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**Metabolic engineering of *Synechococcus* sp. strain PCC 7002 for the photoautotrophic production of riboflavin (vitamin $B_2$)**

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## Abstract

In times of global warming, photosynthetic microorganisms hold great promise as biotechnological platforms for the environmentally sustainable production of a wide range of commodity chemicals and biofuels. However, little effort has been made to study the capacity of photosynthetic bacteria to produce *fine* chemicals, some of which are required in larger quantities and thus represent attractive targets for carbon-neutral production processes. One of these compounds is the light-sensitive B-vitamin riboflavin, which is used as a food and feed additive for humans and farm animals. This study describes the first photoautotrophic bioprocess for the over-production of a vitamin compound, riboflavin ($B_2$), which was achieved by metabolic engineering of the cyanobacterium *Synechococcus* sp. strain PCC 7002. By expressing the riboflavin biosynthesis genes from *Bacillus subtilis* in *Synechococcus* sp. PCC 7002 under the control of strong cyanobacterial promoters the supernatant concentrations of riboflavin was increased 211-fold compared to wild-type, reaching a final concentration of riboflavin of ~78 µM over the course of 38 days. The light-sensitivity of riboflavin at wavelengths below λ=500 nm was overcome by culturing the production strain under red LED light. Additionally, a *Synechococcus* sp. PCC 7002 strain sensitive to the toxic antimetabolite roseoflavin was generated. Selection of this strain on the toxic riboflavin-analog roseoflavin yielded several roseoflavin-resistant riboflavin-overproducing mutants. Integration of the riboflavin biosynthesis genes into such a parent strain further increased the riboflavin levels, thus showing that the selection approach is compatible with genetic engineering for riboflavin production.

Additionally, the endogenous riboflavin biosynthesis genes of *Synechococcus* sp. PCC 7002 were characterized. Overall, this project lays the foundation to build a potent phototrophic riboflavin production strain and demonstrate that cyanobacteria can serve as biotechnological platforms for the sustainable production of vitamins.

## German Abstract (Zusammenfassung)

In Anbetracht der globalen Klimaerwärmung muss die Biotechnologie neuartige Ansätze für die nachhaltige Produktion von chemischen und biochemischen Wertstoffen erarbeiten. Mit dieser Zielsetzung werden seit einigen Jahren genetisch veränderte photosynthetische Mikroorganismsmen untersucht, die eine ganze Reihe an Grundchemikalien herstellen können. Es sollte allerdings auch die photosynthetische Herstellung von *Fein*chemikalien in Betracht gezogen werden, sofern diese Stoffe in ausreichender Menge benötigt werden um den Forschungs- und Entwicklungsaufwand zu rechtfertigen. Eine dieser Feinchemikalien ist das B-Vitamin Riboflavin, welches eine wichtige Rolle als Ergänzungsmittel in der Nahrungs- und Futtermittel-Industrie spielt. In dieser Studie zeige ich, dass durch genetische Veränderung des Cyanobakteriums *Synechococcus* sp. PCC 7002 eine Überproduktion von Riboflavin erzielt werden kann. Durch Überexpression der Riboflavin-Biosynthese-Gene von *Bacillus subtilis* mithilfe von starken cyanobakteriellen Promotoren konnte im Vergleich zum Ausgangsstamm eine 211-fache Steigerung der Riboflavinproduktion mit einer finalen Konzentration von 78 µM in 38 Tagen erreicht werden. Die Lichtempfindlichkeit des Riboflavin-Moleküls bei Wellenlängen unter $\lambda$=500 nm konnte durch die Kultivierung der Bakterien im Rotlicht umgangen werden. Zusätzlich konnten mithilfe des toxischen Antimetaboliten Roseoflavin einige Riboflavin-überproduzierende *Synechococcus* sp. PCC 7002 Mutanten selektiert und charakterisiert werden. Integration der Ribofavin-Biosynthese-Gene in einen solchen Stamm lieferte eine höhere Riboflavin-Produktion als eine Integration derselben Gene in den wild-typischen Stamm und zeigt damit, dass die Strategie der Selektion mit der genetischen Manipulation kombiniert werden kann. Desweiteren wurden in dieser Studie die endogenen Riboflavin-Biosynthese-Gene von *Synechococcus* sp. PCC 7002 auf ihre Funktionalität hin überprüft.

Mit diesem Projekt wird folglich am Beispiel von Riboflavin gezeigt, dass Cyanobakterien als biotechnologische Platform für die nachhaltige $CO_2$-neutrale Produktion von Vitaminen dienen können.

## Contents

# *Prologue*

*Three billion year ago, Earth's atmosphere was an anoxic mix of gases that would be lethal to many life forms today. Ancient inorganic microorganisms, however, thrived; some using light energy to reduce $CO_2$ to organic matter by stealing away electrons from $H_2$, $H_2S$ or $Fe^{2+}$ [2]. Then, an event occurred that should change the fate of this biosphere completely: oxygenic photosynthesis allowed the first cyanobacteria to efficiently strip electrons from the ubiquitous water molecule [3], releasing oxygen gas a byproduct. The rise of cyanobacteria led to the Great Oxidation Event more than 2 billion years ago (sometimes called the Oxygen Catastrophe) that caused the extinction of many archaic life forms and enabled the evolution of aerobic organisms [4]. At first, most of the released oxygen was captured by the oceans, seabed rocks and land masses - it should take more than a billion years until these sinks were saturated, leading to a massive surge of oxygen in the atmosphere approx. 800 million years ago [5]. Without cyanobacteria slowly building an oxygenic atmosphere, complex life forms would probably never have arisen [6-8].*

# I

# Introduction

## 1.1. Cyanobacteria as host organisms for the production of commercially relevant products

### Cyanobacteria - a general overview

Cyanobacteria are a phylum of gram-negative bacteria capable of performing oxygenic photosynthesis to convert light energy into chemical energy, while fixing atmospheric $CO_2$ into organic matter. Many cyanobacterial species are able to capture nitrogen from inorganic sources [9, 10], therefore marking the starting point of the organic carbon and nitrogen cycle that underlies every sustainable ecosystem. Cyanobacteria are ubiquitously present in marine and fresh-water environments, but also in very extreme natural habitats, ranging from Antarctic conditions [11] to hot springs [12] and lakes of a wide range of salinity and pH [13]. Probably due to different environmental requirements, cyanobacteria display a high degree of morphological diversity, most distinctively separated into unicellular and filamentous strains [14] (Figure 1).

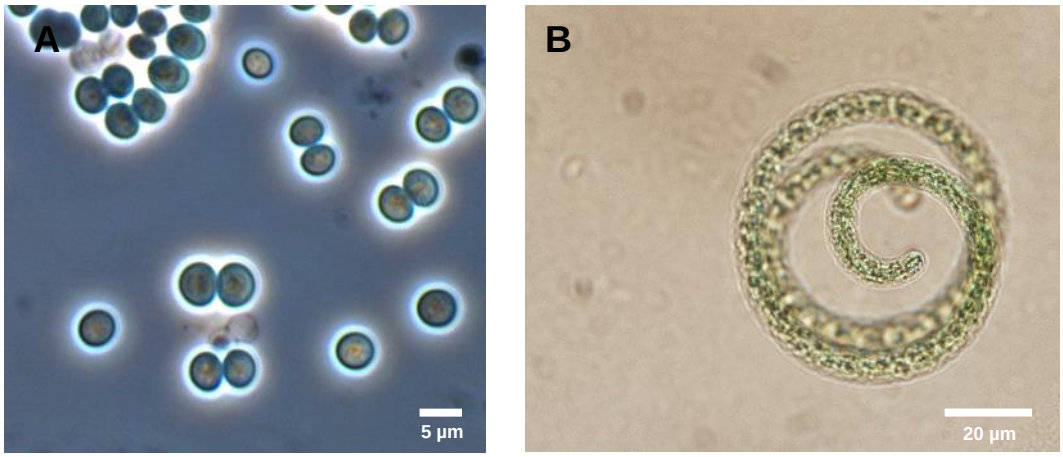



**Figure 1: Example of a unicellular and a filamentous cyanobacterium. (A)** The unicellular laboratory strain *Synechocystis* sp. PCC 6803 and **(B)** the filamentous edible cyanobacterium *Arthrospira* ("*Spirulina*") *platensis*.

Some cyanobacteria are edible and are used as a valuable source of nutrients for humans and animals. The filamentous *Arthrospira ("Spirulina") platensis* has been consumed for centuries in South America and Africa [15, 16] and was claimed a "superfood" attributed with several positive effects on human and animal health [17]. On the other hand,

cyanobacteria also contribute to toxic algal blooms - which are often a result of anthropogenic eutrophication of water bodies - with severe public health impact [18], since a lot of species produce poisonous compounds. However, a considerable number of laboratory strains have been well-characterized and are generally recognized as safe (GRAS).

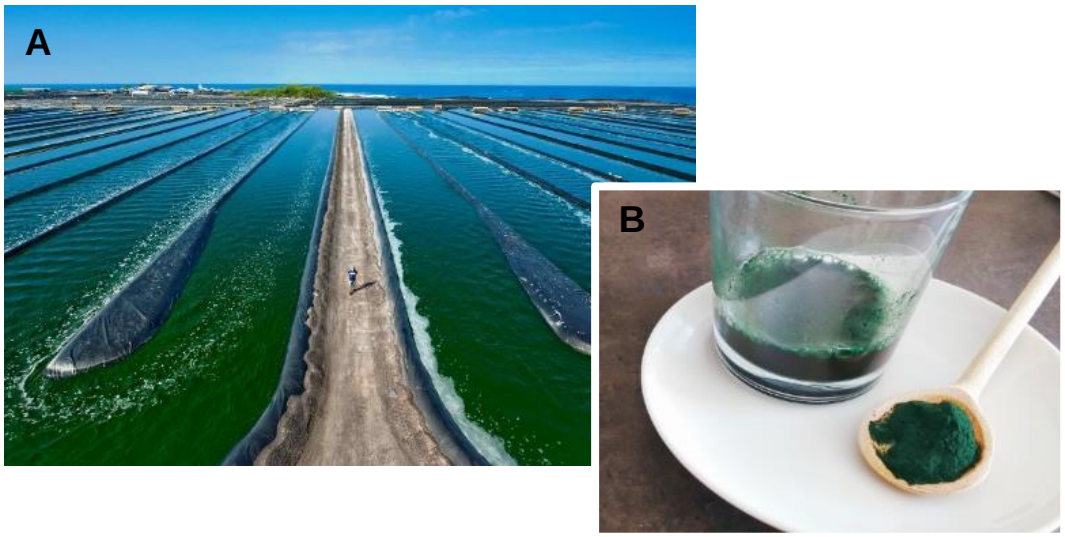



**Figure 2: *Spirulina* as food source for humans and animals. (A)** *Spirulina* farm in Hawaii for large-scale cultivation of *Spirulina* in an open pond system[1]. **(B)** The *Spirulina* product is commercially available as dry powder.

## Engineering cyanobacteria for sustainable production of biofuels and commodity chemicals

With rising concern about the effects of global warming, the carbon-capturing properties of cyanobacteria makes them attractive host organisms for production of commodity chemicals and biofuels, which, to date, are mostly based on fossil sources (gas and oil) and therefore contribute to the greenhouse effect [19]. Compared to eukaryotic algae and plants, which are also under investigation for their potential use as renewable sources for biofuels, cyanobacteria show a more rapid growth and a higher photosynthetic efficiency [20]. With regard to large-scale biotechnological applications, cyanobacteria are favorable over land

[1] Image was taken from http://hawaiianspirulinaworldshealthiestfood.blogspot.com, version 2020-02-28, posted by Jc Lao Carrillo

plants since they do not require arable landmass. This is especially important since biofuels produced by cyanobacteria ("third generation biofuels") do not physically or economically compete with plants used for food and feed production, as it is the case for first- and second-generation plant-based biofuels [21]. Additionally, cyanobacteria were shown to require less water resources than soybean or corn [22]. Utilizing marine species solves the issue of competition for fresh-water resources entirely, at least in locations were salt water is available. Furthermore, some attempts were made to cultivate cyanobacteria in waste-water [23]. In recent years, advances in cyanobacterial biotechnology has led to a great number of engineered strains producing a wide variety of commodity chemicals, ranging from alcohols, fatty acids, sugars, bioplastic precursors, terpenes, and others, which is comprehensively reviewed in [24] and [25].

**Cyanobacteria-derived pharmaceuticals, colorants and fertilizers**

Cyanobacteria do not only hold great promise in the field of bioenergy and production of commodity chemicals, but also provide an interesting source for the discovery of novel substances for pharmaceutical use. An increasing number of compounds with antibacterial, antiviral, or cytotoxic effects, respectively, is currently under investigation, many of which being products through non-ribosomal peptide synthases [26]. Other compounds are investigated for non-biomedical use, including herbicides, algaecides and insecticides [27]. Furthermore, natural pigments that are ubiquitously produced in cyanobacteria, like chlorophylls, carotenoids, and phycobiliproteins could substitute synthetic dyes, e.g. in food coloring and cosmetics [28].

Cyanobacteria also hold great promise for biological remediation of waste-water (e.g. from urban, industrial and agricultural sources), which can have detrimental effects on natural water bodies due to eutrophication [29]. In a controlled environment, however, cyanobacteria biomass recycled from waste water could be used as fertilizer to increase soil fertility due to capturing of inorganic phosphorus, nitrogen and carbon, production of vitamins and symbiotic effects with agricultural plants [30].

**Genetic and metabolic engineering of cyanobacterial species**

Some cyanobacterial species, including the laboratory strains *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, are naturally

competent [31-34], which makes them very susceptible to genetic and/or metabolic engineering. Other species, including the recently described fast-growing strain *Synechococcus* UTEX 2973 [35], can be transformed using e.g. bacterial conjugation or electroporation [36, 37]. Since genetic and/or metabolic engineering of cyanobacteria is a relatively new field of research, the amount of knowledge and tools available for cyanobacterial model species still lags behind well-studied microbial hosts such as *Escherichia coli* or *Bacillus subtilis*. Unfortunately, not all genetic elements well characterized in these species work reliably in cyanobacteria [38]. Therefore, special efforts have been made to expand the toolbox of cyanobacterial biotechnology, e. g. by identifying and characterizing promoters, terminators, ribosomal binding sites, integration sites, riboswitches and other genetic elements [39], as well as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-based genome editing techniques using Cas9 [40, 41], Cpf1 [42] or Cas12a [43].

**Vitamins as potential new target compounds for phototropic bioprocesses**

As described above, metabolic engineering of cyanobacteria has focused mainly on the production of commodity chemicals and biofuels [24], since these compounds are required in large quantities and are still mostly generated from fossil sources. However, as cyanobacterial biotechnology advances, it becomes increasingly interesting to produce other commodity and even fine chemicals employing phototrophic microorganims.

To the best of my knowledge, no attempt has been made to engineer cyanobacteria for the production of vitamins. One reason might be that a some vitamins are light-sensitive [44, 45], making phototrophic production challenging due to long illumination periods required to generate cyanobacterial biomass. Notably, even microbial production of vitamins using heterotrophic host species is advanced to the stage of industrial production only for a few vitamin compounds so far, including vitamin C [46], $B_2$ [47], $B_{12}$ [48, 49] and $K_2$ [50, 51]. Some vitamins, on the other hand, are synthesized chemically [50], even though heterotrophic production strains are currently under investigation [52, 53] and might replace chemical synthesis in the near future.

Engineering cyanobacteria for the purpose of vitamin production would show that the current microbial biotechnology could be developed towards a more sustainable future. To this end, this project was initiated to establish the first genetically engineered cyanobacteria production strain for the B-vitamin riboflavin. This compound was chosen since its

biosynthesis pathway is well-studied in different microorganisms and it is required in large quantities in the food and feed industry. Additionally, genetic engineering of heterotrophic microorganisms for riboflavin productino has proven successful and the corresponding concepts might serve as "blueprints" for a cyanobacterial laboratory strain as well [47].

In the future, another possibility is the use of vitamin-overproducing edible cyanobacteria as animal feed. Microalgal strains like *Arthrospira platensis* are already used as a feed supplement in aquaculture and other industrial farming, partially replacing fish meal [54, 55]. On the other side, attempts were made to enrich the algal diet of fish larvae with riboflavin [56], which is a common additive in aquaculture [57-59] as well as land-based industrial farming [60]. Therefore, generating a riboflavin-overproducing *Arthrospira* platensis strain would be an attractive future goal. However, metabolic engineering of *Arthrospira platensis* is currently challenging, mostly due to difficulties in transformation and the generation of stable mutants [61].

## 1.2. The role of riboflavin in health and disease

Riboflavin (vitamin $B_2$) is an essential micronutrient to maintain proper cellular function. Since mammals do not have the ability to synthesize riboflavin, a sufficient amount has to be taken up with the diet and, to a lesser extent, is derived from the intestinal microbiome. Riboflavin is absorbed mainly in the small and large intestine via carrier mediated transport [62]. Upon absorption, riboflavin is converted into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both are cofactors utilized by flavoenzymes using FMN and FAD to transport electron, hydrogen atoms and hydride ions to perform redox-reactions and other processes [63]. To date, around 90 flavin-utilizing proteins are known in humans; malfunction of many of these proteins is associated with metabolic disorders [64].

Nutrition authorities recommend a daily intake of riboflavin of 1.2 and 1.4 mg/day for adult women and men, respectively [65], to prevent riboflavin deficiency (ariboflavinosis) in the general population. Riboflavin deficiency is associated with de-regulated iron utilization and an increased risk of persistent anemia [66], as well as cataract formation [67]. Other

manifestations include sore throat, skin inflammation, loss of hair, odema of mucous membranes, as well as neurological disorders [68]. Riboflavin supplementation was shown to have a positive impact on clinical symptoms of several illnesses, ranging from migraines [69] to Crohn's disease [70].
Due to enrichment regulations installed by many countries to replenish vitamin losses that occur during industrial processing of food, e.g. rice, corn meal and flour, riboflavin deficiency is rather uncommon in the general population of developed countries. However, mild deficiencies were detected in certain subpopulations, including the elderly [71], pregnant women [72], vegetarians [73], as well as in developing countries [74].

In 2015, riboflavin was synthesized in a quantity of 9 000 tons/year, which was mostly used in the animal feed industry (70%). After initially being produced chemically, microbial fermentation of riboflavin was established by Hoffman-La Roche. As the biological process was shown to be more cost-effective it replaced chemical synthesis in 1998 [75]. An analysis conducted on behalf of the German Federal Environmental Protection Agency revealed that the environmental impact of microbial production is lower in terms of global warming, acidification, ozone creation and cumulated energy consumption, compared to chemical synthesis, but higher in terms of eutrophication. However, the study concluded that the microbial production of riboflavin is overall more sustainable than the chemical process [76].

## 1.3. The riboflavin biosynthetic pathway in *B. subtilis* and other bacteria

The gram-positive firmicute *B. subtilis* is one of the best-studied microorganisms with regard to the riboflavin biosynthesis pathway. In *B. subtilis*, six enzymes are responsible for converting one molecule of guanosine triphosphate (GTP) and two molecules of ribulose-5-phosphate (Ru5P) into riboflavin (Figure 4). Two of these enzymes, RibDG and RibAB, are bifunctional. One step of the riboflavin biosynthesis, namely the conversion from 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (ARP) 5'-phosphate to ARP, is not yet elucidated and might be catalyzed by rather unspecific cellular phosphatases [77, 78]. The riboflavin biosynthesis genes form the transcription unit *ribDGEABHT*, whereas

the function of *ribT* is unknown. The gene *ribFC*, encoding for a bifunctional riboflavin kinase/FMN adenylyltransferase, is not part of this transcription unit. Expression of the genes *ribDGEABHT* is controlled by an FMN-riboswitch, which terminates transcription upon binding of FMN [79]. As a result, intracellular FMN levels are tightly regulated.

The riboflavin biosynthesis genes are naturally expressed in low abundance and the corresponding gene products display low catalytic activity [80], especially the bifunctional RibAB [81], which functions as the "doorman" of riboflavin biosynthesis by introducing both substrates (GTP and Ru5P) into the pathway.

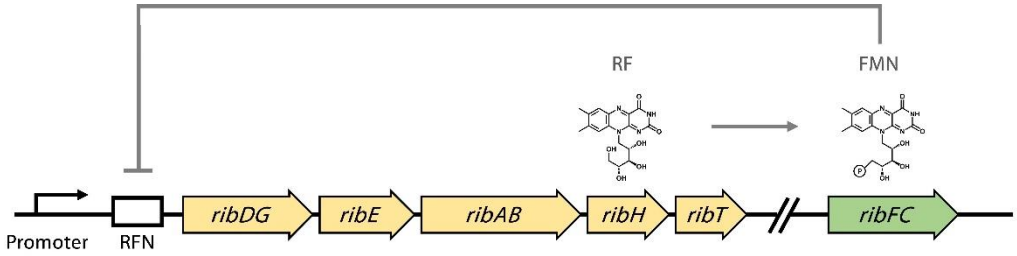



**Figure 3: Regulation of expression of the *ribDGEABHT* genes in *B. subtilis* by an FMN-sensing negative feedback mechanism.** The FMN-sensing aptamer (RFN) is a transcriptional riboswitch that terminates transcription upon bindng of FMN.

Whereas riboflavin biosynthesis is similar in different bacteria, differences occur in localization of the riboflavin biosynthesis genes (operon vs. distributed genes) and regulation (FMN-dependent regulation of the whole operon vs. regulation of only certain genes, transcriptional vs. translational attenuation) [82]. Many bacterial species express flavin transporters to import flavins into the cytoplasm, some of which are even riboflavin-auxotrophic [82, 83]. Other species express flavin exporters, e.g. in the case of *Shewanella oneidensis*, an electrogenic microorganism secreting flavins to mediate extracellular electron transfer [84].

In the phylum of **cyanobacteria**, the aforementioned well-studied species *Synechococcus* sp. PCC 7002, *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. PCC 6803, and others, contain all genes necessary for riboflavin biosynthesis, according to the (automatic) annotation in the NCBI database. However, these genes have never been studied experimentally.

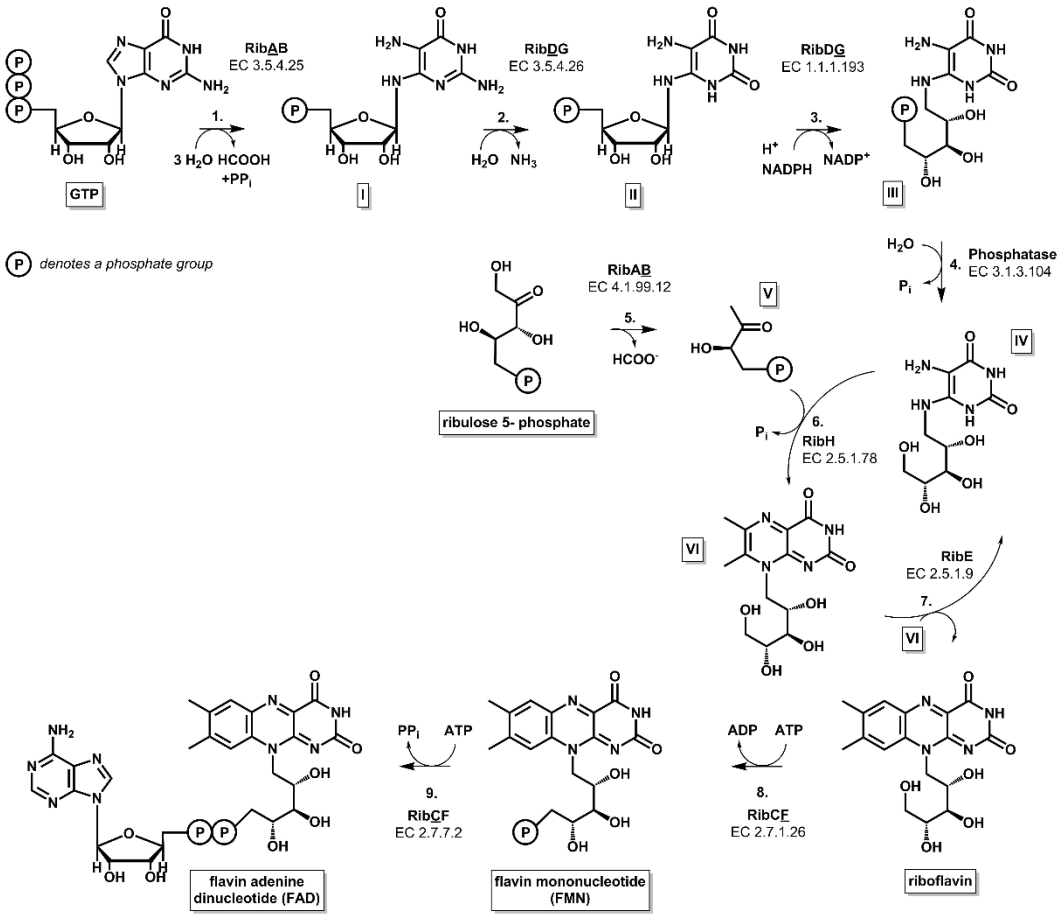



**Figure 4: Riboflavin biosynthesis in bacteria [87].** Riboflavin is synthesized from one molecule guanosine-5'-triphosphate (GTP) and two molecules ribulose-5-phosphate in a seven step enzymatic process (1.-7.). Riboflavin is further processed into the active cofactors FMN and FAD (8.-9.). Enzyme short names are according to the new genetic nomenclature [88]. RibAB functions both as a GTP cyclohydrolase II (step 1, RibA) (EC 3.5.4.25) and a (3S)-3,4-dihydroxy-2-butanone 4-phosphate synthase (step 5, RibB) (EC 4.1.99.12). RibDG is a bifunctional 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate deaminase (step 2, RibD) (EC 3.5.4.26) and a 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate reductase (step 3, RibG) (EC 1.1.1.193). RibH functions as a lumazine synthase (step 6) (EC 2.5.1.78). RibE is a riboflavin synthase (EC 2.5.1.9) dismutating two molecules of VI to give IV and riboflavin (step 7). RibFC is bifunctional, acting as flavokinase (RibF) (EC 2.7.1.26) and FAD synthetase (RibC) (EC 2.7.7.2). An unknown phosphatase catalyzes the dephosphorylation of III to IV (step 4) (EC 3.1.3.104). Intermediates in the riboflavin biosynthesis pathway are: **I**: 2,5-Diamino-6-(5-phospho-D-ribosylamino)pyrimidin-4(3*H*)one; **II**: 5-Amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate; **III**: 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate; **IV**: 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **V**: (3*S*)-3,4-Dihydroxy-2-butanone 4-phosphate; **VI**: 6,7-Dimethyl-8-D-ribityllumazine.

Even though riboswitch regulation occurs in this phylum, e.g. for the vitamin $B_{12}$ biosynthetic pathway [85], no FMN riboswitch has been found in cyanobacteria [86]. Therefore, if and how flavins are regulated in cyanobacteria still remains unclear, even though previously published transcriptomics data in *Synechocystis* sp. PCC 6803 suggests that the riboflavin biosynthesis genes are regulated by light-dark-cycles (see Appendix, section 6.3, p. 130).

## 1.4. Strategies to improve riboflavin biosynthesis in *B. subtilis*

Beside the naturally riboflavin-overproducing fungi *Ashbya gossypii* and *Candida famata*, *B. subtilis* is one of the most extensively engineered species for industrial production of riboflavin [89]. Not being a natural riboflavin overproducer, *B. subtilis* was chosen as a host strain as (i) it is generally recognized as safe (GRAS), (ii) had already been a well-studied host organisms for recombinant expression of proteins, (iii) had been used for other fermentation processes yielding products for human consumption and (iv) had already been developed for the production of purines [90]. A detailed summary of the history of metabolic engineering and bioprocess optimization of riboflavin overproducing microorganisms is given by Hohmann and Stahmann [89].

### 1.4.1. Anti-metabolites for selection of purine and riboflavin overproducing strains

Before the dawn of rational genetic engineering, selection-based strategies were applied to yield metabolite-overproducing strains, often combined with mutagenic reagents or UV radiation. In this fashon, *B. subtilis* wild-type strains were selected on toxic antimetabolites such as 8-azaguanine [91, 92], decoyinine and methionine sulfoxide [93, 94] to recover mutant strains with increased biosynthesis of GTP, one of the precursors of riboflavin.

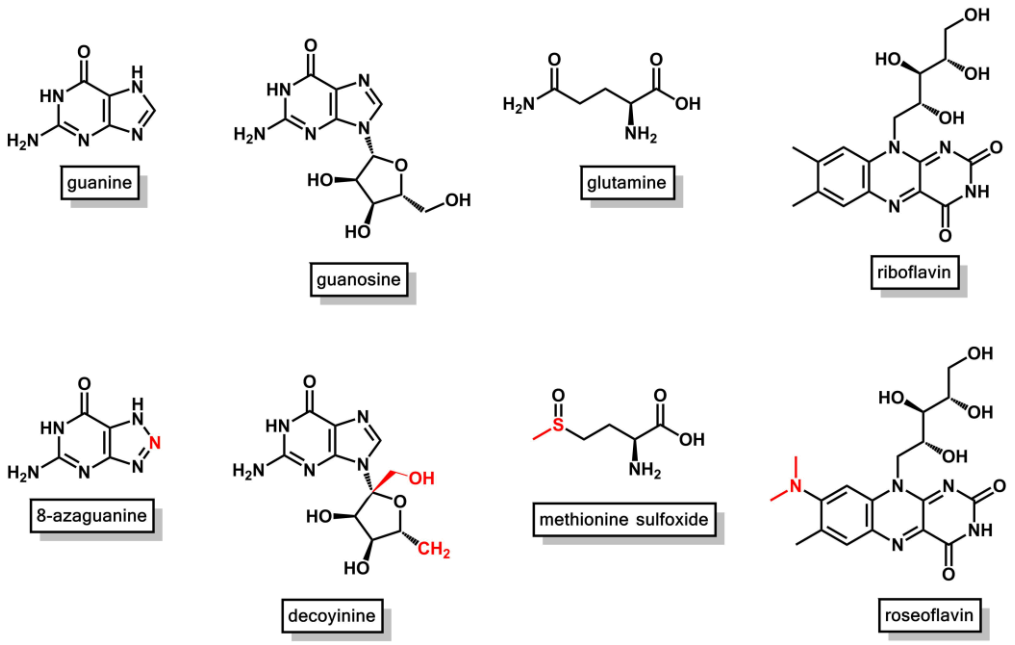



**Figure 5: Subset of antimetabolites used in the selection of purine- and riboflavin-overproducing *Bacillus subtilis* strains.** Comparison of the actual metabolites (top) with their respective toxic anti-metabolites (bottom). Structural differences are highlighted in red.

### 1.4.2. Roseoflavin-selected strains often overproduce riboflavin

The toxic riboflavin analog 8-Dimethylamino-8-demethyl-D-riboflavin (roseoflavin) was first isolated from *Streptomyces davaonensis* [95], displaying antibiotic properties against some gram-positive bacteria [95, 96]. Incubation of different gram-positive species on roseoflavin yielded mutant strains with increased production of riboflavin, as was shown for *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus pumilis* [96], some lactobacillae [97, 98], and others. Notably, these strains are not defined as genetically modified organisms (GMO) which made them valuable microbial hosts for the biotechnological production of riboflavin for the food and feed industry.

Roseoflavin is usually taken up by bacteria via specialized flavin importers [83] and/or *via* penetration of the gram-positive cell wall. The gram-negative cell membrane, however, does not seem to be permeable for roseoflavin in the absence of a flavin importer, as was shown for *E. coli* [99]. Roseoflavin-sensitivity, however, can be induced by heterologous expression of flavin importer proteins [100].

In *B. subtilis*, roseoflavin-resistant mutant strains often show a distinct set of genetic alterations. First, mutations frequently appear in the 5'UTR of the *ribDG* gene, harbouring a riboswitch sensing FMN and RoFMN, the latter being produced from roseoflavin by the endogenous riboflavin kinase [101, 102]. Upon binding of FMN/RoFMN to the endogenous FMN-riboswitch, transcription of the riboflavin biosynthesis operon mRNA is terminated [79]. Some roseoflavin-selected mutant strains contain a de-regulated FMN-riboswitch that allows transcription even at high levels of FMN/RoFMN. Another mutation commonly found in roseoflavin-selected riboflavin-overproducing strains occurs at nucleotide position 820 of the *ribFC* gene (bifunctional riboflavin kinase/FMN adenylyltransferase, EC:2.7.1.26/2.7.7.2). This mutation leads to a reduced activity of RibFC [103]. Therefore, both FMN and RoFMN production is decreased, which subsequently decreases repression of the riboflavin biosynthesis operon, but also reduces the carbon flux from riboflavin to FMN.

### 1.4.3. Genetic engineering of *B. subtilis* for riboflavin production

Attempts to over-express the endogenous riboflavin biosynthesis operon *ribDGEABHT* from plasmids to create riboflavin-overproducing *B. subtilis* strains first resulted in genetic instability. Stable mutants were generating by chromosomally integrating multiple copies of a deregulated riboflavin biosynthesis operon derived from a roseoflavin-selected *Bacillus amyloliquefaciens* strain. Further improvements were made by Perkins et al., using the strong bacteriophage promoter *SPO1* to drive expression of the riboflavin biosynthesis operon. Placing an additional copy of the *SPO1* promoter upstream of the *ribAB* gene further increased riboflavin levels. A *B. subtilis* host strain selected on the antimetabolites 8-azaguanine, decoyinine, methionine sulfoxide and roseoflavin was engineered in this fashion. With this strain, 14 g/L of riboflavin in an 48 hour fermentation were produced with a yield of 7 % (w/w) on the carbon source [93]. Other strains generated were built on the principles described above, for example strains that are free of antibiotic markers, which is of great significance for marketing fermented products in Europe [89].

After the potential of the terminal riboflavin biosynthesis pathway was thought to be exhausted by sufficient overexpression of the riboflavin biosynthesis genes, further emphasis was made to increase energy and precursor supply for the production of riboflavin. For example, to increase the energy supply of a riboflavin-overproducing *B. subtilis* strain,

the efficiency of the oxidative phosphorylation pathway was increased by deleting the terminal *bd* oxidase. The *bd* oxidase translocates only one proton (*H+*) for each electron (*e-*), while other oxidases translocate two *H+* for each *e-*. As a result, the flow of electrons was directed to a more efficient oxidase, resulting in a higher H+ gradient, resulting in a more efficient generation of ATP via a type I ATP synthase. This strategy resulted in a significant increase in biomass and riboflavin production in an industrial fed-batch culture [104].

The carbon flux through the pentose phosphate pathway was increased by overexpressing the gene encoding glucose-6-phosphate dehydrogenase (*zwf*) [105] and glucose dehydrogenase (*gdh*) [106], leading to an increase in ribulose-5-phosphate and, subsequently, phosphoribosyl pyrophosphate (PRPP), the precursor for purine synthesis. The purine biosynthesis pathway was altered by increasing enzyme levels of all enzymes directing the carbon flux to guanosine-monophosphate (GMP), while reducing adenosine-monophosphate [107] production from inosine-monophosphate [92] (Figure 6)

A transketolase (*tkt*) mutation was reported, yielding an enzyme with reduced activity, increasing the concentration of D-ribose [108], which is thought to increase Ru5P precursor supply. To increase riboflavin export, a flavin transporter from *Streptomyces davaonensis* was used to excrete riboflavin into the culture medium, as summarized in [47].

Of all measured taken to increase riboflavin production, the insertion of multiple copies of the riboflavin biosynthesis pathway under a strong promoter led to the highest increase in riboflavin concentration (290-fold) compared to the parent strain [93], while other strategies increased riboflavin only in the range of 1- to 2-fold, compared to their respective parent strain [47].

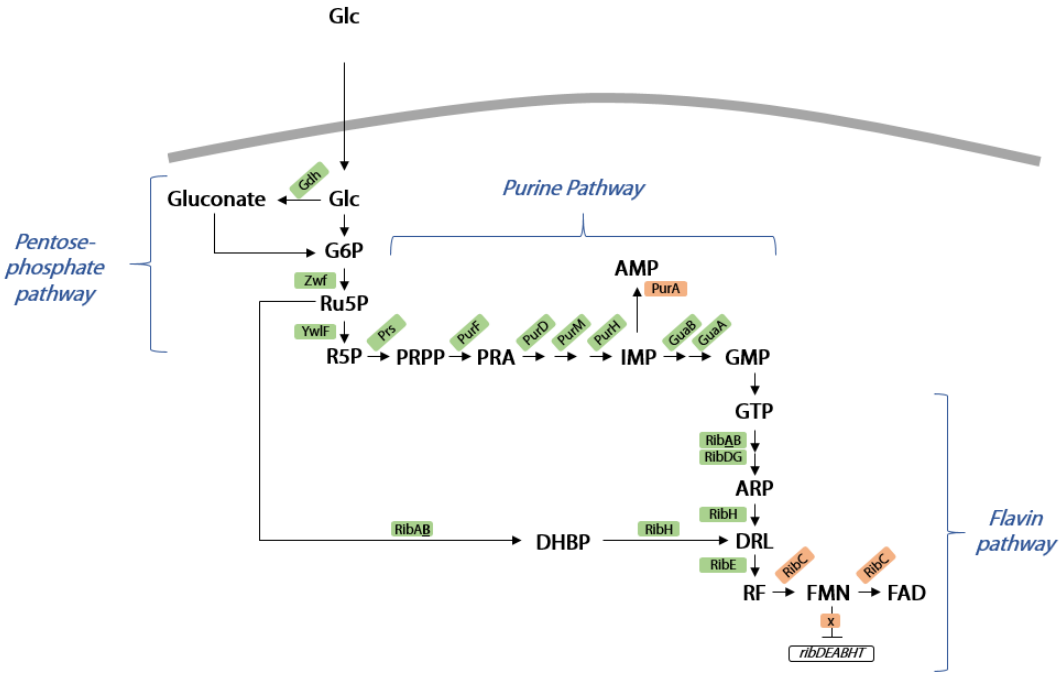



**Figure 6: Increasing the carbon flux from glucose to riboflavin through metabolic engineering of *B. subtilis*, adapted from Schwechheimer, 2016 [47].** Upregulated genes are highlighted in green, downregulated genes are highlighted in red.

Glucose (**Glc**) is converted to ribose-5-phosphate (**R5P**) via the pentose-phosphate pathway. R5P is converted into guanosine triphosphate (**GTP**) via the purine pathway. Ribulose-5-phosphate (**Ru5P**) and **GTP** are elevated due to overexpression of enzymes of the pentose-phosphate and purine pathway. Both serve as precursors of the riboflavin (**RF**) biosynthesis. Increased expression of RF biosynthesis enzymes increases RF levels in the supernatant. Downregulation of RibC activity reduces conversion of RF to FMN and FAD. De-regulation of the FMN riboswitch prevents inhibition of the FMN-sensing riboswitch controlling transcription of the riboflavin biosynthesis genes.

**G6P**: Glucose-6-Phosphate; **Ru5P**: Ribulose-5-Phosphate; **R5P**: Ribose-5-phosphate; **PRPP**: Phosphoribosyl pyrophosphate; **PRA**: 5-Phosphoribosylamine; **IMP**: inosine monophosphate; **AMP**: adenosine monophosphate; **GMP**: guanosine monophosphate; **GTP**: guanosine triphosphate; **ARP**: 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione; **DRL**: 6,7-Dimethyl-8-ribityl-lumazine.

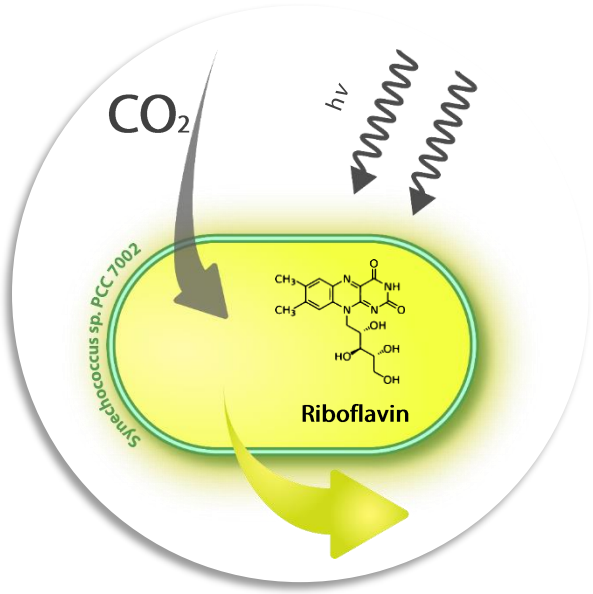
**Gdh**: Glucose dehydrogenase; **Zwf**: Glucose-6-phosphate dehydrogenase; **YwlF**: Ribose 5-phosphate isomerase; **Prs**: PRPP synthetase; **PurF**: glutamine phosphoribosylpyrophosphate amidotransferase; **PurD**: phosphoribosylglycinamide synthetase; **PurM**: phosphoribosyl amino-imidazole synthetase; **PurH**: phosphoribosylaminoimidazole carboxyformyltransferase/inosine-monophosphate cyclohydrolase; **PurA**: adenylosuccinate synthetase; **GuaB**: inosine-monophosphate dehydrogenase; **GuaA**: GMP synthase.

## 1.5. Strategies for riboflavin-overproduction in *Synechococcus* sp. PCC 7002

Some of the successful strategies to increase riboflavin production in *B. subtilis* might be transferred to *Synechococcus* sp. PCC 7002 to generate riboflavin-oveproducing photoautotrophic production strains. First and foremost, one or multiple copies of a riboflavin biosynthesis operon should be integrated into the genome under the control of a strong promoter. When using the *B. subtilis* riboflavin biosynthesis operon, a deregulated gene cluster should be used, therefore either omitting the FMN riboswitch or using a de-regulated FMN riboswitch. Using heterologous riboflavin biosynthesis genes is recommended, since addition of endogenous genes might cause genetic instability. As RibAB is the rate-limiting step of riboflavin synthesis, increasing RibAB by adding a strong promoter in front of the *ribAB* might increase riboflavin production also in *Synechococcus* sp. PCC 7002. Selection and engineering approaches should be combined, i.e. a strain selected on the toxic antimetabolite roseoflavin should be used as a parent strain for expression of heterologous riboflavin biosynthesis genes. A *ribFC* mutation in *Synechococcus* sp. PCC 7002 similar to the *ribFC820* mutation in *B. subtilis* could reduce the flux from riboflavin to FMN/FAD and might increase riboflavin concentrations even in the absence of a FMN riboswitch.

Several strategies applied for *B. subtilis* might not be transferable to *Synechococcus* sp. PCC 7002. The *B. subtilis* production strains utilize fed glucose as their carbon source, thus enhancement of glucose uptake and increasing the carbon flux through the pentose phosphate pathway yielded increased precursor supplied for both Ru5P and GTP (see **Figure 6**). A phototrophic strain, however, assimilates carbon *via* the Calvin-Benson-Bassham (CBB) Cycle, in which Ru5P is a precursor for ribulose-1,5-bisphosphate, which is used by RuBisCO (Ribulose 1,5 Bisphosphate Carboxylase/Oxygenase) to fix atmospheric carbon dioxide. Therefore, increasing the flux from Ru5P to GTP might reduce the productivity of the CBB cycle of the host organism. However, since metabolic engineering for increased precursor supply was not within the scope of this thesis, this hypothesis is only addressed theoretically in the discussion section.

## 1.6. Aim of this PhD project




Cyanobacteria have never been engineered for the production of vitamins. Therefore, the aim of this study was to fill this gap by establishing and characterizing the first phototrophic bioprocess for production of vitamin $B_2$ (riboflavin) in the phototrophic host *Synechococcus* sp. PCC 7002.

# II

# Materials and Methods

## 2.1. Strains, Plasmids and Primers used in this study

A comprehensive list of strains, plasmids and primers used in this study is given in the Appendix sections 6.4 (p. 131), 6.5 (p. 132) and 6.6 (p. 139), respectively.

## 2.2. Standard Equipment and Media

### 2.2.1. Laboratory Equipment

**Table 1: List of devices used in this project**

| Device | Model | Manufacturer |
|---|---|---|
| Autoclave | Varioklav® Dampfsterilisator 175 | H+P Labortechnik, Habermoos, Germany |
| Centrifuge | Heraeus Multifuge X1R | Thermo Fischer, Waltham, USA |
| Electroporesis device (DNA) | Mini-Sub Cell GT + Wide, Power Pac Basic | BIO-RAD, Munich, Germany |
| Electroporesis device (Protein) | Power Pac Basic | BIO-RAD, Munich, Germany |
| FastPrep | FastPrep-24 5G | MP Biomedicals, USA |
| FrenchPress | Constant Cell Disruption System | Constant System, Daventry, UK |
| Gel imager | Molecular Imager® GelDoc™ XR, Quantity One 1D-Analysis software | BIO-RAD, Munich, Germany |
| incubator for cultivation of cyanobacteria | TS 606-G/2 | WTW, Weilheim, Germany |
| LC/MS System | 1200 Infinity series with DAD/FLD and API-ESI 6130 Quadrupole | Agilent Technologies, Waldbronn, Germany |
| Optical Microscope | Leica DMRBE | Leica Microsystems, Wetzlar, Deutschland |
| PCR Thermocycler | C1,000™ Thermal Cycler, dual | BIO-RAD, Munich, Germany |
| Photometer | Ultraspec 3100 pro | Amersham Biosciences, UK |

| | | |
|---|---|---|
| Photometer for DNA quantitation | NanoVue | GE Healthcare, Little Chalfont, UK |
| Plate reader | Genius Pro | Tecan Group, Männedorf, Schweiz |
| Sterile Bench | Variolab Mobilien W 90 | Waldner Laboreinrichtungen GmbH & Co KG, Wangen, Germany |
| Tabletop centrifuge | Eppendorf 5415 R | Eppendorf, Hamburg, Germany |
| Thermomixer | Thermomixer comfort | Eppendorf, Hamburg, Germany |
| Ultracentrifuge | Avanti J-30I | Beckmann Coulter, Brea, USA |
| water bath | IKA® HBR4 digital | IKA GmbH, Staufen im Breisgau, Germany |

### 2.2.2. Chemicals

Standard laboratory-grade chemicals were used for buffers and reagents.
**Lumichrome** (7,8-Dimethylalloxazine, Item No. 20730; CAS No. 1086-80-2, purity >90%) and **lumiflavin** (Item No. 20645, CAS No. 1088-56-8, purity >95%) was purchased from Cayman Chemicals (Ann Arbour, Michigan, USA). **FMN** (riboflavin 5'monophosphate sodium salt hydrate, F8399, purity >95%) and **FAD** (flavin adenine dinucleotide disodium salt hydrate, F6625, purity >95%) was purchased from Sigma Aldrich (now Merck, Darmstadt, Germany). **Roseoflavin** (8-Dimethylamino-riboflavin, Item No. 16001, CAS No. 51093-55-1, purity >95%) was purchased from Cayman Chemicals.
The reagents for the **RibH coupled enzymatic assay** (see section 2.6.3, p. 34) were a gift from Markus Fischer, Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Germany.

### 2.2.3. *Synechococcus* sp. PCC 7002 growth medium AA+

All components for the *Synechococcus* sp. PCC 7002 growth medium AA+ are listed in Table 2. For 1.023 L of AA+ medium, 10 mL of 100x AA+ salts, 1 mL of 1000x trace metal solution, 10 mL of 100x TRIS, 1 mL of 1000x $FeCl_2$ solution and 1 mL of $B_{12}$ solution was added to 1 L of AA+ basic medium. The medium was stored in the fridge for stability and to minimize light exposure.

**Table 2: *Synechococcus* sp. PCC 7002 growth medium AA+ components**

| Media component | Compound | concentration [g/L] | Annotation |
|---|---|---|---|
| AA+ basic medium | NaCl | 18.00 | Autoclave |
| | $MgSO_4$ x 7 $H_2O$ | 5.00 | store at RT |
| 100x AA+ salts | $KH_2PO_4$ | 2.50 | Autoclave |
| | $Na_2EDTA$ x 2 $H_2O$ | 3.00 | Store at 4 °C |
| | KCl | 60.00 | |
| | $NaNO_3$ | 100.00 | |
| | $CaCl_2$ | 13.30 | |
| 1000x trace metal solution | $H_3BO_3$ | 2.86 | Autoclave |
| | $MnCl_2$ x 4 $H_2O$ | 1.81 | Store at 4 °C |
| | $ZnSO_4$ x 7 $H_2O$ | 0.222 | |
| | $Na_2MoO_4$ x 2 $H_2O$ | 0.390 | |
| | $CuSO_4$ x 5 $H_2O$ | 0.079 | |
| | $Co(NoO_3)_2$ x 6 $H_2O$ | 0.0494 | |
| 100x TRIS | Tris(hydroxymethyl)-aminomethan HCl | 100.00 | Adjust to pH 8.2 with NaOH, autoclave, store at 4 °C |
| 1000x $FeCl_2$ solution | $FeCl_2$ x 6 $H_2O$ | 4.05 | filter sterilize (!), store at 4 °C |
| 1000x $B_{12}$ solution | Cobalamin (Vitamin $B_{12}$) | 0.004 (4 mg/L) | Aliquot, freeze, keep thawed aliquots in the dark at 4 °C |

### 2.2.1. M9 minimal medium

**Table 3: M9 minimal medium for growth of *E. coli***

| Media component | Compound | amount per L | Annotation |
|---|---|---|---|
| M9 medium | $Na_2HPO_4$ | 8.0 g | To avoid precipitation of $CaSO_4$ either the $CaCl_2$ has to be added first, or filter-sterilized $MgSO_4$ is added after autoclaving |
| | $KH_2PO_4$ | 4.0 g | |
| | NaCl | 0.5 g | |
| | $NH_4Cl$ | 0.5 g | |
| | Glucose 20 % (w/v) | 20.0 mL | |
| | $MgSO_4$ 1M | 1.0 mL | |
| | $CaCl_2$ 1M | 0.3 mL | |
| | trace element solution 10x | 10.0 mL | |
| 10x trace element solution | EDTA | 5.0 g | Add EDTA first, adjust to pH 7.5, filter sterilize final buffer |
| | $FeCl_3$ x 6 $H_2O$ | 0.83 g | |
| | $ZnCl_2$ | 84.0 mg | |
| | $CuCl_2$ x 2 $H_2O$ | 13.0 mg | |
| | $CoCl_2$ x 6 $H_2O$ | 10.0 mg | |
| | $H_3BO_3$ | 10.0 mg | |
| | $MnCl_2$ x 6 $H_2O$ | 1.6 mg | |

### 2.2.2. Antibiotics

**Table 4: List of antibiotics used in this thesis for selection of recombinant *E. coli* and *Synechococcus* sp. PCC 7002**

| Compound | Stock concentration 1000x [mg/mL] | Final conc. for *E. coli* cultures [µg/mL] | Final conc. for *Synechococcus* sp. PCC 7002 cultures [µg/mL] |
|---|---|---|---|
| Ampicillin (in $H_2O$) | 100 | 100 | not used |
| Kanamycin (in $H_2O$) | 50 | 50 | 50 - 300 |
| Chloramphenicol (in EtOH) | 25 | 25 | 5 - 20 |

## 2.3. Construction and validation of a red LED array

Since riboflavin is not stable under standard cyanobacterial conditions using white light (see section 3.2.1, p. 51), even in combination with a red high-pass filter foil (see appendix section 6.1, p. 124), an LED array device was constructed providing illumination restricted to red light. The LED array was designed under the guidance of Prof. Lamparter, KIT Karlsruhe. Plastic scaffolds were 3D-printed (20x20 cm, containing holes of 5 mm diameter). LEDs were mounted to the scaffold and connected via copper wires. In general, seven orange-red LEDs (630 nm, 2.1 V, 0.02 A) were connected in a row, therefore the maximum voltage and current is 14.7 V and 0.02 A, respectively. Seven of these rows were connected in parallel to fill one scaffold, therefore the maximum current is 0.14 A per scaffold. Four scaffolds were then connected in parallel, resulting in a maximum current of 0.56 A. Similarly, for the dark-red LEDs (700 nm, 2.0 V, 0.05 A), four LEDs were connected in a row (8.0 V) and three rows were connected in parallel (0.15 A) for each scaffold. Connection of scaffolds is in parallel, leading to a maximum current of 0.6 A. To provide electricity, a direct current (DC) power supply is used for each light color, tunable from 0-30 V and 0-5 A, respectively.

The photon density in µE (µmol photons*$m^{-1}$*$s^{-1}$) in a distance of 60 cm from the array was measured using the light meter *LI-250* with the sensor *li-cor quantum Q32318* (LI-COR, USA). Since this device was not always accessible in this lab, a commercial lux meter was used to measure light intensity in lux and subsequently calculate the photon density in µE. The lux-to-µE conversion factor depends on the wavelength and was calculated to be 51±2.6 and 4.3±0.2 for the 630 nm and 700 nm LEDs, respectively. The array is tunable over the range of 2-54 µE and 0.1-7.1 µE, for the 630 nm and 700 nm LEDs, respectively. The light intensity spectrum of the LED array was measured in the Beuermann Lab, Mannheim University of Applied Sciences. It shows distinct peaks at 630 and 700 nm wavelength. Only a marginal light "contamination" in the range of 400 - 500 nm could be detected (Figure 7). Since light is the driving force behind photosynthesis and therefore considered a "nutrient" for cyanobacteria, it has to be taken into account that light distribution in the cyanobacterial growth incubator is not perfectly homogenous. Therefore, it is necessary for the shaking surface to be positioned centrally below the LED incubator, since especially the outskirts of the incubator are poorly illuminated.

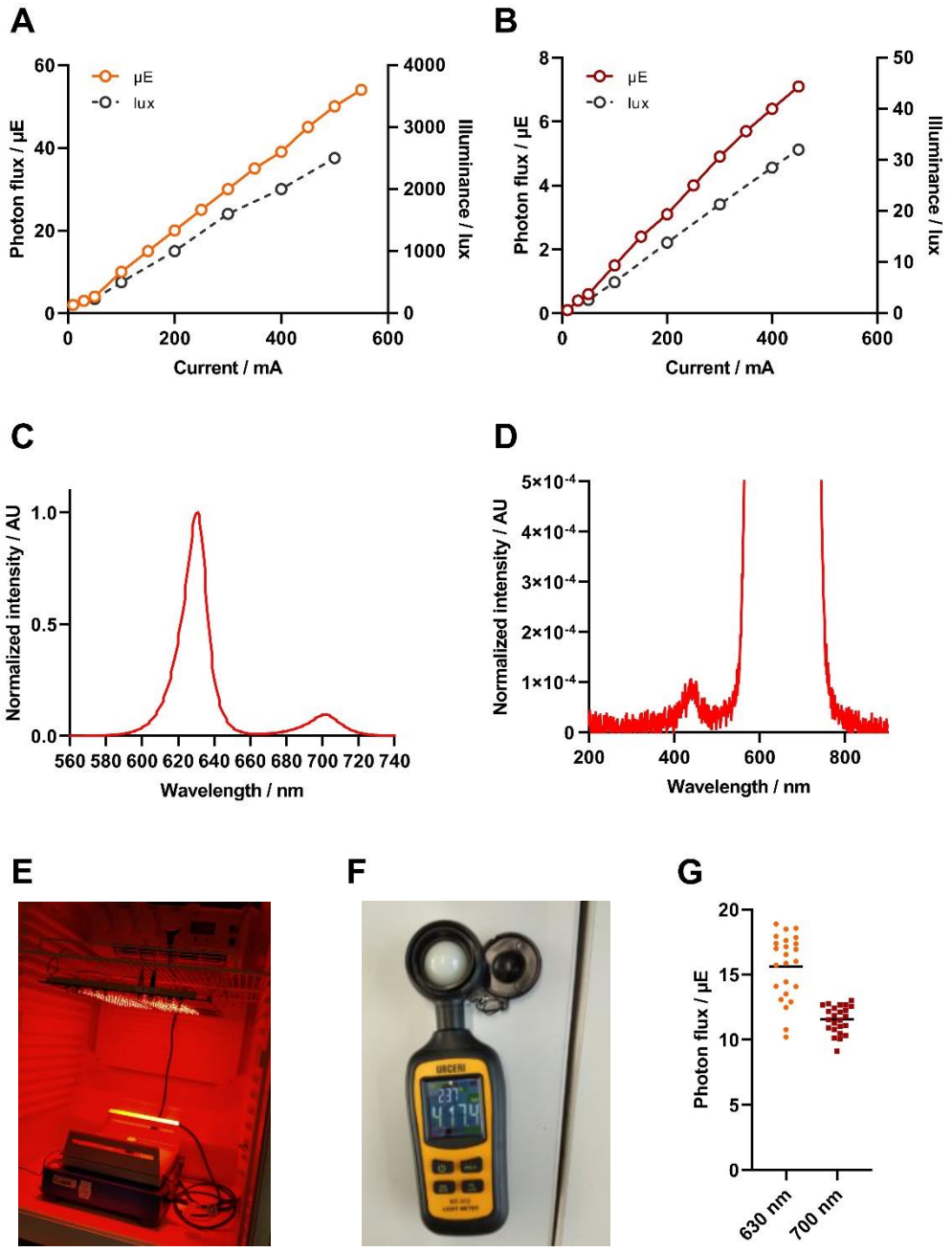



**Figure 7: Construction and validation of a red LED aray. (A)** The photon flux [µE] and illuminance [lux] values for the orange-red 630 nm LEDs and **(B)** the dark-red 700 nm LEDs, respectively, were measured in a range from 10-550 mA. **(C)** Intensity spectrum of the red LED array in standard red-light conditions. **(D)** Only marginal light intensities were measured in the range of 400 - 500 nm which is harmful for riboflavin. **(E)** The LED array was mounted in the incubator 40 cm above the shaking surface. **(F)** The illuminance was measured with a commercial lux-meter. **(G)** Light intensity distributation in the incubator. Each position of four 6-well plates has slightly different intensity, with higher values for plates located in the central position of the shaker.

## 2.4. Microbiological Methods

### 2.4.1. Standard cultivation of *E. coli* strains

If not stated otherwise, *E. coli* strains were cultivated at 37 °C in LB medium. For plate cultures, agar plates were prepared by adding 15 g/L Agar-Agar to LB medium before autoclaving and solidifying in petri dishes. Liquid cultures were grown under constant shaking at 200 rpm. Growth was measured as absorbance at 600 nm ($OD_{600}$) via spectrometer. For storage, cultures were mixed with equal volume of 50 % Glycerol and stored at -80 °C in the strain collection of the Institute for Technical Microbiology at Mannheim University of Applied Sciences (SI Stammsammlung).

### 2.4.2. Preparation of chemically competent *E. coli*

*E. coli* DH5 alpha cells were plated on solid LB growth medium. A single colony was picked and grown in 20 mL LB in a 100 mL Erlenmeyer flask overnight at 37 °C, 220 rpm. The next day, cultures were diluted to $OD_{600}$ of 0.1 in 100 mL LB medium and cultivated in a 300 mL Erlenmeyer flask at 37 °C, 200 rpm, until $OD_{600}$ of 0.6 was reached. Cultures were harvested in 50 mL single use plastic containers (Flacon®), kept on ice for 15 min and centrifuged at 5000 rpm for 15 min at 4 °C. The pellet was resuspended in 16 mL Buffer RF1, stored on ice for 15 min and the suspension was centrifuged again at 5000 rpm for 15 min at 4 °C. The pellet was suspended in 4 mL Buffer RF2 and stored on ice for 15 min. The suspension was aliquoted à 100 µL in 1.5 mL single used plastic containers (Eppendorff®) and stored in -80 °C.

**Table 5: Buffers for preparation of chemically competent *E. coli***

| Buffer | Compound | Amount per 250 mL H2O | |
|---|---|---|---|
| RF1 | RbCl | 3,02 g | Adjust to pH 5.8 with acetic acid |
| | $CaCl_2$ x 2 $H_2O$ | 0,37 g | |
| | Glycerol (100 %) | 37,5 ml | |
| | MnCl x 4 $H_2O$ | 2,48 g | |
| | KAc | 0,74 g | |

| | | | |
|---|---|---|---|
| RF2 | RbCl | 0.3 g | Adjust to pH 6.5 with |
| | $CaCl_2$ x 2 $H_2O$ | 2.75 g | NaOH |
| | Glycerol (100 %) | 37.5 mL | |
| | MOPS (3-(N-morpholino)-propanesulfonic acid) | 0.53 g | |

### 2.4.3. Complementation of riboflavin-auxotrophic *E. coli* strains

Chemically competent cells of the riboflavin-auxotrophic *E. coli* strains BSV11 (Δ*ribB*), BSV13 (Δ*ribE*), BSV18 (Δ*ribA*) [110] and SI#78 (Δ*ribD*) were prepared as described above. Competent cells were transformed with their respective riboflavin biosynthesis gene orthologue from *Synechococcus* sp. PCC 7002, or an empty plasmid control, respectively. The transformation mix was incubated overnight on LB plates containing 200 µM riboflavin and 100 µg/mL ampicillin. Three different colony forming units (cfu) were picked and grown overnight at 37 °C, 220 rpm, in LB medium containing 100 µg/mL ampicillin and 200 µM riboflavin. 2 mL of fresh LB medium, or LB medium supplemented with 200 µM riboflavin, respectively, was inoculated with 1 µL of the overnight culture and cultivated for 16 hours. $OD_{600}$ > 1.0 was considered as growth and therefore successful complementation, whereas $OD_{600}$ < 0.2 was considered growth failure.

### 2.4.4. Standard red-light cultivation of *Synechococcus* sp. PCC 7002

Since cyanobacteria were reported to be very sensitive to detergents, glassware (tubes, Erlenmeyer flasks, Schott® flasks) was incubated in dd$H_2O$ overnight before autoclaving. If not stated otherwise, *Synechococcus* sp. PCC 7002 cultures were grown in AA+ medium supplemented with vitamin $B_{12}$ to a final concentration of 4 µg/L under red-light conditions, as described previously, and constant shaking at 100 rpm. Agar plates were prepared using 15 g/L Agar-Agar in 1 L of AA+ basic medium, before other media components were added. For storage, 475 µL of the culture was added to 25 µL of DMSO (final concentration of 5 %) and stored in the strain collection at -80 °C.

### 2.4.5. Cultivation of *Synechococcus* sp. PCC 7002 in different scales

Some experiments require simultaneous cultivation of a large number of different strains, making it necessary to grow them in a scale smaller than 100 mL Erlenmeyer flasks. In general, *Synechococcus* sp. PCC 7002 can be cultivated in (sterile, non-coated) well plates, with and without shaking. In the experiments described in this thesis, pre-cultures and colonies picked from agar plates were frequently grown in 24-well plates in a total volume of 1 mL. In the experiments describe in section 3.6 (p. 62, GFP reporter assay) and section 3.9 (p. 72, RF operon with different promoters), however, the experimental cultures were cultivated in 6-well plates in a total volume of 5 mL (Figure 8).

Several issues have to be considered when cultivating cyanobacteria in small scale. First, compared to cultures cultivated in Erlenmeyer flasks, cultures cultivated in 6-well plates usually grow faster and reach a higher $OD_{730}$. An explanation for this phenomenon could be that the surface area per volume is higher in 6-well plates, compared to Erlenmeyer flasks, resulting in a more optimal illumination and gas exchange. The shape of the Erlenmeyer flasks probably also cause some of the light to be reflected at the glass surface, whereas the orthogonal orientation of the well plate cover to the light path results in approx. 90% transmission of incoming light (as measured with a lux meter).

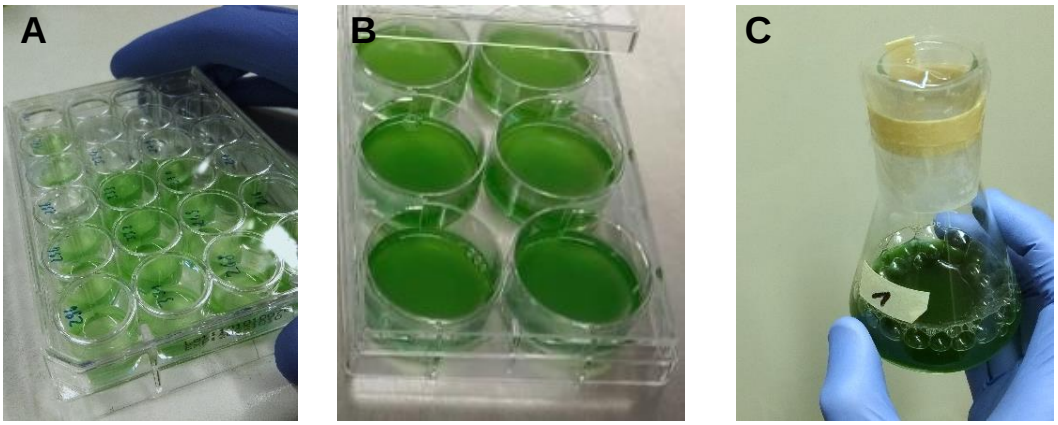



**Figure 8: Growth of *Synechococcus* sp. PCC 7002 in different scales. (A)** Pre-cultures were usually cultivated in 24-well plates. **(B)** The 6-well format was used for experimental cultures in case of high sample number. **(C)** Preferably, cultures were cultivated in 100 mL Erlenmeyer flasks using custom-made transparent caps.

Secondly, evaporation is a major influential factor when cultivating cyanobacteria, since long incubation times inevitably lead to a loss of culture volume that could falsely suggest

a higher $OD_{730}$. This is especially the case for cultures grown in 6-well plates, which have a large surface area per volume. To adjust for evaporation, plates were weighted before sampling and $^1/_6$ of the lost weight was refilled per well using $ddH_2O$. However, this strategy does not adjust for the difference of evaporation that occurs depending on well position (Figure 9 A). Evaporation leads to an increased concentration of cells and media components (e.g. flavins), which is exemplarily shown for a set of recombinant *Synechococcus* sp. PCC 7002 strains in Figure 9 B. Normalization reduces the variability within each set of triplicates (Figure 9 C).

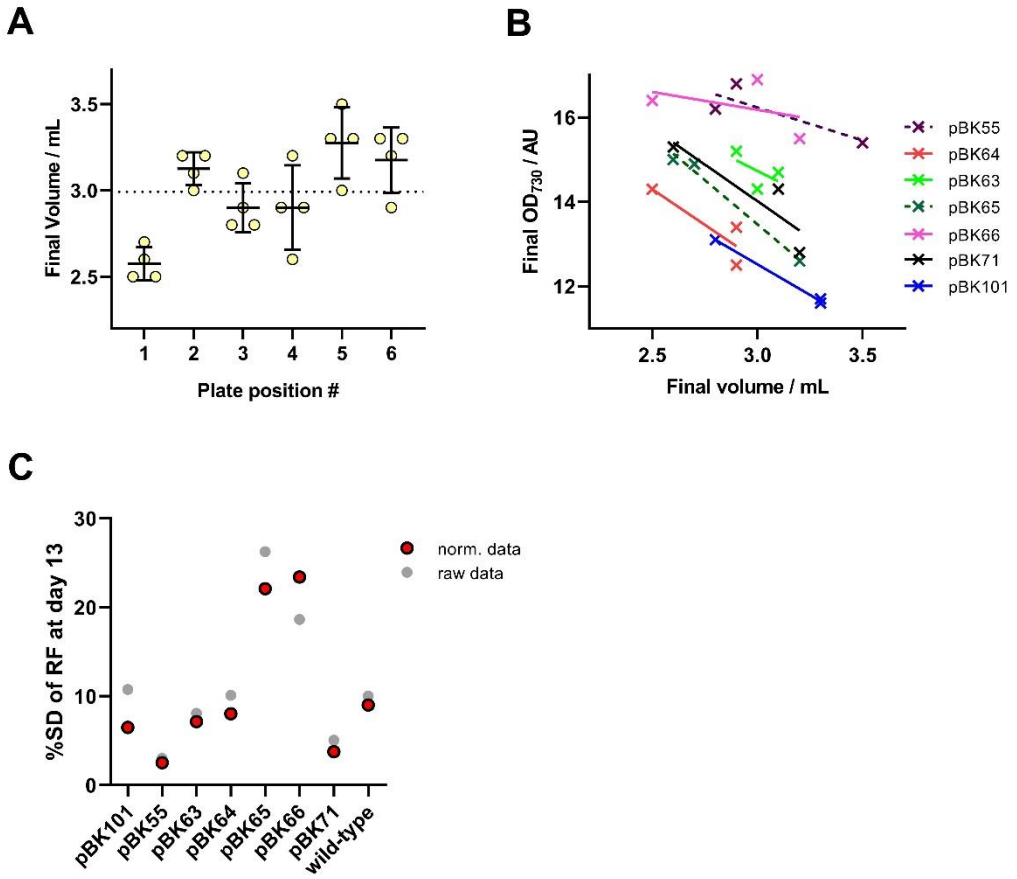



**Figure 9: Normalization of data to adjust for evaporation.** Exemplarily, the riboflavin-producing *Synechococcus* sp. PCC 7002 variants described in Section 3.9, p. 72, are shown. **(A)** The final $OD_{730}$ of the experimental cultures differs depending on the position of the well in the 6-well plate. Wells were numbered from top left to bottom right (horizontal plate). **(B)** Correlation between final volume and the final $OD_{730}$ of each *Synechococcus* sp. PCC 7002 strain illustrates the influence of evaporation on the measured $OD_{600}$ values. **(C)** The relative standard deviation (%SD) of the riboflavin data points at day 13 are given for each strain triplicate before and after normalization. For most strains normalization reduced the variability within each data set.

Due to the evaporation bias, cultivation of *Synechococcus* sp. PCC 7002 in 6-well plates is only recommended in case of a screening procedure using a large number of strains. Whenever possible, Erlenmeyer flasks should be used, since evaporation can be easily adjusted for by weighting each flask separately. Therefore, this cultivation method was chosen for the major experiments described in section 3.10 (p. 75, characterization of best-performing riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains) and section 3.15 (p. 90, transformation of roseoflavin-selected strains with the *B. subtilis* riboflavin biosynthesis operon).

### 2.4.1. Lysis of *Synechococcus* sp. PCC 7002 by bead beating

*Synechococcus* sp. PCC 7002 culture samples were collected in FastPrep tubes (MP Biomedicals, USA) and centrifuged at 2000 x g for 5 min. Subsequently, ~200 µL of glass beads (300 µm) and 200 µL of AA+ medium was added. The lysis was performed using the FastPrep device (MP Biomedicals, USA). Number and duration of cycles, as well as the speed of the FastPrep device depends on the buffer system used. If no lysis buffer is used, the speed was set to 7 m/s and the cells were lysed for 30 sec, kept on ice for 1 min, which iterated two more times.

### 2.4.2. Transformation of *Synechococcus* sp. PCC 7002

*Synechococcus* sp. PCC 7002 is naturally competent and integrates heterologous DNA sequences via homologous recombination [32]. Therefore, a suicide plasmid is required that contains the DNA fragment of interest flanked by homology sequences of about 250 - 1250 bp, displaying increasing integration efficiency with longer homology arms [111]. A selective antibiotic resistance marker needs to be present within the flanking regions, to select for integration-positive clones on agar plates containing the respective antibiotic. In principle, this method of integration can be applied to every genetic locus in *Synechococcus* sp. PCC 7002. However, integration should preferably be targeted to so-called “neutral sites” that have little or no effect on the host strain when the natural sequence is disrupted. For *Synechococcus* sp. PCC 7002, several of these neutral sites were found to be located in

the genome [111, 112], but sites have also been described on the endogenous plasmids pAQ1 and pAQ3 [113].

For this thesis, a suicide plasmid was created for integration of heterologous DNA sequences into "Neutral Site 2" (NS2) at the genomic locus between *SYNPCC7002_A1202* and *SYNPCC7002_A1203* [111], using a kanamycin resistance cassette amplified from the pUR_dualTag plasmid (AG Wilde, Freiburg) and homology arms of approx. 750 bp length amplified from the *Synechococcus* sp. PCC 7002 genomic DNA (***Figure 10*** A). For transformation, 100-500 ng of the plasmid was linearized via restriction enzyme digest, the mix was heated to 80 °C for 5 min to minimize risk of contamination, and subsequently added to ca. 2 mL a fresh *Synechococcus* sp. PCC 7002 wild-type culture of an $OD_{730}$ of ~1.0 in a standard laboratory glass tube. The transformation culture was incubated under standard white or red light conditions. The next day, the transformation culture was centrifuged at 2000 x g for 5 min, resuspended in ca. 200 µL medium streaked on a selective plate containing 100 µg/mL kanamycin, usually resulting in 10 - 100 integration-positive colonies appearing after 6-8 days (Figure 10 B).

Different (and more complex) transformation protocols exist [112, 114], which can be explored in case this straight-forward protocols does not yield enough colonies for the required application.

*Synechococcus* sp. PCC 7002 contains multiple copies of the chromosome and integration usually does not occur into all copies after the first round of selection. Therefore, cPCR of the integrated region initially reveals a mix of the wild-type and integrated fragment (Figure 10 C). Complete segregation, i.e. the complete integration of a heterologous DNA fragment into each copy of the chromosome, can be achieved by collecting a colony from the selective plate and "re-streaking" it on a plate with higher antibiotic concentration. In my experience, 150 and 200 µg/mL kanamycin for two subsequent re-streaks works well to achieve complete segregation of DNA fragments into NS2.

For integration into the locus on the endogenous plasmid pAQ3 (described in section 3.7, p. 66), I recommend kanamycin concentrations of up to 300 µg/mL to achieve complete segregation. However, for the locus on the high-copy endogenous plasmid pAQ1 complete segregation was never achieved with the kanamycin resistance cassette using up to 3000 µg/mL kanamycin.

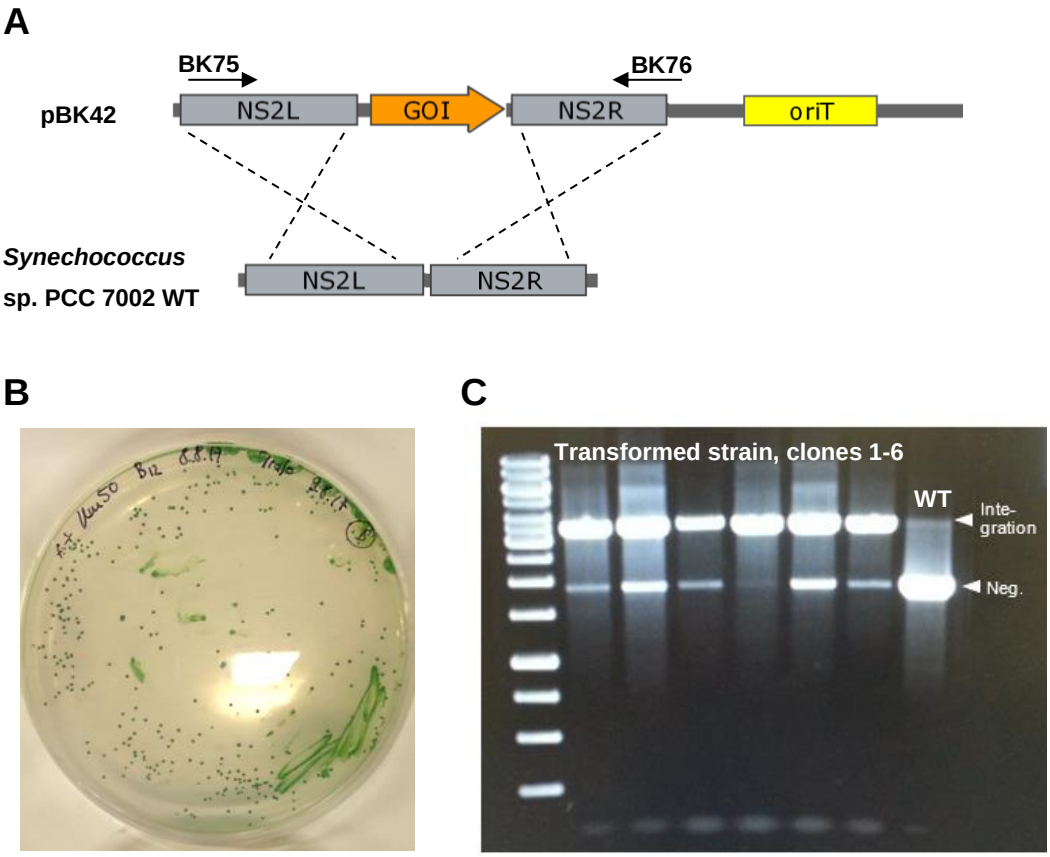



**Figure 10: Transformation of a kanamycin resistance cassette into NS2 of *Synechococcus* sp. PCC 7002. (A)** The plasmid pBK42 contains a kanamycin resistance cassette as a gene of interest (GOI) flanked by homology regions for integration into the genomic locus *SYNPCC7002_A0159* (NS2). **(B)** Integration-positive clones were selected on an AA+ 1% agar plate containing 4 µg/L vitamin $B_{12}$ and 50 µg/mL kanamycin. Positive clones appear as green cfus after several days of incubation. **(C)** Cfus were analysed by cPCR, using primers (BK75/BK76) that bind in the flanking homology regions of NS2. Incomplete segregated clones reveal bands that display both the integrated and the wild-type fragment. L: DNA Ladder 1kb plus; WT: wild-type as template strain.

## 2.5. Molecular Biology Methods

### 2.5.1. Standard cloning procedure in *E. coli* DH5 alpha

If not stated otherwise, molecular cloning was performed in *E. coli* DH5 alpha. DNA fragments of interest were amplified by Polymerase Chain Reaction (**PCR**) using Phusion High-Fidelity Polymerase (Thermo Fisher, Germany) according to the manufacturer's protocol. Amplified DNA fragments were visualized on a 1% **Agarose Gel** using Ethidium bromide (EtBr) staining. Depending on the efficiency of product formation, either the initial PCR reaction mix was purified using a **PCR purification** kit (Thermo Fisher), or the fragment was cut from the gel and purified using a **gel extraction** kit (Thermo Fisher), respectively. The purified DNA solution was quantified using the Nanoview device.

Cloning was performed via **standard restriction enzyme cloning** using Fast-Digest enzymes (Thermo Fisher) as recommended by the manufacturer. Fragments were ligated into an appropriately digested vector backbone using **T4 DNA Ligase** (Thermo) according to the manufacturer's recommendation. For **transformation of ligated fragments**, 5 µl of the reaction mix was added to 50 µL of chemically competent *E. coli* DH5 alpha cells previously thawed on ice for 20 min. For **transformation of purified plasmid**, 1-5 ng of plasmid were used. The mix was incubated for 25 min and subjected to a heat shock at 42 °C for 45 seconds using either a heat block or a water bath. The mix was incubated on ice for 2-5 minutes. Subsequently, 1 mL of LB medium was added and the cells were recovered for 1 hour at 37 °C, 200 rpm, in a 2 mL Eppendorff tube. Cells were centrifuged at 3000 x g for 1 min, resuspended in 100 µL LB medium and spread on a **LB agar plate** containing the appropriate antibiotic. The plate was incubated at 37 °C overnight. If necessary, colony PCR (**cPCR**) was performed by using partial cell mass of the colony forming unit as template for a Dream-Taq mediated PCR. The reaction mix was heated to 95 °C for 2 min before following standard protocol. Colonies were picked and cultivated in 3 mL LB medium in a glass tubes at 37 °C, 220 rpm, for 12-16 hours. Plasmid DNA was extracted using the Plasmid **Miniprep** Kit (Thermo Fisher) according to the manufacturer's protocol. **Sequencing** was performed at LGC Genomics GmbH according to the company's recommendation.

### 2.5.2. Reverse Transcriptase (RT)-PCR

RNA was extracted from cultures grown to an $OD_{730}$ of ~1.5 using the *Quick RNA MiniPrep Plus Kit* (Zymo Research Europe, Germany) according to the manufacturer's recommendations. cDNA was synthesized from ~100 ng total RNA using the *Maxima™ H Minus cDNA Synthesis Master Mix* (Thermo Scientific, Germany) containing random hexamer primers. To rule out DNA contamination, a DNAse digestion step was performed before cDNA synthesis. As a negative control, the reaction was performed omitting the reverse transcriptase (RT). Subsequently, 0.5 µL of the cDNA was PCR-amplified in a 25 µL reaction volume using the *Dream Taq* DNA polymerase (Thermo Scientific, Germany) with specific primers for the respective genes.

### 2.5.3. Extraction of genomic DNA and whole genome sequencing

*Synechococcus* sp. PCC 7002 cultures were grown in 50 mL liquid cultures to an $OD_{730}$ of 1.0-2.0. Cultures were harvested and aliquoted into fractions containing $4*10^8 – 2*10^9$ cells. Samples were centrifuged at 2500 x g for 10 min and the pellet was stored in -80 °C. Genomic DNA was extracted using the *NucleoSpin Microbial DNA Kit* (Macherey-Nagel, Germany). To generate samples meeting the requirements of the λ=260/280 and λ=260/230 ratios of 1.8-2.0 and 2.0-2.2, respectively, the protocol was modified using additional washing steps with buffers BW and B5. Samples were whole-genome sequenced by Eurofins Genomics (Konstanz, Germany) using the INVIEW resequencing method for genomes up to 50 Mb, to take into account the polyploidy of *Synechococcus* sp. PCC 7002 [115]. Variant detection bioinformatics analysis was performed by Eurofins Genomics.

## 2.6. Biochemical Methods

### 2.6.1. Chlorophyll-α extraction from *Synechococcus* sp. PCC 7002

*Synechococcus* sp. PCC 7002 samples were centrifuged at 14000 rpm for 7 minutes in a tabletop centrifuge. The pellet was resuspended in 100% ice-cold Methanol (MeOH) and stored in the fridge for 30 min at 4 °C in the dark. Subsequently, the reaction mix was centrifuged again at 14000 rpm for 7 minutes. Supernatant was analyzed with a spectrometer, measuring absorbance 664 nm. Chlorophyll-α content was calculated using the coefficient $E_{\lambda}$=12.9447 [116].

### 2.6.2. Purification of Strep-tagged RibH from *Synechococcus* sp. PCC 7002

The *ribH* gene from *Synechococcus* sp. PCC 7002 (*SYNPCC7002_A0136*) was cloned into the *StarGate® Acceptor Vector* pPSG_IBA3 (IBA GmbH, Germany) coupled to an N-terminal strep tag (pBK128), or a C-terminal strep tag (pBK129), respectively. An empty pPSG_IBA3 plasmid was used as negative control. Plasmid variants were transformed into *E. coli* BL21(DE3). Cfus were picked from the LB plate, cultivated overnight in 3 mL LB medium containing 100 µg/mL ampicillin, at 30 °C, 200 rpm. Subsequently, the cultures were diluted to $OD_{595}$ of 0.05 in 100 mL LB, using a 300 mL shake flask, and cultivated at 37 °C and 200 rpm. Cultures were induced with 500 µM IPTG at $OD_{595}$ of 0.5-0.6 and grown for 3 hours. Subsequently, cultures were harvested on ice and centrifuged at 4500 x g for 10 min at 4 °C. The pellet was washed with ice-cold dd$H_2O$ and stored at -20 °C until further processing.

On the day of purification, the pellet was resuspended in ice-cold Buffer W pH 8.0, containing one tablet *cOmplete™ protease inhibitor* (Roche, Switzerland) per 50 mL buffer. Cells were lysed using the FrenchPress to obtain the "crude lysate". The samples were centrifuged at 8000 rpm, 4 °C for 15 min to separate major cellular debris. The supernatant was transferred to ultracentrifugation tubes and ultracentrifuged with the rotor JA-30.50 at 30000 rpm, 30 min, at 4 °C. The supernatant was labelled "cleared lysate". Samples were

subsequently purified via a sepharose column (IBA, Germany), using a column volume of 1.5 mL. Strep-Tag purification was done according to the manufacturer's protocol. Protein content in the fractions was analyzed using the standard Bradford method.
Samples were analyzed using standard denaturing SDS PAGE. All samples were stored by adding Glycerol (50 %) to a final concentration of 25 %. For the RibH assay, a final concentration of 5 µM purified protein was used.

### 2.6.3. RibH coupled enzymatic assay

The RibH assay was performed as described previously [78]. All substrates and a purified *B. subtilis* RibH enzyme (positive control) were kindly provided by the group of Markus Fischer, Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Germany.

**Table: Components of the RibH enzymatic assay.** DHBP: 3,4-dihydroxy-2-butanone 4-phosphate; ARPP: 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedion-5'-phosphat; YcsE: *B. subtilis* phosphatase (EC:3.1.3.104); RibH: 6,7-dimethyl-8-ribityllumazin synthase; EDTA: Ethylenediaminetetraacetic acid; TCA: Trichloroacetic acid;

| Compound | Final concentration |
|---|---|
| 10x Buffer | 50 mM Tris-HCl, pH 8.0, 30 mM KCl, 5 mM $MgCl_2$, 1 mM DTT |
| DHBP | 200 µM |
| ARPP | 100 µM |
| YcsE | 0.5 µM |
| RibH | 5 µM |
| → EDTA | 20 mM |
| → TCA | 5 % (w/v) |

The assay was performed in 50 µL reaction volume. After incubation at 23 °C for 10 min, EDTA was added to a final concentration of 20 mM. The mix was further incubated at 37 °C for 20 min. TCA was added to a final concentration of 5 % (w/v). The mix was incubated on ice for 10 min, centrifuged at 14000 rpm for 10 min, filtrated and applied to the HPLC, using the flavin protocol described in the materials and methods section. The

final product 6,7-dimethyl-8-ribityllumazine elutes after ca. 4.7 min and can be detected using the fluorescence detector set to 410 nm excitation and 490 nm emission wavelength.

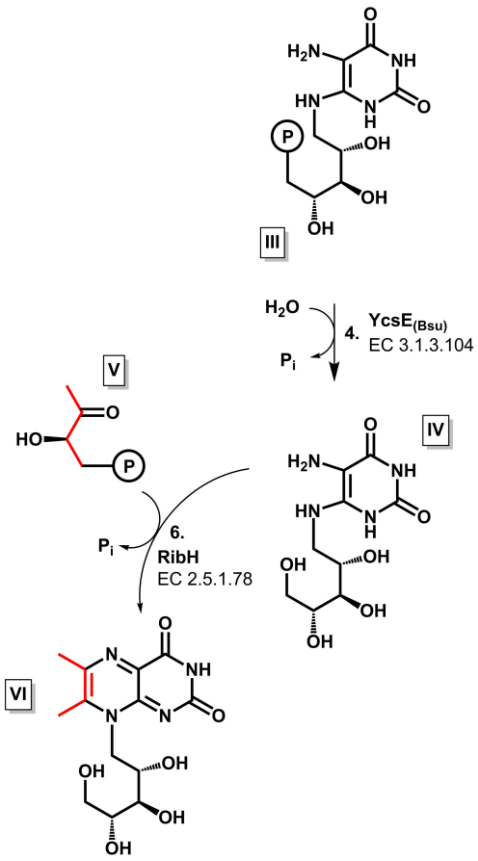



**Figure 11: RibH assay.** The riboflavin biosynthesis enzyme RibH catalyzes the reaction from 5-Amino-6-(D-ribitylamino)uracil (IV) and 3,4- Dihydroxy-2-butanon 4-phosphat (V) to 6,7-Dimethyl-8-(1-D-ribityl)lumazine (VI). Carbon molecules transferred in this reaction are highlighted in red. The *B. subtilis* phosphatase YcsE (EC:3.1.3.104) was used to produce IV from 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione -5'-phosphat (III). The product 6,7-Dimethyl-8-ribityllumazine (VI) was measured by HPLC using the fluorescence detector at 410 nm excitation and 490 nm emission wavelength.

## 2.7. Analytical Methods

### 2.7.1. GFP and flavin measurement *via* plate reader

**GFP** fluorescence was measured using the Tecan GeniusPro® plate reader at 485 nm excitation wavelength and 535 nm emission wavelength. If not stated otherwise, samples were diluted to $OD_{730}$ or $OD_{600}$, respectively, of 0.4 in 100 µL $ddH_2O$ per well of a flat-bottom black 96-well plate (Greiner Bio-One, Frickenhausen, Germany). The gain setting "optimal" was used, therefore calculating the optimal gain for each measurement.
The same protocol was used to get an estimate of **flavins** in the supernatant of cyanobacterial cultures. Since several compounds (e.g. riboflavin, FMN, FAD, and probably other unknown compounds) fluoresce under these conditions, all measurements were considered preliminary and samples were further analyzed with HPLC, if necessary. The instrument gain of the Tecan plate reader was usually set to 25 when analyzing flavins.

### 2.7.2. Flavin measurement by high-performance liquid chromatography (HPLC)

To analyze different flavin compounds from culture supernatant or cell lysate, samples were centrifuged at 14,000 rpm for 10 min. Subsequently, 10% (v/v) of 50% (w/v) trichloroacetic acid (TCA) was added to the supernatant and the samples were centrifuged again for 14,000 rpm for 10 min. Each sample was filtered through a 0.22 µm syringe-filter into a brown HPLC vial and stored at 14 °C until measurement. HPLC analysis was performed on a 1260 Agilent HPLC system using a Kinetex Biphenyl column (Phenomenex, Germany). The column was equilibrated with 85% Buffer A (10 mM formic acid, 10 mM ammonium formate, pH=3.7) and 15% MeOH. The column was heated to 50 °C and loaded with 2-20 µL sample. The HPLC protocol is given in Table 6:

**Table 6: HPLC protocol used for separation of flavins.** The percentage of Buffer A of total buffer composition over time is given. Ultrapure MeOH is used as second buffer component.

| Time / min | % Buffer A |
|---|---|
| 0 | 85 |
| 3 | 77 |
| 3.1 | 73 |
| 5 | 70 |
| 6.5 | 68 |
| 13.5 | 68 |
| 20 | 5 |
| 20.1 | 0 |
| 24.1 | 0 |
| 24.5 | 100 |
| 29.5 | 100 |
| 30 | 85 |
| 35 | 85 |

Using this protocol, flavin compounds can be separated distinctively (Figure 12). In general, flavins were quantified via absorbance measurement at 450 nm. Lumichrome (LC) was measured at 350 nm since it does not absorb at 450 nm. 6,7-dimethyl-8-ribityl-lumazine (LZ) shows an absorbance maximum at 410 nm and was measured with the fluorescence detector set to 410 nm excitation and 490 nm emission (Figure 13).

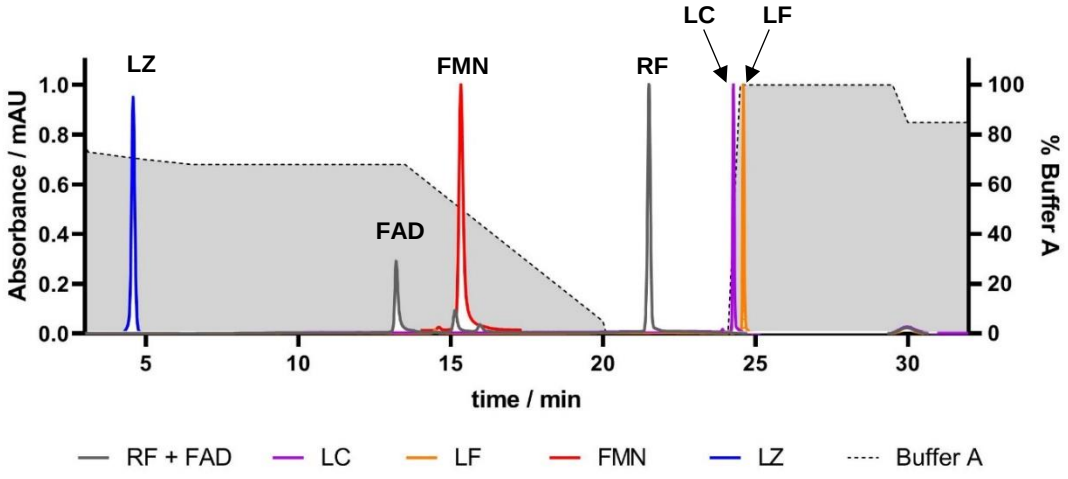



**Figure 12: Overlay of chromatograms of different flavin compounds analyzed in this study.** 6,7-dimethyl-8-ribityl-lumazine (LZ) elutes at ~4.7 min, FAD at ~13.2 min, FMN at ~15.3 min, riboflavin at ~21.5 min, lumichrome (LC) at ~24.3 min, lumiflavin (LF) at ~24.6 min. Exact elution times vary slightly depending on the buffer batch.

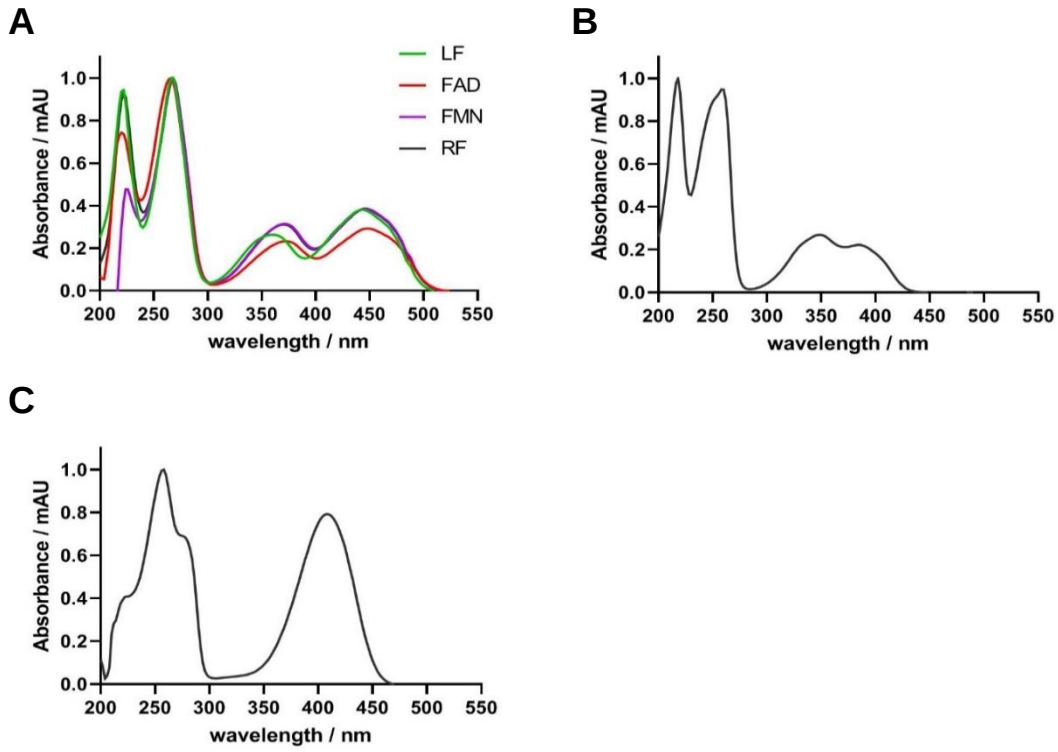



**Figure 13: Absorbance spectrum of flavins measured in this study. (A)** Lumiflavin (LF), FAD, FMN and riboflavin show absorbance maxima at 365 nm and 445 nm. **(B)** Lumichrome shows absorbance maxima at 350 nm and 385 nm. **(C)** 6,7-dimethyl-8-ribityl-lumazine shows one distinct absorbance maximum at 410 nm. The peaks below a wavelength of 300 nm are not useful for the measurement, since a lot of cellular components absorb in this range.

### 2.7.3. Identification of riboflavin biosynthesis enzymes in cellular lysates[2]

**Sample preparation:** Liquid cultures of *Synechococcus* sp. PCC 7002 variants were cultivated in standard red-light conditions until $OD_{730}$ of approx. 2.0. Subsequently, 8 mL of culture was centrifuged at 2500 x g for 5 min and the pellet was resuspended in 400 µL of Buffer W containing one tablet of cOmplete ™ protease inhibitor (Roche, Germany) per 50 mL buffer. Cells were lysed by bead beating as described previously.

[2] All paragraphs in this section were adopted from the Core Facility for Mass Spectrometry & Proteomics, ZMBH Heidelberg, with the permission of Dr. Renata Blatnik.

*E. coli* strains expressing *B. subtilis* riboflavin biosynthesis genes were used as positive control. Cultures were grown over night at 37 °C, 220 rpm and lysed by bead beating. Protein concentration was measured using Bradford.

**SDS PAGE:** A 0.5 µg/µL protein solution was prepared containing 1x Laemmli Buffer and denatured at 95 °C for 15 min. Samples were run on a 10% Bis-Tris gel at the Core Facility for Mass Spectrometry & Proteomics, ZMBH Heidelberg, using 1x MOPS buffer with 10 mM DTT in the gel chamber and native 1x MOPS buffer outside the gel chamber. The gel was run at 80 V for 10 min and at 110 V for 45 min. Fixation, Coomassie staining, destaining and storage was performed by the Core Facility according to their standard protocol. Protein bands of interest were manually excised from gels. The gel pieces were washed once with 60 µL of 1:1 (v/v) 50 mM triethylammonium bicarbonate buffer (TEAB; Sigma-Aldrich, Taufkirchen, Germany) and acetonitrile (ACN; Roth, Karlsruhe, Germany), pH 8.5 for 10 min and shrunk three times for 10 min each in 60 µL ACN and washed in 60 µL 50 mM TEAB, pH 8.5.

**In-gel digestion:** Gel pieces were dehydrated with 60 µL 100% ACN. A total of 70 µL of 8 ng/µL in 50 mM TEAB trypsin solution (sequencing grade, Thermo-Fisher, Rockford, USA) was added to the dry gel pieces and incubated 4 h at 37 °C. The reaction was quenched by addition of 20 µL of 0.1% trifluoroacetic acid (TFA; Biosolve, Valkenswaard, The Netherlands). The resulting peptides were extracted once for 15 min with 50 µL 1:1 (v/v) 50 mM TEAB and ACN, pH 8.5 and once for 15 min in 70 µL ACN. The supernatant from each extraction step was collected and dried in a vacuum concentrator before LC-MS analysis.

**LC-MS:** Nanoflow LC-$MS^2$ analysis was performed with an Ultimate 3000 liquid chromatography system coupled to an Orbitrap Elite or an Orbitrap Q Exactive mass spectrometer (Thermo-Fischer, Bremen, Germany). Samples were delivered to an in-house packed analytical column (inner diameter 75 µm x 20 cm; CS – Chromatographie Service GmbH, Langerwehe, Germany) filled with 1.9 µm ReprosilPur-AQ 120 C18 material (Dr. Maisch, Ammerbuch-Entringen, Germany). Solvent A was 0.1% formic acid (FA; ProteoChem, Denver, CO, USA) in $H_2O$ (Bisolve) and solvent B was composed of 0.1% FA (ProteoChem), 10% $H_2O$ (Biosolve) and 89.9% ACN (Biosolve). Sample was loaded to the analytical column for 20 min with 3% B at 550 nL/min flow rate. Peptides were

separated with 1h linear gradient (3-40% B) with flow rate of 300 nL/min. The Elite mass spectrometer was operated in data-dependente acquisition mode, automatically switching between MS and $MS^2$. MS spectra (m/z 400–1600) were acquired in the Orbitrap at 60,000 (m/z 400) resolution, with an automatic gain control (AGC) target value of $10^6$ and maximal ion injection time (IT) 10 ms. Collision induced dissociation $MS^2$ spectra were generated for up to 15 precursors with normalized collision energy of 35% in the ion trap. The $MS^2$ AGC target value was set to $10^4$ with a maximum IT of 100 ms.
The Q Exactive mass spectrometer was operated in data-dependent acquisition mode, automatically switching between MS, acquired at 60,000 (m/z 400) resolution, and $MS^2$ spectra, generated for up to 15 precursors with normalized collision energy of 27% in the HCD cell and measured in the Orbitrap at 15,000 resolution. The $MS^2$ AGC target value was set to $10^5$ with a maximum IT of 50 ms.

**Protein Identification:** Raw files were analyzed using Proteome Discoverer with the Sequest (Thermo Fisher Scientific, San Jose, USA; version 2.2). Sequest was set up to search against Uniprot *Synechococcus* sp. PCC 7002 database (retrieved in March, 2020), common contaminants and the target proteins RibD, RibE, RibAB, RibH and RibT from *B. subtilis*, with trypsin as the digestion enzyme. A fragment ion mass tolerance was set to 0.50 Da and a parent ion mass tolerance to 10 ppm. Carbamidomethylation of cysteine was specified as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and acetylation of the protein N-terminus were specified as variable modifications. The peptide and protein identity was verified by Scaffold (version Scaffold_4.8.4, Proteome Software Inc., Portland, USA). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm [117] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [118]. Proteins that contained similar peptides and could not be differentiated based on $MS^2$ analysis alone were grouped to satisfy the principles of parsimony.

### 2.7.4. Relative quantitation of riboflavin biosynthesis enzymes in cellular lysates[3]

Samples were prepared as described previously and processed in the Core Facility for Mass Spectrometry & Proteomics, ZMBH Heidelberg as described previously.

**Dimethyl labelling:** Dimethyl duplex labeling was performed according to standard protocol [119]. Briefly, digested peptides from samples were tagged in solution with stable-isotope dimethyl labels comprising regular formaldehyde and cyanoborohydride (28 Da shift, designated "light label") or deuterated formaldehyde and regular cyanoborohydride (32 Da shift, designated "intermediate label") (all reagents from Sigma-Aldrich, Taufkirchen, Germany). Samples were mixed in equal amounts, acidified with TFA such that the pH<2 and desalted with C18 stage tips as described in [120]. Samples were dried in a vacuum centrifuge and stored in -20°C until LC-MS analysis.

**LC-MS measurements** were performed as described above.

**Protein identification and relative quantification of dimethyl labeled samples with MaxQuant:** Raw files were processed using MaxQuant (version 1.5.3.30; [121]) for peptide identification and quantification. $MS^2$ spectra were searched against the Uniprot *Synechococcus* sp. PCC 7002 database (retrieved in March, 2020), common contaminants and the target proteins RibD, RibE, RibAB, RibH and RibT from *B. subtilis* by Andromeda search engine with the following parameters: Carbamidomethylation of cysteine residues as fixed modification and Acetyl (Protein N-term), Oxidation (M) as variable modifications, trypsin/P as the proteolytic enzyme with up to 2 missed cleavages allowed. The maximum false discovery rate for proteins and peptides was 0.01 and a minimum peptide length of 7 amino acids was required. Match between run option was not activated. Quantification mode was with the dimethyl Lys 0 and N-term 0 as light labels and dimethyl Lys 4 and N-term 4 as intermediate labels. All other parameters were default parameters of MaxQuant. Quantitative normalized ratios were calculated by MaxQuant and used for further data analysis.

[3] All paragraphs in this section were adopted from the Core Facility for Mass Spectrometry & Proteomics, ZMBH Heidelberg, with the permission of Dr. Renata Blatnik.

## 2.8. Cloning overview

A comprehensive list of plasmids created in this study is given in Table 16, p. 132). The following section gives an overview about how plasmids were cloned.
All DNA fragments were PCR-amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Germany), digested with High-Fidelity restriction enzymes (Thermo Scientific) and ligated with T4 DNA ligase according to the manufacturer's recommendations.

**Plasmid for integration of heterologous DNA fragments into NS2 of *Synechococcus* sp. PCC 7002:** A shuttle vector for integration of heterologous DNA fragments at the NS2 ("neutral site 2") locus of *Synechococcus* sp. PCC 7002 [111] was cloned based on the high-copy plasmid pIDTsmart (Integrated DNA Technologies, USA). Homology fragments of ~750 nt for homologous recombination were PCR-amplified from the *Synechococcus* sp. PCC 7002 genome. The upstream homologous region *NS2L* was amplified using the primer pair BK31x/BK32x and the downstream homologous fragment *NS2R* was amplified using primers BK33x/BK34x. *NS2L* was digested with High-Fidelity restriction enzymes *Bsa*I/*Nhe*I, *NS2R* was digested with *Nhe*I/*Xho*I. Both fragments were ligated into an *Eco*RI/*Xho*I-digested pIDTsmart backbone, resulting in the plasmid pBK41.

**GFP variants for integration into NS2:** A GFP expression cassette was created on the basis of the pURdualTag vector provided by the group of Prof. Annegret Wilde (Freiburg, Germany). pURdualTag was linearized with *Bsa*I/*Nde*I and re-ligated using the annealed oligonucleotides BK60/BK61. Therefore, the resulting vector pBK22 does not contain the spectinomycin resistance cassette but provides an additional *Nhe*I restriction site necessary for further cloning. The kanamycin resistance cassette *kmR* was amplified from pBK22 using primers BK69new/BK36x, digested with *Spe*I/*Not*I and ligated into pBK41 to create pBK42. The *Synechocystis* sp. PCC 6803 genome served as template for the promoters *cpc560* (PCR-amplified using primers BK79new/BK80new), *psbA2L* (BK81new/BK83new) and *psbA2s* (BK82new/BK83new). The promoter *A2813* was PCR-amplified from the *Synechococcus* sp. PCC 7002 genome using primers BK64new/BK85. The theophylline-inducible *pTrcTheo* promoter sequence "ENYC4" was obtained from Nakahira et al. [122], ordered as a synthetic DNA fragment and PCR-amplified with

primers BK94/BK95. The superfolder GFP [123] gene was PCR-amplified from the original pURdualTag vector using primers BK66new/BK36x. The fragment was digested with *Bsa*I/*Not*I, fused to the aforementioned promoters digested with *Nhe*I/*Bsa*I and ligated into pBK42 digested with *Nhe*I/*Not*I, yielding the GFP-expression plasmids pBK47 (*NS2L_kmR_cpc560_gfp_NS2R*), pBK48 (*NS2L_kmR_psbA2L_gfp_NS2R*), pBK54 (*NS2L_kmR_A2813_gfp_NS2R*), pBK55 (*NS2L_kmR_psbA2s_gfp_NS2R*) and pBK70 (*NS2L_kmR_pTrcTheo_gfp_NS2R*). Also, the fragment *NS2L_kmR_psbA2s_GFP_NS2R* was PCR-amplified from pBK55 using primers BK31x/BK93, digested with *Bsa*I and inserted into the medium-copy vector pKT25 [124] digested with *Eco*RI/*Bgl*II to yield pBK62.

**GFP variants for integration into pAQ1 and pAQ3:**

The homology regions for integration at the loci of the endogenous *Synechococcus* sp. PCC 7002 plasmids pAQ1 and pAQ3 were describe by Xu et al. [125]. For integration at pAQ1, the upstream homology fragment *flankB* (nucleotide 2605-3011 of pAQ1) was PCR-amplified from the *Synechococcus* sp. PCC 7002 wild-type genome with primers BK128/BK129new and digested with *Bsa*I. The downstream homology fragment *flankA* (nucleotide 4196-4707 of pAQ1) was amplified with BK130/BK131 and digested with *Bsa*I. Both fragments were ligated into pKT25 digested with *Eco*RI/*Xba*I, resulting in the plasmid pBK82. The fragment *kmR_psbA2s_gfp* was PCR-amplified from pBK55 using primers BK69new/BK36x, digested with *Spe*I/*Not*I and ligated into pBK82 digested with *Spe*I/*Not*I, resulting in the plasmid pBK83.

For integration at pAQ3, the upstream homology fragment *flankD* (nucleotides 7200-7705 of pAQ3) was PCR-amplified from the *Synechococcus* sp. PCC 7002 wild-type genome with primers BK142/BK143 and digested with *Bsa*I. The downstream homology fragment *flankC* (nucleotide 7706-8359 of pAQ3) was amplified with BK143/BK144 and digested with *Bsa*I. Both fragments were ligated into pKT25 digested with *Eco*RI/*Xba*I, resulting in the plasmid pBK84. The fragment *kmR_psbA2s_gfp* was digested with *Spe*I/*Not*I and ligated into pBK84 digested with *Spe*I/*Not*I, resulting in the plasmid pBK85.

**Riboflavin biosynthesis operon variants for integration into NS2:**

The riboflavin biosynthesis genes *ribDGEABHT* were amplified from the *B. subtilis* sp. substr. 168 using primers BK26/BK84. The operon codes for the bifunctional RibDG

(EC:3.5.4.26, EC:1.1.1.193), RibE (EC:2.5.1.9), the bifunctional RibAB (EC:3.5.4.25, EC:4.1.99.12), RibH (EC:2.5.1.78) and RibT (function not known), thus also including internal promoters and terminators [126]. Since cloning of the *ribDGEABHT* fragment into the high-copy plasmid pIDTsmart was not successful (probably due to toxicity effects of the *ribAB* gene) the *ribDGEABHT* fragment was digested with *Bsa*I/*Not*I, fused to the promoters *cpc560*, *psbA2s* and *A2813*, digested with *Nhe*I/*Bsa*I and ligated into pBK62 (based on pKT25) digested with *Nhe*I/*Not*I to create the *rib* gene expression plasmids pBK63 (*NS2L_kmR_cpc560_ribDGEABHT_NS2R*), pBK64 (*NS2L_kmR_psbA2s_ ribDGEABHT_NS2R*) and pBK65 (*NS2L_kmR_A2813_ribDGEABHT_NS2R*). A variant of pBK64 was created by adding the promoter *cpc560* between the *ribE* and *ribAB* gene. To this end, the fragment *NS2L_kmR_psbA2s_ribDGE* was amplified from pBK64 using primers BK31x/BK155 and the fragment *ribABHT_NS2R* was amplified with BK93/BK156. Both fragments were digested with *Bsa*I, fused to the *cpc560* promoter digested with *Nhe*I/*Bsa*I and ligated into pKT25 digested with *Eco*RI/*Bgl*II to create the plasmid pBK101 (*NS2L_kmR_psbA2s_ribDGE_cpc560_ribABHT_NS2R*). A variant of pBK101 was created containing the chloramphenicol resistance cassette *cmR* amplified from pUC19*_glgA1_cmR* (Wilde group, Freiburg University) with primers BK184/185 and digested with *Bsa*I. The fragment *psbA2s_ribDGE_cpc560_ribABHT* was amplified from pBK101 using BK82new/pBK36x and digested with *Bsa*I/*Not*I. Both fragments were ligated into the pBK62 vector backbone digested with *Spe*I/*Not*I, creating the plasmid pBK126 (*NS2L_cmR_cpc560_ribDGEABHT_NS2R*).

To generate a synthetic *rib* gene transcription unit codon-adapted for *B. subtilis*, a codon-usage table [127] was created using the ten most abundant *Synechococcus* sp. PCC 7002 genes, according to previously published transcriptomics data [128, 129]. The *ribAB* gene was placed upstream of the *ribDG* and the RuBisCO large subunit (*rbcL*) terminator was added downstream of *ribT*. Intergenic regions between the *rib* genes were left unchanged. The codon-adapted fragment was ordered as a synthetic gene (Thermo Scientific, Germany), amplified using BK86/BK90, digested with *Bsa*I/*Not*I, fused to the *psbA2s* promoter digested with *Bsa*I/*Nhe*I and ligated into pBK62 digested with *Nhe*I/*Not*I to create pBK71. The codon-adapted *ribAB* gene was PCR-amplified using primers BK86/BK98, digested with *Nhe*I/*Not*I, fused to the *psbA2s* promoter digested with *Bsa*I/*Nhe*I and ligated into pBK62 digested with *Nhe*I/*Not*I to create pBK66.

**RFN variants for expression in *E. coli*:** The riboflavin biosynthesis operon *ribDGEABHT* was amplified from the *B. subtilis* genome, using primers BK26/BK29, digested with *Ase*I/*Kpn*I and integrated into pAH06 (obtained from Prof. Jeffrey A. Gralnick, University of Minnesota) digested with *Ase*I/*Kpn*I. The resulting plasmid pBK13 does not contain the RFN element and uses the endogenous ribosomal binding site of the *B. subtilis ribDG* gene. Different RFN elements were amplified from *B. subtilis* strains stored in the lab-internal strain collection (SI Stammsammlung): RFN#1 was amplified from the riboflavin-overproducing strain SI#358 using primers BK21/BK23, digested with *Bsa*I and integrated into pAH13 digested with *Ase*I, resulting in the plasmid pBK18. Accordingly, RFN#2 was amplified from the *B. subtilis* wild-type strain SI#21 using BK21/BK23 (pBK19), RFN#5 was amplified from SI#358 using primers BK22/BK24 (pBK20), RFN#6 was amplified from SI#21 using BK22/BK23 (pBK21). A synthetic RFN based on the wild-type sequence containing a 14nt-deletion in the terminator region was created by Ahmed Boumezbeur (pBK19_QC).

**RBS variant for expression in *E. coli*:** Based on pBK13 a variant was created that contains a different RBS upstream of the *ribAB* start codon. The original RBS 5' AAAGGAGGTATATT '3 was replaced with 5' AGAGGAAGATTTGC '3 (RBS#2) via Phusion site directed mutagenesis (Thermo Scientific) using primers BK152/BK154, creating plasmid pBK94.

**Plasmid for integration of *pnuX* at NS2 of *Synechococcus sp. PCC 7002* wild-type:** The *pnuX* gene (*CYL77_00365*) was amplified from *Corynebacterium glutamicum ATCC 13032* with primers BK62new/BK63, digested with *Bsa*I/*Not*I, fused to the *psbA2L* promoter digested with *Nhe*I/*Bsa*I, and cloned into pBK42 digested with *Nhe*I/*Not*I to create pBK52.

**Plasmids for complementation experiments in *E. coli* riboflavin auxotrophic strains:** The pGP380 expression vector for *B. subtilis* and *E. coli* was used as a vector backbone for expression of the *Synechococcus* sp. PCC 7002 riboflavin biosynthesis genes in *E. coli* riboflavin auxotrophic strains. The gene *SYNPCC7002_A0427* (*ribAB)* was amplified from the *Synechococcus* sp. PCC 7002 genome using primers BK120/BK121, *SYNPCC7002_A1465* (*ribDG)* was amplified using BK122/BK123, *SYNPCC7002_A0498* (*ribD-C-terminal domain)* was amplified using BK159/BK160, *SYNPCC7002_A2264*

(*ribE)* was amplified using BK124/BK125, and *SYNPCC7002_A0136* (*ribH)* was amplified using BK126/BK127. All *rib* gene fragments were digested with *Bsa*I and ligated into pGP380 digested with *Bam*HI/*Hin*dIII to create the plasmids pBK75, pBK76, pBK99, pBK77 and pBK78, respectively. Note that genes are expressed with an N-terminal strep-tag. To generate a *SYNPCC7002_A2264* (*ribE)* expression plasmid with a C-terminal strep-tag, the fragment was amplified from the *Synechococcus* sp. PCC 7002 genome using BK176/BK125, digested with *Bsa*I/*Xba*I and ligated into pGP382 digested with *Xba*I/*Hin*dIII to create pBK103. To create expression plasmids for the expression and purification of *Synechococcus* sp. PCC 7002 RibH in *E. coli* BL21(DE3), the N-terminally strep-tagged *SYNPCC7002_A0136* (*ribH)* was amplified from pBK78 using primers BK194/BK195, digested with *Bsm*BI and ligated into the StarGate® Acceptor Vector pPSG-IBA3 (IBA GmbH, Germany) digested with *Bsm*BI (pBK128). A C-terminally tagged variant was created by amplifying the un-tagged *SYNPCC7002_A0136* from pBK78 using BK196/BK197, digesting with *Bsm*BI and ligating the fragment into the *Bsm*BI-digested pPSG-IBA3 backbone (pBK129).

# III

# Results

## 3.1. Selecting a cyanobacterial host strain for the production of riboflavin

Several cyanobacterial strains have been used for basic research and biotechnological applications [24]. These strains can be genetically manipulated and tools for gene expression and regulation are being developed at increasing speed. Since research groups often are specialized on one particular strain, the choice of the microbial host for metabolic engineering might often be driven by availability and expertice rather than a thorough assessments of a strain's advantages and disadvantages for a certain application. As our group was not confined to a cyanobacterial strain at the start of this project, literature research was performed to find an optimal host strain as a chassis for photoautotrophic riboflavin production. The goal was to use a unicellular cyanobacteria that is amenable for genetic engineering, while being fast-growing and easy to cultivate with relatively low risk of contamination.

One of the most well-studied cyanobacterial laboratory strains is the freshwater strain *Synechocystis* sp. PCC 6803. It was first isolated in 1968 [130] and is used as a model organism for study of the photosynthetic mechanism, since it is able to grow under mixotrophic and heterotrophic conditions [131] - an absolute requirement when photosynthesis is impaired. *Synechocystis* sp. PCC 6803 is naturally competent [132]. However, the reported doubling time is longer compared to most cyanobacteria laboratory strains [35]. Furthermore, it contains a high number of genome copies [115], which makes genomic integration of foreign genes especially tedious. Furthermore, as personal communication with the group of Prof. Annegret Wilde (Freiburg, Germany) revealed that the strain is prone to contamination, I rejected it as a candidate for this study.
*Synechococcus elongatus* sp. PCC 7942 is another fresh-water bacterium first transformed in 1970 [133] which has been used extensively to study the circadian clock [134].
A strain recently under investigation is *Synechococcus* UTEX 2973, a fresh-water cynobacterium closely related to *Synechococcus elongatus* PCC 7942, was reported to have the shortest doubling times of all cyanobacterial model strains so far [35]. However, without genetic modifications this strain is not naturally competent and less well-studied compared to the other candidates at the beginning of this research project.

*Synechococcus* sp. PCC 7002 is one of the few marine model cyanobacterial strains and has been extensively studied in the lab of Prof. Donald Bryant, Pennsylvania State University [56]. Compared to *Synechococcus elongatus* sp. PCC 7942 and *Synechocystis* sp. PCC PCC 6803 it has faster doubling time (~2.6 h was reported under laboratory conditions) [129]. *Synechococcus* sp. PCC 7002 is euryhaline and can be cultivated in a wide range of NaCl concentrations, which might reduce the risk of contamination. Its acclimatization to different temperature, salinity and light conditions was studied extensively [129, 135, 136], showing that this strain is a versatile candidate for large-scale biotechnological applications under a wide range of conditions.

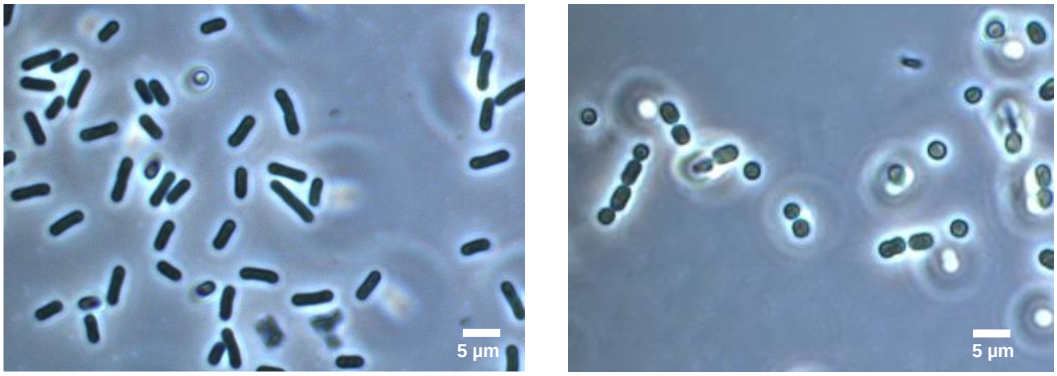



**Figure 14: Morphology of cyanobacterial strains commonly used in research labs.**
**(A)** *Synechococcus* elongatus sp. PCC 7942 displays a rod-shaped phenotype, whereas **(B)** *Synechococcus* sp. PCC 7002 is more roundish and often found in pairs or loose chains of cells. The morphology of *Synechocystis* sp. PCC 6803 is shown in Figure 1 (p. 2).

Genetic engineering of *Synechococcus* sp. PCC 7002 was shown to be rather simple, since the strain is naturally competent [32] and the number of genome copies is relatively low [115]. Integration of foreign genetic fragments occurs via homologous recombination using short homology fragments [111]. Additionally, *Synechococcus* sp. PCC 7002 contains endogenous plasmids [137] with high copy number that can be targeted to achieve significantly higher expression levels compared to expression from loci in the genome [113, 125]. Unfortunately, *Synechococcus* sp. PCC 7002 is not entirely photoautotrophic, since it requires supplementation of 4 µg/L cobalamin (vitamin $B_{12}$). However, a recent study showed that this auxotrophy can be cured [85]. For the aforementioned reasons *Synechococcus* sp. PCC 7002 was chosen as a chassis for overproduction of riboflavin in this study.

## 3.2. Photo-degradation of riboflavin in cyanobacterial growth medium

Cyanobacteria laboratory strains are usually cultivated under artificial white light derived from a fluorescent light-bulb which provides a blend of different wavelengths including ultra-violet and infra-red photons. Riboflavin, on the other hand, is light-sensitive with an absorption spectrum displaying peak maxima at λ=223, 267, 373 and 444 nm in aqueous solutions. Photolysis mostly occurs at the ribityl chain, resulting in diverse photo-degradation products including lumichrome (LC), lumiflavin (LF), formylmethylflavin (FMF), carboxymethylflavin (CMF) and cyclodehydroriboflavin (CDRF) (Figure 15).

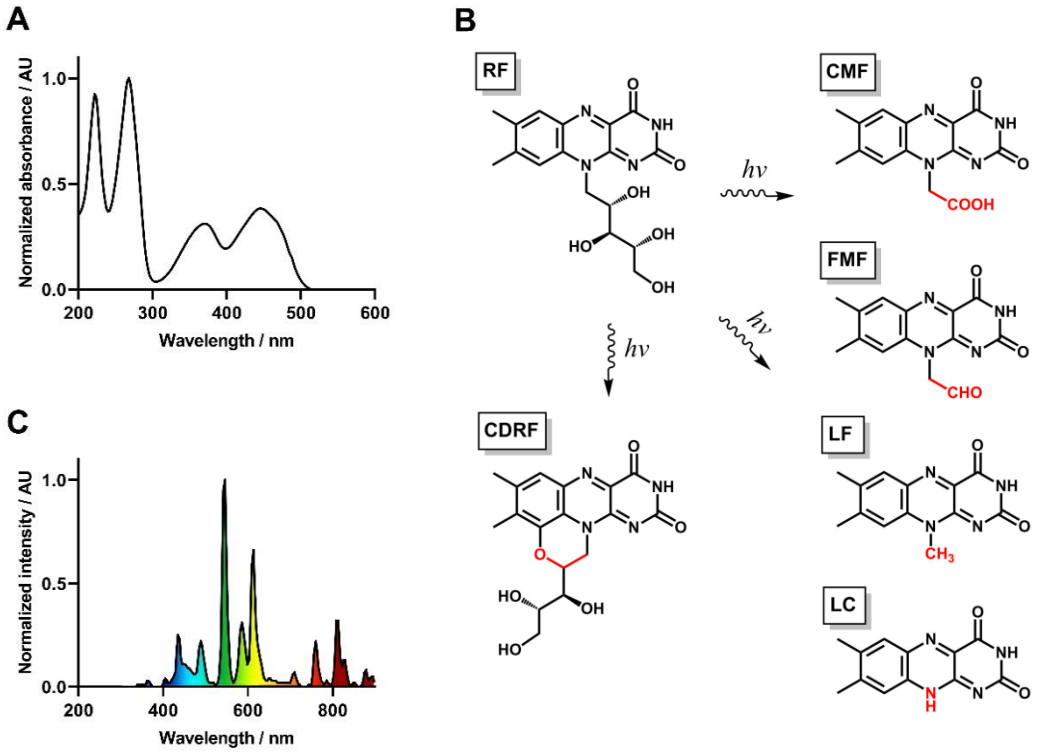



**Figure 15: Light degradation of riboflavin. (A)** Absorbance spectrum of riboflavin in AA+ medium (pH 8.2) shows absorbance maxima in the blue and UV range. **(B)** Major degradation products of riboflavin are lumichrome (LC), lumiflavin (LF), formylmethylflavin (FMF), carboxymethylflavin (CMF) and cyclodehydroriboflavin (CDRF) [44]. **(C)** Light intensity spectrum of a standard white fluorescent light bulb (Philips TLD 18/840) used in this study. Data were acquired in the Beuermann lab (Mannheim University of Applied Science). Colors were added to highlight the visible spectrum.

Formation of riboflavin photo-degradation products in aqueous solution depends on different factors, including pH, buffer and media composition, oxygen content as well as light intensity and wavelength [44].

### 3.2.1. Quantitation of riboflavin degradation under white light conditions

To test the stability of a riboflavin solution in the standard *Synechococcus* sp. PCC 7002 growth medium AA+ (Tris-Buffer, pH 8.2), a riboflavin solution (50 µM) was incubated under white fluorescent light (~40 µE) or in darkness, respectively, at 32 °C and 100 rpm, in triplicates. Samples were taken over the course of 78 hours in white light, and over the course of 42 days in red light. Flavin concentration was measured with HPLC as described in the materials and methods section (p. 36). Since LC and LF were shown to be the most prevalent compounds in aqueous solution under neutral and alkaline conditions [44], only these photoproducts were quantified (Figure 16A).
The riboflavin concentration was fitted using the GraphPad Prism software, applying a one-phase decay non-linear model. The calculated half-life for riboflavin was 0.96 hours in white light and 910 days in dark conditions. As the average growth curve of a cyanobacterial culture in ambient air under similar white light conditions requires ca. 3-4 weeks of incubation time, using a white fluorescent light source would be detrimental for riboflavin stability.

### 3.2.2. Riboflavin is stable under red-light conditions using a customized LED array

It was previously shown that *Synechococcus* sp. PCC 7002 can be cultivated under dichromatic light in the red spectrum, specifically targeting the main photosynthetic pigments phycocyanin (620/630 nm) and chlorophyll-a (680 nm) [139-141]. Growth under monochromatic light was demonstrated as well, with 630 nm being more effective than 680 nm, in terms of growth per illumination [139]. In this study, light-emitting diodes (LEDs) of 630 nm were used as the main light source. Additionally, LEDs of 700 nm were

used in order to specifically excite the P700 reaction center in photosystem I. The construction and properties of the LED-Array are described in the Materials and Methods section 2.3, p. 22. To test the stability of riboflavin under these conditions, a riboflavin solution (50 µM) in AA+ medium was incubated as described above, using the customized red LED array as a source of illumination (Figure 16B). The half-life of riboflavin under these conditions was calculated to be 501 days, which is appropriate for stable overproduction of riboflavin using *Synechococcus* sp. PCC 7002.

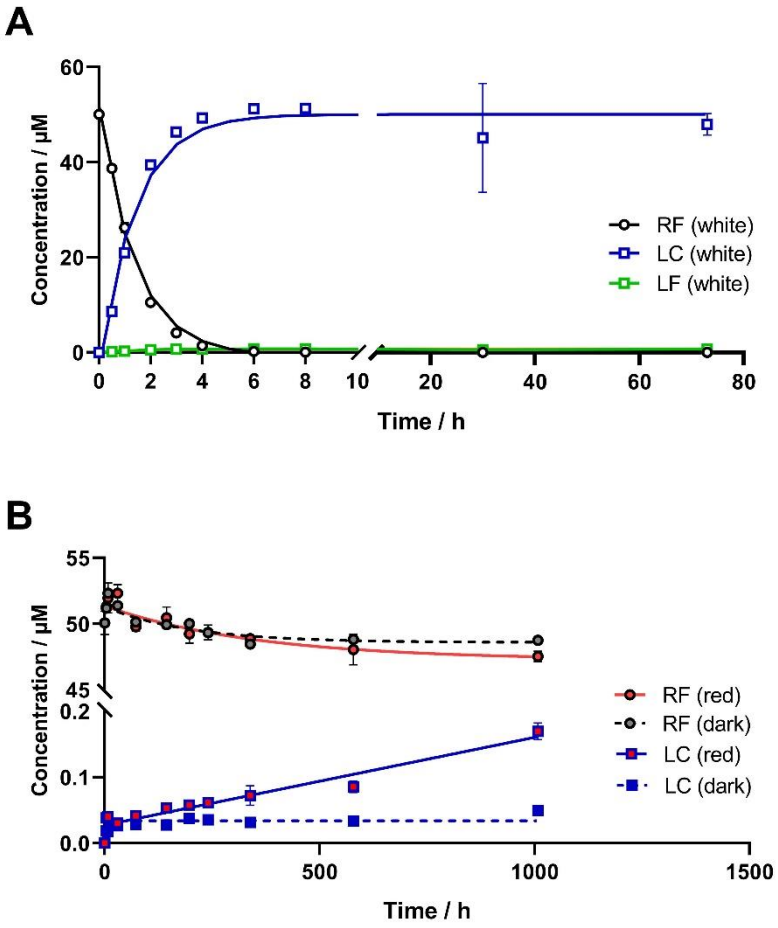



**Figure 16: Riboflavin is degraded under white light but stable under red light conditions. All samples were incubated at 32 °C. (A)** Under white-light conditions, riboflavin (50 µM in AA+ medium) was degraded with a half-life of ca. 1 hour, resulting in formation of lumichrome as major photo-product. Only traces of lumiflavin could be detected (<0.1 µM). **(B)** Riboflavin (50 µM in AA+ medium) is stable under red light and in the dark, with a half-life of of 501 and 910 days, respectively. Only traces of lumichrome could be detected in the sample incubated in red light. Lumiflavin levels were below the detection limit of the HPLC protocol used in this study.

## 3.3. Cultivation of *Synechococcus* sp. PCC 7002 under red-light conditions

To analyze growth of *Synechococcus* sp. PCC 7002 under red light compared to standard white light, a wild-type culture at $OD_{730}$ of 0.1 was cultured in 6-well plates under either monochromatic red light of 630 nm ranging from 6 – 50 µE, dichromatic red-light of 25 µE with a 630:700 nm ratio of 1.0, dichromatic red light of 10 µE with a 630:700 nm ratio of 0.6, and white light (Philips Master TLD 18/840, ~40 µE), respectively. No significant difference in $OD_{730}$ was observed for the different red-light conditions over the course of 15 days (**Figure 17**). However, cells grew to a higher $OD_{730}$ under red light compared to white light. This is in line with the fact that the white light source mainly produces photons of wavelengths that cannot be utilized by cyanobacteria.

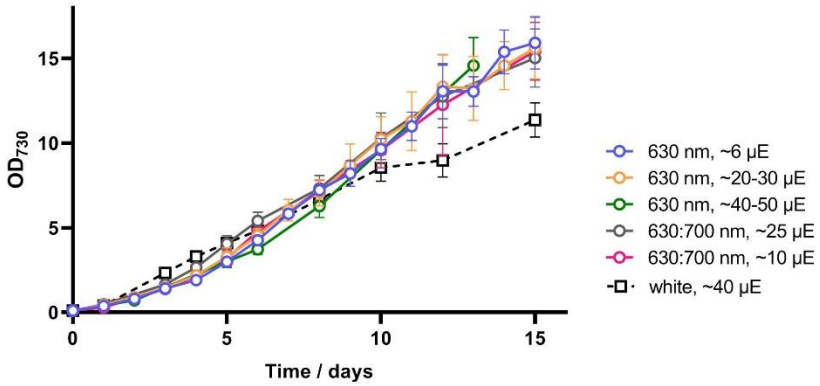



**Figure 17: Growth of *Synechococcus* sp. PCC 7002 wild-type under different red-light conditions, compared to white light.** All samples cultivated in red light show similar $OD_{730}$ over time but reach a higher $OD_{730}$ than the samples cultivated in white light.

As growth characteristics of *Synechococcus* sp. PCC 7002 did not differ significantly under different intensities of red light, the LED array was set to 15 µE for the 630 nm LEDs and to 10 µE for the 700 nm LEDs. This setting was defined as "standard red light conditions" and used for all subsequent experiments conducted under red light.

Note that the illumination was set according to data obtained from cells grown in 6-well plates (Figure 18A). However, cultures grew slower and less dense when a culture volume of 30 mL in 100 mL Erlenmeyer flasks was used (Figure 18B).

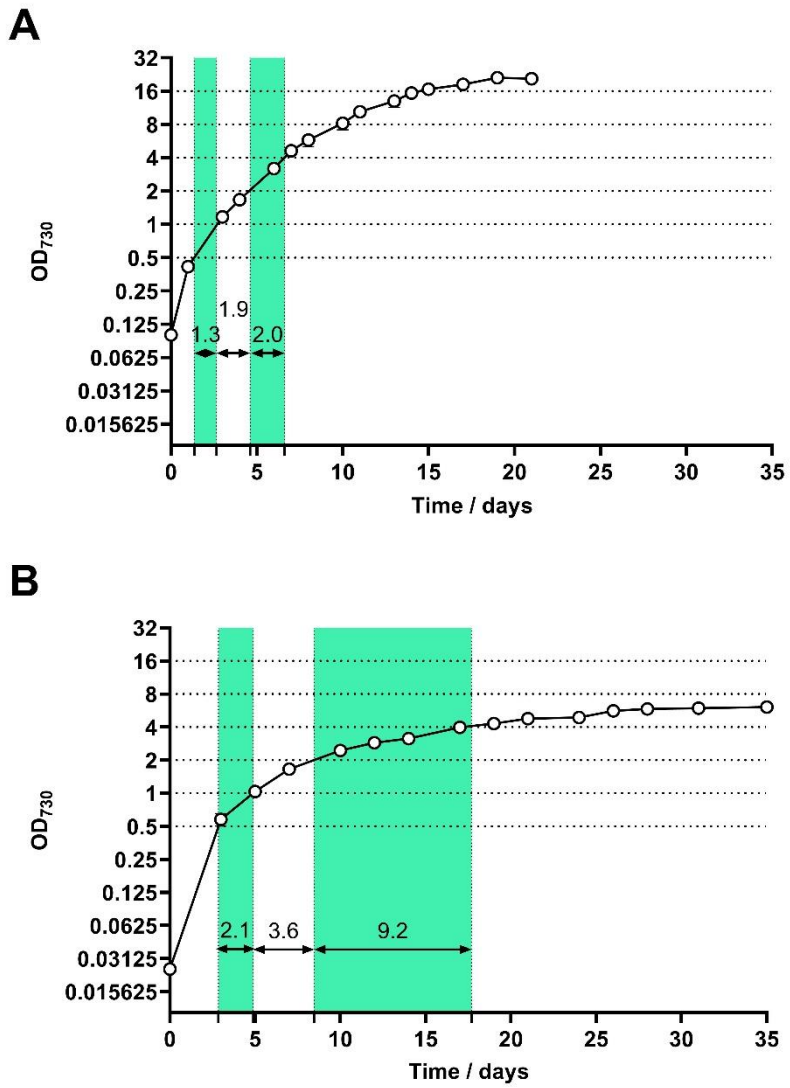



**Figure 18: *Synechococcus* sp. PCC 7002 wild-type grows faster and to a higher $OD_{730}$ in 6-well plates compared to 100 mL Erlenmeyer flasks.** The doubling times in the range of $OD_{730}$ 0.5-1.0, 1.0-2.0 and 2.0-4.0 were calculated. **(A)** The wild-type strain cultivated in 4 mL culture volume in 6-well plates reaches a higher $OD_{730}$ and has shorter doubling times, compared to the wild-type cultivated in 30 mL culture volume in 100 mL Erlenmeyer flasks **(B)**.

## 3.4. Complementation of riboflavin-auxotrophic *E. coli* strains with riboflavin biosynthesis genes from *Synechococcus* sp. PCC 7002

### 3.4.1. Endogenous riboflavin biosynthesis genes in *Synechococcus* sp. PCC 7002

The circular chromosome of *Synechococcus* sp. PCC 7002 contains all genes coding for the enzymes necessary for riboflavin biosynthesis from guanosine-5'-triphosphate (GTP) and ribulose-5'-phosphate (Figure 4, p. 9), as annotated in the NCBI database (GenBank: CP000951.1). Additionally, it contains the gene *SYNPCC7002_A0498*, annotated as "*ribD* C-terminal domain". The protein translation of this gene shows similarities to the C-terminal half of the *B. subtilis* RibDG enzyme (bifunctional deaminase/reductase) and therefore is thought to only have reductase activity (Table 7).

**Table 7: Annotation and genetic context of the *Synechococcus* sp. PCC 7002 riboflavin biosynthesis enzymes.** Transcription units are marked in bold. Antisense orientation of the gene is indicated by brackets ( ).

| Gene | Gene ID | Genomic context |
|---|---|---|
| *ribAB* | *SYNPCC7002_A0427* | *A0428_**A0427**_A0426* |
| *ribDG* | *SYNPCC7002_A1465* | *A1464_(**A1465**)_A1466* |
| *ribE* | *SYNPCC7002_A2264* | *A2263_(**A2264**)_A2265_A2266* |
| *ribH* | *SYNPCC7002_A0136* | *A0165_**A0136**_A0137* |
| *ribFC* | *SYNPCC7002_A2398* | *A2397_**A2398_A2399**_A2400* |

The bifunctional riboflavin kinase/FMN adenylyltransferase (*SYNPCC7002_A2398*) annotated as "*ribF*" displays 33% identity in the amino acid sequence compared to the *B. subtilis* RibCF protein. Except for the RibAB protein, all homologs have a similar size. No homolog was found for the *B. subtilis* RibT protein.

**Table 8: Protein size (aa: amino acids) and sequence identity of the *B. subtilis* and *Synechococcus* sp. PCC 7002 riboflavin biosynthesis enzymes.** Similarities were calculated using the NCBI Blastx tool.

| Protein | *B. subtilis* | *Synechococcus* sp. PCC 7002 | Sequence identity (protein) |
|---|---|---|---|
| RibDG | 362 aa | 372 aa | 38% |
| RibE | 216 aa | 228 aa | 35% |
| RibAB | 399 aa | 554 aa | 61% |
| RibH | 155 aa | 159 aa | 60% |
| RibT | 125 aa | - | - |
| RibFC | 317 aa | 332 aa | 33% |

The riboflavin biosynthesis genes in *Synechococcus* sp. PCC 7002 genes are not organized in an operon, as in many other prokaryotes [82], but scattered all over the chromosome (Figure 19). Also, all riboflavin biosynthetic enzymes are transcribed as single genes, according to the Biocyc database. RibFC, however, forms a transcription unit with *SYNPCC7002_A2399*, which is annotated as a MoxR family ATPase (Table 7) with no apparent functional association to flavin biosynthesis.

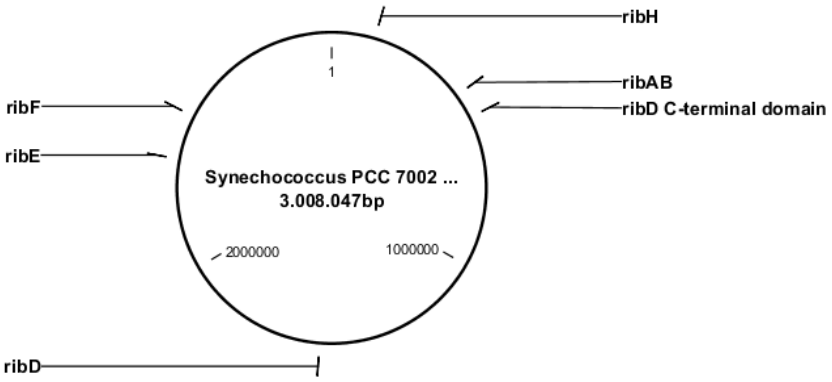



**Figure 19: Circular chromosome of *Synechococcus* sp. PCC 7002 showing the location of the riboflavin biosynthesis genes.**

All riboflavin biosynthesis genes are expressed in low transcript abundance, as indicated by transcriptomics data published previously [128, 129] (Figure 20).

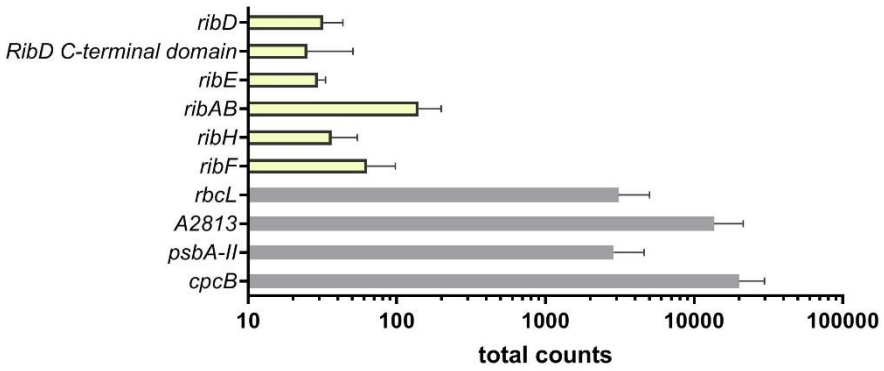



**Figure 20: mRNA transcript abundance of the riboflavin biosynthesis genes in *Synechococcus* sp. PCC 7002 under standard white light growth conditions.** *rbcL: RuBisCO large subunit; A2813: unknown protein, psbA-II: photosystem II D1 subunit; cpcB: phycocyanin beta subunit.* Data was published by Ludwig and Bryant [128, 129]. Promoters of the *A2813*, *psbA-II* and *cpcB* genes were used in this project (section 3.6, p. 62).

### 3.4.2. Complementation of *Synechococcus* sp. PCC 7002 riboflavin biosynthesis genes into riboflavin-auxotrophic *E. coli* strains

To test if the riboflavin biosynthesis enzymes catalyze their annotated function, all riboflavin biosynthesis genes were cloned into the expression vectors pGP380 and pGP382 [142], respectively, fused to a constitutive promoter ($P_{degQ36}$). Note that the pGP380 plasmid contains an N-terminal strep-tag, whereas the pGP382 plasmid contains a C-terminal strep-tag. Plasmids were transformed into the riboflavin auxotrophic *E. coli* strains BSV11 (Δ*ribB*), BSV13 (Δ*ribE*), BSV18 (Δ*ribA*) [110] and SI#78 (Δ*ribDG*) [143]. An empty plasmid was used as control. Each transformation mix was streaked on an LB plate containing 100 µg/mL ampicillin and 200 µM riboflavin. Colonies were grown in 3 mL LB containing 100 µM/mL ampicillin, in the presence or absence of 200 µM riboflavin, respectively, for 16 hours at 37 °C, 200 rpm. Subsequently, cultures were diluted 1/2000 and grown in 3 mL either in the presence or absence of 200 µM riboflavin, for 16 hours at 37 °C, 200 rpm. $OD_{600}$ was measured using a photo spectrometer.

All riboflavin biosynthesis genes from *Synechococcus* sp. PCC 7002 were able to restore growth of their respective auxotrophic *E. coli* strain upon complementation (Figure 21A). As expected, the *ribD C-terminal domain* encoded in *SYNPCC7002_A0498* did not rescue the *ribDG*-deleted strain. Since no *ribG*-deficient strain was available, a possible RibG

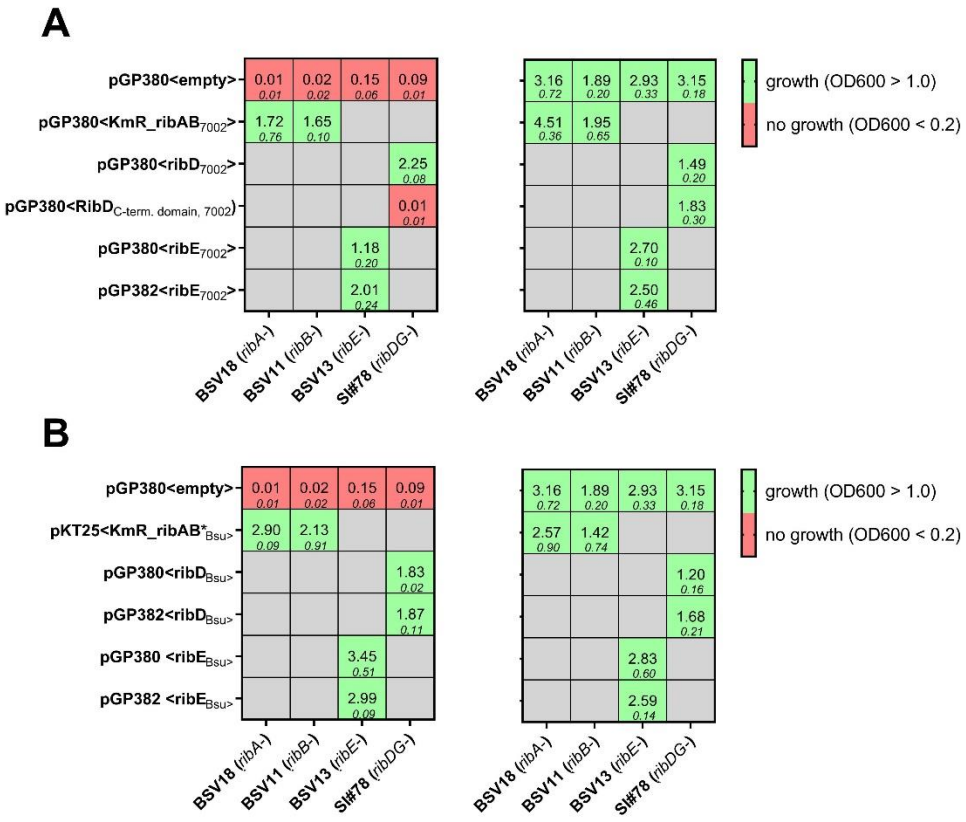



**Figure 21: Function of putative *rib* genes *ribAB*, *ribDG* and *ribE* from *Synechococcus* sp. PCC 7002 were confirmed by complementation of riboflavin-auxotrophic *E. coli* strains.**

**(A)** Riboflavin-auxotrophic *E. coli* strains were complemented with putative *Synechococcus* sp. PCC 7002 *rib* genes and were grown in LB medium (heatmap left), or LB medium supplemented with 200 µM riboflavin (heatmap right). The genes annotated as *ribAB*, *ribDG* and *ribE* in the *Synechococcus* sp. PCC 7002 genome were able to rescue their respective auxotrophic *E. coli* strain, except for *SYNPCC7002_A0498*. **(B)** As a control, *rib* genes from *B. subtilis* were used to complement riboflavin-auxotrophic *E. coli* strains which were cultivated in LB medium (heatmap left), or LB medium supplemented with 200 µM riboflavin (heatmap right). All *rib* genes from *B. subtilis* were able to restore growth in their respective riboflavin-auxotrophic *E. coli* strain.

Final $OD_{600}$ values of overnight cultures are given ± one SD (lower value, italics), whereas an $OD_{600}$ >1.0 was considered as successful growth of the respective culture (green). Growth failure (red) was defined as $OD_{600}$ <0.2. All experiments yielded significant results ($p<0.005$) in a t-test comparing “non-treated” (empty plasmid) with “treated” (plasmid containing the *rib* gene used to rescue the respective auxotrophic *E. coli* strain), except for the complementation of *SI#78* with *SYNPCC7002_A0498*.

function of the gene product could not be investigated. None of the strains grew when transformed with the empty plasmid (negative) control. All strains grew in the presence of 200 µM riboflavin (Figure 21A). The *B. subtilis* riboflavin biosynthesis genes in pGP380 were used as positive control and were all able to rescue their respective auxotrophic strains (Figure 21B).

In the absence of a *ribH*-deleted auxotrophic *E. coli* strain, complementation of the *Synechococcus* sp. PCC 7002 *ribH* gene was not possible. Therefore, RibH activity was measured qualitatively via an enzymatic assay (see following section).

## 3.5. RibH Purification and enzymatic assay

### 3.5.1. Purification of Strep-tagged *Synechococcus* sp. PCC 7002 RibH

Since there was no *ribH*-deficient *E. coli* strain available, the functionality of the *Synechococcus* sp. PCC 7002 endogenous RibH enzyme (6,7-dimethyl-8-ribityllumazine synthase, EC:2.5.1.78) could not be validated via complementation. Therefore, an N-terminally and C- terminally strep-tagged version of the *ribH* gene from *Synechococcus* sp. PCC 7002 (*SYNPCC7002_A0136*) was cloned into the StarGate® Acceptor Vector pPSG_IBA3 (IBA GmbH, Germany). An empty pPSG_IBA3 plasmid was used as negative control. Protein expression and purification was conducted as described in the materials and methods section 2.6.3, p. 34. All elution fractions (Figure 22 A, B) were analyzed by SDS-PAGE and Coomassie staining (Figure 22 C, D). The strep-tagged RibH variants appear as sharp bands at around 16 kDa. The purest fractions are E3 and E4, however, contain a low concentration of protein. Therefore, the fraction E2 (conc. 0.3 mg/mL) was used to perform the enzymatic assay. As negative control, the fraction E2 of the empty-plasmid control was used to exclude enzymatic activity provided by the *E. coli* cellular background. Additionally, water was used as a negative control.

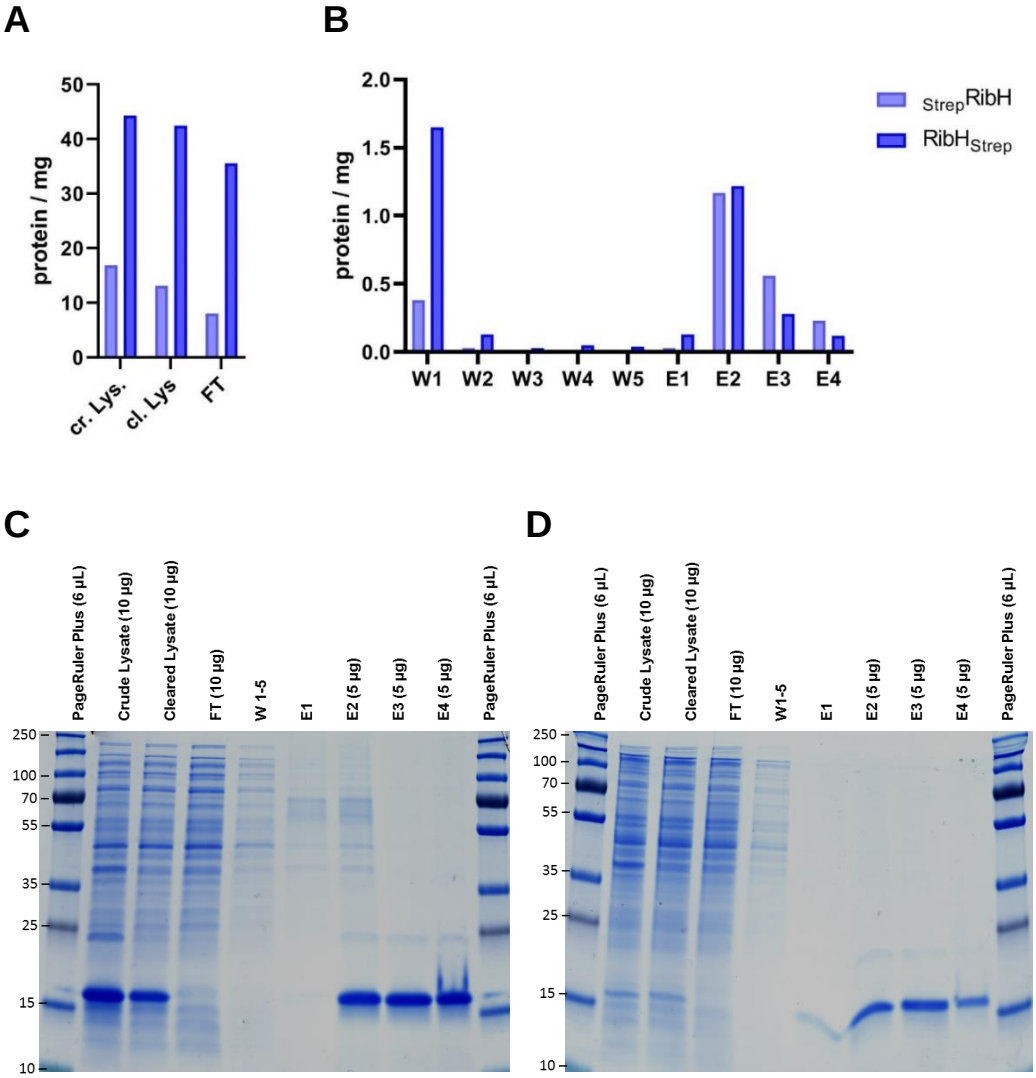



**Figure 22: Purification of Strep-tagged RibH (16 kDa) from *Synechococcus* sp. PCC 7002 expressed in *E. coli* BL21(DE3).**

**(A, B)** Total protein recovered from the different strains in different stages of purification. **(C)** SDS-PAGE analysis for the N-terminally Strep-tagged variant of RibH. **(D)** SDS-PAGE analysis of the C-terminally tagged variant of RibH.

### 3.5.2. Enzymatic assay of the *Synechococcus* sp. PCC 7002 RibH

The Strep-tagged RibH variants described above were analyzed in a enzymatic assay described previously [78], p. 34. Both the N- and C-terminally strep-tagged version of the RibH enzyme from *Synechococcus* sp. PCC 7002 is able to convert IV and V into 6,7-dimethyl-8-ribityllumazine (VI), showing as a distinct peak at ~4.7 min elution time, as measured with the HPLC inbuilt fluorescence detector at 410 nm excitation and 490 nm emission wavelength. The *B. subtilis* RibH was used as a positive control. The negative controls (water and the empty plasmid control fraction E2, respectively) did not show product formation (Figure 23).

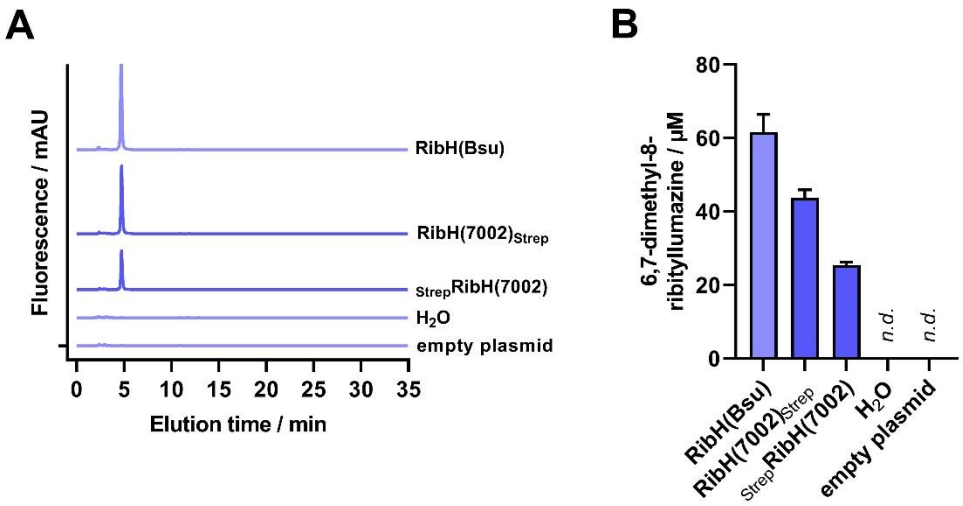



**Figure 23: The *Synechococcus* sp. PCC 7002 gene *SYNPCC7002_A0136* codes for a lumazine synthase (RibH). (A)** HPLC chromatograms of the enzymatic assay reaction mixes reveal the product 6,7-Dimethyl-8-ribityllumazine (VI) when the *B. subtilis* (Bsu) RibH is used. Both the N- and C-terminally tagged *SYNPCC7002_A0136* gene products synthesize 6,7-Dimethyl-8-ribityllumazine and therefore display RibH function. **(B)** Final concentration of 6,7-Dimethyl-8-ribityllumazine in the enzymatic reaction mixes (n=3, error bars represent one SD). No 6,7-Dimethyl-8-ribityllumazine could not be detected in the negative control reactions (n.d.).

## 3.6. Promoter screening in *Synechococcus* sp. PCC 7002 under red-light conditions using a GFP-reporter assay

On the transcriptional level, gene expression is mostly controlled by promoters – regulatory elements located in the 5'UTR of their respective gene(s) - which are themselves either repressed or activated by transcription factors and other regulatory proteins. To engineer metabolite-overproducing bacterial strains, it is therefore important to use promoters yielding high transcript levels of the genes of interest.

Whereas for *E. coli* and other "workhorses" of biotechnological research and application a broad spectrum of native and synthetic promoters have been characterized, only a few strong promoters have been described in cyanobacteria so far [144]. One of these elements is the *psbA2* promoter, driving expression of the photosystem II reaction center protein D1. This promoter has been used by several research groups for the expression of heterologous genes in *Synechocystis* sp. PCC 6803 [145, 146] and was shown to yield moderate to strong expression in other reporter systems in different cyanobacteria [113, 147]. One of the strongest promoters reported so far is the *cpc560* promoter from *Synechocystis* sp. PCC 6803. It drives expression of the C-phycocyanine beta subunit (*cpcB*) gene and consists of two promoter elements and 14 transcription factor binding sites. Using this promoter, expression of a heterologous protein as high as 15% of total protein was reported [148]. Furthermore, the *cpc560* promoter was used already to yield high levels of gene expression in *Synechococcus* sp. PCC 7002 [113, 149].

The promoter *A2813* consists of 500 nt in the 5'UTR of the *SYNPCC7002_A2813* gene, which is presumed to encode a porin, according to the NCBI database (GenBank: CP000951.1). The *A2813* promoter was shown to yield "moderate" expression in *Synechococcus* sp. PCC 7002 while only being moderately influenced by light [111] and was therefore part of the set of promoters tested in this study.

Finally, the *lac* promoter *pTrc* derived from *E. coli* was used as heterogeneous and presumably not light-regulated promoter for gene expression in *Synechococcus* sp. PCC 7002. The *lac* promoter was reported as a strong promoter to drive gene expression of heterologous genes in cyanobacteria already, e.g. for ethylene [150] and butanol production [151]. It was shown to yield high gene expression, but still lower than when using the *cpc560* promoter, in *Synechocystis* sp. PCC 6803 [152]. Furthermore, the *lac* promoter was combined with a theophylline-inducible translational riboswitch [153], yielding a fusion

promoter that can be induced in the presence of theophylline. The system was implemented in *Synechococcus elongatus* sp. PCC 7942 [122] and *Synechocystis* sp. PCC 6803 [154], but, to the best of my knowledge, not in *Synechococcus* sp. PCC 7002 so far.

When working with promoters derived from photosystem genes, dependence on different light conditions (intensity and/or wavelength) has to be considered. For example, the *cpcB* promoter derived from the cyanobacterium *Fremyella displosiphon* was shown to be active in cultures grown in red light, but not in green light [155]. The *Synechococcus* sp. PCC 6803 *psbA2* promoter was shown to be regulated by light intensity (white light), displaying a positive correlation between light intensity and transcript levels, while being inactive in the dark [156]. Therefore, it was necessary to evaluate the strength of the chosen regulatory elements under the aforementioned red-light conditions (630 and 700 nm, as described in section 3.3, p. 53) that are required to culture a riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain. Transformation plasmids were created containing the different promoters (*cpc560*, *psbA2*, *A2813* and *pTrcTheo*) fused to a superfolder GFP [123] and a kanamycin resistance cassette (*kmR*) amplified from the pUR plasmid (Wilde Group, Freiburg). For the *psbA2* promoter, both the 500 bp upstream region (*psbA2L*) and a truncated version (*psbA2s*) were used, whereas the truncated version was expected to yield higher expression levels [147]. The fragments *kmR_[promoter]_gfp* are flanked by ~750 bp sequences labelled *NS2L* and *NS2R*, for homologous recombination at the previously described "neutral site 2" (NS2) [111] (Figure 24).

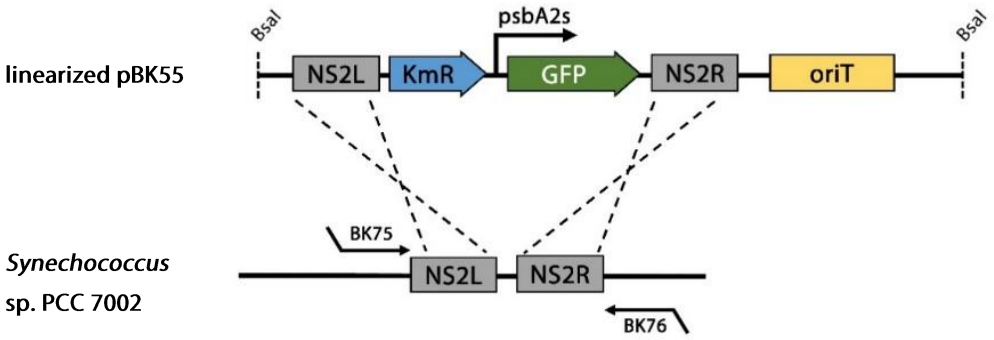



**Figure 24: Integration of the *kmR_psbA2s_gfp* fragment (pBK55) at the NS2 locus of *Synechococcus* sp. PCC 7002. Plasmids were linearized with the restriction enzyme *Bsa*I (*Eco*31I).** Promoter variants contain the *cpc560* (pBK47), *A2813* (pBK54), *psbA2L* (pBK48), *pTrcTheo* (pBK70) promoter instead of the *psbA2s* promoter (pBK55). pBK62 is the non-promoter control.

Transformation was performed as described in the Materials and Methods (p. 28). All strains analyzed in this experiment were completely segregated. Cells were grown in 6-well plates from pre-cultures, and GFP fluorescence was measured at 485 nm excitation and 535 emission wavelength using a plate reader, over the course of a complete growth curve. All $OD_{730}$ and fluorescence values were normalized as described in the Materials and Methods (p. 26).

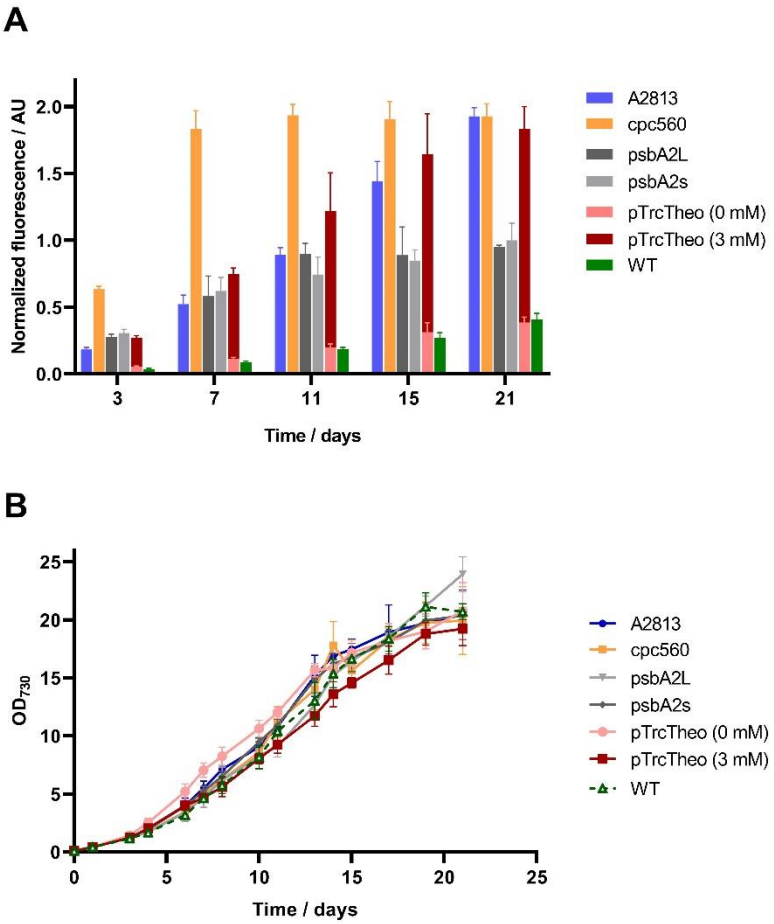



**Figure 25: Characterization of promoters in *Synechococcus* sp. PCC 7002 using a GFP-reporter assay. (A)** GFP fluorescence of all *Synechococcus* sp. PCC 7002 variants expressing GFP from different promoters, as measured by TECAN plate reader, after normalization. **(B)** Cells grew over the course of 22 days until reaching stationary phase. Growth of the GFP-expressing strains is comparable to the wild-type.

As expected, the *cpc560* promoter variant yielded the highest GFP signal at each time point, displaying a striking "headstart" in the early growth phase. The *A2813* promoter was more active in the stationary phase compared to the initial growth phase, as described previously

[111]. Both the long and short version of the *psbA2* promoter yielded moderate expression without a significant difference. The *pTrcTheo* promoter resulted in GFP fluorescence comparable to the *A2813* promoter when induced with 3 mM theophyllin, whereas the non-induced culture gave fluorescence values comparable to the wild-type control (Figure 25A). Therefore, this promoter is a promising tool if tight regulation of heterologous genes is required (e.g. in case of toxicity of a certain gene product). Growth of all GFP variants is similar to the wild-type (Figure 25B).

### 3.6.1. GFP-based analysis of the inducible promoter *pTrcTheo*

The *Synechococcus* sp. PCC 7002 strain carrying the *pTrcTheo* promoter driving expression of GFP from NS2 was cultured in 24-well plates in AA+ medium containing 50 µg/mL kanamycin and different concentrations of theophylline in a range from 0-3000 µM, grown to an $OD_{730}$ of 0.6-0.7 and analysed with the plate reader at 485 nm excitation and 535 nm emission wavelength. The strain expressing GFP from the *A2813* promoter was used as a reference to compare promoter strength to the previous experiment. Fluorescence values of the negative control (*Synechococcus* sp. PCC 7002 expressing *KmR* without GFP from NS2) were subtracted as baseline. Increase in theophylline concentrations yielded an increase in the fluorescent signal (Figure 26). Therefore, the *pTrcTheo* promoter was shown to be a valuable tool when expression levels of heterologous genes needs to be regulated in *Synechococcus* sp. PCC 7002.

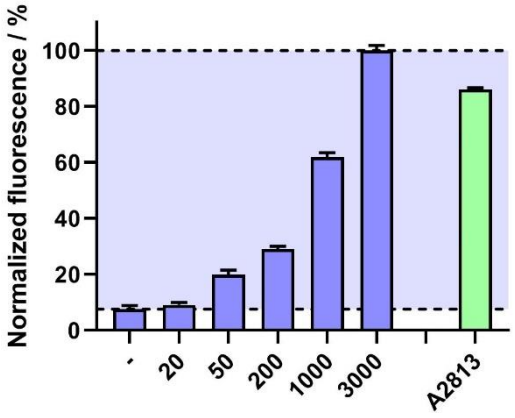



**Figure 26: The *pTrcTheo* regulatory element can be strongly induced with theophylline in Synechococcus sp. PCC 7002.** Expression of GFP with the theophylline-inducible promoter *pTrcTheo* in using different concentrations of theophylline (uninduced, 20, 50, 200, 1000 and 3000 µM). The (uninduced) baseline fluorescent signal is ~8% of the fully released promoter.

## 3.7. Endogenous plasmids for high expression of heterologous genes in *Synechococcus* sp. PCC 7002

It was first described in 1976 that the cyanobacterium *Synechococcus* sp. PCC 7002 (formerly known as *Agmenellum quadruplicatum*) contains several endogenous plasmids additional to its circular DNA [137]. In a doctoral thesis by Yu Xu at the Bryant Lab at Pennsylvenia State University, the following copy numbers of these plasmids were determined [125] (Table 9).

**Table 9: Copy-number of the chromosome and endogenous plasmids of *Synechococcus* sp. PCC 7002 determined in the Bryant lab.**

| | **Copy # p. 31** | **Size / bp** |
|---|---|---|
| chromosome | 6 | 3,008,047 |
| **pAQ1** | **50** | **4,809** |
| **pAQ3** | **27** | **16,076** |
| pAQ4 | 15 | 32,037 |
| pAQ5 | 10 | 38,515 |
| pAQ6 | 6 | 124,029 |
| pAQ7 | 6 | 186,451 |

Thus, pAQ1 and pAQ3 have a much higher copy number than the chromosome, making these endogenous plasmids attractive targets when strong expression of heterologous genes is required. Genetic tools for integration of gene sequences into pAQ1 and pAQ3 via homologous recombination have already been developed [113, 125]. In 2015, the Bryant lab integrated a ~4 kb fragment containing the *phaABEC* genetic operon for synthesis of polyhydroxybutyrate (PHB) into pAQ1, effectively demonstrating that integration of a large DNA fragment is possible into the high-copy plasmid [149]. Proof of integration, however, was shown by PCR amplification using primers binding inside the operon, instead of outside the integration site, therefore not revealing whether segregation is actually complete. In my understanding, complete segregation was also not shown for the genes integrated into pAQ1 by Dr. Yu Xu [125]. This does not seem to be uncommon, as in a study published in 2015 it was also shown for *Synechocystis* sp. PCC 6803 that a locus on the high-copy plasmid pCC5.2 could not be segregated completely [157], suggesting

that the integration either disrupt essential genes or that complete segregation is simply unlikely due to the high copy number. In an attempt to express a previously described GFP reporter cassette from the endogenous plasmids pAQ1 and pAQ3, I constructed integration plasmids for integration of the *psbA2s*-driven *gfp* gene at the loci described previously by Xu et al. [113, 125]. Therefore, the *kmR_psbA2s_GFP* fragment was amplified from pBK55 and fused to homology regions for integration into pAQ1 (resulting in the plasmid pBK83) and pAQ3 (pBK85) contains homology regions for integration into pAQ3. Complete segregation was achieved only for the pAQ3 integration by selecting on 300 µg/mL kanamycin, showing that this genetic locus is a valuable alternative to the NS2 locus for stable over expression of heterologous genes. The pAQ1 integration, on the other hand, did not lead to complete segregation even after increasing the selective pressure of kanamycin by using concentrations of up to 3000 µg/mL (Figure 27).

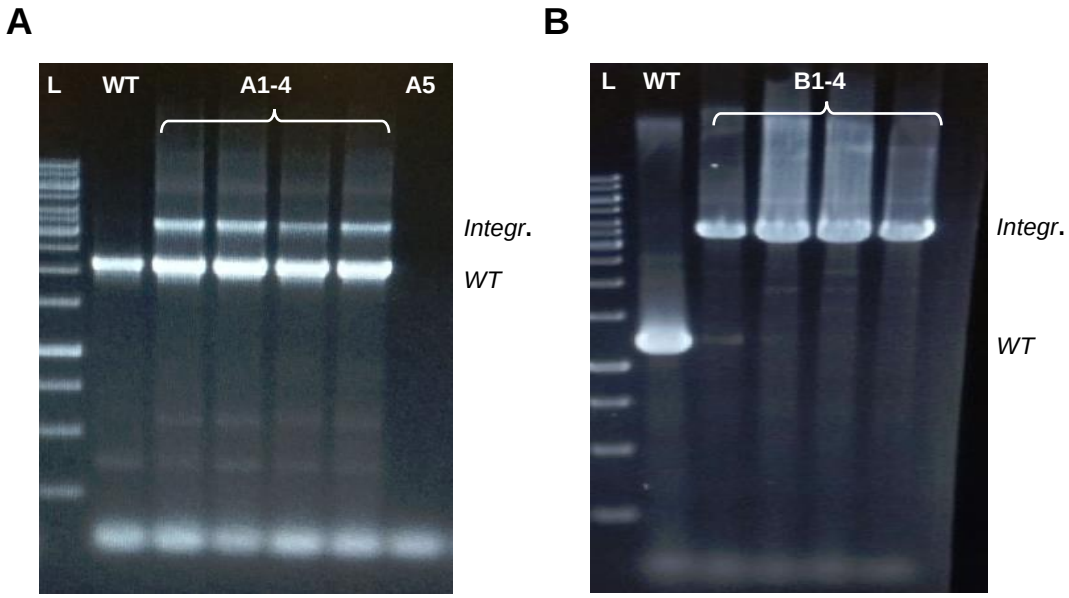



**Figure 27: Integration at pAQ3, but not pAQ1, results in complete segregation of a GFP construct. (A)** cPCR test for integration of the *kmR_psbA2s_GFP* construct in pAQ1 using primers BK118/119. The fragment is not integrated into each copy of the genome and mostly yields the wild-type fragment. **(B)** Complete segregation occurs for integration at the pAQ3 locus. L: 1kb plus ladder. WT: wild-type. A1-4: pAQ1-integrations. A5: water ctrl. B1-4: pAQ3-integrations.

To compare expression from endogenous plasmids with the genetic loci NS2, the previously described *Synechococcus* sp. PCC 7002 mutant strains expressing

*psbA2s_gfp* and *cpc560_gfp* were cultured in comparison to the strains containing integrations in the pAQ1 and pAQ3 plasmids. All strains were incubated in 24-well plates under red-light conditions, using AA+ medium containing 100 µg/mL kanamycin, until $OD_{730}$ of ~2.0 was reached. These precultures were then diluted to $OD_{730}$ of 0.1. Subsequently, $OD_{730}$ and GFP fluorescence was measured at day 2, 4 and 7. The GFP/$OD_{730}$ ratio was calculated and normalized for the value obtained for the strain expressing *psbA2s_GFP* from NS2. The data given in Figure 28 is the average of the normalized GFP/$OD_{730}$ ratios of each strain. Expression from pAQ1 and pAQ3 results in a 4-fold and approx. 2-fold increase in GFP fluorescence per $OD_{730}$, respectively, compared to expression from NS2. Therefore, expression of GFP using the moderately strong *psbA2s* promoter from pAQ3 is almost as high as when using the strong *cpc560* promoter from NS2.

Overall, this experiment shows that gene expression can be increased using a high-copy genetic locus. Especially the pAQ3 locus provides an attractive alternative or addition to the NS2 locus, e.g. for expression of two different heterologous genes in one strain using different antibiotic markers.

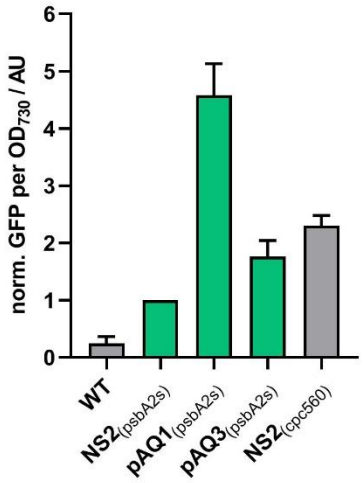



**Figure 28: Expression of GFP from pAQ1 and pAQ3 is higher compared to NS2, under otherwise same conditions.** Expression of the *KmR_psbA2s_GFP* fragment yields highest GFP fluorescence when integrated into pAQ1, even though the strain is not completely segregated (Figure 27A). Expression from pAQ3 is stronger than from NS2. The *cpc560* variant was already described in the previous section and was used as a reference to show the maximal GFP fluorescence that could be produced so far using the NS2 integration (in this lab).

## 3.8. Analysis of FMN-riboswitch variants in *E. coli* BL21(DE3)

The 5'UTR of the *B. subtilis ribDG* gene contains a riboswitch regulating transcription of the riboflavin biosynthesis operon [158, 159] via transcriptional termination upon binding of FMN (Figure 29) [79].

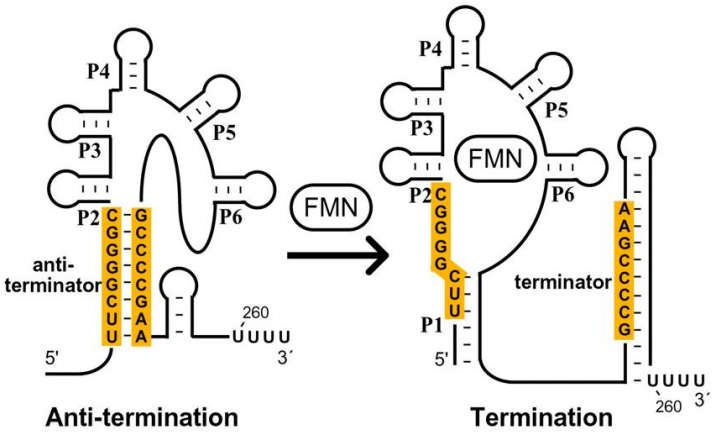



**Figure 29: The FMN-riboswitch from *B. subtilis* induces transcriptional termination of the riboflavin biosynthesis gene operon in the presence of FMN.** Figure was taken from Winkler et al., 2002 [79].

Therefore, including this regulatory region into the *B. subtilis* riboflavin biosynthesis operon *ribDGEABHT* expressed in *Synechococcus* sp. PCC 7002 was thought to limit the production of riboflavin. However, deletion of the whole FMN-regulated riboswitch was shown to have detrimental effects on mRNA stability in *B. subtilis* [93]. However, several "de-regulated" variants of the FMN riboswitch were described that have reduced sensitivity towards FMN and therefore less repressive activity on the riboflavin biosynthesis genes in the presence of FMN. These de-regulated riboswitches contain a G->A mutation in the P5 stem of the riboswitch, and/or a GGG->AAA mutation in the P2 stem, respectively, and are thought to have reduced ability to form the termination structure in the presence of FMN (Figure 29).

To test the effect of these RFN element variants on riboflavin production in the gram-negative bacterium *E. coli*, the RFN variants were PCR amplified from *B. subtilis* strains in the strain collection of the Mack lab (Table 10). The RFN variants were fused to the

riboflavin biosynthesis genes amplified from *B. subtilis* wild-type. The fragment is regulated by a *lac* promoter present on the medium-copy plasmid pBBR1_MCS2. Additionally, a variant was created that contains both the G->A mutation in P2 and the GGG->AAA mutation in P5 (RFN#5), but no terminator area. Another variant is based on the wild-type RFN sequence, however, lacking a short 14-nt sequence necessary to form the terminator structure (contructed by Ahmed Boumezbeur in the Mack lab). The DNA sequences of these variants are aligned in ***Figure 30***. Expression in *E. coli* BL21(DE3) revealed that the variants containing the wild-type RFN (RFN#2), the G->A mutation alone (RFN#1), or the GGG->AAA mutation alone (RFN#6), respectively, produced low levels of flavins (as measured via fluorescence in the TECAN plate reader) and are therefore not truly de-regulated. Highest flavin concentration was found using the variant containing no RFN at all, the variant lacking the second half of the RFN sequence (RFN#5), and the variant lacking the 14-nt region in the terminator sequence (Figure 30 B).

**Table 10: Origin and description of the RFN variants tested in this experiment.** All variants contain the riboflavin biosynthesis operon *ribDGEABHT* from *B. subtilis.*

| **RFN variant** | **Source** | **expression in *E. coli*** | **Description** | **Integration in *Synechococcus* sp. PCC 7002** |
|---|---|---|---|---|
| No RFN | - | pBK13 | - | pBK64 |
| RFN#1 | SI 358 | pBK18 | RF-overproducers, contains G->A in P5 stem | - |
| RFN#2 | SI 21 | pBK19 | Wild-type RFN | pBK117 |
| RFN#5 | SI 358 | pBK20 | Contains GGG->AAA in P2 and G->A in P5, no terminator area | pBK118 |
| RFN#6 | SI 23 | pBK21 | GGG->AAA in P2 | - |
| RFN - Term | SI 21 | pBK90 | 14-nt detetion in terminator region (A. Boumesbeur) | pBK119 |

As the different RFN variants did not increase riboflavin levels compared to the variant omitting the riboswitch, no RFN element was used to generate riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains. However, as it cannot be excluded that *Synechococcus* sp. PCC 7002 responds to the presence of the riboswitch different than *E. coli*, the deregulated variants of the RFN riboswitch should be tested in *Synechococcus* sp. PCC 7002. Plasmids are already cloned, however, have not been transformed yet.

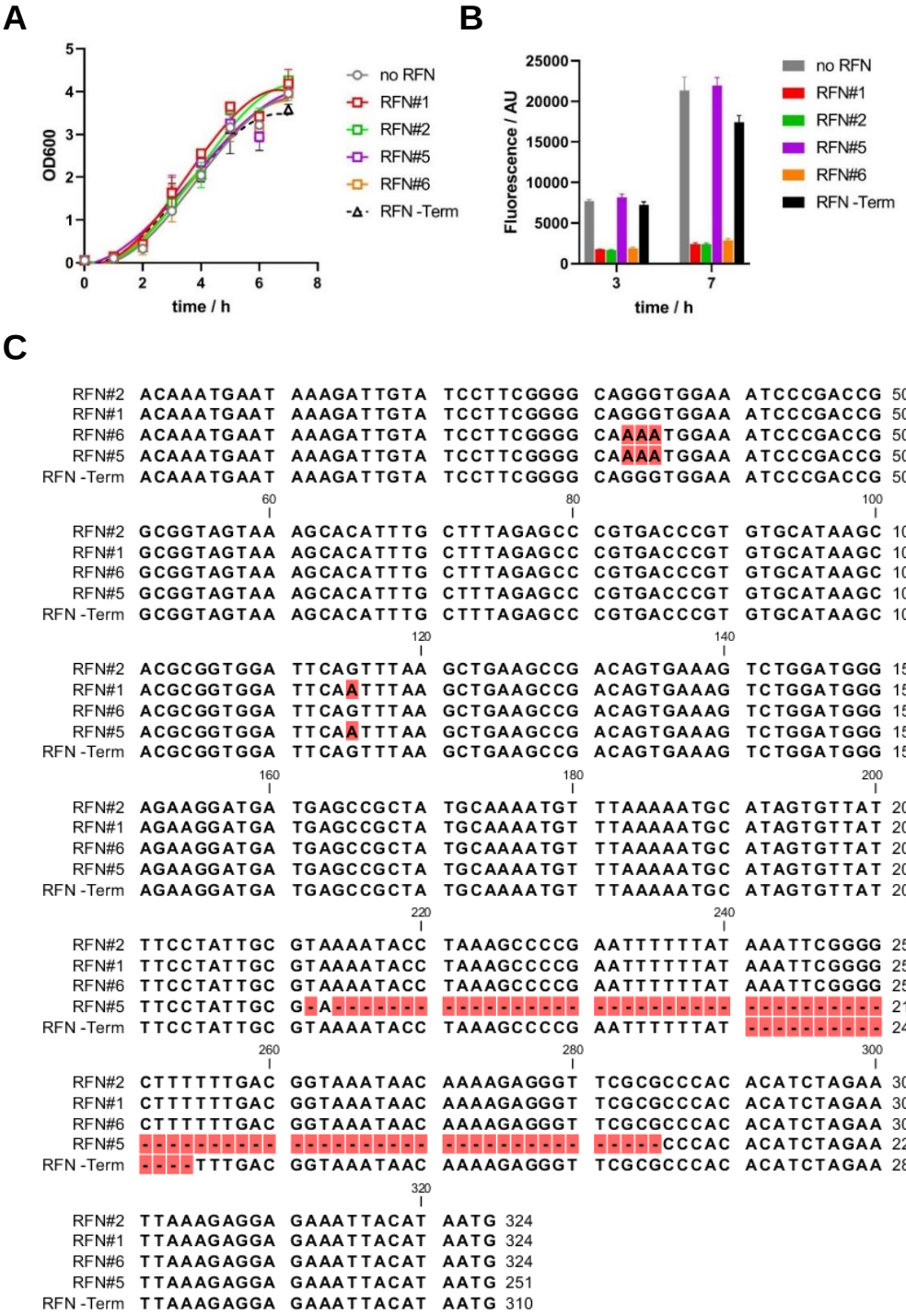



**Figure 30: Characterization of FMN-riboswitch (RFN) variants in *E. coli* BL21(DE3).**

**(A)** All strains grew similar (duplicates, curves: non-linear third-polynomical fit using GraphPad Prism). **(B)** Fluorescence was measured using the TECAN plate reader at excitation 485 nm and emission 535 nm at 3 and 7 hours post-induction with 50 µM IPTG. **(C)** Alignment of all RFN variants analyzed in this experiment. The sequence of the RFN elements is shown up to the start codon of the *ribDG* gene. All variants were aligned to the wild-type RFN (RFN#2).

## 3.9. Screening of *Synechococcus* sp. PCC 7002 variants genetically engineered for increased riboflavin production

Overexpression of the riboflavin biosynthesis genes using a strong promoter was shown to yield the highest increase in riboflavin production in *B. subtilis* compared to the parent strain [89] and was therefore the first strategy to create phototrophic riboflavin overproducing *Synechococcus* sp. PCC 7002 strains.

To this end, the riboflavin biosynthesis transcription unit *ribDGEABTH* was amplified from the *B. subtilis* 168 wild-type strain. It includes the original sequence from the start codon of *ribDG* until 40 nt past the stop codon of *ribT*. Therefore, an endogenous terminator downstream of *ribT* is included. Also, the integration plasmid contains the *terOOP* terminator amplified from the pUR_dualTag plasmid obtained from the Wilde lab (Freiburg, Germany). The FMN-riboswitch upstream of the riboflavin biosynthesis genes was omitted, since the previous experiment showed that its absence did not reduce flavin concentration compared to using a fully de-regulated riboswitch. The transcription unit *ribDGEABHT* was fused to the promoters *cpc560*, *psbA2s* and *A2813*, respectively. The integration plasmids further contain the kanamycin resistance cassette and the flanking regions for integration at NS2, as described previously. Additionally, a variant was created based on the fragment *psbA2s_ribDGEABHT* containing an additional *cpc560* promoter upstream of the *ribAB* gene, a strategy that was already applied in *B. subtilis* [93].
Since codon-adaptation was shown to increase expression of heterologous genes in cyanobacteria [160], the *B. subtilis* riboflavin biosynthesis operon was codon-adapted as described in section 6.1 (p. 124). Both the codon-adapted operon *ribABDGEHT* and the codon-adapted *ribAB* gene, respectively, were used in this experiment (Figure 31A). All plasmids were linearized and transformed into *Synechococcus* sp. PCC 7002 wild-type as described previously (p. 28), and complete segregation was confirmed with cPCR. Pre-precultures were grown from DMSO stocks in 24 well plates until $OD_{730}$ of 2.0. Pre-cultures were diluted to $OD_{730}$ of 0.1 and cultured in 6-well plates in 5 mL AA+ medium containing 50 µg/mL kanamycin and 4 µg/L vitamin $B_{12}$. For the wild-type control, kanamycin was omitted. Samples were lysed by bead beating and flavin concentration was analyzed using HPLC.

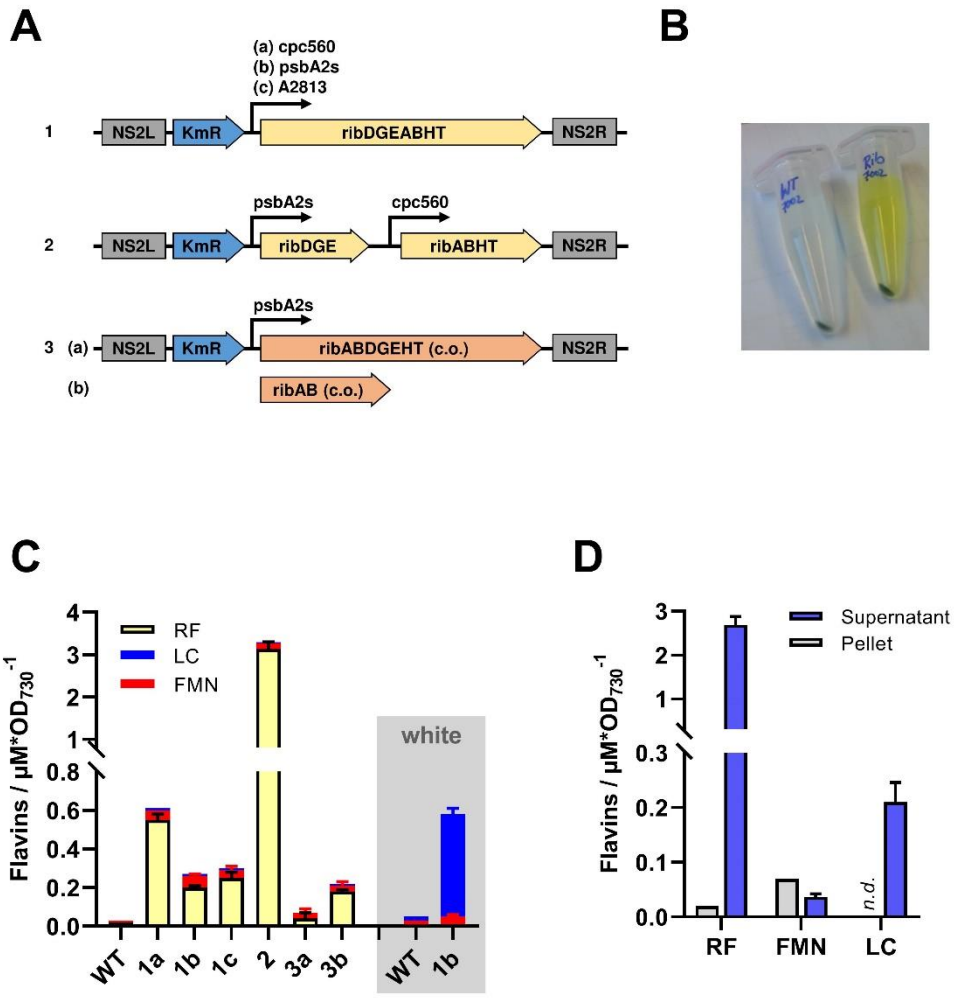



**Figure 31: Recombinant *Synechococcus* sp. PCC 7002 overexpressing *B. subtilis rib* genes overproduce riboflavin. (A)** Design of plasmid constructs for integration of *B. subtilis rib* genes into *Synechococcus* sp. PCC 7002 at NS2. A kanamycin resistance cassette (*KmR*) was used to select for positive clones and to force segregation. The constructs carry the wild-type *rib* genes *ribDGEABHT* from *B. subtilis* in combination with different promoters (1), or using a *psbA2s* promoter upstream of *ribDG* with an additional *cpc560* promoter upstream of *ribAB* (2). Additionally, codon-adapted genes *B. subtlis rib* genes were used fused to the *psbA2s* promoter (3). **(B)** The supernatant of recombinant *rib* gene overexpressing *Synechococcus* sp. PCC 7002 strains is yellow, compared to the wild-type, due to riboflavin production. **(C)** The recombinant *Synechococcus* sp. PCC 7002 strains overproduce riboflavin (measured at day 13 of growth), with highest levels measured for the variant expressing the *B. subtilis rib* genes from the *cpc560* promoter (1a) and the variant containing both the *psbA2s* and the *cpc560* promoter (2). The wild-type strains and the *psbA2s* variant were also cultivated in white light and yield mostly lumichrome. **(D)** Supernatant and pellet of variant 2 (*psbA2s/cpc560*). Riboflavin is mostly found in the supernatant, while most FMN remains in the pellet. Error bars display one SD (n=3). WT: wild-type, RF: Riboflavin, LC: Lumichrome.

Of the variants carrying the *B. subtilis* riboflavin biosynthesis operon with the original codon-usage, the *cpc560* variant yielded the highest flavin concentration, followed by the *A2813* and the *psbA2s* variant, as indicated already in the GFP-based assay (see section 3.6). The codon-adapted version of the riboflavin biosynthesis operon yielded lower flavins levels, compared to the operon with original codon usage containing the same promoter. Interestingly, the codon-adapted *ribAB* yielded higher flavin levels than the whole codon-adapted *rib* operon. However, the highest flavin concentration was measured in the variant expressing the original-codon *B. subtilis* riboflavin biosynthesis operon driven from *psbA2s* containing an additional *cpc560* promoter upstream of *ribAB*.

To measure flavin concentration and composition under white light conditions (~40 µE, 100 rpm, 32 °C), the *Synechococcus* sp. PCC 7002 wild-type and the mutant strain containing *psbA2s_ribDGEABHT* were cultured under otherwise same conditions. These strains yielded higher levels of total flavins, however, mostly consisting of lumichrome (Figure 31C). Analysis of the cellular pellet und the culture supernatant of the riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain expressing the pBK64 fragment (*psbA2s_ribDGEABHT*) cultivated in red light revealed that most (uncharged) riboflavin is found in the supernatant, whereas most (charged) FMN remains in the pellet. This is consistent with observations in other microorganisms overproducing riboflavin and is thought to occur due to passive permeation of riboflavin through the membrane [161].

## 3.10. Detailed analysis of the two best-performing recombinant *Synechococcus* sp. PCC 7002 strains engineered for riboflavin production.

In the previous experiment, different promoters were tested in combination with different variants of the riboflavin biosynthesis operon from *B. subtilis* in order to find variants yielding high flavin concentrations. Since the experiment was performed in small scale using 6-well plates, evaporation control was not optimal. Also, more flavin-measurements should be taken in order to analyze the riboflavin production over time, since it was shown in the GFP-based assay (see section 3.6) that promoter strength varies over the course of a growth curve. Therefore, in order to get more detailed and reliable data, the two *Synechococcus* sp. PCC 7002 strains containing the integration fragment from pBK63 (*NS2L_kmR_cpc560_ribDGEABHT_NS2R*) and pBK101 (*NS2L_psbA2s_ribDGE_ cpc560_ribABHT_NS2R*) were cultivated in 30 mL medium in a 100 mL Erlenmeyer flask. Evaporation was corrected by weighting each flask before measurement. Additionally, chlorophyll-a levels were measured, as chlorophyll-a is associated with overall health and photosynthetic productivity of cyanobacterial cells. The experiment was performed using precultures cultivated in 24-well plates in triplicates, until $OD_{730}$ of ~2.0. The experimental cultures started at $OD_{730}$ of 0.05 and were grown for 38 days.

The wild-type *Synechococcus* sp. PCC 7002 yielded total riboflavin supernatant levels of 0.35 ± 0.03 µM after 38 days of growth. The recombinant riboflavin-overproducing strain expressing *cpc560_ribDGEABHT* produced 19.9 ± 1.0 µM riboflavin, whereas the strain expressing *psbA2s_ribDGE_cpc560_ribABHT* produced 73.8 ± 7.23 µM, respectively (Figure 32A). Therefore, the mutant strains increase flavin production 57- and 211-fold, respectively, compared to the wild-type. While for the strain containing *cpc560_ribDGEABHT* the riboflavin-production per $OD_{730}$ was higher in the first half of growth, the strain containing both the *psbA2s* and the *cpc560* promoters steadily increased riboflavin levels. The amount of the major degradation product lumichrome was found to be 17 % in the mutant strain containing *cpc560_ribDGEABHT* and 9 % in the strain containing *psbA2s_ribDGE_cpc560_ribABHT* (Figure 32B). Lumiflavin and FAD could not be detected from the supernatant. FMN levels remained < 0.2 µM in the wild-type and < 0.8 µM in the recombinant strains (not shown). Both recombinant strains grew similar to the wild-type (Figure 32C). Chlorophyll-a levels per $OD_{730}$ were slightly lower in the

recombinant strains, compared to the wild-type. However, this is only significant in the first half of the growth curve due to large intra-sample variability in the second half.

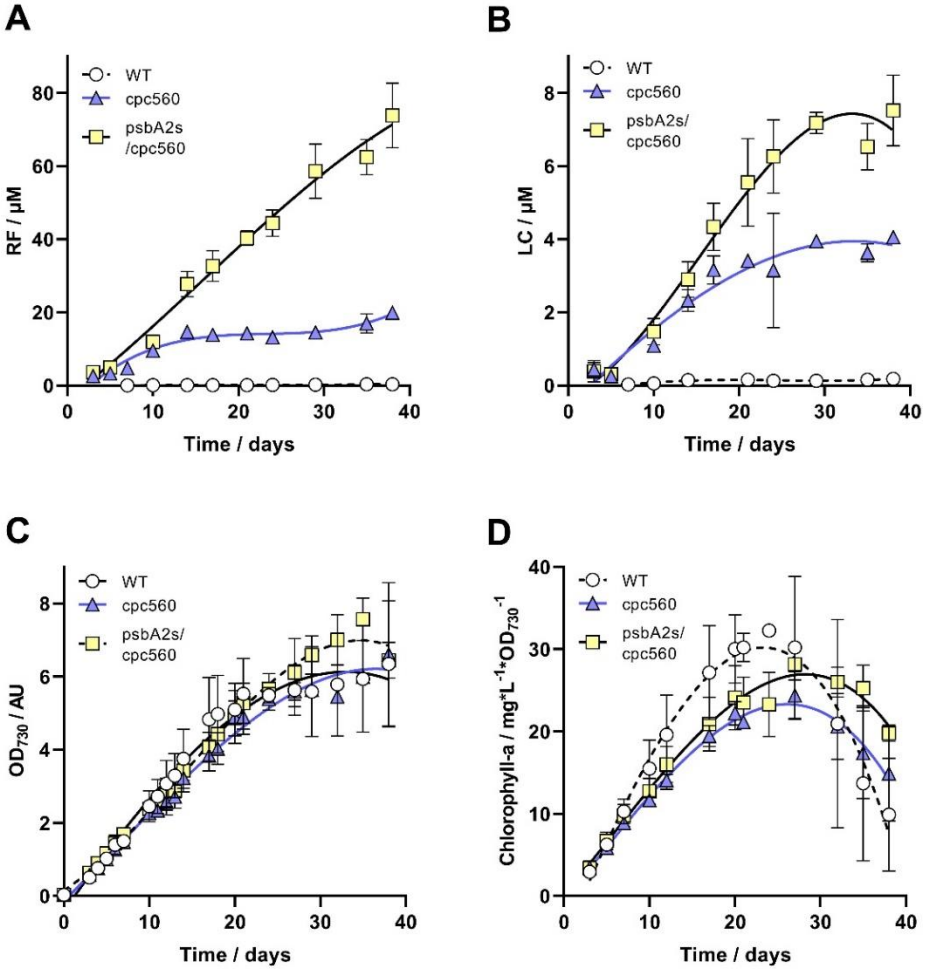



**Figure 32: Comparison of riboflavin and lumichrome levels, growth and chlorophyll-a concentration in the wild-type and recombinant riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains. (A)** Accumulatin of riboflavin over time. The strain expressing *ribDGEABHT* from the *cpc560* promoter ("*cpc560*") accumulates riboflavin mostly in the early phase of growth. The strain expressing the *B. subtilis rib* genes from the *psbA2s* promoter with an additional *cpc560* promoter upstream of *ribAB* (*"psbA2s/cpc560"*) yields highest flavin levels, increasing in a linear fashion. **(B)** Lumichrome formation over time is highest in the *psbA2s/cpc560* strain. **(C)** Growth of the recombinant *Synechococcus* sp. PCC 7002 variants is similar to the wild-type. **(D)** Chlorophyll-a levels are slightly reduced in the recombinant strains. Data points represent averages of triplicates, error bars display one SD. The data were fitted using a fourth-order polynomic non-linear regression in GraphPad Prism. RF: Riboflavin, LC: Lumichrome.

## 3.11. Qualitative analysis of *B. subtilis rib* gene expression in genetically engineered riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains

To confirm the presence of the heterologously expressed *B. subtilis* riboflavin biosynthesis gene transcripts in the recombinant *Synechococcus* sp. PCC 7002 strains, a Reverse-Transcriptase-Polymerase-Chain-Reaction (RT-PCR) experiment was conducted. Total RNA was extracted from liquid cultures in early log phase, using the Zymo Quick-RNA™ Miniprep Kit and reverse-transcribed into cDNA using the *Maxima® First Strand cDNA Synthesis Kit* (Thermo Scientific) with random hexamer primers. As a negative control (to rule out DNA contamination) the reverse transcriptase was omitted. 1 µL of the cDNA was subsequently used as template for a *Dreamtaq*® PCR reaction using primers specific for the respective genes. The *B. subtilis ribDGEABHT* mRNA/cDNA could not be detected as one fragment in the riboflavin overproducing *Synechococcus* sp. PCC 7002. However, the *ribAB, ribDGE* and *ribHT* fragments could be amplified using specific primers. The endogenous *SYNPCC7002_A0427* (*ribAB)* from *Synechococcus* sp. PCC 7002 was amplified as a positive control (Figure 33).

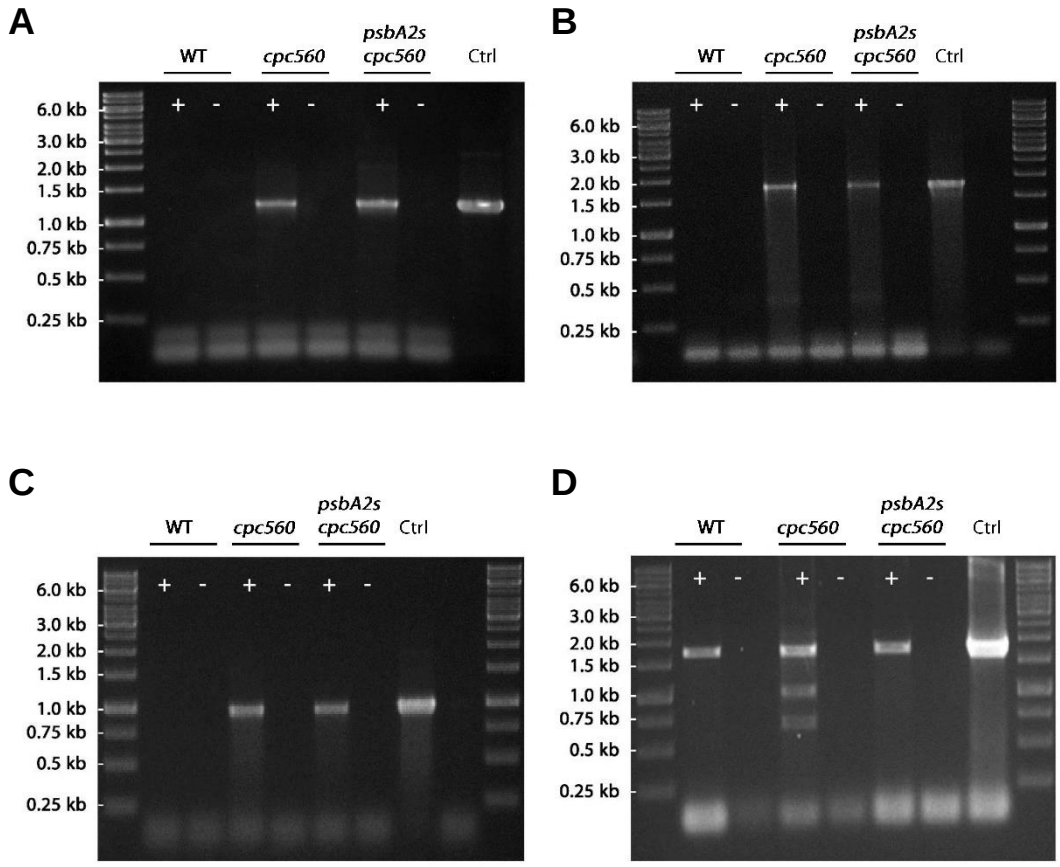



**Figure 33: All heterologously expressed *rib* genes from *B. subtilis* were detected in the recombinant *Synechococcus* sp. PCC 7002. (A)** The *B. subtilis* gene transcript *ribAB* is expressed in the riboflavin-overexpressing *Synechococcus* sp. PCC 7002 strains, but not in the wild-type, as confirmed by RT-PCR analysis (+). The *B. subtilis rib* genes are expressed either from a single *cpc560* promoter, or the promoter combination using both *psbA2s* and *cpc560*. A plasmid encoding the *B. subtilis rib* genes *ribDGEABHT* was used as positive control (Ctrl) for the PCR amplification step. To rule out DNA contamination, cDNA synthesis omitting the reverse transcriptase enzyme was performed (-). **(B)** Accordingly, the *B. subtilis* transcripts of *ribDGE* and **(C)** *ribHT* were detected. **(D)** The transcript of the *SYNPCC7002_A0427* (*ribAB*) gene served as a positive control for the cDNA synthesis step. In this case, a plasmid expressing *SYNPCC7002_A0427* was used to as a positive control for the PCR amplification step.

## 3.12. Identification of *B. subtilis rib* enzymes in a riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain

To identify the heterologously expressed *B. subtilis* riboflavin biosynthesis genes in the riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain expressing *kmR_psbA2s_ribDGE_cpc560_ribABHT* (pBK101) from NS2, a protein identification experiment was performed at the Core Facility for Mass Spectrometry & Proteomics, ZMBH Heidelberg.

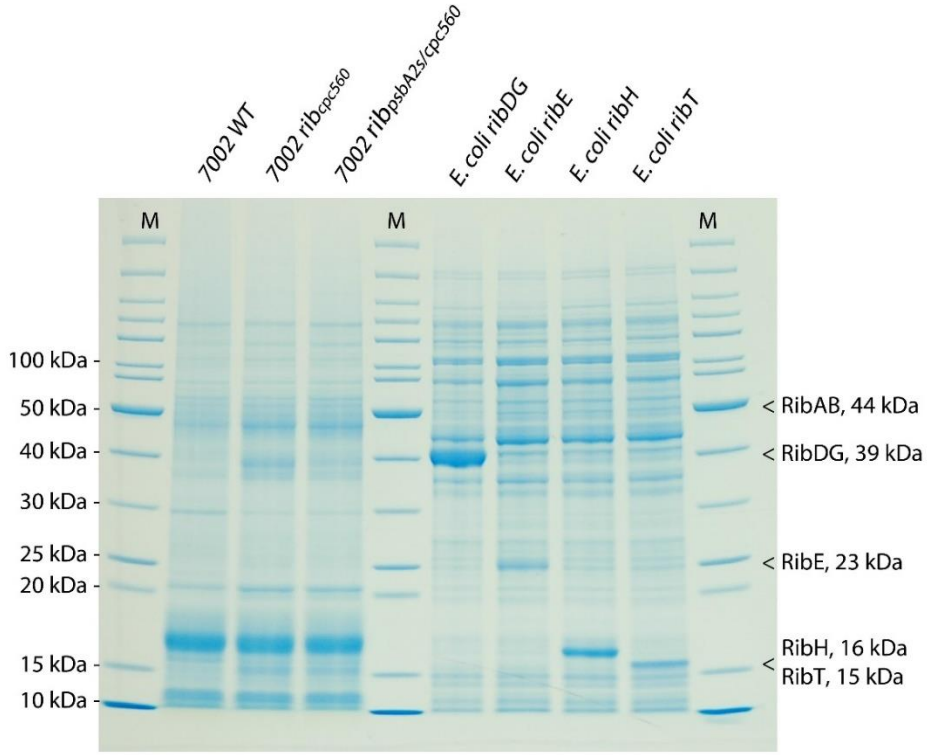



**Figure 34: *B. subtilis rib* enzymes expressed in the recombinant *Synechococcus* sp. PCC 7002 cannot be visualized on a Coomassie-stained SDS-PAGE.** Cellular lysate of *Synechococcus* sp. PCC 7002 (left side of the gel) wild-type and riboflavin-overproducing strains utilizing either the *cpc560* promoter or the *psbA2s/cpc560* promoter combination. For the recombinant strains a faint band is visible which could represent the RibDG enzyme. None of the other *B. subtilis rib* enzymes could be detected. As comparison, cellular lysate of *E. coli* strains expressing *B. subtilis rib* genes from expression plasmids yielded distinguishable bands of the sizes expected for the individual *rib* enzymes (right side of the gel). The *B. subtilis ribAB* gene was not expressed in this experiment. For each sample cellular lysate conaining 10 µg total protein was used.

The recombinantly expressed *B. subtilis rib* enzymes expressed in *Synechococcus* sp. PCC 7002 were not visible on the Coomassie-strained SDS-PAGE. Strep-Tagged *B. subtilis* riboflavin biosynthesis enzymes expressed in *E. coli* BL21(DE3) were used as positive controls to identify the region that should be cut from the gel. *Synechococcus* sp. PCC 7002 wild-type was used as negative control (Figure 34).

In the recombinant *Synechococcus* sp. PCC 7002 strain, all proteins of interest could be identified, along with 220 other unique proteins that can be used as a background to normalize data in a quantitation experiment. The *B. subtilis* riboflavin biosynthesis enzymes were detected in the pooled *E. coli* samples (except for RibAB) and in the recombinant *Synechococcus* sp. PCC 7002 strain, but not in the wild-type control. Quality of the data was sufficient, according to the Core Facility. Several distinct peptides could be detected in high abundance, therefore enabling identification of the proteins of interest with high confidence (Figuer 35).

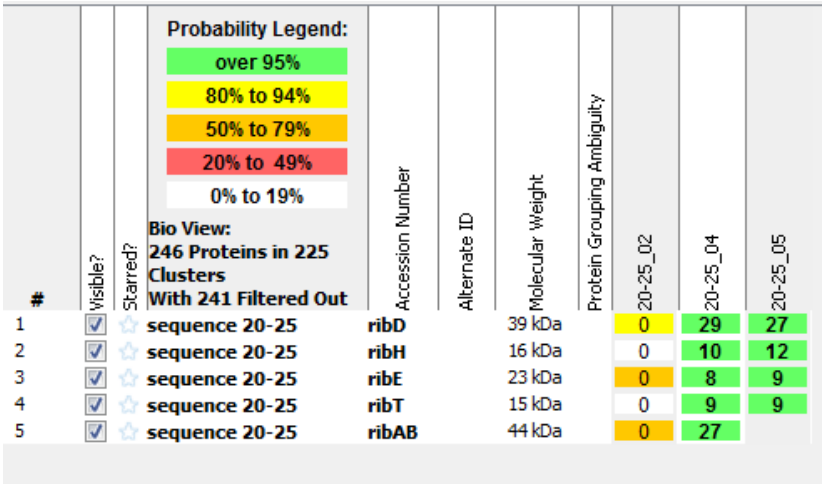



**Figure 35: All heterologously expressed proteins from *B. subtilis* were detected in the recombinant riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain.** For each protein, unique peptides were identified with sufficient coverage (number of individual peptides shaded in green). The *B. subtilis* enzymes were not identified in the *Synechococcus* sp. PCC 7002 wild-type (20-25_02), but were detected in the strain expressing *psbA2s_ribDGE_cpc560_ribABHT* (20-25_04). *B. subtilis rib* genes (except for *ribAB*) were expressed individually in *E. coli* BL21(DE3). These samples were pooled and used as a control sample (20-25_05). All data were visualized using Scaffold_4.10.0 (Proteome Software Inc., Portland, USA).

## 3.13. Quantitation of *B. subtilis rib* enzymes in riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains

Since the *B. subtilis* riboflavin biosynthesis enzymes could be identified using the LC/MS workflow of the Core Facility for Mass Spectrometry and Proteomics at ZMBH Heidelberg, the *B. subtilis rib* enzymes were quantified in different riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains. The strain expressing the *psbA2s_ribDGEABHT* (pBK64) was used as baseline. The strain containing the *cpc560* instead of *psbA2s* promoter (pBK63) was used to analyze the effect of a stronger promoter on *rib* enzyme expression. The riboflavin-overproducing strain expressing *psbA2s_ribDG_cpc560_ribABHT* (pBK101) was used to analyze the effect of the additional promoter on the expression levels of the recombinant *rib* enzymes. Samples were grown from DMSO stocks (biological triplicates), harvested at $OD_{730}$ of 2.0, analyzed with LC/MS and post-processed as described in the materials and methods (section 2.7.4, p. 41).

In comparison to the strain expressing *psbA2s_ribDGEABHT*, the variant containing an additional *cpc560* promoter upstream of the *ribAB* gene yielded a 4-fold higher RibAB expression, thus strengthening the hypothesis that increase of the rate-limiting RibAB enzyme is responsible for the high riboflavin levels detected in this strain. However, this strain also showed a 2-fold increase in RibE and a 2-fold decrease in RibD, which cannot be explained by the addition of the *cpc560* promoter. Also, RibH and RibT levels were not increased even although they were expected to be influenced by the additional *cpc560* promoter (Figure 36). Comparing the *psbA2s_ribDGE_cpc560_ribABHT* variant to the *cpc560_ribDGEABHT* variant also revealed a significant 2.3-fold increase of RibAB in the two-promoter-variant, which most likely explains the 4-fold higher riboflavin-concentrations detected in this strain (section 3.10, p. 75).

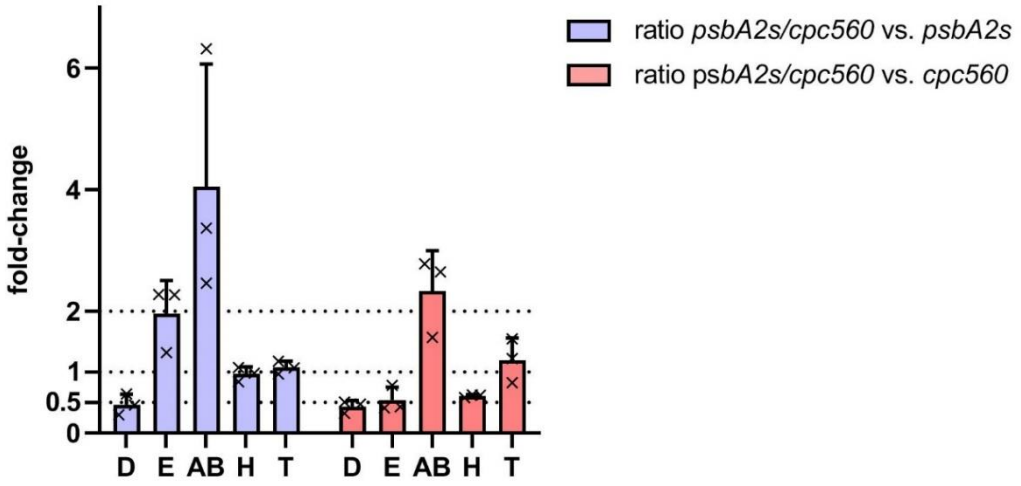



**Figure 36: Comparison of *rib* enzyme levels between different recombinant riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains.** A 4-fold increase in RibAB levels were observed in the strain expressing *psbA2s_ribDGE_cpc560_ribABHT* compared to the strain expressing *psbA2s_ribDGEABHT* without the additional *cpc560* promoter (blue). The variant containing the *psbA2s/cpc560* promoter combination also displayed higher RibAB levels when compared to the strain expressing the *B. subtils rib* genes from a single *cpc560* promoter (red). All data represent averages of triplicates, error bars show one SD. For clarification, individual data points are represented by “x”. A fold-change of 0.5<x<2.0 was not considered significant (see dotted lines).

## 3.14. Selection of *pnuX*-expressing *Synechococcus* sp. PCC 7002 on roseoflavin yields riboflavin-overproducing strains

*Synechococcus* sp. PCC 7002 is a gram-negative bacterium for which, to the best of our knowledge, no flavin import system was found [83]. Therefore, the gene *pnuX*, coding for a flavin importer that was previously used to generate a roseoflavin-sensitive *E. coli* strain [100], was amplified from *Corynebacterium glutamicum*. The gene was integrated at NS2 of *Synechococcocus* sp. PCC 7002 (Figure 37A) and selected via kanamycin as described previously. Transformants were picked from the plate and restreaked until segregation was complete, as confirmed by cPCR (Figure 37B).

To test the roseoflavin-sensitivity of the *pnuX*-expressing *Synechococcus* sp. PCC 7002 mutant strain, compared to the wild-type strain, a defined number of cells was dropped on agar plates containing different concentrations of roseoflavin. Whereas the wild-type grew on roseoflavin concentrations of up to 200 µM, no growth was observed for the *pnuX*-expressing strain when cultivated on >2 µM roseoflavin (Figure 37C).

### 3.14.1. Some roseoflavin-sensitive *pnuX*-expressing *Synechococcus* sp. PCC 7002 overproduce riboflavin

With the goal to select for roseoflavin-resistant (and potentially riboflavin-overproducing) mutants, $2x10^8$ cells of an early log-phase culture of *Synechococcus* sp. PCC 7002 expressing *pnuX* were streaked on a AA+ agar plate containing 100 µM roseoflavin. After two weeks, distinct colony forming units appeared with a frequency of ca. $4*10^{-6}$, whereas the wild-type strain produced a bacterial lawn (Figure 38).

Nine colonies (termed #48-56) were picked from the plate and grown in AA+ medium containing 50 µg/mL kanamycin without roseoflavin in 1 mL medium in 24-well plates. After several days, the culture supernatant was analysed using the TECAN plate reader at 485 nm excitation and 535 nm emission wavelength. Out of nine colonies analyzed, seven showed increased flavin production compared to the parent *pnuX*-expressing *Synechococcus* sp. PCC 7002 strain (Figure 39A).

In order to confirm these results, a biological replicate of this experiment was performed using a fresh *pnuX*-expressing *Synechococcus* sp. PCC 7002 culture streaked on an agar

plate containing 100 µM roseoflavin. Two cultures ("#1new" and "#2new") picked from the plate were grown in AA+ liquid culture medium and analyzed for riboflavin fluorescence using the TECAN plate reader. These strains show similar riboflavin-overproduction compared to the strains described earlier (Figure 39C).

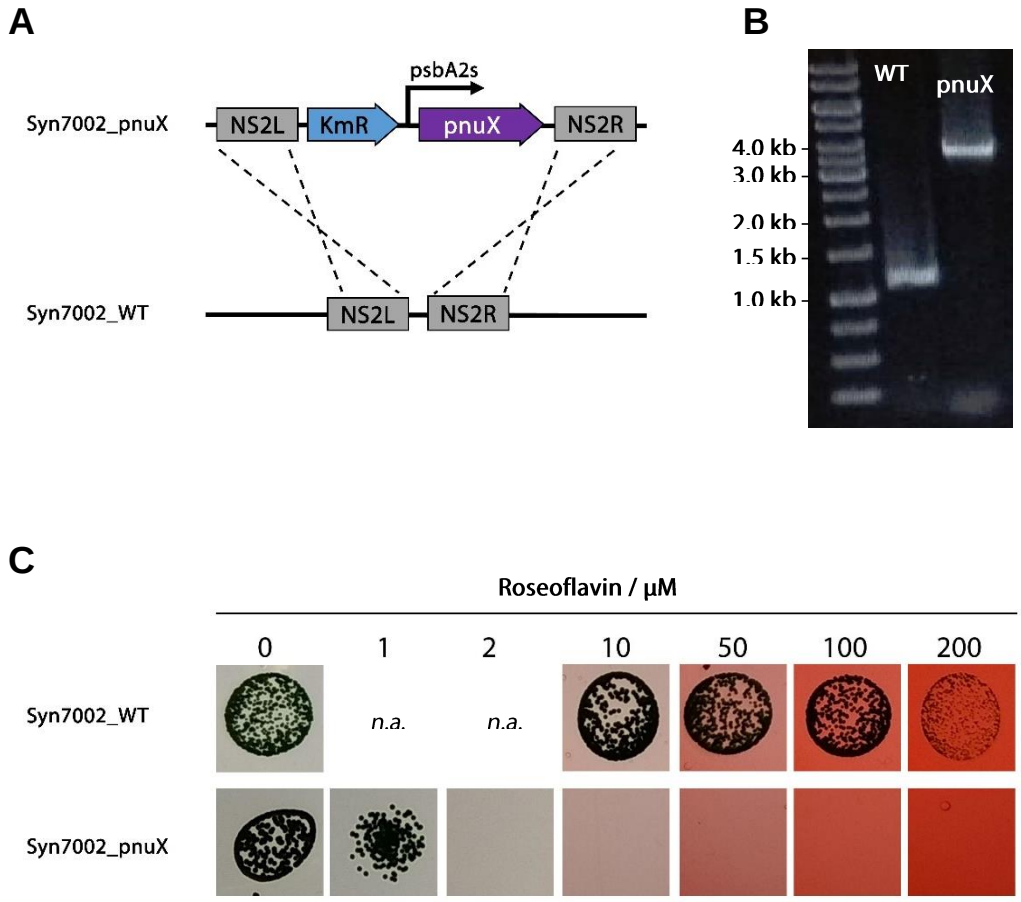



**Figure 37: The flavin importer PnuX from *Corynebacterium glutamicum* induces roseoflavin (RoF) sensitivity in *Synechococcus* sp. PCC 7002. (A)** The *pnuX* gene was integrated at neutral site 2 (NS2) in the *Synechococcus* sp. PCC 7002 genome under the control of the *psbA2* promoter via homologous recombination. **(B)** Transformants were selected on kanamycin until segregation was complete, as confirmed by cPCR using Primers BK75 and BK76 (binding in the NS2L and NS2R, respectively). **(C)** A strongly diluted liquid culture (ca. $10^2$ cells) was dropped on AA+ agar plates containing different amounts of roseoflavin (0-200 µM). Whereas the wild-type strain is still able to grow on 200 µM roseoflavin, growth of the strain expressing *pnuX* is impaired at roseoflavin concentrations > 2 µM.

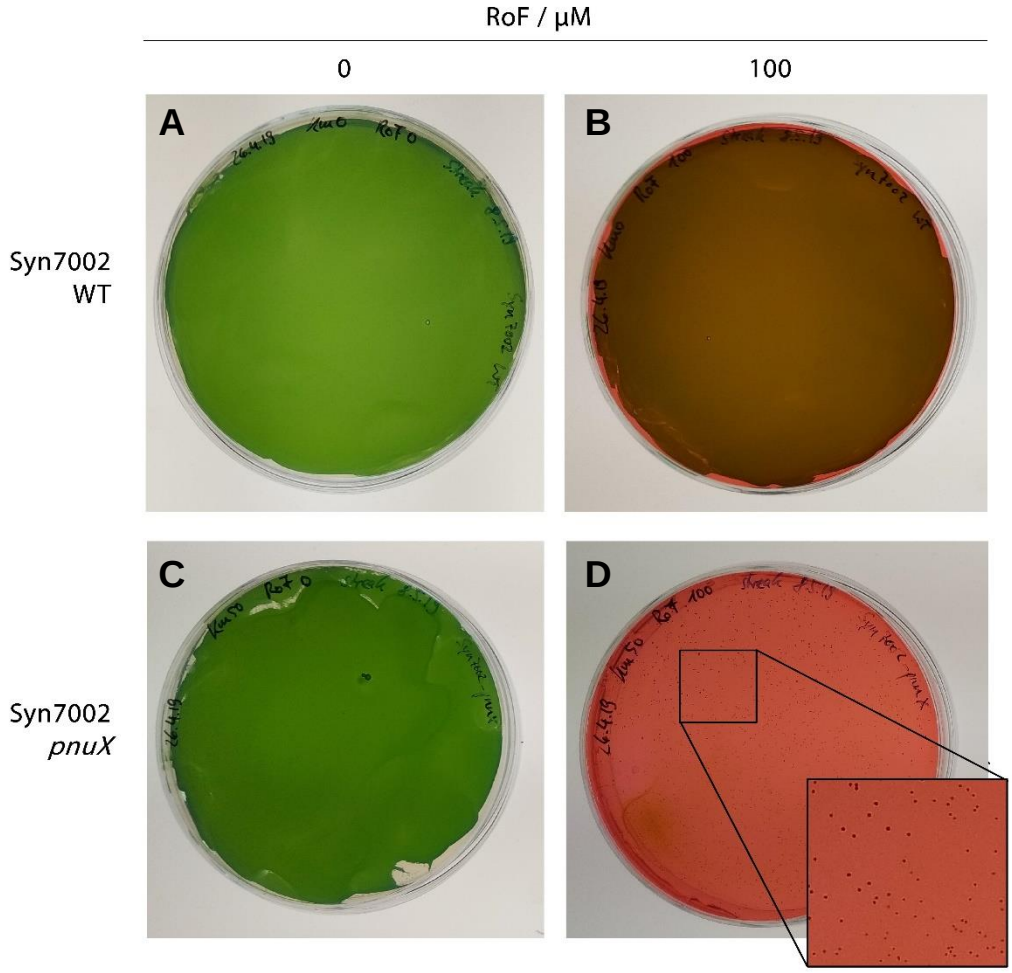



**Figure 38: Selection of roseoflavin (RoF) resistant *Synechococcus* sp. PCC 7002 strains from AA+ plates containing 100 µM roseoflavin. (A)** *Synechococcus* sp. PCC 7002 wild-type plated on AA+ agar plates forms a bacterial lawn. **(B)** 100 µM roseoflavin does not prevent lawn-formation for the wild-type strain. **(C)** The *pnuX*-expressing strain shows lawn formation similar to the wild-type on an agar plate without roseoflavin. **(D)** Selection of the *pnuX*-expressing strain on 100 µM roseoflavin yields distinct colony forming units.

### 3.14.2. Riboflavin-productivity stabilizes at 2.3 µM per $OD_{730}$

Taking into account the polyploidy of *Synechococcus* sp. PCC 7002, which could lead to a loss of acquired mutations over time, the stability of riboflavin-overproduction over several generations was analyzed, using the strains #48-51 and #55-56, as well as the wild-type. To this end, the liquid cultures grown from the original plate (L#1, Figure 39A) were diluted to an $OD_{730}$ of 0.1 and cultivated for 9 days under standard red-light conditions in

AA+ medium without roseoflavin. The culture was then analyzed for flavin fluorescence using the TECAN plate reader. Subsequently, the stationary phase cultures were diluted to an $OD_{730}$ of 0.1 and cultivated for 9 days. This process was iterated two more times. Therefore, riboflavin-productivity was measured over the course of 45 days. Notably, the riboflavin concentration per $OD_{730}$ decreases drastically towards the second liquid culture (L#2), compared to the culture picked directly from the plate (L#1). However, this effect could be due to the different culture conditions, since the cultures picked from the plates were incubated for a longer time. The flavin levels per $OD_{730}$ decrease slightly towards the third consecutive liquid culture (L#3), however, then stabilize at a concentration of 2.3 µM per $OD_{730}$, which is ca. 28-fold higher compared to the wild-type producing 0.08 µM flavins per $OD_{730}$ (Figure 39B).

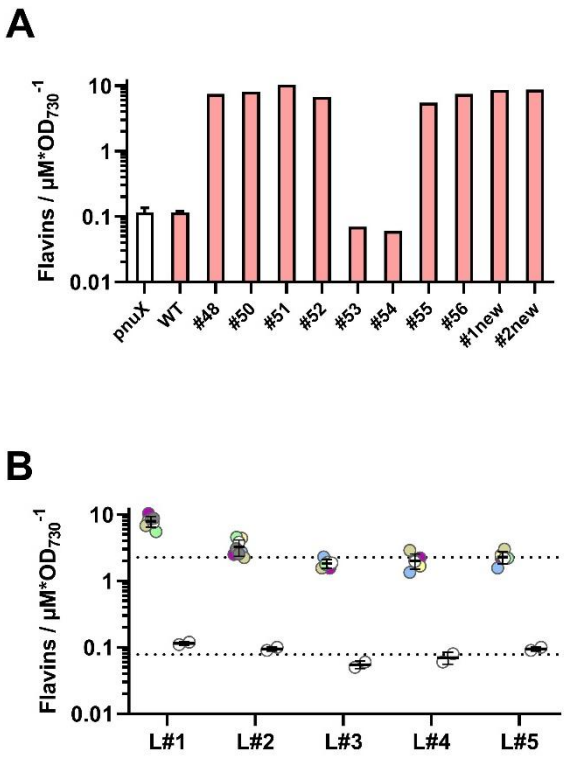



**Figure 39: Roseoflavin-selected *pnuX*-expressing *Synechococcus* sp. PCC 7002 strains overproduce flavins. (A)** *pnuX*-expressing colonies selected on an AA+ agar plate containing 100 µM roseoflavin overproduce flavins (red bars). A wild-type strain cultivated on an agar plate containing 100 µM roseoflavin is included as control (WT), as well as a *pnuX*-expressing strain not selected on roseoflavin (white bar). **(B)** Flavin levels of the roseoflavin-selected riboflavin-overproducing strains decreases initially, but stabilizes at around 2.3 µM per $OD_{730}$ on average (upper dotted line), whereas the wild-type produces 0.08 µM per $OD_{730}$ on average (lower dotted line).

### 3.14.3. No mutations were found in the *pnuX*-, *ribFC*-, and *ribAB*-genetic regions of the riboflavin-overproducing strains

Sequencing analysis of the roseoflavin-resistant riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains #48-52 and #55-56 revealed that the integrated *pnuX*-cassette is intact. The two roseoflavin-resistant strains that did not show riboflavin-overproduction showed a ATG->ATA mutation in the start codon of the *pnuX* gene (strain #53) and a premature stop codon (TGG->TAG, nt269, strain #54), respectively. Therefore, these strains most probably regained their wild-type properties in terms of roseoflavin-sensitivity.
For the riboflavin-overproducing strains, sequencing revealed no mutations in the *ribFC* gene or any of the riboflavin biosynthesis genes, or surrounding genomic region (as defined in Table 7, p. 55). Therefore, the strains #48, #51, #1new and #2new were subjected to whole-genome sequencing.

### 3.14.4. Whole-Genome sequence analysis of roseoflavin-selected riboflavin-overproducing *Synechococcus* sp. 7002 strains

Genomic DNA was extracted from the *Synechococcus* sp. PCC 7002 wild-type (parent) strain, the *pnuX*-expressing parent strain and four of the roseoflavin-selected riboflavin-overproducing mutant that were derived from these strains (#48, #51, #1new and #2new) (Figure 40).

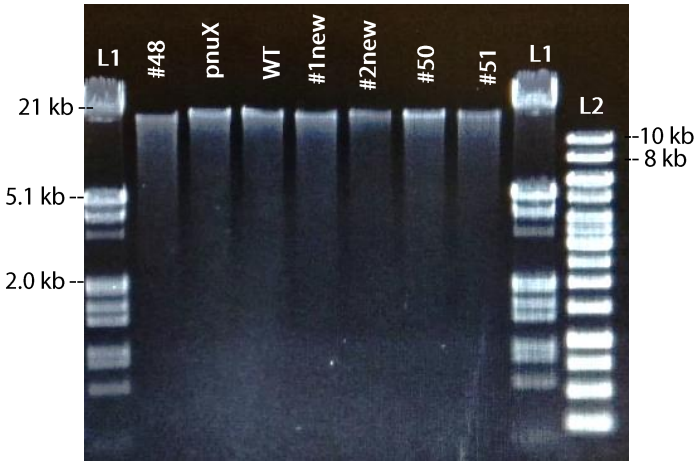



**Figure 40: Agarose gelelectrophoresis with genomic DNA extracted from *Synechococcus* sp. PCC 7002 cultures**. L1: Phage l DNA ladder *Eco*RI/*Hind*III; L2: 1 kb plus DNA ladder

Whole-genome sequencing and bioinformatics analysis was performed at Eurofins Genomics GmbH (Konstanz, Germany). As a reference, the *Synechococcus* sp. PCC 7002 wild-type sequence was used, with contains the genome (CP000951) and all endogenous plasmids (CP000952-CP000957). 118 mutations with up to 100 % mutation frequency were reported for the sequenced *Synechococcus* sp. PCC 7002 wild-type strain, compared to the reference sequence. Using the sequenced wild-type strain as new baseline, several mutations were reported that only appear in the riboflavin-overproducing strains. However, all mutations but one occur with a mutation frequency below 25%. Upon manual inspection using NCBI's Genome Workbench, it became clear that all of these low-frequency mutations reported for the non-wild-type can also be found in the wild-type and *pnuX*-expressing unselected strain with similar frequency, therefore being a false-positive artefact of the sequencing analysis.

Only one (!) mutation was found to occur with 100% mutation frequency in all riboflavin-overproducing strains, but not in the wild-type and the *pnuX*-expressing parent strains. This mutation occurs in the *SYNPCC7002_A0225* gene as a 4-nucleotide deletion at nucleotide position 15-18. These results were confirmed by primer-based (manual) sequencing. The roseoflavin-selected strains #53 and #54 - which "escaped" roseoflavin-selection due to mutation in the *pnuX* gene - also do not carry this mutation. Therefore, the frame-shift mutation in *SYNPCC7002_A0225* is clearly associated with a riboflavin-overproducing phenotype due to roseoflavin-selection (Figure 40).


**Figure 40: Multiple sequence alignment of roseoflavin-selected *pnuX*-expressing *Synechococcus* sp. PCC 7002 and their parent strains. The region surrounding the *SYNPCC7002_A0225* gene was sequenced and reveals a 4-nt deletion in the riboflavin-overproducing strains.** The deletion occurs at nucleotides 15-18 only for the roseoflavin-selected strains that overproduce riboflavin (i.e. #48, #51, #1new and #2new). The parent strains as well as the roseoflavin-selected strains that do not overproduce riboflavin (i. e. #53 and #54) contain the wild-type *SYNPCC7002_A0225* gene.

The *SYNPCC7002_A0225* gene codes for an unknown protein of 141 amino acids in size, annotated as a "TRP repeat protein" as it contains several tetratricopeptide (TRP) structural repeats. The mutation causes a frame-shift resulting in a truncated protein of 77 amino acids. Assuming that the mutated *SYNPCC7002_A0225* gene product is not functional, the effect of a knock-out of *SYNPCC7002_A0225* on the *Synechococcus* sp. PCC 7002 wild-type and the strain expressing *pnuX*, respectively, was tested. A knock-out plasmid was created to replace *SYNPCC7002_A0225* with a chloramphenicol resistance cassette (pBK138) (Figure 41). Integration and complete segregation was confirmed by cPCR. However, no increase in flavin production could be measured in the transformed wild-type and *pnuX*-expressing strain, respectively.

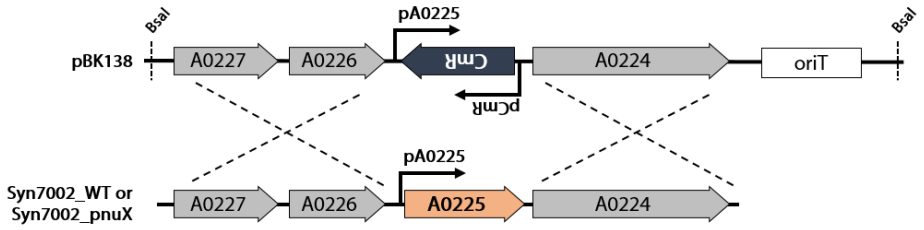



**Figure 41: Replacement of *SYNPCC7002_A0225* with chloramphenicol resistance marker (CmR) in anti-sense orientation.**

Additionally, the genomic region *A0227_A0226_A0225_A0224* was amplified from either the *Synechococcus* sp. PCC 7002 wild-type or from the roseoflavin-selected riboflavin-overproducing strain #48 carrying the *SYNPCC7002_A0225* frame-shift mutation. Both fragments were added to a *Synechococcus* sp. PCC 7002 wild-type culture in a standard transformation experiment. The transformation mix was streaked on AA+ agar plates containing 50 µM roseoflavin. The cfu count of both transformed cultures were comparable, thus not confirming the expectation that transformation with the mutated gene fragment yields a higher number of ex-transformants. A knock-out of the mutated *SYNPCC7002_A0225* gene in a roseoflavin-selected riboflavin-overproducing strain was not yet attempted. This experiment would show whether riboflavin-overproduction could be reversed by reversing the *SYNPCC7002_A0225* mutation. Also, replacement of the native *SYNPCC7002_A0225* with the mutated variant was not yet attempted. Both experiments are necessary to shed light on the role of *SYNPCC7002_A0225* in riboflavin-production of roseoflavin-overproducing *Synechococcus* sp. PCC 7002 strains.

## 3.15. Roseoflavin-selected *Synechococcus* sp. PCC 7002 as chassis for genetic engineering of riboflavin-overproducing strains

In this thesis, two strategies were applied to generate riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains. First, the riboflavin biosynthesis operon from *B. subtilis* was integrated into the genome of *Synechococcus* sp. PCC 7002 (section 3.9, p. 72). The second approach was to integrate the flavin transporter gene *pnuX* from *C. glutamicum* into the genome of *Synechococcus* sp. PCC 7002 in order to induce roseoflavin-sensitivity and subsequently selecting riboflavin-overproducing mutants from an agar plate containing roseoflavin (section 3.14, p. 83). Hypothetically, both approaches are compatible and, combined, was thought to further increase riboflavin production. This was shown already for commercial *B. subtilis* strains that were first selected on toxic anti-metabolites (8-azaguanin, methyl sulfoxide, decoyinine and roseoflavin) and subsequently used as chassis for genetic engineering [93].

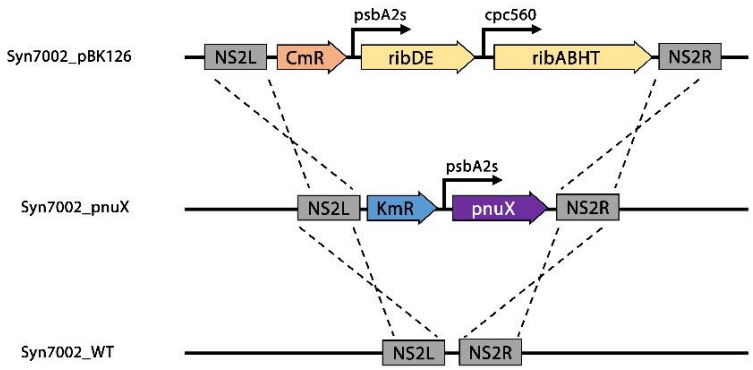



**Figure 42: The *pnuX* gene from the roseoflavin selected *Synechococcus* sp. PCC 7002 strain is "cured" by the riboflavin biosynthesis operon.** In order to force integration, a chloramphenicol cassette (CmR) had been used instead of the kanamycin cassette (KmR) used previously for the riboflavin-overproducing strains.

To test this approach in *Synechococcus* sp. PCC 7002, the goal was to use a roseoflavin-selected riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain as a parent strain for integration of the riboflavin biosynthesis operon from *B. subtilis*. Since the *pnuX* gene - which is fused to a kanamycin resistance cassette and integrated into NS2 for the purpose of roseoflavin-selection - was no longer required, the *pnuX* gene was replaced with the

*B. subtilis* riboflavin biosynthesis operon. Therefore, an integration plasmid was created containing the riboflavin biosynthesis operon *psbA2s_ribDGE_cpc560_ribABHT* amplified from pBK101 fused to a chloramphenicol resistance cassette, flanked by homology regions for integration at NS2 (pBK126). The roseoflavin-selected riboflavin-overproducing strain #48 was transformed with linearized plasmid pBK126. Selection for integration-positive clones was performed using 7.5 µg/mL chloramphenicol. Segregation was forced during several restreaks on agar plates using chloramphenicol concentrations of up to 20 µg/mL. Integration of the riboflavin biosynthesis operon, and therefore loss of the *pnuX* gene, was confirmed by cPCR, as described previously.

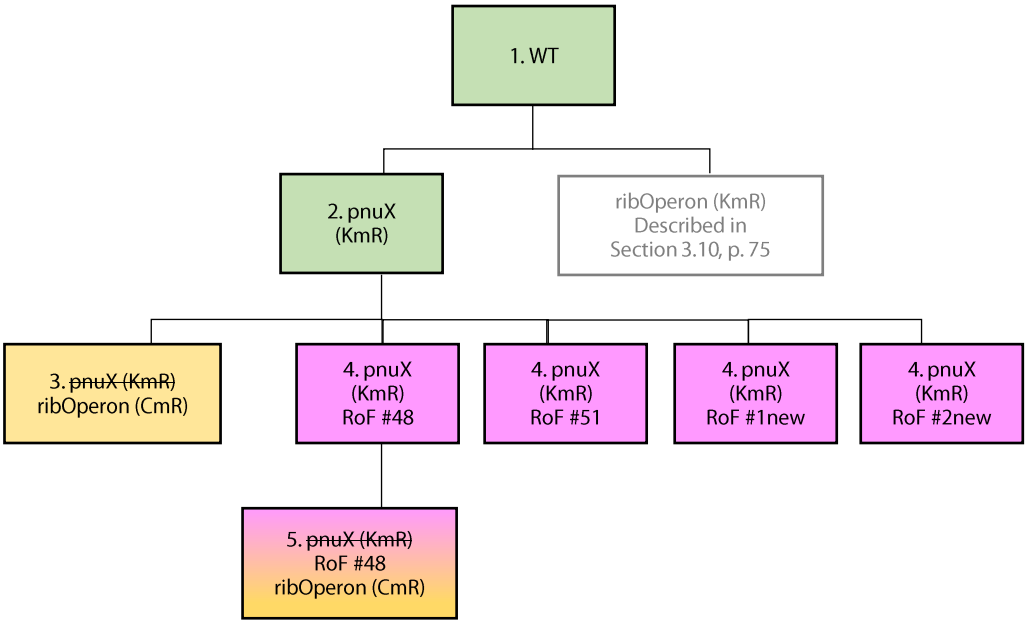



**Figure 43: Lineage of strains used in this experiment.** The *Synechococcus* sp. PCC 7002 wild-type strain (1.) was used as a parent strain to integrate the *pnuX* gene from *C. glutamicum* fused to a kanamycin resistance cassette (2.), as described in section 3.14. The resulting strain yielded the roseoflavin-resistant riboflavin-overproducing strains (4.) #48, #51, #1new and #2new, described in section 3.14.2. The NS2 locus of the parent *pnuX*-expressing strain (2.) was replaced by the riboflavin biosynthesis operon from *B. subtilis* by integration of the plasmid pBK126 (3.), therefore representing the genetic engineering strategy described in section 3.9. As a combination of both approaches, the roseoflavin-selected strain #48 was "overwritten" with the riboflavin biosynthesis operon from *B. subtilis* using plasmid pBK126 (5.).

Growth and flavin production of the wild-type was compared to either the unselected *pnuX*-expressing strain, the roseoflavin-selected riboflavin-overproducing strains, the genetically

engineered strain based on the wild-type parent and the genetically engineered strain expressing the same construct in the roseoflavin-selected strain #48.

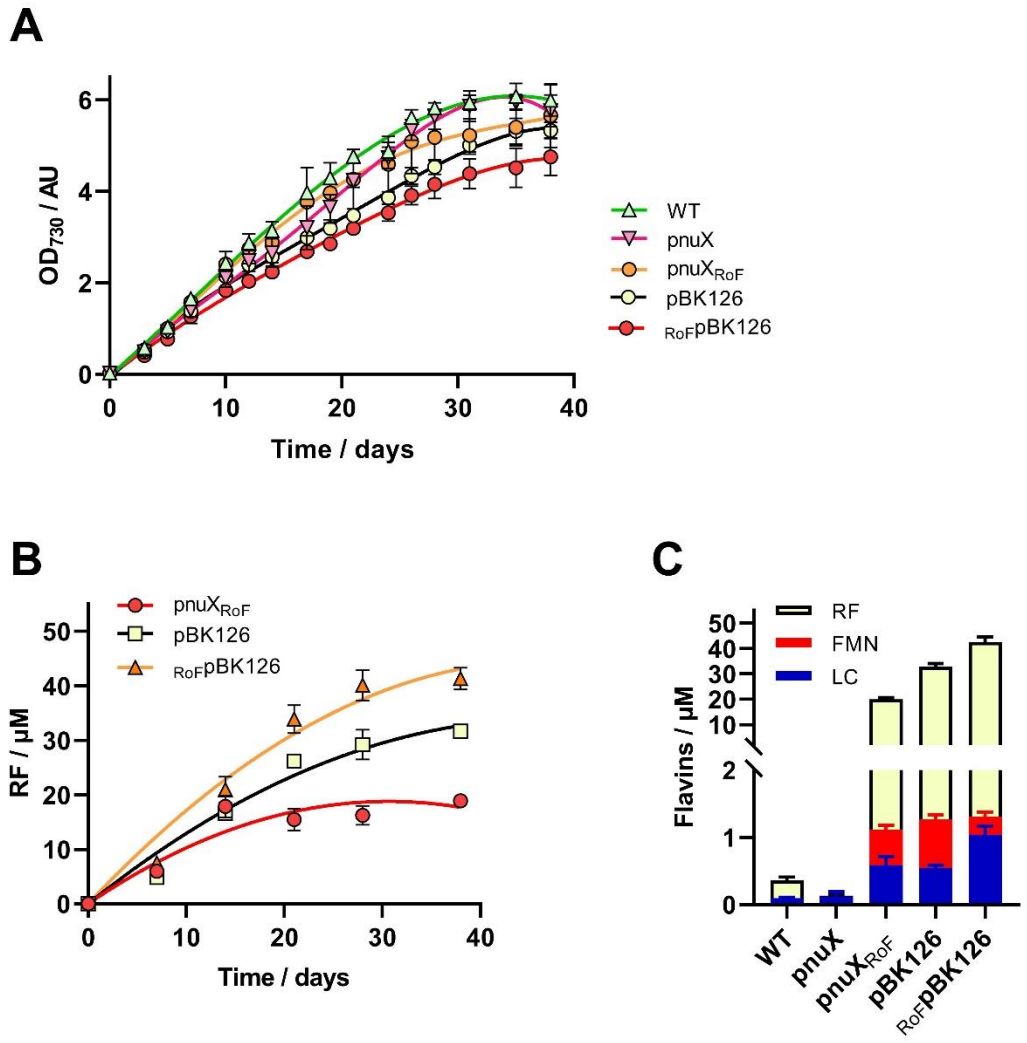



**Figure 44: Roseoflavin-selected *Synechococcus* sp. PCC 7002 as chassis for genetic engineering to yield higher riboflavin levels. (A)** $OD_{730}$ of the wild-type, an unselected ("*pnuX*") and several roseoflavin-selected *pnuX*-expressing strains ("$pnuX_{RoF}$"), as well as an unselected strain expressing the *B. subtilis rib* genes with the *psbA2s/cpc560* promoter combination ("*pBK126*"), and a roseoflavin-selected strain expressing the same construct ("$_{RoF}$*pBK126*"). **(B)** The roseoflavin-selected strain expressing the *B. subtilis rib* genes yields highest riboflavin levels. **(C)** Final supernatant concentrations of riboflavin, FMN and lumichrome. When the roseoflavin-selected strain is used as a parent strain for expression of the *B. subtilis rib* genes ("$_{RoF}$*pBK126*"), riboflavin production is significantly increased.. RF: riboflavin, LC: lumichrome.

All strains were grown in AA+ medium without antibiotic containing 4 µg/L vitamin $B_{12}$ in 100 mL Erlenmeyer flasks over the course of 38 days under standard red-light conditions.

Growth curves suggest that there is a mild growth impairment of the roseoflavin-selected *Synechococcus* sp. PCC 7002 strain expressing the *rib* genes. Growth impairment was less significant in the variant expressing the *rib* genes in a wild-type parent strain. Mild growth reduction was also observed for the roseoflavin-selected strains not expressing additional *rib* genes (Figure 44A). Riboflavin levels were highest for the roseoflavin-selected strain expressing additional *B. subtilis rib* genes, thus proving that the roseoflavin-selected strain can be used as a chassis to further increase riboflavin production but genetic engineering (Figure 44B,C). Lumichrome levels were below 3% of total flavins for all strains (Figure 44C). Note that the total flavin concentration for all strains was lower than for the strain genetically engineered with the pBK101 plasmid, as described in section 3.10, p. 75. One hypothesis is that the different antibiotic resistance marker in pBK126 is responsible for this effect, since all other variables were kept the same.

## 3.16. Comparison of recombinant *Synechococcus* sp. PCC 7002 and *B. subtilis* for overproduction of riboflavin

Recombinant *Synechococcus* sp. PCC 7002 strains engineered for overproduction of riboflavin yield product levels comparable to the early engineered *B. subtilis* strains (Figure 45). Upon replacement of the endogenous *rib* promoter and FMN riboswitch with a strong promoter (*SPO*) and a deregulated FMN riboswitch ($G_{37}G_{38}G_{39}$-> $A_{37}A_{38}A_{39}$, see section 3.8, p. 69) upstream of the *ribDG* gene, the *B. subtilis* WT overproduces riboflavin. Additional mutations decreasing the activity of RibFC and transketolase (*tkt*) further increased riboflavin production. The *Synechococcus* sp. PCC 7002 strains overexpressing the *B. subtilis rib* genes with different promoters yield similar riboflavin levels (Figure 45). However, the *B. subtilis* strains accumulated these levels over the course of 9 hours, whereas the *Synechococcus* sp. PCC 7002 strains were grown for 38 days. Therefore, the *B. subtilis* strain expressing $P_{veg}$*_ribDGEABHT* and carrying the *ribC820* and *tkt* mutations accumulates riboflavin at a rate of 343 µg*$L^{-1}$*$h^{-1}$, whereas the best-performing *Synechococcus* sp. PCC 7002 strain accumulates riboflavin at a rate of 30.5 µg*$L^{-1}$*$h^{-1}$.

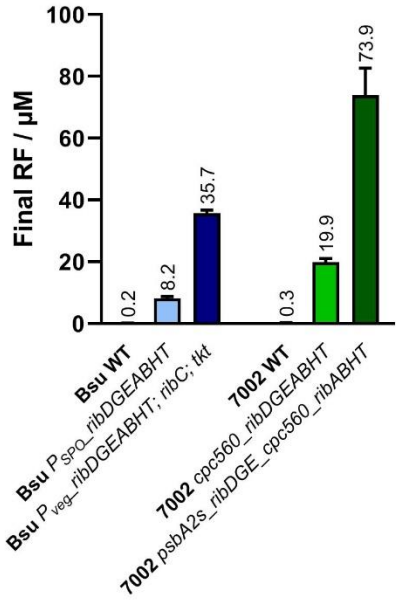



**Figure 45: Comparison of wild-type and recombinant *B. subtilis* and *Synechococcus* sp. PCC 7002 strains engineered for riboflavin production.** *B. subtilis* strains were grown in LB medium at 37 °C, 220 rpm for 9 hours. *Synechococcus* sp. PCC 7002 strains were grown in AA+ medium under red-light conditions, at 32 °C, 100 rpm, for 38 days.

## 3.17. RBS engineering increases riboflavin production in *E. coli*

The ribosomal binding site (RBS) is a sequence upstream of a gene's start codon that is recognized by the ribosome to initiate translation. Highly expressed genes usually have RBSs similar to the consensus sequence determined for this species, whereas poorly expressed genes show only little similarity. Using different RBSs under otherwise same conditions was shown to increase gene expression also in cyanobacteria [147, 160]. In 2009, the Salis Lab (California, USA) published an automated method that predicted the protein expression influenced by different RBS with great accuracy over five orders of magnitude. Subsequently, in a forward engineering approach they showed that rational design of the RBS influences protein expression in a predictably way [163]. Using the Salis Lab online tool ("RBS Calculator"), the total free energy $\Delta G_{tot}$ and translation initiation rate of the RBS of the riboflavin biosynthesis genes from *B. subtilis* was calculated (Table 11). The numbers somewhat reflect the enzyme concentration assumed by Birkenmeier et al. for the riboflavin-overproducing *B. subtilis* strains under production conditions [80], however, could not be confirmed for the *B. subtilis* wild-type due to a lack of absolutely quantified proteomics data. However, the RBS prediction suggests that the translation initiation rate for the RibAB enzyme is approx. one order of magnitude lower than for the RibD and the RibH protein. Therefore, rational design of the RBS upstream of *ribAB* gene towards a RBS with higher $\Delta G_{tot}$ might increase production of RibAB in relation the other riboflavin biosynthesis genes under otherwise same conditions.

**Table 11: Translation initiation rates calculated for the *B. subtilis* riboflavin biosynthesis genes, using the Salis Lab RBS Calculator**

| RBS of gene | $\Delta G_{tot}$ (kcal/mol) | Translation initiation rate |
|---|---|---|
| *ribDG* | -11 | 197371 |
| *ribE* | -3.7 | 5610 |
| *ribAB* | -6.7 | 25167 |
| *ribH* | -12 | 335947 |

In a first attempt to test whether RBS engineering of the *B. subtilis rib* genes might be a promising approach to increase riboflavin production, the original RBS upstream of the *ribAB* gene (expression plasmid pBK13) was replaced by an RBS calculated to have a 3-

fold higher translation initiation rate (pBK94), according to the Salis RBS Calculator tool. Both plasmids are based on the medium-copy plasmid pBBR_MCS2, containing an IPTG inducible *lac*-promoter. Chemically competent *E. coli* BL21(DE3) aliquots were transformed with either pBK13 or pBK94, respectively, and cultivated in LB medium. Subsequently, the experimental cultures were either cultivate without induction, or induced with 20 µM IPTG, at 37 °C, 220 rpm. Flavin concentration was approximated using the TECAN plate reader as described previously. The flavin concentration was 1.30- and 1.65-fold higher for the strain expressing the modified construct (pBK94), compared to the original RBS (pBK13). Therefore, this preliminary data suggest that RBS engineering would be a promising strategy to further increase riboflavin production (Figure 46B).

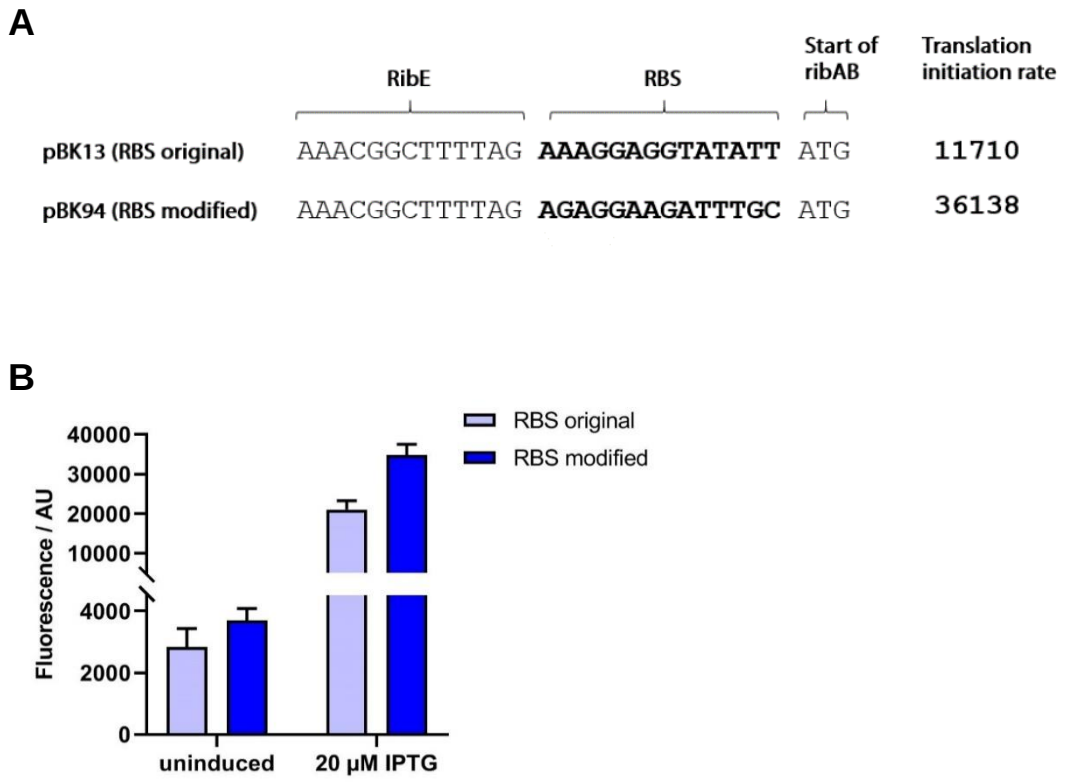



**Figure 46: Increase of flavin production by RBS engineering of the *ribAB* gene in the *B. subtilis rib* operon. (A)** The original RBS was modified to achieve a higher (predicted) translation initiation rate. **(B)** Flavin fluorescence of transformed *E. coli* cultures revealed higher flavin levels for the strain expressing the modified RBS. Transcription of the *rib* operon was either kept basal (uninduced) or was induced with 20 µM IPTG.

# IV

# Discussion

## 4.1. Goals and achievements of this project

The aim of this project was to generate the first phototrophic production strain for vitamin $B_2$ (riboflavin). The marine cyanobacterium *Synechococcus* sp. PCC 7002 was selected as a host strain. To shed light on the endogenous riboflavin production in *Synechococcus* sp. PCC 7002, the native riboflavin biosynthesis genes *ribDG*, *ribE* and *ribAB* were functionally characterized via complementation of auxotrophic *E. coli* strains. The function of the *Synechococcus* sp. PCC 7002 RibH was confirmed via an enzymatic assay. All genes were able to catalyze their annotated function and explains why *Synechococcus* sp. PCC 7002 is phototrophic for riboflavin. Plasmids for integration of heterologous DNA into the genome of *Synechococcus* sp. PCC 7002 were created using DNA sequences for homologous recombination at NS2. The first obstacle was the light-induced degradation of riboflavin under cyanobacterial standard conditions using white light. Adding a low-pass filter did not reduce degradation sufficiently (Appendix section 6.1, p. 124), therefore a red LED array was constructed which allows both stable incubation of riboflavin and growth of the *Synechococcus* sp. PCC 7002 host strain comparable to white-light conditions. Subsequently, a set of promoters was characterized in *Synechococcus* sp. PCC 7002 under red-light conditions using a GFP-based readout. Three of these regulatory elements were fused to the riboflavin biosynthesis operon *ribDGEABHT* amplified form *B. subtilis* and integrated into the genome of *Synechococcus* sp. PCC 7002. The recombinant strains produced strongly elevated riboflavin levels and the concentration correlated with the respective regulatory element's strength assessed in the GFP experiment. Codon-adaptation of the *B. subtilis rib* operon for expression in *Synechococcus* sp. PCC 7002 did not improve riboflavin production. However, addition of a strong promoter upstream of the *ribAB* gene of the *B. subtilis rib* operon (original codon usage) produced highest riboflavin levels. Almost all riboflavin is found in the supernatant, whereas FMN remains in the cell.

Riboflavin concentration peaked 74 µM after 38 days of growth for the best-performing *Synechococcus* sp. PCC 7002 strain expressing *psbA2s_ribDGE_cpc560_ribABHT*, which is a 211-fold increase compared to the wild-type. RNA was extracted and the presence of *B. subtilis rib* gene mRNA transcripts was confirmed using RT-PCR. All heterologously expressed proteins were identified by LS/MS. Protein quantitation of the heterologous riboflavin biosynthesis enzymes revealed significantly elevated RibAB levels in the strains

producing high levels of riboflavin, which is in line with the hypothesis that RibAB represents the rate-limiting step in riboflavin biosynthesis also in the recombinant *Synechococcus* sp. PCC 7002 strains.

The second strategy to create riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains was the selection of mutants on the toxic antimetabolite roseoflavin. *Synechococcus* sp. PCC 7002 was made sensitive to roseoflavin by stable integration of the flavin transporter *pnuX* from *C. glutamicum*. Selection of the *pnuX*-strain on agar plates containing roseoflavin yielded several riboflavin-overproducing strains. Four of these strains were analyzed via whole-genome sequencing and showed a distinct 4-nt deletion in the *SYNPCC7002_A0225* gene, which is associated specifically with the roseoflavin-resistant riboflavin-overproducing phenotype. So far, however, no mechanisms of riboflavin-overproduction could be deduced, and a knock-out of *SYNPCC7002_A0225* did not influence riboflavin production in the wild-type strain.

To prove that the roseoflavin-selection approach can be combined with expression of heterologous *rib* genes, a roseoflavin-selected riboflavin-overproducing *Synechococcus* sp. PCC 7002 was used as a chassis for *B. subtilis rib* gene expression. The resulting strain yielded higher levels of riboflavin compared to the parent strain and compared to a wild-type strain carrying the same construct.

Other experiments were conducted that set the stage to further engineer *Synechococcus* sp. PCC 7002 for riboflavin overproduction. First, the endogenous high-copy plasmids pAQ1 and pAQ3 were targeted for integration of a GFP-expression cassette. The recombinant strains yielded higher fluorescence signals compared to the strains carrying the same GFP-expression cassette at NS2 in the circular chromosome. Therefore, the endogenous plasmids could be used to boost expression of *rib* genes in *Synechococcus* sp. PCC 7002 and therefore increase riboflavin production.

Additionally, an initial experiment in *E. coli* showed that the ribosomal binding site of the *B. subtilis ribAB* gene within the *ribDGEABHT* operon can be altered to increase riboflavin production without the need to add additional copies or target different neutral sites.

## 4.2. Slow growth as disadvantage of cyanobacterial as biotechnological platforms

Compared to heterotrophic microorganisms, e.g. *E. coli* and *B. subtilis*, which have reported doubling times of 20-30 min, even the fastest growing cyanobacteria laboratory strain characterized so far, *Synechococcus* UTEX 2973, was reported to have doubling times of 2.1 hours. This value was determined under specialized laboratory conditions, which include $CO_2$ gassing, optimized temperature and illumination, and cultivation in turbidostat conditions [35]. *Synechococcus* sp. PCC 7002 was reported to achieve doubling times of 2.6 hours under similar conditions. In my experiments, however, when cultivating the wild-type strain in 30 mL culture volume in a 100 mL Erlenmeyer flasks under ambient air, doubling time is < 24 hours only for $OD_{730}$ < 0.5. Doubling in the $OD_{730}$ range of 0.5 - 1.0 takes 2.1 days and increases to 3.6 days in the $OD_{730}$ range of 1.0 - 2.0 (Figure 8, p. 26). Growth in 6-well plates is generally faster, probably due to a lower ratio of culture depth per surface area, which might result in better illumination and gas exchange. Using an incubator capable of supplementing $CO_2$ would most likely shorten all experimental procedures. However, large-scale biotechnological applications also need to be conducted in ambient air (0.04 % $CO_2$) and are further limited by natural light cycles and seasonal lighting [164], suboptimal lighting due to cell shading [165], suboptimal turbidity due to cost-effective agitation, varying temperatures, etc, which all increase the doubling time of the respective strain. Recent studies have demonstrated that the photosynthetic capacity of cyanobacteria and biomass accumulation over time can be increased by genetic engineering. For example, Ducat et al. showed that the photosynthetic efficiency of cyanobacteria is increased when the carbon flux is driven away from the primary metabolism to a storage molecule (e.g. the osmoprotectant succhrose) in *Synechococcus* elongatus sp. PCC 7942 [166, 167]. Additionally, the photosynthetic efficiency of cyanobacteria can be directly improved, as was shown by overexpression of the Calvin-Benson cycle enzymes RuBisCO, sedoheptulose bisphosphatase, fructose bisphosphate aldolase, and transketolase [168]. By installing additional bicarbonate transporters into the membrane it was shown that both oxygen evolution and growth rate could be increased in *Synechocystis* sp. PCC 6803 [169]. These results indicate that the cynobacterial doubling times could be artificially increased in the future, making bioindustrial photoautotrophic production processes more economically attractive.

## 4.3. *Synechococcus* sp. PCC 7002 as a chassis for genetic engineering

*Synechococcus* sp. PCC 7002 has proven to be a reliable platform for genetic engineering in this study. Integration into the genome (NS2) using linearized plasmids usually worked on first trial with sufficient efficiency, following the straight-forward protocol described on p.28. Segregation could be enforced reliably by re-streaking positive clones 1-2 times on increasing antibiotic concentration. No genetic instability (mutations or loss of integrated fragments) was observed when cultivating (completely segregated) strains over the course of several growth curves, even without antibiotics. Integration and complete segregation of DNA fragments into the high-copy plasmid pAQ3 was also successful, therefore expanding the possibilities for integration of heterologous DNA fragments into *Synechococcus* sp. PCC 7002. However, complete segregation of DNA fragments integrated into pAQ1 was not successful, even when using kanamycin concentrations up to 3000 µg/mL. This might be due to the fact that the homology arms described for this integration site flank a considerable portion of the endogenous plasmid which gets lost as a result of integration [113, 125], leaving some doubt whether this particular integration site is actually "neutral". To the best of our knowledge, complete segregation of this locus was never published. However, even an incompletely segregated strain yielded more than 4-fold increase in GFP expression compared to the same construct expressed from the genomic integration site NS2. Therefore, other integration sites or resistance markers should be explored to exploit the high-copy plasmid pAQ1 for expression of heterologous genes.

## 4.4. Expression of *B. subtilis rib* genes in *Synechococcus* sp. PCC 7002

### Total expression levels of *B. subtilis rib* genes in *Synechococcus* sp. PCC 7002 are still very low

The *cpc560* promoter from *Synechocystis* sp. PCC 6803 yielded the strongest fluorescence signal and, when combined with the riboflavin biosynthesis transcription unit *ribDGEABHT* from *B. subtilis*, highest riboflavin production. This is in line with recent

literature, showing that the *cpc560* promoters is one of the strongest promoters described in cyanobacteria so far [148]. However, the previously mentioned study achieved expression of a heterologous protein to levels of up to 15% of total protein, using the *cpc560* promoter driving expression of a codon-adapted gene in *Synechocystis* sp. PCC 6803, which yielded a visible band on a Coomassie-stained SDS-PAGE. In my experiments, however, the gene products of the *ribDGEABHT* operon expressed by *cpc560* are not visible on a standard SDS-PAGE (Figure 34, p.79).

The *pTrcTheo* promoter is a $p_{lac}$-derived promoter fused to a theophylline riboswitch [153]. In an *E. coli* BL21(DE3) expression strain it can be regulated on both the transcriptional and the translational level, using IPTG and theophylline as inducers, respectively. Since *Synechococcus* sp. PCC 7002 does not contain a lac-inhibitor (LacI) protein, regulation of this promoter is only possible on the translational level. The *pTrcTheo* promoter yielded remarkably strong GFP fluorescence in *Synechococcus* sp. PCC 7002 and could be tighly regulated with different theophylline concentrations (p. 65). The basic $p_{lac}$ promoter should be tested in the absence of the theophylline-binding aptamer-forming sequence, since it might be used an alternative constitutive strong promoter not influenced by light.

**The FMN riboswitch was not used to create a riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain**

Surprisingly, the FMN riboswitches of two allegedly riboflavin-overproducing *B. subtilis* strains did not yield higher riboflavin concentrations compared to the wild-type riboswitch, as was shown in *E. coli* (p. 69). Only by deleting the latter half of the riboswitch, or by specifically deleting the 14-nucleotide sequence forming the stem of the terminator loop high flavin levels were produced. However, riboflavin levels in these strains were not significantly higher than when omitting the FMN-riboswitch completely. Therefore, the *B. subtilis* riboflavin biosynthesis transcription unit was integrated into *Synechococcus* sp. PCC 7002 without the FMN-riboswitch. In order to test whether the FMN riboswitch influences *rib* gene expression in *Synechococcus* sp. PCC 7002, a set of plasmids was created to integrate the *B. subtilis rib* genes in *Synechococcus* sp. PCC 7002 under the control of the *psbA2s* promoter regulated by different FMN variants (Table 12). These plasmids have not been transformed yet.

**Table 12: Plasmids for integration of the *B. subtilis ribDGEABHT* containing different FMN-riboswitch variants into NS2 of *Synechococcus* sp. PCC 7002.** Sequence alignment of the FMN riboswitch (RFN) variants is shown in *Figure 30*, p. 71.

| plasmid | integration fragment | description |
|---|---|---|
| pBK64 | *KmR_psbA2s_ ribDGEABHT* | no FMN-riboswitch |
| pBK117 | *KmR_**RFN#2**_psbA2s_ ribDGEABHT* | FMN-riboswitch derived from the wild-type *B. subtilis* |
| pBK118 | *KmR_**RFN#5**_psbA2s_ ribDGEABHT* | FMN-riboswitch containing the G->A and GGG->AAA mutation, deletion of latter half of the riboswitch (including terminator sequence) |
| pBK119 | *KmR_**RFN-Term**_psbA2s_ ribDGEABHT* | wild-type FMN-riboswitch with 14-nucleotide deletion in the terminator stem (designed by A. Boumezbeur) |

**Codon-adaptation of the *B. subtilis rib* genes for expression in *Synechococcus* sp. PCC 7002 did only yield low levels of riboflavin**

Codon-adaptation was shown to increase gene expression [170] also in cyanobacteria [160]. Unexpectedly, codon-adaption of the *B. subtilis rib* genes for *Synechococcus* sp. PCC 7002 (see section 6.1, p. 124) did not increase riboflavin production. In my opinion, it is highly unlikely that the altered codons are actually sub-optimal, as they were all derived from the sequences of highly expressed genes in *Synechococcus* sp. PCC 7002. All codon-adapted genes are intact and should result in the same protein primary sequence as the *B. subtilis* genes. The intergenic regions between the riboflavin biosynthesis genes were kept unchanged, including the endogenous ribosomal binding sites (RBS). Note that for the codon-adapted operon the orientation of the riboflavin biosynthesis genes was changed, placing the *ribAB* gene first, followed by *ribDG*, *ribE*, *ribH* and *ribT*. This was supposed to increase RibAB expression. Codon-adptation altered the sequence of the internal promoters that are located within the *B. subtilis rib* operon [126]. However, since these promoters are not strong in *B. subtilis* and might not even be active in *Synechococcus* sp. PCC 7002, it does not explain low riboflavin production. A possible explanation could be that codon-adaptation altered so-called “pause sites”, which are sequences in the mRNA transcript that cause the RNA polymerase to stop for a certain amount of time, allowing proper protein folding [171]. Therefore, it would be possible that one or several *rib*

enzymes are abundant but misfolded and therefore unfunctional - a hypothesis that can only be tested by protein quantitation (Western Blot or LC/MS analysis).

**Quantitation of *B. subtilis rib* genes in recombinant *Synechococcus* sp. PCC 7002 using LC/MS**

Following the hypothesis that riboflavin production can be increased by increasing RibAB expression, the strong promoter *cpc560* was integrated upstream of the *ribAB* gene in the otherwise unchanged riboflavin transcription unit driven by *psbA2s*, as described in this study. In the absence of the additional *cpc560* promoter (pBK64), the *Synechococcus* sp. PCC 7002 strain yielded 0.2 µM riboflavin per $OD_{730}$. Adding the *cpc560* promoter (pBK101) increased riboflavin production 15.6-fold, to 3.13 µM per $OD_{730}$ (Figure 31, p.73). Proteomics data (Figure 36, p.82) suggest that the increase in riboflavin production is indeed due to an increase in RibAB expression, which is ~4-fold higher in the strain carrying the additional *cpc560* promoter upstream of *ribAB*. Conversely, the *ribH* and *ribT* genes, which are located downstream of *ribAB*, were not expressed at higher rate, as the RibH and RibT enzyme levels were comparable. Also, neither the downregulation of RibD (0.5-fold) nor the upregulation of RibE (2.0-fold) can be explained by the addition of the *cpc560* promoter.

In my opinion it is difficult to assess whether the results of the proteomics analysis actually represent the biological reality, even though error bars (one standard deviation from average, n=3) are low. The different samples analyzed are considered biological replicates, since the cultures were picked as individual cfus from the transformation plate, stored as individual DMSO stocks, grown in individual Erlenmeyer flasks and run on individual lanes in the SDS-PAGE gel. The bands cut from each lane were dimethyl-labelled individually before subjected to the LC/MS workflow. Variability introduced during trypsin digest, dimethyl-labelling and LC/MS run could have an effect, however, should be normalized for using the background protein signals.

Further experiments need to be conducted to correlate expression strength of the *B. subtilis rib* genes with riboflavin production in *Synechococcus* sp. PCC 7002. Transcriptomics analysis would shed light on the question if transcript levels actually increase when a stronger or additional promoter is used. However, transcript abundance is only one factor influencing protein abundance. Many other regulatory elements (e.g. RBS, elongation factors, secondary structures, pause sites, mRNA degradation) could influence the

production of the heterologous *rib* enzymes in *Synechococcus* sp. PCC 7002 and have to be investigated further.

## 4.5. Selection of *Synechococcus* sp. PCC 7002 *pnuX*-strains on roseoflavin

Integration of the flavin transporter *pnuX* from *C. glutamicum* into the genome of *Synechococcus* sp. PCC 7002 induced sensitivity towards roseoflavin. This was surprising for several reasons. First, no riboswitch regulating the expression of flavin biosynthesis genes is known in *Synechococcus* sp. PCC 7002 [86]. The FMN riboswitch was shown to be a likely "target" for mutations to occur when selecting microorganisms on roseoflavin [97]. Second, as the roseoflavin-selection depends on the proper function of the PnuX transporter, it was assumed that loss-of-function mutations in the promoter or gene sequence of *pnuX* would appear frequently and reverse roseoflavin-sensitivity without increasing riboflavin production. Third, as no chemical mutagen or UV-radiation for random mutagenesis was used prior to selection, only mutations were expected occurring naturally in *Synechococcus* sp. PCC 7002. To the best of my knowledge, the mutation rate in this species is not known. Assuming a specific mutation rate of $\mu_b=5.4*10^{-10}$ per basepair per replication, as published for *E. coli* by Drake et al. [172], it would require ca. 540 mL of a culture with an $OD_{730}$ of 1.0 (which contains ca. $1.0*10^8$ cells) to generate mutants with specific mutatation at any given nucleotide. Instead, only ca. $2.0*10^8$ cells were plated on AA+ plates containing 50 µM roseoflavin, yielding several hundred roseoflavin-resistant colonies. Only two out of nine strains analyzed carry loss-of-function mutations in the *pnuX* gene. Seven out of nine strains did indeed overproduce riboflavin. Cultivated in liquid cultures, however, the riboflavin levels per $OD_{730}$ decreased significantly until it stabilized at ca. 2.3 µM per $OD_{730}$. This could be due to the polyploidy of *Synechococcus* sp. PCC 7002 [115] that might cause loss of mutations over time if these mutations are harmful for the host cells. Attempts to segregate possible mutations by re-streaking the cultures multiple times on agar plates containing roseoflavin did not yield stable riboflavin-producing colonies.

Whole-genome sequencing analysis revealed only one mutation that is associated with the roseoflavin-selected riboflavin-overproducing phenotype, which is found in the

*SYNPCC7002_A0225* gene. Knock-out of this gene by replacement with a chloramphenicol resistance cassette, however, did not induce riboflavin-production in the wild-type strain. To shed light on the role of *SYNPCC7002_A0225*, further experiments should be conducted, including (1) a replacement of the endogenous gene with the mutated gene in the wild-type, and (2) *vice versa* replacing the mutated gene in the roseoflavin-selected riboflavin-overproducing strains. Also, it is possible that polar effects influence the expression or stability of *SYNPCC7002_A0224*, which is on an operon with *SYNPCC7002_A0225*. *SYNPCC7002_A0224*, however, codes for a "M48 familiy metallopeptidase" with no apparent connection to riboflavin biosynthesis.

As shown in section 3.15 (p. 90) the roseoflavin-selected riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain #48 can be used as a chassis for integration of the *B. subtilis rib* operon (pBK126: *NS2L_CmR_psbA2s_ribDGE_cpc560_ribABHT_NS2R*), yielding higher flavin levels compared to both the untransformed #48 strain and the wild-type strain expressing the same construct. The overall riboflavin-concentration per $OD_{730}$, however, was lower in the pBK126-transformed #48 strain compared to the wild-type strain transformed with pBK101 (*NS2L_KmR_psbA2s_ribDGE_cpc560_ribABHT_NS2R*), see section 3.10, p. 75. It is possible that the different antibiotic selection markers contribute to this effect. However, this should not confuse the fact that the combination of selection and genetic engineering was successful in *Synechococcus* sp. PCC 7002.

## 4.6. Outlook

Targeted metabolome analysis of all riboflavin and purine precursors and intermediates should be considered to develop more elaborate strategies for increasing riboflavin production in *Synechococcus* sp. PCC 7002. Such an analysis could shed light on the question whether the phototrophic riboflavin production in *Synechococcus* sp. PCC 7002 is limited by precursors supply (GTP, Ru5P), or by shortage or accumulation of (potentially toxic) intermediates due to relative over- or under-expression of individual *rib* genes.
In general, the production of all *rib* enzymes might be further increased by integration of the *B. subtilis rib* operon into the endogenous plasmids (e.g. pAQ1 and pAQ3) of

*Synechococcus* sp. PCC 7002. Integration plasmids were already cloned (e.g. pBK87-91), but not yet successfully transformed. Alternatively, riboflavin biosynthesis might be increased by increasing the levels of RibAB, as RibAB levels determine the riboflavin production of the recombinant riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains. Increased RibAB levels could be achieved either by integrating additional copies of *ribAB* into a different genomic locus of *Synechococcus* sp. PCC 7002, or by increasing *ribAB* expression through different promoters and/or by using different ribosomal binding sites, as proposed by Salis et al. [163]. To prevent depletion of Ru5P (substrate of RibB), which plays an important role in the Calvin-Benson-Cycle of *Synechococcus* sp. PCC 7002 (Figure 47), it might be considered to overexpress an enzyme executing only RibA function (EC 3.5.4.25). A gene coding for such a monofunctional RibA enzyme can be found in *Escherichia coli* (e.g. *CXP41_07115*, GeneBank: CP025268.1). The *pTrcTheo* promoter could generally be used as a regulatory element to fine-tune gene expression and to investigate the relationship between gene dosage and riboflavin output in these novel riboflavin-overproducing strains.

Furthermore, the selection approach could be expanded using different antimetabolites. To the best of my knowledge no purine import system is known in *Synechococcus* sp. PCC 7002, compared to e.g. *E. coli* [174]. Thus, selection on 8-azaguanine would probably require integration of a transporter protein. Interestingly, cyanobacterial strains were cultivated on glutamine as sole nitrogen source [175] and it was shown for some cyanobacteria that amino acids are transported into the cell [176], therefore methyl sulfoxide might be used to select resistant *Synechococcus* sp. PCC 7002 mutants which would not be regarded as gene-modified organisms (GMO).

An additional strategies to increase riboflavin production in *Synechococcus* sp. PCC 7002 is to increase the carbon flux through the purine pathway. Unfortunately, *Synechococcus* sp. PCC 7002 does not seem to contain a protein similar to PurR in *B. subtilis*, which functions as a suppressor molecule for several genes of the purine biosynthesis pathway and can be deleted to increase purine synthesis [177-179]. Also, no purine riboswitch was described for cyanobacteria so far [86]. Therefore, carbon flux through the purine pathway has to be increased by overexpression of the purine biosynthesis enzymes (**Figure 6**, p.14), in order to increase GTP precursor supply.

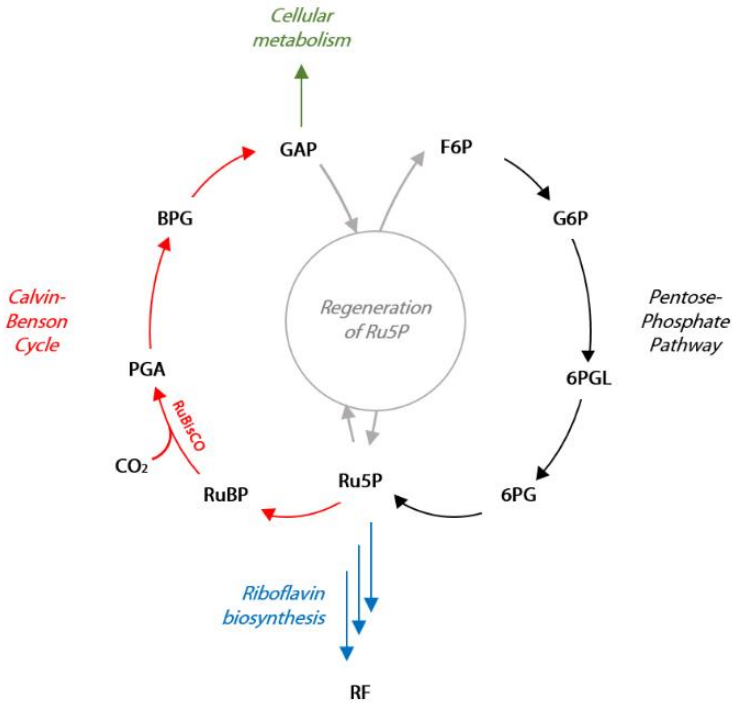



**Figure 47: The riboflavin biosynthetic pathway competes for ribulose-5-phosphate (Ru5P) with the Calvin-Benson Cycle.** Ru5P is converted into ribulose-1,5-bisphosphate (RuBP). The enzyme RuBisCO catalyzes carboxylation of RuBP which leads to two molecules 3-phosphoglyceric acid (PGA). PGA is converted into 1,3-bisphophoglyceric acid (BPG), which is converted to glyeraldehyde-3-phosphate (GAP). Five out of six GAP molecules are regenerated via a complex intertwined pathway catalysed by different aldolases, transketolases, phosphatases, isomerases and epimerases. Ru5P is also derived from the reductive pentose phosphate pathway. Increasing riboflavin biosynthesis might drain Ru5P from the Calvin-Benson Cycle. The scheme was adapted from Waldbauer et al. (2012) [173].

The metabolic flux away from riboflavin could be decreased by reducing the activity of RibFC. In *B. subtilis* a *ribFC820* mutant displaying reduced activity in the RibFC enzyme accumulates riboflavin [89, 103]. As my riboflavin-overproducing *Synechococcus* sp. PCC 7002 strains show increased FMN levels compared to the wild-type (Figure 44, p.92), this strategy might be adopted by mutating the endogenous *ribFC* or exchanging it with the *B. subtilis ribFC820* variant.

# VI

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# VII

# Appendix

## 6.1. Investigating filter foils for stable incubation of aqueous riboflavin solutions

In general, standard cyanobacterial laboratory culturing employs white light originating from a fluorescent light bulb. As described in section 3.2, p. 50, white light illumination causes degradation of riboflavin. An initial strategy investigated in this thesis was to block light below a certain wavelength using a high-pass filter. The future incentive would be to use sunlight as a light source, which would make sense for energy-efficient large-scale cultivation of riboflavin-overproducing cyanobacteria outside the lab. Therefore, a commercial red high-pass filter ("106 primary red", Lee Filters, UK) was purchased. Light intensity distribution of a white halogen lamp was measured in the range of 300-1100 nm. Subsequently, a filter was positioned in the path between light source and the sensor, and transmitted light was measured. The spectrum derived from light (without filter or additional light source) was subtracted as baseline. Transmission-% of the filter is defined as the ratio of the measurements with and without the filters.

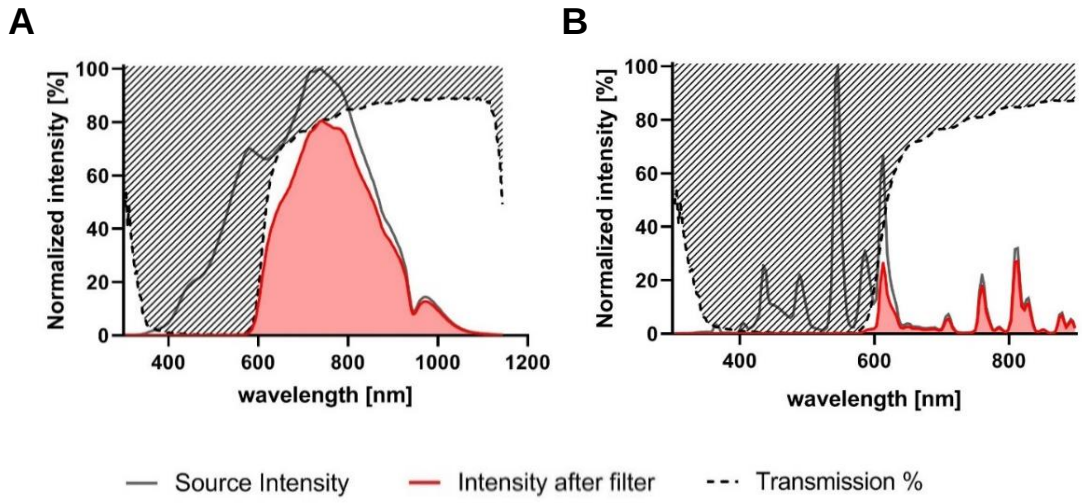


**Figure 48: Transmission of light through a red high-pass filter. (A)** The light intensity spectrum of a halogen lamp was measured before (grey) and after (red) transmission through a red high-pass filter. Transmission-% of the filter was calculated from the ratio between both measurements after baseline subtraction (dotted line and background). **(B)** The light intensity spectrum of a Philips TLD 18W/840 fluorescent lamp was measured (grey). Transmitted light (red) is only a small fraction of the total light emitted. All measurements were conducted in the Beuermann Lab, Mannheim University of Applied Sciences, Mannheim, Germany.

Applying the red filter, light is blocked almost completely in the range from 350-600 nm. Transmission drastically increases from 1% transmission at 576 nm to 60% at 630 nm, and reaches a maximum of 89% at 1016 nm. Since the halogen light source provides wavelengths mostly in the range 350-1100 nm, the transmission of light in the UV spectrum could not be measured accurately with this method. The high percentage of transmission in this area might as well be an artefact caused by ambient light (Figure 48).

The stability of a riboflavin solution (45 µL in AA+ medium) under white light combined with a red-light filter was measured in comparison to an uncovered sample incubated under white light (Philips TLD 18W/840), and in dark conditions, respectively. As described in more detail in section 3.2, p. 50, riboflavin is degraded quickly in white light, but is stable in the dark. Covering the sample with a filter foil increases stability of riboflavin, however, within 190 hours (ca. 8 days), about 30% of riboflavin is degraded (Figure 49).

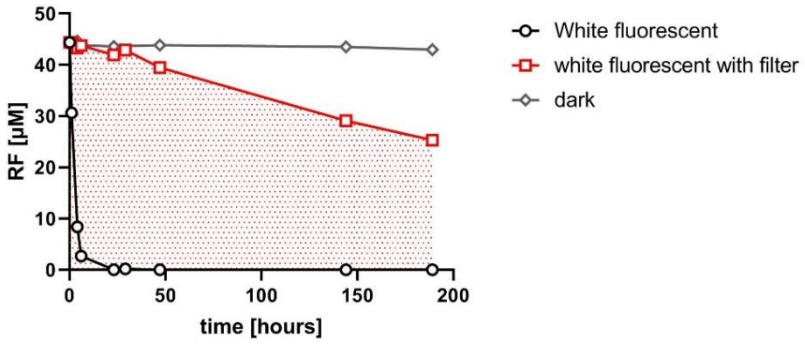


**Figure 49: Applying the red high-pass filter on a riboflavin solution.** A 45 µM riboflavin solution in AA+ medium degrades quickly under white light (black dots). Adding a filter increases stability of riboflavin, however, ca. 30% is lost within 8 days (red squares, shaded area). Riboflavin is stable in the dark.

Culturing the riboflavin-overproducing *Synechococcus* sp. PCC 7002 strain containing *psbA2s_ribDGEABHT* (pBK64) in white light vs white light with filter yields similar results: the major degradation product lumichrome is the most prevalent flavin compound when cultured under white light. Adding the filter stabilizes riboflavin, however, ca. 30% of the flavin content is lumichrome (Figure 50).

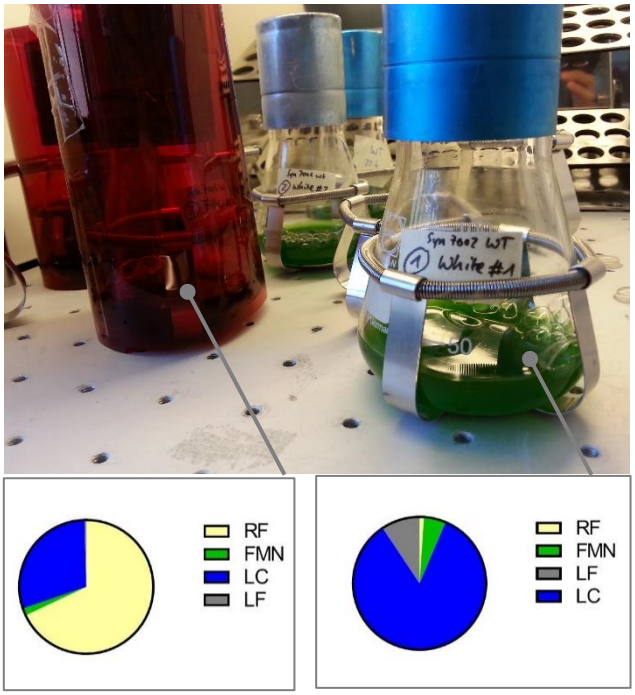


**Figure 50: Example of *Synechococcus* sp. PCC 7002 expressing *psbA2s_ribDGEABHT* cultivated in white light with and without a red-filter foil.** Covered by the red filter foil the strain yields mostly riboflavin (left). An uncovered culture yields mostly lumichrome. RF: Riboflavin, LC: Lumichrome, LF: Lumiflavin.

## 6.2. Codon-adaptation of *rib* genes for expression in *Synechococcus* sp. PCC 7002

Codon-adaptation is a strategy to increase gene expression by using codons that are highly abundant in the respective species. It was shown that codon-adaptation increases both transcript and protein levels [170] and increase product titres in metabolically engineered Synechocystis PCC 6803 strains [160].

A codon-usage table (Table 14) was created from the most highly abundant genes in *Synechococcus* sp. PCC 7002 (Table 13), as derived from transcriptomics data published by Ludwig and Bryant [128, 129]. Several of the codons in the original *B. subtilis rib* operon are not used in the highly abundant *Synechococcus* sp. PCC 7002 genes, suggesting that some of these codons are rare and might hinder expression of the *B. subtilis rib* genes in *Synechococcus* sp. PCC 7002.

**Table 13: Most abundant transcripts in *Synechococcus* sp. PCC 7002 under standard conditions as derived from Ludwig and Bryant**

| Locus_tag | Counts [OD 0.7] | Gene name | Gene product |
|---|---|---|---|
| *SYNPCC7002_A2210* | 70907 | *cpcA* | phycocyanin, alpha subunit |
| *SYNPCC7002_A1418* | 63774 | *psbA* | photosystem q(B) protein |
| *SYNPCC7002_A1961* | 63457 | *psaA* | PS I P700 chlorophyll A apoprotein A1 |
| *SYNPCC7002_A2209* | 52347 | *cpcB* | phycocyanin, beta subunit |
| *SYNPCC7002_A2813* | 48691 | - | S-layer like protein; probable porin |
| *SYNPCC7002_A2531* | 43332 | - | conserved hypothetical protein |
| *SYNPCC7002_A1929* | 43223 | *apcB* | allophycocyanin, beta subunit |

All rare and moderately rare codons in the original *B. subtilis rib* operon sequence were changed to more frequent *Synechococcus* sp. PCC 7002 codons using the platform ATGme [180]. The resulting gene sequence (see annotated sequence below) was ordered, produced and validated by Thermo Scientific.

**Table 14: Codon usage table calculated for *Synechococcus sp. PCC 7002***

http://www.kazusa.or.jp/codon/cgi-bin/countcodon.cgi)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| UUU 15.8( | 58) | UCU 26.2( | 96) | UAU 9.0( | 33) | UGU 3.8( | 14) |
| UUC 41.2( | 151) | UCC 15.3( | 56) | UAC 24.8( | 91) | UGC 2.2( | 8) |
| UUA 5.5( | 20) | UCA 0.8( | 3) | UAA 2.5( | 9) | UGA 0.3( | 1) |
| UUG 13.6( | 50) | UCG 1.4( | 5) | UAG 0.0( | 0) | UGG 19.4( | 71) |
| CUU 17.2( | 63) | CCU 11.7( | 43) | CAU 4.4( | 16) | CGU 18.0( | 66) |
| CUC 36.0( | 132) | CCC 17.7( | 65) | CAC 22.1( | 81) | CGC 8.7( | 32) |
| CUA 7.1( | 26) | CCA 1.4( | 5) | CAA 19.1( | 70) | CGA 1.1( | 4) |
| CUG 12.6( | 46) | CCG 1.1( | 4) | CAG 10.4( | 38) | CGG 1.9( | 7) |
| AUU 20.5( | 75) | ACU 31.1( | 114) | AAU 17.2( | 63) | AGU 5.7( | 21) |
| AUC 34.9( | 128) | ACC 28.4( | 104) | AAC 38.5( | 141) | AGC 16.1( | 59) |
| AUA 0.0( | 0) | ACA 8.5( | 31) | AAA 11.2( | 41) | AGA 1.4( | 5) |
| AUG 20.7( | 76) | ACG 3.5( | 13) | AAG 14.2( | 52) | AGG 0.0( | 0) |
| GUU 33.0( | 121) | GCU 47.7( | 175) | GAU 32.2( | 118) | GGU 64.9( | 238) |
| GUC 15.3( | 56) | GCC 18.6( | 68) | GAC 24.0( | 88) | GGC 30.3( | 111) |
| GUA 16.4( | 60) | GCA 27.8( | 102) | GAA 24.8( | 91) | GGA 4.6( | 17) |
| GUG 7.4( | 27) | GCG 15.6( | 57) | GAG 9.0( | 33) | GGG 4.4( | 16) |

**Sequence of the codon-adapted riboflavin biosynthesis operon**

The sequence of the codon-adapted riboflavin biosynthesis operon is given below, using the color code: pTrcPromoter; *ribAB*, *ribDG*, *ribE*, *ribH*, ribT, *rbcL* terminator

Note that the order of the riboflavin biosynthesis genes was changed in the codon-adapted operon. With the intention to increase expression of the RibAB enzyme and put it under the control of the inducible *pTrcTheo* promoter, the *ribAB* gene was placed upstream of *ribDG*. The 5' UTR of *ribDG* was placed with the 5'UTR of *ribAB*. *ribE*, *ribH* and *ribT* contain their original 5' UTRs. The terminator sequence from the RuBisCO large subunit gene *rbcL* was used to terminate the transcription of the operon.

TTTTAAGCTAGCAGGAAAACCCGATGGTGCCTTTAAATATTCTGAAATGAGCTGTTGACAATTAATCATCCGGCTCGTATAA
TGTGTGGAATTGTGAGCGGATAACAATTTCATACGCTCACAATTTGTACCGGTGATACCAGCATCGTCTTGATGCCCTTGGC
AGCACCCTGCTAAGGAGGCAACAAGATGAGCTTCCACCCCATCGAAGAGGCGCTCGATGCTCTCAAGAAAGGCGAAGTAATC
ATCGTAGTCGACGACGAAGACCGTGAAAATGAGGGTGACTTTGTTGCACTCGCGGAACACGCGACCCCCGAGGTAATCAATT
TCATGGCTACTCACGGTCGTGGCCTCATCTGTACTCCCCTCTCTGAAGAAATCGCGGACCGCCTGGACCTGCACCCTATGGT
TGAGCACAATACTGACTCTCACCACACCGCCTTCACTGTATCTATCGATCACCGTGAAACTAAGACTGGTATTAGCGCTCAA
GAGCGTTCTTTTACTGTCCAGGCACTCCTCGATTCTAAATCTGTACCCTCCGACTTTCAGCGCCCTGGTCACATTTTCCCCC
TCATTGCTAAAAAGGGTGGTGTTCTCAAACGTGCCGGTCACACTGAAGCCGCTGTCGATTTGGCGGAGGCCTGTGGTTCTCC
CGGTGCTGGTGTAATCTGTGAAATTATGAATGAGGATGGTACTATGGCGCGCGTTCCCGAATTGATCGAGATCGCTAAGAAG
CACCAGCTGAAGATGATCACTATCAAGGACCTCATCCAATACCGTTATAACCTCACCACTTTGGTTGAACGTGAAGTTGATA
TTACTCTCCCCACCGATTTTGGTACTTTCAAGGTCTACGGTTACACTAACGAAGTTGATGGCAAAGAACACGTTGCTTTCGT
CATGGGCGACGTCCCCTTTGGTGAAGAACCCGTATTGGTTCGTGTTCACTCTGAATGTCTCACTGGTGACGTTTTCGGTTCC
CACCGTTGTGATTGTGGTCCCCAGCTCCACGCTGCCCTCAATCAAATCGCTGCGGAGGGCCGCGGTGTTCTCCTGTATCTCC
GTCAGGAAGGCCGTGGTATTGGCTTGATTAACAAACTCAAGGCGTACAAACTGCAAGAACAGGGCTACGATACTGTTGAAGC
CAACGAAGCGCTGGGTTTCCTCCCCGATCTCCGTAACTACGGCATTGGTGCTCAAATCCTCCGTGACTTGGGTGTTCGTAAC

ATGAAACTCCTGACCAACAATCCCCGTAAAATTGCTGGTTTGGAAGGTTACGGTTTGAGCATCTCTGAACGTGTCCCCCTGC
AAATGGAAGCCAAGGAACACAATAAGAAATACCTCCAGACTAAGATGAATAAGTTGGGTCACTTGTTGCACTTCTAAAGAGG
AAGATTTGCATGGAAGAATACTACATGAAGCTCGCACTCGATCTCGCAAAACAAGGCGAAGGCCAAACTGAGAGCAACCCTT
TGGTCGGTGCTGTTGTCGTTAAGGACGGTCAAATCGTAGGTATGGGTGCTCACCTCAAGTACGGCGAAGCCCACGCCGAAGT
ACACGCTATTCACATGGCAGGCGCTCACGCAGAAGGTGCTGACATCTACGTTACTTTGGAACCCTGTTCTCACTACGGTAAG
ACTCCTCCCTGTGCTGAACTCATCATCAACTCTGGTATCAAACGTGTCTTCGTCGCTATGCGTGATCCTAATCCCCTCGTCG
CTGGCCGTGGCATTTCTATGATGAAGGAAGCCGGTATCGAGGTACGTGAAGGCATCCTGGCCGACCAAGCCGAACGTCTCAA
CGAAAAATTCCTGCACTTTATGCGTACCGGTCTGCCTTACGTCACTCTCAAGGCCGCAGCAAGCCTGGATGGCAAGATTGCC
ACCTCTACTGGTGATTCTAAGTGGATTACCTCTGAGGCTGCTCGTCAGGACGCTCAGCAATACCGTAAAACTCACCAATCTA
TCTTGGTTGGCGTTGGTACTGTTAAGGCTGATAACCCTAGCCTGACTTGTCGTTTGCCCAATGTTACCAAACAGCCTGTTCG
TGTTATCTTGGATACCGTATTGAGCATTCCTGAAGATGCAAAAGTAATTTGCGATCAGATCGCCCCCACTTGGATCTTTACT
ACTGCTCGTGCTGACGAAGAAAAGAAGAAACGTCTCTCTGCCTTTGGTGTTAATATCTTCACTCTCGAAACTGAACGTATCC
AAATCCCCGATGTACTCAAAATTTTGGCTGAAGAAGGCATTATGTCTGTTTACGTTGAAGGTGGTTCTGCTGTTCACGGTTC
TTTCGTCAAAGAAGGTTGCTTCCAAGAGATCATCTTCTACTTCGCACCCAAGCTCATCGGTGGCACCCACGCACCCTCCCTC
ATTTCTGGTGAAGGTTTCCAATCTATGAAAGATGTCCCTCTGTTGCAGTTTACCGATATCACTCAAATCGGTCGTGACATTA
AGTTGACTGCCAAGCCCACTAAGGAATAAGATGGTGACCATGTTTACCGGTATTATTGAAGAAACCGGCACTATTGAATCTA
TGAAGAAAGCGGGTCACGCTATGGCATTGACCATCAAATGTAGCAAAATTCTTGAAGACGTTCACCTCGGTGACTCTATTGC
AGTTAACGGTATCTGTTTGACCGTTACTGATTTCACTAAGAACCAATTCACCGTAGATGTAATGCCCGAAACCGTTAAGGCT
ACTTCCCTTAATGATTTGACTAAGGGCTCTAAAGTAAACCTTGAACGTGCCATGGCTGCGAACGGTCGTTTCGGTGGCCACT
TCGTTAGCGGTCACGTAGACGGCACCGCGGAGATTACTCGTATTGAAGAAAAGTCTAACGCTGTATACTACGATCTCAAGAT
GGACCCCTCCCTCACCAAAACTCTCGTACTCAAAGGCTCTATCACTGTTGATGGCGTTTCTCTCACTATTTTTGGCCTCACT
GAAGACACCGTAACCATCAGCCTCATCCCCCACACTATCTCTGAAACTATTTTCTCTGAAAAGACTATTGGCTCCAAAGTTA
ACATTGAGTGTGATATGATTGGTAAGTACATGTACCGTTTCCTCCACAAAGCAAATGAAAACAAGACCCAACAAACTATTAC
CAAAGCTTTCCTCTCTGAAAACGGTTTTTAATCACAAATATCACAAAAAAGGATGGGAATCATATGAACATCATTCAAGGTA
ACCTTGTTGGCACCGGCCTCAAGATCGGTATCGTTGTTGGTCGTTTTAACGATTTTATTACCTCTAAATTGCTCAGCGGCGC
GGAAGACGCACTCCTTCGTCACGGTGTAGACACTAACGACATCGATGTTGCCTGGGTCCCCGGTGCTTTCGAAATTCCCTTC
GCGGCCAAAAAGATGGCAGAAACCAAGAAGTACGACGCTATTATCACTTTGGGTACTGTAATTCGTGGTGCGACTACTCACT
ACGATTACGTCTGTAATGAGGCTGCTAAGGGTATCGCTCAAGCTGCGAACACTACCGGTGTCCCCGTAATTTTCGGCATTGT
AACTACTGAGAATATCGAACAGGCAATCGAACGTGCTGGTACTAAGGCCGGTAATAAAGGCGTTGACTGTGCTGTTTCTGCA
ATCGAAATGGCTAATCTCAACCGTAGCTTCGAATAATTTGCTGAAAACAGTTTAAAAATATGGCGAAAATGATATAATGTGA
GAAAACGGATCACCTATTCGTATCCGTTAATAGCAGACTGGACATTTTGGATATAGAGGGGTTTTTATGCTGATTCGTTACA
AGAAATCTTTCGAGAAGATCGCTATGGGTTTGCTGTCTTTCATGCCCAACGAAAAAGATTTGAAACAATTGCAGCAAACCAT
TAAGGACTACGAAACTGATACTGACCGTCAACTCTTCTTGTGGAAAGAAGACGAGGACATTGTCGGTGCAATCGGTGTCGAA
AAAAAGGATAGCGAAGTTGAAATTCGTCACATTTCCGTTAATCCCTCTCACCGTCACCAAGGCATCGGTAAGCAGATGATGG
ACGCGCTCAAGCACCTCTTTAAAACTCAGGTCCTCGTTCCCAATGAGTTGACTCAATCTTTCTTCGAACGTTGTCAAGGTCA
ACAGGATCAGGACATCTCTTACAACAACTAATCTACATTGAATCATGCGGAATGCGATCGCCTTAGGACGGTCGCATTTTTT
GTTTACGTCTAAAATTAGTCGAAATGAGCAGAAGAGCATACATCTGGAAGGCGGCCGCTTTTTTTTTT

## 6.3. Diurnal regulation of *rib* genes in *Synechocystis* sp. PCC 6803

In the work of Saha et al. [181], *Synechocystis* sp. PCC 6803 was cultivated under diurnal conditions (12 hours white light and 12 hours dark). RNA was extracted every two hours over the course of 48 hours and the transcriptome was quantified. I re-analysed the data for expression of *rib* genes, revealing that transcripts of *ribA* and *ribF* are more abundant during the dark period, whereas *ribH* transcript levels were higher during the light period.

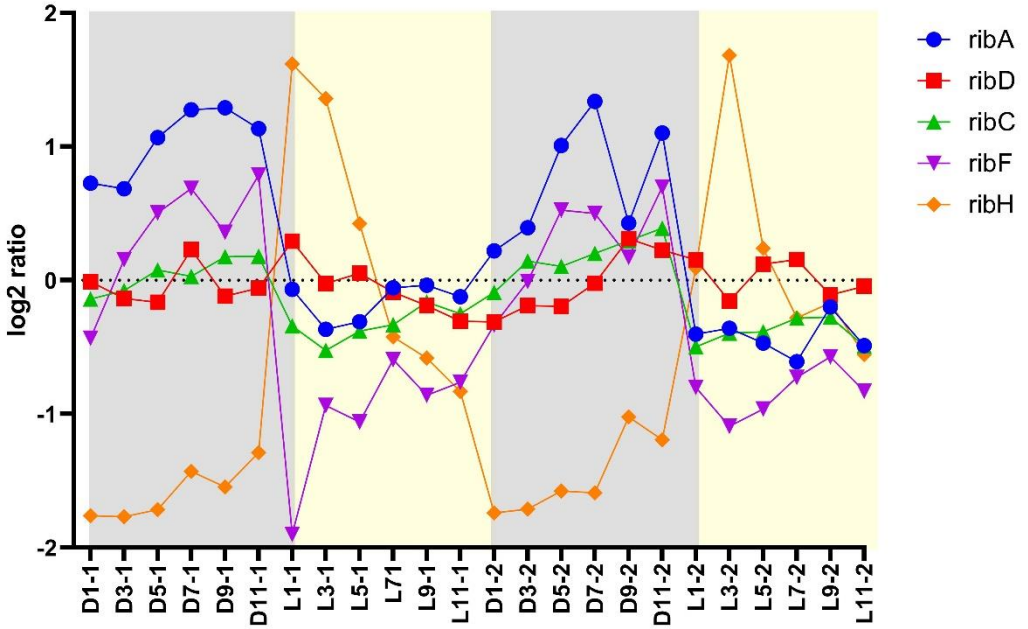


**Figure 51: Transcriptomics data from Saha et al. [181] reveal diurnal regulation of some *rib* genes in *Synechocystis* sp. PCC 6803.** Dark periods are shaded in grey, light periods are not shaded. Total RNA was taken every two hours over the course of two days. ***ribA***: Bifunctional GTP cyclohydrolase II (EC 3.5.4.25) and (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12). ***ribD***: bifunctional 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate deaminase (EC 3.5.4.26) and 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate reductase (EC 1.1.1.193). ***ribF***: bifunctional flavokinase (EC 2.7.1.26) and FAD synthetase (EC 2.7.7.2). ***ribC***: riboflavin synthase (EC 2.5.1.9). ***ribH***: lumazine synthase (EC 2.5.1.78).

## 6.4. List of parent species and strains used in this project

All microbial species and strains used in this project are listed in Table 15. Note that a comprehensive overview of all strains generated in this study is given in Table 16.

**Table 15: List of all microbial species and strains used in, and relevant for, this project. (A)**: Erb Group, MPI for Terrestrial Microbiology, Marburg, Germany. **(B)** WIlde Group, Freiburg University, Freiburg, Germany. **(C)** Wattana Jeamton, King Mongkut's University of Technology Thonburi, Thailand.

| Strain | | Relevant feature(s) | SI # | Used for |
|---|---|---|---|---|
| ***Escherichia coli*** | | | | |
| | DH5alpha | strong replication of plasmids, recombination-deficient, thiamine and arginine auxotrophic | 92 | cloning |
| | Top10 | strong replication of plasmids, recombination-deficient, blue/white-screening, leucine auxotrophic | 234 | cloning |
| | BL21(DE3) | T7 RNA polymerase, strong lac-Repressor *lacI*$^{q}$ | 145 | expression of proteins for purification |
| | BSV11 | permeable to riboflavin, *ribB*-, *Km*$^{R}$ | 314 | Source: [110] complementation of riboflavin biosynthesis genes |
| | BSV13 | permeable to riboflavin, *ribE*-, *Km*$^{R}$ | 316 | |
| | BSV18 | permeable to riboflavin, *ribA*-, *Km*$^{R}$ | 315 | |
| | SI#78 | permeable to riboflavin, *ribDG*- | 78 | |
| ***Synechococcus* sp. PCC 7002 (A)** | | | 1322 | Source of *A2813* promoter |
| ***Synechocystis elongatus* sp. PCC 7942 (A)** | | | 1321 | |
| ***Synechocystis* sp. PCC 6803 (B)** | | | 1320 | Source of promoters *cpc560* and *psbA2* |
| ***Arthrospira platensis* C1 (C)** | | | 2025 | |
| ***Bacillus subtilis* subsp. 168** | | | 19 | Source of riboflavin biosynthesis transcription unit *ribDGEABHT* |
| ***Corynebacterium glutamicum* MB001 (DE3)** | | | 1631 | Source of *pnuX* gene |

## 6.5. List of relevant plasmids used in this project

**Table 16: List of plasmids used in this study.** Plasmids are based on different vector backbones: [a]pIDTsmart (high-copy), [b]pKT25 (medium-copy), [c]pUT18 (medium/high-copy), [d]pUR (medium copy, conjugation), [e]pGP380 (high copy, N-terminal Strep-Tag), [f]pGP382 (high copy, C-terminal Strep-Tag), [g]pPSG-IBA3 (medium-copy, N-terminal Strep-Tag), [h]pAH06 (medium-copy). Plasmids marked in blue were not yet analysed/transformed but might be relevant for further studies. MCS: multiple cloning site. Strep: Strep-Tag.

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pURdualTag[d] | *<KmR; SpecR; sfGFP>* | Wilde Group, Freiburg, Germany | DH5a (1935) | |
| pBK42[a] | *NS2<Km$^R$>* | this study, adapted from [111] | DH5a (1942)<br>*Syn.* 7002 (1668-1670) | |
| pBK82[b] | *pAQ1<empty>* | this study, adapted from [113] | DH5a (1967, 1968) | |
| pBK84[b] | *pAQ3<empty>* | this study, adapted from [113] | DH5a (1977) | |
| pGP380 | *$P_{deqQ}$_Strep_MCS* | [142] | Top10 (356)<br>BSV11 (1749-1751)<br>BSV13 (1994-1996)<br>BSV18 (1779-1781)<br>#SI78 (1761-1763) | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pGP382 | *$P_{deqQ}$_MCS_Strep* | [142] | Top10 (357) | |
| pPSG_IBA3 | | IBA Lifesciences GmbH | DH5a (1646)<br>$BL21_{(DE3)}$ (1658) | |
| **Plasmids for integration of *gfp* into *Synechococcus*** sp. PCC 7002 | | | | |
| pBK47[a] | *NS2<$Km^R$; cpc560_gfp>* | this study | DH5a (1943)<br>*Syn.* 7002 (1671-1673) | |
| pBK48[a] | *NS2<$Km^R$; psbA2L_gfp>* | this study | DH5a (1944)<br>*Syn.* 7002 (1674-1676) | |
| pBK49[a] | *NS2<$Km^R$; gfp>* | this study | DH5a (1948)<br>*Syn.* 7002 (1713) | |
| pBK54[a] | *NS2<$Km^R$; A2813_gfp>* | this study | DH5a (1946)<br>*Syn.* 7002 (1677-1679) | |
| pBK55[a] | *NS2<$Km^R$; psbA2s_gfp>* | this study | DH5a (1947)<br>*Syn.* 7002 (1687, 1688) | |
| pBK83[a] | *pAQ1<$Km^R$; psbA2s_gfp>* | this study | DH5a (1969, 1970)<br>*Syn.* 7002 (1869-1872) | *not* completely segregated |
| pBK85[a] | *pAQ3<$Km^R$; psbA2s_gfp>* | this study | DH5a (1978)<br>*Syn.* 7002 (1873-1876) | completely segregated! |
| pBK123[b] | *pAQ3<$Cm^R$; psbA2s_gfp>* | this study | DH5a (1988)<br>*Syn.* 7002 (1835-1837) | |
| pBK124[b] | *pAQ3<$Spec^R$; psbA2s_gfp>* | this study | DH5a (1989) | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pBK130[a] | *NS2<Cm$^R$; psbA2s_gfp>* | this study | DH5a (2010)<br>*Syn.* 7002 (2015-2018) | |
| pBK131[b] | *pAQ1<Cm$^R$; psbA2s_gfp>* | this study | DH5a (2011) | |
| **Plasmids used for *pnuX* integration and RoF selection in *Synechococcus* sp. PCC 7002** | | | | |
| pBK52[a] | *NS2<Km$^R$; psbA2L_pnuX>* | this study | DH5a (1945)<br>*Syn.* 7002 (1684, 1685) | |
| pBK132[c] | *A0225<A0224_A0225_A0226>*<br>*does not contain antibiotic for selection* | this study | DH5a (2016) | |
| pBK133[c] | *A0225<A0224_A0225mut_A0226>*<br>*does not contain antibiotic for selection* | this study | DH5a (2014) | |
| **Plasmids for integration of riboflavin biosynthesis genes into *Synechococcus* sp. PCC 7002** | | | | |
| pBK63[b] | *NS2<Km$^R$; **cpc560**_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1949, 1950)<br>*Syn.* 7002 (1686, 1687) | |
| pBK64[b] | *NS2<Km$^R$; **psbA2s**_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1951, 1952)<br>*Syn.* 7002 (1688-1690) | |
| pBK65[b] | *NS2<Km$^R$; **A2813**_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1953, 1954)<br>*Syn.* 7002 (1703-1705) | |
| pBK66[b] | *NS2<Km$^R$; A2813_ribAB$_{codon\text{-}adapted}$>* | this study | DH5a (1955)<br>*Syn.* 7002 (1706, 1707) | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pBK67[b] | *NS2<Km$^R$; psbA2s_ribAB$_{7942}$>* | this study | DH5a (1956) | |
| pBK69[b] | *NS2<Km$^R$; P$_{TrcTheo}$_ribABDGEHT$_{codon-adapted}$>* | this study | DH5a (1957) | |
| | | | *Syn.* 7002 (2026) | |
| pBK70[b] | *NS2<Km$^R$; P$_{TrcTheo}$_gfp>* | this study | DH5a (1958) | |
| | | | *Syn.* 7002 (1680, 1681) | |
| pBK71[b] | *NS2<Km$^R$; psbA2L_ribABDEHT$_{codon-adapted.}$>* | this study | DH5a (1959) | |
| | | | *Syn.* 7002 (1708, 1709) | |
| pBK79[b] | ***pAQ1**<KmR; cpc560_ ribDEABHT$_{Bsu}$>* | this study | DH5a (1966) | |
| pBK87[b] | ***pAQ3**<Km$^R$; cpc560_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1971, 1972) | |
| pBK88[b] | ***pAQ3**<Km$^R$; psbA2**L**_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1973, 1974) | |
| pBK89[b] | ***pAQ3**<Km$^R$; A2813_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1975, 1976) | |
| pBK91[b] | ***pAQ1**<Km$^R$; psbA2L_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1979) | |
| pBK92[b] | ***pAQ1**<Km$^R$; A2813_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1980, 1981) | |
| pBK98[b] | ***pAQ3**<Km$^R$; psbA2**s**_ribDGEABHT$_{Bsu}$>* | this study | take from plasmid library | |
| pBK101[b] | *NS2<Km$^R$; **psbA2s**_ribDE$_{Bsu}$_**cpc560**_ribABHT$_{Bsu}$>* | this study | DH5a (1984) | |
| | | | *Syn.* 7002 (1710-1712) | |
| pBK102[b] | ***pAQ1**<Km$^R$; psbA2s_ribDGEABHT$_{Bsu}$>* | this study | DH5a (1985) | |
| pBK115[b] | *pAQ3<Cm$^R$; psbA2s_ribAB$_{7002}$>* | this study | DH5a (1987) | contains part of *ribD* after *ribAB*! |
| pBK125[b] | *NS2<**Cm$^R$**; psbA2s_ribDGE_cpc560_ribABHT$_{Bsu}$>* | this study | DH5a (2006) | |
| | | | *Syn.* 7002 (1818-1820) | |
| | | | *Syn.* 7002 RoF#48 (2012) | |
| pBK127[b] | *NS2<**Cm$^R$**; cpc560_ribDGEABHT$_{Bsu}$>* | this study | DH5a (2007) | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| **Plasmids for complementation of riboflavin biosynthesis genes in *E. coli* riboflavin auxotrophic strains** | | | | |
| pBK75[e] | <$P_{deqQ}$_Strep_***ribAB*$_{7002}$**> | this study | DH5a (1960) | |
| pBK76[e] | <$P_{deqQ}$_Strep_***ribDG*$_{7002}$**> | this study | DH5a (1961) | |
| | | | SI#78 (1990-1992) | |
| pBK77[e] | <$P_{deqQ}$_Strep_***ribE*$_{7002}$**> | this study | DH5a (1962) | |
| | | | BSV13 (1997-1999) | |
| pBK78[e] | <$P_{deqQ}$_Strep_***ribH*$_{7002}$**> | this study | DH5a (1964) | |
| pBK99[e] | <$P_{deqQ}$_Strep_***ribD*$_{7002\text{-}C\text{-}term.domain}$**> | this study | DH5a (1982) | |
| | | | SI#78 (1767-1769) | |
| pBK103[f] | <$P_{deqQ}$_***ribE*$_{7002}$**_Strep> | this study | DH5a (1963) | |
| | | | BSV13 (n.a.) | |
| pBK104[e] | <$P_{deqQ}$_Strep_***ribE*$_{Bsu}$**> | our group | Top10 (369) | |
| | | | BSV13 (2000-2002) | |
| pBK105[e] | <$P_{deqQ}$_Strep_***ribH*$_{Bsu}$**> | our group | Top10 (370) | |
| pBK106[f] | <$P_{deqQ}$_***ribE*$_{Bsu}$**_Strep> | our group | Top10 (373) | |
| | | | BSV13 (2003-2005) | |
| pBK107[f] | <$P_{deqQ}$_***ribH*$_{Bsu}$**_Strep> | our group | Top10 (374) | |
| pBK108[f] | <$P_{deqQ}$_***ribH*$_{7002}$**_Strep> | this study | DH5a (1965) | |
| pBK112[f] | <$P_{deqQ}$_***ribAB*$_{7002}$** > | our group | DH5a (1986) | No strep tag. Used |
| | | | BSV11 (1758-1760) | for complementat. |
| | | | BSV18 (1776-1778) | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pBK113[f] | <$P_{deqQ}$_***ribAB$_{Bsu}$***_*Strep*> | our group | DH5a (406) | |
| pBK114[f] | <$P_{deqQ}$_***ribDG$_{Bsu}$***_*Strep*> | our group | DH5a (372)<br>SI#78 (1991) | |
| **Plasmids for *ribH*-expression in *E. coli* BL21(DE3)** | | | | |
| pBK128[g] | <$P_{T7}$; ***Strep***_*ribH$_{7002}$* | this study | DH5a (2008) | |
| pBK129[g] | <$P_{T7}$; *ribH$_{7002}$*_***Strep***> | this study | DH5a (2009) | |
| **Integration plasmids for alteration of the *SYNPCC7002_A0225* genomic locus** | | | | |
| pBK136[a] | *A0225<A0224_**(pKm$^R$_Km$^R$)**_pA0225_A0226>* | this study | DH5a (2019-2021) | |
| pBK137[a] | *A0225<A0224_**Km$^R$**_pA0225_A0226>* | this study | DH5a (2022-2024) | |
| pBK138[a] | *A0225<A0224_**Cm$^R$**_pA0225_A0226>* | this study | take from plasmid library | |
| **Plasmids to test different versions of the FMN-riboswitch in *E. coli* and *Synechococcus* sp. PCC 7002 (not used in this study)** | | | | |
| pBK13[h] | *$P_{A1}$_ribDGEABHT$_{Bsu}$* | this study | DH5a (1934) | |
| pBK18[h] | *$P_{A1}$_RFN#**1**_ribDGEABHT$_{Bsu}$* | this study | DH5a (1938) | |
| pBK19[h] | *$P_{A1}$_RFN#**2**_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1939) | |
| pBK20[h] | *$P_{A1}$_RFN#**5**_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1940) | |
| pBK21[h] | *$P_{A1}$_RFN#**6**_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1941) | |
| pBK90[h] | *$P_{A1}$_RFN#**2 (no term.)**_ ribDGEABHT$_{Bsu}$* | A. Boumezbeur | take from plasmid library | |

| Plasmid ID (Backbone) | Integration Site <relevant features> | Source or reference | Host Strain (incl. Stammsammlung #) | Annotation |
|---|---|---|---|---|
| pBK117[b] | *pKT25_NS2_KmR_RFN#**2**_psbA2s_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1693) | |
| pBK118[b] | *pKT25_NS2_KmR_RFN#**5**_psbA2s_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1694) | |
| pBK119[b] | *pKT25_NS2_KmR_RFN#**2 (no term.)**_psbA2s_ ribDGEABHT$_{Bsu}$* | this study | DH5a (1695) | |
| **Miscelaneous plasmids that do not fit the above categories** | | | | |
| pBK15 | *pSL2680_cpf1* | [42] | DH5a (1936, 1937) | |
| pBK100[a] | *NS2<Km$^R$; A2813_cpf1>* | | DH5a (1983) *Syn.* 7002 (1696-1698) | *Integration of cpf1 into Syn7002 was successful* |
| pBK94[h] | *P$_{A1}$_ribDGE$_{RBS\#2}$ABHT$_{Bsu}$* | | take from plasmid library | *RBS of ribAB was changed* |

## 6.6. List of relevant primer sequences

**Table 17: List of all nucleotide sequence relevant for this project**

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK20_pAH seq | 2144 | | tcgtct tcacct cgagaaaatttat c |
| BK21_BsaI_AscIx_RFN_for | 2145 | TT GGTCTC T CGCG | gacaaatgaataaagattgtatccttcggg |
| BK22_BsaI_AscIx _RFN(AAA)_for | 2146 | TT GGTCTC T CGCG | gacaaatgaataaagattgtatccttcggggcaaaatggaaatccc |
| BK23_BsaI_AscIx_RFN_rev | 2147 | TT GGTCTC T CGCG | aaccctcttttgttatttaccgtc |
| BK24_BsaI_AscIx _RFN_rev | 2148 | TT GGTCTC T CGCG | tcgcaataggaaataacactatgc |
| BK31x_BsaI_EcoRI_KpnI_NS2L_for | 2149 | TT GGTCTC GAATTC GGTACC | ggttgcgcttttgatatttcttggc |
| BK32x_NheI_NS2L_rev | 2150 | TTA GCTAGC | catttttgagagatgtcttccccgc |
| BK33x_NheI_NotI_NS2R_for | 2151 | TAT GCTAGC TAT GCGGCCGC | cagaaatgaagaacgtagaag |
| BK34x_XhoI_NS2R_rev | 2152 | TTAA CTCGAG | cctgccatcacgttggtatc |
| BK35x_NheI_KmR_for | 2153 | TTTGCTAGC | tttctacgggttcgctgc |
| BK36x_NotI_pUR_rev | 2154 | TTATGCGGCCGC | ttccagatgtatgctcttctgctc |
| BK44_NotI_NheI_pA1_for | 2155 | TTA GCGGCCGC a GCTAGC | cagaatgcttaatgaattacaacag |
| BK45_SpeI_NcoI_pAH06_rev | 2156 | TTA ACTAGT CCATG | gataccgtcgacctcgagg |
| BK46_SpeI_Stop_pnuX_rev | 2157 | TTA ACTAGT | tcagactgtcacagactcctgagc |
| BK54_ribD seq | 2158 | | cgcaagatctaaggccagcttc |
| BK55_pUR seq_KmR | 2159 | | gattacagatcctctagaagaacag |
| BK56_pUR seq-SpecR | 2160 | | gtgaaagttggaacctcttac |
| BK59 seq | 2161 | | cttccagatgtatgctcttctgctc |
| BK60_oligo1 | 2162 | | gcgt gctagc tcaact catatg |

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK61_oligo2 | 2163 | | ta catatgagttgagctagc |
| BK62new_pnuX for | 2164 | TTT GGTCTC T | atgaatcctataaccgaattattagacgcaac |
| BK63_BamHI_Stop_EcoRI_pnuX_rev | 2165 | TAT GGATCC TCA GAATTC | actgactgtcacagactcctgagcttc |
| BK64new_NheI_A2813_for | 2166 | TTAA GCTAGC | ccgatttaagttcaaaaactttattg |
| BK65_A2813 rev | 2167 | CGTCACCATATG | ttgtttttcctcacacattaaggc |
| BK66new_sfGFP for | 2168 | AAA GGTCTC T | atgagcaaaggagaagaacttttcac |
| BK67_pIDTsmart seq for | 2169 | | gtaaaacgacggccagt |
| BK68_pIDTsmart seq rev | 2170 | | caggaaacagctatgac |
| BK69new_SpeI_KmR | 2171 | TTAAT ACTAGT | catcgccacgttgtgtctc |
| BK75_seq NS2 region for | 2172 | | ccgttctaaaccaaggctgc |
| BK75_seq NS2 region rev | 2173 | | catatttaccagactcgtcggctt |
| BK79new_Cpc560 for | 2174 | TTAAT GCTAGC | acctgtagagaagagtccctg |
| BK80new_Cpc560 rev | 2175 | TTT GGTCTC T | tcattgaattaatctcctacttgactttatgagttgg |
| BK81new_psbA2L for | 2176 | TTAAT GCTAGC | gttccagtggatatttgc |
| BK82new_psbA2 short for | 2177 | TTAAT GCTAGC | caggtaaactcttctcaac |
| BK83new_psbA2 rev | 2178 | TTT GGTCTCT | tcatttggttataattccttatgtatttgtcg |
| BK84_ribD(bsu) | 2179 | ATA GGTCTC A | atgagtgaagagtattatatgaagctggccttag |
| BK85_A2813 rev | 2180 | TTT GGTCTCT TCAT | ttgtttttcctcacacattaaggc |
| BK86_ribA(cod.opt) | 2181 | TTT GGTCTC A | atgagcttccaccccatcg |
| BK90_ribOperon rev | 2182 | TTTT GGATCC GCGGCCGC | cttccagatgtatgctcttc |
| BK94_pTrc for | 2183 | TTT GGTCTC A GCTAGC | aactgttgggaagggc |
| BK95_pTrc rev | 2184 | TTT GGTCTC T TCAT | cttgttgcctccttagcaggg |
| BK96_BsaI_ribAB7942_for | 2185 | TTT GGTCTC A ATGA | ctttgccagaagactttcag |

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK97_NotI_ribAB7942_rev | 2186 | TTATGCGGCCG | ctggggtttgtttcgtgtg |
| BK98_ribAB(cod.opt) rev | 2187 | TTATGCGGCCG | cctctttagaagtgcaacaagtg |
| BK103 seq NS2R upstream | 2188 | | gtgaaatccctggcttgc |
| BK118_pAQ1 seq for | 2189 | | gttacagcgtgaccaagc |
| BK119_pAQ1 seq rev | 2190 | | gtttccagtccccatctgtgc |
| BK120_ribAB(7002) for | 2191 | TT GGTCTC AGATCC | gatacagcttggcagtttgattc |
| BK121_ribAB(7002) rev | 2192 | TT GGTCTC AAGC | ttatttgggctgctggaagtgatag |
| BK122_ribD(7002) for | 2193 | TT GGTCTC AGATCC | acctcaaacgctgcccccg |
| BK123_ribD(7002) rev | 2194 | TT GGTCTC TAGCTT | gaattatttgcctggttacgcc |
| BK124_ribE(7002) for | 2195 | TT GGTCTC AGATCC | tttactggattgattcaagccc |
| BK125_ribE(7002) rev | 2196 | TT GGTCTC TAGCTT | cctataaatagccatgttcctggagg |
| BK126_ribH(7002) for | 2197 | TT GGTCTC AGATCC | gctacctttgaaggaacctataacg |
| BK127_ribH(7002) rev | 2198 | TT GGTCTC TAGCTTA | aatctttcccatcagactcgcc |
| BK128_pAQ1_flankB for | 2199 | AT GGTCTC TCTAGAA | ctctcaccaaagattcacctg |
| BK129new_pAQ1_flankB rev | 2200 | AT GGTCTC T TCCT ACTAGT | ctaagcctcctgaataaatctatttatac |
| BK130_pAQ1_flankA for | 2201 | TT GGTCTC T AGGA GCGGCCGC | ctttctcttatgcacagatggg |
| BK131_pAQ1_flankA rev | 2202 | TT GGTCTC GAATTC | ggggttttctcgtgtttaggc |
| BK138_ribD_seq_for | 2203 | | ctgtttcgccgtggacactaag |
| BK139_ribE_seq_rev | 2204 | | gaggtatgcggtattagtc |
| BK140_ribA_seq_for | 2205 | | ccagccagaaatccatacg |
| BK141_ribA_seq_rev | 2206 | | ggaagagaagattgatattggc |
| BK142_pAQ3_flankB for | 2207 | AT GGTCTC TCTAGAA | caaggatgaagccgaactacg |
| BK143_pAQ3_flankB rev | 2208 | AT GGTCTC T TCCT ACTAGT | ctaagagtcagaattttaatttgtc |

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK144_pAQ3_flankA for | 2209 | TT GGTCTC A AGGA GCGGCCGC | ctgactcttagattatttccag |
| BK145_pAQ3_flankA rev | 2210 | TT GGTCTC GAATTC | gcagtcagaactcaaatgatttatc |
| BK152_RBS#2 | 2211 | AAAGGAGGTATATT | atgtttcatccgatagaagaagcac |
| BK153_RBS#10 | 2212 | AAAAAGGAGGTATTATTTT | atgtttcatccgatagaagaagcac |
| BK154_rev_primer | 2213 | | ctaaaagccgttttcgcttaagaaggc |
| BK155_ribE(Bsu) rev | 2214 | TTT GGTCTC ACTAGT | ctaaaagccgttttcgcttaagaagg |
| BK156_ribAB(Bsu) for | 2215 | TTT GGTCTC T ATGA GT | tttcatccgatagaagaagcactgg |
| BK157_Cpf1 for | 2216 | TTT GGTCTC A ATGAGT | atttatcaagaatttgttaataaatatagtttaag |
| BK158_Cpf1 rev | 2217 | TTTT GCGGCCGC | ttagttattcctattctgcacgaactc |
| BK159_ribD_CtermDomain(7002) for | 2218 | TT GGTCTC AGATCC | atgcataggcagcgacccc |
| BK160_ribD_CtermDomain(7002) rev | 2219 | TT GGTCTC AAGC | ctgttattgtttcatgcggtaatggag |
| BK170_NS2 | 2220 | | catcacaagggatactgccac |
| BK171_pAQ1 | 2221 | | caagggcataaaacgtcc |
| BK172 | 2222 | ATAGGTCTCAAT | gataacaatttcacaggcgcg |
| BK173 | 2223 | TTT GGTCTCTTCAT | ccttatgtatttgtcgatgttcag |
| BK176_ribE(7002) with RBS | 2224 | TAT TCTAGA AAAGGAGGAAACAATA | gtgtttactggattgattcaagc |
| BK177new_ribH(7002) with RBS | 2225 | TAT TCTAGA AAAGGAGGAAACAATA ATG | gctacctttgaaggaacctataacg |
| Bk178_Linker_for | 2226 | CCGG | ggatcgaaaggaggaaacaatcatg |
| BK179_Linker_rev | 2227 | GATC | gcatgattgtttcctcctttcgatcc |
| BK180_KmR_for | 2228 | TTTT GGATCC | catcgccacgttgtgtctc |
| BK181_KmR_rev | 2229 | TTTT GGTACC | ggccttgctgttcttctag |
| BK182_pKmR_for | 2230 | AA CCCGGG | catcgccacgttgtgtctc |
| BK183_aphT_rev | 2231 | TTGGATCCCATGATTGTTTCCTCCTTTC GATC | gattagaaaaactcatcgagcatc |

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK184_CmR for | 2232 | TT GGTCTCA CTAGC A | gtcgactacgtaagaggttcc |
| BK185_CmR rev | 2233 | TT GGTCTCT CTAGT | ggcgtttaagggcaccaataac |
| BK186_SpecR for | 2234 | TT GGTCTCA CTAG | attacagatcctctagaagaacag |
| Bk187_SpecR rev | 2235 | TT GGTCTCT CTAGT | ttatttgccgactaccttggtg |
| BK190_KmR seq | 2236 | | cagtttcatttgatgctcgatg |
| BK191_ribAB(7002) seq | 2237 | | gctttgtctaccgtgag |
| BK192_pAQ3 seq | 2238 | | ccatttccgcccaagaat |
| BK193_pAQ3 seq | 2239 | | gttactatcgcctcagtgc |
| BK194_Strep for | 2240 | AATT CGTCTC TAATG | tggagccacccgcagttc |
| BK195_ribH(7002) rev with Stop | 2241 | AATT CGTCTC C TCCC | ttaaatctttcccatcagactcgcc |
| BK196_ribH(7002) for without Strep | 2242 | AATT CGTCTC TAATG | gctacctttgaaggaacctataacg |
| BK197_ribH(7002) without Stop | 2243 | AATT CGTCTC C TCCC | aatctttcccatcagactcgcc |
| BK198_A2397(7002) seq | 2244 | | accaagtaacccaaacctg |
| BK199_ribC(7002) rev | 2245 | | ccacaaggacaagacgaatg |
| BK200new_ribC(7002) up | 2246 | | gatgatttcctcgatggcg |
| BK201_ribAB(7002) up | 2247 | | gtgatcgaaactgccgttaag |
| BK202_ribAB(7002) up | 2248 | | ctacagtatgggcgaagg |
| BK203_ribAB(7002) up | 2249 | | gtcctggagccagataatttc |
| BK204_ribC(7002) down | 2250 | | cgtccctattccctccaagg |
| BK205_ribAB(7002) downstream | 2251 | | attgtgaagggtgatccgaag |
| BK208_A0225 for | 2252 | | cctcgtcctggattacagc |
| BK209_A0225 rev | 2253 | | cttcacgccaataatcacc |
| BK210_A0224 for | 2254 | TTT GAATTC | taccaatttcgccatccgc |

| Project ID | Lab ID # | Overhang Sequence | Binding Sequence |
|---|---|---|---|
| BK211_A0224 rev | 2255 | TT AGATCT TT ACTAGT | taaacaacagtagaggtgattattggcg |
| BK212_pA0225 rev | 2256 | TT AGATCT TT ACTAGT | tgtgggaaatcttttggg |
| BK213_pA0225 for | 2257 | TT AGATCT | gtcatagttgtcattcgggctc |
| BK213.1_A0226 for | 2258 | TT AGATCT A | ttaaaccccaaaagatttcccac |
| BK215_KmR rev | 2259 | TT AGATCT GCTAGC | tgattagaaaaactcatcgagcatc |
| BK216_KmR for with RBS | 2260 | TTT AGATCT | gcttacataaacagtaatacaaggg |
| BK218_A0227 rev | 2261 | TTTT CTCGAG | cttgatcaacaggtcaaattggg |

## 6.7. List of abbreviations

**Table 18: List of abbreviations**

| | |
|---|---|
| 5'UTR | 5' untranslated region |
| Amp | ampicillin |
| *B. subtilis* | *Bacillus subtilis* |
| *C. glutamicum* | *Corynebacterium glutamicum* |
| Cm | chloramphenicol |
| cPCR | colony polymerase chain reaction |
| DAD | diode array detector |
| DMSO | Dimethyl sulfoxide |
| DTT | 1,4-dithio-D-threitol |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| FAD | Flavin adenine dinucleotide |
| FLD | fluorescence light detector |
| FMN | flavin mononucleotide or riboflavin-5'-phosphat |
| GFP | green fluorescent protein |
| HPLC | high-pressure liquid chromatography |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| Km | kanamycin |
| LB | lysogeny broth (medium) |
| LED | light emitting diode |
| MS | mass spectrometry |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| NS2 | neutral site 2 |
| PCR | polymerase chain reaction |
| RBS | Ribosome binding site |
| RF | riboflavin |
| RoF | roseoflavin |
| rpm | rounds per minute |
| RT | room temperature |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| *S. davaonensis* | *Streptomyces davaonensis* |
| SDS-PAGE | Sodium dodecyl sulfat polyacrylamid gel electrophoresis |
| TCA | trichloroacetic acid |
| WT | wild type |

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