YEAST VOL. 11: 945–952 (1995)

Molecular Cloning of the GTP-Cyclohydrolase Structural Gene *RIB1* of *Pichia guilliermondii* Involved in Riboflavin Biosynthesis

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Received 24 March 1995; accepted 24 April 1995

The structural gene of GTP-cyclohydrolase, involved in riboflavin biosynthesis, was cloned from a *Pichia guilliermondii* genomic library. A 1855 bp genomic DNA fragment complementing the riboflavin auxotrophies of an *Escherichia coli ribA* mutant, defective in GTP-cyclohydrolase II, and a *P. guilliermondii rib1* mutant was isolated and sequenced. An open reading frame with the potential to encode a protein of 344 amino acids with a predicted molecular mass of 38 711 Da was detected. The *P. guilliermondii* enzyme shows a high degree of homology to GTP-cyclohydrolases type II from *E. coli* and *Baccillus subtilis* and to GTP-cyclohydrolase from *Saccharomyces cerevisiae*. Functional GTP-cyclohydrolase from *P. guilliermondii* may consist of four identical subunits. The sequence of the *RIB1* gene of *P. guilliermondii* was submitted to the EMBL sequence database and is accessible under Accession Number Z49093.

KEY WORDS — yeast; riboflavin; GTP-cyclohydrolase; DNA sequence

INTRODUCTION

Biochemical aspects of riboflavin biosynthesis have been studied in some detail in yeasts and bacteria (for reviews see: Shavlovskii and Logvinenko, 1988a; Brown and Williamson, 1987). In the initial reaction, GTP-cyclohydrolases (GCH) convert GTP into the pyrimidine precursor of biopterin, tetrahydrofolate (GCH type I) and riboflavin (GCH type II), 2,5-diamino-4-hydroxy-6-ribosylamino-pyramidine-5′-phosphate (Figure 1). In yeast, subsequent steps include in a reductive reaction the formation of 2,5-diamino-4-hydroxy-6-ribitylaminopyrimidine-5′-phosphate (*RIB2*), deamination to 2,4-dihydroxy-5-amino-6-ribitlaminopyrimidine-5′-phosphate (*RIB3*) and dephosphorylation to 2,4-dihydroxy-5-amino-6-ribitylamino-pyrimidine. Another precursor of riboflavin, 3,4-dihydroxy-2-butanone-4-phosphate, is synthesized from ribulose-5-phosphate (*RIB6*). This intermediate reacts with 2,4-dihydroxy-5-amino-6-ribitlaminopyrimidine under the formation of 6,7-dimethyl-8-ribityllumazine (*RIB5*). Ultimately, riboflavin synthase (*RIB7*) catalyses the formation of one molecule of riboflavin and one molecule of 2,4-dihydroxy-5-amino-6-ribitylaminopyrimidine from two molecules of 6,7-dimethyl-8-ribityllumazine.

GTP-cyclohydrolase (*RIB1*) catalyses the initial and rate-limiting step of riboflavin synthesis and is subject to multiple metabolic controls. Properties of GTP-cyclohydrolases II of *Escherichia coli* and *Bacillus subtilis* were thoroughly studied and structural genes coding for these proteins were cloned and sequenced (Foor and Brown, 1975; Mironov *et al.*, 1989; Boretskii *et al.*, 1991, 1992; Richter *et al.*, 1993). The yeast *Pichia guilliermondii* is of great industrial interest because of its high capacity to synthesize riboflavin.

In *P. guilliermondii*, GTP-cyclohydrolase synthesis is repressed by iron and it is transcriptionally regulated by interaction of negative (RIB80, RIB81) and positive (RIB83, RIB84) regulatory

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CCC 0749-503X/95/100945-08

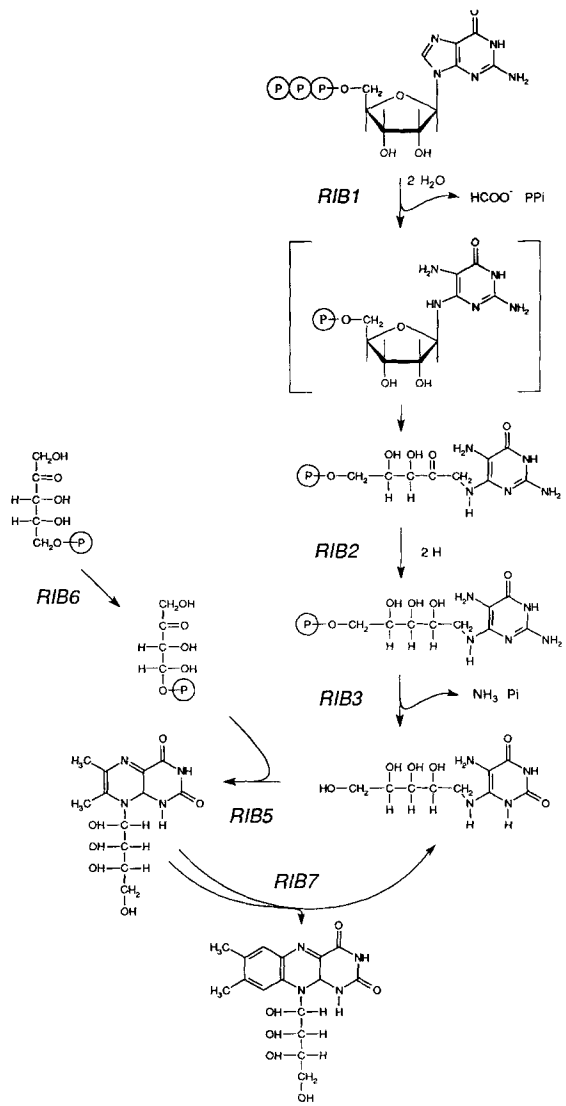


Figure 1. Riboflavin biosynthesis in *P. guilliermondii*. RIB1: GTP-cyclohydrolase; RIB2: 2,5-diamino-4-hydroxy-6-ribosylamino-pyrimidine-5′-phosphate reductase; RIB3: 2,5-diamino-4-hydroxy-6-ribitylamino-pyrimidine-5′-phosphate deaminase; RIB6: 3,4-dihydroxy-2-butanone-4-phosphate synthase; RIB5: 6,7-dimethyl-8-ribityllumazine synthase; RIB7: riboflavin synthase.

factors (Shavlovskii and Logvinenko, 1988a, b). GTP-cyclohydrolase activity is inhibited by FAD and adenine-containing nucleotides (Shavlovskii *et al.*, 1978). Recently, we have cloned the structural gene encoding GTP-cyclohydrolase of *P. guilliermondii* by functional complementation of a *ribA* mutation in *E. coli* (Zakal'skii *et al.*, 1990). The *RIB1* gene of *P. guilliermondii* codes for a predicted protein of 38 kDa, suggesting that the functional 160 kDa GTP-cyclohydrolase consists of four identical subunits. The deduced protein sequence presented here shows significant homologies to GTP-cyclohydrolase from the yeast *Saccharomyces cerevisiae* and in their C-terminal half to the enzyme of *E. coli*. Sequence homologies were also identified to proteins from *B. subtilis* and *Azospirillum brasilense* suggesting close evolutionary relationships in this crucial biosynthetic pathway.

MATERIALS AND METHODS

Strains and culture conditions

P. guilliermondii wild-type strain L2 (*mat*$^-$, *hisx*) and riboflavin-deficient mutant rib1-21 with a defective GTP-cyclohydrolase were used (Zakal'skii *et al.*, 1990). *E. coli* strains XL1 blue and K802 were used to propagate plasmids, and riboflavin-deficient ribA802-81 (Tesliar and Shavlovskii, 1983) and BSV-821 (Boretskii *et al.*, 1991) with a block of GTP-cyclohydrolase II harboring various *RIB1* gene fragments of *P. guilliermondii* were used for functional complementation analysis. Standard LB and YPD media (1% yeast extract, 1% Bacto peptone and 2% glucose) were used to propagate bacteria and yeast cells, respectively (Tesliar and Shavlovskii, 1983). Transformed *E. coli* strains were maintained on media plates containing 100 mg/l ampicillin. Riboflavin-deficient strains of bacteria and yeasts were grown on media containing 50 mg/l and 200 mg/l riboflavin, respectively. Iron was removed from the medium using the 8-hydroxyquinoline method (Cowart *et al.*, 1980), where indicated. For the analysis of flavinogenesis, yeasts were grown in 10 ml minimal medium (Shavlovskii *et al.*, 1980) with great aeration. GTP-cyclohydrolase activity was analysed in cells grown in 200 ml minimal medium for 48–96 h. *P. guilliermondii* cells were cultivated at 29°C.

Determination of GTP-cyclohydrolase activity

Cells of *E. coli* ribA-81 transformed with plasmid pR1 and derivatives (Figure 2) were grown at 28°C on LB medium containing ampicillin. Cell extracts were prepared by ultrasonic treatment (Zakal'skii *et al.*, 1990). *P. guilliermondii* mutant rib1-21 transformed with plasmid pR1 and

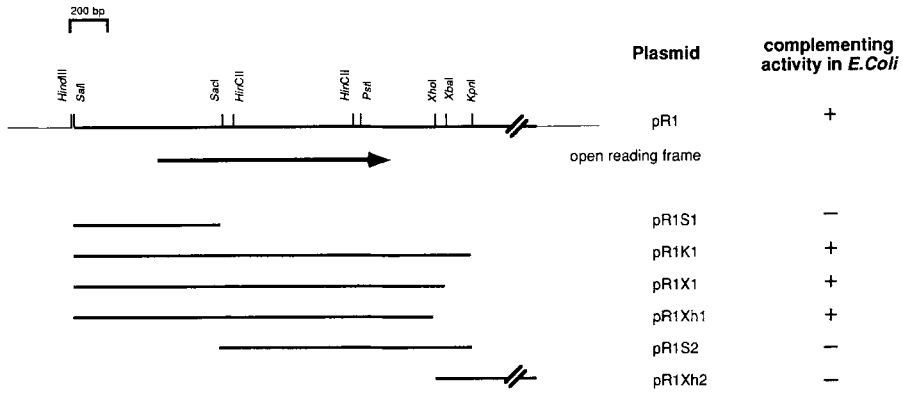


Figure 2. Clones used in this study. Plasmid pR1 harboring a 2·5 kbp genomic DNA fragment from *P. guilliermondii* was isolated by complementation of an *E. coli* ribA mutation (Zakal'skii *et al.*, 1990).

derivatives was grown on selective media, harvested by centrifugation and homogenized with glass beads. After a low-speed clarification, GTP-cyclohydrolase activity was determined in *E. coli* and yeast homogenates by a fluorometric method as described (Zakal'skii *et al.*, 1990). Protein concentration was determined by the Lowry method (Lowry *et al.*, 1956).

Miscellaneous procedures

DNA was transformed into *E. coli* using the procedure described by Cohen (Maniatis *et al.*, 1984). Isolation, restriction, ligation and amplification of plasmid DNA and agarose gel electrophoresis were carried out as described by Maniatis (Maniatis *et al.*, 1984). Competent cells of *P. guilliermondii* were obtained by the lithium acetate method (Ito *et al.*, 1983). Sequencing was performed by the chain-termination method of Sanger (Sanger *et al.*, 1977) using the DyeDeoxy Terminater Cycle Sequencing Kit (Applied Biosystems Inc.) and an automated DNA Sequencer 373A (Applied Biosystems Inc). Sequences were edited and analysed using the GCG program package (Genetics Computer Group, 1994) and compared to sequences contained in GenBank release 78.0, and releases since then, using electronic mail implementations of the Blasta and Blastn programs (Altschul *et al.*, 1990) provided by the National Institute of Biotechnology Information. Sequence alignments were computed using the Multiple Alignment Construction and Analysis Workbench (Schuler *et al.*, 1991).

RESULTS

We have previously isolated a 2·5 kb genomic fragment of *P. guilliermondii* which complemented the riboflavin auxotrophy of an *E. coli* ribA mutant that is defective in GTP-cyclohydrolase II. The gene contained in this fragment also complemented the riboflavin auxotrophy of *P. guilliermondii* rib1 mutants, lacking GTP-cyclohydrolase activity (Zakal'skii *et al.*, 1990). We now present evidence that the cloned gene codes for *P. guilliermondii* GTP-cyclohydrolase, the first committed step in the riboflavin biosynthetic pathway.

Subcloning of the fragment in *E. coli* ribA-81 mutants resulted in a minimal fragment 1·68 kb in length that allowed the cells to grow in the absence of riboflavin in the medium. The same fragment contained in plasmid pR1X1 also complemented the riboflavin deficiency of the GTP-cyclohydrolase-defective *E. coli* strain BSV-821. GTP-cyclohydrolase specific activity was $4{\cdot}95 \times 10^{-5}$ U/mg protein in extracts of *E. coli* ribA-81/pR1X1 transformants and $1{\cdot}8 \times 10^{-5}$ U/mg in extracts of *E. coli* wild-type K802. This result indicates efficient expression of the heterologous *RIB1* gene in *E. coli* and a gene dosage effect by expressing it from a high copy number plasmid.

Plasmid pR1X1 was transformed into the riboflavin-deficient rib1-21 mutant of *P. guilliermondii* and transformants that had the plasmid integrated into their genome were selected for riboflavin prototrophy. Frequency of transformation was about 150 transformants/μg plasmid DNA, using the LiOAc transformation procedure.

Table 1. Flavinogenesis and GTP-cyclohydrolase (GTP-CH) specific activity in *P. guilliermondii* wild-strain L2 and two independent rib1-21 mutants transformed with complementing plasmid pR1X1, harboring the *RIB1* gene (strains 2 and 4).

Strain	Iron conc. (μg/ml)	Cell density (mg/ml)	Riboflavin (μg/ml)	Riboflavin productivity (mg/g cells)	GTP-CH activity (10^{-5} U/mg)
L2	0·01	1·70	7·52	4·42	5·00
	0·20	4·30	0·60	0·14	0·42
2	0·01	2·40	2·88	1·20	1·11
	0·20	4·60	1·76	0·38	0·35
4	0·01	2·30	2·72	1·18	0·83
	0·20	6·00	1·44	0·24	0·35

In Table 1, flavin production in response to the supplementation with iron, as well as GTP-cyclohydrolase specific activities of wild-type strain L2 and two independent transformants are summarized. In *P. guilliermondii* rib1-21/pR1X1 transformants grown in a medium with low iron concentration, we observed sufficient production of riboflavin to support wild-type-like growth; however, the total amount of flavins synthesized was lower than in the wild strain L2 (Table 1). Under conditions of high concentrations of iron in the medium, transformed mutant strains accumulated higher amounts of flavin compared to wild type. This increase, however, was not due to an increased GTP-cyclohydrolase activity under these growth conditions. In the presence of low iron in the medium, GTP-cyclohydrolase specific activity was four to six times lower in transformed strains than in wild-type cells. This result indicates that regulation of flavinogenesis by iron is obscured to some extent in the transformants, suggesting that regulatory elements required for full derepression of *RIB1* expression are lacking in plasmid pR1X1.

The 1·9 kbp insert of plasmid pR1K1 harboring the complementing gene was sequenced. An open reading frame (ORF) with the potential to encode a protein of 344 amino acid residues, with a predicted molecular mass of 38 711 Da was contained in this fragment. DNA and deduced protein sequences are depicted in Figure 3.

The 3′ end of another potential reading frame that had no homologies to any protein in the current databases is located 279 bp upstream of the putative translation start site of the *RIB1* gene, leaving a rather small regulatory region for the GTP-cyclohydrolase structural gene. Two TATAT sequences that could resemble TATA-boxes

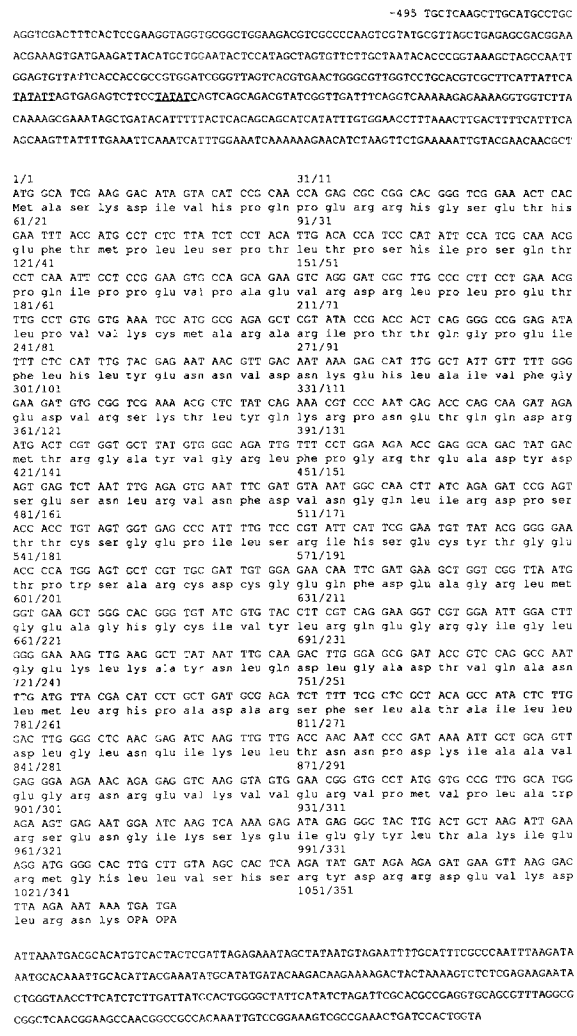


Figure 3. DNA sequence and deduced amino acid sequence of the *P. guilliermondii RIB1* gene. Putative TATA-boxes are underlined.

(Struhl, 1989) are located 237 bp and 216 bp upstream.

The molecular mass of GTP-cyclohydrolase of *P. guilliermondii* as determined by gel filtration is 160 kDa (Shavlovskii *et al.*, 1976), suggesting that the enzyme consists of four identical subunits. A tetrameric complex with the same molecular weight was also identified in extracts from *E. coli* expressing the heterologous protein (Zakal'skii *et al.*, 1990), suggesting that the polypeptide contains structures which promote formation of an active tetramer even under heterologous conditions. In contrast, GTP-cyclohydrolase of *E. coli* functions as a homodimer with each subunit 196 amino acid residues in length (Richter *et al.*, 1993).

The *RIB1* protein sequence from *P. guilliermondii* shows a remarkable degree of homology to a putative GTP-cyclohydrolase from *S. cerevisiae* (Doignon *et al.*, 1993; Baur *et al.*, 1993), with 60% identical amino acids and more than 70% overall homology over the entire length of the protein. The C-terminal half of the *P. guilliermondii* enzyme is also highly homologous to GTP-cyclohydrolases II from *Photobacteria* (Lee and Meighen, 1992; Lee *et al.*, 1994) and *E. coli* (Richter *et al.*, 1993).

Fifty-five amino acids are identical between amino acid residues 167 and 296 of the *P. guilliermondii* enzyme and parts of the predicted proteins from *E. coli* and *B. subtilis* (Figure 4a). Furthermore, a highly conserved region is present between amino acid residues 314 and 326, suggesting the location of functional domains in this area. However, the highest similarity between the two yeast species is observed between amino acids 102 and 162 in *P. guilliermondii* and 71 and 133 in *S. cerevisiae*, respectively, a region which is not contained in any of the bacterial sequences (Figure 4a).

In Figure 4b, alignments of bacterial and yeast GCH and RIB6/ribb enzymes are presented. Interestingly, *gch2* from *Bacillus subtilis* appears to encode a bifunctional enzyme, with C-terminal region homologous to *E. coli, S. cerevisiae* and *P. guilliermondii* GTP-cyclohydrolase proteins and the *N*-terminal region catalysing the RIB6/ribb (*E. coli, Photobacterium phosphoreum*) equivalent reaction in flavin biogenesis (Figure 4b).

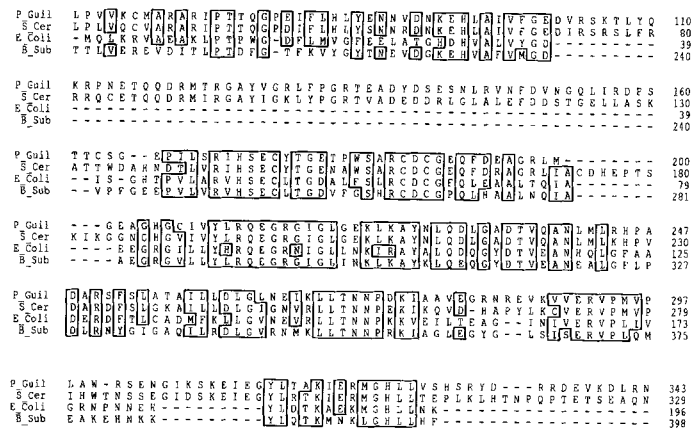
DISCUSSION

P. guilliermondii is an industrial yeast of great interest for the production of flavins. In order to understand the mechanisms that control flavinogenesis in that yeast, we have isolated mutants defective in flavin biosynthesis (Zakal'skii *et al.*, 1990). Here we describe the molecular characterization and identification of *RIB1* gene of *P. guilliermondii*, encoding GTP-cyclohydrolase, the initial step in riboflavin biosynthesis. The predicted enzyme consists of 344 amino acids and is more than 70% homologous to the *RIB1* protein sequence derived from ORF YBL0417 of chromosome II of *S. cerevisiae* (Doignon *et al.*, 1993; Baur *et al.*, 1993). Whereas no homologies were observed in the *N*-terminal region of about 110 amino acids, the rest of the protein is characterized by a high degree of homology to GTP-cyclohydrolases of a number of bacteria, including *E. coli* (Richter *et al.*, 1993), *B. subtilis* (Boretskii *et al.*, 1991), *A. brasilense* (E. Van Bastelaere, V. Keijers and J. Vanderleyden unpublished; GenBank accession number U09869) and *P. phosphoreum* (Lee *et al.*, 1994). GTP-cyclohydrolase from *P. guilliermondii* reveals no obvious homologies to GTP-cyclohydrolases type I, even though they catalyse similar reactions (Katzenmeier *et al.*, 1991; Micka *et al.*, 1991; Gutlich *et al.*, 1992; Nomura *et al.*, 1993; Hatakeyama *et al.*, 1991). By comparing the published sequences of GTP-cyclohydrolases type II we observed interesting differences in the molecular organizations of these enzymes in different organisms.

The functional domain of the *P. guilliermondii* enzyme appears to be located in the region between amino acids 167 and 326, based on multiple sequence alignment to homologous enzymes. *B. subtilis* GTP-cyclohydrolase consists of 398 amino acid residues; however, deletion of the *N*-terminal 128 amino acids does not affect GTP-cyclohydrolase activity (Boretskii *et al.*, 1991), further supporting this notion. Recently it was found that the *N*-terminal sequence of the *B. subtilis* enzyme is very similar to an unidentified protein of *Vibrio harveyi* and to the enzyme catalysing the formation of 3,4-dihydroxy-2-butanone-4-phosphate (Figure 1) in *E. coli* (Richter *et al.*, 1992; Swartzman *et al.*, 1990). This observation suggests that the *B. subtilis* enzyme is bifunctional and may contain both GTP-cyclohydrolase (equivalent RIB1; Boretskii *et al.*, 1992) and 3,4,-dihydroxy-2-butanone-4-phosphate synthase (equivalent RIB6/ribb; Richter *et al.*, 1993) activities.

Sequence alignment of three well-studied GTP-cyclohydrolases reveals a rather conservative

(a)



(b)

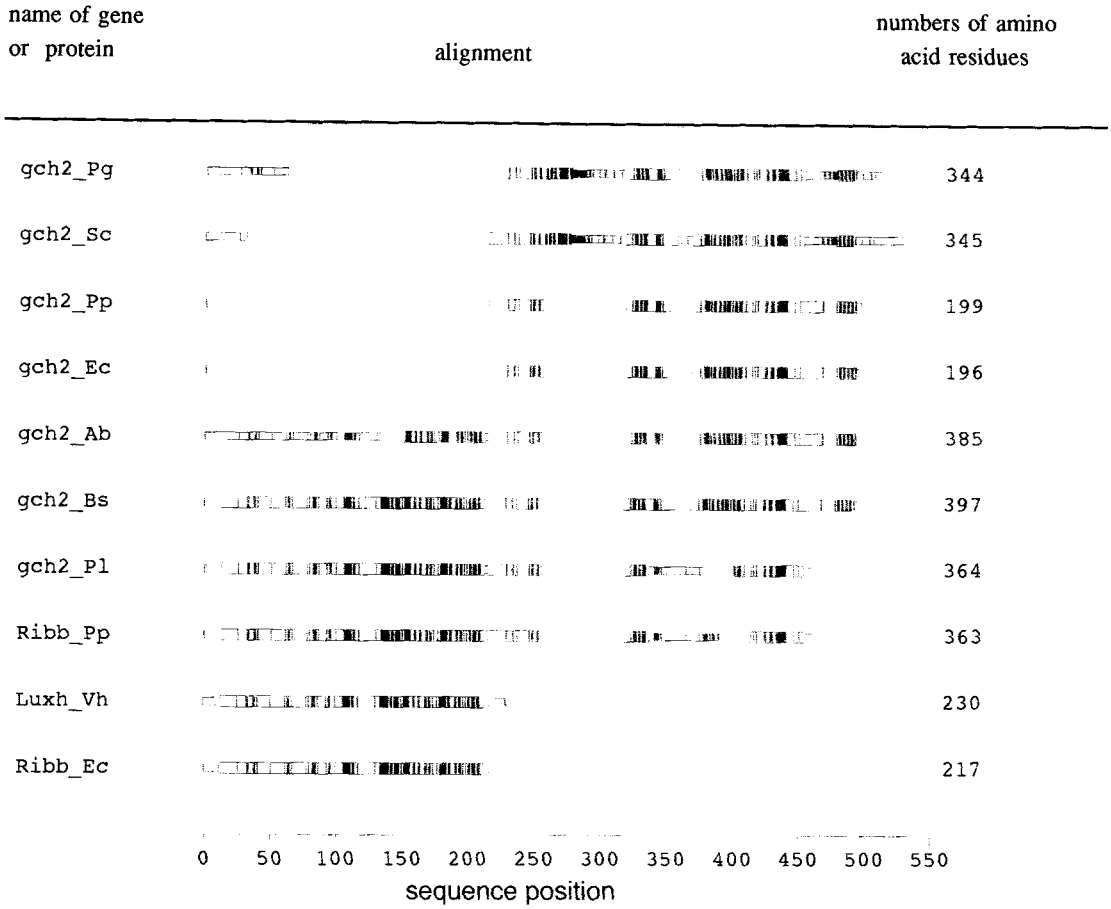


Figure 4. Alignment of various GTP-cyclohydrolases and homologous proteins from yeast and bacterial sources. (a) Representation of sequences of highest homology. Note that the peptide sequence unique to the yeast GTP-cyclohydrolases shares about 50% identity. (b) Alignment using the Multiple Alignment Construction and Analysis Workbench (Schuler *et al.*, 1991). GCH from *Bacillus subtilis* appears to be a bifunctional enzyme, harboring GCH activity "RIB1" in its C-terminal and ribb/RIB6 activity in its N-terminal region. According to these structural similarities, gch2 from *Photobacterium leiognathi* and luxh from *Vibrio harveyi* may rather be ribb/RIB6 analoques. (Pg, *Pichia guilliermondii*; Sc, *Saccharomyces cerevisiae*; Pp, *Photobacterium phosphoreum; Ec, Escherichia coli*; Ab, *Azospirillum brasilense*; Bs, *Bacillus subtilis*; Pl, *Photobacterium leiognathi*; Vh, *Vibrio harveyi*).

arrangement of homologous positions in the alignment region 360–430 (for amino acid numbering in the sequence alignments see Figure 4b). This arrangement of homologous positions is also typical for GTP-cyclohydrolases from *P. phosphoreum* and *A. brasilense*, which were shown to be GTP-cyclohydrolases by functional complementation of an *E. coli* ribA mutant (Lee and Meighen, 1992; Lee *et al.*, 1994). It can be speculated that the region 360–430 may contain the active site of GTP-cyclohydrolases. In contrast, ORF III of the lux operon of *P. leiognathi*, which has been described as a GTP-cyclohydrolase based on its homology to the protein from *B. subtilis*, lacks a significant part of the conserved domain, suggesting that this protein is rather a 3,4-dihydroxy-2-butanone-4-phosphate synthase.

It is well established that flavinogenesis of *P. guilliermondii*, but not of *S. cerevisiae*, is obscured by iron ions (Shavlovskii and Logvinenko, 1988a). Comparison and molecular analysis of regulatory sequences upstream of the respective structural genes should further help to clarify regulation of flavinogenesis in response to iron.

ACKNOWLEDGEMENT

We wish to acknowledge the financial support by the Österreichische Akademische Austauschdienst to O.L-T.

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