CHARACTERIZATION OF BIOFILM FORMATION AMONG *BURKHOLDERIA PSEUDOMALLEI* MUTANTS: *BPS*I, *PPK, RPO*N2, AND *RPO*S

RUNGRAWEE MONGKOLROB

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (BIOCHEMISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2015

Thesis
entitled

CHARACTERIZATION OF BIOFILM FORMATION AMONG *BURKHOLDERIA PSEUDOMALLEI* MUTANTS: *BPSI, PPK, RPON2,* AND *RPOS*

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Biochemistry)
on
November 9, 2015

Miss Rungrawee Mongkolrob
Candidate

Prof. Sakol Panyim,
Ph.D. (Biochemistry)
Chair

Prof. Sumalee Tungpradabkul,
Ph.D. (Molecular Biology)
Member

Asst. Prof. Kittisak Yokthongwattana,
Ph.D. (Agricultural & Environmental
Chemistry)
Member

Mr. Sittiruk Roytrakul,
Ph.D. (Phytochemistry)
Member

Mr. Saranyoo Ponnikorn,
Ph.D. (Biochemistry)
Member

Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

Prof. Skorn Mongkolsuk,
Ph.D. (Biological Science)
Dean
Faculty of Science
Mahidol University

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Rungrawee Mongkolrob
.....
Miss Rungrawee Mongkolrob
Candidate

.....
Prof. Sumalee Tungpradabkul,
Ph.D. (Molecular Biology)
Major advisor

.....
Asst. Prof. Kittisak Yokthongwattana,,
Ph.D. (Agricultural & Environmental Chemistry)
Co-advisor

Sittiruk Roytrakul
.....
Mr. Sittiruk Roytrakul,
Ph.D. (Phytochemistry)
Co-advisor

.....
Prof. Patcharee Lertrit,
M.D., Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

.....
Asst. Prof. Kittisak Yokthongwattana,
Ph.D. (Agricultural & Environmental Chemistry)
Program Director
Doctor of Philosophy Program in Biochemistry
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my special appreciation and tremendous thanks to my advisor Professor Sumalee Tungpradabkul for encouraging my research and allowing me to grow as a research scientist. It is such as an invaluable advice on both researches as well as on my future career as a teacher. I would also like to thank my chair committee, Professor Sakol Panyim, as well as Asst. Prof. Kittisak Yokthongwattana, Dr. Sittiruk Roytrakul and Dr. Saranyoo Ponnikorn for serving as my committee members even at hardship. Your brilliant comments and suggestions make my defense be an appeased moment, thanks to you all. I would especially like to thank Dr. Sakawrat Kanthawong and Assoc. Prof. Suwimol Taweechaisupapong for her assistance with the CDB device at Kon Kean University, Miss Somrudee Chaiyasit at the OLYMPUS Bioimaging center and Mr. Sirichai Kositarat at Central Instrument Facility, Mahidol University for their generous guidance. All of you have been there to support me when I operated and collected all data for my Ph.D. thesis.

I am infinitely indebted to Commission of Higher Education for Ph.D. Scholarship in the program Strategic Scholarships for Frontier Research Network for the financial support enabling me to undertake this program. Many thanks to all colleagues at Department of Biochemistry, Faculty of Science, Mahidol University for a professional training atmosphere and all of ST's Lab members, exclusively PuNong, Farn, Ploy, Su, Bank, Zeez, P'Chain and Prig, who supported me in discussing, writing, and incenting me to strive towards my goal. Finally, I would like to thank P'Tuu, she had been a great mentor for me from the beginning of my research life.

At the end, I would like to express my deepest appreciation to my family and all the teachers who have taught me since my childhood. Words cannot express how grateful I am to my mother and father for all of the sacrifices that you've made on my behalf. Your encouragement supporting me was what sustained me thus far.

Rungrawee Mongkolorb

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RUNGRAWEE MONGKOLROB 5137074 SCBC/D

Ph.D. (BIOCHEMISTRY)

THESIS ADVISORY COMMITTEE: SUMALEE TUNGPRADABKUL, Ph.D.; KITTISAK YOKTHONGWATTANA, Ph.D.; SITTIRUK ROYTRAKUL, Ph.D.

ABSTRACT

Burkholderia pseudomallei is the cause of melioidosis, a fatal tropical infectious disease, which was reported to have a high rate of recurrence even when an intensive dose of antibiotics was used. The biofilm formation is believed to be one of the possible relapse causes because of the ability to increase drug resistance. Extracellular polymeric substance (EPS) in biofilm has been reported to be related to limitation of antibiotic penetration in *B. pseudomallei*. However, the mechanisms through which that biofilm creates a restricting diffusion to antibiotics remains unclear. In this study, the researcher presented a correlation between exopolysaccharide production in biofilm matrix and antibiotic resistance in *B. pseudomallei* by using *bps*I, *ppk*, *rpo*N2 and *rpo*S mutant strains. CLSM revealed a reduction of the exopolysccharide production and disability of the micro-colony formation in *B. pseudomallei* mutants, which paralleled the antibiotic resistance. Different ratios of carbohydrate contents in exopolysaccharide of the mutants was detected, although they had the same components; glucose, galactose, mannose, and rhamnose with no detectable peak found in *bps*I mutant. These results indicated that the correlation between these phenomenal in *B. pseudomallei* biofilm at least resulted from exopolysaccharide which may have been under the regulation of *bps*I, *ppk*, *rpo*N2 or *rpo*S genes.

KEY WORDS: ANTIBIOTIC RESISTANCE / BIOFILM / BURKHOLDERIA PSEUDOMALLEI / EXOPOLYSACCHARIDE

111 pages

การศึกษาคุณสมบัติการสร้างไบโอฟิล์มระหว่างเชื้อ *BURKHOLDERIA PSEUDOMALLEI* ผ่าเหล่าชนิดต่างๆ

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รุ่งรวี มงคลรบ 5137074 SCBC/D

ปร.ด. (ชีวเคมี)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: สุมาลี ตั้งประดับกุล, Ph.D.; กิตติศักดิ์ หยกทองวัฒนา, Ph.D.; สิทธิรักษ์ รอยตระกูล, Ph.D.

บทคัดย่อ

B. pseudomallei เป็นแบคทีเรียอันเป็นสาเหตุของโรค melioidosis โรคติดเชื้อร้ายแรงในเขตร้อนซึ่งได้รับรายงานว่ามีอัตราการเกิดซ้ำสูง ถึงแม้ว่าผ่านการรักษาด้วยยาปฏิชีวนะปริมาณเข้มข้นสูงมาแล้วก็ตาม การสร้างไบโอฟิล์มถูกเชื่อว่าเป็นหนึ่งในสาเหตุที่เป็นไปได้ของการกำเริบของโรค เนื่องจากไบโอฟิล์มทำให้ความสามารถในการต้านทานยาของเชื้อเพิ่มมากขึ้น จากรายงานการศึกษาเกี่ยวกับสารพอลิเมอร์ที่อยู่ภายนอกเซลล์ (EPS) ในไบโอฟิล์ม พบว่ามีส่วนเกี่ยวข้องกับการจำกัดการซึมผ่านของยาปฏิชีวนะใน *B. pseudomallei* อย่างไรก็ตามกลไกที่ไบโอฟิล์มช่วยจำกัดการแพร่กระจายของยาปฏิชีวนะเข้าสู่เซลล์ยังคงไม่ชัดเจน ในการศึกษานี้ เราได้นำเสนอความสัมพันธ์ระหว่างการผลิต Exopolysaccharide ในไบโอฟิล์มและความต้านทานต่อยาปฏิชีวนะใน *B. pseudomallei* 4 สายพันธุ์ ซึ่งถูกกลายพันธุ์ที่ยีน *bps*I, *ppk*, *rpo*N2 และ *rpo*S ภาพของการสร้างไบโอฟิล์มโดยกล้องจุลทรรศน์คอนโฟคอลแสดงถึงการลดลงของการผลิต Exopolysccharide และความไม่สมบูรณ์ของการก่อตัวของกลุ่มเซลล์ในสายพันธุ์ที่ถูกกลายพันธุ์และยังพบว่าความต้านทานยาปฏิชีวนะก็ลดลงในทำนองเดียวกัน นอกจากนี้อัตราส่วนของชนิดคาร์โบไฮเดรตใน Exopolysaccharide ของแต่ละสายพันธุ์ก็ไม่เหมือนกัน ถึงแม้ว่าทุกสายพันธุ์จะมีส่วนประกอบที่เหมือนกันได้แก่ กลูโคส, กาแลคโตส, แมนโนสและแรมโนส แต่ในสายพันธุ์ *bps*I กลับไม่พบแรมโนส จากผลการทดลองชี้ให้เห็นว่าความสัมพันธ์ระหว่างปรากฏการณ์เหล่านี้ในไบโอฟิล์ม ส่วนหนึ่งเกิดจาก Exopolysaccharide ซึ่งน่าจะอยู่ภายใต้การควบคุมของยีนที่ถูกกลายพันธุ์

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LIST OF ABBREVIATIONS

BCSA	*Burkholderia cepacia* selective agar
PSA	*Pseudomonas* selective agar
BPSA	*B. pseudomallei* selective agar
MALDI-TOF MS	Matrix-assisted laser desorption/ionization of time-of-flight mass spectrometry
CAZ	ceftazidime
MRP	meropenem
TMP-SMX	trimethoprim/sulfamethoxazole
EPS	extracellular polymeric substances
CPS	capsular polysaccharides
LPS	lipopolysaccharide
Cdp	c-di-GMP-specific phosphodiesterase
AHL	N-acyl-homoserine lactone
*bps*I	a Lux inducing quorum-sensing homolog gene
ppk	Polyphosphate kinase gene
*rpo*N2	RNA polymerase sigma factor N (54) chromosome 2 gene
*rpo*S	RNA polymerase sigma factor S (38) gene
CV	crystal violet
MVBM	Modified Vogel and Bonner's medium
MHB	Mueller Hinton Broth
CLSM	confocal laser scanning microscopy
CF	cystic fibrosis
CFU/ml	Colony forming unit per milliliter
FITC-conA	fluorescein isothiocyanate-concanavalin A
PI	propidium iodide
CBD	Calgary Biofilm Device

LIST OF ABBREVIATIONS (cont.)

IC50	half maximal inhibitory concentration
GC-MS	gas chromatography-mass spectrometry
TMS	Trimethylsilyl
HMDS	hexamethyldisilazane
TMCS	trimrthylchlorosilane
STD	standard
dRF	detector response factor

CHAPTER I
INTRODUCTION

Burkholderia pseudomallei is a gram-negative aerobic bacterium which widely disperses in soil and, more particularly, in pooled surface water such as in rice paddies (1). This organism is the causative agent of a life-threatening disease of humans known as melioidosis, which has main endemic areas in tropical regions, especially Southeast Asia and northern Australia (1-3). Individuals can become infected by this bacteria via various infection routes; subcutaneous inoculation of an abraded skin, inhalation and aspiration of contaminated dust, sexual transmission, and ingestion of contaminated food or water (1, 3, 4). *B. pseudomallei* has been called 'the great mimicker' due to the clinical manifestations that present with an array of clinical signs and symptoms from acute or localized infection, acute pulmonary infection, acute blood stream infection or septicemia, and chronic suppurative infection. However, the most severe clinical representation is septic shock which mostly found in several pre-existing risk factor host conditions (2, 5). The complicated-clinical manifestations lead to obscurity of correctly diagnosis. Many repots have shown that a failure of proper diagnosis of this disease can lead to adverse outcomes including death, due to the ineffectiveness of the normal empirical antibiotic regimens used for the suspected bacterial sepsis (6-8). *B. pseudomallei* have inherent resistance to many types of antibiotics such as penicillin, ampicillin, first and second generation cephalosporins, gentamicin, etc. A common treatment of melioidosis requires an initial period of intensive treatment, such as ceftazidime (CAZ) and meropenem (MRP), for at least 10 days, continued by at least 3 months of prolonged oral eradication therapy of the appropriate antibiotics such as trimethoprim-sulfamethoxaxole (TMP-SMX) plus doxycycline. Even following the recommended 20-week intensive treatment, the melioidosis mortality rate remains high (40–50%) and relapse is ordinary in endemic areas (9, 10). One cause of reported-drug resistances in *B. pseudomallei* is an ability of biofilm formation, which may be the cause of disease recurrence (10).

The biofilm is a glycocalyx polysaccharide matrix encasing the bacterial population as a barrier to support many functions for the survival of the species, which is adherent to each other and/or to surfaces or interfaces. This extracellular matrix production supports many functions, including formation and maintenance of micro-colony and biofilm structure, enhancement of the biofilm resistance to environmental stress and antimicrobial agents, protection of the bacteria from protozoan grazing, and biofilm nutrition (11). Extracellular polymeric substances (EPS) in biofilms are a variety of macromolecules including DNA, proteins, lipids and polysaccharides, the main structural component, though its composition still depends on the environmental conditions, the age of the biofilm, and the particular biofilm-forming strain. The biofilm exopolysaccharides can be presented in many forms, including cell-bound capsular polysaccharides (CPS), unbound "slime" or free EPS, and as the O-antigen component of lipopolysaccharide (12). The different forms of extracellular polysaccharides in the biofilm have different contributions for the survival of the species (13). Moreover, the process of biofilm formation is complex and can be classified into three major steps: i) phenotypic changes in response to proximity of a surface and subsequent attachment using swimming, swarming or twitching, ii) bacterial communication via quorum sensing system (N-acyl homoserine lactone-AHL), cell aggregation and micro-colony formation, and iii) production of extracellular matrix to form mature three dimensional biofilm structures (14).

Bacteria capable of forming biofilms display enhanced antibiotic resistance compared to their free-living planktonic counterparts. The *B. pseudomallei* biofilm was reported to be a barrier to the diffusion of some antimicrobial agents resulting in limitation of the activity and diffusion of those antibiotics (15), however, this ability in *B. pseudomallei* has not been clearly demonstrated the correlation of bacterial virulence (16, 17). Although the limitation of antibiotic penetration by the *B. pseudomallei* biofilm has been demonstrated, there remains a need to investigate how the biofilm creates the retardation of antibiotic penetration.

Four *B. pseudomallei* mutants defective in the *bps*I, *ppk*, *rpo*N2 *and rpo*S genes were previously constructed and described by our laboratory (18-20). *B. pseudomallei bps*I gene encodes for an auto inducer synthase, which is important for synthesis of quorum-sensing signal molecule, N-acyl-homoserine lactone (AHL) (18).

The polyphosphate kinase gene, *ppk*, encodes an enzyme responsible for synthesis of inorganic polyphosphate from ATP that has been reported to be relevant in an energy supplement for biofilm formation (20). Alternative sigma factor N (sigma 54 or *rpo*N2) (unpublished data) and sigma S (sigma 38 or *rpo*S) are important for nitrogen assimilation and survival under stress conditions respectively (18-20). In addition, previous studies from other gram negative bacteria have shown that these regulatory genes have roles in biofilm antibiotic resistance (21-24).

Therefore, it is interesting to investigate how these four *B. pseudomallei* genes- *bps*I, *ppk*, *rpo*N2 and *rpo*S – involve in biosynthesis of the biofilm which may lead to the ineffective function of the biofilm matrix in a role of antibiotic resistance. The knowledge of resistant mechanism by the biofilm via these four genes of interest may elucidate possible targets to reduce antibiotic resistance in the biofilm *B. pseudomallei.*

CHAPTER II
LITERATURE REVIEWS

2.1 *Burkholderia pseudomallei* and Melioidosis

2.1.1 History and General bacteriology

Burkholderia pseudomallei was originally documented as the causative agent of melioidosis, which is a life-threatening disease of humans, in Myanmar in 1912 by A. Whitmore, English pathologist, and his colleague, C.S. Krishnaswami (25). This bacterium is firstly named as the non-motile *Bacillus pseudomallei,* after identification based on biochemical characteristics and having flagella, it was called *Pseudomonas pseudomallei* (26). Onward to 1992, the bacterium together with other seven members from the *Pseudomonas* species had been classified into new genus called *Burkholderia* genus due to the identification of its specific 16S RNA and lipid and fatty acid composition (27).

B. pseudomallei is a gram-negative, motile, aerobic, nonacid-fast and non-spore-forming bacillus which have 0.5-1 μm in width and 1-2 μm in length. On culture, the bacteria initially form smooth colony and further become wrinkle after 5 days of incubation whose specific character depending on the strain (1). It has many important biochemical characteristics including oxidase positive, nitrate reduction and its ability to assimilate L-arabinose (Ara+) which use to distinguish from the closely related but less pathogenic *B. thailandensis*. *B. pseudomallei,* a clinical strain isolated from a melioidosis patient, can generally grow on laboratory routine media, like Luria-Bertani or Tryptic soy media, but cannot assimilate L-arabinose (Ara-) (28). Additionally, it can also survive in anaerobic condition with the presence of acidic environment and in distilled water for many years. The *B. pseudomallei* genome is composed of two chromosomes with 4.07 Mb and 3.17 Mb which a large chromosome contains many genes associated with core functions for cell growth and metabolism

while the other carries genes encoding for accessory functions such as adaptation and survival in various hostile environments (29).

2.1.2 Epidemiology

Melioidosis has main endemic areas in tropical regions, corresponding approximately to the tropical latitudes between 20°N and 20°S, particularly in northern Australia and Southeast Asia, such as Thailand, Laos, Vietnam, Malaysia and Indonesia (Figure 2.1) (30). Moreover, there are some cases reported in other parts of the world, such as west and east Africa, the Caribbean, Central and South America and the Middle East, which are from returning travelers to areas of endemicity and sporadic autochthonous (1).

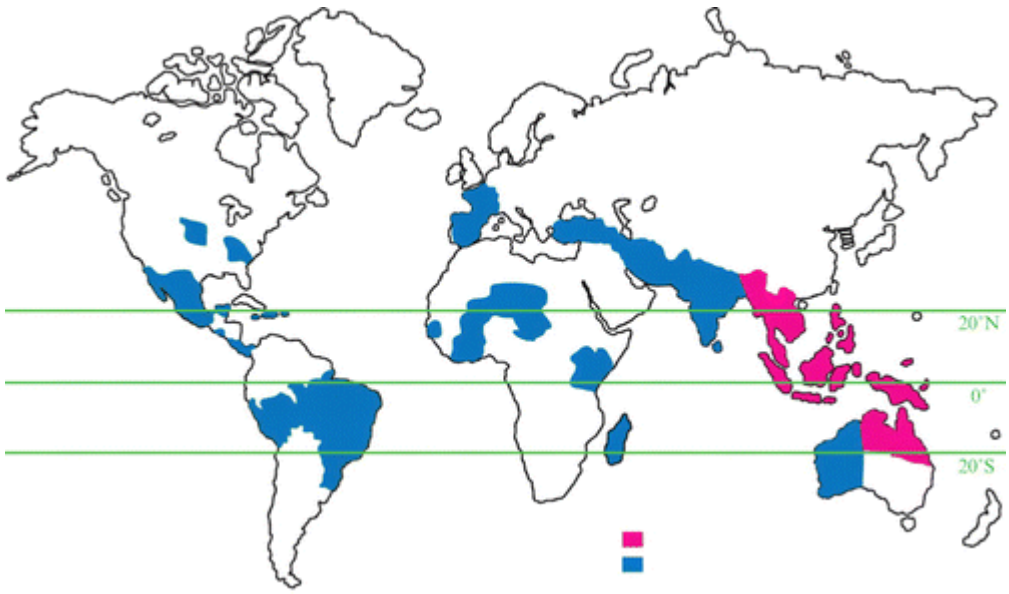

Figure 2.1 World distributions of melioidosis. The major endemic areas are shaded in the pink, while the sporadic autochthonous are showed in the blue (30).

In Thailand, melioidosis is prevalent in northeastern areas, including Khon Kaen, Nakhon Ratchasima, Buri Rum, Udon Thani and especially Ubon Ratchathani with the annual incidence of 4.4 cases per 100,000 (31). Owing to *B. pseudomallei* is widely dispersed in soil and especially in pooled surface water such as in rice paddies,

the highest occurrences are happened in monsoonal and rainy season, particularly with rice farmers. People acquired the bacteria mainly by infection with contaminated food or water through preexisting skin abrasion or ulceration. There are other modes of infection including inhalation of infectious dust and aerosol, together with melioidosis patient affiliation (32, 33). The disease has 60-80% mortality rate and rapid progression with 40% of deaths in septicemias. In addition, *B. pseudomallei* is intrinsically resistance to many common antimicrobial agents such as penicillin, ampicillin, first and second generation cephalosporin, erythromycin, gentamicin and rifampicin (34, 35). Almost 80% of populations in northeast Thailand have developed antibodies to *B. pseudomallei* without any symptoms which might develop into disease later. Even the disease is apparently cured with complete regimen, the relapse can still occur with recalcitrant to further treatment (36). Clinical presentations of Melioidosis varies from subclinical to clinical, similar to other bacterial infections, and virtually every organ can be infected (37).

2.1.3 Mode of acquisition and risk factors

B. pseudomallei typically exhibit three main modes of transmission including ingestion of contaminated water, inhalation of contaminated soil dust and water droplets, and direst skin inoculation through skin abrasions or wounds, which are no proven on the relative contributions of each (Figure 2.2). Normally, like other infectious diseases, an inoculation size and acquisition modes are responsible for disease pattern and severity (1). Although inhalation was firstly thought to be the primary infection mode (38), a study of infectious doses (39) and correlation between case increment and periods of heavy rainfall, associated with pneumonic presentations and increased-severity cases, has been defined suggesting that an increase of inhalation shift during extreme weather events (40). Now inoculation is believed to be the major acquisition mode for *B. pseudomallei*. In the Darwin series, there are 25% of patients having an inoculation injury prior to clinical presentations (1, 41). Even ingestion has been suggested as a mode of infection, there is no defined contribution of this route. Thus ingestion may not be the primary mode of acquisition (1). Laboratory acquired-cases have rarely been reported but the particular biosafety precautions are really important (42-44). Furthermore, other unusual modes of transmission, including

person to person (45) and a possible sexual intercourse have been described (46). Perinatal and vertical transmissions (mother to child) are also possible but quite rare (47, 48). The severe melioidosis can occur in healthy person but the lethal rarely emerges in the absence of risk factors. In a Thai study, an increased risks of melioidosis is associated with variety of risk factors including diabetes mellitus, thalassemia, rheumatic heart disease, chronic lung and renal diseases, male gender, steroid use, consumption of kava (an extract from *Piper methysticum* root) and infection by *Mycobacterium tuberculosis* but rarely human immunodeficiency virus, like HIV (1, 32). The major risk factor for melioidosis is diabetes mellitus based on a high incidence of melioidosis in patients with the disease, which is around 35.5% of total 64% preceding illness cases in Thailand (49).

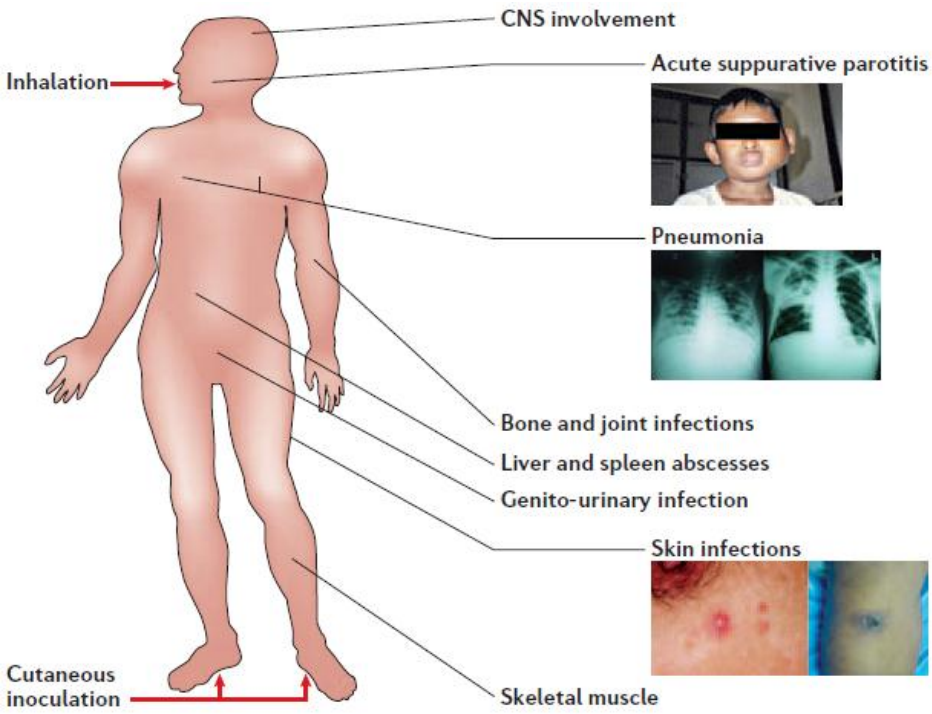

Figure 2.2 Routes of infection and selected clinical features of melioidosis. Several clinical symptoms of the disease travesty to many other gram-negative bacteria resulting in the term “the great mimicker”.(3)

2.1.4 Melioidosis clinical manifestations and severities

Melioidosis has various clinical manifestations mimicking other infections including tuberculosis and typhoid fever, malaria, leptospirosis, septicaemia caused by other gram-negative bacteria and mycotic infections, leading to be termed as "the great mimicker" (Figure 2.2). The most common melioidosis presentation is pneumonia, which is shown in approximately half of all cases. After bacterial inoculated infection, hematogenous spread occurs leading to arising cases of lung-involved pneumonia, which mostly found behind the clinical status (1). The other common manifestation of melioidosis are skin and soft tissue infections which may be the source of systemic infection and may rapidly progressive like other necrotizing fasciitis (50). In endemic regions, septicemia with or without pneumonia is the commonest clinical presentation, however, encephalomyelitis, osteomyelitis, septic arthritis, spinal involvement, localized abscesses in any other deep organ or superficial soft tissue have also been reported (51, 52). Significant clinical presentations in each endemic area have been reported. Male Australian melioidosis patients around 18% exhibit genitourinary infection with prostatic abscesses. Whereas there is no evidence of supportive parotitis in Australia, Thai children patients show 30 to 40% rate of parotitis occurrence (53). There are variety of incubation periods which may range from 2 days to 26 days (32). The definitive classification of melioidosis presentation is still not available, thus it can be an acute, sub-acute, subclinical, and chronic. Melioidosis clinical manifestations and severity variations depend on one or more of three main factors: i) bacterial strain and its presentation of virulent factors, ii) host immune response, and iii) mode of acquisition. Owing to a lot of evidences suggest that the disease pattern is determined from host factors, especially comorbidities and age, as prime importance (1). Relapse after apparently complete regimen is well limned, associated with a similar mortality to that for the first infection, which occurs in 13 to 23% of cases and a median of 6 to 8 months (54). A higher risk of relapse mainly associate with insufficient therapy rather than from re-infection which is evident from molecular typing of patient isolates with recurrent melioidosis (55). The major risk factors of relapse included disease severity, lack of ceftazidime of the first phase, length of the eradication therapy (less than 8 weeks), and antibiotic choices used in the eradication regimen (54, 56).

2.1.5 Laboratory diagnosis

The variety of clinical presentations of melioidosis infection causes difficulty in diagnosis. The gold standard for diagnosis of this disease requires isolation and cultivation of *B. pseudomallei* from non-sterile clinical specimens. Generally, *B. pseudomallei* can grow on non-selective agar, forming small, smooth colonies with a metallic sheen and strong soil smell after 24 to 48 hours and become dry and wrinkled after 3–5 days.

Figure 2.3 *Burkholderia pseudomallei* colony morphologies. Bacterial colony morphotypes were demonstrated on Ashdown's selective medium

These morphologies resemble to other gram-negative bacteria, such as *Pseudomonas stutzeri* (57), therefore, many selective media have been developed for more specific isolation of this organism. There are several types of the selective culture media, including Ashdown's medium, Francis medium, *Burkholderia cepacia* selective agar (BCSA) or *Pseudomonas* selective agar (PSA), and *B. pseudomallei* selective agar (BPSA), however, the most regularly selective medium used for the diagnosis is Ashdown medium (58). Because of inherited resistance to gentamycin, excluding the rare gentamicin sensitive *B. pseudomallei* strains, this antibiotic is used as the primary selective added in those selective agars (59). Within 72 h, colonies on Ashdown's medium will grow at 37°C and become purple due to the neutral red-comassie indicator. Francis medium is modified from Ashdown's medium by increasing in gentamicin concentration (4 mg/L to 8 mg/L) and using bromocresol

purple instead of neutral red indicator, resulting in yellow-colored colonies of cultured *B. pseudomallei* (60). A slightly improved sensitivity for Francis medium was found in a comparative study between these two media using spiked sputum samples. BCSA or PSA are alternatively used in laboratories outside of endemic areas where a rare case of *B. pseudomallei* investigation is occasionally occurred (58). *B. pseudomallei* selective agar (BPSA) is another selective medium developed to improve the discovery of *B. pseudomallei* from contaminated specimens (61), though there is no significant difference in sensitivity for *B. pseudomallei* in a study comparing Ashdown's agar, BCSA and BPSA (62). In order to improve the diagnosis of this disease, biochemical tests, including positive oxidase reaction, reduction of nitrate, arginine dihydrolase and gelatinase activities or oxidation of variety of sugar, are archived for further identification. These two types of test cannot yet provide reliable identification of *B. pseudomallei*. Other test panels, such as the API 20NE and RapID NF Plus systems, were tested to be promising for confirmatory identification, though the poor performance and conflicting reliability had been reported (63, 64). The Vitek automated system (the Vitek 1) has significant reliability and implication for identification of *B. pseudomallei*, which is widely used in non-endemic areas and occasional occurrences (65). However, the presumptive identification for clear bacterial morphologies needs 24 to 72 hours or more for the time for specimen plating and profoundly bacteria growth. A number of techniques with reduction of time consuming for achieved diagnosis have been developed, including antigen detection on specimens or on culture supernatant, antibody detection, molecular techniques, and rapid culture techniques. Latex agglutination for culture identification and direct immunofluoresence from direct specimens has shown appropriate sensitivity and specificity with significant reduction of time consuming. Nevertheless, these techniques are not widely used because of an unavailable specialized facilities and no field testing. The most widely used test for *B. pseudomallei* is indirect hemagglutination (IHA) because it is easy to perform without expensive devices and rapid to diagnose when compared with the culture-based methods (66). As rates of seropositivity in population of endemic areas are high (67), probably because of repeated exposure to *B. thailandensis* and *B. pseudomallei* (68), poor sensitivity and specificity of this IHA is commonly found leading to problematic diagnosis in the

endemic areas. Other sensitive methods having been developed are molecular techniques such as polymerase chain reaction (PCR), dot immunoassay, pulsed-field gel electrophoresis (PFGE) and restricted fragmentation length polymorphism (RFLP), few have been field tested. The detection of regions in 23S rRNA, 16S rRNA and 16S and 23S RNA junction using targeted primers has been evaluated (69-74) with a sensitivity of 100% but a low specificity is a small clinical case (75). The most rapid and lowest costing detection of *B. pseudomallei* is Matrix-assisted laser desorption/ionization of time-of-flight mass spectrometry (MALDI-TOF MS) which require MALDI Biotyper Security Reference Library, that need to be expanded for more reliable identification (76). As many techniques have been developed to achieve the gold standard of *B. pseudomallei* diagnosis, there is no best method that gives the best sensitivity or specificity, thus the conclusive diagnosis of *B. pseudomallei* should be performed with multiple and reliable tests together.

2.2 Melioidosis treatments and Problems

2.2.1 Clinical management: antibiotic therapies

Clinical treatment of melioidosis has two main stages: an intravenous intensive treatment of acute phase, similar to that of any severe gam-negative septicemia, followed by an eradication phase. The common standard treatment requires 2-4 weeks of parental therapy, as initial intensive treatment, continued by 3-6 months of oral eradication therapy. As *B. pseudomallei* has been reported that it is intrinsically resistant to many broad-spectrum antibiotics, most of them are susceptible to newer beta-lactams, ceftazidime (CAZ), imipenem (IPM), meropenem (MRP), piperacillin (PIP), amoxycillin–clavulanate, ceftriaxone and cefotaxime. At the moment, the effective combination of antimicrobial agents used in the intravenous phase of melioidosis treatment is basic of the ceftazidime-based regimens (77-79). The treatment of severe melioidosis using ceftazidime as first-lined therapy shows a significantly lower rate of mortality (80) than those treated with other effective antibiotics, such as amoxicillin/clavulanate or cefoperazone/sulbactam (79, 81).

However, other antibiotics may be used as a second-line therapy in the areas that the ceftazidime-based options are unavailable or contraindicated (82, 83). The alternative antibiotics for the first-line therapy of ceftazidime are carbapenems, including imipenem and meropenem, due to their highly *in vitro* activities (84). The oral antibiotic therapy, a four–drug combination including trimethoprim, sulfamethoxazole, doxycycline, and chloramphenicol, usually takes three months for the minimum, though this is further lengthened in severe infections (85).

Nowadays, licensed vaccine for prevention of melioidosis is still unavailable. Development of the vaccine using much possible antigens is underway of animal model experiments. Therefore, the common precautions require instant cleaning of external wounds after contacting contaminated soil or standing water or avoid contact with those contaminated substances in endemic areas, especially in people with high risk, like a diabetic patient. People who have to work and have a chance in contact with contaminated matters can prevent themselves by using protective clothing such as rubber boots or gloves.

2.2.2 General antibiotic properties

2.2.2.1 β-lactam antibiotics

β-Lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam nucleus (Figure 2.4) in its molecular structure, including penicillin derivative, cephalosporins, monobactams, and carbapenems.

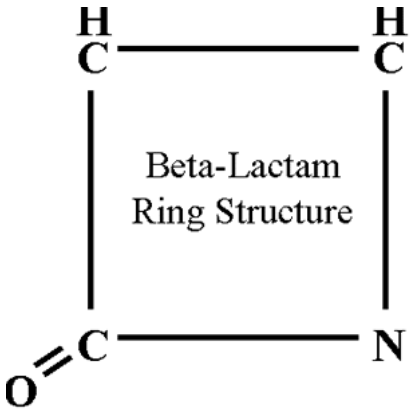

Figure 2.4 The β-lactam ring structure.

The main function of this antibiotic is through interfering with structural crosslinking of peptidoglycans in bacterial cell walls (86). The main β-Lactam antibiotics act by inhibiting synthesis of the peptidoglycan layer of bacterial cell walls and resulting in a weakened and self-degrading cell wall. The β-Lactam antibiotics display effective results with rare resistance, especially as a treatment. Nevertheless, there are 3 main modes of bacterial resistance to β-lactams: broad spectrum reduced sensitivity to β-Lactam; production of a specific ceftazidimase while retaining susceptibility to other third generation cephalosporins; and reduced β-lactamase inhibition by clavulanic acid (87). As it is a broad spectrum of antibiotics and have rare resistant, some of them are the backbone of melioidosis treatment, ceftazidime and carbapenem (meropenem).

2.2.2.2 Ceftazidime

Ceftazidime is a third-generation cephalosporin antibiotic with bactericidal activity and have more effects on gram-negative bacteria than gram-positive bacteria. Ceftazidime is chosen to be the initial treatment of meliodosis because of its high mortality benefits than other antibiotics. Resistance to ceftazidime treatment develops in fewer than 1% of treated cases (2). In Thai patients, ceftazidime treatment has been associated with a 50% lower overall mortality than 74% to 37% of conventional treatment (80). Due to *B. pseudomallei*'s survival ability within phagocytic cells, producing glycocalyx, and forming micro-colonies in the infected tissue, it can cause relapse in melioidosis treatment with ceftazidime and amoxicillin/clavulanic acid. The biofilm of the strain susceptible to ceftazidime also showed high resistance to ceftazidime (88). However, ceftazidime has more effectiveness on melioidosis when compared to the conventional treatment.

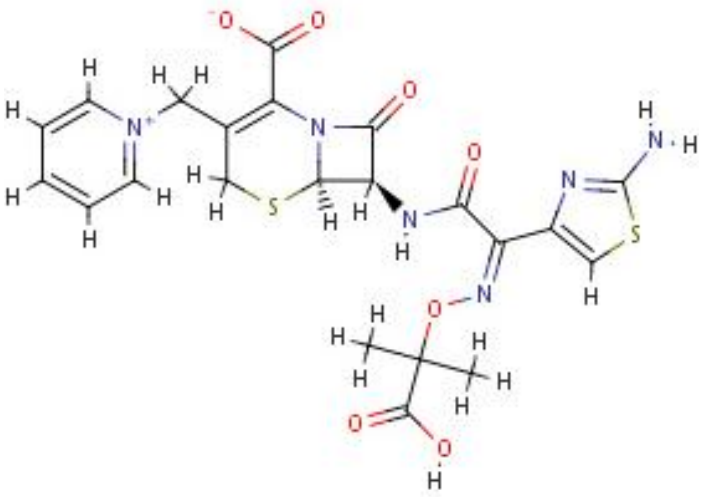

Figure 2.5 Chemical structure of ceftazidime (CAZ)

2.2.2.3 Meropenem

Meropenem, one of carbapenem subclass antibiotics, is an ultra-broad spectrum injectable antibiotic that acts against a variety of gram-positive aerobic, gram-negative aerobic and anaerobic bacteria including extended spectrum beta lactamase (ESBL) producers (89). It inhibits bacterial wall synthesis like other beta-lactam antibiotics but it is highly resistant to degradation by beta-lactamases. Evidences have been reported that meropenem is probably equivalent to imipenem and ceftazidime (35) and may be associated with improved outcomes in the treatment of melioidosis sepsis patients. However, meropenem has some theoretical benefits over ceftazidime in that they are more active in vitro demonstrate a post-antibiotic effect, and are associated with decreased endotoxin release (84). A case study of *B. pseudomallei*'s susceptibility to meropenem and ceftazidime between conventional and intracellular susceptibility test revealed that the susceptibility of *B. pseudomallei* culture collection was reduced to both agents in an intracellular susceptibility test method and also found that meropenem has a lower minimum inhibitory concentration that ceftazidime (90).

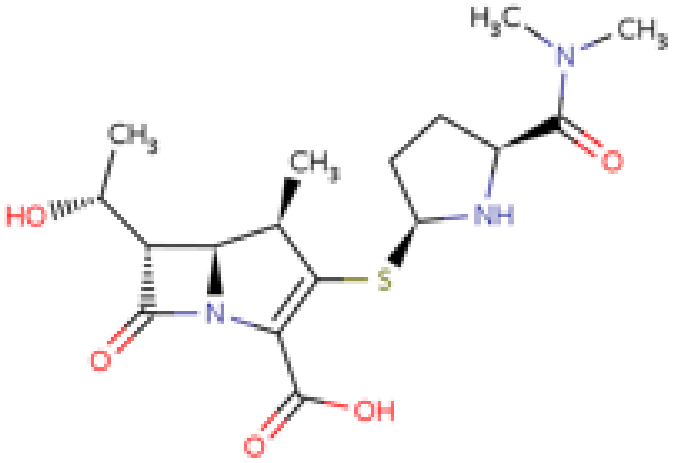

Figure 2.6 Chemical structure of meropenem (MRP)

2.3 Antibiotic resistance in *B. pseudomallei*

2.3.1 History of drug resistance in *B. pseudomallei*

The study of potential antimicrobial agents against *B. pseudomallei* has been examined for a long period of time. The initial *in vitro* susceptibility tests of this bacterium against various antimicrobial agents started in 1970 (91). Such studies have been continuously investigated, since Cheng and Currie (1) had reviewed and reported categories of intrinsically resistant and susceptible antibiotics to *B. pseudomallei.* It has been accepted that *B. pseudomallei* is intrinsically resistant to many antibiotics, including first and second generations of cephalosporins, penicillins, rifamycins, aminoglycosides, qinonolone, colistin, gentamicin and macrolides (10). The resistance of most antibiotics arise from production of chromosomal β-lactamases, the enzyme that hydrolyzed most cephalosporins but it can be inhibited by clavulanate (92, 93). Amoxicillin-clavulanate is an alternative antibiotic for the treatment of children, women and patients with intolerance to the first-line therapy. The most important β-lactamases enzyme is the Bush Jacoby-Medeiros class 2e beta-lactamase BPS-1, encoded by the gene *blaA* (94), which is one of the seven coding genes of Ambler class A, B, and D beta-lactamases found in *B. pseudomallei* (29). Bacteriostatic

antibiotics and the oral antibiotic trimethoprim/sulfamethoxazole (TMP-SMX), with or without doxycycline and chloramphenicol do not appear to be used in the intensive phase (1), even though TMP-SMX have been showed little activity in this phase (80). Acquired resistance to chloramphenicol, doxycycline and TMP-SMX has been observed when each of them was used as monotherapy (34, 35). Moreover, kanamycin also shows effective killing ability to *B. pseudomallei,* however, it is mostly used in research field but not in the clinical treatment (1). Another system conferring macrolide resistance is the efflux system AmrAB-OprA, revealing from transposon mutation analysis, yet it is found only in the high-level aminoglycoside treatment (95). However, the mechanisms of these resistances have not been clearly understood. Noticeably, the distinct phenotypic changes, including derepression of the chromosomal enzyme, an insensitivity to inhibition by beta-lactamase inhibitors, and a betalactamase specific for ceftazidime (87), are the major causes of acquired resistance of antibiotics, yet resistance to ceftazidime and carbapenems remains uncommon (35, 96). Therefore, at present ceftazidime and carbapenems are the chosen drugs for the first-line parental treatment.

2.3.2 Reoccurrence of the disease

B. pseudomallei is reported to cause very high relapse rate compared to other bacterial infection. Although patients have survived the initial intensive phase of the disease, it still has a high change of relapse. Relapse attributable to resistance may occur with either oral or intravenous agents used in the treatment (35). The most important determinants of relapse are choice of an appropriate regimen and duration of the therapy, for example, the patients who have long enough adherences (12-16 weeks) to a suitable therapy will result in 90% decrease risk of relapse (97). As *B. pseudomallei* has also been reported to develop the biofilm, many reports showed that biofilm-forming bacteria can be up to 1,000 times more resistant to antimicrobial agents than their planktonic counterpart (10, 16). In addition, the study of biofilm *B. pseudomallei* on a silastic surface using electron microscopy demonstrated that ceftazidime and TMP-SMX treatment for 24 hours cannot kill bacterial cells inside the biofilm (98). Thus, the relapse might be due to reactivation of the biofilm forming bacteria that made them resist to the antimicrobial agents.

2.4 Biofilm

Biofilms are three-dimensional structures attaching on a solid surface and occupied by communities of bacteria (Figure 2.7) (99). On the other hand; biofilm is a glycocalyx exopolysaccharide shield that contains communities of bacteria. Many bacteria can develop into sessile antimicrobial-agent-resistant biofilm communities such as *Pseudomonas aeruginosa, Porphyromonas gingivalis, Shigella, Salmonella spp, Bacillus cereus* and many strains of *Burkholderia spp* (100).

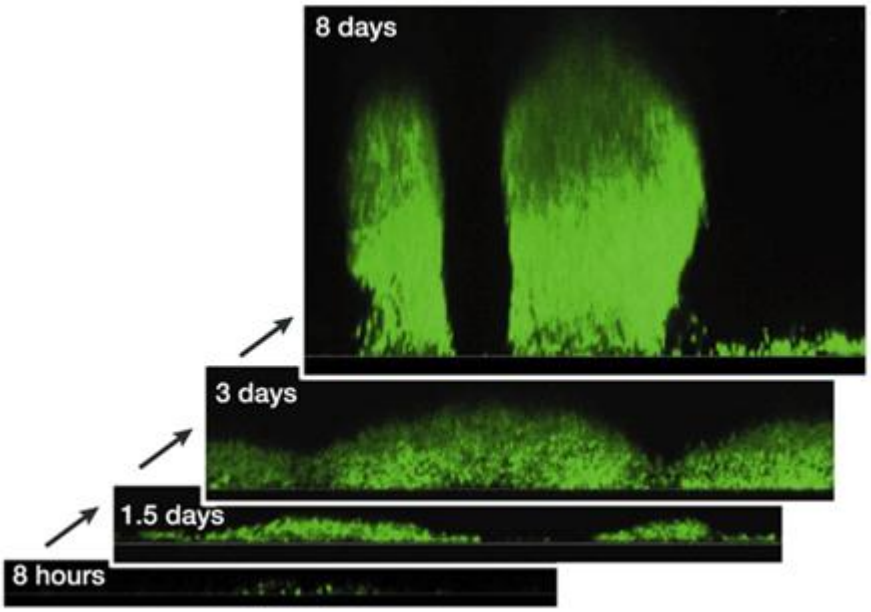

Figure 2.7 Confocal-microscope images of a *P. aeruginosa* biofilm developing over time on a microscope slide (100).

The main purpose of biofilm is to support the survival of the species encased within the exopolysaccharide matrix under variety of hostile conditions. In addition, the extracellular matrix produced by the biofilm-forming bacteria has been shown to interfere with uptake by immune cells. Interestingly, *B. pseudomallei* is one of special cases in this respect because some of its variants posses a true capsule which intervenes with the bacterial opsonization and their being phagocytosis (101). Moreover, the capsule facilitates formation of micro-colonies in which the organism is both protected from antibiotic penetration and phenotypical alteration, resulting in a

decrease in susceptibility (2). As a result, the biofilm has become an extremely important clinical problem since biofilm-forming bacteria exhibit very high resistance upon the exposure of antimicrobial agents and immune system of human hosts. It has been reported that the biofilm-forming bacteria can develop resistant to antibiotics 1,000 times higher than their free-living counterpart (16). Since they have stayed longer in a host body and caused chronic symptoms, these lead to the relapsed infection which is the reason why the incubation periods of melioidosis ranges from 2 days to 62 years. Another problem is that when bacteria stay inside the biofilm, they produce toxic substances that are harmful to the host (36).

2.4.1 Biofilms composition

Biofilm which is defined as community of microorganism those attached to surface and were embedded within the matrix then cause persistent and chronic bacterial infections. *B. pseudomallei* are able to produce extracellular matrix mainly consisting of the highly hydrated glycocalyx that enhances the formation of micro-colony, allows them to adhere nonspecifically to the available surface and resists to the defense system. This extracellular matrix production supports many functions, including formation and maintenance of micro-colony and biofilm structure, enhancement of the biofilm resistance to environmental stress and antimicrobial agents, protection of the bacteria from protozoan grazing, and biofilm nutrition (11). Extracellular polymeric substances (EPS) in biofilms are a variety of macromolecules including DNA, proteins, glycolipids and polysaccharides, the main structural component, though it composition still depends on the environmental conditions, the age of the biofilm, and the particular biofilm-forming strain. The biofilm exopolysaccharides can be presented in many forms, including cell-bound capsular polysaccharides (CPS), unbound “slime” or free EPS, and as O-antigen component of lipopolysaccharide (12). Distinguishing between these polysaccharide forms is often difficult due to transition from the cell-bound capsular polysaccharides to be the unbound “slime” or free EPS and the association between capsular polysaccharides and phospholipids. Cell-bound capsular polysaccharides or CPS are highly hydrated molecules that are often conjugated to the cell surface of the bacterium via covalent attachments to phospholipid, lipopolysaccharide or lipid-A molecules. *B. pseudomallei*

genome contains four operons that responsible for biosynthesis and exportation of the capsular polysaccharide cluster I-IV (CPSI-CPSIV), CPSI of which have an important role in virulence of the bacteria (13, 102). The slime polysaccharide or free EPS that unbound from the cell were studied by Denisov, II., which consisted of galactose, glucose, mannose, rhamnose and two unidentified carbohydrates (103). Moreover, a study of the exopolysaccharide contents in other *Burkholderia* species also showed similar components, such as *Burkholderia cepacia* which the exopolysaccharides were composed of galactose, rhamnose, mannose, glucose, and glucuronic acid (104). The lipopolysaccharide (LPS) of *B. pseudomallei* was structurally characterized and reported to contain two types of O-polysaccharide moieties termed type I O-PS and type II O-PS, which had been shown to be involved in serum resistance, however, there is no reports about the role and the responsible genes of the type I O-PS (105). The different forms of extracellular polysaccharides have different contributions for the survival of the species such as they may promote adherence of bacteria to both surfaces, biotic and abiotic, and other bacterial cells, which may facilitate colonization of a particular niche leading to the biofilm formation and the persistence of the organisms during colonization (13).

2.4.2 Biofilms formation steps

Biofilm formation processes are complex mechanisms that involved in many phenomenal including the phenotypic change in response to the proximity of a surface, the bacteria communication, the cell aggregation and micro-colony formation and the production of extracellular matrix (11). The initial step of biofilm formation is the reversible attachment of free floating bacteria to an appropriated surface after swimming, swarming or twitching which can be either biotic or abiotic surface. The planktonic cells with flagella differentiate into non-motile in the second stage followed by micro-colony formation using the type IV pili-mediated twitching motility (106). Quorum sensing system is used for recruitment between bacteria in order to aggregate together using quorum-sensing effecter molecules. Then, the bacteria accumulate forming-complex biofilm architecture by secretion of extracellular glycocylyx matrix or extracellular polymeric substances (EPS) to form mature three dimensional biofilm structures (14). This exopolysaccharide production is utilized in stabilization the

biofilm structure and become a macrocolony. During this process, the other attracted bacteria continuously come and secrete EPS to form the mature three dimensional biofilm structures. Finally, in the appropriated conditions the mature biofilm can burst and release those biofilm bacteria leading to new-site colonization (Figure 2.8) (107). Moreover, the size of mature biofilm also depends on cell division and recruitment allowing inside bacteria start differentiation. However, in most cases, the biofilm bacteria will be in stationary stage, stop metabolisms and stay inside the biofilm for long periods of time (100). Therefore, the biofilm formation mechanism consists of 5 main processes; 1) bacterial attachment 2) quorum sensing processes and micro-colony formation and 3) secretion of exopolysaccharide 4) maturation of macro-colony and 5) dispersion for new-site colonization.

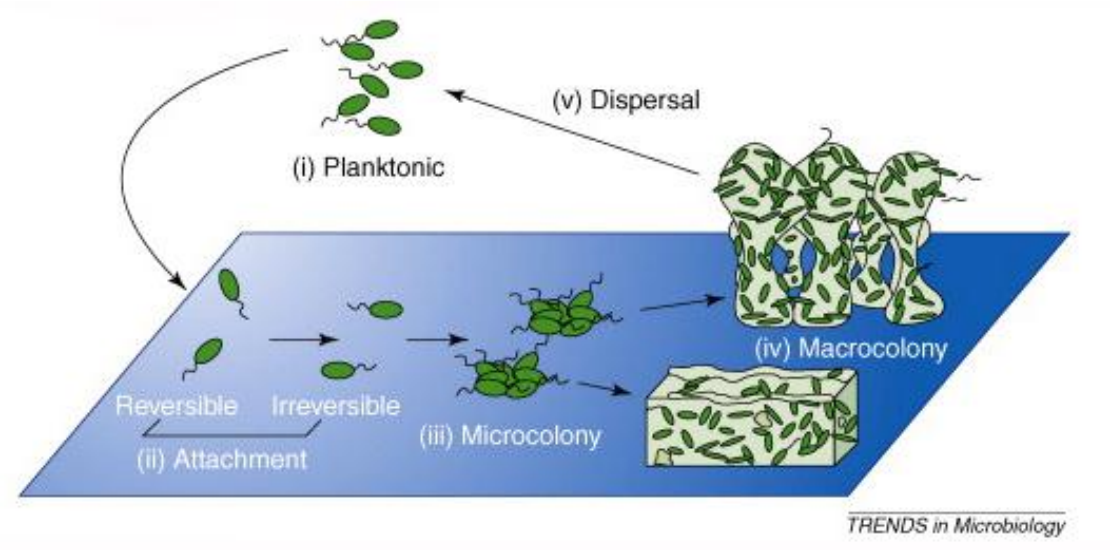

Figure 2.8 Five steps of biofilm development (107)

2.4.3 Molecular regulations of biofilm formation

The complex biofilm formation processes require many systems to control in each step. There are many reports showing genes that regulated the biofilm formation processes. Loprasert, S et al. have shown that *B. pseudomallei* lacking OxyR gene exhibited an increased ability to form biofilms in minimal medium, but not in Luria-Bertani broth suggesting that OxyR gene may play a role in the biofilm formation of *B. pseudomallei* (108). Furthermore, a study by Hwee Siang Lee et al.

showed that CdpA, a major c-di-GMP-specific phosphodiesterase involving in the regulation of intracellular c-di-GMP levels in *B. pseudomallei* KHW displayed a negative regulation on the biofilm formation. The *cdpA* mutant having high intracellular c-di-GMP levels produced more exopolysaccharide and exhibited significant increase in biofilm formation on an abiotic surface (109). The wzm gene encoding a capsular ABC transporter trans-membrane protein, which functions to transport capsular polysaccharide across cytoplasmic membrane, have some roles in the biofilm formation as the *B. pseudomallei* mutant lacking wzm produced less biofilm than the wild-type strain in the minimal medium (110). Moreover, the alternative sigma factor sigma (E) (RpoE) of *B. pseudomallei* which controls the adaptation gene expression to the stress response showed the positive regulation on the ability of biofilm formation in *B. pseudomallei* K96243 (111). Since there have been many reports in genetic regulations of the biofilm formation in *B. pseudomallei*, the authentic mechanisms of biofilm formation are still unrevealed.

2.5 *B. pseudomallei* mutant strains

As *B. pseudomallei* has also been reported to develop the biofilm, thorough studies of genes involving in the mechanisms of biofilm development of this organism may lead to a hope in production of an effective vaccine for the disease. These genes have many important roles in various systems of the cell. In our laboratory, there are 4 *B. pseudomallei* mutants that were differently mutated.

2.5.1 *B. pseudomallei bps*I mutant (constructed by Lumjiaktase, P., 2006)

This mutant lacks auto inducer synthase gene that important for synthesis of quorum-sensing signal molecules, like N-acyl-homoserine lactone (AHL). In quorum sensing system, there are two regulatory genes, autoinducer synthase (*luxI*) and transcriptional activator (*luxR*), which regulate five luciferase structural genes (*luxCDABE*). The LuxI protein synthesizes quorum-sensing signal molecules, which increase the autoinducer concentration in both intracellular and extracellular

compartments, when cell population density is high. At a critical autoinducer concentration, the autoinducer binds the LuxR protein. Then, the lux CDABE operon is activated through the binding of the LuxR-autoinducer complex on its promoter (*lux box*) (112). AHL-dependent quorum sensing systems regulate various functions including bioluminescence, plasmid conjugal transfer, production of virulence factors in pathogens, motility, antibiotic biosynthesis, and biofilm formation (18). Quorum-sensing signal molecules are needed in cell-cell communication in the micro-colony formation step that important for determinant of the ultimate three dimensional architecture of the mature biofilm. Therefore, this gene may have an important role in the second step of biofilm formation.

2.5.2 *B. pseudomallei ppk* mutant (constructed by Tunpiboonsak, S., 2005)

This mutant lacks polyphosphate kinase gene that encodes poly P kinase (PPK), a membrane associated enzyme or a principle enzyme converting ATP to poly P in many bacteria (113). Poly P is a chain of hundreds of phosphate residues linked by high-energy phosphoanhydride bonds (114), that have variety of biological functions depending on species, cells or sub cellular compartments of the host, such as substitution for ATP in kinase reaction, reservoir for Pi, chelation of metals etc. *Ppk1* only exists in prokaryote, not eukaryote, and is similar in many bacteria, including several pathogenic bacteria that cause severe disease in human (115). *PPK* gene is highly conserved among both gram positive and gram negative bacteria and it shows to play a significant role in adaptation to nutritional and environmental stresses, and stationary phase survival. Moreover, it plays an important role in virulence of many pathogenic bacteria, such as motility, quorum sensing, antibiotic resistance, toxic release, and biofilm formation (116). In biofilm formation processes, this gene important for the synthesis of inorganic polyphosphate for ATP could involve in an energy supplement for synthesis of the biofilm exopolysaccharide.

2.5.3 *B. pseudomallei rpo*N2 mutant (constructed by Tunpiboonsak, S., 2010)

The mutant lacks sigma factor 54 of RNA polymerase gene. Sigma factor 54 of RNA polymerase gene or *rpoN2* gene is important for nitrogen assimilation for amino acid metabolism. RpoN (σN) is a special alternative sigma factor due to its absolute requirement for an additional transcriptional activator to initiate RpoN-dependent gene transcription. In *Escherichia coli*, a lot of the RpoN-dependent genes are implicated in nitrogen assimilation and metabolism. In *Vibrio fischeri*, RpoN controls motility and biofilm formation and is also essential for establishing a symbiotic colonization of the host, and in *P. aeruginosa*, RpoN regulates synthesis of alginate, pili, and flagella, which are well-documented virulence factors and also involve in biofilm formation. There are a wide range of processes that are usually not essential for cell survival and growth under favorable conditions controlled by RpoN, including the regulation of genes involved in utilization of unusual carbon sources, flagella motility, O-antigen expression, alginate production, symbiotic colonization, biofilm formation, and the expression of other sigma factors (117).

2.5.4 *B. pseudomallei rpo*S mutant (constructed by Subsin, B., 2003)

This mutant lacks sigma factor 38 of RNA polymerase gene, alternative sigma factor that has an important role in the survival of the species under stress conditions. In gram-negative bacteria, this sigma factor activates expression of genes required in response to various stresses including acid, heat shock, UV light, osmotic, oxidative stresses, and carbon starvation also controls expression of extracellular virulence factors (19). Sigma S factor (σS) of *Escherichia coli* is regulated by transcription, translation and proteolysis, and varying stress conditions differentially affect these levels of control (118). In addition, the alternative sigma-factor RpoS of *P. aeruginosa* is a positive transcriptional regulator of *psl* gene expression, the major structural components of the *P. aeruginosa* biofilm matrix (119). Therefore, *rpo*S gene that has an important role in the stress conditions and stationary phase survival may some effects on the biofilm formation in *B. pseudomallei*.

CHAPTER III
HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

As mentioned earlier, there are many previous reports in other gram negative bacteria showing that the regulatory genes of interest have roles in biofilm antibiotic resistance. Three out of four *B. pseudomallei* mutant, including *bps*I, *ppk*, and *rpo*S, have primary found by Thanwatanaying, P. that lack of these genes particularly influent the ability of biofilm formation. In addition, we have further proven that flagella mediated motility are not essential in the initial attachment of biofilm formation. Sigma factor N (RpoN) is another important regulatory gene that has been reported the roles in nitrogen assimilation in many gram negative bacteria. Furthermore, there are many studies showing that *rpo*N involve in the biofilm formation of other gram negative bacteria, like *P. aeruginosa* and *E.* coli, etc. As *rpo*N2 mutant has recently been constructed in our laboratory, it is interesting to investigate the roles of this gene in the biofilm formation of *B. pseudomallei.* Thus, to confirm whether all of our four genes of interest really involve in the biofilm formation, more information of the biofilm characteristics of all mutant strains need to be clarify.

Huangterakul, C. had studied antibiotic susceptibility of all three *B. pseudomallei* mutants compared to the wild type in planktonic condition. It is clearly showed that the three *B. pseudomallei* mutants had similar drug susceptibility to their wild type. As biofilm forming-bacteria is well-known displayed higher drug resistance than their planktonic counterpart, therefore, it is possible that the different effects on biofilm formation by each mutated gene may have some impacts on the biofilm function as a defender of antibiotics. Extracellular polymeric substances (EPS) of the biofilm are suggested as a major barrier in protection of the bacteria from the hostile environments and contain exopolysaccharides as the principal structural component. For that reason, the components of biofilm exopolysaccharides of the mutants may be

altered from the lack of the mutated genes and may further be the cause of biofilm dysfunction in antibiotic resistance.

We hypothesize that all of the genes of interest; *bps*I, *ppk, rpo*N2 and *rpo*S, should regulate the biofilm formation in *B. pseudomallei.* The lack of these four genes may cause an ineffective biofilm formation which further affects the ability of drug resistance. Moreover, the biofilm resistance mechanism may depend on the matrix that acts as a diffusion barrier for the antibiotic penetration which should be under regulation of the regulatory genes. Finally, from the knowledge of resistant mechanism by the biofilm via these four genes of interest, we may be able to propose the possible biofilm exopolysaccharide biosynthesis pathway of *B. pseudomallei* which may involve with antibiotic resistance in the biofilm of *B. pseudomallei.*

3.2 Objectives

- To compare the amount and density of biofilm formation among *Burkholderia pseudomallei* wild type, *Burkholderia pseudomallei bps*I *mutant, Burkholderia pseudomallei ppk mutant, Burkholderia pseudomallei rpo*N2 *mutant* and *Burkholderia pseudomallei rpo*S *mutant* using two different medium types; Luria-Bertani (LB) and Modified Vogel and Bonner's medium (MVBM)
- To compare the biofilm architecture, thickness and micro-colony formation among *B. pseudomallei* wild type and its mutants
- To test antibiotic susceptibility among *B. pseudomallei* wild type and its mutants growing to form biofilm
- To identify carbohydrate compositions of exopolysaccharide in biofilm between *B. pseudomallei* wild type and its mutants.
- To verify possible correlation between antibiotic resistance and exopolysaccharide composition in *B. pseudomallei* biofilms which may lead to possible mechanism of biofilm formation and try to determine the important of each genes in biofilm formation processes.

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacteria strains and plasmids

Bacterial strains used in this study are listed in Table 4.1. PP844 is a clinical isolate from the blood of a melioidosis patient. The three *B. pseudomallei* mutant strains lacking the *bps*I, *ppk*, *rpo*N2 and *rpo*S genes were previously constructed in our laboratory.

Table 4.1 Bacterial strains and plasmids

Strains	Genotype or relevant characteristic	References
B. pseudomallei		
PP844 (BpWT)	Prototroph, blood culture isolate from a patient at Khon kaen University Hospital	(120, 121)
*bps*IM	PP844 *bps*I mutant	(18)
*ppk*M	NF10/38 *ppk* mutant	(20)
*rpo*N2M	PP844 *rpo*N2 mutant	(unpublished data)
*rpo*SM	PP844 *rpo*S mutant	(19)
Plasmids		
pKBI	pKNOCK-Tc containing a 298 bp internal segment of *B. pseudomallei* bpsI gene	(18)
pKPPK	pKNOCK-Tc containing a 500-bp internal segment of *B. pseudomallei* ppk gene	(20)
pKRpoN2	pKNOCK-Tc containing a 363-bp internal segment of *B. pseudomallei rpo*N2 gene	(constructed by Tunpiboonsak, S., 2005)
pKBS1	pKNOCK-Tc containing a 600-bp internal segment of *B. pseudomallei* rpoS gene	(19)

4.1.2 Antibiotics in resistance analysis

Ceftazidime (CAZ) and meropenem (MRP) were chosen to use in antibiotic susceptibility test because these drugs are currently used in the initial intensive treatment of melioidosis and still have rare resistances to *B. pseudomallei.* In term of antibiotic structures, these two drugs are represented for large and small antibiotics, respectively, to investigate for the effect of drug diffusion through biofilm matrix. All antimicrobial agent powders were prepared as stock solution (10 mg/ml) (see Appendix B) and store at -20°C. The serial dilution of the antibiotics from 1024 µg/ml to 1 µg/ml was done with Mueller Hinton Broth (MHB), an enrich media used for recovering antibiotic-challenged bacteria after the challenging step.

4.1.3 Chemical reagents for biofilm staining and visualization

B. pseudomallei biofilm were stained by crystal violet in colorimetric measurement and visualized using CLSM

- 1% crystal violet (CV) (see appendix B)
- 95% ethanol
- 0.15 M phosphate-buffer saline (PBS pH 7.0) (see appendix B)
- 0.9% NaCl (Normal Saline) (see appendix B)
- 2.5% glutaraldehyde in PBS pH 7.0 (see appendix B)
- 50 µg/ml FITC-con A (see appendix B)
- 8 µM PI in PBS pH 7.0 (see appendix B)
- Prolong Gold Antifade reagent

4.1.4 Chemical reagents for exopolysacchride isolation and GC-MS

- 1 M NaOH (see appendix B)
- concentrated HCl
- 70% ethanol
- 10 mM Sodium bicarbonate and Na_2EDTA (see appendix B)
- 1 M metanolic HCl (see appendix B)
- Tri-Sil reagent; HMDS-TMCS in pyridine
- Hexane

4.2 Methods

4.2.1 Bacterial growth conditions

B. pseudomallei were grown on Luria-Bertani (LB) agar and subcultured into LB broth (see appendix B) contained appropriated drugs. For *B. pseudomallei* mutant strains, tetracycline was added to the media from 5 mg/ml stock solution to final concentration 60 μg/ml (see appendix B). All bacteria were inoculated into appropriated-biofilm-induced media and further statically cultured at 37°C for 24 hours. The other media used in this study are Modified Vogel and Bonner's medium (MVBM) and Mueller Hinton Broth (MHB), a chemically defined medium used to facilitate the formation of biofilm (10) (see appendix B).

4.2.2 Colorimetric measurement of biofilm formation

The extent of biofilm formation was assayed by staining with crystal violet as previously described by O'Toole *et al* (122) with slightly modification. A conventional biofilm formation assay normally used LB as a cultured medium; however, there is another medium that is more specific to the biofilm formation; Modified Vogel and Bonner's medium or MVBM. This media is composed of suitable chemical components for an induction of exopolysaccharide-enclosed micro-colonies at the bacterial cell surfaced which mimic a similar morphology of *P. aeruginosa* infected-bronchial mucus of a CF patient (123). It is interesting to investigate whether two different media cause distinct effects on biofilm formation in *B. pseudomallei.* Thus, the biofilm assay of all *B. pseudomallei* strains were tested in both LB and MVBM. Briefly, a single colony of bacteria cultured on a appropriated LB agar plate was inoculated in 3 ml of LB broth and grown overnight at 37ºC in a 200 rpm shaker incubator (Labcon, Laboratory Marketing Services, Johannesburg, South Africa) as inoculum. The preculture bacteria were subcultured with 0.1% inoculum (v/v) into 10 ml of matching LB broth and MVBM and further incubated at 37ºC until OD_{600} = 0.3 (around 10^8 CFU/ml). Two hundred microliter of 10^8 CFU/ml were transferred to 96 well polystyrene plates (Corning®, NY, USA) with three replicated wells and statically incubated at 37ºC for 24 hours for biofilm formation. Sterile LB broth and MVBM were adjacently added to be negative controls of the experiment. Thereafter,

the supernatant fluid of each plate was aspirated gently to remove non-adherent bacteria. The surface-attached cells were stained with 125 µl of 1% CV for 15 min. The excessed-unbounded crystal violet and unattached cells were gently washed three times with sterile water. The plates were dried and the attached dye in each plate was solubilized by addition 200 µl of 95% ethanol into each well. The biofilm cell-eluted dye was diluted and measured at 540 nm using a microplate reader (Thermo Electron Corporation, MA, USA). Two independent experiments were performed and statistically tested by One-Way ANOVA with the Fisher LSD method at alpha 0.05.

4.2.3 Biofilm Visualization using confocal laser scanning microscopy (CLSM)

A single colony of cultured bacteria was inoculated in 5 ml of MVBM and grown overnight at 37ºC in a 200 rpm shaker incubator (Labcon, Laboratory Marketing Services, Johannesburg, South Africa). The overnight bacterial suspension was measured OD_{600} and diluted into 0.1-0.15 (10^7 CFU/ml). These diluted suspension was further 10-fold diluted and 1 ml of this final dilution was loaded into 24-well microtiter plate having a plastic cover slip underneath and statically incubated at 37ºC for 24 hours. The visualization of biofilm forming bacteria using CLSM was carried out as previously described (20) with slight modifications. Briefly, surface-attached cells were washed two times with 0.15 M PBS pH 7.0 and fixed by 2.5% v/v glutaraldehyde in PBS pH 7.0 at 4˚C for 3 hours. After three washes with PBS, the fixed- died bacterial biofilm was stained with fluorescein isothiocyanate-concanavalin A in distilled water (FITC-ConA; 50µg/ml), which reacts to exopolysaccharide of the biofilm, at room temperature for 15 min. After three times washing, , the bacterial biofilm was stained with propidium iodide (PI) in distilled water 8 µM at room temperature for 45 min. FITC-ConA fluorescein contains a lectin-chain (concanavalin A) that will conjugate to the exopolysaccharide matrixes secreted by the bacteria while PI penetrates only cells with damaged membranes and binds to DNA.

Cells were mounted with 5 µl of Prolong Gold Antifade reagent (Invitrogen, Carlsbad, CA 92008, USA) and visualized, using a FluoView FV10i-DOC confocal microscope (OLYMPUS AMERICA, Melville, NY, USA). Fluorescent probes were excited as follows: FITC-ConA at 488 nm and PI at 535 nm. Two

independent experiments were performed and each *B. pseudomallei* strain was captured with five different fields. All confocal images were digitized and analyzed with FV10-ASW 3.0 Viewer software to obtain biofilm thickness, biofilm integrated fluorescent intensity, bacterial density, and other parameters. Biofilm thickness and exopolysaccharide integrated fluorescent intensity were statistically transformed into logarithm and tested by One-Way ANOVA. All pairwise was tested further by the Fisher LSD method at alpha 0.05.

4.2.4 Antibiotics resistance analysis for planktonic and biofilm conditions using the Calgary Biofilm Device

4.2.4.1 The Calgary Biofilm Device (CBD)

The CDB is available through MBEC Biofilms technology Ltd., Calgary, Alberta, Canada. Antibiotics susceptibility test between planktonic and biofilm promoting conditions within the same strain was performed as described by Ceri *et al.* (124) with slight modifications (10). The CDB (MBEC™ device) is a 96-well microtiter plate with a lid containing 96 pegs sealed on the top. The pegs are designed to sit in the channels of a standard 96-well microtiter plate and channel the flow of medium across the pegs to create consistent shear force in each well, resulting in the formation of equivalent biofilms at each peg site. This phenomenon allows the surfaced-biofilm peg to be exposed to a new antibiotics plate. The planktonic bacteria can shed from the biofilm on the pegs into each well of the challenge plate and serve as the planktonic inoculums for the lowest concentration of antimicrobial agent in which the biofilm-shed planktonic bacterial population could not be established. After antibiotic treatment, the biofilms on the pegs will be removed into each well by sonication and recovered with fresh MHB medium. This recovery will be measured for the minimal biofilm eradication concentration values. The survival planktonic and biofilm bacteria reported as bacterial inhibitions to calculate the half maximal inhibitory concentration or IC50 for comparison between the two conditions.

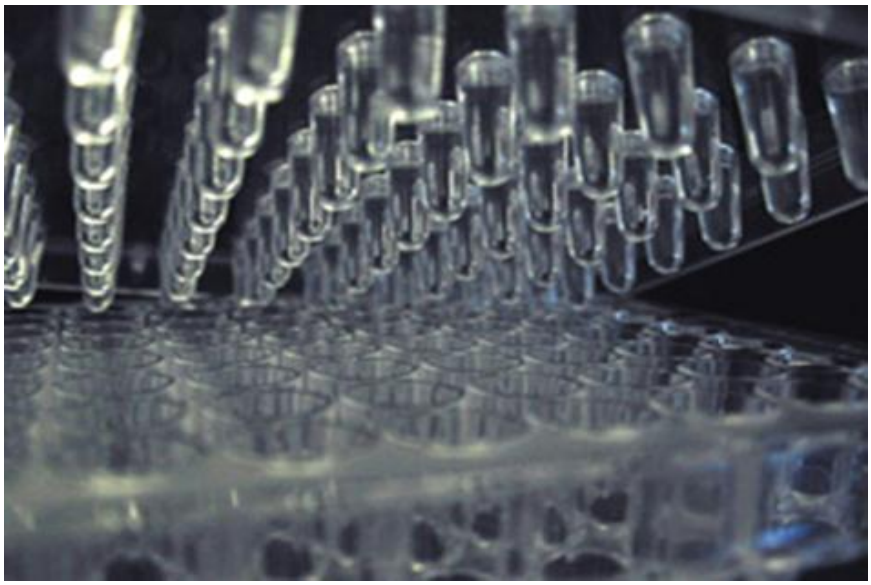

Figure 4.1 The CBD™ device

4.2.4.2 Antibiotic resistance analysis

Bacterial biofilms were formed on each peg by culturing cells in MVBM with specific conditions at an initial bacterial concentration of 10^7 CFU/ml. The final volume (150 µl) of each bacterial suspension was placed in the 96-well microtiter plate. Rows of the plate were arranged with three replicates for each strain, with one row containing only media to serve as a negative control. The plates were incubated with shaking at 100 rpm at 37ºC, for 24 hours. Ceftazidime and meropenem were separately serial diluted in MHB medium in a new 96-well microtiter plate from 1024 µg/ml to 1 µg/ml with the final test volume of 200 µl per well. Antibiotic-free MHB medium were added into one column to serve as the growth control. Biofilm-bacteria present in the biofilm-lid were challenged with ceftazidime and meropenem at 37ºC, 24 hours. Following the challenge step, the peg lid was washed using 0.9% NaCl to remove any residual antimicrobial agents. The turbidity of the challenge plates was checked by using the microtiter plate reader (Thermo Electron Corporation, MA, USA) at 620 nm to determine survival of planktonic bacteria. The peg lids were then placed over a new 96-well microtiter plate containing fresh MHB medium. The peg-attaching biofilm was removed by sonication for 5 minutes. After incubation for an additional 24 hours at 37ºC, the presence of viable bacterial biofilm was determined by monitoring turbidity at 620 nm.

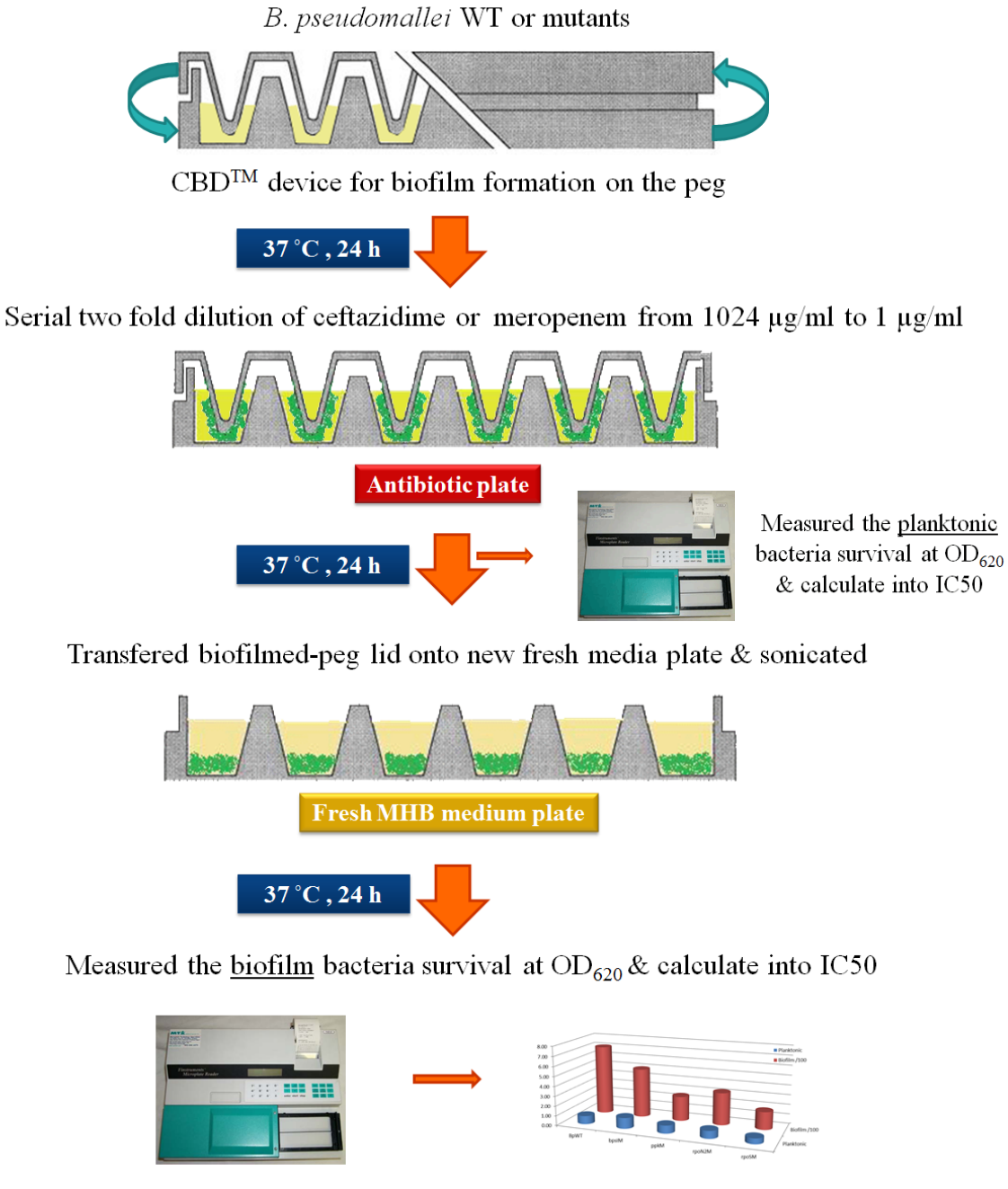

Figure 4.2 Flow chart of an antibiotic susceptibility test

4.2.5 Isolation of biofilm extracellular polysaccharide using ethanol-precipitation

Overnight bacterial cultures were started with 0.5% inoculums into 300 ml cultures of MVBM with specific conditions in each 500 ml flask. The bacteria were statically cultured to facilitate biofilm formation at 37 ºC for 4-5 days; BpWT and *bps*IM require 5-days incubation while *ppk*M, *rpo*N2M, and *rpo*SM demand 4 days. The biofilm-pellicle was gathered from the top of the culture using a 10-ml pipette with final medium volume less than 5 ml. Extracellular matrix isolation was carried out as previously described with slight modification (125). Briefly, the collected biofilm-pellicle was washed with 10 ml of sterile distilled H_2O and further treated with 25 ml of 1 M NaOH. The mixture was vortexed for 30 min to dissolve the polysaccharide mixtures in the pellicle. The treated pellicle was transferred into ultra-centrifuge tubes and further centrifuged at 200,000 g in a SW70 Ti rotor of Optima LE80K ultracentrifuge (Beckman Coulter, CA, USA) at 4°C for 1 h. The supernatant was collected and filtered through a 0.22 μm syringe filter. Then, the NaOH-treated supernatant was neutralized with 30 ml of concentrated HCl, followed by precipitation with ethanol which added into 70% final concentration and finally placed at -20°C overnight. The precipitant was collected by centrifugation at 15,000 rpm in a Sorvall SLA-1000TC rotor of high-speed Refrigerated Centrifuge (Sorvall® RC-5C plus) (Thermo Fisher Scientific, MA, US) for 45 min at 4°C. The washing step was done by the addition of 70% ethanol followed by air drying for 1 hour. After the pellet was dried, they were resuspended in 5 ml of distilled water and lyophilized by using FreezeDry Supermodulyo-230 (Thermo Scientific, MA, US) overnight. The lyophilized products were resuspended again in 5 ml of distilled water and then transfer into dialysis tubes and dialyzed three times against 4 liters of distilled water to liberate salt and detergents. After dialysis, the isolated exopolysaccharides of extracellular matrix were lyophilized again to calculate for total weight of dry exopolysaccharide in each *B. pseudomallei* strain before carbohydrate analysis.

4.2.6 Exopolysaccharide composition analysis

Glycoconjugate composition analysis of exopolysaccharide in extracellular matrix extracted from *B. pseudomallei* was performed using gas chromatography-mass spectrometry (GC-MS). Trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides were generated from the glycoconjugates by acid methanolysis prior to analysis as previously described by Merkle & Poppe *et al.* with slight modification (125, 126). The exopolysaccharide samples, containing 0.5 mg of exopolysaccharide, from each *B. pseudomallei* strain were drying in 13 x 100 mm Teflon-Lined screw caps. Standard Mixtures of monosaccharaides expected to be in the exopolysaccharide were concurrently prepared with an interested sample. Before drying by lyophilization, 20 µg of *myo*-inositol as an internal standard is added into each sample and the standard mixture. The standard mixture was actualized through the whole procedure in parallel with all samples to be analyzed. After lyophilization, the dried sample and standard mixture were added with 500 µl of 1 M metanolic HCl for methanolysis at 85°C for 20 hours in heating block (Thermo Scientific, MA, US). Each of the sample caps must be tightly sealed due to the leakage of methanol or introduction of water into the vessels can lead to incompleteness of the hydrolysis reactions. After complete methanolysis, the methanolic HCl was evaporated at 45°C under a hot air by using Speed Vacuum Concentrator (Thermo Electron Corporation, MA, US) to remove the exceeded methanolic HCl. The methanolyzed samples were washed with 250 µl of methanol, followed by the evaporation at 45°C until the samples were completely dried. The washing and evaporating steps were repeated for one more time. The samples were then N-acetylated by the addition of 40 µl of pyridine and 40 µl of acetic anhydride in 200 µl of methanol and incubated at room temperature overnight to re-acetylated N-acetyl group of amino sugars. The remaining solvent and excess acetylating reagents of N-acetylation were evaporated under air at 45°C. To silylate the methylglycosides generated from methanolysis, 180 µl of the silylating reagent, Tri-Sil® reagent composed of hexamethyldisilazane (HMDS) and trimrthylchlorosilane (TMCS) in pyridine solvent (Pierce, Rockford, IL), was added to each of the dried samples. The volume of Tri-Sil reagent used in each sample must over exceed to the amount of the dried samples in order to the completeness of the silylation. All sample tubes sealed with the screw cap were incubated at 85°C for 30

min. The excess Tri-Sil reagent was evaporated under air at room temperature, which should be carried out with a limited time because they are volatile after the silylation. After evaporation, TMS methylglycosides were rinsed with 1 ml of hexane by vortexing to dissolve the methylglycosides from the pellet, followed by centrifugation at 1,000 g for 3 min at room temperature to alleviate settling of insoluble salts. The supernatant hexane was transferred to 1.5 ml fresh vial glass tubes and evaporated under air at room temperature just until dry. The TMS methylglycosides were ready for GC-MS analysis and then were resuspended with 100 μl of hexane. One micro litter of TMS methylglycosides in hexane was automatically injected (split at 1:50) onto a Agilent Hewlett Packard Gas Chromatograph with auto sampler interfaced with a 5973N MSD (Agilent Technologies, CA, US) using a HP-1 fused silica capillary column (25m x 0.32 mm x 0.17 μm). Identification of the monosaccharide components was based on comparison of the retention times with those of the standard monosaccharides mixture (126). All samples were analyzed in triplicated independent experiments.

4.2.7 Quantitation of Monosaccharide components of analyzed samples

Depending on the nature of the glycoconjugate being analyzed, the standard monosaccharides derivatized simultaneously with the sample can include arabinose, ribose, rhamnose, fucose, xylose, mannose, glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, N- acetylneuraminic acid, galacturonic acid, glucorunic acid, and glucoheptose (126). According to previous studies, Denisov *et al.* has shown components of slime polysaccharide of *B. pseudomallei* unbounded from the cell, which composed of galactose, glucose, mannose, rhamnose and two unidentified carbohydrates. Moreover, CPS III capsule and capsule CP-2 also showed the similar polysaccharide component even the two unidentified carbohydrates are different, which found to be xylose and uronic acid respectively (102, 103). As there are other monosaccharide types available in our laboratory, therefore, the standard monosaccharide mixture relevant to the particular glycoconjugate which were chosen to be analyzed composed of rhamnose, xylose, mannose, glucose, and galactose, glucoronic acid. All of the standard (STD) sugars were prepared as a stock solution at

a concentration of 1 mg/ml in water (see appendix B) except for glucuronic acid that is not available in our laboratory. 1-ml aliquots of each of the chosen sugars are combined in a single tube. Fivefold less *myo*-insitol (200 µl) is added to the mixture. The standard mixture may be frozen. When ready to analyze a sample, 500 µl of the standard mixture is lyophilized, and subjected to parallel derivatization with the sample. The amount (milligrams) of each sugar in the standard mixture is a function of the number of sugars that were mixed together. A standard mixture always be prepared and derivatized in parallel with the samples being analyzed.

Detector response factor (dRF) (the ratio of the peak area of the internal standard to that of each standard sugar) are determined for each standard and are used to calculate the amount of each component of the sample being analyzed. The response factor for each component of the standard mixture is calculated according to the following formula, using mannose as an example.

Response factor

$$= \frac{\text{total peak area for STD (Man) / peak area of internal STD (Myo-inosital)}}{\text{weight of STD (Man) (mg) / weight of internal STD (mg)}}$$

After a peak is identified in the analyzed sample ("unknown") as mannose by comparison of the retention time with those of known standards, it can then be quantified by weight, using the response factor calculated above:

Weight of (mannose) in unknown (mg)

$$= \frac{\text{peak area of (Man) in unknown x weight of } \textit{myo}\text{-inosital in unknown (mg)}}{\text{peak area of } \textit{myo}\text{-inosital in unknown x } \mathbf{\text{Response factor of (Mannose)}}}$$

Due to the distinct capacity of biofilm formation in each *B. pseudomallei* strain, the amount of each monosaccharide in the exopolysaccharides formed in the purified carbohydrate may not correctly represent the effects of mutated genes on the biofilm production capacity. Therefore, the biofilm capacity factors in each strain were calculated from the exopolysaccharide integrated fluorescent intensity according to the

formula, for which wild type strain was designed as the positive control and was used to subtract all of the carbohydrate contents.

Biofilm capacity factors

$$= \frac{\text{exopolysaccharide integrated fluorescent intensity of the mutant}}{\text{exopolysaccharide integrated fluorescent intensity of the wild type}}$$

4.2.8 Statistical tests

P-values of the biofilm thickness, exopolysaccharide integrated fluorescent intensity, IC_{50} values of bacterial inhibition and the carbohydrate contents among *B. pseudomallei* strain were statistically tested by one-way ANOVA followed by the Fisher LSD method at an alpha level of 0.05, using SigmaPlot version 11.0.

CHAPTER V
RESULTS

5.1 Characteristics of biofilm formation in *B. pseudomallei* mutants

5.1.1 Biofilm density assay by crystal violet (CV) staining

To determine the differences between the *bps*I, *ppk*, *rpo*N2, and *rpo*S *B. pseudomallei* mutant strains in terms of biofilm density and thickness was monitored. Biofilm characters were investigated in both LB and MVBM by a colorimetric assay with crystal violet staining and confocal laser scanning microscopy (CLSM). After surface-attached cells were stained with 1% CV, the biofilm-forming cells were solubilized by addition of 95% ethanol. The solubilized-dye was further diluted with sterile water and measured at a wavelength of 540 nm (Table 5.1). The final concentration of biofilm formation of each *B. pseudomallei* strain was normalized with an 95% ethanol blank, calculated with specific dilution factors and averaged as shown in Table 5.2 and was summarized into Figure 5.1.

Table 5.1 The biofilm-associated dye and its dilution factors

Bp Strains	Net OD_{540} of biofilm associated dye							
	LB			Dilution factors	MVBM			Dilution factors
WT	0.323	0.280	0.235	7	0.157	0.126	0.114	-
*bps*I	0.160	0.163	0.171	3	0.427	0.803	0.429	2
ppk	0.105	0.163	0.179	3	0.169	0.180	0.163	-
*rpo*N2	0.142	0.147	0.178	3	0.259	0.230	0.231	2
*rpo*S	0.090	0.183	0.134	3	0.104	0.091	0.088	-

Table 5.2 The final biofilm concentrations and % relative biofilm concentration in all *B. pseudomallei* strains

Bp strains	Final average OD_{540}		% relative biofilm conc. (LB)	% relative biofilm conc. (MVBM)
	LB	MVBM		
WT	1.6366	0.0846	100	100
*bps*I	0.3554	1.0136	21	1198
ppk	0.3084	0.1230	19	145
*rpo*N2	0.3284	0.3876	20	458
*rpo*S	0.2684	0.0466	16	55

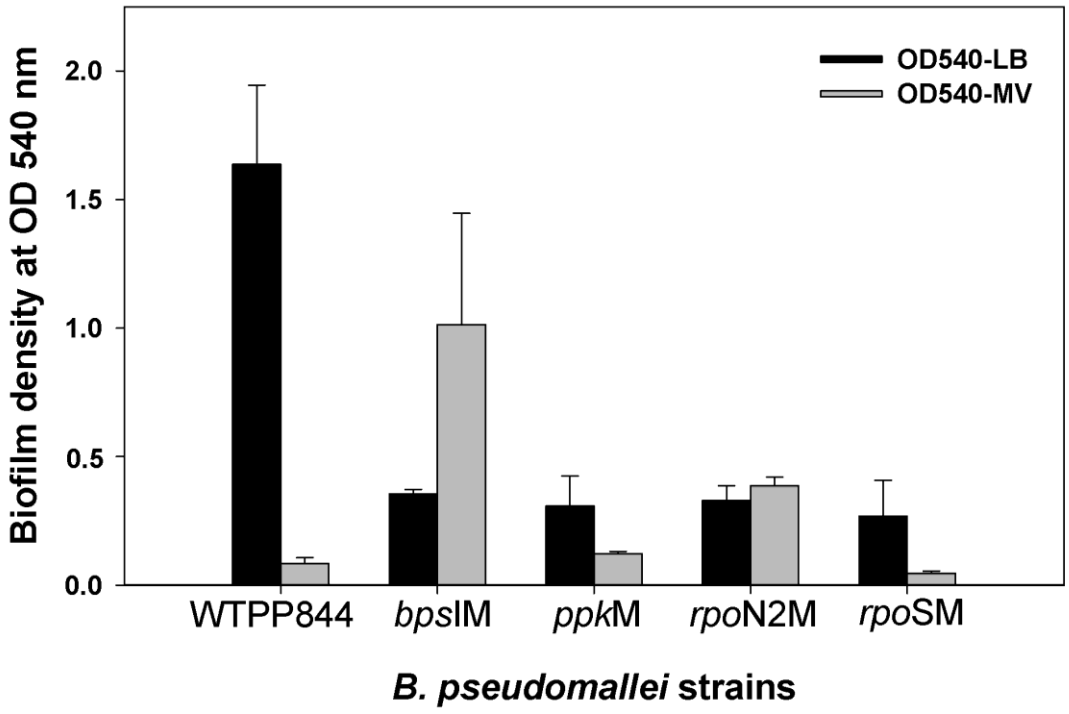

Figure 5.1 Comparison of the final biofilm concentrations in each *B. pseudomallei* strain between two media

In LB medium, each of the *B. pseudomallei* mutant strains significantly exhibited defects in biofilm formation with similar density at around 19% when compared to the wild type. Inconceivably, biofilm formation of all strains in MVBM medium turned into asynchronous results. The *bps*I mutant became the highest biofilm forming strain while the biofilm-forming ability of the wild type was similar to the *rpo*S mutant. This suggests that each media has specific effect on biofilm induction in *B. pseudomallei*. As the results from two media become contrary to each other which may result from each different induction effect on biofilm formation, thus this method may not provide precise information of biofilm formation ability by the bacteria. Therefore, confocal laser scanning microscopy or CLSM was chosen to study more details of biofilm characters.

5.1.2 Visualization of biofilm structure using confocal laser scanning microscope (CLSM)

To investigate characteristic of biofilm formation among *bps*I, *ppk*, *rpo*N2 and *rpo*S *B. pseudomallei* mutant strains in more details, CLSM was used to visualize biofilm architecture, micro-colony formation, thickness and exopolysaccharide production in each of the *B. pseudomallei* strains between LB and MVBM media. It is apparent that all *B. pseudomallei* mutants had distinct defectiveness on an ability of biofilm formation in both media. The fixed-death bacteria were stained with FITC-ConA fluorescein which has lectin-chain of isothiocyanate-concanavalinA that will be conjugate to exopolysaccharide matrix of the bacteria. After washing of exceed FITC-conA, the bacteria were continuously stained with Propidium iodide (PI) that will penetrate into the death cells and bind to bacterial DNA. Five different fields for each *B. pseudomallei* strain were captured in Z-axis direction and used for determination of biofilm thickness and exopolysaccharide integrated fluorescent intensity.

FITC-ConA was excited with blue laser at wavelength 490 nm and emitted green laser at wavelength 525 nm and PI was excited with dark red laser at wavelength 535 nm and emitted the light red laser at wavelength 617 nm (Figure 5.2). Therefore, the exopolysaccharide matrix was represented with green color (Figure 5.2A) and the bacterial cells were shown in red color (Figure 5.2B). CLSM can present a photo merging between each fluorescein dye thus yellow color was representation of the

bacteria cells having their exopolysaccharide matrix around them (Figure 5.2D). The light microscope image of the bacteria was also shown in Figure 5.2C for comparison with the fluorescent images.

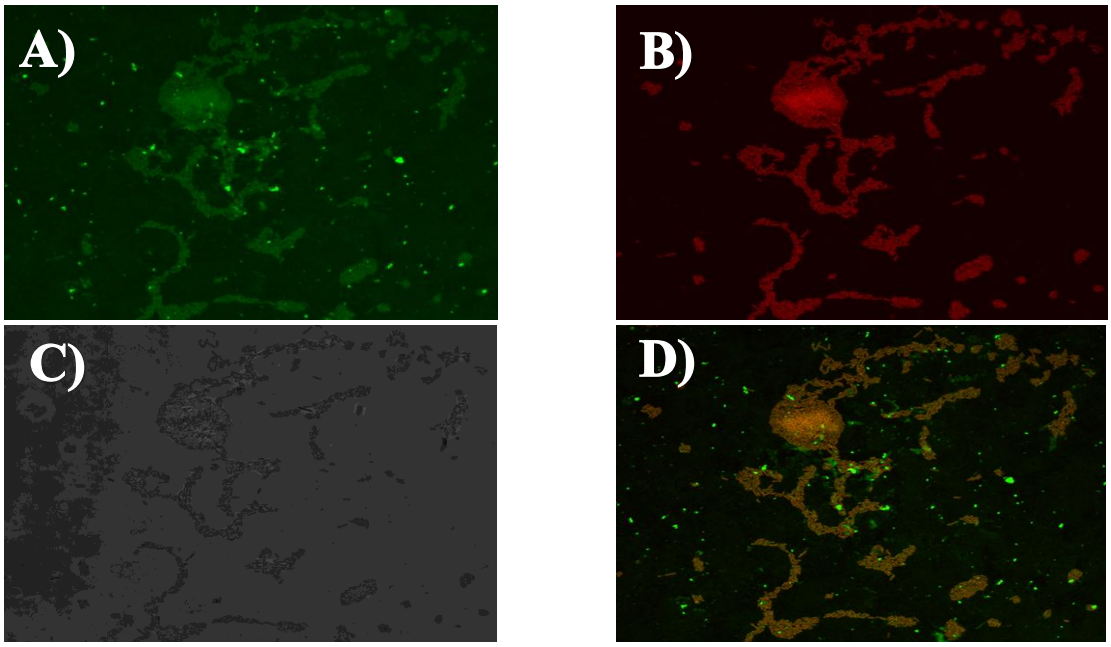

Figure 5.2 Representation of *B. pseudomallei* biofilm by CLSM. The CLSM images of *B. pseudomallei* wild type which also represent micro-colony formation. A) Exopolysacchride matrix was stained with FITC-ConA. B) Bacteria cells which their DNA were stained by PI. C) Bacteria cells from light microscope and D) The merged image between A and B; yellow color represents for bacteria cells having exopolysaccharide matrix.

The CLSM images have elucidated micro-colony formation and thickness of *B. pseudomallei* in Figure 5.3. Five fields for each *B. pseudomallei* strain were captured and biofilm thickness was calculated from the number of pictures taken in every 1 µm form the top along the depth (Z-axis). It is obvious that micro-colonies of *B. pseudomallei* wild type was formed in both LB and MVBM (Figure 5.3A), which is the overlapping of a group of bacterial cells, scattered throughout the slide. Apparently, MVBM can induce micro-colonies formation more than LB media, in terms of size and thickness. Micro-colony thickness of the wild type biofilm in LB

was illustrated at 8.17 ± 1.47 µm which is about two-fold less than the thickness in MVBM (18.77 ± 3.67 µm). Moreover, MVBM can obviously induce more bacterial attachment and spreading onto the surface which should be suitable for the initial step of biofilm formation.

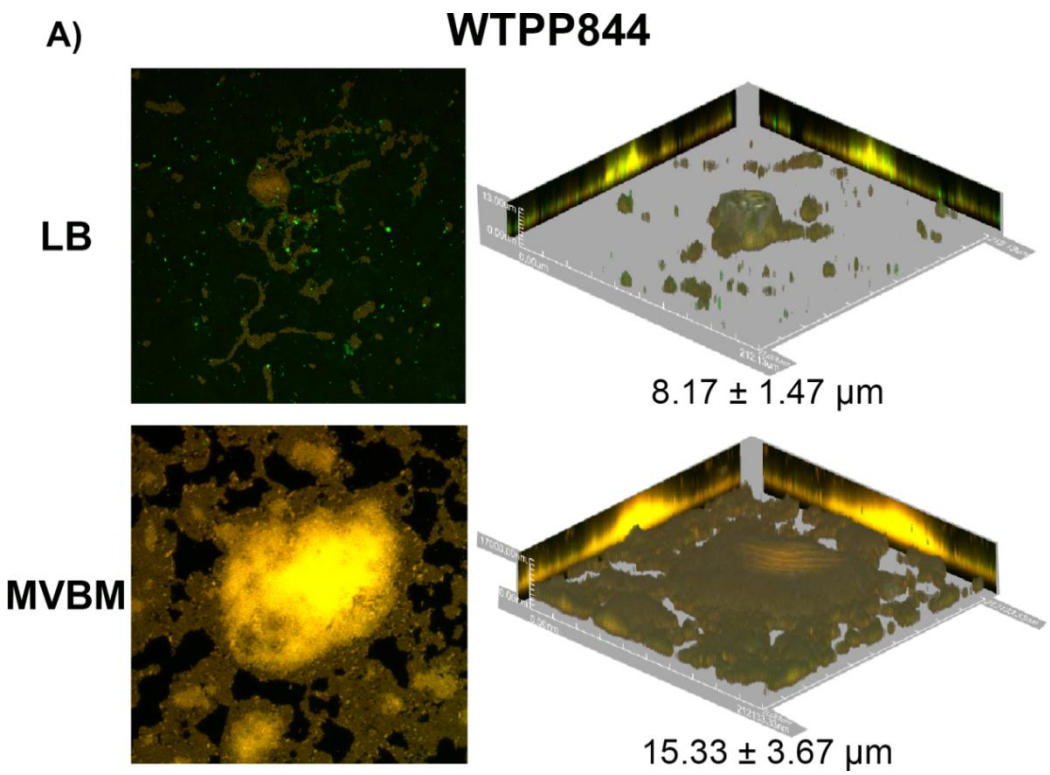

Figure 5.3 Visualization of biofilm production and thickness in *B. pseudomallei* A) wild type, B) *bps*I, C) *ppk*, D) *rpo*N2, and E) *rpo*S mutant strains in both LB and MVBM using CLSM

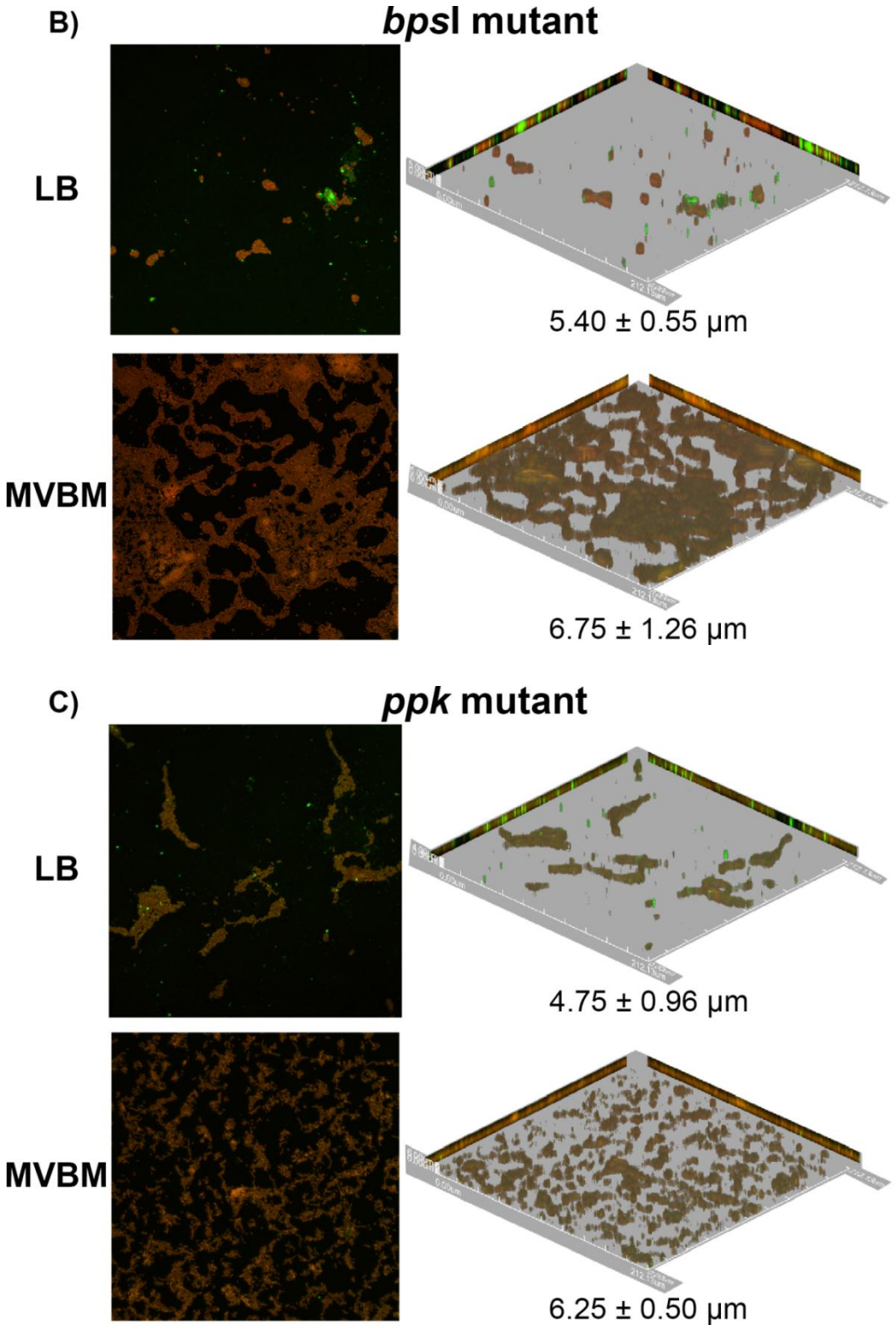

Figure 5.3 (cont.) Visualization of biofilm production and thickness in *B. pseudomallei* A) wild type, B) *bps*I, C) *ppk*, D) *rpo*N2, and E) *rpo*S mutant strains in both LB and MVBM using CLSM

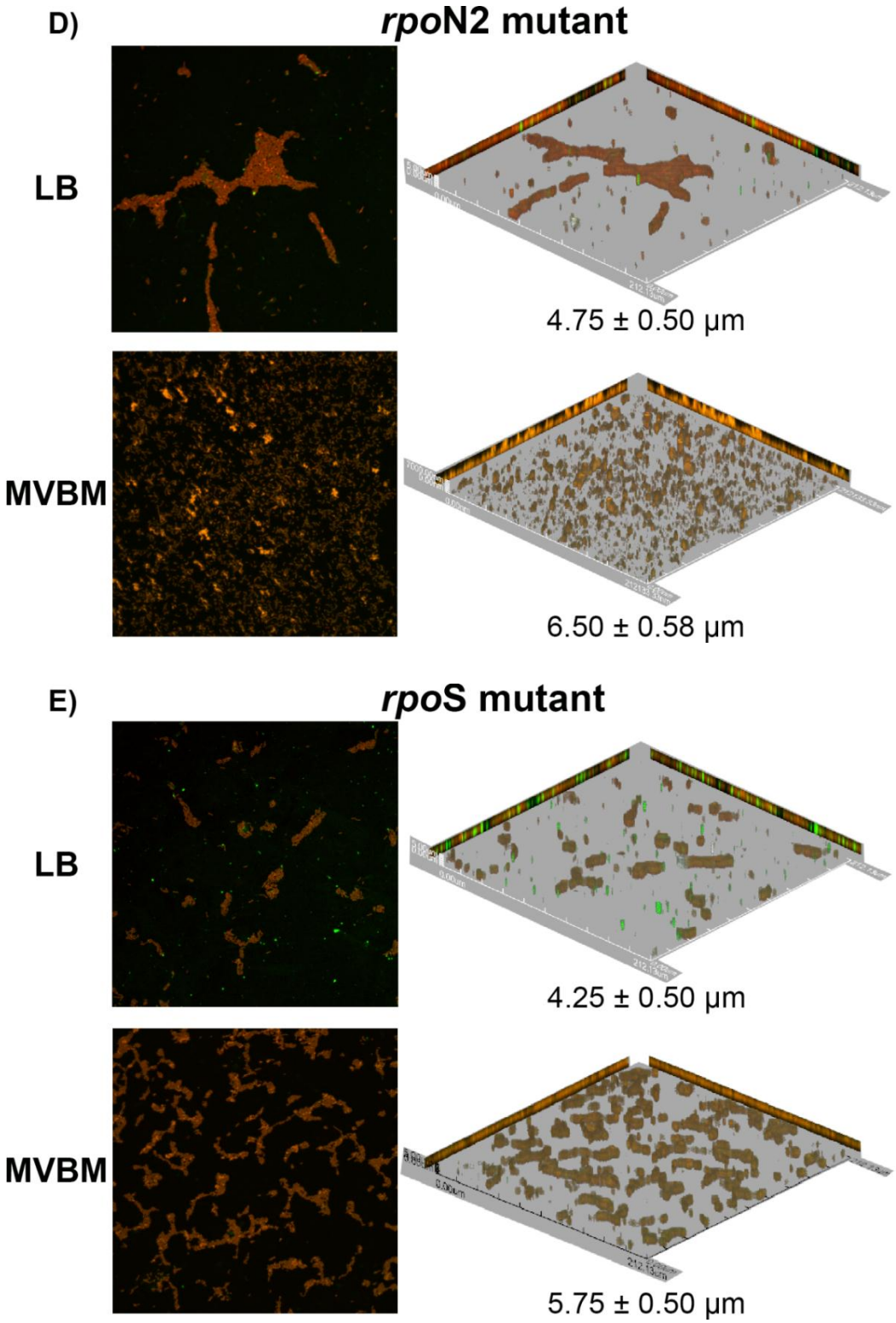

Figure 5.3 (cont.) Visualization of biofilm production and thickness in *B. pseudomallei* A) wild type PP844, B) *bps*I, C) *ppk*, D) *rpo*N2, and E) *rpo*S mutant strains in both LB and MVBM using CLSM

All of the *B. pseudomallei* mutants could not form mature micro-colonies either in LB or MVBM as seen in the CLSM images (Figure 5.3 B-E). They showed extensive bacterial spreading and aggregation with small thickness in contrast to the large-mature micro-colonies seen in the wild type. In all mutants, MVBM still had more capability of surface-attached induction than LB. Furthermore, biofilm thickness of the bacteria cultured in MVBM (around 6 µm) was higher than that of LB (around 4 µm) in all mutant strains confirming that this biofilm-induced media should be more effective and suitable for biofilm study than the other. Every *B. pseudomallei* mutant displayed similar micro-colonies and thickness in both media, though the *bps*I mutant are seem to be higher thickness than other mutants and the *rpo*S mutant had the thinnest biofilm thickness.

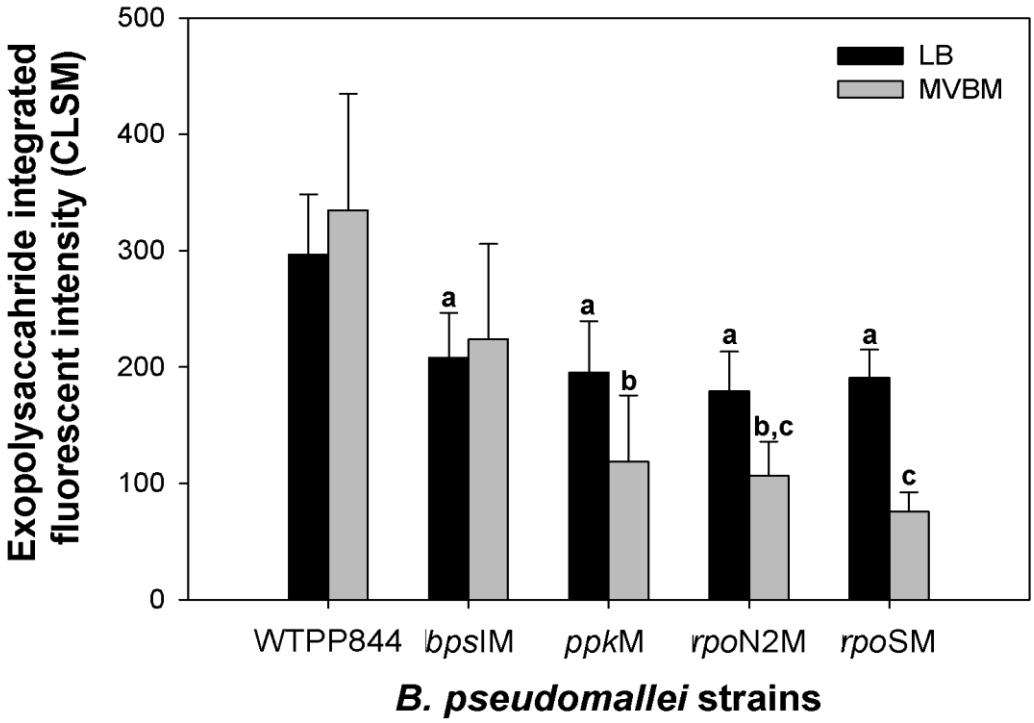

Figure 5.4 The biofilm exopolysaccharide production of *B. pseudomallei*. The averaged exopolysaccharide integrated fluorescent intensity of *B. pseudomallei* biofilms in LB and MVBM media calculated from CLSM. Same alphabet represents for the same group with no significant difference.

The biofilm exopolysaccharide integrated fluorescent intensity was calculated from five-observed fields using FV10-ASW 3.0 Viewer software and averaged into Figure 5.4. The results demonstrate that each of the *B. pseudomallei* mutant strain produced biofilm exopolysaccharide significantly lower than the wild type both in LB and MVBM. However, there is no significant difference between each mutants cultured in LB medium while distinct exopolysaccharide production among the mutants was observed in MVBM, which the *bps*I mutant had higher exopolysaccharide production than the *ppk* mutant followed by the *rpo*N2 and *rpo*S mutants ($p=<0.001$) suggesting that they do have specific effects on the ability of biofilm formation.

It is evident that exopolysaccharide production results obtained here are in good agreement with previous micro-colony formation and biofilm thickness which are apparently shown that MVBM have particular effects on biofilm formation of *B. pseudomallei* more than LB. These finding support our postulation that MVBM is more suitable for biofilm study than LB media as it can induce biofilm phenotype clearer than the other. Furthermore, as biofilm characters in MVBM showed specific ability of each mutant, it indicates that our genes of interest have specific influences on biofilm formation in *B. pseudomallei.*

5.2 Effect of differential biofilm formation ability on antibiotic resistance

5.2.1 Abilities of antibiotic resistance among the biofilm of *B. pseudomallei* mutants

Biofilm formation is known to alter antibiotic resistance in many bacteria (10) however a correlation between biofilm formation and antibiotic resistance in *B. pseudomallei* has not been established. The antibiotic resistance of *B. pseudomallei* mutants was investigated using both planktonic and biofilm forming conditions. The antibiotics susceptibility of each of the *B. pseudomallei* strains was compared in the Calgary Biofilm Device (CDB), a commercial device used for testing antibiotic susceptibility. All *B. pseudomallei* strains were tested simultaneously in 96-well microtiter plates with two independent biological replicates. Ceftazidime (CAZ) and meropenem (MRP) were used as these drugs are currently used in the treatment of melioidosis. Antibiotic concentration was used form 1024 µg/ml to 1 µg/ml with 200 µl of final volume. The OD540 of bacterial survival were measured and plotted into the graph (supplementary, Figure 8.1 and 8.2). Percentage of bacterial inhibition was calculated from the survival and the half maximal inhibitory concentration (IC50) was further identified and shown in Figure 5.5.

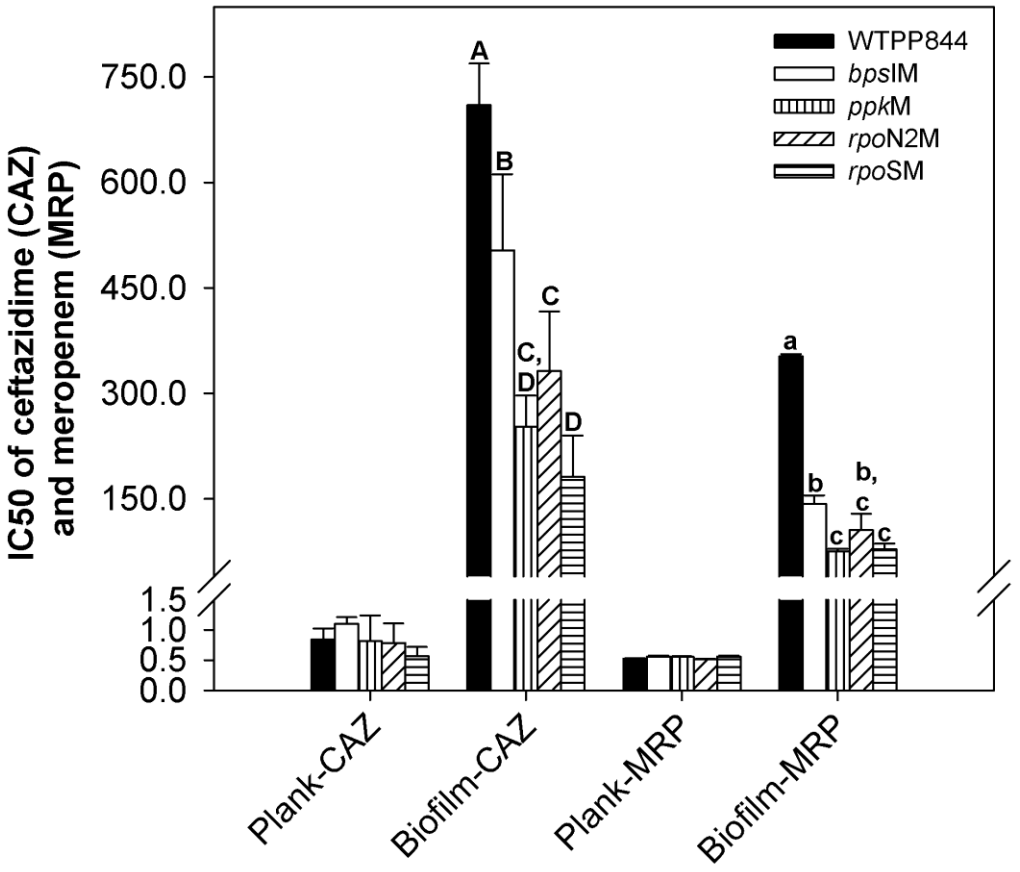

Figure 5.5 Comparison of IC50 between ceftazidime (CAZ) and meropenem (MRP). IC50 of CAZ and MRP treated all of *B. pseudomallei* strains in both planktonic and biofilm conditions. Same alphabet represents for the same group with no significant difference.

All of the *B. pseudomallei* strains in planktonic condition display a similar IC50 of CAZ and MRP, approximately 1-0.5 µg/ml in both treatments. Under biofilm promoting conditions, IC50 values in both drug conditions were dominantly increased up to 100-700 folds compared to their planktonic counterpart. Furthermore, distinct IC50 values between mutants were observed. The IC50 of biofilm-treated with CAZ in *bps*I, *ppk*, *rpo*N2, and *rpo*S mutated strains were approximately 29%, 64%, 53% and 74% lower than the *B. pseudomallei* wide type, respectively, whereas the IC50 of biofilm-treated with MRP in the mutant strains approximately decreased by 60%,

79%, 70% and 77% respectively (P=<0.001) as shown in Figure 5.5. Notably, IC50 values for CAZ roughly two to three folds greater than observed in MRP, which is consistent with results obtained in the previous study (15). Due to specific structure of these two antibiotics showing that CAZ contains more complex and larger structure than MRP, it is possible that a smaller drug can easily diffuse through the matrix acting as diffusion barrier more than a bigger one resulting in the lower IC50 observed in MRP treatment.

The IC50 of biofilm-treated with CAZ shows a substantial difference among biofilm *B. pseudomallei* mutant strains in contrast to the IC50s of biofilm-treated with MRP that are similar to each other. The difference between CAZ IC50s among *B. pseudomallei* mutants suggests that each mutated gene has the different effect on biofilm formation. While the MRP IC50s between the mutants were observed, it may be implied that the smaller structure of MRP is not affected for the diffusion through the altered biofilm extracellular matrix. The *bps*I mutant displayed higher antibiotic resistance than any other mutants in both CAZ and MRP treatment (P=<0.001), indicating that lack of the *bps*I gene was not greatly altered its biofilm formation. The mutants that showed the greatest effect on biofilm mediated antibiotic resistance are *ppk* and *rpo*S as they exhibit the lowest IC50 values for both CAZ and MRP with mutation of the *rpo*S gene displaying the lowest antibiotic resistance. The *rpo*N2 mutant showed an intermediate defect in biofilm mediated resistance with 53% and 70% reductions in IC50 for CAZ and MRP, respectively, even there was no significant different with *ppk* mutant in CAZ treatment and with *bps*I mutant in MRP treatment. Thus, *rpo*N2 appears to have a limited regulatory role in biofilm formation which should be different from *rpo*S gene.

5.2.2 Correlation between the exopolysaccharide production and the antibiotic resistance in the *B. pseudomallei* biofilms

Comparison between the exopolysaccharide production by CLSM and IC 50 values of CAZ and MRP treatments has revealed a similar trend between these two phenomenal. Therefore, it may be implied that there is a possible link between these two phenomenal which may be under the regulation of our investigated genes in *B. pseudomallei* biofilm. Although there is no significant difference between the

capability of antibiotic resistance in biofilm *ppk*, *rpo*N2 and *rpo*S mutants for both antibiotics, they still have particular biofilm formation ability as illustrated in Figure 5.6. This suggests that these four genes cause specific influences on biofilm production which greatly affect the biofilm structure resulting in the reciprocal decrease of antibiotic resistance.

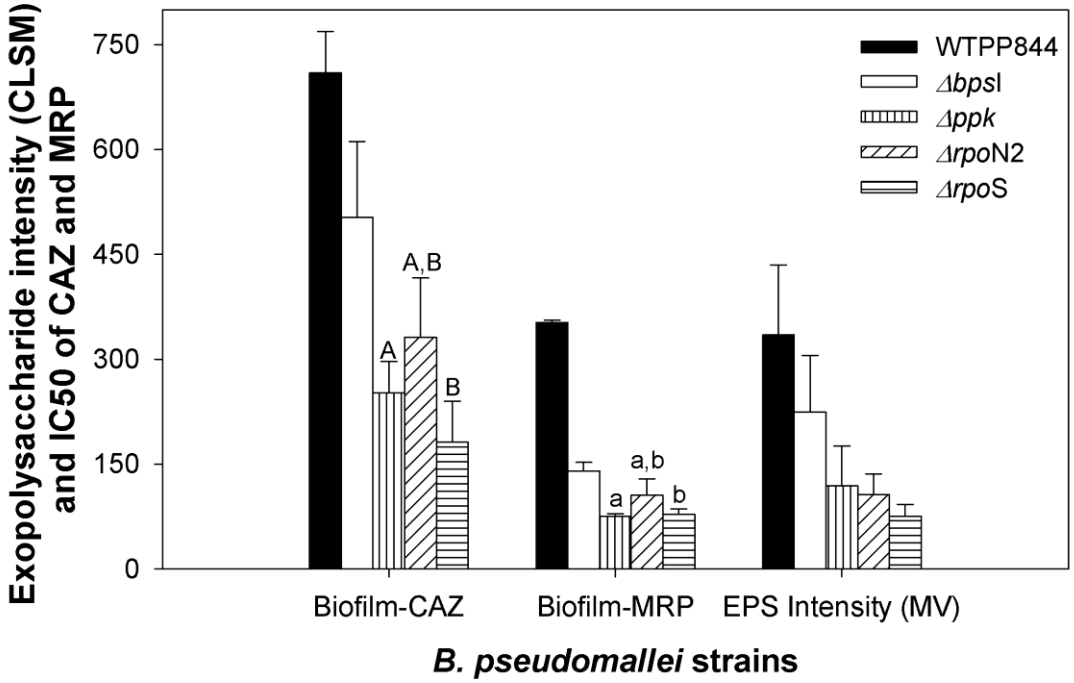

Figure 5.6 Exopolysaccharide productions from CLSM and antibiotic resistance (IC50) of CAZ-and MRP-treated biofilm. Comparisons between exopolysaccharide integrated fluorescent intensity and IC50 of CAZ and MRP treatments in biofilm condition of *B. pseudomallei* wild type and four mutant strains were shown. The same alphabets on the bar graphs are represented within the same group indicating no significant difference. (P = <0.001).

5.3 Exopolysaccharide composition from *B. pseudomallei* mutant strains

5.3.1 Identification of biofilm exopolysaccharide component

Extracellular polymeric substances (EPS) of the biofilm are suggested as a major barrier in protection of bacteria from the hostile environments containing exopolysaccharide as the principal structural component (127). It is possible that the reduction of drug resistance capability in biofilm *B. pseudomallei* mutants may result from the defectiveness of exopolysaccharide architecture. To investigate whether mutation of the *bps*I, *ppk*, *rpo*N2, and *rpo*S genes results in alterations of the carbohydrate composition in biofilm glycoconjugates, the extracellular polysaccharide matrix of biofilms were isolated and ethanol-precipitated. Trimethylsilyl (TMS) derivatives of the lyophilized biofilm glycoconjugates were further analyzed using gas chromatography-mass spectrometry (GC-MS) with triplicate independent samples.

The identification of the monosaccharide components was based on comparison of the retention times with those of the standard monosaccharide mixture. As previously mention above, the standard mixture of expected monosaccharide types, consist of rhamnose, xylose, mannose, glucose, galactose, and glucoronic acid in *B. pseudomallei* biofilm, however, due to the lack of standard glucoronic acid, there are only five standard monosaccharides investigated and total ion chromatogram (TIC) representing retention times of all standard monosaccharides were shown in Figure 5.7. J. Bleton *et al.* had reported the characteristic of natural sugars and uronic acid after methanolysis and trimethylsilylation for composition analysis using GC-MS, which were showed as the identified peaks of α-pyranoside and β-pyranoside owing to the widely known anomerization and ring isomerization processes of sugars (128) (Figure 5.8). To identify the pattern peaks of each standard sugar in the standard mixture of our study, the merged peaks of all interested standard monosaccharide from the reference chromatograms of J. Bleton work was created (Figure 5.9) which showed similar peak patterns to our five standard monosaccharide mixture (Figure 5.7), though, the retention times of all standard sugars does not match to ours. As the previous data from J. Belton *et al.* is not so specific to our results and the revealed peaks were not clearly match to the sugar library of the GC-MS software, the

particular peaks of each standard sugar need the confirmation. Therefore, the pattern peaks of each standard were studied using one monosaccharide at a time (Figure 5.10).

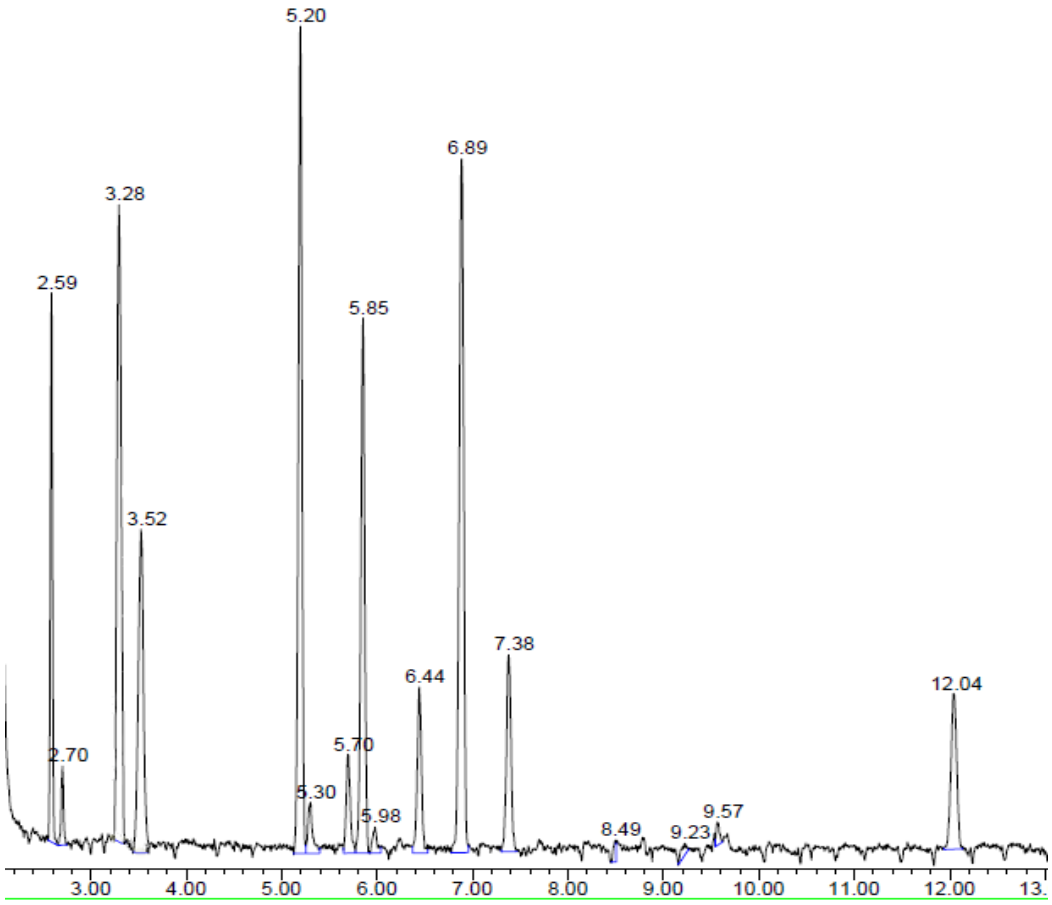

Figure 5.7 The total ion current chromatogram of unidentified five standard monosaccharides. Total ion current profiles of rhamnose, xylose, mannose, glucose, and galactose. Retention time (RT) is shown in min (X-ais).

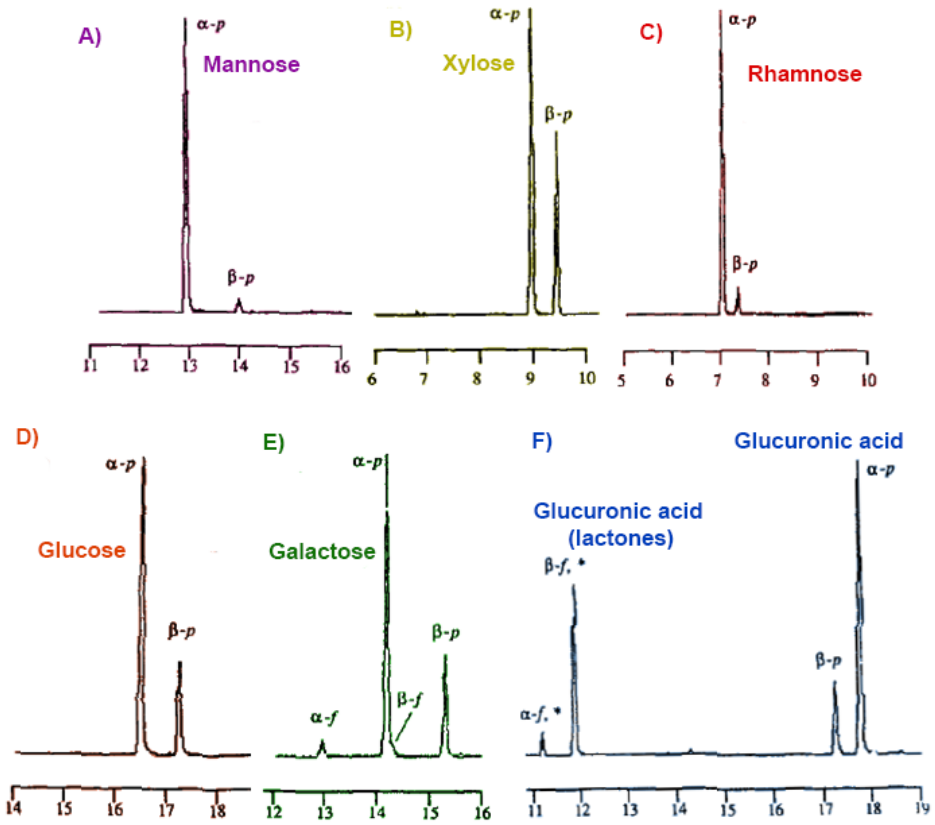

Figure 5.8 The total ion current chromatograms of standard monosaccharides from J. Bleton *et al.* The total ion current chromatograms of each standard monosaccharide in α-pyranoside and β-pyranoside, which showed different retention times from our study, reported by J. Bleton *et al*; A) mannose B) xylose, C) rhamnose, D) glucose, E) galactose, and F) glucuronic acid (lactone forms are labeled with asterisks). Retention time (RT) is in minutes.

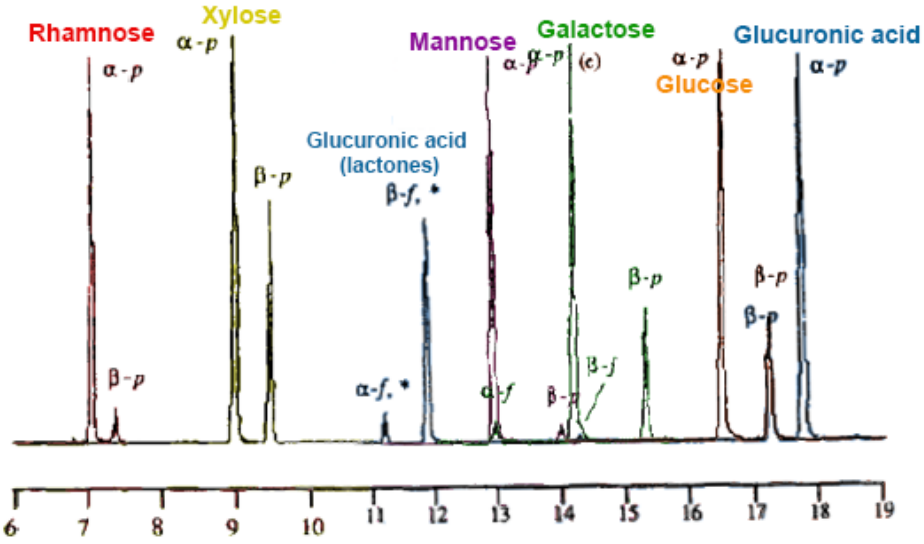

Figure 5.9 Combination of total ion chromatograms of six standard monosaccharides from J. Bleton *et al.*

Each of the standard monosaccharide was individually methanolyzed and trimethylsilylated together with the standard mixture and the extracted exopolysaccharides from all of *B. pseudomallei* strains. All of the samples were further analyzed in GC-MS which finally resulted in pattern peaks of total ion chromatograms. The total ion chromatographic patterns of each of the standard monosaccharide were shown in figure 5.10. The specific retention times of rhamnose with both conformations (α and β) are at 2.60 min and 2.71 min followed by xylose at 3.29 min and 3.25 min. Mannose, galactose and glucose are further secreted out in the retention time period of 5-7 min; mannose at 5.20 and 5.71 min, galactose at 5.30, 5.85, 5.98, 6.44 and 9.57 min, and glucose at 5.90 and 7.39 min, while an internal standard, *myo*-inositol, has a retention time at 12.05 min. This multiple peak pattern of each standard sugar facilitated the identification in the standard mixture. As the result, the sequence of secreted standard monosaccharide is rhamnose, xylose, mannose, galactose and glucose which are almost the same as the reference chromatograms from J. Bleton *et al.* (Figure 5.9). Therefore, the retention times of those standard monosaccharides were used to identify carbohydrate composition of the exopolysaccharide matrix in *B. pseudomallei* biofilm of wild type and mutant strains.

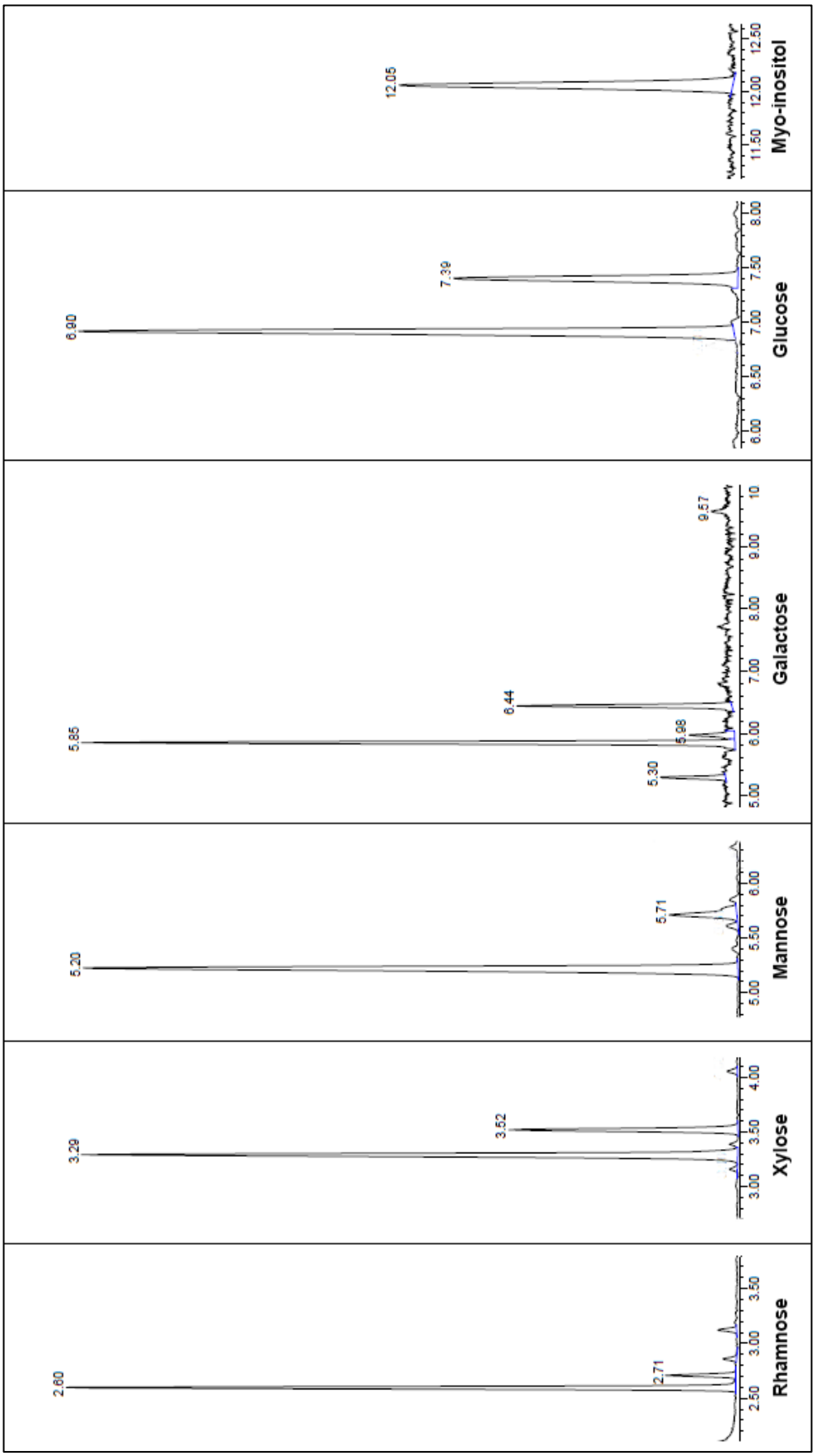

Figure 5.10 Total ion chromatographic patterns of the methanolysis-trimethylsilylation products obtained from individual standard monosaccharides

Total ion chromatogram of extracted biofilm exoplysaccharide of *B. pseudomallei* wild-type was showed in Figure 5.11 which has a similar peak pattern to the standard mixture (Figure 5.7).

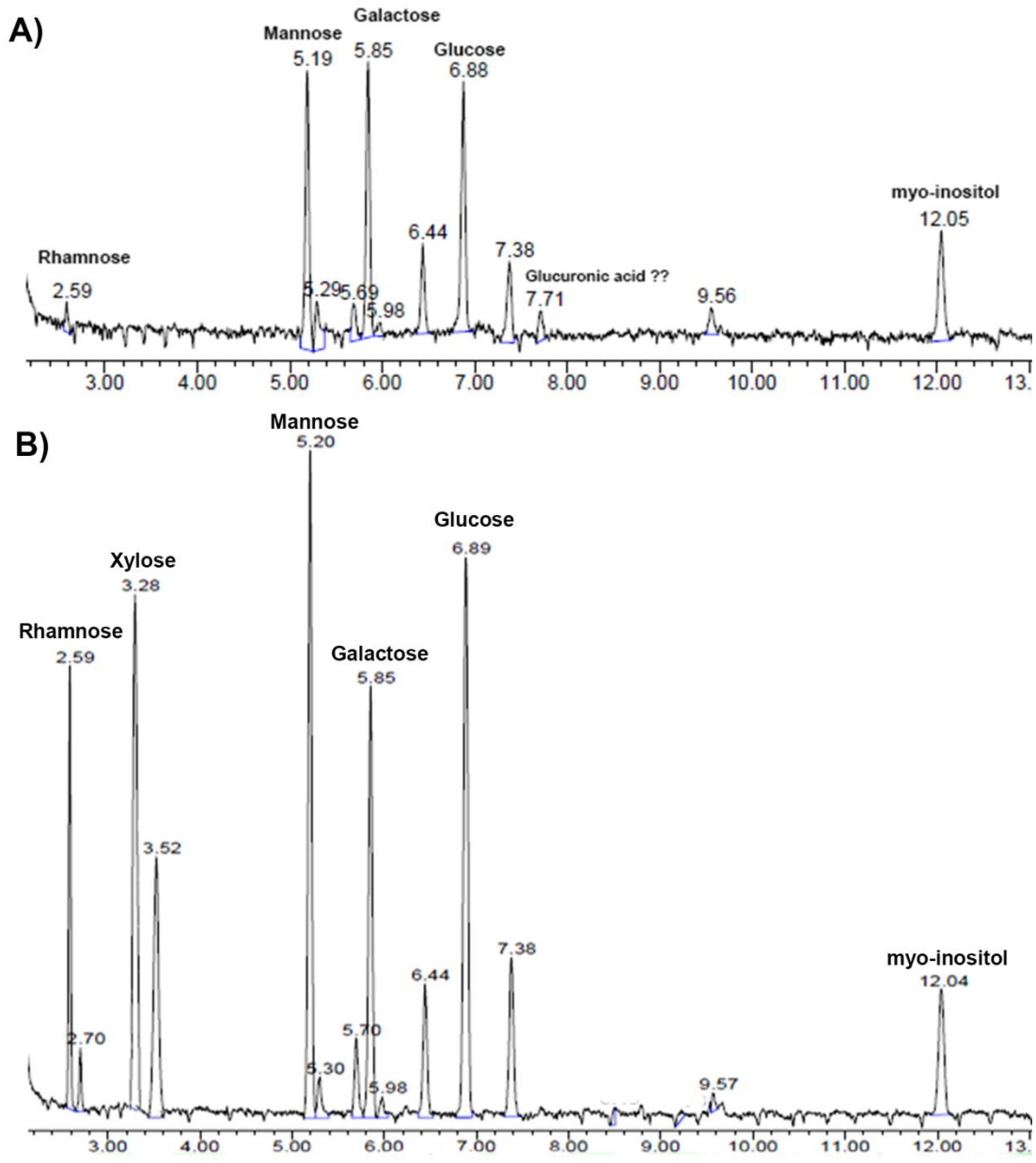

Figure 5.11 Comparison of total ion peaks between extracted biofilm exoplysaccharide and standard mixture. Total ion chromatogram of extracted biofilm exopolysaccharide from *B. pseudomallei* wild type (A) compare to total ion current graph of the identified standard mixture (B).

The chromatogram displays peaks at 2.59, 5.19, 5.29, 5.69, 5.85, 6.44, 6.88, 7.38, 7.71, 9.56 and 12.05. When compared to the retention times of the standard mixture, biofilm exopolysaccharide of *B. pseudomallei* wild type consist of rhamnose (RT = 2.59), mannose (RT = 5.19 and 5.69), galactose (RT = 5.29, 5.85, 5.98, 6.44 and 9.57), and glucose (RT = 6.88 and 7.38). Apparently, there is another peak at 7.71 min that cannot be identified from our standard mixture. From the reference chromatograms of six standard monosaccharides of J. Bleton *et al* (128), the identified peaks of α-pyranoside form of glucuronic acid were presented after the peak of α-pyranoside glucose which is very similar to the peak at 7.71 min in the chromatogram of *B. pseudomallei* wild type. Therefore, I have assumed that this peak may be glucuronic acid. The peak at 12.05 min is *myo*-inositol that was added into every reaction tube as the internal standard.

All of the biofilm exopolysaccharides of *B. pseudomallei* mutants, *bps*I, *ppk*, *rpo*N2, and *rpo*S (Figure 5.12), showed same patterns of total ion peaks to the wild type, suggesting that all of the mutants contain the same carbohydrate components of biofilm extracted exopolysaccharide to the wild type which consist of rhamnose, mannose, galactose, glucose and glucuronic acid. Surprisingly, overall of the revealed peaks of carbohydrate components in all of the mutants showed higher amount than the wild type and there are some peaks that cannot be detected in the mutants. Therefore, the amount of each monosaccharide component was further quantitated to verify the effect of the mutated genes to the exopolysaccharide biosynthesis in *B. pseudomallei* biofilm.

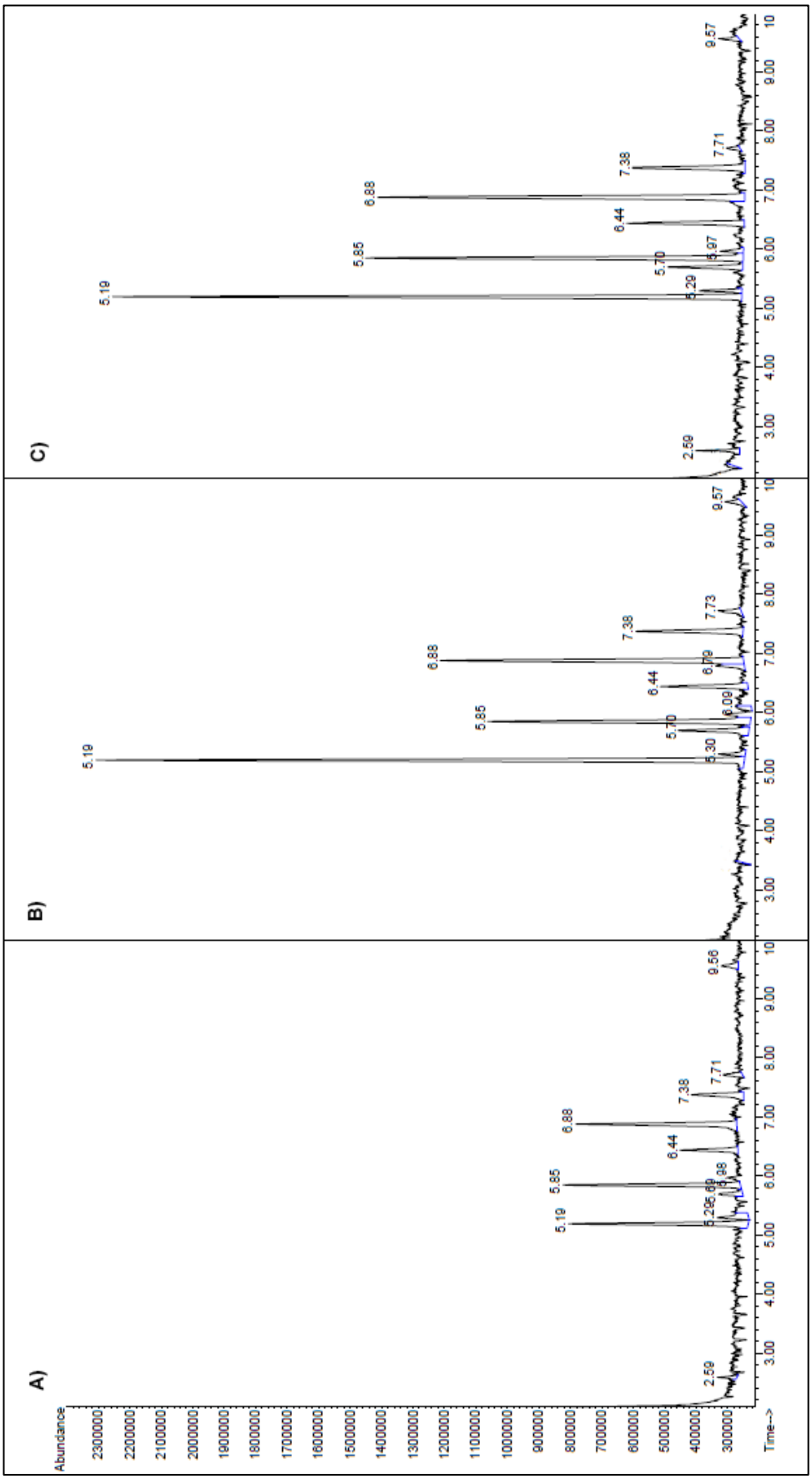

Figure 5.12 Total ion chromatograms of wild type, *bpsI* and *ppk*. Total ion chromatographic patterns from extracted biofilm exopolysaccharide of *B. pseudomallei* wild type (A) and mutant strains (B = *bpsI*, C = *ppk*)

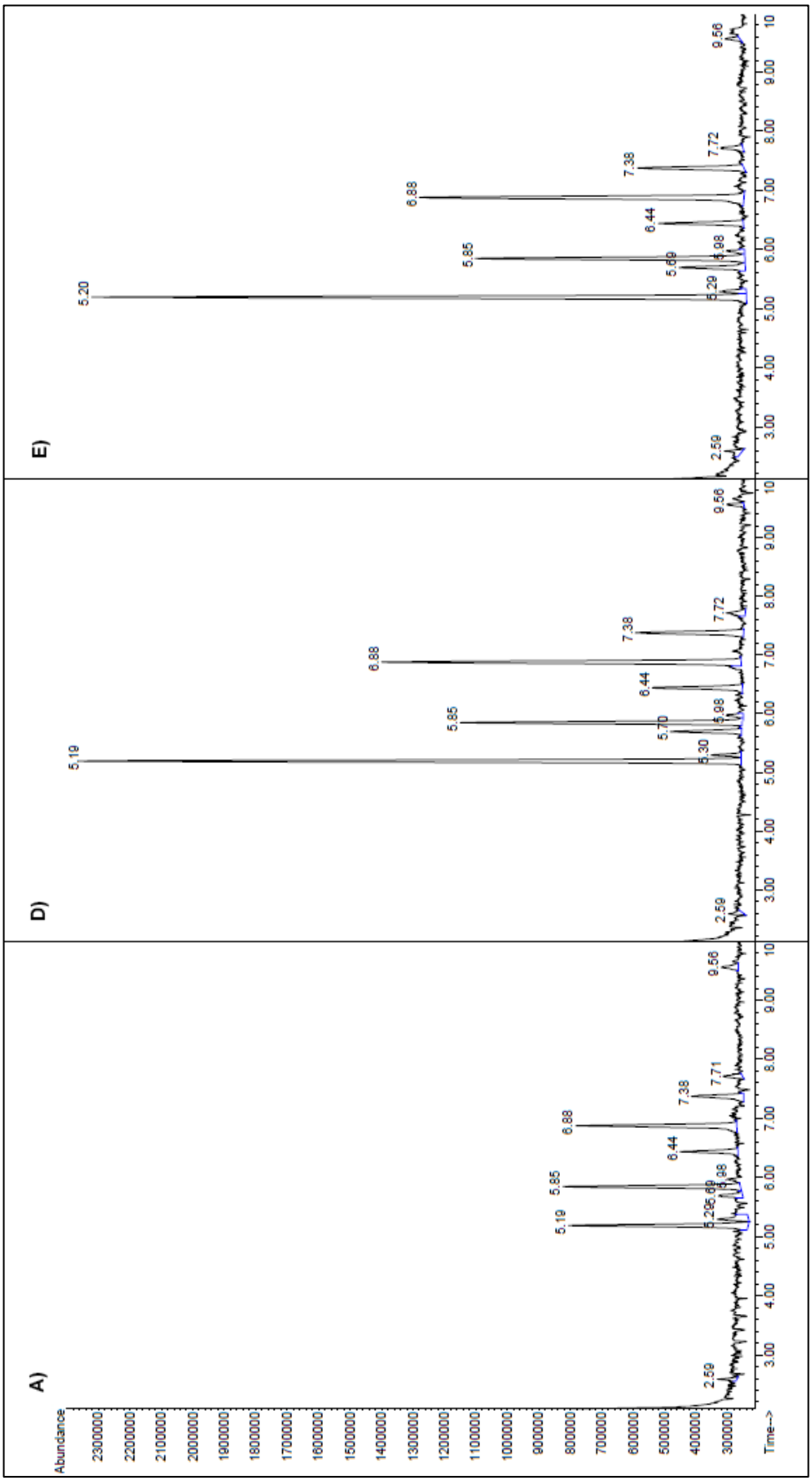

Figure 5.12 (cont.) Total ion chromatograms of wild type, *rpo*N2 and *rpo*S. Total ion chromatographic patterns from extracted biofilm exopolysaccharide of *B. pseudomallei* wild type (A) and mutant strains (D = *rpo*N2, E = *rpo*S)

5.3.2 Quantitation of monosaccharide components of analyzed exopolysaccharides

To quantitate the amount of monosaccharides in unknown sample, the standard mixture of the relevant particular glycoconjugate, consisting of rhamnose, xylose, mannose, galactose and glucose, was subjected to parallel derivatization with the sample and further analyzed in GC-MS. The amount (milligrams) of each sugar in the standard mixture is a function of the number of sugars that were mixed together. The ratio of the peak area of an internal standard, added to the mixture, to that of each standard sugar in the mixture was determined as detector response factor (dRF) are determined as mentioned above (method 4.2.vi) and used to calculate the amount of each component of the sample being analyzed. The total ion chromatogram of each biofilm exopolysaccharide from 0.5 mg of purified biofilm exopolysaccharide material of all *B. pseudomallei* strains revealed the identification of monosaccharide types and percent peak areas which were further used for the weight calculation. After the identification of multiple peaks in the analyzed sample using a comparison of the retention times, weight of identified sugar in unknown (mg) was calculated according to the following formula as mentioned earlier (method 4.2.vi).

The calculation of detector response factors (dRF) of each independent experiment and total peak area of each identified monosaccharide type and internal standard in each experiment which were used for weight calculations were listed into summarized tables as shown in supplementary data (Table 8.1 and Table 8.2). All samples were analyzed in triplicate independent experiments. The average of carbohydrate amount in each *B. pseudomallei* strains from the triplicate independent experiments were summarized into Table 5.3.

Table 5.3 Carbohydrate contents in the exopolysaccharide matrix of the *B. pseudomallei* biofilm

***B. pseudomallei* strains**	**Carbohydrate contents/0.5 mg purified exopolysaccharides (Ratio)**				**Carbohydrate contents/biofilm production capacity**			
	Glucose (µg)	**Galactose (µg)**	**Mannose (µg)**	**Rhamnose (µg)**	**Glucose (µg)**	**Galactose (µg)**	**Mannose (µg)**	**Rhamnose (µg)**
WTPP844	84.27 ± 8.71 (1.00)*	110.61 ± 2.60 (1.31)	68.75 ± 8.41 (0.82)*	26.55 ± 9.09 (0.30)	84.27 ± 8.71	110.61± 2.60	68.75 ± 8.41	26.55 ± 9.91
***bps*IM**	100.91 ± 7.19 (1.00)*	105.14 ± 10.02 (1.04)*	164.61 ± 13.39 (1.63)	ND	67.53 ± 4.81	70.36 ± 6.71	110.16 ± 8.96	ND
***ppk*M**	71.53 ± 39.67 (1.00)*	87.84 ± 39.33 (1.23)*	84.24 ±45.32 (1.18)*	7.03 ± 5.24 (0.1)	25.36 ± 14.06	31.14 ± 13.94	29.86 ± 16.07	2.49 ± 1.86
***rpo*N2M**	99.73 ± 3.23 (1.00)*	105.75 ± 17.46 (1.06)*	148.54 ± 1.06 (1.49)	4.56 ± 0.62 (0.05)	31.71 ± 1.03	33.63 ± 5.55	47.24 ± 0.34	1.45 ± 0.20
***rpo*SM**	105.78 ± 4.67 (1.00)*	114.50 ± 14.86 (1.08)*	152.45 ± 4.92 (1.44)	6.06 ± 1.59 (0.06)	23.89 ± 1.06	25.86 ± 3.36	34.43 ± 1.11	1.37 ± 0.36

ND means no detection of the carbohydrate peak

* Means that there is no significant difference between those monosaccharide ratios.

Table 5.3 illustrates the carbohydrate contents from 0.5 mg of purified biofilm exopolysaccharide material of *B. pseudomallei* wild type and all of the mutant strains. The ratio of the carbohydrate contents in each strain was calculated by using glucose as a calibrator because this sugar is commonly found in other bacterial exopolysaccharide matrices (104, 129, 130). The carbohydrates in the exopolysaccharide matrix of the wild type biofilm are glucose, galactose, mannose, and rhamnose in the ratio 1.00:1.31:0.82:0.30. Noticeably, glucose and mannose are the main components in equal amounts, while galactose also appears to be a major component ($P = < 0.001$), which is consistent with the previous report (104).

The exopolysaccharide matrices of the *B. pseudomallei* mutant strains contain the same carbohydrate contents; however, shifting in the ratios of the contents can be observed. The *bps*I mutant exhibits a different carbohydrate content ratio (1.00:1.04:1.63:ND), in which mannose becomes the major component instead of galactose as in the wild type. Interestingly, the monosaccharide content in this mutant is likely to be increased, while no detectable peak of rhamnose was observed as shown in Table 2. Likewise, the *ppk*, *rpo*N2 and *rpo*S mutants contain distinct carbohydrate content ratios of 1.00:1.23:1.18:0.10, 1.00:1.06:1.49:0.05 and 1.00:1.08:1.44:0.06, respectively, in which no significant difference was found between the glucose, galactose and mannose in the *ppk* mutant (Table 5.3). Similar trends in the carbohydrate ratios were revealed between the *bps*I, *rpo*N2 and *rpo*S mutants, with mannose as the major component. Perceptibly, a remarkable reduction in the rhamnose content can be observed in all of the mutants, especially in the *bps*I mutant. Therefore, it is possible that the genes of interest may be involved in the regulation of rhamnose biosynthesis. As the mannose contents were elevated in both the *bps*I, *rpo*N2 and *rpo*S mutant strains, which also included rhamnose reductions, we speculated that there should be some correlations between mannose and rhamnose synthetic pathways, which is modulated by these genes.

In addition, this carbohydrate composition analysis of the exopolysaccharides was studied from equal amounts of purified carbohydrate material; nevertheless, each *B. pseudomallei* strain has a unique capacity for biofilm formation as previously reported. Thus, to obtain a more precise view of the effects of these genes on the biofilm production capacity, recalculation of the carbohydrate contents

was performed according to the biofilm capacity. The specific biofilm capacity factors were calculated from each exopolysaccharide integrated fluorescent intensity by CLSM, for which the wild type strain was designed as the positive control (supplementary, Figure 8.3), and used to subtract all of the carbohydrate contents as reported in Table 5.3. The subtracted carbohydrate contents among *B. pseudomallei* strains were compared in Figure 5.13. It can be clearly seen in Figure 5.13 that there are shifts in the carbohydrate ratios and deprecated production of the monosaccharide types.

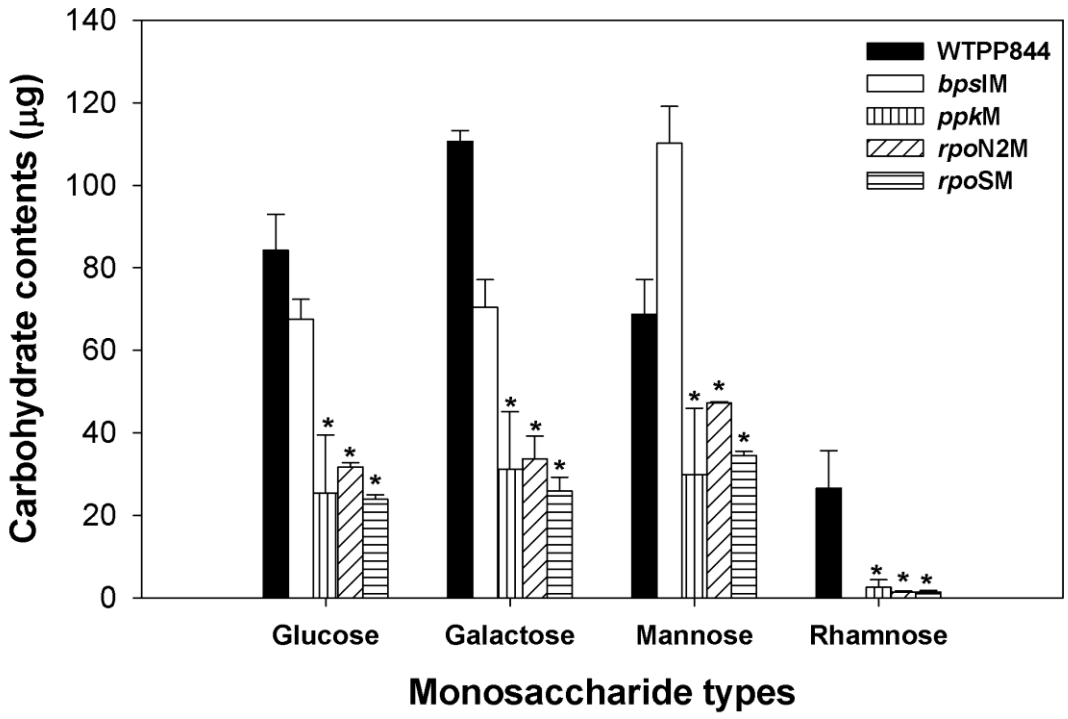

Figure 5.13 Subtracted carbohydrate contents in the exopolysaccharides of all *B. pseudomallei* strains. The carbohydrate contents of the exopolysaccharides produced by *B. pseudomallei* wild type and mutant strains after subtraction from the biofilm production capacity determined by CLSM were compared to each other; ■ = BpWT, □ = *bps*IM, ▥ = *ppk*M, ▨ = *rpo*N2M and ▤ = *rpo*SM. Asterisks indicate that there is no significant difference between those strains within a monosaccharide type.

Noticeably, the glucose and galactose contents were dramatically reduced in all of the mutants, especially in the *ppk*, *rpo*N2 and *rpo*S mutants by approximately 70-72%, 62-70% and 72-77%, respectively. Although the *bps*I mutant displayed reductions in the glucose and galactose contents, it showed only 20% and 36% lower contents compared to the wild type. Together with glucose and galactose production, all of the mutants also showed massive reductions in the rhamnose contents by 91-95% for the *ppk*, *rpo*N2 and *rpo*S mutants, while no rhamnose production was detected in the *bps*I mutant. Surprisingly, although mannose production in the *ppk*, *rpo*N2 and *rpo*S mutants was reduced by 57%, 31% and 50%, respectively, the mannose content of the *bps*I mutant increased by up to 60% compared to the wild type. Consistent with the previous results, the *bps*I mutant has the lower effect on biofilm exopolysaccharide composition than the other mutants because of its higher production of glucose, galactose and mannose. Therefore, these results suggest that the exopolysaccharide compositions of *B. pseudomallei* biofilms are modulated by *bps*I, *ppk*, *rpo*N2 and *rpo*S genes resulting in defects of the core biofilm polysaccharide structure, which may influence antibiotic resistance.

CHAPTER VI
DISCUSSIONS

The biofilm is a glycocalyx polysaccharide matrix encasing the bacterial population as a barrier to support the survival of the species. The biofilm formation requires many regulatory elements responding to the environmental changes, such as the hostile conditions presenting in host environment. Many reports have documented genes that involve in the biofilm formation processes. Studies by Loprasert, S. *et al*. have shown that *B. pseudomallei* lacking the oxidant stress regulator OxyR exhibited an increased ability to form biofilms in minimal medium, but not in a enriched medium such as Luria-Bertani broth (108). Furthermore Hwee Siang Lee *et al.* have described a role for CdpA, a major c-di-GMP-specific phosphodiesterase, in an negative regulation on the biofilm formation. The *cdpA* mutant has elevated intracellular c-di-GMP levels resulting in the enhanced production of exopolysaccharides and a significant increase in biofilm formation (109). Moreover, the *B. pseudomallei* alternative sigma factor sigma E (*rpo*E) which controls adaptations to the stress was shown to be a positive regulator of biofilm formation (111). However, the molecular regulation for the complex multi-step process of biofilm formation remains unclear.

Biofilm-forming bacteria are well-known to be capable of enhancing antibiotic resistance compared to their planktonic counterparts. Extracellular polymeric substances (EPS) in the biofilm have been reported to be a greatest barrier to diffusion for drug penetration into various bacteria (127). Previous studies from other gram negative bacteria have shown that drug resistance in the biofilm partly involves quorum sensing communication and many regulatory genes, including *ppk, rpo*N2 and *rpo*S genes (22-24). Furthermore, the mutants containing defects in *ppk* gene and the quorum sensing system AHL synthase gene, *bps*I or *bps*R, have been previously reported aggravated biofilm formation in other gram-negative bacteria (20, 131). Moreover, the *rpo*N2 and *rpo*S regulators have been reported to control biofilm

formation in others gram negative bacteria, including *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*, *Vibrio anguillarum M3, Escherichia coli,* and *Vibrio fischeri* (21, 22, 117, 119, 132-137). In *B. pseudomallei*, the biofilm was reported to be a diffusion barrier for some of the antimicrobial agents; however, how the biofilm create the ability in retardation of antibiotic penetration is still unrevealed.

6.1 Characteristics of the differential biofilm formation ability and drug resistance among *B. pseudomallei* mutants

Using *B. pseudomallei* strains (*bps*I, *ppk*, *rpo*N2, and *rpo*S) previously constructed and characterized by our group, we have demonstrated a correlation between biofilm production and antibiotic resistance under the regulation of these genes. All of the *B. pseudomallei* mutants displayed the similar defect of the biofilm formation in LB. Surprisingly, the contrary results were found in MVBM, which *bps*I mutant showed higher biofilm forming-ability than any other mutant strains. The result suggests that this two media have different effect on the biofilm formation; however, this CV staining method cannot provide all of the biofilm characteristics. Thus, to confirm the ability of biofilm formation of each *B. pseudomallei* mutant, CLSM was used to verify the other biofilm characters.

From the CLSM results, the *B. pseudomallei* mutants displayed the significant defects in micro-colony formation, biofilm thickness, and exopolysaccharide production in both LB and MVBM media, which apparently illustrated clearer biofilm phenotypes than the other medium. The biofilm formation of *B. pseudomallei* mutants in MVBM demonstrated higher surface attaching abilities and biofilm thicknesses than the mutant biofilms in LB, suggesting that our genes of interest actually have some influences on the biofilm formation and MVBM, the biofilm induced-media, should be more suitable for the biofilm study than the other. Moreover, all *B. pseudomallei* mutants significantly exhibit lower biofilm exopolysaccharide production than the wild type in both media. Nevertheless, MVBM can display the particular effects of the mutated genes to biofilm formation than the other, especially in the *rpo*S mutant. Thus, this confirms that all of the genes of interest have particular repercussions on the biofilm formation in *B. pseudomallei*.

Furthermore, MVBM can provide more insight information of this phenomenon than LB which these results support our assumption to use MVBM as the cultured medium for the biofilm study in this work.

The different biofilm formation abilities of the *B. pseudomallei* mutants have been illustrated the affectation on the antibiotic resistance of the biofilm. As we mentioned earlier that each of the mutants displayed the significant defect in all of the biofilm characteristics, especially in the exopolysaccharide production, the antibiotic resistance of the biofilm of each mutant demonstrated the similar trend of reduction of the defective biofilms, suggesting that the loss of antibiotic resistance ability is a consequence of the biofilm defectiveness due to the mutated gene. This finding extends those of Pibalpakdee, confirming that the *B. pseudomallei* biofilm generates a restrictive diffusion barrier for some types of antimicrobial agents, such as CAZ and imipenem (IMP) (15) and our genes of interest may play some important roles in this phenomenon. The substantial difference in the biofilm-treated IC_{50} values of *B. pseudomallei* wild type was observed between CAZ and MRP. Because CAZ has much complex and larger chemical structure than MRP, it was suggested that the biofilm likely posses a diffusion barrier for larger antimicrobial agents. This corresponds well with previous studies reporting that the biofilm should not be able to limit the diffusion of small antibiotic molecules (138, 139).

The antibiotic resistance among the *B. pseudomallei* strains was substantially different, especially when challenged with CAZ. According to the moderate reduction in biofilm capability and antibiotic resistance in the *bps*I mutant (Figure 5.6), it may be implied that this gene should retained the ability to resist the antibiotic diffusion and have less involvement in biofilm resistance than other genes. Interestingly, the positive regulation of *rpo*S on *bpsI* expression and autoinducer production on a stationary phase of *B. pseudomallei* has been reported by Wongtrakoongate, P. (140). For this reason, it can be assumed that the *rpo*S gene has more influence on the biofilm characteristics than the *bps*I gene which coincides well with both previous data. The *ppk* and *rpo*N2 mutants have an average impact on biofilm formation as exhibited by its intermediate resistance of antibiotics and exopolysaccharide production. Our finding therefore indicates that all of the genes of

interest have specific roles in biofilm formation and antibiotic resistance of *B. pseudomallei*.

6.2 Correlation between antibiotic resistance of the biofilm stage and exopolysaccharide composition in *B. pseudomallei* biofilm

As the major component of extracellular polymeric substance (EPS) of the biofilm is exopolysacharides and EPS has been reported as a diffusion barrier of the biofilm, the decreased antibiotic resistance of *B. pseudomallei* mutants may result from an aberration in the exopolysaccharide structure. In this study, GC-MS analysis of the purified biofilm exopolysaccharides revealed that the mutated genes obviously affect the ratio of carbohydrate composition. The exopolysaccharide matrix of the *B. pseudomallei* wild type biofilm consisted of glucose, galactose, mannose, and rhamnose in the ratio 1.00:1.31:0.82:0.30, which galactose appears to be a major component (P = < 0.001). Previous studies have demonstrated that *B. pseudomallei* produce an acidic, water-soluble exopolysaccharide, with the structure, [-->3)-beta-D-Galp2Ac-(1-->4)-alpha-D-Galp-(1-->3)-beta-D-Galp-(1- ->5)-beta-Kdo-(2-->]n, that can be recognized by the IgG 1 monoclonal antibody 3015. However, this exopolysaccharide was identified as capsular polysaccharide as its repeating unit contains Kdo, a major component of the capsular polysaccharide (141, 142). Our carbohydrate compositions analysis indicates some similarity to the previously described triple-branched heptasaccharide repeating unit of the capsule polysaccharide (CP2) in *B. pseudomallei*, composed of glucose, galactose, mannose, rhamnose, and glucuronic acid. However, the proportion of carbohydrate residues presented in the biofilm exopolysaccharide we identified (glucose, galactose, and mannose) were not similar to the major component of CP2 (glucose, galactose, and rhamnose) (143). Therefore, it is evident that the expolysaccharide identified in this study may not be the capsular polysaccharide, nevertheless, this capsule CP2 may act as the core polysaccharide needed for structural maturation in the biofilm extracellular matrix. Consistent with our observations, Cescutti, P *et al*. has demonstrated that the exopolysaccharide produced by *Burkholderia cepacia* is composed of galactose (major component), rhamnose, mannose, glucose, and glucuronic acid (104).

The same carbohydrate contents were found in all of the *B. pseudomallei* mutant biofilms with the different ratios and the major component. Dramatic reductions in rhamnose production were evidently showed in the biofilm exopolysaccharide matrix of all mutants, especially in the *bpsI* mutant in which rhamnose cannot be detected. As the defective drug resistance in all of the biofilm mutants was previously illustrated, it may be conceivable that the disruption of the biofilm matrix results from the loss of rhamnose leading to the partial disability of the diffusion barrier of the biofilm. Remarkably, the carbohydrate contents of the *bpsI* mutant contains negligible reduction when compared to the wild type, whereas the mannose production is dramatically increased, suggesting that the lack of this mutated gene slightly causes the perturbation of the matrix structure resulting in an moderate impact on the dysfunction of the biofilm antibiotic resistance, which may come from the slight reduction of glucose and galactose together with the absence of rhamnose. Moreover, the dramatic accumulation of mannose found in this mutant cannot help recover the ability of antibiotic resistance, suggesting that glucose, galactose and rhamnose should be the major components of exopolysaccharide in EPS of *B. pseudomallei* biofilm instead of mannose. While the measurable influence of the *bps*I mutation on the biofilm exopolysaccharide compositions led in the intermediate disability of the drug resistance, the *ppk, rpo*N2 and *rpo*S genes caused significant effects on all of the carbohydrate compositions, corresponding to particular extenuations in their antibiotic resistant abilities. From the data gained in this study, it can be implied that the exopolysaccharide composition of the biofilm matrix is a major structural content important for restricting antibiotic diffusion.

6.3 Possible mechanism of exopolysaccharide biosynthesis in *B. pseudomallei* biofilm under regulation of four studied genes

Apparently, the exopolysaccharide matrix from the *bps*I, *rpo*N2 and *rpo*S mutants revealed significant increases in mannose and loss of rhamnose production, indicating that there could be a correlation between the mannose and rhamnose biosynthetic pathways. From the literature reviews, Richuo, J.A. et al has demonstrated the biosynthesis pathways of biofilm exopolysaccharide in *Burkholderia cepacia* IST408. This bacteria has previously been reported its exopolysaccharide was consisted of glucose, galactose, mannose, rhamnose and glucoronic acid, which similar to our finding in *B. pseudomallei,* and required the nucleotide activated sugar as precursors for the synthesis (144). Interestingly, from the synthesis of nucleotide sugar precursors for the exopolysaccharide pathway of *B. cepacia* IST408, it was reported that UDP-galactose is synthesised from UDP-glucose and that GDP-mannose is a precursor for GDP-rhamnose synthesis. Comparative analysis of possible exopolysaccharide biosynthesis enzymes annotated in each step of *B. cepacia* in *B. pseudomallei* was performed by database searching, according to the annotated information of the enzymes in metabolic pathways of *B. pseudomallei*, to determine whether the enzymes involved in activated sugar precursor formation were presented (appendix C). As expected, all of the enzymes needed for the synthesis of activated sugar precursors for the exopolysaccharide biosynthesis were identified in *B. pseudomallei*.

The possible biosynthetic pathway of the nucleotide sugar precursors for biofilm exopolysaccharides production in *B. pseudomallei* according to the work of Richuo J.A. is postulated with all of the annotated enzymes as shown in Figure 6.1. To verify whether our genes of interest may involve in regulations of these exopolysaccharide biosynthetic enzymes, 150 bps upstream regions of each enzyme were used to predict for *bps*I-, *rpo*N- and *rpo*S-dependent promoters by using the Hidden Markov Model (HMM) based method as previously described by Osiriphun, Y (145), with prediction training sets created from *bps*I- and *rpo*S-dependent genes in *B. pseudomallei* (145, 146), while the *rpo*N training set was created from σ54-dependent promoter sequences of *E. coli* (147) (appendix C).

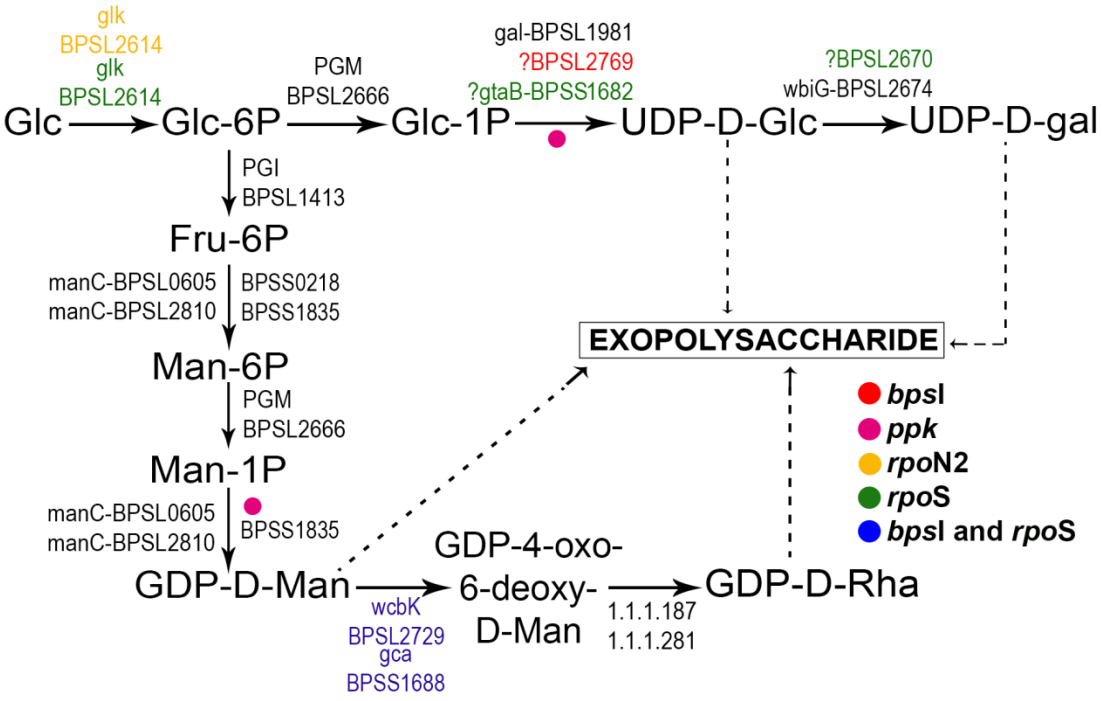

Figure 6.1 Postulated biosynthetic pathways of the nucleotide sugar precursors for the biofilm exopolysaccharides. Enzymes predicted to be under *bps*I regulation (red abbreviations), *rpo*N2 regulation (yellow abbreviations), *rpo*S regulation (green abbreviations) and both *bps*I and *rpo*S regulation (blue abbreviation) are indicated. The steps postulated to be involved with *ppk* function are illustrated with pink spots.

BPSL2769 and wcbK–BPSL2729 or gca-BPSS1688, which is GDP (GCP) -mannose-4,6-dehydratase, were predicted to be under *bps*I regulation because all of them were predictably found to have a lux box-like promoter of the *bps*I gene (Appendix C). BPSL2769 or UTP glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) is responsible for converting glucose-1-phosphate into UDP-D-glucose. While the other two enzymes, wcbK–BPSL2729 and gca-BPSS1688 (GDP-mannose-4,6-dehydratase (EC 4.2.1.47)), are responsible for converting GDP-mannose into GDP-4-oxo-6-deoxy-D-mannose, which is further converted into GDP-rhamnose by GDP-4-dehydro-6-deoxy-D-mannose reductase (EC 1.1.1.281). Corresponding to our observation, the carbohydrate contents found in the *bps*I mutant demonstrated that glucose and galactose contents were slightly reduced and mannose was accumulated. As the two predicted enzymes were found in front of glucose and galactose synthesis, the lack of *bps*I may result in the reduction of these two sugars. Moreover, wcbK–BPSL2729 and gca-BPSS1688 are responsible for converting UDP-mannose into UDP-rhamnose, thus the mannose accumulation may result from dysfunction of this gene. This finding suggests that these two enzymes might be under *bps*I regulation.

There are only three enzymes among all of the postulated enzymes having a chance to be under *bps*I control. However, many of these enzymes, including glucokinase (glk-BPSL2614), UDP-glucose-1-phosphate uridylyltransferase (gtaB-BPSS1682), UDP-glucose 4-epimerase (BPSL2670) and GDP-mannose-4,6-dehydratase (BPSL2729) (Appendix C), were predictably identified the *rpo*S-dependent promoter in front of their starting site, which all of them are involved in either the conversion of UDP-glucose into UPD-galactose or the conversion of GDP-mannose into GDP-rhamnose, especially glucokinase (glk), which functions in the first step of the pathway (Figure 6.1). Both BPSL2769 and gtaB-BPSL1682 predicting to be under *rpo*S control involve in the conversion of glucose-1-phosphate into UDP-glucose. Remarkably, wcbK-BPSL2729 and gca-BPSS1688 were previously predicted to be under *bps*I regulation, they were predictably found to have *rpo*S promoters in front of lux box-like promoters as well. As previously mentioned, *rpo*S has the positive regulation on *bpsI* expression in *B. pseudomallei* (140), thus this supports the assumption that *rpo*S has the superior impact on exopolysaccharide synthesis than *bps*I.

The carbohydrate components of the *rpo*N2 and *rpo*S mutant biofilm are similar in quantities and types. From the promoter prediction of *rpo*N-dependent promoter retrieved from *E. coli* (147), there is only one enzyme predicted to have the *rpo*N-dependent promoter, which is glucokinase (glk-BPSL2614). This possible promoter was found in front of the *rpo*S-dependent promoter previously identified (Appendix C). As shown in Figure 5.13, the carbohydrate contents of *rpo*N2 and *rpo*S mutant biofilms show reciprocal ratios, but the *rpo*N2 mutant contains little higher quantity than the *rpo*S mutant. This result corresponds well with the promoter prediction of *rpo*N2- and *rpo*S-dependent promoters found that *rpo*S predictably regulates many enzymes than *rpo*N2 resulting in the higher effects on exopolysaccharide production of the biofilm.

This observation of *bps*I-, *rpo*N2- and *rpo*S-dependent promoters supports our results that *bps*I, *rpo*N2 and *rpo*S may control the transcription of some biosynthetic enzymes essential to the production of activated sugar precursors for exopolysaccharide biosynthesis, especially *rpo*S, which should be involved in every carbohydrate synthesis, resulting in the significant decreases in all of the carbohydrate contents. Although *rpo*N2 has shown a strong impact on the exopolysaccharide contents as well, it would rather have less effect on this mechanism than *rpo*S which may due to the only one possible regulated enzyme. Meanwhile, *bps*I has specific regulation only on glucose, galactose and rhamnose, which has resulted in the moderate production of biofilm exopolysaccharide. Interestingly, a correlation between quorum sensing and rhamnose synthesis, which is an important polysaccharide in biofilm formation and found to be disappeared in the *bps*I mutant, has been reported. Shrout, J.D. *et al.* have shown that *P. aeruginosa* uses acyl-homoserine lactone signals during cell-cell communication to coordinate the expression of genes responsible for the production of polysaccharides in the biofilm as well as other virulent factors (148). In agreement with our observation, this finding confirms that *bps*I is not only involved in quorum sensing but also in the control of polysaccharide synthesis, especially in rhamnose production. In addition, Gamage has described the function of the *bps*I gene in cell-cell communication for micro-colony formation and determination of the ultimate three-dimensional architecture of the mature biofilm (131). There are some evidences showing that *rpo*N2 and *rpo*S genes

have positive regulations on exopolysaccharide biosynthesis of *V. anguillarum* and *Agrobacterium* (135, 149, 150), nevertheless, the correlation between these two genes and the synthesis of biofilm exopolysaccharide was not been elucidated in *B. pseudomallei*. From our knowledge, this is the first report in which *rpo*N2 and *rpo*S genes have potentialities in particular functions on exopolysaccharide biosynthesis through the regulation of enzymes important for the production of activated sugar precursors.

The carbohydrate content ratio found in the *ppk* mutant biofilm evidently showed significant reduction. Since Tunpiboonsak, S., has postulated the *ppk* functions in supplementing energy for all of the biofilm formation processes, including biofilm formation, maturation and extracellular matrix accumulation (20), this explicit alteration of the exopolysaccharide content ratio supports the assumption of *ppk* function in biofilm stages that *ppk* gene should have particular roles in activation of the sugar precursors at the specific steps as shown in figure 6.1 (pink spots). Polyphosphate kinase is an enzyme responsible for synthesis of inorganic polyphosphate from ATP, which is further used as precursors for synthesis of the activated sugars; thus it is possible that the mutation of this gene may result in the un-activated sugars. From this reason, the exopolysaccharide framework could not be formed and would cause a further dramatic impact on antibiotic resistance of the mutant. Interestingly, the *P. aeruginosa ppk* mutant has also been shown to be deficient in rhamnolipid production and formation and differentiation of biofilm as well as displaying aberrant quorum sensing (106). This evidence supports our assumption that *ppk* gene should involve in the formation of activated sugar precursors of exopolysaccharide production in the biofilm stage of *B. pseudomallei.* In summary, the exopolysaccharides of the biofilm matrix in *B. pseudomallei* are composed of following three major carbohydrate types: glucose, galactose and mannose, which could be controlled by the investigated genes, including *bps*I, *ppk*, *rpo*N2 and *rpo*S genes.

CHAPTER VII
CONCLUSIONS

We studied the characteristic of biofilm formation among *B. pseudomallei* wild type and four mutants in terms of architectures and protection ability in an early biofilm stage. Apparently, all of the genes of interest, including *bps*I, *ppk*, *rpo*N2 and *rpo*S genes have specific roles in the regulation of biofilm formation resulting in the distinct biofilm characters, including lost of biofilm density and micro-colony formation, reduction of biofilm thickness and attachment of biofilm-forming *B. pseudomallei* and exopolysaccharide production in biofilm EPS. Interestingly, the different media result in distinct induction in biofilm formation, which MVBM is more suitable than LB medium for the studying of attaching-biofilm characters. Additionally, every *B. pseudomallei* mutant particularly lost their antibiotic resistance ability corresponding with the exopolysaccharide production intensity calculated by CLSM.

The exopolysaccharide production in the biofilm of *B. pseudomallei* mutants from GC-MS remarkably showed the particular production ability reciprocal to the reduction of drug resistance as well. The same carbohydrate contents of exopolysaccharide in EPS from biofilm all of *B. pseudomallei* strains have been identified, composing of glucose, galactose, mannose, and rhamnose. However, the ratios of each carbohydrate component in the exopolysaccharide of biofilm from *B. pseudomallei* mutant strains are different, in which the *bps*I gene could play specific function in rhamnose synthesis and the connection between mannose and rhamnose biosynthetic pathways can be observed in all of the mutants, particularly for the *bps*I gene mutant. Although rhamnose production cannot be detected in the *bps*I mutant and the accumulation of mannose is significantly increased, this phenomenon cannot compensate the ability of drug resistance by this mutant. Therefore, it may imply that mannose is not the main composition of exopolysaccharide that contribute to strengthen the biofilm structure in terms of a barrier for antibiotic diffusion.

Meanwhile, the *ppk*, *rpo*N2 and *rpo*S genes uniquely influence the synthesis of all of the carbohydrate contents. As the significant reduction of glucose, galactose and rhamnose consistent with the decrement of antibiotic restriction in all mutants had been found, indicating that these three carbohydrate contents, including glucose, galactose and rhamnose, should play an important role in structure skeleton of *B. pseudomallei* biofilm which could be involved in the restriction of antibiotic diffusion. Most notably, to our knowledge, this is the first study in which the *rpo*N2 and *rpo*S gene displayed the particular functions on exopolysaccharide biosynthesis in *B. pseudomallei*, which is relevant to drug resistance. From our finding, they provide insight into the correlation between biofilm extracellular matrix and antibiotic resistance in *B. pseudomallei*, in which the exopolysaccharides should be the main components in creating the diffusion barrier for antibiotics, although the other EPS components may collaborate in order to raise antibiotic resistance in the biofilm.

The postulation of exopolysaccharide synthesis pathways in *B. pseudomallei* has been proposed. All of these investigated genes have a possibility to regulate the transcription of those enzymes responsible for the synthesis of activated sugar precursors, including GDP (GCP) -mannose-4,6-dehydratase (BPSL2769, wcbK–BPSL2729 or gca-BPSS1688), glucokinase (glk-BPSL2614), UDP-glucose-1-phosphate uridylyltransferase (gtaB-BPSS1682), and UDP-glucose 4-epimerase (BPSL2670), of the biofilm exopolysaccharide in *B. pseudomallei*. However, the molecular perception of regulation of exopolysaccharide synthesis through these genes remains to be investigated. This knowledge may contribute to the understanding of antibiotic resistance mechanism in the biofilm of *B. pseudomallei.* Therefore, an important goal for future studies is to determine the molecular insight of such genes on exopolysaccharide production and the roles of other EPS components in drug resistance of the biofilm.

CHAPTER VIII
SUPPLEMENTARY

8.1 Bacterial surviving profiles of all of *B. pseudomallei* strains

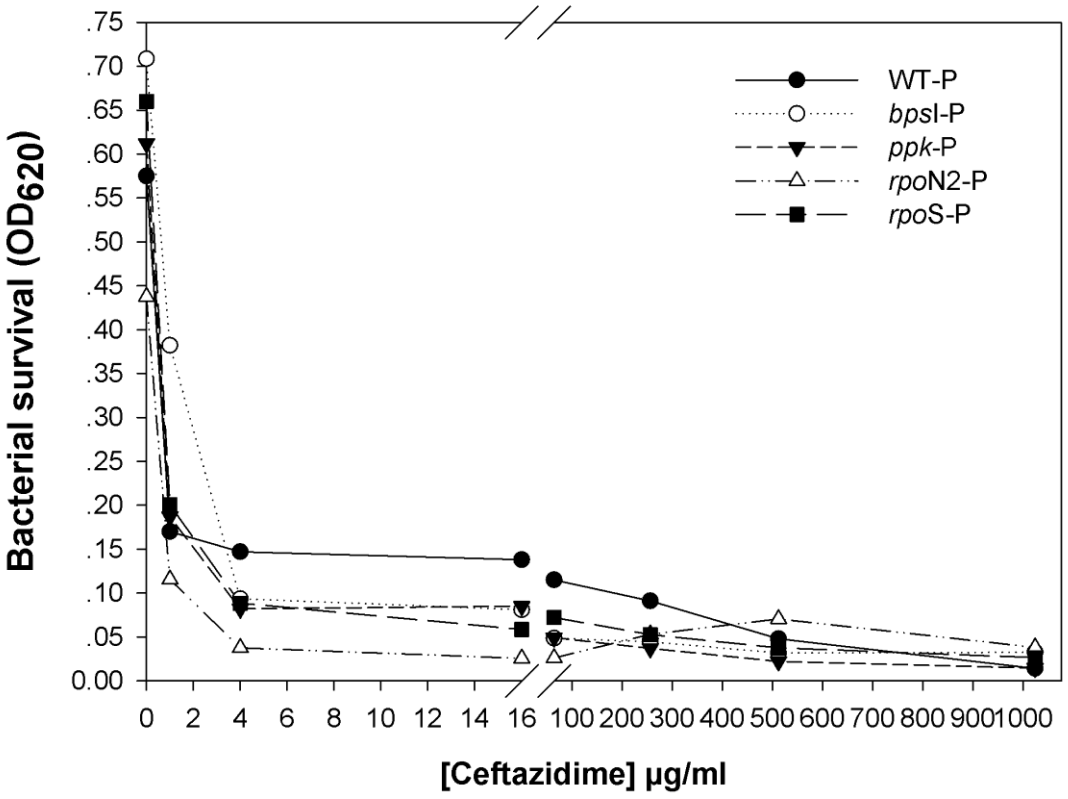

Figure 8.1 Bacterial survival (OD_{620}) at various CAZ concentrations in both planktonic and biofilm conditions

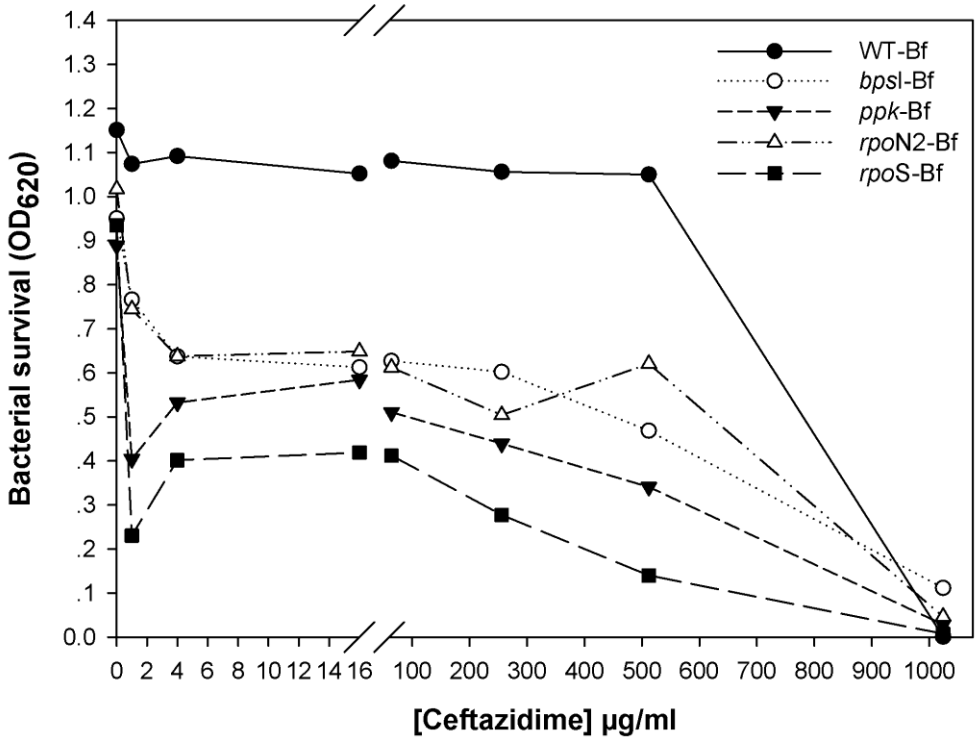

Figure 8.1 (cont.) Bacterial survival (OD_{620}) at various CAZ concentrations in both planktonic and biofilm conditions

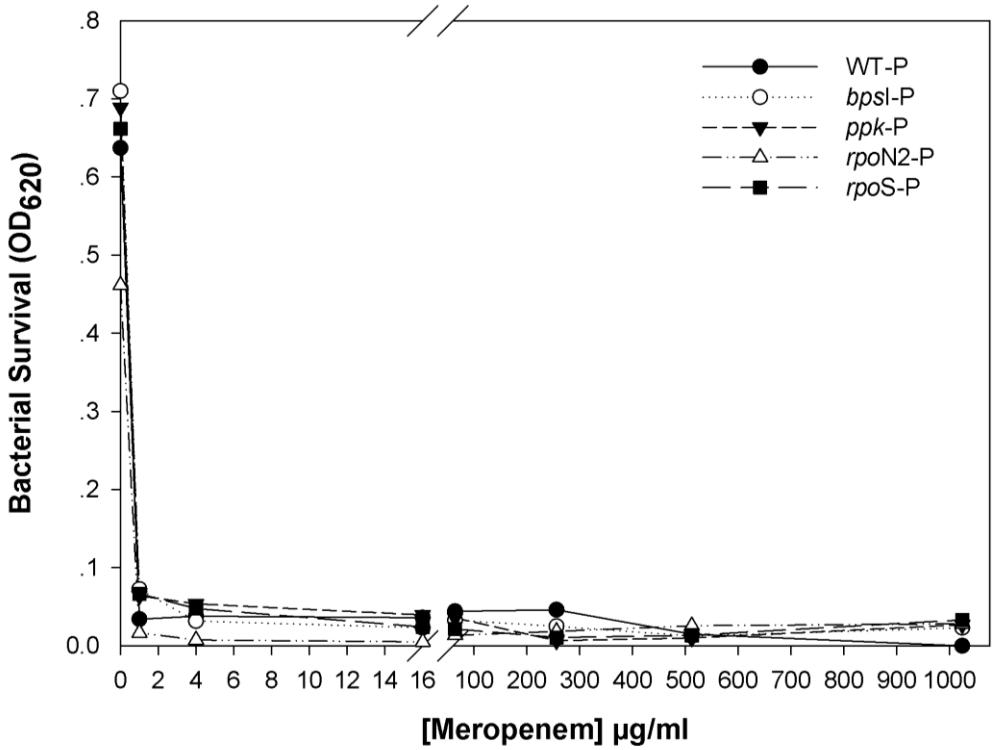

Figure 8.2 Bacterial survival (OD_{620}) at various MRP concentrations in both planktonic and biofilm conditions

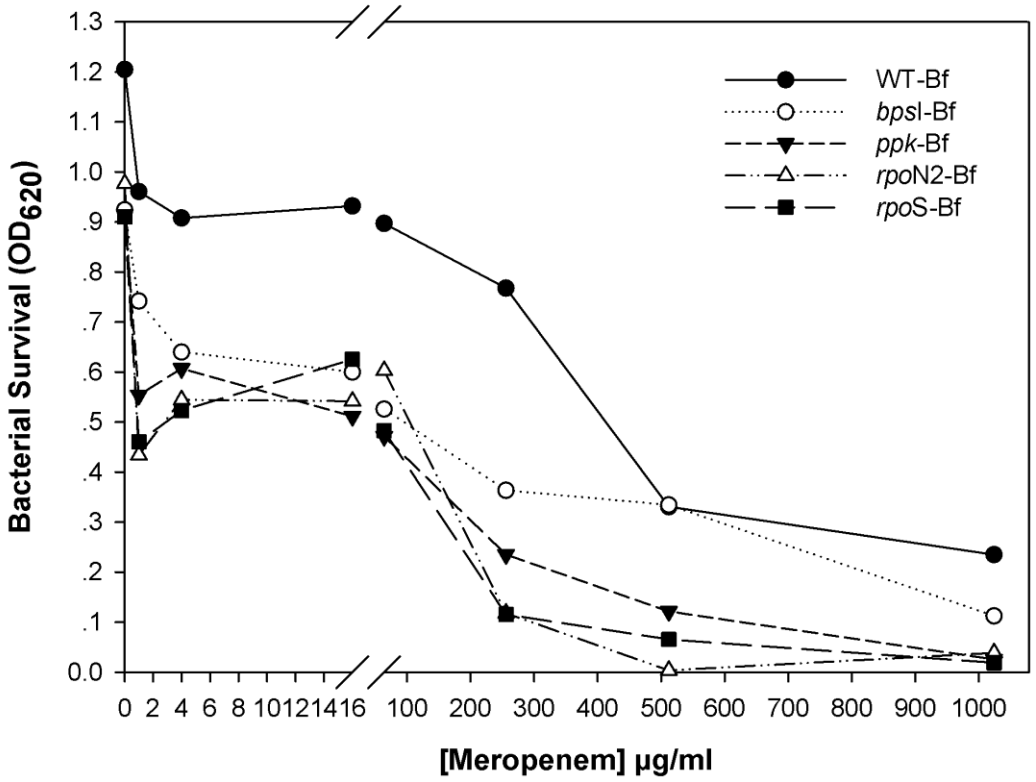

Figure 8.2 (cont.) Bacterial survival (OD_{620}) at various MRP concentrations in both planktonic and biofilm conditions

8.2 Summarized weights of all monopolysaccharide types

Table 8.1 Total peak areas of all of carbohydrate contents. Raw data of total peak areas of every monosaccharide in *B. pseudomallei* strains for three experiments

Experiments	**Total peak areas**				
Sugar types / **Bp strains**	**Glucose** RT = 6.90, 7.39	**Galactose** RT = 5.30, 5.85, 5.98, 6.44, 9.57	**Mannose** RT = 5.20, 5.71	**Rhamnose** RT = 2.59, 2.71	**Myo-inositol** RT = 12.04
WTPP844 (1)	16.93	16.40	11.37	4.45	4.82
WTPP844 (2)	21.77	26.27	19.41	3.84	5.85
WTPP844 (3)	25.20	24.68	24.02	4.97	9.63
*bps*IM (1)	24.91	17.95	39.56	0.00	6.05
*bps*IM (2)	24.59	22.69	36.93	0.00	5.91
*bps*IM (3)	24.21	21.83	39.47	0.00	13.11
*ppk*M (1)	14.54	15.64	16.88	0.77	8.61
*ppk*M (2)	25.42	25.19	28.70	1.37	7.53
*ppk*M (3)	27.65	22.82	31.23	2.59	13.72
*rpo*N2M (1)	24.49	23.12	36.28	0.87	6.47
*rpo*N2M (2)	27.52	21.37	36.52	0.71	7.76
*rpo*N2M (3)	29.34	19.19	43.14	0.88	6.59
*rpo*SM (1)	21.06	18.80	27.81	0.96	4.97
*rpo*SM (2)	27.73	22.3	37.61	0.94	7.11
*rpo*SM (3)	26.01	20.18	41.46	0.85	6.39

Table 8.2 Detector response factors (dRF) of each independent experiment

Standard Monosaccharides	Detector response factors (dRF)		
	(1)	(2)	(3)
Glucose	0.388	0.455	0.359
Galactose	0.303	0.291	0.279
Mannose	0.376	0.260	0.303
Rhamnose	0.269	0.148	0.121

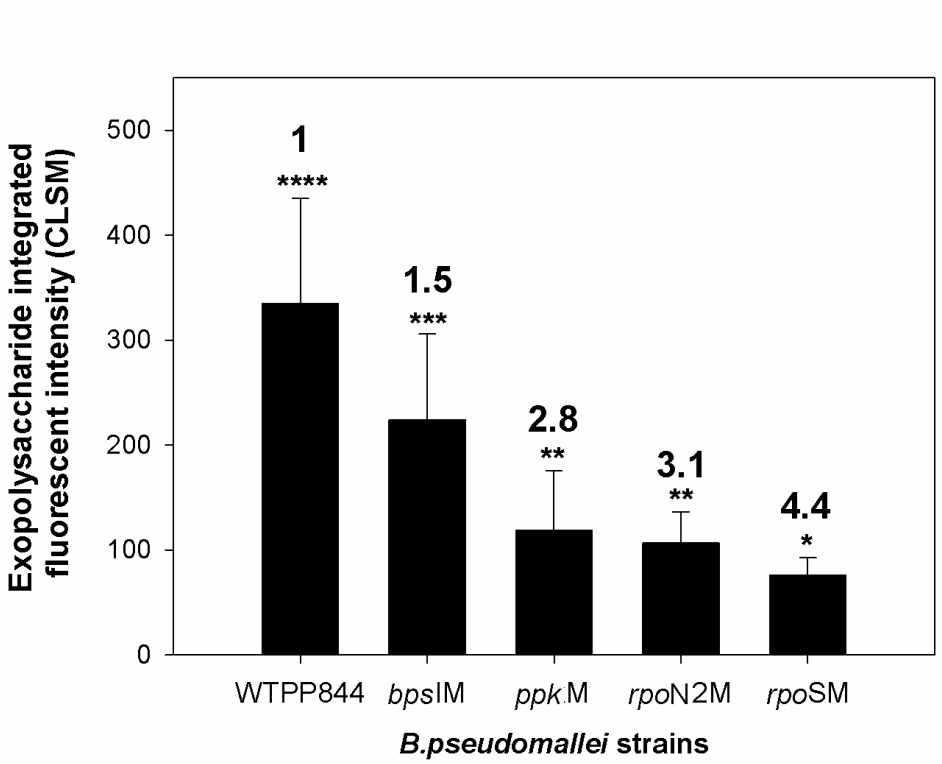

Figure 8.3 The specific biofilm capacity factors of *B. pseudomallei* strains

REFERENCES

1. Cheng, A.C. and B.J. Currie, *Melioidosis: epidemiology, pathophysiology, and management.* Clin Microbiol Rev, 2005. **18**(2): p. 383-416.
2. White, N.J., *Melioidosis.* Lancet, 2003. **361**(9370): p. 1715-22.
3. Wiersinga, W.J., et al., *Melioidosis: insights into the pathogenicity of Burkholderia pseudomallei.* Nat Rev Microbiol, 2006. **4**(4): p. 272-82.
4. Dance, D.A., *Ecology of Burkholderia pseudomallei and the interactions between environmental Burkholderia spp. and human-animal hosts.* Acta Trop, 2000. **74**(2-3): p. 159-68.
5. Wiersinga, W.J., B.J. Currie, and S.J. Peacock, *Melioidosis.* N Engl J Med, 2012. **367**(11): p. 1035-44.
6. Ulrich, R.L., et al., *Role of quorum sensing in the pathogenicity of Burkholderia pseudomallei.* J Med Microbiol, 2004. **53**(Pt 11): p. 1053-64.
7. Dance, D.A., *Melioidosis: the tip of the iceberg?* Clin Microbiol Rev, 1991. **4**(1): p. 52-60.
8. Brent, A.J., et al., *Misdiagnosing melioidosis.* Emerg Infect Dis, 2007. **13**(2): p. 349-51.
9. Patel, N., et al., *Development of vaccines against Burkholderia pseudomallei.* Front Microbiol, 2011. **2**: p. 198.
10. Sawasdidoln, C., et al., *Growing Burkholderia pseudomallei in biofilm stimulating conditions significantly induces antimicrobial resistance.* PLoS One, 2010. **5**(2): p. e9196.
11. Costerton, J.W., et al., *Microbial biofilms.* Annu Rev Microbiol, 1995. **49**: p. 711-45.
12. Bazaka, K., et al., *Bacterial extracellular polysaccharides.* Adv Exp Med Biol, 2011. **715**: p. 213-26.

13. Reckseidler-Zenteno, S.L., *Capsular Polysaccharides Produced by the Bacterial Pathogen Burkholderia pseudomallei,* in *The Complex World of Polysaccharides*, D.N. Karunaratne, Editor. 2012, InTech.
14. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. **284**(5418): p. 1318-22.
15. Pibalpakdee, P., et al., *Diffusion and activity of antibiotics against Burkholderia pseudomallei biofilms.* Int J Antimicrob Agents, 2012. **39**(4): p. 356-9.
16. Taweechaisupapong, S., et al., *Virulence of Burkholderia pseudomallei does not correlate with biofilm formation.* Microb Pathog, 2005. **39**(3): p. 77-85.
17. Songsri, J., et al., *Tn5-OT182 should not be used to identify genes involved in biofilm formation in Burkholderia pseudomallei.* Southeast Asian J Trop Med Public Health, 2012. **43**(1): p. 124-8.
18. Lumjiaktase, P., et al., *Quorum sensing regulates dpsA and the oxidative stress response in Burkholderia pseudomallei.* Microbiology, 2006. **152**(Pt 12): p. 3651-9.
19. Subsin, B., et al., *Role of the stationary growth phase sigma factor RpoS of Burkholderia pseudomallei in response to physiological stress conditions.* J Bacteriol, 2003. **185**(23): p. 7008-14.
20. Tunpiboonsak, S., et al., *Role of a Burkholderia pseudomallei polyphosphate kinase in an oxidative stress response, motilities, and biofilm formation.* J Microbiol, 2010. **48**(1): p. 63-70.
21. Cochran, W.L., et al., *Role of RpoS and AlgT in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide and monochloramine.* J Appl Microbiol, 2000. **88**(3): p. 546-53.
22. Ito, A., et al., *Significance of rpoS during maturation of Escherichia coli biofilms.* Biotechnol Bioeng, 2008. **99**(6): p. 1462-71.
23. Drozd, M., et al., *Contribution of TAT system translocated PhoX to Campylobacter jejuni phosphate metabolism and resilience to environmental stresses.* PLoS One, 2011. **6**(10): p. e26336.
24. Taylor, P.K., A.T. Yeung, and R.E. Hancock, *Antibiotic resistance in Pseudomonas aeruginosa biofilms: towards the development of novel anti-biofilm therapies.* J Biotechnol, 2014. **191**: p. 121-30.

25. Whitmore, A. and C.S. Krishnaswami, *An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon.* Indian Med Gaz, 1912. **92**: p. 262-7.
26. Rogul, M., et al., *Nucleic acid similarities among Pseudomonas pseudomallei, Pseudomonas multivorans, and Actinobacillus mallei.* J Bacteriol, 1970. **101**(3): p. 827-35.
27. Yabuuchi, E., et al., *Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov.* Microbiol Immunol, 1992. **36**(12): p. 1251-75.
28. Smith, M.D., et al., *Arabinose assimilation defines a nonvirulent biotype of Burkholderia pseudomallei.* Infect Immun, 1997. **65**(10): p. 4319-21.
29. Holden, M.T., et al., *Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei.* Proc Natl Acad Sci U S A, 2004. **101**(39): p. 14240-5.
30. Muttarak, M., et al., *Spectrum of imaging findings in melioidosis.* Br J Radiol, 2009. **82**(978): p. 514-21.
31. Suputtamongkol, Y., et al., *The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand.* Int J Epidemiol, 1994. **23**(5): p. 1082-90.
32. Leelarasamee, A. and S. Bovornkitti, *Melioidosis: review and update.* Rev Infect Dis, 1989. **11**(3): p. 413-25.
33. Kunakorn, M., P. Jayanetra, and D. Tanphaichitra, *Man-to-man transmission of melioidosis.* Lancet, 1991. **337**(8752): p. 1290-1.
34. Dance, D.A., et al., *The antimicrobial susceptibility of Pseudomonas pseudomallei. Emergence of resistance in vitro and during treatment.* J Antimicrob Chemother, 1989. **24**(3): p. 295-309.
35. Jenney, A.W., et al., *Antibiotic susceptibility of Burkholderia pseudomallei from tropical northern Australia and implications for therapy of melioidosis.* Int J Antimicrob Agents, 2001. **17**(2): p. 109-13.
36. Wuthiekanun, V. and S.J. Peacock, *Management of melioidosis.* Expert Rev Anti Infect Ther, 2006. **4**(3): p. 445-55.

37. Dance, D.A., *Melioidosis as an emerging global problem.* Acta Trop, 2000. **74**(2-3): p. 115-9.
38. Howe, C., A. Sampath, and M. Spotnitz, *The pseudomallei group: a review.* J Infect Dis, 1971. **124**(6): p. 598-606.
39. Jeddeloh, J.A., et al., *Biodefense-driven murine model of pneumonic melioidosis.* Infect Immun, 2003. **71**(1): p. 584-7.
40. Currie, B.J. and S.P. Jacups, *Intensity of rainfall and severity of melioidosis, Australia.* Emerg Infect Dis, 2003. **9**(12): p. 1538-42.
41. Currie, B.J., et al., *The epidemiology of melioidosis in Australia and Papua New Guinea.* Acta Trop, 2000. **74**(2-3): p. 121-7.
42. Green, R.N. and P.G. Tuffnell, *Laboratory acquired melioidosis.* Am J Med, 1968. **44**(4): p. 599-605.
43. Schlech, W.F., 3rd, et al., *Laboratory-acquired infection with Pseudomonas pseudomallei (melioidosis).* N Engl J Med, 1981. **305**(19): p. 1133-5.
44. Ashdown, L.R., *Melioidosis and safety in the clinical laboratory.* J Hosp Infect, 1992. **21**(4): p. 301-6.
45. Holland, D.J., et al., *Cystic Fibrosis and Burkholderia pseudomallei Infection: An Emerging Problem?* Clin Infect Dis, 2002. **35**(12): p. e138-40.
46. McCormick, J.B., et al., *Human-to-human transmission of Pseudomonas pseudomallei.* Ann Intern Med, 1975. **83**(4): p. 512-3.
47. Abbink, F.C., J.M. Orendi, and A.J. de Beaufort, *Mother-to-child transmission of Burkholderia pseudomallei.* N Engl J Med, 2001. **344**(15): p. 1171-2.
48. Halder, D., et al., *Neonatal meningitis and septicaemia caused by Burkholderia pseudomallei.* Ann Trop Paediatr, 1998. **18**(2): p. 161-4.
49. Suputtamongkol, Y., et al., *Risk factors for melioidosis and bacteremic melioidosis.* Clin Infect Dis, 1999. **29**(2): p. 408-13.
50. Wang, Y.S., C.H. Wong, and A. Kurup, *Cutaneous melioidosis and necrotizing fasciitis caused by Burkholderia pseudomallei.* Emerg Infect Dis, 2003. **9**(11): p. 1484-5.
51. Chaowagul, W., et al., *Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand.* J Infect Dis, 1989. **159**(5): p. 890-9.
52. Currie, B.J., et al., *Neurological melioidosis.* Acta Trop, 2000. **74**(2-3): p. 145-51.

53. Dance, D.A., et al., *Acute suppurative parotitis caused by Pseudomonas pseudomallei in children.* J Infect Dis, 1989. **159**(4): p. 654-60.
54. Currie, B.J., et al., *Melioidosis: acute and chronic disease, relapse and re-activation.* Trans R Soc Trop Med Hyg, 2000. **94**(3): p. 301-4.
55. Haase, A., et al., *RAPD analysis of isolates of Burkholderia pseudomallei from patients with recurrent melioidosis.* Epidemiol Infect, 1995. **115**(1): p. 115-21.
56. Chaowagul, W., et al., *Relapse in melioidosis: incidence and risk factors.* J Infect Dis, 1993. **168**(5): p. 1181-5.
57. Inglis, T.J., et al., *Comparison of diagnostic laboratory methods for identification of Burkholderia pseudomallei.* J Clin Microbiol, 2005. **43**(5): p. 2201-6.
58. Foong, Y.C., M. Tan, and R.S. Bradbury, *Melioidosis: a review.* Rural Remote Health, 2014. **14**(4): p. 2763.
59. Ashdown, L.R., *An improved screening technique for isolation of Pseudomonas pseudomallei from clinical specimens.* Pathology, 1979. **11**(2): p. 293-7.
60. Francis, A., et al., *An improved selective and differential medium for the isolation of Burkholderia pseudomallei from clinical specimens.* Diagn Microbiol Infect Dis, 2006. **55**(2): p. 95-9.
61. Howard, K. and T.J. Inglis, *Novel selective medium for isolation of Burkholderia pseudomallei.* J Clin Microbiol, 2003. **41**(7): p. 3312-6.
62. Peacock, S.J., et al., *Comparison of Ashdown's medium, Burkholderia cepacia medium, and Burkholderia pseudomallei selective agar for clinical isolation of Burkholderia pseudomallei.* J Clin Microbiol, 2005. **43**(10): p. 5359-61.
63. Glass, M.B. and T. Popovic, *Preliminary evaluation of the API 20NE and RapID NF plus systems for rapid identification of Burkholderia pseudomallei and B. mallei.* J Clin Microbiol, 2005. **43**(1): p. 479-83.
64. Inglis, T.J., et al., *Potential misidentification of Burkholderia pseudomallei by API 20NE.* Pathology, 1998. **30**(1): p. 62-4.
65. Lowe, P., C. Engler, and R. Norton, *Comparison of automated and nonautomated systems for identification of Burkholderia pseudomallei.* J Clin Microbiol, 2002. **40**(12): p. 4625-7.

66. Appassakij, H., et al., *Diagnostic value of the indirect hemagglutination test for melioidosis in an endemic area.* Am J Trop Med Hyg, 1990. **42**(3): p. 248-53.

67. Khupulsup, K. and B. Petchclai, *Application of indirect hemagglutination test and indirect fluorescent antibody test for IgM antibody for diagnosis of melioidosis in Thailand.* Am J Trop Med Hyg, 1986. **35**(2): p. 366-9.

68. Kanaphun, P., et al., *Serology and carriage of Pseudomonas pseudomallei: a prospective study in 1000 hospitalized children in northeast Thailand.* J Infect Dis, 1993. **167**(1): p. 230-3.

69. Brook, M.D., B. Currie, and P.M. Desmarchelier, *Isolation and identification of Burkholderia pseudomallei from soil using selective culture techniques and the polymerase chain reaction.* J Appl Microbiol, 1997. **82**(5): p. 589-96.

70. Dharakul, T., et al., *Detection of Burkholderia pseudomallei DNA in patients with septicemic melioidosis.* J Clin Microbiol, 1996. **34**(3): p. 609-14.

71. Kunakorn, M. and R.B. Markham, *Clinically practical seminested PCR for Burkholderia pseudomallei quantitated by enzyme immunoassay with and without solution hybridization.* J Clin Microbiol, 1995. **33**(8): p. 2131-5.

72. Rattanathongkom, A., R.W. Sermswan, and S. Wongratanacheewin, *Detection of Burkholderia pseudomallei in blood samples using polymerase chain reaction.* Mol Cell Probes, 1997. **11**(1): p. 25-31.

73. Sirisinha, S., et al., *Recent developments in laboratory diagnosis of melioidosis.* Acta Trop, 2000. **74**(2-3): p. 235-45.

74. Sura, T., et al., *Polymerase chain reaction for the detection of Burkholderia pseudomallei.* Diagn Microbiol Infect Dis, 1997. **29**(3): p. 121-7.

75. Haase, A., et al., *Evaluation of PCR for diagnosis of melioidosis.* J Clin Microbiol, 1998. **36**(4): p. 1039-41.

76. Inglis, T.J., et al., *Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis for rapid confirmation of Burkholderia pseudomallei in septicemic melioidosis.* Am J Trop Med Hyg, 2012. **86**(6): p. 1039-42.

77. Chetchotisakd, P., et al., *Randomized, double-blind, controlled study of cefoperazone-sulbactam plus cotrimoxazole versus ceftazidime plus cotrimoxazole for the treatment of severe melioidosis.* Clin Infect Dis, 2001. **33**(1): p. 29-34.
78. Simpson, A.J., et al., *Comparison of imipenem and ceftazidime as therapy for severe melioidosis.* Clin Infect Dis, 1999. **29**(2): p. 381-7.
79. Thamprajamchit, S., P. Chetchotisakd, and B. Thinkhamrop, *Cefoperazone/sulbactam + co-trimoxazole vs ceftazidime + co-trimoxazole in the treatment of severe melioidosis: a randomized, double-blind, controlled study.* J Med Assoc Thai, 1998. **81**(4): p. 265-71.
80. White, N.J., et al., *Halving of mortality of severe melioidosis by ceftazidime.* Lancet, 1989. **2**(8665): p. 697-701.
81. Suputtamongkol, Y., et al., *Ceftazidime vs. amoxicillin/clavulanate in the treatment of severe melioidosis.* Clin Infect Dis, 1994. **19**(5): p. 846-53.
82. Samuel, M. and T.Y. Ti, *Interventions for treating melioidosis.* Cochrane Database Syst Rev, 2002(4): p. CD001263.
83. Thibault, F.M., et al., *Antibiotic susceptibility of 65 isolates of Burkholderia pseudomallei and Burkholderia mallei to 35 antimicrobial agents.* J Antimicrob Chemother, 2004. **54**(6): p. 1134-8.
84. Cheng, A.C., et al., *Outcomes of patients with melioidosis treated with meropenem.* Antimicrob Agents Chemother, 2004. **48**(5): p. 1763-5.
85. Inglis, T.J., D.B. Rolim, and J.L. Rodriguez, *Clinical guideline for diagnosis and management of melioidosis.* Rev Inst Med Trop Sao Paulo, 2006. **48**(1): p. 1-4.
86. Holten, K.B. and E.M. Onusko, *Appropriate prescribing of oral beta-lactam antibiotics.* Am Fam Physician, 2000. **62**(3): p. 611-20.
87. Godfrey, A.J., et al., *Pseudomonas pseudomallei resistance to beta-lactam antibiotics due to alterations in the chromosomally encoded beta-lactamase.* Antimicrob Agents Chemother, 1991. **35**(8): p. 1635-40.
88. Vorachit, M., et al., *Antimicrobial resistance in Burkholderia pseudomallei.* Acta Trop, 2000. **74**(2-3): p. 139-44.

89. Kumarasinghe, G., C. Chow, and C. Chiu, *In vitro activity of meropenem against organisms causing serious infections in a Singapore hospital.* Int J Antimicrob Agents, 1997. **9**(2): p. 121-5.
90. Inglis, T.J., et al., *Comparison of the susceptibilities of Burkholderia pseudomallei to meropenem and ceftazidime by conventional and intracellular methods.* Antimicrob Agents Chemother, 2004. **48**(8): p. 2999-3005.
91. Eickhoff, T.C., et al., *Pseudomonas pseudomallei: susceptibility to chemotherapeutic agents.* J Infect Dis, 1970. **121**(2): p. 95-102.
92. Livermore, D.M., et al., *beta-Lactamase of Pseudomonas pseudomallei and its contribution to antibiotic resistance.* J Antimicrob Chemother, 1987. **20**(3): p. 313-21.
93. Ho, P.L., et al., *Characterization of a laboratory-generated variant of BPS beta-lactamase from Burkholderia pseudomallei that hydrolyses ceftazidime.* J Antimicrob Chemother, 2002. **50**(5): p. 723-6.
94. Cheung, T.K., et al., *Cloning and expression of class A beta-lactamase gene blaA(BPS) in Burkholderia pseudomallei.* Antimicrob Agents Chemother, 2002. **46**(4): p. 1132-5.
95. Moore, R.A., et al., *Efflux-mediated aminoglycoside and macrolide resistance in Burkholderia pseudomallei.* Antimicrob Agents Chemother, 1999. **43**(3): p. 465-70.
96. Smith, M.D., et al., *In-vitro activity of carbapenem antibiotics against beta-lactam susceptible and resistant strains of Burkholderia pseudomallei.* J Antimicrob Chemother, 1996. **37**(3): p. 611-5.
97. Huffam, S., et al., *Out of hospital treatment of patients with melioidosis using ceftazidime in 24 h elastomeric infusors, via peripherally inserted central catheters.* Trop Med Int Health, 2004. **9**(6): p. 715-7.
98. Vorachit, M., et al., *Resistance of Pseudomonas pseudomallei growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole.* Antimicrob Agents Chemother, 1993. **37**(9): p. 2000-2.
99. Woods, D.E., et al., *Current studies on the pathogenesis of melioidosis.* Microbes Infect, 1999. **1**(2): p. 157-62.

100. Fuqua, C. and E.P. Greenberg, *Listening in on bacteria: acyl-homoserine lactone signalling.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 685-95.

101. Meluleni, G.J., et al., *Mucoid Pseudomonas aeruginosa growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients.* J Immunol, 1995. **155**(4): p. 2029-38.

102. Reckseidler-Zenteno, S.L., et al., *Characterization of the type III capsular polysaccharide produced by Burkholderia pseudomallei.* J Med Microbiol, 2010. **59**(Pt 12): p. 1403-14.

103. Denisov, I., *[The polysaccharide component of Pseudomonas pseudomallei slime]*. Zh Mikrobiol Epidemiol Immunobiol, 1985(11): p. 23-6.

104. Cescutti, P., et al., *Structural study of the exopolysaccharide produced by a clinical isolate of Burkholderia cepacia.* Biochem Biophys Res Commun, 2000. **273**(3): p. 1088-94.

105. Reckseidler-Zenteno, S.L., *Capsular Polysaccharides Produced by the Bacterial Pathogen Burkholderia pseudomallei*, in *The Complex World of Polysaccharides*, D.D.N. Karunaratne, Editor. 2012, InTech. p. 127-152.

106. Rashid, M.H., et al., *Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9636-41.

107. Monds, R.D. and G.A. O'Toole, *The developmental model of microbial biofilms: ten years of a paradigm up for review.* Trends Microbiol, 2009. **17**(2): p. 73-87.

108. Loprasert, S., et al., *The Burkholderia pseudomallei oxyR gene: expression analysis and mutant characterization.* Gene, 2002. **296**(1-2): p. 161-9.

109. Lee, H.S., et al., *CdpA is a Burkholderia pseudomallei cyclic di-GMP phosphodiesterase involved in autoaggregation, flagellum synthesis, motility, biofilm formation, cell invasion, and cytotoxicity.* Infect Immun, 2010. **78**(5): p. 1832-40.

110. Yuen, C.W., et al., *Construction and characterization of a Burkholderia pseudomallei wzm deletion mutant.* J Microbiol Biotechnol, 2012. **22**(10): p. 1336-42.

111. Korbsrisate, S., et al., *The Burkholderia pseudomallei RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation.* FEMS Microbiol Lett, 2005. **252**(2): p. 243-9.

112. Engebrecht, J., K. Nealson, and M. Silverman, *Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri.* Cell, 1983. **32**(3): p. 773-81.

113. Zhang, H., K. Ishige, and A. Kornberg, *A polyphosphate kinase (PPK2) widely conserved in bacteria.* Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16678-83.

114. Shi, X., N.N. Rao, and A. Kornberg, *Inorganic polyphosphate in Bacillus cereus: motility, biofilm formation, and sporulation.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17061-5.

115. Rao, N.N., M.R. Gomez-Garcia, and A. Kornberg, *Inorganic polyphosphate: essential for growth and survival.* Annu Rev Biochem, 2009. **78**: p. 605-47.

116. Chen, W., R.J. Palmer, and H.K. Kuramitsu, *Role of polyphosphate kinase in biofilm formation by Porphyromonas gingivalis.* Infect Immun, 2002. **70**(8): p. 4708-15.

117. Saldias, M.S., et al., *Burkholderia cenocepacia requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages.* Infect Immun, 2008. **76**(3): p. 1059-67.

118. Hengge-Aronis, R., *Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase.* Microbiol Mol Biol Rev, 2002. **66**(3): p. 373-95, table of contents.

119. Irie, Y., et al., *Pseudomonas aeruginosa biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA.* Mol Microbiol, 2010. **78**(1): p. 158-72.

120. Wuthiekanun, V., et al., *Biochemical characteristics of clinical and environmental isolates of Burkholderia pseudomallei.* J Med Microbiol, 1996. **45**(6): p. 408-12.

121. Anuntagool, N., et al., *Lipopolysaccharide from nonvirulent Ara+ Burkholderia pseudomallei isolates is immunologically indistinguishable from*

lipopolysaccharide from virulent Ara- clinical isolates. Clin Diagn Lab Immunol, 1998. **5**(2): p. 225-9.

122. O'Toole, G.A., et al., *Genetic approaches to study of biofilms.* Methods Enzymol, 1999. **310**: p. 91-109.

123. Lam, J., et al., *Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis.* Infect Immun, 1980. **28**(2): p. 546-56.

124. Ceri, H., et al., *The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms.* J Clin Microbiol, 1999. **37**(6): p. 1771-6.

125. Friedman, L. and R. Kolter, *Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix.* J Bacteriol, 2004. **186**(14): p. 4457-65.

126. Merkle, R.K. and I. Poppe, *Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/mass spectrometry.* Methods Enzymol, 1994. **230**: p. 1-15.

127. Martin, C., et al., *Strategies for antimicrobial drug delivery to biofilm.* Curr Pharm Des, 2015. **21**(1): p. 43-66.

128. Bleton, J., et al., *Characterization of neutral sugars and uronic acids after methanolysis and trimethylsilylation for recognition of plant gums.* Journal of Chromatography A, 1996. **720**(1–2): p. 27-49.

129. Denisov, II, *[The polysaccharide component of Pseudomonas pseudomallei slime]*. Zh Mikrobiol Epidemiol Immunobiol, 1985(11): p. 23-6.

130. Friedman, L. and R. Kolter, *Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms.* Mol Microbiol, 2004. **51**(3): p. 675-90.

131. Gamage, A.M., et al., *N-Octanoylhomoserine lactone signalling mediated by the BpsI-BpsR quorum sensing system plays a major role in biofilm formation of Burkholderia pseudomallei.* Microbiology, 2011. **157**(Pt 4): p. 1176-86.

132. Sheldon, J.R., et al., *Role of rpoS in Escherichia coli O157:H7 strain H32 biofilm development and survival.* Appl Environ Microbiol, 2012. **78**(23): p. 8331-9.

133. Gualdi, L., L. Tagliabue, and P. Landini, *Biofilm formation-gene expression relay system in Escherichia coli: modulation of sigmaS-dependent gene expression by the CsgD regulatory protein via sigmaS protein stabilization.* J Bacteriol, 2007. **189**(22): p. 8034-43.

134. Wolfe, A.J., et al., *Vibrio fischeri sigma54 controls motility, biofilm formation, luminescence, and colonization.* Appl Environ Microbiol, 2004. **70**(4): p. 2520-4.

135. Hao, B., et al., *Role of alternative sigma factor 54 (RpoN) from Vibrio anguillarum M3 in protease secretion, exopolysaccharide production, biofilm formation, and virulence.* Appl Microbiol Biotechnol, 2013. **97**(6): p. 2575-85.

136. Thompson, L.S., et al., *The alternative sigma factor RpoN regulates the quorum sensing gene rhlI in Pseudomonas aeruginosa.* FEMS Microbiol Lett, 2003. **220**(2): p. 187-95.

137. Xu, K.D., et al., *Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed Pseudomonas aeruginosa biofilms.* FEMS Microbiol Lett, 2001. **199**(1): p. 67-71.

138. Singh, R., et al., *Penetration of antibiotics through Staphylococcus aureus and Staphylococcus epidermidis biofilms.* J Antimicrob Chemother, 2010. **65**(9): p. 1955-8.

139. Rodriguez-Martinez, J.M., S. Ballesta, and A. Pascual, *Activity and penetration of fosfomycin, ciprofloxacin, amoxicillin/clavulanic acid and co-trimoxazole in Escherichia coli and Pseudomonas aeruginosa biofilms.* Int J Antimicrob Agents, 2007. **30**(4): p. 366-8.

140. Wongtrakoongate, P., S. Tumapa, and S. Tungpradabkul, *Regulation of a quorum sensing system by stationary phase sigma factor RpoS and their co-regulation of target genes in Burkholderia pseudomallei.* Microbiol Immunol, 2012. **56**(5): p. 281-94.

141. Nimtz, M., et al., *Structure of an acidic exopolysaccharide of Burkholderia pseudomallei.* Eur J Biochem, 1997. **250**(2): p. 608-16.

142. Steinmetz, I., et al., *Exopolysaccharides of Burkholderia pseudomallei.* Acta Trop, 2000. **74**(2-3): p. 211-4.

143. Kawahara, K., S. Dejsirilert, and T. Ezaki, *Characterization of three capsular polysacharides produced by Burkholderia pseudomallei.* FEMS Microbiol Lett, 1998. **169**(2): p. 283-7.

144. Richau, J.A., J.H. Leitao, and I. Sa-Correia, *Enzymes leading to the nucleotide sugar precursors for exopolysaccharide synthesis in Burkholderia cepacia.* Biochem Biophys Res Commun, 2000. **276**(1): p. 71-6.

145. Osiriphun, Y., et al., *Identification and characterization of RpoS regulon and RpoS-dependent promoters in Burkholderia pseudomallei.* J Proteome Res, 2009. **8**(6): p. 3118-31.

146. Kiratisin, P. and S. Sanmee, *Roles and interactions of Burkholderia pseudomallei BpsIR quorum-sensing system determinants.* J Bacteriol, 2008. **190**(21): p. 7291-7.

147. Zhao, K., M. Liu, and R.R. Burgess, *Promoter and regulon analysis of nitrogen assimilation factor, sigma54, reveal alternative strategy for E. coli MG1655 flagellar biosynthesis.* Nucleic Acids Res, 2010. **38**(4): p. 1273-83.

148. Shrout, J.D., et al., *The contribution of cell-cell signaling and motility to bacterial biofilm formation.* MRS Bull, 2011. **36**(5): p. 367-373.

149. Ruffing, A.M. and R.R. Chen, *Transcriptome profiling of a curdlan-producing Agrobacterium reveals conserved regulatory mechanisms of exopolysaccharide biosynthesis.* Microb Cell Fact, 2012. **11**: p. 17.

150. Li, X., et al., *RpoS and indole signaling control the virulence of Vibrio anguillarum towards gnotobiotic sea bass (Dicentrarchus labrax) larvae.* PLoS One, 2014. **9**(10): p. e111801.

APPENDICES

APPENDIX A
LIST OF CHEMICALS AND INSTRUMENTS

All chemicals used in the experiments were analytical grade. Names and sources of chemicals are listed below.

1. Chemicals

Chemicals	**sources**
Absolute ethanol (C_2H_5OH)	Merck
Arabinose ($C_5H_{10}O_5$)	Sigma
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	Fluka
Citric acid anhydrous ($C_6H_8O_7$)	Fluka
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
Fucose ($C_6H_{12}O_5$)	Sigma
Galactose ($C_6H_{12}O_6$)	Sigma
Glucose ($C_6H_{12}O_6$)	Sigma
Glutaraldehyde $OHC(CH_2)_3CHO$	Sigma
Hexane (C_6H_{14})	BDHprolabo
Hydrochloric acid (HCl)	Scharlau
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Mannose ($C_6H_{12}O_6$)	Sigma
Methanol (CH_3OH)	BDHprolabo
Methanolic hydrochloric acid (Met-HCl)	Sigma
Myo-inosital ($C_6H_{12}O_6$)	Sigma
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Prolong Gold Antifade reagent	Invitrogen
Pyridine (C_5H_5N)	Merck

Rhamnose ($C_6H_{12}O_5$)	Sigma
Ribose ($C_5H_{10}O_5$)	Sigma
Sodium ammonium hydrogen phosphate ($NaNH_4HPO_4.4H_2O$)	Merck
Sodium bicarbonate ($NaHCO_3$)	J.T. Baker
Sodium chloride (NaCl)	BDHprolabo
Sodium ethylene diamine tetra-acetic acid (Na_2EDTA)	Merck
Sodium hydrogen phosphate (Na_2HPO_4)	Merck
Sodium hydroxide (NaOH)	Merck
Tri-Sil reagent (HMDS+TMCS/pyridine)	Pierce
Xylose ($C_5H_{10}O_5$)	Sigma

2. Media, dyes, antibiotics and supplements

Media, dyes, antibiotics and supplements	**sources**
0.22 µm Syringe filters	Jet Biofil
96-well plates	Nunc
Casein peptone	Criterion
Ceftazidime (CAZ)	S.Bheasach
Conical centrifuge tubes 15 ml, 50 ml	Corning
Crystal violet	Math C&B
D-glucose	Sigma
Fluorescein isothiocyanate-concanavalin A (FITC-ConA)	Sigma
Glass flask 500 ml	Pyrex
Glass tubes with Teflon-lined screw cap 13x100 mm	Pyrex
Glass vials 1.5 ml with hole-screw cap and PTFE/silicone septum	Agilent
Insert 250 µl	Agilent
Meropenem (MRP)	AstraZeneca
Microcentrifuge tubes 1.5 ml	Extragene
Mueller Hinton Broth (MHB)	Criterion

Nutrient agar	Criterion
Plastic cover slips	SPL Life Sci
Plastic culture dishes 90x15 mm	Corning
Plastic cuvette	Brane
Plastic pipette 10 ml	Corning
propidium iodide (PI)	Sial-Sigma
Sorvall centrifuge tubes 500 ml	Sorvall
Tetracycline	Sigma
The Calgary Biofilm Device (CBD)	MBEC
Ultra-centrifuge polycarbonate tubes	Beckman
Volumetric flask 5, 10 ml	Pyrex
Yeast extract	Criterion

3. Instruments

Instruments	**sources**
Autoclave machine	Sturdy
Autopipette 1-2 µl	Gilson
Autopipette 200-1000 µl	Gilson
Autopipette 20-200 µl	Gilson
Autopipette 2-20 µl	Gilson
BSL2 Safety cabinet	ESCO
Electric balancer	Denver
FluoView FV10i-DOC confocal microscope	Olympus
Freeze dryer	ThermoSci
Freezer -20°C	Sanyo
Freezer -80°C	Brunswich
Gas chromatography-mass spectrometry (GC-MS)	Agilent
Heating box	ThermoSci
Incubator	Memmert
Labofuge 400R centrifuge	Heraeus
LE80K ultracentrifuge	Beckman

Magnetic stirrer	Stiroheater
Micro-centrifuge	Biofuge
Microtiter plate reader	Thermo-Elect
pH meter	Satorious
Shaker incubator	Labcon
Sonicator	FisherSci
Sorvall SLA-1000 Refrigerated Centrifuge	FisherSci
Spectrophotometer U-1900	Hitachi
Speed vacuum concentrator	Thermo-Elect
Vortex	SciIndustries

APPENDIX B
REAGENTS AND MEDIA

1. Antibiotic stocks

1.1. Tetracycline (5 mg/ml)

Tetracycline powder	25	mg
Absolute ethanol	5	ml

Cover a conical centrifuge tubes 15 ml with aluminum foil in order to protect the solution form the light

Dissolve and stir the powder in absolute ethanol for 15-30 min, until completely dissolved.

Store at -20°C (Light prevention)

1.2. Ceftazidime (10 mg/ml)

Ceftazidime pentahydrate	55.55	mg
Solvent solution		
Na_2CO_3	5	mg
Sterile-distilled water for injection	5	ml

Dissolve in solvent solution

Filter through disposable 0.22 µm Syringe filters

Store at -20°C (Light prevention)

1.3. Meropenem (10 mg/ml)

Meropenem trihydrate	55.55	mg
Sterile-distilled water for injection	5	ml

Dissolve in distilled water

Filter through disposable 0.22 µm Syringe filters

Store at -20°C (Light prevention)

2. Media

2.1. Luria-Bertani (LB) agar containing 60 µg/ml tetracycline (100 ml)

Casein peptone	1	g
Yeast extract	0.5	g
NaCl	0.5	g
Nutrient agar	1.5	g
Distilled water	100	ml

Autoclave at 121°C for 15 min, add 1.2 ml of 5 mg/ml tetracycline

Store at 4°C before preparation of agar plates

2.2. LB broth containing 60 µg/ml tetracycline (200 ml)

Casein peptone	2	g
Yeast extract	1	g
NaCl	1	g
Distilled water	100	ml

Autoclave at 121°C for 15 min, add 1.2 ml of 5 mg/ml tetracycline

Store at 4°C (Light prevention)

2.3. Modified Vogel and Bonner's medium (MVBM) (500 ml)

$MgSO_4.7H_2O$	0.1	g
Citric acid anhydrous	1	g
$NaNH_4HPO_4.4H_2O$	2.66	g
K_2HPO_4	5	g

Dissolve in 459.5 ml of distilled water, autoclave at 121°C for 15 min

36% $CaCl_2.2H_2O$	3.6	g/10ml
25% D-glucose	12.5	g/50ml

Add 0.5 ml of filter-steriled 36% $CaCl_2.2H_2O$ and 40 ml of filter-steriled 25% D-glucose

Store at 4°C

2.4. Mueller Hinton Broth-MHB medium (500 ml)

MHB powder	10.5	g
Distilled water	500	ml

Dissolve in distilled water and autoclave at 121°C for 15 min

Store at 4°C

3. Reagents for antibiotic resistance analysis

3.1. 0.9% NaCl (Normal Saline) (100ml)

NaCl	0.9	g
Distilled water	100	ml

Dissolve in distilled water and autoclave at 121°C for 15 min

Store at room temperature

4. Staining and fixing solutions for biofilm visualization

4.1. 1% Crystal violet (50 ml)

Crystal violet	0.5	g

Dissolve in 50 ml of distilled water, filter through Whatman™ grade 4 filter paper

Store at room temperature (Light prevention)

4.2. 0.15 M phosphate-buffer saline (PBS) pH 7.0 (1L)

NaCl	0.8	g
Na_2HPO_4	1.15	g
KCl	0.2	g
$KH_2 PO_4$	0.2	g

Dissolve in 900 ml of 3B water; adjust pH with conc. HCl to 7.0

Autoclave at 121°C for 15 min, Store at room temperature

4.3. 5% glutaraldehyde stock solution in PBS pH 7.0

glutaraldehyde 25% in H_2O	5	ml
0.15 M PBS pH 7.0	20	ml

Mixing together and store in a light protective bottle at 4°C

Working solution: 2.5% glutaraldehyde in PBS pH 7.0

4.4. 1 mg/ml FITC-con A stock solution in 3B steriled-H_2O

Lyophilized FITC-con A	2	mg
3B steriled-H_2O	2	ml

Working solution: 50 µg/ml FITC-con A in 3B steriled-H_2O

Store in a light protective bottle at -20°C

4.5. 8 µM PI working solution in PBS pH 7.0

1 mg/ml PI	100	µl
0.15 M PBS pH 7.0	1900	µl

Mixing together and store in a light protective bottle at 4°C

5. reagents for exopolysacchride isolation and GC-MS

5.1. 1 M NaOH (100ml)

NaOH pellets	4	g
Distilled water	100	ml

Dissolve in distilled water prior to use.

5.2. 10 mM Sodium bicarbonate

$NaHCO_3$	0.252	g
Distilled water	300	ml

Dissolve in distilled water prior to use.

5.3. 10 mM Na_2EDTA

Na_2EDTA	1.117	g

Distilled water	300	ml

Dissolve in distilled water prior to use.

5.4. 1 M metanolic HCl (15ml)

3M metanolic HCl	5	ml
Methanol	10	ml

Dissolve 3M metanolic HCl into methanol in a brown glass bottle

Store at 4°C (light prevention)

APPENDIX C

Promoter prediction of enzymes annotated to be involved in the postulated exopolysaccharide biosynthesis pathways in *B. pseudomallei*

The biosynthesis of biofilm exopolysaccharide in *B. cepacia* requires the nucleotide activated sugar as precursors for the synthesis. As the composition biofilm exopolysaccharide in *B. cepacia* was similar to our finding in *B. pseudomallei*, a comparison of enzymes responsible for the synthesis of nucleotide activated sugar between *B. cepacia* and *B. pseudomallei* were performed according to the annotated information of the enzymes in metabolic pathways of *B. pseudomallei* proposed in KEGG database (http://www.kegg.jp/kegg-bin/show_pathway?bps00040 and http://www.kegg.jp/kegg-bin/show_pathway?bps00051).

After the identification of conceivable enzymes regulating the exopolysaccharide production, the upstream regions of these enzymes were used for the prediction of promoter binding sites of the interested regulators. In accordance with Osiriphun, Y's work (145), HMMer program version 2.3.2 (Hidden Markov Model based method) is software used for analysis of the possible DNA binding sites of *bps*I, *rpo*N2 and *rpo*S regulators from the number of previously reported promoters which have been created as training sets. The lists of promoters used as the training sets of *bps*I and *rpo*S were retrieved from *B. pseudomallei* according to Kiratisin, P. and Osiriphun, Y.(145, 146), respectively, while the *rpo*N training set was created from σ54-dependent promoter sequences of *E. coli* (147) as shown in three tables below. Briefly, HMM formats of each training set were created using the HMMbuild.exe package of the HMM program and were used in searching for DNA binding sites of each regulator along the 150 bps upstream region of the predicted enzymes by using HMMsearch.exe package in the program.

List of LUX-box like promoters used in prediction training set for the *bps*I promoter prediction of postulated enzymes responsible for the synthesis of nucleotide activated sugar

LUX-box like consensus	sequences
>bpsI1	CCCTGTAAGGGTTAACAGTT
>bpsI2	ACCTGTA-GAAAATCGTAGT
>bpsI3	TCGTGTC-GCGC-SSCSGCC

List of DNA binding sites of RpoS used in prediction training set for the *rpo*S promoter prediction of postulated enzymes responsible for the synthesis of nucleotide activated sugar

RpoS DNA binding sites	sequences
>BPSS1830	CTACGAT
>BPSS1722	CTAAACT
>BPSS1722(2)	CTATCTT
>BPSL0328	CTATAAT
>BPSL2613	CTACAAT
>BPSS1183	CTAATGT
>BPSL2507	CTACGTT
>BPSL2169	CTATAAT
>BPSS0407	CTACGCT
>BPSS0004	CTAAAAT
>BPSS0004(2)	CTACTGT
>BPSL1269	CTAAACT
>BPSL3215	CTACGCT
>BPSL2910	CTACGCT
>BPSL2910(2)	CTACCGT
>BPSL2917	CTATTTT
>BPSS2288	CTACGCT
>BPSS1399	CTAAGGT
>BPSL3008	CTATAGT
>BPSL3008(2)	CTACGTT
>BPSL2298	CTAAAAT
>BPSL2298(2)	CTATTGT
>BPSS1782	CTATATT

RpoN DNA binding sites	sequences
>BPSS2346	CTATGCT
>BPSL0599	CTATGAT
>BPSL1549	CTACACT
>BPSL3012	CTATCTT
>BPSL3012(2)	CTAGGGT
>BPSS0212	CTAACCT
>BPSS0213	CTAACCT
>BPSS0683	CTAGACT
>BPSS2055	CTACTGT

List of DNA binding sites of RpoN from *E. coli* used in prediction training set for the *rpo*N2 promoter prediction of postulated enzymes responsible for the synthesis of nucleotide activated sugar

RpoN DNA binding sites	sequences
>glnK	TGGCACACCGCTTGCAAT
>glnA	TGGCACAGATTTCGCTTT
>glnH	TGGCACAGATTTCGCTTT
>nac	TGGCAAGCATCTTGCAAT
>pspA	TGGCACGCAAATTGTATT
>dcuD	TGGCAGGGTTTTCTCTTT
>zraP	TGGCACGGAAGATGCAAT
>prpB	TGGCACACCCCTTGCTTT
>zraS	TGGCATGATTTCTGCTGT
>hycA	TGGCACAAAAAATGCTTA
>atoD	TGGCACTCCCCTTGCTAT
>fdhF	TGGCATAAAAGATGCATA
>ibpB	TGGTAAATGGTTTGCTGT
>astC	TGGCACGAACCCTGCAAT
>rtcB	TGGCACGACGGTTGCAAT
>hydN	TGGCATGATTTGTGAATG
>hypA	TGGCACAATTATTGCTTG
>ygiG	TGGCGCAATCCCTGCAAT
>yahE	TGGAAGCGATTGTGCTTA
>yaaU	AGGCGCTGGAGCTGCTGG
>yhjC	TGGTTAGTACGCATGCAA
>abgB	TGGCCCGCGTGCAGCAAC

RpoN DNA binding sites	sequences
>crl	TGGTAAAACAGTTGCATC
>nikA	TGGCAAATCGTCAGCGTA
>emrD	TGGCGTATATCTGGCTAA
>ycdM	TGGCATCCGCTTTGCAAA
>ycjJ	TGGAGCGCGGGCGGCAAC
>yaiS	TGGCGCATCGCTTGCTCG
>ygfK	TGGCAAGAGTGGTGCGAT
>aslB	TGGTAGCGCAACTGGTTT
>b2710	TGGCACGCAATCTGCAAT
>yhfZ	TGGCCTGACGCAGGCCGC
>yjcG	TGGCGGGCGAACGGCGAA
>yejH	TGGCATACTATTAGCAGA
>ddpX	TGGCATGAGATCTGCATA
>htpG	TGGAACAGCGTCTGGCAG
>xdhA	TGGCGTAAATCTTGCCTG
>rhaD	AGGCCTACAGGTCGGCAA
>acrD	TGGCTCGTACCTTGCCGC

The *bps*I, *rpo*N and *rpo*S promoter prediction results of postulated enzymes responsible for the synthesis of nucleotide activated sugar in *B. pseudomallei.*

1. BPSL2769

LUX-box: 2769L-140ups: domain 1 of 1, at -80 bps to -62 bps: score -8.0, E = 1

->cctgtagaga.tcctggt<-

cc gta +g+ tcc ++

61 CCGGTATCGCcTCCCCTC 78

2. BPSL2729 (wcbK)

LUX-box: 2729-wcbK-140ups: domain 1 of 1, at -81 bps to -61: score -4.4, E = 0.96

->cctgtagagatcctggt<-

c+t t+gaga++ctg+t

60 CGTTTCGAGAAACTGCT 76

rpoS: 2729-wcbK-140ups: domain 1 of 1, at -17 bps to -12 bps: score -8.9, E = 1

->CTAa.T<-

CTAa T

124 CTAAtT 129, belongs to *rpo*S promoter group 1

3. BPSS1688 (gca)

LUX-box: 1688s-140ups: domain 1 of 1, at -93 bps to -75 bps: score -6.0, E = 0.98

->cctgtagaga.tcctggt<-

cctg +g+g+ c+ g++

48 CCTGACGCGCgGCGCGCC 65

rpoS: 1688s-GCA-140ups: domain 1 of 1, at -32 bps to -26: score -8.3, E = 1

->CTA.a.T<-

CT +a T

110 CTTcAtT 116, belongs to *rpo*S promoter group 3

4. BPSL2614 (glK)

rpoN: 2614-glk-150ups: domain 1 of 1, at -27 bps to -11: score -6.5, E = 0.99

->tGGca.g.a.tttgc.at<-

tGGc ++ + g +

124 TGGCCaAcGaCAAGGgGA 141

rpoS: 2614-glk-150ups: domain 1 of 1, at -32 bps to -26: score -8.3, E = 1

->CTA.a.T<-

C A ++T

142 CGAaGcT 148, belongs to *rpo*S promoter group 3

5. BPSL1682 (gtaB)

rpoS: 1682L-gtaB-140ups: domain 1 of 1, at -21 bps to -14: score -7.7, E = 1

->CTA.a.T<-

C A+a+T

120 CAAcAcT 126, belongs to *rpo*S promoter group 3

6. BPSL2670

rpoS: 2670L-140ups: domain 1 of 1, at -57 bps to -50: score -5.9, E = 0.98

->CTA.a.T<-

TAt +T

84 TTATCAT 90, belongs to *rpo*S promoter group 2

BIOGRAPHY

NAME Miss Rungrawee Mongkolrob

DATE OF BIRTH 31 August 1986

PLACE OF BIRTH Bangkok, Thailand

INSTITUTIONS ATTENDED Mahidol University, 2008-2015
Doctor of Philosophy
(Biochemistry)
Chulalongkorn University, 2004-2007
Bachelor of Science
(Biochemistry)

RESEARCH GRANT Ph.D. Scholarship in the program Strategic Scholarships for Frontier Research Network from Commission of Higher Education

E-MAIL scrmr303@gmail.com

PUBLICATIONS

Tunpiboonsak S., **Mongkolrob R.**, Kitudomsub K., Thanwatanaying P., Kiettipirodom W. , Tungboontina Y., and Tungpradabkul S. Role of a *Burkholderia pseudomallei* Polyphosphate Kinase in an Oxidative Stress Response, Motilities, and Biofilm Formation, J Microbiol. (2010); 48(1): 63-70.

Mongkolrob R., Taweechaisupapong S., and Tungpradabkul S. Correlation between biofilm production, antibiotic susceptibility and exopolysaccharide composition in *Burkholderia pseudomallei bps*I, *ppk*, and *rpo*S mutant strains, Microbiol Immunol. (2015); doi: 10.1111/1348-0421.12331. [Epub ahead of print]