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Physiological characterization of a pyrimidine auxotroph exposes link between uracil phosphoribosyltransferase regulation and riboflavin production in *Ashbya gossypii*

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ABSTRACT

The blockage of the *de novo* pyrimidine biosynthetic pathway at the orotidine-5′-phosphate decarboxylase level was previously demonstrated to affect riboflavin production in the industrial producer fungus *Ashbya gossypii*. However, the molecular basis for the unusual sensitivity to uracil displayed by the pyrimidine auxotroph *A. gossypii Agura3* was unknown. Here, uridine was shown to be the only intermediate of the pyrimidine salvage pathway able to fully restore this mutant's growth. Conversely, uracil, which is routinely used to rescue pyrimidine auxotrophs, had a dose-dependent growth-inhibitory effect. Uracil phosphoribosyltransferase (UPRT) is the pyrimidine salvage pathway enzyme responsible for converting uracil to uridine monophosphate in the presence of phosphoribosyl pyrophosphate (PRPP). Characterization of the *A. gossypii* UPRT, as produced and purified from *Escherichia coli*, revealed that uracil concentrations above 1 mM negatively affected its activity, thus explaining the hypersensitivity of the *Agura3* mutant to uracil. Accordingly, overexpression of the AgUPRT encoding-gene in *A. gossypii Agura3* led to similar growth on rich medium containing 5 mM uracil or uridine. Decreased UPRT activity ultimately favors the preservation of PRPP, which otherwise may be directed to other pathways. In *A. gossypii*, increased PRPP availability promotes overproduction of riboflavin. Thus, this UPRT modulation mechanism reveals a putative means of saving precursors essential for riboflavin overproduction by this fungus. A similar uracil-mediated regulation mechanism of the UPRT activity is reported only in two protozoan parasites, whose survival depends on the availability of PRPP. Physiological evidence here discussed indicate that it may be extended to other distantly related flavinogenic fungi.

Introduction

The use of auxotrophies as tools for genetic modification of microbial strains is widely employed in molecular microbiology. However, there are some recognized problems associated with their use, such as a lack of clarity regarding how much nutrient supplementation is required for each auxotrophy and microorganism [1,2]. Auxotrophy for pyrimidines, resulting from blockage of the *de novo* pyrimidine biosynthetic pathway at the orotidine 5′-phosphate decarboxylase level (encoded by *URA3* or corresponding ortholog in non-yeast species) or at the orotate phosphoribosyltransferase level (encoded by *URA5* or homologs), is one of the most used among microbes [1–3]. As standard procedure, these mutants require the supply of exogenous uracil to rescue their growth, thus being termed uracil auxotrophs.

Uracil enters the pyrimidine salvage pathway and is converted to uridine monophosphate (UMP) in a one-step reaction catalyzed by uracil phosphoribosyltransferase (UPRT), thus re-establishing the biosynthesis of pyrimidines (Fig. 1). While efficient in restoring growth of the majority of the pyrimidine auxotrophs, the supply of exogenous uracil is unable to fully overcome the effects of this auxotrophy in some fungi, which require the supply of exogenous uridine. Among them are *Candida albicans* [3], *Ashbya gossypii* (*syn. Eremothecium gossypii*) [4], *Debaryomyces hansenii* (*syn. Candida famata*) [5], *Candida guilliermondii (syn. Pichia guilliermondii*) [6], *Aspergillus fumigatus* [7] and *Aspergillus nidulans* [8]. Despite the importance of this apparently uncommon phenotype, it is poorly understood at the molecular level.

A. gossypii is a filamentous fungus of considerable industrial and biological significance, due to its capacity to overproduce riboflavin

Abbreviations: UMP, uridine monophosphate; UPRT, uracil phosphoribosyltransferase; AFM, Ashbya Full Medium; MM, minimal medium; SC, synthetic complete; TrxA, thioredoxin A; PRPP, phosphoribosyl pyrophosphate; 5-FC, 5-flucytosine; 5-FU, 5-fluorouracil; GTP, guanosine-5-triphosphate

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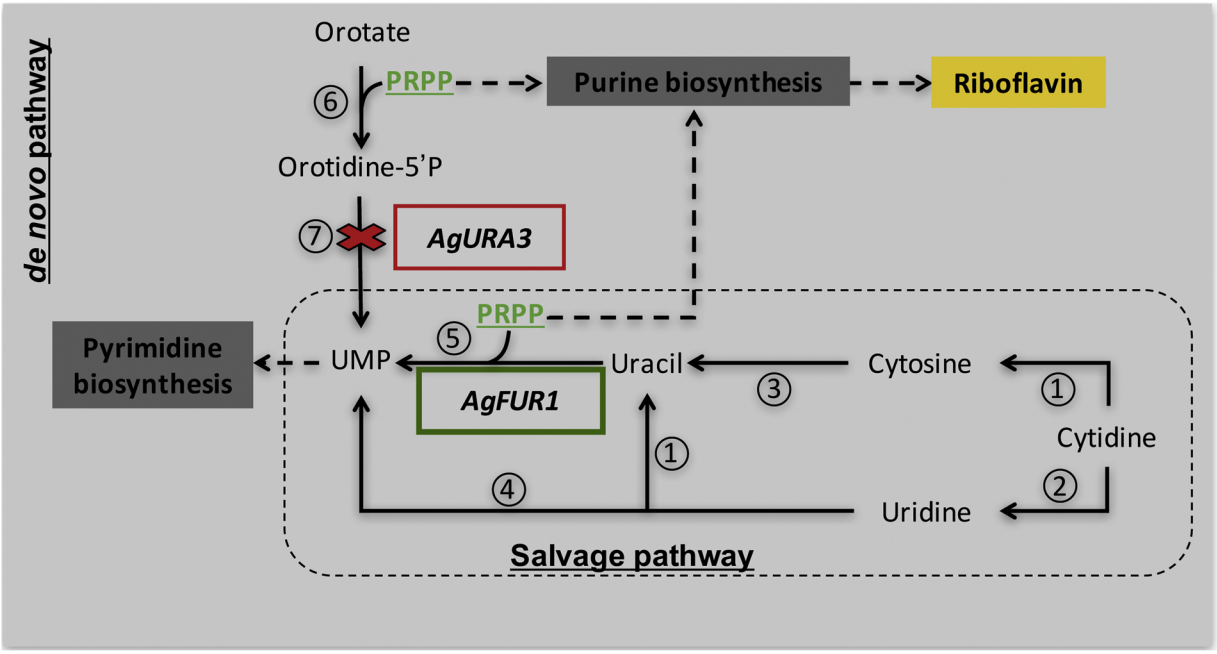

Fig. 1. Schematic representation indicating the *de novo* and salvage pathways of pyrimidine ribonucleotides. Red × marks where the *de novo* pyrimidine biosynthetic pathway is blocked in the *A. gossypii Agura3* uracil auxotroph. Dashed arrows indicate a multi-step pathway. UMP: uridine monophosphate. Numbers indicate the enzymes involved in each step of the pyrimidine pathways. 1: nucleosidase/uridine nucleosidase; 2: cytidine deaminase; 3: cytosine deaminase; 4: uridine kinase; 5: uracil phosphoribosyltransferase; 6: orotate phosphoribosyltransferase; 7: orotidine-5'-phospate decarboxylase.

(vitamin B2) and to its unique genetic and biological features [9,10]. The genetic blockage of its pyrimidine pathway at the *AgURA3* level (orotidine 5'-P to UMP) leads to increased riboflavin production on standard Ashbya Full Medium (AFM), a phenotype that is reverted when extra uridine/uracil (≥0.5/1 mM) is added to the medium [11]. Unlike *ura3* mutants from the closely related yeasts *Saccharomyces cerevisiae* and *Kluyveromyces* sp. [12], the *A. gossypii Agura3* mutant not only requires the supply of exogenous uridine to fully overcome its auxotrophy, but its growth is also inhibited by high uracil concentrations [4,11]. Thus, the *ura3* mutation, a common genetic alteration among microorganisms, has a profound impact not only on the growth but also on the physiology of this fungus.

In order to elucidate the molecular mechanism(s) underlying the unusual phenotypes of this *A. gossypii* pyrimidine auxotroph, here its growth was analyzed on media containing different pyrimidine supplements, envisioning the detection of bottlenecks in the pyrimidine salvage pathway. This allowed the identification of limitations at the AgUPRT level (Fig. 1). Characterization of recombinant AgUPRT revealed a type of metabolic enzyme regulation previously unknown in *A. gossypii*. Moreover, common features with other organisms, such as unrelated protozoan parasites [13,14] and other flavinogenic fungi, were identified and discussed, thus extending the impact of this study.

Materials and methods

Strains and media

The *A. gossypii Agura3* strain (*Agura3*Δ::*lox*P) [4], a uridine/uracil auxotroph derived from the parental strain *A. gossypii* ATCC 10895, was used in this study. For *AgFUR1* overexpression, the *AFR052C* ORF (NCBI Reference Sequence: NM_210953.1) was isolated from the *A. gossypii* genome by PCR (5'cggaattcATGaaaagttttagtgcccg and 5'ccgctcgagTCAtatgcagtagtaccgg) and cloned between the *Eco*RI and *Xho*I restriction sites of the plasmid pFMT [15], under the regulation of the strong constitutive promoter *AgTEF*. The resulting plasmid (pFMT*AgFUR1*) was then transformed into the *Agura3* strain as previously described [15]. Stock cultures of these strains were maintained as spores suspended in Spore Buffer (200 g L^{-1} glycerol, 8 g L^{-1} NaCl and 0.25% (v v^{-1}) Tween 20) at −80 °C. Spores were prepared as previously described [15] with minor modifications. The mycelium was digested with 4 mg mL^{-1} of Lysing Enzymes from *Trichoderma harzanium* (Sigma-Aldrich) for 2 h at 37 °C. Agar-solidified minimal medium (MM; 6.7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose and 1 g L^{-1} $CaCO_3$), synthetic complete (SC) medium [16] (without uracil) buffered with 1 g L^{-1} $CaCO_3$ and AFM (10 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 20 g L^{-1} glucose, 1 g L^{-1} myo-inositol) were used for the growth of *A. gossypii* strains. Where indicated, 200 μg mL^{-1} geneticin (G418) was added to the medium for plasmid maintenance. *Escherichia coli* NZY5α and NZYBL21 (DE3) (NZYTech) were used for plasmid construction and recombinant protein production, respectively. *E. coli* strains were grown in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl) supplemented with 100 μg mL^{-1} ampicillin for selection.

Radial growth conditions for A. gossypii strains

Agar-solidified MM, SC or AFM supplemented with uracil, uridine, cytosine or cytidine at 0, 1, 4 or 5 mM was inoculated with 10 μL of a suspension of spores (10^7 spores mL^{-1}) and incubated at 30 °C for 8 days. Colony radial growth [17] was determined daily by measuring the diameter of colonies in 90 mm diameter Petri dishes in two perpendicular directions, through two guide lines previously drawn on the lower outer face of the plates. Three biological replicas were used for each condition. Images of the colony morphology were recorded using a Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) with the Image Lab 4.0 software at day 8 of growth. The lag phase corresponded to the time (in days) that no growth was observed beyond the inoculum halo. The maximum radial growth rate was obtained through the slope of the trend line of a linear regression applied to the radial growth curve

Table 1
Maximum radial growth rates and lag phases of *A. gossypii Agura3* on agar-solidified MM supplemented with different concentrations of intermediates from the pyrimidine salvage pathway. Data are representative of three biological replicates. Radial growth rates are expressed as mean ± standard deviation. Superscripts represent significant differences ($p < 0.05$) between values of the same column (supplement concentration). n.d., not determined, because for this condition growth was only observed in the last day (day 8), which impeded determination of the maximum radial growth rate; –, no growth beyond the inoculum halo.

	Maximum radial growth rate (mm day^{-1})			Lag phase (days)		
	1 mM	4 mM	5 mM	1 mM	4 mM	5 mM
Uridine	10.9 ± 1.4^{a}	10.8 ± 0.3^{a}	12.9 ± 0.1^{a}	3	2	2
Uracil	5.6 ± 0.2^{b}	2.9 ± 0.1^{d}	n.d.	3	3	7
Cytosine	–	4.2 ± 0.3^{c}	6.5 ± 0.5^{b}	–	5	4
Cytidine	6.9 ± 0.7^{b}	6.4 ± 0.6^{b}	6.5 ± 0.6^{b}	5	3	3

excluding the lag phase. The percentage of relative growth was determined by normalizing the maximum radial growth of each condition by the maximum radial growth obtained with 5 mM uridine.

AgUPRT sequence analysis, recombinant production and purification

Multiple protein sequence alignment was performed with Clustal Omega [18] (https://www.ebi.ac.uk/Tools/msa/clustalo/) using default parameters and FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the phylogenetic tree produced by Clustal Omega. Amino acid sequences were retrieved from NCBI (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/). The full-length *AgFUR1* cDNA, with codons optimized for recombinant expression in *E. coli* and flanked by *Nco*I and *Xho*I recognition sites at the 5'- and 3'-end, respectively, was synthesized by NZYTech (Lisbon, Portugal). The *AgFUR1* synthetic gene was excised from the carrying plasmid by digestion with *Nco*I and *Xho*I and ligated to the pETM20 vector (EMBL) in fusion with the N-terminal thioredoxin A (TrxA) and His_6 tags (linked to the cloning site by a TEV protease recognition sequence) [19], and the construct was transformed into chemically competent NZY5α *E. coli* cells (NZYTech). The AgUPRT expression plasmid (pETM*AgFUR1*) was then transformed into the *E. coli* expression strain NZYBL21 (DE3) (NZYTech). A transformant colony was picked and grown in 100 mL of LB medium containing 100 µg/mL ampicillin to an OD_{600nm} of 0.5, and protein expression was induced with 0.2 mM IPTG for 16 h at 18 °C. Cells were recovered by centrifugation (at 4 °C for 15 min at 10,000 rpm) from 20 mL culture fractions and lysed with NZY Bacterial Cell Lysis Buffer (NZYTech) supplemented with 1 mM PMSF, according to the manufacturer's instructions. Soluble cell-free extracts were collected by centrifugation and incubated with 0.5 mL of HisPur™ Ni-NTA Resin (Thermo Fisher Scientific) for purification of the recombinant fusion protein. Purification was conducted according to the manufacturer's instructions as given for the batch method, using 50 mM Tris pH 8.0, 150 mM NaCl (Tris-NaCl buffer) with 20 mM imidazole as equilibration buffer, and Tris-NaCl buffer with 40 mM or 300 mM imidazole as washing and elution buffer, respectively. For TrxA-His_6 partner removal, the purified fusion protein was digested with TEV-His_6 protease overnight at 4 °C at the ratio of 1:20 (w w^{-1}) in equilibration buffer. Elution buffer was previously exchanged with equilibration buffer using a PD MidiTrap G-25 column (GE Healthcare). The cleaved AgUPRT was then purified from the fusion tags and protease by reverse purification using the same resin and above-mentioned protocol, but in which the target protein was collected from the resin washing steps. Purified protein was analyzed by SDS-PAGE using 15% (w v^{-1}) acrylamide gels, followed by BlueSafe staining (NZYTech). Predicted size for cleaved AgUPRT was 26.3 kDa. Imidazole was removed from the protein using the PD MidiTrap G-25 column (GE Healthcare). The concentration of recombinant AgUPRT was estimated from the absorbance at 280 nm using the respective molar extinction coefficient (ε = 13,785 M^{-1} cm^{-1}). Purified AgUPRT was stored at 4 °C until use.

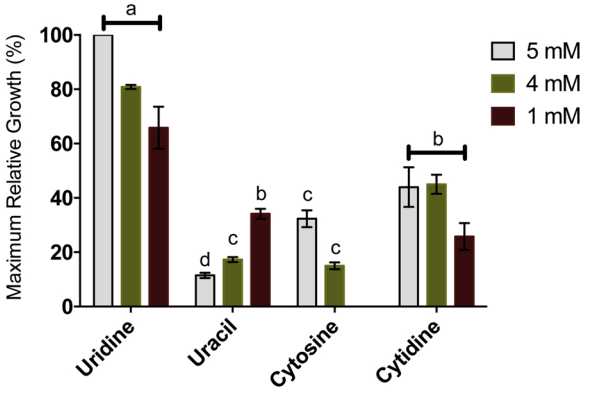

Fig. 2. Maximum relative growth of *A. gossypii Agura3* on agar-solidified MM supplemented with different concentrations of intermediates from the pyrimidine salvage pathway. The percentage of maximum relative growth was normalized to the radial growth of the best condition tested (5 mM uridine). Data are representative of three biological replicas after 8 days of growth. Letters represent significant differences ($p < 0.05$) between bars with the same supplement concentration.

Enzyme activity assays

AgUPRT enzyme activity was determined spectrophotometrically by measuring the conversion of uracil into UMP as previously described [14,20]. To determine the effect of uracil concentration on AgUPRT activity, the assay mixture consisted of TMD 50 buffer (50 mM Tris−HCl, 5 mM $MgCl_2$, and 2 mM DTT, pH 7.5) with 1 mM phosphoribosyl pyrophosphate (PRPP) and uracil concentrations ranging from 10 µM to 1.25 mM. To assess the influence of different PRPP concentrations on AgUPRT activity, the assay mixture consisted of TMD 50 buffer with 0.5 mM uracil, and PRPP concentrations ranging from 25 µM to 2 mM. For each condition tested and prior to the assay, the background absorbance was set to zero to remove any response caused by substrates or reagents. Enzyme activity measurements were performed using a V-560 UV/Vis Spectrophotometer (Jasco) at 30 °C, and reactions were initiated by the addition of approximately 2 µg of enzyme to the assay mixture (1 mL final volume in a 1 cm quartz cuvette). The assays were based on the differential molar absorption coefficients between uracil and UMP at 280 nm (Δε = 1407.5 M^{-1} cm^{-1}). An increase in absorbance was observed due to the formation of UMP, which was monitored for up to 2 min. Initial rates were calculated from the linear part of each reaction progress curve, converted to specific enzyme activity (µmol min^{-1} $mg_{protein}^{-1}$) and then plotted against substrate concentration.

Statistical analysis

GraphPad Prism for IOS version 6.0 was used for all statistical analyses. Differences between *A. gossypii*´s growths were tested by two-way ANOVA followed by Tukey's multiple comparison test. The analysis of differential AgUPRT activity was conducted by one-way ANOVA followed by Dunnett´s multiple comparison test. Statistical significance was established at $p < 0.05$ for the comparisons.

Results and discussion

The growth of *A. gossypii Agura3* can only be fully restored by the

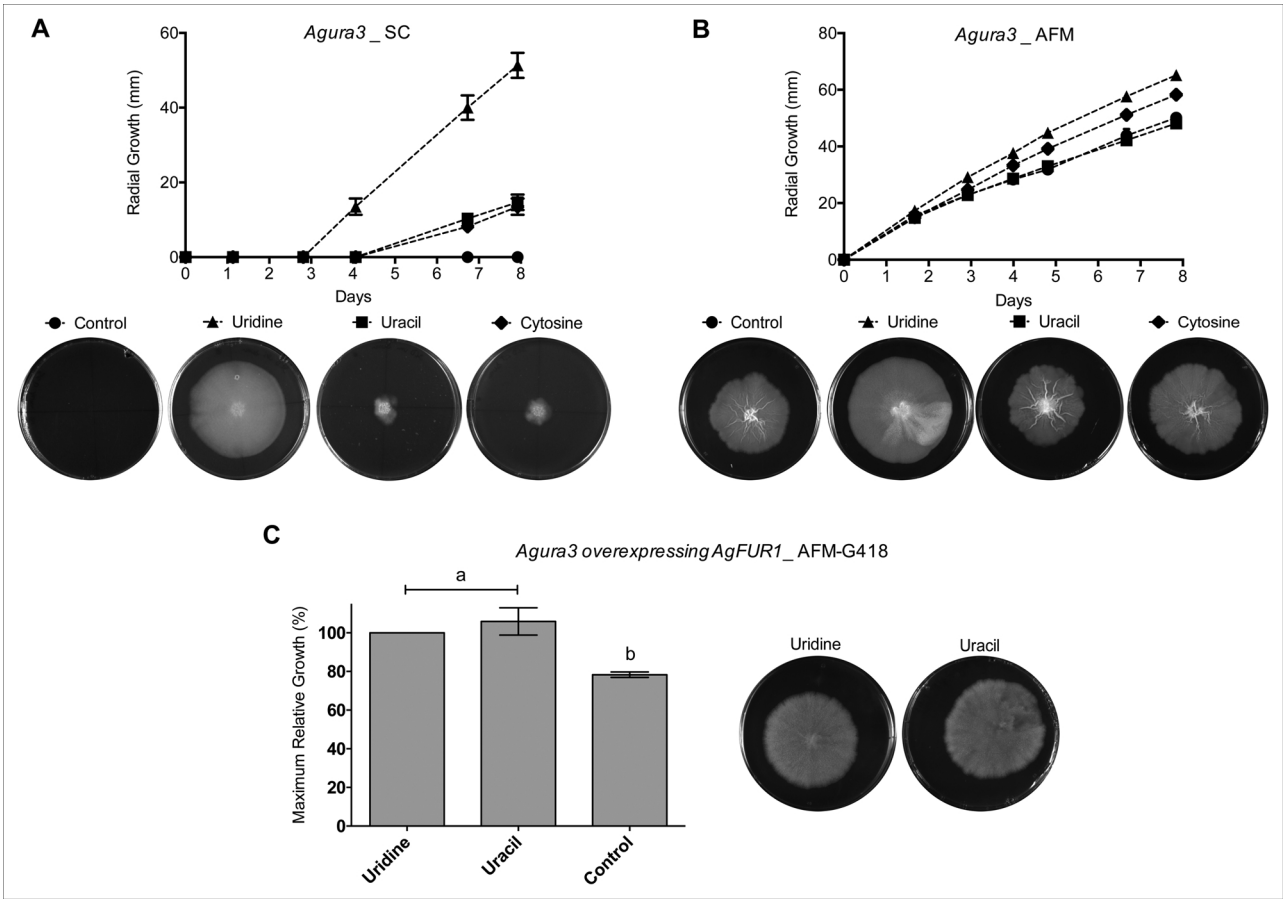

Fig. 3. Radial growth curves and colony morphologies of *A. gossypii Agura3* on agar-solidified SC (A) or AFM (B) supplemented with 5 mM uridine, uracil or cytosine. (C) Maximum relative growth and colony morphology of *A. gossypii Agura3* overexpressing the gene Ag*FUR1* on agar-solidified AFM supplemented with 5 mM uridine or uracil. Letters represent significant differences ($p < 0.05$) between bars (supplementation). The control conditions represent media without added supplement. The growth observed in AFM without any added supplement is due to the presence of small quantities of various pyrimidines in the complex constituents of this rich medium. Data represent the average ± standard deviation from three (A and B) or two (C) biological replicates. (Where not shown, error bars were smaller than the symbols). Images were recorded after 8 days of growth.

addition of uridine to the medium [4], being inhibited by high uracil concentrations [11]. A similar phenotype has been reported for uracil auxotrophs of *A. nidulans* [8]. Although not experimentally demonstrated, the growth-inhibitory effect of high uracil concentrations observed in *A. nidulans* pyrimidine auxotrophs was proposed to be linked with misincorporation of uracil into the DNA. This seems not to be the case for *A. gossypii*, since the parental strain of *Agura3* (ATCC 10895) did not display deficient growth when grown at high concentrations of uracil (Supplementary Figure A1) and no evidence was found for genomic DNA damage under these conditions using different DNA electrophoretic techniques (data not shown). Therefore, *per se*, the presence in the medium of a high content of the pyrimidine supplement is not deleterious for growth of this fungus, becoming detrimental only when uracil metabolism is required. Thus, this study focused on the characterization of the *A. gossypii* pyrimidine salvage pathway which according to the available genomic information (the Ashbya Genome Database) [21] has two independent routes by which UMP can be synthesized, one via AgUPRT and the other via uridine kinase, which converts uridine directly into UMP (Fig. 1).

Growth-rescuing effect of various pyrimidine supplements on A. gossypii Agura3

In an attempt to identify potential rate-limiting steps in the *A. gossypii* pyrimidine salvage pathway, the colony radial growth of *A. gossypii Agura3* was characterized on agar-solidified minimal medium (MM) supplemented with different concentrations (1, 4 or 5 mM) of one of the four intermediates in the pyrimidine salvage pathway, namely uracil, uridine, cytosine or cytidine (Table 1; Fig. 2). This range of concentrations was chosen based on previous results showing that this strain's growth is highly hampered by uracil concentrations ≥5 mM and minimally affected by ≤1 mM uracil [11]. In addition to MM, two other media with distinct basal composition, one defined (SC) and one rich (AFM), were also supplemented with 5 mM uracil, uridine or cytosine and used to test the growth of *A. gossypii Agura3* (Fig. 3A and B). Uracil is usually supplied at concentrations below 5 mM to rescue the growth of uracil auxothrophs of other fungi. Thus, to confirm that the growth-inhibitory effect of uracil is not common among fungal pyrimidine auxotrophs, we tested the growth of the frequently used *S. cerevisiae* CEN.PK113-5D (*ura3*-), *Yarrowia lipolytica* Po1f (*leu- ura- Δaep Δaxp SUC+*) and *K. marxianus* KMS2 (*ura3*-) in SC and verified that their growth was not affected by up to 10 mM uracil in the medium (Supplementary Figure A2).

Regardless of the medium and supplement concentration, uridine always performed the best in rescuing the growth of the *A. gossypii Agura3* mutant (Table 1; Figs. 2, 3 A, 3B). In MM, even with the lowest concentration tested (1 mM), the radial growth of *Agura3* on uridine was superior to that obtained with 5 mM of any other pyrimidine supplement (Table 1; Fig. 2). Uridine supplementation also produced the shortest lag phases (Table 1; Fig. 3A). In contrast, uracil failed to completely rescue the growth of the *Agura3* mutant. Moreover, increasing concentrations of this supplement were increasingly

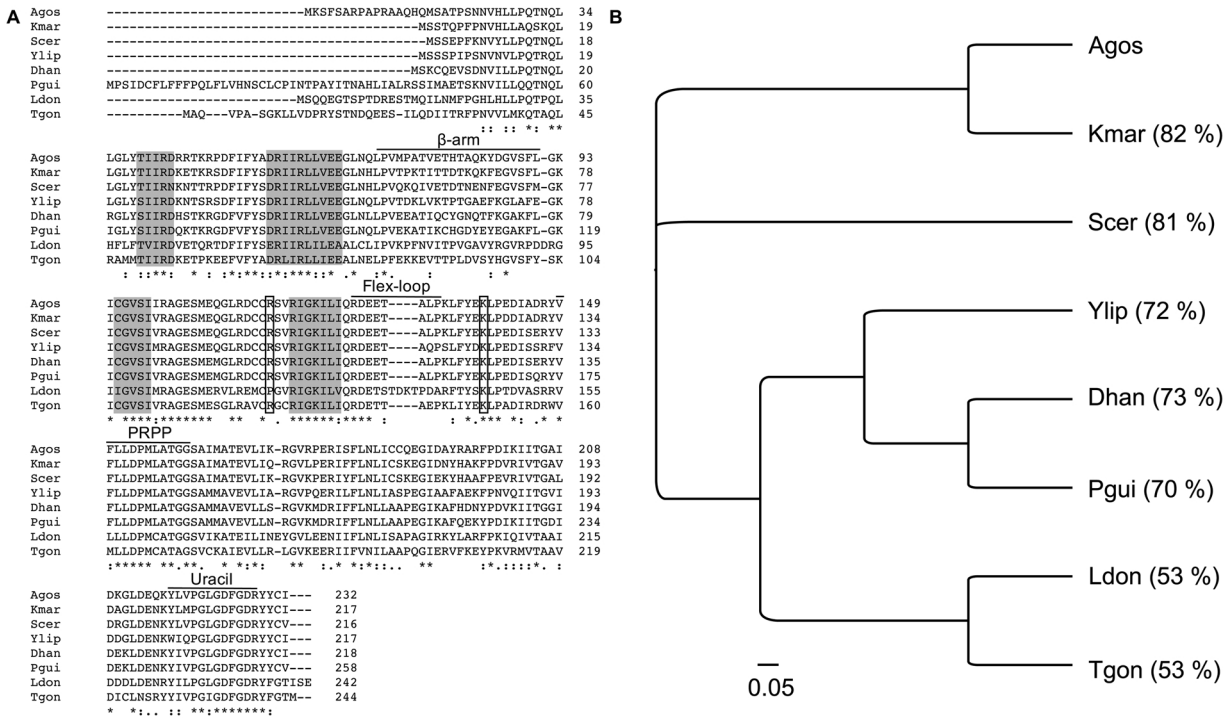

Fig. 4. Alignment of AgUPRT with other UPRTs and corresponding phylogenetic tree. (A) UPRT amino acid sequences from *A. gossypii* (Agos, **NP_985599**), *K. marxianus* (Kmar, **XP_022674246**), *S. cerevisiae* (Scer, **NP_011996**), *Y. lipolytica* (Ylip, **XP_506088**), *D. hansenii* (Dhan, **XP_002770455**), *P. guilliermondii* (Pgui, **XP_001482408**), *L. donovani* (Ldon, **XP_003864273**) and *T. gondii* (Tgon, **XP_002364470**). Four conserved regions from human to prokaryotes are shaded in grey [22]. The residues that constitute a β-arm, a flex-loop and the PRPP and uracil binding sites identified previously [23] are marked with black lines. Boxed residues represent positions where mutations were reported to increase resistance to 5-Flucytosine or 5-Fluorouracil. Asterisks (*) represent fully conserved residues, colons (:) represent residues with strongly similar properties and periods (.) represent residues with weakly similar properties. (B) Phylogenetic tree resultant from the alignment performed by ClustalO, which was drawn with FigTree v1.4.3. Indicated values represent the percentage of sequence identity to AgUPRT.

detrimental for the growth of this auxotroph (Table 1; Fig. 2, 3 A). Cytosine, which is metabolized into uracil before conversion to UMP through the AgUPRT route (Fig. 1), was also unable to fully restore growth (Figs. 2,3), but a positive correlation was observed between cytosine concentration and growth (Table 1; Fig. 2). On the other hand, cytidine, which can be metabolized into UMP via UPRT or uridine kinase (Fig. 1), exceeded uracil and cytosine, but not uridine, in rescuing growth (Table 1; Fig. 2). Morphologically, the colonies grown on media supplemented with uracil or cytosine presented an irregular shape, contrasting with the normal circular shape displayed on uridine-supplemented media (Fig. 3A, B). In addition, wrinkled colonies were observed on rich AFM supplemented with uracil or cytosine, whereas smooth flat colonies were found in plates with uridine (Fig. 3B).

Uridine can be directly metabolized by *A. gossypii* into UMP without going through the AgUPRT route. Thus, altogether, these results identified the AgUPRT-catalyzed reaction as the likely rate-limiting step in the *A. gossypii* pyrimidine salvage pathway responsible for the dose-dependent detrimental effect of uracil on the growth of *Agura3*. Indeed, overexpression of the AgUPRT encoding-gene in *Agura3* led to improved growth on AFM containing 5 mM uracil, to an extent that the maximum radial growth obtained with uracil was no longer statistically different from that obtained with the same amount of uridine (Fig. 3C).

AgUPRT sequence analysis, recombinant production and purification

AgUPRT is a 232 amino acids-long protein encoded by the gene *AFR052C* (syntenic homolog of *S. cerevisiae* gene *FUR1*) (Fig. 4A). Highly conserved regions across UPRTs from organisms of different kingdoms and even superkingdoms [22] are also conserved in AgUPRT (shaded gray in Fig. 4A). The AgUPRT amino acid sequence shares high identity (˜ 80%) with the UPRTs from closely related yeasts, such as *S. cerevisiae* and *K. marxianus*, and 53% with the well-characterized UPRTs from the distantly related protozoan parasites *Leishmania donovani* and *Toxoplasma gondii* (Fig. 4B). Curiously, despite belonging to closely related parasites, the UPRTs from *L. donovani* and *T. gondii* share less identity between each other (49%) than with that of *A. gossypii* (Fig. 4B).

Important secondary structures described for the *T. gondii* UPRT, as well as the binding regions for its substrates, PRPP and uracil [23], are indicated in Fig. 4A. While the PRPP and uracil binding regions are highly conserved across species, the β-arm and the flex-loop zones only share a degree of conservation across closely related species (Fig. 4A). In *C. albicans*, resistance to 5-flucytosine (5-FC) was associated with decreased UPRT activity due to the substitution of the arginine residue corresponding to R_{126} in AgUPRT by cysteine (C) [24]. None of the 8 UPRTs aligned here have a cysteine in that position, the arginine residue being conserved among 7 of them (Fig. 4A). 5-FC is an antifungal prodrug that is metabolized by cytosine deaminase into 5-fluorouracil (5-FU), which relies on its catalysis by UPRT to exert toxicity [24,25]. In *Leishmania infantum*, substitution of the lysine corresponding to K_{139} in AgUPRT by a threonine (T) was found to contribute to increased resistance to 5-FU [25]. This lysine is also conserved among the UPRTs aligned in Fig. 4A.

Since the *in silico* analysis of the AgUPRT did not highlight any particular feature that could account for limitations in its enzymatic activity, experimental characterization of this enzyme was carried out. The *AgFUR1* gene with codons optimized for expression in *E. coli* was cloned into an expression plasmid and subsequently expressed in *E. coli* BL21. AgUPRT was initially produced as an insoluble protein at induction temperatures of 37 and 18 °C (data not shown), but by N-

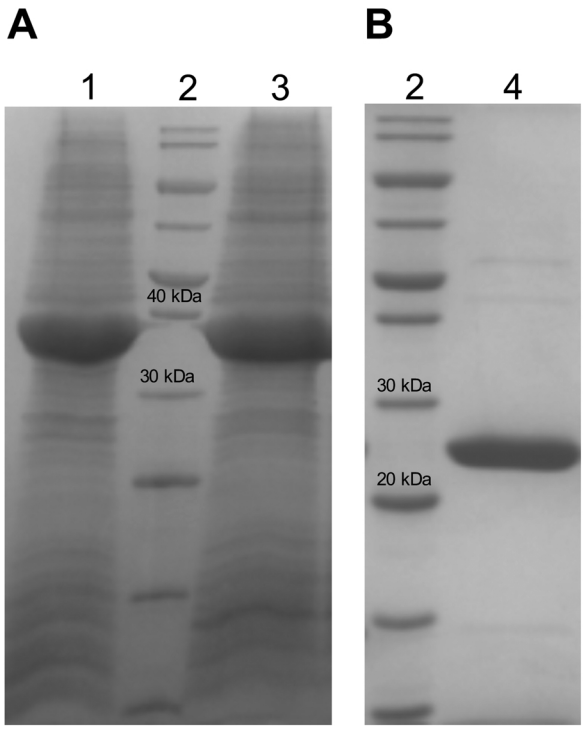

Fig. 5. SDS-PAGE analyses of the production (A) and purification (B) of AgUPRT from *E. coli*. Lane 1, crude extract of cells producing AgUPRT fusion with TrxA (TrxA-AgUPRT); Lanes 2, molecular weight markers; Lane 3, supernatant containing the soluble protein fraction after lysis of the TrxA-AgUPRT producing cells; Lane 4, AgUPRT cleaved and purified from TrxA-AgUPRT.

terminal fusion to TrxA it was subsequently expressed mostly in soluble form (Fig. 5). Over 2 mg of purified TrxA-AgUPRT fusion protein was obtained per 20 mL of bacterial culture induced at 18 °C with IPTG. AgUPRT was then cleaved efficiently from TrxA with TEV protease and further purified (Fig. 5), with an overall yield of 0.3 mg of pure AgUPRT per mg of fusion protein. The cleaved and purified AgUPRT could be observed in SDS-PAGE gel as a single band of ~26 kDa, consistent with its calculated molecular weight (Fig. 5), and was stable when stored at 4 °C in Tris-NaCl buffer for at least 1 week.

AgUPRT activity is negatively affected by high uracil concentrations

The purified recombinant AgUPRT was enzymatically active, catalyzing the phosphoribosylation of uracil to UMP in the presence of PRPP (Fig. 6). Maximum activity was obtained with 0.4-0.5 mM uracil in the presence of 1 mM PRPP, decreasing thereafter with increasing uracil concentrations (Fig. 6A). On the other hand, activity in the presence of 0.5 mM uracil and at varying concentrations of PRPP was maximal at 0.75–1 mM PRPP, remaining constant thereafter (Fig. 6B). As shown in Fig. 7, uracil concentrations ≥1 mM negatively affected AgUPRT activity in a statistically significant way, such that with 1.25 mM uracil, catalytic activity was approximately 80% of maximum. This dose-dependent effect of uracil on AgUPRT activity could not be circumvented by the addition of more PRPP (data not shown). These observations are in line with what was observed in terms of growth of the *A. gossypii Agura3* mutant, for which uracil concentrations above 1 mM were increasingly deleterious (Table 1; Fig. 2).

UPRT is an enzyme that is highly conserved across species [26] and has been characterized in several organisms, from prokaryotes [27] to humans [22]. Among them, the UPRTs from the protozoan parasites *L. donovani* and *T. gondii* have also been described to be negatively affected by high uracil concentration [14]. Moreover, the growth of an *L. donovani* pyrimidine auxotroph was shown to be (i) inhibited by increasing concentrations of uracil, (ii) not completely rescued by cytosine, and (iii) only fully restored when uridine was used as supplementation [13], thus revealing a surprising, but also striking similarity to what was observed here with *A. gossypii Agura3*.

The conversion of uracil to UMP catalyzed by UPRT is dependent on the presence of PRPP, an essential precursor shared by the purine and pyrimidine pathways, both *de novo* and salvage pathways [28,29]. In turn, the purine biosynthetic pathway is essential for the production of guanosine-5-triphosphate (GTP), one of the precursors for riboflavin production. Indeed, a direct correlation between increased availability of PRPP and riboflavin overproduction by *A. gossypii* was previously demonstrated [30], which may explain the riboflavin-overproducing phenotype displayed by the *Agura3* mutant [11]. Overproduction of riboflavin is a key differentiating trait of *A. gossypii*, not only at the biotechnological level [9] but also ecologically, as it has been suggested that *A. gossypii* overproduces riboflavin as a detoxifying [31,32] and protective mechanism [33]. Although the regulatory mechanisms involved in riboflavin overproduction remain largely unknown, the biological relevance of the negative regulation of AgUPRT activity by high uracil concentration is most likely related with this trait, for which it

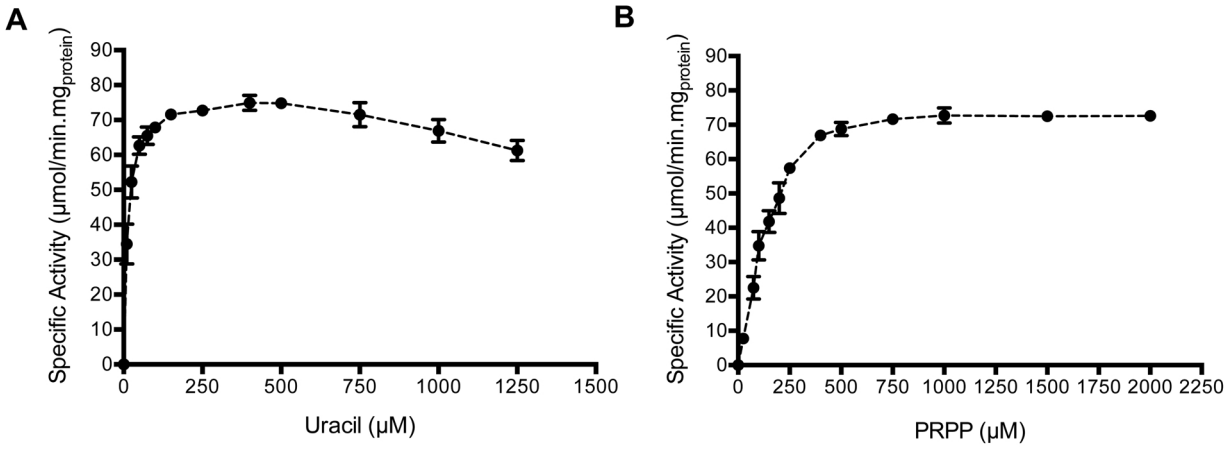

Fig. 6. AgUPRT activity at varying concentrations of uracil and PRPP. Specific AgUPRT activity was determined spectrophotometrically as a function of uracil concentration in the presence of 1 mM PRPP (A), and as a function of PRPP concentration in the presence of 0.5 mM uracil concentration (B). Data represent average ± standard deviation from three experimental replicates.

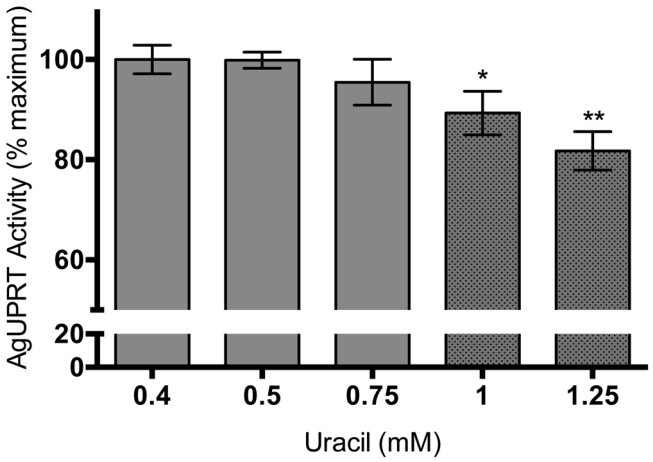

Fig. 7. Effect of increasing uracil concentrations on AgUPRT activity. AgUPRT activity was determined spectrophotometrically at a set-saturating PRPP concentration (1 mM) and represented as percentage of the maximum activity. Data represent average ± standard deviation from three experimental replicates. Asterisks represent significant differences (* $p < 0.05$; ** $p < 0.01$) compared to maximum activity.

may well contribute as a mechanism to preserve PRPP for purine (and ultimately riboflavin) biosynthesis. Apart from ATP, which is present at mM levels, the average physiological concentrations of purines and pyrimidines for the routine functioning of the cell are in the μM range [34]. Therefore, after these requirements are satisfied, PRPP can be channeled for the generation of precursors (GTP) for the production of riboflavin, which in *A. gossypii* is highly active in the late stages of growth and can easily reach very high concentrations (up to 20 g L^{-1}) [35,36].

Evidence supporting this hypothesis can be found in the almost perfect parallel with the events in L. *donovani* and *T. gondii*. Since protozoan parasites lack the *de novo* purine biosynthetic pathway, they must scavenge nutritionally essential purines from their hosts [13], which they do through the purine salvage pathway, activity of which relies on two other PRPP-dependent enzymes. Therefore, the biological relevance for the presence in these parasites of a uracil-mediated regulation mechanism of UPRT has been linked with the increased need of PRPP for purine biosynthesis [14]. Although the PRPP needs of each organism are for different purposes, the fact that *A. gossypii*, *L. donovani* and *T. gondii* have UPRTs which, after reaching a maximum activity, are negatively affected by increasing uracil concentrations strongly indicates that this mechanism may exist to favor purine synthesis when the pyrimidine requirements are satisfied.

A further independent and remarkable support to this hypothesis is the fact that uracil auxotrophs from other highly flavinogenic fungi, such as the yeasts *P. guilliermondii* and *D. hansenii*, are also unable to grow well in medium supplemented with uracil alone [5,6]. These reports indicate that the hypersensitivity to uracil observed in *A. gossypii Agura3* extends to pyrimidine auxotrophs of other flavinogenic fungi and suggest that the UPRT modulation mechanism also exists in these fungi, just as the phenotypes and corresponding molecular mechanisms observed in *L. donovani* extend to other protozoan parasites (*e.g., T. gondii*) [14].

Conclusions

Here, it is shown that high uracil concentrations negatively affect AgUPRT activity, which can explain the growth impairment observed in the *A. gossypii Agura3* auxotroph when increasing concentrations of this pyrimidine intermediate are present in the medium. Until now, this phenomenon had been only reported to exist in protozoan parasites. The present findings demonstrate that this type of mechanism exists beyond the requirement for survival and can be extended to microorganisms that are phylogenetically distant from parasites. Moreover, since uracil auxotrophs are regularly used as backgrounds in genetic and metabolic engineering strategies, awareness is raised to these unusual phenotypes that may interfere with the correct assessment of strain modification outcomes.

From a biotechnological point of view, understanding the mechanisms behind the biosynthesis of high-value products by natural microbial producers is essential to develop stable and long-lasting biotechnological processes. After years of improving riboflavin production yields and titers in *A. gossypii*, the unraveling of new regulatory mechanisms involved in its biosynthesis will ensure the maintenance of this paradigm process in the successful industrial track. Contributing to that, the modulation mechanism of the AgUPRT activity reported here reveals a new way of economizing essential metabolic precursors for riboflavin overproduction by *A. gossypii*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2018.12.004.

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