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Mutation of *recA* gene of *Vibrio cholerae*: Towards the development of attenuated *Vibrio cholerae* vaccine strains

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Biomedicine

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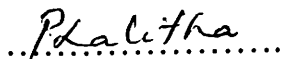16150, Kubang Kerian, Kelantan
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CERTIFICATE

This is to certify that the dissertation entitled

"Mutation of *recA* gene of *Vibrio cholerae*:

Towards the development of attenuated *Vibrio cholerae*

vaccine strains "

is the bonafide record of research work done by

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ABSTRACT

Despite over hundred years of study, the intestinal pathogen *Vibrio cholerae* still causes epidemic outbreaks in areas of the world. The endemic areas include India, Asia, Africa, the Mediterranean, and more recently, South and Central America, Mexico and the United States (Faruque *et al*, 1998). This Gram-negative bacterium colonizes the human intestine and causes potentially fatal diarrhoeal disease- the Cholera.

A number of genes associated with the virulence of *Vibrios* had so far been identified by researchers of cholera. These genes are gaining popularity among researchers especially in the manipulation of them towards the production of an effective cholera vaccine.

In this research, however, the *recA* gene was the center of interest. It was reported by Ghosh *et al* (1985) that the *Vibrio cholerae* have a RecA system that is analogous to the *Escherichia coli* RecA system. The RecA protein is the central enzyme for homologous recombination, DNA strand exchange, and DNA repair mechanism (Kuzminov, 1999). In the development of live attenuated, oral cholera vaccine, the mutation of *recA* gene was strongly recommended by some researchers to render the vaccine strains recombination deficient (Goldberg and Mekalanos, 1986; Taylor *et al*, 1994; Kenner *et al*, 1995; Kaper *et al*, 1994; Boyd and Waldor, 1999). Hence, the general approach of study is to mutate the *recA* gene in existing vaccine strain (VCUSM-2) to stabilize and prevent the strain from further acquiring the undesirable gene when released to the environment.

The study began with a deletional mutation in the 1.2kb *Vibrio cholerae recA* gene previously cloned onto the vector, pTOPO2.1. The *Bst*EII was recognized as the unique restriction enzyme site which existed twice in the middle of *Vibrio cholerae recA* gene at the location of 710bp and 782bp. Upon restriction with *Bst*EII, a small fragment of gene with the

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size of 72bp was deleted. The sticky overhang was polished and this was followed by a self-blunt end ligation of the linearized plasmid. The deletional mutation of *recA* gene produced a +1 frameshift mutation starting from the ligation junction to the down stream of *recA* gene. As a result a stop codon was introduced at 748bp of the gene, and hence results in a truncated *recA* gene product.

The successfully mutated *Vibrio cholerae recA* gene was then subcloned onto the pBluescriptIISK before it was transferred onto the pCVD442, a conjugatable suicide plasmid for conjugation with the cholera vaccine strain, VCUSM-2.

INTRODUCTION

The history of cholera

The modern history of cholera was recorded back in 1817, when cholera spread out of India and began the first of its seven pandemics. For the past centuries, the cholera bacterium had spread worldwide to the Middle East, South and Central America, Mexico and the United States (Faruque *et al.*, 1998; Faruque *et al.*, 2002; Reidl and Klose, 2002; Pourshafie *et al.*, 2002). Cholera is a boarder-less disease for no country can defy the invasiveness of cholera into its territory, let it be the underdeveloped, developing or well-developed countries.

It has been generally accepted that there are seven recorded pandemics of cholera in history. The first four pandemics were not known of their exact etiological agent, as it could not be recognized at that moment of time. The last three pandemics, however, were recognized to be the *V. cholerae* serogroup O1 (Faruque *et al.*, 2002; Reidl and Klose, 2002).

The seventh pandemic which began in 1961 in Indonesia was caused by the O1 serogroup of *Vibrio cholerae* and it continues until today (Lan and Reeves, 2002). It was not until 1992, large outbreaks of cholera began in Bangladesh and India which were found to be caused by a previously unrecognized serogroup of *Vibrio cholerae*. The bacterium resembled the *Vibrio cholerae* O1 in both the cultural and biochemical characteristics, producing the same virulence cholera toxin but did not agglutinate with the O1 antisera. The isolation of this new strain of vibrios were designated as O139, synonym as the Bengal strains. Since then, its presence had been reported from 11 countries in the South–East Asia region and became a threat of cholera outbreaks alongside with the *Vibrio cholerae* O1 (Faruque *et al.*, 2002).

The bacterium

The cholera vibrios are Gram-negative, slightly curved rods that belong to the family *Vibrionaceae*. They are facultatively anaerobic, non-spore forming, and motile with a single polar flagellum. *Vibrio cholerae* can grow well in alkaline condition up to pH10 but its growth is retarded when the pH drops below 6.0.

Vibrio cholerae is classified serologically based on the heat-stable somatic 'O' antigen on the surface of bacterium. By agglutination test with specific antisera, the *Vibrio cholerae* is classified into different serogroups. Currently more than 200 'O' serogroups has been described. The O1 serogroup can be further divided into different serotypes: Ogawa, Inaba and Hikojima based on specific antigenic formulas. However, there is no recognizable serotype given to the O139 serogroup so far.

It is the O1 or O139 strain which often found to be responsible for major outbreaks. The ability of the O1 and O139 serogroup to produce the cholera toxin (CT) is an essential determinant of its virulence. Only those which carry the *ctx* gene elements are capable of producing the cholera toxin and this *ctx* gene is actually acquired from a bacteriophage, the CTXΦ (Reidl and Klose, 2002; Faruque *et al.*, 1998).

Epidemiology, pathogenesis and impact of cholera outbreaks in the world

Outbreaks of cholera had been well documented by the World Health Organization (WHO). In year 2002, 40 countries worldwide had officially reported 123,986 cholera cases and claimed 3,763 deaths (Chaignat, 2004). However, the actual statistical value of morbidity and mortality may be under reported due to surveillance limitations.

Cholera is often associated with poor sanitation, overcrowding population, war and famine, all of which are often linked to the third world countries. However, cases of cholera

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are no longer restricted to the third world countries. As the bacteria exist as natural inhabitant of aquatic ecosystem, they are capable of invading the human system via contaminated food or drink without boundaries (Reidl and Klose, 2002)

Upon uptake of contaminated food or water, the bacteria adhere and penetrate into the mucosal lining of intestine. It then colonized the ileum and secreted the cholera toxin. The B subunit of the cholera toxin binds to the ganglioside G_{M1} receptor on the mucosal cells to allow the entrance of A subunit of cholera toxin into the cell. Activation of the A subunit will increase the level of intracellular cAMP and causes prolonged hypersecretion of water and electrolytes, resulting in massive watery diarrhea (Reidl and Klose, 2002). The disease is characterized by typical clinical symptoms such as passage of rice-watery stool, followed by vomiting, dehydration, hypovolemic shock and acidosis. Those inflicted lose gallons of water in just one day, and death could easily occur if treatment is not given promptly.

Despite the human suffering, cholera outbreak also causes panic, disrupting the social and economic structure as well as deterring the development of affected communities. For example, the cholera outbreaks in Peru in 1991 had cost the country USD770 million due to food embargoes and adverse effects on tourism (Chaignat, 2004).

Diagnosis, treatment and control measures

The clinical diagnosis is suggested by strikingly severe, watery diarrhea with additional signs and symptoms as described above. An immediate wet mount of watery stool sample or hanging-drop preparation of rectal swab in alkaline peptone water may reveal the characteristic 'darting' motility of *Vibrio cholerae* under light microscope. This movement can be stopped by specific antisomatic antibody. The gold standard laboratory diagnosis of cholera is by performing the rectal swab culture on the Thiosulfate-Citrate-Bile salt-Sucrose

(TCBS) medium whereby the sucrose fermenting *Vibrio cholerae* appeared as large, yellow, smooth, round and glistening colonies against a bluish-green background. Other methods like slide agglutination test with specific antiserum, fermentation test (oxidase positive), fluorescent antibody test, and the more recent PCR based rapid detection techniques have also been applied in specialized laboratory to detect *Vibrio cholerae*.

Treatment of cholera involves the rapid replacement of fluid and ion loss by intravenous infusion of the isotonic solutions. To bypass the need for sterility or intravenous invasion, the oral rehydration salt (ORS) can be administered orally in the less severe cases of cholera. The ORS is a simple regimen to revert the loss of water and electrolytes in severely diarrheal patient. It is especially useful for the third world countries where medical devices are inadequate. Besides, ORS is also convenient for immediate home treatment which is often life saving to a cholera patient. It was reported that a simple ORS treatment can reduced the mortality rate of cholera up to ten fold (Faruque *et al.*, 1998). Most antibiotics play no significant value in treatment of cholera although some, for instance tetracycline may shorten the duration of diarrhea and reduced fluid loss.

The WHO had recommended the improvement of water supply and sanitation as the most sustainable approach to prevent cholera. In terms of human behaviour in relation to personal hygiene and proper food handling, community health education plays an important role in this aspect (Chaignat, 2004). Preventative measure by using vaccine against cholera is gaining popularity for the prospective protection of population at risk in the endemic areas. It is believed that the oral cholera vaccine has greater values over the parenteral vaccine as it mimics the natural invasion of the bacteria into human body to elicit a mucosal immune response.

The importance of *recA* gene

The *recA* gene produces a 38 kDa RecA protein. The RecA protein is the key enzyme which involves in homologous recombination by catalyzing the pairing of a foreign ssDNA into the complementary region of dsDNA in a bacterium. On the other hand, it is also a co-protease for activating the LexA protein in self-repair mechanism when there is injury in the bacterial DNA. Under normal circumstances, LexA binds to the SOS boxes of DNA which represses the expression of both the *lexA* and *recA* gene. Upon injuries to the DNA, the RecA protein will be triggered and it functions as a co-protease to cleave the LexA (Vierling *et al.*, 2001).

The *recA* gene homologs had been cloned from many different bacterial species and the sequence as well as function of *recA* gene was found out to be highly conserved among bacteria (Vierling *et al.*, 2001). The ultimate involvement of *recA* gene in homologous recombination and DNA repair mechanism is perhaps the most striking features that lured scientists into the study of *recA* gene in many microorganisms.

One drawback of the live oral vaccine is the doubt of its safety after being shed by the vaccinees to the environment. It is feared that these vaccine strains, although attenuated in the first place might somehow regain the virulence gene from the wild type strains in environment and further undergoes undesirable mutation (Taylor DN *et al.*, 1994; Kenner JR *et al.*, 1995; Kaper JB *et al.*). The concern becomes a string that leads researchers into the study of *recA* gene in *V.cholerae*. By the mutation of *recA* gene in *V.cholerae*, it is hoped that the mutation will render the organism homologous recombination deficient. It will then become an environmentally safe vaccine strain that could no longer undergo any natural genetic alteration when release to the environment.

REVIEW OF LITERATURE

To manipulate and mutate the *recA* gene is like a safety measure in the development of live cholera vaccine. The *recA* mutant will prevent the vaccine strains from picking up possible virulence genes when release to the environment. Since the discovery of *recA* system in *Vibrio cholerae* dated back in 1985 by Ghosh and colleagues, they had created a new window in the cholera research. In their study, the researchers had designed a probe from the *recA* gene of *Escherichia coli* and then performed the Southern blot hybridization with the *Vibrio cholerae* genome. This had successfully revealed a complementary sequence which bind with the probe. The sequence hence was purported as the possible *recA* gene of *Vibrio cholerae*. Next, they pursued to examine if the gene had properties of *recA* proteolytic function in SOS response. By using the DNA damaging agent (Furazolidone), the SOS-like response (as measured by beta phage induction) was observed.

Following the discovery of *recA* gene in *Vibrio cholerae*, a pilot study of the *recA* gene mutation was carried out by Mekalanos and Goldberg (1986). The study was triggered when they realized the cholera toxin gene (CTX) was only found in the toxigenic strains but not in the non-toxigenic strains of *Vibrio cholerae*. Besides, this CTX gene existed at multiple location of the cholera chromosome and capable of undergoing tandem duplication and amplification (Mekalanos, 1983). This indicated active recombination events frequently occurred in the *Vibrio cholerae* and causing it virulence. For further study of this recombination event, they tried to mutate the *recA* gene to produce a *recA⁻ Vibrio cholerae* strain which is deficient in homologous recombination.

First, they randomly cut the *Vibrio cholerae* genome into fragments with *Sau*3A and cloned them into the *Bam*HI site of a plasmid, pBR327. This had produced a plasmid-based

library of the *Vibrio cholerae* genome. The plasmids were then transformed into a special *Escherichia coli* host to which its *recA* gene was deleted. By using the complementation method, they grown the bacteria in media added with DNA damaging agents, Methyl methane sulfonate (MMS). Only the vector which carried the *recA* gene of *Vibrio cholerae* is capable of complementing the *Escherichia coli* host for its resistancy to the MMS. In this way, they had successfully isolated the *recA* gene from the genomic library, and they performed a 4-base pair insertional mutation into the *Xba*I site within the *recA* gene. This had introduced a nonsense mutation which leads to the production of a truncated RecA protein. The mutated *recA* was then transferred to the *Vibrio cholerae* genome by spontaneous recombination. The *recA⁻ Vibrio cholerae* strain was produced and it became susceptible to the damaging effects of UV light and was impaired in homologous recombination activity.

In the same year, the team presented another paper sequel to the previous research mentioned above, on the effect of *recA* mutation for the cholera toxin gene amplification and deletion events (Mekalanos and Goldberg, 1986). Here, they had inserted a kanamycin resistant cassette into the *ctx* loci of *Vibrio cholerae*. Under normal circumstances, *Vibrio cholerae* is sensitive to kanamycin not more than 150µg/ml. However, with the introduction of the kanamycin resistant gene, the bacteria spontaneously became highly resistant and capable of growing on 3mg/ml of kanamycin in media. When performing a Southern blot analysis, they realized that *ctx* element was amplified up to 20 fold. The same approach was repeated for *Vibrio cholerae* with a mutated *recA* gene. They then found out that the amplification process was completely inhibited in the absence of a functional *recA* gene. This clearly showed that the *recA* gene had an effect on *ctx* gene amplification and was essential for the process to happen. On the other hand, they had also isolated spontaneous kanamycin

sensitive colonies. They noticed that spontaneous deletion of the *ctx* genes happened in both the *recA+* and *recA⁻ Vibrio cholerae* strains, however it occurred at 21-fold lower in the *recA-* strains.

Another study carried out by Hamood AN and colleagues (1986) had also successfully isolated and characterized the *Vibrio cholerae recA* gene. Their method of *recA* isolation was similar to the study of Mekalanos and Goldberg (1986) as described above. The *recA* gene was a 3.6kb fragment restricted from the *Vibrio cholerae* chromosomal DNA. They then performed a DNA hybridization experiment to detect homology between the *Escherichia coli recA* and the *Vibrio cholerae recA* but failed to prove the homology between them. Nevertheless, they had observed that RecA protein from both different species shared a common epitope as the antiserum produced against purified *Escherichia coli recA* cross-react with the *Vibrio cholerae recA* gene. In this study, however, no mutation of the *Vibrio cholerae recA* gene was performed.

As it moves to 1994, another research team by Stroeher and colleagues had cloned the *recA* gene of O1 *Vibrio cholerae*. A deletional mutation was then performed in the *recA* gene and the resulting mutant showed typical sensitivity to UV light exposure and DNA damaging agents as well as impaired in homologous recombination. They also performed the infant mouse cholera model by introducing the animal with *Vibrio cholerae recA-* strains. They concluded that the *recA* deletion mutant of *Vibrio cholerae* do not show altered virulence in the animal in causing cholera and hence it is ideal for complementation study. This finding is supportive to the mutation of *recA* gene in production of live cholera vaccine.

On the contrary, a research group by Kumar *et al* (1994) had disclaimed the fact by saying that *recA* mutation would indeed reduce the adherence and colonization of *Vibrio*

cholerae. Here they constructed a frameshift mutation of the *recA* gene of both classical and El Tor strains of *Vibrio cholerae* and subsequently filter mating the mutated *recA* with the wild type *Vibrio cholerae*. When experimenting the adherence and colonization studies of the mutated *recA* in animal model, they found out that the *recA* mutants shown 65% decrease in the adherence of rabbit intestine and marked decrease in the colonization of infant mice intestine.

However, the importance of *recA* mutation in the production of live vaccines is revealed in a study carried out by Kaper and colleagues (1994). The research group had produced an attenuated live *Vibrio cholerae* O1 vaccine strain, namely CVD 103-HgR. This live vaccine strains had raised the concern of many who question if the bacteria is capable of reacquire the virulence gene via horizontal gene transfer. To clear the dark clouds and stamp out suspicion, Kaper and colleagues had performed a series of mating experiments under a variety of *in vivo* and *in vitro* condition to testify the possibility of gene reacquisition in their vaccine strains. They mated their vaccine strain CVD 103-HgR with three wild type of *Vibrio cholerae* O1 strains in different biotypes and serotypes. All the wild type strains were marked with antibiotic resistant gene. The *in vitro* mating condition were also varied in terms of donor/ recipient ratio (10:1 and 1:10), broth mating and plate mating, as well as mating in the suspension of different water samples from the environment. Of all, none of the *in vitro* mating experiments yielded transconjugants after screening with antibiotic sensitivity.

On the other hand, the *in vivo* mating experiment was carried out by inoculation of a cocktail of CVD 103-HgR and wild type *Vibrio cholerae* into the suckling mice model. The experiment also showed no transconjugant after a 24-hour *in vivo* incubation period. Although all data showing negative results, the group however did not deny that natural transfer of *ctx*

genes into their vaccine strains would never occur. This statement had inadvertently support the mutation of *recA* gene in cholera vaccine development for it will render the vaccine strain stable when release into the environment.

More importantly, this approach will permanently close the door of suspicion among the disputing researchers and assure the publics of the safety measure undertaken in the genetic manipulations of live vaccine strains.

LACUNAE IN THE LITERATURE

When the Bengal strain of *Vibrio cholerae* (O139) first emerged in 1992, it was not given much attention from researchers who thought it was just another non-O1 passerby which causes occasional cholera-like disease. Not until this Bengal strain began to show its persistency in causing large cholera outbreak in parallel with the O1 serogroup, the researchers came to realize that the O139 had determined to stay. The O139 is now considered as the second etiologic agent of cholera by researcher worldwide (Faruque *et al.*, 2002).

Due to the negligence of the O139 *Vibrio cholerae* in the earlier time of its occurrence, the research carried out on this strain was much as less compared to the O1 strain. The research on the *recA* gene of O139 *Vibrio cholerae* is even scarce. So far there is no published work on the cloning and mutation of *recA* gene of the O139 *Vibrio cholerae* yet.

OBJECTIVE OF THE STUDY

The objective of study is to perform a deletional mutation on the *recA* gene of O139

Vibrio cholerae and then sub-clone it onto conjugatable suicide plasmid, pCVD442 for the

use of conjugation with the candidate vaccine strains (VCUSM-2) in future.

MATERIALS AND METHODS

1. Simulation with Vector NTI program

Vector NTI is a versatile bio-informatic tool which functions as a database manager for a personal collection of nucleic acid and protein sequence profile. It is widely used as a tool for remote and local database searching, virtual cloning, oligonucleotide designing, identification of restriction enzyme site, gel electrophoresis modeling *etc.*

In this study, the Vector NTI was used to identify the unique restriction enzyme site in the *Vibrio cholerae recA* gene. The entire restriction enzyme available in the database was selected. The restriction pattern of *recA* gene was examined to identify the unique restriction enzyme which restricts the *recA* gene in the middle of its open reading frame while not restricting the vector. Following this, a deletional mutation was simulated in the *recA* gene with the unique restriction enzyme site chosen.

2. Media preparation

2.1 Preparation of Luria Bertani (LB) agar

LB agar was prepared by dissolving 15g of tryptone (Merck), 5g of yeast extract (Oxoid), 10g of Sodium chloride (Amresco) and 15g of agar (Amresco) into 750ml of deionised distilled water. The pH was adjusted up to 7.0 by using 1M of sodium hydroxide and the final volume was made up to 1000ml. It was then autoclaved at 121°C for 15 minutes before pouring into sterile Petri plates with 25ml each. The media was cooled down to 40°C before the addition of appropriate antibiotic. The autoclaved media was stored in cool room for not more than one month.

2.2 Preparation of Luria Bertani (LB) broth

LB broth was prepared by dissolving 15g of tryptone, 5g of yeast extract and 10g of Sodium chloride into 750ml of deionised distilled water. The pH was adjusted up to 7.0 by using 1M of sodium hydroxide and the final volume was made up to 1000ml. It was then aliquoted 10ml each into universal bottles and autoclaved at 121°C for 15 minutes and stored at room temperature.

2.3 Concentration of antibiotics

The Ampicillin and Kanamycin were two antibiotics used in this study for isolation of bacteria host carrying the plasmid with antibiotic resistant gene. They were added into the LB agar or broth with the suitable concentration as recommended.

Table 1: Concentration of antibiotics used in LB agar and LB broth

Antibiotics	Final concentration
Ampicillin	100 µg / ml
Kanamycin	50 µg / ml

3. Restriction enzyme digestion

3.1 Restriction enzyme and its principle

Restriction enzymes (also known as restriction endonuclease) are isolated from bacteria that used them as a defense mechanism against viral infection. They are many restriction enzymes available, and each has a specific DNA recognition sequence. Wherever this particular sequence occurs along the chromosome, the enzyme cleaves the sugar phosphate backbones of DNA and produces fragments.

The restriction enzymes used in this study were obtained from Fermentas, USA as shown in Table 2 below:

Table 2: Restriction enzymes used in this study

Restriction enzymes	Cleavage site	Optimal buffer	Optimal temperature
*Eco*RI	5'...G AATTC...3' 3'...CTTAA G...5'	*Eco*RI unique buffer	37°C
*Bst*EII	5'...G GTNACC...3' 3'...CCANTG G...5'	Buffer O$^+$	37°C
*Sal*I	5'...G TCGAC...3' 3'...C AGCT G...5'	Buffer O$^+$	37°C
*Sac*I	5'...GAGCT C...3' 3'...C TCGAG...5'	*Sac*I unique buffer	37°C

3.2 Methodology

The restriction digestion of DNA was performed in a 0.5ml tube. The reaction mixture was prepared by adding the following reagents as shown in Table 3. The volume was made up to 20μl and mixed well before incubated at 37°C for 2 to 4 hours. Upon completion of digestion, the restriction pattern was studied by loading the sample in agarose gel electrophoresis. When double digestion was performed, the most appropriate buffer as recommended by the manufacturer was used.

Table 3 : Preparation of the restriction digestion reaction mix

Contents	Volume
Sterile deionised distilled water	15.0 μl
10X buffer	2.0 μl
Restriction enzyme	1.0 μl
DNA	2.0 μl
TOTAL	**20.0 μl**

3.3 T4 DNA polymerase treatment

Following restriction digestion of DNA, the T4 DNA polymerase enzyme (Fermentas, USA) was used to polish the sticky end of restriction product in order to convert it into a blunt end product. This was done by first heat inactivation of the restriction digestion mixture, followed by the addition of 1µl of T4 DNA polymerase and 0.2µl of dNTPs (10 mM). It was then incubated at 16°C (the lower compartment of refrigerator) for 20 minutes followed by heat inactivation at 65°C for 15 minutes.

4. Agarose gel electrophoresis

4.1 Principle of agarose gel electrophoresis

The DNA product from restriction analysis or Polymerase Chain Reaction (PCR) can be visualized by agarose gel electrophoresis. Electrophoresis is a process of separating molecules based on their size and charge in an electric field. The support medium used is a thin gel made up of agarose.

When the agarose polymerized, it forms a matrix with holes in between the interlocking fibers. This allows the DNA to sieve through the gel when electrical current is applied. Due to the phosphate group in DNA, it is negatively charged in the electric field and hence it moves from cathode to anode. The velocity of movement for a DNA molecule will depend on its size. The smaller molecule moves faster than the larger molecule as it can sieve through the gel matrix easily whilst the larger molecule will have hindered movement when passing through the small holes in gel.

The concentration of agarose gel used in this study was 0.8%. This concentration may vary depends on the desired resolution power to visualize the bands.

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4.2 Preparation of 50X Tris Acetate EDTA (TAE) stock

A 50X stock solution of TAE was prepared by dissolving 121g of tris-base in deionised distilled water and adding 50ml of 0.5M EDTA and 28.5ml glacial acetic acid. Deionised distilled water was added to make a final volume of 500ml and stored at room temperature. A working solution (1X TAE) was prepared by diluting 20ml of stock solution with 980ml deionised distilled water.

4.3 Preparation of 0.8%TAE agarose gel

By using the weighing scale, 0.8g of agarose gel (Pronadisa) was weighed and placed in a 250ml capacity conical flask. Then 100ml of 1X TAE was added into the flask and the agarose powder was dissolved with brief mixing. It was then boiled for 3 minutes in microwave. Once it was completely dissolved, the flask was allowed to cool to 50°C before addition of 0.5µl of Ethidium bromide (10mg/ml). The solution was mixed well and immediately poured into the agarose gel tray which was pre-assembled with desirable gel comb.

4.4 Running of agarose gel electrophoresis

After the agarose gel polymerized, it was transferred to an electrophoresis tank filled with 1X TAE buffer. The DNA sample to be visualized was placed in a new tube and added with gel loading buffer in 1:5 ratio. After gentle mixing, the samples were loaded into the wells of the gel. 4µl of the molecular weight marker (100bp or 1kb ladder) were loaded as well. The gel must submerge fully beneath the buffer and electrophoresis was run at 100V for 1 hour. When completed, the DNA bands were visualized under UV transilluminator. The Ethidium bromide added to the gel will intercalate with the DNA and upon exposure to UV light, the DNA fluores as orange bands.

4.5 Gel elution

After visualization with the UV transilluminator, the desired band of DNA can be eluted from the gel for further manipulation. In this study, the elution was carried out by using the ULTRAFREE-DA kit from Milipore. The agarose gel containing the desired DNA was carefully cut out with a sterile surgical blade and transferred into the elution tube. The tube was then centrifuged at 12,000rpm for 15 minutes at room temperature. Within the tube contained a filter which separates the DNA from the gel. The eluted DNA is stored at -20°C for further manipulation.

5. Ligation

5.1 Principle and methodology of ligation

Ligation of DNA occurs when two DNA termini are covalently linked to each other with the help of T4 DNA ligase. The T4 DNA ligase is an enzyme which catalyses the synthesis of phosphodiester bond between the 3'-hydroxyl and 5'-phosphoryl ends of DNA. It requires the Mg^{2+} and ATP as co-factors.

In this study, the T4 DNA ligase from Fermentas, USA was used. There are two different methods of ligation, namely the blunt end ligation and the sticky end ligation. Both methods were used in this study.

For blunt end self-ligation of the linearized vector, all the contents mentioned in Table 4 were added into a microcentrifuge tube, briefly vortex and spin down. The reaction was then incubated at 16°C (lower compartment of refrigerator) for overnight. On the next day, 1μl of T4 DNA ligase was added and incubated at room temperature for another hour. The ligation mix was then ready for transformation.

As for the sticky end ligation, the restricted vector and insert as well as distilled water (Table 5) were added into a clean microcentrifuge tube and incubated at 56°C for 5 minutes. Following this 1 0X l igation b uffer a nd T 4 D NA ligase were a dded and i ncubated a t 1 6°C (lower compartment of refrigerator) for overnight. On the next day, 1μl of T4 DNA ligase was added and incubated at room temperature for another hour. The ligation mix was ready for transformation.

Table 4: Blunt end ligation of linearized pTOPO2.1-*recA* (self-ligation)

Contents	Volume
Linearized pTOPO2.1-*recA*	5 μl
10X ligation buffer	2 μl
T4 DNA ligase	1 μl
Distilled water	12 μl
TOTAL	**20 μl**

Table 5: Sticky end ligation of restricted vector and insert

Contents	Volume
Vector	2 μl
Insert	10 μl
10X ligation buffer	2 μl
T4 DNA ligase	1 μl
Distilled water	5 μl
TOTAL	**20 μl**

6. Transformation

6.1 Principle of transformation

Transformation is a process whereby bacteria can uptake foreign DNA from the environment. For transformation to occur, the bacteria need to be 'competence'. Some bacteria are naturally competence and readily undergo transformation, for instance the *Haemophilus, Bacillus, Neisseria, Escherichia* species and etc. On the other hand, the transformation can also be induced by making the bacteria 'competence'. This can be done via physical or chemical induction. The most common method used are Calcium chloride method, Polyethylene glycol method or by electroporation.

6.2 Methodology of competent cell preparation

For Calcium chloride method, a single isolated colony from freshly streaked plate was inoculated into 2ml LB plain broth and incubated at 37°C in the static condition. On the next day, 300μl of the overnight culture were taken and inoculated into a 50ml LB broth. The culture was incubated in the shaker (250rpm, 37°C) for 3 to 4 hours. As the bacterial growth reached an optical density (OD 600nm) of 0.5, the culture was immediately put on ice to stop the growth. It was then centrifuged at 3000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet was gently resuspended with 6 ml of ice-cold 100mM Magnesium chloride. It was then incubated in ice for 45 minutes.

Then, the suspension was again centrifuged at 3000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet was gently resuspended with 3ml of ice-cold 100mM Calcium chloride. Both the chemicals altered the permeability of bacterial cell membrane and making it competence for transformation.

The competent cells can be used immediately for transformation. If storage is desired, 450μl of glycerol (15% of the 3ml competent cell suspension) is added to the competent cell as cryopreservative and aliquoted 150μl each into sterile microcentrifuge tube for storage at -70 °C.

The growth of bacteria was strictly monitored so as not to exceed 0.5 (OD600) and the entire preparation was carried out on ice as the cells were extremely sensitive against heat.

6.3 Methodology of transformation

For the Calcium chloride method of transformation, the suitable volume of ligation mixture or plasmid to be transformed was added into the competent cells and incubated on ice for 20 minutes. Then, heat shock was given to the cells by incubating the tube at 42°C for 30 to 45 seconds. This step is important to increase the permeability of cell by opening up the pores of membrane. Then the tube was immediately placed on ice and kept for 2 minutes. This is another critical step and the transfer to ice should be quickly done as prolong heating would kill the cells. Next, 350μl of fresh LB broth was added into the tube and incubated at 37°C for 1 hour. The LB broth was added to provide nutrient to the cells for recovery from the heat shock. During incubation, any plasmid which successfully transformed into the competent cells would express the antibiotic marker gene for selection of successful transformation later. After incubation, an appropriate amount of cells was inoculated on LB agar containing antibiotics which corresponding to the antibiotic marker gene in plasmid. The plates were incubated overnight at 37°C.

7. Patching and lysate preparation

A successful transformation on the LB agar must be further confirmed for the insert of interest. For this reason, around 12 colonies were chosen randomly from the plate and patched on a freshly prepared agar plate added with the same corresponding antibiotic. This was done to maintain the isolated colony as a stock for further use in future.

Patching was done by touching the colony with a stab inoculating wire and inoculated on the LB agar, as shown in Figure 1. After patching, the same loop was suspended in 20μl of distilled water in a 0.5ml tube for the lysate preparation. Then, the lysate was added with 2 drops of mineral oil and boiled at 100°C for 10 minutes. The content was then briefly spun down and 2μl from the lysate was used as template for PCR screening to confirm the insert of interest.
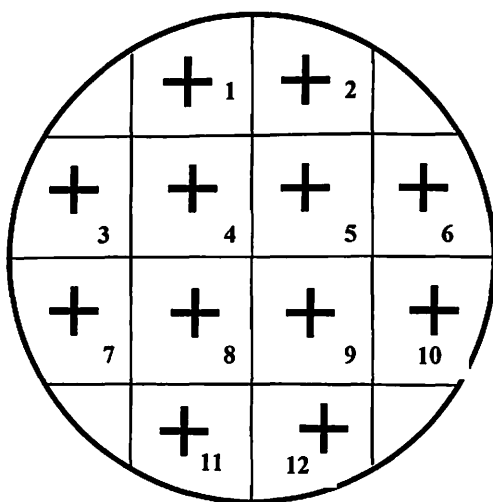
Figure 1: Patching on the LB agar