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**STUDYING BACTERIAL POPULATION
DYNAMICS DURING BIOFILM DISPERSAL
AND EFFICACY OF JUGLONE AS
ANTIBIOFILM AGENT IN A MICROFLUIDICS
DEVICE**

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Studying bacterial population dynamics
during biofilm dispersal and efficacy of
Juglone as antibiofilm agent in a microfluidics
device

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A thesis submitted in partial fulfilment of the
requirements for the degree of Doctor of
Philosophy

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*), a Gram-negative pathogen, often causes infection in patients with impaired host immune systems. *P. aeruginosa* can adhere to surfaces and form biofilms to survive as a community. *P. aeruginosa* infections are difficult to treat not only because their ability to develop resistance to antibiotics but also because its biofilm blocks the effective treatment of infections.

Over 80% of chronic infections are caused by biofilms. Biofilms are composed of aggregates of bacteria and their secretion of extracellular polymers (EPS). Biofilm infection is challenging to treat because biofilm matrix protects the bacteria from being killed by antibiotics. It is the primary contributor to most persistent infections and can help bacteria to develop antibiotic resistance. The life cycle of a biofilm is divided into four steps, bacterial attachment, colonization and secretion of extracellular matrix, biofilm maturation and biofilm dispersal, the bacteria cells resume the planktonic phenotype that can reattach to a fresh surface and begin a new life cycle. There are two important systems that mediate the life cycle of biofilms, the c-di-GMP second messenger system and the Quorum sensing (QS) system. QS system

enables bacteria to communicate, which is important for forming biofilms. And the upregulation and decrease of intracellular c-di-GMP levels will regulate the formation and dispersal of biofilms.

Since the biofilm life cycle ends with biofilm dispersal, biofilm dispersal is believed to be an effective method of eliminating biofilm. There are two biofilm dispersal methods proposed for clinical treatment, namely chemically-dominated biofilm dispersal (CID), and enzymatic-dominated disassembly (EDA). CID induces active biofilm dispersal using chemical stimuli, such as nitric oxide (NO), while EDA targets the biofilm matrix for enzymatic degradation of biofilm matrix and direct destruction of the biofilm, such as glycoside hydrolases.

However, there is currently limited knowledge about the differences between CID biofilm dispersal or EDA disassembly. Moreover, there is a lack of 3D biofilm models that are appropriate for studying and observing biofilm dispersal and recolonization in fresh areas. Therefore, to explore the mechanisms and population dynamics of biofilm dispersal, it is necessary to design a new model to study the dispersal and recolonization of biofilms, which can also be used to evaluate the differences between CID-dominated biofilm dispersal and EDA-

dominated biofilm disassembly.

Microfluidics is used to develop models for studying biofilms as a low-cost, high-throughput research tool that allows direct real-time observation of biofilms. And because the liquid environment in microfluidics can be precisely controlled, it is possible to study the effects of environmental changes on biofilms in the microfluidic model under well-defined conditions.

Therefore, there are three aims of this study. [1] A biofilm-dispersal-then-recolonization (BDR) microfluidic model needs to be developed that can be used to study biofilm dispersal and recolonization. [2] The difference between biofilm dispersal and recolonization between CID biofilm dispersal and EDA biofilm degradation will be analyzed and evaluated in the BDR model. [3] The synergistic use of a new QS inhibitor Juglone with antibiotics to eliminate biofilms will be evaluated in the BDR model.

The BDR device developed in this study was divided into two chambers, a primary chamber for observing biofilm dispersal and a secondary chamber for observing enbacteria recolonization, which is controlled by a valve between the two chambers. Then the CID-dominated biofilm

dispersal experiments and EDA-dominated biofilm disassembly experiments were performed in the BDR device. The results showed that CID dispersed the biofilm more rapidly than EDA. In addition, there is also a difference in the ability of bacteria released by CID and EDA to recolonize. The released bacteria from CID could not immediately recolonize, in contrast, the bacteria aggregates released by EDA are able to recolonize within 2 hrs. Furthermore, human lung fibroblasts (HLF) were used as an *in vitro* model in this study. It is a cell that plays a key role in the lung's response to infection. The *Caenorhabditis elegans* (*C. elegans*) was also used as a model of infection in this study. It is particularly useful in infection research as it shares many conserved genes and signaling pathways with humans. Both HLF and *C. elegans* are useful infection model systems, providing a platform to explore host-pathogen interactions and allowing treatment of various therapeutics to be evaluated. In this study, it was found that the bacteria released by CID were less cytotoxic to the 3D-HLF spheroids and *C. elegans* infection models compared to EDA because they were unable to recolonize within 2 hrs.

Juglone is a natural compound that binds to the residues of PqsR receptor to interfere with the pqs QS system. The efficacy of Juglone

and colistin in the synergistic treatment of *P. aeruginosa* was evaluated. The results showed that synergistic treatment with Juglone and colistin significantly reduced biofilm and prevented biofilm cell recolonization. Moreover, through the combination of Juglone with colistin, biofilms could be effectively eliminated, and inflammatory responses reduced in a Medaka fish *in vivo* model.

This project contributes to the understanding of biofilm dispersal processes and microbial responses to changes in the environment. The BDR microfluidic device was developed in this project that allows for the study of biofilm dispersal and recolonization in a controlled and real-time manner. The device offers advantages such as rapid biofilm formation, evaluation of drug concentrations and responses, and the inclusion of cell cultures and *C. elegans* within the device for studying host responses. The findings of this project have significant implications for the development of more effective therapeutic strategies against biofilm-mediated infections in both clinical and environmental settings. Additionally, the project also identifies Juglone as a QS inhibitor that can inhibit biofilm formation and reduce bacteria recolonization. The study shows that a combinatorial therapy of Juglone and colistin can effectively eliminate biofilms and prevent the

recolonization of bacteria in both *in vitro* and *in vivo* infection models.

Publications arising from the thesis

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Abbreviations

CID:	chemically induced dispersion
EDA:	enzymatic disassembly
<i>P. aeruginosa:</i>	<i>Pseudomonas aeruginosa</i>
CF:	cystic fibrosis
QS:	Quorum Sensing
PCN:	Pyocyanin
AHL:	Acyl homoserine lactone

PQS:	<i>Pseudomonas</i> Quinolone Signal
IQS:	An integrated QS system
acyl-HSL:	N-acylated homoserine lactone
OdDHL:	<i>N</i> -3-oxo-dodecanoyl-homoserine Lactone
BHL:	<i>N</i> -butanoyl-homoserine lactone
HHQ:	2-heptyl-4-quinolone
EPS:	Extracellular polysaccharide
eDNA:	Extracellular DNA
c-di-GMP:	Cyclic di-GMP
DGC:	Di-guanylate cyclase
PDE:	Phosphodiesterase
BDR:	<i>Biofilm disperse-then-recolonize</i>
GFP:	Green fluorescence protein
MIC50:	Minimum inhibitory concentration 50%
<i>C. elegans</i>:	<i>Caenorhabditis elegans</i>

Chapter I. Introduction

1.1 *Pseudomonas aeruginosa*

1.1.1 *P. aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a frequently encountered Gram-negative bacterium that possesses opportunistic pathogenic properties. ⁽¹⁾ The bacterium is rod-shaped and has a long flagella at one end, and many pili around the bacterium can be observed under electron microscopy ^(2, 3).

P. aeruginosa is highly adaptable to the environment. Since the first isolation of *P. aeruginosa* from a wound pus in the late 19th century, it has been widespread in a variety of natural and clinical settings ^(4, 5), including human-polluted environments such as sewage, oil spills, swimming pools and water contaminated with biocides ⁽⁵⁾. It is well-adapted to various environments due its the extensive genetic repertoire and mosaic genomic structure ⁽⁶⁾. Over 500 regulatory genes have been discovered within the PAO1 genome, indicating a significant number of genes are employed in controlling various processes, like the production of virulence factors, biofilms formation ^{(7,}

⁸⁾. *P. aeruginosa* strains possess genomes that are larger in size than the majority of bacteria that have been sequenced ^(8, 9). The genome size of this species can range from 5.5 to 7 Mbp, showcasing variability within the species itself ⁽⁸⁾.

P. aeruginosa is a versatile opportunistic pathogen. It is commonly found in hospital settings involving burns, wounds, meningitis, endocarditis and microbial keratitis ⁽¹⁰⁾. As the primary bacterium responsible for cystic fibrosis (CF) disease, it displays a typical phenotype in all CF lungs, including mucus, lipopolysaccharide modification, flagellar and hair loss, and upregulation of antibiotic efflux ^(11, 12).

The pathogenesis of *P. aeruginosa* infection depends on the variety of virulence factors it produces, which include endotoxin, exotoxin A, pyocyanin and many pathogenic factors like elastase and rhamnolipids ⁽¹³⁾. The production of these virulence factors is coordinated by a bacteria density monitoring the mechanism called Quorum Sensing (QS) ⁽¹³⁾. In addition, *P. aeruginosa* infections are becoming increasingly complicated, mainly because *P. aeruginosa* is highly multidrug-resistant. For example, the resistance of *P.*

aeruginosa to penicillin, cephalosporins and carbapenems is the most common^(14, 15).

1.1.2 Process of *P. aeruginosa* infection

P. aeruginosa infections are divided into acute and chronic infections. Acute infections spread rapidly and can result in high mortality rates. In contrast, chronic infection is caused by biofilms, and may require longer treatment courses and may be more difficult to treat.

A complete *P. aeruginosa* infection consists of three stages: (1) bacterial adherence and colonization; (2) localized infection; (3) disseminated systemic disease ^(13, 16).

Initially, bacteria can easily infect injured body regions such as damaged skin or mucosal barriers (e.g. trauma, surgery or indwelling devices) or damaged immune defences (AIDS, cystic fibrosis) ^(13, 17). Pili, flagella, and alginate slime, which produce extracellular polysaccharides, help *P. aeruginosa* to adhere to surfaces ⁽¹³⁾. At this stage, the infection develops into acute or chronic, depending on whether the bacteria are able to produce a biofilm ⁽¹⁸⁾.

After the bacteria have successfully attached to the surface of the

body, *P. aeruginosa* will produce proteases that damage tissue components and produce cytotoxins that attack eukaryotic cells such as neutrophils ⁽¹⁹⁾. A typical *P. aeruginosa* virulence factor involved in infection, pyocyanin (PCN) is a blue-green redox-active secondary metabolite that interferes with cellular functions, such as cellular respiration and growth ⁽²⁰⁾. *P. aeruginosa* infects localized tissues of the body while forming a biofilm that is difficult to eliminate ⁽²¹⁾, and the bacteria spread through the bloodstream and invade the entire body ⁽¹³⁾.

Acute infection is caused by large amounts of extracellular virulence factors that destroy the body's tissues and enter the body's blood vessels, causing multiple organ failure and death. Some virulence factors, such as exotoxin A, exoenzyme S, and elastase, play an essential role in the virulence of *P. aeruginosa* ⁽¹⁸⁾. The strain's virulence will be reduced significantly if these factors are deficient. Exotoxin A can cause damage to host tissues, and help bacteria to invade host tissues, and also inhibit the host immune system ⁽²²⁾. It was shown that purified exotoxin A was highly lethal to mice ⁽²³⁾. Extracellular enzyme S can directly damage tissue and affect the capacity of bacteria to spread during infection ⁽¹⁸⁾. Elastases such as

LasB elastase can dissolve a variety of proteins including elastin and fibronectin of blood vessels, disrupting tissue components and interfering with host defence mechanisms ⁽²⁴⁾. Most bacterial infections, such as upper respiratory tract infections, skin and wound infections, urinary tract infections, intestinal infections and pneumonia, are acute. There are also serious and life-threatening infections such as septicemia, meningitis, and severe pneumonia ⁽²⁵⁾.

Chronic infections are usually caused by bacterial biofilms, which contrast with the planktonic bacteria in acute infections ⁽¹⁸⁾. This process produces fewer virulence factors and has a lower mortality rate than acute infections ⁽²⁴⁾. Chronic infections can occur in patients with diseases that cause defects in the main defence barrier (innate immunity) and are difficult to cure with antibiotics ⁽²⁶⁾. Chronic infections are persistent ⁽²⁷⁾. Pathogenic bacteria in biofilms can survive with the host for long periods, as in the case of CF lung and chronic wounds ⁽¹⁸⁾. The persistent *P. aeruginosa* biofilm in the airways of CF patients causes continuous inflammation during chronic lung infections, which leads to tissue damage, necrosis of the lung tissue, and, ultimately, the hosts' death ⁽²⁸⁾.

1.2 Quorum Sensing (QS) system

Quorum Sensing (QS) is a communication mechanism between bacteria through the production of small diffusible molecules that can be detected by surrounding organisms⁽²⁹⁻³¹⁾. N-Acylhomoserine lactones (AHLs) are the most common QS signal in Gram-negative bacteria, and increased concentrations of AHL induce the activation of transcriptional regulators ^(31, 32). The QS system can regulate the expression of virulence genes and significantly affect cellular virulence, the formation of biofilms and the acquisition of antibiotics resistance in *P. aeruginosa* ^(30, 33).

When bacteria reach a specific density, the QS system may regulate the release of several virulence factors, including exotoxin A, rhamnolipids, chlorophyllin, and lipase, which significantly influences the severity of acute and chronic *P. aeruginosa* infections ^(24, 34-36). Thus, the QS system is a virulence determinant for *P. aeruginosa*.

The *las* QS system, *rhl* QS system, Pseudomonas Quinolone Signal (PQS) QS system and an integrated QS system (IQS) are currently known to compose the QS system in *P. aeruginosa*.

1.2.1 The *las* and *rhl* systems

las and *rhl* are both n-acylated homoserine lactone (acyl-HSL) signaling systems ^(33, 37). The auto-inducer synthase LasI in the *las* system and the auto-inducer synthase RhII in the *rhl* system produce AHL signal molecule *N*-3-oxo-dodecanoyl-homoserine Lactone (OdDHL) auto-inducer and *N*-butanoyl-homoserine lactone (BHL) auto-inducer, respectively ^(33, 36). The inducer OdDHL binds to receptor protein LasR to form the complex LasR-OdDHL, which activates *lasI* and the expression of other downstream genes, like the *rhlR* and *rhlI* genes in the *rhl* system, and the *pqsR* and *pqsABCDH* genes in the PQS system ^(33, 36). The receptor protein RhIR of the *rhl* system is activated to form a complex RhIR-BHL that is essential for the transcriptional expression of target genes ⁽³⁷⁾. Although the regulators of the *las* and *rhl* QS systems are different, both systems can regulate the transcription encoded by *lasB* ⁽³⁸⁾.

Moreover, both regulatory systems regulate approximately 1-10% of *P. aeruginosa* genes ^(37, 38), such as elastase, exotoxin A, lectins and pyocyanin ⁽³³⁾. Elastase regulated by the *las* QS system could lead to degradation of elastin, collagen, and other extracellular matrix proteins

⁽³⁹⁾. And the *las* system also regulates exotoxin A, which also causes cell death ⁽²²⁾. Exotoxin A of *P. aeruginosa* binds to animal cells, enters through endocytosis, and can be processed by furin ⁽⁴⁰⁾. The toxin fragments are retrogradely transported to the cytosol, blocking protein synthesis then causing cell death ⁽⁴⁰⁾. Examples of virulence factors regulated by the *rhl* system include lectins and pyocyanin ⁽³⁷⁾. Lectins induce ciliary paralysis in the airways ⁽⁴¹⁾, while pyocyanin inhibits host cell respiration through oxidative actions, leading to oxidative stress and ciliary paralysis in the airways ⁽⁴²⁾. Additionally, pyocyanin can delay the inflammatory response to *P. aeruginosa* infection by damaging neutrophils ⁽⁴²⁾.

1.2.2 The PQS system

The third intercellular QS signaling system is the *pqs* QS system and it is integrated with other systems of the QS system ⁽⁴³⁾. The QS signals include 2-heptyl-4-quinolone (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS) ⁽⁴⁴⁾. The PQS synthesis cluster consists of *pqsABCD*, *phnAB* and *pqsH* ⁽³⁷⁾. PQS can directly activate the *pqsABCDE* manipulator by binding directly to the LysR-type transcriptional regulator PqsR ⁽⁴⁴⁾. HHQ is a precursor of PQS, which is transmitted

between bacteria ⁽⁴⁵⁾. The genes required for synthesizing HHQ need to be activated by the transcriptional regulator PqsR. *PqsA* activates o-aminobenzoate to form o-amino benzoyl coenzyme A and is the first step in PQS biosynthesis ^(37, 46). *PqsB*, *PqsC* and *PqsD* mediate the conversion of o-aminobenzoate to HHQ by incorporating β -keto-undecanoic acid ^(37, 46, 47). Then HHQ is converted to PQS by the flavin-dependent monooxygenase *pqsH* ^(37, 46, 47). The PQS system is controlled by the *las* system, and PqsR expression is also regulated by LasR, as mentioned before.

The *pqs* signaling system is also involved in regulating virulence factors during bacterial infection. Mutations in the *pqs* system lead to reduction in biofilm formation and decrease in virulence factors such as pyocyanin, elastase, and PA-IL lectin ^(37, 48). Moreover, the precursor of PQS, HHQ, and its derivative HQNO, 4-hydroxy-2-heptylquinoline-N-oxide, are frequently present in the sputum, bronchoalveolar fluid, and mucopurulent secretions of CF patients ^(37, 49). This indicates the significance of the PQS system in *P. aeruginosa* infection.

1.2.3 The IQS system

IQS is an integrated QS system. Its structure is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde ⁽³⁷⁾, but the receptor protein has not been identified. The absence of IQS leads to decrease in PQS levels and reduced signaling of the inducer BHL of the *rhl* system. Consequently, the virulence factor production is affected and the levels are reduced ⁽³⁷⁾. IQS was found to be able to integrate environmental stress cues with QS networks ^(37, 50). It is able to monitor both bacterial population density and phosphate limitation pressure to ⁽³⁷⁾ regulate the production of virulence factors ⁽⁵⁰⁾. This integration allows bacteria to use QS and stress signals to increase virulence and overcome host defences ⁽⁵⁰⁾. Phosphate depletion is a common stress during infection ⁽⁵¹⁾, and the presence of IQS triggers a significant enhancement in virulence factor production ⁽⁵⁰⁾. In addition, IQS can compensate for mutations in the *las* system, coordinate population physiology, regulate downstream quorum sensing systems, and maintain bacterial competitiveness in interactions with the host ⁽³⁷⁾.

The QS system can control the production of virulence factors and is associated with the biofilms that cause chronic infection. When IQS is damaged, there is reduced production of PQS and BHL signaling molecules ⁽³⁷⁾, and virulence factors such as pyocyanin, rhamnolipids

and elastase are also reduced ^(37, 50).

The *las*, *rhl*, *pqs* and *iqs* systems of *P. aeruginosa* are interconnected to form an integrated QS system that coordinates intercellular communication in bacteria ⁽⁵⁰⁾. The QS system involves the coordination of intercellular communication through signalling molecules that accumulate in the surrounding environment as bacterial density increases ⁽⁵²⁾. When the concentration of signalling molecules increases to a threshold level, they bind to receptor proteins, thereby activating the genes related to biofilm formation ⁽⁵³⁾. This suggests that the QS system plays a key role in the development of biofilm-associated infections.

1.3 Biofilms

Bacteria can attach to different surfaces and form biofilms in early infections. Biofilms are complex aggregates formed in the bacterial self-generated extracellular polymer (EPS) matrix and are widely found in the natural environment ⁽⁵⁴⁾. This model is essential to sustaining survival in difficult living conditions, such as temperature fluctuations and low nutrient availability ^(54, 55). By forming biofilms, bacteria can evade the immune system and resist antimicrobial

treatments. Studies have shown bacteria within biofilms display antibiotic resistance that exceeds 1000 times of that of planktonic bacteria ^(55, 56).

In recent years, chronic infections caused by biofilms are estimated to account for approximately 80% of bacterial infections ⁽⁵⁷⁾. However, biofilm infections are difficult to treat because the bacteria in the biofilm can develop antibiotic resistance, causing conventional antibiotic therapy to become ineffective. In addition, biofilms are hiding places for bacterial cells, which are released from the biofilm at the end of life cycle and then recolonized, which facilitates the development of secondary infections. Biofilms become a critical way for bacteria to compete, survive and dominate ⁽⁵⁵⁾. Thus, the eliminating of biofilm infections has become more complex, especially in chronic infectious diseases.

1.3.1 Composition of biofilms

Biofilms are matrix-enclosed communities comprised 5-35% of bacteria cells and 50-80% EPS matrix ^(58, 59). *P. aeruginosa* secretes biomolecules such as polysaccharide, proteins, extracellular DNA (eDNA), and ions to form a biofilm EPS matrix ^(60, 61). The EPS matrix

is the primary structure of the biofilm and maintains the bacterial community, protects the bacteria, provides nutrition and facilitates intercellular communication ^(55, 62).

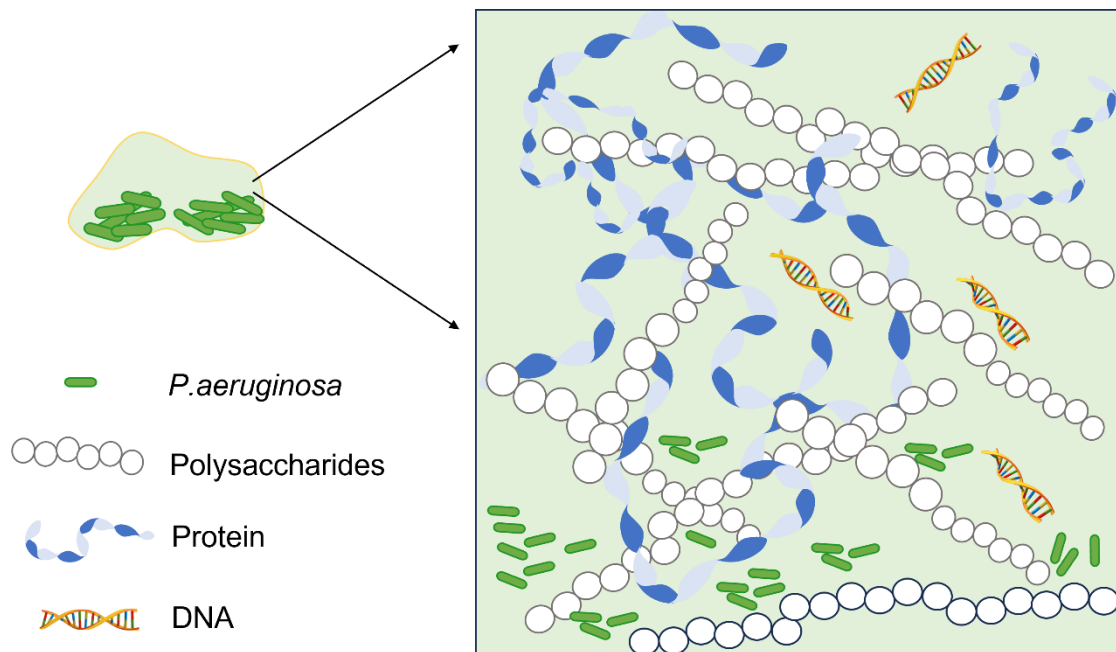


Figure 1.1. The constituents of the biofilm matrix. Polysaccharides, DNA, and proteins are distributed among bacteria in the biofilm, together they form the extracellular matrix of the biofilm.

1.3.1.1 Polysaccharides

Extracellular polysaccharides, as the main component of EPS, are involved in structural stabilization, surface attachment and biofilm

formation in the *P.aeruginosa* biofilm matrix. There are three main extracellular polysaccharides, Pel, Psl and alginate ⁽⁶³⁾.

The Pel, which is a cationic polysaccharide polymer consisting of partially deacetylated N-acetyl-D-glucosamine and N-acetyl-D-aminogalactose, is known to be involved in initiating surface attachment and maintaining the integrity of biofilms ^(55, 64). It has been shown that Pel can promote resistance to aminoglycoside antibiotics and colistin in biofilm bacteria ⁽⁶⁵⁾. Besides, the importance of pel is not only in initiating and maintaining cell-cell interactions in biofilms but it can also play a role in cell-surface adhesion when alginate production is insufficient ^(64, 66).

Psl is a neutral pentasaccharide that is, usually composed of D-glucose, D-mannose and L-rhamnose ^(55, 67). It is located in the periphery of the mushroom-like structure in the biofilm to maintain the stability and function of the biofilm structure ⁽⁶⁷⁾. Similar to Pel, Psl plays a role in protecting biofilm bacteria from antimicrobial agents and phagocytosis by neutrophils, which makes it is the one of the most effective defence mechanisms for the persistence of infection ^(55, 68). In addition, Psl can be the signal to trigger the activity of two

enzymes, SiaD and SadC, they are responsible for the production of c-di-GMP, which is an intracellular secondary messenger molecule ⁽⁶⁹⁾.

Another polysaccharide is alginate, which is a negatively charged polymer formed from residues of gulonic and mannose acids. ⁽⁷⁰⁾. This polysaccharide has a variety of essential functions for promoting biofilm maturation, preventing cell phagocytosis, regulating cell-to-cell interaction, and slowing down the spread of antibiotics in biofilm ^(55, 71, 72).

1.3.1.2 Extracellular Proteins

The proteins in the biofilm matrix are divided into non-enzymatic proteins and enzymes. Non-enzymatic proteins in the matrix, such as lectins, pili, fimbriae and flagella, maintain the formation and stability of the polysaccharide matrix network and the connection between the bacterial surface and the extracellular EPS ⁽⁷³⁾.

Lectins are carbohydrates-binding proteins with selectivity for sugars, and LecA and LecB are the two lectins found in EPS so far ⁽⁷⁴⁾. LecA and LecB are related to bacterial infection and biofilm formation. LecA is required for *P. aeruginosa* to form biofilms on polystyrene and stainless-steel surfaces ⁽⁷⁵⁾. LecB is required to form biofilms on glass

surfaces ⁽⁷⁶⁾. LecA and LecB are also necessary for biofilm formation. Compared to wild-type PAO1, biofilms formed by LecA mutant had reduced substrate coverage, while LecA overproducing strains showed more biofilm formation ⁽⁷⁷⁾. LecB mutant formed biofilms with reduced thickness and surface coverage ⁽⁷⁵⁾. However, the mechanism of exacting LecA and LecB secretion is still not known.

CdrA is an adhesin involved in the formation of *P. aeruginosa* biofilm and the bacterial self-aggregation ⁽⁷⁸⁾. It is capable of braking down into smaller protein fragments that are released into the culture supernatant ⁽⁷⁴⁾. It is a matrix protein that binds to the extracellular polysaccharide Psl, which is involved in regulating the *P. aeruginosa* biofilms formation on abiotic surfaces ⁽⁷⁹⁾. The biofilms formed by CdrA-deficient strain are more thinner and less structured than those formed by the wild type, and the over production of CdrA causes the increase in self-aggregation of bacteria self-aggregation in liquid culture ^(80, 81). This CdrA-mediated auto aggregation phenomenon depends on polysaccharide Psl ⁽⁸²⁾. The Psl-deficient strains were unable to self-aggregate, while CdrA overproduction made it possible for the Pel-deficient strains to self-aggregate ⁽⁷⁴⁾. CdrA is polyvalent and can bind to different polysaccharides ⁽⁸³⁾. For example, the

addition of exogenous mannose can reduce the phenotype of self-aggregation, as CdrA is less bound to the mannose residues in Psl ⁽⁸⁰⁾.

Extracellular enzymes are ubiquitous in biofilms ⁽⁸⁴⁾. It has been shown that extracellular enzymes can accumulate in biofilm matrices through interaction with extracellular polysaccharides, generating an activated matrix that can be considered an external digestive system ⁽⁷³⁾. For example, the lipase in the *P. aeruginosa* EPS matrix binds to alginate, which results in increased enzyme stability and amounts ⁽⁸⁵⁾. Extracellular enzymes have a variety of functions, such as degrading biopolymers to supply nutrients to bacteria and working as virulence factors during infection ⁽⁸⁶⁾. Extracellular elastases and lipases can feed biofilms by degrading host tissues and in this capacity they are used as virulence factors, like alkaline protease, elastase and lipase LipA ⁽⁸⁷⁾.

1.3.1.3 Extracellular DNA

Extracellular DNA (eDNA) is a DNA molecule released into the EPS matrix by active cell lysis or bacterial cell death ⁽⁸⁸⁾. The eDNA is critical in the formation and stabilization of biofilms. It can interact with other molecules (such as proteins and polysaccharides) to form

complex network structures that contribute to the adhesion and stability of biofilms ⁽⁷³⁾.

In addition, eDNA may also play an important ecological role in microbial communities. It can be used as a signalling molecule to direct biofilms' aggregation and formation ⁽⁸⁹⁾. Moreover, eDNA can influence microorganisms' genetic diversity and adaptability and promote evolution and adaptation through mechanisms such as horizontal gene transfer ⁽⁹⁰⁾.

1.3.2 The biofilm life cycle

The biofilm life cycle can be divided into four stages. In the first stage, bacterial cells adhere to the surface by their appendages, such as flagella and type IV hyphae ⁽⁵⁵⁾. Next, the bacterial cells lose their motility and transfer to irreversible attachment while producing EPS ^(55, 57). During the third stage, the attached bacteria gradually colonize into more structured microcolonies, further developing into extensive three-dimensional mushroom-like structures. This biofilm structure is characteristic of biofilm maturation ^(55, 57). Research has shown that swarming movements affect the structure of the biofilm, with highly motile bacteria producing smooth biofilms and less motile cells

producing mushroom-like biofilms ⁽⁹¹⁾. In the fourth stage, the biofilm begins to age, and enzymes in EPS degrade the matrix to release dispersed cells ^(55, 92). These bacterial cells can colonize new surfaces to form biofilms after reverting to the planktonic mode ^(57, 93).

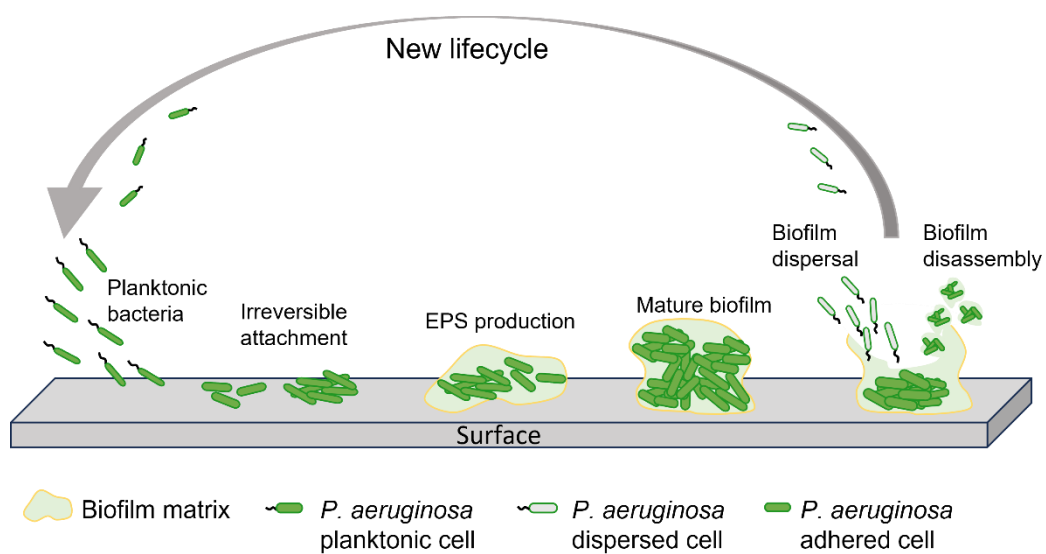


Figure 1.2. Biofilm life cycle. Biofilms begin with bacterial adhesion and production of EPS, followed by the formation of microcolonies that mature into three-dimensional structures in the third stage. Finally, the biofilm disperses, and releases dispersed cells. The dispersed cells revert to planktonic cells after a while and the life cycle begins again.

1.3.3 QS system and biofilm formation

P. aeruginosa produces a variety of virulence factors via the QS system to regulate the biofilm formation, including with flagella, exotoxins, pyocyanin and others ⁽⁹⁴⁾. For example, PqsR is required for flagella swarming in PAO1 to form biofilms ⁽⁴⁴⁾, this is important to start biofilm lifecycle. Biofilm lifecycles could be initiated by the QS system of bacteria through (1) surface attachment, such as the association of bacteria with surfaces; (2) the production of extracellular polymers that help bacteria to aggregate and form biofilms; and (3) the synthesis of biosurfactants, e.g. rhamnolipids, that regulates the interaction of bacteria with their environment and facilitates bacterial attachment during biofilm formation and dispersal ⁽⁹⁵⁾. Moreover, the QS system can influence the structure and morphology of biofilms. For example, the biofilms formed by bacteria lacking QS are flat and immature, whereas those formed by wild-type strains are mushroom-shaped on LB agar plates ^(96, 97).

1.3.4 Cyclic di-GMP (c-di-GMP) signaling

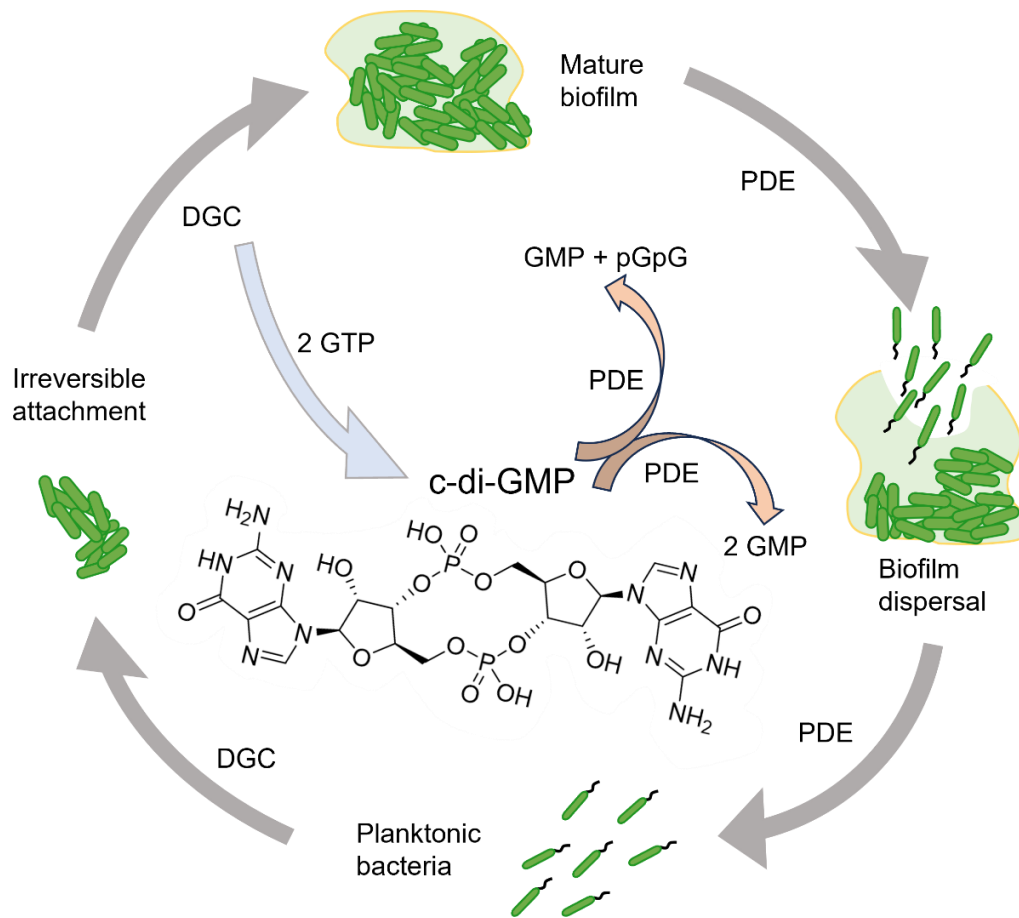


Figure 1.3. The c-di-GMP signaling system regulates the life cycle of biofilms. DGCs and PDEs are related to the synthesis and degradation of intracellular c-di-GMP and regulate the biofilm life cycle. When the concentration of c-di-GMP increases, bacteria begin to attach to fresh surfaces and start the biofilm cycle. Whereas decreased levels of c-di-GMP lead to biofilm dispersal.

In addition to the QS system, a signaling system based on the secondary messenger cyclic di-GMP (c-di-GMP) also controls the biofilm lifecycle. C-di-GMP is an intracellular secondary signalling

molecule that coordinates the lifestyle transition of bacteria from motility to sessility or from sessility to motility ^(98, 99). Determinants of biofilm formation regulated by c-di-GMP also include flagellar rotation, contraction of type IV hyphae, synthesis of extracellular polysaccharides, expression of surface adhesins, antimicrobial resistance and the production of other responses to stress and secondary metabolites, and also involve biofilm dispersal ⁽⁹⁸⁻¹⁰⁰⁾.

The intracellular concentration of c-di-GMP signalling influences the formation and dispersal of biofilms. High intracellular concentrations of c-di-GMP stimulate the production of matrix components, such as adhesins and exopolysaccharide, to form biofilms. Low concentrations of c-di-GMP promote bacterial motility and dispersal ⁽⁹⁸⁾.

The concentration of c-di-GMP in cells is related to the rate of its synthesis and degradation ⁽⁹⁸⁾. It is synthesized from two molecules of GTP by the enzyme di-guanylate cyclase (DGC) and degraded by phosphodiesterase (PDE) to guanosine 5'-phosphate-(3'-5')-guanosine (pGpG) and GMP ⁽⁹⁸⁾. The DGC and PDE enzymes have an effect on bacterial swarming motility ⁽¹⁰⁰⁾. As c-di-GMP has an inverse relationship with motility, DGC activity (production of c-di-GMP)

will inhibit motility, while PDE activity (degradation of c-di-GMP) will promote motility ⁽¹⁰¹⁾. During *P. aeruginosa* biofilm formation, it was found that at least five DGCs specifically control the transition from planktonic to surface-associated growth: WspR, SadC, RoeA, SiaD and YfiN/TpbB ^(98, 102-104). And the DGCs GcbA and NicD or the PDE DipA (Pch), RbdA and NbdA could regulate the dispersal of the biofilm ^(98, 104, 105).

DGC and PDE catalytic domains are the GGDEF and EAL or HD-GYP domains, respectively. DGC activity is associated with the GGDEF domain, which is named following the amino acid sequence motif that is an essential component of the enzyme active site ⁽¹⁰⁶⁾. DGC is a complex of two identical molecules, and GGDEF is a specific region of the DGC molecule with a catalytic site on a homodimer. The catalytic site will bind to two GTP molecules and catalyze the conversion to c-di-GMP. Mg^{2+} is required in the catalytic reaction to provide the necessary environmental conditions and catalytic capacity ^(98, 107).

EAL and HD-GYP are two structural domains and they are so named because of the specific conserved motifs in the amino acid sequence ⁽¹⁰⁶⁾. These amino acid sequences are essential for the enzyme's

activity. PDE enzymes are responsible for degrading c-di-GMP and converting it into other metabolites, like 5'-pGpG and GMP ⁽⁹⁸⁾. The amino acid motifs in the EAL and HD-GYP structural domains provide the functions required for enzyme catalysis, thus, the degradation of c-di-GMP can be efficiently catalyzed. The active EAL structural domain of PDE is a monomeric enzyme that can linearise c-di-GMP to 5'-pGpG, which is then further degraded by non-specific cellular PDE, and this process is catalyzed by Mg ²⁺ or Mn ²⁺ and inhibited by Ca ²⁺ and Zn ²⁺ ⁽¹⁰⁶⁾. The second type of c-di-GMP-specific PDE enzyme is the HD-GYP domain protein, which destroys the phosphodiester bond in c-di-GMP to produce 5'-pGpG and further degrades 5'-pGpG to GMP ⁽¹⁰⁷⁾.

The sensory domain of c-di-GMP facilitates the translation of various environmental signals into c-di-GMP levels ⁽⁹⁵⁾. Environmental and cellular signals regulate the expression of DGC and PDE, and DGC and PDE are sensitive to different input signals through their N-terminal sensory domain and balance c-di-GMP levels through their antagonistic activities ^(108, 109). Most of the GGDEF, EAL and HD-Gyp domains are connected to various N-terminal sensory input domains, which means that many environmental and cellular signals are linked

with the c-di-GMP signalling network to sense external signals, including oxygen and redox conditions, and various extracellular substances ^(110, 111).

1.4 Biofilm infection of *P. aeruginosa*

The infection of *P. aeruginosa* biofilms often happens in clinical conditions, particularly in healthcare facilities, hospitals and patients ⁽¹¹²⁾. It will infect many different tissues and organs of the body, including skin wounds, respiratory tracts, urinary tracts, wounds and central venous catheters ⁽²⁵⁾. *P. aeruginosa* infections often become chronic and recurrent through biofilm formation ⁽¹¹³⁾. *P. aeruginosa* biofilm infections are challenging to eradicate primarily because of their resistance to antibiotics and the barrier function of biofilms in intercepting antibiotic molecules ⁽¹¹⁴⁾.

1.4.1 Antibiotics resistance of *P. aeruginosa* biofilms

P. aeruginosa bacteria have shown resistance to a wide range of antibiotics. It is a highly adaptive bacterium with the largest genome and can act through modulators in intrinsic, acquired or adaptive resistance ^(33, 115). Also, the sticky gel-like EPS matrix in *P. aeruginosa*

biofilms acts as a barrier for bacteria by preventing the penetration of external substances ⁽¹¹⁶⁾. They help the bacteria community to develop antibiotic resistance, for example, high bacterial cell density within biofilms increases the frequency of mutations, as well as the accumulation of the antibiotic-degrading enzyme (β -lactamase) and the lower metabolic rate of dormant bacteria in the biofilm ⁽¹¹⁷⁾. Therefore, it is difficult to eliminate biofilm infections with antibiotics.

1.4.1.1 Intrinsic antibiotic resistance

The extracellular membrane of *P. aeruginosa*, the efflux pump system and its self-produced antibiotic-inactivating enzymes constitute the mechanism of intrinsic drug resistance ^(118, 119). Gram-negative bacteria have outer membranes, which surround a relatively thin layer of peptidoglycan to allow the substrate required for bacterial growth to pass through ⁽¹²⁰⁾. The outer membranes have an unusual composition of lipid molecules (lipid A) which are bonded covalently to the polysaccharide, reducing membrane fluidity, and thus increasing permeability ^(120, 121). The permeability of *P. aeruginosa* outer membrane is low and effectively inhibits most small molecule

antimicrobial agents available on the market, such as β -lactams antibiotics⁽¹²²⁾. The reduction in molecular permeability is beneficial for β -lactamases in *P. aeruginosa*^(120, 123). When an β -lactams antibiotic molecule enters, the enzyme inactivates it with optimal efficiency⁽¹²⁰⁾.

1.4.1.2 Acquired antibiotic resistance

Mechanisms for developing acquired resistance in *P. aeruginosa* include horizontal gene transfer and chromosomal gene mutations^(33, 119). Chromosomal mutations can lead to acquired resistance, including resistance to β -lactams, fluoroquinolones, aminoglycosides and polycationic antimicrobial agents⁽³³⁾. Antibiotic modifying enzymes are an important mechanism of acquired resistance, and common types include phosphorylation, acetylation and adenylation^(33, 124). These mechanisms can lead to chemical changes or destruction of antibiotics, making them ineffective⁽³³⁾. For instance, aminoglycosides are antibiotics that inhibit the growth and reproduction of bacteria by disrupting their protein synthesis⁽¹²⁵⁾. *P. aeruginosa* modifies the structure of aminoglycoside antibiotics by producing a transferable aminoglycoside modifying enzyme (AME)

that inactivates the antibiotics ⁽¹²⁶⁾. Therefore, appropriate therapeutic strategies are required to combat resistance development effectively. These include the rational use of antibiotics, improved disease prevention and control, and the development of new antimicrobial agents.

1.4.1.3 Adaptive resistance

Adaptive resistance is a reversible phenotypic trait and is distinct from intrinsic and acquired resistance ^(33, 115, 127). It can be mediated by environmental factors like media, pH, oxygen, temperature changes, nutritional deficiencies and other environmental stresses ⁽³³⁾. In addition, *P. aeruginosa* regulators have been implicated in antibiotic resistance and virulence ⁽¹¹⁸⁾. It is important to understand adaptive resistance to develop antibacterial therapies to prevent infection and infection transfer.

Antibiotic resistance of *P. aeruginosa* biofilms is one of the most important types of adaptive resistance and is a significant characteristic of biofilms ⁽¹²⁸⁾. *P. aeruginosa* bacteria grown in biofilms have been shown to have lower sensitivity to antimicrobial agents and host immune responses compared to planktonic bacteria due to

physical barrier, the reduction of metabolic activity and gene expression changes ⁽¹¹⁶⁾. The bacteria, even those without intrinsic resistance or protective mutations, can also develop insensitivity to antibiotics when grown in biofilms ⁽¹¹⁹⁾. Research has demonstrated that bacteria within biofilms exhibit antibiotic tolerance levels ranging from 100 to 1,000 times higher than those of planktonic bacteria ⁽¹¹⁶⁾. Biofilm-mediated resistance is not related to genetic mutations but is an adaptive mechanism that allows antibiotic sensitivity to be rapidly re-established when bacteria are no longer protected by biofilms ⁽¹²⁹⁾. The mechanism of biofilm-mediated antibiotic resistance is mainly to prevent the antibiotic from penetrating into the bacterial cells, adjust the microenvironment (pH, oxygen concentration) in the biofilm in order to inhibit antibiotic effects and induce slow growth of biofilm cells. Biofilm bacteria induce adaptive stress response by sensing antibiotics and subsequently develop resistance to protect bacteria from antibiotics ^(119, 130).

P. aeruginosa has the most complex regulation network of bacteria, its biofilm also has the significant ability to develop or acquire new resistance mechanisms to new antibiotics, so the overuse and misuse of antibiotics may pose a critical risk to public health ⁽¹¹⁹⁾. Therefore,

new treatment strategies have to be developed to target *P. aeruginosa* biofilm infections.

1.5 Exploring new therapies against *P. aeruginosa* biofilms

Due to the use of multiple antibiotic resistance strategies by *P. aeruginosa* biofilms, research has been conducted to develop novel treatments. Various anti-biofilm agents have been developed to eliminate biofilms with the regulatory mechanisms of biofilms and the components of biofilms. These anti-biofilm agents eliminate biofilms in different ways, and then bacteria dispersed from the biofilms can be treated synergistically with low concentrations of antibiotics to eliminate bacterial infection ⁽¹³¹⁾.

1.5.1 Anti-biofilm agent targeting QS

With increased population density in biofilms, population signals can reach sufficient levels for QS to activate biofilm maturation and dispersal by a coordinated approach ⁽¹³²⁾. In *P. aeruginosa*, QS-controlled AHL and PQS signalling can regulate the production of rhamnolipids. Rhamnolipids are involved in the maintenance of biofilms pathways for nutrients transport ⁽¹³³⁾. And rhamnolipids are

responsible for the mushroom shaped biofilm formation by promoting bacteria twitching motility ⁽¹³⁴⁾. However, once rhamnose is in abundance, it will cause the *P. aeruginosa* biofilm to disperse in advance ⁽¹³²⁾. In addition, exogenous addition of purified *P. aeruginosa* rhamnolipids to wild-type biofilms also caused bacterial detachment ⁽¹³⁵⁾. Thus, exogenous rhamnose can serve as an anti-biofilm agent against QS targets.

1.5.2 Chemically induced dispersal

Studies of the mechanisms of bacterial dispersal from biofilms to eliminate biofilms have begun. Biofilm dispersal is the elimination of biofilms by driving biofilm cells actively into the dispersed state through the stimulation of dispersal agents. ⁽¹³⁶⁾. However, because biofilm bacteria are attached and enclosed by EPS, making dispersal difficult. ⁽¹³⁷⁾. It has been identified various biological factors that compel bacterial cells to disperse from biofilms, and these reasons are divided into an active process of seeding dispersion and a passive process associated with shearing ⁽¹³⁷⁾.

At the end of the biofilm life cycle, the biofilm begins to age and can no longer provide sufficient nutrients for bacteria growth in it. At that

time, the bacteria will disperse from the biofilm ⁽⁵⁷⁾. Many active dispersal are induced by modifying environmental factors, such as nutrients, oxygen supply, nitric oxide, pH and chemicals ⁽¹³⁷⁾.

Chemically induced dispersal (CID) is through bacteria respond to environmental changes and external stresses ⁽⁵⁷⁾. When bacteria detect these changes and stresses, they communicate through signalling channels and molecules to activate dispersal. CID-dominated biofilm dispersal is primarily associated with two major regulatory systems of bacterial biofilms: the QS system and c-di-GMP signalling.

Biofilm dispersal has been shown to be related to the QS system. QS signaling molecules can induce biofilm dispersal by activating genes that promote detachment from the biofilm and by disrupting the structural integrity of the biofilm matrix ⁽¹³⁸⁾. For example, in *P. aeruginosa* biofilms, rhamnolipids are QS-regulated biosurfactants that are secreted to induce detachment during dispersal ⁽⁵⁷⁾. These biosurfactants break down the biofilm matrix, making it easier for bacteria to detach and disperse ⁽¹³⁹⁾.

Another way to induce biofilm dispersal is to regulate the c-di-GMP

levels. DGC produces c-di-GMP, which PDE then degrades. Thus, by inducing the PDE expression, the c-di-GMP level can be reduced, leading to biofilm dispersal ⁽⁵⁷⁾ .

1.5.2.1 Anti-biofilm agents targeting c-di-GMP signalling

The c-di-GMP is closely linked to the life cycle of biofilms and the transition from the more virulent stage of acute infection to the less virulent form of chronic infection ^(98, 140). It has been shown that biofilm cells can disperse when *P. aeruginosa* biofilm-grown cells are treated with toxic compounds, which may be due to the defence response of the bacterial cells ^(141, 142). This process requires the environmental sensor BdlA, which initiates the degradation of c-di-GMP, then leading to the disruption of the biofilm ⁽¹⁴³⁾.

Since PDE is essential for the c-di-GMP degradation, PDE activators can also act as anti-biofilm agents. Although stimulation of PDE activity has more potential to reduce c-di-GMP levels in bacteria than inhibition of DGC activity, there has been limited research on the discovery of compounds with stimulating PDE activity.

Nitric oxide (NO) is the most commonly used PDE activator and suppliers of it have been shown to have the effect of inducing biofilm

dispersal, such as SNP (Sodium Nitroprusside) ⁽¹⁴⁴⁾. NO stimulates the PDE activity of *P. aeruginosa*, leading to an overall reduction in intracellular c-di-GMP and dispersal ⁽⁵⁷⁾.

Another PDE activator is the compound H6-335-P1. It mediates the activation of *bifA* gene involved in the encoding of PDE in *P. aeruginosa*, then effectively reduces the level of c-di-GMP in *P. aeruginosa* to result in the dispersion of bacterial biofilms ⁽¹⁴⁵⁾.

DGC is also essential for c-di-GMP synthesis. DGC inhibitors can effectively eliminate biofilms. It has been shown that ebselen, a small molecule compound, disrupts DGC activity in *P. aeruginosa* by covalently modifying a cysteine residue near the c-di-GMP binding site ⁽¹⁴⁶⁾. The ebselen oxide also inhibits the c-di-GMP generation by the same covalent mechanism ⁽¹⁴⁷⁾.

The 2'-F-c-di-GMP is another *P. aeruginosa* DGC inhibitor. And it is an analogue of c-di-GMP, a potent DGC inhibitor in which the fluoride replaces the 2'-OH of c-di-GMP ⁽¹⁴⁸⁾. This compound also inhibits phosphodiesterase (PDE), and its inhibitory effect on PDE is more potent than its impact on DGC, and causes high concentration of c-di-GMP ⁽¹⁴⁶⁾.

1.5.3 Enzymatic disassembly (EDA)

Another method is disassembly, which disrupts or degrades biofilm matrix by shear forces directly ⁽⁵⁷⁾. The bacteria in the biofilm will be exposed after biofilm disruption, and the infection can be treated with a combination of antibiotics. As the EPS is the main component of biofilms, enzymatic disassembly (EDA) can be applied to eliminate them.

The enzymatic disassembly degrades EPS matrix by enzymes to release the bacteria from biofilms, such as glycoside hydrolases, deoxyribonucleases and protein hydrolases ⁽¹⁴⁹⁾. For example, proteases can degrade adhesive and extracellular proteins of biofilms, weakening their adhesion and structure to result in biofilm disassembly ⁽¹⁵⁰⁾.

Besides, cellulase can destroy the biofilms of *Staphylococcus aureus* and *P. aeruginosa* in wounds ⁽¹⁵¹⁾. Cellulase can hydrolyze Psl to smaller saccharide molecules to destroy the structure of Psl ⁽¹⁵²⁾. Moreover, it can break the organized structure formed by Psl on the surface of bacteria ⁽¹⁵²⁾. By hydrolyzing Psl and disrupting its helical distribution, cellulase can reduce the concentration of Psl, which has a

negative impact on the formation and stability of bacterial biofilms ⁽¹⁵³⁾.

This can inhibit the bacterial growth, attachment and survival ^(152, 153).

1.5.4 Development of other anti-biofilm agents

Furthermore, there are also a variety of methods that are effective in eliminating biofilms, such as phages, photodynamic therapy, nanotechnology, natural plant compounds.

Phages have been shown to disrupt the *P. aeruginosa* biofilm. Phages are natural viruses that infect bacteria. They replicate within their host bacterial cells, causing the number of phages to increase and then destroying more bacteria ⁽¹⁵⁴⁾.

Nanotechnology has been employed to combat biofilms. Nanoparticles can easily and selectively penetrate the structural barriers of biofilms and target the matrix components of biofilms to effectively eliminate planktonic bacteria, antibiotic-resistant species, and biofilm structures ⁽¹⁵⁵⁾. The combination of anti-biofilm agents with nanoparticles has been observed to enhance anti-biofilm activity ⁽¹⁵⁶⁾.

Photodynamic therapy (PDT), which is used in the anti-cancer field, can also be used to eliminate bacterial biofilms. The mechanism of

PDT is that the fluorescent photosensitizer would produce reactive oxygen species (ROS) when exposed to light to induce death of cancer cells or bacteria ⁽¹⁵⁷⁾. Studies have shown that antimicrobial photodynamic therapy (aPDT) can cause an increase in intracellular reactive oxygen species in *P. aeruginosa* biofilms. ⁽¹⁵⁸⁾. Moreover, aPDT-dominated biofilm eradication is followed by ROS-induced oxidative stress, biofilm matrix reduction, adhesion loss and compositional changes ⁽¹⁵⁹⁾.

Juglone is a natural naphthoquinone compound that is derived from various parts of the walnut tree, including the roots, stems, leaves, and fruits ⁽¹⁶⁰⁾. It has been found to possess several beneficial properties, including antibacterial, antiviral, antifungal, and antioxidant activities ⁽¹⁶¹⁾. Previous studies have demonstrated that Juglone exhibits antibacterial effects and specifically inhibits the growth of Gram-positive bacteria, such as *Staphylococcus aureus* ⁽¹⁶²⁾. These findings suggest that Juglone may have potential as an antibacterial agent.

1.6 QS inhibitors

In the case of chronic infections caused by biofilms, bacteria

dispersed by biofilms are often a significant reason for secondary infections ⁽¹⁶³⁾. This is because the bacteria released by biofilms will revert to a planktonic status, recolonize the surface and start a new life cycle ⁽¹⁶⁴⁾. Thus, it is important to inhibit bacteria from forming biofilms.

Antibiotics are generally preferred for killing planktonic bacteria and inhibiting biofilm formation. Some traditional antibiotics, such as tobramycin, ciprofloxacin and mucormycin, significantly inhibit the formation of *P. aeruginosa* biofilms ⁽¹⁶⁵⁾. However, due to the increasing resistance of *P. aeruginosa* bacteria to antibiotics, the development of new antimicrobial agents is essential.

In addition to antibiotics, blockage of the QS system is an important strategy to prevent biofilms. *P. aeruginosa* uses the QS system to control virulence factor and biofilm formation. Therefore, it is viable to inhibit biofilm formation by developing biofilm inhibitors targeting the QS system. For example, flavonoid, a product of natural plant metabolism, is a natural antagonist of LasR and RhlR, which can significantly reduce the binding capacity of the QS regulatory gene promoter of *P. aeruginosa*, resulting in biofilm inhibition ⁽¹⁶⁶⁾.

Small molecule QSIs are synthetic compounds that can interfere with QS, such as signal synthesis, signal binding or signal transduction ⁽¹⁶⁷⁾. For example, 5-fluorouracil is an antimetabolite that inhibits *P. aeruginosa* QS by interfering with the synthesis of QS signaling molecules ⁽¹⁶⁸⁾. The cinnamaldehyde also reduces the DNA binding capacity of the response regulator LuxR ⁽¹⁶⁹⁾. Moreover, synthetic analogues of HHQ and PQS have been developed to interfere with *P. aeruginosa* QS and inhibit biofilm formation ⁽¹⁷⁰⁾. PqsR is the receptor protein for PQS, and some QS inhibitors can reduce biofilms by binding to its ligand-binding domain ⁽¹¹⁹⁾. Vanillin is an example of this kind of QS inhibitor, and it has been shown to inhibit the PQS QS system and associated virulence by binding to the active site of PqsR, and then to against *P. aeruginosa* infections ⁽⁵⁷⁾.

Enzymatic QSIs are enzymes that can degrade QS signal molecules, thereby inhibiting QS ⁽¹⁷¹⁾. The AHL lactases are enzymes that can hydrolyse the lactone ring of the AHL signalling molecule, thereby inactivating it ⁽¹⁷²⁾. For example, AiiA from *Bacillus spp.* and QsdA from *Ralstonia eutropha* ⁽¹⁷³⁾. The AHL acylases are enzymes that hydrolyse the amide bond of the AHL signalling molecule, thereby inactivating it. PvdQ from *P. aeruginosa* is an AHL acylase ⁽¹⁷⁴⁾. The

enzymes of AHL oxidases and reductases can modify the structure and then change the activity of AHL signalling molecules ⁽¹⁷⁵⁾. For example, the AHL oxidase QuiP of *P. aeruginosa* can modify the QS signalling molecule, resulting in the reduction of activity ⁽¹⁷⁵⁾.

1.7 Microfluidics-based cultivation methods

Microfluidic chips are microchips that integrate the sample preparation, reaction, separation and detection ⁽¹⁷⁶⁾. In contrast to conventional laboratories, they allow the adjustment of the microenvironment to the requirements, such as the flow of microfluidics in microchannels, to improve the efficiency of experiments ⁽¹⁷⁷⁾. Microfluidic chips are widely used in fields such as single-cell analysis, genetic engineering and proteomics, and they provide in-depth study of bacteria and fungi ⁽¹⁷⁶⁾.

1.7.1 Experimental approaches to cultivate biofilms

The most commonly used method for static biofilm incubation is the micro-titration plate static biofilm model. This method can provide a high throughput screen for mutants with attachment defects or evaluate the effect of different treatments or compounds on

attachment or biofilm formation ⁽¹⁷⁸⁾.

Another way is to grow biofilms in the Calgary Biofilm Device (CBD), which consists of a polystyrene cover with 96 pegs and a standard 96-well micro-titration plate ⁽¹⁷⁹⁾. By culturing biofilms on the surface of the pegs, researchers can observe and evaluate the phenotypic characteristics of different microbial strains. These characteristics may include growth rate, cell aggregation, adhesion capacity, biofilm structure and antibiotic resistance ⁽¹⁸⁰⁾.

However, there are some common drawbacks of traditional biofilm culturing methods, such as 96-well plates, CBD and Petri dishes. For example, traditional methods lack 3D structure and complexity ⁽¹⁸¹⁾. Traditional 96-well trays and Petri dishes are based on a 2D planar culture system that does not provide a complex 3D environment and structure ⁽¹⁸²⁾. For some biofilms, particularly those with complex structures and functions, such 2D culture conditions can not truly simulate their natural environment.

In addition, the limited supply of oxygen and nutrients is a disadvantage of the conventional way to culture biofilms ^(183, 184). Conventional 96-well trays and Petri dishes may not provide sufficient

supply of oxygen and nutrients due to limitations in the size and volume of the wells or Petri dishes. This can result in slow, non-uniform biofilm growth or nutrient deficiencies ⁽¹⁸⁴⁾. However, the use of microfluidics to culture biofilms can provide sufficient nutrients through flow and remove metabolic wastes ⁽¹⁸⁵⁾. Flow chamber biofilms can be continuously monitored in real-time, allowing the observation of biofilm formation and development over time ⁽¹⁸⁶⁾. Hence, a high throughput device with stable flow that can rapidly form 3D biofilms needs to be developed.

1.7.2 Microfluidic chips

The materials used to manufacture the microfluidic chips are mainly silicon or polymers such as polydimethylsiloxane or PDMS, polymethyl methacrylate or PMMA ⁽¹⁸⁷⁾. There are microfluidic grooves and channels in the chips for sample collection and fluid flow ⁽¹⁸⁸⁾. It contains a small platform for the channel system, which is connected to the media reservoir by microtubes ⁽¹⁸⁹⁾. Microfluidic chips are highly complex two- (2D) and three-dimensional (3D) microstructures that combine sample preparation, reaction, separation and detection ⁽¹⁹⁰⁾. They are popular in the laboratory for their high analytical throughput,

high sensitivity, ability to handle and process reduced volumes of reagents, small carbon footprint and low cost ^(187, 191).

1.7.3 Microfluidics in microbiology

Microfluidic technology offers several advantages in culturing bacteria. Microfluidic devices allow almost instantaneous changes in the environmental conditions within the device to study adaptation of bacteria ⁽¹⁹²⁾. It is possible to set specific conditions to enable the growth of several species of bacteria, including low-nutrient bacteria, which is still a challenge for traditional 2D cultures ^(182, 193). In addition, microfluidics is high flexibility in design, which makes it suitable for a wide range of research methods ⁽¹⁹⁴⁾. Microfluidics can be used to study bacterial phenotypes at the single cell level, which can provide insight into the molecular and cellular microbiology of host-bacteria interactions ⁽¹⁸²⁾.

1.7.3.1 Biofilm formation

Bacterial biofilms are highly dynamic and sensitive to the environment. The advantages of the microfluidic model are beneficial for the formation of bacterial biofilms, such as spatial and temperature control and precise concentration gradients ^(182, 195).

Biofilms are often cultured through conventional methods, such as culture flasks, Petri dishes or well plates ⁽¹⁸²⁾. However, the moldability of microfluidic chips can provide a similar growth environment *in vivo* for biofilms. As the optical transparency of the microfluidic model allows visualization and analysis by microscopy, it can be combined with microscopy techniques to track the changes in bacterial biofilms ⁽¹⁹⁶⁾. In addition, microfluidics integrates multiple experimental processes into a single platform, shortening the experimental time and thus reducing the number of operations performed by researchers, which reduces the risk of contamination and technical variability compared to traditional methods ⁽¹⁸²⁾.

Microfluidic devices for biofilm growth are already available. For example, A microfluidic device consisting of microfluidic channels and distributed pneumatic pumps has been developed to provide continuous or intermittent fluid flow to 96 individual biofilms ⁽¹⁹⁷⁾. And it successfully was applied to test the sensitivity of PAO1 biofilm to different antimicrobial agents. ⁽¹⁹⁷⁾.

1.7.3.2 Antibacterial agents

Microfluidics has also been developed for drug screening. Traditional

drug screening takes time and is costly, but as a miniaturized technology, microfluidics provides high analytical efficiency and high throughput capabilities ⁽¹⁹⁰⁾. The multi-channel and array design enables high throughput and can increase screening efficiency by hundreds of times ⁽¹⁹⁸⁾. Moreover, microfluidic devices are small and many functions can be integrated into a single chip, so it also reduces costs and reagent consumption ⁽¹⁹⁹⁾.

1.8 Knowledge gaps

1. Optimization of biofilm growth environment: Although recent advances in biofilm modelling have incorporated 3D feature components of biofilms, most studies have focused on the use of flat surfaces as the primary objective. This oversimplified concept is due to the failure to consider the multifactorial and interdependent mechanisms that are related to biofilm formation. Therefore, most of *in vitro* models currently available focus on surfaces, including mechanical deformation and geometry and nutrient availability. The currently known biofilm models also have several limitations, including the long growth time of the biofilm and the choice of method (including real-time monitoring) to assess biofilm formation and maturity.

Therefore, it is crucial to establish high-throughput biofilm arrays that are capable of growing 3D biofilms in a short period of time in an *in vivo*-like environment, and it can also monitor the process from biofilm formation to biofilm maturation and dispersal.

2. Observability of biofilm activity: There are currently very few biofilm models that are suitable for comprehensive studies and observations of biofilm dispersal and recolonization in fresh surfaces. Most biofilm models are 2D and focus on the screening of antimicrobial agents ⁽²⁰⁰⁾. There are few studies on the dispersal and recolonization of 3D biofilms. Biofilm dispersal and recolonization is a dynamic process involving the movement, adhesion and disassembly of biofilm matrix. However, in most biofilm models, the dynamic behavior of biofilms cannot be observed and continuously captured and analyzed in real time.

3. Dynamic analysis of biofilm activity: The increase in superbugs and antibiotic-resistant infections caused by biofilms has been posing a risk to the health of the world. Therefore, biofilm dispersal is very important for the study and treatment of bacterial diseases. Although there are some studies on bacterial biofilms on microfluidic devices,

they are mainly focused on bacteria-surface interactions and screening for antibiotics. So far, there are no related studies on biofilm induced dispersion and biofilm disassembly within a microfluidic model. Hence, a high throughput array microfluidic model can be established to study the dispersion of biofilms.

4. Study of bacteria released by biofilm: Both biofilm dispersal and disassembly are promising therapeutic strategies for the elimination of biofilm-mediated human infections. Lack of knowledge regarding the microorganisms released during biofilm dispersal or breakdown, however, prevents anti-biofilm strategies from being effectively used in environmental and clinical settings.

1.9 Hypotheses

1. The microfluidic device designed in this project can rapidly grow bacterial biofilms, and can be used for study of induced dispersal and disassembly of these biofilms.
2. There are differences in the population dynamics and recolonization behavior of bacterial biofilms after dispersal and disassembly.
3. The antibacterial agent can remove biofilms and reduce bacterial

biofilm infections. In microfluidic models, it can be used in synergistic combination with antibiotics to enhance inhibition of virulence expression and eradicate biofilm infections.

1.10 Objectives

1. To develop a continuous-flow microfluidic device that enables the rapid biofilm cultivation, biofilm dispersal and recolonization of fresh surfaces for observation.
2. To evaluate the differences in bacterial population dynamics between CID and EDA when bacteria depart biofilms and recolonize fresh surfaces using *P. aeruginosa* as proof-of-concept.
3. To evaluate Juglone as a novel QS-inhibiting and antibiofilm agent in combination with antibiotics to eliminate biofilms and reduce bacterial dissemination.

1.11 Project significance and innovation

1. The microfluidic model developed in this project can be used to evaluate novel antibiofilm agent-antibiotic combination therapies. Due to the stable fluid that provides consistent nutrients in the device,

biofilms can form rapidly, and the effects of drugs on biofilms can be observed in real-time through high-throughput arrays, which have the potential to screen drugs rapidly.

2. This project could improve the understanding of biofilm dispersal and microbial responses to environmental changes. This has important implications for the understanding of biofilm formation, development and stability and will contribute to the development of effective control and elimination biofilms.
3. By understanding the bacteria behavior and interactions during biofilm dispersal, new bacterial targets can be identified, and potential targets for future new drug development can be identified.

Chapter II. Material and Methods

2.1 Bacteria culture

Luria-Bertani (LB) medium (Difco, Becton Dickinson and Company, USA) and ABTGC medium were used for *P. aeruginosa*. ABTGC medium consists of AB minimal medium, 2.5 mg/L thiamine, supplemented with 0.2% (wt/vol) glucose and 0.2% (wt/vol) casamino acids [83]. Bacterial strains were inoculated in 2 ml of LB medium containing appropriate antibiotics for plasmid maintenance at 37 °C with shaking at 200 rpm overnight.

Table 2.1 Bacterial strains and plasmids in this study

Strain/ plasmid	Description	Source/ Reference
<i>P. aeruginosa</i>		
PAO1	Prototypic nonmucoid wild-type strain	(201)
PAO1/ <i>p_{lac}-gfp</i>	Streptomycin resistance, Tn7-transposon is present in PAO1, with constitutively expressed	(202)

	GFP	
PAO1/ <i>p_{cdrA}-gfp</i>	Carb ^r , PAO1 carrying the c-di-GMP reporter plasmid.	(203)
PAO1/ <i>P_{BAD}-YhjH/p_{lac}-gfp</i>	Streptomycin resistance, Tn7-transposon (<i>p_{lac}-gfp</i>)-carrying PAO1/ <i>P_{BAD}-YhjH</i> with constitutively expressed GFP.	(204)
<i>ΔbdIA/p_{lac}-gfp</i>	Streptomycin resistance, <i>ΔbdIA</i> carrying the Tn7-transposon (<i>p_{lac}-gfp</i>), with constitutively expressed GFP.	This study
<i>ΔfliM/p_{lac}-gfp</i>	Streptomycin resistance, <i>ΔfliM</i> carrying the Tn7-transposon (<i>p_{lac}-gfp</i>), with constitutively expressed GFP.	This study
<i>ΔPelA/p_{lac}-gfp</i>	Streptomycin resistance, <i>ΔPel</i> carrying the Tn7-transposon (<i>p_{lac}-gfp</i>), with constitutively expressed GFP.	(205)
<i>ΔPsIBCD/p_{lac}-gfp</i>	Streptomycin resistance, <i>ΔPsI</i> carrying the Tn7-transposon (<i>p_{lac}-gfp</i>), with constitutively	(205)

	expressed GFP.	
$\Delta PeI\Delta PsI/BCD/p_{lac^-}gfp$	Streptomycin resistance, $\Delta PeI/PsI$ carrying the Tn7-transposon ($p_{lac^-}gfp$), with constitutively expressed GFP.	(205)
$\Delta pqsA /p_{pqsA^-}gfp$	Gentamycin resistance, $\Delta pqsA$ carrying a $pqsA$ -gfp reporter fusion	(206)
$\Delta pqsR/p_{pqsA^-}gfp$	Gentamycin resistance, $\Delta pqsA$ carrying a $pqsA$ -gfp reporter fusion	(206)
$\Delta pqsR/p_{lac^-}pqsABCDE/p_{pqsA^-}gfp$	Gentamycin resistance, $\Delta pqsR/pqsABCDE$ carrying a $pqsA$ -gfp reporter fusion.	(206)

2.2 BDR model design and fabrication

The microwells layer and gradient generator were made by the backside lithography procedure ⁽²⁰⁷⁾. The channel layer was obtained from a 3D printing mold. Polydimethylsiloxane (PDMS), used to create the microfluidic device, was created using a Sylgard 184 silicone elastomer kit from Dow Corning in the United States by completely

combining the base resin and curing agent in a ratio of 10:1 by weight. The cured PDMS moulds underwent plasma treatment and were left in a vacuum desiccator for 24 hrs before being subjected to trichloro(1H,1H,2H,2H-perfluorooctyl) silane (Sigma-Aldrich, # 448931, Germany). Using plasma technology, the various layers were joined together, and they were then baked for two hours at 70°C. The valve was created using 3D printing technology directly.

2.3 Biofilm formation in 8-well chamber

The bacteria were added at a ratio of 1:1000 to the 8-well chamber containing ABTGC then incubated at 37°C for 12 hrs. After treatment with the agent, the bacteria were stained with Propidium iodide (PI, Invitrogen™) dye, then wash 2 times with 0.9% NaCl. Images were then captured with Leica TCS SP8 MP Confocal Microscope.

2.4 The formation of biofilms in the microfluidics device

The bacterial culture grown in LB Broth for 12 hrs in 37°C was diluted with ABT minimal medium containing 30 mM glucose and 5 g/L casamino acids (ABTGC) medium ⁽²⁰⁸⁾ at 1:100. The BDR device was disinfected with 75% alcohol and removing bubbles before use. First,

the valve was closed. Then a sterile syringe was used to inject 100 μ l of diluted bacterial solution into the dispersion channel. Allow the bacteria to settle in the microwells for 20 minutes. In order to continuously supply nutrients to the biofilm growth, and the appropriate shear force of the water flow could help the biofilm growth, we chose to use flow rate of 4 ml/hr. ABTGC medium was injected by a syringe pump (New Era Pump Systems, Farmingdale, USA) from the water inlet with flow rate of 4 ml/hr for 12 hrs to form biofilms in a 37°C room. The valve is then opened, allowing the bacteria that were previously trapped in the biofilm to recolonize the secondary channel.

2.5 CID and EDA assay

Biofilms were cultured 12 hrs continuously with 4 ml/hr ABTGC flow. 150 μ M SNP (Sigma-Aldrich, Germany) and 5% (w/v) L-arabinose (Sigma-Aldrich, Germany) were used as dispersal agents for treating biofilms. As biofilm disturbing agents, Sigma-Aldrich, Germany's Cellulase (100 units/ml) and 12.5 units/ml pectinase (from *Aspergillus aculeatus*, Sigma-Aldrich, Germany) were utilized. The biofilm dispersal agents and disrupting agents continuously flowed into the BDR device at 4ml/hr through the syringe pump in 37°C ⁽²⁰⁹⁾. The bacterial fluid

flowing out of the dispersal channel was collected for measuring c-di-GMP level and confocal imaging.

2.6 Establishment of the mathematical modelling

The mathematical model was established together with Mr. Hua Junhao a collaborator from CityU. There were two sections to the modelling program: data processing; and video processing. In the video processing part, the program could be divided into four steps. Firstly, the program splited each frame of the video into images. Secondly, the program converted these images to grayscale. Thirdly, the program set a global threshold ($T=30$) and used the threshold function to convert pixels greater than 30 to 255, convert those pixels less than 30 to 0. In order to save the value into a text file, the program would then counted the amount of white pixels in each image.

In the data processing part, the program would normalize the beginning value to the same intensity. Polynomial regression and exponential regression were the regression formulas that MATLAB employed. The regression formula showed blow:

First order polynomial regression:

$$y = ax + b \quad (1)$$

Second order polynomial regression: control

$$y = ax^2 + bx + c \quad (2)$$

Fourth order polynomial regression:

$$y = ax^4 + bx^3 + cx^2 + dx + e \quad (3)$$

Exponential regression:

$$y = a \cdot e^{bx} \quad (4)$$

The parameters that needed to be fitted were a, b, c, d and e . Video frame numbers were represented by x , while normalised intensity was represented by y .

The R^2 value assessed how well a model was able to predict data. The higher the value of R^2 , the better the model was at predicting the data.

The equation below could be employed for calculating the value of R^2 :

$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (5)$$

The $\hat{y}$ calculated values of regression equation, $\bar{y}$ was the mean value of y .

2.7 Quantification of bacterial numbers via CFU

Cell suspensions were serially diluted in saline and transferred to LBA agar plates (5 technical replicates), and incubated at 37°C for 12 h. Counted the colonies growing on the Petri dishes and tabulated with $\text{CFU /ml} = \text{number of colonies} \times \text{dilution factor} / \text{volume}$ ⁽²¹⁰⁾.

During the bacterial dispersal, dispersed bacteria were collected at the water outlet of the dispersal channel, and CFU of the dispersing bacteria was performed. After the bacterial dispersion process, the dispersal channel and the recolonization channel were repeatedly rinsed with 1 ml of saline, respectively, to make the biofilms in the microwells fall off into the saline, and then CFU was performed. Experiments were performed in triplicate, and the result was shown with the mean $\pm$ standard deviation.

2.8 Quantification of c-di-GMP levels in biofilms by ELISA kit

The 1 ml of 0.9% NaCl was injected into the dispersal chamber using a sterile syringe, then dislodge the biofilms in the microwells, and then

the liquid was transferred to a 1.5 ml centrifuge tube. And then the bacteria were lysed for 5 minutes at 40% amplitude using a probe sonicator (SFX 550, SSE-1, Branson, Emerson, USA) with a 30 second on/30 second off output. According to the manufacturer's instructions, a c-di-GMP ELISA kit (LMAI, Shanghai, China) was used to test the c-di-GMP concentration, and a microplate reader (Tecan, Infinite M1000 Pro, Switzerland) was used to measure it at OD 490 nm. Biofilm video captured by Time-Lapse Function of confocal microscope.

2.9 Quantification of GFP fluorescence from cdrA-gfp biosensor

The software ImageJ was used to measure the fluorescence levels of green fluorescent protein (GFP) and pyoverdine in biofilm images. The formula is corrected total cell fluorescence (CTCF) = integrated density - (area of selected cells X mean fluorescence of background readings). The level of fluorescence is positively correlated with the number of biofilm cells.

2.10 Confocal imaging

Brightfield, GFP, and pyoverdine fluorescence in Z-stacks were all monitored by a Leica TCS SP8 Confocal Microscope system with the

10x objective to photograph all microscopic images of biofilms. ImageJ, LAS X and Imaris were used to process the images. Experiments were performed in triplicate.

2.11 Coating Pel and Psl exopolysaccharides on microwells

The extracted 10 µg/ml Pel and Psl polysaccharides were coated on the surface of the microwells for 16 hrs at 4°C and dried for 2 hrs at room temperature. The *Δpe/AΔps/BCD/p_{lac}-gfp* was used to incubation of biofilms on microtiter wells coated with Pel or Psl exopolysaccharides.

2.12 Human lung fibroblasts culture

Human lung fibroblasts (HLF, ATCC) were cultured in T25 flasks (Thermo Fisher Scientific) with a density of 1×10^6 cells/ml for 72 hrs in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at the incubator with 37°C, 5% CO₂.

2.13 Establishment of HLF 3D spheroids model in BDR device

To generate 3D spheroids, the 20 µl droplets were dropped onto the

lids of hydrated 60 X 15 mm cell culture dishes (SPL) and incubated at 37°C, 5% CO₂ for 24 hrs ⁽²¹¹⁾. The spheroids were stained by Cell Tracking Deep Red Fluorescence stain (Invitrogen™) before transferred to the secondary chamber of the BDR device by a sterile syringe and needle, then incubated for 20 minutes at 37°C, 5% CO₂ to encourage adherence to the microwells bottom.

The infection was observed directly at 0 hrs, 2 hrs and 5 hrs with the Leica TCS SP8 MP using 10x objective and 40X zoom. Propidium iodide (PI, Invitrogen™) was used to stain dead cells.

2.14 *C. elegans* culture

By distributing the bacteria on nematode growth medium (NGM) agar plates at 37°C for 16 hours, *E.coli* OP50 was employed to make worm feed. ⁽²⁰⁵⁾. To increase the population, *C. elegans* was cultivated on an OP50 plate for 72 hours at room temperature.

2.15 Establishment of *C. elegans* *in vivo* infection model

The length of the *C. elegans* in stage L2 and L3 (0.3 mm-0.75 mm) is adapted to the volume of the microwells (length X width X height: 250

$\mu\text{m} \times 150 \mu\text{m} \times 150 \mu\text{m}$) ⁽²¹²⁾. The stages of L2 and L3 *C. elegans* were transferred to the secondary chamber of the BDR device. Then the valve was opened to allow the flow to enter the recolonization channel to infect the *C. elegans*. The experiment was performed at 25°C.

The infection was observed directly at 3 hrs, 6hrs and 12hrs with the CLMS using 10x objective. The 20 *C. elegans* were collected at each time point and performed CFU.

2.16 Molecular docking

The water molecules were deleted from PqsR (4JVC) by AutoDock Tool v.1.5.7 ⁽²¹³⁾. The PyMol by Schrödinger v. 2.5 was used to find the polar hydrogens. The amino acid residues round primary ligand NHQ and polar hydrogens of PqsR were searched, then docking was started. The BIOVIA Discovery Studio was used to detect and compare the docking sites of PqsR for ligand NHQ and Juglone. Then the docking images was generated by PyMol.

2.17 Half-maximal inhibitory concentrations (IC₅₀) and the minimum inhibitory concentration (MIC)

The bacterial culture grown in LB Broth for 12 hrs at 37°C was diluted with ABT minimal medium containing 30 mM glucose and 5 g/l casamino acids (ABTGC) medium ⁽²⁰⁸⁾ with different concentrations of Juglone (0-10µg/ml) in 96 wells plate. The plate was then overnight incubated in a 37°C Tecan Infinite 200 Pro microplate reader (Tecan Group Ltd, Männedorf, Switzerland), with OD600 analysis performed every 15 minutes.

2.18 Quantification of PQS concentration

As previous study ⁽²⁰⁶⁾, we inoculated the PAO1 culture overnight at 37 °C shaking incubator, then diluted the bacteria in ABTGC medium with different concentrations of Juglone for 7 hours at 37 °C shaking incubator to produce PQS. Then the 100µl supernatant was added into 96 wells plate and mixed with 100µl $\Delta pqsA/p_{pqsA}\text{-}gfp$, $\Delta pqsR/p_{pqsA}\text{-}gfp$ and $\Delta pqsR/p_{lac}\text{-}pqsABCDE/p_{pqsA}\text{-}gfp$ mutants, respectively. The OD600 and GFP value of 96-well plate was measured by the microplate reader overnight at 37 °C every 15 min.

2.19 Swarming motility assay

The swarming experiment was performed on the 0.5% LB agar plates⁽²⁰⁶⁾. The 5 µl PAO1 overnight culture was directly placed on the top of the centre part of the agar. Then the plates were incubated in the 37 °C incubator overnight.

2.20 Pyocyanin production

The PAO1 was inoculated in 10 ml ABTGC with various concentrations of Juglone and cultured in 37 °C shaking incubator overnight. The ABTGC was used as negative control, the chloroform was added into filtered supernatant of overnight culture, the upper layer was discarded and 0.2M HCl was added to extracted pyocyanin. Then measured the OD520 value.

2.21 Medaka fish infection model

The medaka fish infection model was previously developed by Dr. Sylvia, Liu Yang and used in this study. As previously described⁽²¹⁴⁾, before surgery, larval fish (1 month age) were first anesthetized with 0.015 µM tricaine mesylate (Sigma-Aldrich, #MS-222, USA). Next, the

skin wound (~100 µm) on the tail of the fish was gently scratched with a sterile syringe needle (26G × 1 1/2", 0.45n × 13 mm, Terumo, Japan) under an optical stereomicroscope and then infected with PAO1. A bacterial culture (OD600 = 1) was incubated with a volume ratio of 1:100 of water for 1 hr to achieve bacterial colonization. The infected fish were then incubated in fresh water for 24 hours to help establish infection.

After establishing the fish infection model, the Medaka tail was treated with 0.1 µg/ml colistin and 0.125 µg/ml Juglone for 2 hrs and then imaged using the SP8 confocal microscope. Tail samples were collected and crushed through an ultrasound machine (SFX 550, SSE-1, Branson, Emerson, USA).

2.22 Quantification of fish cytokines by ELISA kit

Fish were assayed for innate immune cytokines (TNF-α and IL-6) using an ELISA kit (Bosun, Jiangsu, China) and measured at OD 450 nm by an enzyme marker (Tecan, China) Infinite M1000 Pro, Switzerland). Cytokine concentrations were normalized by protein concentrations, which were measured at OD 280 nm with a Nanodrop (Thermofisher, NanoDrop One, ND-One-W, USA). CFUs were performed.

2.23 Statistical analysis

The mean $\pm$ standard deviation was used to express the results. Data groups were analyzed using one-way ANOVA and Student's t-test to assess the relationships between independent variables. and p-values were calculated. Each experiment was conducted in triplicate for reproducibility and did technical replicates, resulting in the mean $\pm$ standard deviation being presented for the results of each independent trial.

Chapter III Establishment of biofilm disperse-then-recolonize (BDR) microfluidics model

3.1 Summary

Some traditional methods of biofilm formation, such as 2D well plates and Calgary devices ⁽¹⁷⁹⁾, all happen in static liquid environment, which is quite different from the biological environment. Although some microfluidic biofilm formation devices are currently being developed, such as flow cell systems ⁽¹⁸⁰⁾, Microfluidic flow chambers (MFCs) ⁽²¹⁵⁾ and BioFlux device ⁽¹⁹⁷⁾, most microfluidic devices form 2D biofilms on a flat surface, and little has been described about the recolonization of biofilms after dispersal ⁽²¹⁶⁾. Hence, a microfluidic model device that allowed the study of 3D biofilm dispersal and recolonization was developed in this study.

The biofilm disperse-then-recolonize (BDR) microfluidic model was developed to evaluate both biofilm dispersal and recolonization. The BDR device consisted of a gradient generator, an upstream primary channel and a downstream secondary channel. The gradient generator allowed the mixing of two media in a concentration gradient, and their flowing into the upstream primary channel and downstream secondary

channel, each of which contained 300 microwells in which biofilms could be grown.

In order to observe the growth of biofilms, a p_{cdrA} -*gfp* biosensor was used to monitor the expression of *cdrA*, a key gene involved in biofilm formation and dispersal in the *P. aeruginosa* ⁽⁷⁹⁾. The green fluorescent protein (GFP) reporter gene is controlled by the *cdrA* promoter. As c-di-GMP is involved in regulating the formation and dispersal of *P. aeruginosa* biofilms ⁽¹⁰⁰⁾, it has also been used to evaluate the biofilm growth and dispersal activities.

According to the results of the biofilm fluorescence image, CFU and the fluorescence intensity of biofilms in the microwells, bacteria could form biofilms in microwells within 12 hrs. This result was verified by increased expression of biofilm markers. After that, the primary chamber and secondary chamber were tested by physiological dispersal within 2 hrs after biofilm formation in the primary chamber.

Finally, the concentration gradient generator was also tested with tobramycin. Tobramycin has been used for many years to treat *P. aeruginosa* infections as it is one of the antibiotics that maintains a high level of activity against *P. aeruginosa* ⁽²¹⁷⁾. In this study, 8 different

concentrations of tobramycin were generated by a concentration gradient generator to treat the preformed biofilms.

3.2 Results

3.2.1 Establishment of the BDR device

The BDR was designed with a structure consisting of four key components: a gradient generator, a valve, a seal layer and a microwells layer (Figure 3.1a). The BDR device was made of PDMS and the valves of it were made by 3D printing technology ⁽²¹⁸⁾. The initial molds for the gradient generator and the microwell layer were made using a diffuser back-side lithography procedure. The conical microwells were 250 x 150 x 150 μm (length x width x depth) and each array contained 300 microwells. The sealing layer and valve were made of a polylactic acid (PLA) material by 3D printing.

In order to verify the generation of concentration gradients, red and blue food dyes were flowed into the BDR device and COMSOL software was used to simulate the concentration gradients (Figure 3.1c). Concentration distributions were also quantified using SYTO 9 fluorescent dyes (Figure 3.1d). The results showed that the BDR device had worked and successfully generated eight different concentrations through the gradient

generator. In the middle channels, the two colors were mixed with similar ratios. The result of quantified fluorescence intensity in each channel showed that the fluorescence ratio in the middle channel was approximately 46%.

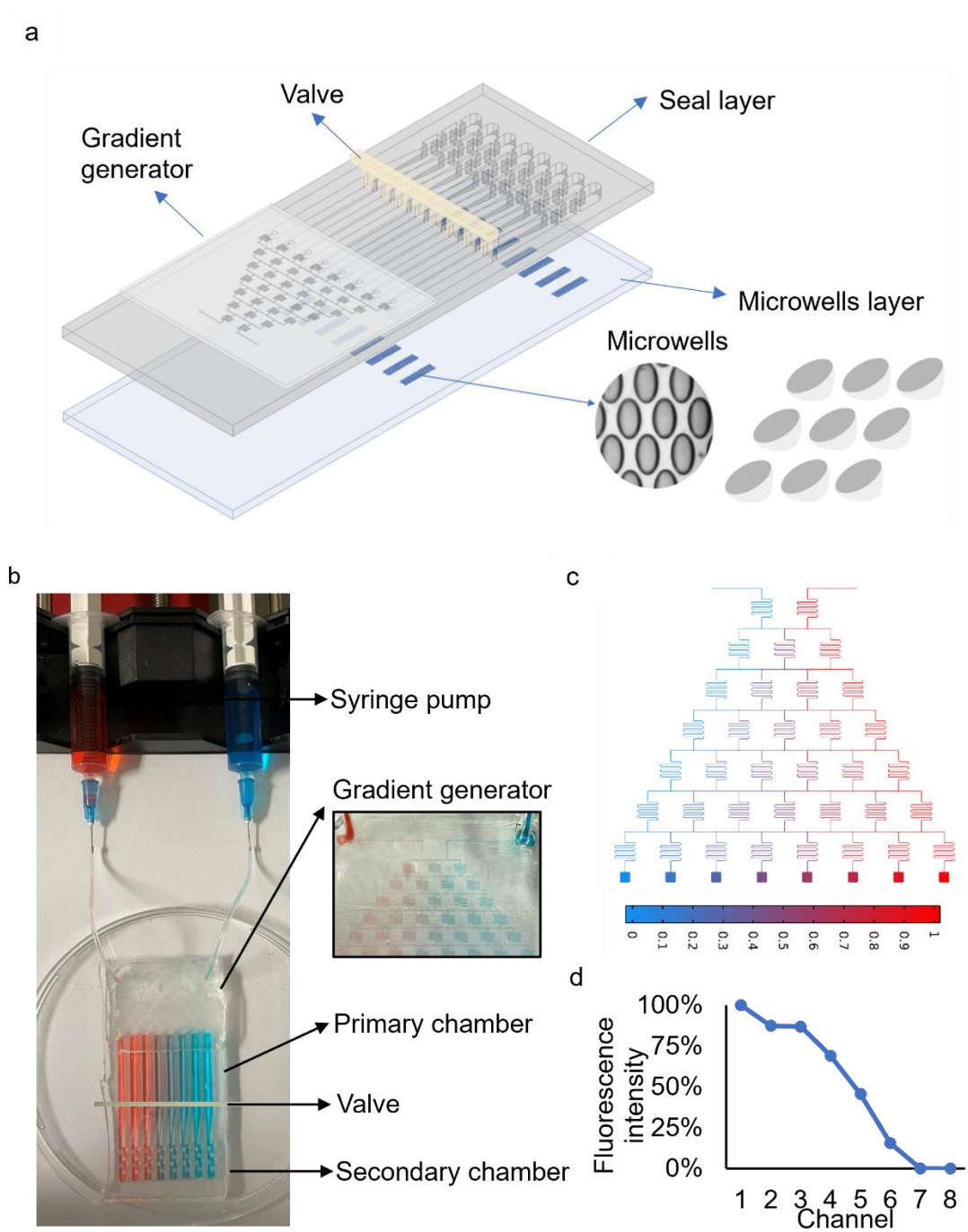


Figure 3.1 Establishment of BDR device. (a) The structure of the BDR device design includes a gradient generator, seal layer, valve and

microwells layer. (b) A working image of the complete BDR device with red and blue dyes, including syringe pump, gradient generator, valve, primary chamber and secondary chamber. (c) Flow simulation diagram of a microfluidic chip, with the flow rate indicated in the bottom legend. (d) The concentration gradient generator can distribute the SYTO 9 fluorescent stain into different concentrations (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

3.2.2 The BDR device for biofilm dispersal and recolonization

In this BDR device (Figure 3.2), the two media were mixed with a concentration gradient through a gradient generator. By adjusting the concentration, the concentration distribution could be controlled. The sealing and microwells layers had multiple channels for inducing fluid flow into the different channels. Through the distribution of the channels, the device was divided into two parts: the primary chamber for biofilm formation and dispersal, and the secondary chamber for bacterial recolonization.

The primary chamber was a straight channel for biofilms formation and dispersal. The secondary chamber was designed with a serpentine channel that could be used to collect dispersed bacteria and observe if

they could recolonize on microwells. The connection of the upper and lower parts was controlled by a valve. When the valve was closed, the liquid in the straight channel flowed out from the outlet hole at the end of the channel. When the valve was opened, the liquid would flow to the serpentine channel.

At the bottom of the device, the microwells layer provided a space for bacteria to grow. Microwells could mimic the *in vivo* environment, in which bacteria could grow and form 3D biofilms ⁽²¹⁸⁾. The channels shaped by the sealing and microwells layers enabled accurate fluid distribution so that the media would flow in a predictable direction while providing a suitable environment for bacterial growth and biofilm formation. The valves played a role in connecting the two chambers in the device. By turning the valves on or off at the appropriate time, the primary and secondary chambers could be controlled, and then the recolonization process of dispersed bacteria would be observed.

The device could be used to simulate and observe the dispersal and recolonization behavior of bacteria biofilms within different treatments, and to understand the dynamic process of biofilm development.

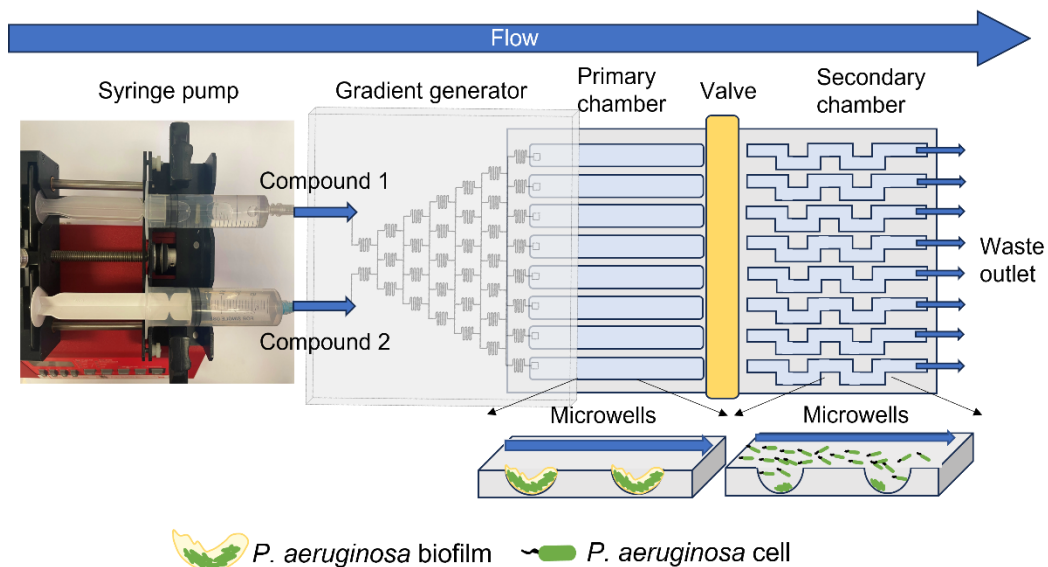


Figure 3.2. Schematic diagram of BDR device in work. The two flows were mixed by a concentration gradient generator and then flow into eight channels, where biofilms grow in the microwells, and bacteria released from the biofilms flowed into a second chamber for recolonization. (The patent pending number is PWG/PA/1529/6/2023.)

3.2.3 Verification of biofilm formation in the BDR device

To verify the formation of biofilms in the BDR device. PAO1/*p_{lac-gfp}* was grown in the BDR device with a continuous media flow of 4 ml/hr. According to the findings of the study ⁽²²⁰⁾, it was demonstrated that cells exhibited adhesion up to a flow rate of 4 ml/hr. Beyond this threshold, cell adhesion was compromised, indicating that a flow rate of 4 ml/hr served as a critical point for maintaining cell adhesion.

Consequently, this specific flow rate of 4 ml/hr was employed in order to ensure the preservation of the biofilm structure while providing an adequate supply of nutrients. The images of microwells in BDR devices were taken at 0, 6, 12 and 24 hrs (Figure 3.3a), and then the fluorescence levels were quantified (Figure 3.3b). In addition, the CFU assay was performed to evaluate bacterial numbers (Figure 3.3c). The results showed that biofilms could be formed in the BDR device in only 12 hrs. It was further confirmed by the increased expression of the biofilm markers *p_{cdrA}-gfp* biosensor and c-di-GMP (Figures 3.3d and 3.3e).

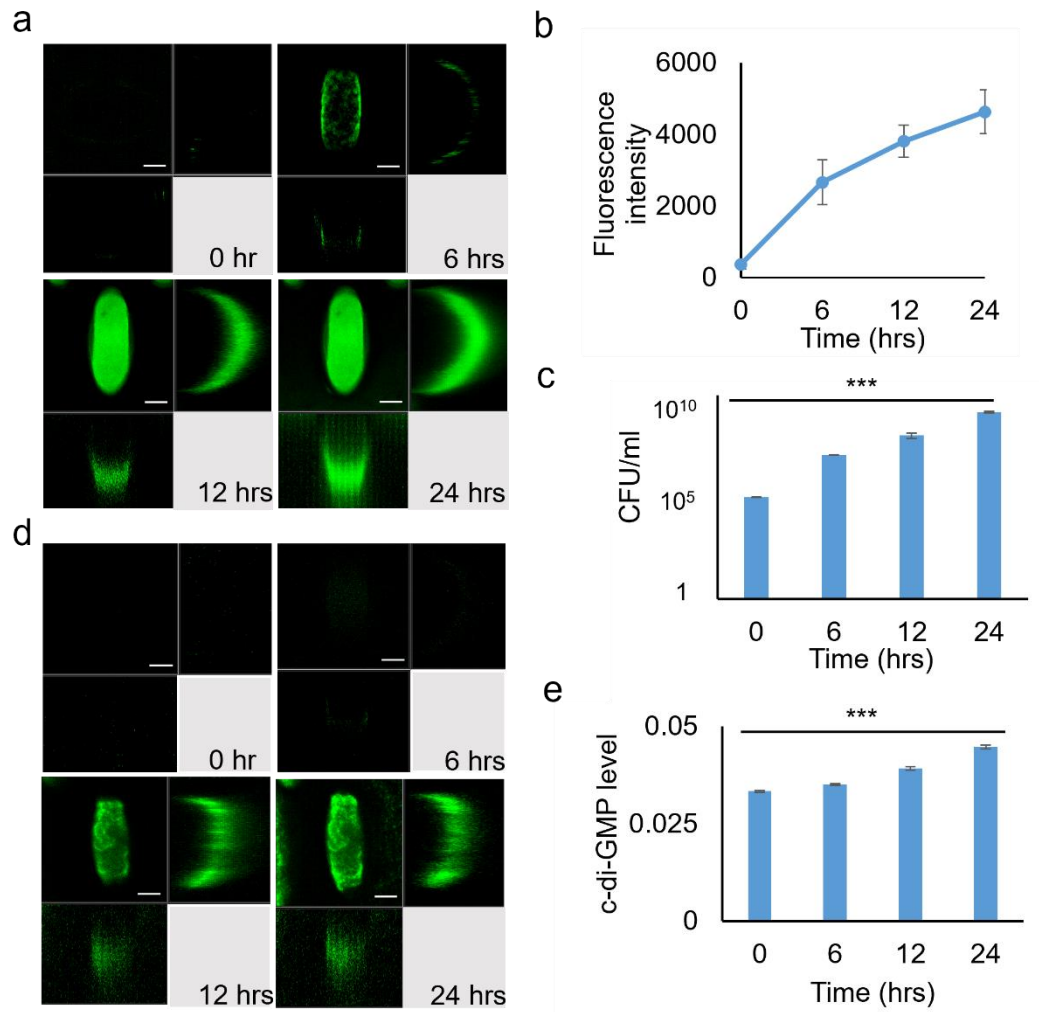


Figure 3.3 Biofilm formation over time in microfluidics chip. (a) The images of PAO1/p_{lac}-gfp biofilm formation at 0 hrs, 6 hrs, 12 hrs, and 24 hrs. (b) The fluorescence intensity of PAO1/p_{lac}-gfp biofilm. (c) CFU results of PAO1/p_{lac}-gfp biofilm. (d) Biofilm biomarkers p_{cdrA}-gfp are expressed in biofilms at 0 hrs, 6 hrs, 12 hrs, and 24 hrs. (e) The c-di-GMP level (ng/ml) at 0 hrs, 6 hrs, 12 hrs, and 24 hrs were quantified by ELISA. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The

scale bar in all images was 50 μm . *** $P < 0.001$. (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

3.2.4 Tracking of physiological biofilm dispersal in microfluidics chip

Firstly, biofilms that were incubated for 12 hrs were observed by a confocal microscope for 2 hrs and with an ABTGC flow at 4 ml/h at 37°C. The results of images and fluorescence intensity showed that the biofilm did not decreased after 2 hrs without the anti-biofilm agents (Figures 3.4a-b). Subsequently, 2-hour time-lapse images were analyzed to obtain trajectories of bacterial movement. The trajectories showed that bacteria had stayed in the microwells for a period of time and then left (Figure 3.4c), and the trend of the bacterial populations leaving the biofilm at a velocity over time was demonstrated (Figure 3.4d).

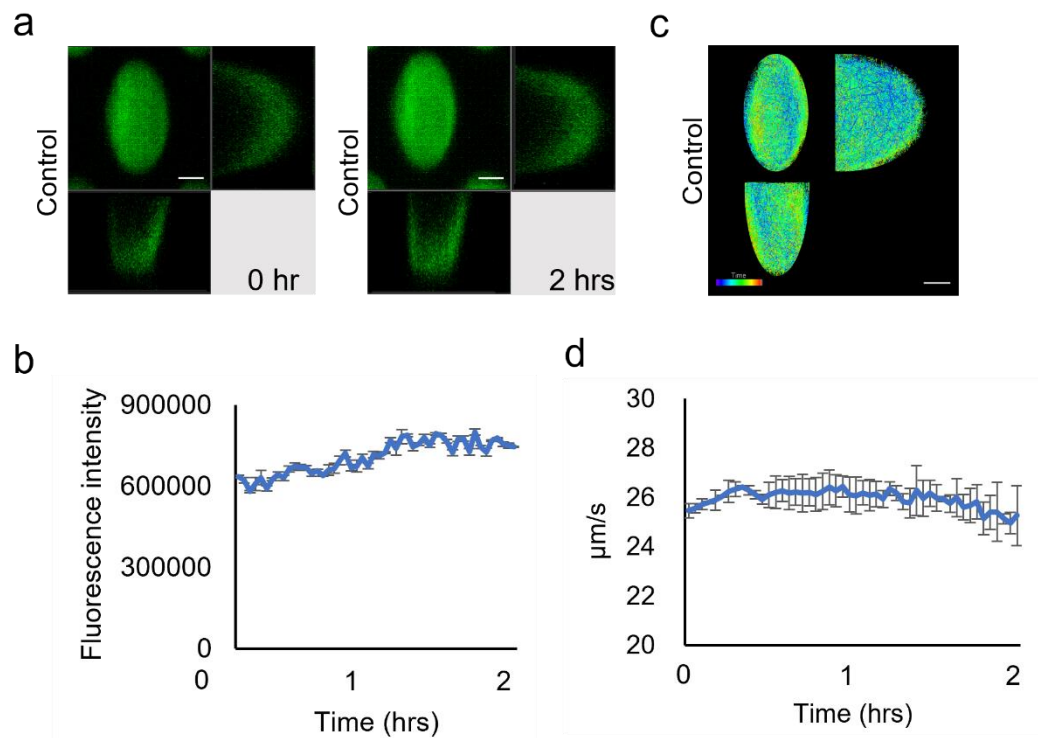


Figure 3.4. Tracking of physiological biofilm dispersal in microwells. (a) Physiological biofilm dispersal over time. (b) The biomass changes of recolonization over time. (c) Time resolved bacteria trajectories of physiologically biofilm dispersed cells. (d) The velocity of reduction in bacterial populations over time. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar in all images was 50 μm . (Reprint with permission from Ma Y, Deng Y, 2023

(219))

3.2.5 Verification of recolonization chamber in BDR device

It was confirmed that the primary chamber used for observing biofilm dispersal worked. The second chamber for recolonization was subsequently tested. The movement trajectories of bacteria in the recolonization chamber was analyzed. In the recolonization chamber, the confocal images showed that the bacteria released from physiologically biofilm dispersal were able to rapidly recolonize within 2hrs in the microwells with the physiological biofilm dispersal (Figure 3.5a). This was confirmed by quantification of the *gfp* fluorescence intensity (Figure 3.5b). The analysis of bacterial trajectories also showed that the bacteria recolonized and multiplied in the microwells within 2 hrs (Figure 3.5c). The velocity of bacterial recolonization remained consistent with the velocity in the dispersal chamber, both in the range of 25 to 28 $\mu\text{m/s}$ (Figure 3.5d). These results indicated that the serpentine chamber effectively trapped the bacteria and allowed them to grow in the microwells.

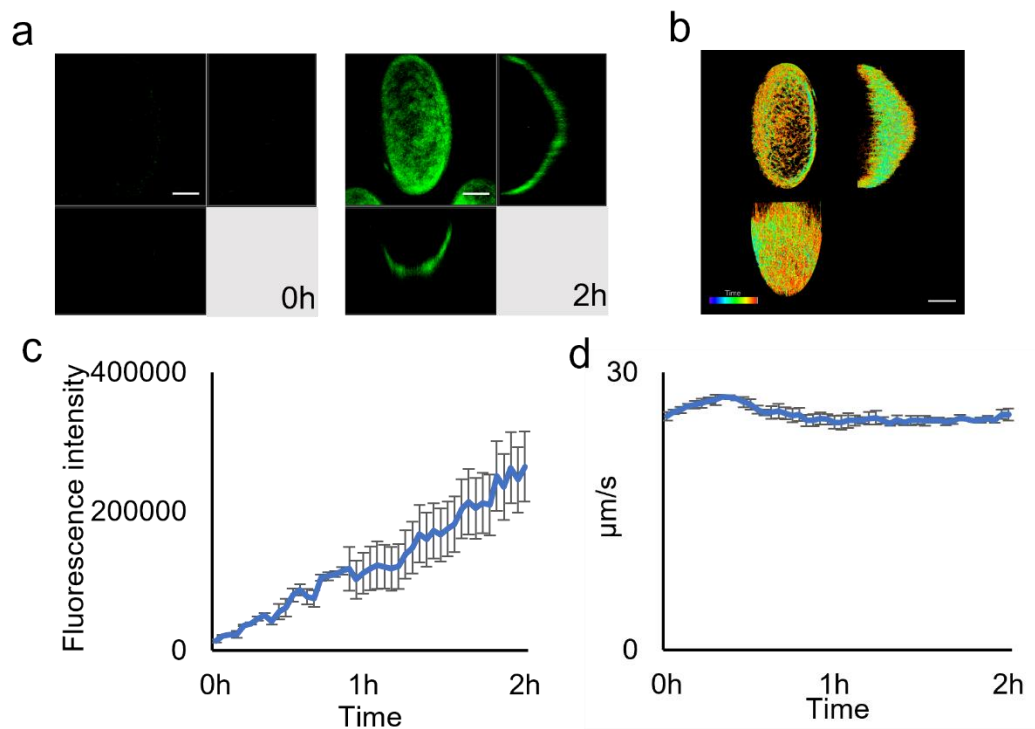


Figure 3.5. Recolonization tracking of physiological biofilm

bacteria in microwells. (a) Recolonization of physiological biofilm

bacteria over time. (b) Time-resolved bacteria trajectories of

recolonization cells. (c) The biomass changes of recolonization over

time. (d) The recolonization velocity of biofilm bacteria. Experiments

were performed in triplicate, at least 3 images were captured for each

replicate, the representative images were shown. The scale bar in all

images was 50 μm . (Reprint with permission from Ma Y, Deng Y, 2023

(219).)

3.2.6 Antibiotics flow through concentration gradient generator can kill biofilms

Next, in order to verify the effectiveness of the concentration gradient in the distribution of anti-biofilm agents, 30 µg/ml of tobramycin was used to flow into the concentration gradient generator to kill the preformed biofilm in the primary chamber. Based on the results of the fluorescence distribution of SYTO 9, 30 µg/ml of tobramycin was diluted in the concentration gradient generator by ABTGC to 8 different concentrations as shown in Table 3.1. Confocal images of the biofilm at 2 hrs showed that as the concentration of tobramycin increased, the biofilm reduced (Figure 3.6a). The biofilm CFU results in the primary chamber showed that the tobramycin could be diluted by the concentration gradient generator to generate different concentrations to kill biofilms (Figure 3.6b).

Table 3.1 Concentrations of tobramycin in 8 channels generated by the concentration gradient generator.

Channel	1	2	3	4	5	6	7	8
Concentration (µg/ml)	0	0.24	4.61	13.67	20.71	26.03	26.71	30.00

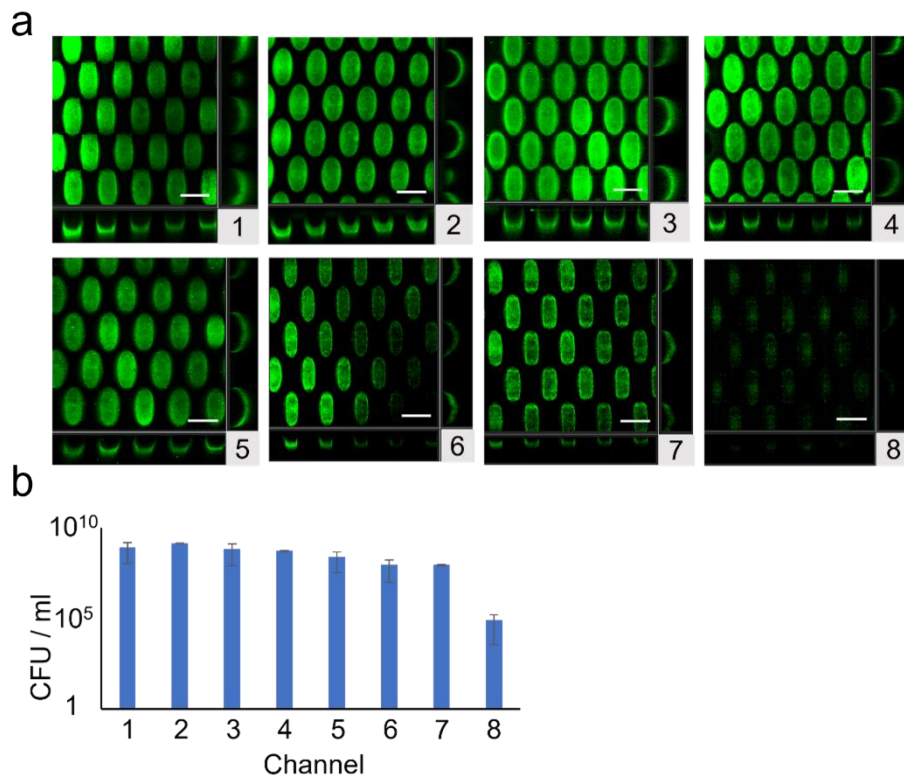


Figure 3.6. The use of tobramycin in the BDR device. (a) Confocal images of biofilms in channels 1-8 respectively after being treated with different concentrations of tobramycin generated by the concentration gradient generator. The numbers on the images represent the channels. The scale bar in images was 50 μm . (b) CFU results for biofilms in 8 channels. Experiments were performed in triplicate, the averages and s.d. from triplicate experiments were shown. (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾.)

3.3 Discussion

Bacterial biofilm infections are difficult to eliminate ⁽¹⁴⁷⁾. Therefore, in order to evaluate the effectiveness of anti-biofilm strategies and to screen for effective anti-biofilm drugs, culturing biofilms *in vitro* is necessary. Bacterial biofilm cultures in common use are mainly static cultures, where bacteria are grown in the media in a static state to form a biofilm ⁽²²¹⁾. Containers such as culture flasks, Petri dishes and well plates are usually used. Static culture is suitable for rapid biofilm formation but has the disadvantages of non-uniform biofilm structure and limited nutrient availability ⁽²²²⁾.

There are also currently some dynamic fluidic biofilm cultures using microfluidics. Microfluidic technology combines micro-channels and controlled flow conditions to enable the precise control of bacterial biofilm formation. Microfluidic cultures can provide microscale flow environments and precise control of parameters such as temperature, oxygen and nutrient supply ⁽²²³⁾. It is able to simulate the formation of bacterial biofilms under physiological conditions ⁽²²⁴⁾. Compared with other microfluidic models, such as microfluidic agarose channel devices ⁽²²⁵⁾ and microfluidic wound model ⁽²²⁶⁾, the BDR model in this

study has embedded microwells that allow biofilms to grow in it, which enables the formation of 3D biofilms with the stable flow, and the biofilm formation time is shorter. And it can be easily observed with confocal microscopy. In addition, in contrast to other biofilm culture models, the BDR device can also be used to study models for the biofilm dispersal and recolonization (Table 3.2).

Table 3.2. Comparison of current biofilm culture models and BDR model.

	Traditional 2D culture (peg lids or wells)	Flow chamber model	Our microfluidic model
2D/3D model	2D	3D	3D
Biofilm formation	Long (24 hrs)	Long (24 hrs)	Short (12 hrs)
Media flow	Static (200 μ l – 15 ml)	Continuous (unlimited volume)	Continuous (unlimited volume)
Waste and dead cells	Accumulated	Removed as nutrients are	Removed as nutrients are

		replenished by flow	replenished by flow
Throughput array	Low (1-96)	Low (1-3)	High (more than 300 per channel)
Real-time monitoring	No	Yes	Yes
Application	Biofilm formation and genetic screening	Biofilm formation observation and agents screening	Observation of biofilm formation, dispersal and recolonization; Agents screening; Synergistic therapy.

The c-di-GMP levels were used as an indicator to verify whether biofilms were growing in the BDR device. As mentioned before, high c-di-GMP concentrations in cells have been shown to be associated with biofilm formation ⁽⁹⁸⁾. The *cdrA* gene could respond to c-di-GMP levels in *P. aeruginosa* ⁽²²⁷⁾. Therefore, the fusion of *cdrA* as a promoter with *gfp* could be used as a reporter gene for c-di-GMP in *P. aeruginosa*

biofilm. The confocal images of the *p_{cdrA}-gfp* biosensor and the c-di-GMP levels showed that a mature 3D biofilm could be formed in 12 hrs. This demonstrates that this BDR device could be applied to culture 3D biofilms in the flow state and within a shorter period.

Thus, the physiological dispersal process of biofilms can be further observed with the Time-lapse function of confocal laser scanning microscope (CLSM). According to previous studies, wild-type *P. aeruginosa* relied on flagellar motility at a velocity of 30-50 $\mu\text{m/s}$ ⁽²²⁸⁾. The result of this study found that the biofilm biomass was increased over 2 hrs and the velocity of biofilm cells leave was shown to remain at a steady level around 30 $\mu\text{m/s}$. This indicated that the amount of biofilm dispersal was less than the increase in the absence of anti-biofilm agent treatment, and that the velocity of biofilm dispersal was related to its own velocity of movement ⁽²²⁸⁾.

During the biofilm physiological dispersal, the valve of the BDR device was opened, which allowed the released biofilm bacteria to recolonize in the secondary chamber. The results demonstrated that biofilm bacteria grew and multiplied at a steady velocity in the second chamber. This indicated that the BDR device could be used to directly observe

and study the process of biofilm dispersal and recolonization.

The BDR device was also developed to screen and test anti-biofilm agents. The 8 different concentrations of anti-biofilm agents generated by the concentration gradient generator could be used to rapidly test effectiveness of anti-biofilm agents. Tobramycin was used to kill biofilms in the BDR device. In this experiment, the concentration gradient generator successfully produced 8 different concentrations to kill biofilms. This demonstrated that it was feasible to use the BDR device in the testing and screening of anti-biofilm agents.

3.4 Conclusions

In conclusion, the elimination of bacterial biofilm infections is a major challenge. The biofilm cultures *in vitro* are necessary to evaluate anti-biofilm strategies and to screen for effective antibiofilm agents. While ordinary static cultures are also capable of forming biofilms, those biofilms suffer from inhomogeneous structure and limited nutrient availability. In contrast, dynamic fluidic biofilm cultures using the BDR microfluidic mode could offer precise control of flow conditions, temperature, oxygen, and nutrients, simulating physiological conditions and enabling the formation of 3D biofilms with stable flow in a shorter

time. This model allowed the formation of biofilms that could be observed easily by confocal microscopy and provided a novel platform for studying the process of biofilm dispersal and recolonization.

The use of c-di-GMP levels as an indicator in the BDR device confirmed mature 3D biofilm formation within 12 hrs. The results imaged by Time-lapse function of the CLSM further revealed the physiological dispersal process of the biofilm, with the velocity of biofilm dispersal found to be related to its own movement velocity. Additionally, the BDR device allowed direct observation and study of the process of biofilm dispersal and recolonization during the biofilm physiological dispersal.

Moreover, the BDR device had the potential to be applied to screen and test anti-biofilm agents. The concentration gradient generator facilitated the testing of anti-biofilm agent effect on biofilms. In the case of tobramycin, it successfully produced 8 different concentrations, demonstrating the feasibility of using the BDR device for anti-biofilm agent screening.

In summary, the BDR model provided a versatile platform for studying biofilm formation, dispersal, and recolonization, as well as for rapid and

efficient screening of anti-biofilm agents, offering promising insights for combating persistent biofilm infections.

Chapter IV Dynamics of biofilm dispersal

4.1 Summary

CID and EDA, the two main methods of biofilm dispersal, were tested in this study. CID is a method that induces bacteria to actively escape from biofilm by chemical signals, then destroying the biofilm, while EDA is a process that used enzymes to degrade the biofilm of polysaccharides in the EPS matrix. In order to explore the differences between CID and EDA, biofilm elimination experiments were performed by CID and EDA methods in a BDR device and the velocity, biomass and the released bacterial populations were analyzed.

Sodium Nitroprusside (SNP), as a donor of Nitric Oxide (NO), has been shown to induce the dispersal of *P. aeruginosa* biofilms ⁽¹⁴²⁾. NO was one of the first molecules identified as a biofilm dispersion agent. It reduces the concentration of c-di-GMP by activating PDE. The reduction in c-di-GMP concentration is followed by an increase in the expression of matrix degrading enzymes ⁽²²⁹⁾. In addition, arabinose could be used to regulate the intracellular c-di-GMP levels of the PAO1/p_{BAD}-*yhjH* biofilms. The PAO1/p_{BAD}-*yhjH* strain contain a pJN105 plasmid carrying the *araC*-P_{BAD} promoter, which could be induced by

arabinose. The PDE gene *yhjH* could be integrated with the promoter and therefore the PDE of the PAO1/*p_{BAD}-yhjH* could be activated by arabinose to regulate the c-di-GMP level in bacteria ⁽²⁰⁴⁾.

Cellulase and pectinase were used in biofilm disassembly by EDA, cellulase and pectinase acted as glycosidic hydrolases ⁽²³⁰⁾, which had been shown to degrade biofilms by disassembling the glycosidic bonds of the polysaccharides in the biofilm matrix ⁽²³¹⁾.

4.2 Results

4.2.1 CID Induced active biofilm dispersal.

In the process of inducing biofilm dispersal, individual cells were released from the biofilm and subsequently left the microwells. Confocal images showed that the biofilms treated with NO and arabinose for 2 hrs were less than that in 0 hrs. (Figure 4.1a). This was confirmed by subsequent results for the quantification of fluorescence intensity (Figure 4.1b). The cell trajectory showed that most bacteria were released by the biofilm and then left the microwells within 1 hrs (Figure 4.1c). At the beginning of dispersal, CID-induced biofilm bacteria had similar motility to the physiologically dispersed cells, but

the motility of the biofilm bacteria in the microwells gradually decreased at 2 hrs, which indicated that the bacteria had dispersed significantly during the initial stage and left the microwells (Figure 4.1d). In addition, the results of the mathematical model showed that the biofilm biomass in the CID method decreased with exponential decay. This meant that in the initial phase of the experiment, the number of bacteria present had decreased rapidly at a rate proportional to the number of bacteria present at that time. However, the rate of reduction slowed down over time, indicating that fewer bacteria were dispersed from the biofilm at later stages. This demonstrated that many bacteria had dispersed in the initial phase and the dispersal had slowed down in the later phases (Figure 4.1e; Table 4.1).

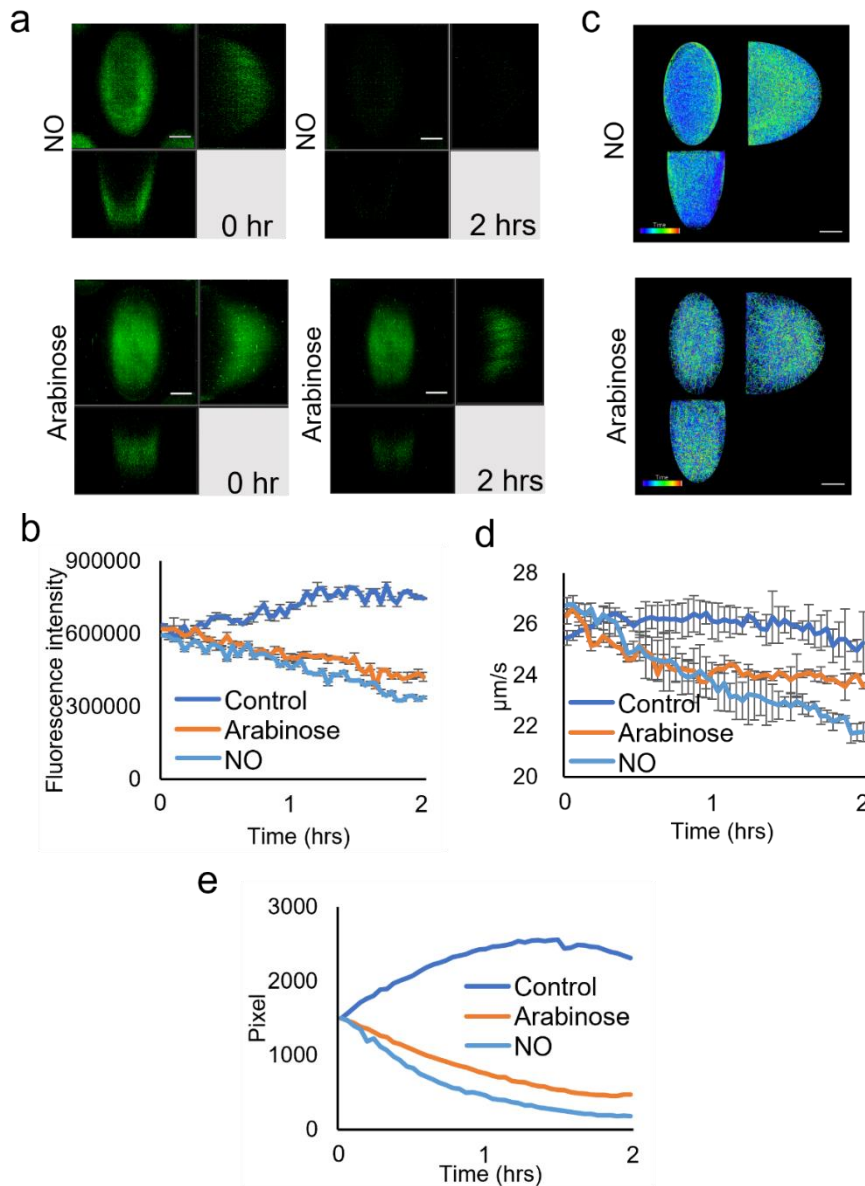


Figure 4.1. Dynamics of biofilm dispersal dominated by c-di-GMP signalling reduction (a) Confocal images of CID dominated biofilm dispersal. (b) Biomass reduction over time. (c) Bacteria trajectories of CID within 2 hrs. (d) The movement velocity of bacterial populations over time. (e) Mathematical modelling of CID with an exponential decay.

Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar in all images was 50 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

Table 4.1 Mathematical model for CID

	Equation	Control	Arabinose	NO
Polynomial	$y = ax^2 + bx + c$	0.9954		
	$y = ax + b$		0.9495	0.8825
Exponential	$y = a \cdot e^{bx}$		0.9957	0.9954

(Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

4.2.2 Biofilm disassembly causes biofilm reduction

The two enzymes, cellulase and pectinase, were used to degrade the biofilm. The confocal images showed that treatment biofilms with pectinase and cellulase for 2 hrs was less than for 0 hr (Figure 4.2a). The biomass results showed that the biomass of the enzyme-treated biofilm also decreased within 2 hrs (Figure 4.2b). Bacterial trajectory images showed that bacteria did not leave the micropores immediately after biofilm decomposition (Figure 4.2c). The velocity at which bacteria

left the biofilm was high at the beginning, but then decreased immediately (Figure 4.2d). And the EDA-dominated biofilm disassembly was shown as a polynomial decay in the mathematical model (Figure 4.2e; Table 4.2), suggesting a steady decline in biofilm biomass over time, which was slow and over an extended period.

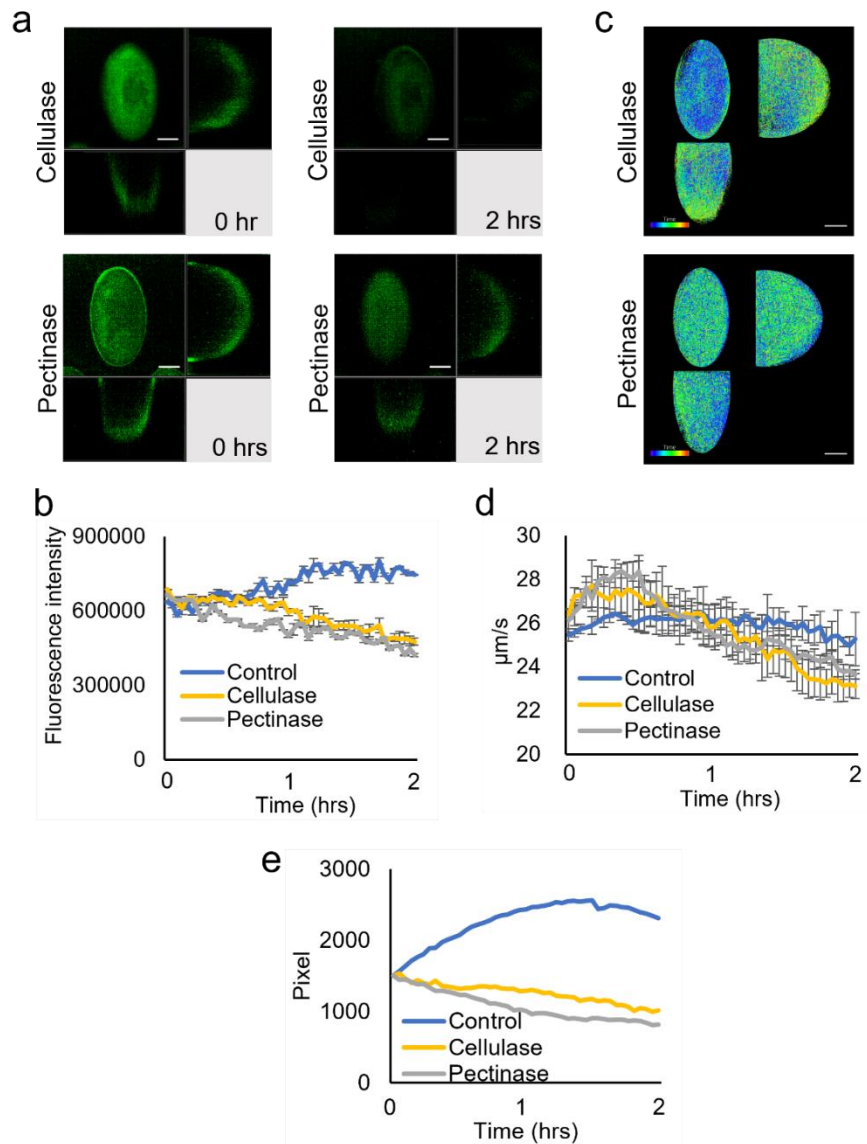


Figure 4.2. Dynamics of biofilm disassembly. (a) EDA dominated biofilm disassembly images. (b) Fluorescence intensity reduction over time. (c) Bacteria trajectories of EDA within 2 hrs. (d) The movement velocity of bacterial populations over time. (e) Mathematical modelling of EDA with polynomial decay. Experiments were performed in triplicate,

at least 3 images were captured for each replicate, and the representative images were shown. The scale bar in all images was 50 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

Table 4.2 Mathematical model for EDA

	Equation	Control	Cellulase	Pectinase
Polynomial	$y = ax^2 + bx + c$	0.9954		
	$y = ax + b$		0.9426	0.9356
Exponential	$y = a \cdot e^{bx}$		0.9675	0.9310

(Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

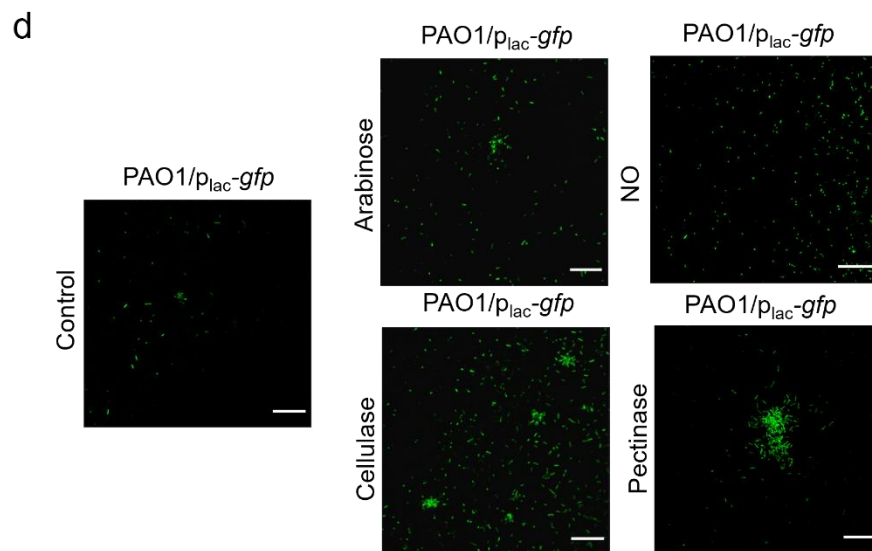
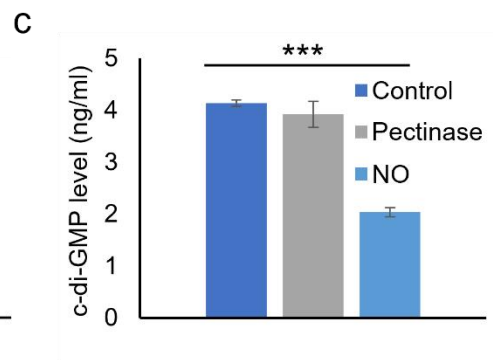
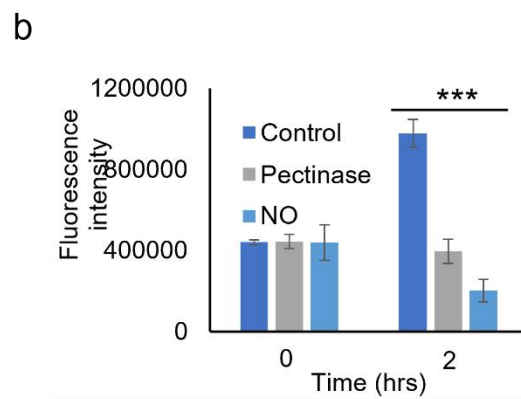
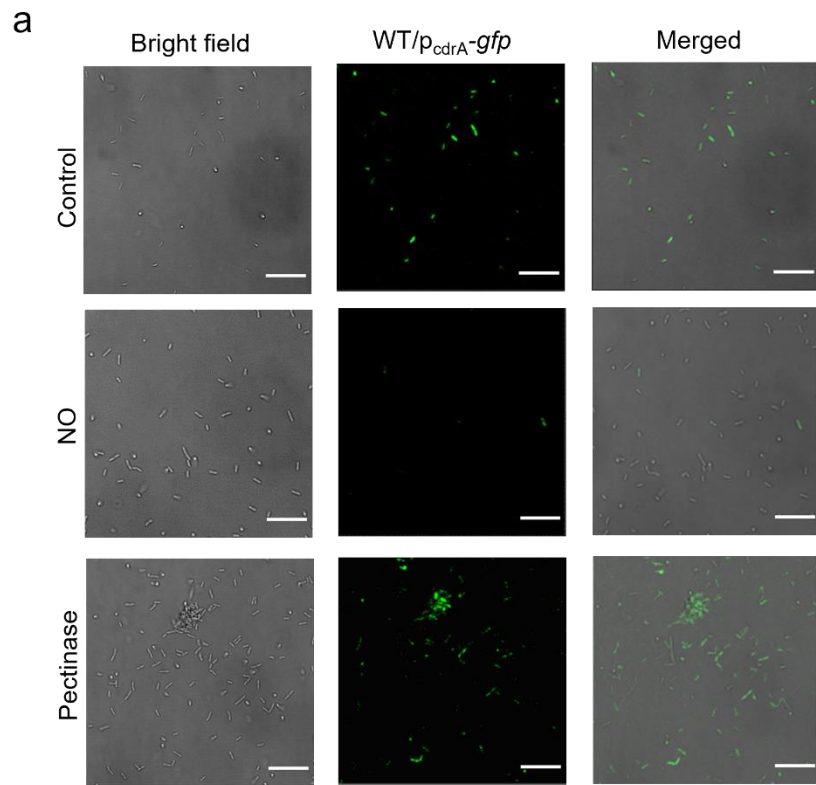
4.2.3 Differences between CID and EDA revealed by c-di-GMP levels and mutants

To investigate the differences between CID and EDA, a series of experiments was conducted to show the changes in c-di-GMP levels in biofilm bacteria. The expression of the *cdrA* gene was positively regulated by c-di-GMP in *P. aeruginosa* ⁽²⁰⁴⁾. The p_{cdrA} -*gfp* fusion gene was formed by fusing the promoter of the *cdrA* gene with the *gfp* gene. As a result, the promoter would prompt the expression of the p_{cdrA} -*gfp* fusion gene when the *cdrA* gene was positively regulated by c-di-GMP.

Thus, the PAO1/*p_{cdrA}-gfp* strain was chosen for the experiment and it was found that after induction of active biofilm dispersal, biofilm bacteria differed significantly from physiologically dispersed and EDA-disintegrated bacteria (Figure 4.3a). Fluorescence intensity measurements (Figure 4.3b) and comparison of intracellular c-di-GMP levels (Figure 4.3c) showed that the CID-induced bacteria had significantly lower c-di-GMP than the physiological group and the disassembled biofilms. It was also observed that most of the released biofilm bacteria dispersed by CID were shown as single individuals, whereas the EDA-treated biofilm released many small fragments (Figure 4.3d).

To further confirm the differences in c-di-GMP between CID biofilm dispersal and EDA biofilm disassembly, $\Delta bdlA$ mutant was used in experiments with CID and EDA. The gene *BdIA*, which is controlled by c-di-GMP signaling, is a chemosensory protein that it can regulate PDE activity and respond to external signals that affect dispersion, such as various nutrients, NO, ammonium chloride and heavy metals ⁽²³²⁾. Studies have shown that *BdIA* inactivation can impair the dispersal behavior of *P. aeruginosa* biofilms ⁽¹⁴³⁾. Here, it was shown that the $\Delta bdlA$ biofilm could not be dispersed from biofilms by NO, but could be

degraded by pectinase (Figure 4.3e). In addition, since the biofilm dispersal was mediated by flagella ⁽²³³⁾, $\Delta fliM$, a flagellar-deficient mutant ⁽²³⁴⁾, was chosen for the experiments to explore the effect of flagella in CID dispersal and EDA biofilm disassembly. The results indicated that the dispersed bacteria of the $\Delta fliM$ mutant were unable to leave the microwells voluntarily, while the biofilm in the microwells was reduced by pectinase degradation (Figure 4.3f).



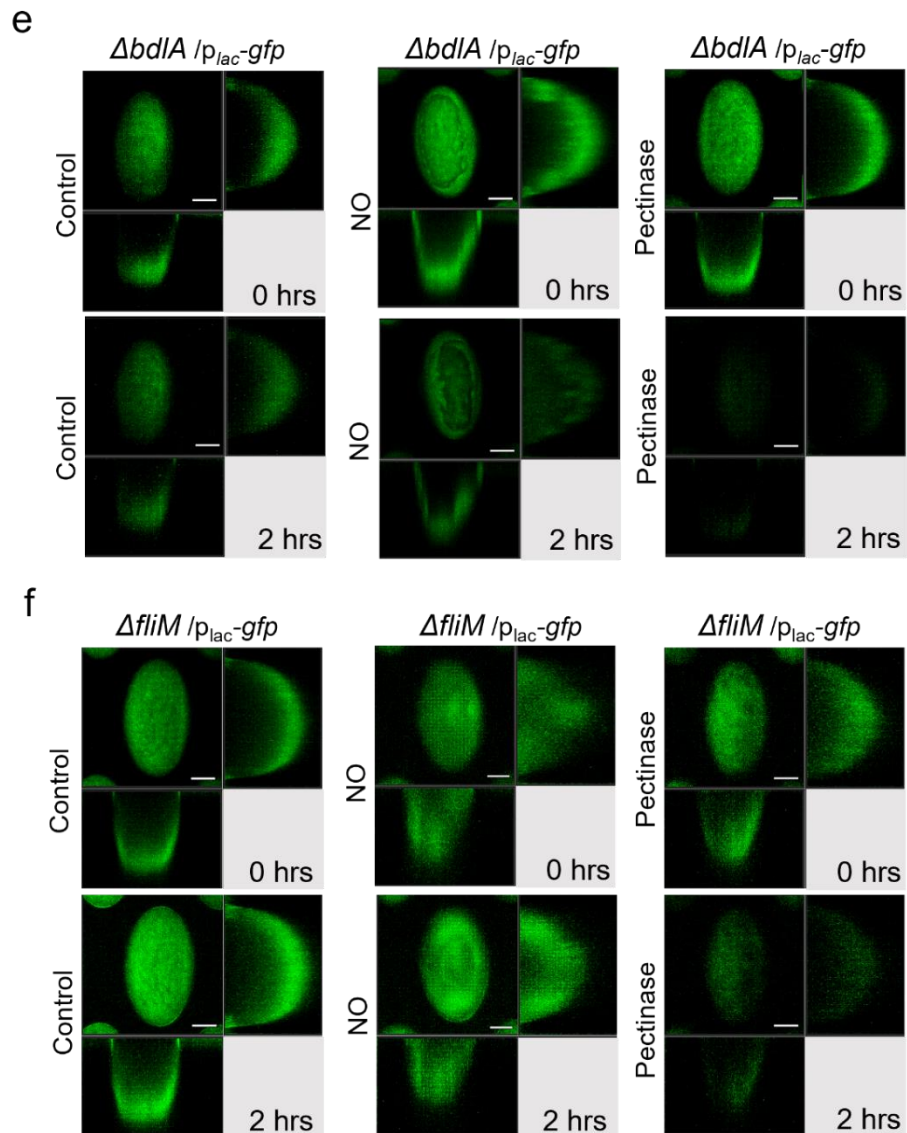


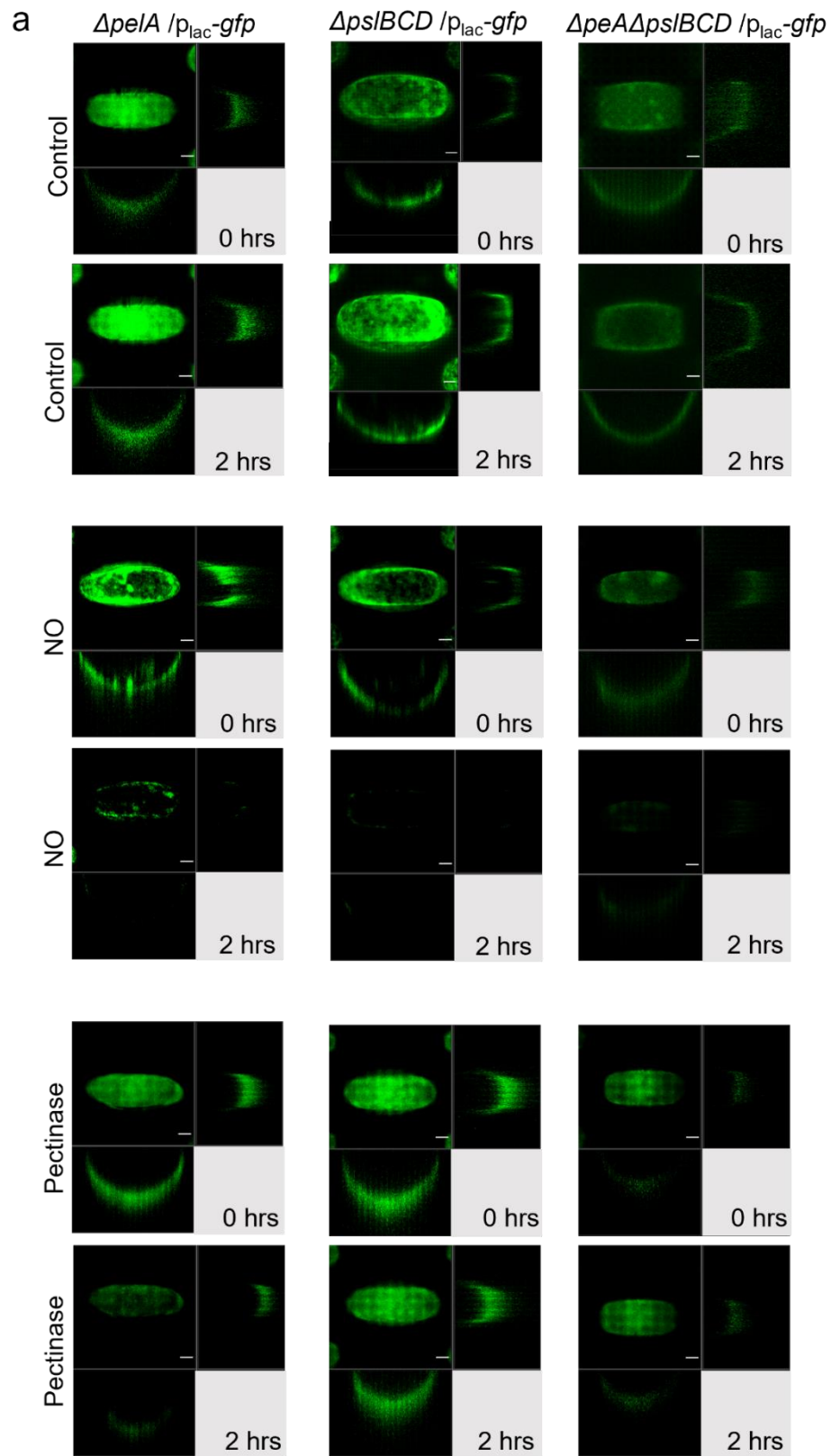
Figure 4.3. Different physiologies between bacterial cells generated by induced biofilm dispersal and those by biofilm matrix. (a) Biofilm dispersed PAO1/p_{cdrA}-gfp cells, when dispersed from the biofilm, showed a decrease in GFP expression, whereas cells that remained in the biofilm continued to exhibit GFP expression. The scale

bar was 10 μm . (b) $P_{\text{cdrA-gfp}}$ fluorescence level represented the reduced c-di-GMP levels in biofilm CID and high c-di-GMP levels in EDA treated biofilms. The averages and s.d. from triplicate experiments were shown. *** $P < 0.001$. (c) Significant reduction of c-di-GMP levels in CID biofilm. The averages and s.d. from triplicate experiments were shown. *** $P < 0.001$. (d) Bacteria that were released by biofilm CID mostly existed as single cells. The scale bar is 10 μm . (e) Less biofilm dispersal was observed in the ΔbdIA mutant, but biofilm could be efficiently disassembled in the ΔbdIA mutant. The scale bar is 50 μm . (f) The ΔfliM mutant showed an absence of biofilm dispersal. However, the ΔfliM mutant displayed efficient biofilm disassembly. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar is 10 μm . *** $P < 0.001$ (Reprint with permission from Ma Y, Deng Y, 2023 (219)).

4.2.4 Pectinase-mediated biofilm disassembly degrades Psl polysaccharides

After identifying that CID-dominated biofilm dispersal required the contribution of *bdIA* and flagella, in order to confirm the polysaccharide

in EPS matrix required for EDA-dominated biofilm disassembly, experiments were conducted on *pelA*-deficient, *pslBCD*-deficient and *pelApslBCD*-deficient strains, respectively. The confocal images showed that pectinase could degrade the $\Delta pelA$ biofilm but did not degrade that of $\Delta pslBCD$ (Figure 4.4a). Next, the 10 $\mu\text{g/ml}$ exogenous Pel and Psl polysaccharide were used to coat the microwells. The $\Delta pelA\Delta pslBCD$ mutant was used to grow with Pel or Psl polysaccharide flowed with ABTGC media and pectinase, respectively. Then it was found that the formed biofilms of Psl polysaccharide coated channel were less than no coated channel and Pel polysaccharide coated channel (Figure 4.4b). Subsequent quantification of fluorescence intensity in microwells also showed that the biomass of Pel-coated wells was higher than that of Psl-coated biomass after pectinase treatment (Figure 4.4c). The CFU results also demonstrated that the pectinase treated Psl coated channels had fewer bacteria than the Pel coated channels (Figure 4.4d). This proved that the EDA dominated biofilm disassembly was through degrading the polysaccharide.



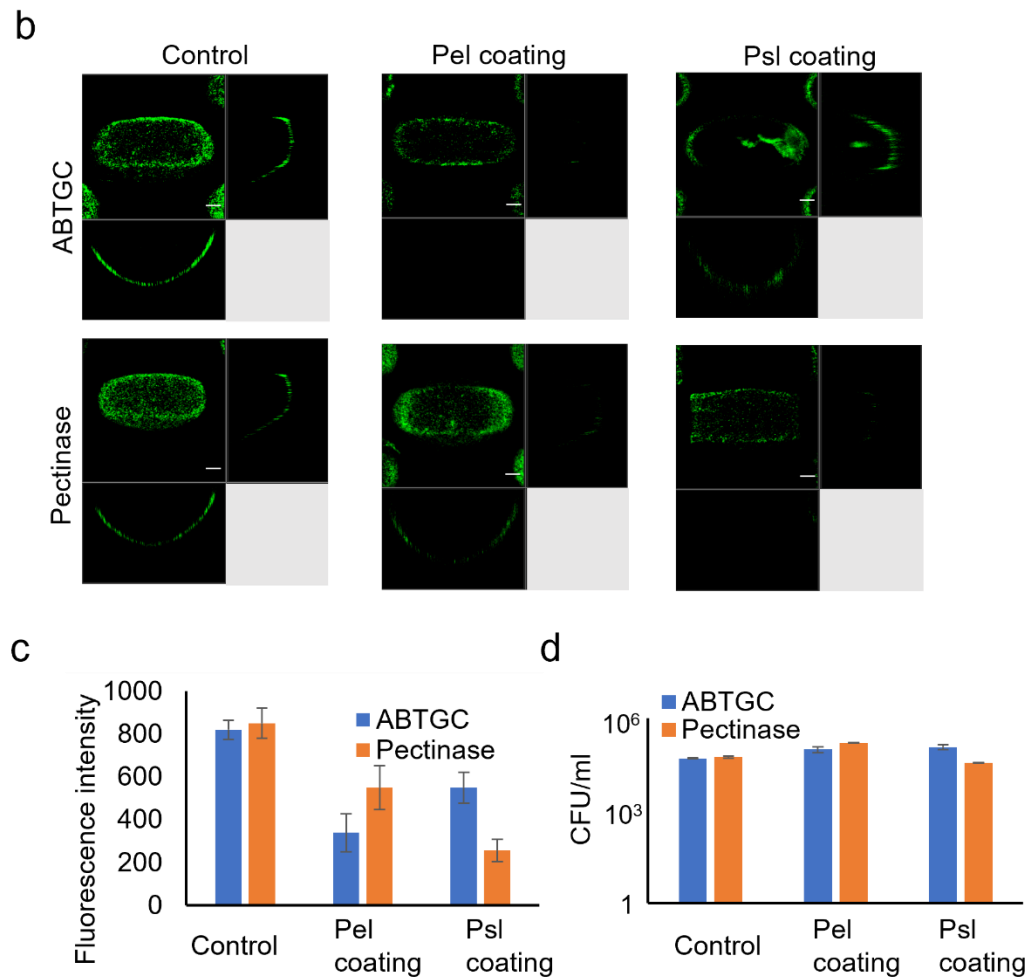


Figure 4.4. Pectinase degrades psl in EPS. (a) NO could induce the mutants' biofilms dispersal, and Psl could be degraded by pectinase. The scale bar is 50 μ m. (b) Experiments with Pel and Psl polysaccharide coated microwells. The scale bar is 50 μ m. (c) Biomass for $\Delta pel/\Delta ps/BCD$ grew in pel and psl polysaccharide coated microwells. (d) CFU result for $\Delta pel/\Delta ps/BCD$ grew with pel and psl

polysaccharide. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

4.2.5 CID and EDA did not kill biofilm bacteria

In addition, PI stains were used to label dead cells to explore whether bacteria released by CID and EDA remain viable for recolonization. The results showed that the biofilm bacteria released by both methods were alive (Figure 5.6a). Therefore, the ability of all bacteria to recolonize and multiply was not affected.

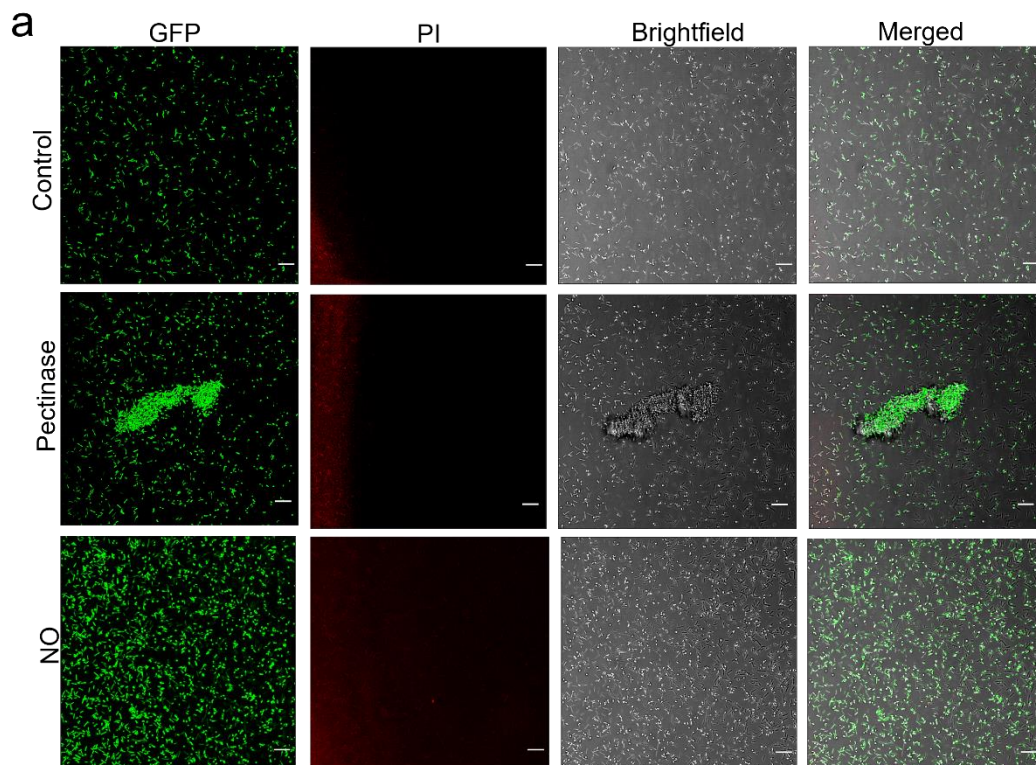


Figure 4.5. Biofilm bacteria labelled by PI stain. (a) PI stain to label biofilm bacteria with GFP released by CID and EDA. The scale bar is 20 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

4.3 Discussion

Currently, biofilms account for 80% of all bacterial infections ⁽⁵⁷⁾. This study discussed the difference between the CID-dominated biofilm dispersal and EDA-dominated biofilm disassembly.

NO and *yhjH* genes were used to reduce the c-di-GMP levels in order to induce active biofilm dispersal. After treatment with NO and arabinose, the biofilm biomass was significantly reduced. It was previously shown that biofilms formed in flow chamber were rapidly and completely dispersed after inducing YhjH phosphodiesterase ⁽²³⁵⁾. This was also demonstrated in this study through analyzing velocity, trajectory and mathematical model. The velocity of the measured bacterial population leaving the biofilm was similar to that of the physiologically dispersed bacteria at the beginning, but it gradually decreased over time. The combination of this with the trajectory images of the captured biofilm bacteria within 2 hrs indicated that bacteria released by CID-led biofilm dispersal mainly happened in the early

stages and left the microwells. According to the mathematical model, the CID-dominated biofilm dispersal decayed with an exponential pattern. This also meant that the bacteria released from the biofilm had left the microwells at an early stage of biofilm dispersal, which was the same as that presented by the trajectory image. Besides, in static models, NO and *yhjH* still could induce biofilm dispersal ⁽²³⁶⁾.

EDA dominated biofilm disassembly could also significantly reduce biofilm within 2 hrs. The velocity of the biofilm-degrading bacterial population leaving the biofilm was initially higher than that of the physiologically dispersed bacterial population, but it also decreased significantly over time. This might be due to the fact that cellulase and pectinase had caused disruption of the entire biofilm to release a large number of biofilm bacteria during the initial biofilm disassembly. However, as the biofilm was continuously disassembled by the enzymes, fewer biofilm bacteria were released and consequently the velocity of biofilm bacterial populations leaving the microwells decreased. Besides, the trajectory picture of the captured biofilm bacteria showed that the released biofilm bacteria did not leave the microwells rapidly after the biofilm was disassembled by the enzymes, resulting in a steady polynomial decay of the biofilm biomass.

Both CID and EDA biofilm dispersion could reduce biofilm, however, there were some differences between these two methods. Previous findings had shown that the biofilm degradation process was mediated by the cleavage of the biofilm matrix, rather than through the motility of the cells themselves ⁽²³⁷⁾. In this study, similar conclusions were demonstrated. CID-dominated biofilm dispersal required the motility of the bacteria themselves, whereas EDA-dominated biofilm disassembly directly degraded the biofilm matrix. The biofilm bacteria released by CID appeared as single individuals, whereas EDA disassembly resulted in many small bacterial aggregates. This might be related to the fact that biofilm active dispersal required reduced levels of c-di-GMP ⁽¹⁰⁰⁾. The results in this study showed there was a significant decrease in the levels of intracellular c-di-GMP in the bacteria released by the CID-dominated biofilm dispersal, but that c-di-GMP levels in the bacteria after biofilm disassembly were similar to those in biofilm cells.

Meanwhile, the $\Delta bdIA$ strain, which lacked the c-di-GMP signaling control gene *bdIA*, also demonstrated that the biofilms could not be dispersed by CID, but could be disassembled by pectinase. This would also indicate that CID-dominated biofilm dispersal was an active process through the biofilm bacteria to reduce c-di-GMP levels and

then leave the biofilm, as biofilm dispersal was associated with low levels of c-di-GMP ⁽¹⁴²⁾. Since the bacterial dispersal is flagella-dependent ⁽²³⁸⁾, the $\Delta fliM$ mutant with deficient flagella was used to explore the differences between CID and EDA. The results showed that CID-dominated biofilm dispersal failed to reduce biofilm due to the lack of flagella, whereas EDA achieved biofilm reduction by disassembling the biofilm. This result also indicated that the CID-dominated biofilm dispersal was an active process while the EDA-dominated biofilm disassembly was a passive method.

The cellulase and pectinase used in EDA biofilm disassembly targeted the polysaccharides of biofilms ^(231, 239). As the main polysaccharides in biofilms were Pel and Psl ⁽²⁴⁰⁾, CID and EDA experiments were performed using Pel-deficient and Psl-deficient mutants respectively, and the results showed that the biofilms of $\Delta pelA$ could still be disrupted by pectinase treatment, while the biofilms of $\Delta pslBCD$ that lacked Psl were not disrupted by pectinase disassembly. This explained that pectinase could disassemble the Psl polysaccharide in biofilms. The addition of exogenous Pel and Psl to the biofilm-deficient $\Delta pelA\Delta pslBCD$ mutant enabled the formation of the biofilm, and then treatment with pectinase also could disrupt the biofilms that were

formed with the addition of exogenous Psl. These findings also indicated that the disassembly of EDA-dominated biofilms relied on enzymes that specifically targeted the components of the biofilm matrix.

4.4 Conclusions

CID and EDA, were compared in this chapter. Both methods showed effectiveness in reducing biofilms, but they presented distinct differences.

CID biofilm dispersal induced active biofilm reduction by decreasing c-di-GMP levels through the use of NO and *yhjH* genes. The released bacteria displayed a pattern of exponential decay and predominantly appeared as single individuals. This active process depended on flagella and low levels of c-di-GMP, as indicated by the results with the $\Delta bdlA$ mutant.

EDA biofilm disassembly was studied by the use of cellulase and pectinase enzymes that targeted the Psl polysaccharides in biofilms. The initial disassembly caused a sudden release of a large number of biofilm bacteria and many small bacterial aggregates. Over time, the disassembly continued steadily, leading to a polynomial decay of

biofilm biomass. Enzymes targeting the biofilm matrix components were crucial for EDA-dominated biofilm disassembly.

The differences between CID and EDA methods suggested variations in the release process of bacteria and the nature of biofilm reduction. CID was an active process driven by bacteria to reduce c-di-GMP levels, while EDA was a passive method relying on enzyme-mediated disassembly. The findings also highlighted the importance of intracellular c-di-GMP levels, flagella, and biofilm matrix components in the respective mechanisms.

In summary, while both CID and EDA demonstrated biofilm reduction capabilities, they operated through distinct mechanisms, released different forms of bacteria, and relied on specific factors for their effectiveness. Understanding these differences would contribute to a broader knowledge of biofilm elimination strategies and may guide the development of targeted approaches for combating biofilm-related infections.

Chapter V Recolonization of bacteria released from CID and EDA biofilms dispersal

5.1 Summary

The lifecycle of a biofilm starts with the recolonization of bacteria on fresh surfaces after dispersal; thus, the bacteria released during biofilm dispersal are an important factor in causing reinfection. The difference between biofilms in CID dispersal and EDA disassembly was evaluated previously.

To observe the difference in recolonization of bacteria released during CID and EDA dispersal, the valve of the BDR device was opened during the biofilm dispersal to connect the biofilm dispersal chamber with the recolonization chamber. Then the dispersed bacteria could recolonize to form a new biofilm in the secondary chamber. The bacteria were observed using the confocal microscope, and virulence was tested. The result showed that bacteria released from biofilm dispersal and disassembly were still viable by comparing the trajectory, velocity and biomass differences.

Furthermore, in order to evaluate the impact of biofilm-released

bacteria on the host, *in vitro* and *in vivo* infection models were employed in this study. Infection models are widely used in research on the interaction between infectious pathogenic microorganisms and host organisms, pathogenic mechanisms, immune responses and the development of new drugs and vaccines. Typical infection models include cell culture models, animal models, worm models, and synthetic organism models ⁽²⁴¹⁻²⁴⁴⁾.

Cell culture models established by cultured cells *in vitro* can be used to study the invasion, proliferation and pathogenesis of pathogenic microorganisms as well as the immune response of cells to pathogenic microorganisms ⁽²⁴⁵⁾. Since 2D models lack tissue complexity and physiological relevance, 3D cell culture models are beginning to be developed. Compared to traditional 2D cell culture models, 3D models better simulate the complexity of tissues and organs in living organisms. The 3D models have great potential to bridge the gap between cell-based discovery studies and animal models ⁽²⁴⁶⁾. It is already being used to study host-pathogen interactions and human disease progression, as well as to develop new drugs and therapies.

C. elegans is a commonly used model organism with a small body size, a short life cycle, a relatively simple nervous system and genetic background ⁽²⁴⁷⁾. *C. elegans* has a complete physiological system, including digestive, reproductive, endocrine, sensory and neuromuscular systems ⁽²⁴⁸⁾. This enables it to be an excellent model organism for studying toxicity and drug effects. The effects of chemicals, drugs or environmental factors on the biological system can be assessed by measuring the nematode's survival rate, growth and development, reproductive capacity, behavioral responses, and other indicators.

An *in vitro* model based on human lung fibroblasts (HLF) and an *in vivo* model of *C. elegans* were established in this study. It helped to explore the virulence of the released bacteria to the host and contributed to developing future treatments for biofilm-associated infections and diseases. The results showed that bacteria released by CID and EDA generated different virulence effects on these models.

5.2 Results

5.2.1 Bacteria released by CID biofilm dispersal could not recolonize new surfaces effectively

Compared with the bacteria released by physiological dispersal, the bacteria released from CID-dominated biofilm dispersal were unable to recolonize in the microwells (Figure 5.1a). The measured fluorescence intensity also showed no bacteria multiplied in the microwells within 2 hrs (Figure 5.1b). This result was also indicated in the trajectory diagram (Figure 5.1c). During the CID-dominated biofilm dispersal, the velocity of bacterial movement was consistent with the velocity of bacteria released by physiological dispersal in the microwells (Figure 5.1d). Mathematical models were also developed for recolonization (Figure 5.1e), and it demonstrated physiologically dispersed bacteria recolonized and multiplied in the microwells with an exponential functional mode, which showed a proportional increase in the rate of bacterial cells over time. In contrast, the bacteria released by CID failed to recolonize on the new surfaces (Table 5.1).

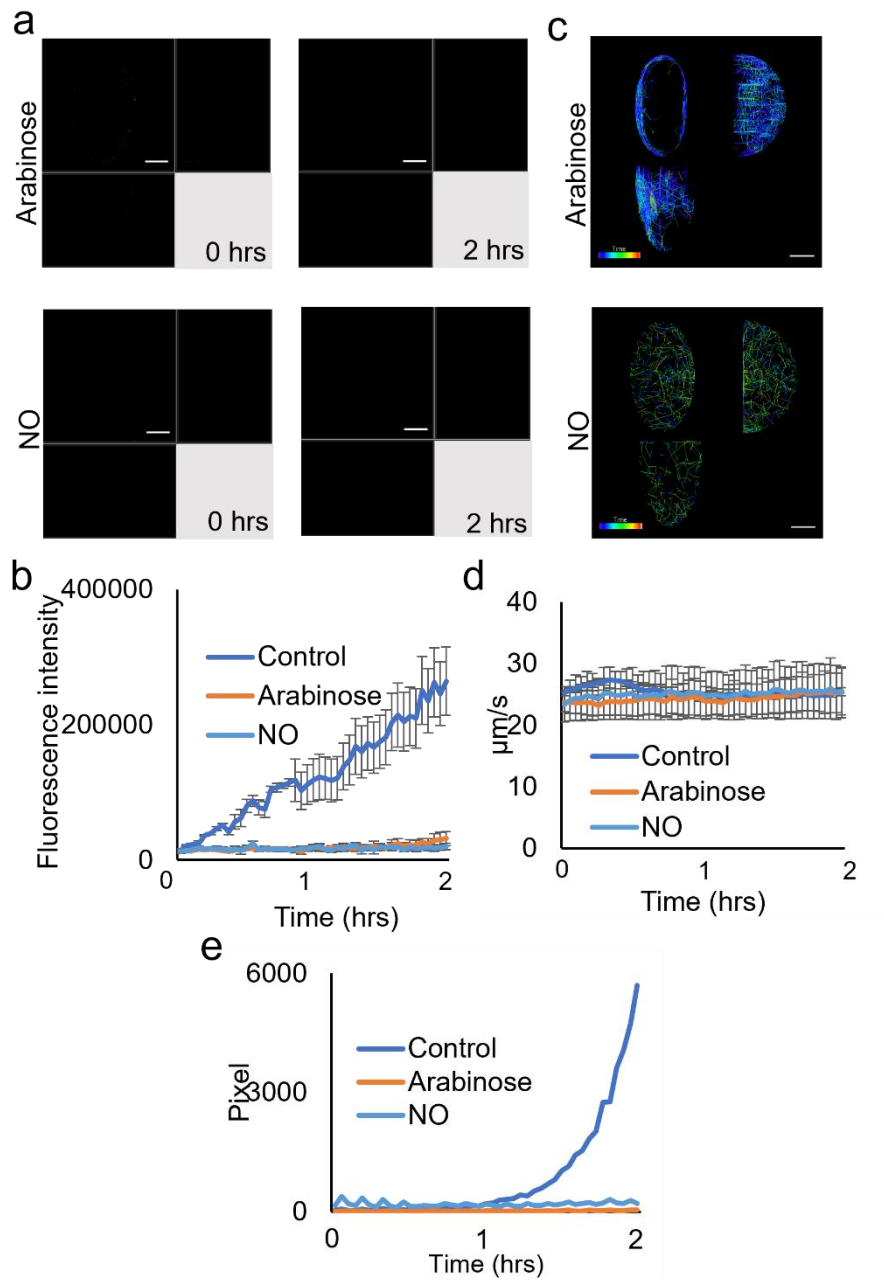


Figure 5.1. Recolonization dynamics of biofilm dispersed cells mediated by CID. (a) Confocal images of recolonization of biofilm-dispersed cells over time. (b) Induced biofilm-dispersed cells' time-

resolved bacterial recolonization trajectories. (c) Biofilm biomass (GFP intensity) did not increase over time. (d) Over time, cells in the biofilm dispersed at steady velocity. (e) The mathematical model for CID biofilm dispersal. Experiments were performed in triplicate, at least 3 images were captured for each replicate, and the representative images were shown. The scale bar is 50 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

Table 5.1 Recolonization mathematical model of CID released bacteria

	Equation	Control	Arabinose	NO
Polynomial	$y = ax + b$	0.5977	0.5469	0.01068
Exponential	$y = a \cdot e^{bx}$	0.9978	0.6049	0.01235

(Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

5.2.2 Bacterial aggregates released by EDA biofilm disassembly recolonize on new surfaces

In contrast to the recolonization of CID biofilm dispersal, bacteria released by EDA biofilm disassembly could be recolonized (Figure 5.2a), although the magnitude of recolonization was lower than in the physiological dispersal (Figure 5.2b). From the bacteria tracking trajectories (Figure 5.2c), it was found that those aggregates released from biofilm disassembly mainly recolonized at around 1 hr and that the

velocity of recolonization was higher than physiologic dispersal (Figure 5.2d). The mathematical model showed that the fitted curve for recolonization was the exponential pattern for EDA biofilm disassembly bacteria (Table 5.2), which was compared to physiological dispersal, although it was lower in magnitude (Figure 5.2e).

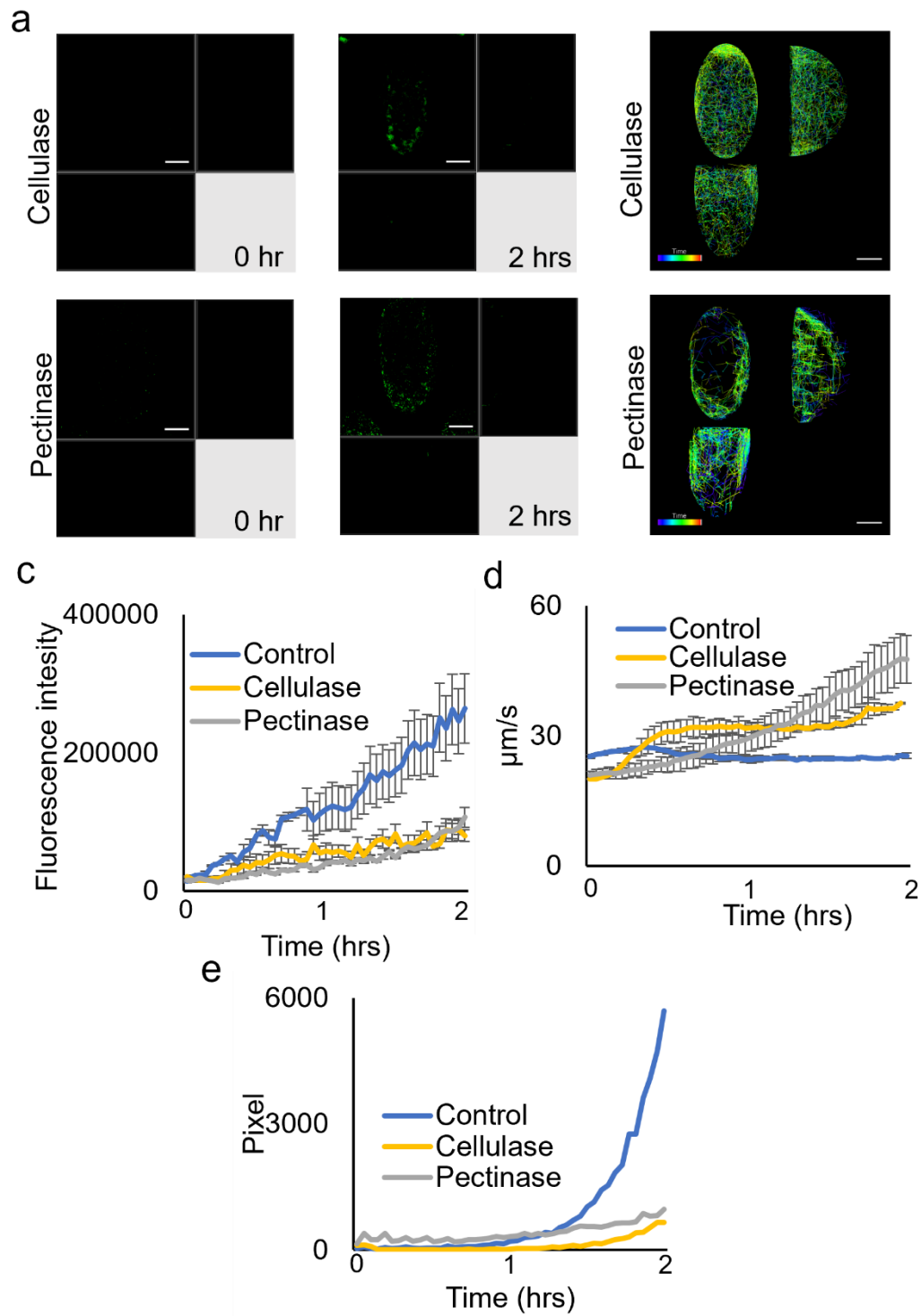


Figure 5.2. Recolonization dynamics of biofilm disassembled bacteria.

(a) Recolonization images of biofilm disassembled over time. (b)

Disassembled cells' time-resolved bacterial recolonization trajectories. (c) Biofilm biomass (GFP intensity) did not increase over time. (d) Over time, biofilm aggregates were released at constant velocities. (e) The mathematical model for CID biofilm disassembly. Experiments were performed in triplicate, at least 3 images were captured for each replicate, and the representative images were shown. The scale bar is 50 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

Table 5.2 Recolonization mathematical model of CID-released bacteria

	Equation	Control	Cellulase	Pectinase
Polynomial	$y = ax + b$	0.5977	0.518	0.7561
Exponential	$y = a \cdot e^{bx}$	0.9978	0.9636	0.8843

(Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾)

5.2.3 The effect of bacteria released by biofilm dispersal on the HLF spheroids

As bacteria released after biofilm dispersal were an important reason for secondary infection, in order to explore the impacts of recolonization on the host of bacteria released from biofilm dispersal, the infection models on HLF 3D spheroids were established for simulating and

observing the effects of bacteria *in vitro* model after biofilm dispersal.

The second chamber attached with HLF 3D spheroids was connected to the primary chamber during biofilm dispersal, which enabled the released bacteria to infect the 3D spheroid model for 2 hrs. Both physiologically dispersed bacteria and EDA released aggregates could infect cells rapidly, but the bacteria dispersed by CID could not recolonize on the cells (Figure 5.3a). Bacteria released by CID-dominated biofilm dispersal were less deadly to the HLF spheroids model than physiological dispersal and EDA disassembly (Figure 5.3b), this may be due to the fact that the bacteria released by the CID did not recolonize the new surface.

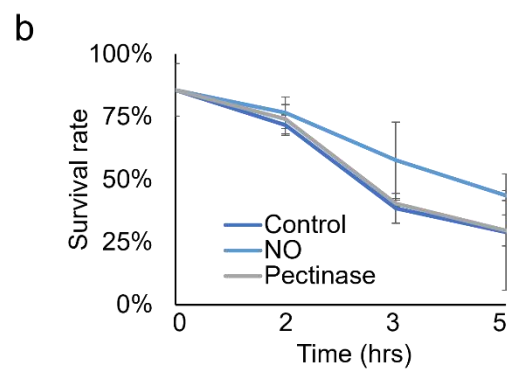
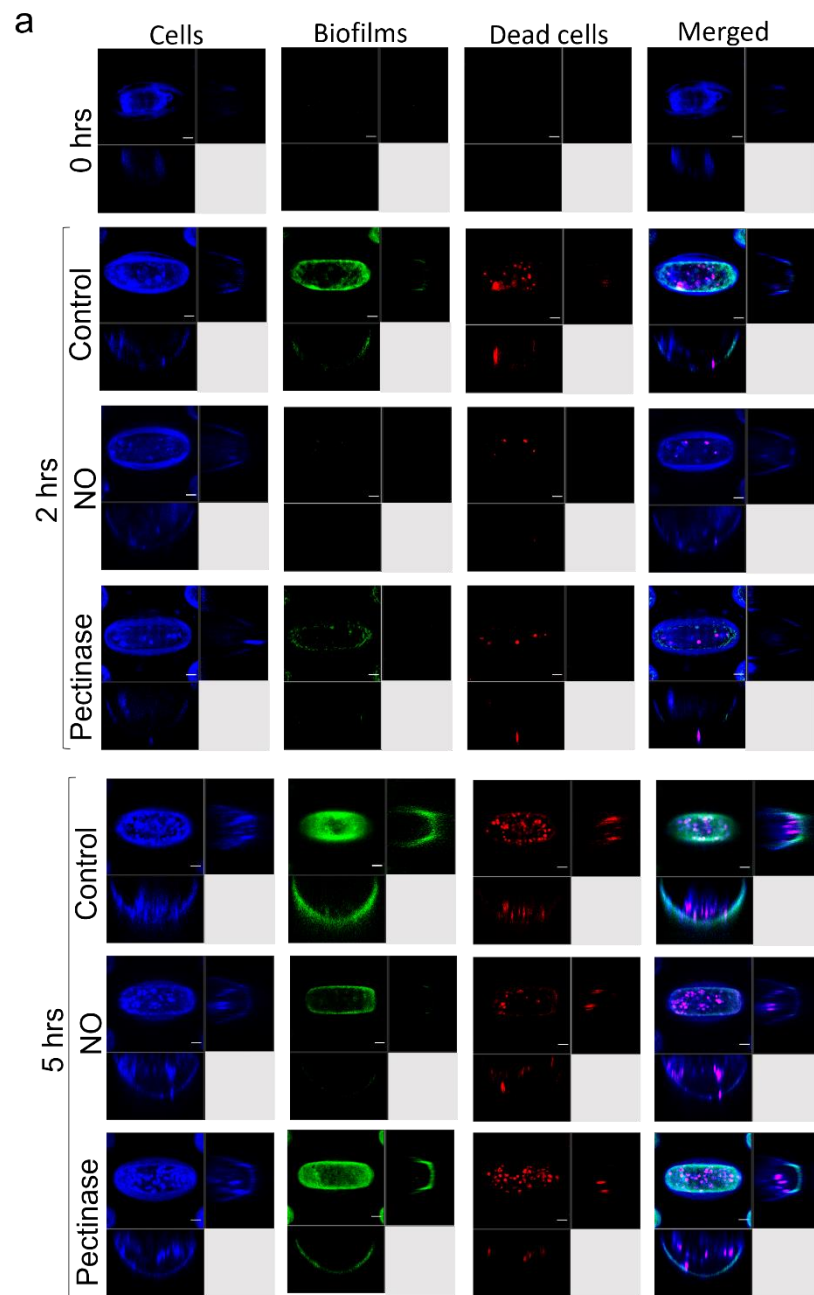


Figure 5.3. Recolonization of released bacteria on the HLF 3D spheres infection models in BDR device. (a) Images of HLF 3D spheres infected with bacteria released by different methods mediated biofilm dispersal at 0 hrs, 2 hrs, 3 hrs and 5 hrs. (b) Mortality of HLF 3D spheres at 0 hrs, 2 hrs, 3 hrs and 5 hrs. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar is 50 μm (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

5.2.4 The effect of bacteria released by biofilm dispersal on the *C. elegans*

To further explore the virulence of biofilm-released bacteria *in vivo*, an infection model for *C. elegans* was established. Biofilm dispersal experiments were then performed, which allowed the released biofilm bacteria to flow into the recolonization channel containing *C. elegans*. The confocal images showed that the biofilm bacteria released by EDA were more fed by *C. elegans* at 6 hrs and 12 hrs (Figure 5.4a). This may be due to the bacteria released by EDA disassembly were able to recolonize new surfaces more quickly and were able to be captured by *C. elegans*. It was found that biofilm bacteria induced to disperse by

NO were less deadly to *C. elegans* than pectinase. Moreover, NO-induced biofilm bacterial dispersal caused less lethal to *C. elegans* than pectinase (Figure 5.4b); this may also be related to the ability of biofilm bacteria to recolonize new surfaces.

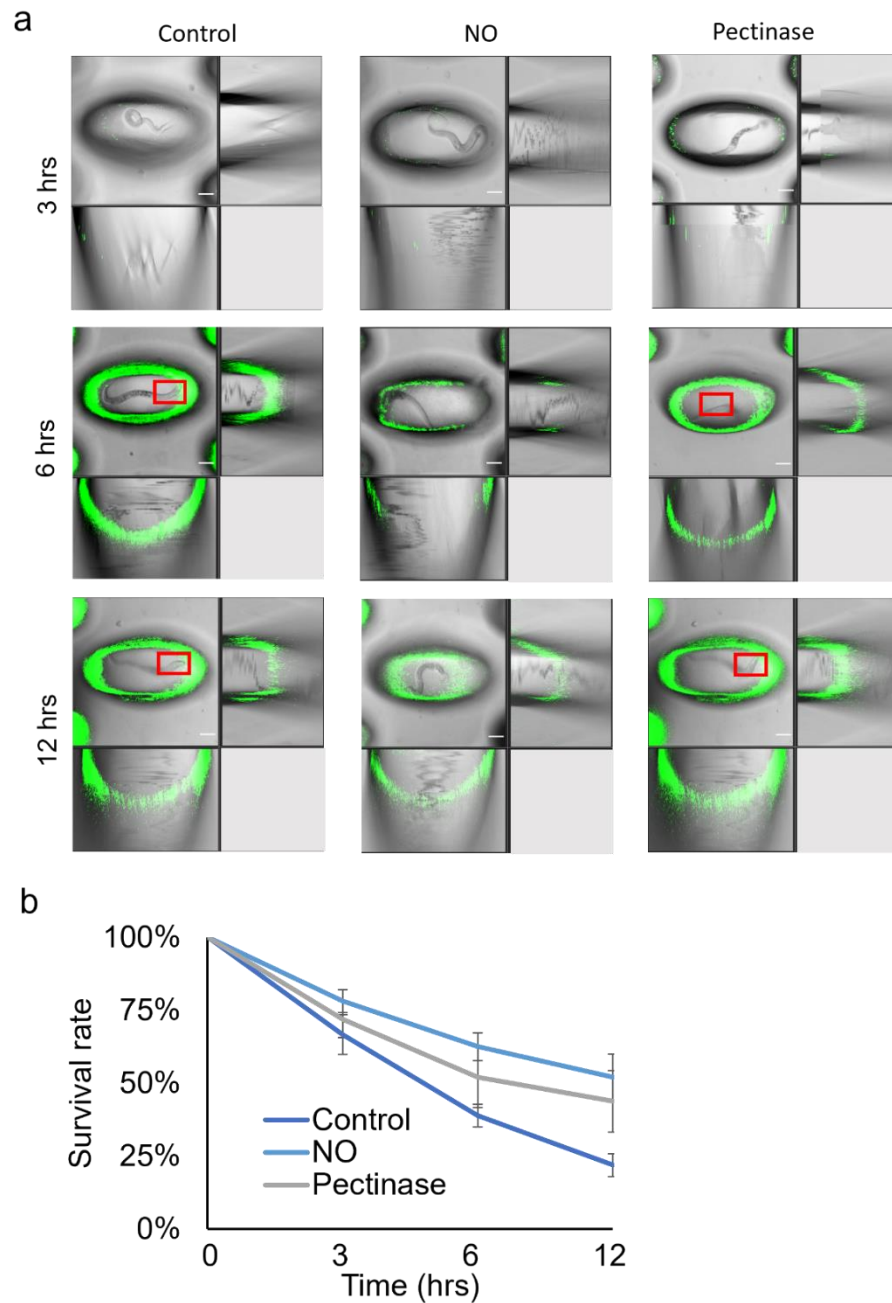


Figure 5.4. Recolonization of released bacteria on the *C. elegans* *in vivo* infection models in BDR device. (a) Image of *C. elegans* was

infected by bacteria released from different methods mediated biofilm dispersal at 3 hrs, 6 hrs and 12 hrs. Bacteria in *C. elegans* were labelled with red boxes. The scale bar is 50 μm . (b) Mortality of *C. elegans* at 0 hrs, 3 hrs, 6 hrs and 12 hrs. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

5.2.5 Differences in settlement of released biofilm bacteria without flow

As previously mentioned, the majority of biofilm bacteria released by CID were single bacteria, whereas those released by EDA were mostly small bacterial aggregates, and there was also a difference in the ability to recolonize on infection models for HLF spheroids and *C. elegans*. To explore whether the reason for these differences was related to the settlement speed, biofilm-released bacteria were collected and observed under confocal microscopy for differences in settlement over 2 hrs periods.

Confocal images showed that the bacterial aggregates released by EDA settled at the beginning, while those released by CID could still

not settle and grow at 2 hrs (Figure 5.5a). Quantifying the fluorescence intensity of the settled bacteria showed that the EDA-released biofilm bacteria settled significantly faster than the CID-released bacteria (Figure 5.5b). Next, bacteria released by CID and EDA were collected and added to chambers covered with HLF spheroidal models, and the mortality of cells were recorded using confocal microscopy. The confocal images showed that bacteria released by CID without flow caused more cell death than EDA without flow (Figure 5.5c). This was confirmed by the cell viability results (Figure 5.5d). The reason may be that the bacteria released by NO-induced biofilm dispersal could adhere to the cells without flow, then lead to cell death because of the high virulence of the bacteria released from CID-induced biofilm dispersal.

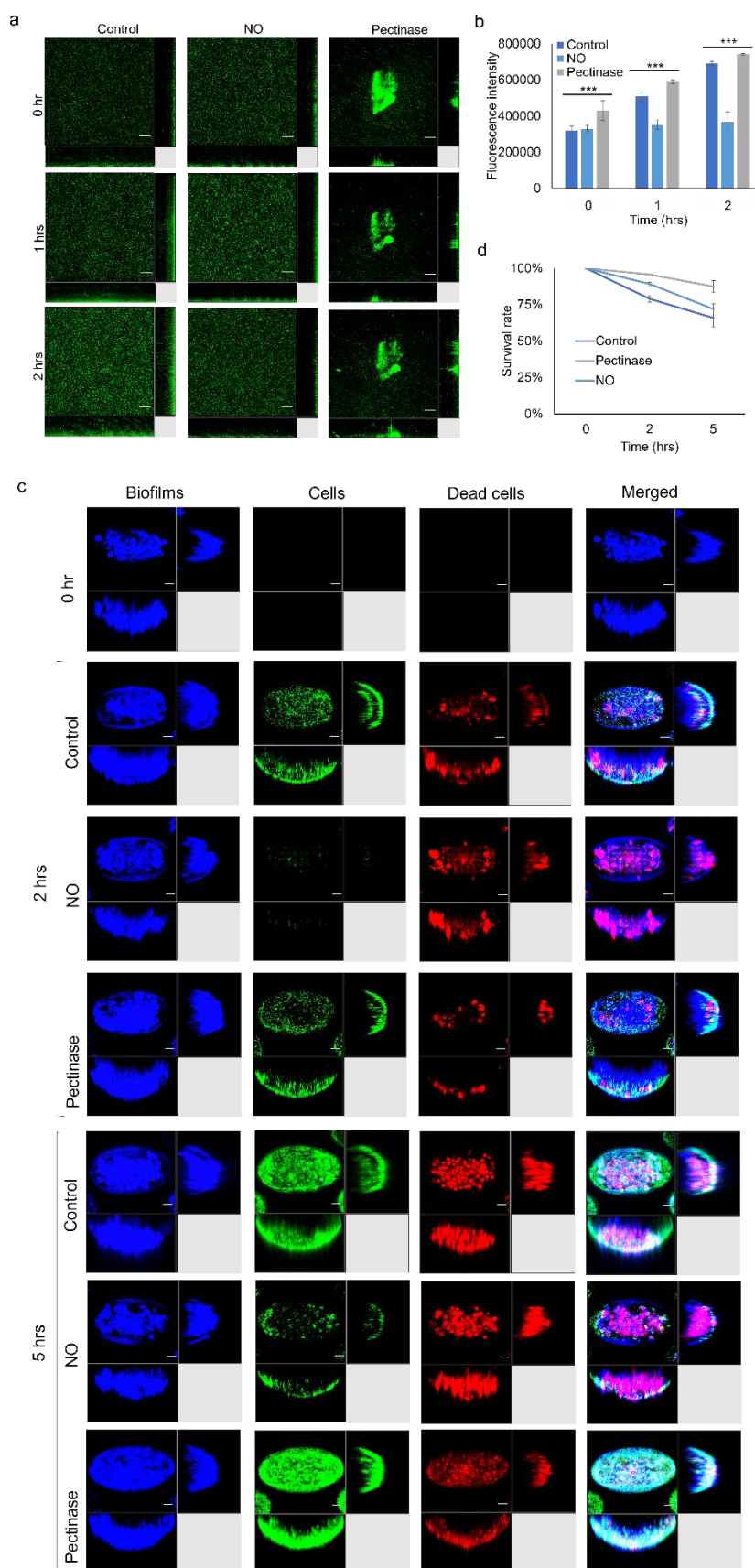


Figure 5.5. Differences in the settling of bacteria released by CID and EDA without flow. (a) Confocal images of released bacteria in an 8-well chamber at 0 hr, 1 hr, 2 hrs. The scale bar is 10 μ m. (b) Biomass of bacteria settled on the bottom at 0hr, 1hr, 2hrs. The averages and s.d. from triplicate experiments were shown. ***P < 0.001. (c) Confocal images of HLF spherical infection model without flow at 0 hr, 2 hrs, 5 hrs. (d) Lethality of HLF from bacteria released by biofilm dispersal without flow at 0 hr, 2 hrs, 5 hrs (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

5.2.7 Clinical isolates tested in the BDR device

It has been shown previously that biofilm dispersal and disassembly could be performed in the BDR device. To verify that the BDR device could also apply to clinical strains, biofilm dispersal and recolonization experiments were conducted with PA14, CF173 and CF273, the clinical isolates of *wspF* mutations collected from CF patients ^(249, 250). In this experiment, since CF173 and CF273 are pro-biofilm-forming clinical isolates⁽²⁴⁹⁾, thus, higher concentrations of SNP and pectinase were used than previously. The confocal images of CF173 showed that NO and pectinase could reduce biofilms (Figure 5.6a). The results of

biofilm dispersal by NO and biofilm disassembly by pectinase within 2 hrs were demonstrated by quantifying the fluorescence intensity in microwells (Figure 5.6b). In addition, the results of bacterial recolonization by biofilm release within the recolonization chamber were similar to those of PAO1 (Figure 5.6c). Besides, the dispersal and recolonization results of CF273 were similar to those of CF173, as both NO and pectinase reduced biofilm (Figure 5.6d). The biomass showed that after 2 hrs, the biofilm with NO-induced dispersal decreased the most (Figure 5.6e), and there were fewer bacteria recolonized than physiological dispersal and pectinase treatment (Figure 5.6f). In addition, PA14 was also tested as a clinical isolate that could not generate Psl polysaccharides ⁽²⁴⁰⁾. Since pectinase disassembled Psl polysaccharides in the biofilm matrix, the biomass of PA14 biofilm was not decreased within 2 hrs, whereas the CID dominated by NO could be effective in dispersing the biofilm (Figure 5.6g, 5.6h). These bacteria were still unable to recolonize within 2 hrs released by NO-induced biofilm dispersal compared to biofilm bacteria released after physiological dispersal and pectinase treatment (Figure 5.6i).

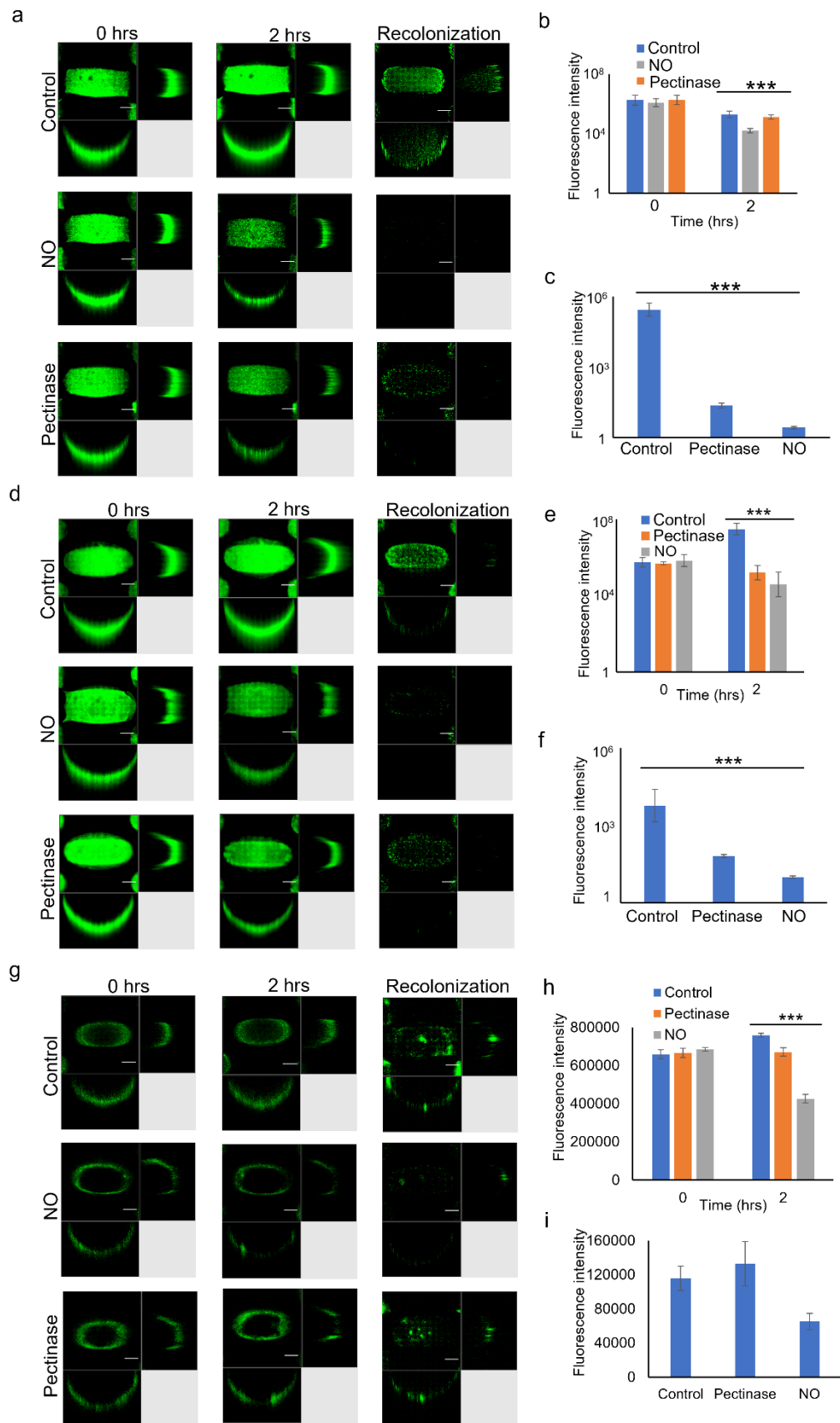


Figure 5.6. Biofilm dispersal and disassembly of Clinical samples

in the BDR device. (a) Confocal images of CF173 biofilms dispersal and recolonization in BDR device. The scale bar is 50 μm . (b) Biomass of CF173 biofilms at 0 hr and 2 hrs. The averages and s.d. from triplicate experiments were shown. $***P < 0.001$. (c) Biomass of CF173 biofilms in recolonization chamber. The averages and s.d. from triplicate experiments were shown. $***P < 0.001$. (d) Confocal images of CF273 biofilms dispersal and recolonization in BDR device. The scale bar is 50 μm . (e) Biomass of CF273 biofilms at 0 hr and 2 hrs. The averages and s.d. from triplicate experiments were shown. $***P < 0.001$. (f) Biomass of CF273 biofilms in recolonization chamber. (g) PA14 biofilms dispersal and recolonization in BDR device. The scale bar is 50 μm . (h) Biomass of PA14 biofilms at 0 hr and 2 hrs. The averages and s.d. from triplicate experiments were shown. $***P < 0.001$. (i) Biomass of PA14 biofilms in recolonization chamber. The averages and s.d. from triplicate experiments were shown. $***P < 0.001$. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown (Reprint with permission from Ma Y, Deng Y, 2023 ⁽²¹⁹⁾).

5.3 Discussion

At the end of the biofilm life cycle, the bacteria leave the biofilm, and after converting to a planktonic status, they can recolonize the fresh surface and start a new biofilm life cycle ⁽²⁵¹⁾. CID biofilm dispersal and EDA biofilm disassembly showed differences in the process and the biofilm bacteria released. To further explore the differences in recolonization of released biofilm bacteria from these two biofilm dispersal methods, the recolonization process was recorded and analyzed, and an HLF spheroid *in vitro* infection model and *C. elegans in vivo* infection model were established to evaluate the virulence of the released biofilm bacteria.

Biofilm bacteria released by CID-dominated biofilm dispersal could not recolonize the microwells within 2 hrs; this was due to the c-di-GMP of the dispersed bacteria remaining at low levels, causing the dispersed cells to maintain a dispersed phenotype and be unable to reattach ⁽⁹³⁾. During the observations, the movement velocity of the CID-released biofilm bacterial population was consistent with that of the physiologically dispersed released biofilm bacteria, and the trajectory in the microwells also showed that the CID-released biofilm bacteria could

not recolonize and multiply in the microwells. According to previous studies, biofilm bacteria induced to disperse could not recover their planktonic state to recolonize fresh surfaces for at least 6 hrs ⁽⁹³⁾. This resulted in a higher survival rate of the host cells as the CID-released biofilm bacteria were temporarily unable to reinfect the host cells with the flow. Also, bacteria were less observed in the esophagus of *C. elegans*, because CID-released bacteria did not recolonize and could not be captured by *C. elegans*. This result was consistent with a previous study, that the bacteria released by induced biofilm dispersal had spread into the bloodstream and accumulated in the spleen, and also showed no adverse effects in mice ⁽²³⁶⁾. Thus, the organism with flow could tolerate highly virulent bacteria with low c-di-GMP levels released by biofilm dispersal. However, bacteria with CID-induced dispersal released may require higher concentrations of antibiotics to kill them, as it has been shown that bacteria with low c-di-GMP released from induced dispersal were more resistant to the antimicrobial peptide colistin ⁽²⁰⁴⁾.

However, unlike CID-released biofilm bacteria, EDA-released biofilm bacteria could recolonize and multiply in microwells within 2 hrs. According to the analysis of the mathematical model, the multiplication

of EDA-released biofilm bacteria tended to increase exponentially, similar to the multiplication pattern of physiologically dispersed released bacteria. This suggested that the EDA-released biofilm bacteria could normally recolonize the new surface and multiply, although at a lower rate than the physiological dispersed-released bacteria, possibly due to the inhibitory effect of enzymes on the biofilm bacteria ⁽²⁵²⁾. However, combination therapy with EDA and antibiotics may reduce secondary infection. It has been shown that glycoside hydrolases are compatible with antibiotics and enhance the antibacterial activity of colistin ⁽²⁵³⁾. Therefore, the bacterial aggregates released by EDA are more susceptible to being killed by antibiotics.

Since EDA-released biofilm bacteria could normally recolonize new surfaces, and most of the EDA-released bacteria were small aggregates, they were more likely to settle to the bottom of microwells, resulting in higher lethality for the HLP spheroid model and were more easily captured by *C. elegans*.

Since the experiment had been performed with flow, HLF spheroids were prepared in a new recolonization chamber to explore the results generated without flow. The CID and EDA released biofilm bacteria

were added and observed for a period of time; the results were different from before with flow. The bacteria released by NO-dominated biofilm dispersal were more lethal to HLF than pectinase. The results suggest that CID-induced dispersal released biofilm bacteria were more highly virulent to cells than EDA due to the low c-di-GMP level⁽²⁵⁴⁾. A similar conclusion was found in a previous study that bacteria with low c-di-GMP levels were highly virulent⁽²⁰³⁾.

Based on the results of the previous studies, CID-dominated biofilm dispersal differed from EDA-dominated biofilm disassembly at the recolonization within 2 hrs. In order to confirm whether these results were applicable to other *P. aeruginosa*, clinical isolates were tested. The results of experiments on clinical isolates CF173 and CF273 showed that CID and EDA effectively reduced biofilms, although higher concentrations were required to treat them. Furthermore, CID-dominated biofilm dispersal of released bacteria could not recolonize the new surface within 2 hrs. These results were consistent with those demonstrated in the wild type. It indicated that the CID and EDA biofilm elimination methods could also be used for other *P. aeruginosa*.

5.4 Conclusion

In conclusion, the study between CID and EDA methods revealed differences in the recolonization process and the behavior of released biofilm bacteria. CID-released biofilm bacteria were unable to recolonize within 2 hrs due to their low c-di-GMP levels, resulting in a dispersed phenotype that prevented reattachment. This lack of recolonization had positive implications for host cell survival and reduced bacterial presence in *C. elegans*. On the other hand, EDA-released biofilm bacteria demonstrated the capacity to successfully recolonize and multiply within the microwells, although at a lower rate compared to physiologically dispersed bacteria. The small aggregate form of EDA-released bacteria facilitated their settling at the bottom of microwells, leading to higher lethality in the HLF spheroid model and increased capture by *C. elegans*. However, due to the high virulence of bacteria released by CID biofilm dispersal at low levels of c-di-GMP, the lethality of CID-released bacteria to cells without flow was higher than that of EDA-released bacteria.

The study also extended to clinical isolates of *P. aeruginosa*, where both CID and EDA methods effectively reduced biofilms with higher

concentrations for treatment in BDR devices. Moreover, the inability of CID-released bacteria to recolonize the new surface within 2 hrs was consistent across different *P. aeruginosa* strains, confirming the applicability of CID and EDA biofilm elimination methods for other clinical isolates.

Overall, these results highlight the distinct characteristics of CID and EDA regarding recolonization ability and bacterial behavior. Understanding these differences contributes to developing targeted strategies for biofilm elimination, potentially improving treatment outcomes for biofilm-related infections caused by *P. aeruginosa* and other bacterial pathogens.

Chapter VI Juglone is a novel antibacterial agent targeting biofilm infections

6.1 Summary

Bacteria recolonization after biofilm dispersal is an important reason for secondary infection. Antibiotic treatment is still the main clinical option. However, it has become increasingly challenging to treat bacterial infections due to the development of drug-resistant strains, and therefore, developing new antimicrobial agents is one of the effective ways to eliminate bacterial biofilm infections.

The QS system is a regulatory system for bacterial biofilm formation; therefore, the development of antimicrobial agents that damage the QS system will effectively inhibit or eliminate bacterial infections. In this study, the Juglone (5-hydroxy-1,4-naphthoquinone), a pure compound of Walnut, was screened as a QS inhibitor by molecular docking. Juglone inhibits the growth of bacterial biofilms by binding to the active site of PqsR. On this basis, the Medaka fish model of *P. aeruginosa* infection was developed and treated with colistin and Juglone for combinatorial treatment.

Colistin, an antimicrobial peptide, can disrupt the bacterial outer membrane by targeting lipid A of the lipopolysaccharide (LPS) ⁽²⁵⁵⁾. This will lead to an increase in bacterial permeability and kill bacteria ⁽²⁵⁵⁾. However, forming outer membrane vesicles, in which PQS is involved, protects the bacteria from the impact of colistin ⁽²⁰⁶⁾. Thus, as a QS inhibitor, Juglone can work synergistically with colistin to inhibit and eliminate the *P. aeruginosa* biofilms.

The results showed that the combination of Juglone and colistin reduced biofilm infections. Therefore, Juglone may be a novel antibacterial agent to treat *P. aeruginosa* infections.

6.2 Results

6.2.1 Juglone inhibits *P. aeruginosa*

In order to verify whether the Juglone has an inhibition effect on the PQS of the QS system, a preliminary screen was performed using the PAO1/*p_{pqsA}-gfp*. PqsA is an important enzyme involved in the PQS synthesis; thus, the pqsA promoter was fused to green fluorescent protein to be a transcriptional biosensor that can be used to measure the expression of *pqs* QS ^(206, 256). The result showed that Juglone is an

inhibitor of the PQS. The minimum inhibitory concentration (MIC) against the PAO1 growth was approximately 5 µg/ml (Figure 6.1a). In addition, the expression of *p_{pqsA}-gfp* was gradually inhibited with increased concentrations of Juglone (Figure 6.1b), and the half-maximum inhibitory concentration (IC₅₀ value) for inhibition of the expression of *p_{pqsA}-gfp* was calculated to be approximately 0.3 µg/ml (Figure 6.1c).

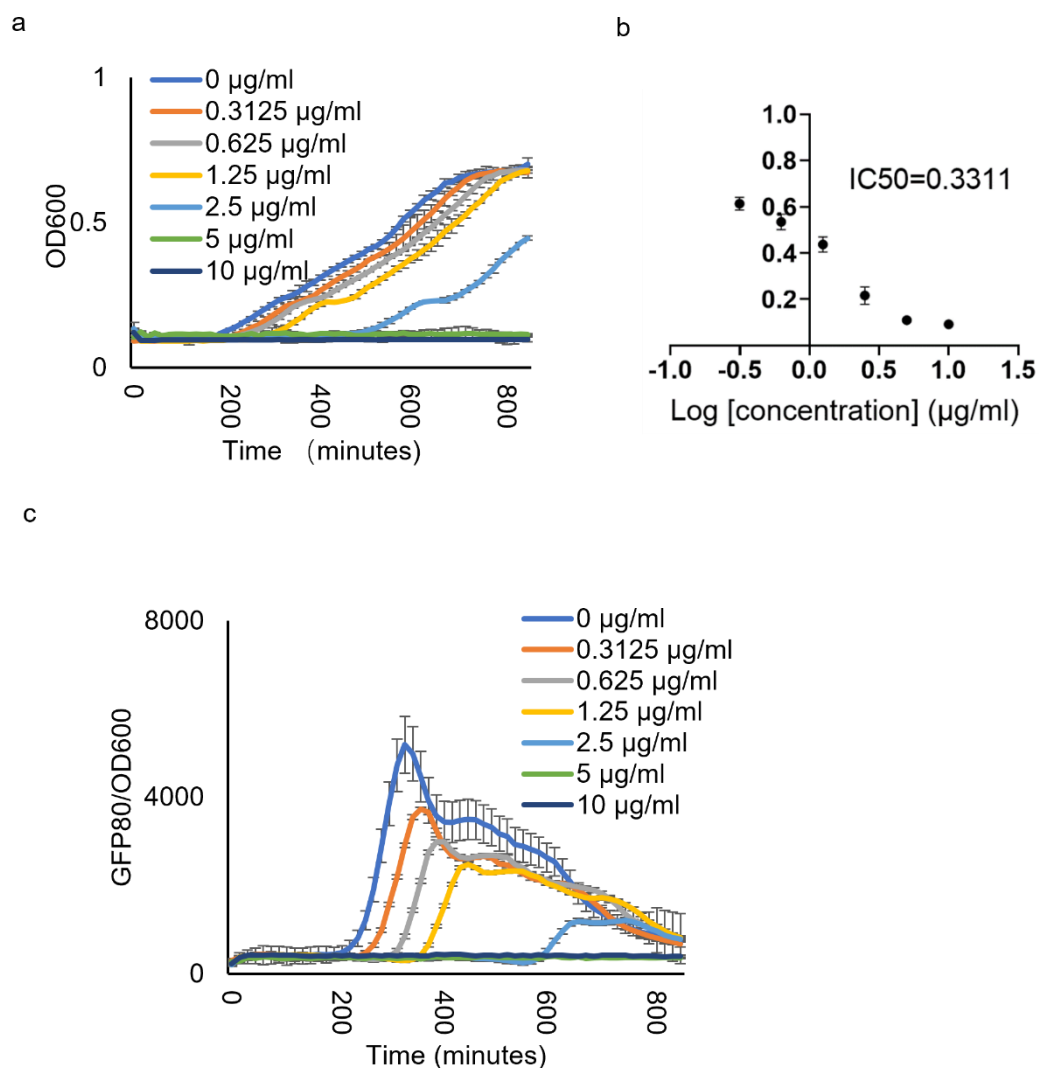


Figure 6.1. Juglone inhibits QS system. (a) PAO1 was inhibited from growing in various concentrations of Juglone. (b) The half-maximal inhibitory concentration (IC50) of Juglone. (c) Inhibitory effect of various concentrations of Juglone on *p_{pqSA}-gfp* expression. Experiments were performed in triplicate, and the averages and s.d. from triplicate

experiments were shown.

6.2.2 Inhibition of PqsR by Juglone

Since Juglone was confirmed to inhibit the expression of *p_{pqsA}-gfp*. In order to confirm whether it could inhibit the production of *pqs* signaling, the PQS production would be evaluated. To quantify the amount of PQS produced and secreted by bacteria treated with Juglone, the *ΔpqsA/p_{pqsA}-gfp*, which cannot synthesize PQS signalling molecules, was added to the supernatant of PAO1 cultures treated with different concentrations of Juglone. And the relative PQS concentrations of PAO1 showed by GFP expression decreased with increasing Juglone concentration (Figure 6.2a), indicating that Juglone could reduce *pqs* signaling.

In order to further confirm whether Juglone inhibits the synthesis of PQS signaling molecules or the response gene, 10 μM exogenous PQS were added with *ΔpqsA/p_{pqsA}-gfp* to cultures containing different concentrations of Juglone, respectively. The results showed that the expression of *p_{pqsA}-gfp* also reduced with the increase of Juglone concentrations, which meant Juglone may inhibit the *pqs* response instead of biosynthesis (Figure 6.2b).

Therefore, to verify whether Juglone could inhibit the response gene PqsR, the PqsR deficient mutant, was employed to be treated with Juglone. The result showed that Juglone did not affect the expression of *pqsA-gfp* (Figure 6.2c). In addition, $\Delta pqsR/p_{lac}-pqsABCDE/p_{pqsA}-gfp$ was also used to confirm the result, as it is unable to respond to PQS, but with overexpression of the PQS operon ⁽²⁰⁶⁾. The result also illustrated that Juglone did not affect the GFP expression (Figure 6.2c). Therefore, it was confirmed that Juglone inhibited the *pqs* QS system through PqsR.

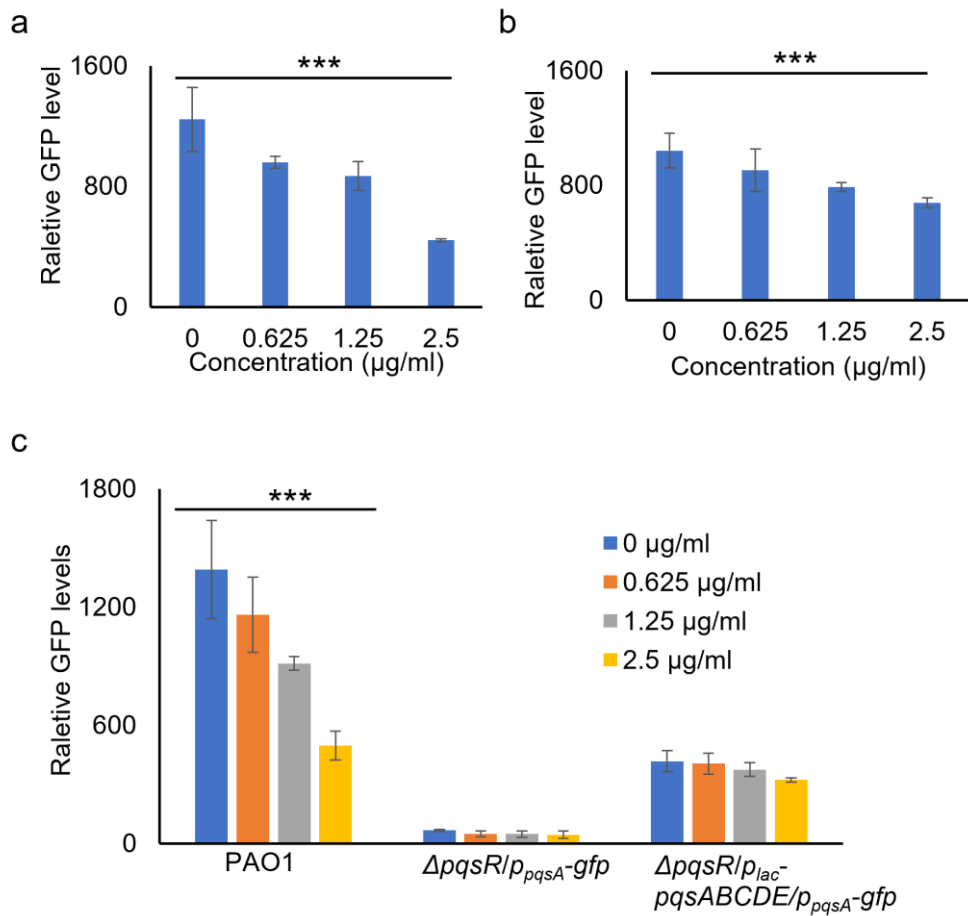


Figure 6.2. Inhibition of PQS production by Juglone. (a) relative reduction of PQS using $\Delta pqsA/p_{pqsA}-gfp$. (b) relative GFP quantification of $\Delta pqsA/p_{pqsA}-gfp$ with exogenously added 10 μM PQS in varying concentrations of Juglone. (c) Relative GFP quantification of PAO1, $\Delta pqsR/p_{pqsA}-gfp$, $\Delta pqsR/p_{lac}-pqsABCDE/p_{pqsA}-gfp$ in varying concentrations of Juglone. Experiments were performed in triplicate, and the averages and s.d. from triplicate experiments were shown. ***P < 0.001.

6.2.3 Molecular Docking of Juglone to PqsR

After confirming that Juglone interfered with the PQS, molecular docking was conducted to confirm the active binding site of Juglone and PqsR. In the PQS QS system of *P. aeruginosa*, PQS bonded directly to the LysR-type transcriptional regulator PqsR, which then activated the pqsABCDE operon to regulated activities such as biofilm, virulence factor production and flagellar motility ⁽²⁵⁷⁾. The PqsR receptor protein could be activated by a variety of 2-alkyl-4-quinolones, including PQS, HHQ, C9-PQS and NHQ ⁽²¹³⁾. NHQ binds to PqsR at active residue sites such as L207 and L208 ⁽²¹³⁾. The binding sites of NHQ (yellow) and the Juglone (green) in the PqsR active site (grey) were compared through the molecular docking software Autodock Vina (Figure 6.3a), and showed that the Juglone could bind to PqsR at both the L207 and L208 sites (Figure 6.3b and 3c).

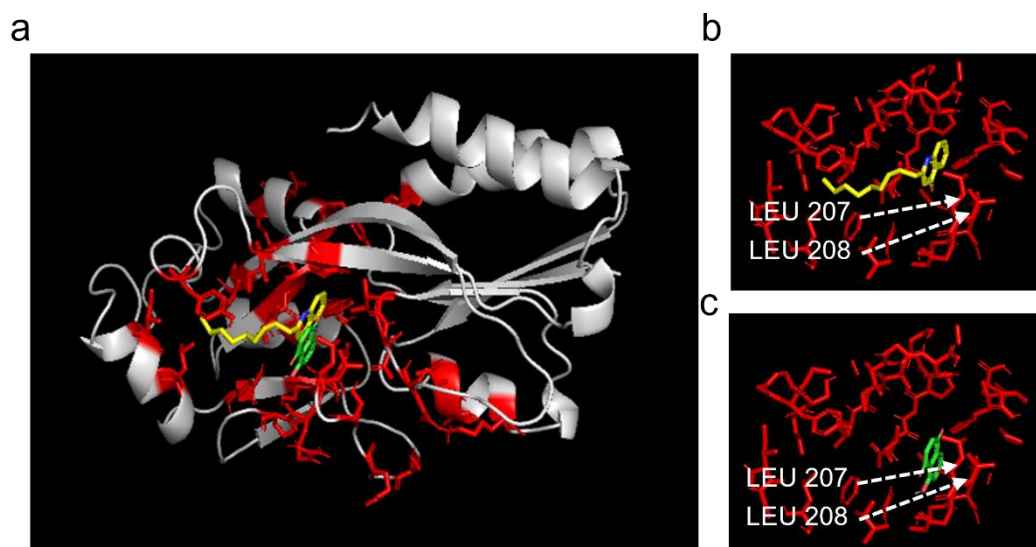


Figure 6.3. Molecular docking of Juglone to PqsR. (a) Superimposed comparison of NHQ and Juglone binding to PqsR. (b) NHQ binding to PqsR, with -6.9 kcal/mol binding affinity of L207 and L208. (c) Juglone binding to PqsR, with -6.6 kcal/mol binding affinity of L207 and L208.

6.2.4 Inhibition of PQS-controlled virulence factors

P. aeruginosa produces a variety of virulence factors, including flagella, exotoxins, pyocyanin and others ⁽⁹⁴⁾. The PqsR is required for flagella swarming in PAO1 to form biofilms ⁽⁴⁴⁾. Thus, the effect on inhibiting the swarming of PAO1 on LBA plates was verified. The result showed that 2.5 µg/ml Juglone could effectively inhibit the twitching motility (Figure 6.4a and 4b). Pyocyanin synthesis was regulated by the pqsR gene ⁽²⁵⁸⁾. The quantity of pyocyanin was measured that was produced by *P.*

aeruginosa at different concentrations of Juglone. The results showed that the amount of pyocyanin decreased with increased concentrations of Juglone (Figure 6.4c, 4d).

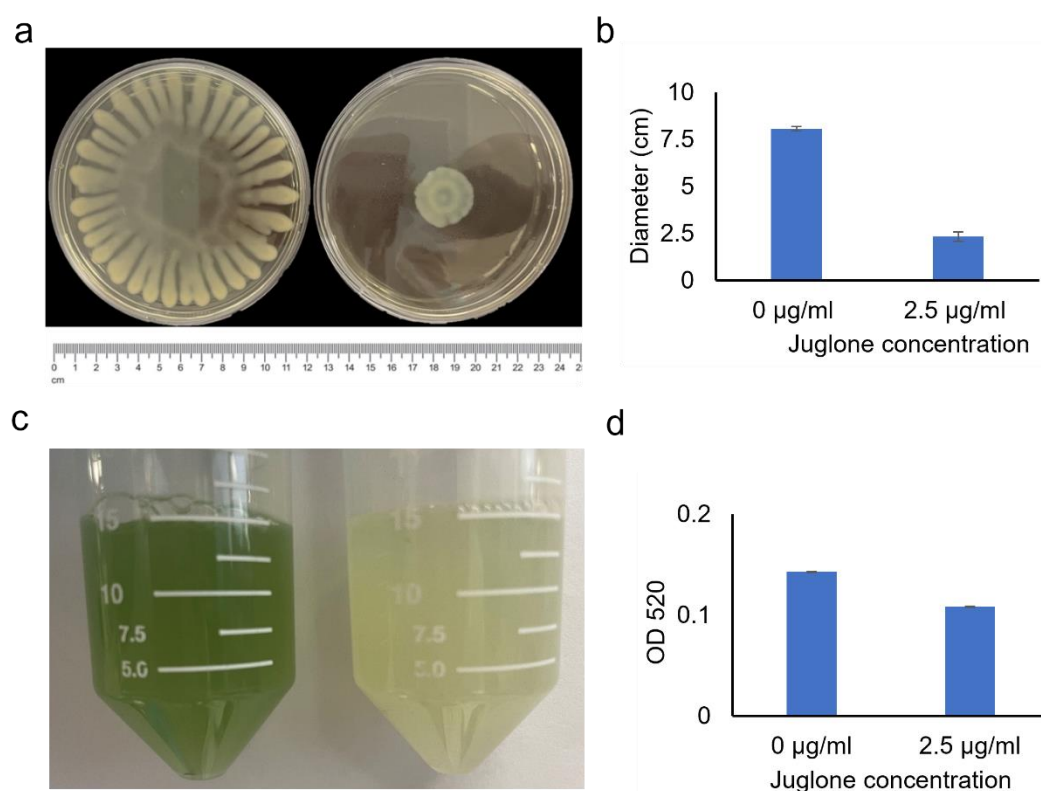


Figure 6.4. Inhibition of PQS-controlled virulence factors by Juglone. (a) Images of swarming motility by *P. aeruginosa* with Juglone treatment. (b) Diameter of the swarming area by *P. aeruginosa* with Juglone treatment. (c) Pyocyanin concentration. (d) Relative pyocyanin concentration. Experiments were performed in triplicate, and the averages and s.d. from triplicate experiments were shown. *** $P < 0.001$.

6.2.5 Colistin-Juglone combinatorial therapy eliminated biofilm.

The antibiotic-resistant characteristics of biofilms present a considerable impediment to effective pharmacotherapy due to the protective nature of their extracellular matrix ⁽²⁵⁹⁾. Thus, developing new therapeutic models, such as combinatorial therapy, would improve the effectiveness of treatment. Combinatorial therapy with colistin and anti-biofilm agents is a potential strategy for treating bacterial infections. The perturbation of the bacterial outer membrane by colistin may facilitate the uptake of anti-biofilm agents, allowing the agent to kill the bacteria easily.

Various concentrations of colistin and Juglone were initially tested to observe their inhibitory effects on PAO1 biofilms, respectively. The results showed that 1 µg/ml of colistin monotherapy or 1.25 µg/ml of Juglone monotherapy had limited effects on inhibiting biofilm formation and eliminating preformed biofilms but were more effective when used combinatorial therapy than alone. The dead bacteria were stained by PI dye. Confocal microscopy was used to observe the effect of Juglone and colistin in inhibiting biofilm formation and eliminating the preformed biofilm (Figure 6.5a). The fluorescence intensity was measured and

showed that colistin-Juglone combinatorial therapy effectively inhibited biofilm formation (Figure 6.5b). The CFU was performed to quantify the bacteria number, illustrating that the combinatorial use of Juglone and colistin was more effective in inhibiting biofilms (Figure 6.5c).

Then biofilm elimination experiments were performed to verify whether Juglone and colistin would effectively kill formed biofilms. Various concentrations of colistin and Juglone were initially tested to observe their eliminative effects on biofilms, respectively. The confocal images revealed that the monotherapies of 1.25 $\mu\text{g/ml}$ of Juglone or 1 $\mu\text{g/ml}$ of colistin could eliminate a part of the biofilm bacteria, while their combinatorial therapy showed a significant increase in efficacy compared to their monotherapies (Figure 6.5d). This result was consistent with biomass (Figures 6.5e and 5f). The CFU result also showed a decrease in biofilm bacteria after treatment of the formed biofilm with Juglone and colistin (Figure 6.5g).

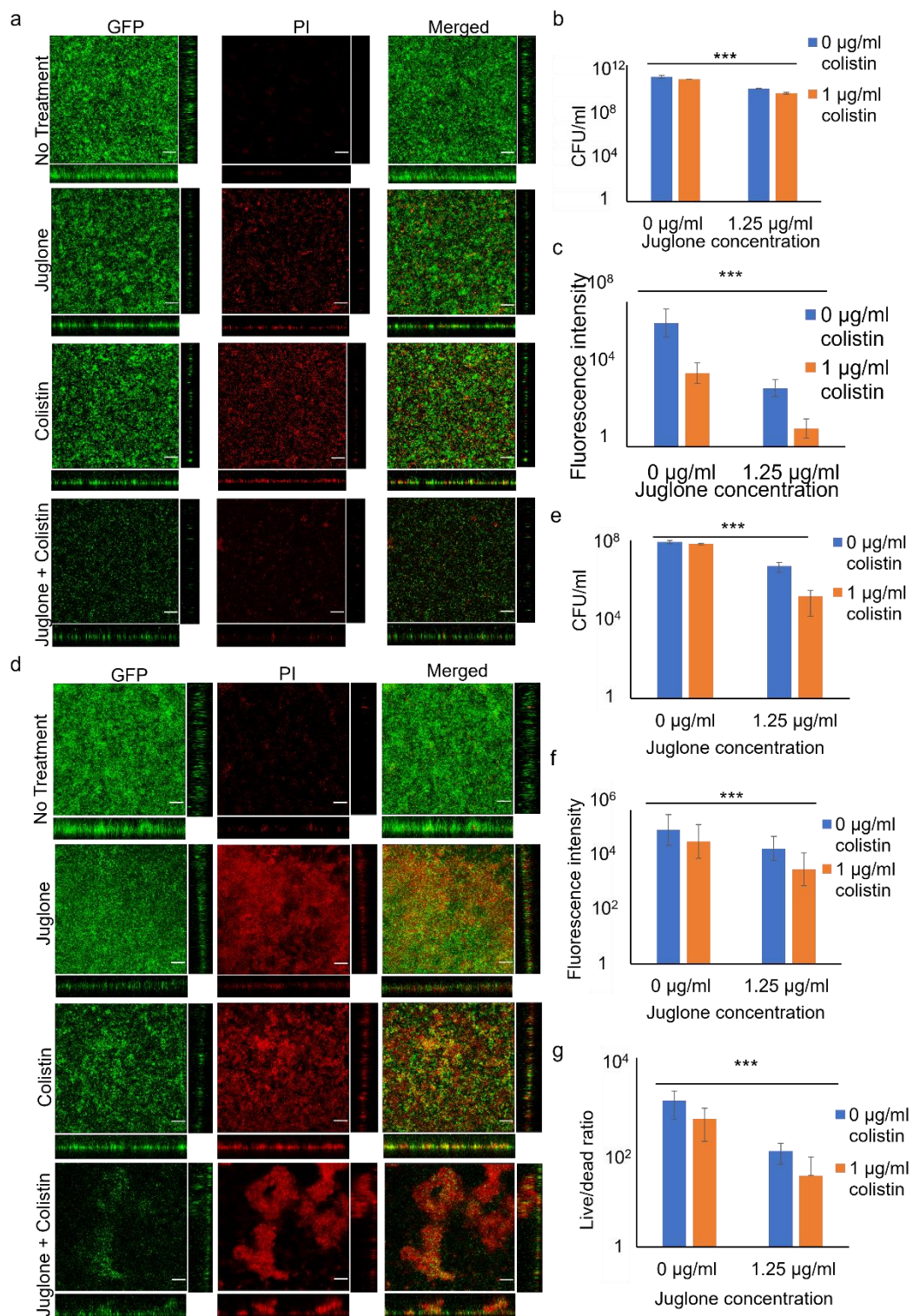


Figure 6.5 Combinatorial treatment of colistin with Juglone could

inhibit and eliminate biofilm. (a) Confocal images for inhibition of biofilm. (b) CFU result for inhibition of biofilm formation. (c) Biomass of biofilm with combinatorial treatment. (d) Confocal images for eliminating pre-formed biofilms. (e) Biomass of biofilm after combinatorial treatment. (f) The live/dead ratio of eliminating pre-formed biofilms. (g) CFU result of eliminating pre-formed biofilms. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar in all images was 50 μm . The averages and s.d. from triplicate experiments were shown. *** $P < 0.001$.

6.2.6 Combinatorial therapy eliminates biofilm of Clinical isolates.

In addition, in order to test whether Juglone and its combinatorial treatment with colistin could work on clinical samples, two clinical samples, CF173 and CF273, were used to perform the biofilm killing assay. As before, PI dye was used to stain dead cells. The confocal images of CF173 (Figure 6.6a) and CF273 (Figure 6.6b) showed that Juglone and colistin could effectively kill biofilms. Quantification of GFP showed that the combinatorial effect significantly increased biofilm mortality (Figure 6.6c and 6d). The live/dead ratios for CF173 and

CF273 also showed a significant decrease in biofilm survival after treatment with colistin and Juglone (Figure 6.6e and 6f). This was also demonstrated by the CFU results (Figures 6.6g and 6h).

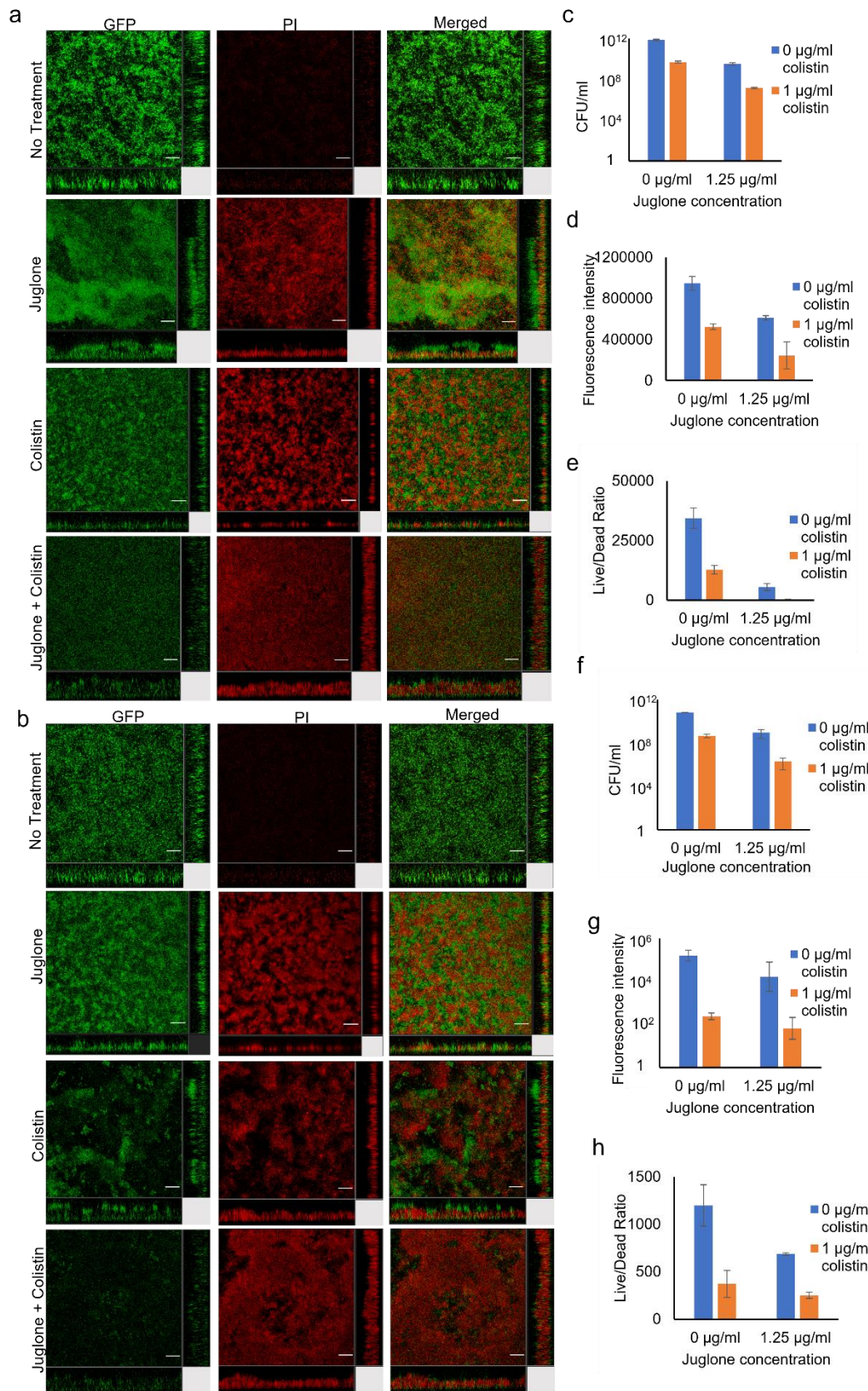


Figure 6.6 Synergistic treatment of colistin with Juglone on clinical samples. (a) Confocal images of treating CF173 pre-formed biofilms. (b) Confocal images for treating CF273 pre-formed biofilms. (c) Biomass of CF173 biofilm with synergistic treatment. (d) Biomass of CF273 biofilm after synergistic treatment. (e) The live/dead ratio of eliminating CF173 biofilms. (f) The live/dead ratio of eliminating CF273 biofilms. (g) CF173 CFU results for killing biofilms. (h) CFU result of eliminating CF273 biofilms. Experiments were performed in triplicate, at least 3 images were captured for each replicate, the representative images were shown. The scale bar in all images was 50 μ m. The averages and s.d. from triplicate experiments were shown. ***P < 0.001.

6.2.7 Combinatorial treatment of Juglone and colistin enhances efficacy in BDR device

Subsequently, the combinatorial treatment of Juglone and colistin was tested in a BDR device. The effective concentrations of colistin and Juglone were first tested in monotherapy in the BDR device to eliminate biofilm. The results showed that 15 μ g/ml of colistin monotherapy and 20 μ g/ml of Juglone monotherapy effectively eliminated biofilm in the BDR device. This concentration was higher

than previously used because the biofilm formed in the BDR device was 3D shaped and had a more complex and thicker biofilm structure than in a 2D surface ⁽²⁶⁰⁾. Thus, higher concentrations were required for the treatment. Based on the results of the quantified fluorescent dye concentrations shown in Figure 2.1d, 30 µg/ml of colistin and 30 µg/ml of Juglone were mixed to generate eight concentrations to have flowed into the channel. These eight combinatorial concentrations of colistin and Juglone from the 1st to the 8th channel were shown in Table 6.1.

Table 6.1 Colistin and Juglone concentrations used in channels 1 to 8 of the BDR device

Channel	1	2	3	4	5	6	7	8
Colistin (µg/ml)	30	26.2	26	20.7	13.7	4.6	0.02	0
Juglone (µg/ml)	0	0.02	4.6	13.7	20.7	26	26.2	30

The confocal images showed that the combinatorial treatment of the 5th channel was more effective than other combinatorial of colistin and Juglone (Figure 6.7a), and the concentrations were 13.7 µg/ml of colistin and 20.7 µg/ml Juglone. In the case of colistin at a concentration of 15 µg/ml and Juglone at 20 µg/ml alone, the combined

treatment in channel 5 demonstrated similar concentrations but achieved a similar level of biofilm elimination as the higher concentration of Juglone in the 8th channel. In addition, the combined treatment of channel 5 used lower concentrations of colistin and Juglone than the other combined concentrations, causing a higher level of biofilm elimination (Figures 6.7c and 7d). In the second chamber, the bacteria could not recolonize within 2 hours at any concentration due to inhibition by Juglone and colistin (Figure 6.7b). The biomass results also demonstrated the inability of bacteria to recolonize and multiply in the second chamber (Figure 6.7c).

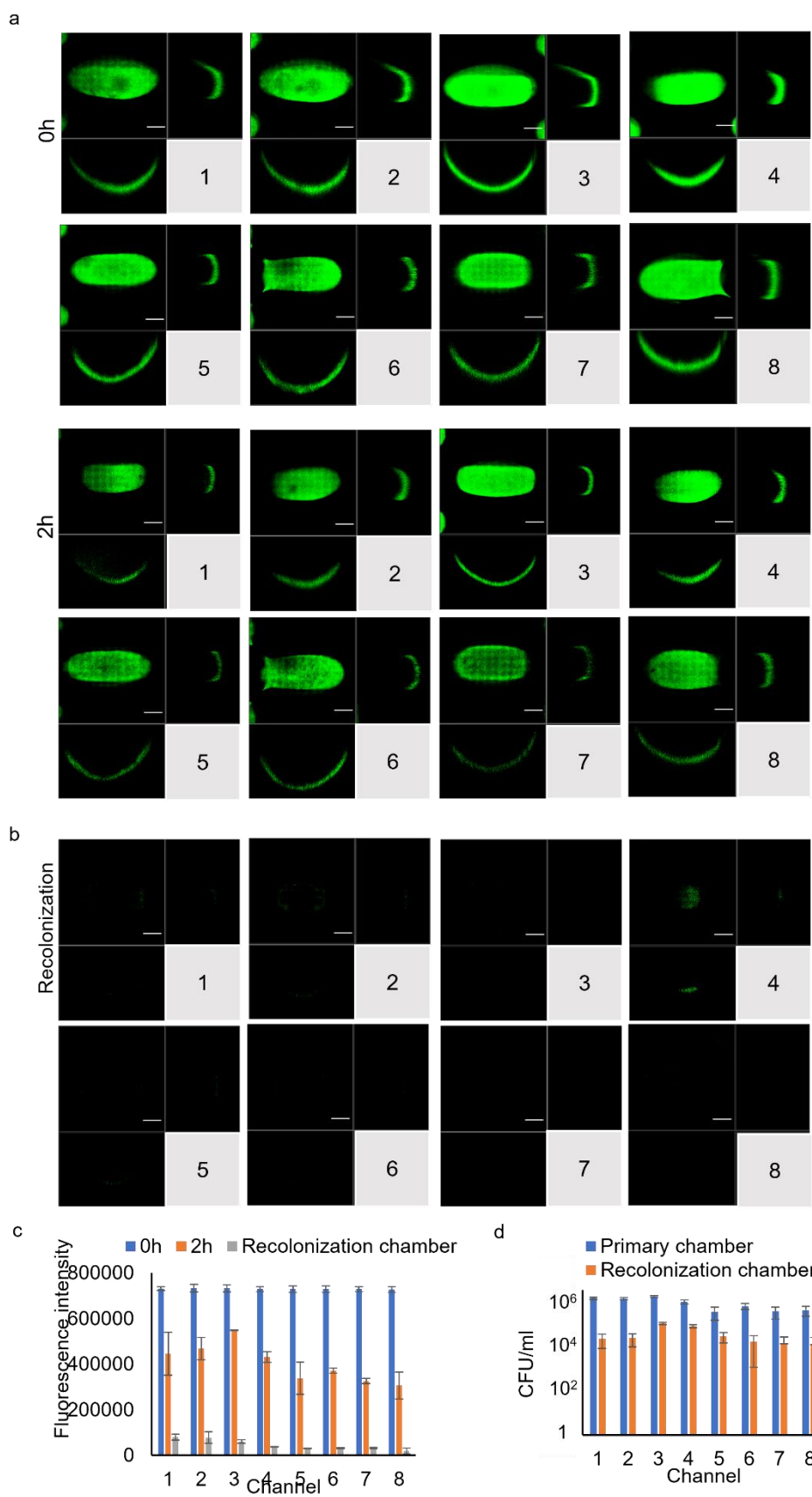


Figure 6.7. The effect of combinational therapy in the BDR device.

(a) Before and after images of colistin and Juglone mixed in ratio to treat biofilm for 2 hrs. The number inset of each image represented channel order. (b) Confocal images of recolonization channels within 2 hrs. The number represents channel order. (c) Biomass for the primary chamber at 0 hr, 2 hrs, and the recolonization chamber. (d) CFU results for primary chamber and recolonization chamber. Experiments were performed in triplicate, at least 3 images were captured for each replicate, and the representative images were shown. The scale bar in all images was 50 μm . The averages and s.d. from triplicate experiments were shown.

6.2.8 Effective *in vivo* biofilm elimination using colistin and Juglone in a Medaka fish infection model

As one of the most used experimental fish models in biomedical research, the Medaka has a genome with 24 pairs of chromosomes that are homologous to vertebrates, including humans ⁽²⁶¹⁾. Medaka is not only easy and inexpensive to propagate, but more importantly, the immune system of Medaka larvae is similar to that of humans ⁽²¹⁴⁾. For example, Medaka has various types of immune cells that are found in

humans, including macrophages, neutrophils, and lymphocytes ⁽²⁶²⁾. In addition, the Medaka larvae are mostly transparent, enabling all development stages to be easily observed and microbial infections to be visualized ^(214, 263).

To verify whether the compound is effective for *in vivo* infection, the wound infection model of the Medaka fishtail was employed ⁽²¹⁴⁾. In this study, HLF spheroids were initially used for toxicity testing of Juglone, and the results showed that Juglone had a low lethality to the cells at the concentrations used (Figure 6.8a).

Medaka larvae was then used as infected hosts; the concentrations of colistin and Juglone used on the *in vivo* model were both approximately 100 times lower than in the *in vitro* experiments; due to the small size of the Medaka larvae, the tail infection formed less biofilm than *in vitro* mode. The PAO1 infection model in Medaka larvae was treated with colistin and Juglone and imaged by CLSM (Figure 6.8b). The results of biomass measurements demonstrated that biofilm residual remained after treatment with Juglone and colistin monotherapies, while the combination of Juglone and colistin was more effective than monotherapies (Figure 6.8c). The levels of

pyoverdine, the main iron scavenging metabolite of *P. aeruginosa* biofilms, were also quantified to evaluate the effectiveness of combinatorial therapy. The results showed that levels of pyoverdine were lower in wounds treated with monotherapies than in untreated wounds and that the combination of Juglone and colistin was also effective in reducing pyoverdine (Figure 6.8d). Moreover, the pro-inflammatory cytokines IL-6 and TNF- α were quantified using ELISA, and the results showed a significant upregulation of these cytokines in the infected fish model (Figure 6.8e and 8f). The results for CFU showed that while treatment with Juglone and colistin monotherapies also reduced biofilm, the combination of treatments resulted in a significant reduction in CFU levels (Figure 6.8g).

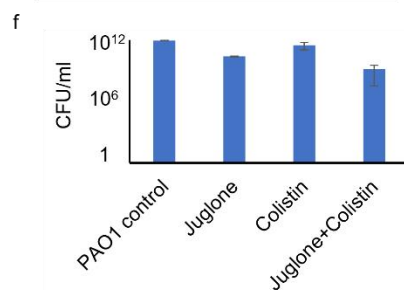
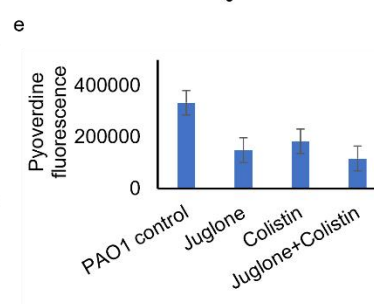
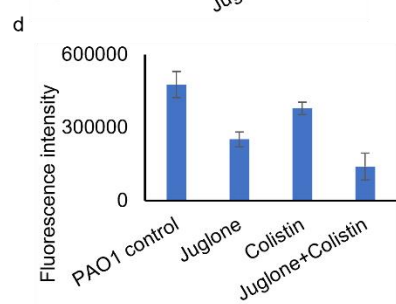
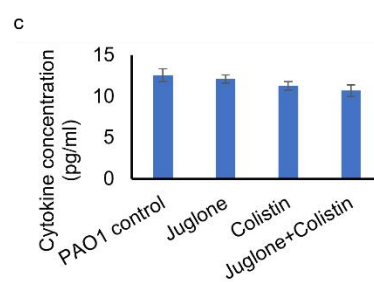
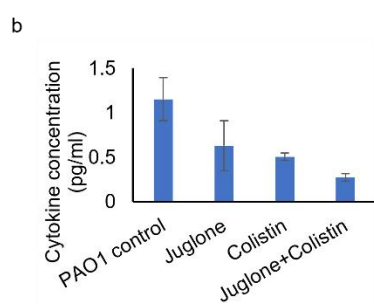
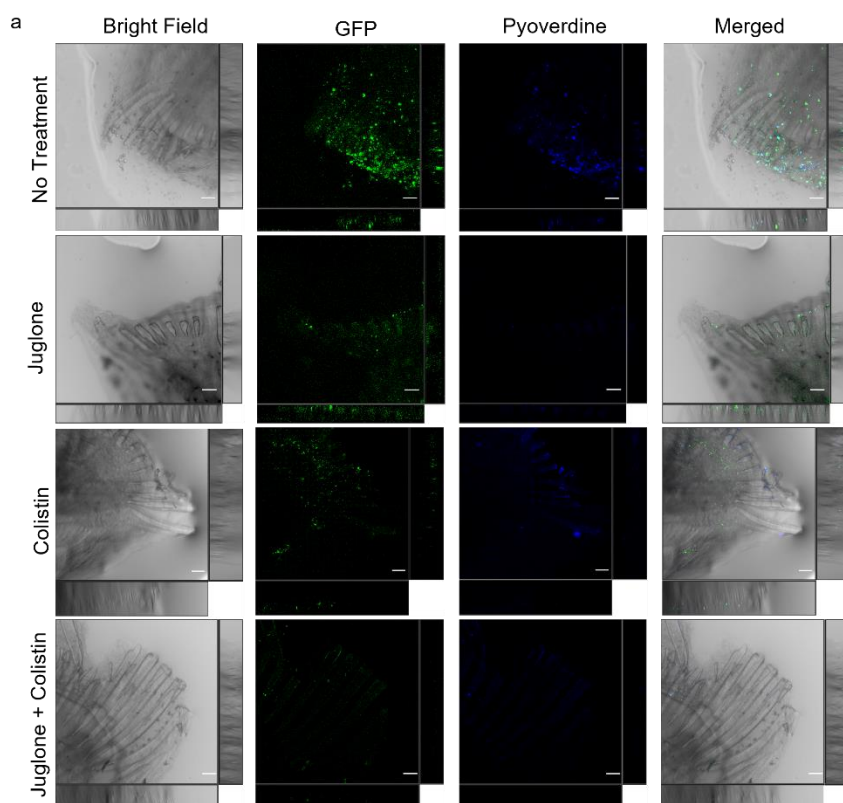


Figure 6.8. Synergistic treatment of colistin with Juglone could eliminate biofilm on Medaka fish. (a) Confocal images for biofilm on Medaka tails. (b) Cytokines 1L-6 in Medaka fish with PAO1 infection. (c) Cytokines Tnf- α in Medaka fish with PAO1 infection. (d) GFP biomass of biofilm with synergistic treatment. (e) Pyoverdine biomass of biofilm after synergistic. (f) CFU result of eliminating pre-formed biofilms. Experiments were performed in triplicate, at least 3 images were captured for each replicate, and the representative images were shown. The scale bar in all images was 50 μm . The averages and s.d. from triplicate experiments were shown. ***P < 0.001.

6.3 Discussion

The PQS is a crucial component of the QS system of *P. aeruginosa*. The inhibition of PQS has been shown to attenuate virulence and decrease the expression of various virulence genes, such as pyocyanin and biofilm formation ⁽²⁶⁴⁾. The development of inhibitors of the QS system can help inhibit biofilm formation by disrupting communication between microorganisms and their ability to coordinate gene expression. This can prevent the formation of protective substrates and make bacteria more susceptible to antimicrobial treatments. The PQS

QS system is an important regulator of bacteria and can influence biofilm production and virulence factors. PqsR is the receptor for PQS, and the mutation of PqsR would result in the absence of any production of AQS and pyocyanin ⁽²⁶⁵⁾. Therefore, it is feasible to develop anti-biofilm agents by disrupting the PQS QS via PqsR.

In this study, Juglone was screened by molecular docking. The *p_{pqsA}-gfp* was used as a biosensor, and it was determined that Juglone could inhibit the PQS system of wild type PAO1 and the production of PQS signaling molecules.

While determining whether Juglone inhibits the PQS biosynthesis or inhibits the response gene, the $\Delta pqsA$ mutant, which cannot synthesize PQS itself, was used, remaining with *p_{pqsA}-gfp* as the biosensor. It was found that Juglone could inhibit the fluorescent expression of *p_{pqsA}-gfp* even with the same concentration of exogenous PQS added. In contrast, the fluorescent expression of *pqsA-gfp* could not be inhibited by Juglone, as shown in the experiments using a $\Delta PqsR$ mutant that could not respond to PQS. This indicated that Juglone would not inhibit the synthesis of PQS but would inhibit PQS response genes via PqsR.

The molecular docking was performed further to identify the binding

sites for Juglone and PqsR, and it was determined that Juglone could bind to the LEU207 and LEU208 active sites of PqsR. Compared to previous studies, the most active natural ligand NHQ binding sites of PqsR also included LEU207 and LEU208 ⁽²¹³⁾. Therefore, it was confirmed that Juglone inhibits PQS signaling by binding to the active site of PqsR. Furthermore, as PQS could regulate the motility of *P. aeruginosa* ⁽⁴⁴⁾, Juglone caused a reduction in motility and less pyocyanin production in *P. aeruginosa* by inhibiting PQS signaling was also demonstrated in this study. Thus, Juglone could be a *pqs* inhibitor.

Juglone, as a *pqs* inhibitor, could inhibit biofilm formation. Several studies have shown that combining antibiotics with *pqs* inhibitors could improve treatment efficacy and reduce the development of antibiotic resistance, reducing the reliance on antibiotics alone as the primary therapy for bacterial infections ⁽²⁶⁶⁾. Colistin has been shown to have an improved ability to eliminate biofilm and improve therapeutic efficacy when combined with specific anti-biofilm agents, like vanillin ⁽²⁰⁶⁾.

Therefore, colistin was used in combination with Juglone to assess whether Juglone had the same effect. First, biofilm formation inhibition experiments were performed, and the results demonstrated that the

combination of Juglone and colistin could significantly inhibit biofilm formation compared to monotherapy; this was followed by biofilm elimination experiments, which showed that combining Juglone and colistin killed more biofilms. In this study, the concentration of Juglone and colistin were unable to completely inhibit or eliminate biofilms when used in monotherapy, but the effect was significantly improved when used in combination and almost completely inhibited or eliminated biofilms. This proved that Juglone had the potential to be used in combination with antibiotics for the treatment of *P. aeruginosa* infections.

Hence, a biofilm model in the BDR device was established to evaluate the effect of Juglone and colistin in eliminating biofilm in an *in vitro* 3D infection model. In this BDR device model, the effective concentrations of Juglone and colistin were initially tested, respectively. Notably, the effective concentration of the anti-biofilm agent is higher due to the more complex structure of the biofilm formed in the microwells than in the 2D surface. Subsequently, Juglone and colistin were mixed into different combinations of eight concentrations by a concentration gradient generator and then used to treat the biofilms formed within the primary chamber. The results showed that the ability of Juglone and

colistin to eliminate biofilms at certain concentration combinations was improved compared to higher concentrations of Juglone and colistin monotherapy; this suggested that a combination of low concentrations of colistin and Juglone could be effective as a high concentration of monotherapy in 3D *in vitro* infection model.

In order to evaluate the effect of the combination treatment on infection *in vivo*, the infection model of the tail of the medaka larvae was developed. The concentrations of Juglone and colistin used were lower than those used previously due to the small size of the larvae and the less biofilm in the tail. Results from experiments with *in vivo* models indicated that the combination therapy of Juglone and colistin was more effective in eliminating *P. aeruginosa* biofilms than the monotherapy. The combination therapy was also shown to effectively reduce the inflammatory response in fish by quantifying the inflammatory cytokines TNF- α and IL-6 in medaka larvae. These results suggested that the combination therapy of Juglone and the antibiotic colistin had potential as a treatment option for *P. aeruginosa* infections, particularly in the case of biofilm formation.

6.4 Conclusions

In conclusion, this study provided insights into the inhibitory effect of Juglone on the PQS system of *P. aeruginosa*. The findings suggested that Juglone inhibited the expression of PQS-responsive genes mediated by PqsR. Molecular docking studies supported the binding of Juglone to the active site of PqsR, further elucidating the mechanism of inhibition.

In addition, the combination of Juglone and the antibiotic colistin showed improved efficacy in inhibiting biofilm formation and eliminating biofilm compared to monotherapy. This combination therapy had shown potential in improving treatment efficacy and reducing the development of antibiotic resistance. In an *in vitro* three-dimensional infection model, the combination of low concentrations of Juglone and colistin showed comparable efficacy to high concentrations of monotherapy. In addition, in an *in vivo* infection model, the combination therapy effectively eliminated *P. aeruginosa* biofilms and reduced the inflammatory response.

These findings suggested that Juglone might be a potential therapeutic option for *P. aeruginosa* infections, particularly in biofilm formation

cases. In addition, using combination therapy with Juglone and antibiotics could provide an approach to treat *P. aeruginosa* infections, potentially reducing reliance on antibiotics monotherapy and helping to prevent the development of antibiotic resistance.

However, further studies and clinical trials are needed to validate the efficacy and safety of Juglone-based treatments in human patients. The information from this study may pave the way for developing new antimicrobial strategies against the PQS QS system of *P. aeruginosa*.

Chapter VII Conclusion and future works

7.1 Conclusions

Since the elimination of bacterial biofilm infections presents a significant challenge, in order to develop effective strategies and identify potential antibiofilm agents, it is crucial to utilize *in vitro* biofilm cultures. Accordingly, this study developed the BDR device to investigate the dispersal and recolonization of biofilms. While conventional static cultures can also form biofilms, they often show structural heterogeneity and limited nutrient availability. Microfluidic models, on the other hand, in addition to these advantages, the BDR microfluidic model of dynamic fluidic biofilm culture developed in this study also provides other advantages.

First, implementing the BDR microfluidic model provided an effective way to observe biofilm dispersal and recolonization using confocal microscopy. In the BDR device, the formation of mature 3D biofilms in microwells within 12 hrs was confirmed using c-di-GMP levels as an indicator; the time for biofilm formation was less than in other microfluidic models. Furthermore, the application of the BDR model combined with the time-lapse function of the confocal microscope

allowed the physiological process of biofilm dispersal to be observed, which revealed a correlation between the velocity of biofilm dispersal and the velocity of its self-mobility.

Another important advantage of the BDR device was its potential application in screening and testing anti-biofilm agents. The device included a concentration gradient generator, which facilitated mixing anti-biofilm agents into eight different concentrations to evaluate their efficacy against biofilms. The feasibility of the BDR device for screening potential anti-biofilm agents was successfully demonstrated through tobramycin.

Therefore, the BDR microfluidic model was a multifunctional platform for studying all aspects of biofilm biology, including formation, dispersal and recolonization. In addition, it provided a rapid and effective way of screening and evaluating potential anti-biofilm agents.

Hence, the BDR device was used in the experiments to evaluate the dispersal pattern of biofilms. Two methods, CID and EDA were compared in the BDR device to eliminate bacterial biofilms. The results found that both methods effectively reduced biofilm but showed different characteristics and mechanisms.

In this study, CID induced active biofilm reduction by lowering c-di-GMP levels through the activation of NO and *yhjH* genes. The released bacteria exhibited an exponential decay pattern, mainly appearing as individual entities. This active process relied on flagella and low c-di-GMP levels, as demonstrated by the results of the $\Delta bdlA$ mutant.

On the other hand, EDA biofilm disassembly was caused by cellulase and pectinase, which are enzymes that target the Psl polysaccharide in the biofilm. The initial disassembly caused the sudden release of many biofilm bacteria, forming small bacterial aggregates. Over time, the disassembly process could continue steadily, leading to a polynomial decay of biofilm biomass. In EDA-dominated biofilm disassembly, the enzymes that target biofilm matrix components played a key role.

It can be concluded that CID involved an active process motivated by bacteria to reduce c-di-GMP levels, whereas EDA relied on enzyme-mediated disassembly as a passive approach. The findings also highlighted the importance of intracellular c-di-GMP levels, flagella and biofilm matrix components in their respective mechanisms.

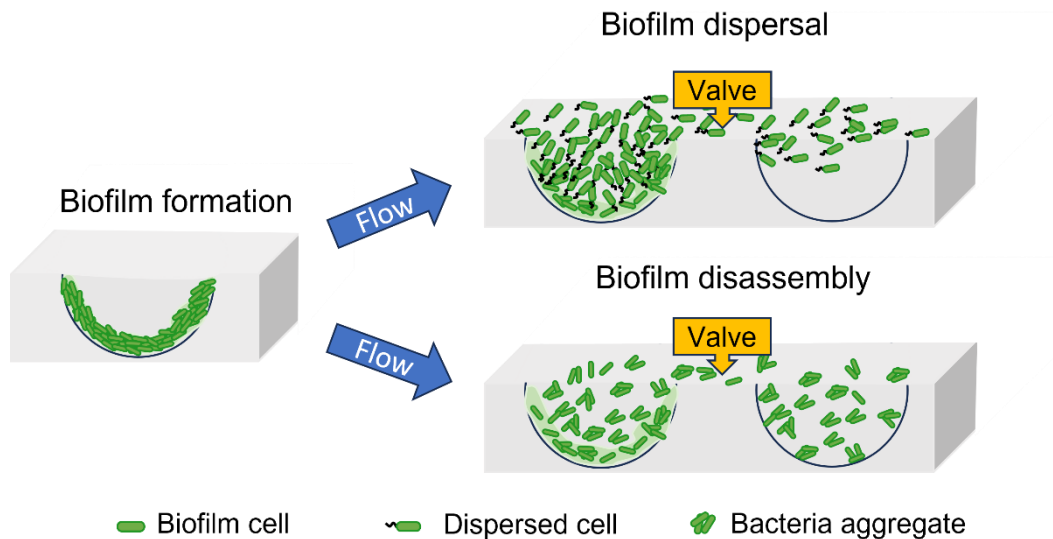


Figure 7.1 The differences between CID and EDA. CID-dominated biofilm dispersal activated bacteria dispersal by decreasing intracellular c-di-GMP levels, and released bacteria could not reattach on new surfaces within a short period. EDA-dominated biofilm disassembly directly disrupted the biofilm matrix, releasing small bacterial aggregates that could rapidly attach to new surfaces.

While both CID and EDA approaches demonstrated the ability to reduce biofilms, they operated through different mechanisms, releasing bacteria in different forms and relying on specific factors for their effectiveness, such as c-di-GMP and biofilm matrix polysaccharides. It was helpful to understand these differences to provide a more extensive insight into biofilm elimination strategies and may guide the development of targeted approaches to combat biofilm-associated

infections. In this study, both CID-dominated biofilm dispersal and EDA-dominated biofilm disassembly could reduce the biofilm biomass, but compared to EDA-dominated biofilm disassembly, biofilm bacteria released by CID dispersal were not able to recolonize and form biofilms within the short period. Thus, CID-induced biofilm dispersal effectively inhibited secondary infections caused by biofilm dispersal. However, the virulence of CID-released bacteria was higher than that of EDA-released biofilm bacteria, which would also cause secondary damage to the host. These findings emphasized potential targets and approaches for the development of anti-biofilm agents.

In addition, differences between the CID and EDA methods were also related to the recolonization behavior of the released biofilm bacteria. CID-released biofilm bacteria showed an inability to recolonize within 2 hrs due to their low c-di-GMP level, which resulted in a dispersed phenotype and hindered reattachment. This limited recolonization capacity positively impacted host cell survival and resulted in a reduction of bacteria in *C. elegans* with flow. In contrast, EDA-released biofilm bacteria demonstrated the ability to successfully recolonize and multiply within the microwells, although at lower velocity than physiologically dispersed bacteria. The EDA-released bacteria

aggregates could facilitate their settlement at the bottom of the microwells, leading to increased lethality in the HLF spheroid model and were easily captured by *C. elegans*. However, in the absence of flow, CID-released bacteria presented significantly higher lethality in HLF spheroid compared to EDA-released biofilm bacteria, which was consistent with previous studies that suggested that the bacteria with low c-di-GMP levels released through induced biofilm dispersal had higher virulence ⁽²⁶⁷⁾.

These findings were further validated in clinical isolates of *P. aeruginosa*, where both CID and EDA methods effectively reduce biofilms. In addition, the inability of CID-released bacteria to recolonize new surfaces within 2 hrs was also observed, which confirmed the feasibility of the CID and EDA biofilm elimination methods for other clinical isolates in different strains of *P. aeruginosa*.

These results emphasized the different characteristics of the CID, and EDA approaches regarding recolonization behavior. It was helpful to understand these differences to develop targeted biofilm elimination strategies that could potentially improve treatment efficacy for biofilm-

associated infections caused by *P. aeruginosa* and other bacterial pathogens.

Additionally, in order to develop novel anti-biofilm agents, Juglone was screened from a library of natural compounds, and its inhibition of the *P. aeruginosa* PQS system was studied. The results showed that Juglone effectively repressed the expression of PQS responsive genes by PqsR. Molecular docking studies provided further evidence confirming the binding of Juglone to the active site of PqsR and elucidating the mechanism of inhibition.

Also, the combination of Juglone and the antibiotic colistin showed more efficacy in inhibiting biofilm formation and eliminating biofilms compared to monotherapy. This combination therapy showed positive results in improving therapeutic efficacy and reducing the development of antibiotic resistance. In the *in vitro* 3D infection model formed in BDR device, the combination of low concentrations of Juglone and colistin showed comparable efficacy to high concentrations of monotherapy. In addition, in an *in vivo* medaka infection model, this combination therapy effectively eliminated *P. aeruginosa* biofilms and reduced the associated inflammatory response.

These findings suggested that Juglone could be a potential treatment option for *P. aeruginosa* infections, particularly those involving biofilm formation. Furthermore, combination therapy using Juglone and antibiotics was a viable approach to combat *P. aeruginosa* infections, potentially reducing dependence on antibiotics alone and helping to prevent antibiotic resistance.

In conclusion, the study focused on: [1]. The development of a continuous flow microfluidic device allowed rapid biofilm incubation, biofilm dispersal and recolonization of fresh areas to be observed; [2]. The evaluation of differences in bacterial population dynamics between CID and EDA when bacteria left the biofilm and recolonized fresh areas, using *P. aeruginosa* as a proof of concept; [3]. The evaluation of Juglone as a novel QS inhibitor and anti-biofilm agent to be used in combination with antibiotics to eliminate biofilms and reduce bacterial transmission. The results obtained in this study may provide potential prospects in the field of drug discovery for developing novel antimicrobial strategies, especially targeting the QS system of *P. aeruginosa*.

However, there are some limitations in this study. The study primarily relied on *in vitro* biofilm cultures using the BDR microfluidic device. While this system offers advantages in control and observation, it may not fully replicate the complex and dynamic nature of biofilm formation and recolonization in natural environments or *in vivo* settings. Therefore, further studies using more diverse and realistic models specific to the aims of the study are necessary.

Secondly, the study mainly focused on the biofilm dynamics and efficacy of CID and EDA about *P. aeruginosa*. Although the results showed that CID effectively eliminated the biofilm, previous studies have shown that the reduction of c-di-GMP leads to increased virulence and resistance to colistin ^(93, 204). Colistin is often used as the most effective antibiotic against multi-resistant bacteria ⁽²⁶⁸⁾; thus, it is possible that bacteria released from CID-dominated biofilm dispersal may require a higher concentration of antibiotics to kill them. The applicability of these findings to other bacterial species or biofilm-related infections remains to be explored. Different bacteria may exhibit variations in their biofilm behavior, mechanisms of dispersal, and response to antibiofilm agents. For example, *P. aeruginosa* and *Staphylococcus aureus* are two common biofilm-forming bacteria, but

they have different biofilm formation and dispersal mechanisms. *P. aeruginosa* produces a number of extracellular matrix components, including alginate and Psl, that help to stabilize the biofilm and promote cell-cell interactions ⁽²⁶⁹⁾. Meanwhile, *Staphylococcus aureus* produces a protein called Bap that helps to mediate biofilm formation and contributes to biofilm stability ⁽²⁷⁰⁾. Therefore, it is important to conduct additional research encompassing a wider range of bacterial pathogens to gain a more comprehensive understanding.

Besides, the EDA-dominated biofilm disassembly in this study tested only two enzymes, cellulase and pectinase, and different enzymes may have different results. It was found that treatment of *P. aeruginosa* biofilms with DNase I and dispersin B resulted in a significant reduction in biofilm biomass ^(271, 272). DNase I targets extracellular DNA within biofilms ⁽²⁷³⁾, while dispersin B targets EPS that help to stabilize the biofilm matrix ⁽²⁷⁴⁾. Therefore, these enzymes targeting different components in the biofilm matrix can also be tested in the BDR device, and the difference between enzymes targeting different components of the biofilm matrix can be observed.

Additionally, while the study demonstrated the potential of Juglone as an anti-biofilm agent and its combinational effect with antibiotics, further research is required to fully elucidate the mechanisms of action, optimize dosages, and assess potential side effects in animals. Furthermore, factors such as host immune responses, biofilm localization in different tissues, and interactions with other microbial species can influence the effectiveness of anti-biofilm strategies. Therefore, further investigations using animal models and clinical trials are needed to validate the safety and efficacy of these approaches.

In summary, while the findings presented in this study provide valuable insights into biofilm dynamics and the potential of antibiofilm agents, some limitations should be considered. Further research using diverse models and clinical studies is necessary to validate and extend these findings, ensuring their applicability in real-world scenarios and developing effective strategies against biofilm-associated infections.

7.2 Future works

The BDR microfluidic model, combined with the CID and EDA approaches, has shown promise in providing insights into different strategies for eliminating biofilms. The gradient generator is an

important part of the BDR model because it generates concentration gradients testing compounds' effectiveness in inhibiting and killing biofilms. Therefore, the concentration gradient generator should be optimized to improve the precision and accuracy of the concentration gradient, such as adjusting the flow rate, temperature and time of the compounds.

However, further investigation is required to evaluate the effectiveness of these approaches *in vivo*. While the BDR microfluidic device has advantages in biofilm research, future studies should aim to validate findings in more realistic models, such as animal models and clinical trials.

Animal models can provide valuable insights into the effectiveness of biofilm elimination strategies ⁽²⁷⁵⁾. They provide a more complex host environment than *in vitro* models and can help assess the safety and feasibility of clinical applications ⁽²⁷⁶⁾. The effectiveness of CID and EDA biofilm elimination strategies can therefore be assessed in animal models.

First, biofilm infection can be established in an animal model. The animal model should be infected with biofilm-forming pathogens using

appropriate infection techniques. The biofilm elimination strategies are then implemented in the infected animal model using appropriate dosing and delivery methods, including intravenous injection, oral gavage, subcutaneous injection, or intraperitoneal injection ⁽²⁷⁷⁾. Different delivery methods can influence the efficacy, specificity, and toxicity of treatments and are often chosen based on the experimental design and the type and size of the animal model being used. Bacteria are then detected and quantified by several assays, such as CFU assay, quantitative real-time PCR (qPCR), immunofluorescence microscopy and flow cytometry ⁽²⁷⁸⁾.

In addition, other antibiofilm approaches should be considered. For example, PDT may be a promising therapy for treating biofilm infections, especially when combined with other therapies ⁽²⁷⁹⁾. This method has been shown to effectively combat various types of bacteria and biofilm structures, including those formed by bacteria in implanted materials or animal models ⁽²⁸⁰⁾. PDT is a non-invasive treatment method involving a photosensitizer and light of a specific wavelength to induce localized damage to bacterial cell components and eliminate the bacteria biofilms ⁽²⁸¹⁾. In PDT, the photosensitizer is used to generate reactive oxygen species (ROS) that can damage and kill bacterial cells

⁽²⁸²⁾. The photosensitizer is activated by exposure to light of a specific wavelength, transferring energy to oxygen molecules in surrounding tissues, generating ROS ⁽²⁸²⁾. Since biofilms are complex ecosystems that may require combination therapy for effective elimination, studying the synergistic effects of PDT and other antibacterial agents, such as antibiotics or enzymes, can help develop more effective combination therapies for biofilm infections. Therefore, the potential synergistic effects of PDT with CID and EDA can also be investigated.

Phages are expected to be used as a biocontrol measure against bacterial infections, including biofilms ⁽²⁸³⁾. They are naturally occurring viruses that target and kill bacteria ⁽²⁸⁴⁾. As a result, they are emerging as a promising alternative to traditional antibiotics ⁽²⁸⁴⁾. It has been found that the use of phage cocktails containing six different phages against strains of *S. aureus*, *P. aeruginosa* and *E. coli* isolated from clinical specimens was effective in reducing and preventing biofilm formation ^(285, 286). Therefore, it is necessary to investigate the phage application in preventing biofilm formation, including evaluating their effectiveness. In addition to exploring different phage cocktails, research is needed to determine the optimal phage delivery method and dosage. This research will include evaluating the effectiveness of

different phage delivery methods, such as topical application, systemic injection, or other routes of administration ⁽²⁸⁷⁾. There is also a need to determine the optimal dose of phages to ensure they effectively prevent biofilm formation without causing adverse effects.

In addition, this research could be extended to eliminating biofilms by physical disruption, such as high-frequency sonication, which has been used for the physical disruption of biofilms ⁽²⁸⁸⁾. However, the optimal therapeutic parameters of sonication (e.g., frequency and intensity) can be optimized to determine the most effective conditions for disrupting different biofilms. Besides, physical disruption can be combined with other treatments (e.g., antibiotics or chemicals) to enhance biofilm eradication.

Furthermore, it has been shown that macrophages can induce biofilm dispersal through their phagocytic activity ⁽²⁸⁹⁾. By consuming bacteria within the biofilm, macrophages can create voids within the structure of the biofilm, which can lead to its breakdown and dispersal ⁽⁶⁶⁾. Additionally, macrophages can produce enzymes and other factors that can degrade the extracellular matrix that holds the biofilm together, further contributing to its disintegration ⁽²⁹⁰⁾. Also, macrophages can

induce biofilms to end their life cycle early ⁽⁹³⁾. However, the mechanisms between the macrophages and biofilms have not been investigated. Therefore, future work could also include exploring the potential mechanisms by which macrophages eliminate biofilms and induce their dispersal, in order to further investigate their role in these processes. The interaction between macrophages and biofilms can first be explored by performing co-culture experiments and observing the dynamic interactions between them. The molecular pathways and enzymes involved in macrophage-induced biofilm dispersal are then identified by gene expression analysis and ELISA assays ⁽¹⁴²⁾. For example, the transcriptome analysis of macrophages exposed to biofilms can identify differentially expressed genes and pathways involved in biofilm dispersal ⁽²⁹¹⁾; ELISA assays can determine the activity levels of various enzymes in macrophages after exposure to biofilms, like protease, which are associated with biofilm dispersal ^(292, 293).

Reference list (citation style: Vancouver)

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