Regulation of Riboflavin Biosynthesis in *Bacillus subtilis* Is Affected by the Activity of the Flavokinase/Flavin Adenine Dinucleotide Synthetase Encoded by *ribC*

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This work shows that the *ribC* wild-type gene product has both flavokinase and flavin adenine dinucleotide synthetase (FAD-synthetase) activities. RibC plays an essential role in the flavin metabolism of *Bacillus subtilis*, as growth of a *ribC* deletion mutant strain was dependent on exogenous supply of FMN and the presence of a heterologous FAD-synthetase gene in its chromosome. Upon cultivation with growth-limiting amounts of FMN, this *ribC* deletion mutant strain overproduced riboflavin, while with elevated amounts of FMN in the culture medium, no riboflavin overproduction was observed. In a *B. subtilis* *ribC*820 mutant strain, the corresponding *ribC*820 gene product has reduced flavokinase/FAD-synthetase activity. In this strain, riboflavin overproduction was also repressed by exogenous FMN but not by riboflavin. Thus, flavin nucleotides, but not riboflavin, have an effector function for regulation of riboflavin biosynthesis in *B. subtilis*, and *ribC* seemingly is not directly involved in the riboflavin regulatory system. The mutation *ribC*820 leads to deregulation of riboflavin biosynthesis in *B. subtilis*, most likely by preventing the accumulation of the effector molecule FDN or FAD.

Various *Bacillus* species have gained increasing importance as host strains for industrial fermentation processes for, e.g., proteases, purine nucleotides, or vitamins (1, 37, 39). Recently, a commercially attractive riboflavin production process using *Bacillus subtilis* Marburg 168 as a host strain has been developed. This strain was optimized for riboflavin production by means of classical mutagenesis procedures and genetic engineering techniques (34). The genes encoding the riboflavin biosynthetic enzymes of *B. subtilis* were found to be clustered in a single 4.3-kbp operon (rib operon) which was mapped at 209° of the *B. subtilis* chromosome (29, 30, 33). The gene products of the rib operon (RibG, RibB, RibA, and RibH) catalyze the conversion of GTP and ribulose-5-phosphate to riboflavin (2, 5, 33).

An untranslated rib leader sequence of almost 300 nucleotides is present in the 5′ region of the rib operon between the transcription start and the translational start codon of the first rib gene (ribG). One class of riboflavin-overproducing *B. subtilis* mutants identified contained single-point mutations, designated ribO mutations, at various positions in the 5′ half of the rib leader sequence (23, 31). A potential rho-independent terminator is present at the 3′ end of the rib leader sequence (23, 29), suggesting that riboflavin biosynthesis in *B. subtilis* may be regulated by a transcription attenuation mechanism.

In a second class of riboflavin-overproducing mutants, designated ribC mutants, the chromosomal lesions were mapped at 147° (25). It was suggested that the riboflavin regulatory system in *B. subtilis* consists of a cis-acting operator element in the rib leader sequence, defined by the ribO mutations, and a trans-acting DNA- or RNA-binding repressor protein encoded by the ribC gene (26).

The ribC gene was recently cloned and sequenced. Surprisingly, the gene was found to have significant sequence similarities to bifunctional bacterial flavokinases/flavin adenine dinucleotide synthetases (FAD-synthetases) (12, 17). Flavokinases (EC 2.7.1.26) catalyze the conversion of riboflavin to FMN; FAD-synthetases (EC 2.7.7.2) convert FMN to FAD (3). In the present work, it is shown that ribC encodes a bifunctional flavokinase/FAD-synthetase which is essential for *B. subtilis* flavin metabolism. The gene product of a *ribC* mutant allele (*ribC*820), which leads to riboflavin overproduction in *B. subtilis* RB52 (12), has drastically reduced enzymatic activity. It is demonstrated that FMN and/or FAD, but not riboflavin, act as effector molecules controlling riboflavin biosynthesis. We present a model which explains the riboflavin overexpression phenotype of *B. subtilis* ribC mutant strains.

MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are described in Table 1. *B. subtilis* 1012 is a wild-type strain with respect to riboflavin biosynthesis (35). *B. subtilis* 1012MM002 contains *Escherichia coli ribC* at sacB. *B. subtilis* 1012MM003 contains *Saccharomyces cerevisiae FADI* at sacB. *B. subtilis* 1012MM004 contains *E. coli ribC* at sacB, and *B. subtilis* *ribC* is replaced by a neomycin cassette. *B. subtilis* 1012MM006 contains *S. cerevisiae FADI* at sacB, and *B. subtilis* ribC is replaced by a neomycin cassette. *B. subtilis* 1012mm087 has a mutation within the ribo region which leads to riboflavin overproduction (23). *B. subtilis* RB52 (34) has a mutation within the *ribC* gene (*ribC*820) (12) that as well leads to riboflavin overproduction. *B. subtilis* RB52MM100 contains *B. subtilis* ribC at sacB. *B. subtilis* RB52MM110 contains *B. subtilis* ribC820 at sacB. All genes that were introduced at sacB (*ribC*, *ribC*820, *ribF*, and *FADI*) are under the control of the medium-strength P*aux* promoter (20). *B. subtilis* RB52p210 contains the *B. subtilis* ribC locus at sacB. *B. subtilis* RB52p211 contains the *B. subtilis* ribC820 locus at sacB (11). In the latter two strains, *ribC* and *ribC*820 are under the control of the original promoter. *E. coli* XL1-Blue (10) and BL21 (40) were used as hosts for gene cloning and expression experiments, respectively. *E. coli* BL21MM01 contains *ribC* on plasmid pJF119HE (15). *E. coli* BL21MM02 contains *ribC*820 on plasmid pJF119HE.

All bacteria were grown aerobically at 37°C on Luria-Bertani (LB) medium or Spizizen's minimal medium (38). FMN (100 μM) was added for the growth of the auxotrophic *B. subtilis* strain 1012MM006. Commercially available FMN (sodium salt; Sigma F2253) was >99% (wt/wt) 5′-FMN after additional purification by high-pressure liquid chromatography (HPLC).

**Construction of plasmids.** The plasmids used in this study are described in Table 1. Plasmid pBluescript II SK– (Stratagene) was used for cloning procedures. Plasmid pJF119HE (15) was used for the overexpression of *ribC* in *E. coli*.

For gene expression, *ribC* was amplified by PCR from chromosomal DNA of *B. subtilis* 1012, using oligonucleotides BamMM1 (5′-GGCTTAAAGGATCCGGGGCCGCGGGCTGG) and EcoMM3 (5′-GCTCCGGGGGTAATTGAC1C1139GGATTGCCAAGCAATGG1123-3). The superscript numbers refer to
Plasmids tides XhoMM8 (5\' GCTGAGC146-3) and EcoMM5 (5\' -1572CGGTTT1571GATC1570-3) from E. coli, was used as a template. The plasmid pMM60 was constructed by ligation of EcoMM3 (5\' -1012GCAAGCTTTGACTG3') and EcoMM2 (5\' -125GGAGCACCATATGCA3') with EcoMM1 (5\' -125GGAGCACCATATGCA3') and EcoMM2 (5\' -125GGAGCACCATATGCA3') and EcoMM2 (5\' -125GGAGCACCATATGCA3'). The plasmid pMM60 was constructed by ligation of digested PCR fragments of ribC, neo, and rpsO (in that order) into XhoI/Sedigested pBluescript II SK. The integration/expression vector pXI12 was used for the functional introduction of genes into the sacB locus (296°) of B. subtilis. DNA amplification, cloning, and sequencing. DNA amplification and cloning were performed according to standard protocols (36). Plasmids pMM01 and pMM02 were proof-sequenced with Taq polymerase (Boehringer). Primary structures were aligned by using MegAlign version 1.05 (DNASTAR, Inc., Madison, Wis.).

**Heterologous expression of ribC.** E. coli BL21 was transformed with pMM01 (ribC wild type) and pMM02 (ribC20), and the resulting strains (BL21MM01 and BL21MM02) were aerobically cultivated at 37°C on LB medium. Gene expression was stimulated by adding 1 mM isoprpossphatholipidacyclonoropside (IPTG) after the culture had reached an optical density at 600 nm of 1.4. After 1 h of further aerobic incubation, cells were harvested by centrifugation.

**Purification of overproduced RibC from E. coli.** All procedures were carried out at 0 to 4°C except for the column chromatography steps, which were carried out at room temperature. Frozen cell paste (5 g) of E. coli BL21MM01 and BL21MM02 was resuspended in 25 ml of cold 100 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol. Cells were sonicated for 2 min at a power of 70 W in a Banson model 450 sonicator equipped with a 3/4-m. flat tip. All subsequent centrifugation steps were performed at 18,000 \times g and 4°C.

**Centrifugation for 45 min removed cell debris and unbroken cells.** The resulting supernatant was made 40% (wt/vol) in ammonium sulfate, and the precipitated proteins were collected by centrifugation. The supernatant was resuspended in 10 ml 100 mM Tris-HCl (pH 8.0). The solution was dialyzed twice
for 16 h every time against 5 liters of 50 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol. After dialysis, an aliquot (0.2 ml) of this solution was applied to a Mono S HR 5/5 cation-exchange column (Pharmacia) previously equilibrated with 50 mM potassium phosphate (pH 6.5). The flow rate was 0.5 ml/min. The bound protein was washed with 13 ml of this buffer, and elution was carried out by applying 33 ml of a linear gradient from 0 to 400 mM sodium chloride in the same buffer. RibC was eluted from the column at 10 ml (200 mM sodium chloride). Aliquots of the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue R-250. The apparently homogeneous fractions were tested directly for flavokinase activity and stored at −20°C. The enzyme was stable for at least 2 weeks under these conditions.

Protein determination. Protein was estimated by the method of Bradford (8), using the Bio-Rad protein assay and bovine serum albumin as a standard.

Protein sequence analysis. N-terminal sequencing of RibC was done by the method of Hewick et al. (19).

Mass spectroscopy and gel filtration experiments. Mass spectroscopy of the purified protein was performed as described earlier (42). The molecular masses of the native enzymes (RibC wild type and mutant) were estimated by chromatography on a Superdex 200 HR 10/30 column (Pharmacia) with 100 mM potassium phosphate (pH 7.5)–100 mM NaCl and a flow rate of 0.5 ml/min. RibC wild type eluted at 16.015 ml (34.176 kDa), and the RibC mutant eluted at 16.012 ml (34.228 kDa). The standard proteins were RNase A (13.7 kDa) eluted at 17.66 ml, chymotrypsinogen A (25 kDa) eluted at 17.00 ml, ovalbumin (43 kDa) eluted at 15.24 ml, albumin (67 kDa) eluted at 14.19 ml, aldolase (158 kDa) eluted at 13.01 ml, catalase (232 kDa) eluted at 12.92 ml, ferritin (440 kDa) eluted at 11.15 ml, and thyroglobulin (669 kDa) eluted at 9.57 ml.

Enzyme assay. HPLC analysis of flavins, and preparation of cell extracts. Flavokinase activity was measured in a final volume of 1 ml of potassium phosphate (pH 7.5) containing 50 μM riboflavin, 3 mM ATP, 15 mM MgCl₂, and 10 mM Na₂SO₄ (32). The mixture was preincubated at 37°C for 5 min, and the reaction was started by addition of the enzyme. After appropriate time intervals, an aliquot was removed and applied directly to a HPLC column (Nucleosil 10 C₁₈; 4.6 by 250 mm; Macherey & Nagel). The following solvent system was used: 0.1 M potassium phosphate (pH 7.5) containing 50 mM ammonium formate (pH 3.7). Detection was carried out with a fluorescence detector (excitation, 470 nm; emission, 530 nm; Waters Associates, Inc.). Flavokinase activity is expressed as nanomoles of FMN formed from riboflavin and ATP.

Cell extracts of B. subtilis strains were prepared as follows. Cells of an overnight culture (50 ml) were collected by centrifugation, and the cell pellet was resuspended in buffer A and sonicated for 15 min. After centrifugation (18,000 × g, 20 min), an aliquot of the supernatant was directly used in the flavokinase assay.

Riboflavin secretion was monitored by HPLC as described above. Overnight cultures were centrifuged, and an aliquot of the supernatant was applied to an HPLC column.

Gene integration into B. subtilis sacB. Transformation-competent B. subtilis 1012 was transformed with PstI-linearized pPM20 and pPM30 (1 μg each) by the previously described two-step procedure (13). The erythromycin-resistant transformants were RB52MM100 and RB52MM110. RB52MM100 and RB52MM110 were RB52MM100 and RB52MM110.

Recombination. Replacement of ribC by a neomycin cassette via double-crossover recombination. B. subtilis 1012MM003 was transformed with SacI-linearized pMM60 (1 μg), and the resulting strain was RB52MM2004. B. subtilis 1012MM003 was transformed by using SacI-linearized pMM60 (1 μg), and the resulting strain was RB512MM006.

Results

Purification and structural characterization of B. subtilis RibC from the wild-type strain 1012 and of the mutant RibC820 from the riboflavin-overproducing strain RB52. The wild-type ribC and mutant ribC820 genes from B. subtilis were separately overexpressed in E. coli, and the corresponding gene products were purified to apparent homogeneity (Fig. 1). The apparent molecular mass of 36 kDa of wild-type RibC as determined by SDS-PAGE (Fig. 1, lane 6) and the molecular mass of 35.665 kDa obtained from mass spectroscopy were in accordance with the molecular mass of 35.665 kDa that was deduced from the 951-bp ribC open reading frame (12). Thus, when overproduced in E. coli, RibC does not contain a covalently bound cofactor. From gel filtration experiments, under nondenaturing conditions, an apparent molecular mass of 34.2 kDa for the wild-type RibC and for the mutant RibC820 was deduced, suggesting a monomeric structure for each protein.

N-terminal sequence analysis of the riboflavin-overproducing strain RB52 revealed a single sequence (MKTIHTHITPHL) and confirmed the N terminus that was tentatively deduced from DNA sequence data earlier (12). Thus, ribC starts with the codon GTG, as is true for about 10% of the B. subtilis genes (18).

RibC is a flavokinase/FAD-synthetase, and the enzymatic activity of the mutant RibC820 is drastically reduced. RibC has significant similarities (>30% identity) to bifunctional bacterial flavokinases/FAD-synthetases, suggesting that RibC is the corresponding enzyme in B. subtilis producing the essential coenzymes FMN and FAD (12). The pure wild-type RibC protein showed a specific flavokinase activity of 580 U/mg of protein (Table 2), which is comparable to the specific flavokinase activity (450 U/mg) reported for a flavokinase from Breibacterium ammoniagenes (28). In addition to FMN, FAD was detected as a second product of the RibC-catalyzed enzymatic reaction (Fig. 2). Thus, RibC is a bifunctional enzyme, as is true for the flavokinase/FAD-synthetases from B. ammoniagenes (28) and E. coli (24).

The flavokinase activity of the pure mutant protein RibC820 from the riboflavin-overproducing strain RB52 was reduced to about 1% of the activity of wild-type RibC (Table 2 and Fig. 2). RibC is essential for growth of B. subtilis. The ribC820 mutation does not lead to an FMIN/FAD auxotrophic B. subtilis strain, although the flavokinase activity of the protein encoded by the mutated gene was not detected as a second product of the RibC-catalyzed enzymatic reaction (Fig. 2).

TABLE 2. Purification of wild-type RibC and mutant RibC820 from cell extracts of overproducing E. coli strains

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Wild-type RibC</th>
<th>RibC820</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavokinase activity</td>
<td>Purification (fold)</td>
<td>Flavokinase activity</td>
</tr>
<tr>
<td>Cell extract</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (40–70% [wt/vol]) purification</td>
<td>140</td>
<td>2</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>580</td>
<td>8</td>
</tr>
</tbody>
</table>

* Measured in a final volume of 1 ml of potassium phosphate (pH 7.5) containing 50 μM riboflavin, 3 mM ATP, 15 mM MgCl₂, and 10 mM Na₂SO₄ and expressed as nanomoles of FMN produced per minute per milligram of protein.
Thus, RibC is essential for the flavin metabolism of the heterologous flavokinase/FAD-synthetase-encoding gene at sacB. The resulting ribC deletion strain 1012MM006 was auxotrophic for FMN, indicating that FMN can be taken up by B. subtilis from the culture medium. No complementation was observed by using exogenous riboflavin. Mutants lacking FAD-synthetase activity could not be rescued by adding FAD to the medium.

Overexpression of wild-type ribC and ribC820 at sacB suppresses the riboflavin overproduction phenotype of the B. subtilis ribC820 strain RB52. The wild-type ribC gene driven by the medium-strength vegI promoter was introduced at the sacB locus of the riboflavin-overproducing B. subtilis strain RB52, which carries the ribC820 mutation. Again, the pXI12 integration/expression system was used. The flavokinase activity of cell extracts of the resulting strain RB52MM100 was drastically increased (54 U/mg of total cellular protein) compared to the recipient strain RB52 (flavokinase activity below the detection limit) or a wild-type B. subtilis strain (0.21 U/mg of total cellular protein). RB52MM100 did not secrete riboflavin into the culture medium. The wild-type ribC gene at the sacB locus under the control of its original promoter (strain RB52p210) also suppressed riboflavin secretion of RB52 (11).

As in the previous experiment, the mutant ribC820 gene was introduced into the chromosome of RB52. The resulting strain RB52MM110 contained two copies of ribC820, one at the ribC locus and the other at sacB under the control of the vegI promoter. This strain showed flavokinase activity (0.16 U/mg of total cellular protein), which was comparable to the flavokinase activity of a ribC wild-type strain (0.21 U/mg of total cellular protein). RB52MM110 did not secrete riboflavin into the culture medium. The ribC820 gene at sacB under the control of its original promoter (strain RB52p211) did not suppress riboflavin secretion of RB52 (11). In conclusion, wild-type ribC is dominant over mutant ribC820, preventing deregulation of riboflavin biosynthesis in ribC820 mutants. Overexpression of the mutant gene ribC820 can restore the wild-type phenotype as well. These results suggest a negative regulatory role of RibC in riboflavin biosynthesis.

FMN or FAD but not riboflavin is an effector molecule regulating riboflavin biosynthesis in B. subtilis. The ribC deletion mutant 1012MM004, which contains the ribF-encoded flavokinase/FAD-synthetase from E. coli (see above), did not overproduce riboflavin. This was also true for the FMN auxotrophic ribC deletion mutant 1012MM006 carrying the S. cerevisiae FAD1 gene (see above) when cultivated with 10 μM FMN. Upon cultivation with 0.1 to 1 μM FMN in the culture medium, however, significant amounts of riboflavin (>10 μM) were secreted (Fig. 3). The low FMN concentrations in the culture medium allowed only suboptimal growth of the bacteria, most likely due to FMN shortage or FMN depletion in the cells. These results indicate that repression of riboflavin overproduction occurs in the absence of B. subtilis RibC. The critical parameter determining the rate of riboflavin biosynthesis seemingly is the intracellular FMN or FAD concentration.

Since the flavokinase/FAD-synthetase of the B. subtilis ribC820 mutant strain has a drastically reduced enzymatic activity, the intracellular flavin nucleotide levels should be lower in the mutant strain than in a B. subtilis wild-type strain. The low nucleotide levels might be the reason for the riboflavin overexpression phenotype of ribC820. Consequently, addition of FMN to the culture medium should prevent riboflavin over-
production and secretion. In fact, in the presence of >10 μM FMN, almost complete repression of riboflavin secretion from *B. subtilis* ribC820 was observed (Fig. 4). FMN did not prevent riboflavin secretion from a *B. subtilis* ribO mutant (1012mro87) with a deregulating cis-acting mutation in the rib leader sequence (Fig. 4). Addition of 10 μM riboflavin had no repressive effect on riboflavin secretion.

These results indicate that FMN and/or FAD, but not riboflavin, act as effector molecules for regulation of riboflavin biosynthesis. With the data presented here, it cannot be distinguished whether both FMN and FAD or only one of the two flavin nucleotides has an effector function. The riboflavin overproduction phenotype of *B. subtilis* ribC820 can be explained by the reduced flavokinase/FAD-synthetase activity encoded by the mutant ribC820 gene, which prevents accumulation of inhibitory FMN and/or FAD levels.

**DISCUSSION**

*B. subtilis* strains carrying mutations in the ribC gene overproduce riboflavin and secrete the vitamin into the culture medium. Deregulation of riboflavin biosynthesis in these mutants strains was assigned previously to a defect in a putative RibC apo-repressor protein (26). The wild-type RibC protein was thought to negatively regulate riboflavin biosynthesis in combination with riboflavin, FMN, and FAD, which should act as corepressors (9). Cloning and sequencing of the ribC gene, however, revealed that the primary structure was similar to that of a number of genes encoding bifunctional bacterial flavokinases/FAD-synthetases (12, 17). In the present work, it is directly shown that RibC has flavokinase/FAD-synthetase activity. Furthermore, it is shown that RibC plays an essential role in the flavin metabolism of *B. subtilis*: a ribC deletion mutant is not viable unless a heterologous bifunctional flavokinase/FAD-synthetase gene (e.g., *E. coli* ribF) or a heterologous FAD-synthetase gene (e.g., *S. cerevisiae* FADI) in combination with exogenous FMN is present. Since the FMN but not the FAD requirements of *B. subtilis* ribC deletion mutants could be met by exogenous supply, it is reasonable to assume that FMN but not FAD can be imported by *B. subtilis*.

After having established the function of RibC in flavin metabolism, we investigated a possible direct regulatory role of the protein in riboflavin biosynthesis. A dual role of a protein in coenzyme metabolism and gene regulation would not be without precedence: the birA gene products of both *E. coli* (14) and *B. subtilis* (7) catalyze the transfer of biotin to the biotin carboxyl carrier protein, generating the physiologically active form of biotin. Together with biotin as a corepressor, *E. coli* BirA also acts as a repressor of the biotin biosynthesis genes (6).

A corresponding function in flavin metabolism and rib gene regulation was envisaged for RibC. However, the data presented here show that riboflavin biosynthesis in two *B. subtilis* mutants lacking the ribC gene is not deregulated, which excludes a direct repressive function of RibC in the regulation of the rib operon, e.g., by physical interaction with the rib operator. This conclusion is based on the very likely assumption that the unrelated *S. cerevisiae* FADI gene product does not have such a hypothetical direct repressive function on riboflavin biosynthesis in the *B. subtilis* mutant 1012MM006. It seems also very unlikely that ribF from *E. coli*, in which riboflavin biosynthesis is constitutive (4), should have a direct repressive function on riboflavin biosynthesis in the *B. subtilis* mutant 1012MM004.

Riboflavin overproduction by the FMN auxotrophic ribC deletion mutant 1012MM006 is affected by the FMN concentration but not by the riboflavin concentration in the culture broth, suggesting that FMN and/or FAD, but not riboflavin, have an effector role in regulation of riboflavin biosynthesis. This conclusion is supported by the observation, that riboflavin secretion in the ribC820 mutant strain RB52 is repressed by exogenous FMN but not by riboflavin. It is conceivable that the intracellular FMN or FAD concentration in the mutant ribC820 strain is lower than that in wild-type cells, due to the drastically reduced flavokinase/FAD-synthetase activity of the ribC820 gene product. The low flavin nucleotide concentration is sufficient for bacterial growth but does not exceed the threshold level inducing repression of riboflavin biosynthesis. As a result, riboflavin, which has no activity as a coenzyme and no corepressor function, is overproduced in the ribC820 mutant strain.

Recently, a mutation designated ribR was mapped at 236° of the *B. subtilis* genome. This mutation was reported to suppress riboflavin secretion of ribC mutant strains. It was suggested that the hypothetical gene product of the affected ribR gene together with RibC forms a putative multimeric rib repressor protein (26). The N terminus (110 amino acids) of RibR (16) shows significant sequence similarity (50% identity) to the C
terminus of RibC and other bifunctional bacterial flavokinases/FAD-synthetases (27). Kitatsuji et al. (24) reported that the flavokinase activity of the E. coli flavokinase/FAD-synthetase was associated with the C terminus. Thus, ribR might encode a flavokinase-like protein. According to our present work, RibC is seemingly the only flavokinase/FAD-synthetase present in vegetative B. subtilis wild-type strains cultivated under the usual laboratory conditions. To explain why ribR acts as a suppressor mutation in ribC mutants, one could hypothesize that the ribR mutation activates the dormant flavokinase activity of the ribR gene product or allows ribR expression in vegetative B. subtilis cells. This could lead to an increase in the intracellular FMN or FAD concentration of ribC mutants and suppression of the riboflavin overproduction phenotype. All riboflavin-overproducing B. subtilis strains isolated so far either carry a lesion in the flavokinase/FAD-synthetase-encoding ribC gene or have mutations within the cis-acting ribO leader region of the rib operon. This could mean that the selection and screening methods applied were not suited for isolation of other than these two types of deregulated mutants. Attempts to isolate new types of deregulated mutants and to identify additional components of the riboflavin regulatory system are ongoing. The lack of deregulated mutants with a lesion in a putative repressor protein could also mean that such a repressor protein does not exist and that the flavin nucleotides would negatively affect riboflavin biosynthesis in the absence of a trans-acting protein.

REFERENCES