Journal of Industrial Microbiology & Biotechnology (1999) 22, 1–7
© 1999 Society for Industrial Microbiology 1367-5435/99/$12.00
http://www.stockton-press.co.uk/jim

GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are rate-limiting enzymes in riboflavin synthesis of an industrial *Bacillus subtilis* strain used for riboflavin production

M Hümbelin[1,2,3], V Griesser[1], T Keller[4], W Schurter[5], M Haiker[1], H-P Hohmann[1,3], H Ritz[6], G Richter[6], A Bacher[6] and APGM van Loon[1]

[1]*F Hoffmann-La Roche Inc, Grenzacherstrasse 124, CH-4070 Basel, Switzerland;* [6]*Department of Organic Chemistry and Biochemistry, Technical University of Munich, Lichtenbergstrasse 4, D-85747 Garching, Germany*

One of the proteins encoded by the riboflavin operon of *Bacillus subtilis*, RibA, was identified as the rate limiting enzyme in an industrial riboflavin producing strain. An additional single copy of the *ribA* gene was introduced into the *sacB* locus of the riboflavin production strain and was expressed constitutively from the medium strength *vegI* promoter. This led to improved riboflavin titers and yields of riboflavin on glucose of up to 25%. Both enzymatic activities of RibA, the 3,4-dihydroxy-2-butanone 4-phosphate synthase activity located in the N-terminal half of the protein and the GTP cyclohydrolase II activity of the C-terminal domain, are necessary for the improved riboflavin productivity.

Keywords: *Bacillus subtilis*; riboflavin; 3,4-dihydroxy-2-butanone 4-phosphate synthase; GTP cyclohydrolase II

Introduction

Several thousand tons of riboflavin (vitamin B2) are produced yearly and are consumed mainly as food and feed additives. In the past few years a number of producers have developed biotechnological processes to replace the more costly chemical synthesis of the compound. Beside the economic advantage, there is also an ecological benefit in that the biotechnological production uses renewable sources, is more environment friendly and yields a product of equal or superior quality [17].

A cost-effective biotechnological riboflavin production process requires a microorganism which is an extreme overproducer of the compound. One microorganism which was turned into an excellent producer strain by means of classical mutagenesis and genetic engineering is *Bacillus subtilis*, a Gram-positive bacterium which is not a natural overproducer of riboflavin [11]. Selection for resistance against purine analogues and methionine sulfoxide yielded mutants with enhanced synthesis of guanosine triphosphate (GTP), one of the precursors of riboflavin (Figure 1a). A mutant with deregulated riboflavin synthesis was obtained through selection for resistance to roseoflavin, a riboflavin analogue. This mutation lies in *ribC*, the gene for riboflavin kinase, which is located outside of the riboflavin operon [6]. All these mutations are combined in the strain RB50, a *B. subtilis* 168 derivative [11]. Genetic engineering involved the construction of two riboflavin operons with strong phage promoters and the integration of these modified operons, pRF69 and pRF93, into the genome of strain RB50. The arrangement of the integrated operons permitted amplification [8] and the presence of antibiotic resistance markers on pRF69 and pRF93 allowed the selection of cells with high copy numbers of the modified riboflavin operons. The final strain was named RB50::$[pRF69]_n$::$[pRF93]_m$ Ade^+ [11] (n and m represent the copy numbers of pRF69 and pRF93).

To further increase the riboflavin productivity of RB50::$[pRF69]_n$::$[pRF93]_m$ Ade^+, it was then necessary to focus on the various possible bottlenecks. One hypothetical bottleneck is the limitation in the supply of one of the precursor molecules, ribulose-5-phosphate or GTP (Figure 1a). In this case an increased flux into the pentose phosphate cycle could overcome the limitation.

A second hypothetical bottleneck is a limitation in one or more of the six enzymatic activities encoded by the first four open reading frames of the riboflavin operon (Figure 1b). In this case the limitation could be relieved by expressing higher levels of the limiting protein. Several attempts to simultaneously overexpress all the proteins encoded by the riboflavin operon to still higher levels than those reached in RB50::$[pRF69]_n$::$[pRF93]_m$ Ade^+ failed. This suggested that one or several Rib-enzymes might exert a negative effect on the cell when expressed above a certain level. If the limiting enzyme had such a negative effect, it would be very difficult, if not impossible, to identify it as the bottleneck. However, in the case that the limiting enzyme has no inhibitory activity, overexpression of the individual protein should reveal the identity of the bottleneck.

[2]Correspondence to M Hümbelin at his present address[3]
[3]Present address: Roche Vitamins Inc, Building 102/4th floor, 340 Kingsland Street, Nutley, NJ 07110-1199, USA
[4]Present address: Novo Nordisk A/S, Novo Alle, 2880 Bagsvaerd, Denmark
[5]Present address: PharmaPart AG, Weberstrasse 10, CH-8036 Zürich, Switzerland
Received 16 June 1998; accepted 30 October 1998

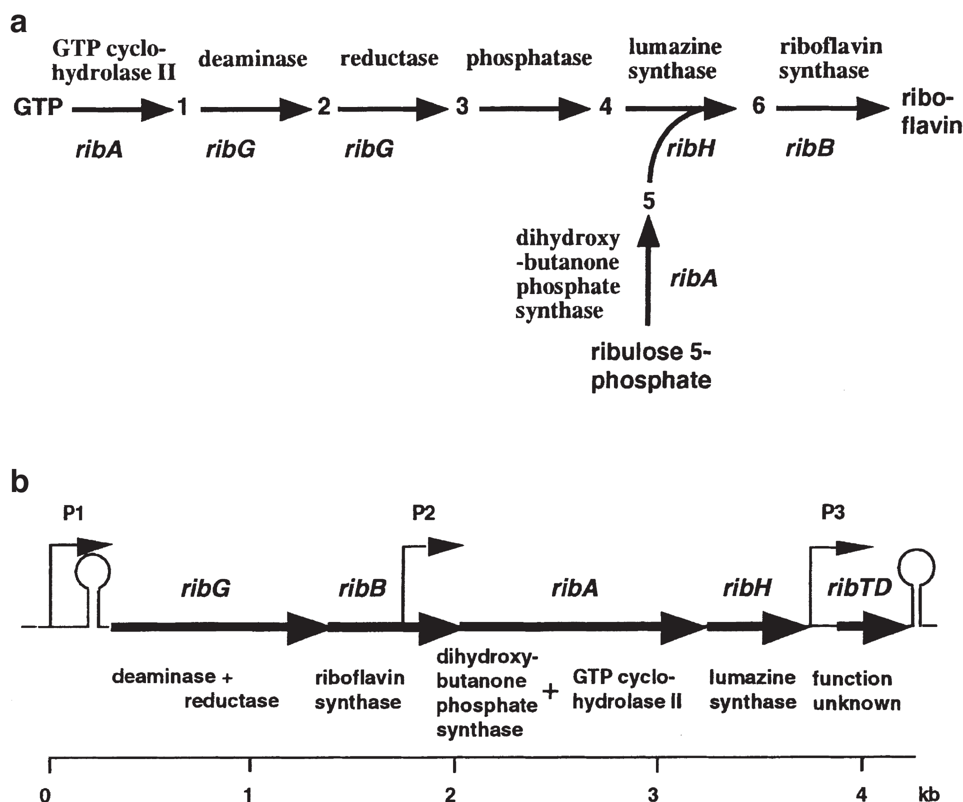

Figure 1 Pathway of riboflavin biosynthesis and the riboflavin operon of *B. subtilis*. (a) Biosynthesis of riboflavin in *B. subtilis*. The enzymatic conversions are symbolised by arrows. The names of the enzymes and the names of the corresponding *rib* genes encoded by the riboflavin operon are indicated. The phosphatase converting compound 3 to compound 4 is not encoded by the riboflavin operon and has not yet been identified. The compounds are: **1**: 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate; **2**: 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; **3**: 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate; **4**: 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione; **5**: L-3,4-dihydroxy-2-butanone-4-phosphate; **6:** 6,7-dimethyl-8-ribityllumazine. For more details see Refs [2] and [3]. (b) The riboflavin operon of *B. subtilis*. The *rib* genes are represented as thick arrows and the functions of their products are indicated below. *ribG* and *ribA* each encode two enzymatic functions of the riboflavin biosynthetic pathway. *ribA* encodes in its 5′-half the 3,4-dihydroxy-2-butanone 4-phosphate synthase and in its 3′-part the GTP cyclohydrolase II [3,14]; *ribG* encodes the deaminase activity in the 5′ region and the reductase activity in the 3′ region [13]. Thin arrows indicate transcription initiation from the promoters P1, P2 and P3. Potential rho-independent transcription stops are symbolised by stem loop structures. The scale is of the length of the P1 transcript of 4277 nt [1,9].

Here, we identify RibA, whose two enzymatic activities commit the precursors GTP and ribulose-5-phosphate to riboflavin biosynthesis (Figure 1), as the limiting Rib-protein in RB50::$[pRF69]_n$::$[pRF93]_m$ Ade^+. Expression of additional RibA leads to an up to 25% increase in riboflavin yield.

Materials and methods

Construction of the vector pXI12 (Figure 2a)

The backbone of the vector is the *Eag*I-*Aat*II fragment of pBR322 containing the ampicillin resistance gene, the origin of replication and the *rop* gene [4] (position 945 to 4283 in sequence with accession number J01749). The pBR322 sequence is flanked by two sequences derived from the levansucrase gene (*sacB*) of *B. subtilis* [16] (*sacB*-5′: position 729 to 1266 and *sacB*-3′: position 1336 to 1794 in sequence with accession number X02730) which serve as homology regions for insertion into the chromosome. As a selectable marker the erythromycin resistance gene *ermAM* from the plasmid pAMβ1 [5] was introduced (position 107 to 1091 in sequence with accession number Y00116). The promoter driving the transcription of the cloned gene is the medium strength, constitutive *vegI* promoter from *B. subtilis* [10] (position 30 to 101 in sequence with accession number J01552). The *cryT* transcriptional terminator is from *B. thuringiensis* [18] (position 268 to 380 in sequence with accession number M13201). The ribosome-binding site (underlined) and the polylinker stretch including the translational start codon (bold) within the *Nde*I site (CATATG) was introduced between the *vegI* promoter and the *cryT* terminator as synthetic DNA with the sequence CTCGAGAATTAAAGGAGGGTTTCAT**ATG** AATTCGGATCCCGGG. The restriction enzyme sites *Pme*I, *Aat*II, *Nhe*I and *Eag*I are derived from PCR primers and were introduced to link the DNA segments described above.

Construction of pXI16

The expression of certain genes cloned in pXI12 might not be tolerated in *E. coli*. To avoid potential problems, a variant of pXI12 was created in which the *vegI* promoter is interrupted by a short sequence. To construct this modified vector, named pXI16, a T to C point mutation was introduced between the −35 and the −10 regions of the *vegI*

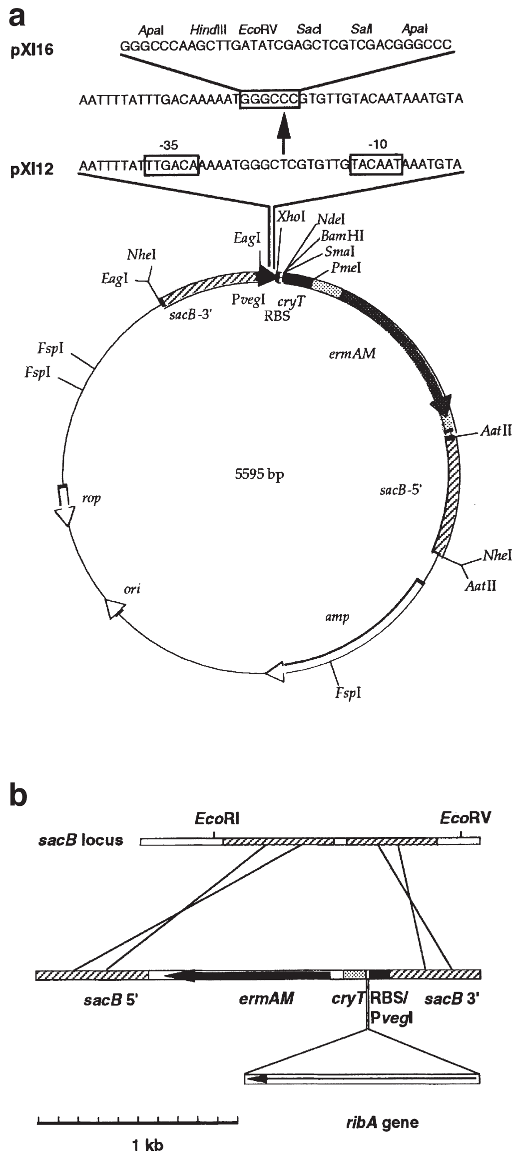

Figure 2 Insertion of *rib* genes into the *sacB* locus of *B. subtilis* with the help of pXI plasmids. (a) Plasmids pXI12 and pXI16. The plasmid is represented by a circle. The sites for relevant restriction enzymes are indicated. The important elements of the vector are labelled as follows: P *vegI:* medium strength, constitutive promoter from *B subtilis*; RBS: synthetic ribosome-binding site; *cryT*: transcriptional terminator from *B. thuringiensis; ermAM:* constitutively expressed erythromycin resistance gene; *sacB*-3′ and *sacB*-5′: homology regions for integration via homologous recombination, derived from the levan sucrase gene of *B. subtilis*; *amp*: ampicillin resistance gene from pBR322; *ori*: origin of replication from pBR322; *rop*: *rop* gene from pBR322. The direction of some of the elements is indicated by arrows. Above the plasmid, the −35 and −10 regions of the *veg* promoter are shown. The arrow points to the T to C mutation which was introduced to create the *Apa*I site (box in middle line) in pXI16. The uppermost line shows the sequence in pXI16 interrupting the promoter and the introduced restriction sites. (b) Integration of the *ribA* gene into the *sacB* locus of *B. subtilis*. The *sacB* locus is schematically shown in the upper part. The sites for the restriction endonucleases *Eco*RI and *Eco*RV are indicated. The homology regions present in the pXI clones are represented as hatched boxes. The lower part shows the pXI plasmid without the pBR322 derived section, with the *ribA* gene cloned between the *Nde*I and *Bam*HI sites. The integration of the pXI derived DNA via double cross-over recombination is indicated.

promoter to create an *Apa*I site and a 30-bp polylinker was then introduced (Figure 2a).

Cloning of the rib genes in E. coli

The *rib* genes were amplified with the polymerase chain reaction (PCR) from *B. subtilis*-derived DNA using the GeneAmp DNA amplification reagent kit (PE Applied Biosystems, Foster City, CA, USA) following the instructions of the manufacturer. The primers were synthesized on a model 392 DNA/RNA synthesizer (PE Applied Biosystems) and had *Nde*I and *Bam*HI sites included for cloning into the pXI vectors. The *Nde*I sites included the translational start codons, ensuring proper expression of the genes from pXI12, and the *Bam*HI sites were introduced immediately after the stop codons of the *rib* genes. An internal *Nde*I site in *ribG* was eliminated by introducing a silent point mutation. The PCR fragments were digested with *Nde*I and *Bam*HI and ligated with *Nde*I/*Bam*HI cleaved pXI16. The *rib* genes were sequenced to verify the absence of PCR-derived mutations.

Construction of variants of the B. subtilis strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ with individual rib genes inserted in the sacB locus

After isolation of the pXI16 plasmids with the *rib* genes inserted, the DNA was cut with *Apa*I, the 30-bp insert was deleted and the *vegI* promoter was reconstituted by ligation. The plasmids were then linearised by cutting with *Fsp*I, which cleaves only in the pBR322-derived sequence (Figure 2a), and the *rib* genes were introduced into transformation-competent *B. subtilis* cells following the described two-step procedure [7]. Since the strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ cannot be made competent for transformation, *B. subtilis* strain 1012 [15] was chosen as an intermediate host. Erythromycin-resistant clones had the *rib* gene and the erythromycin-resistance gene *ermAM* inserted in the *sacB* locus by double cross-over in the homology regions *sacB*-3′ and *sacB*-5′ (Figure 2b). The modified *sacB* locus was then introduced into RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ by transduction with the phage PBS1 using standard procedures [7]. The strain with an additional *ribA* gene in the *sacB* locus was named VB2XL1.

Fermentative production of riboflavin

Evaluation of the riboflavin-overproducing *B. subtilis* strains was conducted in 15-litre Biostat ED 10 vessels (Braun Biotech International, Melsungen, Germany) in carbon-limited fed-batch fermentations. Important physical parameters were controlled using a digital control unit (Braun Biotech International) and monitored via a PC-based multi-fermentor-control system (Braun Biotech International). To determine biomass development and riboflavin accumulation, samples were collected during the fermentation. The biomass was assayed by measuring the optical density at 540 nm and riboflavin content was determined by HPLC. The modified riboflavin operons pRF69 and pRF93 of all strains were amplified [8] prior to fermentation.

Determination of riboflavin by HPLC
One volume of 1 M NaOH was added to a sample of fermentation broth and vortexed for 30 s to dissolve the riboflavin crystals. The mixture was then diluted 125-fold with phosphate-buffer (pH 6.5), filtered through a 0.45-μm pore size filter and fractionated over a 4-mm × 125-mm LiChrospher 100, RP-18 (5-μm) column (Merck KGaA, Darmstadt, Germany) equilibrated with 5% acetonitrile. The column was eluted with a linear gradient of acetonitrile from 5% to 70% in 7 min and the riboflavin was monitored at 403 nm.

Determination of enzyme activities

Preparation of cell lysates: The cells of a 5-ml fermentation sample were collected by centifugation at 3000 × *g* for 10 min at 4°C in a swinging bucket rotor. Some of the cells (typically 100–400 mg) were transferred to a microfuge tube containing 1 ml PBS. Care was taken not to transfer riboflavin which collected at the bottom of the tube, below the cell pellet. The cells were centrifuged at 6000 × *g* for 5 min at 4°C in a microfuge and the pellets were resuspended in 1 ml 15% sucrose, 50 mM Tris/HCl (pH 7.5) containing 10 μg DNase I, 10 μg RNase A and proteinase inhibitor (Complete Protease Inhibitor Cocktail, Roche Molecular Biochemicals, Mannheim, Germany). The cells were opened by sonication and the lysate was centrifuged at 12 000 × *g* for 20 min at 4°C in a microfuge. The supernatant was sterilized by passing it through a 0.45-μm pore size filter and kept frozen at –20°C until further use. The total protein concentration of the samples was determined as described [12].

Assay of GTP cyclohydrolase II activity: The enzyme reaction was carried out in 100 mM Tris/HCl (pH 8.5) containing 5 mM $MgCl_2$ and 5 mM dithiothreitol at a substrate concentration of 1 mM GTP. Cell lysate containing between 4 and 40 μg protein was added to the assay which had a final volume of 100 μl. Samples were incubated at 37°C for 30 min. The reaction was stopped by adding 100 μl of a solution of 1% (v/v) diacetyl in 100 mM EDTA (pH 8). The mixture was heated to 90°C for 1 h and precipitated protein was removed by centrifugation for 10 min at 13 000 × *g*. 6,7-Dimethylpterin was determined by reversed phase HPLC on a Nucleosil 10RP18 column (4 × 250 mm) with 40% (v/v) methanol containing 100 mM ammonium formate at a flow rate of 2 ml min^{-1} (t_R = 2.4 min; excitation 365 nm; emission 435 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate per hour at 37° C.

Assay of 3,4-dihydroxy-2-butanone 4-phosphate synthase activity: The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.0), 10 mM $MgCl_2$, 5 mM ribose 5-phosphate (sodium salt). The substrate ribulose 5-phosphate was produced *in situ* from ribose 5-phosphate by the action of phosphoriboisomerase (5 μl enzyme—20 μmol min^{-1} ml^{-1} in 20 mM phosphate buffer—were added per 100 μl standard assay). The product of the 3,4-dihydroxy-2-butanone 4-phosphate synthase was converted to 6,7-dimethyl-8-ribityllumazine by lumazine synthase which was added at a concentration of 0.1 mg ml^{-1}. Ten microlitres of 10 mM 5-amino-6-ribitylamino-2,4 (1H,3H)-pyrimidinedione (in 20 mM dithiothreitol), the second substrate of lumazine synthase, were added per 100 μl standard assay. Samples were incubated without cell lysate for 15 min at 37°C to produce sufficient substrate for the subsequent reaction, which was then started by the addition of the cell lysate (containing between 4 and 40 μg protein). After incubation at 37°C for 30 min in the dark, the 3,4-dihydroxy-2-butanone 4-phosphate synthase reaction was stopped by the addition of 15 μl 200 mM EDTA (pH 8.0). The mixture was incubated in the dark for another 15 min at 37°C to complete the formation of the lumazine. Twenty-five microlitres of 40% (w/v) trichloroacetic acid were added and denatured protein was removed by centrifugation at 13 000 × *g* for 10 min. 6,7-Dimethyl-8-ribityllumazine was determined by reversed phase HPLC on a Nucleosil 10RP18 column (4 × 250 mm) with 10% (v/v) methanol containing 30 mM formic acid at a flow rate of 2 ml min^{-1} (t_R = 3.2 min). The effluent was monitored fluorometrically (excitation 408 nm; emission 487 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 3,4-dihydroxy-2-butanone 4-phosphate per hour at 37°C.

Results and discussion

Strain VB2XL1 contains an additional ribA gene in the sacB locus
Strain VB2XL1 is derived from strain RB50::[pRF69]$_n$ ::[pRF93]$_m$ Ade$^+$ and carries an additional copy of the *ribA* gene encoding two enzymatic activities of the riboflavin biosynthetic pathway (Figure 1). The additional *ribA* gene is under the control of the *vegI* promoter and was introduced into the *sacB* locus of *B. subtilis* by double cross-over via a pXI vector (Figure 2) as described above. The additional *ribA* gene in VB2XL1 leads to enhanced activities of the 3,4-dihydroxy-2-butanone 4-phosphate synthase (encoded by the first half of *ribA*) and the GTP cyclohydrolase II (encoded by the second half of *ribA*) of about 3- and 6-fold, respectively (Figure 3). Analogously, the other *rib* genes and the N- and C-terminal halves of *ribA* were introduced separately into the *sacB* locus of strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$. Southern blot analysis of all strains confirmed that each contains a single copy of the respective *rib* gene correctly introduced into the *sacB* locus by double cross-over. Expression of the additional *rib* genes was verified by Northern and Western blot analyses (data not shown).

Strain VB2XL1 produces up to 25% more riboflavin as compared to its parent strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$
The influence of the additional *rib* genes inserted into the *sacB* locus on growth behaviour and riboflavin productivity was tested in 15-L fermentors. To assure maximal expression of the *rib* enzymes the modified operons pRF69 and pRF93 were amplified. Samples were drawn at various time-points during the fermentations and analyzed for cell growth (OD_{540}) and riboflavin content (determined by HPLC). The riboflavin titres and yields on glucose of 15

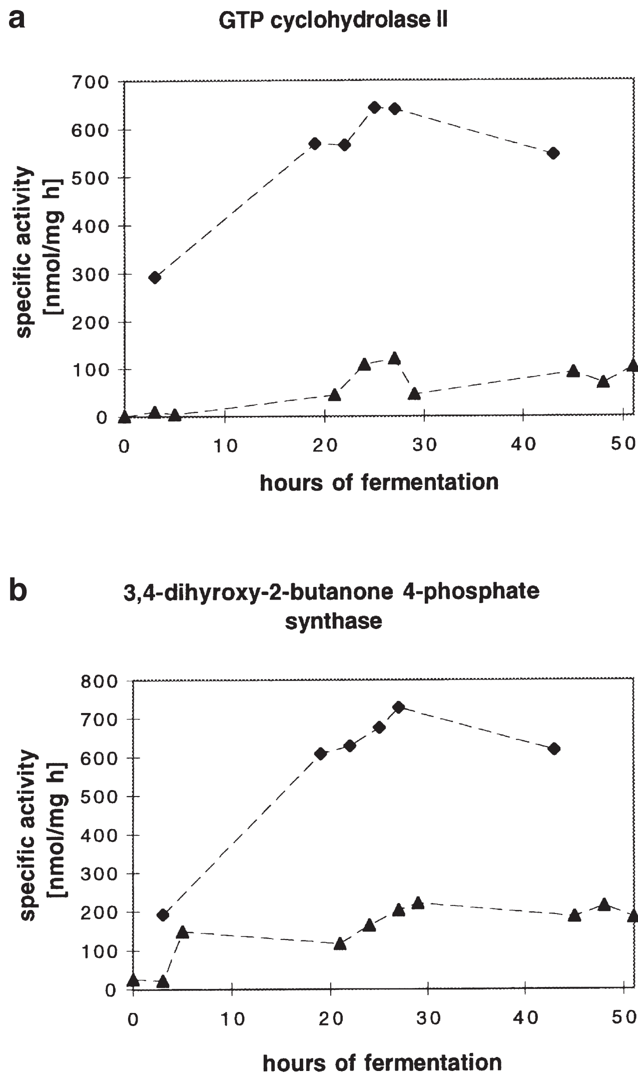

Figure 3 GTP cyclohydrolase II (a) and 3,4-dihydroxy-2-butanone 4-phosphate synthase (b) activities in the strains RB50::[pRF69]$_m$::[pRF93]$_n$ Ade$^+$ and VB2XL1. The strains RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ (▲) and VB2XL1 (◆) were grown in 15-litre bioreactors, samples were taken at various times and the GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase activities were determined as described in the experimental protocol section.

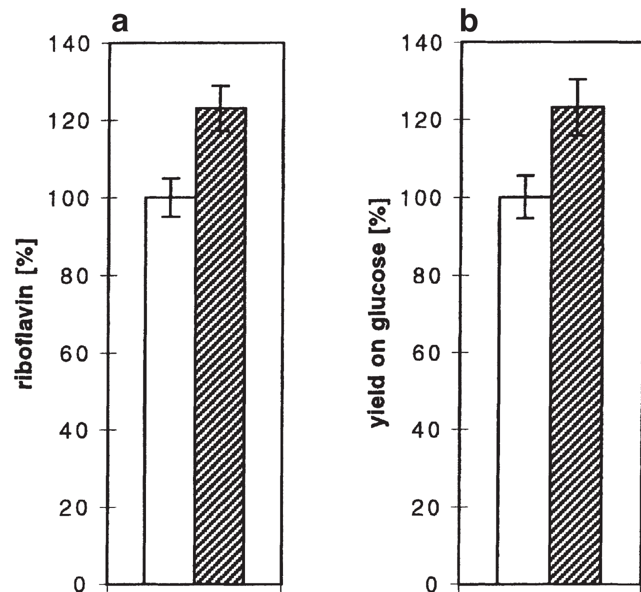

Figure 4 Improvement of riboflavin titres and yield on glucose with VB2XL1. The average riboflavin titres (a) and yields on glucose (% glucose converted to riboflavin (b) of fermentations with the strains RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ (white columns; n = 8) and VB2XL1 (hatched columns; n = 15) are represented graphically. The average values with strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ were set as 100%. The error bars indicate the standard deviations.

independent fermentations with VB2XL1, compared with the eight best results obtained with RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$, are presented in Figure 4. The average increase in riboflavin and yield on glucose was 23%. The potential of VB2XL1 to produce more riboflavin than RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ is still higher since a comparison of the best fermentations of the two strains shows an increase of almost 25%. No further increase in riboflavin productivity could be obtained when a second *ribA* gene was introduced into the *amyE* locus of VB2XL1, suggesting that the RibA protein is no longer limiting for riboflavin synthesis in VB2XL1.

The strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ has more than 15 copies of the *ribA* gene encoded by the amplified operons pRF69 and pRF93. In comparison, VB2XL1 possess only one *ribA* gene more than RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$. The fact that this additional copy results in a 3- to 6-fold increase in the activities of the *ribA* encoded enzymes suggests that the *vegI* driven *ribA* gene inserted into the *sacB* locus is much more efficiently expressed than the other *ribA* genes. This could result from more efficient transcription or translation or improved mRNA stability or a combination thereof. In addition, it might be that a reduced transcription of the riboflavin operon is the consequence of intolerance of high levels of some Rib-proteins. This hypothesis is supported by the observation that synthesis of additional RibB protein has a negative effect on cell growth and synthesis of additional RibG induces cell-lysis after 30 h (Figure 5a). These effects are reproducible whereas the slightly enhanced lysis in the example shown with the strain synthesizing additional RibH (Figure 5a) is not seen in all fermentations with that strain. The reduced growth of the RibB strain is accompanied by lower riboflavin production (Figure 5b) whereas expression of an additional *ribG* or *ribH* gene had no effect on riboflavin synthesis.

Both enzymatic activities of RibA are needed for the productivity increase

To test whether only one of the enzymatic activities of RibA is limiting for riboflavin production, strains analogous to VB2XL1 were constructed that contained only the first half of *ribA*, encoding the 3,4-dihydroxy-2-butanone 4-phosphate synthase activity, or the second half, encoding the GTP cyclohydrolase II activity, in the *sacB* locus of the strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$. An example of a fermentation with each of these strains is presented in Figure 5c and d. Interestingly, each strain containing only the first or second half of the *ribA* gene in *sacB* showed a reduced riboflavin productivity. Furthermore, the strain

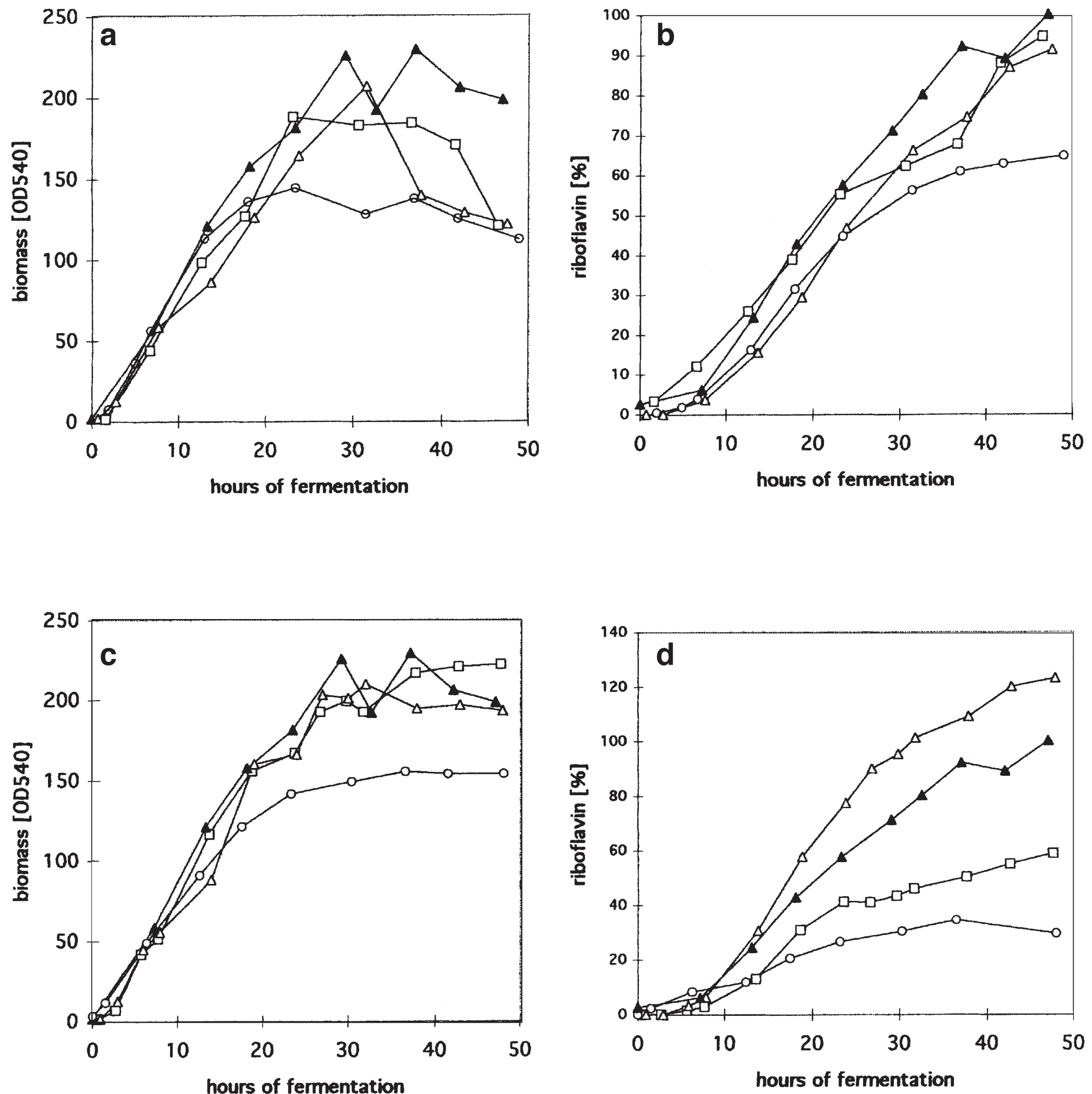

Figure 5 Effect of the introduction of *rib* genes into the *sacB* locus of RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ on cell growth and riboflavin production. Fermentations were carried out with the strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ (—▲—) or its derivatives having the *ribG* (panels a and b: —△—), the *ribB* (panels a and b: —○—), the *ribH* (panels a and b: —□—), the 3,4-dihydroxy-2-butanone 4-phosphate synthase encoding 5′-half of *ribA* (panels c and d: —□—), the GTP cyclohydrolase II encoding 3′-half of *ribA* (panels c and d: —○—) or the entire *ribA* (panels c and d: —△—) inserted in the *sacB* locus. The modified operons pRF69 and pRF93 of all strains were amplified. Panels a and c show the OD_{540} values of samples taken during the fermentations and the corresponding riboflavin titres are plotted in panels b and d. The riboflavin titre reached at the end of the fermentation with strain RB50::[pRF69]$_n$::[pRF93]$_m$ Ade$^+$ was set as 100%.

expressing additional GTP cyclohydrolase II activity showed impaired cell growth, suggesting that an excess of GTP cyclohydrolase II activity exerts a negative effect on cell viability. In conclusion, the concomitant expression of both enzyme activities encoded by the *ribA* gene was essential to improve the production of riboflavin.

References

1 Azevedo V, A Sorokin, SD Ehrlich and P Serror. 1993. The transcriptional organization of the *Bacillus subtilis* 168 chromosome region between the *spoVAF* and *serA* genetic loci. Mol Microbiol 10: 397–405.
2 Bacher A. 1991. Biosynthesis of flavins. In: Chemistry and Biochemistry of Flavoenzymes (F Müller, ed), pp 215–259, CRC Press, Boca Raton, Florida.
3 Bacher A, S Eberhardt and G Richter. 1996. Biosynthesis of riboflavin. In: *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology (FC Neidhardt, R Curtiss III, JL Ingraham, ECC Lin, KB Low, B Magasanik, WS Reznikoff, M Riley, M Riley, M Schaechter and HE Umbarger, eds), pp 657–664, American Society for Microbiology, Washington, DC.
4 Bolivar F, RL Rodriguez, PJ Greene, MC Betlach, HL Heynecker and HW Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95–113.
5 Brehm J, S Salmond and N Minton. 1987. Sequence of the adenine methylase gene of the *Streptococcus faecalis* plasmid pAMβ. Nucl Acids Res 15: 3177.
6 Coquard D, M Huecas, M Ott, JM van Dijl, APGM 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.
7 Cutting SM and PB Vander Horn. 1990. Genetic analysis. In: Molecular Biological Methods for *Bacillus* (Harwood and Cutting, eds), pp 27–74, John Wiley & Sons, Chichester, UK.

8 Jannière L, B Niaudet, E Pierre and SD Ehrlich. 1985. Stable gene amplification in the chromosome of *Bacillus subtilis*. Gene 40: 47–55.
9 Mironov VN, AS Kraev, ML Chikindas, BK Chernov, AI Stepanov and KG Skryabin. 1994. Functional organization of the riboflavin biosynthesis operon from *Bacillus subtilis SHgw*. Mol Gen Genet 242: 201–208.
10 Moran CP, N Lang, SF LeGrice, G Lee, M Stephens, AL Sonenshein, J Pero and R Losick. 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol Gen Genet 186: 339–346.
11 Perkins JB, A Sloma, T Hermann, K Theriault, E Zachgo, T Erdenberger, N Hannett, NP Chatterjee, V Williams II, GA Rufo Jr, R Hatch and J Pero. 1998. Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin. J Ind Microbiol Biotech 22: 8–18.
12 Read SM and DH Northcote. 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. Anal Biochem 116: 53–64.
13 Richter G, M Fischer, C Krieger, S Eberhardt, H Lüttgen, I Gerstenschläger and A Bacher. 1997. Biosynthesis of riboflavin: characterization of the bifunctional deaminase-reductase of *Escherichia coli* and *Bacillus subtilis*. J Bacteriol 179: 2022–2028.
14 Richter G, R Volk, C Krieger, H-W Lahm, U Rötlisberger and A Bacher. 1992. Biosynthesis of riboflavin: cloning, sequencing, and expression of the gene for 3,4-dihydroxy-2-butanone 4-phosphate synthase of *Escherichia coli*. J Bacteriol 174: 4050–4056.
15 Saito H, T Shibata and T Ando. 1979. Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. Mol Gen Genet 170: 117–122.
16 Steinmetz M, D Le Coq, S Aymerich, G Gonzy-Treboul and P Gay. 1985. The DNA sequence of the gene for the secreted *Bacillus subtilis* enzyme levansucrase and its genetic control sites. Mol Gen Genet 200: 220–228.
17 van Loon APGM, H-P Hohmann, W Bretzel, M Hümbelin and M Pfister. 1996. Development of a fermentation process for the manufacture of riboflavin. Chimia 50: 410–412.
18 Wong HC and S Chang. 1986. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc Natl Acad Sci USA. 83: 3233–3237.