Transcriptional regulation of 3,4-dihydroxy-2-butanone 4-phosphate synthase

T. Schlösser, G. Schmidt† and K.-P. Stahmann

Author for correspondence: K.-P. Stahmann. Tel: +49 2461 612843. Fax: +49 2461 612710.
e-mail: p.stahmann@fz-juelich.de

Institut für Biotechnologie 1,
Forschungszentrum
Jülich GmbH, D-52425
Jülich, Germany

The filamentous hemiascomycete *Ashbya gossypii* is a strong riboflavin overproducer. A striking but as yet uninvestigated phenomenon is the fact that the overproduction of this vitamin starts when growth rate declines, which means that most of the riboflavin is produced in the stationary phase, the so-called production phase. The specific activity of 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase, the first enzyme in the biosynthetic pathway for riboflavin, was determined during cultivation and an increase during the production phase was found. Furthermore, an increase of *RIB3* mRNA, encoding DHBP synthase, was observed by competitive RT-PCR in the production phase. The mRNAs of two housekeeping genes, *ACT1* (encoding actin) and *TEF* (encoding translation elongation factor-1 α), served as standards in the RT-PCR. Reporter studies with a *RIB3* promoter–*lacZ* fusion showed an increase of β -galactosidase specific activity in the production phase. This investigation verified that the increase of *RIB3* mRNA in the production phase is caused by an induction of promoter activity. These data suggest that the time course of riboflavin overproduction of *A. gossypii* is correlated with a transcriptional regulation of the DHBP synthase.

Keywords: DHBP synthase, riboflavin, *Ashbya gossypii*, fungi, gene regulation

INTRODUCTION

The filamentous hemiascomycete *Ashbya gossypii* (Ashby & Nowell) (Guilliermond, 1928) is a plant pathogen and was first isolated from cotton (Ashby & Nowell, 1926), but is also able to infect other plants (Phaff & Starmer, 1987). What makes the fungus interesting is its ability to overproduce riboflavin (vitamin B₂) (Wickerham *et al.*, 1946). Yields of up to 15 g l⁻¹ have been reported for strains used in industry for riboflavin production (Bigelis, 1989). Nowadays, *A. gossypii* competes with two other organisms, *Candida famata* and *Bacillus subtilis*, for the title of best tool in the biotechnical process (Stahmann *et al.*, 2000).

Although the biosynthesis of riboflavin has been elucidated in several organisms (Bacher *et al.*, 2000), nothing is known about transcriptional regulation of the corresponding genes in *A. gossypii* or other eukaryotes. In

B. subtilis, a Gram-positive prokaryote, the riboflavin genes are clustered in a single operon which is transcribed into a polycistronic mRNA (Mironov *et al.*, 1994). Mironov and coworkers showed that the steady-state level of the transcript is negatively regulated by riboflavin. The assumption that RibC could function as a repressor on the operator region has been refuted (Mack *et al.*, 1998). RibC and RibR (Solovieva *et al.*, 1999) encode bifunctional and monofunctional flavin kinases, respectively, forming FMN and FAD, which seems to have an effect on the regulation of riboflavin synthesis.

Riboflavin biosynthesis has two roots in the metabolism of the cell. One starts with GTP and the other with ribulose 5-phosphate (Bacher, 1991). In parallel reactions GTP is converted by a GTP cyclohydrolase II into 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5-phosphate and ribulose 5-phosphate is converted by 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase into DHBP. With respect to regulation of riboflavin synthesis, DHBP synthase should be of greater importance because this enzyme is needed twice for the formation of one riboflavin molecule while GTP cyclohydrolase II is only required once. Furthermore, some

† Present address: Tosoh Biosep GmbH, Zettachring 6, D-70567 Stuttgart, Germany.

Abbreviations: ARS, autonomously replicating sequence; DHBP, 3,4-dihydroxy-2-butanone 4-phosphate.

biosynthetic pathways are controlled by transcriptional regulation of the first enzyme. For the mammalian squalene synthase it has been shown that the mRNA level of the corresponding gene, *FDFT1*, is regulated by cholesterol status and by the cytokines TNF- α and IL-1 β (Robinson *et al.*, 1993; Tansey & Shechter, 2000). Transcriptional regulation has also been described for the squalene synthase gene *ERG9* in *Saccharomyces cerevisiae* (Kennedy *et al.*, 1999). In *Gibberella pulicaris*, which produces toxic trichothecene, the first enzyme in the biosynthetic pathway is also transcriptionally regulated (Hohn *et al.*, 1993).

In *Arabidopsis thaliana* (Herz *et al.*, 2000) and *B. subtilis* (Hümbelin *et al.*, 1999) DHBP synthase and GTP cyclohydrolase II are part of bifunctional proteins, whereas in *Escherichia coli* and *A. gossypii* two genes encode two separate enzymes (Richter *et al.*, 1992, 1993; Revuelta *et al.*, 1995). Recently, the first crystallization of a monofunctional DHBP synthase has been reported for *Magnaporthe grisea*. The amino acid sequence of the *M. grisea* enzyme is 48.1% identical to the primary structure of the *A. gossypii* enzyme (Liao *et al.*, 2000).

In this study we focused on regulation of DHBP synthase, which is encoded by *RIB3* in *A. gossypii*. First, evidence concerning regulation of riboflavin overproduction was reported by Pfeifer *et al.* (1950). They used glucose as the carbon source and found that riboflavin overproduction started after glucose had been exhausted from the medium. To show that catabolite repression by glucose is not the regulation mechanism, we chose fermentation conditions in which glucose was present over the whole cultivation period. A pronounced separation between growth phase and riboflavin production phase was found. We show an increase in specific activity of DHBP synthase in the production phase which correlates with transcriptional regulation of the *RIB3* gene.

METHODS

Strains and cultivation conditions. *A. gossypii* strain ATCC 10895 was maintained on solid medium (Stahmann *et al.*, 1994). Inocula for batch fermentations were cultivated in liquid medium, consisting of 10 g yeast extract l⁻¹ and 10 g glucose l⁻¹ (HA medium), and incubated at 28 or 30 °C in 500 ml shake flasks, each containing 100 ml medium, on a rotary shaker (Certomat H; B. Braun) at 120 r.p.m. For batch fermentations used to determine DHBP synthase activity, 30 ml of an overnight culture was used to inoculate a 3 l bioreactor (Braun Biostat E; B. Braun) containing 1.5 l HA medium. The initial pH value for both media ranged from 6.4 to 6.7. Fermentations were run at 28 °C with an aeration rate of 1 vol. vol.⁻¹ min⁻¹. The stirrer speed was set to 100 r.p.m. Antifoam Dehsan Z 2611 (Henkel) was added to the medium to a final concentration of 0.2 ml l⁻¹.

For fermenter and shake-flask experiments, used to assay β -galactosidase specific activity and *RIB3* transcript levels, a mineral salt medium (Monschau *et al.*, 1998) was used with 1 g yeast extract l⁻¹, 15 mM glycine and 20 g glucose l⁻¹ as carbon source.

For batch cultivations used to assay β -galactosidase specific activity, a 5 l bioreactor (Labfors System; Infors) was inoculated with 50 ml of an overnight culture containing 5 l mineral salt medium. The gas flow was set to 5 l min⁻¹ and the pO_2 , controlled by stirring speed (650 $\pm$ 350 r.p.m.), was set to a minimum of 80%. Fermentations were run at 30 °C.

Shake-flask experiments were performed at 30 °C under the conditions described for the inocula. For selective growth of transformants the aminoglycoside geneticin (Roche) was used at final concentrations of 400 μ g ml⁻¹ for solid media and 50 μ g ml⁻¹ for liquid cultures.

Escherichia coli strain DH5 α [*supE44* Δ *lacU169* (ϕ 80*lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used for general cloning methods (Hanahan, 1985).

DNA techniques. For cloning, transformation and plasmid isolation standard techniques were used (Sambrook *et al.*, 1989). Southern blotting was performed with digoxigenin (DIG)-labelled probes using the DIG-Labeling and Detection Kit as recommended by the supplier (Roche). For the isolation of genomic DNA the DNeasy Plant Maxi Kit (Qiagen) was used. For one preparation 1 g wet mycelium was homogenized in liquid nitrogen and processed as described in the manual.

RT-PCR. After 8 and 24 h of cultivation the volume of two shake flasks was pooled and the mycelia were used for total RNA isolation. The isolation of total RNA was carried out with the RNeasy Mini Kit (Qiagen). Cell lysis was performed with 0.2 g mycelium, 1 ml glass beads (diam. 0.5 mm) and 1 ml lysis buffer in a bead mill (Type MM2; Retsch) for 5 min at maximum intensity for one probe. Additionally a DNase treatment on the column was performed with the appropriate Qiagen system. Total RNA was eluted with 50 μ l H₂O.

Total RNA (5 μ g) was used for one RT reaction with Super Script II reverse transcriptase (Life Technologies). The reaction was performed at 44 °C with an oligo-dT₁₅ primer. After the RT reaction the preparation was made up with H₂O to a volume of 100 μ l, of which 2 μ l was used as a template in the following PCR.

PCRs were carried out in a volume of 50 μ l with 4 U *Taq* polymerase (Roche), 5 μ l reaction buffer, 2 μ l each primer (10 pmol μ l⁻¹) and 1 μ l dNTP mix (10 mM each dNTP). Primers were made according to the *RIB3* sequence (EMBL accession no. A46562; *RIB3* forw, 5'-AAGCCCATGCACT-GATATCG-3'; *RIB3* rev, 5'-GCAAGACCGTGCTTCTT-GC-3'), the *TEF* sequence (encoding translation elongation factor-1 α ; X73978; *TEF* forw, 5'-GCCATCTTGATCATT-GCTGG-3'; *TEF* rev, 5'-TTGACTTCAGTGGTGACACC-3') and the *ACT1* sequence (encoding actin; AJ131713; *ACT* forw, 5'-CTTCTACGTGTCCATTTCAGG-3'; *ACT* rev, 5'-AGAGAGGCCAAGATAGAACC-3'). PCR preparations were overlaid with mineral oil (Sigma) and *Taq* polymerase was added by a hot start procedure in the break mode of the thermal cycler (PTC-100; MJ Research). The amplification protocol was performed as follows: initial denaturation for 1 min at 94 °C; 27 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, and a final elongation period for 10 min at 72 °C. After the PCR, 8 μ l 6 $\times$ DNA loading buffer was added. Twenty microlitres of this solution was loaded on a 2% agarose gel for separation of the fragments.

Transformation of *A. gossypii* and isolation of spores. The transformation of wild-type mycelia was carried out by the method described in Monschau *et al.* (1998). The obtained mycelia were suspended in 1 ml 0.9% NaCl with 10 mg Glucanex ml⁻¹ (Novo) and incubated for 1 h at room tem-

Fig. 1. Chromosomal integration of the β -galactosidase reporter together with a geneticin resistance marker in front of the *ICL1* locus of *A. gossypii*.

perature. After a centrifugation step (5 min, 3000 g) the pellet was washed three times with 0.9% NaCl. The pellet was resuspended in 500 μ l 0.9% NaCl. After the addition of 500 μ l paraffin, the emulsion was shaken and stored at 4 °C for phase separation. The paraffin phase, containing the spores, was checked microscopically.

Construction of the reporter plasmid pTS-07. The *lacZ* gene was cut out from the plasmid pAG-110 (Kurth *et al.*, 1992; Steiner & Philippsen, 1994) by *Bam*HI and cloned in the *Bam*HI site of pBR322, leading to plasmid pTS-02. By using primer ts2 (5'-TTTCTCGAggatccGGCCCCGCTAGCGCCGTCGTTTTACAACGTCG-3') an *Apa*I and an *Nhe*I site (double-underlined) were incorporated behind the *Bam*HI site (lower case letters) for directional cloning of the *RIB3* promoter. The second primer, ts1 (5'-GGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTCTCGAGTTT-3') contained, like ts2, an *Xho*I site (underlined) for religation of the PCR product after *Xho*I digestion, resulting in pTS-04. Afterwards the *Bam*HI fragment was excised from pTS-04 and cloned in the *Bam*HI site of pUC18, resulting in pTS-05. Genomic *A. gossypii* wild-type DNA was used for PCR amplification of the *RIB3* promoter. Primers ts3 (5'-TTTGGTACCGGGCCCTTCTTGACGGTCGTTTCTGAA-3') and ts4 (5'-TTTGGTACCGCTAGCTGTCATGTTGCTTGGTTTGTCG-3') contained, besides the *Apa*I and the *Nhe*I sites (double-underlined), a *Kpn*I site (single-underlined) for religation of the PCR product, resulting in pTS-03. The sequence of the *RIB3* gene has been published by Revuelta *et al.* (1995). The *RIB3* promoter was excised with *Apa*I and *Nhe*I from plasmid pTS-03 and cloned in *Apa*I- and *Nhe*I-digested pTS-05. Finally, the *Bam*HI fragment containing the

lacZ gene under the control of the *RIB3* promoter was cloned in the *Bam*HI site of plasmid pAG-100 (Kurth *et al.*, 1992; Steiner & Philippsen, 1994), generating pTS-07. Sequencing of the transition from the promoter to the *lacZ* gene revealed successful in-frame cloning.

Construction of pTS-10 for chromosomal integration of the *RIB3*placZ fusion. In a first step a fragment was amplified by PCR, consisting of the *RIB3*placZ reporter and the geneticin resistance cassette (Fig. 1). By using PCR, in which the plasmid pTS-07 served as template, *Bgl*II sites (underlined) were added at the end of the fragment by the oligonucleotides ts10 and ts11 (ts10, 5'-TTTAGATCTGGATCCGGGCCCTTCTTGCA-3'; ts11, 5'-TTTAGATCTGCCGTCCTGTCAGTCAAGTCAGC-3'). The fragment was cloned between the sequence for a serine tRNA and the *ICL1* gene, encoding isocitrate lyase in *A. gossypii* (Maeting *et al.*, 1999). A 2.9 kb *Sph*I genomic fragment containing these sequences was cloned into the common cloning vector pUC18. Via PCR a *Bgl*II restriction site was added at position 245 (corresponding to EMBL accession no. AJ010727) into the 2.9 kb genomic fragment containing the serine tRNA sequence and the *ICL1* gene. The *Bgl*II fragment was cloned into this *Bgl*II site, generating plasmid pTS-10.

Determination of total riboflavin, mycelial dry mass and glucose. Procedures described by Monschau *et al.* (1998) were used for the determination of total riboflavin and mycelial dry mass. Instead of lysing enzyme, 100 μ l Glucanex (25 mg ml⁻¹) was used for cell disruption. The analysis was carried out by HPLC according to Schmidt *et al.* (1996). Glucose was determined in the culture filtrate with a UV test purchased from Roche.

Determination of DHBP synthase activity. For cell extract preparation mycelia were harvested by filtration (MF membrane filter, 0.8 µm pore size; Millipore) and resuspended in homogenization buffer (100 mM Tris/HCl buffer, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 1 mM PMSF, 10 µM antipain, 10 µM leupeptin) at a ratio of 2 ml (g mycelial wet wt)⁻¹. Cells were disrupted in a French press (Aminco) under 19000 p.s.i. (131 100 kPa) and the homogenate was centrifuged at 20000 g for 30 min. An aliquot (1.5–2.0 ml) of the supernatant was applied onto a Sephacryl S-200-gel permeation column (40 × 1.6 cm; Pharmacia). The column was developed using S200 buffer (50 mM Tris/HCl buffer, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 150 mM NaCl, 0.02% NaN₃) at a flow rate of 18 ml h⁻¹. A 200 µl aliquot of each fraction (1.8 ml) was checked for DHBP synthase activity by adding ribose 5-phosphate (Sigma) and pentose phosphate isomerase (Sigma) to a final concentration of 25 mM and 2 U, respectively, yielding a final volume of 240 µl. Samples were incubated at 37 °C for 4 h. To dephosphorylate the substrate and the formed products, 5 U alkaline phosphatase (Roche) was added with further incubation at 37 °C for 2 h. Finally, assay mixtures were subjected to an ultrafiltration step using Ultrafree-MC 10 centrifugation units (Millipore). The resulting filtrates were analysed for 3,4-dihydroxy-2-butanone by HPLC (Merck) using a Polyspher OAKC column (300 × 7.8 mm; Merck), which was developed isocratically with 5 mM H₂SO₄ at a flow rate of 0.4 ml min⁻¹. The column temperature was set to 45 °C. A UV detector (λ = 188 nm) and a series-connected RI detector (Merck) were employed to monitor the effluent. One unit of enzyme activity catalyses the formation of 1 nmol DHBP h⁻¹.

Determination of β-galactosidase activity. A cell-free crude extract was used to determine β-galactosidase activity. Mycelia were harvested by filtration and suspended in dissolution buffer consisting of buffer A (5% glycerol, 5 mM Tris, 10 mM KCl, pH 7.5) 30% glycerol and Complete (1 pill per 50 ml; Roche), a protease inhibitor mix. After a French press (Aminco) passage at 20000 p.s.i. (138000 kPa) the crude extract was obtained as supernatant by centrifugation at 20000 g for 15 min at 4 °C. The assay was based on a method described by Miller (1972). A mixture of 800 µl buffer A, 200 µl ONPG solution (4 mg ONPG ml⁻¹ in 100 mM KH₂PO₄) was incubated with an appropriate amount of crude extract at 30 °C. The reaction was stopped after 2 min and further time points with 500 µl 1 M Na₂CO₃ and the absorbance was measured at 420 nm. An extinction coefficient (ε) of 6.11 × 10⁵ M⁻¹ cm⁻¹ was used. The amount of total protein was determined by the method of Bradford (1976) with BSA as standard.

RESULTS

Correlation of DHBP synthase specific activity with riboflavin overproduction

Determination of DHBP synthase activity was not possible in crude extracts. Therefore fractionation of proteins was performed with a Sephacryl S-200 column to remove phosphatase activity present in the crude extracts leading to a reduction of the substrate ribose 5-phosphate needed in the assay. DHBP synthase activity was detectable in fractions 25–31 (Fig. 2).

After establishing the enzyme assay for DHBP synthase, analysis was focused on the time course of DHBP synthase activity during a fermenter cultivation in HA

Fig. 2. Elution profile from Sephacryl S-200 chromatography with a crude extract from *A. gossypii*. DHBP synthase activity (●) was detected by HPLC analysis. ○, Absorption of total protein of each fraction.

Fig. 3. DHBP synthase specific activity (Δ) and total riboflavin (▲) during cultivation of *A. gossypii* on HA medium. Glucose (not shown), the main carbon source, was exhausted from the medium after 20 h and biomass (not shown) reached a maximum of 4.6 g l⁻¹. The data shown were obtained in a single cultivation. In an independent experiment (data not in graph) enzyme specific activity increased from 12 nmol h⁻¹ (mg protein)⁻¹ before riboflavin production to a maximum of 145 nmol h⁻¹ (mg protein)⁻¹ during riboflavin production.

medium. This rich medium was selected to obtain enough biomass for the enzyme assay. During fermenter cultivation over 77.5 h the specific activity of DHBP synthase increased from 2.5 nmol h⁻¹ (mg protein)⁻¹ after 22 h to 156 nmol h⁻¹ (mg protein)⁻¹ at the end of fermentation (77.5 h; Fig. 3). In correlation with the increase in the specific activity of DHBP synthase, production of riboflavin was observed. The production of riboflavin increased from 1.2 mg l⁻¹ after 22.5 h of cultivation to 13 mg l⁻¹ at the end of fermentation.

Interestingly, in the growth phase from 0 to 20 h only a basal activity of DHBP synthase was observed. In the first 22.5 h only 9% of the total amount of riboflavin had been formed. The conclusion of this time course

Fig. 4. Relative *RIB3* mRNA concentration in comparison to *TEF* or *ACT1* mRNA levels in growth phase (8 h) and production phase (24 h) analysed by RT-PCR. Either one or two pairs of oligonucleotides were used. The upper part of the figure shows the RT-PCR products using *TEF* and *RIB3* oligonucleotides. The *RIB3* products have a size of 585 bp and the *TEF* products a size of 530 bp. The lower part displays the same experiment using *ACT1* oligonucleotides. The *ACT1* products have a size of 654 bp. Lane M, 100 bp molecular mass marker. The results shown were derived from a typical experiment. Two independent reproductions were performed. One of them was analysed with *ACT1*, the other with *TEF* oligonucleotides (results not shown).

experiment is that overproduction of riboflavin in *A. gossypii* might be caused by regulation of DHBP synthase.

Analysis of *RIB3* mRNA levels in growth and production phase

RT-PCR was performed to study whether the increase in specific activity of DHBP synthase in the production phase is due to an increase in the concentration of the *RIB3* transcript. The fungus was cultivated for 8 h to obtain mycelium in the growth phase and 24 h to obtain cells in the riboflavin production phase. *ACT1* mRNA or *TEF* mRNA served as a standard in the RT-PCR. The transcript of the β -actin gene is one of the most frequently used controls in quantitative and semi-quantitative PCR assays, but it has been reported that this mRNA is subject to modulation (Suzuki *et al.*, 2000). Therefore we decided to use the gene for the translation elongation factor 1 α as a standard. The *TEF* gene is highly expressed in *A. gossypii* (Steiner & Philippsen, 1994) and, furthermore, it has been shown that *TEF* has a more homogeneous expression than actin when used as a positive control in *in situ* hybridization assays (Gruber & Levine, 1997). The results of RT-PCR indicated an increase of *RIB3* mRNA in the riboflavin production phase after 24 h cultivation (Fig. 4). In such PCRs where *RIB3*- and *TEF*- or *ACT1*-

specific oligonucleotides were used in a competitive reaction, the signals of the *RIB3* PCR products increased in comparison to the signals of the standards. When the PCRs were performed without *RIB3* primers an approximately equal expression level of *TEF* and *ACT1*, indicated by equal signal intensities, was observed.

RIB3 promoter-*lacZ* reporter studies for *RIB3* expression

As well as regulation of gene expression by a controlled promoter activity, regulation may be due to a change in mRNA stability. To study whether the observed increase in *RIB3* mRNA in the riboflavin production phase is due to regulation of the promoter activity a reporter plasmid was constructed in which the *lacZ* gene of *E. coli* (Kalnins *et al.*, 1983) served as the reporter gene. It has been stated that the reporter plasmid pAG-110 is suitable for promoter studies of the *TEF* gene in *A. gossypii* (Steiner & Philippsen, 1994). In this plasmid the *lacZ* gene is under the control of the *TEF* promoter. We used plasmid pAG-110 and replaced the *TEF* promoter with the *RIB3* promoter from *A. gossypii*, generating pTS-07 (see Methods and Fig. 1). Plasmid pTS-07 was used for transformation of *A. gossypii* wild-type and several transformants were obtained. The transformants were cultivated for 8 and 24 h to assay β -galactosidase specific activity (Table 1). Geneticin was used in these cultivations to be sure that the plasmid remained present in the strains. All strains transformed with the plasmid pTS-07 (named AgpTS07-) showed an increase in β -galactosidase specific activity in the riboflavin production phase. The mean increase of β -galactosidase specific activity from 8 to 24 h of cultivation was 8.5-fold. The control strain transformed with pAG-110 revealed a weak increase in the production phase in two out of three cases and a decrease in the third. The mean values and standard deviations revealed clearly that there is no significant increase in β -galactosidase specific activity in the AgpAG-110 strain.

Plasmids pAG-110 and pTS-07 contain autonomously replicating sequence (ARS) elements from *S. cerevisiae*, which make extrachromosomal replication in *A. gossypii* possible (Wright & Philippsen, 1991). Therefore, we could not rule out the increase in specific activity of β -galactosidase in the production phase being due to a change in the copy number of pTS-07. In fact, it has been shown that plasmids containing ARS elements are freely replicative in *A. gossypii* (Wright & Philippsen, 1991). By DNA hybridization we found that a single copy of plasmid pTS-07 was integrated into the *A. gossypii* chromosome in all transformants (data not shown). Only the DNA of AgpTS07-B and AgpTS07-C gave an additional signal for extrachromosomal plasmids. In keeping with the finding that homologous recombination is preferred in *A. gossypii* (Steiner *et al.*, 1995), integration of the plasmid at the *RIB3* locus was observed by PCR (data not shown). To confirm that the increase in β -galactosidase specific activity in the production phase was not due to higher copy numbers of

Table 1. β -Galactosidase specific activity of plasmid (*AgpTS07*-) and site-directed (*AgRIB3placZ*-) transformants in growth phase (8 h) and riboflavin production phase (24 h)

After 8 h a mean biomass of $0.3 \pm 0.1 \text{ g l}^{-1}$ and after 24 h a mean biomass of $2.8 \pm 0.3 \text{ g l}^{-1}$ was obtained.

Transformant (<i>Ag</i> -)	β -Galactosidase specific activity [U (mg protein) $^{-1}$]							
	1st measurement		2nd measurement		3rd measurement		Mean $\pm$ SD	
	8 h	24 h	8 h	24 h	8 h	24 h	8 h	24 h
WT	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	–	–
pAG-110	2.41	3.36	2.06	4.84	3.97	2.00	2.81 ± 1.02	3.40 ± 1.42
pTS07-A	≤ 0.001	0.007	–	–	–	–	–	–
pTS07-B	≤ 0.001	0.004	–	–	–	–	–	–
pTS07-C	0.21	2.05	0.34	1.25	–	–	0.28 ± 0.09	1.65 ± 0.57
pTS07-32	0.012	0.085	–	–	–	–	–	–
pTS07-BDS	0.20	4.04	0.23	1.93	0.31	1.35	0.25 ± 0.06	2.44 ± 1.42
pTS07-BDS2	0.30	4.74	–	–	–	–	–	–
pTS07-22DS	0.56	3.89	–	–	–	–	–	–
pTS07-22DS2	0.54	3.86	–	–	–	–	–	–
<i>RIB3placZ</i> -1	0.14	1.67	0.06	1.76	0.08	0.53	0.09 ± 0.04	1.32 ± 0.69
<i>RIB3placZ</i> -3	0.63	3.75	0.31	1.35	–	–	0.47 ± 0.23	2.55 ± 1.70
<i>RIB3placZ</i> -4	0.07	0.38	–	–	–	–	–	–
<i>RIB3placZ</i> -5	0.08	0.49	–	–	–	–	–	–

the reporter plasmid caused by a popout, a defined integration of the reporter construction without an ARS element was performed.

Analysis of site-directed mutants carrying the *RIB3placZ* reporter with respect to genotype and β -galactosidase specific activity

A linearized form of plasmid pTS-10 was used for transformation of *A. gossypii* wild-type. A double cross-over event between the entire 2.9 kb *SphI* homologue sequence should lead to chromosomal integration of the reporter and the resistance marker in front of the *ICL1* locus (Fig. 1). Several transformants were obtained and analysed for genomic integration of the reporter and the geneticin resistance cassette. In Fig. 5 the result of a Southern blot experiment is shown for the wild-type and the transformant *AgRIB3placZ*-1. Chromosomal DNA of both strains was cut with *SphI*, giving a 2.9 kb fragment for the wild-type and a 4.3 kb fragment for the transformant because of an *SphI* restriction site between the *lacZ* gene and the geneticin resistance cassette. The result of Southern blotting clearly revealed the shift from 2.9 kb for the wild-type to 4.3 kb for the transformed strain, *AgRIB3placZ*-1. Above the 4.3 kb fragment an additional band indicated hybridization of the probe with a second putative serine tRNA locus in the *A. gossypii* genome (Fig. 5). Besides Southern blotting, the correct integration of the reporter and the geneticin resistance cassette was confirmed by PCR (data not shown).

Four independently transformed strains with defined genomic integration of the reporter fusion (named

AgRIB3placZ-) were cultivated in shake flasks for 8 and 24 h and the specific activity of β -galactosidase was measured (Table 1). In all strains an increase of β -galactosidase specific activity in the riboflavin production phase was determined. Taking all measurements together, the mean increase in specific activity was tenfold. The results are comparable with those obtained with strains carrying pTS-07. It was clearly shown that a single integration of the *lacZ* reporter in the *A. gossypii* genome is sufficient to see the induction of the *RIB3* promoter. Furthermore, it is striking that the induced *RIB3* promoter is highly active compared to the activity of the *TEF* promoter.

Fermenter cultivation of *AgRIB3placZ*-1

For a better resolution of the growth and riboflavin production phases, strain *AgRIB3placZ*-1 was cultivated in a 5 l fermenter with the same medium used in shake-flask experiments. The results, shown in Fig. 6(a, lower part) indicated low *RIB3* promoter activity in the early and exponential growth phases. The expression of the *lacZ* reporter was induced in the late growth phase as indicated by an increase in β -galactosidase specific activity. The specific activity reached a maximum in the riboflavin production phase of about 8 U (mg protein) $^{-1}$. A correlation between increase in β -galactosidase specific activity and riboflavin production was observed. After induction of reporter expression, riboflavin production started with a short delay and reached a maximum of about 60 mg (g mycelial dry mass) $^{-1}$ at the end of fermentation. Glucose depletion as a trigger for riboflavin production could be excluded: at the end of the fermentation, after 73 h, approximately 12 g

Fig. 5. Genotype verification of the transformant *AgRIB3placZ-1* by Southern blotting. Chromosomal DNA of *A. gossypii* wild-type (lane W) and *AgRIB3placZ-1* (lane T) was digested by *SphI* and probed with an *SphI*–*BglII* fragment derived from pT5-10. Lane M, DIG-labelled molecular mass marker; bands from top to bottom are 23130, 9416 and 6557 bp.

glucose 1^{-1} was still left in the medium (Fig. 6a, top). Yeast extract in the medium was growth-limiting. The experiment was completed by analysing *RIB3* mRNA levels during cultivation in comparison to *TEF* mRNA levels. The result of the RT-PCR is shown in Fig. 6(b). While the *TEF* mRNA level remained constant during cultivation, an increase in *RIB3* mRNA was observed in the late growth phase. This result was in keeping with the increase in β -galactosidase specific activity, also detected in the late growth phase. The loss of the *RIB3* signal at the end of the cultivation period (after 73 h) could be explained by replacement of dead cells by hyphae germinated from spores. This was visible by microscopical investigation of the culture.

DISCUSSION

Here we report for the first time the detection of DHBP synthase activity in *A. gossypii*, although this organism was used to investigate the four-carbon precursor needed for the riboflavin molecule (Plaut, 1954; Bacher *et al.*, 1982, 1983, 1985). The reason for the difficulties in detection is interfering enzymes which have to be removed. Volk & Bacher (1988, 1990) developed the assay in the yeast *Candida guilliermondii* and DHBP synthase activity has to date also been measured in *Arabidopsis thaliana* (Herz *et al.*, 2000) and the prokaryotes *B. subtilis* (Hübelin *et al.*, 1999) and *E. coli* (Richter *et al.*, 1992). The range of activity found in *A. gossypii* is comparable with that reported for the other organisms and, although rather low when compared with specific activities of enzymes of central metabolism, it is sufficient to explain the observed riboflavin production rate.

Until now, regulation of riboflavin synthesis has only been studied at the mRNA level in *B. subtilis*. We showed regulation of DHBP synthase at the activity and mRNA levels as well as by a β -galactosidase reporter experiment. Independently, on each level an approximately tenfold induction was shown during the production phase in comparison to the growth phase. Since *RIB3* encodes the first enzyme in the quantitatively more important branch of the riboflavin biosynthetic pathway, its regulation might be sufficient to explain riboflavin overproduction. However, the five remaining genes, *RIB1*, *RIB2*, *RIB4*, *RIB5* and *RIB7*, also have to be studied.

The scattering observed in the reporter activities determined was not due to strain instability or copy number effects of the plasmid used. The variability also occurred in strains where the reporter was integrated in the *A. gossypii* chromosome. The critical point seems to be the use of shake flasks, because the fermenter cultivation, where optimal conditions could be chosen, showed maximum activities which were never reached in a shake flask. In any case, the relative induction is visible despite the scattering of the absolute activities. Furthermore, plasmid-transformed strains can be used for deletion analysis of the *RIB3* promoter or reporter constructions of the other *RIB* genes. In the case of a coordinated induction of several *RIB* genes, a possible clustering, which has been shown for primary as well as secondary metabolite pathway genes (Keller & Hohn, 1997), has to be investigated in *A. gossypii*.

Glucose repression, which is a general mechanism for adaptation of metabolism to carbon source availability in the environment (see reviews by Ronne, 1995;

Fig. 6. (a) Fermenter cultivation of the mutant strain *AgRIB3placZ-1*. Glucose (∇), CO_2 in the exhaust gas ($\blacktriangledown$), mycelial dry mass ($\square$), β -galactosidase specific activity ($\blacksquare$) and total riboflavin ($\blacktriangle$) during cultivation are shown. The graphs shown represent a typical experiment. This was repeated once, revealing that the kinetics of induction in relation to growth and riboflavin, which is the essential point, were reproducible. In three further experiments β -galactosidase specific activity was $0.03 \pm 0.02 \text{ U (mg protein)}^{-1}$ when CO_2 in the exhaust gas was increased to 0.05% and $5.7 \pm 1.9 \text{ U (mg protein)}^{-1}$ when maximal riboflavin concentration was reached (means $\pm$ SD; data not shown in graph). (b) Results of RT-PCR showing *RIB3* mRNA concentrations relative to *TEF* mRNA levels during cultivation. Either one or two pairs of oligonucleotides were used. Arrows indicate *RIB3* products with a size of 585 bp and *TEF* products with a size of 530 bp.

Gancedo, 1998; Carlson, 1999) in yeast and other fungi, is probably not the regulatory mechanism likely to explain the kinetics of *RIB3placZ* expression and riboflavin overproduction. The arguments are, first, that more than $10 \text{ g glucose l}^{-1}$ were left in all cases when maximal *RIB3placZ* expression and riboflavin production were determined. Second, the specific productivity [$> 60 \text{ mg riboflavin (g biomass)}^{-1}$] was considerably high and cannot be expected to be enhanced by other carbon sources. Third, induced *RIB3placZ* expression reached the same order of magnitude as the *TEFplacZ* control. The situation is different in the case of the regulation of secondary metabolism in *Penicillium chrysogenum*, where the first gene in penicillin

biosynthesis, *acvA*, encoding δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine synthetase, is repressed by glucose (Revilla *et al.*, 1984, 1986). It is striking that in *Aspergillus nidulans*, *acvA* is not repressed, but the second gene, *ipnA*, is repressed by glucose (Brakhage, 1992). Recently, the glucose repressor CRE1 was shown to regulate *pcbC* and *cefEF*, two genes of cephalosporin synthesis in *Acremonium chrysogenum* (Jekosch & Kück, 2000). Although riboflavin is a primary metabolite, its overproduction has no known function for growth and therefore it can be classified as a product of secondary metabolism. This point of view justifies a comparison with the regulation of antibiotic synthesis pathways. Besides the *ICL1* promoter (Maeting *et al.*, 1999), the *RIB3* promoter is only the second regulated promoter to be described in *A. gossypii*. It might become a useful tool for the construction of strains overexpressing genes of flux-limiting enzymes in the riboflavin production phase.

An interesting subject for further research is the trigger for riboflavin overproduction. In fermenter cultivations riboflavin overproduction started at declining growth rates. The ideal tool for investigation might be a chemostatic cultivation with the reporter strain *AgRIB3placZ-1* at different growth rates.

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