# MOLECULAR CLONING OF THE PROMOTER OF VIRULENCE GENES *virB* and *virE* FROM *AGROBACTERIUM TUMEFACIENS*

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# MOLECULAR CLONING OF THE PROMOTER OF VIRULENCE GENES *virB* and *virE* FROM *Agrobacterium tumefaciens*

By

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## ABSTRACT


## MOLECULAR CLONING OF THE PROMOTERS OF VIRULENCE GENES *virB* AND *virE* FROM *Agrobacterium tumefaciens*

**Eliza Loo Po-Iian**

The *lac* operon is the most widely used expression vector due to its availability and ease of use. However, the *lac* expression system has its weaknesses whereby it is leaky and expensive. This leads to the idea of constructing acetosyringone-inducible expression system to overcome the limitations of the *lac* operon. The construction of acetosyringone-inducible expression vector involves the cloning of sensor (*virA)* and transcriptional activator (*virG*) and the cloning of *vir* promoters. The focus of this project is on the cloning of *vir* promoters. The method used to clone the *vir* promoters into target plasmid pD15 and pD17 is site-directed mutagenesis (SDM) whereby a megaprimer is required to prime SDM. The megaprimer is generated through PCR whereby it carries the *vir* promoters and sequence homologous to the target plasmid. SDM then introduce the *vir* promoters as mutations into the plasmids concurrently replacing the existing promoter in the plasmid. The isolation of *vir* promoters and production of megaprimers were successfully carried out. However, the SDM was unsuccessful mainly due to the lack of optimization. Several measures such as the size of target plasmid, requirements of SDM, size of mutation and the primer design were taken into consideration to resolve the problem. If the SDM optimization could be successfully carried out, a modified plasmid with *vir* promoter would be generated.

The construct of a functional acetosyringone-inducible expression vector would be completed once *virA*, *virG* and the *vir* promoters are cloned in a same vector.

# ACKNOWLEDGEMENTS

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## DECLARATION

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

____________________________

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**APPROVAL SHEET**

This project entitled "**MOLECULAR CLONING OF PROMOTERS OF VIRULENCE GENES *virB* AND *virE* FROM *Agrobacterium tumefaciens***" was prepared by ELIZA LOO PO-IIAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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# PERMISSION SHEET

It is hereby certified that **ELIZA LOO PO-IIAN** (ID No: **08ADB05300**) has completed this final year project entitled "**MOLECULAR CLONING OF THE PROMOTERS OF VIRULENCE GENES *virB* AND *virE* FROM *Agrobacterium tumefaciens*"** supervised by Dr. WONG HANN LING from the department of Biological Science, Faculty of Science.

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

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Amp | Ampicilin |
| CAP | Catabolite Activator Protein |
| Cm | Chloramphenicol |
| cAMP | Cyclic Adenosine Monophosphate |
| Kan | Kanamycin |
| LB | Luria Bertani |
| MCS | Multiple Cloning Site |
| SDM | Site-directed Mutagenesis |

# CHAPTER 1

# INTRODUCTION

## 1.1 The Inducible Gene Expression System

There are two types of bacterial gene expression system- the constitutive gene expression system and the inducible gene expression system. Both systems serve the same function to express a particular gene. The main difference between these two systems is the consistency of transcription.

The constitutive gene expression system refers to the system where the gene product is made at all times without the need of activator or repressor. In other words, the RNA polymerase constantly transcribes the gene's mRNA regardless of the extracellular conditions or environmental influences (Abedon, 1997). This expression system usually belongs to the housekeeping gene, which gene products are required on a regular basis to maintain the cellular functions and structures.

An inducible gene expression system is a type of facultative gene expression system. This means the system only expresses its genes when they are required. For instance, the well studied *lac* operon only produce the enzymes ß-galactosidase, lactose permease and lactose transacetylase when the lactose or its isomers, called activators are present in the extracellular environment. When no activators are present, the *lac* operon represses its expression system. This mechanism helps the bacteria in conservation of energy for other essential processes such as replicating and healing (Sadava, Heller, Orians, Purves, & Hillis, 2007).

## 1.2 The Acetosyringone-inducible Expression System in *Agrobacterium tumefaciens*

The natural acetosyringone-inducible system belongs to *Agrobacterium tumefaciens.* The *A. tumefaciens* utilizes acetosyringone produced by wounded plants to transfer part of its Ti plasmid, the T-DNA into the plant cell. The ability of the T-DNA transfer in the presence of acetosyringone later revealed that it was the action of the virulence (*vir*) genes located in the Ti plasmid. The *A. tumefaciens* carries Ti plasmid that encodes genes for the synthesis and catabolism of nopaline. There are 6 *vir* genes in the nopaline Ti plasmids which are *virA, virB, virC, virD, virE,* and *virG* compared to octopine Ti plasmids which carry an additional *virF* (Rogowsky, Close, Chimera, Shaw & Kado, 1987). The promoters of these *vir* genes were further studied to be used in this project to construct a new acetosyringone-inducible system.

The key components required to build this inducible expression vector include a sensor or receptor, transcriptional activator and promoter. A sensor functions to detect the presence of the inducer and then send signals to a transcriptional activator, which will bind to the inducible promoter to drive the transcription of the gene downstream. In the acetosyringone-inducible system, it was found that the *vir* gene that is responsible for sensing the inducers such as phenol, aldose monosaccharide and phosphate is *virA* and the transcriptional activator that works together with VirA is VirG. VirG functions to serve as a 'regulatory protein' to stimulate the transcription of other *vir* genes as shown in Figure 1.1 (Rogowsky et al., 1987). Signals received from the sensors will then stimulate the transcriptional activator to trigger the RNA polymerase to transcribe *vir* promoter mRNAs which will be translated to proteins to

express the gene. There are 4 *vir* promoters each starts the transcription of proteins with specialized functions.

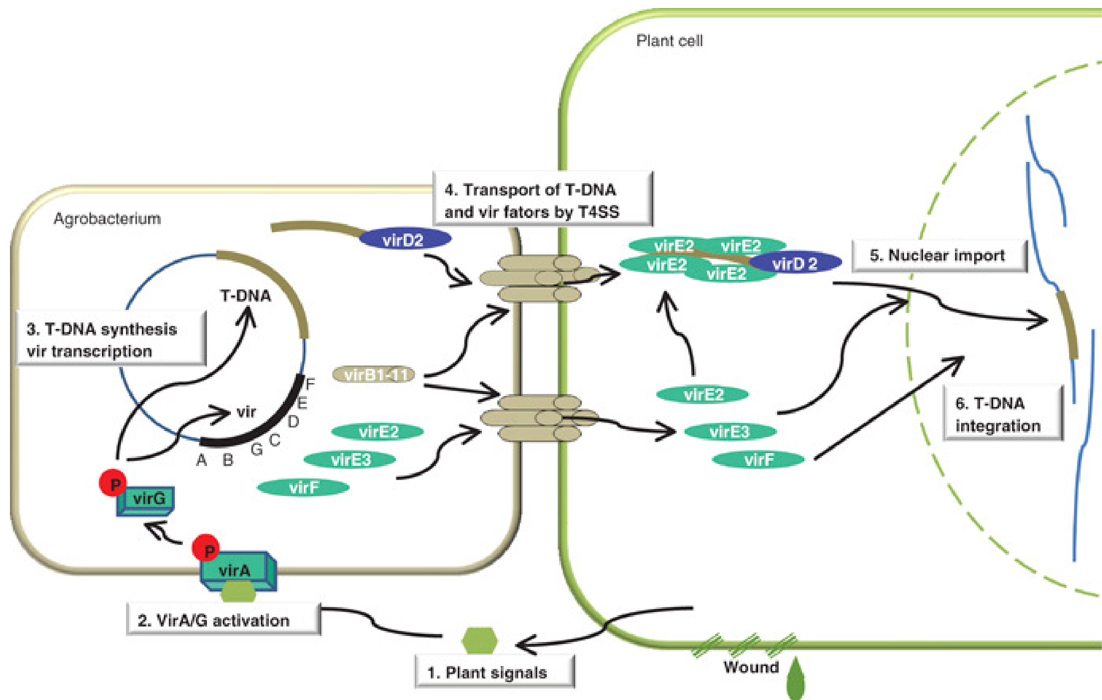



**Figure 1.1: A schematic diagram showing the functions of the *vir* genes.** (Adapted from Pitzschke & Hirt, 2010).

The *virA* functions to receive signal from inducer and send them to *virG* which in turn will stimulate the other *vir* promoters.

## 1.3 The Significance and Objectives of this Study

This project focuses on the cloning of two of these four *vir* promoters, the *virB* and *virE* promoters

The objectives of this project are:

I. To clone the *virB* and *virE* promoters of *A. tumefaciens.*

II. To replace the T7 promoter of pD15 and pD17 plasmids with *virB* and *virE* promoters.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 The Operon

### 2.1.1 The Lac Operon

The *lac* (for lactose) operon is a mechanism for metabolizing lactose. It is a cluster of three structural genes encoding proteins involved in lactose metabolism, namely the ß-galactosidase encoded by *lacZ*, lactose permease encoded by *lacY* and lactose transacetylase by *lacA*. ß-galactosidase converts lactose into glucose whereas lactose permease and lactose transacetylase are proteins required for utilization of lactose by the bacterium (Parker, 2001). Bacteria carrying this system conserve energy by making these three proteins only when necessary, that is only when lactose are available to be metabolized (Sadava et al., 2007). This operon can be both positive and negative regulators. It works as a positive regulator when CAP is involved whereby cAMP-CAP protein complex activate the inactive CAP site. In the negative regulation, an active repressor halts the functioning of the operon. In this case, the *lac* repressor blocks the production of mRNA, hence the expression of the genes (Griffths, Miller & Suzuki, 2000).

The *lac* operon is an inducible operon. Inducible means the ability of the operon to synthesise a specific enzyme in response to the appearance of its substrate (Lewin, 2008). The mechanism to which how the *lac* operon works easily describes the *lac* operon as an inducible operon. Generally, the transcription starts with binding of the RNA polymerase and DNA-binding protein to a specific DNA binding site known as

the promoter. This promoter is located upstream of the three structural genes, *lacZ, lacY* and *lacA*, in that order. Once bound to the promoter, the RNA polymerase transcribes all the three genes into mRNA which will then be translated into proteins. This ought to take place without the presence of the repressor.

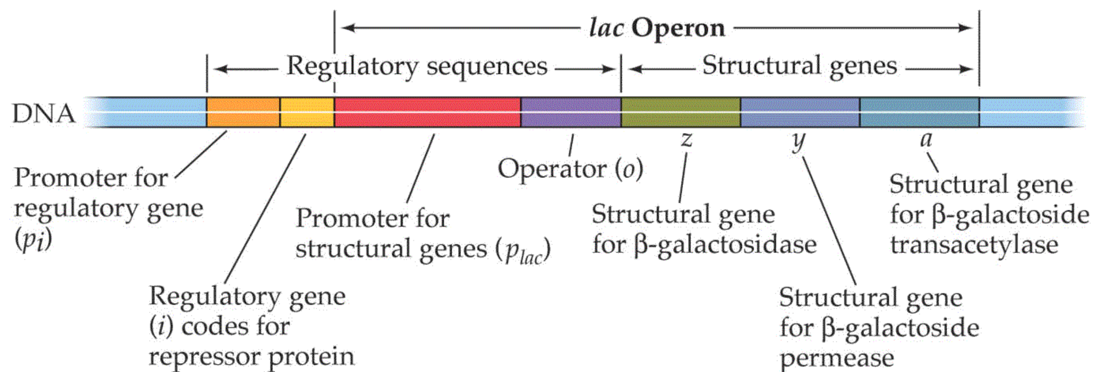



**Figure 2.1 : The of *lac* operon of *E. coli*.** (Adapted from Sadava et al., 2007).

Looking deeper into the mechanism of the *lac* operon, there are two regulators functioning together. This dual control system responds to glucose and lactose individually. The control systems are the CAP (Catabolite Activator Protein) which responds to glucose and the operator works in respond to lactose. The CAP site is located adjacent to the promoter and it allows the binding or unbinding of cAMP (cyclic adenosine monophosphate) to activate or inactivate it. In the absence of glucose, cAMP level is high in the cell. The cAMP then binds to the CAP, which increases the production of ß-galactosidase to a great extent. The cAMP-CAP protein complex then binds to the CAP site hence, activates the CAP site. An activated site bends the operon, allowing the RNA polymerase to associate with the promoter

(Campbell & Reece, 2005). Similarly, in the present of glucose, cAMP level drop low in the cell. This prevents the binding of cAMP to CAP and thus, unable to activate the CAP site. Therefore, the RNA polymerase does not associate with the promoter.

The second regulatory system involves the operator and a repressor protein known as the *lac* repressor. The repressor is controlled by a small-molecule inducer and the natural inducer for the *lac* operon is ß-1,6-allolactose (Lewin, 2008). This repressor protein has two binding sites. One of the binding sites is the operator site in which binding to this site cover a part of the promoter region, thus prevents the binding of RNA polymerase onto the promoter. The other binding site of the *lac* repressor protein is part of the inducer (Lewin, 2008). When the inducer is present, it binds to the repressor protein and inactivates the repressor in a way that it reduces the repressor's affinity to bind to DNA (operator). The repressor will then be released from the operator, allowing RNA polymerase to associate with the promoter and hence, transcribes *lacZ* and subsequently, *lacY* and *lacA*.

### 2.1.2 The Limitations of *lac*-inducible Gene Expression System

Section 2.1.1 mentioned that the *lac* operon uses the CAP control mechanism in response to glucose. In this regulation, when the glucose concentration is high in the cell, the glucose interacts with lactose permease encoded by *lacY* and blocks the import of lactose molecules into the cell. The absence of lactose forbids the hydrolysis of lactose into allolactose, hence effectively inactivates the operon. In the event of inducer exclusion, the present of inducer or lactose in the cell will not activate the operon due to the low level of ß-galactosidase (Patterson, 2009).

Other scientists, however, reported otherwise. A typical gene expression involves several stages mainly the transcription process followed by translation into proteins. Similarly, the expression of the *lac* operon involves these two main stages. Once an inactive operon comes in contact with the inducer, the transcription process is stimulated. The *lac* mRNA is unstable and has a half life of only 3 minutes (Lewin, 2008). This feature allows the induction to be reverse almost instantaneously. Therefore, once the inducer is absent, the transcription of mRNA falls rapidly back to the basal level. Due to the declining amount of mRNA present in the cell, the translation of *lac* mRNA into enzymes (ß-galactosidase) ceases as well after the removal of the inducer. On the contrary, the synthesized ß-galactosidase persists longer in the cell due to the stability of the enzyme. As a result, ß-galactosidase remains at the induced level for a longer period (Lewin, 2008). Figure 2.2 summarizes the transcription and translation process upon induction and removal of inducer.

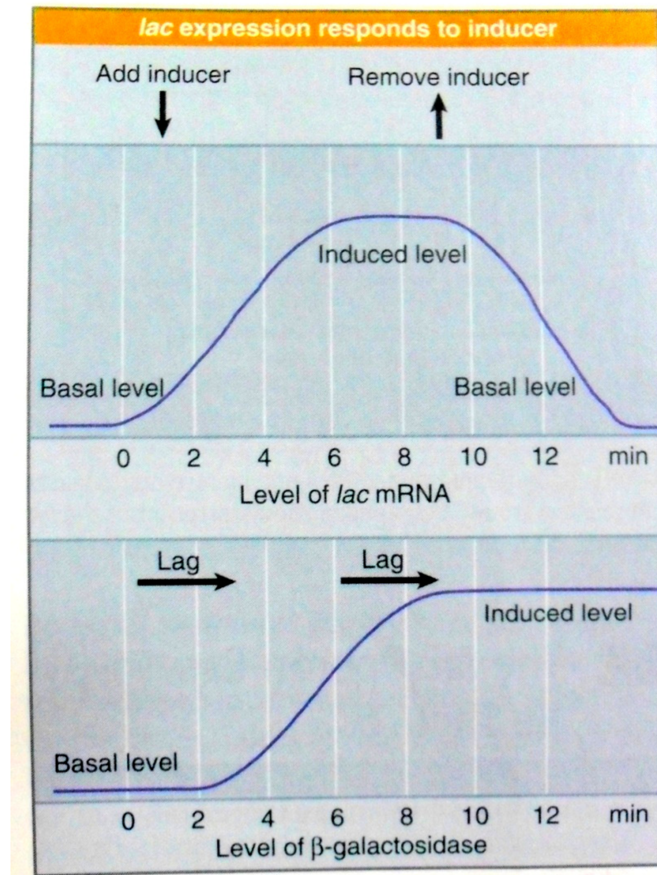



**Figure 2.2 : Transcription and translation level of the *lac* operon upon induction and removal of inducer.** (Adapted from Lewin, 2008).

The upper part of the figure shows the transcription level and lower part of the figure shows the translation level respectively against the period it stays at the induced level.

This information gives an idea that the *lac* operon may have background expression or leaky. Leaky, in this context means the operon may express itself even when it is not induced, producing an inaccurate result when it is used to measure an expression of a particular gene.

*In vitro*, IPTG (Isopropyl β-D-1-thiogalactopyranoside) is often used as an inducer for the *lac* operon. IPTG is an isomer of allolactose, which means it is structurally similar to allolactose and is able mimic the activity of allolactose. The only alteration IPTG

has is an additional sulfur group on the carbon chain, transforming it to gratuitous inducer. A gratuitous inducer induces the synthesis of an enzyme but will not be metabolised (Lewin, 2008). This feature makes IPTG the most prefered chemical to use since it would not interfere with the study of cell's system. Although the use of IPTG is common in many laboratories, IPTG is not economical hence, cannot be used on industrial scale. A bottle of 200 mM IPTG from one of the leading Life Science and High Technology companies, Sigma Aldrich® costs RM 290.01, which is RM 6.08 per gram. Besides, IPTG gives a highly intense responses (Mihasan, Ungureanu & Artenie, 2007) and it is highly toxic (Viitanen, Vasala, Neubauer & Alatossava, 2003), hence, inappropriate for high-end projects as it has the potential to kill the cells and cause impurities that could not be removed through downstream processes or require additional purification steps. IPTG is said to be *par excellence* (Lewin, 2008) with allolactose, further encourage background expression or unintended induction.

These disadvantages of using *lac* operon expression system brought up the idea of using other molecule or chemical inducible operon, in this project, the acetosyringone-inducible expression system.

### 2.1.3 Advantages of Acetosyringone-inducible Gene Expression System

Acetosyringone is a phenolic compound naturally secreted by plants upon wounding. In the modern days, this chemical compound can also be synthesised from acetovanillone (Crawford, Eaton, & Pepper, 1956). Acetosyringone is volatile and it is one of the main chemicals involved in plant-pathogen interaction (Baker et al., 2005). In a typical plant transformation experiment, the bacteria *A. tumefaciens* are usually used to infect the plants due to the fact that these bacteria carry T-DNA in the

tumour-inducing plasmids or Ti plasmids. The T-DNAs have the ability to transfer and incorporate into the plant genome. Another important segment in the Ti plasmid which carries the T-DNA is the virulence (*vir*) genes. These genes take charge from of how the T-DNA is excised from the plasmid to transporting and protecting the T-DNA, as briefly described in the introduction and will be in more details in 2.2. These *vir* genes are activated by acetosyringone.

Section 2.1.2 explained the problems encountered with *lac* operon, one of the main problems is being leaky. Unlike lactose, acetosyringone is not a metabolite produced by bacterial cells. Therefore, if it is used as an inducer for an operon, there will be no fear of leakiness. The only possibility for acetosyringone to be present in a bacterial system is when it is provided extracellular.

Usage of acetosyringone as an inducer for an operon may also come in handy when it is used on *A. tumefaciens*. As much as the usage of *A. tumefaciens* is wide spread among scientists for plants transformation, researchers projected a drawback in this method. Apparently, transferring foreign genes with *A. tumefaciens* relates to poor survival rates of target cells due to *A. tumefaciens* hypersensitive responses (de la Riva, González-Cabrera, Vázquez-Padrón & Ayra-Pardo, 1998). One successful method used to overcome this drawback was to use antioxidant compounds to prevent necrosis. A study by another group of researchers showed that acetosyringone when used in high concentration may inhibit the growth of *A. tumefaciens* at the same time loses their virulence (Fortin, Nester & Dion, 1992). In this study, the researchers treated cultures of *Agrobacterium spp.* with high concentrations of acetosyringone and found that different strains of *Agrobacterium* respond to different concentrations of

acetosyringone but in general, at high concentration of acetosyringone, they lose their virulence and their growth were inhibited. This, therefore, offers an advantage to the acetosyringone-inducible expression vector system where acetosyringone is used as an inducer and when the concentration is slightly increased, the host (*A. tumefaciens*) becomes inactive before the target cells undergo necrosis, hence, higher cell survivability.

## 2.2 The Virulence Genes of *A. tumefaciens*

### 2.2.1 Ti Plasmid

Tumour-inducing plasmid, more commonly known as Ti plasmid or pTi is the plasmid found in *Agrobacterium spp.* This plasmid is 206, 479 bp long and it has 196 genes that code for 195 types of proteins. As briefly mentioned in Section 2.1.3, the Ti plasmid carries a region of T-DNA important for integration of foreign genes into the new host (plant) cell. The T-DNA carries genes that cause tumour formation in plant cells. The focus of this project, however, is the virulence (*vir*) genes.

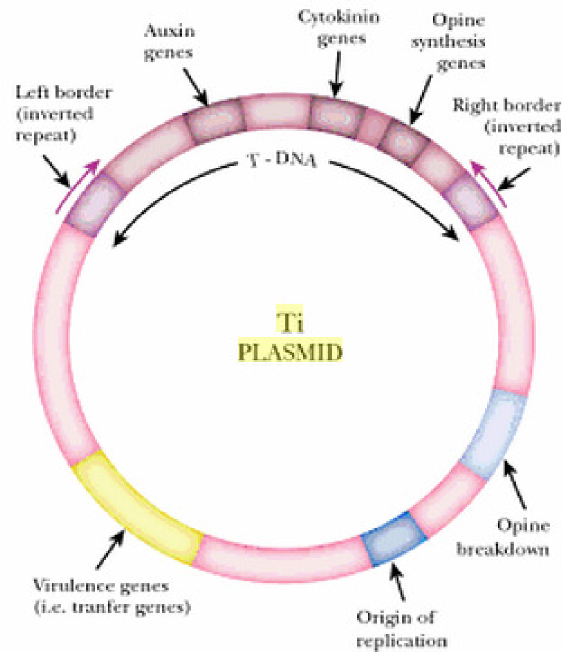



**Figure 2.3 : The general structure of Ti plasmid**. (Adapted from Clark, 2005).

The *vir* region of the Ti plasmid is responsible for coding of proteins important for the processing, transfer and integration the T-DNA into plant cell. The *vir* region of Ti plasmid for nopaline Ti plasmid (as used in this project) consists of different *vir* genes which are *virA, virB, virC, virD, virE,* and *virG* (Rogowsky et al., 1987). Each of these genes are polycistronic operons, which means they code for more than just one type of protein, for instance, the *virE* produces $virE_1$ and $virE_2$ proteins and *virB* produces proteins $virB_1$ to $virB_{11}$. Upstream of each of these *vir* operons is a *vir* box (Schrammeijer et al., 2000), which is a large 12 kb fragment of consensus sequence where it acts as a *cis*-regulator to which the virG protein binds to express a *vir* gene ( Nester & Verma, 1993).

Each of the *vir* gene codes for proteins that perform different functions on different stages of transporting the T-DNA into the plant cell. In this project, only the promoter

of two genes, *virB* and *virE* were each used in the process of constructing acetosyringone-inducible expression vector.

### 2.2.2 *virB* and *virE* Promoters

Promoter is a specific sequence of nucleotides on a gene fragment whereby the RNA polymerase binds to initiate a transcription (Dale & von Schantz, 2008). They may also contain enhancer or repressor to boost or repress the regulation and are typically located on the upstream of the genes they regulate.

According to a research, the *virB* and *virE* promoters are the most highly expressed promoters (Rogowsky et al., 1987). In this research, a team of researchers fused all the different *vir* promoters to separate *lux* operon and induced the promoters with acetosyringone. The *lux* operon genes codes for luminescent proteins which enables the researchers to measure the degree of expression by light production. Results of this research showed that the two most highly expressed promoters are *virB* and *virE* as shown in graph in Figure 2.4. To be qualified as a good expression vector, the vector should be able to produce large amount of mRNA, hence protein. Therefore, these two highest expressing promoters are used in the construction of acetosyringone-inducible expression vector to obtain optimum results.

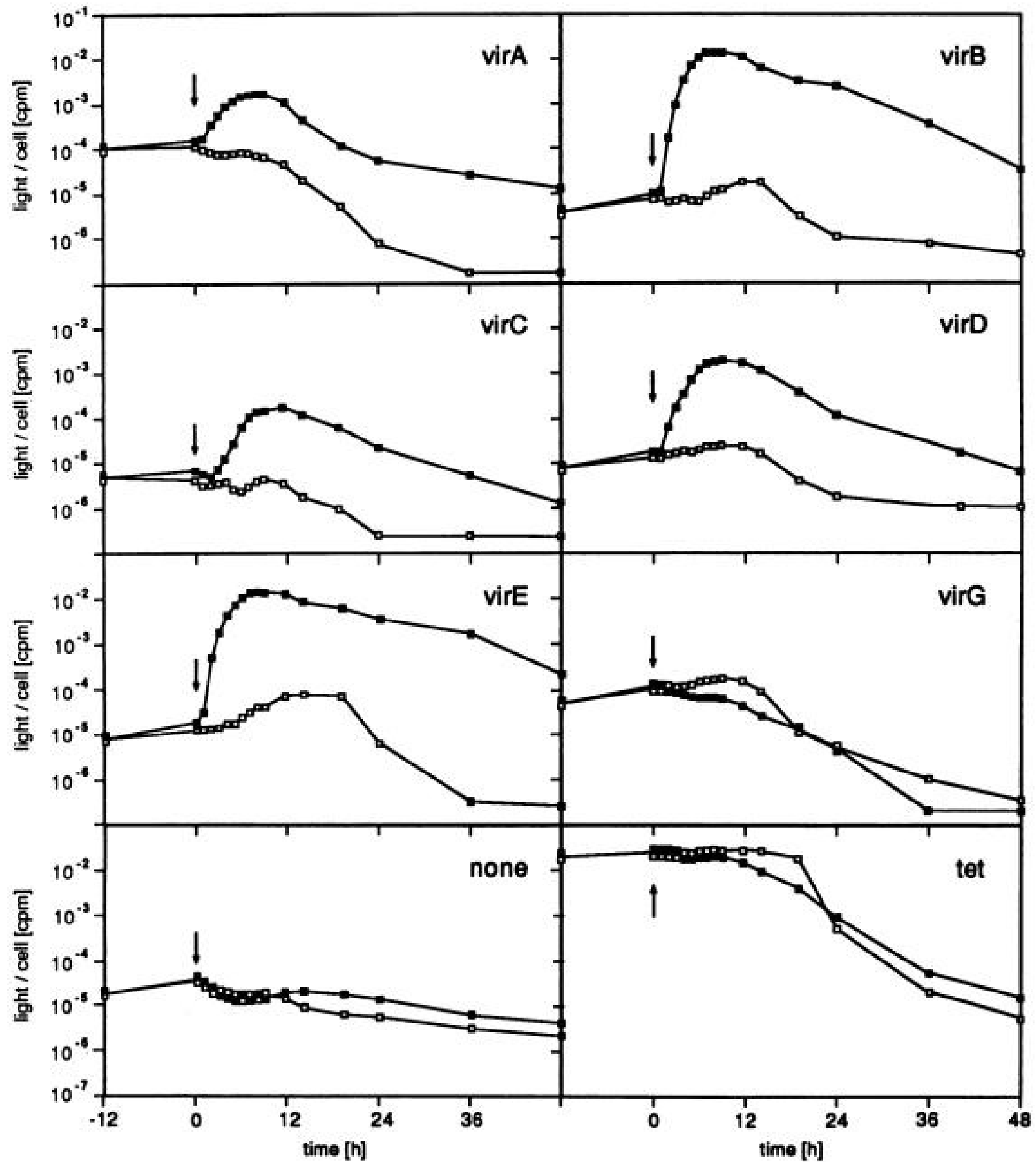



**Figure 2.4 : Expression of *lux* gene directed by *virA, virB, virC, virD, virE, virG* promoters and two controls (labelled 'none' and 'tet').** (Adapted from Rogowsky et al., 1987).

Expression level is determined by measuring the light production (luminescent) when promoters are induced with acetosyringone.

## 2.3 The Construction of an Acetosyringone-inducible Gene Expression System

### 2.3.1 The Conventional Cloning Techniques

The long-established method for gene cloning is straight forward but it requires more steps hence, more time and effort. Usually, gene cloning starts with copying the gene from a source, the genome or a larger piece of gene, and amplify the gene using PCR. The PCR products will then be cleaved to produce sticky ends (or sometimes, blunt ends) to complement the ends of the vector or plasmid to which the gene will be inserted. After cleaving the gene of interest with the suitable restriction enzyme, the gene is ligated into the plasmid. Since there will be chances for the plasmids to self-ligate, a screening process will be carried out to select the plasmids with gene of interest. This is done by inserting the plasmids (both with gene of interest and self-ligated) into a bacterial host in a process called transformation. There are several applicable screening methods. One of which is called the blue-white screening where the principle behind this technique is to screen for bacteria that is not able to encode a functional ß-galactosidase. This is because the MCS for the insertion of foreign gene is located in the gene fragment that partially codes for ß-galactosidase. Therefore, plasmids with insert cause disruption to the production of ß-galactosidase. The inability of the bacteria to code for a functional ß-galactosidase causes the colony to turn white and easily distinguished when they are grown on X-gal.

Another commonly used method to screen for plasmids with genes of interest is through the antibiotic selection method. In this method, the plasmid carries an antibiotic resistant gene and the insert is usually placed in the antibiotic marker gene region, disrupting the gene. Thus, bacteria which are able to grow on antibiotic selection plates are those carrying the plasmid without the desired gene. The desired

bacteria are then obtained by tracing back to a replica plate (Dale & von Schantz, 2008).

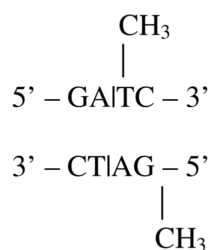
Though this established cloning technique is widely used, it is not applied in this project to construct acetosyringone-inducible expression vector. Instead of just constructing a new expression vector, this project is also designed to test a different cloning method, which is to create a mutation on the readily available plasmids.

### 2.3.2 Site-Directed Mutagenesis

The strategy used in the construction of this new expression system is called site-directed mutagenesis (SDM). It is simply a PCR based mutagenesis technique that introduces a specific mutation either an insertion or a deletion to a precise locus. In this project, the SDM is used to insert, at the same time, delete a fragment of gene into pD15 and pD17 plasmids.

Most mutagenesis techniques require linear single stranded mutant DNA fragments to be cloned into plasmids in order to propagate. An SDM technique commonly known as QuikChange® method (Wang & Malcolm, 1999) offers the capability to introduce a direct mutation onto the plasmid without the need to clone the gene of interest into a separate vector. This technique also eliminates the need for specific restriction sites or repeated transformation. It is a rapid process utilizing a set of oligonucleotide primers which are complementary to the sense and anti-sense strand of the gene at the same time containing the desired mutation (Reece, 2004). The double stranded plasmids are used as templates where the primers will anneal and extend during the temperature cycle generating newly synthesized modified plasmids (Figure 2.5).

The newly synthesized plasmids will not be methylated, hence providing a feature to distinguish between the modified and unmodified plasmids which are usually *Dam* methylated. Digestion with *DpnI* removes the unmodified parental plasmids leaving the modified plasmids for the following transformation protocol. *DpnI* is an endonuclease that specifically cleaves methylated and hemimethylated DNA at the specific the recognition site as below (Reece, 2004):




The products from *DpnI* digestion are then transformed into highly competent bacterial cell using electroporation to seal the nick in the newly synthesised plasmids and to propagate the plasmids with desired mutation. Since the unmodified plasmids will be degraded and will not be transformed into the cells, the bacteria colonies that grow on the agar plates are bacteria harbouring the modified plasmids.

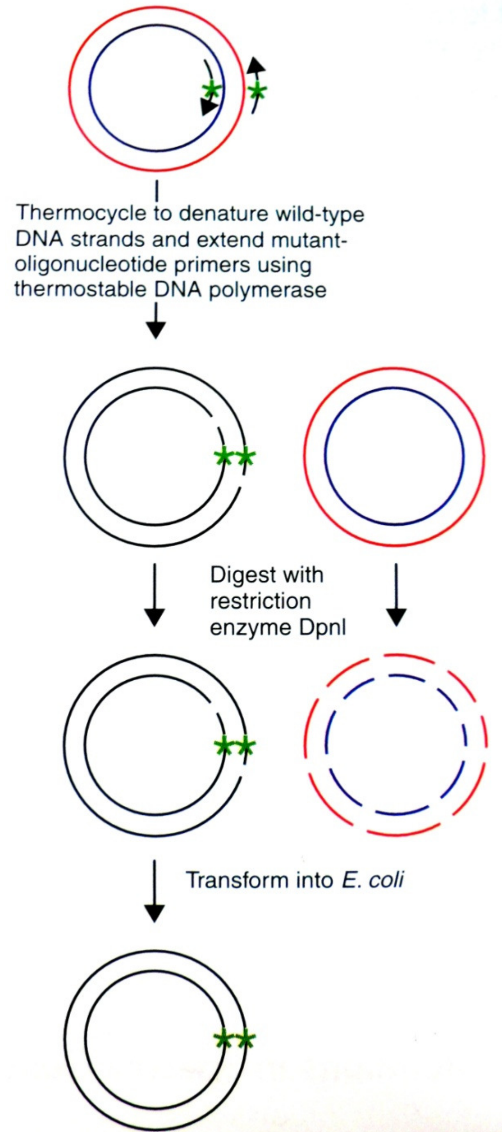



**Figure 2.5 : A schematic diagram summarizing the QuikChange® mutagenesis process.** (Adapted from Reece, 2004).

CHAPTER 3

# MATERIALS AND METHODS

## 3.1 List of Materials and Apparatus Used

**Table 3.1 : List of apparatus and equipments used during the course of the project and their respective manufacturers.**

| Apparatus/Equipment/Machine | Manufacturer |
|---|---|
| ABJ Analytical Balance | KERN |
| Autoclave sterilizer | HIRAYAMA |
| Desktop computer | Acer |
| Electrophoresis System | TAKARA BIO INC. (Electrophoresis system Mupid®-2plus) |
| Electroporator | Eppendorf (Electroporator 2510) |
| Electroporation cuvette | Eppendorf |
| Electronic balance | KERN |
| Hotplate stirrers | Stuart |
| Incubator | Memmert |
| Imaging system | UVP (MultiDoc-It Digital Imaging System) |
| Laminar hood flow | ESCO Laminar Flow Cabinet (Model: AHC-4DI) |
| Microcentrifuge | Thermo Electron Corporation (Sorvall Legend Micro 17 Centrifuge) |
| Micropipette | ViPR Ω Mechanical Pipette ( 0.5 - 10µl), (10-100 µl), (100-1000 µl), Eppendorf (0.1-2.5 µl), ThermoScientific (1-10 µl), (10-100 µl), (100-1000 µl), Gilson (Pipetman) (2-20 µl), (50-200 µl), (200-1000 µl) |
| Microwave | Sharp |
| Nanospectrophotometer | Implen |
| PCR thermocycler | Bio-RAD (MyCycler™ Thermo Cycler) |
| Refrigerator | Toshiba |
| Shaking incubator | N-Biotek |
| Spectrophotometer | BIO-RAD SmartSpec™ |
| Spinning evaporator | EYELA Centrifugal Evaporator cve-2000 |

| | |
|---|---|
| Table Top Refrigerated Centrifuge | Dynamica (Velocity 14R centrifuge) |
| Water bath | Memmert |

**Table 3.2 : List of materials used during the course of the project and their respective manufacturers.**

| Chemicals/Materials | Manufacturer |
|---|---|
| 100 bp DNA marker | 100bp DNA Ladder One (Code 07908-75) |
| 1 kb DNA marker | Fermentas GeneRuler 1kb DNA ladder 0.5µg/ml |
| 95% Ethanol | Copens Scientific (M) Sdn. Bhd. |
| Absolute ethanol | HmbG® Chemical |
| Acetic acid | SYSTERM® |
| Adenine hemisulfate salt, minimum 99% | SIGMA |
| Agarose powder | Vivantis |
| Ampicilin sodium | Wako |
| Boric acid | QRëC™ |
| Calcium chloride dehydrate | QRëC™ |
| DNA polymerase | Takara PrimeSTAR HS DNA polymerase (Code R010A Lot N170/DA) 2.5u/µl, Fermentas Taq DNA Polymerase (recombinant) (Lot 00046416 #EP0402) 5u/µl |
| DNA polymerase buffer | Fermentas 10X Taq Buffer with $(NH_4)_2SO_4$ (Lot 00044197 #EP0402), Takara 5X PrimeSTAR Buffer- $Mg2^+$ plus (Lot A2701A) |
| dNTP | TOYOBO (2mM) |
| Ethidium bromide | BIO BASIC INC. |
| Ethylenediaminetetraacetic Acid (EDTA) | QRëC™ |
| Gentamycin sulfate | BIO BASIC INC. |
| Glucose (dextrose) | Rdeh |
| Glycerol | QRëC™ |
| Hydrochloric acid | MERCK |
| Isopropanol | MERCK |

| | |
|---|---|
| Kanamycin sulfate | Wako |
| Magnesium chloride | Fermentas 25mM $MgCl_2$ (Lot 00037863), QRëC™ |
| PCI (Phenol/Chloroform/Isoamylalcohol) | CALBIOCHEM |
| Potassium acetate | MERCK |

**Table 3.2 (continued) : List of materials used during the course of the project and their respective manufacturers.**

| Chemicals/Materials | Manufacturer |
|---|---|
| Primer: *virB* | SDL proVirB (forward primer), SDR proVirB (reverse primer) |
| *virE* | SDL proVirE (forward primer), SDR proVirE (reverse primer) |
| colony PCR primer | F-DT-106 (forward primer) |
| Sodium acetate | SYSTERM® |
| Sodium chloride | SYSTERM® |
| Sodium hydroxide | R & M |
| Streptomycin Sulfate | Wako |
| Tetracycline hydrochloride | Nacalai Tesque |
| Tris-HCl | BIO BASIC INC. |
| Tris | Vivantis |
| Trypyone | CONDA pronadisa |
| Yeast Extract | Scharlau |

## 3.2 Preparation of Competent Cells

### 3.2.1 Calcium Chloride Competent Cells

Calcium chloride competent cells are used for heat shock transformation. Materials needed for this procedure are *Esherichia coli (E. coli)* DB3.1, LB broth, $CaCl_2$, $MgCl_2$ and glycerol.

A single colony was picked with a sterile toothpick from agar plate and the colony was inoculated in 2 mL of LB broth in a 15 mL Falcon tube. The tube was incubated in 37°C with agitation overnight. On the following day, the optical density for the broth containing bacteria was measured at the wavelength of 600 nm ($OD_{600}$). Bacterial broth was diluted with fresh LB broth to $OD_{600}$ of about 0.1 and then left to incubate in 37°C with agitation until the $OD_{600}$ is in the range of 0.4-0.6 (usually takes 2-3 hours).

In the mean time, the transformation buffer was prepared. The buffer contained 20 mM of $CaCl_2$ and 80 mM of $MgCl_2$. In this project, 20 microcentrifuge tubes, each containing 200 µL of cells summing up to 4000 µL in were prepared. Since the stock solution of both $CaCl_2$ and $MgCl_2$ is 1 M, only 80 µL of $CalCl_2$ and 320 µL of $MgCl_2$ from their respective stock solution are needed to be diluted to the required 20 mM and 80 mM solution. The solution was topped up to 4000 µL with distilled water.

Once $OD_{600}$ is between 0.4 and 0.6, broth was spun down at 9000 rpm for 5 minutes at room temperature to pellet the bacterial cells and remove the LB broth. Supernatant was discarded while the pellet was resuspended with equal volume of 0.1 M $CaCl_2$ to wash the pellet. The mixture was spun again at the same parameters, supernatant discarded and the pellet resuspended with $CalCl_2$. These steps were repeated for a final

time but instead of washing the pellet with $CaCl_2$, the transformation buffer was then added into the tube and the pellet was resuspended. 175 µL of this mixture and 25 µL of 80% chilled glycerol (after which diluted to 10%) was added into each microcentrifuge tube. The tubes were then frozen in liquid nitrogen and stored at -80°C.

### 3.2.2 Electrocompetent Cells

Electrocompetent cells are used for transformation through the electroporation method. The materials needed for this procedure are *E .coli* DB3.1, 10% glycerol and LB broth.

The procedure for the inoculation and growing of *E. coli* is as described in Section 2.2.1. Once $OD_{600}$ is between 0.4 and 0.6, broth was spun down at 9000 rpm for 10 minutes at 4°C to pellet the bacterial cells and remove the LB broth. Supernatant was discarded while the pellet was resuspended with equal volume of 10% glycerol. These steps were repeated for another 3 times, each time reducing the volume of 10% glycerol added by half. The final mixture was aliquot into 10 microcentrifuge tubes each containing 40 µL of mixture. They were then frozen in liquid nitrogen and stored at -80°C.

## 3.3 Cell Competency Test

### 3.3.1 Heat-Shock Transformation

Heat-shock transformation induces the entrance of foreign plasmid into a host cell. The materials needed for this procedure are the plasmids (pD17 and pD15), recipient bacterial cell (previously induced $CaCl_2$ competent cells), LB broth and 100 µg/mL Amp and 12 µg/mL Cm antibiotic selection agar plates.

Antibiotics were prepared from the stock according to the desired concentration and spread uniformly onto separate agar plates and labelled accordingly. Plates were then incubated in 37°C for at least 30 minutes to ensure complete antibiotic absorption by agar. 500 µL of LB broth was pipetted into a microcentrifuge tube and incubated in 37°C as well.

Fifty microliters of *E. coli* DB3.1 and 1.0 µL of plasmid was pipette into a microcentrifuge tube. The tube was flicked gently to mix the two solutions and then incubated in ice for 15 minutes. Cells were then heat-shocked by incubating the tube in 42°C water bath for a short 45 seconds and then quickly put back into ice incubation for another 2 minutes. After the incubation, the pre-warmed 500 µL of LB broth was mixed into the cells and plasmid mixture and was incubated in a 37°C shaking incubator for 30 minutes. After that, the microcentrifuge tube containing the mixture of transformed cells and LB broth was spun down at 14,000 rpm for 5 minutes at room temperature to pellet the cells and remove excess LB broth. 450 µL of supernatant was discarded. The pellet was resuspended with the remaining 50 µL supernatant and plated on 100 µg/mL Amp agar plate. The plate was incubated in 37°C overnight.

On the following day, a single colony from the 100 µg/mL Amp plate was picked up with a sterile toothpick and streak onto the 12 µg/mL Cm antibiotic selection agar plate. The plate was incubated in 37°C overnight.

### 3.3.2 Electroporation

Cells need to be transformed with plasmid with specific selection marker in order to determine their competency. Materials needed for this procedure are *E. coli* DB3.1, pUC19 and LB broth.

One mililiter of LB broth was incubated in 37°C. While incubating, 1 µL of pUC19 plasmid was added into 40 µL of *E. coli* DB3.1 electrocompetent cells in a 1.5 mL microcentrifuge tube. The tube with the mixture was incubated in ice for 3 minutes. Mixture was then pipette into the electroporator cuvette and any air bubbles were removed. The cell and plasmid mixture was electroporated at 1600 volts. After electroporation, the pre-warmed 1 mL of LB broth was added into the cuvette to resuspend the cells. The content of the cuvette was then pipette into a 1.5 mL microcentrifuge tube which will be incubated in 37°C with agitation for 30 minutes. After the incubation, the tube was spun at 14,000 rpm for 5 minutes. The supernatant was discarded until only 50 µL was left. The pellet was resuspended in the remaining supernatant.

### 3.3.3 Cell Competency Test (Serial Dilution)

It is important to ensure that the cells (*E. coli* DB3.1) are very competent especially for site-directed mutagenesis. Materials needed for this procedure are 100 µg/mL Amp selection agar plates and deionised distilled water.

Serial dilution was performed to test the cell competency. Electrocompetent cells can usually grow up to $10^{10}$ colonies per µg plasmid whereas $10^6$ colonies per µg plasmid for $CaCl_2$ competent cells. However, since *E. coli* DB3.1 is a strain where it is difficult to induce its competency, its electrocompetency was only tested up to $10^6$ colonies per µg plasmid. The stock was diluted with distilled water as shown in Figure 3.2 to obtain a range of $10^6$ to $10^8$ dilutions. The 3 bacterial dilutions along with the stock as control were plated on 4 separate 100 µg/mL ampicilin selection agar plates.

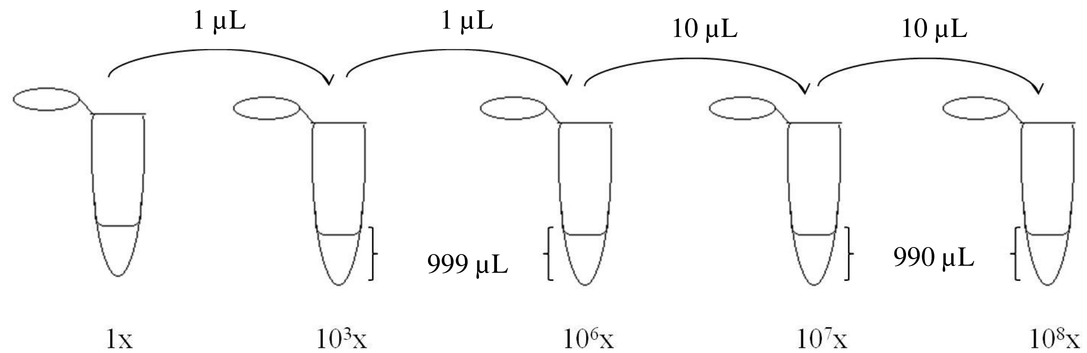



**Figure 3.2 : Serial dilution from stock solution (1x) to obtain up to $10^8$ dilutions.**

## 3.4 Propagation of Plasmids

### 3.4.1 Transformation of Plasmids into Competent Cells

pD15 and pD17 were transformed into $CaCl_2$ competent cells as procedure described in Section 3.3.1.

### 3.4.2 Plasmid Extraction using Promega Kit

In this step, plasmids inserted into bacterial cells during transformation are extracted and purified in order to increase the amount of plasmids. The material needed for this procedure are bacterial culture (*E. coli* DB3.1 with insert pD17 or pD15), Promega Cell Lysis Buffer, Promega Neutralization Solution, Promega Endotoxin Removal Wash, Promega Column Wash Solution and Promega Elution Buffer.

Six hundred microliters of bacterial culture grown in LB broth was transferred into a 1.5 mL microcentrifuge tube. 100 µL of Cell Lysis Buffer was added into the tube. The two solutions were mixed by inverting the tube. 350 µL of cold (4-8°C) Neutralization solution was added into the tube once the solution changed from opaque blue to clear blue. Neutralization solution was mixed by inverting the tube a

couple of times. Once the solution turned yellow, the tube was spun at 14,000 rpm for 3 minutes.

The supernatant was transferred into a PureYield™ Minicolumn provided by the kit. The minicolumn was placed into a PureYield™ Collection Tube and centrifuged at 14,000 rpm for 15 seconds. The flowthrough was discarded. 200 µL of Endotoxin Removal Wash was added into the minicolumn and was spun again at 14,000 rpm for 15 seconds. Then, 400 µL of Column Wash Solution was added into the minicolumn and centrifuged at 14,000 rpm for 30 seconds. Finally, the minicolumn was transferred to a 1.5 mL microcentrifuge tube and 30 µL of Elution Buffer was added into the minicolumn. The minicolumn was allowed to stand for 1 minute before it was spun at 14,000 rpm for 15 seconds. The eluent in the microcentrifuge tube was stored in -20°C.

0.7% agarose gel (0.14 g agarose powder in 20 mL TBE buffer) was prepared and gel was run with 80 V for 40 minutes to determine if the correct plasmid was extracted.

## 3.5 Isolation of *virB* and *virE* Promoters

### 3.5.1 Growth of *Agrobacterium tumefaciens* GV3101

The Ti plasmid pMP90 from the *A. tumefaciens* GV3101 was used as the template DNA for PCR amplification of *virB* and *virE* promoter genes. The materials needed for this procedure are *A. tumefaciens* GV3101 stock, LB broth and Kan selection plate.

A clean and sterile toothpick was dipped into *A. tumefaciens* GV3101 stock solution and streaked onto Kan selection plate. The plate was wrapped with aluminium foil for better growth. The bacteria were left to grow in 42°C for 2 days. After 2 days, a single colony was picked with a clean and sterile toothpick and inoculated in 2 mL LB broth overnight at 37°C shaking incubation.

### 3.5.2 DNA Extraction

Since the pMP90 Ti plasmid is huge, genomic extraction method was used instead of the usual plasmid extraction method. The materials needed for this procedure are DNA Extraction solution, isopropanol, 70% ethanol, PCI (phenol/chloroform/IAA) and RNase.

Four hundred microliters of DNA Extraction solution were added into equal volume of *A. tumefacien* cells. DNA Extraction solution is made up of 200 mM Tris-HCl (pH 8.0), 250 mM NaCl, 25 mM EDTA and 0.5% SDS. DNA Extraction solution and cells in a microcentrifuge tube was centrifuged at 15,000 rpm for 10 minutes. The supernatant was recovered and added with equal volume of chilled PCI. The solution mixture was vortex and centrifuged at 15,000 rpm at 4°C for 5 minutes. The supernatant was recovered and pipette into a new microcentrifuge tube. 300 µL of isopropanol was added into the tube and mixed. The tube was then centrifuged at 15,000 rpm for 10 minutes and the supernatant was discarded. The DNA pellet was washed with 150 µL of ethanol and then dried in vacuum centrifuge. The dried pellet was dissolved in 50 µL of TE buffer.

### 3.5.3 Purification of DNA by Ethanol Precipitation

By purifying DNA extracted, RNAse used during extraction is removed. This step is important to obtain a more pure DNA solution and more accurate concentration. The materials needed for this procedure are PCI, sodium acetate, absolute ethanol, 70% ethanol and TE buffer.

Equal volume of PCI mixed into the PCR product treated with RNase. The two solutions were vortex and then centrifuged at 14,000 rpm for 4 minutes at 4°C. The supernatant was recovered and transferred into a new tube. 10 volumes of sodium acetate and 2.5 volumes of absolute ethanol were added into the supernatant. The tube was left to stand on bench for 30 minutes before it was centrifuged at 14,000 rpm for 30 minutes at 4°C. This time, the supernatant was removed and 10 volumes of 70% ethanol were added into the tube. The tube was centrifuged at 14,000 rpm for 5 minutes at 4°C. Again, the supernatant was removed and the pellet was resuspended with 50 µL of TE buffer.

### 3.5.4 Isolation and Amplification of *virB* and *virE* Promoters by PCR

Genes of interest need to be isolated and then amplified in order to obtain enough amounts (concentration) for site-directed mutagenesis. The materials needed for this procedure are DNA template (Ti plasmid pMP90), DNA polymerase, DNA polymerase buffer, dNTPs, a set of forward and reverse primers, DMSO and deionised distilled water.

Primers used for *virB* are SDL proVirB as forward primer and SDR proVirB as reverse primer, whereas primers for *virE* are SDL proVirE as forward primer and SDR

proVirE as reverse primer. Each PCR tube was allocated with the total amount of 25 µL of reaction mixture as in Table 3.3. After the process, the PCR products were run on a 2.0% agarose gel (0.4 g agarose powder in 20 mL TBE buffer) at 80 V for 45 minutes.

**Table 3.3 : Reaction mixture of PCR reaction for amplification of *virB* and *virE* promoters.**

| **Solution** | **Amount (µL)** |
|---|---|
| DNA template | 0.50 |
| Takara 5X PrimeSTAR Buffer- $Mg2^+$ plus | 5.00 |
| 2 mM TOYOBO dNTP | 2.50 |
| 10 mM Primer 1 (forward primer) | 1.00 |
| 10 mM Primer 2 (reverse primer) | 1.00 |
| Takara PrimeSTAR HS DNA polymerase | 0.25 |
| DMSO (2.0%) | 0.50 |
| $dH_2O$ | 14.25 |
| Total | **25.00** |

The conditions used for the PCR was as follow:

96°C 15 seconds

Denaturation

| | | | |
|---|---|---|---|
| | 96°C | 15 seconds | 25 cycles |
| Annealing | 60°C | 15 seconds | |
| Elongation | 70°C | 60 seconds | |
| Denaturation | 96°C | 15 seconds | 5 cycles |
| Annealing | 65°C | 15 seconds | |
| Elongation | 70°C | 60 seconds | |
| | 70°C | 60 seconds | |
| | 10°C | ∞ seconds | |

### 3.5.5 Purification of PCR Product by Ethanol Precipitation

Purification of PCR products purpose is to remove excessive reaction mixture elements such as primers, dNTPs and polymerases before the product are used for the next reaction. Besides, this step enables mixing of several tubes of PCR product to increase the overall concentration of the PCR product. The procedure for this step is as described in Section 3.5.3.

## 3.6 Gene Insertion into Plasmids and Cells

### 3.6.1 Site-Directed Mutagenesis (SDM)

SDM inserts the gene of interest into the plasmids. The materials needed for this procedure are high concentration of PCR product from Section 3.5.4, target plasmid (pD17 and pD15), DNA polymerase, DNA polymerase buffer, dNTPs, DMSO and deionised distilled water. Purified PCR products from Section 3.5.4 serve as primers for this reaction. The reaction mixture of SDM is given in Table 3.4.

**Table 3.4 : Reaction mixture of SDM.**

| **Solution** | **Amount (µL)** |
|---|---|
| Purified PCR product | 5.25 |
| Takara 5X PrimeSTAR Buffer- $Mg2^+$ plus | 2.50 |
| 250 mM TOYOBO dNTP | 2.50 |
| Takara PrimeSTAR HS DNA polymerase | 0.50 |
| $dH_2O$ | 14.25 |
| Total | **25.00** |

The PCR conditions used are as follow:

| | | | |
|---|---|---|---|
| | 96°C | 60 seconds | |
| | 96°C | 30 seconds | |
| Denaturation | 60°C | 60 seconds | 20 cycles |
| Annealing | 70°C | 390 seconds | |
| Elongation | 70°C | 420 seconds | |
| | 10°C | ∞ seconds | |

### 3.6.2 *DpnI* Digestion

*DpnI* digests the plasmids without gene inserts. The materials needed for this procedure are *DpnI*, 10x *DpnI* buffer, PCR product from Section 3.6.1, and distilled water. The reaction mixture for *DpnI* digestion is as shown in Table 3.5 below. All tubes were then incubated in 37°C water bath overnight.

**Table 3.5 : Reaction mixture for *DpnI* digestion.**

| Solution | Amount (µL) |
|---|---|
| DpnI | 2.0 |
| 10x DpnI buffer | 5.0 |
| PCR product | 10.0 |
| $dH_2O$ | 33.0 |
| Total | **50.0** |

### 3.6.3 Purification of PCR Products after *DpnI* Digestion by Ethanol precipitation

The purpose of this step is to purify the PCR products from Section 3.6.2. The procedure is as described in Section 3.5.3.

## 3.7 Screening for Plasmids of Interest

### 3.7.1 Transformation by Electroporation

This step purpose is to insert the purified PCR products from Section 3.6.3 into pD17 and pD15. The procedure is as described in Section 3.3.1.

### 3.7.2 Colony PCR

Colony PCR amplifies and screens for the genes of interest from the plasmid in the cell (*E. coli* DB3.1). The materials needed for this procedure are DNA polymerase, DNA polymerase buffer, dNTPs, a set of forward and reverse primers and deionised distilled water.

The forward primer used was F-DT-106 whereas the reverse primers used were SDL proVirB for *virB* gene and SDL proVirE for *virE* gene. The reaction mixture for this colony PCR tube is given in Table 3.6.. After the process, the PCR products were run on a 2.0% agarose gel at 80 V for 45 minutes.

**Table 3.6 : Reaction mixture for colony PCR.**

| Solution | Amount (µL) |
|---|---|
| Takara 10x Ex-Taq buffer | 1.00 |
| 2mM TOYOBO dNTP | 1.00 |
| 10µM primer 1 (forward primer) | 1.00 |
| 10µM primer 2 (reverse primer) | 1.00 |
| Takara Ex-Taq polymerase | 0.05 |
| $dH_2O$ | 5.95 |
| Total | 10.00 |

The conditions used for this reaction are as follow:

| | | | |
|---|---|---|---|
| | 94°C | 180 seconds | |
| | 94°C | 30 seconds | |
| Denaturation | 55°C | 60 seconds | 35 cycles |
| Annealing | 72°C | 35 seconds | 35 cycles |
| Elongation | 72°C | 300 seconds | 35 cycles |
| | 10°C | ∞ seconds | |

# CHAPTER 4

# RESULTS

## 4.1 Electrocompetent Cells Competency Test

After transforming electrocompetent *E. coli* DB3.1 with pUC19, the overnight incubation in 37°C results in colonies as shown in Figure 4.1.

Given the number of colonies on plate are approximately 350, the concentration of pUC19 (plasmid) used was 10 pg/µL (only 1 µL used, hence 10 pg), and the dilution factor is 2.5, the competency of electrocompetent *E. coli* DB3.1 can be calculated as below:

Cell competency = Number of colonies x Concentration of DNA (µg) x Dilution factor

$= (35 \times 10) \times (10 \times 10^{5}) \times 2.5$

$= 8.75 \times 10^{8}$ cfu/µg plasmid

An acceptable competency for electrocompetent cell ranges between $10^{6}$ and $10^{10}$. Therefore the electrocompetent *E. coli* DB3.1 cells prepared was sufficiently competent for the cloning experiments.

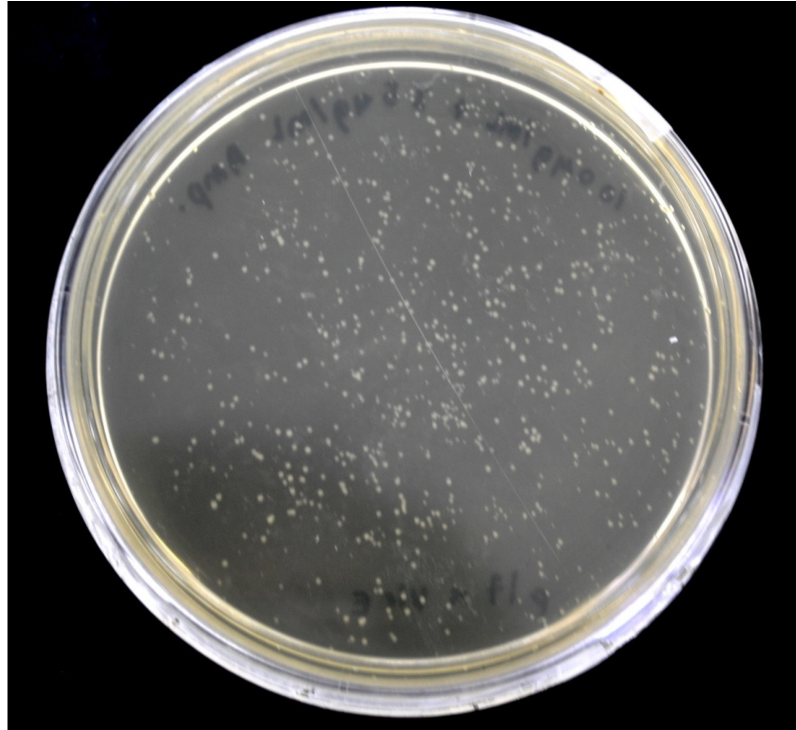


**Figure 4.1 : Electrocompetent *E. coli* DB3.1 transformed with pUC19 on 2x YT with 100 µg/mL Amp selection plate.**

## 4.2 Propagation of Plasmids

### 4.2.1 Transformation of E.coli BD3.1 with pD15 and pD17

The plasmids pD15 and pD17 was used to transformm *E. coli* DB3.1 using the heat shock transformation method and then the transformants were plated onto 2x YT with 35 μg/mL Amp. The plate was then incubated at 37°C overnight. A lower concentration of Amp was used to induce growth of transformants. The transformant colonies on the Amp selection plates are shown in Figure 4.2.

Since both pD15 and pD17 carry two types of antibiotic resistance genes which are Amp and Cm, a transformant colony was picked and purified by dilution streaking on a fresh 2x YT plate with 12 μg/mL Cm. The purpose of this step is to confirm the transformants are indeed carrying pD15 or pD17 and not contaminants.

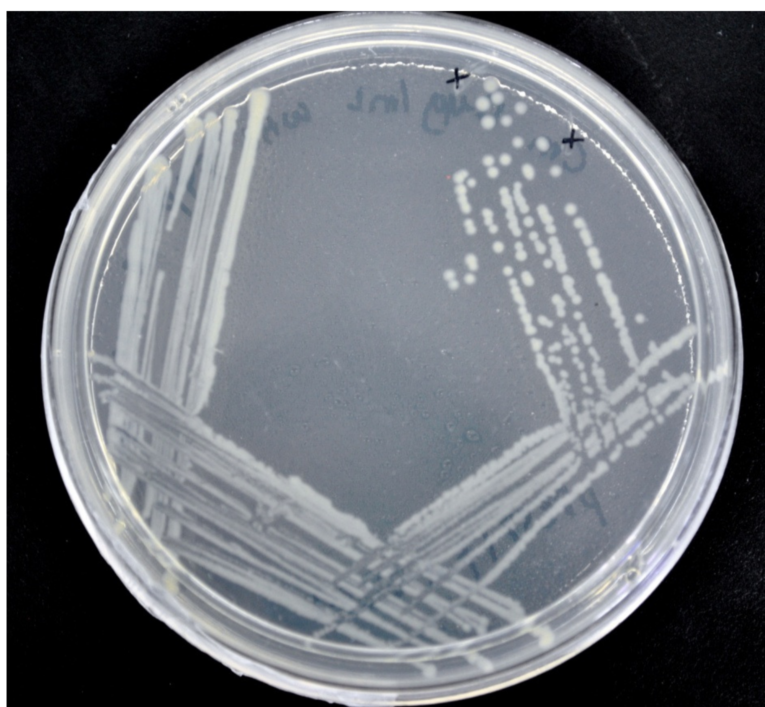


**Figure 4.3 : Dilution streaking of *E .coli* DB3.1 transformed with pD17 plated on 2x YT with 12 μg/mL Cm**.

### 4.2.2 pD15 and pD17 Extraction

A single colony of *E. coli* DB3.1 transformed with pD15 and *E. coli* DB3.1 transformed with pD17 on 2x YT plate with 12 µg/mL Cm (as mentioned in Sections 4.2.1 and 4.2.2) was picked out of their plates respectively. They were inoculated in separate 3 mL of 2x YT broth at 37°C overnight. The plasmids were then extracted out and purified using ethanol precipitation. The purified plasmids were then electrophoresised on a 0.7% agarose gel at 80 V for 45 minutes. The resulting gel image is as Figure 4.4.

From Figure 4.4, two separate single bands show that both pD15 and pD17 was successfully extracted and being no smearing signifies the plasmid extracted was pure. The data obtained from nano-spectrometry analysis on both plasmids are tabulated in Table 4.1 below. Although pure DNA samples should have an A260/A280 (purity) ratio of 1.8, 2.0 is an acceptable value to proceed with this project.

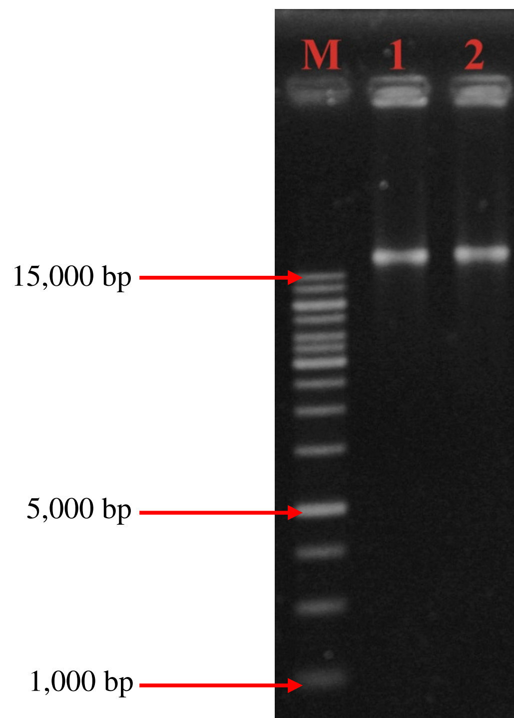



**Figure 4.4 : Electrophoresis gel image showing extracted and purified pD15 and pD17 plasmids on a 0.7% agarose gel.**

Lane M : 1,000 bp DNA ladder
Lane 1 : Purified pD15 plasmid
Lane 2 : Purified pD17 plasmid

**Table 4.1 : A260, A280, purity and concentration of plasmids pD15 and pD17 as obtained from nano-spectrometry.**

| | **pD15** | **pD17** |
|---|---|---|
| A260 | 0.017 | 0.020 |
| A280 | 0.008 | 0.006 |
| A260/A280 | 2.125 | 2.077 |
| Concentration (ng/μL) | 42.5 | 67.5 |

## 4.3 Isolation of v*irB* and v*irE* Promoters

### 4.3.1 DNA Extraction from *A. tumefaciens* GV3101

The DNA of *A. tumefaciens* was extracted as mentioned in Section 3.5.2 and purified as mention in Section 3.5.3. The extracted DNA was run on a 0.7% agarose at 80 V gel for 45 minutes. Figure 4.5 shows the electrophoresis gel image after the DNA extraction and purification of *A. tumefaciens* GV3101.

The single band obtained with no smearing in Figure 4.5 shows that the DNA was successfully extracted and pure. The data obtained from nano-spectrometry analysis on the extracted and purified DNA is tabulated in Table 4.2 below. A slightly lower A260/A280 (purity) ratio implies that there is a minor RNA contamination. Overall, the purity obtained is still an acceptable ratio to be classified as a pure DNA sample and to proceed with the experiment.

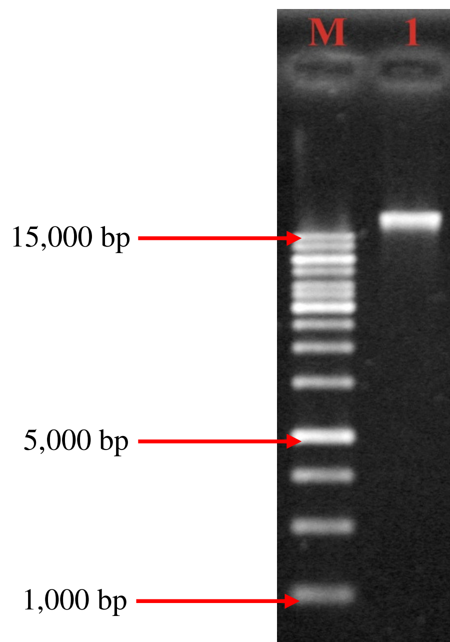



**Figure 4.5 : Electrophoresis gel image showing extracted and purified DNA from *A. tumefaciens* GV3101 on a 0.7% agarose gel.**

Lane M : 1,000 bp DNA ladder
Lane 1 : Purified *A. tumefaciens* GV3101 DNA

**Table 4.2 : A260, A280, purity and concentration of DNA extracted from *A. tumefacines* GV3101 as obtained from nano-spectrometry.**

| | ***A. tumefaciens* GV3101 DNA** |
|---|---|
| A260 | 0.187 |
| A280 | 0.110 |
| A260/A280 | 1.701 |
| Concentration (ng/µL) | 82.5 |

### 4.3.2 PCR Amplification of *virB* and *virE* Promoters

The product from Section 4.3.1 was used to obtain the two promoters of interest, *virB* and *virE*. After running procedure from Section 3.5.4 and purified them as mentioned in Section 3.5.5, the resulting gel image for *virB* is as shown in Figure 4.6 and *virE* as shown in Figure 4.8.

As summarized in Figure 4.7, *virB* promoter is 183 bp long and there are 20 extra bases for plasmid annealing, hence, the expected band should be 223 bp. As indicated in Figure 4.6, the PCR product bands are located slightly higher than the 200 bp band of the ladder. This indicates the *virB* promoters were successfully amplified. In each lane of Figure 4.6, the PCR different concentrations of DMSO were tested and all resulted in a right band of expected size.

As shown in Figure 4.9, the *virE* promoter is 191 bp long and the annealing primer is 40 nucleotides long. Therefore, the expected PCR product should be 231 bp long. From Figure 4.8, the *virE* promoter bands are slightly higher than the 200 bp band of the ladder. This suggests the *virE* promoters were successfully amplified. Two lanes of *virE* are amplified for higher yield.

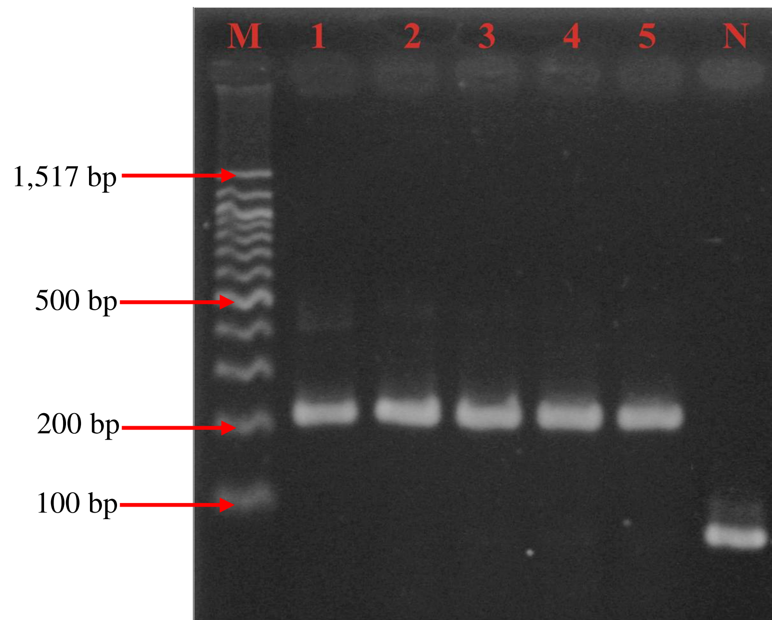



**Figure 4.6 : Electrophoresis gel image showing PCR product of *A. tumefaciens* GV3101 using F-SDR proVirB and F-SDR proVirB primers.**

| | |
|---|---|
| Lane M | : 100bp DNA Ladder |
| Lane 1 | : *virB* (No DMSO added into PCR reaction) |
| Lane 2 | : *virB* (0.5% DMSO added into PCR reaction) |
| Lane 3 | : *virB* (1.0% DMSO added into PCR reaction) |
| Lane 4 | : *virB* (2.0% DMSO added into PCR reaction) |
| Lane 5 | : *virB* (4.0% DMSO added into PCR reaction) |
| Lane N | : Negative control |

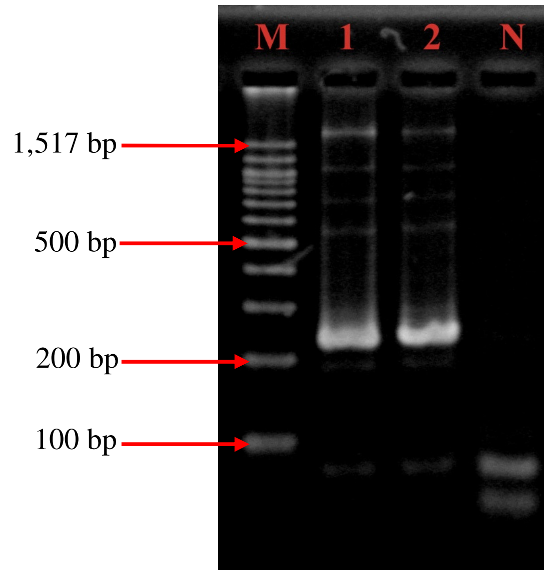



**Figure 4.8 : Electrophoresis gel image showing PCR product of *A. tumefaciens* GV3101 using F-SDR proVirE and F-SDR proVirE primers.**

| | |
|---|---|
| Lane M | : 100bp DNA Ladder |
| Lane 1 | : *virE* (PCR product) |
| Lane 2 | : *virE* (PCR product) |
| Lane N | : Negative control |

### 4.3.3 Ethanol Purification of Amplified *virB* and *virE* Promoters

The products from Section 4.3.2 were purified with method as described in Section 3.5.3. The purified genes were then run on a 2.0% agarose gel for 45 minutes at 80 V. Figure 4.10 shows the electrophoresis outcome.

Having no smear for both lanes 1 and 2 (respectively for *virB* and *virE* promoters) indicate that the purification process was successfully carried out and that contamination was minimal. Further analysis using nano-spectrometry on *virB* and *virE* promoters is tabulated as in Table 4.3. The A260/A280 ratio of the amplified *virB* and *virE* promoter fragments were 1.782 and 1.762 indicating the recovered DNAs were sufficiently pure for cloning experiments.

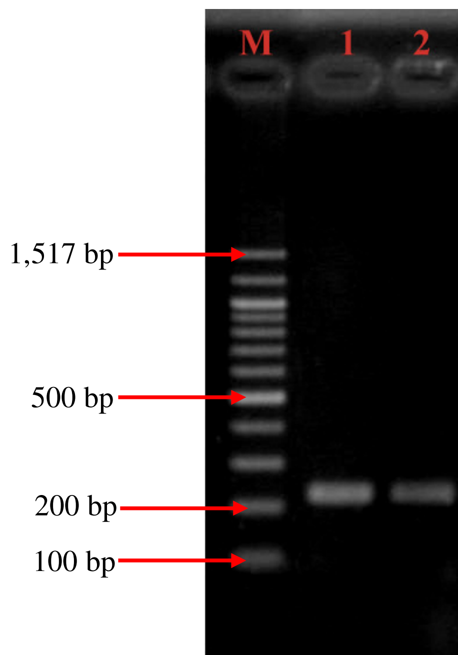



**Figure 4.10 : Electrophoresis gel image showing purified *virB* and *virE* promoters.**

Lane M : 100bp DNA Ladder
Lane 1 : Purified *virB*
Lane 2 : Purified *virE*

**Table 4.3 : A260, A280, purity and concentration of *virB* and *virE* after purification process respectively as obtained from nano-spectrometry.**

| | ***virB*** | ***virE*** |
|---|---|---|
| A260 | 0.271 | 0.141 |
| A280 | 0.152 | 0.080 |
| A260/A280 | 1.782 | 1.762 |
| Concentration (ng/μL) | 96.0 | 70.5 |

## 4.4 Screening for Modified Plasmids

### 4.4.1 Transformation

Protocol in Section 3.7.1 was carried out to insert the transformed plasmids with genes of interest into *E .coli* DB3.1. Figure 4.11 (A)-(D) shows the outcomes of the transformation. Fifteen colonies from each plate were then picked for colony PCR screening. Each colony was streaked on a master plate as shown in Figure 4.12 (A)-(D) below.

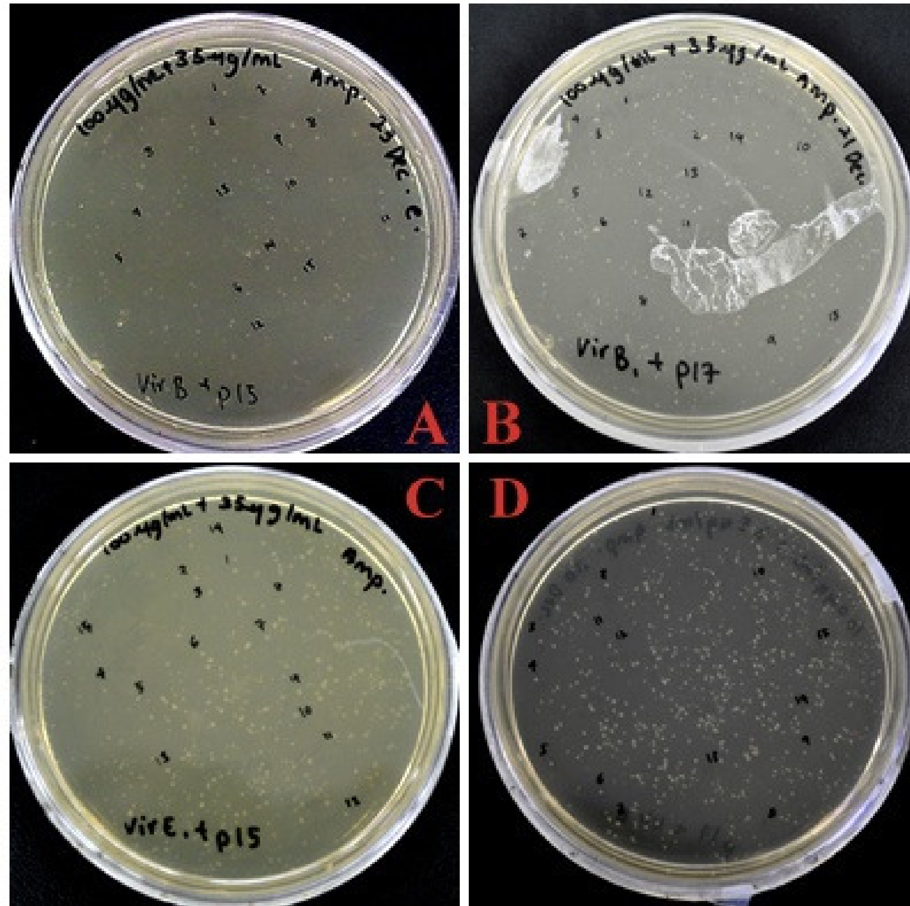



**Figure 4.11 : Each plate showing colonies resulting from transformation of *E.coli*. DB3.1 with plasmids A) pD15 with *virB* promoter, B) pD17 with *virB* promoter, C) pD15 with *virE* promoter, and D) pD17 with *virE* promoter.**

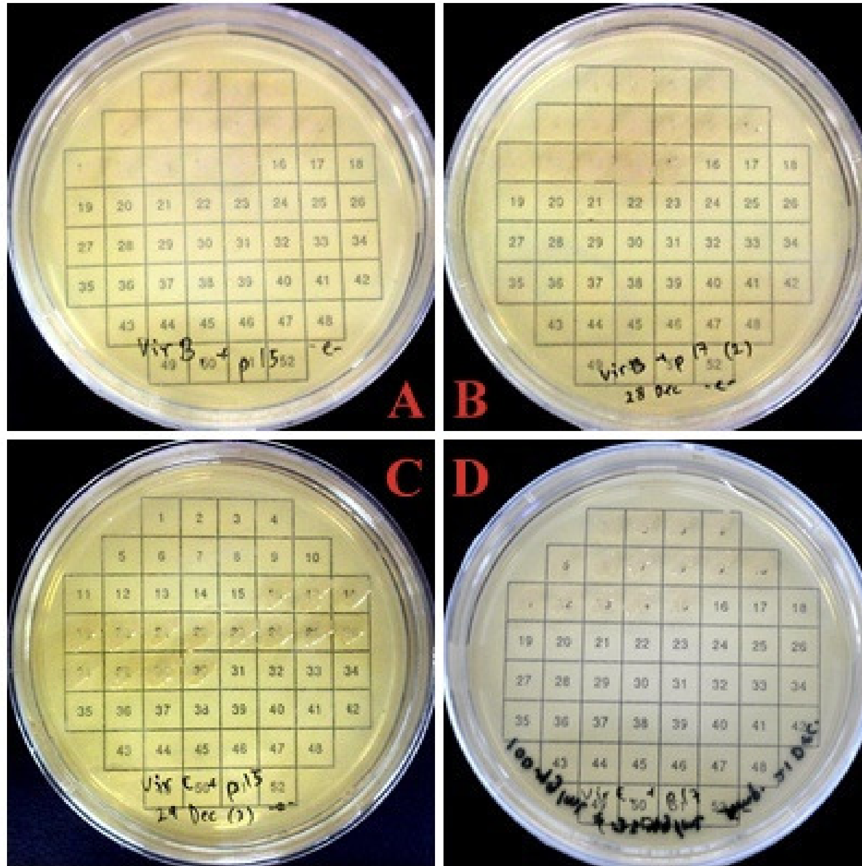
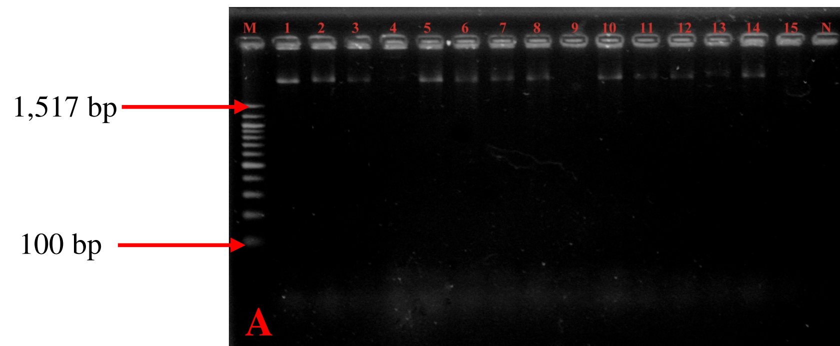


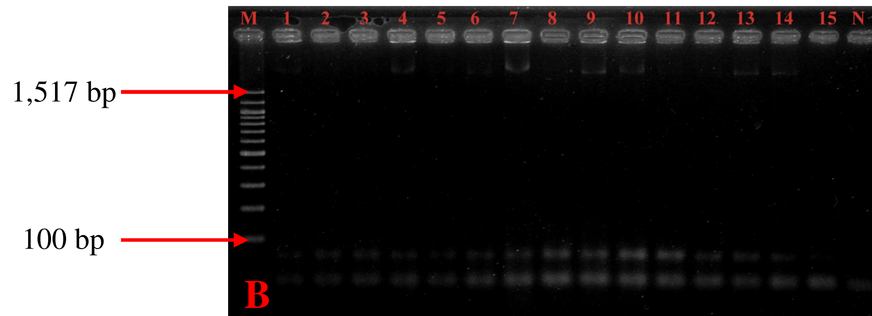

**Figure 4.12 : Master plates for transformation of *E.coli*. DB3.1 with plasmids A) pD15 with *virB* promoter, B) pD17 with *virB* promoter, C) pD15 with *virE* promoter, and D) pD17 with *virE* promoter.**

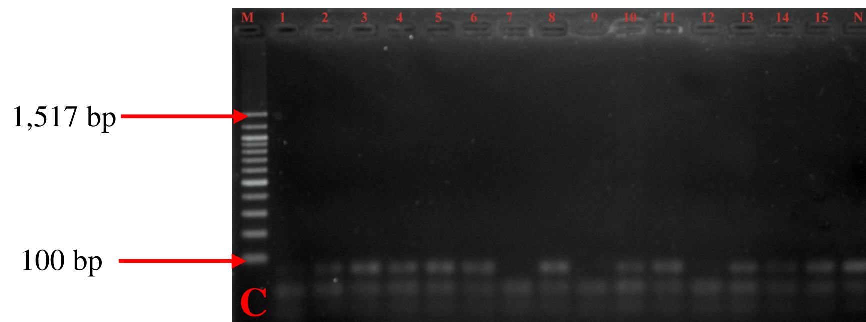
### 4.4.2 Colony PCR

Using method in Section 3.7.2, each colony on the master plates were screened using colony PCR. The agarose gel image for each screening is as Figure 4.13 (A)-(D) and the content of the lanes is as stated in Table 4.4.

As stated in Section 4.3.2, *virB* and *virE* prmoters are 223 bp and 231 bp long respectively. Their bands should fall just slightly above the 200 bp band of the ladder. However, these bands were not seen in Figure 4.13 (A)-(D). On the other hand, bands seen in Figure 4.13 (A)-(D) fall above the 1,517 bp (highest) band of the ladder and below the 100 bp (lowest) band of the ladder. Judging from the size, the genomic DNA of the bacteria has size larger than 1,517 bp thus, they ought to fall above the highest band of the ladder. The primers used are 40 bp long hence, primer-dimers formed during the PCR fall below the lowest band of the ladder.



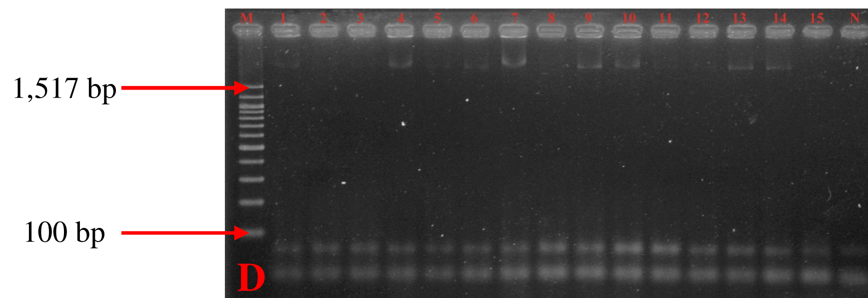




**Figure 4.13 : Electrophoresis gel image showing results of colony PCR screening for A) *virB* promoter in pD15, B) *virB* promoter in pD17, C) *virE* promoter in pD15, and D) *virE* promoter in pD17.**

**Table 4.4 : Table summarizing the lanes and master plate from which they origin of electrophoresis images of Figure 4.13 (A)-(D).**

| Figure | Master Plate (Figure) | Lanes | | |
|---|---|---|---|---|
| | | **M** | **1-15** | **N** |
| 4.11 (A) | pD15 with *virB* promoter (4.10 (A)) | 100 bp DNA | Colonies 1-15 from respective master plates | Negative control |
| 4.11 (B) | pD17 with *virB* promoter (4.10 (B)) | | | |
| 4.11 (C) | pD15 with *virE* promoter (4.10 (C)) | | | |
| 4.11 (D) | pD17 with *virE* promoter (4.10 (D)) | | | |

# CHAPTER 5

# DISCUSSION

## 5.1 Competent Cells

DNAs require external assistance to enter bacterial cells into order to replicate or have their genes expressed. These assistances may come in either chemical or physical method, in which both methods require competent cells. Competent cells are bacterial cells 'equipped' with the ability to take up extracellular DNA from the environment. They are measured in terms of transformation efficiency whereby the higher the competency of the cells, the higher the transformation efficiency. Highly competent cells accept extracellular DNAs more readily and result in better transformation efficiency. Likewise, cells with low competency do not take in extracellular easily and result in fewer numbers of transformants.

Cells used for chemical transformation methods usually require less competent cells because the chemical methods are less harsh and may not result in high cell death. The transformation efficiency using competent cells prepared for chemical method is usually in the range of $10^5$ to $10^6$ cfu/μg of plasmid DNA (Sambrook & Russell, 2001). The physical method, electroporation as used in this project requires highly competent cells. Electroporation involves electrical pulses to destabilize and create pores on the cell membrane through which plasmid DNA are able to enter the cells. This method is harsher compared to chemical method, hence reducing the number of surviving cells for DNA uptake. Therefore, electroporation require the use of highly competent cells in order to compensate for the low cell survival.

The preparation of highly competent cells is tedious which is why cells which are able to provide transformation efficiency as high as 1 $x10^{10}$ cfu/µg DNA are more commonly available commercially. Transformation of larger plasmids also affects the transformation efficiency. Transforming larger plasmids, which are more susceptible to shearing, would lower the transformation efficiency. In this project the size of plasmids used is 2.86 kb and the calculation in Section 4.1 shows that the transformation efficiency of the plasmids into *E. coli* DB3.1 prepared was 8.75 x $10^8$ cfu/µg DNA. Taking the size of the plasmids and that the competent cells were lab-prepared into consideration, the transformation efficiency indicates that the bacterial cells prepared are highly competent and excellent for cloning experiments.

## 5.2 Isolation of *vir* Promoters

The isolation of *vir* promoters using PCR generates a set of megaprimers, used to prime the SDM. F-SDR proVirB and R-SDR proVirB were used to isolate *virB* promoter and F-SDR proVirE and R-SDR proVirE were used to isolate *virE* promoter from the Ti plasmid. Unlike the usual primers used to amplify a certain DNA fragment, the primers used to generate the megaprimers contain sequences complement to the respective *vir* promoters and sequences complementary to the plasmids pD15 and pD17 where the promoters will be introduced. The primers will partially anneal to the Ti plasmid and amplify the *vir* promoter in a way that the sequences complementary to the Ti plasmid will anneal to the DNA template whereas the non-complementary sequences will not anneal during the first cycle. During the second cycle, however, the entire primer will anneal to the DNA fragment amplified in the first cycle and be extended. This results in *vir* promoters with additional sequences

complementary to pD15 and pD17 on both ends. Figure 5.1 is a schematic diagram of the isolation of the *vir* promoters.

Under normal laboratory growth conditions, the Ti plasmid copy number is approximately one per chromosome (Cho & Winnans, 2005). A very low copy number plasmid usually results in very low yield amplification. This is because there are lesser DNA templates for amplification. However, electrophoresis gel images in Section 4.3.2 (Figures 4.7 and 4.8) show that the amplifications of *vir* promoters were successfully carried out even with very low copy number of Ti plasmids. In fact, Table 4.3 in Section 4.3.3 shows that the yield obtained is sufficient to perform SDM which requires large numbers of primers (which are the *vir* promoters).

Besides, having two types of *vir* promoters to isolate, different sets of primers were used. As mentioned, F-SDR proVirB and R-SDR proVirB were used to isolate *virB* promoter and F-SDR proVirE and R-SDR proVirE were used to isolate *virE* promoter from the Ti plasmid. Since each primer is unique in their design, each set of these primers require individual optimize conditions for PCR. Without optimization, the amplified fragments may be heterogenous in size and migrate through the agarose gels as smear rather than as a discrete band (Sambrook & Russell, 2001). From the electrophoresis gel images (Figures 4.7 and 4.8), the PCR products appear as discrete band, signify the optimization for each primer was accomplished.

## 5.3 Insertion of *vir* Promoters into pD15/pD17

### 5.3.1 The Principles Behind *vir* Promoter Insertion with SDM

The idea is to introduce the *vir* promoters as a mutation into the pD15 and pD17 plasmids using SDM. As described in Section 5.2, the megaprimers are generated from Ti plasmids and they are *vir* promoters flanked by two regions that complement the pD15 and pD17 plasmids. These complementary regions are located on the upstream and downstream of the T7 promoter. During the PCR, the plasmids will denature, allowing the megaprimers to anneal, but because the *vir* promoter is not complementary to the T7 promoter, a loop will form in between the complementary regions. The DNA polymerase will then extend the primers and the whole plasmid, omitting the T7 promoter. Thus, the resulting PCR products are plasmids with *vir* promoters instead of T7 promoters. Figure 5.2 shows the SDM process.

### 5.3.2 Screening for Modified Plasmids

In order to screen for the modified plasmids (plasmids with *vir* promoters), two screening steps which are the *DpnI* digestion and colony PCR were carried out as described in Section 3.6.2 and 3.7.2 respectively and the result for colony PCR screening is shown in Section 4.4.2.. The primers used for the colony PCR screening process are F-DT-106 as forward primer for both *virB* and *virE* promoters but reverse primers R-SDR proVirB for *virB* promoter and R-SDR proVirE for *virE* promoter. If the *vir* promoters are successfully inserted into the plasmids, F-DT-106 will anneal 106 bp upstream the *vir* promoters and the reverse primers will to their respective *vir* promoters and amplify the *vir* promoters. On the contrary, if the *vir* promoters were not found in the plasmids, the primers will have no region to anneal on the plasmid and will anneal to each other, forming primer-dimers.

Figure 4.13 (A)-(D) shows that the bands fall above the 1, 517 bp band and/or below the 100 bp band of the ladder. Since the expected band for the *vir* promoters is in the range of 200 bp, this observation concludes that no *vir* promoters were amplified. The larger DNA fragments that migrated slower on the gel were the genomic DNA of *E. coli* whereas the smaller DNA fragments that migrated faster were the primer-dimers formed.

### 5.3.3 Troubleshooting

#### 5.3.3.1 Inefficiency in Amplifying DNA Fragments Longer than 2-3 kb

The sizes of plasmids where the *vir* promoters are incorporated are 7013 bp for pD15 and 6354 bp for pD17. Standard PCR conditions are sufficient to amplify fragments up to 2 kb but the conditions may not be optimum for fragments larger than 2 kb (Sambrook & Russell, 2001). Since the plasmids used for SDM are larger than 2 kb, further optimization is required in order to successfully insert the *vir* promoters into the plasmids.

Amplification of long DNA fragments may lead to undesirable damage on the template and product due to the high temperature. Since the plasmids are large, hence, more nucleotides, the damaged DNAs in the buffer will alter the capability of the buffer to maintain a control over the pH. Therefore, the buffer could not provide an environment with the optimum pH for the amplification. Besides, amplification of a large DNA fragment may also imply difficulties in denaturing the DNA for primer annealing. Elevating the denaturation temperature and time may increase sensitivity and allow complete DNA denaturation but this may put activity of the DNA

polymerase at risk (Miesfeld, 1999). The average extension time used in any PCR amplification is 1 minute per kb of DNA template (Sambrook & Russell, 2001) and this condition was applied for the SDM performed. However, it was suggested that for PCR with large template, an additional 30 seconds should be included (Foord & Rose, 1994). Thus, the short extension time may not be sufficient for the DNA polymerases to extent the entire plasmid.

Furthermore, a large DNA template may decrease the processivity of the DNA polymerase. Processivity is a measure of the average number of nucleotides added to the growing stand by the DNA polymerase per association or disassociation with the template. When a large fragment is used as the template, the chances of DNA polymerase to disassociate from the template and then reassociate are higher. This leads to a higher rate of incorporating mismatches to the daughter strand. Incorporation of incorrect bases at the 3' end of the daughter strand may cause the DNA polymerase to seize up, resulting in undesired PCR products (Sambrook & Russell, 2001). Therefore, due to the usage of a large plasmid as DNA template for SDM, more detailed optimization is required to successfully insert the *vir* promoters into the plasmids.

**5.3.3.2 Requirements for SDM**

SDM requires the use of high concentrations of primers in order to successfully incorporate the mutagenic primers into the plasmid (Sambrook & Russell, 2001). However, there is no formula to calculate the exact concentration of primers needed for a reaction. Trial and error is employed instead. Low concentration of primers will not result modified plasmids whereas high concentration will result in formation of

primer-dimers and smearing on the agarose gel. This requires optimization on annealing temperature whereby theoretically, the annealing temperature should be elevated to increase specificity of primer annealing. Increasing the annealing temperature would, at the same time, reduce the yield (Miesfeld, 1999). Thus, further optimization on concentration of primers needed for a reaction need to be carried out independently.

Another requirement of SDM is to use large number of amplification reactions (Sambrook & Russell, 2001). Having more cycles, however, will lead to higher rate of error in the PCR product. In other words, increasing the number of cycles during PCR increases the chances for not only the desired mutagenesis but also the unwanted mutagenesis to take place. A method to overcome the production of unwanted mutagenesis is to limit the number amplification cycles (Sambrook & Russell, 2001). Section 3.6.1 mentioned that the number of cycles performed for SDM was limited to only 20. This may not be sufficient to insert the *vir* promoters into the plasmids.

#### 5.3.3.3 Large Mutation Size Lowers the Efficiency of SDM

Generally, the larger the size of the mutation, the lower of efficiency of the mutagenesis. It was reported that mutations involving several hundreds of nucleotides, as in this project, experience a 50 fold decrease in efficiency (Sambrook & Russell, 2001). There are two main reasons for the decrease in efficiency. First of all, large mutation involves large mutagenic primers and the stability of these primers when they anneal to the homologous regions on the DNA template decreases in proportion to the distance between the regions (Figure 5.3). Secondly, a large primer increases the chances for partial annealing on incorrect sequence on the template. Consequently,

these lead to reduction in the efficiency of SDM to produce the desired mutation on the plasmids.

# CHAPTER 6

# CONCLUSIONS

. The methodology applied in the insertion of *vir* promoters into new plasmids is site-directed mutagenesis (SDM) whereby the *vir* promoters are introduced as mutation into the plasmids. The introduction of the *vir* promoters as mutation concurrently displaces the original T7 promoter from the plasmids. From the course of this project, one of out the two objectives mentioned was successfully achieved. The *virB* and *virE* promoters were successfully cloned from *A. tumefaciens* Ti plasmid regardless of difficulties involving low copy Ti plasmid number and individual optimizations required for each set of primer used for the isolation of the promoters. Unfortunately, the *vir* promoters were unable to be introduced into the plasmids due to four main factors which are the oversized mutation and plasmid, the conflicting requirements of SDM and the primer design. Further optimization is required in order to successfully produce modified plasmids with *virB* and *virE* promoters.

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