ELSEVIER

FEMS Yeast Research 5 (2005) 829–837

FEMS Yeast Research

www.fems-microbiology.org

Positive selection of mutants defective in transcriptional repression of riboflavin synthesis by iron in the flavinogenic yeast *Pichia guilliermondii*

Yuriy R. Boretsky [a], Kostyantyn Y. Kapustyak [a], Lyubov R. Fayura [a], Oleh V. Stasyk [a], Mykola M. Stenchuk [a], Yaroslav P. Bobak [b], Lyudmyla B. Drobot [b], Andriy A. Sibirny [a,c,*]

[a] *Department of Molecular Genetics and Biotechnology Institute of Cell Biology, NAS of Ukraine, Drahomanov Street, 14/16, Lviv 79005, Ukraine*
[b] *Department of Cell Signaling, Institute of Cell Biology, NAS of Ukraine, Drahomanov Street, 14/16, Lviv 79005, Ukraine*
[c] *Institute of Biotechnology, Rzeszow University, Poland*

Received 9 February 2005; received in revised form 5 March 2005; accepted 5 March 2005

First published online 27 April 2005

Abstract

It is known for many years that iron represses synthesis of riboflavin (RF) and most of RF-synthesizing enzymes in several yeast species, known as flavinogenic yeasts. However, the mechanism of such repression is not known. We have found that iron represses transcription of *RIB1* and *RIB7* genes coding for the first and the last enzymes of RF biosynthesis in the model flavinogenic organism *Pichia guilliermondii*. To decipher molecular mechanisms of iron-dependent repression, isolation and study of the regulatory mutants defective in corresponding regulation is desirable. However, no suitable methods for isolation of such mutants were previously available. We have produced a single-point transition mutation in the *RIB1* gene. The corresponding *rib1-86* mutant exhibits leaky phenotype and is unable to grow in iron-sufficient minimal medium without exogenous RF. However, it can grow in minimal iron-deficient medium without RF, or in iron-sufficient medium upon introduction of the previously-isolated regulatory mutation *rib81*, which leads to increase in RF production. Using the *rib1-86* mutant as parental strain, a collection of mutants able to grow in iron-sufficient medium without exogenous RF has been isolated. The mutants appeared to be defective in regulation of RF biosynthesis and iron homeostasis and were divided into six new complementation groups. Study of one corresponding mutant, *red6*, showed derepression of *RIB1* mRNA synthesis in iron-sufficient medium.
© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Yeast *Pichia guilliermondii*; Riboflavin biosynthesis regulation; GTP cyclohydrolase II; Mutability; Iron

1. Introduction

It is known for many years that iron regulates RF synthesis in several yeast species, known as flavinogenic yeasts. The list includes *Pichia* (*Candida*) *guilliermondii*, *Candida famata* (*Debaryomyces hansenii*), the pathogenic yeast *Candida albicans* and some other species [1–4]. Some of the flavinogenic yeasts possess very high biosynthetic potential, which is illustrated by the fact that mutants isolated from *C. famata* belong to the strongest RF overproducers and are used for industrial RF production [5]. Mechanisms by which iron regulates RF synthesis are poorly understood. The good model organism for such studies is the sporogenous yeast *P. guilliermondii*, because methods for hybridization and

* Corresponding author. Tel.: +380322 740363; fax: +380322 721648.
E-mail addresses: boretsky@biochem.lviv.ua (Y.R. Boretsky), sibirny@biochem.lviv.ua (A.A. Sibirny).

1567-1356/$22.00 © 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.femsyr.2005.03.007

analysis of meiotic spore progeny, as well as DNA transformation, have been developed for this species [6–8]. It has been demonstrated that iron inhibits RF synthesis in *P. guilliermondii* by repression of all (with exception of the second one) enzymes involved in RF production [9]. However, the mechanisms and the level at which iron represses synthesis of enzymes involved in RF production have not been identified yet. Several mutants defective in iron-dependent repression of RF synthesis have been isolated in *P. guilliermondii*, designated as *rib80*, *rib81*, *hit1* [10–12], though the methods for isolation of such mutants were laborious and time-consuming.

Earlier, the *P. guilliermondii rib1-86* RF-deficient mutant lacking the activity of the first enzyme of RF synthesis, GTP cyclohydrolase, has been isolated [13]. It was found that some revertants isolated from the *rib1-86* mutant appeared to be suppressor mutants [14]. This paper contains molecular characteristics of the *rib1-86* mutation and shows how it can be used for simple positive selection of the mutants derepressed in RF production in iron-sufficient medium. The corresponding regulatory mutants are preliminarily characterized. We demonstrate that iron regulates RF synthesis at transcriptional level, repressing production of *RIB1* and *RIB7* mRNAs, and that such transcriptional repression is alleviated in the regulatory mutants *rib81* and *red6*.

2. Materials and methods

2.1. Strains and plasmids

Pichia guilliermondii strains used in this study are listed in Table 1. For plasmid construction and propagation *Escherichia coli* strain *DH5α* (*lacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *supE44 relA1 deoR Δ(lacZYA-argF)U169*) has been used. *E. coli* strain *WA802-81* (*F*$^-$ *e14*$^-$ (*McrA*$^-$) *lacY1 supE44 galK2 galT22 rfbD1 metB1 mcrB1 hsdR2* ($r_k^- m_k^+$) *rib1*), defective in the GTP cyclohydrolase II gene, was used in complementation experiments [8]. The plasmid p19R1 carrying the *P. guilliermondii RIB1* gene coding for GTP cyclohydrolase II was described earlier [8].

Table 1
Yeast strains used in this study

Strain	Genotype	Ref. of source
ATCC9058	Wild type	[7]
L2 (ATCC201911)	*MAT*$^-$ *his-17*	[7]
9D303	*MAT*$^+$ *ade 2-19, rib 1-86*	[13]
LV381	*MAT*$^-$ *hit1-1 his-17*	[12]
LV107	*MAT*$^+$ *rib80-22 arg-1*	[6,11]
LV158	*MAT*$^-$ *rib81-131 his-17*	[6,10]
LV 461	*MAT*$^-$ *red1-2 ade2-19*	[14]
LV 462	*MAT*$^-$ *red2-32 his-17*	[14]
LV 463	*MAT*$^-$ *red3-26 ade2-19*	[14]
LV 464	*MAT*$^-$ *red4-37 ade2-19*	[14]
LV 465	*MAT*$^-$*red5-4 ade2-19*	[14]
LV 466	*MAT*$^-$ *red6-8 ade2-19*	[14]

2.2. Growth conditions and media

Yeast cells were grown on *YPD* or synthetic Burkholder medium supplemented with amino acids (40 mg l^{-1}), bases (20 mg l^{-1}) and RF (200 mg l^{-1}) if required [6]. Iron-deficient media contained about 0.18 μM of iron. Iron was removed from the medium with 8-hydroxyquinoline as described earlier [15]. Iron-supplemented media contained 3.6 μM iron added as ammonium ferrous sulfate hexahydrate. Yeast cells were grown in Erlenmeyer flasks on a gyro shaker (200 rpm) at 30 °C. *E. coli* strains were grown in Luria–Bertrani medium (LB) at 37 °C supplemented with RF (200 mg l^{-1}) or ampicillin (100 μg ml^{-1}) if necessary. Yeast hybridization and subsequent spore progeny analysis was done as described [7].

2.3. Biochemical analyses

RF was assayed fluorometrically. Activity of GTP cyclohydrolase II was determined by a fluorometric method as described earlier [16]. Protein concentration was determined by the Lowry method [17]. Ferric reductase activity and total iron content in cells were assayed by the α,α′-dipyridile method as described [12].

2.4. DNA manipulations and transformation procedures

The 2.1-kbp DNA fragment carrying the GTP cyclohydrolase II gene was amplified by a polymerase chain reaction using primers Rib1for (5′-GGCTCT-AGATTTCACTCCGAAGGTAGG-3′) and Rib1rev (5′-CGCGGATCCTAGAGTCGACACACATCA-3′) and chromosomal DNA of *P. guilliermondii rib1-86* strain as a template. The obtained DNA fragment was purified using the QIAGEN (Chatsworth, CA) PCR purification kit, digested with BamHI and XbaI, and cloned into pUC19 vector cut with the same endonucleases. Three independent recombinant plasmids were sequenced using an ABI automated DNA sequencer model 373A (GMI, Inc., Ramsey, MN). A homology search and alignments were performed with the aid of the BLAST and ClustalW 1.8 programs (available online at www.ncbi.nlm.nih.gov/BLAST/index.html and www.searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Construction of the C207V mutant allele of the *RIB1* gene will be described elsewhere. For yeast electroporation, a modified protocol of Becker and Guarente was used [18]. Yeast cells were grown in rich medium (YPD) to an optical density $OD_{600} \leqslant 0.7$, chilled on ice and pelleted at 4000*g* for 10 min. The cells were

washed with 0.1 M Li acetate, twice with ice-cold water, and twice with 1-M saccharose. They were then re-suspended in 1-M saccharose to a final concentration of 5×10^9 cells ml^{-1}. Aliquots of 200 µl were dispensed into 1.5-ml tubes. Plasmid DNA (0.05–0.20 µg in 1–2 µl of TE buffer) was added and mixed gently. The mixture was transferred into prechilled 2-mm electroporation cuvettes. Electroporation was performed as follows: resistance 400 Ohms; capacitance 25 µF; voltage 2.3 kV. Cells were washed out from the cuvettes with 1-M saccharose, plated on selective medium and incubated at 30 °C for 3 d.

2.5. RNA analysis

Cells were grown to middle-log phase on synthetic media containing defined concentrations of iron. Total RNA was extracted from these cells as described [19]. For the Northern-blot analysis, RNA was denatured with formamide, separated in 1.5% agarose gel containing formaldehyde, and blotted to nylon membrane (Hybond^{tm}-N+, Amersham, Little Chalfont, UK). The Rib1 probe was generated by PCR with primers 5′-GAAACTCACGAATTTACC-3′ and 5′-GTGCG AATCTGCATATGAATAG-3′, using plasmid p19R1-86 as a template. The 0.42 kb KpnI fragment of the *P. guilliermondii* structural gene coding for RF synthase (GenBank AY138984) was used as a Rib7 probe. The probes were labeled with ^{32}P radionuclide using a random-priming kit (Gibco-BRL, Carlsbad, CA). Northern blotting and hybridization were carried out essentially as described [3]. The filters were then exposed to X-ray film at −70 °C for 2 d prior to developing. After hybridization, *RIB1* and *RIB7* mRNA levels were quantified using the Scion (Frederick, MD) software (Scion Image version 4.0.2). Values for mRNA signals were normalized with volume values for ethidium bromide stained rRNAs on the membranes.

3. Results and discussion

3.1. Molecular cloning of the rib1-86 allele of the GTP cyclohydrolase II structural gene

Earlier, the *P. guilliermondii RIB1* gene coding for GTP cyclohydrolase II has been cloned and sequenced [8] (GenBank Acc. No. AX009185). Based on these data, we designed primers to amplify the *rib1-86* mutant allele of the gene. The 2.1 kb DNA fragment was amplified and cloned into pUC19 vector as described in Section 2. In contrast to the plasmid p19R1 carrying the wild-type (WT) allele of the *RIB1* gene, the newly-constructed recombinant plasmid (named p19R1-86) purified from three independent *E. coli DH5α* transformants did not complement RF deficiency in *E. coli rib1* and *P. guilliermondii rib1* mutants lacking GTP cyclohydrolase II activity. Analysis of nucleotide sequences of these three plasmids revealed the same single-point mutation G620A (numbering from the first nucleotide of the initiator ATG codon) in the structural gene. This transition converts a cysteine codon TGT to a tyrosine codon TAT, resulting in C207Y substitution in the predicted amino-acid sequence of GTP cyclohydrolase II (Fig. 1).

3.2. C207Y substitution in P. guilliermondii Rib1p leads to leaky RF deficiency

Alignment of several known GTP cyclohydrolases reveals that the mutated position (C207) is usually occupied by a valine residue (Fig. 1). In contrast, a cysteine residue was found at this position in GTP cyclohydrolases II of *P. guilliermondii*, *Debaryomyces hansenii* and *Candida albicans*, species that exhibit iron-dependent regulation of flavinogenesis. Substitution of tyrosine-207 by valine in the *rib1-86* allele of the *RIB1* gene restored activity of GTP cyclohydrolase II. Plasmids carrying 207V allele of the gene complemented RF deficiency in both *E. coli* and *P. guilliermondii* mutants deficient in GTP cyclohydrolase II, but all yeast transformants were unable to oversynthesize RF on iron-depleted media (data not shown).

The functional role of the cysteine-207 residue remains unknown.

Recently, it has been reported that the catalytic site of GTP cyclohydrolase II from *E. coli* contains one zinc ion (per subunit) coordinately bound by cysteine residues C54, C65, C67 [20]. They correspond to the C176, C187, C189 cysteine residues in the *P. guilliermondii* enzyme but not to C207 (Fig. 1). Since the active site of the enzyme is not affected, most likely C207Y substitution incompletely inactivates *P. guilliermondii* GTP cyclohydrolase II or, renders it unstable. In favor of this hypothesis, regulatory mutation *rib81* partially suppressed *rib1-86* mutation: double mutant *rib1-86 rib81* was RF-prototroph but could not oversynthesize RF [13,14].

Earlier it was shown that iron deficiency causes a 10–20 times increase in GTP cyclohydrolase II activity in the *P. guilliermondii* WT strain [6,10]. RF production and activity of GTP cyclohydrolase II under these conditions both can reach values reported for the constitutive RF-overproducing mutant *P. guilliermondii rib81* [10]. We found that iron deficiency stimulates growth of the *rib1-86* strain in the absence of RF (Fig. 2). The *rib1-86* mutant did not accumulate RF in the culture medium, whereas the WT strain accumulated about 8 mg of RF per 1 g of cell dry weight in the culture medium under these conditions. In contrast, no growth of *P. guilliermondii rib1-86* was observed on the medium containing a physiological concentration of iron but

```
                                                                      *
Pgu(1-86)   172 IHSECYTGETAWSARCDCGEQFDEAG-----------RLMGEAGHGYIVYLR-QEGRGIG
Pgu(wt)     172 IHSECYTGETAWSARCDCGEQFDEAG-----------RLMGEAGHGCIVYLR-QEGRGIG
Cal         180 IHSECYTGETAWSARCDCGEQFDEAG-----------RIMGEAGHGCMVYLR-QEGRGIG
Dha         158 IHSECYTGETAWSARCDCGEQFDEAG-----------RIMGNDGHGCIVYLR-QEGRGIG
Sce         144 IHSECYTGENAWSARCDCGEQFDRAGRLIACDHEPTSNIKGGNGHGVIVYLR-QEGRGIG
Kla          75 IHSECYTGETAWSARCDCGEQFDRAGKLISVERE--GDIVGGNGHGVIVYXR-QEGRGIG
Spo         167 IHSECYTGETAWSARCDCGEQLD AA -----------LISEEGNGVIIYLR-QEGRGIG
Pfl          65 LHSECLTGDALFSQRCDCGSQLEGALK-----------AIAREGRGVLLYLR-QEGRGIG
Vvu         216 LHSECLTGDALFSARCDCGFQLAKALQ-----------NIVAEGAGVLLYLR-QEGRGIG
Eco          50 VHSECLTGDALFSLRCDCGFQLEAALT-----------QIAEEGRGILLYHR-QEGRNIG
Abr         241 LHSECFTGDLLAGLRCDCGQQLRGAIA-----------EIARHGSGVLLYLA-QEGRGIG
Ath         221 VHSECLTGDIFGSARCDCGNQLALSMQ-----------QIEATGRGVLVYLRGHEGRGIG
```

Fig. 1. Sequence comparison of GTP cyclohydrolases II. Pgu, *Pichia guilliermondii*; Cal, *Candida albicans*; Dha, *Debaryomyces hansenii*; Sce, *Saccharomyces cerevisiae*; Kla, *Kluyveromyces lactis*; Spo, *Schizosaccharomyces pombe*; Pfl, *Pseudomonas fluorescens*; Vvu, *Vibrio vulnificus*; Eco, *Escherichia coli*; Abr, *Azospirillum brasilense*; Ath, *Arabidopsis thaliana*. ∗ – position of C207Y mutation.

without RF. Thus, it can be supposed that iron deficiency or the *rib81* regulatory mutation cause hyperexpression of the *RIB1* gene (and possibly of other *RIB* genes), leading to suppression of RF deficiency in *rib1-86* strain.

Although the *rib81* mutation and iron deficiency suppressed RF auxotrophy in *rib1-86* mutant, we could not determine activity of GTP cyclohydrolase II in double *rib1-86 rib81* mutant. Maybe, activity and/or stability of GTP cyclohydrolase II carrying C207Y substitution are low and activity of the enzyme could not be determined due to insufficient sensitivity of the method used (data not shown). Despite that, the results suggested that identified mutation *rib1-86* (G620A) leads to leaky RF deficiency that can be partially suppressed by hyperexpression of RF biosynthesis genes due to a regulatory mutation or iron deficiency.

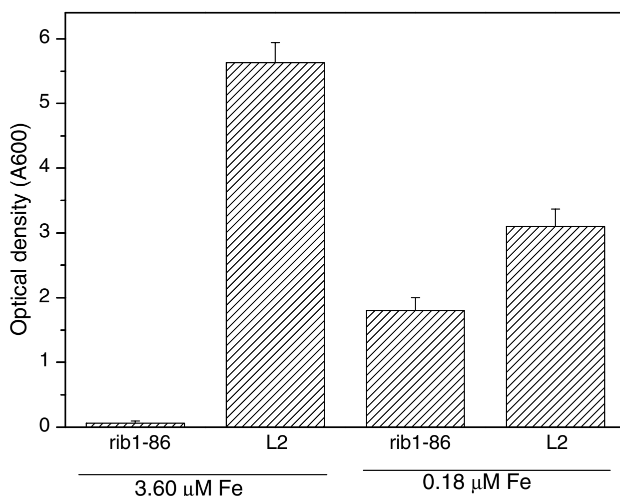

Fig. 2. Dependence of growth of *P. guilliermondii* mutant strain *rib1-86* on iron supplementation. Both cultures were grown overnight in a synthetic medium supplemented with 200 mg RF ml^{-1} and 0.18 μM iron. Cells were harvested, washed three times with the same medium but without RF, inoculated ($OD_{600} = 0.001$) into fresh synthetic media (without RF) supplemented with 3.6 and 0.18 μM of iron, respectively. Cultures were grown on a shaker for 3 d at 30 °C and OD_{600} was measured. Strain L2 was used as a WT. Values shown are the means of three independent experiments.

3.3. rib1-86 *As a tool for isolation of regulatory mutations affecting RF biosynthesis and metal homeostasis*

In contrast to other *P. guilliermondii rib1* mutants, the *rib1-86* strain spontaneously reverted to RF prototrophy at high frequency (Fig. 3). Based on the results described above, we hypothesized that some of such revertants could carry mutations affecting regulation of RF biosynthesis. To check this hypothesis, we isolated and analyzed a collection of spontaneous revertants (RF prototrophs) derived from *rib1-86*.

It has been reported earlier that colonies of the *P. guilliermondii* RF-overproducing mutants *rib80* and *hit1* are characterized by both elevated ferrireductase and overall nonspecific reductase activities. Their colonies were stained red on a medium supplemented with 2,3,5-triphenyltetrazoliumchloride (TTC), due to reduction of TTC to insoluble red triphenylformazan [11,12,21,22]. Therefore, in order to facilitate identification of suppressor regulatory mutations and to distinguish them from intralocus reversion in *rib1-86*, RF-deficient synthetic medium supplemented with 40 mg l^{-1} TTC was used to select for spontaneous revertants. Cells of the *rib1-86* strain were pre-grown in YPD medium supplemented with RF, pelleted, washed once with water and plated on selective medium (10^8 cells per plate). Colonies of RF prototrophs appeared after 6–15 days of incubation at 30 °C (Fig. 3). Some of them were stained red. Complementation analysis revealed that all of them carried a *rib81* mutation that provided suppression of RF deficiency. But elevated reducing activity toward TTC was apparently due to additional mutations, since *rib81* mutants did not exhibit this phenotype [10]. To check that, we crossed each newly-selected mutant with previously characterized *hit1* and *rib80* strains and analyzed the phenotype of the resulting diploids. As a result, 30 strains that carry regulatory mutations different from previously-known mutations *hit1* and *rib80* were identified (Fig. 4).

In order to segregate newly-obtained secondary mutations, each of the selected red RF prototrophs was crossed with the isogenic wild-type strain. Haploid

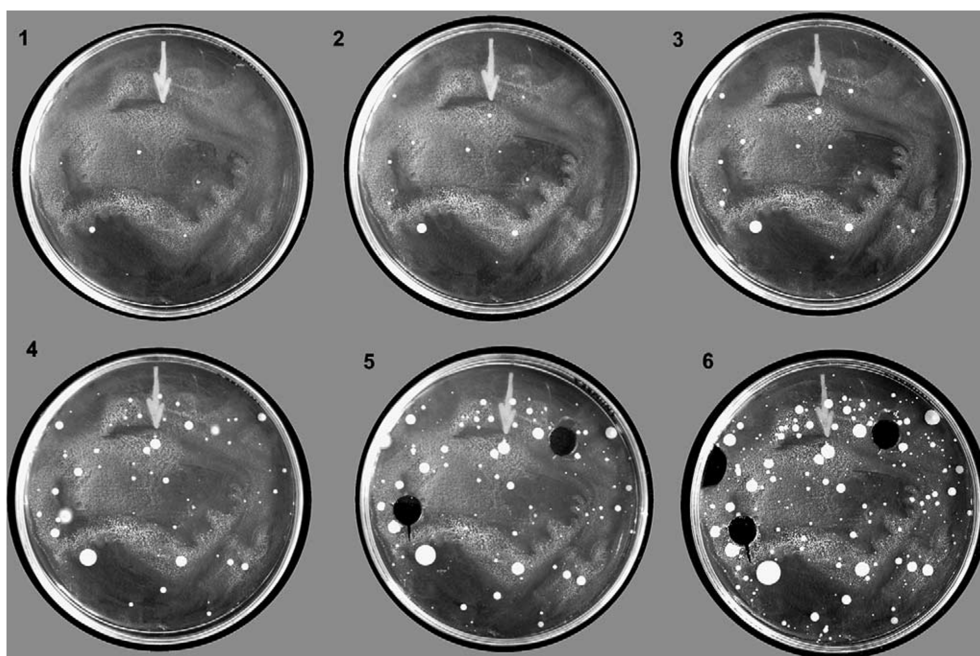

Fig. 3. Kinetics of appearance of RF revertants of *P. guilliermondii rib1-86* mutants. Cells of the strain were pre-grown on YPD medium containing 200 mg l^{-1} of RF to late-log phase, pelleted, washed twice and re-suspended in sterile water. 3×10^8 Cells were plated on each plate and incubated for 15 d at 30 °C. Photos were taken after 5, 7, 9, 11, 13 and 15 d of incubation (numbered 1, 2, 3, 4, 5 and 6 correspondingly).

segregants, red stained on the medium supplemented with TTC, were isolated and analyzed by crossing to the *rib1-86* parental strain as well as to the *rib81* strain. The progeny (complementary to both mutations, *rib81* and *rib1-86*) of both mating types (and carrying auxiliary markers) was identified and used for subsequent analysis. Each of the novel mutants was crossed to WT and corresponding mutations were found to segregate in a 1:1 fashion (data not shown), as expected for a single recessive mutation in a haploid genetic system. To determine the number of genetic loci represented by our mutants, we crossed each mutant to the others and screened diploids for TTC reduction phenotype. By this means, we found that the 30 strains represented mutants in six independent loci, named *RED1* to *RED* 6 (*red*uction) (Fig. 4).

Relative to the WT strain, all *red* mutants had elevated levels of RF production and increased activity of GTP cyclohydrolase II (Table 2). Also, *red* mutants exhibited increased ferric/cupric reductase activity and higher non-hemin iron content (Table 2). They were more sensitive to transition metals than the WT strain [14]. Thus, *red* mutations had pleiotropic effect on *P. guilliermondii* metabolism, as reported before for the mutations *hit1*, *rib80* and *rib81* [10–12].

It should be noted that selected *red* mutations could not suppress RF deficiency in the *rib1-86* mutant without a concomitant *rib81* mutation. The high rate of appearance of two independent mutations in one haploid cell could be explained by long incubation under selective pressure, since it has been reported that the rate of mutation in starved yeast cultures can reach 10^{-3} [23,24]. Also, it might be supposed that the original mutation *rib1-86* increased the mutability of the strain.

To check this hypothesis, we studied frequencies of spontaneous mutations in strain *rib1-86* and two independent transformants of this strain carrying the integrated WT allele of the *RIB1* gene. The L2 strain was used in these experiments as a WT strain. Whereas the *rib1-86* strain produced resistant colonies at a frequency of 1.5×10^{-7} on the medium supplemented with riboflavin, 5-fluoroorotic acid (FOA) and uridine, WT strain L2 exhibited a five times lower frequency of mutation (about 3×10^{-8}) (Fig. 5). Introducing the WT allele of the *RIB1* gene decreased the mutation rate of *rib1-86* strains to a level similar to that of the WT strain. In addition, the spontaneous mutation frequency in the *rib1-86* strain on the synthetic medium supplemented with 125 μM canavanine (i.e., appearance of resistant colonies) was 8–10 times higher than in the isogenic WT strain (data not shown). Together with the data on RF prototrophs selection (see above) these results tentatively suggested that C207Y substitution in GTP cyclohydrolase II leads not only to RF auxotrophy but also to increased mutation frequency in the strain.

It has been shown earlier that *E. coli* GTP cyclohydrolase II as well as MutT protein can remove pyrophosphate from 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) [25], which efficiently induces T:A → G:C and G:C → T:A transversions. It is thought that the produced 8-oxo-dGMP is further eliminated from the nucleotide pool [26]. In the case of

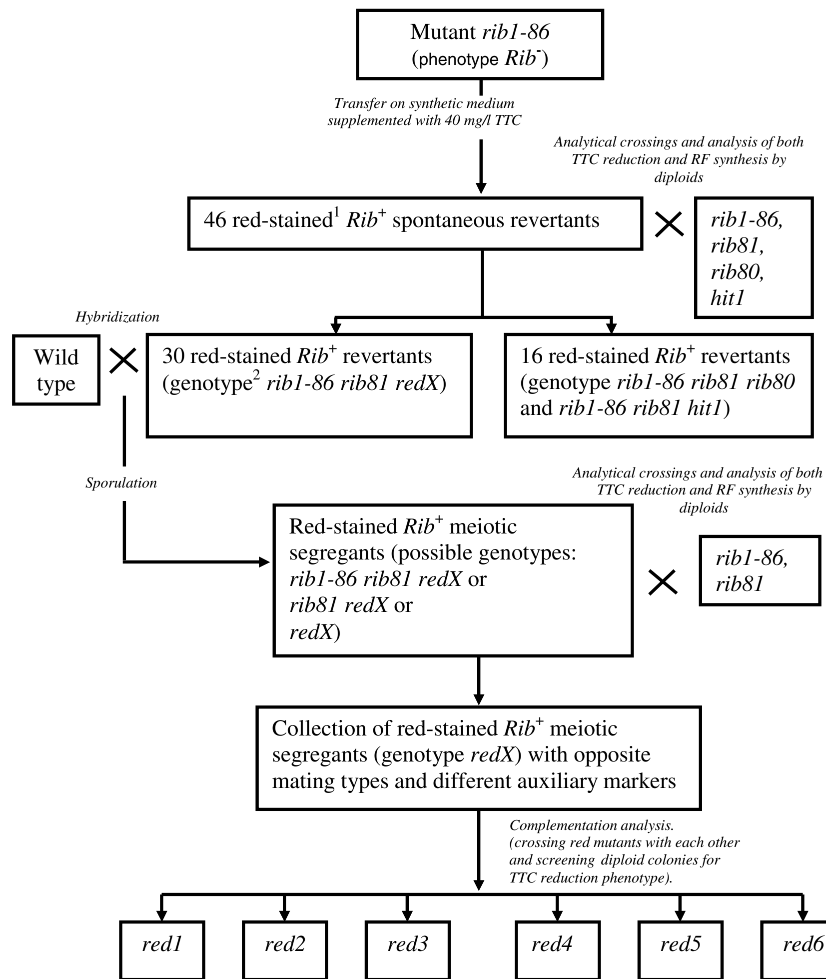

Fig. 4. Scheme of selection of *P. guilliermondii red* mutants. 1 – "red stained" – means that colonies of a strain appear red-colored on media supplemented with 40 mg l^{-1} TTC; 2 – in order to simplify the figure, mating types and auxiliary markers of the strains are not shown.

Table 2
RF production, GTP cyclohydrolase II activity, iron content and ferrireductase activity in *red* mutants and wild-type strain

Strains	RF production, μg (mg dry cells)$^{-1}$	GTP cyclohydrolase activity E (mg protein)$^{-1} \times 10^{-5}$	Fe content, μg (g dry cells)$^{-1}$	Rate of Fe^{+3} reduction, nM Fe (mg dry cells)$^{-1}$
WT	0.2 ± 0.05	0.7 ± 0.17	56.2 ± 4.32	4.2 ± 0.23
red1	0.4 ± 0.06	1.6 ± 0.16	288.7 ± 16.47	20.2 ± 1.62
red2	0.3 ± 0.07	2.2 ± 0.16	186.1 ± 8.28	12.8 ± 0.92
red3	0.4 ± 0.05	2.1 ± 0.14	194.2 ± 9.14	12.1 ± 0.97
red4	0.5 ± 0.08	2.1 ± 0.28	244.5 ± 12.31	22.2 ± 1.27
red5	0.8 ± 0.07	2.6 ± 0.19	276.3 ± 14.21	22.5 ± 1.80
red6	0.9 ± 0.07	2.8 ± 0.24	298.9 ± 16.24	24.1 ± 0.93

Values shown are the means of three independent determinations.

Δ*mutT* background, GTP cyclohydrolase II deficiency significantly increased the frequency of spontaneous mutation in *E. coli* [25]. It is also known that the *E. coli RIBA* gene, encoding GTP cyclohydrolase II, is a member of the *soxRS* regulon, which is induced by superoxide-generating agents [27]. Thus, *E. coli* GTP cyclohydrolase II can serve as an additional means (able to partially suppress *mutT* deficiency) to inactivate 8-oxo-dGTP [25].

In yeast, transcription of genes involved in oxidative stress response is upregulated by transcription factor Yap1 [28]. The promoter region of the *S. cerevisiae* GTP cyclohydrolase II gene contains a number Yap1-binding sites. Despite of some inconsistency in the data, it can be assumed that expression of this gene under oxidative stress is increased [29,30]. Our analysis of the promoter region of the *P. guilliermondii* GTP cyclohydrolase II structural gene revealed Yap1-like binding

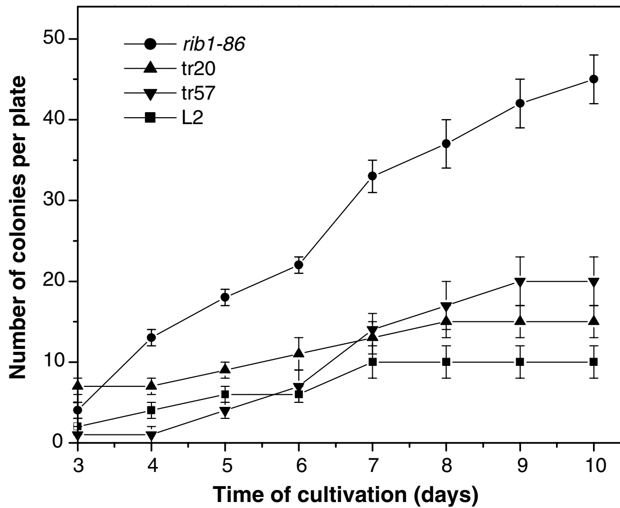

Fig. 5. Frequency of the appearance a 5′-fluoroorotic acid (FOA)-resistant *P. guilliermondii* mutants. Cells of the strains were pre-grown on YPD medium containing 200 mg l^{-1} of RF (if required) to late-log phase, pelleted, washed and re-suspended in sterile water. 3×10^8 Cells were plated on each plate and incubated for 10 d at 30 °C. Synthetic medium (0.67% YNB, 2% glucose, 0.8% CSM-ura) supplemented with RF 200 mg l^{-1}, uridine – 50 mg l^{-1}, FOA-1 g l^{-1} and 15 g^{-1} of agar was used. Strain L2 was used as a WT reference strain. Values shown are the means of three independent experiments.

sites TTAGTCA, TTGCCAA, TTAGTGA, TTACAAA, TTACTCA, located 286, 233, 167, 133 bp upstream of the ATG translational start codon, respectively. This suggests that expression of the gene could be enhanced under oxidative stress in order to protect DNA synthesis from oxidized nucleotides. In support of the existence of such regulation, *P. guilliermondii* increases RF production in response to oxidative stress [31].

Thus, it could be supposed that in *P. guilliermondii* GTP cyclohydrolase II is involved in degradation of 8-oxo-dGTP, a potent mutagenic substrate for DNA synthesis. This specific approach to reduce the quantity of mutations may serve as a supplement to DNA mismatch recognition and repair machinery in yeast [32,33]. To prove the involvement of yeast GTP cyclohydrolases II in degrading 8-oxo-dGTP, it is necessary to study both frequency of spontaneous mutation in *rib1* knockout strains and substrate specificity of the enzymes purified from different yeasts.

3.4. Iron represses transcription of RIB1 *and* RIB7 *genes in the wild-type strain of* P. guilliermondii *but not in the regulatory mutants* rib81 *and* red6

Inhibiting action of iron on RF production in flavinogenic yeasts is known for 60 years [1], although the mechanism of such inhibition is not known. Inhibition of RF production by iron in *P. guilliermondii* results from repression of the first, third, fourth, fifth and sixth reactions of RF production, whereas the second enzyme is synthesized constitutively [9]. The molecular level at which iron represses RF-synthesizing enzymes has not been elucidated. In some schemes, it has been assumed that iron regulates transcription of the corresponding genes and that regulatory mutants possess

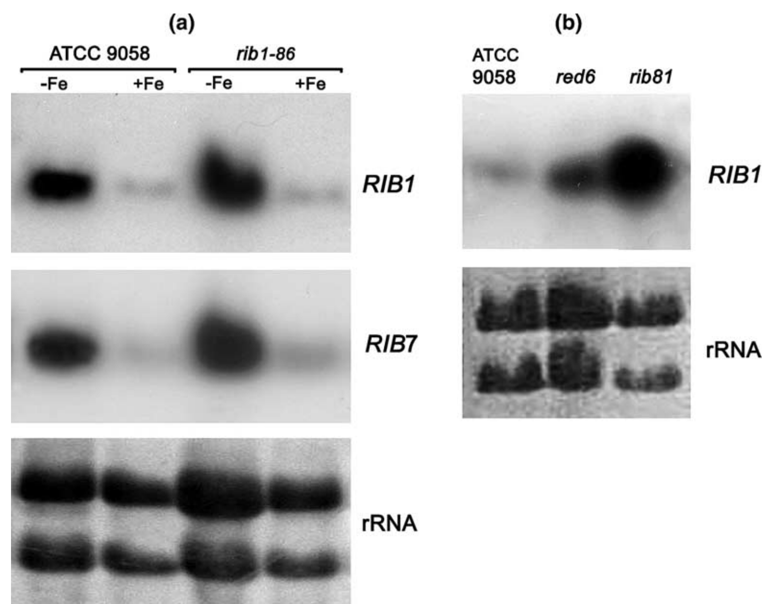

Fig. 6. Effect of iron supplementation and mutations *red6*, *rib81* on expression of *RIB1* and *RIB7* genes in *P. guilliermondii*. The RNA from each sample was analyzed by Northern blotting as described in Section 2. rRNA was detected by ethidium bromide staining as a loading control. (a) Iron-dependent expression of *RIB1* and *RIB7* genes in *P. guilliermondii rib1-86* mutant and wild-type strain ATCC9058. Cells were grown on synthetic media supplemented with 0.18 μM iron (designated as −Fe) or 3.6 μM iron (designated as +Fe). (b) Expression of the *RIB1* gene in *P. guilliermondii* mutants *red6*, *rib81* and wild-type strain ATCC9058. Cells were grown on synthetic medium supplemented with 3.6 μM iron as described in Section 2.

constitutively-derepressed enzymes of RF production in iron-rich medium [6,9]. We decided to test this hypothesis experimentally by assaying the level of *RIB1* and *RIB7* mRNAs in *P. guilliermondii* cells grown in iron-rich and iron-deficient media.

Results of Northern blotting demonstrated that cells of the *rib1-86* mutant as well as of the wild-type strain exhibited relatively low levels of *RIB1* gene expression in medium supplemented with 3.6 μM iron (Fig. 6(a)). Decrease of iron concentration in the medium caused at least a tenfold increase in *RIB1* mRNA levels in both strains. A very similar pattern of regulation was observed for the *RIB7* gene coding for RF synthase (Fig. 6(a)). As expected, the regulatory mutation *rib81* also dramatically increased the level of *RIB1* mRNA. These results suggested that an increase in RF production by *P. guilliermondii* caused by iron starvation or regulatory mutation correlates with an elevated level of mRNAs of key enzymes involved in this biosynthetic pathway. We conclude that regulation of RF biosynthesis by iron in *P. guilliermondii* occurs at the transcriptional level.

In the next experiment, we tested expression of the *RIB1* gene in one of *red* mutants, namely, *red6*. In iron-supplemented synthetic medium, the *red* 6 mutant exhibited a lower level of *RIB1* transcript relative to the formerly isolated regulatory mutant *rib81*, although about five times higher than in a WT strain (Fig. 6(b)).

Thus, iron strongly regulates transcription of genes involved in RF biosynthesis in the wild-type strain of *P. guilliermondii*. Such regulation is impaired in mutants isolated earlier (*rib81*) and described in this study (*red6*), as transcription of RF structural genes is elevated significantly in these mutants in iron-deficient medium.

It is known that different yeast species possess different modes of action of iron-sensing transcriptional factors. For example, transcriptional activation of certain genes under iron deficiency in *S. cerevisiae* is provided by the transcriptional activators Aft1p and Aft2p [34,35]. In contrast, transcription of a similar set of genes is inhibited under iron-repletion conditions by the transcriptional repressor Fep1p in *Schizosaccharomyces pombe* [36,37].

The mechanism of iron-dependent transcriptional regulation of RF biosynthetic genes in *P. guilliermondii* still needs to be elucidated. The simple method for positive selection of mutants impaired in iron-dependent repression of RF biosynthesis developed in this work, opens a possibility to decipher the molecular mechanisms of such regulation in *P. guilliermondii*.

Acknowledgments

The authors thank Dr. Dmitrenko V. and Prof. Dr. Kavsan V. (Kiev) for their expert assistance in Northern-blotting experiments.

References

[1] Tanner, F., Vojnovich, C. and Lanee, J.M. (1945) Riboflavin production by *Candida* species. Science 101, 180–183.
[2] Demain, A.L. (1972) Riboflavin oversynthesis. Annu. Rev. Microbiol. 26, 369–388.
[3] Knight, S.A., Lesuisse, E., Stearman, R., Klausner, R.D. and Dancis, A. (2002) Reductive iron uptake by *Candida albicans*: role of copper, iron and the TUP1 regulator. Microbiology 148, 29–40.
[4] Voronovsky, A.Y., Abbas, C.A., Dmytruk, K.V., Ishchuk, O.P., Kshanovska, B.V., Sybirna, K.A., Gaillardin, C. and Sibirny, A.A. (2004) *Candida famata* (*Debaryomyces hansenii*) DNA sequences containing genes involved in riboflavin synthesis. Yeast 21, 1307–1316.
[5] Stahmann, K.P., Revuelta, J.L. and Seulberger, H. (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Appl. Microbiol. Biotechnol. 53, 509–516.
[6] Sibirny, A.A. (1996) Chapter VII. *Pichia guilliermondii* In: Nonconvential Yeasts in Biotechnology (Wolf, K., Ed.), pp. 255–272. Springer-Verlag, Berlin, Heidelberg.
[7] Sibirny, A.A., Shavlovsky, G.M., Ksheminskaya, G.P. and Naumov, G.I. (1977) Hybridization and meiotic segregation in the paraffin-utilizing yeast *Pichia guilliermondii* Wickerham. Genetika 13, 314–321 (in Russian).
[8] Boretsky, Y., Voronovsky, A., Liuta-Tehlivets, O., Hasslacher, M., Kohlwein, S.D. and Shavlovsky, G.M. (1999) Identification of an ARS element and development of a high-efficiency transformation system for *Pichia guilliermondii*. Curr. Genet. 36, 215–221.
[9] Shavlovskii, G.M. and Logvinenko, E.M. (1988) Supersynthesis of flavins in microorganisms and its molecular mechanism. Prikl. Biokhim. Mikrobiol. 24, 435–447 (in Russian).
[10] Shavlovskii, G.M., Babiak, L.Ia., Sibirnyi, A.A. and Logvinenko, E.M. (1985) Genetic control of riboflavin biosynthesis in *Pichia guilliermondii* yeasts. The detection of a new regulator gene RIB81. Genetika 21, 368–374 (in Russian).
[11] Shavlovskii, G.M., Fedorovich, D.V., Kutsiaba, V.I., Babyak, L.Ya. and Stenchuk, M.M. (1992) Participation of RIB80 gene in regulation of riboflavin biosynthesis and iron transport in yeast *Pichia guilliermonlii*. Genetika 28, 25–32 (in Russian).
[12] Fedorovich, D., Protchenko, O. and Lesuisse, E. (1999) Iron uptake by the yeast *Pichia guilliermondii*. Flavinogenesis and reductive iron assimilation are co-regulated processes. Biometals 12 (4), 295–300.
[13] Shavlovskii, G.M., Stenchuk, N.N. and Kshanovskaya, B.V. (1991) The effect of the regulatory mutation of the *RIB1* locus on riboflavin biosynthesis in *Pichia guilliermondii*. Biopolimery i Kletka 6, 96–99 (in Russian).
[14] Stenchuk, N.N. and Kapustiak, K.E. (2003) The red mutations impair the regulation of flavinogenesis and metal homeostasis in yeast *Pichia guilliermondii*. Genetika 39, 1026–1032 (in Russian).
[15] Cowart, R.E., Marquardt, M.P. and Foster, B.G. (1980) The removal of iron and other trace elements from a complex bacteriological medium. Microbiol. Lett. 13, 117–122.
[16] Shavlovskii, G.M., Logvinenko, E.M. and Zakal'skii, A.E. (1983) Purification and properties of GTP-cyclohydrolase II of the yeast *Pichia guilliermondii*. Biokhimiia 48, 837–843 (in Russian).
[17] Lowry, O.H., Rosenberg, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
[18] Becker, D.M. and Guarente, L. (1991) High-efficiency transformation of yeast by electroporation. Methods Enzymol. 194, 182–187.
[19] Wise, J.A. (1991) Preparation and analysis of low molecular weight RNA and small ribonucleoproteins. Methods Enzymol. 194, 405–415.

[20] Kaiser, J., Schramek, N., Eberhard, S., Puttmer, S., Schuster, M. and Bacher, A. (2002) Biosynthesis of vitamin B2. An essential zinc ion at the catalytic site of GTP cyclohydrolase II. Eur. J. Biochem. 269, 5264–5270.

[21] Bochneri, B. and Savageau, M. (1977) Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Appl. Environ. Microbiol. 33, 434–444.

[22] Bernas, T. and Dobrucki, J. (2000) The role of plasma membrane in bioreduction of two tetrazolium salts, MTT, and CTC Arch. Biochem. Biophys. 380 (1), 108–116.

[23] Hall, B.G. (1992) Selection-induced mutations occur in yeast. Proc. Natl. Acad. Sci. USA 15 (89), 4300–4303.

[24] Rosenberg, S. (2001) Evolving responsively: adaptive mutation. Nat. Rev. Genet. 2, 504–515.

[25] Kobayashi, M., Ohara-Nemoto, Y., Kaneko, M., Hayakawa, H., Sekiguchi, M. and Yamamoto, K. (1998) Potential of *Escherichia coli* GTP cyclohydrolase II for hydrolyzing 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. J. Biol. Chem. 273, 26394–26399.

[26] Maki, H. and Sekiguchi, M. (1992) MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature 355, 273–275.

[27] Koh, Y.S., Chung, W.H., Lee, J.H. and Roe, J.H. (1999) The reversed SoxS-binding site upstream of the rib A promoter in *Escherichia coli*. Mol. Gen. Genet. 261, 374–380.

[28] Fernandes, L., Rodrigues-Pousada, C. and Struhl, K. (1997) Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. Mol. Cell. Biol. 17, 6982–6993.

[29] Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell. 11 (12), 4241–4257.

[30] Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A. (2001) Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell. 12 (2), 323–337.

[31] Protchenko, O.V., Boretsky, Y.R., Romaniuk, T.M. and Fedorovych, D.V. (2000) Oversynthesis of riboflavin by yeast *Pichia guilliermondii* in response to oxidative stress. Ukr. Biokhim. Zh. 72, 19–23.

[32] Harfe, B.D., Minesinger, B.K. and Jinks-Robertson, S. (2000) Discrete in vivo roles for the MutL homologs Mlh2p and Mlh3p in the removal of frameshift intermediates in budding yeast. Curr. Biol. 10, 145–148.

[33] Bruner, S.D., Nash, H.M., Lane, W.S. and Verdine, G.L. (1998) Repair of oxidatively damaged guanine in *Saccharomyces cerevisiae* by an alternative pathway. Curr. Biol. 26, 393–403.

[34] Rutherford, J.C., Jaron, S. and Winge, D.R. (2003) Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. J. Biol. Chem. 278, 27636–27643.

[35] Van Ho, A., Ward, D.M. and Kaplan, J. (2002) Transition metal transport in yeast. Annu. Rev. Microbiol. 56, 237–261.

[36] Pelletier, B., Beaudoin, J., Mukai, Y. and Labbe, S. (2002) Fep1, an iron sensor regulating iron transporter gene expression in *Schizosaccharomyces pombe*. J. Biol. Chem. 277, 22950–22958.

[37] Pelletier, B., Beaudoin, J., Philpott, C.C. and Labbe, S. (2003) Fep1 represses expression of the fission yeast *Schizosaccharomyces pombe* siderophore-iron transport system. Nucleic Acids Res. 31, 4332–4344.