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Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin

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Recombinant DNA engineering was combined with mutant selection and fermentation improvement to develop a strain of *Bacillus subtilis* that produces commercially attractive levels of riboflavin. The *B. subtilis* riboflavin production strain contains multiple copies of a modified *B. subtilis* riboflavin biosynthetic operon (*rib* operon) integrated at two different sites in the *B. subtilis* chromosome. The modified *rib* operons are expressed constitutively from strong phage promoters located at the 5′ end and in an internal region of the operon. The engineered strain also contains purine analog-resistant mutations designed to deregulate the purine pathway (GTP is the precursor for riboflavin), and a riboflavin analog-resistant mutation in *ribC* that deregulates the riboflavin biosynthetic pathway.

Keywords: riboflavin; metabolic engineering; *Bacillus subtilis*

Introduction

Riboflavin is an essential vitamin that is required by all bacteria, animals and plants; it is a precursor to the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Riboflavin is synthesized by plants and microorganisms; it is not produced by higher animals, which must acquire it from their diet. Riboflavin is produced commercially for use as a food and feed additive. Both chemical synthesis, starting with ribose, and fungal fermentations of *Ashbya gossypii*, *Eremothecium ashbyii*, or *Candida flareri* [1,18,19,45] are used for the commercial production of riboflavin. Here we describe the genetic engineering of a bacterial strain to produce high yields of riboflavin in a short fermentation cycle.

Bacillus subtilis was the organism of choice to develop a bacterial route to the commercial production of riboflavin for a number of reasons. First, many products produced in *Bacillus* species are classified as GRAS (Generally Regarded As Safe) and *Bacillus* species have been used previously for fermentative synthesis of products used in the food and feed industries [12]. Second, classical mutagenesis had been used previously to develop strains of various *Bacillus* species that were capable of producing significant quantities of inosine or guanosine (20–40 g L^{-1}) [12,15,40,55,56]. Since GTP is a precursor of riboflavin synthesis [3], this indicated that *Bacillus* species could be engineered to direct the necessary high levels of carbon to the riboflavin pathway. Third, the riboflavin biosynthetic genes of *Bacillus subtilis* have been sequenced and shown to be organized in a single operon, *ribGBAH* [3,38,44,52]; transcription of these genes is controlled by at least two promoters recognized by the vegetative form (σ^A) of *B. subtilis* RNA polymerase [37,43,44]. Introduction of a plasmid containing the *rib* operon into an appropriate strain of *B. subtilis* resulted in enhanced riboflavin production [54]. Finally, *B. subtilis* was the species of choice because advanced molecular genetic and genetic engineering technology were available for use in this strain.

The overall strategy to develop a commercial riboflavin production strain of *B. subtilis* was to combine classical genetic mutant selection and fermentation improvement with genetic engineering of the riboflavin biosynthetic genes that deregulated and increased their level of expression. The expression of the *rib* genes was increased by mutating a regulatory gene (*ribC*), by replacing or bypassing two different promoter/regulatory sites with strong, constitutive promoters, and by increasing the copy number of the genes. To ensure a sufficient supply of GTP, purine analog-resistant mutants were selected [41]. Finally, to develop a low-cost fermentative route to riboflavin production, fermentation conditions were developed for rapid production of riboflavin from a glucose-based medium.

Materials and methods

Bacterial strains, plasmids, and growth conditions

B. subtilis RB9 derived from GP205 [51] was used as the starting strain for isolation of purine and analog-resistant mutants. *B. subtilis* strains used to test integration vectors for riboflavin production were IS75 [16], 1A382 [29], and 62178 [14]; the *rib*$^+$ host to test *lacZ* fusions was PY79 [59]. Plasmids pUC19 [58] and pIC20R [31] were used for cloning into *E. coli*. Plasmids pRF8, pRF34, and pRF36 are derivatives of pBR322 containing the wild-type riboflavin operon and flanking sequences [44]. In pRF34 and pRF36, a 1.7-kb cassette containing the chloramphenicol-resistance (*cat*) gene from pC194 [21] and several unique restriction sites were inserted between a pair of *Nco*I sites upstream of the *rib* operon. Plasmid pNH202 contains the constitutive phage SPO1 promoter from the *Eco*RI* fragment 15 (P_{15}) [28]. *E. coli* strains were grown on Luria-

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Bertani (LB) medium [7,49] prepared without glucose. Competent *E.coli* was prepared by the method of Inoue *et al* [24] or purchased from Gibco BRL, Rockville, MD, USA. *B. subtilis* cells were grown on Tryptose Blood Agar Base (TBAB; Difco Laboratories, Detroit, MI, USA) plates, in Veal Infusion/Yeast Extract (VY) broth [9], or in Spizizen's minimal salts (SS) medium containing 0.4% sodium glutamate [53]. Competent *B. subtilis* was prepared, stored, and transformed as described by Dubnau and Davidoff-Abelson [13]. Plasmid DNA from *E. coli* was isolated by alkaline-SDS lysis [8] and further purified using CsCl-ethidium bromide density gradients.

Isolation of purine and riboflavin-analog resistant mutants

Azaguanine (Az^r) and decoyinine (Dc^r)-resistant mutants were isolated by subjecting cells to ethyl methyl sulfonate (EMS) mutagenesis using standard procedures [36], and plating recovered colonies on SS agar containing 500 μg ml^{-1} azaguanine or 100 μg ml^{-1} decoyinine. Spontaneous methionine sulfoxide (MS^r)-resistant and roseoflavin (RoF^r)-resistant mutants were isolated by streaking colonies on SS agar containing 10 mg ml^{-1} methyl sulfoxide or 100 μg ml^{-1} roseoflavin. Mutants were streaked on medium containing the same analog to confirm resistance.

Detection and measurement of riboflavin

Riboflavin production of individual colonies was detected by exposing the colonies to long-wave UV light (366 nm) and observing yellow fluorescence. Accumulation of riboflavin from shake-flask and fermentation cultures was measured by reverse-phase HPLC. Shake-flask cultures were grown for approximately 24 h in 25 ml of SS medium at 37°C; fermentation conditions are described below. Cell-free supernatants were fractionated over a 4.6-mm × 250-mm Vydac C_{18} column equilibrated with 1% ammonium acetate (pH 6.0). The column was eluted with a linear gradient of methanol and the riboflavin peak was monitored at 254 nm. Authentic riboflavin elutes at the mid-point of the gradient. Riboflavin concentrations were calculated by comparing the integrated peak of the sample to those of riboflavin standards (Sigma, St Louis, MO, USA).

RNA isolation and Northern blots

Total RNA was extracted from cells grown in 25 ml SS medium to early logarithmic stage as described by Igo and Losick [23]. Contaminating chromosomal DNA was removed with RQ1 RNA-free DNAse (Promega, Madison, WI, USA). The RNA was stored in RNAsin (Promega) at –80°C to prevent degradation of the RNA.

Total cellular RNA was separated on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membrane filters using standard procedures [49]. Short DNA probes (17–18 oligonucleotides) homologous to *ribG* or *ribA* were end-labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) as described by the manufacturer. Hybridization was done overnight at 42°C in 5 × SSC (1 × SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50% formamide, 1 × Denhardt's solution, 100 μg ml^{-1} denatured salmon sperm DNA [49]. Blots were washed twice at room temperature, first in 1 × SSC, 0.1% SDS and then in 0.25 × SSC, 0.1% SDS. RNA markers, ranging from 0.36 kb to 9.49 kb (Promega), were used to estimate mRNA size.

Construction of lacZ transcriptional fusions

Transcriptional fusions were constructed using pZΔ327 [60], a derivative of pBR322 containing a *lacZ* reporter gene with a ribosome-binding site derived from the *spoVG* gene of *B. subtilis*. Plasmid pRF36 was digested with *Hin*dIII to obtain a 2.0-kb fragment containing $ribP_1$, the regulatory region *ribO*, and the 5′ end of *ribG*, and a 1.3-kb fragment containing $ribP_2$ (embedded in the *ribB* gene) and the 5′ end of *ribA*. Both fragments were ligated to *Hin*dIII-cut pZΔ327 to give pRF59 and pRF56, respectively. To construct a (P_{15}-*rib*)-*lacZ* fusion, pRF50 (see below) was digested with *Eco*RI and *Bam*HI to obtain a 0.45-kb fragment containing the P_{15} promoter and the first 10 codons of *ribG*. This fragment was mixed with *Hin*dIII-cut pZΔ327 and both fragments were treated with the Klenow fragment of DNA polymerase to make flush ends; ligation of the fragments yielded pRF70. Each fusion was integrated into the modified SPβ prophage SPβ*c2del2*::Tn917::pSK10Δ6 to generate specialized transducing phage [61]. Plasmids pRF56, pRF59, and pRF70 were first transformed in *dcm*/*dam E. coli* mutant GM119 to allow complete digestion with *Bal*I enzyme. The linearized plasmids were then transformed into ZB493 [61], selecting for colonies resistant to 5 μg ml^{-1} chloramphenicol. Specialized transducing lysates containing each fusion were obtained by heat induction at 50°C. Infection of *B. subtilis* strains with the SPβ transducing lysates were carried out using standard procedures [11], and lysogens were selected at either 5 μg ml^{-1} chloramphenicol or 1 μg ml^{-1} erythromycin plus 25 μg ml^{-1} lincomycin.

Construction of integration vectors containing wild-type or P_{15}-modified rib operons

An integration vector (pRF39) with the wild-type *rib* operon, was first constructed by inserting a *rib*-containing 6.5-kb *Xba*I-*Eco*RI from pRF36 into pUC19. To integrate and amplify this integration vector into the *B. subtilis* chromosome, a 1.7-kb cassette containing a chloramphenicol resistance gene (*cat*) was inserted into a unique *Eco*RI site, yielding pRF40 (Figure 1).

A brief description of the construction of vectors with P_{15}-modified *rib* operons is given below; details of the constructions including the sequences of the oligonucleotides used are described elsewhere [44]. To replace the wild-type $ribP_1$ promoter and *ribO* regulatory site with P_{15}, two oligonucleotides were synthesized that recreated the DNA sequence from the *Bgl*II site within the 5′ end of *ribG* up to a position just before the *rho*-independent transcription terminator within the leader region. Ligation of the annealed oligonucleotides to a fragment containing the remainder of *ribG* and the 5′ end of *ribB* yielded pRF46. A 0.4-kb fragment containing P_{15} from pNH202 was then placed just upstream from the RBS of *ribG*, to generate a plasmid (pRF48) with P_{15} directing the transcription of the entire *ribG* gene, and the 5′ end of *ribB*. The remainder of the riboflavin operon was then added, yielding pRF49.

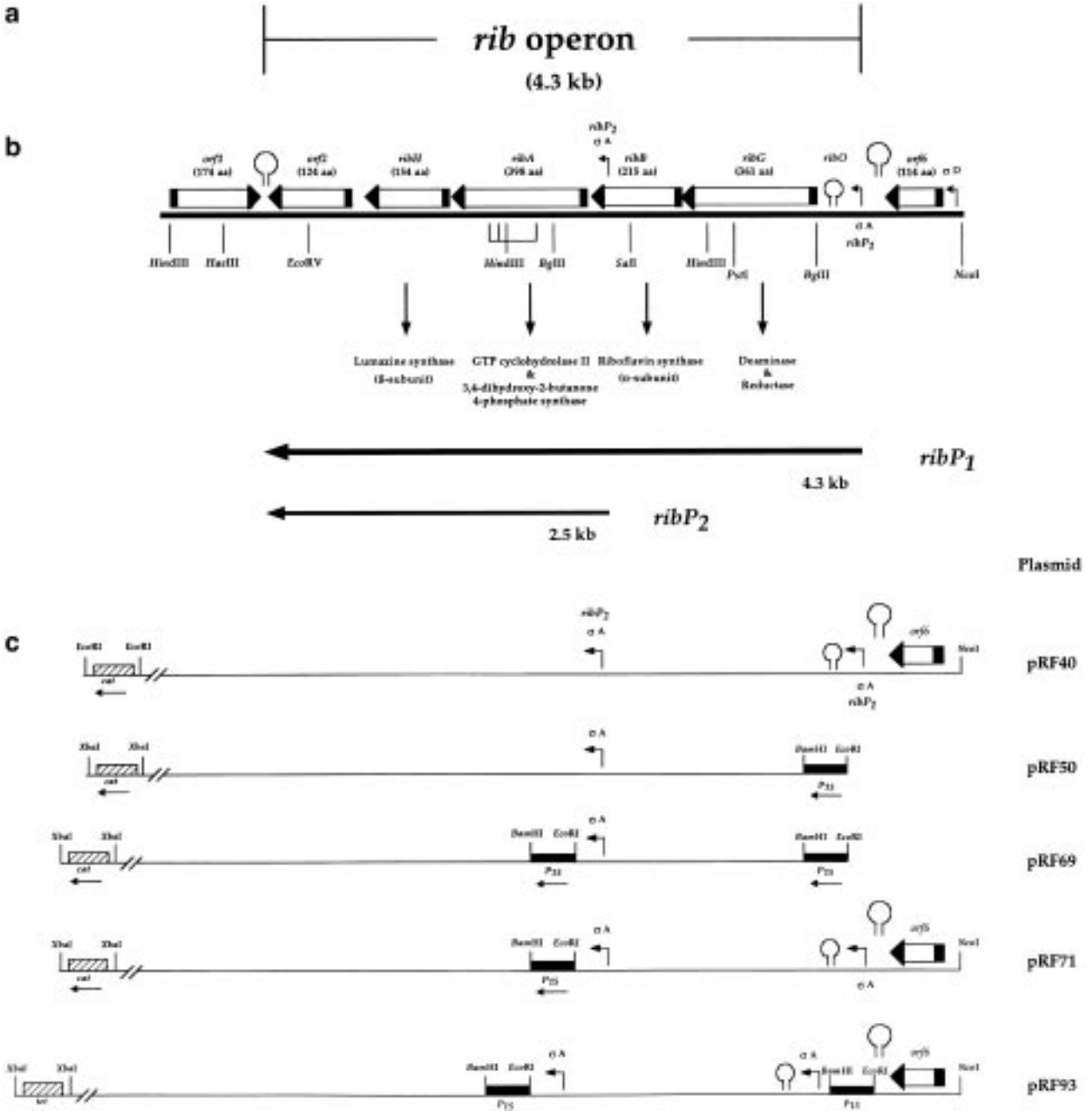

Figure 1 The *B. subtilis* riboflavin operon and integration cassettes containing either the wild-type or P_{15} promoter-modified riboflavin operons. (a) The location of the structural genes, *ribG*, *ribB*, *ribA* and *ribH*, σ^A promoter regions, *ribP*$_1$ and *ribP*$_1$ and *ribP*$_2$, and the *ribO* regulatory site. *orf1*, *orf2*, and *orf6* appear not to be involved in riboflavin synthesis [44,46]. (b) The *rib*-specific polycistronic RNA transcripts detected by Northern hybridization. (c) Replacement or bypass of the native *rib* promoters and *ribC*/RoF^r-dependent regulatory sites with a constitutive P_{15} promoter (see also Materials and Methods). Each P_{15} modified *rib* operon was derived from pRF40 containing the wild-type operon on a 6.5-kb *Xba*I-*Bam*HI fragment. At the end of each modified *rib* operon is a selectable chloramphenicol-resistance gene (*cat*) or tetracycline-resistance gene (*tet*) that allows for integration and amplification of the cassette into the chromosome of *B. subtilis*. Symbols: ■, *Bacillus* ribosome binding sites (RBS); ↰, start sites of transcription for σ^A-recognized promoters, *ribP*$_1$ and *ribP*$_2$; ♀, putative *rho*-independent transcription termination sites (the ♀ located within *ribO* is postulated to be part of a transcriptional termination/anti-termination regulatory mechanism). Not all restriction enzyme sites are shown.

Supernatants of *E. coli* cultures containing pRF49 were yellow indicating the production of riboflavin. A 1.7-kb *cat* cassette was then inserted into the unique *Xba*I site of pRF49, yielding pRF50 (Figure 1).

To place the P_{15} promoter fragment after *ribP*$_2$, two pairs of complementing oligonucleotides were prepared that placed *Bam*HI and *Eco*RI sites into the small intercistronic region between *ribB* and *ribA*. One pair recreated the sequence from a *Dra*I site within the 5′ end of *ribA*, past the start codon of *ribA*, and placed a *Bam*HI site within the 3′ portion of the intercistronic region just before the beginning of *ribA*. The other pair recreated the sequence from a

*Cla*I site within the 3′ end of *ribB*, past the stop codon of *ribB*, and placed a *Eco*RI site within the 5′ portion of the intercistronic region just after the end of *ribB*. The 3′ portion of the engineered intercistronic region was connected first with the last three genes of the riboflavin operon, yielding pRF64 in which the *Bam*HI site is placed immediately upstream of the RBS of *ribA*.

The 5′ portion of the intercistronic region plus the 3′ end of *ribB* was then connected to the 5′ end of the 0.4-kb P_{15} promoter fragment yielding pRF63. This 470-bp fragment containing the P_{15} promoter with the 3′ end of *ribB* was connected to the *Bam*HI-containing fragment containing the last three genes of the operon described above to reconstitute pUC19 plasmids with two different riboflavin operons. One plasmid contained the riboflavin operon with the wild-type $ribP_1$ promoter and regulatory region and the P_{15} promoter upstream of *ribA*. Another plasmid contained the riboflavin operon with two P_{15} promoters, one at the beginning of the operon and a second upstream of *ribA*. Cassettes containing the *cat* genes were then inserted into the unique *Xba*I sites of both plasmids to yield integration vectors pRF69 and pRF71 (Figure 1).

The P_{15} promoter was also introduced within the riboflavin operon without removing the native *rib* promoter and regulatory region. A synthetic 55-bp DNA oligomer containing *Eco*RI, *Sma*I, and *Bam*HI restriction sites was first introduced into a 30-bp non-essential region between the $ribP_1$ promoter and an upstream putative rho-independent transcription site (Figure 1) using site-directed mutagenesis. After rejoining the modified *rib* promoter region to the *rib* structural genes of the operon, a 0.4-kb fragment containing P_{15} from pNH202 was inserted between the *Eco*RI and *Bam*HI restriction sites upstream of the $ribP_1$ promoter. This engineered *rib* operon was further modified to contain a second copy of the $ribP_1$ promoter upstream of *ribA*. This was done by replacing the 2.0-kb *Bgl*II fragment containing the native *ribB-ribA* region with a 2.4-kb *Bgl*II fragment containing the $ribP_1$ promoter upstream of *ribA* (described above). The 1.7-kb *cat* cassette was then inserted into the unique *Xba*I site yielding pRF89. Plasmid pRF93 (Figure 1) was constructed by replacing the 1.7-kb *Xba*I *cat* cassette with a 2.4-kb cassette containing the *tet* gene derived from the *Bacillus* plasmid pBC16 [6].

Integration of P_{15}-engineered rib operons at bpr

The *tet* cassette was first inserted into *bpr*, a non-essential gene of *B. subtilis* encoding bacillopeptidase F [51], to provide a site of homology for integration of P_{15}-modified *rib* operons. *Eco*RV-digested pKT2 containing the *bpr* gene was ligated to a 2.4-kb *tet* cassette, yielding pKT2-*tet*. This gene disruption was inserted into the *B. subtilis* chromosome by transforming a riboflavin-deregulated strain with linearized pKT2-*tet*, and selecting for colonies on TBAB medium with 10–15 μg ml^{-1} tetracycline. The *rib* operon was then deleted from one of these Tc^r colonies, RB54, to ensure that the integration vectors would insert at *bpr*. A 7.2-kb *Xba*I fragment containing the *rib* operon and flanking regions was removed from plasmid pRF34 by digesting the plasmid with *Xba*I and ligating the cut DNA under dilute DNA concentrations. The resulting plasmid pRF82 was linearized and transformed into RB54 selecting for colonies that grew on TBAB medium containing 5 μg ml^{-1} chloramphenicol. Cm^r colonies were screened for riboflavin auxotrophy and one Rib$^-$ Cm^r colony, RB55, was recovered. Southern blots confirmed the deletion of the *rib* operon and the insertion of *tet* into *bpr*.

RB55 was used as a recipient host to integrate *tet*-containing integration vectors with P_{15}-modified *rib* operons (eg pRF93) into the *bpr::tet* locus. This was done by transforming RB55 with plasmid DNA and selecting for Rib$^+$ transformants on minimal medium. All Rib$^+$ transformants produced a bright yellow pigment, indicating the production of riboflavin; Southern blots also confirmed the presence of these vectors at *bpr*. PBS1 transduction was then used to transfer the integrated P_{15}-modified *rib* operons to RB50 containing pRF69 at the *rib* locus, selecting for tetracycline-resistant colonies.

Fermentation conditions

Two shake-flask seed cultures were prepared prior to fermentation. The first seed culture consisted of 25 ml of riboflavin minimal medium (RMM; SS medium supplemented with 0.2 g L^{-1} each of casamino acids and yeast extract (Difco)) in a 300-ml baffled flask. After growth at 37°C for 8 h, 6 ml of the growing culture were used to inoculate 300 ml of fermentation medium supplemented with approximately 3.5% glucose and 7% maltose in a 2-liter flask [44]. After incubation for 12 h at 37°C, the contents were transferred to 7 L of fermentation medium in a 14-liter Chemap bench scale fermentor. Both seed culture media contained either chloramphenicol or a combination of chloramphenicol and tetracycline.

All fermentations were grown using carbon-limited fed-batch growth conditions with computer control of dissolved oxygen concentrations as previously described [44]. The initial carbohydrate was exhausted between 4–8 h of growth, at which time the carbohydrate feeding was initiated. The rate of carbohydrate feeding was computer-controlled to maintain the dissolved oxygen at 15 ± 5% of saturation throughout the remaining fermentation. Typically, fermentation was completed after 48 h.

Results

Isolation of strains containing multiple purine analog-resistant mutations

Purine analogs, 8-azaguanine and decoyinine, and the glutamine antagonist methionine sulfoxide were used to generate mutations in the purine biosynthetic pathway of *B. subtilis* to maximize the metabolic flow of carbon to GTP. A *purA60* mutation was used to block the flow of IMP to AMP and related purine compounds. Strains containing different combinations of these mutations were obtained as diagrammed in the flow chart in Figure 2.

Azaguanine-resistant mutants that contained defects in purine regulation were first isolated from an EMS-mutagenized *B. subtilis* 168 strain using a strategy similar to that described by Ishii and Shiio [25,26]. Az^r mutants appeared at a frequency of 9×10^{-6}. Az^r mutants were also resistant to 8-azaxanthine and were found to grow on hypoxanthine suggesting that resistance to azaguanine was the result of increased expression of the purine biosynthetic

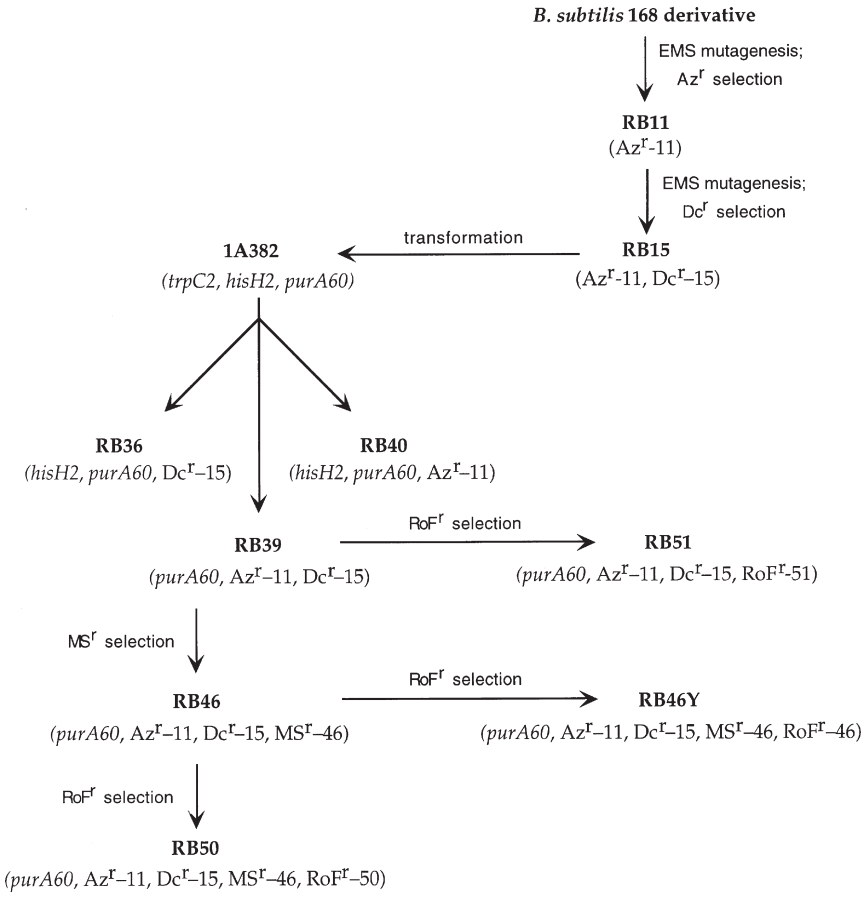

Figure 2 Flow chart diagramming the isolation of *B. subtilis* 168 strains with different combinations of relevant purine and riboflavin analog-resistant mutations.

genes and not in a block in the purine transport system [50]. One such mutant, RB11 (*spoOA*, Az^r-11) was mutagenized again with EMS and decoyinine-resistant mutants were obtained at a frequency of 1×10^{-5}. Resistance to decoyinine is reported to result from changes in GMP synthetase (the *guaA* gene product) that enhance conversion of XMP to GMP [33,34]. Enzymatic tests to determine the alterations in the purine pathway were not performed.

The Az^r and Dc^r mutations from one mutant, RB15 (*spo0A*, Az^r-11, Dc^r-15), were transferred into *B. subtilis* 1A383 (*trpC2, hisH2, purA60*) by DNA congression to separate the purine analog-resistant mutations from any unwanted EMS-induced mutations. In addition, *purA60* mutants are defective in adenylsuccinate synthetase activity, causing a block in synthesis of AMP from IMP [29]. Consequently, we anticipated that combining the two purine analog-resistant mutations with the *purA60* mutation should further increase the metabolic flow from IMP to GTP. Among Trp$^+$ transformants, 3.3% were also Dc^r (eg RB36), 2.3% were Az^r (eg RB40), and 0.3% were both Dc^r and Az^r. A Trp$^+$ Dc^r Az^r transformant, RB39, was found to be His$^+$ and Spo$^-$, indicating that the *his*$^+$ and *spo0A* alleles from RB15 were also introduced into 1A383 by congression. RB39 failed to grow on either guanine or hypoxanthine confirming the presence of the *purA* mutation. Several spontaneous methionine sulfoxide-resistant mutants of RB39 were next isolated, of which RB46 (*purA60, spo0A*, Az^r-11, Dc^r-15, MS^r-46) was saved for further study. Methionine sulfoxide-resistant mutations are speculated to improve the metabolic flow from IMP to XMP by increasing IMP dehydrogenase activity, and to limit degradation of IMP to inosine by decreasing 5′-nucleotidase activity [32]. Riboflavin was not detected when colonies of RB9, RB11, RB15, RB39, and RB46 were exposed to long-wave UV light.

Isolation of riboflavin analog-resistant mutants

Several strains containing multiple purine analog-resistant mutations were selected for resistance to the riboflavin analog roseoflavin in order to deregulate expression of the riboflavin biosynthetic genes [4,35]. Spontaneous RoF^r mutants were recovered at a frequency of 5×10^{-5}. RoF^r mutants from RB36 and RB40 (Figure 2), all showed a low level of fluorescence on minimal media agar plates when exposed to long-wave UV light (366 nm), indicating some riboflavin production. This level of fluorescence was similar to RoF^r mutants of 1A382 containing only the *purA60* mutation. Interestingly, RoF^r mutants from RB39 and RB46 produced a different phenotype with respect to riboflavin production. Among the RoF^r colonies that produced some level of fluorescence, approximately 0.5–1% of the colonies produced an intensely fluorescent yellow colony. This fluorescent-yellow colony phenotype was stable and corre-

lated with a higher level of riboflavin production. Minimal medium shake flask cultures of two such examples, RB51 arising from RB39 and RB50 from RB46, contained about 40 mg L^{-1} and 30 mg L^{-1} of riboflavin, respectively. By comparison, RB46 and one of the low level fluorescent RoF^r mutants obtained from RB46, produced 7 and 14 mg L^{-1} of riboflavin, respectively, when grown in minimal medium shake flask cultures. The RoF^r mutation of RB50 has been recently mapped to the *ribC* locus which has been shown to encode riboflavin kinase [10,17,30].

Based on these results, both the Az^r-11 and Dc^r-15 mutations in RB39 appeared to be necessary for producing higher levels of riboflavin (presumably by overproduction of GTP), since no intensely fluorescent RoF^r colonies were found in strains containing only the Az^r-11 or Dc^r-15 mutation. The MS^r-46 mutation did not appear to contribute significantly to higher riboflavin production because colonies with an intensely fluorescent phenotype could be obtained in non-MS^r strains such as RB51.

To determine whether the RoF^r mutation deregulates transcription of the *rib* operon, restriction fragments containing the two σ^A promoters that control transcription of the riboflavin operon, $ribP_1$ and $ribP_2$ [43], were fused to a reporter *lacZ* gene as described in Materials and Methods. SPβ specialized transducing phage carrying either $ribP_1$-*lacZ* or $ribP_2$-*lacZ* were constructed and used to insert the fusions into a *B. subtilis* prototroph (PY79) and RB50 carrying the RoF^r mutation as described in Materials and Methods. As shown in Table 1, the overall expression of $ribP_1$-*lacZ* in wild-type cells was low, but nevertheless showed riboflavin-specific regulation: β-galactosidase was repressed in the presence of riboflavin (2 μg ml^{-1} final concentration) and induced 1.5 to 2.5-fold in its absence. However, in the presence of the RoF^r mutation, β-galactosidase activity was induced 10 to 15-fold. Similar results were obtained when the $ribP_1$-*lacZ* fusion was introduced by integration of a circular plasmid by Campbell-like recombination at the *rib* locus (data not shown). In addition, comparison of RB50 and RB46 containing the Campbell-integrated fusion showed that higher constitutive β-galactosidase levels were caused by the RoF^r mutation and not by the purine-analog resistant mutations (data not shown).

The RoF^r mutation also appeared to deregulate expression of the $ribP_2$-*lacZ* fusion (Table 1). Expression of $ribP_2$-*lacZ* in wild-type cells was extremely low with or without the presence of riboflavin, so it was not possible to determine whether riboflavin levels regulated $ribP_2$ activity. However, β-galactosidase activity of the fusion was induced 5 to 7-fold in RB50. These results indicated that the RoF^r mutation deregulates expression of the *rib* genes by increasing the level of transcription from both the $ribP_1$ and $ribP_2$ promoters.

Increasing the gene dosage of wild-type riboflavin biosynthetic genes increases riboflavin production

Plasmid pRF8 containing the entire wild-type riboflavin operon on a 10-kb *Eco*RI fragment in the *E. coli* vector pBR322 with a *cat* (chloramphenicol acetyltransferase) gene selectable in *B. subtilis* was transformed into four different *B. subtilis* strains: the riboflavin overproducer RB50, the RB50 parents RB46 and 1A382, and IS75 (*metB5*, *leuA8*, *hisA1*) a common *B. subtilis* 168 laboratory strain. Since pRF8 cannot replicate in *B. subtilis*, chloramphenicol-resistant cells arose by integration of the entire plasmid into the native *rib* locus. The copy number of the integrated pRF8 cassette was increased by selecting for colonies that grew at higher chloramphenicol concentrations [27]. In each strain, colonies were able to grow up to 60 μg ml^{-1} of chloramphenicol. Neither RB46, 1A382, or IS75 (none of which contain the RoF^r mutation) containing amplified pRF8 (having the wild-type $ribP_1$ and $ribP_2$ promoters) displayed the riboflavin overproduction phenotype (ie yellow, fluorescent colonies). Conversely RB50::$[pRF8]_n$ which contains the RoF^r mutation yielded colonies that were more fluorescent yellow than RB50 without the amplified *rib* genes. This finding indicated that amplification of the *rib* genes alone was not sufficient to overproduce riboflavin; overproduction also required the *ribC* mutation, which deregulates expression of the riboflavin biosynthetic genes. In minimal medium shake-flask cultures supplemented with adenine, RB50::$[pRF8]_n$ resistant to 60 μg ml^{-1} chloramphenicol produced approximately 10-fold more riboflavin (0.4–0.7 g L^{-1}) than RB50 (0.02–0.05 g L^{-1}). Similar levels of riboflavin were produced when the size of the amplified *rib* cassette was reduced from 10 kb in RB50::$[pRF8]_n$ to 6.5 kb in RB50::$[pRF40]_n$ (Figure 1; data not shown).

Table 1 Expression of SPβ::*rib-lacZ* transcriptional fusions in wild-type and a *ribC* mutant of *B. subtilis*

Expt	Strain	Relevant genotype	β-galactosidase specific activity[a]	
			+ Riboflavin (2 μg ml^{-1})	− Riboflavin
1	PY79/$ribP_1$-*lacZ*	*rib*$^+$	18	31
	RB50/$ribP_1$-*lacZ*	*rib*$^+$*ribC*	280	280
	PY79/$ribP_2$-*lacZ*	*rib*$^+$	0.4	0.4
	RB50/$ribP_2$-*lacZ*	*rib*$^+$*ribC*	2.1	2.9
2	PY79/(P_{15}-*rib*)-*lacZ*	*rib*$^+$	1690	2040
	RB50/(P_{15}-*rib*)-*lacZ*	*rib*$^+$ *ribC*	1540	1800

[a]Calculated according to Miller [36].

Replacement of rib promoters with SPO1 phage promoters

We attempted to further increase transcription of the *rib* genes by either replacing or bypassing the native *rib* promoters and *ribC*/RoF^r-dependent regulatory sites with constitutive P_{15} promoters derived from the *Bacillus* bacteriophage SPO1.

To introduce strong constitutive promoters into the *rib* operon, short synthetic DNA linkers containing unique *Eco*RI and *Bam*HI restriction sites were placed either 14 base pairs upstream from the ribosome-binding site of *ribG*, the first gene of the operon, or within the polycistronic region between *ribB* and *ribA*, as described in Materials and Methods. It was not possible to remove $ribP_2$ since this promoter was embedded within the *ribB* gene. Ligation of restriction fragments containing the bacteriophage SP01 early gene promoter P_{15} [28] to these sites resulted in integration vectors with three different promoter-modified operons (Figure 1): a riboflavin operon with P_{15} replacing the native $ribP_1$ promoter and regulatory region (pRF50), an operon with the native $ribP_1$ promoter and regulatory region and P_{15} upstream of *ribA* (pRF71), and an operon with two P_{15} promoters, one upstream of *ribG* and another upstream of *ribA* (pRF69). Transformation of these plasmids into *E. coli* DH5α cells resulted in colonies that produced a bright yellow pigment, which was subsequently shown to be secreted riboflavin. Conversely, *E. coli* cells harboring pRF8, which contained just the wild-type *rib* operon, did not secrete significant levels of riboflavin (ie white colonies). These results indicate that the P_{15} promoter was functioning to promote constitutive production of riboflavin biosynthetic enzymes.

To assess the ability of the P_{15}-driven riboflavin operons to produce riboflavin in *B. subtilis*, pRF50, pRF69 and pRF71 were integrated and amplified into four different *B. subtilis* strains: the riboflavin overproducer RB50, the RB50 parents RB36 and RB46, and 62178 containing a defective GMP reductase (*guaC*) that reduces conversion of GMP to IMP [41]. Only the modified *rib* operon with both the 5′ and internal P_{15} promoters (pRF69) produced bright fluorescent yellow colonies indicative of riboflavin in *B. subtilis* strains containing one or more purine mutations. The Dc^r-15 mutation appeared to contribute significantly to riboflavin production whereas the Az^r-11 mutation did not. Interestingly, colonies with the most yellow fluorescence were observed when any of the three vectors were introduced into RB50 containing the roseoflavin and purine analog-resistant mutations. These results indicated that the *ribC* mutation was still required for maximum riboflavin production.

To measure the transcription level of the P_{15} promoter, a SPβ transducing phage carrying *lacZ* fused to the 5′ end of the P_{15}-modified *rib* operon from pRF50 was constructed and introduced into PY79 (*rib*$^+$) and RB50 carrying the *ribC* mutation as described in Materials and Methods. As shown in Table 1, the (P_{15}-*rib*)-*lacZ* fusion generated extremely high levels of β-galactosidase activity in both wild-type and *ribC* deregulated strains (1500–2100 Miller units), which were approximately 4–8 fold higher than levels from the *ribC*-deregulated $ribP_1$-*lacZ* fusion. These results are as expected for a strong constitutive σ^A promoter of *B. subtilis*.

Northern hybridization experiments were also used to examine the level and size of P_{15}-specific *rib* transcripts. Total cellular RNA isolated from RB50::$[pRF50]_n$ Ade$^+$ (Figure 3, lane 3) was isolated and hybridized to a probe that corresponds to the 5′ end of *ribG*. Surprisingly, full-length transcripts (>4.0 kb) were not observed. Instead, the probe strongly annealed to a series of smaller transcripts with molecular weights of 0.6, 0.9, 1.1, 1.3 and 2.1 kb, which decreased in intensity as they got larger. These mRNA species were not detected with a probe corresponding to the 5′ end of *ribA* (data not shown), indicating that these transcripts only encompassed all or part of the first two structural genes, *ribG* and *ribB*. These transcripts were not artifacts of the RNA isolation procedure because hybridization of the probes to total RNA from RB50::$[pRF8]_n$ containing amplified, wild-type *rib* operons produced transcripts of the expected size [2]: a full-length transcript of 4.2 kb originating from $ribP_1$ (lane 1) and a 2.5-kb transcript originating from $ribP_2$ encompassing the last three genes of the operon (lane 2). Analysis of similar P_{15}-driven *rib* operons constructed using PCR-synthesized fragments produced a similar pattern of transcripts [20]. Conversely, the expected full length transcript (2.3 kb) originating from the second internal P_{15} promoter was observed when mRNA isolated from RB50::$[pRF71]_n$ (Figure 3, lane 4) or RB50::$[pRF69]_n$ Ade$^+$ (data not shown) was probed with the *ribA*-specific probe, confirming that the last three *rib* genes were under transcriptional control of the internal P_{15} promoter.

Production of riboflavin by fermentation

Riboflavin overproducers were analyzed for riboflavin production in 14-liter Chemap bench scale fermentors using carbon-limited fed-batch growth conditions with computer control of dissolved oxygen concentrations as described in Materials and Methods. Medium composition was optimized during the course of these experiments so that increases in riboflavin production described below resulted from both media and genetic improvements.

Since all the engineered *B. subtilis* riboflavin production strains contained the *purA60* mutation, initial studies were conducted to optimize the amount of adenosine required for fermentation. The strains were unstable in their requirement for adenosine, and Ade$^+$ revertants were recovered at a frequency of between 0.1 and 1%. Ade$^+$ revertants were not isolated from RB50 alone, suggesting that appearance of these revertants was enriched in strains producing high levels of riboflavin. More important, Ade$^+$ revertants grew faster and produced 25% more riboflavin than their Ade$^-$ parent. Fermentation of Ade$^+$ revertants of RB50::$[pRF8]_n$ produced 6.3 g L^{-1} riboflavin in 49 h (Table 2). Slightly higher levels of riboflavin (7.4 g L^{-1} in 48 h) were produced when the size of the *rib*-containing fragment was reduced from 10 kb (pRF8) to 6.5 kb (pRF40).

The highest levels of riboflavin production were observed with RB50 strains containing P_{15}-engineered *rib* operons. Amplified strains of RB50::$[pRF50]_n$ Ade$^+$, RB50::$[pRF71]_n$ Ade$^+$, and RB50::$[pRF69]_n$ Ade$^+$, produced 11.0 g L^{-1}, 10.5 g L^{-1} and 14 g L^{-1} riboflavin,

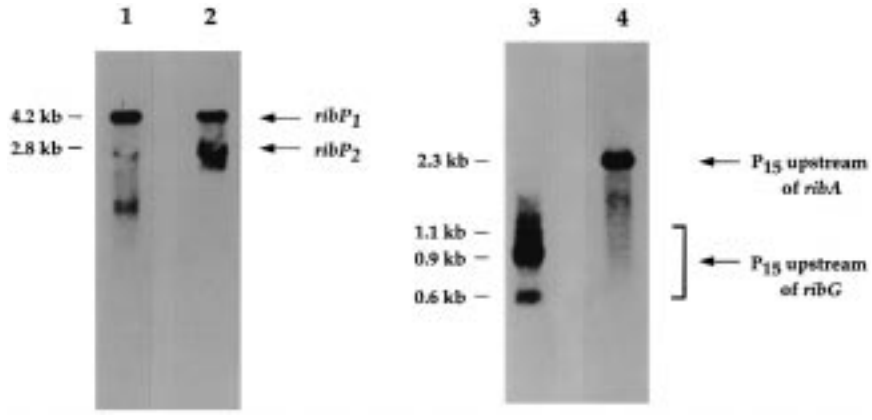

Figure 3 Northern blots showing *rib*-specific mRNAs transcribed from either wild-type or P_{15} promoters. Total RNA was isolated from strains grown in minimal medium in the absence of riboflavin as described in Materials and Methods. Seven micrograms of RNA were separated on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membrane filters; the filters were incubated with synthetic oligonucleotides specific to either *ribG* (lanes 1 and 3) or *ribA* (lanes 2 and 4). Lanes: 1 and 2, RB50::$[pRF40]_n$; 3, RB50::$[pRF50]_n$; 4, RB50::$[pRF71]_n$. Unlabeled RNA markers, ranging from 0.36 to 9.49 kb, were used to estimate mRNA size.

Table 2 Production of riboflavin by fermentation of RB50 containing integrated copies of wild-type and P_{15}-engineered *rib* operons

Strain	Media	Riboflavin (g L^{-1})
RB50::$[pRF8]_n$ Ade^+	RBF-42	6.3
RB50::$[pRF40]_n$ Ade^+	RBF-42	7.4
RB50::$[pRF50]_n$ Ade^+	RBF-42	9.0
RB50::$[pRF50]_n$ Ade^+	RBF-150	11.0
RB50::$[pRF71]_n$ Ade^+	RBF-150	10.5
RB50::$[pRF69]_n$ Ade^+	RBF-150	14.0

[a]See Reference [44] for fermentation media composition.

respectively (Table 2). Under light microscopy, yellow crystals were visible in the fermentation broth; subsequent analysis confirmed that these crystals were pure riboflavin [57]. Riboflavin titers increased with increased expression of the *rib* genes suggesting that expression of the *rib* biosynthetic genes was limiting for riboflavin production. Consequently, we speculated that higher riboflavin titers were possible if additional copies of the P_{15}-driven *rib* operons could be introduced into the *B. subtilis* chromosome.

Introduction of a second P_{15}-driven rib operon at the bpr locus

Using the method described in Materials and Methods, plasmid pRF93 was integrated into the *bpr* locus of RB50::[pRF69], generating a strain containing two chromosomal loci with amplifiable P_{15}-modified *rib* operons. Plasmid pRF93 is a derivative of pRF69 in which the 5′ P_{15} promoter was positioned upstream of $ribP_1$ and the *ribO* regulatory region, and the *cat* gene was replaced by the *tet* gene (Figure 1). Integration of pRF93 into the *bpr* locus occurs by Campbell-like recombination between *tet* sequences on the plasmid and the chromosome. The copy-number of both pRF69 and pRF93 cassettes was increased by selecting for colonies that grew at higher levels of both chloramphenicol and tetracycline. An Ade^+ revertant of the resulting strain RB50::$[pRF69]_n$::$[pRF93]_m$ was then tested for riboflavin production in bench scale fermentors as described above. Riboflavin yields were significantly higher than titers obtained with RB50::$[pRF69]_n$ Ade^+ under similar fermentation conditions.

Discussion

The enzymatic activities required to catalyze the biosynthesis of riboflavin from GTP and ribulose-5-phosphate are encoded by four genes (*ribG, ribB, ribA,* and *ribH*) in *B. subtilis* (Figure 4). These genes are located in an operon the gene order of which differs from the order of enzymatic reactions. GTP cyclohydrolase II activity which catalyzes the first step in riboflavin biosynthesis, synthesis of structure **2** from GTP, is encoded by the third gene in the operon, *ribA*. The RibA protein also contains a second enzymatic function which synthesizes a four-carbon unit (structure **7**) from ribulose-5-phosphate [47,48] that is utilized in a later step (lumazine synthase reaction). The second and third enzymatic steps, deamination of the pyrimidine ring of structure **2** and the subsequent reduction of the ribosyl side chain to form structure **4**, are controlled by another bi-functional enzyme encoded by the first gene of the operon *ribG* [46]. The penultimate step in riboflavin biosynthesis, condensation of the four-carbon unit with structure **5**, is catalyzed by lumazine synthase, the product of the last *rib* gene, *ribH*. Riboflavin synthase which controls the last step of the pathway, conversion of structure **8** to riboflavin, is encoded by the second gene of the operon, *ribB*. The dephosphorylation step of structure **4** is hypothetical. Evidence for a specific phosphatase has not been obtained; however it is possible that a phosphatase of broad substrate specificity may catalyze this step [3]. Transcription of the four riboflavin genes is primarily controlled by the $ribP_1$ promoter and regulatory region located at the 5′ end of the operon. In addition, the last two *rib* genes in the operon, *ribA* and *ribH*, are also transcribed from a second promoter ($ribP_2$) and regulatory region. It is probably not accidental that *ribG* and *ribA* encode bi-functional enzymes. This may represent a mechanism by which *B. subtilis* coordinates the synthesis of these biosynthetic activities. Finally, *ribH* is followed by an open read-

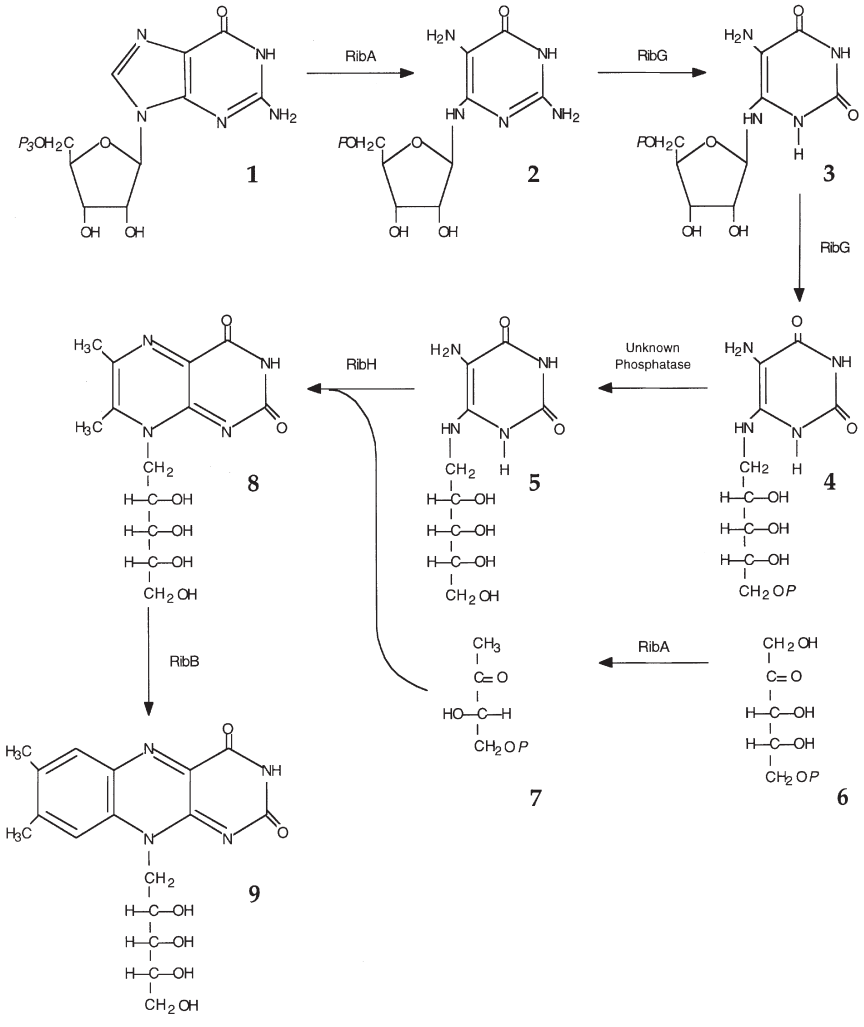

Figure 4 The riboflavin biosynthetic pathway of *B. subtilis*. The corresponding intermediates shown are those produced by *E. coli* and *B. subtilis*: structure **1**, guanosine triphosphate (GTP); structure **2**, 2,5-diamino-6-(ribosylamino)-4 (3H)-pyrimidinone-5′-phosphate; structure **3**, 5-amino-6-ribosylamino)-2,4 (1H, 3H)-pyrimidinedione-5′-phosphate; structure **4**, 5-amino-6-(ribitylamino)-2,4(1H, 3H)-pyrimidinedione-5′-phosphate; structure **5**, 5-amino-6-(ribitylamino)-2,4 (1H, 3H)-pyrimidinedione; structure **6**, ribulose-5′-phosphate; structure **7**, 3,4-dihydroxy-2-butanone 4-phosphate; structure **8**, 6,7-dimethyl-8-ribityllumazine; structure **9**, riboflavin. Structures are adapted from Bacher [5].

ing frame, *orf2*, previously referred to as *ribTD*. The function of this open-reading frame is not known and its gene product is not required for riboflavin synthesis [44,46].

The expression of *rib* genes is regulated by the *ribC* gene whose product has recently been shown to be flavin kinase [10,17,30]. Missense mutations in *ribC* (such as RoF^r-50) that alter the activity of flavin kinase, greatly enhance the expression of genes from both the $ribP_1$ and $ribP_2$ promoters as shown here with the *lacZ* transcriptional fusions. Exactly how *ribC* mutations affect the transcription of the *rib* operon is not known. However, there is a strong *rho*-independent transcription terminator between $ribP_1$ and the first gene in the operon, *ribG*, suggesting a termination/antitermination mode of control that is indirectly affected by the *ribC* gene product [30,39,44]. Furthermore, a number of *cis*-acting constitutive mutations have been mapped to the leader region between the promoter and terminator [39]. The 3′ end of the leader region also contains a small open reading frame (50 amino acids) of unknown function that overlaps the terminator [39,44,52].

In the engineered *rib* vector pRF50, the entire $ribP_1$ promoter and putative regulatory region were deleted and replaced with a constitutive phage SPO1 promoter, P_{15}. Surprisingly, pRF50, did not yield a bright, yellow fluorescent colony when integrated in the genome of wild-type *B. subtilis*. We found that the $ribP_2$ promoter and regulatory region also needed to be bypassed with a second constitutive promoter to yield significant riboflavin overproduction in a wild-type cell. Northern blots confirmed the need for replacing or bypassing both promoters to achieve enhanced transcription of all four *rib* genes. Unlike wild-type *B. subtilis* which accumulated small amounts of an RNA transcript that covered the entire *rib* operon, cells with pRF50 accumulated large amounts of shorter transcripts that covered primarily the first two genes of the operon. The second P_{15} promoter engineered upstream of *ribA* was required to obtain significant accumulation of RNA transcripts that covered the last two *rib* genes in the operon.

Even in the presence of a strain with multiple, integrated copies of the pRF69 vector with two P_{15} promoters, the

ribC mutation was still required for maximum production of riboflavin. This probably resulted in part from the retention of the wild-type $ribP_1$ and $ribP_2$ promoters after recombination between the chromosomal *rib* operon and the pRF69 integration vector containing the engineered *rib* operon. Depending on the site of recombination, the amplified *rib* cassette would contain multiple copies of either the wild-type $ribP_2$ promoter alone or of the P_{15} promoter introduced downstream of $ribP_2$. Most frequently it was the $ribP_2$ promoter alone that was amplified [42]. Conversely, because the pRF93 vector utilizes the *tet* gene as the source of homology for integration and amplification at the *bpr* locus, only the P_{15} promoter introduced downstream of $ribP_2$ is amplified with this vector. In addition to deregulating the activity of the wild-type *rib* promoters, mutations in the riboflavin kinase may also enhance riboflavin production by decreasing the amount of riboflavin converted to FMN and FAD [30].

We observed that fermentation of RB50::$[pRF69]_n$: :$[pRF93]_m$ containing multiple copies of modified, deregulated *rib* operons at two different sites in the *B. subtilis* genome, produced significantly more riboflavin than strains containing only one amplifiable engineered *rib* operon. This indicated that in a strain deregulated for purine and riboflavin production, the activity of the riboflavin biosynthetic enzymes is the rate limiting step in riboflavin biosynthesis. Additional fermentation analysis of this strain is described by Hümbelin *et al* [22] in this issue. Moreover, these investigators report that a further increase in riboflavin titers is observed when a single copy of a constitutively expressed *ribA* gene is introduced into RB50::$[pRF69]_n$::$[pRF93]_m$. This suggests that expression of *ribA* is rate-limiting for riboflavin production in *B. subtilis*.

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