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EXPERIMENTAL ARTICLES

Effect of the *rib83* Mutation on Riboflavin Synthesis and Iron Acquisition in the Yeast *Pichia guilliermondii*

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Abstract–Monogenic *rib83* mutation blocked riboflavin oversynthesis in the yeast *Pichia guilliermondii* and lowered iron acquisition by cells, their ferric reductase activity, and the growth rate in iron-deficient media. Mutants with the combined mutations of *rib83* with *rib80* and *rib81* (the last two mutations impair the negative control of riboflavin synthesis and thus cause its oversynthesis) were unable to depress the enzymes of flavinogenesis (GTP cyclohydrolase and riboflavin synthase) or overproduce riboflavin in both iron-deficient and iron-sufficient media. This suggests that *rib83* mutation is epistatic with respect to *rib80* and *rib81* mutations. The *RIB83* gene may positively control both riboflavin synthesis and iron acquisition in the yeast *P. guilliermondii.*

Key words: yeast *Pichia guilliermondii*, riboflavin biosynthesis, iron transport, regulatory genes.

The synthesis of riboflavin (vitamin B_2) in the yeast *Pichia guilliermondii* is regulated with the involvement of iron ions [1]. The wild-type strains of this yeast grown in media with optimal concentrations of iron synthesize riboflavin in small amounts but produce it in large amounts under the conditions of iron deficiency. The deficiency of iron ions enhances their uptake from the medium by an order and leads to the oversynthesis of riboflavin due to a derepression of almost all enzymes involved in flavinogenesis, except ferric reductase. After elaborating the methods of hybridologic analysis [2], the yeast *P. guilliermondii* became a suitable model to study the genetic regulatory mechanisms of riboflavin synthesis and iron transport in eukaryotic organisms.

The negative regulation of riboflavin synthesis in *P. guilliermondii* is implemented by the regulator genes *RIB80* [3], *RIB81* [4], *HIT1* [5], and *RED1* [6]. The mutation of any of these loci derepresses, even if incompletely, the synthesis of riboflavin, so that it is overproduced in spite of a normal content of iron in the medium. Furthermore, such mutations substantially enhance the uptake of iron by yeast cells, which suggests that the regulation of riboflavin synthesis and iron acquisition in *P. guilliermondii* is coordinated.

In our earlier paper [7], we reported on the *rib83* and *rib84* mutations that made yeast cells incapable of enzyme derepression and riboflavin oversynthesis under the conditions of iron deficiency. These data were interpreted as indicative of the involvement of the *RIB83* and *RIB84* genes in the positive regulation of riboflavin synthesis in the yeast *P. guilliermondii*. However, other important properties of these genes remained unstudied.

This work was undertaken to answer the following questions: How is the *rib83* mutation inherited? Does this mutation influence iron acquisition? How does this mutation interact with the *rib80* and *rib81* mutations?

MATERIALS AND METHODS

Experiments were carried out with the following *Pichia guilliermondii* strains: ATCC201911(MAT$^-$ *hisX-17*), LV109 (MAT$^-$ *rib80-22 hisX-17*), LV158 (MAT$^-$ *rib81-131 hisX-17*), LV250-1(MAT$^+$ *rib83-13 argX-1*). Here, MAT is the mating type locus; *his* and *arg* are the loci that determine the cell requirement for histidine and arginine, respectively; *rib83* is the mutant allele of the positive regulator gene; and *rib80* and *rib81* are the mutant alleles of the negative regulator genes. The other strains mentioned in the paper were obtained by hybridizing the respective mutants with the wild-type strain and isolating meiotic hybrid segregants with the desirable genotype. The genetic analysis of *P. guilliermondii* was carried out as described by Sibirnyi *et al.* [2].

The yeast strains were grown at 30°C in shaken (200 rpm) Erlenmeyer flasks with synthetic Burkholder medium [8] supplemented, if required, with the respective amino acids (40 mg/l). Metal ions were removed from the cultivation media by treating them with 8-hydroxyquinoline. The yeast biomass was measured turbidimetrically using an FEK-56M photoelectrocolorimeter (light filter no. 6; 3-mm-path-length cuvette).

The concentration of riboflavin in the medium was measured using an EF-3M fluorimeter. GTP cyclohydrolase and riboflavin synthase were assayed in cell-free extracts as described by Logvinenko *et al.* [9, 10].

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Table 1. Activity of GTP cyclohydrolase and riboflavin synthase in the wild-type and mutant *P. guilliermondii* strains

Strain	Relevant genotype*	Fe in medium, mg/l	Activity, 10^{-5} U/mg protein	
			GTP cyclohydrolase	Riboflavin synthase
ATCC	Wild type	0.20	0.32 ± 0.03	2.7 ± 0.2
201911		0.01	14.20 ± 1.50	11.0 ± 1.4
LV109	*rib80*	0.20	1.15 ± 0.17	4.2 ± 0.6
		0.01	16.10 ± 1.35	8.4 ± 2.3
LV158	*rib81*	0.20	10.19 ± 1.11	5.6 ± 0.5
		0.01	21.40 ± 2.35	20.0 ± 1.5
LV111-2	*rib80 rib81*	0.20	16.90 ± 3.00	7.1 ± 0.5
		0.01	22.50 ± 2.54	23.6 ± 1.9
LV250-1	*rib83*	0.20	0.30 ± 0.04	2.3 ± 0.2
		0.01	0.42 ± 0.06	3.8 ± 0.3
S183-3	*rib83 rib80*	0.20	0.24 ± 0.03	2.9 ± 0.3
		0.01	0.66 ± 0.09	4.4 ± 0.7
LV291-1	*rib83 rib81*	0.20	0.20 ± 0.04	2.0 ± 0.3
		0.01	0.54 ± 0.07	3.6 ± 0.6
LV154-3	*rib83 rib80 rib81*	0.20	0.26 ± 0.03	3.0 ± 0.4
		0.01	0.70 ± 0.07	5.1 ± 0.7

* Only the mutant alleles of the regulatory genes of flavinogenesis are presented.

The ferric reductase activity of cells was measured using the method described by Fedorovich *et al.* [11]. In this method, the concentration of the Fe(II)– α,α'-bipyridyl complex was measured at 522 nm using an SF-46 spectrophotometer. The rate of the ^{55}Fe uptake by cells was measured as described by Fedorovich *et al.* [12]. The iron content of cells was determined with α,α'-bipyridyl [13].

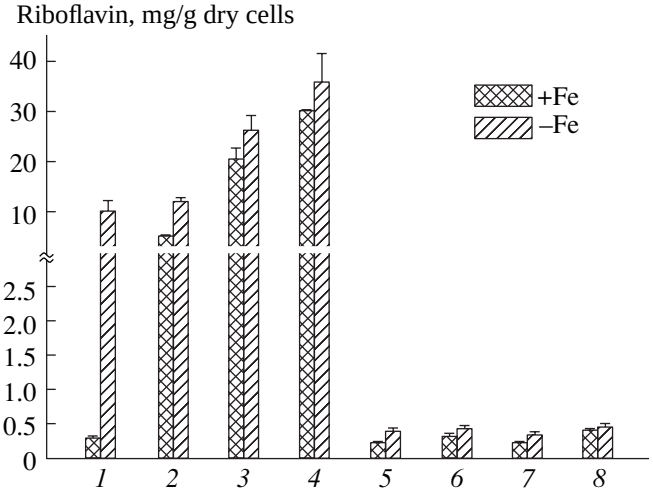

Fig. 1. Biosynthesis of riboflavin by (*1*) the wild-type *P. guilliermondii* strain and its mutants (*2*) *rib80*, (*3*) *rib81*, (*4*) *rib80 rib81*, (*5*) *rib83*, (*6*) *rib83 rib80*, (*7*) *rib83 rib81*, and (*8*) *rib83 rib80 rib81* cultivated in media with a high (+Fe) and low (–Fe) content of iron ions.

RESULTS

Data on the activity of GTP cyclohydrolase and riboflavin synthase, which catalyze, respectively, the first and last steps of riboflavin synthesis in *P. guilliermondii*, are shown in Table 1. In the *rib83* mutant strain LV250-1, as opposed to the wild-type strain ATCC 201911, iron deficiency in the medium (0.01 μg Fe/ml) did not cause derepression of the enzymes mentioned. This fact may explain the inability of strain LV250-1 to overproduce riboflavin under the conditions of iron deficiency (Fig. 1).

Hybridologic analysis showed that 39 of the randomly taken 75 meiotic segregants of the "*rib83* × wild type" hybrid synthesized 0.4–0.8 μg/ml riboflavin in the iron-deficient medium and, thus, were incapable of riboflavin oversynthesis obviously due to the presence of the *rib83* mutation. The other 36 meiotic segregants grown in the iron-deficient medium synthesized riboflavin in amounts from 15 to 76 μg/ml and, therefore, were capable of riboflavin oversynthesis. All hybrids of the first-type segregants with the *rib83* mutant strains were incapable of riboflavin oversynthesis in the iron-deficient medium, confirming the presence of the *rib83* mutation in these segregants. The observed proportion (39 : 36) between the two types of the meiotic segregants of the "*rib83* × wild type" diploid hybrid is close to (1 : 1), suggesting that the *rib83* mutation is monogenic and located in the nucleus.

The regulatory *RIB80* and *RIB81* genes of *P. guilliermondii* are involved not only in riboflavin oversyn-

Table 2. The rates of the ^{55}Fe uptake by the wild-type and mutant *P. guilliermondii* strains cultivated in media with a high (+Fe) and low (–Fe) content of iron ions

Relevant genotype	Number of strains studied	Cell radioactivity, 10^3 cpm/mg dry cells		–Fe/+Fe
		+Fe	–Fe	
Wild type	3	14.4 ± 0.7	164.7 ± 9.0	11.4
rib83	3	11.5 ± 0.9	45.8 ± 2.7	4.0
rib81	3	36.5 ± 2.4	385.8 ± 23.4	10.6
rib83 rib81	2	20.9 ± 1.7	60.7 ± 6.3	2.9

thesis but also in iron transport in this yeast [3, 4]. Measurements of the iron uptake rate for the wild-type and *rib83* mutant cells showed that these two types of cells almost do not differ in the intensity of the ^{55}Fe uptake in an iron-sufficient medium containing 0.2 μg Fe/ml. In the case of the iron-deficient medium, the increase in the iron uptake rate of the wild-type cells was nearly three times that of the *rib83* mutant cells (Table 2), indicating that this mutation impairs iron transport in cells grown in the iron-deficient medium. The increase in the iron uptake rate of the double *rib83 rib81* mutant was lower than in the case of the wild-type and *rib81* mutant cells. Therefore, the *rib83* mutation is epistatic with respect to the *rib81* mutation.

The decrease in the rate of the ^{55}Fe uptake by the *rib83* mutant may be related to the impairment of the ferric reductase of *P. guilliermondii* [11]. The reduction of Fe(III) to Fe(II) is necessary for the active transport of iron into cells; therefore, the impairment of ferric reductase may affect yeast growth and flavinogenesis. To verify this suggestion, we measured the growth rate, flavinogenesis, ferric reductase activity, and the iron content of the wild-type and *rib83* mutant cells (strains 43-D88/37 and 60-D88/37, respectively) incubated in the iron-deficient and iron-sufficient media. In the latter medium, these parameters did not considerably differ for both strains (data not presented).

At the same time, the growth of the mutant strain in the iron-deficient medium was poorer than that of the wild-type strain (after 24 h of growth, the biomasses of these strains differed by about 1.5 times). Moreover, due to a diauxic growth of the wild-type strain, its biomass after 140 h of growth reached 3 mg/ml (Fig. 2), whereas the biomass of the *rib83* mutant remained at a level of 0.7 mg/ml. Similar diauxic growth under iron deficiency was observed for the wild-type *Candida guilliermondii* strain, which secreted riboflavin into the growth medium during the first logarithmic growth phase and took it up during the second logarithmic phase [14]. Unlike *C. guilliermondii* cells, the wild-type *P. guilliermondii* cells synthesized and secreted riboflavin continuously (Fig. 2).

As for the ferric reductase activity of yeast cells grown in the iron-deficient medium (Fig. 3), it tended to decrease during the first 45 h of growth of both wild-type and mutant strains. However, in later cultivation terms, the ferric reductase activity of the wild-type strain began to increase, whereas that of the mutant *rib83* strain remained at a low level. In accordance with these data, the iron content of the wild-type cells at the stage of secondary growth did not decrease and amounted to 20–25 μg/g dry cells.

In the double *rib83 rib80* and *rib83 rib81* mutants, the *rib83* mutation blocked the derepression of GTP cyclohydrolase and riboflavin synthase and, consequently, riboflavin oversynthesis under both iron-deficient and iron-sufficient conditions (Table 1 and Fig. 1). A similar effect of the *rib83* mutation was observed for the triple *rib83* rib80 *rib81* mutant, despite a synergistic effect of the combined *rib80 rib81* mutation on riboflavin synthesis [15]. Therefore, the *rib83* mutation is epistatic with respect to both the *rib80* and *rib81* mutations.

DISCUSSION

Our previous study [7] showed that the regulatory *RIB83* gene is involved in the positive control over riboflavin synthesis in *P. guilliermondii.* The experimental results presented in the given paper refine that the *rib83* mutant allele is epistatic with respect to the *rib80* and *rib81* genes involved in the negative control over riboflavin synthesis. The epistasis of the *rib83* mutation is observed even if the three factors causing riboflavin oversynthesis, i.e., iron deficiency, the *rib80* mutation, and the *rib81* mutation, act simultaneously.

Like the *RIB80*, RIB81, *HIT1*, and *RED1* genes, the *RIB83* gene is polyfunctional, i.e., it is involved in the control of not only flavinogenesis but also iron transport. It can be suggested that this gene is an important element of a mechanism coordinating the supply of iron and riboflavin to the respiratory chain of *P. guilliermondii.*

It is known that the diauxic growth of a culture is a consequence of its adaptation to unfavorable growth conditions. The inability of the *rib83* mutant to grow diauxically in the iron-deficient medium can be explained by its inability to raise ferric reductase activity and thus to satisfy its requirement for iron ions. The key role of the *RIB83* gene may lie in the maintenance of the oversynthesis of riboflavin. Indeed, as Straube suggests [14], riboflavin promotes the acquisition of iron through its chelation and normalizes the metabo-

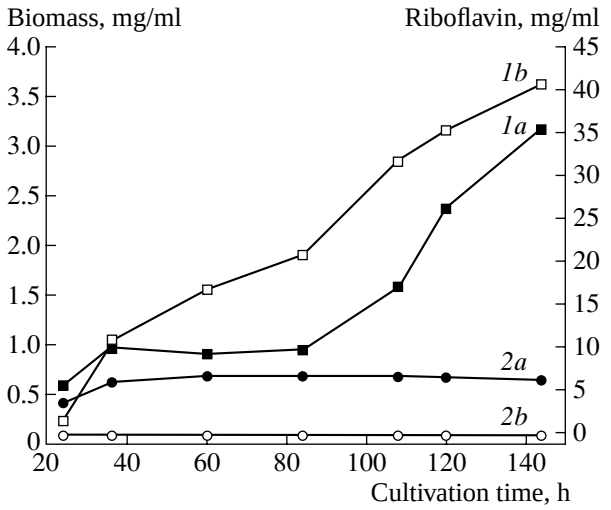

Fig. 2. (*a*) Biomass and (*b*) riboflavin concentration in the culture liquids of (*1*) the wild-type strain 43-D88/37 and (*2*) the *rib83* mutant strain 60-D88/37 of *P. guilliermondii* grown under the conditions of iron deficiency.

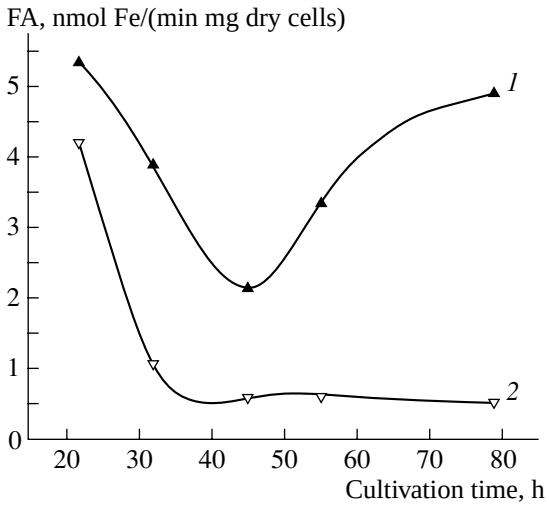

Fig. 3. The ferric reductase (FA) activity of (*1*) the wild-type strain 43-D88/37 and (*2*) the *rib83* mutant strain 60-D88/37 of *P. guilliermondii* grown under the conditions of iron deficiency.

lism of cells through the maintenance of their redox potential at a favorable level. In *Helicobacter pylori*, reduced riboflavin may be involved in the reduction of Fe(III) to Fe(II), thus promoting iron transport into the cells [16]. The active reduction of riboflavin was observed for the *P. guilliermondii hit1* [5] and *rib80 rib81* mutants. According to our earlier observations, the fraction of Fe(III) in the total iron content of *P. guilliermondii* cells increases when they actively synthesize riboflavin [17]. All these data indicate that riboflavin plays an important role in iron metabolism.

The mechanisms of action of the *RIB83* gene on riboflavin synthesis and iron transport in *P. guilliermondii* remain unclear. The *rib83* mutation is not manifested phenotypically under iron-sufficient conditions, indicating that the *RIB83* gene is necessary only under iron-deficient conditions, when this regulator gene induces its target genes. It is still unknown which of the *P. guilliermondii* genes involved in iron metabolism are controlled by the *RIB83* gene.

The molecular mechanisms of iron transport have been studied in more detail for the yeast *Saccharomyces cerevisiae*, whose iron transport is positively regulated by two genes, *AFT1* [18] and *MAC1* [19]. The targets for these regulatory genes are the structural genes *FRE1* and *FRE2* coding for ferric reductases and the structural gene *FET3* coding for copper-dependent oxidase. The *MAC1* gene also controls the expression of the *CTT1* gene coding for cytoplasmic catalase. The mutation of both regulatory genes lowered, even under iron sufficiency, the ferric reductase activity and iron transport. In addition, the *mac1* mutation causes respiratory deficiency and supersensitivity to heavy metal ions. It remains unknown whether or not the *AFT1* and *MAC1* genes are involved in the regulation of riboflavin synthesis in *S. cerevisiae*.

The possibility cannot be excluded that the absence of the phenotypic manifestation of the *rib83* mutation under the conditions of iron sufficiency is due to the fact that the selection method that we used [7] allowed the leaky *rib83* mutations to be detected only under the conditions of iron deficiency. The complete mutation of the *RIB83* gene, which is a positive regulator of riboflavin biosynthesis and iron transport in *P. guilliermondii* cells, may drastically diminish their viability or even make them nonviable.

Further investigation of this and related problems can provide better insight into the role of the *RIB83* gene in the system supplying two cofactors, iron and riboflavin, to the respiratory chain of *P. guilliermondii*. Taking into account that *RIB83* is a key gene controlling the synthesis of riboflavin, such knowledge will make it possible to intensify the selection of active riboflavin producers. Such producers can be used, for instance, in the poultry industry to enrich the poultry diet with vitamin B_2, which, reportedly, beneficially influences the growth of chicken [20].

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