SITE DIRECTED MUTAGENESIS ON HIGHLY CONSERVED AMINO ACID RESIDUES OF CHOLINE KINASE GENE FROM *Neisseria meningitidis*

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by

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LIST OF SYMBOLS

≈	Almost equal to
α	Alpha
β	Beta
℃	Degree Celsius
µ	Micro
ml	Microliter
n	Nano
%	Percent
v/v	Volume to volume
w/v	Weight to volume

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
BSL-2	Biosafety level 2
Bp	Base pairs
CK	Choline kinase
CKIs	Choline kinase inhibitors
CTH	Choline transporter
CCT	CTP: phosphocholine cytidylyltransferase
CDP-choline	Cytidine 5'-diphosphocholine
GMQE	Global model quality estimate
HC-3	Hemicholinum 3
hCKα	Choline kinase alpha
hCKβ	Choline kinase beta
H_2O	Distilled water
IMD	Invasive meningococcal disease
IRIS	Invasive Respiratory Infection Surveillance
LPS	Lipopolysaccharide
LB	Luria-Bertani
$MgSO_4$	Magnesium sulphate
NmCK	*Neisseria meningitidis* CK
PCho	Phosphorylcholine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI3K	Phosphatidylcholine 3-kinase

RE	Restriction enzyme
TE	Transformation efficiency
TAE	Tris acetate-EDTA
UK	United Kingdom
USA	United States of America
Qiagen	QIAquick Gel Extraction Kit
X g	Fold gravity

MUTAGENESIS TERARAH TAPAK PADA JUJUKAN ASID AMINO AMAT TERPELIHARA GEN KOLINA KINASE DARIPADA *Neisseria meningitidis*

ABSTRAK

Neisseria meningitidis menyebabkan penyakit mudah berjangkit yang dikenali sebagai penyakit meningokokus invasif（IMD） yang boleh membawa kematian. Cara terbaik untuk merawat IMD adalah dengan mengambil rawatan antibiotik. Walau bagaimanapun, *N. meningitidis* telah menghasilkan rintangan terhadap antibiotik yang ada pada masa kini. Kolina kinase yang dikodkan oleh gen *licA* dalam *N. meningitidis* (NmCK) merupakan salah satu enzim yang terlibat dalam sintesis membran sel dan perencatan enzim ini pada bakteria yang lain telah menunjukkan kesan antimikrobial yang memberangsangkan. Walau bagaimanapun, ciri biokimia asas dan perencat NmCK masih belum diketahui. Kajian ini bertujuan mengenalpasti dan mengubah suai jujukan amino asid penting pada NmCK yang mungkin boleh dijadikan sebagai sasaran perencatan mengguna mutagenesis PCR-terarah-tapak dua langkah. Dua asid amino aspartat (D153 dan D170) yang terletak di dalam motif CK NmCK telah dikenalpasti sebagai amat terpelihara dan dipilih untuk mutagenesis kepada alanina. Mutagenesis PCR-terarah-tapak dua langkah telah berjaya menghasilkan jujukan lengkap gen D153A NmCK tanpa sebarang mutasi yang tidak diingini. Dua fragmen mutasi D170A telah dihasilkan untuk kombinasi menjadi jujukan lengkap NmCK. Pemodelan molekul untuk NmCK asal dan mutan D153A juga telah dijalankan dan struktur model menunjukkan mutasi amino asid tunggal tidak mengubah struktur keseluruhan enzim ini. Walau bagaimanapun, hipotesis bahawa mutasi ini mengubah poket pengikat kolina telah dibuat. Kesimpulannya, gen D153A NmCK boleh

digunakan untuk ekspresi protein mutan bagi mengkaji kepentingan jujukan asid amino ini dalam pemangkinan dan pengikatan perencat. Struktur model yang terhasil boleh digunakan untuk kajian pelabuhan molekul dengan perencat yang berpotensi.

SITE DIRECTED MUTAGENESIS ON HIGHLY CONSERVED AMINO ACID RESIDUES OF CHOLINE KINASE GENE FROM *Neisseria meningitidis*

ABSTRACT

Neisseria meningitidis causes high transmissible disease known as invasive meningococcal disease (IMD) that is fatal. The best way to treat IMD is by antibiotics treatment. However, *N. meningitidis* has developed resistance to currently available antibiotics. Therefore, it is essential to discover new antimicrobial agents to combat the antibiotic resistant *N. meningitidis* strains. Choline kinase in *N. meningitidis* (NmCK) encoded by *licA* gene is one of the enzymes involved in cell membrane synthesis and inhibition of this enzyme in other bacteria has shown promising antimicrobial effect. However, basic biochemical properties and inhibitors of NmCK are still unknown. The present study aims to identify and modify important amino acid residues for NmCK catalysis that could be targeted for inhibition by performing two-step PCR site directed mutagenesis. Two aspartates (D153 and D170) in the CK motif of NmCK have been identified as highly conserved and chosen for mutagenesis into alanine. Two step PCR site-directed mutagenesis has successfully produced the full length D153A NmCK gene sequence without any unwanted mutation. Two fragments of D170A mutation have also been produced for subsequent combination into full length NmCK. Molecular modeling of wild type and D153A mutant NmCK was also performed and the model structures showed that the mutation of single amino acid did not change the overall structure of this enzyme. However, it is hypothesized that the mutation could alter the choline binding pocket. In conclusion, the D153A

NmCK gene can be used for expression of mutant protein to study the importance of this residue in catalysis and inhibitor binding. The model structures generated can be used for molecular docking studies with potential inhibitors.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Neisseria meningitidis causes invasive meningococcal disease (IMD) which is fatal and highly transmissible worldwide. Although antibiotic is the best treatment against *N. meningitidis*, this organism has started to develop antibiotic resistance (Tzeng & Stephens, 2021). New antimicrobial agents are required to combat this pathogenic bacterium. *N. meningitidis* choline kinase (CK) enzyme contributes to the formation of bacterial membrane and survival. Therefore, inhibiting the activity of CK is a good strategy to develop new generation of antimicrobial agents against antibiotic resistant strains of *N. meningitidis*.

Knowledge about the biochemical pathways involved in the bacterial membrane and cell wall synthesis has been generated in the past decades. Involvement CK in bacterial membrane phospholipid synthesis has also been shown. However, more detailed study of bacterial CK such as its important domain and sequences for catalysis has not been carried out.

The main purpose of this study is to mutate the coding sequences of two conserved amino acids predicted to be important for the activity of *N. meningitis* CK (NmCK) and to compare the model structures of the wild type and mutant NmCK. This work is

expected to provide basic information for further research towards the design of specific and potent NmCK inhibitors to be used as drugs against antibiotic resistant *N. meningitidis*.

1.2 Rational of study

Neisseria meningitidis has been proved to develop antimicrobial resistance (AMR) that will increase its survivability and decrease its susceptibility against various types of antibiotic. Therefore, new antimicrobial agent needs to be discovered and established to combat against AMR *N. meningitidis*. Many research studies have found out that CK is a potential and suitable target to be studied. It is important to characterize the amino acids involved in the catalysis of NmCK so that inhibitors specifically bind to these catalytically important amino acids could be designed for effective enzyme activity inhibition and subsequent retardation of bacterial growth.

1.3 Objectives

1.3.1 General objectives

To perform two-step PCR site-directed mutagenesis on highly conserved amino acid residues of *Neisseria meningitidis* CK (NmCK)

1.3.2 Specific objectives

1. To compare the model structures of wildtype and mutant NmCK
2. To predict for catalytically important amino acids in NmCK at the conserved regions of this enzyme
3. To mutate the highly conserved amino acids residues by two-step PCR site-directed mutagenesis

CHAPTER 2

LITERATURE REVIEW

2.1 Neisseria meningitidis

Neisseria meningitidis is an aerobic and facultative anaerobic bacterium. It is classified as a gram-negative bacterium because it has a peptidoglycan between its outer and inner membrane. It is also known as meningococcal due to its coffee-bean diplococci shape. *N. meningitidis* belongs to the member of β-proteobacteria, which includes another prominent human pathogen known as *Neisseria gonorrhoeae*. *N. meningitidis* was first discovered by Weichsel Baum back in 1887 from a patient that has suffered meningitidis while analyzing his cerebrospinal fluid. *N. meningitidis* infects only human being exclusively. *N. meningitidis* grow best at 35-37℃ due to it being a fastidious organism (Rouphael and Stephens, 2012).
Kovac's oxidase test and carbohydrate utilization are used to confirm the identity of *N. meningitidis*. Carbohydrate utilization need to be carried out if oxidase test shows positive result (*Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria Meningitidis, Streptococcus Pneumoniae, and Haemophilus Influenzae W H O M a n u a l* **(2),** 2011). Once carbohydrate utilization shows positive result as well, serological test needs to be performed. Biosafety level 2 (BSL-2) practices are required for isolating *N. meningitidis*.

Factor H binding protein binds with human Factor H that will allow *N. meningitidis* to be able to survive in blood stream (Pizza and Rappuoli, 2015). Factor H binding protein mostly expressed in meningococcal serogroup B. Factor H is a member of

complement activation regulator that regulates alternate pathway of complement system which is directed towards pathogen. However, the factor H binding protein will bind to factor H and inhibit its activity.

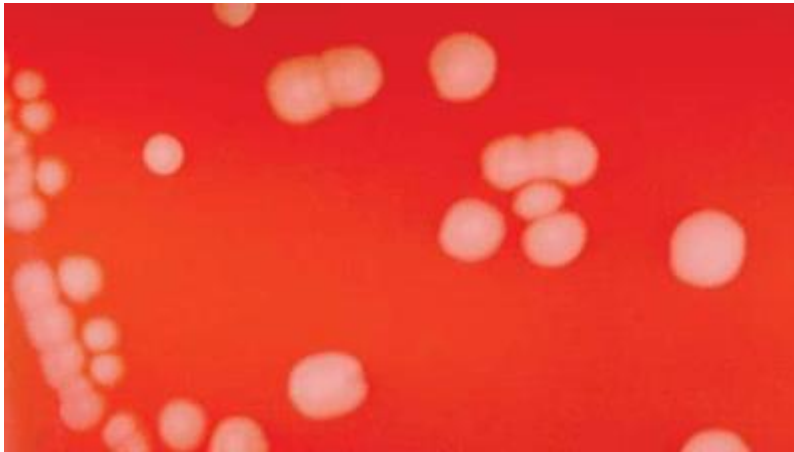

Figure 2.1 *N. meningitidis* colonies on blood agar plate (Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria Meningitidis, Streptococcus Pneumoniae, and Haemophilus Influenzae W H O M a n u a l **(2)**, 2011)

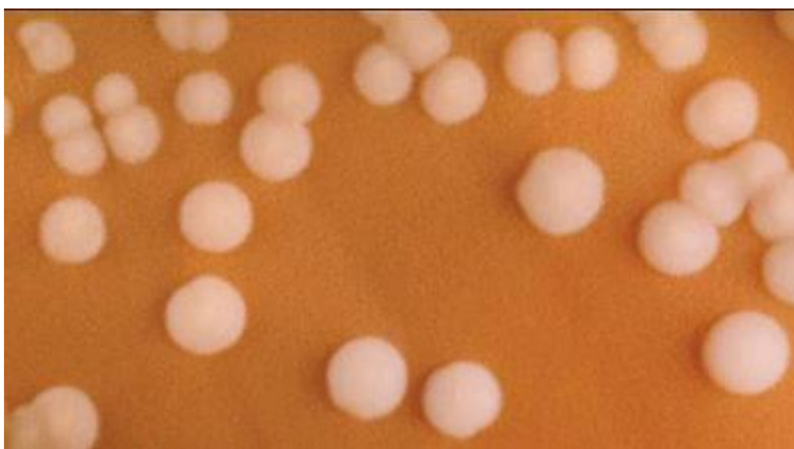

Figure 2.2 *N. meningitidis* colonies on chocolate agar plate (Laboratory Methods for the Diagnosis of Meningitis Caused by Neisseria Meningitidis, Streptococcus Pneumoniae, and Haemophilus Influenzae *W H O M a n u a l* **(2)**, 2011)

2.2 *N. meningitidis*: Pathology, prevalence, and treatment

The term “meningitidis” refers to the inflammation of the meninges or cerebrospinal fluid that protects human brain and spinal cord. *N. meningitidis* caused invasive meningococcal disease (IMD) that is highly transmissible among humans worldwide (Rostamian et al., 2022). *Neisseria meningitidis* is supposed to be a harmless commensal bacterium in adults and this is called being ‘a carrier’. However, there are disease-causing strains that can be highly transmissible. There is a total of 12 serogroups (A, B, C, E, H, I, K, L W135, X, Y, Z) of *N. meningitidis*, some are encapsulated and some or not (Schmitz & Stratton, 2014). Most invasive *N. meningitidis* are encapsulated which possess polysaccharide capsule which can help to survive the human immune response. Among the 12 serogroups, serogroup A, B, C, W135, X and Y cause most of the infections worldwide. In African meningitidis belt, serogroup A is the most prevalent out of so many serogroups (Sato et al., 2022). The route of infection is either through oral or nasal secretions. Therefore, individuals that has close contact with each other such as family and friends have higher chance of getting infected (Soriani, 2017). *Neisseria meningitidis* strains are mostly susceptible to various Gram-negative bacteria. Invasive Respiratory Infection Surveillance (IRIS) had collected 5877 samples from 21 laboratories from 21 countries from 2018 to 2020 (Brueggemann et al, 2021). It can be easily noticed that the isolate count collected from these laboratories has decrease significantly from 2019 to 2020. The underlying reason is due to the coincidence with COVID-10 containment measures in each country Meningococcal disease can cause various complications especially against nerve and brain. The most straight forward way to treat meningococcal disease caused by *N. meningitidis* is using antibiotics. However, mortality rate remains at 10-15% even with antibiotic treatment and healthcare (Tzeng & Stephens, 2021). The main

risk groups of meningococcal disease include young children and immunosuppressed patients.

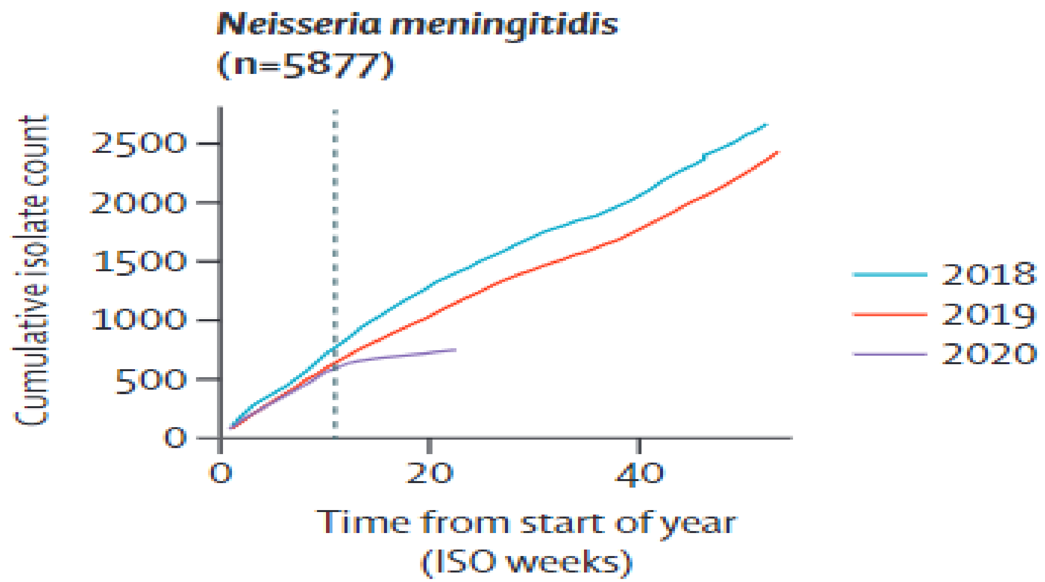

Figure 2.3 Cumulative number of invasive disease cases caused by *Neisseria meningitidis* collected by Invasive Respiratory Infection Surveillance laboratories each week from Jan 1, 2018, to May 31, 2020 (Brueggemann et al., 2021)

2.3 Antibiotic resistance in pathogenic bacteria

The use of antibiotics to treat diseases caused by various bacteria has been a common and straightforward way. However, due to the overuse of antibiotics, bacteria have developed resistance to these antibiotics and became a major source of morbidity and mortality worldwide (Reygaert, 2018). Although most of the bacteria resist only certain antibiotics, it is believed that if the overuse of antibiotics against them is not controlled well, or scientist are not able to discover new antimicrobial agent, these bacteria will increase susceptibility to more antibiotics in the future, known as developing multidrug resistance. Laura Willerton found out that there are rare cases where *N. meningitidis* is resistant to antibiotics in 2021. However, reduced susceptibility and resistance to penicillin have started to be noticed globally. There are several mechanisms of antibiotics resistance for bacteria, which includes (1) limiting uptake of drug. (2) inactivating a drug, (3) modifying a drug target and (4) active drug efflux (Reygaert, 2022). Due to some structural difference between gram positive and negative bacteria, there are some variation among these mechanisms between these two types of bacteria.

A study regarding antibiotic resistance was made to review published study systematically from 2000 to 2020 in the world (Rostamian et al., 2022). Finally, they found out that the overall resistance is to common antibiotics such as ceftriaxone, cefotaxime, ciprofloxacin was low. Yet, they also found out that *N. meningitidis* is less sensitive to the first line of antibiotics except penicillin.

2.4 Cell wall/membrane synthesis in bacteria

Cell wall is an important structure of bacteria that dictates cell shape (Huang et al., 2008). Its primary role is to serve as a primary stress-bearing and shape maintaining element in bacteria. Not only cell wall has its primary role, it also helps to regulate bacterial functions and most importantly, cell growth (Scheffers & Pinho, 2005). Lipopolysaccharide (LPS) can be found in the outer-membrane of Gram-negative bacteria that provides structural integrity of bacteria. Peptidoglycan synthesis is the most important biogenesis pathways for all bacteria. The peptidoglycan layer is made up of polysaccharides with alternating *N*-acetylglucosamine and *N*-acetylmuramic acid saccharide groups.

2.5 Choline kinase in human and bacteria (lice gene and its product)

In human, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two most abundant phospholipid species. Both PE and PC are the final biosynthesis products of two branches of Kennedy pathway. Choline kinase (CK) is an enzyme that catalyzes the very first reaction in the Cytidine 5'-diphosphocholine (CDP-choline) pathway which involves the phosphorylation of choline to form phosphorylcholine (PCho) in PC biosynthesis. ATP and magnesium ions (2+) are both important components. PC is the major phospholipid in mammalian cellular membranes. Selective inhibition of CK will cause simultaneous attenuation of MAPK and PI3K/AKT signaling. (Quartieri et al.,2021). CTP: phosphocholine cytidylyltransferase (CCT) is specifically catalyze the primary regulatory step in Kennedy pathway. This choline branch of Kennedy pathway is also very essential to various medically relevant bacteria such as *Strepotococcus pneumoniae, Haemophilus influnzae,* and *Neisseria meningitidis*; pathogenesis of bacteria requires the support of

activation of choline to CDP-choline (Zimmerman et al., 2019). There are two types of genes that encodes human choline kinase which are choline kinase alpha (hCKα) and choline kinase beta (hCKβ) (Arlauckas et al., 2016). hCKα plays an important role in cell cycle, cell apoptosis regulation and transformation. Overexpression of hCKα can be easily detected in tumor cells. This is because high level of CK expression is highly associated with malignant transformation, invasion, and metastasis, especially particular in breast cancer (Nimmagadda *et al*, 2009). By getting this conclusion have led to the development of new therapeutic interventions that is able to inhibit CK, especially when combat against bacteria that has developed multidrug resistance that is not susceptible to various type of antibiotics.

2.6 Inhibitors for human choline kinase as anticancer agents

Changes in CK activity will affect the rate of PC synthesis. In mammalian tumors, overexpression of CK and phosphocholine can be observed easily. Therefore, it is believed that CK is playing an essential role in cell transformation. Observed increased level of phosphocholine are caused in part to the growth factor-activated Ras and phosphatidylcholine 3-kinase (PI3K) signaling cascades that stimulates CK in the choline branch of Kennedy pathway. Lung, breast, colon, ovaries tumors are found out to be activated by CK. Therefore, CK has been proposed to serve as prognostic marker for cancer progression and a great target to develop novel cancer therapeutic agents.

Hemicholinum 3 (HC-3), a CK inhibitor has been previously reported to show selective antitumor activity (HernaÂ ndez-Alcoceba et al., 1997). The mode of action of CK inhibition was resulted in defects in PC synthesis, that causes the increased level of ceramide in intracellular level which further promotes apoptosis.

2.7 Enzyme inhibitors as antimicrobial agents

Enzyme is an essential molecule that accelerate chemical reactions that happen in living things. These chemical reactions convert substrate molecules into products. Substrates has their own unique active sites that allow enzymes to bind it and increases the reaction. Almost 100% of metabolic processes require the aid of enzyme to speed up the fast enough so that life of living things can be sustained. The mechanics of enzyme used to increase the rate of chemical reactions is through lowering that their activation energy. If the activation energy of the process cannot be lowered down by enzyme, the conversion of substrate to product will take millions time more. Enzyme inhibitor is a molecule that is able to interfere the reaction between an enzyme and substrate by blocking its activity. There are several types of enzyme inhibition mechanics. Firstly, a competitive inhibitor is a molecule that directly binds to the active site of a substrate that avoid the binding on enzyme to the particular substrate, known as blocking. Secondly, non-competitive inhibitor is a type of inhibitor that binds to an allosteric site of the substrate which is not the active site. The reason being is this non-competitive inhibitor is able to change the structure of the substrate, process known as structural shift, that caused the active site structure to be altered and the enzyme cannot bind to the active site. Lastly, uncompetitive inhibitor can bind only to substrate-enzyme complex that directly stop the reaction. The discovery of novel enzyme inhibitor used to combat against bacterial infections is very essential because bacteria have started to develop antibiotics resistance against certain types of antibiotics. As mentioned early, CK is essential for bacteria to survive in human cells and caused infection against human. Therefore, instead of consuming antibiotics as a treatment that will eventually lead to development of AMR against more types of

antibiotics for bacteria, we can use the mechanics of enzyme, substrate and enzyme inhibitors to create an alternative way of inhibiting the reaction between CK and its substrate to increase the bacteria's survivability. Therefore, it is believed that discovering suitable CK inhibitor is a great way to combat bacteria.

2.8 Important domains and amino acid residues in choline kinases

Choline is essential for animals because cell requires PC to grow and divide (1). Up to 95% of total choline pool in most tissues are used up for biosynthesis of PC via the Kennedy pathway. In mammalian cells, there are two different CK genes which are CKα and CKβ that encode for three types of CK isoforms, CKα1, CKα2 and CKβ. Choline kinase was first demonstrated in yeast by J. Wittenberg and A. Kornberg back in 1953. Amino-terminal regions between species are not well conserved amongst CKs. However, sequence alignments show highly conserved regions. The carboxyl-terminal portion has a highly conserved region known as Brenner's motif which is similar to many other enzymes that catalyzes phosphotransfer reactions. On the other hand, the second highly conserved region of amino acids in CK which is known as CK motif. By observing the figure above, it can be easily shown that H, N, D and N amino acid residues in Brenner's motif and I, D, E, Y and N amino acid residues in CK motif are highly conserved which is consistent among different CK genes (Khalifa et al., 2020).

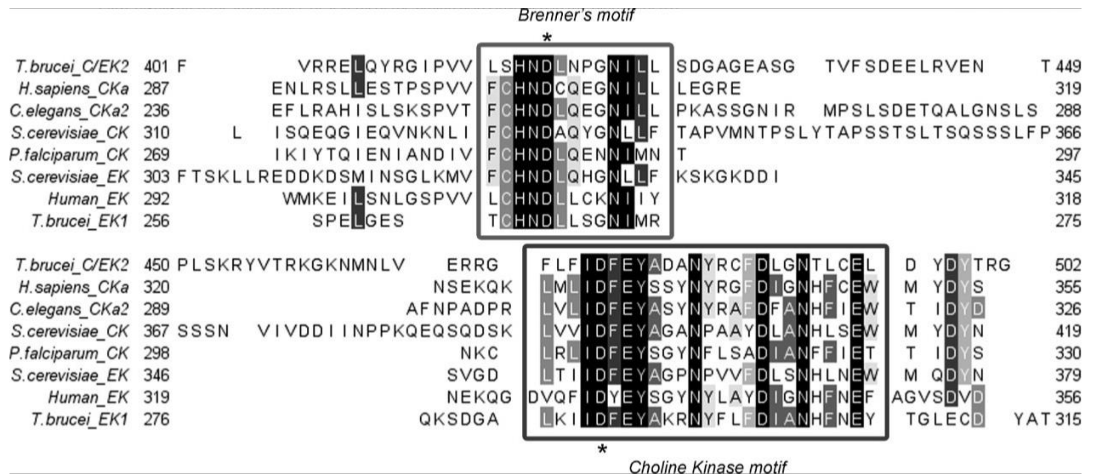

Figure 2.4 Sequence alignment of the region containing the Brenner's and the choline kinase motifs of previously characterized C/EKs. *H. sapiens* (CKα, P35790 and EK, Q9HBU6), *C. elegans* (CKα2, Q22942), *S. cerevisiae* (CK, P20485 and EK, Q03764), and *T. brucei* (EK1, AM939568 and CEK2, AM939569). The boxes highlight the choline kinase motif and Brenner's phosphotransferase motif. The two Asp residues marked by an asterisk when mutated generate catalytically inactive mutants. Residues are shaded according to identity and similarity (Khalifa et al., 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 List of chemicals

Chemicals used for this study are listed in Table 3.1.

3.1.2 List of kits and consumables

Kits and consumables used in this study are listed in Table 3.2.

3.1.3 List of equipment

Equipment used in this study is listed in Table 3.3.

3.1.4 List of buffers and reagents

Buffers and reagents used in the present study are listed in Table 3.4.

3.1.5 List of primers

Primers used in this study are listed in Table 3.5.

3.1.6 Escherichia coli Strain

The *E. coli* strains used is XL10-Blue

3.1.7 List of plasmids used

The plasmid construct was generated by our research group by cloning the *licA* gene encoding *Neisseria gonorrhea* choline kinase (NmCK) gene into the *Nde*I and *BamH*I restriction sites of pET-14b plasmid (Novagen).

3.1.8 List of software used in this study

The software used in this study are listed in Table 3.6

Table 3.1 List of chemicals

Name	Manufacturer	Place of origin
Agarose	Vivantis	Selangor, Malaysia
*Bam*HI-HF (20000U/mL)	New England Biolabs	USA
Polyethylene glycol 8000	Sigma-Aldrich	USA
Glycerol	Sigma-Aldrich	USA
$MgSO_4 \cdot 7H_2O$	BDH Chemicals	UK
(0.2% w/v) Glucose	1st base	Selangor, Malaysia
LB broth	Miller	Darmstadt, Germany
LB agar	Miller	Darmstadt, Germany
*Nde*I (20000U/mL)	New England Biolabs	USA

Table 3.2 List of kits and consumables

Name	Manufacturer	Place of origin
QIAquick Gel Extraction kit	Qiagen	Hilden, Germany
FavorPrep Plasmid Extraction Mini Kit	Favorgen	Austria
Disposable cuvette (Uvette)	Eppendorf	Germany
Microcentrifuge tube (1.5mL)	Axygen	USA
Micropipette tips	Bioseen	Malaysia
Latex examination gloves	Ansell Healthcare	USA
Syringe	Becton Dickinson	USA

Table 3.3 List of equipment

Name	Manufacturer	Place of origin
Micropipette	Gilson	Wisconsin, USA
pH meter	Mettler Toledo	Greifensee, Switzerland
Deep freezer (-80℃)	Ilshin	Seoul, Korea
Freezer (-20℃)	Leisherr	Germany
Refrigerator (4℃)	Sharp	Japan
Elite 200 power supply	Wealtec Bioscience Co.,Ltd	United States
Gel electrophoresis tank	Wealtec Bioscience Co.,Ltd	United States
Microwave oven	Samsung	Seoul, Korea
pH meter	Mettler Toledo	Greinfensee, Switzerland
Thermal cycle	Biorad	USA
UV-transilluminator	Spectroline	Westbury, New York
Vortex mixer	Erla	Selangor, Malaysia
Biophotometer plus	Eppendorf	Hamburg, Germany
Analytical balance	Mettler Toledo	Greifensee, Switzerland
Incubator+Shaker	Thermo Fisher Scientific	USA

Table 3.4 List of buffers and reagents

Name	Manufacturer	Place of origin
TAE buffer (10x)	Promega	USA
6x DNA loading dye	New England Biolabs	USA
1 kb DNA ladder	New England Biolabs	USA
100bp DNA ladder	1st base	Selangor, Malaysia
Flurosafe DNA stain	1st base	Selangor, Malaysia
DreamTaq buffer	Thermo Fisher Scientific	USA
dNTP Mix	Thermo Fisher Scientific	USA
25 mM $MgCl_2$	Fermentas	USA
DreamTaq buffer	Thermo Fisher Scientific	USA

Table 3.5 List of primers

Name	Sequence	Place of origin
pET-14b T7 forward primer	5’-TAA TAC GAC TCA CTA TAG G-3’	IDT, Malaysia
pET-14b T7 reverse primer	5’-CTA GTT ATT GCT CAG CGG-3’	IDT, Malaysia
D153A NmCK-F	5’-CAT GTC ACA ATG CGC TCG TTC CGG AG-3’	IDT, Malaysia
D153A NmCK-R	5’-CTC CGG AAC GAG CGC ATT GTG ACA TG-3’	IDT, Malaysia
D170A NmCK-F	5’-CTT TCC TTT ATT GCG TGG GAA TAC AG -3’	IDT, Malaysia
D170A NmCK-R	5’-CTG TAT TCC CAC GCA ATA AAG AAA AG-3’	IDT, Malaysia

Table 3.6 List of software

Name	Purpose	Webpage
Webcutter 2.0	Determination of restriction sites	http://heimanlab.com/cut2.html
SWISS-MODEL	Molecular modelling and comparison of model structures	https://swissmodel.expasy.org
PyMOL	Protein 3D structure superimpositio n	https://pymol.org/2/
Clustal Omega	Multiple sequence alignment	https://www.ebi.ac.uk/Tools/msa/clustalo/
NCBI	Gene sequence search	https://www.ncbi.nlm.nih.gov
Blastp	Sequence confirmation	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Pr oteins

3.2 Methods

3.2.1 Preparation of *E. coli* competent cells

A 0.5 ml volume of an overnight *E. coli* culture in LB medium was inoculated in 50 ml of solution A (10 mM $MgSO_4$, 0.2% (w/v) glucose in LB-medium) and incubated at 37°C with shaking until optical density of 0.3 to 0.5 at 600 nm was reached. The cells were kept on ice for 10 minutes and pelleted by centrifugation at 1500x g for 10 minutes at 4°C. The cells were kept on ice in all the subsequent steps. A 0.5 ml volume of solution A (precooled to 4°C) was used to gently resuspend the cells and 2.5 ml of precooled solution B (36% (v/v) glycerol, 12% (w/v) polyethylene glycol 8000, 12 mM $MgSO_4$ in LB-medium and filter sterilized) were added and gently mixed without vortexing. The cell suspension was divided into 100 µl fractions in 1.5 ml microtubes and stored at –80°C.

3.2.2 Transformation of NmCK plasmid DNA into *E. coli* cells

The competent cells prepared from previous step were thawed on ice for about 10 minutes. Ten microliter of NmCK plasmid construct, pET-14b-NmCK, (149.7 ng/µL) were added to the cell suspension and mixed gently. The cells were incubated on ice for about 20 minutes before being subjected to heat shock at 42°C in water bath for 1 minute. The cells were cooled on ice for 1 to 2 minutes and 900 µl of LB medium were added. The cell suspension was incubated at 37°C with shaking for 1 hour. A 100 µl volume of the cell suspension were spread on LB agar plate with selective antibiotic and incubated at 37°C overnight.

3.2.3 Determination of transformation efficiency

Transformation efficiency (TE) refers to the number of colony-forming units (cfu) that can be produced by transforming 1µg of plasmid into given volume of competent cells. The equation used to calculate TE is shown below:

$$\frac{\text{Number of Colonies on Plate (df)}}{\text{Amount of DNA plated (ng)}} \quad \text{X } 1000 \text{ ng/µg}$$

3.2.4 Purification of plasmid DNA

Plasmid DNA extraction and purification was done using FavorPrep™ Plasmid Extraction Mini Kit. 35ml of well-grown bacteria culture was transferred to a 1.5ml microcentrifuge tube and centrifuged at 11,000 x g for 1 minute to pellet the cells and supernatant was discarded completely. 200µL of FADP1 Buffer (RNase A added) was added to cell pellet and resuspended completely through pipetting. 200µL of FADP2 Buffer was then added and gently inverted for 7 times and incubated at room temperature 3 minutes to lyse the cells. 300µL of FADP3 Buffer was then added and gently inverted for 7 times immediately after 3 minutes to neutralize the lysate. After that, sample was centrifuged at full speed (~18,000xg) for 5 min to clarify the lysate. At the same time, FADP column was placed in a collection tube provided. After 5 minutes of centrifugation, supernatant was transferred to the FADP column and centrifuged at 11,000x g for 30 seconds. Flow-through was discarded and column was placed back to the collection tube. 400µL of WP Buffer was then added to the FADP column and centrifuged at 11,000 x g for 30 seconds. Flow-through was then discarded and column was placed back to the collection tube again. 700µL of Wash buffer

(96% ethanol added) was added to the FADP column and centrifuged at 11,000 x g for 30 seconds. Flow-through was discarded and column was placed back to the collection tube. Additionally, sample was centrifuged at full speed (~18,000xg) for 3 minutes to dry the FADP column. FADP column was placed on a new 1.5ml microcentrifuge tube. 75μL of elution buffer was added to the membrane center of the FADP column and let it stand for 1 minute. Finally, sample was centrifuged at full speed (~18,000 x g) for 1 minute to elute the pET-14b-NmCK plasmid and stored at -20℃. 1μL of purified pET-14b-NmCK plasmid was diluted in 49μL to be measured for its concentration.

3.2.5 Restriction digestion of DNA (for plasmid and PCR product)

Restriction digestion of DNA in this research was used for different purposes. Firstly, restriction digestion using suitable restriction enzyme (RE) was used to screen for positive clone. Secondly, RE was used to obtain linearized pET-14b backbone and NmCK gene separately. RE candidates were chosen by using Webcutter 2.0 under the website http://heimanlab.com/cut2.html. The chosen candidates for digesting the pET-14b-NmCK enzyme are *BamH*I and *Nde*I.

Firstly, digestion reaction sample was prepared to confirm the plasmid obtained from the colony is positive which includes 1μg of purified pET-14b-NmCK, 1 μL of *Bam*HI, 1μL of *Nde*I, 5 μL of Cutsmart® buffer and 39 μL of distilled water which was loaded into a 1.5ml microcentrifuge tube and incubated at 37℃ overnight.

Secondly, a total of 80 μL digestion reaction sample was prepared to confirm the plasmid obtained the pET-14b backbone which includes 14.99μg of purified pET-14b-NmCK, 1 μL of *Bam*HI, 1μL of *Nde*I, 8 μL of Cutsmart buffer and 25 μL of distilled

water which was loaded into a 1.5ml microcentrifuge tube and incubated at 37℃ overnight.

3.2.6 Agarose gel electrophoresis

Agarose gel [1%(w/v)] was prepared to analyze the results of positive colony screening, linearization of pET-14b backbone and PCR products from 2 step PCR site-directed mutagenesis.

1g of agarose was mixed with 100ml of 1x TAE buffer and boiled in a microwave oven and cooled down to around 50℃ in room temperature until solidified. 1.5μL of Flurosafe DNA was added and mixed by swirling. DNA ladders used include Gene Ruler 1kb DNA ladder and Quick-Load 100bp DNA ladder. TAE buffer was filled in the gel electrophoresis tank before performing agarose gel electrophoresis. For positive clone screening purpose, samples with mass between 2 to 20μL was added with 2μL 6X gel loading dye buffer before loading to sample wells. For linearizing pET-14b backbone, samples with mass 45μL was added with 8μL 6X gel loading dye buffer before loading to sample wells. For PCR products, samples with volume 5μL was added with 1μL gel loading dye buffer before loading to sample wells. Gel electrophoresis was run under 100V and 2A for 35 minutes after loading all the samples into the gel. The gel was then visualized using UV-transilluminator.

3.2.7 Gel extraction and purification of plasmid DNA

After performing agarose gel electrophoresis for linearization of pET-14b backbone and PCR products. Gel extraction and purification is performed for further ligation purpose by using QIAquick Gel Extraction Kit (Qiagen) for linearized pET-14b plasmid backbone and full length D153A NmCK gene. The gel was viewed under UV transilluminator and the band of interest was excised using a clean scalpel. The weight of each DNA gel fragments was determined in a 1.5 mL microcentrifuge tube. 3 volumes of buffer QG was added for 1 volume of gel slice by assuming 100mg equals to 100µL for the rest of the protocol. The samples were then incubated at 50℃ for 10 minutes and vortexed every 2 to 3 minutes during incubation. After dissolved, 1 volume of isopropanol was added to 1 volume of gel slice and mixed. QIAquick spin column was placed in a provided 2ml collection tube. The sample was applied onto the QIAquick spin column and centrifuged at full speed for 1min. Flow-through was discarded and QIAquick spin column was placed back on the same column. 0.5ml of Buffer QG was added onto the QIAquick spin column and centrifuged at full speed for 1min. Flow-through was discarded and QIAquick spin column was placed back on the same column again. 0.75 mL of Buffer PE was added to QIAquick spin column and centrifuged at full speed for 1 min to wash. Flow-through was discarded and QIAquick spin column was placed back on the same column again then centrifuged at full speed for 1 minute. QIAquick spin column is then placed on top of a new 1.5ml microcentrifuge tube. 50µL of Buffer EB (10mM Tris·Cl, pH 8.5) was added onto the center of the QIAQuick membrane and the column was centrifuged for 1 minute at maximum speed. The sample in the 1.5ml microcentrifuge tube was stored at -20℃.

3.2.8 Two-step PCR site directed mutagenesis

3.2.8(a) Preparation of PCR mixture

In the first round of polymerase chain reaction (PCR), two separate PCR were carried out to amplify the mutated left and right halves of the *licA* gene. In the first reaction tube, pET-14b universal forward primer was combined with the reverse mutagenesis primer to generate the left half fragment. In the second reaction tube, forward mutagenesis primer was combined with pET-14b universal reverse primer to generate the right-half fragment. A second round of PCR was run to combine the left and right fragments by using the pET-14b universal forward and reverse primers and the PCR products (left and right fragments obtained above) as template. The two PCR fragments were able to form the full length *licA* gene containing the desired mutation due to overlapping region introduced into the mutagenesis primers.

The PCR mixture contains 5µL of DreamTaq PCR buffer, 1 µL of dNTPs, 1 µL of Taq polymerase, 3 µL of $MgCl_2$ and 35.5 µL of distilled water, 1.25µL of each primer (10 pmol/µl), 2 µL of original pET-14b-NmCK plasmid as template (200 ng/µl), 1 µL of dNTPs (10 mM), 3 µL of $MgCl_2$ (25 mM) and 35.5 µL of distilled water to a total volume of 50 µl. The PCR cycling protocol is described in section 3.2.8.2 below.

A positive control of PCR mixture was prepared by combining 1.25µL of pET-14b universal forward and reverse primer each, 5µL of dreamTaq PCR buffer, 1 µL of dNTPs, 1 µL of Taq polymerase, 2 µL of original pET-14b-NmCK plasmid as template, 3 µL of $MgCl_2$ and 35.5 µL of distilled water. A negative control of PCR mixture was

prepared without the template which is combined with 1.25µL of pET-14b universal forward and reverse primer each,

3.2.8(b) PCR cycling protocol

All rounds of PCR were following the 3-step PCR protocol using the BioRad thermal cycler. For the first step, the PCR mixtures were run at 94℃ for 10 minutes. For the second step, it was run at 94℃ for 1 minute, 55℃ for 1 minute and 72℃ for 1 minute, all in 35 cycles manner. Lastly, the mixture was run at 72℃ for 5 minutes. This protocol applied to both the generation of left and right-hand products, and the combination PCR product.

3.2.9 Molecular modelling and comparison between wildtype and mutants model structures

SWISS-MODEL was used to determine the model of wildtype and mutant model and comparison was made between them. The template used is pneumococcal *lic*A in complex with choline (Protein data bank number: 4r7b.1). 3D superimposition between two proteins were done using TM Align.