Chapter 6
Pichia guilliermondii

Andriy A. Sibirny and Yuriy R. Boretsky

Contents

Abstract *Pichia guilliermondii* (asporogenous strains of this species are designated as *Candida guilliermondii*) is the model organism of a group so named "flavinogenic yeasts" capable of riboflavin oversynthesis during starvation for iron. Besides, some strains of this species efficiently convert xylose to xylitol, an anti-caries sweetener. However, there are also pathogenic *C. guilliermondii* strains. This species has been used for studying enzymology of riboflavin synthesis due to overproduction of participating enzymes and intermediates under iron-limiting conditions as well as for identification of genes of negative and positive action involved in such a regulation. Besides, *P. guilliermondii* was used for identification and studying the properties of the systems for active transport of riboflavin in the cell (riboflavin permease) and out of the cell (riboflavin "excretase"). The genetic line of *P. guilliermondii* with high fertility has been selected and the methods of classic genetics (hybridization and analysis of meiotic segregation) have been developed. More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors with recessive

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and dominant markers, several transformation protocols including that for gene knock out. Recently, the genome of this yeast species was sequenced and become publicly available (http://www.broad.mit.edu).

Keywords Flavinogenic yeasts, riboflavin, iron-limiting condition, riboflavin, permease, *Pichia guilliermondii*

6.1 Introduction

Pichia guilliermondii (asporogenous strains of this species are designated as *Candida guilliermondii*) belongs to the group so named "flavinogenic yeasts" capable of riboflavin oversynthesis during starvation for iron (Tanner et al., 1945). Other yeast species which can be referred as "flavinogenic" are: *Candida famata* (other names: *Candida flareri, Torulopsis candida*, teleomorph is known as *Debaryomyces hansenii*), *Schwanniomyces occidentalis* and *Candida albicans* (Shavlovsky and Logvinenko, 1988a; Knight et al. 2002). *P. guilliermondii* is often considered as the model organism for the group of flavinogenic yeasts as only for this flavinogenic yeast species methods both of classic and molecular genetics have been developed (Sibirnyi et al., 1977b; Boretsky et al., 1999). Besides, some strains of this species efficiently convert xylose to xylitol, anti-caries sweetener (Canettieri et al., 2001). However, there are also pathogenic *C. guilliermondii* strains (Krcmery and Barnes, 2002). *P. guilliermondii* has been used for studying enzymology of riboflavin synthesis due to overproduction of participating enzymes and intermediates under iron-limiting conditions as well as for identification of genes of negative and positive action involved in such a regulation. Besides, *P. guilliermondii* was used for identification and studying the properties of the systems for active transport of riboflavin into the cell (riboflavin permease) and out of the cell (riboflavin "excretase"). The genetic line of *P. guilliermondii* with high fertility has been selected and the methods of classic genetics (hybridization and analysis of meiotic segregation) have been developed (Sibirnyi et al., 1977b, c). More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors with recessive markers and several transformation protocols including that for gene knock-out. Since 2005 genome of this yeast species was sequenced and become publicly available (http://www.broad.mit.edu).

6.2 Classification of *Pichia guilliermondii,* Physiology and Research History

Pichia guilliermondii Wickerham represents a collection of sporogenous strains which formerly were classified as the asporogenous species *Candida guilliermondii* (Cast.) Langeron a. Guerra (Wickerham and Burton, 1954; Wickerham, 1966). This

means that each strain of *C. guilliermondii* which is able to hybridize with any strain of *P. guilliermondii* must be transferred to the latter species, for example, even the type strain C. *guilliermondii* ATCC 9058 must now be considered as *P. guilliermondii* (Sibirny et al., 1977b). It is not known at the moment, how many strains really belong to species *Candida guilliermondii*, i.e. are unable to hybridize.

According to web site of Broad Institute, *Candida guilliermondii* (teleomorph *Pichia guilliermondii*) is a rarely observed pathogen, accounting for a few percent of all candidemias (see: *http://www.broad.mit.edu/annotation/genome/candida_guilliermondii/Info.html#t0*). However, for cancer patients, *C. guilliermondii* is the primary cause of fungemia. *C. guilliermondii* is more distantly related to *C. albicans* than *C. tropicalis*, but is more closely related than *C. lusitaniae*. *C. guilliermondii* shares in common with *C. albicans*, *C. tropicalis*, and *C. lusitaniae* the fact that CTG encodes serine rather than leucine, which is a marked distinction from other more distantly related hemiascomycetes including *Ashbya gossypii*, *Saccharomyces cerevisiae*, and *Candida glabrata*. Statement that "*C. guilliermondii* is haploid and has retained a complete sexual cycle including meiosis and sporulation" is contradictory to common view that genus *Candida* includes only asporogenous species.

Earlier, the genetics of *P. guilliermondii* was studied almost exclusively in the former USSR (mostly in Lviv and Kiev, Ukraine), and the former GDR (in Greifswald). The interest to genetic studies of this species was based primarily on its ability to utilize hydrocarbons as sole carbon and energy source (Shchelokova et al., 1974) and production of single-cell protein from hydrocarbons. Formerly, the industrial strain used for that purpose was erroneously identified in the USSR as *Candida guilliermondii,* however, it had been later re-identified as *Candida maltosa* (Bykov et al., 1987). The second reason is the potential of *P. guilliermondii* to convert xylose to xylitol, an anti-caries sweetener (Sene et al., 2001; Carvalho et al., 2002; Rodrigues et al., 2006). This yeast species appears to be the most effective organism for bioconversion of xylose into xylitol and is able to utilize even hemicellulosic hydrolysates obtained by acid hydrolysis (Canettieri et al., 2001). Another reason causing an interest to this yeast species is potential pathogenic properties of some clinical isolates classified as a C*andida guilliermondii* (Krcmery and Barnes, 2002; de Vos et al., 2005). It should be stressed that no cases of candidiasis caused by laboratory strains of *P. guilliermondii* were noted during decades of work with this species, at our institute. Moreover, all patented *P. guilliermondii* strains were properly checked before patenting, and no one of them was found to be pathogenic to laboratory animals. Thus, it can be stated that type strains of *P. guilliermondii* belong to GRAS organisms (Generally Recognized As Safe) and can be used as a model strains for different purposes.

An ability of *P. guilliermondii* strains to overproduce riboflavin (vitamin B_2) during growth in iron-deficient media (Tanner et al., 1945) attracts an especial interest (Shavlovsky and Logvinenko, 1988b). 146 strains of *P. guilliermondii* out of 147 analyzed were able to excrete a yellow pigment, identified as riboflavin, during cultivation in iron-deficient medium (Shavlovsky et al., 1978). Thus, the ability to overproduce riboflavin in the cultural liquid during cultivation in iron-deficient media, could be a species characteristic of *P. guilliermondii*. Such ability is known

for several yeast species that belong to different genera *(Candida famata (Debaryomyces hansenii), Debaryomyces subglobosus, Schwanniomyces occidentalis, P. guilliermondii*) (Tanner et al., 1945; Shavlovsky and Logvinenko, 1988b). Although this phenomenon was first described in 1945, neither the physiological role, nor the mechanisms of iron-dependent regulation of flavinogenesis are known. *P. guilliermondii* can be considered as the model organism for this group, due to development of the methods for classic and molecular genetics of this species. Unlike *D. hansenii* and other representatives of this group, *P. guilliermondii,* being heterothallic species, exists in both haploid and diploid states and can be induced to mate and sporulate. Genetic lines producing large amounts of spores have been isolated and used to study the regulation of riboflavin biosynthesis in this yeast species (Sibirny et al., 1977b). More recently, nucleotide sequence of the genome of this yeast species become publicly available at http://www.broad.mit.edu. Cells of *P. guilliermondii* are heterogeneous, mostly elongate in shape (approx. 2 × 10 μ), sometimes forming a pseudomycelium. The natural habitat of the species is diverse. A study of 140 strains of *P. guilliermondii* isolated from natural habitats showed that the most frequent source of their isolation is oil-containing soil (123 strains); others were isolated from plant leaves, lake water, and cow paunch (Zharova et al., 1980).

All known strains do not utilize lactose, starch, and inositol, whereas they differ in their ability to utilize D-ribose, D-arabinose, D-cellobiose, D-melibiose, salicin, L-rhamnose, L-sorbose, and dulcitol. All known strains utilize hydrocarbons (natural mixtures or n-hexadecane) as sole source of carbon and energy (Kreger van Rij, 1970). Growth on other respiratory substrates, such as ethanol, glycerol, succinate, or citrate, is satisfactory. *P. guilliermondii* is a typical representative of aerobic yeasts, and cannot grow under strictly anaerobic conditions. The standard growth temperature for *P. guilliermondii* is 30°C. The upper limit is near 42°C. Standard media for yeast cultivation can be used for laboratory cultivation of *P. guilliermondii* (YEPD or YEPS in which glucose is substituted by sucrose, are used as "complete" media). As minimal medium, modified Burkholder medium is used (Burkholder, 1943; Shavlovsky et al., 1978). Other standard media can also be used in work with *P. guilliermondii* (Sibirny et al., 1977b).

6.3 Biosynthesis of Riboflavin and Its Regulation

6.3.1 Elucidation of Riboflavin Biosynthetic Pathway

As mentioned before, *P. guilliermondii* was the only genetically studied eukaryote able to regulate riboflavin biosynthesis in response to iron concentration in the medium. This organism was used for identification of structural genes of flavinogenesis as well as for studying mutations impairing a regulation of this biosynthetic pathway.

Using strains of opposite mating types of *P. guilliermondii* genetic lines, 114 riboflavin-deficient mutants were isolated after UV irradiation. Biochemical study based on analysis of accumulated riboflavin intermediates (and their derivatives)

divided them into four biochemical groups. Complementation analysis of 106 mutants revealed 7 complementation classes *(RIB1* to *R1B7).* Later one of them (*RIB4*) was omitted as a double (*rib1 rib5*) mutant. The strains of the biochemical group, accumulating no specific products, corresponded to complementation group I (*rib1*). They lost activity of GTP cyclohydrolase II which catalyses first reaction of riboflavin biosynthesis. *S*trains accumulating blue fluorescenting compounds (apparently derivatives of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5′-phosphate and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione) were combined into biochemical group 2 which comprises two complementation classes *RIB2* and *RIB3*. Strains able to grow in the medium supplemented with 3,4-dihydroxybutanone (this compound directly react with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione yielding 6,7-dimethyl-8-ribityllumazine – immediate precursor of riboflavin) were combined into biochemical group 3 which also comprises two complementation classes, namely *RIB5* and *RIB6*. And finally, mutants accumulating 6,7-dimethyl-8-ribityllumazine were combined into group 4 which corresponds to complementation class *RIB7*. They were shown to be defective in a gene specifying riboflavin synthase that catalyses last step of riboflavin biosynthesis (Shavlovsky et al., 1979). Biochemical and genetic studies of *P. guilliermondii* riboflavin auxotrophs showed complete identity of known steps of riboflavin biogenesis with those in *S. cerevisiae*. Later, detailed enzymological analysis of *P. guilliermondii* riboflavin auxotrophs was carried out. Such analysis, by the way, helped to identify new enzymes catalyzing, unstudied steps of riboflavin biosynthesis (Logvinenko et al., 1985). Also, well characterized *P. guilliermondii* riboflavin auxotrophs were used to facilitate identification of genes coding for enzymes of riboflavin biosynthesis in *Candida famata* (Voronovsky et al., 2002; Voronovsky et al., 2004). The formation of 6,7-dimethyl-8-ribityllumazine was studied using extracts of *rib5* and *rib6 P. guilliermondii* mutants with impaired synthesis of proteins P1 and P2, respectively. It was shown that synthesis of 6,7-dimethyl-8-ribityllumazine took place in extracts of *rib5* mutant (active P1 protein) in the presence of 2,4-dihydroxy-5-amino-6-ribitylaminopyrimidine and the compound formed from ribulose-5-phosphate by extracts of *rib6* mutant (active P2 protein). No lumazine was formed in extracts of *rib6* mutant from pyrimidine substrate and ribose-5-phosphate preincubated pre-incubated with extracts of *rib5* mutant. It was decided that P1 protein (the product of RIB5 gene) participates in the biosynthesis of 6,7-dimethyl-8-ribityllumazine from 2,4-dihydroxy-5-amino-6-ribitylaminopyrimidine and an aliphatic intermediate, which is formed from ribulose-5-phosphate, under the action of P2 protein (the product of RIB6 gene). Thus, it was shown that ribulose-5-phosphate is a second precursor of riboflavin (Logvinenko et al., 1987). Later the aliphatic precursor formed from ribulose-5-phosphate was determined as 3,4-dihydroxy-2-butanone-4-phosphate (Volk and Bacher, 1988, 1990).

At present reactions of this biochemical pathway is studied in details (Bacher, 1991). The initial step in the biosynthetic pathway is the opening of the imidazole ring of GTP catalyzed by the enzyme, GTP cyclohydrolase II (Shavlovsky et al., 1980). The product of this enzyme has been reported to be 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate. This intermediate is converted

to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of side chain reduction, ring deamination, and dephosphorylation. The hypothetical enzyme involved in the dephosphorylation of 5-amino-6-ribitylamino 5′-phosphate is still unknown. The conversion of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione to 6,7-dimethyl-8-ribityllumazine by the enzyme, 6,7-dimethyl-8-ribityllumazine synthase, requires a second substrate, 3,4-dihydroxy-2-butanone 4-phosphate, which is obtained from ribulose 5-phosphate by the catalytic action of 3,4-dihydroxy-2-butanone-4-phosphate synthase. Finally, 6,7-dimethyl-8-ribityllumazine is converted to riboflavin by a dismutation reaction catalyzed by riboflavin synthase (Bacher, 1991; Fischer and Bacher, 2005). Thus, one mole of GTP and two moles of ribulose 5-phosphate are required to biosynthetically generate one mole of riboflavin. A scheme of riboflavin biosynthesis is given in Fig. 6.1.

Finally riboflavin is converted into flavin mononucleotide and then into flavin adenine dinucleotide (FMN and FAD correspondingly), which are universally required for indispensable redox reactions in all cellular organisms. The first reaction is catalyzed by riboflavin kinases. The second reaction is catalysed by FAD synthetases (FMN adenylyltransferase). UTP, GTP, ADP and CTP, besides ATP, can be used as a phosphate donors by the kinase purified from *P. guilliermondii* (Kashchenko et al., 1978). In contrast only ATP was utilized by the enzyme purified from *Bacillus subtilis*. This enzyme preparation also differs from other flavokinases by specificity to reduced form of flavins. It catalysed formation of FMN-H2 and FAD-H2 from reduced riboflavin and ATP, but not the synthesis of FAD from FMN and ATP (Kearney et al., 1979). One can suggests that in general eukaryotes possess two different enzyme catalyzing conversion of riboflavin to FAD whereas bacteria often possess a bifunctional enzyme which exhibit both flavokinase and FMN adenylyltransferase activities (Hagihara et al., 1995; Mack et al., 1998; Santos et al., 2000).

6.3.2 Regulation of Riboflavin Biosynthetic Pathway

Several groups of the mutants affected in the regulation of riboflavin biosynthesis by iron ions were isolated in *P. guilliermondii*, which differed genetically and physiologically. Original approaches were used for isolation of derepressed mutants. One of them is based on selection of the mutants resistant to the riboflavin structural analog, 7-methyl-8-trifluoromethyl-10-(1′-D-ribityl)isoalloxazine. Since wild-type strains of *P. guilliermondii* are resistant to riboflavin analogs due to their slow penetration into the cell, mutants with multiple sensitivity to antibiotics and antimetabolites (Sibirny et al., 1977a, b) were used as initial strains for selection (Shavlovsky et al., 1980b).

Besides, it was found that high concentrations of sulphate or phosphate anions induce increasing sensitivity to antibiotics and antimetabolites (including riboflavin analogs) of the wild-type strains of *P. guilliermondii* and of other yeast species (Sibirny and Shavlovsky, 1981); it was possible to isolate mutants resistant to riboflavin analogs directly from wild-type strains on plates containing high sulphate

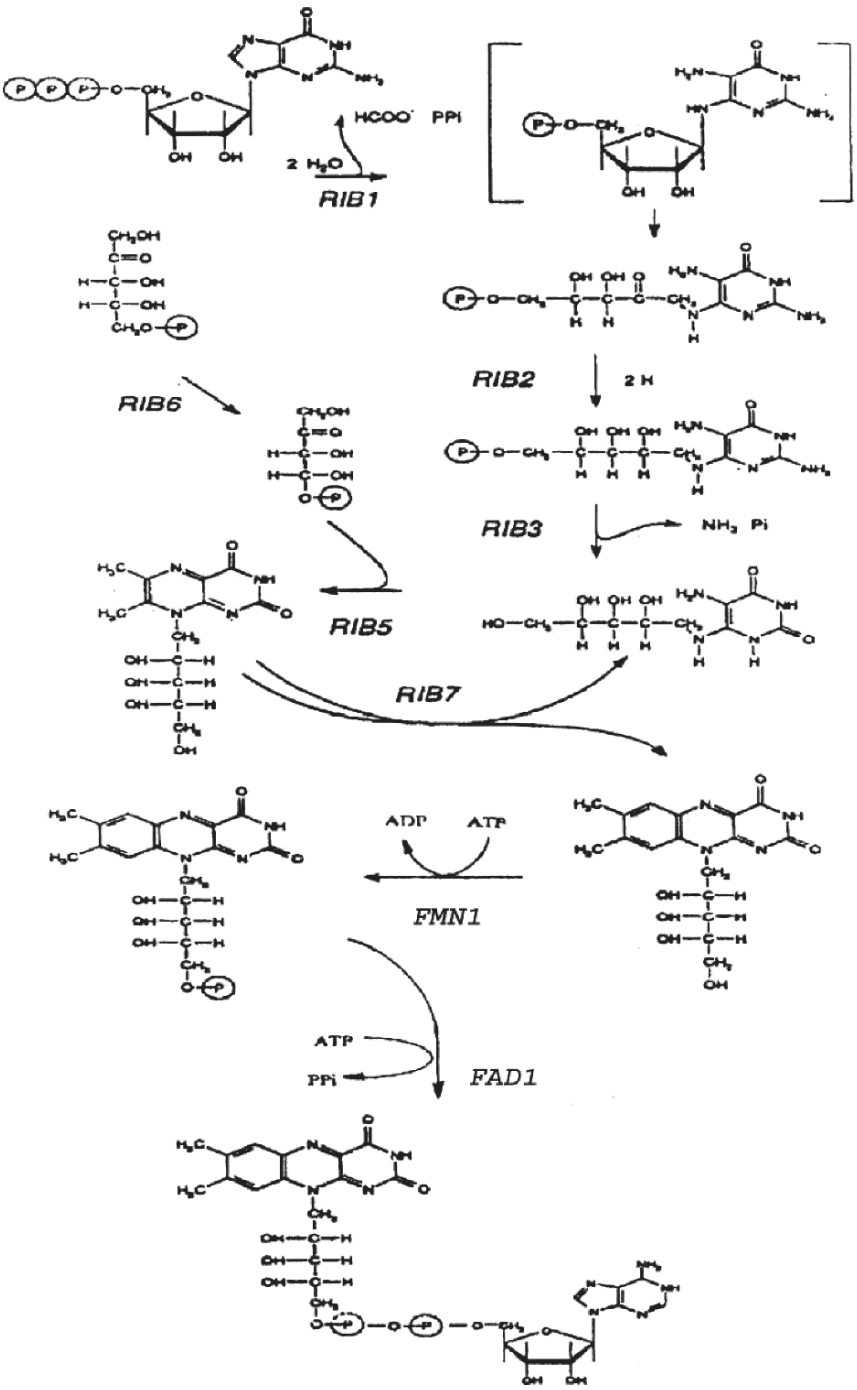

Fig. 6.1 Riboflavin biosynthesis

concentrations (Shavlovsky et al., 1985a). Additionally, derepressed mutants were isolated from previously selected temperature sensitive *rib1* mutants as pseudorevertants able to grow in iron-rich media at the elevated (restrictive for its *rib1* parental strains) temperature (Shavlovsky et al., 1985a). A third approach for isolation of riboflavin-derepressed mutants used leaky *rib2* auxotrophs as initial strains. Activity of the second enzyme of flavinogenesis in the leaky *rib2* strain was so low that they grew without exogenous riboflavin only in iron-deficient media, where other enzymes were derepressed. Selection of leaky *rib2* mutants capable of growing in iron-rich medium without riboflavin allowed the isolation of regulatory mutations enhancing riboflavin production in *P. guilliermondii* (Shavlovsky et al., 1982).

As a result of the use of three above-mentioned methods, riboflavin-overproducing mutants forming two groups of complementation (designated *rib80* and *rib81*) were isolated. Selected mutants over-synthesized riboflavin and exhibited significantly enhanced rate of radioactive iron uptake. Both groups of mutations appeared to be recessive, monogenic and possessed nuclear localization. (Shavlovsky et al., 1982, 1985a, b). Phenotype of *rib80* mutants depends on carbon source in the medium. They did not overproduce riboflavin and did not hyper accumulate iron when glucose in the medium is substituted by dulcitol, glycerol or succinate (Shavlovsky et al., 1990). It was speculated that the corresponding *RIB80* and *RIB81* genes together encode a heterooligomeric regulatory protein complex of negative action (Shavlovsky et al., 1982, 1988a).

Later, in 1991, *P. guilliermondii rib1* riboflavin deficient mutant able to grow in media containing 10 times lower concentration of riboflavin (20 mg l^{-1}) was selected. Cells of this strain actively reduced riboflavin, methylene blue, 2,3,5-triphenyltetrazoliumchloride, they also possessed high activity of ferrireductase and hyperaccumulated iron in the cells. This mutation (designated *hit1* – high iron transport) was shown to cause riboflavin overproduction when introduced in *P. guilliermondii* riboflavin prototrophic strains (Stenchuk et al., 1991; Fedorovich et al., 1999).

In addition, selection of *P. guilliermondii* mutants defective in regulation of RF biosynthesis and iron homeostasis representing 6 new groups of complementation (designated *red1-red6*) was reported recently (Stenchuk and Kapustiak, 2003).

It should be noted that all mutations mentioned above possess pleiotropic effect on *P. guilliermondii* metabolism. All of them cause defects in regulation of iron acquisition and lead to oxidative stress, in addition to riboflavin overproduction (Shavlovskii et al., 1992; Fedorovich et al., 1999; Protchenko et al., 2000).

It was shown that expression of *RIB1* and *RIB7* genes coding for the first and the last enzymes of RF biosynthesis is significantly enhanced in both *red6* and *rib81* mutants. Results of Northern blotting demonstrated that cells of the wild-type strain exhibited low levels of *RIB1* and *RIB7* genes expression when the medium is supplemented with 3.6 μM iron. Decrease of iron concentration in the medium caused at least 10-fold increase in mRNA levels both of the genes analyzed. Obtained results suggested that an increase in RF production by *P. guilliermondii* cells caused by iron starvation or regulatory mutations correlates with elevated level of mRNAs of key enzymes involved in this biosynthetic pathway. Thus, it can be supposed that regulation of riboflavin biosynthesis by iron in *P. guilliermondii* occurs mainly at the transcriptional level (Boretsky et al., 2005).

The *P. guilliermondii* regulatory mutants *rib83* and *rib84,* unable to overproduce riboflavin in iron-deficient medium, were also isolated (Shavlovsky et al., 1989). They were selected as mutants that have lost the ability to excrete riboflavin in an iron-deficient medium. It has been suggested that the corresponding genes encode regulatory proteins of the positive control involved in expression of structural genes of riboflavin synthesis. Double *rib80 rib83* and *rib81 rib83* mutants were unable to overproduce riboflavin, which suggests on epistasis of the *RIB83* gene over the genes *RIB80* and *RIB81*. Notably, cells of *rib83* mutants grown under iron deficiency conditions possess decreased iron transport when compared to the wild-type strain (Stenchuk et al., 2001). One may suggest that mutation *rib83* blocks high affinity iron transport in *P. guilliermondii.* In favor of this assumption, growth rate of *rib83* mutants is significantly decreased in iron deficient media as compared to the wild-type strain. Possibly, mutations *rib83* inactivates an unknown transcription factor required for expression of genes involved in riboflavin biosynthesis and high affinity iron transport in *P. guilliermondii.* It could be speculated that products of genes of positive and negative type of action form a cascade system for proper transmission of the regulatory signals and provide coordinated regulation of iron acquisition and riboflavin biosynthesis that is essential for *P. guilliermondii* viability.

Unfortunately, no regulatory genes of riboflavin synthesis were isolated so far, therefore molecular mechanisms of iron-dependent regulation of riboflavin synthesis remain to be quite speculative. The difficulties in regulatory gene cloning arose from the absence of differences in growth patterns between wild-type strains and regulatory mutants in most of tested media. To overcome this problem, methods of insertional mutagenesis can be useful. Recent communication from this lab on successful development of method for insertional mutagenesis in another flavinogenic yeast, *C. famata*, and identification of several regulatory genes of positive action, involved in riboflavin synthesis (Dmytruk et al., 2006) makes cloning of regulatory genes of riboflavin synthesis in *P. guilliermondii* the immediate task for the future research.

6.4 Riboflavin Transport

Most of the wild-type strains and the riboflavin-deficient mutants of *P. guilliermondii* are incapable of riboflavin-mediated transport from the medium (Sibirny et al., 1977d). It should be noted that riboflavin-deficient mutants of *P. guilliermondii* required for optimal growth addition to the medium up to 200 mg l^{-1} of riboflavin. This concentration is close to saturated solutions of this vitamin (near 400 mg l^{-1} in neutral water solution).

Using riboflavin auxotrophs, the secondary auxotrophs growing in media containing only 2.0 mg l^{-1} of riboflavin were selected. They were shown to possess multi drug sensitivity. However, selected mutants were unable to accumulate significant amounts of the vitamin in the cells due to uptake of this vitamin from the medium (Sibirny et al., 1977a).

After an additional step of mutagenesis, *P. guilliermondii* mutants able to grow in media containing less than 0.3 mg l^{-1} of riboflavin were selected. On media

containing 100–200 mg l^{-1} of riboflavin they accumulated significant amounts of intracellular riboflavin turning color of the colonies to yellow. This observation suggested on existence of the system for active transport of the vitamin in such mutants.

Two independent riboflavin-deficient mutants MS1–3 and RA68–2 which grew at very low concentration of exogenous vitamin and possessed riboflavin transport systems with different properties designated as RF-permease I and RF-permease II, respectively, have been isolated (Sibirny et al., 1977d; Shavlovsky and Sibirny, 1985).

Properties of RF-permease I were studied using strain MS1–3 and riboflavin prototrophs, isolated from it. Riboflavin uptake via RF-permease I was saturable and displayed an apparent *Km* of 0.17 mM and velocity 0.27 nmoles/(min × mg of dry weight cell). The pH optimum was at 5.8. Transport system was very specific as did not catalyzed transport of several structural analogs of riboflavin and FMN and FAD were not transported by this system at all. Activity of RF-permease I rises 30–50 fold in 4 hours after substitution of glucose by sucrose, maltose or other α-glucosides in the medium. It was found out the coordinate regulation of RF permease I and α-glucosidase and identified genes of negative and positive control involved in such regulation (Sibirny et al., 1979; Sibirny and Shavlovsky, 1984a). Genetic analysis revealed three unlinked regulatory genes designated *RFP80, RFP81* (of negative action (recessive mutations *rfp80, rfp81*), and *RFP82,* a gene of positive action (dominant mutations $RFP82^c$) that are involved in regulation of riboflavin transport. The recessive mutations *rfp82* were isolated as meiotic segregants from an intragenic recombination between two $RFP82^c$ – alleles or as mutants resistant to riboflavin analogues in a medium with the riboflavin permease inducer sucrose. Interallelic complementation was found within the *RFP80* and *RFP82* loci, and the corresponding maps were constructed (Sibirny and Shavlovsky, 1984a). The epistasis-hypostasis test showed that the gene *RFP82* acts after the gene *RFP80.* A model for the action of regulatory products of the identified genes, which form a cascade system in expression of riboflavin permease and a-glucosidase, was presented (Sibirny and Shavlovsky, 1984a).

It was shown that cells of MS1–3 strain grown on sucrose are able to uptake riboflavin even without exogenous energy source in the reaction mixture, however, inhibitors of energy metabolism (uncouplers of oxidative phosphorylation, inhibitors of ATPase and others) strongly inhibited riboflavin uptake. These data suggest on existence of system of active transport of riboflavin, which differs from that described in *S. cerevisiae* (Perl et al., 1976; Reihl and Stolz, 2005). Ability to transport exogenous riboflavin appeared to be a semidominant feature since colonies of diploid hybrids (between strain MS1-2 and the wild-type strain) exhibited slightly yellow color after long incubation on solid media containing 200 mg l^{-1} of riboflavin. Concentration of intracellular riboflavin in MS1-3 strain could reach up to 6 g l^{-1}. Significant part of the vitamin was accumulated in vacuoles as crystals. It was shown that riboflavin transport by this strain is significantly reduced by glucose, sorbose but not by fructose, galactose and mannose when added to the reaction mixture.

After additional steps of UV mutagenesis the mutant 9i, derivative of the strain MS1-3, was selected. This mutant was able to grow in media containing 0.005 mg l^{-1}

of riboflavin. Riboflavin uptake by this strain displayed an apparent *Km* of 0.031 mM and maximal velocity was 1.0 nmoles/ (min × mg of dry weight cell). This strain has been successfully used to develop a method for microbiological assay of riboflavin and for new method for measurement of riboflavin kinase activity (Sibirny and Shavlovsky, 1984b; Kashchenko et al., 1991).

In contrast to RF-permease I, RF-permease II activity (strain *RA68–2*) did not depend on type of carbon source (sucrose or glucose) in the media. Both sucrose and glucose did not effect RF-permease II activity when added to the reaction mixture (Shavlovsky and Sibirny, 1985; Sibirny, 1986). Velocity of riboflavin transport via RF-permease II is 10 times lower as compared to the first transport system.

The mechanism of evolution of these transport systems, which are cryptic in the wild-type strains, is not known. In addition to riboflavin permeases, P. *guilliermondii* possesses a riboflavin-excreting system which is present also in the wild-type strains (Shavlovsky et al., 1977; Sibirny et al., 1978). Riboflavin permease II and riboflavin excretase are apparently synthesized constitutively (Sibirny et al., 1977d, 1978). Mutants defective in riboflavin excretase were isolated. If to combine genetic defect in riboflavin excretase and overexpression of riboflavin synthesis, resulted strain accumulated in cells huge amounts of riboflavin which exceeded normal amount of this vitamin in yeast cells up to 1000 times. Cells of such mutants can be used as rich source of vitamin in the content of animal feed (Sibirny and Shavlovsky, 1984b; Sibirny, 1986).

6.5 Genetic Techniques

6.5.1 Life Cycle

All known natural or collection strains of *P. guilliermondii* are heterothallic (Wickerham and Burton, 1954; Kreger van-Rij, 1970; Shchelokova et al., 1974; Zharova et al., 1977). The mating types were designated mat^+ and mat^- (Sibirny et al., 1977b). Homothallic natural or collection strains are not described, though some of meiotic segregants of the hybrids obtained by protoplast fusion manifest a homothallic phenotype. Such a phenotype was designated as pseudohomothallic ($mat^{+,-}$) as these strains segregate mat^+ and mat^- clones during cultivation in complete synthetic medium. The pseudohomothallic phenotype was completely eliminated in the meiotic pedigree of $mat^{+,-}$ x mat^+ or $mat^{+,-}$ x mat^- hybrids (Sibirny, 1986). It was hypothesized that such pseudohomothallic ($mat^{+,-}$) strains are aneuploids.

6.5.2 Sexual Crosses

Cell conjugation between prototrophic strains of opposite mating types was observed on wort agar (Zharova et al., 1977). Study of sexual hybridization between auxotrophic mutants showed that most efficient matings occurred in solid media

with sodium acetate or tomato juice (Sibirny et al., 1977b). Hybridization was not observed in complete media. Thus, optimal conditions for crossing of *P. guilliermondii* appeared to be poor starvation media. Later, such a conclusion found support during the investigation of hybridization conditions for other yeast species. It was shown that yeast species found in natural habitats as haploids (haplonts) efficiently cross in poor media, whereas diplontic species (found in nature as diploids) hybridize predominantly in rich media (Naumov et al., 1980, 1981).

The following procedure of sexual hybridization is generally used for *P. guilliermondii* (Sibirny et al., 1977b). Auxotrophic strains of opposite mating types with complementary nutritional requirements are grown as a streak on complete YEPD or YEPS media. The strains are crossed on plates with acetate medium (sodium acetate, 1%; potassium chloride, 0.5%), incubated for 2–3 days, and then are replica-plated onto minimal medium. The prototrophic hybrids are formed at the contact sites of the streak cross.

6.5.3 Protoplast Fusion

Hybridization can also be obtained by protoplast fusion (Sibirny et al., 1982; Klinner and Böttcher (1984). In this case, hybrids were obtained between auxotrophs belonging to opposite or to the same mating type. The maximal frequency of hybridization by protoplast fusion was near 2×10^{-2} (Sibirny et al., 1982).

In addition to intraspecific *P. guilliermondii* hybrids, protoplast fusion was used for isolation interspecific hybrids with *Pichia kudriavzevii* and *Hansenula polymorpha* (Sibirny et al., 1982; Kashchenko et al., 1987).

Sexual or protoplast fusion hybrids of *P. guilliermondii* appeared to be very stable mononuclear diploids during growth on synthetic or complete media, and did not sporulate under such conditions (Zharova et al., 1980; Klinner and Böttcher, 1984; Sibirny, 1986). It is interesting to note that ploidy of the protoplast fusion hybrids never exceeded the diploid level (Klinner and Böttcher, 1984; Büttner et al., 1985). It was suggested that protoplast fusion hybrids appeared to be aneuploids in many cases.

Hybrids of *P. guilliermondii* were able to sporulate on acetate media. Most hybrids produced only one to two spores after 5–6 days of incubation at room temperature, but some pairs of strains gave diploids, which produced up to 40% asci with spores (Sibirny et al., 1977b). Incubation at 30°C depressed the sporulation. Five other media tested did not induce sporulation (Sibirny, 1986).

6.5.4 Protocol for Isolation and Fusion of Protoplasts

Cells are cultivated in sugar-mineral medium containing yeast extract (0.5%), 0.05 M potassium phosphate buffer, pH 6.0, and growth factors for auxotrophs (40 μg ml^{-1}). Cultivation is run for 16–24 h to middle exponential growth phase (cell mass

0.7–l.0 mg dry weight ml^{-1}). Cells are sedimented by centrifugation and washed twice with water.

Cells (50–100 mg dry weight ml^{-1}) are incubated in the following mixture: 0.05 M Tris-HCl, pH 7.0; 0.4 M $CaCl_2$; 0.01 M dithiothreitol; Zymolyase 20000 (2 mg ml^{-1}; Seikagaku Corp.) or β-Glucuronidase (10–15 mg ml^{-1}; Sigma) for 30– 40 min at 37°C with periodic mixing. Control of protoplasting is monitored using phase contrast microscopy. Yield of protoplasts usually reached 100%.

Protoplasts are separated from incubation medium by centrifugation at 2000 rpm for 5 min at 4°C, twice washed with cold (4°C) 0.4 M $CaCl_2$, and then are resuspended in the same solution at 7.5 mg dry weight ml^{-1}.

Suspensions of a pair of auxotrophic mutants containing 50×10^6 of protoplasts ml^{-1} are mixed in a 1:1 ratio, sedimented by centrifugation, and resuspended in the medium inducing protoplast fusion (25% polyethylene glycol 6000, containing 0.1 M $CaCl_2$, in 0.05 M Tris-HCl buffer, pH 8.6). Final concentration of the protoplasts of both strains is 50×10^6 ml^{-1} mixture. The obtained suspension is incubated for 20 min at 30°C with periodic mixing.

The protoplast mixture is spread onto a surface of an agar medium containing an osmotic stabilizer (1 M sucrose or 1 M sorbitol). Any additional pouring of top-overlaid mild agar is not necessary for *P. guilliermondii.*

6.5.5 Analysis of Meiotic Segregants

Asci of hybrids contain as a rule two spores, sometimes one-spored asci appeared, while three- or four-spored asci were seldom found. Asci have oval or elongate forms, while spores are characterized by round or hat-shaped forms (Sibirny et al., 1977b). Preferential production of two-spored asci is the result of degeneration of several nuclei formed during meiosis (Zharova et al., 1977, 1980).

Meiotic segregants of sexual hybrids, which produced abundant amounts of spores frequently, gave diploids characterized by low spore frequency. A genetic line, i.e. haploid strains hybrids of which produced a large amount of spores, was selected by inbreeding; using sister crosses between several consecutive pedigrees of meiotic segregants (Sibirnyi et al., 1977c). The strains denied from this genetic line easily crossed and sporulated, producing up to 60% of two-spored asci. Unfortunately, strains which appeared to be capable of producing four-spored asci were not isolated. Thus, tetrad analysis is impossible for *P. guilliermondii.* Electron microscopic studies showed that, during meiosis, diploid cells formed three or four nuclei but only part of them were surrounded by a spore envelope and formed spores. Apparently the other nuclei degenerated (Zharova et al., 1977, 1980).

Several methods of elimination of vegetative diploid cells for random spore analysis were developed. The method, based on selective killing of diploid vegetative cells by elevated temperature, (55°C and 60°C), was unsuccessful. More appropriate appeared to be the method based on eliminating vegetative cells by vaseline oil or killing diploid cells by ethanol or diethyl ester. The most suitable method,

which used 20% ethanol to eliminate non-sporulated hybrid cells for random spore analysis, is given below. Survival of spores of *P. guilliermondii* genetic line is equal to 86% (Sibirny et al., 1977b, c; Sibirny, 1986).

6.5.6 Protocol for Random Spore Analysis

A sporulating diploid cell suspension (2–2.5 mg dry weight ml^{-1}) is incubated at 30°C for 2 h in helicase (β-Glucuronidase) solution (10–15 mg ml^{-1}) for digestion of asci envelopes. Then the suspension is gently homogenized in a glass homogenizer to separate the spores, diluted to a cell concentration of approx. 0.6 mg dry weight ml^{-1}, and 1/5 part (by volume) of ethanol is added. The suspension is incubated with permanent shaking for 10–12 min and, after dilution with 20% ethanol, is spread onto YEPD medium.

P. guilliermondii does not contain its own plasmids. Therefore, plasmids based on a *Saccharomyces cerevisiae* vector were used for *P. guilliermondii* transformation at the beginning (Kunze et al., 1985; Zakal'skii et al., 1990; Logvinenko et al., 1993). Efficiency of transformation was low (10–20 transformants mg^{-1} of plasmid DNA), suggesting that autonomously replicating sequences (ARS-elements) derived from *S. cerevisiae* are not functional in *P. guilliermondii* cells.

6.6 Transformation

P. guilliermondii genes *RIB1* and *RIB7* coding respectively for GTP cyclohydrolase II and riboflavinsyntase were cloned by functional complementation of corresponding *E. coli* riboflavin deficient strains (Zakal'skii et al., 1990; Logvinenko et al., 1993). Later nucleotide sequence both of the genes was determined and was found to encode polypeptides sharing significant homologies with the corresponding enzymes in other organisms (Liauta-Teglivets et al., 1995; Boretsky, unpublished data). It was observed that efficiency of transformation of *P. guilliermondii rib1* mutants with plasmids containing the *RIB1* gene was about 150 transformants mg^{-1} of plasmid DNA. In contrast efficiency of transformation of *P. guilliermondii rib7* mutants with plasmids containing the *RIB7* gene was only 10–20 transformants mg^{-1} of plasmid DNA. Analysis of nucleotide sequences of the cloned *RIB1* gene revealed A + T rich sequence containing two stretches of homology to yeast ARS consensus sequence. Cloning of the 3′-located sequence into plasmid carrying the *RIB7* gene increased efficiency of transformation of *P. guilliermondii rib7* mutants by two orders of magnitude and provide autonomous replication of constructed plasmid. Thus, *P. guilliermondii* ARS-elements was identified and had been used to construct *P. guilliermondii/ E. coli* shuttle vectors (Boretsky et al., 1999).

Using constructed plasmids a several protocols of yeast transformation were tested and properly modified in order to increase transformation efficiency of *P. guilliermondii*. At present, lithium acetate transformation protocol provided

efficiency up to 1000 transformants μg^{-1} of DNA of a replicative plasmid whereas spheroplast transformation or electroporation both routinely give 3–5 × 10^4 transformants μg^{-1} of DNA of a replicative plasmid. Protocols for these procedures are given below.

A modified protocol reported by Ito is currently used to treat the *P. guilliermondii* cells with lithium acetate (Ito et al., 1983). Yeast cells were grown overnight aerobically in YPD medium to an optical density of $OD_{600} \leq 0.5$ and pelleted at 3,000 g for 10 min. Usually we use 100–150 ml of the medium. The cells were washed with water, pelleted and re-suspended in 10 ml of 0.1 M lithium acetate, TE buffer pH 7.5 (LiAc/TE buffer). After incubation (1 hour at 30°C) they were pelleted and re-suspended in fresh LiAc/TE buffer to a final concentration of 5 × 10^9 cells ml^{-1}. 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 of 50% PEG in LiAc/TE buffer 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, pelleted, re-suspended in 1 ml YPD and incubated at 30°C for 1 h. Finally the cells were pelleted again, re-suspended in 150 µl of 1 M sucrose, plated on a selective medium and incubated at 30°C for 3–6 days.

Spheroplast transformation was done using a modified protocol for *Pichia pastoris* transformation (Cregg et al., 1985). Yeast cells were grown aerobically overnight in YPD medium to an optical density of $OD_{600} \leq 0.3$ and pelleted at 3000 g for 10 min. Usually we use 200–350 ml of the medium. The cells were pelleted at 3000 g for 10 min washed with water, pelleted and re-suspended in 10 ml of 1 M sucrose, 50 mM dithiothreitol, 25 mM EDTA, pH 8.0. After 15 min incubation at room temperature cells were pelleted again, washed 2 times with 1 M sucrose and re-suspended in 1 M sucrose, 25 mM EDTA, 100 mM sodium citrate pH 5.8. Zymolyase (or lyticase) was added and cells were incubated for 10–40 min (30°C, slow agitation) in order to get approximately 5–10% of spheroplasted cells. More intensive treatment with an enzyme increases percentage of spheroplasts, but significantly reduces efficiency of transformation. Thus, quantity of an enzyme used for the treatment of cells should be justified before. Cells are pelleted at 2,000x g for 5 min, washed twice with 1 M sucrose, once with 1M sucrose, 10 mM $CaCl_2$ and re-suspended in the last solution to a final density 2 × 10^8 cells ml^{-1}. To re-suspend the pelleted cells and spheroplasts slowly and repeatedly draw them into a pipette and release them into the tube was done. 100 µl aliquots of the cell suspension were dispensed into 1.5 ml tubes and 0.1–1 µg of transforming DNA together with 5 µg of single strand carrier DNA were added. After 20 min of incubation at 25°C 1 ml of solution of 20% PEG 3350, 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$ was added and immediately (gently, avoid vortexing) mixed. The mixture was incubated for additional 15 min at 25°C. Spheroplasts and cells were pelleted (1000x g for 10 min), re-suspended in 1 M sucrose, 10 mM $CaCl_2$ and incubated for 30 min at 25°C. Aliquots of the suspension were plated on selective medium containing 1 M sucrose and incubated for 3–6 days at 30°C.

For yeast electroporation, a modified protocol of (Becker and Guarente, 1991) was used. Yeast cells were grown aerobically overnight in a rich medium (YPD) to an optical density of $OD_{600} \leq 0.5$, chilled on ice and pelleted at 3000x g for 10 min.

All subsequent manipulations should be done at 2–4°C. 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^{-1}. Aliquots of 200 µl were dispensed into 1.5 ml tubes. Plasmid DNA (0.05–0.50 µg in 1–2 µl of TE buffer) was added and mixed gently. The mixture was transferred into prechilled 1 mm electroporation cuvettes. Electroporation was performed as follows: resistance – 200 Ω; capacitance – 25 µF; voltage – 1.8 kV. The cells were washed out from the cuvettes with 1 ml of YPD medium, incubated for 1 hour at 30°C, pelleted and re-suspended in 1 M sucrose, plated on selective medium and incubated at 30°C for 3–5 days.

6.7 Plasmid Rescue

All constructed *P. guilliermondii/ E. coli* shuttle vectors and plasmids are rather unstable in the yeast cells. Even after 5–8 divisions under selective pressure only 5–30% of cells of transformants contain a plasmid bearing selective marker. Never the less plasmid DNA can be rescued from *P. guilliermondii* transformants. Fresh colony of a transformant was inoculated in 3 ml of YPD medium and grown at 30°C for approximately 15 hours. Cells were pelleted, washed with water, and resuspended in 0.3 ml of water. 0.6 ml of acetone was added. After 10 min incubation cells were pelleted, re-suspended in 1 ml of acetone and incubated for 15 min at 37°C. Washed cells were pelleted at 5000g for 7 min, dried and re-suspended in 0.2 ml of 50 mM Tris-HCl buffer pH 8.0, 50 mM EDTA, 0.5% SDS, 100 mM NaCl. The cell suspension was incubated for 30 min in ice-water bath with occasional shaking. Cells were removed by centrifugation; 0.02 ml of 3 M potassium acetate pH 5.0 were added to supernatant and mixed. To precipitate DNA two volumes of prechilled ethanol were added and mixture was incubated at 0°C for 1 h. DNA was pelleted by centrifugation washed with 70% ethanol, dissolved in 20 µl of TE buffer and used for transformation of an *E. coli* strain.

Calculations based on comparison of *E. coli* transformation efficiencies suggested that about 0.001–0.01 µg of the plasmid DNA could be purified from *P. guilliermondii* transformants using procedure described above.

6.8 Construction of Knockout Strains

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 reported for this yeast species. We isolated *P. guilliermondii ura3* auxotrophs using a positive selection procedure with 5-fluoroorotic acid and used them as recipients for transformation experiments (Boretsky unpublished). To identify whether the *URA3* gene was impaired in FOA-resistant strains we constructed a pAGU3 plasmid 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. Despite the presence of PgARS, the efficiency of transformation of the *P. guilliermondii ura3* auxotrophs was very low: 40–50 transformants per 1 μg of DNA. A reason for that could be ambiguity of CUG codon in *P. guilliermondii* like it was reported for many *Candida* species (Sugiyama et al., 1995). To avoid this problem we substituted the single CUG codon encoding a conservative leucine residue (L45) in the *URA3* gene by another leucine codon, CUC. The efficiency of the transformation of *P. guilliermondii ura3* auxotrophs with the newly constructed pAGU34 plasmid that carries the modified gene was 100 fold increased when compared to the pAGU3 plasmid. This result can suggest that the CTG codon codes for serine (like in *Candida* species) and not leucine in *P. guilliermondii* cells (Boretsky unpublished).

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.

Three plasmids designated p72, p724D and p724R were constructed in which *RIB7* structural gene was replaced by the modified *S. cerevisiae URA3* gene as described above. Plasmid p72 was digested with *Bam*HI and *Pae*I restriction endonucleases generating the *rib7::URA 3–2,3* deletion cassette. Plasmids p724D and p724R were digested with *Bam*HI restriction endonuclease generating the *rib7::URA3–2,8D* and *rib7::URA3–2,8R* deletion cassettes, respectively.

The appropriate *ura3* mutant of *P. guilliermondii* was transformed with these cassettes using the spheroplasting procedure, Li-Ac method and electroporation. To get a larger number of transformants, the electroporation procedure was used in the initial experiments. Efficiency of transformation was approximately 1000 transformants per 1 μg of DNA for all cassettes. However, no stable riboflavin deficient recombinant strains were selected among approximately 400, 280 and 200 transformants obtained with cassettes *rib7::URA3–2,3 rib7::URA3–2,8D* and *rib7::URA3–2,8R*, respectively, after the electroporation procedure. Similar results were obtained when the spheroplasting procedure was applied for transformation – no stable riboflavin deficient strains were selected among 116 transformants checked. 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. No riboflavin auxotrophs were selected with the cassette *rib7::URA3–2,3* that harbors a shortened terminator sequence of the *RIB7* gene (Fig. 6.2a).

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

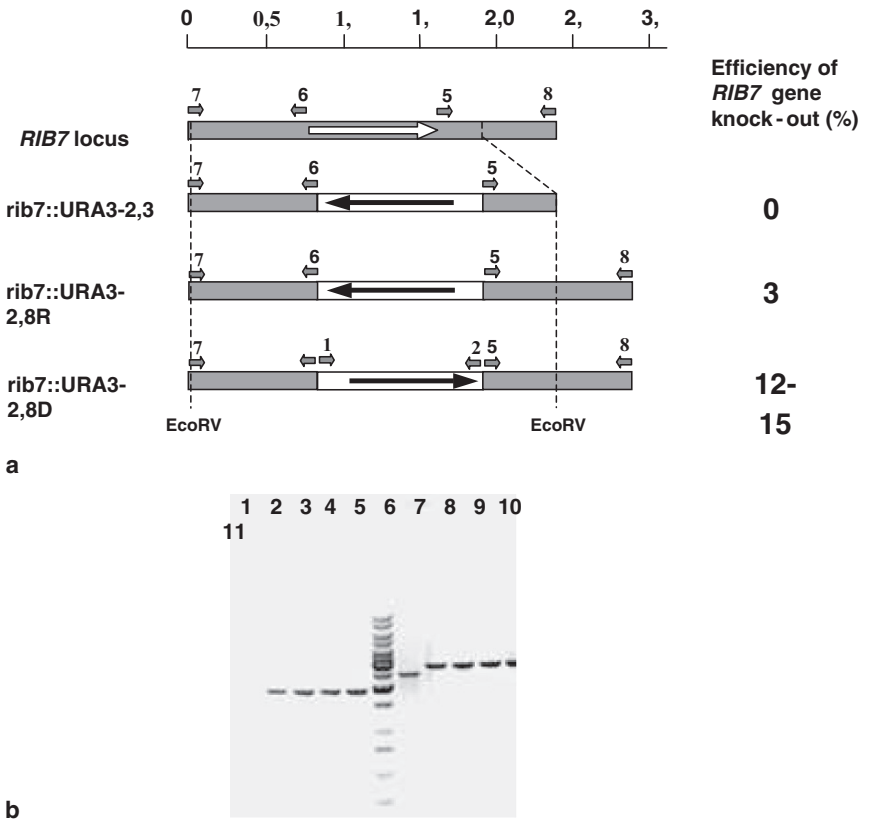

Fig. 6.2 Deletion of the *RIB7* gene in *P. guilliermondii*. (**a**) Scheme of cassettes used to delete *RIB7* gene. ⇒- *RIB7* structural gene of *P. guilliermondii.* ➡- *URA3* structural gene of *S. cerevisiae.* (**b**) Checking of knock-out recombinant strains by means of PCR Lane 1 – DNA-signals obtained 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* correspondingly, (primer 7 and primer 2); lane 6–1 kb DNA ladder "Fermentas" (from the top: 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb, 0.25 kb); lane 7 – DNA-signals obtained with recipient strain (primer 7 and primer 8); lanes 8, 9, 10, 11 – DNA-signals obtained with recombinant strains *Δrib7–1*, *Δrib7–2*, *Δrib7–3*, *Δrib7–4* correspondingly (primer 7 and primer 8)

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 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) also gave expected results: 2.4 kb and 2.8 kb signals in the cases of the recipient and recombinant strains respectively (Fig. 6.2b). Taken together, the obtained results suggest that in these transformants the *rib7::URA3–2,8D* deletion cassettes integrated into genome by homologous recombination which lead to a knock-out of the *RIB7* structural gene. To further prove the feasibility of this approach, we

constructed a deletion cassette *rib1::URA3–10* (that contains 1.6 kb and 1.4 kb promoter and terminator sequences of the *RIB1* gene respectively) and transformed it into the same recipient strain using the lithium acetate procedure. Efficiency of transformation was approximately 10000 transformants per 1 μg of DNA. Only 2 of 2000 transformants checked exhibited riboflavin deficiency. Deletion of *RIB1* gene was confirmed by means of PCR using total DNA of these transformants and a set of appropriate primers. 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. Thus, two genes were deleted successfully in *P. guilliermondii* (Boretsky unpublished). 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.

6.9 Conclusions

In contrast to other yeast species that overproduce riboflavin under conditions of iron deprivation, *P. guilliermondii* exists in both haploid and diploid forms, can be easily stimulated to mate and sporulate. Genetic line producing large amounts of spores was selected. More recently, tools for molecular genetic studies of *P. guilliermondii* have been developed which include collection of host strains, vectors and several transformation protocols including that for gene knock-out. Genome of *P. guilliermondii* has been sequenced recently and is publicly available. Thus, *P. guilliermondii* seems to be an attractive model for studying the specific iron-dependent regulation of cellular metabolisms, more specifically, control of riboflavin biosynthesis. Besides, some strains of this species efficiently convert xylose to xylitol, anti-caries sweetener. Production both of riboflavin and xylitol are of great industrial interest. But regulation of these metabolic pathways by *P. guilliermondii* has to be studied in details. At present, even the most active mutants of *P. guilliermondii* produce much less riboflavin than the other industrial producers, based on yeast *C. famata*, bacterium *B. subtilis* and fungus *Ashbya gossypii*. Selection of the more active *P. guilliermondii* riboflavin producers is possible, but will demand a strong increase in specific activity of GTP cyclohydrolase, which apparently limits flavinogenesis and the enhancement of this process by the purine precursor, GTP. The mutants of *P. guilliermondii* capable of active riboflavin transport efficiently accumulate this vitamin from diluted solutions inside the cells. It is possible to use such mutants for concentration of riboflavin from cultural liquids of weak producers. System of energy-dependent riboflavin excretion of riboflavin, which differs from that of riboflavin permease has been found in *P. guilliermondii.* Mutants defective in riboflavin excretase have been isolated which accumulate huge amounts of riboflavin inside the cells. It is suggested that obtaining the riboflavin-enriched yeast biomass as a source of riboflavin would be a much cheaper procedure than other possible methods for concentration of this vitamin from diluted solutions.

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