CHARACTERISATION OF *bla*-TEM PROMOTER SEQUENCE FROM *Enterobacter cloacae*

KIN ZI XIAN

BACHELOR OF SCIENCE (HONS) BIOTECHNOLOGY

FACULTY OF SCIENCE

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By

KIN ZI XIAN

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ABSTRACT

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KIN ZI XIAN

Extended-spectrum beta-lactamases (ESBLs) are a group of rapidly evolving enzymes that contribute to the growing number of disease outbreaks globally. ESBL-producers pose challenging disease control issues as the choice for antimicrobial treatment is often limited due to their multi-resistance to many antibiotics. The purpose of this study was to amplify and characterise the *bla*-TEM gene from *Enterobacter cloacae*. PCR amplification was performed using *bla*-TEM specific primers and a DNA fragment with expected size of 870 bp was observed. Based on BlastX alignment search, the translated DNA sequence was identical to the hypothetical protein from *Clostridium nexile*, which carries the coding region for beta-lactamase enzymes. Subsequently, genome walking methodology was used to characterise the unknown sequences flanking the *bla*-TEM gene. The *Eco*RV gene library was subjected to nested PCR amplification using gene specific primers and adaptor primers. Amplification of upstream DNA fragments in both attempts yielded four distinct DNA sequences of various sizes, which were designated as GW1, GW2, GW3 and GW4. Sequence analysis showed that only sequence GW4 was able to overlap and join the *bla*-TEM gene, extending beyond the primer annealing site. The *bla*-TEM

upstream sequence of GW4 consists of 1076 bases in length. An open reading frame of 861 bp corresponded to the *bla*-TEM gene was predicted and was further verified as TEM-116. Apart from the Shine-Dalgarno sequence, the -35 and -10 promoter regions were also predicted in the upstream sequence analysis. The putative promoter regions were identical to the promoter *P3*, corresponding to a relatively weaker promoter activity. The downstream DNA fragments were not characterised due to non-specific PCR amplification. In future, more gene libraries will be constructed and DNA sequences of GW4 can be subjected to two-pass sequencing to obtain a longer and more reliable sequences for detailed analysis.

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- Last but not least, I would like to thank my friends and family for their moral support and assistance in any aspect during completion of this project.

DECLARATION

I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

Name: KIN ZI XIAN

APPROVAL SHEET

This project report entitled "**CHARACTERISATION OF *bla*-TEM PROMOTER SEQUENCE FROM *Enterobacter cloacae"*** was prepared by KIN ZI XIAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. CHOO QUOK CHEONG) Date: ……..

Supervisor

Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **KIN ZI XIAN** (ID No: **09ADB03351**) has completed this final year project entitled **"CHARACTERISATION OF *bla*-TEM PROMOTER SEQUENCE FROM *Enterobacter cloacae*"** under supervision of Dr. Choo Quok Cheong from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I do not prepare it within six (6) months time from this date provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

_____

(KIN ZI XIAN)

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LIST OF ABBREVIATIONS

A	Absorbance
BDGP	Berkeley Drosophila Genome Project
Blast	Basic Local Alignment Search Tool
bp	Base pair
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra-acetate
ESBLs	Extended-spectrum beta-lactamases
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-bertani
$MgCl_2$	Magnesium chloride
nif	Nitrogen fixation gene
NNPP	Neural Network Promoter Prediction
OD	Optical density
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Shine-Dalgarno sequence

spp.	Species
TDNN	Time-delay neural network
TE	Tris-EDTA
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Eating raw and minimally processed vegetables is an essential part of the diet for most of the people around the world, especially for the vegetarians and health-conscious consumers. From 1988 to 1996, yearly consumption of raw fruits and vegetables in United States increased by almost 20 pounds per person (Buck et al., 2003). Nevertheless, numerous disease outbreaks caused by viruses, bacteria and parasites have been linked epidemiologically to consumption of raw fruits and vegetables. According to a risk assessment done by Centre for Food Safety (2006), the disease outbreaks associated with the consumption of raw vegetables had been on the rise in many developed countries over the last few years. Data on foodborne disease outbreaks from 2009 to 2010 from Centers for Disease Control and Prevention (2011) reported 1527 foodborne outbreak diseases, resulting in 1184 hospitalizations, 29444 illness cases and 23 death cases.

Pathogens can contaminate raw vegetables in several ways. These include application of wastewater and improper composted manures to soils in which vegetables are grown. Improper handling during harvesting and storage process also encourages harbouring of bacteria on vegetables. *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter* are some of the pathogens associated with fruits and vegetables, leading to numerous outbreaks of foodborne illness (World Health Organization 2013). The foodborne illness became more widespread with

bacteria acquiring resistance to pesticides or antibiotics normally used in agriculture field.

The global emergence of antibiotic-resistant bacteria is due to the increased and continuous usage of antibiotics by human being. These antibiotic-resistant pathogens are more difficult to be detected by routine laboratory assays. Hence, this will cause delay in diagnosis and application of suitable antimicrobial treatment (Dhillon and Clark 2011). There is also growing concern for the lack of new antibiotics, especially for the antibiotic-resistant Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) (Boucher et al., 2009). The ESBL-producing organisms can confer resistance to beta-lactam antibiotics, such as penicillins and cephalosporins. There is limitation of treatment options as these organisms also exhibit co-resistance to many other classes of antibiotics (Rawat and Nair 2010). Carbapenems are the choice of treatment for the infection caused by ESBL-producing organisms. However, the carbapenem-resistant isolates which produce carbapenemases were first detected in the United State in 2010 (Minnesota Department of Health 2011).

The ESBLs have various genotypes, with the most common being SHV, TEM, and CTX-M. Sequencing of *bla*-TEM genes in Enterobacteriaceae revealed several types of *bla*-TEM genes (Tristram et al., 2005). Genetic context of *bla*-TEM genes will be useful in understanding the antibiotic mechanisms of these antibiotic resistance organisms. The research in the past few years revealed that the *bla*-TEM gene was present in the plasmids, either in the complete transposon or part of TnA

transposon that included the gene (Bailey et al., 2011). TnA usually appears to be truncated by IS26 among the partial forms (Wain et al., 2003; Cain et al., 2010). Another recent report by Cobos et al. (2013) showed that the TnA transposons of beta-lactamase TEM-1 and TEM-182 were inserted between *tfc20* and *tfc21* genes after analyzing the flanking regions of *bla*-TEM genes in selected isolates. These genes are typically correlated with conjugative and integrative elements in *Haemophilus* spp., which are the origins of the isolates. Expression of *bla*-TEM genes are also observed to be controlled by four different promoters *P3*, *Pa/Pb*, *P4* and *P5* found upstream of corresponding genes. Both TEM-1 and TEM-30 were shown to have increased activity with the presence of promoters *P3*, *Pa/Pb* and *P4* at their upstream regions (Lartigue et al., 2002).

Hence, genetic characterisation of ESBL gene is an effective mean to identify and study the gene. Genome walking methodology can be used to characterise the unknown sequences flanking the gene. The objectives of this study were:

- To amplify the *bla*-TEM gene from *Enterobacter cloacae*,
- To characterise the PCR-amplified *bla*-TEM gene fragment using cloning and sequencing methodology,
- To determine the unknown sequences flanking the *bla*-TEM gene using genome walking methodology.

CHAPTER 2

LITERATURE REVIEW

2.1 Consumption of Raw Vegetables

Raw vegetables refer to vegetables which have not been cooked before consumption. Vegetables are rich in vitamins, minerals and dietary fibres and essential for good health. Cohort studies have shown the inverse relationship between high consumption of fruit and vegetables with coronary heart disease (He et al., 2007). Rich sources of vitamins, fibers, polyphenols and other bioactive phytochemicals in fruits and vegetables may lead to lower coronary heart disease risk. Cooked vegetables will lose their water-soluble and heat-sensitive bioactive compounds (Danesi and Bordoni 2008; Ruiz et al., 2008). Some of the beneficial enzymes will be destructed in this cooking process.

Therefore, raw vegetables are beginning to be favoured by more consumers. Based on the statistical report by Organic Trade Association (2011), sales of organic food and beverages in 2010 showed 7.7 percent growth over sales in 2009, with fresh fruits and vegetables leading all the way. Nevertheless, consumption of raw vegetables is associated with foodborne illness. A variety of bacterial pathogens, parasites and enteric viruses may reside on the fresh vegetables (Centre for Food Safety 2006).

2.2 The Emergence of Foodborne Illness

Foodborne illness refers to illness resulted from the consumption of contaminated food. The resultant symptoms include nausea, diarrhea, fever and vomiting. Raw vegetables have high potential to serve as vehicles for human diseases. Based on recent outbreak data from Center for Science in the Public Interest in America between 2001 and 2010, fresh produce was the top category responsible for foodborne illness, attributing to 696 outbreaks and 25,222 illnesses (DeWaal and Glassman 2013).

Bacteria capable of causing disease such as *Bacillus cereus*, *Clostridium botulinum* and *Listeria monocytogenes* are normal soil inhabitants (World Health Organization 2013). On the contrary, intestinal tracts of animals harbour bacteria such as *Shigella*, *Salmonella*, *Campylobacter* and *Escherichia coli* which tend to contaminate raw vegetables through animal manure, sewage or improperly treated irrigation water. Vegetables are more likely to be contaminated when they grow on the soil which is fertilized with improperly treated animal waste or irrigated with contaminated water. Unhygienic processing, preservation and marketing practices may also contribute to the contamination. This results in foodborne illness when the contaminated raw greens are consumed as there is no high temperature during cooking process to destruct the harmful pathogens.

Outbreaks of foodborne illness due to raw fruits and vegetables often occur in developing countries due to poor processing and handling practices. However, disease outbreaks associated with consumption of raw fruits and vegetables have

increased in some industrialized countries due to increased food import and changes in dietary habits (Altekruse et al., 1997). In United States, the number of foodborne illness per year which was related with consumption of raw vegetables and fruits had doubled during past two decades prior to 1999 (Fan et al., 2009). High-quality raw vegetables can be supplied to consumers all around the world with the advances in agronomic practices, processing and marketing strategies. Hence, these practices help to expand the geographical distribution and occurrence of foodborne illness associated with contaminated fresh produce (World Health Organization 2013).

2.2.1 Enterobacteriaceae

Members of Enterobacteriaceae family are Gram-negative rods and facultative anaerobes that can ferment glucose, often with gas formation. Examples of familiar pathogens are *Escherichia coli*, *Salmonella*, *Klebsiella* and *Shigella*. The name of this family is derived from enterobacterium, which means intestinal bacterium (Lund et al., 1999). Others than human and animal intestines, they are also found in soil, water and plants. They can be found among flora associated with growing vegetables. Hence, raw vegetables are susceptible to this family of bacteria and cause illness to the consumers.

Urinary tract infection is the most common infection caused by Enterobacteriaceae. They also synthesize exotoxins which can lead to diarrhea and body fluid loss (Falagas and Karageorgopoulos 2009). According to Centers for Disease Control

and Prevention (2011), Enterobacteriaceae was responsible for most foodborne outbreaks from 2009 to 2010. *Salmonella* contributed to the highest percentage of outbreak-related hospitalizations (49%), followed by Shiga toxin-producing *Escherichia coli* (16%). The groups of bacteria which are often found on growing vegetables are coliforms or faecal coliforms such as *Klebsiella* and *Enterobacter* (Duncan and Razzell 1972; Splittstoesser et al., 1980; Zhao et al., 1997).

2.2.2 *Enterobacter cloacae*

The genus *Enterobacter* from Enterobacteriaceae family was recognized in 1970s, with *Enterobacter cloacae* and *Enterobacter aerogenes* as the established species. It is the most common genera that can be found on vegetable samples, especially *Enterobacter cloacae* (Rico et al., 2003). This species is a Gram-negative rod and uses peritrichous flagella for movement. Although *Enterobacter* species primarily are not human pathogens, *Enterobacter cloacae* has been associated with broad range of clinical syndromes (Haryani et al., 2008). It causes various infections such as urinary tract, lower respiratory tract, wounds and all manner of other nastiness. It is also isolated from the small intestine of patient with acute diarrhea. The occurrence of *Enterobacter cloacae* in human infections is greater than that of *Enterobacter aerogenes* (Lund et al., 1999). Hence, this species of bacteria is viewed as potential agents of foodborne disease.

Paterson et al. (2005) showed that *Enterobacter cloacae* was the most common species isolated in a antimicrobial susceptibility testing of *Enterobacter* species

worldwide. *Enterobacter cloacae* has intrinsic resistance to ampicillin and narrow-spectrum cephalosporins. It also gains resistance to expanded-spectrum cephalosporins through a high frequency of mutation (Sanders and Sanders 1983; Then 1987). Hence, treatment of *Enterobacter cloacae* infections becomes complicated due to the emergence of multi-resistant strains.

2.3 Antibiotics

Antibiotics, synonymously known as antibacterials, are agents that are produced by microorganisms to destruct or slow down the growth of other microorganisms (Nordqvist 2009). Antibiotics were first discovered by Alexander Fleming in 1928 when he noticed that the bacterial growth was affected by penicillin produced by a mold, *Penicillium notatum*. More than 100 different types of antibiotics have been recognized nowadays and applied by doctor to cure mild to severe infections (Johnson 2012).

Antibiotics kill microorganisms such as bacteria, fungi and parasites. However, they are ineffective against viruses. Antibiotics can be rather large and complex organic molecules (Todar 2012). Hence, they may need as much as 30 separate enzymatic steps for synthesis. Despite of the action by other antibiotics, most of the microorganisms are not affected by their own antibiotics. Their antibiotics can work on closely-related strains.

Antibiotics can be classified in several ways. Based on type of activity, antibiotics are grouped as bacteriostatic if they inhibit bacterial growth reversibly and bactericidal if they kill bacteria directly (Mayer 2010). Antibiotics also can be categorized as narrow or broad spectrum. Narrow spectrum antibiotics work on specific types of bacteria. On the other hand, broad spectrum antibiotics act on structures or processes which are common to wide range of bacteria. Furthermore, antibiotics also can be classified based on chemical structure, where antibiotics within a structural class share same patterns of effectiveness and toxicity generally. The main classes of antibiotics are beta-lactams, tetracylines, fluoroquinolones, macrolides and aminoglycosides. Each class consists of multiple drugs and each drug is specific in some way.

2.3.1 Beta-Lactam Antibiotics

One of the world's major biotechnology markets is represented by beta-lactam antibiotics, especially penicillins and cephalosporins (Chandel et al., 2008). Beta-lactam compounds comprised more than half of all commercially available antibiotics when measured by sales up to 2003 (Elander 2003). They share similar feature, beta-lactam ring, in their structures. This antibiotic covers a large category, including penicillin, cephalosporin, carbapenem and monobactam.

Actions of beta-lactam antibiotics are bactericidal. They interfere with bacterial growth by inactivating penicillin-binding proteins (PBPs) which are involved in final stages of peptidoglycan biosynthesis (Spratt and Cromie 1988). The

peptidoglycan layer is important for integrity of structural cell wall while PBPs are transpeptidase enzymes which catalyze the cross-linking of the peptidoglycan. Beta-lactam antibiotics exert their antibacterial effect by binding to PBPs and hence disrupting cell wall synthesis. This will cause the cell pressure to rise considerably until the cell is lysed and destroyed. Hence, this antibiotic can safely disrupt biosynthesis of bacterial cell wall without affecting existing body cells in animals as animal cells do not have cell walls.

2.3.1.1 Ampicillins

Ampicillin is an antibiotic which belongs to penicillin group of beta-lactam drugs and has been widely used to cure bacterial infections since 1961. It is an aminopenicillin which has a broad spectrum of activity compared to natural penicillin (Wenzler et al., 2003). Ampicillin affects Gram-positive organisms such as streptococci and staphylococci. It is also effective against some Gram-negative microbes such as coliforms, *H. influenza* and *Proteus* spp.

The presence of an amino group in molecular structure of ampicillin helps to distinguish it from penicillin. This amino group helps ampicillin to penetrate the outer membrane of Gram-negative bacteria. Ampicillin exerts its antibacterial effect by acting as competitive inhibitor of enzyme transpeptidase, which is needed for cell wall synthesis. Besides, ampicillin also inhibits chloroplasts division of the glaucophytes and moss *Physcomitrella patens* (Kasten and Reski

1997). In research field, ampicillin resistance gene is widely used as selectable marker in molecular cloning to verify insert of desired gene into the plasmid.

2.4 Evolution of Antibiotic Resistance

Although antibiotics have revolutionized medicine in many aspects, the use of these drugs has led to the emergence of resistant strains. Antibiotic resistance occurs when the microbes alter in some way that lessens or eliminates effectiveness of drugs used to cure the infections. Any effective therapeutic agent is always compromised by the potential development of resistance to that particular compound since it is being applied (Davies and Davies 2010). Data from European Centre for Disease Prevention and Control (2012) demonstrates that there is increasing trend of multi-resistance to many antibiotics in both *Klebsiella pneumonia* and *Escherichia coli* in most European countries. The emergence of antibiotic resistant strains is due to misuse and overuse of antibiotics, expanding from therapeutic cure to large-scale farming of animals. Wenzel and Edmond (2000) stated that 23 million kg of antibiotics are used annually in the United States, where 50% is used in medical field and 50% is used in farming, animals and aquaculture.

Bacteria may exhibit resistance to antibiotics through several mechanisms. First, bacteria can acquire gene encoding enzyme that inactivates the antibiotic before it can do harm. Second, bacteria may develop efflux pump that pumps out the antibiotics before reaching target site. Thirdly, bacteria can acquire several genes

for metabolic pathway which synthesizes altered bacterial cell walls that lack of binding site for antibiotics or acquire mutations that block antibiotics from intracellular target site via down regulation of porin genes (Tenover 2006).

2.4.1 Beta-Lactamase and the Extended-Spectrum Beta-Lactamase

Since beta-lactam antibiotics have long history in the treatment of bacterial infections, their usage is accompanied by the development of resistance in target organisms. Beta-lactamases, especially in Gram-negative microbes, are major determinant for development of this resistance (Poole 2004). Beta-lactamases are the most common single cause of bacterial resistance to beta-lactam antibiotics (Livermore 1995). Beta-lactamases are enzymes that cleave the beta-lactam ring in the structure, and thus deactivating the compound's antibacterial effects. Genes coding for these enzymes can be carried on bacterial chromosomes, which are inherent to the organisms, or can be plasmid-mediated with the potential to be transferred between bacterial population (Dhillon and Clark 2011).

When the bacteria conferred resistance to one type of beta-lactam antibiotic, new derivatives of antibiotics were developed by researchers and were named cephalosporins, carbapenems and monobactams (Mulvey 2006). However, the bacteria continued to evolve and alter existing beta-lactamase enzymes to break down the new compounds. The enzymes capable of hydrolyzing newer derivatives are called extended-spectrum beta-lactamases (ESBLs) and were first observed in early 1980's (Mulvey 2006).

ESBLs are beta-lactamases which confer bacterial resistance to the penicillins; first-, second- and third-generation cephalosporins; and aztreonam (except cephamycins or carbapenems) by hydrolysis of these antibiotics (Paterson and Bonomo 2005). These enzymes are inhibited by beta-lactamase inhibitors such as clavulanic acids. ESBLs can be found in a variety of Enterobacteriaceae species, particularly *Klebsiella pneumonia, Klebsiella oxytoca* and *Escherichia coli*. They can also be produced by *Pseudomonas aeruginosa* and other Enterobacteriaceae strains such as *Proteus, Enterobacter, Citrobacter, Serratia marsescens, Morganella morganii, Burkholderia cepacia* and *Capnocytophaga ochracea* (Bradford 2001; Thomson 2001).

There are various genotypes of ESBLs, with the most common are the SHV, TEM and CTX-M types (Dhillon and Clark 2011). Since ESBL enzymes are plasmid-mediated, the genes coding for the enzymes can easily move between different bacteria. Other than the genes coding for ESBL enzymes, most plasmids also carry genes that confer bacterial resistance to several non-beta-lactam antibiotics. Infections caused by ESBL-producing organisms have higher morbidity, mortality and greater fiscal burden. Hence, they contribute to serious global public health issues.

2.4.2 TEM-Type ESBLs

TEM-1 is the beta-lactamase which is most commonly found in Gram-negative bacteria. It was first discovered in Greece in the 1960s and was named TEM after

the patient, Temoniera, from whom it was isolated (Bradford 2001). Native TEM-1 beta-lactamase confers resistance to ampicillin, penicillin and first-generation cephalosporin such as cephalothin. Up to 90% of ampicillin resistance in *Escherichia coli* is due to synthesis of TEM-1 (Livermore 1995). Subsequently, TEM-2, the first variant described, is distinguished from TEM-1 by the substitution of a lysine for a glutamine at position 39 (Matthew and Hedges 1976). TEM-2 has similar substrate profile as TEM-1 and hence is not considered an ESBL. TEM-3 is the first TEM-type beta-lactamase with ESBL phenotype that was originally reported in 1989 (Sougakoff et al., 1988). Since that first report, more than 90 additional TEM derivatives have been described. Although majority of new derivatives are ESBLs, some beta-lactamases are inhibitor-resistant enzymes.

Each TEM-derived ESBL has a slightly different substrate profile, where one ESBL will hydrolyze a specific extended-spectrum cephalosporin more effectively than another ESBL (Rupp and Fey 2003). The subtle differences in substrate profile of many ESBLs cannot be used to differentiate between enzymes. Instead, analysis of the amino acid sequence is needed for discrimination.

2.4.3 The *bla* Gene and *bla*-TEM Gene

The *bla* gene codes for enzyme beta-lactamase and confers bacterial resistance to ampicillin. Hence, it is also known as *amp* (Clark 2005). The *bla* gene conferring such resistance can be found on bacterial chromosome or plasmid (Roy et al., 1984;

Simpson et al., 1986). The *bla* gene encoding TEM-1 beta-lactamase is the most common ampicillin resistance marker being applied in molecular biology. It is usually used as reporter gene to provide resistance to beta-lactam antibiotics to the transformed bacteria.

Most ESBLs are derived from TEM and SHV beta-lactamases through point mutations within the *bla*-TEM and *bla*-SHV genes, giving rise to extended-spectrum antibiotic resistance (Bush et al., 1995; Bradford 2001). The structural genes for TEM-1 penicillinases are named *bla*-TEM-1a and *bla*-TEM-1b while the structural gene for TEM-2 is designated as *bla*-TEM-2 (Goussard and Courvalin 1991). The *bla*-TEM gene nomenclature was proposed by Goussard and Courvalin on the basis of the sequences of structural *bla*-TEM genes and their promoters.

2.5 Genetic Characterisation and Polymerase Chain Reaction (PCR)-based Genome Walking Methodology

Characterisation refers to description of quality or character of an individual. In terms of genetics, it refers to the detection of variation due to differences in either DNA sequences or specific genes or modifying factors (Vicente et al., 2005). It also involves description of attributes that involve specific DNA sequences. Genetic characterisation with molecular techniques can reveal differences in genotypes and hence has higher efficiency in detection of genes than phenotypic approaches. Isolating and determining the nucleotide sequences flanking the known regions is part of the studies of genetic characterisation. It may be useful in

identification of regulatory sequences outside complementary DNA (cDNA) coding regions and gaps in genome sequencing projects.

Genome walking is a molecular procedure that can be used to identify nucleotide sequences flanking known regions. Genome walking comprises several polymerase chain reaction (PCR)-based methods, in which an oligonucleotide specific for the known sequence is coupled with an oligonucleotide derived from the adopted genome walking strategy (Volpicella et al., 2012). The methods are classified into three groups based on their first step: restriction-based (R-GW) method, primer-based (P-GW) method and extension-based (E-GW) method (Leoni et al., 2011). R-GW method requires preliminary restriction digestion of genomic DNA and ligation of restriction fragments to DNA-cassettes. Next, P-GW method is characterised by the use of various walking primers, containing either random or degenerate sequences, coupled to a sequence specific primer. In E-GW method, extension of sequence specific primer and subsequent 3'-tailing of resulting single-stranded DNA that provides substrate for final PCR amplification was used.

The increasing number of sequencing projects helped to compile enormous amount of sequence data which can be used to develop specific gene markers and thus identify novel functional variation (Han et al., 2004). The flexibility of genome walking strategies makes it beneficial for analysis of large libraries and identification of specific sequences where whole genome sequencing projects have not been undertaken (Leoni et al., 2011).

2.5.1 Characterisation of *bla*-TEM Promoter Sequence

Several studies have been conducted to characterise the *bla*-TEM gene and its promoter region. TEM derivatives could be differentiated from each other by the types of promoters, namely weak promoter *P3*, two strong overlapping promoters *Pa/Pb* and promoter *P4* (Leflon et al., 2000). Another promoter *P5* was suggested by Lartigue et al. (2002), which has similar -10 promoter region as promoter *P3*.

Genome walking strategy was applied in a study by Vignoli et al. (2006) to sequence a small ColE1 plasmid encoding TEM-144. The *bla*-TEM coding sequence was found in antimicrobial resistance region zone. The *bla*-TEM promoter was found upstream of *bla*-TEM and belonged to the weak promoter type *P3*. In another study done by Tristram et al. (2005), polymorphic nucleotides of *bla*-TEM genes of TEM-positive strains were distinguished using single nucleotide specific PCR. The *bla*-TEM genes were found to be associated with the promoter *P3*, the promoters *Pa/Pb* or a novel promoter produced due to a 135 bp deletion and a G162T substitution.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strain

This project was conducted using the bacterial strain *Enterobacter cloacae* isolated from cucumber (Ong 2012). This bacterial strain was maintained in a stab culture and was designated as strain UW2.

3.1.2 Chemical Reagents and Equipments

The chemical reagents and equipments used in the project were provided by Department of Biological Science, Universiti Tunku Abdul Rahman. These chemical reagents and equipments were obtained from Molecular Biology, Microbiology and Final Year Project Laboratories. Some equipments were also accessible from General Biology and Postgraduate Laboratories. The details of materials used were listed in Table 3.1.

CHAPTER 4

RESULTS

4.1 Total DNA Extraction

Total DNA was successfully isolated from UW2 bacterial isolate using GeneJET Genomic DNA Purification Kit (Fermentas). By using Nanodrop 1000 Spectrophotometer, the concentration of total DNA was measured as 191 ng/µl. On the other hand, the A_{260}/A_{280} purity was measured as 1.873, which was within the range for good-quality DNA (1.8 – 2.0). The gel image to assess purified total DNA was shown in Figure 4.1.

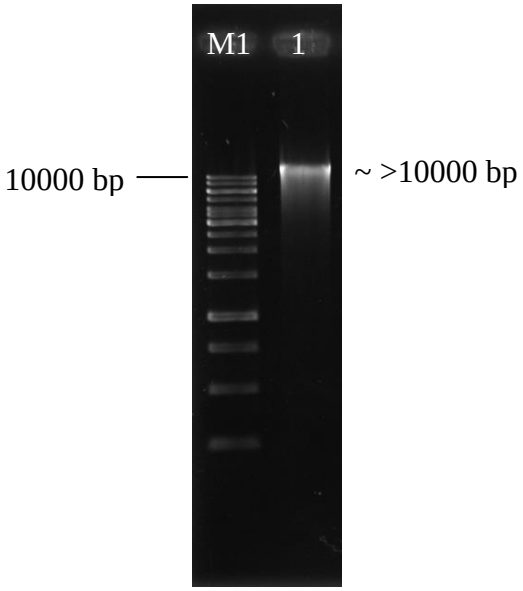

Figure 4.1: Gel electrophoresis image for total DNA extraction.

Lane M1 is the 1 kb ladder (0.5µg) while Lane 1 represents the total DNA.

CHAPTER 5

DISCUSSION

5.1 Total DNA Extraction

In this study, total DNA was extracted from UW2 bacterial isolate using commercial kit, which allows DNA to be extracted more conveniently and less time-consuming. The total DNA extracted had a considerably low concentration of 191 ng/µl. Low DNA yield was possibly due to the bacterial cells which were not completely lysed. The Proteinase K digestion at 56℃ could be prolonged for better cell lysis. Absorbance ratio at 260 nm and 280 nm was used to evaluate DNA purity, where a ratio of around 1.8 indicates pure DNA. In this study, total DNA had considerably high purity, with A_{260}/A_{280} ratio of 1.873. As shown in Figure 4.1, total DNA demonstrated high molecular weight when assessed on agarose gel. Slight smearing below the band indicated some DNA degradation which was probably due to shearing during isolation procedure.

5.2 Molecular Characterisation of PCR Fragment

5.2.1 PCR Amplification of Target Gene and Agarose Gel Purification

In this study, a standard PCR condition with 30 cycles was used for amplification of *bla*-TEM homologous gene. The result for PCR amplification was shown in Figure 4.2 (a). No product was amplified in negative control, indicating absence of contamination. An expected band of 870 bp indicated that the target gene with

CHAPTER 6

CONCLUSION

In characterisation of *bla*-TEM gene of UW2 strain of *Enterobacter cloacae*, a readable DNA sequence of 868 bp was obtained by overlapping the sequences obtained in two-pass sequencing with T7 and SP6 primers. The BlastX alignment analysis showed that 100% similarity was achieved and corresponded to the hypothetical protein from *Clostridum nexile*, harbouring TEM beta-lactamase within its coding region. It also showed considerably high similarity of 99% to beta-lactamase from other organisms.

GenomeWalker gene library was constructed using *Eco*RV-digested restriction fragments for the characterisation of unknown sequences flanking the *bla*-TEM gene. Upstream DNA sequences were characterised by amplification products of various sizes for DNA sequencing. Nevertheless, only sequence GW4 could overlap with the characterised *bla*-TEM gene to give a sequence of 1076 bp. The predicted open reading frame corresponded to the *bla*-TEM gene region of a TEM-116. Analysis on the upstream sequence showed the presence of a purine rich Shine-Dalgarno sequence preceding the open reading frame of *bla*-TEM 116 gene fragment. Next, the predicted 5' transcriptional start site, together with -35 and -10 promoter regions were located to be within several base pairs from the Shine-Dalgarno sequence. On the other hand, characterisation of downstream

DNA fragments was unsuccessful, which was probably due to large size of DNA fragments of *Eco*RV gene library that were unsuccessfully amplified using PCR.

Unknown DNA sequences flanking the *bla*-TEM gene can be further characterised by constructing other GenomeWalker gene libraries using other restriction enzymes. Besides, another strategy can be developed to employ long range PCR for obtaining longer DNA fragments.

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