

Engineering *Escherichia coli* for Carotenoid Biosynthesis

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Chapters 1 and 2 of this thesis comprise background information and literature reviews on isoprenoid production and membrane engineering. Chapter 1 and Chapter 2 were prepared solely by myself.

Chapter 3 is based on a manuscript to be submitted for publication, in which I am the first author. Figures 3.1, 3.3, and 3.4 were prepared by Eileen M. Bates, and Figure 3.2 was prepared by Dylan Perrera. The p10-aramgs plasmid was constructed by Andreea M. Palage. I conducted all the other work, including research investigation, data collection, and analysis for Figures 3.5 to 3.9. Dr. Valerie Ward initiated the project, provided guidance and supervision, and reviewed the manuscript. This project was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canada Foundation for Innovation (CFI).

Chapter 4 was prepared by myself, including research investigation, data collection and analysis. The leaky strain used in this chapter was prepared by Dr. Reza Roushan. Dr. Valerie Ward initiated the project, provided guidance and supervision.

Collaborative work with MASc student Rachel J. Blanchard from Dr. Tizazu Mekonnen's research group has already been published in 'Advanced Sustainable Systems', with the title "Modified Cellulose Nanocrystals Enabled Antimicrobial Polymeric Films," in which I am the third author having conducted the antimicrobial tests. Details are included in Appendix E.

Abstract

This research integrated the isopentenol utilization pathway (IUP) into the *E. coli* chromosome and utilized various promoters to optimize lycopene production. Additionally, the research evaluated the effects of overexpressing monoglycosyldiacylglycerol synthase (MGS) and diglucosyldiacylglycerol synthase (DGS) from *Acholeplasma laidlawii*, to induce the formation of intracellular membrane vesicles, potentially increasing the cell's capacity to store hydrophobic compounds like carotenoids. In a second strategy, the application of knock-out mutants for Braun's lipoprotein (*lpp*) in *E. coli* led to the production of extracellular membrane vesicles to avoid intracellular enzyme accumulations.

Results demonstrated that integrating the IUP in the chromosome significantly improved lycopene yields compared to traditional pathways and the plasmid system. The overexpression of MGS and DGS resulted in increased intracellular lipid content but did not significantly enhance carotenoid production beyond IUP-expressing strains. Notably, knocking out *lpp* yielded a 3.3-fold increase in extracellular lycopene production. Overexpressing MEP pathway enzymes, including IDI, GGPPS, CrtI, CrtB, DXS, IspD, IspF, IspG, and IspH in the leaky strain, further boosted lycopene yields.

This research underscores the potential of genetic and metabolic engineering to optimize isoprenoid production in microbial hosts, paving the way for more efficient and scalable production methods for these valuable compounds. The findings highlight the efficacy of the IUP and extracellular production strategies in overcoming traditional pathway limitations and enhancing yields, thereby contributing to the broader application of microbial biosynthesis in industrial and pharmaceutical contexts.

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Table of Contents

Author’s Declaration	ii
Statement of Contributions	iii
Abstract	iv
Acknowledgements	v
List of Figures	xi
List of Tables.....	xii
List of Abbreviations.....	xiii
Chapter 1 – Overview	1
1.1 Research Background.....	1
1.2 Research Motivation and Objectives.....	1
1.3 Thesis Outline	4
Chapter 2 – Literature Background.....	6
2.1 Different Hosts for Recombinant Protein Expression.....	6
2.2 Cultivation Conditions	7
2.3 Proteins in Different Pathways.....	8
2.4 Intracellular Vesicles.....	11
2.5 Extracellular Secretion	11
2.6 Protein Quantification	12

2.6.1 Cell disruption	13
2.6.2 Protein separation, concentration, and isolation	13
2.7 Carotenoid Extraction	16
2.8 Other Analytical Methods	17
2.8.1 Gas chromatography (GC)	18
2.8.2 High-performance liquid chromatography (HPLC)	18
Chapter 3 – Engineering Intracellular Production of Carotenoids in <i>Escherichia coli</i>	20
3.1 Abstract	20
3.2 Introduction	20
3.3 Materials and Methods	24
3.3.1 Strains and routine cultivation.....	24
3.3.2 Cloning	25
3.3.3 Chromosomal insertion	27
3.3.4 Western Blot.....	28
3.3.5 Production of isoprenoids.....	29
3.3.6 Carotenoid extraction	30
3.3.7 Total lipid content by direct transesterification to FAME	31
3.4 Results.....	32
3.4.1 Construction of IUP strains	32
3.4.2 Expression of membrane modulating enzymes.....	36

3.4.3 Effect of MGS and DGS on cell morphology	38
3.4.4 Lycopene production in MGS and DGS strains.....	39
3.4.5 Effect of MGS and DGS on β -carotene production	42
3.5 Discussion	43
3.6 Concluding Remarks.....	44
Chapter 4 – Engineering the Membrane for Extracellular Production of Carotenoids in <i>Escherichia coli</i>	45
4.1 Introduction.....	45
4.2 Materials and Methods	45
4.2.1 Strains and routine cultivation.....	45
4.2.2 Cloning.....	46
4.2.3 Preparation of electrocompetent cells	47
4.2.4 Electroporation	48
4.2.5 Production of isoprenoids.....	49
4.2.6 Carotenoid extraction	49
4.3 Results.....	50
4.3.1 Lycopene production in the leaky strain	50
4.4 Concluding Remarks.....	54
Chapter 5 – Conclusions and Future Recommendations	55
5.1 Conclusions.....	55

5.2 Future Recommendations	55
5.2.1 Short-term recommendations	55
5.2.2 Long-term recommendations	56
Letters of Copyright Permission	57
References	62
Appendices	77
Appendix A: Plasmid Maps and Sequence Maps	78
Appendix B: Equations for Data Analysis	93
Appendix C: Representative FAME Curves	94
Appendix D: Raw Data from Analysis of Microscopic Images	95

List of Figures

Figure 1.1 Extracellular production of lycopene. Created with BioRender.com.....	4
Figure 2.1 Lycopene synthesis pathways for prokaryotes and eukaryotes [38, 41].	10
Figure 3.1 Strategy for membrane engineering and overexpression of lycopene or β -carotene using the IUP pathway	23
Figure 3.2 Sequencing results of the promoter region of the knock-in IUP strains.....	32
Figure 3.3 A. Dry cell weight and B. lycopene production in plasmid and chromosomal IUP systems 24h after induction with 0.1 mM IPTG and 25 mM isoprenol	33
Figure 3.4 Characterizing IUP strains expressing the lycopene synthesis pathway	34
Figure 3.5 Western blot confirming expression of MGS and DGS in the membrane fraction of <i>E. coli</i>	35
Figure 3.6 Effect of MGS/DGS on DCW and lipid content of the cells.	36
Figure 3.7 Compound microscope images of <i>E. coli</i> without MGS expression (A) and <i>E. coli</i> with MGS expression (B)..	38
Figure 3.8 Effect of MGS or DGS expression on lycopene production in proD and trc strains, WT (NC), and the plasmid-based IUP expression (PC) system.	40
Figure 3.9 A. Dry cell weight and B. β -carotene concentration and C. β -carotene content of strains with and without MGS induction.	42
Figure 4.1 Comparison of wild-type strain and Δlpp leaky strain for dry cell weight (g/L).	50
Figure 4.2 Comparison of wild-type strain and Δlpp leaky strain for lycopene production.....	51
Figure 4.3 Comparison of wild-type strain and Δlpp leaky strain for total carotenoid content (mg/g).....	52

List of Tables

Table 2.1 Comparison of Common Protein Expression Systems.	7
Table 2.2 Main Techniques Used for Cell Disruption. Reproduced with permission from [49, 50]..	13
Table 2.3 Comparison of Different Protein Quantification Techniques.....	16
Table 2.4 Comparison of Different Lycopene Extraction Methods.	17
Table 2.5 Comparison of Different Analytical Settings for Measurement of Carotenoid Production.....	19
Table 3.1 List of <i>E. coli</i> Strains and Plasmids Used in This Work.....	24
Table 3.2 List of Primers Used in This Chapter	26
Table 3.3 Results from Cell Length and Width Analysis	38
Table 3.4 Summary of Effects of Chromosomal Insertion and MGS or DGS Expression on Specific Lycopene Content.	43
Table 4.1 List of <i>E. coli</i> Strains and Plasmids Used in This Work.....	45
Table 4.2 List of Primers Used in This Chapter	46

List of Abbreviations

ADP – Adenosine Diphosphate

alDGS – Diglucosyldiacylglycerol Synthase

alMGS – Monoglucosyldiacylglycerol Synthase

Amp – Ampicillin

Ara – Arabinose

ATP – Adenosine Triphosphate

CDP-ME – 4-Diphosphocytidyl-2-C-methylerythritol

CDP-MEP – 4-Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate

CK – Choline Kinase

Cm – Chloramphenicol

CrtB – Phytoene Synthase

CrtE – Geranylgeranyl Pyrophosphate Synthase

CrtI – Phytoene Desaturase

DCW – Dry Cell Weight

DMAPP – Dimethylallyl Pyrophosphate

DXP – 1-Deoxy-D-xylulose 5-phosphate

DXS – 1-Deoxy-D-xylulose 5-phosphate Synthase

ELISA – Enzyme-Linked Immunosorbent Assay

FAME – Fatty Acid Methyl Ester

FPP – Farnesyl Pyrophosphate

G3P – Glyceraldehyde 3-phosphate

GC-FID – Gas Chromatography with Flame Ionizing Detector

GlcDAG – α -Glucosyl-Diacylglycerol

GPP – Geranyl Pyrophosphate

GGPP – Geranylgeranyl Pyrophosphate

GGPPS – GGPP Synthase

HMBPP – 4-Hydroxy-3-methyl-butenyl 1-diphosphate

HPLC – High-Performance Liquid Chromatography

IDI – Isopentenyl-Pyrophosphate Delta Isomerase

IPK – Isopentenyl Phosphate Kinase

IPP – Isopentenyl Pyrophosphate

IPTG – Isopropyl β -D-1-Thiogalactopyranoside

IspA – FPP Synthase

IspD – 2-C-Methyl-D-erythritol 4-phosphate cytidyl transferase

IspF – 2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase

IspG – 2-C-Methyl-D-erythritol-2,4-cyclodiphosphate reductase

IspH – 4-Hydroxy-3-methyl-butenyl 1-diphosphate Reductase

IUP – Isopentenol Utilization Pathway

Kan – Kanamycin

LB – Luria Broth

MEC – 2C-Methyl-D-erythritol 2,4-diphosphate

MEP – 2-C-Methyl-D-Erythritol-4- Phosphate Pathway

MVA – Mevalonate Pathway

RT – Room Temperature

sd – Standard Deviation

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sec – General Secretary

SOC – Super Optimal Broth with Catabolite Repression

Spec – Spectinomycin

SRP – Signal Recognition Particle

Tat – Twin Arginine Translocation

WHO – World Health Organization

Chapter 1 – Overview

1.1 Research Background

Isoprenoids are a class of chemicals containing over 50,000 different compounds [1]. These natural products have a variety of industrial uses, such as flavorings, fragrances, and pesticides, as well as numerous medicinal applications [1]. The medicinal use of isoprenoids by ancient people can be traced back to 4,500 years ago [2]. Ancient Romans utilized plant oils and latex for certain treatments, the main effective components of which are now identified as diterpene derivatives [3]. Artemisinin, recently isolated from traditional Chinese herbal medicine by Chinese scientists, has demonstrated high effectiveness in malaria treatment [2]. Currently, the World Health Organization (WHO) estimates that 80% of the global population relies on traditional remedies for their primary healthcare [4]. However, these remedies are typically produced in small quantities in their native hosts and the chemical synthesis is not systematic. Biosynthesis in a microbial host like *E. coli* offers a promising route for synthesizing and producing these compounds [1].

1.2 Research Motivation and Objectives

Isoprenoids are synthesized from the five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [1]. In eukaryotes, these compounds are synthesized via the mevalonate pathway (MVA), while in prokaryotes, they are generated through the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) [5]. Both pathways pose challenges for engineering due to their reliance on multiple cofactors and oxygen-sensitive enzymes. Moreover, they are tightly regulated and compete with essential cellular pathways for precursors [1].

To address these challenges, the isopentenol utilization pathway (IUP) was developed [1]. This pathway employs two successive phosphorylation reactions, facilitated by a promiscuous choline kinase (*ck*) from *Saccharomyces cerevisiae*, and an isopentenyl phosphate kinase (*ipk*) from *Arabidopsis thaliana*, to convert isoprenol and/or prenol into IPP and DMAPP [1]. The IUP pathway enhances isoprenoid production by over 17-fold, decoupling isoprenoid synthesis from central carbon metabolism, and relies solely on ATP [1].

Carotenoids, being highly hydrophobic, can be stored in lipid droplets due to their fat-soluble nature [6, 7]. The study conducted by [8] demonstrates the possibility of enhancing membrane capacity through the expression of monoglycosyldiacylglycerol synthase (*mgs*) or diglycosyldiacylglycerol synthase (*dgs*) sourced from *Acholeplasma laidlawii*. In *E. coli*, the overproduction of either *mgs* or *dgs* leads to the accumulation of intracellular membrane vesicles [8]. The IUP was integrated into the chromosome of *E. coli* MG1655 (DE3) using different promoters of varying strengths, and was used in this study to compare the specific yield of lycopene. Additionally, the impact of *mgs* and *dgs* expression on lipid content on a dry basis was evaluated in the wild-type strain. Comparisons were made among the production of lycopene or β -carotene, in combination with *mgs* or *dgs*, across wild-type, plasmid-based IUP, and chromosomal IUP strains.

Negative feedback inhibition is a regulatory mechanism observed in both MEP and MVA pathways, wherein downstream intermediates exert inhibitory effects on upstream enzymes. For example, IPP inhibits 1-deoxy-D-xylulose 5-phosphate (DXP) synthase [9], while IPP, DMAPP, geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) inhibit mevalonate kinase [10]. This

feedback mechanism can constrain lycopene production, necessitating cell harvest and lysis for product recovery, thereby presenting challenges for continuous isoprenoid production.

The extracellular production of these compounds holds promise for streamlining purification processes and enabling continuous production. To address the limitations posed by feedback inhibition, a strategy leveraging the efflux behavior of methylerythritol cyclodiphosphate (MEC) in response to overexpression of specific pathway enzymes has been proposed [11]. This strategy involves tagging *ispG/H* enzymes with a transit peptide, particularly the twin-arginine translocation (Tat) signal peptide, to facilitate their transport via the Tat pathway, owing to the iron-sulfur protein nature and folded conformation of these enzymes.

Moreover, the generation of knock-out mutants targeting Braun's lipoprotein (*lpp*) using single-guide RNA (sgRNA) has been demonstrated to result in the production of extracellular membrane vesicles [12]. Transformation with a lycopene plasmid, along with plasmids encoding *ispG/H* with a transit peptide and *dxs-ispD/F*, is anticipated to yield enhanced lycopene production. Figure 1.1 demonstrates the proposed method for extracellular lycopene production.

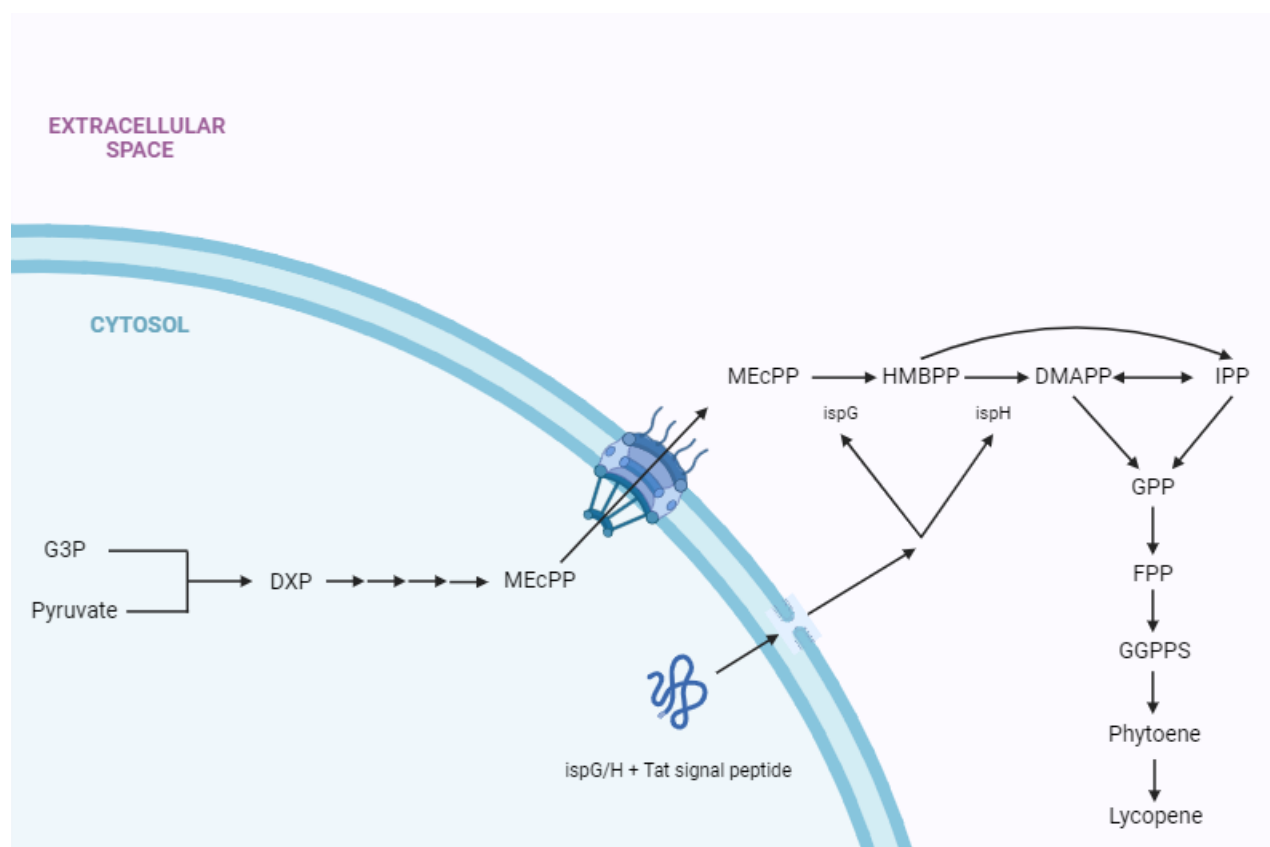


Figure 1.1 Extracellular production of lycopene. Created with BioRender.com.

1.3 Thesis Outline

The first chapter serves as an introductory section to this thesis, outlining the research background, motivation, and objectives. The second chapter provides a comprehensive overview of the relevant literature background. It begins with a discussion about different hosts used for recombinant protein expression and various cultivation conditions for *E. coli*. Subsequently, it delves into proteins involved in different pathways of lycopene production, both intracellular and extracellular secretion. Furthermore, the chapter discusses techniques and analytical tools for protein quantification, carotenoid extraction, and analysis of organic products. Chapter 3 is under preparation for publication to a refereed journal. This chapter explores the potential for

increasing the production yield of lycopene through engineered *Escherichia coli* by expressing monoglycosyldiacylglycerol synthase (MGS) or diglucosyldiacylglycerol synthase (DGS) and inserting the upstream isopentenol utilization pathway (IUP) into the chromosome. Chapter 4 explores a strategy to enhance lycopene production by employing a leaky strain, generated through a knock-out mutation targeting the lipoprotein to facilitate the extracellular secretion of key enzymes in the MEP pathway. Finally, Chapter 5 summarizes the concluding remarks and includes recommendations for future investigation. Section ‘Letters of Copyright Permission’ includes copyright licenses and related permissions for reproducing figures throughout this thesis.

In addition, six appendices (A to E) at the end of this thesis provide complementary information. Appendices A to D include plasmid maps and sequence maps, equations for data analysis, representative FAME curves and raw data. Appendix E contains detailed information about a paper already published, representing a side-project during this thesis.

Chapter 2 – Literature Background

2.1 Different Hosts for Recombinant Protein Expression

Different hosts for genetic recombinant protein expression have been commonly applied in various situations requiring prokaryotic and eukaryotic proteins due to pharmaceutical, medical, and industrial uses [13, 14]. Advantages and disadvantages of each host system are summarized in Table 2.1. *E. coli* has been chosen for carotenoid-related protein expression for several reasons, including cost-effectiveness, rapid growth rate for reducing research cycle, and ease of genetic manipulation, despite some limitations of the bacterial system [15, 16, 17, 18]. These reasons also explain why bacterial hosts are commonly used for genetic modification and protein expression in scientific research. However, handling bacterial hosts, such as *Salmonella*, requires more restrictive regulations due to endotoxins and exotoxins they produce [19]. Additionally, the lack of post-transcriptional and post-translational modifications and the possibility of inclusion body formation can potentially lead to protein misfolding, decreasing protein expression efficiency [20, 21, 22]. Based on the comparison in Table 2.1, yeast can be another suitable option for protein production. Besides the advantages mentioned above for *E. coli*, which can also be applied to yeast, yeast poses relatively lower risks and fewer safety concerns to public [20, 23, 24]. Microalgae and mammalian cells are considered reasonable candidates for protein production requiring glycosylation or other post-translational modifications, but these can be time-consuming and costly [20, 21, 22, 24, 25].

Table 2.1 Comparison of Common Protein Expression Systems.

Characteristic	Bacteria	Yeast	Microalgae	Mammalian cells	Ref.
Cost	Low	Low	Low	High	[20, 24]
Growth Rate	Fast	Fast	Fast	Slow	[20, 24]
Glycosylation	None	Incorrect	Correct	Correct	[20, 24]
Genetic Manipulation	Easy	Easy	Difficult	Difficult	[23, 24]
Safety	Low	High	High	Medium	[20, 24]
Secretion	Low	Medium	High	High	[25]
Protein Productivity	Medium	High	Low	Medium to high	[20, 24]
Inclusion Bodies	Present	Present	Absent	Absent	[21]

2.2 Cultivation Conditions

Different cultivation conditions, namely, osmolarity, pH, temperature, aeration rate, and medium composition, have notable effects on *E. coli* growth rate, cell morphology, gene expression, and protein production [26, 27, 28]. Research by [28] indicates that *E. coli* can thrive across a pressure spectrum of 1-400 atm, owing to the relatively low sensitivity of cells to shear stress, with a temperature range of 23-40 °C. Interestingly, the average cell length experiences a marked increase at pressures between 250 and 400 atm [24]. Typically, *E. coli* grows optimally at 37°C under aerobic conditions with a pH of 7.0 [29] and an osmotic pressure of 0.3 OsM [30]. However, certain plasmids, such as the plasmid pAC-BETAipi used in this study, requires lower temperatures for expression, as exemplified by its activity at 30°C [31]. Additionally, proteins like IspG/H, mentioned in Section 2.2, are iron-sulfur proteins sensitive to oxygen [32], potentially performing better under anaerobic conditions.

Noor et al. (2013) examined the correlation between temperature and various culture media on *E. coli* growth [33]. Notably, at a temperature of 45°C, only sluggish growth was

evident on nutrient agar, while no observable growth occurred on LB agar or M9 agar. Conversely, at 37°C, rapid cellular proliferation was observed across all three media types. Moreover, at lower temperatures such as 30°C or 25°C, moderate growth was observed on nutrient agar and LB agar, whereas growth on M9 agar remained slow. These observations underscore the joint influence of temperature and nutrient composition on optimizing cellular growth dynamics.

2.3 Isoprenoid Biosynthesis Pathways

In the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (IspD), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), and isopentenyl-pyrophosphate delta isomerase (IDI) are recognized as rate-limiting enzymes [34, 35]. Thus, overexpressing genes encoding these four enzymes with strong promoters holds promise for enhancing the MEP pathway and increasing isoprenoid production, particularly lycopene as investigated in this thesis. The initial stage of the MEP pathway involves condensing two types of three-carbon compounds, pyruvate and glyceraldehyde 3-phosphate (G3P), into 1-deoxy-D-xylulose 5-phosphate (DXP) via enzyme IDI. IspD and IspF facilitate the formation of 4-diphosphocytidyl-2C-methyl D-erythritol (CDP-ME) from 2C-methyl-D-erythritol 4-phosphate (MEP) and the conversion of 4-diphosphocytidyl-2C-methyl D-erythritol 2-phosphate (CDP-MEP) into 2C-methyl-D-erythritol 2,4-diphosphate (MEC), respectively [36]. IDI mediates the interconversion between isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which serve as precursors for synthesizing various isoprenoids. In the MVA pathway, typically found in eukaryotic organisms, DMAPP is generated solely through the isomerization of IPP by the enzyme IDI [2]. However, in the MEP

pathway, the final step is catalyzed by 4-Hydroxy-3-methyl-butenyl 1-diphosphate reductase (IspH), yielding both IPP and DMAPP. Nonetheless, while IDI is non-essential for organisms utilizing the MEP pathway, it plays a role in maintaining the balance between IPP and DMAPP to fulfill cellular demands across diverse conditions [37].

Two additional critical enzymes involved in the formation of DMAPP and IPP are 2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase (IspG) and IspH [38]. IspG, existing as a homodimer, is an iron-sulfur cluster protein that facilitates a reductive dehydration reaction, converting MEC to 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMBPP), which is then transformed into DMAPP and IPP by the enzyme IspH, another iron-sulfur protein [37]. Like most iron-sulfur proteins, both IspG and IspH are sensitive to oxygen due to decreased availability of ferric iron and direct oxidation of sulfur trafficking intermediates, which can disrupt their structure [32, 39]. Employing a combination of approaches, such as co-expression of IspG with the *isc* operon and utilization of different reduction systems, has led to a significant improvement in IspG activity by several orders of magnitude [37]. Likewise, optimization of iron-sulfur cluster maturation and reduction systems has the potential to further enhance IspH activity by several orders of magnitude [37].

In accordance with the research conducted by [1], it is suggested that CrtE (GGPP synthase), CrtB (phytoene synthase), or CrtI (phytoene desaturase) may act as limiting factors in lycopene biosynthesis. This inference arises from the observed accumulation of the upstream compound IPP, despite the presence of a higher copy of the enzyme production plasmid. Consequently, the researchers illustrate that substituting CrtE with GGPPS sourced from *T. canadensis* can potentially enhance lycopene yield with a sufficient supply of glucose. The flow chart depicting both the MEP and MVA pathways is demonstrated in Figure 2.1.

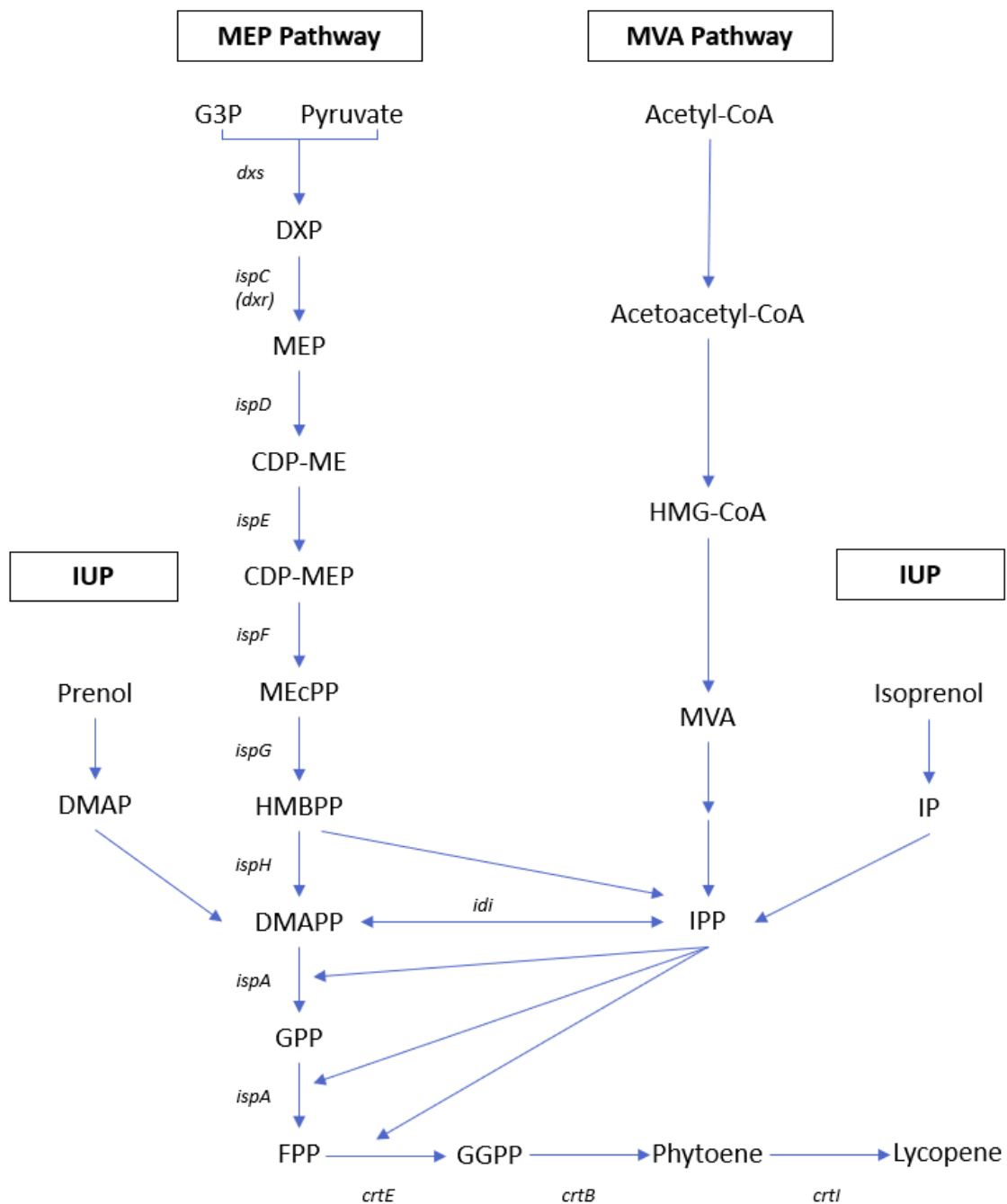


Figure 2.1 Lycopene synthesis pathways for prokaryotes and eukaryotes [37, 40].

2.4 Lycopene Localization

Researchers have discovered that the hydrophobicity of carotenoids enables their storage and accumulation within the lipid bilayer of *E. coli* cell membranes [6, 7, 41]. Overexpressing a foreign lipid glycosyltransferase, derived from *Acholeplasma laidlawii*, has been found to generate a substantial amount of cytoplasmic membrane vesicles, thereby creating more space for carotenoid storage. Notably, typical glycosyltransferases studied by [8] include monoglucosyldiacylglycerol synthase (alMGS) for synthesizing the nonbilayer glucolipid α -glucosyl-diacylglycerol (GlcDAG), and diglucosyldiacylglycerol synthase (alDGS), which shares similar characteristics. Subsequent studies by [42] have demonstrated that overexpressing the *almgs* gene in engineered *E. coli* enhances the production of β -carotene and lycopene. However, to date, no research has investigated the correlation between overexpression of the *aldgs* gene and lipid content, subsequently elevating carotenoid yield.

2.5 Extracellular Secretion

The secretory production of recombinant proteins targeting the periplasm and extracellular space has a positive impact on isoprenoid production [43]. However, under standard laboratory conditions, wild-type *E. coli* generally does not naturally secrete extracellular proteins [44]. Cytoplasmic proteins necessitate genetic modifications involving specialized pathways or membrane alterations to facilitate localization to the extracellular space. For prokaryotes, two primary protein secretion pathways transport proteins from the cytoplasm to the periplasm: the general secretory (Sec) pathway and the twin arginine translocation (Tat) pathway [45]. The Sec pathway comprises two sub-transport systems for transporting unfolded proteins with a Sec

signal peptide. The first system transports pre-translational proteins with a signal peptide recognized at the translating ribosome by the ribosome-bound signal recognition particle (SRP), and then guided via the SRP receptor to the Sec translocon. The second system facilitates the transport of post-translational unfolded proteins, which are then guided toward the Sec translocon by the ATPase, SecA. The Tat pathway transports folded proteins containing a Tat signal peptide without requiring the facilitation of guiding proteins.

For protein transport from the periplasm to the extracellular space, the primary strategy involves disrupting cellular structural components, including the peptidoglycan cell wall or the cross-linking between the cell wall and the outer membrane. According to research conducted by [46], this can be achieved by removing genes such as *lpp/pal*, which encode lipoprotein responsible for the covalent cross-linkage between the outer membrane and the peptidoglycan; or *mrcA/B*, which encodes peptidoglycan synthetase. Another study, conducted by [47], revealed that exporting recombinant proteins can be achieved by extracellular membrane-bound vesicles through the expression of full-length human α -synuclein (α Syn) in *E. coli*. The primary advantage of this method is the isolation from cell culture and long-term storage, especially for insoluble, toxic, or disulfide-bond-containing proteins.

2.6 Protein Quantification

Protein quantification process requires several steps, including cell disruption, separation of soluble and insoluble proteins, concentration of proteins, isolation of the protein fractions, protein purification, and protein quantification [48]. This section discusses each step and the commonly used methods for them.

2.6.1 Cell disruption

Cell disruption can be categorized into mechanical approaches and non-mechanical approaches. Mechanical approaches include high-pressure homogenization, bead mill, grinding, and ultrasonication. Non-mechanical approaches encompass physical methods such as osmotic shock, chemical methods including detergents or pH extremes, and biological methods including cell lytic enzymes [49]. These methods are summarized in Table 2.2. In typical biological laboratories, mechanical methods such as high-pressure homogenizers or bead mills are frequently employed, while non-mechanical methods like the use of sodium hydroxide are favored due to their low cost and suitability for large-scale production [48].

Table 2.2 Main Techniques Used for Cell Disruption. Reproduced with permission from [49, 50].

Cell Disruption	Mechanical	Homogenization, bead mill, grinding, ultrasonication, pressure cell	
	Non-mechanical	Physical	Osmotic shock, temperature extremes, gas decompression
		Chemical	pH extremes, detergents, antibiotics, solvents
		Biological	Cell lytic enzymes, autolysis, phage, wall inhibitors

2.6.2 Protein separation, concentration, and isolation

Proteins can be separated based on solubility in different solvents [51]. Centrifugation is widely employed to separate the protein-rich aqueous phase from the solid phase. However, parameters set for separating cellular proteins by centrifugation, such as high acceleration and low temperatures, might hinder large-scale separation [48, 52]. Concentrating proteins is sometimes

an optional step, depending on biomass concentration. Ultrafiltration is extensively used for protein concentration and isolation, based on the different molecular weight cutoff of the membrane, which retains desired proteins while allowing impurities to pass through the membrane [53]. Other methods like precipitation and spray-drying are also commonly used to concentrate proteins [48].

2.6.3 Purification

The primary technique used for protein purification is chromatography, which offers several options such as ion-exchange chromatography, reversed-phase chromatography, hydrophobic interaction chromatography, affinity chromatography, and size exclusion chromatography [53, 54].

Ion exchange chromatography stands out as a widely utilized method in protein purification. Its principle is the reversible binding of charged molecules to a stationary phase with oppositely charged functional groups [55]. This technique showcases notable advantages, including high resolution, facilitating the efficient separation of closely related molecules based on their charge disparities. Additionally, ion exchange chromatography demonstrates a remarkable scalability suitable for large-scale operations [56]. However, one notable concern is the possibility of non-specific binding of certain molecules to the resin, which can lead to reduced yields and decreased purity [55]. Nonetheless, this challenge can be effectively mitigated through complementary utilization alongside affinity chromatography and ultrafiltration techniques. Affinity chromatography, based on specific binding interactions between an immobilized ligand and its binding partner, further purifies proteins with high specificity, selectivity, and fast purification, albeit with potential cost implications due to resin expenses [57].

2.6.4 Quantification

Protein quantification is a fundamental assessment of protein concentration and purity. This section reviews four commonly used techniques for protein quantification: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blot, Enzyme-Linked Immunosorbent Assay (ELISA), and UV Absorbance.

SDS-PAGE is a widely employed method for protein separation based on molecular weight through a polyacrylamide gel under an electric field [53, 58]. This principle exploits the differences in surface charge of proteins, causing smaller proteins to migrate faster than larger ones, resulting in effective separation.

Western Blotting involves the transfer of separated proteins onto a hydrophobic membrane followed by probing with specific antibodies [53]. The working principle relies on the binding of antibodies to target proteins immobilized on the membrane. Visualization of antibody-bound proteins is achieved through colorimetric or chemiluminescent detection methods, allowing for specific and sensitive detection of target proteins [59, 60].

There are several types of ELISA, including direct, indirect, sandwich, and competitive ELISA [53, 61, 62, 63]. The working principle involves the specific binding of detection antibodies to the target protein, followed by the addition of enzyme substrate to produce a detectable signal proportional to the amount of bound target protein.

UV Absorbance for Protein Quantification relies on the absorption of UV light by proteins at 280 nm due to the presence of aromatic side chains [53, 64]. The working principle involves measuring the absorbance of UV light by protein samples using a spectrophotometer. Protein concentration is determined based on the Beer-Lambert law, correlating absorbance to

protein concentration using a standard curve. Table 2.3 compares the advantages and disadvantages of the four protein quantification techniques discussed above.

Table 2.3 Comparison of Different Protein Quantification Techniques.

Technique	Advantages	Disadvantages	Ref.
SDS-PAGE	Simple, fast, high sensitivity, minimal sample loss, excellent protein separation, versatile applicability	Limited dynamic range, less sensitive Coomassie staining	[53, 58]
Western Blot	High sensitivity, quantitative analysis, specificity	Requires specific antibodies, time-consuming, high cost, labor-intensive	[53, 59, 60]
ELISA	High sensitivity, specificity, quantitative measurement, high-throughput capability	Requires specific antibodies, variability in results due to antibody binding affinity	[53, 61, 62, 63]
UV Absorbance	Simple, fast, versatile applicability	Low accuracy, lack of specificity, susceptible to interference, necessitates standard curve calibration	[53, 64, 65]

2.7 Carotenoid Extraction

Lycopene, a carotenoid compound, has been the subject of extensive scientific inquiry, with researchers investigating various laboratory extraction methodologies, such as super critical fluid extraction and solvent extraction, among others, for subsequent analytical assessment [66]. The predominant technique employed in laboratory settings is solvent extraction, colloquially known as liquid-liquid extraction, which exhibits variability based on the selection of lycopene sources and quantities, organic solvent compositions and proportions, solvent volumes, temperatures, and extraction durations across different investigative studies. Notably, [67] and [68] conducted

thorough comparative analyses of disparate extraction conditions, culminating in the identification of optimal factor settings conducive to enhanced lycopene yields. A comprehensive overview of these diverse solvent extraction approaches is presented in Table 2.4.

Table 2.4 Comparison of Different Lycopene Extraction Methods.

Author	Emenhiser et al. 1995 [69]	Xu et al. 2013 [67] *	Poojary et al. 2015 [68] *
Lycopene resource	Homogenized grapefruit	Red grapefruit	Tomato
Sample volume	12 g	10 g	1 g
Solvent ratio	Hexane, ethanol, and acetone (50:25:25) with 15 ml water	Petroleum ether, acetone, and 95% ethanol (2:1:1, v/v/v) with 2% (w/v) dichloromethane and 0.05% (w/t) butylated hydroxytoluene	Acetone and n-hexane (1:3)
Solvent volume	100 ml	30 ml	40 ml
Temperature	Room temperature (RT)	30 °C	20 °C
Extraction time	5 min	30 min	40 min

* The most optimal methods are reported

2.8 Other Analytical Methods

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are two powerful analytical techniques used for compound analysis [70, 71], typically for fatty acids methyl esters (FAME) [72, 73, 74] and carotenoid [75, 76] in this study, respectively. This section briefly discusses these analytical tools.

2.8.1 Gas chromatography (GC)

Gas chromatography (GC) is a highly effective analytical technique utilized primarily for the separation and quantification of volatile organic compounds [53, 77]. The fundamental principle of GC lies in the differential partitioning of compounds between a stationary phase and a gaseous mobile phase as they flow through a chromatographic column [78]. In this process, the gaseous mobile phase, comprising a carrier gas along with air and fuel, facilitates the movement of analytes through the column. Various detectors are employed in GC systems to detect and quantify separated compounds accurately. Among the detectors commonly used are mass spectrometry (MS), electron capture detector (ECD), flame ionization detection (FID), and thermal conductivity detection (TCD) [53, 78]. Of these, FID has been particularly effective for the quantification of fatty acids methyl esters (FAME) by GC, demonstrating its utility and reliability in compound analysis [79, 80, 81].

2.8.2 High-performance liquid chromatography (HPLC)

HPLC is a frequently used method for determining purity. Similar to GC, HPLC separates compounds based on their differential interaction with a stationary phase and a mobile phase flowing through a column [53, 82, 83]. Various analytical settings outlined in research papers for the measurement of carotenoid production are compared and summarized in Table 2.5.

Table 2.5 Comparison of Different Analytical Settings for Measurement of Carotenoid Production.

Author	Pupin et al. 1999 [84]	Ma et al. 2022 [85]	Gong et al. 2020 [86]
Column	C18 (250×4.6 mm, 5 µm)	C18 (250×4.6 mm, 5 µm)	C18 (250×4.6 mm, 5 µm)
Temperature	22 °C	40 °C	30 °C
Wavelength	450 nm	450 nm	476 nm
Mobile Phase	Acetonitrile: methanol: 1,2 dichlorethane (60:35:5) added with 0.1% BHT, 0.1% TEA, 0.05 M ammonium acetate in methanol	Acetonitrile: methanol: isopropanol (5:3:2)	A (methanol: acetonitrile: dichloromethane, 21:21:8) and B (methanol/water, 1:9)
Flow Rate	1 ml/min	1 ml/min	0.8 ml/min
Gradient	For the entire spectral range	For the entire spectral range	0 to 18 min (80–100% A and 20–0% B), 18–40 min (100% A and 0% B), 40–45 min (100–80% A and 0–20% B), and 45–50 min (80% A and 20% B)

Chapter 3 – Engineering Intracellular Production of Carotenoids in *Escherichia coli*

3.1 Abstract

Carotenoids are a class of highly hydrophobic compounds synthesized by plants in limited quantities. This chapter explores the potential for increasing the production yield of lycopene, a typical carotenoid compound, through engineered *Escherichia coli*. Given that lycopene biosynthesis occurs within microbial hosts and is subsequently stored within lipid membranes, our study focuses on impact of inducing membrane vesicles on lycopene yield by expressing monoglycosyldiacylglycerol synthase (MGS) or diglycosyldiacylglycerol synthase (DGS) from *Acholeplasma laidlawii* and inserting the upstream isopentenol utilization pathway (IUP) into the chromosome. The effect of MGS and DGS on lipid production in the cell was quantified. Our results show that inserting the IUP pathway into the chromosome increased the specific lycopene yield by 2.1-fold compared to the plasmid-based system when using a proD constitutive promoter and 2.0-fold increase using the inducible trc promoter. Expression of MGS or DGS had no effect on lycopene content in the control strains and resulted in significantly decreased production in the strains expressing a chromosomal copy of the IUP pathway. The expression of MGS and DGS resulted in a small increase of 31% and 33% (w/w) in lipid content, respectively, and therefore did not increase the lipid storage capacity on a dry weight basis.

3.2 Introduction

Isoprenoids are a large and diverse category of natural compounds, consisting of more than 50,000 unique molecules produced across kingdoms [87]. These secondary metabolites are

naturally present in low concentrations, but have a variety of important industrial applications, such as pharmaceuticals, fragrances, flavours, cosmetics, pigments and pesticides [87]. Even with very efficient extraction and purification procedures, natural production cannot keep up with market demand [5]. Chemical synthesis methods have been proposed to ameliorate this, but these methods typically involve complex reactions that produce lower quality products and environmentally damaging waste [5]. Because of this, synthetic biology and metabolic engineering approaches are being developed to increase productivity.

All isoprenoids are synthesized by successive condensation of the five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [88]. These precursors are typically produced through either the eukaryotic mevalonate pathway (MVA) or the prokaryotic 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) [88]. Both pathways are difficult to engineer, due to their reliance on central carbon metabolism [1]. These pathways are highly regulated, compete with vital cellular processes for starting materials, require multiple cofactors, and use oxygen-sensitive enzymes [1].

The isopentenol utilization pathway (IUP) was invented to solve some of the challenges with precursor synthesis [1]. This pathway uses 2 successive phosphorylation reactions to convert isoprenol and/or prenol into IPP and DMAPP via a promiscuous choline kinase (CK) from *Saccharomyces cerevisiae*, followed by an isopentenyl phosphate kinase (IPK) from *Arabidopsis thaliana*. In addition, an isopentenyl-pyrophosphate-delta isomerase (IDI) is included to interconvert IPP and DMAPP, balancing their relative concentrations. This pathway increases isoprenoid production more than 17-fold, completely separates isoprenoid synthesis from central carbon metabolism, and only relies on a single cofactor, ATP [1].

Carotenoids are highly hydrophobic and they are stored in membranes during heterologous production [5]. This further complicates their synthesis, as overproduction reduces membrane fluidity, killing the cells, and placing an upper limit on yield [5]. One proposed solution is to increase the capacity of the membrane by remodelling by the expression of monoglycosyldiacylglycerol synthase (*mgs*) or diglucosyldiacyl-glycerolsynthase (*dgs*) from *Acholeplasma laidlawii* [8, 89]. In *E. coli*, overproduction of MGS or DGS fills the cytoplasm with membrane vesicles [8]. The exact mechanism of vesicle formation by these enzymes is not known, but MGS is thought to bind the inner leaflet of the membrane bilayer and change lipid packing density [8]. This difference in density between the two monolayers is thought to create bends in the membrane that are pinched off, forming a vesicle. MGS causes production and insertion of a foreign glucolipid into the membrane causing changes to overall lipid composition, but DGS did not result in accumulation of this exogenous glycolipid and was still capable of forming intracellular membrane vesicles [8].

Some attempts to engineering the membrane of *E. coli* have yield modest increases in lycopene and β -carotene production, although increasing the intracellular lipid contents in lipid bodies of oleaginous yeasts have resulted in more promising results than those in bacterial systems [90]. In previous works, *mgs* expression resulted in a modest 12% increase in lycopene production [43]. Overexpression of enzymes in the diglyceride-3-phosphate synthesis pathway (PlsB, PlsC, DgkA) in addition to the expression of *mgs* resulted in a further 13% increase in specific lycopene production [43]. In another work, the hydrophobic polymer, polyhydroxybutyrate (PHB) was produced in *E. coli* with the goal of creating a hydrophobic sink for lycopene storage, however, while lycopene was localized preferentially in the PHB bodies, there was no reported increase in lycopene production [91].

In this study, the IUP pathway was inserted into the chromosome of *E. coli* MG1655 (DE3) with different promoters varying in strength and the specific yield of lycopene was compared (Figure 3.1). The effect of *mgs* and *dgs* expression on the lipid content on a dry basis was also assessed in the wild-type strain. Production of lycopene or β -carotene along with *mgs* or *dgs* in wild-type, plasmid-based IUP and chromosomal IUP strains was compared.

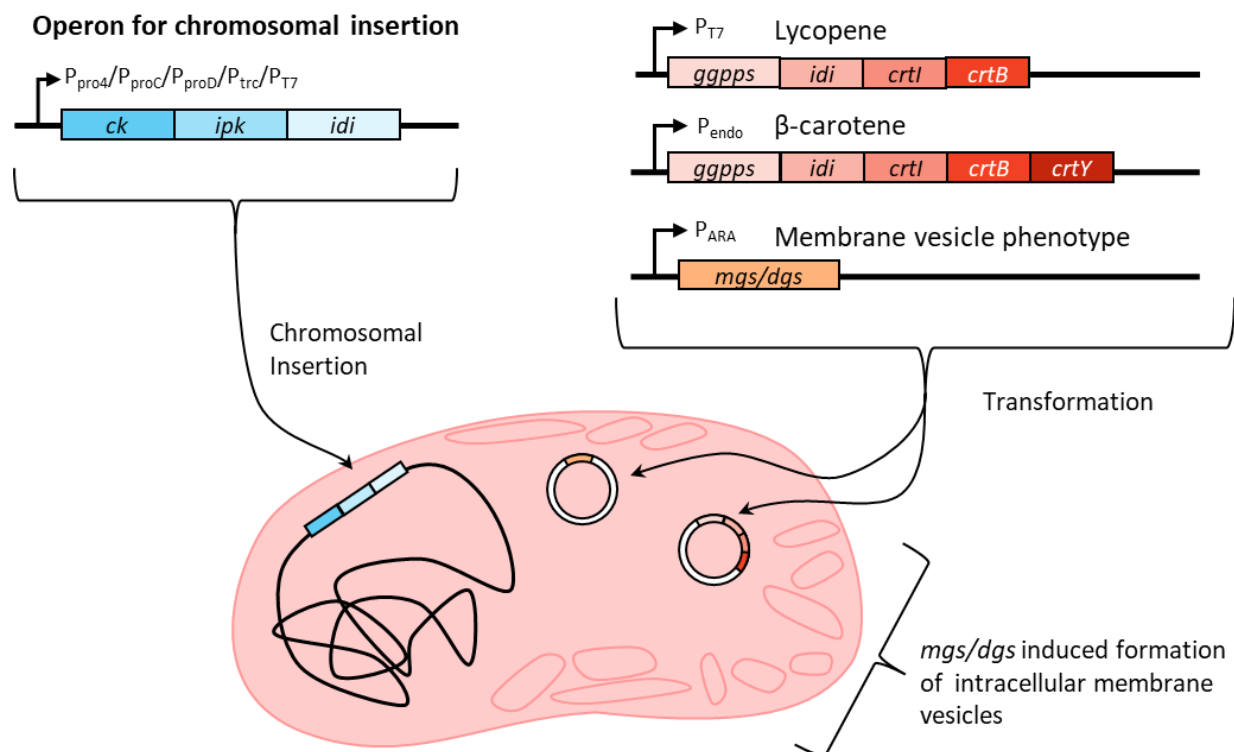


Figure 3.1 Strategy for membrane engineering and overexpression of lycopene or β -carotene using the IUP pathway. The IUP pathway was inserted into the chromosome near the arabinose operon of *E. coli* using the different promoters listed. Either lycopene or β -carotene were expressed from a plasmid using either an inducible T7 promoter or the endogenous promoter for β -carotene from *P. agglomerans*. MGS or DGS were expressed using an arabinose promoter on a separate plasmid.

3.3 Materials and Methods

3.3.1 Strains and routine cultivation

E. coli strains were obtained from the source indicated in Table 3.1. All strains were stored as glycerol stocks at -80°C. Strains were grown in LB media (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 37°C and 200 rpm for routine purposes. SOC media (20 g/L tryptone, 5 g/L yeast extract, 3.603 g/L NaCl, 1.2037 g/L MgSO₄, 0.186 g/L KCl and 0.9521 g/L MgCl₂) was used for cell recovery after electroporation. Appropriate kanamycin (Kan^R), ampicillin (Amp^R), and spectinomycin (Sp^R), were supplemented in the media at a final concentration of 50 µg/mL, 100 µg/mL, and 50 µg/mL.

Table 3.1 List of *E. coli* strains and plasmids used in this work.

Host/Strain	Description	Reference
NEB-5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	NEB
MG1655 (DE3)	<i>ΔendA ΔrecA (λ DE3)</i>	Addgene #37853
pro4IUP	<i>ΔendA ΔrecA (λ DE3), yabP::P_{Pro4} (ck, ipk, idi)::rluA, Kan^R</i>	This study
proCIUP	<i>ΔendA ΔrecA (λ DE3), yabP::P_{ProC} (ck, ipk, idi)::rluA, Kan^R</i>	This study
proDIUP	<i>ΔendA ΔrecA (λ DE3), yabP::P_{ProD} (ck, ipk, idi)::rluA, Kan^R</i>	This study
T7IUP	<i>ΔendA ΔrecA (λ DE3), yabP::P_{T7lac} (ck, ipk, idi)::rluA, Kan^R</i>	This study
trcIUP	<i>ΔendA ΔrecA (λ DE3), yabP::P_{trclac} (ck, ipk, idi)::rluA, Kan^R</i>	This study
PC	MG1655(DE3), pSEVA228pro4IUP	
Plasmids	Description	Reference
pSEVA228-pro4IUP	RK2, Kan ^R , P _{pro4} (<i>ck, ipk, idi</i>)	[1]
p5T7-lycipi-ggpps	pSC101, Sm ^R , P _{T7lacUV} (<i>ggpps, idi, crtI, crtB</i>)	[1]
pAC-BETAipi	p15A, CmR, (<i>crtEBIY, idi</i>)	Addgene #53277
p20-ara-mgs	pBR322, Amp ^R , P _{araB} (<i>mgs</i>)	This study

p20-ara-dgs	pBR322, Amp ^R , P _{araB} (<i>dgs</i>)	This study
p20-ara-mgs-his	pBR322, Amp ^R , P _{araB} (<i>mgs-6xhis</i>)	This study
p20-ara-dgs-his	pBR322, Amp ^R , P _{araB} (<i>dgs-6xhis</i>)	This study
pCBFD1	pBR322, Amp ^R , P _{araB} (<i>cbfd1</i> , <i>hbfd1</i>)	Addgene #53364
pTargetF	pBR322, Sm ^R sgRNA empty scaffold	Addgene #62226
pTarget- <i>yabP</i>	pTargetF with <i>yabP</i> -sgRNA and <i>yabP/rluA</i> homology region	This study
pTarget- pro4	pTarget- <i>yabP</i> with <i>yabP</i> /P _{Pro4} (<i>ck</i> , <i>ipk</i> , <i>idi</i>), Kn ^R / <i>rluA</i>	This study
pTarget-proC	pBR322, Sm ^R sgRNA- <i>yabP</i> / P _{ProC} (<i>ck</i> , <i>ipk</i> , <i>idi</i>), Kn ^R / <i>rluA</i>	This study
pTarget-proD	pBR322, Sm ^R sgRNA- <i>yabP</i> / P _{ProD} (<i>ck</i> , <i>ipk</i> , <i>idi</i>), Kn ^R / <i>rluA</i>	This study
pTarget-T7	pBR322, Sm ^R sgRNA- <i>yabP</i> / P _{T7lac} (<i>ck</i> , <i>ipk</i> , <i>idi</i>), Kn ^R / <i>rluA</i>	This study
pTarget-trc	pBR322, Sm ^R sgRNA- <i>yabP</i> / P _{trclac} (<i>ck</i> , <i>ipk</i> , <i>idi</i>), Kn ^R / <i>rluA</i>	This study
pCas	repA101(Ts) Kan ^R P _{cas-cas9} P _{araB-Red} <i>lacI</i> ^q P _{trc} -sgRNA-pBR322	Addgene #62225
pCas-Cm	repA101(Ts) Cm ^R P _{cas-cas9} P _{araB-Red} <i>lacI</i> ^q P _{trc} -sgRNA-pBR322	This study

Notes: sg-RNA-*pMB1*, sgRNA with a crRNA sequence for targeting the *pMB1* region. sg-RNA-*yabP*, sgRNA with a crRNA sequence for targeting a region between *yabP* and *rluA* (cut site: 59 549) and an editing template with a 1.0 kb region homologous to either side of the cut site. sgRNA-*yabP*:: P_{Pro4} (*ck*, *ipk*, *idi*), Kn^R:: *rluA*, sgRNA with a crRNA sequence targeting a region between *yabP* and *rluA* (cut site: 59 549) and an editing template with two 0.5 kb regions homologous to either side of the insertion site and a 4.5 kb P_{Pro4} (*ck*, *ipk*, *idi*), Kn^R insertion. Plasmid maps can be found in Appendix A.

3.3.2 Cloning

All vectors were constructed using the routine cloning protocol and the primers described in the supplementary materials. The genes *mgs* and *dgs* from *Acholeplasma laidlawii* were codon optimized for *E. coli* and synthesized by Twist Biosciences. They were cloned into the backbone of pCBFD1 containing the arabinose promoter/repressor, ampicillin resistance marker, and the origin using Gibson assembly to create p20-aramgs. To create the pTarget plasmids used to knock-in the IUP pathway, the online tool ChopChop [92] was used to generate a sgRNA spacer

sequence “AAATTCGCTGTGGAGCA’GGAAGG” targeting the region between yabP and rluA (NC_000913.3[59126-59687]) for insertion. The antibiotic selection marker of pCas9 was changed from kanamycin to chloramphenicol making pCas9-Cm by Gibson assembly to make the system compatible with other plasmids. A 1 kbp region surrounding the junction of yabP and rluA was amplified from MG1655 (DE3) using Q5 polymerase (NEB) and added to pTargetF by Gibson assembly along with the sgRNA spacer sequence to create pTarget-yabP. Then the IUP pathway genes were amplified from pSEVA228-pro4IUP along with a kanamycin resistance marker and inserted into the homology region of pTarget-yabP so that they are flanked by yabP and rluA using Gibson assembly. The promoter was varied by altering the Gibson primers. All constructs were verified by sequencing. Primers used in this chapter are listed in Table 3.2.

Table 3.2 List of primers used in this chapter.

Cloning step	Primer name	Primer sequence (5’ - 3’)
Integration	Int_CP_fwd	gtactcagacgttgacgtg
Integration	Int_CP_rev	ggaatacgaccggaccgag
pCas-Cm	BB_Cm_fwd	taacggcaaagcagtcagagtagaatag
pCas-Cm	BB_Cm_rev	acgcctggtggtcagaccaagtgttactc
pCas-Cm	CasCmC_2_fwd	gggtctgctatgtggtgcta
pCas-Cm	CasCmC_2_rev	ggtgagctggtgatatggga
pCas-Cm	CasCmC_fwd	gggtgatgctgccaacttac
pCas-Cm	CasCmC_SP1	gggtgatgctgccaacttac
pCas-Cm	Cm_fwd	ttggtctgaccaccaggcgtttaagggc
pCas-Cm	Cm_rev	ctctgactgcttgccgttacgcaccac
pTargetT step 1	HMR_fwd	tgaagatccttaacctgggcgtgctacc
pTargetT step 1	HMR_rev	agatcctttaagcagtatgtggcccg
pTargetT step 1	Ori_fwd	catactgctttaaggatctaggtgaagatc
pTargetT step 1	Ori_rev	ccttctgctccacagcgaatttactagtattatacctaggactg
pTargetT step 1	PT1C_fwd	aggcgagatcaccaagtag
pTargetT step 1	PT1C_rev	cagcagattacgcgcagaaa
pTargetT step 1	SP1	gcctgatgcggtatttctcc

pTargetT step 1	Spec_fwd	aaattcgctgtggagcaggaagggttttagagctagaaatagcaag
pTargetT step 1	Spec_rev	gccaggttaaggatcttcacctagatc
pTargetT step 2	IUP_Pro4_fw d	ctgtggagcatttacgggcatgcataagg
pTargetT step 2	IUP_ProCD_f wd	tattcaggaggagaccacaacg
pTargetT step 2	IUP_rev	attccctcccagggttatgcagcggaaaag
pTargetT step 2	IUP_T7_fwd	taatacgaactactataggggaattgtgagcggataacaattcccattcaggagg accacaacg
pTargetT step 2	IUP_trc_fwd	ttgacaattaatcatccgctcgtataatgtgtggaattgtgagcggataacaattcta ttcaggaggagaccacaacg
pTargetT step 2	PT_BB_fwd	cataaccctgggaagggaattaccgaatg
pTargetT step 2	PT_BB_Pro4_ rev	tgcccgtaaagtctccacagcgaattttg
pTargetT step 2	PT_BB_ProC _rev	cgttggtgtctccctgaataatcatacagccttatgcatgcccgtaaagtctccaca gcgaatttttg
pTargetT step 2	PT_BB_ProD _rev	cgttggtgtctccctgaataattatacagccttatgcatgcccgtaaagtctccacag cgaatttttg
pTargetT step 2	PT_BB_T7_re v	ggggaattgttatccgctcacaattcccctatagtgagtcgtattatgctccacagcg aatttttg
pTargetT step 2	PT_BB_trc_re v	gaaattgttatccgctcacaattccacattatacagccggatgattaattgtcaat gctccacagcgaatttttg
pTargetT step 2	PT2_SP1	tccggatatgaacaaactgca
pTargetT step 2	PT2C_CP_fw d	tcgccaaccagactgctaatt
pTargetT step 2	PT2C_CP_rev	tcgatccctgttcttggt
pCBFD1 backbone	bb_fwd	cgatgagggaatgataagtctgcgatgg
pCBFD1 backbone	bb_rev	gggcggattgcttattccagatgcgtgc
mgs/dgs fragment	dgsmsgs_fwd	ctggaataagcaatccgccctcactacaac
mgs/dgs fragment	dgsmsgs_rev	gacttatcattccctcatcgacgccagag

3.3.3 Chromosomal insertion

Insertions were performed using a modified version of a published protocol [93]. pCas-Cm and pTarget plasmids containing each version of the IUP pathway were transformed into MG1655 (DE3) using electroporation. The transformants were recovered in LB broth with Kan, Sm, and

Cm at 30°C for 24-48 h. Then, 15 mL of broth was centrifuged for 15 min and the cell pellet was resuspended in LB with Kan and 0.5 mM IPTG and incubated overnight at 30°C to cure the pTarget plasmid. Then another 10 mL of LB was added and the culture was incubated overnight at 42°C to cure pCas-Cm. After curing, 10m L of the culture was spread onto an LB plate and streaked for single colonies. Colonies were screened for the insertion using colony PCR (Described in SI) and streaked onto three separate plates each containing a single antibiotic (Kan, Sm, Cm) to confirm the plasmids were cured. The PCR product was purified and sent for sequencing to confirm the insertion was correct. Each new strain (Table 3.1) was then made electrocompetent and transformed with combinations of p5T7-lycipi-ggpps, pAC-BETAipi, p20-ara-mgs, p20-ara-dgs.

3.3.4 Western Blot

To confirm expression of DGS and MGS, strains expressing the his-tagged versions were grown to 0.5 OD600 and induced with 5g/L of arabinose and incubated at 37°C, for 24h. Cultures (25 mL) were centrifuged at 2,300 x g for 15 min at 4°C and the cell pellet was resuspended and washed using deionized water. The pellet was resuspended in 25 mL of PBS (50 mM, pH 7.4) and sonicated (Qsonica Misonix Sonicator XL-2000) for 5 min at 22.5 Hz. The lysates were centrifuged for 10 min at 2,300 x g. The supernatant containing the soluble protein was prepared for SDS-PAGE by combining 25 µL with 1 µL of DNase, and 5 µL of Laemmli buffer containing 5% v/v β-mercaptoethanol. The insoluble pellet after centrifugation was resuspended in TE/SDS buffer (10 mM Tris HCl, 1 mM EDTA, 1% w/v SDS, pH 8) and combined with the same as the soluble protein fraction. Samples were boiled for 10 min at 95 °C.

Samples were then loaded to pre-cast SDS-PAGE gels (12%, Biorad Canada) and run at 125V for 120 min. The gel was then soaked in 1L of protein transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 15 min. Proteins were transferred to the nitrocellulose membrane using electroblotting (Trans-Blot Turbo Transfer System, Biorad) for 25 min at 25 V and 0.4 A. The blot was briefly rinsed with PBS (50 mM, pH 7.4) then washed with TBST (10 mM Tris-HCl, 100 mM NaCl, 0.05% v/v Tween 20, pH 8.0) for 5 min at RT. Non-specific binding was blocked by incubating the membrane in 5% w/v nonfat milk for 1h with gentle shaking. The membrane was then incubated in TBST with 5% w/v nonfat milk containing 1:1000 dilution of Anti-His antibody (Catalog No. 501122712, Fisher Scientific) overnight at 4°C. The membrane was then washed three times with TBST for 5 min each and then incubated with Alexa Fluor™ 488 conjugated secondary antibody (Catalog No. A-21141, Thermo Fisher) in TBST with 5% w/v non-fat milk at a 1:10,000 dilution for 1 h at RT. The membrane was then washed three times as before and imaged using a Biorad ChemiDoc using a 700/50 filter.

3.3.5 Production of isoprenoids

Glycerol stocks were streaked onto LB agar plates (15 g/L agar) with the appropriate antibiotic and grown overnight at 37 °C. Seed cultures were prepared by transferring one colony into LB broth and grown overnight at 37 °C. Flasks containing M9 media (4 g/L glucose, 6.8 g/L Na₂HPO₄·7H₂O, 3g/L KH₂PO₄, 0.5 g/L NaCl, 1.0 g/L NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂) with Wolfe's Trace elements [94] containing the appropriate antibiotics were inoculated with 1% v/v seed culture in triplicate. Growth was monitored using absorbance at 600 nm (OD₆₀₀). In experiments requiring induction of gene expression, IPTG was added to a final concentration of

0.1 mM when the culture reached an OD₆₀₀ of 0.5. When required, 25 mM of isoprenol was also added when OD₆₀₀ = 0.5. Induced cultures were grown for 24 hours at 37 °C and 200 rpm, unless otherwise indicated. Dry cell weight was measured by centrifuging 2 mL of bacterial culture at 9,000 rpm for 2 min. The supernatant was removed, the cells were washed with ultrapure water and the pellets were transferred to a preweighed microcentrifuge tube. The solution was centrifuged, and the supernatant was removed. The cell pellets were frozen overnight and dried for 16 h using a Labconco lyophilizer. The dried pellets and tube were weighed, and the difference was reported as dry cell weight (DCW; g/L).

3.3.6 Carotenoid extraction

To quantify carotenoids, 1 mL of culture was centrifuged at 9,000 rpm for 2 min in a dark microcentrifuge tube to prevent photodegradation. The supernatant was removed, and the cell pellet was resuspended in 1 mL of a 1:1 volume ratio of ethanol and acetone. The tubes were incubated for 1 h at room temperature in the dark with occasional mixing by inversion every 15 min. The tubes were centrifuged at 9,000 rpm for 2 min to remove cell debris and 200 µL was transferred into a 96-well plate. The absorbance was read at 475 nm using a plate reader (BioTek). The blank reading was subtracted from the absorbance and the carotenoid concentration was determined by dividing the absorbance by 0.0816 L/mg [95]. Carotenoid content was determined by dividing concentration by DCW. All the equations used for data analysis are summarized in Appendix B.

3.3.7 Total lipid content by direct transesterification to FAME

The fatty acid methyl ester (FAME) content by weight was determined for triplicate cultures using a slightly modified standard FAME laboratory analytical procedure (LAP) from the National Renewable Energy Laboratories (NREL) [96]. Approximately 10 mg of each sample was transferred into a glass vial with a stir bar, and the exact weight was recorded. 25 μ L of a 10 mg/mL methyl nonadecanoate (C19:0Me) standard was added to each sample using a gas tight syringe. Using a micropipette, 200 μ L of 2:1 (v/v) chloroform:methanol and 300 μ L of 0.6 M HCl:methanol was added to each sample. Vials were sealed and placed in a boiling water bath for 1 hour with vigorous stirring. Then, they were removed from the water bath and allowed to cool to room temperature. Once cool, 1.0 mL of hexane was added to each sample using a gas tight syringe. Then, samples were returned to the stir plate and allowed to mix for 1 hour without heating. The aqueous and organic layers were allowed to separate, and 450 μ L of the hexane layer was transferred to an autosampler vial containing 50 μ L of a 1 mg/mL methyl heptadecanoate (C17:0Me) standard. FAME was separated and analysed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30m, 0.25 mm, 0.25 μ m). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min⁻¹ to 200°C, 3°C min⁻¹ 220°C, and hold for 10 min. Individual FAMES were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C19:0Me and

dividing the total by the weight of cells used in the assay. An image of representative FAME quantification curves can be found in Appendix C.

3.4 Results

3.4.1 Construction of IUP strains

Expression of heterologous proteins from the chromosome increases the genotype stability and reduces the metabolic burden of maintaining extrachromosomal DNA compared to plasmid-based systems [97]. It also eliminates the need to use antibiotics to maintain the plasmid which is too expensive to use on a large-scale [97]. When there is only a single copy of the insert, promoter strength will play an important role in the expression of the heterologous genes. Alternatively, the genes can be inserted multiple times to improve expression level [14]. Five strains of *E. coli* expressing the IUP pathway under the control of constitutive (pro4, proC, proD) and inducible promoters (trc, T7) with varying strength were created using a CRISPR-cas9/ λ_{red} system [17, 98]. Integration of the IUP pathway between *yabP* and *rulA* was confirmed by colony PCR and sequencing (Figure 3.2). The strains were then transformed with p5T7-lycippi-ggpps which results in the production of the carotenoid lycopene that can be quantified using UV/Vis spectroscopy.

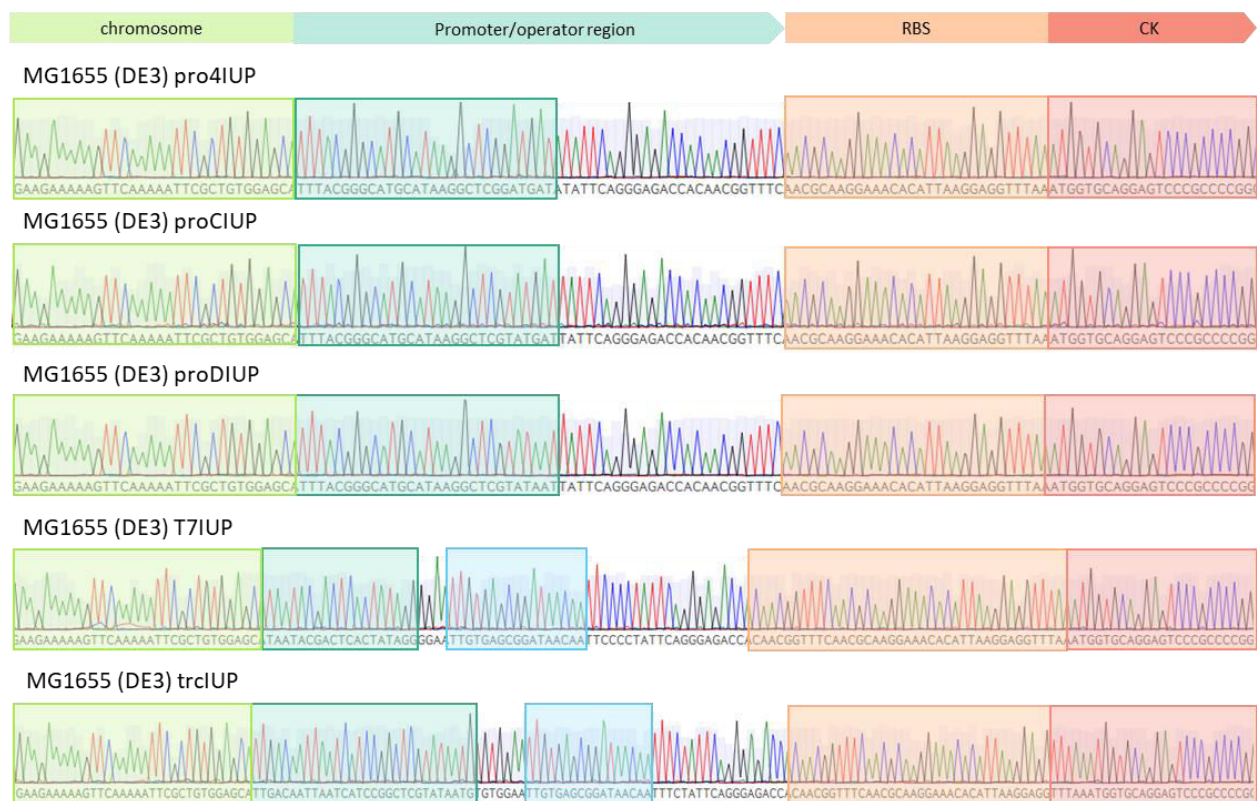


Figure 3.2 Sequencing results of the promoter region of the knock-in IUP strains. Green indicates the portion of the chromosome, turquoise/blue the promoter or operator regions, orange the ribosome binding site (RBS), and red the start of the choline kinase gene (CK).

Initial results show that the integration of the IUP pathway in the chromosome resulted in high levels of lycopene production in M9 minimal media using every promoter (Figure 3.3). Interestingly, lycopene titres were consistently in the range of 7.4-9.4 mg/L regardless of the promoter used and there were no statistical differences between strains ($p > 0.05$ compared to positive control). However, when the dry cell weights of each strain were observed, there were significant differences (Figure 3.3A). The proC and proD strains exhibited significantly lower growth than the plasmid-based PC ($p < 0.01$, 2 tailed T-test, $n=3$) as did the trcIUP strain ($p < 0.05$, 2 tailed T-test, $n=3$). As a result of the lower growth rates, the lycopene contents of the cells (specific yield) varied significantly and integration of the IUP in the chromosome had a

positive effect on lycopene production (Figure 3.3B – dark bars). The best producers were the trcIUP and proDIUP strains making 17.1 ± 3.2 mg/g and 17.6 ± 2.2 mg/g of lycopene respectively. This is a 2-fold increase in lycopene content compared to the positive control (8.4 ± 0.3 mg/g).

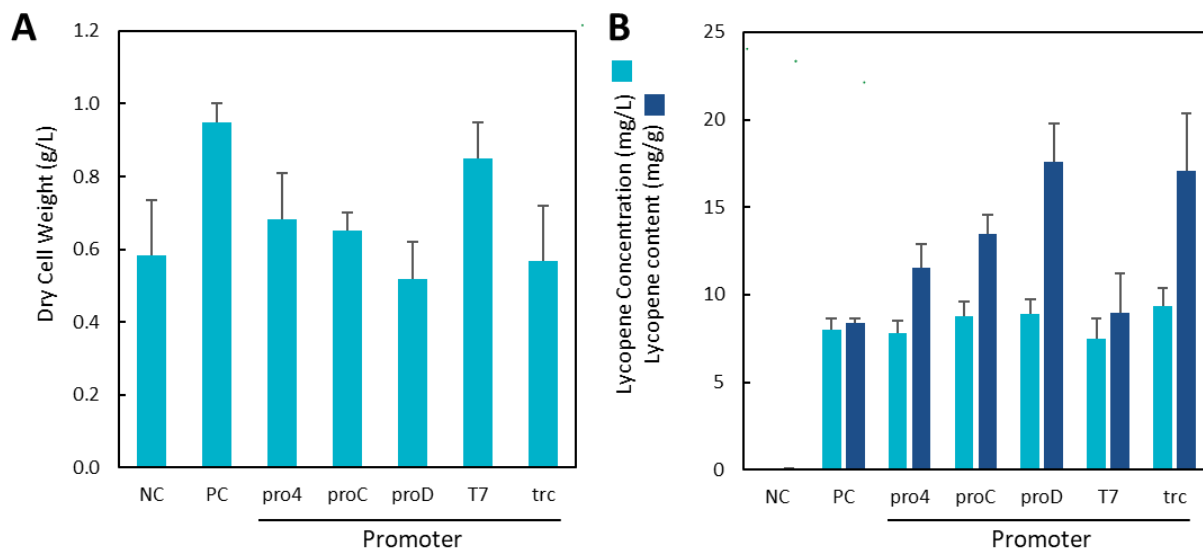


Figure 3.3 A. Dry cell weight and **B.** lycopene production in plasmid and chromosomal IUP systems 24h after induction with 0.1 mM IPTG and 25 mM isoprenol. Light bars represent the lycopene titre (mg/L) and the dark bars represent the lycopene content (mg/g). PC represents the positive control plasmid-based IUP strain and the negative control (NC) is wild-type MG1655(DE3) without the IUP pathway.

Further investigation of the growth characteristics of these strains show that when the lycopene pathway is expressed along with the IUP pathway on the plasmid system (PC), dry cell weight of these cultures also increases. While the use of constitutive promoters pro4/C/D for chromosomal expression of the IUP did not increase the dry cell weight (DCW). In all cultures the production of lycopene steadily increased for the first 12h (Figure 3.4), after which the productivity decreased slightly. Interestingly, growth lasted for only 8h in the PC, whereas

T7IUP strains and cell death might have occurred in the proD strain, which had a lower final DCW than any other culture.

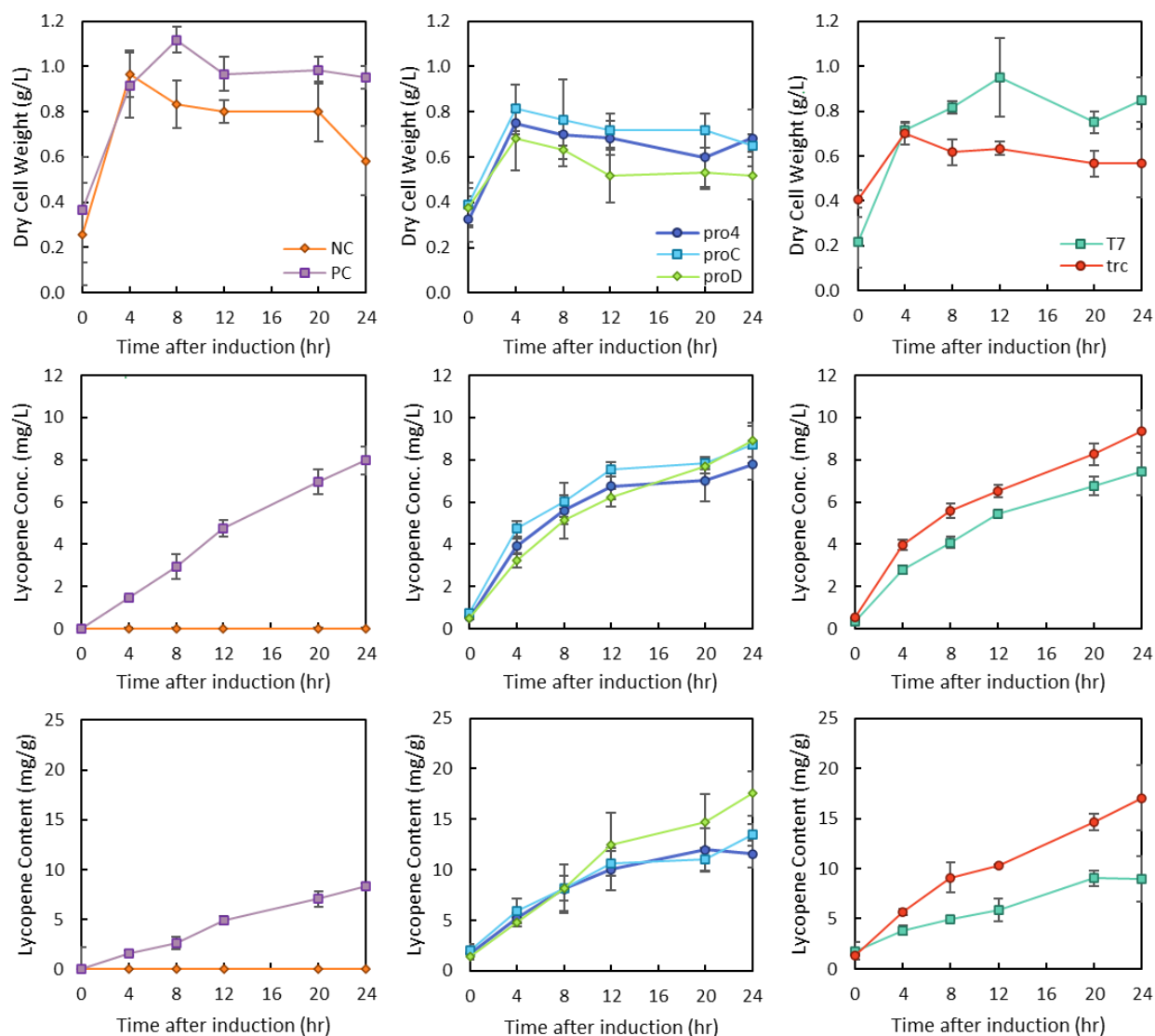


Fig 3.4 Characterizing IUP strains expressing the lycopene synthesis pathway. Changes in cell growth is shown in the top row of panels. Changes in lycopene titres over time are shown in the middle panels, and changes in lycopene productivity are shown in the bottom row of panels. The promoters used are indicated in the legends; pro4/C/D are constitutive promoters, whereas trc/T7 are IPTG inducible promoters. PC refers to the plasmid-based IUP system, while NC refers to WT *E. coli*.

3.4.2 Expression of membrane modulating enzymes

Phytoene desaturates (*crtI*) is the enzyme responsible for the formation of lycopene from its colourless intermediate phytoene and is a membrane-peripheral enzyme [99]. Increasing the membrane area could have two impacts on lycopene production by increasing the storage capacity of the cell for the products of this pathway (phytoene and lycopene) as well as increasing the area available for CrtI to associate with the inner membrane. To investigate this possibility, MGS and DGS from *A. laidlawii* were expressed to form intracellular membrane vesicles. First, the MGS (45 kDa) [8] and DGS (40 kDa) [100] expressions were confirmed by western blot to be present in the insoluble membrane fraction (Figure 3.5). Then, *E. coli* containing either the MGS or DGS expression cassette only, were grown in M9 media with varying concentrations of their inducer arabinose to see the effect of these genes on DCW and lipid content of the cells (Figure 3.6).

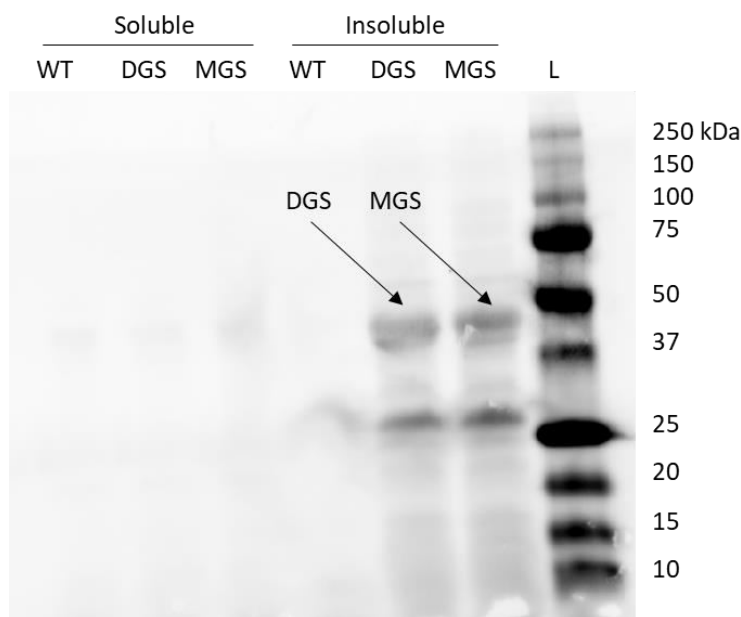


Figure 3.5 Western blot confirming expression of MGS and DGS in the membrane fraction of *E. coli*. DGS (lane 1) and MGS (lane 2) were his-tagged and immunoblotted using anti-His antibodies and compared to the ladder (lane 3).

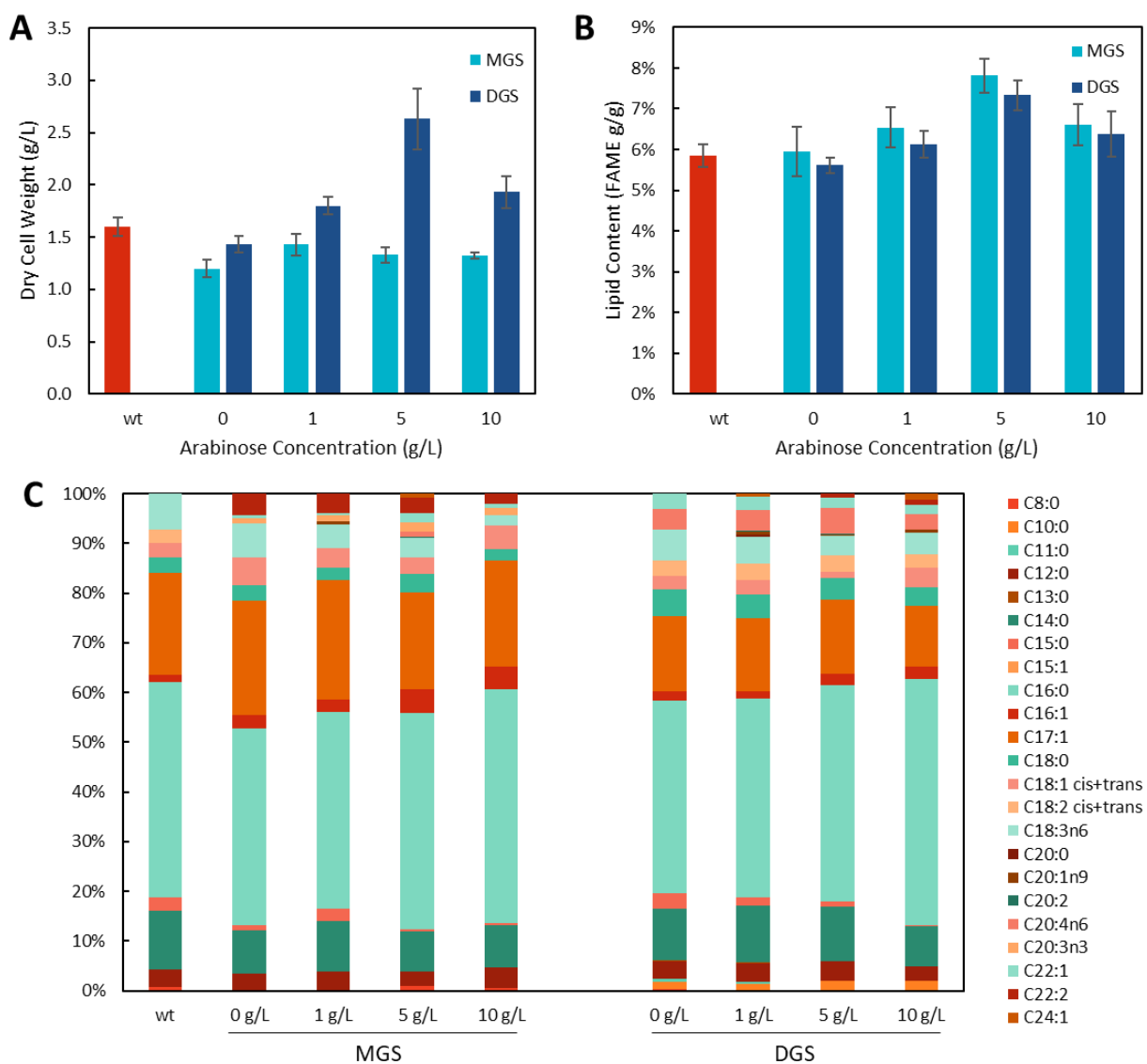


Figure 3.6 Effect of MGS/DGS on DCW and lipid content of the cells. **A.** Dry cell weights and **B.** lipid content of *E. coli* expressing MGS or DGS with different concentrations of the inducer, arabinose. The wild-type control (wt) did not contain any plasmids. **C.** FAME lipid composition.

For MGS, the DCW was slightly lower for MGS than for the WT strain and no changes were seen with increasing arabinose concentrations. However, for DGS, DCW was significantly higher in cultures induced with 5 g/L of arabinose (Figure 3.6A). With the formation of intracellular vesicles, one might expect to see an increase in lipid content of the cells. However,

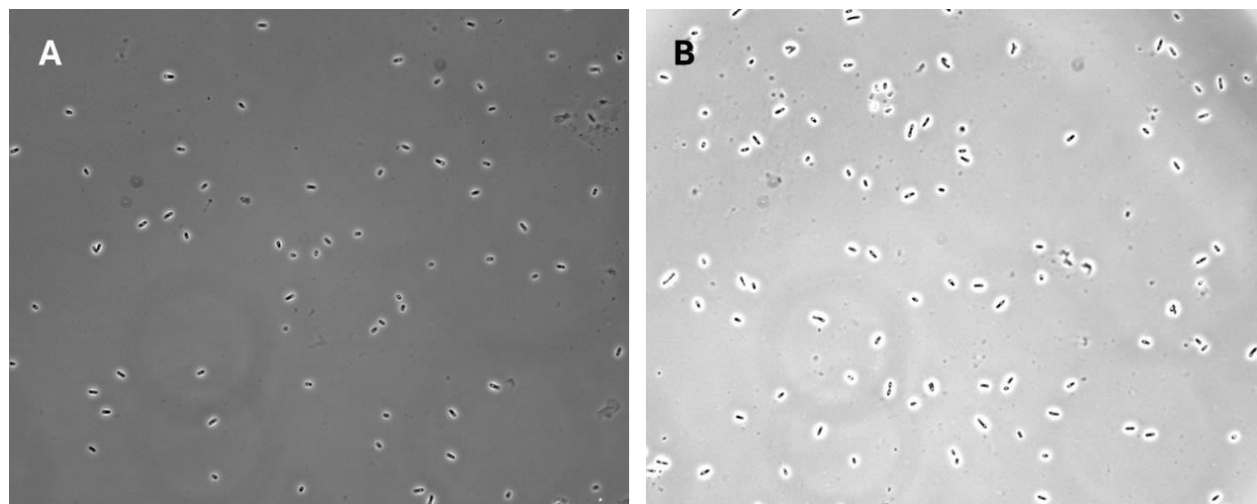
total lipids were determined by transesterification using an acid catalyst which can convert both triacylglycerides and phospholipids to fatty acid methyl esters (FAME), and only a small increase in FAME was found for DGS and MGS with arabinose induction (Figure 3.6B). It is unknown how the formation of intracellular vesicles affects the cell size/volume and it is possible that cells grow larger and lower cell densities (cells/mL) result in the same DCW for the WT and MGS/DGS cultures effectively resulting in no appreciable increase in lipid content. The lipid composition was also found to be similar in all samples (Figure 3.6C), however, both MGS and DGS expressing strains did form C16:1 which increased with increasing arabinose concentration and C22:2 which was not present in the WT strain.

3.4.3 Effect of MGS and DGS on cell morphology

Eriksson et al. (2009) demonstrated that strong expression of MGS or DGS results in elongated cells, as observed through cryogenic transmission electron microscopy (cryo-TEM) images [8]. To validate this conclusion, compound microscopy at 100x magnification was employed to examine two strains: the wild-type with no MGS expression (Figure 3.7A) and a strain expressing MGS via the plasmid p20-ara-mgs-his (Figure 3.7B). The microscopy images were processed using CellProfiler and Ilastik software. A one-way ANOVA was conducted to analyze cell length and width. The results indicated that the f-ratio for length was 2.62988 with a p-value of 0.106984, suggesting no significant difference at $p < 0.05$. Conversely, the f-ratio for width was 32.70681 with a p-value of < 0.00001 , indicating a significant difference at $p < 0.05$. Therefore, based on these results, no significant elongation of cells can be concluded. The statistical results are presented in Table 3.3, and supplementary data are provided in Appendix D.

Table 3.3 Results from cell length and width analysis.

	WT	Recombinants
Strain	MG1655 (DE3) proD-IUP	MG1655 (DE3) proD-IUP
Plasmid	p5T7-lycipi-ggpps	p5T7-lycipi-ggpps + p20-ara-mgs-his
Total cell counted	64	87
Average length (pixel)	17.11382 (sd=3.69)	18.30825 (sd=4.97)
Average width (pixel)	8.713499 (sd=0.96)	7.661052 (sd=1.22)
Average aspect ratio	0.74415	0.86769

**Figure 3.7** Compound microscope images of *E. coli* without MGS expression (A) and *E. coli* with MGS expression (B).

3.4.4 Lycopene production in MGS and DGS strains

To see if co-expression of MGS or DGS increased the production of lycopene, the plasmid for lycopene production along with either the DGS or MGS plasmids were transformed into the proD-IUP and trc-IUP strains and well as the wild-type strain containing only the endogenous MEP pathway (NC) or the wild-type strain containing the pSEVApro4-IUP plasmid (PC) (Figure 3.8). Again, with 5 g/L of inducer, expression of DGS increased the DCW of the wild-type culture (NC, Figure 3.8A) but not in the other strains. Expression of the IUP and lycopene genes with MGS or DGS resulted in significantly higher lipid levels than the wild-type strain (NC), as

confirmed by one-way ANOVA test ($p < 0.05$). Notably, the trc+MGS strain exhibited superior lipid production and this strain also exhibited higher lipid content (Figure 3.8B & C). However, lycopene production decreased in all of the strains when compared to the strains only expressing lycopene biosynthesis pathway (Figure 3.3), with the PC lycopene content decreasing from 8.4 ± 0.3 mg/g to 6.8 ± 0.5 mg/g for DGS and no change for MGS (8.6 ± 2.6 mg/g). For the proD strain, lycopene content decreased from 17.6 mg/g to 12.5 mg/g for MGS and 10.4 mg/g for DGS. A similar decrease was seen in the trc strain. One possible explanation for the decrease could be the formation of inclusion bodies. When inclusion bodies form, they can have profoundly negative effects on the metabolism of the host cell including other recombinant proteins being expressed such as those from the IUP and lycopene biosynthesis genes. Inclusion bodies are also more likely to form with the expression of membrane proteins like DGS/MGS particularly when expressed from strong promoters.

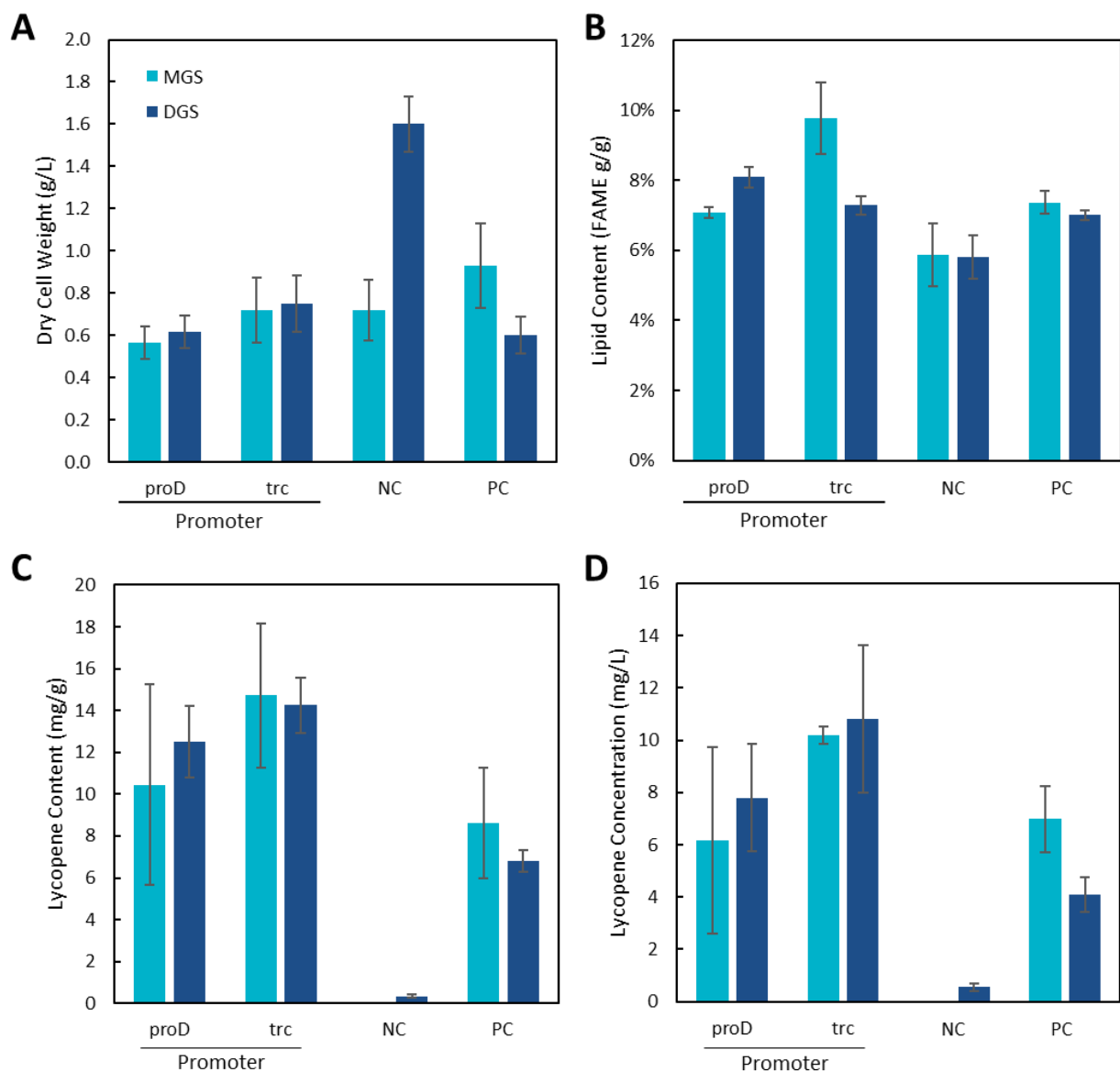


Figure 3.8 Effect of MGS or DGS expression on lycopene production in proD and trc strains, WT (NC), and the plasmid-based IUP expression (PC) system.

Interestingly, DGS expression also resulted in a detectable amount of lycopene in the WT strain (Figure 3.8C & D), when lycopene is usually undetectable without modifications to the endogenous isoprenoid pathway, confirming that DGS expression does have a positive effect on lycopene production. In contrast, DGS expression decreased the lycopene volumetric production in the positive control system where lycopene production is much higher. This may indicate that

DGS and MGS expression only have minor effects in highly productive carotenoid producing strains and it may validate our hypothesis that inclusion bodies are being formed in the more highly engineered strains expressing a number of recombinant proteins simultaneously.

3.4.5 Effect of MGS on β -carotene production

Curious as the effect on different carotenoid products which would have different solubilities in the cell membrane, MGS was expressed alongside the pathway for β -carotene production (Figure 3.9). In contrast to the lycopene strains, production of β -carotene must be conducted at a lower temperature (30°C). In this scenario, DCW decreased in the strains expressing the IUP from a plasmid or from the chromosome (Figure 3.9A). No significant differences in β -carotene content were observed (Figure 3.9 C), however, the proD strain did see an increase in β -carotene concentration (7.2 mg/L vs. 9.8 mg/L) in strains induced for MGS expression (Figure 3.9B), but this only served to offset the decrease compared to the wild-type strain (NC). One possible explanation for the difference in productivity compared to the lycopene strains could be the difference in optimal temperatures of the IUP pathway and the β -carotene pathway. The increase in lycopene production in IUP strains was minor compared to the wild-type strain at 30°C, while it was significant at 37°C (unpublished results).

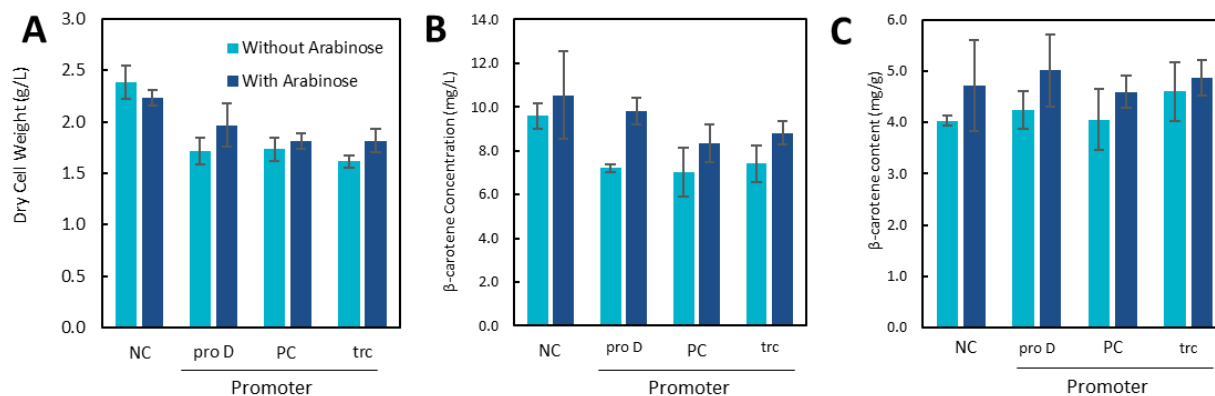


Figure 3.9 A. Dry cell weight and B. β-carotene concentration and C. β-carotene content of strains with and without MGS induction.

3.5 Discussion

Notably, the investigation conducted by [43] also aimed at enhancing lycopene production through the expression of MGS. In addition, they overexpressed *plsb* and *plsc* genes to boost phospholipid production via the diglyceride-3-phosphate synthesis pathway, resulting in a modest 13% increase in lycopene yield. They achieved slightly better results by combining this pathway with MGS; lycopene production improved, with a 32% increase observed when both pathways were employed. Meanwhile, they inserted the *mgs* gene into the chromosome, potentially explaining the higher gene expression compared to plasmid insertion, as was done in our study. However, it's worth noting that the strain might require more time for membrane engineering to take effect. This is evidenced by their 96-hour expression period resulting in higher lycopene production with the *mgs* gene, compared to the 48-hour expression period utilized in their study. Table 3.4 summarize the effects of chromosomal insertion and MGS or DGS expression on specific lycopene content.

Table 3.4 Summary of Effects of Chromosomal Insertion and MGS or DGS Expression on Specific Lycopene Content.

Plasmid system		Ch(trcIUP)		Ch(proDIUP)	
8.4 mg/g		17.1 mg/g		17.6 mg/g	
+ MGS	+ DGS	+ MGS	+ DGS	+ MGS	+DGS
8.64 mg/g	6.79 mg/g	14.7 mg/g	14.3 mg/g	10.4 mg/g	12.5 mg/g

3.6 Concluding Remarks

Overall, insertion of the IUP pathway into the chromosome had a large effect on the specific yield of lycopene at 37°C (2.2-fold increase for proD), however, this effect was not observed for production of β -carotene at 30°C. Expression of MGS and DGS from *A. laidlawii* to induce intracellular lipid production did slightly alter the lipid profile of the *E. coli* strains and resulted in minor increases in lipid content, but did not increase carotenoid production in the IUP expressing strains. DGS expression did have a positive impact on carotenoid expression in the wild-type strain increasing the lycopene content from undetectable levels to 0.3 mg/g. However, this was outweighed by the decrease in carotenoid yields in the IUP strains expressing MGS or DGS.

Chapter 4 – Engineering the Membrane for Extracellular Production of Carotenoids in *Escherichia coli*

4.1 Introduction

Lycopene, a carotenoid known for its significant health benefits and commercial value, encounters challenges in achieving high-yield production due to negative feedback inhibition within the MEP pathway. Specifically, the accumulation of IPP, a downstream intermediate, inhibits the upstream enzyme DXP [10]. To address this issue, this chapter explores a strategy to enhance lycopene production by employing a leaky strain, generated through a knock-out mutation targeting the lipoprotein responsible for covalent cross-linkage between the outer membrane and the peptidoglycan layer. This genetically modified strain facilitates the extracellular secretion of key enzymes in the MEP pathway, including DXS, IspD, IspF, IspG, and IspH. By effluxing these overproduced rate-limiting enzymes into the extracellular space, the accumulations of intracellular enzymes are reduced, thereby alleviating IPP-induced feedback inhibition. This chapter presents experimental results validating this strategy and discusses the broader implications of extracellular enzyme production in microbial metabolic engineering.

4.2 Materials and Methods

4.2.1 Strains and routine cultivation

E. coli strains were obtained from the source indicated in Table 4.1. All strains were stored as glycerol stocks at -80°C. Strains were grown in LB media (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 37°C and 200 rpm for routine purposes. SOC media (20 g/L tryptone, 5 g/L

yeast extract, 3.603 g/L NaCl, 1.2037 g/L MgSO₄, 0.186 g/L KCl and 0.9521 g/L MgCl₂) was used for cell recovery after electroporation. Where appropriate, chloramphenicol (Cm^R), kanamycin (Kan^R), ampicillin (Amp^R), and spectinomycin (Sp^R), were supplemented in the media at a final concentration of 25 µg/mL, 50 µg/mL, 100 µg/mL, and 50 µg/mL, respectively.

Table 4.1 List of *E. coli* Strains and Plasmids Used in This Work.

Host/Strain	Description	Reference
JM109	recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ ⁻ , Δ (lac-proAB), [F', traD36, proAB, lacIqZΔM15]	Addgene #49761
JM109-Δ <i>lpp</i>	recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ ⁻ , Δ (lac-proAB), [F', traD36, proAB, lacIqZΔM15], Δ <i>lpp</i>	By Reza M. Roushan
Plasmids	Description	Reference
p10-tac-dxs-ispDF	p15A, CmR, P _{tac} (<i>dxs</i> , <i>ispD</i> , <i>ispF</i>)	This study
pARA-ispGH	pBR322, Amp ^R , P _{araB} (<i>ispG</i> , <i>ispH</i>)	This study
p5T7-lycipi-ggpps	pSC101, Sp ^R , P _{T7lacUV} (<i>ggpps</i> , <i>idi</i> , <i>crtI</i> , <i>crtB</i>)	[1]
pAC-BETAipi	p15A, CmR, (<i>crtEBIY</i> , <i>idi</i>)	Addgene #53277
pCBFD1	pBR322, Amp ^R , P _{araB} (<i>cbfd1</i> , <i>hbfd1</i>)	Addgene #53364
pJOE7706.1	Km ^R , <i>rop</i> , <i>rep</i> , P _{tac} <i>lacI</i> ^q , <i>rrnB</i> , <i>eGFP</i>	Addgene #135075

Note: Plasmid maps can be found in Appendix A.

4.2.2 Cloning

All vectors were constructed using the routine cloning protocol and the primers described in Table 4.2. The gene *dxs*, *ispD*, *ispF*, *ispG* and *ispH* were cloned from *E. coli* JM109 by PCR. The gene *lacI* from plasmid pJOE7706.1, whereas *dxs*, *ispD*, and *ispF* were cloned into the backbone of pAC-BETAipi containing the chloramphenicol resistance marker, and the p15A origin using Gibson assembly to create p10-tac-dxs-ispDF. Subsequently, *ispG* and *ispH* were cloned into the backbone of pCBFD1 containing the arabinose promoter/repressor, ampicillin

resistance marker, and the origin using Gibson assembly to create pARA-ispGH. All constructs were verified by sequencing.

Table 4.2 List of primers used in this chapter.

Cloning step	Primer name	Primer sequence (5' - 3')
Plasmid 1	p15-bb_fwd	accaggcggttaagggcac
Plasmid 1	p15-bb_rev	gcaagggtaccgctgaattg
Plasmid 1	lacIq_tac_fwd	aattcagcgggtaaccttgctatagggcaattgcgttg
Plasmid 1	lacIq_tac_rev	tgtaattccgggtcatatgtatatctccttc
Plasmid 1	ispD_fwd	acatatgaccggaattaacatggcaacc
Plasmid 1	ispD_rev	gcgtttcactattcgattatgtattctcc
Plasmid 1	terminator_fwd	taatgccgaatagtgaacgccgtagcgc
Plasmid 1	terminator_rev	gggtgcccttaaacgcctggtaggggtattgtctcatgagcg
Plasmid 2	pARA-bb_fwd	agttatgcgaccatgccgaactcagaag
Plasmid 2	pARA-bb_rev	cgccgggtgatcggtatggagaaacagtag
Plasmid 2	ispG_fwd	tccatacccgatcaccggcgagtaacag
Plasmid 2	ispG_rev	gggtatatctccttattaaaggagccatgcgggttcaatc
Plasmid 2	ispH_fwd	ctttaataaggagatataccactggaggcgtaacatg
Plasmid 2	ispH_rev	ttcggcatggtcgcataacttaggctgc
Plasmid 1	p10-tac-ispD-f	tcattaaggcaacaaaatgaagtaaggaggtatttttatggcaaccactcatttg
Plasmid 1	p10-tac-ispD-r	gtgaaattgttatccgctc
Plasmid 1	DXS-f	tgagcgggataacaatttcacgttaaggaggttttttatgagtttgatattgccaataacccg
Plasmid 1	DXS-r	ttatgccagccaggccttg
Plasmid 1	ispF-f	tcaaggcctggctggcataacataaaggaggttttttatgcgaattggacacgggttttg
Plasmid 1	ispF-r	tcattttgtgccttaatgagtagc

4.2.3 Preparation of electrocompetent cells

To prepare electrocompetent *E. coli* cells, a 5 mL seed culture was grown from a pre-made glycerol stock in a sterile Falcon tube at 37°C with shaking at 250 rpm. Meanwhile, a stock solution containing 20% v/v glycerol and 15 g/L of mannitol was prepared and sterilized via autoclaving. After overnight incubation, when the culture reached maximum cell density, 200 mL of fresh LB media was inoculated with the seed culture to achieve an OD600 of

approximately 0.01. This culture was divided into two 500 mL Erlenmeyer flasks and incubated at 30°C with shaking at 200 rpm until the OD600 reached between 0.4 and 0.6. The flasks were then chilled on ice for 30 minutes. The cultures were transferred to four 50 mL Falcon tubes and centrifuged at 4°C and 3720 rpm for 15 minutes. The cells were resuspended in 10 mL of cold water per tube and then combined into two of the tubes. Using a 10 mL serological pipette, 10 mL of the glycerol and mannitol stock solution was slowly added to the bottom of each tube, leaving a small amount of solution in the pipette to prevent mixing of the two phases. The tubes were centrifuged again under the same conditions and the cells were resuspended in 200 µL of the glycerol and mannitol stock solution. The concentrated cell suspension was aliquoted into 50 µL portions in sterile microtubes and stored at -80°C.

4.2.4 Electroporation

A portion of electrocompetent cells was thawed on ice along with a correctly sequenced plasmid suspension. Once thawed, the cell suspension was transferred to a chilled electroporation cuvette with a 1 mm gap, and 1 µL of the DNA suspension was added. For multiple plasmids, 1 µL of each plasmid suspension was included. After ensuring the absence of bubbles, the cuvette was placed in an electroporation chamber and pulsed at 1.8 kV and 25 µF capacitance. Immediately after pulsing, 1 mL of SOC media was added to resuspend the cells, and the mixture was transferred to a sterile tube for incubation at 37°C for 2 hours. Subsequently, 100 µL of the culture was spread onto an LB plate containing the appropriate antibiotics and incubated at 37°C for 24 hours. Successfully transformed colonies were used for glycerol stock preparation and future experiments.

4.2.5 Production of isoprenoids

Glycerol stocks were streaked onto LB agar plates (15 g/L agar) with the appropriate antibiotic and grown overnight at 37 °C. Seed cultures were prepared by transferring one colony into LB broth and grown overnight at 37 °C. Flasks containing M9 media (4 g/L glucose, 6.8 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3g/L KH_2PO_4 , 0.5 g/L NaCl, 1.0 g/L NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2) with Wolfe's Trace elements [94] containing the appropriate antibiotics were inoculated with 1% v/v seed culture in triplicate. Growth was monitored using absorbance at 600 nm (OD_{600}). In experiments requiring induction of gene expression, IPTG was added to a final concentration of 0.1 mM when the culture reached an OD_{600} of 0.5. Induced cultures were grown for 24 hours at 37 °C and 200 rpm, unless otherwise indicated. Dry cell weight was measured by centrifuging 2 mL of bacterial culture at 9,000 rpm for 2 min. The supernatant was removed, the cells were washed with ultrapure water and the pellets were transferred to a preweighed microcentrifuge tube. The solution was centrifuged, and the supernatant was removed. The cell pellets were frozen overnight and dried for 16 h using a Labconco lyophilizer. The dried pellets and tubes were weighed, and the difference was reported as dry cell weight (DCW; g/L).

4.2.6 Carotenoid extraction

To quantify carotenoids in the cell pellet, 1 mL of culture was centrifuged at 9,000 rpm for 2 minutes in a dark microcentrifuge tube to prevent photodegradation. The supernatant was removed, and the cell pellet was resuspended in 1 mL of a 1:1 volume ratio of ethanol and acetone. The tubes were incubated for 1 hour at room temperature in the dark with occasional

mixing by inversion every 15 minutes. Then, the tubes were centrifuged at 9,000 rpm for 2 minutes to remove cell debris, and 200 μ L was transferred into a 96-well plate.

To quantify carotenoids in the supernatant, 1.0 g of freeze-dried sample and 400 mL/g of a 1:3 volume ratio of acetone and n-hexane were added to the 15 mL falcon tube. The tubes were incubated for 40 minutes at room temperature in the dark with occasional mixing by inversion every 5 minutes. Then, the extracts were washed with 10 mL of aqueous 1.0 M NaCl solution with vigorous shaking for 15 minutes. Subsequently, the tubes were centrifuged at 9,000 rpm for 2 minutes to remove cell debris and left to stand for 15 minutes for the separation of polar and non-polar phases [68]. Then 200 μ L of the polar phase (upper layer) was transferred into a 96-well plate.

The absorbance was read at 475 nm using a plate reader (BioTek). The blank reading was subtracted from the absorbance, and the carotenoid concentration was determined by dividing the absorbance by 0.0816 L/mg [95]. Carotenoid content was determined by dividing the concentration by DCW. All the equations used for data analysis are summarized in Appendix B.

4.3 Results

4.3.1 Lycopene production in the leaky strain

Braun's lipoprotein (LPP) plays a crucial role in maintaining cell envelope integrity by forming covalent cross-links between the outer membrane and peptidoglycan layer [101]. Disruption of the *lpp* gene results in a periplasmic leaky phenotype, which can facilitate continuous lycopene production under appropriate nutrient conditions. This study compares lycopene production in a wild-type strain (JM109) and a *lpp* knock-out (Δlpp) strain, designated as LPP in Figures 4.1 to

4.3, using different combinations of plasmids. The plasmids include p5T7-lycipi-ggpps, which overproduces IDI, GGPPS, CrtI and CrtB; p10-tac-dxs-ispDF, which overproduces DXS, IspD and IspF; and pARA-ispGH, which overproduces IspG and IspH. These plasmids are designated as p5, p10, and pA in Figure 4.1 to 4.3, respectively.

Figure 4.1 shows the dry cell weight (DCW) of the wild-type and leaky strains at 24 and 48 hours with different plasmid insertions. At the 24-hour mark, the cell density of the leaky strain is significantly lower than the wild-type when both p5T7-lycipi-ggpps and pARA-ispGH plasmids are inserted. No significant difference in DCW is observed under other conditions.

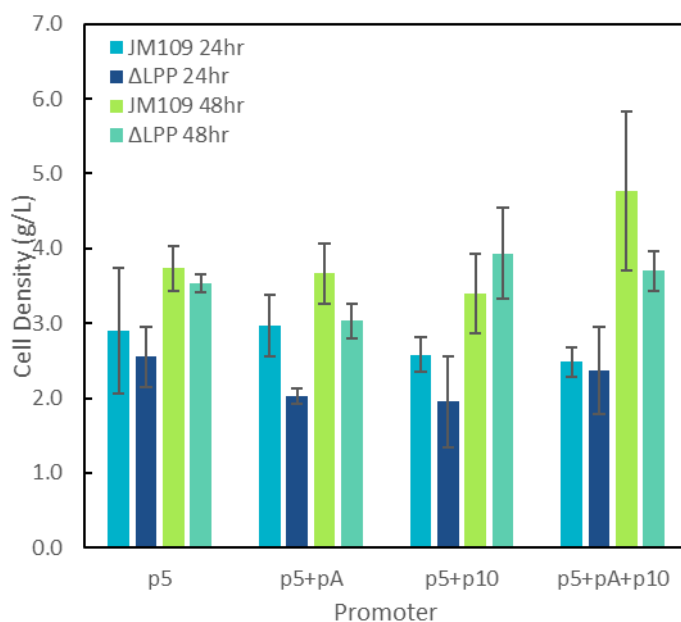


Figure 4.1 Comparison of wild-type strain and Δlpp leaky strain for dry cell weight (g/L).

Figure 4.2A and 4.2B depict intracellular lycopene production extracted from cell pellets. The leaky strain with overexpression of DXS, IspD, and IspF, shows a lower intracellular lycopene content compared to the wild type. However, no significant difference is observed

when proteins IspG and IspH are overexpressed. Figure 4.2C and 4.2D present extracellular lycopene yields, extracted from the supernatant of the cell culture. Both lycopene content and concentration in the supernatant are markedly higher in the leaky strain than in the wild-type strain, except when all three plasmids are inserted simultaneously. These results collectively suggest that knocking out the lipoprotein gene (*lpp*) promotes extracellular lycopene production.

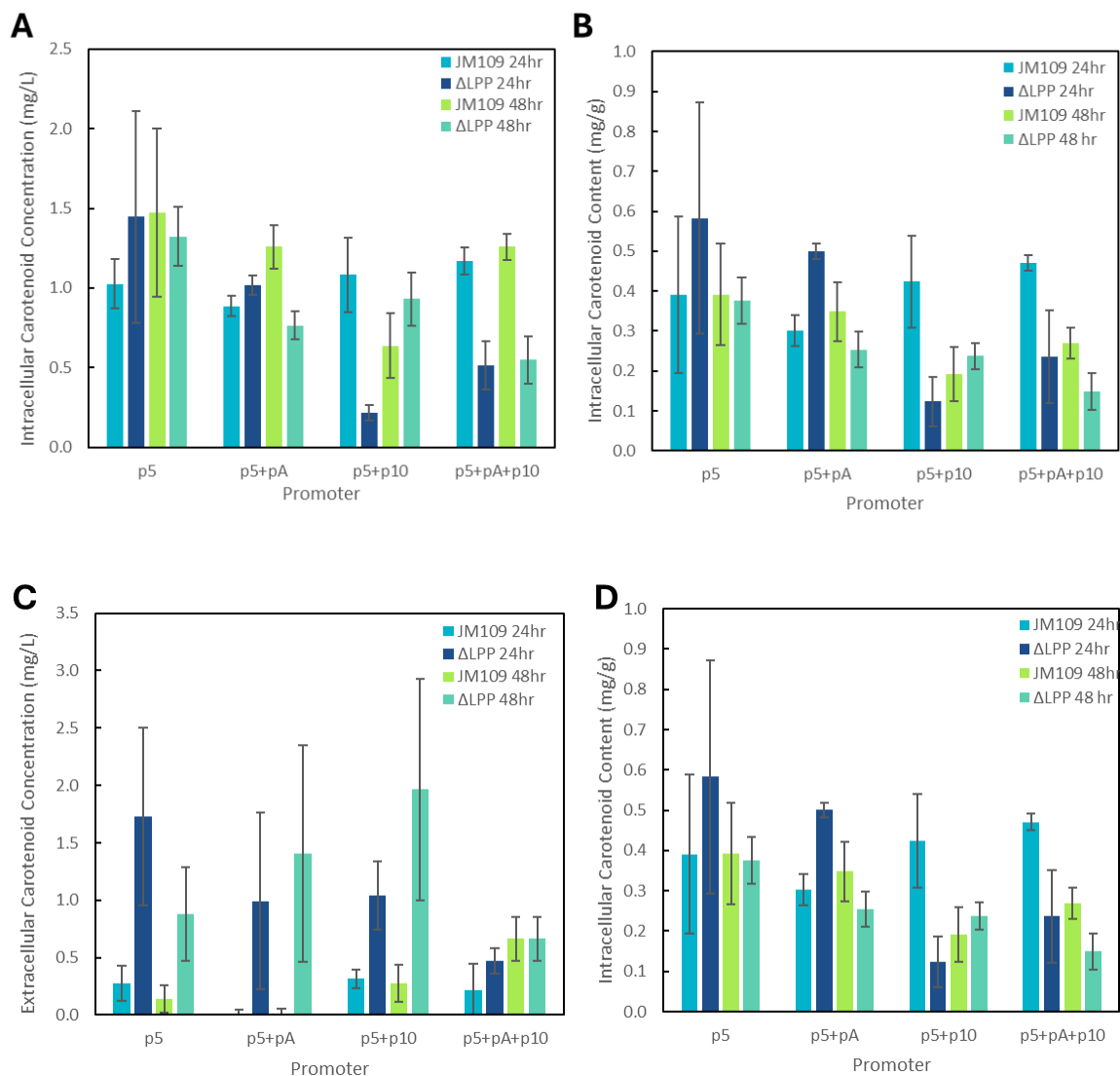


Figure 4.2 Comparison of wild-type strain and Δlpp leaky strain for lycopene production. **A.** Intracellular carotenoid concentration (mg/L) **B.** Intracellular carotenoid content (mg/g) **C.** Extracellular carotenoid concentration (mg/L) **D.** Extracellular carotenoid content (mg/g).

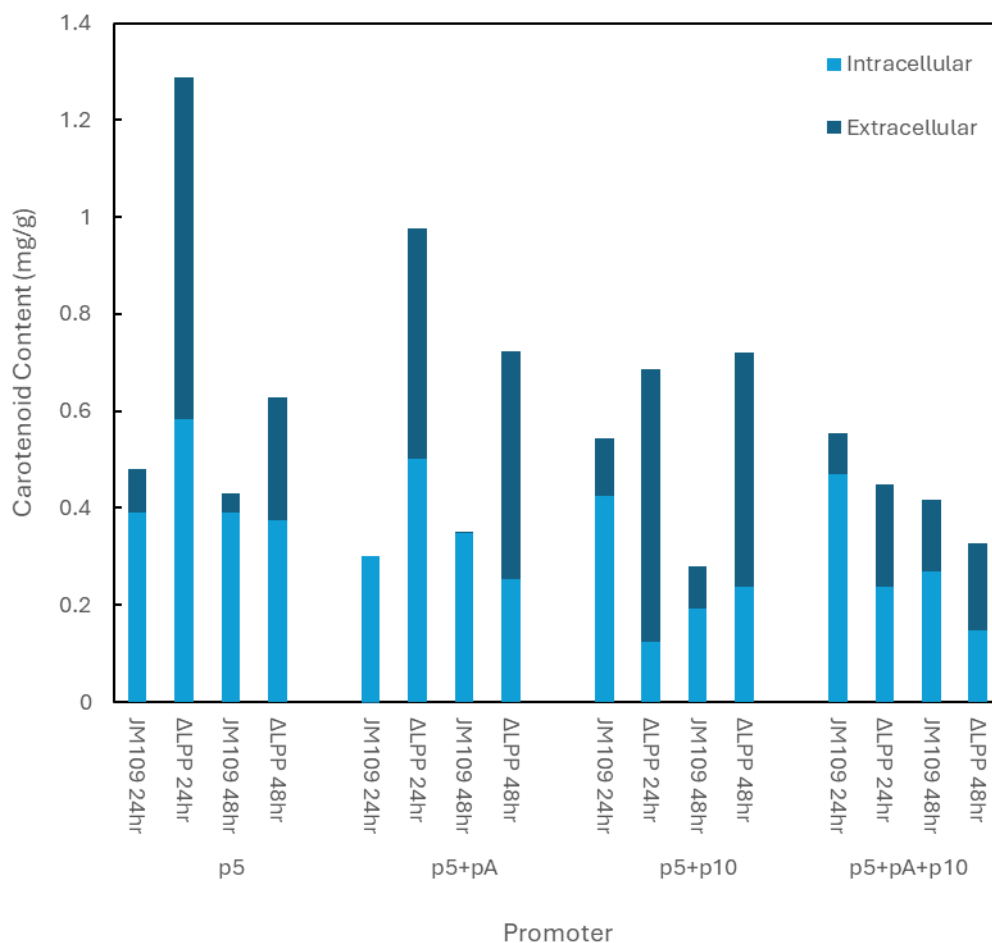


Figure 4.3 Comparison of wild-type strain and Δlpp leaky strain for total carotenoid content (mg/g). p5 represents plasmid p5T7-lycipi-ggpps, p10 represents plasmid p10-tac-dxs-ispDF, pA represents plasmid pARA-ispGH.

Figure 4.3 shows the total carotenoid content yield. The graph indicates higher overall lycopene production in the leaky strain with plasmid combinations of p5T7-lycipi-ggpps, p5T7-lycipi-ggpps + p10-tac-dxs-ispDF, and p5T7-lycipi-ggpps + pARA-ispGH.

The findings indicate no increase in extracellular lycopene production or overall lycopene yield when all three plasmids are inserted into the leaky strain compared to the wild-type strain. Moreover, adding p10-tac-dxs-ispDF or pARA-ispGH plasmids did not significantly enhance lycopene yield compared to the insertion of p5T7-lycipi-ggpps alone. This might be attributed to the fact that IspG and IspH are iron-sulfur proteins requiring specific twin-arginine translocation (Tat) signal peptides to be transported from the cytoplasm to the periplasmic space.

4.4 Concluding Remarks

In summary, the knock-out of the lipoprotein gene (*lpp*) leads to a maximum 3.3-fold increase in lycopene yield. Overexpressing IDI, GGPPS, CrtI, and CrtB via the p5T7-lycipi-ggpps plasmid, as well as combining this plasmid with p10-tac-dxs-ispDF (overexpressing DXS, IspD, and IspF) or pARA-ispGH (overexpressing IspG and IspH), enhances lycopene production in the leaky strain. However, no significant difference in lycopene production is observed when all three plasmids are used simultaneously in the leaky strain compared to the wild-type strain.

Chapter 5 – Conclusions and Future Recommendations

5.1 Conclusions

This research investigated the potential for both intracellular and extracellular overproduction of isoprenoids, specifically focusing on lycopene. By implementing an alternative optimized pathway, known as the IUP, instead of the traditional MEP pathway in *E. coli*, significant improvements were observed. This optimization involved converting isoprenol and/or prenol into IPP and DMAPP, which are crucial intermediates in lycopene biosynthesis. Additionally, the expression of MGS and DGS from *Acholeplasma laidlawii* induced intracellular lipid production. Although this slightly altered the lipid profile of the *E. coli* strains and resulted in minor increases in lipid content, it did not enhance carotenoid production beyond the levels achieved by strains expressing the IUP. For extracellular lycopene production, knocking out the lipoprotein gene (*lpp*) resulted in up to a 3.3-fold increase in lycopene yield. Furthermore, overexpressing MEP pathway-related enzymes, including IDI, GGPPS, CrtI, CrtB, DXS, IspD, IspF, IspG, and IspH—significantly boosted lycopene production in the leaky strain.

5.2 Future Recommendations

5.2.1 Short-term recommendations

Following the successful insertion of N-terminal signal peptides into the genes *ispG* and *ispH* to target the periplasmic space, it is essential to repeat the carotenoid extraction and analysis described in Section 4.3 using the newly constructed plasmid. This modification is anticipated to

result in a substantial increase in extracellular lycopene production in the supernatant. Carotenoids should be further analyzed and quantified utilizing HPLC to ensure accurate measurement. Additionally, the overexpression of proteins DXS, IspD, IspF, IspG, and IspH should be validated through Western blot analysis to confirm successful gene expression.

5.2.2 Long-term recommendations

The impact of other genes that can induce outer membrane leakage, such as *pal*, *mrcA*, and *mrcB*, should be assessed and compared with the current results obtained from the *lpp* knock-out experiment regarding lycopene production. Moreover, different combinations of these gene knock-outs should be explored to identify the most effective genetic modifications.

The strategies employed in this research for engineering the membrane to enhance carotenoid production, specifically lycopene and β -carotene, should be extended to the production of other carotenoids that possess higher commercial or medical value, such as cannabinoids. Furthermore, these membrane engineering strategies should be adapted and applied to other organisms, including microalgae and yeast, to diversify the scope of bioengineering applications.

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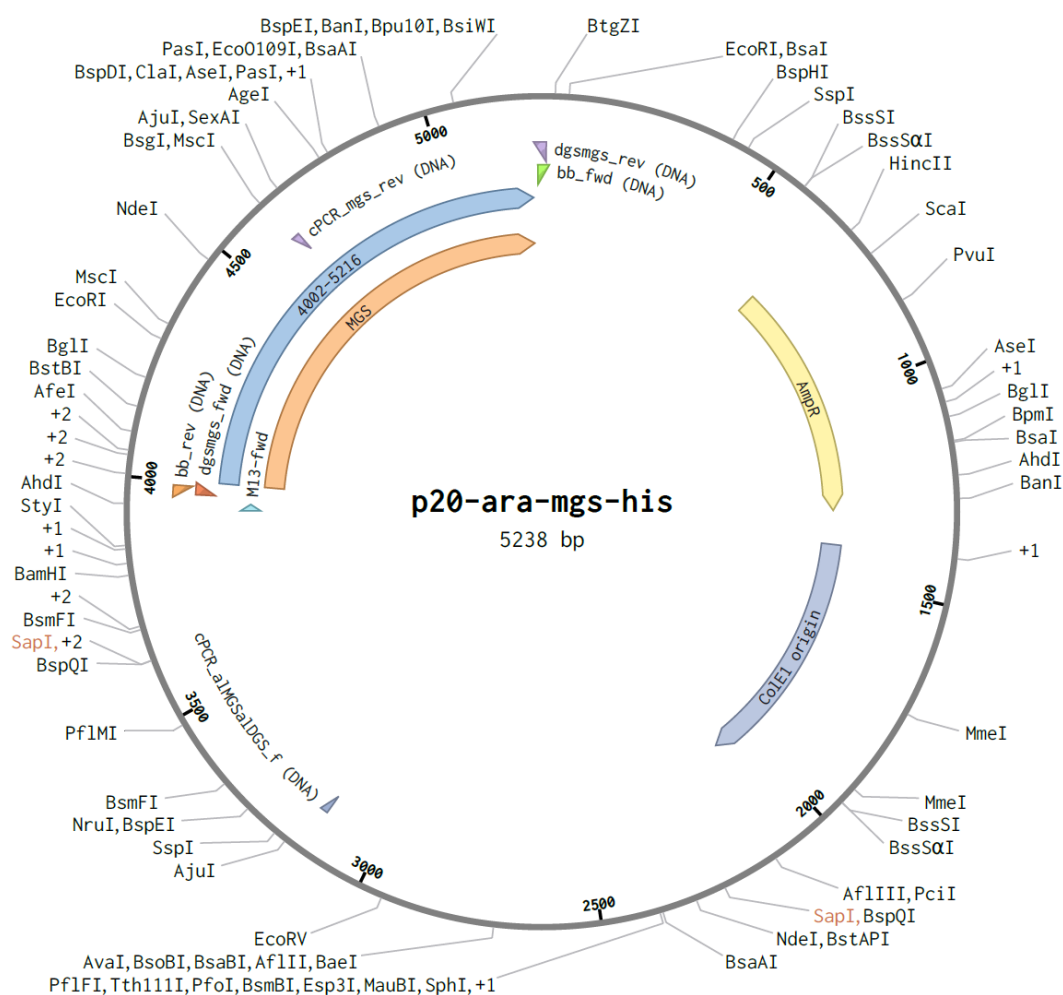
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Appendices

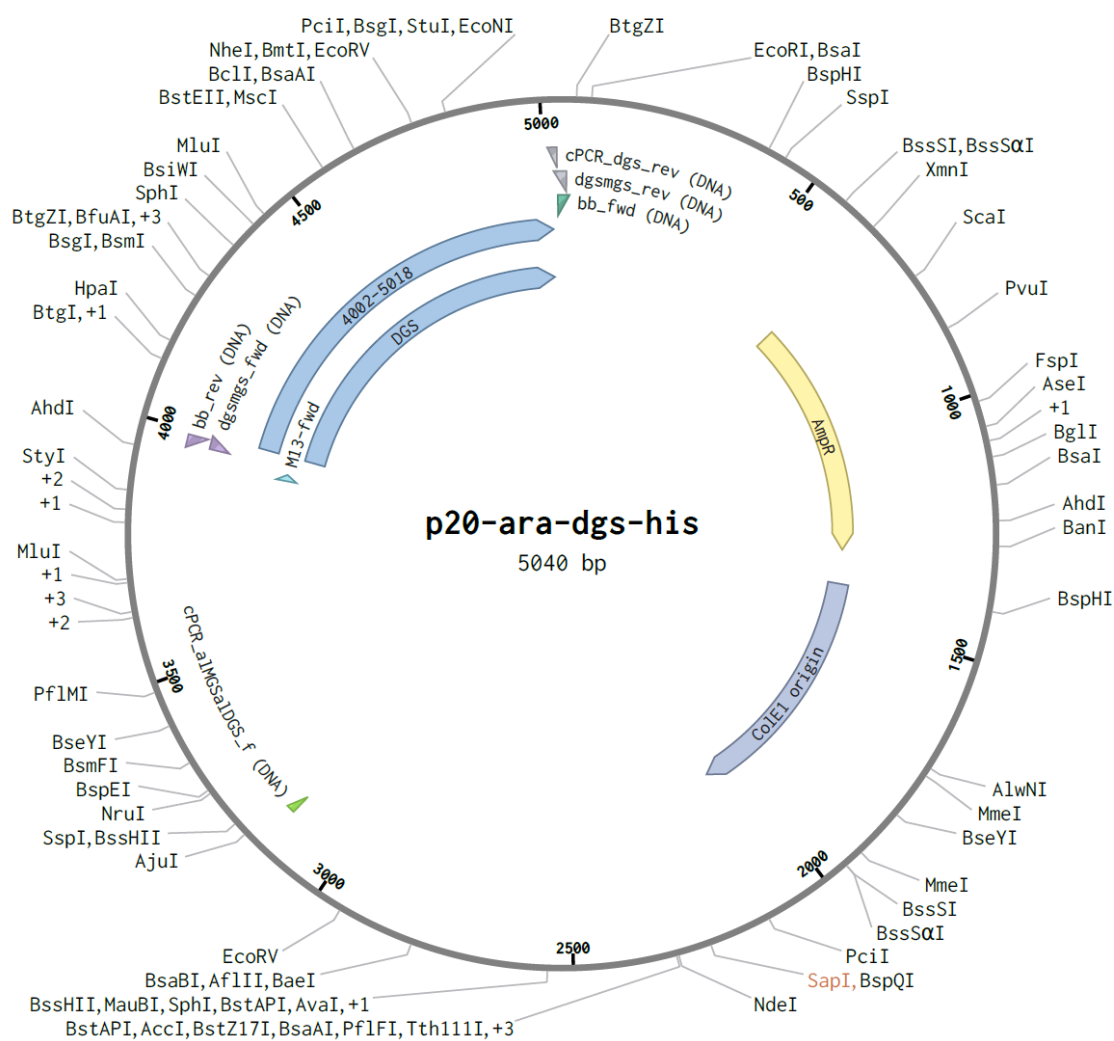
Appendix A includes maps of the plasmids utilized in this research, specifically p20-ara-mgs-his, p20-ara-dgs-his, p5T7-lycipi-ggpps, pCBFD1, pAC-BETAipi, pSEVA228-pro4IUP, pJOE 7706.1, p10-tac-dxs-ispDF, and pARA-ispGH, as well as synthesized gene sequences, including alMGS and alDGS, that were employed in this study. Appendix B summarizes all the equations used for data analysis. Appendix C demonstrates representative curves from FAME quantification by GC. Appendix D contains the raw data from the analysis of microscopic images using the Ilastik application. Finally, Appendix E provides information on the paper that has been published.

Appendix A: Plasmid Maps and Sequence Maps

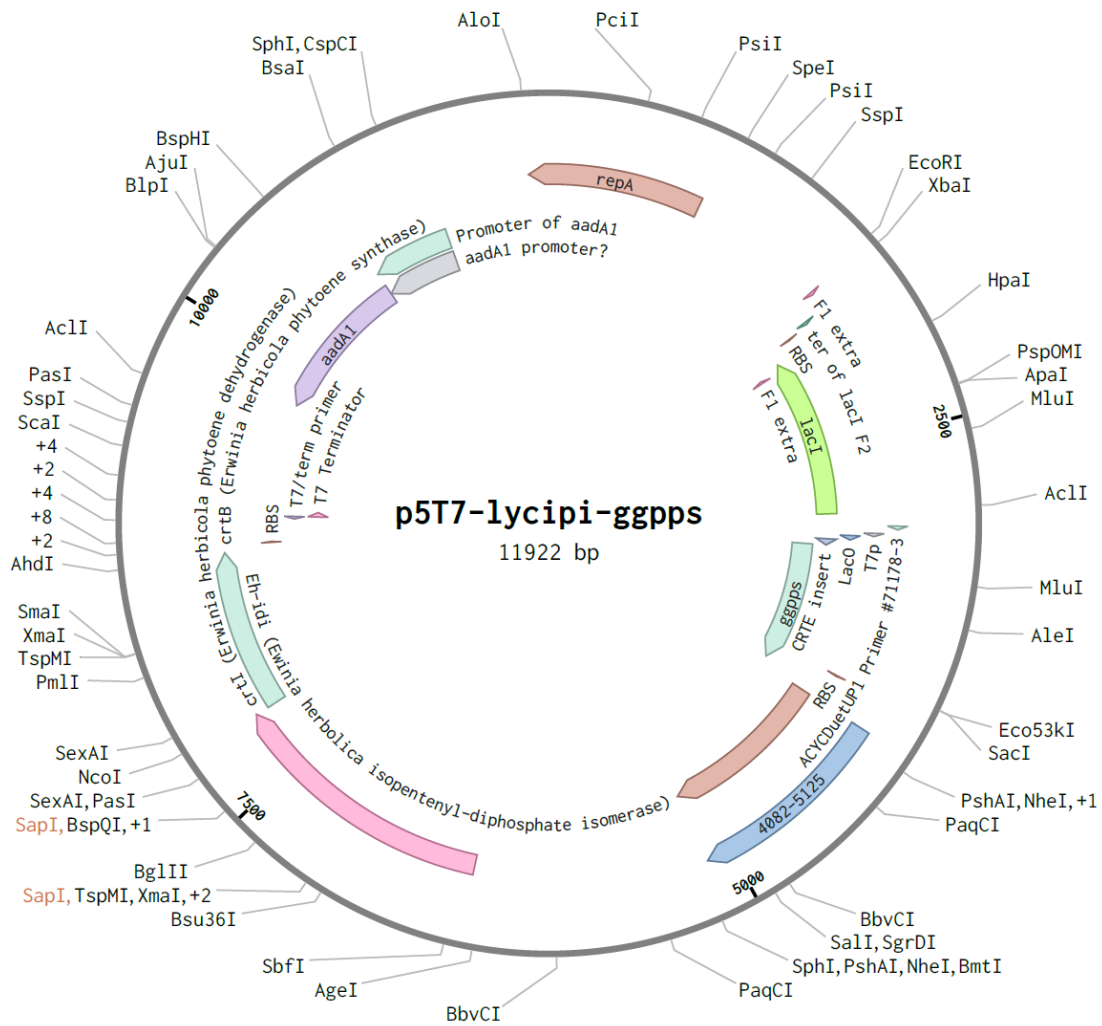
p20-ara-mgs-his (5238 bp)



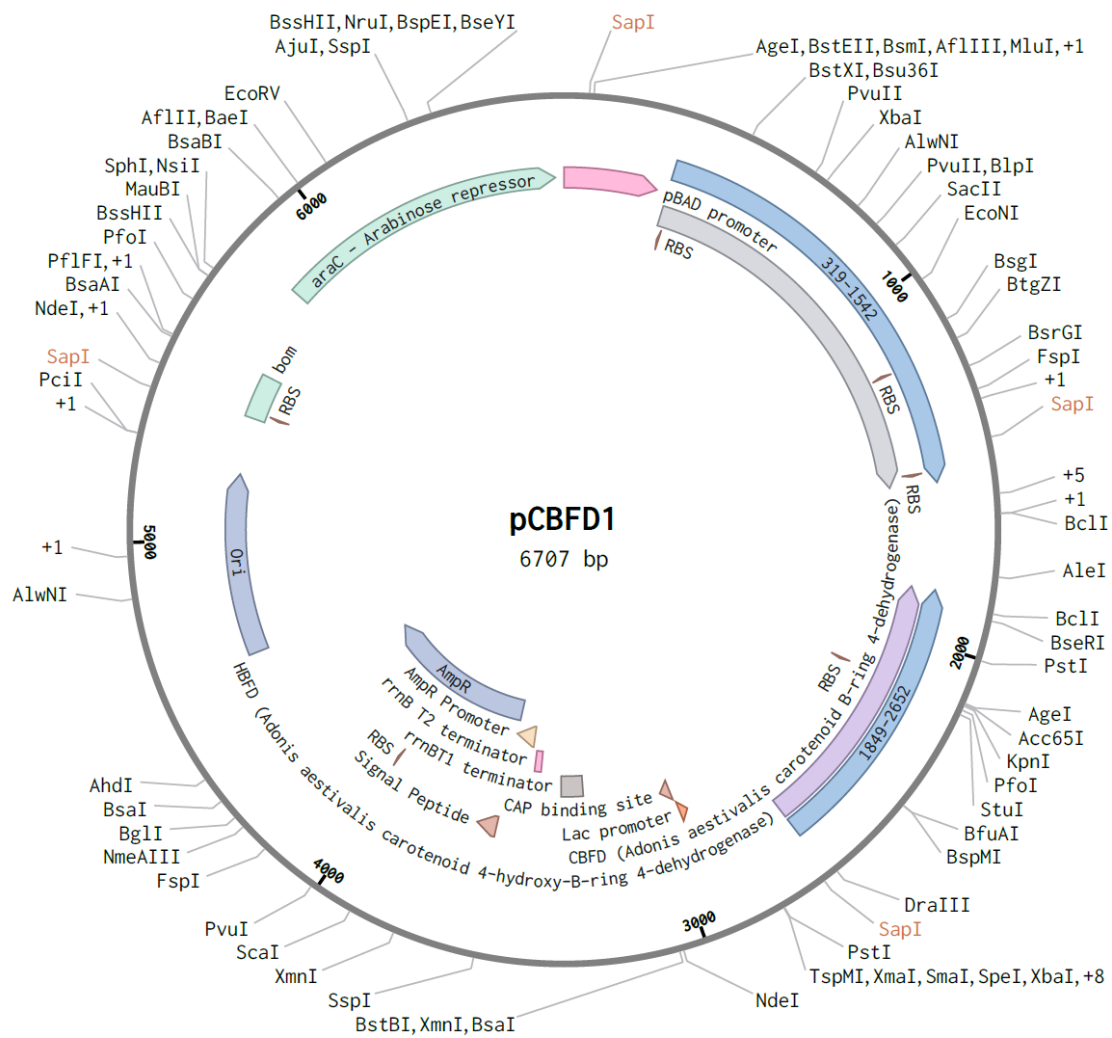
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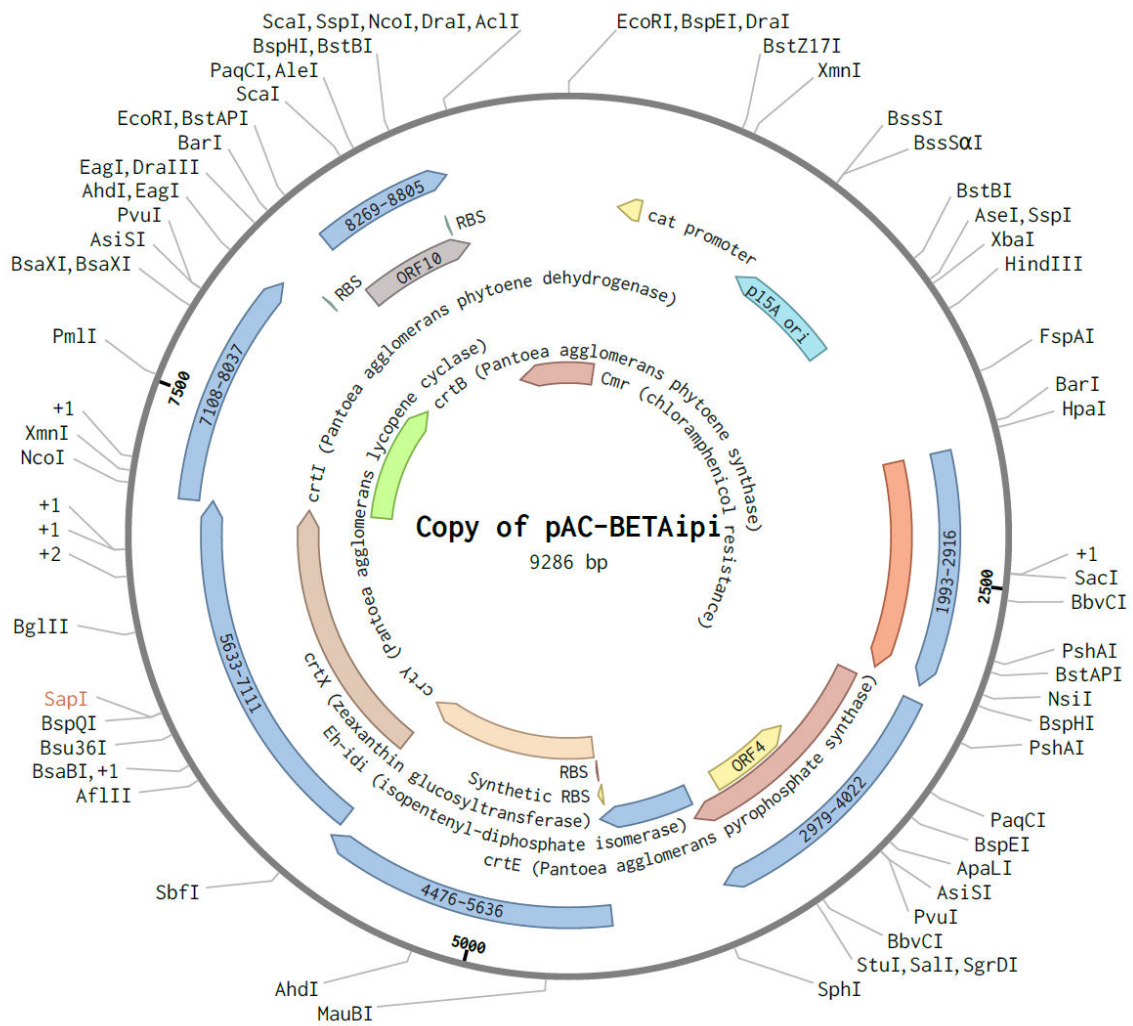
p5T7-lycipi-ggpps (11922 bp)



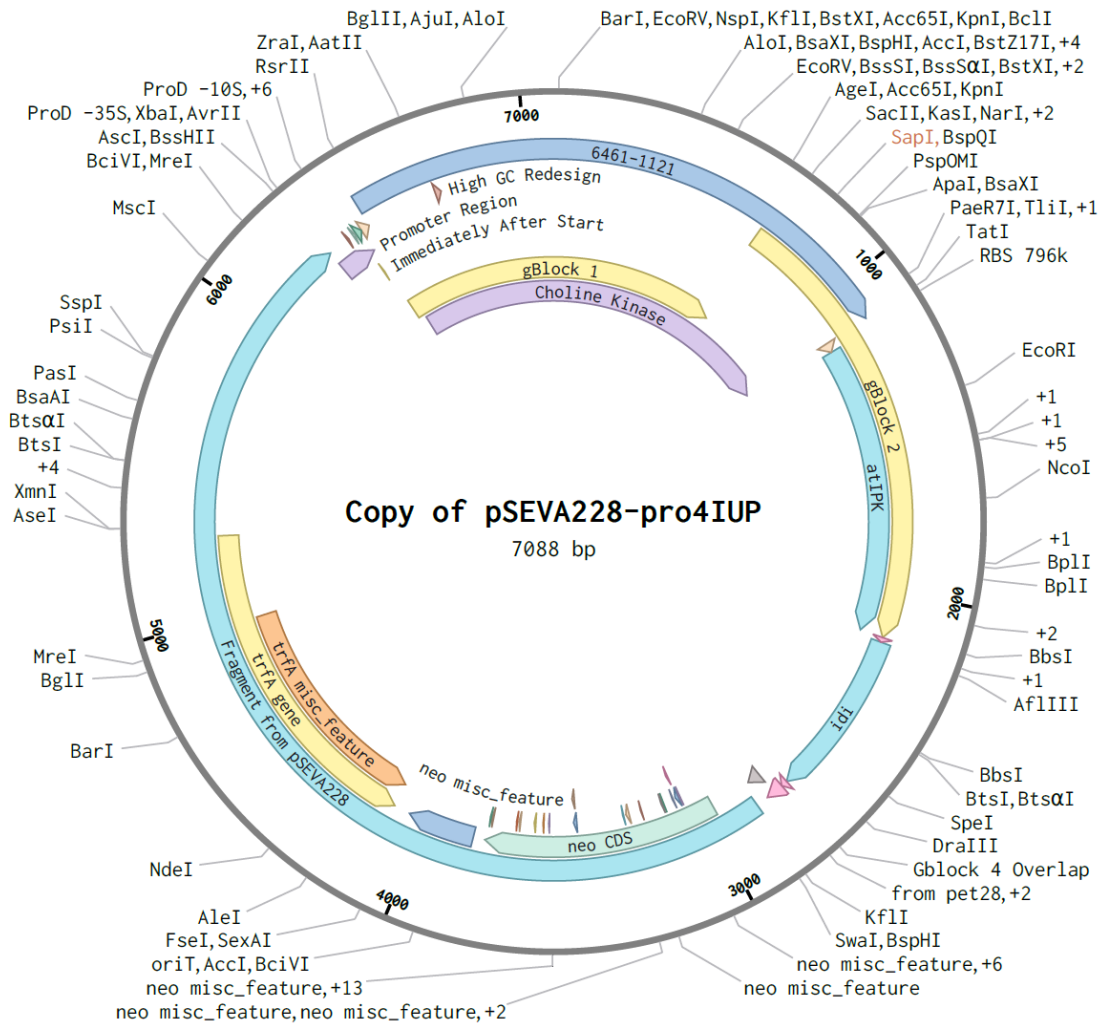
pCBFD1 (6707 bp)



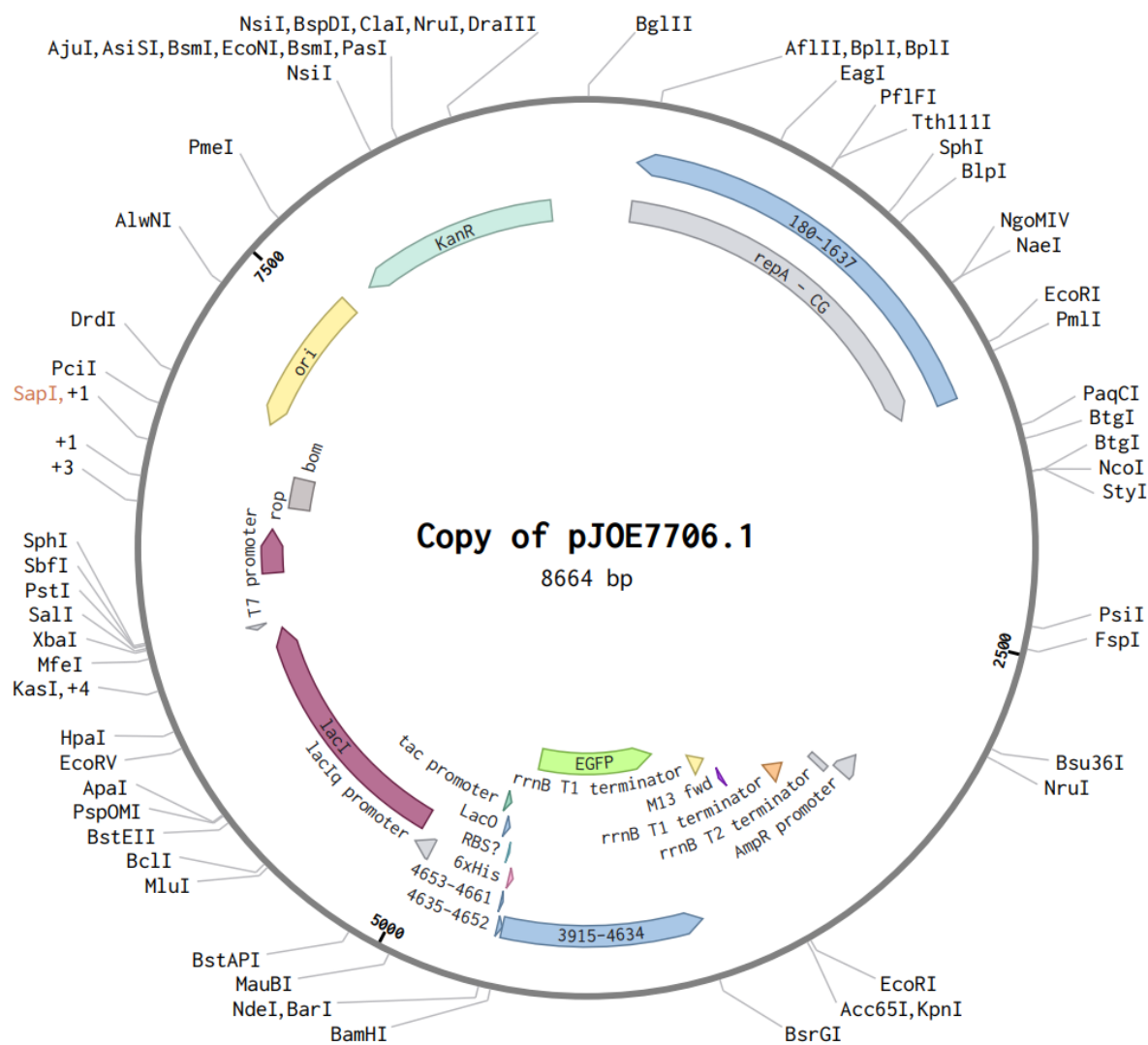
Copy of pAC-BETAipi (9286 bp)



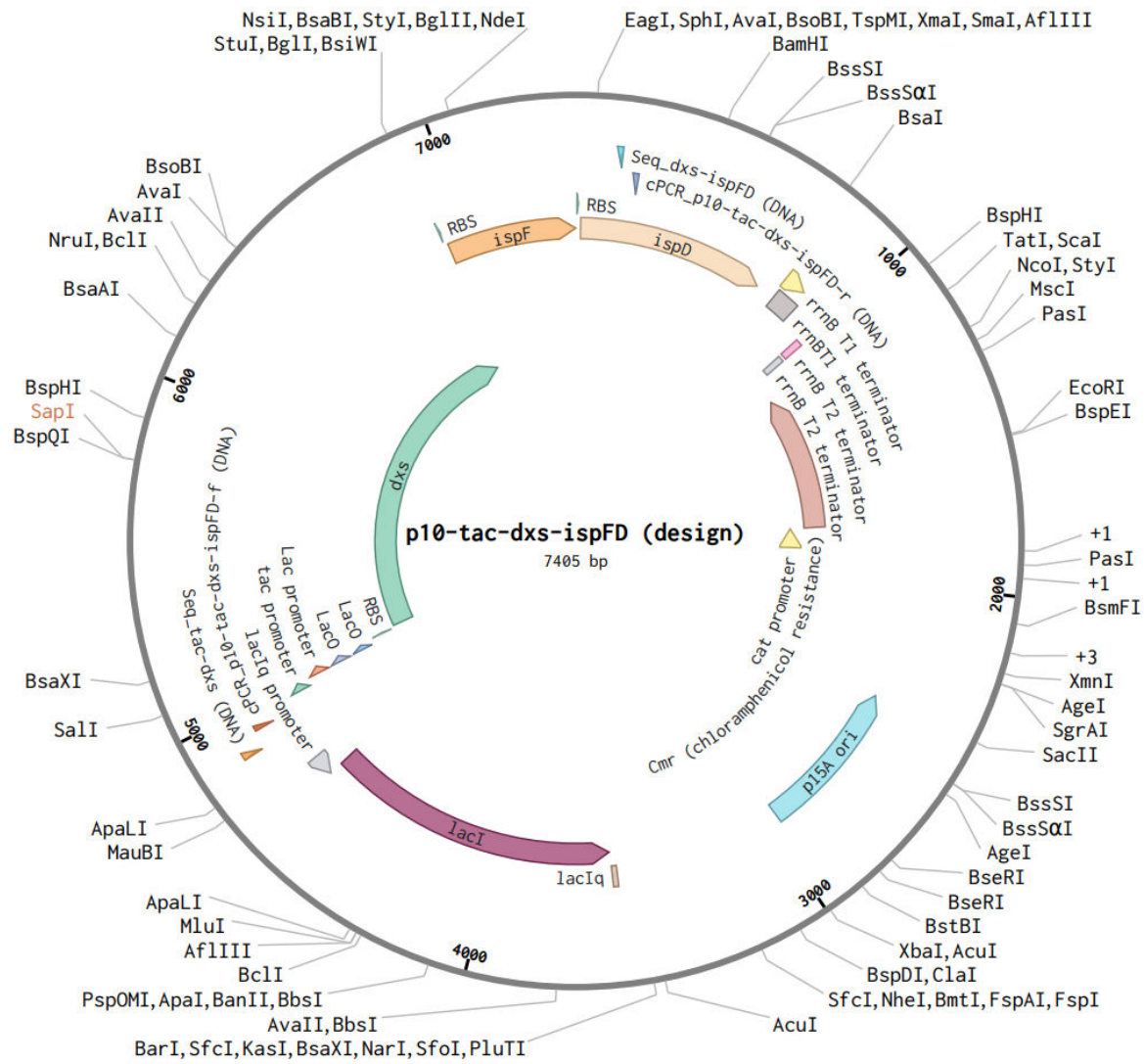
Copy of pSEVA228-pro4IUP (7088 bp)



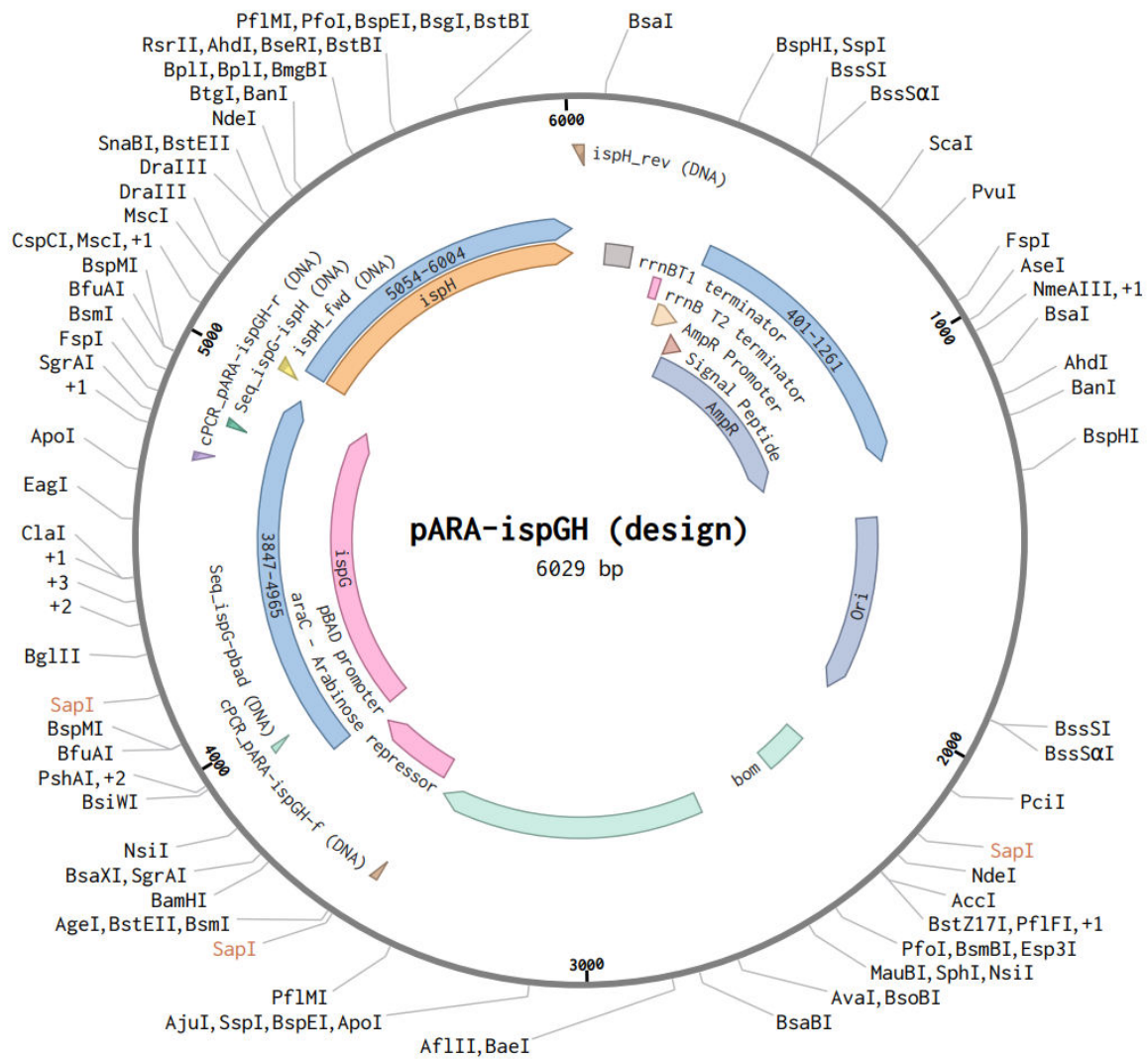
Copy of pJOE7706.1 (8664 bp)



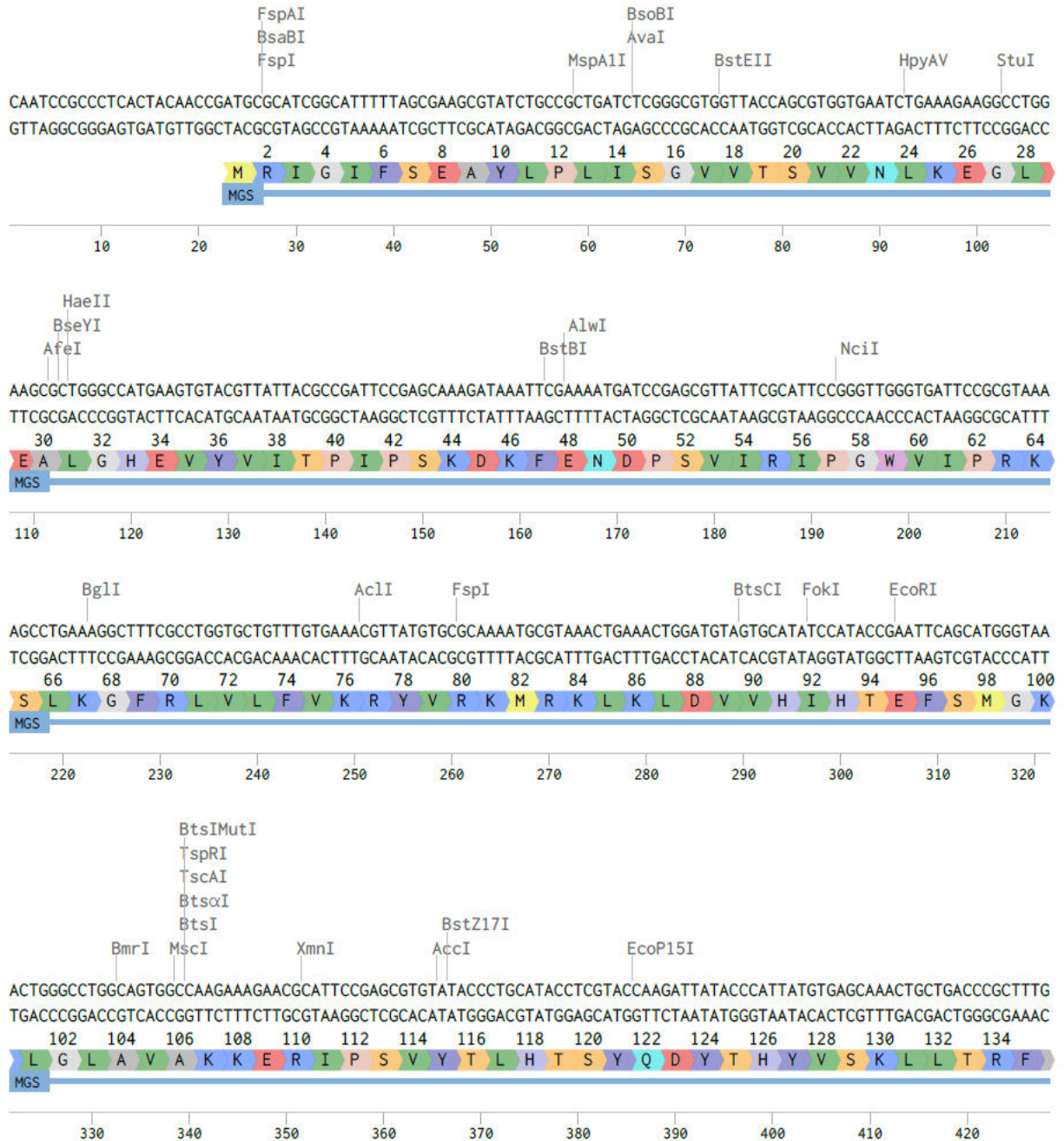
p10-tac-dxs-ispFD (design) (7405 bp)

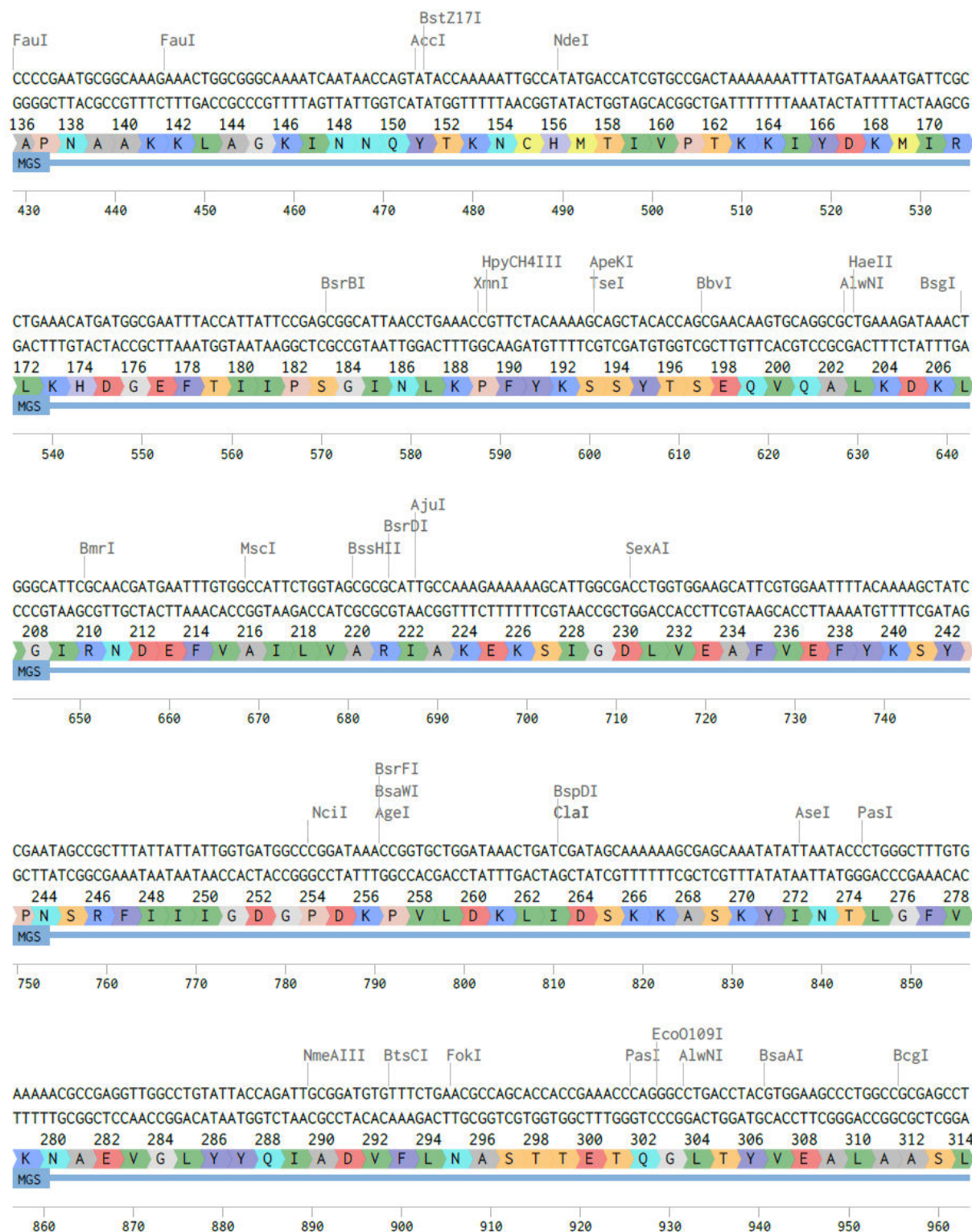


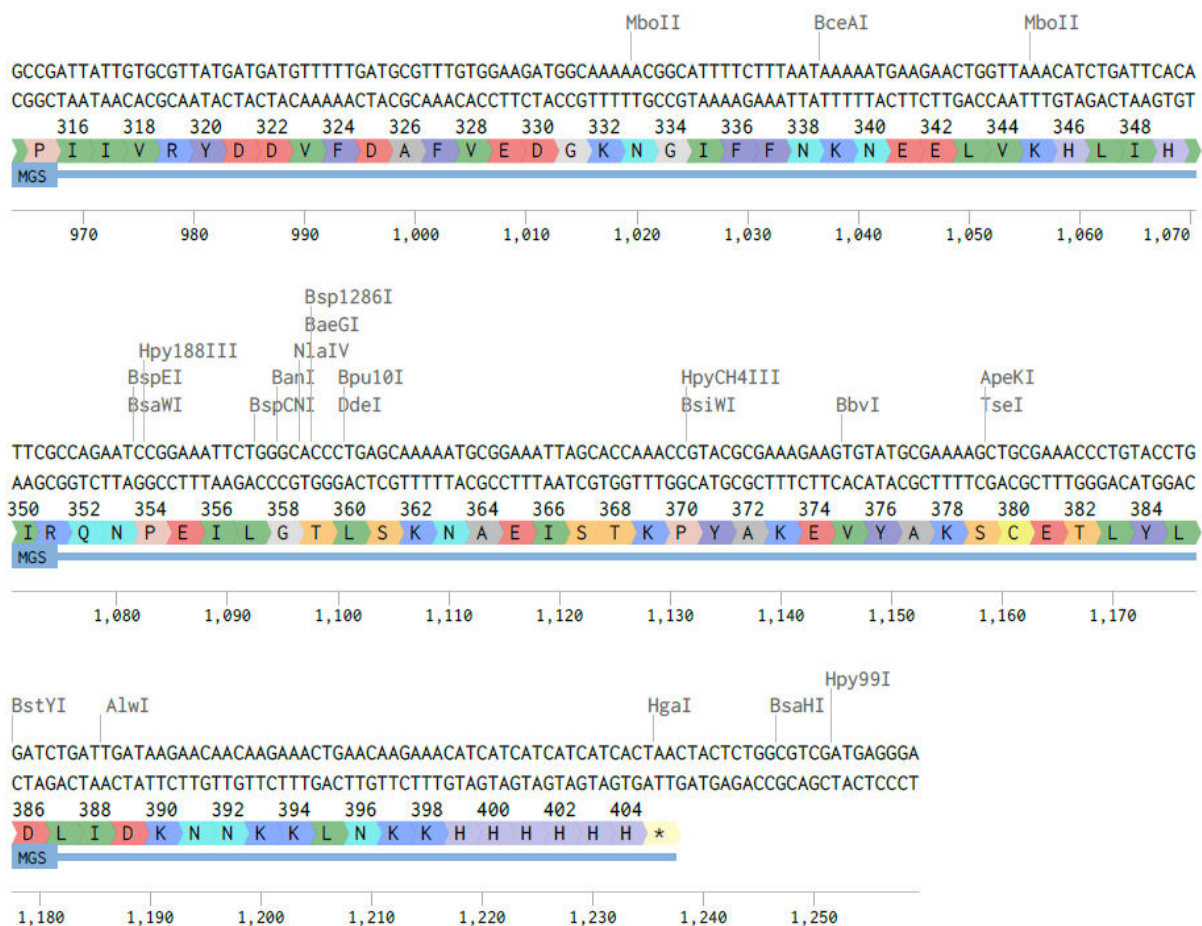
pARA-ispGH (design) (6029 bp)



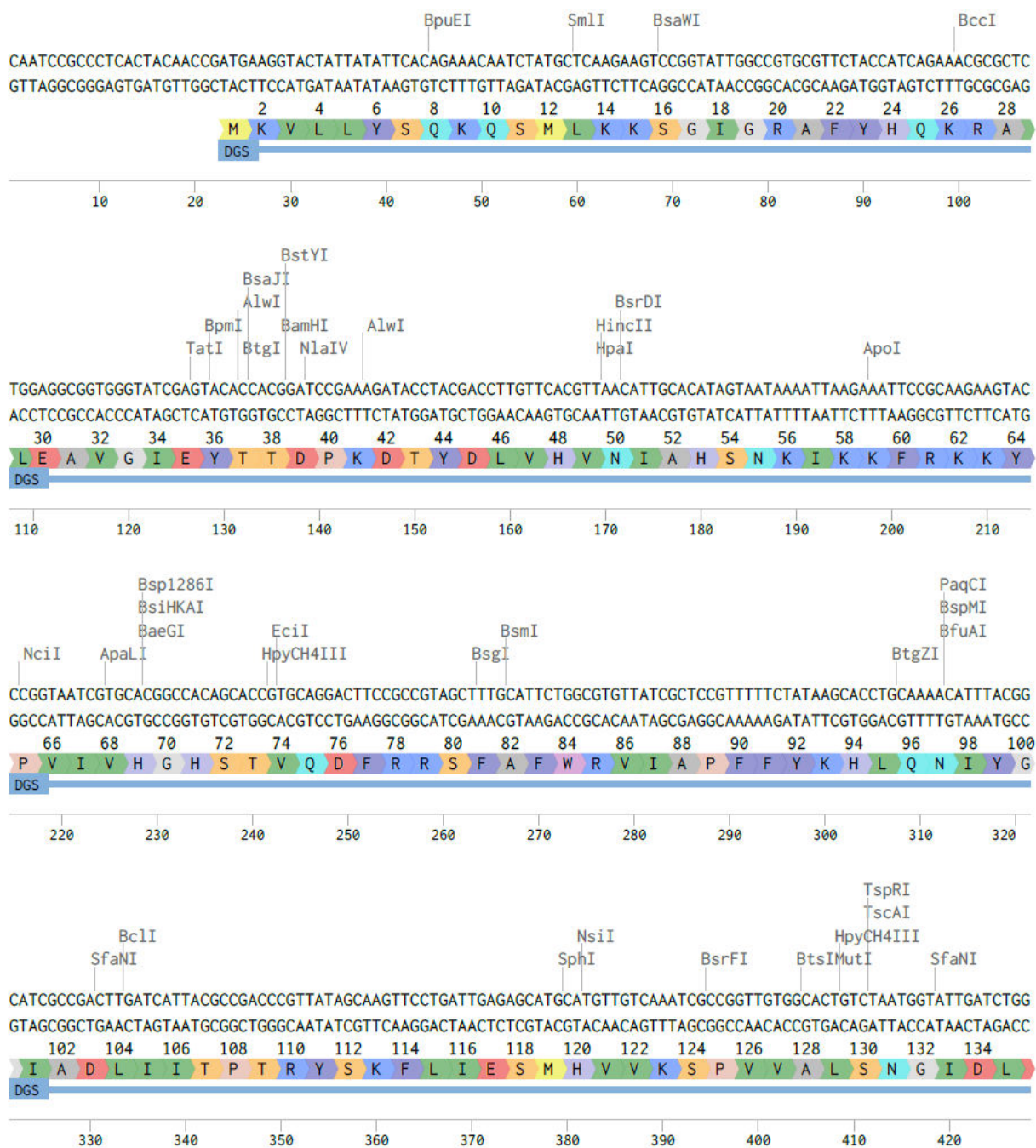
alMGS fragment from Twist (1259 bp)

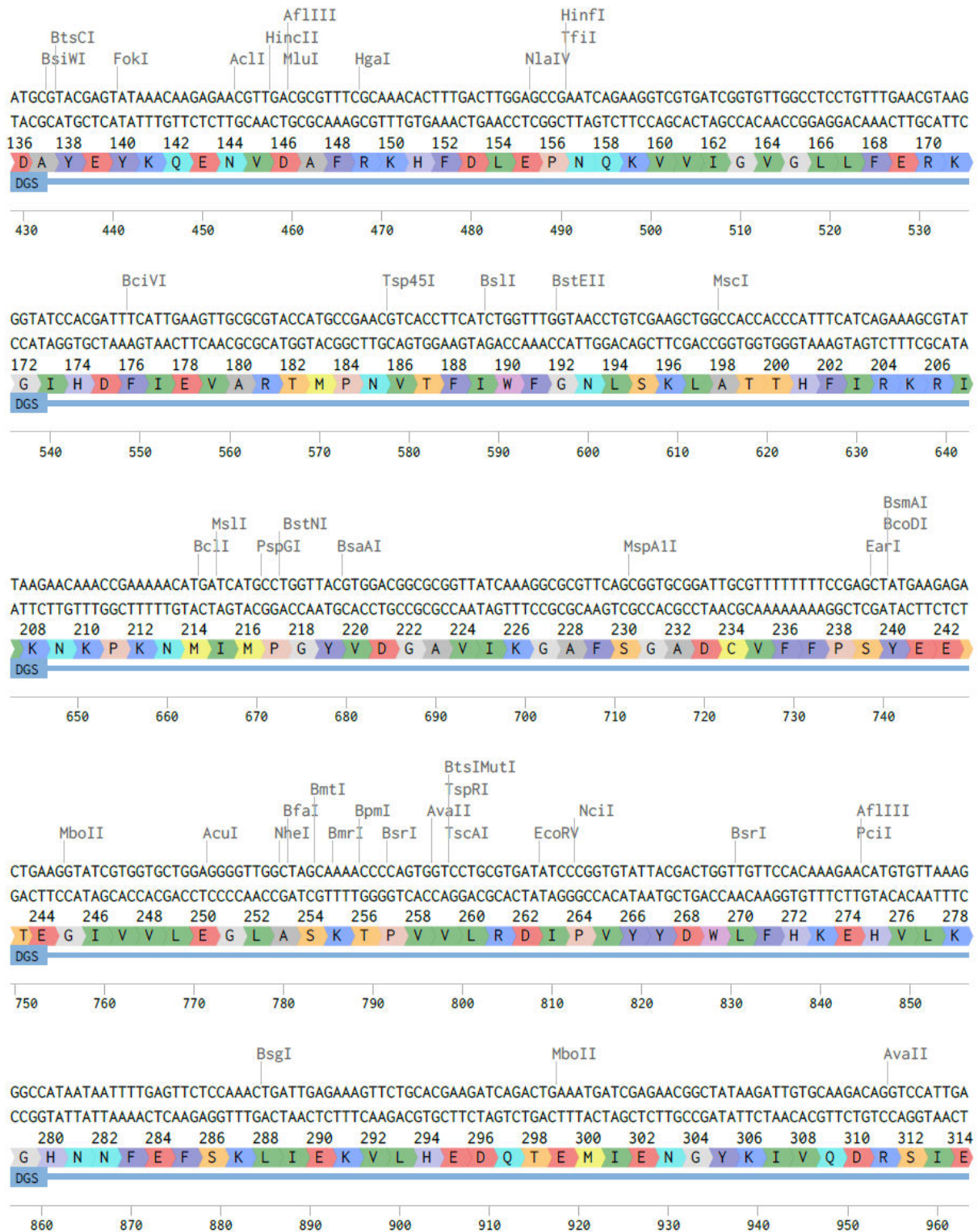


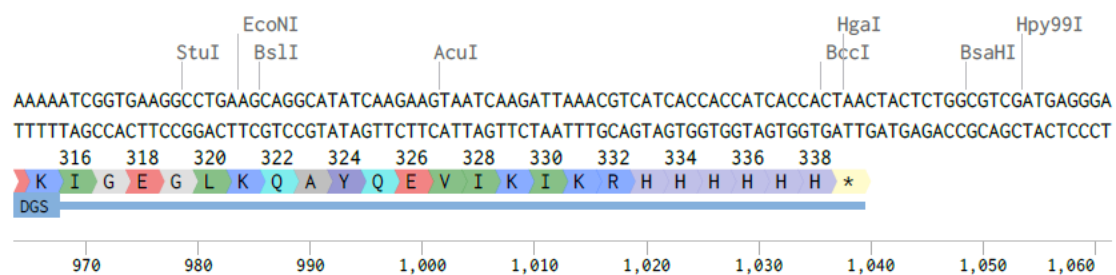




alDGS fragment from Twist (1061 bp)







Appendix B: Equations for Data Analysis

E1: Dry Cell Weight

$$Cell\ Density(g/L) = \frac{Tube\ With\ Pellet\ Weight(g) - Empty\ Tube\ Weight(g)}{0.002L}$$

E2: Carotenoid Concentration [95]

$$Carotenoid\ Concentration\ (mg/L) = \frac{A_{475nm\ sample} - A_{475nm\ blank}}{0.0816L/mg}$$

E3: Carotenoid Content

$$Carotenoid\ Content(mg/g) = \frac{Carotenoid\ Concentration(mg/L)}{Cell\ Density(g/L)}$$

E4: FAME Extraction Efficiency

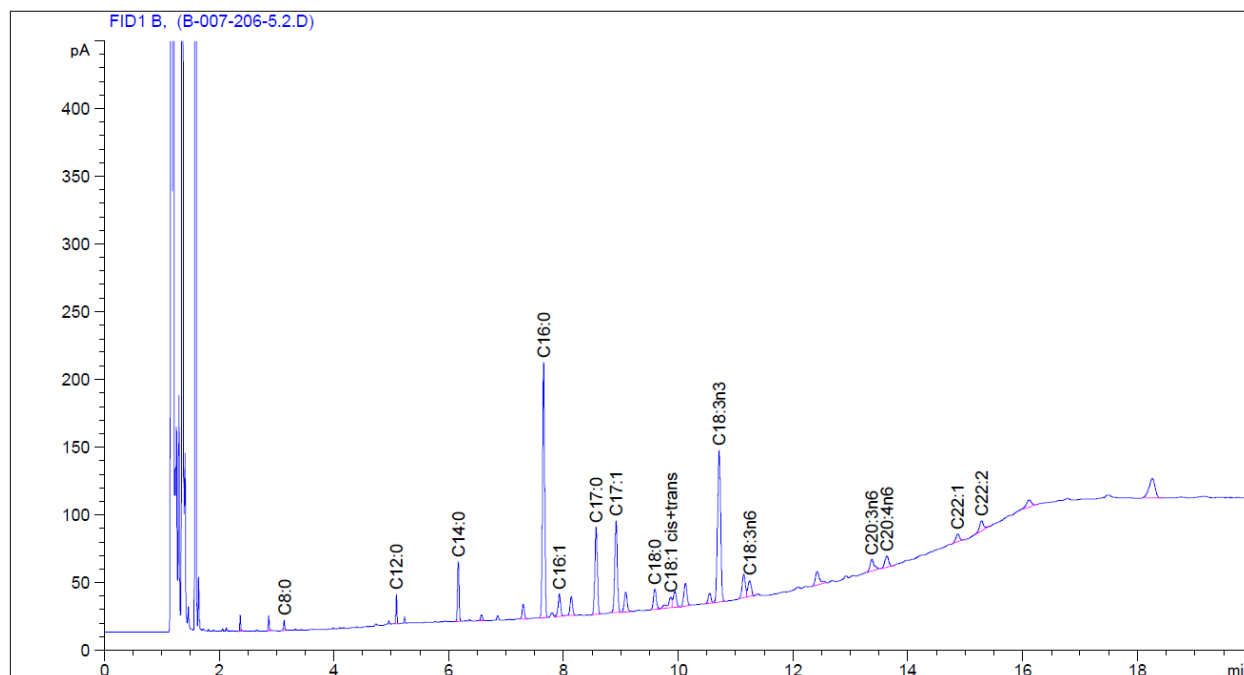
$$FAME\ Extraction\ Efficiency = \frac{[C19:0]_{Measured}}{[C19:0]_{Standard}} \times 100\%$$

E5: FAME Content

$$FAME\ Content(\% \text{ weight}) = \frac{[FAME]_{Total} - [C19:0]_{Measured} - [C17:0]}{Extraction\ Efficiency \times Sample\ Weight} \times 100\%$$

Appendix C: Representative FAME Curves

Figure below presents representative FAME quantification curves obtained through GC analysis. The sample analyzed is *E. coli* MG1655 (DE3) harboring the plasmid p20-ara-mgs-his, which was induced with 5 g/L of arabinose. The detailed analytical results are discussed in Section 3.4.2.



Appendix D: Raw Data from Analysis of Microscopic Images

# of Cells	WT		Recombinants	
	MajorAxisLength	MinorAxisLength	MajorAxisLength	MinorAxisLength
1	17.62466	8.210163	19.80401	6.888976
2	19.77943	8.548619	14.62185	7.744738
3	12.42931	7.816616	31.89995	7.1766
4	13.83318	10.34182	14.78114	7.717737
5	19.06305	8.251499	17.49556	7.732461
6	22.97873	9.118274	16.17546	7.398299
7	23.19634	9.747306	15.54488	6.598232
8	14.62711	8.259662	26.11408	9.360259
9	14.60947	8.164141	12.61346	6.868584
10	15.66021	8.519127	20.483	7.794856
11	15.86118	8.281662	22.44444	6.934472
12	15.12425	8.09149	12.77929	9.427233
13	15.15276	8.718325	12.86323	8.214005
14	18.96264	11.88549	21.00679	7.491207
15	22.60614	8.212236	21.70605	7.679578
16	18.67099	8.542301	23.67284	6.953426
17	17.99413	8.210432	18.59743	7.105914
18	22.67015	9.310166	15.33312	8.093103
19	16.29111	8.022689	14.03151	7.298858
20	14.86962	8.418001	14.95725	8.081386
21	13.19272	8.514429	15.15608	7.525686
22	14.90047	8.210987	18.16848	7.653
23	19.29894	8.128137	11.00607	9.172846
24	15.50269	8.627646	24.94961	7.783533
25	18.35027	8.82837	10.18374	9.314373
26	14.86505	10.87003	24.58637	7.059913
27	21.21162	8.640139	12.91379	7.07025
28	20.08088	8.382622	15.67426	8.070102
29	20.09074	8.458479	14.00313	6.799945
30	12.8217	8.741392	12.21568	11.76622
31	15.42951	8.729465	32.93082	7.460722
32	17.8039	7.668429	21.4743	7.953311
33	15.99883	8.85201	14.85502	7.632811
34	22.6362	14.09514	11.84713	7.370676
35	12.4266	8.033733	13.27434	7.861009
36	12.07701	8.110201	13.07046	7.684351
37	13.47509	8.782168	22.5051	7.662068
38	18.73601	8.69665	20.84999	7.024965
39	13.11875	7.952161	15.56154	7.400737
40	20.92303	8.186685	12.01968	6.79786

41	10.96993	8.558631	15.8907	7.691802
42	14.14916	8.474958	14.41696	7.843172
43	14.04293	8.564109	9.53129	8.53727
44	14.16435	8.159981	25.74171	7.168699
45	14.69824	8.765105	13.08065	8.306938
46	19.82322	8.339358	16.67327	7.888747
47	16.6668	8.42878	18.51392	7.10915
48	20.80069	8.668589	17.73825	7.538421
49	15.1554	8.219388	20.17555	9.212042
50	23.81388	9.203029	23.25088	6.760973
51	20.89201	8.062239	26.50137	6.886317
52	19.82438	9.198573	21.25807	9.877513
53	19.06304	8.70575	24.53267	12.01509
54	12.75106	8.281178	23.01609	5.445612
55	20.87247	8.825486	19.08681	7.636805
56	12.98361	8.821116	22.10006	6.977122
57	18.26639	8.12792	15.51111	6.756955
58	19.47618	8.539588	15.46238	7.59911
59	12.65185	8.442337	26.06469	6.591558
60	12.65927	9.231114	20.79892	13.33106
61	13.32621	9.071834	22.10903	7.311668
62	22.47131	8.196407	15.59151	7.624902
63	13.29946	8.697942	19.73944	7.233878
64	27.51814	8.901601	16.17571	8.131945
65			19.54545	7.598199
66			17.02709	6.592617
67			27.83625	8.324791
68			16.62053	6.684274
69			19.29765	6.728978
70			20.02457	10.64299
71			19.29573	6.984359
72			11.75167	7.337306
73			16.32346	7.097869
74			13.72301	6.670073
75			23.62684	6.915843
76			17.91761	6.89863
77			28.41417	6.796553
78			20.83281	6.85198
79			15.72876	7.711615
80			21.72451	7.451685
81			12.41138	7.243988
82			13.0071	6.92712
83			12.33449	7.105871
84			16.30852	5.996569

85	23.2439	7.45758
86	18.22034	6.633466
87	20.46994	6.758087