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A script for initiating molecular biology studies with non-conventional yeasts based on *Saccharomycopsis schoenii*

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ABSTRACT

Non-conventional yeasts (NCYs), *i.e.* all yeasts other than *Saccharomyces cerevisiae*, are emerging as novel production strains and gain more and more attention to exploit their unique properties. Yet, these yeasts can hardly compete against the advanced methodology and genetic tool kit available for exploiting and engineering *S. cerevisiae*. Currently, for many NCYs one has to start from scratch to initiate molecular genetic manipulations, which is often time consuming and not straight-forward. More so because utilization of *S. cerevisiae* tools based on short-flank mediated homologous recombination or plasmid biology are not readily applicable in NCYs. Here we present a script with discrete steps that will lead to the development of a basic and expandable molecular toolkit for ascomycetous NCYs and will allow genetic engineering of novel platform strains. For toolkit development the highly efficient *in vivo* recombination efficiency of *S. cerevisiae* is utilized in the generation and initial testing of tools. The basic toolkit includes promoters, reporter genes, selectable markers based on dominant antibiotic resistance genes and the generation of long-flanking homology disruption cassettes. The advantage of having pretested molecular tools that function in a heterologous host facilitate NCY strain manipulations. We demonstrate the usefulness of this script on *Saccharomycopsis schoenii*, a predator yeast with useful properties in fermentation and fungal biocontrol.

1. Introduction

Saccharomyces cerevisiae is undoubtedly the premier microorganism for the production of various goods including bulk chemicals such as bioethanol and biofuels, fine chemicals and pharmaceuticals (Favaro et al., 2019; Hu et al., 2019; Nielsen, 2019). This yeast can serve as a platform strain for a huge variety of chemicals, aromas and fragrances – *e.g.* those belonging to the class of terpenoids - far beyond what yeasts can naturally produce (Moser and Pichler, 2019). With new capabilities in metabolomics and synthetic biology rational strain design can increase the speed in the design and building new strains so that the bottleneck is moved more to the test and analyze phases of the cycle (Marcellin and Nielsen, 2018). As with major agricultural crops we currently rely on only a few fungal species to carry the burden. However, there is a large biodiversity of yeast strains that could serve as future platform strains. This includes filamentous fungal species that produce secondary metabolites, *e.g.* antibiotics, but also other yeasts that are capable of compound overproduction at a level not feasible in *S. cerevisiae* without major engineering efforts. Classical examples of non-*cerevisiae* production strains are *Ashbya gossypii* to produce riboflavin/ vitamin B_2 and *Aspergillus niger* for the production of citric acid (Aguiar et al., 2015; Revuelta et al., 2017; Cairns et al., 2019; Tong et al., 2019). Thus, it is obvious that non-conventional yeasts need to be developed to a level comparable to *S. cerevisiae* to be able to exploit their enormous potential in a sustainable biobased economy (Lopez et al., 2015; Rebello et al., 2018; Straathof et al., 2019).

However, non-conventional yeasts are quite different from *S. cerevisiae* so that molecular genetic tools invented for this species cannot readily be transferred to them. Thus, to embark on even the most basic engineering steps requires redevelopment of tools, which can be a frustratingly slow progress involving a lot of trial and error experimentation. Advancing a new system to a platform production strain is thus time consuming and costly.

The needs for establishing molecular biology in a new fungal system will be very similar: genetic engineering tools for DNA-mediated transformation and molecular genetic manipulations are required. Thus, a functional dominant selection marker is a key step in this process. Often synthetic genes are constructed that bear no homology to the strains' genome. This should facilitate homologous recombination for targeted gene deletion experiments (Hegemann et al., 2014; Dohn

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et al., 2018). The expression of this marker gene, however, requires a promoter that is functional in the target strain.

Genetic manipulation of a species requires an efficient protocol for transformation. Regularly used protocols for yeasts are electroporation or the Lithium acetate/single-strand carrier DNA/polyethylene glycol method (Gietz and Schiestl, 2007; Kawai et al., 2010), while other methods rely on protoplast-mediated transformation, *Agrobacterium*-mediated transformation, electroporation or a biolistic method (Li et al., 2017). For each species there are modifications and adaptations required to increase the efficiency. In *S. cerevisiae*, for example, the Lithium acetate method includes a heat-shock at 42 °C (Gietz et al., 1995). A similar protocol has been used for *Candida albicans* transformation, however, for improved efficiency the heat shock should be adjusted to 44 °C (Walther and Wendland, 2003).

Reporter genes are used to analyze the expression level of genes or the subcellular localization of proteins. The most prominent in use are the green fluorescent protein (*GFP*), luciferase or β-galactosidase activity assays (based on *LacZ* expression) (Hitchcock et al., 2006; Sturtevant, 2009).

To be able to initiate gene-function analyses, a method for the targeted disruption of genes is required. For systems with a highly efficient homologous recombination (HR), *e.g.S. cerevisiae, Candida albicans or Schizosaccharomyces pombe,* PCR-based gene targeting methods can be employed (Wach et al.;, 1994; Bahler et al., 1998; Wilson et al., 2000). Other less amenable systems may benefit from a deletion of a Ku70 homolog of the Non-Homologous End-Joining pathway to increase the HR frequency (Ninomiya et al., 2004; Koh et al., 2014). Alternatively, in even more challenging systems the length of the homologous regions flanking the selectable marker can be increased (Taneja et al., 2004). The ease of genome and transcriptome sequencing allows to embark on an omics-based characterization of a non-conventional yeast to generate large scale datasets (Junker et al., 2019). From these datasets sufficient information can be easily derived to generate long flanking homology regions for gene deletion experiments.

Here we report a script for embarking on molecular genetic studies with a non-conventional yeast that should be broadly applicable in other ascomycetous species. We work with *Saccharomycopsis* predator yeasts that are able to penetrate and kill fungal prey cells *via* penetration pegs. *Saccharomycopsis* species are only distantly related to *S. cerevisiae* but have been found in traditional fermented beverages and thus have dual-purpose application potential in fermentation and biocontrol.

2. Material and methods

2.1. Strains and culture conditions

All yeast strains used and generated in this study (Table 1) were grown in standard YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C. Solid media plates were prepared with 2% agar. For selection, YPD plates supplemented with 200 µg/ml G418/geneticin, hygromycin or clonNAT, or minimal media lacking leucine (6.7 g/L yeast nitrogen base with NH_4SO_4 and 0.69 g/L complete synthetic medium leucine drop-out mixture) were used. Promoter activity was tested with a drop of X-gal (dissolved in *N,N*-dimethylformamide at a concentration of 20 mg/mL) on YPD plates. For plasmid propagation *Escherichia coli* DH5α was used and grown at 37 °C in 2xYT (1.6% bacto tryptone, 1% yeast extract, 0.5% NaCl) supplemented with ampicillin for selection.

2.2. Molecular techniques

The Lithium acetate transformation method was used for yeast transformation (Schiestl and Gietz, 1989). Plasmid DNA was prepared using the PureYield Plasmid Midiprep System (Promega, Fitchburg, USA). The homology regions flanking the *MSB2* ORF were amplified from genomic DNA using primers 122 and 123 for the 5′ homology region and 124 and 125 for the 3′ homology region. PCR fragments of homology regions were cloned into pGEM (Promega, Madison, USA). The *msb2* mutants were verified by diagnostic PCR using primers 132 and 133 for the internal fragment, 134 and 135 for the G1-G2 verification band, and 136 and 137 for the G3-G4 verification band (for list of primers see Table 3). All primers were obtained from Sigma Aldrich (Overijse, Belgium). All PCR and restriction digestion products were analyzed by gel electrophoresis separation on 1% agarose gels, stained with GelRed (Genaxxon Bioscience, Germany).

2.3. In vivo recombination in S. cerevisiae to generate plasmids

The set of six pRS shuttle plasmids containing the reporter constructs were generated by *in vivo* recombination of promoter sequences flanked by adaptor sequences and a vector backbone containing the *Streptococcus thermophilus lacZ* gene. The 1 kb-long upstream regions of six *S. schoenii* ORFs (*SsTEF1, SsGPD, SsENO1, SsYOL152W, SsMET25* and *SsHSC82*) were amplified from genomic DNA using primers 100–111 containing 45 bp adaptor sequences to facilitate recombination. The vector backbone was prepared by removing the *Ashbya gossypii TEF1* promoter from the pRS417-AgTEF1-lacZ plasmid by restriction digestion with *Kpn*I/*Xho*I. *S. cerevisiae* BY4741 strain was co-transformed with the pair of promoter and backbone fragments, for each construct. Transformants were selected on YPD plates supplemented with G418/Geneticin using the *GEN3* marker on the vector backbone.

To generate the *YES1-3* markers, pRS415 plasmid was linearized by digesting with *Sma*I. *SsTEF1* promoter region was amplified from the pRS417-SsTEF1-lacZ plasmid with primers F-YESx-TEF1 (112) and R-YESx-TEF1 (113) that add 45 bp adaptor regions. The antibiotic-resistance ORFs were amplified with primer pairs 114–115, 116–117 and 118–119; and from plasmids pUC57-SAK1, pUC57-HYG3 and pUC57-SAT3, respectively for the *YES1*, *YES2* and *YES3* constructs. For *YES4* and *YES5* marker genes, *S. cerevisiae* was co-transformed with the circular pRS415-YES2 plasmid and the PCR products for the two *kanR* ORFs amplified with the primers 120 and 121 from plasmid pFA-kanMX for *YES4* and with the same primers from plasmid pUC57-CTGfree-kanR (containing a synthetic construct in which four CTG positions were changed to TTA) for *YES5*. The *S. cerevisiae* transformants were selected on appropriate antibiotics for each marker to confirm recombination and functionality.

The disruption cassette for *S. schoenii MSB2* ORF was constructed by co-transformation with four fragments. The vector backbone was pRS415 linearized by *Sma*I. The two flanking homology regions to the ORF were amplified from the pGEM plasmids, this time with primers 126–129 to add 45 bp adaptor sequences. Finally, the fourth fragment,

Table 1
Strains used and generated in this study.

Strain	Genotype	Source
BY4741	*MATa; his3Δ1; leu2Δ0; met1Δ0; ura3Δ0*	Euroscarf
Saccharomycopsis schoenii	Wild-type	Westerdijk Fungal Biodiversity Institute, Utrecht, NL
S. schoenii MSB2 deletion strain #1	*MSB2/msb2::SAK1*	This study
S. schoenii MSB2 deletion strain #2	*MSB2/msb2::SAK1*	This study

Table 2
Plasmids used and generated in this study.

Plasmid	Features	Source
pGEM-T	pGEM-T Easy Vector System	Promega
pRS415	*amp, LEU2*	(Simons et al., 1987)
pRS415-YES1	*amp, LEU2, YES1*	This study
pRS415-YES2	*amp, LEU2, YES2*	This study
pRS415-YES3	*amp, LEU2, YES3*	This study
pRS415-YES4	*amp, LEU2, YES4*	This study
pRS415-YES5	*amp, LEU2, YES5*	This study
pRS415-MSB2 disruption cassette	*amp, LEU2, SAK1*	This study
pRS417-AgTEF-lacZ	*amp*, *GEN3*, *lacZ*	(Dünkler and Wendland, 2007)
pRS417-SsTEF-lacZ	*amp*, *GEN3*, *lacZ* driven by *S. schoenii TEF1* promoter	This study
pFA-kanMX6	*kanMX6*	(Wach et al., 1994)
pUC57-SAK1	*SAK1*, *amp*	GenScript
pUC57-HYG3	*HYG3, amp*	GenScript
pUC57-SAT3	*SAT3, amp*	GenScript
pUC57-CTGfree-kanR	*kanR ORF, amp*	GenScript

SAK1 marker, was amplified from plasmid pUC57-SAK1 with primers 130 and 131. Transformants were selected on minimal media lacking leucine. Plasmids obtained by *S. cerevisiae in vivo* recombination were propagated and verified in *E. coli*. All plasmids constructed and used in this study are listed in Table 2.

2.4. Microscopy

The pseudohyphal growth on mat formation plates were imaged with an Axioplan 2 Imaging microscope (Zeiss) equipped with an Orca Hamamatsu digital camera.

3. Results

3.1. In silico construction of synthetic selectable marker genes

Here we describe a fast *in silico* approach that we employed to generate synthetic marker genes for a NCY. To decide on suitable dominant selectable markers, we determined the sensitivity of *Saccharomycopsis schoenii* to various antibiotics. This indicated that *Saccharomycopsis* species are sensitive to the antibiotics G418/geneticin, hygromycin and nourseothricin/clonNAT. For these antibiotics there are resistance genes available. In the next step the resistance gene ORFs need to be fused to suitable promoters. The availability of draft genome sequence information and a preliminary annotation of the NCY genome allows for the simple sequence retrieval of target gene sequences. Recently we published the draft genome sequence of *S. schoenii* (Junker et al., 2019). From the *S. schoenii* genome we selected three DNA-polymerase II dependent promoters regulating the *S. schoenii* homologs of the *S. cerevisiae ACT1, PGK1* and *HEF3* genes. This choice was motivated by the high degree of conservation of these genes amongst ascomycetes. Based on our finding that *Saccharomycopsis* species employ the alternate translation of CTG into serine instead of leucine we sought to codon optimize the open reading frame (ORF) of

Table 3
Primers used in this study.

Primer	Name	Sequence (5' → 3')
100	F-TEF1	**cgccagggttttcccagtcacgacgttgtaaaacgacggccagtg**ctcgagtccagaataacatcaaatc
101	R-TEF1	**caatctttggatcgtttaaataagtttgaatttttttcagtcatgtt**ctataaaaaatgttagtatggag
102	F-GPD	**cgccagggttttcccagtcacgacgttgtaaaacgacggccagtg**ctcgagattgggcacgtaaccgag
103	R-GPD	**gtttaaataagtttgaatttttttcagtcatgttcatgga**cccgggatgaatgattatagatgtgtttag
104	F-ENO1	**tgtaaaacgacggccagtgagcgcggtatacgactcactatag**cccgggggtacccctagagcctgttctttat
105	R-ENO1	**ctttggatcgtttaaataagtttgaatttttttcagtcatgttcataaa**ctcgagtgtggacagctatgttgg
106	F-YOL152W	**tgtaaaacgacggccagtgagcgcgtaatacgactcactatag**cccgggggtacccaatagcaagattacca
107	R-YOL152W	**ctttggatcgtttaaataagtttgaatttttttcagtcatgttcataaa**ctcgagctttagtcaatatgtttatttatc
108	F-MET25	**tgtaaaacgacggccagtgagcgcgtaatacgactcactatag**cccgggggtaccgagtgcctaattatctgc
109	R-MET25	**ctttggatcgtttaaataagtttgaattttcagtcatgttcataaa**ctcgagtatagtaaaaaagaaggtaaaat
110	F-HSC82	**tgtaaaacgacggccagtgagcgcgtaatacgactcactatag**cccgggggtaccgctacctgtttttgtttgc
111	R-HSC82	**ctttggatcgtttaaataagtttgaatttttttcagtcatgttcataaa**ctcgagttttgcagtgagtaggaatac
112	F-YESx-TEF1p	**tgtaaaacgacggccagtgagcgcgcgtaatacgactcactatag**gaagcttcgtacgctgcaggtc**ggatcccccgggggcgcgcc**gtccagaataacatcaaatc
113	R-YESx-TEF1p	**gttggagttcaaacgtggtctggaaacgtgagtcttttccttaccc**tataaaaaaatgttagtatggag
114	F-YES1(kanORF)	ggtaaggaaaagactcacgtttcca
115	R-YES1(kanORF)	**ggaaacagctatgaccatgattacgccaagcgcgcaattaaccct**tctgatatcatcgatgaattcgagctcgtttaaacattggtaatag
116	F-YES2(hphORF)	**ctgacttttgtcttgttatggactccatactaacatttttttata**gaaaaaaccagaattgactgctacttc
117	R-YES2(hphORF)	**ctgatatcatcgatgaattcgagctcgtttaaacattggtaatag**gaccacctttgattgtaaatag
118	F-YES3(sat2ORF)	**ctgacttttgtcttgttatggactccatactaacatttttttatag**aaaatttctgttattcctgaacaag
119	R-YES3(sat2ORF)	**ctgatatcatcgatgaattcgagctcgtttaaacattggtaatag**gaccacctttgattgtaaatag
120	F-kanORF	**ctgacttttgtcttgttatggactccatactaacatttttttatag**ggtaaggaaaagactcacg
121	R-kanORF	**acaaatctaataaagtgaaaatctccccctttcacacttcacatatgt**tagaaaaactcatcgagcatc
122	F-5'flank	caagcgtatcacaacgtgctcc
123	R-5'flank	ctgaggtcgaactgcttgaag
124	F-3'flank	gaagacccaatggatgatgttg
125	R-3'flank	ccaccaaagtcttatggatgc
126	F-5'flank + adaptor	**tgtaaaacgacggccagtgagcgcgcgtaatacgactcactatagcccggg**caagcgtatcacaacgtgctcc
127	R-5'flank + adaptor	**gacctgcagcgtacgaagcttcaggcatgacacgagtgatgtcat**ctgaggtcgaactgcttgaag
128	F-3'flank + adaptor	**ctcgaattcatcgatgatatcagaattcttttctgtatgtaa**gtctgaagacccaatggatgatgttg
129	R-3'flank + adaptor	**ggaaacagctatgaccatgattacgccaagcgcgcaattaaccctcccggg**ccaccaaagtcttatggatgc
130	F-SAK1	atgacatcactcgtgtcatgcctgaagcttcgtacgctgcaggtc
131	R-SAK1	agacttacatacagaaagaattctgatatcatcgatgaattcgag
132	F-MSB2internal	cccagagtaccactgatgc
133	R-MSB2internal	cagaagcagcaatagtagatgg
134	G1-MSB2	tcatcctgtttgattctgcg
135	G2-SAK1	cacataccgttagattcgtag
136	G3-SAK1	gcaattccacttgatgcttgatg
137	G4-MSB2	ctgcaacacatgagatagtcc

Adaptor sequences are in bold. Restriction sites for *Xho*I (CTCGAG), *Kpn*I (GGTACC), *Sma*I (CCCGGG), *Eco*RI (GAATTC) and *Eco*RV (GATATC) are underlined.

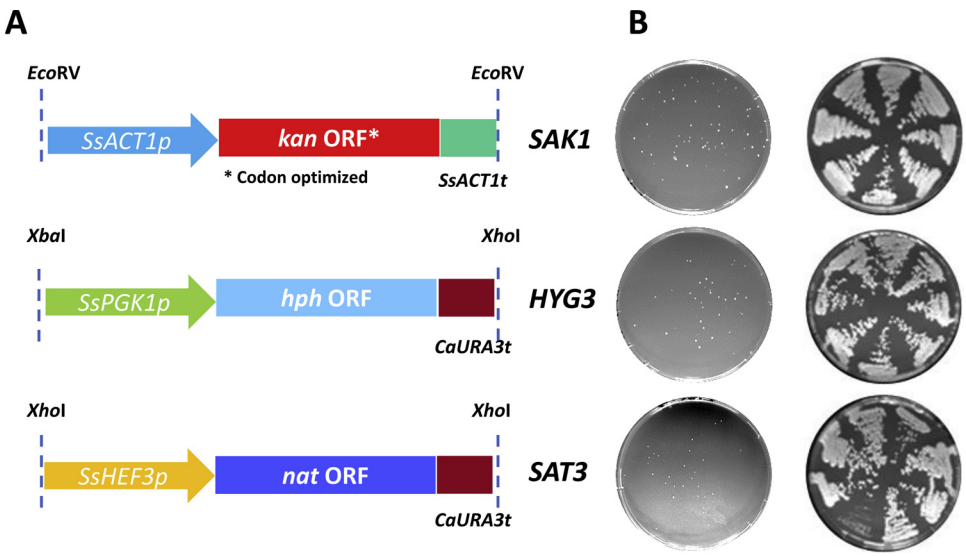


Fig. 1. *In silico* design of novel synthetic marker genes. (A) Promoter sequences were derived from the *S. schoenii* genome sequence and fused with the indicated open reading frames of the antibiotic resistance genes followed by a short terminator. Markers were then synthesized (Genscript, Piscataway, NJ USA). (B) Marker genes were linearized by using the indicated restriction enzymes and transformed into *S. schoenii.* Eight primary transformants for each marker gene (left set of petri plates) were restreaked on new selective plates indicating stable transformation (right set of petri plates).

one selectable marker. To this end we generated a codon usage table of *S. schoenii* strongly expressed genes based on the *ACT1, TEF1, TUB1* and *HHF1* ORFs. With this information the kanamycin resistance gene ORF derived from *kanMX* (used for *S. cerevisiae* transformations) was codon optimized. Additionally, the four CTG codons were changed into standard leucine encoding TTA codons. The resulting synthetic gene, *SAK1*, therefore consists of the *S. schoenii ACT1* promoter and terminator and the codon optimized *kan*-ORF (Fig. 1). Similarly, the *SsPGK1* promoter was fused with the hygromycin ORF and the *SsHEF3* promoter with the nourseothricin ORF, each followed by the *CaURA3* terminator. This generated the synthetic markers *HYG3* and *SAT3*, respectively (Fig. 1). These marker genes were obtained as custom company synthesized DNA-fragments.

We used the Lithium acetate/single strand carrier DNA/PEG transformation protocol of *S. cerevisiae* and obtained transformants for all three selectable marker genes in *S. schoenii* (Fig. 1). The locus of integration of the marker genes has not been analyzed but was presumed to be at random places in the genome. The transformation efficiency varies considerably, but usually 20 transformants per transformation with up to 10 µg of DNA could be obtained.

3.2. Generation of a reporter gene construct

The following sections describe how the highly efficient *in vivo* recombination system of *S. cerevisiae* can be utilized for the generation and testing of various constructs for subsequent use in a NCY. Promoter regions are less well defined compared to ORFs. Thus, when picking a promoter region there is a chance that it does not contain all sequences required for activation. In our case we generated three synthetic marker genes that were functional, however, *SAK1* was not functional in *S. cerevisiae.* To increase the chance of identification of functional NCY promoters we aimed at isolating heterologous promoters that are also functional in *S. cerevisiae*. This simplifies shuttling of constructs between the *S. cerevisiae* construction platform to the NCY.

To this end we used a *S. cerevisiae* shuttle vector harboring a *LacZ* reporter gene that was previously also used in *C. albicans* and is thus free of CTG codons (Uhl and Johnson, 2001). We selected several *S. schoenii* genes based on RNAseq transcriptomics data and conservation to *S. cerevisiae* (Junker et al., 2019), amplified their 1 kb regions upstream of their ATG start codon *via* PCR and added short flanks as adaptors for *in vivo* recombination in *S. cerevisiae* to generate the promoter-*LacZ* fusions. This is a fast and reliable seamless cloning approach and readily yields the desired constructs. Using X-gal as a substrate for β-galactosidase the activity of the reporters in *S. cerevisiae* could be demonstrated. The strongest *S. schoenii* promoter in this heterologous assay was obtained from the *TEF1* gene, while a *SsMET25-LacZ* fusion construct showed no activity, which is expected as the homologous *S. cerevisiae* promoter would also be off under the conditions tested (Fig. 2). The plasmids can easily be retrieved from *S. cerevisiae*, amplified in *E. coli* and then linearized for transformation into a NCY. By this we also demonstrated that the *SsTEF1-lacZ* reporter gene construct was also active in *S. schoenii* (Fig. 2C).

3.3. Generation of a bi-functional marker gene constructs

When generating constructs based on PCR products and *in vivo* recombination in *S. cerevisiae* it would be convenient to be able to test the functionality of the individual parts before transferring them into a NCY. Therefore, we set out to generate new marker genes based on the *SsTEF1* promoter fused to the resistance gene ORFs we used before. Since the *SsTEF1* promoter is functional in *S. cerevisiae,* the novel *YES1*, *YES2* and *YES3* markers generated by *in vivo* recombination could be directly selected for in *S. cerevisiae* (Fig. 3).

As a functional assay for the CTG codon reassignment in *Saccharomycopsis*, two markers, *YES4* and *YES5* were generated. The two markers are identical except for the presence of four CTG codons in *YES4*, which were converted to TTA codons in *YES5*. While both markers were found to be functional in *S. cerevisiae,* transformants could be obtained in *S. schoenii* only with the CTG-free *YES5* marker (Fig. 3).

3.4. Generation of long-flanking disruption cassettes by S. cerevisiae in vivo recombination recombination

We initially attempted transformation of *S. schoenii* with short-flank PCR products in which up to 100 bp of terminal homology regions to the target genes were added to the selectable marker by PCR. Since these attempts were unsuccessful, we aimed at increasing the homology region to up to 1500 bp. In the following we present the general design of this approach for a *S. schoenii* gene. To this end we picked an *S. schoenii MSB2* homolog, which in *S. cerevisiae* encodes a mucin family member that is involved in various signaling pathways. To generate a disruption cassette for *MSB2*, 5′- and 3′- flanking homology regions directly upstream of the start codon and downstream of the stop codon of the *MSB2* ORF were amplified from genomic DNA. These PCR products can either be cloned or directly used for a second PCR to add the adaptors required for *in vivo* recombination in *S. cerevisiae*. These two PCR products are then co- transformed with a linearized yeast shuttle vector (*e.g.* pRS415) and a NCY selectable marker (in this case still

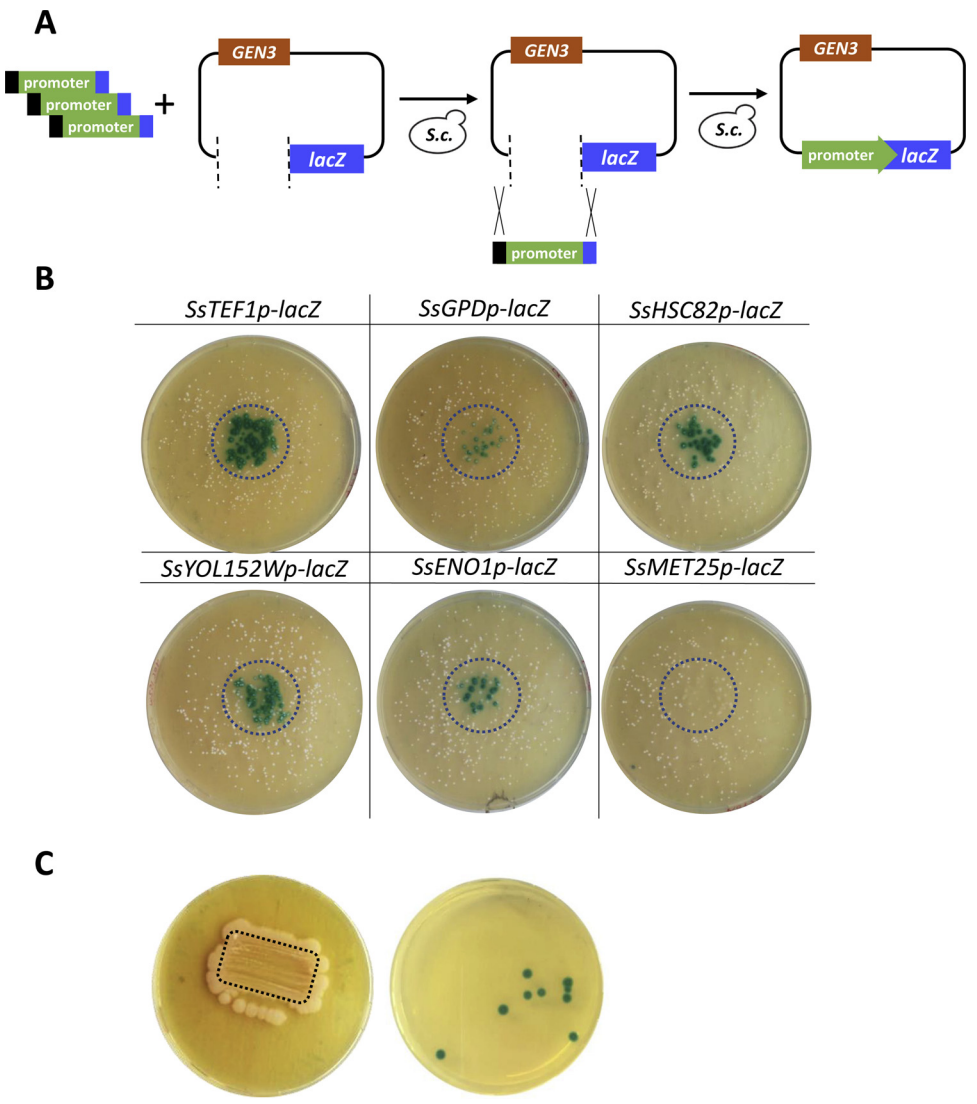


Fig. 2. Identification of heterologous promoter function in *S. cerevisiae*. (A) Several promoter fragments were obtained by PCR on genomic *S. schoenii* DNA and introduced in a reporter gene plasmid using *S. cerevisiae in vivo* recombination. (B) In the center of the primary *S. cerevisiae* transformation plates a drop of X -gal was spotted and the conversion of X-gal into a blue dye indicates LacZ expression and promoter activity. (C) Absence of β-galactosidase activity in wildtype *S. schoenii* cells to which X-gal was applied in the marked area (left plate). *S. schoenii* transformants harboring the *SsTEF1*-promoter-*LacZ* reporter gene construct were treated with X-gal. All nine transformants displayed β-galactosidase activity (right plate).

SAK1 even though *YES1* would be recommendable) into *S. cerevisiae* (Fig. 4A). In *S. cerevisiae* plasmid constructions with four or five fragments can be routinely accomplished. This approach allows exact design and generation of disruption cassettes and in this case we added terminal restriction sites to release the disruption cassette from the shuttle vector prior to transformation of *S. schoenii*. The construct was verified by diagnostic PCR and then shuttled through *E. coli* for plasmid propagation (Fig. 4A).

3.5. Transformation of S. schoenii and deletion of MSB2

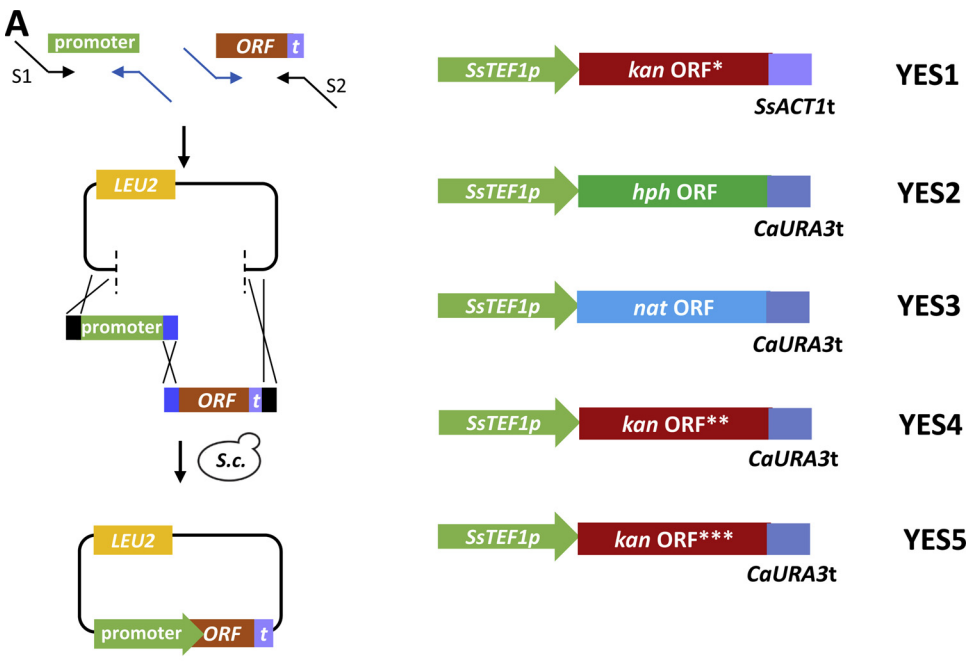
To transform *S. schoenii*, the *MSB2*-disruption cassette was released from the plasmid backbone by restriction digest with *Sma*I. We used the standard lithium acetate/single strand carrier DNA/PEG transformation method from *S. cerevisiae* to transform *S. schoenii.* The method has not been optimized for use in *Saccharomycopsis*. However, we obtained a small number of transformants that were checked for stable resistance to the antibiotic G418 when propagated in liquid culture (Fig. 4B). Initial transformants were analyzed by diagnostic PCR and two out of 10 transformants showed the correct marker integration bands (G1-G2 and G3-G4) with the absence of an internal band corresponding the *MSB2* ORF (I1-I2) (Fig. 4C). These results, *i.e.* the homozygous deletion of *MSB2*, are consistent with our observation that homothallic *S. schoenii* cells are preferentially haploid in their vegetative life cycle and sporulate upon zygote formation. The G1-G2 and G3-G4 diagnostic PCR bands were also cloned into pGEM and sequenced to verify exact integration of the disruption cassette at the target locus (our unpublished results). Taken together, these data indicate the first successful targeted disruption of a gene in the NCY *S. schoenii*.

3.6. Phenotypic characterization of S. schoenii msb2 mutant strains

In *S. cerevisiae MSB2* encodes a protein involved in signal transduction and pseudohyphal growth. Homozygous deletion of *MSB2* in diploid cells reveals reduced radial growth/spreading of the mutant on low nitrogen plates (Cullen et al., 2004). We therefore compared filamentous growth of the two *msb2* mutants with the wildtype on low strength agar plates (Fig. 5). This showed a reduced ability of the *msb2* mutant for pseudohyphal growth. This was particularly visible at the colony edges. Here only limited protrusions were found in *msb2* colonies whereas the wildtype showed ample pseudohyphal growth (Fig. 5B).

4. Discussion

The inevitable conversion of our petrol-based economy to a bio-based economy requires utilization of hitherto untapped resources. NCYs are non-*cerevisiae* strains that provide us with a metabolic biodiversity that may be useful either for specific compound production or when further developed as platform strains to produce more diverse



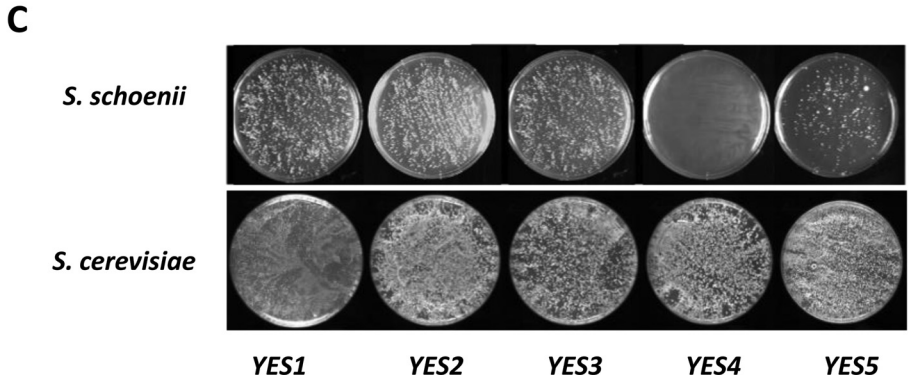


Fig. 3. Generation of a set of shuttle marker genes. (A) The *in vivo S. cerevisiae* platform was used to assemble promoter and ORF + terminator fragments into a yeast shuttle vector. (B) Based on the *S. schoenii TEF1* promoter, *YES1* – *YES5* markers were generated harboring different resistance gene ORFs. (*) *YES*1 contains a codon optimized *kan*-ORF adapted to the *S. schoenii* codon usage of highly expressed genes. *YES4* (**) and *YES5* (***) contain the original *kanMX*-ORF with the sole difference that four CTG codons were converted to TTA codons in *YES5*. (C) Transformation plates showing the result of the transformation of the *YES1-YES5* markers in either *S. schoenii* or *S. cerevisiae*. Based on the alternative codon usage of *Saccharomycopsis* species (CTG translated as serine instead of leucine) *YES4* is not active in *S. schoenii*.

and engineered metabolites (Lobs et al., 2017). This is of particular interest if value-added chemicals can be produced from low-value substrates (Mironczuk et al., 2016). Introductory level knowledge on NCYs can be obtained by using multi-omics approaches that provide a most detailed general overview on gene models, their transcriptional regulation and protein expression patterns under fermentation conditions (Czajka et al., 2017; Junker et al., 2019).

However, there are few examples of microbes as natural overproducers of compounds that can be used on a commercial scale, one of them is *Ashbya gossypii* with its potential to excessively overproduce riboflavin/vitamin B_2 (Aguiar et al., 2017). This indicates that microbial production platforms need to be engineered to produce compounds at large scales in an economically competitive manner. Novel tools such as CRISPR/Cas9 have been introduced successfully in several NCYs including *Pichia pastoris/Komagataella phaffii* (methylotropic, used for heterologous protein expression and secretion) *Kluyveromyces lactis* (lactose utilization and protein secretion) and *Y. lipolytica* (lipid production) (Horwitz et al., 2015; Schwartz et al., 2016; Gassler et al., 2019). Nevertheless, these tools require the prior establishment of a basic tool kit.

We set up a simple script to initiate molecular biology studies with ascomycetous NCYs. The highly efficient *in vivo* recombination system of *S. cerevisiae* provides a platform for generation and validation of constructs to be used in NCYs. This allows complete freedom in design and fast assembly of parts without depending on restriction recognition sites and has been utilized in different systems with great success (Lin et al., 2015; Shih et al., 2016; Chandran and Shapland, 2017). Individual elements can be tested in *S. cerevisiae,* which is a major advantage of this protocol. This provides proof that the tools are functional before they are tested in a NCY and reduces the experimental uncertainties to the efficiency of the transformation protocol.

The transformation efficiency of *S. cerevisiae* is extremely high. However, in our hands the same protocol (Lithium acetate/ss DNA/PEG) yielded several orders of magnitude less transformants in *S. schoenii*. The optimization of a transformation protocol will be a crucial step in NCY genetic engineering. To this end, several other transformation methods can be tested including electroporation, protoplast-mediated or *Agrobacterium*-based transformation and biolistic methods (Li et al., 2017). To promote homologous recombination (HR), using split but overlapping marker fragments has been used successfully (Fairhead et al., 1996). This requires HR to reassemble the marker and it is hypothesized that as HR is already activated this will also improve gene targeting. The transformation efficiencies vary considerably between experiments and comparing the use of selectable markers, *e.g.SAK1*, with the use of disruption cassettes based on *SAK1*. Disruption cassettes are usually at least twice as large as the marker due to the long flanking homology regions. Thus, treatment of the cell wall to facilitate uptake of the transforming DNA may be a target for

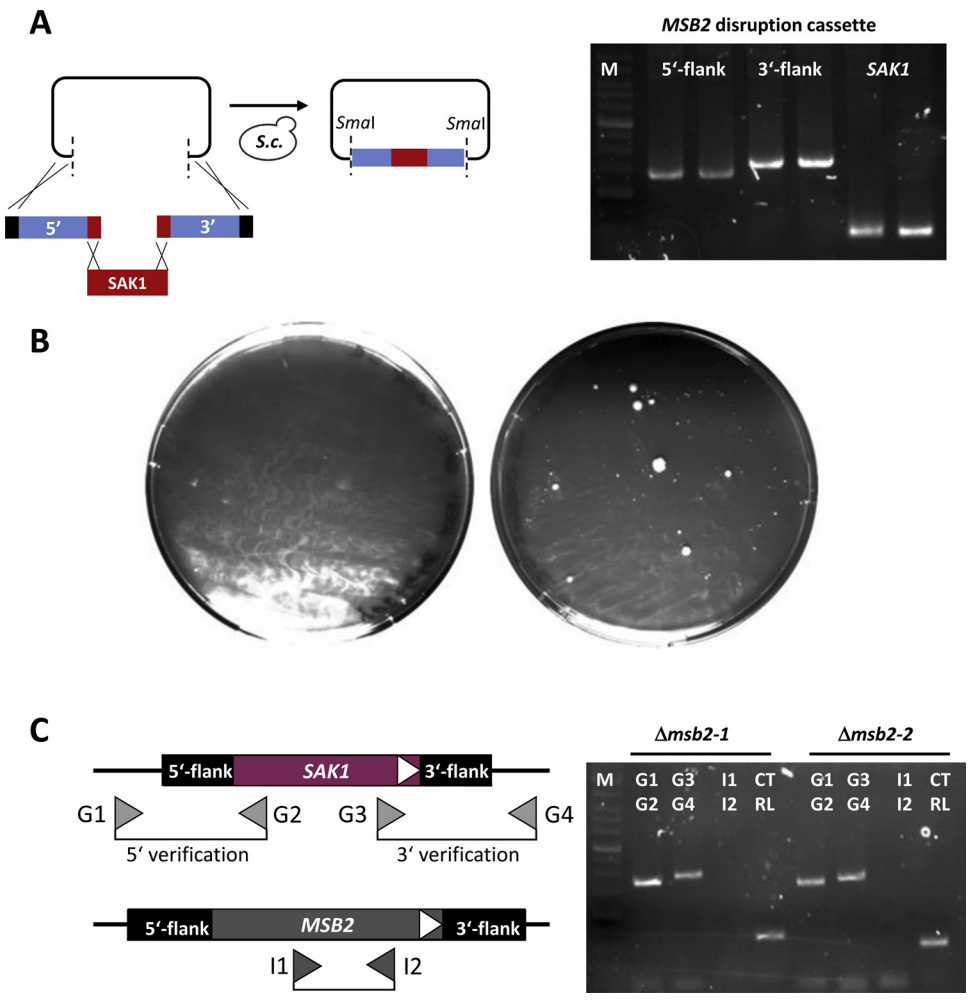


Fig. 4. Generation and use of long-flank homology region containing disruption cassettes. (A) The *in vivo S. cerevisiae* platform was used to assemble disruption cassettes consisting of two long-flanking homology regions and the selectable marker *SAK1* into a yeast shuttle vector. The incorporation of the individual fragments for an *MSB2* disruption cassette was verified by diagnostic PCR (GelRed stained agarose gel on the right). (B) Transformation of a *Sma*I-digested and linearized *MSB2* disruption cassette into *S. schoenii* yielded a few primary transformants (right plate) compared to the negative control (left plate). (C) Diagnostic PCR for verification of correct integration of the *MSB2* disruption cassette in the two *msb2* mutants.

optimization. Often, however, HR in NCYs is not very efficient and by far less efficient than in *S. cerevisiae*. In several cases deletion of the NHEJ-pathway, by deleting Ku70 has helped to drastically increase HR efficiency (Ninomiya et al., 2004; Naatsaari et al., 2012; Dai et al., 2019 and references therein). An opposite approach is the overexpression of *RAD51*, encoding a central gene for HR (Liu et al., 2004). We have recently engineered lager yeast strains overexpressing *RAD51*, which was sufficient to promote PCR-based gene targeting using short-flanking homology regions. This now paves the way for accelerated gene function analyses in this economically valuable hybrid yeast strains (Bernardi et al., 2019).

Our interest in the genus *Saccharomycopsis* is twofold. First, species of this genus, *e.g.S. fibuligera* and *S. fermentans* have been isolated from various spontaneous fermentations and thus have a long tradition to cope with artificial brewing environments (Carroll et al., 2017; Huang et al., 2019). For *S. fermentans* we recently showed a flocculation behavior very similar to *S. cerevisiae*. This behavior is based on Ca^{2+}-dependent flocculation likely carried out *via* an expanded gene family of telomere associated *FAS* genes (Hesselbart et al., 2018; Bernardi et al., 2018). We currently analyze the fermentation properties of various *Saccharomycopsis* species, particularly looking at the aroma compound formation of these NCYs.

The second fascinating aspect of *Saccharomycopsis* biology is their necrotrophic mycoparasitism, *i.e.* their ability to penetrate and kill fungal prey species (Lachance and Pang, 1997; Lachance et al., 2000, 2012). We have recently embarked on a more detailed analysis of this predation behavior. We could show that a wide variety of human fungal pathogens belonging to the *Candida* clade and including *C. auris* fall prey to *S. schoenii* (Junker et al., 2018). The analysis of several *Saccharomycopsis* genomes indicated that organic sulphur auxotrophy – basically methionine auxotrophy – provides a key hint that starvation triggers predation (Junker et al., 2017, 2019; Hesselbart et al., 2018). In a detailed omics study we could determine that genes involved in sulphur metabolism are upregulated in *S. schoenii* under starvation conditions but that this upregulation is alleviated in the presence of prey suggesting that feeding on prey cells overcomes this starvation (Junker et al., 2019).

To better understand the molecular mechanisms governing these biological features gene-function analyses are required. With the framework presented in this study including the generation of tools for targeted gene deletions we can now embark on functional analyses of genes, *e.g.* those identified in previous genome/transcriptome studies such as a *FLO8* homolog that may be responsible for *FAS* gene expression similar to *ScFLO8* in *S. cerevisiae* (Bernardi et al., 2018).

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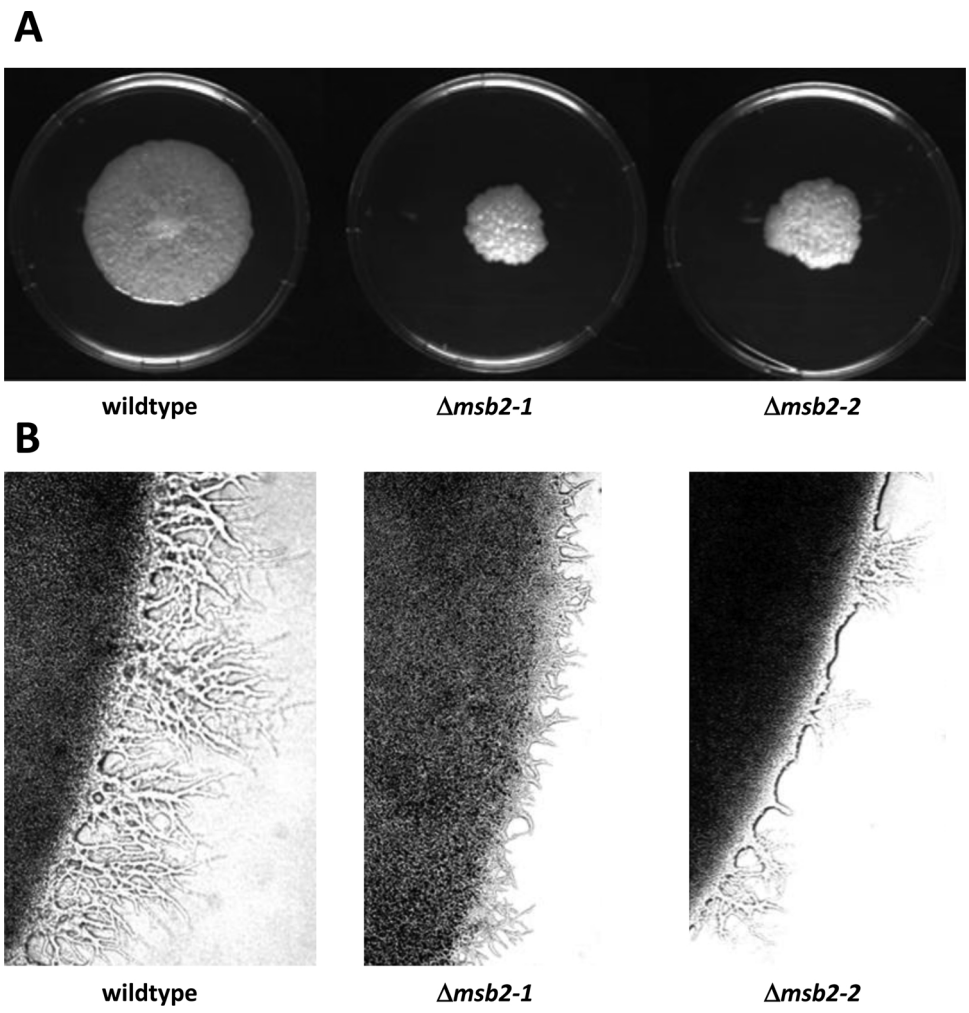

Fig. 5. Phenotypic characterization of *msb2* mutants. (A) Low strength agarose plates (0.3% agar) were used to compare radial growth of the wildtype and the two *msb2* mutant strains. Petri dishes were imaged after seven days of growth. (B) Micrographs of colony edges of the wildtype and the *msb2* mutants to visualize pseudohyphal growth differences.

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