ELUCIDATING THE MECHANISM OF ACTION OF CAFFEIC ACID PHENETHYL ESTER (CAPE) VIA TRANSCRIPTOMIC PROFILING OF *Burkholderia pseudomallei* STRAIN K96243

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UNIVERSITI SAINS MALAYSIA

2019

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by

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Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

August 2019

ACKNOWLEDGEMENT

First and foremost, praise be to Allah S.W.T, the Almighty and the Most Gracious, for granting me strength, determination and willpower to complete my research journey.

I would like to express my sincere gratitude to my dearest supervisor, Dr. Suriani Mohamad, for giving me an opportunity to conduct this research under her precious guidance. Her visions, knowledge, and motivations were the driving force towards the completion of this research. It was truly a great privilege and honor for me to be under her supervision. Special thanks to my colleagues in Lab 414, Mardani, Matthlessa, Suhaimi, and Haida, who helped and guided me during those early days in the Microbiology lab. To my fellow lab mates Zawani and Nafiu, thank you for always being there for me during the hard times. I would also like to express my sincere appreciation to my beloved family, especially to both of my parents who have always supported me in achieving my dreams. Their sacrifices and utmost efforts in providing me with financial and emotional supports have allowed me to be where I am today. I would like to thank MyBrain15 Scholarship for providing financial aid during my first year of studies. Last but not least, I owe my deep gratitude and heartfelt thanks to the special person who has helped me in learning new knowledge in Bioinformatics and has provided endless mental support throughout my rough days.

I specially dedicate this thesis to my father, Abu Hasan Mohamad Noor and my mother, Hashimah Puteh.

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LIST OF ABBREVIATIONS

B. pseudomallei	*Burkholderia pseudomallei*
$BaCl_2$	Barium chloride anhydrous
CAPE	Caffeic acid phenethyl ester
CAPA	Caffeic acid phenethyl amide
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazine
cDNA	Complementary deoxyribonucleic acid
COG	Clusters of orthologous group
DEG	Differentially expressed genes
DEPC	Diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EPI	Efflux pump inhibitor
GC content	Guanine-cytosine content
HCl	Hydrochloric acid
HMDS	Hexamethyldisilazane
INT	*p*-iodonitrotetrazolium
MDR	Multidrug resistant
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
$MgCl_2$	Magnesium chloride

mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RND	Resistance-Nodulation-Cell Division
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
spp.	Species (plural)
TAE	Tris-acetate-EDTA
TEMA	Tetrazolium microplate assay
T3SS	Type III secretion system
T6SS	Type VI secretion system
UV	Ultraviolet

LIST OF UNITS AND SYMBOLS

bp	Base pairs
°C	Degree Celsius
CFU/ml	Colony forming unit per milliliter
Cq	Quantification cycle
E	Amplification efficiency
g	Gram
g/mol	Grams per mole
hr	Hour
kb	Kilo base pairs
L	Liter
min	Minute
µg	Microgram
µl	Microliter
ml	Milliliter
µM	Micromolar
ng	Nanogram
nm	Nanometer
psi	Pound-force per square inch
rpm	Revolutions per minute
V	Volt
× g	Acceleration due to gravity
% v/v	Percent volume/volume
% w/v	Percent weight/volume

PENENTUAN MEKANISME TINDAKAN ASID KAFEIK FENETIL ESTER (CAPE) MELALUI PROFIL TRANSKRIPTOM *Burkholderia pseudomallei* STRAIN K96243

ABSTRAK

Burkholderia pseudomallei adalah sejenis bakteria patogen Gram-negatif yang menyebabkan melioidosis, sejenis penyakit berbahaya dan endemik di kawasan Asia Tenggara, terutamanya Malaysia dan Thailand. *B. pseudomallei* mempunyai kelaziman rintangan terhadap pelbagai kelas antibiotik, seperti aminoglikosida, makrolida, β-laktam, dan sefalosporin. Kewujudan protein pam pembawa keluar yang mampu mengepam keluar antibiotik dari sel serta kebolehtelapan membran bakteria yang rendah yang menghalang penetrasi antibiotik menyumbang kepada kelaziman rintangan antibiotik. Faktor-faktor tersebut merumitkan proses terapi antibiotik untuk penyakit melioidosis. Oleh itu, antara strategi berkesan untuk meminimumkan tahap rintangan bakteria adalah menggunakan sebatian yang dapat menguatkan aktiviti antibiotik. Dalam kajian ini, potensi asid kafeik serta terbitannya iaitu asid kafeik fenetil ester (CAPE), asid klorogenik, dan asid kafeik fenetil amida (CAPA) untuk bertindak sebagai pembantu antibiotik (adjuvan) telah dinilai. Hasil kajian mendapati gabungan kepekatan sub-hambatan CAPE dengan gentamisin atau kanamisin berjaya mengurangkan MIC antibiotik tersebut dalam *B. pseudomallei* sebanyak empat kali ganda dan dua kali ganda. Imbasan mikroskopi elektron menunjukkan keadaan sel bakteria yang dirawat dengan CAPE terjejas dan mengalami perubahan fenotip yang ketara, seperti kecacatan bentuk, penyusutan saiz, serta pembentukan bonjolan dan lekukan pada membran. Untuk memahami dengan lebih mendalam tentang mekanisme tindakan CAPE, pemprofilan transkrip RNA digunakan

untuk menentukan kesan pendedahan CAPE kepada bakteria. Melalui analisis transkriptom *B. pseudomallei* yang telah dirawat dengan CAPE, sebanyak 658 gen telah dikenal pasti mengalami perbezaan dalam regulasi berbanding dengan sel yang tidak terawat. Antara gen yang dikenal pasti memainkan peranan dalam tindak balas kepada CAPE adalah mekanisme pemerolehan zat besi, seperti biosintesis dan pengangkutan siderofor, enzim reduktase ferrat, protein pengangkut zat besi, dan protein penyimpan zat besi. Ini menunjukkan bahawa CAPE mungkin bertindak sebagai kelator besi yang menyebabkan bakteria mengalami kekurangan zat besi. Kelompok gen lain yang umumnya dikaitkan dengan keterbatasan zat besi, seperti metabolisme nitrogen, sistem rembesan bakteria, fosforilasi oksidatif, dan pernafasan juga mengalami perbezaan regulasi dalam sel yang dirawat dengan CAPE. Gen yang mengekod protein pam pembawa keluar, protein porin, dan enzim yang terlibat dengan proses degradasi asid hidroksisinamat mengalami peningkatan regulasi, menunjukkan bahawa bakteria cuba mengehadkan penetrasi CAPE dan mengurangkan kepekatannya di dalam sel. Selain itu, regulasi gen yang mengekod pengatur global Crp/Fnr, protein yang mengawal pembahagian kromosom dan sel, dan protein yang mengawal pembentukan lapisan peptidoglikan adalah antara yang mengalami peningkatan regulasi, manakala regulasi terhadap gen yang mengekod protein mengandungi besi, sistem pengambilan heme, dan pengatur global Fis mengalami pengurangan. Data yang diperoleh daripada RNA-seq disahkan melalui PCR-transkripsi terbalik kuantitatif (qRT-PCR). Hasil kerja ini menunjukkan bahawa mekanisme tindakan CAPE dalam *B. pseudomallei* adalah pelbagai, dan mod utama CAPE adalah menghalang penyerapan zat besi serta merencat integriti membran *B. pseudomallei*.

ELUCIDATING THE MECHANISM OF ACTION OF CAFFEIC ACID PHENETHYL ESTER (CAPE) VIA TRANSCRIPTOMIC PROFILING OF *Burkholderia pseudomallei* STRAIN K96243

ABSTRACT

Burkholderia pseudomallei is a Gram-negative bacterial pathogen that causes melioidosis, a life-threatening disease endemic in the regions of Southeast Asia, particularly Malaysia and Thailand. *B. pseudomallei* is intrinsically resistant to wide classes of clinically useful antibiotics, such as aminoglycosides, macrolides, β-lactams, and older-generation cephalosporins. The expression of multiple efflux pumps and the low permeability of the bacterium's outer membrane contribute to its multidrug-resistant (MDR) traits, which further complicate the therapeutic management for melioidosis. Thus, a promising strategy to minimize the resistance level of MDR bacteria is by the use of non-antibiotic helper compounds as an adjuvant to potentiate the antimicrobial activity of antibiotics. In this study, the potential of caffeic acid and its derivatives, i.e. caffeic acid phenethyl ester (CAPE), chlorogenic acid, and caffeic acid phenethyl amide (CAPA) to act as antibiotic potentiators in *B. pseudomallei* were evaluated. We demonstrated that the combination of a sub-inhibitory concentration of CAPE with gentamicin or kanamycin significantly reduced the MIC of these antibiotics in *B. pseudomallei* by four-fold and two-fold, respectively. Scanning electron microscopy further revealed that the fitness of the cells treated with CAPE were compromised, with apparent phenotypic changes to the cell morphology and disruption in the membrane architecture, such as shrunken, deformed shape and formation of membrane blebs and dimples. To decipher the mechanism of action of CAPE, RNA-sequencing was utilized to determine its impact of exposure based

on the transcriptional profiles of *B. pseudomallei*. Transcriptome analysis of CAPE-treated cells indicated significant modulation of gene expression, of which 658 genes were differentially expressed. The genes responsible for iron acquisition mechanisms were highly induced, including siderophore biosynthesis and uptake, ferric reductases, iron transport/utilization-like proteins, and iron storage proteins, implying that the bacterium was deprived of iron. The exposure of CAPE to the nutrient-rich medium might have rendered iron selectively unavailable for the bacterium, which suggest that CAPE might be acting as an iron chelating agent. Other clusters of genes that are generally associated with the condition of iron limitation, such as those implicated in nitrogen metabolism pathway, bacterial secretion system, oxidative phosphorylation, and respiration were also differentially regulated in CAPE-treated cells. Genes encoding efflux pump proteins, porin-related proteins, and hydroxycinnamate degradation enzyme were upregulated, indicating that the bacterium likely attempted to limit the penetration of CAPE and reduce its intracellular concentration. In addition, the expression of genes encoding global regulator Crp/Fnr, partitioning protein, and murein-associated proteins were induced, whereas genes encoding iron-containing proteins, heme uptake system and global regulator Fis were downregulated. The data obtained from RNA-seq were corroborated by quantitative reverse transcription-PCR (qRT-PCR). The results of the present work indicate that the mechanisms of action of CAPE in *B. pseudomallei* are multifarious, in which the major modes involve iron chelation and disruption of bacterial membrane integrity.

CHAPTER 1: INTRODUCTION

1.1 Background of study and problem statement

The Gram-negative *Burkholderia pseudomallei* is a facultative intracellular pathogen that causes severe infections with a broad spectrum of illnesses known as melioidosis. Melioidosis is highly endemic in Southeast Asia, particularly Malaysia and Thailand, as well as in northern Australia and several regions in the subtropics or tropics (Leelarasamee, 2000; Mukhopadhyay et al., 2011; Puthucheary, Lin, & Yap, 1981; Vadivelu et al., 1997; Vuddhakul et al., 1999). *B. pseudomallei* is an environmental saprophyte that naturally resides in wet soil, stagnant water, and rice paddy areas in the prevalent regions (Dance, 1991, 2002; White, 2003). Although it is a saprophytic bacterium, it is capable of causing an opportunistic infection that could rapidly advance to a fatal illness, especially in patients with comorbidities. Infection commonly occurs through inhalation or inoculation of bacteria in the skin breaks or wounds upon contact with contaminated soil or water.

B. pseudomallei is a causative factor for community-acquired pneumonia and septicemia in some of the endemic regions, in which mortality rate is high despite intensive antibiotic treatments (Boonsawat et al., 1990; Chaowagul et al., 1989; Currie et al., 2000; Dance, 1991; Elliott et al., 2005). Clinical presentations of melioidosis are wide-ranging and may resemble other types of disease, thus complicating its diagnosis. General manifestations of melioidosis may include acute pulmonary infection, localized or chronic suppurative infection, acute septicemia or others. It may also present as an asymptomatic infection which the bacterium can stay latent for a long period of time before clinical symptoms are apparent.

B. pseudomallei is able to survive in toxic conditions and is resistant to many antimicrobials or disinfectants used in hospitals and household settings. The bacterium is intrinsically resistant to wide classes of antibiotics, particularly aminoglycosides, β-lactams, macrolides, rifamycins, colistin, and older-generation cephalosporins (Dance et al., 1989; Jenney et al., 2001). The resistance of *B. pseudomallei* towards a broad spectrum of clinically useful antibiotics presents a challenge in the treatment of this disease. Melioidosis is difficult to manage as infection can still result in a rapid fatal outcome even with the use of appropriate antimicrobial agents. Besides, antibiotics typically used in the first-line treatment of Gram-negative bacterial sepsis such as aminoglycosides, ampicillin, or amoxicillin are clinically ineffective in treating melioidosis. Treatment of *B. pseudomallei* also proves to be challenging due to the need for a prolonged course of complex antimicrobial therapy (Cheng & Currie, 2005; Woo et al., 2003). The treatment course for melioidosis comprises two phases: an intensive acute phase and an eradication phase. The first phase of therapy involves intravenous administration of ceftazidime, meropenem, or imipenem for at least 10 to 14 days. The second phase of therapy involves oral administration of trimethoprim–sulfamethoxazole (TMP/SMX) for a minimum of three to six months (Wiersinga, Currie, & Peacock, 2012).

Antibiotic treatments that adhere strictly to the recommended procedures usually result in better outcomes; however, the rates of failure in clinical therapy of melioidosis still remain high (Dance, 2014; Pitman et al., 2015). The bacterium's intrinsic and acquired resistance to antimicrobial agents are among the factors that contribute to treatment failures. *B. pseudomallei* develops resistance to antibiotics via several known mechanisms, such as drug efflux, target modification, enzymatic inactivation of drugs, and reduced permeation (Schweizer, 2012a).

Developing novel classes of antibiotic that have a new mechanism of action is very challenging, costly, and time-consuming. Many new antibiotics that have entered the market are derivatives of old drugs that hold the same key function, which experts believe that these second, third, or fourth generations of antibiotics could only extend the drugs' efficacy by several years (reviewed in Buckland, 2017). In fact, the progress in the discovery of novel antibiotics has slowed down since 1980s. In recent years, a small number of novel antibiotics have been introduced for the treatment of Gram-positive infections; however, no novel classes of antibiotics have been successfully produced for Gram-negative bacteria in the last 40 years (reviewed in Buckland, 2017; World Health Organization, 2014). This was previously due to the perception that the existing armamentarium of antibiotics were sufficient to control bacterial infections. Other factors include huge economic cost and low investment in research and development (R&D) on novel antimicrobials, since major pharmaceutical companies have shifted their focus towards more profitable diseases (reviewed in González-Bello, 2017). Thus, a good alternative in tackling antimicrobial resistance would be minimizing the impact and emergence of antibiotic resistance. This can be achieved through the use of antibiotic adjuvants (also termed 'antibiotic potentiators' or 'resistance breakers'), such as efflux pumps inhibitors, β-lactamase inhibitors, or outer membrane permeabilizers. Adjuvants are co-administered with antibiotics to inhibit bacterial mechanisms of resistance and potentiate the antimicrobial action of antibiotics (Farha & Brown, 2013; Gill, Franco, & Hancock, 2015; Kalan & Wright, 2011; Worthington & Melander, 2013). This combination approach has the potential to reinstate the clinical efficacy of existing antibiotics, broaden the antibiotic spectrum, minimize the effective dose of antibiotics required in a treatment, and render multidrug-resistant (MDR) bacteria susceptible to antibiotics. As antibiotic adjuvants

generally possess weak or no antibacterial activity, bacteria will not develop resistance towards these compounds.

The use of antibiotic adjuvants is a promising approach to decrease the level of resistance in *B. pseudomallei* and restore the effectiveness of antibiotics it is currently resistant to. Antibiotic adjuvants that work as efflux pump inhibitors might be the best candidate, since extrusion of antibiotics by efflux systems is the major mechanism of resistance in this bacterium (Schweizer, 2012b). Efflux inhibition strategies can be accomplished in several ways, for instance, disrupting the source of energy of the pumps, obstructing the interaction between different components of a multi-segmented pump, blocking the binding of antimicrobial agents to the cytoplasmic membrane pumps, or targeting the regulatory genes that control the expression of efflux pumps (Pagès & Amaral, 2009; Poole & Lomovskaya, 2006). An efflux pump inhibitor (EPI) is characterized by its modulating ability to specifically impede the export of antibiotics by bacterial efflux systems, which sometimes might also hinder the export of physiological substrates of the pumps. The rising prevalence of MDR strains has reduced the clinical efficacy of many antibacterial agents; hence, the ability of EPIs to circumvent the efflux of antibiotics will reinstate the clinical utility of several older compounds, increase the potency of antimicrobial agents, and prevent further development of multidrug resistance (reviewed in Kamicker et al., 2008).

Plant-based compounds have emerged as interesting candidates for potential antibiotic adjuvants, which in turn have motivated huge scientific interest in the discovery of novel EPIs from natural sources (Abreu et al., 2016; Abreu et al., 2017; Chusri et al., 2009; Lacmata et al., 2012; Noumedem et al., 2013). Indeed, bioactive compounds derived from natural products have been extensively used for pharmaceutical and medicinal purposes due to their highly recognized benefits. Plants

and herbs have been used since primeval times for the treatment of various ailments including bacterial, viral and fungal infections (Dupont et al., 2006; Temrangsee, Kondo, & Itharat, 2011). Plant resources provide a vast repertoire of structurally complex, chemically diverse, and biologically active phytochemicals. Phenolic compounds like caffeic acid and its derivatives are among the bioactive phytochemicals that are abundantly found across the plant kingdom. They occur naturally in food such as fruits, grains, olive oil, tea, coffee, vegetables and many others (Higdon & Frei, 2006). They have been gaining increasing attention for their broad spectrum pharmacological attributes, which encompass antibacterial, antiviral, anti-diabetic, anti-inflammatory, antioxidant, anticancer, and immunomodulatory effects (Celik & Erdogan, 2008; Celik, Erdogan, & Tuzcu, 2009; Challis & Bartlett, 1975; Gülçin, 2006; Ikeda et al., 2011; Tsai et al., 2012). Interestingly, some of the derivatives of caffeic acid have also been reported to demonstrate strong efflux inhibition activities in Gram-positive bacteria like *Staphylococcus aureus* (Santos et al., 2018; Fiamegos et al., 2011; Michalet et al., 2007). This suggests the potential of caffeic acid derivatives as good EPI candidates considering they are capable of inhibiting MDR efflux pumps, and are readily available and non-toxic for human consumption.

Due to the beneficial prospects of caffeic acid derivatives, the present study was initiated to explore their potential role as antibiotic adjuvants, particularly as efflux pump inhibitors in Gram negative *B. pseudomallei*. One-concentration combination test using tetrazolium microplate assay (TEMA) was employed to investigate whether the combination of a particular test compound with antibiotics caused any reduction in minimum inhibitory concentration (MIC) of the antibiotic. The main focus of this work was to unravel the mechanism of action of a selected test

compound, especially on the regulation of gene expression in *B. pseudomallei* in response to the compound. To achieve this, RNA sequencing (RNA-seq) was utilized to identify the set of protein coding mRNAs that were differentially expressed between two different experimental conditions. Next-generation sequencing (NGS) technologies such as RNA-seq have revolutionized transcriptomic research by producing high-throughput datasets with unprecedented accuracy, sensitivity and precision. It has become the preferred method for gene expression profiling and is rapidly replacing conventional methods like microarrays (Ozsolak & Milos, 2011; Shendure & Ji, 2008; Wang, Gerstein, & Snyder, 2009).

1.2 Research objectives

This study was aimed at evaluating the potential efflux inhibitory activity of caffeic acid, chlorogenic acid, caffeic acid phenethyl amide (CAPA), and caffeic acid phenethyl ester (CAPE) on *B. pseudomallei* strain K96243. Gene expression profiles of *B. pseudomallei* from RNA-seq data derived from control and treated bacterial cells were generated in order to investigate if a selected test compound (CAPE) modulates the expression of multidrug efflux pumps in *B. pseudomallei*, and to reveal the effects that the compound exerts at the transcriptional level. This enables the identification of a set of transcripts that were differentially expressed and allows a better understanding on the possible modes of action of the compound. The main objectives of this present study are outlined as follows:

Objective 1: To assess the antimicrobial activity and efflux inhibition activity of caffeic acid, chlorogenic acid, CAPE, and CAPA against *B. pseudomallei* strain K96243 using tetrazolium microplate assay (TEMA).

Objective 2: To investigate if the selected compound (CAPE) caused any observable changes to the cell morphology and membrane architecture of *B. pseudomallei* using scanning electron microscope (SEM) visualization.

Objective 3: To decipher and elucidate the mechanism of action of CAPE via transcriptomic profiling of treated and untreated *Burkholderia pseudomallei* cells.

CHAPTER 2: LITERATURE REVIEW

2.1 A brief overview on antimicrobial resistance

Antimicrobial resistance is becoming a major public health crisis as its global incidence continues to rise (Lushniak, 2014; Michael, Dominey-Howes, & Labbate, 2014). The World Health Organization (WHO) has listed antimicrobial resistance as one of the biggest health threats in this century (World Health Organization, 2014). It poses adverse consequential impacts on the management and treatment of infectious diseases, as drugs of high clinical importance are gradually becoming less effective against multidrug-resistant (MDR) pathogens. In addition to the increasing levels of resistance, the rate of which newer antimicrobials are being discovered has also severely dropped (Laxminarayan, 2014). If the resistance trends continue to persist in this current state, a post-antibiotic era may perhaps become imminent (Ventola, 2015b, 2015a). Thus, understanding the underlying mechanisms that drive bacterial resistance to antibiotics and developing novel therapeutic approaches are imperative in order to curb the emergence of MDR strains.

The outstanding genetic plasticity of bacteria enables them to adapt and respond to various threats, including the harmful effects of antimicrobial compounds. Bacterial resistance mechanisms are extremely crucial for their continuous adaptability and survival. Through millions of years of evolution, bacteria have evolved complex adaptive mechanisms to survive constant exposure to naturally occurring antimicrobials in the environment. These mechanisms of resistance are attained through mutability or transfer of mobile genetic elements (reviewed in Munita & Arias, 2016). Furthermore, natural selection has resulted in the proliferation and spread

of drug-resistant bacteria, as their drug-sensitive competitors are eradicated under antibiotics stress (Read & Woods, 2014).

2.2 Mechanisms of antimicrobial resistance in bacteria

The current arsenal of antibiotics work against bacteria through several modes of action, such as targeting bacterial DNA replication and repair (RNA polymerase, DNA gyrase), cell-wall biosynthesis, membrane structure, protein synthesis (subunit 30S or 50S of ribosome), and folic acid metabolism (reviewed in González-Bello, 2017). Development of bacterial resistance to antibiotics can be intrinsic or acquired. The low permeability of membrane barriers, the presence of multidrug transporters that can extrude antibiotics, and the lack of target structures for specific antimicrobial actions are among the fundamental characteristics of intrinsic resistance in bacteria (Putman, van Veen, & Konings, 2000; Taylor-Robinson & Bébéar, 1997). On the contrary, acquired resistance is primarily caused by chromosomal mutation in the genome of the bacteria or acquisition of antibiotic resistance determinants through horizontal gene transfer (Nikaido, 1994). The fundamental mechanisms of bacterial antibiotics resistance involve several major modes:

(1) enzymatic inactivation of antibiotics (Wright, 2005),

(2) target site modification (Lambert, 2005),

(3) active efflux of antibiotics, and changes in membrane permeability (Kumar & Schweizer, 2005).

2.2.1 Enzymatic inactivation of drugs

To inactivate antibiotic molecules, many Gram-positive and Gram-negative bacteria are capable of producing enzymes that can degrade or modify the antibiotics, thus rendering them ineffective. For instance, resistance to penicillin and

cephalosporin is mediated through the cleavage of their β-lactam ring by hydrolytic enzymes called β-lactamases; the destruction of the amide bond of β-lactam ring subsequently leads to the degradation of the antibiotics (Bonnet, 2004; Bush, Jacoby, & Medeiros, 1995; Kotra & Mobashery, 1999; Poole, 2004). Antimicrobial compounds like chloramphenicol, macrolides, aminoglycosides, rifampicin, or streptogramin can be inactivated by transferase enzymes through phosphorylation (Matsuoka & Sasaki, 2004; Nakamura et al., 2000; Yazawa et al., 1994), acetylation (Allignet & el Solh, 1995; Schwarz et al., 2004; Vetting et al., 2004), ribosylation (Houang et al., 2003), or nucleotidylation of the antibiotics molecule (Brisson-Noel et al., 1988; Pedersen, Benning, & Holden, 1995). The addition of chemical moieties through enzymatic modification affects the binding of antibiotics to their target which subsequently impedes their activity (Strateva & Yordanov, 2009; Tolmasky, 2000).

2.2.2 Target site modification

Modification of antibiotics target site is another common mechanism of drug resistance in bacteria. This mechanism usually involves target alteration through mutational changes which cause reduction in the affinity of the binding site (Spratt, 1994). A well-characterized example is the amino acid alterations in bacterial penicillin-binding proteins (PBPs) which lead to the increase in resistance to β-lactam antibiotics such as penicillin, amoxicillin, and ampicillin in several bacterial strains (Dowson, Coffey, & Spratt, 1994; Kosowska et al., 2004; Nagai et al., 2002). Resistance to fluoroquinolones is conferred by the presence of mutations in the structural genes that alter enzymes like DNA gyrase or topoisomerase IV, which prevents antibiotics action on the targeted enzymes (Ince et al., 2002; Khodursky, Zechiedrich, & Cozzarelli, 1995).

2.2.3 Drug efflux

Bacteria can actively efflux antibiotics out of the cells and regulate the permeability of their membranes, as part of their fundamental mechanisms of drug resistance (Nikaido, 1994). The extrusion of antimicrobial agents out of the bacterial intracellular milieu is due to the presence of membrane transporter proteins, generally known as efflux pumps. These efflux pumps are capable of transporting out broad classes of antimicrobials, including aminoglycosides, macrolides, fluoroquinolones, and tetracycline, thus lowering antibiotics concentrations inside the cells (Nikaido & Zgurskaya, 1999; Webber & Piddock, 2003). They also possess a variety of other physiological functions (Piddock, 2006). Bacterial efflux pumps can either specifically extrude only one class of antibiotics or non-specifically extrude multiple classes of antibiotics, hence triggering the emergence of MDR phenotypes in bacteria (Poole, 2005). There are five classes of bacterial efflux pump systems, which include the resistance-nodulation-division (RND) superfamily, the major facilitator (MFS) superfamily, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the ATP-binding cassette (ABC) superfamily. The ABC superfamily is a primary transporter which utilizes ATP to drive the binding and extrusion of substrates (Davidson & Maloney, 2007), whereas the RND, MFS, MATE, and SMR families are secondary transporters which depend on proton motive force as a source of energy (Forrest, Krämer, & Ziegler, 2011).

2.2.4 Reduced permeation

In addition to efflux mechanism, bacterial resistance can be further enhanced through changes in membrane permeability. Gram-positive bacteria such vancomycin-intermediate resistant *Staphylococcus aureus* (VISA) strains are capable of increasing

their cell wall thickness to restrict drug penetration, which significantly reduces their susceptibility to vancomycin (Cui et al., 2000). The outer membranes of Gram-negative bacteria are made up of phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet, which act as barriers against the penetration of lipophilic toxic compounds, including antibiotics (Nikaido, 2003). Diffusion and passive transport across the outer membrane are achieved through water-filled channels called porins (Lambert, 2002). To limit the influx of hydrophilic antimicrobial agents, bacteria can reduce membrane permeability by regulating the expression levels of porins to decrease the number or change the types and selectivity of the porin channels (Chevalier, Pagès, & Malléa, 1999; Dé et al., 2001; Hancock & Brinkman, 2002). This will subsequently decrease the rate of diffusion of hydrophilic compounds such as chloramphenicol, β-lactams, tetracycline, and certain fluoroquinolones across the membrane (Pagès, James, & Winterhalter, 2008).

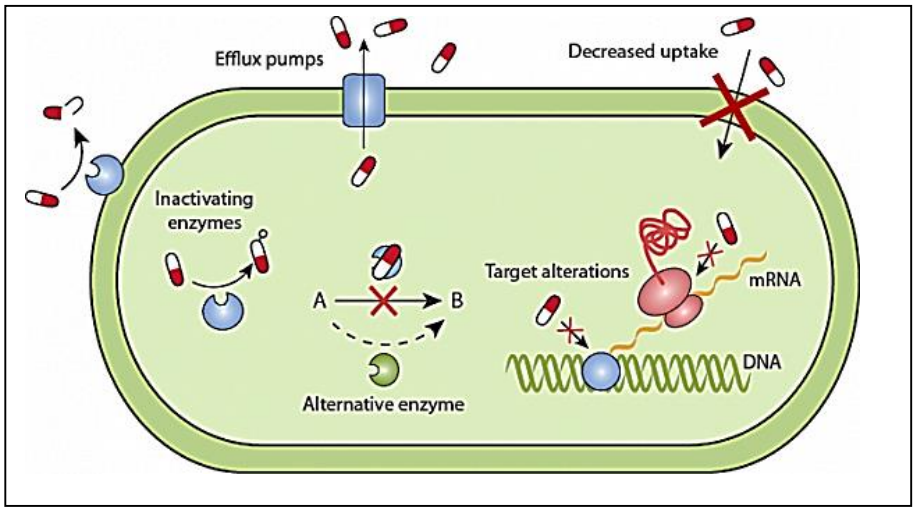

Figure 2.1 Mechanisms of antibiotic resistance in bacteria (Figure adapted from Gullberg, 2014).

2.3 Causes of antimicrobial resistance and possible ways to reduce it

Antimicrobial resistance is a naturally occurring process; however, the inappropriate use of antimicrobial agents tends to accelerate its development in microorganisms through selective pressure. Indeed, continuous overdependence on antibiotics and their uncontrolled usage are the main reasons for the upsurge of bacteria that are predominantly resistant to antibiotics (Davies, 1996). *Burkholderia* spp. such as *B. pseudomallei* are among the pathogens of clinical significance due to their intrinsic resistance to multiple antimicrobial agents and their capacity to acquire complex multidrug resistance (Poole, 2001).

The lack of progress in the development of novel antimicrobials is the cause for the current shortage of new antimicrobial options that can substitute ineffective drugs. This highlights the importance of restoring and maintaining the efficacy of existing antimicrobial agents. A number of strategies have been initiated to control and reverse antimicrobial resistance, such as co-administrating antibiotics with molecules or adjuvants that can block a targeted resistance machinery (Baym, Stone, & Kishony, 2016), strengthening the activity of antibiotics through chemical or structural modifications (Kondo & Hotta, 1999; Malabarba, Nicas, & Thompson, 1997; Sztaricskai et al., 1999), and suppressing the mechanisms that confer persistence (Chen et al., 2011; Rutherford & Bassler, 2012; Smith & Romesberg, 2007) or virulence in bacteria (Allen et al., 2014; Ross-Gillespie et al., 2014; Swoboda et al., 2009).

2.4 *Burkholderia pseudomallei*: Introduction and profiles

B. pseudomallei is a betaproteobacteria which belongs to the family *Burkholderiaceae*. It is characterized as an aerobic, non-spore forming, oxidase-positive, motile bacillus with one or more polar flagella. It is a small-sized bacterium that measures approximately 2-5 µm in length and 0.4-0.8 µm in diameter. The Gram stain of this bacterium shows Gram-negative rods that have bipolar staining with a characteristic "safety pin" appearance. During the early days, it was formerly known by various names such as *Bacillus pseudomallei*, *Bacillus whitmorii*, *Malleomyces pseudomallei*, and *Pseudomonas pseudomallei* (Cheng & Currie, 2005). In 1992, Yabuuchi and his colleague proposed the transfer of seven species from the *Pseudomonas* group to the new *Burkholderia* genus, which included *Pseudomonas mallei*, *Pseudomonas pseudomallei*, *Pseudomonas cepacia*, *Pseudomonas solanacearum*, *Pseudomonas gladioli*, *Pseudomonas caryophylli*, and *Pseudomonas pickettii*; hence, the name *Pseudomonas pseudomallei* was subsequently changed to *Burkholderia pseudomallei* (Yabuuchi et al., 1992).

The bacterium is a soil saprophyte that thrives in wet soil, stagnant water, and rice-farming areas (White, 2003). It grows optimally at a temperature of 37 °C or 42 °C in a neutral or mildly acidic soil medium with pH 6.5 to 7.5 (Chen et al., 2003). In laboratory settings, it is a non-fastidious organism that grows readily on a variety of nutrient media such as Ashdown's agar, Luria medium, Mueller Hinton broth, MacConkey agar, and blood agar. Colony morphology on agar plate is characterized by smooth, translucent, round, and slightly raised appearance after an overnight incubation at 37 °C. Aging colonies show dry, rough and wrinkled morphology with a metallic appearance. The bacterial cultures also exude an earthy, musty odor.

B. pseudomallei is considered a potential agent of biological warfare due to its capability to spread infection by aerosol transmission (Rotz et al., 2002), in addition to its high virulence, mortality rate, and resistance to a wide range of antimicrobial agents. Since 2015, the U.S. Centers for Disease Control and Prevention (CDC) has classified it as a category B bioterrorism agent (https://emergency.cdc.gov/agent/agentlist-category.asp). *B. pseudomallei* can survive in harsh environmental conditions, including nutrient-deprived settings such as low availability of iron (Hantrakun et al., 2016), limited oxygen (Hamad et al., 2011; O'Rourke et al., 2017), and in dehydrated soil with low moisture content (Tong et al., 1996). It can persist in highly acidic environment of pH 4.5 (Dejsirilert et al., 1991), in detergent and disinfectant solutions (reviewed in Cheng & Currie, 2005; Gal et al., 2004), and in high concentrations of salt or oxidative agents. In 2010, it was discovered that *B. pseudomallei* was able to survive in distilled water without any nutrients for at least 16 years, which indicates the ability of the bacterium to endure and adapt in extreme conditions (Pumpuang et al., 2011).

2.4.1 Genome characteristics of *B. pseudomallei*

The complete genome of *B. pseudomallei* strain K96243 has been sequenced by The Wellcome Sanger Institute (Holden et al., 2004). The genome is 7,247,547 basepairs (bp) in size with a G+C content of 68.06%. It is one of the largest and most complex prokaryotic genomes, harboring two circular chromosomes of 4,074,542 bp and 3,173,005 bp, respectively. The larger replicon, known as chromosome 1, consists of 3,460 coding sequences, 53 transfer RNAs (tRNAs) and 9 ribosomal RNAs (rRNAs), whereas chromosome 2 contains 2,395 coding sequences, 8 tRNAs and 3 rRNAs. Chromosome 1 encodes proteins essential for core cellular functions such as cell growth, central metabolism, macromolecular biosynthesis, chemotaxis, and

mobility. Chromosome 2 encodes proteins related to accessory functions which are important for adaptation and survival ability of *B. pseudomallei*, such as regulation of laterally acquired DNA, secondary metabolism, iron acquisition, and osmotic protection (Holden et al., 2004). The regions of genomic island (GI) which make up 6.1% of *B. pseudomallei* genome contain mobile genetic elements acquired through horizontal gene transfer. The GI regions are an important evolutionary feature for *B. pseudomallei* virulence (Holden et al., 2004). A large proportion of *B. pseudomallei* genes are still unannotated or uncharacterized.

The genome of *B. pseudomallei* is made up of various gene clusters that are important for environmental survival and virulence of the bacterium. Quorum sensing (Ulrich et al., 2004), pathogenicity islands, protein secretion systems (type II, type III, type IV, and type VI secretion systems) (Burtnick et al., 2011; DeShazer et al., 1999; French et al., 2011; Gong et al., 2011; Schwarz et al., 2014; Stevens et al., 2002; Winstanley, Hales, & Hart, 1999), drug resistance determinants, and cell surface adhesion (Holden et al., 2004) are among the factors implicated in the virulence and pathogenesis of *B. pseudomallei*. The type III secretion systems (TTSS) are one of the most significant virulent determinants of *B. pseudomallei*, in which the secretion of effector proteins into the cytosol of target cells through its needle-like apparatus is a requisite factor in the invasion of host cells (Muangsombut et al., 2008; Stevens et al., 2002). Certain surface polysaccharides, such as the O-antigenic polysaccharide moiety of *B. pseudomallei* lipopolysaccharides play an important role in facilitating intracellular survival and bacterial evasion from host innate immune systems (Arjcharoen et al., 2007). The capsular polysaccharides help protect *B. pseudomallei* from phagocytic mediated killing, in which it subsequently increases the extracellular persistence of the bacterium (Reckseidler-Zenteno, DeVinney, & Woods, 2005). Other

factors such as flagella (Chua, Chan, & Gan, 2003; Chuaygud et al., 2008) and type IV pili (Essex-Lopresti et al., 2005) are necessary to facilitate bacterial adherence and invasion of host cells.

2.5 History of melioidosis

B. pseudomallei is an etiological agent of melioidosis (Dance, 1991), an acute and life-threatening disease endemic in Southeast Asia, northern Australia, and tropical regions (Rolim et al., 2005; Chaowagul et al., 1989; Currie, Dance, & Cheng, 2008; Howe, Sampath, & Spotnitz, 1971; Kanungo et al., 2002; Puthucheary, Parasakthi, & Lee, 1992; Sexton et al., 1993). This bacterium is known to cause potentially fatal infection in both human and animals, including cows, goats, sheep, horses, pigs, and others (Dance, 1991; Sprague & Neubauer, 2004). The disease was first discovered in Rangoon, Burma by pathologist Alfred Whitmore and his assistant Krishnaswami in 1911, of which they initially described it as "glanders-like" disease (Whitmore, 1913; Whitmore & Krishnaswami, 1912). Stanton and Fletcher then proposed the name melioidosis, which was derived from the Greek words "melis" (distemper of asses) and "eidos" (resemblance) on account of several clinical and pathophysiological similarities between the disease and glanders (Stanton & Fletcher, 1921). Melioidosis was first documented in Malaya by Fletcher following an outbreak involving laboratory animals at the Institute of Medical Research, Kuala Lumpur in 1913 (Stanton & Fletcher, 1925). Some of the earliest cases of human melioidosis were described by Stanton based on two occurrences in Kuala Lumpur in 1917 (Stanton, Flectcher, & Kanagarayer, 1924).

2.5.1 Clinical presentation and physiognomy of melioidosis

Melioidosis can be categorized into the acute, subacute, or chronic forms of illness (Howe et al., 1971). It has a very broad clinical spectrum, varying from asymptomatic infection and localized abscess formation at one end of the spectrum, to disseminated abscesses in multiple organs, fulminant sepsis, shock, and death at the other end of the spectrum (Currie et al., 2004; Jain et al., 2007; Malczewski et al., 2005; Silbermann et al., 1997; Tiangpitayakorn et al., 1997; Walsh et al., 1995; White, 2003; White et al., 1989; Whitmore & Krishnaswami, 1912). The most aggressive form of melioidosis is acute melioidosis, which represents more than 85% of the reported clinical cases (Currie et al., 2010). Mortality rate due to melioidosis is noticeably higher in Southeast Asian countries, with 33% to 65% of documented cases compared to other endemic regions such as Northern Australia (14%) and India (9.5%) (reviewed in Kingsley et al., 2016; Limmathurotsakul et al., 2010). The most common clinical manifestations of melioidosis are acute/subacute pneumonia, acute febrile illness, localized skin or soft tissue abscess, and septicemia (Currie et al., 2000; Kingsley et al., 2016; Smith et al., 1987; White, 2003). Acute septicemic infections often quickly result in death despite intensive antibiotic therapy and have been the causal factor for significant mortality and morbidity in Southeast Asia (Chaowagul et al., 1989). Pneumonia is presented in nearly half of all melioidosis cases (Currie et al., 2000; Meumann et al., 2012) and the acute form of melioidosis pneumonia resembles other types of bacterial pneumonia. Clinical and radiologic features of chronic pneumonia may also mimic pulmonary tuberculosis (Reechaipichitkul, 2004; Vidyalakshmi et al., 2008; White, 2003). Due to its protean manifestations, the infection is always termed “the great mimicker” (Yee et al., 1988). The varying clinical

patterns of melioidosis that lack distinct pathognomonic features often lead to inaccurate diagnosis and management of the disease.

2.5.2 Disease transmission

Infection is commonly acquired through direct contact with soil or muddy water, in which the pathogen enters the human body through skin wounds, cuts, or abrasion (Howe et al., 1971; Whitmore & Krishnaswami, 1912). Other routes of infection may include aspiration, inhalation of airborne dust or droplets, and ingestion of contaminated water (Currie et al., 2001; Dance, 2002). In Southeast Asia, the highest number of diagnosed cases were reported from Thailand (Chaowagul et al., 1989; Leelarasamee, 2000; Vuddhakul et al., 1999), Malaysia (Hassan et al., 2010; Kingsley et al., 2016; Puthucheary et al., 1992; Vadivelu et al., 1997), and Singapore (Chan, Jayaratnam, & Teo, 1985; Lim et al., 1997; Tan, Ang, & Ong, 1990; Tong et al., 2009; Yap et al., 1991, 1995). Majority of the patients were farmers and agricultural workers who contracted the infection during their agricultural activities (Cheng & Currie, 2005; Reechaipichitkul, 2004). The marked seasonal incidence of melioidosis in endemic regions is influenced by seasonal rain and severe weather conditions, such as heavy monsoons, flooding, tsunamis, and typhoons which consequently increase the risk for potential exposure to the pathogen (Apisarnthanarak, Khawcharoenporn, & Mundy, 2012; Bulterys et al., 2018; Chierakul et al., 2005; Currie, Ward, & Cheng, 2010; Ko et al., 2007). Active tourism activities have also been linked to melioidosis infection in travelers who have visited regions where *B. pseudomallei* is pervasive (Svensson et al., 2006; Visca et al., 2001).

2.5.3 Host risk factors

Melioidosis is regarded as an opportunistic infection; hence, individuals with certain medical conditions, primarily those who are immunocompromised or immunosuppressed, are at greater risk of developing severe infections. Among the underlying risk factors for melioidosis include comorbidities such as diabetes mellitus, excessive alcohol consumption, impairment of the immune system, renal failure, chronic lung disease, steroid intake, malignancies, and thalassemia (Currie et al., 2000; Suputtamongkol et al., 1999). Seroprevalence studies indicate that *B. pseudomallei* infection is mostly asymptomatic, though it may quickly progress to a fatal illness in patients with comorbid factors (Ashdown & Guard, 1984; Cheng et al., 2008; Kanaphun et al., 1993; Wuthiekanun et al., 2006). The incubation period for melioidosis is generally one to 21 days after initial exposure, with a median of 9 days to the onset of infection; however, symptoms can rapidly develop within 24 hours with a high inoculum (Currie et al., 2000). In the case of latent infection, the incubation period may take months or years before clinical symptoms are apparent. The progression from asymptomatic infections to the onset of melioidosis usually depends on the host's condition. The bacterium can remain latent inside the host for a long period of time and then reactivate when the infected person is immunocompromised (Currie et al., 2000). The longest documented period of latency from initial exposure to the onset of the disease is 62 years (Ngauy et al., 2005).

2.5.4 Disease latency and relapse

Recurrence of infection is a common feature of melioidosis, in which 5-28% of melioidosis patients have been reported to experience clinical relapse after a prolonged disease-free interval following the completion of antibiotic therapy

(reviewed in Wiersinga et al., 2018). Recrudescence can occur as a result of re-exposure to other strain, or reactivation of the original strain which persisted in a dormant state and was not completely eradicated during previous treatment (Cheng & Currie, 2005; Currie et al., 2010; Maharjan et al., 2005; Suputtamongkol et al., 1993; Yee et al., 1988). Bacterial persistence is believed to be one of the causes for disease latency, antimicrobial treatment failure, and chronic recurrent infections. Several factors influencing *B. pseudomallei* persistence have been identified, including two-gene toxin/antitoxin (TA) systems, metabolic enzymes, and adaptive mutations (Lewis & Torres, 2016). The TA systems mediate the formation of antibiotic tolerant variants, known as persisters, which form stochastically within a bacterial population in response to antimicrobial pressure (Harrison et al., 2009; Keren et al., 2004; Shah et al., 2006). When challenged with a lethal concentration of antibiotics, the TA modules encode a toxin that halts cell growth and lowers metabolic processes, which causes a small fraction of the clonal bacterial population to enter a transient state of dormancy (Christensen et al., 2004; van Melderen, Bernard, & Couturier, 1994). Since antibiotics generally target the actively growing cells, the formation of dormant persisters enables a subpopulation of the bacteria to tolerate and escape the effects of antibiotics (Keren et al., 2004). Nevertheless, much of the knowledge pertaining to the molecular mechanism of bacterial persistence still remains unclear.

The capability of *B. pseudomallei* to remain in a dormant state is also attributed to its intracellular adaptation within the hosts. As a facultative intracellular pathogen, it is able to invade and multiply inside non-phagocytic (epithelial cells) and phagocytic cells (macrophages, leukocytes, monocytes, and neutrophils) (Egan & Gordon, 1996; Jones, Beveridge, & Woods, 1996; Pruksachartvuthi, Aswapokee, & Thankerngpol, 1990). Following internalization, it can escape from the membrane-bound phagosome

into the cytosol (Stevens et al., 2002), where it subsequently polymerizes host actin to invade neighboring cells (Kespichayawattana et al., 2000). The actin-based intracellular motility enables the bacteria to move between cells effectively while avoiding host immune surveillance, which include the effector T cells, circulating antibodies, complement proteins, or other extracellular immune factors (Ray et al., 2009). It has been suggested that the bacterium may also localize to the nucleus of infected cells and use the nuclear compartment as a protective niche for intracellular persistence (Vadivelu et al., 2017).

2.5.5 Detection and identification of melioidosis

Culture and isolation of *B. pseudomallei* from clinical specimens such as blood, urine, pus, skin lesions, sputum, or throat swab remains the gold standard for diagnosis of melioidosis (Anuntagool, Rugdech, & Sirisinha, 1993). Laboratory diagnosis can be difficult due to mixed variation in colony morphology that can lead to incorrect identification (Chantratita et al., 2007; Pumpuang et al., 2011). Besides, isolation from clinical samples often takes time and requires expertise, thus delaying initiation of treatment. Serological tests such as latex agglutination test or indirect haemagglutination test have lower sensitivity in general and are commonly performed for a provisional diagnosis. Enzyme-linked immunosorbent assay (ELISA) is a more convincing serological test (Ashdown et al., 1989). Conventional biochemical methods such as the API 20NE system provide an accurate identification in most of the cases (Amornchai et al., 2007); however, it can also misidentify the bacterium as *Chromobacterium violaceum*, *Burkholderia cepacia* or *Pseudomonas aeruginosa* (Inglis et al., 1998; Lowe, Engler, & Norton, 2002). Molecular identification techniques such as polymerase chain reaction (PCR), pulse field gel electrophoresis (PFGE), random amplification of particle of deoxyribonuclease (RAPD), and

restricted fragmentation length polymorphism (RFLP) are more reliable because of their high specificity, sensitivity, robustness and speed (Kunakorn et al., 2000; reviewed in Raja, Ahmed, & Singh, 2005; Rattanathongkom, Sermswan, & Wongratanacheewin, 1997). Nonetheless, these methods are not routinely accessible for clinical diagnosis.

2.5.6 Treatment of melioidosis

The course of treatment for melioidosis consists of two critical phases. The first one is the intensive acute phase, which typically involves short-term intravenous administration of ceftazidime, meropenem, or imipenem for an average duration of 10 to 14 days; however, severe cases of infection may require longer period of treatment. The next phase of therapy is the eradication phase, which is initiated after positive improvements are seen in patients during the initial treatment. A combination of trimethoprim–sulfamethoxazole (TMP/SMX) is administered orally for at least three to six months, depending on the severity of the infection (Wiersinga et al., 2012). In the case of incompatibility or intolerance towards TMP/SMX, other antibiotics such as amoxicillin/clavulanic acid or doxycycline are recommended (Lipsitz et al., 2012). An optimum choice of drugs and the duration of antibiotic therapy are extremely crucial in avoiding mortality and preventing the relapse of melioidosis (Suputtamongkol et al., 1993). Treatment of *B. pseudomallei* also proves to be challenging due to its natural resistance to numerous antibiotics and the need for a lengthy course of biphasic antimicrobial therapy (Cheng & Currie, 2005; Woo et al., 2003). The incidence of disease relapse and treatment failures still remain high despite the intensive course of recommended antibiotic therapy due to the bacterium's antibiotic resistance machinery, intracellular survival, and ability to exist in a dormant state in the host for an extended duration (Dance et al., 1989; Schweizer, 2012a;

Suputtamongkol et al., 1993; Wong, Puthucheary, & Vadivelu, 1995). Many researches are currently focusing on the discovery and development of an effective vaccine for melioidosis prevention, however none has been commercially developed for clinical use so far (Cheng & Currie, 2005; Dowling, 2013; Peacock et al., 2012).

2.6 Antibiotic resistance in *B. pseudomallei*

The arsenal of antibiotics and therapeutic options available for the treatment of melioidosis are limited because of the intrinsic resistance of *B. pseudomallei* to wide classes of antimicrobial agents. *B. pseudomallei* is inherently resistant to various types of antibiotics such as β-lactams, macrolides, aminoglycosides, colistin, rifamycins, and older-generation cephalosporins (Dance et al., 1989; Jenney et al., 2001). Several mechanisms of antibiotic resistance in *B. pseudomallei* have been previously documented, which include enzymatic inactivation (Godfrey et al., 1991; Livemore et al., 1987), target modification (Viktorov et al., 2008), low membrane permeability (Burtnick & Woods, 1999), and drug extrusion by multidrug efflux systems (Lipsitz et al., 2012; Moore et al., 1999; Schweizer, 2003).

The lipopolysaccharide O-antigen and outer core components on the outer membrane of *B. pseudomallei* function as a permeability barrier to prevent the penetration of cationic compounds, thus contributing to its intrinsic resistance to antibiotics such as polymyxin B (Burtnick & Woods, 1999). PenA, a class A β-lactamase, is accountable for primary resistance to β-lactams in most of the clinical isolates (Godfrey et al., 1991). PenA confers resistance to numerous β-lactam antibiotics such as amoxicillin, ampicillin and carbenicillin. It is chromosomally encoded by *pen*A gene; *in vitro* deletion of *pen*A causes the mutants to become fully susceptible to these antibiotics (Rholl et al., 2011). Overexpression of PenA due to a