SYNTHETIC BIOLOGY AS AN APPROACH TO ENGINEER MICROBIAL CHASSIS FOR THE BIOSYNTHESIS OF VITAMIN B12

Milca R. da C. R. Lins[1*], Gabriel G. Leal[1], Luan C. Carvalho[1] & Yuri B. Caetano[1]

[1]Center for Natural and Human Sciences, the Federal University of ABC, Santo André, Brazil.
** Corresponding author's email address: milca.lins@ufabc.edu.br*

ABSTRACT

The bioprocess for vitamin B12 or cobalamin production relies primarily on microorganisms due to the impossibility of chemical synthesis for industry, involving over 70 reactions. Two major strains, Propionibacterium shermanii and *Pseudomonas denitrificans*, carry out industrial production, reaching a maximum yield of 300 $mg.L^{-1}$. Researchers are leveraging modern molecular biology techniques and synthetic gene circuits to engineer robust microbial platforms capable of producing higher quantities of vitamin B12 sustainably. Synthetic Biology, alongside Biotechnology, offers a novel approach to enhance the production of various metabolites through microbial engineering. Thus, this literature review underscores Synthetic Biology's potential in optimizing the metabolic engineering of strains to produce this compound. Highlighted are innovative strategies, including disrupting the riboswitch regulation mechanism and employing heterologous gene expression through synthetic operon design, to enhance VB12 yields in engineered bacterial strains like *Escherichia coli* and *Bacillus megaterium*. Despite challenges in achieving optimal production levels, advancements in Synthetic Biology hold promise for refining microbial factories and have the potential to revolutionize industrial bioproduction.

Keywords: Cobalamin. Industrial Workhorse. Metabolic Engineering.

1 INTRODUCTION

Biotechnology's premise is to replace unsustainable procedures with sustainable ones, through biological resources adoption and microorganisms, which are designed as chassis for the production of various value-added bioproducts. Within modern biotechnology, Synthetic Biology rose in the early 2000s because of the need to combine foundations from biology, chemistry, computer science, and engineering to reprogram cells, and it has been changing our ability to manipulate and modify living systems rationally.[1,2]

Furthermore, understanding how natural biochemical networks work facilitates the modification of these networks to achieve enhanced production of a bioproduct of interest, forming the foundation of the Metabolic Engineering field.[3] Synthetic Biology enhances the engineering of pathways in a chassis, which is the organism that hosts the synthetic genetic components and provides the cellular machinery and resources to execute the central dogma of molecular biology. Thus, microbial factories are rationally created to produce the desired protein or compound by modifying metabolic pathways.[4] Now, it is possible to apply synthetic gene circuits to insert various genes from different organisms into a microbial platform, redirecting the metabolic flux focused on the bioproduct of interest.[5]

As high-value-added products, vitamins are essential nutrients that play a fundamental role in the metabolism of eukaryotes. Vitamin B12 (VB12) or cobalamin, in particular, performs a key coenzyme role in the mitochondrial pathway for energy production, in the citric acid cycle, amino acid metabolism, methylation-mediated regulation, DNA synthesis, and more biological processes. However, humans and animals do not produce these metabolites, necessitating their acquisition through diet or feeding. VB12 is widely used as a dietary supplement, as an important feed additive, as medicine for treating hematologic and neurological disorders, etc. Some bacteria and archaea synthesize this vitamin through aerobic or anaerobic pathways. Thus, bacteria are commonly used to produce this vitamin at an industrial scale by biotechnological processes.[6,7]

Therefore, this review highlights studies that use the Synthetic Biology approach to increase the production of VB12 by rationally engineered bacteria. To this end, we will discuss the biosynthetic pathway of VB12 production by bacteria, the riboswitch mechanism that regulates genes related to biosynthesis, the microbial platforms that have been engineered to date, and the production levels achieved, as well as the genetic engineering strategies that authors have used to modify the cells.

2 MATERIAL & METHODS

This work represents a brief literature review of published manuscripts regarding the genetic modification of bacteria for vitamin B12 production. The method employed involved a comprehensive literature search and review on this topic on the PubMed platform of the National Center for Biotechnology Information (NCBI) as the primary search resource. The manuscripts chosen were published up to April 2024, and the search terms used included: 'Synthetic Biology', 'vitamin B12', 'bioproduction', 'cobalamin', 'riboswitch', and 'microbial factories' in various combinations. Key articles in the Synthetic Biology area, such as those published by Cameron (2014) and regarding the first synthetic gene circuits, were included.

3 THEORETICAL BACKGROUND

Synthetic Biology improves the efficiency with which biological systems can be designed *in silico*, and built and characterized *in vitro* and *in vivo*.[1] Thus, synthetic gene circuits have been built and employed in many applications, including medical purposes such as therapies, diagnostics,[8] and biosensing,[9] environmental applications such as biosensing,[10] and bioremediation, and the industrial production of value-added compounds.[11] The first synthetic genetic circuits were built and validated in the early 2000s, such as the Toggle Switch, which controls gene expression like a genetic switch with ON and OFF states.[12] Modern and efficient tools and techniques are being developed to achieve Synthetic Biology goals, such as genome engineering by CRISPR-Cas, DNA assembly, regulation of gene expression,[13] etc. Besides, the iterative Design-Build-Test-Learn cycle is applied to achieve success in the construction of these new biological systems.[14]

Synthetic Biology could also be an approach for improving VB12 production by creating a robust chassis. Currently, the bioprocess for VB12 production is primarily carried out using microorganisms, and its chemical synthesis is impractical for industry because it involves more than 70 reactions. Some bacteria can produce VB12 by two pathways: 1) The *de novo* pathway, which involves approximately 30 genes starting from glutamate and can operate under aerobic or anaerobic conditions; 2) The salvage pathway, involving approximately 12 genes and depending on transporters for importing a cobalamin analog.[15]

Vitamin B12 belongs to the cobalamin group and has the most complex structure compared to the other B vitamins (Figure 1). It is composed of a corrin core, where a cobalt ion is connected to a tetrapyrrole ring, and has a 5,6-dimethylbenzimidazole (DMB) in the alpha position. The upper axial ligand in the beta position designates the form of cobalamin, whether it is cyanocobalamin, hydroxocobalamin, methylcobalamin, or 5'-deoxyadenosylcobalamin.[7,16] VB12's complex structure could explain why many steps are required in its chemical synthesis.

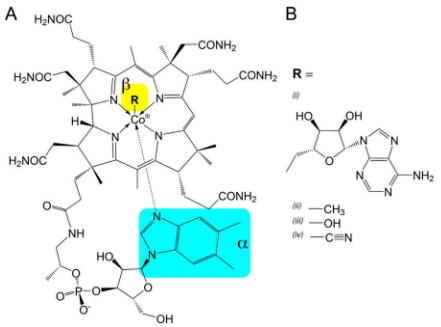

Figure 1 Representation of the vitamin B12 complex structure. A. The structure features a corrin ring, with the alpha-axial position in blue and the beta-axial position in yellow. The 5,6-dimethylbenzimidazole is orientated to the alpha-axial face, and there are variable beta-axial moieties (R) that determine the cobalamin form. B. (i) 5'-deoxyadenosylcobalamin, (ii) methylcobalamin, (iii) hydroxocobalamin, and (iv) cyanocobalamin (Lennon and Batey, 2022).

The most widely used industrial strains for producing VB12 are *Propionibacterium shermanii* and *Pseudomonas denitrificans*, with maximum yields reported to be approximately 300 $mg.L^{-1}$.[7] The genomes of these microorganisms have been genetically manipulated, along with improvements in culture media, supplements, and fermentation conditions. Table 1 provides a comprehensive list of strains engineered for the production of VB12, detailing the genetic modifications made to each strain and highlighting the resulting yield in vitamin B12 biosynthesis. In light of this, further improvements are desirable for the creation of a robust microbial platform that produces greater amounts of this value-added product.

Table 1 Overview of studies that employed bacterial strains for vitamin B12 (VB12) production.

Reference	Chassis	Yield of VB12 achieved	Strategy or innovation for VB12 production
17	*Propionibacterium freudenreichii*	1.7 $mg.L^{-1}$	Multigene expression was achieved by *hemA* from *Rhodobacter sphaeroides* and endogenous *hemB* and *cobA* genes.
18	*Pseudomonas denitrificans*	214 $mg.L^{-1}$	Cultivation of an industrial strain under glucose and betaine as feeding carbon sources. The pH of the fermentation broth was optimized.
19	*Bacillus megaterium*	0.220 $mg.L^{-1}$	cbi operon operates without the downregulation caused by the VB12 riboswitches. Addition of exogenous cobalt in fermentation.
20	*Escherichia coli*	307 $\mu g.g^{-1}$ of cell dry weight	Construction of six synthetic gene clusters with 28 genes from *Rhodobacter capsulatus*, *Brucella melitensis*, *Sinorhizobium meliloti* 32020, *Salmonella typhimurium*, and *Rhodopseudomonas palustris*. Optimization of fermentation conditions.

Despite the low yields, we focused on two manuscripts that used elegant strategies to engineer VB12-producing strains. Moore et al. (2014) aimed to disrupt the downregulation of a gene cluster caused by a riboswitch mechanism. Genes related to the VB12 pathway in *Bacillus megaterium* are controlled by riboswitches which are cis-regulatory RNA located in the 5'-untranslated region of the mRNA, regulating expression of the downstream genes within the transcript. Riboswitches regulate expression at the level of transcription elongation or translation initiation by their ability to bind intracellular metabolites (Figure 2). The engineering of the *de novo B. megaterium* pathway involved constructing the *cbi* operon without the riboswitch sequence. However, this strategy does not yet provide a high production yield of VB12, reaching 0.22 mg.L^{-1}. While the deletion of riboswitches might lead to a decrease in gene expression, it could employ the synthetic RNA strategy to disrupt the aptamer structure of the riboswitch, avoiding cell burden and allowing temporal control expression.[13]

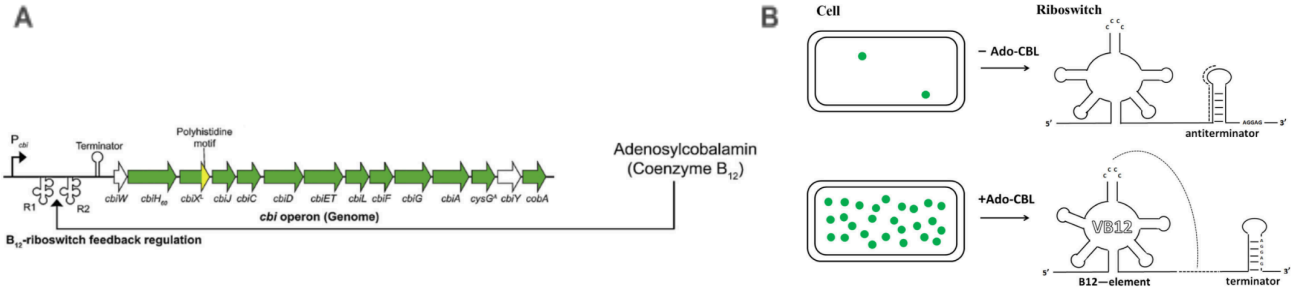

Figure 2 Representation of the adenosylcobalamin pathway in *Bacillus megaterium*. A. Two B12-riboswitches (R1 and R2), which are located upstream of the 14-gene *cbi* operon, modulate gene expression at the level of transcription elongation. B. Representation of the VB12 riboswitch mechanism. In cells containing a low concentration of VB12 (green circle), an antiterminator structure allows transcription of downstream genes within the mRNA. When VB12 reaches a certain threshold concentration and binds to the aptamer domain, a terminator hairpin is formed, attenuating transcription. (Adapted from Moore et al., 2014, and Cai et al., 2018).

Another innovative approach was the engineering of the *de novo* synthetic pathway in *Escherichia coli* strains, which can now produce VB12 at 307 µg.g^{-1} of cell dry weight.[20] The engineered *E. coli* produces vitamin B12 by heterologously expressing 28 genes from *Rhodobacter capsulatus*, *Brucella melitensis*, *Sinorhizobium meliloti* 32020, *Salmonella typhimurium*, and *Rhodopseudomonas palustris*. The genes are organized into six different modules of gene constructions and were inserted into the genome successfully. This strategy demonstrates how synthetic biology can be a useful approach for the construction of new metabolic pathways by building synthetic gene modules, with a greater number of genes and applying modern molecular biology techniques to engineer bacterial genomes. The bottleneck still lies in the VB12 production yield by these strains, but the proof-of-concept of these strategies might inspire the engineering of new robust chassis and non-model organisms.

4 CONCLUSION

The main microorganisms used for the industrial production of vitamin B12 do not yet reach the numbers desirable for industry, as demonstrated above. Thus, these recent innovations demonstrate the potential of Synthetic Biology to improve existing biotechnological processes and rationally engineer strains, boosting the development of microbial production systems. As the area continues to evolve, it holds promise for even more efficient and sustainable methods of producing essential compounds like vitamin B12.

REFERENCES

1 CHEN, Y. Y., GALLOWAY, K. E., SMOLKE, C. D. 2012. Genome. Biol. 13 (240).
2 CAMERON, D., BASHOR, C., COLLINS, J. 2014. Nat. Rev. Microbiol. 12. 381-390.
3 WU, Z., LIANG, X., LI, M., et al. 2023. Microb. Cell. Fact. 22 (76).
4 ADAMS, B. L. 2016. ACS Synth. Biol. 5. 1328-1330.
5 CHO, J. S., KIM, G. B., EUN, H., MOON, C. W., LEE, S. Y. 2022. JACS Au. 2 (8). 1781-1799.
6 CAI, Y., XIA, M., DONG, H., et al. 2018. BMC Biotechnol. 18 (27).
7 BALABANOVA, L., AVERIANOVA, L., MARCHENOK, M., SON, O., TEKUTYEVA, L. 2021. Int. J. Mol. Sci. 22 (4522).
8 YAN, X., LIU, X., ZHAO, C., et al. 2023. Sig. Transduct. Target. Ther. 8 (199).
9 McNERNEY, M. P., PIORINO, F., MICHEL, C. L., STYCZYNSKI, M. P. 2020. ACS Synth. Biol. 9 (2). 402-411.
10 JUNG, J. K., ALAM, K. K., VEROSLOFF, M. S. et al. 2020. Nat. Biotechnol. 38. 1451-1459.
11 LEZIA, A., MIANO, A., HASTY, J. 2021. Proc. IEEE. 110 (5). 613-630.
12 GARDNER, T., CANTOR, C., COLLINS, J. 2000. Nature. 403. 339-342.
13 LINS, M. R. D. C. R., AMORIM, L. A. D. S., et al. 2021. Metab. Eng. 68. 59-67.
14 MARUCCI, L., BARBERIS, M., et al., 2020. Front. Bioeng. Biotechnol. Sec. Synthetic Biology. 8 (942).
15 ACEVEDO-ROCHA, C. G., GRONENBERG, L. S., MACK, M., COMMICHAU, F. M., GENEE, H. J. 2019. Curr. Opin. Biotechnol. 56. 18-29.
16 LENON, S. R., BATEY, R. T. 2022. J. Mol. Biol. 434 (18). 167585.
17 PIAO, Y., YAMASHITA, M., et al. 2004. J. Biosci. Bioeng. 98 (3). 167–173.
18 LI, K. T., LIU, D.H., et al. 2008. Bioprocess Biosyst. Eng. 31. 605-610.
19 MOORE, S. J., et al. 2014. New Biotechnol. 31 (6). 553-561.
20 FANG, H., LI, D., KANG, J. et al. 2018. Nat. Commun. 9. 4917.

ACKNOWLEDGEMENTS

To the Center for Natural and Human Sciences, at the Federal University of ABC, UFABC, the Synthetic Biology Brazilian Network (Rede Brasileira de Biologia Sintética), and the I COBBIND organization commission.