THE EXPRESSION OF A *BURKHOLDERIA PSEUDOMALLEI* ANTIGEN IN MAMMALIAN CELL

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Burkholderia pseudomallei is the causative agent of melioidosis which is a fatal disease of human and animals. This pathogen is an intracellular bacterium, which can survive in both phagocytic and non-phagocytic cells. Similar to other intracellular bacteria, the effective immunity that confers protection depends on the role of cytotoxic T lymphocyte (CTL). To study the role of cytotoxic T lymphocyte response in melioidosis, the assay requires a target cell that expresses the antigen derived from a pathogen. Therefore, the preparation of a target cell expressing *B. pseudomallei* antigen is the initial step for the study of CTL response against this pathogen.

In this study, an antigenic gene, pBps-1, of *B. pseudomallei* was successfully cloned into pcDNA 3.1(+). The pBps-1/pcDNA 3.1(+) recombinant plasmid could express a 18.7 kDa pBps-1 protein in *E. coli*, as detected by SDS-PAGE and Western blot analysis. The recombinant plasmid was subsequently transfected into J774A.1 cell by electroporation. The electroporation condition used for transfection was established by using the expression of reporter gene, green fluorescent protein (GFP), before transfecting this cell line with the obtained recombinant plasmid. However, the expression of pBps-1/pcDNA 3.1(+) in mammalian cell could not be detected by staining with Coomassie brilliant blue or Western blot analysis. Factors that may affect these results were discussed.

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Burkholderia pseudomallei เป็นเชื้อที่เป็นสาเหตุของโรคเมลิออยโดสิสซึ่งก่อให้เกิดการตายทั้งในคนและสัตว์ *B. pseudomallei* จัดเป็นแบคทีเรียซึ่งสามารถอาศัยและมีชีวิตอยู่ได้ภายในเซลล์ชนิดต่างๆทั้งเซลล์ฟาโกซัยท์ และ เซลล์ที่ไม่ใช่ฟาโกซัยท์ได้ ดังนั้นภูมิคุ้มกันที่มีประสิทธิภาพและสามารถป้องกันโรคนี้น่าจะขึ้นอยู่กับบทบาทของ cytotoxic T lymphocyte (CTL) ซึ่งมีบทบาทในการตอบสนองต่อแบคทีเรียที่อาศัยอยู่ภายในเซลล์ชนิดอื่นๆ การศึกษาการตอบสนองของ cytotoxic T lymphocyte ในโรคเมดิออยโดสิสต้องการเซลล์เพาะเลี้ยงที่มีการนำเสนอของแอนติเจนของเชื้ออย่างเหมาะสม ดังนั้นการเตรียมเซลล์เพาะเลี้ยงที่มีการแสดงออกของแอนติเจนของเชื้อ *B. pseudomallei* จึงเป็นขั้นตอนเริ่มต้นของการศึกษาบทบาทของ CTL ต่อเชื้อนี้

การศึกษานี้ได้ทำการโคลนยีน pBps-1 ของเชื้อ *B. pseudomallei* เข้าในพลาสมิด pcDNA 3.1 pBps-1/pcDNA 3.1(+), ที่ได้สามารถสร้างโปรตีนที่มีขนาด 18.7 kDa ใน *E. coli* จากนั้นนำพลาสมิดที่ได้เข้าสู่เซลล์ J774A.1 ด้วยวิธี electroporation เพื่อเตรียมเซลล์เพาะเลี้ยงที่มีการแสดงออกของแอนติเจนของเชื้อนี้ โดยตรวจหาประสิทธิภาพของวิธี electroporation ที่ใช้ทำการทดสอบโดยดูการแสดงออกของ reporter gene ซึ่งได้แก่ green fluorescent protein (GFP) ก่อนทำการ transfection ด้วยพลาสมิด pBps-1/pcDNA 3.1(+) ผลการศึกษาพบว่า pBps-1 ไม่สามารถแสดงออกได้ในเซลล์เพาะเลี้ยง

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LIST OF ABBREVIATIONS

Abbreviation	Term
APS	Ammonium persulfate
bp	Base pair
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocyte
^{o}C	Degree Celcius
DNA	Deoxyribonucleic acid
G 418	Geneticin
gm	Gram
IFN-γ	Gamma interferon
kb	Kilobases
kDa	Kilodaltons
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
M	Molar
mM	Millimolar
2-ME	2-mercaptoethanol
μF	Microfarad
mg	Milligram
μg	Microgram

LIST OF ABBREVIATIONS (continued)

Abbreviation	Term
ml	Millilitre
µl	Microlitre
MOPS	3-[N-morpholino] propanesulfonic acid
PBS	Phosphate-buffered saline
rpm	Revolutions per minute
RNA	Ribonucleic acid
RPMI	Rosswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
TE	Tris-EDTA buffer
TENS	Tris-EDTA-NaOH-SDS
TFBI	Transfomation buffer I
TFBII	Transformation buffer II
TNT	Tris-NaCl-Tween 20 buffer

CHAPTER I

INTRODUCTION

Cumulative evidences suggest that *Burkholderia pseudomallei*, the causative agent of melioidosis, is a facultative intracellular bacteria. Previous studies showed that *B. pseudomallei* can survive and multiply in human phagocytes *in vitro* (1) and also survive in both non-phagocytic and phagocytic cells (2). In addition, the pathogen can persist in a dormant stage in macrophage for months or years (1). Several histopathological studies demonstrated granulomatous lesions and abcessation in both infected animal and human autopsies. These lesions comprised of epithelioid cells and Langerhan's giant cells surrounded by fibrotic zone calcification or purulent exudate that may be seen in the center of the lesion similar to that found in mycobacterial infection (3-4). Furthermore, immunity generated after natural infection by *B. pseudomallei* such as complement or antibodies is ineffective in controlling colonization (5). The pathogen tends to cause chronic infection that often recrudesces after apparent cure of the primary infection (6), which is difficult to eradicate. These evidences suggested that the effective specific immunity against *B. pseudomallei* may involve the role of cell-mediated immunity, particularly cytotoxic T lymphocyte. The requirement of CTL in protective immunity was demonstrated in other intracellular bacteria including *Listeria monocytogenes* and *Mycobacterium tuberculosis* (7-10) and the role of cytotoxic T lymphocyte response against all these pathogens can be carried out by CTL assay. The initial step for detection of CTL to the pathogen requires the preparation of target cells expressing *B. pseudomallei* antigen. In this study, the target

cell for CTL assay was constructed by cloning the antigenic gene of *B. pseudomallei* into mammalian expression vector, pcDNA3.1(+). The recombinant plasmid was subsequently transfected into mammalian cell line by electroporation. The expression of *B. pseudomallei* antigenic gene in mammalian cell was studied by Western blot analysis with rabbit polyclonal antibodies against this protein. Attempts were made to obtain the transfected cells that stably expressed *B. pseudomallei* antigen for further investigation on the role of cytotoxic T lymphocyte response to *B. pseudomallei* antigen in animal model. This study would provide a tool for CTL study and better understanding the host defense mechanism to *B. pseudomallei* and the protective immunity to this pathogen that may be beneficial for development of effective vaccine for melioidosis.

CHAPTER II

OBJECTIVES

The objectives of this study are:

1. To clone a recombinant antigenic gene of *B. pseudomallei* into a mammalian expression vector.

2. To study the expression of recombinant antigen of *B. pseudomallei* in a mammalian cell and prepare target cells for further study on the cytotoxic T lymphocyte response to *B. pseudomallei* antigen.

CHAPTER III

LITERATURE REVIEW

1. Melioidosis

1.1 Clinical manifestations

Melioidosis was first described in Burma in 1912 as a glanders-like disease of man by Whitmore, a British pathologist, and Krishnaswami, an assistant surgeon, working in Rangoon General Hospital (11). It is a potentially fatal disease of human and animals, the disease is endemic primarily in southeast Asia particularly northeast Thailand and northern Australia (5, 12). In northeast Thailand, fatality resulted from septicemic melioidosis is significantly higher than septicemia caused by other pathogens (5). The outbreaks of this disease are particularly prevalent during period of high rainfall. It is thought that during this period, the pathogen is physically leached from its normal environmental habitat of soil and surface water to create a more temporary widespread distribution. In addition, sporadic cases have also been reported in the Philippines, Mexico, Papua New Guinea, Africa, and South America. Melioidosis is a disease of rice farmers, who are the majority of the rural population in this region and associated either with healthy persons or with underlying predisposing conditions, such as diabetes and renal failure (13-14). Furthermore, melioidosis occurs much more commonly in the immunocompromised host. The incidence in both

endemic and non-endemic areas is likely to increase in this era of the AIDS pandemic and global travel (15).

Septicemic melioidosis is a major cause of community-acquired septicemia in northeast Thailand with a high mortality rate of 80-90% with death occurring within 24-48 hours after onset (16). Patients with septicemic melioidosis usually present with a short history of fever and no clinical evidence of focal infection. Most patients are profoundly ill. The clinical course of septicemic melioidosis often deteriorates rapidly, and death often occurs within the first few days after hospitalization. Vuddhakul *et al* (17) surveyed the frequency of *B. pseudomallei* infections in patients attending government hospitals throughout Thailand in 1997 and reported that the burden of patients was greater in the northeastern region than other regions in Thailand. Suputtamongkol *et al* (13) found a total of 617 medioidosis cases in Ubon Ratchatani during 5 years period from 1987-1991. Sixty percent of the patients were septicemic melioidosis and the overall case fatality rate was 44%. A similar study was also carried out by the same group in four other hospitals in northeast Thailand between January and December 1997 (14). Septicemic melioidosis was found in 102 cases of 204 patients with culture-proven melioidosis and the mortality was 36.3%.

Melioidosis encompasses a wide clinical spectrum and can be seen as an inapparent infection, asymtomatic pulmonary infiltration, acute localized suppurative infection, acute septicemic infection, or as a chronic suppurative infection (18). Some melioidosis patients have the clinical symptoms similar to infection by other pathogens. The incubation period is not well defined and perhaps depends on various factors including the number of infecting organisms and their virulence, and the degree of host

immune status, etc. It can be as short as a few days as reported in the case of pneumonia after near drowning, or as long as 26 years (19) or possibly of indefinite duration. One of the most perplexing problems about melioidosis is its ability to remain in dormant stage and emerge in some later time if the conditions are favorable, such as under stress or trauma, which often leads to relapse of the disease (20). A high rate of relapse was also observed upon cessation of antimicrobial therapy and antibiotic resistance was acquired during treatment (21-23). Relapse of the infection occurs in 10% of patients completing a full course of antimicrobial treatment and is more common if patients do not complete the course of oral therapy. Clinical presentation of relapse is as severe as the initial infection, and the mortality associated with relapse is approximately 30% (23). Patients with septicemic melioidosis developed relapse more frequently than patients with localized melioidosis (22) and had a 4-times greater probability of relapse (23). In addition, various stimuli are capable of reactivating the disease, such as following influenza (24), dengue hemorrhagic fever (25-27), operative procedure and burns (28).

Current antibiotic therapy for melioidosis patients, while notably superior to past regimens, remains relatively ineffective and rather expensive for Thai patients. The combination of antibiotic treatment is potentially toxic that can not be used in children and pregnant women. In addition, *in vitro* antagonistic effect between bacteriostatic drugs and the bactericidal activity of ceftazidime that is used for the treatment of severe melioidosis was demonstrated (29). The ineffectiveness of antibiotic therapy resulted from the resistance of the pathogen that may prevent antibiotics entering to the cells by the production of glycocalyx, formation of microcolonies in damage tissues (30) and survival within phagocytic cells (2) rather

than acquisition of plasmids. The *in vitro* study showed that biofilm production of *B. pseudomallei* switched its susceptibility to resistance to ceptazidime and cotrimoxazole (31).

1.2. *Burkholderia pseudomallei*

Burkholderia pseudomallei is the causative agent of melioidosis (11). This pathogen was previouly designated *Bacillus pseudomallei* by Whitmore (32). Afterthat, various propasals were made regarding its nomenclature such as *Pfeiferella whitmori, Bacillus whitmori, Flavobacterium pseudomallei, Actinobacillus pseudomallei, Loefferella whitmori, and Malleomyces pseudomallei* (18,33). Brindle and Cowan (34) classified it into family *Pseudomonadaceae* emphasizing its character as glucose-oxidizer and its possession of polar mono-or multi-trichous morphology, which was further supported electromicroscopically by Wetmore and Gochenour (35). Finally, the presently accepted nomenclature, *Burkholderia pseudomallei* was introduced by Yabuchi *et al.* (36) in 1992.

Yabuchi *et al* (36) transferred seven species of the genus *Pseudomonas* to the genus *Burkholderia* based on the 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid compositions, and phenotypic characteristics. The members of the genus *Burkholderia* are *Burkholderia pseudomallei*, *Burkholderia mallei*, *Burkholderia cepacia*, *Burkholderia caryophilli*, *Burkholderia gladioli*, *Burkholderia picketti* and *Burkholderia solanacearum.*

Burkholderia pseudomallei is an opportunistic, gram-negative bacillus that is motile by means of polar tuft of two to four flagella. It is a facultative aerobe and a natural inhabitant of soil, stagnant water, and rice paddies in areas where it is endemic.

This bacteria can survive for a long period of time in the environment and previous study suggested that it is a facultative intracellular bacterium (37) and has the phenotypic variation that causes the pathogen to adjust and tolerate in different environments for a long time period. *B. pseudomallei* produces various virulent factors that may involve in pathogenesis but their precise role in pathogenesis of melioidosis has not yet been resolved. This pathogen is resistant to lysis by human serum, therefore, it could not be killed by complement although complement can be activated (38). Harley *et al* (39) studied the ultrastructure of *B. pseudomallei* and found that this pathogen was phagocytosed by mouse peritoneal macrophages as well as various cells in tissue cultures but apparently inhibited the normal processes of intracellular killing. The destruction of the phagosomal membrane occurred and the bacterium escaped into the cytoplasm. Infection is probably due to soil or water contamination of skin abrasions or inhalation of *B. pseudomallei* from an environmental source especially people regularly contacted with soil and muddy water (40). The isolation of the organism from water and soil has been reported from Malaysia, Vietnam, Australia, and Thailand (41). In Malaysia, extensive environmental surveys in the 1960s demonstrated the widespread presence of *B. pseudomallei* in water and soil in East and West Malaysia (42-45). In Vietnam, Phung (46) also found *B. pseudomallei* in soil and water. In Australia, there was a survey of water and soil of sheep paddock sampled from clay layers of various depths over a two-year period. The isolation rate of *B. pseudomallei* was much lower than those reported in other countries of Southeast Asia (47). In Thailand, environmental surveys revealed the widespread presence of *B. pseudomallei* in water and soil (48). *B. pseudomallei* could be more frequently isolated from soil samples from the northeastern region when comparing to the other regions

(17). Kanai and Dejsirilert (41) suggested that *B. pseudomallei* is a normal inhabitant of the water and soil, and that the pathogen do not require animals as a maintenance host because of the long survival of the pathogen in the environment even in tap water (49) or soil in moist places (47,50). Therefore, it is not surprising to find that the highest incidences of infection caused by this bacteria occur during the rainy season in tropical regions. A significant percentage of persons succumbing to these infections are known to be rice farmers and their families (32).

2. Immunity to intracellular bacteria

For the reason that *B. pseudomallei* is a intracellular bacteria, therefore immunity to other intracellular bacteria are reviewed as follows.

Intracellular pathogens can be classified into 2 groups, an obligate intracellular pathogen which adapts itself to live in the host and could not survive outside cells, such as viruses, rickettsia, chlamadia, and mycoplasma. The other is a facultative intracellular pathogen, which is able to live either inside or outside of the host cells in phagocytic and/or non-phagocytic cells. This type of pathogens include intracellular bacteria such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Yersinia enterocolitica*, *Salmonella* and *Shigella* spp. Most of the intracellular bacteria are usually resistant to innate immunity. They can avoid being ingested or killed by phagocytes as well as are resistant to complement attack. Therefore, the clearance of these bacteria by antibodies is not effective. Thus, elimination of intracellular bacteria requires specific immune mechanism which is mediated by the mechanism of cellular immunity (51).

Cell-mediated immunity eliminates intracellular bacteria by 2 possible mechanisms, killing of phagocytosed pathogens as a result of macrophage activation by T cell-derived cytokines, particularly INF-γ, and lysis of infected cell by $CD8^+$CTLs. Protein antigens of intracellular bacteria stimulate both $CD4^+$ and $CD8^+$ T cells. Presumably, $CD4^+$ T cells are activated by exogenous antigens that are internalized and presented by class II MHC-expressing APCs to become functional $CD4^+$ helper T cells. The activated $CD4^+$ helper T cells are either of T_h1 phenotype or T_h2 phenotype depending on the microenvironment of cytokines during activation. Several studies indicate that intracellular bacteria induce the activation of T_h 1 phenotype. T_h 1 cells secrete INF-γ which activates macrophage to produce reactive oxygen species and enzymes that kill phagocytosed bacteria. They also stimulate the production of antibody isotypes which activate complement and opsonize the bacteria for phagocytosis (51).

If the bacteria survive within cells and release their antigens into the cytoplasm surrounding them, the antigens will be processed and presented by class I MHC on the surface of infected cells to activate $CD8^+$ CTLs. The activated CTLs can eliminate intracellular bacteria by the secretion of INF-γ that results in macrophage activation and lysis of infected cells. The pathways involved in CTLs mediated cytotoxicity are granule-exocytosis pathway and Fas/Fas ligand pathway. The granule-exocytosis pathway is characterized by the secretion of the lytic protein, perforin, which polymerize to form a pore in the host cell membrane, allowing the entry of the cosecreted granule contents, granzymes, leading to the apoptosis of target cell. The Fas/Fas ligand pathway involves the crosslinking of a surface membrane ligand (Fas

ligand) presented on the antigen activated CTLs and a death receptor (Fas) on the target cells. The interaction between these two molecules initiate a cascade of proteolytic enzymes ultimately resulting in target cells apoptosis and lysis. CTLs mediated lysis of target cell result in the release of bacteria into extracellular environment and subsequently the bacteria are killed by activated macrophage (51). Antigen specific $CD8^+$ T cells also secret a cytokine that has bactericidal activity to inhibit the extracellular growth, such as demonstrated for *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* (52-53).

Optimal protection against intracellular bacteria is regarded as a coordinated interaction between different T cell subsets (54). The role of different T cell subsets in defense mechanism against intracellular bacteria have been investigated by several studies in animal model. These studies indicated a substantial role for $CD8^+$ T cell and CTLs in host defense against intracellular bacteria. Thus, $CD8^+$ T cell probably complement other components of the immune to mount an efficient and long-lasting immunity to intracellular bacteria. The roles of $CD8^+$ T cell against some intracellular bacteria are reviewed as follow

2.1 Cell-mediated immunity to *Listeria monocytogenes*

Listeria monocytogenes has been a pathogen of choice in gene knockout models in mice to assess the role of different immune components in controlling intracellular bacterial infection. Knockout mice with distinct T cell deficiencies demonstrated that $CD8^+$ TCRα/β^+T cells played an important role in clearance of a primary infection and in protective immunity, while $CD4^+$ and TCR γ/δ^+ T cells played a less dominant role

(7). The early adoptive transfer experiments demonstrated that antibody played no role in protective immunity, and that anti-listerial immunity resided within the cellular component of *Listeria monocytogenes*-immunized mice (55-56). The anti-listerial protection resided within the the $CD8^+$ T cell subset while $CD4^+$T cells did not adoptively transfer anti-listerial resistance (8). The role of $CD8^+$ T cells in acquired resistance to this pathogen would suggest that this immune cell subset functions as a cytotoxic T lymphocyte (CTL) population capable of discriminating between infected and non-infected cells, via immunorecognition of MHC class I-presented peptides by CTL (9). Therefore, MHC class I antigen presentation pathway stimulate $CD8^+$ T cells which are cytolytic and mediate acquired resistance to this pathogen. The secreted *Listeria monocytogenes* proteins listeriolysin (LLO) and p60 were the target antigens for $CD8^+$ T cells that can transfer immunity to naive mice (57-58).

2.2 Cell-mediate immunity to *Mycobacterium tuberculosis*

The successful resolution of infection with *Mycobacterium tuberculosis* is believed to involve the induction of CTLs that are capable of killing cell harboring this pathogen. CTLs reactive to *M. tuberculosis* were present in the lungs of patients with tuberculosis (59) and produced high level of INF-γ. Munk ME (60) demonstrated that a 30 kDa protein (p32), which was a mycobacterial secreted antigen, induced T cell proliferation *in vitro* in BCG-vaccinated Caucasian donor. CTLs obtained from these person lysed target cells pulsed with *Mycobacterium tuberculosis*, p32 or synthetic peptide from p32. Mohagheghpour N *et al* (61) showed that the secreted 19 kDa lipoprotein of *M. tuberculosis* was recognized by $CD8^+$ CTLs from tuberculin skin-test positive individuals and had the cytolytic activity against autologous monocytes

infected with H 37Ra strain of *M. tuberculosis*. Denis O *et al* (10) demonstrated that vaccination of BALB/c mice with plasmid DNA carrying the gene for the major secreted mycobacterial antigen 85 A(Ag 85A) from *M. tuberculosis* led to generation of Th_1 helper T cells response and $CD8^+$ mediated cytotoxicity to lyse Ag85-tranfected and peptide pulsed-P815 target cells. In addition, it also conferred significant protection in immunized mice against challenging with lived *M. tuberculosis*. and *M. bovis*.

2.3 Cell-mediated immunity to *Yersinia enterocolitica*

Yersinia enterocolitica is another intracellular bacteria that can elicit $CD8^+$ CTLs response in animal model. Starnbach MN (62) investigated that immuniztion of C57BL/6 mice with Yop 51 protein-coated spleen cells elicited cytotoxic T lymphocytes that could lyse autologous target cells pulsed with Yop 51 peptide, Yop 51 loaded target cells or Yop 51 transfected target cells.

2.4 Cell-mediated immunity to *Salmonella typhi*

Sztein MB *et al* (63) demonstrated that human volunteers orally immunized with attenuated vaccine strains of S. typhi, CVD 908, elicited circulating $CD8^+$ CTLs capable of killing S. typhi-infected autologous EBV-transformed cells. $CD8^+$ CTLs activity was blocked by monoclonal antibodies to human class I MHC but not by monoclonal antibodies to class II MHC antigens. Furthermore, the depletion of $CD8^+$ T cells eliminated or markedly reduced the CTL activity against S. typhi-infected autologous EBV-transformed cells.

In the case of *Burkholderia pseudomallei*, antibodies to this pathogen could be found in melioidosis patients. However, they could not confer protection. There are some studies on the immunity to the pathogen. Miyaki K (64) showed that this pathogen could multiply in unstimulated murine macrophage cell line, J774A.1, and the intracellular bacteria growth within INF-γ-activated macrophages was inhibited by reactive nitrogen intermediates killing mechanism. Santanirand P *et al* (65) demonstrated that resistance to acute infection of this pathogen in Taylor Outbred mice was absolutely dependent upon the production of INF-γ by NK cell INF-γ pathway. Furthermore, several histopathological studies showed that there were several granulomatous lesions in both infected animal and human autopsy. These lesions were comprised of epithelioid cells and Langerhan's giant cells which were surrounded by fibrotic zone calcification or purulent exudate that may be seen in the center of lesion (3,4,65). Santanirand P *et al* examined pathology of the spleen from sublethally infected mice which developed chronic melioidosis and found the infiltration of lymphocytes and phagocytes but the bacteria were not observed.

The findings of effective immunity to other intracellular bacteria and the histopathological study of both infected animal and human by *B. pseudomallei* clearly demonstrated that cell-mediated immunity may play the important role against *B. pseudomallei* via the activation of cytotoxic T lymphocytes responses and the role of cytotoxic T lymphocyte against all intracellular bacteria described above can be investigated by cytotoxicity assay (CTL assay).

3. The study of cytotoxic T lymphocyte response by cytotoxicity assay

Cytotoxicity assay is the study of primed cytotoxic T cells response to specific antigen of pathogens of both animals and human by determining the lysis of antigen expressing target cells, due to their interaction *in vitro*. Lysis of target cells can be quantitated by release of incorporated radioactive ^{51}Cr molecules into the supernatant after recognizing by specific CTL. This radioactive molecule is rapidly incorporated into live cells as chromate ion and reduced in the cytoplasm as chromic ion derivative that is no longer reincorporated when released from dead cells. The quantity of ^{51}Cr release from labeled target cells in a certain period of time can be taken as a measure of the number of target cells lysed by cytotoxic T cells. This simple measurement can thus be used to obtain qualitative information on whether cytotoxic T cells are present and whether target cells are susceptible to lysis.

In principle, any cell type that has the appropriate MHC restriction to tested cytotoxic T cells can be used as targets for measuring CTL activity including activated cells such as lymphoblasts, tissue culture cells, or tumor cells. The preparation of target cells expressed specific antigen for the detection of CTL response by cytotoxicity assay can be done by 3 ways.

3.1 Infection of target cell with pathogen

The particular pathogen is cocultured with target cells at a desirable period to allow the natural infection of the pathogen to occur *in vitro*. The infected target cells will process and present the peptides derived from various proteins of the pathogen in the cytoplasm with MHC class I on the target cell surface. The preparation of the

infected target cells is easy to do but the number of infected cells may be low resulted from the toxicity of the infecting pathogen to the cells. In addition, the appropriate safety precaution must be concerned when infecting the cells with a highly virulent pathogen. The establishment of infected target cells is the initial tool for the study of CTL against the particular intracellular pathogen (9,59,63).

3.2 Delivery of recombinant antigenic gene into target cells

The specific antigen from the antigenic gene can be expressed and act as endogenous protein in the cytoplasm of target cells and subsequently is processed and presented to the surface of target cells with MHC class I the same as *in vivo*. This method is well-define and reproducible. However, time is required for characterization of a selected antigenic gene. This target cell preparation is useful for the detection of CTL response to specific antigen (57,66-68).

Target cell expressing specific antigen of a pathogen can be used to study the role of cytotoxic T lymphocyte response by CTL assay. They can be prepared by cloning and expression of a selected antigenic gene. The selected antigenic gene is cloned into the mammalian expression plasmid vector and transferred into a mammalian cell by a suitable method. The outcome is the generation of mammalian cell expressing a particular antigen from the transferred gene further used as target cell in the assay.

3.3 Peptide pulsed target cells

The synthetic peptide from specific antigen that stimulate CTL response can be pulsed on target cells (58, 69). Therefore, peptide exchange will occur on the target

cells surface in order to replace the synthetic peptide of the specific antigen for the binding to MHC class I on the surface of target cells. However, this process requires the large amount of the synthetic peptide. The preparation of target cells in this way is to define peptide epitopes from specific antigen for stimulation of CTL response.

4. Mammalian expression system

4.1 Plasmid vector for expression of DNA in mammaliam cell

Plasmid vector for expression of DNA in mammaliam cell (70) can be divided into 2 types.

4.1.1 Simple plasmid-based vectors contain both prokaryotic sequences that facilitate the propagation and amplification of recombinant DNA in bacteria and one or more eukaryotic transcription units that are expressed only in eukaryotic cells. The eukaryotic transcription unit consists of non-coding sequences and sequences coding for selectable markers. The components that are used in various plasmid expression vectors are

4.1.1.1 The promoter that mediates the transcription of foreign DNA sequences and critical for efficient expression. Promoters are usually short DNA sequences (<1 kb) that bind endogenous transcription factors. Many eukaryotic promoters contain two types of recognition sequences, the *TATA* box and the upstream promoter elements. The *TATA* box locates 25-30 bp upstream of the transcription initiation site and involves in directing RNA polymerase II to begin RNA synthesis at the correct site. Promoters can be divided into constitutive promoter and inducible promoter. Constitutive promoters are frequently derived from viruses and

induced in response to various signals. They include Simian virus (SV 40) promoter, Rous sarcoma virus promoter, adenovirus major late promoter and cytomegalovirus immediate early promoter. Inducible promoters require inducible substances for the function of them such as glucocorticoid-inducible promoter, metal inducible promoter and heat-shock inducible promoter. The upstream promoter element locates within 100-200 bp upstream of the *TATA* box and determines the rate at which transcription is initiated. Cytomegalovirus and Simian virus 40 (SV40) promoters have been favorite general mammalian promoters for years and many derivatives have been made and are commercially available.

4.1.1.2. Enhancer elements that stimulate transcription and active when placed downstream from the transcription initiation site or at considerable distances from the promoter. Many enhancer elements are derived from viruses. The two enhancer/promoter combination that are active in a broad range of cells are derived from the long terminal repeat (LTR) of Rous sarcoma virus genome and from human cytomegalovirus.

4.1.1.3. Multiple cloning sites (MCS or Polylinker) Plasmid vectors encode a series of restriction endonuclease recognition sites. These sites are engineered for convenient cloning of DNA into a vector at a specific position.

4.1.1.4. Termination and polyadenylation that ensures the efficient polyadenylation and transcription termination of interested mRNA. A sequence in the 3' end of the expression vector, that signals the addition of a poly (A) tail, often stabilizes the mRNA and lead to a better expression. The polyadenylation signal consists of a conserved sequence (AAUAA) 11 to 30 nucleotides upstream of the

polyadenylation site and a GU- or U- rich region downstream. Many different sources of polyadenylation signals are used in the construction of vector and a common one is derived from the SV 40 virus.

4.1.1.5. Selectable markers in eukaryotic cells are the components of plasmid vector for the isolation of cell lines that express transfected gene. Some of the common selectable markers for mammalian cell are

- Resistance to neomycin or hygromycin B, conferred by the bacterial neomycin phosphotransferase gene or hygromycin B phosphotransferase attached to a suitable promoter.

- Resistance to toxic substances such as adenosine nucleotides, conferred by adenosine deaminase gene or difluoromethylornithine, conferred by ornithine decarboxylase.

- Resistance to inhibitors that block nucleotide biosynthesis in cells, conferred by dihydrofolate reductase gene, bacterial xanthine-guanine phosphoribosyl transferase (XGPRT) gene or thymidine kinase gene.

These plasmids do not contain a eukaryotic replicon therefore no episomal amplification of the transfected DNA occur in host cells. Instead, the transfected DNA stably integrates into the genome of the transfected cells for stable expression of the cloned gene.

4.1.2 More complex plasmid vectors that also incorporate elements from the genome of viruses to increase the copy number of the transfected DNA and the efficiency of which foreign proteins are expressed. Elements for replication in these

plasmids are the viral replicon for the episomal replication of plasmid vector in permissive cell types by the appropriate trans-acting factor provided by a gene either on the plasmids or the genome of the host cells. Plasmid vectors containing the replicon of papovaviruses such as SV 40 or polyomaviruses can replicate to extremely high copy number in cells that express the appropriate viral T antigen such as COS cell. Plasmid vector containing replicon of bovine papillomaviruses and Epstein-Barr virus propagates episomally at lower copy number.

pcDNA 3.1(+) plasmid vector is a commercial plasmid used for the construction of DNA vaccine. It contains about 5,428 bp and is designed for high-level stable and transient expression in mammalian hosts. pcDNA 3.1(+) is available with the multiple cloning sites to facilitate cloning. The expression of cloned gene in this vector is mediated by the human cytomegalovirus immediate-early (CMV) promoter/enhancer in a wide range of mammalian cells. In addition, the vector will replicate episomally in cell lines that are latently infected with SV40 or that express the SV40 large T antigen such as COS7. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The plasmid also contains ampicillin-resistance gene (Amp^r), ColEl origin of replication for the selection and amplification of this plasmid in *E. coli* and neomycin-resistance gene (Neo^r) for the selection of stable expression of cloned gene in mammalian cells (71).

4.2 Delivery of plasmid DNA

Several transfection methods have been developed to deliver plasmid DNA for studying the expression of cloned genes in mammaliam cell *in vitro*. These include calcium-phosphate transfection and DEAE-dextran transfection, which rely on the

uptake of condensed plasmid DNA bound to the cell surface by non-specific endocytosis (72). The plasmid DNA is retained in endocytic vesicles and released into cytoplasm of the cell. Electroporation transfers DNA by subjecting cells to a high voltage electrical pulse which causes the formation of temporary nanometer-sized pores in the plasma membrane (73). DNA is taken up directly into the cell cytoplasm and travel to the nucleus, bypassing the endocytic vesicles which sometimes destroy or damage the DNA. This method is versatile and can be adapted to meet the requirement of many cell lines (74), including the cell lines that are refractive to other techniques and is not dependent upon special characteristics of the cell. In addition, DNA can also be incorporated into artificial lipid vesicles (liposomes). Liposome is an amphiphilic molecules which can self-associate with plasmid DNA and condense it into particles by binding of the negatively charged phosphate groups on DNA to the positively charged surface of the liposome. The residual positive charge then presumably mediates binding to negatively charged sialic acid residues on the cell surface resulted in fusion of the liposome with the cell membrane. This fusion results in the delivery of DNA into cell cytoplasm.

All four methods described above can be used for transient expression of the required gene. Three of these, calcium-phosphate transfection, electroporation and liposome-mediated transfection, can be used to efficiently produce cell lines containing stably integrated DNA. DEAE-dextran mediated transfection does not work well when producing stable cell lines but is more reproducible than calcium phosphate transfection when used for transient expression of genes. Electroporation is also very reproducible and convenient but requires more cells than either of the chemical procedures do. It also reduces the need for optimization of transfection efficiency. Optimization of this

protocol for specific cell lines is dependent on voltage/electric field strength and can be performed within a few hours. The transfection efficiency of electroporation is nearly always higher than those achieved by other protocols (75).

4.3 The monitoring of gene transfer

A convenient way to optimize any transfection methods can be done by monitoring of the reporter gene expression in the host cells after transfection of that reporter gene.

The simplest way to monitoring of gene transfer is to assay the transient expression of reporter gene after introduction of DNA. The reporter gene should have minimal or no effects on the physiology of the transfected cell. Ideally, the assay for the reporter gene expression would be extremely sensitive.

The green fluorescent protein (GFP), cloned from the jellyfish *Aequorea victoria*, serves as an easily detected fluorescent tag, requiring no substrates or associated cofactors, and has become widely used as a reporter gene. It also permits highly sensitive and nondestructive monitoring of gene transfer and expression a wide variety of organisms and cell types (76) by using fluorescent microscope. The GFP of *Aequorea victori* is a 238 amino acid polypeptide and is highly fluorescent and stable in many assay conditions (77). The absorption and emission wavelenght of GFP are similar to those of fluorescein. Therefore, It has excitation peaks at 395 nm and 475 nm and emission peak at 509 nm with a small shoulder at 540 nm.

The expression and modification of GFP in mammalian cell has been reported. Chiocchetii *et al* (78) used GFP, under the control of the human hemopexin and mouse

β_1 integrin promoter, as a marker for gene expression in transgenic mice. Subramanian S and Srienc F (79) performed quantitative analysis of transient gene expression in single mammalian cell to determine the ability of GFP to act as a quantitative reporter. They found that green fluorescent can be used for a quantitative measure of GFP in single cell. Mosser DD *et al* (80) used GFP reporters to select tetracycline-regulated cells from a mixed population of cells.

Other reporters used for the monitoring of gene transfer include isotopic assays, such as using chloramphenicol acetyltransferase (CAT) and human growth hormone (hGH) expressing plasmids; and chemiluminescense assays using firefly luciferase and β-galactosidase expression plasmids. Chloramphenicol acetyl transferase (CAT) has been most frequently used. The assay of the expression is relatively easy and quite reliable but can be time consuming. Unlike CAT, the assay of human growth hormone (hGH) used immunological technique which employs commercially available kits that are relatively quick and convenient to use. The assay of hGH is done in the medium rather than in cell extracts like the assay of CAT. In addition, both CAT and hGH could be detected *in vitro* only (75).

Luciferase is another widely used reporter gene and the expression assay is much faster and more sensitive than CAT. The detection of luciferase does not involve radioactivity like the detection of CAT and could be carried out *in vivo* as well as *in vitro*. β-galactosidase is the another reporter gene that is widely used. The assay of this gene can be done *in vivo* and *in vitro* by using either colorimetric assay, fluorometric assay or chemiluminescent assay. The latter gives the most sensitivity when comparing to the others assays (75).

CHAPTER IV

MATERIALS AND METHODS

1. Mammalian expression plasmid

The plasmid vector used for the cloning of *B. pseudomallei* antigenic gene in this study was pcDNA 3.1(+) (Invitrogen, California, USA) (Figure 1). This pcDNA 3.1(+) plasmid vector contains 5,428 bp and is designed for high-level stable and transient expression in mammalian hosts (71). pcDNA 3.1(+) contains multiple cloning sites to facilitate cloning. The expression of a cloned gene in this vector is mediated by the human cytomegalovirus immediate-early (CMV) promoter/enhancer that is functional in a wide range of mammalian cells. In addition, the vector can replicate episomally in cell lines that are latently infected with SV40 or those express the SV40 large T antigen such as COS7. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The plasmid also contains ampicillin-resistance gene (Amp^r), ColEl origin of replication for the selection and amplification of this plasmid in *E. coli* and neomycin-resistance gene (Neo^r) for the selection of stable expression of cloned gene in mammalian cells.

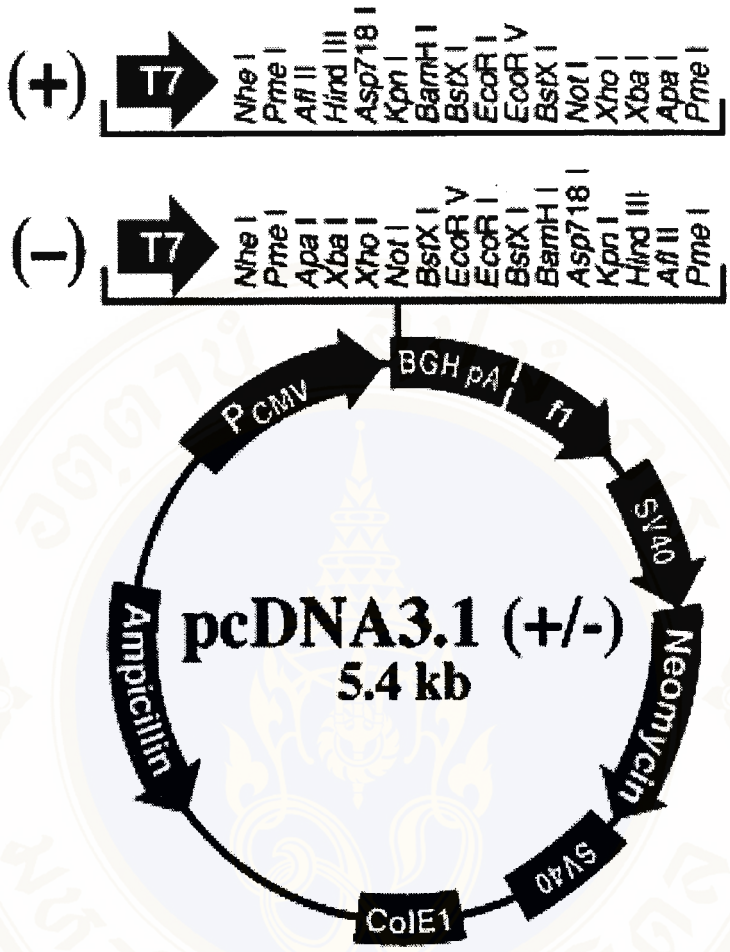


Figure 1. Map of the plasmid vector pcDNA 3.1(+). CMV, the promoter/enhancer; ColEl origin for replication; Amp^r and Neo^r; SV40 origin of replication in cells expressing T antigen; fl filamentous phage origin of replication; BGH and polyA adenylation site; MCS, multicloning site (71)

2. Reporter gene expression plasmid

The monitoring of gene transfer was determined by detecting the expression of a reporter gene. The plasmid used for monitoring the gene transfer by electroporation was pGreen-Lantern-1 (Life Technologies, Maryland, USA) (Figure 2). It has the size of 5,030 bp. This eukaryotic reporter plasmid expresses a modified form of green fluorescent protein (GFP) that has 238 amino acids from *Aequorea victoria* (jellyfish) under the control of CMV early promoter/enhancer. This form of GFP includes a serine to threonine mutation at amino acid position 65 which reduces the Stokes shift resulted in decreasing the distance between the excitation and emission wavelength. The excitation wavelength of this modified GFP is 490 nm and the emission wavelength is 510 nm. Additionally, the codon usage for 80 amino acids had been changed for better employment of the activated tRNA pool found in mammalian cells. Both of these changes increased the ease of visualization, the intensity of light emission and efficiency of expression of this vector in eukaryotic cells.

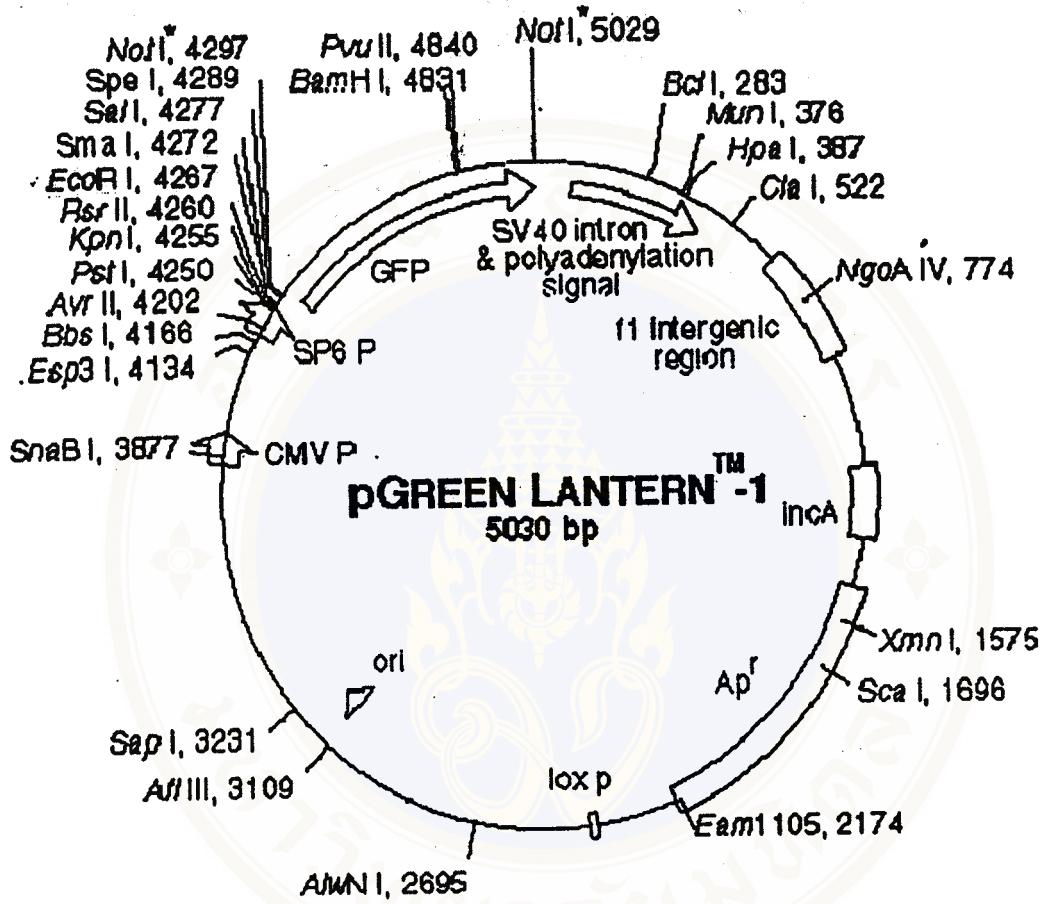


Figure 2. Map of the plasmid vector pGreen-Lantern-1 CMV, the promoter/enhancer; Modified GFP gene, high copy origin for replication; Amp^r; SV40 intron and polyadenylation site

3. The antigenic gene of *B. pseudomallei*

The antigenic gene of *B. pseudomallei* used in this study was pBps-1 gene. pBps-1 gene was obtained from the *B. pseudomallei* genomic DNA library cloning in pKSII(-) plasmid vector at *Bam* HI site (81). This gene has about 510 bp and encodes the protein which has the molecular weight of 18.7 kDa. pBps-1 gene is located at nucleotide position 488-995. The pBps-1 protein strongly reacted with sera from both acute and septicaemic melioidosis patients (81).

4. Mammalian cell

The expression of pBps-1 antigenic gene of *B. pseudomallei* was detected in J774A.1 cell after delivery of pBps-1/pcDNA 3.1(+) into this cell type by electroporation. J774A.1 cell was derived from BALB/c mouse alveolar macrophage cell line. It is a loosely adherent cell and expresses H-2^d haplotype of MHC.

5. Strategy for molecular cloning of *B. pseudomallei* antigenic gene in pcDNA 3.1(+) vector

The *B. pseudomallei* antigenic gene, pBps-1 was recloned from pKSII(-) by digestion with *Not*I restriction endonuclease (New England Biolabs, Hertfordshire, UK) that recognized the hexanucleotide 5' CGGCCG 3' target sequence and *Xho*I (New England Biolabs, Hertfordshire, UK) that recognized the hexanucleotide 5' CTCGAG 3' target sequence within pBps-1. Therefore, *Not*I and *Xho*I digested pBps-1 was cloned into *Not*I and *Xho*I digested pcDNA3.1(+) vector and transformed into *E. coli* strain TG1. *E. coli* that harbored the recombinant plasmid was selected by

electrophoresis of *Not*I and *Xho*I cut fragment and confirmed by automated DNA sequencing (Figure 3).

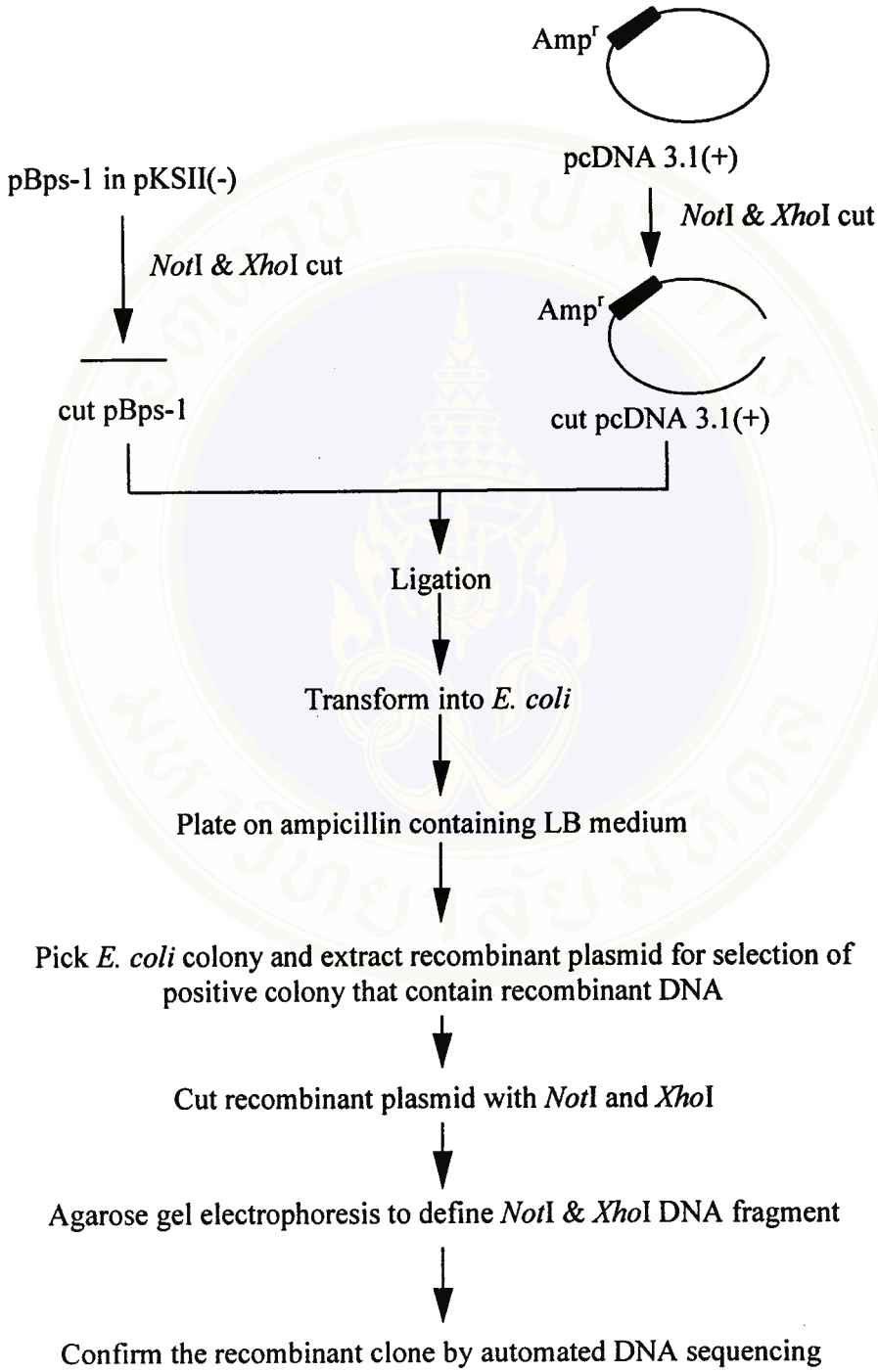


Figure 3. Cloning strategy of *B. pseudomallei* antigenic gene, pBps-1 in pcDNA 3.1(+).

5.1 Preparation of pcDNA 3.1(+) plasmid

pcDNA3.1(+) was obtained by using high quality 50 ml plasmid preparation (82). A single bacterial colony was inoculated into 50 ml of Luria-Bertani media (LB media) (see Appendix) containing 100 μg/ml of ampicillin and incubated overnight at 37°C with continuous shaking. The culture was transferred to a centrifuge tube and centrifuged for 10 minutes at 4,000 rpm to pellet the cells. The pellet was resuspended in 4 ml of ice cold GTE buffer (see Appendix) by vortexing, followed by the addition of 8 ml of freshly prepared TENS buffer (see Appendix). The mixture was mixed by inverting the tube and place on ice for 5 minutes. Six millilitres of 3 M sodium acetate pH 5.2 (see Appendix) was then added. The content was mixed by inverting and incubated on ice for 10 minutes and centrifuged at 4,000 rpm for 15 minutes at 4°C to pellet cell debris and chromosomal DNA. The supernatant was transferred to a fresh tube and 17 ml of isopropanol was added and the mixture was left at -70°C for 15 minutes. The mixture was pelleted at 4,000 rpm for 15 minutes at 4°C. The pellet was resuspended in 2ml of TE buffer pH 8.0 (see Appendix) and added with 2.5 ml of 4.4 M LiCl (see Appendix). The mixture was pelleted at 4,000 rpm for 15 minutes at 4°C and the supernatant was transferred into a new tube. Ten millilitres of absolute ethanol was added into the supernatant and left at room temperature for 15 minutes. The solution was centrifuged at 4,000 rpm for 15 minutes at 4°C to pellet DNA. The DNA pellet was washed once with 1 ml of cold 70% ethanol and resuspended in 400 μl of TE buffer and finally transferred to a new eppendorf tube. The DNA solution was extracted twice with phenol, once with phenol:chloroform:isoamyl alcohol (100:96:4), and finally extracted twice with ether. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and

2.5 volume of absolute ethanol and the pellet was washed with cold 70% ethanol, allowed to dry, and finally resuspended in 50 µl of sterile distilled water. The DNA was stored at –20°C until use.

5.2 Digestion of pcDNA 3.1(+) plasmid

Two µg of pcDNA 3.1(+) was digested with 10 units of *Not*I and 20 units of *Xho*I restriction enzymes for 6 hours at 37°C. The reaction mixture was extracted and purified with Geneclean II kit (Bio101, California, USA) as follows. The reaction mixture was added with 3 volumes of NaI solution and followed by adding 5 µl of glassmilk. The reaction mixture was mixed and placed on ice for 5 minutes. The reaction mixture was briefly centrifuged to pellet the glassmilk. The glassmilk was washed with 300 µl of the NEW Wash buffer 3 times. The digested plasmid was eluted from the glassmilk by adding 10 µl of sterile distilled water, mixed by vortexing and incubate at 50°C for 5 minutes. The digested plasmid was removed from the glassmilk by centrifugation, transferred to a new eppendorf tube and stored at –20°C until use.

5.3 Preparation of pBps-1 antigenic gene for cloning

The antigenic gene, pBps-1 in pKSII(-), was extracted from *E. coli* by alkaline lysis method (83). A single bacterial colony was inoculated into 2.0 ml of LB medium containing 100 µg/ml of ampicillin and incubated overnight at 37°C with continuous shaking. The culture was transferred to a microcentrifuge tube and centrifuged briefly to pellet the cells. The pellet was resuspended in 100 µl of TE buffer pH 8.0 by vortexing, followed by the addition of 300 µl of freshly prepared TENS buffer (see

Appendix). The mixture was mixed by inverting the tube until it became sticky. One hundred and fifty microlitres of 3 M sodium acetate pH 5.2 was then added. The content was mixed by inverting and incubated on ice for 3-5 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C to pellet cell debris and chromosomal DNA. The supernatant was transferred to a new tube and 900 µl of cold absolute ethanol was added and the mixture was left at –20°C for 2 hours. The solution was pelleted at 10,000 rpm for 10 minutes at 4°C. The pellet was washed once with 1 ml of cold 70% ethanol, allowed to dry, and finally resuspended in 20 µl of sterile distilled water.

DNA of pBps-1 antigenic gene in pKSII(-) from the alkali lysis preparation was digested with 10 units of *Not*I and 20 units of *Xho*I restriction enzymes for 6 hours at 37°C. The reaction mixture was electrophoresed in 0.7 % low melting point agarose gel in TBE buffer (see Appendix) at constant voltage of 50 volts. After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide and was visualized under a UV transilluminator for pBps-1 DNA fragment. The pBps-1 band from ethidium bromide-stained agarose gel was excised with a razor blade using a brief visualization under UV light. The excised agarose gel was melt and pBps-1 was extracted from the gel by using Geneclean II kit. The melt agarose gel was added with 0.5 volume of TBE modifier and 4.5 volume of NaI solution, brieftly vortexed, and followed by adding 5 µl of glassmilk. The mixture was mixed by vortexing and placed on ice for 10 minutes. The mixture was then briefly centrifuged to pellet the glassmilk and the glassmilk was washed with 300 µl of the NEW Wash buffer 3 times. The pBps-1 was eluted from the glassmilk by adding 10 µl of sterile water, mixed by vortexing and incubate at 50°C for 5 minutes. The pBps-1 was removed from the

glassmilk by a brief centrifugation, transferred to a new eppendorf tube and stored at -20°C until use.

5.4 Ligation of pBps-1 antigenic gene into digested pcDNA 3.1(+)

*Not*I and *Xho*I digested pcDNA 3.1(+) was mixed with *Not*I and *Xho* I digested pBps-1 antigenic gene in the 15 µl ligation reaction. One µl of T4 DNA ligase (400,000 units/ml) (New England Biolabs) and ligation buffer (50 mM TrisCl pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin) were added to the mixture and incubated at 16°C, overnight. The ligated DNA was used for transformation into competent *Escherichia coli* cells.

5.5 Preparation of competent *E. coli*

Competent *E. coli* strain TG1 was prepared by using a modified rubidium chloride method of Hanahan (84). *E. coli* cells were prepared by inoculating 100 ml of Luria-Bertani media (LB media) with 1 ml of overnight culture of *E. coli*, grown in a flask with good aeration and vigorous shaking (250-300 rpm) at 37°C until an OD_{550} of 0.5 was obtained. The cell suspension was transferred into a pre-cooled tube and cells were pelleted by centrifugation at 3,000 rpm, 4°C, for 10 minutes. The pellet was resuspended in 30 ml of cold TFBI solution (see appendix). After incubation on ice for 10 minutes, cells were pelleted again and resuspended in 4 ml of cold TFBII solution (see appendix). These competent *E. coli* were aliquoted 200 µl each into a cold microcentrifuge tube and stored at –70°C until used.

5.6 Transformation procedure

Transformation was carried out using a procedure described by Mandel and Higa (85) with some modifications. The stored competent *E. coli* were thawed on ice, 15 µl of ligated DNA were added to the 200 µl of competent cells and the mixture was kept on ice for 60 minutes. The mixture was then heat shocked at 42°C for 90 seconds, and chilled on ice for 2 minutes before the addition of 900 µl of LB medium. The transformed cells were incubated at 37°C for 1 hour with continuous shaking, centrifuged at 3,000 rpm, 4°C, for 3 minutes and the pellet was resuspended in 100 µl of LB medium before plating on agar plates with antibiotic medium (Difco Laboratories, MI, USA) containing 100 µg/ml of ampicillin. The plates were incubated at 37°C, overnight. After the incubation period, ampicillin-resistant colonies were grown on the agar plate

6. Selection of recombinant clones

6.1 Isolation and preparation of recombinant plasmid from *E. coli* host cell

A single bacterial colony from the agar plate was picked and inoculated into 2.0 ml of LB medium containing 100 µg/ml of ampicillin and incubated overnight at 37°C with continuous shaking. The recombinant plasmid was extracted from each tube of bacterial culture by the alkali lysis method described above. The extracted recombinant plasmid from each colony of transformed cell was digested with 5 units of *Not*I and 5 units of *Xho*I restriction enzymes for 3 hours at 37°C in order to identify

which bacterial colony harbored pBps-1 gene in pcDNA 3.1(+) by agarose gel electrophoresis.

6.2. Agarose gel electrophoresis

The restriction enzyme digested recombinant plasmid was analysed by agarose gel electrophoresis. Twenty microlitres of digested recombinant plasmid was mixed with 3 µl of 6x loading buffer (see Appendix) and loaded on 1% agarose gel, then electrophoresed in 1x TBE buffer (see Appendix) at constant current of 80 mA. The current was stopped when the blue dye marker reached the bottom of the gel. After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide and was visualized under a UV transilluminator and photographed by Gel Doc 1000 apparatus (Bio-Rad, California, USA). λ-DNA digested with *Hin*d III and DNA ladder were used as the standard markers for estimation of molecular size of the DNA fragments. The fragments of the λ-DNA digested marker were 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56 and 0.12 kb in sizes. The fragments of DNA ladder were 1.5, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 kb in sizes.

6.3 Preparation of recombinant plasmid containing pBps-1 gene for nucleotide sequencing

Bacteria that harbored pBps-1 cloned in pcDNA 3.1(+) from glycerol was inoculated into 5 ml of LB medium containing 100 µg/ml of ampicillin and incubated overnight at 37°C with continuous shaking. Five millitres of overnight culture was transferred to a eppendorf tube and centrifuged briefly to pellet the cells. The pellet was resuspended in 200 µl of GTE buffer by pipetting up and down, followed by the

addition of 300 µl freshly prepared 0.2 N NaOH/1% SDS. The mixture was mixed by inverting the tube and incubated on ice for 5 minutes. Three hundred microlitres of 3 M potassium acetate pH 4.8 was added. The content was mixed by inverting, and incubated on ice for 5 minutes and centrifuged at 8,000g for 10 minutes at room temperature to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a fresh tube and Rnase A was added to the mixture for a final concentration of 20 µg/ml and incubated at 37°C for 20 minutes. After the Rnase A treatment, the mixture was extracted twice with 400 µl of chloroform. The aqueous phase was then transferred to a new tube and the DNA was precipitated with an equal volume of 100% isopropanol. The mixture was immediately centrifuged at 8,000g for 10 minutes at room temperature, the DNA pellet was washed once with 70% ethanol, air dried, and dissolved in 32 µl of deionized water. The plasmid DNA was precipitated by first adding 8 µl of 4 M NaCl, and then adding 40 µl of autoclaved 13% PEG 8000. The mixture was mixed and incubated on ice for 20 minutes. The plasmid DNA was pelleted by centrifugation at 8,000g for 15 minutes at 40°C. The pellet was washed once with 500 µl of 70% ethanol, air dried, and resuspended in 20 µl of deionized water, and stored at -20°C. The concentration and purity of DNA was estimated by agarose gel electrophoresis.

6.4 Nucleotide sequencing with ABI PRISM BigDye^{TM} Terminator

A cycle sequencing reaction was prepared by adding 8 µl of ABI PRISM BigDye^{TM} Terminators (PE-Applied Biosystes, Inc., California, USA), 3.2 pmoles of primers (T7 promoter primer) and approximately 200-500 ng of template DNA in a total of 20 µl volumes. The reaction mixture was subjected to 25 cycles, each

composed of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. Excess dye terminator was removed by adding 16 µl of deionized water and 64 µl of 95% ethanol to each sequencing reaction, and then mixed by inversion for a few time. The mixtures were left at room temperature for 15 minutes to precipitate the extension product, and centrifuge at 10,000g for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed with 100 µl of 70% ethanol. The pellet was dried by inverting the tube onto a paper towel. The tube was resuspended in 25 µl of template suppression reagent, denatured by heat at 95°C for 2 minutes and then left on ice until the reaction was loaded on ABI 310 Genetic Analyser (PE-Applied Biosystems, Inc., California, USA). The DNA was automatically injected into the capillary for 30 seconds at 2.5 kV and subjected to 2 hours of capillary electrophoresis at 12.2 kV at 50°C in the POP-6 polymer. Raw electrophoregram was collected after laser excitation using the Data Collection software (PE-Applied Biosystems, Inc., California, USA). The sequence data was automatically called using the Sequence Analysis software which analyzed the electrophoregram pattern in comparison with the dye matrix file.

6.5 Analysis of the nucleotide sequences

The nucleotide sequence was obtained and verified using the Sequence Navigator software (PE-Applied Biosystems, Inc., California, USA). Sequence analysis was carried out using the Mac Vector software (Oxford Biomolecular Group, Oxford, UK) and compared with nucleotide sequence of pBps-1 gene previously analysed.

7. Expression of recombinant pBps-1/pcDNA 3.1(+) clone in *E. coli*

7.1 Absorption of sera with *E. coli* lysates

One hundred millilitres of *E. coli* cells strain TG1 were grown in LB medium in a flask with good aeration by vigorous shaking (250-300 rpm) at 37°C, overnight. The cell suspension was transferred into a 50 ml tube and cells were pelleted by centrifugation at 3,000 rpm, 4°C, for 10 minutes. The pellet was resuspended in 3 ml of 50 mM TrisCl pH 8.0, 10 mM EDTA pH 8.0. The suspension was frozen and thawed several times, and then lysed by sonication using 15 seconds pulse with 15 seconds interval for a total of 10 minutes (Model W-380, Heat Systems-Ultrasonics, Inc., NewYork, USA) on ice. After sonication, the disrupted cells were removed by centrifugation at 12,000 rpm, 4°C, for 10 minutes, and the supernatant was dispensed into a microcentrifuge tube and stored at –20°C until used.

The expression of pBps-1 cloned in pcDNA 3.1(+) was detected with polyclonal rabbit antibody against pBps-1 protein after pre-adsorption with *E. coli* lysate. The rabbit polyclonal antibody were diluted 1/10 with blocking solution (see Appendix). One hundred microlitres of *E. coli* lysate was added for every 10 µl of rabbit polyclonal antibody. The mixture was incubated for 4 hours at room temperature (86). After centrifugation for 10 minutes at 10,000 rpm, 4°C, the supernatant was transferred to a new tube and stored at 4°C in the presence of 0.05% sodium azide until used for the Western blot analysis.

7.2 Preparation of *E. coli* lysate containing recombinant pBps-1 protein

One hundred microlitres of an overnight culture of recombinant *E. coli* expressing pBps-1 protein was inoculated in 2 ml LB medium containing 100 μg/ml ampicillin and incubated at 37°C, for 4 hours with vigorous shaking. After incubation period, the cells were pelleted by brief centrifugation, washed once with phosphate buffered saline (PBS) (see Appendix) and resuspended in 200 μl of PBS and stored at –20°C until used.

7.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (87) to separate and to determine the molecular weight of the recombinant protein. Electrophoresis was performed using 5% stacking gel and 15% separating gel (see Appendix). Lysate of *E. coli* containing the specific recombinant protein was mixed with 2x sample buffer (see Appendix), then heated in boiling water bath for 5 minutes prior to applying to the gel. Lysate of *E. coli* TG1 harboring pBps-1 cloned in pKSII(-) was used as positive control. Lysate of *E. coli* TG1 and lysate of *E. coli* TG1 harboring pcDNA 3.1(+) were used as negative control. Electrophoresis was performed in a vertical direction with constant voltage of 100 volts. The current was stopped when the blue dye marker reached the bottom of the gel. The gel was then carefully taken out for staining or blotting.

7.4 Coomassie brilliant blue staining

The electrophoresed gel was stained by soaking in 0.25% (w/v) Coomassie Brillant Blue R-250 in 10% (v/v) glacial acetic acid and 45% (v/v) methanol for at least 1 hour. Excess staining was removed by placing the gel in several changes of destaining solution (10% (v/v) glacial acetic acid and 30% (v/v) methanol in distilled water) until the background was clear. The standard protein markers were a mixture of bovine serum albumin (66 kDa), egg albumin (45 kDa) and IgM light chain (25 kDa). The molecular weight of the recombinant protein was determined from SDS-PAGE pattern and calculated from standard curve of standard protein markers (88).

7.5 Western blot analysis

After electrophoresis, the protein was blotted onto nitrocellulose membrane (0.45 μm pore size; Schleicher & Schuell, Dassel, Germany) using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, California, USA) with Bio-Ice cooling unit. A constant voltage of 80 volts was applied for one hour to transfer the protein.

The recombinant protein, pBps-1, expressed in *E. coli* on the nitrocellulose membrane was evaluated by staining with the adsorbed rabbit polyclonal antibody against this protein, and detected by immunoenzyme method as followed. The nitrocellulose membrane was immediately immersed in a large volume of TNT buffer at room temperature for 30 minutes with continuous rotation to remove the debris of polyacrylamide gel. Unoccupied protein binding sites were blocked with blocking solution (see Appendix) at room temperature for 1 hour with continuous rotation. The nitrocellulose membrane was soaked in diluted adsorbed polyclonal antibody (1:50

dilution in 2.5% (w/v) skimmed milk in TNT buffer) and the reaction was allowed to take place at room temperature for 1 hour with continuous rotation. The membrane was then washed 3 times in TNT buffer. Alkaline phosphatase conjugated anti-rabbit immunoglobulins (Sigma, Minesota, USA) (1:250 dilution in 2.5% (w/v) skimmed milk in TNT buffer) was added, followed by incubation at room temperature for 1 hour with continuous rotation. The membrane was again washed 3 times, then the alkaline phosphatase substrate (see Appendix) was added. The enzymatic reaction was allowed to process for 30 minutes in the dark, and was stopped by rinsing the membrane in large volume of water. Positive reaction appeared as a red purple band on the membrane.

8. Transfection of the recombinant plasmid into J774A.1

pGreen-Lantern-1 and recombinant pBps-1/pcDNA 3.1(+) were the plasmid vectors delivered into J774A.1 by electroporation. pGreen-Lantern-1 plasmid vector was used for monitoring the transfection procedure by detection the transient expression of green fluorescence protein (GFP). pBps-1/pcDNA 3.1(+) was the plasmid for detection the stable expression of pBps-1 protein from *B. pseudomallei* in this cell line

8.1 Preparation of J774A.1 cells

J774A.1 kept in liquid nitrogen was thawed at 37°C in water bath and transferred to centrifuge tube containing RPMI 1640 medium (Gibco, Grand Island, USA). Cells were washed by centifugation at 1,000 rpm, 4°C for 5 minutes and the medium was discarded from the cell pellet. The cell pellet was washed again twice with RPMI 1640 medium and cultured in 15 ml of RPMI 1640 medium containing

20% fetal calfserum (Fetal clone I, Hyclone Laboratories, Utah). The medium was changed every 2 days until the desired number of cells were obtained.

8.2 Preparation of the recombinant plasmid DNA used for transfection

The recombinant plasmids were prepared by alkali lysis method from 500 ml culture of *E. coli*. Ten microlitres of *E. coli* TG-1 containing each plasmid was inoculated into 2 ml of Luria-Bertani media (LB media) (see Appendix) containing 100 μg/ml of ampicillin and incubated overnight at 37°C with continuous shaking. One millilitre of the overnight culture was transferred to 500 ml of the same medium and incubated overnight at 37°C with continuous shaking. The 500 ml overnight culture was centrifuged for 10 minutes at 5,000g to pellet the cells. The pellet was resuspended in 15 ml of TE buffer (see Appendix) containing 100 μg/ml Rnase A by vortexing, followed by the addition of 15 ml of freshly prepared TENS buffer (see Appendix). The mixture was mixed by rolling and tilting and placed for 20 minutes at room temperature. Thirty millilitres of 1.32 M potassium acetate pH 4.8 (see Appendix) was then added. The content was mixed by rolling and tilting and incubated for 10 minutes at room temperature and centrifuged at 14,000g for 15 minutes to pellet cell debris and chromosomal DNA. The supernatant was filtrated through Miracloth and collected. One half volume of isopropanol was then added to the filtered clear supernatant and mix by vortexing. The mixture was centrifuged 14,000g for 15 minutes at room temperature. The supernatant was discarded and the pellet was washed with 10 ml of cold 70% ethanol. The pellet was dried and resuspended in 500 μl of TE buffer pH 8 (see Appendix). The DNA was stored at –20°C until use.

8.3 Preparation of linearized pBps-1/pcDNA 3.1(+) for stable transfection

Seventy five micrograms of pBps-1/pcDNA3.1 prepared in 8.2 was digested with *Sal*I restriction enzyme (New England Biolabs, Hertfordshire, UK), that recognized the hexanucleotide 5' GTCGAC 3' target sequence, for 2 hours at 37°C. The reaction mixture was extracted twice with phenol and once with phenol: chloroform (1:1). The DNA was then precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of absolute ethanol and the pellet was washed with cold 70% ethanol, allowed to dry, and finally resuspend in 50 µl of sterile distilled water. The DNA was stored at –20°C until use for transfection.

8.4 Electroporation procedure

Electroporation procedure for transfection of pGreen-Lantern-1 and pBps-1/pcDNA 3.1(+) into J774A.1 was modified from that of Stacey (89). Briefly, $5x10^6$ J774A.1 cells were resuspended in 350 µl of complete RPMI 1640 medium (see Appendix). The suspension of the cells was placed into 0.2 cm electroporated cuvette (Bio-Rad, California, USA) containing 40 µl of 20 µg recombinant DNA. The cells and DNA were mixed by holding the cuvette on the two window sides and flicking the bottom. Electroporation of the cells was done by titrating the voltage at 100, 150 and 200 volts with the capacitance of 950 µF by using Gene Pulser apparatus (Bio-Rad, California, USA). The experiments were performed twice for each condition. The voltage that gave the optimal expression of GFP was chosen for the electroporation of pBps-1/pcDNA 3.1(+). One millilitre of the complete medium was immediately added to the transfected cells. Then, the transfected cells were transferred from the

cuvette into 4 ml of the complete medium and each 1 ml of transfected cells was added to each well of 24 wells tissue culture plate. The transfected cells were incubated at 37°C for 24 hours. Half of the medium was removed from the transfected cells and an equal volume of the fresh warm medium was added. The transfected cells were further incubated for another 24 hours before detecting the expression of GFP or growing the transfected cells in the selective medium containing G 418 for obtaining the resistant cell which stably expressed pBps-1 protein.

8.5 Detection of transient expression of the GFP in transfected J774A.1 cells

pGreen Lantern-1 transfected cells were harvested from each well of tissue culture plate and centrifuged at 1,000 rpm, 4°C for 5 minutes. The volume of the transfected cells from each well was adjusted to 10 µl and added on a clean slide. The expression of GFP of transfected cells was detected by visualization under Axioskop fluorescent microscope (Carl Zeiss, Oberkochen, Germany) and the survival of the transfected cells was determined by staining with 0.2% trypan blue.

8.6 Detection of stable expression of pBps-1 protein in transfected J77A.1 Cells

PBps-1/pcDNA 3.1(+) transfected cells were cultured in complete RPMI 1640 medium containing 250 µg/ml of G 418 after growing in non-selective medium for 48 hours. The selective medium was changed every 4 days during incubation of the transfected cells for 2 weeks. The transfected cells from each well were harvested and centrifuged at 1,000 rpm, 4°C for 5 minutes and the volume of the transfected cells

were adjusted to 100 µl. Ten microlitres of the transfected cells were determined for the resistant cell by staining with 0.2% trypan blue. The rest of the transfected cells was pooled and the cell lysate was prepared as described previously . Lysate of *E. coli* TG1 harboring pBps-1 cloned in pKSII(-) was used as the positive control. SDS-PAGE and Western blot analysis as described above were used to detect the stable expression of pBps-1 protein. The standard protein markers were the low range molecular weight protein markers (Bio-Rad, California, USA). These protein markers composed of phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The molecular weight of the recombinant protein was determined from SDS-PAGE pattern and calculated from standard curve of the standard protein markers (88).

CHAPTER V

RESULTS

1. Cloning of a recombinant antigenic gene of *B. pseudomallei* in mammalian expression vector

An antigenic gene, pBps-1, in pKSII(-) plasmid vector (Stratagene, Cambridge, UK) was prepared and digested with *Not*I and *Xho*I. The pBps-1 fragment of 847 base-pairs in size was observed in 0.7% low melting point agarose gel after electrophoresis of digested recombinant pKSII(-) (Figure 4). The 847 base-pairs fragment was excised from the gel and selected for cloning. The DNA fragment was ligated with *Not*I and *Xho*I digested pcDNA 3.1(+) plasmid (Invitrogen, California, USA) and the ligation product was transformed into *E. coli* strain TG1. A total of 18 colonies of recombinant clones were obtained in ampicillin plate and 10 colonies were selected for identifying pBps-1 cloned in pcDNA 3.1(+).

2. Identification of pBps-1 insert in pcDNA 3.1(+)

Ten colonies of *E. coli* TG1 containing recombinant plasmids were grown on ampicillin plate and cultured overnight in LB broth containing 100 μg/ml ampicillin. Recombinant plasmids were extracted from the overnight culture. The extracted recombinant plasmids from each colony was digested with *Not*I and *Xho*I and electrophoresed in 1% agarose gel to identify *Not*I and *Xho*I-digested pBps-1 fragment using λ-DNA-*Hin*d III and DNA-ladder as markers. Three clones from colonies 5, 8

and 10 containing the 847 bp insert whereas other clones had only the 5,400 bp DNA fragment which were of the *Not*I and *Xho*I-digested vector (Figure 5).

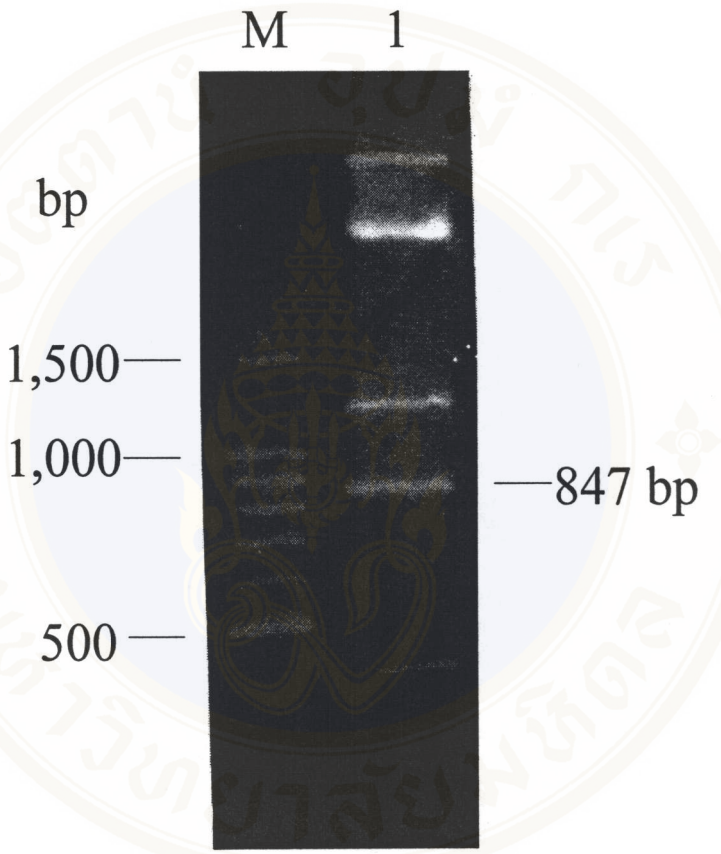


Figure 4 Low melting point agarose gel electrophoresis of *Not*I and *Xho*I-digested recombinant pKSII(-).

Lane M : 100 base-pair DNA ladder marker.

Lane 1 : *Not*I and *Xho*I-digested recombinant pBps-1/pKSII(-).

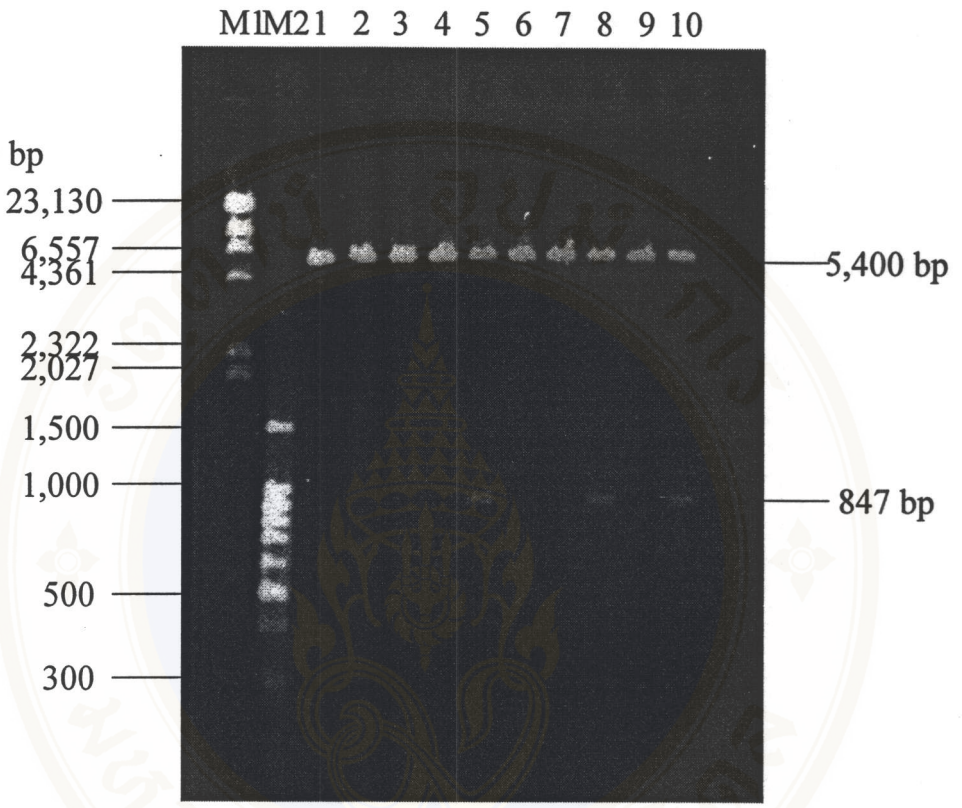


Figure 5. Agarose gel electrophoresis of pcDNA 3.1(+) harboring pBps-1 gene by cleaving plasmid DNA with *Not*I and *Xho*I to obtain the inserted pBps-1. The result showed that clones 5, 8 and 10 had the 847 bp insert.

Lane M1 : λ *Hin*d III marker.

Lane M2 : 100 base-pair DNA ladder marker.

Lane 1-10 : clones 1-10.

3. Nucleotide Sequencing of pBps-1 gene in pcDNA 3.1 (+)

One of the three positive colonies was chosen for further characterization. This recombinant plasmid was used as the DNA template for nucleotide sequencing of this gene by using T7 promoter as a primer in order to confirm the identity and orientation of pBps-1 gene cloned in this vector. The sequence reaction was loaded on ABI 310 Genetic Anlyser (PE-Applied Biosystems, Inc., California, USA). The obtained nucleotide sequences of pBps-1 was verified using the Sequence Navigator software (PE-Applied Biosystems, Inc., California, USA). The nucleotide sequencing of pBps-1 cloned in pcDNA 3.1(+) was compared with the nucleotide sequences of pBps-1 cloned in pKSII(-) which was previously analysed by using the MacVector software (Oxford Biomolecular Group, Oxford, UK). The nucleotide sequences of pBps-1 cloned in pcDNA 3.1(+) showed identity with the nucleotide sequences of pBps-1 cloned in pKSII(-) (Figure 6).

```
                              20                  40                  60
                               *                   *                   *
pBps-1/pKSII(-)     GATCCAGCGGGAAAACATCGGCTCGTGGAAGGAGCGCGGCGATCTCGAGCAGGAGCTCAA

                              80                 100                 120
                               *                   *                   *
pBps-1/pKSII(-)     CCAGTGGATCCGCCAGTACGTCGTCGACATGGACAACCCGTCGCAGAGCGTGCGCAGCCG
pBps-1/pcDNA                                                          CTGGCTAGC

                             140                 160                 180
                               *                   *                   *
pBps-1/pKSII(-)     CCGCCCGCTGCGGCAGGCGCAGATCGTCGTGTCGGACGTCGAGGGCGAACCCGGCTGGTA
pBps-1/pcDNA        GTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGTAATTCTG

                             200                 220                 240
                               *NotI               *                   *
pBps-1/pKSII(-)     TCGCGTGGACATGAAGGTGCGGCCGCACTTCAAGTACATGGGCGCGTTCTTCACGCTGTC
pBps-1/pcDNA        CAGATATCCAGCACAGTG..................................

                             260                 280                 300
                               *                   *                   *
pBps-1/pKSII(-)     GCTCGTCGGCAAGCTCGAAAAGCGCTAGGCGGGCGGGTCGGGCGGATTTCATCGGAAACA
pBps-1/pcDNA        ............................................................

                             320                 340                 360
                               *                   *                   *
pBps-1/pKSII(-)     TTCGCCCGGCCCGGCGAAACGAAAATCAAACGCGCCCGCTCGATAATCGATTCACGCCGA
pBps-1/pcDNA        ............................................................
```

```
                                380                 400                 420
                                 *                   *                   *
pBps-1/pKSII(-)     TGGATTCGGATACCTGACGAACCGCTGCCGGCATCGCACGCGCGACGCCGGCGACGTCGG
pBps-1/pcDNA        ............................................................

                                440                 460                 480
                                 *                   *                   *
pBps-1/pKSII(-)     GTCGAGCCCGGAGCCGTCTTCGTCAACTCGGCGCGCTGCGCCGCCATCACGCAAAAAGGA
pBps-1/pcDNA        ............................................................

                                500                 520                 540
                    start codon  *                   *                   *
pBps-1/pKSII(-)     GAGCGCCATGCTGGCCGGAATATATCTCAAGGTCAAAGGAAAAACCCAGGGGGAAATCAA
pBps-1/pcDNA        ............................................................

                                560                 580                 600
                                 *                   *                   *
pBps-1/pKSII(-)     AGGCTCCGTCGTTCAGGAAGGTCATGACGGGAAAATCCACATCCTCGCCTTCAAGAACGA
pBps-1/pcDNA        ............................................................

                                620                 640                 660
                                 *                   *                   *
pBps-1/pKSII(-)     CTACGACATGCCTGCCAGGCTCCAGGAAGGCCTGACGCCCGCCGCCGCCGCTCGCGGCAC
pBps-1/pcDNA        ............................................................

                                680                 700                 720
                                 *                   *                   *
pBps-1/pKSII(-)     GATCACGTTGACGAAGGAAATGGACAGATCGTCGCCGCAATTCCTGCAGGCGCTCGGCAA
pBps-1/pcDNA        ............................................................

                                740                 760                 780
                                 *                   *                   *
pBps-1/pKSII(-)     GCGCGAGATGATGGAAGAGTTCGAGATCACGATCCACCGTCCGAAGACGGATACAACAGG
pBps-1/pcDNA        ............................................................

                                800                 820                 840
                                 *                   *                   *
pBps-1/pKSII(-)     TGGGGACCTGACCGAACTCCTGTTCACGTACAAGTTCGAAAAAGTGCTGATCACCCACAT
pBps-1/pcDNA        ............................................................

                                860                 880                 900
                                 *                   *                   *
pBps-1/pKSII(-)     GGACCAATACTCGCCCACGCCGCACAAAGACGATAGCAACGGCATCAAGGAAGGCTTGCT
pBps-1/pcDNA        ..........................>
```

Figure 6. The nucleotide sequences comparison of pBps-1/pcDNA 3.1(+) and pBps-1/pKSII(-).

4. Expression of pBps-1 gene in pcDNA 3.1(+)

Since pBps-1 could be constitutively expressed in *E. coli* containing the recombinant plasmid, pKSII(-) (81). It was possible that the expression of pBps-1 was independent from the *lacZ* promoter in pKSII(-) and might have its own intrinsic promoter. Therefore, the positive clone that had been already sequenced was selected to detect its expression in *E. coli*. The expressed protein pBps-1 was demonstrated by SDS-PAGE compared with the lysate of *E. coli* TG1 harboring pBps-1 in pKSII(-) (Figure 7). The presence of the recombinant protein was confirmed by Western bloting using the absorbed rabbit polyclonal antibody to this protein. The recombinant pBps-1 in pcDNA 3.1(+) expressed a protein with a molecular weight of approximately 18.7 kDa similar to pBps-1 protein expressed from pBps-1 in pKSII(-) (Figure 8).

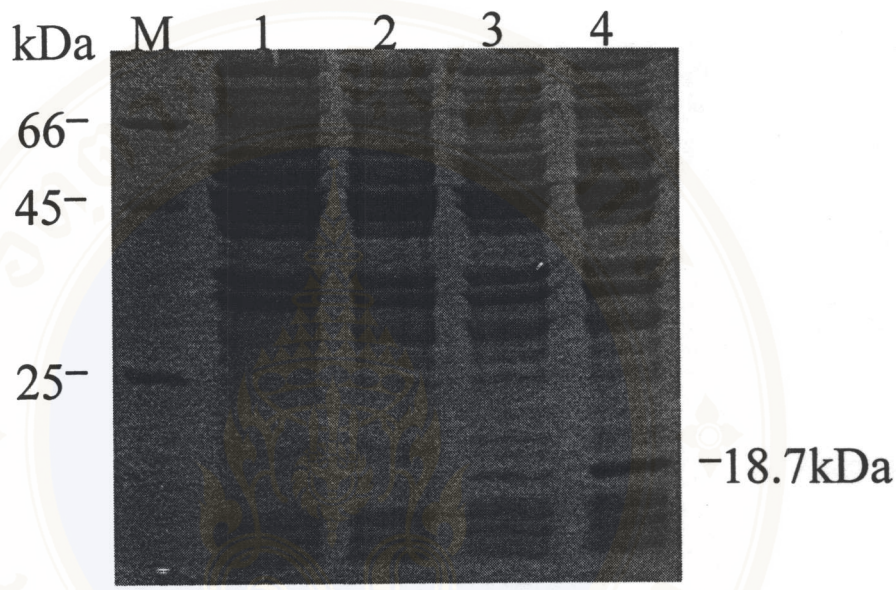


Figure 7. Expression of pBps-1 protein (18.7 kDa in size) was demonstrated by Coomassie brilliant blue staining of *E. coli* protein separated by SDS-PAGE.

Lane M : Molecular weight markers.

Lane 1 : *E. coli* TG-1.

Lane 2 : *E. coli* TG-1 harboring pcDNA 3.1(+).

Lane 3 : *E. coli* TG-1 harboring pBps-1 in pcDNA3.1(+).

Lane 4: *E. coli* TG-1 harboring pBps-1 in pKSII (-).

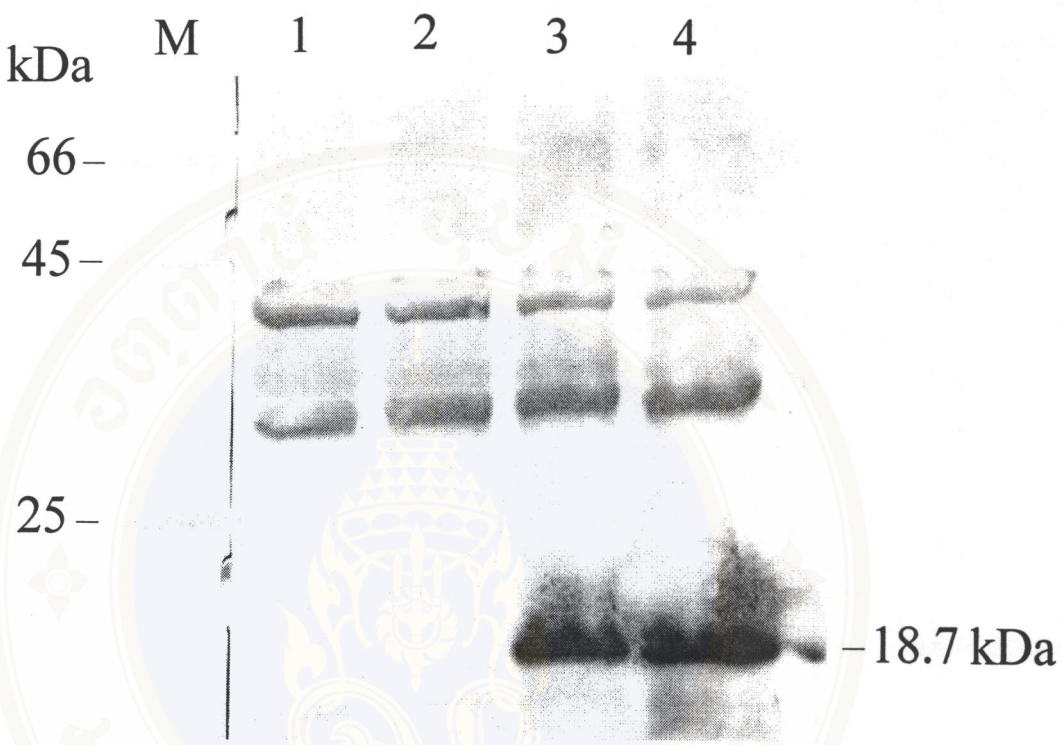


Figure 8. Expression of pBps-1 protein (18.7 kDa) detected by Western blot analysis of the recombinant pBps-1 clone and *E. coli* TG-1 tested with absorbed rabbit polyclonal antibody against pBps-1.

Lane M : Molecular weight markers.

Lane 1 : *E. coli* TG-1.

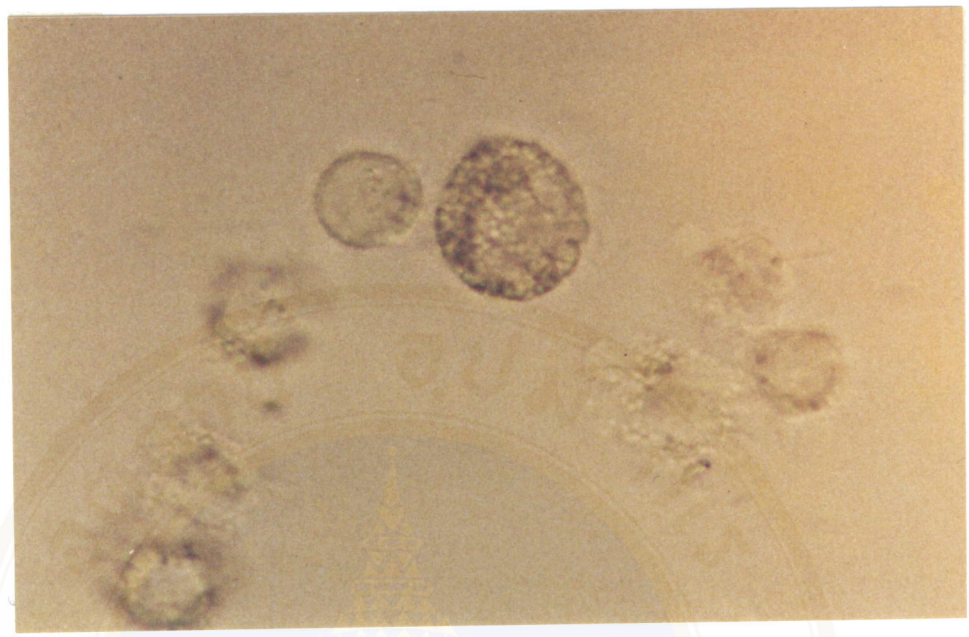
Lane 2 : *E. coli* TG-1 harboring pcDNA3.1(+).

Lane 3 : *E. coli* TG-1 harboring pBps-1 in pcDNA3.1(+).

Lane 4 : *E. coli* TG-1 harboring pBps-1 in pKSII(-).

5. Transfection of J774A.1 cells with pGreen-Lantern-1

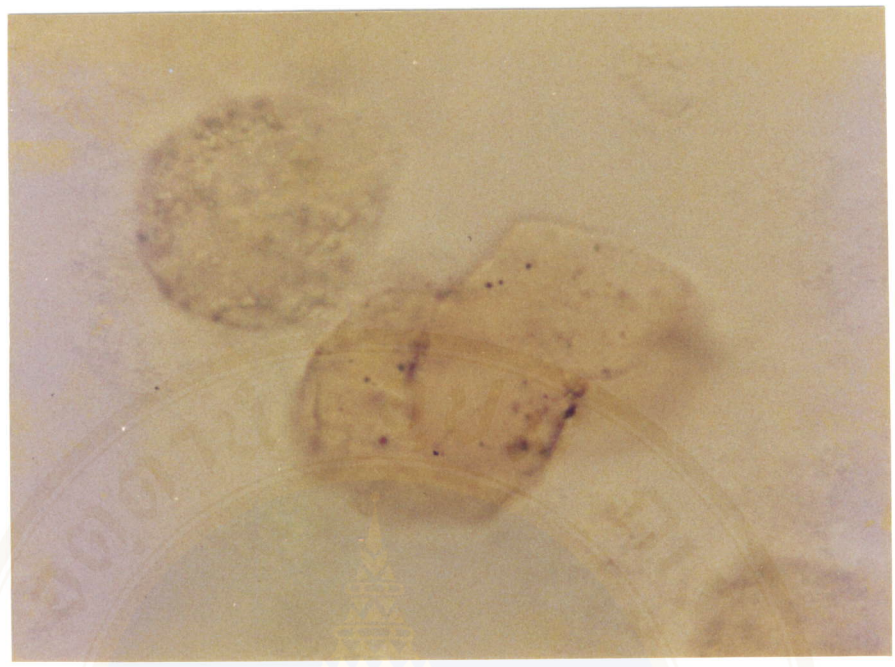
Electroporation of J774A.1 with pGreen-Lantern-1 was used to establish the optimal condition for transfection in J774A.1 cell. Electroporation was done by titrating the voltage at 100, 150 and 200 volts with the capacitance of 950 μF. The experiments were performed twice for each condition. Transfection in J774A.1 was optimized by electroporation with pGreen-Lantern-1 at 200 volt and capacitance of 950 μF and the time constant of 14.4 msec. The total survival cells were 340 cells of the initial $5x10^6$ cells by staining with 0.2% trypan blue. Therefore, the survival rate of transfected cells were 1 in $1.47x10^4$ total cells used for electroporation when transfecting with this condition. The transient expression of GFP could be detected in the transfected cell by visualization under the fluorescence microscope comparing with the untransfected J774A.1 cell, which no any detectable GFP (Figures 9 and 10). The percentage of positive cells that expressed GFP was 8.8% of the survival cells. In addition, the number of the survival cells and GFP positive cells were not different from other two voltages (100, 150 volts) used in the experiment (Data not shown). Electroporation at 200 volts gave the GFP positive cells with strongest fluorescent intensity.



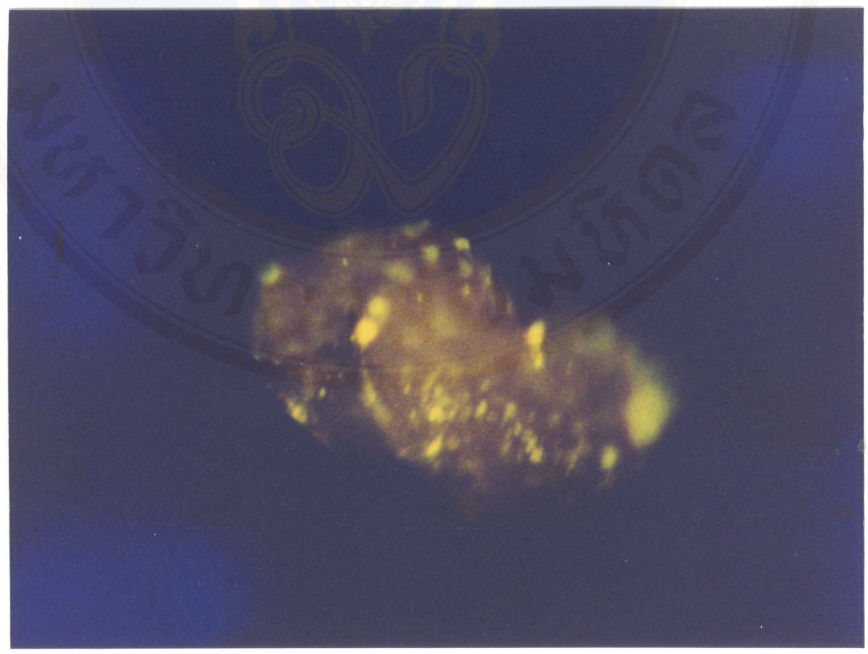
(A)

(B)

Figure 9. Untransfected J774A.1 cell visualized under a light microscope (A) and fluorescent microscope (B) (magnification 400x).



(A)



(B)

Figure 10. The expression of GFP in pGreen-Lantern-1 transfected J 774A.1 cells after 48 hours of electroporation visualized under a light microscope (A) and fluorescent microscope (B) (magnification 400x). The expression of GFP was observed in the transfected cells.

6. Stable expression of pBps-1 gene in J77A.1 cell

The electroporation condition used for pGreen-Lantern-1 in 4. was used for electroporation of linearized pBps-1/pcDNA 3.1(+) into J774A.1. After 48 hours of culturing in non-selective medium, the transfected cells were grown in the selective medium containing 250 μg/ml of G 418 for obtaining the resistant cells that stably expressed this protein. The concentration of 250 μg/ml of the drug was determined by titrating the drug level that could 100% inhibit the growth of J774A.1 cell (Data not shown). The total resistant cells obtained after growing in the selective medium for 2 weeks were approximately 100 by staining with 0.2% trypan blue, which represented 1 in $5x10^4$ in the transfection that had the stable integration of pBps-1/pcDNA 3.1(+) into the chromosome. The total resistant cells obtained was not different when two times electroporation of the recombinant plasmid into this cell line. The rest of the resistant cells were pooled and prepared as cell lysate for detecting the expression of pBps-1. However, the expression of pBps-1 could not be detected in the pooled cell lysate of the transfected cells by staining with Coomassie brilliant blue after separation by SDS-PAGE (Figure 11) or Western blot analysis using the absorbed serum against pBps-1 protein from melioidosis patient (Figure 12). The patient's serum was used instead of rabbit polyclonal antibody because the rabbit polyclonal antibody cross reacted with a protein component of the untransfected J774A.1, which had the molecular weight resemble pBps-1 protein.

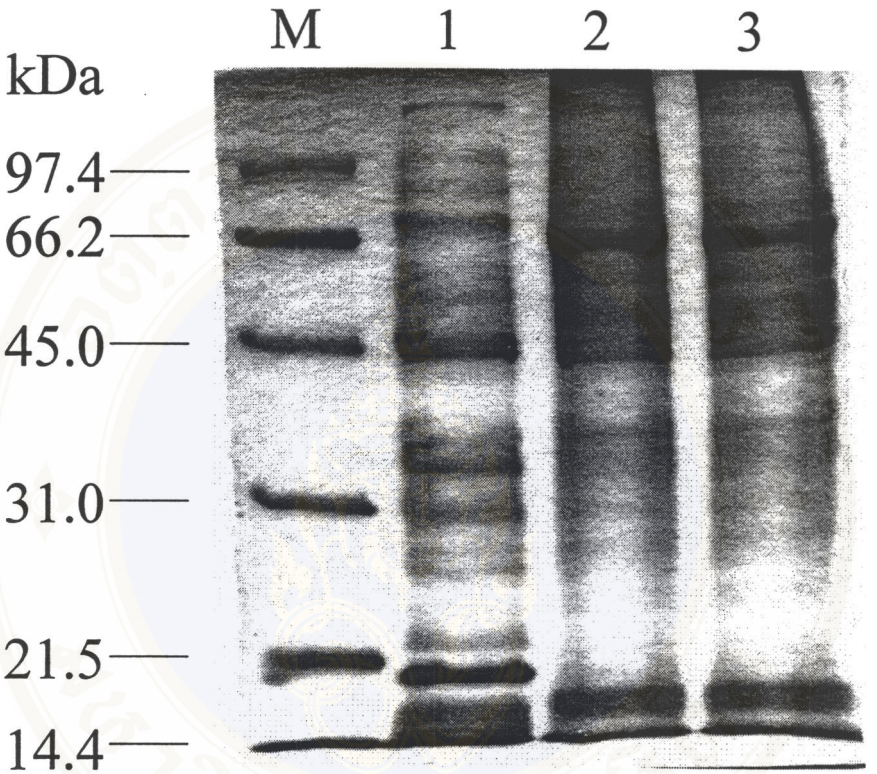


Figure 11. Expression of pBps-1 in J774A.1 cell demonstrated by Coomassie brilliant blue staining after separating by SDS-PAGE. pBps-1 protein was not observed in the transfected cell.

Lane M : Molecular weight markers

Lane 1: *E. coli* TG-1 harboring pBps-1 in pKSII (-).

Lane 2: Untransfected J774A.1 cell.

Lane 3: pBps-1/pcDNA 3.1(+) transfected cell.

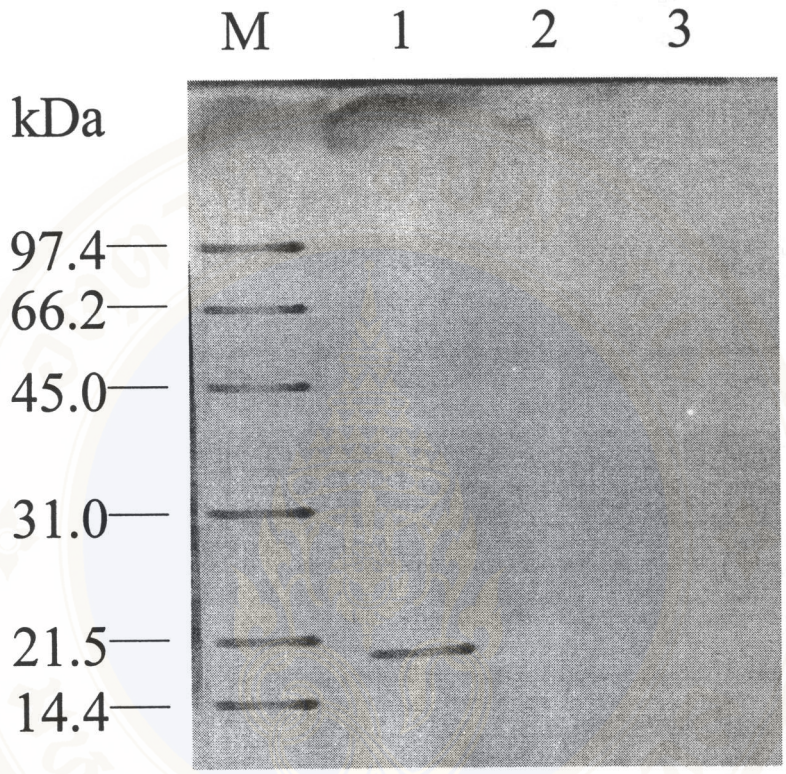


Figure 12. Expression of pBps-1 in J774A.1 cell demonstrated by Western blot analysis with absorbed serum from melioidosis patient reacted with pBps-1 protein. No any detectable 18.7 kDa was found in transfected cell.

Lane M : Molecular weight markers

Lane 1: *E. coli* TG-1 harboring pBps-1 in pKSII (-).

Lane 2: Untransfected J774A.1 cell.

Lane 3: pBps-1/pcDNA 3.1(+) transfected cell.

CHAPTER VI

DISCUSSION

Melioidosis is a fatal disease of human and animals which is endemic in Southeast Asia and Northern Australia. In Thailand, melioidosis is an important public health problem especially in the northeastern part of Thailand. The causative agent, *Burkholderia pseudomallei*, can survive within the phagocytic and non-phagocytic cells, therefore can escape the immunity of the hosts generated after infection including avoidance by attack complement or antibodies. Effective immunity that confers protection and elimination of the disease should involve the role of cell-mediated immunity especially cytotoxic T lymphocyte, like other intracellular bacteria. However, the role of cytotoxic T lymphocyte in melioidosis has not yet been investigated. Limited data on the non-specific cellular immunity and some specific antibodies to LPS and flagellin of the pathogen that confer partial protection have been investigated in animal models (65, 90-91).

To study the role of cytotoxic T lymphocyte against most intracellular pathogens including viruses, parasites and intracellular bacteria, preparation of target cells is the initial step for further analysis of the CTL response to the pathogen.

In this study, transfection of *B. pseudomallei* antigenic gene into a mammalian cell is the method of choice for preparing target cell expressing *B. pseudomallei* antigen, which could present the antigen on the surface of the transfected cell with

MHC class I, similar to that occurs *in vivo*. This method is well-defined and reproducible for preparation of antigen expressing cells for CTL assay. The preparation of the antigen expressing cells to be use as the target cells by this strategy was successfully demonstrated in the investigation of CTL response in *L. monocytogenes* and *M. tuberculosis* (57, 66-68). Moreover, this strategy was also used in the CTL assay of influenza A virus and CTL response to DNA vaccination (92-93). The transfected gene was cloned into mammalian cell expression vector, pcDNA 3.1(+), in order to have this gene expressed in the mammalian cells, therefore, being processed the expressed protein. The pBps-1 antigenic gene was chosen as the model antigen gene of *B. pseudomallei* because this gene encodes the protein that strongly reacts with sera from localized and septicemic melioidosis patients. In addition, pBps-1 gene was already cloned and available in pKSII(-) and all nucleotide sequence and restriction mapping were known for allowing the manipulation of this gene.

The pBps-1 gene was successfully cloned into pcDNA 3.1(+), and confirmed with automated DNA sequencing. The starting codon (ATG) of pBps-1 gene was located at nucleotide position 488 of the 847 bp fragment (Figure 6). The recombinant plasmid, pBps-1/pcDNA3.1(+), could express a 18.7 kDa protein in *E. coli* cells independently from the control of CMV promoter/enhancer in pcDNA3.1(+). This showed that the upstream of the starting codon of pBps-1 gene may possibly have its intrinsic promoter (81) that located between nucleotide position 200 and 487 within the 847 bp cloning fragment.

In order to establish a transfection protocol for J774A.1 cells, pGreen-Lantern-1, a plasmid encoded modified green fluorescent protein (GFP), was used prior to the transfection of pBps-/pcDNA 3.1(+) in J774A.1 cell. GFP was chosen as the reporter gene in the experiment because the transient GFP expression can be easily detected by using only fluorescence microscope, and required no substrates like other reporters. In addition, because different plasmid DNA can enter the cell in a similar efficiency, electroporation allows DNA to enter the cells directly by passing the pores generated after cells exposed to electrical current. Whereas, other transfection methods need chemical reaction to form the DNA particle complex before uptaking DNA into the cells. Therefore the size or the base composition of the plasmid possibly affects the DNA complex formation. During procedure optimization, no significant difference in the number of transfected cells expressing GFP and survival cells when using 3 different voltages (200,150 and 100 volts) used in electroporation condition. However, electroporation at 200 volts gave the positive cells with strongest fluorescent intensity and was chosen for further study.

Stable expression of pBps-1 gene was required because the target cells in CTL assay should permanently express antigen via MHC-class I. pcDNA 3.1(+) could not replicate in J774A.1 transfected cells so that it is unlikely to detect the transient expression of pBps-1 if the copy number of the transfected plasmid is low. Therefore selection of stable expression of pBps-1 gene that has the integration of this gene into the genome of transfected cells was required. G 418 was used for the selection of the transfected cells that stably expressed pBps-1 protein in the experiment because the recombinant plasmid contains neomycin resistance gene. The expression of this drug

resistance gene will detoxify G 418 activity and the resistant cells, which had the integration of the recombinant plasmid into the genome would be obtained.

In the production of the target cells which stably expressd pBps-1 gene, 100 resistant cells were obtained after culturing the transfected cells in selective medium. The ratio of resistant cells was 1 in $5x10^4$ of transfected cells. This showed that the integration of recombinant plasmid was able to occur and number of resistant cells that had the integration of pBps-1/pcDNA 3.1(+) was similar to most cell types that ranging between 1 in 10^4 to 10^5 by using electroporation as the transfection method (75). However, the expression of pBps-1 was not detected by staining with Coomassie brilliant blue after separation by SDS-PAGE or by Western blot analysis.

The most possible factors that may affect this result may involve the electroporation condition, the characteristic of the cell type and the detection method of the expressed gene. Regarding the electroporation condition, it was not known that the recombinant plasmid actually were in the transfected cells after electroporation at this experiment eventhough the optimal electroporation condition used in the experiment was prior established by GFP expression. In retrospect, detection of this gene in the transfected cells should be carried out to confirm this leading to know that DNA could efficiently enough to enter J774A.1 cells.

J774A.1 cells used for the preparation of target cells expressing pBps-1 gene in the study had doubling time more than 18-24 hours after several passages and may not be healthy enough to be transfected. Changing of the cell type should be considered. However, the cell type that can be used as the target cell in a CTL assay is limited due to what type of host chosen as the model for study of the role of cytotoxic T

lymphocyte response. J774A.1 has been used as target cells for the study of cytotoxic T lymphocyte to *Listeria monocytogenes* in BALB/c mice (8, 94) which is the same animal host for further study of CTL response in melioidosis. This cell type was also available in the laboratory at that time that the experiment was carried out. Alternatively, P815 (mastocytoma cell) is another choice but this type of cell was not available at that time. A cell that supports the replication of this plasmid such as COS7 can be used to investigate the transient expression of the pBps-1 gene in mammalian cell before producing pBps-1 expressing target cell for CTL assay.

Concerning the detection method of the expressed gene, cell lysate contained about 90 resistant cells were subjected to SDS-PAGE and Western blot analysis. If that pBps-1 gene could express in the resistant cells, the overall expression of the cell lysate may be at a low level that was lower than the minimal detection range of Coomassie brilliant blue and Western blot analysis (95). Detection of any reporter gene expression by this method should be done to establish the detection range of Coomassie brilliant blue and Western blot analysis. Alternatively, expanding of the resistant cells to be the bulk culture for giving the enough number of resistant cells could be done when using the healthy cell culture. Therefore, it was not advantage to extend culturing of the resistant cells in this experiment.

In addition, the toxicity of the expressed protein to J774A.1 cells may lead to the inability to detect the expression of this gene. Therefore, cloning this gene into other plasmid vector that contains a strong on/off promoter should be employed to prove this factor.

If pBps-1 gene could be demonstrated within the cells, the problem may involve the expression of pBps-1 gene. Other factors may be accounted for the inability to have stable expression of pBps-1 gene including the base composition in the constructed recombinant vector and the characteristic of pBps-1 gene itself.

The nucleotide sequence of 847 bp cloning fragment in the constructed recombinant plasmid may also influence the expression of pBps-1 gene in mammalian cells. The pBps-1 gene is a bacterial gene and bacterial systems translate polycistronic message well. In contrast, translation of most mammalian mRNA may decrease about 10-fold by upstream start sites if an upstream AUG codon is in the wrong reading frame leading to depressing the yield of the protein from the actual site (96). Alternatively, the start codon of the desirable gene is not effected by the upstream AUG if the start codon has the consensus sequence, A/GCCAUGG, (Kozak's sequence) that is preferential bound by 40S ribosome of the mammalian cell (96). This may lead to the low expression or inability of pBps-1 in mammalian cell. Nevertheless there was also evidence that Kozak's sequence did not affect the expression of human fibroblast growth factor (bFGF) in insect cell (97). The 847 bp fragment of pBps-1 contains two upstream AUG codon that are not in the same reading frame and the third AUG that is the actual start codon of pBps-1 gene is not compatible to Kozak's sequence.

In addition, the optimal codon usage of the expressed genes in mammalian and bacterial system may be distinct which result from the compositional pressure and evolution of the organism. The bias of codon usage was found in both prokaryotic and eukaryotic cells (98-101). Therefore, the optimal codon usage in pBps-1 gene

theoretically may differ from the optimal codon usage in the highly expressed gene of mammalian cell and become the rare codons in mammalian system. The rare codons have the potential to retard the movement of ribosomes as the host cell attempts to cope with the translational inefficiently (102). The optimization of codon usage of the eukaryotic genes have been demonstrated when optimal expression in *E .coli* (103-105). Optimizations of the codon usage of the listeriolysin gene of *Listeria monocytogenes* or gp160 and gag gene of HIV-1 were also required for the expression in mammalian cell (94, 106-108). Therefore, although it is a remote possibility, measurement of transcription efficiency in the mammalian cell may be used to evaluate this issue.

All of these factors should be considered for a successful establishment of target cell expressing pBps-1 gene used in the study of cytotoxic T lymphocyte response to this pathogen by CTL assay.

CHAPTER VII

CONCLUSION

The present study was carried out to obatain an expression of *B. pseudomallei* gene in mammalian cell to be used as target cells for cytotoxic T cell study. The 847 bp *Not*I and *Xho*I digested fragment of pBps-1 from *Burkholderia pseudomallei* was sucessfully cloned into pcDNA 3.1(+) at *Not*I and *Xho*I site. The recombinant plasmid was transformed into *E. coli* TG1. Three positive clones that harbored the recombinant plasmid containing 847 bp DNA fragment was obtained and Automated DNA sequencing was used to confirm the identity and orientation of the insert. The expression of pBps-1/pcDNA3.1(+) could be detected in *E. coli* by Coomassie brilliant blue staining after SDS-PAGE and Western blot analysis with rabbit polyclonal antibody against this protein.

The optimization of transfection of mammalian cell by electroporation was determined using the transient expression of GFP in the transfected J774A.1 cells. The optimal expression of GFP was observed when using electrical voltage at 200 volts and capacitance at 950 μF. This electroporation condition was chosen for transfecting of pBps-1/pcDNA 3.1(+).

The transfection of linearlized pBps-1/pcDNA3.1(+) into J774A.1 was carried out and the stably resistant transfected cells were obtained by selecting in the selective

medium after culturing the transfected cells in the selective medium for 2 weeks. However, the expression of pBps-1 protein was not detected in the transfected cell lysate by SDS-PAGE or Western blot. Therefore, target cell expressing antigenic gene of *B. pseudomallei* was not obtained in this experiment. Factors that may affect this results are discussed.

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APPENDIX

GENERAL REAGENTS

Phosphate buffered saline (PBS)

NaCl (Fluka)	8.00	gm
KCl (BDH)	0.20	gm
Na_2HPO_4 (Merck)	1.44	gm
KH_2PO_4 (Merck)	0.24	gm

Dissolve in distilled water, adjust to pH 7.2 with HCl and adjust to 1,000.00 ml final volume with distilled water. Sterilized by autoclaving for 15 minutes at 121°C, 15 lb/square inches.

1.0 M Tris-HCl pH 8.0

Tris base (Sigma)	12.11	gm

Dissolve in distilled water, adjust to pH 8.0 with concentrated HCl and adjust to 100.00 ml final volume with distilled water. Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches.

1.0 M Tris-HCl pH 9.5

Tris base (Sigma)	12.11	gm

Dissolve in distilled water, adjust to pH 9.5 with concentrated HCl and adjust to 100.00 ml final volume with distilled water.

0.5 M EDTA pH 8.0

EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$) (Amresco)	18.6	gm

Dissolve in distilled water, adjust to pH 8.0 with NaOH and adjust to 100.00 ml final

volume with distilled water. Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches.

1.0 M NaOH

NaOH (Fluka)	4.00	gm
Distilled water	100.00	ml

10 % SDS

Lauryl sulfate (Sigma)	10.00	gm
Distilled water	100.00	ml

5.0 M NaCl

NaCl (Fluka)	29.22	gm
Distilled water	100.00	ml

1 M glucose

D(+) glucose anhhydrous (Fluka)	18.02	gm
Distilled water	100.00	ml

1 M $MgCl_2$

$MgCl_2$ (Merck)	20.30	gm
Distilled water	100.00	ml

MEDIA FOR BACTERIAL CULTURE

Luria-Bertani medium (LB medium)

Tryptone (Difco)	10.00	gm
Yeast extract (Difco)	5.00	gm
NaCl (Fluka)	5.00	gm
1 N NaOH	1.00	ml

Dissolve and adjust the volume to 1,000.00 ml with distilled water. Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches.

Ampicillin plate

Antibiotic medium 11 (Difco)	30.50	gm
Distilled water	1,000.00	ml

Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches, left the media cooled down to 50 °C at room temperature, added ampicillin (stock 100 mg/ml) 1 ml, poured the media on petri dish.

REAGENT FOR MOLECULAR CLONING

Transformation buffer I (TFBI)

CH_3COOK (Merck)	1.47	gm
$MnCl_2 \cdot 4H_2O$ (BDH)	4.95	gm
RbCl (Sigma)	6.05	gm

Dissolve in distilled water, adjust to pH 5.8 with 0.2 M acetic acid and adjust to 500.00 ml final volume with distilled water. Sterilized by filtration through a 0.2 μm syringe filter and stored at 4°C.

Transformation buffer II (TFBII)

100 mM MOPS pH 7.0 (Sigma)	50.00	ml
$CaCl_2 \cdot 2H_2O$ (Merck)	5.51	gm
RbCl (Sigma)	0.61	gm
15% (w/v) Glycerol (Sigma)	75.00	ml

Dissolve and adjust the volume to 500.00 ml with distilled water. Sterilized by

filtration through a 0.2 μm syringe filter and stored at 4 °C.

TE pH 8.0 (10 mM TrisCl pH 8.0, 1 mM EDTA pH 8.0)

1 M TrisCl pH 8.0	1.00	ml
0.5 M EDTA pH 8.0	0.20	ml

Dissolve and adjust the volume to 100.00 ml with distilled water.

GTE buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA)

1 M glucose	5.00	ml
1 M Tris-HCl pH 8.0	2.50	ml
0.5 M EDTA	2.00	ml

Dissolve and adjust the volume to 100.00 ml with distilled water.

3.0 M Sodium acetate

Sodium acetate (CH_3COONa) (Merck)	24.61	gm

Dissolve in distilled water, adjust to pH 5.2 with glacial acetic acid and adjust to 100.00 ml final volume with distilled water.

1.32 M potassium acetate

Potassium acetate (CH_3COOK) (Merck)	12.95	gm

Dissolve in distilled water, adjust to pH 4.8 with glacial acetic acid and adjust to 100.00 ml final volume with distilled water.

TENS buffer (10 mM TrisCl pH 8.0, 1 mM EDTA pH 8.0, 0.1 N NaOH, 0.5% SDS)

1 M TrisCl pH 8.0	50.00	μl
0.5 M EDTA pH 8.0	10.00	μl
1 N NaOH	0.50	ml
10% SDS (w/v)	0.25	ml

Dissolve and adjust the volume to 5.00 ml with distilled water.

1xTBE buffer

Tris base (Sigma)	10.80	gm
Boric acid (Fluka)	5.50	gm
0.5 M EDTA (pH 8.0)	4.00	ml

Dissolve and adjust the volume to 1,000.00 ml with distilled water.

6x Gel-loading buffer

Bromophenol blue (Sigma)	25.00	mg
Xylene cyanol FF (Sigma)	25.00	mg
Glycerol (Sigma)	3.00	ml

Dissolve and adjust the volume to 10.00 ml with distilled water.

Ethidium bromide (10 mg/ml)

Ethidium bromide (Sigma)	1.00	gm
Distilled water	100.00	ml

The solution was stored in a dark bottle at 4 °C.

4.4 M LiCl

Lithium Chloride anhydrous (BDH)	18.65	gm
Distilled water	100.00	ml

Sterilized by autoclaving for 15 minutes at 121 °C, 15 lb/square inches

REAGENT FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

2xSample buffer (50 mM TrisCl pH 6.8, 4% SDS (w/v), 20% Glycerol (v/v) and 0.02% Bromophenol blue)

Sodium dodecyl sulfate (SDS, Sigma)	0.40	gm

Glycerol (Sigma)	2.00	ml
1.0 M TrisCl pH 6.8	0.50	ml
Bromophenol blue (Sigma)	2.00	mg

Dissolve and adjust the volume to 10.00 ml with distilled water. One ml of 2-mercaptoethanol (2-ME) was added to 9.00 ml of sample buffer.

10% Sodium dodecyl sulfate (SDS) (w/v)

Sodium dodecyl sulfate (Sigma)	1.00	gm
Distilled water	10.00	ml

10% Ammonium persulfate (APS) (w/v)

Ammonium persulfate (Promega)	1.00	gm
Distilled water	10.00	ml

1.5 M TrisCl pH 8.8 (resolving gel buffer)

Tris base (Sigma)	18.16	gm

Dissolve in distilled water, adjust to pH 8.8 with concentrated HCl and adjust to 100.00 ml final volume with distilled water.

1.0 M TrisCl pH 6.8 (Stacking gel buffer)

Tris base (Sigma)	12.11	gm

Dissolve in distilled water, adjust to pH 6.8 with concentrated HCl and adjust to 100.00 ml final volume with distilled water.

30% Acrylamide-bisacrylamide solution

Acrylamide (Bio-Rad)	29.00	gm
N, N-bismethylene acrylamide (Sigma)	1.00	gm
Distilled water	100.00	ml

The solution was stored in a dark bottle at 4 °C.

Electrode buffer

Tris base (Sigma)	3.02	gm
SDS (Sigma)	1.00	gm
Glycine (Sigma)	14.24	gm

Dissolve in distilled water, adjust to pH 8.3 with 1 N HCl and adjust to 1,000.00 ml final volume with distilled water.

15% Separating gel

30% Acrylamide-bisacrylamide solution	5.00	ml
Distilled water	2.30	ml
1.5 M TrisCl pH 8.8	2.50	ml
10% SDS (w/v)	100.00	µl
10% Ammonium persulfate (w/v)	100.00	µl
TEMED (Bio-Rad)	4.00	µl

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis (Mini-Protein II Electrophoresis Cell, Bio-Rad), overlay with water. Then the gel solution was allowed to polymerize for 30 minutes.

5% Stacking gel

30% Acrylamide-bisacrylamide solution	500.00	µl
distilled water	2.10	ml
1.0 M TrisCl pH 6.8	380.00	µl
10% SDS (w/v)	30.00	µl
10% Ammonium persulfate (w/v)	30.00	µl
TEMED (Bio-Rad)	3.00	µl

After TEMED was added, the gel solution was immediately loaded on the vertical gel electrophoresis. The comb was gently inserted on the top of the gel to make well for sample application. Then the gel solution was allowed to polymerise for 10 minutes.

Staining solution

Coomassie brillaint blue R250 (Fluka)	0.25	gm
Methanol (J.T. Baker)	45.00	ml
Glacial acetic acid (J.T. Baker)	10.00	ml

Dissolve and adjust the volume to 100.00 ml with distilled water.

Destaining solution

Methanol (J.T. Baker)	300.00	ml
Glacial acetic acid (J.T. Baker)	100.00	ml

Dissolve and adjust the volume to 1,000.00 ml with distilled water.

REAGENT FOR IMMUNOBLOT TECHNIQUE

Towbin's buffer

Tris base (Sigma)	3.04	gm
Glycine (Sigma)	14.41	gm
Methanol (J.T. Baker)	200.00	ml

Dissolve and adjust the volume to 1,000.00 ml with distilled water.

Washing buffered (TNT) (10 mM TrisCl pH 8.0, 150 mM NaCl, 0.05% Tween 20)

1 M TrisCl pH 8.0	10.00	gm
5 M NaCl (Fluka)	30.00	gm
Tween 20 (Sigma)	0.50	ml

Dissolve and adjust the volume to 1,000.00 ml with distilled water.

Blocking buffer (5% skimmed milk (w/v) in TNT)

Skimmed milk (Marvel)	5.00	gm
TNT	100.00	ml

Stock Ponceau S solution

Ponceau S	2.00	gm
Trichloroacetic acid	30.00	gm
Sulfosalicylic acid	30.00	gm
Distilled water	100.00	ml

Dilute 1 part of the stock solution with nine part of distilled water to make a working solution. Discard the working solution after use.

Substrate buffer

1 M Tris-HCl pH 9.5 (Merck)	10.00	ml
5 M NaCl (Fluka)	2.00	ml
1 M $MgCl_2$ (Merck)	0.50	ml

Dissolve and adjust the volume to 100.00 ml with distilled water.

Chromogenic substrate (freshly prepared)

Nitro Blue Tetrazolium (Sigma)	50.00	mg

Dissolved in 700 µl of dimethylformamide (Fluka) and adjust hte volume to 1ml with distilled water.

5-Bromo-4-Chloro-3-Indolyl Phosphate (Sigma)	50.00	mg

Dissolved in 1 ml of dimethylformamide (Fluka).

Take 50 mg/ml of 5-Bromo-4-Chloro-3-Indolyl Phosphate 33 µl and 50 mg/ml of Nitro Blue Tetrazolium 66 µl to substrate buffer 10 ml.

REAGENT FOR CELL CULTURE

1x RPMI 1640 medium

RPMI 1640 (Gibco)	52.00	gm
Distilled water	5.00	L
$NaHCO_3$ (Merck)	10.00	gm

Adjust pH with 1N HCL to pH 7.2 and sterilized by 0.45 μm millipore membrane filtration.

Complete medium for culturing J 774A.1

1x RPMI 1640	80.00	ml
Fetal clone I (Hyclone)	20.00	ml
100x penicillin	100.00	μl
100x streptomycin	100.00	μl

0.2% Trypan blue

Trypan blue dye	0.05	gm
Distilled water	5.00	ml

100 mM HEPES buffer

HEPES (Sigma)	0.26	gm

Dissolve in distilled water, adjust to pH 7.3 with HCl and adjust to 1,000.00 ml final volume with distilled water.

20 mg/ml G 418

G 418 (Gibco)	20.00	mg

Dissolved in 1 ml of 100 mM HEPES buffer and sterilized by 0.22 μm filter.

BIOGRAPHY

NAME	Mr. Triwit Rattanarojpong
DATE OF BIRTH	30 July 1965
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INSTITUTION ATTENDED	Mahidol University, 1984-1987: Bachelor of Science (Public Health)
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