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Development of a transformation system for gene knock-out in the flavinogenic yeast *Pichia guilliermondii*

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Abstract

Pichia guilliermondii is a representative of a yeast species, all of which over-synthesize riboflavin in response to iron deprivation. Molecular genetic studies in this yeast species have been hampered by a lack of strain-specific tools for gene manipulation. Stable *P. guilliermondii ura3* mutants were selected on the basis of 5′-fluoroorotic acid resistance. Plasmid carrying *Saccharomyces cerevisiae URA3* gene transformed the mutant strains to prototrophy with a low efficiency. Substitution of a single leucine codon CUG by another leucine codon CUC in the *URA3* gene increased the efficiency of transformation 100 fold. Deletion cassettes for the *RIB1* and *RIB7* genes, coding for GTP cyclohydrolase and riboflavin synthase, respectively, were constructed using the modified *URA3* gene and subsequently introduced into a *P. guilliermondii ura3* strain. Site-specific integrants were identified by selection for the Rib^- Ura^+ phenotype and confirmed by PCR analysis. Transformation of the *P. guilliermondii ura3* strain was performed using electroporation, spheroplasting or lithium acetate treatment. Only the lithium acetate transformation procedure provided selection of uracil prototrophic, riboflavin deficient recombinant strains. Depending on the type of cassette, efficiency of site-specific integration was 0.1% and 3–12% in the case of the *RIB1* and *RIB7* genes, respectively. We suggest that the presence of the ARS element adjacent to the 3′ end of the *RIB1* gene significantly reduced the frequency of homologous recombination. Efficient gene deletion in *P. guilliermondii* can be achieved using the modified *URA3* gene of *S. cerevisiae* flanked by 0.8–0.9 kb sequences homologous to the target gene.

Keywords: Riboflavin biosynthesis; Codon usage; Deletion cassette; Transformation; *URA3* marker; Yeast *Pichia guilliermondii*

1. Introduction

Some yeast species activate riboflavin (vitamin B_2) synthesis in response to iron depletion (Shavlovskii and Logvinenko, 1988). This group, the so called “flavinogenic yeasts”, includes *Pichia guilliermondii*, *Schwanniomyces occidentalis*, *Debaryomyces subglobosus*, the industrially important species *Debaryomyces hansenii* (the anamorph is known as *Candida famata*) and the human opportunistic pathogen *Candida albicans* (Shavlovskii and Logvinenko, 1988; Voronovsky et al., 2002; Knight et al., 2002). Although this phenomenon was first described in 1945 (Tanner et al., 1945), mechanisms of iron-dependent regulation of riboflavin biosynthesis are still unknown.

P. guilliermondii (asporogenous strains of this species are designated as *Candida guilliermondii*) could be a model organism to study mechanisms of iron-dependent regulation of riboflavin biosynthesis (Wickerham, 1966; Sibirny, 1996). Strains of this yeast species are of great industrial interest because of their high capacity to produce riboflavin and a sweetener, xylitol (Sibirnyi et al., 1977; Mussatto et al., 2005). Unlike other species of flavinogenic yeasts, this heterothallic

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Table 1
P. guilliermondii strains used in this study

Strains	Genotype	Source or reference
L2 (ATCC 201911)	*MAT*$^{-}$*hisX*	(Sibirnyi et al., 1977)
hit 1	*MAT*$^{+}$*cytX hit1*	(Stenchuk et al., 1991)
Meiotic segregant *m1*	*MAT*$^{+}$*hisX hit1 ura3*	This study
Meiotic segregant *m2*	*MAT*$^{+}$ *hisX hit1 ura3*	This study
Meiotic segregant *m3*	*MAT*$^{-}$*hisX hit1 ura3*	This study
Meiotic segregant *m4*	*MAT*$^{-}$*hisX hit1 ura3*	This study
Δrib7-1	*MAT*$^{+}$*rib7Δ::URA3 ura3 hisX hit1*	This study
Δrib7-2	*MAT*$^{+}$*rib7Δ::URA3 ura3 hisX hit1*	This study
Δrib7-3	*MAT*$^{+}$*rib7Δ::URA3 ura3 hisX hit1*	This study
Δrib7-4	*MAT*$^{+}$*rib7Δ::URA3 ura3 hisX hit1*	This study
Δrib1-53	*MAT*$^{+}$*rib1Δ::URA3 ura3 hisX hit1*	This study
Δrib1-54	*MAT*$^{+}$*rib1Δ::URA3 ura3 hisX hit1*	This study

hisX — an unidentified mutation causing histidine deficiency in *P. guilliermondii*.
hit1 — an unidentified mutation causing high iron transport in *P. guilliermondii*.
ura3 — a mutation causing uracil and cytosine deficiency in *P. guilliermondii* due to inactivation of orotidine 5′-monophosphate decarboxylase.
cytX — an unidentified mutation causing uracil and cytosine deficiency in *P. guilliermondii*.

species exists in both the haploid and diploid forms, which can be easily stimulated to mate and sporulate (Sibirnyi et al., 1977; Sibirny, 1996). High fertility genetic lines of this species have been isolated (Shavlovskii et al., 1980; Shavlovsky and Sibirny, 1985). They were used for identification of genetic factors involved in iron-dependent regulation of riboflavin biosynthesis and studying both riboflavin uptake and excretion (Shavlovskii et al., 1980, 1985; Sibirny and Shavlovsky, 1984; Shavlovsky and Sibirny, 1985; Sibirny, 1996). *P. guilliermondii* mutant strains defective in regulation of riboflavin biosynthesis also are defective in the regulation of iron acquisition (Shavlovskii et al., 1985; Stenchuk et al., 2001). They form 11 groups of complementation (Stenchuk et al., 2001; Boretsky et al., 2005). Recently, the genome of this yeast species was sequenced and is publicly available (http://www.broad.mit.edu- or http://www.ebi.ac.uk/fasta33/wgs.html).

However, the absence of an exploitable transformation system hampered further identification of molecular mechanisms involved in the regulation of riboflavin biosynthesis in *P. guilliermondii*. Earlier, we reported the transformation of *P. guilliermondii* with autonomously replicating plasmids (Boretsky et al., 1999). The reported system is not applicable for studying the regulation of riboflavin biosynthesis since structural genes of riboflavin biosynthesis were used as selectable markers. Further advances in studying this yeast required the development of molecular tools for gene manipulation. In this paper, we describe the *URA3*-based transformation system and construction of *P. guilliermondii* knockout strains. We anticipate that these tools will have a broad application in deletion or modification of specific *P. guilliermondii* genes.

2. Materials and methods

2.1. Strains, growth conditions and media

P. guilliermondii strains used in this study are listed in Table 1. For plasmid construction and propagation *E. coli* strain *DH5α* (lacZÄM15 recA1 endA1 gyrA96 thi-1 hsdR17($r_K^{-}m_K^{+}$) supE44 relA1 deoR Δ(*lacZYA-argF*)*U169*) has been used. Orotidine 5′-monophosphate decarboxylase deficient *E. coli* strain (*pyrF cys B*) kindly provided by Dr. Beckerich JM. (Laboratoire de Microbiologie et Genetique Moleculaire, CNRS-Institut National Agronomique Paris-Grignon-INRA, 78850 Thiverval-Grignon, France) and *Saccharomyces cerevisiae* strain *YPH499* (*MATα ura3-52 lys2-8019*(*amber*) *ade2-101* (*ochre*) *trp1-63 his3-200 leu2-1*) were used in complementation experiments.

Yeast cells were grown on the complete medium YPS (10 g yeast extract, 20 g peptone, 20 g sucrose, 20 g agar) at 30 °C or synthetic Burkholder medium supplemented with amino acids (40 mg/L), uracil or cytosine (20 mg/L) and riboflavin (200 mg/L) if required (Shavlovskii et al., 1985). *P. guilliermondii ura3* mutants were selected on a medium containing 0.67% YNB (Yeast Nitrogen Base), 2% glucose, 0.08% CSM-Ura (Complete Synthetic Media Without Uracil (BIO 101, Inc.), (Sherman, 1991), 50 mg/L of uracil, 1 g/L of 5′-fluoroorotic acid (FOA) and 20 g/L of agar (Boeke et al., 1984; Kato et al., 1997).

Yeast cells were grown in Erlenmeyer flasks on a gyro shaker (200 rpm) at 30 °C. Yeast strain hybridization and subsequent spore progeny analysis were performed as described (Sibirnyi et al., 1977). *E. coli* strains were grown in Luria-Bertani medium (LB) at 37 °C supplemented with ampicillin (100 μg ml^{-1}) if necessary. Minimal medium M9 supplemented with 0.05% vitamin free casamino acid was used for complementation experiments (Sambrook and Russell, 2001).

2.2. Plasmid construction and analysis

DNA manipulation and transformation of *E. coli* were carried out according to previously published procedures (Sambrook and Russell, 2001). To increase the efficiency of yeast

Table 2
Primers designed in this study

N	Designation	Sequence 5′→3′
1.	Ura31f	CGGGATCCATCATTTTTTTTTTATTC
2.	Ura32r	CGGGATCCGGTAATAACTGATATAATT
3.	URA3-Xh	AGGAATTACTCGAGTTAGTTGAAG
4.	URA3-REV	CAACTAACTCGAGTAATTCCTTG
5.	Fr71	GAAGATCTATTATGAACGAACTTGAG
6.	Re72	GAAGATCTTTACCGTTCTCGATTTAAG
7.	R7BamFo	CTGGATCCAGAGAGTCCAATACATC
8.	ReR7Bam	AAGGATCCTTAAAGTCGATAGGCATTC
9.	PRIf	ATAGATCTATGGTGCCGTTGGC
10.	PRIre	CAAGATCTTGTTCGTACAATTTTTCAGAAC
11.	PJJ_F	AGAACCTGTTGACATGACTCC
12.	PJJre	ATGACGATGGTTTGGATCAGG
13.	JB33	AACCGAAATGGCATGTTGAAC
14.	JB34	GCAGGTTAATGGACGGAAATG
15.	JB31	GCCAGAAGCAACACATCTG
16.	JB32	TGCTTCTCCAGAATACGCCAT

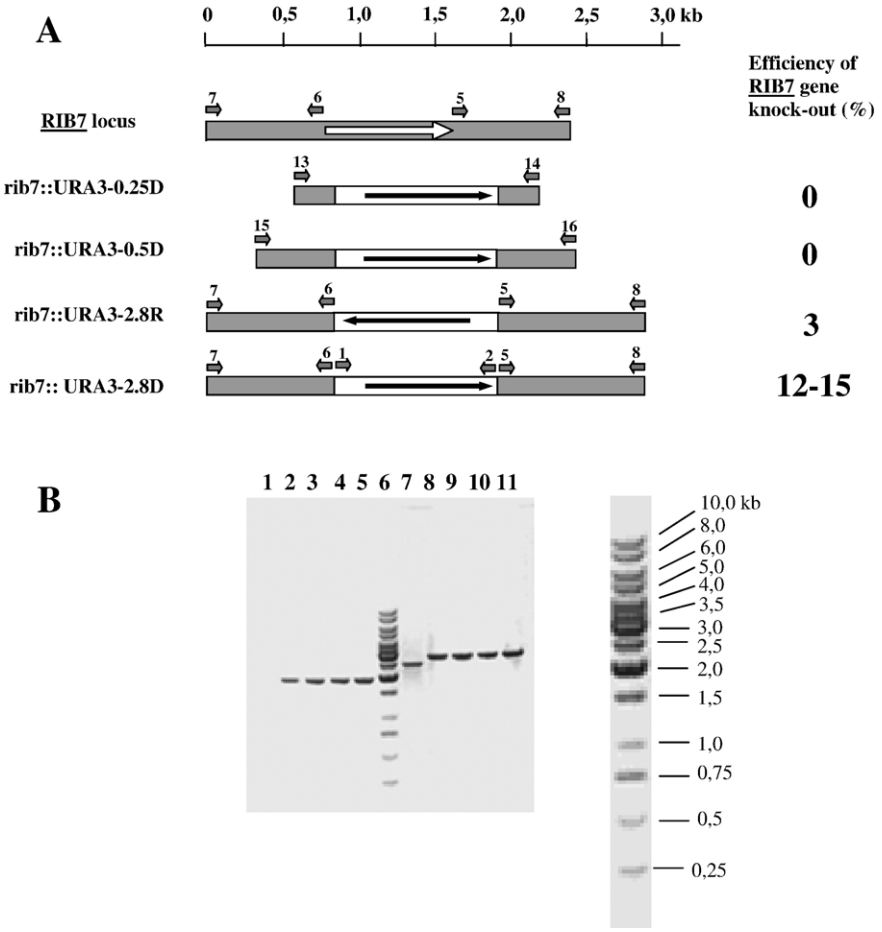

Fig. 1. Deletion of the *RIB7* gene in *P. guilliermondii*. A. Scheme of cassettes used to delete *RIB7* gene. ⇨ *RIB7* structural gene of *P. guilliermondii*. ➡ *URA3* structural gene of *S. cerevisiae*. ▭ 5′ and 3′ untranslated sequences of *RIB7* structural gene. B. Checking of knock-out recombinant strains by means of PCR. Lane 1 — DNA-signals obtained with recipient strain (primer 7 and primer 2; Table 2); lanes 2, 3, 4, 5 — DNA-signals obtained with recombinant strains *Δrib7-1*, *Δrib7-2*, *Δrib7-3*, *Δrib7-4*, respectively, (primer 7 and primer 2; Table 2); lane 6 — 1 kb DNA ladder (Fermentas); lane 7 — DNA-signals obtained with recipient strain (primer 7 and primer 8; Table 2); lanes 8, 9, 10, 11 — DNA-signals obtained with recombinant strains *Δrib7-1*, *Δrib7-2*, *Δrib7-3*, *Δrib7-4*, respectively (primer 7 and primer 8; Table 2).

transformation a 0.82 kb *Hinc*II DNA fragment carrying the *P. guilliermondii* ARS element was cloned into the *Hinc*II site of pUC19. The resulting pAG1 plasmid was used to clone the *URA3* gene. The 1.06 kb DNA fragment carrying the *S. cerevisiae URA3* gene was amplified by polymerase chain reaction using Ura31F and Ura32R primers and the pMT506 plasmid as a template (Cullin and Minvielle-Sebastia, 1994). (Table 2). The obtained DNA fragment was purified, digested with *Bam*HI, and cloned into the *Bam*HI site of the pAG1 vector. The resulting pAGU3 plasmid was used for complementation experiments.

Substitution of single CUG codon by the leucine codon CUC (mutation G135C) was done by two-stage PCR using two degenerate primers. At the first stage 5′ and 3′ ends of the cloned gene were amplified using primers Ura31F with URA3-REV (primer 1 and primer 4; Table 2) and Ura32R with URA3-Xh (primer 2 and primer 3; Table 2), respectively and plasmid pAGU-3 as a template. Primers URA3-REV and URA3-Xh used in these reactions carried mutation G135C (numbering from the first A of the initiator codon) and provided overlapping termini in the PCR products. At the second stage the entire mutated gene was amplified with primers Ura31F and Ura32R (primer 1 and primer 2; Table 2) using the obtained 0.36 kb and 0.75 kb DNA fragments (5′ and 3′ ends of the gene, respectively) as a template. The resulting 1.06 kb DNA fragment was purified, digested with *Bam*HI, and cloned into *Bam*HI site of pAG1 vector. The resulting plasmid called pAGU34, harbours a modified *URA3* gene of *S. cerevisiae* cloned at the same orientation as in pAGU3.

To construct *RIB7* deletion cassettes the 2.4 kb fragment of plasmid pER7-1 (Boretsky et al., 2002) was amplified by PCR using primers R7BamFo and ReR7Bam (Table 2 primers 7 and 8, respectively) thereby introducing *Bam*HI sites at the ends. The PCR product was purified, digested with *Bam*HI restriction endonuclease and cloned into the *Bam*HI site of the pUC19 vector. The constructed pR72 plasmid carries the *RIB7* structural gene with 0.8 kb and 0.9 kb of promoter and terminator sequences respectively. It was used to substitute *RIB7* structural gene by previously modified *S. cerevisiae URA3* gene (see above). Almost the entire sequence of the pR72 plasmid, except the *RIB*7 structural gene was amplified with primers Fr71 and Re72 (Table 2, primers 5 and 6, respectively) thereby introducing *Bgl*II sites at the ends of the PCR product. The PCR

product was purified, digested with *Bgl*II restriction endonuclease and ligated with the 1.06 kb *Bam*HI fragment of pAGU34 plasmid carrying the modified *S. cerevisiae URA3* gene. The resulted plasmids p724D and p724R carried modified *S. cerevisiae URA3* gene inserted (in opposite orientations) between 0.8 kb and 0.9 kb of promoter and terminator sequences of *P. guilliermondii RIB7* gene, respectively.

Deletion cassettes rib7::URA3-0.25D was amplified by PCR using primers JB33 and JB34 (Table 2 primers 13 and 14, respectively) and plasmid p724D as a template (see Fig. 1A in R&D section). Deletion cassettes rib7::URA3-0.5D was amplified by PCR using primers JB31 and JB32 (Table 2 primers 15 and 16, respectively) and plasmid p724D as a template (see Fig. 1A in R&D section). Deletion cassettes rib7::URA3-2.8D was amplified by PCR using primers R7BamFo and ReR7Bam (Table 2 primers 7 and 8, respectively) and plasmid p724D as a template (see Fig. 1A in R&D section). Deletion cassettes rib7::URA3-2.8R was amplified by PCR using primers R7BamFo and ReR7Bam (Table 2 primers 7 and 8, respectively) and plasmid p724R as a template (see Fig. 1A in R&D section). All PCR products were purified and used for transformation.

To construct *RIB1* deletion cassette, the pR plasmid that carries the *RIB1* gene was used (Zakal'skii et al., 1990). Almost the entire sequence of the pR plasmid, except the *RIB1* structural gene was amplified with primers PR1f and PR1re (Table 2, primers N.9 and N.10, respectively) thereby introducing *Bgl*II sites at the ends. The PCR product was digested with *Bgl*II endonuclease and ligated with 1.06 kb *Bam*HI DNA fragment carrying the modified *S. cerevisiae URA3* gene. The resulting pR110 plasmid harbours the modified *S. cerevisiae URA3* gene inserted between 1.6 kb and 1.4 kb of promoter and terminator sequences of *P. guilliermondii RIB1* gene respectively. The constructed plasmid was digested with *Eco*RI endonuclease yielding a rib1::URA3-10 deletion cassette (see Fig. 2A in R&D section).

All recombinant plasmids were sequenced using an ABI automated DNA sequencer model 373A. Homology search and alignments were performed with the aid of the BLAST and ClustalW 1.8 programs (available on-line at http://www.ncbi.nlm.nih.gov/BLAST/index.html and http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

2.3. Transformation procedures

Spheroplast transformation was done as described (Boretsky et al., 1999). A modified protocol was used to treat the cells with lithium acetate (Ito et al., 1983). Yeast cells were grown in YPS medium to an optical density of OD_{600} ≤0.5 (≤ 0.24×10^8

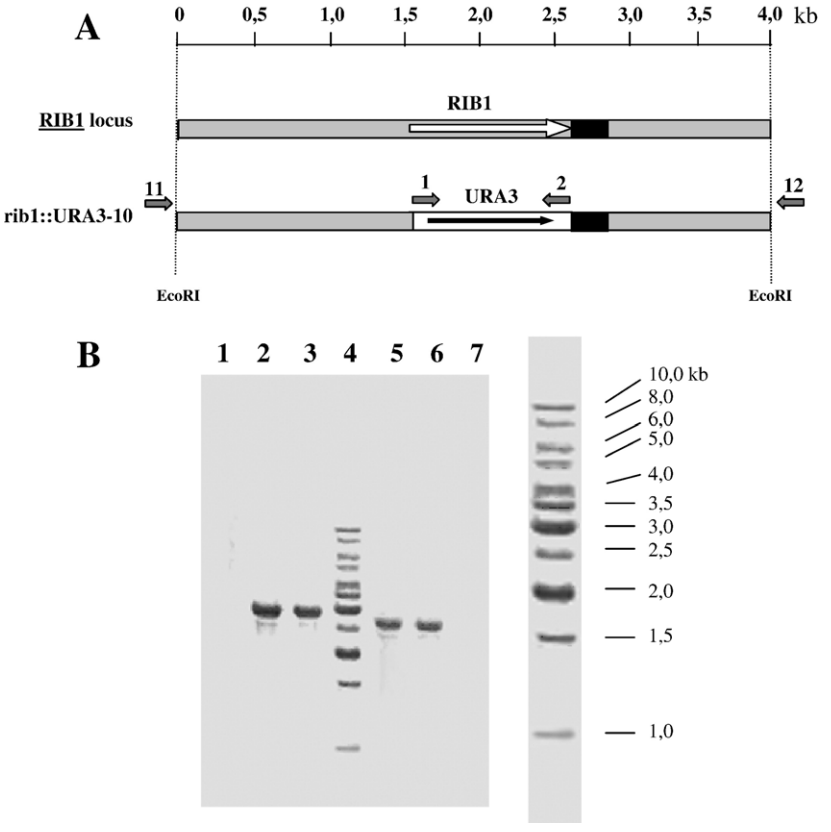

Fig. 2. Deletion of the *RIB1* gene in *P. guilliermondii*. A. Scheme of the cassette used to delete *RIB1* gene in *P. guilliermondii*. ⇨ — *RIB1* gene of *P. guilliermondii*. ➡ — *URA3* gene of *S. cerevisiae*. ■ ARS element of *P.guilliermondii*. ▭ — 5′ and 3′ untranslated sequences of *RIB1* structural gene. B. Checking of knock-out recombinant strains by means of PCR. Lane 1 — DNA-signals obtained with recipient strain (primer 11 and primer 2; Table 2); lanes 2, 3 — DNA-signals obtained with recombinant strains *Δrib1-53* and *Δrib1-54*, respectively (primer 11 and primer 2; Table 2); lane 4 — 1 kb DNA ladder (Fermentas); lanes 5, 6 — DNA-signals obtained with recombinant strains *Δrib1-53* and *Δrib1-54*, respectively (primer 1 and primer 12; Table 2); lane 7 — DNA-signals obtained with recipient strain (primer 1 and primer 12; Table 2).

cells/ml) and collected by centrifugation at 3000 *g* for 10 min. The cells were washed with water, centrifuged and re-suspended in 0.1 M lithium acetate, 10 mM Tris–HCl, 1 mM EDTA pH=7.5 (LiAc/TE buffer). After incubation (1 h at 30 °C) they were pelleted and re-suspended in fresh LiAc/TE buffer to a final concentration of 5×10^9 cells/ml ($OD_{600}\approx100$). Aliquots of 50 μl were dispensed into 1.5 ml tubes. Plasmid DNA (1–10 μg in 1–10 μl of TE buffer) and 250 μl 50% PEG in LiAc/TE were added and mixed vigorously. After incubation at 30 °C for 30 min, the cells were heat-shocked (15 min, 42 °C), chilled in ice for 1 min, harvested, re-suspended in 1 ml YPS and incubated at 30 °C for 1 h. Finally the cells were centrifuged again, re-suspended in 150 μl of 1 M sucrose, plated on a selective medium and incubated at 30 °C for 3–5 days.

For yeast electroporation, a modified protocol of Becker and Guarente was used (Becker and Guarente, 1991). Yeast cells were grown in a rich medium (YPS) to an optical density of $OD_{600}\leq0.5$ ($\leq0.24\times10^8$ cells/ml), chilled on ice and centrifuged at 3000 *g* for 10 min. The cells were washed with 0.1 M Li acetate, 10 mM Tris–HCl, 1 mM EDTA pH=7.5, twice with ice-cold water, and twice with 1 M sucrose. They were then re-suspended in 1 M sucrose to a final concentration of 5×10^9 cells/ml. Aliquots of 200 μl were dispensed into 1.5 ml tubes. Plasmid DNA (0.05–0.50 μg in 1–2 μl of 10 mM Tris–HCl, 1 mM EDTA pH=8.0 buffer) was added and mixed gently. The mixture was transferred into chilled 1 mm electroporation cuvettes. Electroporation was carried out in a Bio-Rad Gene Pulser II (resistance — 200 Ω; capacitance — 25 μF; voltage — 1.8 kV) with a Bio-Rad Pulse Controller II included in the circuit. The cells were washed out from the cuvettes with 1 ml YPS medium, incubated 1 h in 30 °C, centrifuged and re-suspended in 1 M sucrose, plated on selective medium and incubated at 30 °C for 3–5 days.

2.4. PCR analysis of yeast transformants

Total DNA was extracted as described previously, dissolved in TE buffer and used as a template for PCR analysis. (Gojkovic et al., 2000). 25 μl of the PCR mixture consisted of 2.5 μl of 10× *Taq* buffer with ammonium sulphate, 0.5 μl of 100 pM forward primer and 0.5 μl of 100 pM reverse primer, 2 μl of solution of total DNA (0.1 μg/μl) extracted from recombinant strains, 2.5 μl of 2 mM of each dNTP, 2 μl of 25 mM $MgCl_2$, 0.1 μl of 5 μ/μl *Taq* DNA polymerase and 14.9 μl of milli-Q water. PCR was performed with initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 40 s, primer annealing at 51 °C for 45 s and primer extension at 72 °C for 2 or 3 min.

3. Results and discussion

Earlier we reported on the development of a *P. guilliermondii* transformation system based on the use of *rib1* and *rib7* riboflavin auxotrophs as the recipient strains and cloned corresponding genes (*RIB1* and *RIB7* coding GTP cyclohydrolase II and riboflavin synthase, respectively) as the selectable markers (Boretsky et al., 1999). Later we demonstrated that plasmids containing an autonomously replicating sequence located near the *RIB1* gene transform *P. guilliermondii* cells with high efficiency, but they are unstable and integrate into the host genome by nonhomologous recombination (Piniaga et al., 2002). Therefore, this transformation system cannot be applied for studying the regulation of either riboflavin biosynthesis or iron acquisition.

The widely used *URA3* marker appeared to be the most convenient and useful selectable markers for *P. guilliermondii* transformation, since no antibiotic resistance genes are known. Therefore we decided to isolate *ura3* auxotrophs using a positive selection procedure with FOA and to use them as recipients for transformation experiments.

We observed that the wild type strains of *P. guilliermondii* could tolerate up to 0.4 g/l of FOA. FOA-resistant strains selected on medium containing 1 g/l of FOA were not uracil deficient (data not shown). Possibly, FOA resistance of such mutants could be explained by activated pyrimidine biosynthesis. To avoid that, *hit1* strain defective in pyrimidine biosynthesis was used in subsequent experiments. Cells of this strain were pregrown in YPS medium, collected by centrifugation, re-suspended in water and plated (5×10^7 cells per plate) on selective medium containing 1 g/l of FOA (see ""Pls. link to section 2: Materials and Methods Section). Four colonies of FOA-resistant mutants appeared after 12 days of incubation at 30 °C. In order to segregate the *cytX* mutation from newly obtained mutations, which confers FOA resistance, one of the selected mutants was crossed with the *P. guilliermondii* L2 strain. Spores obtained from this diploid were plated again on a selective medium containing FOA. All selected FOA-resistant haploid segregants resulting from this crossing exhibited uracil auxotrophy.

To identify whether the *URA3* gene was impaired in FOA-resistant strains we constructed a pAGU3 plasmid (see M and M) carrying the PgARS and *S. cerevisiae URA3* gene and used it for complementation experiments. The constructed plasmid complemented uracil deficiency in well characterized *S. cerevisiae YPH499* and *E. coli pyrF* strains. Among 7 randomly picked *P. guilliermondii* haploid segregants tested (see ""Materials and Methods Section and Table 1), only 4 (named *m3*, *m4*, *m7*, *m8*,) were transformed to uracil prototrophy by the pAGU3 plasmid. Despite the presence of PgARS the efficiency of transformation of the *P. guilliermondii m3* haploid segregant was very low: 40–50 transformants per 1 μg of DNA. Similar efficiency was reported when *P. guilliermondii* strains were transformed with integrative plasmids (Piniaga et al., 2002) (see Table 3).

As mentioned above *P. guilliermondii* and *C. guilliermondii* differ only in the ability to mate and sporulate (Sibirny, 1996). It is known that many *Candida* species exhibit differences in codon usage when compared to *S. cerevisiae* which uses the universal genetic code (Lloyd and Sharp, 1992). The most significant difference is that the CUG codon, a universal leucine codon, is read as serine in many *Candida* species (Sugiyama et al., 1995). There are 26 leucine residues in the *S. cerevisiae URA3* protein, one of which (namely L45), is located in a highly conservative region and is coded by the CUG codon. Later, when the *P. guilliermondii* genome became available, we found

Table 3
Efficiency of transformation of uracil dependent strains by plasmids pAGU3 and pAGU34

Strains	Efficiency of transformation by plasmid DNA, (transformants/μg DNA)	
	pAGU3	pAGU34
E. coli (*Pyr F*)	2×10^9	2×10^9
S. cerevisiae (*YPH 499*)	5×10^2	5×10^2
P. guilliermondii (*m3*)	5×10^1	5×10^3

The transformation experiments were replicated three times. Data from a representative experiment are shown.

that the *URA3* protein of this yeast species also possessed a leucine residue encoded by the UUA codon in the same position. We may suppose that the low efficiency of transformation could be a result of the formation of partially inactivated OMP decarboxylase, in which L45 is changed to a serine residue due to the translation specificity in the host cells.

To check this hypothesis we substituted CUG codon encoding L45 leucine residue with other leucine codon CUC. Newly constructed plasmid pAGU34 that harbours G135C (numbering from the first A) allele of *S. cerevisiae URA3* gene was used for complementation experiments. (see ""Materials and Methods Section). The efficiency of the transformation of well characterized *ura3* mutants of *S. cerevisiae* and *E. coli* was the same for pAGU3 and for the newly constructed pAGU34 plasmid. In contrast, the efficiency of transformation of *P. guilliermondii m3* meiotic segregant with the newly constructed pAGU34 plasmid was 100 fold increased when compared to the pAGU3 plasmid (Table 2). Thus, substitution of the single leucine CTG codon with other leucine codon (CTC) in the *S. cerevisiae URA3* gene causes a dramatic increase in efficiency of *P. guilliermondii* transformation. The results obtained allow suggesting that the CTG codon codes for serine and not leucine in *P. guilliermondii* cells.

Before the *P. guilliermondii* genome sequenced, only 2 genes, *RIB1* and *RIB7*, were cloned and sequenced. The *P. guilliermondii RIB1* gene coding for GTP cyclohydrolase II has been shown to be partially overlapped with an ARS element which decreases the probability of homologous recombination in this yeast species (Piniaga et al., 2002). Thus, the only target available for an initial knock-out experiment was the *RIB7* gene coding for riboflavin synthase.

Four deletion cassettes were generated in which *RIB7* structural gene was replaced by a modified *S. cerevisiae URA3* gene as described above (Fig. 1A). Deletion cassettes rib7::URA3-0.25D and rib7::URA3-0.5D contained, respectively, 0.25 kb and 0.5 kb both of promoter and terminator sequences of the *RIB7* gene. Deletion cassettes rib7::URA3-2.8D and rib7::URA3-2.8R (differs from each other by opposite orientation of the *URA3* gene) both contained 0.8 kb and 0.9 kb of promoter and terminator sequences of the *RIB7* gene, respectively (Fig. 1A).

The *m3* haploid segregant of *P. guilliermondii* was transformed with these cassettes using the electroporation procedure, spheroplasting and Li–Ac method. To get a larger number of transformants, the electroporation procedure was used in the initial experiments. Efficiency of transformation for all cassettes was approximately 1000 transformants per 1 μg of DNA. However, no stable riboflavin deficient recombinant strains were selected among approximately 280, 240, 300 and 200 transformants obtained with cassettes rib7::URA3-0.25D, rib7::URA3-0.5D, rib7::URA3-2.8D and rib7::URA3-2.8R, respectively. Similar results were obtained when the spheroplasting procedure was applied for transformation. No stable riboflavin deficient strains were selected among 400 transformants (approximately 100 transfomants for each cassette). In contrast, approximately 12% of stable riboflavin auxotrophs were selected among uracil prototrophs when the recipient strain was transformed with rib7::URA3-2,8D cassette using the lithium acetate procedure. Efficiency of transformation was approximately 200 transformants per 1 μg of DNA. The number of riboflavin auxotrophs was decreased to 3% when using deletion cassette rib7::URA3-2,8R that has an opposite orientation of the *URA3* gene (Fig. 1A). No riboflavin auxotrophs were selected with the cassette rib7::URA3-0.25D and rib7::URA3-0.5D 2,3 that harboured a shortened promoter and terminator sequence of the *RIB7* gene (Fig. 1A).

In order to confirm deletion of the *RIB7* gene, riboflavin auxotrophs obtained with rib7::URA3-2,8D cassettes were further checked. For that purpose, two PCR amplifications were performed using the total DNA of transformants as a template. 1.9 kb DNA fragments were obtained with primers 7 and 2 (see Table 2) when total DNA of selected recombinant strains was used as a template whereas no signal was generated in the case of recipient strain. Another set of primers (namely 7 and 8; see Table 2) also gave expected results: 2.4 kb and 2.8 kb signals in the cases of the recipient and recombinant strains, respectively (Fig. 1B). Taken together, the obtained results suggest that in these transformants the rib7::URA3-2,8D deletion cassettes integrated into the genome by homologous recombination which lead to a knock-out of the *RIB7* structural gene.

To prove the feasibility of this approach, we constructed a deletion cassette rib1::URA3-10 and transformed it into the same recipient strain using the lithium acetate procedure. Efficiency of transformation was approximately 10,000 transformants per 1 μg of DNA. Only 2 of 2000 transformants checked exhibited riboflavin deficiency. 2.9 kb and 1.6 kb DNA fragments were amplified using total DNA of these transformants with two pairs of primers: pJJ_F, Ura32r (11 and 2) and Ura31f, pJJre (1 and 12) respectively. No signal was obtained with those sets of primers when the DNA of the recipient strain was used as a template (Fig. 2B). Thus, we have successfully deleted two genes in *P. guilliermondii*. The decreased efficiency of *RIB1* knock-out could be a suggestion that the ARS element adjacent to the GTP cyclohydrolase structural gene reduces the efficiency of homologous recombination in this yeast species.

P. guilliermondii Wickerham represents a collection of sporogenous strains which were formerly identified as the asporogenous species *C. guilliermondii* (Cast) Langeron a. Guerra. This means that each strain of *C. guilliermondii* which is able to mate and sporulate should be transferred to the species *Pichia guilliermondii* (Sibirny, 1996). Our results allow suggesting that

in *P. guilliermondii,* the CUG codon is decoded as a serine, as has been reported for many *Candida* species. Substitution of the 45th CUG codon in the *S. cerevisiae URA3* gene by a CUC codon caused significant increase in transformation efficiency of *P. guilliermondii ura3* strains. The modified *S. cerevisiae URA3* gene appears to be a useful marker for gene replacement experiments in *P. guilliermondii*. Structural genes coding for riboflavin synthase and GTP cyclohydrolase were successfully deleted using this approach, despite the fact that introduced DNA integrated into the *P. guilliermondii* genome mainly by nonhomologous recombination. Notably, the method of DNA delivery affects the rate of production of desired integrants in *P. guilliermondii*: Lithium acetate procedure only provided selection of stable riboflavin deficient recombinant strains. The developed transformation system can be used for identification of genes involved in the regulation of riboflavin biosynthesis and for other studies that require functional analysis of *P. guilliermondii* genome.

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