IN SILICO RECONSTRUCTION OF BIOFILM FORMATION PATHWAYS AND IDENTIFICATION OF PUTATIVE BIOFILM-RELATED SMALL RNAS OF Salmonella enterica serovar Typhi

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by

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PEMBINAAN SEMULA METABOLIK PEMBENTUKAN BIOFILEM SECARA *IN SILICO* DAN PENGENALAN ANGGAPAN RNA KECIL BIOFILEM YANG BERKAITAN DENGAN *Salmonella enterica* serovar Typhi

oleh

NG FUI LING

Tesis yang diserahkan untuk

memenuhi keperluan bagi

Ijazah Sarjana Sains

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AHL	Acyl-homoserine lactone
bp	Base pair
BLAST	Basic Local Alignment Search Tool
c-di-GMP	Cyclic diguanylate monophosphate
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharide or extracellular polysaccharides
Gal	Galactose
GDP	Guanosine 5′-diphosphate
Glc	Glucose
Hep	ADP-D-glycero-D-manno-heptose
Kdo	3-Deoxy-D-manno-2-octulosonat
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MeSH	Medical Subject Headings
NCBI	National Center for Biotechnology Information
nt	Nucleotide

OMP	Outer membrane protein
PCR	Polymerase chain reaction
QS	Quorum sensing
R-LPS	Rough lipopolysaccharide
rpm	Revolution per minute
S-LPS	Smooth lipopolysaccharide
sRNA	Small ribonucleic acid
TBE	Tris/Borate/EDTA
Und-P	Undecaprenyl phosphate
UDP	Uridine 5′-diphosphate
UDP-Glc	Uridine diphosphoglucose
xg	Centrifuge force

LIST OF SYMBOLS

%	percentage
ºC	degree Celcius
µl	microliter

Pembinaan Semula Metabolik Pembentukan Biofilem secara *In Silico* dan Pengenalan Anggapan RNA Kecil Biofilem yang Berkaitan dengan *Salmonella enterica* serovar Typhi

Abstrak

Salmonella enterica serovar Typhi menyebabkan demam kepialu dan membentuk biofilem dalam pundi hempedu manusia. Biofilem ialah satu pengagregatan kompleks mikroorganisme yang melekat dan tumbuh di atas struktur permukaan. Perisian Pathway Studio digunakan untuk memetakan tiga komponen laluan-laluan utama pembentukan biofilem *S.* Typhi (exopolysaccharida, lipopolisakarida, oligosakarida dan rantai O-antigen). Analisis perbandingan pembentukan biofilem telah dilakukan antara *S.* Typhi CT18, *Salmonella enterica* serovar Typhimurium LT2 , dan *Pseudomonas aeruginosa* PAO1 untuk menentukan laluan-laluan regulatori yang dipulihara. Ia telah menentukan bahawa penderiaan korum, flagelum sintesis, multidrug sistem pengaliran keluar, curli biosintesis , dan dua komponen sistem isyarat adalah dipulihara di antara ketiga-tiga mikroorganisme ini. Pembinaan semula laluan-laluan menunjukkan CsgD yang mengatur pengeluaran dua mejar exopolysaccharida (selulosa dan curli), dan menghalang pembentukan flagelum melalui cGpGp (juga dikenali sebagai c-di-GMP). Protein SdiA didapati untuk mengawal fimbriae dan flagelum, kepatuhan pada sel-sel epitelium, multidrug toleransi , dan penderiaan korum. Sejumlah 21 RNAs kecil biofilem yang berkaitan dianggap telah dikenal pasti dan pemencilan dibuat dengan menggunakan *S.* Typhimurium ATCC 14028.

In silico* Reconstruction of Biofilm Formation Pathways and Putative Identification of Related Small RNAs of *Salmonella enterica

Abstract

Salmonella enterica serovar Typhi causes typhoid fever and forms biofilm in the human gallbladder. Biofilm is a complex aggregation of microorganisms adhering and growing on the surface of a structure. The Pathway Studio software was used to map out three major components of *S.* Typhi's biofilm formation pathways (exopolysaccharide, lipopolysaccharide, oligosaccharide and O-antigen chain). Comparative analysis of biofilm formation was performed among *S.* Typhi CT18, *Salmonella enterica* serovar Typhimurium LT2, and *Pseudomonas aeruginosa* PAO1 to determine the conserved regulatory pathways. It was established that quorum sensing, flagella synthesis, multidrug efflux system, curli biosynthesis, and the two-component signaling system are conserved among these three microorganisms. The reconstructed pathways showed that CsgD regulates the production of two major exopolysaccharides (cellulose and curli), and inhibits the formation of flagellum through cGpGp (also known as c-di-GMP). The protein SdiA was found to regulate fimbriae and flagella, adherence on epithelial cells, multidrug tolerance, and quorum sensing. A total of 21 biofilm-related putative small RNAs were identified and isolation was done using *S.* Typhimurium ATCC 14028.

CHAPTER 1

Introduction

Biofilm is a mat of microorganisms that stick to each other on surfaces. Microbes that live in biofilm are resistant to classical antibiotics. The growth of microorganisms in biofilms are the implications of adaption towards the environment and it marks the enhanced resistance to antibiotics as well as antimicrobial agents (Gilbert *et al.*, 1997). Understanding the structure, morphology and the processes for the formation of microbial biofilms will allow us to find out methods in treating various microbial diseases. Network and pathway modeling based approaches are vital in our understanding of disease progression and drug responses. One of the outcomes of the *in silico* approach is to find biomarkers of biofilm. Biomarkers are indicators, signals, or early warnings of the specific diseases. It is an exciting area of research where the measurement of gene expression allows for different stages of diseases to be monitored and specific drug can be used.

My thesis is focused on the biofilm formation of *Salmonella* Typhi. Typhoid is caused by *S.* Typhi. Humans are the only reservoir for this microorganism (Ong *et al.*, 2010). It is transmitted through the feces of typhoid carriers. The understanding of the regulation of biofilm in typhoid carriers is crucial. The following literature review will describe the biofilm phenomenon, its characteristic, structures and the regulation.

CHAPTER 2

Literature Review

2.1 Bacterial Biofilms

2.1.1 Biofilm Defined

Biofilm is a mat of microorganisms that stick to each other on surfaces. The discovery of biofilm was first introduced by Antonie van Leeuwenhoek through his simple microscope that was used to observe the scrapings from teeth surfaces in the late eighteenth century (Donlan and Costerton, 2002).

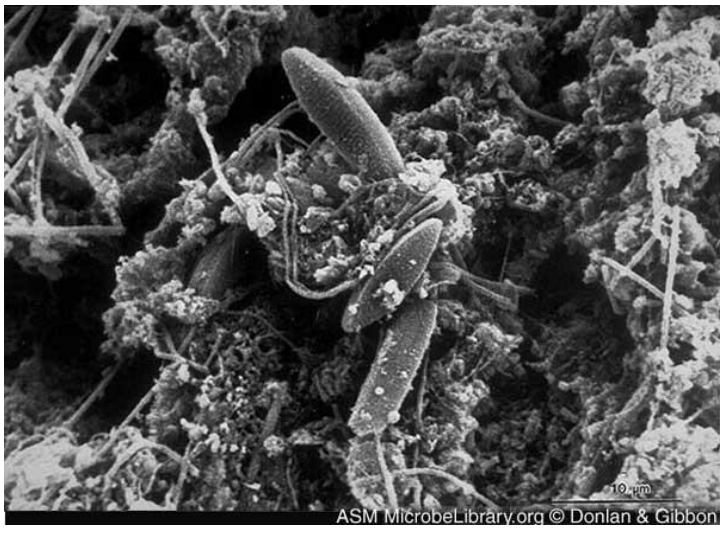

Figure 2.1 Electron microscopy of the development of biofilm on mild steel surface from industrial water system (Adapted from DONLAN, R. M. 2002. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.*,8**,** 881-90. Figure 1, page 882).

Biofilm can be formed from single or multiple types of microorganisms living together in a community. Figure 2.1 is an example of multiple types of microorganisms in a biofilm. These bacterial cells produce a matrix which is known as exopolysaccharide or extracellular polysaccharides (EPS). EPS plays an important role as physiological integrated community (Costerton, 2009a). Biofilm can be found in a variety of places including in aquatic environments such as river rocks, animals, and plants as well as technical systems such as filters, reservoirs, pipelines, and heat exchangers. Moreover, medical devices and even on certain organs in the body are capable to become pathogenic ecosystem for biofilm formation. About 80% of bacteria that form biofilm are the main cause of chronic infections. Typhoid carriers, biofilm formed by *S.* Typhi in the gallbladder, and cystic fibrosis by biofilm formation of *Pseudomonas aeruginosa* in the lungs are the examples of the chronic infections.

2.1.2 Biofilm properties

2.1.2.1 Attachment for colonization

The attachment process begins when planktonic cells get in touch with the surfaces. There are few crucial criteria that enable the process of attachment. One of the criteria is the capability of translocation to the surface (Kjelleberg and Givskov, 2007a). As the planktonic cells approach the surface, either moving randomly or by taxis to the chemical or physical properties (Grimaud, 2010; Nadell *et al.*, 2009), the cells must overcome the repulsive forces that occur between the liquid and the surfaces before attachment occurs. This initial attachment is entirely a physicochemical process

(Grimaud, 2010) and both surfaces are overall negative charges (Denyer *et al.*, 1993). Hence, to overcome these repulsive forces, the bacteria may require to have a variety of swimming appendages, move passively towards the surface via Brownian motion or random motion, and perhaps, flow along the fluid environment (Kjelleberg and Givskov, 2007a). In other words, attractive forces are needed to overcome the repulsive forces in order for the attachment (Denyer *et al.*, 1993) (Table 2.1).

The properties of the substratum plays an important role in the attachment process (Donlan, 2002). The cell surface properties such as the hydrophobicity, and the irregular surfaces or the roughness of the surface, and interface characteristics play the main factors (Grimaud, 2010). The rougher the surface, the higher chance for the biofilm to be grown on that surface (Donlan, 2002). The hydrophobic surfaces, such as plastics, are the most favorable surfaces for the development of biofilm instead of hydrophilic surfaces. Thus, surface hydrophobicity plays an important role in the attachment (Denyer *et al.*, 1993; Donlan, 2002; Tahmourespour *et al.*, 2008). When hydrophobic interaction increases, the nonpolar nature of either one or both surfaces also increases (Donlan, 2002). With the increase of nonpolar nature, the repulsive forces will be easily overcome and encourage the irreversibly attachment (Donlan, 2002). In Gram-positive bacteria, the hydrophobicity of the cell surfaces are determined by the length of mycolic acids while for Gram-negative bacteria, it is based on lipopolysaccharides (Bendinger *et al.*, 1993; Grimaud, 2010). Studies showed that chain length or the removal of either mycolic acids (Gram-positive) or lipopolysaccharide (Gram-negative) highly influence the interaction with hydrocarbons (Al-Tahhan *et al.*, 2000; Grimaud, 2010) and hence, affect the hydrophobicity of the surfaces.

Table 2.1 Interactions that occur during the attachment of biofilm (Adapted from DENYER, S. P., HANLON, G. W. & DAVIES, M. C. 1993. Mechanisms of Microbial Adherence. *In:* DENYER, S. P., GORMAN, S. P. & SUSSMAN, M. (eds.) *Microbial Biofilms: Formation and Control.* Blackwell Scientific Publication. Table 2.1 page 15)

Type of Interactions	Type of forces	Characteristic of the forces
Reversible	van der Waals Electrostatic	Long range, weak, low specificity
Irreversible	Dipole-dipole Dipole-induced dipole Ion-dipole Ionic Hydrogen bonds Hydrophobic	Short range, generally high specificity

2.1.2.2 Nutrient availability - for favorable niche

Nutrient availability is one of the important factors for attachment of biofilm. Nutrients are obtained from the aqueous phase (Grimaud, 2010). The growth of bacteria is simply dependent on nutrient availability and limiting resources. In biofilm, the outermost cells receive nutrients by diffusion and will grow faster than the inner cells (Nadell *et al.*, 2009). The thickness of biofilm is determined by the rate of the cells to consume the nutrients and the concentration level of the nutrient in the environment (Nadell *et al.*, 2009). Different concentration of nutrients and different diffusion rate of the cells in consuming the nutrients will determine the irregularity of the surface of the biofilm. In the outermost layer of biofilm, oxygen consumption is sufficient and

consequently, aerobic metabolism occurs on the surface of biofilm (Nadell *et al.*, 2009). As the depth of the biofilm increases, the cells within the biofilm are switched into anaerobic conditions which eventually lead to the growth of obligate anaerobes (Nadell *et al.*, 2009).

2.1.2.3 Environmental Stress - as defense

The formation of biofilm depends on the type of stress in the environment. Biofilms are normally resistant to shear forces, such as the strong flow of water in the pipelines (Jefferson, 2004). When biofilms are formed in the low-sheared environment, they can be loosely attached and detached from the surfaces (Donlan and Costerton, 2002). However, if the biofilms are grown in the high-sheared environment, they have a tendency to resist the mechanical forces, for example biofilm that formed in the gas pipeline. In general, a shear stress environment could determine the strength of the biofilm and its density as well (Hall-Stoodley and Stoodley, 2002; Liu and Tay, 2001). Unlike planktonic cells, they can withstand a variety of antibiotics, phagocytosis, nutrient depletion, temperature, pH differentiation as well as oxygen radicals (Jefferson, 2004). Because of this unique characteristic to resist antibiotics and phagocytosis, biofilms are a major health threat. In addition, the flow velocity also affects the biofilm formation. This normally occurs in the reservoirs or pipelines in the industrial area. The higher the velocity, the thinner the depth of the biofilm. This is also known as hydrodynamic boundary layer (Donlan, 2002).

With those existing environmental stresses, exopolysaccharide (EPS), the sticky gel-like matrix, is needed as a shield to protect the biofilm especially in the process of biofilm formation. EPS may contain DNA that plays in defense particularly in resistance for antibiotics, phagocytosis, pH differentiation and temperature.

2.1.2.4 Collective Behavior - for community

Collective behavior has been well discussed in a number of microorganisms with different environmental conditions (Davey and O'Toole G, 2000). Collective behavior can be carried out with not only a single species but multispecies to perform vital functions (Davey and O'Toole G, 2000). One of the most interesting examples of collective behavior is quorum sensing.

Quorum sensing (QS) is a cell-to-cell communication mechanism which allows bacteria to coordinate the regulation of gene expression in response to changes in cell-population density (Svenningsen *et al.*, 2009). Bacterial communication is facilitated by the production and subsequent recognition of small signaling molecules (autoinducers) which regulate the important phenotypes, including conjugation, transformation, bioluminescence, biofilm formation, swarming motility, antibiotic biosynthesis, and virulence factor production (Teasdale *et al.*, 2009). These autoinducers can be produced from microorganims of the same species or microorganisms with different genera (Kaper and Sperandio, 2005). When the concentration of an autoinducer reaches a critical threshold, the bacteria will alter their gene expression in order to detect and respond to this signal (Kaper and Sperandio, 2005). The most described autoinducers are acyl-

homoserine lactone (AHL) in Gram-negative bacteria and peptide signaling Gram-positive bacteria (Parsek and Greenberg, 2005; Spoering and Gilmore, 2006). According to Donlan (2002), mutants that are unable to produce autoinducers will form a thinner layer of biofilm and it is easier to be removed compared to the wild type. Moreover, quorum sensing can be considered as the alternative for diffusion sensing. Under unfavorable conditions, the cells will secrete the small molecules and ultimately will detach from the surfaces to find a new place. Therefore, secretion of small molecules shows that quorum sensing is vital for attachment and detachment of the cells in the biofilm formation.

Lateral gene transfer also takes place in the biofilm as it is an excellent closely-pack environment of exchanging extrachromosomal DNA (Donlan, 2002; Jefferson, 2004; Nadell *et al.*, 2009). The close intact of cell-to-cell and less shearing encourage the exchange and eventually lead to high speed of conjugation process and uptake of DNA than planktonic cells (Donlan, 2002; Jefferson, 2004). This benefits the bacteria as they could exchange antibiotic resistance genes among themselves. Perhaps, this is another reason why biofilm is resistant to most of the antibiotics.

There are four factors that affect the formation of biofilm; attachment, nutrient availability, environmental stress and collective behavior (Table 2.2). Different factors will contribute different functions such as attachment occurs for bacterial colonization, nutrients availability for biofilm to be formed in a favorable place, various environmental stresses will increase the resistance of biofilm and collective behavior will increase the bacteria community.

Table 2.2 Factors that affect biofilm formation

Colonization	Favorable Niche	Defense	Community
Attachment	Nutrients availability	Environmental Stress	Collective behavior
interaction substratum	concentration rate of diffusion	antibiotics phagocytosis nutrient depletion temperature pH differentiation	quorum sensing horizontal genes transfer

2.1.3 Process of Biofilm Formation

There are three distinct stages in the formation of biofilm for most species: surface attachment, development of microcolonies, and mature biofilm embedded in EPS (Davey and O'Toole G, 2000; Stanley and Lazazzera, 2004). The architecture of the mature biofilm, which is shown in Figure 2.2, proposed that environmental factors are involved in the formation of biofilm. In general, the biofilm formation is initiated with the attachment of the swimming cells triggered by the environmental conditions to form a multicellular structure.

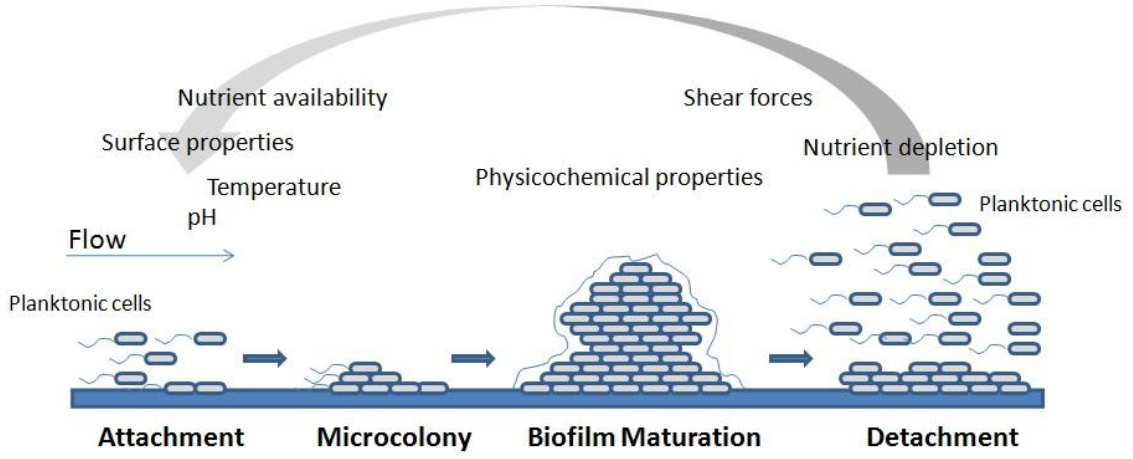

Figure 2.2 General model for the formation of biofilm structure. The planktonic cells are attached on a favorable surface and form a microcolony which leads to biofilm maturation. Exopolysaccharide is formed and surrounds the colony during biofilm maturation. Detachment of the cells occurs when there are unfavorable conditions involved. (Adapted from STANLEY, N. R. & LAZAZZERA, B. A. 2004. Environmental signals and regulatory pathways that influence biofilm formation. *Mol. Microbiol.*, 52**,** 917-24, Figure 1, page 518)

2.1.3.1 Attachment

When the bacteria approach a solid-liquid interface closely, the motility of bacteria tend to slow down and form a transient association with the surface and other cells before the attachment takes place (Prakash *et al.*, 2003). As has been mentioned above, during the adhesion of bacteria to the surface, cell-surface interaction was formed to overcome the forces generated by both the bacteria and surface. In this stage, the function of flagella, fimbriae, and pili are vital for surface motility. However, different species require different structures for attachment. In general, flagella is vital to overcome the repulsive forces rather than have a role in adhesion (Prakash *et al.*, 2003). Once the bacteria reach the surface, flagellated appendages such as fimbriae and pili

take over the attachment role. Fimbriae is vital in cell-to-surface hydrophobicity while pili is important for host-cell colonization (Prakash *et al.*, 2003).

2.1.3.2 Development of Microcolonies

To proceed to the development of microcolonies, cell-surface interaction is not sufficient. A stable cell-cell interaction is also required to hold the microcolony (Stanley and Lazazzera, 2004). In this stage, signal transduction is needed for the formation of surface motility structures, for example the pili, and also the production of exopolysaccharides which mediates the stabilization of cell-cell interaction. Quorum sensing becomes important in this stage. The bacteria secrete the signal molecules and when the signal molecules reach a threshold, the genes that control EPS production will be stimulated (Prakash *et al.*, 2003). Signals that are transmitted at the initial stage of biofilm formation and the development of microcolonies are important in adaptation to the environment that is favored by the bacterial itself. Different bacteria will respond to different environmental conditions with different growth patterns.

2.1.3.3 Formation of Mature Biofilm

The maturation of biofilm is regulated by several signals such as quorum sensing, catabolism repression, and starvation. Studies showed that in this stage, biofilm has developed properties such as increased of antibiotic tolerance, development of EPS, increased resistance to UV light, and also increased production of secondary metabolites.

Adaptation to these developments involves a great number of bacterial species to optimize the acquisition of nutrients. It has been proposed that in this stage pillars with water channels were formed in order to allow the incoming nutrient and outgoing of the waste (Danese *et al.*, 2000; Stanley and Lazazzera, 2004).

2.1.3.4 Detachment of the cells

In this stage, the cells in the biofilm will migrate to become planktonic cells. This condition occurs especially when the environment is not suitable. The detachment could be triggered by internal factors, for example quorum sensing, or external factors such as the shear forces or nutrient depletion (Donlan, 2002). There is not much known about the mechanism, function, or regulation of detachment from the biofilm.

2.1.4 Biofilm Structure

2.1.4.1 Exopolysaccharide

In the biofilm, bacteria are embedded in an extracellular matrix known as exopolysaccharide or extracellular polysaccharide (EPS) (Donlan and Costerton, 2002). The matrix is usually produced by the bacteria themselves (Flemming and Wingender, 2010). EPS can be hydrophilic or hydrophobic (Donlan, 2002). EPS is composed of approximately 97% of water (Karatan and Watnick, 2009; Sutherland, 2001) and this makes it highly hydrated. Thus, with this highly hydrated matrix, desiccation of the biofilm can be prevented (Donlan, 2002). Besides water, it also consists of protein, DNA,

polysaccharides, lipids, and other biopolymers made by different bacteria under various conditions (Flemming and Wingender, 2010).

Polysaccharides are an important element of biofilm matrices. Its chemical and physical properties could vary based on the monomer units and also its glycosidic linkages, for example α-1,6 β-1,4 or β-1,3 (Pamp *et al.*, 2007). The most famous polysaccharide that has been studied is poly-N-acetylglucosamine (PNAG) (Karatan and Watnick, 2009; Pamp *et al.*, 2007). This polysaccharide is produced by *Staphylococcus aureus*. Polysaccharide intercellular adhesion (PIA) is another type of polysaccharide produced by *Staphylococcus enteridis* (Pamp *et al.*, 2007). Cellulose is an example of a polysaccharide which is widely described in *E. coli* and *Salmonella* spp. (Pamp *et al.*, 2007). With the support of curli fimbriae, the strength and integrity of the biofilm is stronger and the chances of growing under different types of conditions are higher (Pamp *et al.*, 2007). *P. aeruginosa* produces at least three different types of polysaccharides; alginate, Pel, and Psl (Flemming and Wingender, 2010; Pamp *et al.*, 2007). According to Femming and Wingender (2010), alginate is not crucial for biofilm formation of *P. aeruginosa* but it plays an important role as mechanical stability when it comes to the formation of microcolonies and mature biofilms. Unlike alginate, Pel and Psl are important in biofilm formation in non-mucoid strains when the alginate gene is not expressed (Flemming and Wingender, 2010). Both Pel and Psl are important in maintaining the biofilm structure especially at the later stage of development (Parsek and Tolker-Nielsen, 2008). Pel, which is rich in glucose, is responsible for the biofilm development at air-medium interfaces and biofilms that were attached to surface. Psl, a repeating pentasaccharide which contains D-mannose, D-glucose and L-rhamnose, is

involved in the attachment stage where Psl is anchored to the cell surface in a helical pattern (Flemming and Wingender, 2010). Another type of polysaccharide, known as Vibrio polysaccharide (VPS), is produced by *Vibrio cholerae* (Pamp *et al.*, 2007).

EPS plays an important role in forming the morphology and internal structure of the biofilm, as it determines the physicochemical properties of the biofilm. It forms three dimensional, gel-like, highly hydrated, and locally charged biofilm matrices. The properties such as mechanical stability, binding of water, diffusion, adsorption, mass transport, and optical properties caused by EPS are crucial for the construction of biofilm (Flemming and Wingender, 2010). In most of the cases, when EPS is not present in the biofilm, bacteria still attach to the surfaces but multilayer biofilms are unable to grow (Karatan and Watnick, 2009).

2.1.4.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is found at the outer leaflet and is an important component of the outer membrane in Gram-negative bacteria (Rojas *et al.*, 2001). It is an amphiphilic molecule, a lipid- and fat- loving molecule (Gronow *et al.*, 2010). LPS is a complex glycolipid which can be divided into three regions; lipid A, core polysaccharide and O-polysaccharide which is also known as O-antigen (Sperandeo *et al.*, 2009).

Figure 2.3 is the schematic representation of lipopolysaccharide structure. This lipopolysaccharide structure is known as ‘smooth lipopolysaccharide’ (S-LPS). The name was taken from the ‘smooth’ and shiny morphology of the bacterial colony (Costerton, 2009b). Lipid A is a lipophilic membrane anchor which is located in the

outer membrane (Costerton, 2009b; Gronow *et al.*, 2010). Core oligosaccharide, which consists of inner and outer core regions, is the connecter between lipid A and the O-polysaccharide. The inner core oligosaccharide is rich in negative charges (Muller-Loennies *et al.*, 2007). This characteristic is obtained from 3-Deoxy-D-manno-2-octulosonate (Kdo) and ADP-D-glycero-D-manno-heptose (Hep) in the form of a phosphate substituent (Costerton, 2009b). According to Gronow and Brade (2001), this characteristic is important to strengthen the LPS monolayer. Ionic bridges form in this case to link the molecules by divalent cations such as Mg^{2+} and Ca^{2+}. In most bacteria, O-polysaccharide is attached to the outer core oligosaccharide. With the complete presence of lipid A, core oligosaccharide, and O-antigen, smooth colonies appear. O-polysaccharide consists of repeating units (O-unit) of monomers and a single glycosidic link (Costerton, 2009b). In *Salmonella*, the monomers are galactose, rhamnose, mannose, and abequose (Wang *et al.*, 1996). O-polysaccharide extends from the surface and forms a protective layer. The long chain of O-polysaccharide will provide complement-mediated serum killing resistance. Thus, with this property, the O-polysaccharide becomes a virulence factor in most bacteria (Costerton, 2009b).

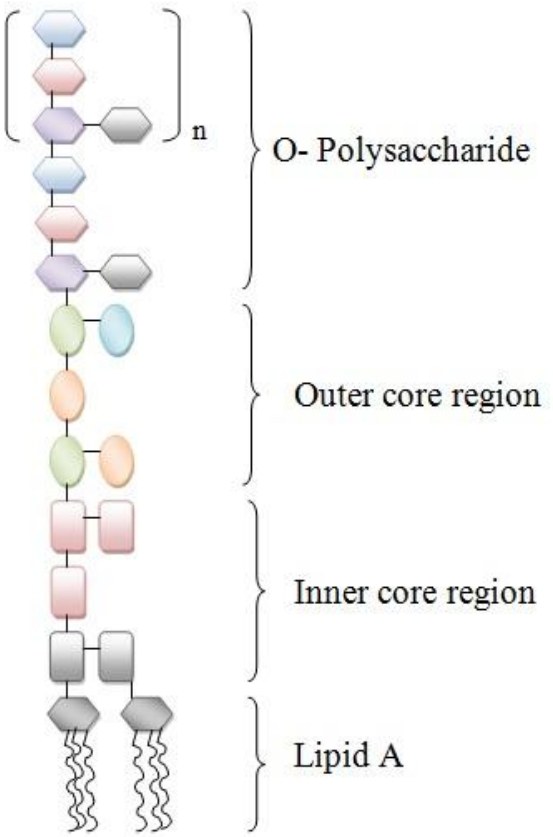

Figure 2.3 Schematic representation of lipopolysaccharide structure. It consists of lipid A, core oligosaccharide (inner core and outer core), and O-antigen. (Adapted from CAROFF, M. & KARIBIAN, D. 2003. Structure of bacterial lipopolysaccharides. *Carbohydr. Res.*, 338**,** 2431-47. Figure 1, page 2433)

On the other hand, those colonies that lack the smooth characteristic will be known as 'rough LPS' (R-LPS). This is the truncated LPS where the mutants possess a defective O-polysaccharide. These mutants have defects in certain biosynthesis steps which are unable to attach or produce the O-polysaccharide (Muller-Loennies *et al.*, 2007). One of the examples for the truncated LPS is *E. coli* K-12. It carries the defect in O-polysaccharide which does not show the smooth morphology. Those mutants that have the missing core oligosaccharide and O-polysaccharide are known as

lipooligosaccharide (LOS) (Costerton, 2009b). The extension oligosaccharide is attached at the inner core oligosaccharide. Thus, the inner core is conserved but the outer core and the O-polysaccharide are variable among Gram-negative bacteria (Gronow *et al.*, 2010).

As has been mentioned above, the inner core region is rich in negative charge. The formation of ionic bridges by divalent cations is important in minimizing the electrostatic repulsion (Costerton, 2009b). Thus, LPS plays a role in helping to stabilize the membrane of Gram-negative bacteria (Gronow *et al.*, 2010). Besides, LPS plays an important role in outer membrane integrity. The features of LPS allow the outer membrane to act as a barrier to prevent the entry of toxic molecules. The length of O-polysaccharide is essential in absorbing the nutrients and important in resistance to many different detergents and antibiotics (Christie, 2010). This will increase the percentage of bacteria to survive in different types of environments. The focus of my research is specifically towards *Salmonella*.

2.2 Genus *Salmonella*

Genus *Salmonella* is a Gram-negative pathogenic bacteria that is mostly isolated from animal host (White *et al.*, 2006).The genus is composed of *Salmonella bongori*, which can be found in cold-blooded animals and *Salmonella enterica*, which is a six subspecies division that is divided based on O antigen and H antigen (Anjum *et al.*, 2005; Sabbagh *et al.*, 2010). A small fraction of *Salmonella* serovars, approximately 60%, are pathogenic to human and are mostly from *S. enterica* subspecies I (Anjum *et al.*, 2005).

S. enterica serovar Typhimurium (*S.* Typhimurium) and *S. enterica* serovar Typhi (*S.* Typhi) are the most well known examples of *S. enterica* subspecies I which are involved in human infections (Sabbagh *et al.*, 2010). *S.* Typhimurium is the main cause for gastroenteritis in human and is non-typhoidal *Salmonella* (Richards *et al.*, 2010; Sabbagh *et al.*, 2010). However, it causes typhoid-like infection in mice. Hence, it serves as a laboratory model to study typhoid fever (Richards *et al.*, 2010). *S.* Typhi is mainly pathogenic to human only and is responsible for typhoid fever (Richards *et al.*, 2010). 22 million individuals are estimated to be infected with *S. enterica* serovar Typhi each year. From the total infected patients, there are approximately 200,000 deaths (Charles *et al.*, 2009; Crump *et al.*, 2004). Nevertheless, according to Anjum *et al.* (2005), comparison of genome sequences between *S.* Typhimurium LT2 and *S.* Typhi CT18 has shown that roughly 89% of the genes are conserved.

2.3 *Salmonella* and Biofilm

Signal transduction through protein-protein interactions is one of the main systems in controlling various biological processes. Comprehensive studies in this regulation can facilitate the understanding of what had happened in biofilm during its development. *S.* Typhi can exist in asymptomatic carrier state in the human gallbladder, suggesting that there is a connection between the bacteria and individuals with gallbladder abnormalities (Prouty and Gunn, 2003). In the liver, the bacteria are being shed into the gallbladder. The bacteria interact with bile that is produced by the liver to digest the lipids by emulsification. However, *Salmonella* is protected from the high

concentration of bile which consists of bile acids, cholesterol, phospholipids and bilirubin (Prouty *et al.*, 2002). Antibiotics treatments are not effective in eliminating the biofilm in the gallbladder. The only option to get rid of the biofilm is by removing the gall stone by surgery (Prouty *et al.*, 2002). Recent studies show that small RNAs (sRNAs) are involved in most of the cellular networks in bacteria, which include biofilm regulation, quorum sensing, bacterial virulence, and also responses to environmental stresses.

2.4 small RNA and Biofilm

RNAs that do not translate into protein are known as non-coding RNAs or functional RNAs (Chang *et al.*, 2010). In bacteria, these RNAs are ~50 to 600 nt long (Gottesman *et al.*, 2006) and are generally encoded in the intergenic region of chromosomes (Vogel, 2009). Although sRNAs are located in the intergenic region, it cannot be precluded that these sRNAs are expressed or regulate the protein coding sequence (Liu *et al.*, 2009). Their synthesis is regulated by the bacterial stress response or virulence condition. These sRNAs regulates gene expression by base pairing to its mRNA target and affecting the translation process of the mRNA. In *Vibrio cholerae*, quorum sensing is controlled by four sRNAs known as quorum regulatory RNAs (Qrr1-4) (Svenningsen *et al.*, 2009). Qrr sRNAs function with Hfq, an RNA chaperone to control the translation of the target and in this case, the target is HapR, a transcription factor of quorum sensing. Recently, the genome of *S.* Typhi has been sequenced. Transcriptomic analysis on *S.* Typhi identified 97 putative non-protein coding sRNA

(Chinni *et al.*, 2010). Among these putative non-proteins coding sRNA, some are involved in biofilm formation as well as in multidrug resistance of *S.* Typhi. Thus, discovery of sRNAs in biofilm regulation of *Salmonella* is expected to be similar with other bacteria as most of the sRNAs often appear to regulate the similar functions (Svenningsen *et al.*, 2009).

2.5 Metabolic and Regulatory Pathway

A metabolic pathway is a chain of chemical reactions that are linked to one another in a cell (Jeong *et al.*, 2000). To enhance the rate of chemical reactions, a set of enzymes are required which act as catalysts. Almost all metabolic reactions require enzymes to catalyze the chemical reactions and produce the end products. Figure 2.4 shows a simple metabolic pathway which has two reactions, four types of compounds (C1, C2, C3, and C4), two types of enzymes, one substrate, one intermediate which can be substrate and product, and finally, end product.

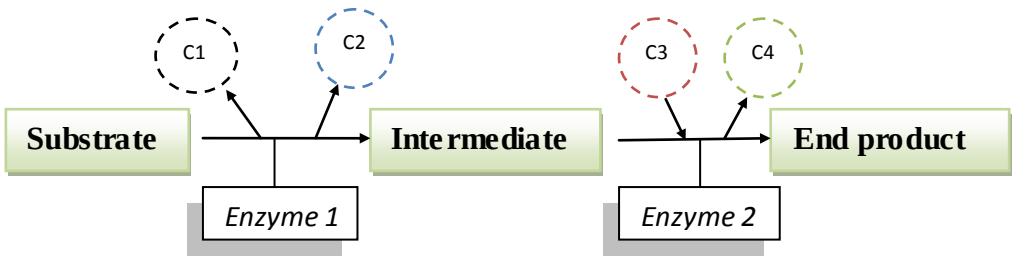

Figure 2.4 A standard and simple metabolic pathway that consists of two reactions

Figure 2.5 is an example of a standard and simple gene regulatory pathway. In a gene regulatory pathway, a gene or its encoded protein is the sensor for environmental signals (such as high salt concentration). This sensor initiates the signal transmission via a series of transmitter and regulators (CpxR, CsgB and CsgA). Regulators ultimately up- or down-regulate the gene expression.

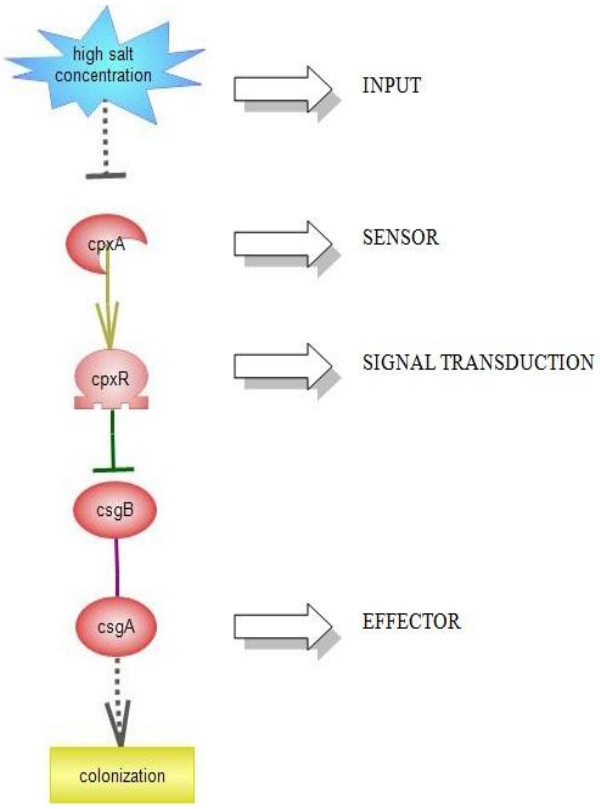

Figure 2.5 An example of gene regulatory pathway

2.6 *In silico* Metabolic Pathway Reconstruction

To understand a biological function, genes have to be incorporated into a phenotypical context. Thus, reconstruction of metabolic pathways is crucial in predicting gene function and the reactions in an organism. The organism-specific metabolic pathways are reconstructed based on the enzyme-gene and reaction-enzyme information (Ma and Zeng, 2003). One enzyme may catalyze different reactions and one reaction may be catalyzed by different enzymes. Metabolites (ATP, ADP, Pi, H_2O, NADH, and NAD^+) are used as carriers for transferring electrons in a metabolic pathway.

Many databases and tools are available from the internet including EcoCyc, MetaCyc, BioCyc, Kyoto Encyclopedia of Genes and Genomes (KEGG), Metabolic Pathways Database (MPW), and What Is There (WIT) (Covert *et al.*, 2001). These genomic databases and metabolic reconstruction websites provide access to the annotated genome sequences. Metabolic maps in KEGG and EcoCyc are the reference of the organism-specific database.