Master's thesis

NTNU
Norwegian University of Science and Technology
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Exploration of selectable markers and the effect of fatty acid synthase inhibitors on fatty acid synthesis in *Aurantiochytrium* sp.

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Preface and acknowledgements

This thesis was completed from August 2018 to May 2019, as the result of a 5-year master's degree at the Department of Biotechnology and Food Science at NTNU, Trondheim. The thesis and work were supervised by Professor Helga Ertesvåg and was done as a part of the Auromega project, a research collaboration between NTNU and SINTEF on thraustochytrid omega-3 fatty acid production. It has been very educational and interesting, and I have acquired valuable knowledge which I will appreciate throughout my life.

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Samandrag

Thraustochytridar er heterotrofe, marine protistar, som har blitt kommersielt interessante dei siste tiåra sidan dei har ein høg produksjon av docosahexaensyre (DHA), som er ei langkjeda fleirumetta omega-3 feittsyre (LC-ω3-PUFA), i tillegg til skvalen og karotenoidar. LC-ω3-PUFA er essensielle feittsyrer og verdsette for sine mange helsemessige fortrinn, og sidan den noverande forsyninga i verda ikkje er nok til å dekke eit aukande forbruk, må alternative kjelder utviklast. Eit alternativ er oljer frå thraustochytridar, og *Aurantiochytrium* er ein av thraustochytride genera som har vist høgast DHA produktivitet. Det er derfor interessant å utvikle stammar av dette genuset til å auke DHA produksjonen og for å betre forstå biokjemien av DHA syntesen.

Ulike seleksjonsmarkørar blei utforska til å bli brukt på *Aurantiochytrium* sp. T66 og S61, sidan ein fungerande seleksjonsmetode enno ikkje eksisterer for desse stammane. Effektane av dei potensielle selektive sambindingane 5-fluoroorotsyre og 5-fluorocytosin på veksten av T66 og S61 blei undersøkte, noko som viste at stammane er resistente mot desse sambindingane. Dermed kan dei ikkje brukast som selektive sambindingar for desse stammane. Resistensen mot 5-fluorocytosin var ikkje overraskande, sidan eit BLAST søk viste at T66 truleg ikkje har eit gen som koder for cytosin deaminase, eit enzym som omgjer sambindinga til den giftige sambindinga 5-fluorouracil. Dette tyder derimot på at 5-fluorocytosin kan brukast til motseleksjon i desse stammane, noko som kan vere interessant dersom ein fungerande grunnleggjande seleksjonsmetode blir etablert.

Resistensgen mot dei antibiotiske stoffa cykloheksimid og zeocin (høvesvis eit mutert gen som koder for det ribosomale proteinet L44, og *Sh ble* genet) blei også utforska som potensielle seleksjonsmarkørar. Ved elektroporering av T66 og S61 med desse gena, var falske positive eit tilbakevendande problem. I tillegg blei transformasjonar av *Sh ble* genet, der også ein kombinasjon av elektroporering og rysting med glasskuler blei testa, vist å ikkje vere vellykka etter testing av nokre resulterande koloniar ved hjelp av PCR amplifikasjon. Dette tyder på at transformasjonsmetoden og seleksjonsprosedyrene må utviklast vidare.

Ein annan potensiell seleksjonsmetode er komplementering av auxotrofe mutantar. For å utforske dette blei 10 000 koloniar frå ein mutert kultur av *Aurantiochytrium* sp. T66 screena for auxotrofe mutantar ved hjelp av replikaplating på rikt og minimalmedium, og fem koloniar som ikkje kunne vekse på minimalmedium blei dyrka med kvar av dei 20 proteinogeniske aminosyrene, uracil eller adenin. Ein mutant vaks noko betre på uracil og aspartat, men dette er truleg ikkje eit resultat av éin mutasjon. Ein annan mutant vaks noko betre på glutamat og glutamin, som kan ha tyda på problem med nitrogenassimilering på grunn av ein glutamat/glutamin auxotrofi. Dyrking av denne mutanten med rikeleg glutamat auka ikkje veksten, og det blei dermed ikkje stadfesta at denne mutanten var ein glutamat auxotrof. Ingen klar effekt blei observert av å dyrke dei andre mutantane med aminosyrer, uracil eller adenin, altså blei ingen auxotrofe mutantar stadfesta.

I thraustochytridar er hovudsporet for DHA syntese gjennom eit dedikert enzymkompleks. For å betre forstå korleis feittsyresyntase og DHA syntase systema fungerer i forhold til kvarandre, blei effekten av ulike feittsyresyntaseinhibitorar på feittsyreprofilen til T66 celler undersøkt ved hjelp av massespektrometri. Ingen av inhibitorane auka DHA produksjonen, men nokre av dei hadde ein effekt på andre feittsyrer. Dette tyda på at inhibering av feittsyresyntase ikkje auka fluxen gjennom DHA syntasen.

Contents

Abstract

Thraustochytrids are heterotrophic, marine protists, which have become commercially interesting in the past decades because of their high productivity of docosahexaenoic acid (DHA), which is a long-chain polyunsaturated omega-3 fatty acid (LC-ω3-PUFA), as well as squalene and carotenoids. LC-ω3-PUFAs are essential fatty acids and appreciated for their many health benefits, and as the current world supply is not enough to cover an increasing consumption, alternative sources must be further developed. An alternative is oils from thraustochytrids, and *Aurantiochytrium* is one of the thraustochytrid genera which have shown the highest DHA productivity. It is therefore interesting to develop strains of this genus to enhance the DHA production and to better understand the biochemistry of the DHA synthesis.

Different selection markers to be used in *Aurantiochytrium* sp. strains T66 and S61 were explored, as a successful selection method does not yet exist for these strains. The effects of the potential selective compounds 5-fluoroorotic acid and 5-fluorocytosine on T66 and S61 growth, were evaluated, which showed that the strains are resistant to these compounds. Thus, they could not be used as selective compounds in these strains. The resistance to 5-fluorocytosine was not surprising, as a BLAST search showed that T66 most likely does not harbor a gene encoding cytosine deaminase, an enzyme which transforms the compound to the toxic compound 5-fluorouracil. However, this suggests that 5-fluorocytosine could be used as a counterselective compound in these strains, which could be relevant if a functional basic selection procedure is established.

Resistance genes to the antibiotics cycloheximide and zeocin (a mutated gene encoding ribosomal protein L44 and the *Sh ble* gene, respectively) were also explored as potential selection markers. After electroporation of T66 and S61 with these genes, false positives were a recurring problem. In addition, the transformations of the *Sh ble* gene, in which a combination of electroporation and agitation with glass beads was also tested, did not seem successful from testing a few resulting colonies by PCR amplification. This indicates that the transformation method and selection procedures must be further developed.

Another potential selection method is the complementation of auxotrophic mutants. To explore this, 10 000 colonies of mutant *Aurantiochytrium* sp. T66 were screened for auxotrophic mutants by replica plating on rich and minimal media, and five colonies which failed to grow on minimal medium were cultivated with each of the 20 proteinogenic amino acids, uracil or adenine. One mutant grew slightly better on uracil and aspartate, which most likely did not result from one mutation. Another mutant grew slightly better on glutamate and glutamine, which might have indicated problems with nitrogen assimilation due to a glutamate/glutamine auxotrophy. Cultivating this mutant with an abundance of glutamate did not improve the growth, and thus it was not established that it was a glutamate auxotroph. No apparent effect of cultivating the other mutants in the presence of any amino acids, uracil or adenine was observed, and thus no

auxotrophic mutants were established.

In thraustochytrids, the major pathway for DHA synthesis is by a dedicated enzyme complex. To further understand how the fatty acid synthase and DHA synthase systems function in relation to each other, the effect of different fatty acid synthase inhibitors on the fatty acid profile of T66 cells was evaluated by mass spectrometry. None of the inhibitors enhanced DHA production, although some of them affected other fatty acids. This indicated that inhibition of fatty acid synthase did not increase the flux through DHA synthase.

1 Introduction

1.1 Thraustochytrids

Thraustochytrids are unicellular, heterotrophic, saprophytic, obligate marine protists abundant in seawater and sediments, first discovered in 1934 (Aasen et al., 2016; Marchan et al., 2018). They are classified as Labyrinthulomycetes, a class of fungus-like and mostly marine unicellular organisms (Leyland et al., 2017). Furthermore, they belong to the kingdom of Stramenopila, characterized by the formation of zoospores with two flagella (cell appendages used for motility). Zoospores are spores produced for the purpose of propagation and are in thraustochytrids released from a sporangium, which is produced from a vegetative cell by progressive cell divisions while the cell wall remains intact (Leyland et al., 2017; Marchan et al., 2018). There have been some changes in the taxonomy of thraustochytrids over the past years, due to a weak correlation between morphology and 18S ribosomal DNA phylogeny (Marchan et al., 2018). For example, in 2007, Yokoyama and Honda proposed to reassign some of the species in the genus *Shizochytrium* to the new genera *Aurantiochytrium* and *Oblongichytrium* (Yokoyama & Honda, 2007). The latter, in addition to the genus *Althornia*, are as of recently not considered a part of the thraustochytrid family anymore, although some scientists still consider them as thraustochytrids. Based on life cycle, morphology, phylogenic analysis, and biochemical marker profiles (polyunsaturated fatty acids and carotenoids), there are now nine accepted genera of the thraustochytrid family, and some strains and groups remain unclearly classified (Marchan et al., 2018).

1.1.1 Industrial production of polyunsaturated fatty acids, squalene, and carotenoids from thraustochytrids

Some thraustochytrid strains have become commercially valuable because they have a relatively high production of long-chain polyunsaturated omega-3 (ω3) fatty acids (LC-ω3-PUFA), squalene or carotenoids. LC-ω3-PUFAs have several double bonds in their hydrocarbon chains, one of which is located between the third and fourth carbons, counted from the ω carbon (the carbon furthest away from the carboxyl group). The nomenclature Cx:y specifies the number of carbons (x) and double bonds (y) in the hydrocarbon chain of fatty acids (Nelson & Cox, 2013, p. 357-363). LC-ω3-PUFAs are essential fatty acids and are often obtained in the diet in the form of docosahexaenoic acid (DHA; C22:6) and eicosapentaenoic acid (EPA; C20:5) (Salem Jr & Eggersdorfer, 2015). They are widely recognized for their health benefits, including preventing cardiovascular diseases, being anti-inflammatory, and being important in early neural development and immune function (Swanson et al., 2012). However, it has been stated that the current world supply of ω3 fatty acids is not enough to cover the human recommended intake in the world (Salem Jr & Eggersdorfer, 2015). Additionally, fish and fish oil, the major dietary source of DHA/EPA, now often contain environmental

contaminants (Hauvermale et al., 2006). Therefore, efforts have been raised to develop more alternative sources of DHA/EPA, e.g., marine microorganisms and microscopic animals, and oil from genetically modified plants (Salem Jr & Eggersdorfer, 2015). Squalene, an intermediate in the biosynthesis of sterols, is widely used as an additive in medicinal compounds and as a hydrating agent in cosmetics, among other applications. Its primary source has been shark liver oil, but a more environmentally friendly source is desired. Carotenoids are pigments mostly produced by plants and microorganisms, and are used as coloring agents in food, feed, pharmaceuticals, and cosmetics. Currently, commercial carotenoids are mostly chemically produced or obtained from plants, with which microbial production has not yet been cost competitive (Aasen et al., 2016).

Thraustochytrid strains with high DHA productivity can accumulate 50–70% of cell dry weight (CDW) of lipids, where DHA constitutes 30–70% of total fatty acids. The thraustochytrid genera which have shown the highest productivities are *Aurantiochytrium*, *Shizochytrium* and *Ulkenia*. Thraustochytrids are some of the few known organisms in which LC-ω3-PUFAs constitute a large part of their storage lipids. Most lipid accumulating microorganisms have storage lipids composed of mainly saturated fatty acids, and LC-ω3-PUFAs are more often a part of the membrane lipids of marine microorganisms. Lipid-accumulating microorganisms generally accumulate lipids as triacylglycerols (TAGs) when an essential nutrient, often nitrogen, is limiting and organic carbon is abundant. The nutrient limitation restricts cell division, and the organic carbon gets assimilated into TAGs. In thraustochytrids, lipid accumulation can be initiated when nitrogen is limiting, although accumulation also takes place in rich medium when nitrogen is accessible (Aasen et al., 2016).

Because of the high DHA productivity, industrial production of DHA-rich oils from thraustochytrids started in the 1990s, and it is of commercial interest to optimize this production. The latter has been the main motivation for most of the research done on thraustochytrids. For example, the biochemical pathways of DHA synthesis in thraustochytrids have been investigated, and researchers have worked to optimize the process and growth conditions for DHA production (Aasen et al., 2016). Additionally, the genomes of *Aurantiochytrium* sp. strain T66 and other thraustochytrid strains have been sequenced, and many of the genes predicted, with the aim to investigate which genes are relevant for the DHA synthesis (Liu et al., 2016). This will be useful in mapping the biochemical DHA synthesis, and to see how it is regulated. The genome sequences are also necessary to do genetic manipulation of thraustochytrids, for research purposes or to make the production of DHA and other products more efficient. Furthermore, the sequencing has revealed a high GC content (62.8% in T66) in the thraustochytrid genome (Liu et al., 2016). This is useful to know in case a transgene is to be expressed in thraustochytrids, as the GC content affects gene expression, in accordance with the codon usage of the organism (Kudla et al., 2009). To succeed with genetic manipulation, an applicable expression/transformation system must exist for the species, which has previously proven difficult to procure for thraustochytrids (Aasen et al., 2016).

1.2 Potential selective compounds and selectable markers for thraustochytrids

When introducing DNA into an organism, it is desirable to be able to detect which cells have taken up the DNA, and potentially which cells are expressing an introduced gene of interest (GOI). This is commonly done by including a selectable marker gene, which preferably is dominant, with the DNA introduced. By introducing a gene which confers resistance to a selective compound which is toxic or growth inhibitory to the wild type cells, the transformants will be selected for in the presence of the compound (negative selection). Transformants can also be selected by positive selection, for example by introducing a selectable marker gene encoding an enzyme which can digest a substrate which the wild type cells cannot utilize (Prateesh & Vineetha, 2015). An alternative selection method is to select for the cells which do not harbor a specific gene, known as counterselection. This can be done by the use of a counterselectable marker, which confers sensitivity to a counterselective compound. Hence, in the presence of this compound, the cells holding the counterselectable marker are selected against (Reyrat et al., 1998). Selection of mutant diploid organisms can be more challenging than of haploids, as the selectable marker must be dominant. Otherwise, the organism must be homozygous for the selectable marker (Sadowski et al., 2008).

1.2.1 Genetic engineering of thraustochytrids

Transgenes have previously been transferred into thraustochytrid cells using the physical transformation methods particle bombardment, electroporation (Marchan et al., 2018), and a combination of electroporation and agitation with glass beads to weaken the cell wall (Adachi et al., 2017). Particle bombardment involves acceleration of high-density particles, usually made of gold, tungsten or platinum, covered with DNA, into the cells. During electroporation, an electrical field generates pores in the cell membrane, thereby allowing uptake of DNA (Rivera et al., 2014). Infection by *Agrobacterium tumefaciens* has also been successfully used to transfer DNA into thraustochytrids (Marchan et al., 2018), and involves the transfer of DNA into recipient cells by conjugation (Frandsen, 2011). It is also possible that transformation methods that have been successful in photosynthetic microalgae could be successful in thraustochytrids as well (Aasen et al., 2016).

There are only a few selection markers which have been successful in selection of transgenetic thraustochytrids, including resistance genes for antibiotics including G418, hygromycin, paromomycin, chloramphenicol, zeocin, cycloheximide (Aasen et al., 2016), neomycin (Kobayashi et al., 2011), and blasticidin (Matsuda et al., 2012). The concentrations of the respective selection compounds required for selection have been strain-dependent (Aasen et al., 2016). To make the transformed thraustochytrids integrate the transgene into their chromosome, a method that has been successful is to flank the genetic material introduced into the cells with 18S ribosomal DNA (rDNA) sequences, so that it is integrated via 18S rDNA-targeted homologous recombination (Hong et al., 2013). This is a

process in which two DNA sequences with similar or identical regions anneal and exchange sequence information between the similar regions, by DNA synthesis (Court et al., 2002). Although genetic manipulation has been a success in a few thraustochytrid strains, it has generally been challenging, and the success of different methods seems to depend on the strain or genus (Aasen et al., 2016).

1.2.2 5-Fluoroorotic acid

5-fluoroorotic acid (5-FOA) is a chemical used in selection of yeast, as it kills cells with a functional URA3 gene (wild type). Selective concentrations used for yeast are 174 mg/L (Tani et al., 2013), 750 mg/L (Gleeson et al., 1990) and 1000 mg/L 5-FOA (Nakazawa et al., 2016). The URA3 gene encodes orotidine 5'-monophosphate (OMP) decarboxylase, which catalyzes the conversion of 5-FOA to the cytotoxic compound fluorodeoxyuridine (Thermo Fisher Scientific, n.d.-a). Fluorodeoxyuridine is toxic due to its inhibition of thymidylate synthase, an essential enzyme in DNA synthesis. It is also metabolized into compounds which can be incorporated into and interfere with RNA (National Center for Biotechnology Information, n.d.). The native function of the enzyme OMP decarboxylase is to catalyze the last step in pyrimidine biosynthesis, the conversion of OMP into uridine 5'-monophosphate (UMP) (Sievers & Wolfenden, 2002). Hence, an organism defective of this enzyme is a uracil auxotroph, and resistant to 5-FOA, and can therefore grow in medium containing 5-FOA, supplemented with uracil (Thermo Fisher Scientific, n.d.-a).

1.2.3 5-Fluorocytosine and cytosine deaminase

5-fluorocytosine (5-FC) is a drug that is toxic to cells with cytosine deaminase enzyme activity, an enzyme which native function is to convert cytosine to uracil by deamination for pyrimidine salvage. The enzyme can also convert 5-FC to the toxic compound 5-fluorouracil (5-FU), which can be metabolized into products which inhibit DNA or protein synthesis, by inhibiting thymidylate synthase or by incorporating into RNA, respectively (Young & Purton, 2014). Genes encoding cytosine deaminase (FCY1 in *Saccharomyces cerevisiae*, and *codA* in *E. coli*) has been used as counterselectable markers, together with 5-FC as counterselective compound at 1-2 g/L, in yeast and bacteria in which the wild type organisms lack cytosine deaminase enzyme activity (Nakazawa & Honda, 2015; Young & Purton, 2014). Most bacteria and yeast show cytosine deaminase activity, but mammalian cells and cells of higher plants do not (Young & Purton, 2014). In an organism in which the wild type harbors the functional gene encoding cytosine deaminase, 5-FC could also be used to select for mutants containing a genetic fragment of interest, by disrupting the gene encoding cytosine deaminase upon an integration of the fragment into the chromosome, thereby making the cell 5-FC resistant. The disruption could be done by homologous recombination targeting this gene, as with 18S rDNA targeted homologous recombination described in section 1.2.1.

1.2.4 Cycloheximide

Cycloheximide, produced by *Streptomyces griseus*, is an inhibitor of eukaryotic protein synthesis, more specifically, the elongation phase of translation. It does this by binding the large ribosomal subunit and inhibiting translocation of tRNA and mRNA (Schneider-Poetsch et al., 2010). This leads to cell growth arrest and cell death (Sigma-Aldrich, n.d.-b). Resistance to 50 µg/mL cycloheximide, conferred by a mutated version of the gene encoding ribosomal protein L44 (RPL44, a component of the large ribosomal subunit), where proline 56 is replaced with glutamine, has been used as a selectable marker in *Aurantiochytrium* sp. KRS101 (Hong et al., 2013). This mutation changes one of the target proteins of cycloheximide, thus making it unable to bind (Kawai et al., 1992).

1.2.5 Zeocin

Zeocin is a commercially available antibiotic that is effective against both prokaryotes and eukaryotes (Benko & Zhao, 2011). It belongs to the bleomycin/phleomycin family, and its mechanism of action is to intercalate into and cleave the DNA using reactive radicals, thus causing cell death (Oliva-Trastoy et al., 2005). The bleomycin/phleomycin family of antibiotics is isolated from *Streptomyces*, and zeocin is a derivative of phleomycin D1, which is isolated from *Streptomyces verticillus*. It is copper-chelated in its inactive form, but upon entering the cell, the Cu^{2+} is reduced to Cu^{1+} and removed by sulfhydryl compounds, thus activating the antibiotic (Thermo Fisher Scientific, n.d.-c). The *Sh ble* gene, isolated from *Streptoalloteichus hindustanus* (Sh) (Bennett et al., 1998), confers resistance to zeocin (as well as phleomycin), as the gene product binds to the antibiotic and thereby prevents its action. Therefore, the gene has been used as a selective marker together with zeocin (Oliva-Trastoy et al., 2005) at concentrations of 50-300 µg/mL for yeast cells (Benko & Zhao, 2011; Thermo Fisher Scientific, n.d.-c), and 1.5-100 µg/mL for thraustochytrids (Cheng et al., 2011; Ren et al., 2015), including *Aurantiochytrium* sp. (Suen et al., 2014). A high salt concentration inhibits the antibiotic activity of zeocin, and a high cell density affects the zeocin selection efficiency, which is why it is recommended to use low salt media and plate at low cell densities when using zeocin as a selective compound (Thermo Fisher Scientific, n.d.-c).

1.2.6 Auxotrophic mutants

Auxotrophic mutants have acquired a mutation in a gene encoding an enzyme essential in the synthesis of an essential metabolite, e.g., an amino acid, vitamin, purine or pyrimidine, and are therefore dependent of a supplement of this specific metabolite for growth. The wild type of the species is prototrophic, and thus able to synthesize this metabolite autonomously, and can therefore grow on minimal media without the metabolite (Snustad & Simmons, 2012, p. 170-171, 341). This phenomenon has been utilized as a genetic tool in molecular biology. By

complementing the defective gene with the wild type allele in the auxotrophic mutant, reversing it to a prototroph, the revertants can be selected for by cultivation in minimal medium (Pronk, 2002). This has been done in both bacteria (Yadav, 2007) and yeast (Hashimoto et al., 2005; Gleeson et al., 1990), although auxotrophic mutants of diploid organisms can be challenging to obtain, because two genes must be defective for them to be auxotrophs since it is a recessive trait (Hashimoto et al., 2005). Auxotrophic yeast mutants have been produced by random mutagenesis (Hashimoto et al., 2005; Gleeson et al., 1990) and by site-directed mutagenesis (Kitamoto et al., 1990). They have been isolated by enrichment using drugs that select for the auxotrophs (for example 5-FOA) (Gleeson et al., 1990; Kitamoto et al., 1990), or by doing conventional replica plating on rich and minimal media, where the auxotrophs grow on rich but not minimal medium (Hashimoto et al., 2005). Hashimoto et al. proposed loss of heterozygosity (LOH) as an explanation for their frequency of auxotrophic mutants in yeast strains, which was higher than expected because the strains were diploid. They proposed that this might have happened after an initial induced mutation in one of the alleles, or that some of the strains were heterozygous (mutant in one of the alleles) beforehand, and that the mutagenesis might have induced LOH, possibly by recombination (Hashimoto et al., 2005). LOH is a mutation event that renders the cell homozygous in a locus, that is, both alleles become equal (Zhu et al., 1992).

A set of 20 amino acids are the monomers which combine by peptide bonds to form proteins. Amino acids consist of a carboxyl group, an amino group, and a variable side chain bound to a carbon atom (the α carbon). Purines and pyrimidines are cyclic, nitrogenous bases and the variable components of nucleotides, which are important energy carriers in cells and monomers of DNA and RNA. An essential ingredient in the synthesis of amino acids and nucleotides is nitrogen, which is assimilated into biomolecules through the amino acids glutamate and glutamine. Glutamate is an amino group donor in the synthesis of most other amino acids, including glutamine, which again provides amino groups for some amino acids and a diversity if biomolecules. Amino acids again serve as a source of nitrogen for nucleotide synthesis. Intermediates in the glycolysis, the citric acid cycle, or the pentose phosphate pathway serve as precursors in amino acid synthesis. Some amino acids share parts of their synthetic pathways before they separate, and some amino acids are synthesized with other amino acids as precursors. The amino acid synthetic pathways may intertwine in these manners with the synthesis of other biomolecules as well. For example, histidine is produced from purine precursors, and aspartate serves as a precursor in pyrimidine synthesis (Nelson & Cox, 2013, p. 888-916).

1.3 Fatty acid biosynthesis

Fatty acids, which are carboxylic acids of 4 to 36 carbons long hydrocarbon chains, are precursors for many lipids, including storage (for energy) and membrane lipids (Nelson & Cox, 2013, p. 357-363). Fatty acids may also serve as signaling molecules and membrane anchors for proteins, among other functions (Herbst et al., 2018). Fatty acids can be saturated, meaning that the hydrocarbon chain contains no double bonds between the carbons, or unsaturated, where the chain contains one or more double bonds. Different unsaturated fatty acids vary in the positions of the double bonds (Nelson & Cox, 2013, p. 357-363).

A common type of storage lipids in eukaryotes is triacylglycerols (TAGs), which also serve as a depot for essential and non-essential fatty acids in addition to energy (Coleman & Lee, 2004). They consist of three fatty acids bound to a glycerol molecule by ester bonds. Naturally occurring TAGs are most commonly composed of different kinds of fatty acids, although they may also consist of the same kind. TAGs are present in the form of microscopic droplets in the cytosol of most eukaryotic cells (Nelson & Cox, 2013, p. 357-363).

Fatty acids are also a major constituent of many membrane lipids, which form a lipid bilayer in biological membranes. The types of lipids vary between different organisms and membranes. A common type is glycerophospholipids, composed of two fatty acids linked to the two first carbons of glycerol by ester bonds, and a polar or charged group bound to the third carbon by a phosphodiester linkage (Nelson & Cox, 2013, p. 357-363).

1.3.1 Fatty acid synthase

Fatty acids are synthesized in all organisms by the enzyme complex fatty acid synthase (FAS). The synthesis occurs by successive addition of two-carbon units from the three-carbon intermediate malonyl-CoA, to a carbohydrate-derived precursor, in which CO_2 is produced as a by-product. The initial substrate is the two-carbon compound acetyl-CoA, which is the first acyl group in the growing fatty acid chain and also carboxylated to form malonyl-CoA (Herbst et al., 2018). FASs are divided into two major types: type I, generating fatty acids in vertebrates and fungi, and type II, a dissociated system generating fatty acids in plants and bacteria (Nelson & Cox, 2013, p. 834-843). Type I FAS contains seven different domains which each hold different active sites for different reactions. It is further divided into yeast FAS (yFAS) and animal FAS (metazoan FAS, mFAS). These types are distinguished by slightly different domains, in addition to differing in the number of polypeptide chains. The mFAS consists of one single polypeptide, and the yFAS consists of two, in which three of the domains are located on the α subunit, and the other four on the β subunit (Herbst et al., 2018).

In eukaryotes, the FAS complex is found in the cytosol (Nelson & Cox, 2013, p. 834-843). However, mitochondria have their own FAS system, which resembles a bacterial type II system and consists of several different polypeptides harboring the different active sites. Several details of mitochondrial fatty acid synthesis remain unraveled, but it has been shown that it is crucial for mitochondrial function (Kastaniotis et al., 2017).

The reaction cycle catalyzed by yFAS, and its domain organization, are shown in figure 1.1, as this is most related to the thraustochytrid FAS. A specific acetyltransferase (AT) domain loads the acetyl moiety of acetyl-CoA onto a different domain, the acyl carrier protein (ACP), to which the growing substrate is tethered during iterative decarboxylative condensations with malonyl-CoA, catalyzed by ketosynthase (KS). The loading of malonyl-CoA is performed by malonyl-palmitoyl transferase (MPT). The intermediate yielded from condensation is further modified by ketoreductase (KR), dehydratase (DH) and enoylreductase (ER), the latter containing flavin mononucleotide (FMN). Reducing power is supplied by NADPH. These modifications yield a fully saturated acyl intermediate, which is further elongated in subsequent cycles. MPT unloads palmitate (C16:0) or stearate (C18:0), the final product after seven or eight cycles, respectively. In metazoan fatty acid synthesis, both the initial acetyl moiety and the malonyl-CoA are loaded by malonyl-acetyl transferase (MAT), instead of AT and MPT in yFAS. The final product palmitate is unloaded by a thioesterase (TE) domain, and the ER does not contain FMN. Apart from this, the fatty acid synthesis by yFAS and mFAS are alike (Herbst et al., 2018). As the elongation takes place by addition of two-carbon units, mostly even-numbered fatty acids are generated by FAS (Nelson & Cox, 2013, p. 834-843).

To produce a variety of fatty acids, the palmitate or stearate produced by FAS may be further elongated by elongases, which are enzymes with KS, KR, DH and ER activities (Aasen et al., 2016), and desaturated by desaturases, forming double bonds between carbons (Nelson & Cox, 2013, p. 834-843). However, the production of LC-PUFAs is catalyzed by a dedicated polyketide synthase (PKS) enzyme complex rather than FAS in some marine microorganisms (Hauvermale et al., 2006).

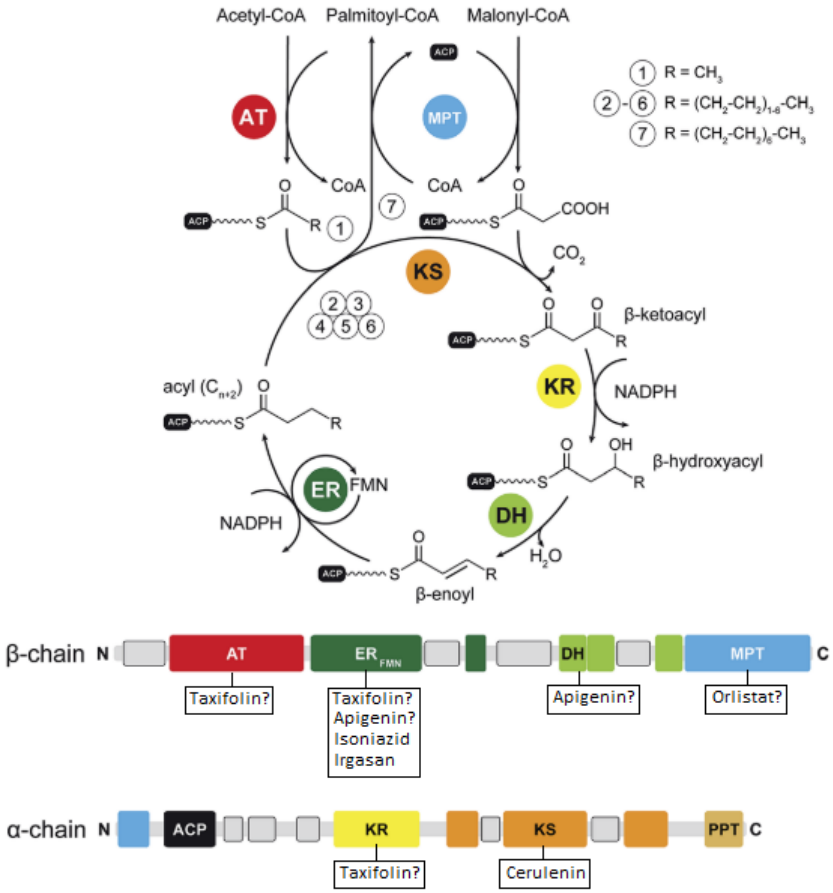


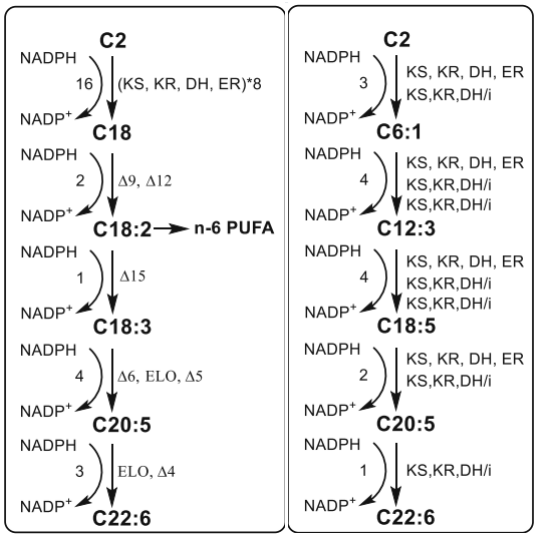
Figure 1.1: The cycle for fatty acid synthesis catalyzed by yeast fatty acid synthase (yFAS), and the domain organization on its two polypeptide chains (lower panel). The figure is copied (but modified) from Herbst et al. (2018). AT loads the initial two-carbon unit from acetyl-CoA onto ACP, which is then condensed by KS with a two-carbon unit from malonyl-CoA, loaded by MPT. The generated β-ketoacyl intermediate is modified by KR, DH, and ER, generating an acyl intermediate which is further elongated in succeeding cycles. After seven or eight cycles, the final product palmitate or stearate, respectively, is unloaded by MPT (here, seven cycles are illustrated). The domains different FAS inhibitors are inhibiting are also indicated in the lower panel. Taxifolin, apigenin, and orlistat are indicated with a question mark as it is somewhat unclear which domain they might inhibit in this enzyme. AT: acetyltransferase, MPT: malonyl-palmitoyl transferase, KS: ketosynthase, KR: ketoreductase, DH: dehydratase, ER: enoylreductase.

1.3.2 Fatty acid synthesis in thraustochytrids

There are two pathways for fatty acid synthesis in thraustochytrids. The standard FAS enzyme complex produces shorter, saturated fatty acids (mostly C14:0 and C16:0), and a PKS enzyme complex produces DHA (Aasen et al., 2016). Hauvermale et al. showed in 2006 that the FAS found in the thraustochytrid genus *Schizochytrium* is similar to the yeast FAS (yFAS) in sequence homology and domain organization, but the *Schizochytrium* FAS has all the domains on one single polypeptide (Hauvermale et al., 2006). The PKS enzyme complex is

probably the main pathway for DHA production in high DHA producing strains, and the high productivity of DHA in many thraustochytrid species is connected with this alternative PKS pathway. The PKS enzyme complex also synthesizes docosapentaenoic acid (DPA; C22:5 ω3), also a fatty acid characteristic for thraustochytrids. Thraustochytrids also produce low levels of other unsaturated fatty acids, including EPA, arachidonic acid, C16:1, C18:1 and C18:2. These are synthesized from modification of fatty acids produced by FAS, by elongases or desaturases (Aasen et al., 2016).

Some of the steps of the PKS pathway for fatty acid synthesis remain unresolved. However, it is known that the production takes place by iterative elongation with malonyl-CoA and acetyl-CoA as the initial substrate, analogous to the steps performed by FAS. In addition, the PKS pathway also uses NADPH as reducing power. An essential difference is that the reduction step by ER of FAS, in which a double bond is removed, is omitted in the PKS pathway. Hence, less reducing power is needed, and there is no need for desaturation by desaturases after the fatty acid synthesis (Aasen et al., 2016). The FAS and PKS pathways for DHA biosynthesis are compared in figure 1.2.



(a) (b)

Figure 1.2: The alternative pathways for biosynthesis of docosahexaenoic acid (DHA; C22:6) in thraustochytrids. Copied from Aasen et al. (2016). The number of NADPH needed for the reactions are shown to the left of the arrows, and the enzyme activities are shown to the right. KS: ketosynthase, KR: ketoreductase, DH: dehydratase, ER: enoylreductase, DH/i: bifunctional dehydratase and trans-cis isomerase. **(a)** The fatty acid synthase (FAS) pathway (to C18), before modification by elongases (ELO) and desaturases (Δ, the number indicating which bond, counted from the carboxyl end, is desaturated). The branching point to ω6 (n-6) PUFAs is also indicated. **(b)** The polyketide synthase (PKS) pathway.

As previously mentioned, optimization of the process and growth conditions for DHA production in thraustochytrids has been a task for researchers. In fermentation studies of thraustochytrids with an abundance of nitrogen, the DHA fraction of TFA has been observed to be higher during exponential growth than during lipid accumulation. As suggested by Aasen et al., this is probably because phospholipids constitute a higher share of the total lipids during exponential growth, and they have a higher content of DHA than storage lipids. Another parameter which has shown to affect the production rate of DHA in thraustochytrids is the availability of oxygen. Zero dissolved oxygen in the medium has shown to increase the DHA fraction of TFA, relative to an abundance of oxygen. According to Aasen et al., this could be explained in part by a lower FAS productivity during oxygen limitation, as this enzyme seems to be more affected by this than PKS (Aasen et al., 2016).

1.3.3 Fatty acid synthase inhibitors

There is a range of different inhibitors of FAS, inhibiting different domains or by other means. These have been used as, e.g., tools in research, antimicrobial agents, and in medicines. The inhibitors used in this study are described next, and which domains of FAS they presumably inhibit are indicated in figure 1.1.

1.3.3.1 Apigenin

Apigenin, or 4',5,7-trihydroxyflavone, is a flavone (a class of flavonoids, which are phenol compounds found in plants (Panche et al., 2016)) abundant in many fruits and vegetables. It has been recognized for its low toxicity and many health benefits, for example lowering blood pressure, being antioxidant, anti-inflammatory, antibacterial, and antiviral, and having anti-carcinogenic effects (Yan et al., 2017). The latter has, among other factors (Yan et al., 2017), been associated with its inhibitory effect on FAS, as this enzyme is overexpressed in many cancers (Brusselmans et al., 2005). Apigenin does so by leading to a downregulation of the FAS protein expression (Bumke-Vogt et al., 2014). It has also shown to inhibit enzymes in the type II FAS pathway, more exactly FabI of the protozoan *Plasmodium falciparum* and FabZ of *Helicobacter pylori*, corresponding to the enoylreductase (ER) and dehydratase (DH) of type I FAS, respectively (Zhang et al., 2008). In studies of its FAS inhibition in human cancer cells, apigenin has been tested in a range of concentrations from 0.5 to 100 μM (Brusselmans et al., 2005; Bumke-Vogt et al., 2014). Apigenin also has several other physiological activities, including inhibition of human estrogen synthetase, inhibition of cell proliferation by cell cycle arrest, induction of apoptosis (Sigma-Aldrich, n.d.-a), and inhibition of a family of human aldo-keto reductases (which participate in several physiological reactions) (Zemanova et al., 2015). It has also been studied for its bioactivity against pathogenic fungi, in which it showed to affect membrane integrity of *Candida albicans* at a minimal inhibitory concentration of approximately 20 μM (Lee et al., 2018).

1.3.3.2 Taxifolin

Taxifolin, also known as dihydroquercetin, is a flavonoid compound found in onion, milk thistle, and some tree barks. It is also present in some medicaments, including Venoruton® and Legalon™, the latter being an extract from the seeds of milk thistle. Taxifolin is an antioxidant (Weidmann, 2012), but also has anti-carcinogenic activities, partly because of its FAS inhibitory activity (Brusselmans et al., 2005; Weidmann, 2012). To investigate the latter, Brusselmans treated cancer cells with 25 μM taxifolin (Brusselmans et al., 2005). In a study from 2004, it was indicated that the mechanism of FAS inhibition by taxifolin was by competing with acetyl-CoA for its active site (Li & Tian, 2004). It has also been shown that taxifolin inhibits the FabG and FabI reductases of bacterial type II FAS, which correspond to the ketoreductase (KR) and enoylreductase (ER) of the FAS pathway, respectively (Zhang & Rock, 2004).

1.3.3.3 Orlistat

Orlistat is a drug mostly used as a treatment for human obesity, as it deactivates intestinal lipase and inhibits the absorption of fat in the intestines (Baretić, 2013). However, its activity as an FAS inhibitor has also been investigated, especially in cancer research, as FAS is strongly linked to tumor progression. For this purpose, orlistat has been tested in a range of concentrations from 0.75 to 100 μM in treatment of cancer cells (Kridel et al., 2004). More specifically, it is an inhibitor of the thioesterase (TE) domain of FAS (Kridel et al., 2004), which function is to release the final palmitate product from ACP of animal FAS (Herbst et al., 2018). In yFAS, this task is performed by another domain, namely malonyl-palmitoyl transferase (MPT), hence it is unclear whether orlistat inhibits this enzyme. If so, it is reasonable to surmise that it might inhibit the MPT domain. The effect of orlistat on lipid productivity in the high lipid producing microalgae *Nannochloropsis* has also been tested, in which it increased the lipid productivity at a concentration of 40 nM (Franz et al., 2013).

1.3.3.4 Cerulenin

Cerulenin, isolated from the fungus *Cephalosporum caerulens* (Lupu & Menendez, 2006), is an antifungal antibiotic, which works by inhibiting the biosynthesis of fatty acids by binding and irreversibly inhibiting the condensing ketosynthase (KS) domain of FAS systems. Due to its FAS inhibition, it has been used to investigate the distinctive FAS and PKS pathways of fatty acid synthesis in thraustochytrids, and how the synthesis of different fatty acids is divided between them. From this, Hauvermale et al. found that cerulenin concentrations up to 25 μM inhibited ^{14}C-acetate incorporation in short-chain fatty acids (produced by FAS), but not in PUFAs (DHA and DPA). In addition, at concentrations up to 5 μM, the ^{14}C-acetate incorporation in PUFAs was higher than with no cerulenin in the medium (Hauvermale et al., 2006). In the study by Hauvermale et al., as well as in further studies on the effect of cerulenin on DHA synthesis in thraustochytrids, cerulenin has been tested at concentrations from 0.5 to 200 μM (Chaung et al., 2012; Chen et al., 2016; Hauvermale et al., 2006).

1.3.3.5 Isoniazid

Isoniazid, also known as isonicotinic acid hydrazide (INH), is a medication used to treat tuberculosis. It works by inhibiting lipid and nucleic acid synthesis in *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Timmins & Deretic, 2006). To suppress lipid synthesis, isoniazid inhibits enoyl reductase (ER) (InhA in *Mycobacterium tuberculosis*) of fatty acid synthesis (Cheng et al., 2016; Marrakchi et al., 2000), for which purpose it must be activated. In the treatment of tuberculosis, the activation is catalyzed by the mycobacterial peroxidase enzyme KatG. Reactive species from this activation form adducts with NAD^+ and $NADP^+$, which then inhibit ER (Timmins & Deretic, 2006). Because of its inhibitory activity of FAS, the effect of isoniazid on DHA production in thraustochytrids has been studied. From testing isoniazid concentrations in the medium of 0.1 to 50 mM, Cheng et al. concluded that it enhanced the DHA production in *Aurantiochytrium* sp. (Cheng et al., 2016).

1.3.3.6 Irgasan

Irgasan, alternatively triclosan, is a broad spectrum antimicrobial trichlorinated diphenyl ether, commonly used in household products such as toothpaste and hand disinfecting soap, to inhibit the growth of microorganisms (Riber et al., 2016). It is also possible that it has an anti-carcinogenic effect (Sigma-Aldrich, n.d.-c). Irgasan works by inhibiting the enoylreductase (ER) of FAS, by binding to it and NAD^+, forming a stable complex which inhibits the ER activity (Riber et al., 2016). Its FAS inhibition activity has inspired a study of the effect of irgasan on DHA productivity in thraustochytrids. From testing irgasan concentrations in the medium of 0.1 to 100 μM, Cheng et al. concluded that irgasan also enhanced the DHA production in *Aurantiochytrium* sp. (Cheng et al., 2016).

1.4 The purpose of this work

Since thraustochytrids are commercially interesting because of their high DHA productivity, further development of tools to optimize this production is desirable. *Aurantiochytrium* is one of the thraustochytrid genera which have shown the highest DHA production, and is therefore interesting for further development.

An approach to optimize the DHA productivity is by genetic manipulation of strains, or to use genetic manipulation to study the functions of genes that are important for DHA synthesis. However, thraustochytrids have appeared to be challenging to manipulate genetically, and few methods for transformation and selection have been successful. Selection is also a challenge because thraustochytrids are diploid (Iwasaka et al., 2018). Another approach is to regulate the thraustochytrid culture conditions, which could also be useful to gain knowledge of the biochemistry of DHA synthesis.

The purpose of this work was to test selective compounds and selectable markers on *Aurantiochytrium* sp. strains T66 and S61, as a functional selection method does not yet exist for these strains, as well as to optimize the growth conditions of strain T66 for DHA synthesis by including FAS inhibitors in the growth medium. The effect of the potential selective compounds 5-FOA and 5-FC on the thraustochytrids was to be tested, and if they inhibited their growth, the individual genes making them sensitive could be disrupted to be used as selectable markers. Additionally, constructs containing the resistance gene to cycloheximide or zeocin (the mutated gene encoding RPL44 and the *Sh ble* gene, respectively) were to be cloned, to be transformed into strain T66 and potentially S61. Different promoters and terminators were to be tested in combination with the resistance genes, to see if they could express the genes and thus make the transformed cells cycloheximide or zeocin resistant, respectively. Furthermore, a mutant strain of *Aurantiochytrium* sp. strain T66 was to be screened for auxotrophic mutants to potentially be used for selection of transformants. This was to be done by evaluating the growth of different colonies on rich and minimal medium. Clones which failed to grow on minimal medium was to be tested for growth in minimal medium supplemented with an amino acid, uracil or adenine, and clones which grew in this medium was to be tested by PCR to identify the prospective mutation which made them auxotrophic. In addition, the effect of the FAS inhibitors apigenin, taxifolin, orlistat, cerulenin, isoniazid, and irgasan on T66 DHA production was to be investigated by analyzing the lipid and fatty acid content of cells cultivated in their presence, to see if they could improve DHA productivity.

2 Materials and methods

2.1 Media and solutions

2.1.1 Yeast extract–peptone–dextrose–sea salt (YPDS) medium

20 g/L peptone
10 g/L yeast extract
Deionized H_2O to 500 mL

The mixture was sterilized by autoclaving for 20 minutes at 121 °C. Then, 50 mL of autoclaved 40% (w/v) glucose, 200 mL autoclaved deionized H_2O and 250 mL of sterile (filtered with 0.2 µm filter) 7% Tropic Marin® sea salt classic was added per liter of media, generating a salt concentration of 1.75% in the final medium. For a medium with lower salt concentration (0.5%), 70 mL sterile 7% tropic marine sea salt was added per liter of media. If not stated otherwise, the medium with the higher salt concentration was used. For medium used for agar plates, 24 g/L agar was added before autoclaving, and streptomycin and ampicillin was added to a final concentration of 200 mg/L after autoclaving.

2.1.2 Minimal medium (MM)

40 g/L glucose
0.7 g/L NH_4Cl
0.3 g/L KH_2PO_4
18 g/L Na_2SO_4
0.25 g/L $MgSO_4 \cdot 7H_2O$
0.2 g/L $CaCl_2 \cdot 2H_2O$
0.4 g/L KCl
6.1 g/L tris base
5.8 g/L maleic acid
Deionized H_2O

The pH was adjusted to 7.0 using 10 M NaOH before the solution was sterilized with 0.2 µm filter. Before inoculation, 5 mL/L trace mineral solution 1 (TMS-1) and 1 mL/L vitamin mixture 1 was added. For agar plates, deionized H_2O was added to 500 mL before the pH adjustment and sterile filtering. Then, 500 mL autoclaved agar (48 g/L), TMS-1 and vitamin mixture 1 was added, and streptomycin and ampicillin were added to a final concentration of 200 mg/L.

2.1.3 Fat accumulation medium

This medium was prepared in the same way, with the same components, as minimal medium, except for adding 0.3 g/L yeast extract before sterile filtering. Before inoculation, 5 mL/L TMS-1 and 1 mL/L vitamin mixture 1 was added.

2.1.4 Luria Broth (LB) medium

10 g/L NaCl
10 g/L tryptone
5 g/L yeast extract
Deionized H_2O

The solution was autoclaved for 20 minutes at 121 °C. For Luria agar (LA) medium, 20 g/L agar was added before autoclaving.

2.1.5 Psi medium

5 g/L yeast extract
20 g/L tryptone
5 g/L $MgSO_4$
Deionized H_2O

Using 1 M KOH, the pH was adjusted to 7.6, and the solution was autoclaved at 121 °C for 20 minutes.

2.1.6 Transformation buffer 1 (TFB1)

2.94 g/L KAc
12.1 g/L RbCl
1.47 g/L $CaCl_2 \cdot 2H_2O$
10 g/L $MnCl_2 \cdot 4H_2O$
150 mL/L glycerol
Deionized H_2O

The pH was adjusted to 5.8 with 10% acetic acid. The solution was then sterilized by filtration with 0.2 μm filter.

2.1.7 Transformation buffer 2 (TFB2)

2.1 g/L MOPS
11 g/L $CaCl_2 \cdot 2H_2O$
1.21 g/L RbCl
150 mL/L glycerol
Deionized H_2O

The pH was adjusted to 6.5 with dilute NaOH, and the mixture was sterilized by filtration with 0.2 μm filter.

2.1.8 50 mM phosphate buffer

2.87 g/L KH_2PO_4
5.03 g/L K_2HPO_4
3.86 g/L dithiothreitol (DTT)
14.61 g/L ethylenediaminetetraacetic acid (EDTA)
Double-distilled H_2O

The solution was sterile filtered with 0.2 μm filter.

2.1.9 Trace mineral solution 1 (TMS-1) with chloride salts

2.28 g/L $FeCl_2$ (beads)
0.27 g/L $CuCl_2 \cdot 2H_2O$
0.21 g/L $ZnCl_2$
0.15 g/L $MnCl_2 \cdot 2H_2O$
10 mg/L $Na_2MoO_4 \cdot 2H_2O$
17 mg/L $CoCl_2 \cdot 6H_2O$
50 mL/L concentrated HCl
Deionized H_2O

The solution was sterile filtered with 0.2 μm filter.

2.1.10 Trace mineral solution 1 (TMS-1) with sulfate salts

5 g/L $FeSO_4 \cdot 7H_2O$
0.390 g/L $CuSO_4 \cdot 5H_2O$
0.440 g/L $ZnSO_4 \cdot 7H_2O$
0.150 g/L $MnSO_4 \cdot H_2O$
10 mg/L $Na_2MoO_4 \cdot 2H_2O$
20 mg/L $CoCl_2 \cdot 6H_2O$
50 mL/L concentrated HCl
Deionized H_2O

The solution was sterile filtered with 0.2 μm filter.

2.1.11 Vitamin mixture 1

0.05 g/L Thiamin HCl
0.005 g/L Cobalamin
Deionized H_2O

The solution was sterile filtered with 0.2 μm filter.

2.2 Plasmids

A list with descriptions and references for the plasmids used to clone constructs in this work is given in table 2.1. A general plasmid map of pEMR04 and the plasmids cloned (and some used in transformation of thraustochytrids) are shown in figure 2.1, and the promoters, GOIs, and terminators they contain are given in table 2.2. The plasmids contain either the mutated gene encoding RPL44 described in section 1.2.4, or the *Sh ble* gene described in section 1.2.5. When the terminator from pBGP1 was used, the *Pichia pastoris* autonomous replication sequence (PARS1) (Lee et al., 2005) was also included in the vector, to be sure that the whole terminator was included.

The TEF1 and GAP promoters (pTEF1 and pGAP, respectively) are strong constitutive (generates gene expression in all conditions) promoters from yeast, originally controlling the expression of a TEF gene (translation elongation factor) and a GAP gene (glyceraldehyde-3-phosphate dehydrogenase), respectively (Nevoigt et al., 2006; Waterham et al., 1997). These promoters have previously been used to express genes in thraustochytrids (Aasen et al., 2016), although the pGAPs tested in this work are not the same as the one which has been used in thraustochytrids (Hong et al., 2013). The first pGAP used (from pEMR04) was from the plasmid pPPE31 (Laila Berg, unpublished), which is a modified version of pBGP1. Eventually, the unmodified version of pGAP was used, to test this as well.

The GME, 8444, 4480 and 5903 promoters are from the *Aurantiochytrium* sp. T66 genome. pGME originally controls a gene encoding a protein annotated as GDP-mannose 3,5-epimerase (GME). p8444 controls a gene encoding ribosomal protein (RP) S23, p4480 controls a gene encoding RPL10a-1, and p5903 controls a gene encoding RPS0.

The yeast terminators AOX1 and CYC1 (AOX1tt and CYC1tt, respectively) are originally terminating the transcription of an AOX gene (alcohol oxidase) and a CYC (cytochrome c) gene, respectively (Curran et al., 2013; Hong et al., 2011; Zaret & Sherman, 1984). These terminators have previously been used for transcription termination in thraustochytrids (Cheng et al., 2011; Hong et al., 2013).

Table 2.1: Descriptions and references for the plasmids used to clone constructs in this work.

Plasmid	Description	Reference
pEMR04	Contains 18S cassette with single intron version of RPL44 gene, pGAP and AOX1tt	E-Ming Rau
pEMR05	RPL44 cDNA in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pEMRp1	pTEF1 in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pEMRp2	pGME in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pEMRp3	p8444 in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pEMRp4	p4480 in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pEMRp5	p5903 in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pBGP1	Vector with another version of pGAP, as well as the *Sh ble* gene in combination with pTEF1 and CYC1tt	(Lee et al., 2005)
pMAT5	*Sh ble* gene from pBGP1 in pCR® - Blunt II -TOPO® vector	E-Ming Rau
pMAT7	*Sh ble* gene and CYC1tt from pBGP1 in pCR® - Blunt II -TOPO® vector	E-Ming Rau

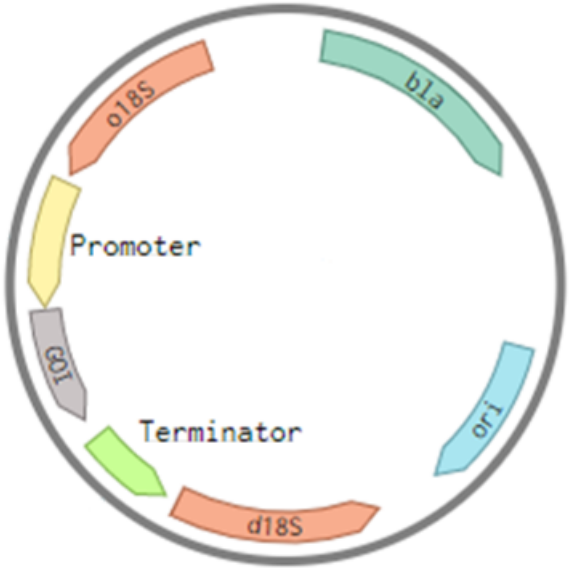


Figure 2.1: A general plasmid map of pEMR04 and the constructs cloned in this work, showing the 18S cassette in which the variable regions promoter, GOI (gene of interest), and terminator are contained. The plasmids also contain a gene encoding ampicillin resistance (bla), and a yeast origin of replication (ori).

Table 2.2: The promoters, genes of interest (GOI) and terminators contained in the 18S cassettes of the plasmids cloned in this work, as well as the parent plasmids containing their backbones.

Plasmid	**Promoter**	**GOI** [1]	**Terminator** [1]	**Parent plasmid**
pMAT1	GAP from pEMR04	RPL44 cDNA from pEMR05	AOX1	pEMR04
pEMR04p3	8444 from pEMRp3	Single intron RPL44	AOX1	pEMR04
pMAT1p1	TEF1 from pEMRp1	RPL44 cDNA	AOX1	pMAT1
pMAT1p2	GME from pEMRp2	RPL44 cDNA	AOX1	pMAT1
pMAT1p3	8444 from pEMRp3	RPL44 cDNA	AOX1	pMAT1
pMAT1p4	4480 from pEMRp4	RPL44 cDNA	AOX1	pMAT1
pMAT1p5	5903 from pEMRp5	RPL44 cDNA	AOX1	pMAT1
pMAT3	TEF1 from pBGP1	*Sh ble* from pBGP1	CYC1 from pBGP1	pEMR04
pMAT4	GAP from pEMR04	*Sh ble* from pBGP1	CYC1 from pBGP1	pEMR04

[1] From the parent plasmid when not stated otherwise.

Table 2.2: (continued)

Plasmid	Promoter	GOI [1]	Terminator [1]	Parent plasmid
pMAT4n	GAP from pBGP1	*Sh ble*	CYC1	pMAT4
pMAT10	GAP from pBGP1	Single intron version of RPL44	AOX1	pEMR04
pMAT6	GAP from pMAT10	*Sh ble* from pMAT5	AOX1	pMAT10
pMAT8	GAP from pMAT10	*Sh ble* from pMAT7	CYC1 from pMAT7 and AOX1	pMAT10

[1] From the parent plasmid when not stated otherwise.

2.3 Bioinformatic tools

2.3.1 BLAST

Basic Local Alignment Search Tool (BLAST) is an algorithm that compares regions of biological sequences by generating a certain score of similarity. It aligns both protein and nucleic acid queries to sequence databases (Altschul et al., 1990). There are several modes of operation when using BLAST, including tblastn, which aligns protein sequences with translated nucleotide sequences from a nucleotide database, and blastp, which aligns protein sequences with protein databases (Gertz et al., 2006). Additionally, BLAST calculates the statistical significance of matches when it generates an "expect value"(e-value), which is an estimate of how many times a given score would be generated by chance (Madden, 2013).

2.3.2 Benchling

Benchling is a web-based research platform with several available tools, including the "Molecular Biology Suite", which is a set of digital molecular biology tools. These tools can be used to design primers and other sequences, align sequences, and translate between DNA and protein sequences, among other purposes. They can also be used to digest DNA sequences with different restriction enzymes digitally, and map and digitally clone plasmid sequences by different virtual laboratory techniques, including restriction enzyme-based cloning (Benchling, Inc, n.d.).

2.4 Experimental procedures

2.4.1 Cultivation of microorganisms

2.4.1.1 Thraustochytrids

Aurantiochytrium sp. strain T66 (Jakobsen et al., 2007) or S61 (Jakobsen et al., 2008) stored at -80 °C were streaked out on a YPDS plate, or 100-1000 µL of a liquid culture of T66 or S61 were plated out on a YPDS plate, using a rod or 5-10 3 mm diameter glass beads per plate.

100-250 µL of mutagenized *Aurantiochytrium* sp. strain T66 cells with 72.9% or 11.1% survival after mutagenesis, stored at -80 °C, were plated on a YPDS plate. When plating the strain with 11.1% survival, a $0.4 \cdot 42$ mm needle was used to separate aggregated cells when plating, to assure clonality of colonies. The cells were randomly mutagenized by SINTEF, using nitrosoguanidine.

The plates were incubated at room temperature for 3-14 days until colonies appeared.

Manual picking with sterile toothpicks or automatic picking using a Genetix QPix II colony picker with "Christmas tree" picking heads was done to pick colonies. To sterilize the picking heads between colonies, they were dipped two times in 80% ethanol, heated to evaporate the ethanol, and then dipped in 0.1% tween80 at 3 °C to cool the picking heads. The cooling step could also be left out.

To make a liquid culture, cells from the plates were inoculated in YPDS medium and incubated in a shake flask (baffled if 250 mL or more) at 25 °C and 170 rpm for 24 hours or more.

2.4.1.2 *E. coli*

Escherichia coli (*E. coli*) DH5α (Chen et al., 2018) was used to purify cloned constructs from each other. To make a liquid culture, a colony from an LA plate, or cells stored at -80 °C, was inoculated in LB-amp (100 mg/L), and incubated at 37 °C and 225 rpm over night.

2.4.2 Quantification of growth

The growth of thraustochytrids and *E. coli* in liquid media was quantified by measuring optical density at a wavelength of 600 nm (OD_{600}), using a helios ε spectrophotometer. If the OD_{600} was over 0.5, the sample was diluted for a more accurate measurement.

2.4.3 Digestion of DNA by restriction enzymes

Restriction enzymes catalyze the cleavage of specific DNA sequences, originally as a part of the defense against foreign DNA (from bacteriophages) in bacteria (Pray, 2008). Restriction enzymes were used in this work to digest plasmid DNA at specific sites, to generate specific fragments. Suitable enzymes for specific digestions were chosen by doing digital digestions in benchling.

Reaction mixture:
1 µg DNA
5 µL 10x buffer
0.5-2 µL restriction enzyme
Double-distilled H_2O to 50 µL

The buffer used varied with the specific restriction enzymes used and was specified and supplied by the vendor of the enzyme. Some digestions required two different enzymes, in which cases the most suitable buffer for both the enzymes was used, or the buffer was regulated after the first digestion. The reaction mixture was incubated for at least 15 minutes and up to overnight at the optimal temperature for the restriction enzymes. The duration and temperature of the incubation were also specified by the enzyme vendor.

2.4.4 Ligation of DNA by ligase

To fuse DNA fragments (insert and backbone) when cloning constructs, T4 DNA ligase was used, an enzyme which fuses DNA ends with complementary overhangs.

Reaction mixture:
At least 1 µL insert
0.5-2 µL backbone
2 µL 10x T4 DNA ligase buffer
1 µL T4 DNA ligase
Double-distilled H_2O to 20 µL

The reaction was set up so that the molar ratio of insert:vector was 3:1 or higher. The reaction mixture was incubated for 10 minutes at room temperature.

2.4.5 DNA gel electrophoresis

DNA gel electrophoresis is a method for separating DNA molecules based on size, using an electrical field. A solution of the molecules is loaded in a well in an agarose gel, closest to the negative electrode. Because DNA is negatively charged (the phosphate backbone), it will migrate towards the positive electrode in the gel. The smallest molecules travel through the pores in the agarose meshwork most easily, while the larger molecules must squeeze through the pores. Thus, the smaller molecules travel farther in the gel than the larger ones. To see the sizes of the fragments separated, a standard with fragments of known sizes is loaded on the same gel, and to visualize the DNA fragments, the gel is usually stained with a dye which binds DNA specifically (Clark & Pazdernik, 2013, p. 111-113).

In this work, DNA gel electrophoresis in 0.8% agarose gel, or 2% agarose to separate fragments under 500 bp from each other, was performed. The agarose was dissolved in 1xTAE buffer (Tris-acetate-EDTA) prior to solidification. The gel also contained 50 µL/L GelGreen® or GelRed®, a dye which is excited by UV light when bound to DNA. GelGreen® was used when fragments were to be cut out of the gel, as it is also excited by blue light so that one can avoid being exposed to UV light when excising fragments from the gel. In other cases, GelRed® was used, as this dye is more sensitive for staining DNA (Biotium, n.d.). The electrophoresis was performed in 1xTAE buffer at 100 volts for 30-90 minutes, depending on the sizes of the fragments and the difference in size between fragments to be separated. As a standard, λ DNA digested with the restriction enzyme PstI was used, shown in appendix A.

2.4.6 Purification of DNA fragments from agarose gel

The Monarch DNA Gel Extraction Kit from New England BioLabs Inc. was used to purify DNA fragments from the agarose gel after gel electrophoresis, and the protocol version 1.1–10.14 from the kit was followed. The columns in this kit, which binds DNA, can isolate up to 5µg DNA. The DNA fragment of interest was excised from the gel, and four volumes of gel dissolving buffer was added to the piece of gel before it was incubated at 55 °C until the gel was dissolved, vortexing at intervals. The dissolved gel was loaded onto a column placed in a collection tube, and it was centrifuged with closed cap for one minute at 16000 g (as all the centrifugation steps in the protocol from this kit). Then, 200 µL DNA wash buffer was added, and it was again centrifuged for one minute, to wash away impurities. The washing step was repeated before the column was transferred to a 1.5 mL microcentrifuge tube, and 6 µL DNA elution buffer was added to the column matrix. After one minute, the column and microcentrifuge tube were centrifuged for one minute to elute the DNA. The concentration of DNA in the eluate was determined by measuring the absorbance at 260 nm using NanoDrop One from Thermo Fisher Scientific. The purified DNA was stored at -20 °C.

2.4.7 Purification of DNA fragments from a digestion reaction

2.4.7.1 Using Monarch PCR & DNA Cleanup Kit

The version 1.2–3.16 protocol from the Monarch PCR & DNA Cleanup Kit was followed to purify DNA fragments from digestion reactions. The columns in this kit, which binds DNA, can isolate up to 5µg DNA. The digestion reaction mixture was diluted with DNA cleanup binding buffer at the ratio 2:1 (binding buffer:reaction mixture), and then loaded onto a column placed in a collection tube, which was centrifuged with closed cap for one minute at 16000 g (as for all of the centrifugation steps from this kit). Then, the column was washed for impurities twice, transferred to a 1.5 mL microcentrifuge tube and the DNA was eluted, in the same way as described in section 2.4.6. However, in this protocol, 6 µL or more DNA Elution buffer was added to the column, depending on the DNA concentration and amount of sample that was desirable. In addition, the elution buffer was here heated to approximately 50 °C before use, because of the large sized DNA (≥10 kb). The concentration of DNA in the eluate was determined by measuring the absorbance at 260 nm using NanoDrop One from Thermo Fisher Scientific. The purified DNA was stored at -20 °C.

2.4.7.2 By ethanol precipitation

When a larger amount of DNA fragments was needed (around 100 µg), DNA from a digestion reaction mixture was purified by ethanol precipitation. 100 µL digestion reaction mixture, 11 µL 3 M sodium acetate at pH 5.2, and 277.5 µL 100% ethanol (or this ratio) were mixed well together, and incubated at -20 °C for at least 2 hours to precipitate DNA. The solution was then centrifuged at 4 °C and 12700 rpm for 15 minutes, and the resulting DNA pellet was washed with 1 mL 70% ethanol and centrifuged again for 10 minutes at 12700 rpm. The ethanol was poured off and the DNA pellet dried before it was dissolved in double-distilled H_2O (amount depending on desired concentration and amount of sample). The DNA-concentration was determined by measuring the absorbance at 260 nm using NanoDrop One from Thermo Fisher Scientific. The purified DNA was stored at -20 °C.

2.4.8 Isolation of plasmid DNA from *E. coli*

For validation of cloned constructs, i.e., when a small amount of plasmid DNA was needed, plasmids from transformed *E. coli* DH5α cells were isolated and purified according to the version 1.0.0 of the protocol from ZymoPURE^{TM} Plasmid Miniprep kit from Zymo Research. To isolate a larger amount of plasmid DNA, e.g., to be transformed into thraustochytrids, the protocol from ZymoPURE^{TM} II Plasmid Midiprep kit from Zymo Research was followed. These plasmid isolation kits yield purified plasmid DNA via a spin column-based method. According to the manufacturer, the columns in the Miniprep kit can isolate up to 100 µg plasmid DNA, whereas the columns in the Midiprep kit can isolate up to 400 µg.

For the Miniprep kit, a 2 mL culture of *E. coli* DH5α cells transformed with ligated plasmid DNA was centrifuged at 16000 g (the same as all the centrifugation steps in this kit) for 15-20 seconds. For the Midiprep kit, a 50 mL culture was spun down at 3400 g for 10 minutes. The supernatants were discarded. For both the kits, the pellets were resuspended by pipetting with P1 buffer, and then alkaline P2 buffer was added before the solution was incubated at room temperature for 1-2 minutes, to lyse the cells. The lysate was neutralized by adding ice-cold p3 buffer and mixing thoroughly, before incubation for 1-2 minutes at room temperature. The volumes used of P1, P2 and P3 buffers are given in the kit manuals. From here, the procedures from the two kits were different.

The procedure for the Miniprep kit continued with centrifuging the sample for 2-4 minutes. The supernatant was transferred to a Zymo – Spin^{TM} IIN column which binds the plasmid DNA, placed in a collection tube, and this was centrifuged for 30 seconds. To wash away impurities from the column, 200 µL endo-wash buffer was added, and the column was centrifuged for 30 seconds. Another washing step was carried out with 400 µL plasmid wash buffer, before centrifugation for one minute. The column was then transferred to a 1.5 mL microcentrifuge tube, and 30 µL DNA elution buffer was added to the column matrix. The column was incubated for one minute at room temperature, before centrifugation for 30 seconds to elute the plasmid DNA.

The Midiprep kit sample was transferred to a ZymoPURE^{TM} syringe filter. After the precipitate had floated to the top after 5-8 minutes, approximately 20 mL clear lysate was transferred to a 50 mL tube through the syringe filter. 8 mL binding buffer was added to the lysate and mixed thoroughly, and the mixture was added to a Zymo – Spin^{TM} III-P Column Assembly (also containing a column matrix that binds the plasmid DNA) placed onto a vacuum manifold. When adding anything to the column, the vacuum was off. The vacuum was then turned on until the liquid had passed through the column. To wash away impurities from the column, 2 mL wash 1 was added and then vacuumed. The wash step was then repeated two times with 2 mL wash 2. The column was then transferred to a collection tube and centrifuged at $\geq$10000 g for one minute to remove residual wash buffer. Then, the column was transferred to a 1.5 mL microcentrifuge tube, and 200 µL elution buffer was added to the column matrix. After two minutes, the column was centrifuged at $\geq$10000 g for one minute to elute the plasmid DNA.

The concentration of plasmid DNA in the eluates was determined by measuring the absorbance at 260 nm using NanoDrop One from Thermo Fisher Scientific. The isolated DNA was stored at -20 °C.

2.4.9 Isolation of DNA from thraustochytrids

2.4.9.1 Crude genomic DNA extraction

This procedure is based on the method to extract crude genomic DNA from yeast, described by Lõoke et al. (2011). A thraustochytrid colony was picked from a YPDS plate and suspended (by vortexing) in 100 µL 200 mM LiOAc, 1% SDS solution. This suspension was incubated at 70 °C for 5 minutes to lyse the cells, before 300 µL 100% ethanol was added and the solution was vortexed to precipitate the DNA. Then, the solution was centrifuged at 15000 g for three minutes, and the pellet with DNA and cell debris was washed with 1 mL 70% ethanol for one minute (by turning the tube). The ethanol was poured off, and the pellet was left to dry before it was dissolved in 100 µL double-distilled H_2O. The cell debris was spun down at 15000 g for 15 seconds, and the DNA was now in the supernatant. The sample was stored at -20 °C.

2.4.9.2 Extraction of genomic DNA

Genomic DNA was isolated using the MasterPure^{TM} Complete DNA and RNA Purification Kit, according to the protocol for cell samples and precipitation of total DNA. Cells from 0.5 mL liquid culture of thraustochytrids were spun down at 13000 rpm for one minute before they were resuspended to a volume of 25 µL by vortexing for 10 seconds. 300 µL of tissue and cell lysis solution, containing 1 µL Proteinase K, was added to the cells and mixed thoroughly. The suspension was incubated at 65 °C for 15 minutes while vortexing every 5 minutes, before it was kept on ice for 3-5 minutes. Then, 150 µL of MPC protein precipitation reagent was added to the solution which was then vortexed at full speed for 10 seconds. Cell debris was spun down at 4 °C and 16000 g for 10 minutes. 500 µL isopropanol was added to the recovered supernatant, and the tube was inverted 30-40 times to precipitate the DNA. The DNA was then spun down at 4 °C and 16000 g for 10 minutes. The isopropanol was poured off, and the pellet was rinsed two times with 70% ethanol which was removed afterward using a pipet, as well as air drying the pellet. The DNA was resuspended in 50 µL elution buffer from ZymoPURE^{TM} Plasmid Miniprep kit from Zymo Research. The DNA-concentration was determined by measuring the absorbance at 260 nm using NanoDrop One from Thermo Fisher Scientific, and the isolated DNA was stored at -20 °C.

2.4.10 Transformation

Transformation is a term for any method to transfer naked DNA into cells. This DNA may be incorporated into the chromosome of the recipient cell, and transferred genes may be expressed. Cells that are able to take up naked external DNA are said to be competent. Some species of bacteria are naturally competent and have genes encoding proteins which aid uptake of the DNA (Chen & Dubnau, 2004). Cells which are not naturally competent can be transformed using biological, chemical or physical methods, often to introduce recombinant DNA (for example a foreign gene controlled by a native promoter). The cell membrane and cell wall are barriers that must be crossed to transform cells (Rivera et al., 2014).

To transform *E. coli* DH5α with plasmid DNA, chemical transformation was used. This is a method where ion treatment is used to make cells competent. Treatment with divalent cations, most often Ca^{2+}, and often in combination with monovalent ions, for example Rb^{+}, has been most effective. The divalent cations are thought to bind both the negatively charged surface of the cell and the negatively charged DNA, canceling the repulsion between the DNA and the cell surface. Furthermore, the binding of the calcium ions to the cell membrane changes the permeability of the membrane. The ion treatment is followed by a heat-shock treatment which causes the cells to take up DNA, the mechanism of which is unclear. However, it is thought that the increased kinetic forces of molecules outside the cells are contributing to push the DNA inside the cells (Asif et al., 2017).

To transform *Aurantiochytrium* sp. strains T66 and S61, electroporation or a combination of electroporation and bead treatment was used. During electroporation, an electrical field causes changes in the membrane potential and stretching of the cells, thereby generating pores in the cell membrane, through which the DNA can be taken up. Different variable parameters have an impact on the transformation efficiency, such as pulse length, type and duration of the electrical field, the created membrane potential, DNA form and concentration, and the tolerance of the cells to the electroporation (Rivera et al., 2014). To enhance the transformation efficiency, electroporation can also be done in combination with cell wall weakening methods, such as agitation with glass beads (Adachi et al., 2017), chemical treatments or treatments with cell wall degrading enzymes. Agitation with glass beads is simple, fast and cheap, but the degree of damage to both cells and DNA can be relatively high (Rivera et al., 2014).

2.4.10.1 Making competent *E. coli* DH5α cells

One mL of a liquid *E. coli* DH5α culture was inoculated in 100 mL Psi medium and incubated at 37 °C until an OD_{600} of 0.4 was reached. The culture was then kept on ice for 15 minutes before the cells were spun down (4000 rpm at 4 °C for 5 minutes). The cells were resuspended in 40 mL ice-cold TFB1, and then incubated on ice for 5 minutes and spun down again. They were then resuspended in 3 mL ice-cold TFB2 and aliquoted in 1.5 mL Eppendorf tubes (100 µL in each) before they were frozen immediately by submerging them in liquid nitrogen for approximately 10 seconds. The aliquots were stored at -80 °C.

To calculate the transformation efficiency of the competent *E. coli* DH5α cells, an aliquot of cells was transformed with 361 ng of plasmid DNA (pMAT4). 100 µL of dilutions of 10^{-2} and 10^{-3} were plated, which resulted in 199 and 32 colonies, respectively. Based on the 10^{-3} plate, the transformation yielded approximately 300 000 CFU/mL (32 CFUs $\cdot 10^4 \approx$ 300 000 CFU/mL). Since 361 ng DNA was added to 1 mL, the transformation efficiency was calculated to be approximately $8.3 \cdot 10^5$CFU/µgDNA:

$$\frac{300\ 000\ \text{CFU/mL}}{361\text{ng}/1000\text{ng}/\mu\text{g}} = 8.3 \cdot 10^5 \text{CFU}/\mu\text{g}$$

2.4.10.2 Transformation of competent *E. coli* DH5α

Competent *E. coli* DH5α cells were transformed with cloned constructs, to purify the individual plasmids from each other, as well as for storage of successful constructs. DNA (1 µL if transforming plasmid DNA, 10 µL if transforming a ligation) was added to an aliquot of competent cells which had been thawed on ice for 5-10 minutes beforehand. It was mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42 °C for 45 seconds before they were kept on ice for three minutes. The heat shocked cells were inoculated in 0.9 mL LB medium and incubated at 37 °C and 225 rpm for one hour. 100 µL of cells were plated on LA containing 100 mg/L ampicillin, and the rest of the cells were spun down at 12000 g for 30 seconds. The supernatant was discarded, and the cells were resuspended in 100 µL LB and plated. The plates were incubated at 37 °C overnight.

2.4.10.3 Transformation of thraustochytrids by electroporation

This procedure is based on the method described by Hong et al. (2013) and was used to transform selection markers into thraustochytrid cells. A liquid thraustochytrid culture, cultivated for 26-28 hours, was incubated on ice for 5 minutes before the cells were spun down (4000 g at 4 °C for 15 minutes). The supernatant was discarded, and the cells were resuspended in 25 mL ice-cold deionized water and spun down again. This wash with ice-cold water was repeated once more, and then the cells were resuspended in 25 mL 50 mM phosphate buffer to generate protoplasts (cells lacking a cell wall). The suspension was rotated at 20 rpm in a PTR-35 Multi-Rotator from Grant-bio, for 30 minutes at room temperature, before the cells were spun down. The cells were then rewashed with 25 mL ice-cold deionized water and then with 10 mL ice-cold deionized water. Then, the cells were resuspended in ice-cold 1 M sorbitol to a volume which generated the desired cell density. 10 µL linearized DNA was added to 200 µL of the cells, and as a negative control, 10 µL DNA elution buffer from Monarch PCR & DNA Cleanup Kit or double-distilled H_2O (if ethanol precipitation was used to isolate the DNA from the digestion) was added to an aliquot of cells. They were then incubated on ice for 10 minutes. For electroporation, the cells and DNA were transferred to precooled 2 mm gap cuvettes, and electroporated with a voltage of 2000 V, a resistance of 200 Ω and a capacitor capacity of 25 µF, using a GenePulser

Xcell^{TM} from Bio-Rad. Immediately after electroporation, 1 mL YPDS medium was added to the cells before they were transferred to 5 mL YPDS medium in a 50 mL tube and incubated overnight at 25 °C and 170 rpm. The cells were then spun down at 4000 g for 5 minutes and resuspended in YPDS medium to a volume which gave an appropriate cell density for plating. The suspension was spread out on YPDS plates containing antibiotics, and without antibiotics for positive control.

2.4.10.4 Transformation of thraustochytrids by a combination of glass bead treatment and electroporation

This procedure is based on the method described by Adachi et al. (2017) and was used to transform selection markers into thraustochytrids. A liquid thraustochytrid culture of known OD_{600} was incubated on ice for 5 minutes, before the cells were spun down (4000 g at 4 °C for 15 minutes), and resuspended in YPDS medium to 400 µL per aliquot. 300 mg 0.5 mm diameter glass beads, which were acid washed and autoclaved beforehand, were added to 400 µL cell suspension in a 1.5-mL microcentrifuge tube, which was then vortexed at full speed for 10-30 seconds. The cells were then spun down (for 5 minutes when in microcentrifuge tubes) and washed once with 1 mL sterile 1.75% Tropic Marin® sea salt classic, and then three times with 1 mL sterile 50 mM sucrose. The cells were spun down after each wash step, and the supernatants were discarded. Finally, the cells were resuspended in 50 mM sucrose, to a total volume of 200 µL, to give an OD_{600} of 0.64, before 10 µL linearized DNA, DNA elution buffer from Monarch PCR & DNA Cleanup Kit or double-distilled H_2O for negative control, was added. Then, the suspension of cells and DNA was transferred to precooled 1 mm gap cuvettes, and electroporated with a voltage of 500 V, a resistance of 200 Ω and a capacitor capacity of 25 µF, using a GenePulser Xcell^{TM} from Bio-Rad. Immediately after electroporation, 1 mL YPDS medium was added to the cells, before transfer to 5 mL YPDS medium in a 50 mL tube. The cells were then incubated overnight at 25 °C and 170 rpm. The suspension was then centrifuged at 4000 g for 5 minutes, and the cells were resuspended in YPDS medium to a volume which gave an appropriate cell density for plating. The cells were then spread out on YPDS plates containing antibiotics, and without antibiotics for positive control.

2.4.11 PCR

Polymerase chain reaction (PCR) is a method for amplification of specific DNA sequences through iterative reaction cycles of three steps; template denaturation, primer annealing, and elongation by a DNA polymerase. This method can produce millions of copies of the target sequence in a short time and is illustrated in figure 2.2. The primers are the foundation on which the polymerase initiates DNA synthesis, as it attaches the first nucleotides to them. Two primers are needed, one complementary to one end of the target sequence, on one of the DNA strands, and the other complementary to the other end on the other DNA strand (figure 2.2). In this way, they also provide the specificity of the amplification, and the primer sequences are designed by the experimenter. They are usually 15 to 20 bases long,

and the longer they are, the more specific they are. In the first step of PCR, the template DNA is denatured by heating to 90 °C or so for 1-2 minutes. Then, the temperature is lowered to 50-60 °C to allow the primers to anneal to their complementary sequence in the template. In the elongation step, the temperature is adjusted to the polymerase used, which then synthesizes complementary DNA strands starting from the primers. The polymerase must be thermostable to tolerate the high temperature of the denaturation step and is often isolated from thermophilic bacteria. The new DNA molecules synthesized during the cycles also serve as templates for generating more DNA molecules. The PCR is performed in a thermal cycler, an instrument which cycles through the different temperatures (Clark & Pazdernik, 2013, p. 164-171).

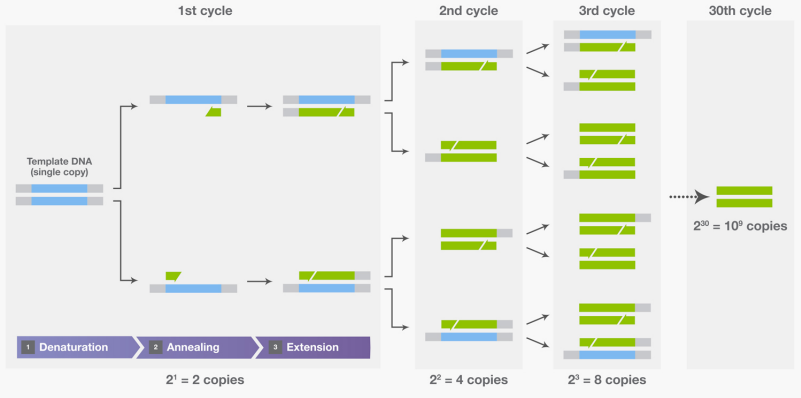


Figure 2.2: The iterative amplification of DNA sequences by PCR, showing the three steps of each cycle (Thermo Fisher Scientific, n.d.-b).

To see if thraustochytrid cells had been successfully transformed with the *Sh ble* gene, PCR was performed on their genomic DNA to see if the gene was amplified. The sizes of the fragments generated by the primer pairs used are given in table 2.3. The sequences of the primers are given in appendix B.

Table 2.3: The expected size of the fragments amplified by the primer pairs used in PCR to amplify the *Sh ble* gene.

Primer pair	**Fragment size (bp)**
Ble-F and Ble-SgrDI-R	438
Ble-F and Ble-ter-SgrDI-R	748
zeo sjekk F and Ble-SgrDI-R	394
zeo sjekk R and pTEFpspOMI-F	845

Reaction mixture:
5 µL 5x Q5 Reaction buffer
0.5 µL dNTPs, 10 mM
1.25 µL forward primer, 10 µM
1.25 µL reverse primer, 10 µM
1 µL template DNA
0.25 µL Q5 high-fidelity DNA polymerase
5 µL 5x Q5 high GC enhancer
Double-distilled H_2O to 25 µL

The PCR was performed in a C1000 Touch^{TM} thermal cycler from Bio-Rad, with the following program:

Initial denaturation: 98 °C for 3 minutes
25-35 cycles:
    Denaturation: 98 °C for 10 seconds
    Annealing: 61-72 °C for 30 seconds
    Elongation: 72 °C for 30 seconds to 1 minute
Final extension: 72 °C for 2 minutes

The temperature of the annealing step depended on the melting temperature of the primers, which again is dependent on their length and base composition. The duration of the elongation step depended on the length of the expected PCR product, based on the fact that the rate of polymerization by Q5 high-fidelity DNA polymerase is 1 kb / 20-30 seconds, according to the manufacturer. The sample was then cooled to 4 °C, and the final product was stored at -20 °C.

2.4.12 Extraction of lipids from thraustochytrids

A modified Bligh and Dyer (1959) method using a Precellys homogenizer was used to isolate total lipids from thraustochytrid cells, for analysis of the lipid content and fatty acid composition of the cells. A 5-10 mL sample from a liquid thraustochytrid culture was spun down at 5000 rpm and 4 °C for 5 minutes. The supernatant was discarded, and the cells were washed in 5 mL ice-cold 0.9% NaCl and spun down again. The cells were then washed in 5 mL ice-cold double-distilled H_2O, spun down, and the pellet was stored in -20 °C before it was freeze-dried at -103 °C for 24 hours, using a VirTis BenchTop Pro freeze dryer from SP Scientific. The freeze-dried cells were stored at -20 °C until lipid extraction.

Approximately 20 mg freeze-dried cells were added to a 2 mL vial with sleeve, containing 500 mg 1.4 mm zirconium oxide beads. A cap with o-ring was used. 1000 µL methanol:chloroform (2:1) was added, and the sample was homogenized in a Precellys 24 homogenizer from Bertin instruments for $3 \cdot 30$ seconds at 6500 rpm with 15 seconds pause, while kept at approximately 15 °C using N_2 gas. Then, 333 µL chloroform was added, and the sample was shaken at 1500 rpm for one minute before 333 µL deionized H_2O was added, and the sample was shaken again.

The sample was then centrifuged at 14000 rpm and 16 °C for 10 minutes. 550 µL of the lower chloroform phase containing the lipids was transferred to a pre-weighed 1.5 mL glass vial (transparent HPLC, Holger Technology). The chloroform was evaporated on a heating block at 40 °C while flushing with N_2 gas to avoid oxidation, and the transparent HPLC vial was placed in an exicator overnight before it was weighed. The extracted lipids were stored at -20 °C.

2.4.13 Hydrolysis of lipids to free fatty acids and extraction of the fatty acids

Evaporated samples containing 3-10 mg lipids extracted from thraustochytrids was hydrolyzed to free fatty acids (FFA) to be analyzed by mass spectrometry. One mL 2 M KOH in 95% ethanol or deionized H_2O was added to a sample before it was flushed with N_2 gas, capped with a PTFE lined cap, and incubated at 70 °C for 90 minutes for hydrolysis. The sample was then transferred to a 13 mL polypropylene tube, which was pre-rinsed in methanol to minimize palmitate and stearate contamination from the tube (Yao et al., 2016). One mL 4 M H_2SO_4 was added to extract the K^+ to the acidic aqueous phase, and the sample was mixed before 2 mL dichloromethane (DCM) was added for FFA extraction. The sample was whirl-mixed for 60 seconds and then centrifuged at 4000 g for 10 minutes. 200 µL of the DCM phase was transferred to a 1.5 mL glass LC-vial, flushed with N_2 gas, capped with a PTFE lined cap, and stored at -20 °C.

3 Results

3.1 Exploration of selectable markers to be used in *Aurantiochytrium* sp. strains T66 and S61

Since a functional selection method for *Aurantiochytrium* sp. T66 and S61 does not exist, different possible selection markers were investigated here. The potential selective compounds 5-FOA and 5-FC were looked into by testing the effect they had on T66 or S61. In addition, resistance genes for the selective compounds cycloheximide and zeocin were cloned into constructs and transformed into thraustochytrids to be tested as selection markers.

3.1.1 The effect of 5-FOA on thraustochytrids

To test the effect of 5-FOA on their growth, and thus if it could be used to select for uracil auxotrophic mutants (section 1.2.2), *Aurantiochytrium* sp. strains T66 and S61 were plated on YPDS plates containing 500 mg/L, 1000 mg/L or no 5-FOA. The number of colonies on the plates after three days of incubation are shown in table 3.1. These results demonstrate that T66 and S61 were able to grow on 5-FOA, and are thus resistant. Thus, 5-FOA cannot be used as a selective compound for thraustochytrids. As there was little or no growth on the positive control plates without 5-FOA, a lower dilution of cells was plated on positive control plates when testing their response to 5-FC, described next.

Table 3.1: The number of *Aurantiochytrium* sp. T66 and S61 colonies counted on YPDS plates with or without different concentrations of 5-fluoroorotic acid, plated with different dilutions of cells.

Culture		5-FOA concentration (mg/L)		
		0	**500**	**1000**
T66	**10^{-2}**	- [1]	355	500+
	10^{-4}	3	1	0
	10^{-6}	0	0	0
S61	**10^{-2}**	- [1]	54	12
	10^{-4}	0	0	0
	10^{-6}	0	0	0

[1] Not plated.

3.1.2 Exploration of 5-FC as a selective compound

3.1.2.1 Searching for cytosine deaminase in thraustochytrids using BLAST

To investigate if *Aurantiochytrium* sp. strain T66 has a gene encoding the enzyme cytosine deaminase, which confers sensitivity to 5-FC as mentioned in section 1.2.3, a BLAST search was done. If the strain has this gene, it indicates that it is sensitive to 5-FC, and hence 5-FC could, in theory, be used to select for 5-FC resistant mutants. Cytosine deaminase protein sequences from *Candida albicans*, *Saccharomyces cerevisiae* (Erbs, Exinger, & Jund, 1997), and *Nannochloropsis gaditana* (GenBank ID: EWM21163.1), were BLASTed against protein sequences from strain T66 (whole-genome shotgun contigs database) using tblastn. These searches generated lower scores than 80, a coverage of 20-70%, and e-values higher than 10^{-14}, indicating that T66 does not have any proteins that resemble the cytosine deaminases from these organisms. Protein sequences from T66 that were automatically annotated as cytosine deaminase, "probable cytosine deaminase", or "deaminase activity" were also BLASTed against the reference proteins and model organism databases using blastp. This generated similar results, in that there were no apparent similarities with cytosine deaminase domains from other proteins, which suggests that T66 does not have cytosine deaminase activity. Hence, 5-FC is probably non-toxic to T66 cells, but its effect on the cells was tested to examine this further.

3.1.2.2 The effect of 5-FC on thraustochytrids

To test if 5-FC could be used to select for mutants by testing the effect of 5-FC on their growth, *Aurantiochytrium* sp. strain T66 was plated on YPDS plates containing 1000 mg/L, 2000 mg/L or no 5-FC. Here, strain S61 was not tested, as it is genetically similar to T66, and this result is probably more dependent on what genes the strains have, as opposed to the test of their response to 5-FOA, which depended on other factors. The resulting colonies on the 5-FC plates were smaller than on the 0-plates. The number of colonies on the plates after three days of incubation are shown in table 3.2, which indicate that the thraustochytrids are resistant to 5-FC. This means that neither 5-FC can be used in selection of mutant thraustochytrids containing a GOI, and other methods should be explored.

Table 3.2: The number of *Aurantiochytrium* sp. T66 colonies counted on YPDS plates with or without different concentrations of 5-fluorocytosine, plated with different dilutions of cells.

Dilution	5-FC concentration (mg/L)		
	0	**1000**	**2000**
$\mathbf{10^{-2}}$	62	59	57
$\mathbf{10^{-3}}$	7	2	1

3.1.3 Cloning of constructs containing resistance genes for cycloheximide and zeocin

To investigate if cycloheximide or zeocin could be used as selective compounds for thraustochytrids, several plasmid constructs containing a mutated gene encoding RPL44 or the *Sh ble* gene, were cloned to be transformed into thraustochytrids to see if these genes rendered them cycloheximide or zeocin resistant, respectively (sections 1.2.4 and 1.2.5). The genes were cloned in combination with different promoters and terminators, to test a range of levels of gene expression. The backbones and inserts of the constructs to be cloned were cut out of their respective parent plasmids, using appropriate restriction enzymes which generated compatible cohesive ends at the DNA cut sites for T4 DNA ligase to fuse. The digestion reactions were subjected to DNA gel electrophoresis to separate the fragments (not shown), and the backbones and inserts were excised from the gel, purified, and ligated. The ligations were transformed into competent *E. coli* DH5α cells, and plasmid DNA was extracted from 2-6 resulting colonies (depending on the number of colonies on the plates relative to the negative control plates representing cells transformed with ligations without the insert). The plasmid DNA was digested with appropriate restriction enzymes to generate an identifiable fragment size pattern, and then subjected to DNA gel electrophoresis, to confirm that the plasmid was the correct construct. Electrophoresis was also performed on the parent plasmid containing the backbone, on the same gel and digested with the same set of enzymes, as a negative control to distinguish this from the correct construct. The restriction enzymes used to verify the constructs, and the expected fragments from these digestions, are shown in table 3.3.

Table 3.3: The restriction enzymes used to verify the cloned constructs, and the expected fragment sizes from the digestion of the correct constructs and the re-ligated parent plasmid containing the backbone.

Construct	**Enzymes**	Expected fragments	
		Correct construct	**Re-ligated parent plasmid**
pMAT1	BamHI-HF and KpnI-HF	3579, 1994	3571, 1470, 683
pEMR04p3	pspOMI and SbfI	4352, 1014, 280, 179	4352, 1372
pMAT1p1	BbsI-HF and NotI-HF	3776, 1163, 607	4410, 1163
pMAT1p2	NcoI and pspOMI	5063, 208	5063, 510
pMAT1p3	NcoI and SpeI-HF SalI-HF and SfoI	5078, 596 4197, 935, 542	5078, 495 4197, 1376
pMAT1p4	NcoI and SpeI-HF SalI-HF and SfoI	5078, 627 4197, 1508	5078, 495 4197, 1376
pMAT1p5	NcoI and SpeI-HF	5078, 779	5078, 495
pMAT3	BbsI-HF and XhoI	3844, 1052, 822	4606, 822, 296
pMAT4	EcoRV-HF and NcoI	3994, 1288, 440	3998, 1726
pMAT4n	EcoRV-HF and PshAI	3855, 1288, 580	4434, 1288
pMAT10	NheI-HF and BglII	4937, 788	4937, 420, 367
pMAT6	SgrAI and XhoI DraIII	4141, 822, 645 2404, 2006, 1198	4903, 822 2803, 2006, 916
pMAT8	SgrAI and SalI-HF DraIII	4197, 1134, 588 2715, 2006, 1198	4197, 1528 2803, 2006, 916

3.1.3.1 Constructs containing the cycloheximide resistance gene

The result from the verification of the pMAT1 construct, extracted from six *E. coli* clones, is shown in figure 3.1. This indicates that five of the constructs were correct, as they had the expected fragment pattern (table 3.3), and one of them (lane 2) was likely the re-ligated parent plasmid, as it had the same fragment pattern as the negative control. Liquid cultures of the transformed *E. coli* cells containing the constructs in lanes 1 and 3 were stored at -80 °C.

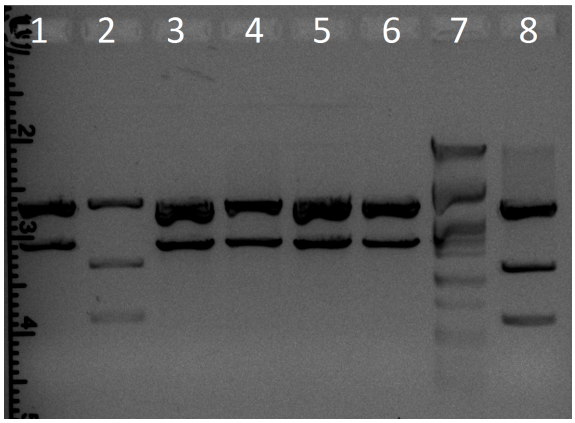


Figure 3.1: The result from the verification of the pMAT1 construct, extracted from six *E. coli* clones (lanes 1-6, each lane represents one clone). Lane 8 contains the digested parent plasmid (pEMR04), and lane 7 contains the lambda PstI standard, shown in appendix A.

The result from the verification of the pEMR04p3 construct, extracted from six *E. coli* clones, is shown in figure 3.2. This indicates that all of the examined constructs were correct. The smallest fragment of 179 bp (table 3.3) was not visible in any of the lanes, presumably due to the small size. Liquid cultures of the transformed *E. coli* cells containing the constructs in lanes 1 and 5 were stored at -80 °C.

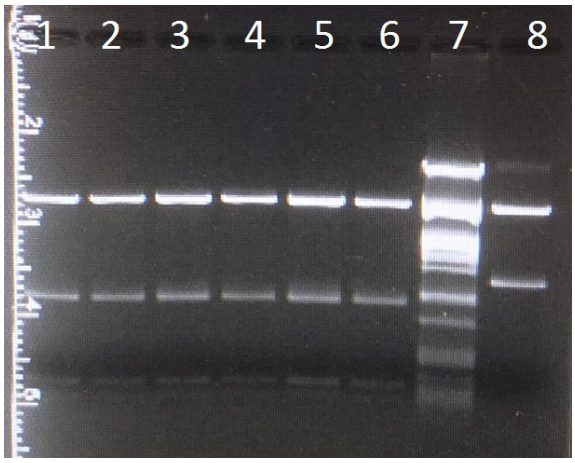
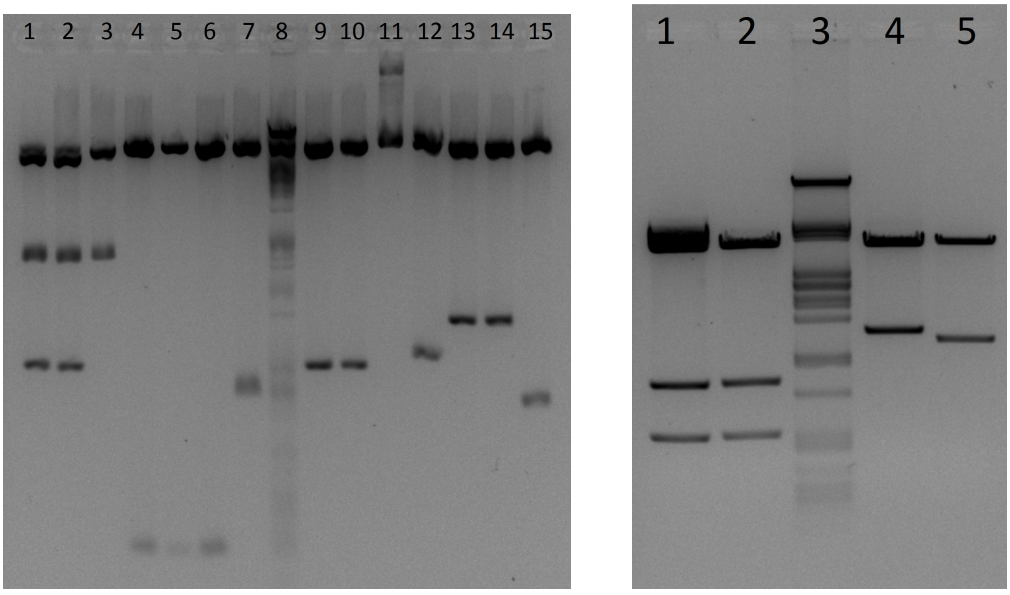


Figure 3.2: The result from the verification of the pEMR04p3 construct, extracted from six *E. coli* clones (lanes 1-6, each lane represents one clone). Lane 8 contains the digested parent plasmid (pEMR04), and lane 7 contains the lambda PstI standard, shown in appendix A.

The result from the verification of the derivative constructs of pMAT1 is shown in figure 3.3. For each construct (pMAT1p1, pMAT1p2, pMAT1p3, pMAT1p4 and pMAT1p5), two *E. coli* colonies were examined to hold the correct construct, except for pMAT1p2, where three colonies were examined. Figure 3.3 a demonstrates that all of the constructs tested to be pMAT1p1, pMAT1p2 and pMAT1p5 were correct, and transformed cells containing these were stored at -80 °C. As for pMAT1p4, the construct in lane 11 was not correct, and the other one tested (lane 12) was difficult to distinguish from the constructs tested to be pMAT1p3. Therefore, another test of these constructs was done using a different set of restriction enzymes, to distinguish them from each other (figure 3.3 b). This demonstrated that they were the correct constructs, and cells containing them were stored at -80 °C.



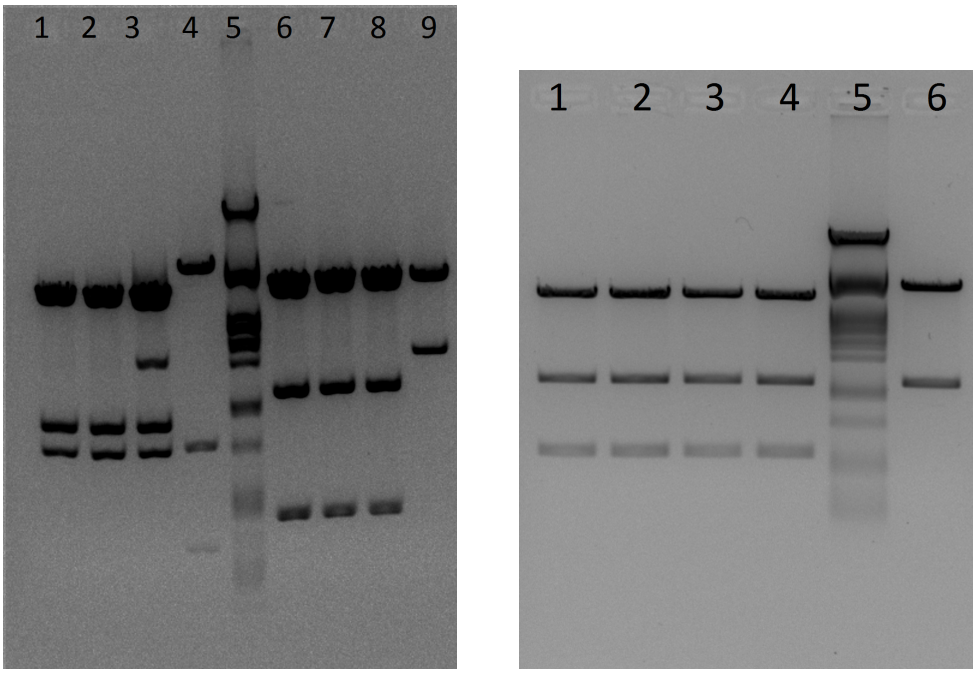
(a) (b)

Figure 3.3: The result from the verification of the derivative constructs of pMAT1. One lane represents one *E. coli* clone. **(a)** Lanes 1 and 2 contain constructs cloned as pMAT1p1, lanes 4-6 as pMAT1p2, lanes 9 and 10 as pMAT1p3 (digested with NcoI and SpeI), lanes 11 and 12 as pMAT1p4 (digested with NcoI and SpeI), and lanes 13 and 14 as pMAT1p5. Lanes 3, 7, and 15 contain the digested parent plasmid (pMAT1). Lane 8 contains the lambda PstI standard, shown in appendix A. **(b)** The result from the verification of the constructs pMATp3 and pMATp4 using the restriction enzymes SalI-HF and SfoI. Lanes 1 and 2 contain constructs cloned as pMAT1p3 and lane 4 as pMAT1p4. Lane 5 contains the digested parent plasmid (pMAT1), and lane 3 contains the lambda PstI standard, shown in appendix A.

Transformations of *Aurantiochytrium* sp. T66 with the different constructs containing the cycloheximide resistance gene were carried out by E-Ming Rau and terminated because of problems with false positives.

3.1.3.2 Constructs containing the zeocin resistance gene

The result from verification of the constructs pMAT3 and pMAT4, extracted from three *E. coli* clones for each construct, is shown in figure 3.4 a, and the result from verification of the pMAT4n construct extracted from four clones, is shown in figure 3.4 b. These results indicate that all of the constructs examined were correct, although one of the pMAT3 constructs generated one additional band (lane 3 in figure 3.4 a). This additional band was probably a result of incomplete digestion, as the size of this fragment is the same as the two smallest fragments combined (1052+822). Transformed *E. coli* holding the pMAT4n constructs in lanes 2 and 4, and all of the pMAT3 and pMAT4 constructs tested, were stored at -80 °C.



(a) (b)

Figure 3.4: **(a)** The result from verification of the constructs pMAT3 (lanes 1-3) and pMAT4 (lanes 6-8). Each lane represents one *E. coli* clone. Lanes 4 and 9 contain the digested parent plasmid (pEMR04), and lane 5 contains the lambda PstI standard, shown in appendix A. **(b)** The result from verification of the pMAT4n construct, extracted from four *E. coli* clones (lanes 1-4, each lane represents one clone). Lane 6 contains digested parent plasmid (pMAT4), and lane 5 contains the lambda PstI standard, shown in appendix A.

The result from the verification of the pMAT10 construct, extracted from three *E. coli* clones, is shown in figure 3.5. Based on this, all three constructs tested seemed to be the correct ones, and *E. coli* cells containing the constructs in lanes 2 and 3 were stored at -80 °C.

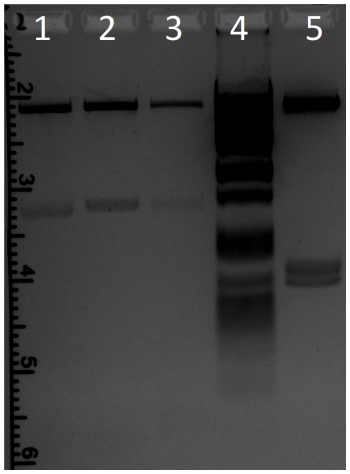
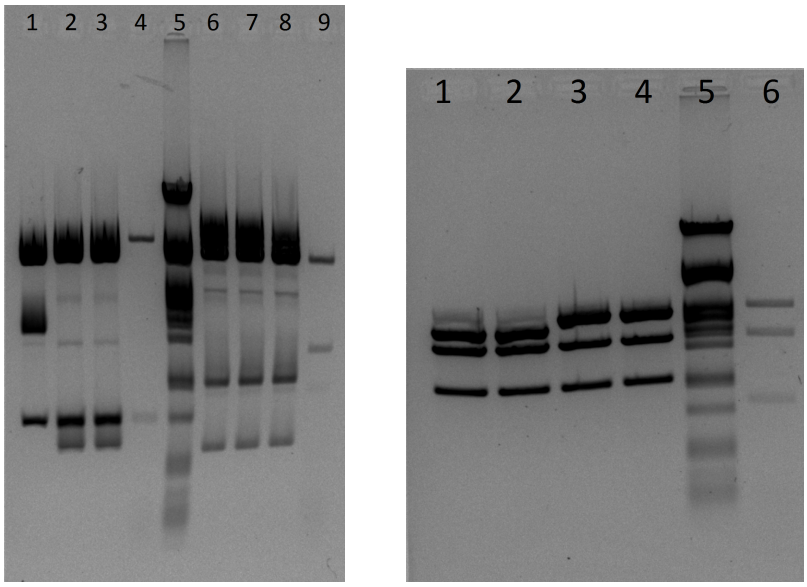


Figure 3.5: The result from the verification of the pMAT10 construct, extracted from three *E. coli* clones (lanes 1-3, each lane represents one clone). Lane 5 contains digested parent plasmid (pEMR04), and lane 4 contains the lambda PstI standard, shown in appendix A.

The result from the verification of the pMAT6 and pMAT8 constructs, extracted from three *E. coli* clones for each construct, is shown in figure 3.6. Figure 3.6 a shows that all three of the constructs tested to be pMAT8, as well as two of the constructs tested to be pMAT6 (lanes 2 and 3, lane 1 looked more like the parent plasmid), were most likely the correct ones. However, there were a few faint, unspecific bands in addition to the expected ones, which is why these constructs were tested again with different restriction enzymes (figure 3.6 b). In this test, the constructs were verified, and transformed cells holding them were stored at -80 °C.

The pMAT10 construct was made as a vector to hold the unmodified version of pGAP, to be used in the cloning of pMAT6 and pMAT8. It was therefore not used for transformation. The pMAT8 construct was made as a backup, to test having both the AOX1 and CYC1 terminators next to each other, but was neither used for transformation. The rest of the constructs containing the *Sh ble* gene were used in transformations of thraustochytrids, described next.



(a) (b)

Figure 3.6: The result from the verification of the constructs pMAT6 and pMAT8. One lane represents one *E. coli* clone. **(a)** Lanes 1-3 contain constructs cloned as pMAT6 (digested with the enzymes SgrAI and XhoI), lanes 6-8 contain constructs cloned as pMAT8 (digested with SgrAI and SalI-HF), and lanes 4 and 9 contain the digested parent plasmid (pMAT10). Lane 5 contains the lambda PstI standard, shown in appendix A. **(b)** The result from verification of the constructs pMAT6 and pMAT8 using the restriction enzyme DraIII. Lanes 1 and 2 contain constructs cloned as pMAT6, lanes 3 and 4 as pMAT8, lane 6 contains the digested parent plasmid (pMAT10), and lane 5 contains the lambda PstI standard, shown in appendix A.

3.1.4 Transformation of the *Sh ble* gene into thraustochytrids

To see if the *Sh ble* gene (described in section 1.2.5) could render *Aurantiochytrium* sp. strains T66 and S61 cells zeocin resistant, and thus be used as a selection marker, wild type cells were electroporated, with or without treatment with glass beads to weaken the cell wall, with linear DNA fragments containing the gene. The DNA fragments were made by cutting pMAT3, pMAT4, pMAT4n and pMAT6 (table 2.2) with the restriction enzymes KpnI-HF and NotI-HF, and isolating the fragments from the digestion reaction. The fragments were controlled to be correct by DNA gel electrophoresis (not shown).

As described in section 1.2.5, the cell density plated affects the selection activity of zeocin, which is why different cell densities were tested for plating, along with testing of which zeocin concentration to use. To do this, different wild type cell densities were plated out on lower salt YPDS plates with different zeocin concentrations. Lower salt YPDS plates were used due to the inhibitory a effect high salt concentration has on the antibiotic activity of zeocin (section 1.2.5). Plates with a sufficiently low cell density to be able to select for single mutant colonies which might appear when plating out transformed cells, were considered an appropriate cell density and zeocin concentration.

3.1.4.1 Transformation of pMAT3 and pMAT4 into *Aurantiochytrium* sp. strain T66 by electroporation

In the first transformation, a liquid culture of *Aurantiochytrium* sp. strain T66 was electroporated with linear DNA from pMAT3 or pMAT4 (at this time, pMAT6 was not cloned yet). This was the first time the transformation protocol was tested by us, and we did not focus on the cell density for plating here. For pMAT3, 4.1 µg DNA was added to an aliquot of cells (10 µL of 406 ng/µL DNA), and 3.8 µg DNA from pMAT4 was added to another aliquot. 200 µL of electroporated and recovered cell suspension was plated out per low salt YPDS plate containing 75 µg/mL zeocin, or no zeocin for positive control. The zeocin concentration used was based on experiments testing the growth of wild type T66 on zeocin, done by E-Ming Rau and SINTEF. The resulting positive control plates were overgrown. The degree of growth after 8-14 days on the resulting plates containing zeocin was too extensive to count colonies, and the growth on the negative control plates (plated with cells electroporated with elution buffer) was not significantly less. This suggests that the cell density on the plates was too high for the zeocin to have any significant effect on the cell growth, or / in combination with that the zeocin concentration was too low. Thus, a test of cell density to plate and zeocin concentration to use when doing transformations was done, described next.

3.1.4.2 Test of zeocin concentration and cell density for plating

To test different cell densities for plating on different concentrations of zeocin, a liquid culture of *Aurantiochytrium* sp. strain T66 cultivated for 26-28 hours and concentrated 2, 4, 6, 8 and 10 times, was plated on lower salt YPDS plates with 75 or 100 µg zeocin per mL, or no zeocin for positive control. The unconcentrated culture was also plated. The cells were concentrated by spinning them down at 4000 g for 5 minutes, and then resuspending them in a suitable volume of YPDS medium. The resulting positive control plates were overgrown, and the resulting growth on the plates containing zeocin is shown in figure 3.7. Of these plates, the 2 times concentrated plate containing 75 µg/mL zeocin (figure 3.7 b) showed an appropriate degree of growth, and therefore this approximate cell density was plated out on this zeocin concentration when doing the next transformation. On the plates in figure 3.7, some areas were overgrown, possibly because of high cell densities in these areas, which indicates uneven plating of cells. Thus, instead of a metal rod, glass beads were used to plate cells in subsequent transformations and zeocin tests, to spread the cells more evenly.

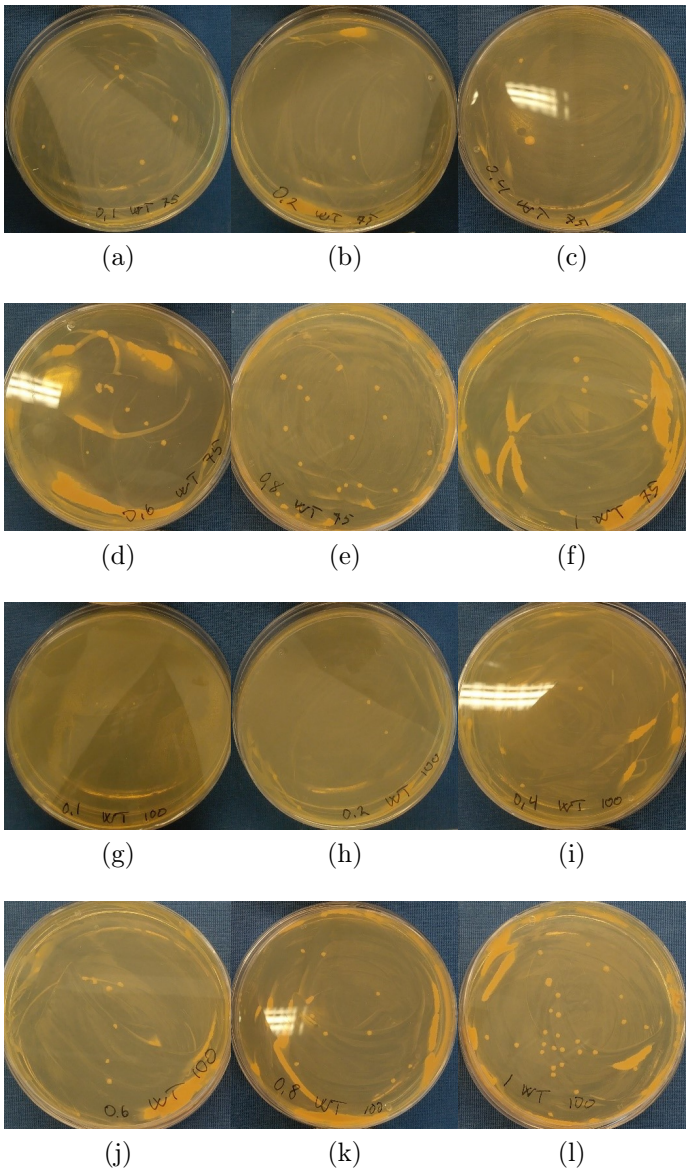


Figure 3.7: The resulting degree of growth from plating different densities of wild type *Aurantiochytrium* sp. strain T66 cells on lower salt YPDS plates with 75 (**(a)-(f)**) or 100 (**(g)-(l)**) µg/mL zeocin, 8 days after plating. **(a) and (g)** Unconcentrated culture, or concentrated **(b) and (h)** 2, **(c) and (i)** 4, **(d) and (j)** 6, **(e) and (k)** 8 and **(f) and (l)** 10 times.

3.1.4.3 Transformation of pMAT3 into *Aurantiochytrium* sp. strain T66 by electroporation

The next transformation was done of a liquid *Aurantiochytrium* sp. T66 culture with 5.7 µg linear DNA from pMAT3. Only one plasmid was used for the transformation to keep the number of plates manageable, because of the low cell density required. After electroporation and recovery, 150 µL cell suspension was plated out per low salt YPDS plate containing 75 µg/mL zeocin. As the previous positive controls were overgrown, a 10^{-2} dilution was plated on a YPDS plate

without zeocin, to be able to count the CFUs in the positive control. This resulted in approximately 3000 colonies, meaning approximately $3 \cdot 10^5$ cells were plated per plate. Representative plates containing zeocin from this transformation are shown in figure 3.8, which shows that the numbers of colonies on the plates compared to the numbers of colonies on the negative control plates (with cells transformed with elution buffer) were not significantly different from each other. This suggests that the transformation was unsuccessful. However, a few more early colonies appeared on the transformant plates than on the negative control plates, which were tested by PCR amplification.

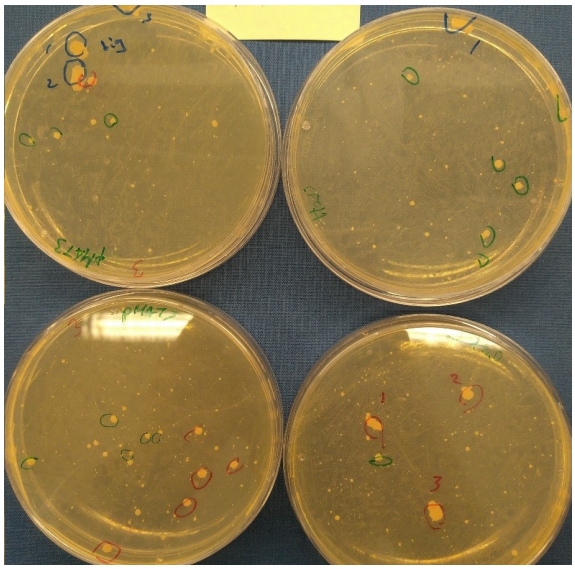


Figure 3.8: Representative low salt YPDS plates with 75 µg/mL zeocin, plated with *Aurantiochytrium* sp. strain T66 transformed with a linear fragment from pMAT3 containing the *Sh ble* gene, to see if it made the cells zeocin resistant. The picture is taken 12 days after plating, and the two plates on the left are with cells transformed with pMAT3, and the two plates on the right are negative control (cells transformed with elution buffer). The colonies marked in blue were the largest colonies 6 days after plating, and the colonies marked in red were slightly smaller colonies. Colonies marked in green appeared after the blue and red ones. DNA from the colonies numbered 1 and 2 (in red on the negative control) were subjected to PCR to see if they had the *Sh ble* gene.

Both crude genomic DNA and genomic DNA were extracted from the colonies numbered 1 and 2 in figure 3.8, both from the pMAT3 plates and the negative control plates (marked in red on the negative control). This DNA was amplified by PCRs with primer pairs complementary to flanking sequences of the transformed DNA sequences, to see if the *Sh ble* gene had been incorporated into the genome of the cells, in which case it would be amplified in the PCR. Genomic DNA from wild type *Aurantiochytrium* sp. strain T66 and pMAT3 plasmid DNA was used as negative and positive controls, respectively.

The crude DNA from the transformants was tested with several different primer pairs, and the results were ambiguous because bands of PCR products emerged inconsistently. This inconsistency raised the suspicion of contamination, and thus

PCR samples with double-distilled H_2O instead of template was included as negative controls. These did not show any PCR product, which minimized the probability of contamination. Genomic DNA from the transformants was also tested with one primer pair, which gave similar results as the crude extracts. The result from testing with the primer pair Ble-F and Ble-SgrDI-R was the least ambiguous and is shown in figure 3.9. PCR samples containing both positive control and crude genomic DNA extracted from electroporated cells were also included in this test to avoid false negatives in case the crude DNA extracts contained inhibitors of the PCR. These reactions also gave PCR products from the positive control, indicating that the crude extracts did not contain any such inhibitors. In this test (figure 3.9), the PCR product was visible only in the lanes containing the positive control, although there were a few weak, unspecific bands, which could be caused by unspecific binding of the primers. This result indicates that the *Sh ble* gene was not incorporated in any of the clones tested, although they were seemingly zeocin resistant. A potential problem could be that the sample size was quite small, which is why a higher cell density was tested in the next transformation. In the following transformations and cell density tests, the cell density was also calculated more accurately, as this seemed to be an important factor.

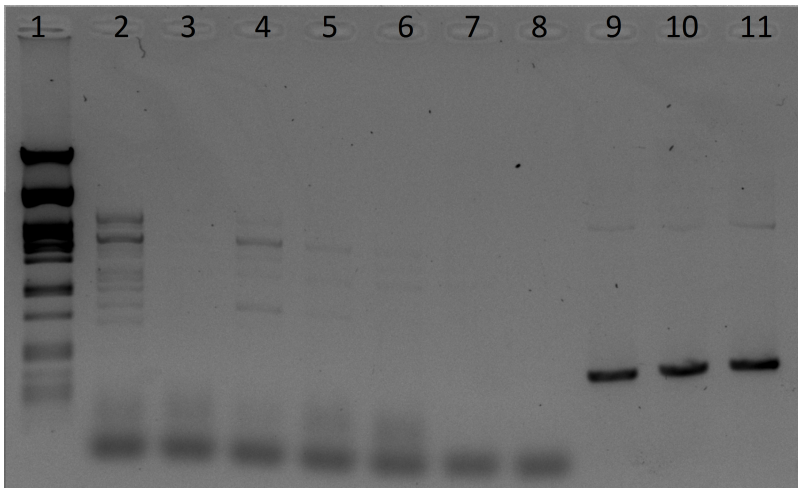


Figure 3.9: The result from testing if *Aurantiochytrium* sp. T66 transformants harbored the *Sh ble* gene, using the primer pair Ble-F and Ble-SgrDI-R. Lane 1 contains the lambda PstI standard, shown in appendix A. Lanes 2 and 3 contain PCRs of crude genomic DNA extracted from colonies 1 and 2, respectively, from cells electroporated with pMAT3. Lanes 4 and 5 contain PCRs of crude genomic DNA extracted from colonies 1 and 2, respectively, from the negative control from the same transformation. Lanes 6 and 7 contain PCRs of crude genomic and genomic DNA, respectively, extracted from wild type *Aurantiochytrium* sp. T66, and lane 8 contains a negative control PCR with double-distilled H_2O instead of template. Lanes 9 and 10 contain PCRs of pMAT3 plasmid DNA together with crude genomic DNA extracted from electroporated cells, where lane 9 represents colony 1 electroporated with pMAT3, and lane 10 represents colony 1 from the negative control. Lane 11 contains a PCR of pMAT3 plasmid DNA (positive control).

3.1.4.4 Transformation of pMAT3, pMAT4n and pMAT6 into *Aurantiochytrium* sp. strain T66 by electroporation

Next, a liquid T66 culture with an OD_{600} of approximately 0.85 was electroporated with 2.5, 4.3, or 3.6 µg linear DNA from pMAT3, pMAT4n (instead of pMAT4 to test the different version of pGAP), or pMAT6, respectively. After recovery, approximately $2.1 \cdot 10^6$ cells (150 µL cell suspension) were plated out per low salt YPDS plate with 75 µg/mL zeocin. For each transformation, a 10^{-3} dilution was also plated on a YPDS plate without zeocin, on which the resulting colonies were counted to be approximately 2000-2500 per plate. The number of colonies on the resulting plates containing zeocin is shown in table 3.4, which does not show a clear difference in number between the transformants and the negative control. Still, the colonies that appeared first (the largest ones) were tested by PCR amplification.

Table 3.4: The number of resulting colonies of different sizes after plating *Aurantiochytrium* sp. strain T66 cells electroporated with pMAT3, pMAT4n, and pMAT6.[1]

	Large	**Medium**	**Smaller**
Negative control [2,4]	2	20	133
pMAT3 [2,5]	8	24	307
pMAT4n [2,5]	3	19	322
pMAT6 [3,5]	1	17	271

[1] The colonies were counted 11 days after plating cells on low salt YPDS plates with 75 µg/mL zeocin, to see if the *Sh ble* gene rendered the cells zeocin resistant. The total number of colonies on each plate was approximately 200 (not shown), including colonies of smaller sizes than the ones showed here.
[2] Total number of colonies from 15 plates.
[3] Total number of colonies from 13 plates.
[4] Cells transformed with elution buffer.
[5] Linearized DNA.

Crude genomic DNA extracted from the large colonies on the resulting plates from this transformation (table 3.4) was amplified by PCR with the primer pair Ble-F and Ble-SgrDI-R to see if the transformed DNA had been incorporated into the genomic DNA of the transformed cells. DNA gel electrophoresis was performed on the PCR products, and the result is shown in figure 3.10, in which the PCR product was only sufficiently visible as a band in the lane containing the positive control. This, again, suggests that the *Sh ble* was not incorporated into the genome of the transformed cells, suggesting that the colonies were false positives, which indicates that the selection pressure was not high enough. Therefore, another test of plating different wild type cell densities on low salt YPDS plates of different zeocin concentrations was done, described next.

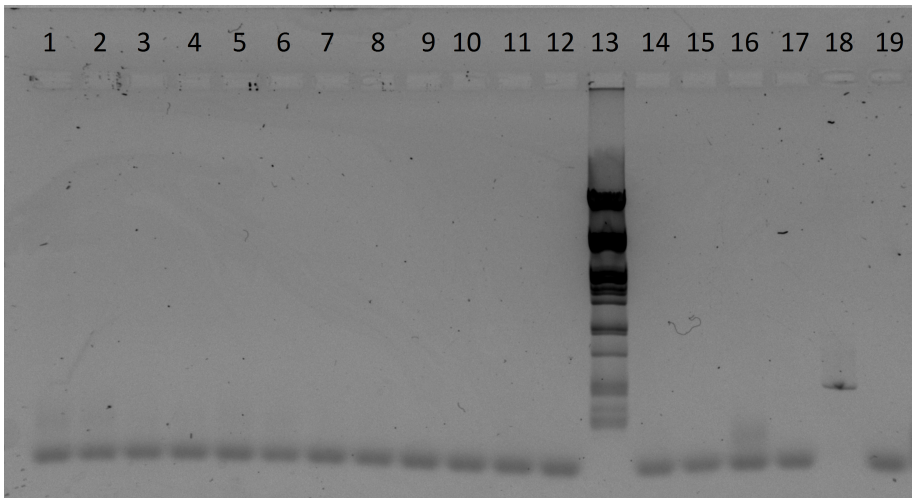


Figure 3.10: The result from testing if *Aurantiochytrium* sp. T66 transformants harbored the *Sh ble* gene, using the primer pair Ble-F and Ble-SgrDI-R. Lanes 1-8 contain PCRs of crude genomic DNA extracted from 8 respective clones electroporated with pMAT3. Lanes 9-11 contain PCRs of crude genomic DNA from 3 respective clones electroporated with pMAT4n, lane 12 contains a PCR of crude genomic DNA from a clone electroporated with pMAT6, and lanes 14 and 15 contain PCRs of crude genomic DNA from two respective negative control colonies from the same transformation. Lanes 16 and 17 contain PCRs of crude genomic and genomic DNA, respectively, extracted from wild type *Aurantiochytrium* sp. strain T66. Lane 18 contains a PCR of pMAT3 plasmid DNA, lane 19 contains a negative control PCR with double-distilled H_2O instead of template, and lane 13 contains the lambda PstI standard, shown in appendix A.

3.1.4.5 A second test of zeocin concentration and cell density for plating

As the zeocin concentration of 75 µg/mL seemed to be too low, another test of zeocin concentration and cell density for plating electroporated thraustochytrids was done with both *Aurantiochytrium* sp. strains T66 and S61. The following cell densities of wild type cells were plated on lower salt YPDS plates containing 100, 150 or 200 µg zeocin per mL: $3 \cdot 10^6$, $6 \cdot 10^6$ and $1.2 \cdot 10^7$ T66 cells per plate, and $4 \cdot 10^5$, $8 \cdot 10^5$ and $1.6 \cdot 10^6$ S61 cells per plate. For positive control, 10^{-3} dilutions of each cell density were plated on YPDS plates without zeocin. The resulting number of colonies on these plates coincided with the cell densities plated. The growth on the zeocin plates, 8 days after plating, indicated that the 100 µg/mL zeocin plates with $3 \cdot 10^6$ T66 cells and $8 \cdot 10^5$ S61 cells per plate had a low enough degree of growth to select for transformants. Thus, this zeocin concentration and cell density were used when doing the next transformations. However, the plates were also observed 21 days after plating, shown in figures 3.11 and 3.12. This was done to see the degree of selection pressure after a longer time, in case it took some time for transformants to appear when plating these. These results showed that at a zeocin concentration of 100 µg/mL, false T66 positives would appear after this long time, while the selection pressure on S61 would still be adequate.

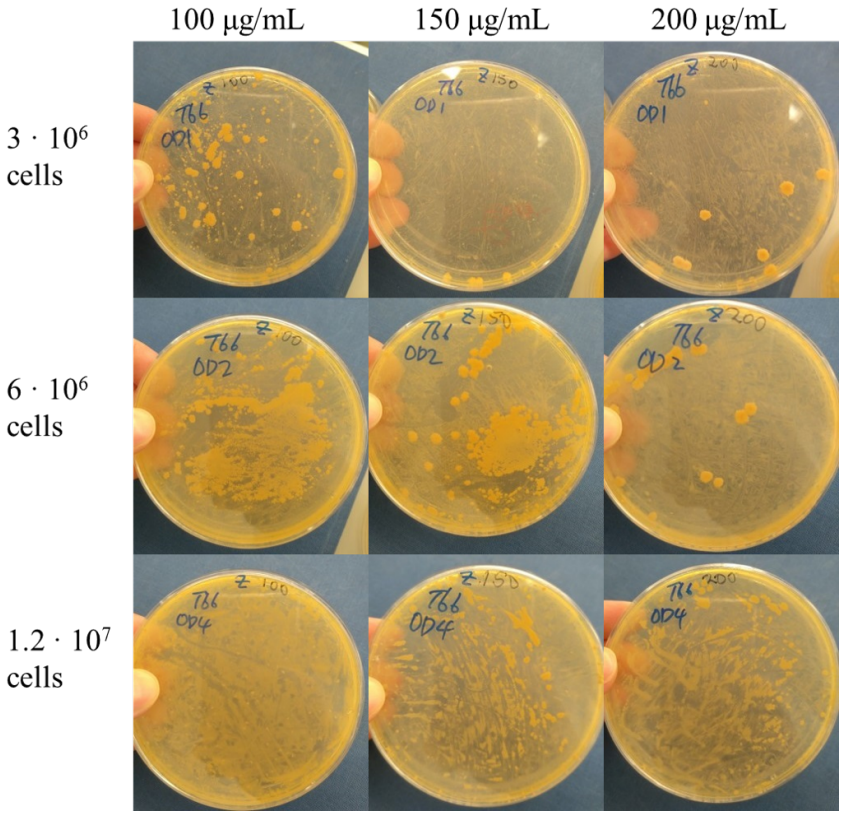


Figure 3.11: The resulting degree of growth 21 days after plating approximately $3 \cdot 10^6$, $6 \cdot 10^6$, or $1.2 \cdot 10^7$ cells of wild type *Aurantiochytrium* sp. T66 per lower salt YPDS plate with 100, 150 or 200 µg/mL zeocin.

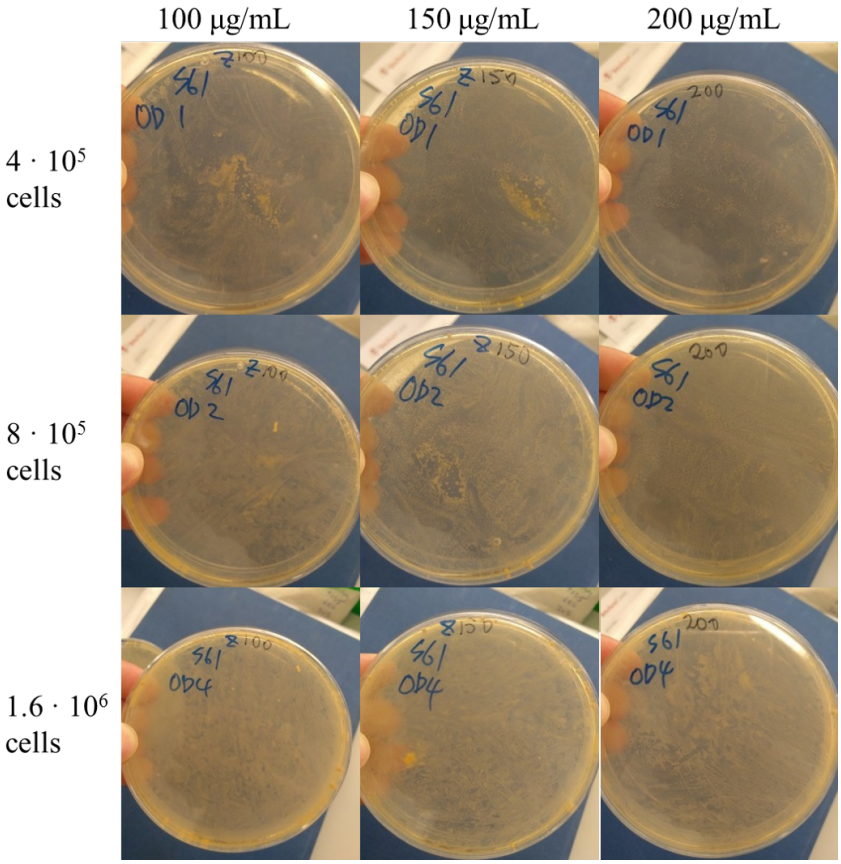


Figure 3.12: The resulting degree of growth 21 days after plating approximately $4 \cdot 10^5$, $8 \cdot 10^5$, or $1.6 \cdot 10^6$ cells of wild type *Aurantiochytrium* sp. S61 per lower salt YPDS plate with 100, 150 or 200 µg/mL zeocin.

3.1.4.6 Transformation of pMAT3 and pMAT6 into *Aurantiochytrium* sp. strains T66 and S61 by electroporation

Next, both *Aurantiochytrium* sp. strain T66 and S61 was transformed, to see if transformation of S61 could be more successful. Only two constructs were used for the transformations to keep the number of plates manageable. A liquid T66 culture with an OD_{600} of approximately 0.5, and a liquid S61 culture with an OD_{600} of approximately 1, were electroporated with linear DNA from pMAT3 (10.9 µg) or pMAT6 (10.45 µg). After incubation overnight, approximately $3 \cdot 10^6$ cells of strain T66 and $4 \cdot 10^5$ cells of strain S61 were plated on each low salt YPDS plate with 100 µg/mL zeocin. 10^{-3} dilutions were also plated on YPDS plates without zeocin, which resulted in approximately 3000 colonies per plate. The number of colonies on the resulting zeocin plates is shown in table 3.5. These results did not indicate successful transformations since the number of colonies from cells electroporated with DNA was not higher than the number of colonies on the negative control plates. Because of this, no PCR amplification of DNA from the transformed cells was done.

3.1.4.7 Transformation of pMAT3 into *Aurantiochytrium* sp. strain T66 by a combination of electroporation and bead treatment

To test if weakening the cell wall by bead treatment affected the transformation efficiency, a liquid culture of *Aurantiochytrium* sp. strain T66 with an OD_{600} of approximately 0.5 was transformed with pMAT3 (10.9 µg) by a combination of electroporation and glass bead treatment. To test this different method, it felt unnecessary to use more than one construct. After electroporation and recovery overnight, approximately $3 \cdot 10^6$ cells were plated on each low salt YPDS plate containing 100 µg zeocin per mL. A 10^{-3} dilution was also plated on a YPDS plate without zeocin, which resulted in approximately 3000 colonies. The number of colonies on the resulting zeocin plates is shown in table 3.5, which also indicates an unsuccessful transformation, because there were not significantly more colonies from cells electroporated with DNA, than from the negative control. It seemed that the transformation protocol for these thraustochytrid strains needed to be further optimized, which is why a test of the survival after different durations of glass bead treatment of the cells was done.

The plates which the results in table 3.5 represent were observed over a longer period (22 days) than previous transformations, to see if transformants might appear later. This transformation was done before the finding from the previous test of zeocin concentration, that 21 days after plating, the selection pressure of 100 µg/mL zeocin was too low for strain T66. The result in table 3.5 confirms this.

Table 3.5: The average number of new colonies on each plate at different time points after plating *Aurantiochytrium* sp. strain T66 and S61 transformed with pMAT3 and pMAT6.[1]

		Day 7	Day 14	Day 22	Total
T66	**Negative control** [2,5]	2	12	44	58
	pMAT3 [2,6]	2.7	13.7	34.7	51
	pMAT6 [2,6]	2.3	16	35	53.3
	Negative control beads [3,5]	4.5	23.5	35	63
	pMAT3 beads [3,6]	0.5	10	17.5	28
S61	**Negative control** [2,5]	4.7	0.3	1	6
	pMAT3 [4,6]	0	0	0	0
	pMAT6 [4,6]	0	0	0.17	0.17

[1] The cells were plated on low salt YPDS plates containing 100 µg zeocin per mL, after electroporation with or without bead treatment, to see if the *Sh ble* gene rendered the cells zeocin resistant.
[2] Average number on each of 3 plates.
[3] Average number on each of 2 plates.
[4] Average number on each of 6 plates.
[5] Cells transformed with double-distilled H_2O.
[6] Linearized DNA.

3.1.4.8 Test of bead treatment of thraustochytrids

A test of different durations of bead treatment of *Aurantiochytrium* sp. strain S61 was done, to examine the effect this treatment had on cell survival. As the treatment was used in combination with electroporation to weaken the cell wall, it was expected to have some effect on cell survival. If it does not affect the survival at all, this suggests that it does not have a large effect on the cell wall integrity either. A liquid *Aurantiochytrium* sp. S61 culture with an OD_{600} of 0.66 was split into 1.5 mL aliquots, which were spun down at 4000 g for 5 minutes. The cells were resuspended in YPDS medium to 400 µL. 300 mg acid washed and autoclaved 0.5 mm diameter glass beads were added to each aliquot, and the different aliquots were vortexed at full speed for 10, 15, 20, 25, 30 or 40 seconds. The cells, as well as a 10^{-3} dilution for each treatment period, were then plated on low salt YPDS plates. After 4 days, approximately 400 colonies were counted on all of the plates plated with 10^{-3} dilutions, which indicates that the different durations of bead treatment did not have a significant effect on cell survival. Strain T66 has previously proven to be as resilient as strain S61, and therefore it is plausible that it would tolerate this treatment just as well. The transformation protocol still seemingly needs further optimization before a successful transformation can be done, and this task was continued by E-Ming Rau. Therefore, another possible mechanism for selecting mutant *Aurantiochytrium* sp. strain T66 and S61 was explored, described next.

3.2 Screening for auxotrophic mutants

A mutagenized strain of *Aurantiochytrium* sp. strain T66 with 11.1% survival after mutagenesis was screened by replica plating for amino acid, uracil or adenine auxotrophic mutants, which could potentially be used for selection of mutant cells. The decision to screen a strain with 11.1% survival rate was based on the method by Hashimoto et al., in which strains with 8.1-22% survival rates were screened (Hashimoto et al., 2005).

3.2.1 Survival of mutants after freezing

Firstly, the survival of the mutagenized strains after freezing was evaluated, to be able to calculate the approximate amount of cell culture to plate of the strain to be screened to get an appropriate density of colonies per plate. This was done by plating 100 µL each of dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of mutagenized T66 cells with 72.9% survival after mutagenesis on YPDS plates. Two dilution series were plated. A different mutagenized strain was used (less mutagenized), to not waste the one to be screened, as the impact of freezing on survival was assumed to be approximately the same for both strains. The number of colonies on the resulting plates three days after plating is shown in table 3.6. From this, it was estimated a survival rate of approximately $6.7 \cdot 10^5$ CFUs per mL frozen culture. A survival rate of $3.4 \cdot 10^5$ CFU/mL after freezing was estimated for this strain by

SINTEF, by comparison with the survival of wild type cells after freezing. Thus, plating this strain after freezing gave 97% more CFUs than expected.

Table 3.6: The number of colonies on YPDS plates three days after plating two independent dilution series of a mutagenized culture of *Aurantiochytrium* sp. T66 with 72.9% survival after mutagenesis.

Dilution	Dilution series	
	1	**2**
$\mathbf{10^{-2}}$	- [1]	239
$\mathbf{10^{-3}}$	57	77
$\mathbf{10^{-4}}$	8	4
$\mathbf{10^{-5}}$	2	2

[1] Not countable.

3.2.2 Test of wild type growth on minimal medium

Then, to see if wild type T66 cells were able to grow on MM, to be able to screen for mutants by replica plating on MM plates, cells from a YPDS plate were inoculated in 50 mL MM in a 250 mL baffled shake flask. The culture was incubated at 25 °C and 170 rpm, and growth was observed after approximately 24 hours.

3.2.3 Test of automatic colony picking

Automatic colony picking was tested on wild type T66 cells, to see if this method could be used to pick mutant colonies for replica plating. One mL each of dilutions of $2 \cdot 10^{-3}$ and 10^{-3} of a liquid T66 culture with an OD_{600} of approximately 4 were plated on $25 \cdot 25$ cm YPDS plates to give a suitable density of colonies (approximately 500 colonies per plate). Three days after plating, $2 \cdot 96$ colonies were picked and inoculated in YPDS medium, then MM, then YPDS medium again in flat-bottomed 96-well plates with 120 µL medium in each well. The series of inoculation in different media was done to see the frequency of failed inoculations in one or more of the media. MM was included to verify that the cells were able to grow in MM in 96-well plates. YPDS medium was inoculated after this, to see if any general failed growth in MM was due to failed inoculation, or because the cells could not grow in MM (if they also failed to grow in the YPDS inoculated after MM, the lack of growth in MM would be more likely due to failed inoculation). For the two sets of 96 colonies picked, two different sterilization procedures of the picking heads were followed before picking and after the inoculation, to then dip them in YPDS medium in 96-well plates to evaluate the effectiveness of the sterilization. For the first set, the sterilization included cooling of the picking heads, which it did not for the second set. The plates were incubated at 25 °C and 600 rpm for three days before the growth was evaluated. The plates which were inoculated after sterilizing the picking heads showed no

growth in any of the wells, demonstrating that the sterilization was effective. For the plates inoculated with colonies, the growth in the different media is shown in table 3.7. The frequency of successful inoculations was generally higher in the plates inoculated after cooling of the picking heads, and in these plates, only approximately 35% of the colonies picked were successfully inoculated during both the two first inoculations, which would be the number of inoculations for screening mutants (MM and YPDS medium). This percentage is too low because most of the colonies screened would not be inoculated in either one or both of the media. Therefore, manual picking with toothpicks was used for screening instead.

Table 3.7: The number of colonies inoculated successfully in the different media after automatic picking. [1]

	Medium (inoculation) number							
	1	**2**	**3**	**1+2**	**1+3**	**2+3**	**All**	**None**
Cooling [2]	21	20	2	29	5	2	5	12
No cooling	13	36	2	14	1	2	0	28

[1] Media number 1 and 3 were YPDS and medium number 2 was MM.
[2] The picking heads were cooled down after evaporation of ethanol used for sterilization.

3.2.4 Screening by replica plating

Since the automatic colony picking did not work, the mutagenized strain of *Aurantiochytrium* sp. strain T66 with 11.1% survival after mutagenesis was screened for mutants by manual replica plating. Cells were plated on 14 cm YPDS plates to give maximally 300 colonies per plate, based on the approximated survival after freezing (section 3.2.1). After 3-7 days, 10 000 colonies were picked and replica plated on MM and YPDS plates. Of these, 78 failed to grow on MM, and were re-tested on MM plates. Only five of these failed to grow on MM again, and they also showed slow growth on YPDS medium. These five clones were re-streaked on a MM plate, over a larger area to better see any potential growth, as well as on MM plates supplemented with 18 mg/L adenine, 76 mg/L uracil or one amino acid, to test for auxotrophy. All 20 proteinogenic amino acids were tested and were present at 76 mg/L, except for leucine which was present at 380 mg/L in the medium. The concentrations of adenine, uracil and amino acids were based on their concentrations in yeast synthetic drop-out medium supplements from Sigma-Aldrich. After seven days, three of the clones showed relatively evident growth on the MM plate, and they did not grow significantly better on any amino acids. The other two clones showed very faint growth on MM, one of which grew slightly better on uracil and aspartate, and the other one grew slightly better on glutamate and glutamine. This opened a possibility that the latter clone could be a glutamate/glutamine auxotroph, which would result in problems in assimilating nitrogen into biomolecules, as this takes place through these amino acids (section 1.2.6). Potential enzymes which might be dysfunctional or underexpressed in this mutant are glutamine synthetase and glutamate synthase, which constitute the major pathway for assimilation of ammonium into

glutamate. Glutamine synthase, present in all organisms, catalyzes the production of glutamine from glutamate and ammonium, which is the first step of the assimilation into glutamate. In bacteria and plants, the second step is catalyzed by glutamate synthase, in which glutamate is produced from amination of α-ketoglutarate with glutamine as an amino group donor (Nelson & Cox, 2013, p. 888-916). Most likely, T66 harbors glutamate synthase, as it has genes which have been annotated as encoding glutamate synthase, as does the thraustochytrid *Hondaea fermentalgiana* (GenBank ID: GBG31165.1).

As glutamate and glutamine are important entry points for nitrogen into biomolecules, it might not be enough with a small supplement in the medium to support the growth of a glutamate/glutamine auxotroph properly. Therefore, the mutant suspected to be an auxotroph of this kind was inoculated in MM supplemented with 5 g/L sodium glutamate, to test if it grew better with more glutamate. Approximately the same amount of cells of the same clone was also inoculated in YPDS medium and MM, to compare the growth. The OD_{600} measured after three and four days is shown in table 3.8, which demonstrated that the mutant did not grow better with an abundance of glutamate. Thus, it was not established that it was a glutamate auxotroph.

Table 3.8: The OD_{600} (growth) after three and four days of a potential glutamate auxotroph cultivated in YPDS, MM, and MM supplemented with 5 g/L sodium glutamate (Glu).

	YPDS	**MM**	**Glu**
Day 3	0.212	0.006	0.010
Day 4	0.460	0.007	0.016

Note: The OD_{600} of wild type T66 after two days of cultivation in YPDS and MM media has been measured to be 2 and 7.8, respectively (not shown).

3.3 Fatty acid synthase inhibitors

One key issue in enhancing the *Aurantiochytrium* sp. strain T66 DHA production, is to channel more malonyl-CoA to DHA synthesis. Therefore, it would be interesting to understand further how the FAS and the PKS systems function. For this purpose, the effect of FAS inhibitors on T66 fatty acid production, and specifically DHA production, was investigated by analyzing fatty acid profile by mass spectrometry.

3.3.1 Growth experiment

Firstly, the effect on the growth of T66 by different concentrations of the FAS inhibitors apigenin, taxifolin, orlistat, cerulenin, isoniazid, and irgasan, was examined. This was done to see if, and at which concentration, the inhibitors were likely to inhibit the FAS of the thraustochytrids, and thus have an effect on their fatty acid content. If they had a significant effect on the fatty acid synthesis, it would also affect the synthesis of phospholipids and thus the membrane synthesis

(section 1.3), and thereby affect the growth. One mL of a liquid T66 culture with an OD_{600} of 1.67 was inoculated in each batch of 50 mL fat accumulation medium containing 200 mg/L streptomycin/ampicillin and apigenin, taxifolin or cerulenin at different concentrations. Two mL of a T66 culture with an OD_{600} of 1.12 was inoculated in each batch with orlistat, isoniazid or irgasan at different concentrations. Control cultures without any inhibitors were also started. The cultures were incubated in 250 mL baffled flasks at 25 °C and 170 rpm for seven days, while OD_{600} was measured every day. The resulting growth curves are shown in figures 3.13 and 3.14. These showed that all of the inhibitors, except for taxifolin, seemingly had a concentration-dependent inhibitory effect on T66 growth, and based on this, the concentrations given in table 3.9 of the different inhibitors were used when investigating their effect on T66 fatty acid synthesis. As taxifolin seemed to enhance the growth the higher the concentration, this inhibitor was not examined further.

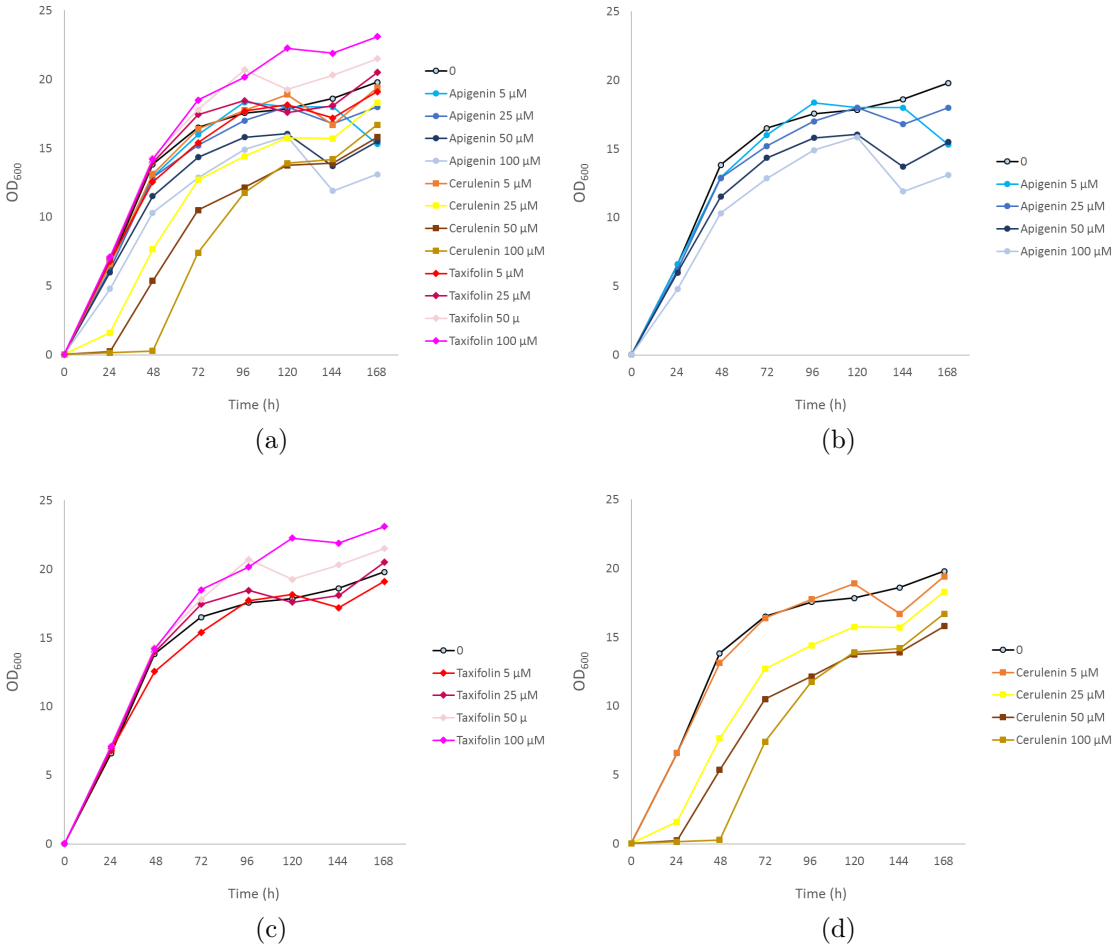


Figure 3.13: The growth (OD_{600}) of *Aurantiochytrium* sp. strain T66 treated with different concentrations of the FAS inhibitors apigenin, taxifolin and cerulenin, measured every day over a period of seven days. The inhibitor concentrations are indicated to the right of the curves, and the culture containing no inhibitors is marked 0. Displayed are the growth curves representing **(a)** all three inhibitors, **(b)** apigenin, **(c)** taxifolin, and **(d)** cerulenin.

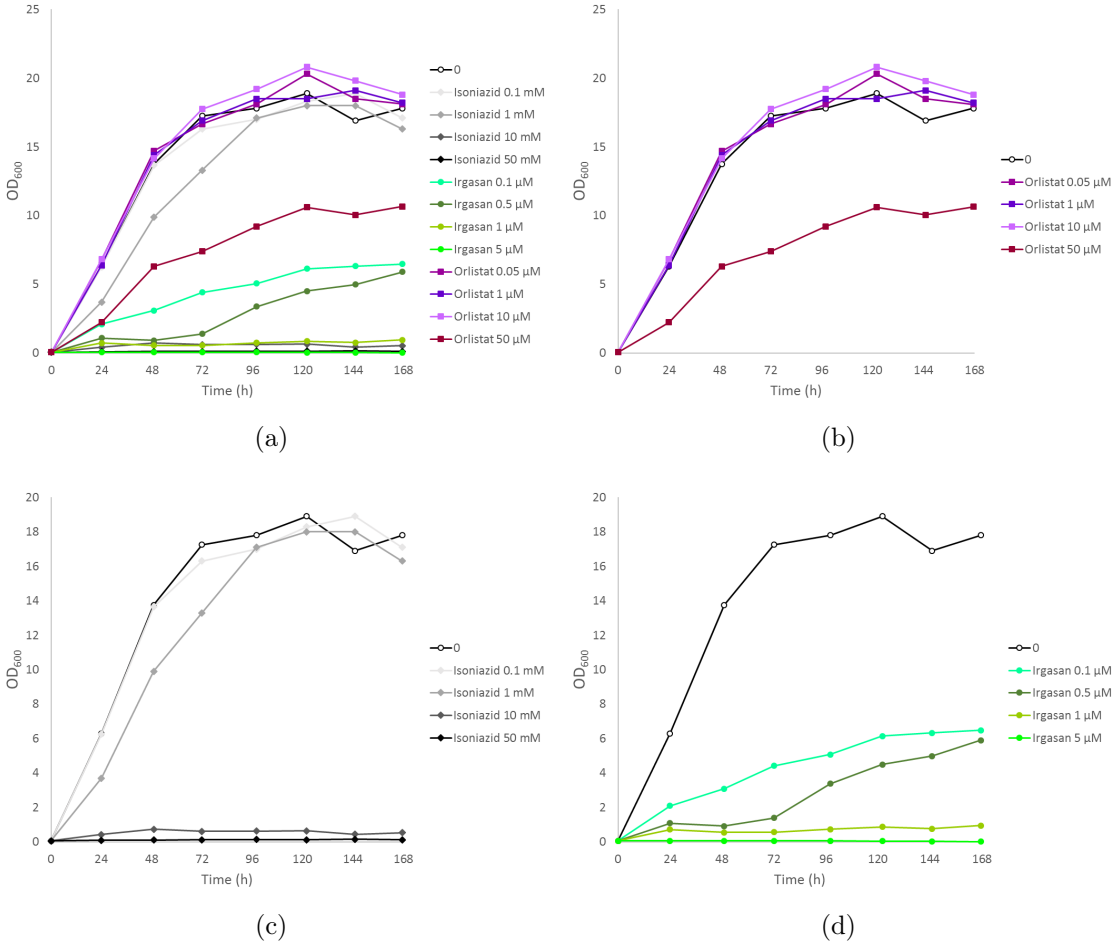


Figure 3.14: The growth (OD_{600}) of *Aurantiochytrium* sp. strain T66 treated with different concentrations of the FAS inhibitors orlistat, isoniazid and irgasan, measured every day over a period of seven days. The inhibitor concentrations are indicated to the right of the curves, and the culture containing no inhibitors is marked 0. Displayed are the growth curves representing **(a)** all three inhibitors, **(b)** orlistat, **(c)** isoniazid, and **(d)** irgasan.

3.3.2 Analysis of lipid content and fatty acid composition

The effects of the FAS inhibitors apigenin, orlistat, cerulenin, isoniazid, and irgasan on T66 lipid content and fatty acid composition were then examined. For each batch, 1.5 mL of a liquid T66 culture with an OD_{600} of 1.46 was inoculated in 50 mL fat accumulation medium containing zero or one of the inhibitors, at the concentrations given in table 3.9. Three parallels for each inhibitor, as well as for the cultures without any inhibitor, were inoculated. For this experiment, no antibiotics were added to the cultures, as it could affect the analysis. However, 200 mg/L streptomycin/ampicillin was used in the preculture. The cultures were incubated in 250 mL baffled flasks at 25 °C and 170 rpm for seven days, while OD_{600} was measured every day. 10 mL from each culture were harvested after two days, and 5 mL were harvested after five and seven days, and lipids were extracted

from these samples. The growth curves and lipid fractions of cell dry weight (CDW) of these cultures are shown in figure 3.15, and the fat-free cell mass and lipid content of the cell cultures are plotted in figure 3.16. These results indicate that the FAS inhibitors had little or no effect on the lipid production of the cells, except for orlistat, which seemed to somewhat inhibit the lipid accumulation. The growth curves indicate that the FAS inhibitors all had an inhibitory effect on the growth of the cells at the concentrations used, but the plots of fat-free cell mass do not indicate this, except for orlistat.

Table 3.9: The concentrations of FAS inhibitors used in the growth medium when testing their effect on lipid and fatty acid production in *Aurantiochytrium* sp. strain T66.

Inhibitor	**Concentration (μM)**
Apigenin	150
Orlistat	50
Cerulenin	100
Isoniazid	2000
Irgasan	0.075

The lipids were hydrolyzed to free fatty acids, which were then analyzed by mass spectrometry after separation by supercritical fluid chromatography by Zdenka Bartosova and Marit Hallvardsdotter Stafsnes, who were in the process of developing the method. The raw data of the fatty acid analysis is shown in appendix C, including concentrations of fatty acids in the dichloromethane phase after hydrolysis and extraction, which are not absolutely quantifiable because only one internal standard was used for all the fatty acids. However, the relative quantification among the fatty acids, and thus the fatty acid percentages of total fatty acids, are reliable.

When doing the hydrolysis, a blank with no lipids added was also included and analyzed by mass spectrometry, to detect any contamination. This demonstrated a presence of palmitate (C16:0) and stearate (C18:0) (most likely from the plastic equipment used), and small amounts of DHA and C16:1 in the samples. The contamination constituted a significant part of the total fatty acids in the samples when the lipids were hydrolyzed using KOH dissolved in water (results not shown), and the hydrolysis was therefore repeated by Zdenka Bartosova and Marit Hallvardsdotter Stafsnes, using ethanolic KOH instead. This gave a higher yield of fatty acids, and thus the contamination did not constitute a significant part of the total fatty acids in these samples.

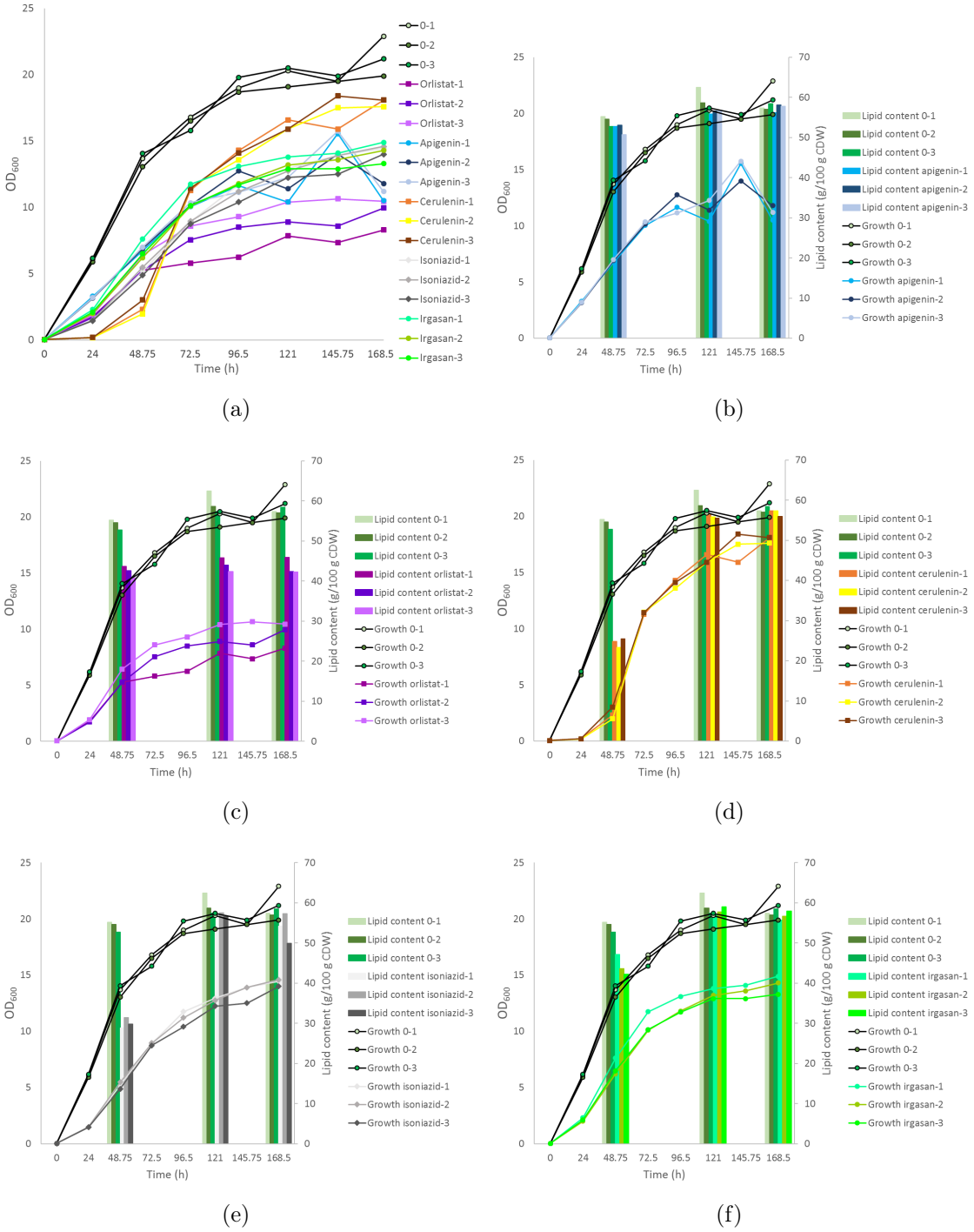


Figure 3.15: The growth and lipid content of *Aurantiochytrium* sp. strain T66 treated with different FAS inhibitors over a period of seven days. The growth was measured every day, and the lipid content was measured after two, five, and seven days. For each inhibitor, as well as for the zero-cultures (0) containing no inhibitors, three parallels were done, indicated to the right of the curves. **(a)** The growth curves representing all of the inhibitors. Displayed are the growth curves and lipid content representing **(b)** apigenin, **(c)** orlistat, **(d)** cerulenin, **(e)** isoniazid, and **(f)** irgasan.

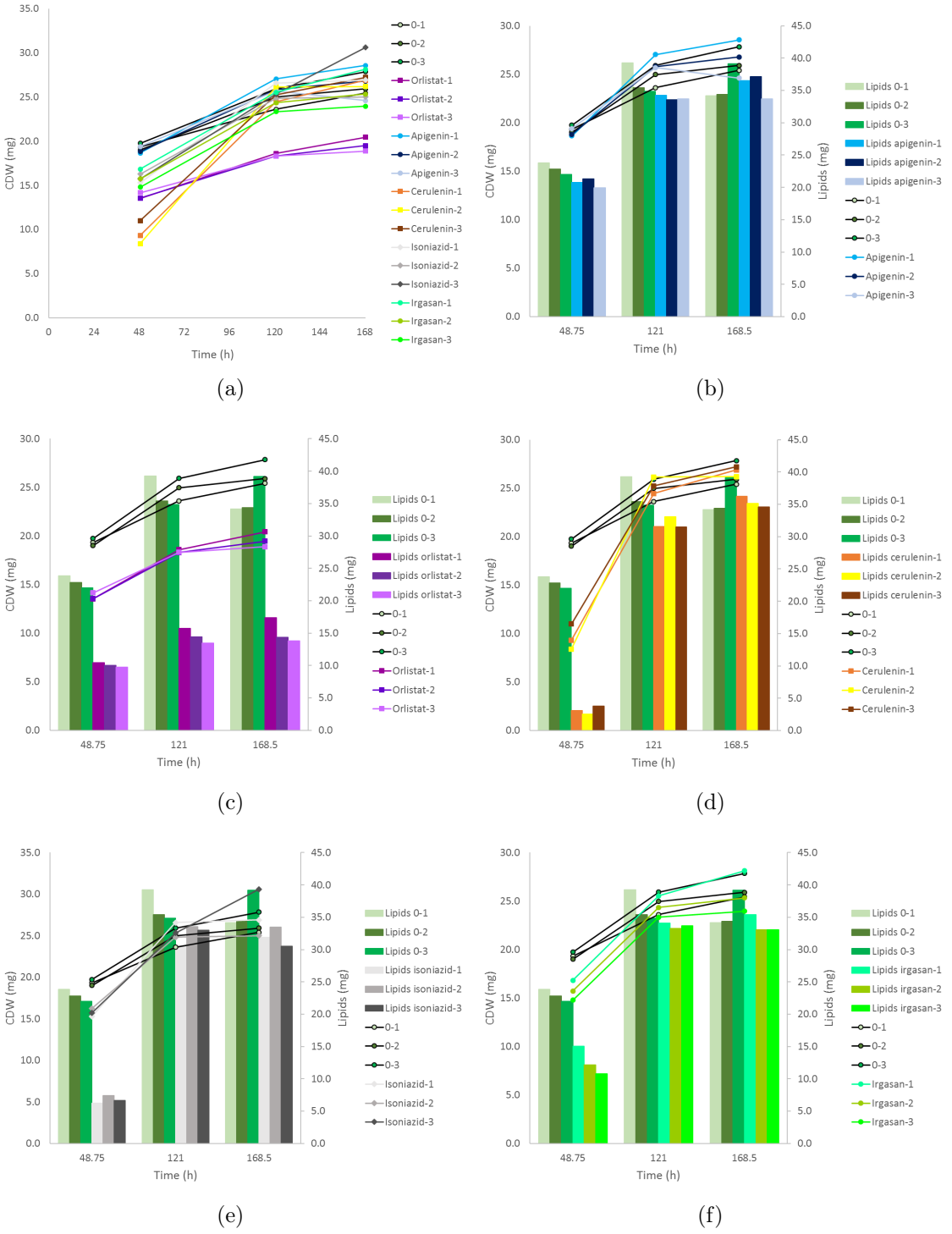
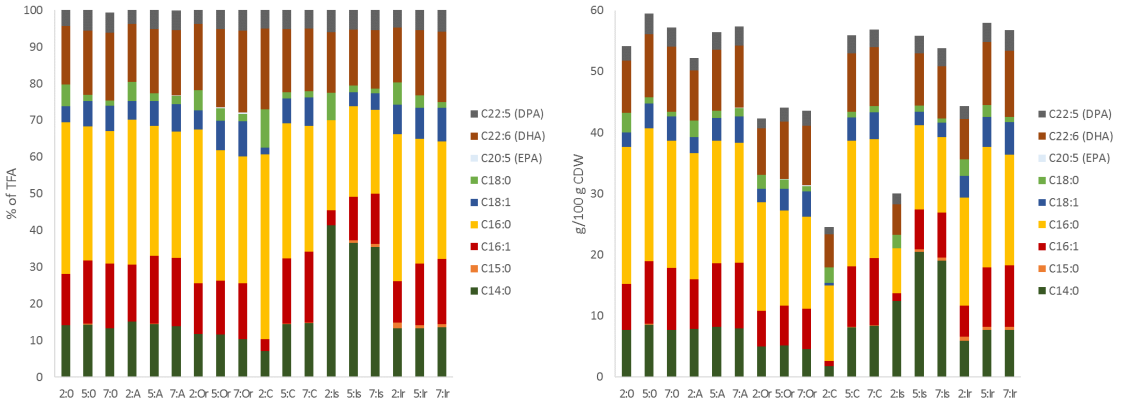


Figure 3.16: The fat-free cell dry weight (CDW) and lipid content in 5 mL *Aurantiochytrium* sp. strain T66 culture containing different FAS inhibitors and cultivated over a period of seven days. The samples were collected after two, five and seven days. For each inhibitor, as well as for the zero-cultures (0) containing no inhibitors, three parallels were done, indicated to the right of the diagrams. **(a)** The fat-free CDW representing all of the inhibitors. Displayed are the fat-free CDW and lipid content representing **(b)** apigenin, **(c)** orlistat, **(d)** cerulenin, **(e)** isoniazid, and **(f)** irgasan.

The fatty acid profiles of the cells are shown in figure 3.17, and the standard deviations (shown in appendix D) were generally around 10% or less of the respective average values for the fatty acids which constituted a significant part of the TFA. The fatty acid profiles indicate that the FAS inhibitors did not seem to enhance DHA production, although some of them had some effect on the fatty acid composition. The weights in figure 3.17 b were calculated by taking the percentage of total fatty acids (figure 3.17 a) of the amount of lipids produced per CDW (figure 3.15). However, the fraction of glycerol and other components of total lipids, and the variation of the weight of fatty acids, were not included in this calculation. Thus, these values are not accurately reflecting the actual weight of fatty acids produced by the cells, but they are sufficient to compare the amount of the different fatty acids produced by the different cultures.



(a) (b)

Figure 3.17: The fatty acid profiles of *Aurantiochytrium* sp. strain T66 treated with different FAS inhibitors over a period of seven days, sampled after two (2:), five (5:) and seven (7:) days. The values are the average of three parallels and the standard deviations are shown in appendix D. 0: no inhibitors, A: apigenin, Or: orlistat, C: cerulenin, Is: isoniazid, Ir: irgasan. **(a)** The percentage of the different fatty acids of total fatty acids (TFA). **(b)** The weight (g) of the different fatty acids produced per 100 g cell dry weight (CDW).

4 Discussion

The aim of this work was to explore methods to select for mutants and to enhance DHA production in *Aurantiochytrium* sp. strain T66.

4.1 Exploration of selectable markers to be used in *Aurantiochytrium* sp. strain T66 and S61

Exploration of the compounds 5-FOA, 5-FC, cycloheximide, and zeocin to be used as selective compounds together with their respective resistance genes, did not result in the establishment of a successful selection method in strains T66 or S61. The cells were resistant to 5-FOA and 5-FC, and during transformation of cycloheximide and zeocin resistance genes, false positives were a recurring problem. This resistance indicates that the strains are resilient and resistant to several antibiotics, including nystatin (Jakobsen et al., 2007). It is unclear what causes the resistance, but it might indicate that they harbor an efflux pump that pumps toxic compounds out of the cell after uptake (Blanco et al., 2016). Another explanation could be that they do not fully take up the antibiotics, or in the case of 5-FOA, the native substrate of OMP decarboxylase might compete with 5-FOA for binding of the enzyme active site (Nelson & Cox, 2013, p. 200-213), and thus 5-FOA does not become fully transformed to the toxic compound fluorodeoxyuridine (section 1.2.2). In theory, T66 and S61 should be sensitive to 5-FOA, as it is transformed to a toxic compound by an enzyme involved in *de novo* pyrimidine synthesis, which all living organisms have (Nelson & Cox, 2013, p. 888-916). The resistance to 5-FC is probably explained by the BLAST search which indicated that T66 most likely does not have a gene encoding cytosine deaminase, which confers 5-FC sensitivity (section 1.2.3). The result from the BLAST search was somewhat unexpected, as most yeast show cytosine deaminase activity. However, T66 did show slower growth in the presence of 5-FC, the reason for which is unclear. 5-FC itself is not toxic to the cells, although it is possible that it decreases their growth in some way. Although it is not suitable to be used in basic selection of T66 mutants, there is still a possibility that 5-FC could be used as a counterselective compound (section 1.2.3).

No successful zeocin resistant transformants were obtained from transformations of *Aurantiochytrium* sp. strains T66 and S61. It is uncertain why, and there are several parameters used in the transformation method that could be unfitting for T66 and S61. The electroporation parameters which have reported to be successful in transformation of thraustochytrids have varied by strain or genus (Cheng et al., 2011; Hong et al., 2013; Ren et al., 2015), and it is thus uncertain if the parameters used here, which were based on methods used for other *Aurantiochytrium* strains (Adachi et al., 2017; Hong et al., 2013), are working for strains T66 and S61. It is possible that the electroporation had little effect on the cells, or that they were too damaged. It is also uncertain if the buffers used were suitable. However, it is possible that some DNA was transformed into the cells,

and that a problem was lack of incorporation into the genome. The frequency of recombination might not be high enough in these strains, or there might not be a very strong preference for homologous recombination between the genome and linear DNA fragments (Smith et al., 1993). Furthermore, if only a small amount of DNA is taken up by the cells, the chance of incorporation into the genome is small compared to when a large amount of DNA is taken up.

However, there is a possibility that actual successful transformants were overlooked because they were not tested, because the large number of false positives made it impracticable to test all the positives. The number of false positives might have been a consequence of an adaption of the cells or weak selection pressure. However, for another *Aurantiochytrium* sp., zeocin has been successfully used as a selective compound at 50 µg/mL (Suen et al., 2014). The selection pressure was a challenge to adjust, as it seemed adequate when testing the zeocin concentration on wild type cells, but when plating transformants, still several false positives appeared. The reason for this is unknown, although the electroporated cells were treated differently than the wild type cells before plating, and were maybe stressed from the electroporation. Perhaps the treatment of the electroporated cells somehow prepared some cells for excretion of zeocin by a prospective efflux pump. Alternatively, it could be possible that the treatment could alter the mutation frequency of the cells, and thereby generate spontaneous mutants. However, this is not a clear explanation for the growth observed. In addition, a spontaneous mutation rendering the cells zeocin resistant is not very likely, because its mechanism of action is to cleave the DNA (section 1.2.5), not to target a protein that could be easily changed by altering a few amino acids.

Since the transformations were unsuccessful, it was impossible to evaluate the different promoters and terminators of the different zeocin resistance constructs, and their effect on the expression of the *Sh ble*. It is uncertain if the promoters used led to an expression of the gene, and it is possible that successful transformants were obtained, but the resistance gene was not expressed. Such transformants would not appear on the zeocin plates unless they were false positives. However, the *Sh ble* gene, and the promoters (GAP and TEF) and terminators tested, have been used successfully in thraustochytrids. Thus, it is reasonable to suspect that they would work in T66 and S61 as well. Nevertheless, it is possible that the past success of these elements was genus or strain-dependent, such as the transformation methods which have been successful in thraustochytrids. The failure of the transformations was also the reason why pMAT8 was not used for transformation, as it was made as a back up in case there were problems with expression of the resistance gene. However, because of the problem with false positives, it did not become relevant to test pMAT8.

4.2 Screening for auxotrophic mutants

Screening for auxotrophic *Aurantiochytrium* sp. T66 mutants by replica plating on MM and YPDS plates revealed no simple auxotrophic mutants among the 10 000 colonies screened (section 3.2). Based on the results from Hashimoto et al., if T66 had the same mutation frequency from nitrosoguanidine as the sake yeast strains they used from UV-mutagenesis (Hashimoto et al., 2005), 5-20 auxotrophic mutants would have been expected when screening 10 000 colonies. However, T66 is probably different from the sake yeast strains used in that study in many ways, and thus it is not surprising that the same frequency of mutants was not obtained.

The decision to screen the strain with 11.1% survival after mutagenesis was also based on the results from Hashimoto et al., and it is not certain that this survival rate (degree of mutagenesis) gave the highest possibility of obtaining auxotrophic mutants in T66. Perhaps the culture was too extensively mutagenized, and therefore several genes were mutagenized in most of the cells, or perhaps it was not enough mutagenized, and thus the frequency of auxotrophic mutants was too low to detect any. Hashimoto et al. also used a different method to mutagenize their strains than what was used in this work (UV mutagenesis and nitrosoguanidine, respectively) (Hashimoto et al., 2005), which could affect the composition of mutants (and mutations) generated (Wong et al., 2006).

Furthermore, the genome size of the strains to be screened is also a factor. The yeast strains screened by Hashimoto et al. are strains of *S. cerevisiae* (Hashimoto et al., 2005), which has a genome of approximately 12.5 Mbp (Link & Olson, 1991), while *Aurantiochytrium* sp. strain T66 has a genome of 43 Mbp (Liu et al., 2016). The larger genome of T66 leads to a smaller chance of a specific gene of interest becoming mutated during random mutagenesis, and thus a lower frequency of auxotrophic mutants would be expected (Drake et al., 1998). Thus, the problem of generating homozygous mutations in diploids by random mutagenesis was not overcome in thraustochytrids, as it has been in yeast (section 1.2.6).

It is possible that a single mutation can render cells auxotrophic for several amino acids or metabolites, as some of them share parts of their synthetic pathways (section 1.2.6). Any mutants of this kind might have been overlooked, as the clones were not cultivated in the presence of two or several amino acids/uracil/adenine simultaneously.

The five mutant clones which first failed to grow on MM showed a very faint growth when streaked out on a larger area on MM plates, which, in theory, should not be possible if they were auxotrophs (section 1.2.6). In theory, this growth could be due to an activity of moonlighting proteins, which can be involved in processes that are unrelated to their native function (Gancedo & Flores, 2008), and thus somehow be able to support a very low degree of growth of auxotrophic mutants on minimal medium. Regardless, it is not likely that these mutants were auxotrophic, as no clear effect of adding amino acids to their medium was observed. It is unclear what mutations these might have, and it is possible that they have several genes mutated. The slow growth could be a consequence of one or more mutations

leading to severe downregulation or impairment of an essential gene.

However, one of the mutants grew slightly better on glutamate and glutamine, indicating that its mutations had something to do with these metabolites, and raising suspicion that it might be a glutamate/glutamine auxotroph. This auxotrophy would result in problems with assimilating nitrogen into biomolecules (section 1.2.6). The faint growth of this mutant on MM could be due to another reaction that is able to incorporate ammonium into organic compounds, for example, L-glutamate dehydrogenase can synthesize glutamate from α-ketoglutarate and ammonium. This pathway only contributes modestly to the assimilation of ammonium into glutamate (Nelson & Cox, 2013, p. 888-916), and would therefore perhaps not be enough to fully support growth. Because it was suspected to be a glutamate auxotroph, this mutant was tested additionally by inoculation in MM with an abundance of glutamate, as it is plausible that a small supplement of glutamate or glutamine was not sufficient to support its growth. However, as this did not significantly improve the growth (table 3.8), the clone was not established as a glutamate auxotroph.

Another mutant grew somewhat better on uracil and aspartate, which indicates that its mutations had something to do with these. This is somewhat coherent, as aspartate is a precursor for UMP and other pyrimidine nucleotides (Nelson & Cox, 2013, p. 888-916). However, it seems inconceivable that it could be both an aspartate and a uracil auxotroph by only one mutation. If the synthesis of aspartate is defected, it would also result in a scarcity of uracil. However, a supplement of uracil would not relieve the scarcity of aspartate, because the synthesis of UMP from aspartate is not reversible, and thus a synthesis of aspartate from uracil is most likely not possible (Nelson & Cox, 2013, p. 888-916). This does not fit with the fact that uracil also improved the growth of this mutant if it only had one mutation. Thus, this mutant is most likely not useful as a tool in selection of transformants, because the auxotrophy could probably not be reversed by complementation of only one gene (section 1.2.6).

4.3 Analysis of the effect of fatty acid synthase inhibitors on *Aurantiochytrium* sp. strain T66

Figure 3.17 shows that the cells treated with FAS inhibitors did not produce a larger amount of PKS products (DHA and DPA) than untreated cells. The cells treated with orlistat did show a slightly larger fraction of DHA of total fatty acids throughout the incubation time, but as these cells produced a smaller amount of lipids in general, the amount of DHA produced was not higher than the amount produced by untreated cells (figure 3.17 b). The earliest time point sample of cells treated with cerulenin also showed a somewhat larger fraction of DHA. However, cells cultivated in the presence of cerulenin displayed a longer lag phase than the other cultures. Since phospholipids constitute a larger fraction of the total lipids before lipid accumulation starts, and phospholipids have a higher content of DHA than storage lipids (section 1.3.2), the resulting DHA-profile from these cells was

probably growth phase-dependent. In addition, these cells produced a significantly smaller amount of lipids, and thus the amount of DHA was not very large.

It is unclear why the FAS inhibitors did not improve the production of DHA. It could be that the FAS was not sufficiently inhibited to give this effect, or not inhibited at all, although it is not certain if the DHA production would increase if the FAS was inhibited. Besides, as the inhibitors did not seem to affect the production of biomass at the concentrations tested, except for orlistat (figure 3.16), it is possible that FAS inhibition would be more evident at different concentrations. It is also possible that the inhibitors have an inhibitory effect on the PKS enzyme complex, which orlistat and cerulenin have shown to have in some organisms (Flores-Sanchez & Gang, 2013; Hiltunen & Söderhäll, 1992; Ohno et al., 1975). In addition, Hauvermale et al. showed that cerulenin did seem to inhibit the PKS of *Schizochytrium* sp. at concentrations of 25 μM or higher. The concentration threshold for inhibition of FAS seemed to be lower (1-5 μM), and this resulted in an enhancement of DHA fraction at concentrations where FAS was inhibited but not PKS. Thus, the DHA enhancement resulted from lower concentrations of cerulenin than what was used in this work (100 μM), which might have had an inhibitory effect of both PKS and FAS, and thus the DHA fraction did not increase. However, the overall lipid production did not decrease, which does not support this. Furthermore, Hauvermale et al. measured the direct incorporation of ^{14}C from acetate over a time course of only 45 minutes (Hauvermale et al., 2006), whereas I looked at the fatty acid profile of cells treated with cerulenin over a period of several days. It is possible that this short time effect of cerulenin would not show after several days.

Isoniazid and irgasan have also previously shown to enhance the DHA fraction of total fatty acids in thraustochytrids (Cheng et al., 2016) over a period of 4 days, and this effect was not achieved in this work. However, the irgasan concentrations used by Cheng et al. (0.1-100 μM) were mostly in the range where the growth of T66 was severely inhibited, an effect which was not seen on the *Aurantiochytrium* sp. strain they used. This can reflect a difference between these strains, which is also probably reflected in the different effects of irgasan on the fatty acid contents of the strains. The different concentrations of isoniazid showed nearly the same growth inhibition pattern on T66 as on the strain used by Cheng et al., but the effect on DHA and lipid production was not the same. This could also be a reflection of differences between the strains, although a factor could also be the less defined medium used by Cheng et al., with tryptone and more yeast extract than what was used in this work (Cheng et al., 2016), which could also be a factor in the different effect seen by irgasan.

The first time point sample of cells treated with cerulenin also showed a lower fraction of C14:0 fatty acids than the untreated cells (figure 3.17). C14:0 is a product of FAS (section 1.3.2), and an inhibition of FAS by cerulenin might be an explanation for this effect, although the later samples of cells treated with this inhibitor did not show this effect. The early growth phase is also a potential explanation.

The cells treated with isoniazid produced a larger amount of C14:0, and a somewhat smaller amount of C16 fatty acids, than the untreated cells. It is uncertain what this is a consequence of since all of these are FAS products. Additionally, the cells treated with isoniazid or irgasan produced a small amount of C15:0, which was still larger than the untreated cells. C15:0 is not a common FAS product, because it is odd-numbered (section 1.3). Odd-numbered fatty acids can be synthesized by using propionyl-CoA as a precursor instead of malonyl-CoA (Horning et al., 1961; Nagai et al., 1971). However, it is unclear how inhibition of FAS could lead to this. It is possible that the substrate affinity of KS was affected, or the synthesis of propionyl-CoA or malonyl-CoA, although it is not, to my knowledge, reported that isoniazid or irgasan have these effects. Since isoniazid-treated cells produced more shorter fatty acids (C14 and C15) and less C16, a more standard product of FAS (section 1.3), it might indicate that the fatty acid synthesis often terminated prematurely in these cells.

Other than the mentioned differences, the fatty acid compositions of the different cultures were quite similar. The differences observed, also in lipid content and growth, could be a reflection of several differences between the inhibitors, for example, which domains or by which mechanisms they inhibit (section 1.3.3). Isoniazid and irgasan inhibit the same domain of FAS, but differences in mechanisms of action or binding site might account for their different effects.

Furthermore, it is unknown if and which effect the FAS inhibitors might have on mitochondrial FAS (section 1.3), which complicates the interpretation of the results. As stated by Kastaniotis et al., it is likely that the type II FAS KS (the type in mitochondrial FAS) is affected by the same inhibitors which affect the KS of type I FAS, as their structures are similar to each other. In fact, it has been shown that the KS of mitochondrial FAS was inhibited by cerulenin (Kastaniotis et al., 2017). However, it is not clear what effect an inhibition of mitochondrial FAS might have on the fatty acid profile of the T66 cells, although it would be expected to synthesize only a minor fraction of the total fatty acids.

The results from the total growth experiment done before analysis of lipid and fatty acid composition (figures 3.13 and 3.14) indicated that taxifolin had a concentration-dependent enhancement of growth (figure 3.13 c). Thus, the effect on total lipids and fatty acid content of T66 cells by this inhibitor was not examined further, because it is not likely that it had an inhibitory effect on FAS when it enhanced growth rather than inhibit it. It is unclear what caused this effect, and it was most likely a consequence of some mechanism other than FAS inhibition, for example a nutritional effect. Taxifolin has several other activities than FAS inhibition, and several of these and their mechanisms are not fully unraveled (Weidmann, 2012). Cells treated with apigenin seemed to proliferate more slowly than cells treated with no inhibitors, in the post-exponential growth phase (figure 3.13 b). This is the phase where T66 has shown to begin the extensive lipid accumulation (Jakobsen et al., 2008), which might correlate to the inhibition of growth in this phase. However, the inhibition of growth by apigenin might also be due to its cell cycle arrest activity (section 1.3.3.1). Apigenin inhibited the growth to a relatively low degree in the concentrations tested, and

therefore a higher concentration was used to test its effect on fatty acid content, as it was more likely to have a more significant effect on FAS (table 3.9). Cerulenin had a concentration-dependent inhibitory effect on growth, and a higher cerulenin concentration resulted in a significantly longer lag phase (figure 3.13 d). The latter indicates that cerulenin generates a growth condition which requires more extensive adaptation by the cells (Rolfe et al., 2012). The highest concentration tested (100 μM) significantly inhibited the growth, and was therefore used for further investigation of the effects of cerulenin on T66. The three lowest concentrations of orlistat tested generated similar growth curves as the culture containing no inhibitors (figure 3.14 b). However, the highest concentration tested (50 μM) significantly inhibited the growth and was used for further investigation. Although it was unclear which domain of the thraustochytrid FAS orlistat would inhibit, and if it would inhibit it at all (section 1.3.3.3), it is clear that it had some effect on the cells. Isoniazid had a weak inhibitory effect at 0.1 mM and 1 mM, and inhibited the growth almost completely at 10 mM and 50 mM, which was not desirable when doing further studies of the effects of isoniazid on T66 cells. Therefore, a concentration of 2 mM was used for further studies. Irgasan had a strong inhibitory effect on growth, which was completely inhibited at the highest concentration tested (5 μM). Less significant inhibition of growth than what was exerted by the lowest irgasan concentration tested (0.1 μM), was desirable for further studies of its effects on T66, and thus a slightly lower concentration (0.075 μM) was used for further investigation.

The growth curves from the T66 cultures from which lipid and fatty acid content were analyzed, indicated that the FAS inhibitors all had a significant inhibitory effect on growth in the concentrations tested (figure 3.15). However, the amount of fat-free cell mass produced by the cultures did not indicate inhibition of growth by the inhibitors, except for orlistat, which led to a reduction in the production of fat-free cell mass as well (figure 3.16). However, the fat-free cell mass did reflect the lag phases induced by isoniazid, irgasan, and especially cerulenin. The inconsistencies of the indication of growth by OD_{600} and fat-free cell mass would be explained if the cells in the 0-cultures accumulated more lipids, and thus became larger and gave a higher optical density. However, figures 3.15 and 3.16 show that the cells treated with inhibitors (except for orlistat and the first samples treated with isoniazid and irgasan) produced approximately the same amount of lipids as the cells treated with no inhibitors. Thus, it must have been some other factor or factors influencing the optical density, e.g., the shape of the cells or inhibition of pigment production. These inconsistencies suggest that also the results from the total growth experiments before lipid and fatty acid analysis, testing the effect of the FAS inhibitors on T66 growth (figures 3.13 and 3.14) might not have been reflecting the actual growth of the cells.

Again, an exception in the lipid production of the cultures was orlistat, which did inhibit the lipid production to a certain degree (figures 3.15 and 3.16), and thus it seems that orlistat had an inhibitory effect of the T66 FAS. The smaller amount of lipids produced by cells treated with isoniazid, irgasan, or cerulenin for two days, was probably because these cells were in an earlier phase of the growth than the cells treated with no inhibitors at this time point. Thus, the lipid accumulation had probably not started properly yet, as this activity is dependent on growth phase (Jakobsen et al., 2008). This shows that I should have measured the lipid content at an earlier time point as well, to detect and compare the start of lipid accumulation in all of the cultures, including the 0-cultures.

4.4 Further studies

As the transformations of *Aurantiochytrium* sp. strains T66 and S61 in this work were not successful, it would be useful to attempt to adapt the transformation method so that it would work for these strains. This could be done by testing the effect of different electroporation parameters on the cells. The percentage of dead cells after electroporation can be examined, to evaluate if the voltage is too high or too low. Whether the cells take up DNA during electroporation could also be examined, by for example staining the DNA. Further testing of bead treatment of the cells could also be done to see if it could be used in combination with electroporation for successful transformations. Extension of the duration or increasing the vigor of the bead treatment could be interesting, to see if it will affect the cells. If successful transformations of T66 or S61 are achieved, the different constructs containing the *Sh ble* gene in combination with different promoters and terminators could be transformed into T66 or S61 to optimize the expression of the *Sh ble* gene, if this is necessary. Furthermore, if a successful basic selection method for T66 or S61 is established, further studies could be done to explore the possibility of using 5-FC as a counterselective compound. Potential genes encoding cytosine deaminase to be used as the counterselectable markers must be investigated, and candidates are FCY1 of *S. cerevisiae*, *codA* of *E. coli* (section 1.2.3), and a cytosine deaminase encoding gene from *N. gaditana* (section 3.1.2.1). The gene should preferably have a high GC content, as the GC content of the T66 genome is relatively high (section 1.1.1). Alternatively, a codon-optimized gene may be synthesized. A candidate gene could be transformed into T66 or S61, to see if it results in 5-FC sensitivity, in which case it could be used as a counterselectable marker.

It might be interesting to screen a T66 strain which has been mutagenized to a higher or lower degree than the strain screened in this work, to see if it has a higher frequency of auxotrophic mutants. This could be done by manual replica plating or by an optimized procedure of automatic colony picking. Should the mutants obtained in this work, which grew somewhat better on glutamate and glutamine or uracil and aspartate, appear to be interesting, sequencing their genomes could be done to find out which mutations they harbor. It could, for example, be interesting to investigate the amino acid and pyrimidine nucleotide metabolism in T66.

Since the concentrations tested of the FAS inhibitors apigenin, cerulenin, isoniazid, and irgasan, did not have a significant effect on the biomass or DHA production of T66, it might be interesting to investigate their effect at different concentrations. Perhaps a different method for quantification of growth than measuring optical density should be used, as it did not seem to reflect the growth of T66 treated with FAS inhibitors accurately. An alternative method could be to measure the amount of protein per mL. It might also be interesting to explore the pre-selection method used by Cheng et al., using irgasan/isoniazid in combination with cold stress to select for mutants with an enhanced DHA production (Cheng et al., 2016).

Furthermore, as the results from this work indicate that lowering the flux through FAS did not enhance the production of PKS products, it could be useful to examine the PKS of strain T66 further, to see what could enhance its DHA production. This could be done by bioinformatic studies, and as done by Hauvermale et al. (2006) in *Schizochytrium* sp., by expression of the T66 PKS in *E. coli* to evaluate the enzyme activity.

5 Conclusion

Establishing a functional selection method for *Aurantiochytrium* sp. strain T66 or S61 was not achieved in this work. An impediment was that the cells showed resistance to the selective compounds tested and appeared as false positives during transformations of resistance genes, a problem that has to be overcome when searching for suitable selection markers for these strains. An explanation for the resistance observed might be an expression of an efflux pump by the cells, although the resistance to 5-FC is probably explained by an absence of a gene encoding cytosine deaminase, which converts 5-FC to a toxic compound. However, it is still possible to use 5-FC and a gene encoding cytosine deaminase for counterselection, when a basic selection procedure is established for the strains. Transformations of the *Sh ble* gene into T66 and S61 seemed to be unsuccessful regardless of the false positives, as no transformants with the gene incorporated into the genome were obtained. This shows that the transformation method must be further tested and developed, to be able to transform T66 or S61 with the *Sh ble* gene to see if it renders them zeocin resistant.

No auxotrophic mutants which could have been complemented in a selection procedure were obtained after screening 10 000 colonies of mutagenized T66. This was probably due to the fact that T66 is diploid, and thus the frequency of homozygous mutations by random mutagenesis is low. A mutant obtained which grew slightly better on glutamate and glutamine than on MM was suspected to be a glutamate/glutamine auxotroph. However, it did not grow significantly better with an abundance of glutamate and was therefore not established as a glutamate auxotroph. Another mutant grew slightly better on uracil or aspartate, but it is unlikely that it was auxotrophic for both uracil and aspartate by only one mutation, and thus it was probably a more complicated mutant. Sequencing must be done to find out what genes are mutated in these mutants.

Thus, establishing an effective selection procedure for T66 and S61 is a challenge, and this must be a subject for further investigation to be able to do genetic manipulation of the strains.

None of the FAS inhibitors tested enhanced the DHA production of T66, although cerulenin, isoniazid, and irgasan did affect the production of other fatty acids. Furthermore, the FAS inhibitors apigenin, cerulenin, isoniazid, and irgasan did not affect the lipid production of T66, which treatment with the FAS inhibitor orlistat did. The effects of the inhibitors could be due to FAS not being inhibited, or / in combination with other activities of the inhibitors, for example PKS inhibition. If FAS was inhibited by these inhibitors, these results show that decreasing the flux through FAS did not increase the flux through PKS, and further investigation of PKS must be done to reveal what could increase its activity.

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Appendices

A The lambda PstI standard

The standard used to evaluate sizes of DNA fragments in DNA gel electrophoresis in this work, the lambda PstI standard, is shown in figure A.1.

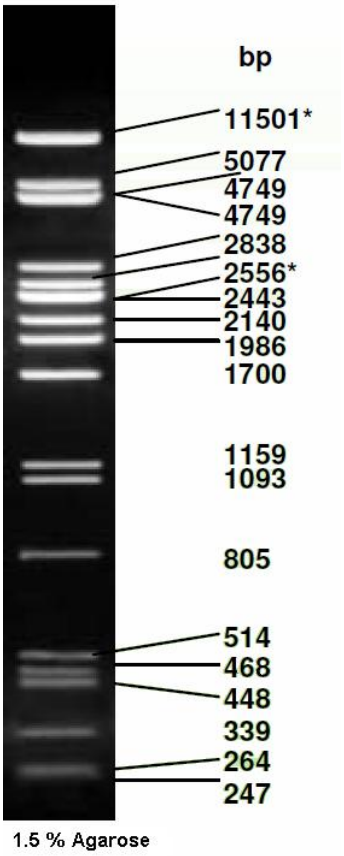


Figure A.1: The lambda PstI standard (GeneON.com).

B Primers

The primers used in this work are shown in table B.1.

Table B.1: The primers used in this work.

Primer	**Sequence (5'-3')**
Ble-ter-SgrDI-R	AATCGTCGACGCGAAGCTTAGCTTGCAAATTAAAG
Ble-F	CGGCATAGTATAATACGACAAGGTG
Ble-SgrDI-R	TAACGTCGACGGCCGCGTCGGACGTGTCAG
zeo sjekk F	AGTTGACCAGTGCCGTTCC
zeo sjekk R	CACGAAGTGCACGCAGTTG
pTEFpspOMI-F	ATTGGGCCCCACACACCATAGCTTC

C Fatty acid analysis raw data

The concentrations of the different fatty acids in the dichloromethane phase, and the fatty acid fractions, given from Zdenka Bartosova and Marit Hallvardsdotter Stafsnes, are shown in tables C.1 and C.2, respectively.

Table C.1: Concentrations (μM) of the different fatty acids in the dichloromethane phase after hydrolysis of lipids from T66 cells treated with FAS inhibitors, from samples taken after two (2:), five (5:) and seven (7:) days of cultivation. 0: no inhibitor, A: apigenin, Or: orlistat, C: cerulenin, Is: isoniazid, Ir: irgasan, Bl1: first injection of blank, Bl2: second injection of blank.

	C14:0	**C15:0**	**C16:1**	**C16:0**	**C18:1**	**C18:0**	**C20:5**	**C22:6**	**C22:5**
2:0-1	782	0	767	2502	262	408	0	855	241
2:0-2	781	0	810	2227	211	332	0	873	234
2:0-3	985	0	940	2744	319	317	0	1142	314
2:A1	982	0	1046	2704	315	357	0	1039	275
2:A2	1114	0	1189	2860	393	450	0	1145	302
2:A3	1298	0	1264	3310	439	340	0	1366	267
2:Or1	407	0	522	1610	170	218	0	697	132
2:Or2	955	0	1157	3037	434	316	0	1373	307
2:Or3	685	0	748	2532	316	377	0	1039	217
2:C1	220	0	105	1864	51	442	0	754	164
2:C2	379	0	132	2637	99	427	0	1333	302
2:C3	365	0	216	2306	99	514	0	923	223
2:Is1	1641	0	137	999	0	423	0	645	238
2:Is2	1971	0	222	1165	0	259	0	801	284
2:Is3	1442	0	137	837	0	222	0	580	206
2:Ir1	1065	110	913	3019	610	496	0	1152	377
2:Ir2	845	95	724	2634	562	432	0	973	316
2:Ir3	989	132	860	3082	597	381	0	1146	340
5:0-1	2355	40	2839	5935	1189	270	17	2828	915
5:0-2	2843	54	3513	7035	1306	493	25	3252	1103
5:0-3	2145	19	2518	5735	1029	104	13	2784	869
5:A1	719	0	916	1841	315	0	0	895	260
5:A2	1489	4	1933	3749	732	466	0	1837	503
5:A3	1956	23	2440	4474	907	244	0	2295	701
5:Or1	1185	0	1533	3580	725	223	28	2098	505
5:Or2	943	0	1155	2611	675	170	22	1711	427
5:Or3	1032	0	1351	3585	775	578	33	2000	492
5:C1	1688	19	2058	4108	794	89	0	2004	621
5:C2	2164	34	2631	5779	1002	539	0	2528	757
5:C3	1745	23	2162	4484	831	97	0	2115	625
5:Is1	3088	64	988	1965	321	96	0	1315	437
5:Is2	2812	57	939	1970	282	159	0	1182	397
5:Is3	3966	78	1235	2728	443	265	0	1593	583
5:Ir1	1372	79	1689	3275	831	102	0	1874	576

Table C.1: (continued)

	C14:0	C15:0	C16:1	C16:0	C18:1	C18:0	C20:5	C22:6	C22:5
5:Ir2	1850	145	2354	4583	1131	693	0	2391	764
5:Ir3	1317	81	1715	3777	893	450	0	1791	534
7:0-1	1059	0	1290	2790	547	107	0	1495	429
7:0-2	1397	0	1846	3651	683	115	0	1860	576
7:0-3	1275	0	1636	3278	623	127	0	1597	494
7:A1	1579	0	2114	3952	782	339	0	1996	620
7:A2	1579	0	2218	4019	900	170	18	2095	620
7:A3	1783	18	2359	4323	1018	326	24	2288	690
7:Or1	827	0	1128	2491	663	109	23	1559	387
7:Or2	686	0	1036	2444	711	109	31	1659	409
7:Or3	667	0	1056	2368	649	195	24	1504	379
7:C1	1729	27	2475	4360	1065	209	0	2229	668
7:C2	1474	0	1912	3216	680	134	0	1628	488
7:C3	1759	22	2194	4058	913	280	0	1915	569
7:Is1	1522	52	1889	3230	855	80	0	1956	595
7:Is2	1464	104	1959	3381	1112	164	0	2130	668
7:Is3	1159	82	1578	3098	858	228	0	1726	541
7:Ir1	3878	112	1521	2536	510	62	0	1801	618
7:Ir2	4345	112	1715	2880	549	251	0	1947	681
7:Ir3	3055	57	1156	1913	375	75	0	1344	461
Bl1	0	0	36	229	0	111	0	46	0
Bl2	0	0	36	310	0	111	0	45	0

Table C.2: Fatty acid percentages of TFA in T66 cells treated with or without FAS inhibitors, from samples taken after two (2:), five (5:) and seven (7:) days of cultivation. 0: no inhibitor, A: apigenin, Or: orlistat, C: cerulenin, Is: isoniazid, Ir: irgasan, Bl1: first injection of blank, Bl2: second injection of blank.

	C14:0	**C15:0**	**C16:1**	**C16:0**	**C18:1**	**C18:0**	**C20:5**	**C22:6**	**C22:5**
2:0-1	13.4	0.0	13.2	43.0	4.5	7.0	0.0	14.7	4.1
2:0-2	14.3	0.0	14.8	40.7	3.9	6.1	0.0	16.0	4.3
2:0-3	14.6	0.0	13.9	40.6	4.7	4.7	0.0	16.9	4.6
2:A1	14.6	0.0	15.6	40.2	4.7	5.3	0.0	15.5	4.1
2:A2	14.9	0.0	16.0	38.4	5.3	6.0	0.0	15.4	4.0
2:A3	15.7	0.0	15.3	40.0	5.3	4.1	0.0	16.5	3.2
2:Or1	10.8	0.0	13.9	42.9	4.5	5.8	0.0	18.6	3.5
2:Or2	12.6	0.0	15.3	40.1	5.7	4.2	0.0	18.1	4.0
2:Or3	11.6	0.0	12.7	42.8	5.3	6.4	0.0	17.6	3.7
2:C1	6.1	0.0	2.9	51.8	1.4	12.3	0.0	20.9	4.6
2:C2	7.1	0.0	2.5	49.7	1.9	8.0	0.0	25.1	5.7
2:C3	7.9	0.0	4.7	49.6	2.1	11.1	0.0	19.9	4.8
2:Is1	40.2	0.0	3.4	24.5	0.0	10.4	0.0	15.8	5.8
2:Is2	41.9	0.0	4.7	24.8	0.0	5.5	0.0	17.0	6.0
2:Is3	42.1	0.0	4.0	24.4	0.0	6.5	0.0	16.9	6.0
2:Ir1	13.8	1.4	11.8	39.0	7.9	6.4	0.0	14.9	4.9
2:Ir2	12.8	1.4	11.0	40.0	8.5	6.6	0.0	14.8	4.8
2:Ir3	13.1	1.8	11.4	40.9	7.9	5.1	0.0	15.2	4.5
5:0-1	14.4	0.2	17.3	36.2	7.3	1.6	0.1	17.3	5.6
5:0-2	14.5	0.3	17.9	35.8	6.7	2.5	0.1	16.6	5.6
5:0-3	14.1	0.1	16.5	37.7	6.8	0.7	0.1	18.3	5.7
5:A1	14.5	0.0	18.5	37.2	6.4	0.0	0.0	18.1	5.2
5:A2	13.9	0.0	18.0	35.0	6.8	4.3	0.0	17.1	4.7
5:A3	15.0	0.2	18.7	34.3	7.0	1.9	0.0	17.6	5.4
5:Or1	12.0	0.0	15.5	36.2	7.3	2.3	0.3	21.2	5.1
5:Or2	12.2	0.0	15.0	33.9	8.7	2.2	0.3	22.2	5.5
5:Or3	10.5	0.0	13.7	36.4	7.9	5.9	0.3	20.3	5.0
5:C1	14.8	0.2	18.1	36.1	7.0	0.8	0.0	17.6	5.5
5:C2	14.0	0.2	17.0	37.4	6.5	3.5	0.0	16.4	4.9
5:C3	14.4	0.2	17.9	37.1	6.9	0.8	0.0	17.5	5.2
5:Is1	37.3	0.8	11.9	23.7	3.9	1.2	0.0	15.9	5.3
5:Is2	36.1	0.7	12.0	25.3	3.6	2.0	0.0	15.2	5.1
5:Is3	36.4	0.7	11.3	25.0	4.1	2.4	0.0	14.6	5.4
5:Ir1	14.0	0.8	17.2	33.4	8.5	1.0	0.0	19.1	5.9
5:Ir2	13.3	1.0	16.9	32.9	8.1	5.0	0.0	17.2	5.5
5:Ir3	12.5	0.8	16.2	35.8	8.5	4.3	0.0	17.0	5.1
7:0-1	13.7	0.0	16.7	36.2	7.1	1.4	0.0	19.4	5.6
7:0-2	13.8	0.0	18.2	36.1	6.7	1.1	0.0	18.4	5.7
7:0-3	14.1	0.0	18.1	36.3	6.9	1.4	0.0	17.7	5.5

Table C.2: (continued)

	C14:0	C15:0	C16:1	C16:0	C18:1	C18:0	C20:5	C22:6	C22:5
7:A1	13.9	0.0	18.6	34.7	6.9	3.0	0.0	17.5	5.4
7:A2	13.6	0.0	19.1	34.6	7.7	1.5	0.2	18.0	5.3
7:A3	13.9	0.1	18.4	33.7	7.9	2.5	0.2	17.8	5.4
7:Or1	11.5	0.0	15.7	34.7	9.2	1.5	0.3	21.7	5.4
7:Or2	9.7	0.0	14.6	34.5	10.0	1.5	0.4	23.4	5.8
7:Or3	9.7	0.0	15.4	34.6	9.5	2.8	0.3	22.0	5.5
7:C1	13.5	0.2	19.4	34.2	8.3	1.6	0.0	17.5	5.2
7:C2	15.5	0.0	20.1	33.7	7.1	1.4	0.0	17.1	5.1
7:C3	15.0	0.2	18.7	34.7	7.8	2.4	0.0	16.4	4.9
7:Is1	35.1	1.0	13.8	23.0	4.6	0.6	0.0	16.3	5.6
7:Is2	34.8	0.9	13.7	23.1	4.4	2.0	0.0	15.6	5.5
7:Is3	36.2	0.7	13.7	22.7	4.4	0.9	0.0	15.9	5.5
7:Ir1	15.0	0.5	18.6	31.7	8.4	0.8	0.0	19.2	5.8
7:Ir2	13.3	0.9	17.8	30.8	10.1	1.5	0.0	19.4	6.1
7:Ir3	12.5	0.9	17.0	33.4	9.3	2.5	0.0	18.6	5.8
Bl1	0.0	0.0	8.6	54.2	0.0	26.2	0.0	10.9	0.0
Bl2	0.0	0.0	7.1	61.9	0.0	22.1	0.0	8.9	0.0

D Standard deviations from fatty acid analysis

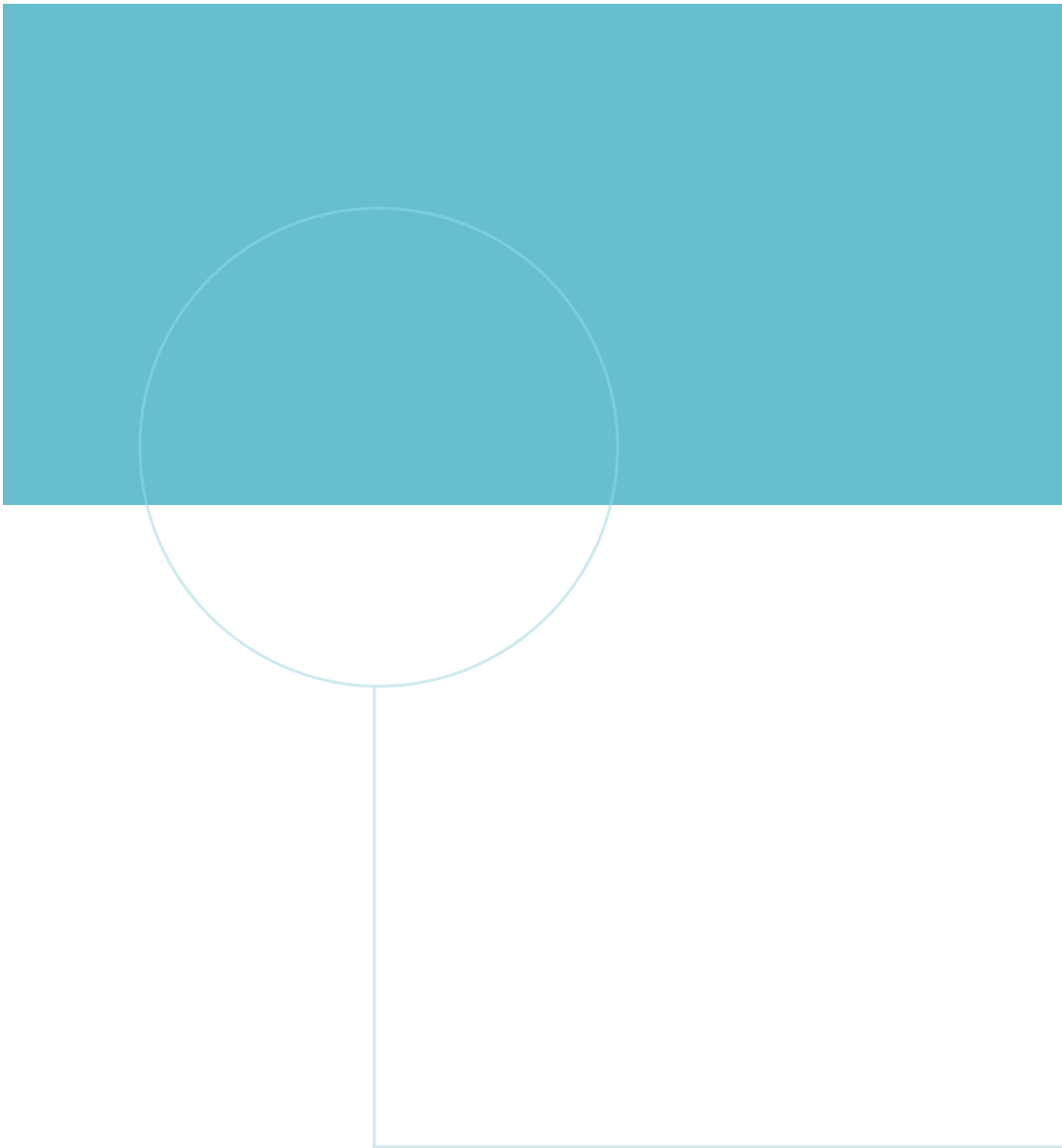
The standard deviations of the fatty acid percentages of TFA from T66 cells treated with FAS inhibitors, are shown in table D.1. The standard deviations of the amounts (g) of fatty acids produced per 100 g CDW of T66 cells treated with FAS inhibitors, are shown in table D.2.

Table D.1: The standard deviations of the fatty acid percentages of TFA from T66 cells treated with or without FAS inhibitors, from samples taken after two (2:), five (5:) and seven (7:) days of cultivation. 0: no inhibitor, A: apigenin, Or: orlistat, C: cerulenin, Is: isoniazid, Ir: irgasan.

	C14:0	**C15:0**	**C16:1**	**C16:0**	**C18:1**	**C18:0**	**C20:5**	**C22:6**	**C22:5**
2:0	0.59	0.00	0.81	1.36	0.45	1.17	0.00	1.10	0.26
2:A	0.54	0.00	0.35	1.01	0.35	0.98	0.00	0.62	0.49
2:Or	0.89	0.00	1.30	1.60	0.62	1.14	0.00	0.49	0.27
2:C	0.88	0.00	1.15	1.24	0.37	2.18	0.00	2.78	0.60
2:Is	1.06	0.00	0.68	0.18	0.00	2.57	0.00	0.68	0.12
2:Ir	0.47	0.19	0.40	0.98	0.37	0.83	0.00	0.23	0.19
5:0	0.20	0.08	0.68	0.98	0.32	0.92	0.02	0.87	0.07
5:A	0.55	0.09	0.34	1.53	0.30	2.18	0.00	0.47	0.36
5:Or	0.94	0.00	0.92	1.43	0.71	2.10	0.03	0.93	0.28
5:C	0.41	0.02	0.55	0.71	0.25	1.56	0.00	0.68	0.27
5:Is	0.65	0.03	0.38	0.82	0.23	0.65	0.00	0.64	0.13
5:Ir	0.76	0.15	0.51	1.51	0.19	2.10	0.00	1.19	0.41
7:0	0.76	0.00	0.84	0.13	0.17	0.15	0.00	0.85	0.11
7:A	0.17	0.06	0.36	0.56	0.57	0.78	0.10	0.25	0.06
7:Or	1.03	0.00	0.56	0.09	0.41	0.76	0.06	0.92	0.20
7:C	1.00	0.11	0.66	0.46	0.61	0.52	0.00	0.57	0.19
7:Is	0.73	0.17	0.04	0.21	0.12	0.76	0.00	0.36	0.08
7:Ir	1.24	0.24	0.77	1.34	0.87	0.84	0.00	0.40	0.14

Table D.2: The standard deviations of the amounts (g) of fatty acids produced per 100 g CDW of T66 cells treated with or without FAS inhibitors, from samples taken after two (2:), five (5:) and seven (7:) days of cultivation. 0: no inhibitor, A: apigenin, Or: orlistat, C: cerulenin, Is: isoniazid, Ir: irgasan.

	C14:0	**C15:0**	**C16:1**	**C16:0**	**C18:1**	**C18:0**	**C20:5**	**C22:6**	**C22:5**
2:0	0.19	0.00	0.45	1.19	0.22	0.70	0.00	0.41	0.09
2:A	0.13	0.00	0.37	0.53	0.17	0.57	0.00	0.12	0.30
2:Or	0.37	0.00	0.68	0.86	0.23	0.45	0.00	0.47	0.12
2:C	0.25	0.00	0.32	0.68	0.10	0.62	0.00	0.43	0.10
2:Is	0.78	0.00	0.26	0.36	0.00	0.68	0.00	0.39	0.11
2:Ir	0.53	0.06	0.43	0.60	0.22	0.47	0.00	0.34	0.20
5:0	0.45	0.05	0.69	0.81	0.37	0.54	0.01	0.55	0.12
5:A	0.33	0.05	0.21	0.70	0.20	1.23	0.00	0.21	0.20
5:Or	0.58	0.00	0.65	0.87	0.29	0.86	0.01	0.65	0.16
5:C	0.27	0.01	0.34	0.29	0.15	0.87	0.00	0.40	0.17
5:Is	0.40	0.01	0.30	0.96	0.13	0.39	0.00	0.22	0.10
5:Ir	0.32	0.09	0.13	1.19	0.15	1.23	0.00	0.56	0.19
7:0	0.37	0.00	0.53	0.34	0.12	0.09	0.00	0.42	0.06
7:A	0.12	0.05	0.34	0.35	0.40	0.43	0.06	0.33	0.21
7:Or	0.68	0.00	0.52	0.75	0.14	0.31	0.02	0.38	0.14
7:C	0.55	0.07	0.51	0.15	0.35	0.28	0.00	0.45	0.19
7:Is	0.93	0.11	0.52	0.95	0.17	0.45	0.00	0.54	0.23
7:Ir	0.56	0.14	0.23	1.06	0.54	0.50	0.00	0.14	0.30



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