THE ROLES OF FERRIC UPTAKE REGULATOR (Fur) FOR PLANT ROOT ATTACHMENT BY *Burkholderia* sp. USMB20

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THE ROLES OF FERRIC UPTAKE REGULATOR (Fur) FOR PLANT ROOT ATTACHMENT BY *Burkholderia* sp. USMB20

by

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PERANAN PENGAWAL ATUR PENGAMBILAN FERIK (Fur) UNTUK PELEKATAN AKAR TUMBUHAN OLEH *Burkholderia* sp. USMB20

ABSTRAK

Pelekatan adalah langkah awal bakteria semasa proses kolonisasi pada akar tumbuhan. Bakteria memerlukan besi sebagai komponen yang penting untuk pertumbuhan dan proses-proses sel. Pengawal atur pengambilan ferik (Fur) merupakan pengawal atur global yang mengawal pengambilan ferum untuk sel bakteria. Kajian ini mengkaji peranan Fur oleh *Burkholderia* sp. USMB20 dalam mengawal pengambilan ferum semasa pelekatan pada akar tumbuhan. Pencilan USMB20 mutan tanpa Fur (USMB20Δ*fur*) digunakan sebagai perbandingan. USMB20 mencapai tahap pelekatan tertinggi dalam kepekatan ferum pada 50 µM. Sebaliknya, USMB20Δ*fur* menunjukkan tahap pelekatan sel yang lebih rendah. Mikroskop electron penskanan (SEM) juga menunjukkan pelekatan USMB20 pada permukaan akar adalah sangat padat berbanding USMB20Δ*fur*. Kajian ini menunjukkan Fur telah mempengaruhi mekanisma pelekatan bakteria pada akar tumbuhan. Analisis ke atas protein menggunakan electroforesis gel dua dimensi (2DE) serta pengecaman peptida mendedahkan bahawa Fur mengawal penghasilan beberapa protein semasa pelekatan USMB20 pada akar tumbuhan tetapi tidak dikenalpasti pada USMB20Δ*fur*. Protein-protein ini dikenal pasti sebagai caperon molekular GroEL, faktor pemanjangan TU, xilose isomerase dan alkyl hidrogenase peroksida. Berdasarkan asai ekspresi gen melalui analisa lansung kualitatif PCR, gen *groEL*, *tuf*, *xylA* dan *ahpC* mengalami pertambahan ekspresi pada USMB20 semasa perlekatan pada akar tumbuhan tetapi tiada peningkatan ekspresi pada USMB20Δ*fur*. Untuk setiap protein dan gen yang dikenal pasti meningkat semasa pelekatan, jujukan

kotak Fur telah dijumpai di kawasan hadapan promoter gen. Fur mengenal kawasan ini sebelum memulakan transkripsi gen-gen ini. Ini menunjukkan kemungkinan gen adalah di kawal atur oleh Fur. Sebagai kesimpulan, Fur adalah penting dalam mengawal atur pengambilan ferum dan beberapa protein penting yang diperlukan semasa pelekatan pada akar tumbuhan oleh USMB20.

THE ROLES OF FERRIC UPTAKE REGULATOR (Fur) FOR PLANT ROOT ATTACHMENT BY *Burkholderia* sp. USMB20

ABSTRACT

Attachment is an early step during bacterial plant root colonization process. Bacteria need iron as an essential component for its growth and cellular processes. Ferric uptake regulator (Fur) is a global regulator that controls bacterial iron uptake. This study investigated the roles of *Burkholderia* sp. USMB20's Fur in controlling iron uptake during plant root attachment. USMB20 Fur minus mutant (USMB20Δ*fur*) was used as comparison. The observation showed USMB20 recorded highest number of attached cell on root surface in 50µM of ferric concentration, where as USMB20Δfur recorded less numbers of attached cell. Scanning Electron Microscope (SEM) showed dense attachment of cells on the root surface compared to the USMB20Δ*fur*. This study has demonstrated that Fur influenced several mechanisms that enhanced bacterial attachment on root. Analysis of protein using Two Dimension Electrophoresis (2DE) and peptide sequencing revealed that Fur controlled the up regulation of several USMB20 proteins during USMB20 attachment nevertheless down regulation for USMB20Δ*fur*. These proteins were identified as molecular chaperone groEL, elongation factor TU, xylose isomerase and alkyl hydrogenase peroxide. Based on relative qualitative gene expression *via* qPCR, genes (*groEL*, *tuf*, *xylA* and *ahpC*) coding for these Fur-regulated proteins were discovered to be induced in USMB20 during root attachment, while no gene expression was observed in USMB20Δ*fur*. Additionally, each up regulated gene had putative Fur-box consensus sequence. The Fur would recognize this region prior to initiation of the transcription

of these genes. In summary, Fur is important in controlling iron uptake and regulating several essential proteins during plant root attachment by USMB20.

CHAPTER 1

1.0 INTRODUCTION

Attachment of bacteria on the plant root surface is a crucial step for successful colonization. Consequently, it promotes plant growth and nodulation for leguminous plant via symbiotic relationships of both symbionts. The primary attachment stage constitutes beneficial contact between a surface and planktonic microorganisms. During the process of attachment, the bacteria must be brought into close proximity of the surface, propelled either randomly or in a directed fashion *via* chemotaxis and mobility (Prakash *et al.*, 2003). To stabilize and secure the attachment, attached bacteria produced extracellular components known as biofilm (O'Toole *et al.*, 2000; Harrison *et al.*, 2006; Uppuluri *et al.*, 2010). It was also reported that iron is also an essential factor for bacterial host colonization and infection especially in animal host (Beisel, 1977; Horsburgh *et al.*, 2001). In contrast, limited studies and little works have been carried out to observe iron effect on plant host-microbe colonization. Earlier report by Molina et al., (2005) and Molina et al., (2006) showed the importance of iron acquisition for microbial attachment of *Pseudomonas putida* KT2440 on roots of corn. It was also revealed that TonB protein which is important in iron uptake system enhanced plant root attachment. Most studies on bacterial host colonization and infection indicated that low and high iron level will negatively affect the colonization based on bacterial biofilm formation observations (Musk *et al.*, 2005; Yang *et al.*, 2007; Aubert *et al.,* 2008; Patriquin *et al.,* 2008). Majority of iron in environment is in oxidation form, which is from ferrous (Fe^{2+}) to ferric (Fe^{3+}). Thus, bacterial need a mechanism to transport iron.

Most bacteria have Ferric Uptake Regulator (Fur) system to transport and uptake ferric for intracellular usage (Stojiljkovic and Hantke, 1995; Harvie *et al.*, 2005) and also involved in infection process. For that reason, this study will uncover the role of Fur in controlling iron uptake during bacterial attachment for plant root colonization. Fur initially was defined as an iron-responsive repressor. Consequently, it was found as a global regulator that control various bacterial functions such as cellular processes including non iron acquisition related function (Cha *et al.*, 2008). Besides, Fur also controlled the expression involved in siderophore-mediated iron uptake and cellular processes; including metabolic pathways, acid tolerance, chemotaxis, oxidative stress response, electron-transport systems and energy metabolism (Quatrini *et al.*, 2005; Zhang *et al.*, 2005; Cha *et al.,* 2008). Studies on *Pseudomonas* sp. and *Burkholderia* sp. reported that Fur was important in virulence and infection properties such as in toxin production, haemolysin production, adhesion activity and iron homeostasis during oxidative stress (Horsburgh *et al.*, 2001; Cha *et al.*, 2008). Thus, Fur is conserved in *Burkholderia* strains and looking at this opportunity, this experiment will investigate the role of Fur for bacterial root attachment by *Burkholderia* sp.

The *Burkholderia* genus is well known for its pathogenicity towards humans and animals (Brett and Woods, 2000; Urban *et al*., 2004). There are also beneficial *Burkholderia* species that have great potential in agriculture and some species are involved in nodule formation with plants (Minerdi *et al.,* 2001; Moulin *et al.,* 2001; Hirsch *et al*., 2011; Salwani *et al.,* 2012). A lot of plant associated *Burkholderia* have recently been isolated from a variety of legumes (Chen *et al.,* 2003, 2005a, 2005b; Barrett and Parker, 2005, 2006; Elliott *et al.,* 2007a, 2007b; Sprent, 2008). These *Burkholderia* sp. may also bring virulence characteristics that could infect and

colonize the host plant (Harvie *et al.*, 2005; Angus *et al.*, 2014). Therefore, the pathogenicity characteristics had been 'transformed' toward mutualism relation for instance symbiotic-pathogenic evolution (Marchetti *et al.*, 2010; Angus *et al.*, 2014). The infection properties such as flagella formation, chemotaxis, motility, 1-aminocyclopropane-1-carboxylate (ACC) deaminase production (to lower down the plant defense system; ethylene) and pili were involved for bacterial attachment. When bacteria interact with the root, attachment will occur and form biofilm for stable colonization and initiate symbiotic relationship with host.

In order to uncover the roles of Fur during bacterial root attachment, Fur minus mutant of USMB20 (USMB20Δ*fur*) which was constructed by Faisal (2014) was used as comparison since it was unable to express the *fur* gene. The ability of USMB20 to attach on the plant root will be examined and compared with the mutant USMB20Δ*fur*. The physiological activities by USMB20 that are involved during attachment were carried out. These would reveal a finding of Fur functions in bacterial attachment during this process on plant root. This study also determined the role of Fur effects on bacterial proteins and genes expression during planktonic and plant root attachment.

1.1 Objectives:

1. To observe root attachment ability of *Burkholderia* sp.USMB20 under varying ferric concentrations.

2. To compare the protein profiles of *Burkholderia* sp.USMB20 and its Fur minus mutant (USMB20Δ*fur*) in root-attached and planktonic cells.

3. To quantify the gene expression of proteins regulated by Fur during root attachment.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Bacteria plant root attachment

Bacteria-plant interaction needs various mechanisms to mediate the attachment process. The first phase of attachment is weak, reversible, and involves unspecific binding with plant lectins, calcium-binding bacterial protein with bacterial surface polysaccharide. The second attachment step requires the synthesis of bacterial cellulose fibrils that causes a tight and irreversible binding of the bacteria to the roots. Attachment observation of *Azospirillum brasilense* to cereals roots can also involve two different steps (Rodríguez-Navarro *et al.*, 2007). Bacterial surface proteins, capsular polysaccharide and flagella appear to govern the first binding step while extracellular polysaccharide is involved in the second step. Outer cell surface proteins and pili are implicated in the adherence of *Pseudomonas* species to plant roots (Fujishige *et al.*, 2006a; Rodríguez-Navarro *et al.*, 2007).

Arabidopsis thaliana roots also produced root exudates (plant polysaccharide) that serve as a signal for *B. subtilis* colonization for biofilm development. Plant polysaccharides are main sources for bacteria to synthesis bacterial EPS for biofilm matrix. Biofilm genes are needed for plant root colonization by specific bacteria and provide insights into how bacterial matrix synthesis may be triggered by the plant (Beauregard *et al.*, 2013)

2.2 Bacterial appendanges for adhesion and attachment

Several adhesions components of bacteria such as pili, fimbriae and curli are important for bacterial autoaggregation and enhancement of biofilm formation on abiotic surfaces in addition to their receptor recognition faculty. Other bacterial appendages that had been report in early reports include bristles, cilia, filaments, fimbriae, fibrillae, pili or needles (Duguid and Anderson, 1967). Over the years, the terms pili (Latin, hairs), fimbriae (Latin, threads), filament and needle (type III secretion apparatus) have been used (Cornelis, 2006).

Chaperone Usher (CU) pili is short appendages with length of 2 μM and width of 7-8 nM. CU pili is responsible for the synthesis of linear multisubunit pili or fimbriae mainly found in Enterobacteriaceae such as *Pseudomonas, Haemophilus, Bordetella, Xylella, Burkholderia, Acinetobacter and Ralstonia* species (Sauer *et al,* 2004). Currently, in this advance genomic era, most reports revealed that bacterial cell surface appendages are consisted with CU pili. CU pili is also important as a virulence factor responsible for specific host attachment (Wright *et al*, 2007; Zavialov *et al*, 2007).

Type IV pili are pilin polymers produced by many Gram negative bacteria. Type IV pili are 1–4 μM in length and 5–8 nm in diameter that can resist stress forces (Merz *et al*, 2000; Maier *et al*, 2002). It is also essential for bacterial virulence, including auto-aggregation, adhesion, twitching motility, biofilm formation and cellular invasion (Craig *et al*, 2004; Burrows, 2005). Unlike curli and CU pili, type IV pilus assembly requires large assembly machinery and energy from ATPs (Fronzes *et al.*, 2008).

Other adhesions components, such as fimbriae and curli (1 μM x 4-7 nm) also confer bacterial autoaggregation to enhance biofilm formation on abiotic surfaces. Curli was identified in a class of bacterial filaments expressed on the outer surfaces of Enterobacteriaceae (Olsen *et al*, 1989; Barnhart and Chapman, 2006). It was reported as 'thin aggregative fimbriae' that contain major proteinaceous component of the extracellular matrix including cellulose. Cellulose will associate with Congo red dye and this method can be used to identify its appearance (Zogaj *et al*, 2001; Chapman *et al*, 2002). In host adhesion by bacteria study, curli has a role in bacterial pathogenesis by promoting cell adhesion and invasion. Studies in mice reveal curli fibres can enhance amyloidosis in the host by acting as a cross-seeding nuclei (Gophna *et al*, 2002; Lundmark, 2005).

2.3 Bacterial Biofilm

Bacterial biofilm is a bacterial community in which cells are embedded in a matrix of extracellular polymeric compounds attached to a surface (Branda *et al.,* 2005). In biofilm, bacteria are protected by the matrix from deleterious conditions (Davey and O'Toole, 2000). This favorable condition provides stable bacterial colonization of plants and animals. Bacteria cells are imbedded in biofilm matrix that consists of major components of biofilm such as exopolysaccharide (EPS), extracellular DNA and water. Other bacterial surface components that are important for autoaggregation and biofilm development are flagella and quorum-sensing signals (Costerton, 1995; Schembri and Klemm, 2001). Biofilm formation involves several stages to develop stable biofilm architecture (Figure 2.1). Free floating bacteria (planktonic) search for surfaces to initiate attachment. Then the attached cells start to produce biofilm matrix which consist of exopolysaccharides as the major component.

At this stage, biofilm becomes more stable at the maturation stage. The end of biofilm maturation is dispersion of bacterial cells due to lack of growth resources (Hall-Stoodley *et al.*, 2004; Harrison *et al.*, 2006; Lasa, 2006).

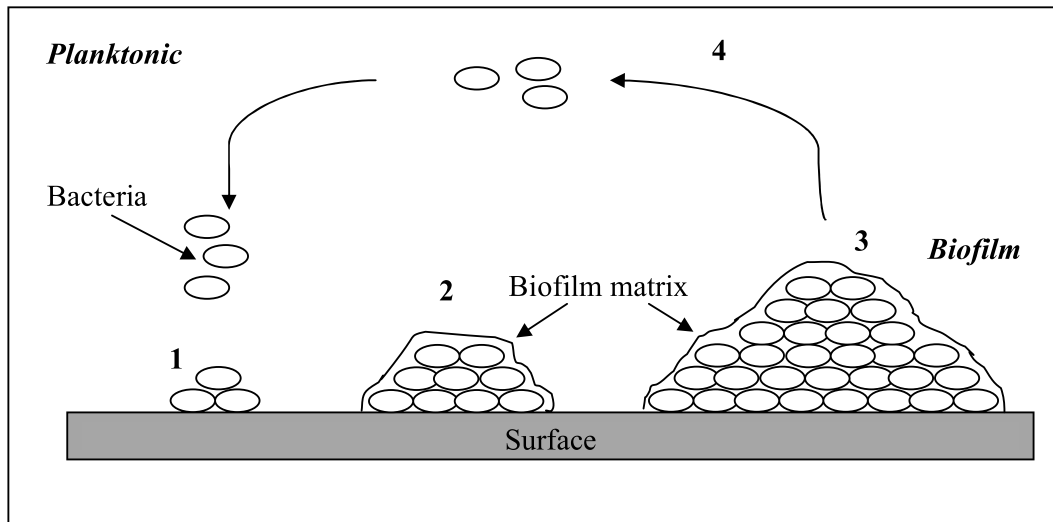

Figure 2.1: Schematic illustrations of stages involved in biofilm formation and development. Stage 1: initial bacterial attachment to the surface; 2: production of the biofilm matrix; 3: maturation of biofilm architecture; 4: dispersion of bacterial cells from the biofilm (Hall-Stoodley *et al.*, 2004; Harrison *et al.*, 2006; Lasa, 2006)

2.4 Biofilm production on rhizosphere for bacterial colonization

Rhizosphere is the area around the plant roots. This was termed by Hiltner in 1904 (Hiltner, 1904; Hartmann *et al.*, 2008). It consists of microorganisms and root secretions. Some of these microorganisms are plant pathogens and also live with plants as mutualists (symbiosis). Bacteria that colonize the roots and promote plant growth are known as plant growth-promoting rhizobacteria. Plant growth promoting rhizobacteria (PGPR) associated with plant root surfaces are known to contribute towards increase in plant yield by mechanisms such as improved mineral uptake,

phytohormone production (Biswas *et al*., 2000; Ryu *et al*., 2003; Fujishige *et al*., 2006b) and pathogen suppression by producing antibiotics (Mazzola *et al*., 1992).

Colonization of microorganisms in the rhizosphere were reported to be the most microorganisms that often exist in biofilms when colonizing plant surfaces (Rovira *et al*., 1974). Other advanced studies discovered that bacterial interactions and formation of biofilms on the root surface involve complex mechanisms (Kearns *et al*., 2005; Stanley and Lazazzera, 2005; Rudrappa *et al*., 2007). Transmission electron microscopy has shown the presence of fibrillar material around rhizobia attached to the root surfaces (Fujishige *et al.*, 2006b). Attachment is an initial step for the biofilm development on the root surface, involving various mechanisms and diverse surface molecules of both partners to mediate in this process (Rodríguez-Navarro *et al.*, 2007).

2.5 Importance of iron for bacteria

Iron is an essential nutrient for bacterial growth and is crucial for bacterial energy production, nucleotide synthesis, regulation of gene expression and important component of many enzymes. In tropical soil, level of iron is in a range of 25 to 160 mg/kg (Shamsuddin et al., 2009) and in the extremely insoluble oxidized ferric form (Fe^{+3}). Therefore, many bacteria produce siderophores which are small organic molecules that they excrete and bind with ferric. The ferric-siderophore complexes are internalized in to bacteria cells by dedicated transport systems (Lopez and Crosa, 2007). Thus, iron acquisition is strictly controlled. In Enterobacteriaceae this control is mediated by Fur protein (ferric uptake regulator) (Schäffer *et al.*, 1985; Stojiljkovic et al., 1994; Baichoo and Helmann, 2002).

2.5.1 Bacterial siderophore

Most bacteria produce and secrete iron chelator molecules known as siderophore to control their iron requirements (Neilands, 1995). Siderophores are water-soluble, low-molecular-weight molecules that bind ferric ion with high affinity. *B. pseudomallei* produces siderophore called malleobactin that belongs to the hydroxamate class (Alice, 2006). Siderophore is capable of removing iron from both transferrin and lactoferrinn in human body (Yang *et al*., 1993). The molecular basis of coordinate regulation by iron has been studied most thoroughly in *E. coli*. In this organism, coordinate regulation of gene expression by iron depends on the regulatory gene known as *fur*.

2.6 Importance of iron for bacterial biofilm formation

Iron regulation of biofilm formation has been demonstrated in many bacterial species such as *Burkholderia cepacia, Pseudomonas aeruginosa*, *Escherichia coli* and *Vibrio cholera* (Lin *et al*., 2012). Most studies indicated that limited iron negatively affects bacterial biofilm formation while elevated levels of iron also compromise biofilm formation (Musk *et al.*, 2005; Yang *et al.*, 2007). Patriquin et al. (2008) reported that, iron levels below 8 μM were needed to effect iron limitation and the attendant negative impact on biofilm formation; so clearly, iron limitation and iron excess both adversely affect biofilm formation in *P. aeruginosa* K1120. Indeed, they also noted a reduction in biofilm formation at 64 μM of $FeCl_3$. Previous studies have suggested that iron limitation effect the biofilm formation, though these generally relied on the use of metal chelators (e.g., lactoferrin) to impose iron restriction (Singh *et al*., 2002; Singh, 2004). Planktonic cell growth also declined with declining Fe levels, though only at concentrations below 1 μM, indicating that the iron needs for

biofilm cells are greater than those of planktonic cells. A mutant of *P. aeruginosa* unable to synthesize pyoverdine (siderophore) was unable to produce high biofilm yield. This shows the importance of siderophore in controlling iron acquisition in biofilm cells. It showed that biofilm cells were sensing iron limitation effects and responsible for biofilm reduction (Patriquin *et al.*, 2008).

In healthy humans the lower respiratory tract as well as mucosa, contains a very low free iron concentration (10^{-12} µM), while in Cystic fibrosis (CF) patients sputum iron concentration is very high with mean value of 63 µM. Accumulation of iron would cause subsequent clinical complications such as production of reactive oxygen species that causes lung disorder, increases bacterial growth and virulence effects. Therefore, the iron availability is an important signal to bacteria counteract by leaving the motile free-living forms and entering into a new lifestyle, biofilm. Consequently this may facilitate penetration of host epithelial barriers, establish colonization and cause infection (Berlutti *et al.*, 2005) Thus, biofilm formation are positively iron-modulated in bacteria.

2.7 Ferric uptake regulator (Fur)

The *fur* gene will transcript to Fur protein. Fur was first observed to repress the transcription of genes that code for components of ferric uptake systems found in *E. coli* by Stojiljkovic et al. (1994). Fur is a small protein (15–18 kDa) containing many highly conserved regions important to its functions (Braun and Hanke, 1990; Coy *et al*., 1994). Analysis of bacterial genome sequences indicated that some bacteria have multiple Fur proteins (Bsat *et al*., 1999). *B. subtilis* has at least three Fur structural homologues. These proteins have diverse functions, suggesting that many functions of Fur and Fur-like proteins are yet to be identified (Bsat *et al*., 1999;

Loprasert, 2000). The clinical isolate, *B. pseudomallei* P844 had identified single copy of *fur* gene (Loprasert *et al*., 2000).

The Fur protein represses the transcription of iron-regulated promoters in response to an increasing intracellular iron concentration. In the presence of iron, inactive Fur binds to ferrous ions (Fe^2+) and becomes an active transcription regulator or repressor that binds to a conserved DNA region known as Fur box. Fur box is located in the vicinity of the gene's promoter (Figure 2.2). The N-terminus of Fur is responsible for DNA-binding, whereas the C-terminus is involved in dimerization of the protein (Stojiljkovic and Hantke, 1995).

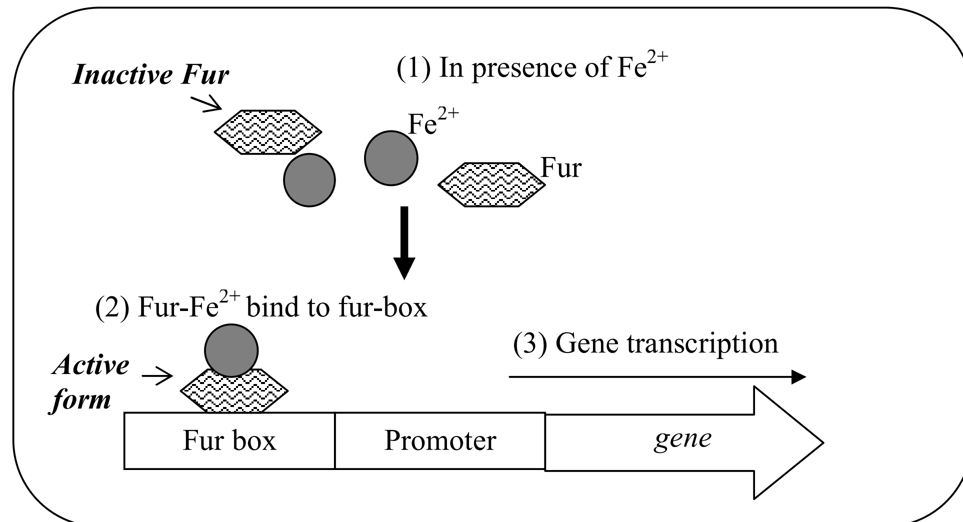

Figure 2.2: Schematic of Fur in inactive form to active form to initiate gene transcription in presence of ferrous iron.

Fur also control and regulate bacterial systems that are related to iron acquisition (Pohl *et al.*, 2003). Fur also was reported to regulate other genes that are not directly related to iron acquisition such as those encoding hemolysin, Shiga-like toxin, manganese superoxide dismutase and oxidative stress reponse (Hassett *et al*., 1996; Escolar *et al*., 1998; Horsburgh *et al*., 2001; Cha *et al*., 2008).

Regulation of gene expression by iron occurs in a number of pathogenic organisms. Additional regulatory proteins may be added on the Fur repressor to

provide the fine-tuning necessary for the optimum regulation of individual virulence genes in response to iron and other environmental signals. Fur and iron concentration are part of the more global control system regulating not only iron assimilation but also several other factors involved in pathogen mechanisms (Litwin and Calderwood, 1993, Loprasert *et al*., 2000). Many research on bacterial virulence of *Pseudomonas* and *Burkholderia* reported that Fur is important in virulence properties such as in toxin production, haemolysin production, adhesion activity and iron homeostasis during oxidative stress.

2.8 Regulatory gene

A regulatory gene is involved in other genes expressions. The *fur* gene is one of the regulatory gene in bacteria. Bacterial regulatory gene control genetic expression for optimum condition in bacterial living system. Thus, the organism would function effectively and efficiently. The regulatory gene may respond to environmental factors and pressures. In some cases, a regulatory gene acts as an activator, turning a gene or group of genes on so that the expression can occur. For example, when the bacteria are exposed to antibiotics, the activator in a bacterium activates a gene for antibiotic resistance. In contrast, regulatory gene may act as repressors, turning genes off so that they cannot express (Maeda *et al.,* 2000; Helmann, 2002; Paget and Helmann, 2003).

2.9 Fur Box

Fur box is a short consensus sequence (19 bp) in front of the upstream intergenic region of *fur*-regulated gene. Fur will bind specifically at Fur box region and trigger bacterial gene expression (De Lorenzo *et al.*, 1987; Baichoo and Helmann, 2002; Pohl *et al.*, 2003). Numerous strategies have been employed to find new Fur

binding sites in the promoter region of suspected iron-regulated genes that are known as Fur box (Chen *et al.*, 2007). Stojiljkovic et al. (1994) created a successful 'Fur titration assay' (consensus sequence-based technique) to locate new Fur binding sites using an *fhuF:lacZ* fusion and Fur consensus sequence containing plasmid titrant on MacConkey plates for *E.coli* (Stojiljkovic *et al.*, 1994). Alternatively, McHugh et al., (2003) used mRNA transcriptional profiles to determine the iron- and Fur-regulated genes in the absence of iron or Fur protein. Another method for finding Fur-regulated genes is to use molecular information theory to locate new binding sites (Schneider and Mastronarde, 1996; (Chen *et al.*, 2007). Multiple alignments were performed on the interest conservation sites (consisted Fur box) and to obtain a set of aligned binding sites. The sequence logo was generated based on this aligned binding sites.

2.10 Flagella

Bacteria move by a variety of mechanisms, but the most studied form of bacterial motility involves the assembly and rotation of propeller-like flagella. Each flagellum is assembled from the inside-out starting with a "basal body" inserted into the cytoplasmic membrane (Macnab, 2003). Flagellum assembly starts with the basal body by secretion apparatus subunits of the axle-like rod that extends through the peptidoglycan and outer membrane. This is followed by formation of short curved hook and long helical filament called flagellin. Flagella biosynthesis requires 30 proteins that are approximately 2% of a cell's metabolic resources (Macnab, 1996; Smith and Chapman, 2010).

In addition to synthesis expenditure, motile bacteria must make further investment by consumption of ion motive forces to power flagellar rotation (Macnab, 1996). The basal body is connected to a motor complex consisting of rotor and stator

(MotA and MotB) to activate the flagellum (Blair and Berg, 1990; Zhou *et al*., 1998; Gabel and Berg, 2003). This system consumes high energy to activate and power the bacterial movement through liquid and swarming over solid surfaces (Darnton *et al*., 2007; Kearns, 2010).

There are three bacterial mode; swimming, swarming and twitching. Swimming motility is a movement in liquid environments by individual cells independently perceiving chemical signals that trigger adaptive chemotactic responses. Swarming is characterized by a multicellular movement of bacteria that migrate above solid substrates while twitching is surface movement of bacteria that is powered by pili subsequently help to attach to the surface and pulling the cell closer to the attachment site (Calvio *et al.*, 2005).

2.11 Bacterial motility for biofilm formation

Bacteria motility is regulated during the transition to attach on the surface to initiate biofilm development (Guttenplan and Kearns, 2013). The motile bacteria became nonmotile when transitioning to the biofilm stage as bacteria are nonmotile in the biofilm. In support of the notion that the motility-to-biofilm transition is important, mutations in many regulatory genes have diametrically opposed effects on biofilm formation and motility (Yildiz *et al*., 1999; Blair *et al*., 2008; Verstraeten *et al*., 2008). The small cytoplasmic signaling molecule cyclic-di-GMP (c-di-GMP) is a key regulator of the motility-to-biofilm transition. Higher levels of c-di-GMP in bacteria cells had trigerred activation of biofilm formation (Romling *et al*., 2005; Guttenplan and Kearns, 2013).

2.12 Importance of iron in bacterial motility

Iron plays important roles in activating flagella motor and regulating filament transcription. This flagella synthesis related to Tol-Pal system requires outer-membrane for stability (Lazzaroni et al., 1999). It comprises five envelope proteins; TolQ, TolR, TolA, TolB and Pal. These proteins will recognize and energize flagellar motor proteins; MotA and MotB. TolA which is similar homolog to TonB3 protein (dependent siderophore receptor) responsible for iron uptake for activating flagellar motor system. These three systems are important as ion potential-driven molecular flagella motors (Cascales *et al.*, 2001).

Iron also influences lateral flagellar gene expression through type III secretion system (T3SS). Lateral flagellar gene expression was examined in addition to T3SS gene expression because iron limitation is one known signal inducing swarming motility. Additionally, iron also signals together with calcium in the regulation of gene sets that are relevant for surface colonization and infection (Gode-Potratz *et al.*, 2010). Iron also affects bacterial twitching motility. Twitching motility is surface motility mediated by type IV pili (Mattick, 2002) and also implicated in biofilm development (Klausen *et al*., 2003). Reduced biofilm formation in response to iron limitation has previously been attributed to enhanced twitching motility. In iron limited condition, enhanced twitching motility was observed, while caused reduction in biofilm formation by *P. aeruginosa* (Patriquin *et al*., 2008). Singh et al. (2004) also reported that the negative impact of iron limitation on biofilm formation coincides with its stimulation of twitching motility (Singh, 2004). Other related study, in iron limitation, timing of rhamnolipid and quorum sensing signal expressions are shifted to the initial stages of biofilm formation and resulted in increased bacterial surface motility. This finding highlighted the importance of biosurfactant production

(rhamnolipid) in enhancing surface motility of bacteria under iron-limiting conditions (Glick *et al.*, 2010).

2.13 Bacterial exopolysaccharide (EPS)

Many bacteria produce exopolysaccharides (EPSs), which play a wide range of roles in their biology. Besides their contribution to the fitness of the producing microorganism to their ecological niche, EPSs are often important virulence determinants produced by pathogens of plants, animals, and humans. EPSs were shown to be important for root colonization in many bacterial species, such as for *Azospirillum brasilense, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Agrobacterium tumefaciens, Sinorhizobium meliloti* and *Pseudomonas fluorescens* (Bais, 2004; Ramey, 2004; Martinez-Gil, 2010). The production of EPS in extracellular matrix is crucial for plant colonization as cells defective in either component could not form biofilms on the root. Additionally, the role of plant exudates on bacterial biofilm formation also stimulates colonization by beneficial microorganism. The plant pectin, arabinogalactan and xylan would be triggered by plant growth-promoting bacteria to initiate first step of attachment and consequently form biofilm. This strong effect on biofilm formation was specific to these three plant cell wall components that induced pellicle formation (Beauregard *et al.*, 2013).

Cepacian is the major EPS produced by a large percentage of clinical isolates of the *Burkholderia cepacia* complex (*Bc*c) (Cunha *et al*., 2004; Herasimenka *et al*., 2008). Cepacian is composed of a branched acetylated heptasaccharide repeat unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid. Several studies have pointed out cepacian as a virulence factor contributing to the overall pathogenicity of Bcc members and thus to their success as pathogens. For

instance, Conway et al. (2004) have shown that the EPS produced by a mucoid *B. cenocepacia* clinical isolate interfered with phagocytosis of bacteria by human neutrophils and facilitated bacterial persistence in the mice model of infection. Sousa et al. (2007) have shown that mutants defective in cepacian production were less virulent than the wild-type cepacian-producing strain or completely avirulent. Cepacian was also found to inhibit neutrophil chemotaxis and the production of reactive oxygen species, both essential components of the innate host defenses (Bylund *et al*., 2006). The persistence of infections has been correlated with the ability of bacterial pathogens to form biofilms. Studies performed with cepacian-defective mutants have demonstrated that, although not required for the initiation of biofilm formation, cepacian is required in formation of thick and mature biofilms (Cunha *et al*., 2004; Ferreira *et al.,* 2010).

2.14 Effect of iron in exopolysacharide production

P. aeruginosa and *Burkholderia* are the leading models for the investigation of EPS production specifically for biofilms. In limited iron condition, bacterial motility is enhanced, while biofilm formation is reduced. This is because production of rhamnolipid and quorum sensing signals in EPS activated twitching motility (Singh *et al*, 2004; Patriquin *et al*., 2008). Under iron-limiting growth conditions (<5 μM) and iron-replete conditions (>10 μM), more than 50% of *P. aeruginosa* isolates from chronic CF pulmonary infections expressed increased levels of extracelluar alginate and mucoidy EPS. These data indicate that alginate production and mucoidy, in contrast to other types of biofilms produced by *P. aeruginosa*, are substantially enhanced under iron limitation (Wiens *et al*., 2014). In presence of high concentration of iron, reduction of extracellular DNA production in biofilm matrix of *P. aeruginosa*

were reported (Matsukawa and Greenberg, 2004; Allesen-Holm *et al*., 2006; Yang *et al*., 2007).

2.15 *Burkholderia* sp.

The *Burkholderia* genus is well known for its pathogenicity. The notorious *B. pseudomallei* and *B. mallei* cause human melioidosis and animal glanders, respectively (Galyov *et al*., 2010). *B. cepacia* and its closely related species opportunistically infect cystic fibrosis patients (Mahenthiralingam *et al*., 2005). In contrast, there are also beneficial species that have great potential in agriculture and some species even form nodules with plants (Gyaneshwar *et al.,* 2011). Currently, all the species ranging from notorious pathogen to beneficial symbiont are all classified into the same genus of *Burkholderia*.

Burkholderia have recently been isolated from a variety of legumes (Chen *et al.,* 2003; Barrett and Parker, 2005, 2006; Elliott *et al.,* 2007a, 2007b). Peix *et al.* (2001) and Park *et al.* (2010) had reported that *B. vietnamiensis* has previously been shown to be capable of enhancing plant growth, promoting indirect nodulation, reacting as an antifungal and also promoting phosphorus mobilization. Moulin *et al*. (2001) found that genus *Burkholderia* in the *β*-proteobacterial could form nodules on legumes in Africa and South America. Nodulation gene, *nod* have been identified in *Burkholderia* group by Chen *et al.,* 2005a. Thus, several *Burkholderia* sp. was included in nodulating-bacteria and recently has been known as rhizobia (Sprent, 2008). The numbers of identified genera and species of rhizobia have increased significantly in recent years. Besides, over the past ten years, bacteria outside of this family have been found and isolated from nodules of legume plants (Willem, 2006; Chen *et al.,* 2007).

Burkholderia sp. USMB20 was isolated from root nodules of *Mucuna bracteata. M. bracteata* is a legume cover crop in Malaysia plantation and it can be grown without extensive fertilization if nodulated by effective rhizobial strains. This *Burkholderia* species is also able to form nodule when it is inoculated onto *M. bracteata* (Salwani *et al*., 2012). It has been proven that *Burkholderia* sp. USMB20 is a nitrogen fixer as deduced previously based on the H_2 evolution analysis (Salwani *et al.,* 2012). During N_2-fixing process, H_2 is released from legume root nodules and known as H_2 evolution. Measurement of H_2 evolution from the nodulated roots of legumes is one of the accurate methods of assaying nitrogenase activity (Hunt and Layzell, 1993). Additionally, *de novo* analysis of draft genome found that *Burkholderia* sp. USMB20 contains only one *fur* gene (NCBI GeneBank Asession no. JTAN00000000, Taxonomy ID: 1571773). This gene had been mutated; therefore USMB20 *fur* minus mutant was used as comparison in this study to observe the role of Fur in bacterial attachment on the plant's root.

2.16 *Mucuna bracteata*

Mucuna sp. has commonly known as velvetbean. The taxonomic of *M. bracteata* is as follows:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Subfamily	:	Faboideae
Tribe	:	Phaseoleae
Genus	:	*Mucuna*
Species	:	*bracteata*

The advantageous of *M. bracteata* are vigorous and fast growth. It is easy to establish and needs low labor and chemical requirements for high biomass production. It can be used as a cover crop because of its competitive ability against competing weed growth *via* the presence of chemicals and physical suppression. Thus, it is suitable to apply in agro farming systems especially in rubber and oil palm plantations (Mathews, 1998; Mendham *et al.,* 2004). *Mucuna* sp. can be grown without extensive fertilization if nodulated by an effective rhizobial strain (Ojo, 2001). The advantage of legume covers in fixing atmospheric nitrogen via symbiotic relationship with rhizobia that associated with the root system by producing nodules (Giller, 2001). This association will eliminate the fertilizer application in field.

Figure 2.3: *M. bracteata* at oil palm plantation.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Bacteria strains

Burkholderia sp. USMB20 (USMB20) which isolated from *M. bracteata*'s root was used and observed for this study. USMB20 *fur* minus mutant strain (USMB20Δ*fur*) was used as comparison. A defective *fur* mutant was constructed from USMB20 by Faisal (2014). The USMB20Δ*fur* constructed by using homologous recombination by replacing *fur* gene start codon with red fluorescence protein (RFP). As compared to the wild type USMB20Δ*fur* had preliminary shown defect in motility, reduced in biofilm formation and lack of quorum sensing signal.

Figure 3.1 summarizes the work done in this study to reveal the roles of Fur in bacterial attachment on plant's root.

3.1.1 Bacterial growth medium (M9 minimal salt medium)

All strains were grown in M9 minimal salt medium. The medium consisted of (g/l) Na_2HPO_4, 5.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; $CaCl_2$, 0.028; $MgSO_4$, 0.12; glucose, 5g; agar, 15g; pH, 6.8 (Glick *et al*., 2004). The medium was dissolved and autoclaved at 121 ^oC, 15 psi for 15 min. The bacteria were cultured in sterile M9 broth medium at 28 ^oC and shaken at 180 rpm for 48 h. Bacterial stock culture was prepared in 50% (v/v) glycerol and kept at -20 ^oC for storage. The mutant strain USMB20Δ*fur* used as comparison for this study was also grown in M9 medium.

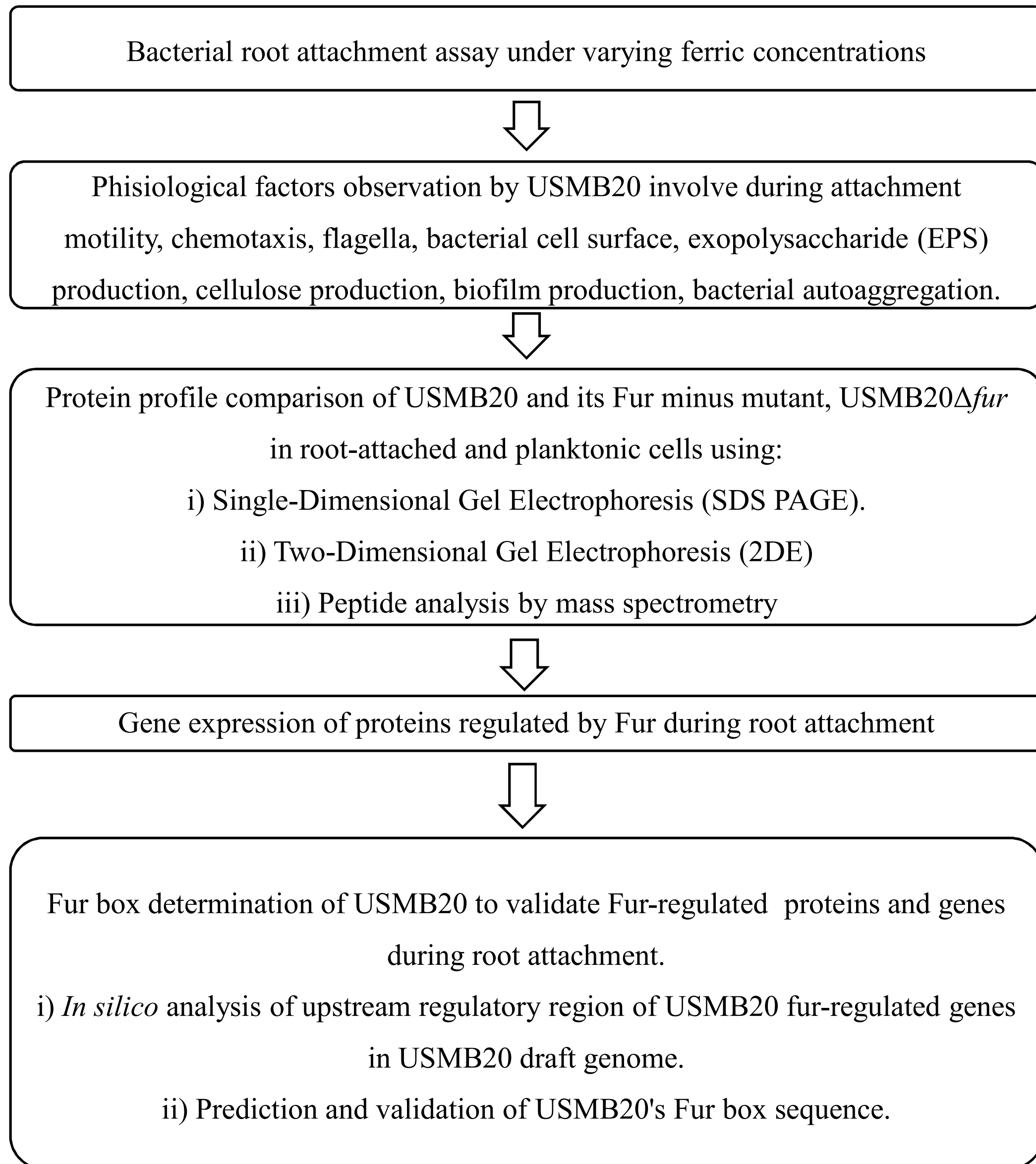

Figure 3.1: Flow chart of experimental analysis to observe the roles of Fur regulator for plant root attachment by *Burkholderia* sp. USMB20

3.2 Colonization of *M. bracteata* seedlings with USMB20

3.2.1 *M. bracteata*'s seed

Seeds of *M. bracteata* were purchased from Sime Darby Seeds and Agricultural Services Sdn. Bhd., Sitiawan, Perak.

3.2.2 Surface sterilization and germination of *M. bracteata* seeds

Seeds of *M. bracteata* were surface sterilized with 95% ethanol for 10 s to remove waxy materials and trapped air. Then, ethanol was drained off and 0.1% (w/v) mercuric chloride ($HgCl_2$) was poured. The contents were swirled gently for 1 min to make sure the seeds and $HgCl_2$ were in proper contact. The seeds were then rinsed five times with sterile distilled water. Finally, seeds were placed on 1% (w/v) water-agar medium. Germination process was carried out in the dark condition at room temperature for five days (Somasegaran and Hoben, 1994).

3.2.3 Preparation of inoculum

A single colony of each USMB20 and USMB20Δ*fur* were inoculated into individual 100 ml of sterilized M9 broth medium and cultured at 28 ^{o}C by shaking at 180 rpm for 48 h.

3.2.4 Inoculation of USMB20 onto *M. bracteata* seedlings

Both bacterial cultures were grown in M9 iron-free broth medium. The bacterial suspension of 0.1 at OD_{600} (10^5 cfu/ml) was prepared by diluting the cells with M9 broth medium supplemented with different ferric chloride ($FeCl_3$) concentrations; 0, 25, 50, 75 and 100 µM (Appendix 1). The bacterial suspension (5 ml) was transferred in to sterile test tube (12 x 2.5 cm) and followed by transferring