The ribR gene encodes a monofunctional riboflavin kinase which is involved in regulation of the *Bacillus subtilis* riboflavin operon

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A 3.5 kb EcoRI-BamHI fragment of *Bacillus subtilis* chromosomal DNA carrying the ribR gene, involved in regulation of the *B. subtilis* riboflavin operon, was cloned in the *B. subtilis*-Escherichia coli shuttle vector pCB20. DNA sequence analysis of this fragment revealed several ORFs, one of which encodes a polypeptide of 230 amino acids with up to 45% sequence identity with FAD synthetases from a number of micro-organisms, such as Corynebacterium ammoniagenes, *E. coli* and Pseudomonas fluorescens, and also to the ribC gene product of *B. subtilis*. The ribR gene was amplified by PCR, cloned and expressed in *E. coli*. Measurement of flavokinase activity in cell extracts demonstrated that ribR encodes a monofunctional flavokinase which converts riboflavin into FMN but not to FAD, and is specific for the reduced form of riboflavin.

Keywords: *Bacillus subtilis*, flavinogenesis, riboflavin, operon regulation, flavokinase

INTRODUCTION

The *Bacillus subtilis* riboflavin operon comprises a cluster of five non-overlapping genes that encode the enzymes which catalyse the reactions for *de novo* riboflavin biosynthesis starting from GTP. The operon is located at 209° on the *B. subtilis* genetic map. The functional organization of the riboflavin operon has been described in detail in several articles (Perkins & Pero, 1993; Mironov et al., 1994).

The expression of the operon is negatively regulated by the product of the ribC gene, which is situated at 147° on the *B. subtilis* chromosome and appears to encode a flavin-activated aporepressor. A possible repressor binding site is situated in a region of 294 bp between the promoter and the first structural gene of the operon (Kil et al., 1992). Mutations in this region designated ribO mutations are cis-dominant and, like ribC mutations, have a phenotype of riboflavin overproduction, resulting in accumulation of the vitamin in the growth medium.

While the exact mechanism of regulation is still unknown (Azevedo et al., 1993) it is evident that the regulatory system of the operon is not limited to the ribC gene and the ribO regulatory region. We have examined *B. subtilis* mutants which carry the constitutive ribCl mutation and are partially resistant to the riboflavin analogue 7,8-dimethyl-10-(O-methylacetoxime)-isoalloxazine (MO), in which the ribityl moiety is replaced with -CH₂-CH=O-N-O-CH₃ (Fig. 1). A number

Abbreviations: FAD and FMN are used generically to include both oxidized and reduced forms; MO, 7,8-dimethyl-10-(O-methylacetoxime)-isoalloxazine.

The EMBL accession number for the sequence referred to in this paper is Y09721.

![Fig. 1. Structure of the riboflavin analogue 7,8-dimethyl-10-(O-methylacetoxime)-isoalloxazine (MO).](image-url)
of these mutants had completely lost the ability to oversynthesize riboflavin but retained the original ribC1 mutation in the chromosome (Kreneva & Perumov, 1994). This was interpreted as the restoration of regulatory activity due to a mutation at a previously unknown locus, resulting in expression of a product that could also regulate expression of the riboflavin operon of B. subtilis. This effect is only seen in ribC constitutive mutants and not in the background of ribO regulatory mutations.

Two mutations which decrease the constitutive expression of the B. subtilis riboflavin operon (designated ribR mutations) were found to be linked to position 236° on the B. subtilis genetic map. Results of transduction and transformation experiments have demonstrated the following order of markers: pheA–apt6–ribR–azlB–aroD4 (Kreneva & Perumov, 1995).

There is a further complication in the pattern of regulation of flavinogenesis in B. subtilis. Previous results have shown that the ribC gene not only encodes a regulator of expression of the riboflavin operon but that the ribC gene product is also the bifunctional enzyme flavokinase/FAD synthetase which produces FMN and FAD from the reduced form of riboflavin (Coquard et al., 1997; Gusarov et al., 1997). This multifunctional role of the ribC gene product increased our interest in ribR and, in this paper, we describe the cloning and sequencing of this gene and functional characterization of its product.

**METHODS**

**Strains and growth conditions.** B. subtilis RK222he (hisH ribO82 recE4), RK399he (hisH ribC1 recE4), RK282he (hisH ribC1 ribR5 recE4), RK5he (hisH ribO335 recE4), RK6he (hisH ribO186 recE4), RK612 (hisH ribC1) and H25 (hisH) and Escherichia coli DH5α [supE44 lacZΔM15 (808lacZΔM15) bsdR17 recA1 endA1 gyrA96 thi-1 relA1] and BL21 (DE3) [F−ompT bsd spha (rpsL mna) gal dcm] were used in this study. Strains RK222he, RK399he, RK282he, RK5he, RK6he, RK612, H25 and DH5α were from the collection of the Department of Molecular and Radiation Biophysics, Petersburg Nuclear Physics Institute, and BL21 (DE3) was from the collection of the Department of Biochemistry, Imperial College.

**B. subtilis** was cultivated in Spizizen minimal medium supplemented with the required nutrients (Anagnostopoulos & Spizizen, 1961), and E. coli was grown on Luria–Bertani (LB) medium (Sigma). To select for resistance markers, 10 µg erythromycin ml−1 and 5 µg analogue MO ml−1 were added to the medium.

For enzyme assays, cell cultures (200 ml) were grown in Spizizen minimal medium supplemented with 0.02% casein hydrolysate at 37°C to an OD660 of 1.0, harvested by centrifugation, washed and resuspended in 0.1 M Tris/HCl buffer, pH 8.0. Cell extracts were made by ultrasonic disintegration (UDN-1) at a frequency of 44 kHz in 50 mM Tris/HCl, 0.1 mM EDTA, 1 mM DTT buffer (pH 8.0) at 0°C, centrifuged at 10000 g for 10 min, and the supernatant was used for the assays.

**Plasmids.** The plasmid cloning vector pCB20 which can tolerate inserts of up to 20 kb in length (Avakov et al., 1990) was kindly provided by Dr Y. Yomantas (State Scientific Research Institute for Genetics and Selection of Industrial Micro-organisms, Moscow). For expression of cloned genes, vector pRSET-5d (Novagen) was used.

**Chemicals.** Riboflavin and FMN were obtained from Serva; [14C]riboflavin was obtained from Amersham; 6,7-dimethyl-8-ribityllumazine was obtained from Serva, and MO was synthesized by the modified method of Fall & Petering (1956). All other chemicals were obtained from Sigma and Serva and were of the highest quality available.

**DNA manipulation.** Chromosomal DNA was isolated according to the method of Saito & Miura (1963), and plasmid DNA was isolated according to Sambrook et al. (1989). E. coli cells were transformed by the method of Mandel & Higa (1970), and B. subtilis by the method of Anagnostopoulos & Spizizen (1961). Restriction, ligation and dephosphorylation enzymes were purchased from ‘Fermentas’ and used according to the manufacturer’s instructions.

The PCR was carried out in an Eppendorf MicroCycler E apparatus using BioTaq polymerase (Bioline). Thirty-five cycles were performed as follows: 20 s at 94°C, followed by 20 s at 55°C, and finally 1 min at 72°C. Synthetic oligonucleotide primers were obtained from Pharmacia Biotech. For DNA sequencing, both strands of DNA were sequenced using the radioactive chain-termination method (Sanger et al., 1977) using T7 DNA polymerase with appropriate primers. Sequences were analysed using the computer programs DNASIS and Prosis (Hitachi).

**Riboflavin accumulation experiments.** Vitamin accumulation in the growth medium was measured spectrophotometrically by monitoring the A260 (Bresler et al., 1972).

**Enzyme assays.** Riboflavin synthetase activity was determined as described previously (Bresler et al., 1972) using 6,7-dimethyl-8-ribityllumazine as substrate. One unit of activity corresponds to the formation of 1 nmol riboflavin min−1 (mg total protein)−1. FMN synthetase (flavokinase) activity was determined in crude cell extracts by the method of Hagiwara (Nakagawa et al., 1995). The reaction mixture (1-0 ml) contained 5 mM riboflavin, 5 mM ATP, 10 mM MgCl2, 20 mM NaF, 10 mM Na2SO4 and supernatant from cell disruption (0.1 ml) in 100 mM Tris/HCl buffer (pH 8.0). Riboflavin was maintained in a reduced state by addition of dithionite and the reaction mixture was covered with a thin layer (about 1 mm) of mineral oil. Assays were carried out at 37°C for 10 min and reactions were stopped by boiling for 5 min in a water bath. A control assay was boiled without incubation.

Flavins were separated from the reaction mixture by HPLC on a reversed phase Separation C18 column (150 × 3.3 mm). The mobile phase was 5 mM ammonium acetate in 20% (v/v) ethanol, pH 6.0, at a flow rate of 0.5 ml min−1 (Gusarov et al., 1997). Flavins were detected and quantified using a 420-AC fluorescence detector (Waters).

**Radiolabelling was also used for measurement of flavokinase activity** (Bresler et al., 1977). In this case, [14C]riboflavin (800–1000 c.p.m. nmol−1) was added to the reaction mixture and aliquots (10–20 µl) were removed at various times and applied to Whatman 3MM chromatographic paper. Riboflavin (Rf 0.4) and FMN (Rf 0.85) were separated by ascending chromatography in water saturated with isoamyl alcohol and located under UV light. Spots were cut out and their radioactivity content was determined by scintillation counting in JS-107 scintillator (Chimfarm). One unit of flavokinase activity corresponds to the formation of 1 nmol FMN min−1 (mg total protein)−1.
Protein concentrations were determined by the method of Bradford (1976) using reagents from Bio-Rad.

**RESULTS AND DISCUSSION**

**Cloning and function of ribR**

Wild-type *B. subtilis* cells can grow in the presence of 8–10 μg riboflavin analogue MO ml⁻¹. In contrast, *ribC* mutants are more sensitive and can tolerate only 1·5–3·5 μg analogue ml⁻¹. The *ribR* mutants (in a *ribC* background), in which a degree of control of riboflavin production is restored, also regain tolerance to MO and can grow at concentrations of about 5–6 μg ml⁻¹ (Kreneva & Perumov, 1994). This phenotypic difference was, therefore, used for cloning of the *ribR* gene.

Chromosomal DNA from strain RK282 (*ribC1 ribR5*) was partially digested with EcoRI, ligated with the EcoRI-digested shuttle vector pCB20 and transformed into strain RK222he. Transformants were selected on MO plates and one clone (from approximately 50 obtained) was chosen for further study. EcoRI mapping of the recombinant plasmid, referred to as pSI1, gave fragments of 1·0, 1·5 and 10·5 kb in addition to the vector, corresponding to a total insert size of 13·0 kb. Clones obtained by transformation of pSI1 into recipients containing the *ri6C* mutation, and selection for the vector-borne erythromycin resistance, had reduced levels of riboflavin production compared to the superproduction of the *ribC* hosts. This correlated with a decrease in the level of riboflavin synthetase activity. For example, the presence of pSI1 in strain RK222he (*ribC862*) decreased riboflavin accumulation from 31 pg ml⁻¹ to less than 1·0 pg ml⁻¹ and for strain RK399he (*ribC1*) accumulation decreased from 28 pg ml⁻¹ to less than 1·3 pg ml⁻¹. Riboflavin synthetase activity changed from 0·55–0·65 units to 0·04–0·06 units, which is only two- to threefold higher than the basal level in wild-type cells.

Riboflavin synthetase is the enzyme which converts 6,7-dimethyl-8-ribityllumazine to riboflavin (Perkins &
I. EcoRI-BamHI restriction fragment derived from one in Fig. 2, which shows that plasmids containing a 3.5 kb 70

Fig. 4. Heterologous expression of the ribR gene product (RibR) analysed by SDS-PAGE. Lanes: 1 and 6, size markers (bovine albumin, 67 kDa; egg albumin, 43 kDa; chymotrypsinogen, 25 kDa; myoglobin, 18 kDa); 2, cell-free extract of BL21(DE3); 3, cell-free extract of BL21(DE3) with pRSET-5d construct containing ribR, non-induced; 4 and 5, BL21(DE3) with pRSET-5d construct containing ribR after 2 h induction with IPTG.

Subcloning and sequence analysis

Following more detailed restriction mapping the insert in pSI1 was subcloned to generate a family of plasmids in the pCB20 vector and transformed into B. subtilis strain RK399he (ribC1). Each of the resulting clones was tested for resistance to the analogue MO and for the ability to repress constitutive riboflavin synthesis resulting from the ribC1 mutation. The results are given in Fig. 2, which shows that plasmids containing a 3.5 kb EcoRI–BamHI restriction fragment derived from one end of the insert in pSI1 conferred both of these phenotypes, presumably due to the expression of the ribR gene. This allowed us to proceed with the determination of the nucleotide sequence of the EcoRI–BamHI fragment to identify the ribR gene.

Sequence analysis of the entire 3.5 kb fragment revealed several ORFs (results not shown). One ORF of 690 bp in length, starting from position 1960, encodes a polypeptide of 230 amino acid residues with a significant degree of homology to the FAD synthetases from other micro-organisms, such as Corynebacterium ammoniagenes, E. coli and Pseudomonas fluorescens (Nakagawa et al., 1993), and to the ribC gene of B. subtilis (Gusarov et al., 1997) (Fig. 3). Particularly given the homology to ribC, we assumed that this was probably the ribR gene.

All of the FAD synthetases to which the presumed ribR gene product showed homology are bifunctional enzymes catalysing the two consecutive, ATP-dependent reactions involved in conversion of riboflavin via FMN to FAD. However, amino acid sequence comparison with these FAD synthetase homologues shows that the new ORF encodes a protein which is almost 100 amino acids shorter than all of the bifunctional FAD synthetases. Additionally, this protein can be clearly divided into two fragments, with all of the N-terminal end (residues 1–112) having homology with the C-termini of the FAD synthetases, starting near residue 200. The overall homology in this region is up to 45% with a high degree of conservation in all eight amino acid sequences compared (Fig. 3) between residues 14 to 25 (numbering with respect to the ribR gene product). Five amino acid residues are completely conserved, namely glycine (G19), proline (P21), threonine (T22), alanine (A23) and asparagine (N24), while residues at positions 15 (small), 16 (positive charge) and 18 (aliphatic) are functionally conserved. A SWISS-PROT search based only on the C-terminal part of the predicted polypeptide (about 108 residues) did not reveal any significant similarity to other sequences available in the database.

The observed ORF has no evident promoter immediately upstream or terminator immediately downstream. However, a typical ribosome-binding site is present. The sequence gTTGCGATttttttaactcatagTATAATt (possible −10 and −35 sequences are shown in capitals), which looks very much like a ε33 (vegetative sigma factor)-dependent promoter, was found approximately 1500 bp upstream from the ORF, which suggests that the ribR gene is part of an operon-like structure.

Using site-specific mutagenesis on the FAD synthetase gene from E. coli, it was shown that the kinase activity of the protein is associated with the C-terminal region, and FAD synthetase with the N-terminal region (Kitatsuji et al., 1993). Two ATP-binding sites have been described (Nakagawa et al., 1995). The region of homology between the predicted ribR gene product and the C-terminal region of the ribC product starts at an ATP-binding site (residues 4–11; Fig. 3). This strongly suggests that ribR encodes a flavokinase.
Is the ribR gene product a flavokinase?

Flavokinase activity was measured in cell extracts as described above. In the case of strain H25 which has a basal level of riboflavin, FMN and FAD synthesis, flavokinase activity was no higher than 0.01 units. In strain RK612, which carries the ribC1 mutation, flavokinase activity was immeasurably small, which was to be expected, because the ribC riboflavin-constitutive mutants are unable to synthesize FMN (Gusarov et al., 1997). However, the flavokinase activity was approximately 0.08 units in strain RK612p, bearing the pSI13 plasmid. While, quantitatively, this was not very high, it was a significant increase in flavokinase activity over the controls.

To confirm that the ribR gene does indeed encode a flavokinase, it was necessary to amplify the level of expression by cloning the ribR gene behind a strong inducible promoter.

Subcloning and expression of the ribR gene in E. coli

To overexpress the ribR gene we used the pET-system in which expression is driven from a T7 RNA polymerase provided by the host (Studier et al., 1990). The pRSET-5d vector was used as it contained convenient NcoI and EcoRI restriction sites in the multiple cloning site (MCS) (Schoepfer, 1993). The ribR gene was amplified by PCR using mismatched primers to create the required NcoI (CCATGG) restriction site in the 5'-end flanking region and EcoRI site (CTTAAG) at the 3'-end. As a result of the construction, the polypeptide expressed had an additional three N-terminal amino acids, methionine (ATG), alanine (GCT) and phenylalanine (TTT), in comparison to the native gene product. However, as the EcoRI site was situated after the stop codon, the C-terminus remained intact.

After initial isolation and screening for ribR recombinants in DH5α, plasmid DNA from a single positive clone was isolated and introduced into BL21(DE3) by transformation. A single colony of the BL21(DE3) clone containing the recombinant pRSET-5d was grown in 8 ml LB medium containing 500 μg carbenicillin ml⁻¹ to an optical density of 0.5–0.6, centrifuged, resuspended in the same medium and IPTG was added to 1 mM final concentration to induce expression.

After 2–3 h incubation cells were collected by centrifugation, resuspended in 1 ml 50 mM Tris/HCl, 0.1 mM EDTA, 1 mM DTT buffer, pH 8.0, and disintegrated by sonication. Cell debris was separated by centrifugation and the supernatant was used for activity determination and SDS-PAGE analysis.

SDS-PAGE revealed that synthesis of a 26 kDa polypeptide was enhanced in cell extracts from induced, compared to non-induced cells (Fig. 4). A mass of 26 kDa would require a coding region of approximately 700 bp, which is consistent with the size of the insert in the pRSET-5d construct. Flavokinase activity in this extract was 60 units, considerably higher than the activity expressed from pSI1 in B. subtilis and also 600-fold higher than the activity in BL21(DE3) without induction.

Despite this high level of flavokinase activity, no traces of FAD were detected in the assays (Fig. 5). Thus it is clear that the ribR gene encodes a monofunctional (with respect to enzyme activity) flavokinase and is devoid of
FAD synthetase activity. The conversion of riboflavin into FMN was only observed when the riboflavin was in the reduced form. Reduced flavins are also the substrates for the bifunctional flavokinase/FAD synthetase from B. subtilis (Kearney et al., 1979). Thus, despite the homology to the flavokinase component in bifunctional enzymes being only 45%, this is sufficient to preserve discrimination between the reduced and oxidized forms of riboflavin. Addition of 3.5 µM MO ml⁻¹ to the reaction mixture reduced the flavokinase activity to 53% of its initial value, but there was little further inhibition at increasing concentrations (up to 10 µM ml⁻¹), despite the fact that growth of wild-type B. subtilis is strongly inhibited at this concentration of analogue (Kreneva & Perumov, 1994).

The exact mechanism of the regulation brought about by the ribR gene product will need further analysis. However, increasing the amount of the mutant ribC gene product in cells had no influence on the repressive effect of the ribR gene product. In addition, ribR expression has no influence on riboflavin overproduction in strains carrying ribO mutations. This means that the ribR gene product does not interfere directly in riboflavin biosynthesis, but functions by restoring an activity normally provided by the ribC-encoded protein. As the ribC mutant strains are flavokinase-deficient it is likely that this restoration occurs, not by direct interaction with the ribC gene product, but as a result of the increase in FMN concentration in the cells.

Sequence analysis shows that the ribR gene is probably part of an operon. Sequence comparison between wild-type and mutant copies of the ribR gene revealed that the mutations do not lie in the structural part of the gene. This suggests that the expression is regulatory, leading to increased production of the ribR gene product in cells and, as a consequence, increased production of FMN.

ACKNOWLEDGEMENTS

We are grateful to Drs C. K. N. Chan Kwo Chion and R. Jackson for advice and fruitful discussions, and are pleased to acknowledge the financial support of the Royal Society of London in facilitating this collaboration.

REFERENCES


Received 27 July 1998; accepted 4 September 1998.