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Biosynthesis of Riboflavin: Characterization of the Bifunctional Deaminase-Reductase of *Escherichia coli* and *Bacillus subtilis*†

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The *ribG* gene at the 5′ end of the riboflavin operon of *Bacillus subtilis* and a reading frame at 442 kb on the *Escherichia coli* chromosome (subsequently designated *ribD*) show similarity with deoxycytidylate deaminase and with the *RIB7* gene of *Saccharomyces cerevisiae*. The *ribG* gene of *B. subtilis* and the *ribD* gene of *E. coli* were expressed in recombinant *E. coli* strains and were shown to code for bifunctional proteins catalyzing the second and third steps in the biosynthesis of riboflavin, i.e., the deamination of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (deaminase) and the subsequent reduction of the ribosyl side chain (reductase). The recombinant proteins specified by the *ribD* gene of *E. coli* and the *ribG* gene of *B. subtilis* were purified to homogeneity. NADH as well as NADPH can be used as a cosubstrate for the reductase of both microorganisms under study. Expression of the N-terminal or C-terminal part of the RibG protein yielded proteins with deaminase or reductase activity, respectively; however, the truncated proteins were rather unstable.

The initial steps in the pathway of riboflavin biosynthesis are shown in Fig. 1. Briefly, the first committed step is catalyzed by GTP cyclohydrolase II and involves the release of formate and of pyrophosphate from GTP (compound 1). The product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (compound 2), is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (compound 5) in two steps involving the reduction of the ribosyl side chain and the deamination of the pyrimidine ring. Early studies with the yeast *Saccharomyces cerevisiae* showed that the reduction of the side chain precedes the deamination of the ring, and 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate (compound 4) was proposed as an intermediate (18). This sequence of reactions was later confirmed by enzymatic in vitro studies with *Ashbya gossypii* and *S. cerevisiae* (10, 11, 17).

On the other hand, enzyme studies with *Escherichia coli* indicated that the deamination reaction precedes the reduction of the ribosyl side chain. An enzyme catalyzing the deamination of compound 2 under formation of 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (compound 3) was purified 200-fold from cell extract of *E. coli* (6). It did not accept the dephosphorylated form of compound 2 as substrate. An enzyme catalyzing the reduction of compound 3 conducive to formation of compound 5 was also purified 200-fold from *E. coli* extract. This enzyme required NADPH as cofactor and was unable to utilize the dephosphorylated form of compound 3 as substrate.

In *Bacillus subtilis*, all enzymes of riboflavin biosynthesis are specified by an operon located at a position of 210° on the chromosome (7). The *rib* operon has been sequenced independently by two research groups and was shown to contain five open reading frames, designated *ribG*, *ribB*, *ribA*, *ribH*, and *ribT* (15, 20). The gene *ribB* codes for riboflavin synthase (22), *ribH* codes for lumazine synthase (12), and *ribA* codes for a bifunctional protein with GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase activity (21a).

Perkins et al. (20) noted that the *ribG* gene located at the 5′ end of the operon shows some similarity to deoxycytidylate deaminases from bacteriophage T2 and *S. cerevisiae* (13, 14) and proposed that the *ribG* gene could code for the deaminase converting compound 2 to compound 3. They further proposed that the gene *ribT* could code for the reductase catalyzing the conversion of 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (compound 3) to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (compound 5) (20). The present paper shows that the *ribG* gene of *B. subtilis* and the homologous *ribD* gene of *E. coli* (24) code for a bifunctional deaminase-reductase of the riboflavin pathway.

MATERIALS AND METHODS

Materials. Primers were synthesized by MWG-Biotech (Ebersberg, Germany). The Geneclean II kit was from Bio 101 (La Jolla, Calif.), and the pBluescript II SK$^-$ vector was obtained from Stratagene. The plasmids pRF2, pNCO113, and p602/22 were a gift of A. van Loon, Hoffmann-La Roche AG, Basel, Switzerland. 6,7-Dimethyl-8-ribityllumazine (1), 6,7-dimethyl-8-ribityllumazine 5′-phosphate (8), 6,7-dimethyllumazine (2), and 6,7-dimethylpterin (2) were prepared by published procedures. QAE cellulose QA92 was obtained from Whatman. Butyl Sepharose 4 FF and Superdex 200 were obtained from Pharmacia Biotech. NADH, NADPH, and glucose 6-phosphate were obtained from Boehringer, Mannheim, Germany.

GenBank-EMBL accession numbers. The *ribG* gene of *B. subtilis* (accession no. X51510) has been sequenced by Mironov et al. (15) and by Perkins et al. (20). The *ribD* gene of *E. coli* was reported as an open reading frame with homology to *ribG* by Taura et al. (24) under accession numbers X64395 and S47077.

Proteins. Restriction enzymes were obtained from New England Biolabs and Pharmacia Biotech. T4 DNA ligase was obtained from Gibco-BRL, and *Taq* DNA polymerase was obtained from Eurogentec. Alkaline phosphatase and DNase were obtained from Boehringer, Mannheim, Germany. Lysozyme and glucose 6-phosphate dehydrogenase were obtained from Sigma. GTP cyclohydrolase II of *E. coli* was isolated from a recombinant *E. coli* strain (21).

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are summarized in Table 1. The plasmids pNCO113 and p602/22 (shuttle vector for *E. coli* and *B. subtilis*) (Fig. 2) were kindly provided by A. van Loon, Hoffmann-La Roche AG. The shuttle vector p602/-CAT was constructed by removal of a part of the chloroamphenicol acetyltransferase gene from the expression plasmid p602/22 (16). The kanamycin resistance gene is retained in this plasmid.

Culture media. *E. coli* cells were grown in Luria-Bertani (LB) medium. Riboflavin-deficient mutants of *E. coli* require very high concentrations of riboflavin for growth. The vitamin was added to culture media at a concentration of 400 mg/liter and was dissolved by autoclaving at 121°C for 20 min.

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† Dedicated to Helmut Simon on the occasion of his 70th birthday.

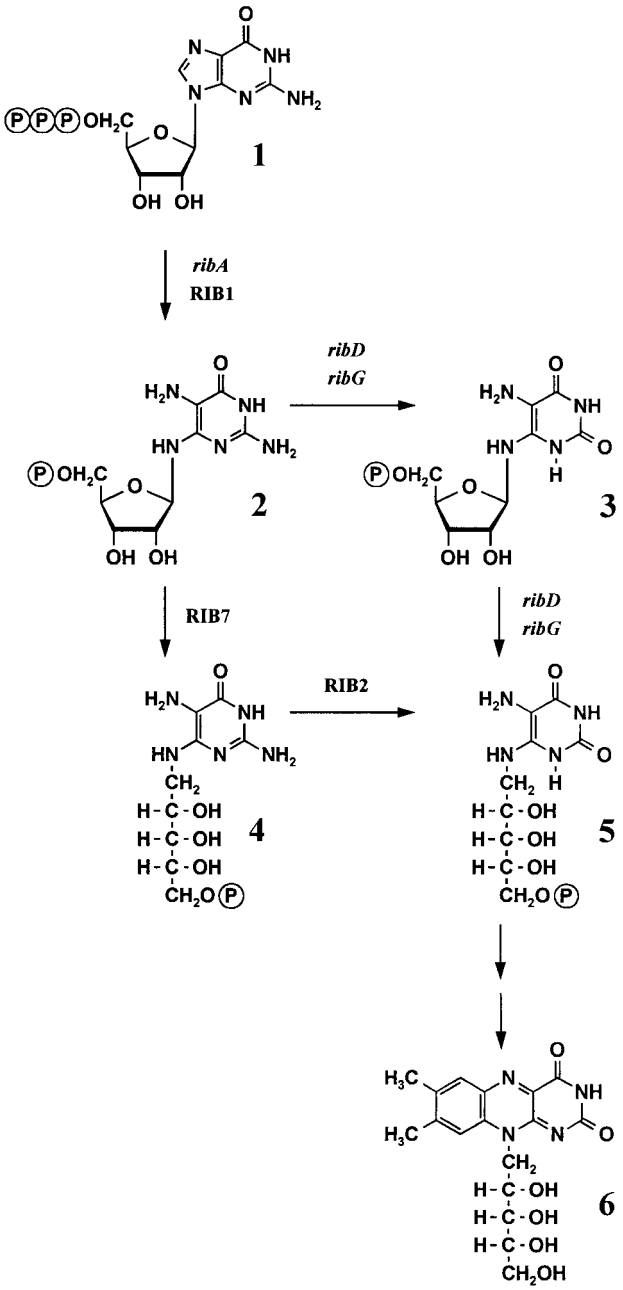

FIG. 1. Biosynthesis of riboflavin. GTP cyclohydrolase II is specified by *ribA* in *E. coli* and *B. subtilis* and by *RIB1* in *S. cerevisiae*; deaminase is specified by *ribD* in *E. coli*, by *ribG* in *B. subtilis*, and by *RIB2* in *S. cerevisiae*; reductase is specified by *ribD* in *E. coli*, by *ribG* in *B. subtilis*, and by *RIB7* in *S. cerevisiae*. 1, GTP; 2, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate; 3, 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; 4, 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate; 5, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; 6, riboflavin.

Enzyme assays. A method for assaying the deaminase and reductase of *E. coli* based on ^{14}C-labeled GTP has been described by Burrows and Brown (6). A different assay method was developed in the course of our studies.

Briefly, substrate 2 for pyrimidine deaminase (Fig. 3) was obtained from GTP with recombinant GTP cyclohydrolase II of *E. coli*. In order to assay the deaminase activity, substrate 2 was incubated with the bifunctional deaminase-reductase in the absence of reduced pyrimidine nucleotide cofactor. Enzyme product 3 was converted to 6,7-dimethyllumazine (compound 8) by reaction with diacetyl. Remaining substrate 2 was converted to 6,7-dimethylpterin (compound 7) by this procedure.

The substrate for reductase is obtained by treatment of GTP with GTP cyclohydrolase II and recombinant RibD protein of *E. coli*, yielding compound 3 (Fig. 3), which was separated from protein by ultrafiltration. NADPH used as cosubstrate for reductase was regenerated in situ by glucose 6-phosphate dehydrogenase. The product of the reductase, compound 5, was converted to 6,7-dimethyl-8-ribityllumazine 5′-phosphate (compound 9) by treatment with diacetyl, and remaining substrate was converted to dimethyllumazine (compound 8). Products were determined by fluorescence-monitored high-performance liquid chromatography (HPLC). A detailed description of these procedures follows.

Determination of deaminase activity. A solution containing 10 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 30 mM dithiothreitol (DTT), 5 mM GTP, and 2 mg of recombinant GTP cyclohydrolase II per ml was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.5 M EDTA to a final concentration of 25 mM. Aliquots of 40 μl were added to a solution (20 μl) containing 600 mM potassium phosphate (pH 7.8) and protein. The mixture was incubated at 37°C for 30 min. A solution (50 μl) containing 1% (vol/vol) diacetyl and 15% (wt/vol) trichloroacetic acid was added, and the mixture was incubated at 95°C for 1 h. Fluorescent products were separated by reverse-phase HPLC with a column of Nucleosil RP18 (4 by 250 mm) and an eluent containing 10% (vol/vol) methanol and 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. The effluent was monitored fluorometrically (excitation, 330 nm; emission, 435 nm). The retention volumes of 6,7-dimethyllumazine (compound 8) and 6,7-dimethylpterin (compound 7) were 22.5 and 12.5 ml, respectively.

Determination of reductase activity. A solution containing 10 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 30 mM DTT, 5 mM GTP, 100 μg of recombinant GTP cyclohydrolase II, and 100 μg of recombinant RibD protein of *E. coli* (see below) in a total volume of 50 μl was incubated at 37°C for 30 min. The solution was passed through a Centricon C30 cartridge (Amicon) to remove protein. The ultrafiltrate was mixed with a solution containing 600 mM potassium phosphate buffer (pH 7.8), 2 μM NADPH, 0.25 mM glucose 6-phosphate, and 0.5 U of glucose 6-phosphate dehydrogenase at a ratio of 5:1. Aliquots (60 μl) of this solution were mixed with the protein solution (40 μl) to be assayed for reductase activity. The mixture was incubated at 37°C for 30 min. A solution (50 μl) containing 1% (vol/vol) diacetyl and 15% (wt/vol) trichloroacetic acid was added, and the mixture was incubated for 1 h at 37°C. 6,7-Dimethyl-8-ribityllumazine 5′-phosphate (compound 9) was monitored by reverse-phase HPLC with a column of Nucleosil RP18 (4 by 250 mm) and an eluent containing 7% methanol and 30 mM formic acid. The flow rate was 2 ml/min. The effluent was monitored fluorometrically (excitation, 408 nm; emission, 485 nm). The retention volume of compound 9 was 7.0 ml.

Construction of expression plasmids for the *ribD* gene of *E. coli* and the *ribG* gene of *B. subtilis*. The *ribD* gene was amplified by PCR with chromosomal DNA from *E. coli* RR28 as template, the oligonucleotide RibD1 as forward primer, and the oligonucleotide RibD2 as reverse primer (Fig. 4 and 5). The GTG start codon in the native *E. coli* sequence was replaced by ATG in the expression construct. The *ribG* gene of *B. subtilis* was amplified with the plasmid pRF2 as template and the primers RibG1 and RibG2. Each amplification product served as template in a second PCR with the universal forward primer P2, which is complementary to the ribosome binding site of the expression vector p602/-CAT, and the reverse primer RibD2 (for the *E. coli* gene) or RibG2 (for the *B. subtilis* gene). The PCR products were cleaved with *Eco*RI and *Bam*HI and were ligated into the expression vector p602/-CAT which had been digested with *Eco*RI and *Bam*HI. The expression plasmids designated p602-RibD and p602-RibG were transformed into *E. coli* M15(pGB3) cells carrying the pGB3 repressor plasmid for the overexpression of *lac* repressor protein. Kanamycin (15 μg/ml) and ampicillin (150 μg/ml) were added for maintenance of both plasmids in the host strain. Clones were analyzed by PCR screening with the universal primer P2 and the selective primer RibD2 or RibG2. Both constructs were monitored by restriction analysis and by DNA sequencing of the 5′ and the 3′ ends of the plasmid inserts.

Construction of expression plasmids for N-terminal domains of the RibG protein of *B. subtilis*. Sections at the 5′ end of the *ribG* gene were amplified by PCR with plasmid pRF2 as template and with primers shown in Fig. 5 and Table 2. The primer G-N1 created an *Nco*I site at the 5′ end of the *ribG* gene. Each of the reverse primers G-DE1 to G-DE5 introduced a *Bam*HI site. Codons for the amino acids A179, K115, V121, V148, and G158 were changed to stop codons by the respective primers. The PCR fragments were ligated into a pBluescript II SK$^-$ vector which had been digested with *Eco*RV and subsequently tailed with dTTP and *Taq* DNA polymerase. The plasmids were transformed into *E. coli* XL1-blue cells and were analyzed by restriction analysis. Clones containing the deaminase domains were then cleaved with *Nco*I and *Bam*HI. The resulting fragments were purified using the Geneclean II kit and were then ligated into a *Nco*I-*Bam*HI-digested pNCO113 expression vector, yielding plasmids pNCO-G-DE1 to pNCO-G-DE5, which were transformed into *E. coli* M15(pREP4).

Construction of an expression plasmid for a C-terminal domain of the RibG protein of *B. subtilis*. The 3′ part of the *ribG* gene was amplified by PCR with plasmid pRF2 as template. The forward primer (G-RED) introduced a *Bsp*HI site at the codon for residue M113 (Fig. 4; Table 3). The reverse primer was RibG2. The amplificate was ligated into a pBluescript II SK$^-$ vector which had been digested with *Eco*RV and subsequently tailed with dTTP and *Taq* DNA polymerase. The resulting plasmid was transformed into *E. coli* XL1-blue cells. The plasmid was reisolated and was digested with *Bsp*HI and *Bam*HI. An 820-bp

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tet^r)]	5
M15(pGB3)	*lac ara gal mtl recA*$^+$ *uvv*$^+$ (pGB3; *lacI* Amp^r)	A. van Loon
M15(pRep4)	*lac ara gal mtl recA*$^+$ *uvr*$^+$ (pRep4; *lacI* Kan^r)	25
RR28	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA*	9
Rib1	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Rib2	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Rib3	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Rib4	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Rib6	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Rib8	F$^-$ *thi pro lac gal ara mtl xyl supE44 endA hsd* (r$^-$ m$^-$) *pheS recA ribD*	21
Plasmids		
pBluescriptIISK$^-$	Cloning vector	Stratagene
pNCO113	Expression vector	23
p602/22	*E. coli-B. subtilis* shuttle vector	A. van Loon
p602/-CAT	p602/22 with deleted *cat* gene	16
pRF2	pBR322 with a 10-kb *Eco*RI fragment containing the *rib* operon of *B. subtilis*	20
p602-RibD	p602/-CAT with the *ribD* gene of *E. coli*	This study
p602-RibG	p602/-CAT with the *ribG* gene of *B. subtilis*	This study
pNCO-G-DE1	pNCO113 with a *ribG* fragment (1–178)[a]	This study
pNCO-G-DE2	pNCO113 with a *ribG* fragment (1–114)[a]	This study
pNCO-G-DE3	pNCO113 with a *ribG* fragment (1–120)[a]	This study
pNCO-G-DE4	pNCO113 with a *ribG* fragment (1–147)[a]	This study
pNCO-G-DE5	pNCO113 with a *ribG* fragment (1–157)[a]	This study
pNCO-G-RED	pNCO113 with a *ribG* fragment (113–361)[a]	This study

[a] Amino acid residues of the *ribG* gene specified by the plasmid.

fragment was isolated from an agarose gel with the Geneclean II kit and was ligated into a *Nco*I-*Bam*HI-digested pNCO113 expression vector, yielding plasmid pNCO-G-RED.

DNA sequencing. Sequencing was performed by the Sanger dideoxy chain termination method with a model 373A DNA sequencer from Applied Biosystems, Foster City, Calif. *E. coli* cells for DNA isolation were grown overnight in LB medium containing 150 μg of ampicillin and 15 μg of kanamycin per ml. DNA was isolated by the modified alkali-sodium dodecyl sulfate method described by Birnboim and Doly (4) with Nucleobond AX-100 columns from Macherey & Nagel, Düren, Germany. Plasmids were sequenced using the oligonucleotides S1 (pNCO), S2 (p602/-CAT), and S3 (Table 3).

Purification of recombinant RibD protein of *E. coli* and RibG protein of *B. subtilis*. The recombinant deaminase-reductase of *E. coli* and *B. subtilis* could be purified by the same procedure. Recombinant *E. coli* strains carrying hyperexpression plasmids with the *ribD* gene of *E. coli* or the *ribG* of *B. subtilis* were grown in LB medium containing ampicillin (150 mg/l) and kanamycin (15 mg/l). At an optical density at 600 nm of about 0.6 to 0.8, isopropylthiogalactoside was added to a final concentration of 0.5 mM. The suspension was incubated for 5 h with shaking. The cells were harvested by centrifugation (4,500 × *g*, 15 min, 4°C), washed with 154 mM NaCl, and stored at −20°C.

Frozen cell mass (4 g) was thawed in 40 ml of a solution containing 50 mM Tris hydrochloride (pH 8.0), 0.3 mM phenylmethylsulfonyl fluoride, 40 mg of lysozyme, and 40 mg of DNase. The mixture was incubated at 37°C for 5 h. The suspension was centrifuged (27,000 × *g*, 20 min, 4°C), and the supernatant was dialyzed overnight against 25 mM Tris hydrochloride (pH 9.0). The solution was placed on a column of QAE cellulose QA92 (10 by 2.5 cm) which had been equilibrated with 25 mM Tris hydrochloride (pH 9.0). The column was developed with a gradient of 25 to 500 mM NaCl containing 25 mM Tris hydrochloride (pH 9.0). Fractions were collected, and ammonium sulfate was added to a final concentration of 1 M. The solution was applied to a column of butyl Sepharose

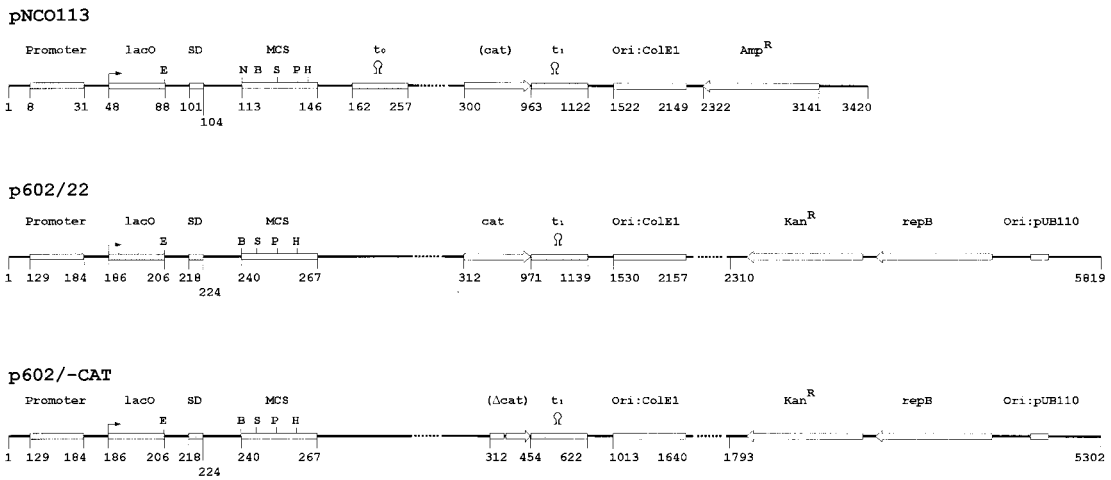

FIG. 2. Expression vectors used in this study. SD, ribosome binding site; MCS, multiple cloning site; t_0,t_1, terminator sequences; (cat), *cat* gene inactivated by a frameshift mutation; (Δ*cat*), *cat* gene inactivated by partial deletion. Selected restriction sites are indicated by single letters: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Sal*I. Transcription start sites are indicated by arrows. Translation starts at bp 114 in plasmid pNCO113 and bp 233 in the vectors p602/22 and p602/-CAT.

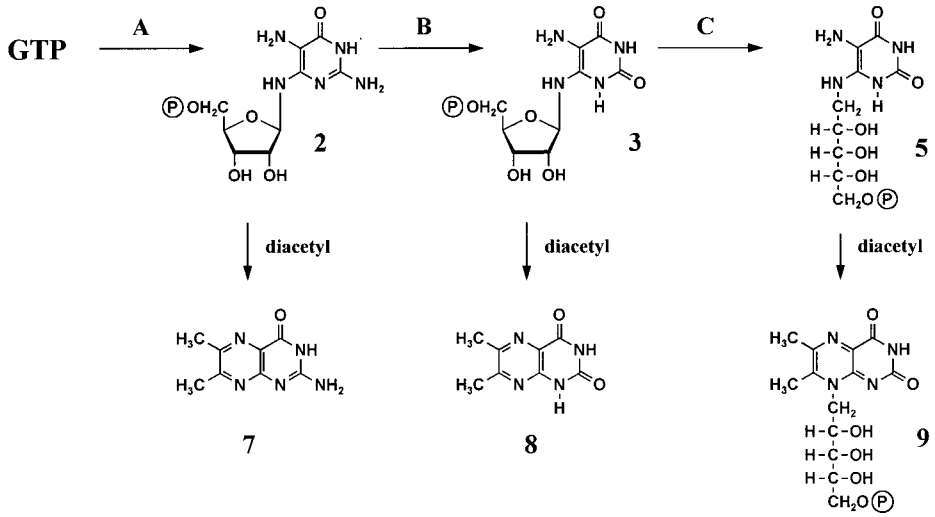

FIG. 3. Assay of deaminase and reductase activities. A, GTP cyclohydrolase II; B, deaminase; C, reductase; 2, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate; 3, 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; 5, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; 7, 6,7-dimethylpterin; 8, 6,7-dimethyllumazine; 9, 6,7-dimethyl-8-ribityllumazine 5′-phosphate.

4 FF which was developed with a linear gradient of 1 to 0 M ammonium sulfate in 50 mM Tris hydrochloride (pH 9.0; total volume, 500 ml). Fractions were combined, and the protein solution was concentrated by ultracentrifugation for 20 h at 160,000 × *g* and 4°C. Aliquots of 1.5 ml containing 10 to 15 mg of protein were passed through a Superdex 200 column (1.6 by 60 cm), which was developed with 100 mM Tris hydrochloride (pH 8.5). Fractions were concentrated by ultrafiltration (Amicon C30) or ultracentrifugation (160,000 × *g*, 20 h, 4°C).

RESULTS

Figure 6 shows an alignment of the deduced sequences for the proteins encoded by the *ribG* gene of *B. subtilis* and the *ribD* gene of *E. coli* located in close proximity to the *nusB* gene at 442 kb on the *E. coli* chromosome. The sequences show 39% identity.

Perkins and Pero had noted earlier (19) that the 5′ end of the *B. subtilis* gene is similar to deoxycytidylate deaminase of bacteriophage T2 and *S. cerevisiae* (13, 14) and suggested tentatively that *ribG* could specify an enzyme catalyzing the deamination of compound 2. Moreover, it was proposed that the *ribT* gene of the 5′ end of the *rib* operon could code for the enzyme catalyzing the reduction of compound 3 (pyrimidine reductase). More recently, we found sequence similarity with the *RIB7* gene (3) of *S. cerevisiae.*

The early steps of the riboflavin pathway are somewhat different in eubacteria and in yeasts (Fig. 1). Circumstantial evidence indicated that the enzyme specified by the *RIB7* gene of *S. cerevisiae* catalyzes the reduction of the ribosyl side chain. We concluded tentatively that a similarity between the yeast gene *RIB7* and the bacterial genes *ribD* and *ribG* could imply that the bacterial proteins serve both as deaminase and reductase catalyzing the conversion of compound 2 to compound 5 in two reaction steps.

Gene expression. In order to verify these presumptive catalytic activities of the bacterial genes, expression plasmids were constructed for the *ribD* gene of *E. coli* and the *ribG* gene of *B. subtilis* as described in Materials and Methods. Recombinant *E. coli* strains harboring plasmids with the entire *ribD* or *ribG* gene produce 40-kDa proteins, accounting for about 20% of cellular protein. The cell extracts showed high deaminase as well as reductase activity.

Enzyme studies. The recombinant RibG and RibD proteins of *B. subtilis* and *E. coli* were purified to apparent homogeneity by the same experimental procedure involving anion-exchange

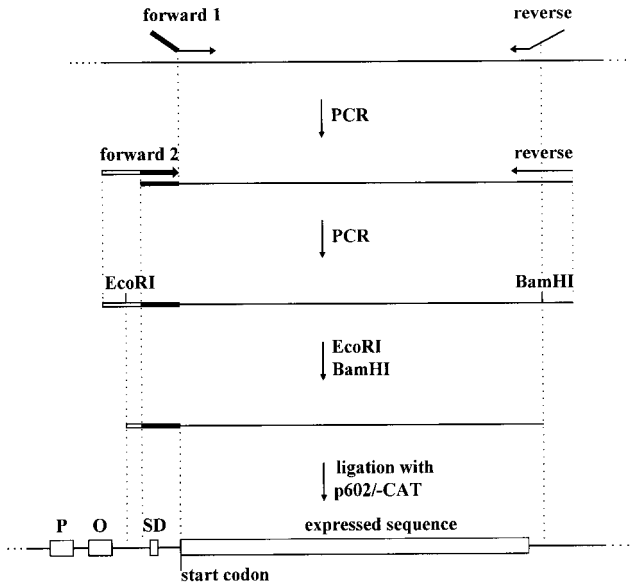

FIG. 4. Construction of expression plasmids for the *ribG* and *ribD* genes. P, promoter; O, *lac* operator; SD, ribosome binding site. Different forward primers (but the same reverse primer) were used in the subsequent PCR amplifications.

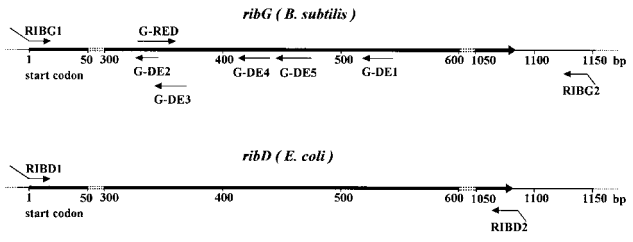

FIG. 5. Primers used for construction of expression plasmids.

TABLE 2. Construction of expression plasmids for the *ribD* and *ribG* genes of *E. coli* and *B. subtilis* as well as for partial deletions of the *ribG* gene

Plasmid	Primer[a]		Template	Amino acid residues
	Forward	Reverse		
p602-RibD	RibD1, P2	RibD2	Chromosomal DNA	1–367
p602-RibG	RibG1, P2	RibG2	pRF2	1–361
pNCO-G-DE1	G-N1	G-DE1	pRF2	1–178
pNCO-G-DE2	G-N1	G-DE2	pRF2	1–114
pNCO-G-DE3	G-N1	G-DE3	pRF2	1–120
pNCO-G-DE4	G-N1	G-DE4	pRF2	1–147
pNCO-G-DE5	G-N1	G-DE5	pRF2	1–157
pNCO-G-DE1	G-RED	RibG2	pRF2	113–361

[a] See Table 3 for primer sequences.

TABLE 4. Purification of the recombinant deaminase-reductase proteins of *E. coli* and *B. subtilis*[a]

Protein	Vol (ml)	Activity (U)[b]	Protein amt (mg)	Sp act (μmol/mg-h)[b]
RibG (*B. subtilis*)				
Cell extract	75	1,717	270	6.4
QA92	600	1,020	108	9.4
Butyl-Sepharose 4 FF	120	340	35	9.6
Superdex 200	40	297	30	9.9
RibD (*E. coli*)				
Cell extract	70	2,590	350	7.4
QA92	480	1,210	112	10.8
Butyl-Sepharose 4 FF	138	787	69	11.4
Superdex 200	38	630	30	21.0

[a] Only deaminase activity was monitored during purification.
[b] Pyrimidine deaminase.

chromatography on QAE cellulose followed by gel permeation chromatography (Table 4).

The catalytic properties of purified recombinant proteins are summarized in Table 5. The kinetic properties of *E. coli* and *B. subtilis* proteins are similar. Both enzymes can use NADH as well as NADPH as cosubstrate.

Domain structure. Expression plasmids directing the synthesis of N-terminal or C-terminal parts of the RibG protein of *B. subtilis* were constructed. Crude cell extracts of recombinant strains expressing at least 147 amino acid residues of the N-terminal part had deaminase activity (specific activity of 2 μmol mg^{-1} h^{-1} in crude cell extract) and no reductase activity (Table 6). The enzyme activity decreased rapidly with time, and the recombinant proteins could not be purified.

Cell extract of a recombinant strain producing a peptide with 248 C-terminal amino acid residues of the RibG protein showed reductase activity (0.5 μmol mg^{-1} h^{-1}) and no deaminase activity. Again, the protein could not be purified due to the low stability. It follows that the RibG protein of *B. subtilis* consists of two domains which can fold independently, yielding enzymatically active, monofunctional proteins, albeit of very poor stability.

The *E. coli ribD* mutants Rib6 and Rib8 (21) could be complemented by the plasmid pNCO-G-RED coding for the reductase domain. The *ribD* mutants Rib1, Rib2, Rib3, and Rib4 could be complemented only by plasmids directing the synthesis of the entire RibD or RibG protein. None of the *ribD* mutants studied could be complemented by constructs directing the synthesis of the deaminase domain.

DISCUSSION

Burrows and Brown (6) reported the partial purification of two different enzymes with deaminase and reductase activities, respectively, from cell extract of an *E. coli* wild strain. Based on gel filtration experiments, the deaminase was assigned an approximate molecular mass of 80 kDa, and the reductase was assigned an approximate molecular mass of 37 kDa. Hypothetically, a reductase of reduced molecular mass could be obtained from the RibD protein by proteolytic processing in vivo. Assuming that the hypothetical protease is present in a relatively low amount, the majority of recombinant protein in the recombinant hyperexpression strain could remain unprocessed.

Perkins et al. (20) had noted that the *ribG* gene of *B. subtilis* has a putative internal ribosome binding site followed by an ATG codon specifying methionine 113. A similar motif is also present in the *ribD* gene of *E. coli* (putative ribosome binding site followed by ATG codon specifying methionine 127). Thus, it was conceivable that both genes could yield a full-length protein and also a shorter C-terminal segment with reductase activity by differential translation.

The expression of partially deleted *ribG* genes of *B. subtilis*

TABLE 3. Oligonucleotides used for the construction of expression plasmids and for sequencing[a]

Oligonucleotide	Type	Cutting site	Primer sequence
P2	Forward 2	*Eco*RI	5′ *AC ACA GAA TTC ATT AAA GAG GAG AAA TTA ACT ATG* 3′
S1	Sequencing		5′ *GTG AGC GGA TAA CAA TTT CAC ACA G* 3′
S2	Sequencing		5′ *GTA TAA TAG ATT CAA ATT GTG AGC GG* 3′
S3	Sequencing		5′ *CT CCA TTT TAG CTT CCT TAG CTC CTG* 3′
RibD1	Forward 1		5′ *GAG GAG AAA TTA ACT ATG* CAG GAC GAG TAT TAC 3′
RibD2	Reverse	*Bam*HI	5′ **TAT TAA** GGA **TCC** TCA TGC ACC CAC TAA ATG CAG 3′
RibG1	Forward 1		5′ *GAG GAG AAA TTA ACT ATG* GAA GAG TAT TAT ATG 3′
RibG2	Reverse	*Bam*HI	5′ TTT CAT GGA TCC GAT TGT GCC 3′
G-N1	Forward	*Nco*I	5′ AAA C**CC ATG** GAA GAG TAT TAT ATG AAG C 3′
G-DE1	Reverse	*Bam*HI	5′ A **GGA** TCC **CTA** ATC CTG TCT TGC AGC 3′
G-DE2	Reverse	*Bam*HI	5′ **A GGA** TCC **GCT A**GC TGA TCC CTC 3′
G-DE3	Reverse	*Bam*HI	5′ **A GGA TCC TTA AGC TA**G CCA GCT TCT TTC 3′
G-DE4	Reverse	*Bam*HI	5′ A GGA TCC **A**GT AC**T A**AA GGC CTG TCC TC 3′
G-DE5	Reverse	*Bam*HI	5′ **A** GGA **TCC TCA** GCT GGC AGC CG 3′
G-RED	Forward	*Bsp*HI	5′ GGG ATC A**T**C ATG ATG AAA GAA GC 3′

[a] Bases complementary to vector sequences are shown in italics. Cutting sites used are underlined. Mutated bases are shown in boldface type.

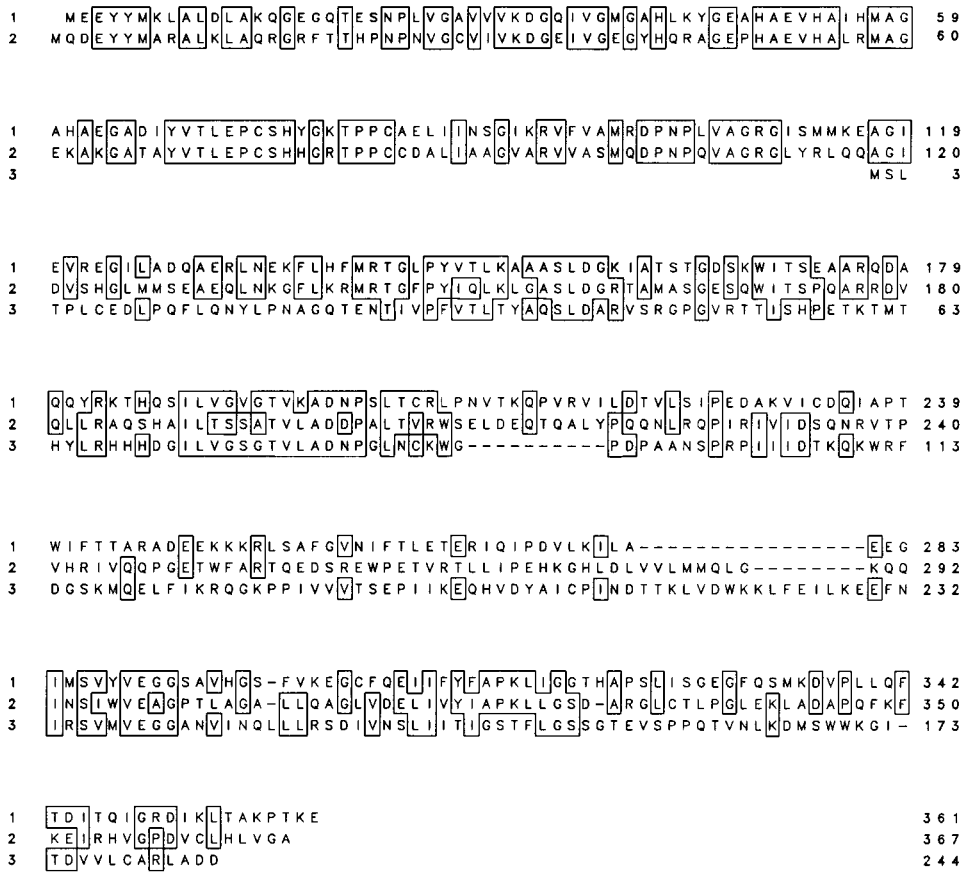

FIG. 6. Alignment of amino acid sequences predicted by the following genes: 1, *ribG* of *B. subtilis*; 2, *ribD* of *E. coli*; and 3, *RIB7* of *S. cerevisiae.*

yields proteins with either deaminase or reductase activity, as shown by enzymatic analysis and by complementation patterns observed with different *ribD* mutants. These data suggest that the RibG protein consists of two domains which can fold independently. More specifically, the N-terminal 147 amino acids are sufficient for deaminase activity, and the C-terminal 248 amino acids are sufficient for reductase activity. However, the stability of the recombinant proteins obtained by partial deletion of the *ribG* gene is very low.

A deletion analysis of the *ribD* gene of *E. coli* has not yet been performed. It remains uncertain whether either of the hypotheses mentioned above (proteolytic processing or translational restart) is sufficient to explain the discrepancies between our findings and those of the earlier studies by Burrows and Brown. However, our data show unequivocally that the *ribD* gene of *E. coli* can specify a 40-kDa peptide with both catalytic activities under study. The same is true for the *ribG* gene of *B. subtilis.*

Recombinant RibT protein failed to catalyze the reduction of compound 3. The metabolic role of the RibT protein remains unknown, but it is unlikely to be involved in the riboflavin biosynthetic pathway.

TABLE 5. Kinetic properties of recombinant deaminase-reductase proteins of *E. coli* and *B. subtilis*[a]

Protein	Enzyme activity (μmol mg^{-1} h^{-1})		
	Deaminase	Reductase	
		NADPH	NADH
RibG	9.9	0.6	2.0
RibD	21.3	6.7	7.5

[a] Specific activities at high substrate concentration (approximately 4 mM compound 2 for deaminase activity; approximately 2 mM compound 3 for reductase activity; 2 mM NADH or NADPH).

TABLE 6. Enzyme activities of strains with expression plasmids containing the genes coding for deaminase and reductase of *E. coli* and *B. subtilis*

Plasmid	Amino acid residues	Relative mass (Da)	Enzyme activity	
			Deaminase	Reductase
p602-RibD	1–367	40,338	+	+
p602-RibG	1–361	39,305	+	+
pNCO-G-DE2	1–114	12,175	−	−
pNCO-G-DE3	1–120	12,803	−	−
pNCO-G-DE4	1–147	15,989	+	−
pNCO-G-DE5	1–157	16,959	+	−
pNCO-G-DE1	1–178	19,164	+	−
pNCO-G-RED	113–361	27,410	−	+

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