



Yeast Functional Analysis Report

Tools and methods for genetic analysis of *Saccharomyces castellii*

Eimantas Astromskas and Marita Cohn*

Department of Cell and Organism Biology, Lund University, Lund, Sweden

*Correspondence to:

Marita Cohn, Department of Cell
and Organism Biology, Lund
University, Sölvegatan 35,
223 62 Lund, Sweden.
E-mail: marita.cohn@cob.lu.se

Abstract

The budding yeast species *Saccharomyces castellii* has provided important new insights into molecular evolution when incorporated in comparative genomics studies and studies of mitochondrial inheritance. Although it shows some diversity in the specific molecular details, several analyses have shown that it contains many genetic pathways similar to those of *S. cerevisiae*. Here we have investigated the possibility of performing genetic analyses in *S. castellii*. We optimized the LiAc transformation protocol to achieve 200–300 transformants/μg plasmid DNA. We found that the commonly used plasmids for *S. cerevisiae* are stably maintained in *S. castellii* under selective conditions. Surprisingly, both 2μ and *CEN/ARS* plasmids are kept at a high copy number. Moreover, the kanMX cassette can be used as a resistance marker against the selective drug geneticin (G418). Finally, we determined that the *S. cerevisiae* *GAL1* promoter can be used for the activation of transcription in *S. castellii*, thus enabling the controlled overexpression of genes when galactose is present in the medium. The availability of these tools provides the possibility of performing genetic analyses in *S. castellii*, and makes it a promising new model system in which hypotheses derived from bioinformatics studies can be experimentally tested. Copyright © 2007 John Wiley & Sons, Ltd.

Received: 8 December 2006
Accepted: 26 February 2007

Keywords: yeast; hemiascomycetes; *Saccharomyces*; *Saccharomyces castellii*; *Naumovia castellii*; genetic tools

Introduction

The yeast *Saccharomyces cerevisiae* has a long history of investigation and is the genetically best-studied eukaryotic organism today. However, many biological questions cannot be answered using only one species. The breakthrough in genomic sequencing provided many new yeast genomes, which have opened the way for comparative genomics studies. The accumulating bioinformatics information urges for further experimental analyses to prove the hypotheses, leading to the request to perform genetic analyses in species other than the traditional model organisms. More importantly, new alternative model organisms will provide new ways to approach problems that cannot be solved in *S. cerevisiae*.

Saccharomyces castellii (syn. *Naumovia castellii*; Kurtzman, 2003) is a member of the *sensu lato* group of *Saccharomyces* species that are more distantly related to *S. cerevisiae*. Currently there are several different isolates of *S. castellii* available in various culture collections. The collection of Centraalbureau voor Schimmelfculturen (CBS) contains nine isolates and the Agricultural Research Service Culture Collection (NRRL) contains six isolates (see Materials and methods for description). Despite this high number of isolates, most *S. castellii* studies have been performed using two main strains: the type strain CBS 4309 (NRRL Y-12 630) and CBS 4310 (NRRL Y-12 631). The genome of *S. castellii* has been sequenced (95%), and has generated interesting results on gene and intergenic sequence evolution in several comparative

genomics studies (Beskow and Wright, 2006; Butler *et al.*, 2004; Cliften *et al.*, 2003, 2006, 2001; Wolfe, 2006). Significantly, it was helpful in the more accurate dating of the genome duplication event in the *Saccharomyces* lineage, where the whole genome duplication (WGD) was found to have occurred before *S. castellii* diverged from *S. cerevisiae* (Cliften *et al.*, 2006).

The *S. castellii* type strain has a genome size of 9.7 Mb and pulse field gel electrophoresis studies showed that it has nine chromosomes (Petersen *et al.*, 1999). This is a decreased size compared to *S. cerevisiae*, which has a 13 Mb genome size, carried on 16 chromosomes. However, a genome-wide gene analysis showed that after WGD, *S. castellii* and *S. cerevisiae* have retained roughly the same number of genes (≈ 5350 vs. ≈ 5770) (Cliften *et al.*, 2006). Interestingly, it was found that preferentially genes encoding regulatory transcription factors were maintained during the mass gene loss that followed the WGD (Beskow and Wright, 2006). Thus, the mean number of regulatory transcription factors is higher in post-WGD species than in pre-WGD species, implicating the development of a more extensive gene regulation network for programming gene expression in post-WGD species.

Although there are species-specific differences in which genes that have been kept, most of the duplicated genes ($\approx 85\%$) were already lost before the speciation (Cliften *et al.*, 2006). Thus, many of the genetic pathways would be predicted to be similar in these species, e.g. the mating type regulation pathway. To test the possibility for interspecies crosses, J. Piskur and colleagues developed heterothallic strains from the parental CBS 4310 strain, in which they also introduced auxotrophic markers (see Materials and methods for details; Marinoni *et al.*, 1999; Naumov *et al.*, 1998; Petersen *et al.*, 2002). Crossing studies showed that *S. castellii* can be successfully mated with *S. cerevisiae*, forming viable hybrids, although the efficiency was lower than observed in intraspecies mating. Moreover, *S. cerevisiae* mating type tester strains exhibit sensitivity to *S. castellii* pheromones (Marinoni *et al.*, 1999). These experimental data were recently confirmed by comparing *MAT* locus sequences of the two species (Butler *et al.*, 2004). It was found that *S. castellii* has a very similar organization of the *MAT* locus, containing the HO endonuclease gene and both silent mating type

cassettes (Butler *et al.*, 2004). Together, experimental and bioinformatics studies suggest that *S. castellii* regulates mating type in much the same way as *S. cerevisiae*, allowing for direct use of *S. cerevisiae* genetic tools for *S. castellii* analysis.

An interesting peculiarity is that no sequences similar to the *S. cerevisiae* centromeric elements (CDE I, II, III) were found within the *S. castellii* genome sequence (Cliften *et al.*, 2006). Although the undisputed evidence for their lack would need to await the complete sequencing of its genome, or alternatively some experimental investigation, it raises very interesting perspectives on the evolution of functional centromeric sequences. Furthermore, sequence analysis of the *S. castellii* mitochondrial genome has revealed some interesting differences and similarities to *S. cerevisiae* mitochondria. Although the *S. castellii* mitochondrial DNA is only 25.7 kb (compared to 75–85 kb of *S. cerevisiae*; reviewed in de Zamaroczy and Bernardi, 1985), more than a half of the sequence encodes genes (Petersen *et al.*, 2002). Thus, the actual number and composition of the genes encoded by *S. castellii* mtDNA is very close to that of *S. cerevisiae* (Marinoni *et al.*, 1999). More importantly, *S. castellii* was found to form petites, even though the suppressivity of these mutants was lower than it is in *S. cerevisiae*, suggesting a different mechanism for *S. castellii* petites mtDNA inheritance (Langkjaer *et al.*, 2003).

One of the research areas where *S. castellii* has been found to contribute extensively as a new model organism is the telomere biology field. The *S. castellii* telomere sequence is a regular repeat (TCTGGGTG) which resembles the human telomeric repeat (Cohn *et al.*, 1998). Moreover, the *S. castellii* telomerase enzyme is highly processive *in vitro*, which was a critical feature for the initial identification of the yeast telomerase enzyme (Cohn and Blackburn, 1995). In contrast, the *S. cerevisiae* telomeres contain highly irregular sequences abbreviated (TG_{1–3}; reviewed in Louis and Vershinin, 2005) and harbour a non-processive telomerase (reviewed in Lue, 2004), features that make it problematic to decipher the molecular details of the telomere maintenance pathways in *S. cerevisiae*. Here, *S. castellii* is able to offer an advantageous alternative, where: (a) the regular telomere repeats direct the exact positioning of telomeric proteins and allow for the determination of the total number of bound telomeric proteins;

(b) the processive telomerase gives a better resolution in telomerase assays, which allows for detailed studies of telomerase biochemistry. In spite of particular differences between *S. cerevisiae* and *S. castellii* telomere structure, they have a very similar telomere maintenance and function overall, which is supported by the analyses done on the *RAP1* and *CDC13* telomere binding protein homologues from both species (Rhodin *et al.*, 2006; Wahlin and Cohn, 2002). Together, these features implicate *S. castellii* as a good candidate model organism for telomere biology studies, encompassing homologous genetic pathways but differing in the molecular details. Having the ability to do genetic analyses in two parallel systems will highly benefit research.

In order to develop *S. castellii* as a model organism for genetic analyses, there is a need to develop genetic tools, such as plasmid systems and selection markers. In this study we have established some general methods for doing genetic analyses in *S. castellii*: (a) we have shown that plasmids developed for *S. cerevisiae* can be efficiently used for *S. castellii*; (b) we have optimized the transformation protocol for introducing these plasmids; (c) we have determined that the geneticin (G418) resistance cassette *kanMX* can be used as a selection marker; and (d) we have shown that the *S. cerevisiae* *GAL1* promoter can be effectively regulated in *S. castellii* to induce transcription of a downstream gene.

Materials and methods

Strains of *Saccharomyces castellii* in culture collections

The culture collection of CBS contains the following isolates: CBS 1579, CBS 2248, CBS 2913, CBS 3006, CBS 2007, CBS 4309 (type strain, the same as NRRL Y-12 630), CBS 4310, CBS 4906 and CBS 7188. NRRL contains the isolates: NRRL Y-12 630 (type strain, the same as CBS 4309), NRRL ETC FFLY-282, NRRL ETC FFLY-283, NRRL ETC FFLY-285, NRRL Y-12 631 (the same as CBS 4310), NRRL Y-17 050 (the same as CBS 2913). Interestingly, different isolates of *S. castellii* exhibit different karyotypes, such as different sizes of chromosomes or even different chromosome numbers (Spirek *et al.*, 2003). The two strains CBS 4309 (NRRL Y-12 630) and CBS

4310 (NRRL Y-12 631) both have nine chromosomes (Petersen *et al.*, 1999; Spirek *et al.*, 2003).

Strains in this study

Escherichia coli DH5 α was used for all the cloning experiments. The strain of *S. castellii* used was Y320: *MAT α* , *ura3*, *ho*.

To study different genetic mechanisms in *S. castellii*, J. Piskur and collaborators introduced auxotrophic markers into *S. castellii* (Supplementary Figure 1). As a basis, the highly fertile strain CBS 4310 was used in the selection for a well-sporulating diploid strain (Y174) (Jure Piskur, personal communication). This strain was screened further to select for a strain that effectively forms tetrads (Y188; Marinoni *et al.*, 1999). To create a stable heterothallic strain, the Y188 spores were mutagenized with ethylmethanesulphonate (EMS) and haploids with a *ho* phenotype were selected, giving rise to the Y239 strain (Naumov *et al.*, 1998). This was followed by another EMS mutagenesis step to introduce the auxotrophic markers *met* (strain Y257; Marinoni *et al.*, 1999) and *arg* (strains Y252 and Y258; Petersen *et al.*, 2002). The *ura3* mutation was introduced via EMS in the diploid Y188 leading to Y235 (Petersen *et al.*,

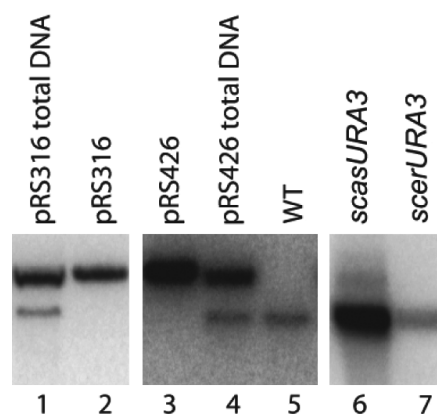


Figure 1. Copy number of *S. cerevisiae* plasmids in *S. castellii*. Total DNA was extracted from the *S. castellii* Y320 strain transformed with the plasmids pRS316 and pRS426, respectively (lanes 1 and 4). The DNA was cleaved with *Bam*HI and *Bsr*GI, blotted and hybridized with the *scasURA3* gene. As controls, the purified plasmids (lanes 2 and 3) and the genomic Y320 DNA (lane 5) were cleaved with the same enzymes. Normalization of the hybridization signals were done by hybridizing the *scasURA3* probe to the PCR products of the *scasURA3* (lane 6) and *scerURA3* (lane 7) genes, and comparing the signal intensities

2002). The spores of Y235 (*ura3⁻*, *HO*) were crossed to Y258 (*arg*, *ho*), the resulting diploids were sporulated and the haploids with *ura3* and *ho* markers were selected as the Y319 and the Y320 strains (Petersen *et al.*, 2002).

Media and culture conditions

Yeasts were grown on YPD medium containing 1% w/v yeast extract, 2% w/v peptone and 2% w/v glucose. Uracil drop-out plates contain 26.7 g/l minimal SD-base (BD-Biosciences), 0.77 g/l *ura⁻* DO supplement (Clontech) and 2% agar (Merck). Resistance to geneticin was tested on YPD containing 75 mg/l geneticin (Gibco, potency 741 µg/mg). For the analysis of the *GALI* promoter, 1.7 g/l yeast nitrogen base (Difco) was mixed together with 0.77 g/l uracil drop-out supplement, 5 g/l ammonium sulphate, 2% glucose or galactose, and 2% agar. The yeast cells were grown at 25 °C.

Sporulation test

Two *S. castellii* colonies of different mating types were mixed on an YPD plate and incubated overnight (16 h) at 25 °C. The mix was transferred to a sporulation plate (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) and incubated at 25 °C for 5–7 days. The presence of spores was examined microscopically and with a 302 nm UV light (spore containing colonies will fluoresce).

Transformation

A single *S. castellii* colony was grown in 50 ml YPD medium until the cell density reached $OD_{600} \approx 0.8$ ($\sim 3 \times 10^7$ cells/ml). The cells were harvested by centrifugation at $1200 \times g$ for 10 min, washed once with 50 ml sterile water and once with 25 ml transformation buffer I (0.1 M Li acetate, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The pellet was resuspended in 250 µl transformation buffer I to obtain the concentration $\sim 6 \times 10^9$ cells/ml. The sample DNA to transform was mixed with 20 µg freshly denatured salmon sperm DNA in a total volume of 10 µl. This DNA was mixed with 50 µl competent cells ($\sim 3 \times 10^8$ cells) and 300 µl transformation buffer II (40% PEG 3350, 1× transformation buffer I). The

transformation mix was incubated at 25 °C for 30 min and transferred to 42 °C for 15 min. After the heat shock, the cells were washed twice with 1 ml sterile water and grown on the selective plates for 3–4 days.

Copy number of plasmids

In order to estimate the plasmid copy number in the Y320 strain transformed with pRS316 or pRS426, total DNA was prepared using the Wizard genomic DNA preparation kit (Promega). The DNA (1.5 µg or 0.5 µg) was cleaved with *Bam*HI and *Bsr*GI, electrophoresed and blotted to Hybond-XL membrane (Amersham), using 0.4 M NaOH as a transfer buffer. The *S. castellii URA3* gene (*scasURA3*) was amplified using primers 5'-TGTCGGCATTAAACATATACCC and 5'-TTATGCTGACTTACCAC. The PCR product was labelled with α -³²dCTP, using Rediprime™ (Amersham Biosciences), to produce the *scasURA3* probe. The membrane was hybridized overnight at 60 °C (0.25 M Na₂HPO₄, 7% SDS, 1 mM EDTA), washed for 5 min at room temperature (100 mM Na₂HPO₄, 2% SDS) and 20 min at 60 °C and screened with a phosphorimager (Bio-Rad). The data were analysed using Quantity One software (Bio-Rad).

Cloning of the *kan^r* gene and the kanMX cassette

The kanMX geneticin resistance cassette was amplified from pFA6 (Wach *et al.*, 1994) using the primers 5'-AAGGCCTTGATCACCGGTAAGCT-TGCCTCGTCCCC and 5'-AAGGCCTTGGCCA-CTAGTGGGCGCAGAGCCGTC. The PCR product was cleaved with *Stu*I and ligated into the *Sma*I site of pRS316. The resulting construct, pRS316-kanMX, was transformed into the *S. castellii* Y320 strain.

The open reading frame of the *kan^r* gene was amplified from the pFA6 plasmid (Wach *et al.*, 1994) using the primers 5'-GGGGTACCACCATG-GGTAAGGAAAAG ACTCACG and 5'-CCGGT-ACCTGATATTACTTTCTGCGC. The amplicon was ligated into the *Kpn*I site of the pYES2 plasmid (Invitrogen) and the resulting pYES2-*kan^r* construct was transformed into the *S. castellii* Y320 strain.

Results and discussion

Growth conditions for *S. castellii*

As described in Materials and methods, the *S. castellii* Y320 strain is a haploid *ura3* strain developed by J. Piskur and colleagues from the parental diploid homothallic strain CBS 4310 (Petersen *et al.*, 2002). We analysed the general growth conditions for this strain at different temperatures and on different types of media. The *S. castellii* Y320 strain grows well on standard rich YPD medium at 25 °C. The generation time was determined to be 120 min under these conditions, and at an optical density of 0.1 OD₆₀₀ units the cell density corresponded to approximately 2×10^6 cells/ml. The Y320 strain does not grow at all at 37 °C, which is in accordance to the behaviour of the parental strain CBS 4310 and the type strain CBS 4309, whereas the growth rate at 30 °C is only slightly lower than at 25 °C. Moreover, the Y320 strain grows on a medium containing galactose as the sole carbon source. When the Y320 strain (*ura3*) is complemented with a plasmid containing the *S. cerevisiae* *URA3* gene, it shows a good growth on synthetic uracil drop-out medium. The *ura3* auxotrophic marker is stable and we did not detect any revertants on uracil drop-out plates when plating control transformation mixtures. Furthermore, the Y320 strain shows a good growth on plates containing 5-fluoro-orotic acid (5-FOA).

The stability of the *ho* mutation was tested by a sporulation analysis. Several colonies of the Y320 strain were mixed and incubated overnight on a YPD plate and then streaked and incubated on a sporulation plate (1% KAc). No ascii could be detected in this analysis, showing that it is a stable heterothallic strain. In contrast, a diploid *S. castellii* strain (Y235) produced a good amount of ascus spores under the same conditions. Overall, with the exception of the growth temperature preference, the general nutritional and growth abilities of the *S. castellii* Y320 strain are highly similar to those of *S. cerevisiae*. Moreover, the Y320 strain can easily be mated with another haploid *S. castellii* strain with the mating type *MATa*, leading to an efficient production of ascus spores on a subsequent sporulation plate. Together, these features show that *S. castellii* can be handled and used for basic genetic analyses in much the same way as *S. cerevisiae*.

Stability of *S. cerevisiae* plasmids in *S. castellii*

Plasmid vectors are invaluable tools for genetic analyses, providing the possibility to study the effect of introducing a new or manipulated gene into an organism. Many different vectors with different functions and features have been developed for *S. cerevisiae*. Since no naturally occurring vectors have yet been isolated from *S. castellii*, our aim was to investigate the possibility of using those already developed for *S. cerevisiae*. In order to serve as a faithful and reliable tool for genetic analysis, it is necessary to determine the stability and copy number of these plasmids within *S. castellii* cells. The *S. cerevisiae* *URA3* gene has proved to be very useful as a marker gene, as it is possible to combine a positive selection for cells containing the *URA3* marker on uracil drop-out plates with a subsequent selection for the loss of the plasmid on 5-FOA medium. This provides a convenient way to easily get rid of the introduced gene and obtain the phenotype in one single streak. When the *S. castellii* Y320 strain was transformed with the integrational plasmid pRS306 (*URA3*; Sikorski and Hieter, 1989), it was able to grow on uracil drop-out medium with a growth rate similar to that on YPD medium. Thus, the *S. cerevisiae* *URA3* gene functionally complements this deficiency and also functions as a marker gene in *S. castellii*.

Several different types of vector constructs have been developed to be used as *S. cerevisiae* shuttle plasmids. Yeast integrational plasmids (YIp) do not carry any replication origin and need to be inserted into the genome in order to be genetically transmitted. Yeast episomal plasmids (YEpl) are kept extrachromosomally, due to the incorporation of a replication origin. The pRS316 (*CEN6*, *ARSH4*, *URA3*) replication is based on the function of the autonomously replicating sequence (*ARS*; Sikorski and Hieter, 1989), resulting in 1–2 copies/cell (Schneider and Guarente, 1991). It also contains the functional centromere element (*CEN*) and has ~57% stability after 10 generations in non-selective medium. The pRS426 (2 μ , *URA3*) replication and stability is based on the 2 μ replication origin and it is maintained as a multicopy plasmid (~20 copies/cell; Christianson *et al.*, 1992). The stability of pRS426 is ~50% after 10 generations in non-selective medium.

We transformed the Y320 strain with the pRS316 and pRS426 plasmids and tested at least four

transformants of each plasmid for their stability. The transformants were inoculated in rich non-selective medium (YPD) and after 10 generations the cells were spread on YPD and uracil drop-out plates, respectively. After 3 days, colonies were counted and the plasmid stability index was expressed as the ratio (number of colonies on uracil drop-out plates : number of colonies on YPD) $\times 100\%$. The average stability in *S. castellii* was determined as 35% for pRS316, and 12% for pRS426. Our results suggest that these plasmids are less stable in *S. castellii* than in *S. cerevisiae*. A plausible explanation for the reduced stability would be that the replication origins and centromere sequences are not properly functioning in *S. castellii*. Indeed, in a search for centromeric sequences related to the *S. cerevisiae* *CEN* element, no such sequence could be found in the *S. castellii* partial genomic sequence (Cliften *et al.*, 2006). Furthermore, there are no reports of the existence of a 2μ -related plasmid in *S. castellii*. However, in contrast to the situation for some other *Saccharomyces* (Talarek *et al.*, 2004) and non-*Saccharomyces* species (Wang *et al.*, 2001), these plasmids were stable in *S. castellii* under selective conditions, and can therefore be efficiently used as genetic tools. The instability of these vectors under the non-selective conditions could be used for the spontaneous removal of the plasmids when a counter-agent (such as 5-FOA) is not available.

Copy number of the plasmids

Depending on the type of replication origin present, the copy number can differ greatly between different kinds of vectors. In *S. cerevisiae* the 2μ plasmids (pRS426) are maintained as high copy number plasmids, while the yeast *ARS* plasmids (pRS316) are usually copied only once in each cell division cycle. However, the exact copy number for a particular plasmid could differ between strains. By analogy, when introducing *S. cerevisiae* vectors into *S. castellii* we would expect the copy number of the plasmid to differ between the two species. Thus, our next aim was to determine the number of the *S. cerevisiae* plasmids present in the *S. castellii* Y320 strain transformed with pRS316 and pRS426, respectively.

To quantify the plasmids we made use of the *URA3* marker present in the plasmid, and we

related the amount of this to the amount of the *URA3* gene present in the *S. castellii* genome. Total DNA extracted from Y320 transformants was cleaved with *Bam*HI and *Bsr*GI and transferred to a nylon membrane. The membrane was hybridized with the labelled *S. castellii* *URA3* gene (*scasURA3*) and the signals analysed using a phosphorimager. Thereafter, the signal intensity of the single copy genomic *scasURA3* was compared with the intensities of the *S. cerevisiae* *URA3* (*scerURA3*) plasmid copies. Although the *URA3* genes are 74% identical in these two species, the hybridization had to be normalized for the difference in binding affinity of the labelled *scasURA3* to the respective *scasURA3* (genomic) and *scerURA3* (plasmid) copies. The hybridization controls showed a 12.6 times higher signal intensity to *scasURA3* when hybridizing to the same amounts of PCR product of *scasURA3* (Figure 1, lane 6) and *scerURA3* (Figure 1, lane 7). Thus, using this PCR hybridization control, the total number of plasmids was calculated as follows:

$$12.6(\text{scerURA3 in plasmid/scasURA3 genomic})$$

The hybridization results showed that the *scerURA3* in pRS316 gave a 7.2 times higher signal than the genomic copy of *scasURA3*, whereas the signal from pRS426 was 2.9 times higher than that from *scasURA3*. Put into the above equation, this translates to ~ 90 copies/cell of pRS316, and ~ 35 copies/cell of pRS426. This result was surprising, since pRS316 is maintained as 1 copy/cell in *S. cerevisiae*. The difference between the species could be explained by the lack of a replication regulation of the *S. cerevisiae* *CEN/ARS* plasmids in *S. castellii*. Interestingly, the pRS426 was replicated in *S. castellii* as a high copy plasmid, with the copy number only slightly higher than that in *S. cerevisiae* (~ 20 copies/cell; Christianson *et al.*, 1992). In conclusion, even though the regulation apparently differs, both the *ARS* and the 2μ sequences of *S. cerevisiae* function as replication origins in *S. castellii*. However, whether the *CEN* element is functional cannot be concluded from this study, since the pRS316 is kept as a high copy plasmid and is therefore not dependent on a functional centromere for its segregation.

Transformation optimization

The most commonly used method for *S. cerevisiae* transformation is the lithium acetate method. It is based on an incubation of the cells with lithium acetate and PEG, followed by a heat shock, which results in uptake of the foreign DNA (reviewed in Gietz and Woods, 2001). Using this method, we were able to transform linear DNA fragments into *S. castellii*, with a transformation efficiency that was roughly similar to that for *S. cerevisiae*. In a preliminary analysis, using similar constructs as for *S. cerevisiae*, we found that the majority of the integrational events seem to occur at loci other than the one specifically included within the construct. This indicates that non-homologous end-joining (NHEJ) is a more efficient pathway in *S. castellii* than homologous recombination, which is similar to the situation in most other organisms (Hentges *et al.*, 2006; Kegel *et al.*, 2006). However, a more statistical analysis will need to be performed to obtain conclusive evidence.

Using the lithium acetate method, we were also able to transform circular *S. cerevisiae* plasmids into *S. castellii*, but with a much lower transformation efficiency than for *S. cerevisiae*. In order to find the optimal conditions for plasmid transformation into *S. castellii*, we tested different parameters of the lithium acetate transformation protocol: (a) amount of carrier DNA; (b) heat shock time; and (c) heat shock temperature. These are all important parameters for obtaining high efficiency transformation. At least three independent colonies of the Y320 strain were transformed in each experiment.

The effects on the Y320 transformation efficiency when varying the amount of freshly denatured salmon sperm DNA are shown in Figure 2. It was found that carrier DNA was an essential component for high-efficiency transformation, because no addition or a very small amount (2 µg) gave a very inefficient transformation. On the other hand, a very high amount of the carrier DNA also reduced the efficiency. Thus, the best results were obtained using ~20 µg salmon sperm DNA with $\sim 3 \times 10^8$ cells of the Y320 strain (Figure 2).

During the heat shock, PEG and lithium acetate increase the permeability of the cell wall, allowing DNA to enter the cells. Because different yeast strains require different times for the shock, we tested a number of different shock durations to find

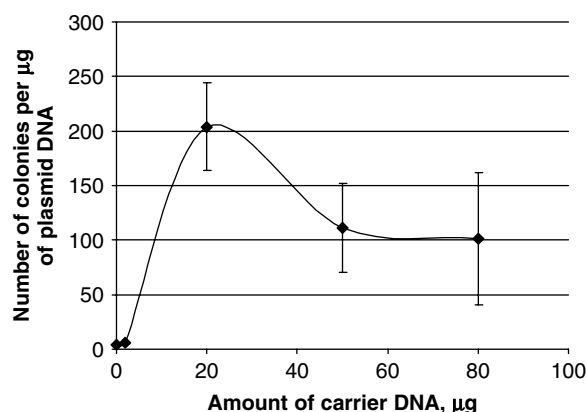


Figure 2. Effect of the amount of carrier DNA on the *S. castellii* Y320 transformation efficiency. For each transformation $\sim 3 \times 10^8$ cells were mixed with 1 µg pRS316 plasmid and selected on uracil drop-out plates. 0, 2, 20, 50 and 80 µg freshly denatured salmon sperm DNA were used in the respective transformations. The heat shock was performed for 15 min at 42 °C. The data represents averages of at least three independent experiments \pm SD

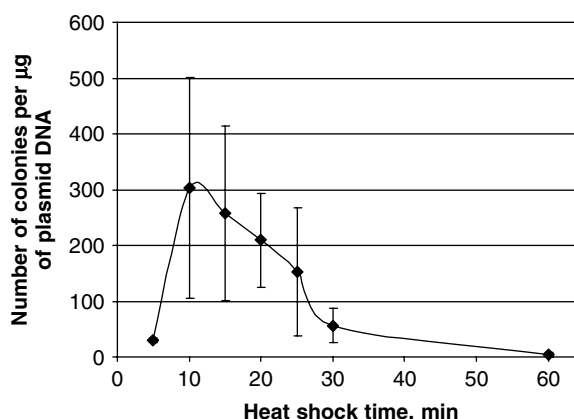


Figure 3. Effect of the heat shock time on the *S. castellii* Y320 transformation efficiency. For each transformation $\sim 3 \times 10^8$ cells were mixed with 1 µg pRS316 and 20 µg salmon sperm DNA. The heat shock was performed at 42 °C for 5, 10, 15, 20, 25, 30 and 60 min. The transformants were selected on uracil drop-out plates. The data represents averages of at least three independent experiments \pm SD

an optimal one for the Y320 strain (Figure 3). The results showed that the maximum efficiency was obtained at 10–15 min. Increasing the heat shock time beyond 15 min steadily reduced the efficiency until almost no transformants were obtained at 60 min. Longer periods of 120 and 180 min were also tested (results not shown) but these treatments

resulted in zero transformants. This finding is rather surprising, since heat shock prolongation increases transformation efficiency of *S. cerevisiae*, up to at least 180 min (Gietz and Woods, 2002).

For most *S. cerevisiae* strains, the optimal growth temperature is 30 °C. In contrast, the *S. castellii* Y320 strain has an optimal growth temperature of 25 °C (Naumov *et al.*, 1998). We therefore asked whether the optimal heat shock temperature may also vary for different species and thereby increase the efficiency of the transformation. The heat shock temperature for *S. cerevisiae* transformation is 42 °C and we tested a range of temperatures from 36 °C to 44 °C for the Y320 transformation (Figure 4). The result showed that the optimal temperature for *S. castellii* was in a very narrow range at around 42 °C. When decreasing or increasing it by 2 °C the transformation efficiency was reduced dramatically. Thus, the optimal heat shock temperature for the Y320 transformation was found to be the same as for *S. cerevisiae*.

In summary, for the three parameters tested, we found that the following conditions were optimal when transforming $\sim 3 \times 10^8$ *S. castellii* Y320 cells with the lithium acetate method: ~ 20 μ g freshly denaturated salmon sperm DNA and a heat shock at 42 °C for 10–15 min (see Materials and methods for the full protocol). Together with 1 μ g plasmid DNA, this resulted in 200–300 *S. castellii* transformants. Despite these optimizations,

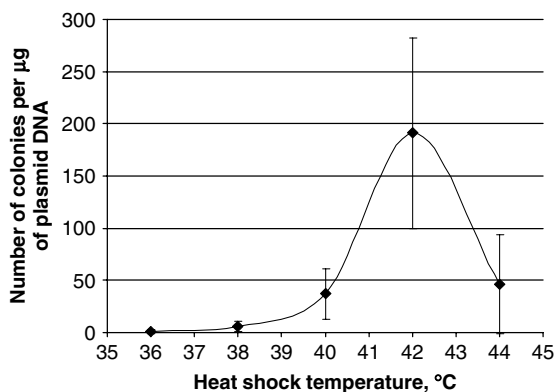


Figure 4. Effect of the heat shock temperature on the *S. castellii* Y320 transformation efficiency. For each transformation $\sim 3 \times 10^8$ cells were mixed with 1 μ g pRS316 and 20 μ g salmon sperm DNA and selected on uracil drop-out plates. The heat shock time was for 15 min at the respective temperatures 36 °C, 38 °C, 40 °C, 42 °C and 44 °C. The data represents averages of at least three independent experiments \pm SD

the transformation of *S. cerevisiae* plasmids into *S. castellii* did not reach the level obtained for *S. cerevisiae*. The optimized *S. cerevisiae* transformation results in 2.55×10^6 transformants/1 μ g plasmid (Gietz and Woods, 2006). This result, together with our results on plasmid stability and copy number, could indicate that not every *S. castellii* transformant keeps the introduced plasmid during the initial generations after transformation, thus reducing the number of the transformants.

Analysis of geneticin resistance marker

The *S. castellii* Y320 strain has an auxotrophic marker, *ura3*, which is used for the positive selection of *URA3*-plasmids on uracil drop-out medium (Petersen *et al.*, 2002). In addition, this auxotrophy gives the opportunity to rid the cell of the *URA3* plasmids when put on a 5-FOA medium. However, to enable more sophisticated genetic analysis, it would be useful to have other additional selective markers available. Geneticin (G418) is a commonly used antibiotic and we therefore tested whether it could also be used as a selective marker for *S. castellii*. Generally, selection against *S. cerevisiae* requires 200 mg/l geneticin (Goldstein and McCusker, 1999), but the optimal concentration of the geneticin in the medium can be very different for various species and strains (range 25–400 mg/l). Thus, we tested which concentration that would effectively inhibit the growth of the Y320 cells (Figure 5). We found that a geneticin concentration as low as 30 mg/l was enough to kill the cells. However, different geneticin batches may have different toxicity levels, and therefore we routinely use a geneticin concentration of 75 mg/l in our selection experiments.

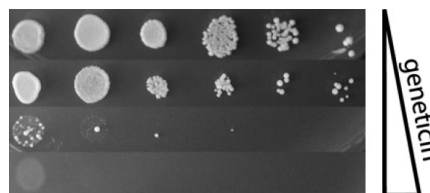


Figure 5. Effect of geneticin (G418) on the *S. castellii* Y320 growth. YPD media was mixed with geneticin at the respective concentrations of: 0, 10, 20 and 30 mg/l (rows 1–4). To examine the optimal concentration of the geneticin for complete growth suppression, an overnight culture of Y320 cells was spotted in 10-fold dilution series on each concentration of geneticin

Next we examined whether the geneticin resistance cassette used in *S. cerevisiae* can induce resistance in *S. castellii* as well. The *kan^r* gene, which was originally discovered in the Tn903 transposon in *E. coli* (Oka *et al.*, 1981), induces resistance to geneticin and kanamycin in *S. cerevisiae*. To express the *kan^r*, it is fused to the *TEF* promoter and the terminator of *Ashbya gossypii*, creating the kanMX cassette, which is constitutively expressed when introduced in *S. cerevisiae* (Wach *et al.*, 1994). In order to test the effect of this cassette in *S. castellii* Y320 strain, we PCR-amplified the entire construct (including promoter, *kan^r* gene and terminator) and ligated it into the pRS316 vector. The resulting construct, pRS316-kanMX, was transformed into the Y320 strain and transformants were plated on uracil drop-out plates containing 75 mg/l geneticin. A comparison to the control plate without geneticin shows that the kanMX cassette confers a complete resistance to the drug (Figure 6, rows 5 and 6). The cell size is somewhat decreased on geneticin plates, and an incubation of 3–4 days is needed for the colonies to reach a reasonable size. Thus, our results showed that the kanMX geneticin resistance cassette could be successfully used as a marker for *S. castellii*, in combination with geneticin as the selective agent, providing a useful tool for *S. castellii* genetic analyses.

Expression vector (*GAL1* promoter) analysis

Different genetic assays require the possibility for a controlled activation of the expression of the gene

under study. One of the most commonly used promoters for induced expression in *S. cerevisiae* is the *GAL1* promoter. This promoter is suppressed when glucose is present in the medium and it gives a strong overexpression of the downstream gene when activated by galactose (Johnston, 1987). Most conveniently, the level of the expression can be regulated by varying the galactose concentration in the medium (Li *et al.*, 2000). A recent bioinformatics study of the *S. castellii* genome (Cliften *et al.*, 2006) showed that *S. castellii* contains most of the genes required to utilize galactose, but the *GAL3* gene is missing. Interestingly, the genes *GAL1*, *GAL7*, transcription activator *GAL4* and repressor *GAL80* are all duplicated in the *S. castellii* genome. The presence of the genes included in the galactose catabolic pathway implicates a possibility for using the *GAL1* promoter expression system in *S. castellii*. To test directly whether *S. castellii* can induce expression from the *S. cerevisiae GAL1* (*scerGAL1*) promoter, we took advantage of the Y320 sensitivity to geneticin (Figure 5) and its ability to grow on a synthetic medium containing galactose as the sole carbon source (Figure 6, right panel). As our previous results showed that 2 μ plasmids were efficiently replicated and maintained in *S. castellii*, we picked the pYES2 vector (2 μ , *URA3*, *GAL1* promoter, *CYC1* terminator) for this experiment. The *scerGAL1* promoter was fused to the *kan^r* gene to produce the pYES2-*kan^r* construct. The construct was transformed into the Y320 strain and selected on uracil drop-out plates. To induce the expression, the *kan^r* transformants

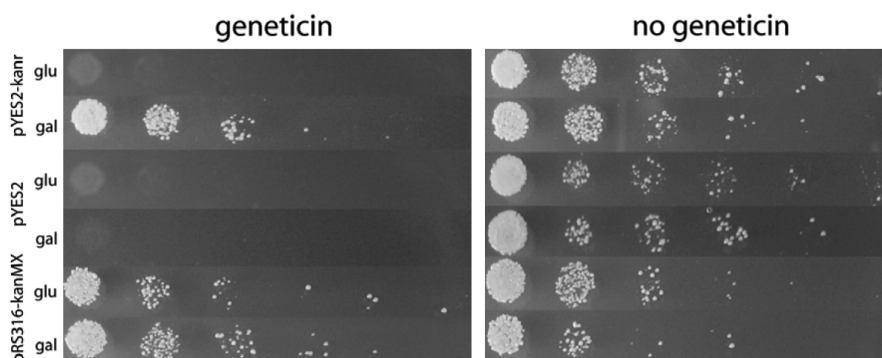


Figure 6. Analysis for the function of the *S. cerevisiae GAL1* promoter in the *S. castellii* Y320 strain. Y320 cells were spotted in 10-fold dilution series on uracil drop-out yeast nitrogen base plates, with (left) or without (right) the addition of 75 mg/l geneticin. The respective glucose (glu) and galactose (gal) carbon sources are indicated on the left. Rows 1 and 2, Y320 transformed with the pYES2-*kan^r* construct; rows 3 and 4, Y320 transformed with the empty pYES2 vector; rows 5 and 6, Y320 transformed with the pRS316-kanMX construct (*kan^r* gene expressed from the *TEF* promoter)

were spotted on uracil drop-out plates containing 2% galactose. These plates also contained geneticin (75 mg/l), thereby inhibiting the growth of any cells not expressing the *kan^r* gene (Figure 6, left, row 2). In parallel the transformants were replica spotted on plates with 2% glucose (for *GAL1* repression), with and without geneticin (Figure 6, row 1).

In the negative control experiment, Y320 was transformed with an empty pYES2 vector (Figure 6, rows 3 and 4). No growth was detected on the geneticin plates, showing that the vector itself could not convey resistance to the drug. The Y320 strain transformed with the pRS316–*kanMX* construct (Figure 6, rows 5 and 6) was used as the positive control, showing that the cells can grow on galactose plates containing geneticin when constitutively expressing the *kan^r* gene from the *TEF* promoter.

Our results showed that the Y320 strain transformed with the pYES2–*kan^r* construct survived on the geneticin/galactose medium, while no resistance was observed on the glucose medium (Figure 6, left, rows 1 and 2). Since the resistance was induced only when the carbon source was galactose, this verifies that the *kan^r* gene expression was induced from the *scerGAL1* promoter. Together, this shows that the *scerGAL1* promoter can be successfully induced by galactose, and that it can serve as an important tool to regulate gene expression in *S. castellii*.

In summary, we have characterized the *S. castellii* Y320 strain in terms of growth parameters, mating type stability and compatibility with some genetic tools commonly used for *S. cerevisiae*. First, we optimized the transformation procedure for introducing plasmid DNA into *S. castellii*. Despite lower transformation efficiency than *S. cerevisiae*, the lithium acetate transformation can be efficiently used to transform *S. castellii*, giving 200–300 transformants when 1 µg plasmid DNA is used. Second, we analysed the *S. cerevisiae* vectors pRS316 and pRS426 in *S. castellii* and found that they were replicated and stably maintained under selective pressure in this species, providing the tool to introduce new genetic material. Third, we found that the *kanMX* cassette conveys resistance to the antibiotic geneticin and can be used as a very efficient selective marker when incorporated into a plasmid. Finally, we showed that the *S. cerevisiae* *GAL1* promoter was induced in *S. castellii* in the

presence of galactose. Since there was no expression on glucose medium, this induction was tightly regulated. This allows the *GAL1* promoter to be used for experimental regulation of gene expression in *S. castellii*, enabling the studies of *in vivo* effects of introduced genes. Together, these analyses showed that, despite the evolutionary distance between *S. cerevisiae* and *S. castellii*, these species share a lot of similar genetic features. This fact allows us to use the well-developed *S. cerevisiae* genetic tools and to take advantage of *S. castellii* in studying the specific genetic features of this species. With its whole genome (95%) sequenced, *S. castellii* has already provided a lot of useful information on genome evolution and will undoubtedly continue to contribute important insights into the molecular evolution of gene functions. Using alternative model systems will be necessary for the full understanding of diversity, but will also be crucial in order to verify the postulated general concepts of molecular cell biology.

Acknowledgements

We are grateful to Jure Piskur for the gift of the *S. castellii* Y320 strain and all the helpful advice. This work was supported by grants from the Carl Trygger Foundation, the Nilsson–Ehle Foundation and the Erik Philip-Sörensen Foundation. E.A. was supported by a scholarship from the Sven and Lilly Lawsky Foundation.

References

- Beskow A, Wright AP. 2006. Comparative analysis of regulatory transcription factors in *Schizosaccharomyces pombe* and budding yeasts. *Yeast* **23**: 929–935.
- Butler G, Kenny C, Fagan A, *et al.* 2004. Evolution of the MAT locus and its Ho endonuclease in yeast species. *Proc Natl Acad Sci USA* **101**: 1632–1637.
- Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.
- Cliften P, Sudarsanam P, Desikan A, *et al.* 2003. Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. *Science* **301**: 71–76.
- Cliften PF, Fulton RS, Wilson RK, Johnston M. 2006. After the duplication: gene loss and adaptation in *Saccharomyces* genomes. *Genetics* **172**: 863–872.
- Cliften PF, Hillier LW, Fulton L, *et al.* 2001. Surveying *Saccharomyces* genomes to identify functional elements by comparative DNA sequence analysis. *Genome Res* **11**: 1175–1186.
- Cohn M, Blackburn EH. 1995. Telomerase in yeast. *Science* **269**: 396–400.

- Cohn M, McEachern MJ, Blackburn EH. 1998. Telomeric sequence diversity within the genus *Saccharomyces*. *Curr Genet* **33**: 83–91.
- de Zamaroczy M, Bernardi G. 1985. Sequence organization of the mitochondrial genome of yeast—a review. *Gene* **37**: 1–17.
- Gietz RD, Woods RA. 2001. Genetic transformation of yeast. *Biotechniques* **30**: 816–820, 822–826, 828 passim.
- Gietz RD, Woods RA. 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350**: 87–96.
- Gietz RD, Woods RA. 2006. Yeast transformation by the LiAc/SS Carrier DNA/PEG method. *Methods Mol Biol* **313**: 107–120.
- Goldstein AL, McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.
- Hentges P, Ahnesorg P, Pitcher RS, *et al.* 2006. Evolutionary and functional conservation of the DNA non-homologous end-joining protein, XLF/Cernunnos. *J Biol Chem* **281**: 37517–37526.
- Johnston M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* **51**: 458–476.
- Kegel A, Martinez P, Carter SD, Astrom SU. 2006. Genome-wide distribution of illegitimate recombination events in *Kluyveromyces lactis*. *Nucleic Acids Res* **34**: 1633–1645.
- Kurtzman CP. 2003. Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygorhizula*. *FEMS Yeast Res* **4**: 233–245.
- Langkjaer RB, Casaregola S, Ussery DW, Gaillardin C, Piskur J. 2003. Sequence analysis of three mitochondrial DNA molecules reveals interesting differences among *Saccharomyces* yeasts. *Nucleic Acids Res* **31**: 3081–3091.
- Li J, Wang S, VanDusen WJ, *et al.* 2000. Green fluorescent protein in *Saccharomyces cerevisiae*: real-time studies of the *GAL1* promoter. *Biotechnol Bioeng* **70**: 187–196.
- Louis EJ, Vershinin AV. 2005. Chromosome ends: different sequences may provide conserved functions. *Bioessays* **27**: 685–697.
- Lue NF. 2004. Adding to the ends: what makes telomerase processive and how important is it? *Bioessays* **26**: 955–962.
- Marinoni G, Manuel M, Petersen RF, *et al.* 1999. Horizontal transfer of genetic material among *Saccharomyces* yeasts. *J Bacteriol* **181**: 6488–6496.
- Naumov GI, Naumova ES, Marinoni G, Piskur J. 1998. (Genetic analysis of *Saccharomyces castellii*, *S. exiguus* and *S. martiniae* yeasts). *Genetika* **34**: 565–568.
- Oka A, Sugisaki H, Takanami M. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. *J Mol Biol* **147**: 217–226.
- Petersen RF, Langkjaer RB, Hvidtfeldt J, *et al.* 2002. Inheritance and organisation of the mitochondrial genome differ between two *Saccharomyces* yeasts. *J Mol Biol* **318**: 627–636.
- Petersen RF, Nilsson-Tillgren T, Piskur J. 1999. Karyotypes of *Saccharomyces sensu lato* species. *Int J Syst Bacteriol* **49**(4): 1925–1931.
- Rhodin J, Astromskas E, Cohn M. 2006. Characterization of the DNA binding features of *Saccharomyces castellii* Cdc13p. *J Mol Biol* **355**: 335–346.
- Schneider JC, Guarente L. 1991. Vectors for expression of cloned genes in yeast: regulation, overproduction, and underproduction. *Methods Enzymol* **194**: 373–388.
- Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.
- Spirek M, Yang J, Groth C, *et al.* 2003. High-rate evolution of *Saccharomyces sensu lato* chromosomes. *FEMS Yeast Res* **3**: 363–373.
- Talarek N, Louis EJ, Cullin C, Aigle M. 2004. Developing methods and strains for genetic studies in the *Saccharomyces bayanus* var. *uvarum* species. *Yeast* **21**: 1195–1203.
- Wach A, Brachat A, Pohlmann R, Philippsen P. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.
- Wahlin J, Cohn M. 2002. Analysis of the RAP1 protein binding to homogeneous telomeric repeats in *Saccharomyces castellii*. *Yeast* **19**: 241–256.
- Wang TT, Choi YJ, Lee BH. 2001. Transformation systems of non-*Saccharomyces* yeasts. *Crit Rev Biotechnol* **21**: 177–218.
- Wolfe KH. 2006. Comparative genomics and genome evolution in yeasts. *Phil Trans R Soc Lond B Biol Sci* **361**: 403–412.