CHAPTER II

REVIEW AND RELATED LITERATURES

Melioidosis : historical review

Melioidosis is an infectious disease which was first reported in 1912 in Rangoon, Burma[17]. At a *post mortem* examination, the disease shows pathological features similar to glanders, an abscess-forming infection caused by *Burkholderia mallei*. Patients who are or were drug addicts often have pneumonia and pus at some parts of their bodies. The isolated bacteria have been shown to grow on peptone agar like the glander bacillus, which is lethal to guinea pigs, but several other typical features were absent. The organism has been shown by microscopy to be motile. Whitmore and Krishnaswami proposed the name *Bacillus pseudomallei* for this new discovered bacterium.[17] There are many names for this disease reported in the literature, including Whitemore's disease, morphia infector's septicemia, or Rangoon beggar's disease. This disease was named melioidosis in 1921, a name taken from the Greek meaning having a resemblance to the distemper of asses[18]. The most reported name of the organism in the literature is *Pseudomonas pseudomallei*, although it has also been reported under many names, including *Bacillus pseudomallei, Bacillus whitmori, Pfeifferella pseudo-mallei, Malleomyces pseudomallei, Flavobacterium pseudomallei*, and *Actino-bacillus pseudomallei* and, most recently, *Burkholderia pseudomallei* accord-ing to new data on 16s RNA sequence, DNA-DNA homology, lipids, and fatty acids[19].

The bacteria in this genus include both animal and plant pathogens such as *B.cepacia*, *B.pseudomallei*, *B.mallei*, *B.gladioli*, *B.cocovenenans*, *B, andropogonis*, and *B.caryophylti*. The name Burkholderia originated from a scientist named W.B. Burkholderia who discovered the causative agent of omnion disease (*B.caryophylti*)[19]. The first melioidosis case in Thailand was reported in 1955[20]. In 1985, over 800 cases were documented[5]. During the past 20 years, in north-eastern Thailand, melioidosis accounted for 20% of all community acquired septicaemias. Currently in our country, there are 500-1000 melioidosis cases reported per year[21,22], and melioidosis is one of the infectious disease still considered to be a public health problem in the country. Those with acute septicemic melioidosis have a high mortality rate as much as approximately 90% if untreated. The treatment requires a combination of drugs over a long term, which can be very expensive[21,22].

There is also a high percentage of relapses. Vaccine development is urgently required for people in endemic areas who face daily exposure to this bacterial organism in the environment[21,22].

Biology of *B.pseudomallei*

The microorganism is a motile, aerobic, non-spore forming gram negative bacillus. A gram stain shows bipolar staining which resembles a safety pin, although this is not specific for *B.pseudomallei*. The morphology in clinical specimens is extremely variable[23]. The organism grows aerobically on most agar media such as blood agar, McConkey agar, and eosin methylene Blue(EMB) and produces clearly visible colonies within 24 hours at 37°C. On Blood agar plate the organism has a unique character, rugose or like a cornflower head.This feature can be clearly seen in certain agars such as

Ashdown agar, which is a simple agar containing crystal violet, glycerol and gentamycin, and is commonly used to culture organisms from contaminated specimens such as sputum [24]. In general the organism can be isolated from clinical specimens that are usually sterile or only slightly contaminated by using general media such blood agar[23]. For the general characteristics , this organism are oxidase positive, utilizes glucose through the oxidative pathway, multitrichous flagella, producing gas from nitrate, and arginine dihydrolase and gelatinase decomposition. The unique biochemical features of *B.pseudomallei* are the reaction of A/N (yellow color on the slant) in triple sugar iron(TSI) agar, a musty earthy odor, a sensitivity to chloramphenicol, kanamycin and tetracyclin, and resistance to gentamycin, colistin and cephalothin[23].

Recently, a new biotype of *B.pseudomallei* was isolated from the soil. This new biotype has distinct characteristics, for instance it can use arabinose sugar as a substrate and shows low virulence in an animal model[25,26]. This biotype was named *B.thailandensis* as a new species[27]. This finding led to the study of melioidosis pathogenesis becoming more complicated. The optimum temperature for growth of *B.pseudomallei* *in vitro* is 37-42°C, and most workers have been unable to grow the organism at temperatures below 21°C. However, *B.pseudomallei* has demonstrated a high versatility and adaptability even though it cannot produce spores. *B.pseudomallei* has an unusually wide range of potential metabolic substrates. Carbon storage is achieved under conditions of substrate excess by using information of cytoplasmic inclusions composed of polyhydroxybutyrate. These inclusions account for the safety-pin appearance of gram-stain stained bacilli. *B.pseudomallei* can utilize nitrate under anaerobic conditions as an alternative terminal electron accepter[28]. It can be isolated from environmental samples such as soil, stagnant water, water treatment plants or rice paddies. *B.pseudomallei* has been isolated from soil

samples at a depth of 90 cm[28]. The organism can survive without any nutrients in distilled water for several years. The species tolerates a wide pH spectrum, temperature range and ultraviolet radiation[29]. Recently, it has been reported that viable *B.pseudomallei* can be detected using epifluororescent supravital stains at pH too low to allow detection of bacteria by conventional viable count [30]. There are other reports of soil which are positive by *B.pseudomallei* specific PCR[31], but which do not contain culturable bacteria, which raises the possibility of survival of environmental *B.pseudomallei* in a viable but non-culturable (VBNC) state. The isolation of the organism from water and soil has been reported mainly from Malaysia, Vietnam, Australia and Thailand[28].

The entire sequences of *B.pseudomallei* was sequenced by the Welcome Trust Sanger centre. The genome is relatively large at 7.24 Mb, divided uneqaully between 2 chromosomes, with chromosome 1 of 4.07 Mb and chromosome 2 of 3.17 Mb. The average percentage of G + C is 68%. It was estimated that there are a total of 5,600 genes with average length of each gene at 1031 bp[32].

Ecology and Epidemiology

The major known endemic areas of melioidosis are Southeast Asia and northern Australia between latitudes 20^{O}S and 20^{O}N[33]. Melioidosis cases have been increasingly reported from other areas around the world such as the Indian Subcontinent, central Africa, central and south America, the Caribbean and some Pacific islands during the last two decades. Most patients in western countries have visited an endemic area as tourists or as service personnels or contact with immigrants or visitors from an endemic area[33,34]. *B. pseudomallei* can cause disease in a wide range of animals, including cows, buffaloes, sheep, goats, birds, crocodiles, etc. However, the *B. pseudomallei* organism

has not been isolated from the stool or urine of the reported animals, and therefore melioidosis not classified as a zoonosis [7].

Meliodosis cases have been reported from every part of Thailand, with the highest number of cases coming from the northeastern area. Most victims have been in contact with wet soil or contaminated surface water. These data raise questions concerning the role of *B. pseudomallei* in the soil and also melioidosis infection rates. The distribution of *B. pseudomallei* in soil collected from the four non-urban regions of Thailand and the frequency of *B. pseudomallei* infections in patients attending government hospitals throughout Thailand in 1997 were surveyed. The organism was recovered in 4.4%, 6.1%, 20.4%, and 5.9% of the soil samples collected from the northern, central, northeastern and southern regions, respectively. The infection rate in patients attending government hospitals in the northeastern region (137.9 per 100,000 patients) was significantly higher than those in the northern (18 per100,000), central (13.4 per 100,000) and southern (14.4 per 100,000). The data suggested that melioidosis is associated with the presence of *B.pseudomallei* in the soil [8, 35]. The distribution of arabinose negative(*ara-*) *B.pseudomallei* strains in soil samples of the four areas was also studied at the same time and it was found that the Ara-strain in soil isolates in the northeast was significantly higher than those from other regions[36]. This distribution of more virulent biotype (*ara-*) soil isolates is also a factor contributing to a high prevalence of melioidosis in northeastern Thailand.

Clinical manifestations

The clinical symptoms of melioidosis vary greatly from asymptomatic to an acute fulminant septicemia. There is usually no obvious infected wound or evidence of recent trauma. The disease is characterized by abscess formation[1]. A

classification system for melioidosis was proposed by Punyagupta[5] with five categories:disseminated septicemic, non-disseminated septicaemic, localised, transient bacteremic and probable infection. This last category is based on serological findings. The disseminated septicaemic group accounts for 45% of cases, with mortality rate of 87%. Non-disseminated septicaemic disease is seen in 12% of cases with mortality of 17% and localised disease accounts for 42% of cases with mortality of 9%[5]. The clinical presentations of patients with melioidosis usually fall into one of the five categories described. This type of classification can be helpful for patient management.

Diagnosis of melioidosis

The definitive diagnosis of melioidosis is made by isolation of *B. pseudomallei* from clinical specimens collected from any part of the body[1]. The throat swab culture is 90% sensitive compared with a sputum culture and is especially useful in children or debilitated patients who cannot expectorate[37]. Routine media such as blood agar or McConkey can be used for all specimens except highly contaminated samples such as sputum that needs selective media such as Ashdown.[23,24] There have been many research developments in serological testing and using a molecular approach to speed up the diagnosis of melioidosis. Serological tests such as the indirect haemagglutination assay are used widely, but these methods are not specific; the assay detects antibodies directed against the conserved lipopolysaccharide – the immunodominant group. Serological test can help to exclude melioidosis, or lend support to the diagnosis in areas where prevalence is low, but they are of little use in endemic areas where most of the population is seropositive[38]. Various primer designs, such as regions of 23 S rRNA, the junction between 16S rRNA and 23 S rRNA, or the specific sequence designed from a specific probe for detection of

meliodosis, have been used in PCR for melioidosis detection. None of them has given satisfactory performance compared with the culture method[39]. Melioidosis should be suspected in any severely ill febrile patient with an underlying predisposing condition who lives in, or has travelled from, an endemic areas[1].

Pathogenesis of melioidosis

The mechanism of pathogenesis of melioidosis remains unknown as many other well known bacterial pathogens. Epidemiological datas suggest that the melioidosis patients acquire the *B. pseudomallei* from contaminated soil and water via skin abrasion, inhalation and ingestion[7,28]. However, there are a few percentage of melioidosis patients that had a firmed history of skin lesions, i.e. 5.2%(22/423) in Thailand and 24.2% (8/33) in Australia[40,41]. The other evidences that support this hypothesis are described. The correlations have been found between the number of admitted melioidosis patients in the government hospital in each part of Thailand with the number of *B. pseudomallei* organism isolated from the soil in nearby environmental samples (i.e. the number of soil samples that are positive for *B. pseudomallei*, and the colony forming units of *B. pseudomallei* in soil samples.) The highest number of melioidosis cases has been reported in northeastern part that correlates with the highest isolation rates of both the number of *B. pseudomallei* in soil and number of arabinose negative strain of *B. pseudomallei* in the soil[8,36]. The data solve some previous disputed reports that the high incident rates of melioidosis are high in northeastern but the *B. pseudomallei* isolation rate from the soil are highest in southern part[42].

There is a wide spectrum of clinical presentations resulting from infection with *B. pseudomallei* ranging from acute fulminant septicaemia,

subacute, chronic or subclinical infection[1]. Although the acute melioidosis can be occurred in a strong healthy person, most of them have underlying disease such as diabetes mellitus, renal disease, cirrhosis, thalassaemia, alcoholism or those who are immunosuppressed as the result of either disease or drug treatment[1,38]. However, melioidosis does not seem to be associated with HIV infection[43]. The other unique feature of melioidosis is incubation period that ranges from shortly only 2-3 days to more than 29 years[1]. Thus the factors that contribute to the development of melioidosis may consist of the route of inoculation, inoculation size, virulence of the infecting strain, immune competence and genetic predisposition of the host. The specific roles of these determinants in the pathogenesis of melioidosis in human remain for further investigation.

Two biotypes of *B. pseudomallei* have been reported in the natural soil as arabinose positive and arabinose- negative strain. Although one melioidosis case has been reported to cause by arabinose- positive strain, nearly all of reported melioidosis patients were caused by arabinose- negative strain[44]. The biotype of *B. pseudomallei* in the soil may play a role in diverse clinical presentations of melioidosis. Currently, how the ability to assimilate arabinose sugar of the *B. pseudomallei* relates to the ability to cause a disease is unknown. The arabinose biotype expression may just be an expressed phenotype that link to some virulence genes of *B. pseudomallei.*

The *B. pseudomallei* has been shown to resist to complement, lysosomal defensins and cationic peptides. The organism also has been demonstrated to produce many extracelluar substances such as protease, lipase, lecithinase, catalase, peroxidase, superoxide dismutase, hemolysin, a cytotoxic exolipid and siderophore[6,45,46]. The role of each determinant in melioidosis remains further investigation. *B. pseudomallei* produces a glycocalyx polysaccharide capsule. This capsule genes cluster has been

demonstrated to be a major virulent determinant of *B. pseudomallei.* The mutagenesis of the capsule gene cluster *of B. pseudomallei* markly reduce the virulence in animal model[47]. The role of this capsule polysaccharide in melioidosis pathogenesis remains unknown. The capsule may facilitates formation of microcolonies in which the organism is both protected from antibiotic penetration and phenotypically altered, resulting in reduced susceptibility to antibiotic[48].

B. pseudomallei is a facultative intracellular organism that has ability to invade both phagocytic and non-phagocytic cells. This bacterial organism survives inside several eukaryotic cell lines and is seen within the phagocytic cells in pathological specimens[49,50]. This ability may play an important role in high relapse rates of melioidosis and pathogenesis of melioidosis. After internalisation, *B. pseudomallei* escapes from a membrane-bound phagosome into the infected cell cytoplasm and then forms membrane protrusions by inducing actin polymerisation at one pole. The actin protrusion from the infected cell membrane mediate spread of the organism from cell to cell[51]. The infected cells have been demonstrated to not express inducible nitric oxide synthase (iNOS) at a detectable level and release significantly lower amount of tumor necrosis factor alpha(TNF-α-)[52]. This datas support the survival of bacterial organism inside the cell. *B. pseudomallei* also has been reported to contain at least 3 type three secretion system (TTSS) clusters in the genome. This well known virulent genes may play a role in this survival strategy[53].

Most of acute fulminant septicaemic melioidosis patients have an underlying disease such as diabetes mellitus and chronic renal failure. The host immunity may also play an important role with the severity of the disease. In animal experiment, interferon-gamma is essential for host-defense, but other components are almost certainly important. Septicaemic melioidosis is

associated with a vigorous inflammatory cytokine response. The high level of pro-inflammatory, anti-inflammatory cytokines, tumour necrosis factor (TNF), interleukin-6, interleukin-10, interferon-gamma and interleukin-18 are found. The concentrations of interleukin-6 or interleukin-10 are independent predictors of mortallity[54,55,56]. The CXC chemokines interferon-gamma-inducible protein 10 and monokine induced by interferon-gamma, and the serine protease granzymes A and B, are also notably raised in septicaemic melioidosis[57,58]. The 308 TNF promoter polymorphism (TNF2 allele) is related to disease severity. Melioidosis has been positively associated with HLA class II DRB1*1602 in Thailand[59,60]. This allele was associated significantly with septicaemic melioidosis (10% in melioidosis group in comparison with 4.8% in control group); this association was independent of confounders such as diabetes mellitus.

An approach to detection of *in vivo* expressed genes

There are only a few numbers of bacteria that cause a disease in human being. These bacterial pathogens have specific ability to colonize and proliferate in a special area of the host environment that can compete with the normal bacterial flora. Such bacterial factors are generally termed as virulence factors. The genes that encoded the virulence factors are called virulence genes. The definition of virulence genes are broadly including all genes contributing to infection as well as disease, with the exception of house keeping genes which encode functions that required for efficient multiplication on non-living substrates[61]. However, in some situation, the definition of virulent genes could not be clearly separated from non-virulent genes. The molecular form of Koch's postulates may be applied as follows; the phenotype or property under investigation should be associated with pathogenic members of a genus or

pathogenic strains of a species; specific inactivattion of the gene(s) assoicated with the suspected virulence trait should lead to a measurable loss in the pathogenicity or virulence; reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity; the induction of specific antibodies to a defined gene product should neutralize pathogenicity. In this case, when antibodies against a certain molecule protect an animal from disease, this is accepted as sufficient to call such a factor a virulent factor[61]. In fact, one phenotype may require a group of bacterial genes expression as a network. The changeable phenotype from mutation of one virulent gene may not be observed in *in vitro* experiment[62,63].

The bacterial pathogenesis is a complex and dynamic process. The virulence mechanism can be divided into various steps which each step usually involves different virulence genes. The expression of genes in the host is regulated by many changeable environment factors that could not be simulated *in vitro* condition. The bacteria in the host behave differ from *in vitro* condition such as requiement of iron for growth, optimal temperature and pH[64]. The cloning and characterization of *in vivo* expressed genes are important for understanding the molecular pathogenesis of bacterial pathogen. The classical study of virulence genes is carried out by the generation of random mutants then searching for avirulent phenotype. This approach is useful but only a limited number of genes are discovered[65]. This technique is improved by using tageted mutagenesis. However, one drawback of this approach is that the genes are induced within the host and are not expressed under normal laboratory conditions[66,67].

With progressive development in molecular biology and immunology, the bacterial pathogenesis study are focused on detection of the *in vivo* expressed genes. The numbers of some smart techniques have been demonstrated to be valuable tools such as In Vivo Expression Technology

Technology(IVIAT)[11,68], Signature tagged Mutagenesis(STM)[12] and Differential Fluorescent Induction(DFI). [13] Currently, with advance in bioinformatic program, the increase number of DNA data in GenBank and the high sensitive detection chemiluminesent protocol, let the immunoscreening approach to be a promising one to detect the *in vivo* expressed genes.

In vivo expression technology (IVET)

IVET is the first practical strategy described for selecting bacterial genes expressed preferentially during infection of an animal host. Random bacterial DNA inserts are cloned upstream of a promoterless reporter gene (such as purA::lacZY) and introduced into the bacterial chromosome of an avirulent purA- strain by homologous recombination. Since purA-strains cannot grow in the host, bacteria can replicate only if they contain a suitable promoter expressed *in vivo*. The bacteria surviving growth in the animal are then screened on agar plates in search of purA-lacZ fusions that are silent under laboratory *in vitro* conditions. This identification of *ivi* genes is dependent upon an arbitrary criterion for the absence of gene activity in laboratory-grown bacteria[11]. IVET has allowed the identification of hundred of ivi genes in a diverse range of bacterial pathogens including *Salmonella enterica sv. typhimurium, Pseudomonas aeruginsa, Staphylococcus aureus, Vibrio cholerae* and *Candida albicans*. To date, over 100 of *ivi* genes have been found that fall in to four broad categories: regulator, metabolic/physiological, stress response and unknown function. While mutations in selected *in vivo* induced genes lead to a decrease in virulence, the role of many of these genes remains unclear[68].

IVET applications have not been limited to animal models, but also involved cultivated cells for pathogens such a *S. typhi* and *S. typhimurium*. The original IVET strategy has been modified to replace the *pur* gene with *cat* and *tnp* gene that can be more applicalbe to other bacterial pathogens.

IVET limitation[65,66] have also be seen such as;

1. A large number of identified ivi genes are not specific for virulence.
2. It does not indicate at which point the bacterial genes are required whether they are transiently induced or expressed *in vivo* at all the time.
3. It requires the bacterial clone to survive in the host until they can be recovered from the organ.
4. A large number of *ivi* gene belong to metabolic genes.

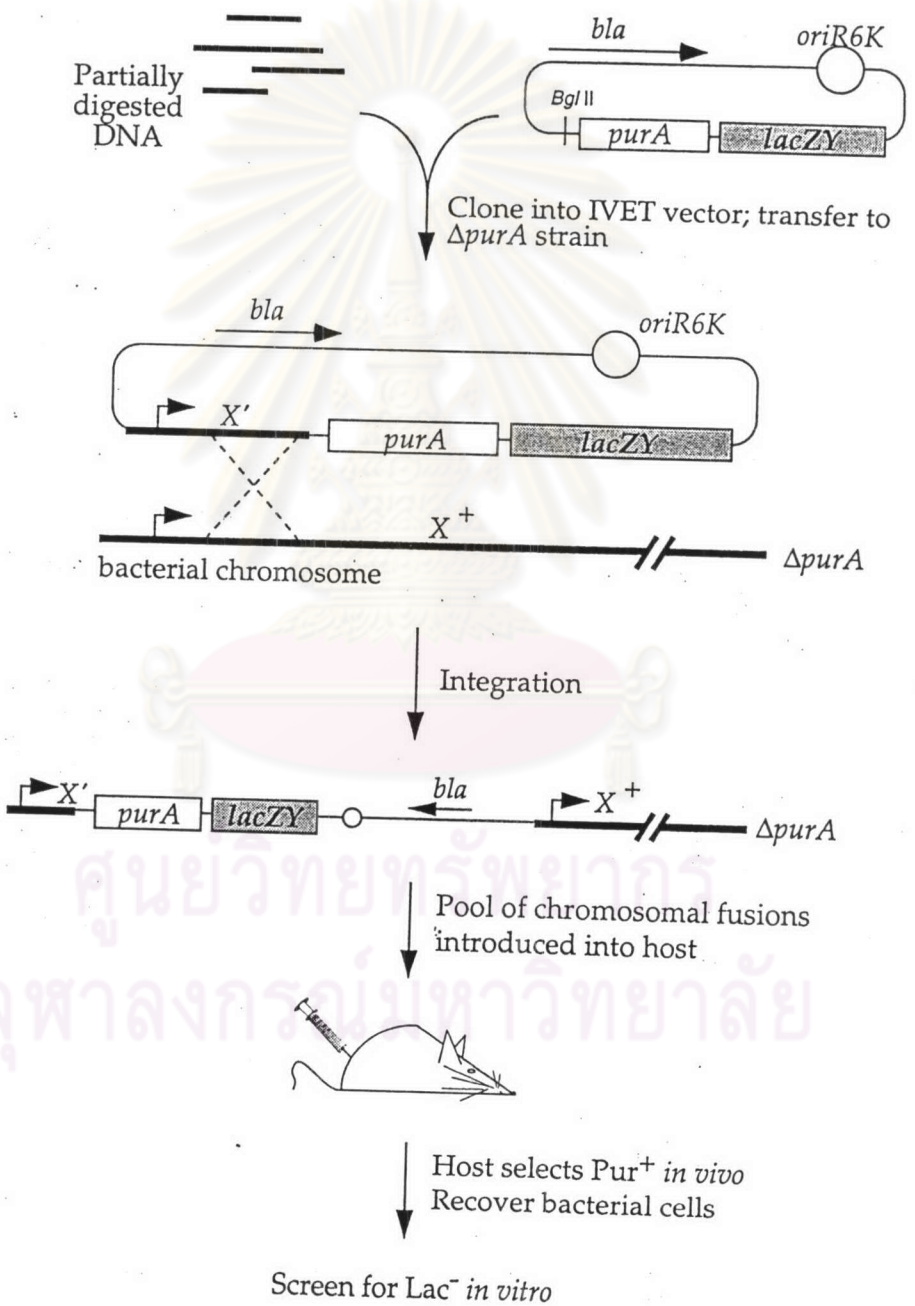

Figure 1 Schematic reprsentation of IVET strategy[66]

Signature-tagged mutagenesis (STM)

This system uses a negative selection strategy to identify avirulent strains created by transposon mutagenesis[12]. Each transposon is tagged with a unique oligonucleotide sequence that allows for individual clones to be identified from a large pool of mutant strain. The basic steps of this approach include: constructing a large pool of transposons, each individually tagged with a randomly generated, unique sequence; generating a collection of tagged transposon mutant strains, each of which is distributed in a separated well in a standard microtiter dish; passing pools of mutants through animal model of infection to provide negative selection against strains with attenuated virulence (i.e. disruptions in essential genes involved in reaching or surviving with the infected organ); recovering the surviving virulent bacteria; amplifying and lebeling the tagged sequence within each transposon insert using the PCR; identifying avirulent strains missing from the recovered pool of mutant. The last step is accomplished by comparing the hybridization patterns produced by radiolabeled tags amplified from the input library and the recovered bacterial survivors to DNA dot blots derived from the input pool. The tags present in mutants deficient in pathogenic genes are absent from the final pool. This technique could simultaneously screen a large pools of mutants in a single animal. STM differs from IVET in that STM identifies functions required for survival within the host[70,71].

STM has been applied to various bacterial patghogen such as *S. typhimurium, V. cholerae, Y. enterocolittica, S. aureus, Streptococcuc pneumoniae.* Some virulence genes such as type three secretion pathogenicity islands was unexpected to be detected as necessary for *in vivo Salmenella* growth[70]. With screening more than 20,000 mutants from 14 bacterial species, STM can identify more than 400 genes of *in vivo* attenuated mutant. Only a few genes encoding

known virulent factors were identified. The discovered genes were genes encoding proteins involved in cellular functions such as metabolism, transport, replication and repair.

Some limitations of STM approach has been reported as follows[65,70,71] :

1. Transposon may not be fully integrated to the bacterial chromosome that let the mulation occur at some specific area of the chromosome.

2. The mutation of gene that encodes toxin production may results in trans-complementable phenotype.

3. The mutation probably may not be saturated

4. STM may not identify mutations causing small or even moderate reductions in survival mutation in genes that are critical for causing disease but do not appreciably affect survival of the bacterium in the host for example ,deletion of the cholera toxin genes does not affect the bacterium's ability to colonize the host

5. STM excludes essential genes for bacterial growth, as an insertion in such a gene produces a lethal phenotype.

Limitation of IVET and STM

According to their strategies, some limitations of both STM and IVET have been reported .They could not detect those genes that express too strong *in vitro* to be classified as *in vivo* induced i.e., toxin genes and extracellular binding protein. Not all genes that are expressd *in vivo* are required for survival *in vivo* and some genes that are required for growth *in vivo* may also be expressed *in vitro*. It does not indicate at which point the bacterial genes are required (whether they are transiently induced or expressed *in vivo* at all the time)[65,66].

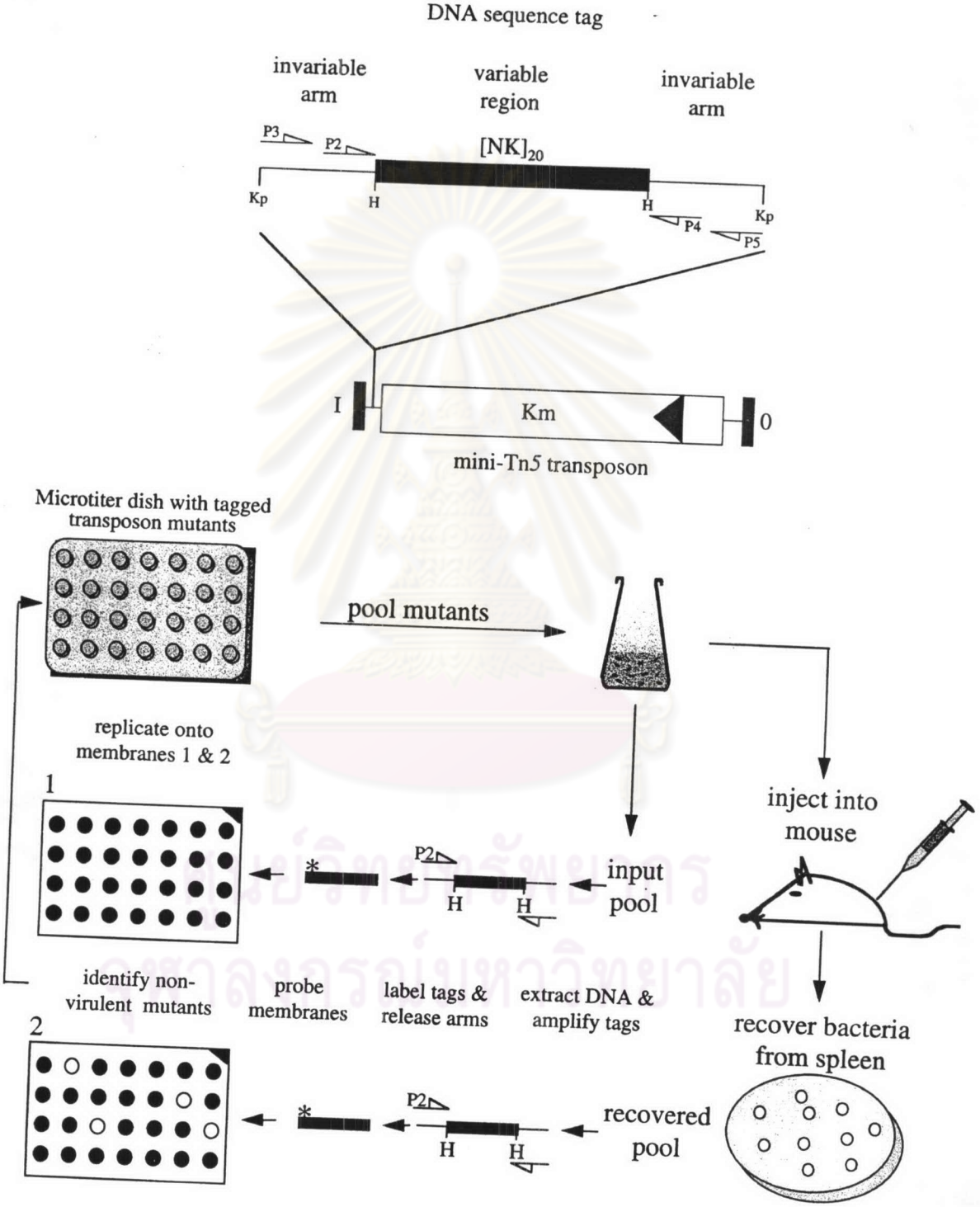

Figure 2 Schematic representative of STM strategy[66]

Differential Fluorescence induction (DFI)

This technique uses a green fluorescent protein (gfp) as a reporter to identify genes expressed in tissue culture such as macrophage cell[13]. DFI involves the cloning of random fusions of genomic DNA fragments upstream of a promoter-less gfp gene. The library of fusions is maintained in plasmids and not recombined into the bacterial chromosome. The library of random promoters fused to gfp are subjected to tissue culture cells. Fluorescence activated cell sorting (FACS) is then used to enrich for green fluorescent mamalian cells allowing isolation of gfp-expressing bacteria[13]. Lysis of the tissue cell and growth of the bacterial population on ordinary laboratory media yielded a population of both fluorescent and non-fluorescent bacteria. The latter population contains gfp gene fusions that are silent under laboratory conditions and is used to infect the tissue culture cell at a ratio such that each cell is infected with at most one bacterium. Sorting tissue cells that emit a fluorescent signal after bacterial infection provided a bacterial population that contained gfp fusions specifically activated in the host cell's intracellular environment. DFI has been applied to isolate genes that are preferencetailly expressed within macrophage of *S. typhimurium.* Fourteen macrophage-induced gene have been identified. Some of them have been reported to comprise essential plasmid or chromosomal genes for *in vivo* survival including components of type III secretion system necessary for survival[65,66]. A number of gram negative and gram positive bacterial pathogens have been studied using DFI, including *E. coli, Listeria monocytogenes, Staphylococcus aureus* and *Streptococcus pneumoniae.*

DFI is like IVET, will not identify genes which are expressed *in vitro* and are also important for virulence *in vivo*. Further experiments are always needed to determine whether ivi genes are crucial for bacterial virulence and survival in the host. Although most of the well known classical virulence genes have not been recovered by this DFI approach. DFI is still a useful tool for identifying bacterial virulence factors as well as a mean of elucidating the microenvironment encountered by pathogens upon infection.

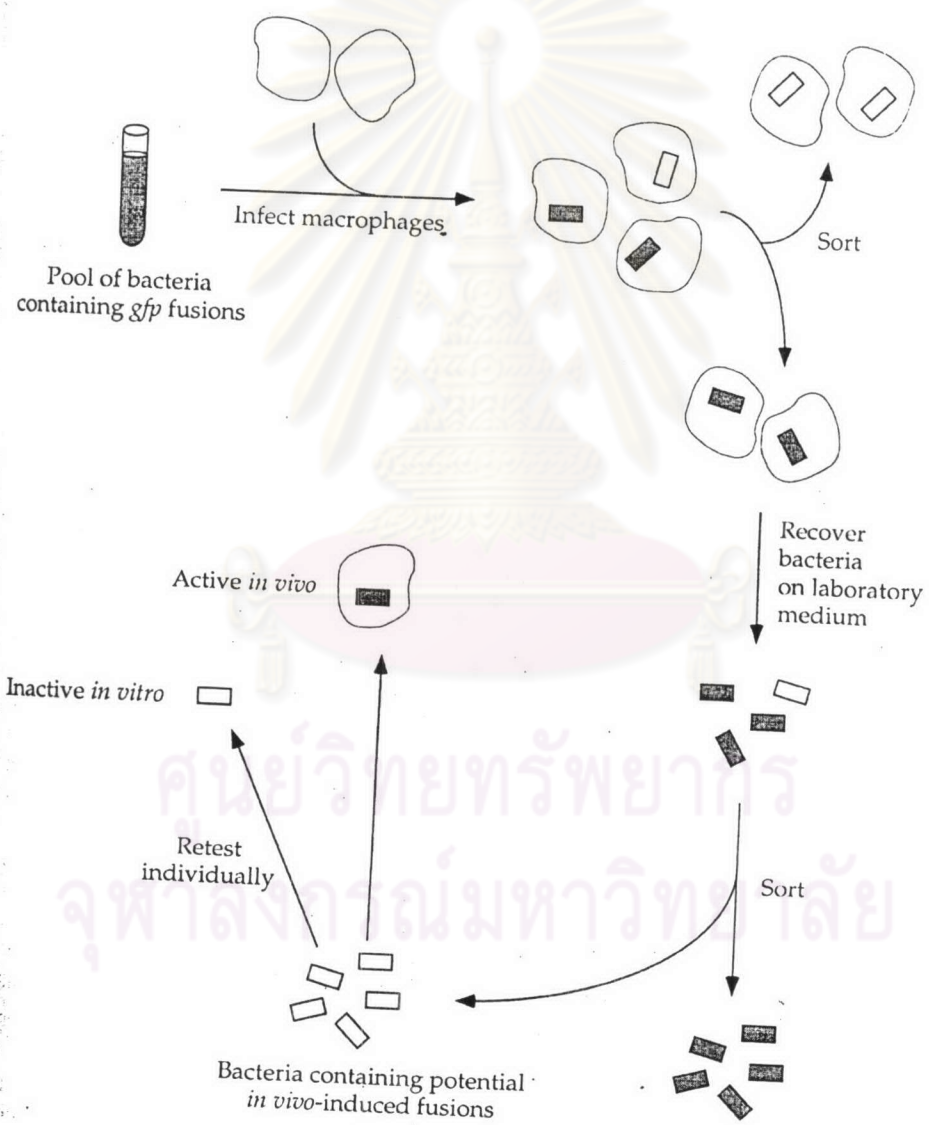

Figure 3 Schematic representation of DFI strategy[66]

Limitation of IVET, STM and DFI

Although some new insights have been demonstrated from isolated *ivi* genes profiles, some limitations also have also been noted such as; they depend on the use of animal models of infection or tissue culture that might not closely approximate the human conditions. Erroneous conclusion may be drawn by extrapolation of results from animal model to human infection. In addition, the genetic systems may not be available to some domestic bacteria.

Some known virulence determinants have been shown to be expressed *in vivo* of *S. typhimurium* pathogenesis study such as *spvB*(IVET); *sas H(DFI); spvA, spvD, spvR* (STM). However, some known virulence genes that expected to be expressed *in vivo* but could not be identified such as *toxR/toxS* of *V. cholerae* in the intestine of infected mice by IVET; staphylococcus toxin in mice by STM. This raises the possibility that some genes described as putative virulence determinants *in virtro* may not produced *in vivo* and are not essential for virulence[65,66].

An approach to isolating *in vivo* expressed gene by reacting with antibodies produced by infection.

Host immune response has long been used as an indicator of vaccine efficiency. During bacterial infection process, many of bacterial genes are expected to be expressed to overcome the host defense system, colonization and proliferation. Some of encoded protein of bacterial *in vivo* expressed genes could induced specific immune response[61]. There are very few research works that have used induced specific antibodies to probe *in vivo* expressed gene by immunoscreening. One reason may be lack of a sufficient sensitve detection system in the past time. Recently with progressive development in bioinformatics computer programs and molecular biology in combination of drastically increase of DNA sequence data in the GenBank, let this approach to be a possible strategy. The immunoscreening stategy has been applied in *Borrelia burgdorferi* infection in mice model. The library was screened with the absorbed serum from actively infected mice and immunized heat –killed *B. burgdorferi* mice. One gene (p21) encoded a protein with molecular weight of 20.7 kDa was isolated to be reacted with only the actively infected mouse serum. This gene was confirmed to be expressed only *in vivo* by northern blot and RT-PCR of the transcribed RNA[15]. *In vivo* induced antigen technology (IVIAT), use a pooled absorbed serum from patient preabasorbed with *in vitro* grown pathogen in immunoscreening[16]. IVIAT approach main target is elimination of *in vitro* induced antibodies from the pooled serum. The pooled absorbed serum is expected to contained only antibodies specific to protein in vivo induced. The *ivi* genes identified by this strategy would encode only *in vivo* induced antigen. Currently, the IVIAT approach is being tested in some bacterial pathogens such as *Actinobacillus actinomycetemcomitans*, *Pseudomonas aeruginosa* and *Candida albican* infections[16]. IVIAT has also

been applied in *M.tuberculosis*[72]. IVAT is expected to provide a valuable new tool to address pathogenic mechanisms of a large number of human pathogens.