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著者	Jeong Bo-Young, Wittmann Christoph, Kato Tatsuya, Park Enoch Y.
journal or publication title	Journal of Bioscience and Bioengineering
volume	119
number	1
page range	101-106
year	2014-08-14
出版者	Elsevier
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URL	http://hdl.handle.net/10297/7902

doi: 10.1016/j.jbiosc.2014.06.014

**Comparative metabolic flux analysis of an *Ashbya gossypii* wild
type strain and a high riboflavin-producing mutant strain**

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Abstract

In the present study, we analyzed the central metabolic pathway of an *A. gossypii* wild type strain and a riboflavin over-producing mutant strain developed in a previous study in order to characterize the riboflavin over-production pathway. ^{13}C -Metabolic flux analysis (^{13}C -MFA) was carried out in both strains, and the resulting data were fit to a steady-state flux isotopomer model using OpenFLUX. Flux to pentose-5-phosphate (P5P) via the pentose phosphate pathway (PPP) was 9% higher in the mutant strain compared to the wild type strain. The flux from purine synthesis to riboflavin in the mutant strain was 1.6%, while that of the wild type strain was only 0.1%, a 16-fold difference. In addition, the flux from the cytoplasmic pyruvate pool to the extracellular metabolites, pyruvate, lactate, and alanine, was 2-fold higher in the mutant strain compared to the wild type strain. This result demonstrates that increased guanosine triphosphate (GTP) flux through the PPP and purine synthesis pathway (PSP) increased riboflavin production in the mutant strain. The present study provides the first insight into metabolic flux through the central carbon pathway in *A. gossypii* and sets the foundation for development of a quantitative and functional model of the *A. gossypii* metabolic network.

[Keywords: *Ashbya gossypii*; Riboflavin; ^{13}C Metabolic flux analysis; Central carbon metabolism; Mutant]

INTRODUCTION

Riboflavin (RIB), also known as vitamin B₂, is widely distributed in plants and microorganisms, where it serves as a precursor for flavin adenine dinucleotide and flavin mononucleotide synthesis. Flavin adenine dinucleotide and flavin mononucleotide have important cellular roles as co-factors for dehydrogenases and oxidoreductases (1). However, animals, including humans, cannot synthesize RIB, and hence, this nutrient must be supplied through foods and dietary supplements (2, 3). Currently, RIB is produced by fermentation using microorganisms such as the fungi *Ashbya gossypii* and *Eremothecium ashbyii*, the yeast *Candida flaveri*, and the bacterium *Bacillus subtilis* (4). *A. gossypii*, which is capable of RIB overproduction, has been used in industrial RIB production. Importantly, the *Ashbya* genome has already been sequenced and annotated (5), which may accelerate the improvement of RIB production in this organism. In order to improve RIB production, many researchers have developed high RIB-producing *A. gossypii* strains through mutagenesis or metabolic engineering (6–9).

Systems biology combines datasets associated with gene expression, such as those from transcriptomics and proteomics, with data from metabolomics and metabolic flux analysis (MFA, fluxomics) (10). This combination of “omics” techniques potentially represents the most powerful approach for understanding and manipulating cellular metabolism (10, 11). ¹³C-MFA provides a direct measure of the metabolic phenotype by quantitatively analyzing *in vivo* carbon flux in metabolic networks (12). Cells are incubated with a carbon isotope (¹³C)-labeled precursor, and the ¹³C-labeling data in metabolic intermediates and end-products are measured and their deviation between simulation and experiment is minimized by iterative variation of the free fluxes until the optimum fit is obtained (13, 14). Fluxes from ¹³C mass isotopomer data and extracellular flux measurements are fit to a steady-state flux isotopomer model using OpenFLUX based on the Elementary Metabolite Units (EMU) decomposition

algorithm (15, 16). OpenFLUX has previously been used for ^{13}C -MFA of lysine-producing *Corynebacterium glutamicum* and succinate-producing *Basfia succiniciproducens* (17, 18). However, it has never been used for fungal metabolic flux analysis.

The metabolism of RIB over-production in *A. gossypii* is a matter of great interest because this fungus has been used for industrial RIB production. However, despite numerous studies, a clear and unequivocal description of fluxomics in *A. gossypii* has not yet been provided. Here, ^{13}C -MFA was performed in an *A. gossypii* wild type strain and a RIB over-producing mutant strain, and differences in metabolism between the wild type and mutant strains were analyzed. The present study revealed differences in carbon metabolic flux in the RIB-producing phase between the wild type and mutant strains.

MATERIALS AND METHODS

Strains and maintenance The *A. gossypii* wild type strain ATCC 10895 and the *A. gossypii* mutant strain W122032 were used in this study. The *A. gossypii* mutant strain was isolated by disparity mutagenesis in our previous study and was shown to produce RIB at levels nine-fold higher than the wild type strain when vegetable oil was used as the sole carbon source (6). Both strains were maintained as frozen spore suspensions in 10% (w/v) glycerol at -80°C . Batch cultures were maintained in 500-mL baffled shake flasks containing 50 mL of growth medium on a rotary shaker at 28°C with agitation at 120 rpm.

Media YD medium (pH 6.8) containing 10 g/L yeast extract and 10 g/L glucose was used for glycerol stocks. The seed culture and preculture were maintained in complex medium containing 20 g/L glucose, 25 g/L corn steep liquor (CSL), 1.0 g/L K_2HPO_4 , and 5.0 g/L peptone (19). The main cultivation was performed in chemically defined minimal medium (pH 6.8) containing 50 mM glucose as a carbon source, 1.5 g/L asparagine, 0.75 g/L

KH₂PO₄, 0.1 g/L myo-inositol, and mineral ions (4.4 mg/L CoCl₂·6H₂O, 18.0 mg/L MnCl₂·4H₂O, 44.0 mg/L ZnSO₄·7H₂O, 10.1 mg/L MgSO₄·7H₂O, 27.0 mg/L FeCl₃·6H₂O, 21.9 mg/L CaCl₂·6H₂O, and 2.7 mg/L CuSO₄·5H₂O). The stock solution for the mineral ions was autoclaved separately for 20 min at 121°C and cooled to room temperature prior to addition. All chemicals were of analytical grade and were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), or Sigma (St. Louis, USA). For tracer experiments, naturally labeled glucose was replaced with 99% [1, 2-¹³C] glucose (99%, Cambridge Isotope Laboratories, Andover, USA). To resolve the metabolic fluxes of interest, two parallel experimental set-ups were chosen for the labeling studies (20).

Cultivation Seed culture and preculture were incubated in complex medium (21) for 48 and 72 h, respectively, at 28°C. *A. gossypii* strains from glycerol stocks (10% v/v) were used for seed culture and were used to inoculate 10 mL complex medium in 100-mL baffled shake flasks. The seed culture was then used to inoculate the preculture (10% v/v). Subsequently, the cells were harvested by centrifugation at 1006 × g for 10 min, washed with sterile distilled water, and used to inoculate the main culture in a 500-mL baffled shake flask containing 50 mL minimal medium. Physiological studies of the quantification of growth and production characteristics were performed using triplicate cultures. Cultures incubated with tracer ¹³C-labeled GLC were maintained in 100-mL baffled shake flasks containing 10 mL minimal medium. All cultures were inoculated with exponentially growing cells from the preculture and were incubated at 28°C and agitated at 120 rpm on a rotary shaker (Certomat BS-1/50 mm, Sartorius, Göttingen, Germany).

Measurements of dry cell weight (DCW) and extracellular metabolites DCW was measured in triplicate. Harvested cells were washed twice with sterile distilled water and then

dried at 100°C until a constant weight was reached. For quantification of organic acids and glucose the culture supernatants were filtered microfilter (Minisart 0.2 µm, Sartorius). Organic acid was measured by HPLC (Elite Lachrome HITACHI Ltd., Japan) using a Metacarb 67H column (250 mm × 4.6 mm, 5 µm, VWR-Hitachi, Darmstadt, Germany) with 1 mM H₂SO₄ as the mobile phase and a flow rate of 0.8 mL/min at 70°C. Glucose was measured using a biochemical analyzer (YSI 2700 SELECT, Ohio, USA). To quantify RIB concentration, 0.8 mL of the culture broth was thoroughly mixed with 0.2 mL 1 N NaOH. A 0.4-mL aliquot of the resulting solution was neutralized with 1 mL 0.1 M potassium phosphate buffer (pH 6.0), and the optical density of the solution at a wavelength of 444 nm was measured. The RIB concentration was calculated using a conversion factor of 127.297 mg-RIB·(L · unit of optical density)⁻¹ (22).

¹³C-Labeling analysis MFA with GC-MS is usually performed as stationary flux analysis, whereby the metabolism is in metabolic and isotopomer steady state. For ¹³C-MFA, cells from the tracer culture were harvested at 7 d of culture time by filtration of 5 mL of culture using a cellulose acetate filter (pore size, 20 µm; Sartorius). After removal of excess medium by washing twice with cold distilled water, the pellets were resuspended in 500 µl 6 M HCl and incubated for 24 h at 100°C to hydrolyze proteins. The hydrolysates were clarified by filtration (0.2 µm, Ultrafree MC, Millipore, Bedford, MA, USA) and dried under nitrogen gas. The resulting proteinogenic amino acids were resolved by the addition of 50 µl dimethylformamide (Sigma, USA) and 0.1% pyridine (Sigma, USA) and were derivatized using 50 µl of the silylation reagent, *N*-methyl-*N*-(*tert*-butyl-dimethylsilyl)-trifluoroacetamide (MBDSTFA) (Macherey-Nagel, Düren, Germany), at 80°C for 30 min prior to gas chromatography and mass spectrometry (GC-MS) analysis. Analysis of the labeling pattern of amino acids from the hydrolyzed cellular protein was carried out using GC-MS (HP Agilent 7890 Gas Chromatograph, Quadrupole Mass Selective Detector 5973;

Agilent Technologies, Waldbronn, Germany). All of the samples were measured first in scan mode, thereby excluding isobaric interference. For enhanced data analysis, the relative fractions of the mass isotopomers of interest were then analyzed in duplicate in selective ion monitoring (SIM) mode (16).

Metabolic flux modeling and metabolic flux calculations The metabolic network model of the central metabolism of RIB-producing *A. gossypii* cells was reconstructed based on a previously described model (23, 24), which is based on the compartmentalized metabolic network of *Saccharomyces cerevisiae* based on previous reports. The model consists of *A. gossypii* central carbon metabolic pathways, including glycolysis, the PPP, the TCA cycle, and the PSP, which was constrained using the measured extracellular uptake fluxes, as well as intracellular fluxes estimated from the ^{13}C isotopomer data. The cytosol and the mitochondrion were regarded as separate compartments, and separate pools for pyruvate, oxaloacetate, and acetyl CoA were included in each of the two compartments. The pathways of anabolism, including amino acid, organic acid, and RIB biosynthesis, were revised by inspection of metabolic flux studies in different bacteria (25, 26), yeasts, and other fungi (27–29). Metabolic flux was calculated using the yield coefficients based on the metabolic network reactions. Fifty-eight stoichiometric reactions, including uptake reactions for GLC (V_1) and ASN (V_2), were considered. Taking biomass concentration and measured physiological yield coefficients into account, 13 measurements of flux from anabolic precursors into biomass were included (Supplementary Table S1). Overall, 24 metabolite balances were formulated from stoichiometric reactions (Supplementary Table S2). A total of 83 reactions were considered in order to construct a metabolic network model of ^{13}C -MFA of *A. gossypii* (Supplementary Table S3). All flux values were normalized with respect to the glucose uptake rate. Metabolic flux values were estimated using the MATLAB-based modeling software OpenFLUX (15). Statistical analysis of the resulting fluxes was carried

Supp.
Tables S1,
S2 and S3

out using a Monte Carlo approach (20), which is capable of providing precise information regarding the error distributions of flux parameters.

RESULTS

Growth and RIB production by *A. gossypii* wild type and mutant strains The time course for cell growth and consumption of glucose (GLC) and asparagine (ASN) by wild type and mutant *A. gossypii* cultures is shown in Fig. 1A and B. Growth was coupled with consumption of GLC and ASN. The amount of ASN consumed by the wild type strain was 5 times higher than that of the mutant strain, but RIB production was 10-fold less in the wild type strain compared to the mutant (Fig. 1A). RIB production by *A. gossypii* has been reported to start at the end of the growth phase linked to sporulation in wild type cells (30). In contrast, RIB production of the mutant strain was found to begin at the beginning of exponential growth. In addition, the concentration of lactate (LAC) in the mutant strain was significantly increased compared to the wild type strain (Fig. 1B).

Fig. 1

Production of RIB as a product and LAC as a by-product was measured and correlated with the amount of GLC consumed. The yields of RIB and LAC were calculated during the exponential growth phase for each strain when cultured on chemically defined minimal medium. The biomass yields based on consumed GLC were 0.047 and 0.063 g DCW/mmol GLC for the *A. gossypii* wild type and mutant strains, respectively. The specific rates of cell growth, GLC and ASN consumption, and RIB and LAC production of the wild type and mutant strains during the exponential growth phase were shown in Table 1. The RIB, LAC, acetate (ACE), alanine (ALA), and pyruvate (PYR) yields based on GLC consumed were higher in the mutant than in the wild type strain. The RIB yield, $Y_{\text{RIB/GLC}}$ was 16-fold higher in the mutant compared to the wild type. It is also notable that $Y_{\text{LAC/GLC}}$ and $Y_{\text{PYR/GLC}}$ were 4.4 and 2.9 times higher in the mutant strain than in the wild type strain. In contrast, the alpha-

Table 1

ketoglutarate (AKG), glutamate (GLU), and succinate (SUC) yields in the mutant strain were relatively lower compared to the wild type strain. The overall yield coefficients for the mutant were significantly different from the wild type under the same culture conditions. The primary differences observed were in production of RIB and its main by-products, including LAC and PYR from the pyruvate pool.

Construction of the metabolic network of *A. gossypii* To quantify metabolic flux, [1, 2-¹³C]-labeled GLC and unlabeled ASN were used as the sole carbon and nitrogen sources, respectively. Physiological yield coefficients (Table 1) and measured mass isotopomer fractions (Tables 2 and 3) were calculated for construction of related metabolic flux models.

Tables 2
and 3

RIB is produced by *A. gossypii* cells cultured on medium containing vegetable oil as a sole carbon source through the β -oxidation pathway, the glyoxylate cycle, the TCA cycle, gluconeogenesis, the pentose phosphate pathway (PPP), the purine synthesis pathway (PSP), and the RIB synthesis pathway (1, 31). In contrast, when GLC is used as a carbon source, RIB is produced through glycolysis (the Embden-Meyerhof-Parnas pathway, EMP), the PPP, the PSP, and the RIB synthesis pathway. In this study, the metabolic model for growth of *A. gossypii* on GLC minimal medium was constructed. In this model, the β -oxidation pathway, glyoxylate cycle, and other pathways were omitted due to the use of GLC as a carbon source.

Analysis of metabolic fluxes of *A. gossypii* wild type and mutant strains The corresponding stoichiometric matrix was calculated, and the results revealed that all metabolic balances were linearly independent. Together with the 43 mass isotopomer fractions (Tables 2 and 3), stoichiometric reactions and carbon fluxes into biomass were available for analysis of metabolic flux. The intracellular flux distributions of wild type and mutant strains are shown in Fig. 2. The relative metabolic fluxes were normalized with respect to the specific GLC uptake rate. Glycolysis and the PPP were used for catabolic and anabolic metabolism of GLC, respectively. In wild type *A. gossypii*, 30% of G6P was

Fig. 2

converted to P5P via the PPP, whereas 65.7% was channeled through the EMP. In contrast, 39.2% of G6P was used to generate P5P via the PPP, and 57.1% was channeled through the EMP in the *A. gossypii* mutant strain. The excess carbon in the PPP, totaling 9.2% in *A. gossypii* wild type strain and 11.7% in the mutant strain, was channeled back to the EMP via F6P and GAP. Carbon flux to the PPP in the mutant strain was not significantly increased compared to that of the wild type strain. In contrast, the flux from the cytosolic pyruvate (PYR_{cyt}) pool to extracellular pools of PYR, LAC, and ALA (PYR_{ex}, LAC_{ex}, and ALA_{ex}) in the mutant strain was 31.6%, a two-fold increase over the wild type strain. The flows of PYR_{cyt} to the extracellular PYR_{ex}, LAC_{ex}, and ALA_{ex} pools in the mutant strain were 16.8%, 6.6%, and 1.8%, respectively, representing a 2–4 fold increase compared to the wild type strain. Consistent with these observations, the intracellular flows of PYR_{cyt} to OAA_{cyt} in the mutant strain were 30.4%, while those in the wild type were 49.1%. Thus, the overall fluxes associated with the EMP in the wild type strain were more connected with the TCA cycle than those in the mutant strain.

The ASN_{ex} flux in the *A. gossypii* wild type strain was 39.4%, which was higher than that of the mutant. The major fraction of ASN was channeled through aspartate (ASP) into the TCA cycle, and the resulting OAA_{cyt} formed through anaplerosis was transported into the mitochondria oxaloacetate (OAA_{mit}). The carbon flux from OAA_{cyt} into the TCA cycle in the wild type strain was 84.5%, but it was 57.5% in the mutant strain. In addition, the normal flux from SER and THR to GLY was 1.3% in the wild type strain, while it was 3.4% in the mutant, suggesting that the mutant strain was more active in supplying GLY. Usually, RIB production by *A. gossypii* is limited by GLY, an early precursor that is required in the PSP (31). GTP is derived from the precursors GLY and P5P (32). Consequentially, this flux analysis indicated that the supply of GTP in the mutant strain was higher than that in the wild type strain, which led to improved RIB production in the mutant in parallel with increased

LAC production.

DISCUSSION

A. gossypii cultured in complex medium can utilize vegetable oils as a carbon source for RIB production, and these oils can be metabolized through the glyoxylate cycle and β -oxidation (33). However, in this study, the relative flux of carbon through all metabolic pathways except the glyoxylate cycle and β -oxidation was investigated because GLC was used as a carbon source. The mutant, RIB-overproducing strain grew more slowly ($\mu = 0.78 \text{ d}^{-1}$) than the control strain ($\mu = 1.01 \text{ d}^{-1}$) and required a 1-day lag phase. To compare carbon flux between the *A. gossypii* wild type and mutant strains, based on the flux distributions, approximately 39.2% of carbon from GLC was channeled into the PPP in the *A. gossypii* mutant, which was higher than that of the wild type (30%). In TCA cycle, pyruvate flux in the mutant strain was 20% lower than that in the wild type. Carbon flux from purine synthesis to RIB in the mutant strain was 16-fold higher than that in the wild type strain, which agreed with the 16-fold higher RIB yield of the mutant than that of wild type. This result demonstrates that increased GTP flux through the PPP and PSP increased RIB production in the mutant strain. This study confirmed that GLY and folate derivative production is critical for RIB production in *A. gossypii*, as the flux in the mutant was directed to production of GLY and a folate derivative, 5, 10-methylenetetrahydrofolate in parallel with its RIB production (34). GLY is the precursor for GTP, which is a precursor for folate, and another folate derivative, 10-formyltetrahydrofolate, is utilized in purine metabolism as a C1 carbon carrier. ASN, which was used as the sole nitrogen source in this study, is also utilized in purine metabolism for the production of phosphoribosylaminoimidazole-succinocarboxamide. In addition, ASN might be used as carbon source. Therefore, OAA_{cyt} and THR made from ASN were constructed into the metabolic model. These results suggest that the PSP is

connected to GLY, folate, and ASN metabolism for RIB production in *A. gossypii*. The phosphoribosylaminoimidazole-succinocarboxamide synthase (ADE1, AER221Wp) expression in the mutant has been shown to be enhanced more than 5-fold over wild type strain during RIB production in a complex medium containing yeast extract and rapeseed oil (6). Thus, the results of this study are in agreement with previous proteomics results for the mutant strain used in this study. Interestingly, in the mutant strain, LAC_{ex} was highly over-produced as a result of increased flux from the PYR_{cyt} pool.

¹³C-MFA based on OpenFLUX was carried out to compare the central metabolism in high RIB-producing *A. gossypii* between wild type strains. However, we found large differences between estimated and measured relative mass isotopomer distributions of metabolites in both strains (Tables 2 and 3). The residual sum of squares (RSS) in wild type and mutant were 0.034 and 0.047, respectively, suggesting that the used model in this study is not reliable enough to analyze RIB producing MFA in *A. gossypii* in detail. Therefore, we set V8-13, V19, V29, V32 and V33 as reversible reactions to refine this metabolic network. The relative mass isotopomer distributions of metabolites in both strains were recalculated, and resulting RSS values in wild type and mutant were 0.044 and 0.030, respectively. This indicates that other factors affected the accuracy of the flux distribution. More detailed analysis of MFA in *A. gossypii* strain requires more accurate MFA method with various respects.

In the present study, *A. gossypii* metabolic flux analysis was investigated using ¹³C-MFA in order to compare metabolic flux between the wild type and mutant strains. Carbon flux from purine synthesis to RIB in the mutant strain was 16-fold higher than that in the wild type strain. This result demonstrates that increased GTP flux through the PPP and PSP increased RIB production in the mutant strain. In order to improve RIB production it is one option to reinforce gene expressions related to PPP and PSP pathways. On the other hand,

industrial RIB producer uses vegetable oil as carbon source, meaning glyoxylate cycle is important. Unfortunately in this work we can't reveal carbon flux of glyoxylate cycle, but if it is available it would be possible to increase oil consumption for RIB improvement. Because it is easy to identify mutated genes of RIB-overproducing mutant by genome analysis. Although ^{13}C -MFA based on OpenFLUX was not highly accurate in *A. gossypii*, analysis of carbon flux from the EMP to the RIB synthesis pathway revealed important differences between the *A. gossypii* wild type and mutant strains. These results set the stage for further rational development of new RIB-overproducing strains.

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FIGURE LEGENDS

FIG. 1. Time course of *A. gossypii* cultivation in 500-mL Erlenmeyer flasks. *A. gossypii* wild type (A) and mutant (B) strains were cultured on minimal medium containing GLC and ASN as carbon and nitrogen sources, respectively. Closed circles, DCW; open circles, RIB concentration; closed triangle, LAC concentration; closed squares, GLC concentration; open squares, ASN concentration.

FIG 2. *In vivo* carbon flux distribution in the central metabolic pathway of wild type (top) and high RIB-producing *A. gossypii* (bottom) strains. *A. gossypii* wild type and mutant strains were cultured on GLC and ASN. The fluxes were determined from the best fit of GC-MS labeling of amino acids from cellular proteins in cultures cultivated with [1, 2-¹³C]-labeled GLC and unlabeled ASN using a comprehensive approach of combined metabolite balancing and isotopomer. For reversible reactions, the direction of the net flux in square boxes is indicated by an additional arrow beside the corresponding reaction. Numbers indicate flux reversibility. All fluxes are presented as a molar percentage of the specific GLC uptake rate. The data are presented as flux $\pm$ random error. Abbreviations: 3PG, glycerate 3-phosphate; AcCoA, acetyl-CoA; ACE, acetate; ACETAL, acetaldehyde; AKG, α -ketoglutarate; ALA, alanine; ANAPL, anaplerotic pathway; ASN, asparagine; ASP, aspartate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; EMP, Embden–Meyerhof–Parnas pathway; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GLC, glucose; GLU, glutamate; GLY, glycine; GTP, guanosine triphosphate; LAC, lactate; MAL, malate; MFA, Metabolic flux analysis, MTHF, methylenetetrahydrofolate; OAA, oxaloacetate; P5P, pentose 5-phosphate; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; PSP, purine synthesis pathway; PYR, pyruvate; RIB, riboflavin; S7P, sedoheptulose 7-phosphate; SER, serine; SUC, succinate; TCA, tricarboxylic acid cycle;

417 THR, threonine; V₁-V₅₈, stoichiometric reactions. Subscripts; cyt; cytosol; ex, extracellular;
418 mit, mitochondria.

TABLE 1. Specific growth rate and yield parameters for wild type and mutant A.

strains.

Parameter	Wild type	Mutant
μ [d ⁻¹]	1.014 ± 0.003	0.783 ± 0.001
Specific consumption rate of GLC* [mmol/g DCW/d]	21.574 ± 0.002	12.429 ± 0.003
Specific consumption rate of ASN* [mmol/g DCW/d]	15.134 ± 0.005	2.333 ± 0.004
Specific production rate of RIB* [mmol/g DCW/d]	0.018 ± 0.002	0.116 ± 0.002
Specific production rate of LAC* [mmol/g DCW/d]	0.809 ± 0.002	1.656 ± 0.001
<i>Yield coefficients</i>		
$Y_{x/GLC}$ [g/mmol]	0.047 ± 0.001	0.063 ± 0.004
$Y_{RIB/GLC}$ [mmol/mmol]	0.001 ± 0.000	0.016 ± 0.002
$Y_{LAC/GLC}$ [mmol/mmol]	0.038 ± 0.001	0.168 ± 0.001
$Y_{ACE/GLC}$ [mmol/mmol]	0.028 ± 0.003	0.037 ± 0.001
$Y_{AKG/GLC}$ [mmol/mmol]	0.053 ± 0.002	0.047 ± 0.006
$Y_{ALA/GLC}$ [mmol/mmol]	0.006 ± 0.001	0.018 ± 0.001
$Y_{GLU/GLC}$ [mmol/mmol]	0.014 ± 0.003	0.014 ± 0.006
$Y_{PYP/GLC}$ [mmol/mmol]	0.023 ± 0.003	0.066 ± 0.002
$Y_{SUC/GLC}$ [mmol/mmol]	0.009 ± 0.006	0.005 ± 0.003

* Obtained in exponential growth phase.

Data are presented as the mean of the specific rate ± standard error from three parallel cultivation experiments.

TABLE 2. Relative mass isotopomer fractions of amino acids from the cellular proteins of *A. gossypii* wild type strain cultivated on 99% [1, 2-¹³C] glucose and unlabeled asparagine.

Amino acid		Mass isotopomers			
		M ₀	M ₁	M ₂	M ₃
Alanine	Calc	0.418	0.144	0.352	0.086
(<i>m/z</i> 260)	Exp	0.444 ± 0.002	0.158 ± 0.003	0.301 ± 0.003	0.097 ± 0.004
Valine	Calc	0.249	0.202	0.297	0.140
(<i>m/z</i> 288)	Exp	0.294 ± 0.010	0.147 ± 0.009	0.302 ± 0.015	0.120 ± 0.017
Aspartate	Calc	0.429	0.219	0.207	0.106
(<i>m/z</i> 418)	Exp	0.392 ± 0.003	0.243 ± 0.002	0.196 ± 0.003	0.116 ± 0.005
Glutamate	Calc	0.253	0.227	0.270	0.143
(<i>m/z</i> 432)	Exp	0.187 ± 0.003	0.203 ± 0.000	0.261 ± 0.003	0.185 ± 0.004
Serine	Calc	0.364	0.290	0.254	0.091
(<i>m/z</i> 390)	Exp	0.376 ± 0.004	0.267 ± 0.003	0.253 ± 0.003	0.104 ± 0.000
Phenylalanine	Calc	0.157	0.122	0.272	0.168
(<i>m/z</i> 336)	Exp	0.191 ± 0.002	0.137 ± 0.001	0.243 ± 0.002	0.152 ± 0.001
Glycine	Calc	0.586	0.312	0.102	
(<i>m/z</i> 246)	Exp	0.559 ± 0.002	0.302 ± 0.001	0.139 ± 0.012	
Tyrosine	Calc	0.136	0.121	0.253	0.177
(<i>m/z</i> 466)	Exp	0.166 ± 0.006	0.137 ± 0.008	0.229 ± 0.010	0.156 ± 0.002
Threonine	Calc	0.429	0.220	0.206	0.106
(<i>m/z</i> 466)	Exp	0.395 ± 0.001	0.243 ± 0.002	0.197 ± 0.003	0.113 ± 0.005
Lysine (<i>m/z</i> 431)	Calc	0.168	0.235	0.255	0.184
	Exp	0.191 ± 0.002	0.181 ± 0.006	0.242 ± 0.001	0.176 ± 0.006
Arginine	Calc	0.187	0.233	0.257	0.175
(<i>m/z</i> 442)	Exp	0.207 ± 0.005	0.205 ± 0.005	0.236 ± 0.002	0.170 ± 0.006

The data shown represent experimental GC-MS results with standard deviation (Exp) and values predicted by the solution of the mathematical model corresponding to the optimized set of fluxes (Calc). Amino acids were analyzed by GC-MS as MBDSTFA derivatives.

TABLE 3. Relative mass isotopomer fractions of amino acids from the cellular proteins of *A. gossypii* mutant strain cultivated on 99% [1, 2-¹³C] glucose and unlabeled asparagine.

Amino acid		Mass isotopomers			
		M ₀	M ₁	M ₂	M ₃
Alanine	Calc	0.423	0.152	0.343	0.082
(<i>m/z</i> 260)	Exp	0.473 ± 0.001	0.154 ± 0.003	0.289 ± 0.000	0.084 ± 0.000
Valine	Calc	0.243	0.196	0.301	0.143
(<i>m/z</i> 288)	Exp	0.333 ± 0.002	0.146 ± 0.001	0.296 ± 0.002	0.106 ± 0.003
Aspartate	Calc	0.604	0.226	0.122	0.037
(<i>m/z</i> 418)	Exp	0.607 ± 0.001	0.224 ± 0.002	0.118 ± 0.001	0.038 ± 0.003
Glutamate	Calc	0.242	0.238	0.269	0.147
(<i>m/z</i> 432)	Exp	0.218 ± 0.001	0.208 ± 0.003	0.263 ± 0.001	0.170 ± 0.003
Serine	Calc	0.401	0.248	0.261	0.090
(<i>m/z</i> 390)	Exp	0.415 ± 0.005	0.235 ± 0.004	0.254 ± 0.004	0.097 ± 0.000
Phenylalanine	Calc	0.175	0.136	0.281	0.165
(<i>m/z</i> 336)	Exp	0.234 ± 0.002	0.142 ± 0.000	0.245 ± 0.000	0.145 ± 0.002
Glycine	Calc	0.597	0.304	0.098	
(<i>m/z</i> 246)	Exp	0.588 ± 0.000	0.266 ± 0.000	0.146 ± 0.000	
Tyrosine	Calc	0.151	0.135	0.262	0.175
(<i>m/z</i> 466)	Exp	0.200 ± 0.010	0.142 ± 0.007	0.235 ± 0.008	0.152 ± 0.003
Threonine	Calc	0.462	0.224	0.191	0.091
(<i>m/z</i> 466)	Exp	0.432 ± 0.002	0.241 ± 0.000	0.187 ± 0.004	0.098 ± 0.001
Lysine	Calc	0.155	0.239	0.257	0.189
(<i>m/z</i> 431)	Exp	0.193 ± 0.001	0.176 ± 0.004	0.235 ± 0.001	0.179 ± 0.004
Arginine	Calc	0.181	0.238	0.260	0.176
(<i>m/z</i> 442)	Exp	0.195 ± 0.014	0.236 ± 0.016	0.185 ± 0.001	0.156 ± 0.006

The data shown represent experimental GC-MS results with standard deviation (Exp) and values predicted by the solution of the mathematical model corresponding to the optimized set of fluxes (Calc). Amino acids were analyzed by GC-MS as MBDSTFA derivatives.

Supplementary materials

Table S1. Precursor stoichiometry for biomass formation

	Wild type ($\mu=1.014\text{ d}^{-1}$) (mmol/g DCW)	Mutant ($\mu=0.783\text{ d}^{-1}$) (mmol/g DCW)
G6P	4.009 ± 0.612	3.731 ± 0.600
GLY	1.186 ± 0.100	1.104 ± 0.100
P5P	1.114 ± 0.301	1.037 ± 0.303
E4P	0.809 ± 0.400	0.753 ± 0.401
G3P	0.219 ± 0.100	0.203 ± 0.100
SER	0.588 ± 0.504	0.547 ± 0.100
PEP	1.618 ± 0.503	1.506 ± 0.509
PYR _{cyt}	5.330 ± 0.600	4.960 ± 0.600
AcCoA _{cyt}	6.002 ± 0.200	5.585 ± 0.200
AcCoA _{mit}	0.908 ± 0.110	0.844 ± 0.100
OAA _{cyt}	1.717 ± 0.100	1.597 ± 0.100
AKG	3.121 ± 0.200	2.904 ± 0.200
THR	1.176 ± 0.301	1.095 ± 0.305

Data were calculated based on reference (1) and are shown average $\pm$ standard deviation.

TABLE S2. Stoichiometry of metabolic reactions in riboflavin-producing *Ashbya gossypii*.

Metabolite	Overall reaction
Glucose 6-phosphate (G6P)	$V_1 - V_9 - V_{14} - V_{46} = 0$
Fructose 6-phosphate (F6P)	$V_8 - V_9 + V_{17} - V_{18} + V_{19} = 0$
Pentose 5-phosphate (P5P)	$V_{14} - V_{19} - V_{47} - 2V_{15} + 2V_{16} - V_{20} = 0$
Erythrose 4-phosphate (E4P)	$V_{17} - V_{18} - V_{19} - V_{48} = 0$
Sedoheptulose 7-phosphate (S7P)	$V_{15} - V_{16} - V_{17} + V_{18} = 0$
Glyceraldehyde 3-phosphate (GAP)	$V_9 + V_{10} - V_{11} + V_{15} - V_{16} - V_{17} + V_{18} - V_{49} = 0$
Glycerate 3-phosphate (G3P)	$V_{11} - V_{12} - V_{20} - V_{21} = 0$
Phophoenol-pyruvate (PEP)	$V_{12} - V_{13} - V_{50} = 0$
Pyruvate cyt (PYR _{cyt})	$V_{13} - V_{31} - V_{34} - V_{40} - V_{41} - V_{45} = 0$
Pyruvate mit (PYR _{mit})	$V_{33} - V_{25} + V_{30} = 0$
Acetyl-CoA mit (AcCoA _{mit})	$V_{25} - V_{26} - V_{57} = 0$
Acetyl-CoA cyt (AcCoA _{cyt})	$V_7 + V_{36} - V_{56} = 0$
α -ketoglutarate (AKG)	$V_{26} - V_{27} - V_{42} - V_{43} - V_{51} = 0$
Succinate (SUC)	$V_{27} - V_{28} - V_{44} = 0$
Malate (MAL)	$V_{28} - V_{29} - V_{30} = 0$
Oxaloacetate mit (OAA _{mit})	$V_{29} - V_{26} + V_{32} = 0$
Oxaloacetate cyt (OAA _{cyt})	$V_3 - V_4 - V_6 + V_{31} - V_{32} - V_{55} = 0$
Guanosine triphosphate (GTP)	$V_{20} - V_{24} = 0$
Serine (SER)	$V_{21} - V_{22} - V_{53} = 0$
Threonine (THR)	$V_5 + V_6 - V_7 - V_{54} = 0$
Acetaldehyde (ACETAL)	$V_{34} - V_{35} = 0$
Acetate (ACE)	$V_{35} - V_{36} - V_{37} = 0$
Aspartate (ASP)	$V_2 - V_3 + V_4 - V_5 = 0$
Glycine (GLY)	$V_7 + V_{22} - V_{23} - V_{52} = 0$

Table S3. Metabolic network for *A. gossypii*. The model file is present with constraints to simulate riboflavin production from glucose.

Glucose uptake

v1 GLC_EX (abcdef) → G6P (abcdef)

Asparagin, threonine and glycine metabolism

v2 ASN_EX (abcd) → ASP (abcd)
v3/ v4 ASP (abcd) ↔ OAA_{cyt} (abcd)
v5 ASP (abcd) → THR (abcd)
v6 OAA_{cyt} (abcd) → THR (abcd)
v7 THR (abcd) → GLY (ab) + AcCoA_{cyt} (cd)

Glycolysis

v8 G6P (abcdef) → F6P (abcdef)
v9 F6P (abcdef) + ATP (X) → DHAP (abc) + G3P (def)
v10 DHAP (abc) → G3P (cba)
v11 G3P (abc) → 3PG (abc) + ATP (X) + NADH (X)
v12 3PG (abc) → PEP (abc)
v13 PEP (abc) → PYR_{cyt} (abc) + ATP (X)

Pentose phosphate pathway

v14 G6P (abcdef) → P5P (abcde) + CO₂ (f) + 2NADPH (2X)
v15/v16 P5P (abcde) + P5P (fghij) ↔ S7P (fgabcde) + G3P (hij)
v17/v18 S7P (abcdefg) + G3P (hij) ↔ E4P (defg) + F6P (abchij)
v19 E4P (abcd) + P5P (efghi) → F6P (efabcd) + G3P (ghi)
v20 P5P (abcde) + 3PG (fgh) + 2MTHF (ij) → GTP (abcdefghij)
v21 3PG (abc) → SER (abc)
v22 SER (abc) → GLY (ab) + MTHF (c)
v23 GLY (ab) → MTHF (a) + CO₂ (b)

Riboflavin Formation

v24 GLY → RIB_EX (B*¹)

TCA cycle

v25	PYR _{mit} (abc)	→	ACCOA _{mit} (bc) + CO ₂ (a) + NADH (X)
v26	AcCoA _{mit} (ab) + OAA _{mit} (cdef)	→	AKG (abcde) + CO ₂ (f) + NADPH (X)
v27	AKG (abcde)	→	0.5SUC (bcde) + 0.5SUC (edcd) + CO ₂ (a) + NADH (X) + ATP (X)
v28	SUC (abcd)	→	MAL (abcd) + NADH (X)
v29	MAL (abcd)	→	OAA _{mit} (abc) + NADH (X)
v30	MAL (abcd)	→	PYR _{mit} (abc) + CO ₂ (d) + NADPH (X)

Transport reactions cytosol-mitochondria

v31	PYR _{cyt} (abc) + CO ₂ (d) + ATP (X)	→	OAA _{cyt} (abcd)
v32	OAA _{cyt} (abc)	→	OAA _{mit} (abc)
v33	PYR _{cyt} (abc)	→	PYR _{mit} (abc)
v34	PYR _{cyt} (abc)	→	ACETAL (bc) + CO ₂ (a)

Formation of AcCoA in the cytosol

v35	ACETAL (ab)	→	ACE (ab)
v36	ACE (ab)	→	AcCoA _{cyt} (ab)
v37	ACE	→	ACE_EX (B ^{*1})

CO₂ formation

v38/v39	CO ₂ (a)	↔	CO ₂ _EX (a)
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Biomass formation

v40	PYR _{cyt}	→	PYR _{cyt} _EX (B ^{*1})
v41	PYR _{cyt}	→	LAC_EX (B ^{*1})
v42	AKG	→	AKG_EX (B ^{*1})
v43	AKG	→	GLU_EX (B ^{*1})
v44	SUC	→	SUC_EX (B ^{*1})
v45	PYR _{cyt}	→	ALA_EX (B ^{*1})
v46	G6P	→	G6P_B (B ^{*1})
v47	P5P	→	P5P_B (B ^{*1})
v48	E4P	→	E4P_B (B ^{*1})
v49	G3P	→	G3P_B (B ^{*1})
v50	PEP	→	PEP_B (B ^{*1})
v51	AKG	→	AKG_B (B ^{*1})
v52	GLY	→	GLY_B (B ^{*1})

v53	SER	→ SER_B (B ^{*1})
v54	THR	→ THR_B (B ^{*1})
v55	OAA _{cyt}	→ OAA _{cyt} _B (B ^{*1})
v56	AcCoA _{cyt}	→ AcCoA _{cyt} _B (B ^{*1})
v57	AcCoA _{mit}	→ AcCoA _{mit} _B (B ^{*1})
v58	PYR _{cyt}	→ PYR _{cyt} _B (B ^{*1})

Amino acid synthesis

v59	GLY (ab) + MTHF (c)	→ SER (abc) (S ^{*2})
v60	PYR _{mit} (abc) + PYR _{mit} (def)	→ VAL (abcde) + CO ₂ (f) (S ^{*2})
v61	E4P (abcd) + PEP (efg)	→ SHKM (efgabcd) (S ^{*2})
v62	SHKM (abcdefg) + PEP (hij)	→ CHRM (abcdefghij) (S ^{*2})
v63	CHRM (abcdefghij)	→ PHE (hijbcdefg) + CO ₂ (a) (S ^{*2})
v64	CHRM (abcdefghij)	→ TYR (hijbcdefg) + CO ₂ (a) (S ^{*2})
v65	CHRM (abcdefghij)	→ ANTHR (abcdefg) + PYR (hij) (S ^{*2})
v66	ANTHR (abcdefg) + P5P (hijkl)	→ CPADR5P (abcdefghijkl) (S ^{*2})
v67	CPADR5P (abcdefghijkl)	→ INDG (abcdfghijkl) + CO ₂ (e) (S ^{*2})
v68	INDG (abcdefghijk)	→ IND (abcdefgh) + G3P (ijk) (S ^{*2})
v69	IND (abcdefgh) + SER (ijk)	→ TRP (abcdefghkji) (S ^{*2})
v70	PYR _{mit} (abc) + PYR _{mit} (def)	→ ISV (abefc) + CO ₂ (d) (S ^{*2})
v71	ISV (abcde) + AcCoA _{mit} (fg)	→ LYS (abcdeg) + CO ₂ (f) (S ^{*2})
v72	AKG (abcde) + CO ₂ (f)	→ ARG (abcdef) (S ^{*2})
v73	AKG (abcde) + AcCoA _{mit} (fg)	→ LYS (abcdeg) + CO ₂ (f) (S ^{*2})
v74	OAA _{cyt} (abcd) + MTHF (e)	→ MET (abcde) (S ^{*2})
v75	P5P (abcde) + MTHF (f)	→ HIS (edcbaf) (S ^{*2})
v76	3PG (abc)	→ CYS (abc) (S ^{*2})
v77	PYR (abc)	→ ALA (abc) (S ^{*2})
v78	OAA _{cyt} (abcd)	→ APN (abcd) (S ^{*2})
v79	OAA _{cyt} (abcd)	→ ASP (abcd) (S ^{*2})
v80	PYR _{mit} (abc) + OAA _{mit} (defg)	→ ILE (defbfgc) + CO ₂ (a) (S ^{*2})
v81	AKG (abcde)	→ GLU (abcde) (S ^{*2})
v82	AKG (abcde)	→ GLN (abcde) (S ^{*2})
v83	AKG (abcde)	→ PRO (abcde) (S ^{*2})

*1 “B” means the reaction that is to be excluded from isotopomer balance, typically

reactions that drain biomass precursors.

*2 “S” means the reaction that is to be excluded from metabolite balance. This type of reaction is used to map label distribution of the measured metabolites to their precursors.

Precursor

3PG	glycerate 3-phosphate
ACE	acetate
AcCoA	acetyl-CoA
ACETAL	acetaldehyde
AKG	α -ketoglutarate
ALA	alanine
ASN	asparagine
ARG	arginine
ASP	aspartate
ANTHR	anthranilate
CHRM	chorismate
CPADR5P	1-(o-carboxyphenylamino)-1-deoxyribose 5-phosphate
CYS	cysteine
DHAP	dihydroxyacetone phosphate
E4P	erythrose 4-phosphate
F6P	fructose 6-phosphate
GAP	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
GLC	glucose
GLU	glutamate
GLN	glutamine
GLY	glycine
GTP	guanosine triphosphate
HIS	histidine
ILE	isoleucine

IND	indole
INDG	indole-3-glycerolphosphate
ISV	isovaline
LAC	lactate
LYS	lysine
MAL	malate
MET	methionine
MTHF	methylenetetrahydrofolate
OAA	oxaloacetate
P5P	pentose 5-phosphate
PEP	phosphoenolpyruvate
PHE	phenylalanine
PRO	proline
PYR	pyruvate
RIB	riboflavin
S7P	sedoheptulose 7-phosphate
SER	serine
SHKM	shikimate
SUC	succinate
THR	threonine
TRP	tryptophan

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