Received: 30 September 2022 | Accepted: 10 February 2023

DOI: 10.1111/1751-7915.14239

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

RESEARCH ARTICLE

Metabolic engineering of thermophilic *Bacillus methanolicus* for riboflavin overproduction from methanol

Vivien Jessica Klein[1] | **Luciana Fernandes Brito**[1] | **Fernando Perez-Garcia**[1] | **Trygve Brautaset**[1] | **Marta Irla**[2]

[1]Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway

[2]Department of Biological and Chemical Engineering, Aarhus University, Aarhus, Denmark

Correspondence
Marta Irla, Department of Biological and Chemical Engineering, Aarhus University, Aarhus C 8000, Denmark.
Email: marta.irla@bce.au.dk

Funding information
Department of Biotechnology and Food Science at the Norwegian University of Science and Technology; The Research Council of Norway within ERA CoBioTech, Grant/Award Number: 327216

Abstract

The growing need of next generation feedstocks for biotechnology spurs an intensification of research on the utilization of methanol as carbon and energy source for biotechnological processes. In this paper, we introduced the methanol-based overproduction of riboflavin into metabolically engineered *Bacillus methanolicus* MGA3. First, we showed that *B. methanolicus* naturally produces small amounts of riboflavin. Then, we created *B. methanolicus* strains overexpressing either homologous or heterologous gene clusters encoding the riboflavin biosynthesis pathway, resulting in riboflavin overproduction. Our results revealed that the supplementation of growth media with sublethal levels of chloramphenicol contributes to a higher plasmid-based riboflavin production titre, presumably due to an increase in plasmid copy number and thus biosynthetic gene dosage. Based on this, we proved that riboflavin production can be increased by exchanging a low copy number plasmid with a high copy number plasmid leading to a final riboflavin titre of about 523 mg L^{-1} in methanol fed-batch fermentation. The findings of this study showcase the potential of *B. methanolicus* as a promising host for methanol-based overproduction of extracellular riboflavin and serve as basis for metabolic engineering of next generations of riboflavin overproducing strains.

INTRODUCTION

Riboflavin, also known as vitamin B_2, is a yellow, water-soluble pigment and a common precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Averianova et al., 2020; Balasubramaniam et al., 2019; Massey, 2000; Pinto & Zempleni, 2016; Revuelta et al., 2016; Schwechheimer et al., 2016). FMN and FAD are flavocoenzymes, which are essential for all living organisms due to their involvement in both redox reactions and the metabolism of carbohydrates, fats, ketone bodies and proteins (Revuelta et al., 2016). In contrast to plants and many microorganisms, animals, including humans, do not synthesise riboflavin and must obtain it from dietary sources (Balasubramaniam et al., 2019; Fischer & Bacher, 2005; Revuelta et al., 2016, 2017; Schwechheimer et al., 2016). The commercial production of riboflavin used to be performed mainly by chemical synthesis which is nowadays fully replaced by microbial fermentation due to its economic and ecological advantages (Averianova et al., 2020; Revuelta et al., 2017). Using biotechnological processes *in lieu* of chemical synthesis leads to the reduction in waste production, energy requirements and the replacement of mineral-oil-based raw materials with more sustainable counterparts, hence represents an ecological advantage without diminishing the product quality (Stahmann et al., 2000; van Loon et al., 1996). The filamentous fungus *Ashbya gossypii* and the yeast *Candida famata* are naturally occurring overproducers of riboflavin

(Averianova et al., 2020; Perkins et al., 1999; Stahmann et al., 2000), while Gram-positive *Bacillus subtilis* was turned into an excellent production strain with riboflavin titres reaching up to 26.5 gL^{-1} in optimized glucose-based fermentation through the use of classical mutagenesis and strain engineering (Lee et al., 2006; Oraei et al., 2018; Perkins et al., 1999; Shi et al., 2009, 2014; Wang et al., 2014).

Currently, the alternatives to sugar-based feedstock conventionally used in the biotechnological industry are intensely sought-after for several reasons, for example competition for resources (land and water) with the food, feed and fuel industries of conventionally used feedstock (Fradj et al., 2016; Linton & Niekus, 1987; Muscat et al., 2020; Olah, 2005; Wendisch et al., 2016). One of such alternatives is methanol, a cheap, water-soluble, non-food, chemically pure compound that can be used as carbon and energy source by methylotrophic microorganisms. Methanol is easy to transport and store, has a reduced hazard of explosions during biotechnological processes compared with gaseous raw materials such as methane and synthesis gas, and is completely utilized during microbial fermentations, an important feature that facilitates downstream processing of bioproducts (Brautaset et al., 2007; Drejer et al., 2020; Irla, Nærdal, et al., 2016; Marx et al., 2012; Nærdal et al., 2015; Sonntag et al., 2015; Whitaker et al., 2015). Due to the fact that methanol is a flammable compound with a flash point of 12.2°C and ignition temperature of 470°C, it requires special considerations regarding safety of handling, explosion protection and use of electrical equipment (Offermanns et al., 2014). However, its boiling point of 65°C and melting point of −96°C warrant that methanol can be stored in tanks, distributed via pipelines and transported by tank cars (Offermanns et al., 2014). Additionally, thanks to its miscibility with water handling of accidents involving methanol (spills or fire) is facilitated, and for that reason, it can be used as a feedstock on large-scale fermentations (Liu et al., 2019).

With an annual production of over 110 million metric ton yr^{-1} worldwide in about ninety methanol plants, methanol is one of the most prevalent chemicals (Al-Douri et al., 2017). Nowadays, (grey) methanol is primarily produced in a two-step process in which methane is converted to syngas (Adnan & Kibria, 2020) but green methanol (bio-methanol) can also be generated from carbon-based feedstocks such as biomass via anaerobic digestion, coal, natural gas and CO_2 in power-to-methanol process (Table S1; Dalena et al., 2018). The price of green methanol is almost a double of grey methanol and triple of sugar feedstocks, and therefore not commercially competitive (Cheng et al., 2019; Martin, 2021). However, its use as a feedstock for the production of vitamins may be beneficial due to its sustainability and improved reception by consumers.

Methylotrophic microorganisms utilize methanol as sole carbon source for growth and are up-and-comers of the bioprocess industry (Pfeifenschneider et al., 2017). *Bacillus methanolicus* is a Gram-positive, facultative methylotrophic bacterium with a high methanol conversion rate and an optimal growth temperature between 50°C and 53°C (Schendel et al., 1990). In *B. methanolicus*, sporulation is controlled by the stage zero sporulation protein A (Spo0A) like in *B. subtilis*; thus, it can be hypothesized that the general stress response is regulated similarly in both organisms (Piggot & Hilbert, 2004; Schultenkämper et al., 2019). Sporulation in *B. methanolicus* occurs due to exposition to different types of stress, for example temperature shift from 50°C to 37°C; however, *B. methanolicus* is not prone to sporulate during typical fed-batch fermentations at 50°C (Schendel et al., 1990). The use of thermophilic bacteria in bioprocesses is very often advantageous due to reduced reactor cooling requirements, the decrease in contamination risks in comparison with mesophilic conditions, applicability of processes with high temperatures in tropical regions and the increased efficiency caused by increased catalytic activity of some enzymes (Abdel-Banat et al., 2010; Brautaset et al., 2007; Jakobsen et al., 2009; Schendel et al., 1990). The increase in the temperature of ethanol fermentation from starchy materials by only 5°C leads to the significant overall cost reduction in this process (Abdel-Banat et al., 2010). In recent years, the knowledge base on metabolism of *B. methanolicus* has increased (Carnicer et al., 2016; Heggeset et al., 2012; Irla et al., 2015; Irla, Heggeset, et al., 2016; Müller et al., 2014). The biochemical background of methanol utilization is well described in *B. methanolicus* and starts with its oxidation to formaldehyde by a methanol dehydrogenase, followed by formaldehyde assimilation via the RuMP (ribulose monophosphate) cycle (Arfman et al., 1989; Brautaset et al., 2004; de Vries et al., 1990, 1992; Jakobsen et al., 2006; Yurimoto et al., 2005). Understanding the regulation of carbon flux in the central metabolism is important for predicting genetic alterations necessary to improve the production yield for cells growing on methanol, as exemplified in recent metabolic engineering approaches (Drejer et al., 2018; Fernandes Brito et al., 2021; Irla et al., 2020; Irla, Heggeset, et al., 2016; Jakobsen et al., 2006; Pfeifenschneider et al., 2017).

Metabolites of the RuMP cycle, which also belong to the non-oxidative pentose phosphate pathway (PPP), play an important role in the biosynthesis of riboflavin (Figure 1). Riboflavin biosynthesis consists of several enzymatic steps initiating from guanosine 5′-triphosphate (GTP) and ribulose 5-phosphate (Ru5P) (Figure 1) (Liu et al., 2020). In *B. subtilis*, Ru5P is isomerized to ribose 5-phosphate (R5P) by R5P isomerase (encoded by *ywlF*) (Liu et al., 2020; Yang, Sun, Fu, et al., 2021) while in *B. methanolicus* this reaction belongs to the RuMP

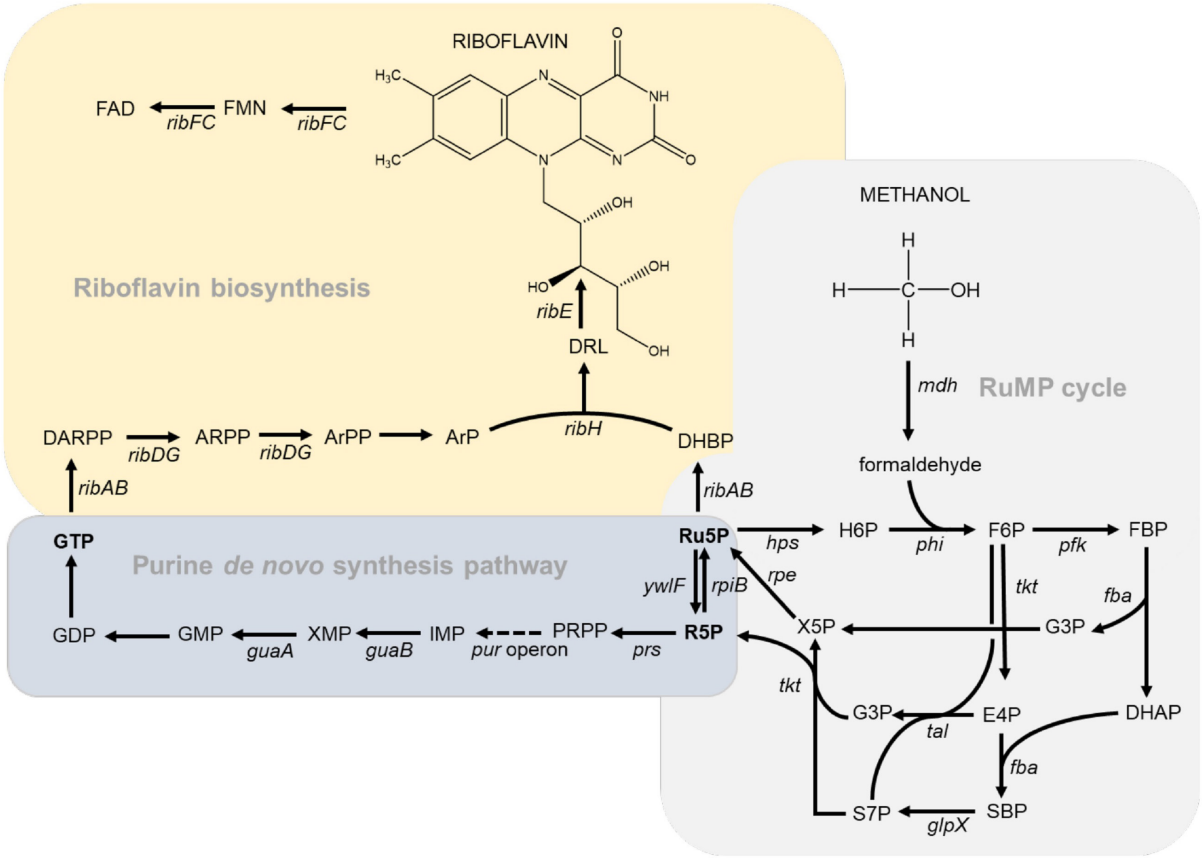

FIGURE 1 Schematic overview of the main genes that play a role in the riboflavin biosynthesis from methanol. The riboflavin biosynthesis pathway in *Bacillus subtilis* and its putative counterpart in *B. methanolicus* (yellow chart). The de novo purine biosynthesis pathway of *B. subtilis* and its putative counterpart in *B. methanolicus* is presented in a blue chart. The methanol assimilation pathway in *B. methanolicus* is presented in a grey chart. Genes: *ribFC*, bifunctional flavokinase/FAD synthetase; *ribE*, riboflavin synthase; *ribAB*, bifunctional enzyme GTP cyclohydrolase II/3,4-DHBP synthase; *ribDG*, bifunctional deaminase/reductase; *ribH*, lumazine synthase; *mdh*, methanol dehydrogenase; *ywlF*, ribose 5-phosphate isomerase in *B. subtilis*; *rpiB*, ribose 5-phosphate isomerase in *B. methanolicus*; *hps*, 3-hexulose-6-phosphate synthase; *phi*, 6-phospho-3-hexuloisomerase; *pfk*, 6-phosphofructokinase; *rpe*, ribulose-5-phosphate 3-epimerase; *tkt*, transketolase; *fba*, fructose-bisphosphate aldolase; *guaA*, GMP synthetase; *guaB*, IMP dehydrogenase; *pur* operon; *prs*, PRPP synthetase; *tal*, transaldolase; *glpX*, fructose-1,6-bisphosphatase; Metabolites: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; DRL, 6,7-dimethyl-8-ribityllumazine; DARPP, 2,5-diamino-6-ribosyl-amino-4(3H)pyrimidinedione 5′-phosphate; ARPP, 5-amino-6-ribosyl-amino-2,4(1H,3H)pyrimidinedione 5′-phosphate; ArPP, 5-amino-6-ribityl-amino-2,4(1H,3H)pyrimidinedione 5′-phosphate; ArP, 5-amino-6-ribityl-amino-2,4(1H,3H)pyrimidinedione; DHBP, 3,4-dihydroxy-2-butanone-4-phosphate; GTP, guanosine 5′-triphosphate; Ru5P, ribulose 5-phosphate; H6P, 3-hexulose 6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GDP, guanosine 5′-di-phosphate; GMP, guanosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; IMP, inosine 5′-monophosphate; PRPP, 5-phospho-α-D-ribosyl-1-pyrophosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; E4P, erythrose 4-phosphate; DHAP, dihydroxyacetone phosphate; S7P, sedoheptulose-7-phosphate; SBP, sedoheptulose-1,7-bisphosphate. Dashed arrows indicate multiple enzymatic steps, and continuous arrows represent single enzymatic steps. Bold metabolites indicate precursors of riboflavin.

cycle, and during growth on methanol, its reverse direction is favoured (Figure 1) (Brautaset et al., 2004; Heggeset et al., 2012). Subsequently, 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) synthetase (encoded by *prs*) catalyses the conversion of R5P into PRPP, which is an entry point of the de novo purine biosynthetic pathway (Figure 1) (Liu et al., 2020; Shi et al., 2014; Yang, Sun, Fu, et al., 2021). PRPP is converted to inosine 5′-monophosphate (IMP) in a chain of reactions, and then, IMP is further transformed to xanthosine 5′-monophosphate (XMP), catalysed by IMP dehydrogenase encoded by *guaB* (Figure 1) (Shi et al., 2014). Guanosine 5′-monophosphate (GMP) synthetase (encoded by *guaA*) catalyses the amination of XMP to GMP, GMP is converted to guanosine 5′-di-phosphate (GDP) and further to GTP (Shi et al., 2014; Yang, Sun, Fu, et al., 2021).

In *B. subtilis*, the enzymes that belong to the riboflavin biosynthetic pathway are encoded by the genes *ribDG*, *ribE*, *ribAB*, *ribH* and *ribT*, which constitute an operon while, based on the genetic homology, in *B. methanolicus*, a putatively similar pathway exists short of *ribT* gene (Figure 1) (Pedrolli et al., 2015). The riboflavin biosynthesis pathway was described for *B. subtilis* starting with the bifunctional enzyme GTP cyclohydrolase II/3,4-DHBP synthase (encoded by *ribAB*) which catalyses

the conversion of GTP into 2,5-diamino-6-ribosyl-amino-4(3H)pyrimidinedione 5′-phosphate (DARPP) and the formation of 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) from Ru5P (Figure 1) (Averianova et al., 2020). DARPP is further transformed to 5-amino-6-ribityl-amino-2,4(1H,3H)pyrimidinedione (ArP) via deamination and reduction reactions, catalysed by the bifunctional deaminase/reductase (encoded by *ribDG*), and dephosphorylation by an unknown phosphatase (Figure 1) (Averianova et al., 2020; Pedrolli et al., 2015; Revuelta et al., 2016). This is followed by the condensation of ArP with 4-dihydroxy-2-butanone-4-phosphate (DHBP) to form 6,7-dimethyl-8-ribityllumazine (DRL), catalysed by lumazine synthase encoded by *ribH* (Figure 1) (Averianova et al., 2020). Finally, riboflavin is formed through dismutation of DRL (Figure 1) (Averianova et al., 2020). This reaction step is catalysed by riboflavin synthase, which is encoded by *ribE* (Figure 1) (Averianova et al., 2020). Riboflavin is further converted to FMN and FAD catalysed by the bifunctional flavokinase/FAD synthetase encoded by *ribFC* (Figure 1) (Averianova et al., 2020; Revuelta et al., 2016) and cannot actively be exported by *B. subtilis* (Hemberger et al., 2011). The terminal *rib* operon gene, *ribT*, was recently revealed to encode a GCN5-related N-acetyltransferase, which transfers the acetyl group from acetyl coenzyme A to numerous substrates (Srivastava et al., 2018). RibU catalyses the uptake of riboflavin from the growth medium and acts as riboflavin transporter (Vogl et al., 2007).

In this study, we aim to improve the native riboflavin production in *B. methanolicus* during methanol-based growth through the overexpression of either the putative native riboflavin biosynthetic pathway or heterologous pathways.

EXPERIMENTAL PROCEDURES

Strains, plasmids and primers

Escherichia coli DH5α was used as a general cloning host and *B. methanolicus* MGA3 was the expression host. The following strains were the source of genomic DNA for cloning of the *rib* operon: *B. licheniformis* MW3, *B. subtilis* 168 and *B. methanolicus* MGA3. Used plasmids in this study are listed in Table 1, created strains in Table 2 and primers are shown in Table 3.

TABLE 1 Bacterial plasmids used in this study.

Plasmid	Relevant characteristics	References
pBV2xp	Kan^R; pHCMC04 derivative for gene expression under the control of the inducible xylose promoter from *B. megaterium*, theta replicating, low copy number plasmid	Drejer et al. (2020)
pBV2xp-rib^{Bl}	Kan^R; pBV2xp derivative for expression of *ribDG*, *ribE*, *ribAB*, *ribH* derived from *B. licheniformis* under control of the inducible xylose promoter from *B. megaterium*, theta replicating, low copy number plasmid	This study
pBV2xp-rib^{Bm}	Kan^R; pBV2xp derivative for expression of *ribDG*, *ribE*, *ribAB*, *ribH* derived from *B. methanolicus* under control of the inducible xylose promoter from *B. megaterium*, theta replicating, low copy number plasmid	This study
pBV2xp-rib^{Bs}	Kan^R; pBV2xp derivative for expression of *ribDG*, *ribE*, *ribAB*, *ribH* derived from *B. subtilis* under control of the inducible xylose promoter from *B. megaterium*, theta replicating, low copy number plasmid	This study
pTH1mp	Cm^R; derivative of pTH1mp-lysC for gene expression under control of the *mdh* promoter. The *lys*C gene was replaced with multiple cloning site, low copy number plasmid	(Irla, Heggeset, et al., 2016)
pTH1mp-*rpe-tkt*	Cm^R; pTH1mp derivative for expression of *rpe* (encoding ribulose-5-phosphate 3-epimerase) and *tkt* (encoding transketolase) under control of the *mdh* promoter, low copy number plasmid	This study
pTH1mp-*prs-gua*AB	Cm^R; pTH1mp derivative for expression of *prs* (encoding ribose-phosphate pyrophosphokinase) and *gua*AB (encoding guanosine monophosphate and inosine monophosphate) under control of the *mdh* promoter, low copy number plasmid	This study
pUB110Smp	Kan^R; gene expression under control of the *mdh* promoter from *B. methanolicus*, rolling circle replicating, high copy number plasmid	(Irla, Heggeset, et al., 2016)
pUB110Sxp	Kan^R; gene expression under control of the inducible xylose promoter from *B. megaterium*, rolling circle replicating, high copy number plasmid	This study
pUB110Sxp-rib^{Bl}	Kan^R; pUB110Sxp derivative for expression of *ribDG*, *ribE*, *ribAB*, *ribH* derived from *B. licheniformis* under control of the inducible xylose promoter from *B. megaterium*, rolling circle replicating, high copy number plasmid	This study
pUB110Sxp-rib^{Bs}	Kan^R; pUB110Sxp derivative for expression of *ribDG*, *ribE*, *ribAB*, *ribH* derived from *B. subtilis*; gene expression under the control of the inducible xylose promoter from *B. megaterium*, rolling circle replicating, high copy number plasmid	This study

TABLE 2 List of *B. methanolicus* strains created in this study.

Abbreviated strain name	Recombinant *B. methanolicus* strains created in this study
BV_Ctr	MGA3(pBV2xp)
BV_Rib^{Bl}	MGA3(pBV2xp-*rib*^{Bl})
BV_Rib^{Bm}	MGA3(pBV2xp-*rib*^{Bm})
BV_Rib^{Bs}	MGA3(pBV2xp-*rib*^{Bs})
BV_Rib^{Bl} (TH-Ctr)	MGA3(pBV2xp-*rib*^{Bl}) (pTH1mp)
BV_Rib^{Bl} (TH-Ru5P)	MGA3(pBV2xp-*rib*^{Bl}) (pTH1mp-*rpe-tkt*)
BV_Rib^{Bl} (TH-GTP)	MGA3(pBV2xp-*rib*^{Bl}) (pTH1mp-*prs-gua*AB)
UB_Ctr	MGA3(pUB110Sxp)
UB_Rib^{Bl}	MGA3(pUB110Sxp- *rib*^{Bl})
UB_Rib^{Bs}	MGA3(pUB110Sxp-*rib*^{Bs})

Molecular cloning

The *E. coli* DH5α competent cells were prepared according to the previously described calcium chloride protocol (Green & Rogers, 2013) or purchased from New England Biolabs. All standard molecular cloning procedures were carried out as described in Sambrook and Russell (2001) or according to manuals provided by producers. Genomic DNA from *B. licheniformis* MW3, *B. subtilis* 168 and *B. methanolicus* MGA3 was isolated as described in Eikmanns et al. (1994) and further uses for the PCR amplification of their native *rib* operons.

The genes *prs*, *guaAB* or *tkt* and *rpe* were PCR amplified from the genomic DNA of *B. methanolicus*. CloneAmp HiFi PCR Premix (Takara) was used to amplify the PCR products which were purified by using QIAquick PCR Purification Kit from Qiagen. For the separation of DNA fragments, 0.8% SeaKem LE agarose gels (Lonza) were used. DNA fragments and plasmid DNA were isolated using QIAquick Gel Extraction Kit (Qiagen) and Monarch Plasmid Miniprep Kit (New England Biolabs, T1010L), respectively. Via isothermal DNA assembly, the PCR-amplified *rib* operons were joined with the digested pBV2xp (cut with SacI and BamHI) and the PCR-amplified genes *prs*, *guaAB* or *tkt* and *rpe* were introduced into the digested pTH1mp vector (cut with XbaI and BamHI) (Gibson et al., 2009). As a result, the plasmids pBV2xp-*rib*^{Bl}, pBV2xp-*rib*^{Bm}, pBV2xp-*rib*^{Bs}, pTH1mp-*rpe-tkt* and pTH1mp-*prs-guaAB* were created (Table 1). The vector pUB110Sxp was constructed in multiple steps. First, pUB110Smp was digested with XbaI and PstI to remove the *mdh* promoter. Then, the promoter was exchanged by isothermal DNA assembly-mediated insertion of the xylose inducible promoter, which was previously PCR amplified from the pBV2xp plasmid using the primers XP1 and XP2 (Table 3). The same DNA assembly method was used to join the digested pUB110Sxp plasmid (cut with SacI and BamHI) and the *rib* operon derived from *B. licheniformis* to create the plasmid pUB110Sxp-*rib*^{Bl} (Table 1). To create the plasmid pUB110Sxp-*rib*^{Bs}, the empty vector pUB100Smp was cut with restriction enzymes PstI and NdeI in order remove the *mdh* promoter, and then, the empty vector was assembled with the PCR-amplified fragment of the pBV2xp-*rib*^{Bs} plasmid that included the xylose inducible promoter and its regulator (Table 5) (Gibson et al., 2009). All isothermal DNA assemblies were followed by a heat shock transformation into *E. coli* DH5α (Sambrook & Russell, 2001). GoTaq DNA Polymerase (Promega) was used for colony PCR and subsequently sequences of cloned DNA fragments were confirmed by Sanger sequencing performed by Eurofins Genomics. All *B. methanolicus* strains were made electrocompetent and transformed with the mentioned plasmids via electroporation as described previously (Jakobsen et al., 2006). The vectors pTH1mp, pTH1mp-*rpe-tkt* and pTH1mp-*prs-guaAB* were used for the transformation of the BV_Rib^{Bl} strain, leading to the strains BV_Rib^{Bl} (TH-Ctr), BV_Rib^{Bl} (TH-Ru5P) and BV_Rib^{Bl} (TH-GTP), respectively. RNA regulatory elements within the operons of the riboflavin genes tested have been deleted to prevent a potential effect on gene expression. All such created strains are listed in Table 2.

Media and growth conditions

The cloning host *E. coli* DH5α was routinely grown in shake flasks in Luria-Bertani (LB) medium at 37°C. For the riboflavin production experiments, *B. methanolicus* strains were grown in 250 mL baffled shake flaks in 50 mL of MVcM minimal medium supplemented with 50 $\mu g\,mL^{-1}$ of kanamycin and/or 5 $\mu g\,mL^{-1}$ chloramphenicol for recombinant strains according to their genetic make-up. Sublethal concentration of chloramphenicol of 0.5 $\mu g\,mL^{-1}$ was added to increase the copy number of pBV2xp plasmid (carrying kanamycin resistance gene). MVcM medium contained the following compounds, in 1 L of distilled water: K_2HPO_4, 4.09 g; $NaH_2PO_4{*}H_2O$, 1.49 g; $(NH_4)_2SO_4$, 2.11 g and was adjusted to pH 7.2 before autoclaving for 20 min at 121°C. (Schendel et al., 1990). The minimal medium was supplemented with 1 mL of 1 M $MgSO_4{*}7H_2O$ solution, 1 mL trace elements solution and 1 mL vitamin solution. Trace elements solution contained the following, in 1 L of distilled water: $FeSO_4{*}7H_20$, 5.56 g; $CuSO_4{*}2H_2O$, 27.28 mg; $CaCl_2{*}2H_2O$, 7.35 g; $CoCl_2{*}6H_2O$, 40.50 mg; $MnCl_2{*}4H_2O$, 9.90 g; $ZnSO_4{*}7H_2O$, 287.54 mg; $Na_2MoO_4{*}2H_2O$, 48.40 mg; H_3BO_3, 30.92 mg; HCl, 80 mL. In 1 L of distilled water, the vitamin solution contained 0.1 g of: biotin, thiamine hydrochloride, riboflavin, calcium D-pantothenate, pyridoxine hydrochloride, nicotinamide; 0.02 g of 4-aminobenzoic acid, and 0.01 g

TABLE 3 Primers used for the construction of all recombinant *B. methanolicus* MGA3 strains of this study.

Primer name	Sequence	Function
VJFW	TCTAATCCTTCTAAAAAATATAATTTAGAAAACTAAG	Sequencing primer for pTH1mp: FW
VJRW	GGTGCGGGCCTCTTCGCTATTACG	Sequencing primer for pTH1mp; RV
PXPF	TGTTTATCCACCGAACTAAG	Sequencing primer for pBV2xp; FW
BVXR	CCGCACAGATGCGTAAGGAG	Sequencing primer for pBV2xp; RV
RI04	TTCACTTAAGGGGGAAATGGCAAATGGAAGAGTATTATATG AAGCTGGCCTTAGAT	*rib*^{Bs} operon for pBV2xp; FW primer
RI05	ACGACGGCCAGTGAATTCGAGCTTTATTCAAATGAGCGGTT TAAATTTGCCAT	*rib*^{Bs} operon for pBV2xp; RV primer
RI06	TTCACTTAAGGGGGAAATGGCAAATGCACGATCATGAGTATATGAA	*rib*^{Bm} operon for pBV2xp; FW primer
RI07	ACGACGGCCAGTGAATTCGAGCTTTATCCAATTTGTCTGAC TAAATTCGCCATTTC	*rib*^{Bm} operon for pBV2xp; RV primer
RI08	TTCACTTAAGGGGGAAATGGCAAATGACGGATGCGCATTATATG AATCTTGCATTGG	*rib*^{Bl} operon for pBV2xp; FW primer
RI09	ACGACGGCCAGTGAATTCGAGCTTTATTGAAGCGACCGGGTCAGGTT	*rib*^{Bl} operon for pBV2xp; RV primer
RI10	TAAACAATTACATAAATAGGAGGTAGTACATATGCCCA ACCAATATCTAGAC	*prs* for pTH1mp; FW primer
RI11	TTAATCAAAAAGTGTGCTGACAGACTGCTCTTC	*prs* for pTH1mp; RV primer
RI12	TCAGCACACTTTTTGATTAAGTAAACAATTACATAAATAGGAGGTAG TAAGAATGTGGGAAAATAAGTTTGTAAAAGAA	*guaB* for pTH1mp; FW primer
RI13	TTAAGATAATGAGTAATTCGGAGCTTC	*guaB* for pTH1mp; RV primer
RI14	CGAATTACTCATTATCTTAAGTAAACAATTACATAAATAGGAGGTAG TAAGAATGCCAGGAAAAACAGAGTTGCAAAACCAGGA	*guaA* for pTH1mp; FW primer
RI22	TAGACCTATGGCGGGTACCATATGTTATTCCCACTCAATTGTTG CTGGCGGTTTGCTTG	*guaA* for pTH1mp; RV primer
RI18	TAAACAATTACATAAATAGGAGGTAGTACATATGATCAAGATTG CACCTTCTATTCTTTC	*rpe* for pTH1mp; FW primer
RI19	TCAATTTCTAATTTTTGCAATGGCTTGAC	*rpe* for pTH1mp; RV primer
RI20	TTGCAAAAATTAGAAATTGAGTAAACAATTACATAAATAGGAGGTAG TAAGAATGCTCCAACAAAAAATAGATATTGATCAGTT	*tkt* for pTH1mp; FW primer
RI21	TAGACCTATGGCGGGTACCATATGTTAGAGAAGCTTTTTAA AATGAGAAACGACAT	*tkt* for pTH1mp; RV primer
XP1	TTACGCCAAGCTTGGCTGCACCATGGACGCGTGACGTGAAA	Forward primer for pUB110Sxp
XP2	CCAGTGAATTCAAGCTCTAGACGACGGCCAGTGAATTCGAG	Reverse primer for pUB110Sxp
PUB1	TTCACTTAAGGGGGAAATGGATGACGGATGCGCATTATATG AATCTTGCATTGG	Forward primer for pUB110Sxp-*rib*^{Bl}
PUB2	GACGACGGCCAGTGAATTCGTTATTGAAGCGACCGGGTCAGGTT	Reverse primer for pUB110Sxp-*rib*^{Bl}
RI36	ATTACGCCAAGCTTGGCTGCACTAACTTATAGGGGTAACACTTA	Forward primer to amplify the xp-*rib*^{Bs} fragment for pUB110Sxp-*rib*^{Bs}
RI37	CTAGACCTATGGCGGGTACCATATTATTCAAATGAGCG GTTTAAATTTGCC	Reverse primer to amplify the xp-*rib*^{Bs} fragment for pUB110Sxp-*rib*^{Bs}

of: folic acid, vitamin B_{12} and lipoic acid (Schendel et al., 1990). For precultures, MVcM was supplemented with 0.25 g L^{-1} of yeast extract, designated as MVcMY. The precultures were grown overnight at 50°C and 200 rpm and were used as inoculum for the riboflavin production cultures at an initial OD_{600} of 0.1 or 0.15. All cultivations were performed with the same conditions in triplicates. The growth of *B. methanolicus* cells was determined by the measurement of OD_{600} with a cell density meter (WPA CO 8000 Biowave) in an interval of 2 h until of 26 h or 28 h. After 4 h (approximately two generational doubling times), each culture was induced with 1 mL of 50% of xylose, since the recombinant genes were expressed from a xylose inducible promoter (Nguyen et al., 2005).

To test whether the growth of *B. methanolicus* is affected by riboflavin supplementation, *B. methanolicus* MGA3 wild-type strain was grown in MVcM media described above supplemented with gradually increasing riboflavin concentrations and growth rates were determined. Therefore, MVcM was supplemented with 0 mg L^{-1}, 6.25 mg L^{-1}, 12.5 mg L^{-1}, 25 mg L^{-1} or 50 mg L^{-1}

riboflavin. It is worth to notice that the 50 mg L^{-1} riboflavin stock was prepared, dissolved in MVcM and kept at 37°C while stirring to dissolve completely. Further concentrations did not enable the complete solving of riboflavin. The riboflavin stocks were sterilized by filtration.

Riboflavin quantification with HPLC

To quantify riboflavin in the supernatants of *B. methanolicus*, production cultures were centrifugated for 5 min at room temperature and 7830 rpm using a 5430R Centrifuge (Eppendorf). The supernatant was aliquoted and stored at −20°C. Sample preparation for the riboflavin quantification with high-performance liquid chromatography HPLC was based on Petteys and Frank (2011), with some modifications. Firstly, frozen supernatants were thawed, and thereof, 500 µL was added to 1 mL of 15% trichloroacetic acid (TCA) for protein precipitation. After mixing the samples gently for 1 min, they were incubated at 25°C for 20 min, protected from light. Afterwards, the samples were centrifugated for 20 min at 7830 rpm and 4°C, and then, 1 mL of the supernatant was transferred into HPLC vials and 150 µL of 2 M K_3PO_4 was mixed to each vial for pH adjustment. Riboflavin was quantified as described in Gliszczynska-swigło and Koziołowa (2000) and Petteys and Frank (2011), with some modifications. A HPLC analysis was performed using a symmetry®C_{18} Column (100 Å, 3.5 µm, 4.6 mm × 75 mm, Waters) and a 2475 HPLC Multi Fluorescence Detector (Waters) at an excitation wavelength of 370 nm and an emission wavelength of 520 nm. As mobile phase 73% of 5 mM ammonium acetate (pH 6) and 27% of methanol was used with a flowrate of 0.8 mL min^{-1} and an injection volume of 1 µL. The runtime was 8 min. Column temperature was set at 25°C and the sample temperature at 4°C. The concentration of riboflavin was calculated by means of linear regression, based on riboflavin standards.

Fed-batch fermentations

All fed-batch fermentations were performed in Applikon 1 L bioreactors with an initial culture volume of 750 mL. MVcMY was used as growth medium supplemented with 0.144 g L^{-1} biotin, 0.01 g L^{-1} vitamin B_{12}, 1 M $MgSO_4$*$7H_2O$, trace metal stock (as described earlier), 50 µg mL^{-1} kanamycin and 150 mM methanol. Precultures were cultivated as described above and used as inocula for an initial OD_{600} of 0.2. The fermentations were performed at 50°C with an initial agitation at 200 rpm and an initial aeration rate of 0.5 vvm (volume of gas/volume of liquid/minute). During the fermentation, the level of dissolved oxygen was maintained at 30% saturation by automatic adjustment of the agitation speed. The pH value was adhered to a value of 6.5 by automatic addition of 12.5% (wt/vol) NH_3 or 10% H_3PO_4. At the beginning of the fermentation, a drop of antifoam agent (Sigma Antifoam 204) was added. During the fermentation, methanol levels were monitored online via the Prima BT gas analyser in order to maintain a methanol concentration at 150 mM through the manually controlled supplementation of methanol feed. The methanol feed solution contained 50 mL of feed trace metal stock mixed with 1 L of methanol–water solution (1:9; v/v) (Jakobsen et al., 2009). The feed trace metal stock was prepared as described in Jakobsen et al. (2009). Methanol feed composition described was used in control conditions. In addition, we tested two different feed compositions: MVS where methanol feed was supplemented with 0.144 g L^{-1} of biotin and 0.01 g L^{-1} of vitamin B_{12} and CVS where methanol feed was supplemented with 0.25 g L^{-1} of yeast extract. The cell growth was monitored by measuring the OD_{600} every 2 h and a sample for riboflavin quantification by HPLC measurement was taken every 4 h. All fermentations were run until rDOS values increased from 30% to 65% (around 36 h). The change in fermentation volume was considered in the calculations of riboflavin concentrations.

RESULTS

Assessment of *B. methanolicus* as a potential host for riboflavin overproduction during methylotrophic growth

In this study, we experimentally confirmed the functionality of a riboflavin biosynthesis operon in *B. methanolicus* comprised of four genes *ribDG*, *ribE*, *ribAB* and *ribH* through its overexpression in *B. methanolicus* that led to the overproduction of riboflavin (Table 4). The expression of the *rib* operon in *B. methanolicus* is controlled by a single promoter (Figure 2A), which can be considered relatively strong, as the transcripts of *rib* operon genes were previously classified into highly

TABLE 4 Riboflavin titres and growth rates of the recombinant strains.

Strain	Production titre [mg L^{-1}]	Growth rate [h^{-1}]
BV_Ctr	0.01 ± 0.00	0.33 ± 0.00
BV_Rib^{Bl}	1.32 ± 0.30	0.30 ± 0.01
BV_Rib^{Bm}	3.07 ± 0.26	0.42 ± 0.00
BV_Rib^{Bs}	15.91 ± 1.18	0.29 ± 0.01

Note: The recombinant strains were cultivated in minimal methanol medium for 28 h, OD_{600} was monitored, and growth rates were determined. Mean values of biological triplicates and standard deviations are presented. For each strain, the riboflavin titres (mg L^{-1}) were determined via HPLC analysis.

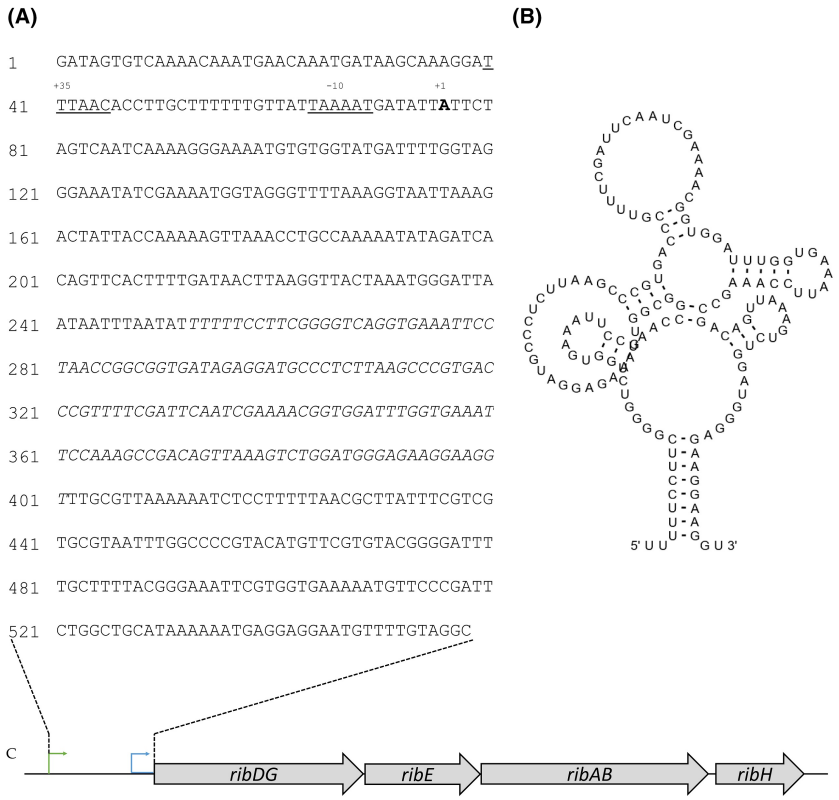

FIGURE 2 *Rib* operon structure, sequence of regulatory elements upstream of *rib* operon in *B. methanolicus* MGA3 (A) and predicted structure of FMN riboswitch (B). Underlined sequences indicate putative promoter, bold letter indicates transcription start site, italic sequence indicates putative riboswitch, native promoter marked in green, and synthetic P_{xyl} (together with Shine-Dalgarno sequence) marked in blue. Predicted riboswitch structure was generated by R2DT using the lysine riboswitch template provided by Rfam (Griffiths-Jones et al., 2003; Sweeney et al., 2020).

abundant category (Irla et al., 2015). The length of mRNA of the *rib* operon is 3935 bp whereof 483 nt belong to the 5′ untranslated region (5′ UTR), which places it among six longest 5′ UTRs in the transcriptome of *B. methanolicus*. Long 5′ UTR are known to carry regulatory elements and, in fact, the 5′ UTR upstream of *rib* operon contains a putative FMN riboswitch presented in Figure 2B (Irla et al., 2015).

It was previously determined that riboflavin supplementation is not necessary to support growth of *B. methanolicus* indicating that the native riboflavin biosynthesis pathway is active in this organism (Jakobsen et al., 2009). Additionally, we showed that the *B. methanolicus* empty vector control strain, BV_Ctr (Table 2), produces detectable levels of extracellular riboflavin in flasks ($0.01 \pm 0.00\,mg\,L^{-1}$), supporting the assumption regarding the activity of the native riboflavin biosynthesis pathway (Table 4).

We next tested the response of *B. methanolicus* to increasing concentrations of riboflavin in the cultivation broth to assess the potential effect of riboflavin overproduction on the growth of *B. methanolicus* in minimal methanol medium, using a maximal concentration of $50\,mg\,L^{-1}$ riboflavin due to limited solubility of commercial riboflavin. We observed about 25% decrease in the growth rate of *B. methanolicus* when growth medium was supplemented with $50\,mg\,L^{-1}$ in comparison with the control conditions (data not shown), and this result could indicate that riboflavin accumulation beyond this level might be a hindering factor for cell growth of riboflavin overproducing *B. methanolicus* strains.

Recombinant expression of various *rib* operons resulted in riboflavin overproduction in *B. methanolicus*

Recombinant *B. methanolicus* strains were created in this study in order to establish methanol-based overproduction of extracellular riboflavin in this bacterium. The *rib* operons derived either from the homologous donor *B. methanolicus* or heterologous donors *B. licheniformis* and *B. subtilis*, were cloned under control of the xylose inducible promoter into a low copy number, Θ-replicating vector pBV2xp (Table 1) to obtain the strains BV_Rib^{Bm}, BV_Rib^{Bl} and BV_Rib^{Bs}, respectively (Table 2). *Bacillus subtilis* was selected as genetic donor because it is a known riboflavin producer (Sauer et al., 1998; Wang et al., 2014), while *B. licheniformis* was chosen due to its optimal growth between 48°C and 51°C, which is in similar temperature range as for *B. methanolicus* (Warth et al., 1978). The wild-type *B. methanolicus* strain MGA3 harbouring the empty vector pBV2xp, here denoted BV_Ctr, represented the control strain for this experiment (Table 2).

All production strains and the control strain were cultivated in minimal methanol medium. The growth rate of the recombinant *B. methanolicus* strains BV_Ctr, BV_Rib^{Bl} and BV_Rib^{Bs} was similar (approximately $0.30\,h^{-1}$), while for unknown biological reason, the strain BV_Rib^{Bm} presented a growth rate of $0.42\,h^{-1} \pm 0.00$ which is typically observed for wild-type *B. methanolicus*. The empty vector control strain BV_Ctr produced $0.01 \pm 0.00\,mg\,L^{-1}$ of riboflavin (Table 4).

Comparatively, the riboflavin titres in the recombinant strains BV_Rib^{Bl}, BV_Rib^{Bm} and BV_Rib^{Bs} were higher, with 1.32 ± 0.30 mg L^{-1} riboflavin produced by BV_Rib^{Bl} and 3.07 ± 0.26 mg L^{-1} by BV_Rib^{Bm} (Table 4). The highest riboflavin titre was produced by the BV_Rib^{Bs} strain (15.91 ± 1.18 mg L^{-1}), which represents a substantial increase in comparison with the titre in the control strain (Table 4). Our results should also indicate that all genes of these operons are functionally expressed in *B. methanolicus*.

Further metabolic engineering of recombinant *B. methanolicus* strain to increase metabolic flux towards riboflavin precursors

We chose the recombinant strain BV_Rib^{Bl} with the lowest extracellular riboflavin titre among the *rib* operon overexpressing strains as background for testing the impact of strategical overexpression of genes involved in the biosynthesis of riboflavin precursors, which could render a high riboflavin producer from a poor-performing parental strain. To achieve this aim, we targeted the biosynthesis of several riboflavin precursors, C5 sugar phosphates—R5P and Ru5P, and a purine nucleoside triphosphate—GTP (Figure 1). In order to improve Ru5P and R5P biosynthesis, we cloned the genes encoding enzymes involved directly in their formation, *rpe* encoding ribulose 5 phosphate 3-epimerase and *tkt* encoding transketolase (Figure 1), into the pTH1mp vector under control of the *mdh* promoter. The pTH1mp plasmid has a rolling circle plasmid mode that is compatible with the θ-replication in the plasmid pBV2xp (Table 2) (Irla, Heggeset, et al., 2016). As a result, the plasmid pTH1mp-*rpe-tkt* was developed and used to transform the strain BV_Rib^{Bl} (Tables 2 and 4), whereby strain BV_Rib^{Bl} (TH-Ru5P) was created (Tables 2 and 5). In order to redirect carbon flow towards GTP, we cloned *prs* gene encoding PRPP synthetase and *guaAB* operon encoding GMP synthetase and IMP dehydrogenase in pTH1mp, generating the plasmid pTH1mp-*prs-guaAB* (Table 5). Our hypothesis was that the overproduction of early and final parts of the GTP biosynthesis pathway (Figure 1) might have a positive effect on riboflavin production level. The plasmid pTH1mp-*prs-guaAB* was used to transform the strain BV_Rib^{Bl} (Tables 1 and 6) to create the strain BV_Rib^{Bl} (TH-GTP) (Tables 2 and 6). The *B. methanolicus* strain BV_Rib^{Bl} transformed with the empty vector pTH1mp, named BV_Rib^{Bl} (TH-Ctr), was used as control in the testing of these strains (Tables 2 and 5).

BV_Rib^{Bl} (TH-Ctr) and BV_Rib^{Bl} (TH-Ru5P) exhibited similar growth rates of 0.29 ± 0.02 h^{-1} and 0.30 ± 0.01 h^{-1}, respectively, in minimal medium, and BV_Rib^{Bl} (TH-GTP) grew at slightly lower rate of 0.24 ± 0.00 h^{-1} for unknown reason (Table 5). Both the control strain BV_Rib^{Bl} (TH-Ctr) and the strain BV_Rib^{Bl} (TH-Ru5P) produced similar riboflavin titres, of 45.44 ± 2.73 mg L^{-1} and 47.57 ± 5.81 mg L^{-1}, respectively (Table 5). In comparison, the riboflavin titre produced by the BV_Rib^{Bl} (TH-GTP) strain was roughly twofold lower with a riboflavin overproduction titre of (25.81 ± 1.92 mg L^{-1}) (Table 5). Unexpectedly, the riboflavin titre for the BV_Rib^{Bl} (TH-Ctr) strain (Table 5) was 34-fold higher under the conditions tested here compared with that for the parental strain BV_Rib^{Bl} (Table 4). The addition of 5 μg mL^{-1} chloramphenicol as pTH1mp selection marker in growth medium of BV_Rib^{Bl} (TH-Ctr) was the only disparity between growth conditions of those strains, and we decided to further experimentally investigate any effects of chloramphenicol supplementation on riboflavin production.

Supplementation with sublethal chloramphenicol concentrations increases riboflavin overproduction in strain BV_Rib^{Bl}

Due to the difference in riboflavin titre observed between strains BV_Rib^{Bl} and BV_Rib^{Bl} (TH-Ctr) (Tables 4 and 5) potentially being caused by chloramphenicol supplementation, we decided to investigate whether the addition of sublethal concentration of chloramphenicol

TABLE 5 Riboflavin titre and growth rates of recombinant strains that possess two expression vectors.

Strain	Production titre [mg L^{-1}]	Growth rate [h^{-1}]
BV_Rib^{Bl} (TH-Ctr)	45.44 ± 2.73	0.29 ± 0.02
BV_Rib^{Bl} (TH-Ru5P)	47.57 ± 5.81	0.30 ± 0.01
BV_Rib^{Bl} (TH-GTP)	25.81 ± 1.92	0.24 ± 0.00

Note: The recombinant strains were cultivated in methanol minimal medium for up to 26 h, OD_{600} was monitored, and growth rates were calculated. For each strain, the overproduction of riboflavin (mg L^{-1}) was quantified via HPLC. BV_Rib^{Bl} (TH-Ctr) is the control strain and harbours, apart from pBV2xp-*rib*^{Bl} plasmid, the empty vector pTH1mp, whereas BV_Rib^{Bl} (TH-Ru5P) contains pBV2xp-*rib*^{Bl} and pTH1mp-*rpe-tkt* (Table 2). Mean values of biological triplicates and standard deviations are presented.

TABLE 6 Effect of sublethal levels of chloramphenicol in the culture broth on riboflavin titre.

Strain	Conditions	Production titre [mg L^{-1}]	Growth rate [h^{-1}]
BV_Ctr	-chr	0.01 ± 0.00	0.33 ± 0.00
	+chr	0.01 ± 0.00	0.27 ± 0.01
BV_Rib^{Bl}	-chr	1.32 ± 0.30	0.30 ± 0.01
	+chr	2.56 ± 0.36	0.22 ± 0.02

Note: The strains BV_Ctr and BV_Rib^{Bl} were cultivated on methanol minimal medium supplemented with 50 μg mL^{-1} kanamycin and either no chloramphenicol (−chr) addition or addition of 0.5 μg mL^{-1} chloramphenicol (+chr) in the growth medium. The riboflavin overproduction (mg L^{-1}) was quantified via HPLC. Technical triplicates with standard deviation are shown.

results in a positive effect on riboflavin production in strains where the *rib* operon is expressed from Θ-replication pBV2xp plasmid. This assumption was based on the findings of Begbie et al. (2005), who have established that the supplementation of media with sublethal levels of chloramphenicol may lead to an increase in the plasmid copy number of pMB1-based pBR322 vector in *E. coli* (Begbie et al., 2005). Based on its similarity to *E. coli*-derived ColE1 and *Bifidobacterium longum*-derived pFI2576, pMB1 is considered to be a θ-replication plasmid (Moon et al., 2009).

A riboflavin production experiment with the strain BV_Rib^{Bl} was performed with a minimal medium supplemented with 50 μg mL^{-1} kanamycin, to assure the maintenance of the pBV2xp-*rib*Bl plasmid (Table 2), and either addition of 0.5 μg mL^{-1} chloramphenicol in test condition or no chloramphenicol supplementation as control. The sublethal concentration of chloramphenicol was previously established (data not shown). The growth of BV_Rib^{Bl} in medium with chloramphenicol supplementation led to twofold increase in accumulation of riboflavin in comparison with the control growth condition without additional chloramphenicol (Table 6). We noticed decreased growth rates for BV_Ctr and BV_Rib^{Bl} grown with supplementary chloramphenicol compared with control cultivation without chloramphenicol supplementation (Table 3). This was expected since antibiotics inhibit bacterial growth (Martínez, 2017).

Expression of *rib* operon using high copy number plasmid causes further increase in riboflavin overproduction

We hypothesized that the chloramphenicol supplementation affected the copy number of θ-replication pBV2xp due to similar observations reported before (Table 6) (Begbie et al., 2005). To test this hypothesis, we decided to express the riboflavin biosynthetic operon with the pUB110 plasmid, with reported copy numbers of 30–50 and 25 ± 1 in *B. subtilis* and *B. methanolicus*, respectively (Gryczan et al., 1978; Irla, Heggeset, et al., 2016). We created plasmids named pUB110Sxp-*rib*^{Bl} and pUB110Sxp-*rib*^{Bs} (Table 2) through introduction of the *rib* operons derived from *B. licheniformis* or *B. subtilis*, respectively, into the high copy number vector pUB110Sxp with a xylose inducible promoter. The empty vector control *B. methanolicus* strain UB_Ctr harbours pUB110Sxp, whereas UB_Rib^{Bl} and UB_Rib^{Bs} carry pUB110Sxp-*rib*^{Bl} and pUB110Sxp-*rib*^{Bs}, respectively (Tables 2 and 7). For the control strain UB_Ctr, a riboflavin production titre of 0.34 ± 0.20 mg L^{-1} was achieved. We noticed that this value is higher compared with riboflavin titres produced by the other empty vector control strains tested in this study (Tables 4 and 6), for which we have no explanation. However, for the strains UB_Rib^{Bl} and UB_Rib^{Bs}, final titres of 6.34 ± 0.29 mg L^{-1} and 180.85 ± 2.61 mg L^{-1}, respectively, were achieved (Table 7). These values represent 5- and 11-fold increase in riboflavin titre in comparison with BV_Rib^{Bl} and BV_Rib^{Bs}, respectively, which are based on low copy number θ-replication plasmids derived from pBV2xp (Table 4). These increased riboflavin titres (Tables 4 and 7) are in agreement with the hypothesis that supplementation with chloramphenicol leads to the increase in plasmid copy number and thus causes gene dose increase and following increase in riboflavin overproduction.

TABLE 7 Riboflavin titre and growth rates of recombinant strains based on the high copy number vector pUB110Sxp.

Strain	Production titre [mg L^{-1}]	Growth rate [h^{-1}]
UB_Ctr	0.34 ± 0.20	0.29 ± 0.01
UB_Rib^{Bl}	6.34 ± 0.29	0.22 ± 0.02
UB_Rib^{Bs}	180.85 ± 2.61	0.33 ± 0.00

Note: The recombinant strains were cultivated in methanol minimal medium for 28 h, OD_{600} was monitored, and growth rates were calculated. For each strain, the titre of riboflavin (mg L^{-1}) was quantified via HPLC. The plasmid UB_Ctr is designated as the control strain for the high copy number plasmid strains and contains the empty vector pUB110Sxp, whereas UB_Rib^{Bl} contains pUB110Sxp-*rib*^{Bl}. Mean values and standard deviations of technical triplicates are presented.

Methanol-based riboflavin overproduction by recombinant *B. methanolicus* strains in fed-batch fermentations

To further test the capacity of the constructed *B. methanolicus* strains for riboflavin overproduction, BV_Rib^{Bs} and UB_Rib^{Bs} were cultivated in a lab-scale fed-batch fermentation under different conditions potentially supporting extracellular riboflavin accumulation. We chose these two strains because they achieved the highest riboflavin titres among the tested low and high copy number plasmid-borne riboflavin overproducing strains. Moreover, we wanted to investigate the influence of a high copy number plasmid on riboflavin overproduction on bioreactor scale. In the control condition, the fermentation medium was supplemented with 0.25 g L^{-1} of yeast extract and 150 mM methanol during the batch phase. The methanol feed solution used in fed-batch phase was composed of 50 mL of feed trace metal stock mixed with 1 L of methanol–water solution (1:9; v/v). Additionally, two other growth conditions were tested. In the condition referred to as complex vitamin supplementation (CVS), 0.25 g L^{-1} of yeast extract was supplemented to the methanol feed solution, while in the condition called minimal vitamin supplementation (MVS), 0.144 g L^{-1} biotin and 0.01 g L^{-1} vitamin B_{12} were added to the methanol feed solution. The supplementation

with yeast extract was tested because we have previously observed that it supports the growth of *B. methanolicus*. Under the conditions tested, similar riboflavin titres were achieved for BV_Rib^{Bs} strain of 36.07 mg L^{-1}, 32.54 mg L^{-1} and 31.76 mg L^{-1} and volumetric productivities of 1.5 mg L^{-1} h^{-1}, 2.7 mg L^{-1} h^{-1}, 2.0 mg L^{-1} h^{-1} in control, MVS and CVS conditions, respectively (Figure 3). A more stable growth and a prevention of riboflavin degradation were observed for CVS condition, whereas increased productivity was detected for MVS condition compared with the control (Figure 3).

For the strain UB_Rib^{Bs}, we used control conditions and yeast extract supplementation to the feed solution (CVS), because of its positive effect on growth stability and riboflavin productivity that occurred during the BV_Rib^{Bs} strain fermentation. In CVS conditions, a riboflavin titre of 522.65 mg L^{-1} was achieved for UB_Rib^{Bs} which is twofold higher compared with control condition, with a production titre of 281.30 mg L^{-1} (Figure 4), whereas the volumetric productivities for these two conditions are 16.1 mg L^{-1} h^{-1} and 11.7 mg L^{-1} h^{-1}, respectively. The riboflavin titre of UB_Rib^{Bs} in CVS condition during the fed-batch fermentation in bioreactor is threefold higher compared with the final overproduction titre achieved in flask fermentation of the same strain (Figure 4, Table 7).

In this study, the highest riboflavin overproduction titre was achieved in bioreactor scale by the strain UB_Rib^{Bs} in CVS condition (522.65 mg L^{-1}). For this strain, the final OD$_{600}$ in bioreactor cultivation increased 19-fold in comparison with flask cultivation and we have expected equivalent increase in riboflavin titre in bioreactor cultivation in comparison with flask cultivation, leading to a predicted titre in the range of 3500 mg L^{-1}. Notwithstanding our predictions, the titre achieved was

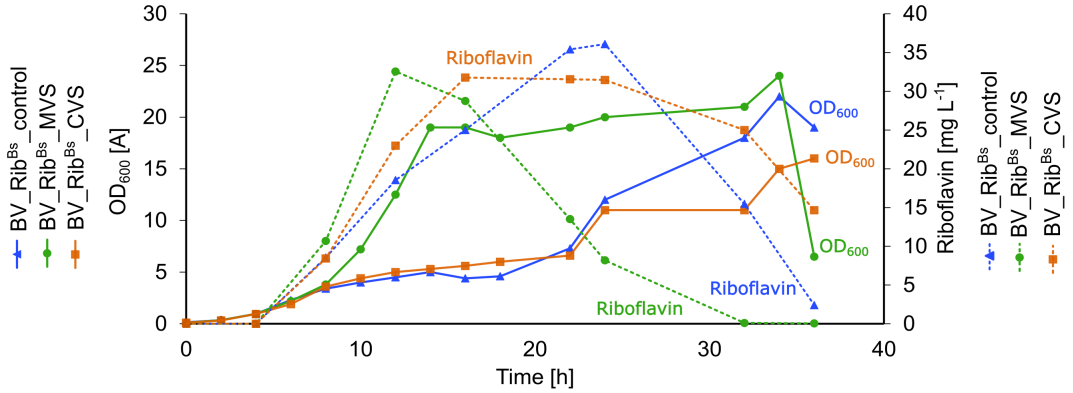

FIGURE 3 Fed-batch fermentations with BV_Rib^{Bs} using 150 mM of methanol as substrate (control) supplemented with yeast extract (CVS) or biotin and vitamin B$_{12}$ (MVS). Control conditions without the supplementation of yeast or biotin and vitamin B$_{12}$ to the growth broth are depicted with a triangle, growth conditions with additional biotin and vitamin B$_{12}$ (MVS) are shown with a circle marker and the supplementation of yeast to the growth medium (CVS) is represented by a square marker. Solid lines depict bacterial growth (OD$_{600}$), whereas dashed lines show the riboflavin overproduction in mg L^{-1}.

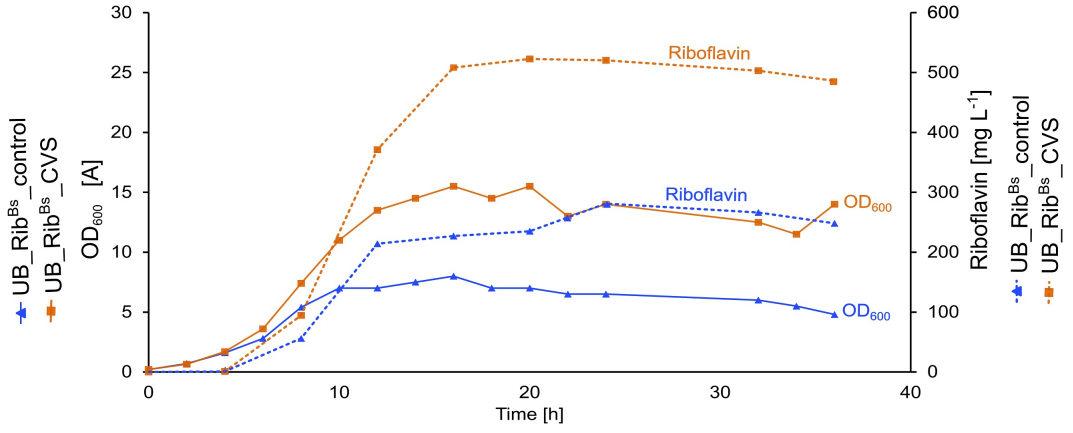

FIGURE 4 Fed-batch fermentations with UB_Rib^{Bs} using 150 mM of methanol as substrate (control) or supplemented with yeast extract (CVS). Control conditions without the supplementation of yeast extract to the growth broth are depicted with a triangle and the supplementation with yeast extract to the growth medium is represented by a square marker. Solid lines depict bacterial growth (OD$_{600}$), whereas the dashed lines show riboflavin production (mg L^{-1}).

sevenfold lower than expected indicating that production titres achieved in flasks cultivation is not directly transferable to bioreactor conditions. Likewise, despite a sixfold increase in the final OD_{600} value for the strain BV_Rib^{Bs} cultivated in bioreactor in comparison with flasks was observed, a riboflavin titre only doubled between these conditions from $15.91 \pm 1.18\,mg\,L^{-1}$ in flask cultivation to $31.76\,mg\,L^{-1}$ in bioreactor cultivation (Table 4, Figure 3). We noticed that the OD_{600} values in bioreactor fermentations were lower compared with previously published bioreactor fermentations of *B. methanolicus* where OD_{600} of 53 was achieved (Heggeset et al., 2012). This could be a result of manual flow adjustment instead of the automated one.

DISCUSSION

In this study, overproduction of riboflavin from methanol was achieved in the thermophilic *B. methanolicus* MGA3 as an expression host. In our approach, we created a series of recombinant strains based on *rib* operons derived from different microbial donors, various expression vectors and their combinations. Among the strains tested in the initial screening, the one carrying *B. subtilis*-derived operon performed best with a final titre of $15.91 \pm 1.18\,mg\,L^{-1}$ in flask cultivation. In a similar study where another thermophilic host, *Geobacillus thermoglucosidasius* DSM2542, was engineered for riboflavin overproduction through overexpression of either native or heterologous *rib* operons, the overexpression of *B. subtilis*-derived *rib* operon did not lead to an increase in the riboflavin titre in comparison with the control strain. However, the overexpression of native *rib* operon or *rib* operon derived from thermophilic *Geobacillus thermodenitrificans* NG80-2 led to riboflavin titres up to 28.7 $mg\,L^{-1}$ in flask cultivations using $20\,g\,L^{-1}$ of glucose and $5\,g\,L^{-1}$ of yeast extract as carbon source. The riboflavin titre in similar range ($15.91 \pm 1.18\,mg\,L^{-1}$) was obtained in our study for the strain BV_Rib^{Bs} (Table 4); however, much less carbon source was supplemented to the cultivation broth, namely only 6.4 $g\,L^{-1}$ of methanol (Yang, Sun, Tan, et al., 2021).

In *B. subtilis*, the deregulation of gluconeogenesis was described as effective strategy to increase riboflavin precursor supply by redirecting the carbon flux through the oxidative PPP, which resulted in enhanced riboflavin production (Wang et al., 2014). Following this example, we expected that the overexpression of genes coding for enzymes involved in central carbon metabolism of *B. methanolicus* would enhance riboflavin titres. Due to its methylotrophic lifestyle, the RuMP cycle in central carbon metabolism of *B. methanolicus* is composed of the enzymes commonly involved in the PPP in various microbial species (Arfman et al., 1989; Jakobsen et al., 2006; Pfeifenschneider et al., 2020; Stolzenberger et al., 2013). To increase the metabolic flux towards common riboflavin precursors, Ru5P and R5P, we constructed the strain BV_Rib^{Bl} (TH-Ru5P) where two genes involved in the RuMP cycle, *rpe* and *tkt* were overexpressed (Figure 1). The increase in riboflavin titre was observed for the two-plasmid strain BV_Rib^{Bl} (TH-Ru5P) in comparison with single-plasmid parental strain BV_Rib^{Bl}, but it cannot be fully attributed to the putative increased carbon flux towards Ru5P and R5P, because the control strain BV_Rib^{Bl} (TH-Ctr) harbouring the pBV2xp plasmid with *rib* operon and empty vector pTH1mp produced similar titre of riboflavin as BV_Rib^{Bl} (TH-Ru5P) which can correspond to 34-fold increase in comparison with single-plasmid strain BV_Rib^{Bl} (Table 5). This increase could potentially be explained by increase in copy number of a Θ-replicating plasmid pBV2xp in the presence of chloramphenicol based on the findings of Begbie et al. which attribute the increase in copy number of Θ-replicating vectors to supplementation with sublethal concentrations of this antibiotics (Begbie et al., 2005). The exchange of expression vector from low copy number pBV2xp to high copy number pUB110Sxp led to almost fivefold increase in riboflavin accumulation (Table 7) which roughly corresponds to the difference in copy number between these two plasmids (Irla, Heggeset, et al., 2016). This indicates that the use of a high copy number plasmid or increase in plasmid copy number through cultivation conditions are feasible strategies to improve riboflavin accumulation in the culture broth. The plasmid-based system used to create *B. methanolicus* riboflavin-producing strains in this study is similar to those previously applied to create *B. subtilis* and *A. gossypii* riboflavin-producing strains (Averianova et al., 2020). Since the use of plasmids in strain engineering leads to genetic instability, other strategies like for example genome integration of overexpression cassettes can be used to ensure the creation of genetically stable strains (Averianova et al., 2020). The low copy number plasmid pBV2xp is known to be stable and advisable for industrial uses (Irla, Heggeset, et al., 2016). In comparison, pUB110mp, a rolling circle replication plasmid, is less stable than pBV2xp, but has a higher copy number leading to increased extracellular riboflavin production (Irla, Heggeset, et al., 2016).

The highest riboflavin titre in this study was obtained in fed-batch fermentation under CVS condition, in which $522.65\,mg\,L^{-1}$ was produced by UB_Rib^{Bs} (Figure 4). That titre was threefold higher compared with the highest riboflavin titre obtained by UB_Rib^{Bs} in flask cultivation (Table 4). We observed a similar trend in our previous research regarding riboflavin production from seaweed which, like methanol, represents an alternative feedstock to sugar (Pérez-García et al., 2022). In 80% seaweed extract conditions, the recombinant *Corynebacterium glutamicum* strain CgRibo4 produced twofold more

riboflavin in fed-batch cultivations (1291.2 mg L^{-1}) compared with flask fermentations (635.0 ± 40.0 mg L^{-1}) (Pérez-García et al., 2022). Despite the improvement of riboflavin titres between flask and bioreactor-scale fermentations, we observed riboflavin degradation in all of the conditions over the time. Based on the literature, riboflavin is thermostable in acidic environment (Farrer & Mac Ewan, 1954). Despite being a photosensitive compound, it is reported to be relatively stable at 50°C when protected from light (Ahmad et al., 2015; Kearsley & Rodriguez, 1981; Sheraz et al., 2014).

We attempted to achieve increased carbon flux towards GTP by pTH1mp-based expression of the genes *prs*, *guaA* and *guaB* encoding enzymes involved in the formation of GTP in the BV_Rib^{BI} strain but observed lower accumulation of riboflavin in the growth media compared with control conditions (Table 5). The possible explanation of no positive effect on riboflavin accumulation by overexpression of these genes is feedback inhibition of PRPP amidotransferase encoded by *prs*. In *B. subtilis* PRPP amidotransferase was shown to undergo feedback inhibition, and it was speculated that its deregulation could increase the metabolic flux through purine biosynthesis pathway (Shi et al., 2014).

A transcriptome analysis has revealed the presence of two putative FMN riboswitches in *B. methanolicus* which are located upstream of the *rib* operon and *fmnP* gene, the latter being a homologue of *ribU* in *B. subtills*, which is annotated as a riboflavin importer (Irla et al., 2015). Hence, another strategy to develop a strain with higher riboflavin production titres could be the creation of a strain with a full 5′ untranslated region upstream of the *rib* operon to assure mRNA stability (Boumezbeur et al., 2020; Mironov et al., 2008). Importantly, the riboflavin riboswitch needs to be deactivated, for example through the introduction of point mutations, so that it does not affect the translation of the *rib* genes when riboflavin concentration increases (Boumezbeur et al., 2020; Mironov et al., 2008; Shi et al., 2014). Furthermore, it needs to be considered that high cytoplasmic FMN levels lead to decreased expression of the riboflavin biosynthesis and import genes controlled by FMN riboswitches thus to a shutdown of both riboflavin biosynthesis and import (Pedrolli et al., 2015). In *B. subtilis*, the regulator protein RibR counteracts this action by preventing transcription termination and allowing expression of the *rib* operon (Pedrolli et al., 2015). To our knowledge, such regulator gene has not been identified in *B. methanolicus* yet. Therefore, heterologous overexpression of *ribR* in *B. methanolicus* could be beneficial for increased riboflavin overproduction by this organism. Recently, Irla et al. have shown for the first time that the putative lysine riboswitch detected through in silico analysis was in fact functional in *B. methanolicus*, indicating the potential of this type of approach (Irla et al., 2021). Further steps should be taken to improve the precursor supply and inhibit by-product formation to develop strains with higher riboflavin production titres for the methanol-based production of riboflavin. Both *B. subtilis* and *A. gossypii* can uptake extracellular riboflavin, but among those two, only *A. gossypii* can actively export flavins (Förster et al., 2001; Hemberger et al., 2011; Nieland & Stahmann, 2013). We assume that *B. methanolicus* can import riboflavin due to the presence of a gene in its genome encoding a protein with 71% similarity to the riboflavin importer RibU from *B. subtilis*. Therefore, the inactivation of riboflavin importer could be a metabolic engineering strategy for creating an efficient *B. methanolicus* cell factory. We suggest that *B. methanolicus*, similarly to *B. subtilis*, cannot actively export riboflavin due to the lack of a riboflavin transmembrane transporter. To enhance the production of extracellular riboflavin, a *B. methanolicus* strain with the introduced gene *ribM* from *S. davawensis*, encoding the energy-independent flavin transport-catalysing protein could be created, like suggested previously for *B. subtilis* (Hemberger et al., 2011).

In this study, we have shown methanol-based overproduction of extracellular riboflavin in thermophilic conditions. We constructed new microbial strains via metabolic engineering which were tested in lab-scale bioreactors. The best-performing strain achieved a riboflavin titre of 522.65 mg L^{-1} which, to the best of our knowledge, is the highest-ever riboflavin titre from methanol reached by methylotrophic bacteria. Therefore, we added riboflavin to the products portfolio derived from methanol in biotechnological processes.

AUTHOR CONTRIBUTIONS

Vivien Jessica Klein: Conceptualization (equal); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal). **Luciana Fernandes Brito:** Conceptualization (equal); methodology (equal); validation (equal). **Fernando Perez-Garcia:** Conceptualization (equal); formal analysis (equal); investigation (equal); validation (equal). **Trygve Brautaset:** Formal analysis (equal); investigation (equal); validation (equal). **Marta Irla:** Conceptualization (equal); formal analysis (equal); investigation (equal); supervision (equal); validation (equal); writing – review and editing (equal).

ACKNOWLEDGEMENTS

FP-G and VJK were funded by the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology. LFB was funded by The Research Council of Norway within ERA CoBioTech, project number 327216.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Marta Irla https://orcid.org/0000-0001-7097-6656

REFERENCES

Abdel-Banat, B.M.A., Hoshida, H., Ano, A., Nonklang, S. & Akada, R. (2010) High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? *Applied Microbiology and Biotechnology*, 85, 861–867.

Adnan, M.A. & Kibria, M.G. (2020) Comparative techno-economic and life-cycle assessment of power-to-methanol synthesis pathways. *Applied Energy*, 278, 1–23.

Ahmad, I., Anwar, Z., Ahmed, S., Sheraz, M.A., Bano, R. & Hafeez, A. (2015) Solvent effect on the photolysis of riboflavin. *AAPS PharmSciTech*, 16, 1122–1128.

Al-Douri, A., Sengupta, D. & El-Halwagi, M. (2017) Shale gas monetization—a review of downstream processing to chemicals and fuels. *Journal of Natural Gas Science and Engineering*, 45, 436–455.

Arfman, N., Watling, E.M., Clement, W., Oosterwijk, R.J., de Vries, G.E., Harder, W. et al. (1989) Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. *Archives of Microbiology*, 152, 280–288.

Averianova, L.A., Balabanova, L.A., Son, O.M., Podvolotskaya, A.B. & Tekutyeva, L.A. (2020) Production of vitamin B2 (riboflavin) by microorganisms: an overview. *Frontiers in Bioengineering and Biotechnology*, 8, 1–23.

Balasubramaniam, S., Christodoulou, J. & Rahman, S. (2019) Disorders of riboflavin metabolism. *Journal of Inherited Metabolic Disease*, 42, 608–619.

Begbie, S., Dominelli, G., Duthie, K., Holland, J. & Jitratkosol, M. (2005) The effects of sub-inhibitory levels of chloramphenicol on pBR322 plasmid copy number in *Escherichia coli* DH5α cells. *Journal of Microbiology and Immunology*, 7, 82–88.

Boumezbeur, A., Bruer, M., Stoecklin, G. & Mack, M. (2020) Rational engineering of transcriptional riboswitches leads to enhanced metabolite levels in *Bacillus subtilis*. *Metabolic Engineering*, 61, 58–68.

Brautaset, T., Jakobsen, Ø.M., Flickinger, M.C., Valla, S. & Ellingsen, T.E. (2004) Plasmid-dependent methylotrophy in thermotolerant *bacillus methanolicus*. *Journal of Bacteriology*, 186, 1229–1238.

Brautaset, T., Jakobsen, Ø.M., Josefsen, K.D., Flickinger, M.C. & Ellingsen, T.E. (2007) *Bacillus methanolicus*: a candidate for industrial production of amino acids from methanol at 50°C. *Applied Microbiology and Biotechnology*, 74, 22–34.

Carnicer, M., Vieira, G., Brautaset, T., Portais, J.C. & Heux, S. (2016) Quantitative metabolomics of the thermophilic methylotroph *Bacillus methanolicus*. *Microbial Cell Factories*, 92, 1–12.

Cheng, M.H., Huang, H., Dien, B.S. & Singh, V. (2019) The costs of sugar production from different feedstocks and processing technologies. *Biofuels, Bioprod Biorefining*, 13, 723–739.

Dalena, F., Senatore, A., Basile, M., Knani, S., Basile, A. & Iulianelli, A. (2018) Advances in methanol production and utilization, with particular emphasis toward hydrogen generation via membrane reactor technology. *Membranes (Basel)*, 8, 1–27.

De Vries, G.E., Arfman, N., Terpstra, P. & Dijkhuizen, L. (1992) Cloning, expression, and sequence analysis of the *Bacillus methanolicus* C1 methanol dehydrogenase gene. *Journal of Bacteriology*, 174, 5346–5353.

Drejer, E.B., Chan, D.T.C., Haupka, C., Wendisch, V.F., Brautaset, T. & Irla, M. (2020) Methanol-based acetoin production by genetically engineered: *Bacillus methanolicus*. *Green Chemistry*, 22, 788–802.

Drejer, E.B., Hakvåg, S., Irla, M. & Brautaset, T. (2018) Genetic tools and techniques for recombinant expression in thermophilic Bacillaceae. *Microorganisms*, 6, 1–19.

Eikmanns, B.J., Thum-Schmitz, N., Eggeling, L., Lüdtke, K.-U. & Sahm, H. (1994) Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology*, 140, 1817–1828.

Farrer, K.T.H. & Mac Ewan, J.L. (1954) The thermal destruction of riboflavin. *Australian Journal of Biological Sciences*, 7, 73–84.

Fernandes Brito, L., Irla, M., Nærdal, I., Balzer Le, S., Delépine, B., Heux, S. et al. (2021) Evaluation of heterologous biosynthetic pathways for production by thermophilic *Bacillus methanolicus*. *Frontiers in Bioengineering and Biotechnology*, 9, 1–15.

Fischer, M. & Bacher, A. (2005) Biosynthesis of flavocoenzymes. *Natural Product Reports*, 22, 324–350.

Förster, C., Revuelta, J.L. & Krämer, R. (2001) Carrier-mediated transport of riboflavin in *Ashbya gossypii*. *Applied Microbiology and Biotechnology*, 55, 85–89.

Fradj, N.B., Jayet, P.A. & Aghajanzadeh-Darzi, P. (2016) Competition between food, feed, and (bio)fuel: a supply-side model based assessment at the european scale. *Land Use Policy*, 52, 195–205.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., III & Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6, 343–345.

Gliszczynska-swigło, A. & Koziołowa, A. (2000) Chromatographic determination of riboflavin and its derivatives in food. *Journal of Chromatography. A*, 881, 285–297.

Green, R. & Rogers, E.J. (2013) Transformation of chemically competent *E. coli*. *Methods in Enzymology*, 529, 329–336.

Griffiths-Jones, S., Bateman, A., Marshall, M., Khanna, A. & Eddy, S.R. (2003) Rfam: an RNA family database. *Nucleic Acids Research*, 31, 439–441.

Gryczan, T.J., Contente, S. & Dubnau, D. (1978) Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *Journal of Bacteriology*, 134, 318–329.

Heggeset, T.M.B., Krog, A., Balzer, S., Wentzel, A., Ellingsen, T.E. & Brautaseta, T. (2012) Genome sequence of thermotolerant *Bacillus methanolicus*: features and regulation related to methylotrophy and production of L-lysine and L-glutamate from methanol. *Applied and Environmental Microbiology*, 78, 5170–5181.

Hemberger, S., Pedrolli, D.B., Stolz, J., Vogl, C., Lehmann, M. & Mack, M. (2011) RibM from *Streptomyces davawensis* is a riboflavin/roseoflavin transporter and may be useful for the optimization of riboflavin production strains. *BMC Biotechnology*, 11, 119.

Irla, M., Drejer, E.B., Brautaset, T. & Hakvåg, S. (2020) Establishment of a functional system for recombinant production of secreted proteins at 50°C in the thermophilic *bacillus methanolicus*. *Microbial Cell Factories*, 19, 1–16.

Irla, M., Hakvåg, S. & Brautaset, T. (2021) Developing a riboswitch-mediated regulatory system for metabolic flux control in thermophilic *Bacillus methanolicus*. *International Journal of Molecular Sciences*, 22, 1–18.

Irla, M., Heggeset, T.M.B., Nærdal, I., Paul, L., Haugen, T., Le, S.B. et al. (2016) Genome-based genetic tool development for *Bacillus methanolicus*: theta- and rolling circle-replicating plasmids for inducible gene expression and application to methanol-based cadaverine production. *Frontiers in Microbiology*, 7, 1–13.

Irla, M., Nærdal, I., Brautaset, T. & Wendisch, V.F. (2016) Methanol-based γ-aminobutyric acid (GABA) production by genetically

engineered *Bacillus methanolicus* strains. *Industrial Crops and Products*, 106, 12–20.

Irla, M., Neshat, A., Brautaset, T., Rückert, C., Kalinowski, J. & Wendisch, V.F. (2015) Transcriptome analysis of thermophilic methylotrophic *Bacillus methanolicus* MGA3 using RNA-sequencing provides detailed insights into its previously uncharted transcriptional landscape. *BMC Genomics*, 73, 1–22.

Jakobsen, Ø.M., Benichou, A., Flickinger, M.C., Valla, S., Ellingsen, T.E. & Brautaset, T. (2006) Upregulated transcription of plasmid and chromosomal ribulose monophosphate pathway genes is critical for methanol assimilation rate and methanol tolerance in the methylotrophic bacterium *Bacillus methanolicus*. *Journal of Bacteriology*, 188, 3063–3072.

Jakobsen, Ø.M., Brautaset, T., Degnes, K.F., Heggeset, T.M.B., Balzer, S., Flickinger, M.C. et al. (2009) Overexpression of wild-type aspartokinase increases L -lysine production in the thermotolerant methylotrophic bacterium *Bacillus methanolicus*. *Applied and Environmental Microbiology*, 75, 652–661.

Kearsley, M.W. & Rodriguez, N. (1981) The stability and use of natural colours in foods: anthocyanin, β-carotene and riboflavin. *International Journal of Food Science and Technology*, 16, 421–431.

Lee, K.H., Park, Y.H., Han, J.K., Park, J.H., Lee, K.H. & Choi, H. (2006) *Microorganism for producing riboflavin and method for producing riboflavin using the same* (U.S. Patent Nr. 7,166,456, 2007). Alexandria, VA: U.S. Patent and Trademark Office.

Linton, J.D. & Niekus, H.G.D. (1987) The potential of one-carbon compounds as fermentation feedstocks. *Antonie Van Leeuwenhoek*, 53, 55–63.

Liu, S., Hu, W., Wang, Z. & Chen, T. (2020) Production of riboflavin and related cofactors by biotechnological processes. *Microbial Cell Factories*, 19, 1–16.

Liu, W.C., Inwood, S., Gong, T., Sharma, A., Yu, L.Y. & Zhu, P. (2019) Fed-batch high-cell-density fermentation strategies for *Pichia pastoris* growth and production. *Critical Reviews in Biotechnology*, 39, 258–271.

van Loon, A.P.G.M., Bretzel, W., Hümbelin, M. & Pfister, M. (1996) Development of a fermentation process for the manufacture of riboflavin. *Chimia (Aarau)*, 50, 410–412.

Martin, A. (2021) *A step forward for "green" methanol and its potential to deliver deep GHG reductions in maritime shipping*. Washington, DC: International Council on Clean Transportation. https://theicct.org/a-step-forward-for-green-methanol-and-its-potential-to-deliver-deep-ghg-reductions-in-maritime-shipping%E2%80%AF/

Martínez, J.L. (2017) Effect of antibiotics on bacterial populations: a multi-hierachical selection process. *F1000Research*, 6, 51.

Marx, C.J., Bringel, F., Chistoserdova, L., Moulin, L., Farhan Ul Haque, M., Fleischman, D.E. et al. (2012) Complete genome sequences of six strains of the genus *Methylobacterium*. *Journal of Bacteriology*, 194, 4746–4748.

Massey, V. (2000) The chemical and biological versatility of riboflavin. *Biochemical Society Transactions*, 28, 283–296.

Mironov, A.S., Karelov, D.V., Solovieva, I.M., Eremina, S.Y., Errais-Lopes, L., Kreneva, P.A. et al. (2008) Relationship between the secondary structure and the regulatory activity of the leader region of the riboflavin biosynthesis operon in *Bacillus subtilis*. *Russian Journal of Genetics*, 44, 399–404.

Moon, G.-S., Wegmann, U., Gunning, A.P., Gasson, M.J. & Narbad, A. (2009) Isolation and characterization of a theta-type cryptic plasmid from *Bifidobacterium longum* FI10564. *Journal of Microbiology and Biotechnology*, 19, 403–408.

Müller, J.E.N., Litsanov, B., Bortfeld-Miller, M., Trachsel, C., Grossmann, J., Brautaset, T. et al. (2014) Proteomic analysis of the thermophilic methylotroph *Bacillus methanolicus* MGA3. *Proteomics*, 14, 725–737.

Muscat, A., de Olde, E.M., de Boer, I.J.M. & Ripoll-Bosch, R. (2020) The battle for biomass: a systematic review of food-feed-fuel competition. *Global Food Security*, 25, 100330.

Nærdal, I., Pfeifenschneider, J., Brautaset, T. & Wendisch, V.F. (2015) Methanol-based cadaverine production by genetically engineered *Bacillus methanolicus* strains. *Microbial Biotechnology*, 8, 342–350.

Nguyen, H.D., Nguyen, Q.A., Ferreira, R.C., Ferreira, L.C.S., Tran, L.T. & Schumann, W. (2005) Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. *Plasmid*, 54, 241–248.

Nieland, S. & Stahmann, K.P. (2013) A developmental stage of hyphal cells shows riboflavin overproduction instead of sporulation in *Ashbya gossypii*. *Applied Microbiology and Biotechnology*, 97, 10143–10153.

Offermanns, H., Schulz, K., Brandes, E. & Schendler, T. (2014) Substance properties of methanol. In: Bertau, M., Offermanns, H., Plass, L., Schmidt, F. & Wernicke, H. (Eds.) *Methanol: the basic chemical and energy feedstock of the future*. Heidelberg, Germany: Springer Berlin, pp. 171–200.

Olah, G.A. (2005) Beyond oil and gas: the methanol economy. *Angewandte Chemie, International Edition*, 44, 2636–2639.

Oraei, M., Razavi, S.H. & Khodaiyan, F. (2018) Optimization of effective minerals on riboflavin production by *Bacillus subtilis* subsp. subtilis ATCC 6051 using statistical designs. *Avicenna Journal of Medical Biotechnology*, 10, 49–55.

Pedrolli, D.B., Kühm, C., Sévin, D.C., Vockenhuber, M.P., Sauer, U., Suess, B. et al. (2015) A dual control mechanism synchronizes riboflavin and sulphur metabolism in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 14054–14059.

Pérez-García, F., Klein, V.J., Brito, L.F. & Brautaset, T. (2022) From brown seaweed to a sustainable microbial feedstock for the production of riboflavin. *Frontiers in Bioengineering and Biotechnology*, 10, 1–15.

Perkins, J.B., Sloma, A., Hermann, T., Theriault, K., Zachgo, E., Erdenberger, T. et al. (1999) Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin. *Journal of Industrial Microbiology & Biotechnology*, 22, 8–18.

Petteys, B.J. & Frank, E.L. (2011) Rapid determination of vitamin B2 (riboflavin) in plasma by HPLC. *Clinica Chimica Acta*, 412, 38–43.

Pfeifenschneider, J., Brautaset, T. & Wendisch, V.F. (2017) Methanol as carbon substrate in the bio-economy: metabolic engineering of aerobic methylotrophic bacteria for the production of value added chemicals. *Biofuels, Bioproducts and Biorefining*, 11, 719–731.

Pfeifenschneider, J., Markert, B., Stolzenberger, J., Brautaset, T. & Wendisch, V.F. (2020) Transaldolase in *bacillus methanolicus*: biochemical characterization and biological role in ribulose monophosphate cycle. *BMC Microbiology*, 20, 1–13.

Piggot, P.J. & Hilbert, D.W. (2004) Sporulation of *Bacillus subtilis*. *Current Opinion in Microbiology*, 7, 579–586.

Pinto, J.T. & Zempleni, J. (2016) Riboflavin. *Advances in Nutrition*, 7, 973–975.

Revuelta, J.L., Ledesma-Amaro, R. & Jiménez, A. (2016) Industrial production of Vitamin B_2 by microbial fermentation. In: *Industrial biotechnology of vitamins, biopigments, and antioxidants*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 15–40.

Revuelta, J.L., Ledesma-Amaro, R., Lozano-Martinez, P., Díaz-Fernández, D., Buey, R.M. & Jiménez, A. (2017) Bioproduction of riboflavin: a bright yellow history. *Journal of Industrial Microbiology & Biotechnology*, 44, 659–665.

Sambrook, J. & Russell, D.W. (2001) *Molecular cloning: a laboratory manual*, 3rd edition. New York: Cold Spring Harbor Laboratory Press.

Sauer, U., Cameron, D.C., Bailey, J.E. & Zu, C. (1998) Metabolic capacity of *Bacillus subtilis* for the production of purine nucleosides, riboflavin, and folic acid. *Biotechnology and Bioengineering*, 59, 227–238.

Schendel, F.J., Bremmon, C.E., Flickinger, M.C., Guettler, M. & Hanson, R.S. (1990) L-lysine production at 50°C by mutants of a newly isolated and characterized methylotrophic *Bacillus* sp. *Applied and Environmental Microbiology*, 56, 963–970.

Schultenkämper, K., Brito, L.F., López, M.G., Brautaset, T. & Wendisch, V.F. (2019) Establishment and application of CRISPR interference to affect sporulation, hydrogen peroxide detoxification, and mannitol catabolism in the methylotrophic thermophile *Bacillus methanolicus*. *Applied Microbiology and Biotechnology*, 103, 5879–5889.

Schwechheimer, S.K., Park, E.Y., Revuelta, J.L., Becker, J. & Wittmann, C. (2016) Biotechnology of riboflavin. *Applied Microbiology and Biotechnology*, 100, 2107–2119.

Sheraz, M.A., Kazi, S.H., Ahmed, S., Anwar, Z. & Ahmad, I. (2014) Photo, thermal and chemical degradation of riboflavin. *Beilstein Journal of Organic Chemistry*, 10, 1999–2012.

Shi, S., Shen, Z., Chen, X., Chen, T. & Zhao, X. (2009) Increased production of riboflavin by metabolic engineering of the purine pathway in *Bacillus subtilis*. *Biochemical Engineering Journal*, 46, 28–33.

Shi, T., Wang, Y., Wang, Z., Wang, G., Liu, D., Fu, J. et al. (2014) Deregulation of purine pathway in *Bacillus subtilis* and its use in riboflavin biosynthesis. *Microbial Cell Factories*, 13, 1–16.

Sonntag, F., Kroner, C., Lubuta, P., Peyraud, R., Horst, A., Buchhaupt, M. et al. (2015) Engineering *Methylobacterium extorquens* for de novo synthesis of the sesquiterpenoid α-humulene from methanol. *Metabolic Engineering*, 32, 82–94.

Srivastava, R., Kaur, A., Sharma, C. & Karthikeyan, S. (2018) Structural characterization of *ribT* from *Bacillus subtilis* reveals it as a GCN5- related N-acetyltransferase. *Journal of Structural Biology*, 202, 70–81.

Stahmann, P., Revuelta, J.L. & Seulberger, H. (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. *Applied Microbiology and Biotechnology*, 53, 509–516.

Stolzenberger, J., Lindner, S.N., Persicke, M., Brautaset, T. & Wendisch, V.F. (2013) Characterization of fructose 1,6-bisphosphatase and sedoheptulose 1,7-bisphosphatase from the facultative ribulose monophosphate cycle methylotroph bacillus methanolicus. *Journal of Bacteriology*, 195, 5112–5122.

Sweeney, B.A., Hoksza, D., Nawrocki, E.P., Ribas, C.E., Madeira, F., Cannone, J.J. et al. (2020) R2DT: computational framework for template-based RNA secondary structure visualisation across non-coding RNA types. *Nature Communications*, 12, 3494.

Vogl, C., Grill, S., Schilling, O. & Mack, M. (2007) Characterization of riboflavin (vitamin B2) transport proteins from *Bacillus subtilis* and *Corynebacterium glutamicum*. *Journal of Bacteriology*, 189, 7367–7375.

de Vries, G.E., Kües, U. & Stahl, U. (1990) Physiology and genetics of methylotrophic bacteria. *FEMS Microbiology Letters*, 75, 57–102.

Wang, G., Bai, L., Wang, Z., Shi, T., Chen, T. & Zhao, X. (2014) Enhancement of riboflavin production by deregulating gluconeogenesis in *Bacillus subtilis*. *World Journal of Microbiology and Biotechnology*, 30, 1893–1900.

Warth, A.D., Scientific, C. & Ryde, N. (1978) Relationship between the heat resistance of spores and the optimum and maximum growth temperatures of *bacillus* species. *Journal of Bacteriology*, 134, 699–705.

Wendisch, V.F., Brito, L.F., Gil Lopez, M., Hennig, G., Pfeifenschneider, J., Sgobba, E. et al. (2016) The flexible feedstock concept in industrial biotechnology: metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, *pseudomonas*, *bacillus* and yeast strains for access to alternative carbon sources. *Journal of Biotechnology*, 234, 139–157.

Whitaker, W.B., Sandoval, N.R., Bennett, R.K., Fast, A.G. & Papoutsakis, E.T. (2015) Synthetic methylotrophy: engineering the production of biofuels and chemicals based on the biology of aerobic methanol utilization. *Current Opinion in Biotechnology*, 33, 165–175.

Yang, B., Sun, Y., Fu, S., Xia, M., Su, Y. & Liu, C. (2021) Improving the production of riboflavin by introducing a mutant ribulose 5-phosphate 3-epimerase gene in *Bacillus subtilis*. *Frontiers in Bioengineering and Biotechnology*, 9, 1–12.

Yang, Z., Sun, Q., Tan, G., Zhang, Q., Wang, Z., Li, C. et al. (2021) Engineering thermophilic *Geobacillus thermoglucosidasius* for riboflavin production. *Microbial Biotechnology*, 14, 363–373.

Yurimoto, H., Kato, N. & Sakai, Y. (2005) Assimilation, dissimilation and detoxification of formaldehyde, a central metabolic intermediate of methylotrophic metabolism. *Chemical Record*, 5, 367–375.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Klein, V.J., Brito, L.F., Perez-Garcia, F., Brautaset, T. & Irla, M. (2023) Metabolic engineering of thermophilic *Bacillus methanolicus* for riboflavin overproduction from methanol. *Microbial Biotechnology*, 00, 1–16. Available from: https://doi.org/10.1111/1751-7915.14239