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# 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 ribC820* mutant strain, the corresponding *ribC820* 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 *ribC820* leads to deregulation of riboflavin biosynthesis in *B. subtilis*, most likely by preventing the accumulation of the effector molecule FMN 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 (*ribC820*), 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 ribF* at *sacB*. *B. subtilis* 1012MM003 contains *Saccharomyces cerevisiae FADI* at *sacB*. *B. subtilis* 1012MM004 contains *E. coli ribF* 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* 1012mro87 has a mutation within the *ribO* region which leads to riboflavin overproduction (23). *B. subtilis* RB52 (34) has a mutation within the *ribC* gene (*ribC820*) (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*, *ribC820*, *ribF*, and *FADI*) are under the control of the medium-strength $P_{vegI}$ 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 *ribC820* 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 *ribC820* 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′-GTCTAGAGGATCC$^{22}$G GGCTGTTAAAGCCGGC$^{38}$-3′) and EcoMM3 (5′-GCTCCCGGGTGAATT C$^{1139}$GGATTGCCAAGCAATGG$^{1123}$-3′). The superscript numbers refer to

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype, antibiotic resistance, or relevant feature(s)[a] | Reference or source |
|---|---|---|
| Strains | | |
| *B. subtilis* | | |
| 1012 | *leuA8 metB5* | 35 |
| 1012MM002 | 1012 $P_{vegI}$ *ribF ermAM @ sacB* | This study |
| 1012MM003 | 1012 $P_{vegI}$ *FADI ermAM @ sacB* | This study |
| 1012MM004 | 1012 Δ*ribC*Ω*neo* $P_{vegI}$ *ribF ermAM @ sacB* | This study |
| 1012MM006 | 1012 Δ*ribC*Ω*neo* $P_{vegI}$ *FADI ermAM @ sacB* | This study |
| 1012mro87 | Riboflavin-secreting *ribO* mutant ($G^{37}$ GG→$A^{37}$AA) | 23 |
| RB52 | *Dc′-15 ribC820 guaC3 metC7 trpC2* | 34 |
| RB52MM100 | RB52 $P_{vegI}$ *ribC ermAM @ sacB* | This study |
| RB52MM110 | RB52 $P_{vegI}$ *ribC820 ermAM @ sacB* | This study |
| RB52p210 | RB52 *ribC* locus @ *sacB* | 12 |
| RB52p211 | RB52 *ribC820* locus @ *sacB* | 12 |
| *E. coli* | | |
| BL21 | Used for gene cloning and expression | 40 |
| BL21MM01 | *E. coli* BL21 containing pMM01 | This study |
| BL21MM02 | *E. coli* BL21 containing pMM02 | This study |
| XL1-Blue | Used for gene cloning | 10 |
| Plasmids | | |
| pBluescriptII SK− | High-copy-number cloning vector, *amp* | Stratagene |
| pJF119HE | Low-copy-number expression vector, *amp* $P_{tac}$ $lacI^q$ | 15 |
| pMM01 | pJF119HE containing a 1.143-kbp *Bam*HI/*Eco*RI fragment carrying *B. subtilis ribC* | This study |
| pMM02 | pJF119HE containing a 1.143-kbp *Bam*HI/*Eco*RI fragment carrying *B. subtilis ribC820* | This study |
| pMM10 | pIXI12 containing a 1.073-kbp *Nde*I/*Eco*RI fragment carrying *B. subtilis ribC* | This study |
| pMM11 | pIXI12 containing a 1.073-kbp *Nde*I/*Eco*RI fragment carrying *B. subtilis ribC820* | This study |
| pMM20 | pIXI12 containing a 0.960-kbp *Nde*I/*Eco*RI fragment carrying *E. coli ribF* | This study |
| pMM30 | pIXI12 containing a 0.955-kbp *Nde*I/*Eco*RI fragment carrying *S. cerevisiae FADI* | This study |
| pMM60 | pBluescriptSK− containing a 2.609-kbp *Xho*I/*Spe*I fragment carrying the *B. subtilis ribC* locus; *ribC* of this locus was replaced by a neomycin resistance cassette (21) | This study |
| pXI12 | pBR322 *sacB5′ ermAM cryT RBS* $P_{vegI}$ *sacB3′ amp*; *B. subtilis* integration/expression vector | 20 |

[a] Abbreviations: *amp*, ampicillin resistance cassette; @ *sacB*, integration of genes by homologous double-crossover recombination at *B. subtilis sacB* (296°); *Dc<sup>r</sup>-15*, decoyinine resistance; *ermAM*, erythromycin resistance cassette; *FADI*, FAD-synthetase gene from *S. cerevisiae*; *guaC3*, 8-azaguanine resistance; *leuA8*, leucine auxotroph; *metB5*, methionine auxotroph; *metC7*, methionine auxotroph; *neo*, neomycin resistance cassette; $P_{vegI}$, medium-strength *vegI* promoter; *ribC*, *B. subtilis* flavokinase/FAD-synthetase gene; *ribC820*, *ribC* gene with mutation G820A; *ribF*, *E. coli* flavokinase/FAD-synthetase gene; *trpC2*, tryptophan auxotroph.

numbers of the *ribC* sequence in the GenBank database (accession no. Z80835). The oligonucleotides contained heterologous *Bam*HI/*Eco*RI restriction sites to allow cloning of the 1.143-kbp PCR fragment, using the expression vector pJF119HE, to create pMM01. Plasmid pMM02 was constructed in the same way except that *ribC820* was amplified by PCR from chromosomal DNA of *B. subtilis* RB52.

For gene integration of *ribC* into the *sacB* locus of *B. subtilis* RB52, pMM10 was constructed. *ribC* was amplified by PCR using oligonucleotides NdeMM2 (5′-$^{76}$GGTGACCGTCATATGAAGACG$^{96}$-3′) and EcoMM3 and chromosomal DNA of *B. subtilis* 1012 as a template. The oligonucleotides contained heterologous *Nde*I/*Eco*RI restriction sites to allow cloning of the 1.073-kbp PCR fragment, using pXI12. For integration of *ribC820* into the *sacB* locus of *B. subtilis* RB52, plasmid pMM11 was constructed in the same way as described above for pMM10 except that *ribC820* was amplified by PCR from chromosomal DNA of *B. subtilis* RB52.

Plasmid pMM20 was constructed for integration of the *E. coli* flavokinase/FAD-synthetase gene *ribF* (22, 24) (GenBank database entry M10428) into *sacB* of *B. subtilis* 1012. *ribF* was PCR amplified by using oligonucleotides NdeMM4 (5′-$^{598}$GTTTTGAGCCACATATGAAGC$^{619}$-3′) and EcoMM5 (5′-$^{1572}$CGGTTTGAATTCATAACAGGC$^{1554}$-3′) and chromosomal DNA of *E. coli* M15 (41) as a template. The oligonucleotides contained heterologous *Nde*I/*Eco*RI restriction sites to allow cloning of the 0.960-kbp PCR fragment by using pXI12 (20).

Plasmid pMM30 was constructed for integration of the FAD-synthetase gene *FADI* (43) from *S. cerevisiae* into *sacB* of *B. subtilis* 1012. *FADI* was PCR amplified by using oligonucleotides NdeMM6 (5′-$^{125}$GGAGCACCATATGCAGCTGAGC$^{146}$-3′) and EcoMM7 (5′-GGAA$^{1074}$TTCCTCCGTTAAACCGTAC$^{1056}$-3′) and chromosomal DNA of a wild-type *S. cerevisiae* strain as a template. The oligonucleotides contained heterologous *Nde*I/*Eco*RI restriction sites to allow cloning of the 0.955-kbp PCR fragment, using pXI12.

The *ribC* gene was replaced by a neomycin cassette via double-crossover recombination. Adjacent homologous regions to the gene *ribC* within the *ribC* locus (147°) of the *B. subtilis* chromosome were *truB* (5′) and *rpsO* (3′). *truB* was amplified by PCR from *B. subtilis* 1012 chromosomal DNA, using oligonucleotides XhoMM8 (5′-CCGCTCGAGCTCAAAAGAAGGAGTG-3′) and EcoMM9 (5′-CGGAATTCACAGAACGGTCACC-3′). *rpsO* was amplified by PCR from *B. subtilis* 1012 chromosomal DNA with oligonucleotides PstMM10 (5′-ACTGCAGCTTGCAACGCACGC-3′) and SpeMM11 (5′-GGACTAGTCGCAGATACGTAAGAAG-3′). The neomycin cassette was amplified by PCR using plasmid pBEST501 (21) as a template and oligonucleotides EcoMM12 (5′-CGGAATTCGCTTGGGCAGCAGGTCG-3′) and PstMM13 (5′-AACTGCAGTTCAAAATGGTATGCG-3′). The plasmid pMM60 was constructed by ligation of digested PCR fragments of *truB*, *neo*, and *rpsO* (in that order) into *Xho*I/*Spe*I-digested pBluescript II SK−.

The integration/expression vector pXI12 (20) 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 (*ribC820*), and the resulting strains (BL21MM01 and BL21MM02) were aerobically cultivated at 37°C on LB medium. Gene expression was stimulated by adding 1 mM isopropylthiogalactopyranoside (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 80 W in a Branson model 450 sonicator equipped with a 3/4-in. flat tip. All subsequent centrifugation steps were performed at 18,000 × *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 made 70% (wt/vol) in ammonium sulfate and centrifuged, and the deep yellow pellet was resuspended in 10 ml 100 mM Tris-HCl (pH 8.0). The solution was dialyzed twice

for 16 h each 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 $MgCl_2$, and 10 mM $Na_2SO_3$ (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 an HPLC column (Nucleosil 10 $C_{18}$; 4.6 by 250 mm; Macherey & Nagel). The following solvent system was used at a flow rate of 2.5 ml/min: 25% (vol/vol) methanol–100 mM formic acid–100 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 washed with 100 mM potassium phosphate (pH 7.5)–1 mM dithiothreitol–0.1 mM EDTA (buffer A). The cells were resuspended in buffer A and sonicated for 5 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 *Pst*I-linearized pMM20 and pMM30 (1 μg of each) by the previously described two-step procedure (13). The erythromycin-resistant transformant *B. subtilis* strains obtained by homologous double-crossover recombination into the *B. subtilis sacB* locus (296°) were selected (1012MM002 and 1012MM003). Integration was checked by PCR. *B. subtilis* RB52 was transformed with *Pst*I-linearized pMM10 and pMM11 (1 μg of each); the resulting strains were RB52MM100 and RB52MM110.

**Replacement of *B. subtilis ribC* by a neomycin cassette via double-crossover recombination.** *B. subtilis* 1012MM002 was transformed with *Sca*I-linearized pMM60 (1 μg), and the resulting strain was 1012MM004. *B. subtilis* 1012MM003 was transformed by using *Sca*I-linearized pMM60 (1 μg), and the resulting strain was 1012MM006.

## 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 35.668 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 pure wild-type RibC revealed a single sequence (MKTIHITHPHL) 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).

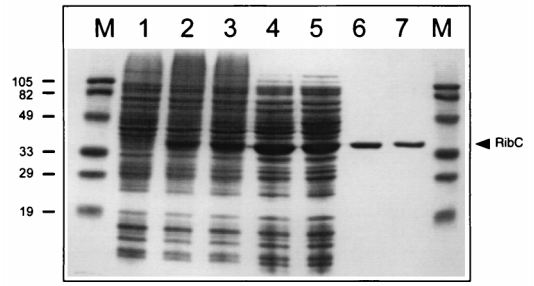


FIG. 1. Overproduction and purification of *B. subtilis* wild-type RibC and mutant RibC820. SDS-PAGE of cell extracts of IPTG-induced *E. coli* strains harboring the *B. subtilis ribC* wild-type (*E. coli* BL21MM01; lane 2) and *ribC820* mutant (*E. coli* BL21MM02; lane 3) genes. As a control, lane 1 shows a cell extract of an IPTG-induced *E. coli* BL21 strain harboring the expression vector pJF119HE without insert. Lanes 4 and 5 show RibC wild type and mutant, respectively, after $(NH_4)_2SO_4$ purification. Cation-exchange eluates of RibC wild type and mutant are shown in lanes 6 and 7. Lanes marked M are molecular weight markers (in kilodaltons).

**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 *Brevibacterium 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 flavokinases/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 FMN/FAD auxotrophic *B. subtilis* strain, although the flavokinase activity of the protein encoded

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

| Purification step | Wild-type RibC | | RibC820 | |
|---|---|---|---|---|
| | Flavokinase activity[a] | Purification (fold) | Flavokinase activity | Purification (fold) |
| Cell extract | 70 | 1 | 1 | 1 |
| $(NH_4)_2SO_4$ (40–70% [wt/vol]) purification | 140 | 2 | 2 | 2 |
| Cation exchange | 580 | 8 | 6 | 6 |

[a] Measured in a final volume of 1 ml of potassium phosphate (pH 7.5) containing 50 μM riboflavin, 3 mM ATP, 15 mM $MgCl_2$, and 10 mM $Na_2SO_3$ and expressed as nanomoles of FMN produced per minute per milligram of protein. FAD synthetase activity could not be exactly measured because exogenous, highly purified 5′-FMN was not accepted as a substrate.

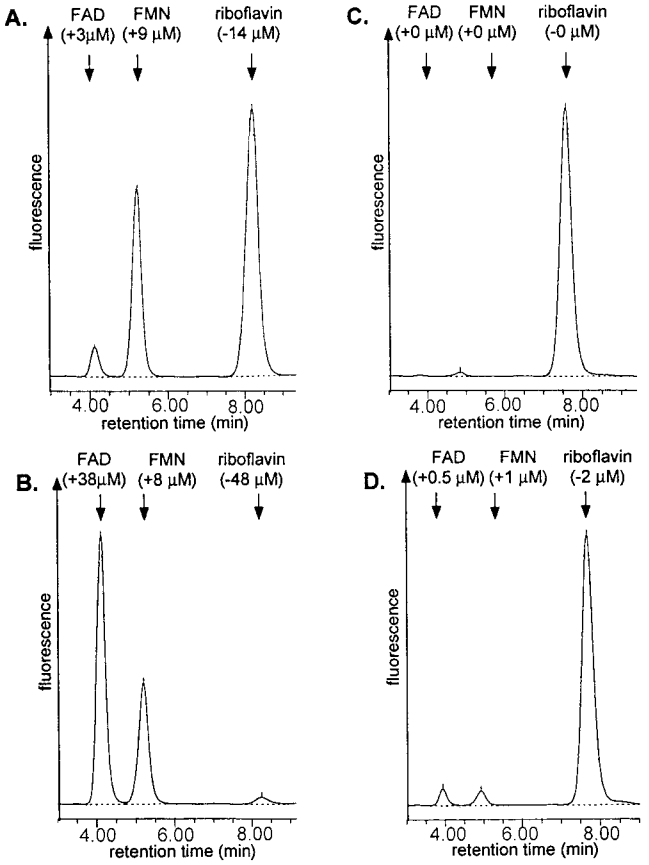


FIG. 2. HPLC chromatograms of the products of flavokinase/FAD-synthetase assays. Assay mixtures containing 50 μM riboflavin, 3 mM ATP, 15 mM $MgCl_2$, and 10 mM sodium sulfite ($Na_2SO_3$) were preincubated at 37°C for 5 min. Pure wild-type RibC (A and B) or mutant RibC820 (C and D) (3 μg of each) was added, and the mixtures were incubated for another 5 (A and C) or 30 (B and D) min. An aliquot was removed and separated on an HPLC column (Nucleosil 10 $C_{18}$; 4.6 by 250 mm; Macherey & Nagel). The following solvent system was used at a flow rate of 2.5 ml/min: 25% (vol/vol) methanol–100 mM formic acid–100 mM ammonium formate (pH 3.7). The reaction was monitored with a fluorescence detector (excitation, 470 nm; emission, 530 nm; Waters Associates). The chromatograms show three clearly resolved peaks of riboflavin (8 min), FMN (5 min), and FAD (4 min). Numbers in parentheses indicate decrease of riboflavin and increase of flavin nucleotides during the enzyme assay.

by *ribC820* is drastically reduced. This could mean that *B. subtilis* contains another flavokinase/FAD-synthetase activity complementing the RibC820 defect. Alternatively, the residual activity of RibC820 might be sufficient to meet the FMN/FAD requirements of *B. subtilis*. Attempts to replace *ribC* in a *B. subtilis* wild-type strain (*B. subtilis* 1012) or in the *ribC820* mutant strain (*B. subtilis* RB52) by a selectable neomycin resistance gene failed, although FMN and FAD were added to the culture medium during the transformation experiments. However, the *ribC* gene could be removed from the *B. subtilis* genome after introduction of the *E. coli ribF* gene encoding a flavokinase/FAD-synthetase. For chromosomal integration of *E. coli ribF* at the *B. subtilis sacB* locus and expression of the heterologous gene, the pXI12 expression/integration vector was used. The resulting *ribC* deletion mutant 1012MM004 had no requirement for FMN or FAD and did not overproduce riboflavin. In summary, transformants devoid of the *ribC* gene could be obtained only from a parent strain that contained a heterologous flavokinase/FAD-synthetase-encoding gene at *sacB*. Thus, RibC is essential for the flavin metabolism of *B. subtilis*. The remaining flavokinase/FAD-synthetase activity of the mutant protein RibC820 within *B. subtilis* RB52 is sufficient for growth under standard growth conditions.

The *ribC* gene could also be deleted in a strain that carried the *S. cerevisiae FADI* gene at the *sacB* locus. The *FADI* gene, which was provided with the pXI12 integration/expression system, encodes an FAD-synthetase. 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-

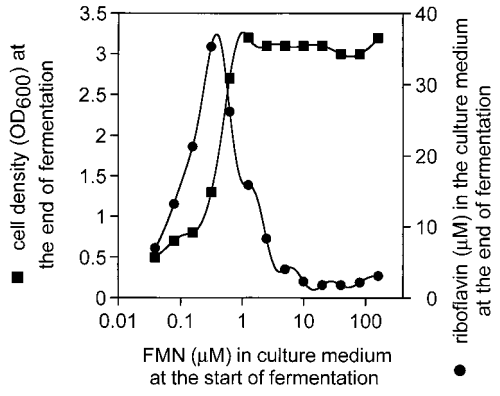


FIG. 3. FMN-dependent riboflavin overproduction in *B. subtilis* 1012MM006. The FMN auxothropic *ribC* deletion mutant *B. subtilis* 1012MM006 was cultivated for 16 h in the presence of the indicated amounts of FMN. Riboflavin in the culture medium at the end of fermentation was determined by HPLC analysis as described for Fig. 2. Cell optical density at 600 nm ($OD_{600}$) at the end of fermentation was estimated photometrically.

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 mutant 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*.

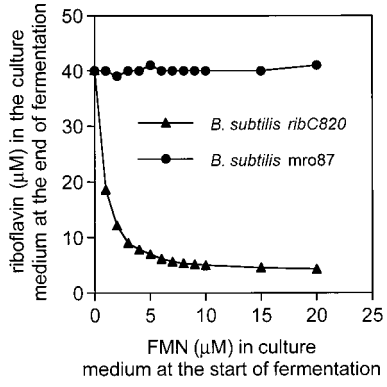


FIG. 4. Exogenuous FMN represses riboflavin overproduction in a *B. subtilis ribC820* (triangles) but not in a *B. subtilis* 1012mro87 (*ribO* mutant, circles) strain. A *B. subtilis ribC820* strain and strain 1012mro87 (*ribO* mutant) were cultivated for 16 h in growth medium supplemented with indicated amounts of FMN. Riboflavin in the supernatant was determined by HPLC as described for Fig. 2. Cell growth was estimated photometrically at 600 nm.

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


1. **Arbige, M. V., B. A. Bulthuis, J. Schultz, and D. Crabb.** 1993. Fermentation of *Bacillus*, p. 871–895. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis*. American Society for Microbiology, Washington, D.C.
2. **Bacher, A.** 1991. Biosynthesis of flavins, p. 215–259. *In* F. Müller (ed.), Chemistry and biochemistry of flavoenzymes, vol. 1. CRC Press, Boca Raton, Fla.
3. **Bacher, A.** 1991. Riboflavin kinase and FAD synthetase, p. 349–370. *In* F. Müller (ed.), Chemistry and biochemistry of flavoenzymes, vol. 1. CRC Press, Boca Raton, Fla.
4. **Bacher, A., S. Eberhardt, and G. Richter.** 1996. Biosynthesis of riboflavin, p. 657–664. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
5. **Bacher, A., K. Eisenreich, K. Kis, R. Ladenstein, G. Richter, J. Scheuring, and S. Weinkauf.** 1993. Biosynthesis of flavins, p. 147–192. *In* H. Dugas and F. P. Schmidtchen (ed.), Bioorganic chemistry frontiers, vol. 3. Springer-Verlag, Berlin, Germany.
6. **Barker, D. F., and A. M. Campbell.** 1981. The *birA* gene of *Escherichia coli* encodes a biotin holoenzyme synthetase. J. Mol. Biol. **146:**451–467.
7. **Bower, S., J. Perkins, R. R. Yocum, P. Serror, A. Sorokin, P. Rahaim, C. L. Howitt, N. Prasad, S. D. Ehrlich, and J. Pero.** 1995. Cloning and characterization of the *Bacillus subtilis birA* gene encoding a repressor of the biotin operon. J. Bacteriol. **177:**2572–2575.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72:**248–254.
9. **Bresler, S. E., E. A. Glazunov, T. P. Chernik, T. N. Schevchenko, and D. A. Perumov.** 1975. Investigation of the operon of riboflavin biosynthesis in *Bacillus subtilis*. V. Flavin mononucleotide and flavin-adenine dinucleotide as effectors in the riboflavin biosynthesis operon. Genetika **9:**84–91.
10. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
11. **Coquard, D.** 1996. Diploma thesis. Ecole Superieure de Biotechnologie de Strasbourg, Illkirch, France.
12. **Coquard, D., M. Huecas, M. Ott, J. M. van Dijl, A. P. G. M. van Loon, and H.-P. Hohmann.** 1997. Molecular cloning and characterisation of the *ribC* gene from *Bacillus subtilis*: a point mutation in *ribC* results in riboflavin overproduction. Mol. Gen. Genet. **254:**81–84.
13. **Cutting, S. M., and P. B. Vander Horn.** 1990. Genetic Analysis, p. 27–74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*, modern microbiological methods. John Wiley & Sons, Chichester, England.
14. **Eisenberg, M. A., O. Prakash, and S.-C. Hisiung.** 1982. Purification and properties of the biotin repressor—a bifunctional protein. J. Biol. Chem. **257:**15167–15173.
15. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
16. **Gusarov, I.** 1996. GenBank database entry Y09721.
17. **Gusarov, I. V., Y. I. Yomantas, Y. I. Kozlov, R. A. Kreneva, and D. A. Perumov.** 1996. Cloning and nucleotide sequence of regulator gene of the *Bacillus subtilis* riboflavin operon. EMBL databank entry X95312.
18. **Hager, P. W. and J. C. Rabinowitz.** 1985. Translational specificity in *B. subtilis*, p. 1–29. *In* D. Dubneau (ed.), The molecular biology of the *Bacilli*. Academic Press, Inc., New York, N.Y.
19. **Hewick, R. M., M. W. Hunkapillar, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256:**7990–7997.
20. **Hümbelin, M., V. Griesser, T. Keller, W. Schurter, H. P. Hohmann, and A. P. G. M. van Loon.** Unpublished results.
21. **Itaya, M., K. Konda, and T. Tanaka.** 1989. A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17:**4410.
22. **Kamio, Y., C. K. Lin, M. Regue, and H. C. Wu.** 1985. Characterisation of the *ileS-lsp* operon in *Escherichia coli*. Identification of an open reading frame upstream of the *ileS* gene and potential promoter(s) for the *ileS-lsp* operon. J. Biol. Chem. **260:**5616–5620.
23. **Kil, Y. V., V. N. Mironov, I. Y. Gorishin, R. A. Kreneva, and D. A. Perumov.** 1992. Riboflavin operon of *Bacillus subtilis*—unusual symmetric arrangement of the regulatory region. Mol. Gen. Genet. **233:**483–486.
24. **Kitatsuji, K., S. Ishino, S. Teshiba, and M. Arimoto.** 1993. Process for producing flavine nucleotides. European patent application 0 542 240 A2.
25. **Kreneva, R. A., and D. A. Perumov.** 1990. Genetic mapping of regulatory mutations of *Bacillus subtilis* riboflavin operon. Mol. Gen. Genet. **222:**467–469.
26. **Kreneva, R. A., and D. A. Perumov.** 1996. Genetic mapping of an additional regulatory locus of the riboflavin operon of *Bacillus subtilis*. Genetika **32:**1623–1628.
27. **Mack, M.** 1997. Unpublished results.
28. **Manstein, D. J., and E. F. Pai.** 1986. Purification and characterization of FAD synthetase from *Brevibacterium ammoniagenes*. J. Biol. Chem. **261:**16169–16173.
29. **Mironov, V. N., M. L. Chikindas, A. S. Krayev, A. I. Stepanov, and K. G. Skryabin.** 1990. Operon organization of riboflavin biosynthesis in *Bacillus subtilis*. Dokl. Akad. Nauk SSSR **312:**237–240.
30. **Mironov, V. N., A. S. Krayev, B. K. Chernov, A. I. Stepanov, and K. G. Skryabin.** 1989. Complete nucleotide sequence of riboflavin biosynthesis operon from *Bacillus subtilis*. Dokl. Akad. Nauk SSSR **305:**482–486.
31. **Mironov, V. N., D. A. Perumov, A. S. Kraev, A. I. Stepanov, and K. G. Skryabin.** 1990. Unusual structure of *Bacillus subtilis rib*-operon regulatory region. Mol. Biol. (Moscow) **24:**256–261.
32. **Nielsen, P., P. Rauschenbach, and A. Bacher.** 1983. Phosphates of riboflavin and riboflavin analogs: a reinvestigation by high-performance liquid chromatography. Anal. Biochem. **130:**359–368.
33. **Perkins, J. B. and J. G. Pero.** 1993. Biosynthesis of riboflavin, biotin, folic acid, and cobalamin, p. 319–334. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis*. American Society for Microbiology, Washington, D.C.
34. **Perkins, J. B., J. G. Pero, and A. Sloma.** 1990. Riboflavin overproducing strains of bacteria. European patent application 0 405 370 A1.
35. **Saito, H., T. Shibaat, and T. Ando.** 1979. Mapping of genes determining nonpermissiveness and host specific restriction to bacteriophages in *B. subtilis* Marburg. Mol. Gen. Genet. **170:**117–122.
36. **Sambrook, J., E. F. Fritsch, and T. E. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Shiio, I.** 1989. Production of primary metabolites, p. 191–211. *In* B. Maruo and H. Yoshikawa (ed.), *Bacillus subtilis*: molecular biology and industrial application, Kodansha Ltd. and Elsevier Science Publishers, Tokyo, Japan.
38. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
39. **Stepanov, A. I., V. G. Zdanov, A. J. Kukanova, M. J. Chaikinson, P. M. Rabinovic, J. A. V. Iomantas, and Z. M. Galuskina.** 1983. Verfahren zur Herstellung von Riboflavin. Offenlegungsschrift DE 3420310/A1.
40. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Mol. Biol. **189:**113–130.
41. **Villarejo, M. R., and I. Zabin.** 1974. Beta-galactosidase from termination and deletion mutant strains. J. Bacteriol. **120:**466–474.
42. **Wilm, M., and M. Mann.** 1996. Analytical properties of the nanoelectrospray ion source. Anal. Chem. **68:**1–8.
43. **Wu, M., B. Repetto, D. M. Glerum, and A. Tzagoloff.** 1995. Cloning and characterization of *FAD1*, the structural gene for flavin adenine dinucleotide synthetase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**264–271.