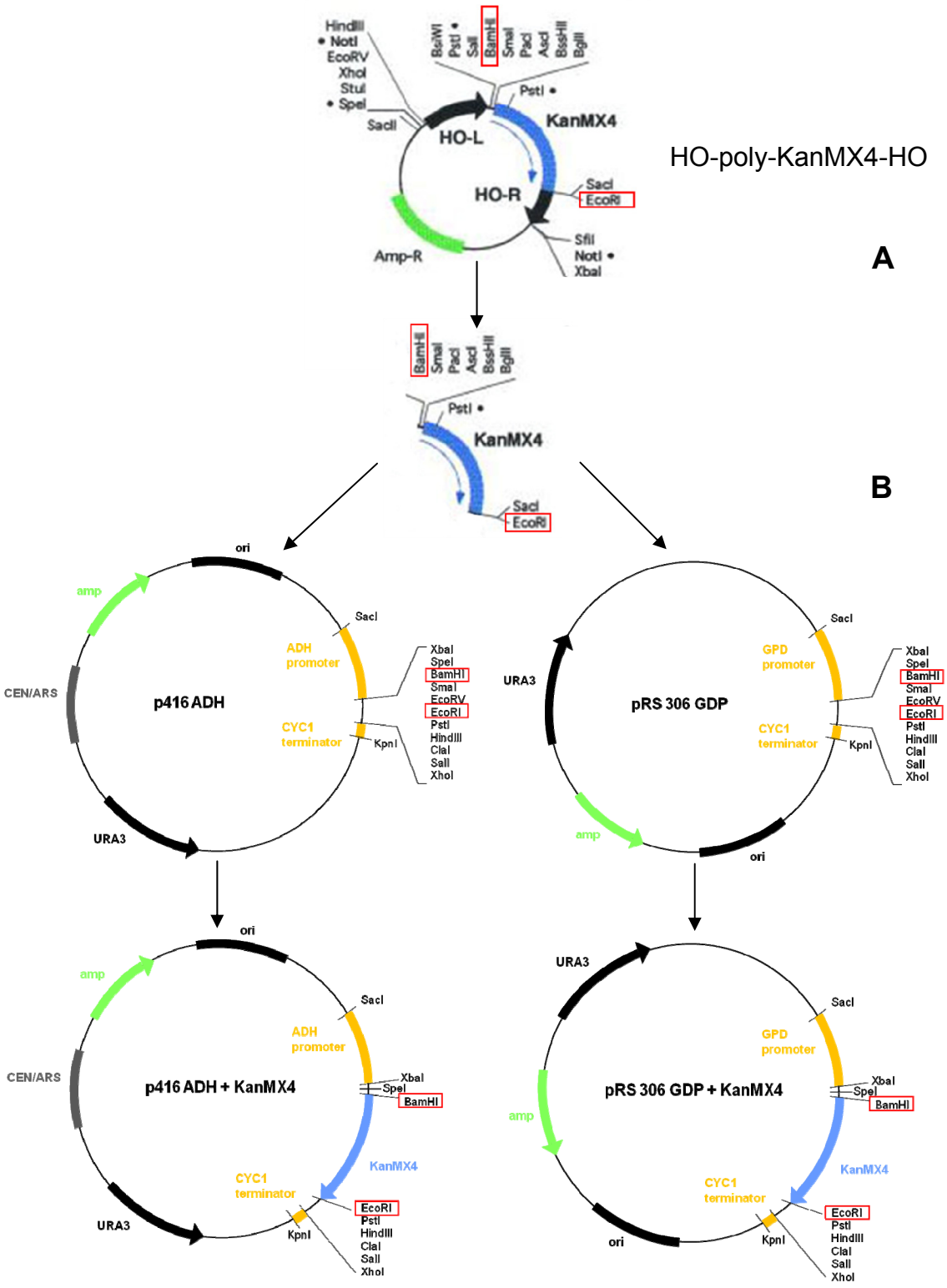

Figure 10: Cloning of the KanMX4 selectable marker into p416 ADH and pRS306 GPD.
A Digestion of HO-poly-KanMX4-HO[61] with EcoRI and BamHI followed by separation of the fragment containing the KanMX4 cassette from HO-poly-KanMX4-HO via agarose gel electrophoresis and extraction of the KanMX4 fragment from agarose gel.
B Ligation of the KanMX4 fragment into the EcoRI and BamHI restriction sites of p416 ADH and pRS306 GPD.

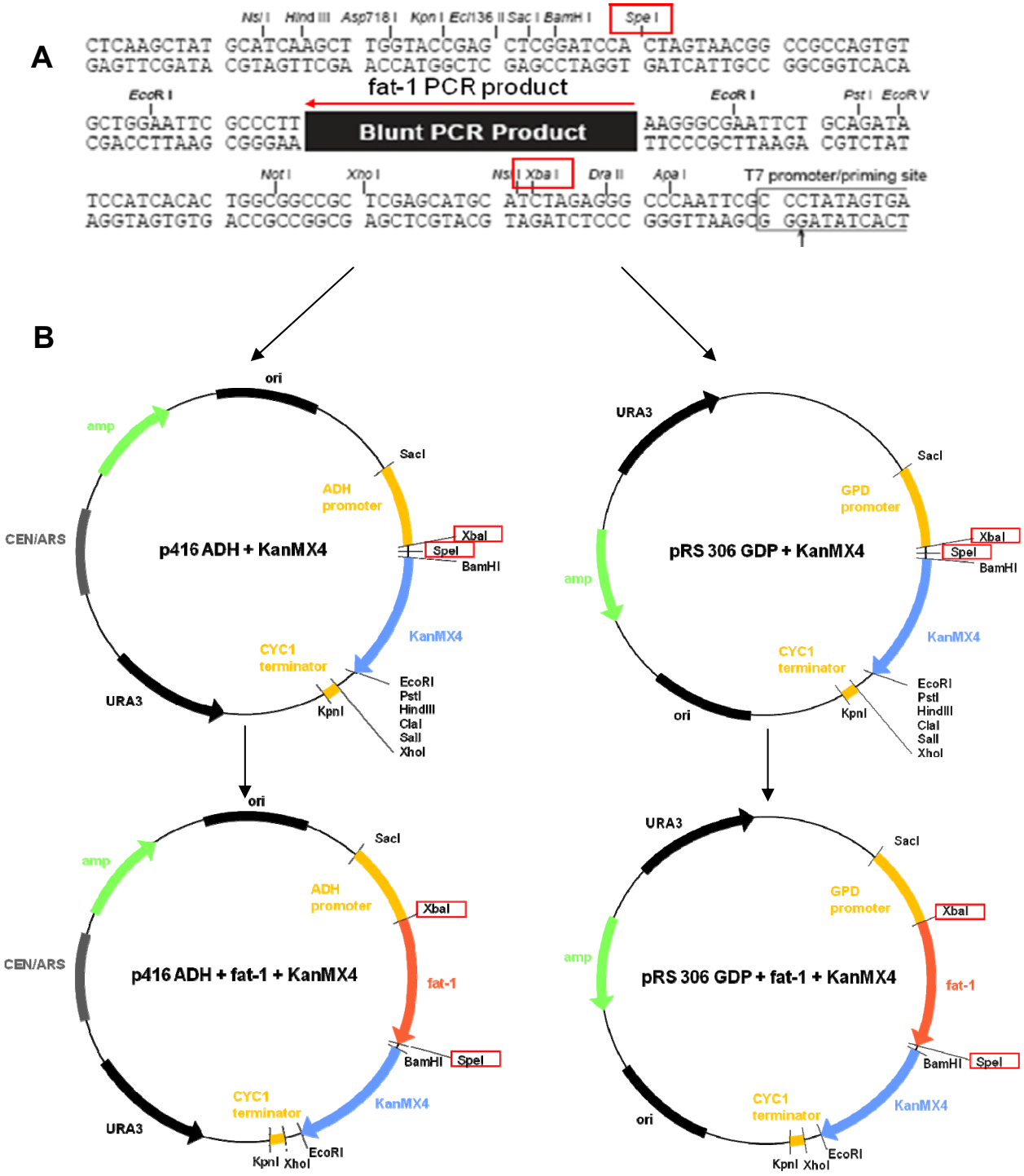

Figure 11: Cloning of the fat-1 gene into p416 ADH + KanMX4 and pRS306 GPD + KanMX4.
A Shown is the MCS of pCR-Blunt II-TOPO[56] with the integrated fat-1 fragment. Digestion of pCR-Blunt II-TOPO + fat-1 with XbaI and SpeI followed by separation of the fragment containing fat-1.
B Ligation of the fat-1 fragment into the XbaI and SpeI restriction sites of p416 ADH + KanMX4 and pRS306 GPD + KanMX4 plasmids.

In order to prove proper insertion of the fat-1 fragment into p416 ADH + KanMX4 and pRS306 GPD + KanMX4, plasmid DNA was extracted from different colonies grown on selective medium and analyzed by PCR using “fat1for” and “fat1rev” primers for amplification of an approximately 500 bp large part of the fat-1 gene. Plasmid DNA showing positive PCR results as in part A of Figure 13 was further examined by restriction digestion with XbaI and SpeI endonucleases. Colonies with plasmids showing a band at approximately 1,250 bp (Figure 13, parts B and C) after restriction digestion contained the expected fat-1 gene and were amplified for further experiments.

After verification of the plasmids constructed by PCR and restriction digest, all plasmids were sequenced at the MGH sequencing core facility. The primers used for sequencing p416 ADH + fat1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4 are detailed in Materials and Methods. Additionally, “ADH1 forward primer 1”, “ADH1 reverse primer 1”, “ADH1 forward primer 2”, and “ADH1 rv 2” were used for sequencing up- and downstream regions of the ADH promoter of p416 ADH + fat1 + KanMX4 while “GPDp rv 1” and “GPDp fw 1” allowed sequencing of the GPD promoter region of pRS306 GPD + fat-1 + KanMX4.

Gels

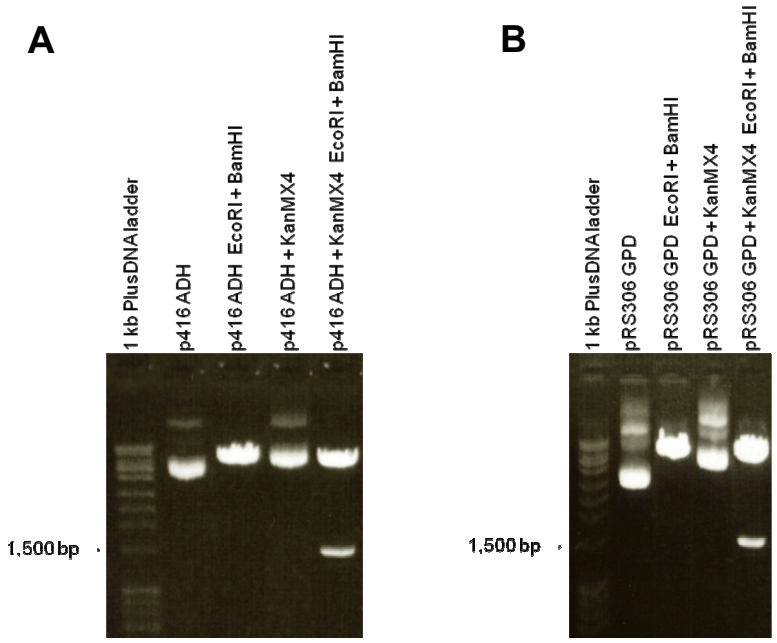

Figure 12: Confirmation of successful cloning of the KanMX4 cassette into p416 ADH and pRS306 GPD by EcoRI + BamHI double digest and agarose gel electrophoresis.
A From left to right: 1 kb Plus DNA ladder, undigested p416 ADH, p416 ADH digested with EcoRI and BamHI, undigested p416 ADH + KanMX4, p416 ADH + KanMX4 digested with EcoRI and BamHI. Fragment at around 1,500 bp is the expected insert of the KanMX4 cassette.
B From left to right: 1 kb Plus DNA ladder as marker, undigested pRS306 GPD, pRS306 GPD digested with EcoRI and BamHI, undigested pRS306 GPD + KanMX4, pRS306 GPD + KanMX4 digested with EcoRI and BamHI. Fragment at around 1,500 bp is the expected insert of the KanMX4 cassette.

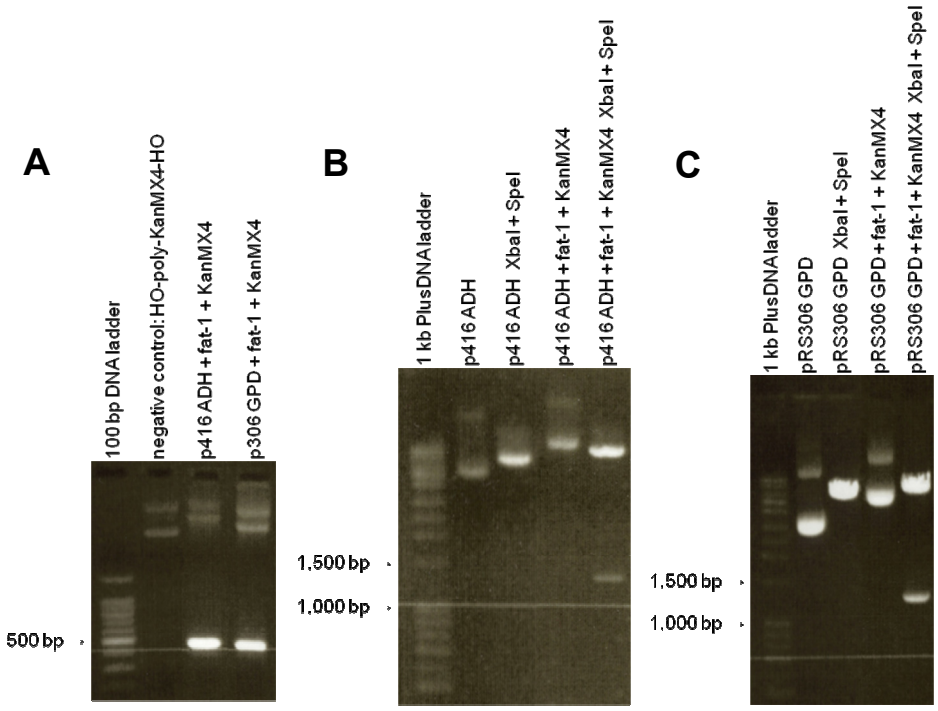

Figure 13: Verification of p416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4.
A PCR amplification of parts of fat-1 using "fat1for" and "fat1rev" primers.
From left to right: 100 bp DNA ladder, negative control (HO-poly-KanMX4-HO), p416 ADH + fat-1 + KanMX4, pRS306 GPD + fat-1 + KanMX4. P416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4 show a band at around 500 bp, confirming the presence of the fat-1 gene.
B XbaI + SpeI double digest, agarose gel electrophoresis.
From left to right: 1 kb Plus DNA ladder, undigested p416 ADH, p416 ADH digested with XbaI and SpeI, undigested p416 ADH + fat-1 + KanMX4, p416 ADH + fat-1 + KanMX4 digested with XbaI and SpeI. Fragment at around 1,250 bp is the expected insert of the fat-1 gene.
C XbaI + SpeI double digest, agarose gel electrophoresis.
From left to right: 1 kb Plus DNA ladder, undigested pRS306 GPD, pRS306 GPD digested with XbaI and SpeI, undigested pRS306 GPD + fat-1 + KanMX4, pRS306 GPD + fat-1 + KanMX4 digested with XbaI and SpeI. Fragment at around 1,250 bp is the expected insert of the fat-1 gene.

Sequencing results

Sequencing results of p416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4 are shown in Figure 14 and Figure 15.

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CATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTCAGGAATTCATGGTCGCTCAT
TCCTCAGAAGGGTTATCCGCCACGGCTCCGGTCACCGGCGGAGATGTTCTGGTTGATGCTCGTGCATCTC
TTGAAGAAAAGGAGGCTCCACGTGATGTGAATGCAAACACTAAACAGGCCACCACTGAAGAGCCACGCAT
CCAATTACCAACTGTGGATGCTTTCCGTCGTGCAATTCCAGCACACTGTTTCGAAAGAGATCTCGTTAAA
TCAATCAGATATTTGGTGCAAGACTTTGCGGCACTCACAATTCTCTACTTTGCTCTTCCAGCTTTTGAGT
ACTTTGGATTGTTTGGTTACTTGGTTTGGAACATTTTTATGGGAGTTTTTGGATTCGCGTTGTTCGTCGT
TGGACACGATTGTCTTCATGGATCATTCTCTGATAATCAGAATCTCAATGATTTCATTGGACATATCGCC
TTCTCACCACTCTTCTCTCCATACTTCCCATGGCAGAAAAGTCACAAGCTTCACCATGCTTTCACCAACC
ACATTGACAAAGATCATGGACACGTGTGGATTCAGGATAAGGATTGGGAAGCAATGCCATCATGGAAAAG
ATGGTTCAATCCAATTCCATTCTCTGGATGGCTTAAATGGTTCCCAGTGTACACTTTATTCGGTTTCTGT
GATGGATCTCACTTCTGGCCATACTCTTCACTTTTTGTTCGTAACTCTGAACGTGTTCAATGTGTAATCT
CTGGAATCTGTTGCTGTGTGTGTGCATATATTGCTCTAACAATTGCTGGATCATATTCCAATTGGTTCTG
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ACCCAATACGGGTACAAATCTCAAGTGAACTACGATTTCTTTGCCCGTTTCCTGTGGTTCAACTACAAGC
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GCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTCACAGACGCGTTGAATTGTCCCCACGCCGC
GCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAA
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CAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGC
TCGATGAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTGTATAG
TTTTTTTATATTGTAGTTGTTCTATTTTAATCAAATGTTAGCGTGATTTATATTTTTTTTCGCCTCGACA
TCATCTGCCCAGATGCGAAGTTAAGTGCGCAGAAAGTAATATCATGCGTCAATCGTATGTGAATGCTGGT
CGCTATACTGCTGTCGATTCGATACTAACGCCGCCATCCAGTGTCGAAAACGAGCTCGAATTCGATATCA
AGCTTATCGATACCGTCGACCTCGAGTCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCC
ACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAG
TTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGC
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CGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCG
CCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATC
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GGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAA
CAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACC
GCATAGGGTAATAACTGATATAATTAAATTGAAGCTCTAATTTGTGAGTTTAGTATACATGCATTTACTT
ATAATACAGTTTTTTAGTTTTGCTGGCCGCATCTTCTCAAATATGCTTCCCAGCCTGCTTTTCTGTAACG
TTCACCCTCTACCTTAGCATCCCTTCCCTTTGCAAATAGTCCTCTTCCAACAATAATAATGTCAGATCCT
GTAGAGACCACATCATCCACGGTTCTATACTGTTGACCCAATGCGTCTCCCTTGTCATCTAAACCCACAC
CGGGTGTCATAATCAACCAATCGTAACCTTCATCTCTTCCACCCATGTCTCTTTGAGCAATAAAGCCGAT
AACAAAATCTTTGTCGCTCTTCGCAATGTCAACAGTACCCTTAGTATATTCTCCAGTAGATAGGGAGCCC
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AACCGCTAACAATACCTGGGCCCACCACACCGTGTGCATTCGTAATGTCTGCCCATTCTGCTATTCTGTA
TACACCCGCAGAGTACTGCAATTTGACTGTATTACCAATGTCAGCAAATTTTCTGTCTTCGAAGAGTAAA
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TTTTTGTTCTGTGCAGTTGGGTTAAGAATACTGGGCAATTTCATGTTTCTTCAACACTACATATGCGTAT
ATATACCAATCTAAGTCTGTGCTCCTTCCTTCGTTCTTCCTTCTGTTCGGAGATTACCGAATCAAAAAAA
TTTCAAGGAAACCGAAATCAAAAAAAAGAATAAAAAAAAAATGATGAATTGAAAAGGTGGTATGGTGCAC
TCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCG
CCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGT
GTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGA

Figure 14: Sequencing results of p416 ADH + fat-1 + KanMX4. The pCR-Blunt II-TOPO fragment with the fat-1 gene is shown in red, with the start and stop codons underlined. The fragment of HO-poly-KanMX4-HO containing KanMX4 is shown in blue. The flanking parts of p416 ADH are shown in black.

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCT
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CATTTTTTTTTTATTCTTTTTTTTGATTTCGGTTTCTTTGAAATTTTTTTGATTCGGTAATCTCCGAACA
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CACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCA
GGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC
AGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCAGTTTATCATTATCAATACTCGCCATTT
CAAAGAATACGTAAATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAAATTAGCCTTTTAA
TTCTGCTGTAACCCGTACATGCCCAAAATAGGGGGCGGGTTACACAGAATATATAACATCGTAGGTGTCT
GGGTGAACAGTTTATTCCTGGCATCCACTAAATATAATGGAGCCCGCTTTTTAAGCTGGCATCCAGAAAA
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CAACTACAGAGAACAGGGGCACAAACAGGCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCC
TGGAGTAAATGATGACACAAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTA
CCTTCTGCTCTCTCTGATTTGGAAAAAGCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAATTATTCCCC
TACTTGACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTATTTCTTAAACTTC
TTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAACTTAGTTTCGACGGATTCT
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CATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTT
GATGCTCGATGAGTTTTTCTAATCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTTGTCATTTG
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TATCAAGCTTATCGATACCGTCGACCTCGAGTCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTC
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TATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTTCTGTACAGACGCGTG
TACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCG
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TGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCG
AGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTAT
TTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA
AACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAA
GAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG
TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTA
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GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG
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TATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAAC
AAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC
ATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

Figure 15: Sequencing results of pRS306 GPD + fat-1 + KanMX4. The pCR-Blunt II-TOPO fragment with the fat-1 gene is shown in red, with the start and stop codons underlined. The HO-poly-KanMX4-HO fragment containing KanMX4 is shown in blue. Black are the flanking parts of pRS306 GPD.

4.1.3.2 Cloning of HO-poly-KanMX4-HO + GPD prom + fat-1t + term

Cloning strategy

Another fat-1 expression plasmid (HO-poly-KanMX4-HO + GPD prom + fat-1t + term) for integration into the yeast genome at the HO locus was constructed using the plasmids pCE8, p426 GPD, and HO-poly-KanMX4-HO. Since HO-poly-KanMX4-HO does not contain a promoter and terminator sequence to regulate the inserted gene's expression, it had to be cloned into the plasmid along with the gene of interest. To achieve this result, the fat-1 gene was initially cloned into p426 GPD, and in a next step the fragment containing the promoter, fat-1 gene, and terminator was inserted into HO-poly-KanMX4-HO.

First of all, pCE8 was digested with EcoRI and XhoI restriction endonucleases to obtain the fat-1 gene which had been cloned into the same restriction sites of that vector. P426 GPD was digested in the same way. After restriction digest and agarose gel electrophoresis, the fragment containing the fat-1 gene was extracted from the agarose gel and ligated into the EcoRI and XhoI restriction sites in p426 GPD to yield p426 GPD + fat-1t (Figure 16).

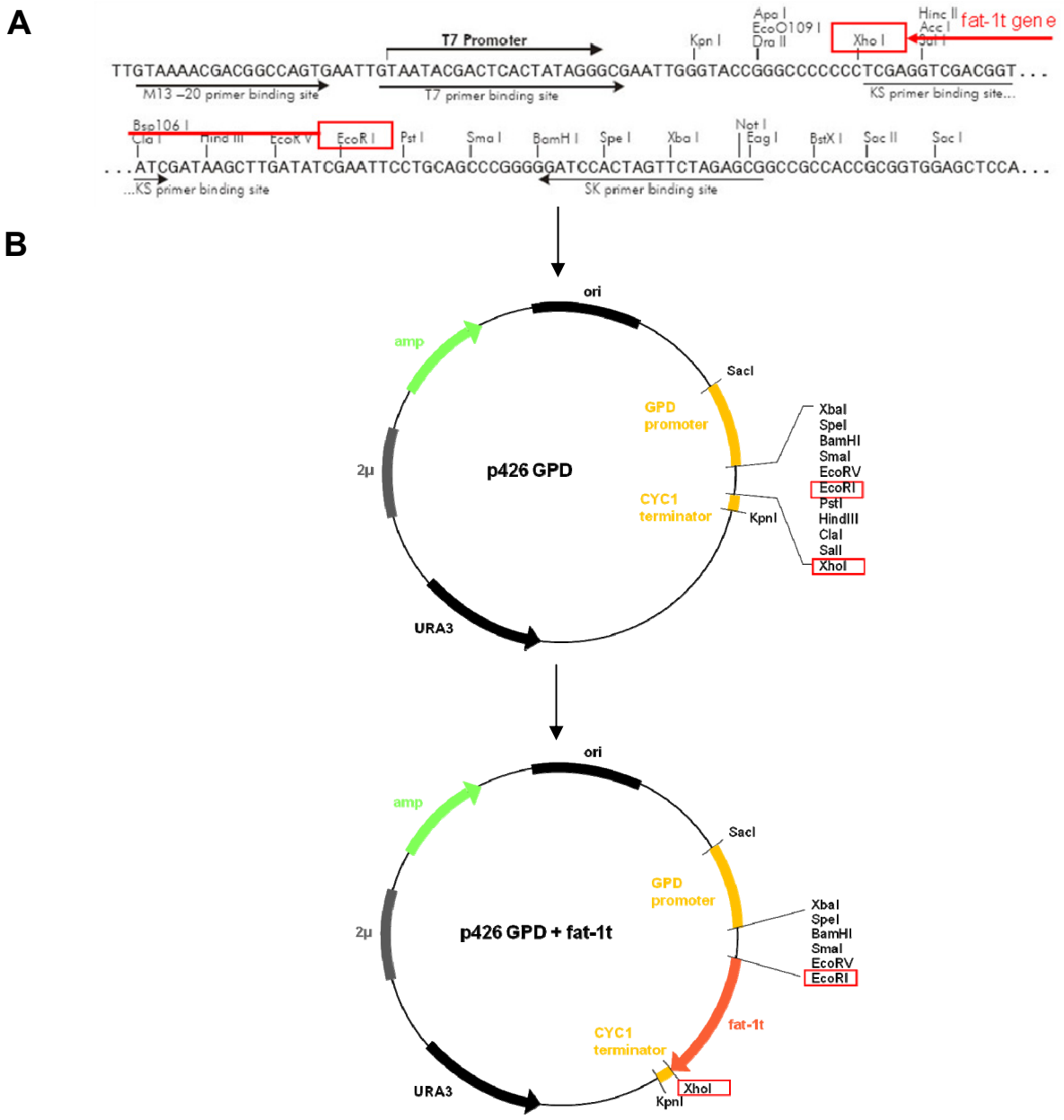

Figure 16: Cloning of the fat-1t gene into p426 GPD.
A Shown is the MCS of pCE8[55] with the integrated fat-1t fragment.
B Ligation of the truncated fat-1 fragment into the EcoRI and XhoI restriction sites of p426 GPD.

Unfortunately, due to a misunderstanding in the laboratory, it became only clear to me after successful cloning of p426 GPD + fat-1t that the fat-1 gene carries an endogenous XhoI restriction site 23 bp upstream of the gene's own stop codon. Therefore the XhoI digest produced a fragment slightly shorter than expected (Figure 17), resulting in a lack of six amino acids and the original stop codon at the C-terminus of the translation product (Figure 18). Fortunately, the p426 sequence itself carries an in-frame stop codon 14 base pairs downstream of the fat-1 gene's XhoI site.

As the active site of the fat-1 protein is not in this area of the amino acid chain but actually at the histidine-coordinated diiron center active site[21] (see Figures 18 and 19), – and in light of the fact that several different plasmid vector approaches were pursued in parallel – cloning of the expression plasmid HO-poly-KanMX4-HO + GPD prom + fat-1t + term was performed anyway and functionality assessed later on in the yeast expression experiments.

```
fat-1      1089  GAACTACGATTTCTTTGCCCGTTTCCTGTGGTTCAACTACAAGCT   1133
fat-1t     1089  GAACTACGATTTCTTTGCCCGTTTCCTGTGGTTCAACTACAAGCT   1133

fat-1      1134  CGACTATCTCGTTCACAAGACCGCCGGAATCATGCAATTCCGAAC   1178
fat-1t     1134  CGACTATCTCGTTCACAAGACCGCCGGAATCATGCAATTCCGAAC   1178

fat-1      1179  AACTCTCGAGGAGAAGGCAAAGGCCAAGTAA                 1209
fat-1t     1179  AACTCTCGAGTCATGTAATTAG                          1200
```

Figure 17: Excerpt of the comparison between the original and truncated (fat-1t) fat-1 gene sequence. The difference in nucleotides is shown in red, the stop codon sequences are underlined.

```
FAT-1      MVAHSSEGLSATAPVTGGDVLVDARASLEEKEAPRDVNANTKQATTEEPRIQ    52
FAT-1t     MVAHSSEGLSATAPVTGGDVLVDARASLEEKEAPRDVNANTKQATTEEPRIQ    52

FAT-1      LPTVDAFRRAIPAHCFERDLVKSIRYLVQDFAALTILYFALPAFEYFGLFGY   104
FAT-1t     LPTVDAFRRAIPAHCFERDLVKSIRYLVQDFAALTILYFALPAFEYFGLFGY   104

FAT-1      LVWNIFMGVFGFALFVVGHDCLHGSFSDNQNLNDFIGHIAFSPLFSPYFPWQ   156
FAT-1t     LVWNIFMGVFGFALFVVGHDCLHGSFSDNQNLNDFIGHIAFSPLFSPYFPWQ   156

FAT-1      KSHKLHHAFTNHIDKDHGHVWIQDKDWEAMPSWKRWFNPIPFSGWLKWFPVY   208
FAT-1t     KSHKLHHAFTNHIDKDHGHVWIQDKDWEAMPSWKRWFNPIPFSGWLKWFPVY   208

FAT-1      TLFGFCDGSHFWPYSSLFVRNSERVQCVISGICCCVCAYIALTIAGSYSNWF   260
FAT-1t     TLFGFCDGSHFWPYSSLFVRNSERVQCVISGICCCVCAYIALTIAGSYSNWF   260

FAT-1      WYYWVPLSFFGLMLVIVTYLQHVDDVAEVYEADEWSFVRGQTQTIDRYYGLG   312
FAT-1t     WYYWVPLSFFGLMLVIVTYLQHVDDVAEVYEADEWSFVRGQTQTIDRYYGLG   312

FAT-1      LDTTMHHITDGHVAHHFFNKIPHYHLIEATEGVKKVLEPLSDTQYGYKSQVN   364
FAT-1t     LDTTMHHITDGHVAHHFFNKIPHYHLIEATEGVKKVLEPLSDTQYGYKSQVN   364

FAT-1      YDFFARFLWFNYKLDYLVHKTAGIMQFRTTLEEKAKAK                  402
FAT-1t     YDFFARFLWFNYKLDYLVHKTAGIMQFRTTLESCN                     399
```

Figure 18: Comparison between the original and truncated (FAT-1t) FAT-1 protein sequence. The difference in amino acids is printed in red. Highlighted and framed are the histidine-rich motifs coordinating the diiron center at the active site.

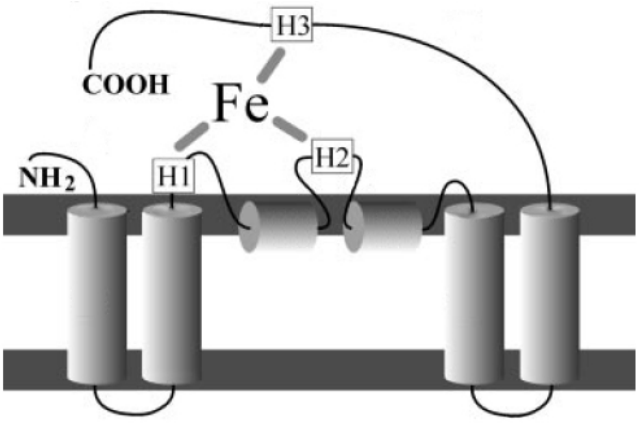

Figure 19: Topological model of membrane-bound fatty acid desaturases such as FAT-1, modified from that of Satasa et al.[21].
H1, H2, and H3 depict the location of histidine-rich motifs involved in coordinating a putative diiron site (Fe).

After electroporation and plasmid DNA extraction from *E. coli* colonies grown under ampicillin selection, PCR and restriction digestion was performed to confirm insertion of fat-1 (see Figure 21).

After that, plasmid DNA from positive clones was amplified and used for cloning of a GPD promoter-fat-1t-CYCI terminator fragment into HO-poly-KanMX4-HO (Figure 20). This fragment was cut from p426 GPD + fat-1t using the SacI and KpnI restriction sites. As visualized in part A of Figure 20, sticky ends created by restriction digestion needed to be blunted in order to produce blunt ends suitable for blunt-end ligation into HO-poly-KanMX4-HO. The DNA polymerase I, large (Klenow) fragment used is a proteolytic product of *E. coli* DNA polymerase I which retains polymerization and 3'→5' exonuclease activity but has lost 5'→3' exonuclease activity[63, 64]. It is able to fill-in 5' overhangs and can remove 3' overhangs, thereby forming blunt ends.
At the same time, HO-poly-KanMX4-HO was digested with SmaI restriction enzyme to obtain a blunt-ended linear plasmid. In order to prevent the blunt ends from self-ligation – again forming a circular vector – ends of the digested HO-poly-KanMX4-HO were dephosphorylated using the calf intestinal alkaline phosphatase (CIP) which catalyzes the removal of 5' phosphate residues required for ligation[65]. The blunt-ended GPD promoter-fat-1t-CYCI terminator fragment was then ligated into the digested and dephosphorylated HO-poly-KanMX4-HO plasmid resulting in the new fat-1 yeast integration plasmid HO-poly-KanMX4-HO + GPD prom + fat-1t + term (see Figure 20, parts B and C).

The new plasmid was introduced into *E. coli* by electroporation and plasmid DNA from colonies grown on LB plates containing ampicillin was screened for the fat-1 insert by PCR using “fat1for” and “fat1rev” primers. Colonies showing amplification of the 500 bp fragment (Figure 22, part A) were further examined to rule out multiple insertion of the GPD promoter-fat-1t-CYCI terminator fragment. For this, digestion by BamHI, SpeI, and NotI restriction endonucleases was performed (Figure 22, part B). The original HO-poly-KanMX-HO plasmid carries one BamHI and two SpeI and NotI restriction sites, leading to a 6,000 bp fragment after BamHI digest and two fragments of 3,000 bp after SpeI and NotI digest, respectively. Since the new anticipated plasmid contained two BamHI restriction sites, the digest was expected to show two bands, one around 1,400 bp, another 6,700 bp in size. By successful cloning of one GPD promoter-fat-1t-CYCI terminator fragment into HO-poly-KanMX4-HO one additional SpeI restriction site could be introduced into the new construct, resulting in three fragments – approximately 2,300, 2,700, and 3,000 bp large. NotI digest was performed to prove insertion of the expected fragment of 2,100 bp, resulting in two fragments of around 3,100 and 5,000 bp size. Colonies with plasmid DNA showing the anticipated results were amplified for further use.

After verification of the constructs by PCR and restriction digest, HO-poly-KanMX4-HO + GPD prom + fat-1t + term was sequenced at the MGH sequencing core facility. The plasmid was only partly sequenced in order to verify the correct insertion of the fat-1 cassette using “forward primer in HO-L”, “reverse primer in KanMX PstI”, “fat-1 seq low 1”, “fat-1 seq up 1”, and “fat-1 seq up 2” primers.

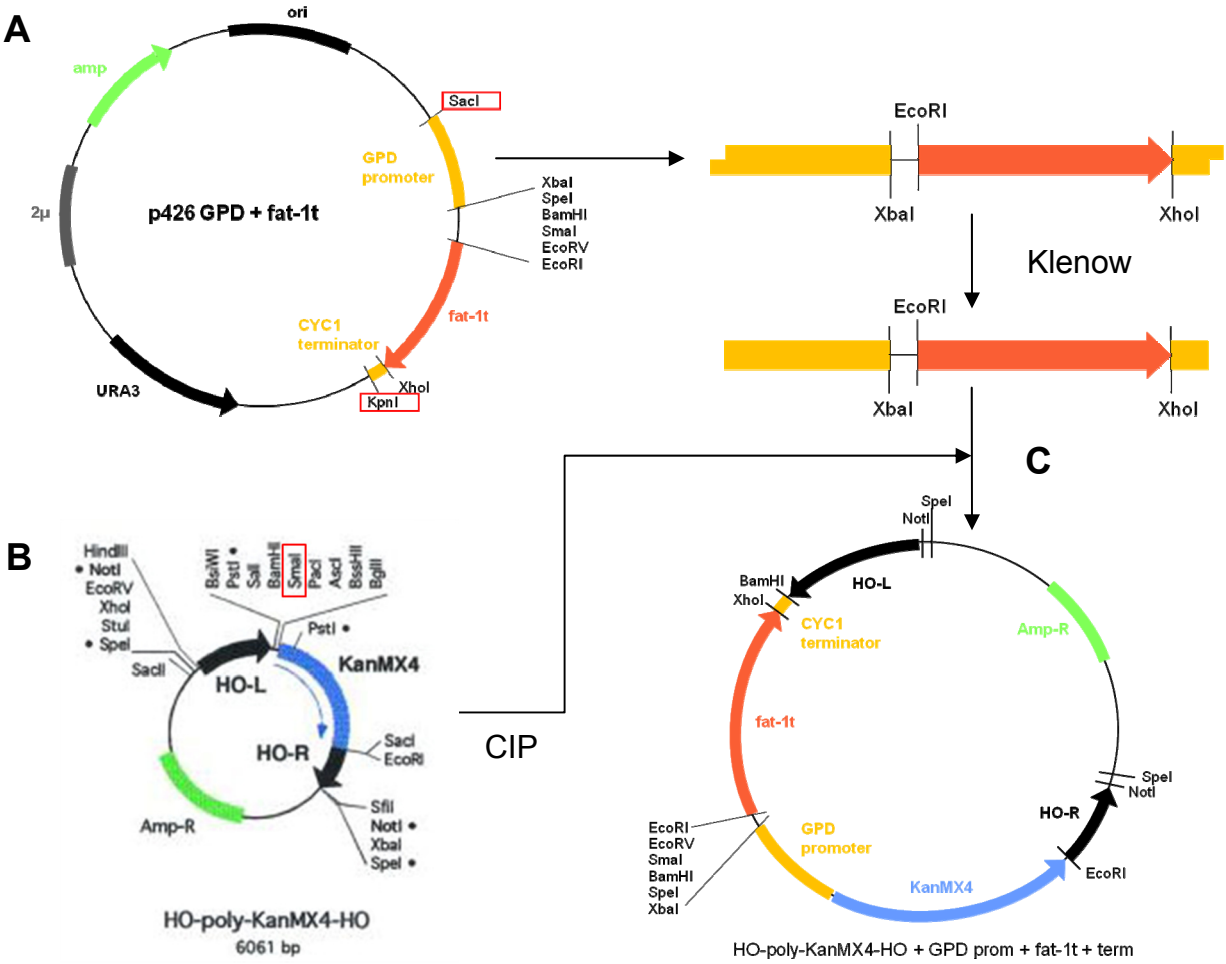

Figure 20: Cloning of the fragment containing the GPD promoter, truncated fat-1 gene, and CYCI terminator into HO-poly-KanMX4-HO.
A Restriction digest of p426 GPD + fat-1t with SacI and KpnI and blunting of the sticky ends using DNA polymerase I, large (Klenow) fragment.
B Restriction digest of HO-poly-KanMX4-HO[61] with SmaI and dephosphorylation of the linear vector using CIP.
C Ligation of the blunted GPD promoter-fat-1t-CYCI terminator fragment with the digested and dephosphorylated HO-poly-KanMX4-HO plasmid.

Gels

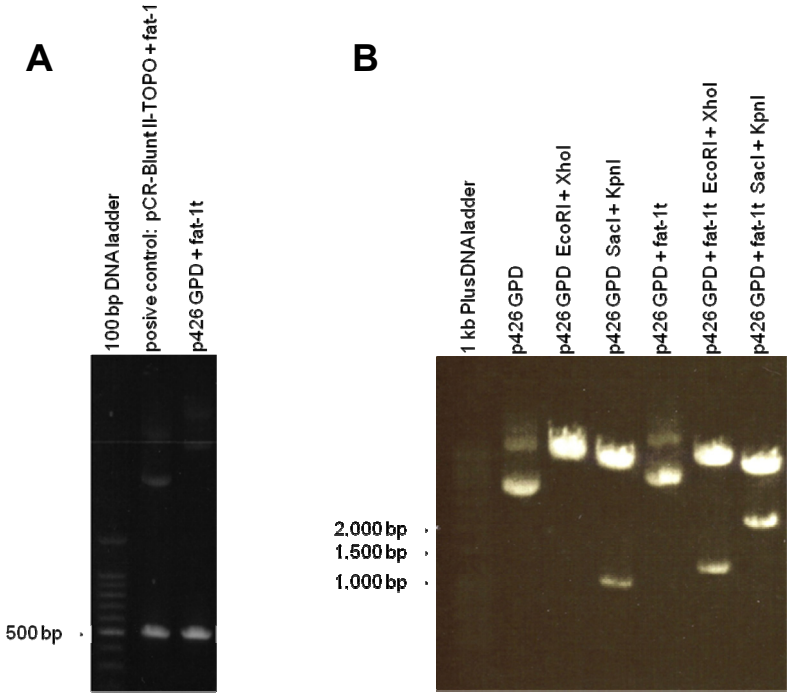

Figure 21: Confirmation of fat-1 insertion into p426 GPD + fat-1t.
A PCR amplification of parts of fat-1 using "fat1for" and "fat1rev" primers.
From left to right: 100 bp DNA ladder, positive control (pCR-Blunt II-TOPO + fat-1), p426 GPD + fat-1t. Band at around 500 bp in p426 GPD + fat-1t confirms presence of the fat-1 gene.
B EcoRI + XhoI and SacI + KpnI double digests, agarose gel electrophoresis.
From left to right: 1 kb Plus DNA ladder, undigested p426 GPD, p426 GPD digested with EcoRI and XhoI, p426 GPD digested with SacI and KpnI, undigested p426 GPD + fat-1t, p426 GPD + fat-1t digested with EcoRI and XhoI, p426 GPD + fat-1t digested with SacI and KpnI. Band at around 900 bp in p426 GPD SacI + KpnI is the GPD promoter, CYCI terminator fragment. Band at around 1,200 bp in p426 GPD + fat-1t Eco RI + XhoI shows the expected insert of the fat-1 gene. Band at around 2,100 bp in p426 GPD + fat-1t SacI + KpnI is the fragment containing GPD promoter, fat-1t gene, and CYCI terminator.

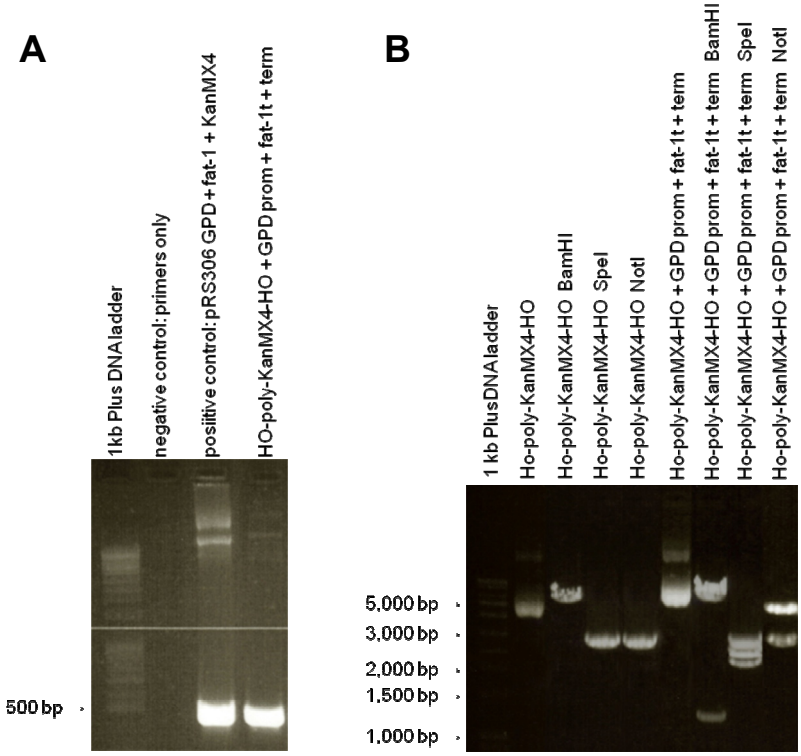

Figure 22: Verification of correct insertion of the GPD promoter-fat-1t-CYCI terminator fragment in HO-poly-KanMX4-HO.
A PCR amplification of parts of fat-1 using "fat1for" and "fat1rev" primers.
From left to right: 1 kb Plus DNA ladder, negative control (primers only), positive control (pRS306 GPD + fat-1 + KanMX4), HO-poly-KanMX4-HO + GPD prom + fat-1t + term. Band at approximately 500 bp in HO-poly-KanMX4-HO + GPD prom + fat-1t + term confirms presence of the fat-1 gene.
B BamHI, SpeI, and KpnI restriction digests.
From left to right: 1 kb Plus DNA ladder, undigested HO-poly-KanMX4-HO, HO-poly-KanMX4-HO digested with BamHI, HO-poly-KanMX4-HO digested with SpeI, HO-poly-KanMX4-HO digested with NotI, undigested HO-poly-KanMX4-HO + GPD prom + fat-1t + term, HO-poly-KanMX4-HO + GPD prom + fat-1t + term digested with BamHI, HO-poly-KanMX4-HO + GPD prom + fat-1t + term digested with SpeI, HO-poly-KanMX4-HO + GPD prom + fat-1t + term digested with NotI.
Band at 6,000 bp in HO-poly-KanMX4-HO BamHI shows the plasmid size of the linear vector. Bands at 3,000 bp HO-poly-KanMX4-HO digested with SpeI and NotI show two fragments of equal size. In contrast, HO-poly-KanMX4-HO + GPD prom + fat-1t + term BamHI shows an additional band at around 1,400 bp, HO-poly-KanMX4-HO + GPD prom + fat-1t + term SpeI two bands of new size at around 2,400 and 2,700 bp, and HO-poly-KanMX4-HO + GPD prom + fat-1t + term NotI an extra one at around 5,000 bp confirming proper insertion of one GPD promoter-fat-1t-CYCI terminator fragment in HO-poly-KanMX4-HO.

Sequencing results

The sequencing results of HO-poly-KanMX4-HO + GPD prom + fat-1t + term are given in Figure 23.

agctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccgcggaagcataaag
tgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttcc
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gtcat

Figure 23: Sequencing results of HO-poly-KanMX4-HO + GPD prom + fat-1t + term. Shown in red is the truncated fat-1 sequence from pCE8, the start and stop codon sequences are underlined. Sequences from p426 GPD are shown in green. In black are the parts of HO-poly-KanMX4-HO, capital letters show parts of the vector verified by sequencing, whereas lower case letters present the rest of the sequence which was added for illustration here.

As already discussed above, sequencing of HO-poly-KanMX4-HO + GPD prom + fat-1t + term revealed that the EcoRI and XhoI restriction digest from pCE8 to obtain the fat-1 gene had produced a fragment lacking the last 21 nucleotides of the fat-1 gene. As shown in the sequence in Figures 17 and 23, there is a stop codon present in the p426 GPD sequence that was able to take over from the endogenous stop codon of the protein, leading to an anticipated transcription and translation product of nearly identical size and with the active site[21] unharmed (see Figures 18 and 19).

4.2 Expression of fat-1 in yeast

4.2.1 Choice of yeast strain

In order to functionally express the fat-1 gene in industrially used wild-type *Saccharomyces cerevisiae*, such a strain needed to be identified (Table 2)[66]. Criteria for strain selection were commercial use in industrial baking, the availability from a company, and diploid state of the yeast.

The *S. cerevisiae* strain Meyen ex E.C. Hansen described as Dutch baker's yeast which is available from ATCC (ATCC number 6037) meets all requirements. Its diploid state was confirmed since it was reported to produce ascospores[67]. The strain was furthermore referred to as transformation host[68].

4.2.2 Yeast transformation

Transformation of *S. cerevisiae* was tested using two different methods – electroporation and lithium acetate. After initial use of different media – YM and YPD medium – positive transformants were selected on YPD plates containing G418, whereas no colonies could be detected on selective YM plates.

In general, transformation was of rather low yield – only few colonies grew after two to five days of incubation subsequent to transformation with p416 ADH + fat-1 + KanMX4 and the NotI restriction enzyme-linearized HO-poly-KanMX4-HO + GPD prom + fat-1t + term. Transformation failed entirely for pRS306 GPD + fat-1 + KanMX4.

Collection number	Reference or source	Description
YS1	ATCC 60223	Super active bakers' yeast
YS2	NL67, N1	Australian bakers' yeast
YS3	ATCC 46523	Bakers' yeast
YS4	ATCC 6037	Dutch bakers' yeast
YS5	Robin Hood	Canadian bakers' yeast
YS6	ATCC 60222	Egyptian bakers' yeast
YS7	Gist Brocades	European bakers' yeast
YS8	Dolomitti	Italian bakers' yeast
YS9	Le Saffre	Singaporean bakers' yeast
YS10	Gold star SA	South African bakers' yeast
YS11	Pakmaya Turkey	Turkish bakers' yeast
YS12	Red star universal foods	USA bakers' yeast
YS13	ATCC 62914	Frozen unbaked bread dough
YS14	NCYC 996	US Patent 4,396,632
YS15	Sungil Korea	Korean bakers' yeast
YS16	Fleishmanns	USA bakers' yeast
YS17	Le Saffre Gold	European bakers' yeast
YS18	Oetker germ	Austrian bakers' yeast
YS19	Basic food corporation	Philippine bakers' yeast
YS20	Julstar korea	Korean bakers' yeast
YS21	Indian yeast company	Indian bakers' yeast
YS22	Asahi	Japanese bakers' yeast
YS23	ATCC 66349	Wild yeast from candied apple
YS24	ATCC 4127	Wild yeast from concord grapes
YS25	ATCC 2360	Wild yeast from kefir
YS26	CBS 7539	Wild yeast from turbid beer
YS27	ATCC 66348	Wild yeast from soil (Japan)
YS28	ATCC 60493	Wild yeast from canned strawberries
YS29	ATCC 42413	Laboratory yeast strain
YS30	ATCC 9896	Laboratory yeast strain
YS31	CBS 2354	Wild yeast from hospital
YS32	ATCC 38554	Wild yeast from fruit juice
YS33	ATCC 56069	Wild yeast fermented cavendish banana
YS34	NCYC 762	Wild yeast from fermentation
YS35	CBS 7002	Wild yeast from alpechin
YS36	ATCC 20867	Alcohol tolerant yeast strain
YS37	X2180–1A	S288C haploid

Table 2: List of yeast strains to compare the fermentative capacities of industrial baking and wild-type yeasts of the species *Saccharomyces cerevisiae* in different sugar media[66].

4.2.3 PCR from yeast cells

Colonies selected on YPD plates containing G418 were cultured in liquid medium and tested for fat-1 by PCR.

Unfortunately, the results for PCR from yeast cells transformed with p416 ADH + fat-1 + KanMX4 demonstrated loss of the plasmid over time. While some colonies showed a fat-1 PCR fragment initially (Figure 24, part A), the PCR band lost intensity and disappeared with increasing generation number.

Reproducible results were achieved in cultures transformed with HO-poly-KanMX4-HO + GPD prom + fat-1t + term (Figure 24, part B).

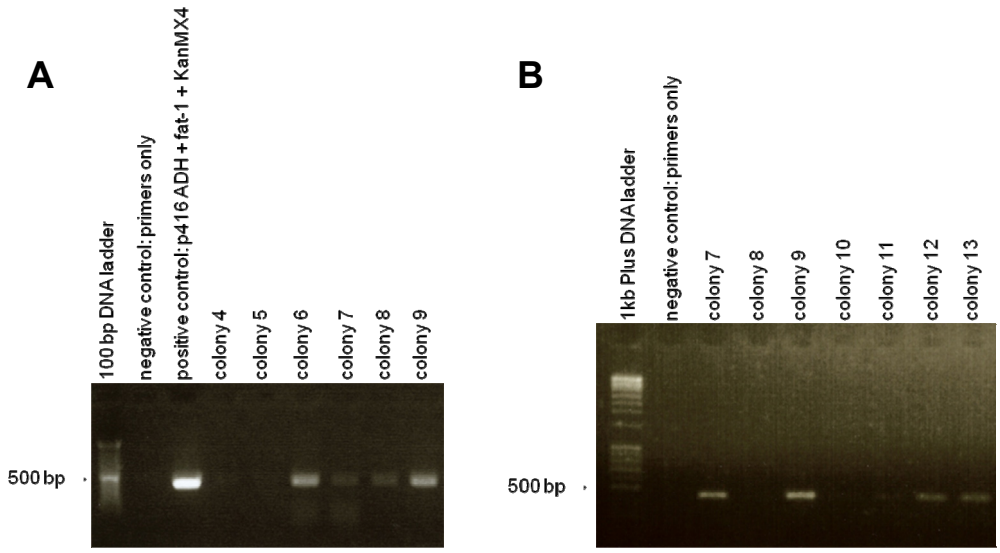

Figure 24: PCR from transformed yeast colonies amplifying parts of fat-1 using "fat1for" and "fat1rev" primers.
A PCR from yeast colonies transformed with p416 ADH + fat-1 + KanMX4.
From left to right: 100 bp DNA ladder, negative control (primers only), positive control (p416 ADH + fat-1 + KanMX4), yeast cells transformed with p416 ADH + fat-1 + KanMX4 colony 4, colony 5, colony 6, colony 7, colony 8, colony 9.
Colonies 6-9 show the approximately 500 bp large amplicon, thereby confirming transformation with the fat-1 gene.
B PCR from yeast colonies transformed with HO-poly-KanMX4-HO + GPD prom + fat-1t + term.
From left to right: 1 kb Plus DNA ladder, negative control (primers only), yeast cells transformed with HO-poly-KanMX4-HO + GPD prom + fat-1t + term colony 7, colony 8, colony 9, colony 10, colony 11, colony 12, colony 13. A positive control was also run but is not shown here.
Colonies 7, 9, 12, and 13 show bands at around 500 bp verifying presence of the fat-1 gene.

4.2.4 Fatty acid analysis from yeast

After incubation of yeast cultures showing positive PCR results with linoleic and arachidonic acid, yeast samples were analyzed by gas chromatography to detect different long-chain fatty acids. Gas chromatograms (Figure 25) of wild-type and transformed yeast samples were compared and the fatty acid composition was determined using fatty acid standards as reference. In wild-type yeast, only endogenous yeast fatty acids and the added linoleic acid and arachidonic acid were detected by GC, whereas two new peaks appeared in the transformed yeast samples. One of them was identified as alpha-linolenic acid (18:3 n-3) as the corresponding fatty

acid from omega-3 desaturation of linoleic acid (18:2 n-6). The other peak could not be distinguished by comparison with fatty acid standards. By confirming presence of alpha-linolenic acid as desaturation product from linoleic acid in the transformed yeast samples, functionality of the omega-3 fatty acid desaturase encoded by the *C. elegans* fat-1 gene was verified. However, desaturation of arachidonic acid to eicosapentaenoic acid was not detected.

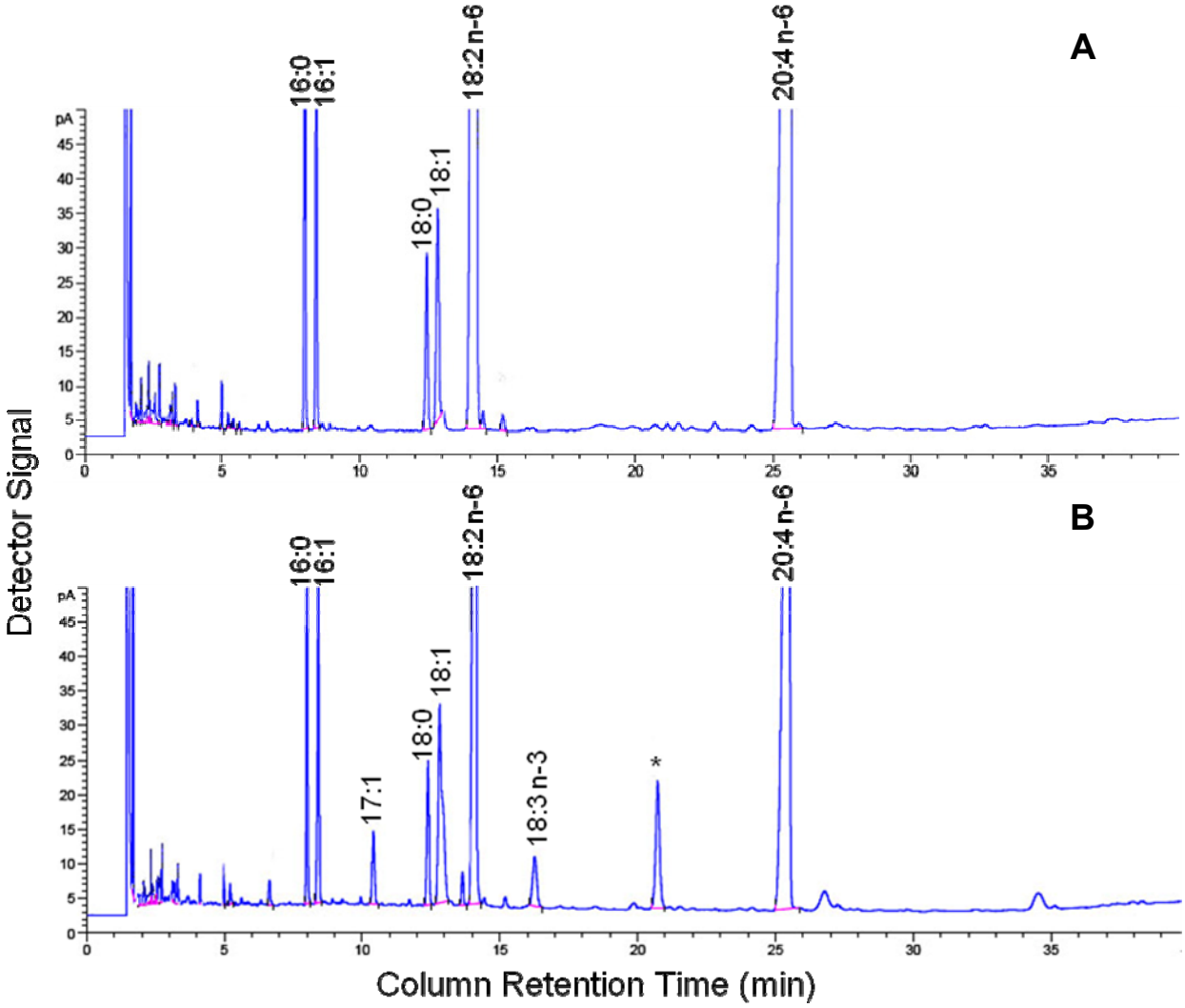

Figure 25: Comparison of the results (gas chromatograms) of wild-type and transformed yeast cells after three days of incubation with linoleic and arachidonic acid.
A Wild-type *S. cerevisiae*. Peaks of the exogenously added linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) as well as the endogenous oleic acid (18:1), stearic acid (18:0), palmitoleic acid (16:1), palmitic acid (16:0), and a range of medium chain fatty acids (not labeled) were detected by GC. In preliminary experiments, heptadecenoic acid (17:1) was also identified as endogenous yeast fatty acid but was not found in the sample shown here.
B *S. cerevisiae* transformed with the fat-1 gene from *C. elegans.* In addition to the peaks detected in **A** and the endogenous heptadecenoic acid (17:1), two other peaks were found in the transformed yeast cultures. One of them could be identified as alpha-linolenic acid (18:3 n-3), the corresponding fatty acid from desaturation of linoleic acid (18:2 n-6). The other peak (labeled with *) could not be distinguished by comparison with fatty acid standards.

5 DISCUSSION

According to several studies, today's Western diets are deficient in omega-3 fatty acids, but include too much omega-6 fatty acids[6-8]. This shift in the omega-6 to omega-3 fatty acid ratio has been implicated in the high incidence of coronary heart disease, hypertension, diabetes, and some types of cancer in the Western world[7, 13], and beneficial effects of omega-3 PUFA have been shown for numerous major diseases in many human studies[7, 8].

Although omega-6 fatty acids are highly abundant in our Western diet, elevating tissue concentrations of omega-3 PUFA relies on continuous dietary intake or supplementation of fats rich in omega-3 fatty acids since omega-6 PUFA cannot be converted into omega-3 fatty acids in the human body[2, 8, 28].

The large-scale supply of omega-3 fatty acids is problematic as they are mainly found in fish and as overfishing leads to depletion of edible fish in the oceans[8]. Additionally, some fish species were found to carry toxic amounts of mercury or other environmental toxins[69]. Encapsulated fish oil supplements have been developed and promoted[70] for providing the benefits of omega-3 fatty acids without the risk of toxicity, but are unlikely to be suited to lifetime daily use because of possible caloric excess[23] and individual intolerance to ingesting high amounts of fish oils[69].

The study presented here demonstrates the creation of expression vectors and functional expression of the *Caenorhabditis elegans* fat-1 gene in a wild-type *Saccharomyces cerevisiae* strain used in the baking industry to establish a possibility for the large scale formation of omega-3 fatty acids in yeast. The presented approach provides a basis for the development of transgenic omega-3 producing organisms either as dietary additives or for industrial utilization and proposes an alternative approach to supply humans with omega-3 PUFA in the future.

5.1 Expression of fat-1 in yeast

In the study presented here, a wild-type *Saccharomyces cerevisiae* strain industrially used in the baking industry was engineered to functionally express the *Caenorhabditis elegans* fat-1 gene. To achieve this, three different yeast expression plasmids containing the fat-1 gene were constructed. These plasmids were then introduced into

the yeast cells by two different modes of transformation. Successful transformation was verified for two of the constructs by growth on selective medium and PCR of the yeast cells. Finally, functional expression of one construct was confirmed by GC analysis of fatty acids from the yeast cultures.

Yeast expression plasmids

Different yeast expression plasmids – centromeric (YCp) and integration (YIp) plasmids – were designed for yeast transformation. Distinct properties of these expression vectors regarding transformation efficiency (high in YCp, low in YIp) and stability were exploited for the experiments.

Two different constitutive promoters – ADH and GPD – were selected for the construction of different yeast expression vectors. Constitutional promoters were chosen over inducible promoters, as the aim of this study was constant fat-1 protein expression to enable permanent omega-3 desaturase activity in the transgenic yeasts.

The *Caenorhabditis elegans* fat-1 gene was obtained in two different ways, by PCR and restriction digest from pCE8.
PCR has the advantage of introducing nucleotide sequences in front and behind the gene of interest by using specially designed primers. In this way, certain restriction sites were created for easier construction of the expression vector. Although PCR sometimes leads to inaccuracies due to a certain error rate of the DNA polymerase, the sequencing results showed no such errors in these experiments.
In contrast, restriction digest with XhoI from pCE8 to obtain the fat-1 gene created a slightly truncated fragment (lacking the last 21 nucleotides of the fat-1 coding sequence). In how far the missing part effects transcription, translation, and functionality of the protein cannot be answered definitively by the experiments shown here. The most probable assumption in light of the GC profiles obtained with the truncated gene is that it does not have an effect at all, as the difference in length and amino acid sequence of the resulting protein is minimal (see Figure 18). Since the GC results revealed LA to ALA conversion after fatty acid incubation of the yeast transformed with the truncated expression plasmid, functionality of the protein was indeed confirmed.

Unfortunately, transformation with the other two fat-1-containing expression plasmids did not result in stable yeast transformants with the – accurate and full length – fat-1 gene. Further experiments to retry transformation with p416 ADH + fat1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4 will be necessary to accomplish this aim in the future.

Yeast transformation

Two different methods of transformation of the wild-type *S. cerevisiae* strain with the designed yeast expression plasmids were chosen. Electroporation and chemical transformation using lithium acetate both led to similar, albeit rather low, transformation efficiencies for the same yeast expression plasmid. Only transformation with one plasmid (pRS306 GPD + fat-1 + KanMX4) was unsuccessful; to retry transformation with pRS306 GPD + fat-1 + KanMX4 will be an aspect of future work.

Furthermore, yeast transformation was only successful on selective plates prepared from YPD medium: Transformants only grew on YPD but not on YM plates containing the same concentration of G418. The reason for this different yield of transformants could be the amount of yeast extract in the growth medium, of which YM medium contains less than one third of that present in YPD medium (personal communication with Bernard Murray, Abbott Laboratories, Chicago, USA). Yeast extract is an autolysate of yeast cells used in preparing microbiological culture media and provides vitamins, amino acids, and nucleotide precursors which the cell would normally synthesize de novo, and yeasts grow much more rapidly in the presence of yeast cell extract hydrolysates[71]. The content of dextrose as supplier of carbon and free energy for biosynthesis[72] is also twice as high in YPD compared to YM medium. In view of these facts it seems conclusive that growth of transformants is facilitated on YPD selective plates.

PCR from yeast cells

Transformed yeast colonies selected on YPD plates containing G418 were tested for the fat-1 gene by PCR. Reproducible and stable results were only achieved in yeast cultures transformed with HO-poly-KanMX4-HO + GPD prom + fat-1 + term. The PCR results from yeast cells transformed with p416 ADH + fat-1t + KanMX4 were

inconsistent with loss of the fat-1 band over longer time spans that the cultures were grown. Especially exposure to varying conditions (e.g. big temperature shifts) resulted in irreproducible results in these cultures. It is therefore likely that the plasmid was lost during cell division, especially when growth conditions were suboptimal. As previously described[50], plasmid stability in recombinant yeast is affected by many genetic and environmental factors, and only integration of the gene into the yeast genome seems to result in relatively stable clones[48, 50, 52].

Fatty acid incubation and analysis

Yeast cultures of the only effective transformation process with HO-poly-KanMX4-HO + GPD prom + fat-1t + term (as demonstrated by repeated positive PCR results for fat-1) were then incubated with the omega-6 PUFA substrates linoleic and arachidonic acid. After three days, yeast samples were analyzed according to their fatty acid content by gas chromatography. Comparison of fatty acid composition of wild-type and transformed yeast samples after fatty acid incubation demonstrated the additional presence of ALA (18:3 n-3) as the corresponding fatty acid from desaturation of LA (18:2 n-6). The expected EPA (20:5 n-3) peak as desaturation product from AA (20:4 n-6) could not be detected by GC analysis. Instead, a second peak which could not be distinguished by comparison with fatty acid standards was found. It can be supposed that the unidentified fatty acid detected by GC analysis is a product of degradation or oxidation of EPA. This assumption results from the fact that PUFA are highly unstable and susceptible to oxidation[28, 73, 74]. Oxidation of lipids is the major cause for the deterioration of PUFA. They form hydroperoxides in the presence of oxygen, which are then further decomposed into a variety of secondary products[75]. When monitoring the stability of unsaturated fatty acids, Boyd et al.[75] found that EPA and DHA were oxidized rapidly, whereas LA was much more stable. They therefore concluded that susceptibility to oxidation appeared to be dependent on the number of double bonds of the fatty acids. These findings are consistent with the research results and give an acceptable explanation why ALA but not EPA could be detected after desaturation of the parental omega-6 fatty acids in yeast cultures expressing the *C. elegans* fat-1 gene. More studies will be necessary in the future to optimize the conditions of these experiments and to validate the formation of omega-3 PUFA observed in the transformed yeast strain.

5.2 Critical assessment of future application

Antibiotic selective marker in food industry

One of the critical aspects when these engineered organisms would gain access to the food industry is the utilization of antibiotics as a selective marker. Although broadly employed in other fields (e.g. food animal production or farming), there are general concerns about utilization of antibiotics in food industry, as this could contribute to increasing resistance to antibiotics in humans. The U.S. Food and Drug Administration has stated[76] that safety of a protein encoded by an antibiotic resistance marker gene should be assessed according to potential toxicity of the protein, the potential to elicit allergenic reactions, and whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficiency of orally administered antibiotics. Further information on 1) whether the antibiotic is an important medication, 2) whether it is frequently used, 3) whether it is orally administered, 4) whether it is unique, 5) whether there would be selective pressure for transformation to take place, and 6) the level of resistance to the antibiotic present in bacterial populations should also be taken into account. Their investigation regarding the utilization of the kanamycin resistance gene – as it was used in the studies presented here – revealed that the aminoglycoside 3'-phosphotransferase II encoded by the kanamycin resistance gene does not have any toxic properties nor does it possess any of the characteristics associated with allergenic proteins, since it is rapidly inactivated by stomach acid, degraded by digestive enzymes and heat labile. In view of the fact that the enzyme would be effectively degraded in the stomach before being able to inactivate orally administered antibiotics, its presence in food will not compromise their therapeutic use. Furthermore, the only antibiotics used in humans inactivated by the enzyme are neomycin and kanamycin, which are hardly ever given orally in clinical practice.

The potential for transformation of the antibiotic resistance gene to gut microorganisms or intestinal epithelial cells is not of significant concern either, as most DNA is degraded through the digestive process and would therefore be unavailable for possible transfer. Even assuming that all microorganisms in the intestine were transformation competent, transformation frequency would still be much less than the frequency of spontaneous mutations leading to kanamycin resistance. Due to the rare application of kanamycin

and neomycin in the clinical setting, selective pressure would be minimal for the development of resistant bacteria.
The U.S. Food and Drug Administration therefore concluded that the kanamycin resistance gene is safe to use as a selectable marker and even suggested it to be the most acceptable among the antibiotic resistance marker genes[76].

Genetically modified organisms in today's practice

The use of genetically modified organisms has been increasing rapidly in medical and agricultural applications. In U.S. supermarkets, at least 60 percent of the food products contain genetically modified organisms[77]. But as consumption rises, so does the concern about these products in public[78]. In order to allow consumers the choice to buy genetically modified food or not, the European Union in 2003 implemented guidelines on the labeling of these products[79]. According to these regulations, all products containing or consisting of or derived from an ingredient, which contains more than 0.9 percent of genetically modified organisms, must be adequately labeled.
In contrast, the new guidelines on genetically engineered animals published in January 2009 by the U.S. Food and Drug Administration[80] still do not require labeling of food from genetically engineered animals.

General acceptance and safety regulations differ regionally; so market access criteria of bioengineered products, such as the newly developed transgenic yeast, would need to be specifically analyzed.
In order to shift the consumer's focus towards the health promoting effects, the transgenic yeast could also be advertised as probiotic.

Yeast as probiotic

Due to its high content of proteins, B complex vitamins, and minerals, yeast has been used as food supplement for many years. Recently it has also been established as probiotic agent for the re-equilibration of the intestinal microflora[81]. The term probiotic refers to live microorganisms that, when administered in adequate amounts, can confer a health benefit on the host[82]. Clinical studies have shown that preparations of the yeast *Saccharomyces boulardii* are effective for the treatment and prevention of several

types of diarrhea and colitis in humans[83], especially in chronic or recurrent diarrhea associated with *Clostridium difficile*[81].

Various mechanisms of controlling pathogenic organisms by probiotic yeast were suggested and reported to involve direct antagonism, action on binding of toxins to intestinal receptors leading to an antisecretory effect, and trophic effects on intestinal cells with stimulation of enzymatic expression and intestinal defense mechanisms[84].

Martins et al.[85] suggest that other *Saccharomyces* species (e.g. *S. cerevisiae*, which is genetically very close to *S. boulardii*) probably have probiotic activity similar to that of *S. boulardii* or even better. Chia et al.[86] successfully applied *S. cerevisiae* concomitantly with antibiotics to patients with relapses of colitis after ineffective antibiotic treatment. Based on their observations, they suggest that this might be inexpensive and safe adjunctive therapy for otherwise immunocompetent patients with relapses of *Clostridium difficile* colitis that are unresponsive to multiple courses of antibiotic therapy.

However, there are some issues regarding probiotics that need to be taken into account:

In order for microorganisms to be considered as probiotics, they must be administered in adequate amounts. For them to reach sufficient concentration to exert therapeutic properties they have to survive passage through the gastrointestinal tract of the host. Therefore, they must overcome biological barriers, including stomach acidity and bile secretion in the duodenum[87]. The results of Pennacchia et al.[88] show survival rates as high as 80 to 90 percent after transit through a simulated human gastrointestinal tract, and results with different *S. cerevisiae* strains – when assessed for their resistance to low pH and bile exposition – support the notion that probiotic yeasts are resistant to gastrointestinal passage[84].

Another big issue is the safety of probiotic treatment. Although yeast is generally regarded as safe, as it has been used in the diet for centuries[86], and its strong evidence for efficacy in antibiotic associated diarrhea[89], there are concerns with regard to safety in particular populations. The most important fear with the use of probiotic yeast is the risk of fungemia and sepsis. This concern is supported by some studies reporting cases of fungemia in patients taking *S. boulardii* supplements[90-93]. A review on this topic by Boyle et al.[94] points out, that all these cases have occurred in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have

described sepsis related to probiotic use in otherwise healthy persons. They propose a list of risk factors for probiotic sepsis (Table 3) which when present should merit caution in using probiotics[94].

Major risk factors:	**Minor risk factors**
1. Immune compromise, including a debilitated state or malignancy 2. Premature infants	1. Central venous catheter 2. Impaired intestinal epithelial barrier, e.g. diarrheal illness, intestinal inflammation 3. Administration of probiotic by jejunostomy 4. Concomitant administration of broad spectrum antibiotics to which probiotic is resistant 5. Probiotics with properties of high mucosal adhesion or known pathogenicity 6. Cardiac valvular disease (*Lactobacillus* probiotics only)

Table 3: Proposed risk factors for probiotic sepsis[94]. The presence of a single major or more than one minor risk factor merits caution in using probiotics.

5.3 Conclusion

The results presented here demonstrate functional expression of the *Caenorhabditis elegans* fat-1 gene in a wild-type *Saccharomyces cerevisiae* strain used in the baking industry and provide a basis for the development of transgenic omega-3 producing organisms. By converting omega-6 to corresponding omega-3 PUFA, these experiments propose an alternative approach to supply humans with omega-3 PUFA in the future in a cost-effective and sustainable way. Furthermore, this approach could not only elevate intake and thereby tissue concentrations of omega-3 PUFA but could also decrease levels of omega-6 PUFA at the same time, thereby balancing the omega-6 to omega-3 fatty acid ratio.

There are two ways by which these modified yeasts could be used to supplement omega-3 fatty acids:
One is to utilize these yeasts as a dietary additive, employing a probiotic strategy in order to achieve the conversion of omega-6 fatty acids from the diet into omega-3 fatty acids in the intestinal tract.

Besides that, the newly developed yeast strain could also be used to produce omega-3 fatty acids from omega-6 containing substrates during fermentation processes, which then would become incorporated into the final food product. Especially in baking industry, vegetable oils rich in LA[95] are used. Since scientists recommended that the

intake of LA should be decreased and ALA intake should be increased[6], the results presented lead to the conclusion that replacement of currently used baker's yeast by the newly developed transgenic one could reach this goal all at once without any alterations of eating habits.
Additionally, if the omega-3 PUFA producing properties of the engineered yeast would be used in fermentation processes, the safety concerns regarding possible side-effects of its uptake – including fungemia and sepsis – would not be an issue.

In view of the fact that *Saccharomyces cerevisiae* is used not only in the baking industry[96, 97], further application of the engineered yeast in brewing and wine making is also conceivable.

However, further research on this topic is necessary. Additional studies are now required in order to broaden our understanding of the newly created transgenic yeast regarding effective conditions for the optimal expression and enzymatic activity of the FAT-1 protein. Future experiments could aim to achieve stable yeast transformation with the plasmids p416 ADH + fat-1 + KanMX4 and pRS306 GPD + fat-1 + KanMX4.
Prospective research should also include in-vivo studies on the change in tissue omega-6 to omega-3 fatty acid ratios and on disease preventing properties of these yeast supplements, since bioavailability of fatty acids from the diet involves a series of physiological processes comprising digestion, absorption, transport, and fatty acid metabolism.
Additional experiments should also comprise safety assessment for probiotic use of these yeasts, which might require further strain adjustments to achieve even better probiotic qualities. Yet, the therapeutic usage of these yeast probiotics should be carefully considered regarding its risk-benefit potential.

6 SUMMARY

Dietary habits adapted by Western societies over the past 100 to 150 years have led to a diet deficient in omega-3, but containing too much omega-6 PUFA. These dietary changes are implicated in the increased incidence of many chronic diseases and some types of cancer. Beneficial effects of omega-3 PUFA have been shown for numerous major diseases in many human studies. The demand for omega-3 fatty acids is therefore increasing, but their natural sources are limited. Many current practices of supplying humans with omega-3 PUFA are cost-ineffective and unsustainable.

Although omega-6 PUFA are highly abundant in the typical Western diet, these cannot be converted into omega-3 fatty acids in the human body, because mammalian cells lack an omega-3 fatty acid desaturase.

This study demonstrates the functional expression of such an omega-3 fatty acid desaturase encoded by the *Caenorhabditis elegans* fat-1 gene in a wild-type *Saccharomyces cerevisiae* strain used in baking industry. Different yeast expression plasmids containing the fat-1 gene were constructed and then introduced into the yeast cells. Successful transformation was verified by growth on selective medium and PCR-analysis of the yeast cells. After exogenous fatty acid incubation, gas chromatographic analysis of fatty acids from the yeast cells showed conversion of linoleic acid (18:2 n-6) to alpha-linolenic acid (18:3 n-3) in the transformed yeast samples, verifying functionality of the protein expressed.

The results presented here thus provide a basis for the development of transgenic omega-3 producing yeasts as dietary supplement or for industrial utilization and suggest an alternative approach of supplying humans with omega-3 PUFA in the future, leading to an increased omega-3 while decreased omega-6 fatty acid intake without the need for changes in habitual diet.

7 ZUSAMMENFASSUNG

In den letzten 100 bis 150 Jahren haben sich die Ernährungsgewohnheiten der westlichen Gesellschaft verändert. Dies führte zu einer Ernährung, die reich an Omega-6-Fettsäuren ist, dafür jedoch einen Mangel an Omega-3-Fettsäuren aufweist. Diese Ernährungsumstellung hat ein erhöhtes Auftreten von vielen chronischen Erkrankungen sowie einigen Arten von Malignomen zur Folge. Die günstigen Auswirkungen von Omega-3-Fettsäuren konnten in zahlreichen Studien für viele bedeutende Krankheiten nachgewiesen werden. Folglich steigt die Nachfrage für Omega-3-Fettsäuren, deren Quellen sind jedoch beschränkt. Viele der heutigen Praktiken, die angewandt werden um die Menschen mit Omega-3-Fettsäuren zu versorgen, sind kostenineffektiv und in dieser Form nicht aufrecht zu erhalten.

Obwohl die typische westliche Ernährungsweise einen Überfluss an Omega-6-Fettsäuren aufzeigt, können diese im menschlichen Organismus nicht in Omega-3-Fettsäuren umgewandelt werden, da Säugetierzellen keine Omega-3-Fettsäure-desaturaseaktivität besitzen.

Diese Arbeit beschreibt die funktionelle Expression einer solchen Omega-3-Fettsäuredesaturase kodiert durch das *Caenorhabditis elegans* fat-1 Gen in einem Wildtypstamm von *Saccharomyces cerevisiae*, der in der Backindustrie eingesetzt wird. Verschiedene Hefeexpressionsplasmide mit dem fat-1 Gen wurden konstruiert und dann in die Hefezellen eingebracht. Eine erfolgreiche Transformation konnte durch Hefewachstum auf Selektivmedium sowie PCR-Analyse der Hefezellen nachgewiesen werden. Nach Inkubation mit exogenen Fettsäuren zeigte sich in der gaschromatographischen Analyse der Fettsäuren aus den Hefezellen eine Umwandlung von Linolsäure (18:2 n-6) in alpha-Linolensäure (18:3 n-3) in den transformierten Hefeproben, welche die Funktionalität des exprimierten Proteins bestätigte.

Die hier präsentierten Ergebnisse bilden somit eine Grundlage für die Entwicklung transgener, Omega-3-Fettsäure-produzierender Hefen als Nahrungsergänzungsmittel oder für deren industrielle Nutzung. Sie stellen daher einen alternativen Ansatz für die zukünftige Versorgung der Menschen mit Omega-3-Fettsäuren dar, welcher ohne eine Änderung der Ernährungsgewohnheiten zu einer erhöhten Omega-3-, bei zugleich verminderter Omega-6-Fettsäureaufnahme beitragen könnte.

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9 FIGURES

10 TABLES

ERKLÄRUNG / STATEMENT OF AUTHORSHIP

„Ich, Katja Lichopoj, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Functional expression of the *C. elegans* fat-1 gene in wild-type yeast“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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LEBENSLAUF / CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.