Genetic and molecular determinants of *Pseudomonas aeruginosa* mono- and dual-species biofilm formation

Keehoon Lee

Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Sang Sun Yoon

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This certifies that the Doctoral Dissertation of Keehoon Lee is approved.

Thesis Supervisor : Sang Sun Yoon

Thesis Committee Member#1: Kyu Ho Lee

Thesis Committee Member#2: Soon Jung Park

Thesis Committee Member#3: Woojun Park

Thesis Committee Member#4: Myeong Heon Shin

The Graduate School
Yonsei University

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ABSTRACT

Genetic and molecular determinants of *Pseudomonas aeruginosa* mono- and dual-species biofilms

Keehoon Lee

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Sang Sun Yoon)

Pseudomonas aeruginosa is an opportunistic pathogen, and it became one of the greatest threats to human health worldwide due to their adaptation ability for various environments and resistance against multiple classes of antibiotics. These characteristics of the bacterium contributed to them to become a major causative agent of healthcare-associated infections (HAIs). They are, also, known to develop robust biofilms. Biofilm is a community of microbes that inhabits on various surfaces and typically surrounded by extracellular matrices (ECM).

Chapter I is a literature review that includes brief history of biofilms, background information of *P. aeruginosa* and its biofilm, biofilm infections and multi-species biofilms.

In chapter II, the investigation was initiated to identify genes that affect the enhancement of the *P. aeruginosa* biofilm by the sub-minimal inhibitory concentrations (MICs) treatment of antibiotics. We screened a transposon (Tn) mutant library of PAO1, a prototype *P. aeruginosa* strain. Among ~5,000 mutants, a *fiuA* gene mutant was verified to form very defective biofilms in the presence of sub-MIC carbenicillin. The *fiuA* gene encodes ferrichrome receptor A, involved in the iron acquisition process. Of note, biofilm formation was not decreased in the *ΔpchΔpvd* mutant defective in the production of pyochelin and pyoverdine, two well-characterized *P. aeruginosa* siderophore molecules. Moreover, the *ΔfiuA,* a non-polar *fiuA* deletion mutant, produced a significantly decreased level of elastase, a major virulence determinant. Mouse airway infection experiments revealed that the mutant expressed significantly less pathogenicity.

In chapter III, we investigate pathogenic factors of the *Pseudomonas aeruginosa* polymicrobial biofilm. Since the introduction of biofilm infection model in medical field, it has discovered that biofilms are responsible for majority of chronic infections. However, the treatments of the chronic biofilm infections are still very limited to surgical removal of the infected sites. Most of the chronic biofilm infections are known to be

polymicrobial infections. *Pseudomonas aeruginosa* and *Enterococcus faecalis* are two of the most spotted bacterial species in biofilm infections but the study of the interactions between these bacteria was very limited. In this investigation, we observed phenotypic changes in the dual-species biofilm of *P. aeruginosa* and *E. faecalis*, such as dramatic enhancement in elasticity of the biofilm, and distinct spatial distribution of each bacterial species in the biofilm. We have found that these phenotypic characteristics were associated with exopolysaccharides (EPS), especially Pel and Psl.

Together, our results suggest that *fiuA* gene has pleiotropic functions that affect *P. aeruginosa* biofilm development and virulence. The targeting of FiuA could enable the attenuation of *P. aeruginosa* virulence and may be suitable for the development of a drug that specifically controls the virulence of this important pathogen. Also, Psl is more associated to the bacteria-surface adhesion and the interaction between *P. aeruginosa* cells to form a structured biofilm, and Pel is more related to interspecies interaction in the polymicrobial biofilm. Therefore, these two EPS can be targets for polymicrobial biofilm infection eradication.

Key words : ***Pseudomonas aeruginosa*, iron, siderophore, ferrichrome receptor A, virulence, *Enterococcus faecalis*, polymicrobial biofilm, elasticity, Pel, Psl**

Chapter I.

Overview of *Pseudomonas aeruginosa* biofilm

I. Brief history of biofilm research

Microbiology has evolved dramatically since Robert Koch introduced Koch's postulates and methods of isolation and pure culture. These techniques have been used in laboratories all over the world and have produced numerous studies for the diagnosis and management of many devastating infectious diseases, such as tuberculosis, cholera, and diphtheria. Because of these important contributions of planktonic pure culture techniques in the health of the human race, these techniques are essential and have been the gold standard for the study of microbes for several decades. However, microbiologists have continuously faced difficulties in eradicating bacteria or growing them in single-species planktonic cultures.

Microbiologists started to realize that it was inadequate to study bacteria in pure planktonic culture in order to understand their natural lifestyle and interactions. The differences between single-species planktonic cultured bacteria and the same bacteria in sessile and mixed species cultures have been characterized due to the development of microscopy technologies. Most bacteria have completely different phenotypes and physiological characteristics when grown in pure planktonic conditions compared to mixed-species sessile conditions.

Since the early 1900s, many descriptions of sessile cultures were made for surface-associated bacteria, marine bacteria attachment on glass surfaces, and many other important observations about sessile bacterial growth conditions.[1,2] The term biofilm had been unofficially used among scientists, but the first official introduction of the term was in the *Microbial Ecology* journal in 1975.[3] Also, the ubiquitous characteristics of biofilms were proposed in the first quantitative examination of bacteria in specific ecosystems by J. W. Costerton and his colleagues in 1978.[4] They discovered an extensively large number of bacteria in the biofilms from the surfaces of rocks from alpine lakes and streams in Montana but found a very small number of planktonic bacteria, and the data was confirmed in different locations.[4] Based on their data, they confirmed that biofilms are a major form of bacterial existence in nature, and the universality of biofilms was suggested and confirmed not only in environmental systems, but also in the industrial and medical fields.[5]

Early in biofilm research, there was a limitation in biofilm observation due to deformation and dehydration during the preparation of samples for bright field or electron microscopy. Thus, biofilms were thought to be a uniform layer of bacteria that were covered in slime. However, in the late 20th century, biofilm observation using confocal laser scanning

microscopy (CLSM) produced a breakthrough in biofilm research through the discovery of defined structures in biofilms such as vertical structural elements and water channels (Fig. 1.1).[6] The discovery of complex biofilm architecture and the presence of water channels in biofilm structures drew the attention of microbiologists because it indicated that biofilms are not just a collection of bacteria but are an actively developed microbial community. The second breakthrough in biofilm research was the discovery of differential gene expression in biofilm bacteria compared to their planktonic counterparts, which indicated refined regulatory systems for biofilm development, and quorum sensing systems were revealed to be one of the regulatory systems for biofilm development.[7,8] After these discoveries, research on biofilms increased exponentially and became a new trend in microbiology.

II. What is biofilm?

Biofilms are generally known as communities of microbes that are attached to certain surfaces that are normally covered with an extracellular matrix (ECM), secreted by the same microbes. The components of the ECM are exopolysaccharide (EPS), extracellular DNA

(eDNA), RNA, proteins, and lipids. The ECMs protect biofilms from harsh environments; as such, bacteria in biofilms are generally more resistant to various disinfectants and antimicrobial substances than their planktonic counterparts.[9,10] This resistant property contributes to numerous biofilm-caused problems in our society. In the industrial field, biofilms cause malfunction of machines, corrosion of facilities, blockage of pipelines, contamination in drinking-water distribution systems, and safety issues in the food industry.[11,12] Because of the problems caused by biofilms, an astronomical amount of money is being spent every year to manage biofilms in these industries. Furthermore, resistance to the host immune response can lead to chronic infections in the host, which threatens many lives worldwide.[9]

However, biofilms are not always bad, and biofilms are positively used in many applications. For example, biofilms are an essential part of the bioremediation process. A biofilm known as sludge is a very important component in the wastewater treatment process, and bioremediation bioreactors contain biofilms that degrade many toxic contaminants and hazardous materials that are generated from various industrial processes.[13,14 15]

III. *Pseudomonas aeruginosa*

Pseudomonas species are ubiquitous in the natural environment and cause disease in both animals and plants. Among the *Pseudomonas* species, *Pseudomonas aeruginosa* is the most well known pathogen that causes human infections. *P. aeruginosa* is a gram-negative bacillus and is known as an opportunistic pathogen. Although the bacterium only causes mild infections, such as *otitis media* or *otitis externa* in healthy individuals, it can also cause serious infections in many different parts of the human body when the immune system is compromised. For example, *P. aeruginosa* is a major cause of mortality in cystic fibrosis (CF) patients. In addition, *P. aeruginosa* also causes bacteremic pneumonia, endocarditis, meningitis, burn wound infections, and sepsis, and these infections are associated with high mortality.[16,17] *P. aeruginosa* is known to produce various virulence factors, including flagella,[18] type IV pili,[19] alkaline protease,[20] elastase,[21] lipopolysaccharide,[22] phospholipase,[23] exotoxin A,[24] pyoverdine,[25] and pyochelin.[25] *P. aeruginosa* has been studied extensively; although many characteristics of this species have been revealed, there are still many aspects of its exact pathogenesis that remain undetermined.[17,26]

Treatment of *P. aeruginosa* infection is facing major challenges due to

the constant emergence of antibiotic-resistant strains. The escalated antibiotic resistance increases the rate of disease occurrence and the mortality due to *P. aeruginosa* infection. *P. aeruginosa* is the most common causative agent of hospital-associated infection (HAI) and the second most common cause of ventilator-associated pneumonia (VAP) in the US.[27] The *P. aeruginosa* genome is larger than many other prokaryotes, and *P. aeruginosa* has an exceptionally large number of regulatory genes in its chromosome, which contributes to the adaptation of this species to various environmental conditions and is closely related to the development of antibiotic resistance.[28,29] Because of its ability to form biofilms, *P. aeruginosa* has become the major cause of HAI. The conversion from the planktonic to the biofilm stage changes gene expression pattern and increases the lateral gene transfer rate on a large scale. These changes are known to contribute to antibiotic resistance.[30-32]

IV. *P. aeruginosa* biofilm development and general characteristics

Biofilm development models have changed several times with the advancement of biofilm research techniques. Advanced experimental techniques revealed that biofilm development consists of three defined stages of initial attachment, maturation, and detachment of biofilms (Fig.

1.1).

1. Attachment of *P. aeruginosa* biofilms

Many early studies on the initial attachment of bacteria suggested the involvement of simple chemical bonds such as Van der Waals forces. However, early stage biofilm development is composed of much more complex events. For example, there are a variety of bacterial structures such as adhesins, type IV pili, and lipopolysaccharide (LPS) that are involved in attachment, and these bacterial structures are specifically regulated by environmental cues.[33,34] Recent studies demonstrated that the initiation of biofilm formation occurs with an increase in c-di-GMP, an intracellular second messenger.[35-39] Many types of environmental cues can cause an increase in c-di-GMP, which activates the production of adhesins and various extracellular matrix products.[35,39] For example, the contact of *P. aeruginosa* to a surface is recognized by the WspA protein, a membrane-bound receptor protein, which creates a signal to produce c-di-GMP and in turn positively regulates the production of CdrA adhesin, Psl, Pel, and alginate in *P. aeruginosa*.[40,41] Biofilm formation is also regulated by small regulatory RNAs (sRNA) in many bacterial species,[42] as Psl and Pel production and the motile-to-sessile switch of *P. aeruginosa* are regulated by sRNA.[43,44]

2. Maturation of *P. aeruginosa* biofilms

After bacteria attach to surfaces or each other, they undergo a series of changes to adapt to the new mode of life. As surface-attached *P. aeruginosa* grow and form microcolonies, they start to produce extracellular matrices (ECMs) and build structures and water channels. As the biofilm matures, the bacteria undergo physiological changes and become much more resistant to stresses from the environment or antibiotics. This biofilm development and maturation are closely related to a signaling system called quorum sensing.[10,28,33]

3. Extracellular matrix of *P. aeruginosa* biofilms

ECMs of biofilms usually consist of EPS, eDNA, proteins, and proteinaceous components, which act as a matrix, adhesive material, and protective barrier.[9,28] There are three identified EPSs in *P. aeruginosa*: Psl, Pel, and alginate.[45] Psl polysaccharide was named for the polysaccharide synthesis locus that was identified in 2004.[46,47] Psl is an important component of the ECM for initiation and maintenance of *P. aeruginosa* biofilms by providing cell-surface attachment and intercellular interactions. In the late stage of biofilm maturation, Psl was shown to accumulate on the outside of three dimensional-structured biofilms.[48,49] This Psl accumulation provides structural support and allows for later

dispersion of the *P. aeruginosa* biofilm.

Pel polysaccharide is an essential component for *P. aeruginosa* to form pellicles at the air-liquid interface and solid surface-associated biofilms.[47,50] The other roles of Pel are to act as a platform for biofilm structure and to provide protection against aminoglycoside antibiotics.[49,51] However, most of these roles depend on the strain of *P. aeruginosa*. The complete biochemical composition of Pel has not yet been identified, but it is known to be a glucose-rich polysaccharide. As mentioned, one of the mechanisms for Psl and Pel production is through the c-di-GMP signaling pathway due to environmental cues, and another postulated Pel production mechanism is involved in the production of LPS.[38-41]

The EPS alginate is the most studied EPS of *P. aeruginosa* biofilms, and it is mainly produced by *P. aeruginosa* strains isolated from CF patients.[49] Alginate is known as a factor used to distinguish mucoid or non-mucoid *P. aeruginosa* biofilms, although it was recently found that Psl also contributes to the mucoid phenotype of the biofilms.[52] Alginate plays many important roles for biofilms. For example, alginate retains water and nutrients and provides antibiotic resistance and immune evasion.[53-55]

Another component of the ECM is eDNA. There are several hypotheses regarding the presence of eDNA in biofilms, such as active secretion, autolysis of bacteria, and release from small membrane vesicles.[48,56] eDNA is known to play several roles in the formation of cation gradients, antibiotic resistance, nutrient source, and early biofilm development.[48,57-59] Also, eDNA is a major proinflammatory factor for *P. aeruginosa* biofilms.[60]

Other than EPS and eDNA, proteins and proteinaceous components also contribute to the formation of biofilm matrix.[48] For example, flagella act as an adhesin to help initial bacterial attachment to the surface.[61] Type IV pili contribute to the formation of mushroom-like biofilm cap structures.[61,62] CdrA adhesin interacts with Psl and increases biofilm stability.[28,41] Cup fimbriae are also one of the proteinaceous components of ECM and play important roles in cell-to-cell interaction during the initial stage of biofilm formation.[28,63]

4. Detachment of *P. aeruginosa* biofilms

The final stage of biofilm development is detachment. There are several types of biofilm detachment mechanisms, sloughing, erosion, and seed dispersal.[34,64,65] These detachment mechanisms are essential to create new biofilms in new niches. The sloughing and erosion mechanisms of

biofilm detachment are called passive detachments and are mediated by shear stress.[34,64] Sloughing is the detachment of a large portion of a biofilm from the original mass, and erosion is a washout of a small portion of biomass or bacteria from the outer surface.[64] Seed dispersal is the active detachment mechanism of *P. aeruginosa* biofilms. In this process, *P. aeruginosa* biofilms release single planktonic cells or microcolonies from the center of the biofilm, leaving an empty cavity.[64] Dispersal of biofilms is closely related to microcolony size. Dispersal starts with spatial differentiation, which is described as the differential localization of motile and non-motile *P. aeruginosa* in the biofilm structure when the biofilm reaches a critical size.[64,65] The motile bacteria locate in the mushroom cavity, and the non-motile bacteria locate at the stalk and walls of the mushroom structure.[64,65] This dispersal mechanism involves ECM degradation and autolysis of a biofilm subpopulation. Biofilm dispersion can also be induced by environmental cues, such as nutrients, oxygen availability, nitric oxide (NO), pH, and various chemicals. For example, a sudden increase in glucose supply can decrease intracellular c-di-GMP, which increases flagella production and induces dispersal.[41] Also, limited oxygen supply can induce biofilm dispersal by enhancing c-di-GMP degradation.[65] NO stimulates

phosphodiesterase (PDE) activity, which decreases intracellular c-di-GMP level in *P. aeruginosa* and leads to dispersal of the biofilm.[65] In addition, there are various chemicals that contribute to the dispersal of *P. aeruginosa* biofilms, such as metal chelators, cis-2-decenoic acid, anthranilate, and other surfactants.[66-68]

5. Quorum sensing (QS) in biofilms

Quorum sensing (QS) is an intercellular communication system that enables bacteria to sense their own population density.[69] QS systems rely on small signaling molecules, N-acyl-homoserine lactones (AHL) for gram-negative bacteria, oligopeptides for gram-positive bacteria, and autoinducer-2 (AI-2) for both classes of bacteria.[69,70] QS systems not only sense population density, but also regulate a variety of traits, such as bacterial phenotype, spatial differentiation in biofilms, motility, and biofilm formation.[71] Genetic expression analysis also revealed that several hundred genes in *P. aeruginosa* are regulated by QS systems.[72]

The role of QS systems in biofilm formation was reported first in 1998 by Davis and his colleagues in *P. aeruginosa* biofilms.[71] They demonstrated that a *lasI* mutant of *P. aeruginosa* only forms flat and undifferentiated biofilms.[71] The *las* system is one of the QS systems, which is involved in sensing and production of N-3-oxo-dodecanoyl

homoserine lactones (N-3-C12-HSL).[69-71] The *rhl* QS system of *P. aeruginosa* senses and regulates the synthesis of N-butanoyl-L-homoserine lactone (C4-HSL).[8] The *rhlI* gene is known to be involved in the formation of mushroom cap structure in *P. aeruginosa* biofilms and in the dispersal of biofilms by controlling the production of rhamnolipids.[69,73] Another QS system of *P. aeruginosa* is the *Pseudomonas* quinolone signal (PQS) system that senses 2-heptyl-3-hydroxy-4-quinolone.[69,74] The PQS system regulates eDNA release in biofilm formation and membrane vesicle production.[74-76]

V. Biofilm infections

Biofilms have become a major issue in the medical field because biofilm infections present high resistance not only to antibiotics, but also to the host immune response.[18,20,27] In addition, microbial pathogen biofilms are major causes of chronic infection.[9] Biofilm-associated infections can be divided into two categories. First are biofilm infections due to indwelling medical devices. For example, there are infections associated with central venous catheters, urinary catheters, prosthetic joints, peritoneal dialysis catheters, pacemakers, contact lenses, and intrauterine devices. The second are direct biofilm infections in host tissues, such as chronic

pneumonia in CF patients, chronic *otitis media*, endocarditis, chronic osteomyelitis, chronic prostatitis, palindromic urinary tract infection, and gingivitis.[77] The major problem with biofilm infections in diverse medical settings is due to their outstanding resistance against various antibiotics and other disinfectants. The microbes in biofilms can be hundreds of times more resistant than their planktonic counter parts.[54] To obtain high antibiotic resistance, microbes in biofilms use several biofilm-specific mechanisms, and these mechanisms are different than those commonly used by planktonic microbes. One of the biofilm-specific antibiotic resistant mechanisms is the physical barrier provided by the ECM that retards the distribution of antibiotics into the biofilm. The distribution rate varies among the types of antibiotics and microorganisms in the biofilm.[54,78] Biofilms also possess a sub-population called persister cells. The persister cells form small colony variants (SCV) that proliferate extremely slowly or stop growth all together. This metabolic arrest could act as a resistance mechanism against strong external stress such as antibiotics.[30] Furthermore, if bacteria develop biofilms due to the starvation-induced stress response, the cells in the inner part of the biofilm are restricted with regard to oxygen and nutrient supply, which leads to the inhibition of growth and

increase of amino acid synthesis for survival. This starvation-induced stringent response plays an important role in enhancing biofilm resistance.[79] Furthermore, glucan production and efflux pumps are known as antibiotic resistance mechanisms in biofilms. Ethanol oxidation, eDNA, and iron acquisition are thought to contribute to antibiotic resistance with mechanisms that are still unknown.[54,80-83]

VI. Multi-species biofilms

In the last three decades, there have been remarkable transitions in microbiology research from the study of pure planktonic cultures to biofilms, which is one step closer to the natural form of microbial living. However, much of biofilm research is still investigating pure single-species biofilms even though pure-species biofilms do not mimic real world microbial biofilms. Multi-species biofilms are the major form in the environment and in the human host. Metagenomic analysis of the human microbiome revealed that there are thousands of bacterial species that reside in the human gastrointestinal tract, oral cavity, respiratory tract, skin, and vaginal tract.[84] Even though most species remain unculturable, they exist and interact with each other. Therefore, it is important to investigate multi-species biofilms and the microbial

interactions that affect biofilm development and host health.

The interspecies interactions within biofilms involve QS systems, metabolic cooperation, or competition, and these interactions result in synergistic or antagonistic effects on the biofilms. Several studies demonstrated that interactions in a polymicrobial biofilm affect the overall characteristics that enhance resistance or virulence.[84-86] For example, microbes in dental plaque undergo spatiotemporal interactions and alter the surroundings in order to promote pathogenic bacterial species to colonize and survive.[87] *Staphylococcus aureus* has been shown to increase infectivity and biofilm development when interacting with *Candida albicans* in serum.[86]

Among the QS systems, the autoinducer-2 (AI-2) system was identified in both gram-negative and gram-positive bacteria and is utilized in interspecies interactions.[88,89] For an instance, an increase in the level of AI-2 concentration induces polymicrobial biofilm formation of *Streptococcus oralis* and *Acinetobacter naeslundii.*[90] Another recently identified QS signal that influences interspecies interactions is the diffusible signal factor (DSF), a fatty acid signal.[91] DSF, secreted by *Stenotrophomonas maltophilia*, enhances polymyxin resistance and biofilm formation of *P. aeruginosa*.[92] In addition to the examples above,

there are many complex interspecies interactions that influence antibiotic resistance, ECM production, or growth.[84,85,93]

VII. Conclusion

It took more than 100 years for microbiologists to recognize that the pure culture system does not represent the actual biology of microbes in natural niches, which led to research in biofilms. Since then, the study of mono-species biofilms has rapidly advanced. This research has provided important physiological and molecular information about biofilm development and characteristics. Even though we have a better understanding of biofilms, there are still many limitations in removal of biofilm from natural environments, industrial sites, or chronic infections. Research on multi-species biofilms and their interspecies interactions is essential to better understand biofilms. However, it is uncertain whether the knowledge and techniques for mono-species biofilm research are appropriate for the study of multi-species biofilms. Several new investigation tools have been introduced in genomics, proteomics, and microscopy for biofilm investigations, but much more research is needed in order to find optimal methods to study multi-species biofilms.

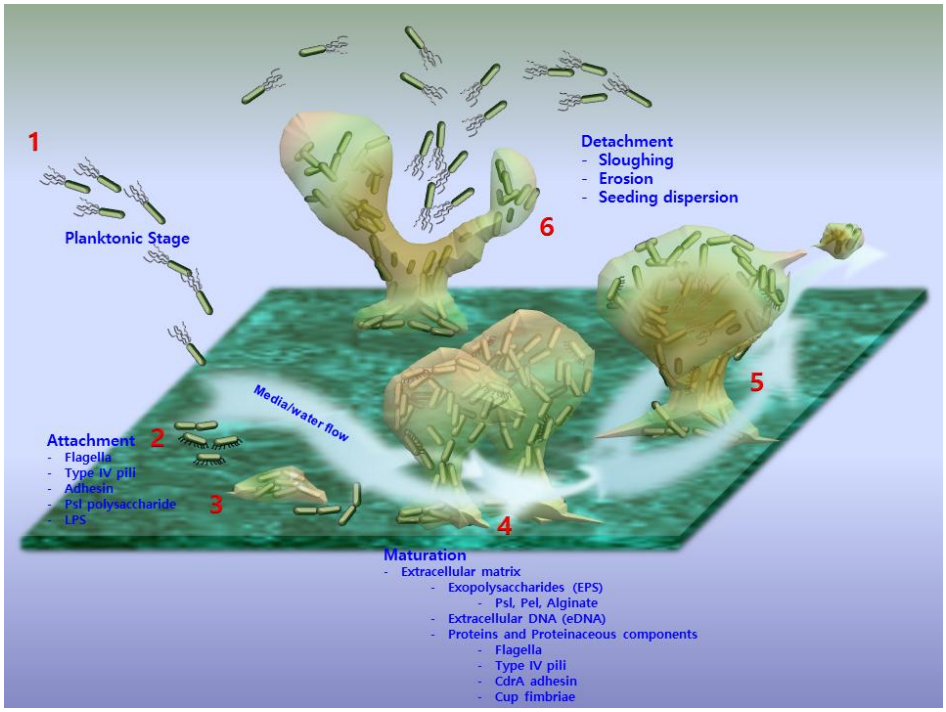

Figure 1.1. Diagrammatic representation of the developmental stages of *P. aeruginosa* biofilm The diagram presents 1) the planktonic stage, 2) attachment of bacteria to a surface, 3) production of ECM, 4) maturation of biofilm structures, 5) spatial differentiation, and 6) biofilm dispersal.

Chapter II

The Ferrichrome Receptor A as a New Target for *Pseudomonas aeruginosa* Virulence Attenuation

The Ferrichrome Receptor A as a New Target for *Pseudomonas aeruginosa* Virulence Attenuation

Keehoon Lee

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Sang Sun Yoon)

I. INTRODUCTION

P. aeruginosa is the most common opportunistic human pathogen. It produces various virulence factors, including flagella, type IV pili, alkaline protease, elastase, lipopolysaccharide, phospholipase, exotoxin A, pyoverdine, and pyochelin.[25] The expression of these various virulence determinants is regulated by complex signal transduction systems in response to changes in the surrounding environment.

A biofilm is defined as a community of microbes that attaches to certain surfaces and is normally covered with self-produced extracellular materials (ECMs). Biofilm infections present high resistance not only to antibiotics, but also to the host immune response.[18,20,27] In addition, biofilms of microbial pathogens are a major cause of chronic infection,

and approximately 80% of all microbial infections are thought to be biofilm-associated.[9] Remarkably, microbes growing in biofilms can be approximately 1,000 times more resistant to antibiotics than their planktonic counterparts.[54] To achieve this high level of antibiotic resistance, biofilm-dwelling microbes use several biofilm-specific mechanisms, such as physical ECM barriers, persister cell formation, and the starvation-induced stress response.[30,54,79,94] Additional antibiotic resistance mechanisms of biofilms include glucan production and efflux pumps. Ethanol oxidation, extracellular DNA, and iron acquisition are also thought to contribute to antibiotic resistance via as yet uncharacterized mechanisms.[54,80-83] The siderophores, pyoverdine and pyochelin are known to play particularly important roles in the evasion of the host immune response and biofilm development.[95-97] The iron uptake system of *P. aeruginosa*, which involves pyoverdin and pyochelin, has been heavily studied. However, information regarding ferrichrome uptake system in *P. aeruginosa* is very limited. Ferrichrome is a heterologous hydroxamate siderophore that is involved in iron acquisition along with pyoverdine and pyochelin in *P. aeruginosa*. Approximately 80% of all ferrichrome is transferred into the cytoplasm through ferrichrome receptor protein A (FiuA).[98] As for ferripyoverdine

and ferripyochelin, energy is required for ferrichrome to be transferred into the cytoplasm. This energy is supplied by an inner membrane protein, TonB1.[25,98,99] TonB1 has been shown to be associated with quorum sensing, which is closely linked to biofilm formation.[100] Previous studies have also identified a link between biofilm formation and iron acquisition.[67,96,101]

Previous studies have shown that biofilm development by *P. aeruginosa* is enhanced in the presence of sub-minimal inhibitory concentration (sub-MIC) treatments of several antibiotics.[102-104] However, the genetic basis underlying this phenomenon has not yet been discovered. In this study, we screened a *P. aeruginosa* transposon mutant library to identify mutants that form defective biofilms in the presence of sub-MIC carbenicillin. We found that a *P. aeruginosa* mutant defective in the ferrichrome receptor A gene (*fiuA*) exhibited dramatic decrease in biofilm formation under sub-MIC carbenicillin concentration but not under the antibiotic non-treated conditions. Importantly, other indications of virulence were also noticeably decreased when the *fiuA* gene was disrupted. This information will potentially be useful in the development of novel therapeutics against *P. aeruginosa* infection.

II. MATERIALS AND METHODS

1. Bacterial strains, plasmids, media, and culture conditions

All bacterial strains and plasmids used in this study are listed in Table 2.1. Bacterial cultures were grown at 37°C in Luria-Bertani medium (LB; 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter). To screen for biofilm-defective mutants at sub-minimal inhibitory concentration (MIC), transposon inserted bacterial strains were grown in 96-well plates containing 100 μl of LB with 9 μg/ml carbenicillin.

Table 2.1. Bacterial strains, plasmids and primers used in this study

Strains, plasmids, and primers	Description	Source
Bacterial strains		
E. coli SM10/λpir	*Donor strain, Kmr, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, kpir*	Lab collection
PAO1	Prototype *P. aeruginosa* laboratory strain	Lab collection
ΔfiuA	PA01 with in-frame deletion of *PA0470* gene	This study
ΔpchΔpvd	Pyochelin and pyoverdine synthesis-defective PAO1 mutant	Lab collection
Plasmids		
pCVD442	sacB suicide vector generated from pUM24	Lab collection
pBTK30	Transposon vector for the construction of a random mutant library, Gm^r	[105]
pJN105c	pJN105 with the gentamicin resistance marker replaced by a carbenicillin resistance marker	[106]
Primers		
fiuA upstream (forward)	5'-AAAGAGCTCTACTGGGCGCTGGACATCA–3'	
fiuA upstream (reverse)	5'-TTCCCGCACCGTTTCTCCAAGCTACAAGT GGTGAGCGACG–3'	
fiuA downstream (forward)	5'-CGTCGCTCACCACTTGTAGCTTGGAGAAACG GTGCGGGAA–3'	
fiuA downstream (reverse)	5'-AAAGAGCTCTCAAGGAA GGCCAGCAGGT –3'	

2. Construction of the *fiuA* deletion mutant

The *fiuA* deletion mutant was created by allelic replacement as previously described.[107] Briefly, flanking sequences (~500 bp) at both ends of the *fiuA* gene locus were PCR amplified with primers listed in Table 2.1. The SacI restriction sites are designated by underlines (Table 2.1). Two inner primers (upstream reverse primer and downstream forward primer) are complementary to each other. In this strategy, the 3' end of the upstream sequence and the 5' end of the downstream sequence are able to anneal during PCR amplification without further treatment. The deletion of the *fiuA* gene was confirmed by PCR, and the resultant strain was designated as *ΔfiuA*.

3. Iron acquisition capability assay

The PAO1, *ΔfiuA,* and pch/pvd mutants were incubated in LB medium at 37°C for 24 hr. Siderophore activities in bacterial supernatants were quantified by SideroTec Kit (Emergenbio, Ireland), an assay kit that detects siderophore in a sample by color change. The assay kit is based on the chrome azurol sulfonate (CAS) assay which was developed by Schwyn and Neilands.[108] The blue reagent turns to purple or pink when

ferric ion is transferred to siderophore, present in a sample.

4. Biofilm growth and quantification

Biofilms were prepared as previously described.[109] Briefly, a static biofilm was prepared by inoculating LB-grown bacterial strains (1:100 dilution) in LB or LB with carbenicillin in 96-well plates (SPL, Korea). The 96-well biofilm plates were incubated at 37°C for 24 hr. Biofilm formation was quantified via the crystal violet (CV) assay, described by O'Toole.[109] The eluted crystal violet stain was diluted (1:5) into a new 96-well plate and the absorbance of each well was read at 550 nm [109].

5. Confocal laser scanning microscopy (CLSM) image analysis of biofilms

Differential interference contrast (DIC) images were acquired using a confocal laser scanning microscope (FV-1000; Olympus Optical Co. Ltd., Japan) equipped with FV10-ASW operating software (ver. 02.01). For CLSM image analysis, biofilms were prepared in 96-well optical bottom plates with a coverglass base (Nunc™, MicroWell™).

After 24 hr of biofilm growth, the planktonic portions of the cultures were discarded and the wells were washed with 0.9% saline. Alternatively, biofilms were grown in coverglass bottom dishes (SPL, Korea) in the same manner as in the 96-well plates. Biofilms were stained with a LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen) and examined with two different wavelength lasers, 488 nm and 594 nm. For three-dimensional (3D) image analysis, Z-stack images were obtained and 3D images were reconstructed using FV10-ASW software.

6. Random Transposon Mutagenesis

For random transposon insertion mutagenesis, PAO1 was conjugated with *E. coli* SM10/λpir cells harboring the pBTK30 plasmid (Table 2.1).[105] Gentamicin-resistant transconjugants were grown on LB agar plates containing 50 μg/mL gentamicin and 50 μg/mL Irgasan (Sigma). Approximately 5,000 mutants were screened. All mutants defective in biofilm development under sub-MIC carbenicillin treatments were selected and their transposon insertion sites were determined by arbitrary PCR.[105]

7. Elastase assay and *Caenorhabditis elegans* survival assay

The level of elastase activity in bacterial culture supernatants was assessed following procedures described elsewhere.[110,111] The levels of elastase in the whole cell lysates and bacterial supernatants were assessed by probing membranes with anti-rabbit elastase antibody as a primary antibody.[112] *P. aeruginosa* virulence assay using *C. elegans* as an infection host was performed as described previously.[113] Briefly, overnight cultures of each bacterial strain were diluted 100-fold into LB broth and cultured until the OD_{600} value reached approximately 1.0. Next, 10 μl of *E. coli* OP50 (negative control strain), PAO1, and *ΔfiuA* suspensions were spotted onto Nematode Growth Media (NGM) agar plates. After 2 hr incubation at room temperature, each plate was seeded with ten L4-stage hermaphrodite worms (three replicates per trial). Plates were incubated at 20 ˚C. Live worms were transferred to a new NGM plate and scored for mortality every 24 hr.

8. Murine airway infection model for *in vivo* virulence experiments

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Yonsei University College of Medicine

(IACUC permit number: 2013-0369-5). To test the virulence of the *ΔfiuA* strain *in vivo*, 8-week-old C57BL/6N inbred female mice (Orient, Korea) were infected with each bacterial strain according to the acute infection protocol.[114] Infections were conducted through the intranasal route by making each mouse inhale 50 μl of the appropriate bacterial culture dilution, applied in a dropwise manner on the nose. Infected mice were returned to the cage and monitored for labored respiration, loss of ability to ambulate, and survival in every 4 hr until 24 hr then euthanized by cervical dislocation after anesthetization for histological observation of the lungs at the humane endpoint. For histological staining, lungs were perfused with sterile PBS and hematoxylin and eosin (H&E) staining was performed according to a standard protocol.[114] For the bacterial burden measurement, another set of mice (n=5) were intranasally infected with 5×10^6 cells of PAO1 or *ΔfiuA*, using the same method as the above. The lungs and spleens from the infected mice were collected at 16 hr after the infection. The harvested left lobes of lungs and spleens were homogenized, and the numbers of bacteria in the organ were measured by viable cell count assay.

9. Statistical analysis

All data are expressed as means ± standard deviations (SDs). The unpaired Student's t-test and one-way ANOVA were used to analyze the significance of all comparisons. P values less than 0.05 were considered to indicate statistical significance. All experiments were repeated at least three times for reproducibility.

III. RESULTS

1. Identification of a mutant, defective in biofilm development under sub-MIC carbenicillin concentrations

The screen for mutants defective in biofilm development under sub-MIC antibiotic concentrations was conducted with 5,000 transposon insertion mutants. All mutants that exhibited growth defects were first eliminated from the screening. Next, crystal violet (CV) assays were performed for all mutants in 96-well plates, and all biofilm-defective mutants were identified. All selected mutants were validated in second round of the CV assays. The transposon insertion site of each selected mutant was determined by arbitrary PCR, followed by DNA sequencing (data not shown). Twelve transposon inserted mutants were left after the growth and biofilm formation screening steps. Six out of the 12 mutants, that showed defective biofilm formation under sub-MIC conditions, have a transposon insertion in the genes, known to affect biofilm formation, such as *pelA, pelD, flgK,* and *gacA* (data not shown). Finally, the transposon mutant PATN715 was selected. The ability of PATN715 to form biofilms was confirmed in normal and sub-MIC carbenicillin treatment conditions using the CV assay (Fig 2.1A) and CLSM image analysis (Fig 2.1B). The PAO1 and PATN715 strains displayed similar

levels of biofilm formation in LB medium. However, the PAO1 biofilm was increased under sub-MIC carbenicillin treatment, whereas the PATN715 biofilm was decreased in this condition (Fig 2.1A). Both CV assay and CLSM analysis presented consistent results. In addition, CLSM image analysis showed bacterial cell elongation in both PAO1 and PATN715 biofilms under antibiotic treatment (Fig 2.1B). The transposon was determined to be inserted at *fiuA* gene based on our arbitrary PCR, and its insertion was further confirmed by PCR (data not shown). DNA sequencing revealed that the transposon was inserted at 2,039 bp downstream from the 5'-end of the *fiuA* gene (data not shown).

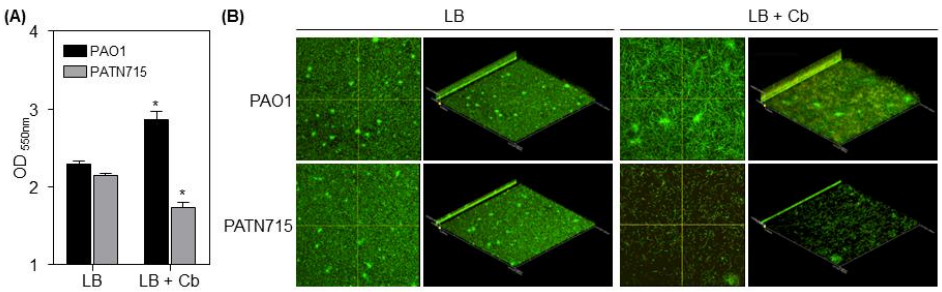

Figure 2.1. Identification of a mutant defective in biofilm development under sub-MIC carbenicillin treatment. (**A**) Crystal violet biofilm assay of PAO1 (black bars) and PATN715 (gray bars) strains grown in LB or LB with 9 μg/ml carbenicillin for 24 hr. Six

replicates were performed; values are expressed as means ± SDs. *P< 0.001 vs. each biofilm grown in LB. (**B**) Transverse confocal scanning laser microscope (CSLM) images and three-dimensional confocal scanning laser microscope (CSLM) images of static biofilms of PAO1 and PATN715 bacteria grown in LB or LB with 9 μg/ml carbenicillin (400x). Biofilms were grown for 24 H.

2. Various virulence-associated phenotypes were affected in *ΔfiuA*, a *fiuA* clean deletion mutant

To better elucidate the effects of *fiuA* gene on bacterial phenotypes, we constructed an in-frame deletion mutant of the *fiuA* gene. Bacterial growth was not affected by the *ΔfiuA* mutation (Fig 2.2A). Both strains, PAO1 and its *ΔfiuA* mutant, entered the exponential phase around 4 hr after inoculation. The doubling times of the PAO1 and *ΔfiuA* strains during the exponential phase were approximately 1.8 hr and 2 hr, respectively.

To determine the role of *fiuA* in the iron acquisition, the siderophore activity of PAO1 and *ΔfiuA* was measured. The qualitative indication for siderophore activity showed the color changes of PAO1 from blue to pink and *ΔfiuA* to purplish color, but there was no color change in *ΔpchΔpvd* sample since the pyochelin and pyoverdine are the major siderophores of *P. aeruginosa* (Fig 2.2B). The quantitative measure of siderophore concentration presented very low amount of siderophore in the *ΔfiuA* supernatant sample (Fig 2.2B). The siderophore concentration of PAO1 supernatant was approximately 10 μg/ml, whereas the concentration of *ΔfiuA* supernatant was around 0.15 μg/ml, and the concentration of *ΔpchΔpvd* was close to zero (Fig 2.2B).

To precisely determine the role of *fiuA* on biofilm development, biofilm developments of different siderophore mutants in two different growth conditions, LB broth and LB broth with sub-MIC carbenicillin, were quantified by the CV assay (Fig 2.3A). The *ΔfiuA* exhibited different patterns of biofilm formation in LB and LB with sub-MIC carbenicillin than the PAO1 and *ΔpchΔpvd* strains. The PAO1 and *ΔpchΔpvd* strains exhibited enhanced biofilm development in the presence of sub-MIC carbenicillin, whereas the biofilm development of the *ΔfiuA* strain was significantly decreased under the same conditions. CLSM image analysis of the biofilms yielded results consistent with those from the crystal violet assay (Fig 2.3B). CLSM Z-stack images revealed that the PAO1 and *ΔpchΔpvd* strains exhibited increased biofilm formation in the presence of sub-MIC carbenicillin conditions, whereas *ΔfiuA* biofilm formation was significantly decreased under the same conditions. Moreover, the compositions of the PAO1 and *ΔfiuA* biofilms were different. Very few dead (red fluorescent) bacterial cells were detected in the *ΔfiuA* biofilm, whereas the PAO1 biofilm contained a considerable amount of dead bacterial cells in its matrix (Fig 2.3B).

To determine whether the *ΔfiuA* strain exhibits any difference in virulence compared with the wild type strain, the amounts of elastase in

the culture supernatants were measured. Elastase is a major virulence factor produced by *P. aeruginosa* that can be detected by SDS-PAGE as a major band migrating at ~33 kDa.[115] First, the Elastin Congo Red assay revealed that the *ΔfiuA* supernatant contained a significantly lower level of elastase activity compared with the PAO1 and *ΔpchΔpvd* supernatants (Fig 2.4A). Western blot analysis revealed that the amounts of elastase in the whole cell lysates from the different strains were not significantly different. However, the amount of elastase in the *ΔfiuA* supernatant was clearly decreased (Fig 2.4B).

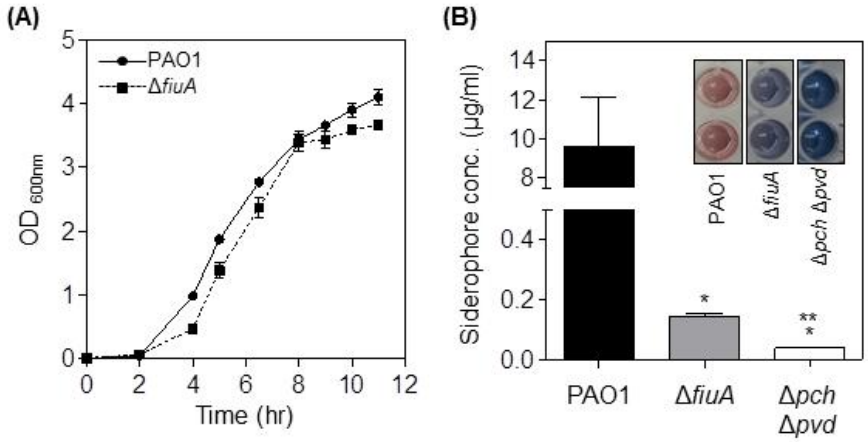

Figure 2.2. *fiuA* deletion effect for growth and siderophore activity of *P. aeruginosa*. (**A**) Growth rates of the PAO1 (solid line) and *fiuA* deletion mutant (*ΔfiuA*, dotted line) strains. Growth was measured by taking the optical density at 600 nm (OD_{600nm}). Three replicates were

performed; data are expressed as mean ± SD. (**B**) Siderophore assay of PAO1, Δ*fiuA*, and *ΔpchΔpvd* double mutant. The color of indicator reagent changes from blue to pink when siderophores exist in the samples. The number of replicates was three, and values of mean ± SD are displayed in each bar. *P< 0.001 vs. siderophore concentration of PAO1. **P< 0.05 vs. siderophore concentration *ΔpchΔpvd* double mutant.

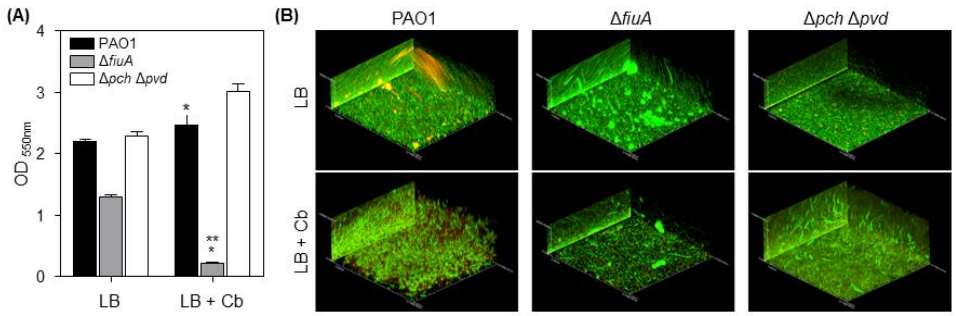

Figure 2.3. Biofilm development of various *P. aeruginosa* siderophore mutants under sub-MIC carbenicillin treatments. (**A**) Effects of sub-MIC carbenicillin treatments on biofilm development in PAO1 and siderophore mutant *P. aeruginosa* strains. Strains shown are PAO1 (black), *ΔfiuA* (gray), and *ΔpchΔpvd* (white). Six replicates were performed; values are expressed as means ± SDs. *P< 0.001 vs. LB-grown biofilm. **P< 0.001 vs. biofilm levels of PAO1 and *ΔpchΔpvd*

mutant in LB + 9 μg/ml carbenicillin. (**B**) Confocal laser scanning microscope images of the biofilm development of indicated *P. aeruginosa* strains with or without sub-MIC carbenicillin treatment. Bacteria were grown in LB (top) or LB + 9 μg/ml carbenicillin (bottom) for 24 hr in a coverglass bottom dish (SPL, Korea). Magnification, 400×.

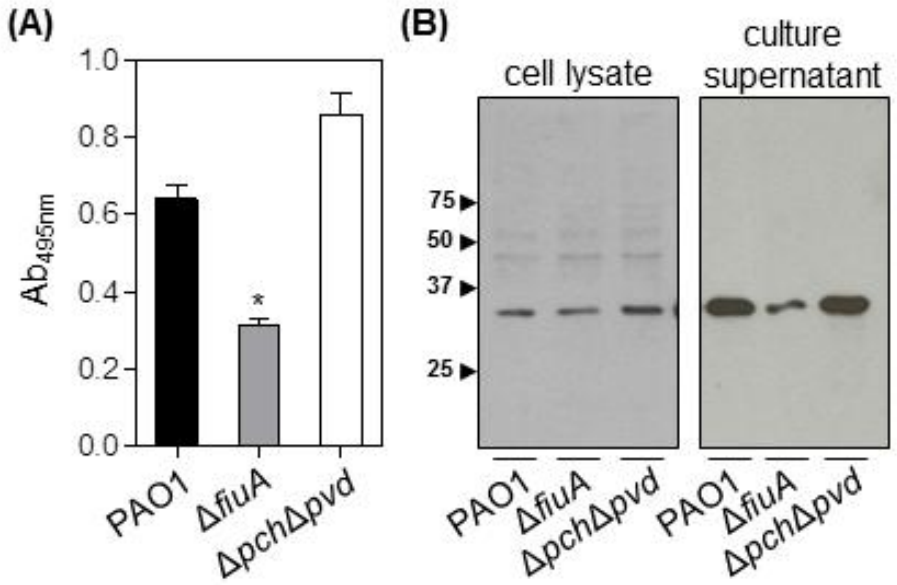

Figure 2.4. Decreased production of elastase by the *P. aeruginosa fiuA* deletion mutant. (**A**) Elastin Congo Red (ECR) assay of the elastase activities in the supernatants of PAO1 (black), *ΔfiuA* (gray), and *ΔpchΔpvd* (white) cultures. Three replicates were performed; values are expressed as means ± SDs. (**B**) Western blot analysis of the elastase

levels in the whole cell lysates and supernatants of PAO1, *ΔfiuA*, and *ΔpchΔpvd* strains.

3. *In vivo* virulence of the *ΔfiuA* mutant was attenuated

We next sought to understand how the deletion of *fiuA* gene affects the virulence of *P. aeruginosa in vivo*. For a preliminary study of *in vivo* virulence, *C. elegans* was used as a model for the multicellular eukaryotic organism. This experiment revealed that the *ΔfiuA*-fed *C. elegans* group has a higher survival rate compared with the PAO1-fed *C. elegans* group (Fig 2.5A). The average lifespans of the PAO1-fed and *ΔfiuA*-fed groups were 8.8 ± 0.8 days and 12.1 ± 0.8 days, respectively, indicating that *C. elegans* grown on *ΔfiuA* bacteria possessed 37.5% longer lifespan than those grown on PAO1 (Fig 2.5A).

Likewise, mice (n=5) infected with PAO1 or *ΔfiuA* cells (5.0×10^6 CFU) showed a contrasting survival rate. Two PAO1-infected mice perished at 22 hr post-infection, whereas all *ΔfiuA*-infected mice survived up to 24 hr (Fig. 2.5B). The survival assay was terminated at 24 hr post-infection, at which all surviving mice were euthanized for histological examination. H&E staining revealed more leukocyte infiltration in the lung tissues of PAO1-infected mice compared with those of *ΔfiuA*-infected mice (Fig. 2.5C). In addition to increased leukocyte infiltration, PAO1-infected mice also exhibited increased damage to the lung connective tissue (Fig. 2.5C). Furthermore, the

bacterial counts in lungs of the mice revealed the definite difference in the infectivity of PAO1 and *ΔfiuA*. The average bacterial load in the lungs of PAO1-infected mice (n=5) was 4.08×10^9 CFU per left lobe of lungs, whereas the average bacteria in the lungs of *ΔfiuA*-infected mice (n=5) was 1.56×10^6 CFU per left lobe of lungs (Fig 2.5D). The average bacterial load in the spleens of PAO1-infected mice (n=5) was 1.14×10^7 CFU per a spleen. Only one *ΔfiuA*-infected mouse presented bacterial count of 3.33×10^4 CFU in its spleen, but bacterial cells were not recovered from the spleen of the other four *ΔfiuA*-infected mice (Fig. 2.5D).

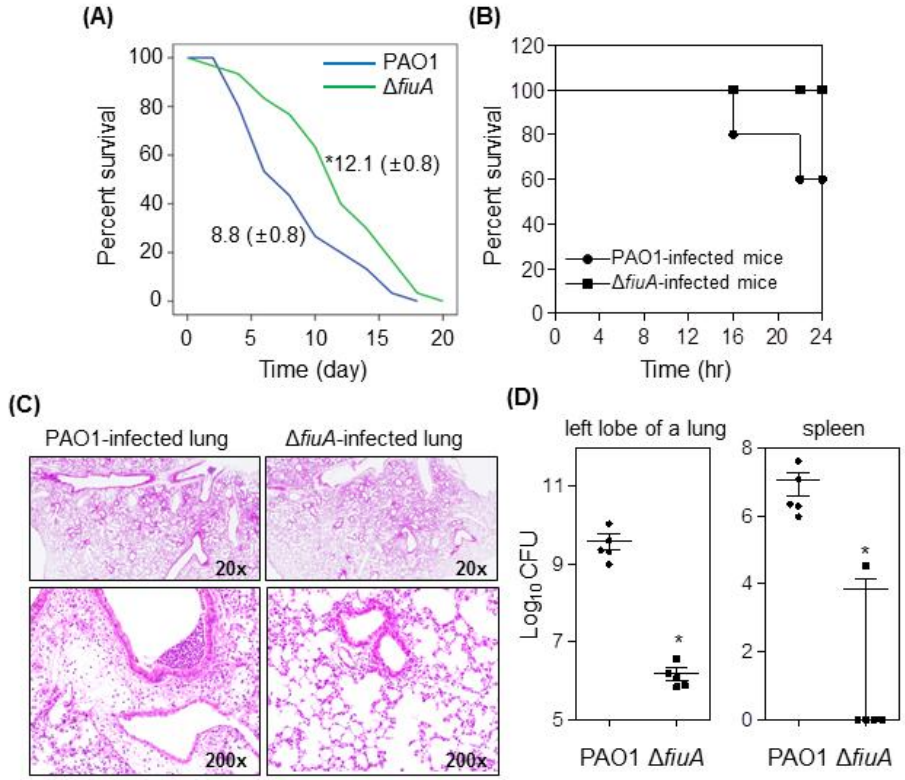

Figure 2.5. *In vivo* virulence analyses of the *fiuA* deletion mutant. (A) *C. elegans* survival curves of the PAO1-fed group (blue line) and the *ΔfiuA*-fed group (green line), and average lifespans of each group of *C. elegans*. *P< 0.01 vs. the survival rate of the PAO1-fed worms. (**B**) Mouse survival rate following infection with PAO1 (circle) or *ΔfiuA* mutant (square). (**C**) Histological analysis of H&E stained lung tissues of intranasally infected mice with PAO1 are shown at 20× and 200× magnifications (Infection dose: 5.0×10^6 CFU). (**D**) Bacterial counts recovered from left lobes of lungs and spleens. The infection dose was 5.0×10^6 cells per mouse of PAO1 or *ΔfiuA*. *P< 0.001 vs. the CFU of the PAO1-infected mice.

IV. DISCUSSION

A previous surveillance study revealed that antimicrobial usage and antimicrobial resistance are strongly correlated with each other.[116] Bacterial resistance to antimicrobial agents is often mediated by the formation of biofilm, a protective mode of bacterial growth.[102,103] In this study, we sought to identify further genetic determinants that are responsible for antibiotic-inducible biofilm formation. After performing a forward genetic screen and subsequent validation processes, we identified the *fiuA* gene, deletion of which resulted in (i) defective biofilm formation under sublethal antibiotic stress, (ii) reduced elastase secretion and (iii) decreased iron acquisition activity.

When PAO1 biofilm was treated with subinhibitory concentration of imipenem, another β-lactam antibiotic, expression of genes encoding AmpC β-lactamase and proteins involved in alginate biosynthesis was increased.[117] Since alginate production occurs in the periplasmic space [118] and β-lactamases, in their mature forms, are also localized in the periplasm, it is highly likely that subinhibitory β-lactam treatment induces changes in the periplasmic environment. FiuA, as an outer membrane protein, interacts with FiuR that is an inner membrane-bound periplasmic protein.[98] Therefore, these notions suggest that (i) functional

FiuA protein may participate in bacterial responses to the subinhibitory β-lactam treatment by orchestrating the periplasmic reprogramming and (ii) the lack of FiuA results in broader consequences that include defective biofilm formation and reduced elastase secretion. Consistent with this idea, the *arr* (aminoglycoside response regulator) gene, encoding a membrane-anchored enzyme, plays an essential role in aminoglycoside-inducible biofilm formation.[102] Collectively, bacterial responses that end up with enhanced biofilm formation and altered virulence are likely accompanied with modifications of cell surface properties in *P. aeruginosa*.

Elastase is a major virulence factor of *P. aeruginosa* that is highly important for the bacterial pathogenesis.[112] Elastin Congo Red (ECR) assays showed that pyochelin and pyoverdine production are not directly related with elastase production (Fig 2.4A). Western blot analysis confirmed the ECR results obtained with the supernatants and also revealed that similar levels of elastase were present in between the cell lysates of PAO1 and the mutant strains (Fig 2.4B). This result indicates that the production of elastase itself is not affected in the mutant strain, but the maturation of elastase may be affected by the deletion of *fiuA* gene. The *ΔpchΔpvd* displayed absolutely no decrease on neither elastase

activity nor elastase production therefore it indicated that iron is not related to the decrease of elastase activity and production in the *ΔfiuA* (Fig 2.4B). The decrease in elastase activity and production could be affected by the deletion of FiuA. Since FiuA is an outer membrane protein, the deletion of FiuA could affect the overall bacterial surface structure, and the alteration in bacterial surface structure could affect the periplasmic proteins, related to secretion process. This suggests a possible pleotropic function of FiuA as a structural scaffold.

The average lifespan of *C. elegans* under normal growth conditions with *E. coli* OP50 bacteria is approximately 10.5 days.[113] We found that *C. elegans* fed on *ΔfiuA* bacteria displayed an even longer lifespan than *C. elegans* fed on *E. coli* OP50 (Fig 2.5A). Moreover, the mice lung histology results confirm that the *ΔfiuA* strain has attenuated virulence, since the higher infiltration rate indicates that the bacterial strain is more virulent [119] (Fig. 2.5C). Four out of 5 *ΔfiuA*-infected mice did not present bacterial infections in the spleens (Fig 2.5D). It indicates that the intranasal infection of *ΔfiuA* is unlikely to cause systemic infections.

Cumulatively, the data presented in this study reveal that the *fiuA* gene, encoded for *P. aeruginosa* ferrichrome receptor A (FiuA), has important functions in addition to its siderophore receptor function.

Previous studies have proposed that *P. aeruginosa* virulence is related to iron acquisition, but most of these studies have focused on pyoverdine and pyochelin, or the ways in which iron transport mechanisms can contribute antibiotic efflux systems.[67,95,96,120-123]. These do not identify the precise role of ferrichrome itself. We identified a direct relationship between FiuA and *P. aeruginosa* virulence. Specifically, we found that FiuA is involved in biofilm enhancement under sub-MIC antibiotic treatment and also in elastase production. However, the exact mechanisms underlying these results are still under investigation.

While the murine airway infection model is a good representation of acute *P. aeruginosa* infection, this model may not accurately represent chronic airway infections, which are the major form of infections in patients with cystic fibrosis.[124] Further studies of the *fiuA* deletion effects in chronic infection models are necessary to fully understand FiuA involvement in *P. aeruginosa* virulence. Despite these limitations, our results clearly demonstrate that FiuA is involved in the production of various *P. aeruginosa* virulence factors.

V. CONCLUSION

In conclusion, this study identified new pleiotropic functions of *P. aeruginosa* ferrichrome receptor A (FiuA) in biofilm formation, elastase

activity, and *in vivo* infectivity. FiuA was found to be dispensable for *P. aeruginosa* growth. This is very important because the use of antibiotics that kill bacteria is known to induce the emergence of new antibiotic-resistant strains, which have the potential to threaten public health worldwide. Therefore, attenuating pathogen virulence instead of killing pathogens is an attractive strategy for novel therapeutics. By targeting FiuA, it may be possible to develop a drug that specifically controls the pathogen virulence.

Chapter III

Molecular determinants for matrix elasticity of the dual-species biofilm of *Pseudomonas aeruginosa* and *Enterococcus faecalis*

Molecular determinants for matrix elasticity of the dual-species biofilm of *Pseudomonas aeruginosa* and *Enterococcus faecalis*

Keehoon Lee

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Sang Sun Yoon)

I. INTRODUCTION

Biofilms are communities of microbes, including fungi, bacteria, and/or archaea that dwell on surfaces and are typically surrounded by extracellular matrices (ECMs).[125] The major characteristics of biofilms are high resistance to antibiotics and other various stresses, high rate of horizontal gene transfer (HGT), quorum sensing, and differential gene expression patterns relative to the planktonic state.[125] Even though it is a very popular concept in microbiology, there are still large areas to be uncovered about biofilms. Biofilms exist on most surfaces and cause serious problems in the medical field as infectious agents and reservoirs for many pathogens.[5,9] Chronic infections are, also, caused by bacteria in a biofilm mode of growth.[9] Due to biofilm characteristics, chronic biofilm infections are very difficult to eradicate.[126] There are two major

categories of biofilm infections. Biofilm infections that are associated with medically implanted devices.[77,127-130] There are also biofilm infections directly associated with tissues, such as chronic otitis media, dental plaque, endocarditis, lung infections in cystic fibrosis patients, urinary tract infections, and chronic wound infections.[84,85,126,131,132] The vast majority of biofilm infections contain more than one species of bacteria, fungus, or other microbes. [85]

Several studies have investigated polymicrobial biofilms that identified the beneficial and synergistic interactions between microbes in a biofilm. The interactions in a polymicrobial biofilm affect its overall function, physiology, or surroundings, which enhances resistance or virulence.[84-86] For example, many microbes exist in dental plaque and these microbes undergo spatiotemporal interactions where one bacterial species attaches to the tooth surface and alters the surroundings to fit the next bacterial species.[87] *Staphylococcus aureus* has shown to increase infectivity, biofilm development, and antibiotic resistance when grown with *Candida albicans* in serum.[86] Also, *Pseudomonas aeruginosa* presented enhanced virulence when incubated with gram-positive bacteria.[133]

Pseudomonas species are gram-negative bacilli and ubiquitous in the environment, some of which can cause disease in both animals and plants. Among the *Pseudomonas* species, *Pseudomonas aeruginosa* is one of common human opportunistic pathogens. It can cause serious infections. *P. aeruginosa* is also very common causative agents of healthcare-associated infection (HAI) and the second most common cause of ventilator-associated pneumonia (VAP)

in the USA. *P. aeruginosa* is known to produce various virulence factors, and expression of these virulence factors is regulated by complex signal transduction systems in response to changes in the surrounding environment, such as biofilm formation.[134]

Enterococci are gram-positive cocci and opportunistic pathogens that are frequently isolated in normal flora of the human gastrointestinal tract, oral cavity, and female genital tract. Enterococci have been reported to readily adhere to various medical devices and produce biofilms.[135] Among enterococcal species, *Enterococcus faecalis* is the most common nosocomial pathogen that typically causes urinary tract infections, peritonitis, bacteremia, infections in abscesses, decubiti and foot ulcers, and endocarditis, and is responsible for approximately 90% of enterococci-related HAIs.[135-137] Furthermore, *E. faecalis* causes serious problems in the clinical area because of its ability to resist various antibiotics.[138]

P. aeruginosa and *E. faecalis* share many similar characteristics and niches and have been found together in clinical samples from humans.[87,130,137] Even though several studies have investigated the synergism in polymicrobial biofilms, only a few studies about polymicrobial biofilms with *P. aeruginosa* and *E. faecalis* have been undertaken. In this research, synergisms of biofilm development were detected including an enhanced elasticity of the *P. aeruginosa* and *E. faecalis* dual-species biofilms. Since this synergistic effect of the dual-species biofilm could enhance the virulence, the molecular elements for the synergistic effects

were investigated. In order to investigate the dual-species biofilm, we constructed mutants with altered exopolysaccharide (EPS) productions and several fluorescence strains. We, also, described the characteristics of a dual-species *P. aeruginosa* and *E. faecalis* biofilm using various techniques. Based on the results, we could suggest the increased elasticity of the dual-species biofilm was due, in part, to the expression of Pel and Psl EPSs by *P. aeruginosa*. In conclusion, we suggest a simple model for dual-species *P. aeruginosa* and *E. faecalis* biofilms development and possible target molecules for more effective eradication of *P. aeruginosa* polymicrobial biofilm infections.

II. MATERIALS AND METHODS

1. Bacterial strains and growth conditions

P. aeruginosa and *E. faecalis* were used as the model bacteria for this polymicrobial biofilm study. *P. aeruginosa* PAO1 (wild type) was used as the basis for all modified *P. aeruginosa* strains used in this investigation. *E. faecalis* OG1RF harboring pMV158GFP (*EF*/green) and pAMβ1 was kindly provided by Prof. Manuel Espinosa Padron (Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain). *E. faecalis* 12448 (Korean Culture Collection of Microorganism (KCCM)) was used for non-fluorescent *E. faecalis* experiments. *P. aeruginosa* harboring pME plasmid vector with transcriptionally fused a dsRED gene after GFP promoter (pME-dsRED) was constructed in the lab, PAO1/red. The *E. faecalis* pMV158GFP possesses tetracycline resistance (Tet^R) marker, and *P. aeruginosa* pME-dsRED have gentamicin resistance (Gm^R) marker for their selection. The bacterial strains and plasmids are described in Table 1. *P. aeruginosa* strains lacking plasmid were grown in Luria Bertani (LB) media at 37°C. *P. aeruginosa* harboring pME-dsRED (PAO1/red) was grown on LB media with 100 μg/ml gentamicin for selection purposes. *E. faecalis* was grown on Brain Heart Infusion broth (BHIB) media and *E. faecalis*

pMV158GFP (*EF*/green) was grown on BHIB media with tetracycline (1 μg/ml) and erythromycin (1 μg/ml) to maintain plasmids

Table 3.1. Bacterial strains and plasmids used in this investigation

Strains and plasmids	Description	Source
Bacterial strains		
PAO1	Prototype *P. aeruginosa* laboratory strain	Lab collection
alg	PAO1 with in-frame deletion of *alg* operon (PA3540-PA3548)	Lab collection
pelA	PAO1 with in-frame deletion of PA3064 gene	Lab collection
psl	PAO1 with in-frame deletion of *psl* operon (PA2231-PA2242)	Lab collection
pelApsl	PAO1 with in-frame deletion of PA3064 gene and *psl* operon	Lab collection
PAO1/dsRED	*PAO1* harboring pME-dsRED	This study
alg/dsRED	*alg* harboring pME-dsRED	This study
pelA/dsRED	*pelA* harboring pME-dsRED	This study
psl/dsRED	*psl* harboring pME-dsRED	This study
pelApsl/dsRED	*pelApsl* harboring pME-dsRED	This study
E. faecalis 12448	*E. faecalis* Type strain (ATCC 19433)	Korean Culture Collection of Microorganisms (KCCM)
E. faecalis/pMV158GFP (*EF*/GFP)	*E. faecalis OG1RF* strain, harboring pMV158GFP and pAMβ1	[139]
Plasmids		
pME-dsRED	Transcriptional fusion of GFP promoter with a gene encoding DsRed, Gm^R	This study
pMV158GFP	pMV158, harbors the GFP gene under the control of the P_M inducible promoter, Tc^R	[139]
pAMβ1	auxiliary plasmid for pMV158GFP transport, Em^R	[139]

2. Biofilm preparation

Biofilms were prepared based on the method of George O'Toole.[109] Briefly, a static biofilm was prepared by inoculating sub-cultured bacterial strains (1:100) in BHIB. BHIB was used for both *P. aeruginosa* strain and *E. faecalis* strain biofilms since *E. faecalis* strains presented retarded growth in LB media where *P. aeruginosa* showed similar growth in both LB and BHIB media (data not shown). Biofilms for quantification and visual observation were prepared in 96-well plates (HM, Korea), biofilms for confocal microscopy were prepared in glass-bottom confocal dishes (SPL, Korea), and biofilms for viscosity measurement and extracellular matrix analysis were prepared in 500 ml cell culture flasks (SPL, Korea).

3. Biofilm analysis

The quantification of biofilms was executed by crystal violet (CV) biofilm assays.[109] Also, the colony forming units (CFUs) of biofilms were measured. The biofilms, grown in 96-well plates, were sonicated with three 10 sec pulses at 40 kHz (Branson 8510 ultrasonic cleaner) to disperse the biofilm-associated bacterial cells. Sonicated biofilm samples (100 μl) were harvested and serially diluted for CFU

measurements.

4. Elasticity and viscosity measurement

Naked-eye observations were performed to assess the elasticity of each biofilm. Biofilms were grown in 96-well plates for 48 hr and dumped by turning the plates upside down. The elasticity of biofilms and their ECMs were observed. The biofilms were grown in 500 ml cell culture flasks and harvested after 48 hr incubation at 37°C. The biofilms and ECMs were transferred to 50 ml conical tubes (HM, Korea) and viscosity was measured using a viscometer (Brookfield digital viscometer model DV-II) with the spindle #6 (RV/HA/HB Series). The viscosity measurement was performed three times for each biofilm sample and the average readings were recorded.

5. Confocal laser scanning microscopy (CLSM) image analysis of biofilms

Differential interference contrast (DIC) images were acquired using a confocal laser-scanning microscope (FV-1000; Olympus Optical Co. Ltd, Japan) equipped with FV10-ASW operating software (ver. 02.01). For CLSM image analysis, biofilms were grown in cover-glass

bottom dishes (SPL, Korea). After 24 hr of biofilm growth, the planktonic portion of the cultures was removed and the plates were washed with 0.9% saline. Bacterial strains harboring fluorescent plasmids were examined at 488 nm and 594 nm for the pMV158GFP and pME-dsRED plasmids, respectively. Styo60® (Life Technologies™) and TOTO-1(Life Technologies™) fluorescent dyes were used for the detection of intracellular DNA and extracellular DNA, respectively. Excitation wavelengths of 652 nm and 514 nm were used for Styo60® and TOTO-1, respectively. Calcofluor white dye was used for EPS detection at 358 nm. For three-dimensional (3D) image analysis, Z-stack images were obtained and 3D images were reconstructed using FV10-ASW software. The ImageJ program was used to analyze the fluorescent intensity of the Z-stack images.[140]

6. Scanning electron microscopy (SEM) of biofilms

The biofilm samples were grown on 12 mm round cover glass and fixed with Karnovsky fixing solution (2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$) for 6 hr. The fixed samples were washed with 0.1 M phosphate buffer for 2 hr and treated with 1% OsO_4 for 2 hr. Then, the biofilm samples were dehydrated in an ascending gradual

series (50 ~100%) of ethanol, infiltrated with isoamyl acetate, and dried (Critical Point Dryer, HCP-2, Hitachi, Japan). The dried biofilm samples were coated with gold via ion sputter at 6 mA for 6 min. The samples were observed using field-emission scanning electron microscopy (FE-SEM; S-800, Hitachi Ltd, Tokyo, Japan)

7. Alginate Assay

Bacterial samples were grown at 37 °C in appropriate media for 24 hr in both planktonic and biofilm modes of growth. The planktonic cultures were centrifuged to obtain supernatants. The supernatant of biofilm samples were carefully harvested by pipetting, and the remaining biofilms were scraped to harvest. The biofilm samples were sonicated and the supernatant harvested. The culture supernatants (20 μl) were mixed with 80 μl of distilled water and assayed for alginate quantification.[141] OD_{600nm} values of bacterial suspensions were used for normalization.

8. Antibiotics resistance of biofilms

The 24 hr mono-species and *P. aeruginosa* and *E. faecalis* dual-species biofilms were prepared. The supernatants were discarded

and fresh media with different concentrations of ciprofloxacin (0, 12.5, 25, and 100 μg/ml) and gentamicin (0, 250, 500, and 2,000 μg/ml) were added and incubated for an additional 24 hr. After antibiotic treatment, the biofilms were harvested and CFUs were determined as previously above.

9. Effect of *E. faecalis* culture derivatives on *P. aeruginosa* biofilm formation

The supernatants of *E. faecalis* were prepared in 5 ml of 16 hr old planktonic and static cultures (both approximately 3.0×10^9 cfu/ml) by centrifugation at 16,000 rpm. Each supernatant samples were supplemented with 25% of 5x BHIB in order to replenish nutrients. The pellets were treated with 2.5% glutaraldehyde to fix the bacterial cells without disrupting the outer membrane. PAO1 and PAEF biofilms were prepared as described previously. PAO1 biofilms with supernatant were prepared with using the *E. faecalis* supernatant samples instead of BHIB. PAO1 biofilms with cells were prepared with addition of the fixed *E. faecalis* cells (approximately 3.0×10^9 cfu/ml). The biofilms were quantified by CV biofilm assay.

10. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to analyze the significance of all comparisons. A *p*-value <0.05 was considered statistically significant. All experiments were repeated at least three times for reproducibility.

III. RESULTS

1. Co-culture and biofilm development of *Pseudomonas aeruginosa* and *Enterococcus faecalis*

The wild type *P. aeruginosa* PAO1 and *E. faecalis* were grown together in BHIB medium. Each bacterial species was differentiated via naked eye observation based on colony phenotypes (Fig. 3.1A). The planktonic growth experiments of individual bacterial samples and co-cultured samples were conducted to determine if one bacterial species affected the growth of the other bacterial species. The CFU/ml was used to plot the data in lieu of optical density due to interference from the excessive production of extracellular materials (Fig. 3.1B, 3.1C). Each bacterial species presented very similar growth patterns and generated the similar number of bacterial cells during growth (Fig. 3.1B). The co-cultured sample presented an almost identical growth pattern as the individually grown cultures (Fig. 3.1C). The results of the growth experiment indicate that *P. aeruginosa* and *E. faecalis* do not affect each other during planktonic growth. Dual-species biofilm development with *P. aeruginosa* and *E. faecalis* was conducted to determine if interactions occur between the two bacterial species during biofilm development. First, the amount of surface biofilm formation was measured using the

standard CV assay (Fig. 3.1D). The data indicate that the *P. aeruginosa* and *E. faecalis* dual-species biofilm showed enhanced biofilm formation compared to the mono-species biofilms (Fig. 3.1D). During biofilm development, a very interesting biofilm phenotype was observed. The biofilm exhibited a highly sticky phenotype when the two bacterial species were incubated together (Fig. 3.2). Thus, the viscosities of the biofilms were measured using a viscosity meter, revealing that the dual-species biofilm presented with significantly higher viscosity than the mono-species biofilms (Fig. 3.1E). Scanning electron microscope (SEM) images also revealed corresponding biofilm phenotypes (Fig. 3.1F). The SEM images of *P. aeruginosa* biofilms showed ECMs, encased and connected bacterial cells where the *E. faecalis* biofilm SEM images showed no ECM with barely any structures. The dual-species biofilm, however, revealed significantly higher amounts of ECM that connected cells of both the same and different species.

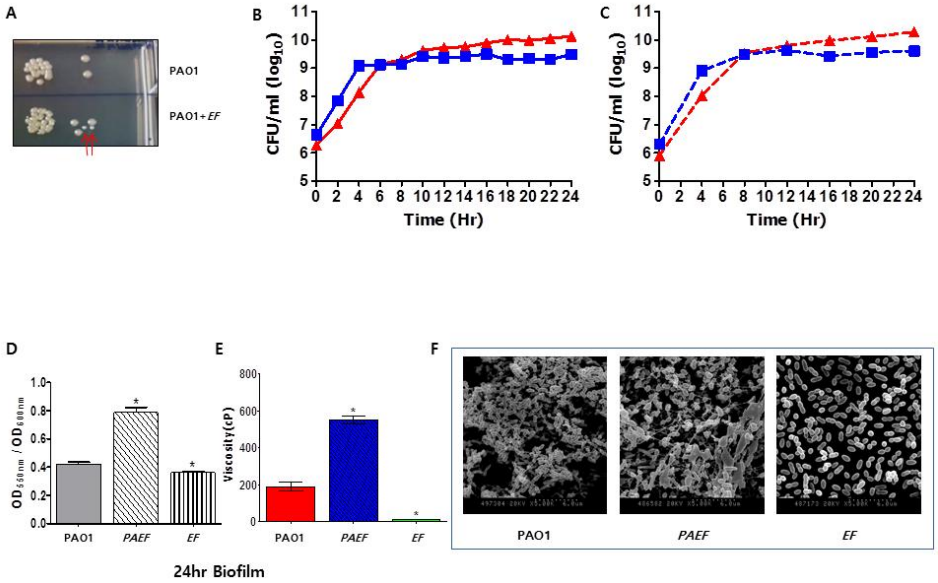

Figure 3.1. Polymicrobial biofilm tests of *P. aeruginosa* and *E. faecalis*. Colony observation of co-cultured *P. aeruginosa* and *E. faecalis* (A). The growth experiment of *P. aeruginosa* (red triangle) and *E. faecalis* (blue square), grown together (B) or individually (C) in a shaking incubator at 37°C. The viable count assay was used to determine the colony forming units (CFU)/ml. The CV biofilm assay of the mono-species and the dual-species biofilms (D). *, $p< 0.001$ versus the biofilm levels of PAO1 and PAEF, EF. Viscosity measurements of the mono- and dual-species biofilms (E). Viscosity appears in units of centipoise (cP). *, $p< 0.0001$ versus viscosity of PAO1 and PAEF, EF. Scanning electron microscope (SEM) images of the mono- and dual-species biofilms of *P. aeruginosa* and/or *E. faecalis* (F). The

magnifications are 5,000x.

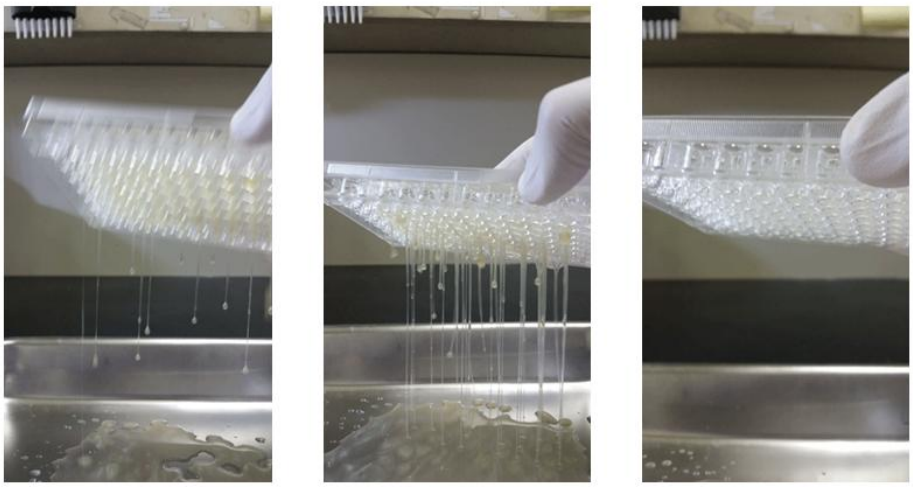

Figure 3.2. Representation of elasticity of Polymicrobial biofilms of *P. aeruginosa* and *E. faecalis*. The elasticity of biofilms was tested by flipping the biofilms upside down. The mono-species biofilms, wild type *P. aeruginosa* (PAO1) and *E. faecalis (EF)*, and the dual-species biofilm with the PAO1 and *E. faecalis* were tested.

2. Construction of fluorescent labeled *Pseudomonas aeruginosa* and *Enterococcus faecalis*

It is very important to distinguish bacterial strains in polymicrobial biofilm investigation to understand the accurate distribution of each species in the biofilm architecture. To distinguish *P. aeruginosa* and *E. faecalis* under confocal laser scanning microscope (CLSM), each bacterium was labeled with different fluorescence. Both bacterial strains harboring recombinant fluorescence plasmids were incubated in the appropriate media, BHIB + Gentamicin (5 μg/ml) + tetracycline (1 μg/ml), to prevent the loss of plasmids during the growth, which did not affect growth or biofilm formation of the bacteria (data not shown). Both mono-species bacterial cultures presented proper fluorescence, red for *P. aeruginosa* pME-dsRED (PAO1/red) and green for *E. faecalis* pMV158GFP (*EF*/green) (Fig. 3.3A, 3.3B). Both bacterial species were clearly distinguishable in the dual-species culture (Fig. 3.3C). Growth experiments were conducted to confirm the same growth pattern as with strains lacking fluorescence constructs. The results show that the fluorescent bacteria had identical growth patterns as the non-fluorescent bacteria (Fig. 3.4A-3.4D) and the biofilm development pattern was also identical (Fig. 3.4E).

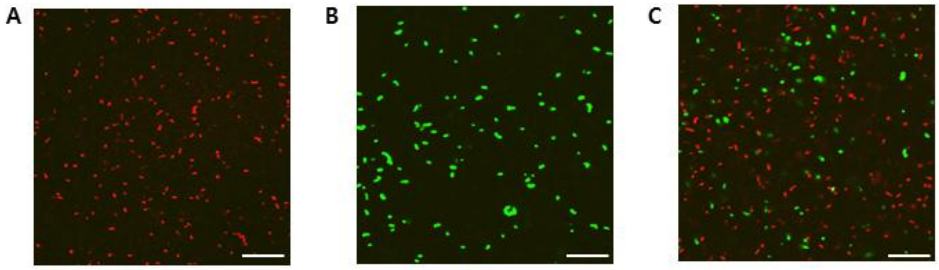

Figure 3.3. Fluorescence microscopy of PAO1/red and *EF*/green. Fluorescence microscopy images of (A) PAO1/red, (B) *EF*/green, and (C) PAO1/red and *EF*/green co-culture. The cultures were incubated at 37°C for 16 hr in Brain Heart Infusion Broth (BHIB) with 5 μg/ml gentamicin and 1 μg/ml tetracycline. The magnifications of the images were 1,000x. Bar size is 10 μm

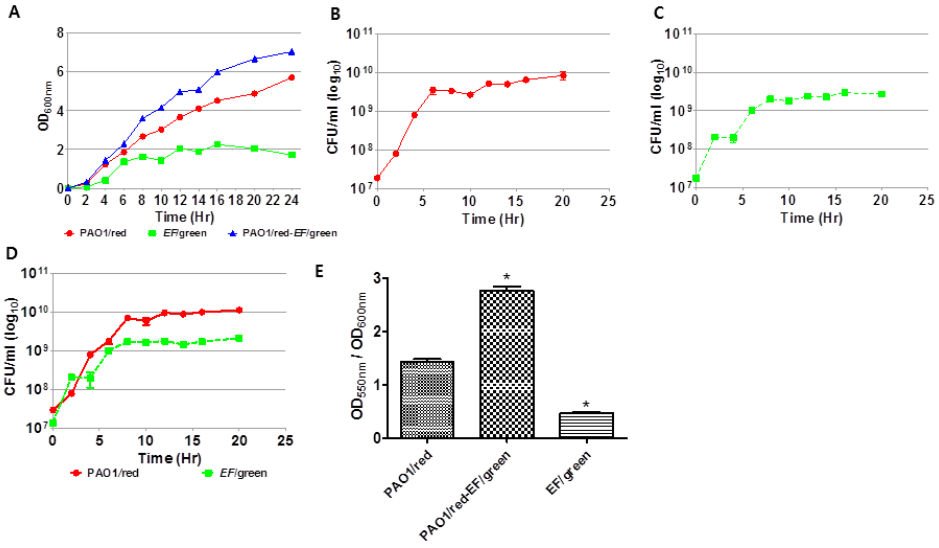

Figure 3.4. Polymicrobial Growth and biofilm development test of PAO1/red and *EF*/green. The growth experiments of PAO1/red (red circle), *EF*/green (green rectangle), and PAO1/red and *EF*/green co-culture (blue triangle). The optical density at 600nm was measured and plotted with 2 hr interval for 24 hr (A). The growth experiments of individually incubated PAO1/red (B) and *EF*/green(C), and co-cultured sample (D). The viable count assay was used in order to determine the colony forming unit (CFU)/ml. The crystal violet biofilm assay of the mono-species biofilms and the dual-species biofilm of PAO1/red and *EF*/green (E). *, P< 0.001 versus the biofilm levels of PAO1/red and PAO1/red- *EF*/green, *EF*/green.

3. Spatial composition of the dual-species biofilm

The biofilms of PAO1/red and *EF*/green were analyzed by CLSM and ImageJ program analysis. The Z-stack images of each biofilm were captured by CLSM, and the fluorescence intensities of each focal plane were analyzed using ImageJ (Fig. 3.5). The CLSM images show a flat PAO1/red biofilm with a height of 100 μm. Fluorescence intensity revealed that most of the biofilm was detected around 15 to 20 μm and gradually decreased (Fig. 3.5A). The *EF*/green biofilm also exhibited a flat biofilm but the height of the biofilm was around 20 to 30 μm, much thinner than the PAO1/red biofilm. In addition, the fluorescence intensity decreased more rapidly than in the PAO1/red biofilm (Fig. 3.5C). The dual-species biofilm exhibited a striking difference in the spatial distribution of each species along the height of the biofilm. The Z-stack images showed a flat *EF*/green biofilm at the bottom of the polymicrobial biofilm with a PAO1/red cluster-structured biofilm on top of the *EF*/green biofilm. The fluorescence intensity measurements showed an stronger *EF*/green fluorescence relative to PAO1/red fluorescence at or below the height of 30 μm, with the PAO1/red fluorescence intensity more dominant than the *EF*/green at planes higher than 30 μm (Fig. 3.5B).

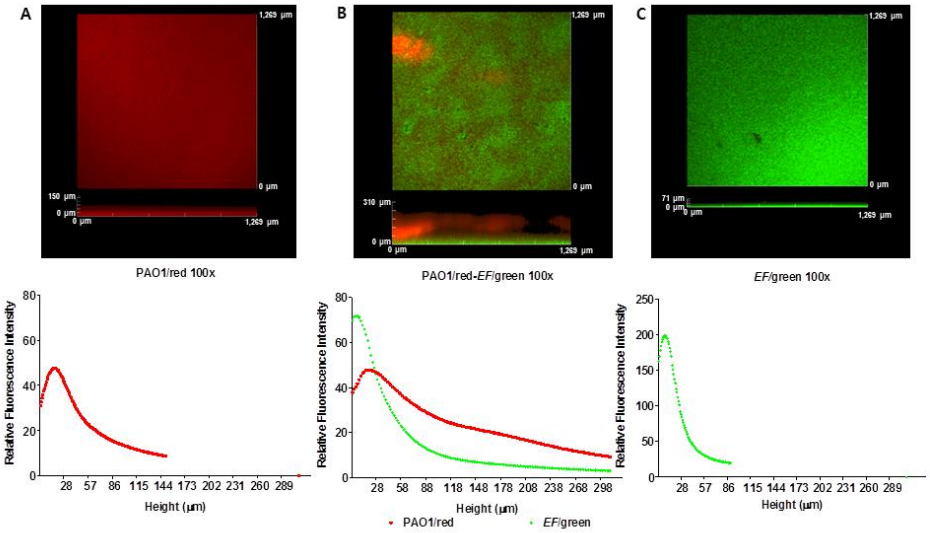

Figure 3.5. CLSM image analysis of mono- and dual-species PAO1/red and *EF*/green biofilms. Confocal laser scanning microscope (CLSM) images and their relative fluorescent intensity measures along the heights of biofilms of (A) PAO1/red, (B) PAO1/red and *EF*/green, and (C) *EF*/green. The biofilms were incubated at 37°C for 48 hr. The relative fluorescence intensities were measured using the ImageJ program.

4. Antibiotic resistances of the dual-species biofilm

The active antibiotic concentrations for each mono-species biofilm were determined (data not shown) and used to measure antibiotic resistance of the dual-species biofilm. Ciprofloxacin was used to inhibit PAO1/red since the pME-dsRED possesses gentamicin resistance, and gentamicin was used to inhibit *EF*/green. The PAO1/red biofilm showed considerably decreased biofilms at 12.5 μg/ml and 250 μg/ml ciprofloxacin and gentamicin, respectively, and the CFU of the mono-species biofilms gradually decreased as the antibiotic concentration increased. The PAO1/red biofilm was eradicated at 50 μg/ml ciprofloxacin and 1,000 μg/ml gentamicin (Fig. 3.6A). The *EF*/green biofilm showed high resistance against the antibiotics but still decreased as the antibiotic concentrations increased (Fig. 3.6B). The dual-species biofilm revealed increased antibiotic resistance of PAO1/red, which presented substantial growth at the 50 μg/ml ciprofloxacin and 1,000 μg/ml gentamicin concentrations (Fig. 3.6A). However, *EF*/green presented with a higher antibiotic susceptibility when it was grown in a dual-species biofilm than in the mono-species biofilm (Fig. 3.6B). This result could be due to micro-environmental changes in the dual-species biofilm, such as pH.

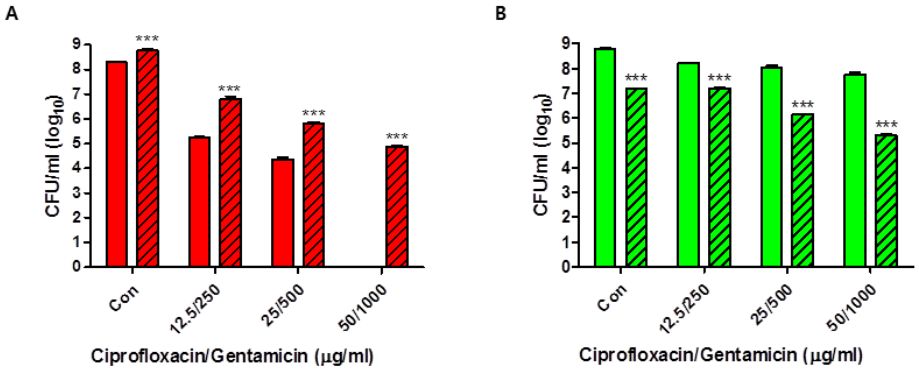

Figure 3.6. Antibiotic resistance of mono- and poly-microbial PAO1/red and *EF*/green biofilms. The antibiotic resistance experiments of (A) mono-species PAO1/red biofilm (red solid) and PAO1/red of the dual-species biofilm (red stripes) (A) and mono-species *EF*/green biofilm (green solid), and *EF*/green of the dual-species biofilm (green stripes) (B) against four different concentrations of ciprofloxacin and gentamicin. ***, $p < 0.0001$ versus the CFU/ml of PAO1/red or *EF*/green of the mono-species biofilms and PAO1/red or *EF*/green of the dual-species biofilms.

5. Effect of extracellular DNA on biofilm development

The dual-species *P. aeruginosa* and *E. faecalis* biofilm presented with higher elasticity than the mono-species biofilms. We directly demonstrated elasticity of the biofilms by dumping the biofilm out of the 96-well plates. The biofilms with higher elasticity presented with a very sticky and slimy phenotype but the biofilms with less elasticity did not show this phenotype (Fig. 3.7). In order to understand which component of the ECM contributes to the enhancement of elasticity in the dual-species biofilm, one of the major components of the ECM, extracellular DNA (eDNA), was examined. The DNA specific dyes Syto60® (red fluorescence) and TOTO-1 (green fluorescence) were used to detect the intracellular and extracellular DNA, respectively, which were observed with CLSM (Fig. 3.7). The *E. faecalis* biofilm presented with a flat biofilm with only red fluorescence, indicating no eDNA present in the biofilm (Fig. 3.7A). The *P. aeruginosa* biofilm image presented both red and green fluorescence, and the merged images revealed that eDNA surrounded the well-established biofilm structure with approximately 250 μm height (Fig. 3.7B). The dual-species biofilm presented with the bottom layer of the biofilm consisting of bacteria and several crevasses, and the crevasses were placed with a slightly thicker

layer (approximately 50 nm) of eDNA (Fig. 3.7C). As compared with the PAO1 mono-species biofilm, an excessive production of eDNA in the dual-species biofilm was not observed in these images while elasticity of the biofilm was dramatically increased. Also, Dnase I treatment showed that the eDNA does not have a critical effect on biofilm development (Fig. 3.8). Therefore, no correlation between elasticity enhancement and eDNA was found in the dual-species biofilm matrix.

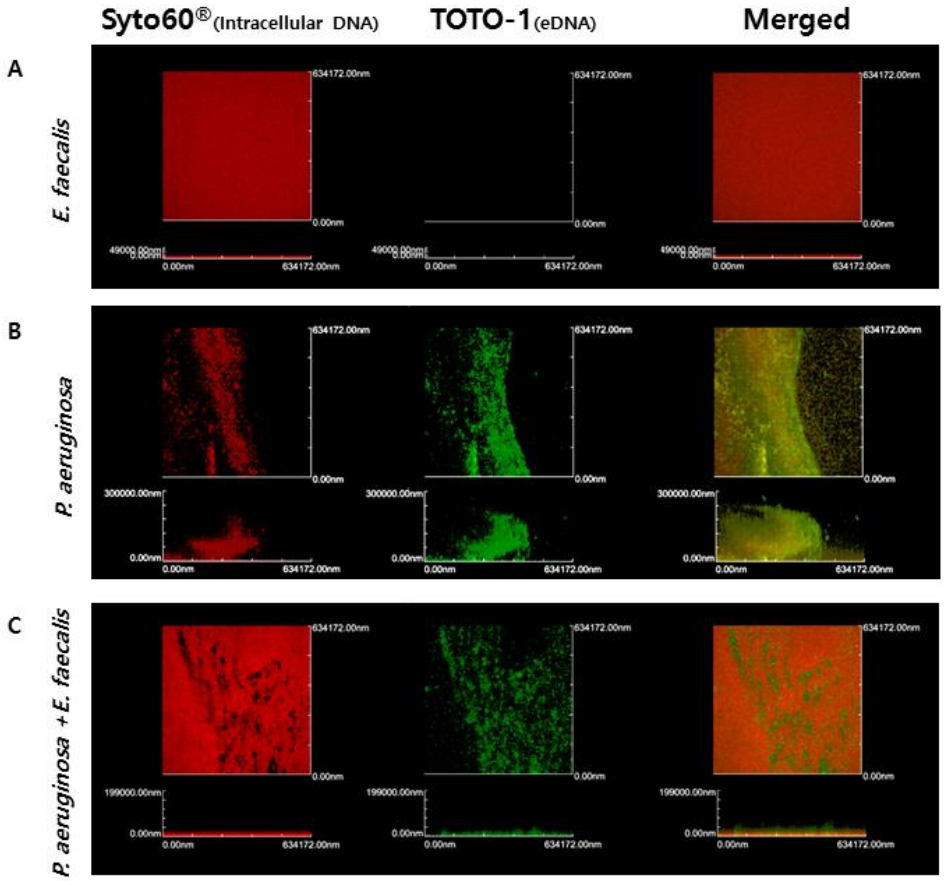

Figure 3.7. Extracellular DNA of mono- and dual-species biofilms of *P. aeruginosa* and *E. faecalis*. The mono-species biofilms of *E. faecalis* (A), and *P. aeruginosa* (B), and the dual-species biofilm of *E. faecalis* and *P. aeruginosa* (C) were stained for intracellular DNA (Styo60®) and extracellular DNA (TOTO-1). The 652 nm land 514 nm wavelengths were used for Styo60® and TOTO-1, respectively.

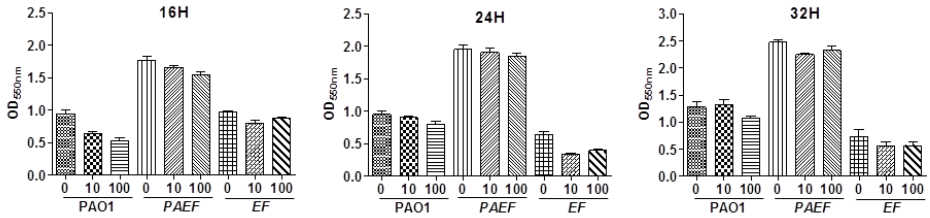

Figure 3.8. DnaseI treatment of mono- and dual-species biofilms of *P. aeruginosa* and *E. faecalis*. Two different concentrations of DnaseI (10 μg/ml and 100 μg/ml) were treated on the biofilms at different time points (16 hr, 24 hr and 32 hr).

6. Effects of *P. aeruginosa* EPSs on elasticity of the dual-species biofilm

Since eDNA did not have a critical effect on biofilm elasticity or development, genes encoding enzymes that produce EPSs were knocked out, and their mono and dual- species biofilm developments were observed. The alginate-negative PAO1, Δ*alg*, presented with less elastic biofilm formation than wild type PAO1, but the dual-species biofilm with *E. faecalis* presented a normal elastic phenotype (Table 3.2 and Fig. 3.9). The amount of alginate was measured in biofilms for *P. aeruginosa*, *E. faecalis*, and an alginate overproduction mutant of *P. aeruginosa*, Δ*mucA*. Alginate production of the dual-species biofilms and Δ*mucA* did not exhibit any increase in alginate production compared to the mono-species biofilms (Fig. 3.10), suggesting that alginate has no significant effect on elasticity increase in the dual-species biofilm. Other EPSs, Pel and Psl are also known to be important for biofilm development of *P. aeruginosa*.[51] The Pel-negative Δ*pelA* PAO1 biofilm showed a similar elastic phenotype relative to the wild type PAO1, and the dual species biofilm, Δ*pelAEF* presented with a very elastic phenotype. The Psl-negative Δ*psl* PAO1 strain showed no elasticity on its biofilm; however, the dual-species Δ*pslEF* biofilm presented with a recovered

elastic phenotype to normal level. The Pel and Psl double negative PAO1, Δ*pelA*Δ*psl*, presented with no elasticity in both the mono- and dual-species biofilms (Table 3.2 and Fig. 3.9). These results suggest that Pel and Psl are involved in biofilm elasticity and that Pel plays a more significant role in the dual-species biofilm elasticity.

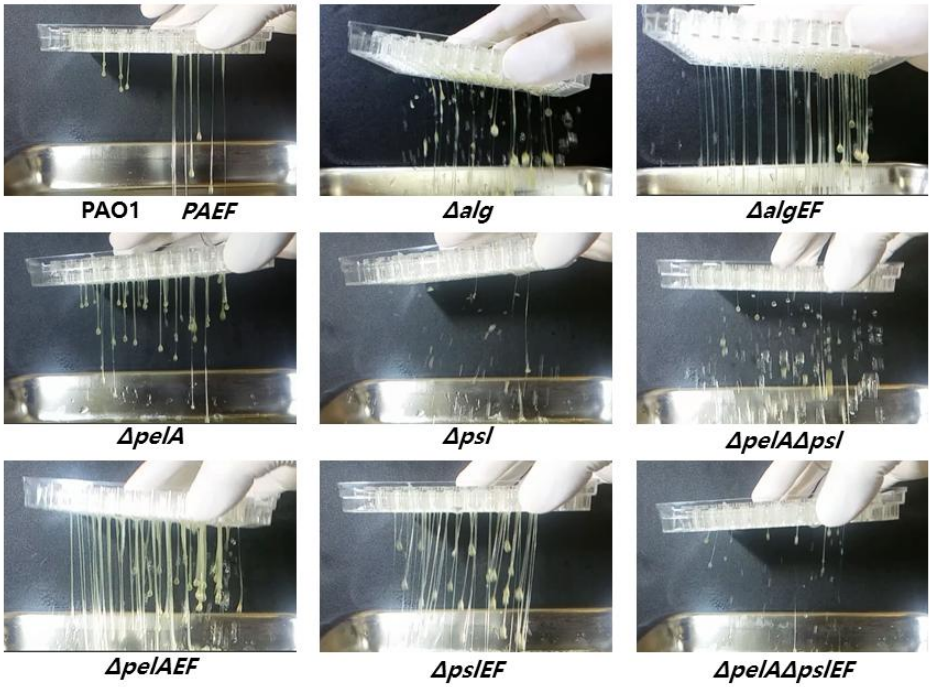

Figure 3.9. Representation of elasticities of biofilms with *P. aeruginosa* EPS mutants. The mono-species biofilms, wild type *P. aeruginosa* (PAO1), alginate negative *P. aeruginosa* (*Δalg*), *pelA* deleted *P. aeruginosa* (*ΔpelA*), *psl* operon deleted *P. aeruginosa* (*Δpsl*), and *pelA* and *psl* operon double negative *P. aeruginosa* (*ΔpelΔApsl*), and the dual-species biofilm with the previous *P. aeruginosa* strains and *E. faecalis* (*EF*) were tested.

Table 3.2. Elasticity experiments of the biofilms

Bacterial strains	Mono-species	Dual-species (with EF)	EPS from *P. aeruginosa*
PAO1	++	+++	Alg+ Pel+ Psl+
Δalg	+	+++	Alg- Pel+ Psl+
ΔpelA	++	+++++	Alg+ Pel- Psl+
Δpsl	-	+++	Alg+ Pel+ Psl-
ΔpelAΔpsl	-	-	Alg+ Pel- Psl-
EF	-	-	N/A

+ : Degree of elasticity (more + indicates higher elasticity), - : No elasticity

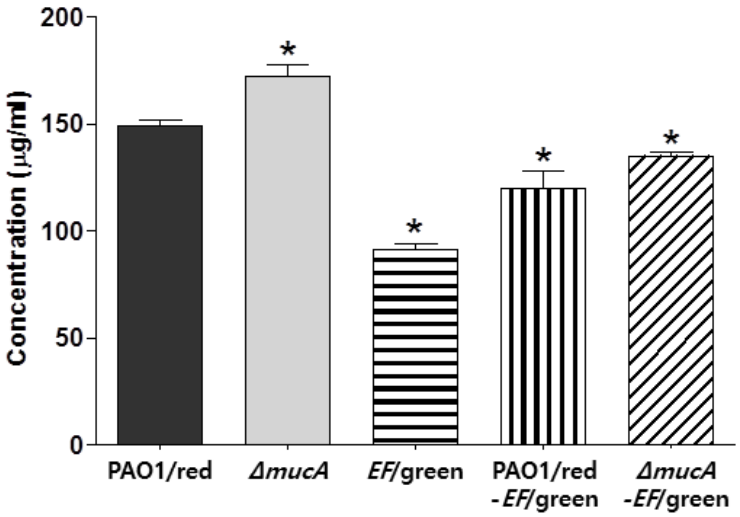

Figure 3.10. Alginate of the mono- and dual-species PAO1/red *and* *EF/*green biofilms. Amount of alginate in the planktonic cultures of PAO1/red, alginate overproducing *P. aeruginosa* (Δ*mucA*) and *EF/*green were measured by Alginate assay (A). *, $p< 0.01$ versus the biofilm levels of PAO1/red and Δ*mucA*, *EF/*green. The amount of alginate in supernatants and biofilms of the mono- and dual-species biofilms was measured (B). *, $p< 0.01$ versus the biofilm levels of PAO1/red and Δ*mucA*, *EF/*green, *PAEF,* Δ*mucAEF*.

7. The role of Pel and Psl on *P. aeruginosa* and *E. faecalis* dual-species biofilms

The Pel-negative PAO1/red, Δ*pelA*/red, presented with normal biofilm structures (Fig. 3.11A). The Psl-negative PAO1/red, Δ*psl*/red, showed flat biofilm development (Fig. 3.11B). However, the double negative mutant for both Pel and Psl, Δ*pelA*Δ*psl*/red, presented with a dramatic decrease in the ability to produce biofilms (Fig. 3.11C). These mutants were also co-cultured with *EF*/green to develop biofilms. The Δ*pelA*/red and *EF*/green biofilm, Δ*pelAEF*, presented with a completely structured Δ*pelA*/red biofilm and *EF*/green biofilms around the Δ*pelA*/red biofilm (Fig. 3.11D). The *Δpsl*/red with *EF*/green biofilm, Δ*pslEF*, showed biofilm formation with both bacteria mixed together quite evenly at the bottom layer of the biofilm with the addition of Δ*psl*/red on top of the bottom layer (Fig. 3.11E). The Δ*pelA*Δ*psl*/red and *EF*/green biofilm, Δ*pelA*Δ*pslEF*, presented with more Δ*pelA*Δ*psl*/red in the biofilm than in the mono-species biofilm (Fig. 3.11F). Interestingly, the Δ*pelA*Δ*psl*/red strain alone could not develop a biofilm but it seemed to be mixed evenly in the *EF*/green biofilm and formed an intact dual-species biofilm, even though the biofilm was still absent of elasticity.

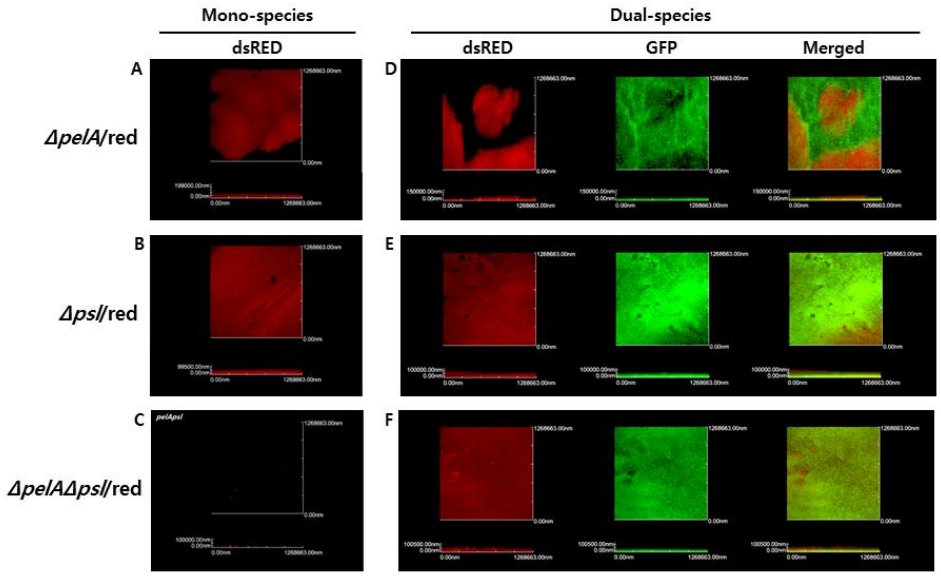

Figure 3.11. Biofilms of *ΔpelA*/red and/or *Δpsl*/red and/or *EF*/green.

The CLSM images of mono-species *ΔpelA*/red (A), *Δpsl*/red (B), *ΔpelAΔpsl*/red (C), the dual-species *EF*/green and *ΔpelA*/red (D), *Δpsl*/red (E), and P *ΔpelAΔpsl*/red (F).

8. Effect of *E. faecalis* culture derivatives on *P. aeruginosa* biofilm formation

24H biofilms of PAO1, PAO1 and *E. faecalis* (PAEF), PAO1 supplemented with supernatant of planktonic *E. faecalis* culture (PA-EF PL sup), PAO1 supplemented with supernatant of static *E. faecalis* culture (PA-EF ST sup), PAO1 supplemented with fixed cells of planktonic *E. faecalis* culture (PA-EF PL cell), and PAO1 supplemented

with fixed cells of static *E. faecalis* culture (PA-EF ST cell) were measured using CV biofilm assay. The results exhibited only PAEF biofilm is statistically different with PAO1 biofilm and the rest of the biofilms were statistically same with PAO1 biofilm (Fig 3.12).

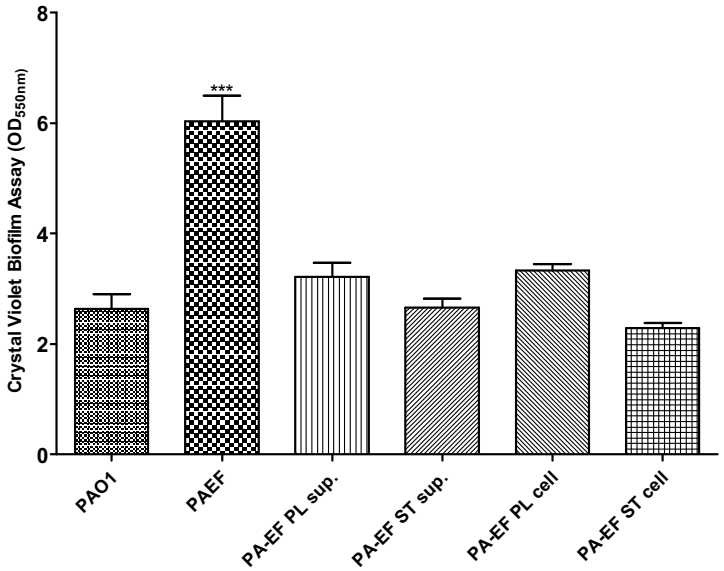

Figure 3.12. Effect of *E. faecalis* culture derivatives on *P. aeruginosa* biofilm formation. Crystal Violet biofilm assay results of 24H biofilms of PAO1, PAO1 and *E. faecalis* (PAEF), PAO1 supplemented with supernatant of planktonic *E. faecalis* culture (PA-EF PL sup), PAO1 supplemented with supernatant of static *E. faecalis* culture (PA-EF ST sup), PAO1 supplemented with fixed cells of planktonic *E. faecalis* culture (PA-EF PL cell), and PAO1 supplemented with fixed cells of static *E.*

faecalis culture (PA-EF ST cell). ***, P< 0.0001 versus the biofilm levels of PAO1.

IV. DISCUSSION

Since the concept of biofilms was introduced in the 1970's, scientists began to understand that microbes have two very different life styles, planktonic and biofilm, and each life style exhibits different phenotypes and gene expression. In the medical field, the previously known concept of infections, Koch's Postulates, could only elucidate acute infections not chronic infections.[9,132] The chronic infection could be explained after the identification of biofilms. The most chronic infections have been revealed to be associated with biofilm infections. Many investigators have uncovered characteristics and mechanisms of biofilm infections since the biofilm infection model was proposed by Costerton and Stewart.[9] However, the eradication of biofilm infections is still extremely difficult, and effective treatments other than surgical removal have not yet been found. One of the major reasons for the hardship of biofilm infection treatment is that the majority of biofilm infections do not consist of a single bacterial species.[85] Biofilms typically consist of multiple species of microbes, and the interactions between microbes in biofilms make these biofilm infections more virulent and hard to treat.[84,137,142] In this investigation, we focused on characterizing and

understanding a *P. aeruginosa* and *E. faecalis* dual-species biofilm, two of the major causative agents of chronic biofilm infections.[126,137,143]

P. aeruginosa and *E. faecalis* were chosen because they belong to two different bacterial groups, gram-negative and gram-positive, respectively, and they both cause biofilm infections in similar areas of human body such as urinary tract and wound infections.[129,131,138,143,144] Preliminary experiments investigating the co-culturability of *P. aeruginosa* and *E. faecalis* showed that each bacterial species was clearly distinguishable (Fig. 3.1A), and neither *P. aeruginosa* nor *E. faecalis* affects each other's growth in planktonic culture conditions (Fig. 3.1B, 3.1C). Interestingly, the CV biofilm assay showed enhanced biofilm formation (Fig. 3.1D), and biofilm viscosity was enhanced in the dual-species biofilm (Fig. 3.1E). In addition, elasticity of the dual-species *P. aeruginosa* and *E. faecalis* biofilm seemed to be much higher compared to the mono-species biofilms (Fig. 3.2). SEM images of the biofilms indicated that there was increased production in the ECM in the dual-species biofilm (Fig. 3.1F). According to these observations, these two bacterial species interact with each other and express synergistic effects on biofilm development.

To elucidate the bacterial interaction in the dual-species biofilm more directly, the PAO1/red and *EF*/green strains, which contain fluorescent

plasmids, were utilized (Fig. 3.3). The dual-species biofilm presented with a more structured PAO1/red biofilm than the mono-species biofilm on top of a flat *EF*/green biofilm layer (Fig. 3.5). This spatial distribution of the dual-species biofilm might be due to the ability to grow under a lack of oxygen. Even though both bacterial species are facultative anaerobes, *E. faecalis* is able to grow using fermentation pathways while *P. aeruginosa* cannot ferment and instead requires nitrogen oxides as an alternative electron acceptor for anaerobic growth.[26,135] Therefore, it could be more efficient for *P. aeruginosa* to locate on top of *E. faecalis* in the dual-species biofilm where there is more oxygen available. The dual-species biofilms were prepared with a 24 hr interval between inoculation times of each bacterium. Regardless of which bacterial species was inoculated first, the biofilms presented same spatial distribution (data not shown).

Antibiotic resistance is much higher in biofilms than in the planktonic counterpart. Several groups have investigated antibiotic resistance in polymicrobial biofilms,[84,145-148] and most of these investigations mentioned increased antibiotic resistance in polymicrobial biofilms. Thus, we conducted antibiotic resistance tests to confirm whether the *P. aeruginosa* and *E. faecalis* biofilms presented with increased antibiotic

resistance. Ciprofloxacin and gentamicin were used because the *P. aeruginosa* biofilm showed reasonable susceptibility against ciprofloxacin and not against gentamicin and *E. faecalis* biofilms presented with the opposite antibiotic susceptibility profile. In this experiment, antibiotic resistance of *P. aeruginosa* was increased but *E. faecalis* presented with a slightly decreased antibiotic resistance (Fig. 3.6). The results indicate that antibiotic resistance is not always increased in polymicrobial biofilms. It was postulated that increased antibiotic resistance of *P. aeruginosa* was due to increased production of the ECM, which probably acts as an extra barrier, and the decreased antibiotic resistance of *E. faecalis* was possibly due to increased metabolism. The optimal growth pH for *E. faecalis* is pH 7.5; however, the pH tends to drop when *E. faecalis* is grown under limited oxygen by switching to fermentation in the biofilm.[135] In a preliminary study, *P. aeruginosa* was shown to elevate the pH to neutral during the co-culturing process, which provides a better pH environment for *E. faecalis* to grow (data not shown). The increased metabolism could contribute to decreased antibiotic resistance. Another reason for the decrease in antibiotic resistance of the *EF*/green biofilm in dual-species biofilms could be the Type VI secretion system (T6SS) of *P. aeruginosa*. The T6SS can

damage the peptidoglycan (PG) layer of the contact *E. faecalis* by secreting the lytic enzymes Tse1 and Tse3,[149] and the PG-damaged *E. faecalis* could be more vulnerable to the antibiotics. Further investigation is necessary to elucidate the precise reason for the increased antibiotic susceptibility *E. faecalis* in the dual-species biofilm with *P. aeruginosa*.

One of the major components of the ECM is eDNA, which is known to play a role in the adhesion of bacterial cells on surfaces with repulsive electronic charge and structure stability.[150,151] Thus, we observed eDNA in the mono- and dual-species *P. aeruginosa* and *E. faecalis* biofilms. The intracellular DNA was stained with Syto60 (red) to observe the distribution of bacterial cells in the biofilms and eDNA was stained with TOTO-1 (green). The molecular size of TOTO-1 is too large to penetrate the bacterial membrane; therefore, it can only stain the DNA outside of bacterial cells. The *E. faecalis* biofilm presented with only red fluorescence, which indicates no eDNA is present in the *E. faecalis* biofilm (Fig. 3.7A). On the other hand, the *P. aeruginosa* biofilm was covered with a substantial amount of eDNA (Fig. 3.7B). However, the production and distribution of eDNA in the dual-species biofilm was not exactly what was expected. First, the amount of eDNA was not increased in the dual-species biofilm compared to the *P. aeruginosa* biofilm (Fig.

3.7C), and the distribution of eDNA in the dual-species biofilm was mainly present in biofilm crevasses, which may indicate that the crevasses of the biofilm were because of *P. aeruginosa,* and the eDNA was present due to the *P. aeruginosa*. To confirm the role of eDNA in biofilm structure, the biofilms were treated with two different concentrations of Dnase I at different time points. Regardless of when and how much Dnase I was used, it did not significantly affect the dual-species biofilm (Fig. 3.8). According to these results, it is presumed that eDNA is not the essential component of the ECM in dual-species biofilm formation.

Another major component of the ECM is the EPS. The *P. aeruginosa* mutants defective in the production of each known EPS, alginate, Pel, and Psl, were constructed, and the elasticity of the biofilms with these mutants and *E. faecalis* were determined (Table 3.2, and Fig. 3.9). In biofilms of each *P. aeruginosa* EPS gene knock out, alginate and Psl seemed to be necessary to obtain the elasticity of the *P. aeruginosa* biofilm; however, the Δ*pelA*Δ*psl* biofilm showed that alginate alone did not express the sticky phenotype of *P. aeruginosa* biofilms (Table 3.2). The Δ*pelA*Δ*pslEF* biofilm showed that alginate is not involved in interspecies interaction with *E. faecalis* for the enhancement of elasticity

(Table 3.2). The Δ*algEF* biofilm result indicated that both Pel and Psl are necessary for the elasticity increase, and the Δ*pelAEF* and Δ*pslEF* biofilms showed that Psl is more associated with elasticity of mono-species biofilm and bacteria-surface adhesion, and Pel is more related to interspecies interactions since the elasticity of Δ*pslEF* biofilm was recovered to normal level (Table 3.2). Even though alginate did not seem to be directly involved in elasticity enhancement, alginate is known to be an important factor for virulence and antibiotic resistance in *P. aeruginosa* biofilms.[54,106] Therefore, we determined alginate production in the mono- and dual-species biofilms via the alginate assay. The results showed no enhancement of alginate in the dual-species biofilm (Fig. 3.10).

Other polysaccharides that are known to have important roles in *P. aeruginosa* biofilms are Pel and Psl.[26,51,152] The CLSM images of Δ*pelA*/red and Δ*psl*/red with *EF*/green biofilms indicated that Psl is more involved in the interaction between *P. aeruginosa* cells to form a structured biofilm and Pel has a greater role in interspecies interaction (Fig. 3.11). The data also confirm that *P. aeruginosa* loses the ability to form biofilm when both Pel and Psl were missing (Fig. 3.11C). However, the Δ*pelA*Δ*pslEF* biofilm showed that Δ*pelA*Δ*psl*/red exists within the

*EF/*green biofilm, which indicates that *E. faecalis* has the ability to weakly interact with *P. aeruginosa* (Fig. 3.11F). However, Δ*pelA*Δ*pslEF* biofilm did not presented any elasticity which indicates elasticity of the dual-species biofilm is solely due to the Pel and Psl (Table 3.2). The exopolysaccharide distribution of the biofilms in the Δ*pelA/*red*,* Δ*psl/*red, and Δ*pelA*Δ*psl/*red mutants and *EF/*green were observed by CLSM using Calcofluor white fluorescent dye (Fig. 3.13) and showed similar patterns of biofilm formation; however, it was difficult to distinguish between Pel, Psl, and alginate in these biofilms. Different approaches are needed to precisely distinguish the distribution of each EPS.

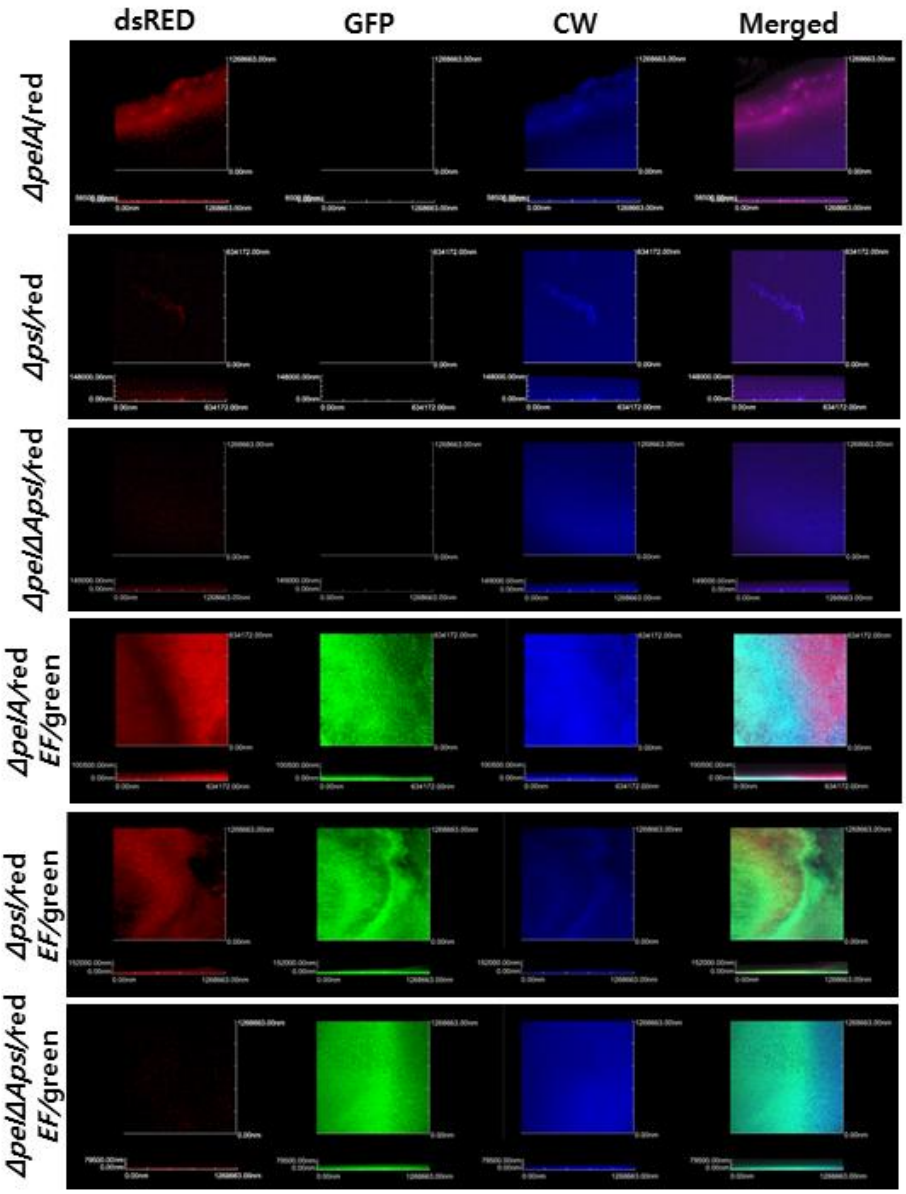

Figure 3.13. The detection of exopolysaccharides (EPS) of the mono- and dual-species biofilms. The mono-species biofilms, *ΔpelA/red*,

Δpsl/red), and *ΔpelΔApsl/red*, and the dual-species biofilm with the previous *P. aeruginosa* strains and *E. faecalis* pMV158GFP (*EF*) were tested. The biofilms were stained with Calcofluor White (CW) for exopolysaccharide detection and observed under CLSM.

V. CONCLUSION

P. aeruginosa and *E. faecalis* can form a dual-species biofilm and have a distinct spatial distribution within the biofilm. *E. faecalis* tends to locate at the bottom of the biofilm and *P. aeruginosa* forms a more structured biofilm on top of the *E. faecalis* biofilm. However, the species were not completely separated in that the biofilms do contain cells of the other species when observed under higher magnifications (data not shown). The dual-species biofilm presents with a much stickier phenotype than the mono-species counterparts, which may contribute to the increase in virulence of polymicrobial biofilm infections. This phenotype is mainly due to the components of the ECM, especially Pel and Psl rather than eDNA and alginate (Fig. 3.14). The results of biofilm formation experiments with supernatants or fixed-cells of *E. faecalis* under planktonic or static culture conditions indicate that the cell-to-cell contact with live *E. faecalis* is essential for expression of the highly viscous phenotype of the dual-species biofilm since none of the biofilms presented increased biofilm formation (Fig. 3.12). This indicates there should be a receptor for *P. aeruginosa* recognition on the *E. faecalis* membrane, and *E. faecalis* may produce signals that trigger Pel and Psl production of *P. aeruginosa*. The identification of the receptor or the

signaling material will contribute to the development of a method to eradicate polymicrobial biofilms with *P. aeruginosa*. Also, targeting the Pel and Psl of *P. aeruginosa* could help eradicate polymicrobial infections more effectively.

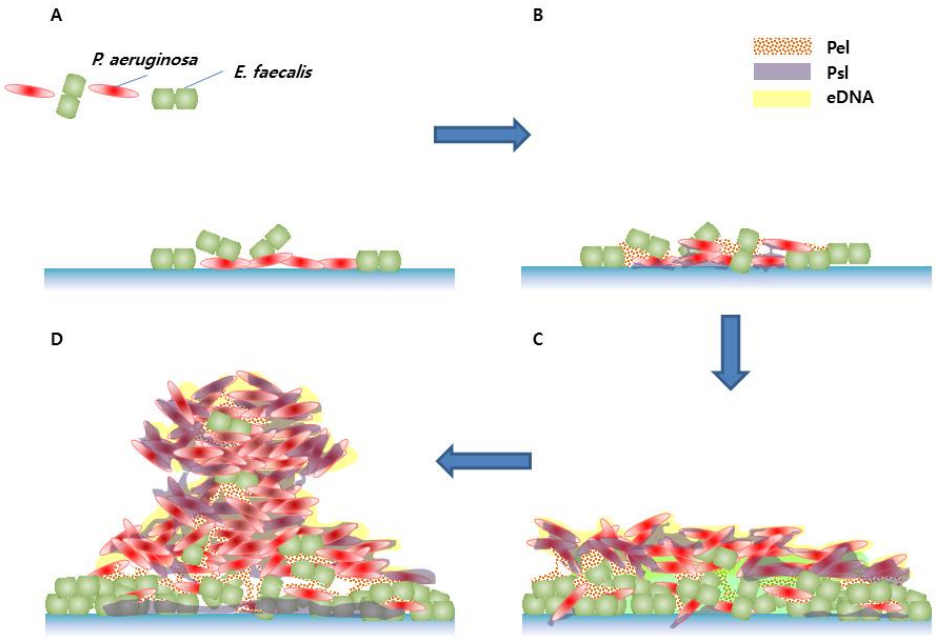

Figure 3.14. Scheme of the dual-species *P. aeruginosa* and *E. faecalis* biofilm development. *P. aeruginosa* and *E. faecalis* attach to a surface without spatial separation (A). During the early stages of dual species biofilm formation, the attached cells proliferate and start producing EPS, Pel (red dots), Psl (purple), and eDNA (yellow) (B). The *P. aeruginosa* and *E. faecalis* start to have distinct spatial distribution (C). In the maturation stage of the dual-species biofilm, *P. aeruginosa* forms a

structured biofilm on top of the *E. faecalis* biofilm, and Psl is associated with the surface and between *P. aeruginosa*, Pel is associated in interspecies interaction between *P. aeruginosa* and *E. faecalis* (D).

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<ABSTRACT(IN KOREAN)>

녹농균(*Pseudomonas aeruginosa*)의 단일종과 복수종의 세균막 형성에 영향을 주는 유전학적 및 분자생물학적 요인

<지도교수 윤상선>

연세대학교 대학원 의과학과

이기훈

녹농균는 기회감염균으로서 다양한 환경에 대한 높은 적응력과 여러 항생제에 대한 저항성으로 인해 인류의 건강에 큰 위협으로 급부상하고 있다. 이러한 균의 특성들은 이 균이 원내감염을 일으키는 주요 원인이 되는데 큰 역할을 하였다. 또한 이 균은 바이오필름을 매우 잘 형성하는 균으로 알려져 있다. 바이오필름은 미생물들의 공동체 집단으로서 다양한 물질의 표면에 존재하며 일반적으로 세포외기질 등에 의해 둘러싸여 있다.

Chapter I 은 바이오필름에 대한 간단한 역사와 녹농균과 녹농균의 바이오필름에 대한 기본 정보, 바이오필름 감염과 multi-species 바이오필름에 대한 literature review로 구성되

어 있다.

Chapter II에서는 녹농균 바이오필름의 형성이 최소생장억제농도 이하의 항생제 처리 시, 오히려 더 증가하는 현상의 원인이 되는 유전자를 찾기 위해 연구가 시작되었다. 녹농균의 protoptye인 PAO1균주의 transposon (Tn) mutant library의 돌연변이를 약 5,000개 정도 검사한 결과, *fiuA* 유전자에 transposon이 삽입된 균주에서 carbenecillin이 최소생장억제농도 이하로 처리되었을 경우, 바이오필름의 형성이 결여됨이 확인되었다. *fiuA* 유전자는 녹농균의 철 이온 획득에 관여하는 ferrichrome receptor A를 코딩하는 유전자이다. 흥미롭게도 녹농균의 잘 알려진 siderophore인 pyochelin과 pyoverdine의 생산이 결여된 *ΔpchΔpvd* 돌연변이에서는 바이오필름 형성이 감소하지 않았다. 그리고 *fiuA* 의 non-polar deletion 돌연변이인 *ΔfiuA* 는 녹농균의 주요 병독인자인 elastase의 생산이 급격히 감소되어 있었고 마우스의 호흡기 감염 결과, 이 돌연변이는 매우 낮은 병원성을 지니고 있음이 확인되었다.

Chapter III에서는 녹농균의 polymicrobial바이오필름에서의 병원성 인자를 연구했다. 바이오필름 감염 모델이 의학계에 소개

된 후, 바이오필름이 대부분의 만성감염의 주요 원인이 된다는 사실이 밝혀졌다. 하지만 여전히 만성 바이오필름 감염의 치료는 환부의 수술적인 제거만이 효과적인 치료방법으로 사용되고 있으며 대부분의 만성감염은 polymicrobial infection으로 알려져 있다. *Pseudomonas aeruginosa*와 *Enterococcus faecalis*는 바이오필름 감염에서 높은 빈도로 동정되는 균들이지만 이 두 박테리아의 상관관계에 대한 연구는 매우 부족한 실정이다.

이 연구에서 우리는 *P. aeruginosa*와 *E. faecalis*의 dual-species 바이오필름에서 급격한 점성(elasticity)의 증가와 각 균의 뚜렷한 공간적인 분포를 관찰했으면 이러한 표현형질들이 *P. aeruginosa*의 exopolysaccharide (EPS), 특히 Pel과 Psl에 관련되어 있음을 확인했다.

종합하면 이번 연구 결과들로 fiuA 유전자가 녹농균의 철 이온의 획득 외에 바이오필름의 형성과 병독성에 영향을 주는 다면발현성 유전자임이 밝혀졌으며 FiuA를 타겟함으로써 녹농균의 병독성을 약화시킬 수 있으며 이를 토대로 녹농균의 병독성을 특이적으로 조절하는 약물의 개발할 수 있다. 또한 이번 연구에서 Psl이 박테리아의 표면부착과 동일 녹농균 사이의 상호작용

에 관련하여 바이오필름의 구조를 형성하는데 관여하고 Pel은 polymicrobial 바이오필름에서 다른 종의 박테리아와의 상호작용 및 부착에 더 관여하고 있다는 것이 확인되었다. 따라서 이 두 가지의 EPS 가 polymicrobial 바이오필름 감염의 제거를 더 용이하게 하는 후보목표가 될 수 있는 가능성이 이번 연구를 통해 제시되었다.

핵심되는 말: ***Pseudomonas aeruginosa*, 녹농균, 철 이온, siderophore, ferrichrome receptor A, 병독성, *Enterococcus faecalis*, polymicrobial 바이오필름, 점성(elasticity), Pel, Psl**

PUBLICATION LISTS

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In Preparation

Lee K, Lee KM, Yoon SS. **Molecular determinants for matrix elasticity of the dual-species biofilm of *Pseudomonas aeruginosa* and *Enterococcus faecalis***