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Biosynthesis of Riboflavin: Cloning, Sequencing, Mapping, and Expression of the Gene Coding for GTP Cyclohydrolase II in *Escherichia coli*

GERALD RICHTER,[1] HARALD RITZ,[1] GERD KATZENMEIER,[1] RAINER VOLK,[1] ANGELIKA KOHNLE,[1] FRIEDRICH LOTTSPEICH,[2] DOROTHEE ALLENDORF,[1] AND ADELBERT BACHER[1]*

Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-8046 Garching,[1] and Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried,[2] Germany

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GTP cyclohydrolase II catalyzes the first committed step in the biosynthesis of riboflavin. The gene coding for this enzyme in *Escherichia coli* has been cloned by marker rescue. Sequencing indicated an open reading frame of 588 bp coding for a 21.8-kDa peptide of 196 amino acids. The gene was mapped to a position at 28.2 min on the *E. coli* chromosome and is identical with *ribA*. GTP cyclohydrolase II was overexpressed in a recombinant strain carrying a plasmid with the cloned gene. The enzyme was purified to homogeneity from the recombinant strain. The N-terminal sequence determined by Edman degradation was identical to the predicted sequence. The sequence is homologous to the 3′ part of the central open reading frame in the riboflavin operon of *Bacillus subtilis*.

Early studies showed that the biosynthesis of riboflavin starts from a purine precursor (for a review, see reference 1). More recently, GTP (compound 1 [Fig. 1]) was identified as the committed precursor (7). An enzyme catalyzing the release of formate and PP_i under formation of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (compound 2) from GTP (Fig. 1) was purified from cell extracts of *Escherichia coli* and was designated GTP cyclohydrolase II (EC 3.5.4.20) (7).

This article reports the cloning of the gene coding for GTP cyclohydrolase II of *E. coli* and the purification of the enzyme to apparent homogeneity from a recombinant strain.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* strains and plasmids used in this study are shown in Table 1.

Materials. Restriction enzymes and T4 DNA ligase were from Bethesda Research Laboratories, the T7 sequencing kit was from Pharmacia, and ^{35}S-Sequetide was from DuPont/NEN. Standard sequencing primers were purchased from Boehringer Mannheim. Synthetic oligonucleotides were synthesized with a Gene Assembler (Pharmacia). The enhanced chemiluminescence direct nucleic acid labelling and detection system was from Amersham, and Zeta Probe nylon membranes were obtained from Bio-Rad.

Isolation of riboflavin-deficient mutants. Cells of *E. coli* RR28 were incubated with ethyl methane sulfonate according to the protocol of Miller (16). Surviving cells were grown on M9 minimal plates containing 400 μg of riboflavin per ml. The phenotype was checked by replica plating on M9 minimal plates without riboflavin.

Library construction and screening. DNA was isolated from *E. coli* RR28 by the procedure of Godson and Vapnek (8) and cleaved with *Eco*RI. The fragments were inserted into the *Eco*RI cloning site of plasmid pUC13. For amplification, the recombinant DNA was transformed into *E. coli* RR28 cells. Plasmid DNA was isolated from 10^4 ampicillin-resistant colonies according to the Triton-lysozyme method of Davis et al. (6). The library was screened by published procedures (23) using the riboflavin-deficient *E. coli* mutant Rib7.

Construction of subclones. Plasmid p7-2 was digested with endonuclease *Ava*I. The band corresponding to a 4.4-kb fragment was excised from an agarose gel, purified with Geneclean (Bio 101), and ligated. *E. coli* Rib7 cells were transformed with the resulting plasmid p7-21. In an analogous experiment, plasmid p7-21 was digested with *Pst*I. The resulting 3.3-kb fragment was ligated to give plasmid p7-23, which was transformed into *E. coli* RR28.

DNA sequence analysis. Plasmid DNA was isolated by using Nucleobond AX columns from Macherey & Nagel (Düren, Germany) according to the manufacturer's instructions or by the method of Holmes and Quigley (11). DNA sequencing was performed with the T7-sequencing kit, ^{35}S-Sequetide labelling mix, and pUC sequencing or reverse sequencing primers or synthetic oligonucleotides. The products after dideoxy sequencing were separated by acrylamide gel electrophoresis. The gels were subsequently dried and autoradiographed.

Mapping. Genomic DNA was digested with the restriction nucleases used by Kohara and coworkers (13). Southern blotting was performed with the enhanced chemiluminescence direct nucleic acid labelling and detection system and Zeta Probe nylon membranes. The 1.7-kb insert of plasmid p7-21 was used as a probe.

Enzyme assay. Assay mixtures contained 200 mM Tris phosphate (pH 8.7), 10 mM GTP, 10 mM $MgCl_2$, and 20 mM dithiothreitol in a total volume of 100 μl. They were incubated at 37°C for 30 min. A solution of 11.4 mM diacetyl (100 μl) was added, and the mixture was incubated at 95°C for 30 min. The diacetyl treatment converted the enzyme product to 6,7-dimethylpterin, which was subsequently measured by reversed-phase high-performance liquid chromatography

* Corresponding author.

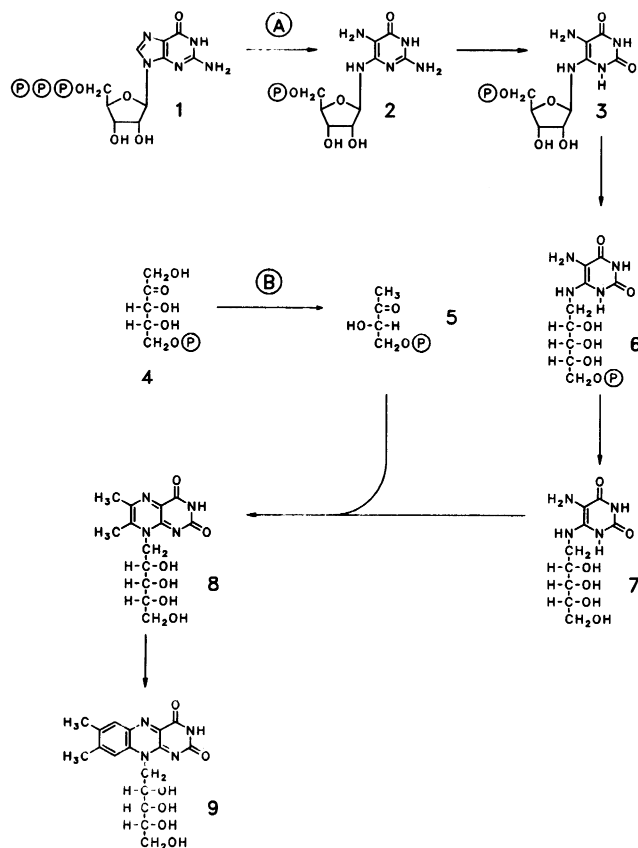

FIG. 1. Biosynthesis of riboflavin (bacterial pathway). (A) GTP cyclohydrolase II; (B) 3,4-dihydroxy-2-butanone 4-phosphate synthase.

(HPLC) on a column of Lichrosorb RP18 (4 by 250 mm). The eluent contained 100 mM ammonium formate and 40% methanol. The effluent was monitored fluorometrically (excitation, 365 nm; emission, 435 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate per h at 37°C.

Protein purification. Mutant strain Rib7 harboring plasmid p7-21 was grown in shaking culture (Luria-Bertani medium containing 150 mg of ampicillin per liter) at 37°C for 16 h.

Frozen bacterial cells (31 g) were thawed in 62 ml of 200 mM Tris hydrochloride, pH 8, containing 2 mM phenylmethylsulfonyl fluoride. Lysozyme (31 mg) and DNase I (0.3 mg) were added, and the mixture was stirred at 37°C for 20 min. The suspension was centrifuged in an SS 34 rotor (Sorvall) at 15,000 rpm at 4°C for 20 min.

Ammonium sulfate (14.8 g) was added to the supernatant (84 ml). The suspension was stirred for 30 min at 4°C. It was centrifuged (Sorvall SS 34 rotor; 15,000 rpm, 20 min), and the precipitate was discarded. Ammonium sulfate (17.0 g) was added to the supernatant (87 ml). The suspension was stirred for 1 h at 4°C. The precipitate was harvested by centrifugation and dissolved in 25 mM Tris hydrochloride, pH 8. The solution was dialyzed three times against 2 liters of 25 mM Tris hydrochloride, pH 8, containing 0.2 mM EDTA, 0.5 mM $MgCl_2$, and 150 mM NaCl.

The dialyzed solution was applied to a column of DEAE-cellulose DE 52 (2.5 by 21 cm) which had been equilibrated with the dialysis buffer. The flow rate was 30 ml/h. The column was developed with 300 ml of equilibration buffer followed by 25 mM Tris-HCl, pH 8, containing 0.2 mM EDTA, 0.5 mM $MgCl_2$, and 200 mM NaCl. Fractions (440 to 570 ml each) containing GTP cyclohydrolase II were collected and dialyzed against 2 liters of 25 mM Tris hydrochloride, pH 8.

The dialyzed protein solution was applied to a column of hydroxyapatite (Bio-Rad; 2.2 by 3.5 cm) which had been equilibrated with the dialysis buffer. The column was developed with 150 ml of dialysis buffer followed by 25 mM Tris hydrochloride, pH 8, containing 10 mM potassium phosphate (flow rate, 32 ml/h). Fractions containing enzyme activity were collected (280 to 320 ml) and concentrated to a volume of 5 ml by ultrafiltration (PM10 membrane; Amicon).

The protein solution was applied to a Sephacryl S-200 column (2.2 by 90 cm) equilibrated with 25 mM Tris hydrochloride, pH 8, containing 2.5 mM EDTA, 5 mM $MgCl_2$, and 100 mM NaCl. The column was developed with the same buffer (flow rate, 12 ml/h). Fractions were combined (135 to 186 ml) and concentrated by ultrafiltration.

Molecular weight estimation. GTP cyclohydrolase II (10 μg) was applied to a Superdex 75 column. The column was developed with the buffer used for Sephacryl S-200 chromatography. The flow rate was 30 ml/h. The apparent molecular weight of the native enzyme was estimated by cochromatography with a mixture of standard proteins (bovine serum albumin [66 kDa], ovalbumin [45 kDa], and lysozyme [14.5 kDa]).

Protein sequencing. The N-terminal protein sequence of the purified protein was determined by automated Edman degradation as described earlier (24).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Reference or source
E. coli		
RR28	*thi leu pro lac ara xyl endA recA hsd* r^- m^- *pheS supE44*	10
Rib7	*thi leu pro lac ara xyl endA recA hsd* r^- m^- *pheS supE44 rib*	This study
BSV18	F^- *ribA18*::Tn*5 thi hsdR*	2
Plasmids		
pUC13	High-copy-number plasmid vector	28
p7-2	pUC13 with a 3.0-kb *Eco*RI fragment	This study
p7-21	pUC13 with a 1.7-kb *Eco*RI-*Ava*I fragment	This study
p7-23	pUC13 with a 0.6-kb *Eco*RI-*Pst*I fragment	This study

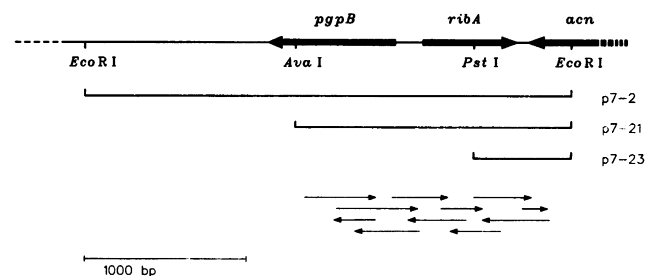

FIG. 2. DNA inserts of plasmids used in this study. The open reading frames coding for GTP cyclohydrolase II are indicated. The sequencing strategy is shown by arrows. The sequenced section enclosed parts of the *pgpB* and *acn* genes, which have been sequenced by Icho (12) and by Prodromou et al. (21).

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CCCATGACTCCCGTTGCCCAGACCAGCAAGATAGCAATGGTTAACGTTCGCCGACGCGGC   60
CACAGCAAACCAACGGCCAGCAGTGCCCAACTGGCAGCAAACATCGTGTGACCGGAAGGA  120
AAGGCAAACCCCGTCTCTTTCTGCCAGTGTGAACGCAAATATTGTGGGATATTTTTCTCT  180
TCAGCCAACTGTTCTTTCACTAGATTTCCGCGTTCTGCTCGCTTTAAAGTGTAGAACTCA  240
TCAACCGGAATATGATGTGTTTTTTCCAGCCAGATAACAAAAGGTCGTGGTTCCTGGACT  300
TTGTCTTTGATCCAGGATTTAACGCCTTGTCCCACAAGGATTGCGGCCGCCAGAATGGCA  360
AATAATACAAAGGCAGCCTTAATGCGAAAACGCAGACACCAGAGAAACCAGCCGAATAAA  420
ATCAAATGTGTAATGACGCCCCAGGGCTGGGTGACAGTTTCAGTAACCCAAAAAGCCGCT  480
TTTAGTAGCCAACTTTGTTCTCCAGGTTGCCAACGCCAGCCAGAAATCCATACGGCTACT  540
GGCATGACAAGCAATAGTGCAGCTCCCACTGCGGTACGTCTGGCAATCGAACGCATGGCC  600
                                                   pgpB <==|
TCTCCTTTTTGATAAGTCCCACAATCATAACTGAAAACGCCAGTTCCAGGAAAAATTGAC  660
AGATTTGTGCCATTCCGTGAACGATCGACGCGTCGTGATTAGGTGAACCCCTTCTCGTTA  720
                                    S.D.        M  Q  L  K  R     5
TGGCAAAATAAGCCAATACAGAACCAGCATTATCTGGAGAATTTCATGCAGCTTAAACGT  780
 V  A  E  A  K  L  P  T  P  W  G  D  F  L  M  V  G  F  E  E     25
GTGGCAGAAGCCAAACTGCCAACCCCATGGGGCGATTTCCTGATGGTGGGATTTGAAGAA  840
 L  A  T  G  H  D  H  V  A  L  V  Y  G  D  I  S  G  H  T  P     45
CTGGCAACCGGACACGATCATGTCGCGCTAGTCTATGGCGATATTTCCGGGCATACCCCG  900
 V  L  A  R  V  H  S  E  C  L  T  G  D  A  L  F  S  L  R  C     65
GTACTTGCGCGCGTCCATTCCGAATGTCTGACCGGTGACGCCCTGTTCAGCTTGCGCTGC  960
 D  C  G  F  Q  L  E  A  A  L  T  Q  I  A  E  E  G  R  G  I     85
GATTGTGGCTTCCAGCTCGAAGCGGCATTGACGCAAATTGCCGAGGAAGGCCGTGGTATT 1020
 L  L  Y  H  R  Q  E  G  R  N  I  G  L  L  N  K  I  R  A  Y    105
TTGCTGTATCACCGTCAGGAAGGTCGTAACATTGGTCTGCTGAATAAAATCCGCGCTTAC 1080
 A  L  Q  D  Q  G  Y  D  T  V  E  A  N  H  Q  L  G  F  A  A    125
GCACTGCAGGATCAAGGTTACGATACCGTAGAGGCTAACCACCAGTTAGGCTTCGCCGCT 1140
 D  E  R  D  F  T  L  C  A  D  M  F  K  L  L  G  V  N  E  V    145
GATGAGCGCGACTTCACTCTTTGCGCTGATATGTTCAAACTCCTTGGCGTCAATGAAGTC 1200
 R  L  L  T  N  N  P  K  K  V  E  I  L  T  E  A  G  I  N  I    165
CGCTTGTTAACCAATAACCCGAAAAAAGTCGAAATTCTGACCGAAGCAGGGATTAATATT 1260
 V  E  R  V  P  L  I  V  G  R  N  P  N  N  E  H  Y  L  D  T    185
GTTGAACGCGTACCATTGATTGTAGGTCGTAACCCCAATAACGAACATTATCTCGATACC 1320
 K  A  E  K  M  G  H  L  L  N  K                               196
AAAGCCGAGAAAATGGGCCATTTGCTGAACAAATAACCCTCTTGCATTGTGTAATTCATT 1380
TGCTTGCCGGAAGCAAAATAACCGGCAAGCAAATAGTTGTTACTTCAACATATTACGAAT 1440
--------->           <--------------         |<== acn
GACATAATGCAAAATGCCGTCGTTCTGGTAGTAGGTCAACTCCGTCGCGGTGTCGATACG 1500
ACAACGGCAGGGTACGACTTCCTGGCTACCATC                            1533
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FIG. 3. Nucleotide sequence of the gene coding for GTP cyclohydrolase II and its flanking regions. The deduced amino acid sequence is shown, and N-terminal amino acids determined by Edman degradation are overlined. The putative ribosome binding site (Shine-Dalgarno [SD] sequence) is double underlined. The genes *pgpB* and *acn* are indicated.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to EMBL and have been assigned accession number X67876.

RESULTS

Cloning. Riboflavin-deficient mutants of *E. coli* were isolated after mutagenesis of *E. coli* RR28 with ethyl methane sulfonate. The specific enzyme defects of these mutants were not determined prior to the cloning experiments.

A gene bank was generated by ligating *Eco*RI-restricted genomic DNA from *E. coli* RR28 into plasmid pUC13. The gene bank was used to transform the mutants, and colonies growing without added riboflavin were selected. Cell extracts of riboflavin-independent transformants were assayed for GTP cyclohydrolase II activity. High enzyme levels in riboflavin-independent transformants of mutant Rib7 were observed. These strains were subsequently shown to contain a plasmid, p7-2, carrying an insert of about 3 kb. Digestion of the plasmid with *Ava*I and ligation produced the plasmid p7-21 (Fig. 2), which retained the ability to complement the riboflavin deficiency of *E. coli* Rib7.

DNA sequence analysis. Plasmid p7-21 contained an insert of 1.7 kb, which was sequenced by a primer walk strategy as summarized in Fig. 2. A total of 1,533 bp on both DNA strands was determined. The DNA segment contains an open reading frame coding for 196 amino acids (Fig. 3).

The predicted amino acid sequence was in line with the result of Edman degradation of isolated GTP cyclohydrolase II (see below). The predicted molecular weight of the polypeptide is 21,836. The open reading frame is preceded by a plausible ribosome binding site (Fig. 3).

Mapping. In order to locate the GTP cyclohydrolase II gene on the physical map of *E. coli* and to determine whether it corresponded to the *ribA* locus, which had previously been mapped to the 28-min region (2, 27), genomic DNA of strain RR28 was digested with the eight restriction endonucleases used by Kohara et al. (13), blotted, and probed with the 1.7-kb insert of plasmid p7-21. The pattern corresponded to

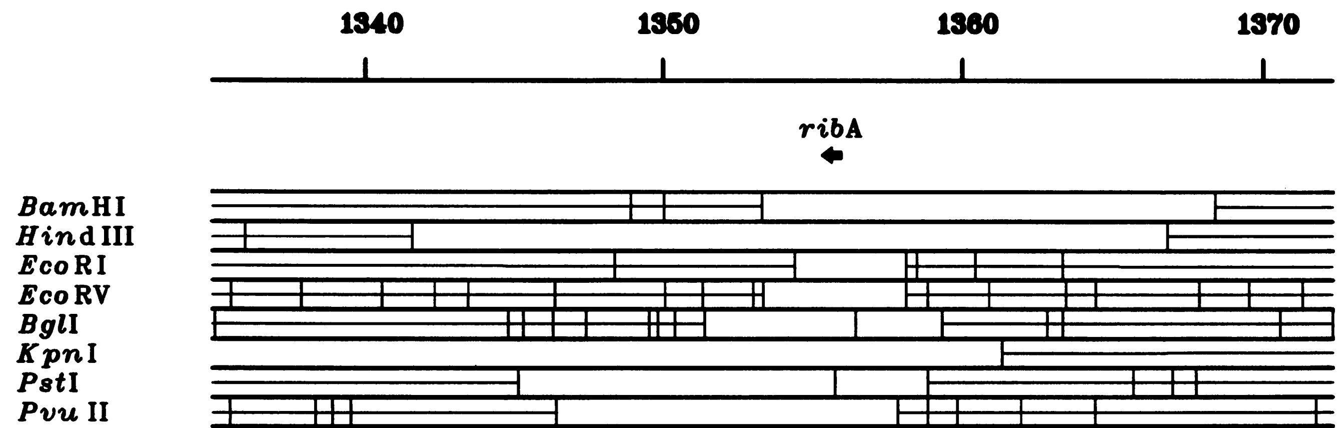

FIG. 4. Restriction map of the 28-min region of the *E. coli* chromosome. The position of the *ribA* gene is indicated. Open bars, restriction fragments which have been mapped by Southern analysis using the cloned 1.7-kb *Eco*RI-*Ava*I fragment of plasmid p7-21 as a hybridization probe. Phages from the Kohara bank (with their minilibrary numbers in parentheses) are indicated.

TABLE 2. Purification of GTP cyclohydrolase II from *E. coli* Rib7 carrying plasmid p7-21

Step	Vol (ml)	Activity (U)	Amt of protein (mg)	Sp act (U/mg)
Cell extract	61	54,000	1,300	42
Ammonium sulfate	20	50,000	800	63
DEAE-cellulose	150	41,000	170	240
Hydroxyapatite	5	20,000	50	400
Sephacryl S-200	6	31,000	14	2,250

the region at 28 min on the *E. coli* chromosome. Plaque blotting of a subset of phages from the Kohara library with the same probe gave a weak signal for phage 13F9 and a strong signal for phage 18B6 (Fig. 4). To check the identity of the *ribA* locus with the gene coding for GTP cyclohydrolase II, we transformed the mutant strain BSV18 (*ribA*) (obtained from B. Bachmann) with plasmids p7-2 and p7-21 as described in Materials and Methods. All transformants could grow on Luria-Bertani medium without added riboflavin. It follows that the gene coding for GTP cyclohydrolase II is identical to *ribA*.

Enzyme purification. *E. coli* strains carrying the plasmid p7-21 show at least a 100-fold increased level of GTP cyclohydrolase II activity. The viability and growth rate of the host are not affected by the presence of the plasmid. In M9 minimal media from cell cultures of *E. coli* RR28 containing p7-21, we detected 200 μg of riboflavin per liter by reversed-phase HPLC. Since we found equal amounts of the vitamin in media from *E. coli* cells carrying the plasmid pUC13, the overproduction of GTP cyclohydrolase II does not show any influence on riboflavin production.

The enzyme was purified to apparent homogeneity from the mutant strain Rib7 harboring plasmid p7-21 by a sequence of ammonium sulfate precipitation and three chromatographic steps as described in Materials and Methods. A typical experiment is summarized in Table 2. The overall purification was about 50-fold. Initial purification experiments yielded protein which gave two closely spaced bands in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The N-terminal sequences of both bands were determined by automated Edman degradation over a length of 13 amino acid residues. The sequences of both proteins were identical and corresponded to the amino terminus predicted from the genomic sequence. We concluded that the size heterogeneity could be a consequence of proteolytic modification at the C terminus. When the protease inhibitor phenylmethylsulfonyl fluoride was included in subsequent purification experiments, the enzyme appeared in SDS gels as a single band of 22 kDa (Fig. 5).

Gel permeation chromatography of purified GTP cyclohydrolase II on a column of Superdex 75 showed an apparent molecular mass of about 45 kDa. This result is in good agreement with the value of 44 kDa reported earlier on the basis of gel permeation chromatography (7). Thus, it appears likely that GTP cyclohydrolase II of *E. coli* is a dimer of identical subunits.

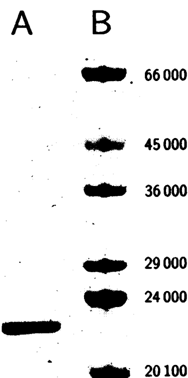

FIG. 5. SDS-polyacrylamide gel electrophoresis (14). Lane A, purified GTP cyclohydrolase II; lane B, marker proteins. Molecular masses (in daltons) of the marker proteins are indicated.

DISCUSSION

The gene coding for GTP cyclohydrolase II of *E. coli* has been cloned by a marker rescue approach on a plasmid carrying a 3-kb *Eco*RI fragment of chromosomal DNA. The recombinant strain had a more than 100-fold increased level of GTP cyclohydrolase II activity. DNA sequencing revealed an open reading frame coding for 196 amino acids. The translated 5′-terminal sequence was identical to the N-terminal sequence of purified GTP cyclohydrolase II. These data show conclusively that the gene codes for the enzyme catalyzing the first step in the biosynthesis of riboflavin.

The gene was mapped to a position at 1,356 kb (28.2 min) of the physical map of the *E. coli* K-12 chromosome on the basis of Southern blots of chromosomal DNA digested with various restriction enzymes. Subsequently, the gene could be localized in phage 18B6 (255) of the Kohara library. The gene is identical to the *ribA* locus reported earlier (2, 27).

The *ribA* gene is preceded by a typical ribosome binding site. A search in the EMBL-GenBank data bank indicated that the region upstream of *ribA* overlaps the presumptive promoter region of the *pgpB* gene (12), which codes for a membrane-bound phosphatase and is read in the direction opposite to that for *ribA* (Fig. 2, 3, and 6). The *pgpB* gene has been reported to code for a membrane-bound phosphatase catalyzing the hydrolysis of at least three different phospholipid-type compounds (12).

The biosynthesis of riboflavin involves the hydrolytic release of phosphate from the pyrimidine intermediate numbered 6 in Fig. 1. The enzyme responsible for the hydrolytic conversion of compound 6 to compound 7 has not been identified. However, it has been shown that compound 6

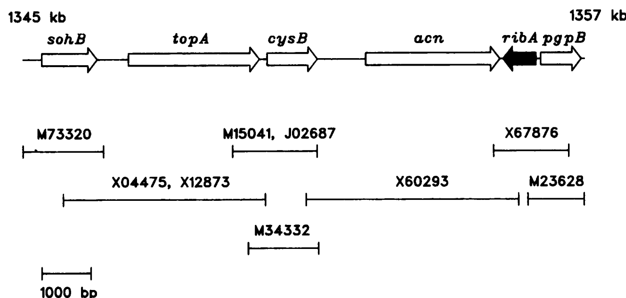

FIG. 6. Genes in the 28-min region of the *E. coli* chromosome. Map positions according to Kohara et al. (13) are indicated at the top. Genes are indicated by arrows. Published DNA sequences are represented by bars with their GenBank-EMBL accession numbers.

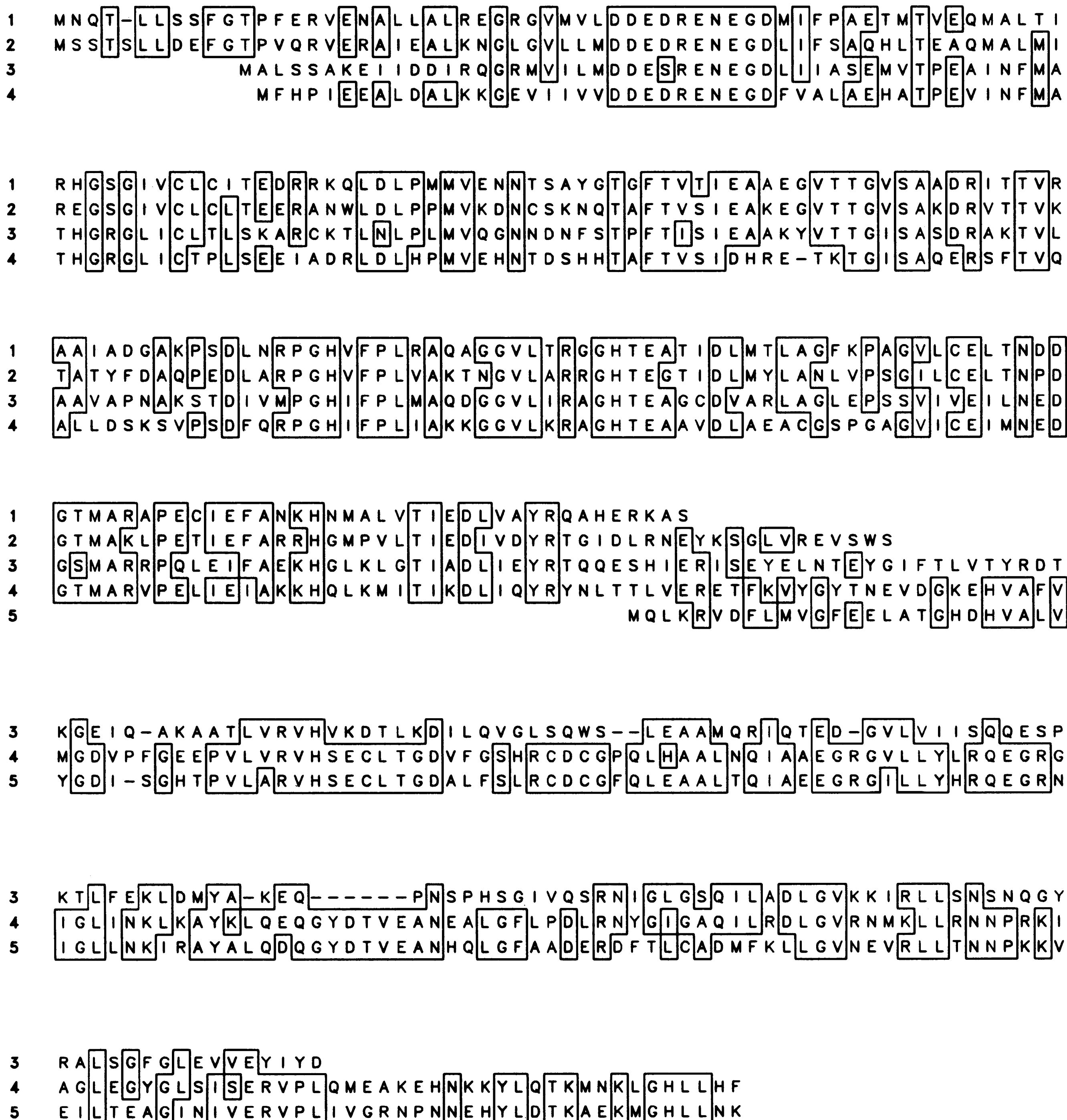

FIG. 7. Alignment of predicted protein sequences. 1, 3,4-dihydroxy-2-butanone 4-phosphate synthase of *E. coli* (RibB); 2, LuxH of *Vibrio harveyi* (26); 3, product of open reading frame II of the *lux* operon of *P. leiognathi* (15); 4, product of open reading frame 3 from the *rib* operon of *B. subtilis* (3,4,17–20); 5, GTP cyclohydrolase II of *E. coli*.

cannot replace compound 7 as a substrate for enzymatic formation of 6,7-dimethyl-8-ribityllumazine (9).

No riboflavin-deficient mutants with a defect of the dephosphorylation step have been reported, and it appears possible that the dephosphorylation of compound 6 can be catalyzed by one or several phosphatases of low substrate specificity. It has also been reported that *pgpB* mutants have no detectable phenotype (5). Thus, it appears unlikely that the phosphatase encoded by *pgpB* is specifically involved in the biosynthesis of riboflavin.

A comparison of the putative promoter regions of the genes *ribA* and *ribB* did not show any detectable similarities suggesting a common binding site for a regulatory protein. Current evidence favors the hypothesis that the genes of

riboflavin biosynthesis are expressed constitutively in *E. coli* (27). Reporter gene insertion into *rib* genes of *Salmonella typhimurium* also suggested constitutive expression in this related organism (30). On the other hand, it should be noted that the riboflavin pathway is tightly regulated in *Bacillus subtilis* (19).

A recent data bank search also revealed that the 3' end of *ribA* is flanked by the *acn* gene coding for aconitase (21). Since the termination of our mapping studies, the map position of the *acn* gene has been reported by others in full agreement with the results of our experiments (21). A presumptive terminator is located between the two genes (Fig. 3).

Contiguous sequence data are now available for a segment extending from kb 1345 to 1357 of the *E. coli* chromosome. The genes located in this segment and the respective accession numbers are given in Fig. 6.

GTP cyclohydrolase II was purified to apparent homogeneity from cell extracts of a recombinant strain carrying the plasmid p7-21, in which it constituted about 2% of the total cellular protein. In an earlier study, the enzyme had been purified about 2,200-fold from *E. coli* wild-type cells (7).

An apparent molecular mass of 45 kDa has been estimated for native GTP cyclohydrolase II of *E. coli* by gel filtration. This suggests tentatively that the protein is a dimer of identical subunits.

In *B. subtilis*, the enzymes involved in the biosynthesis of riboflavin are encoded by an operon located at 209°. The operon has been sequenced and contains five open reading frames (3, 4, 20). Circumstantial evidence suggested that the central open reading frame encompassing a deduced protein sequence of 398 amino acids should code for GTP cyclohydrolase II (18). However, no conclusive evidence has been presented.

Sequence comparison (Fig. 7) shows that the GTP cyclohydrolase II of *E. coli* is homologous to the 3' part of open reading frame 3 of the *B. subtilis* operon. The homology involves 54% identical residues. One gap of one amino acid had to be introduced.

The deduced peptide encoded by open reading frame 3 of *B. subtilis* has about twice the size of the *E. coli* protein. We have shown recently that the 5' part of the *B. subtilis* gene is homologous to the *ribB* gene coding for 3,4-dihydroxy-2-butanone 4-phosphate synthase in *E. coli* (23). This enzyme catalyzes the formation of the four-carbon precursor required for the formation of the xylene ring of the vitamin (29). Thus, the *B. subtilis* protein represents a bifunctional enzyme combining the activities of GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase.

Recently, several open reading frames have been sequenced in the 3' region of the *lux* operon of *Photobacterium leiognathi* (15) which codes for proteins involved in bioluminescence. The 3' moiety of open reading frame II of the *P. leiognathi* operon shows homology to *ribA* of *E. coli*. Moreover, open reading frame II shows homology to the open reading frame 3 of *B. subtilis* over its entire length. It appears likely that the *P. leiognathi* gene codes for a bifunctional enzyme catalyzing the formation of compound 2 and of compound 5 from their respective substrates 1 and 4. The reason for the expression of the *rib* genes in *P. leiognathi* under the control of the *lux* regulon may be that the bioluminescence reaction requires flavin mononucleotide as a cofactor. Thus, activation of the bioluminescence system may be accompanied by an increased demand for riboflavin.

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