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Purine Biosynthesis, Riboflavin Production, and Trophic-Phase Span Are Controlled by a Myb-Related Transcription Factor in the Fungus *Ashbya gossypii*†

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Ashbya gossypii **is a natural riboflavin overproducer used in the industrial production of the vitamin. We have isolated an insertional mutant exhibiting higher levels of riboflavin production than the wild type. DNA analysis of the targeted locus in the mutant strain revealed that a syntenic homolog of the *Saccharomyces cerevisiae BAS1* gene, a member of the Myb family of transcription factors, was inactivated. Directed gene disruption of Ag*BAS1* confirmed the phenotype observed for the insertional mutant, and the Δ*bas1* mutant also showed auxotrophy for adenine and several growth defects, such as a delay in the germination of the spores and an abnormally prolonged trophic phase. Additionally, we demonstrate that the DNA-binding domain of AgBas1p is able to bind to the Bas1-binding motifs in the Ag*ADE4* promoter; we also show a clear nuclear localization of a green fluorescent protein-Bas1 fusion protein. Real-time quantitative PCR analyses comparing the wild type and the Δ*bas1* mutant revealed that Ag*BAS1* was responsible for the adenine-mediated regulation of the purine and glycine pathways, since the transcription of the *ADE4* and *SHM2* genes was virtually abolished in the Δ*bas1* mutant. Furthermore, the transcription of *ADE4* and *SHM2* in the Δ*bas1* mutant did not diminish during the transition from the trophic to the productive phase did not diminish, in contrast to what occurred in the wild-type strain. A C-terminal deletion in the Ag*BAS1* gene, comprising a hypothetical regulatory domain, caused constitutive activation of the purine and glycine pathways, enhanced riboflavin overproduction, and prolonged the trophic phase. Taking these results together, we propose that in *A. gossypii*, Ag*BAS1* is an important transcription factor that is involved in the regulation of different physiological processes, such as purine and glycine biosynthesis, riboflavin overproduction, and growth.**

Ashbya gossypii is a filamentous hemiascomycete of considerable importance in biotechnology due to its natural ability to overproduce riboflavin (vitamin B_2) (5), an essential factor for humans and animals that is frequently used as a food additive (38). *A. gossypii* overproduces riboflavin as a detoxifying and protective mechanism during the late growth phase, when the maximum mycelial mass has been reached (37). Thus, in terms of riboflavin production, two stages can be differentiated during *A. gossypii* culture: a trophic phase when riboflavin production is minimal and the growth rate increases, and a productive phase when the growth rate decreases and riboflavin is overproduced (37). Many physiological and morphological changes occur during the shift from the trophic to the productive phase (27, 37), but the mechanisms triggering the transition are not fully understood. The productive phase is associated with a characteristic intense yellow color of the mycelia, due to the accumulation of the vitamin in the vacuolar compartment (9). During the past few years, different studies have been carried out with a view to improving the excretion of the vitamin (8) and also to increasing the metabolic flux for riboflavin biosynthesis (16, 17, 25, 35).

Riboflavin is synthesized from GTP and ribulose 5-phosphate in a six-step pathway governed by the *RIB* genes (*RIB1* to *RIB5* and *RIB7*) (3). For *A. gossypii*, it has been shown that riboflavin production is correlated with the activity of the Rib3 protein (34). However, enhanced riboflavin overproduction has been reported only when GTP precursors are added to the medium or when the purine or glycine pathways are engineered to provide high concentrations of GTP precursors (16, 21, 25, 35, 38). This indicates that GTP availability is a limiting factor for riboflavin production in *A. gossypii*.

GTP is formed in the cell through the de novo purine pathway (Fig. 1), which starts with the conversion of 5′-phosphoribosyl-1-pyrophosphate (PRPP) to IMP, after which IMP can be transformed into AMP or GMP (30). Alternatively, purine salvage pathways (Fig. 1) allow the interconversion and recycling of purines with the consumption of PRPP (30). The purine salvage pathways contribute to a correct balance between adenyl and guanyl nucleotides. In fact, maintenance of this balance is essential for cell viability; hence, the biosynthesis of purines relies on several regulatory mechanisms at the transcriptional and metabolic levels (4, 10, 11, 13, 14, 22, 40).

In *Saccharomyces cerevisiae*, it has been shown that a complex formed between the transcription factors Bas1 and Bas2 (Pho2) is required for the transcriptional activation of the de novo purine pathway under adenine deprivation (4). In addition, Bas1 and Bas2 also activate the biosynthesis of histidine, glutamine, glycine, and 10-formyl tetrahydrofolate (2, 7), which are purine-related pathways (see Fig. 5 in reference 31).

Current studies have reported that two intermediates of the

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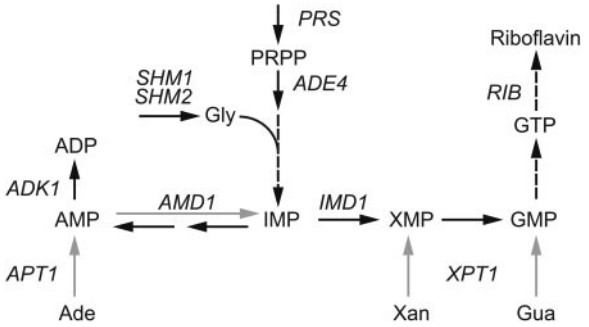

FIG. 1. Schematic representation of the purine biosynthetic pathway. Black arrows designate the de novo pathway, and gray arrows indicate the salvage pathways. Gly, glycine; GMP, guanosine 5′-monophosphate; Ade, adenine, Gua, guanine; Xan, xanthine. The gene names are italicized and correspond to the following enzymatic activities: *PRS*, PRPP synthetase; *ADE4*, PRPP amidotransferase; *SHM1* and *SHM2*, serine hydroxymethyltransferase; *IMD1*, IMP dehydrogenase; *AMD1*, AMP deaminase; *APT1*, adenine phosphoribosyltransferase; *XPT1*, xanthine phosphoribosyltransferase; *ADK1*, adenylate kinase; *RIB*, riboflavin genes.

purine pathway, namely, 5′-phosphoribosyl-4-succinocarboxamide-5-aminoimidazole (SAICAR) and 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole (AICAR), are intracellular signals that promote the interaction between Bas1 and Bas2 and, hence, transcriptional activation (30, 31). Unlike SAICAR, AICAR is also synthesized through the histidine pathway, again confirming the cross talk between purine and histidine biosynthesis (4, 30).

Bas1 is a Myb-related transcription factor comprising an amino-terminal DNA-binding domain that binds to the sequence TGACTC (15, 39), an internal *trans*-activation domain, and a C-terminal domain called the *B*as1 *i*nteraction and *r*egulatory *d*omain (BIRD) for interaction with Bas2 and regulation of the *trans*-activation domain (28). A recent report has suggested that Bas1 is permanently bound to DNA, with the *trans*-activation domain being inactive when adenine is present in the medium. Under conditions of adenine limitation, a regulatory signal, probably dependent on SAICAR and AICAR, increases the Bas1-Bas2 interaction; Bas2 is recruited to the promoters, and the *trans*-activation domains of Bas1 and Bas2 are unmasked for transcriptional activation (31, 36).

Here, we describe the identification and characterization of the transcription factor Bas1 in the filamentous fungus *A. gossypii*. AgBas1p participates in the regulated transcription of genes involved in the biosynthesis of purines and glycine. In addition, different *bas1* mutants showed a significant increase in the production of riboflavin and other growth-related phenotypes. The involvement of Ag*BAS1* in different processes of the *A. gossypii* physiology and its implications for the biotechnological production of riboflavin are discussed.

MATERIALS AND METHODS

***A. gossypii* strains, media, and growth conditions.** The *A. gossypii* ATCC 10895 strain was used and considered a wild-type strain. *A. gossypii* strains were cultured in rich medium (MA2) (9) or synthetic minimal medium (SMM) (35) at 28°C. Amino acids adenine and guanine were purchased from Sigma (Steinheim, Germany) and were used at a final concentration of 0.1 g/liter. A concentration of 250 μg/ml for Geneticin (G418) (Sigma, Steinheim, Germany) or 200 μg/ml for hygromycin B (Phytotechnology Laboratories, Shawnee Mission, Kans.) was used when stated. Sporulation conditions and spore isolations were carried out as previously described (33). Liquid cultures were inoculated with 1×10^6 spores per liter of medium and were performed on a rotary shaker at 120 rpm. The determination of riboflavin production was performed by high-performance liquid chromatography as described previously (35).

Insertional mutagenesis and targeted locus identification. A detailed description of the method has been previously reported elsewhere (33). Briefly, an in vitro transposition reaction using *A. gossypii* genomic DNA digested with PstI and a minitransposon *R* comprising the 5′ and 3′ inverted terminal repeats from the *Himar1* transposon flanking the dominant *G418* resistance marker and the bacterial replicon *ColE1* was performed. After the transposition reaction, the genomic DNA carrying an integrated minitransposon *R* was self-ligated and used to transform the *Escherichia coli* DH10B strain.

To generate insertional mutants, the plasmid library was linearized by enzymatic digestion with PstI and used to transform the wild-type *A. gossypii* strain. The *G418*^{r} *A. gossypii* colonies were sporulated and plated onto selective medium (MA2 plus G418) again. After 4 days, colonies exhibiting a bright yellow color were selected for further analyses. Genomic DNA from the insertional mutants was isolated, digested with XhoI, self-ligated, and transformed into *E. coli* DH10B. The transformants carrying the minitransposon *R* flanked by genomic DNA were selected on LB-kanamycin (50 μg/ml) plates. The identification of the targeted loci was achieved by sequence analysis using primers derived from the left and right ends of the minitransposon *R*, respectively. Determination of the full-length sequences was performed using primer-walking strategies.

Construction of the Δ*bas1* strain. To construct the Δ*bas1* strain, a disruption cassette was engineered. An internal BamHI-SphI fragment of the Ag*BAS1* open reading frame (ORF) was replaced with the *G418*^{r} marker obtained as a BamHI-SphI fragment (33). The disruption module was obtained as a XhoI-BglII fragment with a 356-bp 5′-flanking region and a 520-bp 3′-flanking region homologous to the Ag*BAS1* locus. This fragment was used to transform spores of the wild-type strain of *A. gossypii*. Primary G418^r heterokaryotic transformants were sporulated and, after clonal selection, G418^r Δ*bas1* homokaryotic colonies were isolated. The disruption of Ag*BAS1* was confirmed by analytical PCR and Southern blotting.

RNA extraction, reverse transcription-PCR, and real-time PCR. *A. gossypii* mycelium (200 to 300 mg), previously frozen, was mechanically homogenized in liquid nitrogen using TRIzol reagent (Invitrogen, Carlsbad, Calif.), and total RNA was isolated as described by the manufacturer. RNA was incubated with 20 U of RNase-free DNase I (Roche, Basel, Switzerland), and 5 μg of total RNA was reverse transcribed using an oligo(dT) primer (Isogen, Ijsselstein, The Netherlands) and SuperScript II RT enzyme (Invitrogen, Carlsbad, Calif.).

For real-time quantitative PCR, three serial dilutions (1:10, 1:20, and 1:30) of the synthesized cDNA were amplified with target gene-specific primers (see Table S1 in the supplemental material) using an iCycler iQ system (Bio-Rad, Hercules, Calif.). The following conditions were used: heat activation of DNA polymerase for 15 min at 95°C; 42 cycles of PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s; and a final incubation at 72°C for 10 min. For each PCR product, melting curves were determined according to the supplier's guidelines (Bio-Rad, Hercules, Calif.), ensuring specific amplification of the target gene. Quantitative values were obtained as the threshold PCR cycle number (*Ct*) when the increase in the fluorescent signal of the PCR product showed exponential amplification. The target gene mRNA level was normalized to that of Ag*ACT1* (encoding β-actin) in the same sample. The relative transcription level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{Ag}ACT1})_{\text{condition X}} - (Ct_{\text{target gene}} - Ct_{\text{Ag}ACT1})_{\text{condition Y}}$ (19). The mean of the results was obtained after the $2^{-\Delta\Delta Ct}$ was calculated for three serial cDNA dilutions of each sample in triplicate, and the relative transcription levels were expressed as means ± standard deviations (SD).

N-terminal Ag*BAS1* tagging with GFP(S65T). With PCR methods, an expression module containing a hygromycin resistance marker (*Hyg*^{r}) and the green fluorescent protein S65T [GFP(S65T)] coding region (20) in frame with the Ag*BAS1* ORF under the control of the constitutive promoter of the glyceraldehyde 3-phosphate dehydrogenase-encoding gene Ag*GPD* was constructed. The wild-type Ag*BAS1* locus was replaced with the expression module described above, and the replacement was verified by Southern blot analysis.

Nuclear DNA was stained with the DNA-specific dye Hoechst 33342 (HO) (Sigma, Steinheim, Germany). The fluorescence of the GFP-Bas1p fusion protein in living cells was monitored as previously described (26). Micrographs were acquired using a Photometrics Sensys charge-coupled-device camera coupled to a Leica DMXRA microscope equipped with Nomarski optics and epifluorescence.

Expression and purification of the N-terminal domain of the Bas1p from *A. gossypii*. The DNA sequence corresponding to the N-terminal domain of the AgBas1 (amino acids 1 to 305) was PCR amplified and cloned into the NdeI and

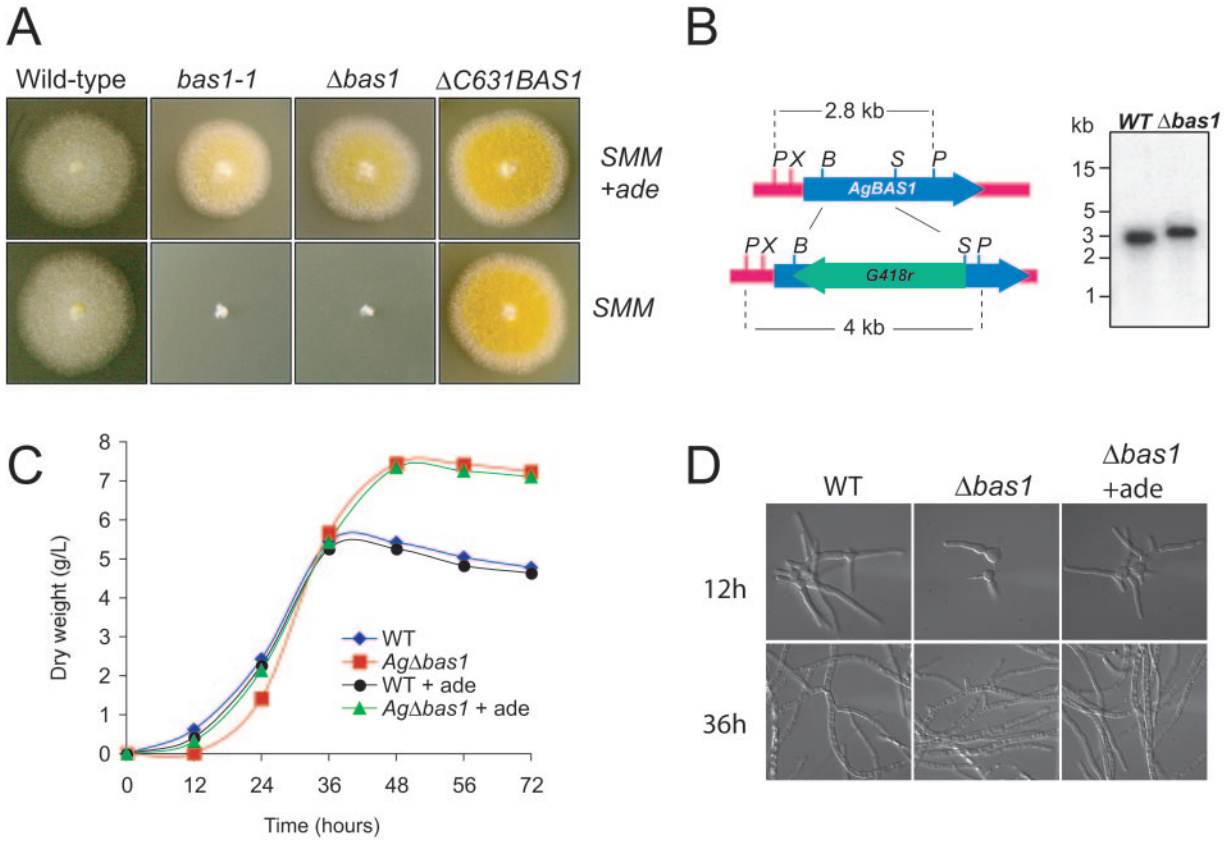

FIG. 2. Characterization of *A. gossypii bas1* mutant strains. (A) Different *A. gossypii* strains were grown on solid SMM or SMM plus adenine (+ade; 0.1 g/liter). The *bas1* mutants exhibit a yellow color due to riboflavin accumulation. (B) Right panel, schematic representation of the wild-type *BAS1* and disrupted *bas1*::*G418*^{r} loci. Left panel, Southern blot analysis to confirm correct *BAS1* disruption. Genomic DNA of the wild-type and Δ*bas1* strains was digested with PstI. P, PstI; X, XhoI; B, BamHI; S, SphI. (C) Growth pattern of *A. gossypii* wild type and Δ*bas1* grown in liquid MA2 rich medium with (+ade) or without adenine supplementation (0.1 g/liter). (D) Microscopic phenotype of *A. gossypii* wild type and Δ*bas1* grown on liquid MA2 rich medium for 12 and 36 h. The germination delay of Δ*bas1* is restored by the addition of adenine (+ade; 0.1 g/liter). WT, wild type.

XhoI site of the pET-28b expression vector (Novagen, Darmstadt, Germany) and used to transform the *E. coli* BL21 (DE3) strain. A single positive colony was inoculated in LB medium plus kanamycin (50 μg/ml) and grown at 37°C until the A_{600} reached 1. Induction of the His-tagged recombinant protein was achieved by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After 1 h of induction, the cells were harvested and resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8; 10 mM Tris-HCl, pH 8; 100 mM NaCl) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were disrupted by sonication for 30 min, and the homogenates were centrifuged at 10,000 × *g* for 10 min. The His-tagged recombinant protein was purified from the supernatant using a Talon (Clontech, Heidelberg, Germany) column, following the manufacturer's instructions. Pure fractions were stored at −20°C until use.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (15). The binding reaction was performed over 30 min at room temperature. In each reaction, 1×10^5 to 5×10^5 cpm of a labeled DNA probe (0.3 to 0.5 pmol) and 0.1 to 1 μg of recombinant His-AgN305Bas1 peptide were used. The binding buffer was 20 mM Tris-HCl, 0.1 mM EDTA, 10% glycerol (vol/vol), 100 mM NaCl, 0.01% Triton X-100 (vol/vol), and protease inhibitor cocktail (Roche, Basel, Switzerland). For competition binding reactions, the unlabeled competitor at 100- and 200-fold molar excess was used. A DNA fragment corresponding to the Ag*GPD* promoter (384 bp) was used as nonspecific competitor. The reaction mixtures were loaded onto a 5 to 8% native polyacrylamide gel in 1× TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA [pH 8.0]), electrophoresed, dried, and exposed to X-ray film at −80°C with an intensifying screen.

Construction of the Δ*C631BAS1* strain. The truncated Ag*BAS1* gene was generated by PCR-mediated modifications. A PCR-derived module containing the 50-bp fragment upstream from the codon encoding amino acid 631 of the AgBas1p followed by the *ScADH1* terminator, the *G418*^{r} marker, and the 50-bp fragment downstream from the Ag*BAS1* stop codon were used to transform wild-type *A. gossypii* spores. The truncated AgΔ*C631BAS1* gene was integrated in the *BAS1* locus. As described above, homokaryotic clones were isolated, and the truncation of Ag*BAS1* gene was confirmed by Southern blotting (see Fig. 7A).

RESULTS

Identification and characterization of a riboflavin-overproducing mutant. From a collection of *A. gossypii* mutants generated by an insertional mutagenesis method developed specifically for the purpose (33), a colony exhibiting a deep yellow color was selected (Fig. 2A); it proved to accumulate 5.5-fold (14.42 mg/g) more riboflavin than the wild-type strain. The presence in the *Himar1*-derived transposon module of a G418 resistance (*G418*^{r}) selectable marker and the *oriC* replication origin allowed the recovery and identification of the genomic integration site (see Materials and Methods for details). Once the integration site had been cloned and sequenced, primers were made to amplify the targeted ORF. DNA analysis revealed that the *Himar1*-derived transposon had integrated into a TA dinucleotide located 697 bp downstream from the initiation codon. A BLAST search in the Ashbya Genome Database (http://agd.unibas.ch/) identified the target as a nonex-

TABLE 1. Riboflavin levels in the different *A. gossypii* strains used[a]

Strain	Concn of riboflavin (mg/g of biomass) at time indicated			
	24 h	48 h	72 h	96 h
Wild type (ATCC 10895)	0.54 ± 0.11	1.44 ± 0.13	2.09 ± 0.16	2.58 ± 0.13
Ag*bas1-1*	0.40 ± 0.08	3.15 ± 0.24	13.47 ± 0.08	14.42 ± 0.10
AgΔ*bas1*	0.49 ± 0.06	3.12 ± 0.11	14.78 ± 0.16	15.31 ± 0.23
AgΔ*C631BAS1*	2.23 ± 0.21	9.76 ± 0.33	23.66 ± 0.36	24.28 ± 0.37

[a] Rich MA2 cultures were initiated from spores of the different *A. gossypii* strains. Ten-milliliter aliquots were removed at different times, and the riboflavin concentration was determined. Experiments were carried out in triplicate, and results are expressed as the average number of milligrams of riboflavin per gram of biomass ± SD.

perimentally characterized ORF (the database identified the gene as *AFR297W*, also designated Ag*BAS1*), a syntenic homolog of the *S. cerevisiae BAS1* gene, which encodes a transcription factor of the Myb family (39). The predicted amino acid sequence of *AFR297W* was aligned with ScBas1 and human c-Myb and showed low identity with ScBas1 (31.4%) and with human c-Myb (13.7%). However, the level of identity in the N-terminal domain was significantly higher (58.8% with ScBas1 and 21.5% with human c-Myb); using the ScanProsite tool (http://www.expasy.org/tools/scanprosite/), it was possible to identify the three signatures distinctive of the Myb family (15) (see Fig. S1 in the supplemental material). We also detected a hypothetical BIRD interaction domain within the Afr297w sequence (residues 630 to 664) compared with ScBas1 BIRD domain, although a low degree of identity (32.6%) was observed (see Fig. S1 in the supplemental material). We thus assumed that *AFR297W* ORF corresponded to the *BAS1* homolog in *A. gossypii* and, consequently, the mutant strain described above was designated *bas1-1*.

Ag*BAS1* inactivation causes riboflavin overproduction, adenine auxotrophy, and an extended trophic phase. To confirm that the accumulation of riboflavin in the *bas1-1* mutant was caused by the inactivation of the Ag*BAS1* ORF, we disrupted it in order to mimic the riboflavin overproduction phenotype found in the *bas1-1* strain. An engineered disruption module containing the dominant *G418*^{r} marker was used (see Materials and Methods). Ag*BAS1* disruption was confirmed by analytical PCR and Southern blotting (Fig. 2B). The new Δ*bas1* strain displayed the same yellow color and the same level of riboflavin production as the *bas1-1* strain (Fig. 2A and Table 1), demonstrating that *BAS1* inactivation was responsible for the increased production of vitamin B_2 in *A. gossypii*.

To further characterize the *bas1-1* and Δ*bas1* strains, we tested their ability to grow on minimal medium. As shown in Fig. 2A, the *bas1-1* and Δ*bas1* strains were unable to grow unless adenine was present in the medium. However, guanine, pyrimidines, or amino acids failed to rescue the wild-type phenotype (not shown). The adenine requirement indicates that *BAS1* is essential for the de novo biosynthesis of purines in *A. gossypii* and that purine salvage pathways are able to restore the synthesis of AMP in a *bas1* mutant when adenine is supplied.

Another interesting phenotype was associated with *BAS1* inactivation. When Δ*bas1* spores were grown on rich medium, a slight delay (4 to 6 h) in the time to germination, together with a significantly long trophic phase (approximately 12 h), was observed compared to the wild type (Fig. 2C-D). Supplementation of the medium with an excess of adenine corrected the delay in the time to germination for Δ*bas1* but failed to reduce the trophic-phase span (Fig. 2C-D).

AgBas1p is localized to the nucleus of *A. gossypii* and binds to the Ag*ADE4* promoter. The subcellular localization of a transcription factor is expected to be nuclear. Accordingly, we examined the subcellular localization of AgBas1 using a *GFP-BAS1* fusion under the control of the Ag*GPD* promoter. The strain carrying the *GFP-BAS1* fusion showed no differences from the wild-type strain in terms of riboflavin production, adenine requirement, and growth (not shown). The GFP-Bas1p fluorescence consistently colocalized with Hoechst 33342 staining of nuclear DNA, confirming that AgBas1p was localized to the nucleus (Fig. 3). This typical nuclear localization was invariably unaffected by the growth phase (Fig. 3).

Another essential feature of a transcription factor is its ability to bind to promoter sequences. We therefore wished to investigate whether AgBas1p was able to bind to the putative Bas1-binding sites located in the Ag*ADE4* promoter (16). The AgBas1 DNA-binding domain (amino acids 1 to 305) was expressed in *E. coli* BL21(DE3) using the pET-28b expression vector, and specific DNA binding of the purified protein to different DNA probes from the Ag*ADE4* promoter was monitored by EMSA analysis. In a first experiment, the complete Ag*ADE4* promoter (363 bp upstream from the initiation codon) was analyzed using a distal 131-bp fragment (fragment A) and a proximal 232-bp fragment (fragment B) as radioactive probes (Fig. 4A). The analysis revealed two different DNA-protein complexes formed with the distal probe (Fig. 4B, lane 2), indicating that two molecules of AgBas1p could be recruited to the Ag*ADE4* promoter. In Fig. 4, lane 2, the upper and lower bands correspond to fragment A, with two and one bound molecules of the AgBas1 DNA-binding domain, respectively. Addition of a 10-fold molar excess of unlabeled fragment completely abolished DNA-protein complex formation,

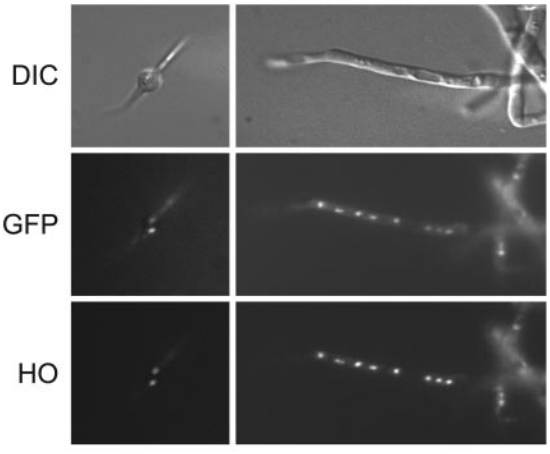

FIG. 3. AgBas1p is localized to the nuclei of *A. gossypii*. An *A. gossyppi* spore (left) and a hypha (right) expressing the GFP-Bas1p were stained with the DNA-selective dye Hoechst (HO) 33342 and viewed under epifluorescence optics. Upper images are the differential interference contrast (DIC) pictures; middle images represent the GFP channel, and lower images correspond to the HO channel of the same field.

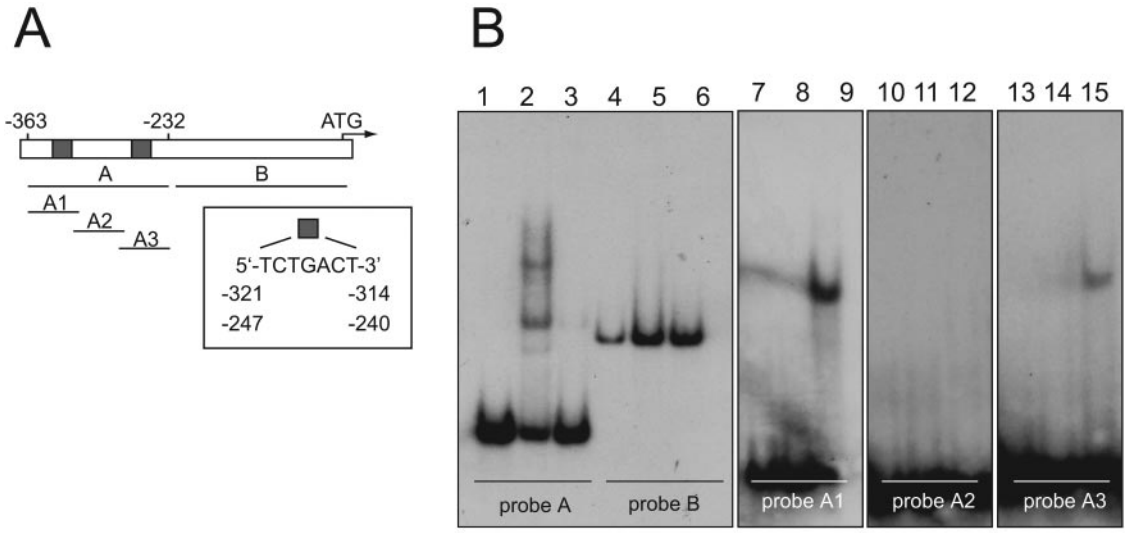

FIG. 4. Identification of the DNA-binding site of AgBas1p by EMSA. (A) Structure of the Ag*ADE4* promoter. Gray boxes indicate the Bas1-binding sites. The different probes used in the assays are depicted. (B) EMSA analyses using different probes: probe A, lanes 1 to 3; probe B, lanes 4 to 6; probe A1, lanes 7 to 9; probe A2, lanes 10 to 12, probe A3, lanes 13 to 15. An excess of the corresponding unlabeled probe was used in lanes 3, 6, 8, 11, and 14. No Bas1 DNA-binding domain peptide was included in lanes 1, 4, 7, 10, and 13.

confirming the specificity of the interaction (Fig. 4B, lane 3). In contrast, a similarly sized (384-bp) DNA fragment corresponding to the Ag*GPD* promoter did not act as a competitor for AgBas1p (data not shown).

When analyzing the sequence of the distal fragment A in detail, we were able to identify two putative heptanucleotide Bas1-binding motifs located at positions −247 and −321 from the start codon (Fig. 4A). Therefore, we explored the ability of the AgBas1 DNA-binding domain to bind to each heptanucleotide. We designed three overlapping oligonucleotides that expanded the fragment A to full-length. Oligonucleotide A1 contained the Bas1-binding motif located at position −321, oligonucleotide A2 did not contain any motif, and oligonucleotide A3 contained the heptanucleotide located at position −247 (Fig. 4A). EMSA analysis revealed that the AgBas1 DNA-binding domain caused gel retardation when combined with oligonucleotide A1 or A3 (Fig. 4B, lanes 9 and 15), but not with A2. Again, an excess of unlabeled probes completely abolished DNA-protein complex formation (Fig. 4B). Based on these results, it may be concluded that AgBas1p is a transcription factor that is able to bind to the two putative Bas1-binding motifs contained in the Ag*ADE4* promoter.

AgBas1p is involved in the adenine-mediated regulation of Ag*ADE4* and Ag*SHM2* in *A. gossypii*. The above results suggested an important role for Ag*BAS1* in the biosynthesis of purine nucleotides and riboflavin. We therefore investigated the transcription profiles of *ADE4* and *SHM2*, which have been reported to be regulated by adenine limitation in *A. gossypii* (16, 35); *PRS1* and *PRS2*, which control the synthesis of the purine precursor PRPP (A. Jiménez, M. A. Santos, and J. L. Revuelta, unpublished results); the *IMD1* gene, which directs the first step of GMP biosynthesis; and all six *RIB* genes in both wild-type and the Δ*bas1* strains.

Total RNA was isolated from cultures of the wild-type and Δ*bas1* strains in the exponential phase (24 h) grown in rich medium, and real-time quantitative PCR was performed to test the transcription levels of the *ADE4*, *SHM2*, *PRS1*, *PRS2*, *IMD1*, and *RIB* genes. As shown in Fig. 5A, *ADE4* and *SHM2* transcription was considerably lower in the Δ*bas1* strain than in the wild-type strain, whereas *PRS1*, *PRS2*, *IMD1*, and the six *RIB* genes were unaffected by the *BAS1* inactivation. This result indicates that Bas1 is a transcription factor that activates the expression of *ADE4* and *SHM2* in *A. gossypii*.

We next analyzed whether the presence or absence of an excess of adenine in rich medium might affect the transcription level of the above genes in the wild-type and Δ*bas1* strains. Our results revealed that the addition of adenine clearly repressed the transcription of *ADE4* and *SHM2* in the wild-type strain (Fig. 5B). In contrast, adenine had no effect on *ADE4* or *SHM2* in the Δ*bas1* mutant, indicating that *BAS1* was essential for the adenine-dependent regulation of both genes. The transcription levels of the other genes analyzed were unaltered by the concentration of adenine in both the wild-type and the Δ*bas1* strains, confirming that Bas1 did not modulate their transcription (Fig. 5B).

Purine and riboflavin genes are inversely regulated during the growth of *A. gossypii*. Riboflavin overproduction in *A. gossypii* occurs during the so-called production phase, when the cultures reach a stationary phase. In addition, the riboflavin-overproducing phenotype of the *bas1* mutants suggests a direct link between the Bas1 function and riboflavin biosynthesis. We therefore checked the transcription pattern of purine and riboflavin genes in the *A. gossypii* wild-type and Δ*bas1* strains along the growth curve in rich medium. Real-time quantitative PCR showed that the mRNA levels of purine genes (*ADE4* and *SHM2*) in the wild type were highest during the trophic phase (12 to 36 h of culture) (Fig. 6, upper panel), when riboflavin production was minimal and growth was active. Thereafter, the transcription of purine genes declined progressively, being lowest during the productive phase (48 to 72 h) (Fig. 6, upper panel). *SHM2* transcription is increased at 72 h, but this effect seems to be independent of Bas1 regulation,

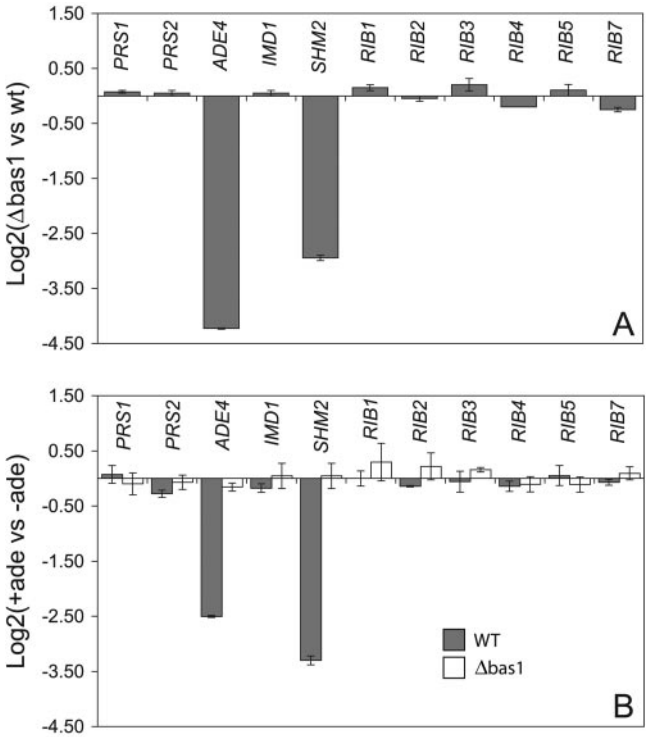

FIG. 5. Comparison of the steady-state transcription levels of purine and riboflavin genes in wild-type and Δ*bas1* strains. (A) Differences in the transcription levels of the genes included in the assays between wild-type and Δ*bas1* strains grown in MA2 rich medium. (B) Relative transcription levels of the indicated genes measured in the wild-type (gray bars) and Δ*bas1* (white bars) strains grown in MA2 rich medium with (+ade) or without (−ade) an excess of adenine (0.1 g/liter). Transcription levels are normalized to the level of Ag*ACT1* mRNA. The ratio of relative transcription of the target gene in panel A was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\Delta bas1} - \Delta Ct_{\text{wild type}}$. The ratio of the relative transcription of the target gene in panel B was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{MA2 + ade}} - \Delta Ct_{\text{MA2 − ade}}$. An average of three separate cDNA dilutions from each target gene were obtained, and the relative transcription levels were expressed as $\log_2 \pm$ SD. vs, versus; WT, wild type.

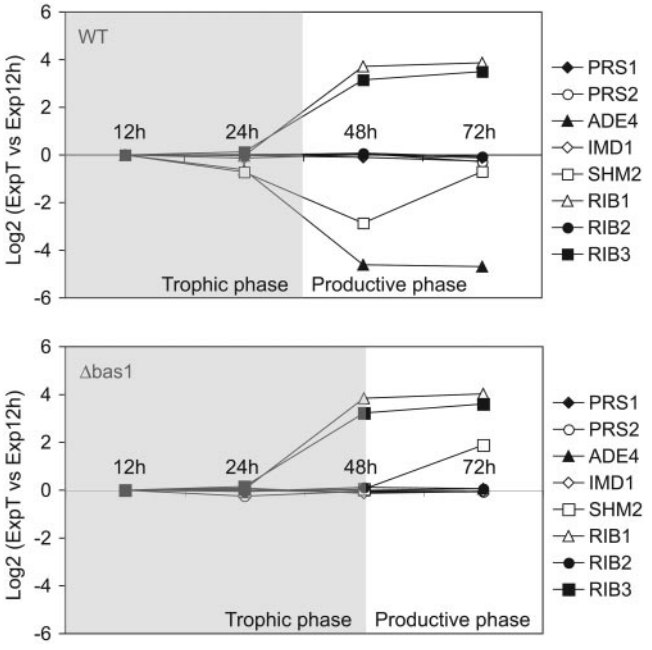

FIG. 6. Transcription profiles of purine and riboflavin genes in wild-type and Δ*bas1* strains during the trophic and productive phases. Transcription profiling of the wild-type (WT) and Δ*bas1* strains grown in MA2 rich medium was achieved at different time points. In the wild-type strain (upper panel), the transcription of *ADE4* and *SHM2* decreased along the growth curve, whereas the transcription of *RIB1* and *RIB3* increased during the productive phase. In the Δ*bas1* strain (lower panel), *ADE4* and *SHM2* show a constitutive deregulated transcription. Transcription levels are normalized with the transcription rate of Ag*ACT1*.Data are representative of four experiments with similar results and are presented as $\log_2$ of the $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{\text{T}} - \Delta Ct_{12\text{ h}}$). T, incubation time (h).

since it also occurs in the Δ*bas1* strain (see below). The *RIB1* and *RIB3* genes were more actively transcribed than the purine genes during the productive phase (Fig. 6, upper panel). In fact, a previous report had already shown an increase in the promoter activity of *RIB3* during the production phase (34). No differences in the transcription pattern of *RIB* genes between the wild type and Δ*bas1* strains were seen (Fig. 6). With regard to purine genes, a deregulated, low, but constitutive transcription was evident in the Δ*bas1* strain throughout the growth curve (Fig. 6, lower panel), demonstrating that Bas1 was essential for accurate regulation of the purine pathway. Remarkably, the transcription levels of purine genes were higher in the Δ*bas1* strain than in the wild type during the productive phase (Fig. 6, lower panel), indicating that a Bas1-independent basal transcription of the de novo purine genes was occurring in the Δ*bas1* strain. However, such a transcription level did not provide enough purines to enable the growth of the Δ*bas1* mutant in the absence of extracellular adenine.

AgBas1 contains a C-terminal regulatory BIRD domain. As described above, we identified a hypothetical C-terminal BIRD domain within the AgBas1p sequence (residues 632 to 743) (see Fig. S1 in the supplemental material). In order to verify the functionality of this regulatory domain, we decided to remove it by PCR-mediated deletion using a *G418*^{r} selectable marker (see Materials and Methods). Homokaryotic G418^r clones carrying the deletion module were confirmed by analytical PCR and Southern blotting (not shown). The new strain lacking the AgBas1 C-terminal domain was designated Δ*C631BAS1*. Deletion of the BIRD domain enhanced the capacity to produce riboflavin—the Δ*C631BAS1* mutant was able to produce almost twice (24.28 mg/g) more riboflavin than the Δ*bas1* mutant and 12-fold more riboflavin than the wild type, hence also affording an intense yellow color to the colonies (Fig. 2A and Table 1). Unlike Δ*bas1*, Δ*C631BAS1* was able to grow in minimal medium without adenine supplementation (Fig. 2A).

The Δ*C631BAS1* strain also displayed a remarkable feature when the spores were cultured in rich medium. Like the Δ*bas1* mutant, Δ*C631BAS1* displayed a longer trophic phase than the wild type; however, germination time was not affected, unlike results for Δ*bas1* (Fig. 7A).

Analysis of the transcription pattern of the purine and riboflavin genes in the Δ*C631BAS1* strain revealed that *ADE4* and *SHM2* transcription was highly activated and that the activation was insensitive to extracellular adenine, since the mRNA

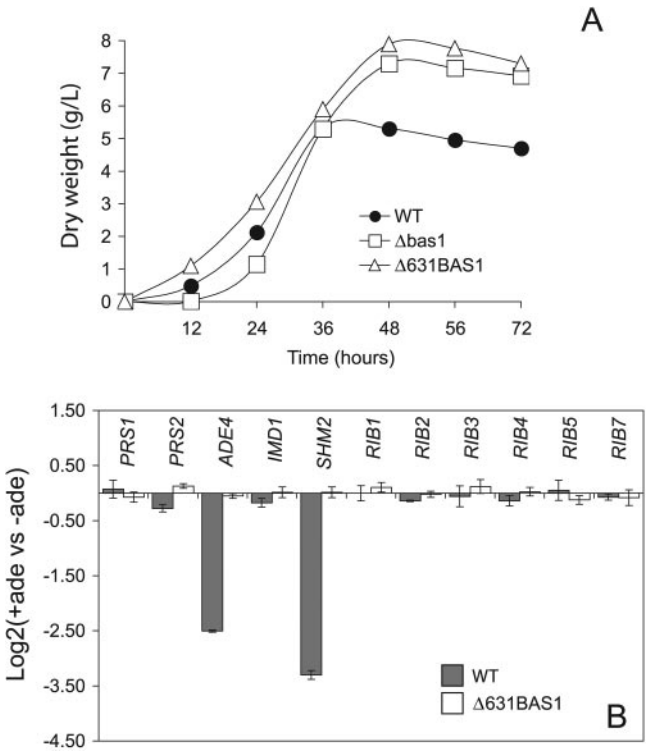

FIG. 7. Characterization of the Δ*C631BAS1* strain. (A) Growth pattern of Δ*C631BAS1* strain grown in liquid MA2 rich medium in comparison with the wild-type and Δ*bas1* strains. (B) Relative transcription levels of purine and riboflavin genes in the wild-type (gray bars) and the Δ*C631BAS1* (white bars) strains grown in MA2 rich medium with (+ade) or without (−ade) an excess of adenine (0.1 g/liter). Transcription levels are normalized to the level of Ag*ACT1* mRNA. The ratio of the relative transcription of the target gene was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\mathrm{MA2\ +\ ade}} - \Delta Ct_{\mathrm{MA2\ -\ ade}}$. An average of three separate cDNA dilutions from each target gene was obtained, and the relative transcription levels are expressed as $\log_2 \pm$ SD.

levels of *ADE4* and *SHM2* were constitutively higher in the Δ*C631BAS1* strain than in the wild type with adenine supplementation (Fig. 7B). However, the other genes analyzed did not show any change in transcription levels when the Δ*C631BAS1* and the wild-type strains were compared (Fig. 7B). Our results suggest that the C-terminal BIRD domain of AgBas1p would be a regulatory domain and that the truncated ΔC631Bas1p form would induce a constitutive transcriptional activation of *ADE4* and *SHM2*, an increase in riboflavin production, and a delayed entry into the productive stationary phase.

DISCUSSION

The overproduction of riboflavin in *A. gossypii* is a physiological process that occurs when active growth finishes and therefore must be somehow linked to the growth phase. It has been suggested that *A. gossypii* overproduces riboflavin as a detoxifying and protective mechanism (37), although the molecular mechanisms triggering the riboflavin overproduction are largely unknown.

In this report, we show that Ag*BAS1* is involved in the regulation of the glycine and purine biosynthesis, which have been shown to increase riboflavin production when added to the medium (21, 38). Furthermore, we constructed different *bas1* mutants that exhibit remarkable differences in their growth patterns and higher levels of riboflavin production than the wild type. In light of this, we propose the Ag*BAS1* gene as a possible candidate for a link between the glycine and purine pathways, the growth profile, and riboflavin overproduction.

Ag*BAS1* was found as the target gene disrupted in a riboflavin-overproducing strain, which had been selected in a screening of random insertional mutants. Directed Ag*BAS1* gene disruption confirmed that *BAS1* inactivation in *A. gossypii* afforded a riboflavin overproduction phenotype. In addition, the Δ*bas1* strain required adenine supplementation, suggesting an important role for Bas1p in the biosynthesis of purines, as described for other organisms (4). The asymmetry of the purine salvage pathways (Fig. 1) explains why only adenine supplementation, but not supplementation with either guanine or xanthine, enables the growth of the Δ*bas1* mutant, since adenyl nucleotides can be transformed into guanyl nucleotides, but not the opposite.

Real-time quantitative PCR experiments comparing mRNA profiles between the wild-type and Δ*bas1* strains showed that AgBas1p is essential for the transcriptional activation of *ADE4* and *SHM2* and also for the adenine-mediated repression of those genes. The role of AgBas1p as a transcription factor is consistent with the nuclear localization of a GFP-Bas1 fusion protein. Additionally, our EMSA experiments showed that the Myb-like DNA-binding domain present in AgBas1p is able to bind the heptanucleotide located in the *ADE4* promoter, which has been reported to be a Bas1-binding motif (15, 16). Such a binding motif has also been found in the Ag*SHM2* promoter (M. A. Santos, L. Mateos, and J. L. Revuelta, unpublished results). Overall, this evidence prompts us to consider AgBas1p to be a transcription factor that regulates purine and glycine biosynthesis, as described for other organisms (4, 6). However, AgBas1p also seems to be either directly or indirectly involved, through the purine pathway, in other physiological processes, such as detoxifying mechanisms or growth and morphogenesis, as discussed below.

Homology analyses identified a C-terminal regulatory domain within the AgBas1p sequence, called the BIRD domain in the yeast ortholog (28), and the complete deletion of this hypothetical BIRD domain in the AgBas1p was achieved. The Δ*C631BAS1* strain produces higher levels of riboflavin than the Δ*bas1* mutant, is able to grow in medium lacking adenine, and shows a constitutive activation of the *ADE4* and *SHM2* genes. In light of these observations, it may be concluded that the C-terminal domain of AgBas1 would be responsible for the adenine sensitivity, for masking the *trans*-activation domain when adenine is present and, presumably, for the Bas2 interaction, as described previously for the yeast BIRD domain (28, 36). In fact, a *ScBAS2* ortholog in *A. gossypii* has recently been identified (L. Mateos, M. A. Santos, and J. L. Revuelta, unpublished results).

The Δ*bas1* and the Δ*C631BAS1* strains also display a prolonged trophic growth phase in comparison with the wild type. In addition, the Δ*bas1* strain exhibits a slight delay in the time to germination, together with other morphological defects in the hyphal structure. These effects may be directly related to the deregulation of the purine pathway in the Δ*bas1* and

Δ*C631BAS1* strains. First, from the results described above, it may be assumed that the de novo purine pathway does not operate properly in the Δ*bas1* mutant and that purines must be formed mainly through the salvage pathways, delaying the germination during the early steps of growth. In fact, adenine supplementation corrected the germination defect in the Δ*bas1* mutant. Second, it has been reported that low levels of intracellular guanyl nucleotides are required for entry into the stationary phase in *S. cerevisiae* and *Bacillus subtilis* (29, 32). The riboflavin overproduction phenotype shown by the Δ*bas1* and the Δ*C631BAS1* strains indicates that a high pool of GTP, the immediate precursor of riboflavin, must exist in both strains, producing a delay in entry into the stationary phase and extending the trophic phase. This hypothesis supports the notion that the guanyl nucleotide pool would be a signal that controls the transition from the trophic phase to the stationary production phase and suggests that an increased riboflavin production and a delayed entry into the stationary phase would be two aspects of the same phenomenon, i.e., a high GTP concentration. Indeed, the Δ*bas1* and Δ*C631BAS1* strains were more resistant to mycophenolic acid than the wild type (see Fig. S2 in the supplemental material), a phenotype associated with high intracellular levels of GTP (1, 23).

Nevertheless, the mechanisms inducing an increased intracellular concentration of GTP, and hence an enhanced riboflavin overproduction, in the Δ*bas1* and the Δ*C631BAS1* strains must be different. Δ*C631BAS1* shows a constitutive activation of the purine pathway that could be responsible for the high GTP levels and the increased riboflavin overproduction observed. Supporting this notion, we have previously demonstrated that the level of transcription of Ag*ADE4* is correlated with the production of riboflavin (16). The explanation for the riboflavin overproduction phenotype in the Δ*bas1* strain is, however, less obvious. As evidenced by its adenine auxotrophy, purines must be formed mostly through the salvage pathways in the Δ*bas1* strain. Under these conditions, the AMP deaminase enzyme activity (Amd1) (24) establishes the asymmetry in the purine salvage pathways and redirects the metabolic flux toward guanyl nucleotides in competition with the adenylate kinase (Adk1) (18). In addition, the enzyme Xpt1 can metabolize xanthine and guanine toward GMP (12) (Fig. 1). The genes encoding these enzymes have been annotated in the Ashbya Genome Database (http://agd.unibas.ch/) as *S. cerevisiae* syntenic orthologs (AGD gene identification numbers *AGR187W*, *ABR204C*, and *ABL070C* for Ag*ADK1*, Ag*AMD1*, and Ag*XPT1*, respectively). However, an *HPT1* homolog, which restores equilibrium by transforming hypoxanthine into IMP in other organisms (41), has not been identified; therefore, hypoxanthine cannot be transformed to IMP in *A. gossypii*. Accordingly, the purine *salvage* pathways in *A. gossypii* may lead to the production of higher amounts of guanyl than adenyl nucleotides; hence, the Δ*bas1* strain, which uses mostly the purine salvage pathways, may be able to synthesize higher amounts of the riboflavin precursor GTP than the wild type. Additionally, the Ag*SHM2* mRNA level during the first steps of growth was lower in the Δ*bas1* strain than in the wild type, and it has been reported that disruption of Ag*SHM2* produces glycine accumulation and enhanced riboflavin overproduction (35). Indeed, riboflavin overproduction in the Δ*bas1* strain was inversely correlated with the *SHM2* transcription level.

In sum, the results presented here suggest that the mechanisms leading to a high concentration of GTP are responsible for riboflavin overproduction in *A. gossypii*. In addition, the BIRD domain found in AgBas1p could be considered an intracellular sensor of the GTP concentration. High levels of GTP could induce a repression signal of the de novo purine pathway through a mechanism in which the Bas1 BIRD domain may be involved, masking the *trans*-activation domain of Bas1. If the BIRD domain is indeed not functional, Bas1 must be constitutively active and the purine pathway must synthesize an excess of GTP, which must be detoxified through riboflavin production. Nevertheless, the *bas1* mutants show several growth alterations affecting the transition from the trophic phase to the productive phase, and these effects indicate that Bas1 could be involved, either indirectly, through the purine pathway, or directly, in the regulation of other hitherto-undescribed growth-related pathways.

This work sheds further light on the complex role of *BAS1* in the physiology of *A. gossypii*. A feasible link between purine and glycine biosynthesis, riboflavin overproduction, and the transition from the trophic to the stationary phase is established. Experiments are currently under way at our laboratory to explore the precise mechanisms underlying some of the effects induced by misregulation of the purine pathway.

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