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EXPERIMENTAL ARTICLES

Restoration of the Wild-Type Phenotype in *Pichia guilliermondii* Transformants

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Abstract—*Pichia guilliermondii* strain with blocked GTP cyclohydrolase II was transformed using replicative plasmids and their fragments containing the structural gene *RIB*1 of this enzyme. Experiments showed that the presence of the ARS element and the promoter region of this gene in the genome of transformants reduces the probability of their reversion to the wild-type phenotype. Different types of recombination in the yeast *P. guilliermondii* are discussed.

Key words: yeast *Pichia guilliermondii*, transformation, recombination types, riboflavin biosynthesis, GTP cyclohydrolase II.

The flavinogenic yeast *Pichia guilliermondii* synthesizes riboflavin in large amounts during its growth in iron-deficient media [1]. The biosynthesis of riboflavin is controlled by at least four negative regulatory genes (*RIB*80, *RIB*81, *HIT*1, and *RED*1) and two positive regulatory genes (*RIB*83 and *RIB*84). The negative regulatory genes also control iron uptake by yeast cells [2–4], which suggests that riboflavin synthesis and iron uptake are subject to coordinate regulation. Taking into account that the products of the aforementioned genes and the mechanism of their action on the expression of the respective structural genes are unknown, the investigation of the promoter and the regulatory regions of these structural genes is of great interest.

The 3'-terminal region of the structural gene of GTP cyclohydrolase II overlaps with the A-T-rich nucleotide sequence that initiates replication in yeast cells (ARS element). The promoter region of this gene also contains an A-T-rich sequence, as well as several potential sites of interaction with the transcription factors [5–7]. The functional activity of these sequences is unknown because of the absence of site-specific mutagenesis technique for *P. guilliermondii*. The application of the standard technique to the site-specific mutagenesis of *P. guilliermondii* is restricted by the possibility of nonspecific recombination in this yeast. The introduction of ARS elements into the plasmids and DNA fragments used for site-specific mutagenesis promotes yeast transformation and allows a great number of recombinant clones to be obtained. However, the possibility cannot be excluded that such an introduction will diminish homologous recombination. All this shows that the study of the integration of autonomously replicating plasmids into the genome of *P. guilliermondii* and the phenotypic analysis of the recombinant clones of this yeast are necessary steps in the investigation of the regulatory sequences of structural genes controlling riboflavin synthesis.

The aim of the present work was to analyze the phenotype of recombinant clones obtained by introducing circular replicative plasmids and their linear fragments into *P. guilliermondii* cells with blocked GTP cyclohydrolase II.

MATERIALS AND METHODS

Experiments were carried out with the *Pichia guilliermondii* ATCC 201911 (L2) MAT(*hisX-17*) strain and its riboflavin-dependent mutant RG-21 ATCC 201912 MAT(*hisX-17 rib1*) strain with blocked GTP cyclohydrolase II [8]. The strains were grown in Burkholder and YEPD media as described by Shavlovskii *et al.* [9].

DNA was isolated, digested, ligated, and subjected to electrophoresis in agarose gel as described by Maniatis *et al.* [10]. Yeast cells were transformed using LiCl [11]. Transformants were selected using YEPD medium without riboflavin. Growth was monitored by measuring culture turbidity in a KFK-2MP photoelectrocolorimeter (light filter no. 6) with a 1-cm-path-length cuvette. Flavins were separated by ascending paper chromatography in 2.5% Na_2HPO_4 and quantified using an EF-3M electrofluorimeter (Russia).

The content of iron in yeast cells was determined according to Kovalev *et al.* [12]. Ferric reductase was assayed with α,α'-dipyridyl, measuring the concentration of the Fe(II)–α,α'-dipyridyl complex with an SF-46 spectrophotometer at 522 nm.

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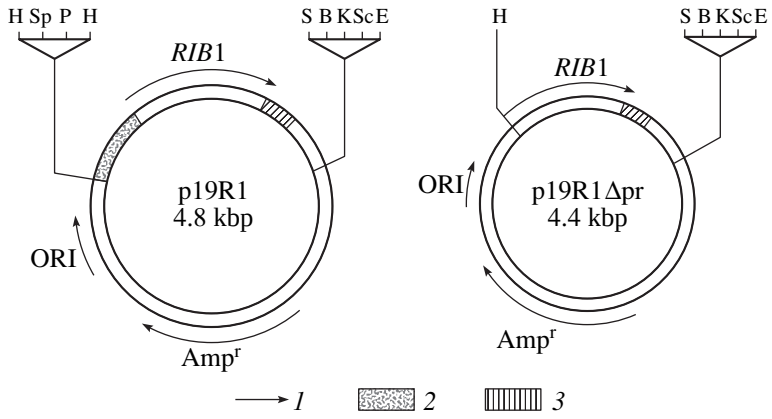

Fig. 1. Recombinant plasmids p19R1 and p19R1Δpr used for the transformation of *P. guilliermondii*. Shown are the following restriction sites: H, *Hin*dIII; Sp, *Sph*I; P, *Pst*I; S, *Sal*GI; B, *Bam*HI; K, *Kpn*I; Sc, *Sac*I; and E, *Eco*RI. Other designations: *1*, the structural gene *RIB*1 of GTP cyclohydrolase II; *2*, the promoter sequence of the *RIB*1 gene; and *3*, ARS element.

RESULTS AND DISCUSSION

To study the integration of autonomously replicating plasmids with the genome of *P. guilliermondii*, the riboflavin auxotroph of this yeast with blocked GTP cyclohydrolase II was transformed using plasmids p19R1 and p19R1Δpr bearing the ARS element of this yeast and the structural *RIB*1 gene coding for GTP cyclohydrolase II. Plasmid p19R1Δpr was derived from plasmid pTC2 [14] by deleting a *Hin*dIII-specific fragment. Plasmid pTC2 differs from plasmid p19R1 in the nucleotide sequence of the promoter region of the structural gene coding for GTP cyclohydrolase II (Fig. 1).

The transformation efficiencies of the riboflavin-dependent mutant *P. guilliermondii rib1* RH-21 strain by plasmids p19R1 and p19R1Δpr were the same (1–1.4×10^2 transformants/μg DNA), indicating that either of the two plasmids bears the ARS element. About 95% of the transformants obtained with plasmid p19R1 and 78% of the transformants obtained with plasmid p19R1Δpr produced extracellular riboflavin in trace amounts and some fluorescent substances dominated by pteridines. The growth rate of recombinant clones was 3 to 7 times lower than that of the parent L2 strain (data not shown).

The phenotypic differences between the recombinant clones and the parent strain may be explained by the altered expression of some genes because of the nonhomologous recombination of transformed DNA fragments in the genome of the recipient strain, as was shown for many lower eukaryotes. For instance, flanking recombination in *Neurospora crassa* gives rise to several copies of a cloned gene in the fungal chromosome [15]. In *Candida glabrata*, introduced DNA is integrated with the yeast genome by the mechanism of nonhomologous recombination, leading to different mutant clones defective, in particular, in the biosynthesis of amino acids [16].

The transformation of *P. guilliermondii* with circular plasmids may be associated with the nonhomologous or single-site recombination of the plasmid DNA [17] and with the formation of multimeric plasmids. This may lead to amplification of the GTP cyclohydrolase II gene and its promoter region and, hence, to the

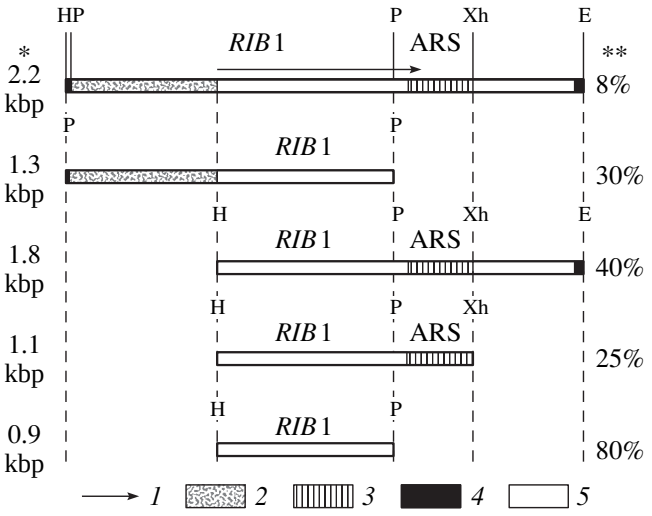

Fig. 2. DNA fragments used for the transformation of the mutant *P. guilliermondii rib1* strain. DNA fragments 2.2 and 1.3 kbp in size were obtained by digesting plasmid p19R1 with the restriction endonucleases *Hin*dIII + *Eco*RI and *Pst*I, respectively. DNA fragments 1.8, 1.2, and 0.9 kbp in size were obtained by digesting plasmid p19R1Δpr with the restriction endonucleases *Hin*dIII + *Eco*RI, *Hin*dIII + *Xho*I, and *Hin*dIII + *Pst*I, respectively. Shown are the following restriction sites: H, *Hin*dIII; P, *Pst*I; X, *Xho*I, and E, *Eco*RI. Other designations: *1*, the structural gene *RIB*1 of GTP cyclohydrolase II; *2*, the promoter sequence of the *RIB*1 gene; *3*, ARS element; *4*, polylinker region; and *5*, *P. guilliermondii* DNA. Fragment sizes are marked by one asterisk (*), and the percentage of transformants with the wild-type phenotype is marked by two asterisks (**).

Flavinogenesis, ferric reductase activity, and iron content in the recombinant *P. guilliermondii* cells

DNA fragment	Clone	Biomass, mg/ml	Flavinogenesis, μg RF/mg dry cells	Ferric reductase, nmol Fe/(mg cells min)	Iron content, μg Fe/g dry cells
	L2	6.9	0.21	1.1	87
	RH-21	–	–	1.3	119
2.2-kbp fragment	155	2.8	0.20	1.9	98
	193	2.7	0.24	0.8	118
	168	2.5	0.16	–	104
	190	2.3	0.15	1.0	96
	197	2.9	0.17	1.6	156
	192	2.5	0.20	1.0	103
1.3-kbp fragment	230	2.3	0.22	–	–
	232	2.6	0.16	–	–
	241	5.0	0.39	–	–
	237	2.5	0.25	–	–
1.8-kbp fragment	224	5.1	0.62	2.3	103
	210	4.8	0.33	1.6	97
	202	3.9	0.39	1.8	45
	211	5.2	0.28	3.5	–
	205	3.6	0.23	1.1	–
	218	3.7	0.15	1.5	95
1.2-kbp fragment	112	3.3	0.28	1.3	77
	140	3.0	0.28	2.1	119
	130	2.5	0.17	1.0	84
	129	2.3	0.24	0.8	80
	137	2.1	0.17	1.2	114
	143	3.0	0.42	2.0	135
	141	2.8	0.08	1.1	–
0.9-kbp fragment	226	3.7	0.36	1.6	90
	228	4.0	0.44	1.4	40

Note: RF is riboflavin. "–" stands for "not determined."

weak expression of other genes involved in riboflavin synthesis because of the deficiency of specific transcription factors [18]. This suggestion is confirmed by the aforementioned fact that most of the recombinant clones accumulated pteridines, which resulted from suppressed riboflavin synthesis.

To diminish the probability of the multicopy integration of plasmids and the extrachromosomal localization of the cloned gene, *P. guilliermondii* was transformed with the linear fragments of plasmids p19R1 and p19R1Δpr containing the *RIB*1 gene (Fig. 2). To obtain such fragments, the plasmid DNA was digested by the respective restriction endonucleases, and the digest was subjected to electrophoresis. The DNA fragments 2.2, 1.8, 1.3, 1.2, and 0.9 kbp in size were eluted from the gel, purified, and used for transformation. The biggest DNA fragment 2.2 kbp in size contained the *RIB*1 gene, the 0.43-kbp promoter region of this gene, and the ARS element with a 3'-flanking nucleotide sequence. Among the riboflavin prototrophs obtained by the transformation of *P. guilliermondii* with the 2.2-kbp DNA fragment, only 8% were found to be close to the wild-type phenotype in growth and riboflavin synthesis characteristics (Fig. 2 and the table). Transformation with the 1.3-kbp fragment lacking the ARS element and its 3'-flanking sequence augmented the relative number of transformants with the wild-type phenotype to 30%. Transformation with the 1.8-kbp fragment lacking the promoter region of the *RIB*1 gene resulted in a relative number of transformants with a wild-type phenotype of 40%. The relative number of transformants with the wild-type phenotype obtained with the

1.2-kbp DNA fragment lacking a 0.6-kbp sequence adjacent to the ARS element decreased to 25%. The small DNA fragment 0.9 kbp in size lacking a promoter and an ARS element transformed the RG-21 cells with a very low efficiency (about 1 transformant/μg DNA). In this case, however, the relative number of transformants with the wild-type phenotype reached 80%.

Taking into account the fact that riboflavin synthesis and iron uptake in *P. guilliermondii* are subject to coordinate regulation, we analyzed yeast transformants for ferric reductase activity and iron content. As can be seen from the table, about 70% of the recombinant clones differed insignificantly from the parent cells in ferric reductase activity and iron content. Some clones (224, 140, and 143) possessed slightly increased ferric reductase activity and some (193, 197, and 143) showed an increased content of iron in the cells. The generation of *P. guilliermondii* recombinants with the altered regulation of iron metabolism is an indication of nonhomologous recombination. At the same time, these recombinants differed from each other in the characteristics of growth and riboflavin synthesis. This indicates that there is no correlation between the size of the DNA fragment used for transformation and the phenotype of the transformants with respect to iron uptake and ferric reductase activity. The absence of transformants with noticeably altered iron uptake is in agreement with the earlier finding that the yeast *P. guilliermondii* possesses not only coordinated regulation of flavin synthesis and iron uptake with the involvement of the *RIB*80, *RIB*81, and *HIT*1 genes but also some mechanisms protecting this yeast from the excessive accumulation of iron [3, 19].

Boretskii *et al.* [17] showed that the introduction of circular integrative plasmids into the mutant *P. guilliermondii* cells with blocked riboflavin synthase restores riboflavin synthesis but not the whole wild-type phenotype. At the same time, the data presented above show that as many as 85% of the transformants obtained with the linear fragments of these plasmids have a phenotype close to the wild type. Therefore, like *C. glabrata* and *N. crassa* [15, 16] and higher eukaryotes, the yeast *P. guilliermondii* may possess different mechanisms for the integration of foreign plasmids into its genome.

As mentioned above, only 8 to 40% of the riboflavin-sufficient recombinant clones of the mutant *P. guilliermondii rib1* strain transformed with DNA fragments bearing the structural gene *RIB*1 and the ARS element exhibit the wild-type phenotype. We can assume that these fragments are nonspecifically integrated into the *P. guilliermondii* genome. Therefore, other linear DNA fragments incapable of autonomous replication should be used for the purposeful transformation of this genome. The transformation of the yeast *P. guilliermondii* with autonomously replicating plasmids resembles the transformation of *Candida* yeasts [18] and higher eukaryotes rather than the transformation of the well studied yeast *Saccharomyces cerevisiae.*

REFERENCES

1. Shavlovskii, G.M. and Logvinenko, E.M., Flavin Oversynthesis in Microorganisms and Its Molecular Mechanisms, *Prikl. Biokhim. Mikrobiol.*, 1988, vol. 24, no. 4, pp. 435–447.
2. Shavlovskii, G.M., Fedorovich, D.V., Kutsyaba, V.I., Babyak, L.Ya., and Stenchuk, N.N., Involvement of the *RIB*80 Gene in the Regulation of Flavinogenesis and Iron Uptake in *Pichia guilliermondii, Genetika*, 1992, vol. 28, no. 9, pp. 25–32.
3. Shavlovskii, G.M., Fedorovich, D.V., and Babyak, L.Ya., The Effect of the Mutation *rib*81 on Riboflavin Synthesis and Iron Uptake in the Yeast *Pichia guilliermondii, Mikrobiologiya*, 1993, vol. 62, no. 5, pp. 897–904.
4. Fedorovich, D., Protchenko, O., 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. Boretsky, Yu., Voronovsky, A., Liauta-Tehlivets, O., Hasslacher, M., Kohlwain, S., and Shavlovsky, G.M., Identification of an ARS Element and Development of a High-Efficiency Transformation System for *Pichia guilliermondii, Curr. Genet.*, 1999, vol. 36, no. 4, pp. 215–221.
6. Baker, R.E., Fitzgerald-Hayes, M., and O'Brien, T.C., Purification of the Yeast Centromere Binding Protein CP1 and a Mutational Analysis of Its Binding Site, *J. Biol. Chem.*, 1989, vol. 264, no. 18, pp. 10843–10850.
7. Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R.D., Iron-regulated DNA Binding by the AFT1 Protein Controls the Iron Regulon in Yeast, *EMBO J.*, 1996, vol. 15, no. 13, pp. 3377–3384.
8. Shavlovskii, G.M., Sibirnyi, A.A., Kshanovskaya, B.V., Koltun, L.V., and Logvinenko, E.M., Genetic Classification of the Riboflavin-Dependent *Pichia guilliermondii* Mutants, *Genetika*, 1979, vol. 15, no. 9, pp. 1561–1588.
9. Shavlovskii, G.M., Zharova, V.P., Shchelokova, I.F., Trach, V.M., Sibirnyi, A.A., and Ksheminskaya, G.P., Flavinogenic Activity of Natural *Pichia guilliermondii* Strains, *Prikl. Biokhim. Mikrobiol.*, 1978, vol. 14, no. 2, pp. 184–189.
10. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Lab., 1982. Translated under the title *Molekulyarnoe klonirovanie*, Moscow: Mir, 1984.
11. Liauta-Teglivets, O., Hasslacher, M., Boretskii, Yu.R., Kohlwein, S.D., and Shavlovskii, G.M., Molecular Cloning of the GTP-Cyclohydrolase Structural Gene *RIB*1 of *Pichia guilliermondii* Involved in Riboflavin Biosynthesis, *Yeast*, 1995, vol. 11, no. 2, pp. 945–952.
12. Kovalev, L.M., Krulikovskaya, L.I., Yakova, V.M., Yashina, O.T., Analysis of Iron in Saccharomycetes with Dipyridyl, *Nauchn. Dokl. Vyssh. Shk.*, 1984, vol. 12, pp. 96–100.

13. Lesuisse, E., Raguzzi, F., Crichton, R.R., Iron Uptake by the Yeast *Saccharomyces cerevisiae*: Involvement of a Reduction Step, *J. Gen. Microbiol.*, 1987, vol. 133, no. 11, pp. 3229–3236.

14. Babyak, L., Bacher, A., Boretsky, Y., Demchyshyn, V., Eberhardt, S., Fedorovych, D., Krieger, C., Luetgen, H., Richter, G., and Van Loon, Overproduction of Riboflavin in Yeast, *Eur. Pat. Appl.*, 1998, no. 98107380, ref. 20071.

15. Case, M.E., Genetic and Molecular Analyses of qa-2 Transformants in *Neurospora crassa*, *Genetics*, 1986, vol. 113, no. 3, pp. 569–587.

16. Cormack, B.P. and Falkow, S., Efficient Homologous and Illegitimate Recombination in the Opportunistic Yeast Pathogen *Candida glabrata*, *Genetics*, 1999, vol. 151, no. 3, pp. 979–987.

17. Boretskii, Yu.R., Petrishin, A.V., Kriger, K., Rikhter, G., Fedorovich, D.V., and Bakher, A., Cloning and Expression of the Riboflavin Synthase Gene of *Pichia guilliermondii*, *Tsitol. Genet.*, 2002 (in press).

18. Kurtz, M.B., Cortelyou, M.W., Miller, S.M., Lai, M., and Kirsch, D.R., Development of Autonomously Replicating Plasmids for *Candida albicans*, *Mol. Cell. Biol.*, 1987, vol. 7, no. 1, pp. 209–217.

19. Fedorovich, D.V., Romanyuk, T.M., Protchenko, O.V., and Gudz', S.P., Synergistic Effect of *rib80* and *hit* Mutations on Riboflavin Biosynthesis and Iron Transport in the Yeast *Pichia guilliermondii*, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 180–184.