ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 1, pp. 55–59. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © L.R. Fayura, D.V. Fedorovych, T.M. Prokopiv, Yu.R. Boretsky, A.A. Sibirny, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 1, pp. 66–71.

EXPERIMENTAL ARTICLES

The Pleiotropic Nature of *rib80, hit1*, and *red6* Mutations Affecting Riboflavin Biosynthesis in the Yeast *Pichia guilliermondii*

L. R. Fayura[1], D. V. Fedorovych, T. M. Prokopiv, Yu. R. Boretsky, and A. A. Sibirny

Institute of Cell Biology, National Academy of Sciences of Ukraine, ul. Dragomanova, 14/16, Lviv, 79005 Ukraine

Received December 15, 2005.

Abstract—The yeast *Pichia guilliermondii* is capable of riboflavin overproduction under iron deficiency. The *rib80, hit1*, and *red6* mutants of this species, which exhibit impaired riboflavin regulation, are also distinguished by increased iron concentrations in the cells and mitochondria, morphological changes in the mitochondria, as well as decreased growth rates (except for *red6*) and respiratory activity. With sufficient iron supply, the *rib80* and *red6* mutations cause a 1.5–1.8-fold decrease in the activity of such Fe–S cluster proteins as aconitase and flavocytochrome b_2, whereas the *hit1* mutation causes a six-fold decrease. Under iron deficiency, the activity of these enzymes was equally low in all of the studied strains.

DOI: 10.1134/S0026261707010080

Keywords: yeasts, regulation of riboflavin biosynthesis, iron accumulation, Fe–S cluster proteins.

In some of yeast species, iron ions are involved in the regulation of production of the flavinogenesis enzymes [1, 2]. In *Pichia guilliermondii*, one of the yeast species most extensively studied in this respect, under iron deficiency the level of transcription of riboflavin (RF) biosynthesis genes increases sharply, thereby increasing the intensity of flavinogenesis [3]. We have previously described the *rib80*, *rib81*, *hit1*, and *red6* mutants of this species, which, aside from the high flavinogenesis intensity, are characterized by changes in iron metabolism. These mutations are recessive, monogenic, and are not associated with the riboflavin biosynthesis genes [1, 3, 4]. It has been previously suggested that the mutations *rib80, rib81, hit1* and *red6* inactivate the hypothetical heterooligomeric repressor protein which controls both riboflavin biosynthesis and the transport of iron ions in *P. guilliermondii* [1]; however, the nature of these mutations and the molecular mechanisms of their functioning are still unknown.

In most yeast species, including *Saccharomyces cerevisiae*, unlike *P. guilliermondii*, iron deprivation does not result in a marked increase in flavinogenic activity [4]. The regulation of RF biosynthesis in *S. cerevisiae* is poorly understood, whereas the control mechanisms which regulate iron uptake are well-known [5, 6]. The regulation of iron assimilation by *S. cerevisiae* is known to be controlled by the Aft1p and Aft2p transcriptional activators [7]. The transcription of the genes involved in iron transport is controlled by these factors and depends on the mitochondrial Fe–S cluster biosynthesis, rather than on the cytosolic iron levels [6]. It is also known that impaired Fe–S cluster synthesis leads to mitochondrial iron accumulation in *S. cerevisiae* and *Candida albicans*. In addition, increased production of vitamin B_2 was noted in *Candida albicans* [8, 9]. The Fe–S clusters and/or the proteins which contain them are probably universal components of the systems which control iron metabolism in yeast cells. Also, it is possible that these factors are involved in the regulation of riboflavin biosynthesis in some yeast species.

This work provides evidence of the pleiotropic nature of the *rib80, hit1*, and *red6* mutations of *P. guilliermondii.* These mutations, in addition to certain abnormalities in the regulation of riboflavin biosynthesis and iron assimilation, cause a decrease in growth rates, structural changes in mitochondria, as well as inhibition of the activity of Fe–S cluster proteins (aconitase and flavocytochrome b_2).

MATERIALS AND METHODS

In our study, we used the yeast strain *Pichia guilliermondii* ATCC 9058 and the *rib80*, *hit1* and *red6* mutants of this species which exhibited impaired regulation of riboflavin biosynthesis [10–12].

The yeasts were grown in the modified Burkholder medium. The medium composition and cultivation conditions were previously described in [13]. The removal

[1] Corresponding author; e-mail: fedorovych@cellbiol.lviv.ua

of metals from the media was performed with the use of 8-oxyquinoline. The yeast biomass was determined turbidimetrically with a Helios Gamma UVG-100105 spectrometer at $\lambda = 600$ nm. Riboflavin concentrations were measured with an EF-3M fluorometer.

The content of iron in the cells and in the mitochondrial fraction was determined colorimetrically with α,α'-dipyridyl [14]. The concentration of the Fe^{2+}–dipyridyl complex was measured with a Helios Gamma UVG-100105 spectrometer at $\lambda = 522$ nm.

Exponential phase cells ($A_{600} = 1.0$) were used for the isolation of protoplasts. To obtain protoplasts, the cell disintegration and differential centrifugation were performed according to the method described by Tan et al. [15]. The efficiency of fractionation was determined according to the activity of the marker protein, the mitochondrial membrane-bound enzyme flavocytochrome b_2, in the pellet and supernatant fractions.

Gel filtration of the cell-free extracts was carried out in a column (1.5 × 10 cm) with Sephadex G-10 at the flow rate of 50 ml/h. The activity of citrate(isocitrate) hydrolyase (EC 4.2.1.3; aconitase) in the column eluents was determined by the rate of isocitrate transformation into *cis*-aconitate according to the method described in [16]. The enzyme quantity required for the synthesis of 1 μmol of *cis*-aconitate in 1 min was accepted as an activity unit.

The activity of L-lactate cytochrome reductase (EC 1.1.2.3; flavocytochrome b_2) was determined in a dialyzed cell-free extract according to the method described in [17]. The enzyme quantity required for the oxidation of 1 μmol of L-lactate in 1 min was accepted as an activity unit. The protein concentration was determined by the method of Lowry et al.

The respiratory activity of the cells was determined by means of an oxygen sensor Biological Oxygen Monitor YS1 Model 5300 at 30°C in oxygen-saturated cell suspensions (0.5 mg/ml) in a mineral medium (pH 5.6) under constant stirring. The respiratory activity was expressed as O_2 amount (μmol) consumed by 1 mg of dry cells in 1 min.

Electron microscopic examination was performed according to [18].

All the experiments were carried out in triplicates. The statistical processing of the results was performed using the Microsoft Office Excel software package.

RESULTS

The mutants *rib80, hit1*, and *red6* are known to exhibit elevated flavinogenic activity, a considerably increased rate of iron transport, and intracellular iron accumulation [10–12]. However, they differ in the level of their flavinogenic activity. While riboflavin production by *rib80* grown in the media with the optimal iron content (3.6 μM) was 3.4 mg RF/g dry cells, the amount of riboflavin produced by *hit1* was about two times lower; in the case of *red6*, it was as low as 0.51 mg RF/g dry cells. As in the wild type strain, decreased iron content in the medium (0.18 μM of iron ions) resulted in an increase in flavinogenic activity in all the studied mutants.

Analysis of the growth of the *P. guilliermondii* mutants exhibiting impaired regulation of riboflavin biosynthesis has shown that, as in the wild type strain ATCC 9058, the exponential growth phase of the mutant cells lasted for 14–16 hours, with a subsequent decrease in growth rates and transition to the stationary phase. The mutants *rib80* and *hit1* differ from the wild strain in their growth rates. The specific growth rate of the strain ATCC 9058 was 0.25 h^{-1}; the generation time was 2.75 h. The specific growth rates of the *rib80* and *hit1* mutants were 0.2 and 0.19 h^{-1}; the generation times were 3.2 and 3.5 h, respectively. The strain carrying the *red6* mutation did not differ from the wild strain in growth kinetics.

Electron microscopic examination demonstrated that the number of mitochondria in the mutants *rib80, hit1*, and *red6* was higher than that in the wild strain (Fig. 1).

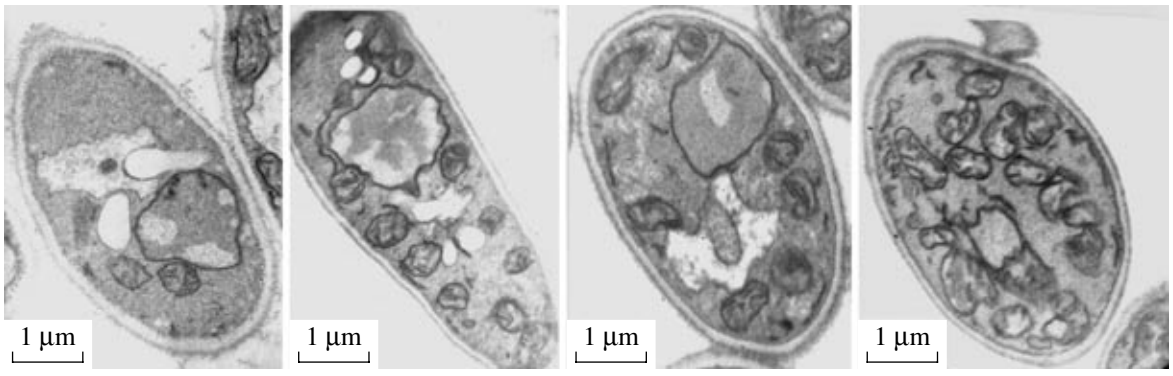

Fig. 1. Ultrathin sections of *P. guilliermondii* cells grown in the medium with 90 μM of iron: (*1*) wild type strain ATCC 9058; (*2*) *rib80*; (*3*) *hit1*; (*4*) *red6*.

The total iron concentrations in the cells of the mutants were two–three times higher than those contained in the cells of the wild type strain (Fig. 2b). The iron content in the mitochondrial fraction of the *rib80, hit1*, and *red6* mutants of *P. guilliermondii* was found to be 2.5–3 times higher than that in the mitochondrial fraction of the wild type strain (Fig. 2a). The ratio of mitochondrial membrane-bound flavocytochrome b_2 contained in the cytosol (supernatant) fraction and in the pellet varied from 16.8 to 22.9. This fact suggests a slight degradation of mitochondria, which could result in a partial loss of iron during isolation. Nevertheless, the results obtained indicate that, in all the studied mutant strains, the iron content in the mitochondria is higher than that in the wild type strain.

Theoretically, high iron concentrations in the mitochondrial fraction of mutant cells could explain their decreased growth rates, for instance, due to the inhibition of their respiratory activity by excessive iron concentrations or to a decrease in the activity of Fe–S cluster proteins, as has been demonstrated in the case of the Δ*yfh1* mutants of *C. albicans* and *S. cerevisiae* [9, 19]. We observed a slight decrease in the specific rate of oxygen consumption by the cells of the investigated *P. guilliermondii* mutants, as compared to the wild type strain both without an exogenous carbon source (Fig. 3) and in the presence of glucose and glycerol (data not shown). As could have been expected, the intensity of oxygen consumption both by the mutant and the wild type cells grown under iron deficiency (0.18 μM) was 1.3–1.5 times lower. Hence, the increase in the iron content in the cells correlates with a decrease in both the growth rate and the respiratory activity of the *rib80* and *hit1* mutants, but not of the *red6* mutant. However, the decrease in the respiratory activity of the studied *P. guilliermondii* strains did not exceed 20–30%, while the rate of oxygen consumption by the Δ*yfh* mutant cells of *C. albicans* was four–five times lower [9].

Figure 4 shows the activity profiles of the two Fe–S cluster proteins, aconitase and flavocytochrome b_2, in the studied strains of *P. guilliermondii* grown in the medium with optimal iron concentration (3.6 μM) and under iron deficiency (0.18 μM). The assayed enzymatic activities of the *rib80* and *red6* mutants was 1.5–1.8 times lower than that of the wild type strain. Iron deprivation caused the decrease in aconitase and flavocytochrome b_2 activity both in the wild type strain and the *rib80* and *red6* mutants. It should be noted that the activity of both enzymes in the mutant *hit1* was four–six times lower, irrespective of iron concentrations in the medium. Figure 4 shows that time of cultivation (24–48 h) had practically no effect on aconitase and flavocytochrome b_2 activities in all the strains. It seems likely that the *rib80* and *red6* mutations do not affect Fe–S cluster synthesis in *P. guilliermondii*, since the decrease in aconitase and flavocytochrome b_2 activity in these strains was not as pronounced as in the mutants of *C. albicans* and *S. cerevisiae* with impaired Fe–S cluster synthesis [6, 8].

DISCUSSION

In the yeast *S. cerevisiae*, a traditional subject of molecular biology research, the expression of the genes involved in iron metabolism is regulated by the Aft1p and Aft2p transcriptional activators [7]. It has been demonstrated that Fe–S clusters, rather than the concentration of cytosolic iron, are the main effectors in the regulation of the corresponding genes [6]. In this species, an inverse correlation was revealed between the activity of Fe–S cluster proteins and the transcription level of *fet3* and *ftr1* genes involved in high-affinity iron transport into cells.

Both in *C. albicans* and *S. cerevisiae*, iron assimilation is generally regulated at the level of transcription of the corresponding genes. However, unlike *S. cerevisiae*, *C. albicans* cells synthesize riboflavin in excessive quantities under iron deficiency [9]. The specific transcription factors regulating the expression of the genes responsible for iron assimilation and riboflavin biosynthesis in this species have not been identified; however

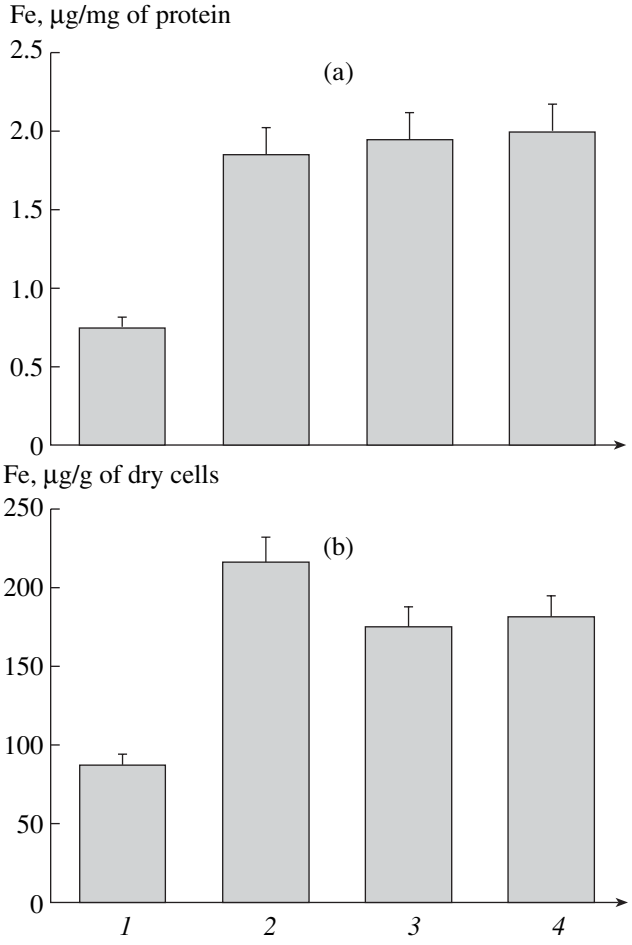

Fig. 2. Concentrations of iron in the mitochondrial fraction (a) and cells (b) of *P. guilliermondii*, wild type strain ATCC 9058 (*1*), and the mutants *hit1* (*2*), *red6* (*3*), and *rib80* (*4*). Growth time 24 h.

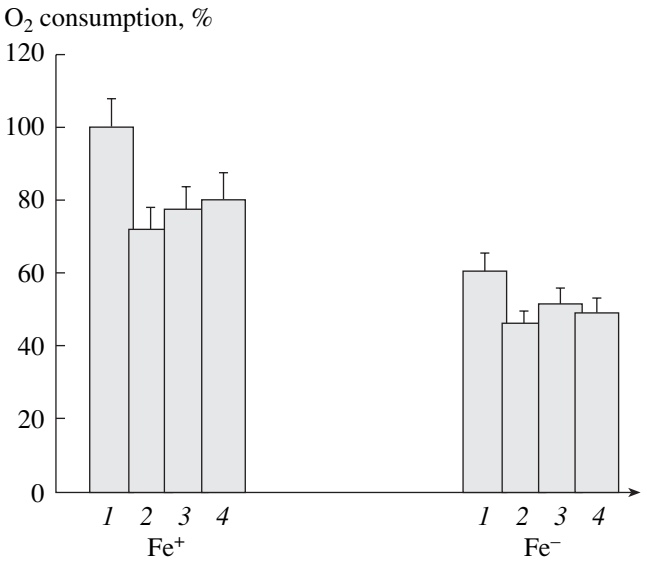

Fig. 3. Oxygen consumption by the cells of *P. guilliermondii* wild type strain ATCC 9058 (*1*) and the mutants *hit1* (*2*), *red6* (*3*), and *rib80* (*4*) grown at various iron concentrations (the incubation mixture did not contain any exogenic substrates). Fe^+ and Fe^-, iron concentrations of 3.6 and 0.18 μM, respectively.

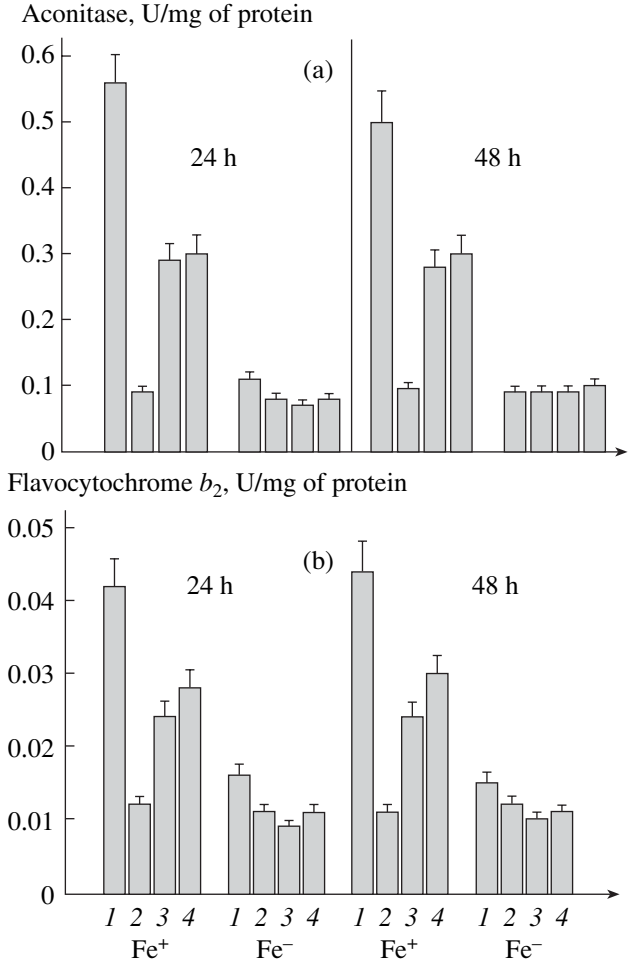

Fig. 4. Aconitase (a) and flavocytochrome b_2 (b) activity in the cell-free extracts of *P. guilliermondii* wild strain ATCC 9058 (*1*), and the mutants *hit1* (*2*), *red6* (*3*), and *rib80* (*4*). Fe^+ and Fe^-, iron concentrations of 3.6 and 0.18 μM, respectively.

it was demonstrated that the yeast global repressor Tup1p is involved in these processes [2].

Mutations of the corresponding genes involved in iron assimilation are responsible for the generation of similar phenotypic properties in *S. cerevisiae* and *C. albicans* [2, 9, 19]. Moreover, many such mutations can be complemented by the insertion of appropriate orthologic genes. Hence, in these yeast species, some elements of the regulatory mechanisms providing cells with iron are quite similar. However, in spite of impressive progress in the study of the regulation processes of iron assimilation, the mechanism of iron sensing in yeasts is unknown.

The previously published data [1, 4, 20], as well as the results of this study, suggest that the regulatory mechanisms providing *P. guilliermondii* cells with iron are similar to those of *C. albicans* [2, 9]. The mutants *rib80, hit1*, and *red* of *P. guilliermondii* were selected for the property "capacity for riboflavin overproduction in the presence of iron" [10–12]. Further investigations into the properties of the mutants have shown that iron deprivation was not responsible for riboflavin overproduction. Moreover, mutant cells accumulate several times more iron than the wild strain. More recently, it was demonstrated that these mutations lead to oxidative stress as well [20].

According to the phenotypic properties (increased concentrations of iron in the cells and in the mitochondrial fraction, high concentrations of malonic dialdehyde, a sharp decrease in aconitase and flavocytochrome b_2 activity), the *hit1* mutant is very similar to the previously described mutants with impaired Fe–S cluster synthesis [6, 8]. The activity of aconitase and flavocytochrome b_2 in the *rib80* and *red6* mutants of *P. guilliermondii* is relatively high. The observed changes in the respiratory activity in all the studied mutants were less significant as in Δ*yfh* cells of *C. albicans* [9].

None of the studied mutations probably affects the gene *yfh*. It can be assumed that Fe–S cluster synthesis in the studied mutants of *P. guilliermondii* is not impaired, and a slight decrease in aconitase and flavocytochrome b_2 activity results from the degradation of labile Fe_4–S_4 groups due to oxidative stress, as had been shown in the case of the *jac1* mutants of *S. cerevisiae* [8].

The observed decrease in the growth rates and the morphological changes of the mitochondria in the mutants *rib80* and *hit1* are a further indication of the pleiotropic nature of these mutations. It is probable that expression of the genes responsible for riboflavin bio-

synthesis correlates with the expression of the genes involved in the iron transport in this yeast species, as in *C. albicans* [9].

The *rib80, hit1*, and *red6* mutants of *P. guilliermondii* possess a number of properties typical of the cells of the wild type strain grown under iron deficiency (riboflavin overproduction, high ferrireductase activity and high transport rates of iron ions, increased concentrations of malonic dialdehyde) [10–12, 21]. This fact may point to the impact of the investigated mutations on the availability of intracellular iron, or to certain abnormalities in the mechanisms of iron sensing. The results obtained indicate the existence of specific common elements involved in the regulation of riboflavin biosynthesis, iron assimilation, and, possibly, in oxidative stress response in *P. guilliermondii.*

The authors thank A.R. Kulachkovskii, Senior Scientist, Ivan Franko National University of Lviv for his help in carrying out electron microscopic examinations.

REFERENCES

1. Shavlovskii, G.M. and Logvinenko, E.M., Role of Iron in the Regulation of Protein Synthesis in Microorganisms, *Usp. Mikrobiol.*, 1988, vol. 22, pp. 108–133.
2. Knight, S.A.B., Lesuisse, E., Stearman, R., Klausner, R.D., and Dancis, A., Reductive Iron Uptake by *Candida albicans*: Role of Copper, Iron and the *TUP1* Regulator, *Microbiology (UK)*, 2002, vol. 148, pp. 29–40.
3. Boretsky, Y.R., Kapustyak, K.Y., Fayura, L.R., Stasyk, O.V., Stenchuk, M.M., Bobak, Y.P., Drobot, L.B., and Sibirny, A.A., Positive Selection of Mutants Defective in Transcriptional Repression of Riboflavin Synthesis by Iron in the Flavinogenic Yeast *Pichia guilliermondii, FEMS Yeast Res*, vol. 5, no. 9, pp. 829–837.
4. Fedorovich, D., Protchenko I., and Lesuisse E., Iron Uptake by the Yeast *Pichia guilliermondii.* Flavinogenesis and Reductive Iron Assimilation Are Co-Regulated Processes, *BioMetals*, 1999, vol. 12, no. 4, pp. 295–300.
5. Philpott, C.C., Protchenko, O., Kim, Y.W., Boretsky, Y., and Shakoury-Elizeh, M., The Response To Iron Deprivation in *Saccharomyces cerevisiae*: Expression of Siderophore-Based Systems of Iron Uptake, *Biochem. Soc. Trans.*, 2002, vol. 30, pp. 698–702.
6. Chen, O.S., Crisp, R.J., Valachovic, M., Bard, M., Winge, D.R., and Kaplan, J., Transcription of the Yeast Iron Regulon Does Not Respond Directly To Iron But Rather To Iron-Sulfur Cluster Biosynthesis, *J. Biol. Chem.*, 2004, vol. 279, pp. 29513–29518.
7. Blaiseau, P.-L., Lesuisse, E., and Camadro, J.M., Aft2p, a Novel Iron-Regulated Transcription Activator That Modulates, with Aft1p, Intracellular Iron Use and Resistance To Oxidative Stress in Yeast, *J. Biol. Chem.*, 2001, vol. 276, pp. 34221–34226.
8. Voisine, C., Cheng, Y.C., Ohlson, M., Schilke, B., Hoff, K., Beinert, H., Marszalek, J., and Craig, E.A., Jac1, a Mitochondrial J-Type Chaperone, Is Involved in the Biogenesis of Fe/S Clusters in *Saccharomyces cerevisiae, Proc. Natl. Acad. Sci. USA*, 2001, vol. 98, pp. 1483–1488.
9. Santos, R., Buisson, N., Knight, A.B., Dancis, A., Camadro, J.-M., and Lesuisse, E., *Candida albicans* Lacking the Frataxin Homologue: a Relevant Yeast Model for Studying the Role of Frataxin, *Mol. Microbiol.*, 2004, vol. 54, no. 2, pp. 507–519.
10. Shavlovskii, G.M., Fedorovich, D.V., Logvinenko, E.M., and Koltun, L.V., Izolation and Characterization of *Pichia guilliermondii* Flavinogenic Strains Carrying the *rib80* (*ribR*) Regulatory Mutation, *Mikrobiologiya*, 1985, vol. 54, no. 6, pp. 919–926.
11. Stenchuk, N.N., Protchenko, O.V., Fedorovich, D.V., and Shavlovskii, G.M., Mutants of *Pichia guilliermondii* with Enhanced capability of Reduction of Riboflavin and Iron Ions, *Genetika*, 1991, vol. 27, no. 3, pp. 561–564.
12. Stenchuk, N.N. and Kapustyak, K.E., The *red* Mutations Impair the Regulation of Flavinogenesis and Metal Homeostasis in Yeast *Pichia guilliermondii, Genetika*, 2003, vol. 39, no. 8, pp. 1026–1032.
13. Shavlovskii, G.M., Zharova, V.P., Shchelokova, I.F., Trach, V.M., Sibirnyi, A.A., and Ksheminskaya, G.P., Flavinogenic Activity of Environmental Strains of the Yeast *Pichia guilliermondii, Prikl. Biokhim. Mikrobiol.*, 1978, vol. 14, no. 2, pp. 184–189.
14. Kovalev, L.M., Krulikovskaya, L.I., Yakova, V.M., and Yashina, O.T., Iron Determination in the *Saccharomyces* Yeasts by the Dipyridine Method, *Nauchn. Dokl. Vyssh. Shkoly*, 1984, vol. 12, pp. 96–100.
15. Tan, X., Titorenko, V.I., van der Kley, I.J., Sulter, G.J., Haima, P., Waterham, H.R., Evers, M., Harder, W., Veenhuis, M., and Cregg, J.M., Characterization of Peroxisome-Deficient Mutants of *Hansenula polymorpha, Curr. Genet.*, 1995, vol. 28, no. 3, pp. 248–257.
16. Romanova, A.K., *Biochemical Methods in the Study of Microbial Autotrophy*, Moscow: Nauka, 1980.
17. Smutok, O., Gayda, G., Gonchar, M., and Schuhmann, W., A Novel L-Lactate-Selective Biosensor Based on Flavocytochrome b_2 from methylotrophic yeast *Hansenula polymorpha, Biosens. Bioelectron.*, 2005, vol. 20, pp. 1285–1290.
18. Kulachkovsky, A.R., Moroz, O.M., and Sibirny, A.A., Impairment of Peroxisome Degradation in *Pichia methanolica* Mutants Defective in Acetyl-CoA Synthetase or Isocitrate Lyase, *Yeast*, 1997, vol. 13, pp. 1043–1052.
19. Chen, O.S., Hemenway, S., and Kaplan, J., Inhibition of Fe-S Cluster Biosynthesis Decreases Mitochondrial Iron Export: Evidence That Yfh 1p Affects Fe-S Cluster Synthesis, *Proc. Natl. Acad. Sci. USA*, 2002, vol. 99, pp. 12321–12326.
20. Shavlovskii, G.M., Fedorovich, D.V., Kutsyaba, V.I., Babyak, L.Ya., and Stenchuk, N.N., Contribution of the *RIB80* Gene To the Regulation of Riboflavin Biosynthesis and Iron Transport in *Pichia guilliermondii, Genetika*, 1992, vol. 28, no. 9, pp. 25–32.
21. Protchenko, O.V., Boretsky, Y.R., Romaniuk, T.M., and Fedorovych, D.V., Overproduction of Riboflavin by Yeast *Pichia guilliermondii* in Response to Oxidative Stress, *Ukr. Biokhim. Zhurnal*, 2000, vol. 72, no. 2, pp. 19–23.