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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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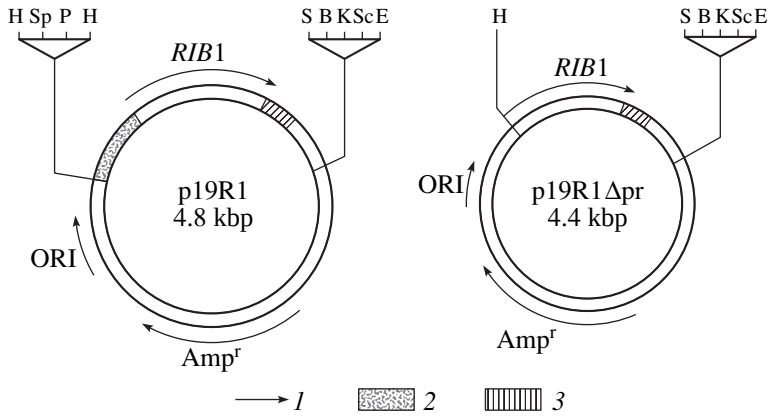

Fig. 1. Recombinant plasmids p19R1 and p19R1Δpr used for the transformation of *P. guilliermondii*. Shown are the following restriction sites: H, *Hin*dIII; Sp, *Sph*I; P, *Pst*I; S, *Sal*GI; B, *Bam*HI; K, *Kpn*I; Sc, *Sac*I; and E, *Eco*RI. Other designations: *1*, the structural gene *RIB*1 of GTP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

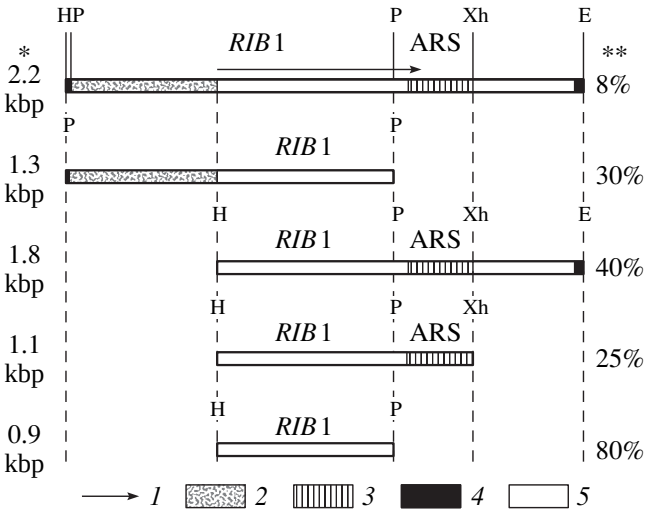

Fig. 2. DNA fragments used for the transformation of the mutant *P. guilliermondii rib1* strain. DNA fragments 2.2 and 1.3 kbp in size were obtained by digesting plasmid p19R1 with the restriction endonucleases *Hin*dIII + *Eco*RI and *Pst*I, respectively. DNA fragments 1.8, 1.2, and 0.9 kbp in size 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 (**).