Metabolic Engineering of *Moesziomyces antarcticus*

Disruption of Mat1 by application of the CRISPR-Cas9 system and conventional gene knock-out methods

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Abstract

Mannosylerythritol lipids have gained increasing attention throughout the last 60 years due to their high yield, application possibilities and self-assembling properties, which are studied to optimise drug delivery in pharmaceuticals. The basidiomycetous yeast *Moesziomyces antarcticus* belongs to a group of microorganisms that excrete MELs in relatively high quantities; up to 40 g/L when grown on vegetable oil. The MELs excreted by *M. antarcticus* are comprised of four different types; di-acetylated MEL-A, mono-acetylated MEL-B and MEL-C and deacetylated MEL-D. In this thesis it was attempted to knock-out the gene Mat1, an acetyltransferase responsible for the acetylation of MEL-D, in order to produce a homogeneous product of MEL-D. The CRISPR-Cas9 system was applied for gene disruption and several successful transformants were obtained. However, Mat1 was not successfully targeted by Cas9 in any of the tested transformants, and therefore the targeted gene was not disrupted. Three alternative methods for gene disruption, based on homologous recombination, were tested for targeting Mat1. A plasmid containing a disruption cassette embedding the nourseothricin resistance gene, with Mat1 homologous flanking regions, was constructed, and transformation into *M. antarcticus* was carried out by electroporation with the plasmid, the disruption cassette, from the digested plasmid, and split markers, constructed from the disruption cassette. To understand the mechanism of acylation of MELs, with fatty acids, an in silico analysis of a potential partial β-oxidation chain shortening pathway in *M. antarcticus* was investigated. The analysis was carried out by comparison with the peroxisomal β-oxidation that takes place in *Homo sapiens* and *Saccharomyces cerevisiae*. Several potential genes were identified in *M. antarcticus* based on protein homology to β-oxidation genes in *H. sapiens* and *S. cerevisiae*.

Declaration

I hereby declare that the project work entitled *Metabolic Engineering of Moesziomyces antarcticus - Disruption of Mat1 by application of the CRISPR-Cas9 system and conventional gene knock-out methods* is an authentic record of my own work carried out at Aalborg University Copenhagen in connection with the devising of my master thesis.

Cathrine Westergaard Nielsen
30 April 2019

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List of Abbreviations

BS	Biosurfactant
C_T	Threshold cycle
CFU	Colony forming unit
crRNA	CRISPR-RNA
DSB	Double strand break
HR	Homologous recombination
LCFA	Long chain fatty acid
MCFA	Medium chain fatty acid
MEL	Mannosylerythritol lipid
NHEJ	Non-homologous end joining
OD	Optical density
PAM	Protospacer adjacent motif
PTS	Peroxisomal targeting signal
rpm	Rounds per minute
RT-PCR	Real-Time PCR
sgRNA	Single guide RNA
TLC	Thin layer chromatography
tracrRNA	Trans-activation crRNA
VLCFA	Very long chain fatty acid

1 General Introduction and Project Background

Surfactants, in general, are considered important chemical products due to their hydrophilic and lipophilic properties. They have a major impact on our everyday lives because of their diversity and many application aspects within detergents, food, cosmetics and many more [10][11]. Surfactants were originally produced from raw materials such as plant and animal oils, however, the synthetic surfactants are currently dominating the market, with two thirds of all produced surfactants originating from petrochemical sources [12]. With the increasing focus on the environment and sustainability as well as the approaching shortage of fossil fuels, it has become necessary once again to look to renewable feedstocks for production of surfactants [13]. As an alternative to the synthetic surfactants, microbial biosurfactants (BS) have gained interest as they display properties that are highly similar to those of the synthetic surfactants, and therefore, they can potentially replace these, while representing a more environmentally friendly option [14]. The microbially produced BS offer several advantages compared to their synthetic counterparts. The main potential of application of microbial BS is within the oil industry for bioremediation of e.g. oil spills, handling of oil sludge and for enhanced recovery of crude oil [15][16]. While synthetic surfactants can be applied in these areas as well, some drawbacks are that they pose an environmental threat as they are non-biodegradeable, and therefore prone to accumulate in the environment, and are toxic to their surroundings [17]. BS, on the other hand, show higher degradability and lower environmental toxicity, as well as tolerance to high temperatures, making them suitable for replacement of synthetic surfactants within many areas of application [16]. Their diversity and the fact that they can be designed to fit certain purposes by changing production parameters such as substrate or production organism, is an important advantage over the synthetic surfactants, making BS suitable for a wide range of purposes [18]. Other beneficial aspects of BS is their digestibility, making them good alternatives to synthetic surfactants within food and cosmetics, and the fact that they can be produced from cheap raw-materials, contributing to low production cost [19].

1.1 Classification of biosurfactants

BSs are amphiphilic compounds, consisting of both a hydrophilic moiety, represented by e.g. a carbohydrate or a carboxylic acid, and a lipophilic moiety, represented by a hydrocarbon such as long chain fatty acids [19]. Biosurfactants are produced by a range of yeasts and bacteria, and can be excreted to facilitate the motility of the microorganism [20]. Whereas the synthetic surfactants are usually classified based on the nature of their polar charge, BSs are classified rather on a basis of their chemical composition and microbial origin [21], and can be divided into the following five main groups;

- glycolipids
- lipopeptides and lipoproteins
- polymeric biosurfactants
- particulate biosurfactants
- fatty acids, phospholipids and neutral lipids

One of the best studied categories of biosurfactants is the glycolipids [22], which consist of carbohydrates containing either aliphatic acids or hydroxyaliphatic acids. Within the glycolipids there are four subcategories; rhamnolipids, mannosylerythritol lipids (MELs), trehalolipids and sophorolipids. Of these four types, MELs have proven throughout the last 60 years, to be one of the most promising glycolipids within industrial application. This is mainly due to the relatively

high yield, compared to other types of BSs, as well as their many application possibilities within pharmaceuticals. One additional point of interest in MELs is their self-assembling properties, which are investigated to be used for optimised drug delivery [20][23]. For these reasons, the production of MELs is under the loop for further investigations throughout this thesis.

1.2 *Moesziomyces antarcticus* as a cell factory

M. antarcticus is a basidiomycetous yeast belonging to the subphylum ustilaginomycotina and the class ustilaginomycetes [24]. It was originally classified as *Candida antarctica*, but has over the years been reclassified, first as *Pseudozyma antarctica* and most recently as *Moesziomyces antarcticus* [4][24]. *M. antarcticus* was initially isolated from a lake within the arctic circle, and it is, among others, the fact that *M. antarcticus* has adapted to grow in cold temperatures, which makes it interesting in the production of chemical products, applicable for low-temperature processes [23][25]. At present, *M. antarcticus* is being used for a commercial production of two cold-adapted lipases CALA and CALB, which are being produced and sold commercially by Novozymes (Denmark) [26]. Another interesting attribute of *M. antarcticus*, and the attribute at focus throughout this thesis, is the fact that it excretes MELs.

1.2.1 Biosynthesis of MELs in *Moesziomyces antarcticus*

MELs are produced by a wide range of microorganisms, among them is the yeast *Moesziomyces antarcticus*, for which the MELs act as a carbon rich energy storage [20]. MELs contain a 4-*O*-β-D-mannopyranosyl-meso-erythritol as their hydrophilic group and a fatty acid and/or an acetyl group as the hydrophobic group, depending on the level of acetylation of the BS [2]. The genes for the biosynthesis of MELs, were originally identified in the dimorphic basidiomycetous yeast *Ustilago maydis*, which is genetically closely related to *M. antarcticus*. The phylogenetic relationship between *M. antarcticus* and *U. maydis* is illustrated in **Figure 6**. The MEL biosythesis in *M. antarcticus*, which can be seen in **Figure 2**, was identified to be carried out in three individual steps, which are catalysed by four enzymes; Emt1, Mac1, Mac2 and Mat1, located in a genecluster found on scaffold 31 of *Moesziomyces antarcticus* [1][27], as illustrated in **Figure 1**. The first step of the biosynthesis is initiated by Emt1, an erythritol/mannose transferase, that catalyses the transfer of GDP-mannose to D-erythritol resulting in the construction of mannosyl-D-erythritol. In the second step of MEL biosynthesis, the acyl transferases Mac1 and Mac2 are responsible for transferring short- and medium-chain fatty acids onto the C-2 and C-3 positions of mannose. The final step is carried out by the acetyltransferase Mat1 which carries out the acetylation of the deacetylated MEL from step 2, and the biosynthesis of MEL is hereby complete [2].

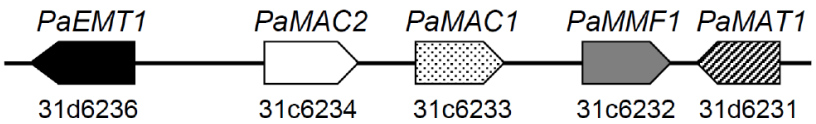


Figure 1: Illustration of the gene cluster responsible for MEL production in *M. antarcticus*, located on scaffold 31 [1].

M. antarcticus produces a mixture of four different types of MELs; MEL-A, MEL-B, MEL-C and MEL-D. The MELs are identified based on their level of acetylation on C-4 and C-6 position of mannose. MEL-A is a di-acetylated MEL featuring two acetyl groups, one on each C-4 and C-6 position, whereas MEL-B and MEL-C are mono-acetylated MELs, with only one acetyl

group on either the C-4 or C-6 position [2]. MEL-D is a deacetylated MEL containing no acetyl groups on either of the C-4 or C-6 positions, and is assembled in the second step of the MEL biosynthesis, hence MEL-A, MEL-B and MEL-C are derivatives resulting from acetylation of MEL-D carried out by Mat1.

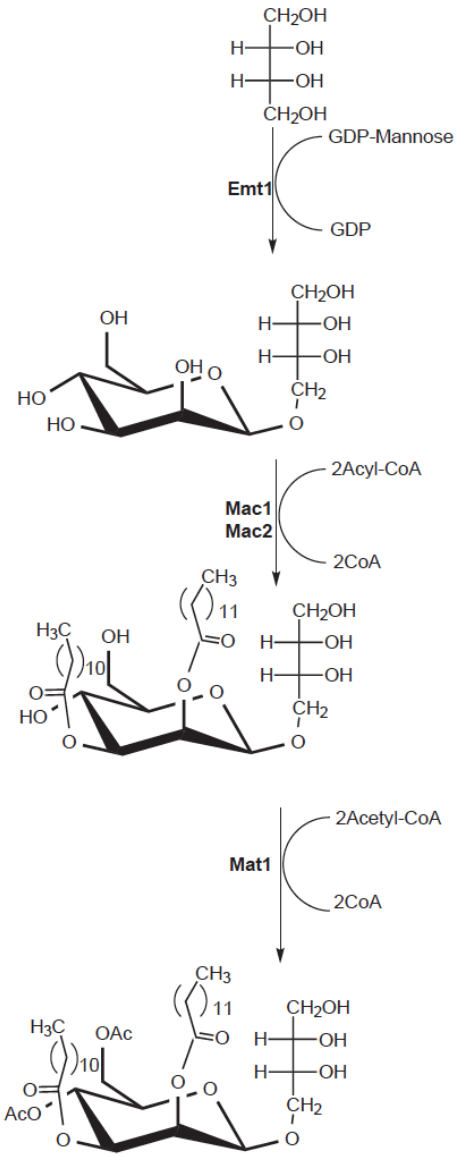


Figure 2: Synthesis pathway of MELs in *M. antarcticus*[2].

1.2.2 Improvements on product yield

Synthetic surfactants can, at the moment, be produced at a lower price and in a larger amount than BSs [11]. In the production of BSs several issues arise and among them is the downstream processing required to recover the product. The glycolipid MEL, produced by *M. antarcticus*, can reach production yields up to 40 g/L when grown on vegetable oils such as soybean oil, however, the extraction of MELs from a growth medium, with an oily substrate remaining, has proved itself challenging [11]. Although vegetable and animal oils can be considered renewable resources, the sustainability of these feedstocks also has to be taken into consideration. With a continuous growth in the Earths population [28], an increase

in the necessity for food is a consequence. Hence the usage of raw materials, suitable for human consumption, for the production of chemical compounds, can be considered ethically unacceptable under such circumstances [29]. Although the interest in BSs is increasing, they cannot yet compete with the synthetic surfactants at an economical level. Especially the cost of substrate has to be considered, as this can account for up to 50% of the total production cost [30]. An advantage, however, of glycolipid BSs, such as MELs, is that they are produced by yeasts, such as *M. antarcticus*, while growing on several substrates that can be considered wastes from other production processes. Of such substrates glycerol, which is obtained as a by-product from biodiesel production [11], and renewable resources such as cellulosic materials that have undergone pre-treatment, are worth mentioning [31].

The drawbacks of BSs relate mainly to the production of these, specifically the cost of production [22]. The production process is troublesome, as BSs are produced microbially, and the process can be difficult to regulate. Additionally, the biological production of surfactants faces a challenge with regards to the yield. Microorganisms that accumulate large amounts of BSs are uncommon, and a prerequisite for a high yield of BSs is the addition of complex media, which adds to the production cost [19]. As mentioned, MELs can be produced from several renewable substrates, one of them being glycerol. Being a waste product, using glycerol for the production of MELs could potentially lower the overall production cost. Another issue in the production and application of BSs in the industry is tailoring them to meet up with the requirements for certain applications [11]. In the basidiomycetous yeast *Ustilago maydis*, it was attempted to knock-out the gene Mat1, which resulted in excretion of the deacetylated type of MEL, MEL-D [1]. As *M. antarcticus* excretes a mixture of MELs, a similar approach could be taken towards disrupting Mat1 in order to homogenise the final product, and thus making it more applicable in certain industrial processes [11].

This thesis consists of three individual parts, with each their own topic in focus. However, they are all connected, as each topic relates to the overall topic; production of MELs in *Moesziomyces antarcticus*. **Part I** is focused on the investigation of a genetic modification of *M. antarcticus* by knock-out of the Mat1 gene by application of the CRISPR-Cas9 gene editing system. **Part II** is somewhat of a continuation of **Part I**, with a similar objective of knock-out of Mat1 following a more conventional approach by constructing a knock-out cassette to induce the molecular change. **Part III** can be regarded as a literature study, in which an understanding of the suggested chain shortening pathway, involved in the acylation of mannosyl-D-erythritol by Mac1 and Mac2, was attained, and several genes, potentially involved in β-oxidation of fatty acids in *M. antarcticus*, were identified, based on protein homology with β-oxidation genes in *Saccharomyces cerevisiae* and *Homo sapiens*.

Part I

Site Directed Disruption of Mat1 in *Moesziomyces antarcticus* Implementing the CRISPR-Cas9 System

1 Introduction

1.1 The CRISPR-Cas9 system

Until recently, genome editing such as insertions or deletions in the genome relied mainly on PCR based methods. However, the CRISPR (clustered regularly interspaced short palindromic repeat) and CRISPR-associated (Cas) protein system, which serves as a defense system in bacteria and archaea against invading DNA from phage and plasmids [32], is able to introduce double strand breaks (DSB) into DNA, with great precision, and hereby cause e.g. gene disruptions or insertions into the genome [33]. The mechanism of the CRISPR system consists of three steps; (1) the adaption of pieces of invading DNA (spacers) into the genome of the host organism in the CRISPR loci, (2) the expression of guide CRISPR-RNAs (crRNA) containing the before mentioned spacers and guiding the CRISPR-Cas complex to the intended location in the invading DNA, (3) the interference of the CRISPR-Cas system, with the invading DNA, in which the CRISPR-Cas complex introduces a DSB in the target locus [34]. Several Cas proteins have been identified in different microorganisms, and recently a new classification method of CRISPR systems has been suggested. The suggested classification deploys three individual CRISPR systems; type I CRISPR systems, type II CRISPR systems and type III CRISPR systems. For all types, it holds true, that Cas1 and Cas2 are believed to be active and play a key role in the function of the CRISPR complex in the integration of short sequences of virus or plasmid DNA, also known as spacers, into the CRISPR loci of the cells genome [35]. However, one main difference between the three system types, is that type II systems differ from type I and type III, in the way that type II systems only require a single Cas9 protein, an endonuclease, in order to carry out their intended function, as opposed to type I and type III, which employ several types of Cas proteins [35]. Due to the simplicity of type II systems, a type II CRISPR system from *Streptococcus pyogenes* has been adapted for genetic engineering of a range of microorganisms.

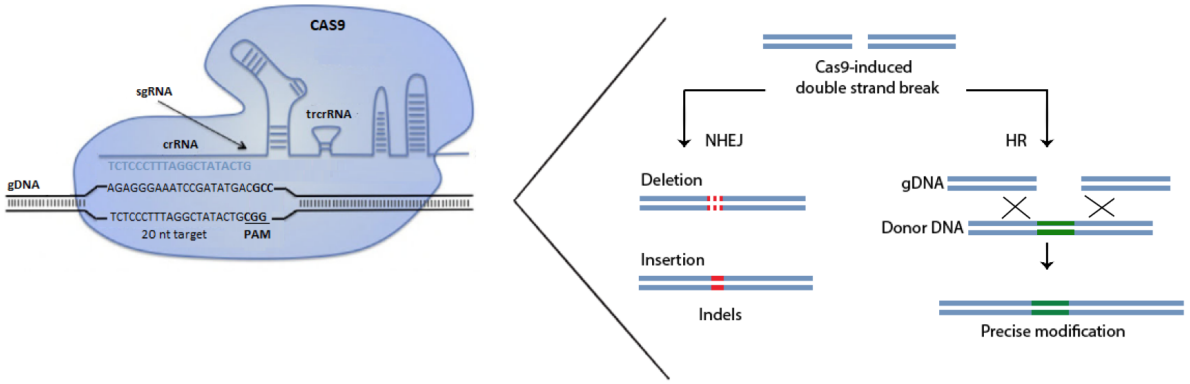


Figure 3: An overview of the mechanism of the CRISPR-Cas9 genome editing system. The Cas9 protein binds with the sgRNA, which guides it to the homologous region of the genomic DNA, where it introduces a double stranded break. The double stranded break is subsequently repaired by either non-homologous end joining or homologous recombination.

The CRISPR-Cas9 complex, developed from *S. pyogenes* for genome editing in other organisms, consists overall of two components, the before mentioned Cas9 protein and a single guide RNA (sgRNA). The sgRNA is a duplex of a crRNA and a trans-activation crRNA (tracrRNA), that together guide the Cas9 nuclease to the targeted location in the DNA, where it introduces a DSB [36]. The crRNA consists typically of 20 basepairs, which are homologous to the target sequence, while the tracrRNA creates a stem loop structure, which binds to the Cas9. Another crucial element of the sgRNA is the protospacer adjacent motif (PAM) sequence, which is found downstream the target DNA sequence. The PAM sequence is recognised by the Cas9 protein, and for Cas9, derived from *S. pyogenes*, the specific PAM sequence in NGG [37]. Following the double stranded break, a mutation in the genome is introduced during the non-homologous end joining (NHEJ) repair, typically resulting in shifting of the open reading frame or the generation of additional stop codons. The result of this being, in both cases, a disrupted gene. Alternatively, a donor sequence can be provided with the sgRNA, resulting in an insertion of the donor sequence into the targeted area by homologous recombination (HR) [5]. An illustartion of the mechanism of the CRISPR-Cas9 system, adapted from *S. pyogenes*, can be seen in **Figure 3**.

1.1.1 Non-homologous end joining

Following a DSB in the DNA, it is crucial for the survival of the cell, that these are repaired efficiently, as unrepaired DSB can lead to apoptosis. One way of repairing such a break, is by NHEJ. An important feature of NHEJ is the fact that, unlike for HR to take place, NHEJ does not require a donor DNA template for the DSB repair to take place. HR is explained in more detail in **Part II Section 1.1**. In the initial step of the NHEJ pathway (**a** in **Figure 4**), the Ku heterodimer binds to the location of the DSB along with other components for NHEJ such as the protein kinase DNA-PKcs. As the Ku heterodimer binds to the sugar backbone rather than the bases of the DNA, it enables it to bind to the DNA at any given location, which makes it a versatile repair system for DSB. Depending on the nature of the DSB, the ends of the DSB may or may not be subject to processing (**b** in **Figure 4**), in order to prepare the end for ligation. DSBs can be caused by a number of reasons which result in the ends needing different types of processing such as filling of gaps by random insertions or deletions (indels). Several

enzymes have been identified, which take part in the preparation of DSB ends for ligation. The final step of the NHEJ pathway (**c** in **Figure 4**), is the ligation of the ends and thereby repair of the DSB, which is carried out by a Ligase IV and XRCC4 complex [38][3].

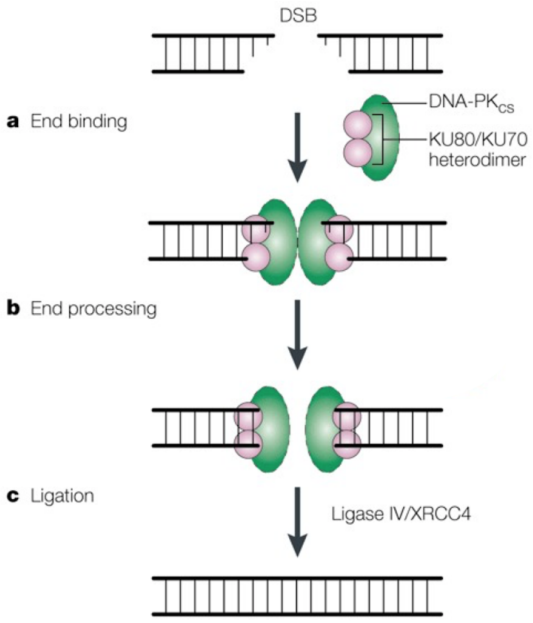


Figure 4: Mechanism of DSB repair by NHEJ [3].

1.2 Application of CRISPR-Cas9 in *M. antarcticus*

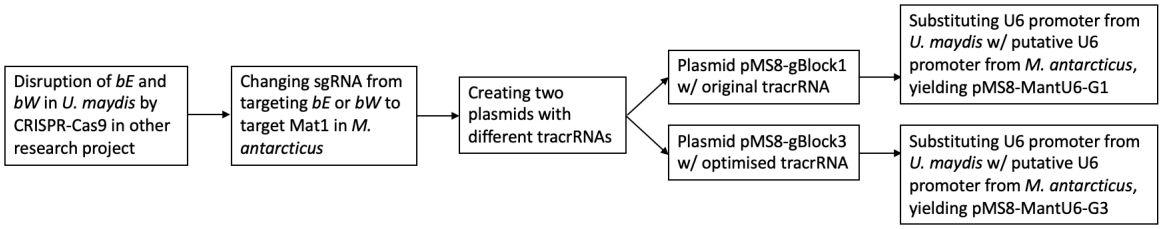


Figure 5: An overview of the work flow of previous work, carried out prior to the preparation of this thesis.

In the basidiomycetous yeast *Ustilago maydis* a recent attempt was made, in another research project, to develop the CRISPR-Cas9 system for genetic engineering of the microorganism. The objective was to disrupt the two genes *bE* and *bW*, involved in the development of a filamentous and pathogenic nature of the fungus [5]. Due to the close genetic relationship between *U. maydis* and *M. antarcticus*, visualised in **Figure 6**, this very approach inspired a similar strategy to be followed for development of a CRISPR-Cas9 gene editing system in *M. antarcticus*. Prior to this project, an investigation into the disruption of the gene encoding Mat1 in *M. antarcticus* was carried out by Cristiano Di Pietro [39]. An overview of the work flow, carried out prior to this project, can be seen in **Figure 5**.

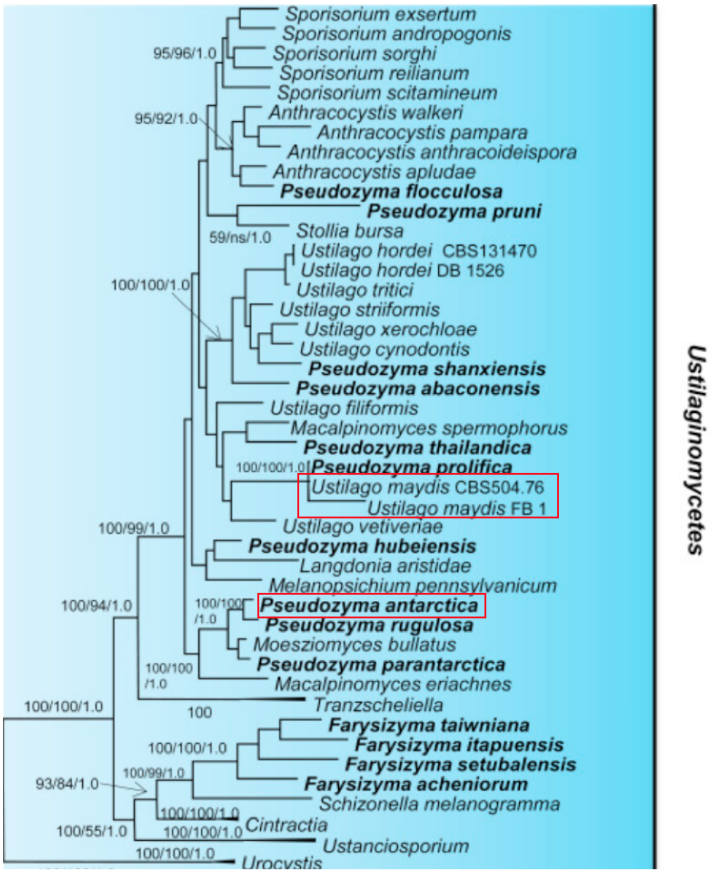
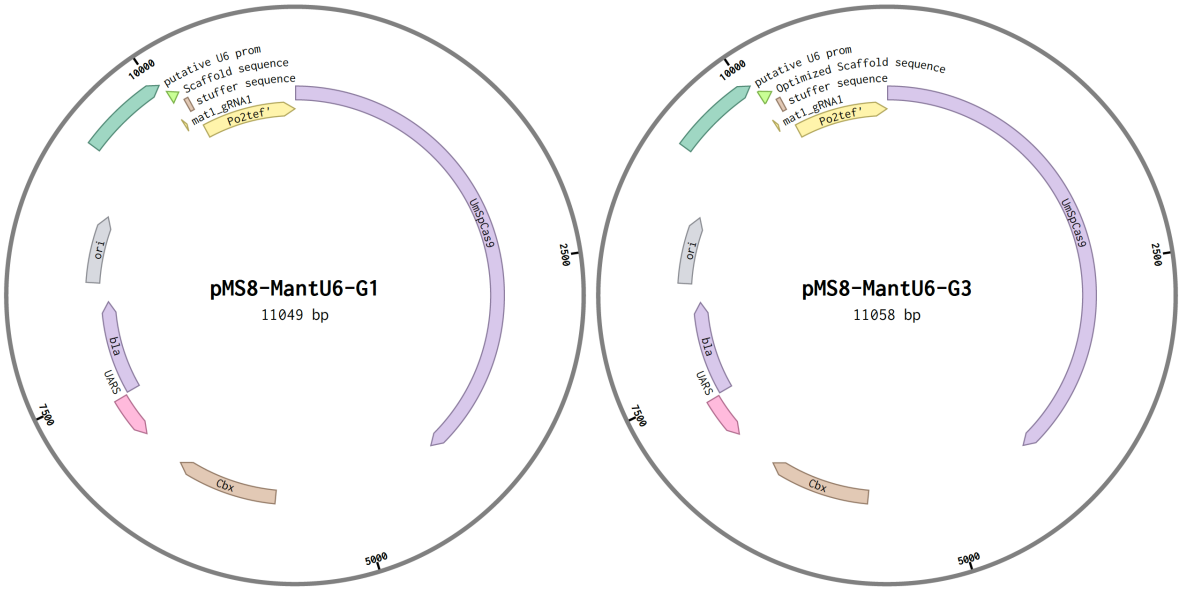


Figure 6: Phylogenetic relationship of species in the class ustilaginomycetes of the ustilaginomycotina subphylum [4].

U. maydis has been reported to excrete MELs, while *Pseudozyma* spp., including *M. antarcticus*, previously *Pseudozyma antarctica* [4], have shown to excrete MELs in large amounts [20]. By disruption of the gene encoding Mat1 in *U. maydis*, it was confirmed that Mat1 is involved in the acetylation of MELs [1]. Therefore, it was decided to develop a system for disruption of Mat1 in *M. antarcticus* by applying the CRISPR-Cas9 system. In the initial attempts of disrupting Mat1 in *M. antarcticus*, two similar plasmids, to that used for targeting *bE* and *bW* in *U. maydis* [5], were developed. The plasmids, pMS8-gBlock1 and pMS8-gBlock3, were constructed to each contain one of two gBlocks. Each gBlock contains a sgRNA, with gBlock1 containing a tracrRNA-crRNA duplex, with the guide crRNA targeting Mat1 in *M. antarcticus* and the tracrRNA from [5], and with gBlock3 containing a tracrRNA-crRNA duplex, with the same guide crRNA targeting Mat1 in *M. antarcticus* but containing an optimised tracrRNA. The sequences of gBlock1 and gBlock3 can be seen in **Appendix A.1** and **A.2**, respectively.



(a) pMS8-MantU6-G1. (b) pMS8-MantU6-G3.

Figure 7: Plasmids used for transformation in **Part I**. pMS8-MantU6-G1 (a) and pMS8-MantU6-G3 (b) plasmids containing the assumed putative U6 promoter from *M. antarcticus* and the sequence for Cas9, optimised for *U. maydis*. (a) contains gBlock1 with a tracrRNA-crRNA duplex with a tracrRNA sequence from [5] upstream the U6 promoter, (b) contains gBlock3 with an optimised tracrRNA sequence.

However, as the initial attempts of transforming pMS8-gBlock1 and pMS8-gBlock3 into *M. antarcticus* did not yield any disruptions of the Mat1, it was hypothesised that the *U. maydis* U6 promoter, used for promoting the gBlocks, was not recognised by *M. antarcticus*. The U6 promoter from *U. maydis* was therefore substituted, in both pMS8-gBlock1 and pMS8-gBlock3, for a putative U6 promoter from *M. antarcticus*, yielding the plasmids pMS8-MantU6-G1 and pMS8-MantU6-G3, seen in **Figure 7**. It is these two plasmids, that are used for the transformations described in **Section 2**. In addition to one of the two gBlocks and the putative U6 promoter, the most essential elements that each plasmid contains is: an otef-promoter for Cas9 (Po2tef), the sequence encoding the Cas9 protein, codon optimized for *U. maydis*, the gene for carboxin resistance (cbx), an autonomously replicating sequence from *U. maydis* (UARS), the gene for ampicillin resistance (bla) and the origin of replication (ori).

1.3 Real-Time PCR for evaluation of gene expression

The following section is greatly based on [6] and [40].

Real-Time PCR (RT-PCR) offers a way of evaluating on the level of gene expression within a cell. Whereas the amplicons of a regular PCR reaction are analysed after ended amplification, the amplicons of an RT-PCR are analysed during the PCR reaction itself, in real time, and the method is therefore referred to as Real-Time PCR.

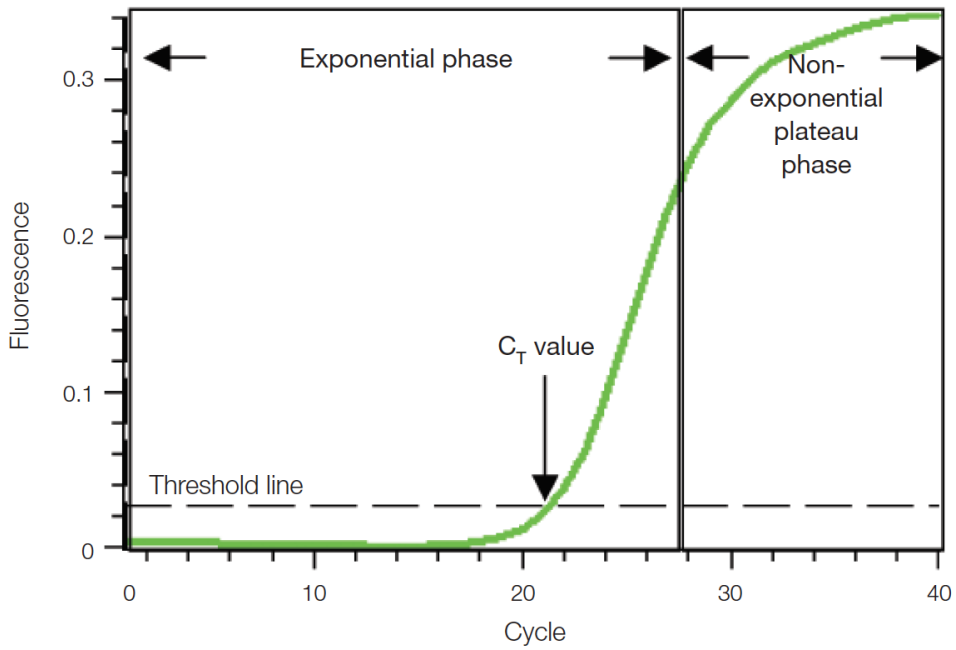


Figure 8: An example plot from an RT-PCR [6]. The number of cycles can be seen on the x-axis, and the corresponding fluorescence is seen on the y-axis.

The measurements that are obtained during an RT-PCR are made possible by the addition of a fluorescent dye, which emits fluorescence when bound to double stranded DNA, that is detected by a RT-PCR machine. The RT-PCR consists of two phases, the exponential phase and the plateau phase. Initially the fluorescence from the dye is undetectable, until a point, called the threshold cycle (C_T), at which it can be detected by the RT-PCR machine. This is illustrated in **Figure 8**. The number of cycles, that it takes for the reaction to reach the C_T, is solely dependent on the amount of template for the PCR reaction initially in the sample, and the C_T value can therefore be used to approximate the amount of template present, and thereby give and impression of the expression level of the gene; if the gene is frequently expressed, the amount of template will be higher, and the C_T value will be low, as a result of this.

As the focus in RT-PCR is to approximate the expression level of genes within a cell, the first step, in preparation for RT-PCR, is to isolate the RNA, as the messenger RNA provides an idea of the expression level of a certain gene. The next step is to carry out a reverse transcription, using a reverse transcriptase, which translates the RNA into cDNA. The cDNA can then subsequently be used as a template for RT-PCR analysis.

1.4 Experimental overview

As the plasmids pMS8-MantU6-G1 and pMS8-MantU6-G1 had already been constructed and a transformation protocol, based on the electroporation method, had been developed in previous work by Daniel Bobkov [41], the initial step of the experimental work, carried out in regards to this thesis, was to transform the previously mentioned plasmids into *M. antarcticus* by electroporation, following the conditions established in the protocol. In addition to transformation by electroporation, a lithium acetate transformation was carried out [42]. During the transformations, the following parameters were tested, in order to optimize them:

- To evaluate on the optimal concentration of antibiotics in the selection medium, an

antibiotic sensitivity test was carried out, in which the sensitivity of *M. antarcticus* towards several different antibiotics at different concentrations was tested.

- YM medium was originally used for the electroporation protocol, however, MEL medium, with glycerol as main carbon source, was also tested for transformation.
- Transformation by electroporation was carried out on day 2, 4, 6 and 9 of fermentation.
- Due to the lack of reproducible results, transformation by electroporation was tested with cells grown in YPD medium for a period of 5 hours [42].

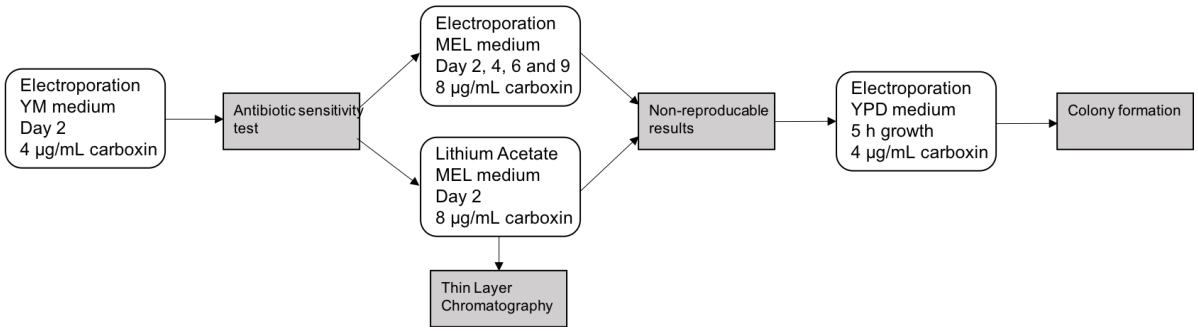


Figure 9: Overview of the experimental work carried out in **Part I** of this thesis.

For the confirmation of transformation, carried out by colony PCR, several primers were used. An overview of the primers can be seen in **Table 1**. The primers in this part were originally developed during previous work on this project [39][41].

Primer name	**Primer sequence**
PMS8_FOR	GCCCCGCTTCAGATCCTGTT
PMS8_REV	GAAAGTCGAGCTCGGTACGG
PMS8_FOR1	CACCCCTGTAGCAGTCTGTCAG
PMS8_REV1	TGAAAGTCGAGCTCGGTACTCG
PMS8Msp_FOR	ATCCATCCGCCCACTTTGC
PMS8-Po2tef_FOR	GAGGGGATCAATTCGACCAA
PMS8-Cas9_REV	TCCTACCGAGTTAGTACCGA
MAT1_FOR	AAGGCGATCTTTGAGGCGTG
MAT1_REV	TTCTCGCTGTCGTCTGACGC
MAT1_FOR2	GAGCTTCAGCAGGGATACCG
MAT1_REV2	CGTTGAACGTGCTAATCCGC

Table 1: Overview of the primers used for the experimental work in **Part I**.

2 Materials and Methods

2.1 Transformation of *Moesziomyces antarcticus* by electroporation and lithium acetate

To transform *M. antarcticus* (PYCC 5048^T), a protocol for electroporation of *M. antarcticus* had been developed [41]. A second transformation was carried out using the lithium acetate transformation [42] and different conditions were investigated for both transformation protocols.

Escherichia coli was used for the propagation of all plasmids used for the experimental work in this thesis. The cells were growth in liquid LB medium (tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L) at 37 ^{o}C and 200 rpm for 16-18 hours before the plasmids were extracted.

2.1.1 Electroporation of *M. antarcticus* cells grown in YM medium

Growth conditions
M. antarcticus cells were inoculated to 10 mL YM medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L) in a 50 mL falcon tube and grown overnight at 28^{o}C and 180 rpm. The following day 1 mL of pre-culture was inoculated to 50 mL YM medium in a 250 mL shake flask and grown for 2 days at 28^{o}C and 180 rpm.

Electroporation
50 mL of cells were harvested by centrifugation for 10 min at 4^{o}C and 4400 rpm and resuspended in 3 mL ice-cold sterile milli-Q water to wash the cells. The washing step was repeated twice, and the cells were resuspended in 250 μL ice-cold 1M sorbitol. 50 μL of the cell suspension was added to a pre-chilled electroporation cuvette along with 8 μg of plasmid and the mixture was vortexed shortly to ensure well mixing. The cells were electroporated at 1.5 kV for 5.1 ms. Immediately after the electroporation, 500 μL of ice-cold 1M sorbitol was added to the cells, and the solution was transferred to a 15 mL falcon tube containing 1 mL glycerol solution (10% w/v). The cells were incubated at 28^{o}C and 180 rpm for 1 hour and 100 μL of the cell suspension was plated onto YM plates containing 4 μg/mL carboxin and incubated at 30^{o}C. Colonies appeared after 2-4 days.

2.1.2 Lithium acetate transformation of *M. antarcticus* cells grown in MEL medium

Growth conditions
M. antarcticus cells were inoculated to 30 mL MEL production medium (yeast extract 1 g/L, $NaNO_3$ 3 g/L, KH_2PO_4 0.3 g/L, $MgSO_4x7H_2O$ 0.3 g/L 12% glycerol (w/v)) in 250 mL erlenmeyer flasks and grown for 2 days for at 28^{o}C and 180 rpm.

Lithium acetate tranformation
50 mL of cells were harvested by centrifugation for 10 min at 4^{o}C and 4400 rpm and resuspended in 4 mL of transformation buffer (40% polyethylene glycerol, 0.2M lithium acetate and 0.1 M dithiothreitol) to wash the cells. The washing step was repeated twice and the cells were resuspended in 100 μL of the transformation buffer. 80 μL of the cell suspension was mixed with 10 μL single stranded DNA (10mg/mL) and 5 μg of plasmid. The mixture was vortexed shortly and incubated for 1 h at 37^{o}C. 200 μL of MEL medium was added to the cell suspension, and 100 μL of cells were spread onto MEL medium plates containing 8 μg/mL carboxin. The plates were incubated at 30^{o}C and colonies started to appear after 2-4 days.

2.1.3 Electroporation of *M. antarcticus* cells grown in YPD medium

Growth conditions
M. antarcticus cells were inoculated to 2 mL of YPD medium (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L) in a 50 mL falcon tube and incubated in a reciprocal shaker at 30 ^{o}C and 180 rpm over night (approximately 16-18 hours). On the following day, 500 μL of the pre-culture was inoculated to 50 mL YPD medium in a 250 mL shake flask and incubated again at 30 ^{o}C and 180 rpm for 5 hours.

Electroporation
50mL of *M. antarcticus* culture was transferred to a falcon tube and centrifuged at 7000 rpm at 20^{o}C for 10 minutes and resuspended in 1 mL 1M sorbitol solution to wash the cells. The washing step was repeated twice before the cells were resuspended in 100 μL 1M sorbitol. 100 μL aliquot of cell suspension was transferred to a pre-chilled electroporation cuvette **(Write company and info)** and 5 μg plasmid was added. The cell suspension was electroporated at 1.5 kV for 5.1 ms and immediately after the electroporation, the cells were diluted in 900 μL YPD medium and transferred to 1 mL YPD medium in a 50mL falcon tube. The cells were recovered at 30^{o} for 0-40hours before plating 100 μL of the cell suspension onto YPD plates containing 4 μg/mL carboxin and incubated at 30^{o}C. After 2-4 days colonies started to appear.

2.1.4 Confirmation of transformation by colony PCR

The confirmation of successful transformation was carried out by colony PCR. From several growing colonies, part of the colony was transferred to an eppendorf tube and mixed with 10 μL 20mM NaOH and dissolved by disrupting the cells using a pipette tip. The solution was heated for 15 minutes at 98^{o}C. The PCR reaction mix was prepared using Dream Taq DNA polymerase and the primers PMS8-FOR and PMS8-REV in 10 μL reaction volumes. The PCR was run as the program in **Table 2** shows and the PCR products were subsequently visualised on a 1% agerose gel.

For each colony PCR, two controls were carried out; a positive control containing DNA from *M. antarticus* and negative control containing water instead of DNA. Additionally, the Mat1 region for each of the selected transformants was amplified using the primers MAT1-FOR and MAT1-REV, following the same directions as stated above.

Step	Temperature (^{o}C)	Time (sec)	
1	95	120	
2	95	30	
3	54	30	
4	72	45	30 total cycles from step 2
5	72	300	

Table 2: PCR program for colony PCR.

2.1.5 Thin Layer Chromatography of transformants

40 transformants were selected at random, inoculated to 2 mL MEL medium in 15 mL falcon tubes and grown for 9 days in a shaking incubator at 28^{o}C and 300 rpm. MELs were extracted from each sample with equal amounts of ethyl acetate and mixed on vortex. The ethyl acetate phase was collected into 1.5 mL eppendorf tubes and evaporated at 80^{o}C. The MELs were

resuspended in 20 μL methanol and 10 μL of the resuspended MEL solution was transferred to the TLC silica plates. The silica plates with loaded samples were placed in a glass chamber containing a mobile phase of chloroform/methanol/MiliQ water in the relation 65:25:4 and left to incubate until the solution had reached the intended travel length (approximately 5 cm from the top edge of the silica plate). To visualize the bands on the plate, it was sprayed with the detection agent containing ethanol/H_2SO_4/H_2O/naphthol in the relation 51:6.5:4:1.5 and subsequently heated to 105^{o}C for 5-10 minutes.

2.2 Effect of medium composition on the morphology of *M. antarcticus*

4 different medium compositions were tested in order to evaluate on the effect of the medium composition on the morphology of *M. antarcticus*. A YM medium with glucose as carbon source, a YM medium with glycerol as carbon source, a MEL production medium with glucose as carbon source and a MEL production medium with glycerol as carbon source were prepared. *M. antarcticus* cells were inoculated to 5 mL of each of the mediums in falcon tubes and grown over night in a reciprocal shaker at 180 rpm and 28^{o}C. 1 mL of cell suspension from each tube was transferred to 50 mL of the specific medium in 250 mL erlenmeyer flasks and cultivated at 180 rpm and 28^{o}C for 3 days. The morphology of the cultures were evaluated in microscope.

2.3 Antibiotic sensitivity test

An antibiotic sensitivity test was carried out, to evaluate the sesitivity of *M. antarcticus* towards specific antibiotics. Phleomycin, hygromycin, carboxin, zeocin, geneticin and nourseothricin were tested in different concentrations and all concentrations were tested with cell suspensions of ODs 2, 4, 6, 8 and 10, respectively. The growth on the plates was evaluated on day 2 and day 4 of incubation at 30^{o}C. YM medium plates were prepared with the antibiotics phleomycin, hygromycin, carboxin and zeocin, and MEL medium plates were prepared with geneticin and nourseothricin, all to yield the concentrations in **Table 3**.

Antibiotic	**Concentrations tested (μg/mL)**
Phleomycin	5, 10, 25
Hygromycin	100, 200, 300, 400, 500
Carboxin	2, 4, 6, 8, 10
Zeocin	25, 50, 100, 200, 400
Geneticin	50, 100, 200, 300, 400
Nourseothricin	100, 200, 300, 400, 500

Table 3: Overview of the tested antibiotics and concentrations.

2.4 RT-PCR primer design

Primers were designed using an online primer design tool provided by NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606INPUT_SEQUENCE=NM_001618.3), and the following considerations were taken into account when designing the primers.

- Primers were designed for the resulting amplicons to be between 75 to 200 bp in length.
- Primers were designed to have a GC content of 50-60%.

- Primers were selected so that the T_m across different primer pairs ranged between 57 ^{o}C to 60 ^{o}C to ensure that the primers would work similarly under the same conditions.
- Two pairs of primers were designed for actin, with one pair flanking an intron, to allow testing for complete DNA degradation during the reverse transcription step.

An overview of the primers designed for RT-PCR can be seen in **Table 4**.

Primer pair	**Primer name**	**Primer sequence (5'→3')**	**Product lenght (DNA, RNA)**
Actin primers	MaAct1FOR1	GTGCGAGTTTTCGCTGTCTC	163bp, 163bp
	MaAct1REV1	TGAACAAGGCTTCGCTGGAT	
Actin primers flanking an intron	MaAct1FOR2	AGCACTGTCGTCATGGAAGC	423bp, 125bp
	MaAct1REV2	TGTGACCGGTTGGAAGGAAG	
Cas9 primers	UmCas9FOR	TCGGACTCGACATCGGTACT	160bp, 160bp
	UmCas9REV	TCAGCGGTTTCTCCTGAGTC	
sgRNA primers	Mat1sgFOR	CCGTACTGGAAGAAACCCAG	91bp, 91bp
	Mat1sgREV	GACTCGGTGCCACTTTTTCA	
Carboxin resistance primers	UmCbxFOR	TCAGCAACGGTCTTCGTACC	176bp, 176bp
	UmCbxREV	CTTGTCAGGGTTCCATCGGT	

Table 4: Overview of primer pairs for qPCR.

2.4.1 Binding of primers

Binding of the primers, designed for RT-PCR, was tested by PCR using gDNA, from *M. antarcticus*, and the plasmids pMS8-MantU6-G1 and pMS8-MantU6-G3. The PCR reactions were prepared using DreamTaq polymerase **(add company name)** and the following settings in **Tabel 5** were used for the reaction program:

Step	**Temperature (^{o}C)**	**Time (sec)**	
1	95	120	
2	95	30	
3	60	30	35 total cycles from step 2

Table 5: PCR program for binding test of qPCR primers.

The PCR products were visualised on a 1% agarose gel.

2.4.2 Total RNA isolation from *Moesziomyces antarcticus*

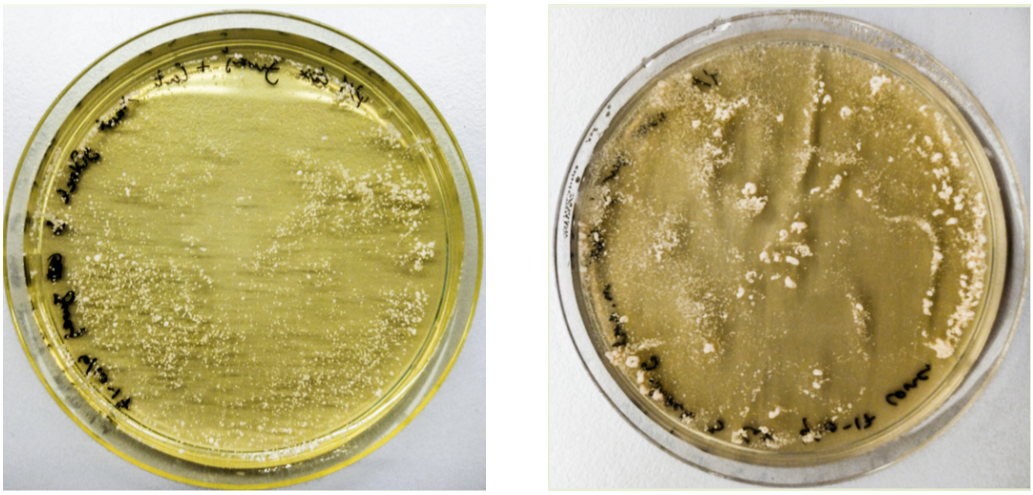
RNA was extracted from *M. antarcticus* with a total RNA isolation kit (AA Biotechnology). Two samples of 20 mg of biomass from *M. antarcticus*, grown for 4 days at 30^{o}C on solid MEL medium, were tested. The kit includes a lysis step in which the cells are lysed, however, the cells in one of the two samples were disrupted in a bead-beater for 30 seconds prior to the lysis step, in order to evaluate on the effectiveness of the lysis step in the kit. 0.8 mL of Fenozol was subsequently added to each sample, and the steps in the isolation protocol, provided by AA Biotechnology, were followed. The extracted RNA was visualised on a 1% agarose gel.

3 Results

3.1 Results of transformation

3.1.1 Electroporation of *M. antarcticus* cells grown in YM medium

In **Figure 10** it can be seen that colonies appear on both the plate containing the transformants and on the control plate making it challenging to distinguish the successful transformants from the background growth.



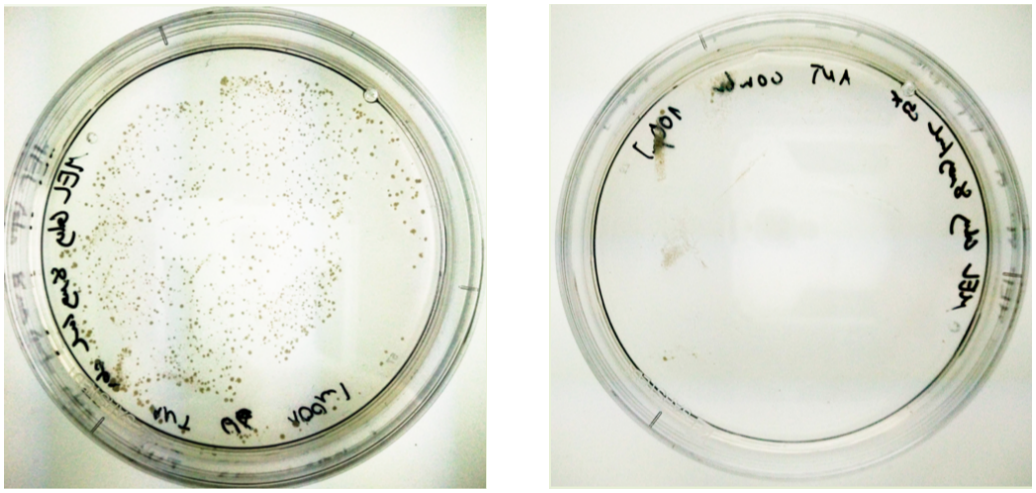
(a) Transformants (b) Control

Figure 10: Results from electroporation of *M. antarcticus* after 4 days of incubation at 30^{o}C on YM medium with 4 μg/mL carboxin. (a) *M. antarcticus* transformed with plasmid DNA. (b) *M. antarcticus* control.

Of the colonies selected for confirmation of transformation by colony PCR, only 1 colony was confirmed to have been successfully transformed with the plasmid.

3.1.2 Lithium acetate transformation of *M. antarcticus* grown in MEL medium

The lithium acetate transformation of *M. antarcticus* resulted in the formation of several colonies on the selection plate after 4 days of incubation at 30^{o}C (**Figure 11a**), while no colonies appeared on the control plate (**Figure 11b**). Approximately 532 colony forming units (CFU) were observed on the plate containing the transformants.



(a) Transformants (b) Control

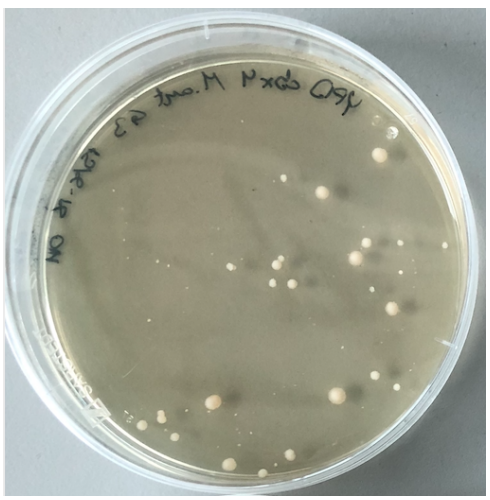
Figure 11: Results from lithium acetate transformation of *M. antarcticus* after 4 days of incubation at 30^{o}C on MEL production medium with 8 μg/mL carboxin. (a) *M. antarcticus* transformed with plasmid DNA. (b) *M. antarcticus* control.

Colonies were selected at random for confirmation of transformation by colony PCR and all selected colonies were confirmed to have been successfully transformed with the plasmid.

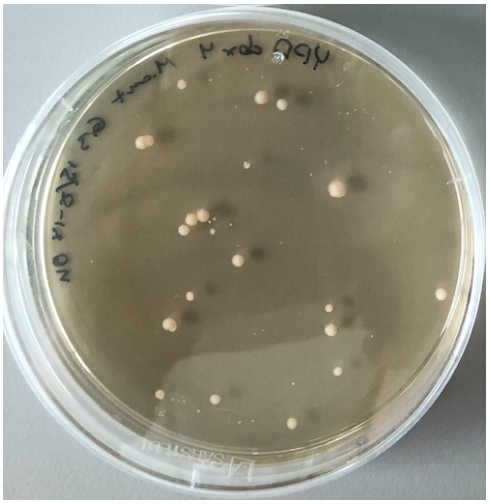
3.1.3 Electroporation of *M. antarcticus* cells grown in YPD medium

The transformations with *M. antarcticus* grown in YPD medium resulted in the formation of colonies, as seen in **Figure 12, 13, 14** and **15**, while no significant growth was observed on the corresponding control plates (not shown).

An average of 22 CFUs were observed on the YPD selection plates in **Figure 12** after 4 days of incubation at 30^{o}C, on plates containing 4 μg/mL carboxin, and with 0 hours of cell recovery after electroporation.



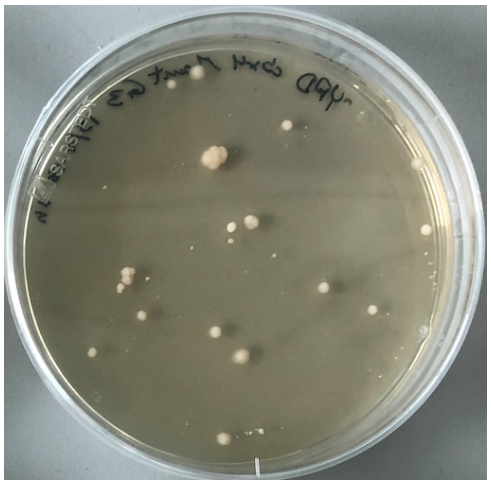
(a) Transformants plate A



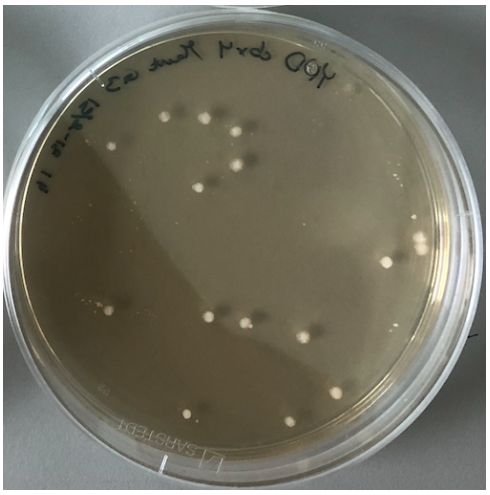
(b) Transformants plate B

Figure 12: Results from electroporation of *M. antarcticus* after 4 days of incubation at 30^{o}C on YPD medium with 4 μg/mL carboxin. 0 hours of cell recovery after electroporation.

Figure 13 shows the formation of colonies of *M. antarcticus* on YPD selection plates containing 4 μg/mL carboxin, after 4 days of incubation at 30^{o}C, with 1 hour of cell recovery after electroporation. An average of 18 CFUs were observed.



(a) Transformants plate A

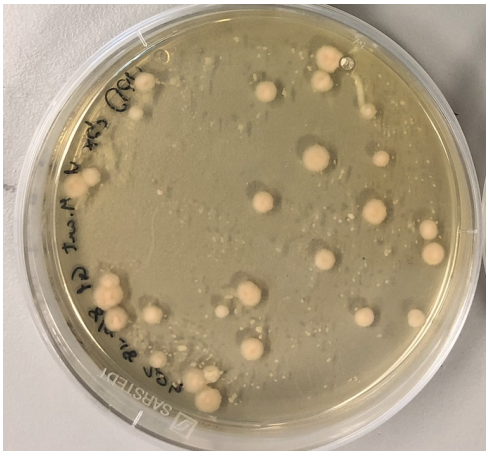


(b) Transformants plate B

Figure 13: Results from electroporation of *M. antarcticus* after 4 days of incubation at 30^{o}C on YPD medium with 4 μg/mL carboxin. 1 hour of cell recovery after electroporation.

The formation of colonies of *M.antarcticus* on YPD selection plates with 4 μg/mL carboxin, after 4 days of incubation at 30^{o}C can be seen in **Figure 14**. The cells were recovered for 20 hours after the electroporation and an average of 24 CFUs were observed.

(a) Transformants plate A

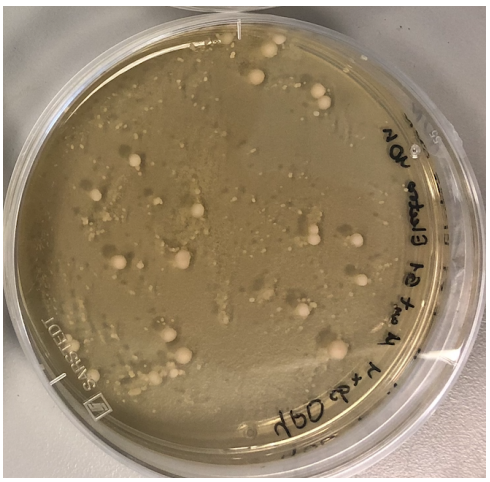


(b) Transformants plate B

Figure 14: Results from electroporation of *M. antarcticus* after 4 days of incubation at 30^{o}C on YPD medium with 4 μg/mL carboxin. 20 hours of cell recovery after electroporation.

In **Figure 15** the formation of colonies of *M. antarcticus*, after 4 days of incubation at 30^{o}C on YPD selection plates with 4 μg/mL carboxin, can be seen. The Cells were recovered for 40 hours at 30^{o}C before plating, and an average of 21 CFUs were be seen.

(a) Transformants plate A

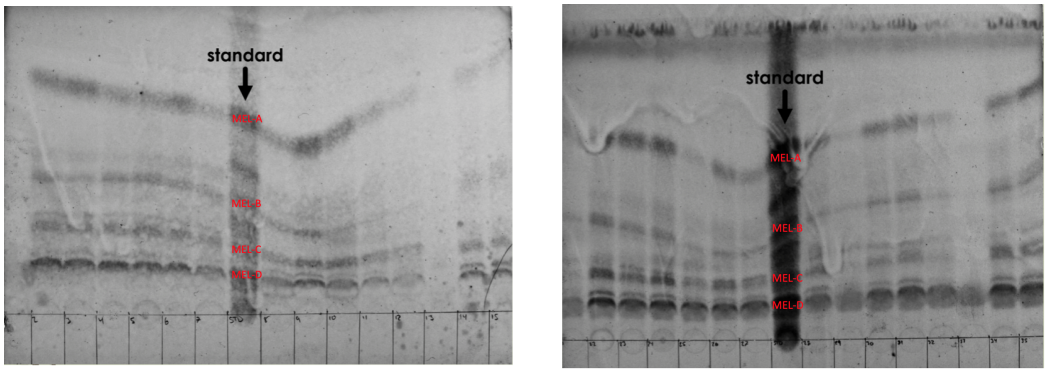


(b) Transformants plate B

Figure 15: Results from electroporation of *M. antarcticus* after 4 days of incubation at 30^{o}C on YPD medium with 4 μg/mL carboxin. 40 hours of cell recovery after electroporation.

3.1.4 Thin Layer Chromatography of transformants

The results from TLC of samples from fermentation with transformed colonies, from the transformation described in **Part I section 2.1.2**, can be seen in **Figure 16**. The standard can be seen in the middle of each of the images **16a** and **16b**. By comparing each of the samples from the transformed colonies to the standard, it can be seen, that there is no observable change in the profile of the excreted MELs.



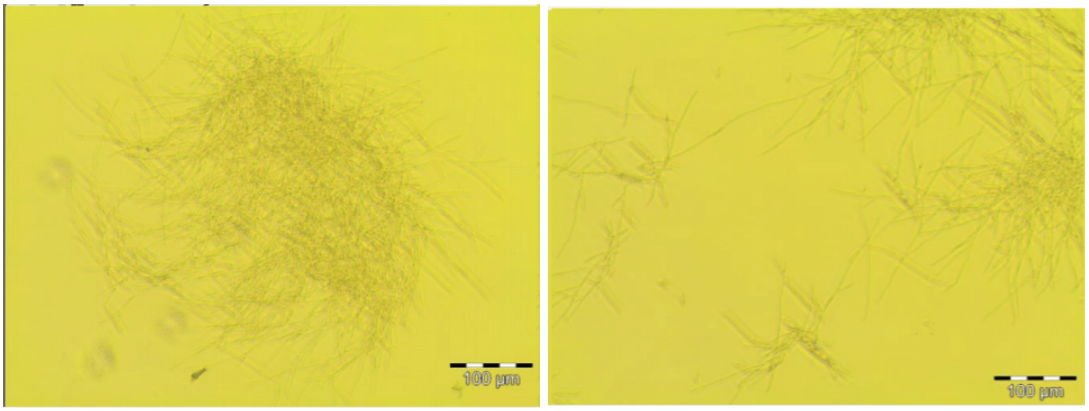
(a) TLC of *M. antarcticus* colonies 1-15

(b) TLC of *M. antarcticus* colonies 21-35

Figure 16: Results from Thin Layer Chromatography of *M. antarcticus* colonies, after 9 days of fermentation in MEL medium.

3.2 Effect of medium composition on the morphology of *M. antarcticus*

In the figures **17a** and **17b** the growth of *M. antarcticus* in YM medium with glucose as carbon source and in YM medium with glycerol as carbon source, can be seen. It can be observed, that the tendency of the growth of *M. antarcticus* is mostly filamentous when growing in YM medium, regardless of the carbon source.

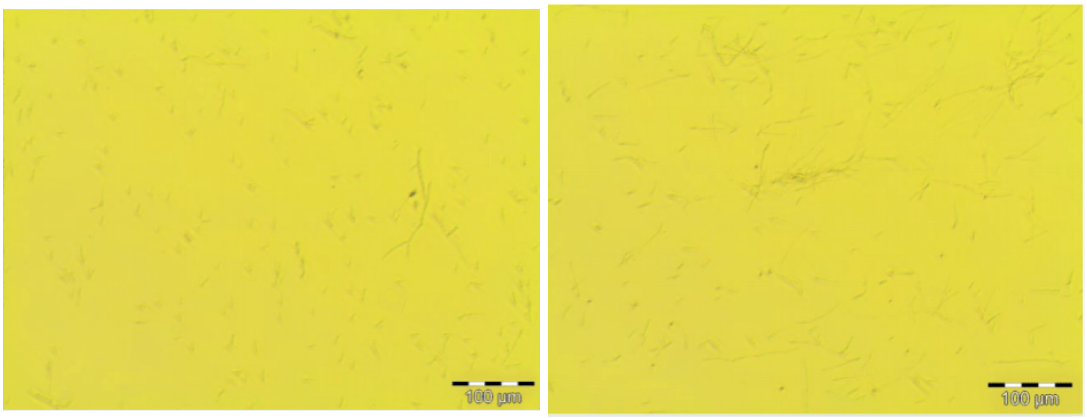


(a) *M. antarcticus* grown in YM glucose

(b) *M. antarcticus* grown in YM glycerol

Figure 17: Morphology of *M. antarcticus* grown in (a) YM medium with glucose as carbon source and (b) YM medium with glycerol as carbon source.

Figure 18 shows the nature of the growth of *M. antarcticus* in MEL medium with either glucose as the carbon source (**Figure 18a**) or glycerol as carbon source (**Figure 18b**). It can be seen that the morphology of *M. antarcticus*, when growing in MEL medium, is yeast like with very few filaments, regardless of the carbon source.



(a) *M. antarcticus* grown in MEL glucose (b) *M. antarcticus* grown in MEL glycerol

Figure 18: Morphology of *M. antarcticus* grown in (a) MEL medium with glucose as carbon source and (b) MEL medium with glycerol as carbon source.

3.3 Results from antibiotic sensitivity test

The growth observed on the plates was defined as:
xxxx = Abundant growth
xxx = Less than abundant growth
xx = Moderate growth
x = Minimal growth
0 = No observed growth

Carboxin
Table 6 shows the variation in growth of *M. antarcticus* after 4 days of incubation at 30 ^{o}C, depending on the correlation between OD and the carboxin concentration.

OD	**2 μg/mL**	**4 μg/mL**	**6μg/mL**	**8μg/mL**	**10μg/mL**
10	xxx	xx	xx	xx	x
8	xxx	xx	xx	x	x
6	xxx	x	x	x	0
4	xxx	x	0	0	0
2	xx	0	0	0	0

Table 6: *M. antarcticus* sensitivity test results for carboxin.

It can be seen from the results of the carboxin sensitivity test in **Table 6** that growth was observed at all ODs for a carboxin concentration of 2 μg/mL and for all carboxin concentrations for ODs of 8 or higher. To completely eliminate growth, a concentration of carboxin higher than 6 μg/mL for ODs of 4 or lower was necessary. An OD of 2 at a carboxin concentration of 4 also resulted in no growth. To avoid growth at concentrations of OD 8 or more, a carboxin concentration higher that 10 μg/mL is needed.

Nourseothricin
Table 7 shows the results from the antibiotic sensitivity test for nourseothricin on day 4 at 30 ^{o}C.

OD	100 μg/mL	200 μg/mL	300 μg/mL	400 μg/mL	500 μg/mL
10	xxx	xx	x	0	0
8	xxx	xx	x	0	0
6	xxx	x	x	0	0
4	xx	x	0	0	0
2	xx	0	0	0	0

Table 7: *M. antarcticus* sensitivity test results for nourseothricin.

The results in **Table 7** show that growth was observed for all ODs at an antibiotic concentration of 100 μ/mL. To eliminate growth on the plates, an antibiotic concentration of 300 μg/mL or higher should be used in combination with ODs lower than 6.

Phleomycin
The results from the antibiotic sensitivity test for phleomycin on day 4 at 30 ^{o}C can be seen in **Table 8**.

OD	5 μg/mL	10 μg/mL	25 μg/mL
10	xxxx	xxx	x
8	xxxx	xx	x
6	xxx	x	x
4	xxx	0	0
2	xx	0	0

Table 8: *M. antarcticus* sensitivity test results for phleomycin.

From the results of the sensitivity test with phleomycin, it can be seen that growth was observed for all ODs at a phleomycin concentration 5 μg/mL. In order to avoid growth completely, a concentration of phleomycin of 10 μg/mL or more was needed for ODs of 4 and lower.

Hygromycin
The results from the sensitivity test for hygromycin on day 4 at 30 ^{o}C can be seen in **Table 9**.

OD	100 μg/mL	200 μg/mL	300 μg/mL	400 μg/mL
10	x	x	x	x
8	x	x	x	x
6	0	0	0	0
4	0	0	0	0
2	0	0	0	0

Table 9: *M. antarcticus* sensitivity test results for hygromycin.

It can be seen from the results in **Table 9** that minimal growth is observed at all concentration of hygromycin with an OD of 10. To eliminate growth completely, when working with hygromycin, it can be seen, that an OD lower than 8 is essential for all hygromycin concentrations tested in this case.

Zeocin
The results from the sesitivity yest for zeocin after 4 days at 30 ^{o}C can be seen in **Table 10**.

OD	25 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL	400 μg/mL
10	xxxx	xxxx	xxx	xxx	xx
8	xxxx	xxxx	xxx	xxx	x
6	xxxx	xxxx	xxx	xx	x
4	xxxx	xxxx	xxx	x	0
2	xxxx	xxx	xx	0	0

Table 10: *M. antarcticus* sensitivity test results for Zeocin.

As it can be seen in **Table 10** the sensitivity test for zeocin shows that growth is observed for all antibiotic concentrations when the OD is 6 or higher and for all ODs when the zeocin concentration is 100 μg/mL or lower.

Geneticin
The results of the antibiotic sensitivity for geneticin on day 4 ant 30 ^{o}C can be seen in **Table 11**.

OD	50 μg/mL	100 μg/mL	200 μg/mL	300 μg/mL	400 μg/mL
10	xxxx	xxx	xxx	x	x
8	xxxx	xxx	xx	0	x
6	xxxx	xxx	x	0	0
4	xxx	xxx	0	0	0
2	xxx	xx	0	0	0

Table 11: *M. antarcticus* sensitivity test results for geneticin.

The results in **Table 11** shows that growth is observed at all antibiotic concentrations with an OD of 10. To efficiently reduce background growth, either an antibiotic concentration of more than 400 μg/mL or a combination of antibiotic concentration of 200 μg/mL and an OD lower than 6 must be considered.

Pictures of growth on the YM plates from day 4 as well as a schematic overview of all results from the sensitivity test from day 2 and day 4 can be found in **Appendix A.3 Figure 32 and 33**.

3.4 Results from RT-PCR

3.4.1 Test of primers for RT-PCR

The results from the test of the primers for RT-PCR can be seen in **Figure 19**. A 100 base pair ladder was used for comparison. Sample 1 shows the amplicons from genomic DNA, extracted from *M. antarcticus*, with the primer pair for MaAct1 1. Sample 2, 3 and 4 show the amplifications of pMS8-MantU6-G1 with the primer pairs Mat1sg, UmCas9 and UmCbx, respectively. Sample 5, 6 and 7 show the amplifications of pMS8-MantU6-G3 with the primer pairs Mat1sg, UmCas9 and UmCbx, respectively.

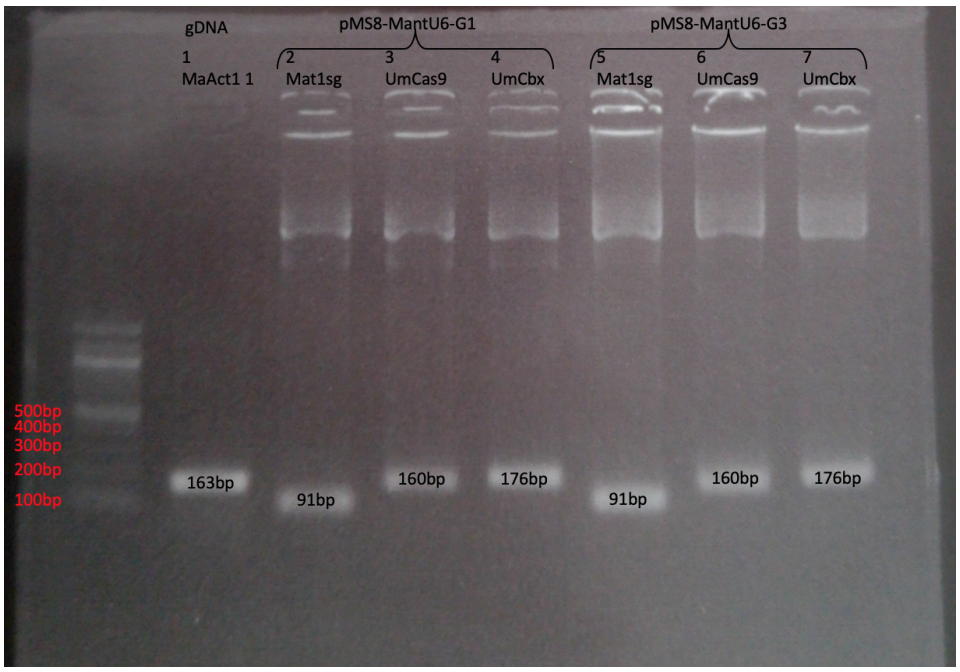


Figure 19: Results obtained from gel electrophoresis of PCR products from the test for bindng of RT-PCR primers.

It can be seen from **Figure 19** that all primer pairs generate amplicons, with band sizes of the expected length, and that no side products are obtained, resulting from secondary structures.

3.4.2 Total RNA isolation

In **Figure 20**, the results from gel electrophoresis of isolated total RNA can be seen. Sample 1 contains the product from RNA extraction with beads, followed by cell lysis and sample 2 contains the RNA product from extraction only with cell lysis. Two clear bands can be seen from the product of sample 1, which are the 28s rRNA and 18s rRNA. No RNA appears to have been extracted from sample 2.

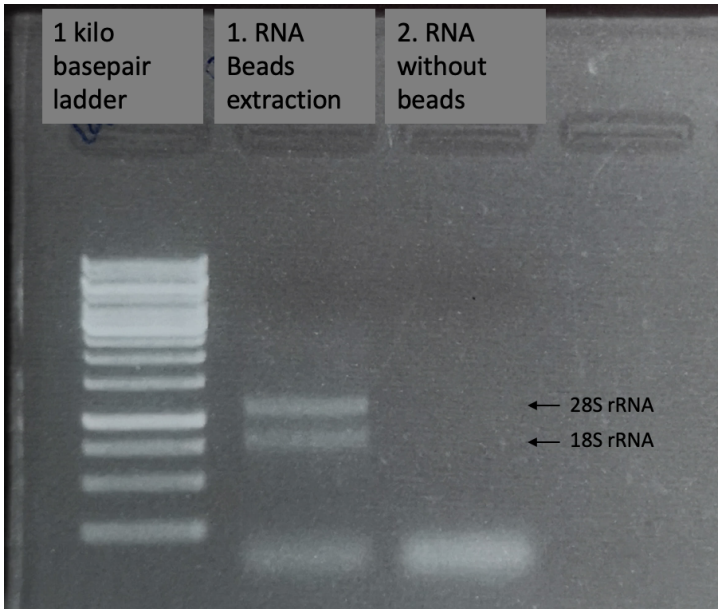


Figure 20: Image of gel from gel electrophoresis of the products from total RNA isolation. For sample 1, two bands of ribosomal RNA can be seen.

4 Discussion

4.1 Transformation

In the initial attempts of transforming the plasmids into *M. antarcticus* by following the transformation protocol developed by Daniel Bobkov [41], only one transformant was obtained, and a significant amount of background growth was observed on the plate, making it difficult to select for transformants. As a result of this, it was decided to carry out an antibiotic sensitivity test for *M. antarcticus*, against several antibiotics including carboxin and nourseothricin among others. The sensitivity test against carboxin showed that *M. antarcticus* can grow on plates with carboxin concentrations up to 10 μg/mL, when the OD is 8 or higher, which explains the high amount of background growth. The problem was solved by increasing the carboxin concentration, of the selection plates, to 8 μg/mL, and in other cases by working with lower ODs.

As the protocol for transformation by electroporation did not yield transformants, a lithium acetate transformation was carried out, which resulted in 532 CFUs; a transformation efficiency of more than 100 CFUs per 1 μg of plasmid DNA, which corresponds well with the results reported by Yarimizu et al. [42]. However, the results obtained in this transformation could not be reproduced, and it was decided to apply the electroporation protocol, described by Yarimizu et al., which resulted in the formation of about 21 CFUs per 5 μg of plasmid DNA. This transformation efficiency is lower than that reported by Yarimizu et al. [42] of 96 CFUs per 5 μg of plasmid DNA. However, Yarimizu et al. are carrying out transformation with a DNA fragment rather than a plasmid, and the difference in size could therefore cause a variation in the efficiency of transfer of DNA across the cell membrane during electroporation.

Due to the more yeast like morphology, which was exhibited when *M. antarcticus* was grown in MEL medium compared to when grown in YM medium, it was decided to continue with MEL medium, after the initial attempts of transformation. However, when performing electroporation as described by Yarimizu et al., the MEL medium was substituted for YPD medium. It is possible that a transformation with cells grown on MEL or YM medium, but otherwise following the same procedure as that used for transformation with YPD medium, could have yielded similar results, with regards to the number of successful transformants. This theory could easily be confirmed or disproved in the lab.

4.2 CRISPR-Cas9 system

From the results of several attempts of transforming the plasmids pMS8-MantU6-G1 and pMS8-MantU6-G3 into *M. antarcticus*, it could be seen that no successful disruptions of the targeted gene, Mat1, occurred. A reason for the unsuccessful implementation of the CRISPR-Cas9 system, could be the efficiency of the sgRNA. The sgRNA was designed and selected by Cristiano Di Pietro [39], using a build-in design tool in Benchling (https://www.benchling.com), based on this particular crRNA having the highest on-target score of 79.1%, among the possible crRNAs for targeting Mat1. However, when analysing the Mat1 genetic sequence for possible crRNAs, using the sgRNA design tool provided by IDT Integrated DNA Technologies, the crRNA, used for the CRISPR-Cas9 system in this thesis, has an on-target score of 65%, while there are several other suggestions for crRNAs with on-target scores up to 96%. The uncertainty of the effectiveness of the selected crRNA, based on the different on-target analysis carried out by the two sgRNA design tools, could indicate that the efficiency of the crRNA used for the experimental work in this thesis, is not sufficient, and as a result of this, no disruption of Mat1 was observed. Furthermore, the fact that two independent

sgRNA design tool score the same sequence with different on-target scores, may indicate that more research into the analysis and design of crRNAs is needed, in order to design sgRNA in an optimal manor.

The expression of Cas9 is essential to the functionality of the CRISPR-Cas9 system. It is the otef-promoter, a modified tef-promoter for *U maydis* [43], that is responsible for the expression of Cas9 in pMS8-MantU6-G1 and pMS8-MantU6-G3. Although this promoter works for expression of Cas9 in *U. maydis* [5], there is a possibility, that in spite of the close genetic relationship between *U. maydis* and *M. antarcticus*, the otef-promoter is not recognised in *M. antarcticus*. The second essential component of the CRISPR-Cas9 complex is the sgRNA. In pMS8-MantU6-G1 and pMS8-MantU6-G3 the original U6 promoter, used for promoting the sgRNA in *U. maydis* [5], was substituted for a putitive U6 promoter from *M. antarcticus*. As the identification of the putative U6 promoter was performed by in silico comparison of the *U. maydis* U6 gene and the *M. antarcticus* U6 gene, it is possible that an error has occurred in the selection of the promoter sequence, which could result in the promoter not functioning properly. The CRISPR-Cas9 system, used for targeting Mat1 in this project, was originally designed by Schuster et al. [5], and the Cas9 was codon optimised for *U. maydis*. Although two microorganisms are closely related such as *U. maydis* and *M. antarcticus*, the codon usage of each microorganism might differ significantly from the other. No analysis of the codon usage of the two microorganisms was carried out in connection to this thesis, however, it is possible that the codon usage by *M. antarcticus* differs from *U. maydis*, to such an extend, that Cas9 cannot be properly transcribed by *M. antarcticus*, which would result in a defect CRISPR-Cas9 system.

An RT-PCR assay was planned as a part of the experimental work, to be carried out in this thesis, through which the expression level of the sgRNA, the Cas9 protein and the carboxin resistance would have been evaluated. The RT-PCR assay might have provided crucial information about which of these previously mentioned problems, were the cause of the lack of an efficient CRISPR-Cas9 system, if any of them. Further investigation into the expression level of the CRISPR-Cas9 components is essential to the continued study of application the CRISPR-Cas9 system for gene disruption in Mat1.

4.3 Conclusion

Based on the transformations carried out in the experimental work of **Part I**, it can be said that the electroporation protocol, developed by Yarimizu et al., is the most efficient, of the protocols tested, in the experimental work of this thesis, for transforming *M. antarcticus*. It can be concluded that *M. antarcticus* is highly sensitive to several of the antibiotics, that were tested. Carboxin, nourseothricin and hygromycin are most efficient as selection markers, inhibiting most growth, under the concentrations and ODs tested. Although several transformants were obtained, it was confirmed by sequencing and TLC assay, that the intended disruption of Mat1 had not occurred in any on the tested transformants. Therefore, it can be concluded that the CRISPR-Cas9 system, tested for disruption of Mat1, is not functional in *M. antarcticus*, as it is currently designed. Further efforts should be made, to identify which component(s) of the CRISPR-Cas9 system is/are currently not functional. This could be done by evaluating on the expression of these through RT-PCR.

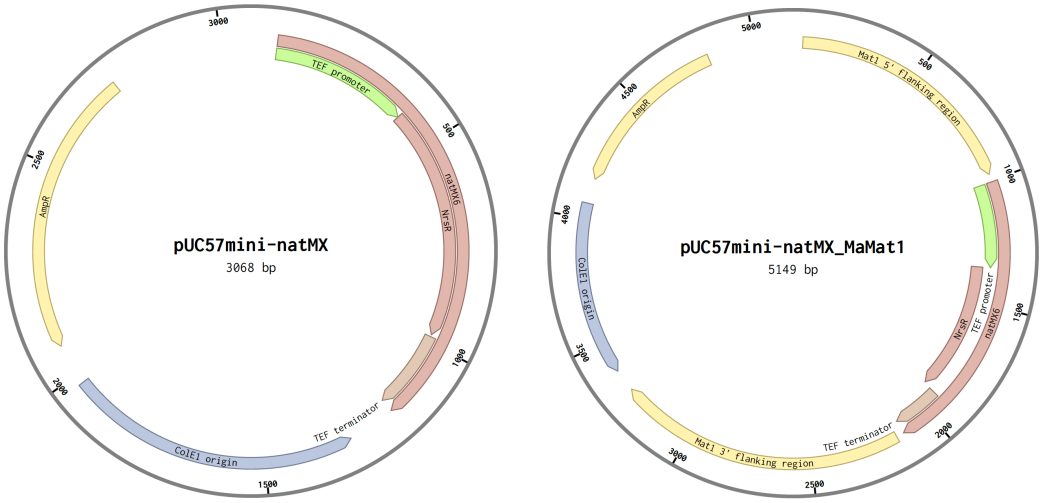
Part II

Site Directed Disruption of Mat1 by Homologous Recombination

1 Introduction

The nourseothricin resistance gene (natMX) has previously been demonstrated to be useful as a selection marker for site directed gene replacement by homologous recombination in *M. antarcticus* [42]. Therefore, a strategy for gene disruption was developed, in which knock-out cassettes embedding the nourseothricin resistance gene, a nourseothricin acetyltransferase from *Streptomyces noursei*, were constructed to target the genes Mat1, Mac1, Mac2 and Emt1.

To target Mat1, the plasmid pUC57mini-natMX-MaMat1 (**Figure 21b**) was designed in silico in benchling, by adding a flanking region both downstream and upstream the natMX gene in the pUCmini-natMX plasmid in **Figure 21a**. Both regions were homologous to the downstream and upstream regions surrounding the Mat1 gene of *M. antarcticus*. For efficient HR the flanking regions were designed to each be a minimum of 900 base pairs in lengt.



(a) pUC57mini-natMX.

(b) pUC57mini-natMX-MaMat1

Figure 21: Plasmids used for transformation in **Part II**. (a) pUC57mini plasmid containing natMX gene from *Streptomyces noursei*. (b) pUC57mini-natMX containing a 5' and a 3' flaking region from *Moesziomyces antarcticus* surrounding the natMX gene.

pUC57mini-natMX-MaMat1 contains an origin of replication for *Escherichia coli* and ampicillin resistance, both necessary elements for replication in *E. coli*. The plasmid also contains the knock-out cassettes, designed to target Mat1 in *M. antarcticus*. However, the plasmid does not contain an autonomously replicating sequence, which means that the plasmid itself cannot be replicated within the *M. antarcticus* cell upon transformation. Due to this, any colonies that appear on the selection plates, following the electroporation, should have the knock-out cassette integrated into the DNA.

1.1 Homologous recombination

Homologous recombination plays a key role, when it comes to ensuring genetic diversity and proper segregation of homologous chromosome pairs during the initial step of meiosis and is initiated by a double stranded break in the DNA. This occurs, as genetic information can be exchanged between two DNA molecules, as illustrated in **Figure 22**, with highest efficiency between DNA molecules of similar sequences. Due to the nature of HR, it can also function as a repair method for the cell during DNA damage, caused by e.g. ionizing radiation and breaks in the replication forks during DNA replication [44][7].

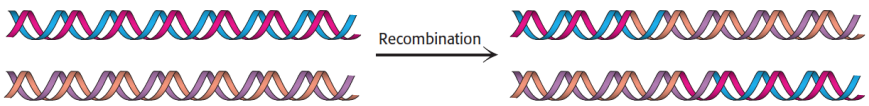


Figure 22: Exchange of genetic information between two DNA molecules through HR [7].

HR is initiated by an endonuclease making a cut to the DNA strand inside the genome, which allows a single stranded binding protein and the enzyme RecA to bind to the single stranded region. This constellation allows for base pairing with a highly similar donor strand and induces a swab of genetic code between the two strands [45]. The mechanism of HR is used, when creating knock-out strains, in which a specific gene is knocked out, by inserting a donor sequence into the targeted gene, or substituting it completely with the donor sequence. This type of gene knock-out can be carried out by transforming a plasmid or a knock-out cassette, containing a donor sequence, e.g. the genetic sequence of an antibiotic resistance gene, flanked by regions that homologous to the regions surrounding the targeted gene, into the microorganism of interest [8]. When HR takes place, it will result in the targeted gene, embedded within the homologous regions, being replaced by the donor sequence, as illustrated in **Figure 23**.

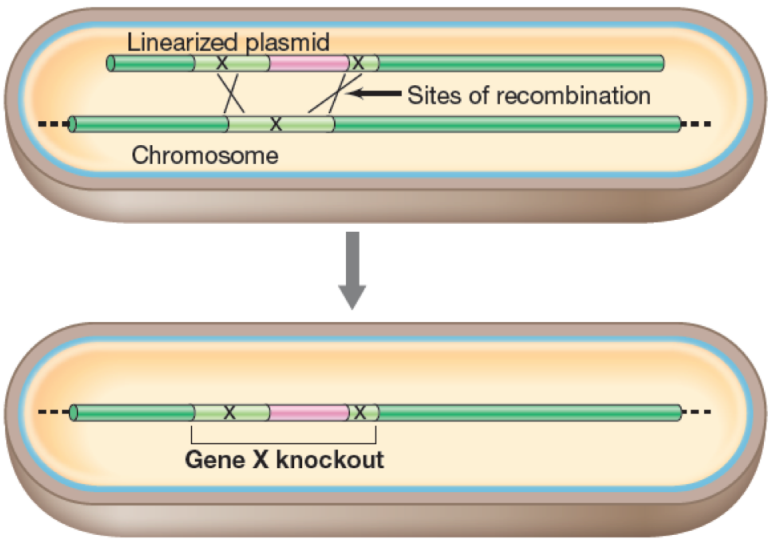


Figure 23: Gene knock-out by homologous recombination between a donor template and the targeted DNA sequence [8].

1.2 Disruption of genes in the MEL biosynthesis

As previously mentioned in section 1.2.1, a knock-out of the Mat1 gene in *M. antarcticus*, would result in the excretion of the deacetylated type of MEL; MEL-D. The main advantage of accumulating a product, consisting of only one type of MEL, is to control the chemical properties of the product and thereby the potential of commercialising the product for a specific use [11]. There are several other genes involved in the MEL biosynthesis, that could as well be targeted in order to tailor the final product of MELs to specific properties and use. The second step of MEL biosynthesis is carried out by the acyltransferases Mac1 and Mac2. These two enzymes are responsible for the acylation of mannose in the mannosylerythritol complex, and a knock-out of either of these genes, or both, could therefore potentially result in the addition of a different chain length of fatty acids, or no addition of fatty acids at all. As Emt1 catalyses the first step in the synthesis of MELs, a knock-out of this gene would theoretically render it impossible for successfully transformed organisms to produce MELs.

1.3 Primer design and gene targeting strategy

pUC57mini-natMX-MaMat1 was designed in such a way, that the homologous regions for Mat1 could easily be substituted with others, in order to target other genes. For each of the genes Mac1, Mac2 and Emt1, primers were designed to generate fragments homologous to the targeted gene with a 30 pb overhang in both the 5' and 3' ends, which is sufficient for Gibson assembly to successfully assemble the fragments [46]. The 30 bp overhangs were homologous to the sequence of pUC57mini-natMX-MaMat1, in the location that the fragments should be inserted, in order to generate several knock-out cassettes, with nourseothricin as selection marker, through Gibson assembly [47]. In order to provide an alternative way of assembling the DNA fragments, each overhang was designed to incorporate a restriction site at each end of the resulting fragments. By constructing the fragments by PCR and subsequently digesting them and/or the plasmid with the appropriate restriction enzyme(s), would allow for ligation of the fragments, and assembly of the knock-out cassettes [48]. An overview of the primers can be seen in **Table 12**.

1.4 Gene disruption by split marker approach

When disrupting a gene by transformation with a disruption cassette, the selection of true transformants, with the HR occurring in the targeted locus, can be somewhat troublesome [49]. Therefore, to optimize the disruption of Mat1 by HR with nourseothricin resistance, an approach using split markers can be tested. The split marker approach is a PCR based method which involves the construction of two separate fragments, each embedding either a 5' or 3' flanking region of the targeted gene, as well as half of the gene intended to replace the targeted gene, as seen in **Figure 24**. In this case, the two fragments together comprise the disruption cassette from pUC57mini-natMX-MaMat1. The primers for constructing the split markers through PCR, are designed in such a way that each of the resulting two constructs have a 40 bp overhang, complementary to the other. The two fragments resulting from the PCR are subsequently transformed into the microorganism simultaneously [50]. As each fragment embeds half of the selection marker, it can be expected, that the transformants will not develop antibiotic resistance, except in the case, that the fragments are successfully incorporated together into the genome, which is facilitated by the 40 bp overhangs. Due to the flanking regions of the split markers, which are complementary to the regions surrounding the targeted gene, the combination of the two fragments and insertion into the genome, is only likely to happen, if the gene is successfully targeted. This approach therefore allows for a more efficient

selection of successful transformants, as it can be expected that for colonies growing, the targeted gene has successfully been replaced by the selection marker.

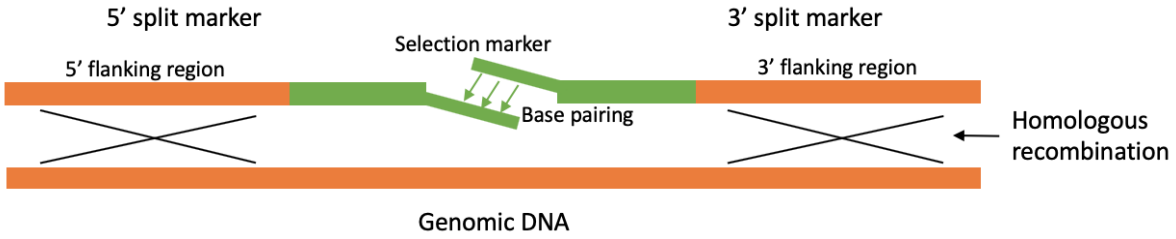


Figure 24: Illustration of gene disruption, by HR, using split markers. The two split markers combine into a complete disruption cassette, by base pairing between the complimentary 40 bp overhangs.

1.5 Experimental overview

In order to disrupt the Mat1 gene in *M. antarcticus* by substituting the Mat1 gene with nourseothricin resistance by HR, three separate transformations were carried out:

- Transformation with pUC57mini-natMX-MaMat1
- Transformation with knock-out cassette
- Transformation with split markers

For each of the three transformations, the following selection for transformants was performed on YPD selection plates with three different concentration of nourseothricin; 50 μg/mL, 100 μg/mL and 200 μg/mL. This was done in order to evaluate on the concentration of nourseothricin needed for obtaining and selecting transformants.

In order to target several genes involved in the MEL biosynthesis in *M. antarcticus*, the primers in **Table 12** were designed, as well as primers for construction of split markers from the Mat1 knock-out cassette in pUC57mini-natMX-MaMat1.

Primer name	Primer sequence	Restriction site
NatMX_Mat1-5'FOR	tgtcgcgaagtcatcaccat	
NatMX_Mat1-5'REV	ggatggggttcaccctctg	
NatMX_Mat1-3'FOR	tctggctggaggtcaccaa	
NatMX_Mat1-3'REV	agatgggcgatatcagatccac	
5MaEMT1_FOR	**gatggtgacactatagaacgcggccgccag**ccggtttcgacactatcggt	NotI
5MaEMT1_REV	**gtcgtcgacctgcagcgtacgaagcttcag**cgtacgatgcgatgaacgtg	HindIII
3MaEMT1_FOR	**gaaaacgagctcgaattcatcgatgatatc**aaactgctgtcgctctgtca	EcoRI
3MaEMT1_REV	**gcggccgcataggccactagtggatctgat**ctttcgcgcttcagtatgcc	SpeI
5MaMAC1_FOR	**gatggtgacactatagaacgcggccgccag**ccgaactgtgagcatgtccg	NotI
5MaMAC1_REV	**gtcgtcgacctgcagcgtacgaagcttcag**aatgggcggtcagaacagg	HindIII
3MaMAC1_FOR	**gaaaacgagctcgaattcatcgatgatatc**gaaaaagatgtgcgagagccg	EcoRI
3MaMAC1_REV	**gcggccgcataggccactagtggatctgat**aggttgaaagatgcggtgga	SpeI
5MaMAC2_FOR	**gatggtgacactatagaacgcggccgccag**cctgtggctcacccagattt	NotI
5MaMAC2_REV	**gtcgtcgacctgcagcgtacgaagcttcag**tggagcgggcgtatctgtaa	HindIII
3MaMAC2_FOR	**gaaaacgagctcgaattcatcgatgatatc**gccgaactgtgagcatgtcc	EcoRI
3MaMAC2_REV	**gcggccgcataggccactagtggatctgat**ttgaatgggcggtcagaaca	SpeI

Table 12: Overview of the primers used for the experimental work in **Part II**. The 30 bp primer overhangs are marked in bold writing.

2 Materials and Methods

2.1 Transformation of *Moesziomyces antarcticus* with pUC57mini-natMX-MaMat1

Growth contitions
M. antarcticus cells were inoculated to 2 mL of YPD medium in a 50 mL falcon tube and incubated in a reciprocal shaker at 30^{o}C and 180 rpm over night. On the following day, 500 μL of the pre-culture was inoculated to 50 mL YPD medium in a 250 mL shake flask and incubated again at 30^{o}C and 180 rpm for 5 hours.

The plasmid pUC57mini-natMX-MaMat1 was designed and ordered from IDT Integrated DNA Technologies.

Electroporation
50mL of *M. antarcticus* culture was transferred to a falcon tube and centrifuged at 7000 rpm at 20^{o}C for 10 minutes and resuspended in 1 mL 1M sorbitol solution to wash the cells. The washing step was repeated twice before the cells were resuspended in 100 μL 1M sorbitol. 100 μL aliquot of cell suspension was transferred to a pre-chilled electroporation cuvette and 4 μg plasmid was added. The cell suspension was electroporated at 1.5 kV for 5.1 ms and immediately after the electroporation, the cells were diluted in 900 μL YPD medium and transferred to 1 mL YPD medium in a 50mL falcon tube.

Selection
24 hours after electroporation, 100 μL of the cell suspension was spread onto YPD selection plates containing either 50 μg/mL, 100 μg/mL or 200 μg/mL nourseothricin and incubated at 30^{o}C. Colonies started to appear after 2-4 days.

2.2 Transformation of *Moesziomyces antarcticus* with disruption cassette

To obtain the fragment with the disruption cassette, pUC57mini-natMX-MaMat1 was digested with the restriction enzyme NotI (New England BioLabs).

Growth conditions
M. antarcticus cells were inoculated to 2 mL of YPD medium in a 50 mL falcon tube and incubated in a reciprocal shaker at 30^{o}C and 180 rpm over night. On the following day, 500 μL of the pre-culture was inoculated to 50 mL YPD medium in a 250 mL shake flask and incubated again at 30^{o}C and 180 rpm for 5 hours.

Electroporation
50mL of *M. antarcticus* culture was transferred to a falcon tube and centrifuged at 7000 rpm at 20^{o}C for 10 minutes and resuspended in 1 mL 1M sorbitol solution to wash the cells. The washing step was repeated twice before the cells were resuspended in 100 μL 1M sorbitol. 100 μL aliquot of cell suspension was transferred to a pre-chilled electroporation cuvette and 800 ng of the fragment was added. The cell suspension was electroporated at 1.5 kV for 5.1 ms and immediately after the electroporation, the cells were diluted in 900 μL YPD medium and transferred to 1 mL YPD medium in a 50mL falcon tube.

Selection
100 μL of the cells were spread onto YPD selection plates containing either 50 μg/mL, 100 μg/mL or 200 μg/mL nourseothricin and incubated for 2-4 days at 30^{o}C, after which colonies started to appear.

2.3 Transformation of *Moesziomyces antarcticus* with split markers

Construction of split markers
The primers NatMX_Mat1-5'FOR, NatMX_Mat1-5'REV, NatMX_Mat1-3'FOR and NatMX_Mat1-3'REV were used to construct split markers, from pUC57mini-natMX-MaMat1, by PCR using Q5 polymerase (New England BioLabs). The reaction was set up according to **Table 13**.

Step	Temperature (^{o}C)	Time (sec)	
1	98	30	
2	98	10	
3	54	30	
4	72	60	35 total cycles from step 2
5	72	120	

Table 13: PCR program for construction of split markers.

Growth contitions
M. antarcticus cells were inoculated to 2 mL of YPD medium in a 50 mL falcon tube and incubated in a reciprocal shaker at 30^{o}C and 180 rpm over night. On the following day, 500 μL of the pre-culture was inoculated to 50 mL YPD medium in a 250 mL shake flask and incubated again at 30^{o}C and 180 rpm for 5 hours.

Electroporation
50mL of *M. antarcticus* culture was transferred to a falcon tube and centrifuged at 7000 rpm at 20^{o}C for 10 minutes and resuspended in 1 mL 1M sorbitol solution to wash the cells. The washing step was repeated twice before the cells were resuspended in 100 μL 1M sorbitol. 100 μL aliquot of cell suspension was transferred to a pre-chilled electroporation cuvette and the PCR products containing the split markers were added. The cell suspension was electroporated at 1.5 kV for 5.1 ms and immediately after the electroporation, the cells were diluted in 900 μL YPD medium and transferred to 1 mL YPD medium in a 50mL falcon tube.

Selection
100 μL of the cells were spread onto YPD selection plates containing either 50 μg/mL, 100 μg/mL or 200 μg/mL nourseothricin and incubated for 2-4 days at 30^{o}C. No formation of colonies was observed after transformation with split markers.

3 Results

3.1 Transformation of *Moesziomyces antarcticus* with pUC57mini-natMX-MaMat1

Figure 25 displays the colonies of *M. antarcticus*, appearing on the YPD selection plates, containing 50 μg/mL nourseothricin, after 4 days of incubation at 30^{o}C.

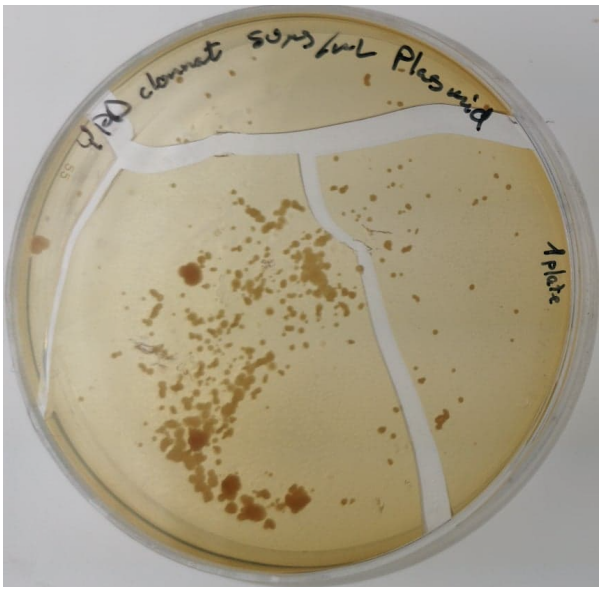


Figure 25: Growth of *M. antarcticus* on YPD plates containing 50 μg/mL nourseothricin, after transformation with pUC57mini-natMX-MaMat1.

It can be seen from the figure, that several colonies have appeared after the electroporation with the pUC57mini-natMX-MaMat1 plasmid.

3.2 Transformation of *Moesziomyces antarcticus* with disruption cassette

The results from transformation of *M. antarcticus* with the fragment containing the disruption cassette, obtained from the pUC57mini-natMX-MaMat1 plasmid, can be observed in **Figure 26**.

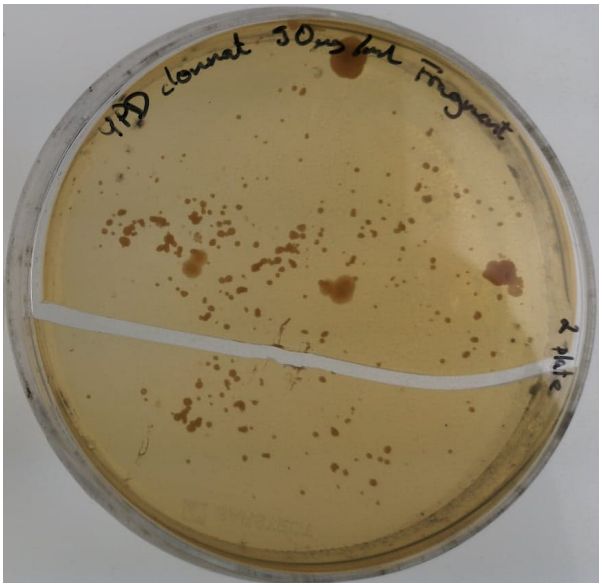


Figure 26: Growth of *M. antarcticus* on YPD plates containing 50 μg/mL nourseothricin, after transformation with the disruption cassette.

It can be seen, that several colonies have appeared after 4 days of incubation at 30^{o}C on YPD selection plates, containing 50 μg/mL nourseothricin.

4 Discussion

As it can be seen in **Figure 25** and **26**, several colonies have formed on the YPD selection plates with 50 μg/mL nourseothricin after 4 days of incubation. It could be assumed, that since the colonies are growing on selection plates, the nourseothricin has successfully been incorporated into the genome of *M. antarcticus*. However, a colony PCR would have to be carried out to confirm the presence of the nourseothricin resistance in the cells, followed by sequencing of the Mat1 region, in order to confirm on-target insertions. Even if it had been confirmed by colony PCR, that the colonies contained the nourseothricin resistance gene, the possibility exists, that the gene had been incorporated into the genome at an off-target site. Yarimizu et al. [42] reported an on-target efficiency of less that 10%, when following a similar approach, which means that several colonies would have to be screened in order to obtain a colony with an on-target insertion. Although the transformants exhibit nourseothricin resistant abilities, this itself cannot be a guarantee that the nourseothricin gene has been incorporated into the genome, as some microorganisms have previously demonstrated to be able to replicate plasmids episomally, without integration into the genome [49]. Episomal replication of the plasmid could be overcome by transformation with split markers, as this type of marker requires a recombination to occur in order to assemble and provide antibiotic resistance, which has indicated an increase in the occurrence of on-target HR [49]. Unfortunately, the transformation with split markers did not yield any colonies. The results of the antibiotic sensitivity test for nourseothricin, which can be seen in **Table 7**, show that *M. antarcticus* is able to grown under the presence of nourseothricin up to concentrations of 200 μg/mL even at low ODs. When examining the selection plates after 4 days of incubation, it was observed, that colonies only appeared on plates with a nourseothricin concentration of 50 μg/mL, and it is therefore possible, that the growth on the selection plates is not transformants but rather background growth of colonies that do not contain the plasmid.

5 Conclusion

It can be concluded, that pUC57mini-natMX-MaMat1 and the disruption cassette, from the digested plasmid, appear to have been successfully transformed into the plasmid. However, a confirmation of the transformation by a colony PCR of the growing colonies was not carried out, and it is therefore necessary to perform such analysis, in order to conclude that the transformations were successful. The split marker approach did not yield any transformants in this case, however, other studies have proven that this method is useful and results in high efficiency of on-target disruptions. The method should therefore be investigated further for the disruption of Mat1 in *M. antarcticus*.

Part III

In Silico Analysis of a Chain Shortening Pathway in *Moesziomyces antarcticus*

1 Introduction

Studies carried out by Kitamoto et al. [51][9] have hinted towards the existence of a chain shortening pathway in *M. antarcticus*, resulting in partial β-oxidation, much like that taking place in the peroxisomes of mammalian cells. This chain shortening pathway is suspected to be involved in the synthesis of fatty acids that constitute the hydrophobic moiety of MELs and the determination of the length of these.

1.1 Chain shortening pathway

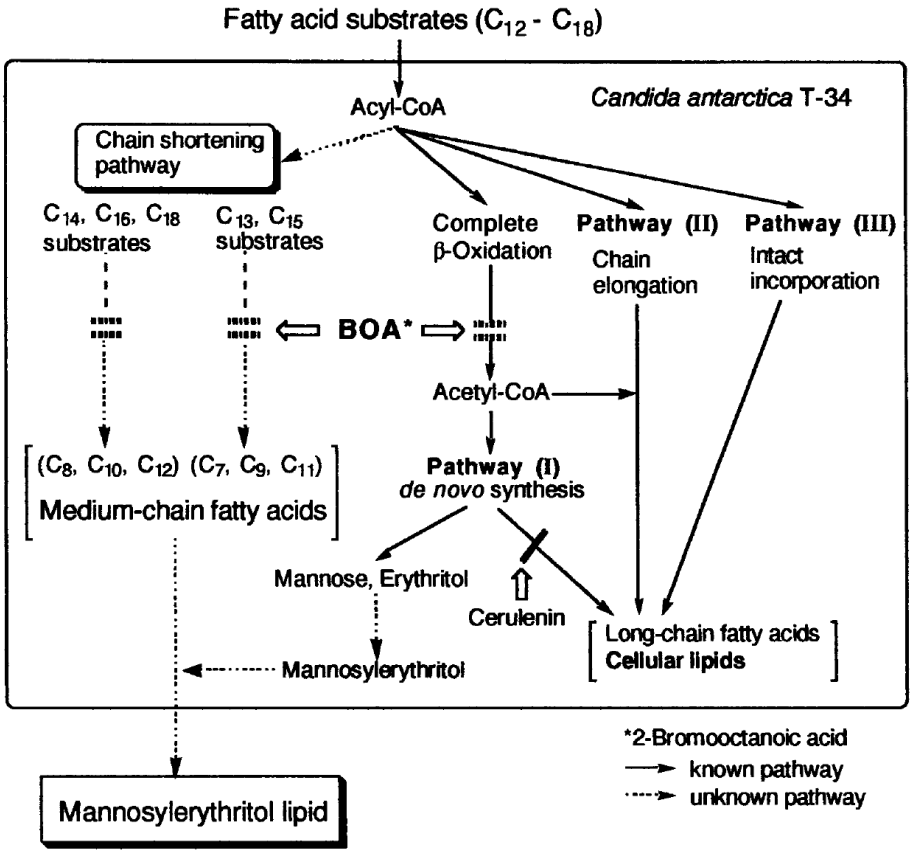


Figure 27: Overview of the pathways involved in the biosynthesis of fatty acids in *M. antarcticus* and the suggested chain shortening pathway [9].

In *M. antarcticus* there are three different pathways, as seen in **Figure 27**, involved in the biosynthesis of fatty acids for MEL production; a *de novo synthesis* pathway, a chain elongation pathway and an intact incorporation pathway. However, due to the fatty acid profile in MELs

produced by *M. antarcticus*, an additional pathway, the chain shortening pathway, has been suggested. In this pathway fatty acids obtained from the provided substrate, undergo partial β-oxidation to result in medium-chain fatty acids that constitute the lipophilic moiety of MELs [9]. It has been shown that the fatty acid profile of MELs is highly dependent on the substrate provided. The chain length of the fatty acids of MELs indicate that these are β-oxidation intermediates of the substrate shortened by one or more C_2 units [51]. To evaluate on the importance of the *de novo synthesis* pathway, the fatty acid profile of MELs were investigated when *M. antarcticus* was grown under the presence of cerulenin, which is a known inhibitor of fatty acid synthesis (*de novo synthesis*). When long chain fatty acids (LCFA) were provided as substrate, the fatty acid profiles of the MELs produced were similar to those obtained without the presence of cerulenin, thus indicating that the fatty acid profile is unaffected by cerulenin and therefore not solely dependent on the *de novo synthesis* pathway [52]. However, the existence of a chain shortening pathway, as well as the genes involved, in *M. antarcticus*, has at this moment, to my knowledge, not yet been documented. As the detailed mechanism of the chain shortening pathway in *M. antarcticus* is yet to be unveiled, it was decided to take a closer look into the mammalian β-oxidation as well as the β-oxidation of *S. cerevisiae*, through a literature study, to identify elements potentially involved in the chain shortening pathway of *M. antarcticus*.

1.2 Mammalian β-oxidation pathway

In mammalian cells, two β-oxidation systems exist; the peroxisomal and the mitochondrial β-oxidation. In the peroxisomal β-oxidation very long chain fatty acids (VLCFA) and LCFA are degraded into medium chain fatty acid (MCFA) intermediates, and the peroxisomal β-oxidation ends at octanoyl CoA. The mitochondrial β-oxidation shortens MCFAs and short chain fatty acids (SCFA) to completion [53]. An overview of the β-oxidation pathway in *H. sapiens* can be seen in **Figure 28**.

In the mitochondria the activated fatty acids undergo several sequences of β-oxidation consisting of four different steps, before being fully oxidised. Each sequence results in the formation of acetyl CoA and acyl CoA, the latter being shortened by two carbon atoms, and acyl CoA will then undergo several more β-oxidation sequences depending on the length of the fatty acid. Fatty acids are activated by CoA to form an acyl CoA, and in the initial step of the fatty acid degradation, acyl CoA is oxidised by an acyl CoA dehydrogenase which introduces a trans double bond between C3 and C2 and gives enoyl CoA. The second step of β-oxidation is a hydration of previously formed trans double bond between C3 and C2 of enoyl CoA to form hydroxyacyl CoA. This process is catalysed by the enzyme enoyl CoA hydratase. The third step of the β-oxidation is the second oxidation reaction that takes place during the β-oxidation sequence, where ketoacyl CoA is formed from hydroxyacyl CoA by the enzyme hydroxyacyl CoA dehygrogenase. In the final step ketoacyl CoA is cleaved by the addition of a CoA molecule to form acetyl CoA and acyl CoA, which is carried out by a thiolase [54].

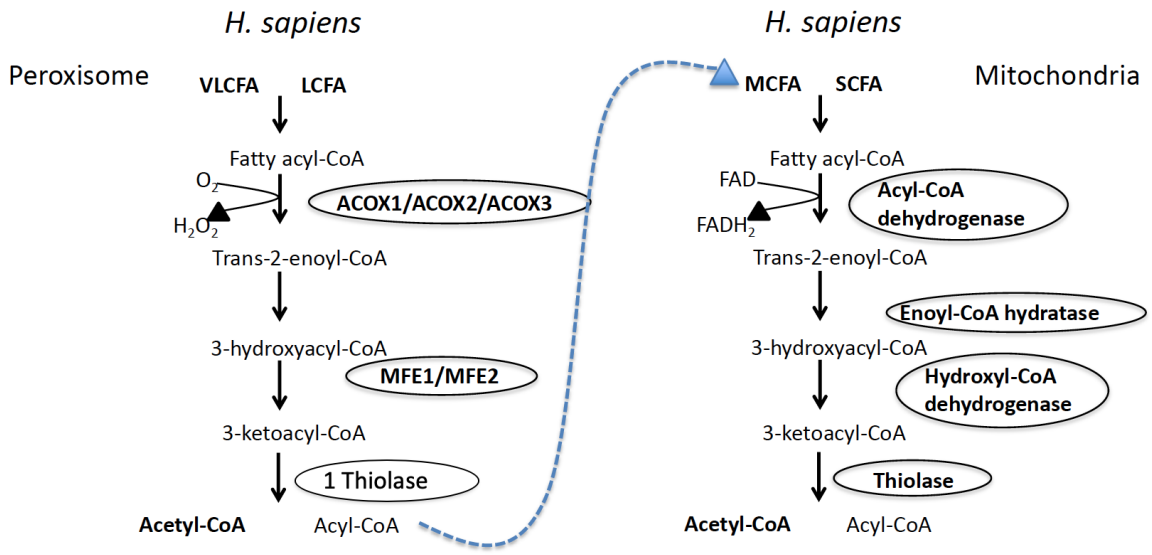


Figure 28: Overview of the fatty acid metabolism of *H. sapiens* taking place in the peroxisome and mitochondria.

The peroxisomal β-oxidation in mammalian cells is in many ways similar to that which takes place in the mitochondria. The two types of β-oxidation differ only in the initial step in which the frist oxidation takes place. As mentioned above an acyl CoA dehydrogenase carries out this step in the mitochondria. The same class of enzymes is involved in the first step of β-oxidation in the peroxisome as well. However, the enzymes involved in this first step in the peroxisome differ from that which catalyses the process in the mitochondria. In the peroxisomes, while catalysing this process, the energy for the oxidation is provided by the addition of $2H^+$ onto O_2 to form H_2O_2 rather than by the addition of $2H^+$ to FAD to form $FADH_2$ as in the mitochondrial β-oxidation [54].

1.3 β-oxidation in Saccharomyces cerevisiae

In order to identify the potential genes involved in a partial β-oxidation pathway in *M. antarcticus*, the mechanism and genes involved in the β-oxidation of *S. cerevisiae* were studied. The β-oxidation of *S. cerevisiae* is in many ways similar to that of *H. sapiens*. However, the β-oxidation in fungi takes place solely in the peroxisomes, and the two therefore differ slightly [55]. In *S. cerevisiae* the energy provided to carry out the initial oxidation step is provided by passing electrons to oxygen to form H_2O_2 as it happens in the peroxisomal β-oxidation of *H. sapiens*.

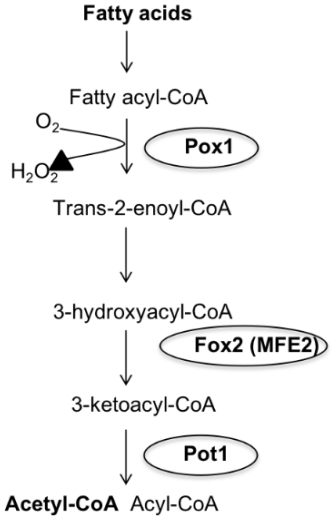


Figure 29: Overview of the β-oxidation pathway of *S. cerevisiae* taking place in the peroxisomes.

The β-oxidation of fatty acids in *S. cerevisiae*, which can be seen in **Figure 29**, initiates by an oxidation of acyl-CoA carried out by the acyl-CoA oxidase, Pox1. This step is similar to that which takes place in the peroxisome of mammalian cells, although the enzyme that catalyses the step is different between the two. Studies of the substrate specificity of Pox1 and mammalian acyl-CoA oxidases have shown that the mammalian peroxisomal acyl-CoA oxidases shows a preference for MCFAs and is also active with LCFAs while Pox1 accepts fatty acids of all chain lengths, allowing the β-oxidation to continue to completion in *S. cerevisiae* [55]. The second and the third step of the β-oxidation pathway is carried out by the multifunctional enzyme Fox2, which is again similar to the multifuntional enzymes MFE1 and MFE2 that carry out the same steps in the peroxisomal β-oxidation in mammalian cells. Finally the thiolytic cleavage in the final step of the β-oxidation is carried out by the enzyme Pot1 and yields an acetyl-CoA and an acyl-CoA, shortened by 2 carbon atoms.

1.4 Confirmation of involvement of putative genes in the chain shortening pathway

Several genes were identified in *M. antarcticus* that could potentially be involved in the chain shortening pathway. The genes were identified based on homology to the protein sequence of the genes already known to take part in the peroxisomal β-oxidation in *S. cerevisiae* and *H. sapiens*. One useful way of identifying the function of a gene, and in this case confirm that the genes, discovered in *M. antarcticus*, are in fact involved in a potential chain shortening pathway, is knocking out the genes and analysing the fatty acid profiles of the MELs for any potential changes [56].

2 Materials and Methods

Genes, potentially involved in the chain shortening pathway of *M. antarcticus*, were identified based on protein homology with annotated genes in *Saccharomyces cerevisiae* and *Homo sapiens*, known to take part in the peroxisomal β-oxidation in these organisms. The analysis was carried out using the platforms UniProt (https://www.uniprot.org) and NCBI protein blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for homology analysis. Furthermore, the identified genes were tested for peroxisomal targeting signals (PTS), which indicates whether the protein, encoded by the gene, takes part in reactions in the peroxisomes. To test for PTS1, a PTS1 predictor provided by IMP Bioinformatics Group (http://mendel.imp.ac.at/pts1/) was used. To confirm that the identified genes are indeed involved in the β-oxidation pathway of *M. antarcticus*, a knock-out of one or more of the identified genes could be carried out. By analysing the secreted MELs obtained from the successful transformants, a change in the fatty acid profile of the produced MELs could either confirm or disprove that the targeted gene is involved in the synthesis of MELs, and if a chain shortening pathway, for MEL production, exists in *M. antarcticus*.

The following criteria were taken into account, when evaluating on the likelihood of the homologous genes, found in *M. antarcticus*, being involved in the chain shortening pathway:

- The genes involved in β-oxidation in *S. cerevisiae* or *H. sapiens* had to be targeted to the peroxisomes, in order to be used for homology analysis in *M. antarcticus*, which was evaluated by using UniProt (https://www.uniprot.org).
- The homology of the genes in *S. cerevisiae* or *H. sapiens* had to cover the sequence of the genes found in *M. antarcticus* without any major gaps within the genes, in order for the discovered gene to be considered likely to have a similar function.
- The genes discovered in *M. antarcticus*, that had not already been identified to be targeted to the peroxisomes, were analysed for PTS.

3 Results

Based on the criteria listed in **Part III Section 2**, an overview of the genes, that were identified to potentially be involved in a chain shortening pathway of *M. antarcticus*, can be seen in **Table 14**. The genetic sequences off all identified genes can be found in **Appendix B**.

Enzyme	**Function**	**Number of homologues in *M. antarcticus***
POX1 (*S. cerevisiae*)	Acyl CoA oxidases	4 Acyl CoA dehydrogenases
ACOX1, ACOX2, ACOX3 (*H. sapiens*)		4 Acyl CoA oxidases
FOX2 (*S. cerevisiae*)	Peroxisomal multifunctional enzyme	1 hydratase-dehydrogenase-epimerase
MFE1, MFE2 (*H. sapiens*)		
Pot1 (*S. cerevisiae*)	3-ketoacyl-CoA thiolase	1
ACAA1 (*H. sapiens*)		3-ketoacyl-CoA thiolase

Table 14: Overview of the genes in *M. antarcticus* that are homologous to those involved in the peroxisomal β-oxidation of *S. cerevisiae* and *H. sapiens*.

4 Discussion

The research carried ot by Kitamoto et al. indicates that a chain shortening pathway plays and active in the acylation of MELs. However, so far no nonequivocal evidence has been provided to confirm this. Several genes were identified in *M. antarcticus* based on protein homology with peroxisomal β-oxidation genes in *S. cerevisiae* and *H. sapiens*. As the enzymes that are involved in the individual steps of the β-oxidation pathway carry out very similar processes across species, it was expected that the protein structure of these enzymes would also be similar, albeit the genetic sequence itself, could vary greatly across the different species. However, it is possible that the identified genes in *M. antarcticus* are not taking part in peroxisomal β-oxidation. For the proteins to enter into the peroxisome a PTS must be present [53]. Two types of PTS are used to target protein to the peroxisome; PTS1 and PTS2. As the identified genes were only evaluated for PTS1, it is possible that other genes, that were discarded due to not harbouring a PTS1, might have carried a PTS2 and therefore still be targeted to the peroxisome.

Another issue, when identifying genes involved in β-oxidation in *M. antarcticus*, is that the *M. antarcticus* genome has not yet been well studied. Although several genes have been auto annotated [27], they have not yet been reviewed, which makes it troublesome to select the genes, when carrying out protein blast. To confirm that the identified genes are in fact active in the peroxisomal β-oxidation and do contribute to a chain shortening pathway, a gene knock-out approach could be taken, similar to that described in **Part II**. Carrying out such gene knock-out could contribute to the understanding of the function of each of the identified genes.

5 Conclusion

Although 10 potential genes were identified, and although the identification was based on homology to the peroxisomal β-oxidation genes in *S. cerevisiae* and *H. sapiens*, none of them were tested for their function, by e.g. gene knock-out. It can therefore neither be concluded that any of the identified genes take part in the β-oxidation of fatty acids, nor that a chain shortening pathway does exist in *M. antarcticus*. The research provided in **Part III** constitutes a starting point for investigations into the chain shortening pathway, however, much more research is yet to be carried out to understand the mechanism of acylation of MELs.

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Appendix A Appendices for Part I

A.1 Genetic sequence of gBlock1

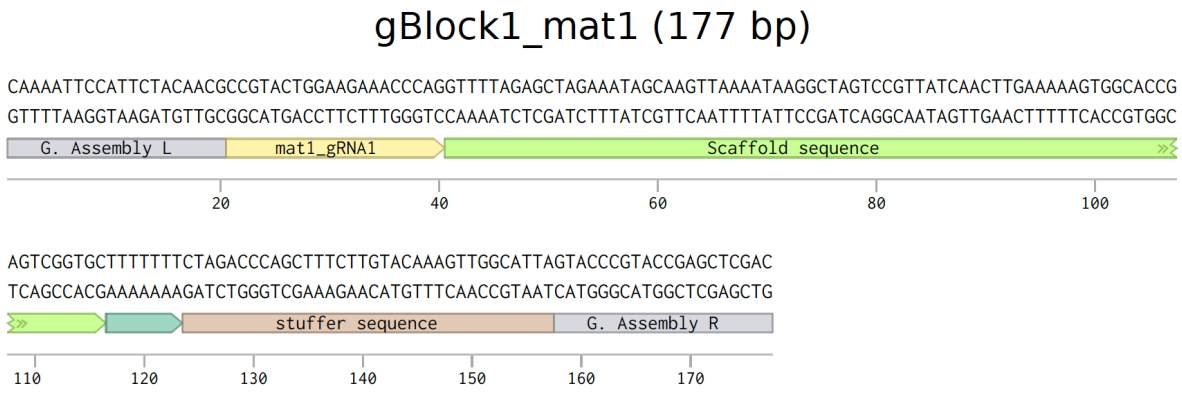


Figure 30: Genetic sequence of gBlock1, encoding the tracrRNA-crRNA duplex constituting the sgRNA of pMS8-MantU6-G1, with the scaffold from [5].

A.2 Genetic sequence of gBlock3

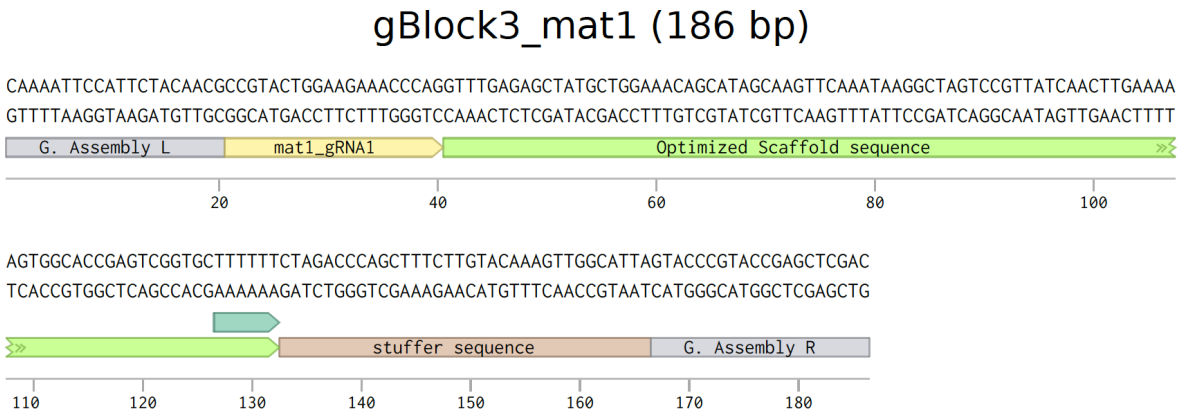
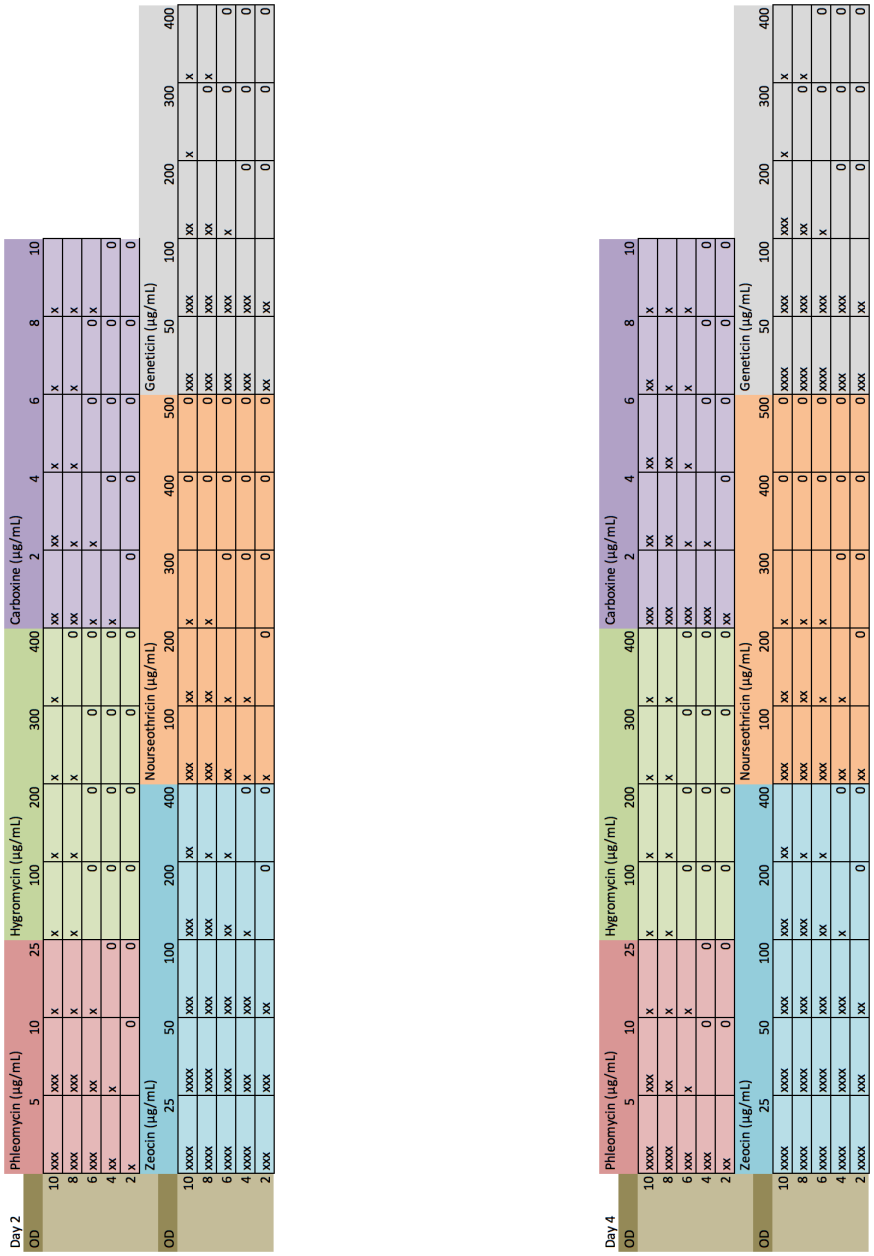


Figure 31: Genetic sequence of gBlock3, encoding the tracrRNA-crRNA duplex constituting the sgRNA of pMS8-MantU6-G3, with the optimised scaffold.

A.3 Antibiotic sensitivity test

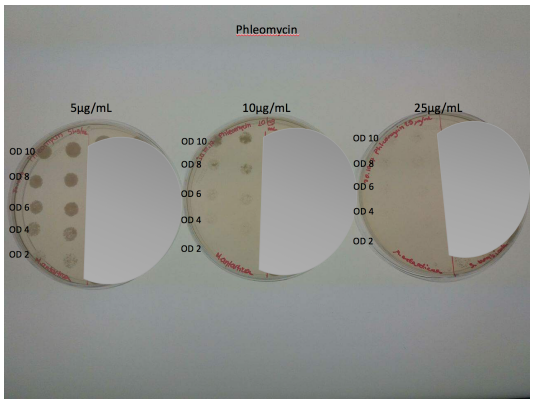
The results from the sensitivity test for phleomycin, hygromycin, carboxin, zeocin, geneticin and nourseothricin at day 2 and day 4 can be seen in **Figure 32**.



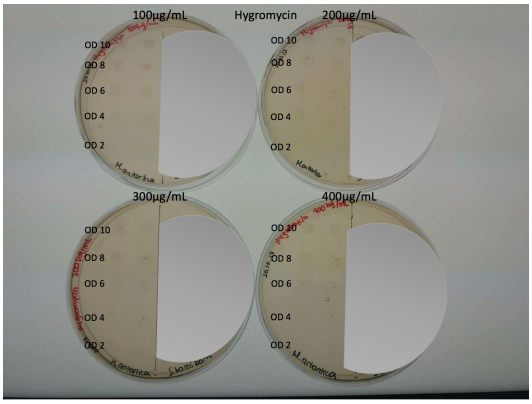
(a) (b)

Figure 32: Results from the sensitivity test for *M. antarcticus* against phleomycin, hygromycin, carboxin, zeocin, geneticin and nourseothricin on day 2 (a) and day 4 (b).

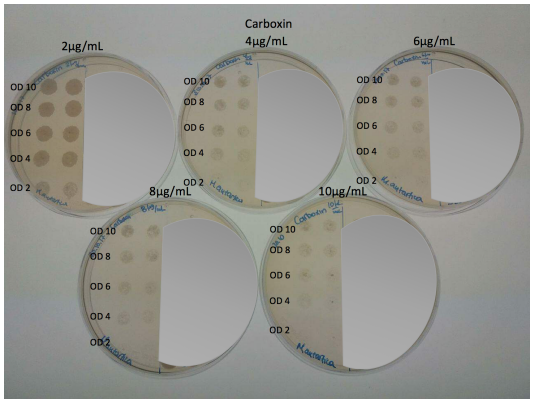
The plates used for the sensitivity test after 4 days of incubation at 30^{o}C can be seen in **Figure 33**.



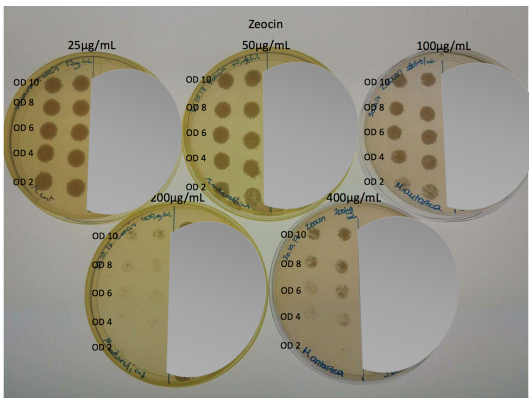
(a) *M. antarcticus* phleomycin sensitivity.



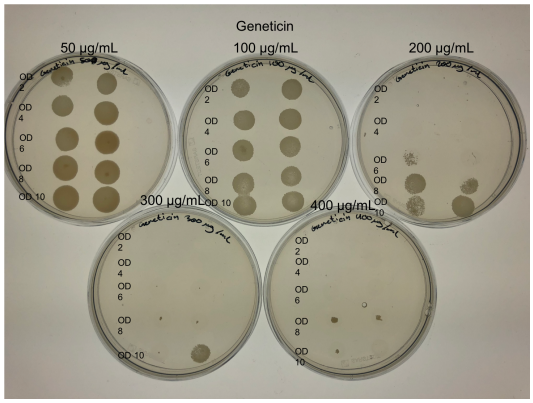
(b) *M. antarcticus* hygromycin sensitivity.



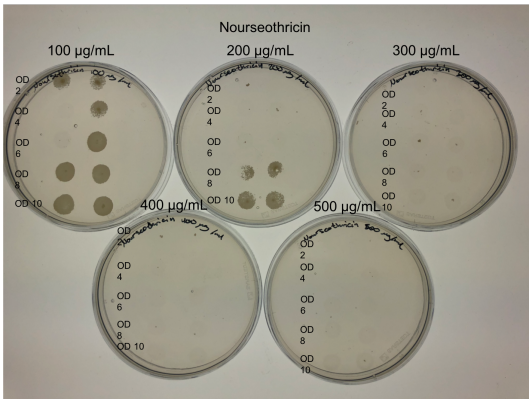
(c) *M. antarcticus* carboxin sensitivity.



(d) *M. antarcticus* zeocin sensitivity.



(e) *M. antarcticus* geneticin sensitivity.



(f) *M. antarcticus* nourseothricin sensitivity.

Figure 33: Growth of *M. antarcticus* cell ODs 2, 4, 6, 8 and 10 on selective plates with varying concentrations of antibiotics.

Appendix B Appendices for Part III

B.1 Putative acyl-CoA dehydrogenases and acyl-CoA oxidases

Genetic sequence of the putative acyl-CoA dehydrogenases and acyl-CoA oxidases in *M. antarcticus*, identified based on protein homology with *S. cerevisiae* and *H. sapiens*.

Short chain acyl coA dehydrogenase
TTAGAGACGCGCGTTCTCGGGCATCATCCTGAGCGCCTGTCGGATGCCGAGATCGGCG
AGCACCTCCTCGGAGCCACCGAGAACGGCGTCAAACTTGTAGGTGCGGTGGAACATCT
CGACAAACTTGCCCATACCGGTCTTTGTGAGGCCGCGGCCGCCAAAGATCTGGACGGC
GCCGTCGGCGACCTCGTGCGAGACGCGCGTGGACCACGACTTGAGCAGGCCGACCTGG
CCAGCGAGGAACTTGGACTGCTGGGCGTAGGACATCTTGCACATCTGGTAGGTCACGT
TTTCGAGCCAGTTCTGTGCGGCCTCGACGAGCGAGATCATCTGCGCCAGCTTCTGGCG
GATGACGGGCTGCGAGGTGAGCGGCTTGCCAAACACCTTGCGCTGGTGGATCCAGAGC
ATGCACTCCTCGCACACGGCGCGGGCGGCGCGGATGGTGGAGCAGCACATGACCCAGC
GCTCGTGGTTGAAGTTGGAGAGGATGTAGTAGATGCCCATGCCGTCCTCGCCGATGAG
GTACTTTTTGGGCACTTGACATTGTCAAACGTGACGTAGGCGGTGCCGGCGGTGGTGG
AGTAGGAGGTCTTGATCTGCTTTGTCTCGACGTTGCCGGCGGTGCGCGGGATGCAGAT
CATGGCGAAGCCCTGGTCGGTCTTGACGGCGGTGGAAAAGTAGTCGGCGAAGGTGCCG
TTGGTGATCCACTTTTTGGTGCCGTTGACAATGTAGTGCTCGCCGTCGTCGGTGAGGG
TGGCGGTGGTGCGAAGGCCCATGACGTCGGAGCCGGCAAAGGCCTCGGAGATGGCGAG
GCAGATAAACTTGTCGCCGGCGAGGACGGGCTCGACAATCTCCTTCTTGAGCTCGTCG
GAGCCAAAGTTCATCACGGGGGGCAGGCCGATGACCATGCCGCCGTTGAGGGCGTCGC
CGAATCCACGCTGTCCGCAGCGGACGAGCTCCTGGGTGAGGATGAGTTCGTGGAAGTA
GTCGAATTCCTCGCCCTTGATGCCGGCAAAGAGGGTGCGTCCGTGGAGGTGCTTGCCG
GGGCCCATGCGCATGGCGTTGATGCCGTTCTTGCCGAGCATCTTGACGAGCTCGACGT
CGGGACGCTTGCCCGAGTCCTCGCAGCGCTGCGCCACCTCGACAATGTGCTCGTCGAC
GTACTTGCGCCACTCGCGCTGGAGGTGGCGGTGGAGTCGTTAAAGTAGGGCGAGTGGT
AGGCGGGGGTGAGCCAGGTGGGCTCTCCGTAGGGGACGCGGGAAGGGCGCCGAGCGGG
TCTGGCTTGAGCTTGGACTGCTCGCCCTTGATGGTGCCGATGATGAGGCGCTGGTACT
GGGGCTGGAGAGGACCTCGCGGCGGTGGAGGCCAAAGAAGGTCTCGGTGGCGTCCTGA
CCGGCGACCTCTTCGTCGTGGAAGACGTTCTCACCACCCGGGTGGAGCTCGGAAAACT
TGGAGACGTTGTAGACCTCGGCGTCGATGACGATCCAGAGGTCGCCCTGCTTGTTGTG
CTTGGCGACCTCCTCGCGCGTGTACTCCTTGCGCTGGGGTGCGGGCGCGCCCTGCTTC
TGTGCAGACAT

Medium chain acyl coA dehydrogenase
TTACTGGGCGTACATCTCCTTGATGTGTCGGGAGATGATGAGCCTCTGGATCTCAGAG
GTGCCCTCGTAGAGACGGAAGATGCGCGCATCGCGGTAGAGCTTCTCGACGGGCGACT
CGGTGTTGAAGCCGAGACCGCCAAAGATCTGAACGGCGGCGTCGACGTTGAAGCCGGC
GGCCTCGGAGGCGCGTGCTTTGGCCATGGAGGCGTAGAAGGAGTTGCGCTGACCCTGG
TCCTTGGTCCAGCACGACTTCCACACGAGGTTGCGCGCCGCCTCGACGTTCATCTGCA
TGTCGGCGAGCATAAAGGCGACGGCCTGATGGTCGATGATGTGCTTGCCCATCGTCTT
GCGCTCCACGGCGTACTTGGCAGCCTCCTCGAGAGCGCGCTGGGCGAGACCGACGGCA
CCGGCAGCGACCAGGGGGCGCGTAATGTCGAATGCACCCATGGCAGTCTTGAAACCGT
CGCCGGGCTTGCCGAGCACGTTCTCCTCGGGCACGACGACGTTCTCGAACGTGATCAT
GCGCGTGTCCGAGCAGCGCTGACCCATGTTGATCTCCTTCTTGCCGATGTGGATACCG
GGCGTGTCGGCATCGACGACAAAGCCGGTCATCGACTTGGCGGCCTTGGCGCTAGGGT
CGCTCTTGGCGAGCACAAAGTACCAGTTGGCCTTGCCACCGTTGGTGATCCACATCTT
GGTGCCGTTGATGACCCACTTGTCGCCCTGCTTCTCGGCCTTGGTGGCGATGTTGGCG

ACGTCCGAGCCGGCGCCGGGCTCGGTGACACAGTAGGCAGCCATGAGGGGCTCCTCGG
TCATGCGGCCGAGGTACTTGGCCTTGGTGGCGTCCGAGGCCGAGACGAGGAGGGGAGC
CTCTGCGAGACCGTTGGCCTCCATGGCGGTCTGGATACCGGTGCATCCGTAGGCGAGC
TCCTCGGAGATCAGGCCACACTCGACGAGACCAAGGCCGGGGCCGCCGTACTTCTCGG
GGATGTGCGTGTTCATGAGACCGAGCTCCCAGCCCTTCTTGAGGATCTCTGTGGGGTA
CTCCATGGTCTGGTCGTGGTGGCGCGCCTGAGGGATGATCTCTTCACGAGCGAACTTG
CGTGCGAGGTCACGGTAGGCCTCCTGGTCCTCGCTGAGGCCGAACGAGACGCCATTGC
TCTCCACGGGTGTAGAGTAGGCTCGGGCGGCACGGGAGGCGGCGAGCGCGGCCTTGTT
GGCCGAGGTGATGGTGCGGCTGCTGCTGGCAACAGCCCTCTTTGCGAAGAGAGACAT

Long chain acyl coA dehydrogenase
ATGTCTGCACAGAAGCAGGGCGCATCCGCCCCCCAGCGCAAGGAGTACACGCGCGAGG
AGGTCGCCAAGCACAACAAGCAGGGCGACCTCTGGATCGTCATCGACGCCGAGGTCTA
CAACGTCTCCAAGTTCTCCGAGCTCCACCCGGGTGGTGAGAACGTCTTCCACGACGAA
GAGGTCGCCGGTCAGGACGCCACCGAGACCTTCTTCGGCCTCCACCGCCGCGAGGTGC
TCTCCAAGCCCCAGTACCAGCGCCTCATCATCGGCACCATCAAGGGCGAGCAGTCCAA
GCTCAAGCCAGACCCGCTCGGCAGCCCTTCGCGCGTCCCCTACGGAGAGCCCACCTGG
CTCACCCCCGCCTACCACTCGCCCTACTTCAACGACTCGCACCGCCACCTCCAGCGCG
AGTGGCGCAAGTATGTCGACGAGAACATCATCGAGGTGGCGCAGCGCTGCGAGGACTC
GGGCAAGCGTCCCGATGTCGAGCTCGTCAAGATGCTCGGCAAGAACGGCATCAACGCC
ATGCGCATGGGCCCCGGCAAGCACCTCCACGGACGCACCCTCTTTGCCGGCATCAAGG
GCGAGGAGTTCGACTACTTCCACGAACTCATCCTCACCCAGGAGCTCGTCCGCTGCGG
CCAGCGCGGATTCGGCGACGCCCTCAACGGCGGCATGGTCATCGGCCTGCCCCCCGTG
ATGAACTTTGGCTCCGACGAGCTCAAGAAGGAGATTGTCGAGCCCGTCCTCGCCGGCG
ACAAGTTTATCTGCCTCGCCATCTCCGAGGCCTTTGCCGGCTCCGACGTCATGGGTCT
CCGCACCACCGCCACCCTCACTGAAGACGGCGAGCACTACATTGTCAACGGCACCAAA
AAGTGGATCACCAACGGCACCTTTGCCGACTACTTTTCCACCGCCGTCAAGACCGACC
AGGGCTTCGCCATGATCTGCATCCCGCGCTCCGCCGGCAACGTCGAGACCAAGCAGAT
CAAGACGTCCTACTCGACCACGGCGGGAACCGCCTACGTCACCTTCGACAATGTCAAG
GTGCCCAAAAAGTACCTCATCGGCGAGGACGGCATGGGCATCTACTACATTCTCTCCA
ACTTTAACCACGAGCGCTGGGTCATGTGCTGCTCCACCATCCGCGCCGCACGCGCCGT
GTGCGAGGAGTGCATGCTCTGGATCCACCAGCGCAAGGTGTTTGGCAAGCCGCTCACC
TCGCAGCCCGTCATCCGCCAGAAGCTCGCACAGATGATCTCGCTCGTCGAGGCTGCGC
AGAACTGGCTCGAAAACGTGACGTACCAGATGTGCAAGATGTCCTACGCCCAGCAGTC
CAAGTTCCTCGCGGGTCAGGTCGGCCTGCTCAAGTCGTGGTCCACGCGCGTCTCGCAC
GAGGTCGCCGACGGCGCCGTCCAGATCTTTGGCGGTCGCGGCCTCACAAAGACGGGCA
TGGGCAAGTTTGTCGAGATGTTCCACCGCACCTACAAGTTTGACGCCGTGCTCGGTGG
CTCCGAGGAGGTGCTCGCCGATCTCGGCATCCGACAGGCGCTCAGGATGATGCCCGAG
AACGCGCGTCTCTAA

Very-long-chain acyl-CoA dehydrogenase
ATGTCCAGTACCGCCAAGCTCACCAAGCCCACCGTTCCATTTTCCGAGGCGCCATGGA
TTCAAGGACTGCCGTCCTCGATCTTCACATCGCCCTCGCATGTCCAATTGCGCGAATG
GTGCCGCGTGTGGTGCGACGAGGTCCTGATCAAGATGGGTGCCAAGTACGAGGAAGCC
GGCGTCATCAATGATGACGAAGCGTACCAGCGCGCTGCCAAGGACGGCGTGCTCTTTG
CTTTCGCCACGGGCGTGCACGTCGACCCCAAGATCGCCAAGATCGCCCAAGCTGCTGG
TGTCGGCCTCCCGGCCGGCATCAAGATGGAGGAATGGAACAACATCCACGACTACATC
ATCTGGGACGAACTCAACCGCTGCGCCAGCCCCGTACTGATGGGCCTGATTGGCGGCC
TCACGTATGGCTCCGGGCCCATCATGCACTTTGCCAGCGACGAGCAGAAGGCACGTTG
GCTTCCCGAGATCTTTGCCGGCCGGAAGCGAATCTGCCTTTCCATCACCGAGCCCCTT
GCCGGTAGCAACGTCGCCAACCTCAGCACCGAGGCGACGCTCATCGAGGAGAATGGAA
AGAAGTTCTACCTCGTTAACGGCAGCAAGAAGTGGATCACCAATGGAATCTACTCGGA

CTGGTTCACCACTGCCGTTCGCACGTCCGGCAAGGCGGGCGAGTCGGGCGGCATTTCG
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GCGGAGCTTCGGGCACGACCATCGTCGAGTTCGACGACGTCAAGGTCCCCATCGAGAA
CCTCATCGGCAAGGAAGGAGAAGGCCTCAAGCTGGTCTTCTTTAACTTCAATCACGAG
CGCATGACGATCGCCTACACAGCCTTGCGCTACGCTAGGGTGTGTATGGAGGATGCGA
TTGCGCACACACGACGACGAGAGGTGTTCGGCAAGATGTTGATCGAGCAGCCGGTGGT
GCGCCACAAAATCGGCCACATGGCGCGCGAGGTGGAGGCGCTGCAAGCCTGGACGGAG
AGCATCATCTTCCAACAGCTCAACATGACGATTGCGCAGTCGAACCTGCTCACCGGTG
GCTCGTGTGCGCTGCTCAAGGCGCACGCCGGTATCGTGCTCGAGAAGGTGGTGCGCGA
GGCGGTGCATCTGCTCGGAGGCATGGGCCTCACCAAGGGCGGCACTGGCGAACGCATC
GAGCGCATCTGGCGCGAAGTCAAGGCGCTCACCGTGCCCGGCGGAAGCGAGGACGTGA
TGATCGACCTGGGCGTGCGCCAGCAGCTCAAGCTCGTCGGCCCCCAGAGCAAGTTCTGA

peroxisomal acyl-coenzyme A oxidase

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GCCCTCTTCCACTCGTAACATCAAGATGGCTTCCAAAAGACCCGCGCCCGAGACCTTG
CAGCCGCGCGACATCCAACAGGAACGCGCGGCATGCTCGTTCGACGTCGACGCCATGT
CGTGCATCCTCCCCGGCTCGCGCACCGAGCGTGACAATGCACGCTGGCTCCTCTCGCT
TCTCAAGGACGACCCGGATCACACTTTTGACAAGGAGGATCGCGTCTTCCAATCTCGC
AACGAACGCTTCCTCAAGGGTCAGCAGGTCTCCCTCCGCTACTTTGAGATCCGCAACC
GCCACGGCCTCGAAAAGAAGGACGCCGACATGCTCCGTCTCTTCACCGACGAGTACCT
TCCCATCCAAGTAGCAGAGAGCATGGCACAGCCCACCCTCATGCGACAGGCAAGCACC
GAACAGTGGGCCGAGTGGGGTCCCATGGTCAGGTCCGGTCGCTGGCTCGGTTGCTACA
TGCAGACCGAGCTCGCACACGGCTCCAACCTCTCGGCACTCAGGACCACCGCCACCTT
CGACCACAACACCGACGAGTGGATCATCAACACTCCAGAGCCGTCTGCCGGAAAGGTG
TGGATCGGAGGCAGTGGTCTCACCGCCACGTACGGCGTGGTCATGGCCAACCTCATCA
TTAGCGGCAAATCGTACGGCATGCACCCCTTCTTGGTTCACCTCCGCTCGCTCAAAGA
CCACACGCTGCTGCCGGGACGTCGCATCATCGAGATGGGCTGTAAGCTTGGCGCGCCT
GCGATGGACAATGGATACACGTGCTTCGACAGTGTTCGCATCCCGCGGTCGCATCTCC
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GACGACTGGTACCTCTGCTCGCCACTCGGCAGCAGCGACGGCCGTGCCTACGAGAGGA

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CCAAAACGGCGTCGTCAAGGGCTACCACGACGGCATCGGTCGCCTCATCCGCGGAGAA
GCGGTCCAGTTTACCGAGCGCGCCAAGCTGTAA

Fatty-acyl coenzyme A oxidase
ATGGCGCCCCCTCGCTTTATCGAGCTCCCCGCGGGTCTCAAGCCCGCCGGTGTCTCCG
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AGCCAGGACCCCGTGTTTGACAAGACCTCCGACGCATACTACGGCCGCGCTGACAAGT
TCCGCCGCGCCATGCTCAAGGACAAGCGCCTCGCCCAGCTCGCAGACGAGCACCAGTG
GGGTCAGACCGAGATCAACATGGCCGAGAAGCTCGCAGACATCCCCGGCCCCATGGGC
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AGGTCTTCTACGAGCCCGCATCCAAGTATCAGATCATCGGATGCTACGCCCAGACCGA
GCTCGGCCACGGCTCCAACGTGCAGGGCATCGAGACCACCGCCACCTGGATCCCCGAG
TCGCAGGAGTGGGAGATCCACAGCCCCACCCTCACCGCCTCCAAGTGGTGGATCGGCG
GCCTCGGCAGGACCGCAGACCACGCCGTCGTCATGGCACAGCTCATCACCAACGGAAA
GAACTACGGCCCCCACCCCTTTGTCGTTCCCATCCGCGACGTCAAGACGCGCGAGGTC
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ACGGCTTCATGCTCTTTGAGCACGTCCGCGTCCCGCACCTCAACCTCCTCGCGCGCTT
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TACGGCACGCTCACCTGGGTGCGCGCCAACATTGTGCGCGATGCCTCCACCGTGCTCA
TGCGCGCCGTCACGGTCGCCATCCGATACTGCGCCGTCCGCAGGCAGTTTGCCGACCG
CGATGCGCCCAAGTTCGGCCAGGACGGAAAGCCGCTCGAGACGCAGGTGCTCGACTAC
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CTCCTCTGGGCATGCCACACCATGGAGCAGGAGTCGGCCGACTTCCTCGCCTCGGGCT
ACATTGACGGCAAGCAGTTCCACCTCCTCGGTTCCAAGGTCCAGGAGATCATGGCCGA
GCTCCGTCCCAACGCCGTGGCGCTTGTCGACGCTTTCGCCGTGCCCGACTATCTCCTC
AACTCGGCCCTCGGCCGTTCGGACGGCAACGTCTACGAGGCGCTCTTCGACTTCGCCC
TCCGCGAGCCGCTCAACTCGGTCAGGTGGAACGTCGATATCAACGACTTGGAGACCAC
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Peroxisomal oxidase
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GTCGCAGGACATCGCCAAGGCGCGTGCCGCCGCCGACTTTGCGCCCGAGCGCATCGAG
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GCAGATCGAGCGATGGGGTAACAAGGCCATGAACCACGAGATCATGGGCTGCTACATG

CAGACCGAGCTCGGTCACGGCACCAACGTCCAGCAGCTCGAGACGACGTGCACCTACG
ACGACGCCTCGGACAGCTTCATCCTCCACTCGCCCACGCTCACCTCGACAAAGTGGTG
GGCCGGCGGCCTCGGCACCACCGCCACCCACGGCGTCGTCCAGGCGCAGCTCATCATC
CACGGCAAGCGCTACGGCCCGCACCTCTTCTTCACCCAGCTGCGTGACATGGACTCCG
GCAAGCTGCTCGACGGCATCGTCGCCGGTGACATTGGTCCCAAGACGTACGGTGCGTT
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ATGCTCTCCAAGCACGCCAAGGTCAAGAAGGGCGGCGAGTACGTCAAGCCGCCCAGCG
ACAAGCTCAGCTACGGCGGCATGATCTTTATCCGTTCGCAGATGATCGACCGTACCGG
CTGGATGCTTTCGCGCGGTGTCACGATCGCTACACGCTACGCGCTCGTGCGTCGTCAG
TTCCGCGACCCTTCGTCGACGGACATCAACGATGTCGAGCGCTCGGTGCTGTCGTACC
CCTCGACTGCGCGCCGTATCTTGCCGCTGCTCGCCAAGGCGTACGCCTACATCCTTGC
GGGCCGCCGCATGAGGACGCTGTACGAGGACATGGCGCAGCAACTCGAGGAGGGCAAC
ACCGAGCTGCTCGCCGACGTCCACGTCGCCTCGTCCTCGCTCAAGGCCTACTGCACCA
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GGCCAAGTCGCACGGCGAGTTTGTCGTCGATCTGTGGTTCGGCGAGGGCGTTCGCGAT
GATGCCGAGTCGTTTGGCGAGCGCGAGGTCAAGTGGCTCAACAAGCTCGTCACGCTGC
ATGCGCTCGTGGCGCTCTCGCGCAACATTACGCCTCTCGTGCTGCCGGCCAGTGCCGG
CCGTACGCTGGCAGGCTATGCCAACGGCCAGGCCATCCTTACGCCCGAGTCGGTGATC
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TCTCGGACGGATTCGGATGGCTCGACTGGGAGCTCGGCAGCAGTCTCGGCAGGAAGGA
CGGCCGCGTGTACGAGCAGCTCATGGCCGACGCAGAGGCCAACCCGCTCAACCACCCC
GGCGCCATCCCCAACCTCGAGACCATCGACAAGGTCGGTGTGTACAAGTACGGCTCCA
ACAACATCGGCAAGGGCGTGCCAGACTGGTACAACGCCGAAATCGGCCCCCTCCTCCG
TGCCGCCGCCAAGCGCGGCGAAGCTTCCCGTCTGTAG

Acyl-CoA oxidase

TTACAGCTTGGCGCGCTCGGTGAACTGGACCGCTTCTCCGCGGATGAGGCGACCGATG
CCGTCGGGGTAGCCCTTGACGACGCCGTTTTCGTCGCGGGCGCCGCGCTCGCCCGAGT
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GCTGCCGAGTGGCGAGCAGAGGTACCAGTCGTCCAGGTCGAAGCTCTCGACGAGTCCC
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CGAGCTGCACGGGTGAGAGAAGCTGGAGCTCAAGGAAGTCGCTGAGCATGTCTGGGCT
CAAGATGCAGTTCTGAATGAGGTGGAAGCGCAGCAGCGCATCGAGCGCGGCAACGTGC
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GCTGCTGCACGAGCTGGGAGAAAGCGTATCCGATCAGGTAGGCTCCGTGGGCGGTCGA
GGCACGCACGGCGAGGTTGGTGTCGATGTGCTTGGATACGCCATCGGCGCCCGCTGTG
CTCGAGCGGTGCTGTGCGAGTCGCTCAACCAGAAGTGCAGCACGGAAGCCGAGCAGCC
CGAGCAAGCTTGCGGGTTGATCGATCTGGTCGACGCCGACAGCCTGCGAACGCTTCGA
AACGGCATCTGCGGAGCCGGACGGAGACGCGACAAGCTCGAGGTAGGCGAGCTCGGGG
TACTGTACCTTGCCGTTGCGCTTGCGCGACTCGTTGAGCTGCTTGACCAAAAAGTTGG
CCGAGGGTCCTGCGAGGAGCATCGAGTTCTCGCCCTCGTAGATGAGGCCGGCGTTGGG
CTGGTTGCGCTCAAAGTCGAAGCCGGCGGCCTGGCTGAAGCCGTGACCGCCCATGCTG
CGACGGCACCGTTCGATGCCGGCGAGCGATTCGTTGGTGCAGACGGCCTTGAGCACGC
TCGAGTAGCCGTGTACGATGGGGAGGAGCGCGCTGTTGCCCTCCGACTCGATCTCGGC
GATCATGCGAGTGTACAGGCGCCGGAGGTGCTGGCTGACAAAGGTGATGGCGTAGGCG

GAGGCAAGGGCAGTGAGCACGCGCGTCTGGACCGAGGCGTAGTCGAGGATCTTGGGCT
CGAGCTGGTCTGGCTGTGTGCTCGAGGTGCCCTGTCGTCGAACGACGGCGTAGCGGAT
CGCAATCGTGGCGGCACGCGCCAGGTGGTGGGCTGCAATCTCGCAGAGGCCGACGCGA
ACGAGGGTCATGGTGCCGCGCGTCATGACTTGGGCGGCGGCATTGCGCTTCTCGTAGA
CGCCGTCTCTGGAGACGGTCTGGAAGCGCTGGAGGAGATGCGAGCGCGGGATGCGAAC
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GGAAGGGGTGCATGCCGTACGACTTTTCGCTGATGATGAGGTTGGCCATGACCACGCC
GTACGTGGCGGTGAGACCGCTGCCTCCGATCCAGACCTTTCCAGCGGACGGCTCTGGA
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GGAGACCTGCTGACCCTTGAGGAAGCGTTCGTTGCGAGATTGGAAGACGCGATCCTCC
TTGTCAAAGATGTGATCCGGATCGTCCTTGAGAAGCGAGAGAAGCCAGCGTGCATTGT
CACGCTCGGTGCGCGAGCCGGGGAGGATGCACGACATGGCGTCGACGTCGAACGAGCA
TGCTGCGCGTTCCTGCTGGATGTCGCGCGGTTGCAAGGTCTCGGGTGCGGGTCTTTTG
GAAGCCAT

B.2 Putative hydratase-dehydrogenase-epimerase

The genetic sequence of the putative hydratase-dehydrogenase-epimerase, identified in *M. antarcticus*, based on protein homology.

ATGTCAGACTTCCCCTCCACCGAGAACGGGAAGATCTCGTTCAAGAACCGCGTCGTCG
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GCTGCCGATGTCGTCGTCGAGGAGATCCAGCAGGCCGGTGGCGAGGCCATCGCCAACT
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CATCGACATCCTCATCAACAACGCCGGTATCCTGCGTGACAAGTCTTTCAAGGCGATG
AGCGACAAGGAGTGGGACCAGATCACGGCCGTCCACATCACCGGCTCGTATGCCTGCG
CCAAGGCCGCATGGCCCCATATGCGCAAGCAGAAGTTCGGCCGCATCATCAACACCTC
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GCCATGATCGGTTTCGGCAAGACGCTCGCCATCGAGGGCGCAAAGTACAACATCCTCT
CCAACGTCCTCGCCCCCGTCGCCGCCAGTCAGCTCACCGCCACGGTCATGCCCCCCGA
GATGCTCGAGAACCTCACGCCCGACTACGTCGTCCCCATTGTCGCCTACCTCGTCTCC
GCCGAGAACAAGGAGGTTTCGGGCCACGTCTTCGAGTGCGGTGCCGGCTTCTTCGCCC
AGGTCCGCCGTGAGCGCAGCCGCGGCGTCGTCTTCAAGACCGACGACTCGTTCACCCC
CGCCGCCGTGCGTGCCAAGATTGACGAGATCCTCGACTTTGACGAGAAGCCCGAGTAC
CCCTTCCGCATCACCGACGCCAACCACATGGAGTTCCTCGAGCGTGCCAAGGAGGCCA
AGTCCAACGACCAGGGCGAAGGCCCCGTCCGTTTCGACGGCAAGACCGTGCTCGTCAC
CGGTGCCGGTGCCGGTCTCGGCCGTGCCTACGCTCACATGTTCGGCAAGCTCGGCGCC
AACGTGGTGGTCAACGACTTCCTCGAGAAGAACGCCGCCGCTGTCGTCGACGAGATCA
AGAAGGCGGGCGGCAAGGCCGCTCCCGCCGTCGGCTCGGTCGAGGACGGCGAGAAGAT
CGTCAAGGCGGCCGTCGACGCCTTTGGCTCGATCCACGTCATCATCAACAATGCCGGT
ATCCTGCGCGACAAGTCGTTCGCCGCCATGTCGGACCAGGAGTGGCACGCCGTGCTCA
ACACGCACCTCCGCGGCACCTACAGCGTCTGCCACGCCGCCTGGCCCATCTTCCAGCA
GCAGAAGTACGGCCGTATCGTCAACACCACCTCGGCCGTCGGCATCTACGGCAACTTT
GGCCAGGCCAACTACTCGACTGCCAAGGCCGGCATCATCGGCTTCACCAACACGCTCG
GCATCGAGGGCAAGAAGTACAACATTCTCGCCAACACGATTGCCCCCAACGCCGGTAC
CGCCATGACCGCCACCATCTGGCCCCAGGAGATGGTCGACGCTTTCAAGCCCGACTAC
GTTGCTCCCATGGTCGGCTACCTCGCCAGCGAGGCCAACGAGGACCTCACCTCGAGCC
TCTTTGAGGTCTCGGGTGGCTGGGTTGCTGCCGTCCGCTGGCAGCGCGCCGGCGGCCA
CTCGTTCAGCCACGGCAAGCCGCCCTCGCCCGAAAAGATTGTCAAGAAGTGGTCCAAG
ATCACCGACTACGAGACCAACCCCTCGTGGCCCACGAGCCCGAGCGACTCGCTCGGCG
ACATTGTCTCGAACTTCGGCCAGGAGGAGGAGGACGAGGATGATGACGACGAGGGCGG
AGCCGGTGGTGACTACAGCGACCCCGAGGACCCCGAGATTGTGCAGAAGGCCAAGAAG
GAGCCCATTGAGGACTCGGAGTTCTCGTACGGCGAGCGCGACGTCATCCTGTACAACC
TCGGCGTTGGTGCCACGGAGAAGGACCTCGACCTTGTGTTTGAGCAGGACGACGACTT
CCGAGCGGTGCCCACGTTTGGTGTGATCCCGCAGTTCATGGCGAGCGGCGGTATCCCT
CTGGACTGGCTGCCCAACTTTAGCCCCATGATGCTGCTGCACGGCGAGCAGTATCTGG
CGATCAAGAAGCCCATCCCCACGAGCGCGACGCTGGTGAACAAGCCCAAGCTGATGGA
GGTGCTGGACAAGGGCAAGGCTGCGGCGGTGACGTCGGTGGTGCACACGTTCGACAAG
GGCAGCGGCGACCTGGTGTTTGAGAGCCAGAGCACCGTCTTCATCCGAGGCTCGGGTG
GCTTTGGCGGCAAGAAGTCGGGCAAGGACCGCGGCGCAGCGTCGGCGGCGAACAAGCC
TCCGTCGCGCAAGGCGGACAAGGTGGTGACGGAGAAGACGACGGATGCGCAGGCTGCG
CTGTACCGTCTGTCGGGCGACTACAACCCGCTGCACATTGACCCCAGCTTTGCGCAGG
TGGGTGGCTTTGACAAGCCCATCCTGCACGGCCTGTGCTCGTTCGGTATCTCGGGCAA
GCACATCTTCCGCGAGTACGGCGCGTACAAGGACATCAAGGTGCGCTTCACCGGCCAC
GTCTTCCCCGGCGAGACGCTCGAGACGAGCATGTGGAAGGAGGGCAACAAGGTCATCT

TCACCACGCGCGTCGTCGAGCGCGACACCCAGGCTCTCGGTGCTGCCGCCGTCACGCT
GGCCGACGAGTAA

B.3 Putative 3-ketoacyl-CoA thiolase

Genetic sequence of the *M. antarcticus* putative 3-ketoacyl-CoA thiolase, obtained by protein homology.

3-ketoacyl-CoA thiolase

ATGTCTGCCGCCCAGCAACGCCACGGAAAGAAGAACATCCTCGCCGAGCACGACGACG
ATGTCGTCATTGTCTCGGCCCTGCGTACCGCCATCACCAAGGCCAAGAAGGGCGGTCT
CGCGCAGTGCGCTCCCGAGGAGATGCTCGGCTGGACCCTCAAGGGTGTCATTGCCGAG
TCCAAGATCGACCCCAAGCTGATTGAGGACGTTGCCGTCGGCACCGTGCTCGCCCCCG
GTGGTGGCTCCACCCAGGCGCGCATGGGCTCGCTCTGGGCTGGCATCCCCAACACTGC
CGGCTGCAACAGCTTGAACCGACAGTGCTCGTCCGGTCTGGCTGCCGTCAACCAGATT
GCTAACCAGATCGCGCTTGGCCAGATCGACATCGGCATTGGCTCTGGTGTTGAGTCGA
TGACGCTCAACTACGGTGCCGGTGTGATGCCCGCCAAAATGTCGGACGCCGTCATGGA
GAACGAGGAGGCAGCCGACTGCATGATGCCCATGGGTATCACCTCGGAGAACGTGGCC
AAGAAGTACAACATCAACCGCCAGAAGCAGGACACCTTTGCCGCCGAGTCGTTCTCTC
GCGCCGCCGCCGCACAGAAGGCGGGCAAGTTCAAGAGCGAGATCGTCACCGTCAAGTA
CACCGACGACGATGGCAACGAGCGCACCGTCGATGCTGACGACGGCATCCGCGAGGGC
GTCACTGTCGAGTCGCTCGGCAAGCTCAAGCCCGCCTTTGCCAAGGACGGATTCACGC
ACGCCGGTAACGCCTCGCAGGTCAGCGACGGTGCCGCCTCGGTCCTGCTCGCTCGCCG
CAGCGCCGCCAAGAAGCACGGCCTGCCCATCATCGGCAAGTTTGTCACGTGTGCCGTG
GTTGGTGTGCCCCCCAACATCATGGGTGTCGGCCCCGCCTACGCCATCCCGCGTCTGT
TCGAGCTCACCGGCCTCACCAAGGACGACATTGACATTTTCGAGATCAACGAGGCCTT
CGCCTCGCAGGCTCTCTTCTCGGTCGAGCACCTCGGCATCGACAAGAAGAAGGTCAAC
CCGGTCGGTGGTGCCATTGCCATGGGCCACCCTCTCGGTGCCACCGGTGCCCGCCAGA
TCGCCACCGGTCTTGCCGAGGCCAAGCGCCAGGGCGGCAAGAAGCTCATCGTCACCTC
GATGTGCGTCGGCACGGGCATGGGTTGTGCCTCGCTCATTGTCTCGGCCTGA

3-ketoacyl-CoA thiolase

ATGGCTCAACGAATCACCCAGCTTGCGTCGCACCTCGACCCACGCTCGTGGTCGGGCA
AGGGCCTGCACGCGCACCAGGCCAAGAACGACTCGGACGTCGTCATCGTCGCCGCGGG
CCGCACGCCGTTCACCAAGGCCTACAAGGGCTCCATGAAGGACGCCAAGTTCGATCTG
CTGTGCTACGAGTTCTTCAAGTCCATCGTTGCGTCCTCGGGCGTCGATCCCAAGCTCA
TCCAGGACATCGTCGTGGGCAACGTGCACAACGATGAGGCGCCCTACTACGTTCGCGC
CGCCGCGCTCGCTGCGGGTATCCCCAACACTACGCCTGCTATCGTCGTCAACAGGTTC
TGCTCCTCGGGTCTCATGGCGATCCGCGCGATCGCTAACGGCATCCAGGCCGGTGAGA
TCGAGTGTGGCCTTGCGTGCGGTATCGAGCACATGTCGACGCAGCCCAAGCGTCCCAC
GATGATCTCGGAGGAGCTGAGCAAGCTCAGCCAGGAGGCGGACGACTGCAAGATGCCC
ATGGGCTGGACCTCGGAGAACGTCGCCAACGACTTTGGCATCTCGAGGCAAAAGATGG
ACGAGTTTGCCGCGCGCTCGCACCAGCGCGCCGTGCAGGCGCAGTCGCAGGGCAAGTT
TGACGCCGAGATCTTCCCCATCTCGCTCCCCACCGTCGCCAAGGACGGCAGTCGTCAG
ACCTCGGTCATTTCCGCCGACGAGGGTCCGCGTGCAGGCACCACCGCAGAGTCGCTCG
GGAAGATTCGTCCCGCTTTCCCACAGTGGGCGCCCGGCAACACGACGGGCGGCAACGC
TTCGCAGATCACCGACGGTGCAGCCGGTGTGATTCTCATGCGTCGATCGCTCGCCAAC
AAGCTCGGCCTCACCATCCTGGGCAAGTACGTCTCGTGTGCCGTCACCGGTCTCGAGC
CTAGGATCATGGGCATTGGCCCCAGCAGTGCCATCCCTGCACTGCTCGAGCAGACGGG
CGTGAGTCAGGACCAGGTGGACCTGTTCGAGATCAACGAGGCCTTTGCAAGCATGTAC
GTCTACTGCGTCGAGAAGCTCGGTCTGGACCCGGAAAAGGTCAATGTCAACGGCGGCG
CATGTGCCCTCGGCCACCCCCTCGGCGCCACGGGTGCAAGGCTCGTCGTCACCGCCCT
CAACGAGCTCAAGAGGAGAAACCAAAAGGTCGCCGTCGTCTCCATGTGCATCGGCCTC
GGCATGGGCGCTGCCGGCCTCATCCTTCGCGAAGACTAG