Kaarel Kurm

Analysis of a putative *Candida albicans* homolog of the mitochondrial recombinase Mhr1 from *Saccharomyces cerevisiae*

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**Abbreviations**

BSA – bovine serum albumin  
CIAP – calf intestine alkaline phosphatase  
DMSO - dimethyl sulfoxide  
EDTA - ethylenediaminetetraacetic acid  
ETC – electron transport chain  
ori – replication origin  
ORF – open reading frame  
HS - hypersuppressive  
kb - kilobase  
PCR – polymerase chain reaction  
mtDNA – mitochondrial desoxyribonucleic acid  
rRNA – ribosomal ribonucleic acid  
TCA – citric acid cycle  
TRIS - tris(hydroxymethyl)aminomethane  
tRNA – transfer ribonucleic acid
Introduction

In eukaryotic cells, mitochondria are a major source of energy production. This function depends on the structural integrity of the mitochondrial (mt) genome. To ensure this, the processes behind mitochondrial DNA (mtDNA) maintenance, such as replication, repair and segregation need to be carried out flawlessly.

So far the budding yeast *Saccharomyces cerevisiae* has been one of the major sources of information on maintenance of the mt genome in yeast due to the relative ease of genetic manipulation in this species. Factors involved in mtDNA maintenance can easily be identified in this yeast, because it can tolerate the loss of its functional mt genome under anaerobic growth conditions in the presence of fermentable carbon sources. Characterisation of the role of those factors in *Saccharomyces cerevisiae* wild type mtDNA maintenance however is made difficult by the fact that the removal of the factor to be studied often causes changes to the mt genome (Contamine and Picard, 2000).

*Candida albicans* from the same family (Saccharomycetaceae) is a different model for mtDNA maintenance, because similarly to more complex eukaryotes it cannot tolerate loss of mtDNA. It is more difficult to genetically manipulate, but the strict need for intact mtDNA allows for examination of the maintenance of wild-type mtDNA. A few homologs to nuclear factors related to mtDNA maintenance in *Saccharomyces cerevisiae* have been characterised in *Candida albicans*. For example, the mtDNA helicase CaHmi1 (Jõers et al., 2007), which is homologous to ScHmi1. CaGcf1, a nonspecific DNA-binding protein (Visacka et al., 2009) is a protein that is homologous to ScAbf2. ScAbf2p has strong affinity to dsDNA (Miyakawa et al., 1995) and has been shown to bend dsDNA, compacting it into structures that resemble mitochondrial nucleoids (Brewer et al., 2003).

*Candida albicans* ORF 19.439 (open reading frame) shows sequence homology with *MHR1* from *Saccharomyces cerevisiae*. In *Saccharomyces cerevisiae* Mhr1 is a recombinase in mitochondria (Ling et al. 1995). While *MHR1* in *Saccharomyces cerevisiae* (*ScMHR1*) has been well studied in recent years (Ling et al., 1995; Ling et al., 2000; Ling et al., 2002; Ling et al., 2004; Ling et al., 2007; Ling et al., 2009; Hori et al., 2009), the possible homolog from *Candida albicans* has not been examined so far. The aim of this thesis was to investigate if Candida albicans orf19.439 could be involved in mtDNA maintenance by assaying if it can complement *ScMHR1*.
1. Literature overview

1.1 Mitochondria

Mitochondria are organelles in eukaryotes. They are separated from the cytoplasm by two membranes that consist of proteins and a phospholipid bilayer. Mitochondria can be divided into five compartments: the outer membrane, the inner membrane, the intermembrane area, the cristae space and the mitochondrial matrix which is the innermost compartment. Cristae space is the space between invaginations of the inner membrane. The matrix is a highly concentrated mix of various enzymes, mitochondrial ribosomes, tRNA and copies of their own mitochondrial genome, the mtDNA (Lodish et al., 2004).

The ability of mitochondria to act as a major energy source for the cell is made possible by the presence of the structural enzymes of the Citric Acid Cycle (TCA) in the matrix and of the Electron Transport Chain (ETC) in the mitochondrial inner membrane. These enzymes oxidize the products of glycolysis – NADH and pyruvate. To do this, they need the presence of oxygen. Generation of ATP via this process is called aerobic respiration or oxidative phosphorylation. In the absence of oxygen ATP can be generated from the products of glycolysis via fermentation but the ATP yield per one molecule of glucose is approximately 15 times lower (Reviewed in Kornberg, 2000).

As a prerequisite of phosphorylative oxidation, pyruvate molecules first pass through the outer mitochondrial membrane passively. After this they are transported through the inner mitochondrial membrane into the mitochondrial matrix via active transport. In the matrix pyruvate is reduced and combined with coenzyme A, resulting in CO₂, acetyl-CoA and NADH.

Acetyl-CoA is the primary substrate of citric acid cycle. One of the enzymes used in the citric acid cycle is anchored to the inner mitochondrial membrane while the other enzymes of this pathway are inside the mitochondrial matrix. Acetyl-CoA is used to transfer the two-carbon acetyl group to the four-carbon acceptor compound oxaloacetate. This forms citrate. During the citric acid cycle two carboxyl groups are oxidated and evaporate as CO₂. The resulting energy is stored in energy-rich cofactors. Per one cycle three molecules of NADH and one molecule of both FADH₂ and GTP are produced (Voet and Voet, 2005).

The Electron Transport Chain (ETC) consists of proteins located in the inner mitochondrial membrane. The high energy molecules NADH and FADH₂ donate their high
energy electrons to the ETC. As the electron from NADH passes through the complex there are 3 sites where a proton is moved from the matrix to the intermembrane space using the incremental releases of energy. Due to a lower reduction potential of FADH$_2$ the electron from this molecule is donated to a later part of the chain and the electron encounters only 2 sites where a proton is moved. This flow of electrons through the ETC causes a proton gradient to form between the mitochondrial matrix and the intermembrane space. The protons can return to the matrix through the ATP synthase. The potential energy of passing protons is used to synthesise ATP from ADP and inorganic phosphate. (Voet and Voet, 2005)

About 95% of the proteins in mitochondria are of nuclear origin (Neupert, 1997). Such proteins are synthesised with a mitochondrial targeting signal, which is usually located in the N-terminal side of the protein. This signal is cleaved from the protein after it has reached the correct intramitochondrial location (Schatz, 1996). The mitochondrial genome codes for certain subunits of the ETC and ATP synthase. For the expression of these necessary genes flawless maintenance of mtDNA needs to be ensured.

Yeasts have proven to be a good target for studies of mtDNA maintenance, *Saccharomyces cerevisiae* for its ability to be easily manipulated and *Candida albicans* because of its strict need of mtDNA that allows investigation of the maintenance of wild-type mtDNA (Contamine and Picard, 2000; Jõers et al., 2007).

### 1.2. *Candida albicans*

The yeast *Candida albicans* is a diploid fungus that is an opportunistic pathogen in humans. It can change between growth as unicellular yeast and multicellular filament (Calderone and Fonzi, 2001).

*Candida albicans* was thought to be asexual until a mating type-like locus was discovered. This locus was very similar to the mating locus of *Saccharomyces cerevisiae* and showed that the key mating regulators are conserved between the two yeasts (Hull and Johnson, 1999). In *Saccharomyces cerevisiae* the mating locus determines the cells mating type. Only cells of different mating type can mate. Genetical manipulation has allowed for showing mating of *Candida albicans*, albeit at a very low frequency. Through mating, tetraploids are formed that have been shown to lose the extra chromosomes randomly, resulting in diploids. This is referred to as parasexual cell cycle (Bennet and Johnson, 2003). So far, *Candida albicans* has not been
shown to undergo meiosis, although its genome has been shown to code several orthologs to genes involved in meiosis of other fungi (Tzung et al., 2001).

1.2.1 Mitochondrial DNA of *Candida albicans*

The mitochondrial genome of *Candida albicans* is approximately 40 kilobases (kb) in size. It codes for 11 proteins that are part of the Citric Acid Cycle or Electron Transport Chain, 3 proteins from the ATP synthase complex, 2 rRNAs and 30 tRNAs (Anderson et al. 2001). Restriction Fragment Length Mapping of *Candida albicans* mtDNA has shown the presence of linear molecules of variable length and molecules with complex structures. No trace of circular molecules was witnessed (Jõers et al., 2007).

Maintenance of the mitochondrial genome in *Candida albicans* is largely unexplored. Recently a homolog to a DNA-binding protein Abf2p that mediates mtDNA compaction into nucleoids in *Saccharomyces cerevisiae* was characterised in *Candida albicans*. The protein, CaGcf1 was identified as a non-specific DNA-binding protein that is localised into the mitochondria and is involved in recombination and replication of mtDNA (Visacka et al., 2009).

In an earlier study a helicase in *Candida albicans*, Hmi1 (CaHmi1) has been characterised and was shown to play a crucial role in the stability of mtDNA, as deletion of both *CaHMI1* alleles resulted in a reversible fragmentation of the mitochondrial genome (Jõers et al., 2007). CaHmi1 was identified as a homolog to the already characterised Hmi1 helicase in *Saccharomyces cerevisiae* where it was shown to be required for maintenance of wild-type mitochondrial DNA (Sedman et al., 2000).

1.3 *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* is a unicellular yeast. It has 2 viable states – haploid and diploid (Sherman, 1991). There are two types of the haploid cells, called mating types “a” and “α”. Haploids of different mating type can conjugate and form diploids. In unfavourable conditions the diploids can sporulate and produce haploid offspring via meiosis (Watson et al., 2004). Vegetative cell division of *Saccharomyces cerevisiae* occurs by budding, a process in which the
daughter is first introduced as an outgrowth from the mother cell. This is followed by meiosis, formation of the cell wall and separation of the cells (Sherman, 1991).

*Saccharomyces cerevisiae* is a good subject for biological research due to its well-defined genetic system, rapid growth [a division time of 90 minutes under ideal conditions (Watson et al., 2004)], an effective transformation system, and a high rate of homologous recombination. A high rate of homologous recombination allows for exact gene disruption and allele-specific replacement (Forsburg, 2001).

### 1.3.1 Mitochondrial DNA of *Saccharomyces cerevisiae*

The size of *Saccharomyces cerevisiae* mtDNA is approximately 80-85 kilobases. This is about twice as big as *Candida albicans* mtDNA, but the coding capacity of both mitochondrial genomes is similar. The *Saccharomyces cerevisiae* mitochondrial genome codes for 7 proteins that take part in ETC and TCA (7 less than in *Candida albicans*), 2 rRNAs and 24 tRNAs (6 less than in *Candida albicans*). As another difference, 2 proteins that are not coded by *Candida albicans* mtDNA are present: Var1p, a ribosome associated protein, and 9S RNA, a component of RNase P (Foury et al., 1998).

There are 20 to 100 copies of the mitochondrial genome per a haploid cell. This constitutes close to 5-15 % of the cell’s total DNA. MtDNA copies are organised into multiple nucleoids per cell with each nucleoid containing a few copies of the mitochondrial genome (Williamson 1976; 1977). The majority of mtDNA in *Saccharomyces cerevisiae* consists of concatemers - variable sized linear head-to-tail multimers of one genomic unit, branched molecules and a fraction of circular molecules (Maleszka et al., 1991).

After mating the mitochondria from both cells merge rapidly and a continuous mitochondrial reticulum is formed that contains mtDNA from both of the parents. (Nunnari et al., 1997). Parental mtDNAs show a high rate of recombination (Wilkie and Thomas, 1973). Heteroplasmy is not maintained for long in the descendant cells, segregating to homoplasmy in 10 to 20 subsequent cell divisions (Dujon et al., 1981).

The mtDNA of *Saccharomyces cerevisiae* contains sequences called ori/rep which are believed to function as origins of replication. Depending on the strain analysed, there are 7 or 8 of those sequences in the mitochondrial genome, 3 or 4 of which are considered to be active (Baldacci et al., 1984). The ori sequence is a 300 base-pair sequence that contains 3 GC-nucleotide rich clusters A, B and C, which are separated by AT-rich sequences. The transcription initiation site was determined to be upstream of the GC cluster C (Baldacci and Bernardi, 1982).
Many components of the replication machinery of *Saccharomyces cerevisiae* mtDNA are well explored, but the mechanisms behind them remain partially speculative. So far there are three hypotheses for the mode of yeast mtDNA replication that have been proposed. The rolling cycle replication (Maleszka et al., 1991), recombination-dependant replication (MacAlpine et al., 1998) and transcription initiated strand-coupled replication (Lecrenier and Foury, 2000). However, topological evidence in favour of one over another mechanism is missing and the proposed models are based on single or concerted functions of different identified protein factors.

*Saccharomyces cerevisiae* is able to grow on fermentable media while having damaged or no mitochondrial genome. Such cells are called petites because of their small growth (Ephrussi et al. 1949). Species who can tolerate having a damaged mitochondrial genome have been termed petite positives (Bulder, 1964). The mitochondrial genomes of petite positives are divided into three categories: $\rho^+$, $\rho^-$ and $\rho^0$. $\rho^+$ cells have a wild-type mitochondrial genome, $\rho^-$ exhibit a mitochondrial genome that contains tandem repeats of a random fragment from the wild-type genome and $\rho^0$ are devoid of mtDNA (Dujon, 1981). Approximately 2% of $\rho^+$ *Saccharomyces cerevisiae* cells turn into $\rho^-$ or $\rho^0$ spontaneously, with this chance increasing in poor growth conditions (Slonimski et al., 1968).

The phenomenon where wild-type mtDNA is outcompeted by $\rho^-$ mtDNA in crosses is called suppressiveness (Ephrussi et al. 1955). If $\rho^+$ mtDNA prevails in all of the progeny cells, the $\rho^-$ strain is called a neutral petite. When 95% or more of the progeny are $\rho^-$ the $\rho^-$ strain that was crossed is called hypersuppressive (HS). It is believed that hypersuppressiveness is caused if the mtDNA fragment maintained contains an active ori/rep, resulting in multiple copies of the ori/rep sequence per mtDNA molecule. This is thought to cause preferential replication of such $\rho^-$ mitochondrial genomes (Blanc and Dujon, 1980). That finding led to the belief that HS strains are a suitable target to study mtDNA replication mechanisms in *Saccharomyces cerevisiae*.

### 1.4 The mitochondrial recombinase Mhr1

*Saccharomyces cerevisiae* MHRI (*ScMHRI*) is a nuclear gene that codes for a protein of 226 amino acids. The mass of this polypeptide is 26.9 kDa. The protein has been shown to be localised into the mitochondrial matrix and the inner mitochondrial membrane (Ling et al., 2002).

Disruption of the *MHRI* gene has been shown to cause increased susceptibility to UV-induced mtDNA damage (Ling et al., 1995). Cells lacking functional Mhr1 protein were also shown to be deficient in mitochondrial homologous recombination (Ling et al., 1995). When
grown on 37°C, cells with disrupted Mhr1 showed damage accumulation in the mitochondrial genome. After the amount of damage reached a certain level respiratory function was lost (Ling et al., 1995).

Accumulation of mtDNA damage has been related to reactive oxygen species, derived from the reactions in the Electron Transport Chain (Richter, 1992). When *Saccharomyces cerevisiae* cells were grown in the presence of malonic acid, an inhibitor to the citric acid cycle that reduces oxidative stress in the mitochondrion, high petite production in the Mhr1 deficient cells was partially suppressed. When azide was added to the growing cells the petite formation rate was enhanced greatly in Mhr1-deficient cells. Azide is an inhibitor to the last enzyme of the Electron transport chain, and causes electron leakage. Wild-type cells showed only a minor increase in petite formation. Together, these results indicated that a function of Mhr1p is related to the repair of damaged mitochondrial DNA (Ling et al., 2000).

In vivo studies have shown that Mhr1p pairs homologous single-stranded and double-stranded DNA, forming a heteroduplex joint. This activity does not need ATP or any other high-energy co-factor (Ling et al., 2002). Heteroduplex joints are general intermediates of homologous DNA recombination (Holliday, 1964). Mhr1-mediated homologous recombination has been proposed to initiate rolling circle DNA replication which could be a pathway for producing the concatemers that are the prevailing form of mtDNA. (Ling et al., 2002). The amount of concatemeric mtDNA molecules depends on Mhr1 activity and labelling experiments have shown that concatemeric DNA is the immediate product of DNA replication (Ling et al., 2004).

In addition it has been proposed that Mhr1 plays a role in segregation of heteroplasmic mtDNA by playing a role of mtDNA transmission into the buds. The required number of mitotic divisions to reach homoplasmy was shown to decrease in the presence of overexpressed Mhr1 and to increase in the absence of functional Mhr1.
2. Experimental part

2.1 Materials and methods

2.1.1 Strains of bacteria and yeast used in this study

Table 1 lists strains of *Escherichia coli* and *Saccharomyces cerevisiae* used or constructed in the course of the present work.

**Table 1: Escherichia coli and Saccharomyces cerevisiae strains used**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Modifications</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR supG φ80delZΔM15 Δ(lacZYA-argF)U169, hsdR17(rlK’ mK’ +), λ-</td>
<td>-</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td><em>E. coli</em> TOP 10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 supG recA1 araD139 Δ(ara-leu)7697 gale15 galK16 rpsLmStr8 endA1 λ-</td>
<td>-</td>
<td>Casadaban and Cohen, 1980</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δmhr1::trp</td>
<td>this study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α Δmhr1</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δmhr1::trp</td>
<td>this study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α pRS316</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>pRS316</td>
<td>this study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α pRS-CaMHR1</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>pRS-CaMHR1</td>
<td>this study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α Δmhr1 +pRS-CaMHR1</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δmhr1::trp+pRS-CaMHR1</td>
<td>this study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> W303α pRS-CaMHR1 Δmhr1</td>
<td>ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</td>
<td>Δmhr1::trp</td>
<td>this study</td>
</tr>
</tbody>
</table>
2.1.2 Media and growth conditions

*Escherichia coli* cells were grown at 37°C in 2xYT (1% yeast extract; 1,6% tryptone; 0,5% NaCl) liquid media or on solid media (2xYT containing 1,7% agar). Liquid cultures were grown on a shaker (Ijeio Tech SI-900 Shaking incubator) at 190 rpm.

*Saccharomyces cerevisiae* cells were grown at 30°C. Different yeast media were used, both in liquid and solid form. The following liquid media were prepared and used as indicated: YPD (1% Yeast extract; 2% Peptone; 2% Glucose;), YPG (1% bacto yeast extract; 2% bacto-peptone; 3% glycerol), Synthetic Complete (SC) with glucose or glycerol (1,34% Yeast Nitrogen Base; 2% glucose or 3% glycerol; 2% of total amino-acid mix; 0,25% leucine; 0,02% adenine; 0,1% histidine; 0,1% lysine; 0,05% uracil; 0,1% tryptophane). For preparation of solid media, 1,7% agar was added.

2.1.3 Plasmid vectors used in this study

Table 2 lists all plasmid DNA vectors that were used or constructed in the current study.

**Table 2: Plasmids used in this work**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Notes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS316</td>
<td>A cloning vector containing URA3, lacZ'/MCS, Amp'</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
<tr>
<td>pRS316ΔSacI</td>
<td>Sacl site is removed from the Multiple Cloning Site (MCS)</td>
<td>this study</td>
</tr>
<tr>
<td>pRS316-P</td>
<td>pRS316ΔSacI with ScMhr1 promoter inserted</td>
<td>this study</td>
</tr>
<tr>
<td>pRS316-PT</td>
<td>pRS316-P with ScMhr1 terminator inserted next to the promoter</td>
<td>this study</td>
</tr>
<tr>
<td>pGEM7-</td>
<td>A cloning vector containing, lacZ'/MCS, Amp'</td>
<td>Promega Corporation</td>
</tr>
<tr>
<td>pGEM7-CaMHR1</td>
<td>pGEM7- with inserted CaMHR1 ORF 19.439</td>
<td>this study</td>
</tr>
<tr>
<td>pRS-CaMHR1</td>
<td>pRS316-PT with CaMHR1 ORF 19.439 inserted between the ScMhr1 promoter and the ScMhr1 terminator</td>
<td>this study</td>
</tr>
<tr>
<td>pBKS-MHR1</td>
<td>pBKS with an insertion of ScMHR1 orf, with 300 bp upstream of the start codon and 300 bp downstream of the</td>
<td>T. Sedman</td>
</tr>
</tbody>
</table>
stop codon

**pUC19-TRP**
pUC19 cloning vector containing TRP cassette, coding for phosphoribosylanthranilate isomerase catalysing a step in tryptophan biosynthesis

**pBKS-Δmhr1TRP**
TRP gene from pUC19-TRP replaces most of the *MHR1* sequence from pBKS-*MHR1*

### 2.1.4 Oligonucleotides used in this work

Oligonucleotides used in this study served as DNA primers for PCR. PCR products were used for cloning and therefore, primers were designed with suitable restriction sites. New restriction sites introduced are indicated in the oligonucleotide name. Nucleotides changed from the original sequence to introduce restriction sites are denoted by small letters in *italics* in the sequence column. Restriction enzyme recognition sites are denoted by underlined sequence. Oligonucleotide primers were obtained from TAG Copenhagen A/S (Denmark).

**Table 3**: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Nucleotide sequence</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ScMHR1prom</em> XbaI</td>
<td>5'-TTGTATCTGAAGTTCTTTATTTTCACACAG-3'</td>
<td>58.6</td>
</tr>
<tr>
<td><em>ScMHR1prom</em> SacI</td>
<td>5'-TTACCTTCATGAGCTGCGCCTGAAATATCG-3'</td>
<td>66.8</td>
</tr>
<tr>
<td><em>CaMHR1</em> SacI</td>
<td>5'-TATTTGAGCATGCGCTATGCACTAGAGAC-3'</td>
<td>66.8</td>
</tr>
<tr>
<td><em>CaMHR1</em> EcoRI</td>
<td>5'-ACAAAAAGAATTCTCAAGCAGTCTCTGTCAGCAG-3'</td>
<td>64.0</td>
</tr>
<tr>
<td><em>ScMHR1term</em> EcoRI</td>
<td>5'-CTTCCTCTGTCATTAGATA-3'</td>
<td>62.7</td>
</tr>
<tr>
<td><em>ScMHR1term</em> XhoI</td>
<td>5’-TCCTCGACTTATTTCTCTCTCAG-3’</td>
<td>64.0</td>
</tr>
<tr>
<td><em>MHR1downPST</em></td>
<td>5'-AGGCTGCAGATGAGATAAGCTCAGTCTCTCTGTCACACCC-3’</td>
<td>69.5</td>
</tr>
<tr>
<td>Trp3out</td>
<td>5'-ATGTAAGCAGAGTGGTGGAGGAGGAG-3’</td>
<td>59.4</td>
</tr>
<tr>
<td>Trp5out</td>
<td>5’-AGCAAGTCAGCGATCGGAATC-3’</td>
<td>57.3</td>
</tr>
</tbody>
</table>
2.1.5 Cloning works

2.1.5.1 PCR of regions of interest
Polymerase Chain Reaction (PCR) was used to amplify *Saccharomyces cerevisiae* Mhr1 promoter (ScMHR1prom) and terminator (ScMHR1term) regions and *Candida albicans* orf19.439 (CaMHR1). The promoter consists of the sequence upstream of the start codon of the Mhr1 gene in *Saccharomyces cerevisiae* (ScMHR1, systematic name YDR296W). The terminator is the sequence downstream of ScMhr1 stop codon. Primers used were ScMhr1promXbaI and ScMhr1promSacI for ScMHR1prom, CaMhr1SacI and CaMhr1EcorI for CaMHR1, ScMhr1termEcorI and ScMhr1termXhoI for ScMHR1term (Table 1).

The PCR reactions were carried out under the following conditions, in a final volume of 20 µl: 1x Pfu buffer (Fermentas); 0,2mM dNTPs; 2,5mM MgSO4; 1pmol of both primers; 0,5U of Pfu polymerase; ~100ng of genomic DNA (*Saccharomyces cerevisiae* DNA for the promoter and terminator region, *Candida albicans* DNA for the ORF). The PCR program consisted of an initial denaturation at 95°C for 3 minutes, after which 29 cycles of 30 seconds of denaturation at 95°C, 30 seconds annealing at 57/60/57°C (promoter/ORF/terminator respectively; according to primer requirements) and 80 seconds of extension at 72°C were carried out, with a final extension step of 3 minutes. Genomic DNA from *Saccharomyces cerevisiae* was prepared as described in 2.1.8.3, genomic DNA from *Candida albicans* was kindly provided by my supervisor Joachim M. Gerhold.

2.1.5.2 DNA modification
Restriction endonucleases were used according to the manufacturers protocols (Fermentas) to digest DNA sequences for cloning.

If needed, removal of single-stranded 3’-overhangs resulting from restriction was carried out by using 3’>5’exonuclease activity of T4 DNA polymerase (Fermentas) in a 20µl reaction containing: 1U of T4 DNA polymerase; 1x T4 DNA polymerase buffer (Fermentas); 0,1mM dNTPs. The mix was incubated at room temperature (RT) for 5 minutes.

Where needed, dephosphorylation reactions with CIAP (Fermentas) were carried out on vectors prepared for ligation of blunt products.

For ligations, T4 DNA ligase (Fermentas) was used. A 20µl reaction consisted of: 1U of T4 DNA Ligase; 1x T4 DNA ligase buffer. 2µl of 50 % PEG4000 and 0,5µl of 2µg/µl BSA were added for blunt-end ligations.
2.1.5.3. DNA clean-up upon modification

For purification of DNA upon modifying reactions, two methods were used: phenol/chloroform extraction or purification from an agarose gel.

The phenol/chloroform extraction was carried out as follows. For sample volumes smaller than 200 µl, the volume of the DNA-containing sample was adjusted to 200µl with sterile MQ. Larger samples were aliquoted accordingly. Dextrane was added, to a final concentration of 0,2mg/ml. 200µl of phenol/chloroform (pH 7,5) were added and the sample was vortexed thoroughly. The watery and phenol/chloroform phases were separated by centrifugation at 3500 rpm for 5 minutes (Eppendorf „Minispin“, F 45-12-11 rotor). The watery upper phase was carefully transferred to a fresh tube. 0,1 volumes of 3M NaOAc and 3 volumes of 100% v/v Ethanol were added, thoroughly mixed and kept at -70°C for 30 minutes to precipitate the DNA. The sample was centrifuged at maximum speed (Eppendorf „Minispin“, F 45-12-11 rotor) for 10 minutes and the supernatant was discarded. The remaining DNA pellet was washed with 300µl of 80% v/v ethanol, centrifuged again at maximum speed and the supernatant was carefully removed. Remaining Ethanol was allowed to evaporate, after which the DNA was taken up in 10-20µl of T

For DNA extraction from agarose gels, the DNA to be purified was separated on 0,8% agarose gels with 300ng/ml ethidium bromide. Correct bands were excised from the gel and DNA was purified from the gel block using a UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Inc) kit according to the manufacturer’s protocol.

2.1.6 Transformation of Escherichia coli, plasmid preparation

2.1.6.1 Transformation protocol

Escherichia coli DH5α or TOP10 were transformed with plasmid DNA as follows. 100µl of heat-shock competent DH5α which had been stored on -70°C were melted on ice. 10µl of a ligation reaction was added, mixed and the cells were kept on ice for 30 minutes. Then, heat-shock at 42°C for 90 seconds was performed after which cells were held on ice for 5 minutes. After this, cells were transferred into 1ml of liquid 2xYT medium supplied with 0.8% Glucose and 20mM MgCl₂, and incubated in a shaker for 15 minutes at 37°C at 175 rpm. After this the cells were concentrated by a centrifuging 2 minutes at 4000 rpm (Eppendorf „Minispin“, F 45-12-11 rotor).
The supernatant was removed, the cells taken up in 200µl sterile H₂O and aliquots of 10-100 µl were plated onto 2xYT plates containing 200µg/ml ampicillin. The plates were incubated at 37°C over night (o.n.).

2.1.6.2 Plasmid purification from E.coli
For plasmid preparation, colonies were randomly picked from transformation plates and inoculated in 3 ml of 2xYT. 1,5ml of a dense over-night culture were centrifuged at 7000rpm for 2 minutes (Eppendorf „Minispin“, F 45-12-11 rotor). The supernatant was removed and the pellet redissolved in 200µl of Sol I. After a thorough vortex 200µl of Sol II were added, the sample tube was gently inverted 10 times and incubated at RT for 5 min.. 200µl of ice-cold Sol III were added and the tube was inverted as above. The mixture was incubated on ice for 10 minutes and centrifuged (Hettich „Mikro200R“ Rotor 2424) at 4°C and 14000 rpm for 15 minutes. The supernatant was carefully transferred to a fresh reaction tube and 0.7 volumes of isopropanol were added, followed by vortexing. The mix was centrifuged at RT for 15 minutes at 14000 rpm. The supernatant was discarded and the remaining pellet was washed with 80% v/v ethanol, centrifuged as before and the supernatant was removed. Remaining ethanol was allowed to evaporate and the pellet was taken up in 30µl of T₁₀E₁.

Solutions used:
**T₁₀E₁**: 10mM TRIS; 1mM EDTA
**Sol I**: 50 mM glucose; 10mM pH8 EDTA; 25mM pH8 Tris; 200µg/ml RNase
**Sol II**: 0,2M NaOH; 1% SDS
**Sol III**: 3M KAc; 0,19M acetic acid

2.1.7 Sequencing
DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech) was used to sequence cloned DNA constructs. Sequencing reactions were carried out in a 10µl volume. 4 different reactions were done to cover the complete cloned sequence. The reactions consisted of 1x buffer; 1,6pmol of primer (1 primer per reaction, in this study primers used were: ScMhr1promXba, CaMhr1SacI, CaMhr1EcoRI, ScMhr1TermXho, Table 4); ~20ng of construct containing plasmid DNA. The sequencing program was 1 minute initial DNA denaturation at
95°C followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 20 seconds and elongation at 60°C for 1 minute.

2µl of salt (1,5M Sodium acetate; 250mM EDTA) with Dextrane and 30µl of 96% Ethanol were added and the mixtures were incubated at -20°C for 15 minutes, centrifuged (Hettich „Mikro200R“ Rotor 2424) for 10 minutes at 14000 rpm at RT and the supernatant was discarded. 100µl of 80% v/v Ethanol were added to samples, mixed and centrifuged at 14000 rpm for 5 minutes. Supernatant was removed and the pellet was dried at 37°C for 10 minutes. The pellet was resuspended in 10 µl of 70% formamide. Sequencing was a commercial service at the Estonian Biocentre (Riia 23B, Tartu, Estonia).

2.1.8 Construction of Saccharomyces cerevisiae strains

2.1.8.1. Saccharomyces cerevisiae transformation protocol
Transformations of Saccharomyces cerevisiae were carried out as follows: Saccharomyces cerevisiae cells from a Glycerol stock were plated onto YPD and incubated at 30°C for 3 days. From the plate a colony was randomly picked and grown overnight in 3ml of liquid YPD. OD600 of the overnight culture was measured with a spectrophotometer (Pharmacia Biotech „Ultrospec2000) and cells from the culture were inoculated into 50ml liquid YPD or Synthetic Complete to get a starting OD600 of 0,1. Cells were grown up to an OD600 of 0,6-0,8 at which the cells were harvested by centrifugation (Hettich „Universal 32R“ 1617 swing-out rotor) at 3200 rpm for 2 minutes at room temperature. Supernatant was carefully removed and the remaining pellet was washed with 50ml of sterile MQ water. Cells were centrifuged as before and washed with 50ml of LiAc0,1T10E1. The cells were centrifuged as above and the supernatant was removed. In parallel, an aliquot of 2 mg/ml salmon sperm DNA (Amresco) in H2O was denatured at 100 °C for 5 minutes an immediately incubated on ice for at least 5 minutes to generate single-stranded carrier DNA.. The yeast cell pellet after centrifugation was taken up in 300µl of LiAc0,1T10E1 (0,1M LiAc; 10mM TRIS; 1mM EDTA )and 50µl of 2mg/ml single-stranded carrier DNA were added to protect the transformed DNA from degradation. 50µl aliquots of competent yeast cells were taken per transformation and ~1µg of the DNA to be transformed was added. The mix was vortexed and incubated at room temperature for 15 minutes. 300µl of 50% PEG4000 were added, the mix was again incubated at room temperature for 15 minutes. Finally 30µl of 100% dimethyl sulfoxide (DMSO) were added and the mix was vortexed for 1-2 seconds after which a heat-shock of 10 minutes at 42°C was performed. Cells were pelleted by
centrifugation at 3000rpm (Eppendorf „Minispin“, F 45-12-11 rotor) and the supernatant was removed. Cells were taken up in 100µl of sterile MQ water and plated to selective SC glucose and glycerol plates.

2.1.8.2 Storage of the constructed strains
After verification, the strains were stored as glycerol stocks. From a fresh plate a colony was picked and inoculated separately into 3ml of YPD and 3ml of YPG. After overnight growth at 30°C in a shaker (Ijeio Tech SI-900 Shaking incubator) at 190 rpm 600µl of cells were taken and 50% Glycerol was added to a final concentration of 20%, vortexed well and snap-frozen in liquid nitrogen. Glycerol stocks were stored at -80°C.

2.1.8.3 Total DNA extraction of Yeast mini-scale preparations
Colonies from a fresh plate were picked and inoculated separately in 3ml of selective SC media. After overnight incubation at 30°C on a shaker (Ijeio Tech SI-900 Shaking incubator) at 190 rpm, the cells were collected by centrifuging 3 minutes at 5000 rpm (Eppendorf „Minispin“, F 45-12-11 rotor). Supernatant was removed, 200µl of lysis buffer (100mM NaCl; 10mM pH8 Tris; 2% Triton; 1% SDS; 1mM EDTA) and ~300µl of 0.5 mm diameter glass beads were added. Cells were broken by vortexing at maximum speed (“Vortex Genie 2”, Scientific Industries, Inc.) for 5 minutes after which another 200µl of the lysis buffer, 200µl of Phenol/Chloroform pH7.5 were added and the tubes were further vortexed at maximum speed for 2 minutes. Tubes were centrifuged at 13400 rpm (Eppendorf „Minispin“, F 45-12-11 rotor) and the upper, aqueous phase was transferred to fresh tubes. 2 volumes of 100% v/v Ethanol were added and mixed carefully to avoid shearing the genomic DNA. After 5 minutes of centrifugation at 13400 rpm the supernatant was removed and the remaining pellet was washed with 500µl of 80% v/v ethanol. The supernatant was removed and remaining ethanol was allowed to evaporate. The pellets were taken up in 20µl of MQ.

1 µl of a 50 times dilution of the extracted DNA was used as PCR template for strain verification by PCR. Primers used are listed in Table 3.

2.1.9 Complementation assay
Two approaches were used to qualitatively and quantitatively determine the ability of CaMHRI to complement the loss of ScMHRI.
For a qualitative assay, cells from glycerol stocks of each strain were plated onto YPD, YPG and if selective pressure for transformed constructs was desired, onto selective SC glucose and SC glycerol plates and grown at 30°C. Colonies from each plate were re-streaked to plates with the identical media and to plates with opposite carbon source than initially used and further grown at 30°C. Plates were documented by digital photography.

For a quantitative assay, growth curves of each strain were constructed by measuring the growth of all strains in YPD and YPG. From fresh selective SC glucose plates a colony of each strain was picked randomly and grown in an overnight culture. The $OD_{600}$ was measured with a spectrophotometer (Pharmacia Biotech „Ultrospec2000) and a dilution of each culture was made into 20ml of YPD and 20ml of YPG to get a starting $OD_{600}$ of 0,1. The dilutions were incubated at 30 °C in a shaker (Ijeio Tech SI-900 Shaking incubator) at 190 rpm and their $OD_{600}$ values were measured every 75 minutes for 11 h. A last time point was measured after o.n. incubation.
2.2 Results

Initial BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi#) for sequences homologous to the nucleotide sequence of *Saccharomyces cerevisiae* MHR1 revealed ORF 19.439 from *Candida albicans* as a possible homolog of the mitochondrial recombinase ScMhr1. Further *in silico* analyses of the amino acid sequences of both ORFs showed 46% identity and 46% similarity of both protein sequences. Based on these findings, further analyses of *Candida albicans* ORF 19.439 were approached.

2.2.1 Cloning of PCR products into a plasmid used for expression of CaMhr1

The promoter and terminator regions of *Saccharomyces cerevisiae* MHR1 (*ScMHR1*prom, *ScMHR1*term) and ORF 19.439 from *Candida albicans* (*CaMHR1*) were produced by PCR as described in 2.1.5.1. Obtained products were checked on 0.8% TAE Agarose gels which contained 300ng/ml ethidium bromide. The expected sizes were 550bp and 655 for *ScMHR1*prom and *ScMHR1*term respectively, and 720bp for *CaMHR1* and the gel confirmed this (Figure 1).

![Figure 1](image)

Figure 1. Agarose gel electrophoresis of obtained PCR products. Ethidium bromide stained agarose gels were used to analyse PCR products. P – *ScMHR1*prom; Ca – *CaMHR1* ORF; T – *ScMHR1*term, w – water control with primers for each reaction but without template DNA, M – DNA size marker.

The objective of cloning was to ligate the PCR products one at a time into pRS316ΔSacI to construct pRS- *CaMHR1*. Plasmid RS316ΔSacI is the *E.coli*-yeast shuttle vector pRS316 with its SacI restriction site removed from the Multiple Cloning Site (MCS). The removal of the MCS
SacI site was necessary to ensure in frame cloning of *ScMHR1prom* and *CaMHRI* via a newly introduced SacI site (Table 3).

Figure 3. Cloning strategy to generate plasmids pRS316ΔSacI and pRS316-P. Depicted are the vectors and the PCR product used. Relevant restriction sites are indicated. Genes are shown as filled arrows, the multiple cloning site (MCS) as a blue box.

Plasmid RS316 was restricted with SacI, Phenol/Chloroform extracted and Ethanol precipitated. The obtained linearised plasmid had 3' overhangs, which were removed with T4 DNA polymerase. After this reaction the plasmid DNA was Phenol/Chloroform extracted again. The blunted ends of the plasmid were ligated with T4 DNA ligase. The plasmid obtained was
named pRS316ΔSacI (Figure 3). Deletion of the SacI site from the MCS was verified by control digestions.

![Figure 4. Cloning strategy to generate plasmids pRS316-PT and pRS316- CaMhr1. Depicted are the vectors and the PCR product used. Relevant restriction sites are indicated. Genes are shown as filled arrows, the multiple cloning site (MCS) as a blue box. CaMHR1 was obtained from pGEM7-CaMHR1 (not shown, described in text).](image)

After double-digestion of pRS316ΔSacI with SmaI and XbaI the plasmid had a blunt end from the SmaI site and a 5’ overhang from the XbaI site. The PCR product ScMHR1prom was digested with XbaI, thus carrying one suitable XbaI overhang and one blunt end due to amplification by Pfu polymerase. The digestion products were extracted from an agarose gel and ligated, resulting in construct pRS316-P (Table 2, Figure 3).
Plasmid RS316-P and ScMHR1term were double-digested with EcorI and XhoI, extracted from an agarose gel and ligated to make the construct pRS316-PT (Table 2, Figure 4). After transformation into *Escherichia coli* transformants were identified from randomly picked colonies by a colony PCR with the primers used to produce ScMHR1term. Liquid cultures were inoculated, pRS316-PT DNA was purified from the cultures. The insertion of ScMHR1term was confirmed by restriction analysis with EcoRI and XhoI.

Cloning of SacI and EcorI digested *CaMHR1* into pRS316-PT vector that was digested with the same enzymes did not yield any colonies. There are two possibilities that could have caused it. It is known that the transformation of larger shuttle vectors like pRS316 is less efficient than that of smaller vectors, so the problem could have been caused by low transformation efficiency. The second possible reason is that *CaMHR1* could be a toxic gene to *E.coli* (Anthony et al. 2004). Therefore EcoRI and SacI cut *CaMHR1* was ligated into a smaller plasmid, pGEM7- cut with the same enzymes to construct pGEM7-*CaMHR1*. The insertion was verified by SacI and EcorI digesion. Successful cloning of *CaMHR1* into pGEM7- proved that the gene is not toxic for *E.coli*. The SacI EcoRI digested *CaMHR1* from pGEM7-*CaMHR1* was ligated into pRS316-PT, resulting in the construct pRS-*CaMHR1* (Figure 4). The construct was transformed into *E.coli* and a colony PCR with the primers ScMhr1promXbaI and ScMhr1termXhoI confirmed the presence of the 3 inserts – ScMHR1prom, CaMHR1 and ScMHR1term.

The ScMHR1prom-*CaMHR1*-ScMHR1term insert in pRS316-CaMhr1 was sequenced as described in 2.1.3. A point mutation was shown at nucleotide position 432 from the start codon of *CaMHR1*, changing T to C. This did not cause a change in the amino acid coded, as both codons AGU and AGC code for serine, also in *Candida albicans*.

### 2.2.2 Construction of a vector containing the DNA sequence used to knock out *ScMHR1*

pBKS-*MHRI* (see Table 2) was double-digested with Eco72I and Bsp119I, the smaller fragment with 643 nucleotides from *ScMHR1* was discarded. The resulting single-stranded 3’ overhang of the Eco72I digest on the remaining plasmid backbone was removed with T4 DNA polymerase. The remaining pBKS backbone carried 300 bp of sequence upstream of the start codon of *S. cerevisiae MHRI* and 47 remaining base pairs of the *ScMHR1* gene, and 220bp of sequence lying downstream of the stop codon (Figure 5).
Figure 5. The cloning strategy to create the pBKS-Δmhr1TRP1. pBKS-Mhr1 was linearised, excising most of ScMHR1. Into the resulting linear plasmid, TRP1 cassette, cut out from pUC19-TRP1, was ligated.

In parallel, pUC19-TRP was digested with EcoRI and Pst to cut out the TRP1 cassette which is used for auxotrophic selection in genetic manipulation of yeast. The DNA fragment containing this cassette was purified from an agarose gel and single-stranded DNA overhangs.
were removed with T4 DNA polymerase. The TRP1 cassette was ligated into the pBKS backbone, resulting in pBKS-Δmhr1TRP (Figure 5), thus carrying a ScMHRI knock-out construct. This construct was cut out of the plasmid via XbaI / XhoI double-digestion and used to transform W303α cells.

2.2.3 Construction of *Saccharomyces cerevisiae* strains

The strains constructed are listed in Table 2.

The *Saccharomyces cerevisiae* wild type (wt) strain used in our laboratory is W303 α/α. In this work, haploid W303α was employed to construct 3 mutant strains.

Transformation of plasmid pRS316 (Table 2) into W303α resulted in the strain W303α pRS316. Transformants were selected for by using selective medium (SC –ura), that lacks the amino acid uracil. The W303α strain has a mutation in the URA3 gene and cannot grow on medium without uracil. Only the cells that are transformants and have URA3 expressed from the plasmid, grow on the plates. The strain was used as a control showing that unmodified pRS316 does not affect growth of *Saccharomyces cerevisiae*.

Transformation of plasmid pRS-CaMhr1 (Table 2) into W303α resulted in strain W303α pRS-CaMhr1. Transformants were selected for by using SC -Ura medium and further confirmed by PCR on total DNA purified from liquid culture. Oligonucleotides used for PCR were ScMHR1promXbaI and ScMHR1termXhoI (Table 4). This strain was constructed to assess whether CaMhr1 can complement loss of ScMhr1 in *Saccharomyces cerevisiae*.

Strain W303αΔmhr1 was created by transforming the TRP1 cassette with flanking DNA as excised from pBKS-Δmhr1TRP into W303α. Transformants were selected for by growth on SC –trp, a medium lacking tryptophane. The W303α strain is unable to synthesise tryptophane by itself, due to having a mutated TRP1 gene. Thus only cells where MHR1 had gotten replaced with TRP1 were viable on the medium. Correct insertion of the deletion construct was confirmed by PCR on purified total DNA from liquid culture using Trp3out and MHR1downPst (Table 3). This strain was constructed to observe the effects of the knock-out of ScMHRI and used in further transformations as described hereafter.

W303α pRS-CaMHRI and W303αΔmhr1 strains were further modified. W303α pRS-CaMHRI was transformed with the knock-out construct from pBKS-Δmhr1TRP to yield W303α pRS-CaMHRI-Δmhr1. Transformants were selected for on SC –ura-trp media and confirmed by
2 different PCRs. First, primers Trp3out and MHR1downPst were used to detect the replacement of MHR1 with TRP and second primers ScMHR1promXbaI and ScMHR1termXhoI were used to show presence of pRS-CaMHR1.

Transformation of W303αΔmhr1 with pRS-CaMhr1 resulted in W303α-Δmhr1-pRS-CaMHR1. This strain was selected for and identified in the same way as previously described for W303α-pRS-CaMHR1-Δmhr1.

2.2.4 Complementation assay
The constructed Saccharomyces cerevisiae strains were analysed qualitatively and quantitatively for growth fitness by two methods as described in 2.1.9.

2.2.4.1 Qualitative growth assay
W303α, W303α-pRS316, W303α-pRS-CaMHR1, W303α-Δmhr1, W303α-Δmhr1-pRS-CaMHR1 and W303α-pRS-CaMHR1-Δmhr1 were plated to solid YPD and YPG media and incubated at 30°C. For all strains, wild type and mutant strains growth could be observed on both fermentable and non-fermentable carbon sources (Figure 6A and B). Surprisingly, as can be seen in Figure 6B, strain W303α-pRS-CaMHR1 expressing native ScMhr1 and heterologous CaMhr1 revealed poor growth on YPG containing non-fermentable carbon source. No differences between wild type and other mutant strains could be observed.
Figure 6. Qualitative growth assay of *Saccharomyces cerevisiae* wild type and ∆mhr1 mutant strains. Plates in A and B are solid YP medium with either glucose (A) or glycerol (B) as the carbon source. Cells of each strain were plated from initial selective SC-glucose plates to A or B and grown at 30°C for 3 days. In A and B, numbers are: 1 – wild type W303α; 2 - W303α pRS316; 3 - W303α pRS-CaMHR1; 4 - W303α ∆mhr1; 5,6,7 - W303α-∆mhr1-pRS-CaMHR1; 8,9 - W303α pRS-CaMHR1-∆mhr1.

2.2.4.2 Quantitative growth assay
Closer examination of the growth fitness of all strains described was carried out by generating growth curves for growth in liquid YPD and YPG (Figure 7A and B). W303α ∆mhr1 cells showed a mild decrease in growth when compared to the wild-type W303α, in both YPD and YPG. The *MHR1* deletion strains with the pRS-CaMhr1 plasmid introduced either before or after the knock-out of *MHR1* did not show an increase in growth compared to the W303α ∆mhr1 strain. One exception was W303α pRS-CaMHR1∆mhr1#2 grown on YPG. In this strain the pRS316-CaMhr1 plasmid had been inserted before the deletion of *MHR1* and it displayed a mild growth advantage over wild type and other mutant strains. The W303α pRS-CaMhr1 strain, where both the endogenous *ScMHR1* and heterologous *CaMHR1* were present, showed the poor growth in YPG (Figure 7B) already observed in the qualitative assay (Figure 6B), and on YPD a mild decrease in growth fitness could also be seen (Figure 7A).
Figure 7. Growth curves of *Saccharomyces cerevisiae* strains W303 α wild-type, W303 α pRS316, W303 α pRS-CaMhr1, W303α Δmhr1, W303α pRS-CaMhr1Δmhr1 and W303α Δmhr1-pRS-CaMhr1 grown at 30°C in liquid YPD (A) and YPG (B). X axis represents time in hours, Y axis represents OD$_{600}$. The standard error is indicated.

2.3 Discussion

One of our work group research subjects covers mechanisms of mtDNA replication in yeast. As outlined before, wild type mtDNA in *Saccharomyces cerevisiae*, a well studied model organism,
may suffer from heavy alterations upon manipulation of factors involved in mt genome maintenance. *Candia albicans* was chosen as a closely related yeast species that in contrast to *S. cerevisiae* cannot lose its wild type mtDNA, thus allowing for examination of an unaltered mt genome in mutant strains (Jõers et al., 2007; Visacka et al., 2009).

Recently, Mhr1 from *S. cerevisiae* (Ling et al., 1995) was shown to be involved in homologous recombination in yeast mitochondria (Ling et al., 1995). Furthermore, homologous recombination has been repeatedly discussed to play a role in mtDNA replication in yeast and *ScMHR1* was related to recombination directed replication initiation (Ling et al., 2007; Hori et al., 2009; Ling et al., 2009).

*Candida albicans* ORF 19.439 was identified as a possible homolog of *ScMHR1*. Alignment of the protein sequences showed 46% identity and 46% similarity, thus ORF 19.439 was termed *CaMHR1*. Since genetic manipulation in *Candida albicans* is rather difficult and time consuming due to its obligatory diploid genome, a possible function of CaMhr1, was first analysed in *Saccharomyces cerevisiae* strains that were constructed to assess if *CaMHR1* can complement loss of *ScMHR1*. Little is known about promoter usage in *C. albicans*. Therefore, *CaMHR1* was put under control of the host (*S. cerevisiae*) promoter region. To logically complete the construct for heterologous expression in different *S. cerevisiae* strains, the terminator region of *ScMHR1* was cloned in frame behind *CaMHR1*. The construct was expressed from a plasmid yeast-*E. coli* shuttle vector pRS316.

It has been shown earlier that knock-out of *MHR1* in *S. cerevisiae* causes reduced growth fitness in rich media (Deutschbauer et al., 2005). However, manipulation of factors active in mtDNA maintenance often affects mt genome stability (reviewed in Contamine & Picard 2000). Therefore, two different strains were generated in order to observe possible differences between knocking out *ScMHR1* prior to presence of *CaMHR1* and *ScMHR1* deletion upon transformation of the *CaMHR1* expressing plasmid.

In a qualitative assay, wild type W303α, W303α Δmhr1, W303α pRS-CaMHR1 and both, W303α pRS-CaMHR1-Δmhr1 and W303α Δmhr1-pRS-CaMHR1 were analysed. Since the phenotype of *MHR1* deletion reported in *S. Cerevisiae* was observed in rich media, glucose and glycerol containing YP media were used. Glucose is a fermentable carbon source that enables *Saccharomyces cerevisiae* to produce energy anaerobically and is thus independent of a functional mt genome. In contrast, glycerol as a non-fermentable carbon source requires yeast to produce energy via oxidative phosphorylation which, in turn needs fully functional mtDNA.
Comparison of media containing one or the other carbon source therefore enables to observe possible changes in mitochondrial respiration due to changes in mtDNA metabolism (Bulder 1964). As demonstrated in Figure 6B, all strains grew equally well on YPD and YPG except for W303α pRS-CaMHRI which carries both, endogenous MHRI and heterologously expressed putative CaMHRI. This strain displayed severe growth inhibition when first grown on YPD and then transferred to YPG which is non-fermentable. The expected decrease in growth fitness of W303α Δmhr1 could not be observed. Therefore, a quantitative assay determining growth curves of the previously investigated strains was carried out. The liquid cultures used in this assay were YPD and YDG as in the previous qualitative assay. Observation of changes in optical density allowed to follow growth fitness more precisely. After prolonged growth (20h) in YPD, W303α showed a mild growth advantage over the other strains, while surprisingly strain W303α pRS-CaMhr1 displayed a weak disadvantage in comparison to the other mutant strains and the wild type. W303α pRS316, wild type strain carrying empty pRS316 plasmid, served as control for possible influences of the vector used to express CaMhr1. This strain revealed a slight disadvantage in comparison to W303α. This phenomenon is occasionally observed in yeast strains propagating plasmid vectors and is therefore readily explained. Strains W303α Δmhr1, W303α pRS-CaMhr1-Δmhr1 and W303α Δmhr1- pRS-CaMhr1 showed similar growth rates to W303α pRS316. Even though standard errors exclude significant differences, the tendencies that can be read out of the growth curves indicate a mild growth disadvantage of ScMHRI deletion strains, while clear complementation by CaMHRI is not observed for growth on glucose containing YP medium.

Slightly reduced growth may account for a reduced energy pool in the absence of fully functional mitochondrial respiration caused by affected mtDNA. To test for this hypothesis, all strains were grown on non-fermentable YPG, thus selecting against loss of mtDNA.

When grown on YPG, wild type W303α cells showed similar growth rates to most mutant strains and W303α pRS316. Interestingly, one out of two W303α pRS316-Δmhr1 strains, in both of which CaMHRI was present before knock-out of ScMHRI, displayed a growth advantage over wild type and other mutant strains, although non-significant as indicated by the standard error. This finding suggested a mild complementation of ScMHRI deletion by CaMHRI. There are two reasons for why the second identical strain did not reveal a similar tendency. It has been observed that sometimes a gene of interest is not being expressed from a plasmid in one clone but still in another (supervisor’s unpublished result), while the cause for this is yet unclear. Thus, either
CaMHR1 was only expressed in one of the two clones or the observed tendency was an artifact as the standard error was rather large. Experiments in the near future will be performed to show whether CaMhr1 protein is expressed in the S. cerevisiae mutant strains generated in the course of this work.

However, W303α pRS-CaMHR1 cells showed an even stronger decrease of growth fitness than they did in YPD medium. This inability to grow on non-fermentable medium indicated a loss of respiratory function and therefore an effect on the mt genome. A similar effect has recently been observed for another Candida albicans protein, Gcf1 (Visacka et al. 2009). This protein, which is a DNA binding protein in C. albicans mitochondria and has a function in mtDNA metabolism is a homolog of Abf2 from Saccharomyces cerevisiae. Abf2 is a DNA binding protein with functions in mtDNA replication and packaging. When both proteins were present in Saccharomyces cerevisiae cells, enhanced loss of wild type mtDNA was observed. It was proposed that this is due to competition of both proteins for the substrate, ultimately resulting in destabilisation of the mt genome. It is therefore plausible to speculate that a similar effect was observed for both Ca and Sc Mhr1 proteins expressed simultaneously in Saccharomyces cerevisiae. If competition of both proteins was the cause of mt genome destabilisation in the present case, this would be observed as a severe growth fitness reduction on non-fermentable carbon sources but only mild or non-observable on a fermentable carbon source like glucose.

Although classical complementation of ScMHRII deletion by CaMHR1 was not observed, most likely due to the rather weak phenotype of MHRII knock-out in S. cerevisiae, the results presented here suggest a function for the gene product of Candida albicans ORF 19.439, CaMhr1p, that may be comparable to its homolog from S. cerevisiae. To further investigate CaMhr1, several experiments are being prepared. Gfp-fusion (green-fluorescent protein) with CaMhr1 will be used to study localisation of the protein and show whether it is transported into mitochondria. In cooperation with a workgroup from Comenius University, Bratislava, CaMHR1 knock-out and conditional mutants will be generated in Candida albicans wild type cells. Such strains will for example be used to investigate possible influences of CaMhr1 on mtDNA metabolism conducting topological studies of C. albicans mtDNA. Furthermore, an existing purification scheme for ScMhr1 from our laboratory will be applied (if needed modified) to heterologously express CaMhr1 in E. Coli, purify protein and characterise its functions in vitro.
Summary

In this study, *Candida albicans* ORF 19.439 (*CaMHR1*) was analysed. It was identified *in silico* as a putative homolog of the *MHRI* gene in *Saccharomyces cerevisiae* (*ScMHR1*). *ScMhr1* localises to mitochondria and has been shown to facilitate homologous recombination, a feature that has lead to the proposal that Mhr1 could initiate rolling cycle replication of mtDNA.

To assess, if the role of *CaMHR1* in mtDNA maintenance could be comparable to that of *ScMHR1*, the gene was cloned into an *E.coli*-yeast shuttle vector, where it was put under the control of *ScMHR1* promoter. The plasmid was transformed into *S. cerevisiae* wild type and *W303α Δmhr1* cells to observe possible complementation. In a first screening, growth assays were performed in rich yeast media containing either a fermentable or a non-fermentable carbon source. The data obtained from qualitative analyses preformed on solid media and quantitative growth curves did not display heterologously expressed *CaMHR1* as being able to clearly complement the deletion of *ScMHR1*. However it could be seen from the performance of the strain *W303α pRS-CaMhr1* that the presence of both, *ScMHR1* and *CaMHR1*, causes a mild decrease in growth fitness on rich glucose (fermentable) medium and a severe inhibition to growth on rich glycerol (non-fermentable) medium.

With respect to a previous finding in another study, this suggests that a function could be shared between the proteins coded by *ScMHR1* and *CaMHR1*, possibly causing competition between the two proteins and subsequently destabilising the mitochondrial genome. Further studies on ORF 19.439 are planned. GFP-*CaMhr1* fusion proteins will be generated to determine if the protein localises to mitochondria. Heterologous expression of *CaMHR1* and purification will be conducted to characterise the functions of the protein *in vitro*. Moreover, to observe if and to what extend mtDNA could be affected knock-out and conditional mutants of *Candida albicans* will be generated.
Saccharomyces cerevisiae mitokondriaalset rekombinaasi Mhr1 oletatava homoloogi pärmist Candida albicans analüüsimine
Kaarel Kurm

Resümee

Mitokondrid on eukarüootide organellid, milles toodetakse põhilise osa päristuumse raku energiast. Selleks on vaja täieliku funktsionaalsusega mitokondriaalset genoomi, sest iga geeniprodukt on vajalik kas valguna, mis võtab reaktsioonidest osa või valguna, mis osaleb teiste valkude sünteesis. Seetõttu, et säilitada mitokondriaalset funktsioone, on vaja mtDNA-d kaitsta kahjulike muutuste eest. See saavutatakse tekkivate mtDNA kahjustuste parandamisega ning mitokondriaalse genoomi veatu replikatsiooni, segregatsiooni ning ekspressiooni kaudu. Saccharomyces cerevisiae on kasutatud mudelorganismina pärmi mitokondriaalse genoomi alalhoidmises osalevate rakutuuma poolt kodeeritavate faktorite uurimisel tänu tema genoomi suhteliselt lihtsale manipuleeritavusele. Selles päristuumses organismis läbi viidud eksperimendid on võimaldanud teadlastel välja pakuda hüpoteese pärmi mitokondriaalse DNA replikatsiooni mehanismide kohta, kuid nende mehanismide täpsem olemus on siiski vaieldav.

Antud töös uuriti Candida albicans avatud lugemisraami (ORF) 19.439 (CaMHR) kui oletatavat homoloogi Saccharomyces cerevisiae geenile MHRI (ScMHR1). On näidatud, et selle ScMHR1 kodeeritav valk paikneb mitokondrites ning viib läbi homoloogset rekombinatsiooni, mistõttu on pakutud, et Mhr1 võiks osaleda veerava ratta replikatsiooni algatamises.

Hindamaks, kas CaMHR1 roll mtDNA alalhoidmises on sarnane ScMHR1-ga, kloneeriti uuritav geen E.coli ja pärmi süstikvektorisse ScMHR1 promooteri kontrolli alla. Konsttrueeritud plasmiid transformeeriti W303α Δmhr1 rakkudesse, et vaadelda võimalikku deleeteritud geeni komplementatsiooni CaMHR1 poolt. Tulemused, mis saadi kvantitatiivsetest ning kvalitatiivsetest kasvu uuringutest ei suutnud selgelt näidata CaMHR1 suutlikust komplementeerida ScMHR1 deletsiooni. Siiski ilmus W303a pRS-CaMhr1 tüve rakude vaatlemisel asjaolu, et mõlema geeni samaaegne olemasolu põhjustab rakude kasvusuutlikkuse nõrga alanemise toitainerikkas süsinikuallikana glükoosi sisaldava sõõtmes ning väga tugeva kasvuvõimekuse alanemise toitainerikkas süsinikuallikana glütserooli sisaldava sõõtmes.
Nähtu annab põhjust oletada, et ScMHRI ja CaMHRI poolt kodeeritavatel valkudel võib olla osaliselt kattuv funktsioon, mistõttu võiks nende valkude vahel tekkida võitlus DNA substraadi pärast, mis viiks mitokondriaalse genoomi destrabiliseerumiseni. Plaanis on viia läbi edasisi uuringuid CaMHRI kohta, et tuvastada, kas antud geeni produkt paikneb mitokondrites, et uurida millised on selle valgu biokeemilised omadused in vitro ning vaadelda, milline mõju on antud geeni välja löömisel Candida albicans mitokondriaalsele genoomile.
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