THE ROLE OF *bcsA* GENE IN CELLULOSE BIOSYNTHESIS OF *BURKHOLDERIA PSEUDOMALLEI*

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THE ROLE OF *bcsA* GENE IN CELLULOSE BIOSYNTHESIS OF *BURKHOLDERIA PSEUDOMALLEI*

by

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TABLE OF CONTENTS

		Page
Acknowledgement		i
Table of contents		iii
List of Figures		viii
List of Tables		x
List of Plates		xi
List of Abbreviations		xii
Abstrak		xv
Abstract		xvii
CHAPTER 1: INTRODUCTION		1
CHAPTER 2: LITERATURE REVIEW		
2.1	The Genus *Burkholderia*	4
2.2	*Burkholderia pseudomallei*	6
2.3	Melioidosis: Epidemiology, manifestations and treatment	8
	2.3.1 Melioidosis: Host pathogen interactions	11
2.4	The genomics of *B. pseudomallei*	14
2.5	Cellulose structure and biosynthesis	16
2.6	Bacterial cellulose and their biological significance	19
2.7	Cellulose synthase	23

2.8	The organization of bcs genes	24
2.9	Regulation of cellulose biosynthesis	28
3.0	Genetic manipulation system in *B. pseudomallei*	31

CHAPTER 3: MATERIALS AND METHODS

3.1	Bacterial strains, vectors and culturing conditions	34
3.2	General molecular biological methods and reagents	34
	3.2.1 Chromosomal DNA extraction	37
3.3	Construction of gene replacement vector pBCS01	38
	3.3.1 PCR amplification of flanking regions of *bcsA* gene	38
	3.3.2 Cloning of amplified recombinant fragment	43
	3.3.3 Transformation of *E. coli* host	44
	3.3.4 Plasmid miniprep	45
	3.3.5 Screening of recombinant plasmid	46
	3.3.6 PCR products and constricts verification	46
3.4	Generation of unmarked *B. pseudomallei* UKMS-01 Δ*bcsA* mutant	46
	3.4.1 Conjugative transfer of gene replacement vector pBCS01 into *B. pseudomallei* UKMS-01	46
	3.4.2 Sucrose resistant of pDM4 derivative vector from *B. pseudomallei* UKMS-01 merodiploids	47
	3.4.3 Verification of putative deletion strains in *B. Pseudomallei* UKMS-01	48

3.5 Characterization of Δ*bcsA* mutant 49

3.5.1 Congo red dye and Calcofluor staining 49

3.5.2 Electron micrscopy 50

3.5.2.1 Scanning electron microscope (SEM) sample preparation using hexamethyl-disilazane (HMDS) method 50

3.5.2.2 Negative staining 50

3.5.3 Growth curve construction 50

3.5.4 Cellulose production assay 51

3.5.5 Aggregation assay 53

3.5.6 Biofilm formation and adherence assays 53

3.5.7 Desiccation survival assay 54

CHAPTER FOUR: RESULTS

4.1 Construction of chromosomal Δ*bcsA* deletion mutant in *B. pseudomallei* UKMS-01 56

4.1.1 Features of the gene replacement vector pDM4 58

4.1.2 Use of pDM4 to generate an unmarked Δ*bcsA* in *B. pseudomallei* UKMS-01 59

4.1.2.1 PCR amplifications of flanking regions of *bcsA* gene and recombinant fragment 59

4.1.2.2 Sequencing of the amplified fragments 61

4.1.2.3 Cloning of recombinant fragment into pGEM®-T Easy 61

4.1.2.4 Subcloning of *Spe*I/recombinant fragment into plasmid pDM4 64

4.1.3 Conjugative transfer of gene replacement vector pBCS01 into *B. pseudomallei* UKMS-01 64

4.1.4 Genetic manipulation of *B. pseudomallei* UKMS-01 via allelic replacement 65

4.1.5 The use of pDM4 to create an unmarked deletion mutant in *B. pseudomallei* 67

4.2 Characterization of Δ*bcsA* mutant 74

4.2.1 Morphotype screen via Congo red and Calcofluor binding 74

4.2.2 Electron microscopy studies of wild type and Δ*bcsA* strains 80

4.2.3 Quantification of cellulose production by digestion with cellulase 84

4.2.4 Roles of cellulose in biofilm formation 89

4.2.5 Roles of cellulose in resistance to desiccation 93

CHAPTER FIVE: DISCUSSION 102

CHAPTER SIX: CONCLUSION 112

REFERENCES 114

APPENDICES

LIST OF FIGURES

		Page
2.1	Different enzymes in the cellulose biosynthetic pathway	17
2.2	Overview of the organizational cellulose biosynthesis operons in different cellulose producing bacteria	26
3.1	Construction of recombinant fragment for allelic replacement via homologous recombination	39
4.1	Overview of research methodology	57
4.2	Plasmid map of pDM4 vector	60
4.3	Illustration of the chromosomal location of primers used for PCR validation and sequencing	69
4.4	Schematic overview of unmarked deletion mutagenesis	70
4.5	Phenotypic characterization of UKMS-01 on agar plate containing Congo red	77
4.6	Phenotypic characterization of Δ*bcsA*01 on agar plate containing Congo red	78
4.7	Screening for calcofluor binding in UKMS-01 and Δ*bcsA*01	79
4.8	Scanning electron micrographs of UKMS-01	81
4.9	Scanning electron micrographs of Δ*bcsA*01	82
4.10	Scanning electron micrographs of both UKMS-01 and Δ*bcsA*01 in planktonic state	83
4.11	Transmission electron micrographs of negatively stained UKMS-01	85
4.12	Transmission electron micrographs of negatively stained Δ*bcsA*01	86

4.13 (a) & (b)	Effect of *bcsA* on biofilm development on plastic surface	91-92
4.14	Phenotypic analysis of air-liquid interface biofilms	94
4.15 (a) & (b)	Cell aggregation properties of UKMS-01 and Δ*bcsA*01 in four different media	96-97
4.16	Growth curves of UKMS-01 and Δ*bcsA*01 in rich and minimal media	98
4.17 (a) & (b)	Desiccation survival assays of UKMS-01 and Δ*bcsA*01 on four different media	100-101
5.1	Genetic organization of putative cellulose biosynthesis genes cluster in *B. pseudomallei* K96243	103
5.2	Genetic organization of regions spanning BPSS0735 in K96243	103
5.3	Multiple sequence alignment output of *bcsA* and BPSS0735	105

LIST OF TABLES

		Page
3.1	Bacterial strains and vectors used in this study	35
3.2	Oligonucleotides primers used in this study	40
3.3	PCR cycling parameters used in this study	41
4.1	Characteristic phenotypes in relation to extracellular matrix components	75
4.2	Quantification of glucose concentration after cellulase digestion	88

LIST OF PLATES

		Page
4.1 (a)	PCR amplification of the flanking regions of *bcsA* and recombinant fragment	62
4.1 (b)	Gel purification of the amplified fragments	62
4.2 (a)	Cloning of the recombinant fragment into pGEM®-T Easy	63
4.2 (b)	Digestion of the pDM4 derivative carrying recombinant fragment with *Spe*I	63
4.3	PCR confirmation of Δ*bcsA* putative mutants	71

LIST OF ABBREVIATIONS

acs	*Acetobacter* cellulose synthase
A-L	air liquid
Amr	antimicrobial resistance
bas	brown and smooth
bcs	bacterial cellulose synthase
bdar	brown, dry and rough
c.f.u.	colony forming unit
cat	chloramphenicol resistance cassette
c-di-GMP	cyclic diguanylate
cel	cellulose
Cm	chloramphenicol
CR-P	plant specific and conserved region
CV	crystal violet
DGC	diguanylate cyclase
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DS	downstream
et al.	and others
FM	floccular material
g	gravity

Gm	gentamicin
GMP	guanosine monophosphate
HMDS	hexamethyldisilazane
HVR	hyper variable region
IPTG	isopropyl-β-D-galactopyranoside
kb	kilo base pairs
kDa	kilo Dalton
Km	kanamycin
lacZ	gene beta galactoside
LB	lysogeny broth/luria-bertani broth
LPS	lipopolysaccharide
Mb	mega base pairs
MCS	multiple cloning site
ml	mililitre
NaCl	sodium chloride
ng	nanogram
ORF	open reading frame
ORF	open reading frame
pas	pink and smooth
PC	physically cohesive
PCR	polymerase chain reaction
pdar	pink, dry and rough
pmol	picomole
ras	red and smooth

rdar	red, dry and rough
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
sacB	gene levansucrase
saw	smooth and white
SEM	scanning electron microscope
spp.	several species
TEM	transmission electron microscope
TTSS	type three secretion system
U	unit
UDP-Glc	uridine diphosphate glucose
US	upstream
UV	ultraviolet
VM	viscous mass
w/v	weight/volume percentage
WA	waxy aggregates
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µg	microgram
µl	microlitre
µm	micrometer

PERANAN GEN *bcsA* DALAM BIOSINTESIS SELULOSA DALAM *BURKHOLDERIA PSEUDOMALLEI*

ABSTRAK

Dua salinan gen selulosa sintase telah dikenalpasti dalam pangkalan data genom *B. pseudomallei* K96243. Kedua-dua gen terletak pada kromosom II dan dilabelkan sebagai rangka bacaan terbuka (ORF) BPSS0735 dan BPSS1577. Perbandingan jujukan menunjukkan kedua-dua gen berkongsi keserupaan dan identiti yang rendah iaitu sebanyak 35% dan 24%. Ini mencadangkan bahawa kedua-dua gen selulosa sintase berasal dari origin yang berbeza. Dalam kajian ini, biosintesis selulosa oleh *B. pseudomallei* telah diselidiki dan kehadiran selulosa dalam bacteria tersebut telah dibuktikan secara eksperimental. Oleh itu, satu kajian telah dijalankan untuk menentukan fungsi BPSS1577 (juga dianotasikan sebagai *bcsA*) dalam biosintesis selulosa bagi *B. pseudomallei*. Mutant delesi *bcsA* telah dijana untuk menentukan samaada *bcsA* terlibat dalam penghasilan biofilem, perlekatan kepada permukaan abiotik dan potensi mandiri terhadap kekeringan. Fungsi BPSS0735 juga boleh disahkan seandainya penghasilan selulosa berlaku dalam mutant delesi. Satu mutant delesi *bcsA* tanpa tanda telah berjaya dijanakan dengan menggunakan vector swa-hapus, pDM4 yang memiliki gen rintang kloramfenikol (*cat*^{R}) sebagai system pemilihan dan gen levan sukrase (*sacB*) sebagai system pemilihan timbal-balik. Mutan Δ*bcsA*01 ini tidak menunjukkan sebarang perbezaan yang ketara dalam penghasilan selulosa berbanding dengan sel jenis liar, seterusnya mengesahkan yang BcsA bukanlah satu-satunya

selulosa sintase yang bertanggungjawab dalam biosintesis selulosa dalam *B. pseudomallei*. Mutan Δ*bcsA*01 mempamerkan keupayaan yang lebih rendah dalam perlekatannya pada plastik, mencadangkan bahawa *bcsA* mungkin memainkan peranan dalam perlekatan kepada permukaan.

THE ROLE OF *bcsA* GENE IN CELLULOSE BIOSYNTHESIS OF *BURKHOLDERIA PSEUDOMALLEI*

ABSTRACT

Two copies of cellulose synthase gene were identified in the genome database of *B*. *pseudomallei* K96243. Both genes are located on the chromosome II and labelled as open reading frame (ORF) BPSS0735 and BPSS1577, respectively. Sequence comparisons revealed that the two genes share low similarity and identity of 35% and 24%, respectively. This suggested that the two cellulose synthase genes are of different origins. In this study, *B*. *pseudomallei* was investigated for the biosynthesis of cellulose and experimental evidences of the presence of cellulose were shown. Hence, a study was conducted to characterize the role of BPSS1577 (also annotated as *bcsA*) in the cellulose biosynthesis of *B*. *pseudomallei*. A *bcsA* deleted mutant was generated to determine whether *bcsA* was involved in biofilm formation, adherence to abiotic surfaces and desiccation survival potential in this bacterium. The functionality of BPSS0735 could be verified if cellulose production is accomplished in the deletion mutant. A markerless deletion mutant of *bcsA* was generated using a suicidal vector that utilized a chloramphenicol resistance gene (*cat*^{R}) as the selectable marker and a levan sucrose gene (*sacB*) as the counter-selectable marker. The Δ*bcsA*01 mutant showed no significant difference in cellulose production compared to wild type cells, thus confirming that BcsA is not the only cellulose synthase responsible for cellulose

biosynthesis in *B*. *pseudomallei*. The Δ*bcsA*01 mutant demonstrated a reduced ability in adherence to plastic suggesting that *bcsA* might play a role in adherence to surfaces.

CHAPTER ONE

INTRODUCTION

Cellulose is naturally viewed as a property of the plant kingdom. It is widely understood that plants produce cellulose as the structural component of their cell wall. However, single-cell organisms such as bacteria also share the same property. The gene encoding the catalytic subunit of cellulose synthase responsible for β-glucan chain polymerization in cellulose biosynthesis was first isolated and identified in *Gluconaceobacter xylinus* (Saxena *et al.*, 1990; Wong *et al.*, 1990) which then laid the basis for subsequent identification of cellulose biosynthesis gene clusters in plants (Pear *et al.*, 1996). Available evidence seemed to support the notion that cellulose synthase enzymes originally belong to bacteria and was later acquired by diverse eukaryotes via lateral gene transfer mechanisms (Saxena and Brown, 2007).

Cellulose biosynthesis machinery is present in a diverse range of bacteria even though the biological significance of cellulose biogenesis in pathogenic, environmental and commensal bacteria is only partially deciphered. The presence of cellulose biosynthesis gene clusters in bacterial genome and the successive events of lateral gene transfer between eukaryotes and within prokaryotes have been widely reported (Saxena and Brown, 2005; Saxena and Brown, 2007). Therefore, it is proposed that selection pressure may favor the maintenance of cellulose biosynthesis gene clusters in bacteria that increase their adaptive fitness in an environmental niche. In *Acetobacteriaceae* and *Rhizobiaceae*, it has been shown that cellulose molecules confer mechanical and chemical protection by maintaining the buoyancy of the cells

at the air-liquid interface (Brown *et al.*, 1976). Likewise, it is also involved in bacterial attachment to their plant host cells (Matthysee, 1983). In colonies of *Enterobacteriaceae*, cellulose is associated with a multicellular morphotype which is also known as red, dry and rough (rdar) morphotype on Congo red plates (Romling *et al.*, 2000). This coordinated multicellular behavior is said to offer various advantages to a bacterial population as compared to single, planktonic cells (Shapiro, 1998). In addition, cellulose is also coiled with the wrinkled spreader phenotype in the *Pseudomonadaceae* (Spiers *et al.*, 2002) which was found to play essential role in the colonization of plant surface (Ude *et al.*, 2006).

B. pseudomallei is a soil-dwelling bacterium that opportunistically infect both human and animals. It is the causative agent of melioidosis, a disease which recorded for up to 50% of mortality rate in certain endemic region. Over the years, the molecular mechanism of pathogenesis in melioidosis and the development of vaccine against melioidosis had grabbed the limelight in researches pertaining to this deadly bacterium. Less attention has been given to the environmental behavior of *B. pseudomallei* despite knowing that this bacterium acquires multifaceted adaptation and survival strategies in natural environments (Stevens and Galyov, 2004). As a result, biological systems which enhance the *Burkholderia* survival outside their host and thereby aiding in the perpetuation of this pathogen might be overlooked.

Since cellulose might act as a stabilizer that confers adaptive fitness to *B. pseudomallei* in the natural environment, an understanding of cellulose biosynthesis system in this bacterium is needed. Intriguingly, the complete genome database of *B. pseudomallei* K96243 indicated the presence of more than one copy of cellulose synthase gene on the chromosome, namely, *bcsA* and BPSS0735. Given the

complexity of this biological system, I have decided to refine the research questions to be addressed in this study. The research objectives are summarized below:

1) To provide experimental evidence that *B. pseudomallei* UKMS-01 is able to produce cellulose.
2) To investigate the role of *bcsA* in cellulose production.
3) To study the resulting effects of *bcsA* deletion on biofilm formation, adherence and desiccation survivability in *B. pseudomallei* UKMS-01.

As such, this study is another effort towards understanding the function of cellulose synthase and the characteristic of cellulose with regard to bacterial behavior. It is hoped that the findings will strengthen our basis on this subject which would allow further studies that ultimately contribute towards the establishment of a theoretical framework of cellulose biosynthesis in *Burkholderia*.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Genus *Burkholderia*

In 1942, the first *Burkholderia sp*. was described by Walter H. Burkholder as a phytopathogenic bacterium (Burkholder, 1942) and it was later renamed as *Pseudomonas cepacia*. For many years, *Burkholderia* spp. was accommodated in the genus *Pseudomonas* due to its ambiguous phenotypic definition. Yabuuchi *et al*. (1992) transferred seven species from the *Pseudomonas* group to the newly established genus *Burkholderia* based on the 16S rRNA, DNA-DNA homologue values, cellular lipid, fatty acid composition and phenotypic characterization. The seven species were *Burkholderia cepacia* (Palleroni and Holmes, 1981), *Burkholderia mallei* (Steele, 1972), *Burkholderia pseudomallei* (Whitmore, 1913), *Burkholderia caryophilli* (Burkholder, 1942), *Burkholderia gladioli* (Mortensen *et al.*, 1988), *Burkholderia picketti* (Ralston *et al.*, 1973) and *Burkholderia solanaceareum* (Buddenhagen *et al.*, 1962). The last two species were subsequently reclassified in the genus *Ralstonia* (Yabuuchi *et al*., 1995). To date, the genus *Burkholderia* contains over 30 species that occupy diverse ecological niches ranging from soil and hospital environment to infected human hosts (Coenye and Vandamme, 2003). All members of the genus *Burkholderia* are aerobic, non-spore-forming Gram negative rod-shape bacteria with a diameter of 0.5 - 1.0 µm and 1.0 - 5.0 µm long. They are able to metabolize glucose oxidatively and use nitrate as an electron acceptor under anaerobic conditions (Garrity, 2005).

Over the years, there has been growing interest within the genus *Burkholderia* due to their agricultural and ecological importance. Several strains which are known as soil bacteria are found to form mutualistic and symbiotic association with plants and colonize roots, stems and leaves (Compant *et al.*, 2008). *Burkholderia vietnamiensis* is widely known for its ability to fix atmospheric nitrogen and promotes nodulation in its plant host (Gillis *et al.*, 1995; Estrada-De Los *et al.*, 2001; Magalhaes Cruz *et al.*, 2001). However, several species in this genus are of major concern due to their pathogenic nature that can cause severe infections in humans and animals. *Burkholderia pseudomallei* and *B. mallei* are the primary pathogens among others and can be potentially used as biological weapons (Rotz *et al.*, 2002; Coenye and Vandamme, 2003). *B. pseudomallei* causes melioidosis in both humans and animals while *B. mallei* is the causative agent of glanders in horses (McGilcray, 1944; Alibasoglu *et al.*, 1986; Smith *et al.*, 1987; Sharrer, 1995; Neubauer *et al.*, 1997). The Type species, *B. cepacia* has great potential in both agricultural and environmental applications (Parke *et al.*, 1991; McLoughlin *et al.*, 1992; Govan *et al.*, 1996; Holmes *et al.*, 1998; Coenye and Vandamme, 2003). However, it also acts as deadly pathogen in immunocompromised patients especially those with cystic fibrosis (CF) (Isle *et al.*, 1984; Govan and Deretic, 1996a).

The complexity and pathogenic nature of the members in this genus have greatly hampered in-depth studies of these microorganisms. However, this situation has improved with the availability of complete genomic sequence data for various strains. The sequencing data indicated that these bacteria consists of multiple replicons genomes with an average GC content of 69%. The total genomic size variably ranges from 4.7 Mb to more than 9 Mb (Wigley and Burton, 2000; Holden

et al., 2004; Nierman *et al.*, 2004; Chain *et al.*, 2006). (Konstantinidis and Tiedje, 2004) has postulated that larger genomes often confer the versatility to dominate in different ecological niches especially those with extreme conditions. Furthermore, it was found that genes involved in regulation and secondary metabolism are significantly enriched while protein translation, cell division, and DNA replication genes were depleted in larger genomes.

Genomic variations have been observed among these phylogenetically closely related species. The presence of >12000 simple sequence repeats within key genes in *B. mallei* (Nierman *et al.*, 2004) and the identification of different genomic islands with atypical GC content as compared to other genomic regions in isolated *B. pseudomallei* strains have been observed (Holden *et al.*, 2004). Genomic variations seemed to diversify the gene contents among the members of this genus and might account for differences in virulence among the members of the genus *Burkholderia*. These observations were further substantiated when Ou *et al.* (2005) demonstrated in *B. pseudomallei* that genomic variations are acquired by means of lateral gene transfer or gene loss events which ultimately shaped their genomes for plasticity, flexibility and versatility (Holden *et al.*, 2004; Nierman *et al.*, 2004; Chain *et al.*, 2006).

2.2 *Burkholderia pseudomallei*

B. pseudomallei was first isolated about 100 years ago (Whitmore and Krishnaswami, 1912). It was found to cause a “glanders” like disease among morphin addicts in Rangoon, Burma (Whitmore and Krishnaswami, 1912; Whitmore, 1913). It is an opportunist pathogen of both humans and animals and has been

classified as category B agent by the United States Centers of Disease Control and Prevention (Rotz *et al.*, 2002). This aerobic, motile and Gram-negative bacillus is an environmental saprophyte commonly isolated from soil, stagnant water, rice paddies and roots of plants in the endemic areas of tropical and subtropical regions (Brett and Woods, 2000; Holden *et al.*, 2004; Stevens and Galyov, 2004). It is catalase and oxidase positive and accumulates β-hydroxybutyric acid during nitrogen starvation (Sprague and Neubauer, 2004).

B. *pseudomallei* can be isolated from clinical and environmental samples using selective media such as Ashdown's and Francis agar media (Ashdown, 1979; Ashdown and Clarke, 1992; Francis *et al.*, 2006). It normally appears mostly as dry and rough colonies on agar media. However, phenotypic switches between rough and smooth colonies were observed in this organism especially both within and between clinical samples (Chantratita *et al.*, 2007). The alteration of phenotype correlates with the adaptation strategy of *B*. *pseudomallei* which involves altered expression of surface determinants which in turn enhance their survivability *in vivo* (Cheng and Currie, 2005; Chantratita *et al.*, 2007). The multiplicity of adaptation and survival strategies confer a selective advantage to the growth of *B*. *pseudomallei* in adverse environmental conditions (Stevens and Galyov, 2004). Being a resilient organism, it can withstand extended nutrient deficient conditions, antiseptic and detergent solutions, low pH environments and dehydration (Cheng and Currie, 2005). However, it is sensitive towards UV irradiation and sunlight (Tong *et al.*, 1996).

Among the members of the genus *Burkholderia*, *B*. *pseudomallei* is closely related to *B*. *thailandensis* and both of them reside in the same environmental niche (Adler *et al.*, 2009). However, *B*. *thailandensis* is less virulent as compared to *B*.

pseudomallei (Brett *et al.*, 1998). This has been widely associated with the presence of a functional arabinose operon in the genome of *B. thailendensis* whereas the same operon was absent in *B. pseudomallei*. It has been postulated that the inability to utilize L-arabinose enhances the survival and persistence of *B. pseudomallei* in animal hosts (Sirisinha *et al.*, 1998; Moore *et al.*, 2004).

2.3 Melioidosis: Epidemiology, Manifestations and Treatment

Melioidosis is an endemic infectious disease in Southeast Asia and Northern Australia. These regions which correspond to the tropical latitudes between 20°N and 20°S are regarded as the major endemic regions for melioidosis (Dance, 1991; Dance, 2000). However, sporadic cases have been documented in Africa, Brazil, Mexico, Philippines, Papua New Guinea and South America (Jones *et al.*, 1996). Recently, studies have reported the expansion of boundaries in the global distribution of melioidosis which now includes the majority of the Indian subcontinent, Southern China, Hong Kong and Taiwan (Cheng and Currie, 2005). Melioidosis accounts for an overall mortality of 50% in northeast Thailand and 20% in Australia. In Thailand, 20% of melioidosis occurs in children under the age of 14 years old and this accounts for 35% of the overall mortality rate of the country (Cheng *et al.*, 2003; White, 2003).

Melioidosis can be acquired through cutaneous inoculation, inhalation and aspiration (Wiersinga and Poll, 2009). The majority of melioidosis are found in individuals who have been exposed to environment which have been contaminated with *B. pseudomallei* be it occupational or recreational (Chaowagul *et al.*, 1989; Currie *et al.*, 2000; Wiersinga *et al.*, 2006). In northeast Thailand, this infection is

particularly prevalent amongst rice farmers and their families who have direct contact with wet soils (Dworkin *et al.*, 2006). Besides that, laboratory-acquired melioidosis has also been reported (Green and Tuffnell, 1968). *B. pseudomallei* often poses higher infection risks in individuals with impaired immunity, the most common being diabetes mellitus and renal failure (Chaowagul *et al.*, 1989). Occasional nosocomial infections have also been reported in intensive care units (White, 2003). Therefore, environment and host factors are central to the disease acquisition of melioidosis (Wiersinga *et al.*, 2006). The finding where frequent occurrence of sepsis and pneumonia are strongly associated with heavy rainfall has suggested that most infections occur during monsoonal seasons, severe weather events and environmental disasters (tsunami in 2004) (Chaowagul *et al.*, 1989; Leelarasamee and Bovornkitti, 1989; Currie *et al.*, 2000; Currie and Jacups, 2003). The flooding of rice paddies causes the bacteria to leach from the soil and increases their chances of exposure to both humans and animals (Brett and Woods, 2000; Currie *et al.*, 2000). Unfortunately, the lack of culture facilities for bacterial isolation in rural endemic regions has greatly thwarted the identification and complete documentation of this disease (Dance, 1991).

Apart of its protean disease manifestations (Yee *et al.*, 1988), melioidosis is commonly present as febrile and septiceamic illness (White, 2003; Holden *et al.*, 2004). The clinical presentations range from acute, fulminant septicemia to chronic deliberating localized infections (White, 2003). This disease is characterized by pneumonia, formation of abscess and bacterial dissemination to distant sites such as liver and spleen (Vatcharapreechasakul *et al.*, 1992; Wong *et al.*, 1995; Puthucheary and Vadivelu, 2002; White, 2003; Leelarasamee, 2004). Virtually, all organs are

affected with liver, spleen, skeletal muscles and prostate being the most common sites (White, 2003). In the most critical case, death can occur within 48 hours as a result of septic shock (Holden *et al.*, 2004). In some instances, this bacterium does not induce clinical symptoms in individual exposed but is capable of remain dormant in its host system, with the longest recorded incubation period being 62 years (Holden *et al.*, 2004; Ngauy *et al.*, 2005).

The treatment of melioidosis is particularly hindered by the intrinsic resistance of *B. pseudomallei* towards an array of antibiotics which include aminoglycosides, beta-lactams, first and second generation cephalosporins, macrolides and polymyxins (White, 2003; Cheng and Currie, 2005). Besides that, *B. pseudomallei* is also highly resistant to the action of cationic antimicrobial peptides which include human neutrophil peptides, protamin sulfate, magainins, melittin and poly-L-lysine (Woods *et al.*, 1999). The antibiotics which *B. pseudomallei* is susceptible to are amoxicillin-clavulanate, chloramphenicol, doxycycline, trimetoprime-selphamethoxazole, ureidopenicillin, ceftazidim and carbapenem (White, 2003; Cheng and Currie, 2005). The recommended antibiotic treatment for melioidosis requires 20 weeks and is divided into intravenous and oral phases (White, 2003; Cheng and Currie, 2005). Relapse is a common phenomenon among the patients of melioidosis even with proper antibiotic treatment (Chaowagul *et al.*, 1993).

Eradication of *B. pseudomallei* from the patients has been the biggest challenge in the treatment process because one quarter of those who survived the initial clinical illness often suffered from recurrence in the later stage (Chantratita *et al.*, 2007). Therefore, prolonged oral therapy is necessary to ensure full clinical

resolution of the infection and subsequently reduce the risk of recrudescence (Dworkin *et al.*, 2006). To date, there is no licensed vaccine available for immunoprophylaxis against melioidosis though flagellin and O-polysaccharide moieties derived from endotoxin properties of *B. pseudomallei* have been proposed to be the most promising candidates for vaccine preparation. Despite the positive outcome in the preliminary studies, the efficacy of these vaccines has to be tested vigorously to ensure active immunization in the future (Woods *et al.*, 1999).

2.3.1 Melioidosis: Host Pathogen Interactions

B. pseudomallei is a facultative intracellular pathogen with broad cellular tropism (Gan, 2005) and can survive and proliferate within phagocytic cells for extended lengths of time (Pruksachartvuthi *et al.*, 1990). Following the internalization of *B. pseudomallei* into targeted cell type, it first lyses the endosome membrane to escape from endocytic vacuole into the cytoplasm (Pruksachartvuthi *et al.*, 1990; Harley *et al.*, 1998a; Harley *et al.*, 1998b; Stevens *et al.*, 2002). Then, it induces actin polymerization at one pole of the bacterial cell which leads to the projection of eukaryotic cell plasma membrane via membrane protrusion. Such protrusion pushes against the adjacent cell, and mediates the intracellular spread of the bacteria (Kepichayawattana *et al.*, 2000; Stevens *et al.*, 2002; Breitbach *et al.*, 2003). When an adjacent cell phagocytoses this protrusion, it protects the subsequent spread of *B. pseudomallei* from the host immune cells by allowing *B. pseudomallei* to elude from secondary vacuoles and thereby multiply intracellularly (Wiersinga *et al.*, 2006).

The pathogenesis of melioidosis has been proposed to be an orchestration of virulence factors in *B. pseudomallei*. During infection, these virulence factors exert a combinatorial effect towards their host system which causes dreadful consequences. The core virulence factors include the type III secretion system (TTSS), capsular polysaccharide, quorum sensing, flagella and lipopolysaccharide (LPS). Other members include type IV pili, the type VI secretion system, siderophore and secreted proteins such as proteases, lipases and haemolysin which play moderate to minor roles in virulence (Whitmore and Krishnaswami, 1912; Colling *et al.*, 1958; Yang *et al.*, 1991; Adler *et al.*, 2009).

Before the internalization of pathogen into infected cell, the ability to adhere to host epithelial cells is an essential virulence mechanism (Woods *et al.*, 1999). In *B. pseudomallei*, type IV pili mediate the attachment of bacteria to host epithelial cells and microcolony formation, opening the gateway to bacterial infection (Adler *et al.*, 2009). During the internalization of *B. pseudomallei* into the infected cell, the type III secretion system is responsible for impairment and corruption of the host-cell processes which consequently promotes bacterial uptake, vacuolar escape and actin-mediated intracellular motility in both phagocytic and non-phagocytic cells (Hueck, 1998; Cornelis and Van Gijsegem, 2000; Plano *et al.*, 2001). Besides that, the type III secretion system has been shown to mediate the formation of multinucleated giant cells by cell fusion (Harley *et al.*, 1998a; Kepichayawattana *et al.*, 2000) and induce apoptosis of infected host cells (Suparak *et al.*, 2005).

Quorum sensing has been previously shown as a functional component in bacterial pathogenicity in various Gram-negative bacteria pathogens (Passador *et al.*, 1993; Gotschlich *et al.*, 2001; Baldwin *et al.*, 2004). Quorum sensing is a cell-

density-dependent communication in bacteria that uses an array of signaling molecules such as N-acyl-homoserine to positively or negatively coordinate the expression of a variety of extracellular products (Fuqua *et al.*, 1994). These include metalloprotease, lipase, hemolysin, siderophores, phospholipase C, antibiotics and biofilm formation (Wiersinga *et al.*, 2006; Vial *et al.*, 2007). In *B. pseudomallei*, disruption of genes encoding quorum sensing resulted in reduced organ colonization, increased time to death and reduced virulence in a Syrian hamster model (Ulrich *et al.*, 2004).

The lipopolysaccharide, an immunodominant antigen in *B. pseudomallei,* has a unique structure as compared to lipopolysaccharide of other Gram-negative bacteria (Matsuura *et al.*, 1996; Utaisincharoen *et al.*, 2000). It is comparatively less capable of activating the inducible nitric oxide synthase (iNOS) system in macrophages, a mechanism responsible for the eradication of intracellular bacteria (Utaisincharoen *et al.*, 2001). Hence, lipopolysaccharide is likely to enhance the intracellular survival of *B. pseudomallei* in phagocytic cells (Adler *et al.*, 2009). The expression of capsular polysaccharide in *B. pseudomallei* is capable of reducing complement factor C3b deposition on bacterial surface and helps to occlude the bacteria from being marked by opsonization (Reckseidler-Zenteno *et al.*, 2005). This is because the opsonization of *B. pseudomallei* will initiate a cascade of host immune response aimed at scavenging intracellular bacteria (Wiersinga and Poll, 2009). Apart of that, the capsule also protects these bacteria from antibiotic penetration via phenotypic alteration (Haussler *et al.*, 1999).

Collectively, it appears that *B. pseudomallei* is particularly adapted to exploit host cells whilst both the host innate and adaptive immune responses work

synergistically towards the eventual clearance of these bacteria and control of melioidosis infection.

2.4 The Genomics of *B. pseudomallei*:

The complete genome of *B. pseudomallei* strain K96243, isolated from a female patient of human melioidosis has recently been sequenced and annotated (Holden *et al.*, 2004). It is a large complex of 7.25 megabase pair (Mb) consisting of two circular replicons, namely chromosome I and chromosome II of 4.07 Mb and 3.17 Mb, respectively (Holden *et al.*, 2004). The genome of *B. pseudomallei* K96243 is of high GC content, with an overall GC content of 68%. Both chromosomes exhibit significant functional partitioning of genes between them (Holden *et al.*, 2004). Essential genes required for cell metabolism and growth are mostly found on chromosome I (core genome) whereas chromosome II (accessory genome) carries genes encoding accessory functions pertaining to bacterial virulence and their survival in different ecological niches (Wiersinga *et al.*, 2006). The genomic contents of the members in the *Burkholderia* family are generally conserved, whereby 86% of *B. pseudomallei* K96243 genome had some matches in all *Burkholderia* strains (Wiersinga and Poll, 2009). The remaining 14% is invariably present across *B. pseudomallei* isolates (Wiersinga and Poll, 2009), highlighting the relative genetic diversity within this species.

Besides genetic diversity, phenotypic diversity is also observed within *B. pseudomallei* of distinct strains. It has been suggested that the phenotypic diversity observed might be due to the presence of genomic islands across isolates (Ou *et al.*, 2005). The analysis of *B. pseudomallei* K96243 genome has identified the presence

of 16 genomic islands which exhibit unusual properties such as G+C content anomalies and frequent dinucleotide signature (Holden *et al.*, 2004). These observations indicated that the DNAs have probably been acquired recently (Ou *et al.*, 2005). In addition, these regions also carry mobile genetic elements such as insertion sequence elements, bacteriophages and plasmids that might alter the gene repertoire of *B. pseudomallei* (Holden *et al.*, 2004; Tumapa *et al.*, 2008). The presence of genomic islands is often associated with clinical strains derived from human patients of melioidosis as compared to environmental isolates; however the role of genomic islands in *B. pseudomallei* pathogenicity has yet to be reported (Sim *et al.*, 2008; Tumapa *et al.*, 2008). Presumably, selection may favor the maintenance of genomic islands because they not only increase bacterial fitness in specialized environmental niches (Tumapa *et al.*, 2008) but they are the key determinants of the genome plasticity in *B. pseudomallei* (Brown and Beacham, 2000).

Over the years, comparative genomics have been conducted extensively among the 3 closely related species, *B. pseudomallei*, *B. mallei* and *B. thailandensis* (Monastyrskaya *et al.*, 2004; Kim *et al.*, 2005; Yu *et al.*, 2006). The comparative analysis deduced that different mechanisms of evolutionary processes might be involved in channeling the divergence of these strains (Ong *et al.*, 2004). Undoubtedly, the availability of whole genome sequence has created a paradigm shift in experimental approaches taken by researchers in their quest to gain further insights into the functional diversity, evolution and pathogenicity mechanisms of *B. pseudomallei* which are vital for infection control and biotechnological applications in agriculture in the near future.

2.5 Cellulose Structure and Biosynthesis:

In many instances, cellulose is cited as the most abundant macromolecule on earth (Ross *et al.*, 1991). It was first thought to be solely produced biologically by plants. However successive research revealed that cellulose is a common component of a great diversity of living organisms (Saxena and Brown, 2005) which include most groups of algae, slime mold (Brown, 1990), certain prokaryotes (both bacteria and cyanobacteria) (Ross *et al.*, 1991; Nobles *et al.*, 2001), and tunicates in the animal kingdom (Kimura and Itoh, 1996). Furthermore, some pathogenic bacteria have also been reported to synthesize cellulose (Zogaj *et al.*, 2001). Cellulose is an unbranched polymer consisting of β-1,4-linked glucose residues (Roelosfsen, 1959). In spite of glucose molecules being the building block of cellulose, a closer inspection of the β-1,4-linked backbone in cellulose suggests that the repeating unit is cellobiose (disaccharide molecule) because each glucose residue is rotated 180° with respect to its neighbouring residue (Brown, 1996).

Initially, the biogenesis of cellulose was thought to be rather simple considering that cellulose is a single linkage homopolymer consisting of only β-1,4-glucan chain. This concept was soon subjected to arguments when loosely wound ribbon of fibrils containing highly structured glucan chains and not individual glucan chains were observed as the end product of cellulose biogenesis in both higher and lower organisms (Haigler, 1985; Delmer, 1987). Hence, it was hypothesized that there must be a synthesizing machinery capable of accomplishing simultaneous polymerization of multiple glucan chains and crystallization of microfibrils. The mystery was finally solved when Brett (2000) reported that the biogenesis of cellulose requires the nearly instantaneous occurrence of three distinct event

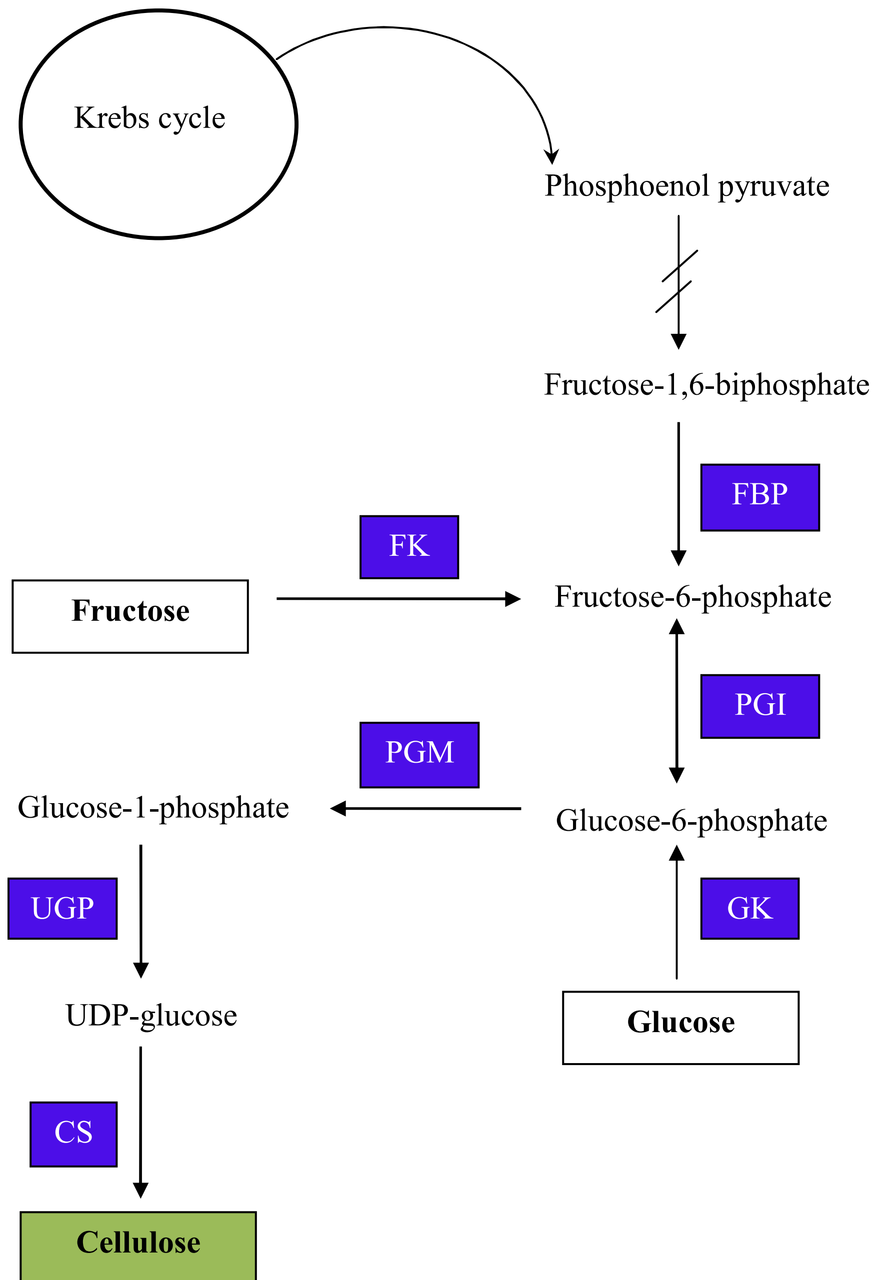

Figure 2.1: Generalized scheme illustrating the role of different enzymes in the pathway leading to the synthesis of cellulose. Interrupted line represents pathway in which intermediate steps are not shown. FBP, fructose-1,6-biphosphate phosphatase (EC 3.1.3.11); PGI, phosphoglucoisomerase (EC 5.3.1.9); GK, glucose kinase; FK, fructose kinase (EC 2.7.1.2); PGM, phosphoglucomutase (EC 5.3.1.9); UGP, UDPG-pyrophosphorylase (EC 2.2.7.9); CS, cellulose synthase (EC 2.4.2.12) (adapted from (Ross *et al.*, 1991; Solano *et al.*, 2002)

polymerization, assembly, and crystallization. The process includes the synthesis of uridine diphosphate glucose (UDP-Glc) which is the immediate sugar nucleotide precursor of cellulose (Swissa *et al.*, 1980). This is followed by glucose polymerization into β-1,4-glucan chains, and assembly of nascent chains into large microfibril structures (Ross *et al.*, 1991). Cellulose synthase, the enzyme responsible for glucose polymerization has been identified as a component of cellulose synthesizing machinery besides ancillary proteins that facilitate precise ordering of glucan chains in the crystalline state (Kimura *et al.*, 1999).

Native cellulose can exist in either two crystalline allomorphs depending on the mode of biosynthesis. These are designated as cellulose I and cellulose II (Franz and Blaschek, 1990). In nature, cellulose I is more commonly found. The β-1,4-glucan chains of cellulose I are synthesized unidirectionally and arranged parallel to each other to form microfibrils whereas the arrangement of glucan chains in cellulose II is random and antiparallel possibly due to the chain folding during synthesis (Gardner and Blackwell, 1974). The addition of hydrogen bonding per glucose residue in glucan chains of cellulose II makes it a more thermodynamically favored allomorph in contrast to the metastable cellulose I (Brown, 2004).

Several techniques from sophisticated microscopy (Frey-Wyssling, 1976), X-ray diffraction (Roelosfsen, 1959) and methylation analysis (Fry, 1988) to simple labeling dyes such as Calcofluor white (Harrington and Raper, 1968), Congo red (Frey-Wyssling, 1976) and purified cellulose-binding domains (Taylor *et al.*, 1996; Levy and Shoseyov, 2002) have been used in the detection and identification of cellulose molecules. Congo red has been shown to bind β(1→4)-D-glucopyranosyl units as well as other extracellular matrix components in several bacteria (Slifkin and

Cumbie, 1988; Zogaj *et al.*, 2001; Spiers *et al.*, 2002) whilst Calcofluor white has an affinity towards β(1→4) and β(1→3) glycosidic bonds of polysaccharides (Spiers *et al.*, 2002). Bacteria that express cellulose display a characteristic pink, dry and rough (pdar) colony morphology when cells are grown on agar medium containing Congo red (Zogaj *et al.*, 2001). A fluorescent phenotype is observed under long-wave UV light when cellulose producing bacteria are grown on Calcofluor agar plates (Zogaj *et al.*, 2001). Though neither dye is specific for cellulose, they are still useful for the preliminary screening of cellulose producing organisms. Apart of these, purified cellulose-binding domains (CBDs) from fungi and bacteria (Taylor *et al.*, 1996; Levy and Shoseyov, 2002) which were proven to be highly specific for cellulose have been increasingly applied in substitution of conventional labeling dyes such as Calcofluor white and Congo red in the field of cellulose studies. The production of cellulose among diverse organisms suggests that cellulose biogenesis may be an ancient evolutionary process that plays a vital role in the successful formation of life on earth. Therefore, fundamental research in the structure, mechanism and evolution of cellulose biosynthesis are worthwhile investigating.

2.6 Bacterial Cellulose and Their Biological Significance:

Bacterial cellulose, in the form of gelatinous pellicle was first described in Gram-negative bacterium *Acetobacter xylinum* by Brown (1886) and further documented by Hestrin and coworkers at the beginning of the twentieth century (Hestrin *et al.*, 1947; Hestrin and Schramm, 1954). Soon, their works initiated extensive consequential studies on bacterial cellulose synthesis with *Gluconacetobacter xylinus* (formerly known as *A. xylinum*) as the archetype. To date,

cellulose production has been reported in a wide range of bacteria which includes *Agrobacterium tumefaciens*, *Rhizobium leguminosarum* bv. *trifolii*, *Sarcina ventriculi*, several species of cyanobacteria and also in gamma-proteobacteria such as *Salmonella* spp., *Escherichia coli*, *Pseudomonas* spp. and *Klebsiella pneumoniae* (Matthysee *et al.*, 1995b; Ausmees *et al.*, 1999; Nobles *et al.*, 2001; Zogaj *et al.*, 2001; Solano *et al.*, 2002; Gerstel and Romling, 2003; Spiers *et al.*, 2003; Zogaj *et al.*, 2003). These organisms have been shown to produce cellulose of different structures depending on the organisms, the synthesizing terminal complexes involved and their culturing conditions (Saxena and Brown, 2005). For instance, under aerobic and static conditions, *G. xylinus* produces cellulose in the form of crystalline fibrils (Brown, 1996) while *E. coli* and *Salmonella* spp. synthesize amorphous cellulose (Le Quere and Ghigo, 2009). In addition, the synthesis of acetylated cellulose has been described in *Pseudomonas fluorescens* (Spiers *et al.*, 2003). Besides morphological variation, they share common features by forming highly inert and hydrophobic nets surrounding the bacteria (Zogaj *et al.*, 2001) that may confer mechanical, chemical or biological protection within natural habitats (Ross *et al.*, 1991).

As a whole, the biological roles of cellulose are mainly associated with the survival, persistence and stabilization of bacterial organisms within the natural environment (Romling, 2002; White *et al.*, 2006). Specifically, it is known as one of the major factors in i) formation of extracellular matrix (Zogaj *et al.*, 2001; Solano *et al.*, 2002), ii) colonization of plants (Matthysee *et al.*, 1981; Matthysee, 1983; Smit *et al.*, 1992; Barak *et al.*, 2007), and iii) persistence in hostile environments such as exposure to UV radiation (Scott Williams and Cannon, 1989), sodium hypochlorite treatment (Solano *et al.*, 2002) and long term desiccation (White *et al.*, 2006).

The formation of biofilm is initiated by the adherence of free living bacterial cells to an exposed surface followed by subsequent growth that occurs concomitantly with the production of a dense network of extracellular matrix (Davey and O'Toole, 2000). The extensive network of exopolysaccharide results in the firm adherence of bacteria to each other and to abiotic surfaces (Kuchma and O'Toole, 2000; Sutherland, 2001b; Gilbert *et al.*, 2002). The interconnection between biofilm formation and extracellular matrix is further proven when mutants unable to synthesize exopolysaccharide are also unable to construct biofilm (O'Toole *et al.*, 2000; Sutherland, 2001a). Intriguingly, in some animal pathogens, cellulose has been shown to be a component of a polysaccharide-rich matrix that contributes to biofilm maturation, multicellular behavior and adherence to animal cells (Scher *et al.*, 2005; Ledeboer *et al.*, 2006; Sandra *et al.*, 2007). The multicellular morphotype confers better ability to withstand competition from newly invading organisms and it is more resilient to antibiotic and biocide treatment (Romling *et al.*, 2000; Zogaj *et al.*, 2001; Zogaj *et al.*, 2003). Further studies on multicellular behavior of these bacteria showed that pellicle formation, cell clumping, extracellular matrix, and biofilm formation are closely associated with the co-expression of cellulose and curli fimbriae (Zogaj *et al.*, 2003). These findings are supported by the fact that a cellulose deficient mutant retained partial ability to form cell aggregates but not a confluent biofilm (Solano *et al.*, 2002). Given that the physical properties of biofilm are highly variable relying on the respective organisms and environmental conditions (Costerton *et al.*, 1996; Davey and O'Toole, 2000; Morris and Monier, 2003; Hall-Stoodley *et al.*, 2004; Ramey *et al.*, 2004), the significant and absolute contribution of cellulose to biofilm formation has yet to be convincingly adduced.

In *G. xylinus*, cellulose pellicle serves as a flotation device which buoys the cells to air liquid interface, maintaining the organisms in an aerobic environment particularly in liquids. Besides, it also protects *G. xylinus* cells from the hazardous effect of UV radiation (Brown, 1996). In addition, cellulose is also known to be involved in the anchoring of several bacterial species to plants (Matthysee *et al.*, 2008), which in certain circumstances, can act as vectors and confer survival advantage to bacterial cells under natural conditions (Barak *et al.*, 2007). For instance, in the cases of *Rhizobium* spp. and plant pathogen *A. tumefaciens*, cellulose is responsible for the irreversible adherence plus aggregation of bacteria at the root tip, for both symbiotic and infectious interaction (Matthysee, 1983; Smit *et al.*, 1992). In parallel research, cellulose is also used by *P. fluorescens* SBW 25 in the colonization of plant surfaces in the phyllosphere and rhizosphere (Ude *et al.*, 2006). On the other hand, the colonization of seeds, sprouts, leaves and fruits of a variety of plants species by *Salmonella enterica*, the Gram-negative bacterium which causes salmonellosis via contaminated food sources have been shown to be facilitated by both cellulose and O-antigen capsule (Barak *et al.*, 2007). Most recently, cellulose synthase mutant of *E. coli* O157:H7 showed a significant reduction in binding to alfalfa sprouts, but not for the binding of *E. coli* O157:H7 to mammalian cells. This finding underscores the fact that cellulose might not be involved in the virulence process, rather it might play pivotal role in the bacterial life cycle outside the animal host, thereby aiding in bacterial transmission (White *et al.*, 2008).

2.7 Cellulose synthase:

Cellulose synthase, a multipolymerizing unit enzyme complex is a family 2 glycosyltransferase that catalyzes the formation of β-linkages (Ross *et al.*, 1991; Campbell *et al.*, 1997). The catalytic subunit of this enzyme is responsible for the elongation of β-glucan chains through a single polymerization step by using UDP-glucose as the substrate (Saxena *et al.*, 1995; Koyama *et al.*, 1997). Usually, the catalytic subunit with the length of 723 to 888 amino acids is encoded by the first gene of the cellulose biosynthesis operon, namely *bcsA* (**b**acterial **c**ellulose **s**ynthase), and in some cases *acsA* (***A****cetobacter* **c**ellulose **s**ynthase) or *celA* (**cel**lulose) (Romling, 2002).

The gene *bcsA* is best conserved among diverse species as compared to other cellulose biosynthesis genes (Romling, 2002). A comparative analysis of cellulose synthases available in the databases concluded that the conserved regions are distributed over two domains, domain A and domain B (Stasinopoulos *et al.*, 1999). Domain A harbors a conserved D, D, D motif which is also known as the UDP binding motif while domain B contains a QXXRQ motif with no known function (Saxena *et al.*, 1995; Saxena and Brown, 2000). Although the catalytic core domains are well conserved, tangible sequence variation has been reported within cellulose synthases of different origins. For example, the hyper variable region (HVR) (Richmond, 2000), the plant specific and conserved region (CR-P), and the zinc finger domain are found only in plant cellulose synthases but absent in proteobacterial and ascidian cellulose synthase (Delmer, 1999).

It has been established that the native form of cellulose synthase molecule is a complex of three protein subunits with sizes of 90, 67 and 54 kDa respectively. This

finding has led to the hypothesis that the bacterial cellulose synthase molecule is a hetero-oligomeric protein complex with the larger (90kDa) band representing an inactive precursor form which then undergoes further processing and yields the 67 and 54 kDa subunits. Both subunits are involved directly in the catalytic and regulatory cores of the enzyme (Ross *et al.*, 1991).

In several strains of *G. xylinus* and *A. tumefaciens*, synthase activity is shown to occur exclusively in the membrane associated fraction (Glaser, 1958), indicating that cellulose synthase is probably an integral membrane protein (Amikam and Benziman, 1989). Therefore, the *in vitro* activity assay of cellulose synthase is often done by incubating the membrane preparation in the presence of UDP-[^{14}C] glucose and Mg^{2+} followed by quantification of radioactivity incorporation into the β-glucan chain (Amikam and Benziman, 1989; Ross *et al.*, 1991).

2.8 The organization of *bcs* genes

It has long been established that distinct genetic elements are required for cellulose biosynthesis in different organisms as observed in the *Acetobactericeae*, the γ and β proteobacteria and the *Rhizobiaceae* (Saxena *et al.*, 1994; Matthysee *et al.*, 1995a; Zogaj *et al.*, 2001). Only two genes are present constitutively within the cellulose biosynthesis operon throughout the species, specifically the genes encoding cellulose synthase and bis-(3’, 5’)-cyclic diguanylic acid (c-di-GMP) binding protein (Romling, 2002).

In general, characterized cellulose biosynthesis operons are represented by *G. xylinus* (*acsABCD*), *E. coli* (*bcsABZC*, *bcsEFG*) and *A. tumefaciens* (*celABC*, *celDE*) respectively (Saxena *et al.*, 1994; Matthysee *et al.*, 1995a; Zogaj *et al.*, 2001). The