CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION

Miss Charintip Yenyuvadee

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry and Molecular Biology
Department of Biochemistry
FACULTY OF SCIENCE
Chulalongkorn University
Academic Year 2019

การแสดงออกร่วมของเอนไซม์ที่ต้านทานการเกิดการยับยั้งแบบย้อนกลับในวิถีการสังเคราะห์ฟีนิลอะลานีนเพื่อเพิ่มการผลิตฟีนิลอะลานีน

น.ส.ชรินทร์ทิพย์ เย็นยุวดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมีและชีววิทยาโมเลกุล ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION
By	Miss Charintip Yenyuvadee
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Associate Professor KANOKTIP PACKDIBAMRUNG, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor TEERAPONG BUABOOCHA, Ph.D.)

..... Thesis Advisor
(Associate Professor KANOKTIP PACKDIBAMRUNG, Ph.D.)

..... Examiner
(Assistant Professor Rath Pichyangkura, Ph.D.)

..... External Examiner
(Assistant Professor Ratree Wongpanya, Ph.D.)

ชรินทร์ทิพย์ เย็นยุวดี : การแสดงออกร่วมของเอนไซม์ที่ต้านทานการเกิดการยับยั้งแบบย้อนกลับในวิถีการสังเคราะห์ฟีนิลอะลานีนเพื่อเพิ่มการผลิตฟีนิลอะลานีน. (CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION) อ.ที่ปรึกษาหลัก : รศ. ดร.กนกทิพย์ ภักดีบำรุง

แอล-ฟีนิลอะลานีน (L-Phe) เป็นกรดอะมิโนจำเป็นที่มีความสำคัญเชิงพาณิชย์ที่ถูกนำมาใช้อย่างแพร่หลายในอุตสาหกรรมอาหารและยา ในปัจจุบันมีความต้องการของแอล-ฟีนิลอะลานีนสูงขึ้นตามความต้องการในการผลิตแอสปาแตมซึ่งเป็นสารให้ความหวานแทนน้ำตาลที่มีแคลอรี่ต่ำ ใน *Escherichia coli* การสังเคราะห์แอล-ฟีนิลอะลานีนถูกควบคุมหลายลำดับขั้น ไอโซฟอร์ม AroG ของ 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) และ chorismate mutase/prephenate dehydratase (PheA) เป็นเอนไซม์ที่สำคัญ 2 ชนิด ที่ถูกยับยั้งแบบย้อนกลับได้โดย L-Phe การแสดงออกร่วมของ *pheA* ที่ต่อต้านการยับยั้งแบบย้อนกลับ ($pheA^{L359D}$) กับยีนอื่น ๆ ที่สำคัญในวิถีการสังเคราะห์ L-Phe ได้แก่ *aroB*, *aroL*, *phedh*, *tktA*, *aroG*, *pheA*, *yddG* และ *glpF* ใน pRSFDuet-1 ($pPTFBLYA^{L359D}$) สามารถเพิ่มการผลิต L-Phe ได้เป็น 3.78 เท่า เมื่อเทียบกับโคลนที่มี $pheA^{wt}$ ($pPTFBLYA^{wt}$) ในงานวิจัยนี้ยีน $aroG^{wt}$ และ *aroG* ที่ต้านทานการควบคุมแบบย้อนกลับ ($aroG^{L175D}$, $aroG^{Q151L}$, $aroG^{Q151A}$ และ $aroG^{Q151N}$) ได้ถูกโคลนเข้า pRSFDuet-1 แล้วทรานส์ฟอร์มเข้า *E. coli* BL21(DE3) พบว่าโคลน $AroG^{Q151N}$ มีแอคติวิตีจำเพาะของ DAHP synthase สูงสุดในภาวะที่มี L-Phe 20 มิลลิโมลาร์ ดังนั้นจึงได้ทำการสร้างโคลนของ *E. coli* BL21(DE3) ที่มี $pBLPTA^{L359D}G^{wt}$ & pYF และ $pBLPTA^{L359D}G^{Q151N}$ & pYF แล้วทำการตรวจวัดการผลิต L-Phe ใน minimum medium ที่มีกลีเซอรอลร้อยละ 6 เทียบกับโคลนที่มี pBLPT & pYF พบว่าโคลน $pBLPTA^{L359D}G^{Q151N}$ & pYF มีการผลิต L-Phe สูงขึ้น 8.7 เท่า และโคลนที่มี $AroG^{Q151N}$ ผลิต L-Phe ได้มากกว่าโคลนที่มี $AroG^{wt}$ 1.2 เท่า

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6072041223 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

KEYWORD: L-Phenylalanine, PheA, AroG, feedback inhibition, Aromatic amino acid pathway

Charintip Yenyuvadee : CO-EXPRESSION OF FEEDBACK RESISTANT ENZYMES IN PHENYLALANINE BIOSYNTHESIS PATHWAY TO INCREASE PHENYLALANINE PRODUCTION. Advisor: Assoc. Prof. KANOKTIP PACKDIBAMRUNG, Ph.D.

L-Phenylalanine (L-Phe) is an important commercial amino acid. It is widely used in food and pharmaceutical industries. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame. In *Escherichia coli*, the synthesis of L-Phe is controlled by the multi-hierarchical regulations. AroG isoform of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) and chorismate mutase/prephenate dehydratase (PheA), two important enzymes, are feedback inhibited by L-Phe. Co-expression of feedback-resistant *pheA* (*pheA*^{L359D}) with other pivotal genes in L-Phe biosynthesis pathway: *aroB*, *aroL*, *phedh*, *tktA*, *aroG*, *pheA*, *yddG,* and *glpF* in pRSFDuet-1 (pPTFBLYA^{L359D}) elevated L-Phe production of *E. coli* BL21(DE3) 3.78 fold in comparison to that of wildtype (pPTFBLYA^{wt}). In this research, wildtype *aroG* (*aroG*^{wt}) and feedback resistant *aroG* genes (*aroG*^{L175D}, *aroG*^{Q151L}, *aroG*^{Q151A} and *aroG*^{Q151N}) were cloned into pRSFDuet-1 and then transformed into *E. coli* BL21(DE3). AroG^{Q151N} clone gave the highest specific activity of DAHP synthase in the presence of 20 mM L-Phe. Therefore, *E. coli* BL21(DE3) containing pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were constructed and their production of L-Phe in 6% glycerol medium were determined in comparison to pBLPT & pYF clone. The presence of PheA^{L359D} and AroG^{Q151N} elevated L-Phe production 8.7 fold while the clone containing AroG^{Q151N} produced L-Phe 1.2 fold higher than AroG^{wt} clone.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....

ACKNOWLEDGEMENTS

This thesis could not be successfully completed without the great carefulness of my advisor Associate Professor Dr. Kanoktip Packdibamrung. Therefore, I would like to thank for her excellent advice, skillful guidance, warmful encouragement, and fruitful discussion throughout the period of my study.

My gratitude is also extended to Associate Professor Dr. Teerapong Buaboocha, Assistant Professor Dr. Rath Pichyangkura and Assistant Professor Dr. Ratree Wongpanya for giving me your precious time on being my thesis's defense committee and for their valuable comments and useful suggestions.

I would like to thank Department of Biochemistry, Faculty of Science where I carried out the research work. Moreover, I would like to thank all members in room 707 in Department of Biochemistry for helping me all the times and making the laboratory enjoyable. Their assistance and suggestions given to this research work have always been constructive.

I would like to thank Sci Super II Grant to develop research potential of the Department of Biochemistry, Faculty of Science, Chulalongkorn University for partial support to my study.

Finally, I would like to express my very profound gratitude to my parents and all members in my family for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. Moreover, I would like to thank all members in BC45 and my special friend for listening, offering me advice, and supporting me through this entire process. This accomplishment would not have been possible without them. Thank you.

Charintip Yenyuvadee

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ala	alanine
aroB	3-dehydroquinate synthase gene
AroG	L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
$AroG^{fbr}$	L-phenylalanine feedback resistant AroG
aroG	L-phenylalanine sensitive isoform of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase gene
$aroG^{fbr}$	L-phenylalanine-feedback resistant 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase gene
aroL	shikimate kinase II gene
Asn	asparagine
Asp	aspartate
bp	base pairs
BSA	bovine serum albumin
^{o}C	degree celsius
CMPD	chorismate mutase/ prephenate dehydratase
DAHP	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylene diamine tetraacetic acid
E4P	erythrose 4-phosphate

fbr	feedback resistant
glpF	glycerol facilitator gene
Gln	glutamine
h	hour
HPLC	high-performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
L	liter
L-Phe	L-phenylalanine
L-Trp	L-tryptophan
L-Tyr	L-tyrosine
LB	Luria-Bertani
Leu	leusine
μg	microgram
μL	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute

mL	milliliter
mM	millimolar
MW	molecular weight
ng	nanogram
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PheA	chorismate mutase/ prephenate dehydratase
pheA	chorismate mutase/ prephenate dehydratase gene
$pheA^{fbr}$	L-phenylalanine feedback resistant chorismate mutase/ prephenate dehydratase gene
phedh	phenylalanine dehydrogenase gene
RNase	ribonuclease
rpm	revolution per minute
s	second
tktA	transketolase gene
Tm	melting temperature
U	unit (μmol/min)
UV	ultraviolet
Val	valine

v/v	volume by volume
wt	wildtype
yddG	aromatic amino acid exporter gene

CHAPTER I

Introductions

1.1 Aromatic amino acid

Aromatic amino acids are amino acids that have an aromatic ring in the side chain. Among the 20 standard amino acids, the following are aromatic: L-tryptophan (L-Trp), L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Aromatic amino acids are the building blocks of proteins. Normally, they constitute less than 10% of the protein. L-tryptophan is the largest and the rarest of the amino acids in proteins [1].

The aromatic amino acids are produced from the shikimate pathway which is found in bacteria, fungi, plants, and some protists. In the three aromatic amino acids metabolism, L-Phe has the highest carbon flux because up to 30% of organic matter constitutes from L-Phe-derived compounds. Plant and microbe can synthesize their own aromatic amino acids to make proteins. In plants, the aromatic amino acids also use as precursors of plant natural products that play a key role in plant growth, development, reproduction, defense and environmental responses. L-Trp is used as a precursor of alkaloids, phytoalexins, indole glucosinolates and auxin. L-Tyr is used as a precursor of isoquinoline alkaloids, pigment betalains and quinones while L-Phe is also used as a precursor of numerous phenolic compounds including flavonoids, tannins, lignans, lignin, and phenylpropanoid/benzenoid volatiles (Figure 1) [2]. For animals, L-Phe and L-Trp are essential amino acids. However, animals have lost the metabolic pathway for aromatic amino acid. They must derive these amino acids through their food and L-Phe can be converted to L-Tyr by Phe hydroxylase. In animals and humans, the aromatic amino acids are used as precursors for the synthesis of biologically and neurologically active compounds that are essential for maintaining biological functions. Nowadays, the aromatic amino acids are important examples of chemical products that can be produced by renewable raw material in microorganisms such as glucose or glycerol [3].

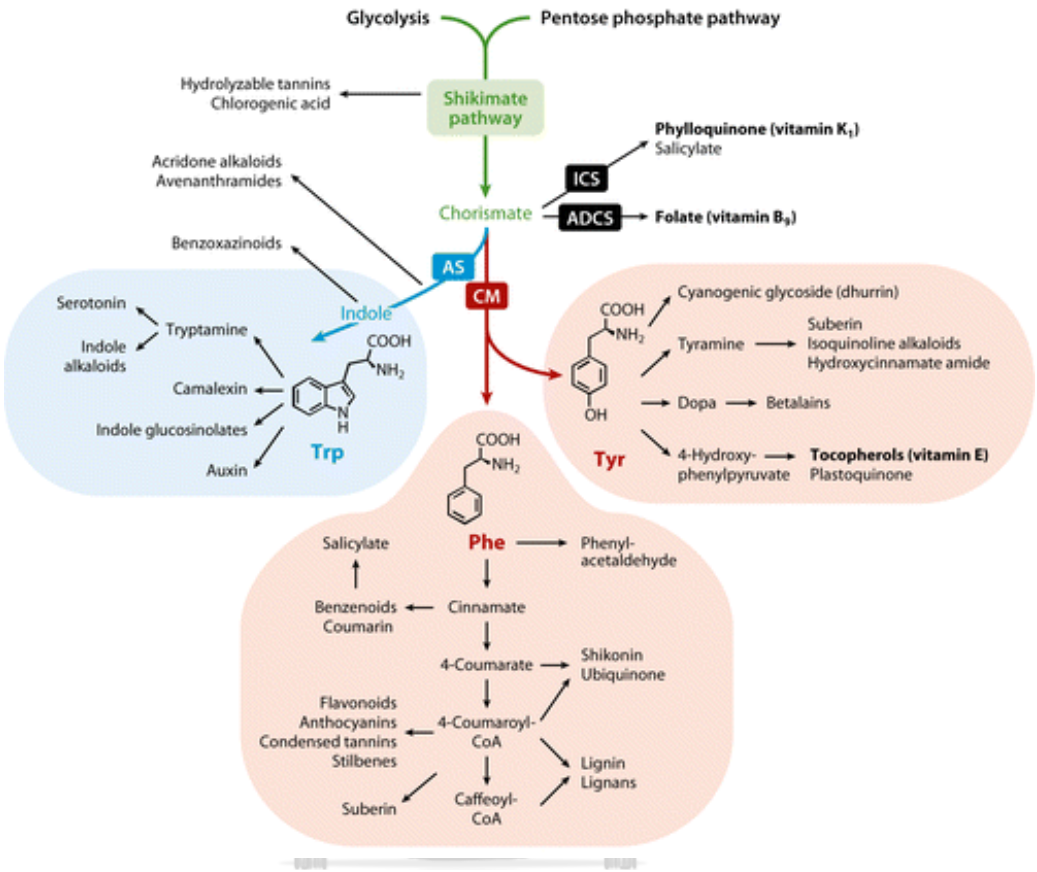


Figure 1. The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in green) produces chorismate, a common precursor for the Trp pathway (blue), the Phe/Tyr pathways (red), and the pathways leading to folate, phylloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diverse array of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (bold). Abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoA, coenzyme A; ICS, isochorismate synthase [2].

1.2 L-Phenylalanine

L-Phenylalanine ($C_9H_{11}NO_2$) is an essential hydrophobic aromatic amino acid. It has a benzyl group as a side chain as shown in Figure 2. It is classified as nonpolar and neutral because of the hydrophobic nature and inert of the side chain. L-Phe is also one of the most important commercial aromatic amino acid for humans and animals [4, 5] . It is provided from diet such as meat, cottage cheese, and wheat germ. In human brain, L-Phe is converted to L-Tyr which is used as a precursor to produce catecholamines such as tyramine, dopamine, epinephrine, and norepinephrine. The neurotransmitter catecholamines act like adrenalin substances that transmit signals between nerve cells and the brain for keeping us awake and alert, reduce hunger pains, function as an antidepressant and help improvement of memory [6-8].

In pharmaceutical industries, L-Phe is used for chemical synthesis of pharmaceutically active compounds like HIV protease inhibitor, anti-inflammatory drugs [9], phenylethylamine, catecholamines [10] and combination with UVA therapy for the treatment of vitiligo [11]. Moreover, L-Phe also used in several psychotropic drugs (mescaline, morphine, codeine, and papaverine). In food industries, L-Phe is used as a supplementary food and a precursor for low calorie sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester) which is 160–180 times sweeter than sucrose [12]. Currently, the requirement of L-Phe is increased according to the great demand for the low-calorie sweetener, aspartame which approximate the world market of US $1.5 billion.

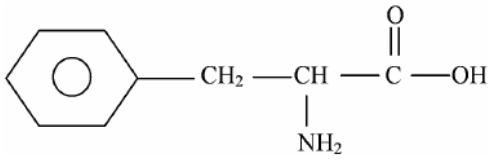


Figure 2. Structure of L-phenylalanine [7].

1.3 L-Phenylalanine production

The L-Phe can be produced by chemical synthesis and bioprocessing such as enzymatic transformation or microbial process [13]. In early industrial process of L-Phe, it was mainly produced by chemical process. However, the chemical synthesis of L-Phe has many disadvantages for example, chemical synthesis uses toxic raw materials that are nonrenewable and generate racemic mixtures of D and L Phe isomers which make it difficult for the purification processes. Furthermore, the process has a high cost and various problems. Therefore, the L-Phe biosynthesis is an attractive alternative since it is a clean technology and uses renewable simple carbohydrates that generates less environmental pollution [14, 15] . More recently, metabolic engineering in *Escherichia coli* (*E. coli*) has been focused because the main metabolisms of *E. coli* have enabled the introduction of such genetic modifications [16]. Furthermore, researchers can use a genetic engineering technique to enhance L-Phe yield [17].

1.4 L-Phenylalanine biosynthesis pathway in *E. coli*

When *E. coli* cell was cultured in medium containing carbon sources such as glucose or glycerol, the carbon sources can be converted to phosphoenolpyruvate (PEP) by glycolysis pathway and erythrose 4-phosphate (E4P) by pentose phosphate pathway. The biosynthesis of aromatic amino acids is started from the condensation of PEP and E4P to D-arabinoheptulosonate7-phosphate (DAHP) by DAHP synthase (EC 2.5.1.54). This step is tightly regulated by its final product, phenylalanine, tyrosine and tryptophan to convert the carbon flow into the shikimate pathway. There are three isozymes of DAHP synthase encoded by *aroF* (L-Tyr-sensitive), *aroH* (L-Trp-sensitive) and *aroG* (L-Phe-sensitive) [18-20].

In the second step of the pathway (shikimate pathway), there are 2 rate limiting enzymes including 3-dehydroquinate (DHQ) synthase encoded by *aroB* and

shikimate kinase II, which is encoded by *aroL*. At chorismite (CHA), the pathways of each aromatic amino acid are separated. To produce L-Phe, chorismate is converted to phenyl pyruvate (PPA) and then PPA is changed to phenylpyruvate (PPY) by bifunctional enzyme chorismate mutase/prephenate dehydratase (CMPD) encoded by *pheA*. This enzyme is feedback regulated by L-Phe. Finally, PPY is converted to L-Phe by amino transferase. The excess L-Phe is excreted by aromatic amino acid exporter encoded by *yddG*. The aromatic amino acid biosynthesis pathway is shown in Figure 3 [21].

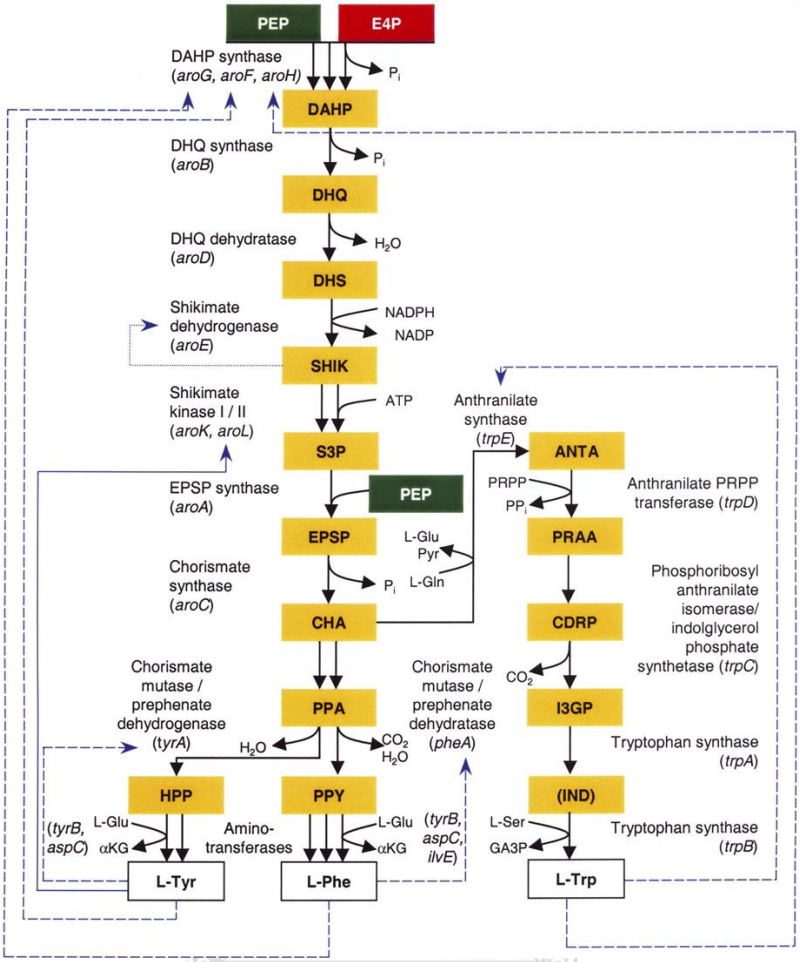


Figure 3. Pathway of aromatic amino acid biosynthesis and its regulation in *E. coli*. To indicate the type of regulation, different types of lines are used: – – –, transcriptional and allosteric control exerted by the aromatic amino acid end products; · · · , allosteric control only; —, transcriptional control only. Abbreviations used: ANTA, anthranilate; aKG, a-ketoglutarate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate; CHA, chorismate; DAHP, 3-deoxy-d-arobino-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSP, 5 enolpyruvoylshikimate 3-phosphate; E4P, erythrose 4-phosphate; GA3P, glyceraldehyde 3-phosphate; HPP, 4-hydroxyphenlypyruvate, I3GP, indole 3-glycerolphosphate; IND, indole; L-Gln, L-glutamine; L-Glu, L-glutamate; L-Phe, L-phenylalanine; L-Ser, L-serine; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; PEP, phosphoenolpyruvate; PPA, prephenate; PPY, phenylpyruvate; PRAA, phosphoribosyl anthranilate; PRPP, 5-phosphoribosyl-a-pyrophosphate; Pyr, pyruvate; SHIK, shikimate; S3P, shikimate 3-phosphate [21].

1.5 L-Phe sensitive DAHP synthase

In *E. coli*, the three isozymes of DAHP synthase are L-Phe sensitive (AroG), L-Tyr sensitive (AroF) and L-Trp sensitive (AroH) which are encoded by *aroG*, *aroF* and *aroH*, respectively [22]. About 80% of total DHAPS activity is made up by AroG while AroF and AroH share 20% and 1% of total activity, respectively [20]. The structure of AroG is a homotetramer whereas AroH and AroF are homodimer. Specific activities of AroG varied widely with different metal ions as follows; Mn^{2+} > Cd^{2+}, Fe^{2+} > Co^{2+}> Ni^{2+}, Cu^{2+}, Zn^{2+} > Ca^{2+}. Moreover, metal variation significantly affectes the apparent affinity for the substrate, E4P, but not for the second substrate, PEP or for feedback inhibition, L-Phe [23].

The first reported 3D structure of DAHP synthase is the crystal structure of AroG complexed with PEP and Pb^{2+} [24]. The tetramer consists of two tight dimers. The monomers of the tight dimer are coupled by interactions including a pair of three stranded intersubunit β-sheets. The monomer is a $(\beta/\alpha)_8$ barrel with several additional β strands and α helices. The PEP and Pb^{2+} are at the C-ends of the β strands of the barrel. Mutations that reduce feedback inhibition cluster 15 Å from the active site, indicating the location of a separate regulatory site (Figure 4).

In 2002, crystal structure of AroG in complex with it inhibitor, L-Phe, PEP and metal ion cofactor, Mn^{2+} was determined to 2.8 Å resolution [25]. L-Phe binds in a cavity formed residues of two adjacent subunits and is located about 20 Å from the closet active site. The mechanism of allosteric mechanism was derived from conformational difference between Phe-bond and Phe-free structures. The inhibitory signal is transmitted from Phe-binding site to the active site of AroG by two interrelated paths. The first path is transmission within a single subunit of clone segments of the protein. The second involves alternative in the contact between subunits. On binding of Phe, AroG loses binding ability to E4P and binds PEP in a flipped orientation.

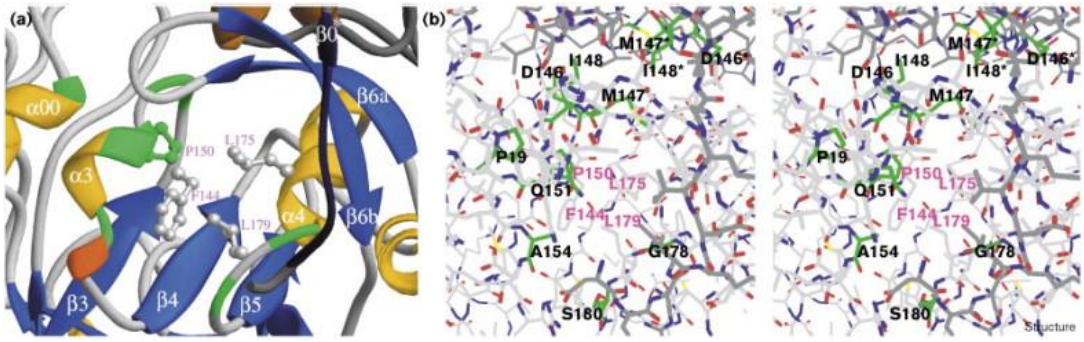


Figure 4. The inferred binding site of Phe in AroG. The proposed binding site of Phe in AroG is delineated by nine mutations–Pro19(α00), Asp146(β3–α3), Met147(β3–α3), Ile148(β3–α3), Pro150(α3), Gln151(α3), Ala154(α3), Gly178(α4–β5), and Ser180(α4–β5) (indicated in green, that eliminate or strongly reduce feedback inhibition. Elements that belong to subunit B are represented in darker colors and labeled with*. Residues forming the inferred pocket for binding Phe are labeled in magenta. (a) Ribbon diagram of the Phe-binding site of subunit A. (b) Stereoview of the Phe-binding site [24].

Hu and coworker (2005) investigated the feedback inhibition site of AroG using 3D structure of AroG co-crystallized with PEP. Phe141, Leu175, Leu179, Phe209, Trp215 and Val221 was replaced by using site-directed mutagenesis. The DAHP synthase activity in the presence of L-Phe from 0 mM to 3 mM showed that L175D was mostly resistant to feedback inhibition. The specific enzymatic activity of L175D at 0 mM of L-Phe increased significantly about 4.46 U/mg when compared with that of wild-type AroG and the relative enzymatic activity remained at 1 mM of L-Phe is about 83.5% [26].

Kikuchi and colleagues also studied the Phe-binding site of AroG. They performed hydroxamine mutagenesis on *aroG*. The mutant A220T, D146N and M147I were partially resistant to Phe inhibition [22]. From this result, Ding and coworker integrated different combinations of two mutation sites into the *aroG*, generating three double-site mutants. The *E. coli* clone possessing $AroG^{A202T/D146N}$ showed the highest enzymatic activity and greatest resistant to feedback inhibition. The relative enzymatic activity of $AroG^{A202T/D146N}$ remained at 20 mM of L-Phe was 96.66% [27].

1.6 Chorismite mutase and prephenate dehydratase (CM-PDT)

The chorismite mutase (CM) and prephenate dehydratase (PDT) (EC 5.4.99.5/4.2.1.51) is one of the allosteric enzymes in the aromatic amino acid biosynthesis pathway. CM-PDT which is encoded by *pheA* catalyzes the second committed step of L-Phe biosynthesis. It can be feedback inhibited by L-Phe. CM-PDT contains 386 amino acids with a molecular mass of 43 kDa. It is a bifunctional enzyme which contains two catalytic domains chorismite mutase domain (residues 1-109) and prephenate dehydratase domain (residues 101-285) and one regulatory-domain (residues 286-386) for L-Phe inhibitor binding site [28-30]. The feedback inhibition regulation of CM-PDT is mediated through allosteric binding of L-Phe which contributes a shift in the aggregation state of the enzyme from an active dimer to

less active tetrameric and octameric species [29]. Inhibition of the prephenate dehydratase activity at 1mM of L-Phe showed that almost total activity was inhibited (85% inhibition). In contrast, chorismate mutase activity was inhibited only 55% [31]. Overexpression of the CM-PDT domain of PheA could improve the metabolic influx to overproduce L-Phe and improve the survival ability under *m*-fluoro-DL-phenylalanine (an analog of Phe) stress [32].

To investigate L-Phe binding site, Nelms and coworker (1992) constructed four mutants which located within codons 304 to 310 of the *pheA* and measured the enzyme activity at various the L-Phe concentrations. They suggested that the recombinant *E. coli* harboring $pheA^{W339P}$ displayed almost complete resistance to feedback inhibition of prephenate dehydratase by L-Phe concentrations up to 200 mM [29].

1.7 Glycerol as a carbon source

Biofuels, such as ethanol and biodiesel, are among the most promising source for the substitution of fossil. About 10% (w/w) glycerol as a main by-product is generated in biodiesel production. The excess glycerol may become an environment problem. The market price of crude glycerol is low with the price of US$ 0.13-0.24 per kilogram [33]. Therefore, glycerol has been considered as a feedstock for new industrial fermentations [34]. Compared to the conventionally fermentation used glucose and sucrose, glycerol is efficient low-cost carbon source. The initial step of glycerol utilization in *E. coli* is the uptake of glycerol molecule into the cytoplasm via protein-mediated glycerol facilitator (GlpF) encoded by *glpF*. Glycerol is trapped by an ATP-dependent glycerol kinase (GlpK) to yield glycerol- 3-phosphate (G3P) which is then oxidized by a membrane-bound ubiquinone-8(UQ8)-dependent G3P dehydrogenase (GlpD) to dihydroxyacetone phosphate (DHAP) that enters glycolysis [35-37].

1.8 Our previous works

Thongchuang (2011) cloned *glpF*, *tktA*, *aroG*, *aroB*, *aroL*, *yddG* from *E. coli* and phenylalanine dehydrogenase gene (*phedh*) from *Bacillus lentus.* Each gene was regulated under T7 promoter in pRSFduet-1. The clone harboring pPTFBLY which contained *phedh*, *tktA*, *glpF*, *aroB*, *aroL* and *yddG* produced high level of L-Phe about 429 mg/L when it was cultured in minimum medium containing 3% glycerol for 240 h. The disadvantage of this clone were slowly growth rate and the colonies changed into flat shape when it was grown on the agar plate because of overexpression of membrane proteins under the regulation of T7 promoter [38]. To solve the problem, Ratchaneeladdajit (2014) applied the dual plasmid system. *phedh*, *tktA*, *aroB*, and *aroL* under T7 promoter was cloned into pRSFduet-1 (pBLPT) while *glpF* and *yddG* were expressed under ara promoter of pBad-33 (pYF). The ara promoter is tight regulated promoter and it uses arabinose as an inducer. After that, these two plasmids were co-transformed into *E. coli* BL21(DE3). The high level of L-Phe production at 746 mg/L was found when the medium was optimized (3.1% glycerol) [39]

Naksusuk (2015) improved the production of L-Phe by overexpression of phenylalanine feedback resistant PheA ($PheA^{fbr}$). Five Leu/Met residues in L-Phe binding pocket were selected for site-directed mutagenesis. The $pheA^{fbr}$ was co-expressed with *phedh*, *tktA*, *aroB*, *aroL, glpF and yddG* in pRSFDuet-1. Among these mutated clones, $pPTFBLYA^{L359D}$ produced the highest concentration of L-Phe at 135 mg/L which was 3.8 fold of that of wildtype PheA clone ($pPTFBLYA^{wt}$) [40] when the clones were cultured in glycerol medium formulated for pBLPT & pYF clone. All genes that were overexpressed in *E. coli* BL21(DE3) for L-Phe production are shown in Figure 5.

Kanoksinwuttipong analysed the amino acid residues that interact with L-Phe in the regulatory site of AroG using the crystal structure of AroG complex with Mn^{2+}, PEP and Phe (code 1KFL in protein Data Bank). The structure was displayed by UCSF

chimera program (Figure 6). Q151 was selected for substitution by Leu, Ala and Asn. The these mutated genes were cloned into pRSFDuet-1 and transformed into *E. coli* BL21(DE3) on phenylalanine feedback inhibition L175D was used as a control [41] .

Therefore, the objectives of this research are

1. To determine the expression of PheA and AroG.
2. To co-express *pheA*^{L359D} and *aroG*^{fbr} with *phedh, tktA, aroB, aroL, glpF* and *yddG* using dual plasmid system of pRSFDuet-1 and pBad33 in order to produce high quantity of L-Phe.

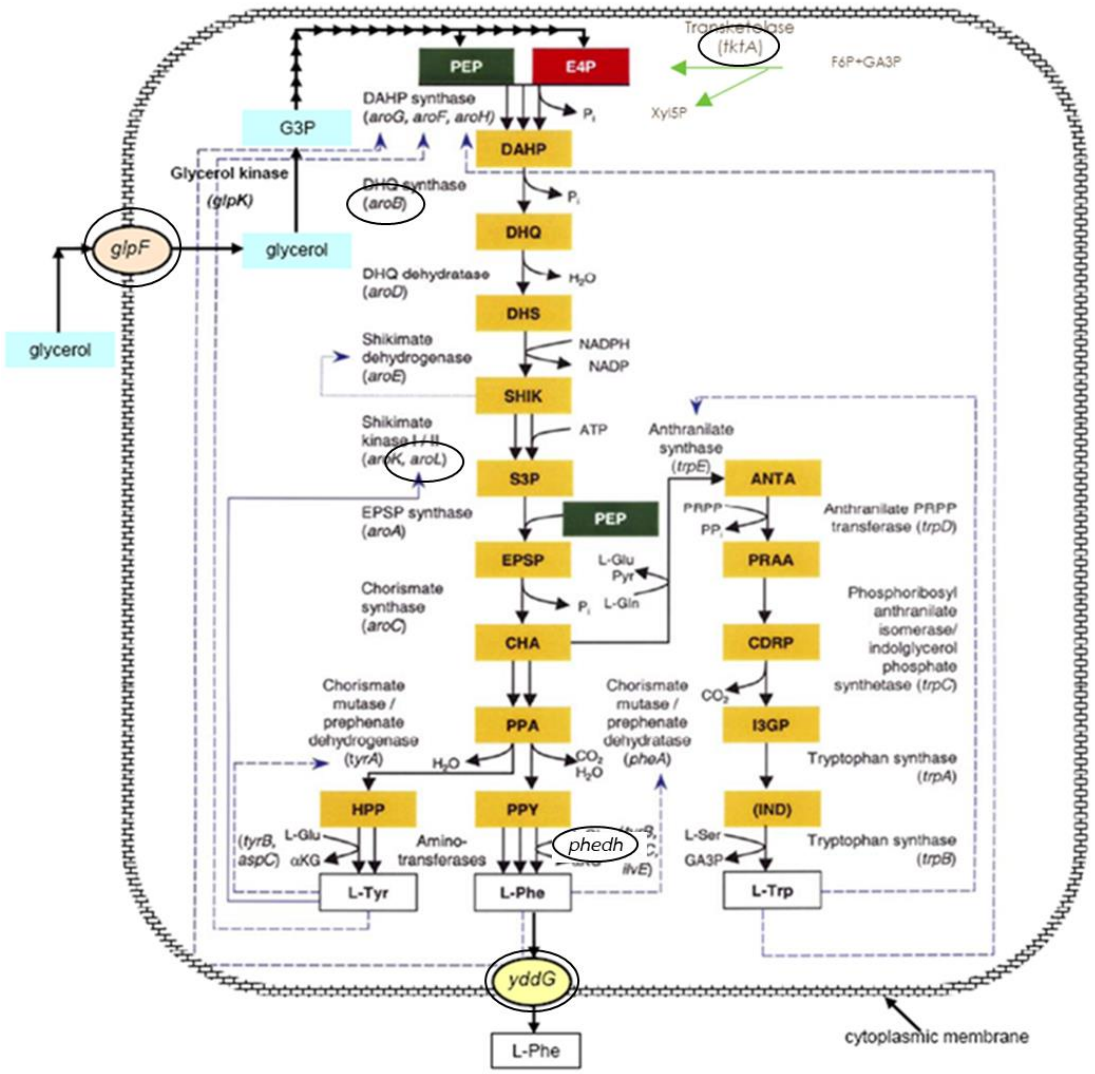


Figure 5. All important genes in this study. Abbreviations used: F6P, fructose 6-phosphate; G3P, glycerol 3-phosphate; GA3P, glyceraldehyde 3-phosphate; *glpF*, glycerol facilitator; *phedh*, phenylalanine dehydrogenase, *yddG*, aromatic amino acid exporter

Source: modified from Bongaert, et al 2001 [21]

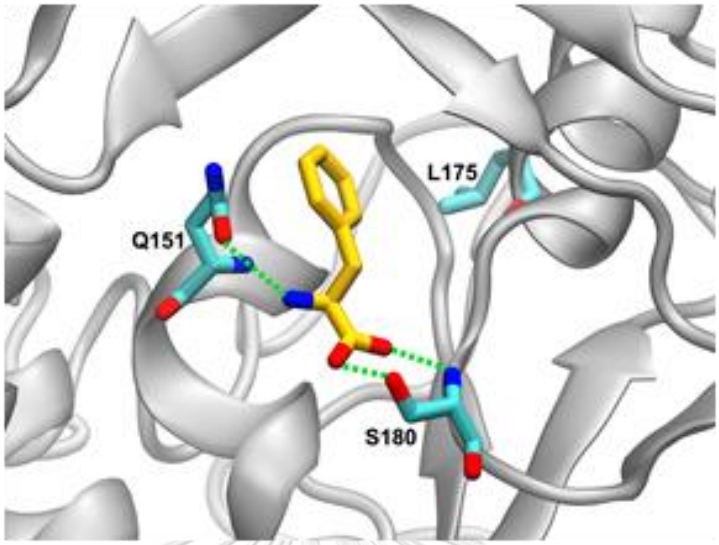


Figure 6. The 3D structure of AroG co-crystallized with inhibitor L-Phe [41].

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave: MLS-3020, SANYO Electric Co, Ltd., Japan

Autopipette: Pipetman, Gilson, France

Analytical Balance: AB135-S/FACT, Mettler Toledo, Germany

Balance: GB1501-S, Mettler Toledo, Germany

Benchtop centrifuge: SorvallBiofuge Primo, Kendro Laboratory Products L.P., USA

Centrifuge, refrigerated centrifuge: Sorvall Legend XTR, Thermo Scientific, USA

Centrifuge, microcentrifuge: Microfuge 22R, Beckman Instrument Inc., USA

Dry bath incubator: MD-01N, Major Science, USA

Electrophoresis unit: Minis-150, Major Science, USA

Electroporator: MicroPulser^{TM} electrophoretor, Bio-Rad Laboratories Inc., USA

Electroporation cuvette: Gene Pulser®/E. coli Pulser® Cuvettes, Bio-Rad, USA

Gel documentation instrument: BioDoc-ItTm Imaging system, UVP, USA

High Performance Liquid Chromatography (HPLC): UFLC, Shimadzu, Japan

HPLC column: Chirex® Chiral 3126 (D)-penicillamine, Phenomenex, USA

Incubator Shaker: Model E24R, New Brunswick Scientific, USA

Incubator Shaker: Innova^{TM}4000, New Brunwick Scientific, USA

Incubator oven: Series04067, Contherm Scientific., Ltd., New Zewland

Laminar flow: HT123, ISSCO, USA

Magnetic stirrer: Model Cerastir CH-1 series, Nickel-electro., Ltd., UK

Membrane filter: 0.45 μm Nylon Membrane Disc, Gs-Tek, USA

Microcentrifuge tube: 1.5 mL, Nest biotechnology, China

Microwave oven: GX-2021M, Galaxy, Korea

Mini personal centrifuge: Model microONE, Tomy Digital Biology Co., Ltd., Japan

PCR tube: thin-well dome-cap PCR tube, MCT-150, Axygen Inc., USA

pH meter: Model S200, Mettler ToledoCo., Ltd., Switzerland

Pipette: Labwarehouse, New Zealand

Pipette tip: Axygen Inc., USA

Sonicator: Vibra cellTm, SONICS & MATERIALS, Inc., USA

Spectrophotometer: BioSpectrometer® kinetic, Eppendorf, Germany

Syringe: 3 mL, 5 mL, 10mL, 20 mL latex free disposable syringe, Nipro Co., Ltd., Thailand

Syringe membrane filter: 0.2 μm Supor® Membrane Acrodisc®, PALL, USA

Thermal Cycler: T100™, Bio-Rad, USA

UV Transilluminator: MacroVueTM UV-25, Hoefer Inc., USA

Vacuum pump: Model number. WP6111560, Millipore Inc., USA

Vortex mixer: TopMix FP15024, Fisher Scientific, USA

2.1.2 Chemicals

Acrylamide: Sigma, USA

Agar: Himedia Laboratories Pvt. Ltd., India

Agarose: SERVA Electrophoresis GmbH, Germany

Agarose: ISC BioExpress, USA

Aluminium sulfate: Univar, USA

Ammonium sulphate: Carlo Erba Reagents, Italy

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromophenol blue: Merck, Germany

Calcium chloride: Scharlau Chemie S.A., Spain

Cobalt sulphate: Sigma, USA

Copper sulfate: Carlo Erba, Italy

Coomassie brilliant blue R-250: Sigma, USA

D-Erythrose 4-phosphate sodium salt: Sigma, USA

Di-sodium hydrogen arsenate: Sigma, USA

6X DNA Loading Dye: Thermo Fisher Scientific Inc., USA

dNTP: Biotechrabbit, Germany

Ethanol (Absolute): RCI Labscan Limited, Thailand

Ethylene diaminetetraacetic acid di-sodium salt (EDTA): Scharlau Chemie S.A., Spain

Glacial acetic acid: Carlo ErbaReagenti, Italy

Glycerol: Analytical Univar Reagent, Ajax finechem, Australia

Glycine: BDH, England

Hydrochloric acid: Carlo ErbaReagenti, Italy

Isopropylthio-β-D-galactosidase (IPTG): Serva, Germany

Iron (II) sulfate heptahydrate: Sigma, USA

β- mercaptoethanol: Acros Organics, USA

Maganese sulfate heptahydrate: Carlo Erba Reagents, Italy

Magnesium sulfate heptahydrate: Carlo Erba Reagents, Italy

Methanol (HPLC grade): RCI Labscan, Thailand

N,N'-methylene-bis-acrylamide: Sigma, USA

N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagents, Italy

Nickel(II) sulfate hexahydrate: Carlo Erba Reagents, Italy

Pancreatic digest of casein: Criterion, USA

10X *pfu* buffer with $MgSO_4$: Biolabs, England

Phenol reagent: Sigma, USA

L-Phenylalanine: Sigma, USA

Phosphoenol pyruvate: Sigma, USA

Potassium di-hydrogen phosphate: Carlo Erba Reagents, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagents, Italy

RedSafe™: Intron Biotechnology, Hongkong

Sodium (meta) arsenite: Sigma, USA

Sodium chloride: Univar, New Zealand

Sodium citrate: Carlo Erba Reagents, Italy

Sodium hydroxy: Carlo Erba Reagents, Italy

Sodium molybdate dihydrate: Carlo Erba Reagents, Italy

Sodium periodate: Sigma, USA

Thiamine hydrochloride: Sigma, USA

2-Thiobarbituric acid: Sigma, USA

Trichloroacetic acid: Sigma, USA

Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagents, Italy

Yeast Extract: Scharlau Chemie S.A., Spain

Zinc sulfate heptahydrate: Carlo Erba Reagents, Italy

2.1.3 Antibiotics

Chloramphenicol: Sigma, USA

Kanamycin: Sigma, USA

2.1.4 Markers

100 base pair DNA ladder: Fermentas Inc., USA

GeneRuler 1 kb DNA Ladder: #SM0311, ThermoFisher Scienctific, Inc., USA

Lamda (λ) DNA /*Hin*dIII: #SM0102, BioLabs, Inc., USA

TriColor protein ladder: Biotechrabbit, Germany

2.1.5 Kits

GenepHlow™ Gel/PCR Kit: Geneaid Biotech Ltd, Taiwan

Presto™ Mini Plasmid Kit: Geneaid Biotech Ltd, Taiwan

2.1.6 Enzymes and restriction enzymes

Pfu DNA polymerase: Biotechrabbit, Germany

Phusion High-Fidelity DNA Polymerase: Thermo Scientific, USA

Restriction enzymes: New England BioLabs, Inc., USA

T4 DNA ligase: Biotechrabbit, Germany

Taq DNA polymerase: Apsalagen, Thailand

2.1.7 Oligonucleotide primers

The oligonucleotide primers were synthesized by Integrated DNA Technologies, Singapore. The oligonucleotide primers used in this study are described in Table 1.

2.1.8 Bacterial strains

E. coli Top10, genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ^- *rps*L(Str^R) *end*A1 *nup*G, was used for cloning and plasmid preparation.

E. coli BL21(DE3), genotype: F^- *omp*T $hsdS_B$ (r_B^-, m_B^-) *gal dcm* (DE3), was used as an expression host for the overexpression of all genes.

2.1.9 Plasmids

pRSFDuet-1 was used for cloning and expression of *aroB*, *aroL*, *tktA*, *phedh*, *aroG* and *pheA* under T7 promoter (Appendix A).

pBAD33 was used for cloning and expression of *glpF* and *yddG* under arabinose pBAD promoter (Appendix B).

All plasmids used in this study are shown in Table 2.

Table 1. The oligonucleotide primers for PCR amplification and DNA sequencing used in this study.

Primer	Sequence	T_m (°C)
For PCR amplification		
aroG_PacI_F	5’-CCTTAATTAATCCCTTATGCGACTCCTGCATTAGG-3’	63.3
aroG_AvrII_R	5’-ACTCCTAGGTTACCCGCGACGCGCTTTCACT-3’	67.0
F_AroG _NcoI	5'-CATGCCATGGTGTATCAGAACGACGATTTACGCATCAA AGAA ATC-3'	65.0
R_AroG_HindIII	5’-CCAAGCTTTTACCCGCGACGCGCTTTCACTGC-3’	62.6
PheA_F2	5’-ATAAGAATGCGGCCGCCGATCCCGCGAAATTAA-3’	61.4
PheA_R	5’-TGATGTACATCAGGTTGGATCAACAGGCA-3’	67.2
For DNA sequencing		
ACYCDuetUP1	5’-GGATCTCGACGCTCTCCCT-3’	60.0
DuetDown1	5’-GATTATGCGGCCGTGTACAA-3’	57.3
F_tktA_aroG_Int	5’-GCTATCGTCGGTATGACCACCTTCGGTGAAT-3’	63.9
T7 terminator	5’-GCTAGTTATTGCTCAGCGG-3’	57.0
Seqduet_R	5’-CGCTTATGTCTATTGCTGGTTTACCGG-3’	59.4

Table 2. Plasmids used in this study.

Plasmids	Characteristics	Source or reference
pAroG	pRSFDuet-1 inserted with *aroG*^{wt} at *Nco*I and *Hin*dIII sites.	This study
pAroG^{fbr}	pRSFDuet-1 inserted with *aroG*^{fbr} (L175D, Q151L, Q151A and Q151N) at *Nco*I and *Hin*dIII sites.	This study
pBLPT	pRSFDuet-1 inserted with *aroB, aroL, phedh and tktA.*	Ratchaneeladdajit, 2014
pBLPTA^{L359D}G^{wt}	pRSFDuet-1 inserted with *aroB, aroL, phedh, tktA, pheA*^{L359D} and *aroG*^{wt}.	This study
pBLPTA^{L359D}G^{Q151N}	pRSFDuet-1 inserted with *aroB, aroL, phedh, tktA, pheA*^{L359D} and *aroG*^{Q151N}.	This study
pBLPTG^{Q151L}	pRSFDuet-1 inserted with *aroB, aroL, phedh, tktA* and *aroG*^{Q151L}.	Ulfah, 2018
pBLPTG^{Q151L}A^{wt}	pRSFDuet-1 inserted with *aroB, aroL, phedh, tktA, aroG*^{Q151L} and *pheA*^{wt}.	This study
pBLPTG^{Q151L}A^{L359D}	pRSFDuet-1 inserted with *aroB, aroL, phedh, tktA, aroG*^{Q151L} and *pheA*^{L359D}.	This study
pDuet_AroG	pRSFDuet-1 inserted with *aroG*^{wt} at *Bam*HI and *Hin*dIII sites.	Kanoksinwuttipong, 2015
pDuet_AroG^{fbr}	pRSFDuet-1 inserted with *aroG*^{fbr} (L175D, Q151L, Q151A and Q151N) at *Bam*HI and *Hin*dIII sites.	Kanoksinwuttipong, 2015
pDuet_pheA^{wt}	pRSFDuet-1 inserted with *pheA*^{wt} at *Hin*dIII and *Afl*II sites.	This study
pDuet_pheA^{L359D}	pRSFDuet-1 inserted with *pheA*^{L359D} at *Hin*dIII and *Afl*II sites.	This study
pPheA^{wt}	pRSFDuet-1 inserted with *pheA*^{wt} at *Not*I and *Bsr*GI sites.	This study
pPheA^{L359D}	pRSFDuet-1 inserted with *pheA*^{L359D} at *Not*I and *Bsr*GI sites.	This study
pYF	pBAD33 inserted with *yddG* and *glpF*.	Ratchaneeladdajit, 2014

2.2 Methods

2.2.1 Construction of pPheA^{wt} and pPheA^{L359D}

To clone *pheA* from pDuet_pheA into pBLPTG^{Q151L}, the restriction sites at 5' and 3' ends of *pheA* have to change from *Hin*dIII and *Afl*II to *Not*I and *Bsr*GI because there are no *Hin*dIII and *Afl*II sites on pBLPTG^{Q151L}

2.2.1.1 Plasmid preparation

The single colony of each *E. coli* BL21(DE3) clones harboring pDuet_pheA^{wt} and pDuet_pheA^{L359D} was cultured in 5 mL LB medium (Appendix C) containing 30 mg/mL of kanamycin and incubated at 37 ºC, 250 rpm for 16-18 h. After that, the cell pellet was harvested by centrifugation at 5,000xg, 3 min. The plasmid was extracted using Presto™ Mini Plasmid Kit as described in Appendix E.

2.2.1.2 Agarose gel electrophoresis

pDuet_pheA^{wt} and pDuet_pheA^{L359D} were analyzed by agarose gel electrophoresis. To prepare agarose gel, 0.8% (w/v) agarose in 1x TBE buffer containing 89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0 was melted in microwave oven. After the gel solution was cooled to 50 - 60 ºC, RedSafe™ was added to make 5% (v/v) of final concentration and then poured into the tray. The DNA samples were mixed with 6x DNA loading dye and then loaded into the well of gel. The electrophoresis was set at 100 volts for 40 min. The DNA bands were detected by UV light of gel documentation instrument. The intensity and size of DNA samples were compared with DNA marker (GeneRuler 1 kb DNA ladder and λ DNA */Hin*dIII).

2.2.1.3 PCR amplification of *pheA^{wt}* and *pheA^{L359D}*

pheA^{wt} and *pheA^{L359D}* (1,161 bp) were amplified from pDuet_pheA^{wt} and pDuet_pheA^{L359D}, respectively, using PheA_F2 forward primer containing *Not*l site and PheA_R reverse primer containing *Bsr*Gl site as listed in Table 2. To increase the level of gene expression, the PheA_F2 primer was designed from 5' end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x *Pfu* reaction

buffer with $MgSO_4$, 1x PCR enhancer, 200 µM of dNTP mix, 1 µM of forward and reverse primer, 1.25 U of *Pfu* polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 ºC for 2 min, 32 cycles of denaturation at 95 ºC for 30 s, annealing at 62 ºC for 45s, extension at 72 ºC for 2 min and final extension at 72 ºC for 5 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit (Appendix F). After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.1.4 Cloning of *pheA*^{wt} and *pheA*^{L359D}

2.2.1.4.1 Preparation of inserts

Each *pheA*^{wt} and *pheA*^{L359D} fragments from section 2.2.1.3 was digested with *Bsr*GI. The 25 µL of digestion mixture contained 1x NEBuffer™ 2.1, 10 U of *Bsr*GI and 200 ng of DNA fragment. The digestion mixture was incubated at 37 ºC for 16 h. After cleaning by GenepHlow™ Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, *Bsr*GI digested *pheA*^{wt} and *pheA*^{L359D} fragments were used as DNA templates for digestion with *Not*I in 1x CutSmart® Buffer. After incubation at 37 ºC for 16 h, purified DNA fragments were confirmed by agarose gel electrophoresis.

2.2.1.4.2 Preparation of vector

pRSFDuet-1 was extracted by using the method described in section 2.2.1.1. The single digestions with *Bsr*GI and *Not*I of pRSFDuet-1 were performed as described in section 2.2.1.4.1. After incubation, linear form of pRSFDuet-1 was confirmed by agarose gel electrophoresis.

2.2.1.4.3 Ligation of inserts and vector

Each purified DNA fragment (section 2.2.1.4.1) was ligated into pRSFDuet-1 (2.2.1.4.2) with vector to insert ratio of 3:1. The 20 µL of ligation mixture contained 89 ng of DNA fragment, 100 ng of vector DNA, 1x T4 DNA ligase buffer and 30 U of T4 DNA ligase. The ligation reaction was incubated at 16 ºC for 16 h. After incubation, the ligation reaction was purified by GenepHlow™ Gel/PCR

Kit. The recombinant plasmids from ligation reaction were called pPheA^{wt} and pPheA^{L359D}.

2.2.1.5 Transformation of recombinant plasmid

pPheA^{wt} and pPheA^{L359D} obtained from section 2.2.1.4.3 were transformed into *E. coli* Top10 by electroporation. In electroporation step, each 5 µL (5 ng) of pPheA^{wt} and pPheA^{L359D} was mixed with 50 µL of competent *E. coli* Top10 cells (Appendix D) and chilled on ice. Each of reaction mixture was transferred to a cold electroporation cuvette. After that, the electroporation cuvette was placed in the chamber and applied for one pulse by electroporator. Five hundred µL of LB medium was added in cuvette to resuspend the transformant cell and transferred to 1.5 mL microcentrifuge tube. After that, the transformant was incubated at 37 ºC, 250 rpm for 1 h. Two hundred µL of transformant was spread on LB agar plate that contained 30 mg/mL of kanamycin and then incubated at 37 ºC for 16-18 h. The construction of pPheA^{wt} and pPheA^{L359D} are shown in Figure 7.

2.2.1.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.1.5 were identified by double digestion with *Not*I and *Bsr*GI. Each single colony of pPheA^{wt} and pPheA^{L359D} clones was picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each recombinant plasmid was digested with *Not*I and *Bsr*GI by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to perform DNA sequencing.

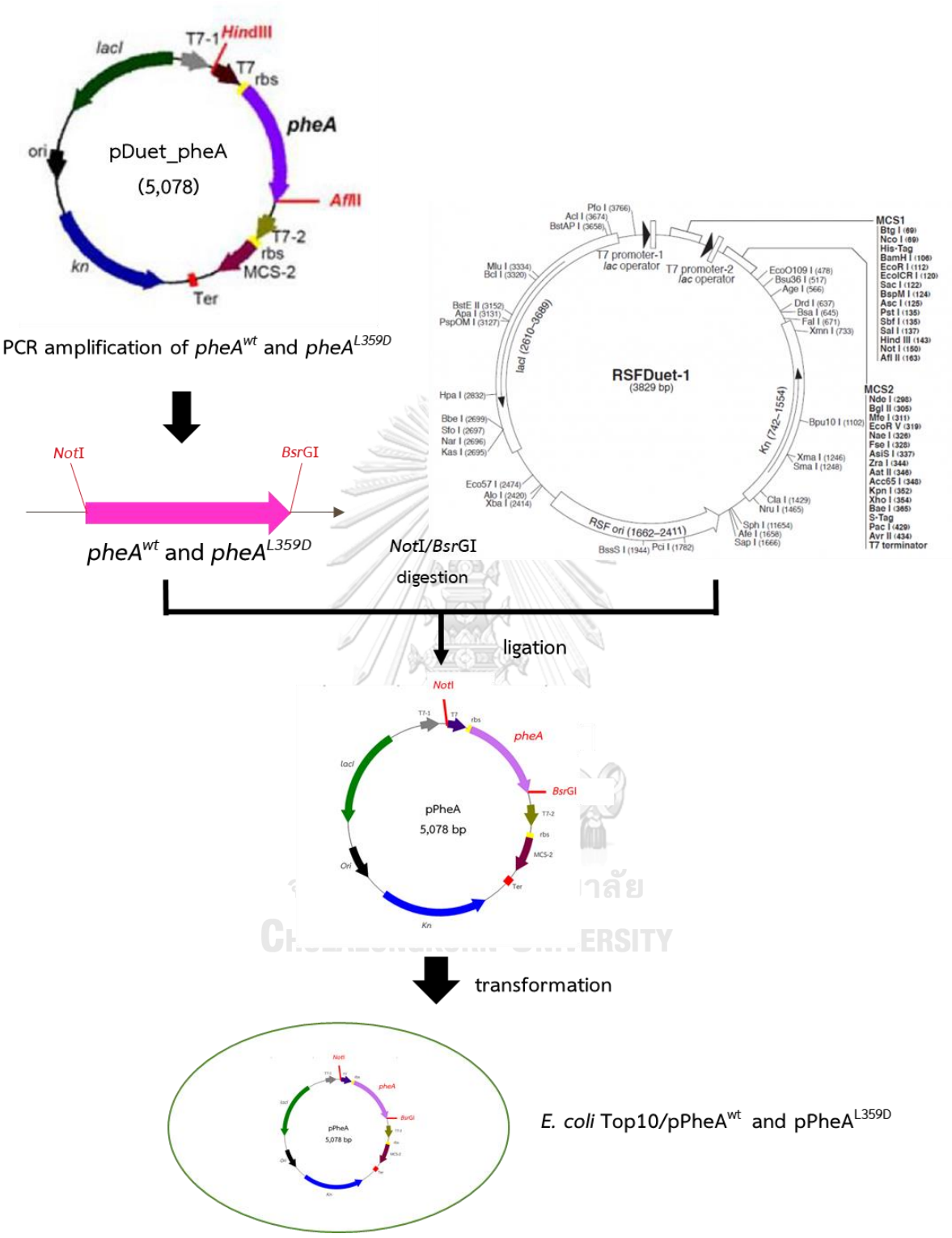


Figure 7. Construction of pPheA^{wt} and pPheA^{L359D}

2.2.1.7 Nucleotide sequencing

The DNA sequencing of recombinant plasmids were performed by Bioneer, Korean Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type *pheA* in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and translated to protein sequence using Genetyx-Win program.

2.2.1.8 Expression of PheA^{wt}

The single colony of pPheA^{wt} clone was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 ºC, 250 rpm for 16-18 h. The cell culture was inoculated into 100 mL of the same medium and cultured in the same conditions. When OD_{600} reached log phase (OD_{600} = 0.6), the expression was induced with 1 mM IPTG for 1-6 h. 1.5 mL of whole cells were harvested at 10,000xg for 3 min and kept for SDS-PAGE analysis.

2.2.1.9 SDS-PAGE analysis

The expression of PheA was confirmed by SDS-PAGE analysis. The SDS-PAGE analysis was performed by the method of Bollag et al.,1996 [42] (Appendix G). The slab gel solution consisted of 12.5% separating gel and 5% stacking gel. Tris-glycine buffer (Appendix G) at pH 8.3 was used for running buffer. For protein loading preparation, the whole cells from section 2.2.1.8 were mixed with 5x sample buffer (Appendix G) and boiled for 15 min. After that, the cell pellets were eliminated by centrifugation at 10,000xg for 15 min. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. The electrophoresis was set at 20 mA per slab gel for 40 min. After running the gel, the SDS gel was stained by the staining solution (Appendix H) with shaking for 30-45 min and then was destained by the destaining solution (Appendix H) with shaking for 30 min. After that, the destaining solution in SDS gel was changed and shaken for overnight. The molecular weight of each protein was analyzed by comparing the band with the protein marker.

2.2.2 Construction of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}

2.2.2.1 pBLPTG^{Q151L} preparation

The pBLPTG^{Q151L} containing *aroB*, *aroL*, *phedh*, *tktA* and *aroG*^{Q151L} was extracted using the method in section 2.2.1.1. The single digestions with *Not*I and *Bsr*GI of pBLPTG^{Q151L} were performed, respectively as described in section 2.2.1.4.1. After incubation, purified linear form of pBLPTG^{Q151L} was confirmed by agarose gel electrophoresis.

2.2.2.2 *pheA*^{wt} and *pheA*^{L359D} preparation

The pPheA^{wt} and pPheA^{L359D} from section 2.2.1.6 were digested with *Not*I and *Bsr*GI as described in section 2.2.1.4.1. After incubation, the *pheA*^{wt} and *pheA*^{L359D} fragments were collected from agarose gel using GenepHlow™ Gel/PCR Kit.

2.2.2.3 Cloning of *pheA*^{wt} and *pheA*^{L359D} into pBLPTG^{Q151L}

After digestion with *Not*I and *Bsr*GI, each *pheA*^{wt} and *pheA*^{L359D} fragment was ligated with pBLPTG^{Q151L} using the method described in section 2.2.1.4.3. After that, the ligation reactions were transformed into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} are shown in Figure 8.

2.2.2.4 Confirmation of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}

The single colonies of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} from section 2.2.2.3 were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. Each of recombinant plasmid was extracted by Presto™ Mini Plasmid Kit. The recombinant plasmids were digested with *Xho*I by the method described in section 2.2.1.4.1. The recombinant plasmids with correct size were identified by agarose gel electrophoresis.

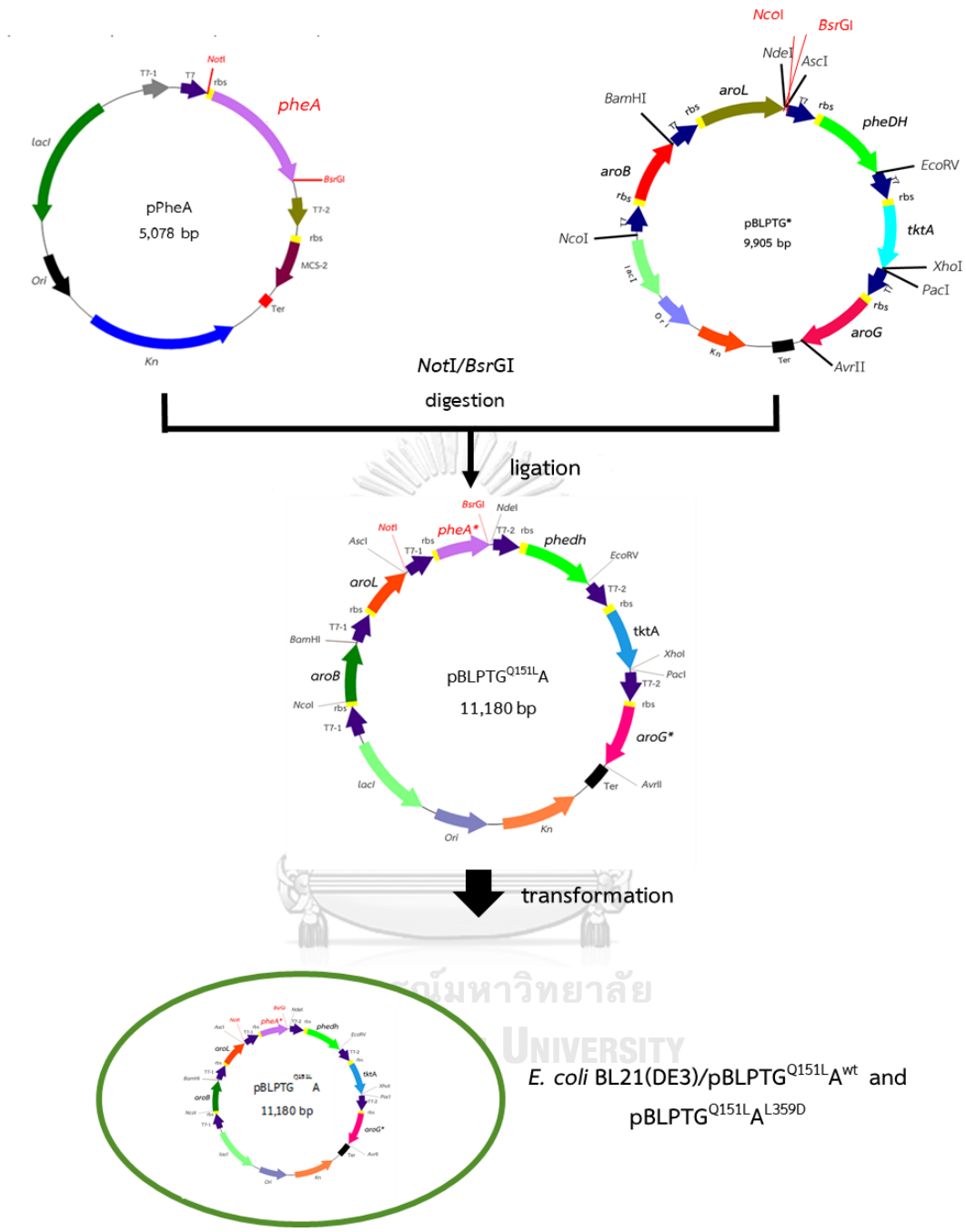


Figure 8. Construction of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}

2.2.3 Expression of AroG

2.2.3.1 Thiobarbiturate assay

DAHP synthase activity was determined by the thiobarbiturate assay method modified from Schoner, 1976 [43] and Liu, 2008 [44]. Each single colony of pAroG^{wt} and pAroG^{fbr} (pAroG^{L175D}, pAroG^{Q151L}, pAroG^{Q151A} and pAroG^{Q151N}) was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and then incubated with shaking at 250 rpm, 37 ºC for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured in the conditions. For shake flask cultivation, the 5% (v/v) of starters were cultured into 200 mL of LB medium and incubated with shaking at 250 rpm, 37 ºC. After the OD_{600} reached 0.6 (log phase), the expression was induced with 1 mM IPTG for 2 h. The cells of each clone were collected by centrifugation at 8,000xg for 10 min and washed with resuspend buffer (0.1 mM KPB, pH 6.5, 200 µM PEP, 0.5 mM DTT, 0.1 mM PMSF and 10 mM EDTA) The cell pellet of each clone was dissolved in resuspend buffer and then broken by ultrasonic cell disruption. The crude extracts were centrifuged at 10,000xg for 20 min to collect the supernatants for dialysis. After dialysis, 1.5 mL of each crude extract was centrifuged at 10,000xg for 20 min to collect the supernatants for assay of enzyme activity.

In thiobarbiturate assay method, the reaction mixture contained 50 mM potassium phosphate, pH 6.5, 5 mM PEP, 2 mM E4P, 0-20 mM L-Phe, 30 µM $MnCl_2$, crude enzyme and H_2O in a total volume of 33.75 µL. The mixture was incubated at 30 ºC for 10 min. The reaction was initiated when the enzyme was added and stopped by addition of 180 µL of 10% (w/v) trichloroacetic acid. After that, the 45 µL of mL of 25 mM $NaIO_4$ in 62.5 mM H_2SO_4 was added in the mixture and incubated at 37 ºC for 30 min. Then, the 45 µL of 2% (w/v) Na_2SO_4 in 0.5 M HCl was rapidly mixed for stopped the reaction and 450 µL of 0.36% (w/v) thiobarbituric acid was added and mixed. The reaction mixture was boiled for 20 min

and then cooled in room temperature. The absorbance at a wavelength 549 nm was measured by spectrophotometer.

2.2.3.2 Protein measurement

The protein concentration of crude extracts were measured using Lowry's method [45]. The 250 µL of crude extract was mixed with 50 µL of solution A and 2.5 mL of solution B and incubated at 30 ºC for 10 min. After incubation, 250 µL of solution C was added and rapidly mixed and then incubated at room temperature for 20 min. The absorbance of protein was detected by spectrophotometer at a wavelength 610 nm. The protein concentration was calculated using the standard curve of BSA. All solutions were prepared as described in Appendix I. The expression of $AroG^{wt}$ and $AroG^{fbr}$ (L175D, Q151L, Q151A and Q151N) under T7 promoter were detected by SDS-PAGE analysis using the method described in section 2.2.1.9.

2.2.4 Reconstruction of pAroG and pAroG^{fbr}

2.2.4.1 Plasmid extraction

The single colonies of *E. coli* Top10 clones harboring pDuet_$AroG^{wt}$ and pDuet_$AroG^{fbr}$ from Kanoksinwutthipong were cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and incubated at 37 ºC, with shaking for 16-18 h. After that, the cell pellets were harvested by centrifugation at 5,000xg for 3 min. The plasmids were extracted using Presto™ Mini Plasmid Kit.

2.2.4.2 Forward primer design

The forward primer containing *Nco*I site at 5' end of *aroG* (5' F_AroG_NcoI) was designed. The ATG of *Nco*I site was used as a start codon of *aroG*.

2.2.4.3 PCR amplification of *aroG^{wt}* and *aroG^{fbr}*

To construct the pBLPTA^{L359D}G, restriction sites at 5' and 3' end of inserted *aroG^{wt}* and *aroG^{fbr}* from pDuet_$AroG^{wt}$ and pDuet_$AroG^{fbr}$ have to change to *Nco*I and *Hind*III, respectively. For PCR amplification of *aroG^{wt}* and *aroG^{fbr}* (1,053 bp), pDuet_$AroG^{wt}$ and pDuet_$AroG^{fbr}$ were used as DNA templates, respectively

Forward primer (F_AroG_NcoI) containing *Nco*I site and reverse primer (R_AroG_HindIII) containing *Hin*dIII site as listed in Table 2 were used. The 50 µL of PCR reaction mixture contained 1x Phusion HF buffer, 1 U Phusion DNA polymerase, 3% DMSO, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 98 ºC for 30 s, 32 cycles of denaturation at 98 ºC for 10 s, annealing at 62 ºC for 10 s, extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.4.4 Preparation of inserts and vector

The *aroG*^{wt} and *aroG*^{fbr} fragments from section 2.2.1.3 and pRSFDuet-1 were digested with *Hin*dIII. The 25 µL of digestion mixture contained 1x NEBuffer^{TM} 2.1, 10 U of *Hin*dIII and 200 ng of DNA template. The digestion mixture was incubated at 37 ºC for 16 h. After cleaning by GenepHlow™ Gel/PCR Kit, each DNA fragment was checked by agarose gel electrophoresis. Next step, *aroG*^{wt}, *aroG*^{fbr} and pRSFDuet-1 fragments digested with *Hin*dIII were used as DNA template for digestion with *Nco*I using 1x CutSmart® Buffer. After digestion, DNA fragments were confirmed by agarose gel electrophoresis.

2.2.4.5 Ligation and transformation

After digestion with *Nco*I and *Hin*dIII, the *aroG*^{wt} and *aroG*^{fbr} fragments were ligated with pRSFDuet-1 using the method described in section 2.2.1.4.3. After that, the ligation reactions were used for transformation into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pAroG^{wt} and pAroG^{fbr} are shown in Figure 9.

2.2.4.6 Confirmation of recombinant plasmid

The plasmids of transformants from section 2.2.4.5 were identified by double digestion with *Nco*I and *Hin*dIII. Each single colony of pAroG^{wt} and pAroG^{fbr} clones was picked up to culture in 5 mL of LB broth containing

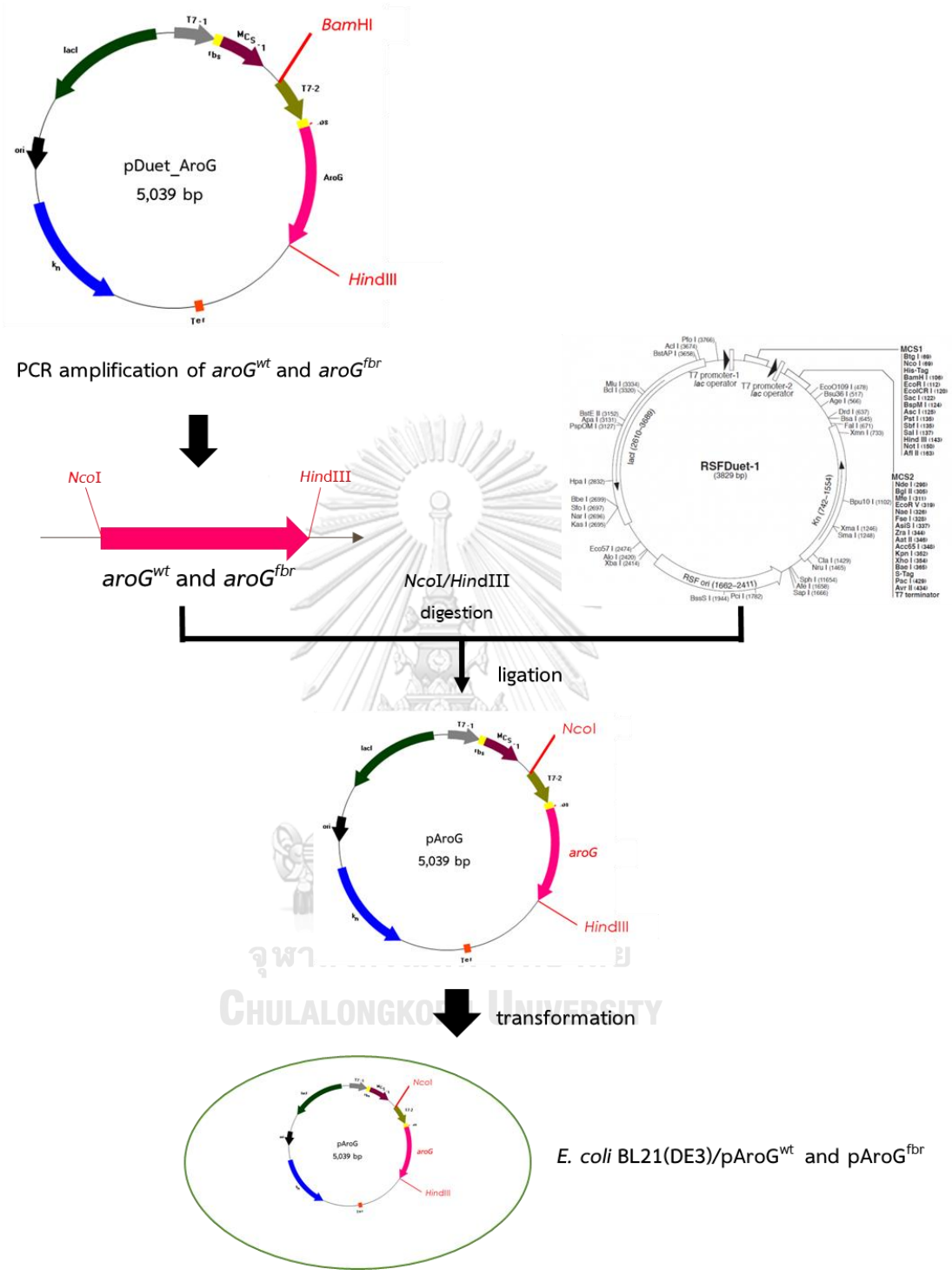


Figure 9. Construction of pAroG^{wt} and pAroG^{fbr}

30 mg/mL of kanamycin and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each of recombinant plasmid was digested with *Nco*I and *Hin*dIII by the method described in section 2.2.1.4.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with the correct size was sent to perform DNA sequencing by Bioneer, Korean using ACYCDuetUP1 as a forward primer and DuetDown1 as a reverse primer as described in section 2.2.1.7.

2.2.5 Construction of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

2.2.5.1 pBLPTA^{L359D} preparation

The recombinant pBLPTGA^{L359D} containing *aroB*, *aroL*, *phedh, tktA*, *aroG* and *pheA* from section 2.2.2.4 was double digested with *Pac*I and *Avr*II. The 25 µL of digestion mixture contained 1x CutSmart® Buffer, 10 U of *Pac*I and *Avr*II and 200 ng of DNA template. The digestion mixture was incubated at 37 ºC for 16 h. After incubation, linear fragment of pBLPTA^{L359D} was separated by agarose gel electrophoresis. The linear form of pBLPTA^{L359D} was purified by GenepHlow™ Gel/PCR Kit. The size of pBLPTA^{L359D} was confirmed by agarose gel electrophoresis.

2.2.5.2 T7_*aroG*^{wt} and T7_*aroG*^{Q151N} preparation

2.2.5.2.1 PCR amplification of T7_*aroG*^{wt} and T7_*aroG*^{Q151N}

To construct pBLPTA^{L359D}G, T7_*aroG* fragments were amplified using forward primer containing *Pac*I and reverse primer containing *Avr*II site at their 5' end. For PCR amplifications, *aroG*^{wt} and *aroG*^{Q151N} (1,053 bp) were amplified from pAroG^{wt} and pAroG^{Q151N}, respectively, using forward primer (aroG_PacI_F) and reverse primer (aroG_AvrII_R). To increase the level of gene expression, the aroG_PacI_F primer was designed from 5' end of T7 promoter. The 50 µL of PCR reaction mixture contained 1x *Pfu* reaction buffer with $MgSO_4$, 1x PCR enhancer, 200 µM of dNTP mix, 1 µM of forward and reverse primers, 1.25 U of *Pfu* polymerase, 1 ng of DNA template and nuclease free water. The PCR condition consisted of initial activation at 95 ºC for 2 min, 32 cycles of denaturation at 95 ºC

for 30 s, annealing at 62 ºC for 45s, extension at 72 ºC for 2 min and the last step of final extension at 72 ºC for 5 min. The PCR products were cleaned by using GenepHlow™ Gel/PCR Kit. After that, the PCR fragments were separated by agarose gel electrophoresis.

2.2.5.2.2 Double digestion with restriction enzymes

Each *aroG* PCR fragment was double digested with *Pac*I and *Avr*II using the method described in section 2.2.5.1.

2.2.5.3 Ligation and transformation

After digestion with *Pac*I and *Avr*II, each *aroG*^{wt} and *aroG*^{Q151N} fragments was ligated with pBLPTA^{L359D} vector using the method described in section 2.2.1.4.3. After that, each ligation reaction was transformed into *E. coli* BL21(DE3) by electroporation method as described in section 2.2.1.5. The construction of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} are shown in Figure 10.

2.2.5.4 Confirmation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

The transformants from section 2.2.5.3 were identified by digestion of their plasmid with *Xho*I. The single colonies of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} were picked up to culture in 5 mL of LB broth containing 30 mg/mL of kanamycin and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. Then recombinant plasmids were extracted by Presto™ Mini Plasmid Kit. Each of recombinant plasmid was digested with *Xho*I by the method in section 2.2.5.1. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to sequence by Bioneer, Korean using F_tktA_aroG_Int as a forward primer and Seqduet_R as a reverse primer as described in section 2.2.1.7.

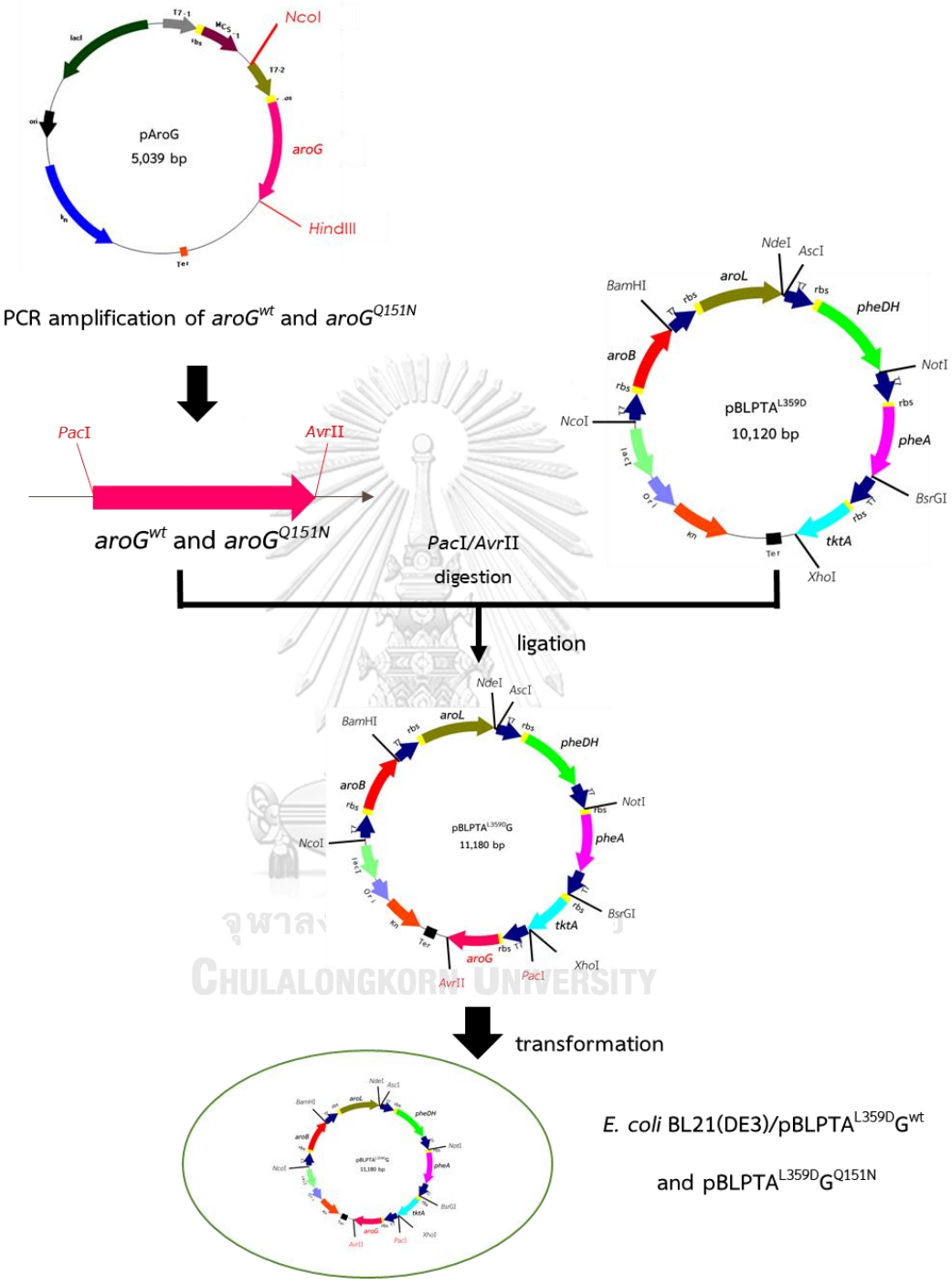


Figure 10. Construction of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

2.2.*6* Co-transformation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} with pYF into *E. coli* BL21(DE3)

The pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} were co-transformed with pYF into *E. coli* BL21(DE3) competent cell using method described in section 2.2.1.5. The transformants were spreaded on LB agar plate containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 ºC for 16-18 h. The growing colonies of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and incubated at 37 ºC with shaking at 250 for 16-18 h. The pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were extracted by Presto™ Mini Plasmid Kit. The recombinant plasmids were confirmed by restriction enzyme digestion. Each of recombinant plasmid was digested with *Bam*HI using method in section 2.2.5.1. The size of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were identified by agarose gel electrophoresis. The colonies containing pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were collected to determine of L-Phe production. The construction of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF are shown in Figure 11.

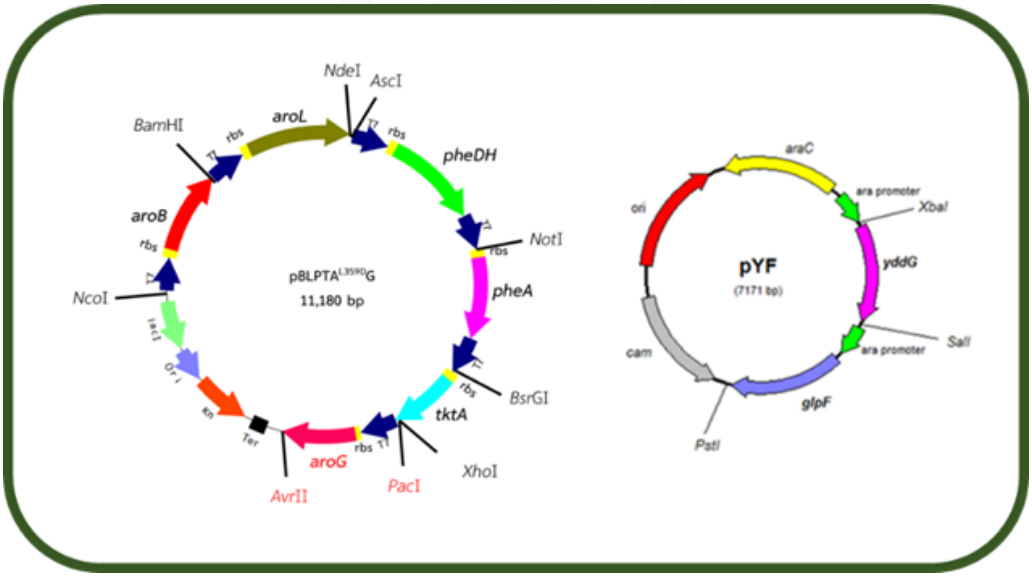


Figure 11. Construction of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF

2.2.7 Determination of L-Phe production by HPLC

Each single colony of pBLPT & pYF, pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF clones from section 2.2.6 was cultured in 5 mL LB medium containing 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol and then incubated at 37 ºC, 250 rpm for 16-18 h. 5% (v/v) of each culture was inoculated into 50 mL of the same medium and cultured of the same conditions. For shake flask cultivation, the 5% (v/v) of starters were separately cultured into 200 mL of minimum medium containing (g/L): 60 glycerol, 42.5 $(NH_4)_2SO_4$, 0.3 $MgSO_4{\cdot}7H_2O$, 0.075 $FeSO_4{\cdot}7H_2O$, 0.015 $CaCl_2{\cdot}2H_2O$, 12 K_2HPO_4, 3 KH_2PO_4, 1 NaCl, 1 Na-citrate, 0.0075 thiamine-HCl and 1.5 mL of trace elements solution contained (g/L): 2.0 $Al_2(SO_4)_3{\cdot}18H_2O$, 3.0 $Na_2MoO_4{\cdot}2H_2O$, 0.75 $CoSO_4{\cdot}7H_2O$, 15 $ZnSO_4{\cdot}7H_2O$, 2.5 $CuSO_4{\cdot}5H_2O$, 0.5 H_3BO_3, 24 $MnSO_4{\cdot}7H_2O$ and 2.5 $NiSO_4{\cdot}6H_2O$ at pH 7.0. The cultures were shaken at 37 ºC, 250 rpm. After the OD_{600} reached 0.6 (log phase), the expression of *yddG* and *glpF* under ara promoter of pBAD33 were induced with 0.02% arabinose. The 1.5 mL of each sample were collected every 24 h for 8 days to measure cell density (OD_{600}) and L-Phe production. The supernatants of each sample were filtrated through 0.22 µm nylon syringe filter. The L-Phe production was measured by HPLC method using Chirex 3126 (D)-penicillamine column. The ratio of 75:25 of 2 mM copper sulfate and methanol was used as a mobile phase and the flow rate was 0.7 mL/min. The peak of L-Phe was detected at wavelength 254 nm. The concentrations of L-Phe were estimated from the standard curve of L-Phe.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Construction of pPheA^{wt} and pPheA^{L359D}

3.1.1 Plasmid extraction

pDuet_pheA^{wt} and pDuet_pheA^{L359D} (5,078 bp) were extracted from *E. coli* Top10. The agarose gel electrophoresis was shown in Figure 12. Then, the pDuet_pheA^{wt} and pDuet_pheA^{L359D} were confirmed by digested with *Afl*II and *Bam*HI. From the result of agarose gel electrophoresis in Figure 13, the DNA fragments were observed around 3.4 kb and 1.2 kb. Therefore, pDuet_pheA^{wt} No.1 and pDuet_pheA^{L359D} No.1 were used for *pheA* amplification.

3.1.2 Amplification of *pheA^{wt}* and *pheA^{L359D}*

The pheA^{wt} and pheA^{L359D} were amplified from pDuet_pheA^{wt} and pDuet_pheA^{L359D}, respectively, using forward primer containing *Not*I site and reverse primer containing *Bsr*GI site. After cleaning by GenepHlow™ Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. The size of *pheA^{wt}* and *pheA^{L359D}* fragments were detected around 1.2 kb as shown in Figure 14.

3.1.3 Cloning of *pheA^{wt}* and *pheA^{L359D}*

The PCR products of *pheA^{wt} and pheA^{L359D}* from section 3.1.2 and pRSFDuet-1 vector were digested with *Not*I and *Bsr*GI. The results of *pheA^{wt}* and *pheA^{L359D}* fragments (1.2 kb) and pRSFDuet-1 linear vector (3.9 kb) after digestion are shown in Figure 15. After that, *pheA^{wt}* and *pheA^{L359D}* fragments were ligated with pRSFDuet-1 linear vector and then transformed into *E. coli* Top10 by electroporation. Four colonies of pPheA^{wt} and five colonies of pPheA^{L359D} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. From Figure 16, the plasmid from pPheA^{wt} transformant No.1, 3 and 4 and pPheA^{L359D} transformant No.1, 2 and 4 moved slower than pRSFDuet-1. Thus, the pPheA^{wt} No.1, 3 and 4 and pPheA^{L359D} No.1, 2 and 4 might harbor the inserted genes. These six plasmids were confirmed by digestion with *Not*I and *Bsr*GI.

From digestion pattern, each recombinant plasmid gave two bands of DNA fragments around 3.9 kb and 1.2 kb as shown in Figure 17. This result confirmed that *pheA*^{wt} and *pheA*^{L359D} were successfully inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

3.1.4 Nucleotide sequencing

To verify the nucleotide sequence of *pheA*^{wt} and *pheA*^{L359D} genes, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuetUP1 as a forward primer and T7 terminator as a reverse primer. The obtained DNA sequences were compared with wild type *pheA* reported by Naksusuk in 2015 using nucleotide blast tools in NCBI and then translated to protein sequence using Genetyx-Win program.

From Figure 18 - 19, nucleotide sequences of *pheA*^{wt} and *pheA*^{L359D} are similar to that of reference nucleotides [40]. Only nucleotide sequence at restriction sites were changed from *Afl*II to *Not*I and *Bam*HI to *Bsr*GI. The correct *pheA*^{wt} and *pheA*^{L359D} were used in the further experiment.

3.1.5 Expression of PheA^{wt}

The expression of PheA^{wt} under T7 promoter was evaluated by SDS-PAGE analysis. The *E. coli* BL21(DE3) harboring pPheA^{wt} from section 3.1.4 was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce *pheA* expression. The whole cells of *E. coli* BL21(DE3) harboring pPheA^{wt} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The protein of *E. coli* BL21(DE3) and *E. coli* BL21(DE3) harboring pRSFDuet-1 were used as a control in lane 1 and 2, respectively. The protein band of chorismite mutase/prephenate dehydratase was detected after 1 mM IPTG induction for 1-6 h in lane 3-8, respectively. The size of recombinant protein was approximately 43 kDa as shown in Figure 20.

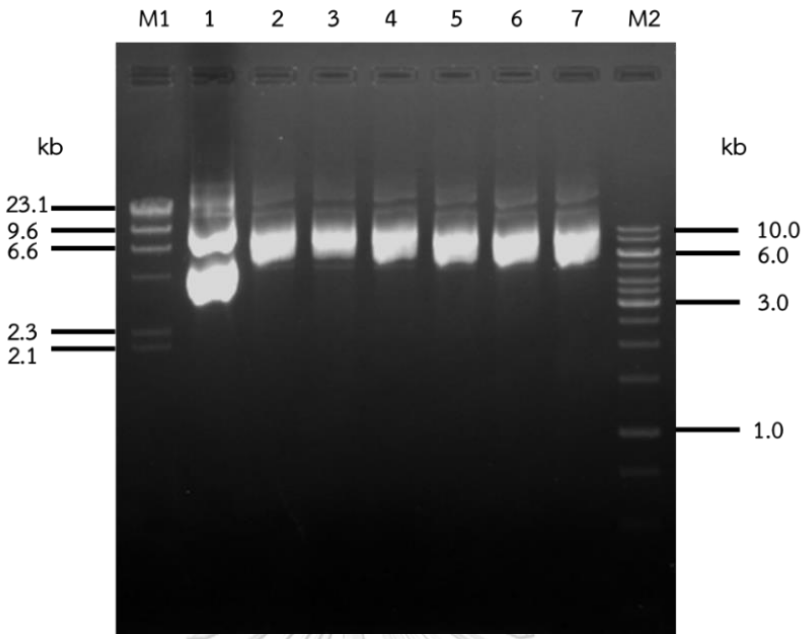


Figure 12. Electrophoretic patterns of pPheA^{wt} and pPheA^{L359D}

Lane M1 : λ DNA /*Hin*dIII marker

Lane 1 : pRSFDuet-1

Lane 2-4 : pPheA^{wt} No.1-3, respectively

Lane 5-7 : pPheA^{L359D} No.1-3, respectively

Lane M2 : Gene Ruler 1 kb DNA ladder

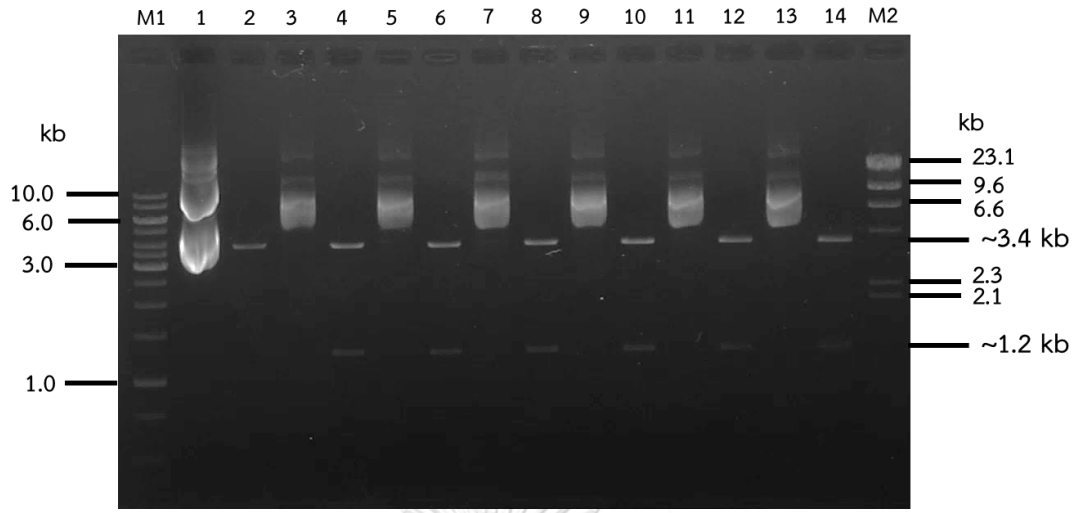


Figure 13. *Afl*II and *Hin*dIII digestion patterns of pPheA^{wt} and pPheA^{L359D}.

Lane M1 : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : *Afl*II/*Hin*dIII digested pRSFDuet-1

Lane 3, 5, 7 : uncut pPheA^{wt} No.1-3 , respectively

Lane 4, 6, 8 : *Afl*II/*Hin*dIII digested pPheA^{wt} No.1-3, respectively

Lane 9, 11, 13 : uncut pPheA^{L359D} No.1-3, respectively

Lane 10, 12, 14 : *Afl*II/*Hin*dIII digested pPheA^{L359D} No.1-3, respectively

Lane M2 : λ DNA /*Hin*dIII marker

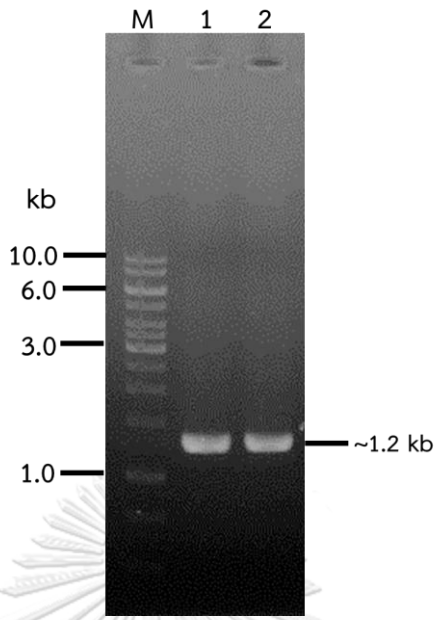


Figure 14. PCR products of *pheA*^{wt} and *pheA*^{L359D}

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : PCR product of *pheA*^{wt}

Lane 2 : PCR product of *pheA*^{L359D}

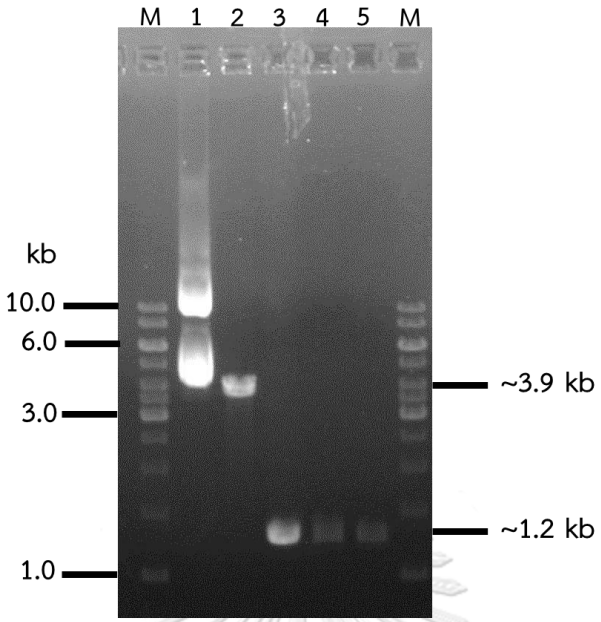


Figure 15. *Not*I and *Bsr*GI digestion patterns of PCR products and pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : *Not*I and *Bsr*GI digested pRSFDuet-1

Lane 3 : uncut PCR product of $pheA^{wt}$

Lane 4 : *Not*I and *Bsr*GI digested PCR product of $pheA^{wt}$

Lane 5 : *Not*I and *Bsr*GI digested PCR product of $pheA^{L359D}$

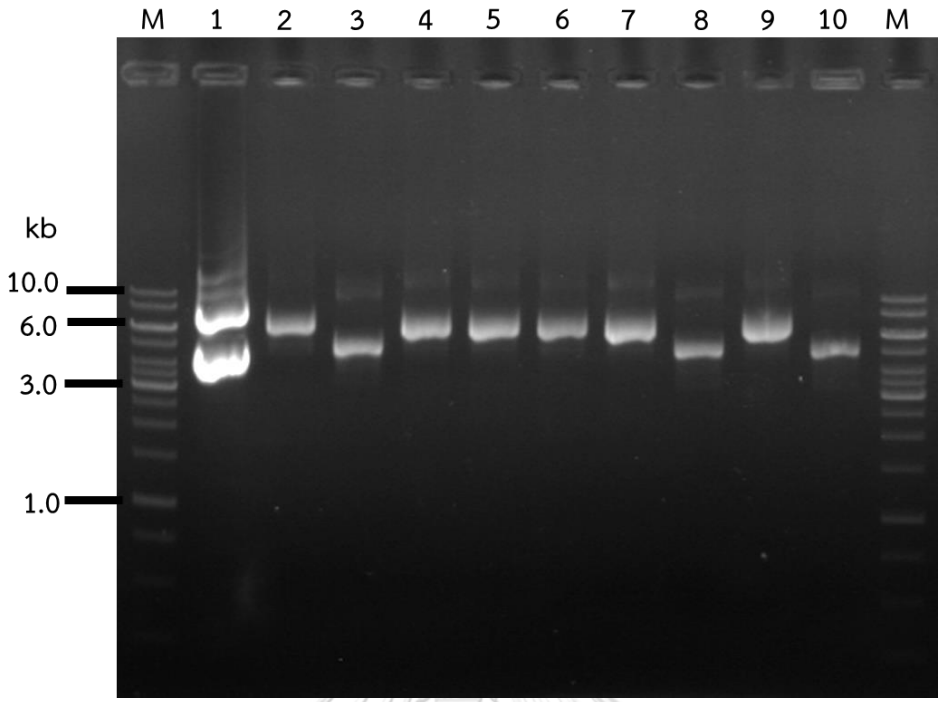


Figure 16. Electrophoretic pattern of plasmid from pPheA transformants.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2-5 : pPheA^{wt} from transformant No.1-4, respectively

Lane 6-10 : pPheA^{L359D} from transformant No.1-5, respectively

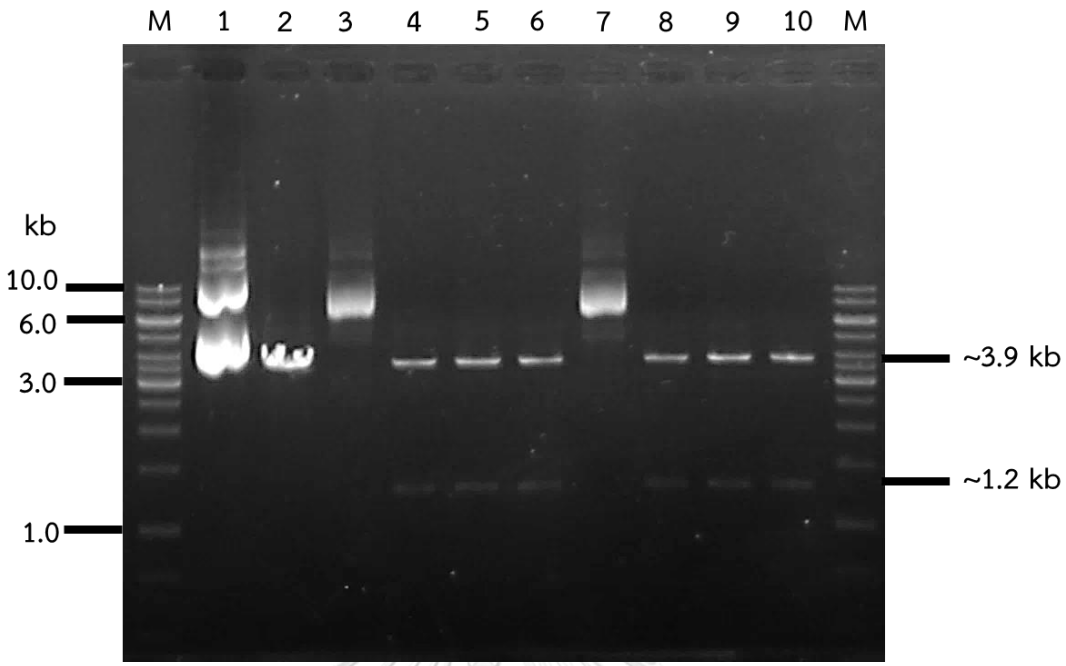


Figure 17. *Not*I and *Bsr*GI digestion patterns of pPheA^{wt} and pPheA^{L359D}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : pRSFDuet-1 digested with *Not*I and *Bsr*GI

Lane 3 : uncut pPheA^{wt} transformant No.1

Lane 4 : *Not*I and *Bsr*GI digested pPheA^{wt} from transformant No.1

Lane 5 : *Not*I and *Bsr*GI digested pPheA^{wt} from transformant No.3

Lane 6 : *Not*I and *Bsr*GI digested pPheA^{wt} from transformant No.4

Lane 7 : uncut pPheA^{L359D} transformant No.1

Lane 8 : *Not*I and *Bsr*GI digested pPheA^{L359D} from transformant No.1

Lane 9 : *Not*I and *Bsr*GI digested pPheA^{L359D} from transformant No.2

Lane 10 : *Not*I and *Bsr*GI digested pPheA^{L359D} from transformant No.4

Score	Expect	Identities	Gaps	Strand
2145 bits(1161)	0.0	1161/1161(100%)	0/1161(0%)	Plus/Plus

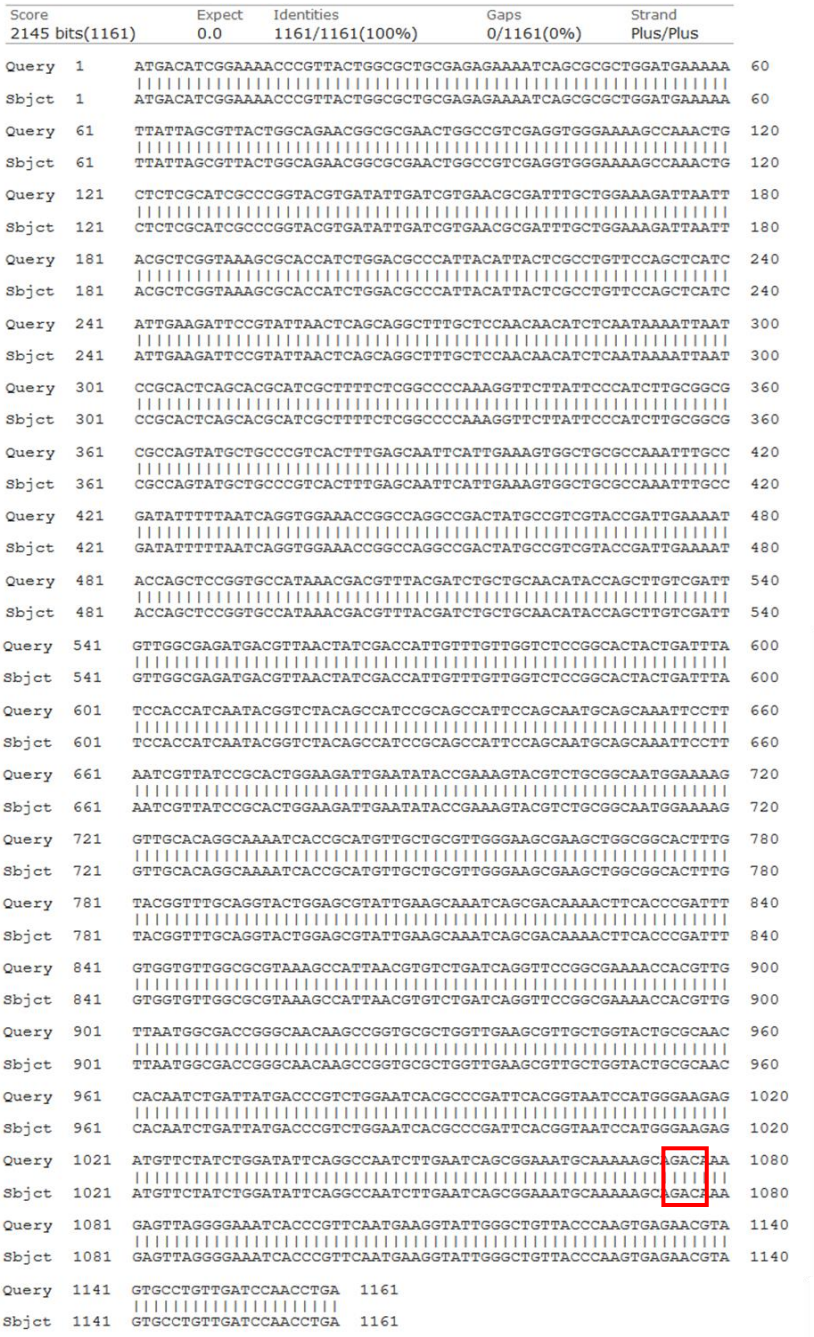
```
Query  1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA  60

Query  61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG  120

Query  121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT  180

Query  181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC  240

Query  241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT  300

Query  301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG  360

Query  361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC  420

Query  421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT  480

Query  481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT  540

Query  541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA  600

Query  601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT  660

Query  661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG  720

Query  721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG  780

Query  781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT  840

Query  841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG  900

Query  901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC  960

Query  961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG  1020

Query  1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA  1080

Query  1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA  1140

Query  1141  GTGCCTGTTGATCCAACCTGA  1161
             ||||||||||||||||||||
Sbjct  1141  GTGCCTGTTGATCCAACCTGA  1161
```

Figure 18. Nucleotide sequence of *pheA*^{wt}

Query represents the nucleotide sequence of *pheA*^{wt} in this work.

Sbjct represents the nucleotide sequence of *pheA*^{wt} reference [40].

The chromatogram of *pheA*^{wt} is shown in Appendix N.



Score	Expect	Identities	Gaps	Strand
2145 bits(1161)	0.0	1161/1161(100%)	0/1161(0%)	Plus/Plus

```
Query  1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA  60

Query  61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG  120

Query  121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT  180

Query  181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC  240

Query  241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT  300

Query  301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG  360

Query  361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC  420

Query  421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT  480

Query  481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT  540

Query  541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA  600

Query  601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT  660

Query  661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG  720

Query  721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG  780

Query  781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT  840

Query  841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG  900

Query  901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC  960

Query  961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG  1020

Query  1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCAGACAAA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCAGACAAA  1080

Query  1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA  1140

Query  1141  GTGCCTGTTGATCCAACCTGA  1161
             |||||||||||||||||||||
Sbjct  1141  GTGCCTGTTGATCCAACCTGA  1161
```

Figure 19. Nucleotide sequence of *pheA*^{L359D}

Query represents the nucleotide sequence of *pheA*^{L359D} in this work.

Sbjct represents the nucleotide sequence of *pheA*^{L359D} reference [40].

Red box represents the mutation of Leu359Asp.

The chromatogram of *pheA*^{L359D} is shown in Appendix O.

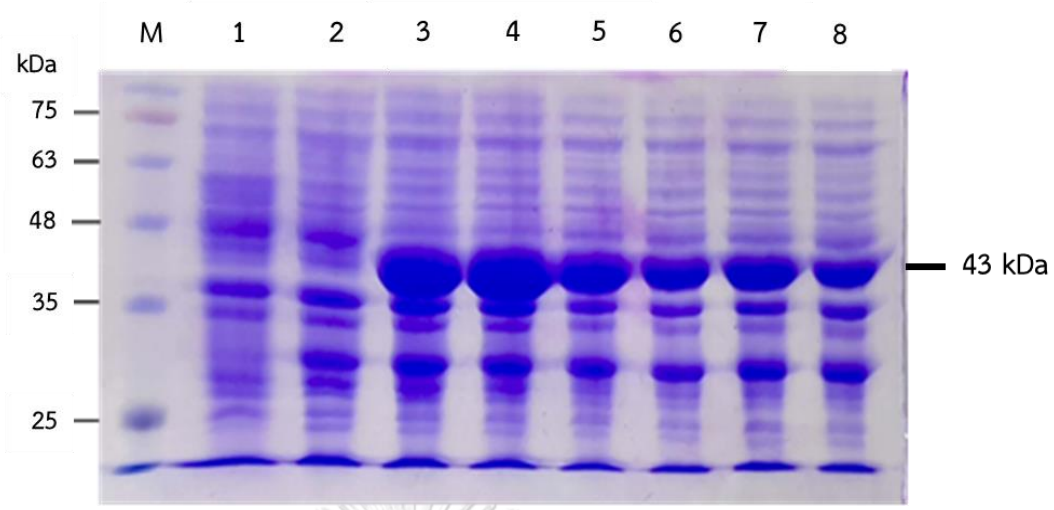


Figure 20. SDS-PAGE of whole cell extracts of *E. coli* BL21(DE3) harboring *pheA*^{wt} after induction with 1 mM IPTG.

Lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : *E. coli* BL21(DE3) after IPTG induction for 1 h

Lane 2 : *E. coli* BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h

Lane 3 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 1 h

Lane 4 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 2 h

Lane 5 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 3 h

Lane 6 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 4 h

Lane 7 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 5 h

Lane 8 : *E. coli* BL21(DE3) harboring pPheA^{wt} after IPTG induction for 6 h

3.2 Construction of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}

The pPheA^{wt} and pPheA^{L359D} from section 3.1.4. were digested with *Bsr*GI and *Not*I. After that, the *pheA^{wt}* and *pheA^{L359D}* fragments were purified from agarose gel using GenepHlow™ Gel/PCR Kit. The pBLPTG^{Q151L} was digested with the same restriction enzymes and purified using same method. From Figure 21, the linear form of pBLPTG^{Q151L} was shown at size around 9.9 kb in lane 3. For *pheA^{wt}* and *pheA^{L359D}* fragments, the DNA bands were obtained at size around 1.2 kb as shown in Figure 22 in lane 3 and 6, respectively. Then, *pheA^{wt}* and *pheA^{L359D}* fragments were ligated with linear pBLPTG^{Q151L} vector and transformed into *E. coli* BL21(DE3). The single colonies of transformants were randomly picked up and cultured in LB broth containing 30 mg/mL of kanamycin. After extraction, the recombinant plasmids were digested with *Xho*I to confirm the positive plasmids of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} as shown in Figure 23. The digestion of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} with *Xho*I gave two DNA bands around 9.9 kb and 1.2 kb as shown in lane 2, 4, 6, 8 and 10. This result showed that the pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D} were successfully constructed.

3.3 Expression of AroG^{wt}

The expression of AroG^{wt} under T7 promoter was evaluated by SDS-PAGE analysis. The *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} from Kanoksinwutthipong was cultured in LB medium containing 30 mg/mL of kanamycin. After cell culture reached log phase, IPTG was added to 1 mM to induce *aroG* expression. The whole cells of *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used as protein molecular weight marker. The whole cell extract of *E. coli* BL21(DE3) harboring pRSFDuet-1 was used as a control in lane 1. The protein bands of *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after 1 mM IPTG induction for 1-6 h are shown in lane 3-8, respectively (Figure 24).

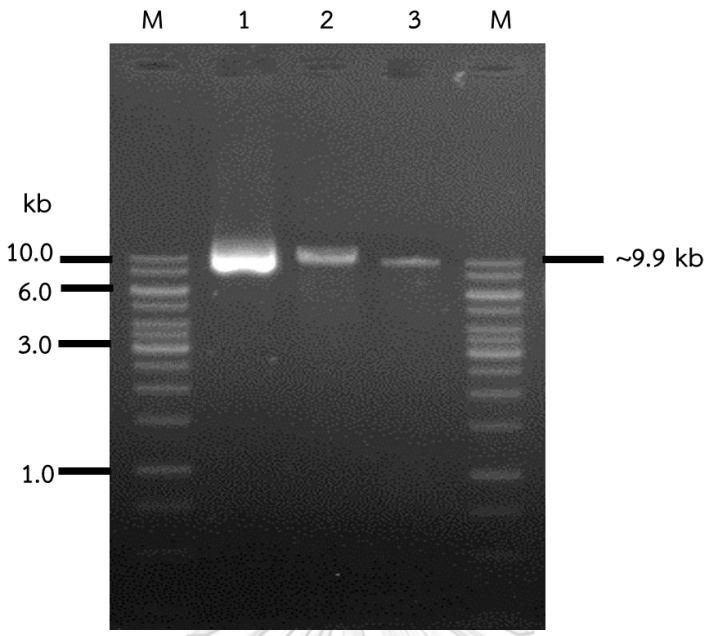


Figure 21. *Not*I and *Bsr*GI digestion pattern of pBLPTG^{Q151L}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTG^{Q151L}

Lane 2 : *Not*I digested pBLPTG^{Q151L}

Lane 3 : *Not*I and *Bsr*GI digested pBLPTG^{Q151L}

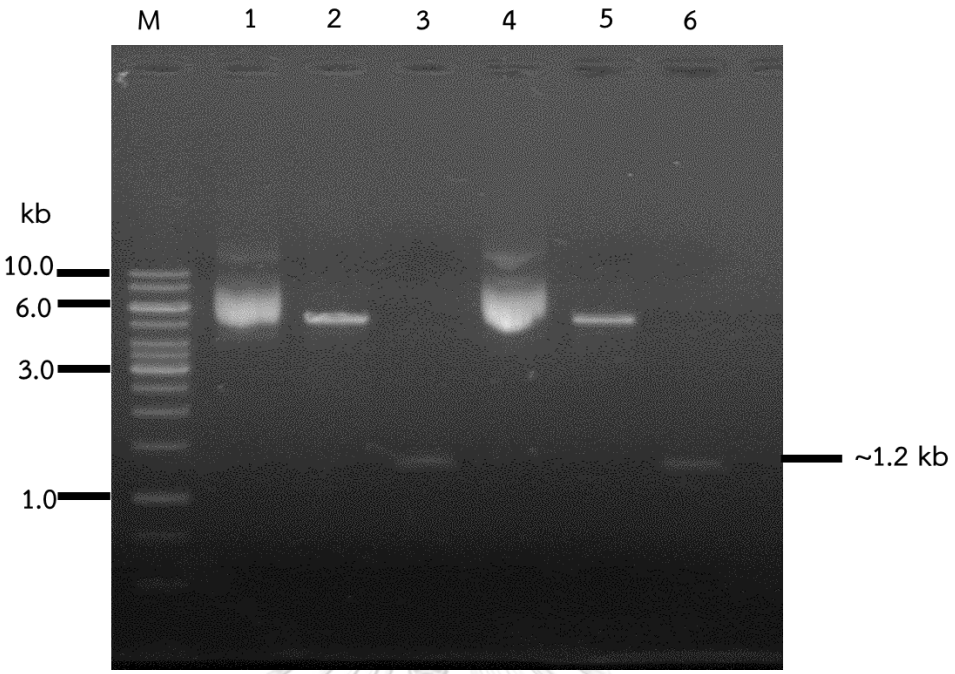


Figure 22. *Not*I and *Bsr*GI digestion patterns of pPheA^{wt} and pPheA^{L359D}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pPheA^{wt}

Lane 2 : *Not*I digested pPheA^{wt}

Lane 3 : *Not*I and *Bsr*GI digested pPheA^{wt}

Lane 4 : uncut pPheA^{L359D}

Lane 5 : *Not*I digested pPheA^{L359D}

Lane 6 : *Not*I and *Bsr*GI digested pPheA^{L359D}

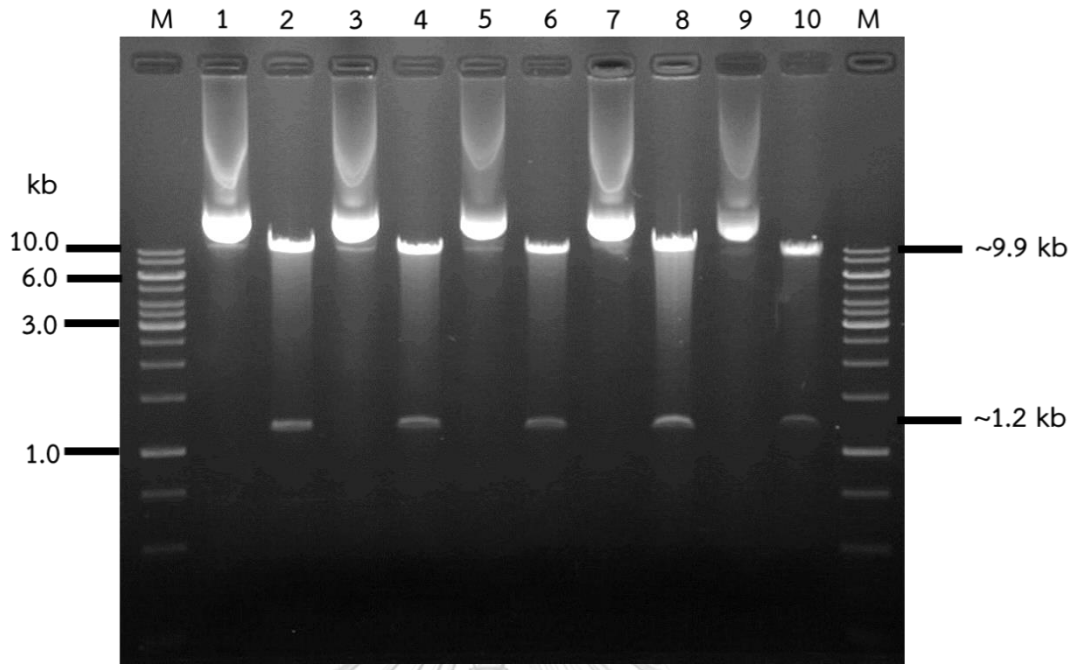


Figure 23. *Xho*I digestion patterns of pBLPTG^{Q151L}A^{wt} and pBLPTG^{Q151L}A^{L359D}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTG^{Q151L}A^{wt} from transformant No.1

Lane 2 : *Xho*I digested pBLPTG^{Q151L}A^{wt} from transformant No.1

Lane 3 : uncut pBLPTG^{Q151L}A^{wt} from transformant No.2

Lane 4 : *Xho*I digested pBLPTG^{Q151L}A^{wt} from transformant No.2

Lane 5 : uncut pBLPTG^{Q151L}A^{L359D} from transformant No.1

Lane 6 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.1

Lane 7 : uncut pBLPTG^{Q151L}A^{L359D} from transformant No.2

Lane 8 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.2

Lane 9 : uncut pBLPTG^{Q151L}A^{L359D} from transformant No.3

Lane 10 : *Xho*I digested pBLPTG^{Q151L}A^{L359D} from transformant No.3

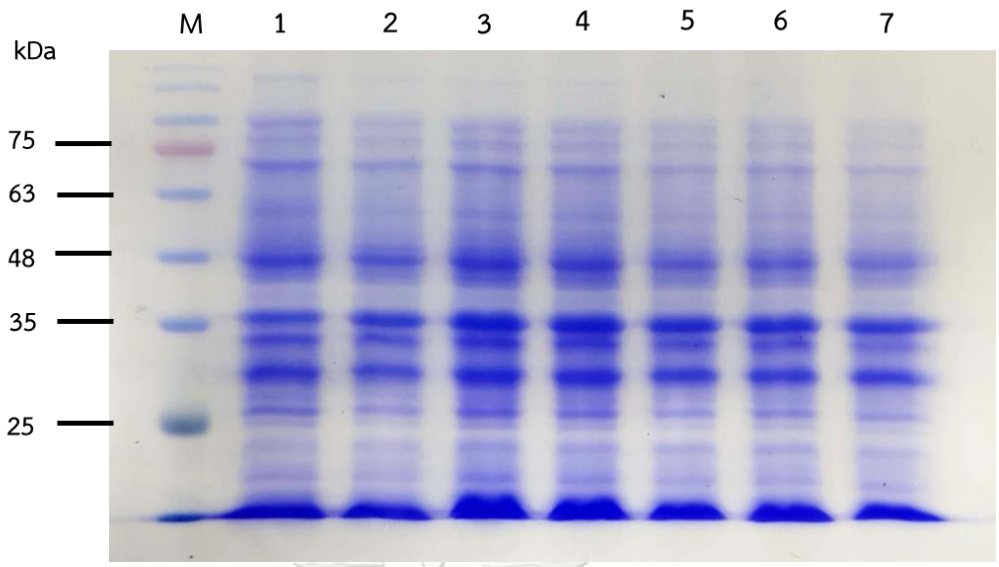


Figure 24 SDS-PAGE of whole cell extracts of *E. coli* BL21(DE3) harboring *aroG*^{wt} after induction with 1 mM IPTG.

Lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : *E. coli* BL21(DE3) harboring pRSFDuet-1 after IPTG induction for 1 h

Lane 2 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 1 h

Lane 3 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 2 h

Lane 4 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 3 h

Lane 5 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 4 h

Lane 6 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 5 h

Lane 7 : *E. coli* BL21(DE3) harboring pDuet_AroG^{wt} after IPTG induction for 6 h

In our previous work, we paid a lot of attempt to determine the expression of AroG by SDS-PAGE analysis and DAHP synthase activity assay. The AroG band could not be observed on SDS-PAGE. Moreover, DAHP synthase activity of the recombinant clone was not differ from that of *E. coli* host cell. At first, we suspected that there were some defects on T7 promoter of pRSFDuet-1 vector used in our laboratory, so *aroG* was subcloned under T7 promoter of pET-28b and again the protein band and activity of AroG could not be detected.

In this research, the sequence of pDuet_AroG was rechecked. The *aroG* was cloned into pRSFDuet-1 between *Bam*HI and *Hin*dIII sites. As shown in Figure 25, the sequence of forward primer was 5'-CGGGATCCATGAATTATCAGA ACGACGATTTACGC-3'. *Bam*HI site is shown in blue and start codon of *aroG* is shown in red. Translation of the gene inserted under T7 promoter-1 is started from Met of His-tag (shown in red box). When *aroG* was inserted, the translation frame of *aroG* was one base shifted and translation was stopped at TGA (shown in green box). Thus, AroG could not be synthesized.

3.4 Reconstruction of pAroG and pAroG^{fbr}

In this part, new forward primer was designed and used for cloning *aroG* into pRSFDuet-1.

3.4.1 Plasmid extraction

pDuet_AroG^{wt} and pDuet_AroG^{fbr} (5,039 bp) were extracted from *E. coli* Top10. The agarose gel electrophoresis is shown in Figure 26. Each recombinant plasmid was used as a template for *aroG* amplification.

Figure 25. Frameshift mutation of *aroG*

A. pRSFDuet-1 vector, red box shows start codon of the recombinant protein.

B. Sequence of inserted *aroG*, *Bam*HI site – blue letter

start codon of *aroG* – red letter

stop codon – green box

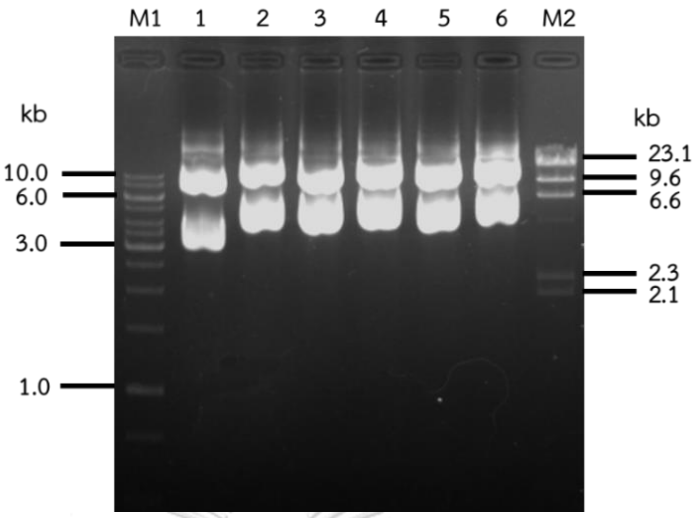


Figure 26. Electrophoretic patterns of pDuet_AroG^{wt} and pDuet_AroG^{fbr}

Lane M1 : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2 : pDuet_AroG^{wt}

Lane 3 : pDuet_AroG^{L175D}

Lane 4 : pDuet_AroG^{Q151L}

Lane 5 : pDuet_AroG^{Q151A}

Lane 6 : pDuet_AroG^{Q151N}

Lane M2 : λ DNA /*Hin*dIII marker

3.4.2 Primer design at 5'end of *aroG*

The new forward primer containing *Nco*I at 5'end was designed. The sequence of new forward primer F_AroG_NcoI is

5'-CATGCCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATC-3'

Blue letter indicates *Nco*I sites while blue underline shows start codon of *aroG.*

3.4.3 PCR amplification of *aroG*^{wt} and *aroG*^{fbr}

The *aroG*^{wt} and *aroG*^{fbr} were amplified from pDuet_AroG^{wt} and pDuet_AroG^{fbr} using forward primer containing *Nco*I site and reverse primer containing *Hin*dIII site. After cleaning by GenepHlow™ Gel/PCR Kit, the PCR fragments were separated by agarose gel electrophoresis. From the result shown in Figure 27, the size of *aroG*^{wt} and *aroG*^{fbr} fragments were detected around 1.1 kb.

3.4.4 Digestion of *aroG*^{wt} and *aroG*^{fbr} fragments and pRSFDuet-1

The *aroG*^{wt} *and aroG*^{fbr} fragments from section 3.4.3 and pRSFDuet-1 vector were digested with *Nco*I and *Hin*dIII. The linear pRSFDuet-1 (3.9 kb) and *aroG*^{wt} *and aroG*^{fbr} fragments (1.1 kb) after digestion are shown in Figure 28-29, respectively. After that, *aroG*^{wt} and *aroG*^{fbr} fragments were ligated to pRSFDuet-1 linear vector and then transformed into *E. coli* Top10 by electroporation. The pAroG^{wt} and pAroG^{fbr} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted and then detected by agarose gel electrophoresis. The pAroG^{wt} from transformant No.1, 3, 4, 5 and 6, pAroG^{L175D} from transformant No.2 - 6 (Figure 30), pAroG^{Q151L} from transformant No.3 and pAroG^{Q151A} from transformant No.4 (Figure 31) as well as pAroG^{Q151N} from transformant No.6 (Figure 32) moved slower than that of pRSFDuet-1.

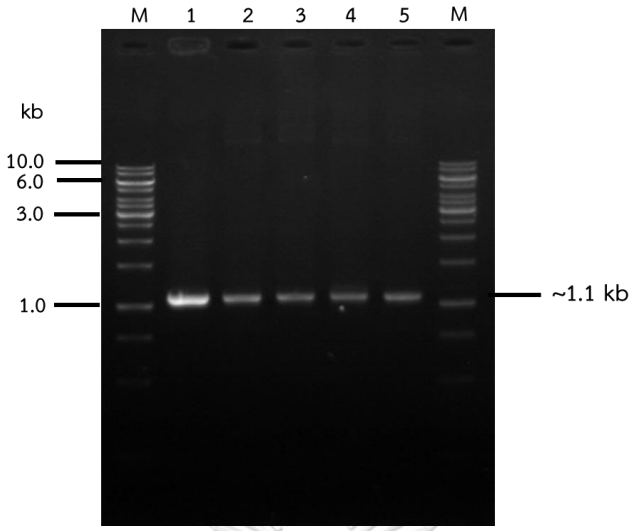


Figure 27. PCR products of *aroG*^{wt} and *aroG*^{fbr}

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : PCR product of *aroG*^{wt}

Lane 2 : PCR product of *aroG*^{L175D}

Lane 3 : PCR product of *aroG*^{Q151L}

Lane 4 : PCR product of *aroG*^{Q151A}

Lane 5 : PCR product of *aroG*^{Q151N}

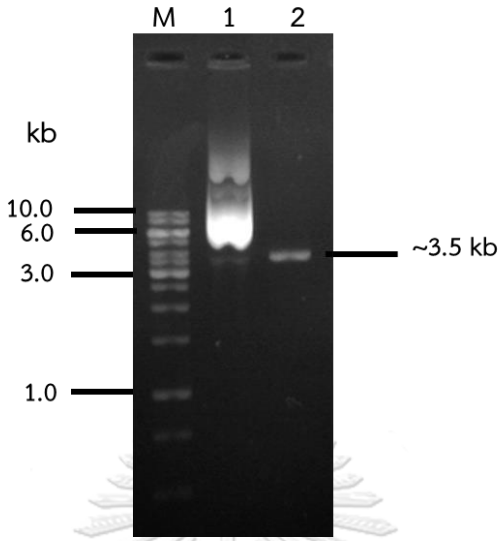


Figure 28. *Nco*I and *Hin*dIII digestion pattern of pRSFDuet-1.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : *Nco*I and *Hin*dIII digested pRSFDuet-1

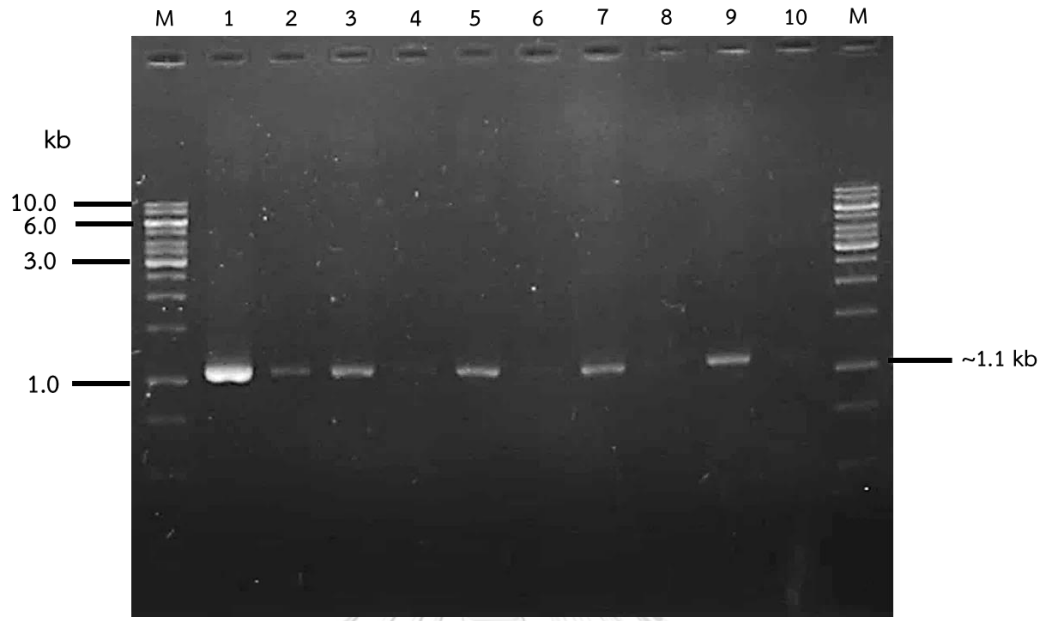


Figure 29. *Nco*I and *Hin*dIII digestion pattern of PCR products.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut PCR product of aroG^{wt}

Lane 2 : *Nco*I and *Hin*dIII digested PCR product of aroG^{wt}

Lane 3 : uncut PCR product of aroG^{L175D}

Lane 4 : *Nco*I and *Hin*dIII digested PCR product of aroG^{L175D}

Lane 5 : uncut PCR product of aroG^{Q151L}

Lane 6 : *Nco*I and *Hin*dIII digested PCR product of aroG^{Q151L}

Lane 7 : uncut PCR product of aroG^{Q151A}

Lane 8 : *Nco*I and *Hin*dIII digested PCR product of aroG^{Q151A}

Lane 9 : uncut PCR product of aroG^{Q151N}

Lane 10 : *Nco*I and *Hin*dIII digested PCR product of aroG^{Q151N}

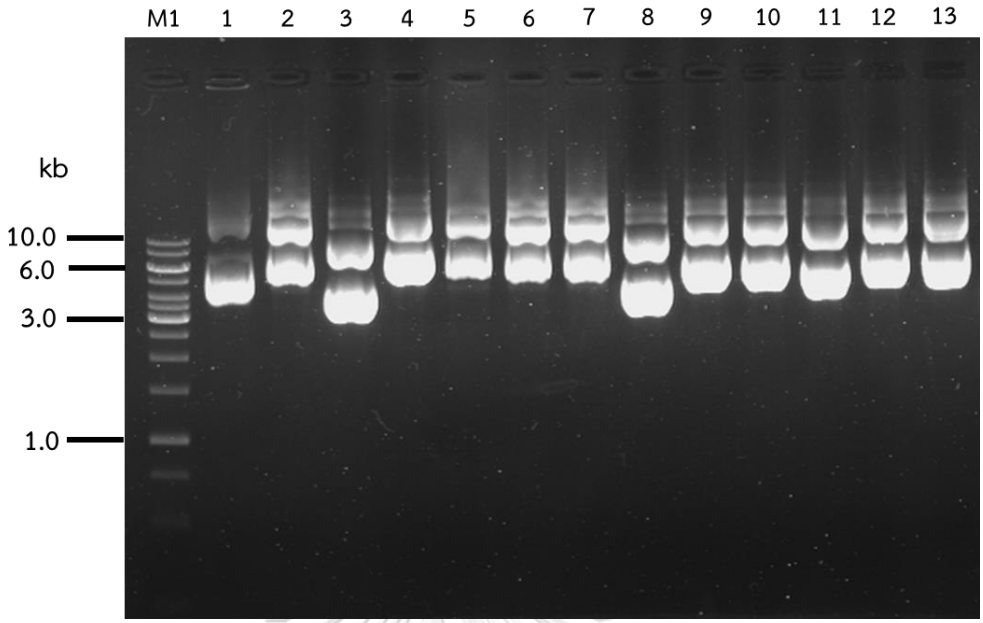


Figure 30. Electrophoretic pattern of plasmid from pAroG^{wt} and pAroG^{L175D} transformants.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2-7 : pAroG^{wt} from transformant No.1-6, respectively

Lane 8-13 : pAroG^{L175D} from transformant No.1-6, respectively

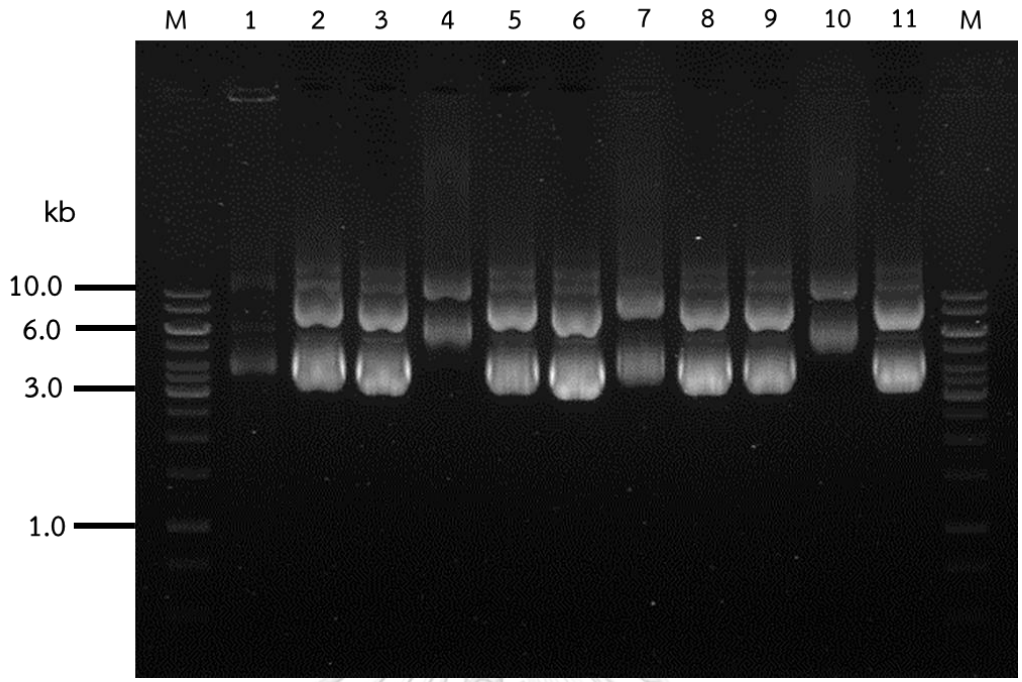


Figure 31. Electrophoretic pattern of plasmid from pAroG^{Q151L} and pAroG^{Q151A} transformants.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2-6 : pAroG^{Q151L} from transformant No.1-5, respectively

Lane 6-11 : pAroG^{Q151A} from transformant No.1-5, respectively

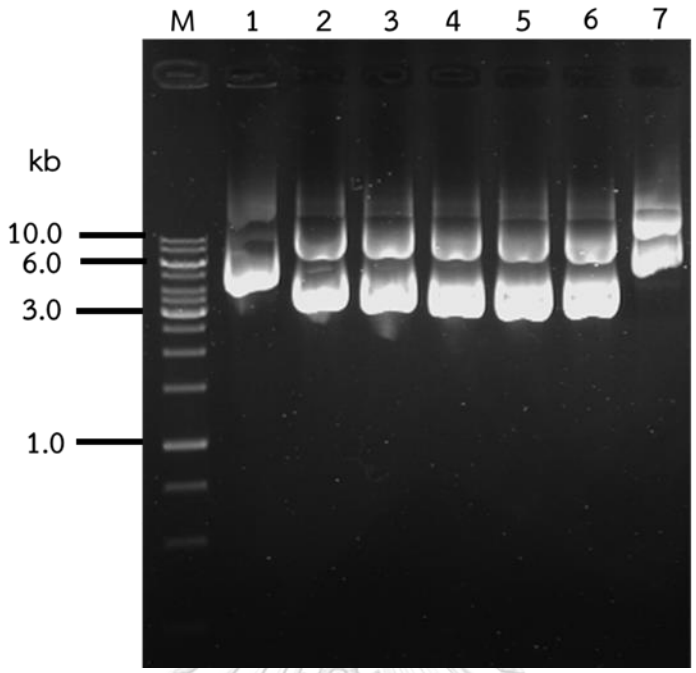


Figure 32. Electrophoretic pattern of plasmid from pAroG^{Q151N} transformants.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : pRSFDuet-1

Lane 2-7 : pAroG^{Q151N} from transformant No.1-6, respectively

Thus, the transformant No.1 from pAroG^{wt}, transformant No.2 from pAroG^{L175D}, transformant No.3 from pAroG^{Q151L}, transformant No.4 from pAroG^{Q151A} and transformant No.6 from pAroG^{Q151L} were confirmed by digested with *Nco*I and *Hin*dIII. From digestion pattern, each transformant gave two bands of DNA fragments around 3.9 kb and 1.1 kb as shown in Figure 33. This result confirmed that *aroG* genes were inserted into pRSFDuet-1. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

3.4.5 Nucleotide sequence of *aroG* genes

To verify the nucleotide sequences of *aroG^{wt}* and *aroG^{fbr}*, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using F_tktA_aroG_Int as a forward primer and Seqduet_R as a reverse primer. The obtained DNA sequences were compared with wild type *aroG* reported by Kanoksinwutthipong in 2014. Genetyx-Win program was used to translate protein sequence [41].

The all of nucleotide sequence of *aroG^{wt}* and *aroG^{fbr}* were changed only at the mutated sites (Figure 34 - 38). The *aroG^{wt}* and *aroG^{fbr}* were used in the further experiment.

3.4.6 Expression of AroG^{wt} and AroG^{fbr}

3.4.6.1 Protein expression

The expression of *aroG^{wt}* and *aroG^{fbr}* under T7 promoter were evaluated by SDS-PAGE analysis. The crude enzyme of AroG^{wt} and AroG^{fbr} were mixed with 5x sample buffer. The supernatants were loaded into the gel. Tri-color protein color was used for protein molecular weight marker. Crude extracts of *E. coli* BL21(DE3) and *E. coli* BL21(DE3) harboring pRSFDuet-1 were used as controls in lane 1 and 2. The protein bands of AroG^{wt} and AroG^{fbr} were detected after 1 mM IPTG induction for 2 h in lane 3-7, respectively. The sizes of recombinant proteins were approximately 38 kDa as shown in Figure 39.

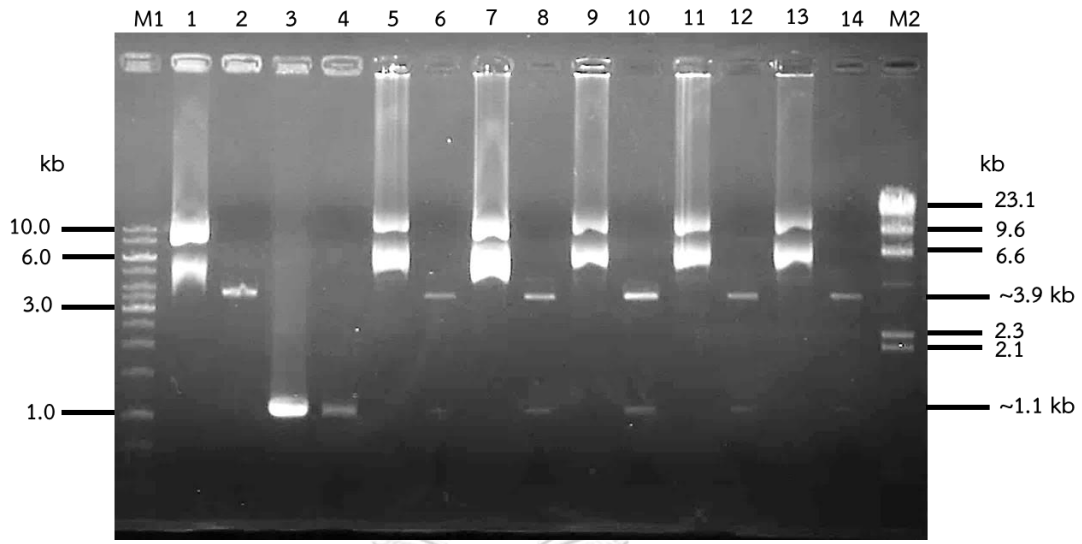


Figure 33. *Nco*I and *Hin*dIII digestion patterns of pAroG^{wt} and pAroG^{fbr}.

Lane M1 : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pRSFDuet-1

Lane 2 : *Nco*I and *Hin*dIII digested pRSFDuet-1

Lane 3 : uncut PCR product of *aroG*^{wt}

Lane 4 : *Nco*I and *Hin*dIII digested PCR product of *aroG*^{wt}

Lane 5 : uncut pAroG^{wt} from transformant No.1

Lane 6 : *Nco*I and *Hin*dIII digested pAroG^{wt} from transformant No.1

Lane 7 : uncut pAroG^{L175D} from transformant No.2

Lane 8 : *Nco*I and *Hin*dIII digested pAroG^{L175D} from transformant No.2

Lane 9 : uncut pAroG^{Q151L} from transformant No.3

Lane 10 : *Nco*I and *Hin*dIII digested pAroG^{Q151L} from transformant No.3

Lane 11 : uncut pAroG^{Q151A} from transformant No.4

Lane 12 : *Nco*I and *Hin*dIII digested pAroG^{Q151A} from transformant No.4

Lane 13 : uncut pAroG^{Q151N} from transformant No.6

Lane 14 : *Nco*I and *Hin*dIII digested pAroG^{Q151N} from transformant No.6

```
        10         20         30         40         50         60
TTATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTT
        70         80         90        100        110        120
TGTTTAACTTTAATAAGGAGATATACCATGGTGTATCAGAACGACGATTTACGCATCAAA
                           M  V  Y  Q  N  D  D  L  R  I  K
       130        140        150        160        170        180
GAAATCAAAGAGTTACTTCCTCCTGTCGCATTGCTGGAAAAATTCCCCGCTACTGAAAAT
 E  I  K  E  L  L  P  P  V  A  L  L  E  K  F  P  A  T  E  N
       190        200        210        220        230        240
GCCGCGAATACGGTTGCCCATGCCCGAAAAGCGATCCATAAGATCCTGAAAGGTAATGAT
 A  A  N  T  V  A  H  A  R  K  A  I  H  K  I  L  K  G  N  D
       250        260        270        280        290        300
GATCGCCTGTTGGTTGTGATTGGCCCATGCTCAATTCATGATCCTGTCGCGGCAAAAGAG
 D  R  L  L  V  V  I  G  P  C  S  I  H  D  P  V  A  A  K  E
       310        320        330        340        350        360
TATGCCACTCGCTTGCTGGCGCTGCGTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATG
 Y  A  T  R  L  L  A  L  R  E  E  L  K  D  E  L  E  I  V  M
       370        380        390        400        410        420
CGCGTCTATTTTGAAAAGCCGCGTACCACGGTGGGCTGGAAAGGGCTGATTAACGATCCG
 R  V  Y  F  E  K  P  R  T  T  V  G  W  K  G  L  I  N  D  P
       430        440        450        460        470        480
CATATGGATAATAGCTTCCAGATCAACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTT
 H  M  D  N  S  F  Q  I  N  D  G  L  R  I  A  R  K  L  L  L
       490        500        510        520        530        540
GATATTAACGACAGCGGTCTGCCAGCGGCAGGTGAGTTTCTCGATATGATCACCCCACAA
 D  I  N  D  S  G  L  P  A  A  G  E  F  L  D  M  I  T  P  Q
       550        560        570        580        590        600
TATCTCGCTGACCTGATGAGCTGGGGCGCAATTGGCGCACGTACCACCGAATCGCAGGTG
 Y  L  A  D  L  M  S  W  G  A  I  G  A  R  T  T  E  S  Q  V
       610        620        630        640        650        660
CACCGCGAACTGGCATCAGGGCTTTCTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGT
 H  R  E  L  A  S  G  L  S  C  P  V  G  F  K  N  G  T  D  G
       670        680        690        700        710        720
ACGATTAAAGTGGCTATCGATGCCATTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCC
 T  I  K  V  A  I  D  A  I  N  A  A  G  A  P  H  C  F  L  S
       730        740        750        760        770        780
GTAACGAAATGGGGGCATTCGGCGATTGTGAATACCAGCGGTAACGGCGATTGCCATATC
 V  T  K  W  G  H  S  A  I  V  N  T  S  G  N  G  D  C  H  I
       790        800        810        820        830        840
ATTCTGCGCGGCGGTAAAGAGCCTAACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAA
 I  L  R  G  G  K  E  P  N  Y  S  A  K  H  V  A  E  V  K  E
       850        860        870        880        890        900
GGGCTGAACAAAGCAGGCCTGCCAGCACAGGTGATGATCGATTTCAGCCATGCTAACTCG
 G  L  N  K  A  G  L  P  A  Q  V  M  I  D  F  S  H  A  N  S
       910        920        930        940        950        960
TCCAAACAATTCAAAAAGCAGATGGATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGT
 S  K  Q  F  K  K  Q  M  D  V  C  A  D  V  C  Q  Q  I  A  G
       970        980        990       1000       1010       1020
GGCGAAAAGGCCATTATTGGCGTGATGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGC
 G  E  K  A  I  I  G  V  M  V  E  S  H  L  V  E  G  N  Q  S
      1030       1040       1050       1060       1070       1080
CTCGAGAGCGGGGAGCCGCTGGCCTACGGTAAGAGCATCACCGATGCCTGCATCGGCTGG
 L  E  S  G  E  P  L  A  Y  G  K  S  I  T  D  A  C  I  G  W
      1090       1100       1110       1120       1130       1140
GAAGATACCGATGCTCTGTTACGTCAACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA
 E  D  T  D  A  L  L  R  Q  L  A  N  A  V  K  A  R  R  G  *
      1150
AAGCTT
```

Figure 34. Nucleotide sequence of *aroG*^{wt}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

The chromatogram of *aroG*^{wt} is shown in Appendix P.

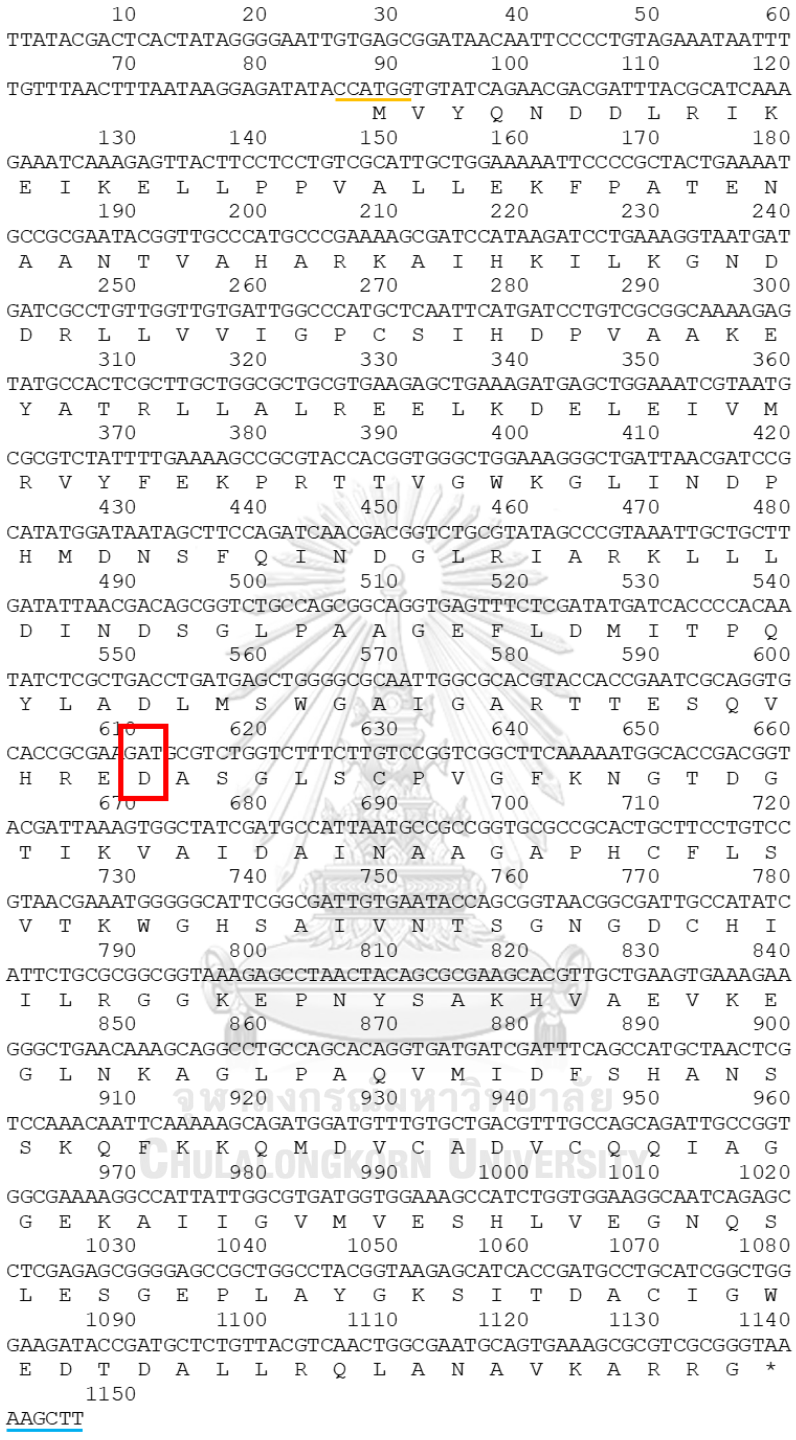


Figure 35. Nucleotide sequence of *aroG*^{L175D}

Orange and blue underlines represent the restriction site of *Nco*I and *Hind*III, respectively.

Red box represents the mutation of Leu175Asp (CTG to GAT).

The chromatogram of *aroG*^{L175D} is shown in Appendix Q.

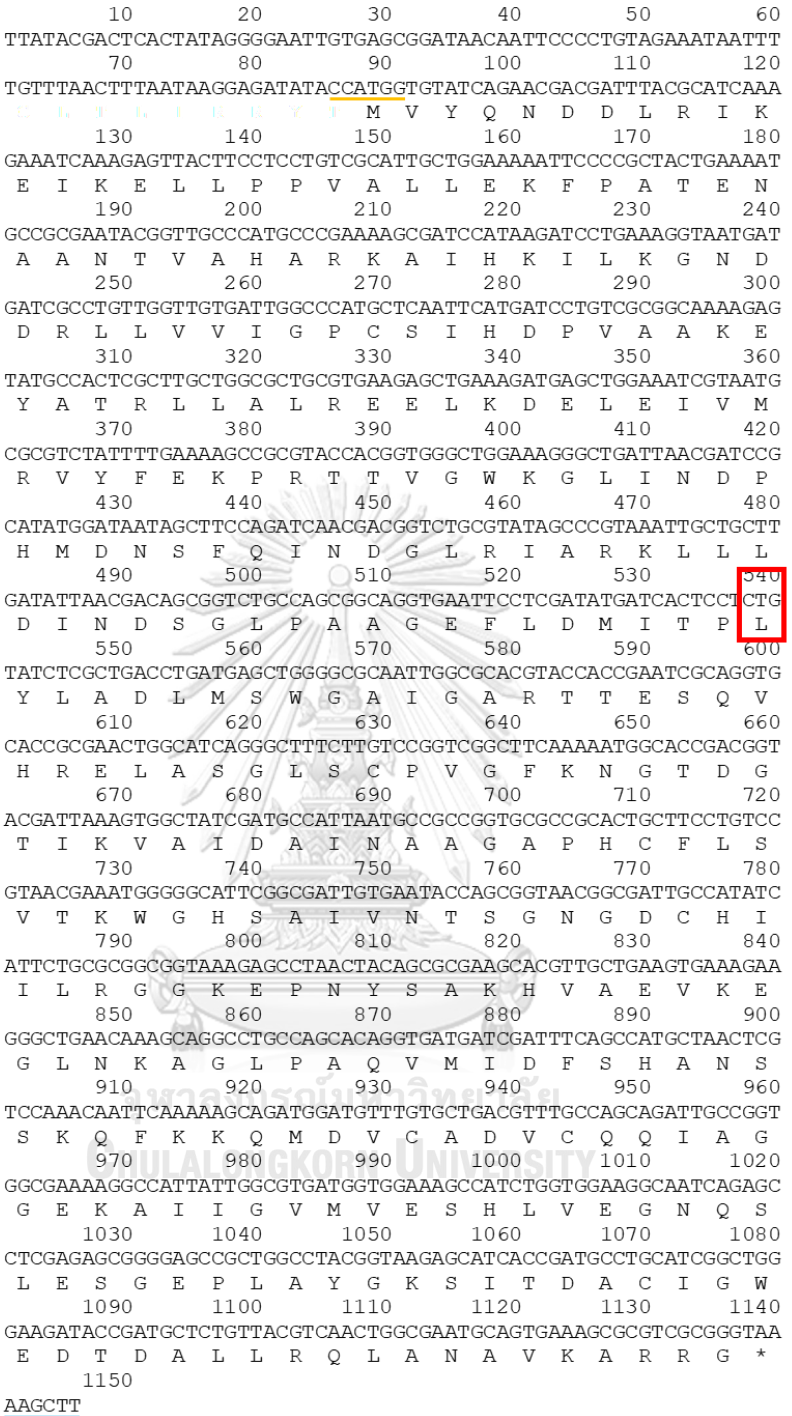


Figure 36. Nucleotide sequence of *aroG*^{Q151L}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Leu (CCA to CTG).

The chromatogram of *aroG*^{Q151L} is shown in Appendix R.

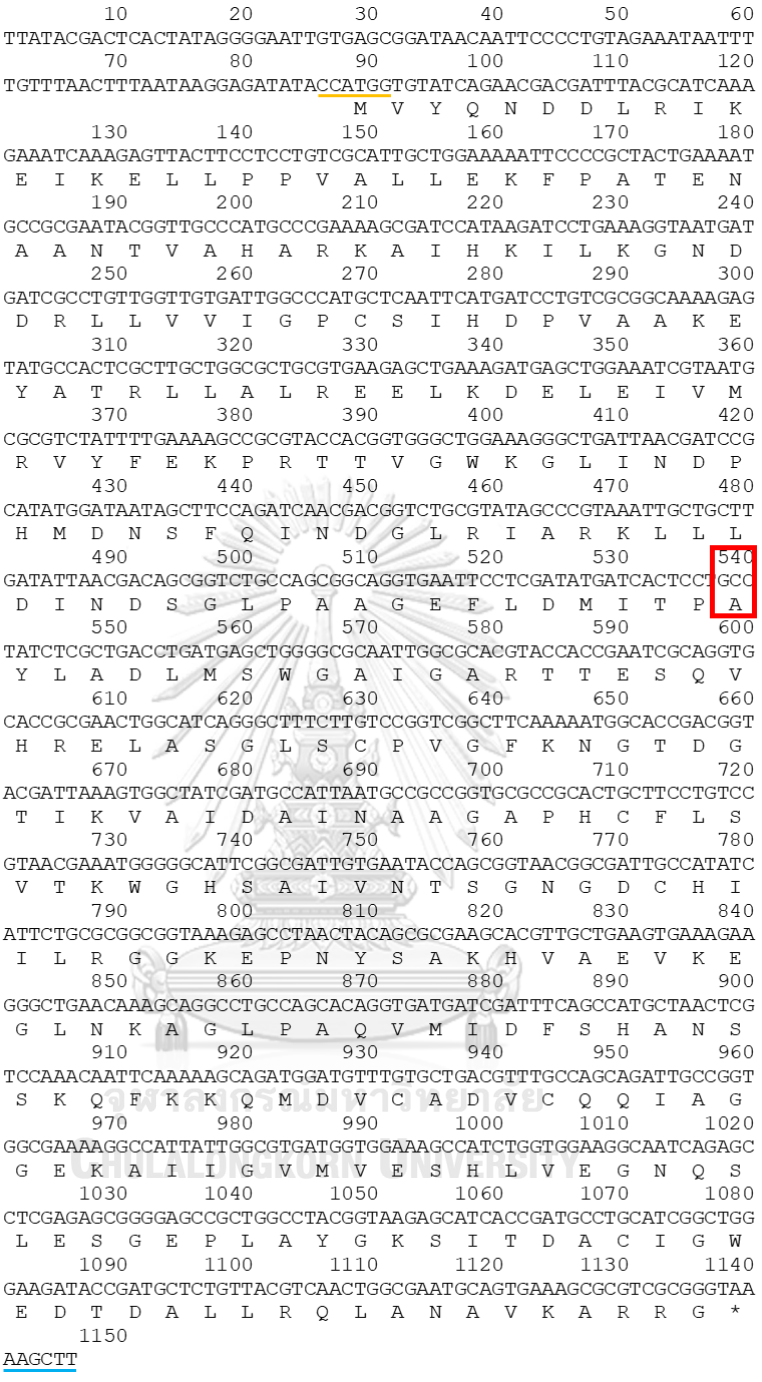


Figure 37. Nucleotide sequence of *aroG*^{Q151A}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Ala (CCA to GCC).

The chromatogram of *aroG*^{Q151A} is shown in Appendix S.

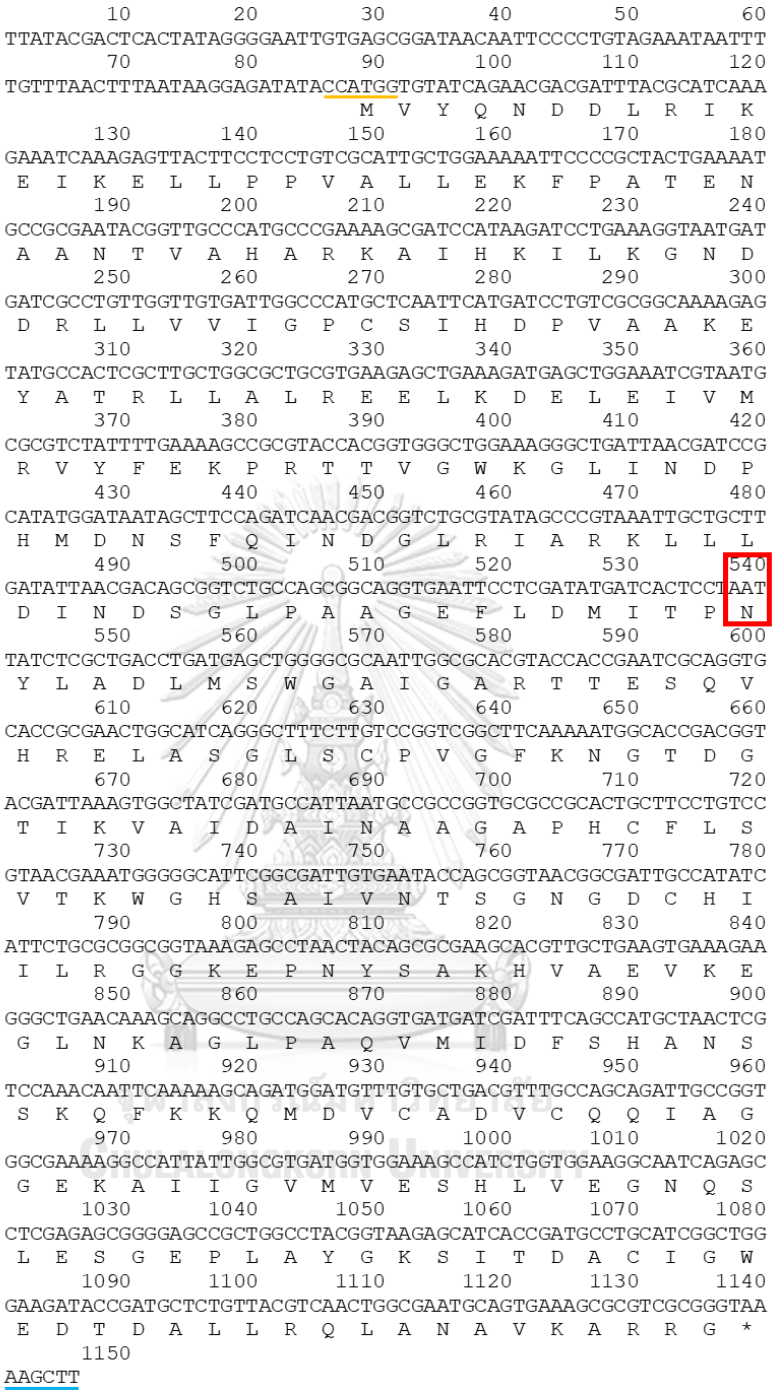


Figure 38. Nucleotide sequence of *aroG*^{Q151N}

Orange and blue underlines represent the restriction site of *Nco*I and *Hin*dIII, respectively.

Red box represents the point mutation of Gln151Asn (CCA to AAT).

The chromatogram of *aroG*^{Q151N} was shown in Appendix T.

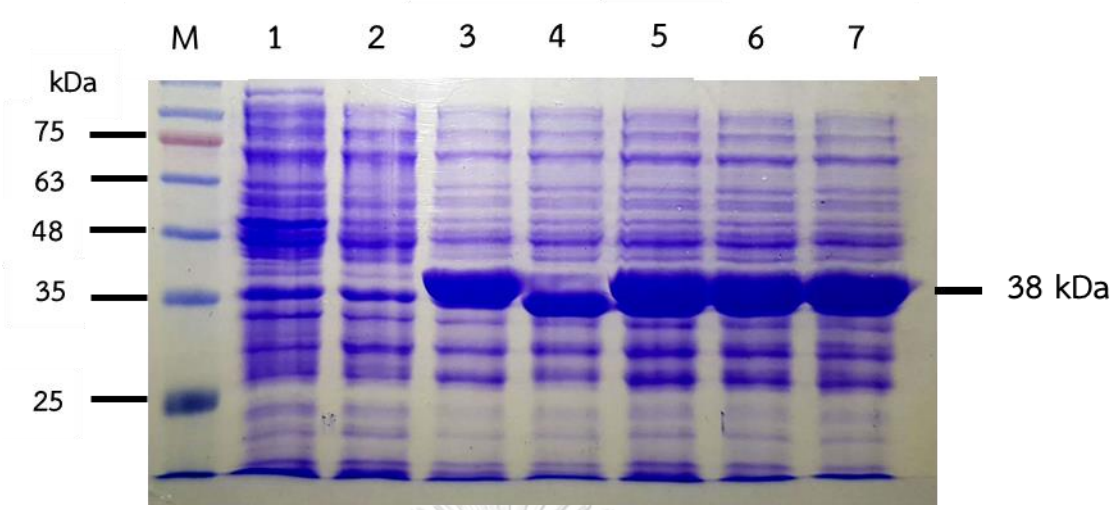


Figure 39. SDS-PAGE of crude extract of *aroG* clones.

lane M : TriColor Protein Ladder (10-180 kDa)

Lane 1 : *E. coli* BL21(DE3)

Lane 2 : *E. coli* BL21(DE3) harboring pRSFDuet-1

Lane 3 : *E. coli* BL21(DE3) harboring pAroG^{wt}

Lane 4 : *E. coli* BL21(DE3) harboring pAroG^{L175D}

Lane 5 : *E. coli* BL21(DE3) harboring pAroG^{Q151L}

Lane 6 : *E. coli* BL21(DE3) harboring pAroG^{Q151A}

Lane 7 : *E. coli* BL21(DE3) harboring pAroG^{Q151N}

3.4.6.2 DAHP synthase activity

In previous studies, Hu and coworkers investigated the feedback inhibition site of AroG using the 3D structure of AroG co-crystallized with PEP. The amino acids were replaced at Phe144, Pro150, Leu175, Leu179, Phe209, Trp215 and Val221. DAHP synthase activity in crude extract of each clone was measured in the presence of L-Phe from 0 mM to 3 mM. The results showed that the mutant at position L175D was mostly resistant to feedback inhibition. L175D enzyme elevated specific enzyme activity at 0 mM phenylalanine from 2.70 U/mg of wild type to 4.46 U/mg and increased of relative enzymatic activity at 1 mM phenylalanine from 8.2% to 83.5% [26]. In 2014, Ding and coworkers constructed three single-site mutant and combined to generate three double-site *aroG*^{fbr} mutant alleles. They analyzed enzymatic activity in all of mutants. The results showed that AroG8/15 had high level of feedback resistance to L-Phe at 20 mM of L-Phe. The relative enzymatic activity of AroG8/15 remained at 20 mM of L-Phe was 96.66% [27].

The amino acid residues that interact with phenylalanine at the regulatory site of AroG are displayed in Figure 40 using Discovery Studio 2020 program. Van der Waal interaction was found between Leu175 and phenylalanine. Among Gln151 as well as Asp6 and Asp7 of the companion tight subunit which form one H-bonding with phenylalanine, we interested in Gln151 since the pocket accommodative the aromatic ring of phenylalanine is formed by hydrophobic side-chains including that of Gln151. To investigate this amino acid residue, structure of AroG when Gln151 was substituted by Ala, Asn and Leu were simulated. All replaced amino acid cannot form H-bonding with phenylalanine (Figure 40B-40D). Differ from Ala and Asn, hydrophobic interaction between Leu151 and phenylalanine was detected.

\

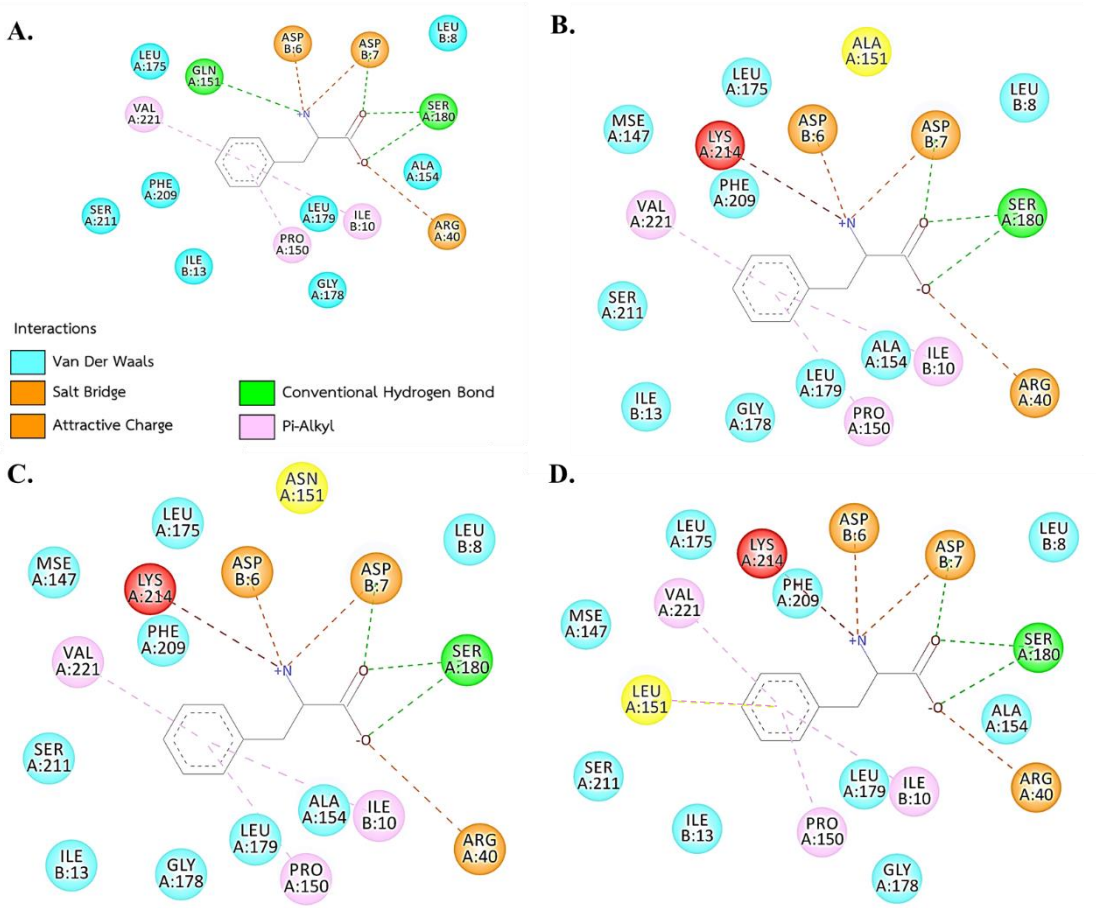


Figure 40. The amino acid residues that interact with phenylalanine at the regulatory site of AroG.

A. AroG^{wt} B. AroG^{Q151A} C. AroG^{Q151N} D. AroG^{Q151L}

DAHP synthase activity of crude extract from AroG clones were assayed for sensitivity to feedback inhibition by L-Phe at a concentration from 0 mM to 20 mM. The results are shown in Figure 41. In the absent L-Phe, all mutated AroG at Gln151 exhibited higher specific activities than $AroG^{wt}$ (1.88 U/mg). In contradiction to the result of Hu and co-worker [26], the control $AroG^{L175D}$ mutant showed lower specific activitiy (0.82 U/mg) than $AroG^{wt}$ (1.88 U/mg). The activity of all recombinant enzymes were decreased in the same pattern when L-Phe was added. Moreover, all AroG mutants at Gln151 showed greater resistance to feedback inhibition when compared with $AroG^{wt}$ and $AroG^{L175D}$. $AroG^{Q151N}$ gave the greatest inhibition pattern at the concentration of L-Phe at 0 - 20 mM. % inhibitions by 20 mM phenylalanine were decreased from 51% of wild type to 12, 16 and 27% for Q151L, Q151N and Q151A, respectively (Table 3). Destruction of two H-bonding between Ser180 and phenylalanine by substitution with Phe (S180F) was reported to decline % inhibition by phenylalanine at concentration of 20 mM from 58% to 7.4% [46]. The result indicated that H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, had a high impact on phenylalanine feedback inhibition. Then, the *aroG*^{Q151N} gene was used for combination with other genes.

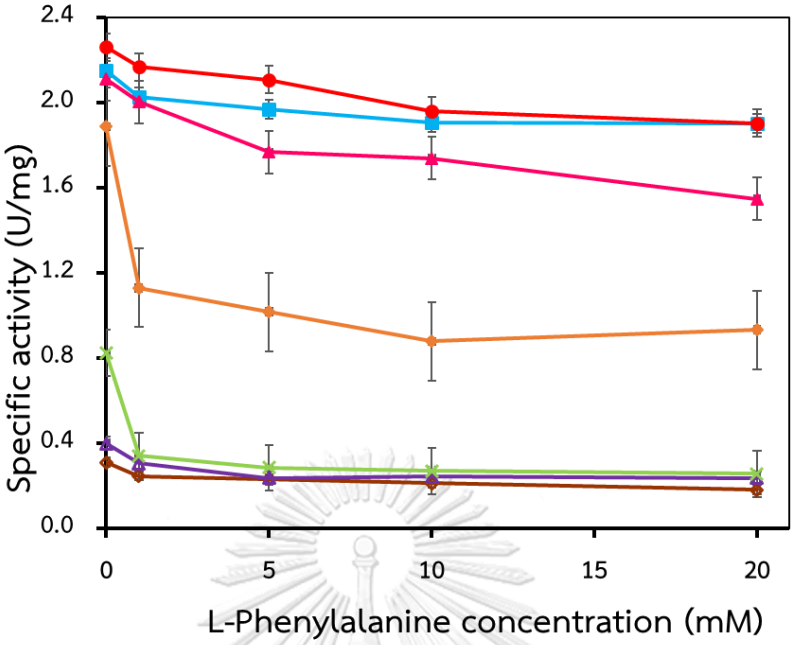


Figure 41. Phenylalanine inhibition pattern of DAHP synthase activities.
The data were received from three independent experiments.
(**opened diamonds**: *E.coli* BL21(DE3), **opened triangles**: *E.coli* BL21(DE3) harboring pRSFDuet-1, **closed diamonds**: *E.coli* BL21(DE3) harboring pAroG^{wt}, **crosses**: *E.coli* BL21(DE3) harboring pAroG^{L175D}, **closed boxes**: *E.coli* BL21(DE3) harboring pAroG^{Q151L}, **closed triangles**: *E.coli* BL21(DE3) harboring pAroG^{Q151A} and **closed circles**: *E.coli* BL21(DE3) harboring pAroG^{Q151N})

Table 3 DAHP synthase activities and feedback inhibition of various AroG clones.

Mutant	Specific activity (U/mg)		%inhibitor
	0 mM L-Phe	20 mM L-Phe	
E. coli BL21(DE3)	0.31 ± 0.04	0.18 ± 0.05	42
pRSFDuet-1	0.40 ± 0.07	0.24 ± 0.04	41
$AroG^{wt}$	1.89 ± 0.39	0.93 ± 0.45	51
$AroG^{L175D}$	0.82 ± 0.40	0.25 ± 0.09	69
$AroG^{Q151L}$	2.15 ± 0.11	1.90 ± 0.05	12
$AroG^{Q151A}$	2.11 ± 0.75	1.55 ± 0.41	27
$AroG^{Q151N}$	2.26 ± 0.06	1.90 ± 0.06	16

3.5 Construction of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

3.5.1 pBLPTA^{L359D} preparation

The pBLPTG^{Q151L}A^{L359D} containing *aroB*, *aroL*, *phedh, tktA*, *aroG* and *pheA* was double digested with *Pac*I and *Avr*II. After digestion, linear fragment of pBLPTA^{L359D} was separated by agarose gel electrophoresis and purified by GenepHlow™ Gel/PCR Kit. From Figure 42, the linear form of pBLPTA^{L359D} vector around 9.9 kb was detected (lane 3).

3.5.2 Amplification of T7_*aroG*^{wt} and T7_*aroG*^{Q151N}

T7_*aroG*^{wt} and T7_*aroG*^{Q151N} fragments were amplified from pAroG^{wt} and pAroG^{Q151N} using forward primer containing *Pac*I site and reverse primer containing *Avr*II site. After cleaning, the PCR fragments were separated by agarose gel electrophoresis. The size of T7_*aroG*^{wt} and T7_*aroG*^{Q151N} fragments were detected around 1.2 kb as shown in Figure 43. Then, PCR fragments were double digested with *Pac*I and *Avr*II. After digestion, T7_*aroG*^{wt} and T7_*aroG*^{Q151N} fragments were purified by GenepHlow™ Gel/PCR Kit. The size of T7_*aroG*^{wt} and T7_*aroG*^{Q151N} fragments were confirmed by agarose gel electrophoresis. From Figure 44, size of T7_*aroG*^{wt} and T7_*aroG*^{Q151N} fragments were around 1.2 kb in lane 2 and 4, respectively.

3.5.3 Cloning of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}

The *aroG*^{wt} *and aroG*^{Q151N} fragments were ligated into pBLPTA^{L359D} linear vector and then transformed into *E. coli* BL21(DE3) by electroporation. The single colonies of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} transformants were randomly picked and cultured in 5 mL of LB broth containing 30 mg/mL of kanamycin. Each recombinant plasmid was extracted, digested with *Xho*I and then detected by agarose gel electrophoresis. From digestion pattern, each recombinant plasmid gave two DNA bands around 10.0 kb and 1.1 kb as shown in Figure 45. This result confirmed that *aroG* genes were inserted into pBLPTA^{L359D}. After that, the nucleotide sequences of the inserts were checked by Bioneer Inc. (Korea).

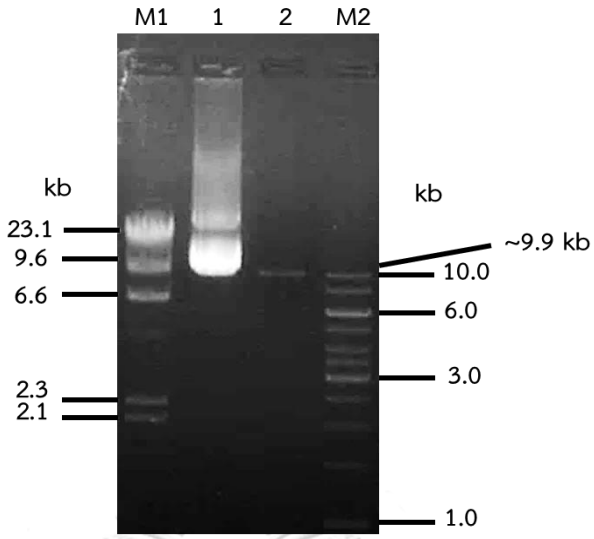


Figure 42. *Pac*I and *Avr*II digestion pattern of pBLPTA^{L359D}.

Lane M1 : Gene Ruler 1 kb DNA ladder

Lane 1 : pBLPTA^{L359D} uncut

Lane 2 : *Pac*I and *Avr*II digested pBLPTA^{L359D}

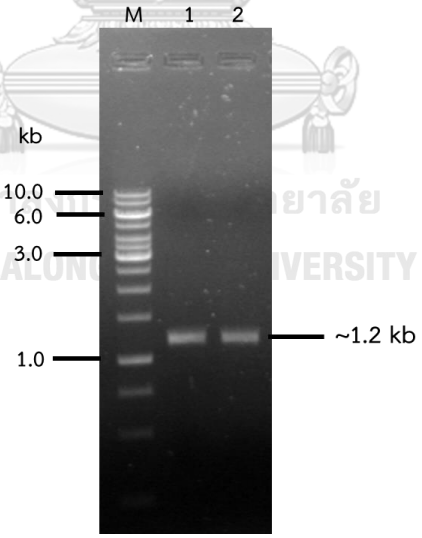


Figure 43. PCR products of *T7_aroG*^{wt} and *T7_aroG*^{Q151N}

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : PCR product of T7_*aroG*^{wt}

Lane 2 : PCR product of T7_*aroG*^{Q151N}

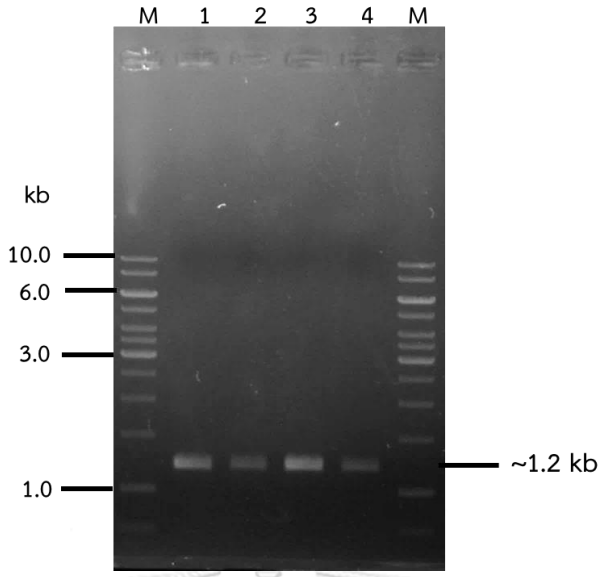


Figure 44. *Pac*I and *Avr*II digestion patterns of PCR products of *T7_aroG*^{wt} and *T7_aroG*^{Q151N}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut PCR product of T7_*aroG*^{wt}

Lane 2 : *Pac*I and *Avr*II digested PCR product of T7_*aroG*^{wt}

Lane 3 : uncut PCR product of T7_*aroG*^{Q151N}

Lane 4 : *Pac*I and *Avr*II digested PCR product of T7_*aroG*^{Q151N}

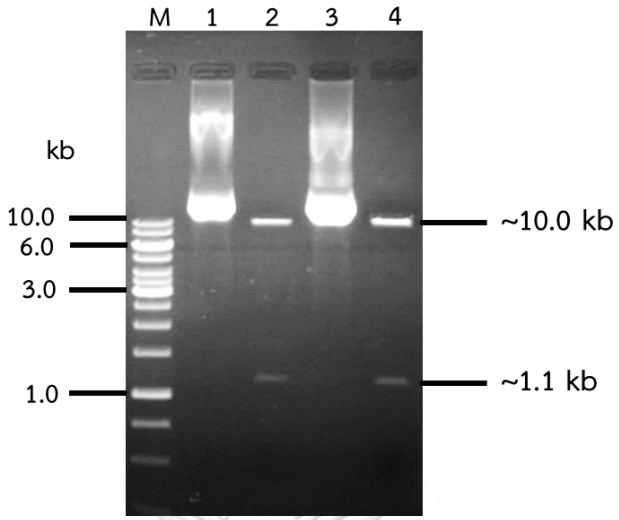


Figure 45. *Xho*I digestion patterns of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N}.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTA^{L359D}G^{wt}

Lane 2 : *Xho*I digested pBLPTA^{L359D}G^{wt}

Lane 3 : uncut pBLPTA^{L359D}G^{Q151N}

Lane 4 : *Xho*I digested pBLPTA^{L359D}G^{Q151N}

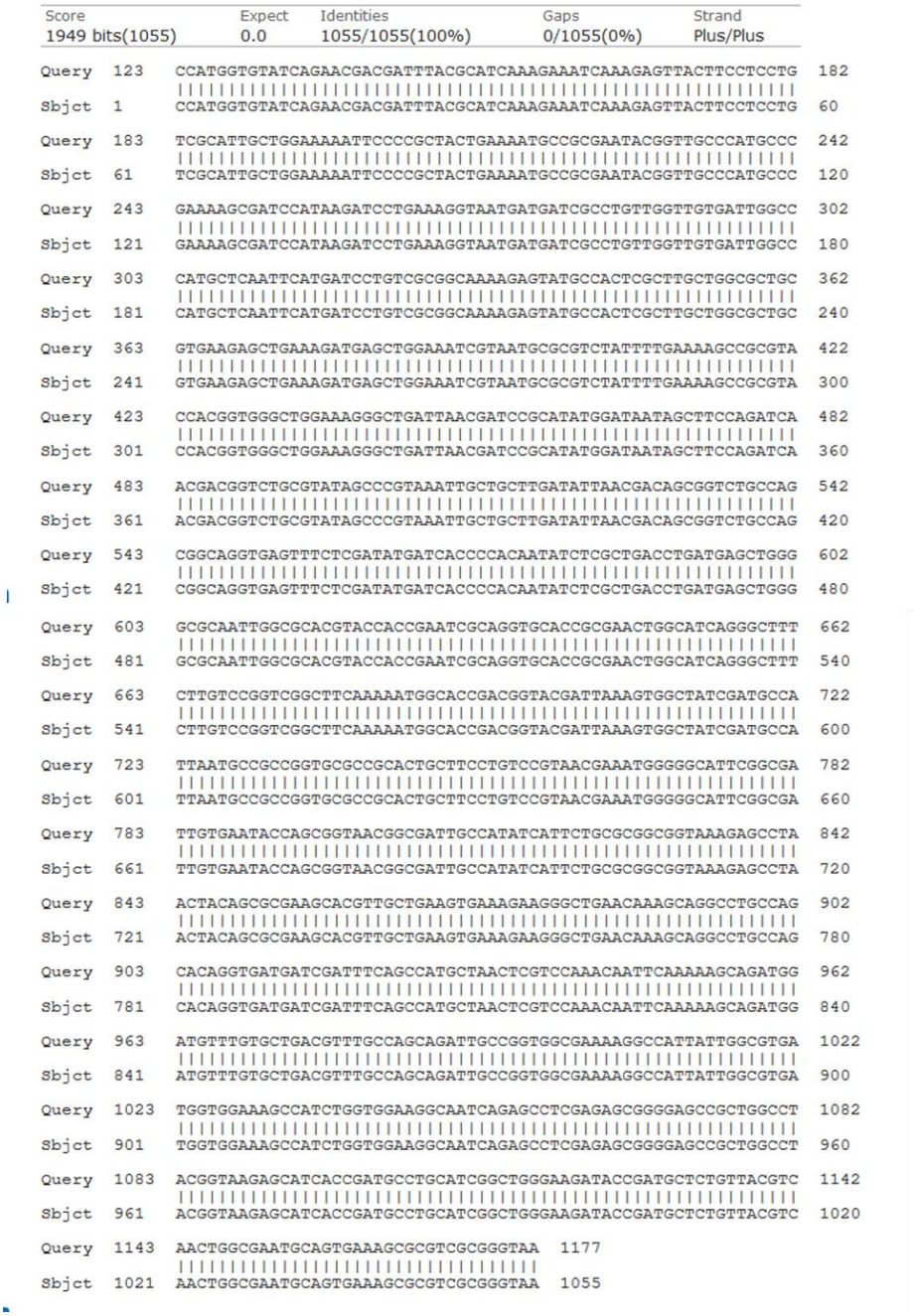
3.5.4 Nucleotide sequencing

To verify the nucleotide sequences of *aroG*^{wt} and *aroG*^{Q151N}, the DNA sequencing of recombinant plasmids were performed by Bioneer, Korean using ACYCDuet1 as a forward primer and DuetDown1 as a reverse primer. The obtained DNA sequences were compared with *aroG* sequence from section 3.4.4 by nucleotide blast tools in NCBI.

The nucleotide sequence of *aroG*^{wt} and *aroG*^{Q151N} showed 100% similarity to these of *aroG* in pAroG (Figure 46 - 47). The correct *aroG*^{wt} and *aroG*^{Q151N} genes were used in the further experiment.

3.6 Co-transformation of pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} with pYF into *E. coli* BL21(DE3)

The correct pBLPTA^{L359D}G^{wt} and pBLPTA^{L359D}G^{Q151N} from section 3.2.3.4 were co-transformed with pYF into *E. coli* BL21(DE3) competent cell using electroporation. The growing transformants of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF clones were picked up to culture in 5 mL LB broth that contained 30 mg/mL of kanamycin and 10 mg/mL of chloramphenicol. After extraction, the pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were confirmed by digestion with *Bam*HI. From Figure 48, pBLPTA^{L359D}G^{wt} & pYF digested with *Bam*HI in lane 10 gave three bands at 10.0 kb, 6.0 kb and 1.1 kb which were same size as pBLPTA^{L359D}G^{wt} (10.0 kb) in lane 2 and pYF (6.0 and 1.1 kb) in lane 6. pBLPTA^{L359D}G^{Q151N} & pYF also gave the same result (lane 12). Then, pBLPTA^{L359D}G^{wt} & pYF in lane 8 and pBLPTA^{L359D}G^{Q151N} & pYF were used to determine L-Phe production.



Score	Expect	Identities	Gaps	Strand
1949 bits(1055)	0.0	1055/1055(100%)	0/1055(0%)	Plus/Plus

```
Query  123   CCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATCAAAGAGTTACTTCCTCCTG  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     CCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATCAAAGAGTTACTTCCTCCTG  60

Query  183   TCGCATTGCTGGAAAAATTCCCCGCTACTGAAAATGCCGCGAATACGGTTGCCCATGCCC  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TCGCATTGCTGGAAAAATTCCCCGCTACTGAAAATGCCGCGAATACGGTTGCCCATGCCC  120

Query  243   GAAAAGCGATCCATAAGATCCTGAAAGGTAATGATGATCGCCTGTTGGTTGTGATTGGCC  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GAAAAGCGATCCATAAGATCCTGAAAGGTAATGATGATCGCCTGTTGGTTGTGATTGGCC  180

Query  303   CATGCTCAATTCATGATCCTGTCGCGGCAAAAGAGTATGCCACTCGCTTGCTGGCGCTGC  362
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CATGCTCAATTCATGATCCTGTCGCGGCAAAAGAGTATGCCACTCGCTTGCTGGCGCTGC  240

Query  363   GTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATGCGCGTCTATTTTGAAAAGCCGCGTA  422
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATGCGCGTCTATTTTGAAAAGCCGCGTA  300

Query  423   CCACGGTGGGCTGGAAAGGGCTGATTAACGATCCGCATATGGATAATAGCTTCCAGATCA  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CCACGGTGGGCTGGAAAGGGCTGATTAACGATCCGCATATGGATAATAGCTTCCAGATCA  360

Query  483   ACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTTGATATTAACGACAGCGGTCTGCCAG  542
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTTGATATTAACGACAGCGGTCTGCCAG  420

Query  543   CGGCAGGTGAGTTTCTCGATATGATCACCCCACAATATCTCGCTGACCTGATGAGCTGGG  602
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CGGCAGGTGAGTTTCTCGATATGATCACCCCACAATATCTCGCTGACCTGATGAGCTGGG  480

Query  603   GCGCAATTGGCGCACGTACCACCGAATCGCAGGTGCACCGCGAACTGGCATCAGGGCTTT  662
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GCGCAATTGGCGCACGTACCACCGAATCGCAGGTGCACCGCGAACTGGCATCAGGGCTTT  540

Query  663   CTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGTACGATTAAAGTGGCTATCGATGCCA  722
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   CTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGTACGATTAAAGTGGCTATCGATGCCA  600

Query  723   TTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCCGTAACGAAATGGGGGCATTCGGCGA  782
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCCGTAACGAAATGGGGGCATTCGGCGA  660

Query  783   TTGTGAATACCAGCGGTAACGGCGATTGCCATATCATTCTGCGCGGCGGTAAAGAGCCTA  842
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   TTGTGAATACCAGCGGTAACGGCGATTGCCATATCATTCTGCGCGGCGGTAAAGAGCCTA  720

Query  843   ACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAAGGGCTGAACAAAGCAGGCCTGCCAG  902
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   ACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAAGGGCTGAACAAAGCAGGCCTGCCAG  780

Query  903   CACAGGTGATGATCGATTTCAGCCATGCTAACTCGTCCAAACAATTCAAAAAGCAGATGG  962
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CACAGGTGATGATCGATTTCAGCCATGCTAACTCGTCCAAACAATTCAAAAAGCAGATGG  840

Query  963   ATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGTGGCGAAAAGGCCATTATTGGCGTGA  1022
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   ATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGTGGCGAAAAGGCCATTATTGGCGTGA  900

Query  1023  TGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGCCTCGAGAGCGGGGAGCCGCTGGCCT  1082
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGCCTCGAGAGCGGGGAGCCGCTGGCCT  960

Query  1083  ACGGTAAGAGCATCACCGATGCCTGCATCGGCTGGGAAGATACCGATGCTCTGTTACGTC  1142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   ACGGTAAGAGCATCACCGATGCCTGCATCGGCTGGGAAGATACCGATGCTCTGTTACGTC  1020

Query  1143  AACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA  1177
             ||||||||||||||||||||||||||||||||||
Sbjct  1021  AACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA  1055
```

Figure 46. Nucleotide sequence of *aroG*^{wt} in pBLPTA^{L359D}G^{wt}

Query represented the nucleotide sequence of *aroG*^{wt} in pBLPTA^{L359D}G^{wt}.

Sbjct represented the nucleotide sequence of in pAroG^{wt}

Score	Expect	Identities	Gaps	Strand
1949 bits(1055)	0.0	1055/1055(100%)	0/1055(0%)	Plus/Plus

```
Query  123   CCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATCAAAGAGTTACTTCCTCCTG  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     CCATGGTGTATCAGAACGACGATTTACGCATCAAAGAAATCAAAGAGTTACTTCCTCCTG  60

Query  183   TCGCATTGCTGGAAAAATTCCCCGCTACTGAAAATGCCGCGAATACGGTTGCCCATGCCC  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TCGCATTGCTGGAAAAATTCCCCGCTACTGAAAATGCCGCGAATACGGTTGCCCATGCCC  120

Query  243   GAAAAGCGATCCATAAGATCCTGAAAGGTAATGATGATCGCCTGTTGGTTGTGATTGGCC  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GAAAAGCGATCCATAAGATCCTGAAAGGTAATGATGATCGCCTGTTGGTTGTGATTGGCC  180

Query  303   CATGCTCAATTCATGATCCTGTCGCGGCAAAAGAGTATGCCACTCGCTTGCTGGCGCTGC  362
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CATGCTCAATTCATGATCCTGTCGCGGCAAAAGAGTATGCCACTCGCTTGCTGGCGCTGC  240

Query  363   GTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATGCGCGTCTATTTTGAAAAGCCGCGTA  422
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GTGAAGAGCTGAAAGATGAGCTGGAAATCGTAATGCGCGTCTATTTTGAAAAGCCGCGTA  300

Query  423   CCACGGTGGGCTGGAAAGGGCTGATTAACGATCCGCATATGGATAATAGCTTCCAGATCA  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CCACGGTGGGCTGGAAAGGGCTGATTAACGATCCGCATATGGATAATAGCTTCCAGATCA  360

Query  483   ACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTTGATATTAACGACAGCGGTCTGCCAG  542
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ACGACGGTCTGCGTATAGCCCGTAAATTGCTGCTTGATATTAACGACAGCGGTCTGCCAG  420

Query  543   CGGCAGGTGAATTCCTCGATATGATCACTCCTAATTATCTCGCTGACCTGATGAGCTGGG  602
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CGGCAGGTGAATTCCTCGATATGATCACTCCTAATTATCTCGCTGACCTGATGAGCTGGG  480

Query  603   GCGCAATTGGCGCACGTACCACCGAATCGCAGGTGCACCGCGAACTGGCATCAGGGCTTT  662
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GCGCAATTGGCGCACGTACCACCGAATCGCAGGTGCACCGCGAACTGGCATCAGGGCTTT  540

Query  663   CTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGTACGATTAAAGTGGCTATCGATGCCA  722
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   CTTGTCCGGTCGGCTTCAAAAATGGCACCGACGGTACGATTAAAGTGGCTATCGATGCCA  600

Query  723   TTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCCGTAACGAAATGGGGGCATTCGGCGA  782
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TTAATGCCGCCGGTGCGCCGCACTGCTTCCTGTCCGTAACGAAATGGGGGCATTCGGCGA  660

Query  783   TTGTGAATACCAGCGGTAACGGCGATTGCCATATCATTCTGCGCGGCGGTAAAGAGCCTA  842
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   TTGTGAATACCAGCGGTAACGGCGATTGCCATATCATTCTGCGCGGCGGTAAAGAGCCTA  720

Query  843   ACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAAGGGCTGAACAAAGCAGGCCTGCCAG  902
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   ACTACAGCGCGAAGCACGTTGCTGAAGTGAAAGAAGGGCTGAACAAAGCAGGCCTGCCAG  780

Query  903   CACAGGTGATGATCGATTTCAGCCATGCTAACTCGTCCAAACAATTCAAAAAGCAGATGG  962
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CACAGGTGATGATCGATTTCAGCCATGCTAACTCGTCCAAACAATTCAAAAAGCAGATGG  840

Query  963   ATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGTGGCGAAAAGGCCATTATTGGCGTGA  1022
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   ATGTTTGTGCTGACGTTTGCCAGCAGATTGCCGGTGGCGAAAAGGCCATTATTGGCGTGA  900

Query  1023  TGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGCCTCGAGAGCGGGGAGCCGCTGGCCT  1082
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TGGTGGAAAGCCATCTGGTGGAAGGCAATCAGAGCCTCGAGAGCGGGGAGCCGCTGGCCT  960

Query  1083  ACGGTAAGAGCATCACCGATGCCTGCATCGGCTGGGAAGATACCGATGCTCTGTTACGTC  1142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   ACGGTAAGAGCATCACCGATGCCTGCATCGGCTGGGAAGATACCGATGCTCTGTTACGTC  1020

Query  1143  AACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA  1177
             ||||||||||||||||||||||||||||||||||
Sbjct  1021  AACTGGCGAATGCAGTGAAAGCGCGTCGCGGGTAA  1055
```

Figure 47. Nucleotide sequence of *aroG*^{Q151N} in pBLPTA^{L359D}G^{Q151N}

Query represented the nucleotide sequence of *aroG*^{Q151N} in pBLPTA^{L359D}G^{Q151N}.

Sbjct represented the nucleotide sequence of *aroG*^{Q151N} in pAroG^{Q151N}

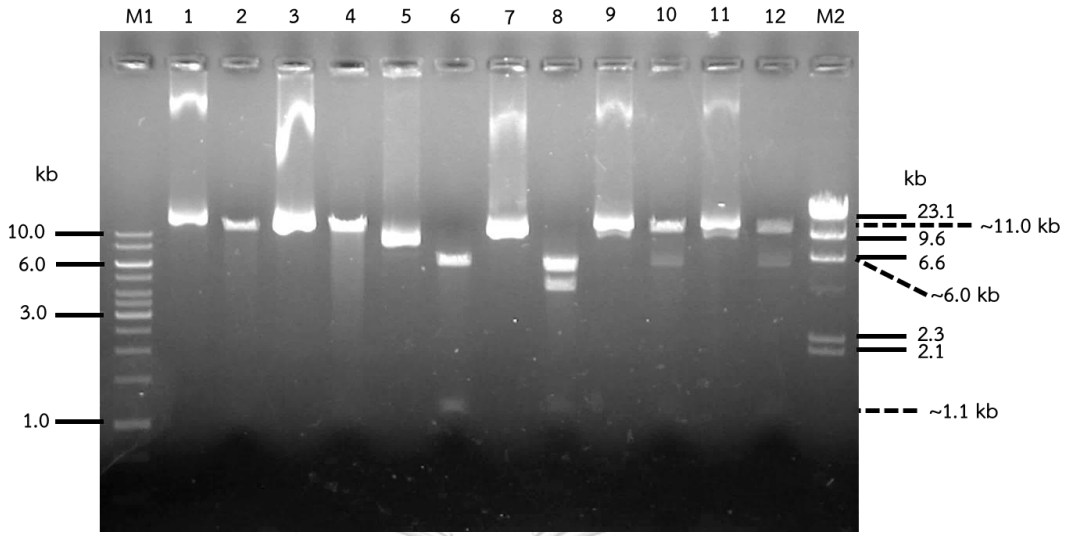


Figure 48. *Bam*HI digestion patterns of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF.

Lane M : Gene Ruler 1 kb DNA ladder

Lane 1 : uncut pBLPTA^{L359D}G^{wt}

Lane 2 : *Bam*HI digested pBLPTA^{L359D}G^{wt}

Lane 3 : uncut pBLPTA^{L359D}G^{Q151N}

Lane 4 : *Bam*HI digested pBLPTA^{L359D}G^{Q151N}

Lane 5 : uncut pYF

Lane 6 : *Bam*HI digested pYF

Lane 7 : uncut pBLPT & pYF

Lane 8 : *Bam*HI digested pBLPT & pYF

Lane 9 : uncut pBLPTA^{L359D}G^{wt} & pYF

Lane 10 : *Bam*HI digested pBLPTA^{L359D}G^{wt} & pYF

Lane 11 : uncut pBLPTA^{L359D}G^{Q151N} & pYF

Lane 12 : *Bam*HI digested pBLPTA^{L359D}G^{Q151N} & pYF

3.7 Production of L-Phe

Each recombinant clone was cultured in 200 mL of minimum medium containing 6% glycerol as a carbon source (Ulfah, 2018). Membrane protein genes (*glpF* and *yddG*) under the tight regulation of ara promoter were induced by 0.02% arabinose. The growth profile measured at a wavelength of 600 nm showed that growth of pBLPTA^{L359D}G^{wt} & pYF and pBLPTA^{L359D}G^{Q151N} & pYF were not significantly different and higher than that of pBLPT & pYF. Cell growth of each clone exhibited the exponential phase until 72 h (Figure 49). L-Phenylalanine production was correlated with phase of cell growth. At 192 h, the recombinant pBLPTA^{L359D}G^{Q151N} & pYF clone gave the highest L-Phe production at 1.95 g/L that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF (0.224 g/L) and pBLPTA^{L359D}G^{wt} & pYF (1.61 g/L), respectively (Figure 50). Addition of recombinant phenylalanine feedback resistant PheA clearly showed to have more impact on L-Phe production than the addition of feedback resistant AroG. PheA (chorismite mutase/prephenate dehydratase) catalyzes a conversion of chorismite to phenylpyruvate through prephenate intermediate that is the key step in determining the L-Phe production while AroG, a main isoform of DAHP synthase, catalyzes the first step of aromatic amino acid biosynthesis pathway. DAHP, the product of AroG can be used not only in the biosynthesis of phenylalanine but also in the syntheses of tyrosine and tryptophan. The cultivating conditions and medium used in this experiment was optimized by Ulfah (2018) for pBLPT & pYF clone. Thus, the composition of glycerol medium as well as culture conditions should be adjusted to improve yield of L-Phe.

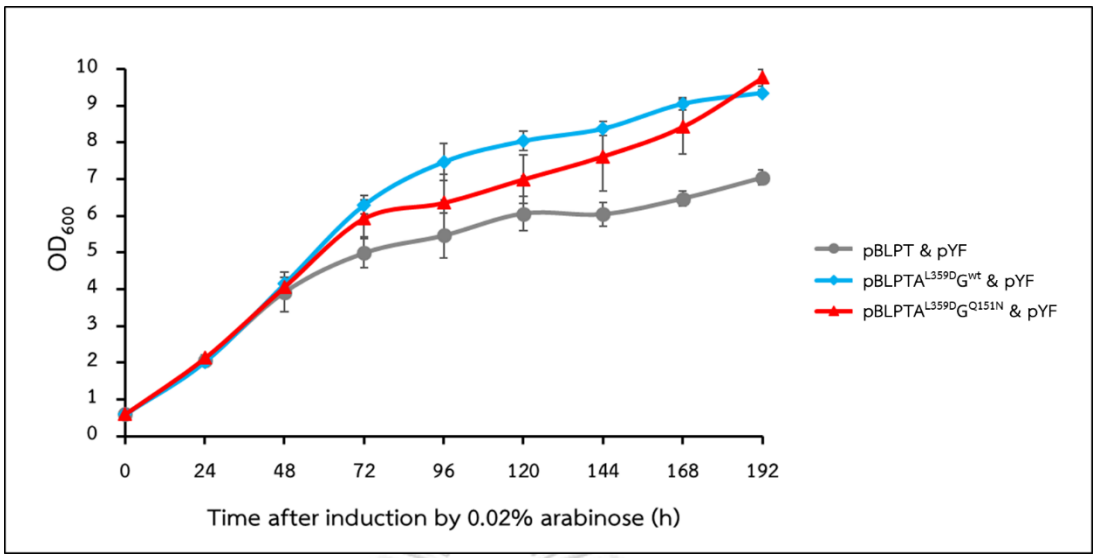


Figure 49. Growth curve of recombinant clones in minimum medium.

The data were received from three independent experiments.

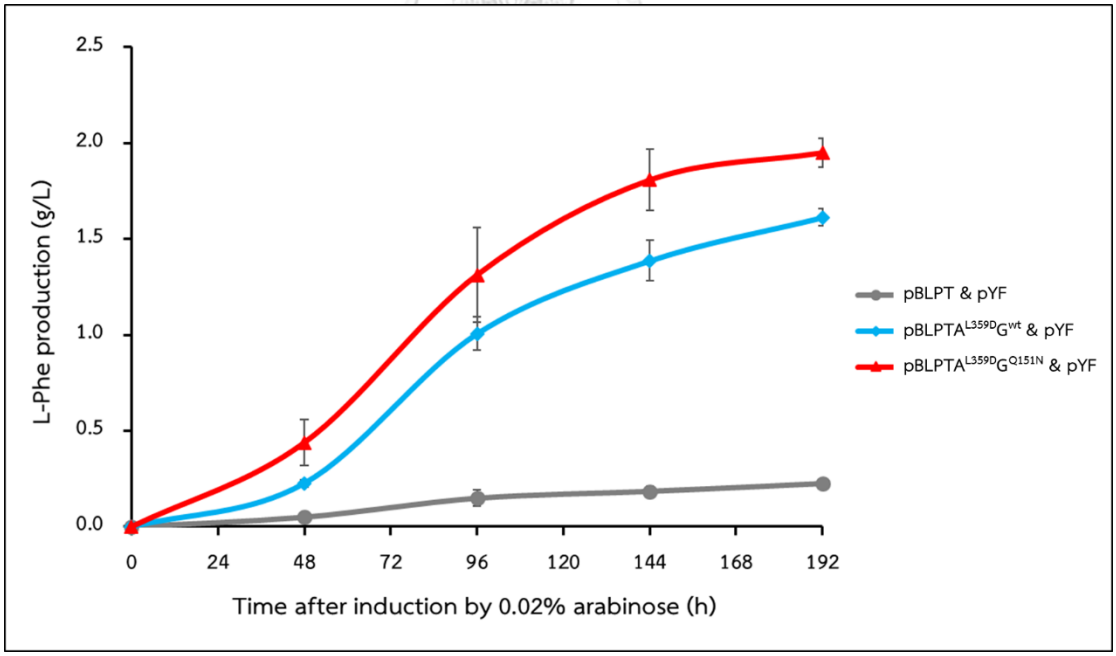


Figure 50. L-Phe production of recombinant clones in minimum medium.

The data were received from three independent experiments.

CHAPTER IV

CONCLUSIONS

1. The expression of PheA in pRSFDuet-1 (pPheA) was confirmed by the appearance of a high intensity protein band around 43 kDa in SDS-gel.

2. Frame shift mutation of *aroG* inserted at *Bam*HI site of pRSFDuet-1 was found. Therefore, pAroG^{wt} and pAroG^{fbr} (pRSFDuet-1 harboring *aroG*^{wt} and *aroG*^{fbr}, respectively) were reconstructed.

3. All obtained *aroG* clones gave the protein band approximately 38 kDa on SDS-gel. The mutated enzymes exhibited slightly higher DAHP synthase activity to the wildtype enzyme and %inhibitions by 20 mM L-Phe were decreased from 51% to 12 – 27%. Thus, H-bonding between Gln151 of AroG and the inhibitor, phenylalanine, has a high impact on phenylalanine feedback inhibition.

4. The recombinant *E. coli* BL21(DE3) clones containing *aroB*, *aroL*, *phedh*, *tktA*, *aroG*, *glpF, yddG* and *pheA* was successfully constructed. After 8 days of fermentation in 6% glycerol medium, pBLPTA^{L359D}G^{Q151N} & pYF clone gave the highest L-phenylalanine production (1.95 g/L) that was 8.7 and 1.2 fold of that obtained from pBLPT & pYF and pBLPTA^{L359D}G^{wt} & pYF clones, respectively. The result revealed that feedback resistant PheA and AroG could elevate L-phenylalanine production.

REFERENCES

1. Wendisch VF: **Amino acid biosynthesis–pathways, regulation and metabolic engineering**, vol. 5: *Springer science & business media*; 2007.
2. Maeda H, Dudareva N: **The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants**. *Annual review of plant biology* 2012, 63:73-105.
3. Báez-Viveros JL, Osuna J, Hernández-Chávez G, Soberón X, Bolívar F, Gosset G: **Metabolic engineering and protein directed evolution increase the yield of L-phenylalanine synthesized from glucose in *Escherichia coli***. *Biotechnology and bioengineering* 2004, 87:516-524.
4. Liu SP, Zhang L, Mao J, Ding ZY, Shi GY: **Metabolic engineering of *Escherichia coli* for the production of phenylpyruvate derivatives**. *Metabolic engineering* 2015, 32:55-65.
5. Yakandawala N, Romeo T, Friesen A, Madhyastha S: **Metabolic engineering of *Escherichia coli* to enhance phenylalanine production**. *Applied microbiology and biotechnology* 2008, 78:283-291.
6. Mahalakshmi R, Jesuraja S, Das SJ: **Growth and characterization of L-phenylalanine**. *Crystal research and technology: Journal of experimental and industrial crystallography* 2006, 41:780-783.
7. Daniel P, Moorhouse S, Pratt O: **Amino acid precursors of monoamine neurotransmitters and some factors influencing their supply to the brain**. *Psychological medicine* 1976, 6:277-286.
8. Fernstrom JD, Fernstrom MH: **Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain**. *The journal of nutrition* 2007, 137:1539-1547.
9. Weiner M, Albermann C, Gottlieb K, Sprenger GA, Weuster-Botz D: **Fed-batch production of L-phenylalanine from glycerol and ammonia with recombinant *Escherichia coli***. *Biochemical engineering journal* 2014, 83:62-69.
10. Silkaitis RP, Mosnaim AD: **Pathways linkingl-phenylalanine and 2-phenylethylamine withp-tyramine in rabbit brain**. *Brain research* 1976,

114:105-115.

11. Siddiqui A, Bhaggoe R, Hu R, Schutgens R, Westerhof W: **L-phenylalanine and UVA irradiation in the treatment of vitiligo**. *Dermatology* 1994, 188:215-218.
12. Liu SP, Liu RX, Xiao MR, Zhang L, Ding ZY, Gu ZH, Shi GY: **A systems level engineered E. coli capable of efficiently producing L-phenylalanine**. *Process biochemistry* 2014, 49:751-757.
13. Zhang C, Zhang J, Kang Z, Du G, Yu X, Wang T, Chen J: **Enhanced production of l-phenylalanine in *Corynebacterium glutamicum* due to the introduction of *Escherichia coli* wild-type gene *aroH***. *Journal of industrial microbiology & biotechnology* 2013, 40:643-651.
14. Berry A: **Improving production of aromatic compounds in *Escherichia coli* by metabolic engineering**. *Trends in biotechnology* 1996, 14:250-256.
15. Wu YQ, Jiang PH, Fan CS, Wang JG, Shang L, Huang WD: **Co-expression of five genes in *E coli* for L-phenylalanine in *Brevibacterium flavum***. *World journal of gastroenterology: WJG* 2003, 9:342.
16. Ikeda M: **Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering**. *Applied microbiology and biotechnology* 2006, 69:615.
17. Wu WB, Guo XL, Zhang ML, Huang QG, Qi F, Huang JZ: **Enhancement of l-phenylalanine production in *Escherichia coli* by heterologous expression of Vitreoscilla hemoglobin**. *Biotechnology and applied biochemistry* 2018, 65(3):476-483.
18. Gottlieb K, Albermann C, Sprenger GA: **Improvement of L-phenylalanine production from glycerol by recombinant *Escherichia coli* strains: the role of extra copies of *glpK*, *glpX*, and *tktA* genes**. *Microbial cell factories* 2014, 13:96.
19. Lin S, Meng X, Jiang J, Pang D, Jones G, OuYang H, Ren L: **Site-directed mutagenesis and over expression of *aroG* gene of *Escherichia coli* K-12**. *International journal of biological macromolecules* 2012, 51:915-919.
20. McCandliss RJ, Poling M, Herrmann K: 3-**Deoxy-D-arabino-heptulosonate** 7-

phosphate synthase. Purification and molecular characterization of the phenylalanine-sensitive isoenzyme from *Escherichia coli*. *Journal of biological chemistry* 1978, 253:4259-4265.

21. Bongaerts J, Krämer M, Müller U, Raeven L, Wubbolts M: **Metabolic engineering for microbial production of aromatic amino acids and derived compounds**. *Metabolic engineering* 2001, 3:289-300.
22. Kikuchi Y, Tsujimoto K, Kurahashi O: **Mutational analysis of the feedback sites of phenylalanine-sensitive** 3-**deoxy-D-arabino-heptulosonate-**7-**phosphate synthase of *Escherichia coli***. *Applied and environmental microbiology* 1997, 63:761-762.
23. Stephens CM, Bauerle R: **Analysis of the metal requirement of** 3-**deoxy-D-arabino-heptulosonate-**7-**phosphate synthase from *Escherichia coli***. *Journal of biological chemistry* 1991, 266:20810-20817.
24. Shumilin IA, Kretsinger RH, Bauerle RH: **Crystal structure of phenylalanine-regulated** 3-**deoxy-D-arabino-heptulosonate-**7-**phosphate synthase from *Escherichia coli***. *Structure* 1999, 7:865-875.
25. Shumilin IA, Zhao C, Bauerle R, Kretsinger RH: **Allosteric inhibition of** 3-**deoxy-D-arabino-heptulosonate-**7-**phosphate synthase alters the coordination of both substrates**. *Journal of molecular biology* 2002, 320:1147-1156.
26. Hu C, Jiang P, Xu J, Wu Y, Huang W: **Mutation analysis of the feedback inhibition site of phenylalanine-sensitive** 3**-deoxy-D-arabino-heptulosonate** 7**-phosphate synthase of *Escherichia coli***. *Journal of basic microbiology: an international journal on biochemistry, physiology, genetics, morphology, and ecology of microorganisms* 2003, 43:399-406.
27. Ding R, Liu L, Chen X, Cui Z, Zhang A, Ren D, Zhang L: **Introduction of two mutations into AroG increases phenylalanine production in *Escherichia coli***. *Biotechnology letters* 2014, 36:2103-2108.
28. Ikeda M, Ozaki A, Katsumata R: **Phenylalanine production by metabolically engineered *Corynebacterium glutamicum* with the *pheA* gene of *Escherichia coli***. *Applied microbiology and biotechnology* 1993, 39:318-323.

29. Nelms J, Edwards R, Warwick J, Fotheringham I: **Novel mutations in the *pheA* gene of *Escherichia coli* K-**12 **which result in highly feedback inhibition-resistant variants of chorismate mutase/prephenate dehydratase**. *Applied and environmental microbiology* 1992, 58:2592-2598.

30. Zhang S, Pohnert G, Kongsaeree P, Wilson DB, Clardy J, Ganem B: **Chorismate mutase-prephenate dehydratase from *Escherichia coli* study of catalytic and regulatory domains using genetically engineered proteins**. *Journal of biological chemistry* 1998, 273:6248-6253.

31. Dopheide TA, Crewther P, Davidson BE: **Chorismate mutase-prephenate dehydratase from *Escherichia coli* K-**12 **II. Kinetic properties**. *Journal of biological chemistry* 1972, 247:4447-4452.

32. Liu SP, Xiao MR, Zhang L, Xu J, Ding ZY, Gu ZH, Shi GY: **Production of L-phenylalanine from glucose by metabolic engineering of wild type *Escherichia coli* W**3110. *Process biochemistry* 2013, 48:413-419.

33. Nicol R, Marchand K, Lubitz W: **Bioconversion of crude glycerol by fungi**. *Applied microbiology and biotechnology* 2012, 93:1865-1875.

34. Wang Z, Zhuge J, Fang H, Prior B: **Glycerol production by microbial fermentation: a review**. 2001, 19:201-223.

35. Sweet G, Gandor C, Voegele R, Wittekindt N, Beuerle J, Truniger V, Lin E, Boos W: **Glycerol facilitator of *Escherichia coli*: cloning of *glpF* and identification of the *glpF* product**. 1990, 172:424-430.

36. Zwaig N, Kistler W, Lin EJJob: **Glycerol kinase, the pacemaker for the dissimilation of glycerol in *Escherichia coli***. 1970, 102:753-759.

37. Murarka A, Dharmadi Y, Yazdani SS, Gonzalez RJ, microbiology e: **Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals**. 2008, 74:1124-1135.

38. Thongchuang M: **Improvement of phenylalanine production in *Escherichia coli* by metabolic engineering process. Ph.D. thesis**. Chulalongkorn University; 2011.

39. Ratchaneeladdajit P: **L-phenylalanine production by recombinant *Escherichia coli* under regulation of T**7 **and ara promoters. Master's thesis**

Chulalongkorn University; 2014.

40. Naksusuk B: **Enhancement of l-phenylalanine production by mutagenesis of *pheA* gene in *Escherichia coli*. Master's Thesis**. Chulalongkorn University; 2015.

41. Kanoksinwuttipong N: **Construction of phenylalanine feedback-resistant** 3-**deoxy-D-arabino-heptulosonate-**7-**phosphate synthase. Senior Project**. Chulalongkorn University; 2014.

42. BOLLAG DM, ROZYCKI, M. D. & EDELSTEIN, S. J. : **Protein methods**. New York, USA, John Wiley-Liss, Inc.; 1996.

43. Schoner R, Herrmann KM: 3-**Deoxy-D-arabino-heptulosonate** 7-**phosphate synthase. Purification, properties, and kinetics of the tyrosine-sensitive isoenzyme from *Escherichia coli***. *Journal of biological chemistry* 1976, 251:5440-5447.

44. Liu YJ, Li PP, Zhao KX, Wang BJ, Jiang CY, Drake HL, Liu SJ: ***Corynebacterium glutamicum* contains** 3-**deoxy-D-arabino-heptulosonate 7-phosphate synthases that display novel biochemical features**. *Applied and environmental microbiology* 2008, 74:5497-5503.

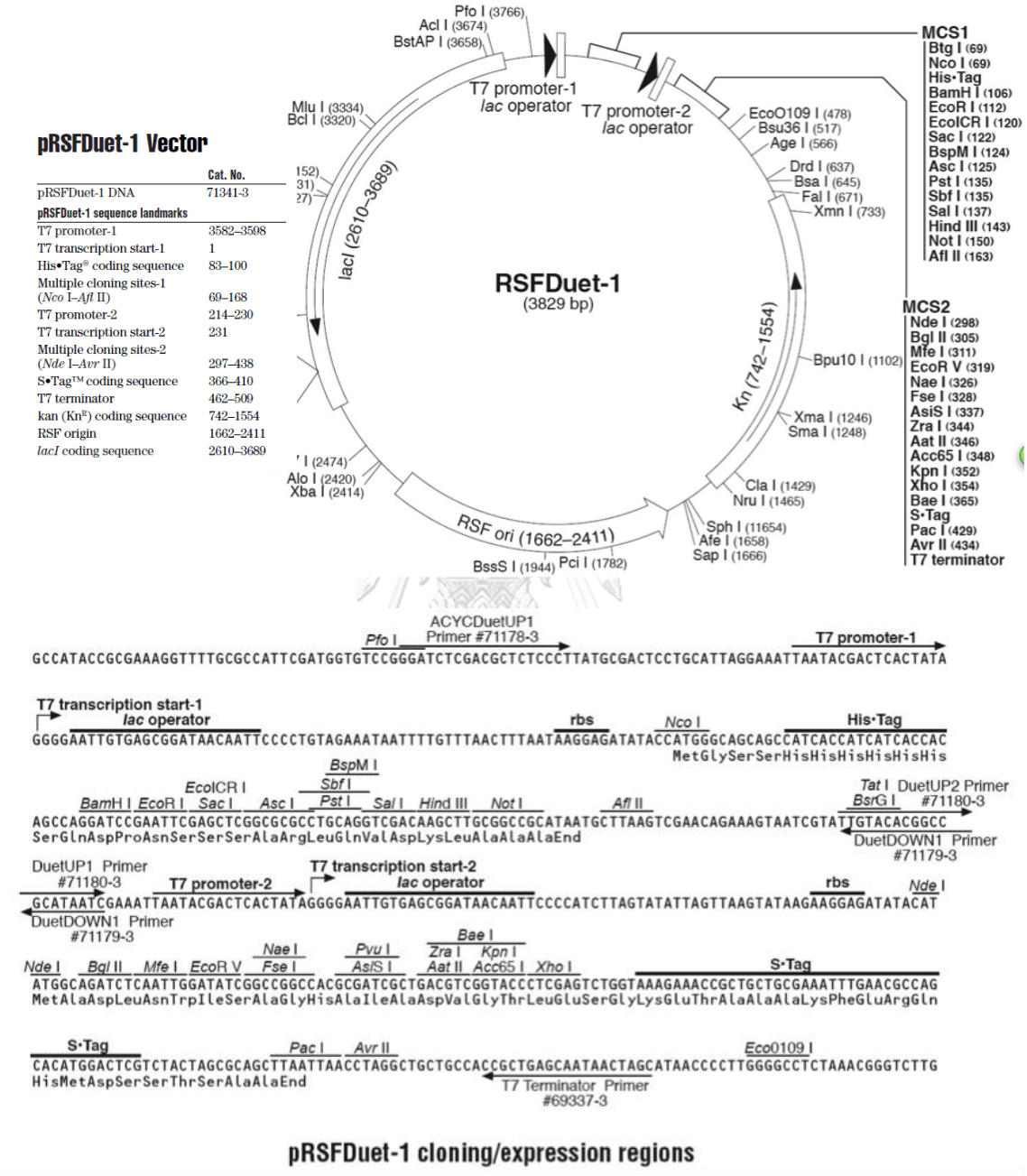
45. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: **Protein measurement with the Folin phenol reagent**. *Journal of biological chemistry* 1951, 193:265-275.

46. Ger YM, Chen SL, Chiang HJ, Shiuan D: **A single Ser-**180 **mutation desensitizes feedback inhibition of the phenylalanine-sensitive** 3-**deoxy-D-arabino-heptulosonate** 7-**phosphate (DAHP) synthetase in *Escherichia coli***. *The journal of biochemistry* 1994, 116:986-990.

APPENDICES

Appendix A

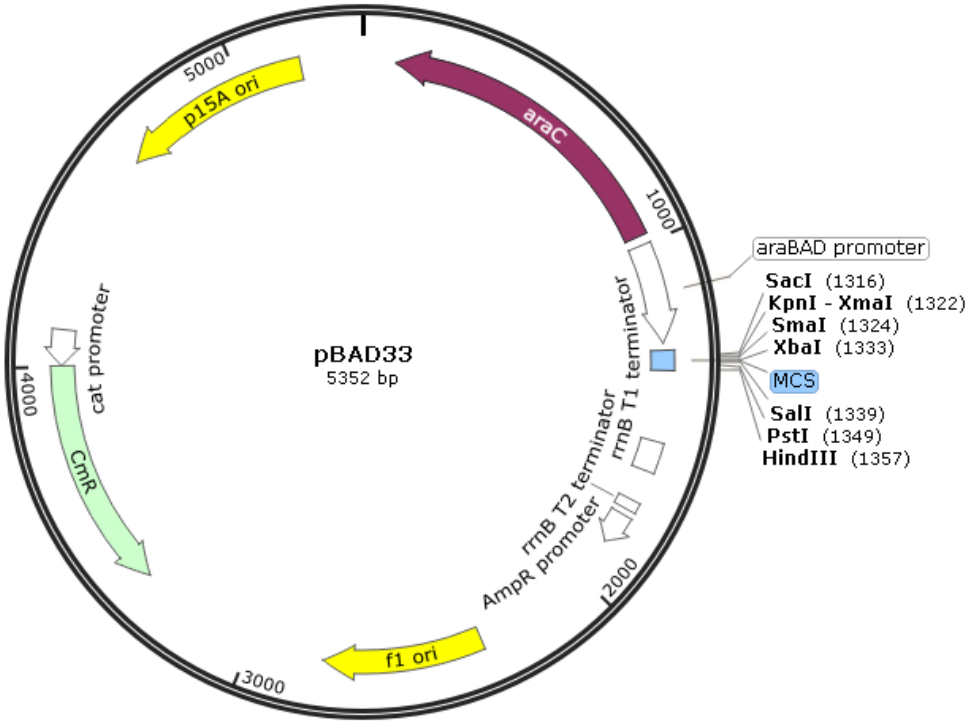
Map of pRSFDuet-1

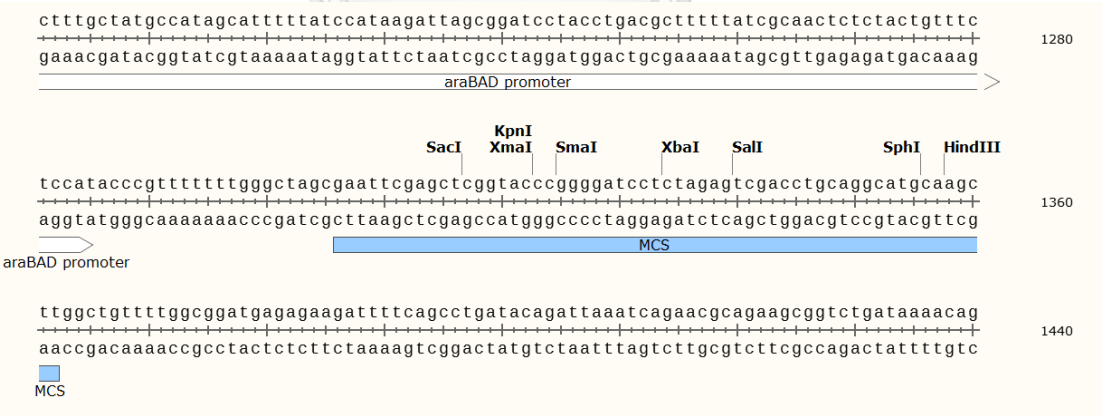


pRSFDuet-1 cloning/expression regions

Appendix B

Map of pBAD-33





Appendix C

Preparation of Luria-Bertani (LB) broth

Luria-Bertani (LB) broth is used for medium growth of *E. coli*. LB medium contained 1% pancreatic digestion of casein, 0.5% NaCl and 0.5% yeast extract. For agar plate, the LB medium is supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclave for 15 minutes at 121 ℃, 15 psi. Then, antibiotic drug (kanamycin, ampicillin or chloramphenicol) was added for selection.

Appendix D

Preparation of competent cell

In this study, we used *E. coli* Top10 and *E. coli* BL21(DE3) as competent cells. Each single colony of *E. coli* Top10 and *E. coli* BL21(DE3) was picked up to culture in 5 ml of LB broth and incubated at 37 ºC with shaking at 250 rpm for 16-18 h. 5% of starter was inoculated into 50 ml of LB broth. The cultures were incubated at 37 ºC with shaking at 250 rpm for 16-18 h. The 5% of each starter was inoculated into 200 ml of LB broth and grown at 37 ºC with shaking at 250 rpm until OD_{600} reached 0.3-0.4. The cultures were chilled on ice and centrifuged at 4ºC, 3,000xg for 10 min. The supernatants were removed and the cell pellets were washed with 2 volume of cool sterilized DI water for 2 times. After that, the cell pellets were washed with 20 ml of cool sterilized 10% glycerol and centrifuged at 4 ºC, 3,000xg for 10 min. Then, the cell pellets were resuspended with cool sterilized 10% glycerol to the final volume 2 ml. Finally, 50 µl of competent cell was aliquoted into microcentrifuge tube and stored at -80 ºC.

Appendix E

Protocol of Presto™ Mini Plasmid Kit

Plasmid extraction was performed using Presto™ Mini Plasmid Kit (Geneaid).

1. Harvesting

The 1.5 ml of cultured bacterial cells was transferred to a microcentrifuge tube and centrifuged at 5,000 x g for 2 min at room temperature. Then, the supernatant was discarded. The harvesting step was repeated using the same 1.5 ml microcentrifuge tube.

2. Resuspension

Two hundred µl of PD1 buffer contained RNaseA was added to the tube containing the cell pellet and then mixed by vortex.

3. Cell Lysis

Two hundred µl of PD2 buffer was added to lyse the cell, mixed gently by inverting the tube and incubated at room temperature for 2 min.

4. Neutralization

Three hundred µl of PD3 buffer was added to neutralize the reaction and then mixed immediately by inverting the tube. After that the supernatant was separated by centrifugation at 10,000 x g for 15 min at room temperature.

5. DNA Binding

All the supernatant was transferred to the PDH column and centrifuged at 10,000 x g for 2 min at room temperature and the flow-through was discarded.

6. Wash

Four hundred µl of W1 Buffer was added into the PDH column. The column was taken centrifuged at 10,000 x g for 2 min to discard the flow-through. After that, 600 µl of wash buffer containing absolute ethanol was added into the PDH column. Centrifugation was performed at 10,000 x g for 2 min at room temperature to discard the flow through follow by centrifugation at 10,000 x g for 3 min at room

temperature to dry the column matrix. The dried PDH column was transferred to a new microcentrifuge tube.

7. Elution

fifty µl of water was added into the center of the column matrix and stood for at least 2 min. Centrifugation was performed at 10,000 x g for 3 min at room temperature to elute the purified DNA.

Appendix F

Protocol of GenepHlow™ Gel/PCR Kit

Purification of DNA fragment using Gel/PCR DNA Fragments Extraction Kit (Geneaid) was performed following these steps:

1. Sample preparation

Gel Dissociation

The 300 mg of agarose gel slice containing relevant DNA fragments was cut and transferred into a microcentrifuge tube. Five hundred µl of Gel/PCR Buffer was added to the sample then mix by vortex. The sample was incubated at 55-60°C for 10-15 min to completely dissolved the gel. After incubation, cool the dissolved sample mixture was cooled to room temperature.

PCR reaction

The 5 volumes of Gel/PCR Buffer was added to the PCR reaction and mixed.

2. DNA Binding

The sample mixture was transferred to the DFH column and centrifuged at 10,000 x g for 2 min. The flow-through was discarded then place the DFH column back in the 2 ml collection tube.

3. Wash

Four hundred µl of W1 buffer was added into the DFH column and centrifuged at 10,000 x g for 2 min and discarded the flow-through. After that, 600 µl of wash buffer contained absolute ethanol was added into the DFH column and stood for 1 min. Centrifugation at 10,000 x g for 2 min was performed to discard the flow-through followed by centrifugation at 10,000 x g for 3 min at room temperature to dry the column matrix. The dried DFH column was transferred to a new microcentrifuge tube.

4. Elution

The dried DFH column was transferred to a new microcentrifuge tube. 20-50 µl of water was added into the center of the column matrix and stood for at least 2 min. The column was centrifuged at 10,000 x g for 3 min at room temperature to elute the purified DNA.

Appendix G

Preparation for SDS-PAGE analysis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 ml with distilled water.

10% (w/v) SDS

Sodium dodecyl sulfate (SDS)	10	g

Dissolved in distilled water to a total volume of 100 ml.

50% (w/v) Glycerol

100% Glycerol	50	ml

Dissolved in distilled water to a total volume of 100 ml.

1% (w/v) Bromophenol blue

Bromophenol blue	100	mg

Brought to 10 ml with distilled water and stirred until dissolved.

The aggregated dye was removed by filtration.

Appendix G (continued)

2. Working solutions

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2	g
N, N'-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 ml with distilled water.

Solution B (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75	ml
10% (w/v) SDS	4	ml
Distilled water	21	ml

Solution C (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50	ml
10% (w/v) SDS	4	ml
Distilled water	46	ml

10% (w/v) Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	ml

Appendix G (continued)

Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	ml
SDS	1	g

Dissolved and adjusted to total volume to 1 liter with distilled water

(final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (w/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	ml
50% (v/v) Glycerol	5.0	ml
10% (w/v) SDS	2	ml
1% (w/v) Bromophenol blue	1	ml
β-Mercaptoethanol	0.5	ml
Distilled water	1.4	ml

Appendix G (continued)

3. SDS-PAGE

12.5% Separating gel

Solution A	4.2	ml
Solution B	2.5	ml
Distilled water	3.3	ml
10% (w/v) Ammonium persulfate	50	µl
TEMED	5	µl

5.0% Stacking gel

Solution A	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10% (w/v) Ammonium persulfate	30	µl
TEMED	5	µl

Appendix H

Preparation for protein staining solution

Staining solution, 1 liter

Coomassie brilliant blue R-250	1.0	ml
Methanol	450	ml
Distilled water	450	ml

Destaining solution, 1 liter

Methanol	100	ml
Glacial acetic acid	100	ml
Distilled water	800	ml

Appendix I

Preparation for Lowry's method solution

Solution A (0.5% copper sulfate and 1% potassium tartate, pH 7.0)

Copper sulfate	0.5	g
Potassium tartate	1	g

Adjusted pH to 7.0 and adjusted to total volume to 100 ml.

Solution B (2% sodium carbonate and 1 N sodium hydroxide)

Sodium carbonate	20	g
Sodium hydroxide	4	g

Dissolved in distilled water to a total volume of 1 liter.

Solution C (phenol reagent)

Folin-Ciocalteu phenol reagent : distilled water is 1:1

Appendix J

Preparation of 10X TBE for agarose gel electrophoresis

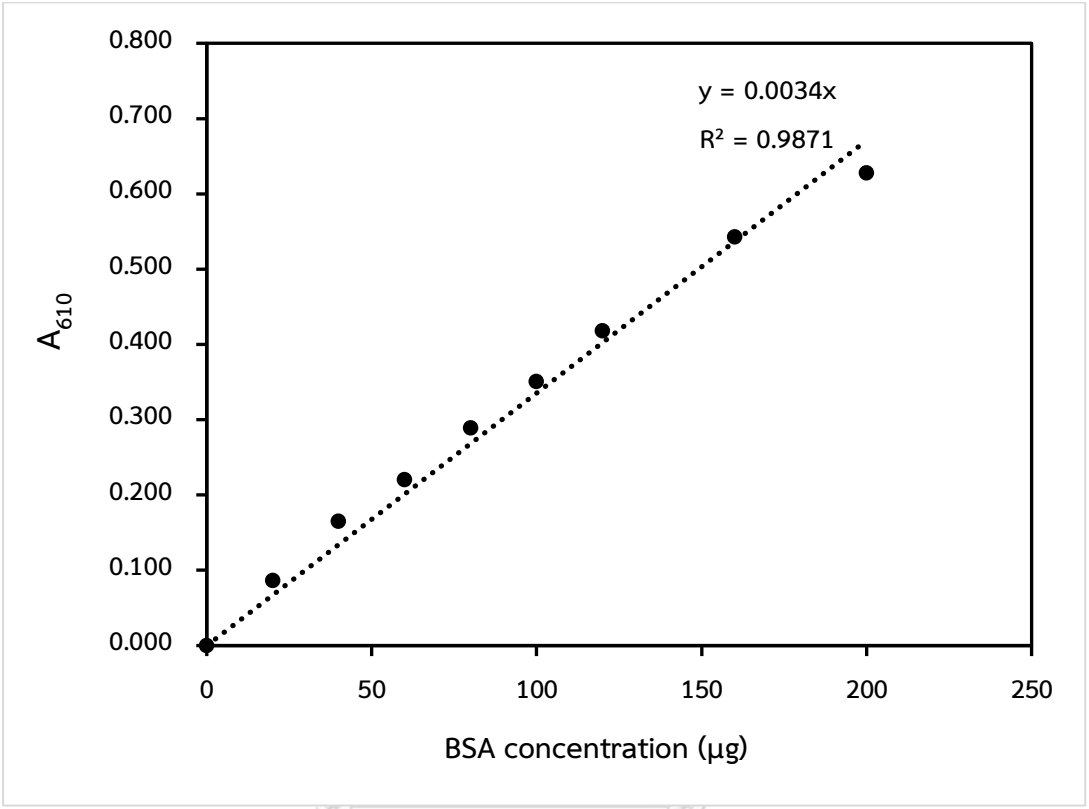
Electrophoresis buffer (10X TBE)

Tris (hydroxymethyl)-aminomethane	54	g
Boric acid	27.5	g
Ethylenediaminetetraacetic acid, disodium salt	9.3	g

Adjust volume to 1 liter with deionized water

Appendix K

Standard curve for protein determination by Lowry's method

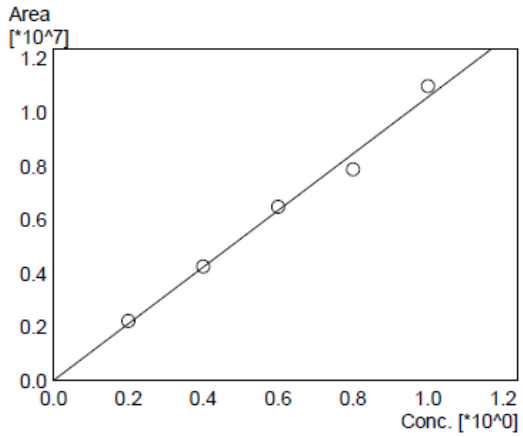


Appendix L

Standard curve for L-Phe determination by HPLC

==== Shimadzu LabSolutions Calibration Curve ====

ID# : 1
Name : L-phe
Quantitative Method : External Standard
Function : f(x)=1.05647e+007*x+45327.1
Rr1=0.9941309 Rr2=0.9882962 RSS=5.287072e+011
MeanRF: 1.072561e+007 RFSD: 5.161007e+005 RFRSD: 4.811853
FitType : Linear
ZeroThrough : Not Through
Weighted Regression : None
Detector Name : PDA-M20A



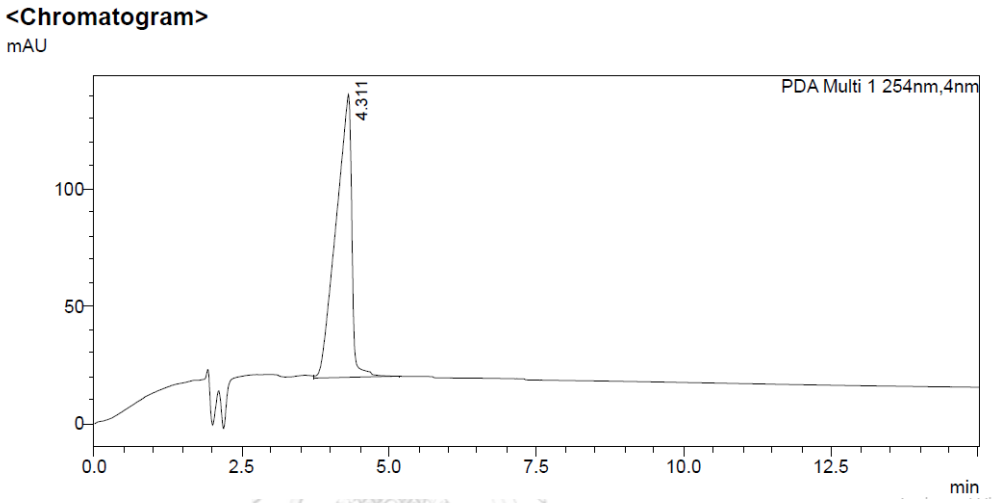
#	Conc.(Ratio)	MeanArea	Area
1	0.2	2246135	2246135
2	0.4	4276915	4276915
3	0.6	6498412	6498412
4	0.8	7900108	7900108
5	1	10999274	10999274

Appendix M

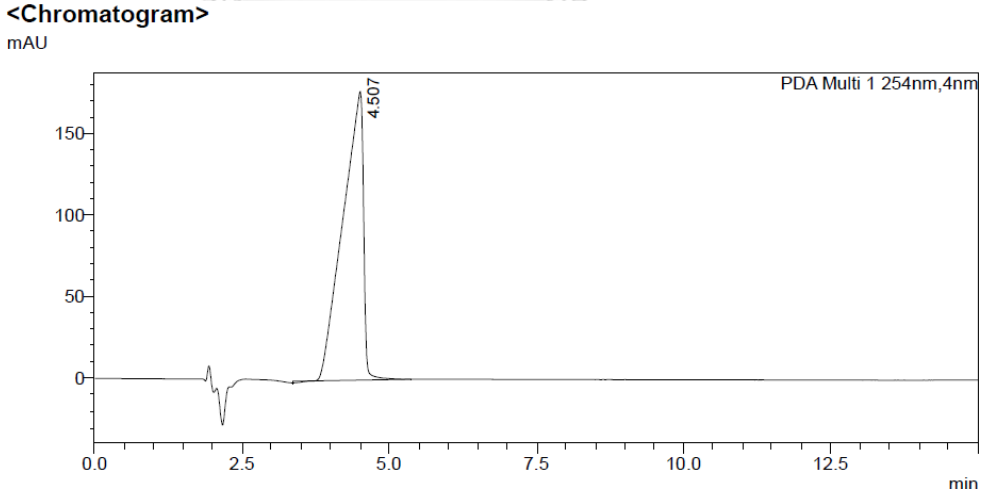
HPLC Chromatogram of L-Phenylalanine

1. L-Phe Standard

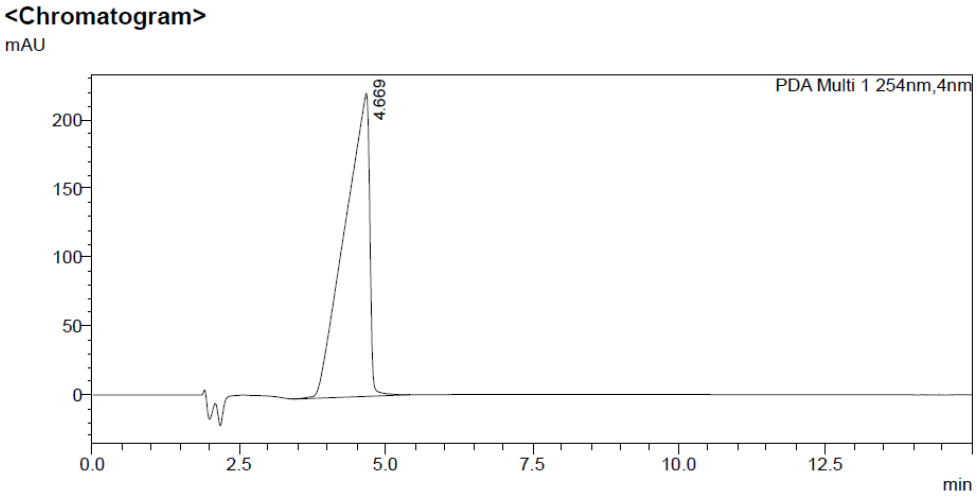
A) L-Phe at 0.2 g/L



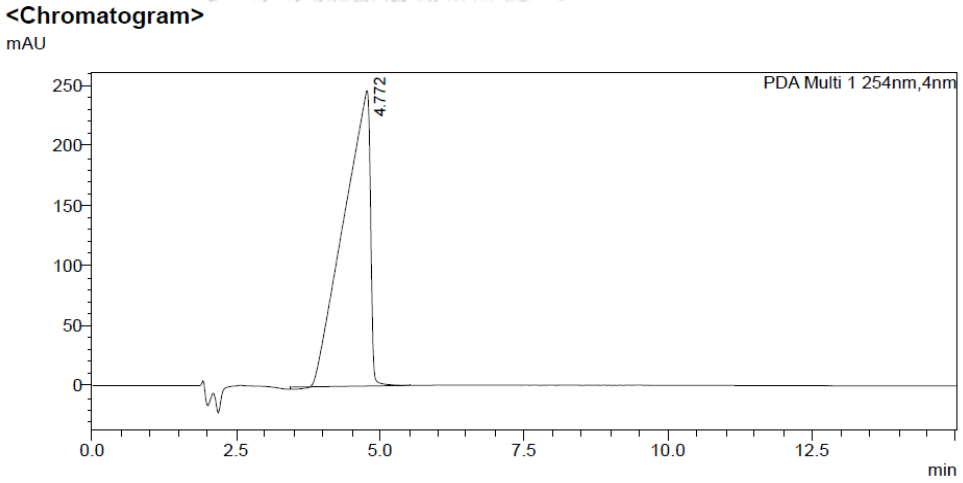
B) L-Phe at 0.4 g/L



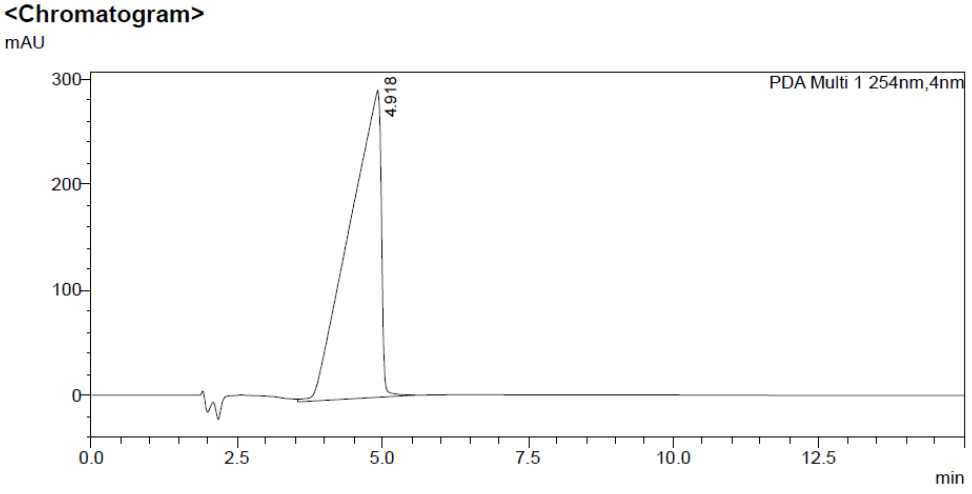
C) L-Phe at 0.6 g/L



D) L-Phe at 0.8 g/L



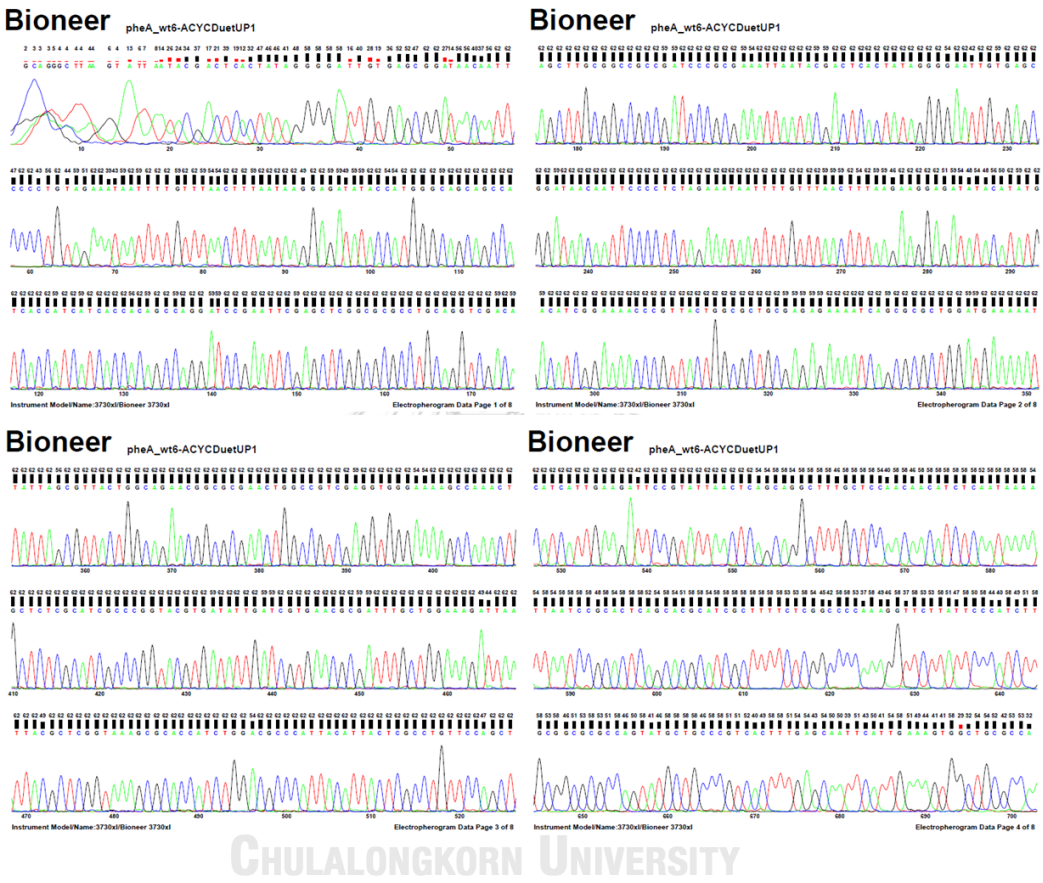
E) L-Phe at 1.0 g/L



Appendix N

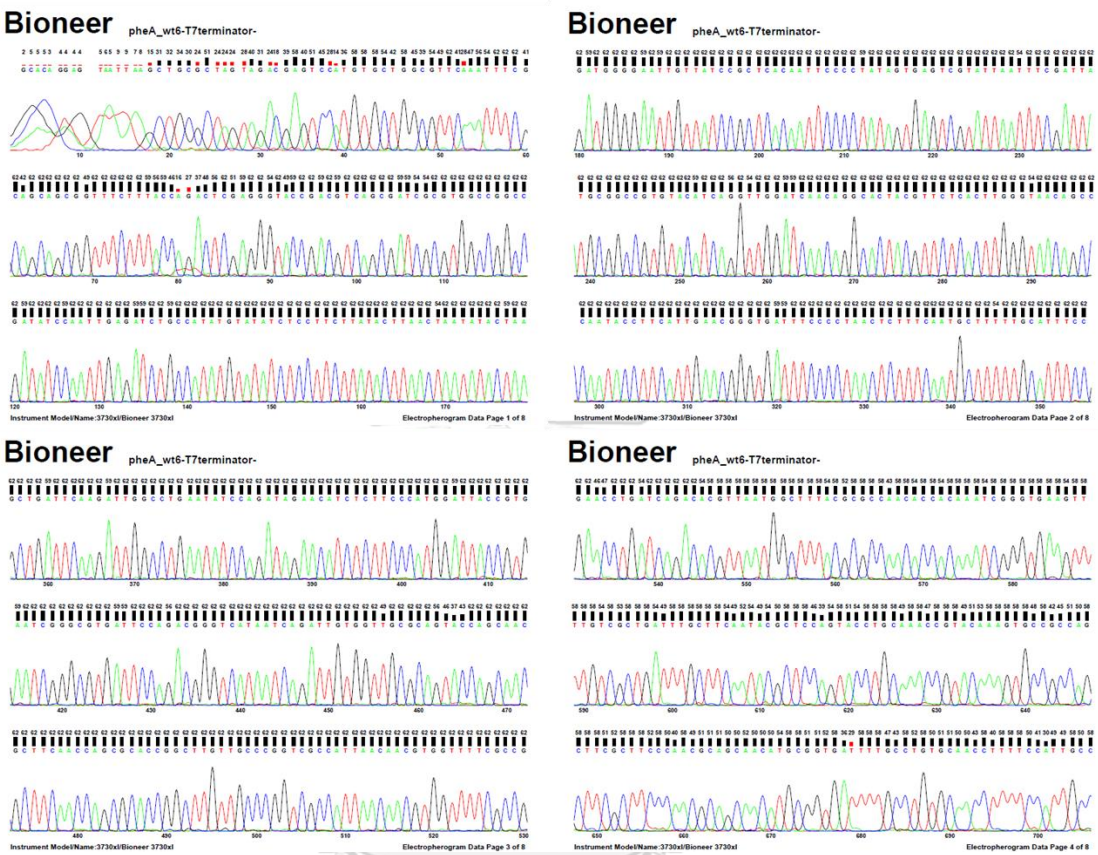
The sequencing chromatogram of *pheA*^{wt} in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers

(A)



Appendix N (continued)

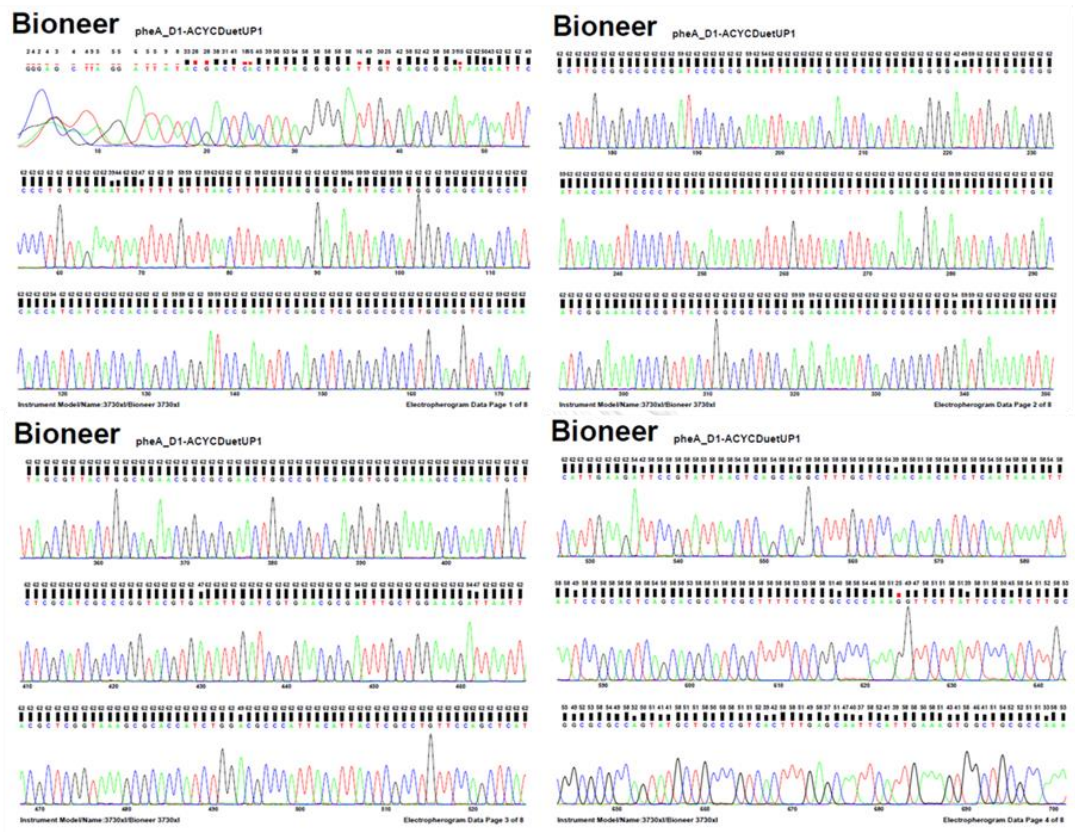
(B)



Appendix O

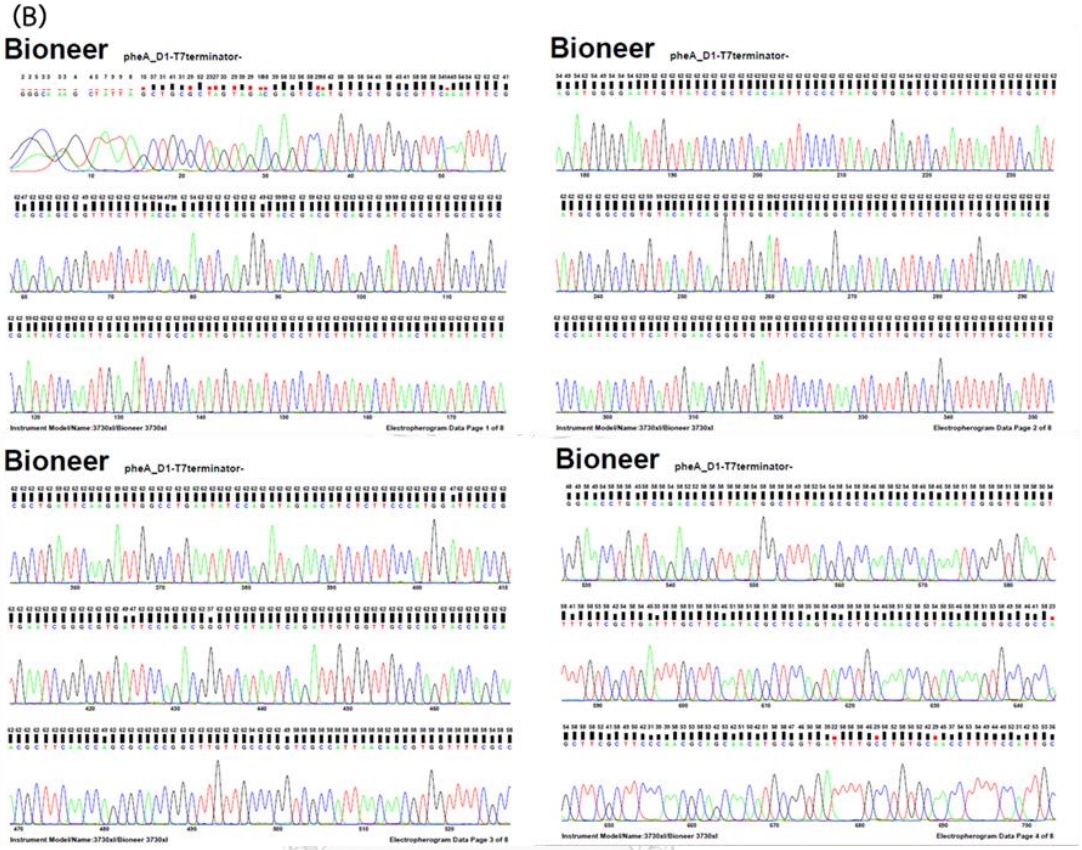
The sequencing chromatogram of *pheA*^{L359D} in pRSFDuet-1 using ACYCDuetUP1 (A) and T7terminator (B) primers

(A)



Appendix O (continued)

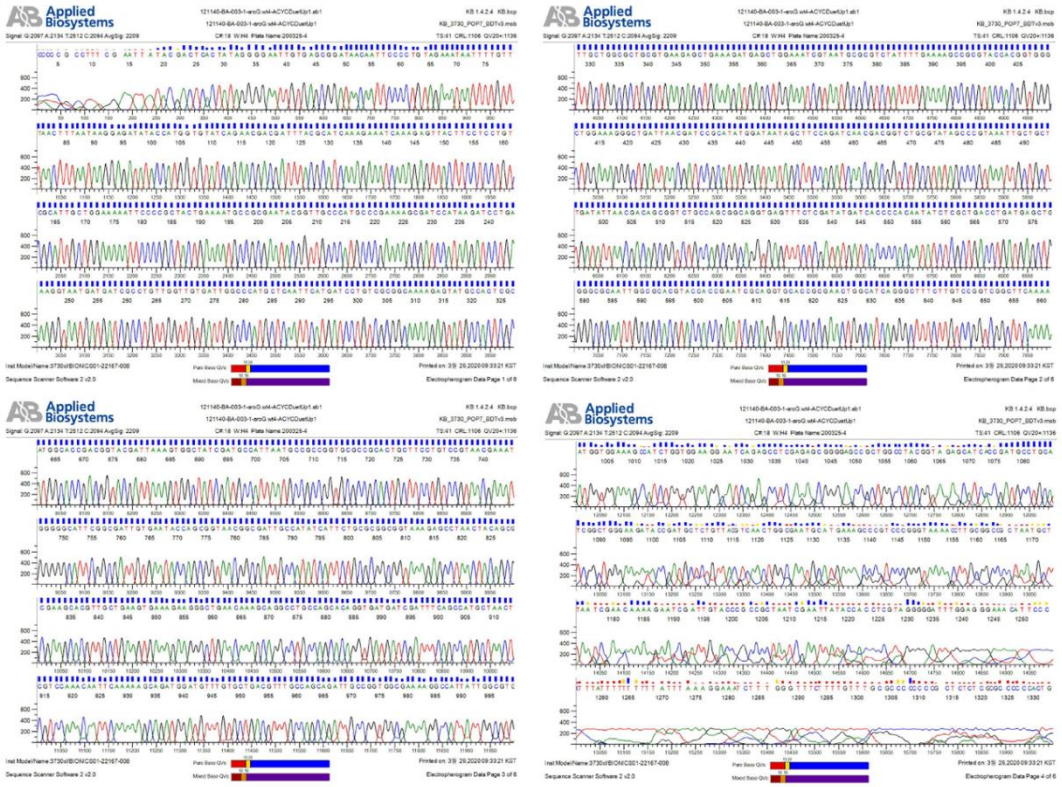
(B)



Appendix P

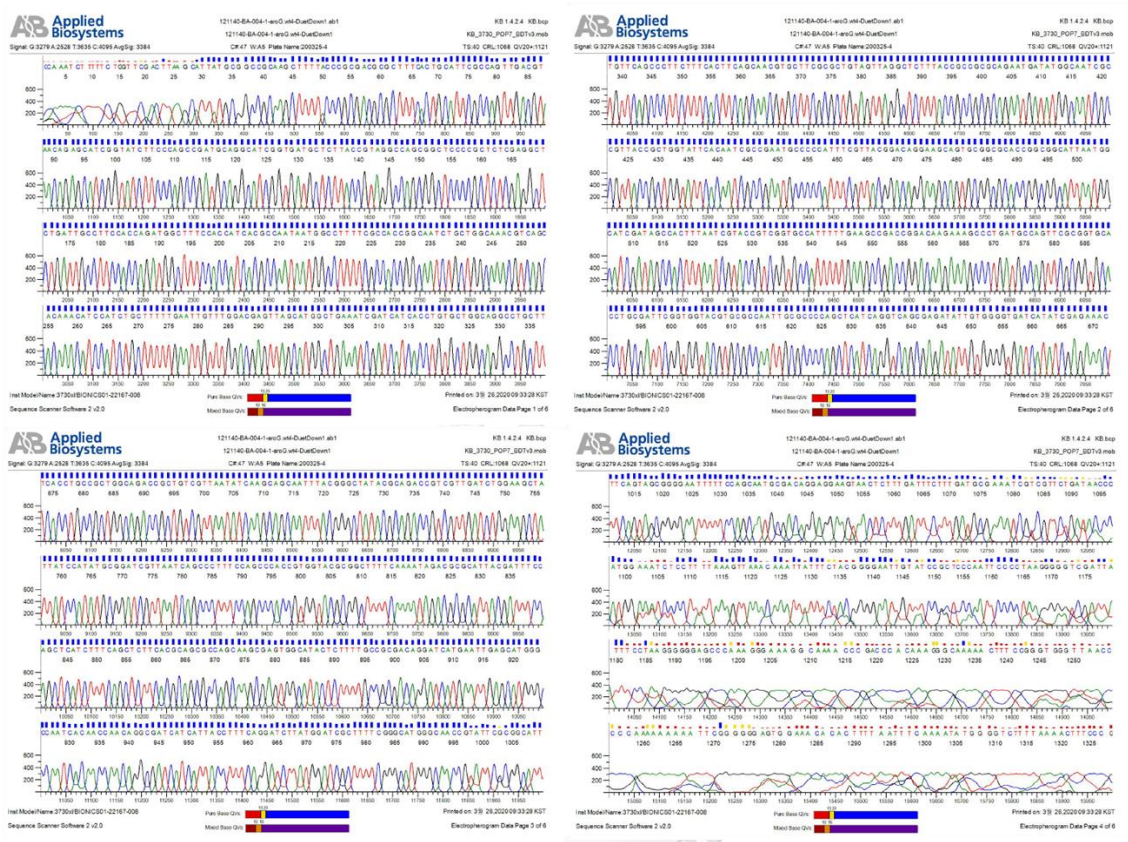
The sequencing chromatogram of *aroG*^{wt} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)



Appendix P (continued)

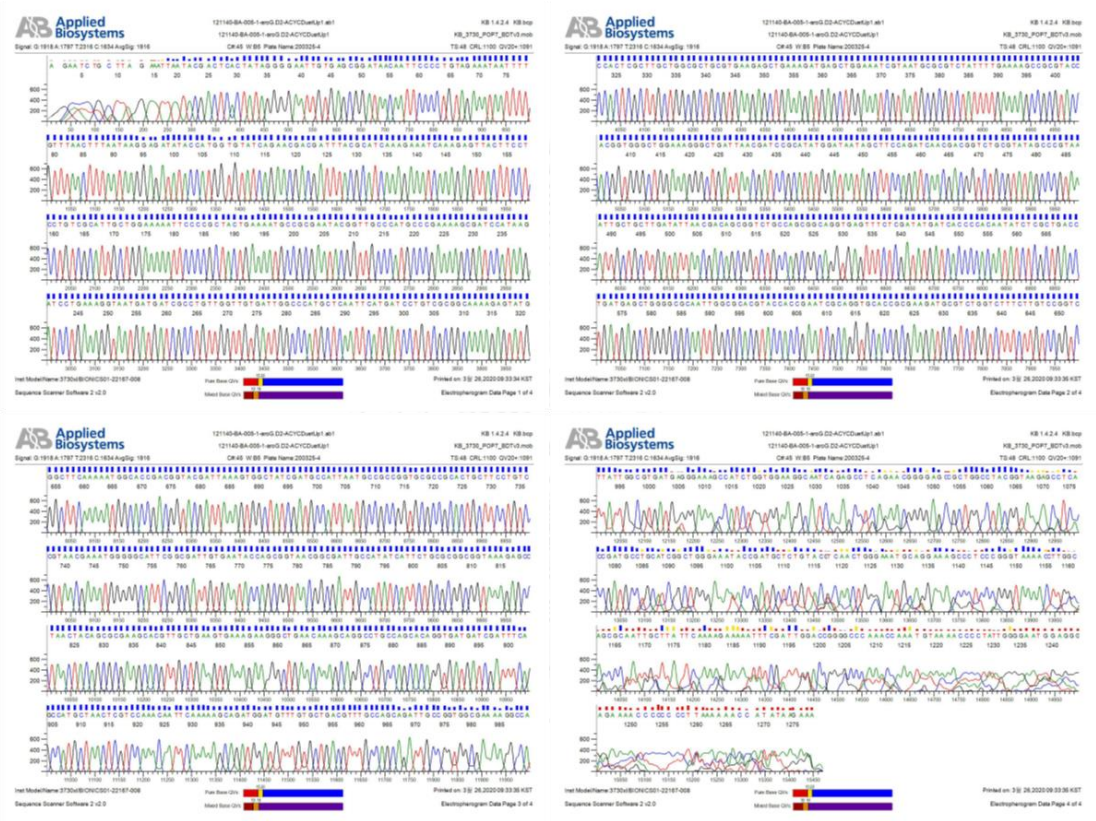
(B)



Appendix Q

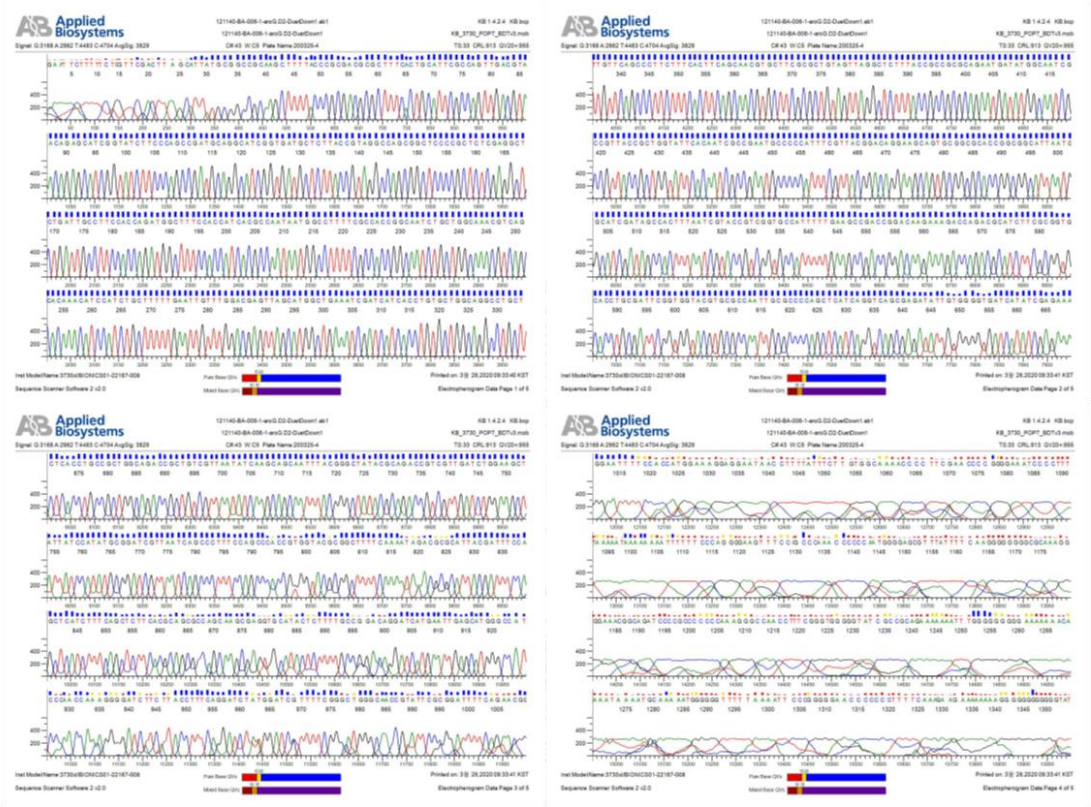
The sequencing chromatogram of *aroG*^{L175D} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)



Appendix Q (continued)

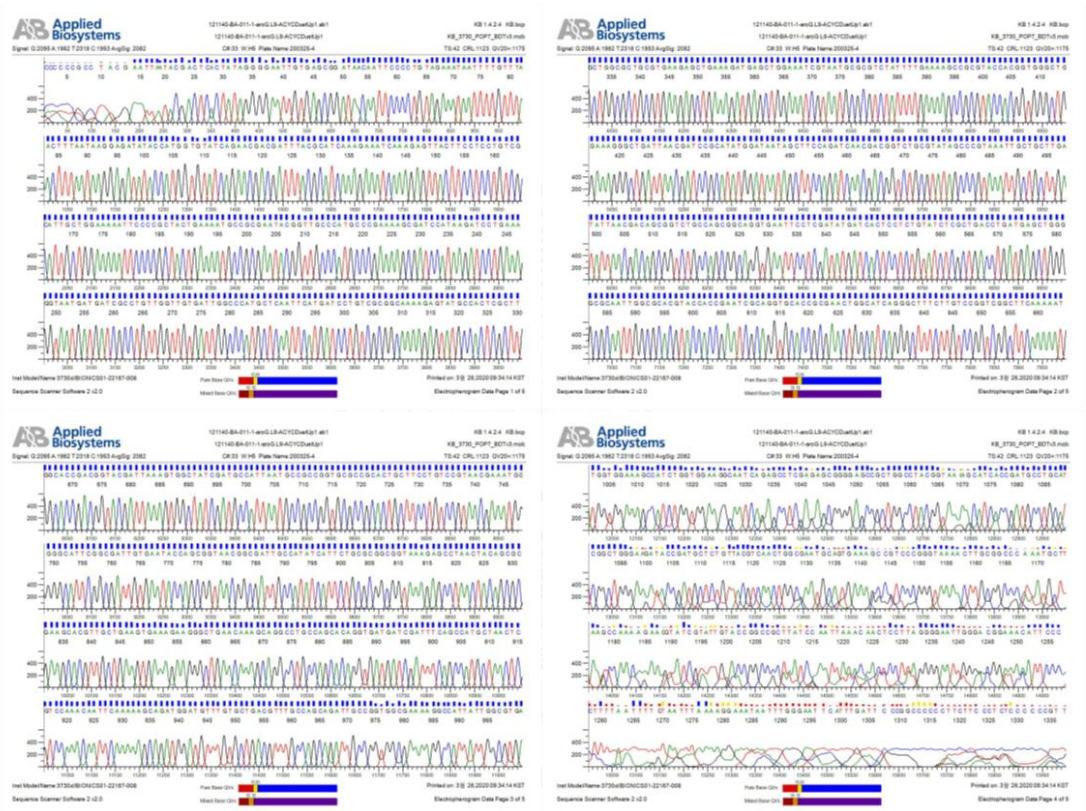
(B)



Appendix R

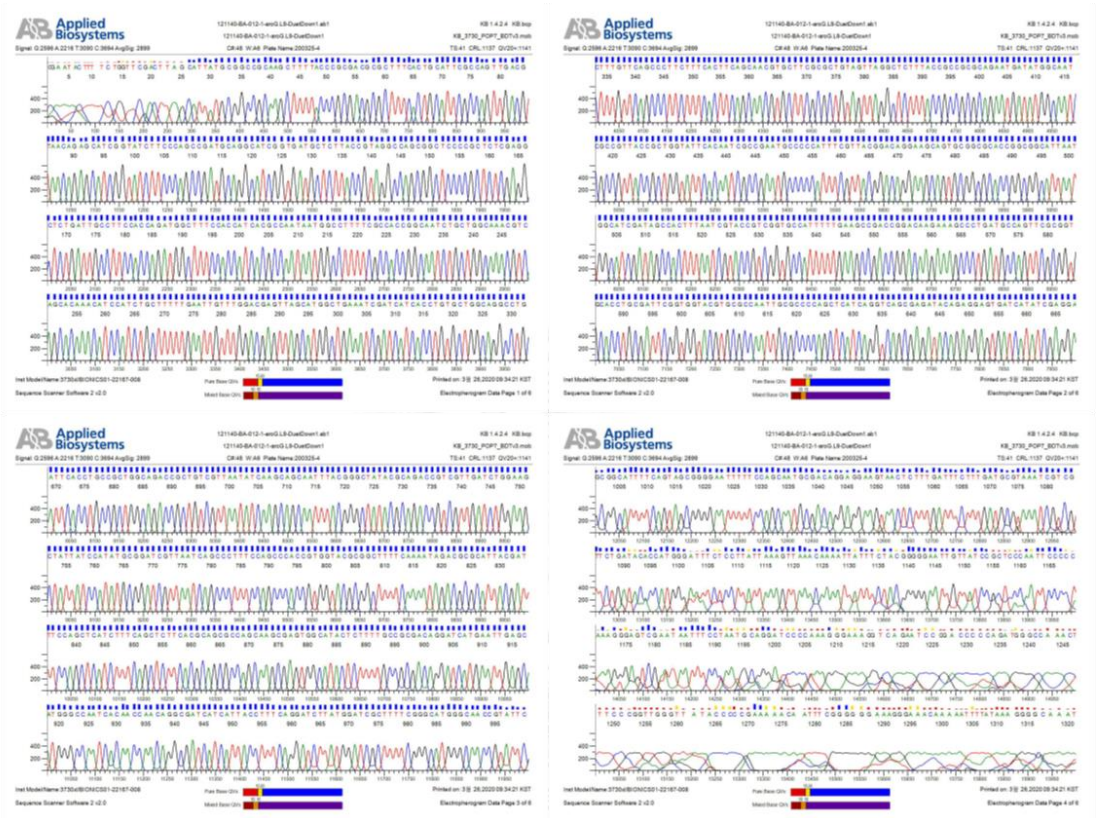
The sequencing chromatogram of *aroG*^{Q151L} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)



Appendix R (continued)

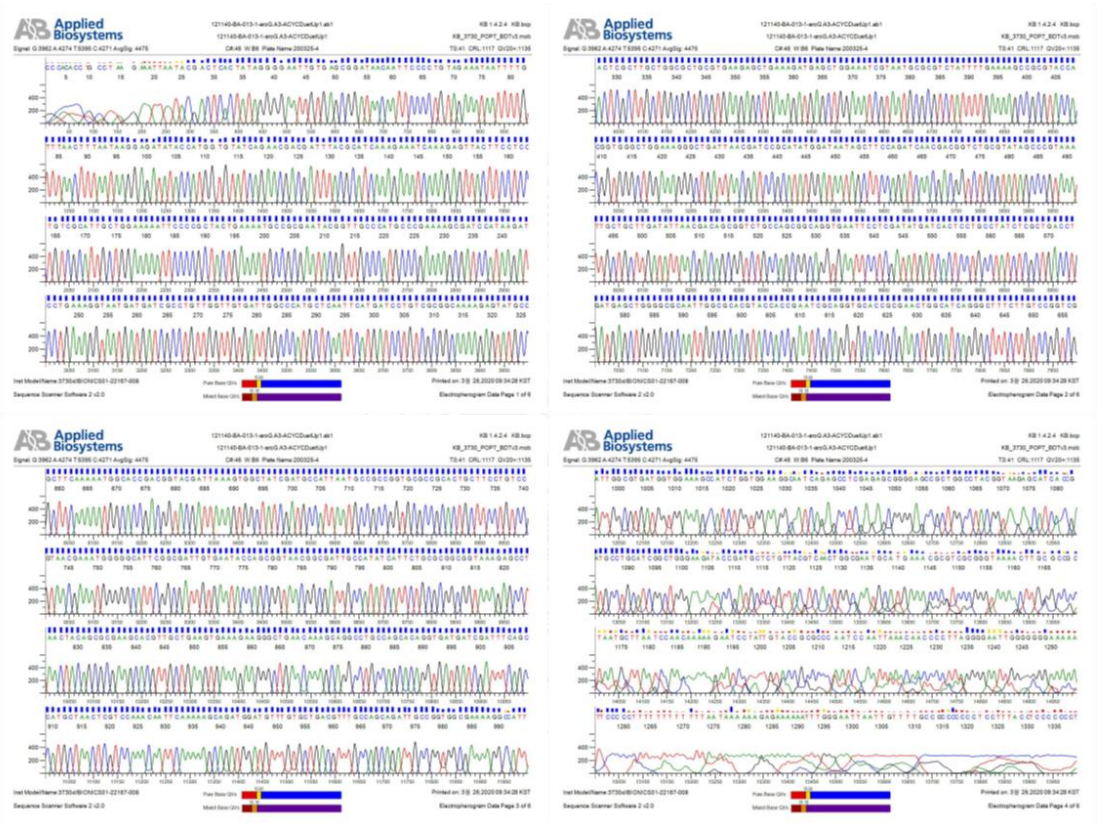
(B)



Appendix S

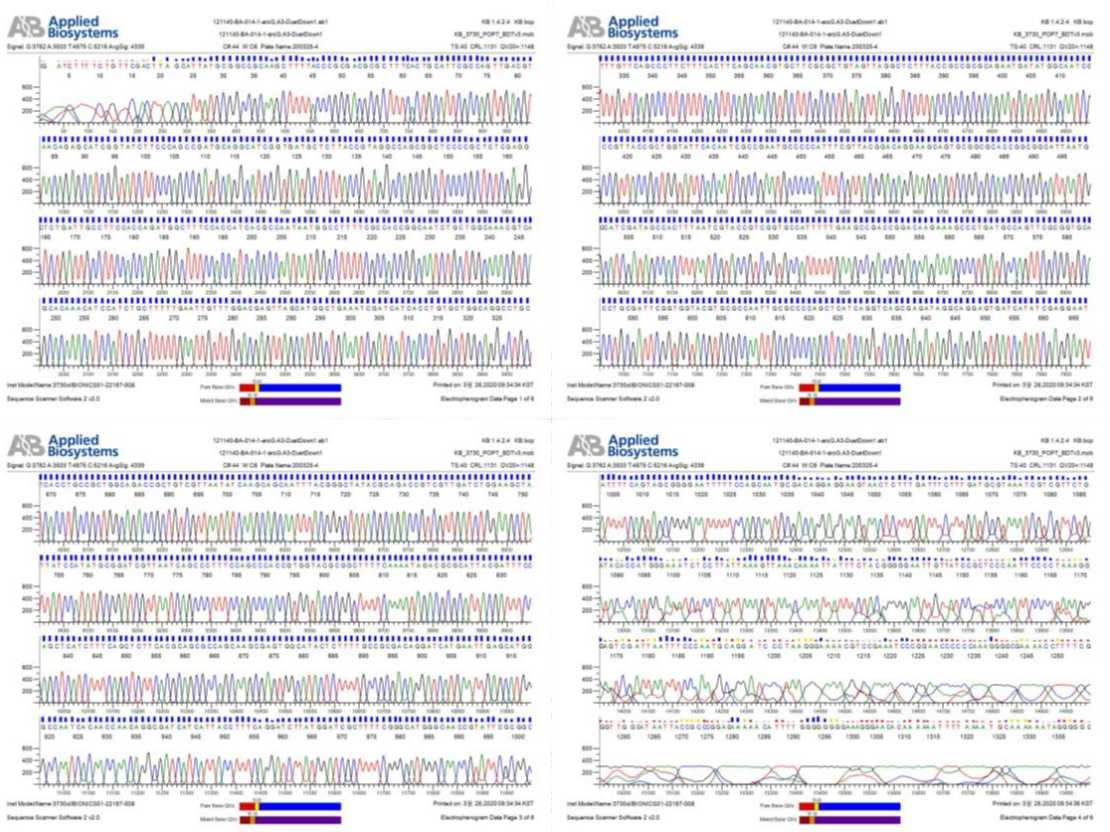
The sequencing chromatogram of *aroG*^{Q151A} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)



Appendix S (continued)

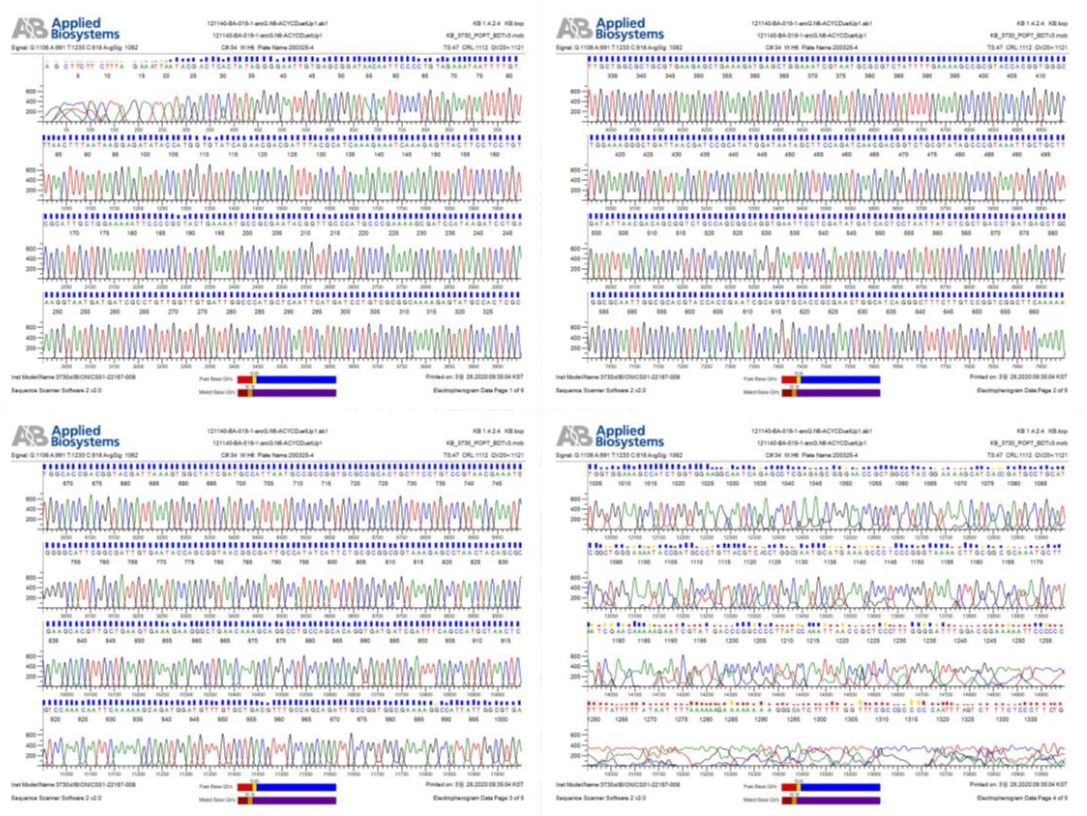
(B)



Appendix T

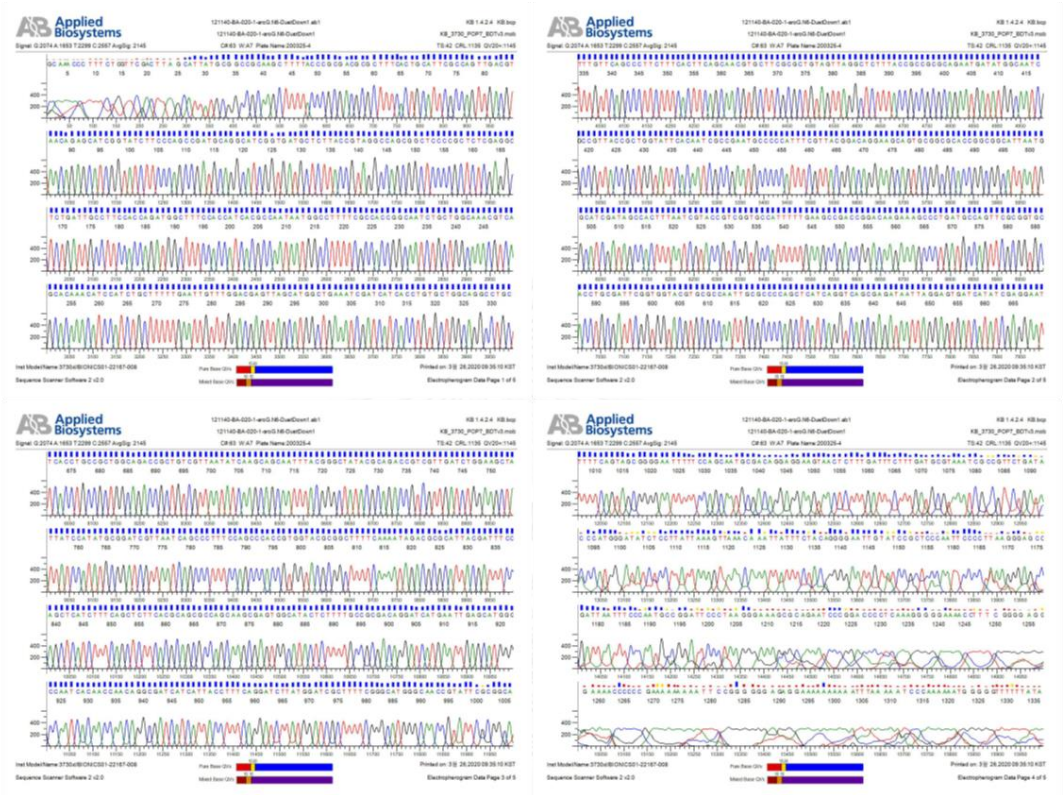
The sequencing chromatogram of *aroG*^{Q151N} in pRSFDuet-1 using ACYCDuetUP1 (A) and DuetDown1 (B) primers

(A)



Appendix T (continued)

(B)



VITA

NAME	Charintip Yenyuvadee
DATE OF BIRTH	1 January 1995
PLACE OF BIRTH	Nakornpathom
INSTITUTIONS ATTENDED	Bachelor of Science (Biochemistry)
HOME ADDRESS	35/269 Supalia vista tiwanon condomenium Krungthep-Nonthaburi Road, Taladkwan, Muang Nonthaburi, Nonthaburi, Thailand 11000
PUBLICATION	Charintip Yenyuvadee, Nichaphat Kanoksinwuttipong, Kanoktip Packdibamrung. Effect of Gln151 on L-phenylalanine feedback resistance of AroG isoform of DAHP synthase in Escherichia coli. ScienceAsia. (summitted on Sep,6 2020)