CONSTRUCTION OF AN OPERON FOR 5-AMINOLEVULINIC ACID BIOSYNTHESIS IN BACTERIA

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ABSTRACT

Biosynthesis is distinguished by its sustainable production methods, utilization of raw materials from renewable sources, mild production conditions, and low waste generation that are typically non-toxic. 5-Aminolevulinic acid (5-ALA) stands as a notable example of a bioproduct obtainable through biosynthesis, due to its diverse range of commercial applications, such as medicine, animal feed, and agriculture. Therefore, the objective of this study was to construct a plasmid through genetic engineering for the overproduction of 5-ALA in a bacterial chassis. To accomplish this goal, a synthetic operon for 5-ALA biosynthesis was constructed and inserted into a plasmid, enabling the subsequent development of a strain capable of producing 5-ALA. In further development, the engineered strains will be cultivated to assess their 5-ALA production capabilities, followed by quantitative analysis. The results will then be compiled and analyzed to evaluate the potential of 5-ALA production by bacterial strains containing the plasmid with the synthetic operon.

Keywords: 5-Aminolevulinic acid. Synthetic Biology. Biosynthesis. DNA assembly.

1 INTRODUCTION

5-Aminolevulinic acid (5-ALA), a non-proteinogenic amino acid, is an important precursor of tetrapyrrolic compounds such as vitamin B_{12}, chlorophyll, and Heme. Its applications encompass photodynamic therapy for tumor treatment, supplementation in animal feed, herbicidal and insecticidal functions, as well as regulation of plant growth.[1] There are two natural biosynthetic pathways for 5-ALA, named C4 and C5. The C4 pathway, present in animals, yeasts, and non-sulfurous purple bacteria, involves the enzyme 5-aminolevulinate synthase (ALAS) catalyzing glycine and succinyl-CoA condensation into 5-ALA in the presence of the cofactor pyridoxal 5'-phosphate (PLP) [2]. In contrast to the C4 pathway, the C5 pathway comprises three main conversions catalyzed by different enzymes. Glutamate, the precursor of the C5 pathway, initially binds to a transfer RNA (tRNA) and forms the glutamyl-tRNA complex, through the activity of the enzyme glutamyl-tRNA synthetase (GluTS), encoded by the *gltX* gene. Next, the complex is reduced to glutamate-1-semialdehyde (GSA) by the activity of the NADPH-dependent glutamyl-tRNA reductase enzyme (GluTR), encoded by the *hemA* gene. The highly reactive intermediate GSA is rapidly converted to 5-ALA through the enzyme glutamate-1-semialdehyde aminotransferase (GSA-AM), encoded by the *hemL* gene. The C5 pathway is found in algae, bacteria, and higher plants.[3]

It is known that the C5 pathway may present some advantages when compared to the C4 pathway. The C4 pathway requires the external addition of glycine, which can increase costs and present challenges in maintaining optimal concentration levels. Excessive glycine concentration can be toxic to host cells and reduce the final yield of 5-ALA. In contrast, the C5 pathway uses glutamate as the precursor, whose titer can be increased through the redistribution of carbon flux from the tricarboxylic acid cycle towards α-oxoglutarate, which can then be converted to glutamate. [1]

To increase the titer of 5-ALA, some studies suggest the overexpression of *rhtA* gene, which encodes the serine/threonine transporter RhtA. [1-4] The broad substrate specificity of the RhtA transporter[2] and the similarity of the physical properties of 5-ALA with proteinogenic amino acids[1] enables the utilization of serine/threonine transporter RhtA to export 5-ALA to the extracellular medium. The advantage of exporting 5-ALA, as well as various target products, is related to the inhibition of target product synthesis caused by the high concentration of this very product [1]. Overexpression of *rhtA* in *E. coli* significantly increased the production of 5-ALA, as the strain expressing *rhtA, hemA,* and *hemL*, showed a 45.9% higher accumulation of 5-ALA than the strain that expressed only *hemA* and *hemL*.[3]

Considering the importance and valorization of 5-ALA and the sustainability added by the biological synthesis, we propose a new combination of genes, grouped in an operon, to engineer a bacterial chassis for biosynthesis of 5-ALA.

2 MATERIAL & METHODS

Considering the presented advantages of the C5 pathway over the C4 pathway, related to precursor supply and costs, the C5 pathway was chosen for the production of 5-ALA. Based on the direct correlation between the enzymes GluTR, GSA-AM, and RhtA and the efficient production of 5-ALA via the C5 pathway[1-4], a synthetic operon containing the genes *hemA, hemL, and rhtA*, in that order, was assembled. For the amplification of the parts by polymerase chain reaction (PCR), genomic DNA was initially extracted from different bacterial species to serve as template for the amplification of the genes.

Modified oligonucleotides were used in the PCR to add restriction sites, the promoter, ribosome binding sites, and a transcription terminator. The *hemA* gene was amplified with a forward primer containing promoter and ribosome binding site sequences. Additionally, a codon for arginine was inserted at position 3 of the amino-terminal end of *hemA*, since this modification was described to improve 5-ALA production by approximately 77% in *E. coli* [5]. The *hemL* and *rhtA* genes were amplified with modified forward primers for the insertion of the RBS in each gene. The reverse primer for *rhtA* had the transcription terminator sequence added. Furthermore, *Bsa*I restriction sites were added to both forward and reverse primers designed for *hemA, hemL, and rhtA*, as well as *Pst*I and *Xba*I sites were introduced into the forward primer for *hemA* and the reverse primer for *rhtA*, respectively.

After all the amplifications, the PCR products of the three genes were confirmed by electrophoresis. This was followed by the purification of each PCR product and the ligation through Golden Gate Assembly. The reaction containing all three parts was incubated with the enzymes *Bsa*I and T4 DNA ligase in cycles alternating temperatures of 37°C and 1f°C. The resulting product was amplified by PCR using the forward primers for *hemA* and the reverse for *rhtA*, and subsequently purified directly from the electrophoresis gel. At the end of the processes, the complete sequence of the amplified and purified *hemA-hemL-rhtA* operon was constructed (Figure 1).

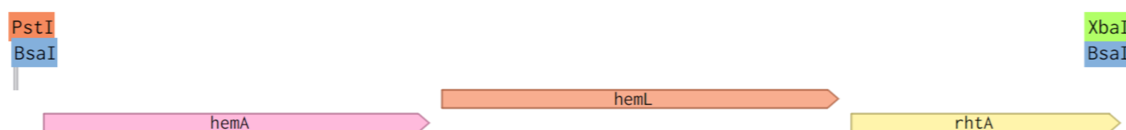

Figure 1 Complete *hemA-hemL-rhtA* operon built via Golden Gate Assembly.

The plasmid pCAT204, used as a vector, was PCR amplified using primers for the insertion of *Bsa*I sites. Following amplification of the plasmid, electrophoresis, purification, and digestion with *Dpn*I were carried out. After the insert and vector had been amplified, they were linked together by a second Golden Gate Assembly. The ligation product was then used for bacterial transformation.

Chemically competent *E. coli* Top10 was transformed by heat shock and the contents of the transformation were plated on LB medium supplemented with antibiotic. Six colonies from the plate were inoculated into test tubes for subsequent plasmid extraction. The extracted plasmids were then digested with *Pst*I, which has only one restriction site in the construct and generates a linearized product, and subjected to electrophoresis to verify the success of the construction.

3 RESULTS & DISCUSSION

The PCR products derived from *the hemA, hemL*, and *rhtA* genes, analyzed by electrophoresis, exhibited the anticipated correct bands on the gel. Confirmation was established by comparing the size of the bands obtained on the gel with the known sizes of the genes, which were 1,385 bp, 1,362 bp, and 1,005 bp for *hemA, hemL*, and *rhtA*, respectively. In the electrophoresis gel (Figure 2), the bands were close to 1.4 kb for both *hemA* (Figure 2A) and *hemL* (Figure 2B), and approximately 1 kb for *rhtA* (Figure 2C), aligning with the expected sizes.

Regarding the amplification of the plasmid, the target region that was amplified was 2,548 bp long. Therefore, the band between 2 and 3 kb shown on the gel in Figure 2D confirms the amplification of the vector.

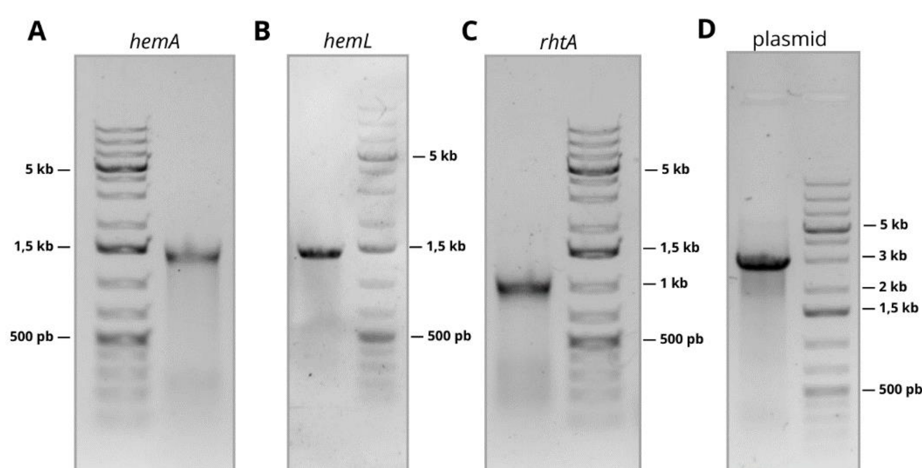

Figure 2 Electrophoresis results of PCR amplifications. **(A)** The *hemA* gene amplification exhibits a band close to 1.4 kb, consistent with the expected size of 1,385 bp. **(B)** Similarly, *hemL* amplification shows a band at 1.4 kb, aligning with its known size of 1,362 bp. **(C)** PCR of *rhtA* reveals a band at 1 kb, matching its expected size of 1,005 bp. **(D)** The band located between 2 and 3 kb confirms the presence of the amplified plasmid, which is 2,548 bp long.

After transforming *E. coli*, plasmids extracted from the 6 colonies underwent linearization via digestion with *Pst*I and were subsequently subjected to electrophoresis. The electrophoresis of the digested plasmids confirmed the successful construction of the plasmid in one of the 6 colonies examined. Specifically, the presence of a band measuring approximately 6 kb, as observed in sample 6 (Figure 3), demonstrated that this colony had been transformed with the plasmid carrying the *hemA-hemL-rhtA* operon

(6,188 bp). In contrast, the other samples (1-5) solely contained the plasmid devoid of the insert (2,548 bp). In samples 1-5, it is likely that the insert failed to properly bind to the vector, leading to the recircularization of the plasmid without the operon. Additionally, this reconnected plasmid remained undigested due to the absence of the *Pst*I site within the vector, which was instead present within the operon. Consequently, the electrophoresis results of samples 1-5 (Figure 3) showed bands representing varying degrees of folding of the recircularized plasmid lacking the insert (2,548 bp).

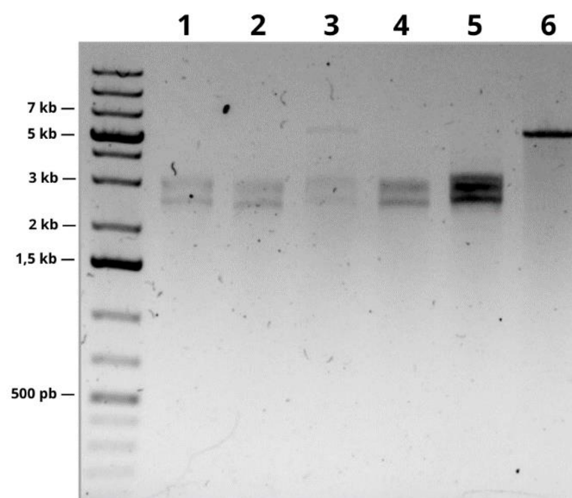

Figure 3 Electrophoretic analysis of the constructed plasmid. Wells numbered 1 through 6 represent samples of plasmids extracted and digested with *Pst*I from six colonies collected for screening. Colonies 1 to 5 did not contain the synthetic operon, whereas colony 6 was successfully transformed with the vector containing *hemA*-*hemL*-*rhtA* (6,188 bp).

4 CONCLUSION

The construction process of the plasmid for 5-aminolevulinic acid production in bacteria has proven to be successful. Each phase of the procedure, including the amplification of the *hemA, hemL*, and *rhtA* genes along with plasmid amplification, as well as the subsequent assembly of the plasmid containing the *hemA*-*hemL*-*rhtA* operon, worked out as expected. In the next steps, the functionality of the operon will be assessed through quantitative analysis to evaluate its efficacy in 5-aminolevulinic acid production. The successful validation of this plasmid holds significant promise for applications in biotechnology and metabolic engineering, offering potential advancements in 5-ALA production.

REFERENCES

[1] JIANG, M. et al. (2022) ‘Natural 5-Aminolevulinic Acid: Sources, Biosynthesis, Detection and Applications’, Frontiers in Bioengineering and Biotechnology. Frontiers Media S.A. Available at: https://doi.org/10.3389/fbioe.2022.841443.

[2] FENG, L. et al. (2015) ‘Metabolic engineering of Corynebacterium glutamicum for efficient production of 5-aminolevulinic acid; Metabolic engineering of Corynebacterium glutamicum for efficient production of 5-aminolevulinic acid’, Biotechnol. Bioeng, 113, pp. 1284–1293. Available at: https://doi.org/10.1002/bit.25886/abstract.

[3] KANG, Z. et al. (2011) ‘Engineering *Escherichia coli* for efficient production of 5-aminolevulinic acid from glucose’, Metabolic Engineering, 13(5), pp. 492–498. Available at: https://doi.org/10.1016/j.ymben.2011.05.003.

[4] LUO, Z. et al. (2022) ‘Synergistic Improvement of 5-Aminolevulinic Acid Production with Synthetic Scaffolds and System Pathway Engineering’, ACS Synthetic Biology, 11(8), pp. 2766–2778. Available at: https://doi.org/10.1021/acssynbio.2c00157.

[5] ZHANG, J. et al. (2017) ‘N-terminal engineering of glutamyl-tRNA reductase with positive charge arginine to increase 5-aminolevulinic acid biosynthesis’, Bioengineered, 8(4), pp. 424–427. Available at: https://doi.org/10.1080/21655979.2016.1230572.

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