Emerging Concepts in Bacterial Biofilms:

Molecular Mechanisms and Control Strategies

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Cambridge Scholars Publishing

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This book first published 2020

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-4261-0
ISBN (13): 978-1-5275-4261-7

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FOREWORD

Glance through the pages of this book and it will show you the tremendous leap in our understanding of bacteria, from the age when they were considered planktonic beings in liquid cultures, to our present appreciation of these microscopic creatures to form functional complexes within a matrix, as biofilms.

I am honored by Dr. Sabu Thomas for having offered me the privilege of introducing the reader to this book on Emerging Concepts in Bacterial Biofilms: Molecular Mechanisms and Control Strategies. I imagine that his choice is driven by the intention to provide a broader framework rather than one of science information alone.

This book provides fascinating insights into biofilms, molecular mechanisms of how they are formed and how they can be controlled. Natural biofilms were first noticed due to their retarding effects on the movement of ships and boats in urban water distribution systems. Soon they were recognized to be significant in infections associated with medical implants and prosthetics. Their relevance to host-pathogen interactions such as colonization of the host tissues, pathogenicity, disease transmission, resistance to host immune responses and drugs remains a major concern to microbiologists and clinicians alike. Therefore this book is timely and meets the important need to understand and develop novel strategies to address issues of bacterial virulence and drug resistance in the context of biofilms.

This comprehensive work is set on the sound foundation of over two decades of Sabu's experience in studying *Vibrio cholerae.* He is undoubtedly one of the leading figures in cholera research in the country. As the book progresses to discuss the clinical implications of biofilm-associated infections, techniques used to study the architecture and dynamics of biofilms, the emerging *omic* approaches to delineate the microbial interactions and the strategies to combat biofilm-associated infections, I am certain that this book will come to be recognized as a reference guide for anyone with a keen interest in the biology of biofilms.

My hearty congratulations to Sabu and his team for this authoritative and comprehensive book; I wish them all the very best.

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PREFACE

In nature, bacteria alternate between a free-swimming, unicellular phase (planktonic) and a sessile, multicellular form, called the biofilm, which are surface-associated microbial communities encased in a self-produced matrix that allows attachment to inert or organic surfaces. Within a biofilm, sessile cells live in a coordinated manner that favors their proliferation as well as successful dispersion and colonization of new niches. This privileged and protected life within biofilms makes it one of the most widely distributed and triumphant modes of existence on Earth. Bacteria behave very differently in biofilm and have a radically different physiology and genetic processes when compared to their planktonic counterparts. Most of the microbes colonising the human body are capable of forming biofilms with enhanced virulence and antimicrobial resistance leading to persistent chronic infections, often associated with medical implants that complicate existing treatment regimes. New studies exploiting the emerging and sophisticated science have dissected the structure of biofilms as well as the genetic, physiological and signal transduction pathways that underpin its development. The recent explosion in metagenomic studies has unravelled the complexity of the microbial population within biofilms and medical microbiologists have started to appreciate the prevalence and involvement of mixed-species biofilms in infections. We have just begun to scratch the surface of polymicrobial biofilms and the properties of the taut matrix that 'houses' the myriad species; yet we are in awe of the intricate interaction and dynamics within these communities that influence antibiotic resistance and pathogenicity. Knowledge of the biology and molecular basis of the complex interplay between bacteria, their impact on host immune response as well as the development of suitable animal models to study polymicrobial biofilms *in-vivo* will be a stepping stone to improving therapeutics and define new targets for disease control.

This volume focuses on the clinical implications of biofilms in healthcare settings and the emerging strategies to inhibit and control bacterial biofilms. It opens by detailing how bacteria communicate with each other via quorum sensing to elaborate biofilms, the challenges faced by clinicians in treating and managing recalcitrant, antibiotic tolerant biofilm infections and the mechanisms for bacterial tolerance and persistence. The

next chapter introduces the remarkable world of mycobacterial biofilms, from the ultra structure to the intriguing role GroEL1 and mycolic acids play in promoting biofilm formation. Subsequent chapters highlight the novel approaches, like nanotechnology, to inhibit or disrupt biofilm as well as elaborate the interactions among bacteria to be exploited as potential targets for treating biofilm-related infections. Finally, the book delves into the cutting edge techniques used to decipher the physiology, structure and composition of biofilms, highlighting the advantages and limitations of several methods.

I hope the book will be useful and exciting to read and instigate more enthusiasm for the subject.

Dr. Sabu Thomas
Chief Editor

ACKNOWLEDGEMENTS

A few people have been instrumental in allowing this project to be completed. Foremost I would like to pen my sincere gratitude to Prof. M. Radhakrishna Pillai, Director of Rajiv Gandhi Centre for Biotechnology, Trivandrum, India for consistent support and encouragement in this endeavor. I also extend my gratitude to the editorial team Dr. Divya M Prabhakaran, Ms. Lekshmi Narendrakumar, Dr. Karthika Suryaletha, Ms. Devika J Das and all my lab members for their constant support and valuable suggestions. Their suggestions and detailed critical analysis greatly improved the final product. I would also like to acknowledge my wife Jiji Peter, Associate Professor and Head of the Dept. of English, St. Gregorious College, Kottarakkara, under the University of Kerala, for taking time to go through the manuscript. This compilation has been developed from various research works conducted in reputed R&D Institutions, Universities and Medical Colleges in India, Nepal and Indonesia by eminent scientists/professors. I am indeed indebted to all of the professors, scientists and young researchers who made valuable contributions to this book. I also sincerely thank Cambridge Scholars Publishing, UK for the excellent editorial assistance and taking keen interest in producing this book. Acknowledgement also goes to the Department of Biotechnology of the Govt. of India.

Dr. Sabu Thomas
Chief Editor

UNDERSTANDING THE MECHANISM OF BIOFILM FORMATION IN MAJOR GRAM-NEGATIVE AND GRAM-POSITIVE PATHOGENS WITH SPECIAL EMPHASIS ON QUORUM SENSING

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Abstract

Established biofilms may consist of microbial cells, exopolysaccharides, an extracellular protein, and extracellular DNA (eDNA). The structure and components of exopolysaccharides, eDNA as well as extracellular protein vary from one bacterium to another. Biofilm formation for each bacterium may be formed regulated by different genetic and environmental factors including the medium, matrix properties, temperature, pH, nutrient availability as well as the presence of organic material. During biofilm formation, bacteria are able to perform cell-to-cell signaling known as quorum sensing activity. In the quorum sensing process, bacteria communicate with one another. Bacterial cells may communicate with each other via quorum sensing activity, which in turn supports the attachment process as well as detachment. QS regulates several processes in some bacteria including pathogenicity, transfer of DNA, production of some secondary metabolites, bioluminescence, sporulation, conjugation, motility, competence, antibiotic production, as well as biofilm formation in response to signaling molecules of quorum-sensing systems. A cell-to-cell communication mechanism known as quorum sensing (QS) has been found to play a role in *P. aeruginosa* biofilm formation, because both QS

and biofilms are impacted by the surrounding environment. QS plays a role in biofilm development as well as biofilm maintenance. In this system, bacteria use signaling molecules. Several Gram-negative bacteria use N-acyl-homoserine lactones (AHLs) as a signal molecule for cell population density. Whereas Gram-positive bacteria use autoinducing peptide (AIP) as their autoinducers. Quorum sensing enables bacteria to communicate with each other at intraspecies and interspecies levels by influencing the gene expression of several genes as well as the behavior of the entire community, although the chemical signals and quorum sensing system differ.

Key words: Biofilm, Environmental factor, Exopolysaccharides, Extracellular DNA, Extracellular protein, Quorum sensing

1. Introduction

Bacteria that live in various environmental conditions naturally form biofilm as the survival strategy to protect the cell from a harsh environment. Biofilms formed in the human body, especially in the digestive tract, are known to have beneficial impacts on human health. For example, *Lactobacillus rhamnosus* GG is a probiotic that can prevent acute diarrhea in children and reduce allergy (Doron et al., 2005). On the contrary, the formation of biofilm by pathogens is of particular concern because it is usually associated with virulence factors. *Streptococcus mutans* can adhere to the oral cavity causing dental plaque (Becker et al., 2002) and *Vibrio cholerae,* the causative agent of cholera, can colonize the human intestine (Tamayo, Patimalla, and Camilli, 2010).

Unlike planktonic cells, biofilms are more resistant to antimicrobial agents. The concentration of antimicrobial agents needed by biofilms is 100-1000-fold higher as compared to planktonic cells. This is due to the static growth and quiescent metabolic activity of cells residing in the anaerobic environment prevailing inside the biofilms (Høiby et al., 2010). For instance, when peroxyacetic acid (PAA) is used as a disinfectant, concentrations of 900 ppm to 1,800 ppm are bacteriostatic and 3,700 ppm to 7,400 ppm are bactericidal to *Salmonella* planktonic cells whereas the concentrations required for inhibiting *Salmonella* biofilms range from 7,400 ppm to 14,700 ppm in bacteriostatic, and 8,300 ppm to 14,700 ppm in bactericidal conditions (Chylkova et al., 2017). In addition, gene expression in biofilm and planktonic cells is also different. Several genes are found up- and downregulated in between 1% and 38% of the complete bacterial genome. For instance, *agr* gene mutations that play a role in cell-

to-cell communication systems and biofilm formation in *Staphylococcus aureus* show a decreased resistance to rifampin (Yarwood et al., 2004).

One of the main factors influencing biofilm occurrence is the quorum sensing process. Quorum sensing (QS) is the process of cell-to-cell communication that influences certain behavior or phenotypic changes when it reaches the critical density point. QS is involved in the regulation of phenotypic bacterial functions, including biofilm formation, bacteriocin production, sporulation, pigment production, bioluminescence and virulence factor regulation (Rutherford and Bassler, 2012; Steiner et al., 2012; Perchat et al., 2016; Banerjee and Ray, 2017; Kaur, Capalash, and Sharma, 2018). In addition, QS plays a role in the dissemination of antibiotic resistance genes. Conjugative plasmid RP4 encodes resistance to kanamycin, tetracycline and ampicillin, that can be transferred between *Escherichia coli* strains. This conjugation can be facilitated by AHLs because its structure is homologous with SdiA proteins. SdiA is known to play a role in the expression of the genes related to the conjugative transfer process (Zhang et al., 2017).

In Gram-positive bacteria, the signal molecule used is short oligopeptides, whereas, in Gram-negative bacteria, it is N-acyl-homoserine lactones (AHLs). Both of them could be involved in interspecies communication. Single planktonic cells produce an autoinducer inside the cell and transmit it across the cell membrane so that, a higher autoinducer concentration outside the cell reflects a higher cell density.

Another quorum sensing system is Autoinducer(s)-2 (AI-2) which is a furanone derivative and connects communication among various taxonomic groups of bacteria (Singh and Ray, 2014; Abisado et al., 2018). The *luxS* gene in *S. mutans* encodes AI-2 which encodes various cellular processes, such as oxidative stress tolerance, tolerance to acids, maturation, and biofilm formation (Wen and Burne, 2004). The co-culture of *E. coli* and *Enterococcus faecalis* induces *lsr* operon in *E. coli*. This operon is affected by AI-2 and influences the formation of a microcolony as the initial step of biofilm formation (Laganenka and Sourjik, 2018). In some Gram-negative bacteria, the AI-2 signaling pathway works parallel and synergistically with other QS pathways, either mediated by AHL or non-AHL molecules. Non-AHL molecules' QS pathways include *Pseudomonas* quinolone signal (PQS), cholera autoinducer-1 (CAI-1) and diffusible signal factor (DSF) (Ng et al., 2012; Tay and Yew, 2013; Reuter, Steinbach, and Helms, 2016; Zhao et al., 2018).

As a cell-to-cell interkingdom communication, bacterial cells produce autoinducer-3 (AI-3). The AI-3 system can detect new autoinducers, such as catecholamines (epinephrine and norepinephrine) produced by the host, which are known to be able to induce bacterial growth. The formation of AI-3 does not depend on *LuxS*. AI-3 also plays an important role in the virulence of bacteria. EHEC, a pathogenic bacterium, can recognize aromatic autoinducer signals, such as catecholamines, and AI-3 produced by normal gastrointestinal microbes to activate virulence genes and colonize host epithelial cells (Pacheco and Sperandio, 2009).

In addition to genetic factors, biofilm formation is also influenced by environmental factors. Osmotic pressure in the biofilm is strongly influenced by the formation of an exopolysaccharide matrix. The difference in osmotic pressure with the external environment plays a role in the uptake of nutrients and in the dispersal process of *V. cholerae* biofilms to colonize the human digestive tract (Yan et al., 2017). Conditions of the gastrointestinal tract such as pH, osmotic pressure, bile and mucin also have an impact on *Lactobacillus* biofilm formation (Lebeer et al., 2007; Aoudia et al., 2015). In *Bacillus subtilis*, *luxS* expression increases in the presence of lactose, which in turn increases the AI-2 production. A high amount of AI-2 is correlated with the increasing *epsA-O* operon and *tapA* that encoded extracellular polysaccharide and amyloid fibers, thus biofilm matrix production is also increased (Duanis-Assaf et al., 2016).

An understanding of the molecular mechanism of biofilm formation can be used to design an alternative substitute for the use of antibiotics to eradicate bacterial diseases. In this chapter, we are going to discuss the molecular mechanisms, quorum sensing and several environmental factors that play a role in biofilm formation by Gram-positive bacteria (*Staphylococcus aureus, Streptococcus*, *Bacillus*, *Lactobacillus*) and also Gram-negative bacteria (*Pseudomonas aeruginosa, Vibrio cholerae, Escherichia coli,* and *Salmonella enterica* serovar Typimurium).

2. Molecular Mechanism of Biofilm Formation in Gram-negative Bacteria

Bacteria in a biofilm structure are more resistant to antibiotics compared with planktonic cells (Svjetlana and Vraneš, 2007). In general, the steps of biofilm formation are adherence/attachment, maturation and dispersion, for both Gram-negative and Gram-positive bacteria. Some of the Gram-

negative bacteria that produce biofilm include *Pseudomonas aeruginosa, Vibrio cholerae, Salmonella enterica* and *Escherichia coli.* The molecular mechanism that regulates the formation of biofilm varies among species and even among different strains of the same species (Monds and O'Toole, 2009). During biofilm formation, bacteria require to communicate with each other through a process called quorum sensing (QS). The intercellular communication relies on small signal molecules. In Gram-negative bacteria, the most intensively investigated signal molecules are *N*-acyl-L-homoserine lactones (AHLs) (Papenfort and Bassler, 2016).

3. Biofilm Structures

Biofilm structures are composed of eDNA, exopolysaccharides and extracellular proteins (Limoli et al., 2015). The extracellular matrix composition of various Gram-negative bacteria is summarized in **Table 1**.

Table 1. Different compositions of biofilm structure

Bacteria	Exopolysaccharide	Extracellular Protein	Extracellular DNA
Pseudomonas aeruginosa	Psl, Pel and alginate are contributed in biofilm formation. Psl contains a repeating pentasaccharide, pel gene cluster synthesis, pel polysaccharide. Alginate is important for structural stability and the protection of biofilms.	Flagella are important for initial cell-to-surface interactions; Pili, the formation of a mushroom-like microcolony.	eDNA was produced; necessary for the utilization of eDNA as a nutrient source.
Vibrio cholerae	Consist of glucose 52.6%, galactose 37% , N-acetylglucosamine 5.1%, mannose 3.8% and xylose 1.5%. Vibrio produce polysaccharide encoded by the *vps* gene.	RbmA (morphology of the rugose colony and biofilm architecture development; Bap (secreted protein associated with biofilm formation) and RbmC.	Regulated by two extracellular nucleases – Dns and Xds.

Escherichia coli	Exopolysaccharides, β-1,6-N-acetyl-D-glucosamine polymer (PGA), colanic acid, and cellulose.	The extracellular matrix component (ECM) consists of a curli protein encoded by the *csg* gene.	The hipBA toxin-antitoxin (TA) system modulates biofilm formation through DNA release.
Salmonella enterica serovar Typhimurium	EPS consist of cellulose and colanic acid. The *wca*M gene is important for the production of EPS components.	The extracellular matrix component (ECM) includes proteins (curli fimbriae and Bap A).	eDNA chelating cation properties activate PhoPQ/PmrAB systems and antimicrobial peptide resistance.

3.1. Exopolysaccharides

The structure and components of exopolysaccharides vary from one bacterium to another. Exopolysaccharide production increases as a response to environmental stress, even though most exopolysaccharides are not biofilm-specific (Rabin et al., 2015). At least three polysaccharides (Psl, Pel and alginate) contribute to biofilm formation in *P. aeruginosa.* Recently, Psl was found to contain a repeating pentasaccharide consisting of D-mannose, D-glucose and L-rhamnose. The *pel* gene cluster (pelA-F, PA3058-PA3064) synthesizes pel polysaccharide, which is rich in glucose and is cellulase-sensitive. On the other hand, alginate is an exopolysaccharide that is important for structural stability and the protection of biofilms. This component is necessary for water and nutrient retention in the matrix of biofilm (Wei and Ma, 2013).

Exopolysaccharides of *V. cholerae* consist of 52.6% glucose and 37.0% galactose, and small amounts of N-acetylglucosamine (5.1%), mannose (3.8%) and xylose (1.5%) (Yildiz and Schoolnik, 1999). The major component of *V. cholerae* biofilm is *Vibrio* polysaccharide (VPS), which is important for three-dimensional biofilm structures. This is encoded by *vps* gene clusters, which consist of two regions, *vpsU*, *vpsA–K*, and VC0916–27 as cluster I and *vpsL–Q* and VC0934–39 as cluster II. These two clusters are separated by the *rbm* gene cluster which encodes proteins for the biofilm matrix and also acts as an intergenic region based on the position (Fong et al., 2010).

Toska, Ho, and Mekalanos (2018) reported that *V. cholerae* synthesizes exopolysaccharides, which protect the cell from attack by the exogenous type 6 secretion system (T6SS). The T6SS system is generally used by

Gram-negative bacteria to attack other bacterial cells and eukaryotes through toxic protein delivery.

Exopolysaccharides of *E. coli* comprise β-1,6-N-acetyl-D-glucosamine polymer (PGA), colanic acid and cellulose. These components are important in the biofilm formation of *E. coli*. PGA is important in biofilm formation due to its role in cell-cell attachment to the surface (Agladze, Wang, and Romeo, 2005). Colanic acid is a protective capsule; it is composed of fucose, glucose, glucuronic acid and galactose. Colonic acid will not be produced when the bacteria are grown in a rich medium. Cellulose production is required to form a rigid biofilm structure.

These components are also produced by other bacteria, including *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis. Inhibition of cellulose production in these bacteria inhibits biofilm formation (Ahmad et al., 2016). Biofilm in *Salmonella* consists of cellulose, colanic acid and O-antigen capsule in non-typhoidal serovars whereas, in serovars Typhi, Dublin, and para-typhi C, the biofilm is composed of cellulose, colanic acid and Vi-antigen [ViAg] (Gunn, Bakaletz, and Wozniak, 2016; Ledeboer and Jones, 2005). In *S. enterica* serovar Typhimurium, the *wca*M gene is important for the production of EPS components (cellulose and colanic acid). Mutations in this gene abolish the biofilm phenotype (Ledeboer and Jones, 2005).

3.2. Extracellular Proteins

Biofilm formation and stabilization require the attachment of some proteins on the cell surface. There are some proteinaceous components in *P. aeruginosa* that are important for biofilm formation including flagella for initial cell-to-surface interactions; pili, for the formation of a mushroom-like microcolony (O'Toole and Kolter, 1998); CdrA for aggregation and increased biofilm stability; and fimbriae, for cell-to-cell interaction and microcolony formation (Wei and Ma, 2013).

The biofilm matrix of *V. cholerae* is composed of RbmA, Bap1, and RbmC. Protein RbmA is required for the morphology of rugose colony and biofilm architecture development. This protein also supports the cell-cell adhesion process (Giglio et al., 2013). Bap1 allows the developing biofilm to adhere to surfaces, while RbmC cooperates with Bap1 in the formation of flexible envelopes that grow as cells divide and stabilize the biofilm (Berk et al., 2013).

ECM in *E. coli* consists of the curli protein encoded by the curli specific gene *csg*. This protein together with the polysaccharide cellulose promotes the host immune system, resistance to desiccation as well as the adherence properties of the cell to the matrix (Hufnagel, DePas, and Chapman, 2015; Smith et al., 2017).

Salmonella biofilm extracellular matrix (ECM) components play a role in biofilm formation. These ECM components contain proteins such as BapA (secreted protein associated with biofilm formation) and curli fimbriae protein (Adcox et al., 2016). Curli protein is the predominant matrix-compound in *S. enterica* serovar Typhimurium biofilms; the disruption of this protein leads to distinct changes in colony morphology (Jonas et al., 2007).

3.3. Extracellular DNA

eDNA also plays an important role in the formation of biofilm (Whitchurch et al., 2002). In *P. aeruginosa*, an eDNA is produced (PA3909) which is necessary for the utilization of eDNA as a nutrient source; therefore, it is confirmed that eDNA is important during the biofilm formation of *P. aeruginosa* (Mulcahy, Isabella, and Lewis, 2014).

Extracellular DNA and the extracellular nucleases in *V. cholerae* contribute to biofilm architecture formation, mainly in the stability of biofilm (Okshevsky and Meyer, 2013). Extracellular DNA is modulated and regulated by two extracellular nucleases, namely Dns and Xds. The *Xds* gene is a member of Pho regulon (Seper et al., 2011; McDonough, Lazinski, and Camilli, 2014). In the biofilm structure for these bacteria, eDNA is known as a matrix component in *Salmonella* biofilm. The eDNA of *S. enterica* serovar Typhimurium has the capability to induce antimicrobial peptide resistance and PhoPQ/PmrAB systems (Wang et al., 2013). This activation happens due to the cation chelating properties of extracellular DNA (Johnson et al., 2013). In *E. coli,* it was found that the hipBA toxin-antitoxin (TA) system modulates biofilm formation through DNA release since the deletion of hipA results in the reduction of the eDNA level in biofilm formation (Zhao et al., 2013).

4. Biofilm formation

Flagella and fimbriae play an important role in the first step of biofilm formation. The formation of biofilm is regulated by different genetic and environmental factors. Genetic studies have shown the role of bacterial

mobility, cell membrane proteins, extracellular polysaccharides and signaling molecules in biofilm formation.

Pratt and Kolter (1998) reported that *E. coli* requires bacterial mobility using flagella and fimbriae for the initial step of biofilm formation. Bacterial mobility enabled by flagella is necessary for establishing the connection between the bacteria and the surface, while the mobility enabled by fimbriae is necessary for the formation of microcolonies. In *E. coli*, type I pili (harboring the mannose-specific adhesin, FimH) are required for the initial interaction and movement on the surface. When adhesin is inhibited, biofilm formation is also inhibited, therefore motility and type I pili are important in the development of biofilm (Pratt and Kolter, 1998).

Adhesins are important for biofilm formation in *V. cholerae.* A flagellum and type IV pilus support the bacterial attachment to the matrix surface. For movement along the surface, they need a flagellum, while exopolysaccharide is important in the three-dimensional biofilm architecture formation (Watnick and Kolter, 1999). MbaA regulates the synthesis of some components of the biofilm matrix in *V. cholerae* (Bomchil, Watnick, and Kolter, 2003). While the RelBE TA system contributes to the colonization process, biofilm formation and resistance to reactive oxygen species (ROS) occur in these bacteria (Wang et al., 2015).

5. Environmental effects

The attachment of bacterial cells is influenced by several factors; not only by motility, growth phase, medium and matrix composition, but also temperature, the presence of organic material, pH and the availability of nutrients. The effects of nutrients and the environment were reported in the biofilm formation of *Salmonella* sp. which included the medium in which they are grown, motility, the growth phase of the cells, the type and properties of the inert material, the presence of organic material, temperature, pH and contact time (Speranza, Corbo, and Sinigaglia, 2011).

The presence of phosphates in the environment showed biofilm reduction in *Listeria monocytogenes*; on the other hand, biofilm formation is induced by the presence of carbohydrates such as trehalose and mannose (Kim and Frank, 1995). Limited nutrients are important for the structural stability of *L. monocytogenes* biofilm through increasing cell death and extracellular DNA release (Cherifi et al., 2017). Biofilm formation timing is influenced by the type and concentration of specific seawater salts in *Vibrio fischeri*

(Marsden et al., 2017). The attachment of *E. coli* to an abiotic surface leads to SOS system induction, also giving a filamentation response, which was triggered when bacteria adhere to the surface for colonization. SOS also contributes to the planktonic cells' genome defense against environmental agents (Costa et al., 2014).

In *V. cholera,* low temperatures also modulate biofilm formation due to the increase in the signaling molecule and the cyclic diguanylate (c-di-GMP) level. This phenomenon also happens for Gram-negative *P. aeruginosa*, but not in Gram-positive *L. monocytogenes* (Townsley and Yildiz, 2015). *cspV* encodes a protein which gives a major regulator response for providing a downshift in temperature so that it controls the cellular processes required for the infectious cycle of *V. cholerae.* This protein regulates the temperature-dependent type VI-mediated interspecies killing and also regulates biofilm formation through modulation of the second messenger cyclic diguanylate (Townsley et al., 2016). The effect of temperature on the formation of biofilm in *L. monocytogenes* showed that biofilm formation is inhibited when the temperature is high (Gorski, Palumeo, and Mandrell, 2003). The effect of temperature was also reported in the biofilm formation of *Salmonella* sp. (Speranza, Corbo, and Sinigaglia, 2011).

Environmental pH is also important for biofilm formation. A study on *V. cholerae* reveals that the optimum pH for the multiplication of this bacteria is 8.2 and a pH lower than 7 reduces biofilm formation due to the inhibition of their mobility by decreasing pH (Hommais et al., 2002). Hostacká, Ciznár, and Stefkovicová (2010) found that biofilm production of *V. cholerae* non-O1 and O1 reached 123% to 316% at pH 7.5 while at pH 8.5 it is 204% to 329% higher (compared with production at pH 5.5). Some bacteria including *Staphylococcus epidermidis* and *E. coli* do not need high pH for the bacterial multiplication process; this is the reason why these bacteria can produce biofilm under an acidic environment such as urethral catheters. Speranza, Corbo, and Sinigaglia (2011) also reported the supported biofilm formation of *Salmonella* sp.

6. Quorum Sensing

Quorum sensing (QS) is the process of bacterial communication via the secretion and detection of autoinducer(s). QS regulates several processes via signaling molecules in some bacteria including pathogenicity, transfer of DNA, production of secondary metabolites, bioluminescence, sporulation, biofilm formation, conjugation, motility, competence and antibiotic

production (Lyon et al., 2001; Miller and Bassler, 2001; Sperandino et al., 2001; Papenfort and Bassler, 2016). Several Gram-negative bacteria use N-acyl-homoserine lactones (AHLs) as signal molecules for the cell population density, which make the bacterial population regulate the expression of several genes and play a major role in biofilm formation.

Quorum sensing in Gram-negative bacteria uses small molecules termed as autoinducers (AIs). These molecules control quorum sensing in Gram-negative bacteria; they are produced from *S*-adenosylmethionine (SAM). Autoinducers will interact with specific receptors which are controlled by the quorum sensing mechanism (Papenfort and Bassler, 2016). Autoinducers are either acyl-homoserine lactones (AHLs) or other molecules. An autoinducer is synthesized in the cell and diffused across the inner and outer membranes. An autoinducer will bind to receptors of cytoplasm which are transcription factors, in the condition where the concentration of AIs is high enough (**Figure 1**). Sometimes AIs are characterized by a two-component of histidine kinase receptors.

Exploration of QS inhibitors or synthetic quorum quenching analogues is very important. One of the recent studies conducted by Haque et al. (2018) developed strategies for the inhibition of quorum sensing virulence factors. El-Hamid et al. (2016) found crude plant extracts with QS inhibitor activities through inhibition of the expression of QS-related genes; thereby inhibition of QS-related genes affects virulence factor production.

6.1. *Pseudomonas aeruginosa*

P. aeruginosa have some QS systems. The first is the las system, in which there are the LasI synthase protein, which is essential for the production of the AHL signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) and the LasR transcriptional regulator which requires 3O-C_{12}-HSL to be an active regulator. Second, the QS system contains RhlI and RhlR proteins. RhlR acts as a transcriptional regulator, while RhlI synthase produces AHL *N*-butyryl-L-homoserine lactone (C_4-HSL) (Gambello et al., 1991; Pearson et al., 1994). These two systems provide a unique target for novel antimicrobial drugs (Smith and Iglewski, 2003).

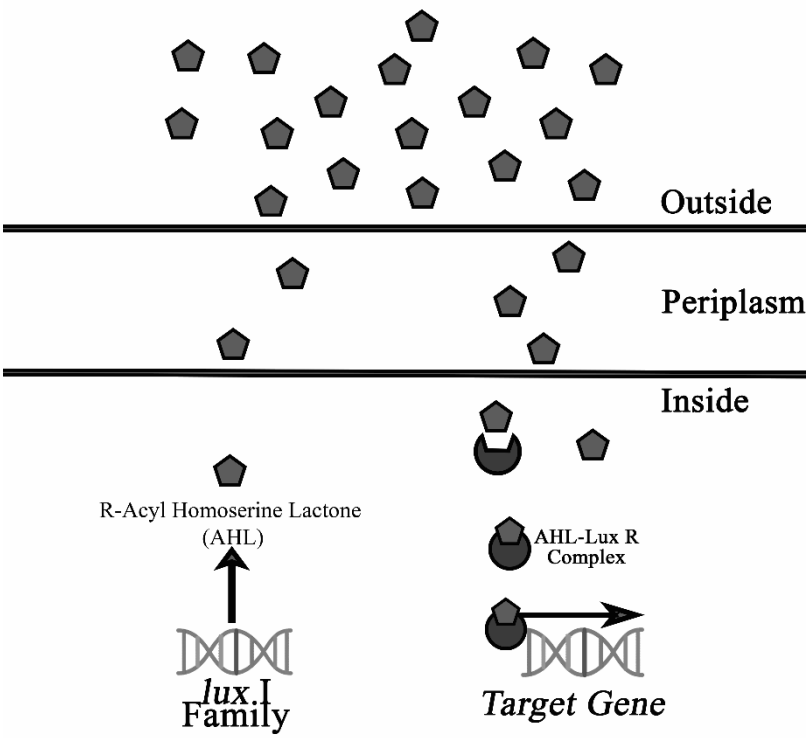

Figure 1. An autoinducer synthase (Lux I) produces QS signals which are usually acyl-homoserine lactones (AHLs). At a high population, density concentration of AHL reaches a threshold and AHL binds LuxR (AHLs receptor). Therefore, the receptor binds a promotor of the target gene and modulates transcription of the target gene

The third QS in these bacteria is PQS which is structurally identified as 2-heptyl-3-hydroxy-4-quinolone, and it is chemically unique from the AHL signals of the *las* and *rhl* systems (Gallagher et al., 2002). The fourth is IQS, a new class of quorum sensing signal molecule identified as 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Lee et al., 2013).

The QS network of *P. aeruginosa* consists of some interconnected signaling mechanisms and it is organized in a multi-layered hierarchy. The QS regulatory network in the bacteria can respond to bacterial population density as well as environmental stress (Lee and Zhang, 2014).

6.2. *Vibrio cholerae*

In *V. cholerae*, QS and 3,5-cyclic diguanylic acid (c-di-GMP), together regulate the formation of biofilm, where QS represses biofilm formation, and c-di-GMP which is an intracellular second messenger activates biofilm formation. HapR plays as a major regulator of the QS system; it can repress biofilm formation via two distinct mechanisms. HapR regulates the transcription of 14 genes which encode proteins for synthesis and degradation of c-di-GMP. Therefore, the reduction of cellular c-di-GMP occurs in a high cell density situation and leads to a decrease in biofilm formation. While in the condition where c-di-GMP increases at high cell density to the level present in the low cell density, QS restores biofilm formation; it is seen that c-di-GMP is important to QS in regulating biofilm formation in *V. cholerae* (Waters et al., 2008).

V. cholerae uses two parallel systems. The first one of an autoinducer is (S)-3-hyroxytridecan4-one (CAI-1 which is synthesized using SAM) while the second one of an autoinducer is AI-2 which is synthesized by LuxS (Rutherford and Bassler, 2012).

V. cholerae expresses its suite of virulence factors at low cell density (Zhu et al., 2002). At low cell density, AphA together with LysR-type transcription factor will activate AphB transcription of tcpPH which is a trans-membrane DNA binding protein. While ToxT is also a transcription factor that functions to activate expression of the gene for cholera toxin and toxin co-regulator. Both of these genes are major virulence factors in *V. cholerae.* Quorum sensing together with other regulators also controls biofilm formation in *V. cholerae,* wherein biofilm formation will be activated at low cell density and will be repressed at high cell density. In the condition of high cell density, quorum sensing represses the production of virulence factors and biofilm thereby facilitating the dispersal of bacteria back to the environment. At low cell density there will be no dispersal of the biofilm formed (Nadell and Bassler, 2011).

Papenfort et al. (2017) reported a new QS autoinducer-receptor pair in *V. cholerae* termed 3,5-dimethylpyrazin-2-ol (DPO). This molecule is made from threonine and alanine, and synthesis depends on the threonine dehydrogenase enzyme (Tdh). DPO acts as an activator through binding to transcription factor VqmA. The complex of VqmA-DPO activates the expression of vqmR which represses genes important for biofilm formation and also toxin production.

6.3. *Salmonella enterica*

For *S. enterica* serovar Typhimurium, a *luxS* gene is necessary for *Salmonella* virulence phenotypes, and the product of this gene also regulates cell density. Deletion of *luxS* also affects other InvF-regulated genes expressed from *Salmonella* pathogenicity island 1 (SPI-1). Therefore, reducing the expression of *invF* and its regulated genes in *Salmonella* cells lacks quorum sensing, resulting in the attenuation of virulence phenotypes (Choi, Shin, and Ryu, 2007).

Monteiro et al. (2011) reported a new signaling pathway in *S. enterica* serovar Typhimurium that plays a role in the turnover of the cell wall and produces biofilm. They also found the specific function of two lytic transglycosylases of family I, MltE and MltC, in the increasing expression of CsgD wherein the transcriptional regulator CsgD is important for regulating biofilm formation.

In *S. enterica* serovar Typhimurium, the luxS gene encoding the AI-2 synthase is involved in the production of the AI-2 molecules from S-adenylmethionine (SAM) (Surette, Miller, and Bassler, 1999). Salmonella pathogenicity island 1 (SPI-1) encodes the type III secretion system which is important for the invasion of the bacteria. Lsr proteins that are important for the processing of phospho-AI-2 will be maximally expressed in the mid-exponential phase (Taga, Miller, and Bassler, 2003). Choi, Shin, and Ryu (2007) reported that the luxS gene is important for the virulence properties of *Salmonella*. When the luxS gene was deleted, the cell density-dependent induction of the *inVF* gene was abolished. LuxS deletion also affects other InvF-regulated genes expressed by *Salmonella* pathogenicity island 1 (SPI-1). The effect of this deletion will be restored by the addition of a synthetic signal molecule or the introduction of a plasmid copy of the luxS gene.

7. Molecular Mechanism of Biofilm Formation in Gram-positive Bacteria

Gram-positive pathogen bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, Streptococci and Enterococci are difficult to treat with antibiotics. Furthermore, if these bacteria form a biofilm, it becomes more difficult to be eliminated by antibiotic therapies and needs a high concentration of antibiotics for treatment (Conley et al., 2003; Dardi and Wankhede, 2014; Moghadam, Pourmand, and Aminharati, 2014; Hashem et al., 2017; Lee, 2017). Biofilm is not only found in the patient

body, but can be found in indwelling medical devices, such as the central venous catheter, urinary catheter and mechanical heart valve (Donlan, 2001).

The formation of biofilm is an important strategy to survive in an unfavorable environment and, on the other hand, to colonize the habitat. *Streptococcus pneumoniae* uses biofilm to avoid complement immunity and to prevent phagocytosis (Domenech et al., 2013).

7.1. Biofilm Structure

The biofilm structure, in general, consists of extracellular polymeric substances (EPS), including exopolysaccharides, extracellular protein (enzymes, EPS-modifying enzymes, cell surface-associated and extracellular carbohydrate-binding protein), extracellular DNA, surfactant and lipids, and water. The function and composition of EPS in various microbial species are not the same (Flemming and Wingender, 2010). The biofilm matrix of several Gram-positive bacteria contains amyloid/amyloid-like protein as found in *Bacillus subtilis*, *Staphylococcus aureus* and *S. mutans* (Hobley et al., 2015). The biofilm matrix creates a unique environment for the microbes. This structure may contribute to cell protection from the antimicrobial compound, host defense, nutrient uptake and the release of cellular material after cell death (Domenech, Garcia, and Moscoso, 2012).

7.2. Biofilm Formation and Regulation

7.2.1. Bacillus

Several *Bacillus* species, including *B. subtilis*, *B. cereus*, *B. thuringiensis* and *B. anthracis* are able to form a biofilm. Exopolysaccharide production is the initial stage for bacteria to adhere to the surface. The major polysaccharide in the biofilm matrix of *B. subtilis* is poly-N-acetyl glucosamine (Roux et al., 2015). This polysaccharide is synthesized by the *epsA-O* operon. Among all genes in the operon, *epsHIJK* is essential for biofilm formation and can be expressed and perform the same function in Gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Roux et al., 2015). Besides EPS, the biofilm matrix of *B. subtilis* needs the extracellular protein, TasA. TasA is the main protein component in the biofilm matrix. This protein is encoded by the *tapA-sipW-tasA* operon (Branda et al., 2006; Cairns, Hobley, and Stanley-Wall, 2014). The function of TasA depends on TapA, which is a TasA accessory/anchoring protein, and SipW, a signal peptidase, to locate

TasA in the extracellular matrix. Both the *epsA-O* and *tasA* operons are regulated by the master regulator SinR (Branda et al. 2006, Kampf et al., 2018). SinR is a transcription repressor that inhibits transcription of the *eps* operon and the *tapA-sipW-tasA* operon. Furthermore, the activity of SinR is controlled by YmdB, showing that YmdB is also essential for biofilm formation (Kampf et al., 2018).

CodY is global transcriptional regulator in *Bacillus* in response to branched-chain amino acid limitation. Several genes are regulated by this regulon, including those involved in biofilm formation (Majedet al., 2016). However, the role of CodY regulation may depend on the growth stage of *Bacillus*. In the early phase, CodY is expected to positively regulate biofilm formation. With the biofilm formation of *B. cereus* ATCC 14579 codY mutants are increased. In contrast, a transposon-inactivated codY mutant of *B. cereus* UW101C decreases the ability to develop biofilm.

7.2.2. Streptococci

S. mutans biofilm formation is influenced by glucosyltransferases (GTFs), glucan-binding proteins (Gbp), surface proteins, namely protein antigen C (PAc), and collagen binding protein (CBP). GTFs function to hydrolyze sucrose to produce glucans, which play a role in the attachment to the tooth surface and determine the stability of the biofilm structure. GBP is important for stabilizing biofilms, binds specifically with glucan and cohesively aggregates. PAc plays a role in attaching to salivary pellicles, and causes virulence in dental caries (Matsumoto-Nakano, 2018). Neuraminidase (NanA) produced by *S. pneumoniae* is involved in biofilm formation.

7.2.3. Enterococci

Biofilm formation in *Enterococcus faecalis* is regulated by several genetic determinants. Expression of the Fsr operon (*fsrABC*) shows important activity in biofilm formation and is considered to be *agr*-like genes because the translated amino acid sequence of FsrA, FsrB, FsrC shows 23%, 23%, and 26% identity or 36%, 38%, and 41% similarity with *Staphylococcus aureus* AgrA, AgrB, and AgrC, respectively (Qin et al., 2000). The mutation of this operon reduces biofilm formation. The Fsr operon is controlled by quorum sensing signaling and it regulates the expression of *gelE-sprE* that synthesizes gelatinase and serine protease, respectively (Qin et al., 2000). The other genetic determinants involved in

E. faecalis biofilm formation are *bee*, *bop*, *epa*, *esp*, *ebpABC*, *ebpR*, *etaR*, *atn*, *dltA*, *salA*, *salB*, and *srtC* (Mohamed and Huang, 2007).

7.2.4. Staphylococci

Polysaccharide and protein factors are important mediators for the attachment of bacterial cells. Polysaccharide intercellular adhesin (PIA) is a major component for intercellular adhesion. PIA biosynthesis is encoded by the *ica* operon that consists of *icaABCD* genes and the *icaR* regulatory gene. The adherence of Staphylococci is also mediated by protein adhesin of the microbial surface component recognizing adhesive matrix molecule (MSCRAMM). Other proteins involved in biofilm formation are, for example, AtlE (in *Staphylococcus epidermidis*) or atl (in *Staphylococcus aureus*), accumulation-associated protein (AAP) and biofilm-associated protein (BAP) (Gotz, 2002).

8. The Role of Quorum Sensing in Biofilm Formation

Gram-positive bacteria show different quorum sensing mechanisms than Gram-negative bacteria. Secreted short peptides (oligopeptide) are used as signal molecules, often called pheromones. This pheromone is encoded in the open reading frame (ORF) and incompletely annotated in the genome sequences. In addition, pheromones can also be obtained from the proteolysis reaction of hydrophobic surface proteins, such as in *S. pyogenes*. Various pheromones are produced by Gram-positive bacteria, such as short hydrophobic peptide and ComS by *S. thermophilus*, AgrD by *S. aureus*, CSP (competence signaling peptide) from *S. pneumoniae*, and PapR from *B. cereus*. Oligopeptide precursors are converted into mature peptides (pheromones) that are about 5-30 amino acids in size by proteolytic or post-translational modifications. Pheromones are secreted out of the cells using specific transporters, interacting with surface enzymes, namely histidine kinase of a two-component system, undergoing phosphorylation, thus inducing a gene expression regulator (Dufour and Lévesque, 2013; Monnet, Juillard, and Gardan, 2014).

8.1. Streptococci

In *Streptococcus*, CSP will activate the com regulon (*comCDE* and *comX*) which plays a role in encoding precursor receptors in the form of the histidine kinase regulator and sigma factor. The CSP precursor is synthesized by ComC. This precursor is translocated across the membrane through the ComAB membrane protein. CSP activates ComD and phosphorylated-ComD

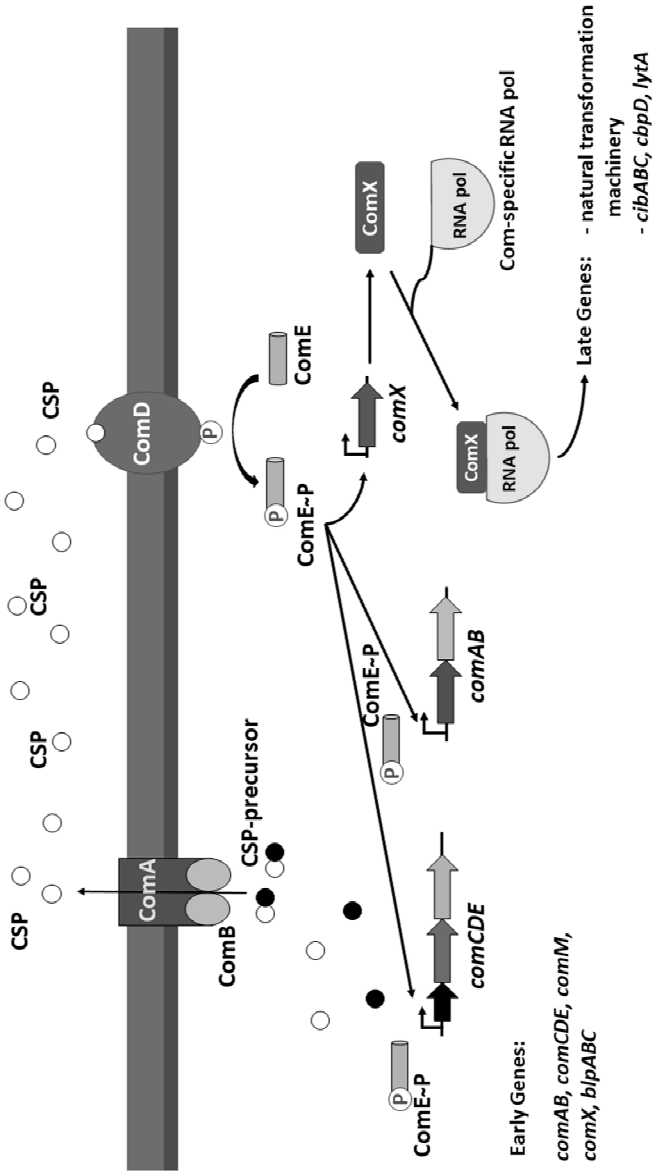

Figure 2. The competence signaling peptide quorum-sensing system in *S. pneumoniae*