The improvement of riboflavin production in *Ashbya gossypii* via disparity mutagenesis and DNA microarray analysis

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**The improvement of riboflavin production in *Ashbya*
gossypii via disparity mutagenesis and DNA
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Abstract We generated a high riboflavin producing mutant strain of *Ashbya gossypii* by disparity mutagenesis using mutation of DNA polymerase δ in the lagging strand, resulting in loss of DNA repair function by the polymerase. Among 1,353 colonies generated in the first screen, 26 mutants produced more than 3 g/l of riboflavin. By the second screen and single colony isolation, nine strains that produced more than 5.2 g/l of riboflavin were selected as high riboflavin producing strains. These mutants were resistant to oxalic acid and hydrogen peroxide as antimetabolites. One strain (W122032) produced 13.7 g/l of riboflavin in a 3-L fermentor using an optimized medium. This represents a nine-fold improvement on the production of the wild type strain. Proteomic analysis revealed that ADE1, RIB1 and RIB5 proteins were expressed at two-fold higher levels in this strain, than in the wild type. DNA microarray analysis showed that purine and riboflavin biosynthetic pathways were up-regulated, while pathways related to carbon source assimilation, energy generation and glycolysis were down-regulated. Genes in the riboflavin biosynthetic pathway were significantly over-expressed during both riboflavin production and stationary phases, for example, RIB1 and RIB3 were expressed at greater than six-fold higher levels in this strain compared to the wild type. These results indicate that the improved riboflavin production in this strain is related to a shift in carbon flux from β -oxidation to the riboflavin biosynthetic pathway.

Keywords *Ashbya gossypii* · Disparity mutagenesis · Riboflavin · Riboflavin biosynthesis

Introduction

Riboflavin is a water-soluble vitamin, also known as vitamin B₂. It is a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential for the transfer of electrons in oxidation-reduction reactions (Schmidt et al. 1996). It is also essential for the health of the mucous membrane in the digestive tract and helps with the absorption of iron and vitamin B6 in the human body and animals.

Fermentation of *A. gossypii* yields an output of more than 4000 metric tons/year, which represents about 50% of commercial riboflavin production in the world (Chotani et al. 2007). Efforts on improving riboflavin production of *A. gossypii* yielded up to 15 g/l riboflavin by chemical mutagenesis more than two decades ago (Bigelis 1989). Riboflavin is synthesized from GTP and ribulose 5-phosphate by RIB genes. From oils, GTP and ribulose 5-phosphate are supplied through β -oxidation, glyoxylate cycle, gluconeogenesis, pentose-phosphate pathway and purine biosynthetic pathway (Fig. 1). Glyoxylate pathway is important for riboflavin synthesis from oils. Isocitrate lyase (ICL, *threo*-D_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) in glyoxylate cycle is the key enzyme involved in this anaplerotic pathway in *A. gossypii* (Schmidt et al. 1996). *A. gossypii* is able to metabolize waste edible oils (Park et al. 2004), converting them into riboflavin via various cycle pathways. We attempted to improve the riboflavin production of *A. gossypii* using classical mutagenesis by UV radiation (Park et al. 2007) or by the chemical mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Tajima et al. 2009), but the riboflavin productivity of these mutants was found to be unstable.

Disparity mutagenesis was introduced by Furusawa (1998) and disparity theory has been developed using computer simulations (Furusawa and Doi 1992). This technology increases the error threshold without the loss of genetic information by many-times division of cells and without damage on cell growth, yielding a large number of advantageous mutants (Shimoda et al. 2006; Abe et al. 2009). The disparity mutagenesis technique is capable of carrying out genomic mutations, including mutations in non-coding regions of the genome. This approach is particularly useful when there is no information on which specific genes or regions of the genome would result in the desired phenotype upon mutation. To obtain genetic diversity, an error-prone DNA polymerase technique is employed. Thus, a mutation is inserted into DNA polymerase δ which is responsible for synthesis of the lagging strands, such that the polymerase loses its DNA repair function (Aoki and Furusawa 2001).

In this study, a high-riboflavin producing strain of *A. gossypii* was generated using disparity mutagenesis technique. Proteomic and DNA microarray analysis demonstrated the up-regulation of genes involved in purine and riboflavin synthetic pathways, which accounts for the nine-fold improvement in riboflavin production observed in the mutant strain, compared with production in the wild type strain.

Materials and methods

Strains and culture methods

A. gossypii ATCC 10895 was used as the wild-type strain (WT) and was grown on YD medium (pH 6.8) containing 1% (w/v) yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan) and 1% (w/v) glucose (Wako Pure Chem. Ind., Ltd., Osaka, Japan) for sporulation and long-term storage at 4°C. YR medium (pH 6.8) containing 1% (w/v) yeast extract and 1% (w/v) rapeseed oil (Wako Pure Chem. Ind. Ltd., Osaka, Japan) was used as the screening medium for riboflavin production in test tube.

For pre-culture of riboflavin production, a seed medium consisting of 30 g/l corn steep liquor (CSL, Nihon Shokuhin Kako Co. Ltd., Tokyo, Japan), 9 g/l yeast extract, and 15 g/l rapeseed oil (pH 6.8) was used. The riboflavin production medium consisted of 60 g/l CSL, 30 g/l gelatin (Wako), 1.5 g/l KH_2PO_4 , 1.5 g/l glycine, mineral ions (2 $\mu\text{g/l Co}^{2+}$, 5 $\mu\text{g/l Mn}^{2+}$, 10 $\mu\text{g/l Zn}^{2+}$, 1 $\mu\text{g/l Mg}^{2+}$), and 50 g/l rapeseed oil (pH 6.8). Mineral ions were prepared as follows, 0.8812 g CoCl_2 , 3.602 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 8.795 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.028 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 200 ml of distilled water, respectively. Each stock solution 1 ml was added to one liter of production medium before autoclave. The flask cultures were prepared in 500-ml Erlenmeyer flasks with a working volume of 50 ml of each medium in a rotary shaker with 7 cm of rotating diameter (BIO SHAKER; Takasaki Inst. Co., Tokyo, Japan) at 220 rpm and 28°C. Riboflavin production was carried out in a 3-L bioreactor (Bioneer-300, Marubishi Co., Ltd., Tokyo, Japan) with a working volume of 1.5 L of production medium. The inoculum dose in each culture was 10% (v/v). Agitation and aeration rates used were 600 rpm and 1 vvm, respectively. Temperature was controlled at $28 \pm 0.5^\circ\text{C}$ but pH was

not controlled. Dissolved oxygen concentration and pH were recorded during the cultivation.

Disparity mutagenesis

Vector construction

Vector for disparity mutagenesis of *A. gossypii* was constructed as follows; *LEU2* (1.2 kb) of YCplac111 (Gietz and Sugino 1988) was excised using *Aat* II and *EcoR* V, and then Geneticin resistance gene cassette (2.5 kb) was inserted into the *Bam*H I restriction site of the multi-cloning site (MCS). The resulting plasmid was designated as YCpG418. The *Ashbya* DNA polymerase δ genome sequence, *POL3* (AFL189W) was obtained from Ashbya database (http://agd.vital-it.ch/Ashbya_gossypii/geneview?db=core;gene=AFL189W). This putative *POL3* consisted of promoter (1 kb), *POL3* (3.3 kb), and terminator (0.6 kb). Two nucleotides of the *POL3* gene were mutated using PCR: 946 bp (A→C) and 952 bp (A→C). The resulting mutated *POL3* (4.9 kb) was inserted into the *Xba* I site of YCpG418, and the resulting plasmid was designated as YCpG418/*pold*^{exo-} (Neo-Morgan, Tokyo; Supplementary file 1). Disparity mutagenesis technology involves transformation of the YCpG418/*pold*^{exo-} and screening of transformants, and the procedures (Fig. 2) are described following sections.

Transformation

Spores of the WT (approximately 1.0×10^6) were grown on YD medium for 27 h. The mycelia were harvested by filtration, washed with distilled water, and suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 25 mM 2-mercaptoethanol. The suspension was incubated at 30°C for 30 min with gentle agitation, and the mycelia were collected by filtration and washed with transformation buffer consisting of 270 mM sucrose, 10 mM Tris-HCl (pH 7.5), and 1 mM MgCl₂. The mycelia were finally resuspended in cooled transformation buffer, and 350 µl of the mycelial suspension was mixed with 1.5 µl of the plasmid YCpG418/*pold^{exo-}*. This plasmid was introduced into the mycelium by electroporation in a Gene Pulser Xcell system (Bio-Rad Lab. Inc., Hercules, CA, USA) at 1.5 kV/cm, 100 Ω, and 25 µF using 2 mm pre-chilled electrocuvettes (Bio-Rad). The post-electroporated mycelia were plated and incubated on a YD plate to regenerate the mycelia at 28°C for 6 h. Subsequently, the mycelia were covered with 20 ml YD medium containing 0.6% agar and 200 µg/ml Geneticin for isolation of the transformants. After 3-6 days incubation, the Geneticin-resistant spores germinated on the bottom and elongated mycelia reached above the surface of the agar. Likewise Geneticin-resistant colonies were picked and transferred into a new YR plate containing 200 µg/ml Geneticin (G418-YR).

Regeneration and screening procedures

The mycelia produced in the G418-YR plate are designated as the first generation. Colonies of the first generation were picked, and transferred to a new G418-YR plate, designated the second generation. This procedure was continued until the 30th

generation, to allow an accumulation of mutations in the mycelia by the overlapping generations.

On the other hand, picked colonies from each generation were also inoculated into 5 ml of YR medium in a test tube and cultured twice in YR medium without Geneticin to release the plasmid from the transformants. Until the 18th generation, YR medium was used, but from the 19th to the 30th generation modified YR medium (containing 2% (w/v) rapeseed oil and 3% (w/v) yeast extract) was used to avoid nutrient depletion. The test tube cultures were carried out at 28°C with 150 rpm for 24 h.

The first screening of mutants was carried out using the YCpG418/*pold^{exo-}*-losing colonies. 100 µl of the YCpG418/*pold^{exo-}*-losing cell suspension was transferred into a new 5 ml YR medium in a test tube, and cultured at 28°C, 150 rpm for 6 d. After culture, the riboflavin concentration was measured and if the colony proved to be a high-riboflavin producer, it was frozen at -20°C for the second screening.

For the second screening the colonies from the frozen stock were transferred into 5 ml of a seed medium in a test tube at 28°C and 200 rpm and shaken for 24 h. Ten per cent of the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 50 ml of the production medium (pH 6.8). Cultivation was conducted at 28°C and 220 rpm for 6 d. All mutant strains were also stored in a -80°C freezer, in the presence of 20% (v/v) glycerol.

Medium optimization

The effect of CSL, yeast extract, rapeseed oil, soybean mill, glycine, alanine, and glutamic acid in the production medium on riboflavin production was investigated using a 2-level factor design. CSL, yeast extract, rapeseed oil, glycine, and glutamic acid were selected as the most effective composition for riboflavin production, and re-optimized using 3-level factor design (Box-Behnken). CSL and yeast extract were extracted to be the most important nitrogen sources and their levels were optimized for riboflavin production by 3-level factor design. Statistical analysis was performed with STATISTICA (StatSoft, Inc., OK, USA), and the regression of the parameters was solved using Mathcad 2001 Professional (Mathsoft Engineering and Education, Inc., MA, USA).

Proteomic analysis

The WT and mutant strains were grown in YR medium in 500-ml Erlenmeyer flasks with a working volume of 50 ml at 28°C for 4 days. The mycelia were harvested, washed twice with sterile distilled water, and suspended in 50 mM potassium phosphate buffer (pH 6.0). Subsequently, the mycelia were sonicated on ice five times at 40 W for 30 s with 1-min intervals, using an ultrasonicator (Sonics & Materials Inc., CT, USA), to extract the intracellular soluble proteins. The sample was centrifuged at 20000 *g* and 4°C for 5 min, and the supernatant containing the soluble proteins was used for two-dimensional electrophoresis proteomic analysis (performed at Shimadzu Techno-Research Inc., Kyoto, Japan). Isoelectric focusing (IEF) was performed between pH 3

and 10 using Immobiline DryStrip gel (GE Healthcare Japan, Tokyo, Japan). After IEF, the gel was equilibrated with sample buffer (6M urea, 20% glycerol, 2% dithiothreitol, 2% sodium dodecyl sulphate 100 mM Tris-HCl pH8.8) and applied to 10-18% polyacrylamide gradient gel. Gels were stained with Coomassie Brilliant Blue (CBB). Some scraps of gels stained with CBB were picked up and proteins were digested by trypsin. Samples were applied to peptide mass fingerprinting (PMF) analysis. For two-dimensional electrophoresis, 250 µg of protein was used.

DNA microarray

The WT and mutants were used for DNA microarray testing. The harvested mycelia were frozen at -80°C and used for RNA extraction. RNA extraction was performed and RNA quality was checked by Nihon Gene Research Lab. Inc. (Sendai, Japan). Custom arrays of *A. gossypii* were manufactured by Roche NimbleGen Inc. (Tokyo, Japan) using 4 × 72K (4 plex) format. For each gene, 6 different oligonucleotides were designed and every oligonucleotide was repeated twice on each slide. Each slide contained all (4726) ORFs of *A. gossypii*. Double-stranded cDNAs were synthesized using oligo-dT and labeled with Cy3 dye. Labeled cDNA was hybridized to custom arrays and data were extracted by NimbleGen software and analyzed by DNASTAR ArrayStar v3.0 (Madison, WI, USA).

Analytical methods

Concentrations of riboflavin, residual oil, and dry cell weight were measured according to previous report (Park et al. 2004). Protein concentration was measured using Protein assay kit II (Bio-Rad Lab. Inc.) based on Bradford method.

Results

Disparity mutagenesis

From the first to the 30th generation, 1,851 Geneticin-resistant colonies were obtained, and of those mutants, 1,353 mutants showing deep yellow colonies were selected as riboflavin producing mutants during the first screening process. From the first to the 18th generation, YR broth medium was used. Between the first and 18st generation, the mutant of 15th generation produced the highest riboflavin concentration. Therefore, from the 19th generation, a modified YR broth medium was used to avoid carbon source depletion. Riboflavin concentration increased between 19th and 23rd generation. However, until 30th generation, riboflavin production decreased. The highest riboflavin concentration was obtained in 23rd generation and no further increase in production was observed in later mutants. The first screening was therefore stopped at the 30th generation. The maximum riboflavin concentration observed in the first screening in test tubes was 2 g/l.

In the second screening process, 26 mutants were picked up from the 1,353 mutants chosen during the first screen. These 26 mutants produced around 3-4 g/L of riboflavin at the second screening in the 500-ml Erlenmeyer flask cultivation. Single

cell colonies of these 26 mutants were isolated and nine colonies were picked as the highest riboflavin producing mutants (Supplementary file 2). Single cell colony isolation was repeated three times and the results of typical 9 mutants are shown in Table 1. Riboflavin concentration increased gradually from 3.5 to 5.8 g/l with repeated isolation. Riboflavin production in two mutants (W114038 and W122044) was less than 4.5 g/l, and they were not screened any further.

The effect of antimetabolites, such as oxalate and hydrogen peroxide, on the riboflavin production of these mutants was investigated. WT120015 and W122032 mutants grown in a medium containing 50 mM of oxalate both produced riboflavin in concentrations of around 60% of the concentration obtained without oxalate supplementation (data not shown). The wild type strain, on the other hand, only produced around 10% of riboflavin when subjected to oxalate supplementation. In the case of hydrogen peroxide (0 to 40 mM), riboflavin production levels of each mutant changed only negligibly, with the exception of the W111024 mutant whose production was reduced to around 60% when hydrogen peroxide levels were highest (data not shown). However, the wild type showed a significant decrease in riboflavin production in the presence of hydrogen peroxide, dropping to 20% with the highest concentrations of hydrogen peroxide tested. These data show that these mutants are resistant to antimetabolites. From these results the W122032 mutant was selected as the best riboflavin producer.

243 The stability of riboflavin production in the W122032 mutant was investigated
244 on a flask scale. A glycerol stock of the W122032 strain was used for seed cultivation.
245 The seed culture was used for its riboflavin production in a 500-ml flask scale and to
246 make a new glycerol stock. This new glycerol stock was designated as the second
247 generation. The second generation of glycerol stock was used for the next cultivation,
248 and stocked the third glycerol stock. These steps were repeated until 14 generations had
249 been generated. The W122032 strain showed good stability, producing riboflavin at
250 concentrations of around 5-6 g/l until the 14th generation (Fig. 3). The standard
251 deviation of riboflavin production from the first to the 14th generation was 0.25,
252 suggesting that the W122032 mutant was a stable producer of riboflavin.

253 Medium optimization and riboflavin production in a bioreactor

254 Conventional media contain gelatin, which is of animal origin, and gelatin is a useful
255 nitrogen source in microbial fermentation. However, to substitute a safe nitrogen source,
256 several nitrogen sources of plant origin were tested and soybean mill was chosen (data
257 not shown). Since the production medium contained rapeseed oil, glycine, CSL, yeast
258 extract, glutamic acid, and mineral components, the composition of the production
259 medium was optimized using Box-Behnken experimental design. Since CSL and yeast
260 extract were the most effective nitrogen sources in riboflavin production, these two
261 nitrogen sources were re-optimized and optimal concentrations of these components

were determined to be 40.3 and 36.1 g/l, respectively. The optimized composition of the production medium is shown in Table 2.

When the W122032 mutant was cultured in the optimized medium in a 3-L fermentor, riboflavin production was significantly improved. Dissolved oxygen level during the culture of W122032 strain was below 10% for 3 days and gradually increased (Fig. 4A) while it was only one day in the wild type strain. pHs of both strains remained 6.5-7.5 throughout the cultures (Fig. 4A). For the riboflavin production, the wild type strain produced 1.5 g/l for 5 days, while the W122032 strain produced 7.2 g/l in conventional medium for 8 days and 13.7 g/l in optimized medium for 9 days, respectively (Fig. 4B). When the culture was repeated 3 months later, under the same conditions, the riboflavin concentration was 13.4 g/l for 9 days (triangles in Fig. 4B). The quantity of rapeseed oil consumed in two cultures of W122032 was 73 and 75 g/l, while it was 79 g/l in the wild type strain. This indicates that this strain is a very stable and high-riboflavin producer in a bioreactor. Riboflavin yield based on the consumed carbon source of the wild type strain was 0.02 (g riboflavin/g consumed carbon source) while that of W122032 was 0.18 g/g. The W122032 mutant had a riboflavin yield nine-fold higher than the wild type strain.

Proteomic analysis

To establish the differences between the wild type and mutant strains, proteomic analysis was performed with about five mutants that produced riboflavin at

282 concentrations higher than 5.7 g/l (Table 1). Proteins that showed significant (5-fold)
283 over- or under-expression compared to that of the wild type strain are listed in Table 3.
284 Enzymes which showed increased expression were phosphoglycerate mutase, which
285 catalyzes the reaction of 2-phosphoglycerate to 3-phosphoglycerate or vice versa in
286 glycolysis, citrate synthase, which catalyzes citrate formation from oxaloacetate in the
287 TCA cycle, and NADP or NADPH binding oxidoreductase and oxidoreductase, which
288 are involved in oxidation and reduction in mitochondria. On the other hand,
289 phosphoribosylaminoimidazole-succinocarboxamide synthase, guanosine-5-
290 triphosphate (GTP) cyclohydrolase II and riboflavin synthase all showed significantly
291 increased expression. Phosphoribosylaminoimidazole-succinocarboxamide synthase
292 catalyzes the formation of [[(S)-2-[5-amino-1-(5-phospho-D-ribosyl)imidazole-4-]], and
293 [[carboxamido]succinate]] (SAICAR) from 5-amino-1-(5-phospho-D-
294 ribosyl)imidazole-4-carboxylate in purine metabolism. GTP cyclohydrolase II is
295 involved in the formation of 2,5-diamino-6-hydroxy-4-(5-
296 phosphoribosylamino)pyrimidine from GTP in the riboflavin biosynthetic pathway.
297 Riboflavin synthase converts 6,7-dimethyl-8-ribityllumazine to riboflavin in the
298 riboflavin biosynthetic pathway. This suggests that phosphoribosylaminoimidazole-
299 succinocarboxamide synthase increases GTP flux from the purine biosynthetic pathway,
300 and GTP cyclohydrolase II increases the flux of 2,5-diamino-6-hydroxy-4-(5-
301 phosphoribosylamino)pyrimidine, which is an intermediate of riboflavin production.
302 This increased carbon flux in the mutants was converted effectively into riboflavin by

the increased activity of riboflavin synthase, which leads to improved riboflavin production in the mutants.

DNA microarray

Gene expression in mutant strains was compared to that of the wild type as shown in Fig. 5. SPS19 and POT1, which are involved in the β -oxidation cycle, showed a two-fold higher expression, while FAA1,4, POX1 and FOX1 had a two-fold lower expression. Of the glyoxylate cycle enzymes, CIT1,2 was expressed at high levels, but MLS1, at low levels. Enzymes in the TCA, glycolysis and pentose phosphate cycles were down-regulated with the exception of CIT1,2 and ZWF1. Enzymes involved in the purine biosynthetic pathway showed a two-fold increased expression with the exception of IMD3,4. Interestingly, RIB1, BIB2, RIB5, and RIB7 were expressed at more than two-fold higher levels than in the wild type. Expression level of RIB3 was a three-fold higher than that of wild type strain. Overall, the gene expressions of purine and riboflavin biosynthetic pathways were up-regulated, but pathways related to carbon source assimilation, energy generation and glycolysis were down-regulated.

Differences in gene expression during logarithmic growth, riboflavin production and stationary phases of the mutant strain were compared with expression in the wild type strain (Fig. 6A and B). Gene expression involved in β -oxidation, glyoxylate and TCA cycles in the mutant strain were decreased compared to expression levels in the wild type strain. In the gluconeogenesis, expression of GLK1, HXK1,2, GMP2,3, PGK1,

ENO1,2, and PYK2 increased 1.5-fold in the mutant strain during the riboflavin-producing phase. PRS1, PRS3 and PRS5 in the pentose phosphate cycle were expressed at three-fold higher levels during the riboflavin production phase compared to the wild type. In the purine biosynthetic pathway, ADE1, ADE5,7 and ADE6 were expressed two- -fold more during the logarithmic growth and stationary phases than in the wild type. Expression of GUA1, YND1 and GUK1 increased only during the stationary phase. In the riboflavin biosynthetic pathway, all enzymes were expressed at significantly higher levels than in the wild type. RIB1 during the riboflavin production and RIB3 during the stationary phase were expressed at levels more than six-fold higher than the wild type. The mutant strain therefore features a highly activated purine and riboflavin biosynthetic pathway, which explains this mutant's improved riboflavin production.

Discussion

In previous studies, we reported our attempts to increase riboflavin production in *A. gossypii* using classical mutagenesis with UV radiation (Park et al. 2007) and chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Tajima et al. 2009). Whilst the filamentous hemiascomycete *A. gossypii* is sensitive to, and is mutated by UV light (Stahmann et al. 2001), the mutant strain gradually lost its excess riboflavin productivity after 2 years of culture and preservation (Park et al. 2007). Chemical mutagenesis using NTG is used for classical strain improvement. *A. gossypii* 13a mutant

was obtained using NTG mutation, and produced three-fold higher than that in the wild type strain. However, the 13a mutant gradually lost its excess riboflavin productivity after preservation for a long period, similarly to the NTU induced mutant (Tajima et al. 2009). Proteomic analysis revealed that the activities of isocitrate lyase (ICL), mitochondrial aldehyde dehydrogenase (ALDH), cytosolic ALDH, threonine aldolase and catalase were increased in the mutant strain. ICL and threonine aldolase are known to be responsible for riboflavin production in *A. gossypii* (Kanamasa et al. 2007; Monschau et al. 1998; Schmidt et al. 1996).

For the purpose of increasing riboflavin productivity, we speculated that disparity mutagenesis, a new genetic mutation technology, may prove to be a useful alternative. In this study, a YCpG418/*pold*^{exo-} vector, which contained mutated *Ashbya* POL3 gene and is responsible for the disparity mutation (Supplementary file 1), was used. The W122032 strain produced 13.7 g/l in optimized medium, which represents the highest levels of riboflavin productivity obtained to date, even when compared to our previous methods (Park et al. 2007; Sugimoto et al. 2010; Tajima et al. 2009). Our results indicate that the technique may be successfully applied in a variety of microorganisms.

Proteomic analysis revealed that ADE1, RIB1 and RIB5 of five mutants were expressed five times higher than in the wild type. ADE1 plays an important role in inosine-5-monophosphate production in the purine biosynthetic pathway. GTP cyclohydrolase II (RIB1) catalyzes the reaction of GTP to 2,5-diamino-6-(5-

phosphoribosyl(amino)-4-pyrimidineone, and supplies carbon flux to the purine biosynthetic pathway. Riboflavin synthase (RIB5) converts 6,8-diemthyl-8-ribityl lumazine to riboflavin. The increased gene expression of this enzyme improves carbon flux from the purine biosynthetic pathway to the riboflavin biosynthetic pathway. This result explains the high riboflavin production observed in the W122032 mutant. Previous reports (Jimenez et al. 2005; 2008) reported that purine biosynthetic pathway is important for riboflavin synthesis in *A. gossypii*. Increase of ADE1 expression corresponds to the results of previous reports.

DNA microarray experiments are consistent with the results of proteomic analysis. Genes in β -oxidation, glyoxylation and TCA cycles in W122032 mutant at the riboflavin-producing phase were down regulated, but genes in gluconeogenesis and pentose phosphate cycles showed increased expression. However, genes in the purine and riboflavin biosynthetic pathway showed increased expression, at levels more than two-fold higher than those of the wild type. This gene expression may increase carbon flux to riboflavin synthesis which is also confirmed by time course studies of gene expression. A previous paper (Karos et al. 2004) reported that transcription rate of ADE1 gene was decreased in *A. gossypii* at the riboflavin-producing phase and on the other hand, transcription rate of 3-phosphoglycerate kinase gene (*PGK1*) was decreased. These results indicate that the purine synthetic pathway is important for riboflavin production in *A. gossypii*. This result corresponds to that of proteomic analysis.

In *Bacillus subtilis* strains, a number of strategies have been used to generate high level riboflavin producing strains. These include enhancement of both gene dosages and the transcriptional levels of the riboflavin operon in mutants (Perkins et al. 1999), the constitutive expression of key genes in the riboflavin biosynthetic pathway (Hümbelin et al. 1999), enhancing generation and reducing maintenance metabolism (Zamboni et al. 2003), increasing precursor supply by modulating carbon flow through the pentose phosphate pathway (Zamboni et al. 2004; Zhu et al. 2006), and deregulation using knockout technology (Tännler et al. 2008). However, these strategies cannot be applied directly to *A. gossypii* strains. The expression of RIB1 and RIB4 of the riboflavin biosynthetic pathway in the mutant was less than that of the wild type strain during the stationary phase. This may limit further improvements in riboflavin production by acting as a bottleneck in the W122032 strain. Over-expression of RIB1 and RIB4 during the stationary phase may be one strategy to further improve riboflavin production in *A. gossypii*. While the riboflavin synthetic pathway in the W122032 strain was increased, genes involved in carbon source assimilation upstream of the riboflavin synthesis pathway were down-regulated. Therefore, increasing the carbon flux to the riboflavin synthetic pathway would also be expected to further improve riboflavin production. Expression of lipases gene in W122032 would be useful for assimilation of the carbon source in β -oxidation cycle.

We have been able to generate a high-riboflavin producing strain of *A. gossypii* using disparity mutagenesis. Proteomic and DNA microarray analysis demonstrated the

405 up-regulation of genes involved in purine and riboflavin synthetic pathways, which
406 accounts for the nine-fold improvement in riboflavin production observed in the
407 W122032 strain, compared with production in the wild type strain.

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411 **Nomenclature**

412 ACO1: acyl-CoA desaturase 1
413 ADE1: phosphoribosylamino-imidazole-succinocarbozamide synthetase
414 ADE4: phosphoribosylpyrophosphate amidotransferase
415 ADE5: 5'-phosphoribosylformyl glycineamide synthetase
416 ADE6: formylglycinamidine-ribonucleotide (FGAM)-synthetase
417 CDC19: pyruvate kinase
418 CIT1,2: citrate synthase
419 ENO1, 2: Enolase 1, 2
420 FAA1,4: fatty acyl-CoA synthetase
421 FBA1: fructose-1,6-bisphosphate aldolase
422 FBP1: fructose-1,6-bisphosphatase
423 FOX1: acyl-CoA oxidase
424 FUM1: fumarase

425 GLK1: glucose-specific glucokinase
426 GND1,2: 6-phosphogluconate dehydrogenase
427 GPM1: phosphoglycerate mutase 1
428 GUA1: guanine monophosphate synthase
429 GUK1: guanylate kinase
430 HXK1: hexokinase
431 ICL1: isocitrate lyase
432 IDH1: NAD⁺-dependent isocitrate dehydrogenase
433 IMD3,4: inosine monophosphate dehydrogenase
434 LSC1,2: succinyl-CoA ligase
435 MDH3: malate dehydrogenase
436 MLS1: malate synthase
437 PCK1: phosphoenolpyruvate carboxykinase
438 PFK1: phosphofructokinase
439 PGI1: phosphoglucoisomerase
440 PGK1: 3- phosphoglycerate kinase
441 POT1: 3-ketoacyl-CoA thiolase
442 POX1: fatty acyl-CoA oxidase
443 PRS1-5: 5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase
444 RIB1: GTP cyclohydrolase I
445 RIB2: deaminase

446 RIB3: 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) synthase,

447 RIB4: 6,7-dimethyl-8-ribityllumazine (lumazine) synthase

448 RIB5: riboflavin synthase

449 RIB7: reductase

450 RKI1: ribose-5-phosphate ketol-isomerase

451 SDH1,2: succinate dehydrogenase

452 SOL1-4: 6-phosphogluconolactonase

453 SPS19: 2,4-dienoyl-CoA reductase

454 TDH3: glyceraldehyde-6-P-dehydrogenase

455 YND1: yeast nucleoside diphosphatase

456 ZWF1: glucose-6-phosphate dehydrogenase

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Figure Legends

Fig. 1 Putative metabolic pathway of riboflavin synthesis in *A. gossypii*.

Fig. 2 Overall procedures for disparity mutagenesis used in the isolation of high riboflavin producing mutants of *Ashbya gossypii*.

Fig. 3 Stability of riboflavin production by the W122032 mutant on a flask scale.

Glycerol stock was used in the culture and after culture a new glycerol stock was prepared, then repeated 14 times.

Fig. 4 Dissolved oxygen (DO) and pH (A), and riboflavin production (B) in the cultures of W122032 mutant and wild type strains using a 3-L bioreactor. Symbols in (A) Time course of DO and pH in the culture of the wild type and W122032 strains: plus, DO of wild type strain; minus, DO of W122032 strain; dotted line, pH of wild type strain; straight line, pH of W122032 strain. MT and WT denote W122032 mutant and wild strains, respectively (B) Riboflavin production in the culture of the wild type and W122032 strains: Symbols: wild type strain in conventional medium (open circles); the W122032 strain grown in conventional medium (closed circles); the W122032 strain grown in optimized medium (open squares); the W122032 strain grown in optimized medium after preservation for 3 months (closed triangles).

Fig. 5 DNA microarray analysis of the W122032 mutant. Gene expression levels are shown in color. Red letters indicate more than three-fold increased expression compared with the wild type strain; pink letters, more than two-fold and less three-fold increased

expression; orange letters, less than two-fold increased expression. Dark blue letters indicate more than three-fold decreased expression to that of wild type strain; purple letters, more than two-fold and less than three-fold decreased expression; light blue letters, less than two-fold decreased expression.

Fig. 6 Time courses of the expression of each gene based on DNA microarray analysis of W122032 mutant. Blue, orange and purple bars denote logarithmic growth phase, riboflavin production and stationary phases, respectively. Logarithmic growth phase of WT and MT was 8 and 16 h, respectively; riboflavin production phase of WT and MT, 16 and 28 h; stationary phase of WT and MT, 36 and 56 h. Red dotted lines indicate expression levels of wild type strain. (A) β -Oxidation, glyoxylate cycle, pentose phosphate and purine biosynthetic pathways are highlighted using dotted lines. (B) Gluconeogenesis, TCA cycle, riboflavin biosynthetic pathways are highlighted using dotted lines.

[illegible]

Supplementary file 1. Plasmid map of YCpG418/*pold*^{exo-}

A 12.3 kb YCpG418/*pold*^{exo-} vector was used for transformation. This vector was constructed from YCplac111 vector (6.1 kb) which has a low copy numbers vector, with deletion of LEU2 molecule (between *AatII*-*EcoRV* sites, 1.2 kb); and insertion of G418 cassette (2.5 kb) in MCS fragment at *BamHI* site. AshbyaPOL3 gene (4.9 kb) fragment was inserted at *XbaI* site of MCS .