Mechanical and functional characterization of extracellular polymeric substances of bacterial biofilms

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**MECHANICAL AND FUNCTIONAL
CHARACTERIZATION OF EXTRACELLULAR
POLYMERIC SUBSTANCES OF BACTERIAL
BIOFILMS**

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**Interdisciplinary Graduate School
Singapore Centre on Environmental Life Sciences
Engineering
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Doctor of Philosophy

2016

Dedicated to my excellent parents, brother, husband and children.

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Abbreviations

3OC ₁₂ -HSL	N-(3-oxododecanoyl)-L-homoserine lactone
AFM	Atomic force microscopy
agr	Accessory gene regulator
AHL	Acyl homoserine lactone
Bap	Biofilm-associated surface protein
c-di-AMP	Cyclic di-adenosine monophosphate
c-di-GMP	Cyclic di-guanylate monophosphate
C ₄ -HSL	N-butyryl-L-homoserine lactone
CDC	Center for Disease Control and Prevention
CSLM	Confocal scanning laser microscopy
DGC	Diguanylate cyclase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
eDNA	extracellular Deoxyribonucleic acid
EPS	Extracellular polymeric substances
gfp	Green fluorescent protein
LB	Luria bertani
MEMS	Micro-electromechanical systems
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
OCT	Optical coherence tomography
OD	Optical density

PDE	Phosphodiesterase
PDMS	Polydimethylsiloxane
PGSE	Pulsed gradient spin echo
PI	Propidium iodide
PIA	Polysaccharide intercellular adhesion
PNAG	Poly- β (1-6)- <i>N</i> -acetylglucosamine (PNAG)
PQS	Pseudomonas quinolone signal
PSM	Phenol-soluble modulins
rfp	Red fluorescent protein
SasG	<i>S. aureus</i> surface protein G
SCV	Small colony variant
TSB	Tryptic soy broth
UV	Ultraviolet

Parameters and units

D	Diffusion coefficient ($\mu\text{m}^2\text{s}^{-1}$)
d	Day
d	Diameter (μm)
E	Young's modulus (Pa or MPa)
f	Force (nN)
γ	Shear strain
G^*	Viscoelastic moduli (Pa)

G'	Storage modulus (Pa)
G''	Loss modulus (Pa)
H	Height (μm)
h	Hour
J	Creep compliance (Pa^{-1})
K	Spring constant ($\text{nN}\mu\text{m}^{-1}$)
k_B	Boltzman constant (JK^{-1})
MSD	Mean squared displacement (μm^2)
min	Minute
ω	Frequency (Hz)
r	Deflection vector (μm)
s	Second
τ	Shear stress (Pa)
τ'	In-phase component of stress (Pa)
τ''	Out-of-phase component of stress (Pa)
t	Time (s)
T	Temperature (K or $^{\circ}\text{C}$)
x	x coordinate (μm)
y	y coordinate (μm)
α	Diffusive exponent

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- Chew SC, Rice SA, Doyle P, Kjelleberg S, Yang L.** 2015. *In situ* Mapping of the Mechanical Properties of Biofilms by Particle-tracking Microrheology. *J Vis Exp* **106**:e53093.
- Chew SC, Teh WK, Kundukad B, Doyle P, Yang L, Rice SA, Doyle P, Kjelleberg S.** 2016. Mechanical signatures of microbial biofilms in micropillar-embedded growth chambers. *Soft Matter* **12**:5224-5232.

List of Talks

Pseudomonas ASM Conference, Sept 8-12 2015, Washington DC.

Asia Women Eco-Science Forum hosted by Amorepacific Corporation, Nov 12-14 2013, Seoul.

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Abstract

Microbial cells can express polymers extracellularly and these polymers are responsible for the assembly of the cells into biofilms. The polymer matrix serves as a physical scaffold that in part serves as the interface between the cells and their environment. One function assigned to the matrix is the trapping or retention of small molecules such as quorum sensing signals or antimicrobials resulting in differential gradients throughout the biofilm. Such gradients are also the result of microbial activity, such as respiration, which can affect local concentrations of nutrients and waste products. This leads to the hypothesis that the mechanical properties of the matrix can affect the behavior and composition of the bacteria, and thus contribute significantly to biofilm physiology and function. By using particle-tracking microrheology, an increase in the elasticity and effective crosslinking of *Pseudomonas aeruginosa* biofilm matrices was attributed to the polysaccharide Psl, providing the first quantitative description of the biophysical properties of Psl. Conversely, the Pel polysaccharide reduced the effective crosslinking within the biofilm, resulting in a more viscous matrix associated with spreading of the biofilms. In particular, when Psl was absent the biofilms were less resistant to shear stress, resulting in the formation of streamers under high flow conditions.

To determine if differences in the properties of these matrix components played an important role in the formation of mixed species biofilms, dual species biofilms were formed with *Staphylococcus aureus* and *P. aeruginosa*. *S. aureus* facilitated biofilm formation and increased cell viability in *P. aeruginosa*. As for the monospecies biofilms, similar rheological roles were observed for the

Pel and Psl exopolysaccharides in dual species biofilms. Whilst Psl was required for the formation of microcolonies, Pel was associated with an increased amount of dead cells with, and the size of, microcolonies. These microcolonies exhibited superdiffusivity within its subregions. Superdiffusivity increases mass transfer, which may have facilitated microcolony expansion in *P. aeruginosa* of the dual species biofilms. Indeed, removal of eDNA by digestion with DNase impaired the dual species biofilm formation and attenuated microcolony formation. The formation of the *S. aureus*–*P. aeruginosa* community biofilm resulted in the specific induction of the *P. aeruginosa* c-di-GMP regulated gene fusion P_{cdrA} -*gfp* within the microcolonies. This expression was dependent on the membrane-associated diguanylate cyclase SadC, suggesting intracellular nucleotide c-di-GMP was involved in microcolony formation.

By using micropillar arrays, the differential pressure during biofilm and microcolony formation in *P. aeruginosa*, as well as biofilms formed by different species, were investigated. For *P. aeruginosa*, differential pressure was greatly reduced in the absence of Pel or Psl. The loss of Pel had a greater impact on biofilm forces, and was likely due to its role in spreading of the biofilm as shown by the microrheological measurements. Thus, the application of two micromechanical tools, that study microscale biophysical phenomena in the biofilm, has enabled the quantitative description of two key polysaccharides that are responsible for formation of the biofilm matrix. This quantitative approach has also resolved distinct roles for the two polysaccharides, for example in the formation of either rigid or soft matrices, potentially explaining why microorganisms encode multiple polysaccharide operons.

1 Introduction

1.1 Background

The field of microbiology has traditionally focused on the study of microbes in pure cultures (single species) and their planktonic (free-floating and singular) state. The practice of using pure cultures was advocated by Robert Koch, who was focused on identifying bacterial pathogens through their isolation and cultivation from diseased organisms (1). The ability to grow bacteria in pure culture provided a means to individually isolate and clone bacterial genes, allowing for the detailed understanding of metabolite pathways, genetics, physiology, epidemiology, medical microbiology and molecular biology. The isolation and characterization of novel bacterial strains continues to deliver many benefits such as natural and biologically useful compounds for the pharmaceutical industry. Examples include antiviral and, antitumor compounds, and polymer-degrading enzymes from bacteria in different ecosystems, such as marine, soil and animal digestive systems (2-4). Isolated and clonal microbial populations however, are rarely found in nature. Instead they exist predominantly as dense, self-organized communities encased in a self-produced matrix, as first alluded to by Antonie van Leeuwenhoek in the 1600s based on his observation of dental plaque. Such communities are commonly called biofilms or flocs (5, 6) and biofilms are ubiquitous in the natural environment, being found on land, water, plants and animals, and in natural and urban settings. Biofilms can host a large diversity of bacterial species, comprising bacteria of distinct metabolic types that contribute to Earth's natural cycling of nitrogen, sulfur and many metals (7). Biofilms have a range of applications as

well where, for example, the metabolic activities of biofilms and flocs have been successfully exploited for wastewater treatment (8). The first recorded use of biofilms, dated to 1893 in Salford, UK, was for the digestion of organic carbon (9). Subsequently biofilms have been used for the bioremediation of land and water (10, 11), the extraction of metals (12), energy production from waste (13, 14), maintenance of plant and soil health for increasing crop yield (15), biocatalysis (16) and production of commercial products (17). While biofilms have many beneficial uses, they can also be detrimental where they are responsible for a range of industrial issues such as biofouling (18) and corrosion (19).

Just as biofilms have positive and negative impacts on industrial processes, they similarly can be good or bad in the host context. Commensal bacteria can form biofilms on mucosal surfaces, such as the intestinal tract, and are thought to behave as a 'virtual organ' that modulates the immune system and facilitates nutrient utilization (20). However, pathogenic bacteria can also form biofilms, which often become the root of many persistent and chronic bacterial infections (6). Examples of biofilm-associated infections of humans are: 1) periodontal disease, which is pandemic and is caused by a multi-species biofilms known as dental plaque (21); 2) chronic respiratory infections in cystic fibrosis patients that eventually cause lung failure (22) and 3) infection and inflammation from medical implants, from pathogenic biofilms that have formed on the surface of catheters, artificial joints, heart valves, and other devices (23). The National Institute of Health (NIH) estimates that 65-80% of microbial infections occurring in the human body are biofilm-mediated (24, 25). In 2000, the Center

for Disease Control and Prevention (CDC) announced biofilms and biofilm-associated infections as two of seven major healthcare obstacles for the medical community in the 21st century. Biofilms are the basis of persistent colonization of hospital facilities, and therefore drive and sustain hospital-acquired infections. In the United States, 2009, hospital-acquired infections are estimated to cost US\$28.4-33.8 billion in direct medical costs (after adjusting to 2007 dollars using the CPI for all urban consumers) (26, 27). Thus, it is important to understand the biofilm formation mechanisms and develop strategies for efficient biofilm control.

1.2 Research gaps and challenges

It is only within the past 30 years that the physiology of the microbial community has been examined in detail. Descriptions of bacteria that have been derived from free-living cells and pure cultures are changing to include complex, self-organized multicellular biofilms. These biofilms can be considered as natural immobilized whole cell catalysts, which may present an alternative method to microbial bioprocesses that usually use the planktonic mode of growth for industrial chemical production and conversion of compounds. While there has been a significant advancement in our understanding of biofilm formation and phenotypes, in particular, structural development, microbial cell-cell communication and the molecular pathways involved in biofilm formation, the extracellular biofilm matrix remains relatively poorly understood in terms of both a detailed understanding of the

biopolymers present as well as how they contribute to the physiological properties of the biofilm matrix. This is despite the matrix being often considered one of the most distinguishing feature of biofilms as compared to planktonic cultures (28) and being a major part of the biofilms that usually accounts for 50-90% of the biofilm's total organic matter (29, 30).

The matrix is comprised of many different components, largely exopolymeric substances that include long chain exopolysaccharides, large adhesin proteins, nucleic acids, humic acids, and lipids (30). Extracellular bacterial structures, such as membrane vesicles, flagella, pili and fimbriae are also considered to be components of the matrix (30). The physicochemical properties of these EPS components are essential in determining the penetration of nutrients into and the diffusion of metabolic products of cells within the biofilm, which in turn affect the biofilm physiology (31, 32). Many EPS components are highly charged and readily absorb water and ions, classified as being hygroscopic (33), which help to protect the biofilm against desiccation and buffer against pH changes. The EPS also buffers the biofilm against starvation by trapping and recycling nutrients from lysed cells, effectively becoming a reserve of carbon, nitrogen and phosphorus containing compounds (30). Furthermore, EPS components can be heterogeneously distributed in the biofilm, creating different microenvironments for the cells (34) that may promote different cell physiological states within the biofilm (see Chapter 2.3 Biofilm physiological heterogeneity). This highlights the need to carry out biofilm investigations at the micro-scale.

Components in the matrix are not just passive structural elements of the biofilm, but rather make up an active and dynamic physical scaffold with

specific binding and catalytic roles in biofilm processes, and signaling roles in biofilm formation that impact on multicellular structural organization and cell physiology. For example, exopolysaccharides left behind by other cells act as trails that can guide cells into microcolonies (35). EPS interactions between different microbial species such as lectin-exopolysaccharide binding are involved in the ordered succession, and spatial organization of species within mixed-species communities (36, 37). Fimbriae and pili have also been shown to be important for mixed species biofilm development, where the fimbriae of *Porphyromonas gingivalis* are required for binding to the SspBI surface polypeptide of *Streptococcus gordonii* (38), and the pili of *P. aeruginosa* engaged in the formation of mixed-species biofilms of *P. aeruginosa* and *S. aureus* (39). Predatory cells of *Myxococcus xanthus* uses the matrix as a medium for collective and directional motility, otherwise known as swarming (28, 40). Under conditions of starvation, *Myxococcus xanthus* can also organize into mound-like structures using matrix components, where cells sporulate and form fruiting bodies (28, 41). The number of different components and interactions that take place in the matrix to contribute to biofilm formation and processes at different times make the analysis and understanding of the biofilm matrix complex and difficult, leading it being referred to as “the dark matter of biofilms” (42).

The matrix also defines the mechanical properties of the biofilm, which directly affect the 3D structure of the biofilms and influences how the biofilm buffers against, deforms and detaches under external forces such as shear. This also has consequences for biofilm stability, dissemination and downstream colonization in flow environments (Figure 1.1). For example, the viscoelastic properties of

the matrix enable biofilms to develop mushroom-shaped microcolonies under low shear conditions, and streamers and ripple beds under high shear conditions (43, 44) (Figure 1.1). The unattached portion of the streamer, known as the streamer tail, is able to stretch out and bridge to new areas. In doing so, it forms a sticky network that captures other biomass and spreads rapidly across surfaces (45). The cohesive strength of the matrix would determine the tensile deformation of the streamer and its subsequent detachment ('sloughing off'), which would then lead to its dissemination and downstream colonization (43).

Figure 1.1. Various types of kinetics (rippling, streaming, detaching, seeding dispersal and rolling) involved in desmination and recolonization in biofilms that depend on the mechanical properties of the biofilm matrix. Reproduction from Montana State University Center for Biofilm Engineering.

The sloughing off of biofilms can be a problem for stable process maintenance in biofilm reactors. The mechanical stability of floccular biofilms will also affect the floc size distribution, which is important in water purification processes as its effects the settlement and separation of biomass from the water.

Thus, just as studies of the molecular pathways have been important for improving our ability to understand biofilms, enhanced knowledge of the biofilm matrix and its properties will also help to unlock new strategies for the utilization and engineering of productive biofilms, and the control and removal of unwanted biofilms.

1.3 Objectives and scopes

The model organism *P. aeruginosa* is known to express two polysaccharides Pel and Psl that are major matrix components and are required for biofilm formation. Studies have indicated that Psl is the more important polysaccharide for biofilm formation and function, wrapping itself around *P. aeruginosa* cells and contributing to cell-surface attachment and signaling (46, 47). In *P. aeruginosa* strains that contain both the *pel* and *psl* operons, Pel mutants are not affected in biofilm initiation whereas Psl mutants show significant defects (48). However, only Pel is expressed in the PA14 strain, whereas both Pel and Psl are expressed in the PAO1 strain where they are considered to have structurally redundant roles (49). In addition, it is likely that Psl and Pel biosynthesis pathways compete for common sugar precursors: both Psl and Pel require the enzyme AlgC to provide the common sugar precursors mannose-1-phosphate and glucose-1-phosphate and induction of Pel results in posttranscriptional reduction of Psl (50). It is apparent that there would be occasions where one exopolysaccharide would be preferably expressed over another. However, the contribution of Pel to the biofilm life cycle in comparison of Psl remains unclear, and there is little detail on how components of the EPS contribute to

biofilm mechanical properties and subsequently affect structure (morphology) and functions.

This study has investigated the mechanical characterization of Pel and Psl polysaccharides of *P. aeruginosa* PAO1, using microrheology and micropillars described in Chapters 1.5.1 Particle-tracking microrheology and 1.5.2 Micropillars. Based on the mechanical roles of Pel and Psl, the impact of Pel and Psl on biofilm morphology (structure) and apparent functional consequences such as surface spreading and microcolony formation is further explored. It addresses the following core questions:

- 1) How Pel and Psl contribute to the viscoelasticity and effective crosslinking of the matrix at different locations and time points during biofilm development,
- 2) How viscoelasticity and effective crosslinking in the matrix affect biofilm morphology and resistance to shear in monospecies flow cell biofilms,
- 3) How the rheological contributions of Pel and Psl impact on the development of multi-species biofilms,
- 4) How are differential pressure within biofilms are affected by Pel and Psl.

2 Literature Review

2.1 Background on the biofilm matrix

2.1.1 Major components of the biofilm matrix

2.1.1.1 Exopolysaccharides

Long chain exopolysaccharides constitute a large fraction of the biofilm matrix of many bacterial species. Examples of biofilm exopolysaccharides include Pel and Psl in *P. aeruginosa* (51, 52), the polysaccharide intercellular adhesin (PIA) in *S. aureus* and *Staphylococcus epidermidis* (53), and cellulose in *Salmonella typhimurium*, *Salmonella enteritidis* and *Escherichia coli* (54, 55). Psl is reported to be a repeating pentasaccharide consisting of D-mannose, D-glucose and L-rhamnose, synthesized by the *psl* operon. Psl was found to have signaling roles by increasing cell contact to the surface and leading the bacteria to gather and build microcolonies on trails where it had been deposited (35). Furthermore, Psl was reported to interact with a large extracellular protein in the matrix, CdrA, and the combination of both CdrA and Psl forms a physical link between individual *P. aeruginosa* cells, leading to aggregation during cultivation in liquid media (56). Psl presents a physical barrier to components of the immune system, by which it can prevent complement-mediated opsonization by neutrophils (57). The Pel exopolysaccharide encoded by the *pel* operon was found to be involved in pellicle formation in *P. aeruginosa* PA14, which does not express the Psl exopolysaccharide (58). A recent study has found Pel to be positively charged and composed of partially acetylated 1-4 glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine that

binds to eDNA in the matrix (59). Pel also provides structural and protective roles to the biofilm (60), but is thought to be redundant within the matrix in *P. aeruginosa* PAO1, where Psl is the primary polysaccharide in the matrix (49). PIA is a positively charged poly- β (1-6)-*N*-acetylglucosamine (PNAG), encoded by the *ica* operon that facilitates biofilm formation in *S. aureus* (61). It has been found to increase *S. aureus* adhesion to human nasal epithelial cells, virulence and lung infection in mouse (53, 62). Cellulose was shown to enhance the resistance of *Salmonella enterica* to desiccation and to prolong the survival of their colonies on plastic surfaces for several months even without the exogenous supply of nutrients (63).

Aside from providing structural and functional roles in the biofilm for bacteria of their own species, exopolysaccharides are also essential for the establishment of multi-species communities. In oral biofilms (dental plaque), *Streptococcus mutans* and anaerobes are the first colonizers of the tooth enamel (64). These initial colonizers are known to produce levans and dextrans as components of the EPS that bind to lectins of other bacterial species, thus establishing the early biofilm community. While there have been recent advances in the isolation and characterization of exopolysaccharides from different microbial species, such studies are still rare and there likely remain a significant number of uncharacterized exopolysaccharides produced by biofilms that play important roles in biofilm formation, development and environmental adaptation in both single and multi-species communities.

2.1.1.2 Proteins and peptides

Cell surface associated proteins also make up a significant component of the biofilm matrix. Examples of cell surface associated proteins are the bacterial appendages pili and aggregative fimbriae, lectins, and cell wall anchored proteins of Gram-positive bacteria. Type IV pili expressing cells form the microcolony cap by the specific interaction of the type IV pili with extracellular DNA (eDNA) of the matrix (see Chapter 1.2.2.3 Extracellular DNA) (65). Thin aggregative fimbriae, which are also known as curli fibers due to their curled morphology in the absence of exopolysaccharides, are produced by most *Salmonella* strains and isolates of *E. coli* as part of their biofilm matrix (66, 67). Curli fibers were also found to have an amyloid like organization, which are characterized by their misfolded protein structure and resistance to denaturation, and the inhibition of amyloid formation also inhibits biofilm formation (68, 69). Surface-attached carbohydrate-binding proteins, such as lectins are able to link the surface of bacterial cells to a substratum as in the case of *P. aeruginosa* (70) or to the exopolysaccharide matrix of other bacteria as in the case of dental plaque forming organisms (21). Cell wall anchored proteins such as Bap (biofilm-associated surface protein) (71), SasG (*S. aureus* surface protein G) (72), and Protein A (73) are used in the proteinaceous matrix of *S. aureus*. Homologues of the Bap protein are also found in many other bacterial species, where they facilitate biofilm formation by functioning as adhesins to surfaces, bacterial cells and host cells (74, 75). The Bap protein has not been found in human isolates of *S. aureus* however, indicating that biofilm formation in human hosts depends on another adhesin (76, 77). Aside from adhesive and structural proteins, other matrix-associated proteins have catalytic functions that can convert molecules to build, maintain or alter the

microenvironment within biofilms. For example, glycosyltransferases found in the matrix of oral biofilms engage in EPS synthesis by producing glucans and levans when sucrose becomes available (36). Glucans and levans provide additional attachment sites for cells and the structural support for microcolony development, thus contributing to growth of plaque (36). Enzymes involved in the degradation of the matrix polymers are also present. Extracellular DNA in *P. aeruginosa* has been found to be degraded by matrix DNases for the utilization of carbon and energy (78), and alginate lyase was found to degrade alginate exopolysaccharides, resulting in increased biofilm sloughing and cell dispersal (79). Some of these enzymes function to digest toxic compounds and antimicrobials before they can reach the cells, such as lipase LipA in digestion of polysorbate 80 (80). Extracellular proteins are also major components in aerobic flocs and granular sludge of wastewater treatment plants (81). A majority of these proteins were found to bind to multivalent cations and organic compounds, and catalyzed the degradation of organic compounds during anaerobic, anoxic and aerobic digestion (82). In *Shewanella oneidensis*, a bacterium that is able to reduce toxic heavy metal ions and absorb radionuclide contaminants, *c*-type cytochromes in the matrix may contribute to the redox activity of the biofilm and extracellular electron transfer reactions (83).

Peptides have been found to act as biosurfactants, which function as key structuring factors of the matrix and are known to play various roles throughout the biofilm life cycle (see Chapter 1.2.2.4 Lipids and Chapter 1.3.1 Biofilm development and life cycle). In the case of *S. aureus*, the phenol-soluble modulins (PSM) peptide has α -helical, amphipathic structure that acts as a biosurfactant and is required for the formation of biofilm channels, cycles of

biofilm detachment and regrowth that are characteristic of *S. aureus*, and control of biofilm expansion.

2.1.1.3 Extracellular DNA

eDNA was first discovered in the biofilm matrix of *P. aeruginosa*, where it was found that the addition of DNase I to early stage biofilms was able to inhibit biofilm formation (84). eDNA was then reported to be released into the biofilm matrix through cell death and autolysis (85, 86) or by release from small vesicles from the outer membrane (see Chapter 2.1.1.4 Lipids) (87). Recently it was shown the explosive cell lysis, mediated via cryptic prophage endolysin activity, resulted in both the liberation of eDNA and production of membrane vesicles (88).

eDNA is involved in the formation of the microcolony cap in *P. aeruginosa*, as a motile subpopulation that make up the cap uses type IV pili to interact with eDNA secreted from a non-motile subpopulation at the microcolony stalk (65). eDNA binds to aminoglycosides and thus shields the bacteria from antimicrobial peptides in *P. aeruginosa* biofilms (89), and protects against exposure to the detergent, SDS (86). A recent report showed that eDNA interacts with the Pel exopolysaccharide at the microcolony stalks of *P. aeruginosa* biofilms, which was suggested to provide structural stability (59). eDNA also interacts with exopolysaccharides in *M. xanthus* biofilms, which enhanced the mechanical strength and adhesive properties of the matrix as investigated by atomic force microscopy (90). eDNA is a significant component in *Staphylococcus spp.* and *Streptococcus spp.* biofilms and treatment with

DNase I was able to disintegrate *S. aureus* biofilms (91). eDNA is released into the matrix of *S. aureus* through murein hydrolase mediated autolysis, encoded by *cidA*. CidA was shown to be important for biofilm adhesion and stabilization *in vitro* and in a murine catheter-associated infection (91). Staphylococcal thermonucleases, encoded by *nuc*, also present in the matrix are able to degrade eDNA and *nuc* mutants develop thicker biofilms with increased levels of eDNA, suggesting that they may function as part of a mechanism for biofilm dispersal in later stages of biofilm development (92).

2.1.1.4 Lipids

Different types of lipids associated with the matrix include glycolipids, phospholipids, neutral lipids, and lipopolysaccharides. Lipids in biofilms generally act as biosurfactants and function to increase surface motility, aid in the uptake of hydrophobic substances and facilitate detachment as well as having antimicrobial and antifungal activity. In *P. aeruginosa*, a class of glycolipids known as rhamnolipids contribute to various stages of biofilm development as a biosurfactant by increasing swarming motility (93), maintenance of water channels and microcolonies in the biofilm by disrupting cell-cell interactions and surface attachment (94), mediating cell detachment (95) and growth inhibition of various bacterial and fungal species (96).

Membrane vesicles are a lipid particulate feature of the matrix. They are derived from the outer membrane of Gram-negative bacteria, composed of mainly lipopolysaccharides, and it is thought that their main purpose is to export cellular components such as nucleic acids (87), extracellular enzymes,

lipopolysaccharides, phospholipids and signaling compounds into the matrix. In *P. aeruginosa*, the hydrophobic signaling compound *Pseudomonas* quinolone signal (PQS) is secreted by membrane vesicles (97).

2.1.1.5 Filamentous phage

Biofilms also support infection by and propagation of bacteriophages, viruses that infect and replicate within bacteria. They have been found to play an important role in gene transfer and regulation of the biofilm life cycle (see Chapter 2.1.1 Biofilm development and life cycle). The filamentous Pf4 bacteriophage within *P. aeruginosa* is a negatively charged filament 2 μm in length and 6-7 nm in diameter. Recently, they have been found to spontaneously assemble the matrix into a more crystalline state by interacting with host and microbial polymers (98). Small colony variants (SCVs) of *P. aeruginosa* produce copious amounts of Pf4 (99) and their matrix was found to have an ordered, liquid crystalline structure that promotes adhesion and enhances tolerance to desiccation and antibiotics (98).

2.1.2 Cohesive interactions between the matrix components

Intermolecular interactions between the different matrix components hold the biofilm together and are responsible for its mechanical stability. The major intermolecular interactions have been categorized and described extensively in Flemming et al., 2013 “Cohesiveness in biofilm matrix polymers” (100) and Flemming et al., 2016 “The Perfect Slime: Microbial Extracellular Polymeric Substances (EPS)” (42). They are described here briefly.

2.1.2.1 London (dispersion) forces

London (dispersion) forces are weak intermolecular forces with binding energy is about 2.5 kJ mol^{-1} . They arise from attraction between the temporary dipoles formed by uneven distribution of electrons around the nucleus, and occur mainly in hydrophobic areas of the biofilm.

2.1.2.2 Electrostatic interactions

Electrostatic interactions are attractive forces between ions or molecules with opposite permanent charges, and induced dipoles. The binding energy of non-ionic electrostatic interactions is usually around 12 to 29 kJ mol^{-1} . Divalent cations, particularly Ca^{2+} , are usually involved in forming salt bridges with polyanions of the matrix, and strongly increase the mechanical robustness of biofilms (101-103). The strength of such salt bridges would be affected by pH conditions of the environment.

2.1.2.3 Hydrogen bonds

Hydrogen bonds are strong intermolecular forces with binding energy around 10 to 30 kJ mol^{-1} and occur mainly between hydroxyl groups, which are commonly found in polysaccharides and water molecules.

2.1.2.4 Entanglements

Mechanical entanglement that result in topological constraints and increased cohesiveness can arise if the polymers in the matrix have molar masses exceeding the critical molar mass (M_C) required for entangling. The M_C is

usually considered as twice the entanglement molecular weight (the average molecular weight of a chain segment between intermolecular junction points, M_e) (104-106). This has been estimated to be above 1.5×10^5 Da for polysaccharides in biofilms (103). It is normally considered that the entanglement of polymers makes the molar mass dependence of the melt viscosity of a polymer change from linear to 3.4 power when $M = Mc$. In solutions of EPS extracted from *P. aeruginosa*, entanglements were found to generate temporary junction points, which had an average lifetime in the order of 17 ms (103). This means that the entanglement network is only elastic to small perturbations lasting less than 17 ms. Entanglements are not expected to play a major role in the overall cohesiveness and mechanical stability of the matrix as biofilms hold mainly water and the concentration of macromolecules varies around 1-3% (w/w) (107, 108).

2.2 Background on biofilm regulation

2.2.1 Biofilm development and life cycle

Genetic studies of monospecies biofilms have shown that biofilms formation and development is a highly regulated process that requires intercellular signaling (109) and typically proceeds in distinct stages (110, 111). Whilst the mechanisms and processes for biofilm development are unique for each species and depend on the environmental conditions, there are some general mechanisms and key processes of biofilm development that can be drawn from the discussion and investigation of the model Gram-negative bacterium *P.*

aeruginosa and model Gram-positive *S. aureus*. The biofilm life cycle of the model Gram-negative bacterium *P. aeruginosa* is typically characterized as preceding in five distinct stages, largely based upon microscopic observation and cell protein analysis of flow cell biofilms (110) (Figure 2.1):

1. Initial attachment, where planktonic bacteria attach to a substratum via non-specific and reversible forces such as van der Waals interactions.
2. Irreversible attachment, where attachment becomes more permanent as *P. aeruginosa* anchor themselves with more specific interactions via cell adhesion structures such as pili (111). The *las* quorum sensing system has been found to be active at this stage (110). The repression of flagellar motility and increased expression of matrix polymers is partly controlled by the *las* and *pqs* quorum sensing systems (see Chapter 2.2.2 Quorum sensing regulation) and the secondary messenger bis-(3',5')-cyclic-dimeric-guanosine monophosphate (c-di-GMP) (see Section 1.3.3.1 ci-di-GMP regulation). In particular, the *P. aeruginosa* polysaccharide Psl has been shown to be important for surface attachment and cell clustering (35).
3. Maturation I, where a thin biofilm of roughly 10 μm thickness is formed out of the multiple layers of cells and cell clusters. Maturation I is accompanied by the activation of the Rhl quorum-sensing system, as determined from the *rhlA* gene reporter, in *P. aeruginosa* (110). Activation of the *rhl* quorum sensing system leads to the production of rhamnolipids that contribute to the biofilm architecture through the maintenance of microcolonies and water channels that facilitate the

supply of nutrients deep into the biofilm (see Chapter 2.1.1.4 Lipids) (94). Both *las* and *rhl* systems are responsible for the production of various extracellular enzymes such as elastase (112) and secondary metabolites such as pyocyanin (113, 114) and pyoverdine (114, 115). Spatially patterned gene expression occurs (32), occasionally resulting in the biofilm developing higher order structures such as mushroom-like microcolonies during maturation II (116).

4. Maturation II, where a thick biofilm has been formed and microcolonies reach 100 μm in height. Protein expression was reported to be the most different during Maturation II, as compared to planktonic cells, with more than 50% of all the proteins being upregulated and less than 7% downregulated, an outcome significantly different from that of maturation I (110). For example, during this stage, the enzyme L-ornithine 5-monooxygenase, involved in pyoverdine synthesis, was at its highest concentration observed for any stage of development (110). Cells can exist within a broad range of physiological states due to the increased structural heterogeneity at maturation II (see Chapter 2.3 Biofilm physiological heterogeneity). Mature biofilms are reported to be more resistant to antibiotic treatment in a drip-flow biofilm model, porcine skin biopsy model, mouse chronic wound model and clinical study (117). Interestingly, the Pf4 phage of *P. aeruginosa* was shown to contribute to microcolony development and differentiation. In particular, microcolonies formed by the phage deletion mutant were smaller than in the wild type and did not undergo the typical microcolony hollowing associated with cell death (118).

Figure 2.1. Biofilm lifecycle based on *P. aeruginosa*. 1) Initial attachment, where planktonic bacteria attach to the substratum by non-specific forces. 2) Irreversible attachment, where clusters of bacteria successfully attach to the substratum via specific cell adhesion structures and secrete EPS. 3) Maturation I, where the biofilm is formed from cell clusters and a thin multilayer of cells. 4) Maturation II, where microcolonies associated with mature biofilms may be formed. 5) Dispersal, where cells leave the biofilm and regain planktonic state. Dispersal is often accompanied by matrix degradation and localized cell death. Bottom panel shows brightfield images of *P. aeruginosa* at the respective stages of biofilm formation (128).

5. During the dispersal stage, microbial cells leave the biofilm to regain their planktonic lifestyle, and dispersal is accompanied by cell death and matrix degradation (119). Dispersal may be triggered by sudden changes in substrate conditions such as starvation (120), sudden increases in organic carbon (110) or a sudden decrease in oxygen concentrations (121). Chemicals such as nitric oxide trigger biofilm dispersal by affecting the intracellular second messenger, cyclic diguanylate monophosphate (c-di-GMP) that regulates biofilm formation (see

Chapter 2.2.3.1 c-di-GMP regulation) (122). Dispersal is also thought to occur as a natural progression to colonize other areas and spread, although key processes have yet to be elucidated. Studies have shown that *P. aeruginosa* naturally produces a compound, *cis*-2-decenoic acid, which is able to disperse and inhibit biofilm development (123).

S. aureus is a model Gram-positive biofilm-forming bacterium although much less is known about the molecular mechanisms regulating the biofilm life cycle of *S. aureus* compared to *P. aeruginosa*. It is a non-motile bacterium, and the following stages have been described for the biofilm cycle of *S. aureus*:

1. Initial adhesion, where *S. aureus* attaches to both abiotic and biotic surfaces by cell wall anchored proteins such as Bap.
2. Proliferation, which involves increased biofilm biomass as a consequence of clonal division and matrix production. In staphylococci, the matrix consists of several secreted polymers such as exopolysaccharides, teichoic acids and proteins as well as eDNA originating from lysed cells through *cidA* expression. PIA facilitates cell aggregation by increasing cell-cell association (40), and eDNA stabilizes the biofilm (91).
3. Detachment and maturation, which is controlled by the Agr quorum-sensing system (see Chapter 2.2.2 Quorum sensing regulation) (124). The Agr quorum-sensing system down-regulates eDNA release into the matrix (125) and up-regulates phenol-soluble modulins (PSMs) production (126). PSMs structure the biofilm through processes such as channel building and control of biofilm thickness (126). PSMs also

mediate detachment of *S. aureus* to facilitate spreading over wet surfaces in a phenomenon known as colony spreading (127).

The two major signaling systems that have been found to regulate biofilm development, quorum sensing and nucleotide signaling, are discussed in further detail below.

2.2.2 Quorum sensing regulation

To coordinate the behavior of individual cells, some bacteria use quorum sensing based extracellular communication systems consisting of small diffusible signaling molecules (129, 130). Several classes of signaling molecules involved in quorum sensing have been described, including N-acyl-L-homoserine lactones (AHLs) (131), which are primarily used by Gram-negative bacteria, and oligopeptides, which are primarily used by Gram-positive bacteria (132). Once the quorum sensing signal molecules reach their threshold concentrations, they activate their specific receptors which are transcriptional regulators, leading to differential expression of a large set of quorum sensing regulated genes to mediate biofilm formation (133).

P. aeruginosa encodes two AHL based quorum-sensing systems, the LasI-LasR and RhII-RhlR systems. LasI is the AHL synthase that synthesizes the AHL signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC₁₂-HSL), which binds to the transcriptional regulator LasR (134, 135). *P. aeruginosa lasI* mutants, which lack a functional signal synthase, are limited in biofilm development, and form flat and undifferentiated biofilms compared to the wild-type *P. aeruginosa* (Figure 2.2) (109, 134).

Figure 2.2. Epifluorescence images of top-down view (top panels) and confocal images of side view (bottom panels) of wild-type PAO1 and *lasI* mutant (PAO-JP1) biofilms grown with or without addition of the autoinducer 3OC₁₂-HSL to the medium. *P. aeruginosa* is *gfp*-tagged and appears green. Reproduction from Davies et al., 1998 (109).

Similarly, in the RhlI-RhlR system, RhlI is the AHL synthase that synthesizes the AHL signal, *N*-butyryl-L-homoserine lactone (C₄-HSL), which binds to the transcriptional regulator RhlR (134, 136). The rhamnolipid biosurfactant is known to be a Rhl quorum sensing regulated product that is mainly synthesized during the later stages of *P. aeruginosa* biofilm development and is important for the maturation of the mushroom-shaped microcolonies as well as biofilm dispersal (95, 137, 138). In addition to 3OC₁₂-HSL and C₄-HSL, *P. aeruginosa* produces a third non-AHL signaling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, also known as the *Pseudomonas* Quinolone Signal (PQS) (139). PQS is synthesized by the *pqsABCDH* operon together with *pqsR*, as the transcriptional regulator and *pqsE*, as the response effector (140). Activation of the PQS quorum sensing system is required for the release of eDNA and initiation of microcolony formation during early stage *P. aeruginosa* flow cell biofilm development (141). In addition to EPS production and biofilm

formation, many other bacterial phenotypes and behavior such as virulence, swarming motility, bioluminescence, sporulation and conjugation are quorum sensing regulated (133).

Many Gram-positive species use oligopeptides that bind to a two-component regulatory system to mediate quorum sensing. This two-component regulatory system consists of a membrane-bound histidine kinase that senses high oligopeptide levels and initiates phosphorylation-dephosphorylation cascades that activate the corresponding response regulator controlled phenotypes such as virulence, sporulation, EPS production and biofilm formation (130). The best-characterized oligopeptide-based quorum sensing system is the accessory gene regulator (*agr*) system in *S. aureus* (124). In this system, *agrD* encodes the precursor of the autoinducing peptide (AIP). It is processed and transported out of the cell by membrane bound transporter AgrB. At high extracellular AIP concentrations, AIP binds to the membrane-bound histidine kinase, AgrC. AgrC then phosphorylates the response regulator AgrA that positively controls virulence. However, quorum sensing negatively regulates biofilm formation of *S. aureus*, with *agr* mutants forming thick biofilms and the addition of AIP-1 resulting in biofilm detachment (Figure 2.3) (142). One explanation for this behavior is that when cell densities are low, biofilm formation in *S. aureus* is initiated to achieve high cell densities. Once high cell density has been reached, *S. aureus* stops biofilm formation, secretes virulence factors and prepares for dispersal. Release of the major EPS component, eDNA, into the matrix has also been found to be negatively regulated by the LytSR two-component regulatory system (125). In contrast to *S. aureus*, quorum-sensing in *Streptococcus*

sanguinis is mediated by the VicRK two-component system which positively regulates eDNA release and biofilm formation (143).

Figure 2.3. (A) A biofilm is not formed when quorum sensing is active in a *S. aureus* *agr*⁺ strain. Biofilm forms in the *S. aureus* *agr*⁺ strain when quorum sensing is repressed by supplementation with glucose (TSBg). Biofilm forms in the *S. aureus* *agr*⁻ strain that is attenuated in quorum sensing, even with glucose supplementation. (B) The addition of exogenous autoinducer peptide-1 (AIP-1) to a 2 d biofilm results in sloughing and detachment of the biofilm over 1-2 d. *S. aureus* is *rfp*-tagged and appears red. Reproduction from Boles *et al.*, 2008 (142).

2.2.3 Nucleotide signaling

2.2.3.1 c-di-GMP regulation

For a wide range of bacteria, biofilm formation is also regulated by the intracellular nucleotide signaling molecule c-di-GMP (144, 145) (Figure 2.4). The intracellular concentration of c-di-GMP operates to modify the expression of genes associated with either biofilm formation or planktonic growth. Typically, a high intracellular c-di-GMP content leads to up-regulation of EPS components and down-regulation of flagella genes, which are key steps in the establishment of a biofilm (144, 146). In contrast, low intracellular c-di-GMP

content increases motility and leads to biofilm dispersal (146). C-di-GMP is synthesized by diguanylate cyclases (DGCs), and degraded by phosphodiesterases (PDEs) (Figure 2.4). Microorganisms often encode multiple DGCs and PDEs and these DGCs and PDEs have been hypothesized to respond to different environmental cues, allowing the bacteria to fine-tune their intracellular c-di-GMP to a wide range of conditions (145, 147).

Figure 2.4. Structure and physiological functions of c-di-GMP. Diguanylate cyclases that have GGDEF domains (red) synthesize c-di-GMP and specific phosphodiesterases that contain EAL or HD-GYP domains (blue) break down c-di-GMP. c-di-GMP facilitates biofilm formation. Reproduction from Hengge, 2009 (144).

C-di-GMP operates by binding to effector proteins (e.g. PilZ domain protein (148)) or c-di-GMP-I riboswitches (149), resulting in conformational changes in the protein or RNA and thus altering the function of those targets to control downstream gene expression. For example, a high intracellular content of c-di-GMP down-regulates *P. aeruginosa* motility via the FleQ effector and up-regulates synthesis of the Pel exopolysaccharide via the PelD effector protein (150, 151). The *P. aeruginosa* $\Delta wspF$ mutant has high intracellular c-di-GMP

content, making it a strong biofilm former (152). In contrast, nitric oxide (NO) was shown to lower the intracellular c-di-GMP content through the activation of PDE activity, ultimately resulting in biofilm dispersal (153). In *Salmonella*, the expression of curli fimbriae (154) and the major exopolysaccharide cellulose is enhanced by high intracellular c-di-GMP concentrations (155).

2.2.3.2 c-di-AMP regulation

S. aureus does not appear to produce or use c-di-GMP to regulate its molecular pathways. However, another type of nucleotide signaling molecule, c-di-AMP has been discovered in *S. aureus* as well as other bacterial species. An absence of c-di-AMP was shown to be deleterious to the growth of *L. monocytogenes* (156) and *Bacillus subtilis* (157), indicating that c-di-AMP controls essential cellular pathways. Recently, c-di-AMP was found to target proteins involved in potassium transport by binding to the phylogenetically widely distributed RCK_C domain, and thus may be important for the regulation of ion transport in many bacteria, green algae and proteobacteria (158). Thus the role of c-di-AMP is distinct from c-di-GMP, whose regulation of motility and production of matrix components have a clear impact on biofilm formation. As c-di-AMP is a newly discovered molecule that potentially regulates many cellular pathways, its implications on biofilm development need to be further studied.

2.3 Biofilm physiological heterogeneity

Cells located at different regions of the biofilm experience different concentrations of metabolic substrates and waste products (32). In example, in

surface-attached biofilms that mainly receive nutrients from the overlying bulk fluid, oxygen and nutrients are readily available to the cells in the outer layer of the biofilm where they directly interact with the aqueous phase. These cells can consume most of the oxygen and nutrients that diffuse into the biofilm and this results in the cells in the deeper parts of the biofilm experiencing oxygen (159) and nutrient depletion and becoming physiologically differentiated from cells at the surface. However, in the case where biofilms are used for particulate degradation (160) and in agar biofilms (161), the biofilm obtains nutrients from the solid substrate, resulting in cells closest to the substratum being in a relatively nutrient rich environment, and cells on the outer part of the biofilm being in a relatively nutrient poor environment.

Nutrient starved cells can exhibit an alternative metabolic state and slower growth rate than the other cells within the biofilm. For example, DNA replication and protein synthetic activity, which can be measured using immunofluorescent detection of pulse-labeled DNA (BrdU labeling) and an inducible green fluorescent protein (*gfp*) construct respectively, show anabolic metabolism. In *S. aureus* and *Staphylococcus epidermidis* biofilms grown aerobically on a membrane, the anabolic zone at the surface was correlated with the oxygen penetration depth as measured by microelectrodes (162) (Figure 2.5). Apart from another thin anabolic zone at the substratum where the biofilm met the membrane and had access to nutrients, the rest of the biofilm was metabolically inactive (162) (Figure 2.5). In another example, *Pseudomonas putida* and *P. aeruginosa* biofilms carrying a growth rate-dependent fluorescent reporter fusion *rrnBP1-gfp*[AGA], showed that cells in the center of

microcolonies in flow cell biofilms decreased in fluorescence intensity once a certain threshold size had been reached (163, 164). These results indicated that the growth activity of cells in the microcolony center was slower than at the microcolony periphery, which has significant implications for the antibiotic tolerance of biofilm cells. For example, antibiotics that target actively growing cells, e.g. ciprofloxacin, tetracycline and tobramycin, are less effective against cells in the interior of the biofilm. In order to target the slow growing subpopulation of the biofilms, a drug with a different mode of action has to be used. An example of a drug that is efficient at targeting the biofilm interior is the polypeptide antibiotic colistin, which acts by solubilizing the bacterial outer membrane (164).

Figure 2.5. Staphylococci biofilms grown on membrane under aerobic conditions showing patterns of cell replication and protein synthetic activity. (A) *S. epidermidis* biofilm with BrdU incorporation and indicate areas with active replication (green). Rhodamine B stain (red) shows total biofilm biomass. (B) *S. aureus* biofilms induced for GFP expression to indicate protein synthetic activity (green) in air. A transmitted light image overlay shows extent of the biofilm biomass. Adapted from Rani et al., 2007 (162).

Besides the matrix contributing to physiological heterogeneity through the overall biofilm structure, different components of the matrix are found to differ in location and development of the biofilm. For example, multiple lectin stains

show that the matrix of microcolonies from river biofilms exhibit distinct binding regions, and may present microdomains that segregate extracellular activities at the microscale (34). The eDNA released in *P. aeruginosa* flow cell biofilms during early stage of biofilm formation localizes at the microcolony center as they become covered by the EPS (84). The Psl exopolysaccharide is mainly localized at the outer layer of the mature *P. aeruginosa* flow cell biofilm microcolonies, and plays an essential role in stabilizing the microcolonies (46). Interestingly, a recent study showed that the Pel exopolysaccharide is mainly localized at the center part of the microcolonies and is associated with the eDNA (59). The fact that the different components in the matrix can create different microenvironments for the cells and biofilms and thereby maintain physiological heterogeneity highlights the need to carry out biofilm investigations at the micro-scale.

2.4 Tools that probe biofilm structure and morphology

Microscopy has been one of the major tools to provide information of biofilm morphology, structure and even phylogeny (165). The most commonly used microscopic technique for biofilm research is confocal laser scanning microscopy (CLSM) (166). CLSM imaging is based excitation and detection of fluorophores placed within the biofilm, and thus employ fluorescent-tagged strains or fluorescent stains that selectively or preferentially bind to specific molecules or species in biofilms and give off emission. Another microscopic

technique used for imaging biofilms but does not rely on fluorescent-tagged strains or fluorescent stains and therefore can visualize biofilms in their natural and unperturbed state is optical coherence tomography (OCT) (167). These two techniques are discussed in further detail below.

2.4.1 Confocal Laser Scanning Microscopy

CLSM employs several laser lines (usually 488, 561 and 633 nm) that excite fluorophores placed within the biofilm (166). In conventional fluorescent microscopy, emission from excited fluorophores below and above the focal plane would also be detected, thus adding background noise to the image. However, CLSM is able to filter the fluorescence emitting from other planes via a pinhole and thus collects fluorescence from the selected focal plane only. A series of 2D images from different focal planes are then scanned and reconstructed to develop a 3D image of the biofilm. In this manner, CLSM is able to provide high quality 3D micrographs of thick samples and boast sub-micrometer resolution that can visualize individual bacterial cells.

In order to observe the location of cells in the biofilm, microbial strains are tagged with fluorescent proteins. The spatial regulation of specific genes within biofilms via fluorescence can also be observed through the transcriptional fusions of specific gene promoters to the fluorescent proteins (168). Live/dead stains such as SYTO62 and propidium iodide that bind to DNA depending on permeability of the cell membrane are useful for cell viability assays.

Another staining technique of particular importance is fluorescent *in situ* hybridization (FISH), which can assess the phylogenetic identity, morphology, spatial distribution and quantity of species in polymicrobial communities (169).

In FISH, fluorescently labeled oligonucleotide probes, which have been designed to be complementary to phylogenetic markers of 16S or 23S rRNA, hybridize with them in permeabilized microbial cells. These probes can be designed to target narrow or broad phylogenetic groups. After incubation and hybridization with the target, unspecific and unbound probes are washed away. The degree of homology between the probe and target, called the stringency of the hybridization, can be adjusted during incubation and washing steps. The biofilm is then observed with CLSM and images analyzed by specific FISH software, such as daime (<http://www.microbial-ecology.net/daime/>).

2.4.2 Optical coherence tomography

OCT is a non-invasive imaging technique that is able to penetrate deeply into thick biological samples and capture 3D images at micrometer resolution without the use of fluorescent-tagged strains and dyes (167). It can probe tissues as deep as 500 μm , which is about 5 times deeper than CLSM. The most advanced OCT instruments have sub-micrometer lateral resolution but axial resolution remains low at 10 μm . Thus unlike CLSM, it cannot visualize bacterial cells.

OCT uses a relatively long wavelength light that allows it to easily penetrate into the sample, such as near-infrared light, from a low-coherence light source to scan across the sample. In conventional microscopy, when an optical beam is directed at biological samples, much of the light is scattered at large angles and this diffusely scattered light is collected which obscures an image. In OCT however, a technique called low coherence interferometry is used to record the optical path length of received photons and reject photons that scatter multiple

times before detection, resulting in clear 3D images of the surface and sub-surface. Thus, OCT investigates the relative optical density distribution of the biofilm, which can be used to indicate density distribution of biofilm. OCT has successfully monitor changes to biofilm structure, formation of gas bubbles and biofilm degradation when flushed with H_2O_2 (170).

While the above techniques have been effective for probing biofilm structure, they are unable to measure the actual mechanical properties of the biofilm without incorporation of other techniques. The techniques that probe the mechanical properties of biofilms are discussed below.

2.5 Tools for biofilm micromechanical characterization

Rheology is the study of the flow or deformation of matter, and many classic mechanical studies of biofilms have focused on defining the rheological properties of such consortia (43, 44). This reflects the practice of growing biofilms under flow conditions, and hence the impact rheological properties will have on mass transfer and detachment processes. Another way to define rheology would be the study of how materials store and release mechanical energy over time as they deform. The stress, $\sigma(t)$ experienced by the material is used as a measure of energy stored by the material, and strain as a function of time, $\gamma(t)$ is used as a measure of deformation. Simple solids store energy, straining and stressing in phase, so that the response of one caused by the other is immediate and results in a spring-like, elastic response. Simple liquids are

able to release energy through viscous flow, and the strain lags stress by a 90° phase lag. Soft materials exhibit behavior in-between the two types of materials, exhibiting some lag in strain. The mechanical basis for deformation may be understood by looking at the way molecules reorganize themselves over time. For example, large molecules or molecular assemblies with high effective crosslinking within the structure are unable to release energy through free movement. When a low shear force is applied to a material, they temporarily deform and store energy as bonds are stretched. As long as the force does not involve breaking of bonds, the atoms do not slip past each other and the deformation reverses. In contrast, small molecular units of viscous fluids are in constant Brownian motion and able to move past each other to easily dissipate energy when force is applied to it. The resistance of the fluid to flow will depend on the strength of attractive forces between molecules, and kinetic energy of the molecules, which depends on the temperature of the system.

The rheology of biofilms is typically measured by mechanical bulk rheometers, which involve placing a sample in between two probing plates, or cone and plate, and applying oscillatory shear strain, $\gamma(t)$ and measuring the resultant shear stress, $\tau(t)$ (101). The oscillatory shear strain is given by:

$$\gamma(t) = \gamma_0 \sin(\omega t) \quad \text{Eq. 2-1}$$

where γ_0 is the initial strain, ω is the frequency of oscillation and t is time. The viscoelastic moduli, G^* can be used to represent the relations between the oscillating stress and strain, and is given by the equation:

$$G^* = G' + iG'' \quad \text{Eq. 2-2}$$

where G' is the storage moduli that behavior of elastic materials, and is the in-phase stress over initial strain:

$$G' = \tau' / \gamma_0 \quad \text{Eq. 2-3}$$

G'' is the loss moduli that dominates the behavior of viscous materials, and is the out-of-phase stress over initial strain:

$$G'' = \tau'' / \gamma_0 \quad \text{Eq. 2-4}$$

Bulk rheology has also been applied on biofilm streamers through application of shear forces to the biofilm via flow in the medium, and measurement of the deformation through the change in biofilm streamer length (44). This technique enabled the rheological properties of biofilms to be measured *in situ* under realistic flow conditions, and revealed that biofilms are elastic and viscoelastic solids at low shear conditions, but have viscoelastic fluid traits at high shear conditions.

Whilst bulk rheology is useful for measuring the overall viscoelasticity of the biofilm, biofilms are by nature heterogeneous. Thus, it is not possible to quantify the viscoelastic properties of the biofilm and to differentiate between the properties of streamers, microcolonies and the flat, undifferentiated parts of the biofilm. Techniques such as particle-tracking microrheology (171, 172) and Rheo-nuclear magnetic resonance (Rheo-NMR) however, are able to probe the local viscoelasticities of the biofilm at the micrometer scale and thus might be a more appropriate technique for rheological investigations of the biofilm. These

techniques are further discussed in Chapter 2.5.1.1 Particle-tracking microrheology and Chapter 2.5.1.2 Rheo-nuclear magnetic resonance.

At both molecular and cellular scales, mechanical forces impact on biological processes, play regulatory roles in gene expression, adhesion, migration and cell fate. For example, fibroblasts respond to mechanical forces on the surrounding collagen matrix through differential expression that promotes the synthesis of connective tissue and inhibition of matrix degradation, thereby increasing elasticity and strengthening the matrix. The genes involved encode for extracellular matrix proteins, fibrogenic growth factors, protease inhibitors, components of focal adhesions and the cytoskeleton, and are known as “mechanosensing genes” (173). In a similar fashion, biofilms are known to increase their elasticity in response to shear forces (43), indicating that mechanosensing is involved in biofilm regulation and EPS production.

Mechanobiology aims to elucidate the mechanisms by which cells sense, transduce, and respond to mechanical forces and thereby modulate their functions (174). Mechanobiology is also involved in the characterization of cellular mechanical properties. Thus far, mechanobiological tools have been developed for and extensively used in the study of mammalian cells, but have been less used in microbial research. Thus microbial research can benefit from the adaptation and application of these tools to our understanding of biofilm structure and function. In general, mechanobiology methods can be divided into active or passive methods (174). Active methods measure the biochemical or mechanical response of a sample to an applied force, also known as force application techniques. Passive methods sense the forces generated by the

sample through deformable substrates or probes, and are also termed as force sensing techniques. Force application techniques include a series of methods, such as micropipette aspiration (175), atomic force microscopy (AFM) (176, 177), optical tweezers (176, 178), magnetic tweezers (176, 179), acoustic tweezers (180), application of compression or shear through mechanical plates or fluid flow (181, 182), ultrasound stimulation (183, 184), electrical field stimulation (185), and micro-electromechanical systems (MEMS) (186). Force sensing techniques include micropillars (187, 188) as well as particle-tracking microrheology (171). AFM and MEMS can also be applied in passive mode to become force-sensing techniques. Micropillars are discussed in further detail in Chapter 2.5.2 Micropillars.

2.5.1 Rheological methods

2.5.1.1 Particle-tracking microrheology

In particle-tracking microrheology, fluorescent particles are placed into the biofilm and act as the mechanical probes. The motion of the particle is tracked by fluorescent microscopy (Figure 2.6A). The mean squared displacement (MSD or $\langle \Delta r^2 \rangle$) is calculated from the particle trajectories, where r is particle displacement, and the $\langle \Delta r^2 \rangle$ is the average of squared distance the particle travels within a certain duration t . For relatively short durations as compared to entire duration of the trajectory, there are many displacement data that can be used to calculate the MSD. For example, for a one min video of a single particle with a frame rate of one frame per sec, there are potentially up to 60 displacement data points for $t = 1$ s. With increasing duration however, less data points are available and the error of the MSD increases (Figure 2.6B). For

example, providing that the particle stays in focus for the entire video and particle trajectory is unbroken, for $t = 60$ s there can only be one displacement data point. To account for the increasing error of the MSD at long time-scales, the MSD is usually be calculated for times corresponding to less than a quarter of the total number of points in the trajectory (189). Multiple particle tracking will also help to increase the number of displacement data points for the MSD calculation (190).

Figure 2.6. (A) 2D particle trajectories of a particle in viscous and elastic medium respectively. The distance a particle explores in a viscous medium increases over time, but is constant in an elastic medium. Each color (navy, blue, cyan, green, yellow, orange, red) represents displacement of equal t . A single trajectory can be broken into several tracks as the particle move out of focus in the z-plane, particularly in viscous mediums. (B) There are many displacement data points that can be used within the trajectory when t is short, and thus error is low at at short time scales. When t is long, larger fractions of the trajectory are used as data points, reducing the number of data points available, resulting in higher errors at long time scales. Adapted from Chew, 2015 (191).

The MSD as a function of time, t ($\langle \Delta r^2(t) \rangle$) can then be analyzed to identify different modes of motion in different regions of the biofilm (Figure 2.7) (172).

For a particle undergoing Brownian motion in two dimensions, the diffusion coefficient (D) of the particle is related to the MSD by the equation:

$$\langle \Delta r^2(t) \rangle = 4Dt^\alpha \quad \text{Eq. 2-5}$$

where α is the diffusive exponent. The MSD has a linear dependence on t for normal diffusion in simple viscous fluids, where $\alpha = 1$. In viscoelastic substances such as polymer solutions, diffusion is random and the MSD does not grow as rapidly as in viscous fluids with $0 < \alpha < 1$. For elastic substances, the MSD is constant with time and $\alpha = 0$. When the MSD stops increasing at longer timescales, confined/corrallated diffusion is taking place and $0 < \alpha < 1$. Superdiffusion can occur by processes such as active transport or flow and results in an upward convex in the MSD curve at $\alpha > 1$. Furthermore, the MSD is inversely proportional to the polymeric crosslinking within the substance (192), which includes chemical bonds, ionic interactions (102, 193) and topological constraints from entanglements of polymer chains (194). These different interactions are collectively known as “effective crosslinking”. The MSD is proportional to the creep compliance, $J(t)$ of the material according to the relation (171, 195):

$$J(t) = \frac{3\pi d}{4k_B T} \langle \Delta r^2(t) \rangle \quad \text{Eq. 2-6}$$

where J = creep compliance, d = particle diameter, k_B = Boltzman constant, T = temperature. Furthermore, the unilateral, complex Fourier transform of the MSD as given in the equation below:

$$\langle \Delta \tilde{r}^2(\omega) \rangle = \int_0^{\infty} dt \exp(-i\omega t) \langle \Delta r^2(t) \rangle \quad \text{Eq. 2-7}$$

is related to the elastic storage $G'(\omega)$ and viscous loss $G''(\omega)$ moduli of the sample through the generalized Stokes-Einstein equation as given below:

$$G'(\omega) + iG''(\omega) = \frac{2k_B T}{3\pi d i \omega \langle \Delta \tilde{r}^2(\omega) \rangle} \quad \text{Eq. 2-8}$$

Figure 2.7. Time dependence of MSD for different diffusional behaviors. Superdiffusion results in an upward convex in the MSD curve and $\alpha > 1$. The MSD has a linear dependence on time in normal diffusion. In viscoelastic substances, anomalous or confined/corralled diffusion occurs due to impediment from chemical crosslinking, London forces, hydrogen bonds, electrostatic interactions or entangled polymers and $0 < \alpha < 1$. In elastic substances, diffusion is confined. This results in an MSD that is constant with time and $\alpha = 0$. Adapted from Ruthardt et al., 2011 (172).

2.5.1.2 Rheo-nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool that investigates structure, dynamics, reaction state, and chemical environment of

molecules based on specific nuclei within the sample that contains spin such as ^1H and ^{13}C . It is able to provide information of order and dynamics at the molecular level such as conformation exchange, phase changes, solubility and diffusion. NMR can also be use for detailed non-invasive imaging at the macroscopic level. NMR has been applied to biofilms to study various microbial processes such as in-vivo metabolic activity (196, 197), heavy metal adsorption (196, 198) and diffusion in the biofilm using the Pulsed Gradient Spin Echo (PGSE) technique (199, 200). In general, PGSE involves a pulsed magnetic field gradient or a sequence of pulsed gradients applied to the sample to orient the nuclei spins to the magnetic field and keep them in phase. During a short delay that allows the spins to return to equilibrium positions, dephasing occurs due spin-spin relaxation and inhomogeneities of the magnetic field. Another pulsed magnetic field gradient or sequence of pulsed gradients that enhances dephasing in a more defined way is applied and the dephasing rate monitored. Without diffusion, a complete re-focusing of all phases can be achieved. However, diffusion leads to an incomplete phase re-focusing and thus an attenuation of the observed NMR signal. The diffusion coefficient can be derived from the value of the signal attenuation.

In 1986, it was suggested by Martins *et al.*, that NMR methods could be used to measure other rheological properties of nematic liquid crystals at the macroscopic level (201). The physical reorientation of the nematic liquid crystal, which would result in realignment of the crystal director, inside the NMR magnet would result in slow evolution of the NMR spectrum, interpretation of which could yield rotational viscoelasticity. This method of ‘Rheo-NMR’ later applied by Goncalves *et al.*, using the ^1H spectra a nematic

liquid crystal (202). Currently, deformation plates like those used in bulk rheology are incorporated into the NMR sample tube. This produces NMR tubes or cells of various types: the cone-and-plate cell, concentric cylindrical Couette cell, four-roll mill cell and biaxial extension cell. These cells fit within the radiofrequency foil of an NMR microimaging probe head, and sample deformation driven by a drive shaft that sits in the bore of the magnet to form the Rheo-NMR device. The velocity field within the rheological cell can then be imaged by conventional NMR imaging, thus providing noninvasive insight of the local rate of sample deformation at the macroscopic level (203).

2.5.2 Micropillars

Micropillars are arrays of flexible, uniformly spaced, vertical cantilevers that can be used to spatially monitor traction forces produced by cells upon adhesion and migration on substrata (Figure 2.8A) (204, 205). In eukaryotic cells, the traction forces are generated by the cytoskeletal motor protein myosin II, which causes re-arrangement of actin filaments when coupled to adhesion sites of the cell (206, 207). To measure traction forces of a cell using micropillars, the cells are cultured on top of the micropillars, and the top and lower planes of the micropillars are imaged by video microscopy. Traction forces applied to the micropillars by the cells cause the micropillar tops to deflect (Figure 2.8B). The deflection of the micropillar, r , is given by:

$$r = \sqrt{[(x_t - x_b)^2 - (y_t - y_b)^2]} \quad \text{Eq. 2-9}$$

where x_t and y_t are x and y positions of the micropillar tops respectively, and x_b and y_b are x and y positions of the micropillar base respectively. Apart for deformation of the PDMS substratum that may result in base tilting or

dislocation of the micropillar (Figure 2.8C) (188), it is usually assumed that the base of the micropillar does not change in its original position as the micropillar is fixed to its substratum. Under such an assumption, it is common to use reference positions of undeflected free micropillars instead as the positions of the micropillar bases.

For micropillars with an aspect ratio (ratio of micropillar height to width) between 1.5-5.0, the deflection vector, r , is related to the horizontal traction force applied on the micropillar top, f , using the Euler-Bernoulli beam bending theory (207) and the following equation:

$$f = Kr \quad \text{Eq. 2-10}$$

where K is the spring constant of the micropillar and given by:

$$K = \frac{3\pi E d^4}{64 H^3} \quad \text{Eq. 2-11}$$

where E is Young's modulus, d is the diameter and H the height of the micropillars (Figure 2.8B). Micropillar diameters typically range from 1 to 5 μm and heights of 1 to 20 μm . For micropillars with an aspect ratio less than 1.5, the deflection of the micropillar is caused more by shearing (Figure 2.8C) and not bending as described in Eq. 2-10. For micropillars with an aspect ratio greater than 5.0, the beam bending can no longer be described by the linear approximation in Eq. 2-10 (188, 207).

Micropillars are usually made from elastomeric materials such as polydimethylsiloxane (PDMS). The micropillars are developed by replica molding of master cantilever templates that are made from silicon wafers using

photolithography techniques. This master may be a negative template (silicon substrate with micro-sized holes) for direct casting of the PDMS micropillars. Alternatively, a positive template (silicon micropillars) may be used to cast additional PDMS negative masters. The micropillars are then cast from these PDMS negative masters instead, which reduce wear and tear on the expensive silicon master. Furthermore, to prevent the elastomer from sticking to the master, the master is usually silanized prior to casting the PDMS. Once the micropillars are fabricated, they are usually cleaned and made hydrophilic by UV ozone treatment. Extracellular matrix proteins may be placed onto the micropillar tops via microcontact printing to help the cell attach to the micropillars. They may also be fluorescently stained to enable detection and determination of micropillar position by fluorescence microscopy.

Micropillars are not expected to detect traction forces generated within a single bacterium as microbial cells are also too rigid (208) as compared to mammalian cells to deform extensively and generate high traction forces within the cell body. Additionally, most laboratory microbial cells are too small to attach multiple micropillars, which is required to generate traction forces and deflection. However, it is possible to use micropillars to study the growth, development and reorganization of bacterial cells in multicellular structures such as biofilms. Previously, micropillar arrays have been able to measure the cooperative retraction forces by bundled type IV pili from a multicellular cluster of *Neisseria gonorrhoeae* (209). Whilst a homogeneous biofilm that grows uniformly around the micropillars and applies uniform pressure on all sides of the micropillars will not produce micropillar deflections, heterogeneous

growth of biofilm are likely to exert pressure differences and generate deflections. Micropillars may also be able to measure the drag on the biofilm from overlying fluid flow when biofilms are grown under flow conditions.

Figure 2.8. (A) A fibroblast (red) sits on top of a micropillar array and subcellular forces from the cell can be measured and spatially mapped by micropillar deflections. (B) Side view of a deflected micropillar. Deflection r is caused by force F from the cell. Micropillar has height H , diameter D and Young's modulus E . (C) Micropillar bending and shear, as well as base tilting and dislocation from substrate deformation contribute to the total deflection of the micropillar. For micropillars with aspect ratios of 1.5-5.0, deflection from micropillar shear is usually ignored. Adapted from Schoen et al., 2010 (188).

3 A spatiotemporal study of the rheological contributions of Pel and Psl in mucoid and non-mucoid *Pseudomonas aeruginosa* biofilms with implications for *Staphylococcus aureus* integration

3.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of nosocomial infections (210). It is able to use three different types of exopolysaccharides as major components of its biofilm matrix. Alginate is often over-expressed in *P. aeruginosa* isolates from cystic fibrosis patients, giving them a mucoidy phenotype (211). The over-expression of alginate protects *P. aeruginosa* from host immune responses and antibiotics (212). However, alginate is not required for biofilm formation in vitro and is often not found in environmental isolates of *P. aeruginosa* (213). In contrast, Psl and Pel have been found to play important roles in biofilm formation (58, 214). Psl is known to be important for attachment and development of surface-attached biofilms (215), whereas Pel has been associated with pellicle formation at the air-liquid interface of standing cultures (58, 214). This indicates that the two polysaccharides differ in structure, density and mechanical properties of their

polysaccharide gel. Rheological methods are appropriate for the investigation of the structure of such polymer gels and poly-disperse solutions. It is hypothesized here that different EPS components may play different rheological roles at different locations and time points of the biofilm. Previous rheological experiments have not mapped local viscoelasticities to specific biofilm structures present within the heterogeneous biofilm, and did not connect molecular components to the rheology of the biofilm (101, 216, 217). Here, passive microrheology was introduced as a tool to investigate the rheological roles of Pel and Psl during development in mucoid and non-mucoid strains of *P. aeruginosa* to provide a more quantitative description of how different matrix components contribute to biofilm structure. The biofilm resistance to shear stress, measured by the extent of streamer formation, and the incorporation of other species into the biofilm in a polymicrobial biofilm model of *P. aeruginosa* and *Staphylococcus aureus*, as a result of changes in biofilm structure by Pel and Psl, were also explored.

3.2 Methodology

3.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 3.1 and were mucoid *P. aeruginosa* strains $\Delta mucA$ (218), and mutants deficient in the production of Psl and or Pel, $\Delta mucA\Delta pelA$ (218), $\Delta mucA\Delta pslBCD$ (218) and $\Delta mucA\Delta pelA\Delta pslBCD$ (218); non-mucoid *P. aeruginosa* strains PAO1 (219), $\Delta pelA$ (48) and $\Delta pslBCD$ (48). Fluorescently tagged strains were constructed via the insertion of a mini-Tn7-eGFP-Gmr cassette as described previously

(220). Overnight cultures of *P. aeruginosa* were grown in 100% Luria Bertani (LB) medium (10 g L⁻¹ NaCl, 5 g L⁻¹ yeast extract and 10 g L⁻¹ tryptone) at 37°C with shaking (200 RPM). Overnight cultures of *S. aureus* 15981 were grown in 100% Tryptic Soy Broth (TSB) medium (17 g L⁻¹ casein peptone, 2.5 g L⁻¹ K₂HPO₄, 2.5 g L⁻¹ glucose, 5 g L⁻¹ NaCl and 3 g L⁻¹ soya peptone).

Table 3.1. List of bacterial strains

Genotype	Phenotype	Description	Reference
<i>P. aeruginosa</i> PAO1Δ <i>mucA</i>	Alg ⁺ Pel ⁺ Psl ⁺	Over-expressing alginate	(218)
<i>P. aeruginosa</i> PAO1Δ <i>mucA</i> Δ <i>pelA</i>	Alg ⁺ Pel ⁺ Psl ⁺	Over-expressing alginate, absence of Pel	(218)
<i>P. aeruginosa</i> PAO1Δ <i>mucA</i> Δ <i>pslB</i> <i>CD</i>	Alg ⁺ Pel ⁺ Psl ⁻	Over-expressing alginate, absence of Psl	(218)
<i>P. aeruginosa</i> PAO1Δ <i>mucA</i> Δ <i>pelA</i> Δ <i>pslBCD</i>	Alg ⁺ Pel ⁺ Psl ⁻	Over-expressing alginate, absence of Pel and Psl	(218)
<i>P. aeruginosa</i> PAO1	Alg ⁻ Pel ⁺ Psl ⁺	Minimal or absence of alginate, wild type	(219)
<i>P. aeruginosa</i> PAO1Δ <i>pelA</i>	Alg ⁻ Pel ⁺ Psl ⁺	Minimal or absence of alginate, absence of Pel	(48)
<i>P. aeruginosa</i> PAO1Δ <i>pslBCD</i>	Alg ⁻ Pel ⁺ Psl ⁻	Minimal or absence of alginate, absence of Psl	(48)
<i>S. aureus</i> 15891		Wild-type	(39)

3.2.2 Cultivation of flow cell biofilms

Overnight *P. aeruginosa* cultures were diluted to a concentration of OD₆₀₀ 0.4 and 350 μL of were the diluted cultures injected into flow cell channels with

dimensions of 1 mm depth, 4 mm width and 40 mm length. The bacteria were allowed to attach to the coverslip substratum for 1 h before M9 medium (48 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 19 mM NH_4Cl , 9 mM NaCl , 2 mM MgSO_4 and 0.1 mM CaCl_2) supplemented with 0.04% glucose (wt/vol) and 0.2% (wt/vol) casamino acids was fed into the chambers at an average flow rate of $5.55 \times 10^{-3} \text{ m s}^{-1}$ using the MASTERFLEX L/S 0752380 peristaltic pump. The biofilm streamers were grown at room temperature 25°C for up to 5 days. For the development of biofilm streamers, a set of steel matrices with mesh size $262.5 \mu\text{m}$ height and $200 \mu\text{m}$ width, and wire thickness of $40 \mu\text{m}$ was fitted into the channels of the flow cell chamber. The bacterial strains allowed to attach to the steel matrices for 1 h before M9 medium was fed into the chambers at a flow rate of $5.55 \times 10^{-3} \text{ m s}^{-1}$ using the MASTERFLEX L/S 0752380 peristaltic pump. The biofilm streamers were grown at room temperature 25°C for up to 4 days. The flow system was prepared as described previously (Figure 3.1) (221).

Figure 3.1. Schematic of the flow cell system: a, medium bottle; b, peristaltic pump; c, bubble trap; d, flow cell; e, waste container. Silicone tubing connects the components. Reproduction from Sternberg et al., 2005 (221).

3.2.3 Confocal laser scanning microscopy

The particle distribution and morphology of biofilms were visualized by CLSM (ZEISS LSM780 Confocal System). Gfp-tagged strains were detected using an argon laser for excitation at 488 nm and detection wavelengths at 500–530 nm; The 1.0 μm particles were detected using a DPSS-561 nm laser for excitation and detection wavelengths at 615-700 nm; 0.5 μm particles were detected using a DPSS-561 nm laser for excitation and detection wavelengths at 580-605 nm; 0.2 μm particles were detected using a argon laser for excitation at 514 nm and detection wavelengths at 550-575 nm. The confocal images were reconstructed and analyzed by the Imaris software package (Bitplane, AG).

3.2.4 Particle-tracking microrheology

Fluorescent particles (Invitrogen, CA) with a diameter of 1.0, 0.5 and 0.2 μm were washed with sterile H_2O and centrifuged at 9391.2 g for 5 min for removal of sodium azide present in the stock solution of the particles. Fluorescent particles were then re-suspended in M9 medium to final concentrations of 2.18×10^6 particles mL^{-1} and fed into the biofilms to allow for incorporation of the particles into the matrix during the course of biofilm development. The motion of the particles as driven by thermal energies ($k_{\text{B}}T$) were recorded using fluorescent microscopy (ZEISS Axio Imager M1) in videos ranging from 1-3 min with a frame rate of approximately 30-75 frames per second. The exposure time of 3 min was short enough to minimize dynamic error, which is usually caused by microscope stage drift. The static error was estimated to be $10^{-5} \mu\text{m}^2$. The dynamic and static error was estimated by monitoring 1.0 μm particles immobilized in 5% agar. The two dimensional particle trajectories were

computed from the videos using public software at <http://physics.georgetown.edu/matlab/>. The particle trajectories were then used to determine the evolution of particle MSDs as time, t progressed. By monitoring the evolution of particle MSDs over t , the viscoelastic properties of the biofilm can be measured. The particle MSD is proportional to the creep compliance as described in Eq. 2-6 in 1.5.1 Particle-tracking microrheology. Low MSD values are a consequence of high degrees of effective crosslinking (192, 195), which may be chemical from covalent bonds, physical interactions such as ionic bonds, and topological constraints from polymer chain entanglement. The two dimensional particle MSDs and creep compliances were calculated from the combined particle trajectories of 5 videos using MATLAB scripts provided by Singapore-MIT Alliance Research Technology.

3.3 Results

3.3.1 Distribution and size selective uptake of particles by the matrix

Mucoid *P. aeruginosa* biofilm cells expressing various combinations of the Psl and Pel exopolysaccharides were grown in a flow cell setup using media containing 1.0, 0.5 and 0.2 μm fluorescent and hydrophilic carboxylated particles. Consistent with previous observations (218), the morphology and development of *P. aeruginosa* ΔmucA (Figure 3.2A) and $\Delta\text{mucA}\Delta\text{pelA}$ (Figure 3.2B) biofilms were similar, forming a thin biofilm with small microcolonies or cell clusters by day 3. The $\Delta\text{mucA}\Delta\text{pslBCD}$ biofilms were less differentiated in morphology and delayed in biofilm development, forming a thicker but flat

biofilm that developed microcolonies only after day 3 (Figure 3.2C). The majority of the $\Delta mucA \Delta pelA \Delta pslBCD$ cells remained in the planktonic phase and was unable to form a biofilm on the coverslip from days 3 to 5 (Figure 3.2D). Mucooid *P. aeruginosa* is known to convert back to its non-mucooid state after long periods of biofilm cultivation (222). To estimate the percentage of non-mucooid revertants in the strains, cells from 5 d biofilms were harvested and colony morphologies observed. All colonies generated by the strains retained their mucooid phenotypes (Figure S3.1, Appendix).

Figure 3.2. Confocal images of day 5 biofilms formed by (A) $\Delta mucA$ (B) $\Delta mucA \Delta pelA$ (C) $\Delta mucA \Delta pslBCD$ strains after being supplemented with 1.0 μm (purple), 0.5 μm (red), 0.2 μm (orange) particles. Biofilms were not formed by (D) $\Delta mucA \Delta pelA \Delta pslBCD$ strains, and most particles were attached to the substratum. Microcolonies, plains and voids are labeled in (A) as an example. Reproduction from (223) with permission from mBio.

Particles were distributed throughout the three biofilm-forming strains, with a greater incorporation of the larger particles over the smaller particles within the microcolonies (Figure 3.3, Figure 3.4). The proportion of 0.5 and 0.2 μm particles were compared to the 1.0 μm particles in the three biofilm forming strains at days 4 and 5 of biofilm development (Figure 3.4A). The ΔmucA biofilm had 2.02 ± 0.41 more 1.0 μm particles than 0.5 μm particles and 4.13 ± 1.24 more 1.0 μm particles than 0.2 μm particles. The $\Delta\text{mucA}\Delta\text{pelA}$ biofilm had 1.85 ± 0.55 more 1.0 μm particles than 0.5 μm particles and 4.81 ± 1.12 more 1.0 μm particles than 0.2 μm particles. The $\Delta\text{mucA}\Delta\text{pslBCD}$ biofilm, which was less differentiated and impaired in biofilm development, displayed less size-selectivity, with 1.52 ± 0.31 more 1.0 μm particles than 0.5 μm particles and 2.44 ± 0.73 more 1.0 μm particles than 0.2 μm particles.

Figure 3.3. Day 5 microcolonies of biofilm forming strains with particle feeding delayed to day 3. (A) The top-left image is the xy-section at top part of microcolony and top-right image is the xy-section at bottom part of the microcolony formed by the ΔmucA strain. The bottom image is the side view of the microcolony with top and bottom positions of in the z-plane. (B) xy-section of top part of $\Delta\text{mucA}\Delta\text{pelA}$ microcolony (C) xy-section of top part of $\Delta\text{mucA}\Delta\text{pslBCD}$ microcolony. Reproduction from (223) with permission from mBio.

To investigate the effect of surface chemistry of particles on their assimilation

into the biofilm, 1.0 μm sulfated particles, which are hydrophobic, were fed to the biofilm and their numbers compared to 1.0 μm carboxylated particles. However, there was no significant difference in the uptake of sulfated and carboxylated particles (Figure 3.4B). By delaying the feeding of particles into the biofilm to day 3, it was found that particles were absent in areas where the biofilms would have already been formed before feeding, such as the bottom layer of the biofilm and the centers of microcolonies. Instead, they were only incorporated in more recent areas of growth such as the top layer of the biofilm, and the exterior of microcolonies (Figure 3.3A). This indicated that the particles entered the biofilm by attachment at the surface and were trapped in the biofilm as the matrix and cells grow around them.

Figure 3.4. Proportion of different types of particles (z-axis) with biofilms on days 4 and 5 for different strains (x-axis). Particle numbers (y-axis) were standardized to one hundred particles of 1.0 μm size and carboxylate modification. (A) The number of 0.5 (red) and 0.2 (orange) μm carboxylated particles to a hundred 1.0 (purple) μm carboxylated particles. (B) The number of 1.0 μm sulfated particles (blue) to a hundred 1.0 μm carboxylated particles (purple). Reproduction from (223) with permission from mBio.

3.3.2 Psl increases effective crosslinking and makes the biofilm elastic, Pel reduces effective crosslinking of biofilm

The viscoelastic properties of the biofilms formed by strains with different combinations of polysaccharides were explored by following the MSD and the creep compliance of the 1.0 μm and 0.5 μm particles embedded within the plains (undifferentiated layers of cells) and microcolonies (see Figure 3.2A for position labels). The particles embedded in the biofilm vibrated at fixed positions, indicating a densely effectively crosslinked viscoelastic to elastic material, whereas particles in void medium moved freely in viscous medium and exhibited Brownian motion (Figure 3.5). The MSD of the particles in the voids (medium) was used as a standard for a viscous material. The temporal changes in viscoelastic properties as the biofilms matured from days 3 and 5 were also quantified.

Figure 3.5. Trajectory of a particle executing Brownian motion in void medium. The inset shows the trajectory of a particle vibrating in the microcolony. Reproduction from (223) with permission from mBio.

The ΔmucA biofilm formed small cell clusters and microcolonies out of a thin plain on day 3. On day 5, the microcolonies had developed large distinct

mushroom structures, indicative of mature microcolonies. Particle-tracking microrheology on the $\Delta mucA$ biofilm was conducted on microcolonies on day 3, mature microcolonies on day 5 and for the plains on day 5. The plain on day 3 was too thin for investigation of rheological properties without significant capillary effects. The MSDs of the 1.0 μm particles were independent of time, which is a characteristic of elastic behavior and were in the order of $10^{-4} \mu\text{m}^2$, indicating that the $\Delta mucA$ biofilm was densely effectively crosslinked (Figure 3.6A). There was a reduction of effective crosslinking density in the microcolonies as they matured from day 3 to day 5. The median creep compliances calculated based on the MSDs were $4.3 \times 10^{-2} \text{ Pa}^{-1}$ for day 3 microcolonies and $2.5 \times 10^{-1} \text{ Pa}^{-1}$ for day 5 microcolonies. The effective crosslinking of day 5 plains was $4.1 \times 10^{-2} \text{ Pa}^{-1}$, which was close to that of day 3 microcolonies, and could be reflective of their less differentiated state.

The development of $\Delta mucA\Delta pelA$ biofilms was similar to $\Delta mucA$ biofilms, and hence day 3 microcolonies, day 5 microcolonies and day 5 plains were investigated. The MSD of the 1.0 μm particles in the microcolonies and the plains were independent of time, indicating that they were elastic. They were also lower than the MSDs in $\Delta mucA$ biofilms, indicating that $\Delta mucA\Delta pelA$ biofilms were more effectively crosslinked than $\Delta mucA$ biofilms. In contrast to $\Delta mucA$, there was no change in MSD as the microcolonies matured. Thus, the viscoelastic properties of the $\Delta mucA\Delta pelA$ biofilms remained similar spatially and temporally. The median creep compliances calculated from the MSDs were $3.6 \times 10^{-2} \text{ Pa}^{-1}$ for day 3 and 5 microcolonies, and $3.2 \times 10^{-2} \text{ Pa}^{-1}$ for day 5 plains.

Figure 3.6. MSDs (left axis) and creep compliance (right axis) of $1.0\ \mu\text{m}$ particles according to location and biofilm age in (A) ΔmucA (B) $\Delta\text{mucA}\Delta\text{pelA}$ (C) $\Delta\text{mucA}\Delta\text{pslBCD}$ (D) $\Delta\text{mucA}\Delta\text{pelA}\Delta\text{pslBCD}$ biofilms, and $0.5\ \mu\text{m}$ particles in (E) ΔmucA (F) $\Delta\text{mucA}\Delta\text{pelA}$ (G) $\Delta\text{mucA}\Delta\text{pslBCD}$ biofilms. (H) MSDs of $1.0\ \mu\text{m}$ particles in $\Delta\text{mucA}\Delta\text{mucA}\Delta\text{pelA}$ and $\Delta\text{mucA}\Delta\text{pslBCD}$ microcolonies. Reproduction from (223) with permission from mBio.

The $\Delta mucA\Delta pslBCD$ strains formed the least differentiated biofilms, consisting of thick plains that allowed for particle-tracking microrheology on days 3 and 5. Microcolonies did not develop by day 3, and thus particle tracking was done on microcolonies at day 5. The plains were much less crosslinked than the plains of the other strains, and had a diffusive exponent $\alpha < 1$, indicating that the biofilm was viscoelastic (Figure 3.6C). The MSD of the microcolonies was lower than that of the plains. The median creep compliances were 1.0 Pa^{-1} for day 3 and 5 plains, and $2.8 \times 10^{-1} \text{ Pa}^{-1}$ for day 5 microcolonies.

$\Delta mucA\Delta pelA\Delta pslBCD$ strain did not form biofilms. A few $1.0 \text{ }\mu\text{m}$ particles were trapped in a loose extracellular material secreted by these cells. The MSD of the $1.0 \text{ }\mu\text{m}$ particles showed a concave shape, indicative of particle confinement (Figure 3.6D).

Microrheology using $0.5 \text{ }\mu\text{m}$ particles was conducted on the various biofilm-forming strains in a similar fashion as for the $1.0 \text{ }\mu\text{m}$ particles. The MSDs of the $0.5 \text{ }\mu\text{m}$ particles in the microcolonies for all strains were lower as compared to the $1.0 \text{ }\mu\text{m}$ particles, indicating that they had become embedded in areas of greater effective crosslinking density as reflective of their smaller size (Figure 3.6E-G). Interestingly, the MSD of the $0.5 \text{ }\mu\text{m}$ particles exhibited more viscous behavior at longer timescales, particularly in the $\Delta mucA$ day 3 microcolonies and $\Delta mucA\Delta pelA$ microcolonies. It is possible that the $0.5 \text{ }\mu\text{m}$ particles were able to pass through the matrix over a long time scales. Surprisingly, the MSD of the $0.5 \text{ }\mu\text{m}$ particles in the $\Delta mucA$ microcolonies at day 3 did not increase by day 5 as was observed for the $1.0 \text{ }\mu\text{m}$ particles, which would have indicated a loss of effective crosslinking (Figure 3.6A,E). However,

the MSD was similar to the MSD of the 0.5 μm particles in the $\Delta\text{mucA}\Delta\text{pslBCD}$ expressing microcolonies in day 5, and supported the conclusion that the rheological contribution of Pel relative to Psl was greater with the microcolony (Figure 3.6G). There were less 0.5 μm particles in the centers of the microcolonies than at the periphery (Figure 3.3), and thus the reduction of effective crosslinking as microcolonies matured as observed for the 1.0 μm particles may only have occurred at the microcolony centers. The 0.5 μm particles did not accumulate in $\Delta\text{mucA}\Delta\text{pelA}\Delta\text{pslBCD}$ biofilms.

To validate whether Psl effectively crosslinked the biofilm more than Pel and that the microcolonies reduced their effective crosslinking as they matured in the absence of alginate, the microrheology of microcolonies of the wild-type PAO1 strain and the mutant strains ΔpelA and ΔpslBCD were investigated using 1.0 μm particles. The day 3 microcolonies of ΔpelA biofilms were the most effectively crosslinked of the strains. The microcolonies of PAO1 showed a reduction in effective crosslinking as they progressed from days 3 to 5. The day 5 microcolonies of the ΔpslBCD mutant were less effectively crosslinked than day 3 microcolonies of PAO1 and ΔpelA biofilms. However, they were more effectively crosslinked than day 5 microcolonies of PAO1 biofilms, which could be reflective of their delayed development. As such, the results indicate that Psl increases the effective crosslinking and makes the biofilm elastic, whereas Pel loosens the biofilm and builds a viscoelastic matrix. In biofilms that express both Pel and Psl exopolysaccharides, Psl has greater contribution to the rheology at the beginning of biofilm development and Pel increases in contribution at later stages of biofilm development.

3.3.3 Rheological contributions determine biofilm resistance to shear and impact streamer formation

Elastic materials reversibly deform in response to force, and thus return to their original shape after force is removed. In contrast, viscous materials irreversibly deform in response to force, increasing the material's tendency to shear. In the case of biofilms, shearing of the EPS often results in the development of streamers. Thus, it is expected that Psl would increase biofilm resistance to shear and decrease streamer formation, whereas Pel would make the biofilm more malleable and facilitate streamer formation.

Figure 3.7. Confocal images of (A) $\Delta mucA$ (B) $\Delta mucA\Delta pelA$ (C) $\Delta mucA\Delta pslBCD$ (D) $\Delta mucA\Delta pelA\Delta pslBCD$ biofilms in the streamer assay. The direction of flow was parallel to the steel matrices and from left to right. Inset shows enlarged view of biofilm. Reproduction from (223) with permission from mBio.

During the cultivation of *P. aeruginosa* exopolysaccharide mutant biofilms on a set of steel matrices, $\Delta mucA$ cells initially formed rough surface-attached biofilms. In the later stages, the biofilms became smooth with enhanced

spreading (Figure 3.7A). The $\Delta mucA\Delta pelA$ strain formed rough, surface-attached biofilms that developed large microcolonies with minimal spreading (Figure 3.7B). $\Delta mucA\Delta pslBCD$ strains formed smooth biofilms with extensive streamer formation that extended between the meshwork. In the later stages, the mesh became clogged with the biofilms (Figure 3.7C). The $\Delta mucA\Delta pelA\Delta pslBCD$ formed little or no biofilm on the steel matrices (Figure 3.7D).

3.3.4 Effective crosslinking and species integration within biofilm communities

The ecological impact of the rheological differences conferred by the exopolysaccharides was explored within a dual-species biofilm comprised of *P. aeruginosa* and *S. aureus*. Specifically, it was hypothesized that the dense effective crosslinking conferred by Psl may reduce integration of other species into *P. aeruginosa* biofilms, whereas a looser matrix conferred by Pel would increase association between two species. Co-cultures of the *P. aeruginosa* $\Delta mucA$ strain and *S. aureus* formed dual-species biofilms composed of two distinct upper and bottom parts. At the bottom part, the $\Delta mucA$ strain associated with *S. aureus* in dual-species microcolonies. At the top part, *P. aeruginosa* was dominant and formed a monospecies layer that capped the microcolonies (Figure 3.8A).

The *P. aeruginosa* $\Delta mucA\Delta pelA$ strain formed microcolonies at the substratum isolated from *S. aureus*, while the $\Delta mucA\Delta pelA$ strain also dominated the upper part of the biofilm, forming a monospecies *P. aeruginosa* layer on microcolonies from both species (Figure 3.8B). The *P. aeruginosa*

$\Delta mucA\Delta pslBCD$ strain was closely associated with *S. aureus* and formed a mixed-species layer with a less pronounced monospecies *P. aeruginosa* cap (Figure 3.8C). The *P. aeruginosa* $\Delta mucA\Delta pelA\Delta pslBCD$ strain did not associate with *S. aureus* and formed small cell clusters. Interestingly, the amount of *S. aureus* was the least when co-cultured with the mutant $\Delta mucA\Delta pelA\Delta pslBCD$ (Figure 3.8D). Thus, Pel was required for the association of *P. aeruginosa* and *S. aureus* in the matrix of dual-species biofilms, while Psl increased the association of *P. aeruginosa* with its own cells, thereby promoting segregation of the two species in the dual-species biofilms. Interestingly without the two exopolysaccharides, *P. aeruginosa* completely outcompeted *S. aureus*.

Figure 3.8. Dual-species biofilms of (A) *P. aeruginosa* $\Delta mucA$ and *S. aureus* (B) *P. aeruginosa* $\Delta mucA\Delta pelA$ and *S. aureus* (C) *P. aeruginosa* $\Delta mucA\Delta pslBCD$ and *S. aureus* (D) *P. aeruginosa* $\Delta mucA\Delta pelA\Delta pslBCD$ and *S. aureus*. *P. aeruginosa* was Gfp-tagged (green) and *S. aureus* was stained with SYTO62 (red). Reproduction from (223) with permission from mBio.

3.4 Discussion

The Pel and Psl exopolysaccharides are major matrix components that are known to contribute to biofilm formation in *P. aeruginosa*. It has been suggested that biosynthesis pathways for Psl and Pel compete with each other for common sugar precursors and that there is differential expression of the exopolysaccharides (50). However, the regulatory conditions for expression of one exopolysaccharide over another, and the distinct roles of the exopolysaccharides is yet unclear. Here, particle-tracking microrheology was used to reveal distinct rheological roles of Pel and Psl polysaccharides and that *P. aeruginosa* can remodel the local mechanical properties of its matrix by differential production of these two exopolysaccharides. Specifically, $\Delta mucA\Delta pelA$ biofilms were elastic and dense with increased effective crosslinking (Figure 3.6B,F), whereas $\Delta mucA\Delta pslBCD$ biofilms were found to be viscoelastic and exhibited less effective crosslinking than the $\Delta mucA\Delta pelA$ biofilms (Figure 3.6C,G). Biofilms of the $\Delta mucA\Delta pelA$ and $\Delta mucA\Delta pslBCD$ mutants did not appear to change significantly in rheological properties across time. The $\Delta mucA$ biofilms expressing both Pel and Psl were elastic, with microcolonies dramatically decreasing in effective crosslinking as they matured, consistent with an increased presence of Pel over Psl in the matrix (Figure 3.6A,E). The reduction in effective crosslinking could be due to decreased expression, release or degradation of Psl at the microcolony center prior to dispersal. Although the generation of the Psl matrix-free cavity would have occurred in $\Delta mucA\Delta pelA$ microcolonies during dispersal, particles would have quickly been lost along with the biofilm without Pel supporting the matrix. Thus Psl increases effective crosslinking and makes the biofilm elastic, whereas

Pel loosens the biofilm. The rheological roles of Pel and Psl found in biofilms of the mucoid *P. aeruginosa* were similar for non-mucoid strains (Figure 3.6H).

The elasticity and increased effective crosslinking conferred by Psl resulted in a rigid biofilm with a deformation-resistant phenotype that resisted spreading and streamer formation but appeared to support microcolony development in a biofilm streamer assay (Figure 3.7B). On the other hand, the reduction of effective crosslinking by Pel increased the malleability of the matrix, which facilitated biofilm spreading and promoted the formation of biofilm streamers in absence of Psl (Figure 3.7A,C).

Using a *P. aeruginosa* and *S. aureus* mixed-species biofilm model, we found that Pel was required for close integration of the two species into mixed-species microcolonies (Figure 3.8A,C). In contrast, Psl was important for *P. aeruginosa* to form single-species biofilms on top of *S. aureus* biofilms (Figure 3.8B). It was also found that biofilms display defined size-selective incorporation of particles in the microcolonies, with preference for the larger 1.0 μm particle, (Figure 3.3, Figure 3.4), which has implications for particle retention in bacteria that are able to produce extracellular micro- or nanoparticles (224). It also has implications for the design of antimicrobial particles in drug delivery.

Hence, the rheological properties of Pel and Psl exopolysaccharides are linked to fundamental biofilm functions such as microcolony development, spreading, and inter-species interactions. A new model for *P. aeruginosa* biofilm remodeling, in which the Psl exopolysaccharide acts as stiff wires to build a dense effectively crosslinked and Pel builds a less effectively crosslinked and

viscoelastic matrix that facilitates lateral spreading of biofilm on the substratum, is proposed (Figure 3.9).

Figure 3.9. Illustration of streamer formation by *P. aeruginosa*, in which Psl forms the surface-attached streamer head and Pel forms the streamer tail. Psl acts as stiff wires that densely crosslinks the matrix, supporting growth fixed at the substratum such as microcolony development. Pel acts as a filler and spread, which is important for expanding to new areas during later stages of biofilm development. Reproduction from (223) with permission from mBio.

The elastic matrix by Psl could limit spreading to habitats and supports growth on-site and microcolony development, whereas the loosely crosslinked matrix by Pel could be important for colonization of new surfaces during the later stages. This would be in complement with previous studies that have shown that Psl is important for cell attachment (225), and initiation of biofilm and microcolony formation (35, 47), and Pel for pellicle formation (non-surface attached biofilm) (214). The model is based upon the early creep investigations of *P. aeruginosa* streamers in a bioreactor (44), and proposes that the firmly

surface-attached streamer head is largely constructed by Psl, whereas the freely oscillating streamer tail is largely constructed by Pel. The model could further validated by staining with Psl specific lectin, HHA from the *Hippeastrum hybrid* plant (226) and Pel specific lectin, WFL from *Wisteria floribunda* plant (59).

The ability of *P. aeruginosa* to switch between different exopolysaccharide production in response to growth conditions has important implications for microbial adaption. In addition, this study indicates that it is possible to exploit natural components of the matrix to alter the biofilm's physical properties. Such strategy may be used for biofilm control and engineering in various healthcare and industrial applications, for example through expression of a highly crosslinked and elastic matrix to limit biofilm proliferation by means of surface spreading and streamer formation, or to resist invasion of other microbial species.

4 Roles of extracellular matrix components in coordinating dual species biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus aureus*

4.1 Introduction

Biofilm formation is a dynamic and complex process which requires coordination of motility, cell-to-cell communication, EPS synthesis, cell-EPS interactions and physiological differentiation (227). To date, our understanding of biofilm formation has been mainly derived from the investigation of model monospecies biofilms. Although this has contributed significantly to our understanding of biofilm formation and function, bacteria in the natural environment most often exist as mixed species biofilm communities (7, 64, 228). For example, in multi-species biofilms such as dental plaque, interactions between different EPS components contributed by different bacterial species play an important role in the establishment of the community (21). In dental plaque, the tooth enamel is first colonized by *Streptococcus mutans* and other anaerobes, which then go on to produce levans and dextrans in the EPS that serve as binding sites for lectins of other bacterial species, thus establishing the early biofilm community (36). In multi-species biofilms involving *P. aeruginosa*, it has also been shown that changes in alginate and Psl production

have a significant impact on mixed species biofilm assembly and the community level resistance of that biofilm to stressors (229). Thus, the contribution of the biofilm matrix represents an important, yet largely under explored feature that mediates interspecies interactions and confers emergent properties to the community.

High levels of intracellular c-di-GMP facilitate matrix synthesis and biofilm formation, while low levels result in biofilm dispersal in *P. aeruginosa* (230). C-di-GMP is known to act on the FleQ transcriptional factor, mediating the derepression of the Pel biosynthetic operon (150), and also increasing expression of the CdrA adhesin that binds directly to Psl (56). The *psl* genes are constitutively expressed in *P. aeruginosa* PAO1, indicating that post-transcriptional mechanisms are involved in its regulation for this strain (230). Interestingly, Psl has been found to stimulate two diguanylate cyclases, SiaD and SadC, through an as yet unknown mechanism, to produce c-di-GMP and stimulate its own biosynthesis, demonstrating that apart from passive structural roles, matrix components can play active roles in EPS regulation and synthesis (47). *Staphylococcus spp.* are commensal bacteria carried by a high percentage of healthy individuals and often cause infections related to chronic wounds and implanted medical devices (231, 232). Both *P. aeruginosa* and *S. aureus* are found in the CF lung (233) and have been utilized as laboratory models for dual-species biofilms (39). *P. aeruginosa* and *S. aureus* have been observed to have an antagonistic relationship, where *P. aeruginosa* reduces the viability of *S. aureus* and is thought to involve *P. aeruginosa* killing by siderophores, as well other genetic, environmental and nutritional factors (234). Furthermore, another study shows that *S. aureus* decreases the transcription of iron-regulated

genes in *P. aeruginosa*, and suggests that *P. aeruginosa* lyses *S. aureus* to use them as an iron source (235). *P. aeruginosa* has been shown to eliminate *S. aureus* by inducing host production of type-II-secreted phospholipase A2, an enzyme with high bactericidal activity towards *S. aureus* but little effect on *P. aeruginosa* (236). Despite this, dual species infections by *P. aeruginosa* and *S. aureus* have been shown to be more virulent and/or result in worse patient outcomes than single-species infections (237-239). In one *in vitro* wound model, *P. aeruginosa* and *S. aureus* have been found to co-exist stably and have enhanced tolerance to antibiotics (240). In the study, host-derived and bacterium-derived matrix components was believed to be responsible for the enhanced tolerance toward antibiotics (240). *P. aeruginosa* is also able to protect *S. aureus* from phagocytosis of *Dictyostelium discoideum* (241).

Biofilms of *S. aureus* contain significant amounts of eDNA in its matrix (91). Similarly, eDNA is required in early stage biofilm formation of *P. aeruginosa* (84) and also involves two polysaccharides, Psl and Pel. In Chapter 3, particle-tracking microrheology showed that differences in the rheological properties of the major matrix polysaccharides Pel and Psl in *P. aeruginosa* affected microcolony building and surface spreading of the biofilm (223). It is also possible that the rheology of the matrix plays a role in multi-species biofilm structure. Therefore various mutants of *P. aeruginosa* that were defective in the production of specific matrix components Pel, Psl and CdrA, and mutants impaired in the production of c-di-GMP through SiaD and SadC diguanylate cyclases, were tested to determine their impact on microcolony structure, rheology and spatial organization of microbial species.

4.2 Methodology

4.2.1 Bacterial strains

The bacterial strains and plasmids used in this study are listed in Table 4.1. *P. aeruginosa* strains were tagged with either mini-Tn7-Strep-Gfp or quorum-sensing and c-di-GMP responsive reporter fusions.

Table 4.1 List of bacterial strains

Strains	Relevant characteristic(s)	Reference
<i>P. aeruginosa</i> PAO1	wild-type strain	(219)
<i>P. aeruginosa</i> PAO1 $\Delta pelA$	PAO1 that does not produce the Pel exopolysaccharide	(48)
<i>P. aeruginosa</i> PAO1 $\Delta pslBCD$	PAO1 that does not express the Psl exopolysaccharide	(48)
<i>P. aeruginosa</i> PAO1 $\Delta cdrA$	PAO1 lacking the extracellular adhesin CdrA	This study
<i>P. aeruginosa</i> PAO1 $\Delta sadC$	PAO1 lacking the membrane bound DGC SadC	This study
<i>P. aeruginosa</i> PAO1 $\Delta siaD$	Gm ^r ; <i>siaD</i> derivative of PAO1 lacking the membrane bound DGC SiaD	This study
<i>P. aeruginosa</i> PAO1 $\Delta sadC\Delta siaD$	PAO1 lacking the membrane bound DGCs SadC and SiaD	This study
<i>P. aeruginosa</i> PAO1/P _{<i>cdrA</i>} - <i>gfp</i>	PAO1 containing the P _{<i>cdrA</i>} - <i>gfp</i> reporter fusion	(242)
<i>P. aeruginosa</i> $\Delta sadC$ /P _{<i>cdrA</i>} - <i>gfp</i>	$\Delta sadC$ containing the P _{<i>cdrA</i>} - <i>gfp</i> reporter fusion	This study
<i>P. aeruginosa</i> $\Delta siaD$ /P _{<i>cdrA</i>} - <i>gfp</i>	$\Delta siaD$ containing the P _{<i>cdrA</i>} - <i>gfp</i> reporter fusion	This study
<i>P. aeruginosa</i> $\Delta sadC\Delta siaD$ /P _{<i>cdrA</i>} - <i>gfp</i>	$\Delta sadC/\Delta siaD$ containing the P _{<i>cdrA</i>} - <i>gfp</i> reporter fusion	This study
<i>S. aureus</i> 15981	wild-type strain	(39)

Overnight cultures of *P. aeruginosa* were grown in 100% LB medium (10 g L⁻¹ NaCl, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ tryptone) at 37°C with shaking (200 RPM). *S. aureus* was grown in 100% TSB medium (17 g L⁻¹ casein peptone, 2.5 g L⁻¹ K₂HPO₄, 2.5 g L⁻¹ glucose, 5 g L⁻¹ NaCl, 3 g L⁻¹ soya peptone) at 37°C with shaking (200 RPM).

4.2.2 Construction of *Pseudomonas aeruginosa* mutants

The $\Delta sadC$, $\Delta siaD$ and $\Delta sadC\Delta siaD$ mutants defective in production of SadC and/or SiaD diguanylate cyclase were constructed by homologous recombination as previously described (47). The $\Delta cdrA$ mutant defective for the CdrA adhesin was constructed by allelic exchange through lambda red recombinase with the Multisite Gateway (Thermo Fisher Scientific, MA) LR expression clone.

4.2.3 Cultivation of static biofilms

For biofilm cultivation, μ -Slide 8 well microscopy chambers (ibidi, Martinsried) were inoculated with 200 μ L of 1:100 diluted overnight cultures of *S. aureus*, or 1:1 (v/v) co-cultures of *S. aureus* and *P. aeruginosa* and incubated for 1 d at 37°C under static conditions. For DNase treatment, the cultures were treated with 200 u mL⁻¹ DNase I at 12 h post-inoculation. The cultures were reincubated at 37°C under static conditions for 10 h to form 22 h biofilms.

4.2.4 Biofilm image acquisition and analysis

Biofilms were visualized using Zeiss LSM780 Confocal Scanning Laser Microscopy. *P. aeruginosa* strains were unlabeled or Gfp-tagged. *S. aureus* 15981 was either Gfp-tagged or unlabeled. For some conditions, the wild-type

P. aeruginosa strains carried the c-di-GMP responsive reporter fusion $P_{cdrA}\text{-gfp}$ (242) in either the wild-type *P. aeruginosa* PAO1 or $\Delta sadC$ and $\Delta siaD$ mutants. Dead cells and eDNA were stained by SYTO62 (243) and YOYO-3 respectively. Gfp was detected using an argon laser for excitation at 488 nm and a broad pass emission filter at 500–530 nm. SYTO 62 and YOYO-3 were detected using a helium-neon laser for excitation at 633 nm and a low pass emission filter at 650 nm. Images were reconstructed using Imaris software package (Bitplane, AG), and the biovolumes calculated using COMSTAT (www.comstat.dk) (244, 245). The Particle Analyzer and Watershed tool in ImageJ was used for cell counting.

4.2.5 Cell and particle tracking

Cell motility and attachment kinetics during the first 18 h after inoculation were studied from a compilation of videos of cells at the substratum in monospecies *P. aeruginosa* culture, and dual-species *P. aeruginosa* -*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta pslBCD$ -*S. aureus* cultures. Videos were taken at 1 h intervals at 0.1 frames per second (fps) for 5 min with a 63 $\times$ objective using fluorescent and brightfield microscopy (ZEISS Axio Observer Z1). For particle tracking and microrheological experiments, fluorescent latex beads with 1.0 μm diameter and carboxylate modification (Invitrogen, CA) were dispersed in TSB to final concentrations of 18.2×10^6 particles mL^{-1} and medium was used to dilute the overnight cultures and grow biofilm. After particle incorporation into the biofilm, particle diffusion was followed using fluorescent microscopy with a 63 $\times$ objective (ZEISS Axio Observer Z1). The motion of particles in the co-cultures was captured in videos of duration 15 min and frame rate of 1-5 fps.

The particle trajectories were obtained with ImageJ (246) plugin Mosaic Particle Tracker (247). The trajectories were pooled together for each biofilm and the mean squared displacements (MSDs) were calculated and analyzed using msdalyzer (248), a MATLAB class for MSD analysis. For a particle undergoing Brownian motion in two dimensions, the diffusion coefficient of the particle, D is related to the MSD as described in Eq. 2-5 of Chapter 2.5.1.1 Particle-tracking microrheology. The values of D were extracted from the linear weighted fit to the MSD for the first 10% of the curve, with goodness of fit $R^2 = 0.96$. The microrheological properties of the sample are proportional to the MSD by $\text{MSD}(t) \propto t^\alpha$, where α is approximate or equal to 1 for viscous fluids, α is approximate or equal to 0 for elastic substances and α lies within intermediate values of 1 and 0 for viscoelastic substances. The values of α were extracted from the logarithmic fit to the MSD for a lag time range of approximately 0.1 s to 100 s. The R^2 values of the curves were greater than 0.96

4.3 Results

4.3.1 Interspecies interactions with *Staphylococcus aureus* initiate *Pseudomonas aeruginosa* clustering, and require both Pel and Psl

To examine how individual bacteria organize themselves into biofilms and the impact of the major polysaccharides of *P. aeruginosa* on the initial stage of biofilm formation, Gfp-tagged wild-type PAO1, and isogenic Pel and Psl mutants were co-cultivated with *S. aureus* 15981 and imaged by confocal

microscopy hourly for the first 18 h. Figure S3.1 depicts the cultures for the first 8 h of biofilm formation every 2 h and Figure 3.1 depicts the cultures during 10-18 h of biofilm formation every 2 h. There was an increase of *P. aeruginosa* cells at surface during first 8 h for all cultures (Figure S4.1) that eventually covered the surface to form a thin layer of cells by 10 h (Figure 4.1).

Figure 4.1. Cellular organization and growth of *P. aeruginosa* during biofilm formation with *S. aureus*. Representative confocal images of cultures of (A) wild-type *P. aeruginosa*, (B) wild-type *P. aeruginosa*-*S. aureus* (C) $\Delta pelA$ -*S. aureus* and (D) $\Delta pslBCD$ -*S. aureus* every 2 h during 10 to 18 h of biofilm formation. *P. aeruginosa* is Gfp-tagged and *S. aureus* are unlabeled.

After 10 h, for monospecies wild-type *P. aeruginosa* control cultures, aggregation into cell clusters was not observed although cracks in the cell layer appeared as it thickened, and were likely nascent void structures as cells left the

surface (Figure 4.1A) For wild-type and polysaccharide *P. aeruginosa* mutants co-cultured with *S. aureus*, *P. aeruginosa* cells were observed to aggregate together at 16 h. Cellular aggregation required some cells to leave the surface to enter into an increasingly dense multi-layered structure, thereby forming voids at their old positions, By 18 h, wild-type *P. aeruginosa* cells in the co-cultures were organized into distinct cell clusters surrounded by voids (Figure 4.1B). The $\Delta pelA$ cells in $\Delta pelA$ -*S.aureus* co-cultures did not cover the substratum as extensively as other strains during the 18 h of static cultivation (Figure 4.1C). This is consistent with the observation that expression of Psl reduces overall surface coverage as bacteria tend to stay only where Psl is deposited and follow Psl trails (35). Similar to wild-type *P. aeruginosa*, $\Delta pelA$ in $\Delta pelA$ -*S.aureus* co-cultures formed cell clusters at 16 h cells. Interestingly, several cell clusters were observed to disperse by 18 h (Figure 4.1C), which was not observed for the wild-type *P. aeruginosa* cultured with *S. aureus* (Figure 4.1B). $\Delta pslBCD$ cells in the co-cultures were unable to form stalks by 18 h and had highest surface coverage out of all the co-cultures (Figure 4.1D).

The bacterial cultures were imaged hourly for 18 h using a combination of video brightfield and epifluorescent microscopy for a more detailed investigation (Figure S4.2 and Figure 4.2). In monospecies wild-type PAO1 cultures and all dual-species *P. aeruginosa*-*S.aureus* co-cultures, *P. aeruginosa* displayed cycles of attachment and detachment after 4-5 h of inoculation (Figure S3.2). *S. aureus* remained attached up to 9-11 h of biofilm development, after which time, *S. aureus* started to detach in all co-cultures, as shown by the stable levels of *P. aeruginosa* numbers at the surface in the

fluorescent images but sudden drop in total attached cells in the brightfield images (Figure 4.2).

Figure 4.2. Fluorescent images showing little change in *P. aeruginosa* numbers at surface between 9 h and 11 h of biofilm development in monospecies *P. aeruginosa* and dual species biofilms. Brightfield images showing reduced number of total cells at 11 h as compared to 9 h of biofilm development in dual species biofilms. *P. aeruginosa* wild-type, Δ*pelA* and Δ*pslBCD* strains are gfp-tagged while the *S. aureus* strain is non-fluorescent.

The cells at the surface at 9 h before detachment and 11 h after detachment were counted and cell loss quantified (Table 4.2). *S. aureus* was not observed to re-attach back to the surface, and *P. aeruginosa* remained loosely associated with the surface for up to 18 h. Cells in control experiments of monospecies *S. aureus* culture did not detach at 9-11 h.

Table 4.2 Total cell loss at surface between 9-11 h of biofilm development.

<i>S. aureus</i>	wtPAO1- <i>S. aureus</i>	$\Delta pelA$ - <i>S. aureus</i>	$\Delta pslBCD$ - <i>S. aureus</i>
0% $\pm$ 0.3%	61% $\pm$ 6%	70% $\pm$ 3%	64% $\pm$ 6%

4.3.2 Competitive interspecies interactions facilitate biofilm formation in *Pseudomonas aeruginosa*, Psl-dependant microcolony formation and Pel-dependant enlargement of microcolonies

Cultures of *P. aeruginosa*-*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta pslBCD$ -*S. aureus* were incubated at 37°C for 1 d to further assess the final morphologies of the co-cultures after biofilms had been formed by confocal microscopy. As the loss of *S. aureus* was observed during the progression of biofilm formation, Gfp-tagged *S. aureus* and unlabeled *P. aeruginosa* were used to determine the proportion of *P. aeruginosa* to *S. aureus* (Figure 4.3). Monospecies cultures of *S. aureus* formed a thick biofilm (Figure 4.3). In contrast, very few *S. aureus* cells could be observed when co-cultured with either wild-type or exopolysaccharide mutants of *P. aeruginosa*. Brightfield imaging of the biofilms revealed that wild-type PAO1 and the $\Delta pelA$ mutant were able to form microcolonies, whereas the $\Delta pslBCD$ mutant could not. Based on the data presented above, *S. aureus* is able to facilitate biofilm formation of *P.*

aeruginosa, although during the later part of dual species biofilm formation, *S. aureus* appears to be killed.

Figure 4.3 Confocal and brightfield images of 1 d biofilms formed by cultures of wild-type *S. aureus*, wild-type PAO1-*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta pslBCD$ -*S. aureus*. *S. aureus* is Gfp-tagged (green) and *P. aeruginosa* is unlabeled. Representative images from three experimental replicates.

Thus, confocal images using Gfp-tagged *P. aeruginosa* strains and unlabeled *S. aureus* were used (Figure 4.4). The biofilms were also stained with nucleic dye STYO62 to access cell viability of the biofilms. The biovolumes of live *P. aeruginosa* and dead cells in the biofilm were then calculated from quantitative image analysis of the confocal images, and summed to determine total biofilm biovolumes (Table 4.3). Monospecies cultures of wild-type *P. aeruginosa* formed a surface-attached biofilm with an average total biovolume per area of $17.7 \pm 0.6 \mu\text{m}^3 \mu\text{m}^{-2}$ and a live *P. aeruginosa* to dead cell ratio of 1.0 ± 0.1 (Figure 4.4, Table 4.3A). When co-cultured with *S. aureus*, the average total biovolume per area increased to $30.3 \pm 5.8 \mu\text{m}^3 \mu\text{m}^{-2}$ with decreased live *P. aeruginosa* to dead cells ratio of 0.8 ± 0.1 . Biofilms from $\Delta pelA$ -*S. aureus* co-cultures had biovolume per area of $28.1 \pm 9.9 \mu\text{m}^3 \mu\text{m}^{-2}$ with increased live *P. aeruginosa* to dead cells ratio of 1.1 ± 0.2 . Co-cultures of $\Delta pslBCD$ and *S. aureus* formed flat biofilms with average total biovolume per area of 30.3 ± 9.7

$\mu\text{m}^3 \mu\text{m}^{-2}$ and highest live *P. aeruginosa* to dead cells ratio of 1.8 ± 0.6 (Figure 4.4, Table 4.3A).

Figure 4.4 Confocal images of 1 d biofilms formed by wild-type PAO1, wild-type *P. aeruginosa*-*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta pslBCD$ -*S. aureus* cultures. *P. aeruginosa* is Gfp-tagged (green). Dead cells are stained with SYTO62 (red). Representative images from three experimental replicates.

Thus, the initial presence of *S. aureus* increased the total biofilm biovolumes by about 1.5-fold as compared to the control monospecies *P. aeruginosa* biofilms. Without the Psl polysaccharide, the dead cell content was very low in $\Delta pslBCD$ -*S. aureus* biofilms. Furthermore, only the wild-type *P. aeruginosa*-*S. aureus* and $\Delta pelA$ -*S. aureus* biofilms were able to develop microcolonies, which were highly concentrated in dead cells. Microcolonies of wild-type *P. aeruginosa*-*S. aureus* cultures had an average total biovolume of 1484 ± 359

μm^3 with a live to dead ratio of 0.8 ± 0.2 (Figure 4.4, Table 4.3B). Microcolonies that developed from $\Delta pelA$ -*S. aureus* co-cultures were smaller with an average total biovolume of $475 \pm 450 \mu\text{m}^3$. Interestingly, microcolonies were found to have a low proportion of dead cells compared to the wild-type, or consist only of dead cells. This resulted in a live *P. aeruginosa* to dead ratio with high standard error of 0.7 ± 0.8 (Figure 4.4, Table 4.3B).

Table 4.3. Biomass and microcolony sizes of 1 d biofilms formed by wild-type PAO1, wild-type PAO1-*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta pslBCD$ -*S. aureus* cultures.

A. Biovolumes of live <i>P. aeruginosa</i> and dead cells of 1 d biofilms				
Strain	<i>P. aeruginosa</i>, Gfp ($\mu\text{m}^3 \mu\text{m}^{-2}$)	Dead cells, SYTO62 ($\mu\text{m}^3 \mu\text{m}^{-2}$)	Total biovolume ($\mu\text{m}^3 \mu\text{m}^{-2}$)	Ratio (Gfp: SYTO62)
Wild-type PAO1	8.8 ± 0.4	8.9 ± 0.6	17.7 ± 0.6	1.0 ± 0.1
Wild-type PAO1- <i>S. aureus</i>	13.7 ± 2.0	16.6 ± 3.8	30.3 ± 5.8	0.8 ± 0.1
$\Delta pelA$ - <i>S. aureus</i>	14.93 ± 5.37	13.16 ± 4.63	28.1 ± 9.9	1.1 ± 0.2
$\Delta pslBCD$ - <i>S. aureus</i>	19.4 ± 6.6	10.9 ± 3.9	30.3 ± 9.7	1.8 ± 0.6
B. Microcolony sizes for 1 d biofilms				
Strain	<i>P. aeruginosa</i>, Gfp (μm^3)	Dead cells, SYTO62 (μm^3)	Total biovolume (μm^3)	Ratio (Gfp: SYTO62)
Wild-type PAO1- <i>S. aureus</i>	804 ± 149	680 ± 221	1484 ± 359	0.8 ± 0.2
$\Delta pelA$ - <i>S. aureus</i>	202 ± 346	273 ± 128	475 ± 450	0.7 ± 0.8

4.3.3 eDNA is essential for microcolony formation in *Pseudomonas aeruginosa*

S. aureus is known to contain significant amounts of eDNA in its matrix (91), and eDNA has been shown to be required for early stage biofilm formation of *P. aeruginosa* (84). To investigate the importance of eDNA during biofilm and microcolony formation, the *P. aeruginosa*-*S. aureus* cultures were treated with DNase I at 12 h of biofilm formation and visualized 10 h later. The biofilms were stained with eDNA dye YOYO-3. DNase I treatment reduced biomass of the biofilms at the surface in monospecies *P. aeruginosa* and all *P. aeruginosa*-*S. aureus* cultures, was able to prevent further formation of microcolonies in wild-type *P. aeruginosa*-*S. aureus* and $\Delta pelA$ -*S. aureus* cultures (Figure 4.5).

Figure 4.5. Confocal images of 22 h biofilms formed by wild-type PAO1, wild-type *P. aeruginosa*-*S. aureus*, $\Delta pelA$ -*S. aureus* and $\Delta psiBCD$ -*S. aureus* cultures treated with DNase I at 12 h, and respective biofilm controls at 12 and 22 h. *P. aeruginosa* is Gfp-tagged (green). eDNA is stained with YOYO-3 (purple). Representative images from three experimental replicates.

4.3.4 Psl effectively crosslinks and confines cells to the microcolonies while Pel allows for superdiffusion and increased cell movement in microcolonies

The physical properties of the biofilms were investigated using passive microrheology with 1.0 μm particles (Figure 4.6, Table 4.4). *S. aureus* monospecies microcolonies and TSB medium were used for comparison to the biofilms formed by the dual-species cultures. The MSDs as a function of time $0.1 \text{ s} \leq t \leq 100 \text{ s}$ were quantified for the microcolonies in dual species cultures comprised of *S. aureus* with either wild-type *P. aeruginosa*, the $\Delta pelA$ mutant or the $\Delta pslBCD$ mutant. The diffusivity of particles within the biofilm, D , and diffusive exponent, α were derived from the MSD curves. Where α is approximately 1, the material (biofilm in this case) is considered to be viscous. An α approximately equal to 0 is indicative of elastic materials, while α values between 0 and 1 suggest the material has viscoelastic properties. The sterile TSB medium had $\alpha = 1.05$ and $D = 1.17 \times 10^{-1} \pm 3.00 \times 10^{-2} \mu\text{m}^2 \text{ s}^{-1}$, which is characteristic of a viscous fluid, whereas microcolonies of monospecies *S. aureus* biofilms were viscoelastic with $\alpha = 0.39$ and diffusivity $1.51 \times 10^{-4} \pm 6.40 \times 10^{-6} \mu\text{m}^2 \text{ s}^{-1}$ (Table 4.4). The introduction of wild-type *P. aeruginosa* to biofilms formed by *S. aureus* resulted in microcolonies with lower particle MSDs and thus were likely more effectively crosslinked and had lower diffusivities $D = 4.78 \times 10^{-5} \pm 1.31 \times 10^{-6} \mu\text{m}^2 \text{ s}^{-1}$ as compared to microcolonies of the monospecies *S. aureus* biofilms. However, the dual species wild-type *P. aeruginosa*-*S. aureus* microcolonies had increased viscous properties with $\alpha = 0.87$ (Table 4.4). Several particles were observed to undergo superdiffusion, which was defined by their directional movement with net displacement of 1-3

μm within a 15 min lag time compared to particle vibrations with net displacement of $< 1 \mu\text{m}$ (Figure 4.6B).

Figure 4.6. Microrheological measurements of wild-type and exopolysaccharide mutants of *P.aeruginosa* biofilms after co-culture with *S. aureus*. (A) MSD curves for wild-type *P. aeruginosa-S. aureus*, $\Delta\text{pelA-S.aureus}$ and $\Delta\text{pslBCD-S. aureus}$ co-cultures. MSD curves for TSB medium and *S. aureus* biofilms are used as standards for comparison. SA in the figure is *S. aureus*. The orange dotted lines indicate goodness of fit to the experimentally determined MSD using a power-law function for the estimation of α . (B) Representative particle trajectories in wild-type *P. aeruginosa-S. aureus*, $\Delta\text{pelA-S.aureus}$ and $\Delta\text{pslBCD-S. aureus}$ co-cultures. The red line in the middle panel indicates a particle undergoing directed motion and superdiffusion, whereas the blue lines indicate particles undergoing non-directional motion or vibration and the yellow lines indicate the non-directional, Brownian movement of a particle. Error bars are standard error.

The $\Delta\text{pelA-S. aureus}$ microcolonies had lower particle MSDs, indicating they were more effectively crosslinked than wild-type *P. aeruginosa-S. aureus*

biofilm microcolonies (Figure 4.6A). They had similar viscosity and diffusivity, with $\alpha = 0.74$ and $D = 5.02 \times 10^{-5} \pm 1.21 \times 10^{-6} \mu\text{m}^2 \text{s}^{-1}$, compared to the wild-type *P. aeruginosa*-*S. aureus* microcolonies (Table 4.4). At longer durations, the MSD curve plateaued (Figure 4.6A), indicating that the particles were restricted to a sub-micrometer region within these microcolonies and were undergoing confined diffusion (Figure 4.6B).

Particles in ΔpslBCD -*S. aureus* co-cultures were not confined and were undergoing Brownian motion, indicating lack of effective crosslinking within ΔpslBCD -*S. aureus* co-cultures (Figure 4.6B). ΔpslBCD -*S. aureus* co-cultures had a diffusivity $D = 2.95 \times 10^{-2} \pm 1.77 \times 10^{-3} \mu\text{m}^2 \text{s}^{-1}$ and $\alpha = 0.41$ (Table 4.4).

Table 4.4. Particle diffusivity and viscoelasticity of co-cultures of wild-type and exopolysaccharide mutants of *P.aeruginosa* with *S. aureus* respectively.

Culture Strains	Region	α	D ($\mu\text{m}^2 \text{s}^{-1}$)
<i>S. aureus</i> (Positive Control)	Microcolony	0.39	$1.51 \times 10^{-4} \pm 6.40 \times 10^{-6}$
Medium (Negative Control)	Void medium	1.05	$1.17 \times 10^{-1} \pm 3.00 \times 10^{-2}$
Wild-type <i>P. aeruginosa</i> - <i>S. aureus</i>	Microcolony	0.87	$4.78 \times 10^{-5} \pm 1.31 \times 10^{-6}$
ΔpelA - <i>S. aureus</i>	Microcolony	0.74	$5.02 \times 10^{-5} \pm 1.21 \times 10^{-6}$
ΔpslBCD - <i>S. aureus</i>	Undifferentiated biofilm	0.41	$2.95 \times 10^{-2} \pm 1.77 \times 10^{-3}$

4.3.5 The membrane associated DGC, SadC, and the CdrA adhesin are required for microcolony formation

Recent studies demonstrated that the Psl polysaccharide is able to stimulate activity of two membrane associated DGCs, SiaD and SadC to elevate intracellular c-di-GMP content and increase cell aggregation in *P. aeruginosa* (47, 249). Since microcolony formation in biofilms formed by *P. aeruginosa*-*S. aureus* required the Psl polysaccharide, the effects of SiaD and SadC on the biofilm and microcolony formation were examined using confocal microscopy and microrheology. CdrA is a *P. aeruginosa* adhesin that is expressed under elevated c-di-GMP levels, and binds to Psl as well as cross-linking Psl polysaccharide polymers to increase structural stability of the biofilm. Therefore, the role of CdrA in microcolony formation was also investigated.

Figure 4.7. Confocal images of 1 d biofilms formed by cultures of $\Delta cdrA$ -*S. aureus*, $\Delta sadC$ -*S. aureus* and $\Delta siaD$ -*S. aureus*. *P. aeruginosa* is *gfp*-tagged (green). Dead cells are stained with SYTO62 (red). Representative images from three experimental replicates.

Co-cultures of *P. aeruginosa* mutants of $\Delta cdrA$ or $\Delta sadC$ with *S. aureus* were unable to form microcolonies after 1 d of biofilm cultivation (Figure 4.7). The microrheology of these two biofilms were similar, based on their MSD curves, D and α values (Figure 4.8, Table 4.5).

Figure 4.8. Microrheological measurements of biofilms formed by *cdrA* and DGC mutants of *P. aeruginosa* biofilms after co-culture with *S. aureus*. MSD curves for $\Delta cdrA$ -*S. aureus*, $\Delta sadC$ -*S. aureus* and $\Delta siaD$ -*S. aureus* co-cultures. MSD curve for wild-type *P. aeruginosa*-*S. aureus* co-culture is plotted for comparison. The orange dotted lines indicate goodness of fit to the experimentally determined MSD using a power-law function for the estimation of α . Error bars are standard error.

Table 4.5. Particle diffusivity and viscoelasticity of co-cultures of CdrA and diguanylate cyclase mutants of *P. aeruginosa* with *S. aureus* respectively.

Culture Strains	Region	α	D ($\mu\text{m}^2 \text{s}^{-1}$)
$\Delta cdrA$ - <i>S. aureus</i>	Undifferentiated biofilm	0.30	$2.54 \times 10^{-4} \pm 1.29 \times 10^{-5}$
$\Delta sadC$ - <i>S. aureus</i>	Undifferentiated biofilm	0.24	$1.46 \times 10^{-4} \pm 7.70 \times 10^{-6}$
$\Delta siaD$ - <i>S. aureus</i>	Microcolony	0.11	$4.66 \times 10^{-5} \pm 1.49 \times 10^{-6}$
$\Delta siaD$ - <i>S. aureus</i>	Undifferentiated biofilm	0.28	$1.77 \times 10^{-4} \pm 3.10 \times 10^{-6}$

For biofilms formed by $\Delta cdrA$ -*S. aureus*, $D = 2.54 \times 10^{-4} \pm 1.29 \times 10^{-5} \mu\text{m}^2 \text{s}^{-1}$ and $\alpha = 0.30$ and for $\Delta sadC$ -*S. aureus* biofilms, the corresponding values were $D = 4.66 \times 10^{-5} \pm 1.49 \times 10^{-6} \mu\text{m}^2 \text{s}^{-1}$ and $\alpha = 0.24$ (Figure 4.8, Table 4.5). This indicated that the microrheology of the biofilms was similar to dual species biofilms of $\Delta pslBCD$ -*S. aureus*. $\Delta siaD$ -*S. aureus* co-cultures were able to establish biofilms consisting of microcolonies (Figure 4.7). These microcolonies had lower MSDs and thus were more effectively crosslinked than $\Delta cdrA$ -*S. aureus* and $\Delta sadC$ -*S. aureus* biofilms (Table 4.5). The diffusivities of $\Delta siaD$ -*S. aureus* microcolonies were similar to wild-type *P. aeruginosa*-*S. aureus* microcolonies at $D = 4.66 \times 10^{-5} \pm 1.49 \times 10^{-6} \mu\text{m}^2 \text{s}^{-1}$. However, unlike wild-type *P. aeruginosa*-*S. aureus* microcolonies, they were elastic with $\alpha = 0.11$. The undifferentiated areas of the $\Delta siaD$ -*S. aureus* biofilms were similar in rheology to $\Delta cdrA$ -*S. aureus* and $\Delta sadC$ -*S. aureus* biofilms, with $D = 1.77 \times 10^{-4} \pm 3.10 \times 10^{-6} \mu\text{m}^2 \text{s}^{-1}$ and $\alpha = 0.28$.

Figure 4.9. Expression of the P_{cdrA} -*gfp* reporter gene fusion in co-culture biofilms formed by *P. aeruginosa* DGC mutants and *S. aureus*. Confocal images of 1 day old mixed-species biofilms formed by *P. aeruginosa* A) $\Delta sadC::P_{cdrA}$ -*gfp*, B) $\Delta siaD::P_{cdrA}$ -*gfp* or C) $\Delta sadC\Delta siaD::P_{cdrA}$ -*gfp* with *S. aureus* 15981. Reporter gene expression is measured by Gfp (green) while dead cells are stained with SYTO62 (red). Representative images from three experimental replicates.

To further validate the role of SadC on *P. aeruginosa* biofilm formation in co-cultures with *S. aureus*, the expression of P_{cdrA} -*gfp* reporter gene fusion in biofilms formed by *P. aeruginosa* DGC mutants and *S. aureus* was examined (Figure 4.9). In accordance with the above results (Figure 4.7, Figure 4.8), SadC was found to be crucial for the expression of the CdrA biosynthetic gene in co-cultures (Figure 4.9).

4.4 Discussion

Bacteria rarely exist as single-cells or as clonal populations in nature but assemble together to form biofilms. Using monospecies cultures of model organisms, researchers have revealed that biofilm formation is a complex process regulated by, among other factors, quorum sensing (109, 250), c-di-GMP signaling (144) and EPS production (30). However, biofilms often include multiple species that function as a cooperative consortium (251), and recent years have witnessed increasing experimental efforts to understand mechanisms of multi-species biofilm formation (252, 253). Additionally, EPS-mediated interspecies interactions were previously shown to affect biofilm community structure and function of *P. aeruginosa*, *Pseudomonas protegens*, and *Klebsiella pneumoniae* (229). In a *Streptococcus mutans* and *Candida albicans* dual-species model, Sztajer et al. showed that the presence of *C. albicans* induced the expression of the *S. mutans* *sigX* quorum sensing regulator, which in turn enhanced EPS synthesis and biofilm formation (254).

We hypothesized that different EPS components might have distinct roles in mediating biofilm formation in co-cultures of multiple microorganisms. Dual-species biofilm models of *P. aeruginosa*-*S. aureus* have indicated that *P. aeruginosa* and *S. aureus* have an antagonist relationship, with *P. aeruginosa* reducing *S. aureus* viability. However, poorer clinical outcomes are associated with co-infections of *P. aeruginosa* and *S. aureus* (237-239), and a recent study has demonstrated that *P. aeruginosa*-*S. aureus* can have synergistic interactions and enhanced antibiotic tolerance (240). Using dual species cultures of *P. aeruginosa*-*S. aureus*, we found that *S. aureus* facilitated the transition of *P. aeruginosa* from the planktonic phase to the biofilm under static conditions. In this model, *P. aeruginosa* was not irreversibly attached throughout the process of initial biofilm formation, whether as monospecies or dual-species cultures, but went through cycles of attachment and detachment up to 9-11 h. At 9-11 h, a significant loss of *S. aureus* was observed (Figure 4.2) and had minimal presence in the mature biofilms (Figure 4.3). Subsequently, cell clustering by *P. aeruginosa* was observed at 16-18 h, which could provide the foundation for the stalks of the nascent microcolonies. In the absence of Pel, the cell clusters would often disperse, indicating this mechanism was impaired (Figure 4.1C). When the dual species biofilms had developed for a period of 1 d, the lack of Gfp-tagged *S. aureus* (Figure 4.3) indicated that *S. aureus* cells were killed by *P. aeruginosa*. This supports other studies that show *P. aeruginosa* reduces *S. aureus* viability (234-236).

Using Gfp-tagged *P. aeruginosa* and SYTO62 to stain dead cells, the amount of biofilm formed and cell viability of the biofilm were measured. The addition of

S. aureus increased the average total biovolumes of *P. aeruginosa*-*S. aureus* biofilms by about 1.5-fold, irrespective of exopolysaccharide mutant (Table 4.3A). *S. aureus* may support *P. aeruginosa* biofilm growth by providing metabolic intermediates that facilitate growth (235). Interestingly cell death was greatly reduced in $\Delta pslBCD$ -*S. aureus* biofilms. Cell death could be important for microcolony formation, as dead cells were found located in the center of wild-type *P. aeruginosa*-*S. aureus* and $\Delta pelA$ -*S. aureus* biofilms, and only these cultures were able to form microcolonies (Table 4.3B). Microcolonies developed from wild-type PAO1-*S. aureus* co-cultures were about 4 times larger than microcolonies from $\Delta pelA$ -*S. aureus* based on total biovolumes (Table 4.3B). Whilst mechanism for this difference is unclear, one possibility is that Pel's binding with eDNA, as observed previously in the stalk portions of microcolonies in monospecies *P. aeruginosa* biofilms (59), resulted in increase incorporation of live *P. aeruginosa* aggregating with the eDNA from dead cells. Indeed, microcolonies developed from wild-type *P. aeruginosa*-*S. aureus* co-cultures comprised of a good mix of live *P. aeruginosa* and dead cells, with live *P. aeruginosa* to dead cell ratio of 0.8 ± 0.2 . Microcolonies developed from $\Delta pelA$ -*S. aureus* co-cultures however were less well mixed in these two populations, and had bipolar characteristics of having microcolonies with a small proportion of dead cells or microcolonies consisting of only dead cells and undetectable amounts of live *P. aeruginosa*. This is reflected by high standard error in the live *P. aeruginosa* to dead cell ratio of 0.7 ± 0.8 .

It is known that eDNA is required during the initial stages of biofilm formation in *P. aeruginosa*, and that the biofilm matrix of *S. aureus* contains significant

amounts of eDNA (91). Lysis of *S. aureus* by *P. aeruginosa* would also release eDNA into the environment. Thus, eDNA contributed by *S. aureus*, may enhance biofilm formation by *P. aeruginosa*. Moreover, a recent study revealed that Pel crosslinks eDNA (59) and as such, biofilm formation may be facilitated in a Pel dependent fashion as supported by the observation that biofilms formed by $\Delta pelA$ -*S. aureus* cultures had the least amount of biomass (Figure 4.4, Table 4.3). When the *P. aeruginosa*-*S. aureus* cultures were treated with DNase I, biofilm formation at the surface was greatly reduced (Figure 4.5). When wild-type *P. aeruginosa*-*S. aureus* and $\Delta pelA$ -*S. aureus* cultures were treated with DNase I during biofilm formation, microcolony formation was attenuated, further supporting this hypothesis.

The mechanical properties of the microcolonies were further investigated by microrheology. The microcolonies developed by wild-type PAO1-*S. aureus* co-cultures were more effectively crosslinked than those of *S. aureus*. This was attributed to the Psl polysaccharide, the absence of which resulted in a significant reduction of effective crosslinking as reflected by the large MSDs. The mechanical crosslinking of the matrix by Psl may play a role in the formation of microcolonies. In contrast, the absence of Pel was associated with increased effective crosslinking of the matrix in microcolonies formed by $\Delta pelA$ -*S. aureus* co-cultures (Figure 4.6A). The rheological roles of Pel and Psl found here were similar to those observed for monospecies biofilms of *P. aeruginosa* formed in flow cells in Chapter 3 (223). It was also found that the presence of Pel resulted in regions of superdiffusivity within the microcolonies, whereas Psl created a subdiffusive environment (Figure 4.6B). A superdiffusive

environment would favor expansion as compared to a subdiffusive environment, and may explain why microcolonies formed by the wild-type *P. aeruginosa* were larger than those formed by the $\Delta pelA$ mutant in the dual-species biofilms. The superdiffusive environment could also be due the increased number of dead cells and eDNA content within the microcolonies when Pel is expressed.

The CdrA adhesin provides structural integrity to the biofilm by aggregating cells through binding to Psl (56). In support of these results, $\Delta cdrA$ -*S. aureus* cultures are unable to form microcolonies and the microrheology of its biofilms were similar to $\Delta pslBCD$ -*S. aureus*. This study also demonstrated that the membrane associated DGC SadC, is crucial for inducing the expression of the *P. aeruginosa* matrix adhesin CdrA synthesis gene (Figure 4.9). Thus, it is suggested that the induction of *cdrA* expression is due to the enhanced synthesis of intracellular c-di-GMP in *P. aeruginosa* cells. This has importance implications for biofilm communities, as it suggests c-diGMP production might be enhanced in the co-cultures compared to monospecies cultures. A higher intracellular c-di-GMP content in *P. aeruginosa* is associated with biofilm formation as well as enhanced resistance towards antimicrobial treatment in the organism by increasing expression and activation of BrlR, a member of the MerR family of multidrug efflux pump activators (255). Increases in c-di-GMP in mixed species biofilms could be one reason why *P. aeruginosa* in mixed species communities show enhanced tolerance to stresses such as surfactants and antibiotics (252).

In summary, this study shows how the EPS contributed by two different species interact to facilitate biofilm formation and affect biofilm physiology and morphology. In cultures of *P. aeruginosa-S.aureus*, eDNA was important for biofilm formation, and treatment with DNase I reduced biofilm biomass and attenuated the formation of microcolonies. Psl was also required for microcolony formation, whereas Pel played a role in increasing total biovolumes of the overall biofilm and microcolonies. The rheological roles of Pel and Psl (223), eDNA in facilitating biofilm formation (84), and Pel's binding to eDNA in the microcolony stalk (59) previously reported for monospecies *P. aeruginosa* biofilm formation appeared to apply also in the biofilm community systems studied here. Thus, it was possible to determine how specific EPS interactions can contribute to overall increases in biomass, cell viability and formation of microcolonies in the biofilm community.

5 Mechanical signatures of microbial biofilms in micropillar-embedded growth chambers

5.1 Introduction

Biofilm formation is a highly regulated process whereby microorganisms employ physiological cooperation and spatial organization to increase both their metabolic efficiency and adaptation to changes in their local environment (32). The result of this process is a multicellular structure that is usually heterogeneous in architecture. Cell growth and death, EPS production and degradation are factors that generate mechanical forces that move biomass to expand or remodel the biofilm. However, research into measuring mechanical forces generated by the biofilm, specifically how they contribute to development of the biofilm structure, is still in its infancy for many biofilms. A recent study has found that *Bacillus subtilis* floating pellicles maintain a low internal stress that drives biofilm spreading after relaxation from confinement and recovery during biofilm ablation (256). For *B. subtilis* grown at the air-solid surface interface, such as on agar media, biofilm spreading is mediated by the osmotic pressure resulting from the EPS absorbing water rather than the internal pressure caused by colony growth (257). Lateral mechanical forces are spatially focused by localized cell death in *B. subtilis*, driving macroscopic movement and vertical buckling of the biofilm matrix. This results in a

wrinkled morphology that may increase resistance to liquid wetting and gas penetration (258, 259). Moreover, the mechanical strength and viscoelasticity of the EPS is expected to impact on external and internal mechanical forces experienced by the biofilm, and thus influence any movement or rearrangement of biomass and affect biofilm structure and morphology. For example, the EPS of *Pseudomonas aeruginosa* biofilms can shear under flow, creating string-like extensions that eventually result in the development of streamers (45). *P. aeruginosa* biofilms are also enhanced in surface spreading and streamer formation when loose viscoelastic matrices are produced, and reduced in surface spreading and streamer formation when more effectively crosslinked elastic matrices are produced (223). As such, it is important to study the mechanical forces or pressure that drive the spreading and self-organization of these microbial populations, which can also provide a conceptual framework for other microbial systems.

Micropillar arrays were developed for the study and measurement of cellular/subcellular traction forces that eukaryotic cells exert upon adhesion to substrates. In the standard micropillar setup and model (Figure 5.1A), the cell is required to adhere to, and therefore leverage on, more than one micropillar for attachment and traction. The micropillar array has been extensively used for studies of cell spreading and single cell migration as well as movement of monolayers of mammalian cells. For eukaryotic cells, the cellular/subcellular traction forces are generated by the cytoskeletal motor protein myosin II, which causes re-arrangement of actin filaments when coupled to adhesion sites of the cell (206, 207). In contrast, apart from the measurement of cooperative

retraction forces by bundled type IV pili in *Neisseria gonorrhoeae*, for which large forces of 200 pN to 1 nN have been recorded (209), micropillars have not been used in force studies of microbial cells. Unlike mammalian cells, microbial cells are considered rigid, with the Young's modulus (E) of live *Escherichia coli* being recorded as 1.9 ± 0.9 to 3.0 ± 0.6 MPa, and 6.1 ± 1.5 MPa for dead *E. coli* (208). As such, they have well-defined shapes that do not deform easily and are unlikely to generate high traction forces within the cell body. In addition, micropillar dimensions are usually in the order of 1-5 μm in diameter, and require sufficient spacing intervals for deflections to be measured. Thus, most microbial cells are too small to attach to and interact with the multiple micropillars required to generate force measurements. However, within the biofilm, cells are collectively held together by an extracellular matrix, of which intercellular forces between cells have been reported to range from 6.5-6.8 nN based upon atomic force microscopy (260). The E for biofilms vary widely, with reported values of 37.82 ± 5.87 kPa (261) and 25.0 ± 2.5 kPa (258) for early stage laboratory biofilms grown on agar, and 200 to 9000 kPa (262) for an environmental biofilm.

The multicellular biofilm can spread over a large area and grow to hundreds of micrometers in thickness. The extracellular matrix secreted by the cells within the biofilm is expected to be the major component that binds to multiple micropillars while holding the cells together (Figure 5.1B). We hypothesized that once the biofilm was formed, forces generated by multicellular microbial behaviour involving cell growth, death, motility and differentiation would create internal mechanical forces or pressure. While a uniform pressure

generates equal lateral forces in all directions and does not deflect the micropillars, the heterogeneous growth and development of the biofilm may produce net mechanical forces or differential pressure sufficient to deflect the micropillars.

This work describes the application of micropillar-embedded growth chambers as a tool for the mechanical characterization of bacterial biofilms. We investigated biofilms formed by several medical and environmental model bacterial species, and mutants of these defective in the production of key matrix components. This includes *E. coli* strains causing urinary tract infections (263), as well as non-virulent strains used in the industrial production of recombinant therapeutics. *P. aeruginosa* is a common opportunistic pathogen. The mucoid type, in which the bacterium overexpresses alginate along with exopolysaccharides Pel and Psl in its matrix, is often isolated from cystic fibrosis patients where it is a major biofilm forming bacterial species (264). *Staphylococcus aureus* and *Staphylococcus epidermidis* often cause biofilm-associated wound infections and device-related infections (265, 266). The environmental bacterium *Shewanella oneidensis* reduces heavy metals and many complex xenobiotics (267). We show here that micropillars can measure forces at the substratum related to biofilm growth and matrix mechanical properties. The various biofilms generate deflection patterns in the micropillar array that are reflective of their growth dynamics and EPS mechanical properties.

5.2 Methodology

5.2.1 Biofilm cultivation

The bacterial strains used in this study include *E. coli* SAR18 F⁺; *P. aeruginosa* PAO1 Δ *mucA*, PAO1 Δ *mucA* Δ *pslBCD* and PAO1 Δ *mucA* Δ *pelA*; *S. aureus* 15981; *S. epidermidis* 1457 and 1457 Δ *atlE*; *S. oneidensis* MR-1 (Table 5.1).

Table 5.1. List of bacterial strains

Strain	Description	Ref.
SAR18 F ⁺	<i>E. coli</i> CSH26 wild-type carrying transfer constitutive IncF plasmids that promotes cell-cell adhesion and biofilm formation mediated by F pili	(268)
PAO1 Δ <i>mucA</i>	Mucoid <i>P. aeruginosa</i> PAO1 expressing alginate, Pel and Psl exopolysaccharides	(269)
PAO1 Δ <i>mucA</i> Δ <i>pelA</i>	Psl mutant of mucoid PAO1, expressing alginate and Pel exopolysaccharides only	(39)
PAO1 Δ <i>mucA</i> Δ <i>pslBCD</i>	Pel mutant of mucoid PAO1, expressing alginate and Psl exopolysaccharides only	(39)
15981	<i>S. aureus</i> wild-type	(39)
1457	<i>S. epidermidis</i> wild-type	(85)
1457 Δ <i>atlE</i>	Autolysin (<i>atlE</i>) mutant of <i>S. epidermidis</i> 1457, unable to release extracellular DNA (eDNA) into matrix	(85)
MR-1	<i>S. oneidensis</i> wild-type	(267)

Overnight cultures of bacterial strains were grown at their optimal temperatures and growth medium to an OD₆₀₀ = 2.0. For *E. coli* SAR18 F⁺, *P. aeruginosa* Δ *mucA*, PAO1 Δ *mucA* Δ *pslBCD* and PAO1 Δ *mucA* Δ *pelA*, growth was at 37°C in Luria Broth (LB) medium. For *S. aureus* 15981, *S. epidermidis* 1457 and *S.*

epidermidis 1457 Δ *atlE*, growth was at 37°C in Tryptic Soy Broth (TSB) medium. *S. oneidensis* MR-1 was grown at 30°C in LB medium. Subsequently for biofilm formation, 10 μ L of overnight cultures were added to the wells of the growth chambers with 190 μ L of medium to a final OD₆₀₀ = 0.1 and incubated for 24 h under static conditions at the optimal temperature of 37°C or 30°C for each organism as described above. The growth curves of the bacterial strains using the above cultivation conditions are provided in Figure S5.1.

5.2.2 Micropillar arrays

PDMS (polydimethylsiloxane) micropillar arrays were moulded from silicon masters made with conventional high-resolution photolithography and deep reactive ion-etching techniques (270). The micropillars had a height (*H*) of 3 μ m, diameter (*d*) of 1.5 μ m and center-to-center spacing of 4 μ m. The Young's modulus (*E*) of the micropillars was 1.0 ± 0.3 MPa as verified using atomic force microscopy. The PDMS micropillar arrays were established at the bottom of growth chambers to form 300 μ L wells, 9.4 mm in width and 10.7 mm in length. The growth chambers were placed in 100% ethanol and sonicated to ensure micropillars remained in upright position and to avoid collapse of the micropillars during introduction of high surface tension fluids. The ethanol solution was replaced with sterile water, and then LB or TSB medium to a final volume of 190 μ L each well.

5.2.3 Microscopy and imaging

For the imaging of micropillar deflections, brightfield Z-stack micrographs of the micropillars after allowing for 24 h of bacterial attachment and biofilm growth were captured (ZEISS Axio Imager M1). Biofilms were stained with

Congo Red (Sigma-Aldrich, Singapore) to improve contrast between the biofilm and micropillars. The positions of the micropillar tops and bottoms were located by their centroid positions using a Gaussian or Mexican Hat filter (background subtraction) in IMARIS software (Bitplane, Zürich). The deflection vector r is given by the difference in micropillar top and bottom positions as given by Eq. 2-9 as discussed in Chapter 2.5.2 Micropillars.

For the biomass quantification of biofilms formed by the respective strains after 24 hours of growth, confocal images of the biofilm were captured (Zeiss LSM780 confocal scanning laser microscope) and analyzed by COMSTAT (www.comstat.dk) (244, 245). Biofilms were cultivated in chambers without micropillars. The Gram-negative bacteria *E. coli*, *P. aeruginosa* and *S. oneidensis* were tagged with the green fluorescent protein (Gfp) for visualization and quantification of live biomass, and stained with propidium iodide (PI) for visualization and quantification of dead biomass. The Gram-positive bacteria *S. aureus* and *S. epidermidis* were stained with SYTO9 and PI for visualization and quantification of live and dead biomass.

5.2.4 Force calculations

In the case where the mammalian cell body is located on top of the micropillars, the micropillar is modeled as a cantilevered beam deflected by a horizontal traction force applied on the micropillar top, f (Figure 5.1A). For small deformations, the force required to deflect the micropillar maybe approximated by Hooke's law using the Euler-Bernoulli beam theory according to Eq. 2-10 and Eq. 2-11 in Chapter 2.5.2 Micropillars.

In the case of the biofilm, the microbial cell does not extend over more than one micropillar. Instead, biofilms form around the micropillars (Figure 5.1B-C). Lateral growth and motion from the biofilm in between the micropillars are expected to apply tension and compression along the entire height of the pillar. A previous study found that biofilms are most stiff and mechanically homogeneous within 10 μm from the substratum (271). In such cases and for short micropillars, such as those used here, the micropillar may be modeled as a cantilevered beam with the biofilm applying a uniform load with total force, f_T across its height (Figure 5.1B). The total force applied to the micropillar is then related to the deflection at the micropillar top according to the equation (272):

$$f_T = \frac{8EI\delta}{H^3} \quad \text{Eq. 5-1}$$

The force, f_T is larger than f by a constant of 8/3. A larger force is expected because more force is required to achieve the same amount of deflection when the force is spread along the length of the micropillar as opposed to being focused at the free end, further from the support. The spring constant K for a uniform force applied along the micropillar height is then:

$$K = \frac{8EI}{H^3} \quad \text{Eq. 5-2}$$

The moment of inertia for a solid beam with a circular cross-section is given by:

$$I = \frac{\pi d^4}{64} \quad \text{Eq. 5-3}$$

Substituting for I in Eq. 5-2 gives:

$$K = \frac{\pi E d^4}{8 H^3} \quad \text{Eq. 5-4}$$

The differential pressure (p) within the biofilm is calculated by dividing the force by the entire cross-section of the micropillar:

$$p = \frac{f_T}{d \times H} \quad \text{Eq. 5-5}$$

Figure 5.1. Micropillar deflection caused by mammalian cell versus biofilm. (A) For mammalian cells, which attach to the top of the micropillar, traction forces are generated by actin re-arrangement that deflect the micropillar. Thus, micropillar deflection is modeled as a beam bending with a lateral force applied at one end. (B) The biofilm grows over and between the micropillars, applying pressure along the entire height of the micropillar. Micropillar deflection is thus modeled as beam bending with uniform load applied along its height. (C) Left: control micropillar array without biofilm. Right: micropillar array covered with biofilm. The area size is $52.4 \times 52.4 \mu\text{m}$.

5.3 Results

5.3.1 Distribution of differential pressure in various biofilms

The bacterial strains attached to the micropillars at the substratum of the growth chambers and formed biofilms that deflected the micropillars over 24 h. The deflection magnitudes of 120 micropillars within a 50 x 50 μm area of substratum from at least three different experimental replicates for each strain were used to calculate the differential pressure applied to the micropillar over 24 h. A histogram was compiled to compare the distribution of pressure differences (Figure 5.2) and the average, maximum and standard deviation of differential pressure were calculated for biofilms of each wild-type and mutant strain (Table 5.2).

Figure 5.2. Distribution of differential pressures within biofilms formed by mucoid *P. aeruginosa*, and wild-type *E. coli*, *S. aureus*, *S. epidermidis* and *S. oneidensis* strains. Frequency of the differential pressure within 0-4 kPa, 4-8 kPa, 8-12 kPa, 12-16 kPa, 16-20 kPa and >20 kPa was indicated, respectively. At least three experimental replicates were used. Error bars are S.D.

For mucoid *P. aeruginosa* (PAO1 Δ *mucA*) and *S. epidermidis*, most of the differences in pressure after 24 h of biofilm growth fell within the 4-8 kPa range. This range accounted for $54.8 \pm 9.2\%$ and $39.0 \pm 7.4\%$, of the pressure differences in *P. aeruginosa* and *S. epidermidis* respectively. For *E. coli* and *S. aureus* respectively, most of the pressure differences fell within the 8-12 kPa range, accounting for $46.4 \pm 5.4\%$ and $39.6 \pm 3.8\%$ respectively. For *S. oneidensis*, a majority of $95 \pm 6\%$ of pressure differences were below 4 kPa. *E. coli* had the highest average differential pressure at 8.5 ± 0.1 kPa. This was then followed by *S. aureus* at 8.0 ± 0.6 kPa, *S. epidermidis* at 7.9 ± 0.6 kPa, *P. aeruginosa* at 7.2 ± 0.2 kPa and *S. oneidensis* at 1.8 ± 0.6 kPa.

Table 5.2. Average, maximum and standard deviation of differential pressure detected in wild-type and mutant strains.

Strain	Differential pressure (kPa)*		
	Average	Maximum	S.D.^
<i>E. coli</i> SAR18 F+	8.5 ± 0.1	16.1 ± 2.7	3.1
<i>P. aeruginosa</i> PAO1 Δ <i>mucA</i>	7.2 ± 0.2	12.0 ± 1.0	2.3
<i>P. aeruginosa</i> PAO1 Δ <i>mucA</i> Δ <i>pelA</i>	3.1 ± 0.7	6.5 ± 1.0	1.2
<i>P. aeruginosa</i> PAO1 Δ <i>mucA</i> Δ <i>pslBCD</i>	6.2 ± 0.3	10.3 ± 1.5	1.8
<i>S. aureus</i> 15981	8.0 ± 0.6	18.7 ± 2.2	3.2
<i>S. epidermidis</i> 1457	7.9 ± 0.6	20.0 ± 3.1	3.7
<i>S. epidermidis</i> 1457 Δ <i>atlE</i>	5.3 ± 0.9	20.5 ± 5.0	3.7
<i>S. oneidensis</i> MR-1	1.8 ± 0.6	5.3 ± 1.6	1.0

*Calculated from 120 micropillars within a 50 x 50 μ m area. Errors are standard error of mean. ^Composite standard deviation of differential pressure. At least three biological replicates were used.

The average differential pressure may be reflective of the heterogeneity within the biofilm of the particular species. In this study *E. coli*, *S. aureus* and *S. epidermidis* formed a heterogeneous biofilm with many microcolonies and channels. Mucoid *P. aeruginosa* also formed heterogeneous biofilms, but with smaller microcolonies and more undifferentiated areas. *S. oneidensis* did not form differentiated biofilms under our growth conditions. While *E. coli* had the highest average differential pressure, *S. aureus* and *S. epidermidis* were able to achieve differential pressures greater than 20 kPa. However, this accounted for less than 1% of the differential pressure. *S. epidermidis* had the highest maximum differential pressure and standard deviation, followed by *S. aureus*, *E. coli*, *P. aeruginosa* and *S. oneidensis*.

Differential pressure arises from the pressure of a growing biofilm biomass exerting itself onto the micropillars. To examine the correlation between differential pressure and the total biofilm biomass generated by the strains, we measured the biomass of static biofilm cultures for each strain (Figure 5.3). The live and dead measurements were combined to determine the total biomass. Among wild-type strains and mucoid *P. aeruginosa*, *S. aureus* and *S. epidermidis* biofilms had the highest total biomass with $5.8 \pm 0.5 \mu\text{m}^3 \mu\text{m}^{-2}$ and $5.4 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$, respectively. *E. coli* displayed the second highest total biomass at $5.1 \pm 1 \mu\text{m}^3 \mu\text{m}^{-2}$. Biofilms of PAO1 Δ *mucA*, *S. aureus* and *S. epidermidis* had significant proportions of dead biomass at $62 \pm 7\%$, $49 \pm 2\%$ and $43 \pm 8\%$ respectively. *E. coli* biofilms displayed a small fraction, $10 \pm 5\%$, of dead biomass. This suggests that cell death was not a major factor for its higher average differential pressure, as would be predicted based on previous

data for *B. subtilis* (258). PAO1 Δ *mucA* and *S. oneidensis* generated the second lowest and lowest total biomass at $3.0 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$ and $1.7 \pm 0.3 \mu\text{m}^3 \mu\text{m}^{-2}$, respectively, and may explain their comparatively lower average differential pressures. These results indicate that although the generation of total biomass could partly account for the differential pressure, other species-specific biofilm matrix factors might also be involved.

Figure 5.3. Live and dead biomass of biofilms formed by various strains in 24 h static cultures.

5.3.2 Matrix components of the biofilm strongly influence differential pressure generated by the biofilm

EPS mechanical properties were hypothesized to influence the deflection of the micropillars. To examine whether micropillars can be used to investigate the impact of specific EPS components on differential pressure in the biofilm, mucoid *P. aeruginosa*, *S. epidermidis* strains, and their respective matrix mutant strains were assessed (Table 5.2 and Figure 5.4). *P. aeruginosa*

ΔmucAΔpelA and *P. aeruginosa ΔmucAΔpslBCD* mutants are unable to synthesize the major *P. aeruginosa* matrix components Pel and Psl exopolysaccharides, respectively (39). Both *S. aureus* and *S. epidermidis* release a large amount of eDNA into their matrix, which contributes significantly to biofilm formation.(85, 91) *S. epidermidis ΔatlE* mutants are unable to release eDNA into the biofilm matrix and hence are expected to produce weaker biofilms (85).

The average and maximum differential pressure measured for the *P. aeruginosa ΔmucAΔpelA* biofilms were 3.1 ± 0.7 and 6.5 ± 1.0 kPa respectively (Table 5.2 and Figure 5.4). The average and maximum differential pressure measured for the *P. aeruginosa ΔmucAΔpslBCD* biofilms were 6.2 ± 0.3 and 10.3 ± 1.5 kPa respectively (Table 5.2 and Figure 5.4). Thus, the loss of the exopolysaccharide Pel significantly impaired the ability of the biofilm to deflect the micropillars, whereas loss of the exopolysaccharide Psl reduced differential pressure to a lesser extent compared to the *P. aeruginosa ΔmucA* parent strain. In the present study, the live and total biomasses of *P. aeruginosa ΔmucAΔpslBCD*, at $3.5 \pm 0.6 \mu\text{m}^3 \mu\text{m}^{-2}$ and $6.0 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$ respectively, was much greater than the *P. aeruginosa ΔmucAΔpelA* strain, at $0.8 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$ and $3.9 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$, respectively, and the *P. aeruginosa ΔmucA* strain, at $1.1 \pm 0.2 \mu\text{m}^3 \mu\text{m}^{-2}$ and $3.0 \pm 0.1 \mu\text{m}^3 \mu\text{m}^{-2}$, respectively (Figure 5.3). This finding agrees with the report that the Pel polysaccharide is important for lateral growth and spreading (223), which would increase the overall pressure in the biofilm. In contrast, the Psl exopolysaccharide increased the cross-linking density and elasticity of the matrix, resulting in increased mechanical stiffness that restricted lateral growth and spreading. Biofilms containing the Pel polysaccharide were also shown to

vary in rheology in different locations and over time, whereas biofilms expressing only Psl were spatially and temporally homogeneous in rheology (223). This may also explain the relative larger contribution of Pel to differential pressure compared to Psl. The expression of both Pel and Psl in *P. aeruginosa* $\Delta mucA$ biofilms had a synergistic effect on increasing pressure differences (Figure 5.4), which may act to improve overall biofilm dynamics and formation.

Figure 5.4. Comparison of quartiles, maximum and minimum of differential pressure data for mucoid *P. aeruginosa*, wild-type *S. epidermidis* and their respective EPS mutant strains. The loss of Pel and Psl in mucoid *P. aeruginosa* reduced the average differential pressure in biofilms, with Pel having greater impact. The loss of Pel also reduced the maximum differential pressure recorded in the biofilms. The reduction of eDNA in *S. epidermidis* reduced the average differential pressure in the biofilms.

eDNA is another well known matrix component crucial for maintaining the biofilm mechanic properties. The *S. epidermidis* eDNA deficient $\Delta atlE$ mutant biofilms showed a decreased ability to deflect the micropillars, with an average differential pressure of 5.2 ± 0.9 kPa compared to 7.9 ± 0.6 kPa for the parental

strain. Despite this, a high differential pressure could still be achieved in the *S. epidermidis* Δ atlE biofilm. Indeed, the maximum differential pressure was 20.5 ± 5.0 kPa, which suggests that other major biofilm matrix components (e.g. polysaccharide intercellular adhesin) played a more important role here in generating pressure differences.

5.3.3 Characteristic deflection patterns of bacterial strains are caused by biofilm heterogeneity and microcolony formation

Deflections of 120 micropillars within a 50×50 μ m area were plotted as vectors (black arrows) on a 2D Cartesian plane for the mucoid *P. aeruginosa* and wild-type strains (Figure 5.5). Micropillars in the control chamber remained undeflected and generated null vectors (Figure 5.5A). *E. coli* SAR18 F⁺ (Figure 5.5B), *P. aeruginosa* PAO1 Δ mucA (Figure 5.5C), *S. aureus* 15891 (Figure 5.5D) and *S. epidermidis* 1457 (Figure 5.5E) developed heterogeneous biofilms that exhibited extensive cell clustering and microcolony formation. The formation of such microcolonies deflected the micropillars in various directions and disrupted the regular micropillar positioning. As a measure of regularity in the deflections and to examine how similar in direction the vectors were across the different biofilms, the cosine similarity of each vector to its right horizontal and lower vertical neighbour was calculated according to the equation:

$$\text{similarity} = \cos \theta = \frac{\mathbf{a} \cdot \mathbf{b}}{\|\mathbf{a}\| \|\mathbf{b}\|} \quad \text{Eq. 5-6}$$

where θ of 0° gives similarity of 1, θ of 90° give similarity of 0 and θ of 180° gives similarity of -1. *E. coli*, mucoid *P. aeruginosa*, *S. aureus* and *S. epidermidis* 1457 biofilms displayed low average similarities of 0.12 ± 0.13 ,

0.11 ± 0.06 , 0.17 ± 0.10 and 0.22 ± 0.07 respectively. The average angles between the neighbouring vectors were nearly orthogonal to each other at $83.2 \pm 8.0^\circ$, $83.8 \pm 3.8^\circ$, $80.0 \pm 5.8^\circ$ and $77.3 \pm 4.0^\circ$ respectively. In contrast to the other species, *S. oneidensis* (Figure 5.5F) formed a thin and homogenous biofilm without any microcolonies and as such, the distance between, and regularity of, the micropillars were largely maintained. On average, the deflection vectors showed a high similarity of 0.79 ± 0.05 and angle of $37.8 \pm 5.1^\circ$ with neighbouring vectors. Thus, the similarity between neighbouring vectors can be a measure of microcolony formation, with a high similarity indicating the presence of very few or no microcolonies.

A red contour, as approximated from a Z-stack of brightfield images, was drawn along the high cell density areas and microcolonies on the deflection vector map (Figure 5.5). For *E. coli* and mucoid *P. aeruginosa* biofilms, micropillars were deflected within the microcolonies as well as for the flat, undifferentiated areas of the biofilm. For *S. aureus* and *S. epidermidis* biofilms, the lengths of the deflection vectors varied widely across the topology of the biofilm. This could be due to the different cluster expansion dynamics of the bacteria. For example, *Staphylococcus* spp. are non-motile and naturally arrange in clusters reflective of their inability to separate after division (273). Biofilm growth is then primarily based on clonal expansion that would exert outward pressure on the micropillars at the growing edges, but less at the focal points of growth. In contrast, *E. coli* and *P. aeruginosa* also utilize surface motility to facilitate the spreading of a developing biofilm and differentiation of flat layer of cells (111, 274, 275).

Figure 5.5. Top image of each panel is a $50 \times 50 \mu\text{m}$ 2D sectional brightfield image of the micropillars deflected by (A) no biofilm or biofilms formed by (B) *E. coli* F+ SAR18 (C) *P. aeruginosa* PAO1 ΔmucA (D) *S. aureus* 15981 (E) *S. epidermidis* 1457 and (F) *S. oneidensis* MR-1. The bottom image for each panel is the deflection vector map. The vectors are depicted by black arrows, enlarged by a factor of 3.5 to increase clarity. The contour of the biofilm is drawn in red, and the blue lines approximate the slope of major edges of the biofilm contour. Images are representative of three experimental replicates.

To further examine whether distinct deflection patterns generated by different species during biofilm growth could be detected, the alignment of deflection vectors at the edges of the biofilm were compared to the slope of the contour (blue line) using the cosine similarity (Figure 5.5). In this case, deflection vectors that point in opposite directions (180° with respect to each other) but lie flat along the slope are considered parallel to the slope. Thus, cosine similarity values are absolute, with a θ value of 180° also giving a similarity of 1. In order of descending similarity and wider angles, the similarities and average angle between the deflection vector and contour were 0.69 ± 0.04 and $46.6 \pm 3.5^\circ$ for *P. aeruginosa*, 0.65 ± 0.01 and $49.8 \pm 0.9^\circ$ for *E. coli*, 0.59 ± 0.03 and $53.9 \pm 2.1^\circ$ for *S. aureus*, and 0.57 ± 0.05 and $55.1 \pm 3.7^\circ$ for *S. epidermidis* respectively. Thus, deflection vectors for the *Staphylococcus* spp. biofilms were less aligned to the biofilm contour as compared to that of *E. coli* and *P. aeruginosa* biofilms, and this pattern may reflect different mechanisms of cluster expansion, i.e. clonal growth vs. surface motility.

5.1 Discussion

There are limited tools available to study biofilm micromechanics and dynamics, with microscopy being one of the primary instruments of investigation. Microscopy is often combined with specialist techniques such as particle (223) and cell-tracking (276), and usually requires high-end microscopes (277) to probe biofilm dynamics. These techniques are labour intensive and time consuming. In addition, cell-tracking within mature biofilms

is difficult because of the inaccuracy in resolving overlapping cells and long experimental times required due to slow cellular dynamics. Other micromechanical techniques used to investigate the physical properties of biological samples usually utilize equipment not available in biological laboratories. Examples of these include atomic force microscopy, optical light or laser traps, micropipette aspiration and magnetic tweezers. In this study, we present micropillar-embedded growth chambers as an experimental tool that can be used with standard, readily available, microscopes.—The bacterial biofilms caused distinct deflection patterns of the micropillar arrays, which can be used to calculate differential pressure in biofilm, as shown here. Biomasses of the growing biofilms were physically constrained by, and exerted pressure onto the micropillars. The heterogeneous development and differentiation of the biofilms resulted in differential pressure that was responsible for deflecting the micropillars. Thus the deflection vector map is both a reflection of heterogeneity and pressure. *E. coli* biofilms had the highest average differential pressure, and *S. oneidensis* biofilms the lowest. Although *S. aureus* and *S. epidermidis* biofilms had similar average differential pressures, the distribution and range of differential pressures experienced within their biofilms were different.

The generation of pressure differences that deflected the micropillar was dependent on EPS characteristics. In general, despite increases in biomass observed in some mutants, removal of any of the major EPS components strongly reduced the pressure differences within the biofilm, as measured here. EPS components that reduce stiffness and increase biofilm malleability are

expected to enhance differential pressure as they increase microbial dynamics, biofilm motion and lateral growth. In *P. aeruginosa*, the Psl polysaccharide has been shown to increase biofilm stiffness, whereas Pel is able to reduce biofilm stiffness and enhance overall malleability (223). In *B. subtilis* biofilms, mechanical forces are focused on areas of weakest matrix stiffness, causing differential pressure and buckling of the biofilm layer (258). EPS components that result in increased variation of biofilm mechanical properties are also expected to increase differential pressure. *P. aeruginosa* biofilms expressing Pel vary more in rheology spatially and temporally as compared to Psl (223).

The formation of high cell density areas and microcolonies in the strains tested was the major factor in disrupting the array and the creation of deflection patterns. Thus, micropillars may be sensitive to the different mechanisms that drive microcolony and cluster expansion for different bacteria. Micropillars may then be coupled with the use of specific mutants to probe, among other things, how EPS components or surface motility that affect biofilm dynamics and microcolony formation translate into pressure differences to affect biofilm morphology. It has been shown that pressure from biofilm expansion in confined spaces is responsible for the wrinkled structure of pellicles at the air-liquid interface (276). Differences in physical pressure generated by different bacterial strains may also have implications for the spatial organization of bacteria in multi-species biofilm communities. Indeed, in Chapters 3 and 4, we have shown that the spatial organization of bacteria in multi-species biofilm communities of *P. aeruginosa* and *S.aureus* is likely to be affected by the

rheological properties of Pel and Psl. Such studies can help in our understanding of the mechanics of biofilm populations or communities (278).

In order to only measure pressure that the substratum biofilms exert in a single direction, one could build elastomeric walls in a microfluidic device or chamber with the biofilm only growing on one side of the wall. Use of fluids with established viscosity in place of the biofilm, and the application of pressure through flow or parallel-plate rheometry may be used to calibrate the system. In a similar setup to the study of Douarche (256), the pressure of the biofilm can also be measure by use of a mobile plate attached to a force sensor, as long friction can be made negligible between the mobile plate and substratum. Finally, surfaces with high aspect ratio structures (tall and thin), such as micropillars, have been employed in biofilm research and industry for their anti-biofouling properties (279). Thus, the methodology described here to measure differential pressure in biofilms can be coupled with such surfaces to produce a ‘smart’ material that detects when biofilms have eventually formed, thus having real application in cleaning and maintenance of equipment of various industries.

6 Summary

6.1 General discussion

Bacteria secrete and employ biofilm associated extracellular polymeric substances (EPS) as a physical scaffold to organize themselves into multicellular structures known as a biofilm. This organization allows for the biofilm to exhibit traits and functions that distinguish the biofilm cells from single cells in planktonic suspensions (28, 29). The condition of multicellularity and structural organization results in phenomena such as physiological differentiation and spatially specific gene expression in cells located in different parts of the biofilm (162, 280). The high cell density environment of biofilms also facilitates inter-species interactions such as cross-talk and metabolite cross-feeding (254, 281) as well as increased rates of horizontal gene transfer (282). This gives rise to traits and functions that are significantly different and not predictable from their single cell counterparts, and such traits are referred to as emergent properties. Salient emergent properties commonly attributed to biofilms include enhanced metabolic cooperativity (7) and increased antimicrobial tolerance, which can contribute to the difficulty in eradicating biofilm based infections. While gene expression differences and physiological status have been well studied in biofilms with respect to development and stress as well as antibiotic tolerance, less is known about how the physical structure contributes to these emergent properties. For example, a recent study has shown that filamentous phage in *Pseudomonas aeruginosa* can interact with microbial polymers in the matrix to form a liquid crystalline structure that promotes biofilm adhesion and enhances tolerance to desiccation and antibiotics (98).

Furthermore, the filamentous phage is mainly involved in the later stages of *P. aeruginosa* biofilm development and the establishment of small colony variants, and is an example of how biofilm mechanical properties are dynamic and change at different stages of biofilm development. The composition of the EPS also changes in response to the environment, with up-regulation of alginate in *P. aeruginosa* to form a mucoid phenotype in cystic fibrosis patients to protect against host immune responses (211, 283) compared to environmental strains of *P. aeruginosa*, which do not produce much alginate (213).

In Chapter 3, particle-tracking microrheology was used to investigate the impact of Pel and Psl polysaccharides, on the rheological properties of mucoid and non-mucoid *P. aeruginosa* flow cell biofilm. It was found that Psl reinforced the young biofilm by increasing the effective crosslinking the matrix and making it elastic. Pel was found to loosen the matrix during the later stages of biofilm development, which may serve to remodel the biofilm into a more fluid and malleable state for spreading. Without Psl, the biofilm was viscoelastic and less resistant to shear stress, resulting in extensive streamer formation under increased flow conditions. Streamers are known to cause more severe blockages and interruption of flow compared to surface-attached biofilms in pipes and porous media (45). Streamers have also been observed to form on the feed spacers of reverse osmosis membranes (284). A further study on the fouling of reverse osmosis membranes show that bacterial strains of the same species use different biopolymers as their major EPS component to foul the surface and respond differently to antimicrobial treatment (285). Thus the mechanical properties of the EPS have far-reaching implications on the

biofouling of industrial systems, such as in the machines for paper manufacture, cooling systems of industrial plants, and on the membranes in water purification systems (286).

Matrix mediated interactions between different bacterial species can effectively ‘cement’ microorganisms onto materials making them extremely difficult to remove, (100), such as in the case of dental plaque (37, 287). Currently, the most effective method for the management and removal of dental plaque involves overcoming cohesive interactions in the matrix through direct mechanical attack, such manual brushing (288) or dental scaling using ultrasonic vibration (289). In *Bacillus thuringiensis* biofilms, a subpopulation of planktonic swimmers must overcome the cohesive strength of the EPS to tunnel through the matrix and infiltrate the biofilm. Swimming velocities were correlated with viscoelastic changes in the biofilm matrix, decreasing with increasing matrix strength. *B. thuringiensis* was able to tunnel through *Staphylococcus aureus* biofilms, and could sensitize the *S. aureus* biofilm to antimicrobials by facilitating solute access to deeper parts of the biofilm via the tunnels (290). As such, the rheological roles of Pel and Psl polysaccharides may also have an impact in multi-species biofilm communities. This was explored in Chapter 3, where Psl was found to increase segregation of mucoid *P. aeruginosa* and *S. aureus* in the dual species biofilms, possibly by tightly mechanically crosslinking mucoid *P. aeruginosa* with its own matrix. Pel was found to increase inter-species association, possibly by loosening the matrix of mucoid *P. aeruginosa* and interacting with the eDNA found in the matrix of *S. aureus*, as Pel was found to crosslink with eDNA (59).

The roles Pel and Psl polysaccharides in multi-species biofilm formation were also further explored using non-mucoid *P. aeruginosa* and the commensal bacterium, *S. aureus* in Chapter 4. Although interactions between non-mucoid *P. aeruginosa* and *S. aureus* were competitive, the presence of *S. aureus* was able to enhance biofilm formation of non-mucoid *P. aeruginosa*, with an overall increase in biomass, cell viability and the formation of microcolonies. The Psl polysaccharide was required for the crosslinking of the biofilm and the formation of microcolonies. Pel was involved in the loosening of the matrix and increasing dead cell content in the microcolonies, although the mechanism for this is unknown. It is possible that dead cells found at the microcolony center could be a source for eDNA, which is later crosslinked by Pel. In support of this hypothesis, it is known that eDNA released in *P. aeruginosa* biofilms during early stage of biofilm formation localizes at the microcolony center as they become covered by the EPS (84). Pel is known to crosslink with eDNA at the microcolony stalk (59) whereas Psl is absent at the microcolony center at later stages of biofilm development (225). The presence of Pel also impacted the diffusivity within the microcolonies, where superdiffusive behavior was found in subregions of the microcolonies. Together with the increasing amount of eDNA of the microcolonies, this process may have been important for facilitating microcolony expansion as Pel expressing microcolonies were larger than microcolonies without Pel by several fold. DNase I treatment of the *P. aeruginosa* and *S. aureus* co-cultures during biofilm formation reduced biofilm biomass and attenuated microcolony formation.

As discussed previously, a prominent feature of the biofilm is its structural

heterogeneity that leads to physiological heterogeneity. The matrix is likely involved in this process, in part by acting as a medium for mass transfer and also by changing its EPS composition according to location and over time as shown in Chapter 3. Previous studies have also shown that, Psl becomes depleted in the center of the microcolony of *P. aeruginosa* biofilms (225). In Chapter 5, the net mechanical forces, or differential pressure that arises from heterogeneous biofilm growth was successfully measured for the first time using micropillar arrays. The differential pressure is transferred via the EPS to deflect the micropillars, and the individual contributions by specific EPS components on differential pressure could be measured. Pel was found to contribute more to differential pressure of the biofilm than Psl. eDNA contributed to differential pressure in *Staphylococcus epidermidis* biofilms. The deflection patterns of the micropillar array are related to the way three-dimensional structures, such as microcolonies, are formed in different bacterial species, and can potentially be used to as informative mechanical signatures for biofilm characterization.

6.2 Concluding remarks

In this thesis, micromechanical methods have been combined with molecular biology to characterize the mechanical properties of two major matrix components, Pel and Psl polysaccharides, and connect their contribution of mechanical properties to biofilm structure and function. Other EPS components, such as eDNA, were examined as well. The different EPS components enabled biofilms to enter different modes of growth such as

microcolony formation and expansion, streamer formation and spreading at the substratum. The different modes of growth were correlated to the mechanical properties of the EPS, which acts as a medium for force and mass transfer. For example, a more viscous matrix allowed for the formation of streamers and enhanced surface spreading. The ability to change mechanical properties of the matrix to allow for various modes of growth are expected to allow microbial cells to better adapt to and survive in changing environments, and it would be interesting to study how the robustness of biofilms depends on such mechanical properties in response to chemical and antibiotic stressors. In summary, understanding how the EPS contributes to different modes of growth in monospecies biofilm populations and multi-species biofilm communities through its mechanical properties enables different strategies for the control and manipulation of biofilms to be developed for the appropriate stage of biofilm growth and condition.

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Appendix

A. Contributorship Statement

In Chapters 3, Seviour T, Kjelleberg S and Rice S initiated the microrheology study and designed the overall study. Yang L provided the bacterial strains and designed the mixed-species experiment. van der Maarel JRC and Doyle P provided software for MSD analysis. Kundukad B provided software for MSD analysis and computed the creep compliances. Chew SC grew the biofilms, performed confocal and particle-tracking experiments, computed and interpreted microrheological data, and designed the streamer experiments. All authors were involved in the interpretation of data and writing of the paper “Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides”, published in 2014, mBio 5:e01536-14.

In Chapter 5, Chew SC, Yang L, Kjelleberg S initiated and designed the overall study. Rice S designed the EPS mutant experiments. Tee SY provided the micropillars. Yang L provided the bacterial strains. Chew SC grew biofilms, designed the model for differential pressure and analyzed the micropillar deflections. Teh WK analyzed the micropillar deflections and measured the growth rate of the strains. Kundukad B measured the E of the PDMS of the micropillars using AFM. Chew SC, Yang L, Kjelleberg S and Rice S were involved in the interpretation of data and writing of the paper, “Mechanical signatures of microbial biofilms in micropillar-embedded growth chambers” published in 2016, Soft Matter, 12:5224-5232.

B. Supplementary Figures

Figure S3.1. Cells from day 5 (A) $\Delta mucA$ (B) $\Delta mucA\Delta pelA$ (C) $\Delta mucA\Delta pslBCD$ (D) $\Delta mucA\Delta pelA\Delta pslBCD$ biofilms re-plated on 100 mm agar plates. All colonies maintain the mucoid phenotype. Top panel are plates at lower density and bottom panel at higher density.

Figure S4.1 Cellular organization and growth of *P. aeruginosa* during biofilm formation with *S. aureus*. Representative confocal images of cultures of (A) wild-type *P. aeruginosa*, (B) wild-type *P. aeruginosa*-*S. aureus* (C) $\Delta pelA$ -*S. aureus* and (D) $\Delta pslBCD$ -*S. aureus* every 2 h during the first 8 h of biofilm formation. *P. aeruginosa* is Gfp-tagged and *S. aureus* are unlabeled. Image areas are $134.95 \times 134.95 \mu m$.

Figure S4.2 Representative images showing attachment and detachment of *P. aeruginosa* during the initial stage of biofilm formation in dual species biofilms. *P. aeruginosa* strains, wild-type, $\Delta pelA$ and $\Delta pslBCD$ are *gfp*-tagged while *S. aureus* is non-fluorescent. Image areas are $52.42 \times 52.42 \mu m$.

Figure S5.1. Mean and standard deviation of planktonic growth rates of the various bacterial strains.