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Microbial Production of Riboflavin Using Riboflavin Overproducers, *Ashbya gossypii*, *Bacillus subtilis*, and *Candida famate*: An Overview

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Abstract In this paper, the microbial production of riboflavin is reviewed and includes descriptions of riboflavin overproducers, and the biosynthesis and details of the key-enzyme genes related to riboflavin. Three kinds of riboflavin overproducers are known; *Bacillus subtilis* and *Candida famate* utilize glucose as a carbon source, but the fungus *Ashbya gossypii* requires plant oil as its sole carbon source. The starting material in riboflavin biosynthesis is guanosine triphosphate (GTP), which is converted to riboflavin through six enzymatic reactions. Though *Bacillus subtilis, Candida famate*, and *Ashbya gossypii* operate via different pathways until GTP, they follow the same pathway from GTP to riboflavin. From the metabolic viewpoint, with respect to improved riboflavin production, the supplementation of GTP, a process-limiting precursor must be considered. The GTP fluxes originate from three sources, serine, threonine and glyoxylate cycles. The development of pathways to strengthen GTP supplementation using biotechnological techniques remains an issue for future research.

Keywords: riboflavin, *Ashbya gossypii*, vitamin B_2, metabolic engineering

INTRODUCTION

Microorganisms produce valuable polymers, such as proteins, nucleic acids, and polysaccharides as well as many smaller molecules useful in human and animal health [1]. The vitamins are an example, and are classified as chemical substances that control and affect the physiological processes, and which are essential for the metabolism and growth of animals, especially mammals. However, these compounds cannot be biosynthesized by mammalian cells, which have lost the capacity to synthesize vitamins, whereas lower organisms retain this ability. Therefore, vitamins are synthesized by a variety of plants and microorganisms, which are nutritional requirements for human and animals. Although many studies have been undertaken in the field of vitamin biosynthesis, progress has been slow because organisms require such small quantities of these compounds.

Riboflavin, the so called vitamin B_2, is widely distributed in plants and animals, and plays an important role in organisms because it is the precursor of flavin mononucleotide (riboflavin 5′-monophosphate, FMN) and flavin adenine dinucleotide (FAD), which function as coenzymes for a wide variety of enzymes in the intermediate metabolism. Therefore, a daily dose of 2 mg of riboflavin is essential for humans. Furthermore the vitamin is useful as an animal feed supplement. Riboflavin is primarily used in the pharmaceutical industry, though some are used in the food industry as an additive, and in the animal feed industry. Thus, vitamins have become one of the important fermentation products in biotechnology industry.

The world consumption of riboflavin was reported to be 1.25×10^6 kg per year, about 30 years ago, again largely for human and animal uses [2]. However, at present, the amount of riboflavin produced is believed to excess 3,000 tons per year. Riboflavin is commercially produced by chemical or by biochemical synthesis, the latter being the preferred route because it is cheaper [3]. As a result, current riboflavin production by fermentation is estimated to be about 2,500 tons per year. This process has the merits of saving half the cost, reducing waste and energy requirements, and using renewable resources like sugar or plant oil [4].

Recently, with the progress of metabolic engineering, many researchers turn their attentions to biological metabolisms to improve the production efficiency of process that produce bioproducts from microorganisms. Riboflavin as an essential compound for humans and animals, and is one of target biotechnical processes, though a vast amount of knowledge has already been accumulated. In this review, we describe and compare riboflavin overproducers, its biochemical and metabolic biosynthesis, and the genes related to key enzymes in riboflavin biosynthesis.

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DEVELOPMENT OF MICROORGANISMS

Riboflavin is a unique vitamin that can be synthesized in large enough amounts by some fungi and bacteria to be exploited successfully on an industrial scale. Many microorganisms have been screened and studied for industrial production. *Clostridium acetobutylicum* was one of the earliest organisms used to produce riboflavin, and its productivity was only 100 mg/L [5]. Some organisms of the genus *Candida* [6-12] were also found to produce riboflavin, and among these, *Candida flareri* achieved about 600 mg/L of riboflavin [13]. But in order to produce higher levels of riboflavin production using these yeasts, inhibition due to iron should be overcome [6]. The biochemical key to riboflavin overproduction appears to involve resistance to the repressive effects of iron. In the biochemical synthesis of riboflavin of normal microorganisms, it appears that iron represses almost all of the riboflavin biosynthetic enzymes, whereas riboflavin or a derivative inhibits the first enzyme of the pathway, GTP cyclohydrolase II. New processes to overcome iron inhibitions and novel mutation techniques [4] that inhibit purine biosynthesis using antimetabolite, tuberculin, in *Candida famate* have recently been developed and give riboflavin yields of 20 to 30 g/L [1,14]. On the other hand, unlike the yeast. Bacteria, iron inhibition is not a factor for the two yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which are natural overproducers of riboflavin. The productivity of these ascomycetes presently is believed to exceed 20 g/L [1]. Both are pathogenic on cotton and form needle-shaped ascospores.

Eremothecium ashbyii

The overproduction of riboflavin by this strain was discovered by Guilliermond and co-workers [15] in 1935. Hence, because it is an overproducer of riboflavin, much work has been undertaken up on E. ashbyii over decades. In the early 1950s, media that supported riboflavin overproduction at 200 mg/L were developed [16-18]. Further addition of Tween 80 and certain proteins, casein or glycine to this allowed yields to reach 1.4 g/L [18]. Currently, yields of 1.5 to 2.5 g/L [5, 19] are possible when very crude sources are used as media.

To increase productivity, 8-azaguanine, an analog of guanine, which is a precursor of the riboflavin biosynthetic pathway, was used. Mutants were obtained to get this compound. The level of vitamin production obtained from the majority of mutants in a synthetic medium containing peptone was 2-4 times higher than the level of the original strain [20].

Recent studies with wild type of *E. ashbyii* have yielded 3.3 g/L of riboflavin using molasses and peanut seed cake as carbon and nitrogen source, respectively [21]. Some studies with yeast-like strains have been undertaken from the viewpoint of morphological observation. The ideal colony for riboflavin production was characterized by its medium growth intensity (colony size, 8-10 mm in diameter over a culturing period of 96 h), intense yellow color, absence of sector formation, and a smooth surface covered by air mycelia [22]. Production of riboflavin using a colony obtained on the basis of these observations reached 1.1 g/L from a starting position of 0.2 g/L, and these results were confirmed by subsequent experiments [23]. The capacity for riboflavin biosynthesis by *E. ashbyii* was characterized by the considerable heterogeneity of the population. This heterogeneity was expressed in a high frequency of splitting off of lemon colony, which had a vitamin production level three times lower than that of the orange colonies. A population of a culture of *E. ashbyii* showed high morphological instability, as well as considerable heterogeneity with respect to its ability to synthesize riboflavin [23]. The relationship between the pH value of the medium and morphological variations was also observed. Highest riboflavin productivity was reported at initial pH of 6.5, using *E. ashbyii* [24,25]. At this constant pH value, morphological variation was shown to involve the gradual increase of swollen hyphal cells and the formation of ascospores [26]. However, obtained at various pH values showed that the highest riboflavin productivity was obtained at pH value of 4.5 to 5.5 and morphological examination revealed that asci and ascospores outnumbered the other forms [26].

Ashbya gossypii

Another riboflavin overproducer, *A. gossypii* was known to yield more riboflavin than *E. ashbyii* [27]. However, with similar constructs and equivalent yield, *E. ashbyii* showed neither nuclear fusion nor meiosis [28]. On the contrary, *E. ashbyii* showed instable in storage, for example during lyphophilization or storage in slant at room temperature [29].

The ability of *A. gossypii* to overproduce riboflavin was discovered in 1946 [30]. The characteristics and fermentation of this strain are very similar to those of *E. ashbyii*. After the discovery of this yeast-like, filamentous fungus, the industrial producer of riboflavin has moved to *A. gossypii*, which has replaced *E. ashbyii* [31]. The first application of *A. gossypii* to industrial scale production was made in 1968 [2]. Since the first quantitive report [30] numerous studies have been carried out to overproduce riboflavin. In early studies, some defined media were established using Tween 80 and purine, and 145 mg/L and later 370 mg/L of riboflavin were produced in use of these media [32,33]. Many media were then developed [34] which contained glucose, vitamins, and amino acids, and mineral salts. In these markedly improved media, the production of riboflavin reached 1 g/L. It was found that glycine was an important component and Tween 80 served to prevent mycelium lysis and stimulator for riboflavin overproduction. Apart from these media, more complex media for industrial application were studied, and using soybean oil as a carbon source and collagenous protein and CSL as nitrogen sources, yields of 5 g/L were obtained [35,36]. The same amount of riboflavin was produced using other newly developed media, which included 3% bone

fat and 2% soybean oil as carbon sources, supplemented with additional soybean oil after 72 h in a 120 h cultivation [37]. Peptone and gelatin were investigated as complex nitrogen sources [35,38], and some waste proteins of animal origin, such as skin and bone glues were also tested [39]. Skin and bone glues contain several important amino acids for riboflavin production, such as glycine (about 20%) and threonine. These amino acids were found strongly stimulate riboflavin synthesis in *A. gossypii* [34,38,40], and in medium containing 3% skin glue as a nitrogen source, and 4% gelatin lard as a carbon source, to which 2.5% gelatin lard was added after 72 and 120 h of cultivation, the amount of riboflavin produced was higher than 5.5 g/L [39].

Microscopical characteristics, such as hyphae, asci, spore and autolysis was found to be varied in *A. gossypii* with various additional components and time courses [41]. Granula and hyaline droplets were described in the other study [42]. In 1990, *A. gossypii* was selected as a subject for fractal geometry, which at that time was being introduced to microbiology [43]. It was the first example of fractal aggregates in biological systems, with a cell as the smallest aggregating unit and the colony as an aggregate. Fractal dimensions increased during growth and demonstrated that the technique might serve as an important way of characterizing the morphology. The experimental results showed that fractal geometry provides a suitable method of describing the biological growth pattern of the *A. gossypii*. On the other hand, with morphological observations made using Nile red staining, found microdroplets in the hyphae of *A. gossypii* [44]. These lipid bodies accumulated neutral lipid as an energy reserve, and the fact was confirmed by separate experiments as follows; when soybean oil was used, the neutral lipid reached about 22% of the mycelium dry weight, while when the mycelium was grown on glucose it produced only 12% triacylglycerol, and these amounts decreased to 3–4% when the glucose declined in the medium. Although many fungi have been found to produce neutral lipid and utilize it, especially during spore germination [45], *A. gossypii* accumulated extraordinarily high amounts of neutral fatty acids. These results showed that the fat metabolism of *A. gossypii* was more efficient than that of using other fungi [44]. The yield enhancing effect of plant oil as a substrate in riboflavin production using *A. gossypii* had been shown earlier [2,25,36].

In terms of using plant oil as the sole carbon source in vitamin formation, the glyoxylate cycle is indispensable because the fatty acids can be degraded by isocitrate lyase, the key enzyme of this anaplerotic pathway, and isocitrate lyase from *A. gossypii* was firstly isolated and characterized [46]. During the production of riboflavin using *A. gossypii*, it was interesting that this enzyme appeared of the start of the riboflavin accumulation [47]. This implies that riboflavin synthesis might begin from storage lipids or this was observed with Nile red, to depended upon isocitrate lyase, which catalyzes the cleavage of isocitrate to glyoxylate and succinate. Itaconate as antimetabolite inhibits this pathway and eventually riboflavin production. For this reason itaconate–resistant mutant grown on soybean oil had a significantly higher isocitrate lyase specific activity, which was accompanied by a large overproduction of riboflavin compared to the wild type strain [47]. Itaconate–resistant mutants were achieved easily using the characteristics of riboflavin. Because riboflavin is yellow and this can be used on agar plates to search for improved riboflavin overproducers, and this method led to *A. gossypii* to riboflavin overproducers being used for industrial applications [4]. When plant oils were used as a carbon source in riboflavin biosynthesis, lipase also becomes important, because the plant oils are degraded to fatty acids and glycerol by an extracellular lipase before their uptake into intracellular organelles. The fungal lipase of *A. gossypii* was hardly detected, because the activity was deactivated by interfacial characteristics [48]. Meanwhile, researches on osmoadaptation mechanisms [49] and vacuole of *A. gossypii* have been much studied [50].

Comparison of the Riboflavin Productivities of Several Microorganisms

In the field of microbial fermentation, there are several riboflavin-producing microorganisms, as shown in Table 1. Among these, the Gram-positive bacteria *Bacillus subtilis*, the yeast *Candida famate*, and the filamentous fungus *Ashbya gossypii* are mainly used for the biosynthesis of riboflavin via large-scale production [4]. *Candida* sp. is a natural overproducer of riboflavin, but these yeasts have to overcome iron inhibition in order to overbiosynthesize riboflavin [31]. *Candida famate* with tubercidine-, and iron-resistance has been developed to the level of plant operation, and it has been recently reported that production of riboflavin reaches to 20–30 g/L using this yeast [1,14]. Like *Candida* sp., bacteria are also inhibited by iron [31]. Among these *Bacillus subtilis* is mainly used to study riboflavin production, especially in terms of biosynthesis [51]. And a recombinant, riboflavin-producing strain of *Bacillus subtilis* was reported to allow productivities of 80 mg/L at 0.3 h^{-1} using a glucose-limited chemostat [52]. To obtain riboflavin production using this Gram-positive bacterium requires at least the deregulation of purine synthesis and a mutation in a flavokinase/FAD-synthase. Currently recombinant *B. subtilis* with these features is in use in large-scale fermentations and producing concentrations exceeding 15 g/L [53].

A. gossypii as a strong overproducer and has been reported to give product yields in the range of 15–20 g/L [1,27]. About 30% of the world's industrial riboflavin output is produced by direct fermentation with *A. gossypii* [27], which has substituted for *E. ashbyii*, although its use in industry has been limited to date [21].

In addition, some experiments have been performed using a minor producer of riboflavin. *Candida guilliermondii*, which produced small amounts (220 mg/L) of riboflavin from liquid brewery waste [54] and *Mycobacterium pheli* also produced small quantities from

Table 1. Summary of the riboflavin productivities of several strains

	Strains	Carbon source	Nitrogen source	Supplements	Max. riboflavin concentration (g/L)	Culture time	References
Bacteria	*Clostridium acetobutylicum*	–	–	–	0.1	–	5
	Bacillus subtilis	Glucose	–	–	0.08	0.3 h	52
Yeast	*Candida flareri*	Glucose, fructose	Urea, glycin, serin, threonine	Guanine, xanthine	0.6	–	13
	Candida guilliermondii	Liquid brewery waste	–	biotin	0.2	3 day	54
	Mycobacterium pheli	Beet molasses	Peptone	–	0.1	6 day	55
Fungi	*Aspergillus terreus*	Beet molasses	Peptone	Asparagine	1.0	16 day	58
	Eremothecium ashbyii	Glucose, fructose, sucrose	Asparagine, serine, threonine	Biotin, thiamine, inositol	0.2	–	16, 17
		Glucose	Caseine, glycine	Tween 80, lecithine	1.4	–	18
		Glucose, sucrose	Soybean meal	–	2.0	–	20
		Glucose, molasses	CSL, dry yeast, meat extract	–	1.0-2.0	4 day	22, 23
		Molasses	Peasnut seed cake	–	3.3	7 day	21
	Ashbya gossypii	Glucose	Asparagine, glycine, tyrosine	Tween 80, hypo-zanthine, biotin, thiamine, nositol	0.4-1.0	–	33, 34
		Corn oil, soybean oil	CSL, yeast extract, collagenous Proteins	–	5.0	–	35, 36
		Soybean oil, bone fat	CSL, gelatin	Glycine	5.0	8 day	37
		Soybean oil	CSL, gelatin, skin glue	Glycine	5.5	8 day	39

beet molasses [55]. A filamentous fungus, *Aspergillus terreus* was isolated from crude oil [56,57] and produced 1 g/L of riboflavin from beet molasses [58].

RIBOFLAVIN BIOSYNTHESIS

There are three kinds of riboflavin overproducers, yeast, gram-positive bacteria, and fungus, the metabolic fluxes of which are shown in Fig. 1. *Candida famata* has a simple pathway from glucose to guanosine triphosphate (GTP), which is the entrance of riboflavin biosynthesis. The *Candida* uses glucose as its carbon source and converts this to ribulose-5-phosphate (Ribu-5P), which is a precursor of L-3, 4-dihydroxy-2-butanone-4-phosphate (DBP). 3-Phosphoglycerate, an intermediate of glycolysis, is also used for the synthesis of glycine that is the precursor of GTP.

Bacilli cells follow a different pathway from yeast, and convert glucose to guanosine monophosphate (GMP), which is also a precursor of GTP.

The most popular riboflavin overproducers are fungi, *A. gossypii* or *E. ashbyii*. Of these two strains, *Ashbya-gossypii* specifically uses plant oil, which is obtained by decomposing fatty acids using extracellular lipase, and the resulting fatty acids enter to the peroxisomes. Biosynthesis begins from acetyl-CoA and forms malate, the precursor of GTP through several enzymatic reactions. On the other hand, citrate an intermediate of the TCA cycle in mitochondria is converted to isocitrate in peroxisomes. Isocitrate is partly converted to glyoxylate, and finally converted to malate.

The resulting metabolites from carbon sources in several riboflavin overproducers enter the riboflavin biosynthetic pathway. Biosynthesis starts from GTP and the final product is riboflavin; the precise fluxes are described as follows.

Guanosine-triphosphate (GTP), as a Precursor of Riboflavin

Fig. 1 shows a proposed model of metabolic flux of riboflavin production in *Ashbya gossypii*. There are many steps from substrate to riboflavin, and the metabolic flux to riboflavin can be separated into specific

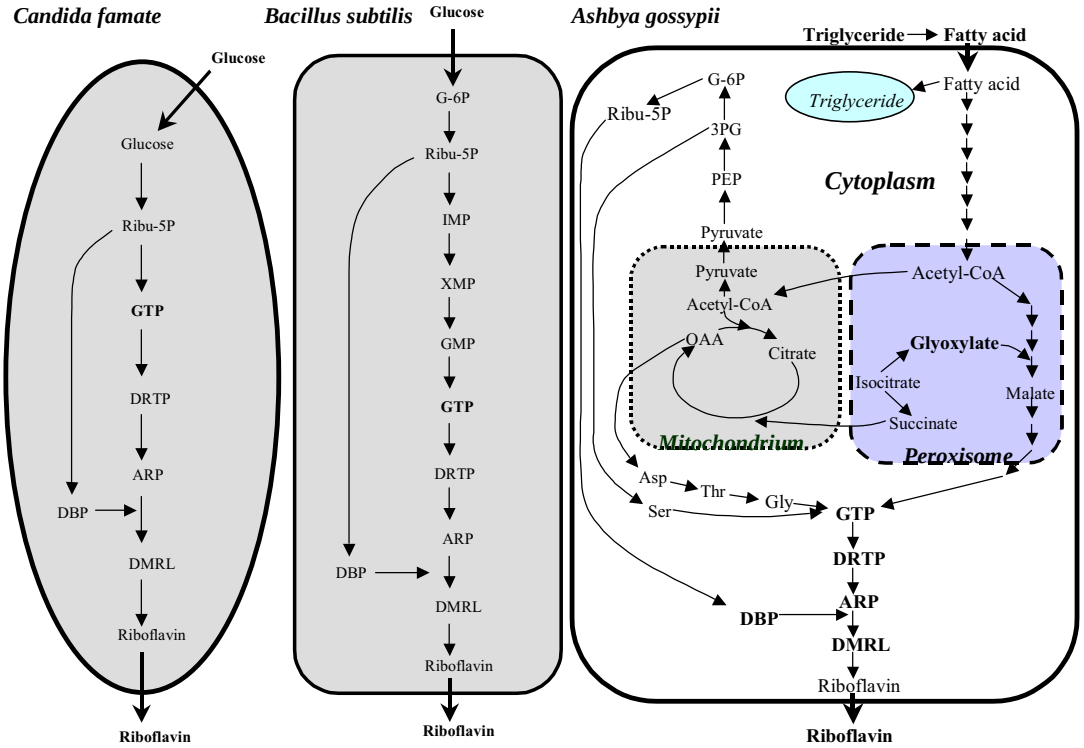

Fig. 1. Proposed model for the metabolic flux for riboflavin production by *Ashbya gossypii*, *Bacillus subtilis*, and *Candida famate*. Abbreviations: G-6P, Glucose-6-phosphate; 3PG, 3-phosphoglycerate; PEP, Phosphoenolpyruvate; Ribu-5P, Ribulose-5-phosphate; OAA, Oxaloactate; Asp, Aspatate; Thr, Thre- onine; Gly, Glycine; Ser, Serine; GTP, Guanosine triphos-phate; GMP, Guanosine monophosphate; XMP, Xanthine monophosphate; IMP, Inosine monophosphate; DRTP, 2, 5-diamino-6-ribosylamino-4 (3H)-pyrimidinedione 5-phosphate; ARP, 5-amino-6-ribitylamino-2, 4 (1H, 3H)-pyrimidine; DBP, L-3, 4-dihydroxy-2-butanone-4-phosphate; DMRL, 6, 7-dimethyl-8-ribityllumazine.

and non-specific reactions. As shown in Fig. 1, most of the metabolites concentrate upon GTP, which is the first precursor of riboflavin. From this compound, six specific reaction steps are required to produce riboflavin. GTP as a purine, which was built de novo from amino acids, tetrahydrofolate derivatives, and CO_2 are well known to stimulate riboflavin overproduction in many organisms.

The sugar moiety is from ribose-5-phosphate from the pentose phosphate pathway. It has been shown that the 4 carbon, 5 carbon, and 7 nitrogen atoms of the purine ring originate from glycine [59]. Therefore, glycine stimulates riboflavin overproduction as a precursor of purine. Then results were obtained with *Candida* sp. [7,9,12] and *A. gossypii* [36]. Glycine in the riboflavin biosynthesis was not associated to cell growth, but only product formation [34], which is thought to be a limiting precursor in riboflavin overproduction.

In early work with *S. cerevisiae*, two pathways of glycine biosynthesis were described as follows [60]; one is a glycolytic pathway starting from the glycolytic intermediate, 3-phosphoglycerate on a glucose medium. The other is a gluconeogenetic pathway starting from glyoxylate, a product of the anaplerotic glyoxylate cycle, on ethanol or acetate medium. Recent work [61] identified an additional glycine biosynthetic pathway, which starts from threonine and act as a major source of glycine in *S. cerevisiae* (Fig. 2).

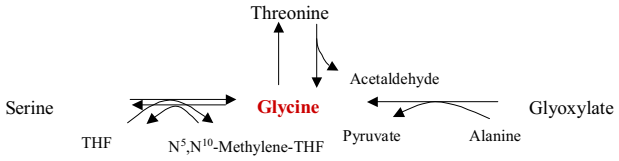

Fig. 2. Glycine biosynthesis in *S. cerevisiae.*

Unlike *Candida* and *Ashbya*, *E. ashbyii* was not stimulated by glycine. Instead, serine and threonine served the same function [62]. It was elucidated that the two compounds are converted to riboflavin through glycine intracellularly [63, 64].

Purine, which is incorporated by glycine, stimulates riboflavin overproduction. Among this xanthine had been postulated for some times to be an intermediate precursor because of its structural similarities. But in the experiments with guanine auxotroph (lacking XMP aminase) *Aerobacter aerogenes* [65,66], *Candida guilliermondii* [67], and *Corynebacterium* sp. [68], it was

proven that the main precursor was a guanine or guanine nucleotide and the conversion of adenine, hypoxanthine and xanthine to riboflavin passed through a guanine or guanine nucleotide.

As research progressed, it was proposed that the guanine precursor is a nucleotide or nucleoside and the first definitive evidence of this was obtained with a mutant of *Samonella typhimurium* [69]. Such results were also obtained with *E. ashbyii* [70-72] and *B. subtilis* [73]. Much work showed that GTP (**I**) is a real immediate precursor of riboflavin. The most conclusive evidence was found in *E. coli* [74], *B. subtilis* [75], the yeasts *Pichia guilliermondii* (*Candida guilliermondii*) [76], and *Torulopsis candida* (*Candida flareri*) [77].

Which Compound Follows GTP?

During biosynthesis, the 8-carbon atom is eliminated as a single carbon unit. This result was obtained with *E. ashbyii* [78]. In the early studies, the compound following GTP was proposed to be 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine, because this compound was accumulated in a riboflavin auxotroph of *S. cerevisiae* [79]. Through intensive researches it has recently been shown that this group is derived from the ribose moiety of GTP, which is incorporated without degradation into the ribityl side chain. These results were obtained with *Salmonella typhimurium* [69], *E. ashbyii* [72,80,81], *Pichia guilliermondii* [82, 83], and *B. subtilis* [73]. As mentioned above, the compound following GTP was suggested to be 2,5-diamino-6-hydroxy-4-ribitylamino-pyrimidine [79]. This compound was established to be 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinedione 5′-phosphate (DRTP) (**II**) in Fig. 3 [84] through 2,5-diamino-6-hydroxy-4-ribosylaminopyrimidine 5′-phos-phate [85].

Two Different Steps to the Next Compound, 5-Amino-6-ribitylamino-2, 4 (1H, 3H)-pyrimidine (ARP)

In the early studies, the next compound was iden-tified using auxotrophs of *Aspergillus nidulans* [86] and *Saccharomyces cerevisiae* [87] as 5-amino-2,6-dihydroxy-4-ribitylaminopyrimidine. Another research group suggested that the compound was 5-amino-2,6-dioxy-4-ribitylaminopyrimidine as the product was accumulated in resting cells of the *E. ashbyii* [88] and a riboflavin-deficient mutant of *B. subtilis* [89]. These compounds are at present known to as ARP (**III**) [84]. Between DRTP (**II**) to ARP (**III**), there are two steps including a deamination and a reduction as shown in Fig. 4. Reduction precedes deamination in one pathway and deamination occurs before reduction in the other pathway. First, reduction before deamination was found in a mutant *S. cerevisiae* [90]. Thereafter the same result was shown in *C. guilliermondii* [82, 84] and *A. gossypii* [91]. The second mechanism involving reduction after deamination shown to operate in *E. coli* [92] and *B. subtilis* [93].

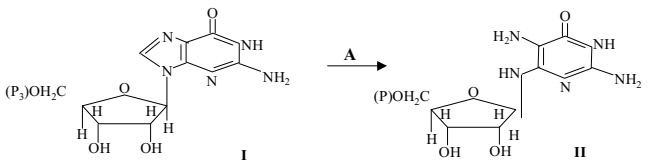

Fig. 3. Biosynthesis from GTP (**I**) to DRTP (**II**). **A** denotes GTP cyclohydrolase **II**.

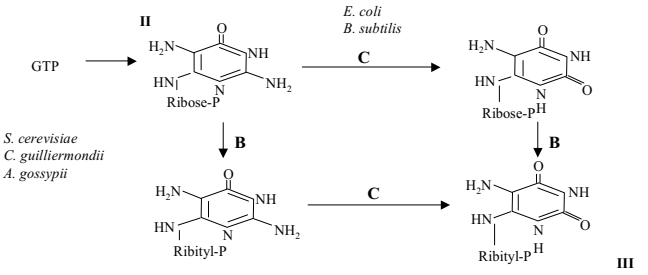

Fig. 4. Two pathways from DRTP (**II**) to ARP (**III**). **B** and **C** denote reductase and deaminase, respectively.

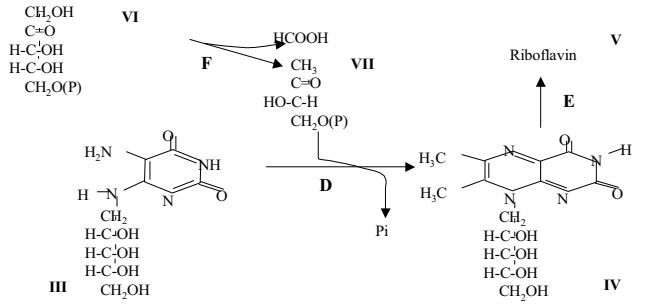

Fig. 5. The origin of the four-carbon moiety as a precursor of DMRL (**IV**). **III** and **IV** denote ARP and DMRL, respectively. DBP (**VII**) is synthesized by DBP synthase (**F**) from D-Ribu-5P (**VI**), one of glycolytic intermediates. **D** and **E** denote DMRL synthase and riboflavin synthase, respectively.

Additional Four Carbon Moiety as a Precursor of Riboflavin

In riboflavin biosynthesis, the pathway from ARP (**III**) to 6,7-dimethyl-8-ribityllumazine (DMRL) (**IV**) in Fig. 5 was the most recently elucidated because of the origin of the four carbon moiety. Significant work was performed on the origin of four carbons. The likely origin of the four carbons was ARP (**III**). Even though much investigating work was performed, the origin of the 4-carbon moiety remained a mystery for a long time. At one time, it was seriously considered that acetoin might be the source of the four carbons from work upon *E. ashbyii* [94,95], and *C. flareri* [96]. But neither acetoin nor acetate is found to be precursors of the dimethylbenzene unit of riboflavin in *E. ashbyii* by labeling experiments [97]. Subsequently its oxidized form, diacetyl was considered because it was known to

condense nonenzymatically with ARP (**III)** to form DMRL (**IV**) [95]. This hypothesis was supported by other experiments with ethanol [98]. Feeding ethanol during the fermentation of *A. gossypii* showed effective stimulation of riboflavin formation. Ethanol was not considered to be directly utilized to form riboflavin, but to be metabolized to form diacetyl through acetaldehyde, pyruvate, and acetoin. Therefore, it was believed that ethanol might play a significant role in the origin of the four carbon unit, but ethanol is currently believed to be precursor of acetyl-CoA that proceeds to isocitrate through the gluconeogenetic pathway. In addition, the incorporation of labeled diacetyl into riboflavin was not sufficient proof of its role as a natural precursor [99]. The stimulation of riboflavin production by adding diacetyl can be explained by another reason, which is that it acts like ethanol rather than as a precursor [100].

On the other hand, glucose and ribose were suggested to be the true donors of the 4-carbon moiety [101], because they incorporated more efficiently than either acetate or pyruvate. Some of the data obtained from measurements of the incorporation of various radioactive compounds were consistent with the view that a pentose compound was the source of the four-carbon unit [99]. Tetraose, formed via the pentose phosphate pathway, was also reported to serve as the precursor of the four-carbon biosynthetic unit [102]. Through various experiments, considerable progress on the origin of the four-carbon moiety was made in studies using *A. gossypii* [103]. It was shown in *in vivo* studies with a variety of ^{13}C-labeled precursors that the C-6α, C-6, C-7, and C-7α of DMRL are biosynthetically equivalent to C-1, C-2, C-3, and C-5 of the pentose phosphate pool. C-4 of the pentose phosphates has no equivalent in lumazine. It was shown that this carbon atom is eliminated by an intramolecular sketal rearrangement [104]. Further studies revealed the same results with *C. guilliermondii* [105] and *B. subtilis* [106]. Several pentose and pentulose phosphates could serve as the second substrate in the reaction to form lumazine. That is, the formation of the four-carbon moiety was deter- mined by the loss of the internal carbon atom 4 from a pentose precursor.

On the other hand, the enzyme that catalyzed the formation of DBP (**VII**) from D-Ribu-5P (**VI**) was reported [107] from *C. guilliermondii*. DBP could yield the lumazine with ARP (**III**). Therefore, this compound was thought to be an obligatory intermediate of riboflavin biosynthesis (Fig. 5).

Conversion of DMRL to Riboflavin

As the nearest precursor of riboflavin (**V**),DMRL (**IV**) in Fig. 5 was identified during the early research period [96,108]. Our understanding of the transformation step between DMRL and riboflavin was elucidated by experiments with *A. gossypii* and *E. ashbyii* [109]. It be came apparent that two moles of DMRL react to give one mole of riboflavin and one mole of ARP (**III**). The

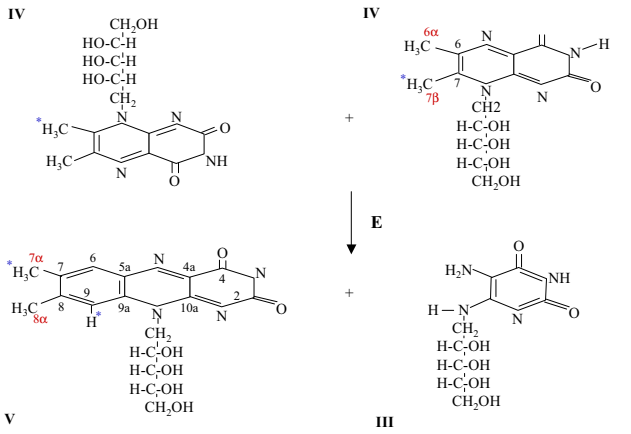

Fig. 6. Bioconversion of DMRL (**IV**) to riboflavin (**V**), which is catalyzed by riboflavin synthase (**E**).

regiospecificity of the reaction have been elucidated by experiments with [7-$^{2}H_{3}$] DMRL as substrate [110]. Riboflavin formed from labeled substrate contained deuterium in positions 9 and 7α. Thus, the two four carbon moieties ultimately form the xylene ring (Fig. 6). This regiospecificity was later confirmed by experiments with ^{13}C-labeled substrates [111] and deuterium-based experimentation [112]. The other product of the reaction, ARP (**III**), was suggested to be reused to form another molecule of DMRL (**IV**) [113], but no firm evidence exists for enzymatic reutilization except for the results obtained with *E. ashbyii* [114], which showed that the ARP (**III**) was reused, at least in part. At present, it has been established that the by-product, ARP, can enter the biosynthesis of riboflavin (**V**) as a precursor because the by-product is also the same biosynthetically and structurally to produced compound from DRTP (**II**).

As mentioned above, the starting materials of riboflavin biosynthesis are GTP (**I**) and Ribu-5P (**VI**), and there are many intermediates on the pathway. On the other hand, several enzymes are concerned in these reactions, and these are described in the following section.

Enzymes Related to Riboflavin Biosynthesis and Regulations of GTP Cyclohydrolase II

On the riboflavin biosynthetic pathway, the starting material, GTP (**I**) is catalyzed by GTP cyclohydrolase II and forms formate and DRTP (**II**) as shown in Fig. 3. The enzyme was found and reported initially a few experiment with *E. coli* [74] and was suggested to be GTP cyclohydrolase II because it differed from a previously studied *E. coli* enzyme known to be involved in folic acid biosynthesis. GTP cyclohydrolase II catalyzes the initial and rate-limiting step of riboflavin biosynthesis and is subject to multiple metabolic controls. Much work has been performed upon the properties of GTP

cyclohydrolase II of *B. subtilis* [115,116] and *E. coli* [117]. In particular, enzyme of *B. subtilis* was a bifunctional enzyme that could also catalyze the formation of 3,4-dihydroxy-2-butanone 4-phosphate from ribulose 5-phosphate [118], and the GTP cyclohydrolase II of *E. coli* and *B. subtilis* was specified by the gene *ribA*. Recently, the gene *RIB1* in *S. cerevisiae*, which encodes GTP cyclohydrolase, was cloned by functional complementation of the corresponding mutation [119,120]. Contemporaneously the structure gene *RIB1* encoding GTP cyclohydrolase II of *Pichia guillermondii* has been cloned by functional complementation of a *ribA* mutation in *E. coli* [121]. The deduced protein sequence obtained showed significant homologies to GTP cyclohydrolase from *S. cerevisiae* and its C-terminal half homologies to the enzyme of *E. coli* and *B. subtilis* [122].

Reductase and Deaminase

The existence of two enzymes involved in the biosynthesis of riboflavin was reported in *E. coli* [92]. These two enzymes catalyzed the conversion reaction of the product by GTP cyclohydrolase II and DRTP (II), to ARP (III). There are two enzymatical steps between the two compounds. First, deamination at carbon 2 of the ring by deaminase was by-passed and instead the ribosylamino group was reduced to a ribitylamino group by reductase. These results are in general agreement with those of *S. cerevisiae* [123]. The major difference is that in yeast, reduction precedes deamination, whereas in *E. coli* deamination occurs first, as shown in Fig. 4. Reductase activity was also shown in the filamentous fungus, *A. gossypii* [91] and in the yeast, *C. guilliermondii* [84].

In the meanwhile, reductase and deaminase in yeast, *S. cerevisiae* [124], is specified by the genes *RIB7* and *RIB2*, respectively, and in other yeast, *Pichia guillermondii* (*Candida guilliermondii*) [122], by *RIB2* and *RIB3*, respectively, but the yeast enzymes have not been studied in detail. On the contrary, the complete sequence of the *B. subtilis* [53,115] and *E. coli* [125] riboflavin operon has been determined. In *B. subtilis*, it is known that the gene *ribG* encodes deaminase and that the gene *ribT* encodes reductase [126], but further study concluded that the *ribG* gene could code for a bifunctional deaminase and reductase [127], which was arrived at by comparing the homology between the 3' end of the *ribG* gene of *B. subtilis* and the *RIB7* gene of *S. cerevisiae* coding for reductase. An *E. coli* gene with sequence homology to the *B. subtilis ribG* gene has also been identified as *ribD* [127,128]. In common with *ribG* of *B. subtilis*, *ribD* of *E. coli* codes for a bifunctional deaminase and reductase in regular order.

DMRL Synthase or the β-Subunit of Riboflavin Synthase

Lumazine, DMRL (**IV**) is biosynthesized from ARP (**III**) and DBP (**VII**) by the enzyme lumazine synthase, as shown in Fig. 5. The gene coding for lumazine synthase in *S. cerevisiae* has been cloned as *RIB4* [129]. Gene *RIB4* is a single copy gene located on the left arm of chromosome XV as indicated by hybridization analysis and its deduced amino acid sequence showed extensive homology to the sequence of the β-subunits of riboflavin synthase from *B. subtilis*. Lumazine synthase occurs as a constituent of the enzyme complex in *B. subtilis*, which consists of 60 β-subunits and 3 α-subunits [130]. The β-subunits indicating lumazine synthase form a capsid with icosahedral 532 symmetry [131,132] and is specified by the gene *ribH* of the riboflavin operon on the *B. subtilis* chromosome [126]. The α-subunits, another component of the riboflavin synthase enzyme complex, formed a trimer that is enclosed in the central core of the icosahedral β-subunit capsid. Lumazine synthase of *E. coli* is specified by a gene-designated *ribE*. The protein is an empty icosahedral capsid consisting of 60 subunits. In contrast to the homologous *B. subtilis* protein, it does not form a complex with riboflavin synthase [133].

Riboflavin Synthase or the α-Subunits of Riboflavin Synthase

The terminal step of riboflavin biosynthesis is catalyzed by riboflavin synthase. This enzyme catalyzes the dismutation of two molecules of the substrate DMRL (**IV**) to yield one molecule each of riboflavin(**V**) and ARP (**III**) as shown in Fig. 6. As mentioned-above, *B. subtilis* has two different riboflavin synthases characterized by α-subunits and β-subunits. The enzyme catalyzing the last step of riboflavin biosynthesis is specified by the gene *ribB* of the riboflavin operon on the *B. subtilis* [126]. The derived amino acids sequence showed internal homology existed between the N-terminal and C-terminal halves of the protein, which suggests that the promoter forms two structurally similar substrate-binding domains [134]. Because, the enzyme binds two molecules of the substrate DMRL, during enzymatic riboflavin production, one mole of the substrate DMRL acts as the donor and the other, as the acceptor of the four-carbon moiety. These two sites are distinguished as the acceptor site binds the product riboflavin, whereas the donor site binds the 2nd product 5-amino-2, 6-dihydroxy-4-ribitylamino-pyrimidine [135,136]. A gene *ribC* encoding mono functional riboflavin synthase in *E. coli* was specified [137] and cloned onto a 6kb DNA segment by marker rescue using a *ribC* mutant of *E. coli*. In common with *E. coli*, *S. cerevisiae* has also a monofunctional ribofla-vin synthase. The gene *RIB5* encoding this enzyme was isolated from a yeast genomic library by functional complementation of a mutant, *rib5-10*, lacking riboflavin synthase activity [138]. The deduced amino acid sequence showed extensive homology to the sequence of the α-subunits of riboflavin synthase of *B. subtilis*. These internal homologous characteristics suggested that the Rip5p subunit contained two structurally related but catalytically different domains.

DBP Synthase

The xylene ring of riboflavin originates by dismutation of the precursor, DMRL (**IV**). The formation of the latter compound requires a four-carbon unit. DBP (**VII**) as the four-carbon unit incorporated into riboflavin (**V**) [107,139]. Ribulose 5-phosphate (**VI**) served as substrate for the formation of DBP (**VII**) as catalyzed by the enzyme DBP synthase (Fig. 5). The enzyme catalyzes the release of carbon 4 of ribulose 5-phosphate as formate, which is accompanied by a complex rearrangement reaction conducive to the formation of DBP from carbon atoms 1,2,3 and 5 of the substrate [139].

As mentioned above in GTP cyclohydrolase II, the ribA encoding GTP cyclohydrolase II in *B. subtilis* could be also specify DBP synthase bifunctionally [118]. In contrast to *B. subtilis*, *E. coli* has two separate genes, *ribB* and *ribA*, that encode the function of DBP synthase [118] and GTP cyclohydrolase II [117], respectively. The *RIB3* gene of *S. cerevisiae* specifying synthase was cloned by the same method that was used to clone the *ribB* in *E. coil* [140].

GENETICS FOR *A. GOSSYPII* AS AN OVERPRODUCER OF RIBOFLAVIN

In riboflavin biosynthesis, there are six specific enzymatic pathways (Fig. 1). However, the metabolic flux to riboflavin is also determined by numerous nonspecific reactions. This means that riboflavin production may be enhanced by supplementation. Ribitol [141], purine [34], or glycine [31,34,36] was studied as enhancers in the early stages of riboflavin production. Among these, glycine is thought to be a limiting precursor in riboflavin overproduction. Glycine supplementation during fermentation with *A. gossypii* doubled riboflavin production at least without growth variations. As mentioned in the riboflavin biosynthesis, there are three routes of glycine enzymatic biosynthesis, among these, serine hydroxymenthyltransferase is the most widespread (Fig. 2). Another enzyme, glutamate glyoxylate aminotransferase (Fig. 2) was found in the mitochondria of a fungus. Thus this enzyme is not co-located with isocitrate lyase in the peroxisomes, which is a main location site of riboflavin metabolic flux. Glycine biosynthesis was discovered in *S. cerevisiae* through the threonine aldolase and later it was understood to be the main reaction [61,142]. Therefore, the gene *GLY1* encoding the threonine aldolase of *A. gossypii* was isolated by heterologous complementation of the glycine-auxotrophic *S. cerevisiae* [143]. In contrast to *S. cerevisiae* the *GLY1* knockout mutant of *A. gossypii* was not auxotrophic for glycine. This means that threonine aldolase plays only a minor role during glycine biosynthesis in *A. gossypii*. Nevertheless, overexpression of *AgGLY1* under the control of the constitute *TEF* promoter and terminator led to a 10-fold increase of threonine aldolase specific activity and a 9-fold increase in riboflavin production when the medium was supplemented with threonine. This strong enhancement could not be achieved by supplementation of glycine alone.

In terms of the metabolism of riboflavin, the glyoxylate cycle as well as glycine biosynthesis is important, because the glyoxylate cycle is essential for the degradation of plant oil in riboflavin production. There are degraded to fatty acids and glycerol by an extracellular lipase [48] and are the preferred substrates for riboflavin biosynthesis because of their yield-enhancing effects [25]. Since the two starting precursors of riboflavin are originated from carbohydrate metabolism, the glyoxylate cycle in cooperation with gluconeogenesis plays an indispensable function in terms of growth and riboflavin production in *A. gossypii*. Therefore, in this metabolic pathway, enzymatic activity of isocitrate lyase, which catalyzes the cleavage of isocitrate to glyoxylate and succinate through a carbon-conserving pathway, is a key point. Recently, a correlation between isocitrate lyase activity and riboflavin production was studied [47] and the isocitrate lyase-encoding gene *AgICL1* was isolated from *A. gossypii* by heterologous complementation of *S. cerevisiae* icl1d mutant [144]. In contrast to *S. cerevisiae ICL1* that is located in the cytosol [145, 146], *AgICL1* is located in the peroxisomes. *AgICL1* was subject to glucose repression and derepressed by glycerol, and was also in part induced by ethanol and acetate and fully induced by soybean oil. These results agree well with the result that soybean oil is an enhanced carbon source in riboflavin production by *A. gossypii.*

On the other hand, using a molecular approach, a study of the structural gene of *A. gossypii* was also undertaken [50]. The structural gene *VMA1* encoding vacuolar ATPase subunit A was cloned. Disruption of this gene of *A. gossypii* could lead to the complete excretion of riboflavin into medium, instead of its retention in vacuolar compartments. Because riboflavin production by *A. gossypii* starts in the late growth phase when septa and vacuoles are formed in the hyphae and concomitantly, vacuoles begin to accumulate large amounts of riboflavin. Therefore *A. gossypii* lacking the intracellular vacuoles can produce more riboflavin without experiencing lethal effects, like *S. cerevisiae*. The same results were obtained with concanamycin A, which inhibits the vacuolar ATPase, which means that riboflavin accumulation to vacuoles depends on the activity of the ATPase.

Summarizing from the genetic viewpoint, the *AgICL1* encoding isocitrate lyase is thought to be a key gene in riboflavin production and the *VMA1* gene disruptant can cause the excretion of riboflavin extracellularly. The former has the ability to promote the main metabolic flux in the total metabolism, the latter has the ability to excrete the final product into the broth.

CONCLUSION

Recently the riboflavin production is shifting from

chemical synthesis to microbial production. The microbial production of riboflavin was reviewed with the viewpoints of biosynthesis, key enzymes, and enzyme related genes. In the metabolic viewpoint for riboflavin overproduction the supplementation method of GTP, a limiting precursor should be developed. Especially in the case of fungi using vegetable oil as a carbon source, the GTP fluxes from glyoxylate cycle, in which case the key enzymes in the glyoxylate cycle need to be strengthen. The research on a gene that is related with key enzyme, *AgICL1* is carrying out to produce riboflavin efficiently. With the progress of these metabolic, biosynthetic, and genetic developments the riboflavin production will be completely replaced by microbial production in a near future.

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