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Chemical Control and Understanding of Horizontal Gene Transfers, Drug-Resistance Development, and Filament and Biofilm Formation

Yuchen Jin

Abstract

Biofilms formed by microbes on surfaces are the sources for persistent infectious diseases and environmental problems. The mechanism and details of how antibiotics promote biofilm formation is largely unknown. For instance, it not clear what stages of biofilm growth are promoted to proceed faster than without antibiotics, what phenotypes of bacteria form in an antibiotic-promoted biofilm, and what different biofilm compositions and structures are caused by the presence of antibiotics. Among other effects, antibiotics can cause bacteria to form filaments of living bacteria. Here, we conduct a real-time study of the adherence of bacteria and antibiotic-induced filamentous bacteria on surfaces and characterize the kinetics of surface adherence of these two forms of bacteria. Studying the effect of different surfaces on promoting filamentous bacteria's adherence on surfaces, we characterize an unexpected correlation between the stage of bacterial growth and the formation and growth of filamentous bacteria on surfaces. Based on these results, we outline the lifestyle of filamentous bacteria and a mechanism by which antibiotics promote biofilm formation.

The drug resistance of bacteria is becoming more severe since antibiotics are first discovered. The development of drug-resistance among bacteria involves spreading and drug-resistant gene "horizontally" between bacteria. These horizontal gene transfers impact the gene composition and evolution of the bacteria, and facilitate the transfer of antibiotic resistance genes. At the molecular level, all three major mechanisms of HGTs, transformation, conjugation and transduction, involve type IV pili appendages on bacterial surfaces. Here, the three mechanisms of horizontal gene transfer are studied. Saturated farnesol derivatives show inhibition effect on tetracycline-enhanced plasmid transformation, ciprofloxacin-enhances PAPI-1 transduction, and PAPI-1 conjugation.

Further study of ciprofloxacin-resistance development in *P. aeruginosa* is conducted by using serial passage assay. The inhibited development of MIC of ciprofloxacin suggest that saturated farnesol derivative inhibits the development of resistance to ciprofloxacin in *P. aeruginosa*.

This thesis also describes the mechanism of pili inhibition by saturated farnesol derivatives. The pili-mediated bacteriophage adsorption is studied, and the results demonstrate that saturated farnesol derivative could cause pili retraction, leading to inhibition of bacteriophage adsorption. More evidence was obtained from other colleagues in Dr. Luk's lab to support the small molecules binding to pili. The MALDI-MS done by Hewen Zheng suggests that saturated farnesol derivative covalently binds to pili. Hewen also performed the alkaline buffer extraction experiment to demonstrate that PAO1 culturing with saturated farnesol derivative resulted in decrease in pili protein expression. Together with the bacteriophage adsorption results, we conclude that saturated farnesol derivative binding to pili cause pili retraction.

Chemical Control and Understanding of Horizontal Gene Transfers, Drug-Resistance Development, and Filament and Biofilm Formation

by

Yuchen Jin

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This thesis is dedicated to my grandparents

who never saw this adventure.

List of Abbreviations

BLI: Biolayer interferometry

Cb^R: Carbenicillin resistance

CdG: Cyclic di-GMP

CFU: Colony-forming unit

Cip: Ciprofloxacin

EPS: Extracellular polymeric substances

Gm^R: Gentamicin resistance

HGT: Horizontal gene transfer

IC50: Half-maximal inhibitory concentrationMIC: Minimal inhibitory concentration

LB: Lysogeny broth

PAPI-1/2: Pseudomonas aeruginosa pathogenicity island ½

PBP: Penicillin binding protein

PFU: Plaque-forming unit

SCV: Small colony variant

Tc^R: Tetracycline resistance

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Chapter 1: Background and Significance

1.1 Antibiotics induce resilient phenotypes

Infectious diseases caused high mortality, leading average life expectancy to be less than 50 years. In 1928, Sir Alexander Fleming discovered penicillin as the first antibiotic in human history. From then on, the revolution of antibiotics begins. Antimicrobial agents are medicines that kill bacteria or keep them from reproducing. Antibiotics could be classified into five groups by mechanism: (1) Inhibition of cell wall synthesis;[1] (2) Inhibition of cell membrane function;[2] (3) Inhibition of protein synthesis;[3] (4) Interference with nucleic acid synthesis;[4] (5) Inhibition of metabolic pathway.[5] For example, beta-lactam such as aztreonam, inhibits proteins (collectively called penicillin binding protein, PBP) that synthesize the crosslinker for bacteria cell wall; aminoglycoside, such as tobramycin, binds to bacterial 30S and 50S ribosome and inhibits protein synthesis; fluoroquinolone, such as ciprofloxacin, inhibits of DNA gyrase to inhibit DNA replication; polymyxin, such as colistin, binds to lipid A of cell membrane of bacteria and causes bacterial death; sulfonamides such as gantrisin, inhibits metabolic pathway.

However, in his Nobel lecture[6] in 1945, Dr. Alexander Fleming, who discover the first antibiotic penicillin in 1928, warned on development of drug resistance by microbes: “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.” Over the years, the healthcare has witnessed such drug resistance at an alarming rate.

What is perhaps more significant is that, at sub-lethal concentrations in laboratory, which is difficult to define in any clinical setting, antibiotics have been discovered to rapidly develop many resilient phenotypes over short period of time, hours to days. These phenotypes include including biofilm,[7-19] filament,[20-26] small colony variants (SCV),[27-29] swarming motility[30] and bacteriophage production.[31-36] All these phenotypes exhibit different levels of drug tolerance, for which the bacteria require a long period time or a higher dose to kill than the wild type bacteria; and drug persistence, for which the bacteria do not get killed and do not grow, but when the antibiotics are removed, the bacteria regrow to a susceptible population with a small persistent population. All these resilient phenotypes are found in both laboratory conditions, and clinical settings, and tolerant bacteria have been demonstrated to be precursor strains for fully stable resistant bacteria that can grow under the treatment of antibiotics.

Biofilms formed by microbes on surfaces are sources of persistent infectious diseases and environmental problems.[7-19] The antibiotics that are used to treat bacterial problems actually often promote the formation of biofilms: sublethal amounts of antibiotics cause significantly larger amounts of biofilm than those formed in the absence of antibiotics, sometimes up to two fold in terms of biofilm mass.[13, 16, 17, 19] This particularly relevant scenario was found in the use of antibiotics to treat biofilms in human hosts, such as in the lungs of cystic fibrosis (CF) patients.[10, 11, 14] These biofilms are extremely difficult to eradicate completely. The mechanisms and details of how antibiotics promote biofilm formation are largely unknown. For instance, it not clear what stages of biofilm growth are stimulated to proceed more rapidly than in the absence of antibiotics, what phenotypes of bacteria form in an antibiotic-promoted biofilm, and what

different biofilm compositions and structures are caused by the presence of antibiotics. For example, Sub-inhibitory concentration of aminoglycosides, such as Tobramycin, could promote 2-fold increase in biofilm mass in wild type PAO1 on abiotic surfaces with live bacteria in it.[37] Also, Tobramycin was reported to promote biofilm by increasing extracellular DNA (eDNA) release (Figure 1.1).[38] Polymyxin promotes biofilm by expression of genes that encode efflux pumps and biofilm-related proteins.[39] Some other antibiotics could trigger the increase of bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG) levels in bacteria, leading to biofilm promotion.[40-42]

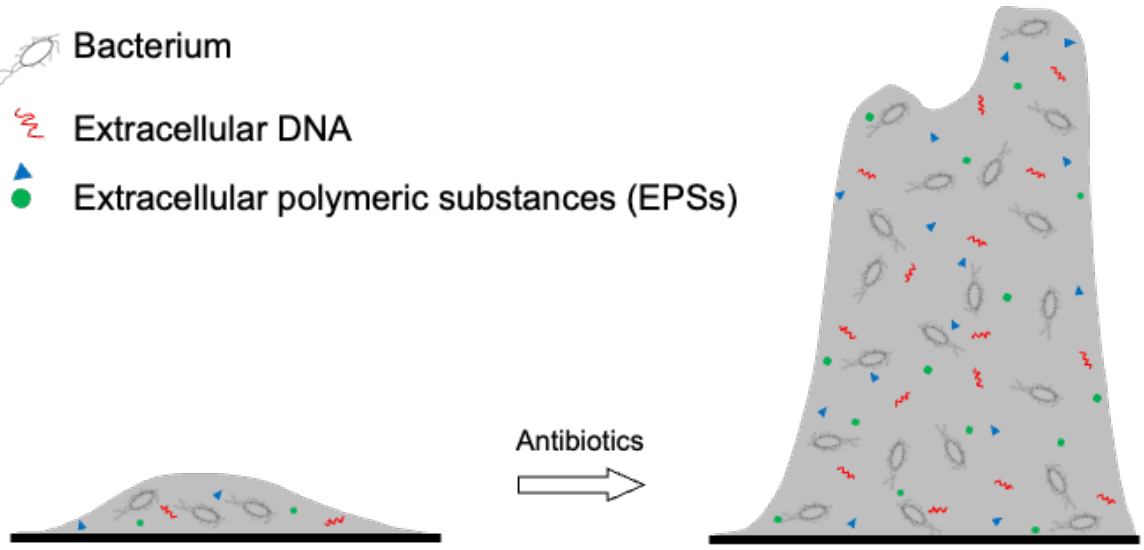


Figure 1.1 Scheme of antibiotics promote biofilm formation

Biofilms formed by microbes on surfaces are the sources for persistent infectious diseases and environmental problems.[9, 13, 15, 19, 43-51] A particular relevant scenario is the use of antibiotics to treat biofilm in human hosts,[47-49] such as biofilms in the lungs of cystic fibrosis (CF) patients.[47-49] These biofilms are extremely difficult to completely eradicate.[47-49] The mechanism and details of how antibiotics promote biofilm formation is largely unknown. For instance, it not clear what stages of biofilm growth are promoted to proceed

faster than without antibiotics, what phenotypes of bacteria form in an antibiotic-promoted biofilm, and what different biofilm compositions and structures are caused by the presence of antibiotics. Among other effects, antibiotics can cause bacteria to form filaments of living bacteria.[22, 25, 52-56] Filamentous bacteria were first isolated and identified from sludge in wastewater.[57] The reason bacteria form filaments was unclear until scientists found that β-lactam antibiotics could induce filamentous bacteria. β-lactam target multiple penicillin-binding proteins (PBPs) and that PBP3 is responsible for cell division.[58] The binding of β-lactam to PBP3 leads to inhibition of bacterial division and to triggering of filament formation.[58] Thus, antibiotics can cause bacteria to form filaments, consisting of living bacteria.[20-26] The impact of filamentous bacteria on biofilm formation is not clear. The effects of surface chemistry on the formation and the growth of filaments are also not well understood. Understanding these factors is likely to be important for controlling biofilm formation[59-65] and for understanding how some antibiotics promote biofilm formation.[17, 19]

Small colony variant (SCV) is a colony morphology of bacteria that has significantly slower growth rate than native bacteria. SCVs were first described as “dwarf colonies” by Kolle and Hetsch in 1906.[66] From then on, more and more SCVs were observed in different bacterial species. SCVs are characterized by their small colony size, slow growth rate and more resistant to antibiotics.[29, 67-71] SCVs were mostly found and discussed in *Staphylococcus aureus*. The first clinical study on *S. aureus* SCVs were conducted by Richard A. Proctor in 1994.[72] Later in 1995, Richard A. Proctor reported their studies on SCVs that were cultured from patients with persistent and relapsing infections.[73] They found that the SCV isolates grew very slowly in culture media and could

be reverted to normal growth with the supplement of nutrient (Figure 1.2). Those SCVs were also reported to be resistant to aminoglycosides.[73]

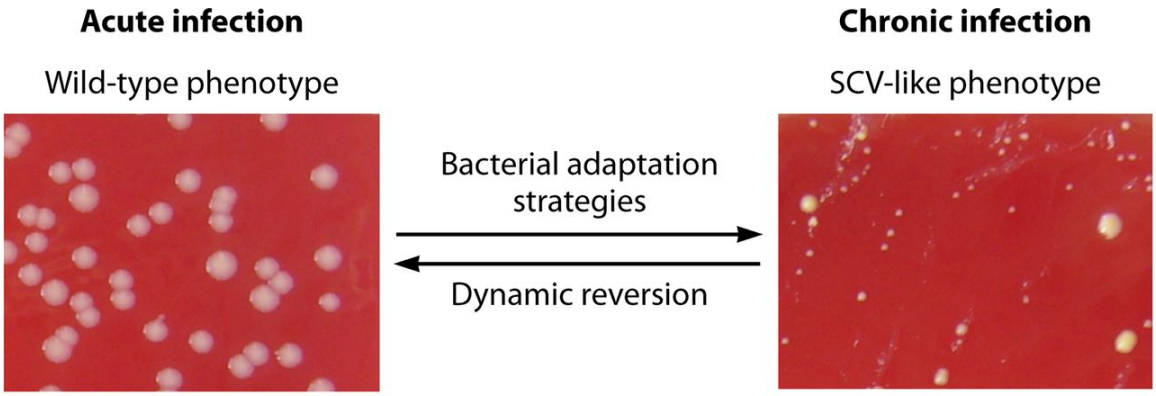


Figure 1.2 Scheme of dynamic SCV development in the course from acute to chronic infection (Citation: *Kahl, B.C., Becker, K. and Löffler, B., 2016. Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. Clinical microbiology reviews, 29(2), pp.401-427.*)

Aminoglycoside antibiotics are knownto induce SCVs.[29, 68, 69, 71, 74] SCVs are also commonly found in biofilms.[67, 69-71, 73] The antibiotic-induced biofilm provides a shelter for slow-growing SCVs that are dormant and highly resistant to antibiotics. For example, rugose small colony variants (RSCVs), the clinical isolates from chronic infections, were found in carbenicillin-promoted *Pseudomonas aeruginosa* biofilm.[71] They found that the RSCV overproduced exopolysaccharide, leading to antibiotic tolerance.[71] The number of SCVs is also reported to increases with the age of biofilm.[69] However, the mechanism of how antibiotic promotes SCV is still unclear.

Swarming motility is one of several motilities conducted by bacteria, others include twitching, swimming and gliding. Bacterial swarming was believed to be powered by rotating flagella (Figure 1.3). However, more studies revealed that pili also played an

important in bacterial swarming.[75, 76] Swarming generally requires an nutrient-rich media that promotes the high growth rate. The softness of the surface such as agar is also critical for enabling swarming. For example, researchers usually prepare agar concentration at 0.5% or less to observe the swarming, while 1.5% agar could solidify the agar surface to inhibit the swarming. Bacteria were reported to synthesize and secret surfactants to spread over surfaces during swarming. For example, *Pseudomonas aeruginosa* synthesizes rhamnolipids to contribute to their swarming motility.[77-79] Antibiotics promoting swarming has been reported in *P. aeruginosa*.[30] The subinhibitory concentration of tobramycin could induce swarming of P. *aeruginosa.*[30]

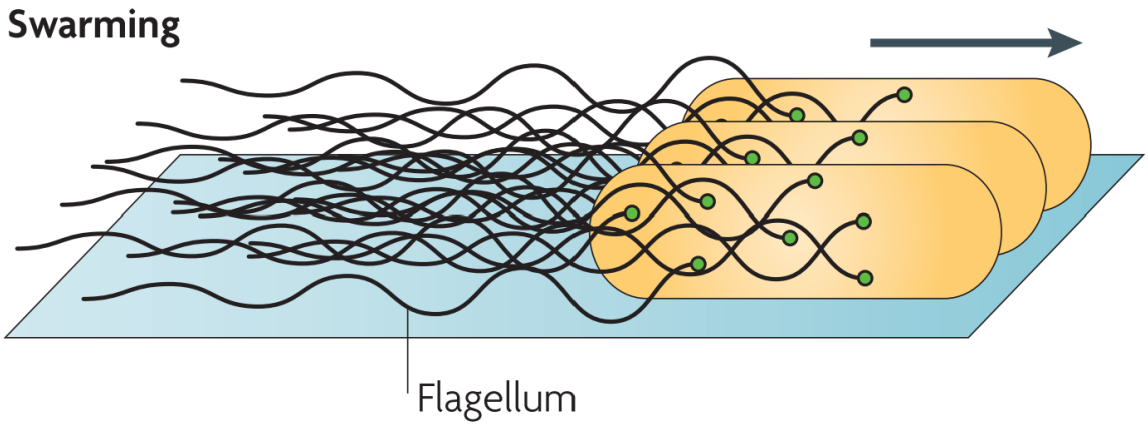


Figure 1.3 Bacterial swarming powered by rotating flagella (Citation: *Kearns, D.B., 2010. A field guide to bacterial swarming motility. Nature Reviews Microbiology, 8(9), pp.634-644.*)

Bacteriophage (or phage) is virus that specifically infect bacteria. When phages replicate in bacteria, lysis of the hosting bacteria can occur. There are estimates of about 10^{31} phages on our planet, classified and reported with new ones by Bacterial and Archaeal Subcommittee (BAVS) within the International Committee on Taxonomy of Viruses (ICTV).[80] In 2021, BAVS has updated and reported 15 families, 31 subfamilies,

734 genera and 1845 species of phages.[81] The well-known families include *Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Inoviridae* (Figure 1.4).[82] There are a variety of phage classifications. For example, phages can be classified based on their genomes that are known to infect *Vibrionaceae*.[83] Phages were also classified based on infection strategies: (1) lytic and non-temperate, (2) chronic and non-temperate, (3) lytic and temperate or (4) chronic and temperate.[84]

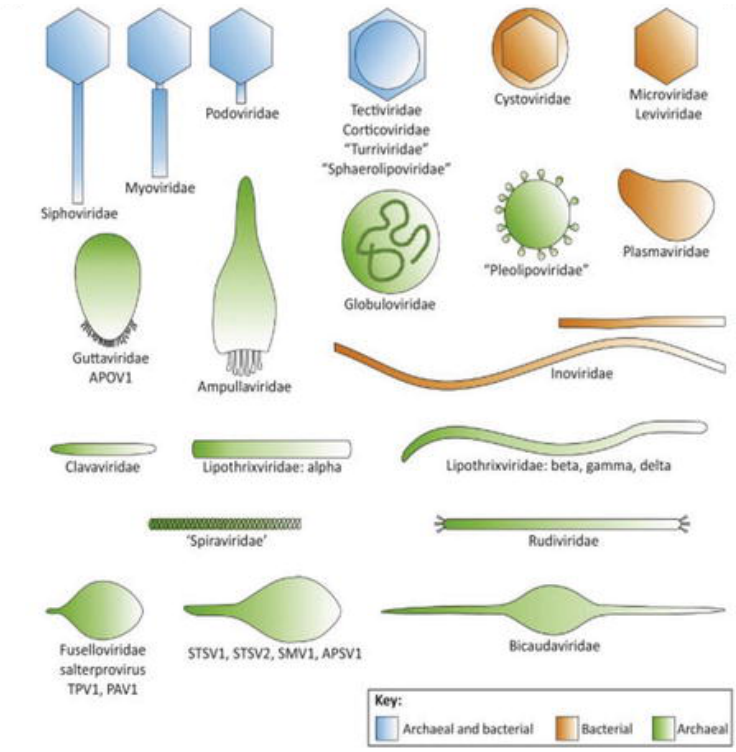


Figure 1.4 Bacteriophage morphotypes (Citation: *White, H.E. and Orlova, E.V., 2019. Bacteriophages: their structural organisation and function. Bacteriophages-Perspectives and Future.*)

In summary, cell wall-related and cell membrane-related antibiotics can induce the filamentous bacteria and contribute to antibiotic-promoted biofilm formation. The protein synthesis-inhibition antibiotics, such as aminoglycoside, can induce small colony variants (SCVs) and promote bacterial swarming motility. The antibiotics (i.e. fluoroquinolone) that

affect DNA synthesis can promote prophage to convert to phage. All current antibiotics have been reported to promote biofilm formation (Figure 1.5).

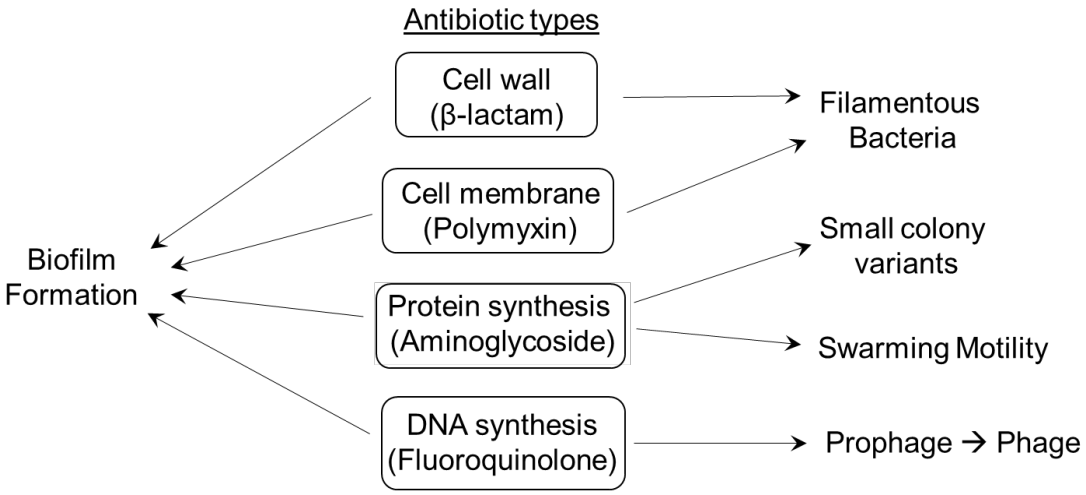
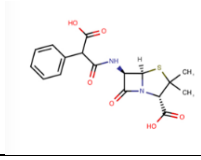
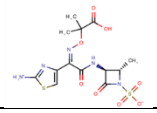
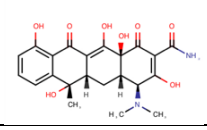
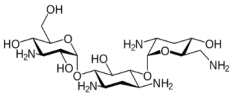
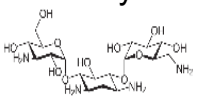
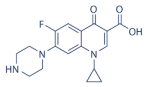
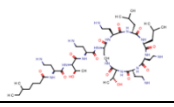


Figure 1.5 Map of antibiotics and promoted bacterial phenotypes that increases their resilience (tolerance, persistence, or resistance). Arrows indicate promotion of the phenotype.

However, our understandings are limited and at its early stage for how antibiotics promote these resilient phenotypes. The receptors for causing resilient phenotypes, unlike killing, are largely unknown (Table 1). β-lactam is well-known to promote biofilm formation among most bacterial species.[7, 8, 14, 17, 19, 85-87] Scientists has revealed that β-lactam can promote biofilm formation by inducing the production of extracellular polymeric substances (EPS),[86] extracellular DNA (ecDNA),[87] inhibiting quorum sensing.[85] Polymyxin also promotes biofilm formation. The receptors for antibiotics promoting these resilient phenotypes is completely unknown. For the formation of filamentous bacteria, binding of β-lactam to penicillin-binding protein (PBP3), without killing, is shown to cause the inability of bacterial division and the formation of filaments. Penicillin-binding proteins (PBPs) are a group of proteins that bind to penicillin or other β-lactams. There are a

variety of PBPs, but only PBP3 is the receptor for filamentous bacteria induction. Tetracycline can promote horizontal gene transfer (HGT), for which, the receptor is still unknown (Table 1). Aminoglycosides promote several resilient phenotypes, including biofilm formation, swarming motility, and SCV formation. Tobramycin promotes these phenotypes in *P. aeruginosa* apparently by regulating the bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG), a global bacterial second messenger (the comprehensive introduction is included in section 1.2). The aminoglycoside response regulator (Arr) gene was identified to mediate the biofilm inducibility (Table 1).[88] Fluoroquinolones were reported to induce lysogenic prophage to exit the chromosome and convert to lytic phage.[89-93] This process directly promotes the transduction mechanism of horizontal gene transfer (HGT), by which antibiotic-resistant genes are spread among bacterial strains. The receptor for prophage induction is also an unknown (Table 1).

Table 1.1 Different classes of antibiotics and the receptors for their promoted resilient phenotypes

Class	Antibiotics	*Known resilient phenotype induced by antibiotics	**Receptor for the resilient phenotype
β-lactam	Carbenicillin 	1) Promote biofilm formation 2) Filament formation	1) biofilm: unknown 2) filament: Penicillin-binding protein (PBP3).
	Aztreonam 		
Tetracyclines	Tetracycline 	Promote horizontal gene transfer (HGT)	Unknown
Aminoglycoside	Tobramycin 	Promote (1) biofilm formation, (2) swarming motility, (3) small colony variants, (4) drug tolerant and persistent populations in biofilm	Arr protein [94, 95]
	Kanamycin 	Promote biofilm formation	Presumably Arr.
Fluoroquinolones	Ciprofloxacin 	Promote biofilm, prophage to phage particles transformation [89-93]	Unknown
Polymyxin	Colistin 	Promote biofilm formation	Unknown

1.2 Resilient phenotypes are correlated with the global secondary messenger, bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG), and the physical conditions the bacteria are in

Bis-(3'-5')-cyclic dimeric guanosine monophosphate or Cyclic di-GMP (cdG) is a global bacterial second messenger that mediates a wide range of bacterial response to external cues and stresses, which are regarded as first messengers.[40-42] Those bacterial responses include biofilm formation,[40, 96] enhanced motilities (swarming, twitching),[30] small colony variants (SCVs) induction[67, 70, 71, 97] and overproduction of metabolite, such as pyocyanin and rhamnolipids (Figure 1.6).[98] These diverse resilient phenotypes are often controlled by or correlated to the high and low levels of the Cyclic di-GMP. The high levels of Cyclic di-GMP in bacteria can be induced by antibiotics treatment, leading to higher antibiotic tolerance in biofilm and promoted SCVs. The low levels of Cyclic di-GMP also show virulence including the promoted motility[30] and overproduction of virulence factors such as pyocyanin and rhamnolipids.[98] Interestingly, both high and low levels of Cyclic di-GMP phenotypes can be promoted by antibiotics at in bacteria under different environmental conditions. For example, sub-lethal dose of tobramycin promotes high levels Cyclic di-GMP phenotypes such as biofilm formation on hard surface. In contrast, sub-lethal dose of tobramycin also promotes swarming motility on soft surface. This phenomenon may indicate that the antibiotic-induced resilient phenotypes are not just chemical condition dependent. It may also be affected by physical conditions, such as the surfaces.

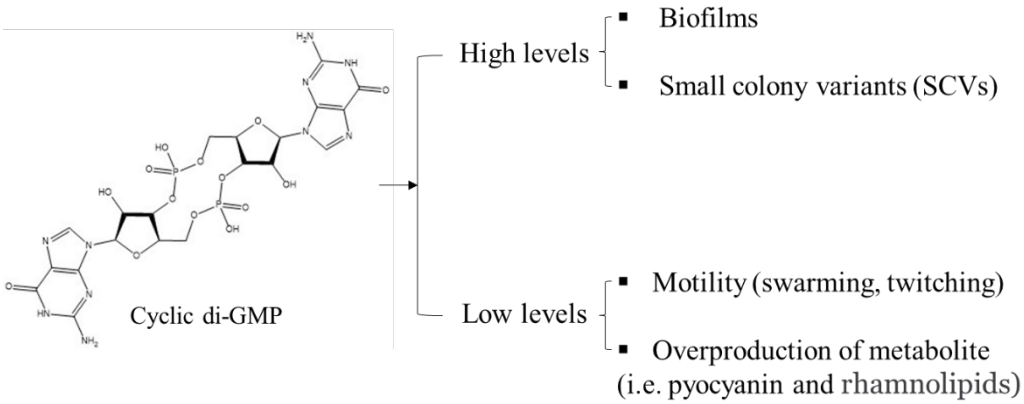


Figure 1.6 Bacteria use Bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG) to mediate response to a wide range of external cues and stresses including biofilm formation, SCV induction, swarming motility and overproduction of metabolite, such as pyocyanin and rhamnolipids.

1.3 Prophage and phage

Phage is a virus that specifically infects bacteria (comprehensive description is included in the section 1.1). Prophage is a phage genome that has been integrated into the host bacterial chromosome. There are two cycles of viral reproduction existing in host bacteria: lytic and lysogenic cycles.[31-36] In lytic cycle, the viral DNA injected by phage is replicating repeatedly using host protein machinery to make viral components, followed by biosynthesis of proteins to assemble new virions. Once the virions have matured, the special viral protein starts to dissolve cell wall to burst the cell to cause host lysis. In lysogenic cycle, the viral DNA is integrated within the host DNA, is not expressed repeatedly, and does not lead to lysis of bacteria. Also, during the usual prokaryotic reproduction, the integrated viral DNA replicates with host DNA to produce the prophage to spread their viral gene materials to daughter cells without causing lysis. However,

lysogenic prophage can exit the chromosome and convert to lytic phage under certain conditions, such as exposing to DNA-damaging antibiotics.[89-93]

1.4 Horizontal gene transfer and antibiotic promotion

Horizontal gene transfer (HGT) is one of the principal routes for bacteria to acquire antibiotic resistance. Bacteria can acquire and exchange DNA materials from and with their environments and other bacterial species rather effectively.[99-116] These HGT impact the gene composition and evolution of the bacteria [115, 117]as well as facilitate the transfer of antibiotic resistance genes.[113, 114, 116] Some mobile genetic elements such as plasmid and *Pseudomonas aeruginosa* pathogenicity island 1 (PAPI-1) can be transferred between bacteria via different mechanisms, such as transformation,[102] transduction[112] and conjugation[106, 107] All those three major mechanisms of HGTs involve type IV pili appendages on *P. aeruginosa* surfaces. While there are multiple ways for a bacterium to uptake DNA materials from its environment,[113] pili appendages have been shown to directly bind and retreat the DNAs close to the bacterial surfaces, and enable the intake of DNAs.[102]

Antibiotics were reported to enhance HGT via different mechanisms.[74, 118-122] For example, streptomycin could enhance transformation in Streptococcus pneumoniae by increasing the fraction of competent cells;[119] β-lactam antibiotics increase DNA uptake by bacteria through inducing cell-wall-deficient forms;[120] tetracycline could enhance conjugation by inducing plasmid-like nonreplicating Bacteroides units (NBUs) that are transferable between different bacteria species.[122] Ciprofloxacin is effective to treat variety of bacterial infections, including *Pseudomonas* infection in cystic fibrosis

patients.[123-125] However, ciprofloxacin-resistance has been observed in *Pseudomonas aeruginosa* isolates both *in vivo* and *in vitro*.[126-128] Also, ciprofloxacin caused the formation of phage-like particles, and it induce the prophage formation.[89-93]

1.5 Type IV pili mediate all three horizontal gene transfer mechanisms: transformation, conjugation and transduction

Transformation is one of the routes for bacterial HGT. Bacteria could take up and express extracellular genetic material from surroundings. Scientists have observed the DNA uptake (transformation) in *Vibrio cholerae* by type IV pili.[102] Pili were described as "gatekeepers" to allow DNA to cross the outer membrane, and the bound DNA could be brought to cell surface with pili retraction.[102] However, the mechanism of how DNA enters the cell is still unclear.

Transduction is mediated by bacteriophage for gene transfer.[109, 111, 129-131] Bacteriophage could infect host bacteria and either incorporate their viral genome into the host genome to replicate as part of the host bacteria, or multiply inside the host bacteria then release new phages into the surroundings. Usually these host bacteria carry plasmids that frequently encode antibiotic resistance.[109, 111] The bacteriophage could either act as a vector to carry genetic elements from donor cells to recipient cells,[130] or increases the concentration of plasmid released from host cell upon lysis.[131]

Conjugation involves the donor strains of bacteria extending their pili to make direct contact with the recipient strains to facilitate the transfer of the DNA.[105, 107] Transduction involves bacteriophage (often from prophage in another bacteria) adsorbing on the pili appendages, and entering the recipient strain by the retraction of pili appendages.[132]

During conjugation, donor cell would use pilus, a thin and tube-like structure,[102, 107, 129, 133-137] to contact recipient cell and retract to bring the two cells together, at which time the donor cell transfers the genetic material to the recipient cell. However, the detail about how genetic material get transferred during conjugation is still unclear. Scientists believe that HGT via conjugation requires direct physical contact between donor and recipient.[105, 108, 129] transfer.[106, 107] They also found that PilS2, one of the type IV pili, was exclusively mediating PAPI-1 transfer.[107]

1.6 A brief summary of the chapters in this thesis

For this thesis, chapter 2 describes how antibiotics induce bacteria to grow in planktonic medium and attach on surface. The characterization of biofilms, particularly in real time, is a challenge. The conventional Crystal Violet dye-staining assay depends on adsorption of dye molecules in the matrix of biofilm materials and does not characterize the internal channel and pore structure of the biofilm.[138] Confocal fluorescent imaging of bacteria expressing fluorescent proteins in a biofilm requires manipulation of the bacteria's biology, and also makes the assumptions that the amount of bacteria in a biofilm is proportional to the biofilm mass and that the distribution of bacteria in biofilm reflects the three-dimensional structure and morphology of the biofilm.[139] Certain clinical strains of bacteria can overproduce large amounts of exopolymers,[140] the structure of which is different from that produced by the wild type. More importantly, both methods require multiple steps of manual treatment of biofilms (washing, staining, extracting the stains) and thus can vary between different individuals conducting the experiment. In contrast, surface spectroscopy methods, such as surface plasmon resonance and

biolayer interferometry (BLI),[141-143] provide real-time and sensitive measurements of molecules attaching to a surface either through physical adsorption or through specific ligand–receptor binding. In particular, BLI measures the optical interference at the tip surface of an optical fiber. This is altered by biomolecule adsorption or biomass deposition, which causes a shift in the interference pattern.[142, 143] The magnitude of the shift is measurable in real time and correlates with the amount of adsorption or deposition of biomolecules or biomass.[143] These surface analytical techniques are well-suited for study of bacterial adherence and biofilm formation, but have not been extensively explored to reveal the details of steps of bacterial adherence.[59, 62, 63]

Biolayer interferometry (BLI) is used to demonstrate that cell-wall antibiotics induce formation of filamentous bacteria and biofilm. Also, we reveal that young (lag phase) bacteria are more efficient to form filaments than fast-growing bacteria. The further study suggests that the growth of antibiotic-induced filamentous bacteria is surface-mediated. The antibiotic-induced biofilm that is composed of filamentous bacteria was observed by Confocal microscopy. The suspended aggregates via filament formation are also observed in the medium.

In chapter 3, the three mechanisms of horizontal gene transfer are studied, including transformation, transduction and conjugation. Saturated farnesol derivatives show inhibition effect on tetracycline-enhanced plasmid transformation, ciprofloxacin-enhances PAPI-1 transduction, and PAPI-1 conjugation. Further study of ciprofloxacin-resistance development in *P. aeruginosa* is conducted by using serial passage assay. The inhibited development of MIC of ciprofloxacin suggest that saturated farnesol derivative inhibits the development of resistance to ciprofloxacin in P. aeruginosa.

Chapter 4 describes the mechanism of pili inhibition by saturated farnesol derivatives. The pili-mediated bacteriophage adsorption is studied, and the results demonstrate that saturated farnesol derivative could cause pili retraction, leading to inhibition of bacteriophage adsorption. More evidence was obtained from other colleagues in Dr. Luk's lab to support the small molecules binding to pili. The MALDI-MS done by Hewen Zheng suggests that saturated farnesol derivative covalently binds to pili. Hewen also performed the alkaline buffer extraction experiment to demonstrate that PAO1 culturing with saturated farnesol derivative resulted in decrease in pili protein expression. Together with the bacteriophage adsorption results, we conclude that saturated farnesol derivative binding to pili cause pili retraction.

Chapter 2. Cell wall-related antibiotics cause lag-phase bacteria to form surface-mediated filaments promoting formation of biofilms and aggregates

2.1 Introduction

Biofilms formed by microbes on surfaces are sources for persistent infectious diseases and environmental problems.[9, 13, 15, 19, 43-51] The antibiotics that are used to treat bacterial problems actually often promote the formation of biofilm: sub-lethal amount of antibiotics cause significantly larger amounts of biofilm than that formed without antibiotics, sometimes up to two-fold in biofilm masses.[13, 19, 50, 51] A particular relevant scenario is the use of antibiotics to treat biofilm in human hosts,[47-49] such as biofilms in the lungs of cystic fibrosis (CF) patients.[47-49] These biofilms are extremely difficult to completely eradicate.[47-49] The mechanism and details of how antibiotics promote biofilm formation is largely unknown. For instance, it not clear what stages of biofilm growth are promoted to proceed faster than without antibiotics, what phenotypes of bacteria form in an antibiotic-promoted biofilm, and what different biofilm compositions and structures are caused by the presence of antibiotics. Among other effects, antibiotics can cause bacteria to form filaments of living bacteria.[22, 25, 52-56] The impact of filamentous bacteria on biofilm formation is not clear. The effects of surface chemistry on the formation and the growth of filaments are also not well understood. Understanding these factors will be important for controlling the biofilm formation,[59, 61, 64, 65, 144-146] and for understanding how some antibiotics promote biofilm formation.[19, 50] Here, we conduct a real-time study of the adherence of bacteria and antibiotic-induced filamentous bacteria on surfaces and characterize the kinetics of surface adherence of these two forms of bacteria. Studying the effect of different surfaces on promoting filamentous bacteria's adherence on

surfaces, we characterize an unexpected correlation between the stage of bacterial growth and the formation and growth of filamentous bacteria on surfaces. Based on these results, we outline the lifestyle of filamentous bacteria and a mechanism by which antibiotics promote biofilm formation.

The characterization of biofilms, particularly in real time, is a challenge. The conventional crystal violet dye–staining assay depends on adsorption of dye molecules in the matrix of biofilm materials, which does not characterize the internal channel and pore structure of the biofilm.[147] Confocal fluorescent imaging of bacteria expressing fluorescent proteins in a biofilm requires the manipulation of bacteria's biology, and also makes the assumptions that the amount of bacteria in a biofilm is proportional to the biofilm mass, and that the distribution of bacteria in biofilm reflects the three-dimensional structure and morphology of the biofilm.[148] Certain clinical strains of bacteria can overproduce large amounts of exopolymers,[149] the structure of which is different than that of the wild type. More importantly, both methods require multiple steps of manual treatment of biofilms (washing, staining, extracting the stains), and thus can vary between different individuals conducting the experiment. In contrast, surface spectroscopy methods, such as surface plasmon resonance and biolayer interferometry (BLI),[150-152] provide real-time and sensitive measurements of molecules attaching to a surface by either physical adsorption or specific ligand-receptor binding. In particular, Bio-layer interferometry (BLI) measures the optical interference at the tip surface an optical fiber, which is altered by the biomolecule adsorption or biomass deposition, and causes a shift in the interference pattern.[150, 151] The magnitude of the shift is measured in real time and correlates with the amount of adsorption or deposition of biomolecules or biomasses.[150]

These surface analytical techniques are well-suited for study of bacterial adherence and biofilm formation, but have not been extensively explored to reveal the details steps of bacterial adherence.[59, 145, 146]

In this work, we use BLI to measure the adherence of bacteria and antibiotics-induced filaments on surfaces. Together with direct microscopy studies, we characterize the lifestyle and stages of filamentous bacteria, including surface-mediated growth, formation of biofilm on surfaces and aggregates in solution, and correlation between stages of planktonic growth and filament formation induced by antibiotics. This work has been published in 2020.[153]

2.2 Results and Discussion

For most biofilm formation on surfaces, the first step is believed to be bacterial attachment to the surface,[43-46] which is facilitated by the adsorption of bacteria's surface protein appendages,[146, 154, 155] followed by micro-colony formation, secretion of exopolymers to form films with internal structures of voids and channels.[46, 155] The surface appendages of bacteria are responsible for nonspecific adsorption on man-made surfaces,[146, 155-158] and for recognizing ligands on hosting mammalian cell surfaces.[146, 158, 159] The kinetics of the adsorption of surface protein appendages on bacteria onto a surface is not well characterized. In comparison to adsorption of free proteins and adhesion of mammalian cells,[160-162] real-time study of bacterial adherence is lacking. Here, we studied the biomass deposition from *E. coli* culture, in real time by using biolayer interferometry (BLI) on surfaces with and without the stress of antibiotics. We also studied

the formation of filamentous bacteria of both *E. coli* and *P. aeruginosa* induced by antibiotics.

2.2.1 Pili-mediated bacterial adherence shows slow rate but large amount of biomass deposition

In order to find out if bacterial adhesion could be monitored by BLI, we first compared the BLI signals of bacterial culture with the signals of free solution proteins on surfaces, An *E. coli* RP437 culture with initial optical density of 0.6 showed a slow rate of biomass deposition but resulted in a large amount of biomass on the surface (Figure 2.1). We observed that adsorption of free solution proteins on surfaces, in general, has a high initial rate of biomass deposition than that from an *E. coli* culture; solution containing 1 mg/mL of lysozyme, and 3 mg/mL of BSA have an initial rate of BLI signal increase of 2.4 and 0.6 nm/min, respectively, whereas the bacterial culture (initial OD_{600} 0.6) has an initial rate of biomass deposition of 0.014 nm/min. However, for the same set of experiments, the final BLI signal caused by bacterial culture can be higher than that by the adsorption of free solution proteins. The final BLI signal for the *E. coli* culture was 0.6 nm, whereas that by 1 mg/mL of lysozyme, and 3 mg/mL BSA were 0.42 and 0.28 nm, respectively (Figure 2.1A). The LB medium itself does not incur any signal increase. These results indicate that both the rate and final amount of the biomass deposition caused by a bacterial culture is grossly different than that by adsorption of free non-assembled proteins at relatively high concentrations (1-3 mg/mL) from solution.

To confirm the bacterial adherence on the surface of the biosensor tips, we firstly re-cultured the biosensor tips that was done monitoring the BLI signal of the bacterial culture. The used biosensor tips were cut and dropped into a fresh LB media, followed by shaking at 37°C. A cloudy media was observed after couple hours, indicating that there were bacteria on the tips. However, the BLI experiment was not conducted in a sterile environment. Thus, the observed bacterial growth in media may not be from the adhered bacteria on surface of the tips. In another study (see Figure 2.19), a slide that has the same material coated surface as biosensor tips (aminopropylsilane coated) was introduced into a *E. coli* RP437 culture. We observed that *E. coli* quickly adhered on the slides. And the filamentous bacteria also grew and adhered on the slides in the presence of antibiotic (see Figure 2.19). These results are consistent with bacteria adhere to the biosensor tips, and suggest that BLI is an effective tool to reveal bacterial adherence on the surface in real time, and to correlate the adherence to the amount of surface appendages on the bacteria.

To examine the effect of surface appendages of bacteria on bacterial adherence on surfaces, we studied a range of different bacterial species for their initial rates of surface adherence without a priori knowledge about the surface appendages. Figure 2.1B shows the BLI signal for culture containing *Serratia marcescens*, *Paenibacillus dendritiformis*, *E. coli* RP437, *E. coli* NEB5α and *E. coli* LE392, all with an initial optical density of 0.6 at the beginning of the BLI monitoring. These strains showed a wide range of initial rates of adherence, and the final amount of biomasses on surface (at the end of 3 h), with *S. marcescens* giving the highest rate and large amount of final biomasses, and *E. coli* LE392 the lowest rate, and the least amount. *E. coli* LE392, a derivative from strain

K-12, does not express fimbriae,[163-165] which is consistent with its low BLI signal results. To further study the importance of fimbriae for *E. coli*,[146, 158, 165, 166] we used *E. coli* HB101, which was constructed to be non-fimbriated and lacked the ability of adhesion,[165, 166] to study its adherence under the same conditions using BLI. *E. coli* HB101 gives significant lower BLI signal than *E. coli* RP437 (Figure 2.2), and with comparable low BLI as the non-fimbriated strain, *E. coli* LE392. These results confirmed the importance of fimbriae for bacterial adherence for *E. coli*.[146, 158]

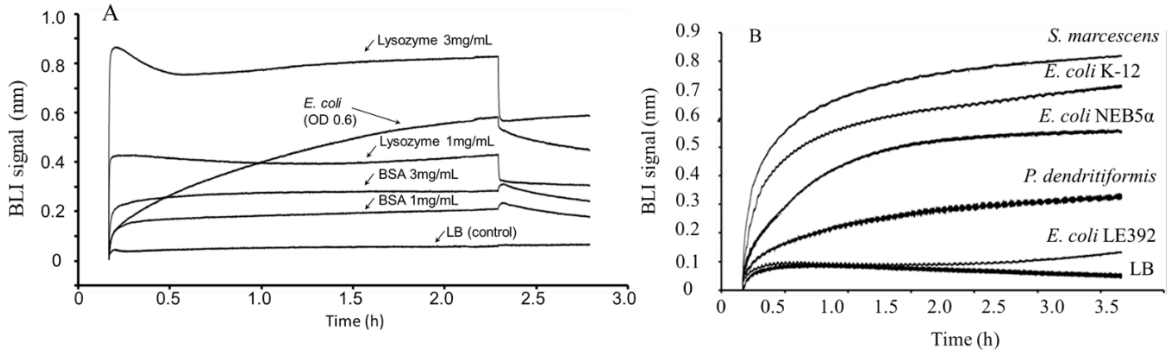


Figure 2.1 (A) BLI signal of *E. coli* RP437 culture (initial OD_{600} 0.6), and protein solutions. Lysozyme and BSA solutions were prepared in fresh LB medium. (B) BLI signals from different bacterial strains of OD_{600} 0.6. Three experimental replicates were performed.

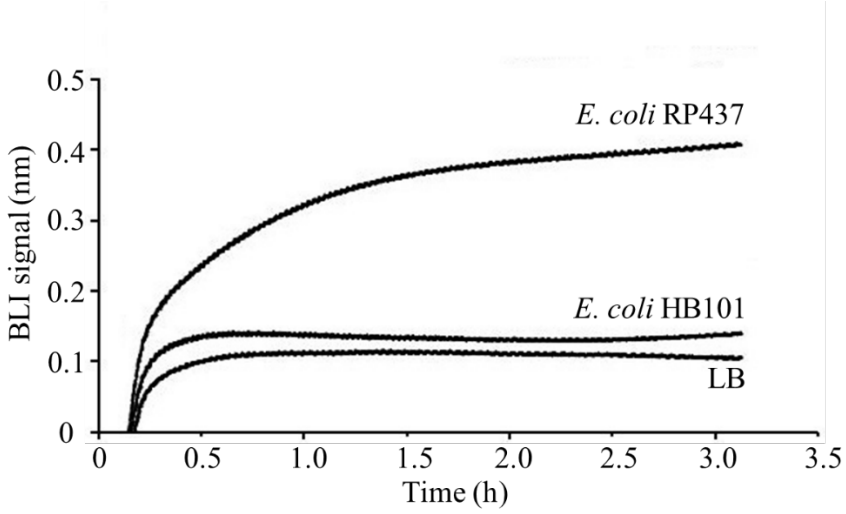


Figure 2.2 Non-fimbriated *E. coli* HB101 gives significant low BLI signal than fimbriated *E. coli* RP437. BLI signal from *E. coli* RP437 and *E. coli* HB101 culture (initial OD600 0.6). Bacterial cultures were prepared in LB medium in falcon tubes, then monitored by BLI at 37 °C.

To examine the effect of bacterial adherence, we studied the BLI signal as a function of different initial bacterial concentrations. Our results demonstrate that the initial rates of BLI signal are proportional to the low concentrations of bacteria (OD_{600} < 0.5) (Figure 2.3). We also found that bacterial adherence on surface is consistent with the adsorption of their surface protein appendages (Figure 2.4). We used transmission electron microscopy (TEM) to examine the strains' surface appendages. All bacteria showed but different amount of surface-associated fibrous attachments. *Serratia marcescens* showed the longest, and the most amount of surface appendages, whereas *E. coli* NEB5α showed fewer appended fibrous attachments. These observation correlates well with the rate of initial biomass adsorption and the final amount of biomass on surfaces by the same bacteria shown by BLI (Figure 1B), for which *S. marcescens* exhibited the highest deposition rate and the largest final BLI signals, and *E. coli* NEB5α showed a lower rate and less amount. We found that *E. coli* LE392 is a strain that does not produce type I fimbriae and has been used to express fimbriae of other species. Consistent with the BLI and TEM results, *E. coli* LE392 exhibited the smallest amount of BLI signal.

The BLI signals from bacterial culture (Figure 2.1B) may come from two sources, the adsorption of secreted proteins, and or the adherence of bacteria on the BLI tip surfaces. Two studies indicate that the BLI signal are more likely from bacteria adherence

than from free protein adsorption in solution. First, the BLI signal from bacterial culture exhibits an unusual character comparing to all known BLI experiments. The BLI signal increased slowly (compare to that from a high concentration protein solution), but increased to a high value at the end of around 2.3 h. We compared to BLI signal from adsorption of a panel of proteins. We found that no protein adsorption showed such character, and neither can we find from literature that show this character from protein adsorption from a solution. High protein concentration can give to such high biomass adsorption (Figure 2.1A), but always accompany with high initial rate of adsorption. Low protein concentration can give low initiate rate of biomass deposition, but never give a large amount of final deposition. This lack of high final biomass is likely due to slow adsorption allows changes of protein conformation to give a thin layer of proteins on surface, making the surface resistant to further protein adsorption.

Second, we note that bacteria adherence is believed to be facilitated by adsorption of protein appendages “grown” on the bacterial surface onto the surfaces in an environment. To examine that the bacteria adherence is supported potentially by pili appendage of bacteria, we tested the BLI signals of two strains *E. coli* RP437 and *E. coli* HB101. The final BLI signal from *E. coli* RP437 was significantly higher than *E. coli* HB101 (Figure 2.2). The only difference between those 2 strains are the surface appendages that enable bacterial adherence. The deficiency of the pili on *E. coli* HB101 is consistent with low BLI signal from bacterial culture of this strain of *E. coli*. Together, the large BLI signals are consistent with adsorption of pili appendages on bacteria, which is a known mechanism for bacterial adherence.

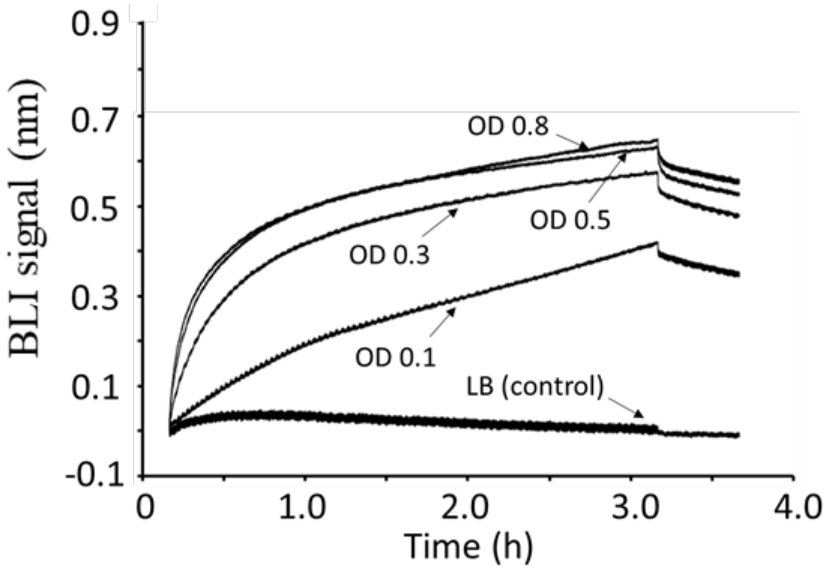


Figure 2.3 The initial rates of BLI signal were proportional to the low concentrations of bacteria (OD_{600} < 0.5). BLI signal of different initial optical densities culture of *E. coli* RP437.

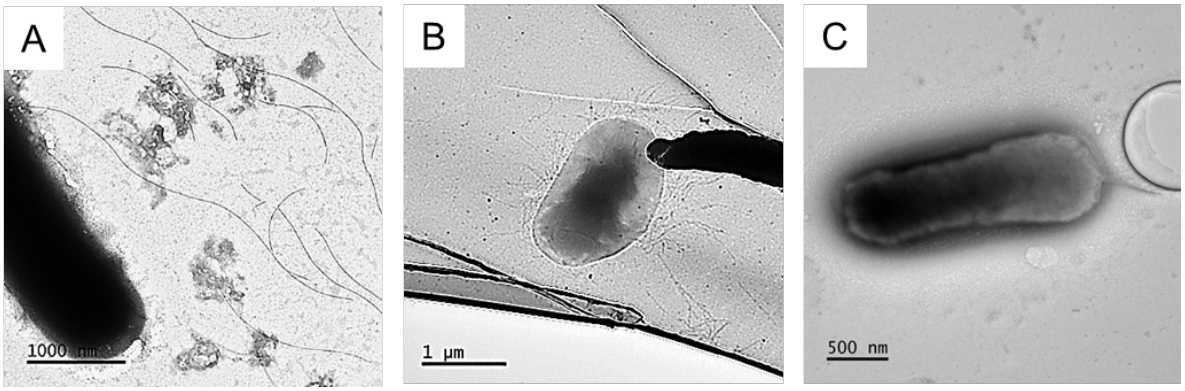


Figure 2.4 Bacterial adherence on surface (BLI signals) was consistent with their surface protein appendages. Transmission electron microscopy (TEM) images of initial OD_{600} 0.6 of different bacterial species. (A) *S. marcescens*, (B) *E. coli* RP437, (C) *E. coli* LE392

2.2.2 Cell wall-related antibiotics promote biomass deposition on surfaces

Antibiotics are known to cause increased formation of biofilms.[13, 19, 50, 51] The mechanism for how antibiotics promotes biofilm formation is not clear. Neither is it clear about which stage of the biofilm formation and growth is impacted the most by the antibiotics. Here, we examined the effect of four antibiotics, carbenicillin, tetracycline, chloramphenicol, and colistin on the initial stage (bacterial adherence) for biofilm formation by *E. coli*. We examined different concentrations of antibiotics, below and above the minimum inhibitory concentration (MIC). We found that carbenicillin (100 μg/mL) and colistin (10 μg/mL) promoted the initial rate and final biomass deposition on surfaces (Figure 2.5), whereas tetracycline and chloramphenicol did not show a noticeable effect for the time period studied. We note that chloramphenicol and tetracycline are reported to promote the biofilm formation by *E. coli*.[51] Thus, while we did not observe an increase of the initial biomass adsorption on the surface, these two antibiotics may promote biofilm development at a later stage. We note that at relatively low concentrations of carbenicillin (9 μg/mL) and colistin (0.1 μg/mL) there were no significant changes in the biomass adsorption when compared to culture without antibiotics. Thus, we focused our studies at 100 μg/mL of carbenicillin. At these concentrations, carbenicillin and colistin caused similar promotion of initial rates of biomass adsorption as that without antibiotics, however, the fast rate of biomass adsorption continued when antibiotic was present for the rest of the 3 h, whereas the biomass deposition started to slow down at about 30 min when antibiotic was absent. These results, at the first glance, seem to suggest that carbenicillin and colistin kill bacteria and cause the release and adsorption of protein debris. To confirm or eliminate this interpretation, we studied the time dependence of OD_{600} in the

presence of carbenicillin, examined the formation of filamentous bacteria, and the effect of antibiotics with young- and fast-growing bacteria on biomass deposition (BLI signals).

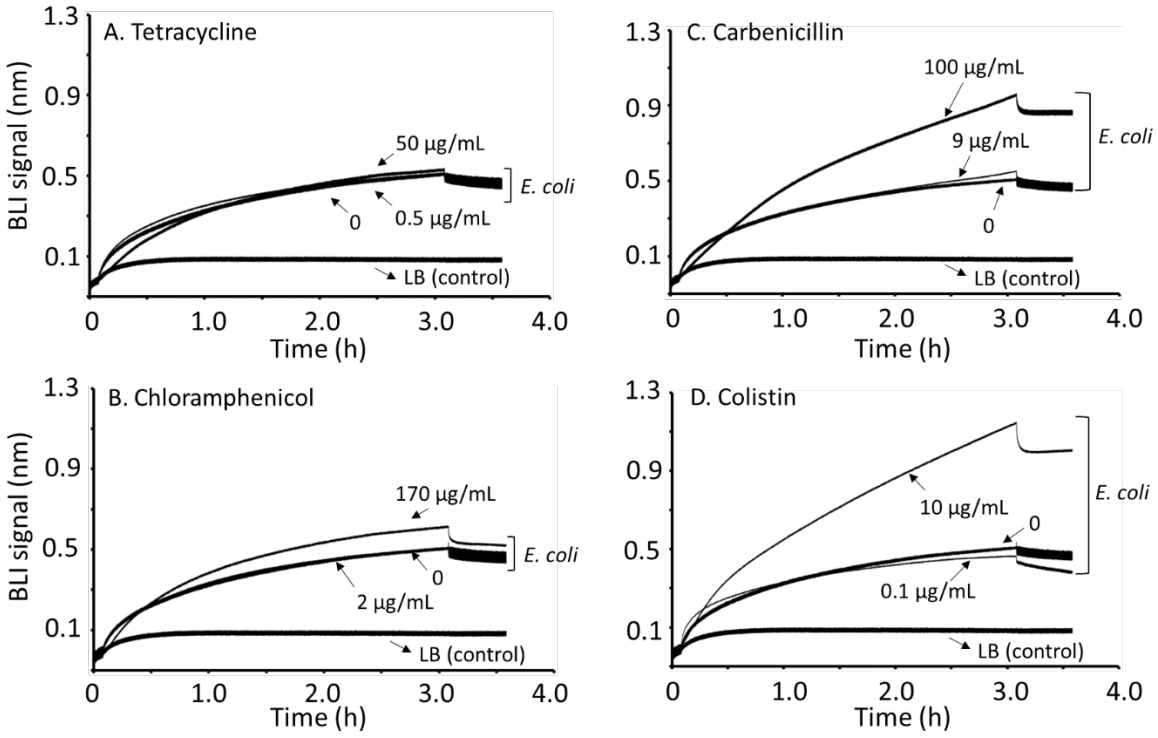


Figure 2.5 BLI signal of *E. coli* RP437 culture (initial OD_{600} 0.6) with and without (A) tetracycline 0.5 µg/mL and 50 µg/mL, (B) chloramphenicol 2 µg/mL and 170 µg/mL, (C) carbenicillin 9 µg/mL and 100 µg/mL, and (D) colistin 0.1 µg/mL and 10 µg/mL Three experimental replicates were performed.

2.2.3 Antibiotics that promote biomass deposition readily cause filament formation

Presence of antibiotic, often at sub-lethal concentration, cause bacteria to form filamentous bacteria, for which a bacterium grows and replicates but does not separate.[53-55] Here, we use light microscopy to directly study the *E. coli* RP437 culture in the presence of carbenicillin under different conditions (Figure 2.6). We observed that culturing of *E. coli* RP437 (initial OD_{600} 0.6) with 100 µg/mL carbenicillin (reported MICs are 16.8-25

μg/mL μg/mL)[167, 168] in a falcon tube with shaking at 250 rpm for 3 h caused the formation of visible suspended aggregates (Figure 2.6A). Sandwiched between microscope glass slides, the sludge showed visible filamentous aggregates that are intertwined with non-filamentous aggregates. These filamentous biomasses are consistent with reported filamentous bacteria.[169, 170] By introducing fresh media without antibiotics, we found that, based on CFU counting, there were about double amount of live bacteria revitalized from a wet aggregate (sludge) mass than from the same mass of the solution (supernatant) from an antibiotic-culture (Figure 2.7).

In a separate experiment, when the bacteria's density reached an OD_{600} of 0.6, we added carbenicillin (to reach 100 μg/mL), and immediately transferred the culture to a polystyrene petri dish, and a glass petri dish, and continued the culture for 3 h without shaking. A suspended film was observed for the culture in the polystyrene petri dish (Figure 2.6B). The culture in the glass petri dish for the same 3 h did not form any visible aggregates or suspended films (Figure 2.6C). Under the microscope, the suspended film showed similar intertwined filaments as that from the culture in shaking falcon tubes, but with fewer non-filamentous aggregates. These results suggest that formation of filaments depends on the materials of the labware used and proceeds more efficiently in plastic labware than in glassware. We believe that the formation of filamentous bacteria is mediated by the bacteria first adhering to a surface.

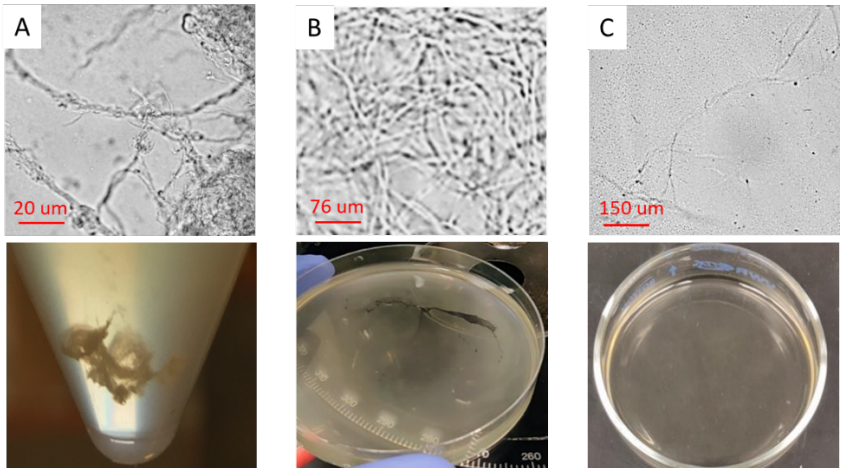


Figure 2.6 *E. coli* RP437 cultures (initial OD_{600} 0.6) were supplemented with carbenicillin (100 µg/mL) and cultured at 37 °C for 3h, in falcon tubes with shaking at 250 rpm, and without shaking in plastic and glass petri dishes. Micrographs of sludge from Falcon tube (A) and of the suspended film from polystyrene petri dish (B), sandwiched between two microscope glass slides. Micrograph of scattered long filament aggregate in the solution in glass petri dish (C). The sample images are shown below the micrograph. More than three experimental replicates were performed by different people at different days.

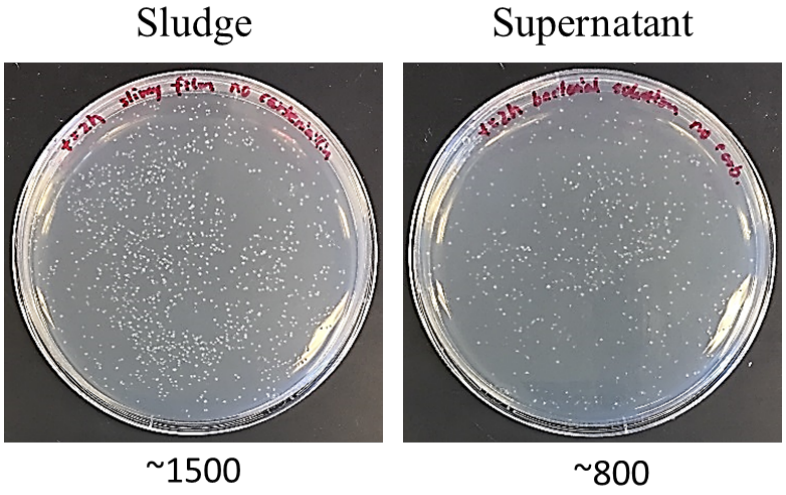


Figure 2.7 About double number of live bacteria were revitalized from a wet aggregate (sludge) mass than from the same mass of the solution (supernatant) from an antibiotic-culture Measuring the number of viable bacteria in the sludge aggregate and in the supernatant of the carbenicillin-treated culture. The sludge aggregate formed and visible

after *E. coli* RP437 (initial OD_{600} 0.6) was cultured with carbenicillin (100 μg/mL) for 3 h. The sludge was pipetted out of the culture and mixed with fresh LB medium. The same weight of the supernatant was pipetted out and mixed with fresh LB medium to reach the same final volume as that of the sludge culture. Both samples were incubated for 2 h at 37 °C, and 100 μL of each sample was spread on an agar plate (1.5% agar (Bacto) in LB without antibiotics). The agar plates were incubated at 37 °C for 12 hours, then incubated at room temperature for 12 hours. The bacterial count is shown below the pictures.

We screened antibiotics for biomass deposition, and for immediate filamentous bacteria formation by *E. coli* RP437. Chloramphenicol and tetracycline did not promote biomass deposition and did not cause filament formation (Figure 2.8); whereas carbenicillin and colistin, both target cell walls, promoted biomass deposition and caused filament formation. We then tested two more β-lactam antibiotics, aztreonam and penicillin, on inducing filamentous bacteria. All three β-lactam antibiotics tested induce filamentous bacteria of *E. coli* RP437 (Figure 2.9). These results are consistent with the knowledge that β-lactams target multiple penicillin-binding proteins (PBPs), and that PBP3 is responsible for cell divisions.[171] We also tested the filaments formation of an *E. coli* strain that is resistant to carbenicillin (*E. coli* RP437 Cb^R) in the presence of carbenicillin. The result shows that carbenicillin (100 μg/mL) does not induce filaments of carbenicillin–resistant *E. coli* RP437. Because the reported MICs of carbenicillin are 16.8 to 25 μg/mL,[167, 168] we also tested sub-MIC (10 μg/mL) of carbenicillin at causing filament formation. Our results suggest that filaments formed at both concentrations, except that more filaments were observed when 10 μg/mL was applied. These results indicate that relevant antibiotics cause filament formation at a wide range of concentrations beyond MIC.

Besides *E. coli* RP437, we also examined the filament formation of *P. aeruginosa*, PAO1, by the stress of the antibiotics in this study. Similar effects of antibiotics were observed on PAO1 as on *E. coli*. Carbenicillin, colistin and aztreonam caused PAO1 to form filaments, whereas chloramphenicol and tetracycline did not (Figure 2.8). Aztreonam is extensively used for CF patients with *P. aeruginosa*,[48] but the effectiveness of aztreonam is still being evaluated[48] (Figure 2.8).

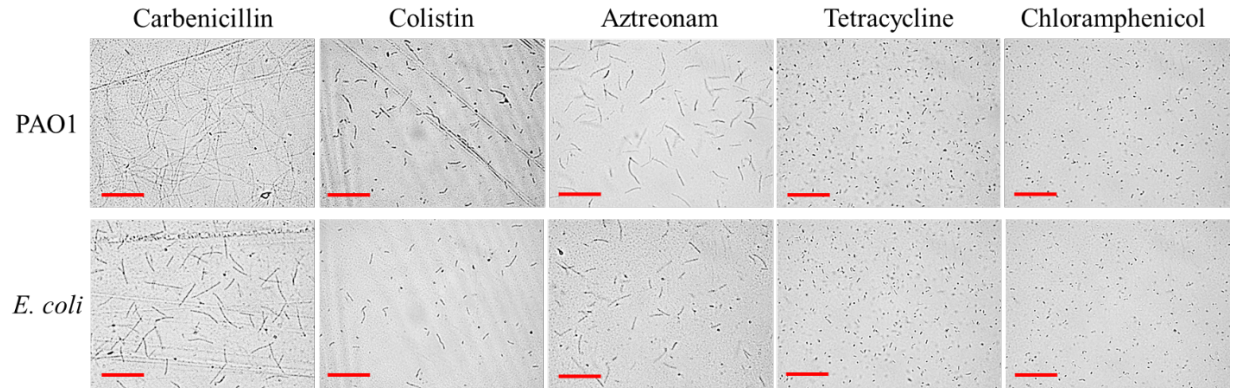


Figure 2.8 Carbenicillin, colistin and aztreonam caused PAO1 and *E. coli* to form filaments whereas chloramphenicol and tetracycline did not. Micrographs of filamentous PAO1 and *E. coli* RP437 (initial OD_{600} 0.2) on APS-coated slides after 2-h culturing with carbenicillin (100 μg/mL), colistin (10 μg/mL), aztreonam (32 μg/mL), tetracycline (50 μg/mL) and chloramphenicol (170 μg/mL) at 37 °C without shaking. Scale bar: 38 μm.

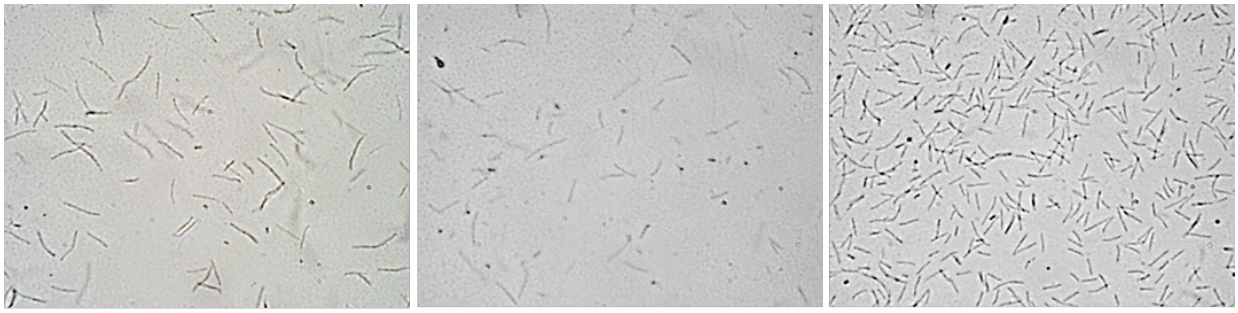


Figure 2.9 β-lactam antibiotics induce filamentous bacteria of *E. coli* RP437. Microscope images of filamentous *E. coli* RP437 (initial OD_{600} 0.6) induced by carbenicillin (100 μg/mL), aztreonam (32 μg/mL) and penicillin (40 μg/m) for 1.5 h in solution. Bacteria were cultured with antibiotics in Falcon tubes at 37 °C with shaking (250 rpm) for 1.5 h, then pipetted some volume of each culture on a glass slide and observed under optical microscope.

2.2.4 Cell wall antibiotics cause young (lag phase) bacteria to form filaments more efficiently than fast-growing bacteria

While studying the biomass deposition from *E. coli* RP437 cultures spiked with carbenicillin, we made a surprising discovery. We added carbenicillin (at 100 μg/mL) to subcultures of *E. coli* RP437 that were grown to different OD_{600} and followed the BLI signal for biomass deposition. We expected the BLI signal to increase with the bacterial density of the subculture (measured by OD_{600}) before we added carbenicillin. On the contrary, both the initial rate of biomass deposition and final amount biomass deposited are inversely related to the initial bacterial density – a lower initial rate and a smaller amount of final biomass deposited were observed for cultures grown to higher densities (Figures 2.10 and 2.5).

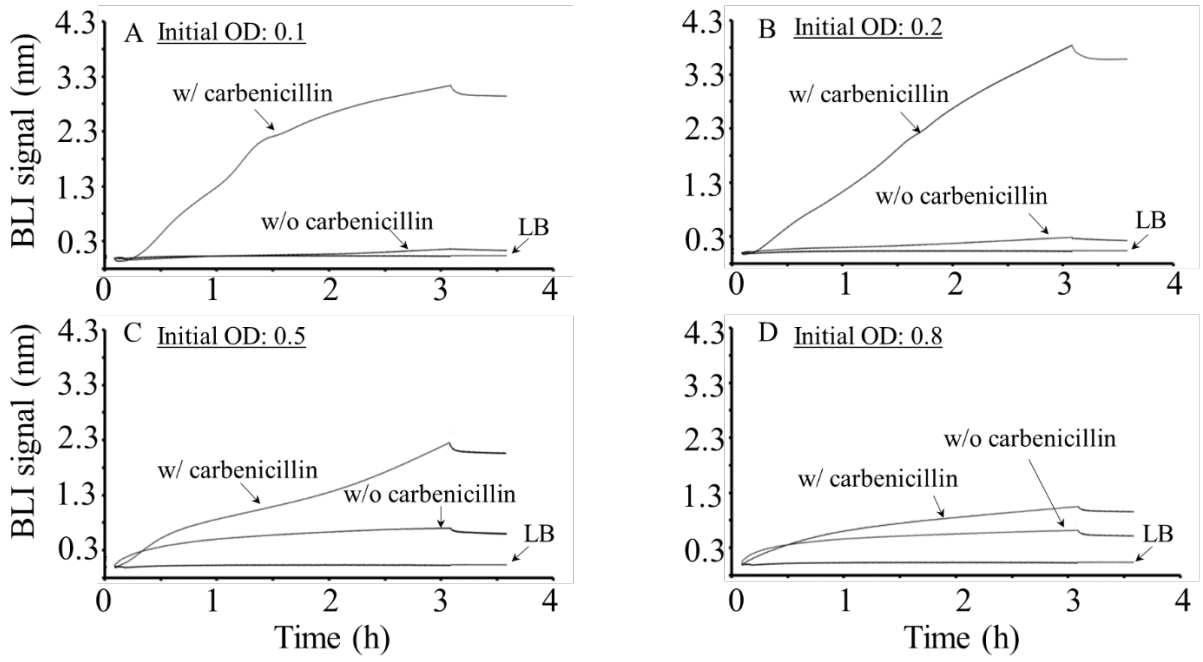


Figure 2.10 BLI signal of *E. coli* RP437 cultures with different initial OD_{600}: (A) 0.1, (B) 0.2, (C) 0.5, and (D) 0.8, with and without carbenicillin (100 μg/mL). Three experimental replicates were performed.

To study the biomass deposition from antibiotic culture as a function of bacterial density, subculture of *E. coli* RP437 (diluted to an equivalent of OD_{600} 0.01) were grown to different densities, with OD_{600} of 0.1, 0.2, 0.5 and 0.8. For these cultures, we added carbenicillin at 100 μg/mL, and immediately monitored the BLI signals for 3 h. We measured the rate of rise of BLI signals at between 30 and 40 min for all eight samples. For cultures with the initial optical density of 0.1, 0.2, 0.5, and 0.8, the rates of BLI signal rising were 0.033 ± 0.006, 0.02 ± 0.004, 0.013 ± 0.005, and 0.007 ± 0.002 nm/min, respectively, when carbenicillin was present; and 0.003 ± 0.0008, 0.006 ± 0.0005, 0.004 ± 0.0004, and 0.004 ± 0.0001 nm/min when there was no antibiotics (Figure 2.11A). The final BLI signals at the end of 3-h culture also reveal an unusual trend. When carbenicillin

was present in culture, the final BLI signals were 3.1 ± 0.4, 3.8 ± 0.6, 2.3 ± 0.3, and 1.0 ± 0.2 nm for cultures with initial optical density of 0.1, 0.2, 0.5, and 0.8 respectively; whereas with no carbenicillin in culture, 0.2 ± 0.05, 0.3 ± 0.08, 0.6 ± 0.06 and 0.6 ± 0.07 nm were obtained, respectively (Figure 2.11B).

The large amount of biomass deposition in the presence of carbenicillin is unlikely to be caused by protein debris adsorption alone because, in the presence of carbenicillin, the final amount of biomass deposition measured by BLI signal also decreased as the bacterial density of the culture increased. Also, the final biomass deposition in the presence of carbenicillin was more than any of those observed by free protein adsorption on surfaces (Figure 2.1A). We also note that the inverse relation is not linear between biomass deposition decrease and bacterial density increase when carbenicillin is present. These results suggest that the primary effect of antibiotics on biomass deposition is the biology of bacteria at different growth stages, rather than the bacterial densities. When the cultures are grown to a low optical density of 0.1 and 0.2, the growth of bacteria is slow; whereas at optical density of 0.5 and 0.8, bacterial growth is fast (see the trace without carbenicillin in Figure 2.10). Because for carbenicillin cultures at low density, the increase of the BLI signal for biomass deposition is linear for the first hour (Figure 2.10A and 2.10B), we hypothesize that this rapid increase of biomass deposition is due to filament growth in the form of elongation on the surfaces.

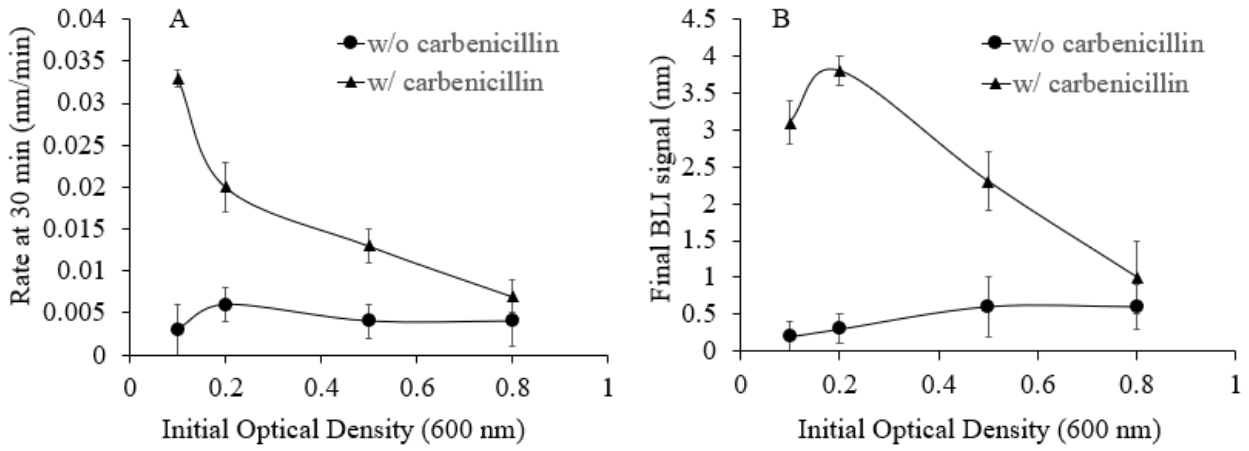


Figure 2.11 Rate of BLI signal increase at 30 min of culture (A) and final BLI signal of the 3-h culture (B) on APS-coated tips from carbenicillin (100 μg/mL) culture having different initial optical density of *E. coli* RP437. Three experimental replicates were performed.

We further studied the optical density of the carbenicillin culture over time. It indicates that the bacteria are being killed but with an unexpected observation (Figure 2.12). In the presence of carbenicillin (100 μg/mL), the optical density increased over the initial period of about 30 min to 1 h, from 0.6 to 0.75, and from 0.2 to 0.4, and from 0.1 to 0.3, and then decreased a steady value of 0.3 (for an initial OD_{600} of 0.6), and of 0.08 (for initial OD_{600} of 0.2 and 0.1). In contrast, without an antibiotic, a typical bacterial growth curve was obtained, showing a lag phase for about 1-2 h, and a fast-growing phase for about 4 h, followed by entering the stationary phase. To explore if the peak in the optical density corresponds to a growth spurt before being killed, we measured the number of live bacteria in the media over 3 h by colony forming units on LB agar plates without antibiotics in the agar, (Figure 2.13). For a subculture grown to OD_{600} of 0.1, the number of bacteria decreased from 6.0×10^7 CFU/mL to 2.8×10^7 CFU/mL over the first 30 minutes, and to 4.0×10^5 CFU/mL for the next 30 min. For the culture with an initial of 0.6, the

number of bacteria decreased from 2.4×10^8 CFU/mL to 1.1×10^8 CFU/mL, and to 3.6×10^7 CFU/mL over the same periods of time. No peak (increase and decrease) in bacterial counts was observed. This result indicates that the initial increase in the optical density is not due to an increase in the number of live bacteria, but likely due to the size or shape changes of some bacteria, or aggregation or formation of filamentous bacteria in the media in response to stress from the antibiotic.

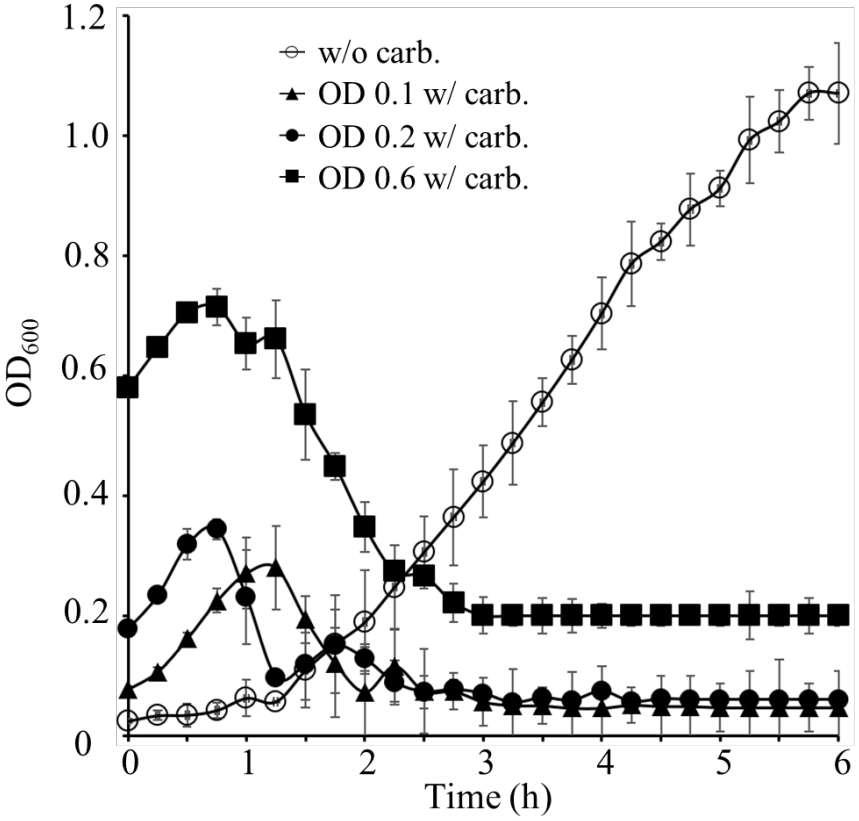


Figure 2.12 Changes in optical density of *E. coli* RP437 cultures on exposure to carbenicillin. An overnight culture was diluted 100-fold, and sub-cultured to OD_{600} of 0.1, 0.2 and 0.6, then each was supplemented with 100 µg/mL carbenicillin. The growth curve of a subculture without carbenicillin was monitored as a control. Three experimental replicates were performed.

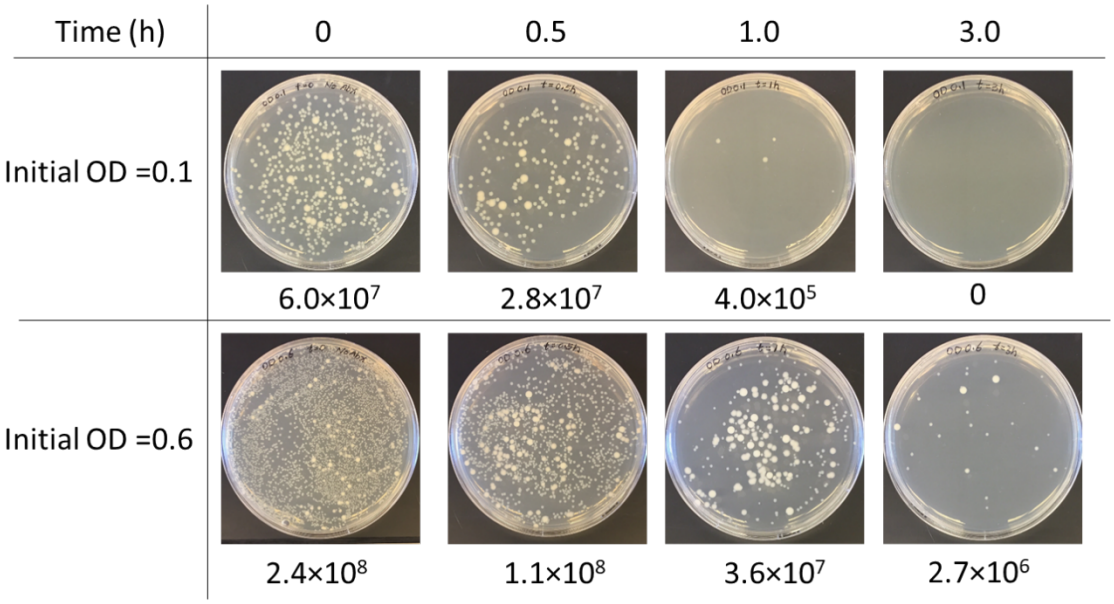


Figure 2.13 The initial increase in the optical density was not due to an increase in the number of live bacteria. After exposure to carbenicillin (100 μg/mL) for different time periods (0 h, 0.5 h, 1.0 h and 3.0 h.), bacterial cultures with initial OD_{600} of 0.1 or 0.6 were diluted 10,000-fold and a 100 μL aliquot of each was spread on hard agar plates (1.5% in LB without antibiotics) to estimate the number of viable cells. The agar plates were incubated at 37 °C for 12 hours, then incubated at room temperature for 12 hours. The number of bacteria (CFU/mL) is shown below each picture.

Most antibiotics,[172-174] such as β-lactams,[173, 174] kill bacteria efficiently only when it reaches fast-growing phase, while the bacteria in slow growth phase may escape antibiotic effects.[172] From the study of filament growth of bacteria adhered on APS-coated slides in carbenicillin containing media, we made an unexpected observation that young (lag phase) bacteria grew much faster as filaments than fast-growing bacteria (Figure 2.14). In these experiments, carbenicillin (100 μg/mL) was added to different bacterial densities (OD_{600} of 0.1, 0.2, 0.5, 0.8), followed by immersing APS-coated slides for 3 min. The slides were then transferred into glass petri dishes containing fresh LB with

carbenicillin (100 μg/mL). We then monitored the attached bacteria on the glass slides for 2.5 h. All four slides shown the growth of filamentous bacteria from adhered bacteria on the surface, but at different rates. The bacteria from a culture with low bacteria density (an OD_{600} of 0.1) had the fastest rate of the elongation of filaments, whereas the bacteria from culture with high densities had lower rates of filament elongation. The formed filaments also appeared to detach from surfaces over time. These results suggest that carbenicillin causes filaments to grow more efficiently for young (lag phase) bacteria at low density than for fast-growing bacteria at high density. In any bacterial culture, the population is not synchronized in growth rate or in their biology, we believe that the filaments observed from fast-growing culture (such as that of an OD_{600} of 0.6) are from the slowing growing population in that culture.

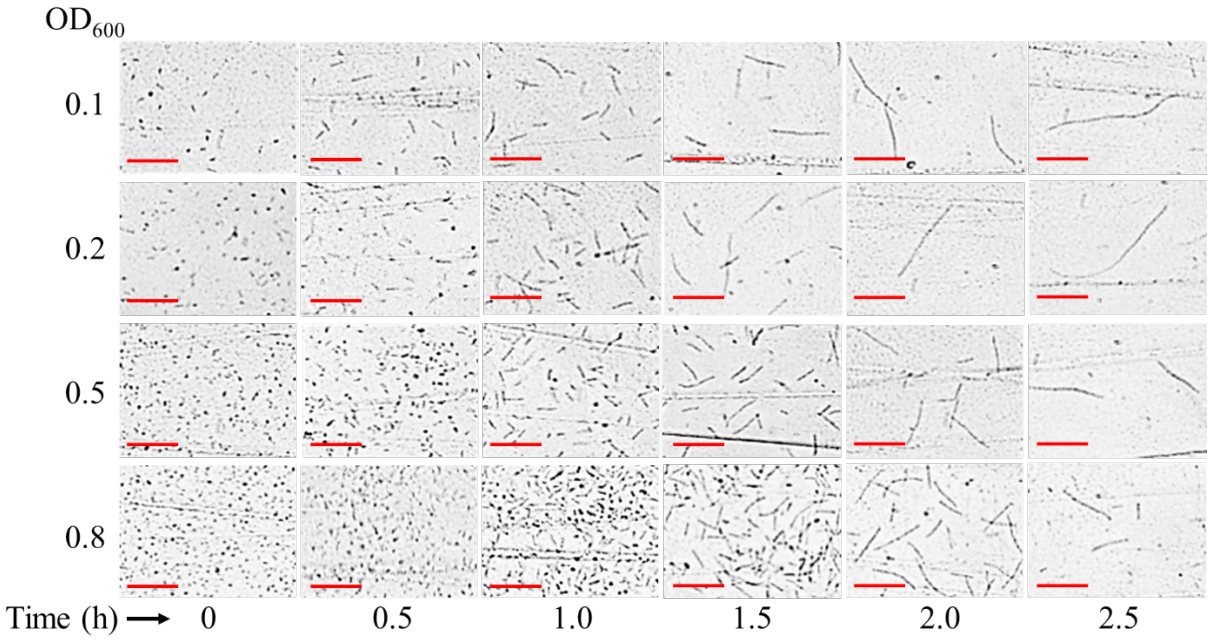


Figure 2.14 Microscope images of growth of filaments from different initial optical densities, 0.1, 0.2, 0.5 and 0.8 of *E. coli* RP437. Four APS-coated slides were immersed in *E. coli* RP437 culture with different initial OD600 (0.1, 0.2, 0.5 and 0.8, respectively) for

3 min, the 4 slides were briefly rinsed with fresh LB, then transferred into 4 glass petri dishes containing fresh LB with carbenicillin (100 μg/mL). The slides were cultured in petri dishes at 37 °C without shaking. The surfaces of APS-coated slides were observed at different times (0 to 2.5 h) by directly putting glass petri dishes under the microscope. Initial OD_{600} of each culture and times of imaging are shown to the left and the bottom of the images, respectively. Scale bars: 38 μm. Three experimental replicates were performed.

In the above experiments, however, the amount of protein debris from dead bacteria adsorbing on surfaces, particularly those from culture with high bacterial density, convolute with the filaments' adherence and growth. To study the filament forming activity by young (lag phase) versus fast-growing bacteria, we subcultured *E. coli* to OD_{600} of 0.1 and 0.4, then diluted the culture of OD_{600} 0.4 to an OD_{600} of 0.1 with fresh medium and added carbenicillin at 100 μg/mL to both cultures. In this way, we compared the bacteria at different growth rates, but with the same density, for their responses to carbenicillin. An APS-coated slide was incubated in each culture for 5 min, transferred to fresh medium with carbenicillin, and growth from attached bacteria was observed over 2 h (Figure 2.15). At first, both young- and fast-growing bacteria showed round bacteria (2 μm) adhered on APS-coated glass slides. For young bacteria (from the subculture of OD_{600} 0.1) with carbenicillin in the culture, the average length of filaments was 3.7 ± 0.6 μm, 8 ± 1.1 μm, 21.3 ± 2.7 μm, 42.6 ± 5.1 μm, and the surface density of filaments was 3.7×10^6, 3.9×10^5, 3.3×10^5 and 3.2×10^4 per cm^2 at time 0.5 h, 1.0 h, 1.5 h and 2.0 h, respectively. For fast-growing bacteria (from a culture of OD_{600} 0.1 diluted from 0.4) with carbenicillin in the culture, more round bacteria were observed without noticeable filaments, and visible filaments were observed at times 1.0 h, 1.5 h and 2.0 h with average length of

filaments 4.8 ± 0.8 μm, 7 ± 1.2 μm, and 12.3 ± 2.4 μm, and the surface density of filaments was 1.4×10^6, 1.2×10^6, and 4.0×10^5 per cm^2, respectively.

These results confirm that antibiotics caused bacteria to grow as filaments on surfaces, and that the filaments formed from the young bacteria grow faster than those formed from fast-growing bacteria. At 1 h, there were more non-filamentous bacteria from the fast-growing bacteria (OD_{600} 0.4) than from the young bacteria (OD_{600} 0.1), but more of the non-filamentous bacteria from culture of OD_{600} 0.4 detached from surfaces over time than those from culture of OD_{600} 0.1. We believe that young bacteria are selected under the stress of carbenicillin to grow as filaments on surfaces without dividing, whereas more actively dividing bacteria are killed.

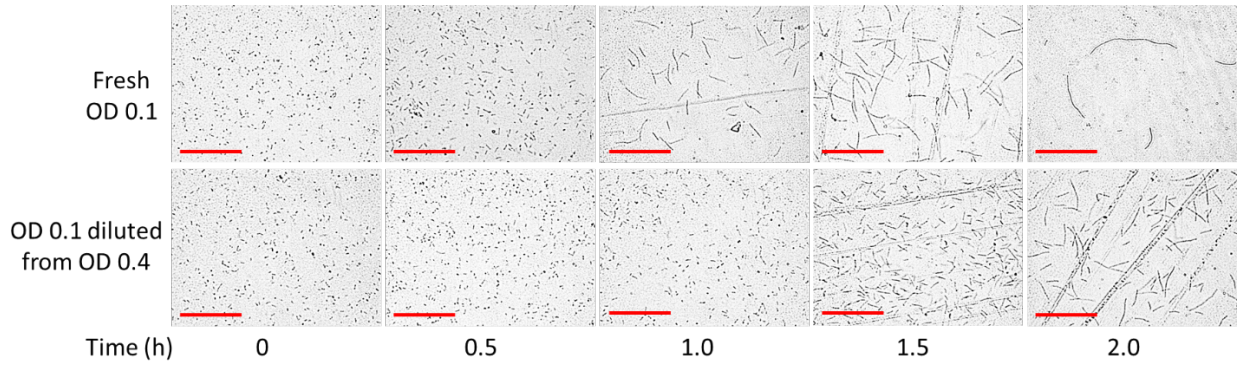


Figure 2.15 Micrographs of APS-coated slides that were immersed in *E. coli* RP437 cultures grown to an initial OD_{600} of 0.1 (top row) or OD_{600} 0.1 diluted from 0.4 for 5 min after carbenicillin was added (to 100 μg/mL). The slides were transferred and cultured in a fresh LB containing 100 μg/mL carbenicillin but no bacteria in glass petri dishes at 37 °C without shaking for different periods of time (0-2 h). See experimental section for details. Scale bar: 38 μm. Three experimental replicates were performed.

To characterize the growth rate of filamentous bacteria on surfaces, we measured the length of filaments on APS surfaces (Figure 2.16). The elongation of filaments' length

seeded from young bacteria (culture in lag phase, OD_{600} 0.1) was about 4.5 times faster than that from fast growing bacteria (culture with an OD_{600} of 0.4 diluted to 0.1). For filaments seeded from culture of an OD_{600} of 0.1, the length increased from 8.0 ± 1.1 μm to 42.6 ± 5.1 μm from 1 to 2 h, giving an estimated elongation rate of 0.58 μm/min, whereas for filaments seed from culture with an OD_{600} of 0.4 (diluted to 0.1), the length increased from 4.8 ± 0.8 μm to 12.3 ± 2.4 μm, an estimated rate of 0.13 μm/min on surfaces.

We also performed the same assay for filaments seeded from cultures with OD_{600} of 0.2 and 0.8. Similarly, filaments from young bacteria (culture with an OD_{600} of 0.2) grew about 4 times faster than filaments seeded from fast-growing bacteria – a culture with an OD_{600} of 0.8, diluted to 0.2 (Figure 2.17 and Figure 2.18).

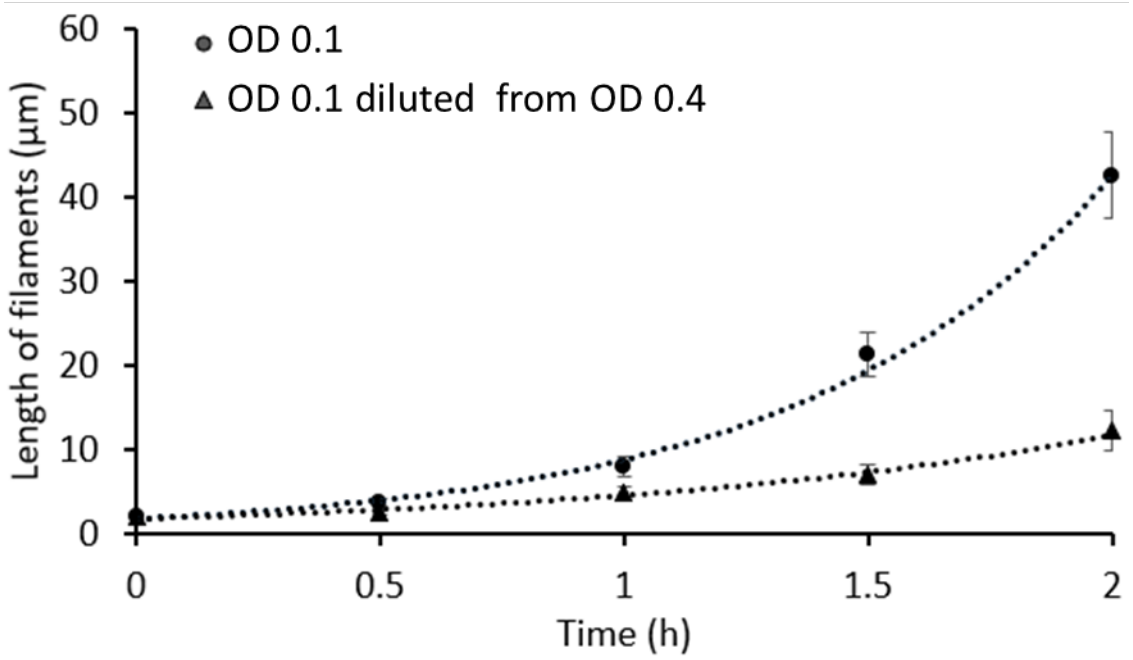


Figure 2.16 The length of filamentous bacteria versus time in LB containing 100 μg/mL carbenicillin. The filamentous bacteria were seeded on APS-coated glass from subcultures of *E. coli* RP437 at an OD_{600} of 0.1, and from an OD_{600} of 0.1 diluted from 0.4.

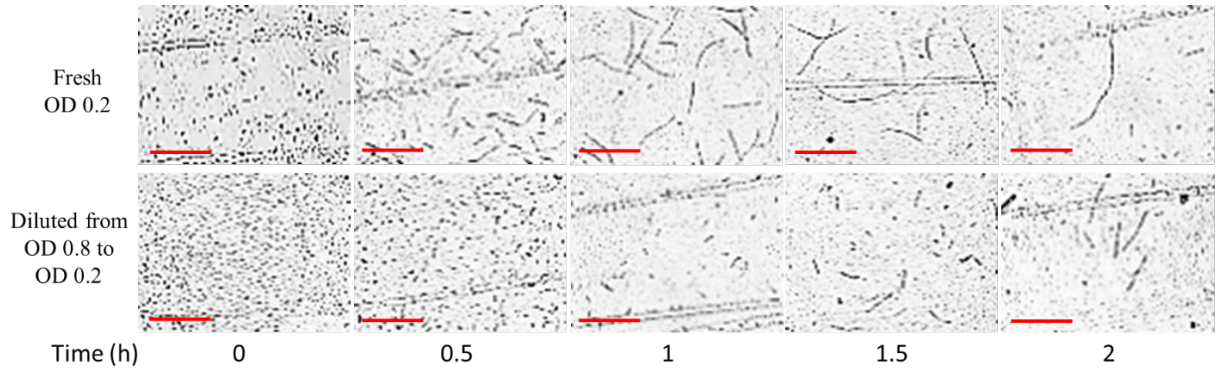


Figure 2.17 The filaments formed from OD 0.8 and OD 0.2 are similar to OD 0.1 and OD 0.4. Micrographs of APS-coated slides that were immersed in *E. coli* RP437 cultures grown to an initial OD_{600} of 0.2 (top row) or grown to 0.8 but diluted to 0.2 at five min after carbenicillin was added (to 100 μg/mL). The slides were transferred and cultured in a fresh LB containing 100 μg/mL carbenicillin but no bacteria in glass petri dishes at 37 °C without shaking for different periods of time (0-2 h). See experimental section for details. Scale bar: 38 μm.

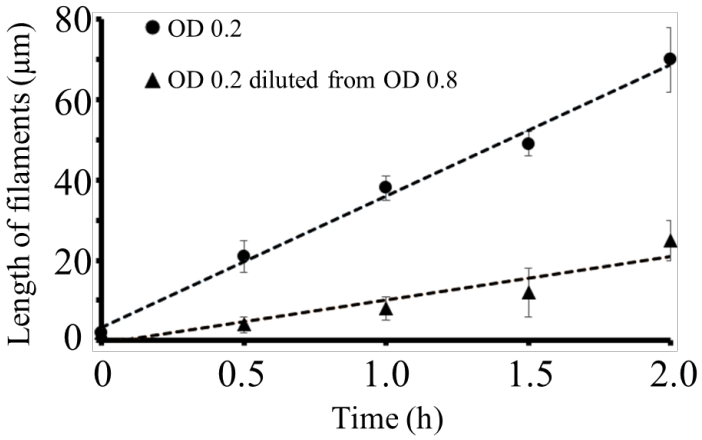


Figure 2.18 Filaments seeded from young bacteria grow at about 0.58 μm/min whereas filaments from fast-growing phase growing at 0.13 μm/min. The length of filamentous bacteria versus time in LB containing 100 μg/mL carbenicillin. The filamentous bacteria

were seeded on APS-coated glass from subcultures of *E. coli* RP437 at an OD_{600} of 0.2, and from an OD600 0.2 but diluted from 0.8.

2.2.5 Growth of antibiotic-induced filamentous bacteria is surface-mediated

At the end of a 3-h culture with carbenicillin, direct observation of surfaces (APS-coated slides, plastic slides, and glass slides) showed full coverage biomass depositions without easily distinguishable filamentous materials. To explore the possible existence of filamentous bacteria on these surfaces, and if surface adherence is required for filament growth, we performed the following experiments. Considering the rapid rise of BLI signal right away when the APS-coated sensor tip was exposed to the bacterial culture containing carbenicillin (Figure 2.10), we briefly immersed (2 ~ 5 min) an APS-coated slide, a polystyrene slide, and a glass slide into a culture right after the antibiotic was added, then rinsed the slides briefly with fresh LB, and transferred them into glass petri dishes containing LB with carbenicillin (100 μg/mL) but without bacteria. Further culturing and examining the surfaces over time, we observed that on both APS-slide and polystyrene plastic, 1 min immersion in carbenicillin-spiked culture and 1-h in carbenicillin-containing medium (without solution bacteria) caused visible adherence of filaments, whereas the glass slide showed significantly less observable filaments (Figure 2.19). We recorded that these filaments move and translocate as living microbes, suggesting that they are living filamentous bacteria.

For antibiotic cultures, the formation of sludge occurs in plastic tubes with shaking, and the suspended films form for culture in plastic petri dishes without shaking, but not in glass petri dishes. For surfaces, more filamentous bacteria were seen on plastic and APS-

coated slides than on microscope glass slides (Figure 2.19). These results suggest that APS-coated and plastic surfaces facilitate more adherence of filaments than glass slides, and that upon adhering on a surface, the filamentous bacteria grow and detach from the surface to facilitate the self-association to form sludge and suspended films. We note that both positively charged APS-coated surfaces and hydrophobic polystyrene surfaces promote protein adsorption, which appears to be a property for facilitating filamentous bacteria adherence and growth.

Few reports have studied filamentous bacteria's attachment to surfaces.[22, 25, 56, 175] Filament formation by bacteria has been shown to increase surface attachment[22] and exhibit different biofilm structure.[175] Their dynamics also respond to the sheer force of fluid they are in contact with.[25, 56] Our results indicate that the growth of filaments in the formation of elongation is promoted by attachment to a surface.

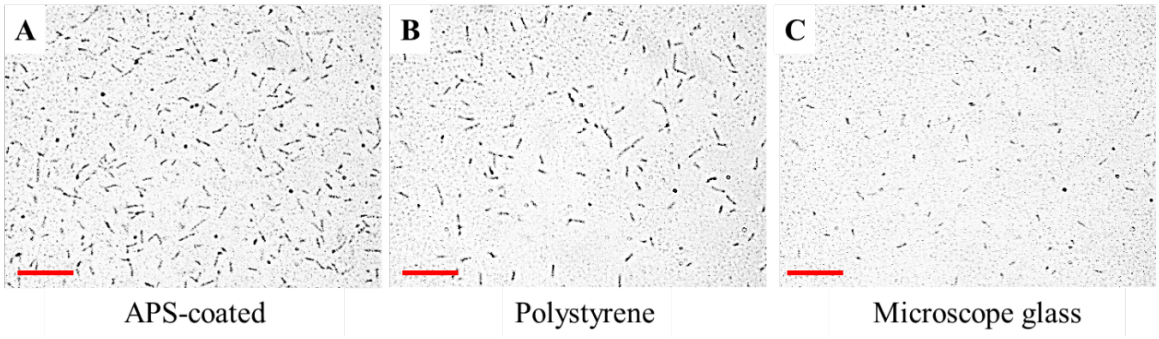


Figure 2.19 Glass slide showed significantly less observable filaments than APS-coated and polystyrene slides. Images of filaments on (A) APS-coated slide (B) polystyrene slide and (C) microscope glass slide. Right after carbenicillin (100 μg/mL final) was added to the *E. coli* RP437 culture (OD_{600} 0.6), the slides were immersed in the culture for 3 min and transferred into glass petri dishes containing fresh LB with carbenicillin (100 μg/mL) but without any bacteria, and were cultured for 1h at 37 °C without shaking. Scale bar: 38 μm.

2.2.6 Cell wall antibiotics cause promoted biofilms and suspended aggregates via filament formation

To examine the effect of antibiotic on biofilm formation under our conditions, we immersed polystyrene slides in cultures of *E. coli* RP437/pRSH103 (initial OD_{600} 0.5) with and without carbenicillin (100 μg/mL) for 24-h at 37 °C with shaking (100 rpm). Images from confocal fluorescence revealed that the biofilm formed in the presence of carbenicillin had almost triple the amount of the biofilm as compared to that without carbenicillin (Figure 2.20). We also confirmed the initial bacterial adherence by confocal fluorescence using *E. coli* RP437/pRSH103. These results confirm the bacterial adherences by filamentous bacteria for biofilm formation and is consistent with optical imaging results of filament attachment on surface, by *E. coli* without fluorescent proteins (Figure 2.21).

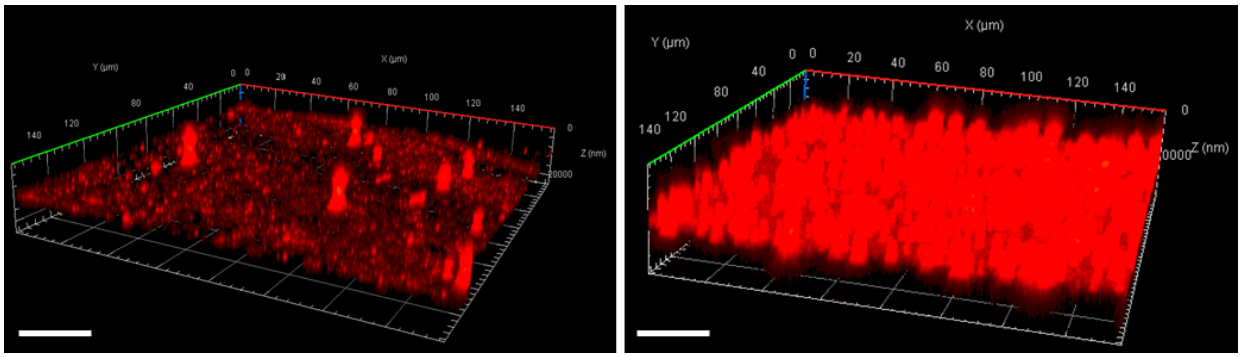


Figure 2.20 Biofilm formed in the presence of carbenicillin had almost triple the amount of the biofilm as compared to that without carbenicillin. Confocal images of biofilm formed by *E. coli* RP437/pRSH103 (initial OD_{600} 0.5) without (left) and with carbenicillin (right, 100 μg/mL) on polystyrene slides in the 6-well plate for 24 h at 37 ℃ with shaking (100 rpm). Polystyrene slides were taken out after 24 h then put on a microscope glass slide

to observe under confocal (the surface of polystyrene slides upside down to the microscope glass slide). Scale bar: 40 nm.

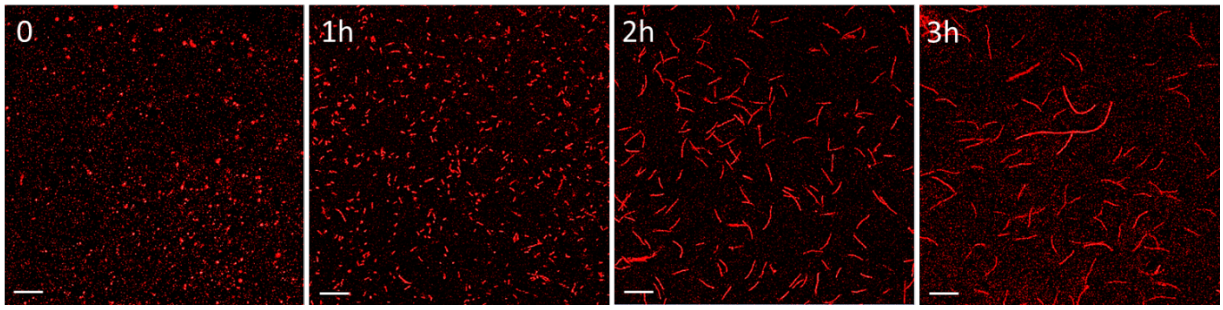


Figure 2.21 *E. coli* RP437/pRSH103 formed similar filamentous bacteria to *E. coli* RP437 on APS-coated surface. Confocal images of filamentous *E. coli* RP437/pRSH103 (initial OD_{600} 0.5) on APS-coated slides. Right after carbenicillin (100 μg/mL) was added to the *E. coli* culture (OD_{600} 0.5), the APS-coated slides were immersed in the culture for 5 min and transferred into glass petri dishes containing fresh LB with carbenicillin (100 μg/mL) but without any bacteria, and were cultured for 0, 1 h, 2 h and 3 h at 37 °C without shaking. Scale bar: 20 μm.

Together with the aggregates observed previously in the *E. coli* culture treated by carbenicillin (Figure 2.6), we believe that the life of filamentous bacteria depends on surface adherence and self-aggregation. When the surface (glass slides) does not support bacterial adherence, the antibiotic-stressed bacteria do not seem to grow as filaments. When there are no bacteria in solution, the filaments grown on surfaces detach over time and form aggregates and films in solution. When there are antibiotic-stressed bacteria in solution, a promoted biofilm is formed, likely along with adsorbed protein debris from dead bacteria, and a suspended biofilm is also formed from detached filaments (Figure 2.22). These results indicate that filamentous bacteria adhere on surfaces and

form biofilm and aggregates. Similar aggregates of filamentous bacteria were observed in sludge formed in septics.[169, 170]

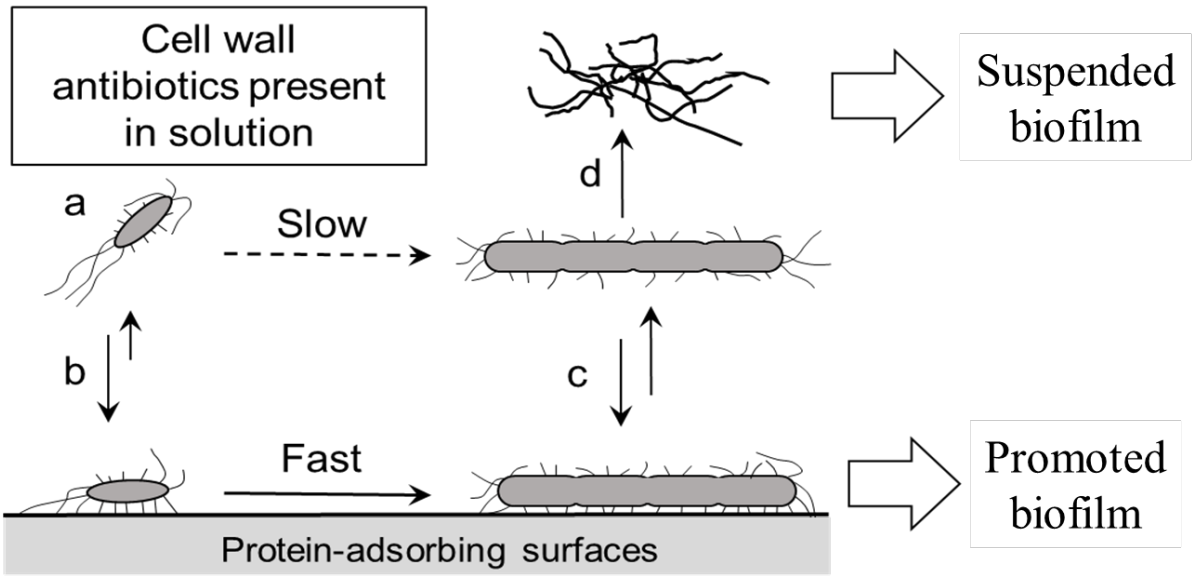


Figure 2.22 Proposed life pattern of filamentous bacteria caused by antibiotics. a. young (lag phase) bacteria. b. Bacterial adherence along with adsorption of protein debris. c. Equilibrium of adherence and detachment of filamentous bacteria from surface that is dependent on bacteria concentration in solution. d. Self-association of filamentous bacteria.

2.2.7 Both protein debris adsorption and filament growth on surface contribute to promoted biomass deposition

Both the growth curve (Figure 2.12) and colony counting (Figure 2.13) indicated significant killing of bacteria by carbenicillin. We examined if the protein debris adsorption from dead bacteria dominates filament growth for the biomass deposition on surfaces over time, and if already formed filaments can adhere and grow on protein–coated surfaces. We first used gel electrophoresis to confirm that there were excessive proteins

in the supernatant when carbenicillin was present (Figure 2.23). To examine the role and the contribution of these proteins to the increase of BLI signals, we first examined the BLI signal of the supernatant of a 3-h *E. coli* RP437 culture with 100 ug/mL of carbenicillin (Figure 2.24A), and compared that to the BLI signal of the 3-h aged *E. coli* RP437 culture with 100 μg/mL. To obtain the supernatant, we sub-cultured the *E. coli* RP437 to an OD_{600} of 0.6, added carbenicillin at 100 μg/mL, and then cultured this solution for 3h. This 3-h carbenicillin treatment caused the OD_{600} to decrease to 0.2. We then added formaldehyde to fix any living bacteria for 16 h, spun down all the solids, and monitored the supernatant for 3 h. We used formaldehyde because the supernatant after removing the bacterial cell pellet still contained viable bacteria without formaldehyde treatment. We found that supernatant from a formaldehyde–treated sample caused a rapid BLI signal increase and plateau quickly in 30 min to a BLI signal close to 1 nm (Figure 2.24A, trace i), a result that is similar to those obtained from adsorption of free proteins (Figure 2.1). Adding formaldehyde (1%) does not cause significant increase in BLI signal (Figure 2.24A, trace ii).

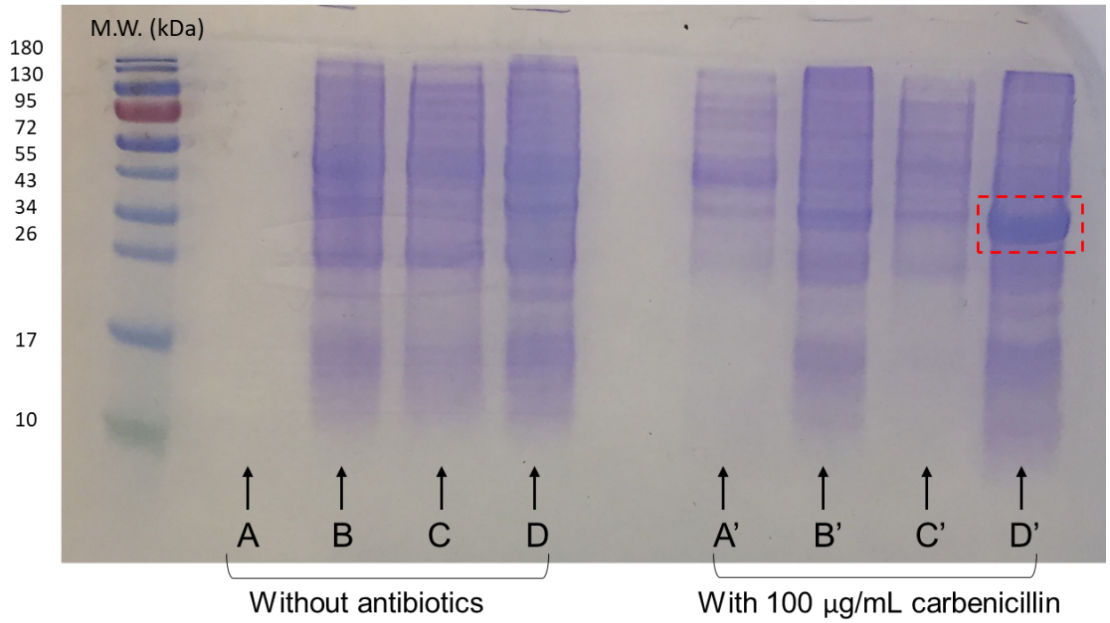


Figure 2.23 Excessive proteins were confirmed in the supernatant when carbenicillin was present. SDS-PAGE of the supernatant and different treatments of the bacterial pellets of *E. coli* RP437 cultures with (A-D) and without (A'-D') 100 µg/mL of carbenicillin. Subcultures of *E. coli* (OD_{600} 0.6) were treated with or without 100 µg/mL of carbenicillin for 3 hours at 37 °C with shaking at 250 rpm (OD_{600} was 0.2 after treatment with carbenicillin). Each subculture was centrifuged at 6,000 rpm for 15 min to separate the supernatant (A and A') from the *E. coli* bacterial pellet. The equal amount (0.2 g) of each pellet was squibbed and resuspended in the equal volume (20 µl) of deionized water to make loading samples B and B', the rest of each pellet (equal amount, 0.2 g) was squibbed and resuspended in equal volume (20 µl) of lysis buffer (10 mL, 0.5 M Tris-HCl, 0.4% SDS) then sonicated at 15-s pulses for ~20 min. Each sonicated mixture was then centrifuged for 20 minutes at 20,000 xg to separate the lysate (C and C') and the bacterial debris (solid pellet with equal amount, 0.2 g), which was squibbed and resuspended in equal volume (20 µl) of deionized water (D and D'). The red box indicates observable over-expressed protein.

For the aged antibiotic-culture of *E. coli* RP437, the bacterial culture from an overnight culture was diluted to an OD_{600} of 0.01 and grown to an OD_{600} of 0.6; then we added carbenicillin at 100 µg/mL and incubated the sample for 3 h at 37 °C with shaking

at 250 rpm. At this stage, we took 5 mL of the culture and added carbenicillin again (5 µL of 100 mg/mL) to 100 ug/mL in culture. Monitoring the BLI signal for another 3 h revealed a two-phase BLI signal profile for both cultures. The first phase showed a rapid increase of BLI signal (2.8 nm/min), almost the same as the BLI signal from the supernatant of formaldehyde-treated culture. The second phase showed a slower increase, but with a large BLI signal (Figure 2.24B, trace iv), which was not observed for any adsorption of free solution proteins in our study. In this second phase, interestingly, adding more carbenicillin actually causes a slightly higher rate of BLI signal increase (Figure 2.24B, trace iii). We note that these aged cultures gave final BLI signals that were larger than with a fresh antibiotic-containing culture (Figure 2.25). We believe that, as the first phase of rapid BLI signal increase is likely due to protein adsorption from lysed bacteria, the large BLI signal in the second phase of aged antibiotic culture is likely due to living bacteria in the solution. Considering the direct observation of filamentous bacteria on surfaces, we believe that this large BLI signal in the second phase is a combination of adherence and growth of filamentous bacteria. We noted that there was a difference between BLI experiments and the microscopy studies. In the BLI experiment, there were bacteria in the antibiotic culture whereas, for the microscopy studies, the adhered bacteria (from a carbenicillin-containing culture) on the surface were cultured in fresh medium with carbenicillin that contains no bacteria.

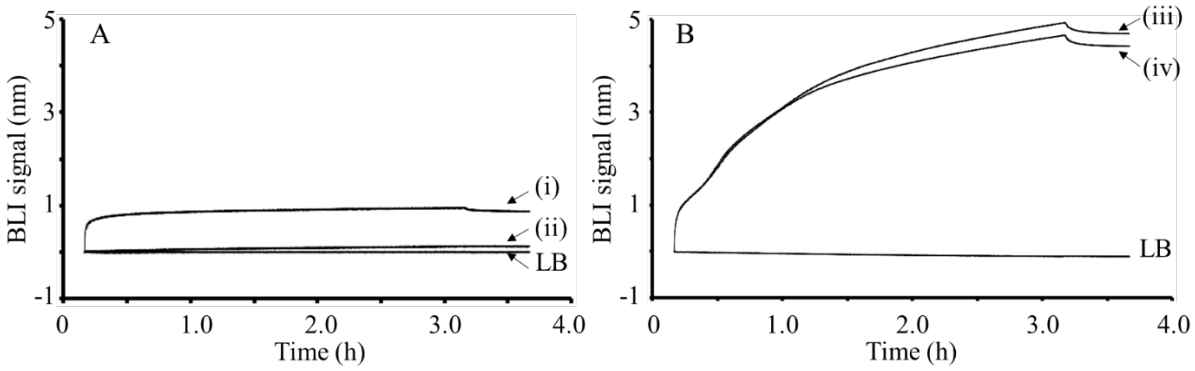


Figure 2.24 (A) *E. coli* RP437 (initial OD_{600} 0.6) was cultured with 100 µg/mL carbenicillin for 3 h, 1% formaldehyde was added for 16 h, all cells/debris were sedimented, then the supernatant was monitored by BLI for 3 h (i). A control monitored 1% formaldehyde in LB (ii). (B) *E. coli* RP437 (initial OD_{600} 0.6) was cultured with 100 µg/mL of carbenicillin for 3 h, resulting in an OD_{600} of 0.2. With (iii) and without (iv) additional carbenicillin added, the samples were monitored by BLI for 3 h. Three experimental replicates were performed.

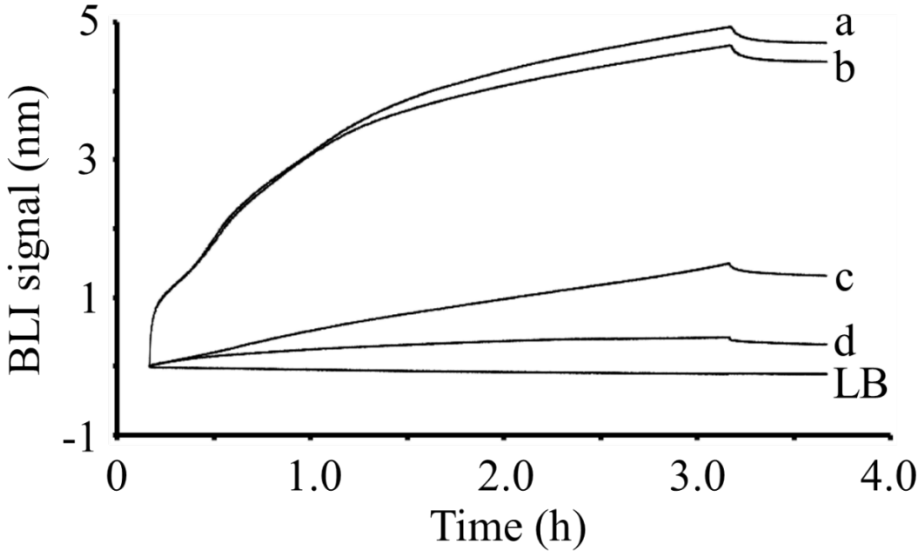


Figure 2.25 The aged cultures gave final BLI signals that were larger than with a fresh antibiotic-containing culture. Comparison of final BLI signals between aged culture and fresh antibiotic-containing culture. *E. coli* RP437 (initial OD_{600} 0.6) was cultured with 100 µg/mL of carbenicillin for 3 h resulting in an OD_{600} of 0.2. Aliquots were transferred to a

96-well plate and monitored for BLI after a second addition of carbenicillin (a) or without (b). As a control, *E. coli* RP437 was cultured without carbenicillin to an OD_{600} of 0.6, then diluted to an OD_{600} of 0.2 and split into two aliquots: one with carbenicillin added to 100 µg/mL (c), the other without carbenicillin (d).

Considering that the filamentous bacteria adhere to surfaces in parallel with protein adsorption, we also examined bacterial adherence on protein coated surface. On APS-tips with Bovine serum albumin (BSA) proteins (0.5 mg/mL and 5 mg/mL), we found that both *E. coli* RP437 cultures with or without carbenicillin resulted in substantial biomass depositions, and the presence of carbenicillin caused about 3–fold increase in biomass depositions (Figure 2.26). Together, these results suggest that antibiotics can promote biofilm formation by two attributes: the adsorption of debris proteins from lysed bacteria, and the enhanced adherence and growth of filamentous bacteria on surfaces.

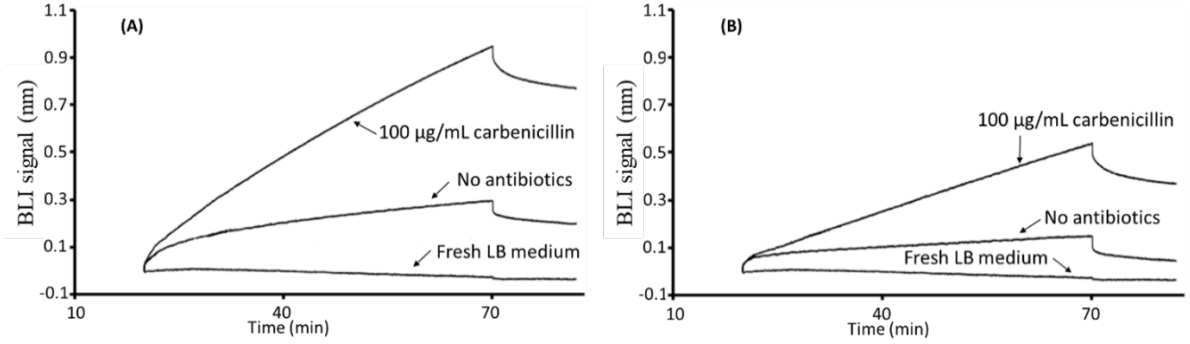


Figure 2.26 The presence of carbenicillin caused about 3–fold increase in biomass depositions on protein coated surface for the *E. coli* RP437 cultures with carbenicillin. BLI signal of *E. coli* RP437 culture with or without antibiotics on APS sensors pre-coated with BSA. APS tips were presoaked in a solution containing BSA at 0.5 mg/mL (A) or at 5 mg/mL (B) for 1 h without taking BLI data, then the pre-soaked tips were transferred into *E. coli* RP437 culture (initial OD_{600} 0.6) with and without 100 µg/mL carbenicillin to monitor BLI signal for 1 h. Fresh LB medium was used as control.

The signaling mechanisms by which antibiotics promote biofilm formation are not entirely clear and are likely different for different classes of antibiotics. Multiple signaling paths may be impacted by antibiotics leading to the promotion of biofilm formation, such as promotion of fimbriae-driven adherence,[13] reduced quorum sensing,[15, 61, 144] increased level of the low bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG),[9] and activated the SOS genes.[176, 177] Among these effects, the key factor for β-lactam is that binding to PBP3 leads to inhibition of bacterial division, and to triggering filament formation,[171] for which the growth is likely mediated by their adherence to a surface.

Our result of surface–driven growth of filaments suggests that the fimbriae/pili-initiated attachment is required for lag-phase bacteria to divide continuously without separation – a correlation yet to be elucidated. A particularly interesting finding is that surface attachment can cause "asymmetric cell divisions" by which two different daughter cells are generated by the bacteria adhered on surfaces.[178-180] Thus, the progeny within each filament grown on surfaces is an interesting topic to explore. Pili-driven surface adherence of bacteria also induces a wide range virulence.[181-183] Whether virulence will be induced by lag-phase bacteria adhering and growing as filaments on surface is another important question to study. Finally, the difference in virulence between filamentous biofilm caused by β-lactam-induced and wild type biofilms, particularly those induced by dispersing bacteria from a biofilm[184], is an important detail to investigate further.

2.3 Conclusion

In this work, we show that certain antibiotics readily cause the formation of filamentous bacteria at concentrations both above and below the MICs. Carbenicillin induces young (for example, in lag phase) bacteria faster and more effectively to form

filamentous bacteria than that of fast-growing bacteria. The growth of filaments in the form of elongation proceeds when the bacteria adhere on a surface, a phenomenon readily observable when the surface-adhered bacteria are in a medium containing only antibiotics, but no bacteria. Filamentous bacteria in these antibiotic cultures form aggregates, suspended films, and promoted biofilms with substantial amount of protein adsorption. When a surface does not adhere bacteria or adheres fewer bacteria, few filaments were observed in the solution, indicating that surface adherence plays a strong role for the growth of filamentous bacteria. These results introduce the unreported lifestyle of filamentous bacteria and could form the basis for a wide range of biological studies, including considerations of engineering applications of filamentous bacteria.

2.4 Materials and Methods

2.4.1 Materials

Lysogeny broth (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.2) was used to grow bacterial strains. Agar plates (1.5% Bacto-agar in LB without antibiotics) were prepared for bacteria counting. 4×Tris buffer (1.5 M, pH 8.8) was prepared by dissolving 181.71 g of Tris base in 800 mL of distilled water followed by adjusting solution to desired pH using HCl (pH = 8.8), then add distilled water until volume is 1 L. Falcon™ 50mL Conical Centrifuge Tubes were from Fisher Scientific. “BLI plates” (Greiner Bio-One E&K Scientific, 96-well, flat bottom, black) and aminopropylsilane (APS)-coated biosensor tips were purchased from ForteBio. The BLI experiments were performed on Octet® RED96 and Octet® RED384 systems. APS-coated slides (Silane Coated Microscope Slides, LabScientific, CAT# 7801B) and Microscope Glass Slides (Fisherbrand, 24 × 60 mm, 0.13 to 0.17 mm, LOT# 072312-9) were used for microscopy

analysis of filaments. Polystyrene petri dishes were from Fisher Scientific (100 × 15 mm, CAT# FB0875712, sterile). Polystyrene slides (~ 2 × 2 cm) were cut from the plastic (polystyrene) petri dish by using a tin snip. Glass petri dish (60 × 20 mm, 10/Pk, CAT# 391-0576) were from VWR International. Lysozyme (≥ 95%), bovine serum albumin (BSA, ≥ 96%), boric acid (≥ 99.5%), carbazole (≥ 95%), colistin (powder), tetracycline (powder, ≥ 98.0% NT), chloramphenicol (powder, ≥ 98% HPLC), aztreonam (powder, ≥ 98% HPLC) were purchased from Sigma-Aldrich. Carbenicillin was purchased from TEKNOVA (100 mg/mL, 25 mL). Sonic dismembrator (model 100) was purchased from Fisher Scientific. Transmission electron microscrope (TEM) grid (Formvar/Carbon on 300 mesh, Ted Pella, Inc.) and 2% Uranyl Acetate Solution (Thomas Scientific) were used for TEM imaging.

2.4.2 Bacterial cultures

Serratia marcescens and *Paenibacillus dendritiformis* were from Agricultural Research Service Culture Collection (NRRL) (Gift from Garza Lab). *E. coli* RP437[145, 185] and *E. coli* NEB5α[186] were from New England Biolabs® Inc. (Gift from Korendovych Lab). Non-fimbriated *E. coli* LE392[163-165] was obtained from the Coli Genetic Stock Center (CGSC, Yale University). *E. coli* HB101[166] was purchased from MCLAB. For each experiment, bacteria were cultured in LB medium at 37 °C overnight at 250 rpm to about optical density of 1.0 (measured by OD_{600}), then sub-cultured to different targeted optical densities.

2.4.3 Methods

General.[150] Each BLI experiment included soaking and transferring the tips consecutively in different solutions for pre-determined periods: preconditioning in LB (10 min),

“association” with protein or bacteria in LB (~3 h) and “dissociation” in just LB (~30 min). 250 µL of each sample (protein or bacterial culture, see below) were transferred into each well of a “BLI plate”. The BLI signals were monitored for pre-determined times at 37 °C with shaking of the “BLI plate” at 100 rpm. BLI monitored fresh LB medium through the pre-determined time as a control.

Protein adsorption and bacterial adherence. Protein solutions of different concentrations (1 mg/mL and 3 mg/mL of lysozyme and BSA) were prepared in fresh LB medium, and filtered (0.45 µm, Cellulose Acetate). Subcultures of different bacterial species: overnight cultures of *Serratia marcescens*, *E. coli* RP437, *E. coli* NEB5α, *Paenibacillus dendritiformis* and *E. coli* LE392 were diluted 100-fold by LB medium, and then grown to OD_{600} 0.6 (or other desired OD_{600}) in Falcon tubes at 37 °C with shaking at 250 rpm with a loose cap before transfer to wells of BLI plates.

Effects of different antibiotics on biomass deposition. Subcultures of *E. coli* RP437 that grew to OD_{600} ~ 0.6 were supplemented with different concentrations of antibiotics in carbenicillin, 9 µg/mL and 100 µg/mL (from a stock of 100 mg/mL); colistin, 0.1 µg/mL and 10 µg/mL (from a stock of 10 mg/mL); tetracycline, 0.5 µg/mL and 50 µg/mL (from a stock of 5 mg/mL); and chloramphenicol, 2 µg/mL and 170 µg/mL (from a stock of 15 mg/mL).

Effect of aged antibiotic culture on biomass deposition. A subculture of *E. coli* RP437 was grown to an OD_{600} of 0.6, and then supplemented with carbenicillin at 100 µg/mL, and cultured at 37 °C for 3 hours at 250 rpm, which resulted with an OD_{600} of 0.2. A volume of 5 mL of this culture was supplemented with 5 µL of 100 mg/mL of carbenicillin. These two cultures, *E. coli* RP437 cultured with 100 µg/mL carbenicillin, with and without

additionally added carbenicillin (5 µL of 100 mg/mL in 5 mL) at 3 h were monitored for BLI signals for additional 3 h.

The growth curve of E. coli RP437 in a culture with carbenicillin. Cultures of *E. coli* RP437 (10 mL) were supplemented with carbenicillin at a final concentration of 100 µg/mL in Falcon tubes. All samples in Falcon tubes were cultured in shaker at 250 rpm and 37 °C for targeted time. Measurement of OD_{600} was performed every 15 min by measuring 200 µL of each bacterial culture in 96-well plate using BioTek™ ELx800™ Absorbance Microplate Reader. A subculture diluted from an overnight culture to give the equivalent of an initial OD_{600} 0.01 without carbenicillin was measured throughout the period of experiment.

Microscopy studies of suspended film and aggregation of filaments. Subculture of *E. coli* RP437 (10 mL, OD_{600} 0.6,) with 100 µg/mL carbenicillin were cultured in a Falcon tube with shaking at 250 rpm and in a polystyrene petri dish without shaking, respectively, at 37 °C for 3 h. Hydrated and slimy aggregates formed in Falcon tubes, and suspended films in polystyrene petri dishes, were pipetted out and sandwiched between two microscope glass slides and examined under a polarizing light microscope.

Microscopy studies of surface-growth of filamentous bacteria. Carbenicillin was added to *E. coli* RP437 subcultures that were grown to OD_{600} of 0.1, 0.2, 0.5, and 0.8. The culture with OD_{600} 0.8 was diluted to 0.2. Right after adding the carbenicillin, 5 mL of culture was transferred to glass petri dishes (6 cm-diameter). An APS-coated slide, a polystyrene slide, and a microscope glass slide were immersed in the same culture for ~3 min. Each substrate was then transferred to separate glass petri dishes containing fresh LB with

carbenicillin (100 μg/mL) and cultured at 37 °C without shaking for targeted time periods. At different time points, the surface of each slide was directly examined in the culture in glass petri dishes under microscope. Polystyrene slides were cut to about 1×1 cm from polystyrene petri dishes by using a tin snip.

Chapter 3: Saturated farnesol derivatives inhibit all three types of horizontal gene transfer mechanisms

3.1 Introduction

The drug resistance of bacteria is becoming more severe since antibiotics were first discovered. The development of drug-resistance among bacteria involves spreading and drug-resistant gene "horizontally" between bacteria.[99-107, 109, 111-117, 132, 187-192] Bacteria can acquire and exchange DNA materials from and with their environments, and from other species rather effectively.[99-107, 109, 111-116, 187, 192] These horizontal gene transfers impact the gene composition and evolution of the bacteria,[115, 117] and facilitate the transfer of antibiotic resistance genes.[113, 114, 116] At the molecular level, all three major mechanisms of HGTs, transformation,[102] conjugation,[106, 107] and transduction,[112] involve type IV pili appendages on bacterial surfaces.

Horizontal gene transfer between bacteria transfers mobile genetic elements[193-195] that are composed of plasmid, integrative and conjugative elements (ICE) and virus (bacteriophage). Bacterial Integrative and conjugative elements (ICEs) were found in both gram-positive and gram-negative bacteria.[194, 195] ICEs are self-transmissible genomic islands that can exist as circular extrachromosomal elements, like plasmid.[195] Also, ICEs transfer between bacteria are phage-associated.[194] Two pathogenicity islands were identified and characterized in *Pseudomonas aeruginosa* as ICEs: *P. aeruginosa* pathogenicity island 1 (PAPI-1, 108 kb) and *Pseudomonas aeruginosa* pathogenicity island 2 (PAPI-2, 11 kb).[106, 107, 196, 197] Both PAPI-1 and PAPI-2 can be integrated at into bacterial chromosome. *Pseudomonas aeruginosa* pathogenicity islands (PAPIs) encode several regulatory genes that control toxins, biofilm formation and antibiotic-resistance

traits.[198, 199] These integrative and conjugative elements (ICEs) were found in virulent strain PA14, and absent from the less virulent strain PAO1,[106, 107, 197] but are transferable between *P. aeruginosa* strains.[106]

Another way that DNAs get transferred between strains are by bacteriophages. Bacteriophages are viruses that infect specifically bacteria. Bacteriophage can be of a lifestyle described as of a lytic cycle or a lysogenic cycle.[31, 195, 200-203] Lytic (virulent) phages infect host bacteria, and then replicate the viral DNA and assemble new phages in host cell with high population of viruses leading to lysis of the host bacteria, resulting in releasing of the new phages. In contrast, lysogenic (temperate) phages do not lyse host bacteria cell. After infection, the DNA from a lysogenic phage is incorporated into the host bacteria's genome and passed on to daughter cells along with the genome. The lysogenic phage DNA that is incorporated in hose cell genome may be excised and converted to the lytic cycle under certain stresses such as antibiotics and UV irradiation.[31] There are over 60 different lysogenic phages found in bacteria, and most *P. aeruginosa* isolates are thought to be lysogenized.[195]

Transformation describes bacteria acquiring gene materials from their surroundings, and can transform into a different phenotype.[99, 100, 102, 103] There are several different ways that facilitate bacterial uptake of DNA materials from the environment,[113]. Heat shock can cause the cells to take up plasmids from surrounding culture.[204] An electromagnetic field can also carry out the bacteria taking plasmid by inducing cell membrane permeability.[205] Calcium chloride is also used to create transient pores on the bacterial cell wall for the entry of foreign DNA.[206] In a natural setting, pili appendages on bacteria have been shown to directly bind and retreat the DNAs close to the bacterial

surfaces, and enable the intake of DNAs (Figure 3.1).[102] DNA uptake (transformation) by type IV pili was directly observed for *Vibrio cholerae*.[102] Pili were described as "gatekeepers" to allow DNA to cross the outer membrane, and the bound DNA could be brought to bacterial surface by pili retraction.[102] However, the details of how pili-attached DNAs enter the bacterial cell is still unclear. Literature describes a two-step process. [102] [100] [207-209] [208, 210] (1) DNAs are retracted with pili through the pores formed by pili machinery into the interior side of the outer membrane. Pili could act as " gatekeepers" [102] to open the outer membrane secretin pore PilQ,[100] allowing for passive DNA diffusion across the outer membrane. This process may happen by the pili appendages retracting into the interior of the bacteria, leaving an open pore in the outer membrane for DNA influx.[207-209] (2) The DNAs that crossed the outer membrane may then be translocated to cross the inner membrane into the cytoplasm through ComA, a bacterial inner membrane protein.[208, 210]

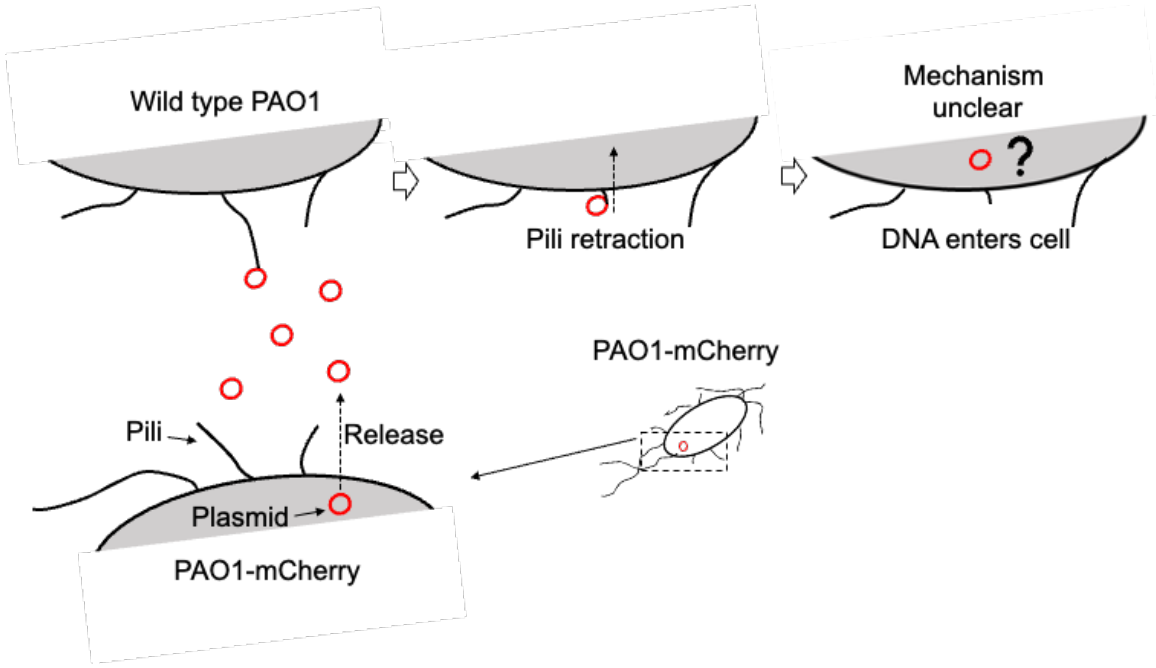


Figure 3.1 Scheme of bacterial transformation. Plasmid is released from donor bacteria into the environment. Recipient bacteria's pili appendages bind and "pick" the plasmid and retract, bringing the DNA to nearby cell membrane. The details of how plasmid enter bacterial cell from near bacterial surface is still unclear.

Transduction is the process that foreign DNA is transferred by bacteriophage adsorption[109, 111, 129-131] Transduction involves one bacterium expressing the DNA encoding the bacteriophages (this DNA is called prophage), which adsorb on the pili appendages of the recipient bacteria. As the pili of the recipient bacteria retract, the adsorbed phages on bacterial surfaces insert their DNA into the recipient cells (Figure 3.2).[132] Bacteriophage could infect host bacteria and either incorporate their viral genome into the host genome to replicate as part of the host bacteria (lysogenic cycle), or multiply inside the host bacteria (lytic cycle) then release new phages into the surroundings. Usually these host bacteria carry plasmids that frequently encode antibiotic resistance.[109, 111] This antibiotic resistance marker enables the bacteriophage to act as a vector to carry

genetic elements from donor cells to recipient cells,[130] and/or to increases the concentration of plasmid in the environment by lysing host cell.[131]

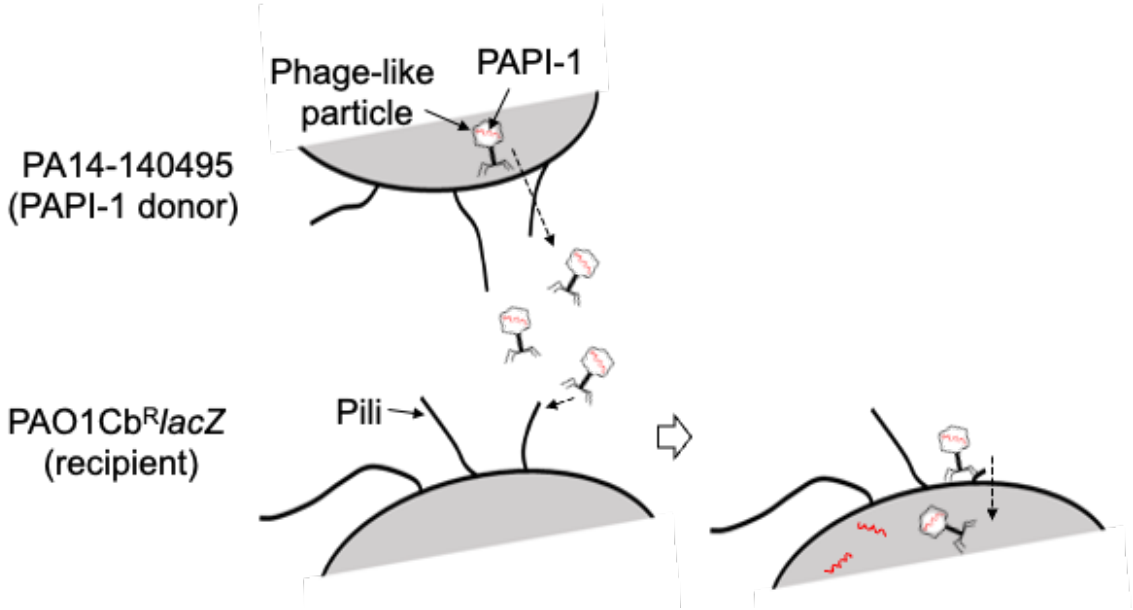


Figure 3.2 Scheme of bacterial transduction. Phage-like particle is biosynthesized and released from donor bacteria through cell lysis. The released phages adsorb on recipient's pili, which retract and bring phage to bacterial surfaces. The phages on bacterial surface infect the recipient bacteria by inserting its DNA through the bacterial membrane and may subsequently be integrated into the bacterial genome.

Conjugation involves the donor strains of bacteria extending their pili to make direct contact with the recipient strains to facilitate the transfer of the DNA (Figure 3.3).[105, 107] During conjugation, the donor cell uses a pilus, a thin and tube-like structure,[102, 107, 129, 133-137] to contact a recipient cell and retract to bring the two cells together, at which time the donor cell transfers the genetic material to the recipient cell. The detail about how genetic material get transferred during conjugation is still unclear, but conjugation is believed to require direct physical contact between donor and recipient.[105-108, 129] A

particular type IV pili, PilS2, was found to exclusively mediate PAPI-1 transfer in *P. aeruginosa*.[107]

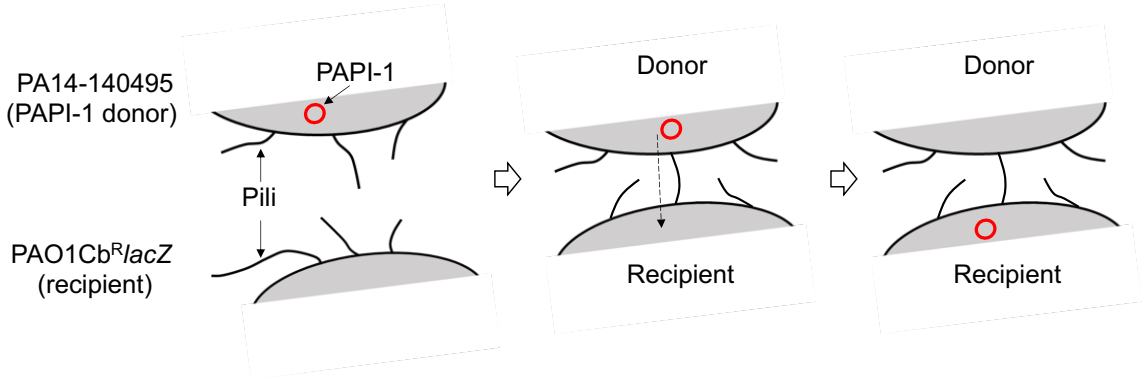


Figure 3.3 Scheme of bacterial conjugation. Donor bacteria's pili appendages make direct contact with the recipient bacteria, facilitating the close contact between two bacteria. Plasmid is transferred from donor to recipient cell through pili. The details of how plasmid enters recipient cell is still unclear.

Horizontal gene transfer is one of the principal routes for bacteria to develop antibiotic resistance. However, there is not many bioassays to monitor this resistance development among bacteria. Serial passage assay is widely used for evaluating the development of antibiotic resistance at progressively increasing concentrations of the tested agents.[189, 191] The development of antibiotic resistance among tested bacteria is assessed by measuring minimum inhibitory concentrations (MICs). In serial passage assay, genetic mutations that causes antibiotic resistance is generally believed to be passed through generations of bacterial proliferation. Such "vertical" transfer of genetic information is believed to be the principal mechanism for the development of drug resistance in multi-passage assays. The contribution from horizontal gene transfer has not been extensively considered and studied. Here, we hypothesized that the development of antibiotic resistance occurring in serial passage culturing may be

substantially enabled by horizontal gene transfers, especially when antibiotics are repeatedly applied with increased dosage.

In this work, we first demonstrate that tetracycline enhances the plasmid transformation for wt PAO1. We show that ciprofloxacin induces phage production by a PA14 strain that carries the gene of *Pseudomonas aeruginosa* pathogenicity island (PAPI-1), and that the phage produced also carries PAPI-1 gene. Then, we show that a class of saturated farnesol derivatized with tetra(ethylene glycols)s and tetra(ethylene glycol)-epoxy (Figure 3.4) inhibit all three horizontal gene transfer mechanisms, transformation, transduction, and conjugation described above for *Pseudomonas aeruginosa* strains. We also demonstrate the inhibition of development of drug resistance during repeated application of the antibiotic, ciprofloxacin, to *P. aeruginosa* over a multi-passage process. Ciprofloxacin, a second generation of fluoroquinolone antibiotic, is widely used to treat infections caused by *P. aeruginosa*. The resistance to Ciprofloxacin has been reported in *P. aeruginosa*.[189] No inhibition of this resistance development has been reported. In this work, we proposed that dosing Ciprofloxacin with $SFEG_4OH$ that bind and inhibit pili appendages can inhibit the development of antibiotic resistance in *P. aeruginosa*.

3.2 Results and Discussion

3.2.1 Past results from Luk group that lead to the hypothesis of chemical inhibition of horizontal gene transfers

Many bacteria are able to translocate as a population to cross surfaces, which is defined as swarming motility.[211] This swarming motility needs functioning bacterial type

IV pili. [212] Previous studies in Dr. Luk's lab on *P. aeruginosa* swarming has shown that saturated farnesol derivative could inhibit bacterial swarming,[213] and the molecule also binds to pili.[214] Those results inspire this work to study if saturated farnesol derivatives could also inhibit HGT mechanisms that are also pili mediated. To study the inhibition of horizontal gene transfers, we studied a class of saturated farnesol derivatives as pili inhibitors, including $SFEG_4OH$, $SFEG_4Oepoxy$ and a control molecule $C_{12}EG_4OH$ (Figure 3.4). While these molecules that were synthesized by Felicia Burns are shown to bind to pili appendages, and inhibit pili-driven swarming motility in Luk lab, [213-215] they have not been studied for their effects on horizontal gene transfers.

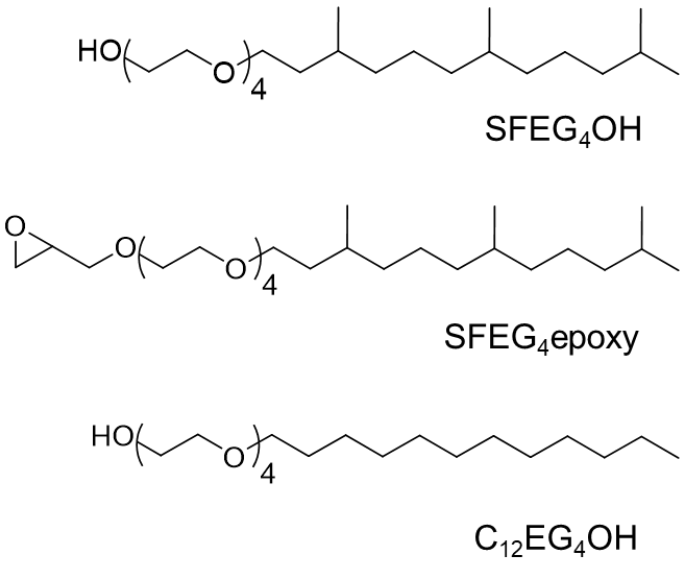


Figure 3.4 Ligands, $SFEG_4OH$, $SFEG_4Oepoxy$; and control molecule $C_{12}EG_4OH$, for binding and inhibiting pili appendages (Molecules were synthesized by Felicia Burns: *Burns, F.N., Alila, M.A., Zheng, H., Patil, P.D., Ibanez, A.C.S. and Luk, Y.Y., 2021. Exploration of Ligand-receptor Binding and Mechanisms for Alginate Reduction and Phenotype Reversion by Mucoid Pseudomonas aeruginosa. ChemMedChem, 16(12), pp.1975-1985*).

3.2.2 Inhibition of tetracycline-enhanced transformation

Transformation is one of HGT mechanisms in bacteria, in which DNA molecules from the environments are taken up by bacteria using pili appendages (Figure 3.5).[102] The pili-bound DNA can be retracted with pili to the cell surface,[102] and be bought into bacterial cell, While the exact details of how pili-bound DNA enter the bacterial cells is not clear, presence of functioning pili has been shown to be necessary.

To examine the inhibition of horizontal gene transfer by transformation, we first confirm the transformation (DNA plasmid uptake from environment) by *P. aeruginosa*. Antibiotics were reported to promote HGT.[74, 118] For example, several antibiotics could promote the expression of conjugation machinery to enhance the conjugation among gram-negative bacteria.[74] We confirmed here that sublethal amount of an antibiotic, tetracycline promoted the transformation of PAO1. We prepare a plasmid-containing supernatant by sonicating the culture of PAO1-mCherry (with tetracycline marker, OD ~1.0; gifted by Dr. George O'Toole), and pelleted and removed the bacterial cell debris. We then mixed the supernatant (5 mL) with the culture of a recipient strain (OD ~1.0, 1 mL), wide type PAO1 culture, with and without the presence of sublethal amount (20 μg/mL) of tetracycline, and cultured for another 24 h. We found that culturing the supernatant from sonicated strain with PAO1-mCherry and wt PAO1 mixture with 20 μg/mL of tetracycline resulted in 1.8 x 10^8 CFU/mL counted on agar plate containing 250 μg/mL of tetracycline, but only 7.2 x 10^2 CFU/mL for culturing the mixture without tetracycline (Figure 3.5). Wild type PAO1 without the supernatant from PAO1-mCherry (Tc^R) did not result in any bacterial growth on the agar plate.

This result indicated that transformation does occur naturally in culture, and that sublethal amount of tetracycline caused an increase of population of tetracycline resistant strains by about 5 logs ($2.7x10^5$). We note that at 20 μg/mL, tetracycline does not kill PAO1 bacteria. Hence, the selection of pre-existing tetracycline-resistant bacteria to grow is unlikely. We believe this increase of tetracycline-resistant strains by 20 μg/mL tetracycline can arise from two different mechanisms. First, the presence of tetracycline promotes transformation: the uptake of DNA from supernatant in the mixed culture by the recipient strains, and the transformed strains are then selected and amplified by the presence of tetracycline. Second, tetracycline at 20 μg/mL may promote spontaneous mutation that cause more tetracycline-resistant strains without killing other bacteria. To find out the mechanism of the promotion, a study is proposed in future work.

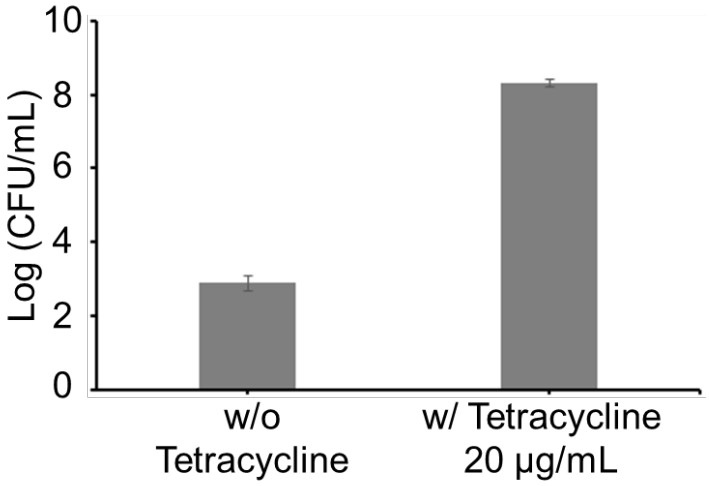


Figure 3.5 Log of colony forming units (CFU) per 1 mL of wt PAO1 culture (24 h) mixed with plasmid-containing supernatant from PAO1-mCherry culture (3 h subculture, sonicated and filtered, and with and without Tetracycline (20 μg/mL). Error bars were obtained from three experimental replicates.

In the presence of 100 μM of $SFEG_4OH$, we found that the transformation enhanced by tetracycline was reduced from 2.0×10^8 to 5.7×10^5 CFU/mL, about a 3-log reduction (Figure 3.6A). A concentration study of $SFEG_4OH$ indicated an IC_{50} around 35 μM (Figure 3.6B). The molecule, $SFEG_4Oepoxy$ (100 μM), showed a similar inhibitory effect, which reduced the tetracycline-enhanced plasmid transformation from 2.0×10^8 to 1.8×10^5, also a 3-log reduction (Figure 3.6A). Both the $C_{12}EG_4OH$ and sodium dodecyl sulfate (SDS) did not show observable inhibition of the plasmid DNA uptake/transformation.

Swarming motility of bacteria is believed to be pili mediated.[211, 212] Previous study of swarming inhibition of PAO1 in Dr. Luk's lab suggested that $SFEG_4OH$ inhibited swarming of PAO1 at around 5 μM in the hydrated agar, whereas $C_{12}EG_4OH$ did not show much swarming inhibition.[213] Those results are consistent with our transformation inhibition results. However, the swarming inhibition occurred at 5 μM that is lower than the concentration needed for inhibiting transformation. We believe that the swarming motility is more sensitive to pili dynamics, relative to control of HGTs. Pili dynamics, extension and retraction, is required for swarming motility. For instance, a mutant pilT, for which the dynamics of pili is impaired does not swarm.[216] We believe that our molecules cause some retraction of pili, but may not be complete for all pili at all times on the bacterial surfaces. While such reduction in pili dynamics inhibit swarming motility entirely, a more complete retraction of pili may be needed to have an effective inhibition of transformation.

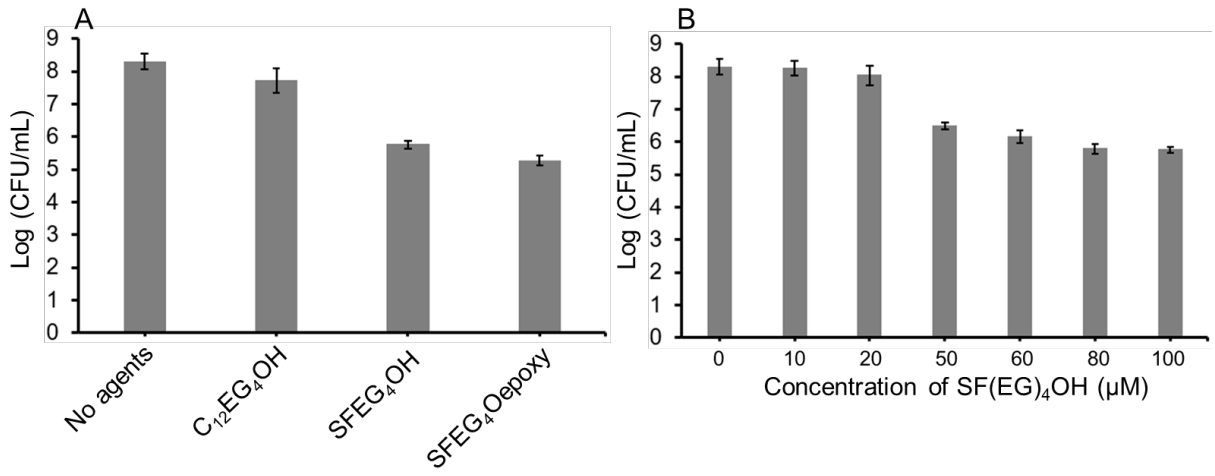


Figure 3.6 Log of colony forming units (CFU) per 1 mL of wt PAO1 culture (24 h) mixed plasmid-containing supernatant from PAO1-mCherry culture (3 h, sonicated and filtered, in the presence of Tetracycline (20 μg/mL), and (A) with 100 μM of $C_{12}EG_4OH$, $SFEG_4OH$ or $SFEG_4Oepoxy$; (B) with different concentrations of $SFEG_4OH$. Error bars were obtained from three experimental replicates.

3.2.3 Ciprofloxacin induces bacteriophage production to enhance PAPI-1 transduction

We use bacteria strains that are established in studying conjugation, which are generously provided by Professor Stephen Lory. [106] These bacteria consist of a donor strain and a recipient strain. The donor strain, *Pseudomonas aeruginosa*, PA14 (PAPI-1), carries *Pseudomonas aeruginosa* pathogenicity island-1 (PAPI-1) that has been incorporated with a gentamicin marker. The recipient strain, PAO1Cb^R*lacZ*, does not harbor PAPI-1. The *lacZ* gene in PAO1Cb^R*lacZ* encodes β-galactosidase that can hydrolyze X-gal, an analog of lactose, into glucose and 5-bromo-4-chloro-3-hydroxyindole, which is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo which has a blue color. The transfer of PAPI-1 introduced gentamicin (Gm^R) marker to the recipient strain, which also

carried carbenicillin (Cb^R) marker. Only PAO1Cb^R*lacZ* that acquired PAPI-1 (Gm^R) could survive on the selective agar containing gentamicin, carbenicillin and X-gal.

Confirmation of ciprofloxacin-induced bacteriophage production by PA14-PAPI-1. Transduction is another HGT mechanism for bacteria to transfer genetic material including antibiotic resistance genes from one to another. The genome of a bacterium often encodes bacteriophages. Under certain stresses, such as antibiotics and UV irradiation, the bacteriophages are expressed, the overpopulation of phages lyse the hosting bacteria, release the phages into environment, and infect other bacteria.[84]

Lysogenic phages insert and integrate their DNA into the circular host's DNA chromosome. This integrated phage DNA is called prophage that is thought as the latent form of a bacteriophage. However, prophage can be excised and converted to virulent bacteriophage, as known as the lysogenic to lytic cycle. Interestingly, the *Pseudomonas aeruginosa* pathogenicity island (PAPI-1) was originally integrated in the chromosome of PA14. After excision, PAPI-1 can convert to an extrachromosomal circular form to be transferred between different *P. aeruginosa* strains. This whole process is similar to the conversion process of prophage to phage in bacteria. Based on the literature, *Pseudomonas aeruginosa* pathogenicity islands (PAPI-1) gene cluster may contain phage DNA, but is not shown or confirmed, perhaps because no one has used ciprofloxacin to stimulate the bacteria. Therefore, we were thinking the PAPI-1 gene cluster may contain phage DNA.

Several antibiotics are reported to induce phage production in *P. aeruginosa* isolates.[89, 202, 217] Specifically, ciprofloxacin at 4 μg/mL induced Liverpool epidemic strain (LES) phages production, which was confirmed by plaque assay.[217] In another study, the

phage tail particles were observed by electron microscopy in the supernatants of *P. aeruginosa* cells treated with ciprofloxacin.[89] Also, antibiotics that cause DNA damage, such as Ciprofloxacin and Mitomycin, are reported to induce the lytic cycle of lysogenic phages.[89, 202]The presence and concentration (titer) of a bacteriophage culture is often determined by a plaque assay.[218] On a double-layer agar plate (0.7% and 1.5% LB agar), bacteria lawn is grown followed by inoculation of bacteriophage-containing culture on the top agar (0.7% LB agar). After couple hours culturing at 37℃, the titers (the clear zone) are observed on agar indicating the plaque-forming units (PFU) of bacteriophage (Figure 3.7).

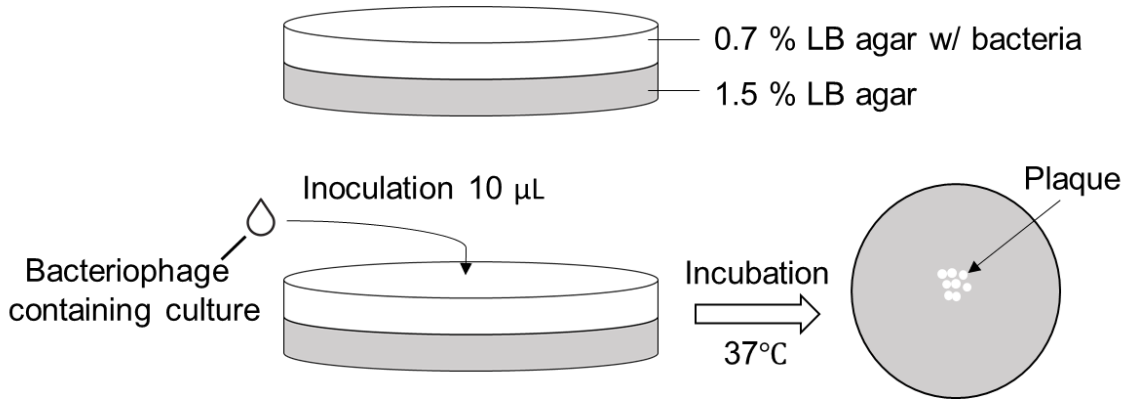


Figure 3.7 Scheme of bacteriophage plaque assay. The double-layer agar consisted of 0.7% LB agar containing bacteria (0.6 OD in LB mixed with 100 μM $SFEG_4OH$) overlay on top of 1.5% LB agar (without bacteria). Bacteriophage (10 μL) containing culture was inoculated on the top agar and was cultured for about 3 hours for plaque formation.

To examine if ciprofloxacin induces bacteriophage production in PA14 (PAPI-1), we first need to test if PA14 (PAPI-1) can generate phage. To confirm the phage induction, we examined five different *P. aeruginosa* strains to grow as bacterial lawn. Wild type PAO1, PAO1-Cb^R*lacZ*, PAO1k, ΔpilA, and PA14 (PAP1-1). *Pseudomonas aeruginosa*

strain incorporated with carbenicillin resistance biomarker and *lacZ* gene, PAO1-Cb^R*lacZ*, will be used for the conjugation assay. PAO1k is a wild type φKMV-sensitive strain.[132] φKMV, a lytic bacteriophage from *Podoviridae* family, requires type IV pili for infections.[219] *Podoviridae* is a family of 130 virus species that are classified into 3 subfamilies, such as Autographivirinae subfamily that includes PhiKMV-like viruses and T7-like viruses.[220-222] *Podoviridae* viruses are short in length and they carry linear double-stranded DNA.[220] PAO1k has been widely used as host for bacteriophage adsorption study.[132] These three strains all may have susceptibility to be lysed by phage. The mutant strain, ΔpilA has impaired production of pilin monomer of pili appendages.[223] As pili is required for phage adsorption and subsequent injection of DNA materials, ΔpilA may not be lysed by phage particles. The PA14 (PAP1-1) is the strain for phage particle production, and thus may have resistance for phage-caused lysis.

First, we found that supernatant of the PA14 (PAPI-1) cultured with ciprofloxacin (0.05 μg/mL, see experimental for details) readily caused plaques on bacterial lawn of wt PAO1, PAO1k, and PAO1Cb^R*lacZ.* Only faint plaques for PA14 (PAP1-1) were observed. However, this supernatant did not cause any plaques on the lawn of ΔpilA mutant (Figure 3.8). These results indicate that the supernatant of PA14 (PAPI-1) contains phage that could cause universal lysis of different strains of *P. aeruginosa*. *P. aeruginosa* PA14 shows some resistance likely because PA14 has be reported to have a CRISPR/Cas adaptive immune system that may help itself to resist phage infection.[224, 225] Bacteria could incorporate phage DNA into their CRISPR cassette to “remember” it. Upon later the same phage infection, the CRISPR cassette would be recruited to match and cleave the phage DNA.[225] The survival of ΔpilA mutant suggest that phage adsorption is pili

mediated. These results are consistent with the hypothesis that ciprofloxacin caused the formation of phage-like particles,[89-93] and the killing of bacteria leading to plaque formation is likely mediated by adsorption of the phage-like particle on pili appendages of *P. aeruginosa*.

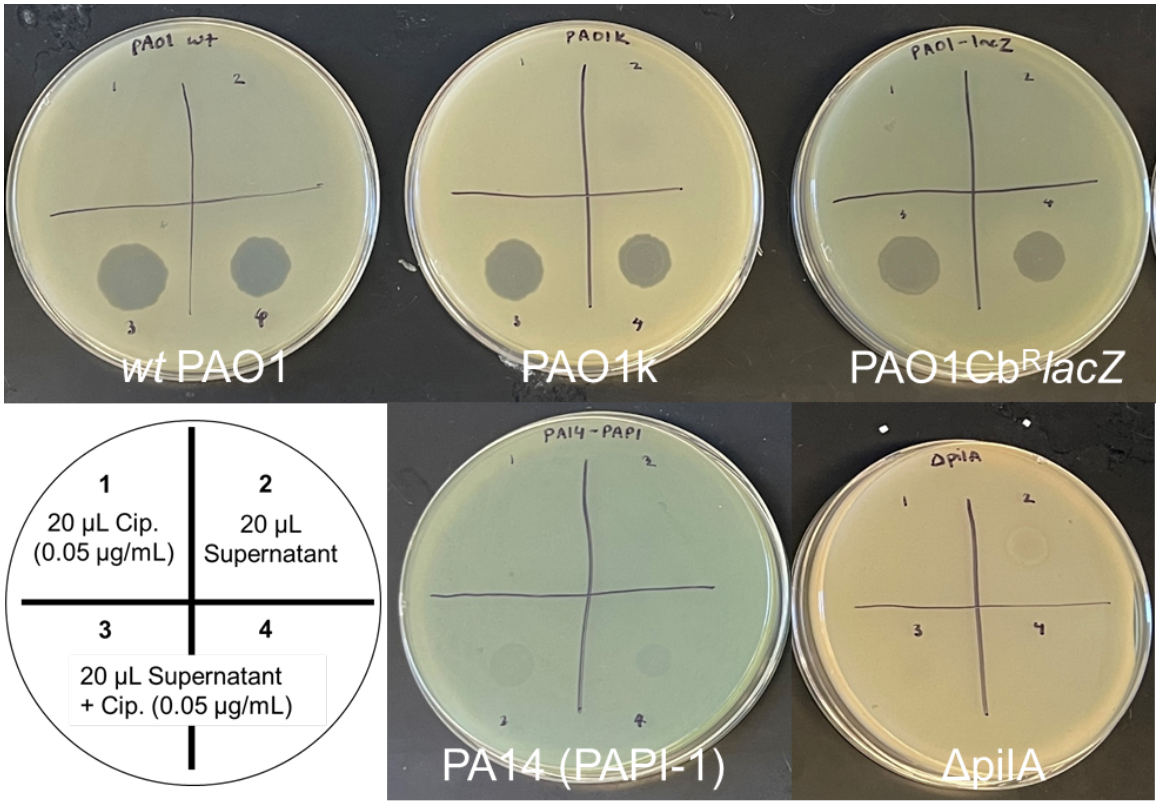


Figure 3.8 Plaque formation on five different bacterial lawn, PA14 (PAPI-1), wt PAO1, PAO1k, PAO1Cb^{R}*lacZ* and ΔpilA. On each lawn, four different inoculation conditions were introduced, (1) 20 µL of 0.05 µg/mL ciprofloxacin solution, (2) 20 µL of bacterial supernatant of PA14 (PAPI-1) without antibiotics, (3 & 4) 20 µL of 0.05 µg/mL ciprofloxacin co-cultured supernatant. Plaque formation on bacterial lawn was photographed after 3 h culturing at 37℃ statically.

Second, we also confirmed that ciprofloxacin promoted transduction of PAPI-1 gene to recipient strains. In order to confirm the mechanism of the ciprofloxacin-enhanced PAPI-1 transfer, we filtered (0.22 μm) the supernatant of PA14 (PAPI-1) to remove all PAPI-1 donor cells. Furthermore, we added universal nuclease (0.5 μL of 10 kU stock) to the supernatant of PA14 (PAPI-1) to remove all genetic materials. Those two procedures reduce or eliminate gene transfer by conjugation or transformation between the supernatant of PA14 (PAPI-1) and PAO1Cb^R*lacZ*.

The *lacZ* gene in PAO1Cb^R*lacZ* encodes β-galactosidase that hydrolyze X-gal, an analog of lactose, into 5-bromo-4-chloro-3-hydroxyindole to show blue color. The transfer of PAPI-1 from PA14 (PAPI-1)_ introduced gentamicin (Gm^R) marker to the recipient strain, which also carried carbenicillin (Cb^R) marker. Thus, on the selective agar containing gentamicin, carbenicillin and X-gal, only PAO1Cb^R*lacZ* that acquired PAPI-1 (Gm^R) could survive and will show blue color. We found that ciprofloxacin (0.05 μg/mL) enhances the PAPI-1 transfer from supernatant of PA14 (PAPI-1) to PAO1Cb^R*lacZ*, and blue colonies were observed (Figure 3.9). Besides the blue colonies, some white colonies were also observed on agar. Those similar white colonies were also observed by Dr. Stephen Lory's group in conjugation assay. They believed that those white colonies were spontaneous mutants.

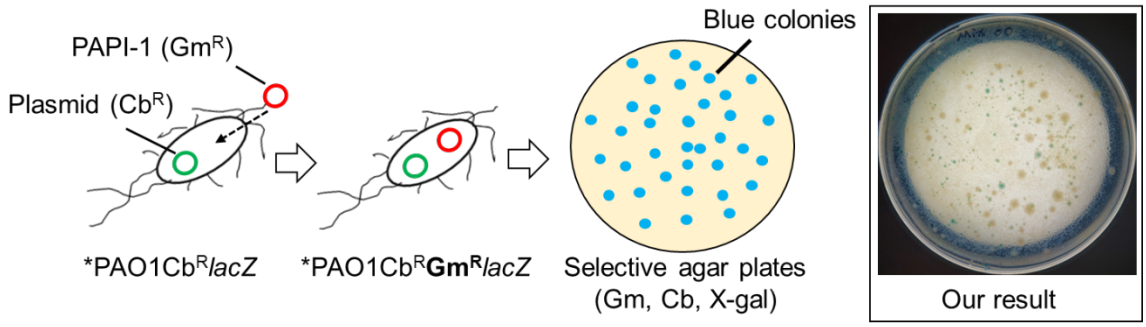


Figure 3.9 Scheme of blue colonies (transductants) formation. PAO1Cb^{R}*lacZ* acquired PAPI-1 gene (GMR) from supernatant of PA14 (PAPI-1). Thus, PAO1Cb^{R}*lacZ* is resistant to both carbenicillin and gentamicin. *lacZ* gene in PAO1Cb^{R}*lacZ* encodes β-galactosidase that can hydrolyze X-gal, an analog of lactose, into glucose and galactose (blue/green color).

We found that culturing the PA14 (PAPI-1) supernatant and PAO1Cb^{R}*lacZ* mixture with 0.05 μg/mL of ciprofloxacin resulted in about 7-fold more survival bacteria on selective agar than the one without ciprofloxacin (Figure 3.10). Because both conjugation and transformation mechanisms were reduced/eliminated, and phage produced by 0.05 μg/mL of ciprofloxacin, the ciprofloxacin-enhanced PAPI-1 transfer was most likely through transduction.

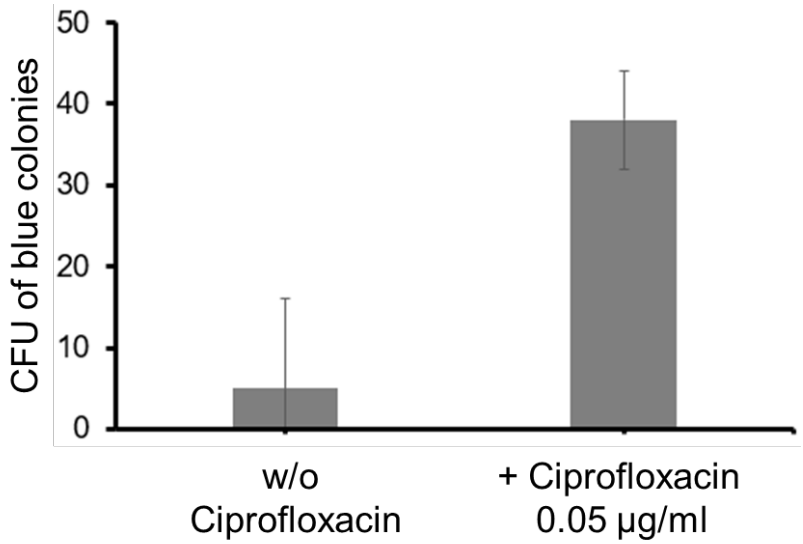


Figure 3.10 Colony forming units (CFU) of blue colonies of PAO1Cb^{R}*lacZ* cultured with cell-free supernatant of PA14 (PAPI-1) for 48 h at 37℃ statically with and without ciprofloxacin (0.05 µg/mL). The supernatant of PA14 (PAPI-1) (initial OD 0.8) was cultured with and without 0.05 µg/mL of ciprofloxacin for 3h at 37℃ statically. Error bars were obtained from three experimental replicates.

3.2.4 Inhibition of ciprofloxacin-enhanced transduction

To examine the inhibition of ciprofloxacin-enhanced PAPI-1 transduction, we applied $C_{12}EG_4OH$, $SFEG_4OH$ and $SFEG_4Oepoxy$ to the system, and the results suggested that $C_{12}EG_4OH$ (100 µM) did not reduce the enhanced PAPI-1 transduction significantly. In contrast, with $SFEG_4OH$ (100 µM) or $SFEG_4Oepoxy$ (100 µM), we got a about 7-fold reduction of the enhanced number of transductants (Figure 3.11).

However, in bacteriophage plaque assay, we observed that the PAO1Cb^{R}*lacZ* was killed by ciprofloxacin-induced phage (Figure 3.8). The phage particles generated by applying ciprofloxacin to a culture of PA14-PAPI suggested that the phage particles were lysogenic in PA14. This result was validated by that fact that these in situ generated phage

particles do not have a strong killing effect on PA14 (Figure 3.8). However, these phage particles did exhibit a strong killing effect for other PAO1 based strains, including wt PAO1, PAO1k and PAO1Cb^{R}*lacZ*, creating plaques on their bacterial lawns, suggesting that the phage particles were lytic to these strains. Since the mixture of phage particles and a culture of PAO1Cb^{R}*lacZ* generated a few blue colonies, these results suggested that the DNA injected into the PAO1Cb^{R}*lacZ* strain could become lysogenic, and represented exactly how a transduction proceeds. In the presence of $SFEG_4OH$, the formation of blue colonies was inhibited. This result was consistent with $SFEG_4OH$ inhibiting the transduction of HGT.

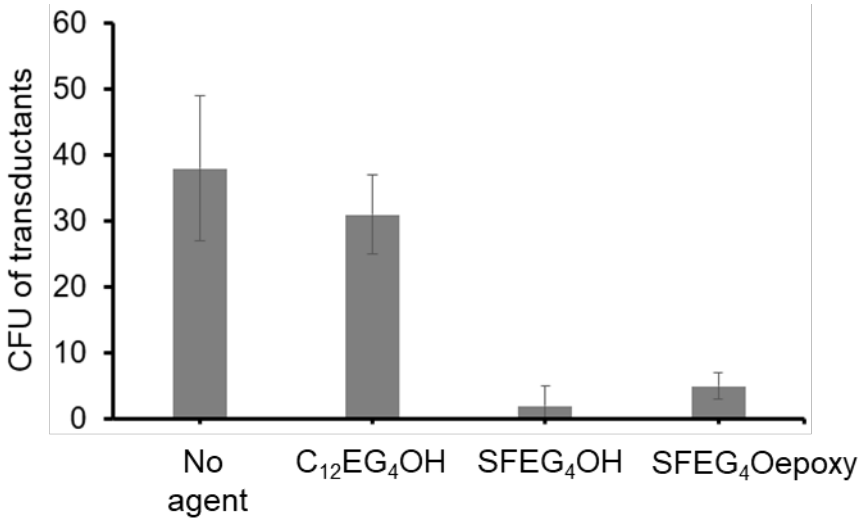


Figure 3.11 Colony forming units (CFU) of PAO1Cb^{R}*lacZ* (blue colonies) with Ciprofloxacin (0.05 μg/mL) in culture in the addition of 100 μM of $C_{12}EG_4OH$, $SFEG_4OH$ or $SFEG_4Oepoxy$ for 48 h at 37℃ statically. PAPI-1 containing supernatant was prepared by co-culturing of PA14 (PAPI-1) with and without 0.05 μg/mL Ciprofloxacin (initial OD 0.8) for 3 h statically before removing the bacteria from supernatant. PAO1Cb^{R}*lacZ* (recipient) was mixed with the that was supplemented with100 μM of $C_{12}EG_4OH$, $SFEG_4OH$ or $SFEG_4Oepoxy$ for 48 h at 37℃ statically. The blue colonies were screened

on agar (Gm, Cb and X-gal) to show PAPI-1 gene transferred into PAO1Cb^R*lacZ*. Error bars were obtained from three experimental replicates.

3.2.5 Inhibition of conjugation

Conjugation is the process that donor and recipient bacteria are physically connected and pulled to each other by pili. To examine the inhibition of conjugation. We adopted an established protocol for studying conjugation efficiency,[106] with the modification of including pili ligand molecules of SFEG$_4$OH and SFEG$_4$Oepoxy. The subculture (~1.05 mL) of PAPI-1 donor strain PA14-140495 (Gm^R) was added to 50 uL of PAO1Cb^R*lacZ* culture (OD ~1.0) that was cultured with 100 uM of SFEG$_4$OH for 3 h (see experimental for details), giving a final concentration of ~4.6 μM. This mixture was cultured statically at 37℃ for 48 h, obtaining a slimy mixture. The slimy mixture (~1.1 mL) was mixed 1 mL of fresh LB and sonicated in a bath for 10 min. The number of transconjugants (blue colonies) in the final mating mixture were screened on a selective agar plate containing 75 μg/mL gentamicin, 150 μg/mL carbenicillin and 40 μg/mL X-Gal.

We note that lacZ is non-transferable from the recipient to the donor strain. Stephen Lory and coworkers reported that the blue colonies were PAO1Cb^R*lacZ* acquired PAPI-1 from PA14, not the other way around.[106] The transfer frequency was calculated by the total number of transconjugants (blue colonies) divided by the total recipients (CFU after 48 h culture at 37 °C). Without including SFEG$_4$OH or SFEG$_4$Oepoxy in the above experiment, we obtained a transfer frequency of 1.1×10^{-5}, which is consistent with the reported value of 6.6×10^{-5}, [106] for which a more concentrated donor culture (OD_{600}= 4.0) was used.[106] We used donor culture with OD_{600} 1.0. When 100 μM of SFEG$_4$OH was present, we obtained a transfer frequency of 5.57×10^{-8}, about a 3-log reduction, whereas

$SFEG_4Oepoxy$ caused a similar inhibition of conjugation transfer, with a transfer frequency to 7.40 x 10^{-7}, about a 2-log reduction (Figure 3.12A). A concentration study showed an IC_{50} of 40 μM of $SFEG_4OH$ at inhibiting the conjugation (Figure 3. 12B). Besides the blue colonies, we also observed some white colonies from spontaneous mutants. We did not test if PAPI-1 conjugation could be enhanced by antibiotics, because without additional antibiotics, conjugation already occurs.

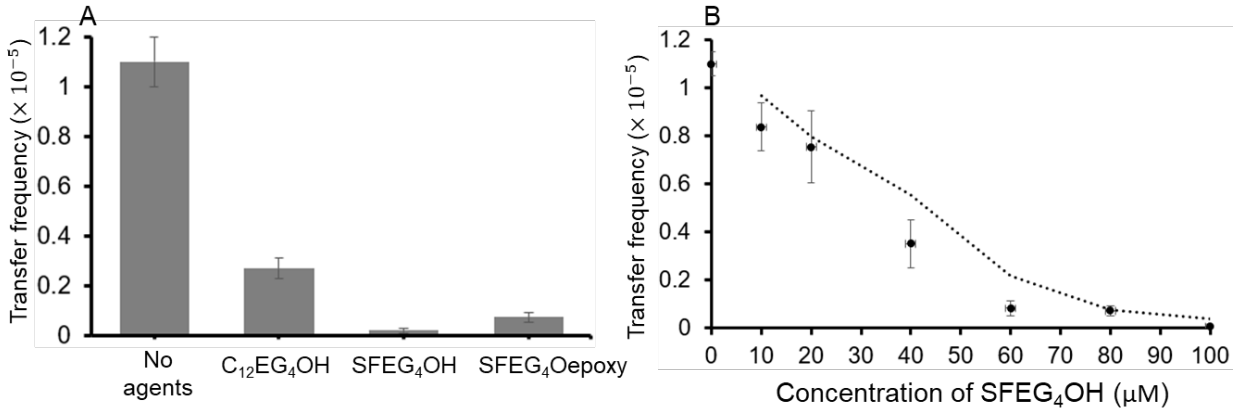
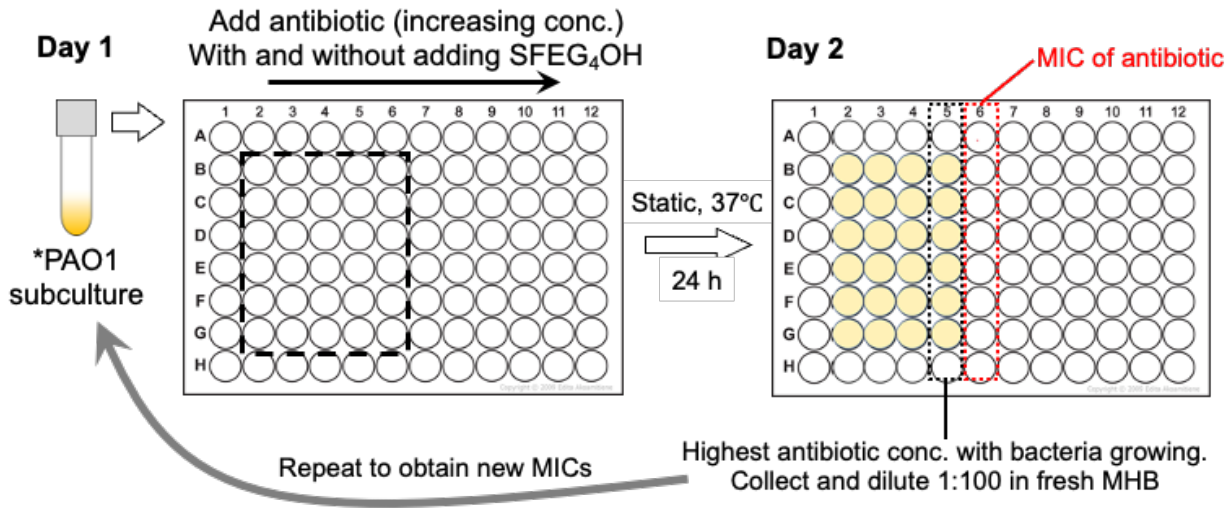


Figure 3.12 Transfer frequency of PAPI-1 from PA14 (PAPI-1) to PAO1Cb^R*lacZ*, (A) in the presence of 100 μM of $C_{12}EG_4OH$, $SFEG_4OH$ or $SFEG_4Oepoxy$; (B) in the presence of different concentrations of $SFEG_4OH$. The transfer frequency was calculated by the total number of transconjugants (blue colonies) divided by the total recipients (CFU after 48 h culture at 37°C). PAO1Cb^R*lacZ* was pre-cultured with small molecules for 3 h at 37°C with shaking at 250 rpm before mating with PA14 (PAPI-1) for 48 h at 37℃ statically. Error bars were obtained from three experimental replicates.

3.2.6 Serial passage assay for evaluating the development of drug resistance

Serial passage assay is widely used in vivo and in vitro to study antibiotic resistance in bacteria.[189] In the assay, bacteria are grown in medium with sub-MIC (sub-inhibitory) concentrations of an antimicrobial agent for a certain time. The MIC of the test antimicrobial agent for the bacteria will increase if the antibiotic resistance develops during the culture period. The development of drug resistance is evaluated by the increase of MIC over multiple cultures (passages).



*PAO1 culture : 1:100 diluted PAO1 subculture (100 µL of overnight culture added to 10 mL of fresh MHB)

Figure 3.13 Scheme of serial passage assay. PAO1 culture for Day 1 was prepared by diluting 100 µL of PAO1 overnight culture with 10 mL of fresh MHB with increasing concentrations of the antibiotic, with increment of MICs, starting from 0.25 x MIC, 0.5 x MIC, 1 x MIC and 2 x MIC in total 200 µL medium. At day 2, the culture (100 µL) with the highest concentration of antibiotic that still show bacterial growth is diluted with 10 mL of fresh MHB and immediately transferred to another plate with addition of adjusted concentrations of the antibiotic (eg. 2 x MIC, 3 x MIC, 4 x MIC, 5 x MIC and 6 x MIC), and repeat the day 1 procedures. The same procedures were repeated for 21 days

(passages). The new MIC of the antibiotic for each passage culture is the lowest concentration of the antibiotic that shows non-growth of bacteria in the column.

The development of ciprofloxacin resistance in *P. aeruginosa* has been reported by serial passage assay previously.[189] To evaluate the effect of $SFEG_4OH$ on inhibiting the development of ciprofloxacin resistance in *P. aeruginosa*, we performed the serial passage assay for wild type PAO1 in the presence of ciprofloxacin with and without 100 μM of $SFEG_4OH$ (Figure 3. 14). The initial concentration of ciprofloxacin used were 0.25 x MIC, 0.5 x MIC, 1 x MIC and 2 x MIC in total 200 μL medium (1 x MIC of ciprofloxacin to PAO1 is 0.4 μg/mL). New MICs of Ciprofloxacin were used for subsequent culture passages.

Without $SFEG_4OH$, the resistance to ciprofloxacin started to develop after 5 passages, and the MIC value increased to 200 μg/mL from 0.4 ug/mL after 13 passages. In the presence of $SFEG_4OH$ (100 μM), the resistance started to show after 11 passages, and the MIC value increased slowly to 20 μg/mL after 21 passages (Figure 3. 14). These results suggest that $SFEG_4OH$ inhibits the development of resistance to ciprofloxacin in *P. aeruginosa*.

The mechanism for resistance development in multi-passage assays is generally regarded to be mutation,[189, 191] and this mutation is passed through generations “vertically”. No comprehensive studies have been reported to reveal the mechanism or the details of the mutation. In previous studies, we found that antibiotics could enhance both transformation and conjugation among bacteria. In serial passage assay, the bacteria were also exposed to ciprofloxacin with the increasing concentrations. This antibiotic stress may cause the promotion of bacterial transformation or conjugation

occurring during the serial passage culturing to transfer the antibiotic-resistance genes horizontally. we believe that horizontal gene transfer is likely a significant contributor to the development of drug resistance in the process of serial passage culturing. Our result suggests that the rapid increase in drug resistant without $SFEG_4OH$ seen starting on the fifth day is largely due to horizontal gene transfer from mutated strain to other strains. In the presence of $SFEG_4OH$, the rise of antibiotic resistance on the 21st day may be mainly the result of “vertical” transfer of mutated gene through generations**.**

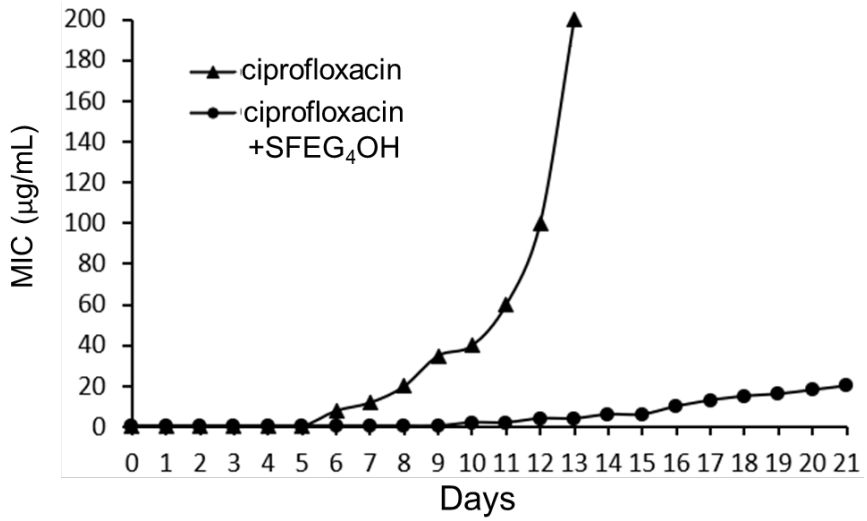


Figure 3.14 The MIC values of ciprofloxacin for wild type PAO1 with and without 100 μM $SFEG_4OH$ after 21 passages (days).

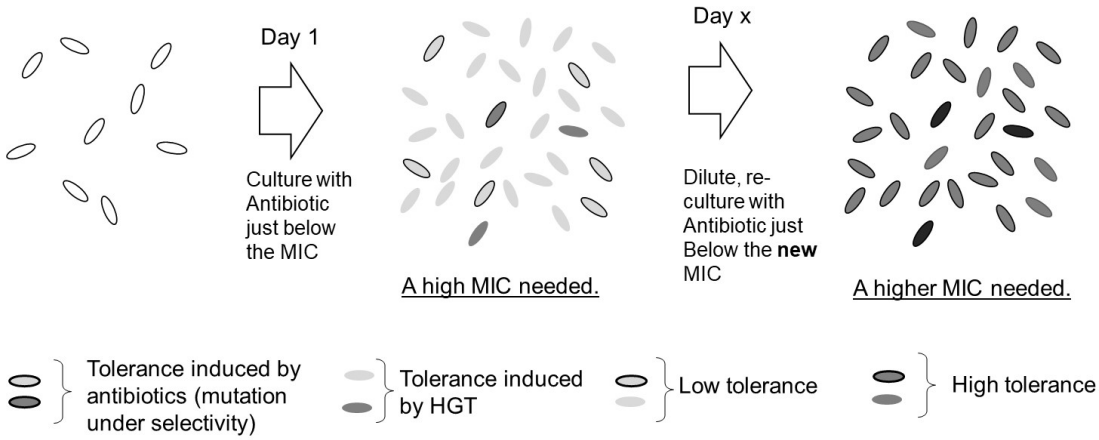


Figure 3.15 Bacteria's evolution during a multi-passage assay without inhibiting HGT.

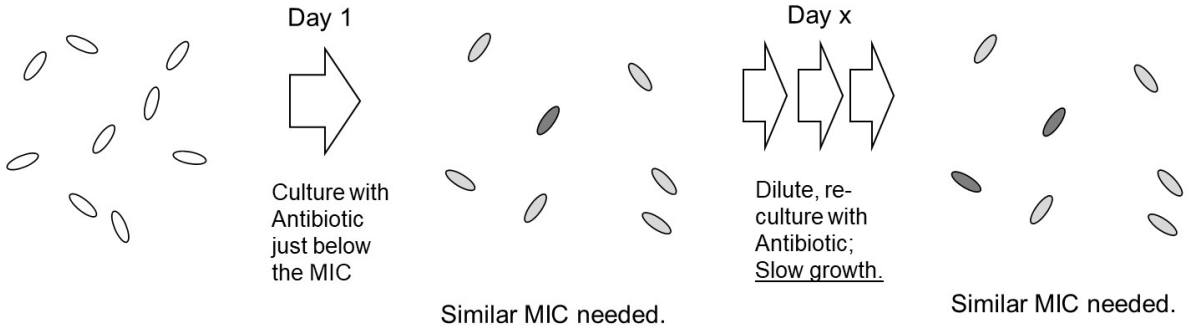
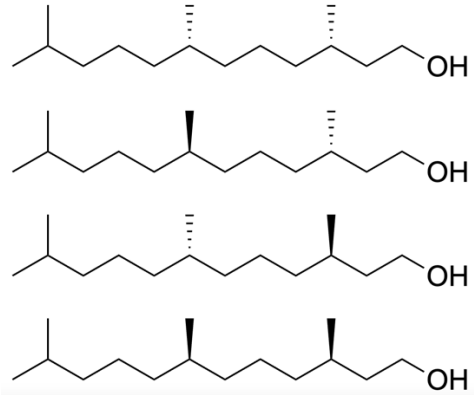


Figure 3.16 Bacteria's evolution during a multi-passage assay with inhibition of HGT. No HGT; Only mutational tolerance occurs; Low epigenetic mutation; Only generational transfer; Slow increase of MIC.

The saturated farnesol derivatives consist of two stereogenic centers, giving a total of four possible stereoisomers as shown below:



The saturated farnesol is synthesized by hydrogenation of trans-farnesol using heterogeneous palladium (Pd) catalyst. While the two double bonds in the trans-farnesol are connected by single bonds, there may still have a weak conformation preference for all trans for the single bonds. This small conformation preference may couple with the approach of farnesol to the Pd catalyst surface, causing a selection of a preferred diastereomer, over the other one that would have been obtained from other conformations. However, because of the diastereomers are of different energy, a prediction of which diastereomer will be preferred based on conformation and catalyst binding is not reliable. But we believe that all four stereoisomers are obtained in the hydrogenation. For the HGT inhibition we observed, the effect may come from one (or more) preferred stereoisomers. The synthesis of individual stereoisomer and the identification of the stereoisomer(s) that is important for the bioactivities is discussed in the future work.

3.3 Conclusion

In this chapter, we studied the three major HGTs and confirmed that antibiotics could enhance plasmid transformation and induce phage transduction. We also demonstrated that saturated farnesol derivatives could inhibit the pili-mediated transformation, transduction and conjugation. In addition, the serial passage assay suggested that supplementing conventional antibiotic treatment with $SFEG_4OH$ can inhibit the development of antibiotic resistance in *P. aeruginosa* during repeated application of antibiotics.

3.4 Materials and Methods

3.4.1 Materials

Lysogeny broth (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.2) was used for bacterial growth. Agar plates (1.5% Bacto-agar in LB with appropriate antibiotics) were prepared for plate counting. Falcon™ 50mL Conical Centrifuge Tubes and sonic dismembrator (model 100) were purchased from Fisher Scientific. Bath sonicator (UC-32D-PRO) was purchased from SRA TruPower. 96-well plates (plastic, flat bottom, sterile) were purchased from Thermo Fisher Scientific. Carbenicillin (100 mg/mL, 25 mL) and Tetracycline hydrochloride (100g, powder) was purchased from TEKNOVA. Colistin sulfate (100 mg, powder) and X-Gal (100 mg, powder) were purchased from Sigma-Aldrich. Ciprofloxacin hydrochloride (5 g, powder) and Gentamicin sulfate (600 I.U/mg, powder) were purchased from Alfa Aesar. Syringe filters (sterilized, 0.22 μm) was purchased from Santa Cruz Biotechnology. Universal Nuclease was from Pierce™ (100kU, Prod# 88702).

3.4.2 Strains, Plasmids, and Culture Conditions

PAO1-mCherry (Tc^R) was given by George A. O'Toole's lab (Dartmouth College). PA14 140495 (PAPI-1) and PAO1Cb^R*lacZ* were given by Stephen Lory's lab (Harvard Medical School). PAO1k, ΔpilA were given by Jeroen Wagemans's lab (KU Leuven, Belgium). All strains were grown in LB medium supplemented with the appropriate antibiotics. PAO1-mCherry (Tc^R) and PAO1-GFP (Cb^R) were served as plasmid donors, while PAO1 wild type was served as plasmid recipient in transformation assays. PA14

140495 (Gm^R) and PAO1*lacZ* (Cb^R) were served as donor and recipient in conjugation assays respectively.

3.4.3 Inhibition of transformation

For transformation, the plasmid donor strain, PAO1-mCherry (Tc^R) was cultured with or without the inhibitor molecules, $SFEG_4OH$ or $SFEG_4Oepoxy$. The donor culture was then sonicated by a tip sonicated to release the DNA plasmids from the bacteria, the bacteria and its debris was removed by centrifugation and filtration. The supernatant containing mCherry (Tc^R) was mixed with the recipient strain, wt PAO1 culture with the presence of $SFEG_4OH$ or $SFEG_4Oepoxy$ and subleathal amount of tetracycline (20 ug/mL) to promote the intake of plasmid. The number of transformed bacteria was quantified by colony forming units on hard agar plate containing 250 ug/mL tetracycline.

To prepare the recipient culture, the inhibitor molecule, $SFEG_4OH$ or $SFEG_4Oepoxy$ (10 mM stock), was added into 1 mL of PAO1 recipient subculture (OD 0.8) to obtain 100 μM, and further cultured for 3 h at 37℃ with shaking (250 rpm).

To prepare the supernatant of the donor strain, an overnight culture of donor strain (PAO1-mCherry) was sonicated using a probe sonicator at power level 1, ON for 5 sec., and OFF for 5 sec., and repeat 6 times; the culture was then centrifuged (4185 g, 15 min) to pellet the bacterial debris. This supernatant of donor strain was filtered with 0.22 μm syringe filter to further remove residual bacterial cells.

For the transfer experiment, the supernatant (5 mL) of donor culture (PAO1-mCherry) was mixed with 1 mL of recipient culture that contained 100 uM of $SFEG_4OH$ or $SFEG_4Oepoxy$.To this mixture culture, 50 μL of 10 mM stock of $SFEG_4OH$ or

$SFEG_4Oepoxy$ was added to maintain 100 μM in the total 6 mL mixture. Tetracycline was also added to the mixture to get a concentration of 20 μg/mL. The same mixed culture was also prepared without adding tetracycline as a control. The mixtures were cultured for 24 h at 37℃ with shaking (250 rpm).

To quantify the plasmid transfer, colony forming units (CFU) of the recipient strains was counted on agar plates containing 250 μg/mL tetracycline.

3.4.4 Confirmation of phage production induced by ciprofloxacin

We use a top agar assay[218] to identify the phage produced from PA14 caused by sublethal amount antibiotic ciprofloxacin killing various strain of *P. aeruginosa*. In this assay, the donor strain that produces the phage was cultured with ciprofloxacin to promote phage production, and then phage was isolated in the supernatant by removing the bacteria by centrifugation and filtering. This phage-containing bacteria-free culture (20 μL) was place on a relative soft top agar gel (0.7 wt% agar), which contains the bacteria strain to be tested for killing by the phage droplets. The top agar was poured and solidified on a "hard" agar gel (1.5 wt% agar). As bacteria grow in the 0.7 wt% top agar gel, the gel turns cloudy. Because phage supernatant solution diffuses into the top agar gel, the phage killing the bacteria will create a clear local zone of plague.

To prepare the bacteria-free supernatant, a subculture (OD_{600} = 0.6) of PA14-140495 (Gm^R) was prepared from an overnight culture. Ciprofloxacin (stock: 1 μg/mL) was added into the subculture to reach final concentration of 0.05 μg/mL, then further culture it for 3 h at 37℃ with shaking at 250 rpm. The culture was centrifuged at 4185 g

and passed through a 0.22 μm filter to obtain bacteria-free supernatant. Another PA14-140495 (Gm^R) supernatant without adding ciprofloxacin was prepared as control.

To prepare the top agar, 3 mL of warm top agar solution (7 g/L agar, 0.5 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract in sterile water) was mixed with 200 μL of PAO1k culture (OD 0.6), or wt PAO1, or PAO1Cb^R*lacZ*, or ΔpilA, and spread on 1.5% "hard" agar plate (LB-Miller, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl ,15 g/L agar, 10-cm in diameter), and cooled for at least 15 min in biosafety level-2 hood to solidify the top gel at ambient temperature.

To examine the plaque formation, the top agar plates were incubated for 3 h at 37 °C followed by inoculation with the bacterial-free supernatant: 20 μL of the supernatant (with or without iprofloxacin), or 20 μL of ciprofloxacin (0.05 μg/ml) solution was placed on the top agar plates statically at 37°C for 12 h to observe plaque formation on bacterial lawn.

3.4.5 Inhibition of transduction

To prepare phage-loaded supernatant from the donor strain, an overnight culture (150 μL) of PAPI-1 donor PA14-140495 (Gm^R) was diluted with 15 mL of LB, and cultured at 37°C with shaking at 250 rpm to reach an OD_{600} of 0.8. Ciprofloxacin (stock 1 μg/mL) was added into this subculture to reach 0.05 μg/mL, and cultured for additional 3 h at 37℃ without shaking; to promote the production of phages. The culture was centrifugated at 4185 g for 10 min to pellet the bacterial cells. The supernatant (10 mL) was filtered through a 0.22 μm filter to further remove bacterial cells, and added with 0.5 μL of

Universal Nuclease (10 kU stock), and incubated for 1 h at ambient temperature; to decompose the DNA molecules in the supernatant.

To prepare the recipient culture, an overnight culture (100 µL) of the recipient strain PAO1Cb^{R}*lacZ* was sub-cultured in 10 mL LB supplemented with and without 100 µM of inhibitor molecule, SFEG$_4$OH (from a stock of 10 mM) at 37°C with shaking (250 rpm) to reach an OD_{600} of 0.6.

For gene transfer by transduction, the prepared supernatant (10 mL) from the PAPI-1 donor was mixed with 1 mL of prepared recipient culture, and further cultured at 37℃ without shaking for 48 h.

To quantify the PAPI-1 transfer, the transductants were counted as blue colonies on selective agar (75 µg/mL gentamicin, 150 µg/mL carbenicillin, and 40 µg/mL X-Gal).

3.4.6 Inhibition of conjugation

We adopted the method of mating in static liquid culture[106] with the modification that inhibitor molecule, SFEG$_4$OH or SFEG$_4$Oepoxy, was cultured with recipient strain (PAO1Cb^{R}*lacZ*), and included in the mating mixture culture.

To prepare the culture of donor strain, PA14-140495 was grown with 75 µg/mL gentamicin in LB medium at 37℃ overnight with shaking (250 rpm). The donor culture (50 µL, OD_{600} 1.0) was further diluted into 1 mL of LB and incubated at 37°C for 2-h with shaking (250 rpm).

To prepare the culture of recipient strain, PAO1Cb^{R}*lacZ* was grown with 75 µg/mL carbenicillin in LB medium at 37°C overnight with shaking (250 rpm). SFEG$_4$OH or

$SFEG_4Oepoxy$ (stock 10 mM) was added to a 50 µL of recipient cultures (OD ~1.0) for a final concentration of 100 µM, and further cultured for 3 h at 37 °C.

For gene transfer by conjugation, the prepared recipient culture (50 uL) was mixed with the prepared donor subculture (1.05 mL) to incubate at 37°C for 48 h statically. The resulting mating culture generated observable slimy substances, and was mixed with 1 mL of fresh LB, and then sonicated in bath for 10 min. The diluted culture in 15 mL falcon tube was immersed a water bath in the sonicator [SRA TruPower, UC-45D-PRO] and sonicated for 10 min with 50% power.

To quantify the PAPI-1 transfer, 100 µL of the final mating mixture was poured and spread on a selective agar containing 75 µg/mL gentamicin, 150 µg/mL carbenicillin and 40 µg/mL X-Gal. The number of transconjugants (blue colonies) was counted after 48 h incubation. The transfer frequency was calculated by the total number of transconjugants (blue colonies) divided by the total recipients (CFU after 48 h culture at 37°C)

3.4.7 Serial passage assay

Serial passage assay. Serial passage assay was adopted as described previously.[189] To prepare the initial bacterial culture, an overnight culture of PAO1 wild type was prepared in MHB in falcon tube at 37°C with 250 rpm shaking. Subculture of PAO1 was prepared by diluting 100 µL of overnight culture into 10 mL fresh MHB (1:100).

For the serial passage experiment, the prepared subculture of PAO1 was transferred into a 96-well plate with adding ciprofloxacin stock solution to get different final concentration (0.25 x MIC, 0.5 x MIC, 1 x MIC, 2 x MIC) in totally 200 µL culture into each well (6 replicates of each concentration were performed in the same column). After

a 24-h incubation in a static incubator at 37°C, the column of wells with the highest concentration of ciprofloxacin that still showed bacteria growth was sub-cultured again: Culture from six wells of this column were taken out and combined, and 100 μL of the combined culture was mixed with 10 mL of fresh MHB (1:100). This freshly diluted culture was transferred into another column of 6 wells with addition of different concentrations of ciprofloxacin. The concentrations were adjusted during the process to compensate for the rising MIC values. The lowest concentration of ciprofloxacin that showed non-growth in column was the new MIC value. The $SFEG_4OH$ (100 μM) was introduced in one of the PAO1 cultures with ciprofloxacin.

To quantify the MIC change for PAO1, this lowest concentration of ciprofloxacin that showed non-growth in column was considered as the new MIC for PAO1 for each passage. The added ciprofloxacin concentrations were increased for the rising MIC values. This process was repeated for 21 times (passages). Two control samples were prepared: (1) the sample of PAO1 culturing with different concentrations of ciprofloxacin alone; (2) the sample of PAO1 culturing with $SFEG_4OH$ alone (100 μM all the time).

Chapter 4. Saturated farnesol derivatives bind to pili causing surface retraction inhibiting related signaling events

4.1 Background and significance of pili appendages of bacteria

4.1.1 Pili structure, assembly, and dynamic (extension and retraction)

Type IV pili are found in many pathogenic bacteria served as an important virulence factor. Type IV pilus, a filament-like appendage on bacterial surface, was reported to mediate bacterial motilities, biofilm formation and horizontal gene transfer. Type IV pilus, an assembly of pilin monomers, has a length of 1000–4000 nm. Pili are are composed of PilA single protein subunit. Pilin has the domain structure for both transmembrane and protein-protein interactions. At the tip of the pilus, there are up to five pilin monomers exposed to serve as receptor binding sites. Previous studies suggest that pili-mediated binding is tip associated. And the binding sites of pilus are only at the tip region. However, scientists observed bacteriophage adsorption happened on both tip and stem of pili. These results suggest that pilus may have multiple binding sites from tip to stem (Figure 4.1).

Type IV pili system, a complex protein assembly, is composed of a filament-like pilin assembly, an inner membrane platform protein, an outer-membrane secretin channel and a cytoplasmic ATPase.[226] The major pilin that forms the filament is PilA in *P. aeruginosa*. The platform protein that exists in inner membrane is PilC in *P. aeruginosa*. The secretin PilQ plays an important role in letting external molecules cross the outer membrane during the pilus retraction. PilT acts as a retraction ATPase to power the pilus retraction, and the PilB serves as assembly ATPase to power the assembly of pili system. The pilin filament is sitting on the ATPase subunit with platform proteins. The ATPase

hydrolyses ATP leading to conformational change and rotation of platform protein. The translation of protein results in the elongation of pilin assembly to force the extension of pilus filament. Also, this motion of extension opens up a gap at the base of the pilus to dock new pilin subunits. Pili retraction depends on PilT, an ATPase family member. PilT binds and hydrolyses ATP to reverse the conformational change of platform protein to release pilin subunits, leading to the collapse of pilin assembly to cause the retraction of pilus into membrane.[136, 226]

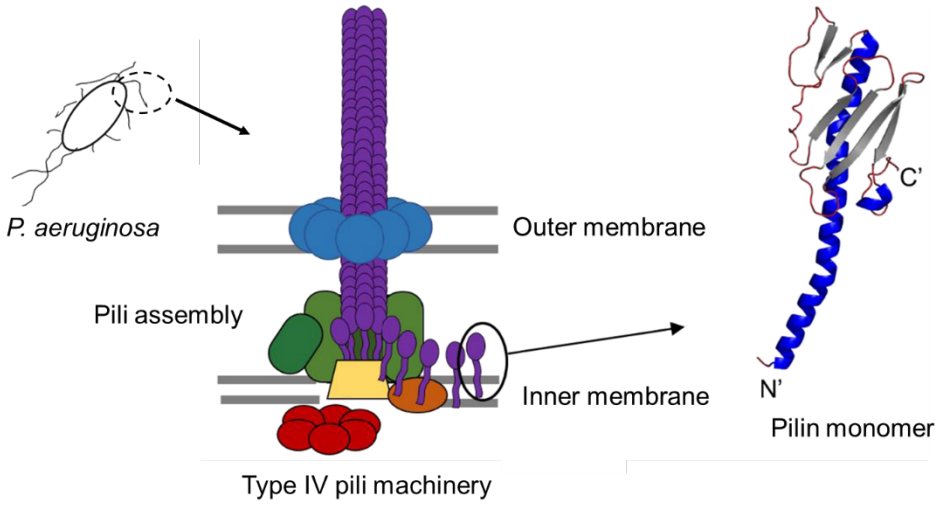


Figure 4.1 The localization of pilin assembly in *P. aeruginosa* and the structure of pilin monomers.

4.1.2 Pili bind to DNA, asialo-GM1 and hydrophobic surfaces

Pili play an important role in binding to DNA, asialo-GM_1 and hydrophobic surfaces. In 2018, Dr. Yves Brun's group first to witness the bacterial DNA uptake through pili extension and retraction.[227] It has been 90 years since bacteriologist Frederick Griffith first proposed the principle of bacterial transformation. Scientists had a hypothesis that DNA transformation in bacteria was mediated by pili. Dr. Yves Brun's group confirmed that pilus-DNA binding initiates transformation. The pilus-bound DNA was retracted with

pilus to cell surface and internalized. Also, they revealed that pilus-DNA binding happened at the tip of pilus. However, the details about how retracted DNA enters cell is still unclear. Pili are involved in horizontal gene transfer, such as natural transformation.[228] Type IV pili of P. aeruginosa are assembled from pilin monomers with 15-kDa.[228, 229] Type IV pili mediate DNA uptake that has been observed in Vibrio cholerae, and the majority of pilus–DNA binding was found to occur at the tip region instead of the sides of pilus.[102] P. aeruginosa type IV pilins are commonly featured with positive charge, that may facility the binding to DNA. Also, the structural study of β-sheet suggests that type IV pili may have interactions with the DNA backbone to mediate DNA binding.[229]

Pili also mediates bacterial adherence to epithelial cells by binding to asialo-GM1, a receptor on host cell. An increased number of asialo-GM1 receptors was found in cystic fibrosis patients' epithelial cells.[230] The first step of bacterial infection is the adherence to host cells, and that step is initiated by the adhesin pili. Several studies have shown that *P. aeruginosa* pili bind to Galβ1-4GlcNAc disaccharide, such as asialo-GM1. The C-terminal domain of the PilA subunit could recognize the disaccharide. Also, the adherence of *P. aeruginosa* to asialo-GM1 receptors is also a tip-associated event.

Pili initiate bacterial biofilm formation by binding to hydrophobic surface. Planktonic bacteria swim in medium and use their pili to sense surface to adhere.

4.1.3 Pili mediate the bacteriophage infection to bacteria

Bacteriophages were first discovered by Frederick W. Twort (1915) and Félix d'Hérelle (1917). are viruses that could infect bacteria and replicate in the host cell, leading to the lysis of the host cell at the end. Bacteriophage is composed of a head

protein shell where the DNA is encapsulated in, and a tail that serves as a needle to inject and transfer DNA into host cell. The tail length varies in different phage species. At the end of the tail, there are couple tail fibers that recognize and bind to the receptors on host cell. Bacteriophage infection is initiated by tail fibers binding to receptors on host cell surface. The lytic cycle caused by bacteriophage infection has five stages, including bacteriophage attachment, DNA entry, DNA replication and protein synthesis, new phage assembly and lysis. The first step of bacteriophage attachment is mediated by pili. Bacteriophage adsorbs on pilus and retracts with pilus to cell surface. Phage cause the pilus shortening was observed on PAO1 surface by electron microscopy.[231] However, the binding region of bacteriophage on pili is still unclear. Scientists have observed pili binding to tip and side of pili.[232, 233] For example, phage f1 can attach to the tip of F pilus, and the tip of F pilus has more than one binding site.[234] Another phage PO4 was reported to bind to side of pili.[233]

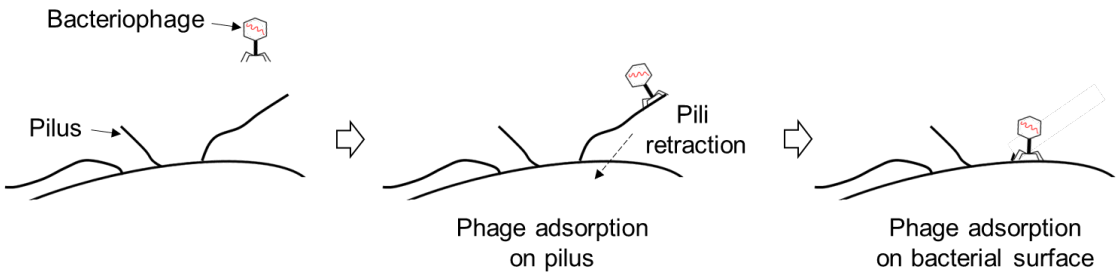


Figure 4.2 Scheme of the initial steps of bacteriophage infection – binding to pili and bacterial surface

4.1.4 Binding of small molecules to pilin monomers

Several studies using TEM have observed small molecules binding to either tip or side of pili. Bacteriophage φ6 was observed to bind to tip and sides of pilus[235] Phage F1 can attach to the tip of F pilus, and more than one phage was found to attach the tip of F pilus simultaneously.[232] It suggested that there were more than one binding site on the tip of pili. Similarly, the spheric RNA phage MS2 and filamentous DNA phage fd bind to the sides of F pili of *E. coli*.[234]

Randall T. Irvin has tried to develop anti-adhesive vaccine targeting pilin protein. They found that pili could bind to the GalNAc(1→4)β-DGal structure of Asialo-GM1, the receptor on epithelial cells for *P. aeruginosa*. They demonstrated that the C-terminal region of pilin is exposed at the tip of the pilus, at which pilus binds to Asialo-GM1.[228, 229] Irvin's group studied the bacterial DNA-binding and concluded that this binding event is pili-mediated.[228] Also, they reported that aliphatic chains of the disaccharide could enhance the binding to the pilin protein. Luk's group has screened several small molecules to study pili inhibition. They designed a novel "bacterial motility enabled" binding assay to reveal that pilin is a receptor for rhamnolipids, an exotoxin that are produced by bacteria. To study the pili-binding mechanisms and the effect, they synthesized the rhamnolipid analogs and examined their effect on controlling pili-mediated bacterial motilities, such as swarming. They revealed that pilin was the protein receptor for synthetic analogs. In the further studies by Circular Dichroism, NMR and Dynamic Light Scattering, they also demonstrated that rhamnolipids and the synthetic analogs could induce pilin's secondary structural change. In the protein-ligand binding

study, they found that the synthetic analogs bind to pilin at low picomolar concentrations, leading to pilin assembly.

4.2 Results and discussion

4.2.1 SFEG$_4$OH cause pili retraction to inhibit bacteriophage adsorption on PAO1k

In order to further study the binding and inhibition of small molecules to pili, we first performed the well-established bacteriophage adsorption assay (Figure 4.2), that is believed to be pili mediated. PAO1k, the wild type φKMV-sensitive strain,[132, 219] was used as host for bacteriophage adsorption assay. In the assay, we prepared top agar containing PAO1k culture (OD 0.1) supplemented with LB-Mg^{2+}. On the meantime, we cultured PAO1k in the presence of bacteriophage φKMV, with and without the addition of SFEG$_4$OH (100 μM). The PAO1k was cultured with bacteriophage φKMV for only 10 minutes before centrifugation and 0.22 μm filtration to remove all bacteria including bacteriophage-bound bacteria and free bacteria. The bacteria-free supernatant was inoculated on the bacterial lawn (top agar) for determining the number of free bacteriophages in the supernatant. The percentage of phage bound to bacteria was calculated as [(titer of added phage − titer in supernatant)/(titer of added phage)] × 100.

We first confirmed the total number of bacteriophages in stock that was 28×10^5 PFU (Figure 4.3A). In the absence of SFEG$_4$OH, the free bacteriophage in supernatant was 22×10^3 PFU after removing the bacterial-bound bacteriophage by centrifugation and filtration (Figure 4.3B). In contrast, the introduction of SFEG$_4$OH (100 μM) in culture brought the free bacteriophage up to 24×10^5 PFU (Figure 4.3C). The bacteriophage adsorption without SFEG$_4$OH was calculated as 98.8%, while the number

reduced to 14.3% in the presence of $SFEG_4OH$ (100 μM). The results suggested that $SFEG_4OH$ (100 μM) reduced the bacteriophage adsorption by about 85%. We Also introduced different concentrations of $SFEG_4OH$ from 10 to 100 μM in the system. The results suggested that $SFEG_4OH$ at 100 μM had the most potency on inhibiting bacteriophage adsorption. The IC50 of inhibiting bacteriophage adsorption by $SFEG_4OH$ was around 60 μM (Figure 4.4B).

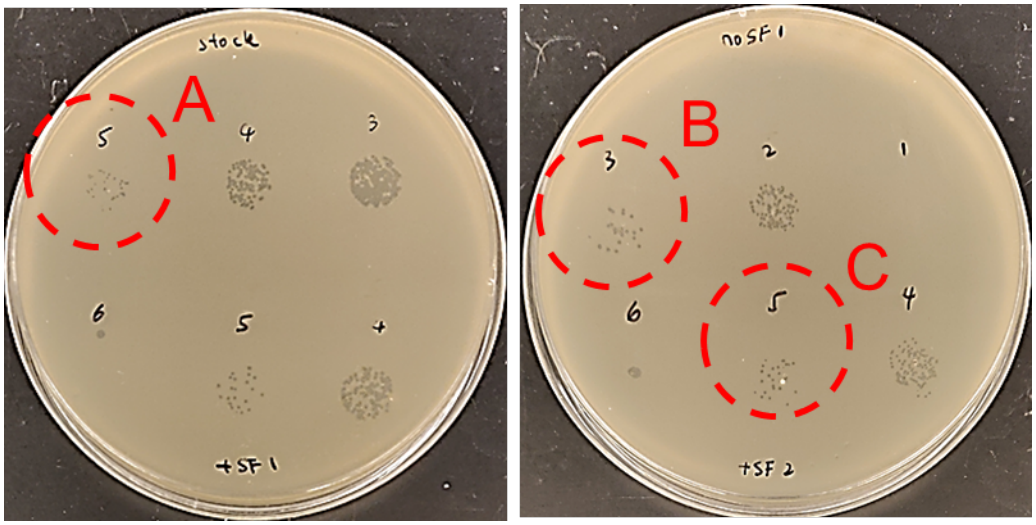


Figure 4.3 Plaque formation on a lawn of PAO1k caused by bacteriophage-containing culture with 10 μL of (A) blank, only bacteriophage stock solution with 10^5 dilution; (B) without $SFEG_4OH$, 103 dilution and (C) with 100 μM $SFEG_4OH$, 10^5 dilution. Different dilutions of each sample were prepared to be inoculated on agar. The (A) bacteriophage stock solution was diluted by 10^3, 10^4 and 10^5-fold before inoculating on the agar; (B) the culture without $SFEG_4OH$ was diluted by 10, 10^2 and 10^3-fold before inoculating on the agar; (C) the culture with 100 μM $SFEG_4OH$ was diluted by 10^4, 10^5 and 10^6-fold before inoculating on the agar (see Figure 3.7 for experimental details).

To study the mechanism of $SFEG_4OH$ inhibition bacteriophage adsorption, we performed the same adsorption assay using *pilT::Tn*, a PAO1 transposon mutant, that is

pili-retraction defective.[216] In the absence of SFEG4OH, *pilT::Tn* showed similar bacteriophage adsorption to PAO1k. However, the introduction of $SFEG_4OH$ (100 μM) did not inhibit bacteriophage adsorption on *pilT::Tn* (Figure 4.4A). We think $SFEG_4OH$ is not likely to block the active site of pili. Also, the alkaline buffer extraction results suggested that bacterial surface protein reduced in the presence of $SFEG_4OH$. Together, we propose that $SFEG_4OH$ cause the pili retraction to inhibit bacteriophage adsorption.

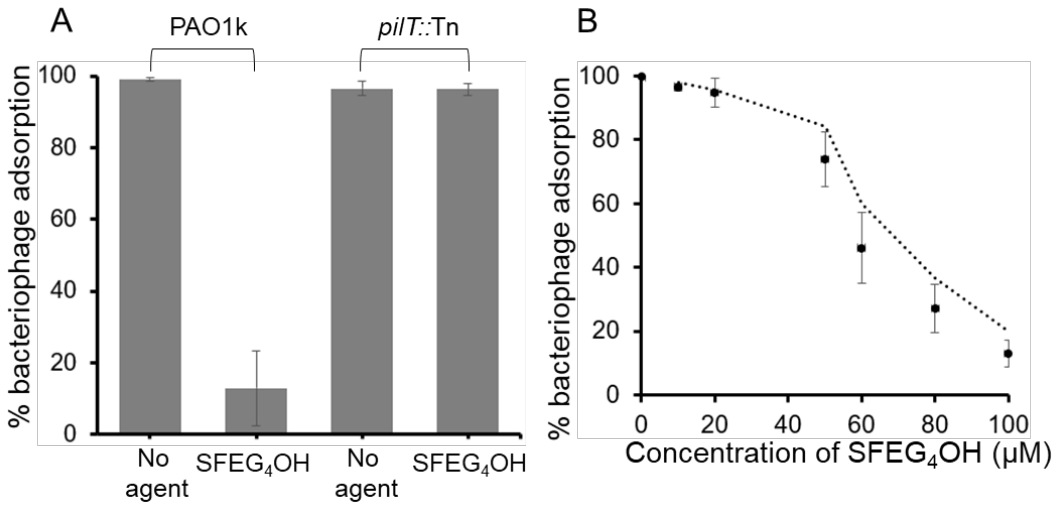


Figure 4.4 (A) Percentage of bacteriophage (φKMV) adsorption on PAO1k and on *pilT::Tn* with and without 100 μM $SFEG_4OH$; (B) Percentage of bacteriophage (φKMV) adsorption on PAO1k with different concentrations of $SFEG_4OH$ (10, 20, 50, 60, 80 and 100 μM). PAO1k or *pilT::Tn* (OD_{600} 0.6) was cultured with phage solution for 10 min at 37 °C with 100 rpm shaking. Error bars were obtained from three experimental replicates.

4.2.2 Disugar-derivatives inhibit bacteriophage adsorption on PAO1k

To study pili inhibition by small molecules further, we screened a class of disugar-derivatized with branched hydrocarbons with well-defined regiochemistry (synthesized by Felicia Burns, a graduate student in Luk Lab), including 2-propylPβM, 3-MeDβM, 3,5-

diMeDβM and 3-Et-7,11-triMeDβM (Figure 4.5). We examined all those molecules on inhibiting bacteriophage adsorption on PAO1k. The results showed that 2-propylPβM, 2-MeNβM and did not inhibit bacteriophage adsorption on PAO1k, whereas 3,5-diMeDβM and 3-Et-7,11-triMeDβM inhibited bacteriophage adsorption by 50% and 60% respectively (Figure 4.6). We believe that the bulky groups closer to disugar structure are important for enabling the small molecules to inhibit pili. Also, the molecule needs to have 12 carbons and substitution group to be effective on inhibiting bacteriophage adsorption. By comparing those 4 small molecules, we found that the substitution group (methyl group) that are closed to the disugar group is important. The more of the substitution group, the more effective on inhibiting bacteriophage adsorption.

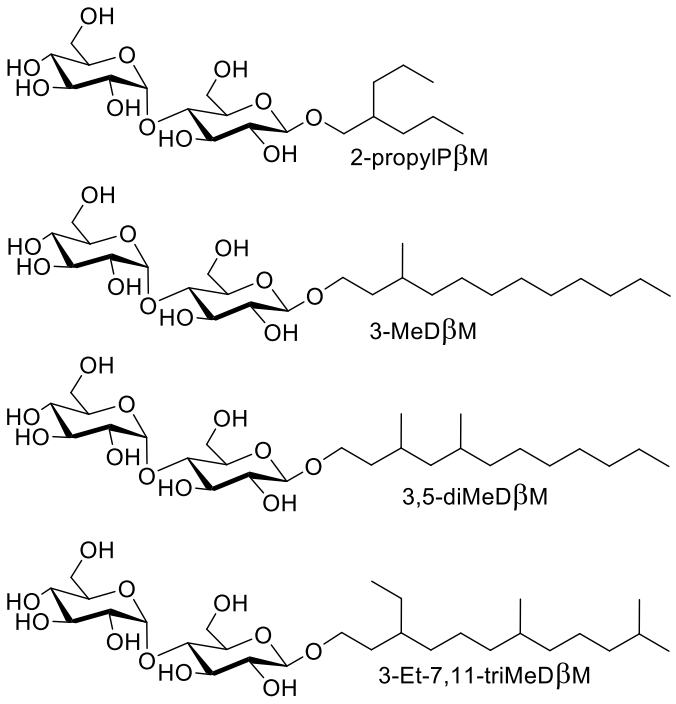


Figure 4.5 Disugar derivatives, 2-propylPβM, 3-MeDβM, 3,5-diMeDβM and 3-Et-7,11-triMeDβM for inhibiting bacteriophage adsorption on PAO1k. The synthesis of those molecules was done by Felicia Burns form Dr. Luk's lab.

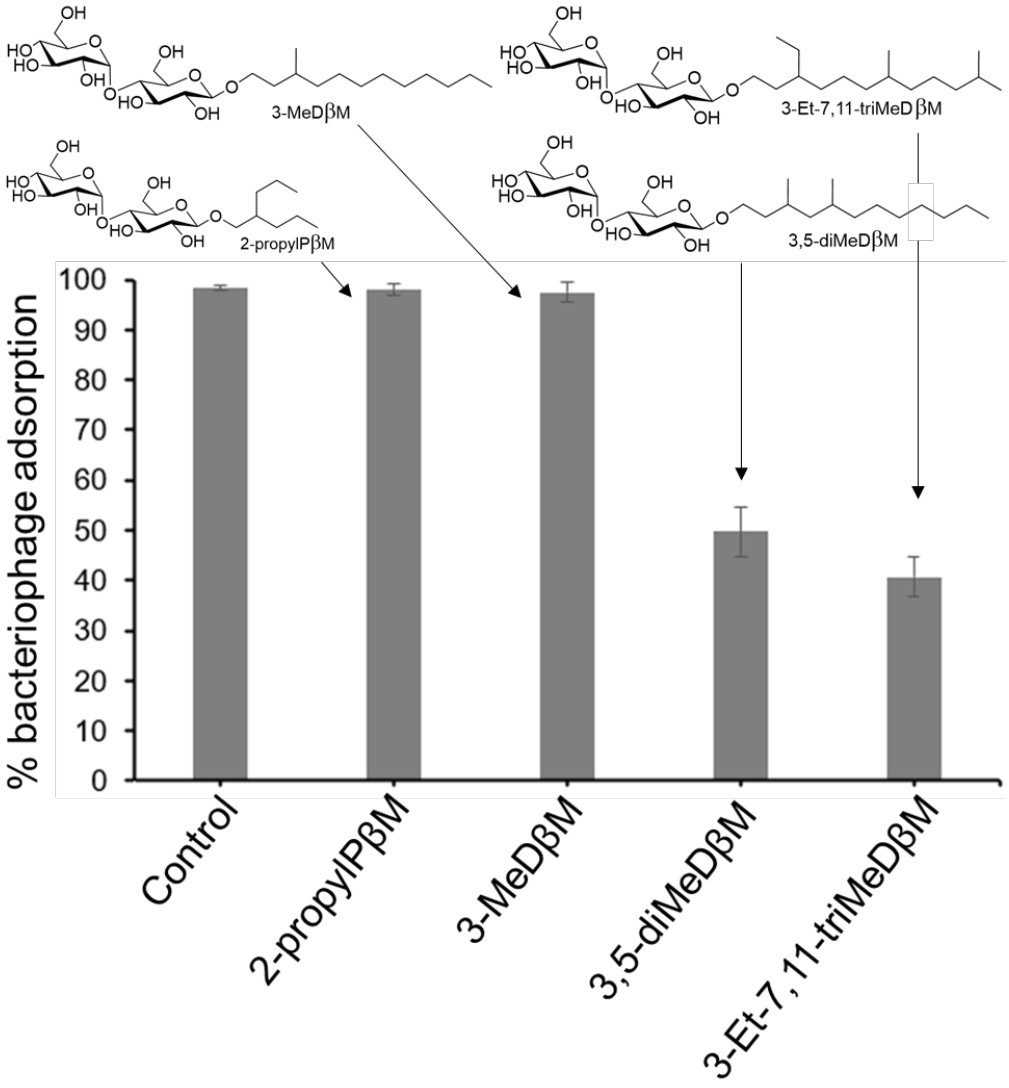


Figure 4.6 Percentage of bacteriophage (φKMV) adsorption on PAO1k with 2-propylPβM, 3-MeDβM, 3,5-diMeDβM and 3-Et-7,11-triMeDβM respectively. Error bars were obtained from three experimental replicates.

4.2.3 Saturated farnesol derivatives delay pili-mediated bacteriophage killing on PAO1k in planktonic media

In addition to the bacteriophage adsorption study, we also explored the bacteriophage killing in planktonic culture with or without 100 μM of $SFEG_4OH$ (Figure 4.7). The bacteriophage was introduced in the PAO1k culture with initial OD 0.5. $SFEG_4OH$ was also added into the culture at the same. The optical density monitoring indicated that $SFEG_4OH$ did not permanently inhibit the killing by bacteriophage. It only delayed the killing by bacteriophage. Phage usually has high affinity to adsorb and bind to the active sites on pili. Thus, $SFEG_4OH$ may be replaced by bacteriophage on binding sites of pili. Another possibility was that $SFEG_4OH$ did not fully block the active binding sites on pili. There were probably non-specific binding sites available for bacteriophage. We think it is highly possible that $SFEG_4OH$ caused pili retraction, a dynamic process including extension and retraction. This dynamic process may cause the $SFEG_4OH$ non-permanent inhibition of bacteriophage adsorption on pili. The other possibility was that bacteriophage was not only binding to pili. Instead, they could also bind to cell surface that was already observed on other bacteria by TEM. This result is consistent with the past finding that anti-pilin antibody can delay the killing of bacteria by an-other predator strain through pili binding.[236]

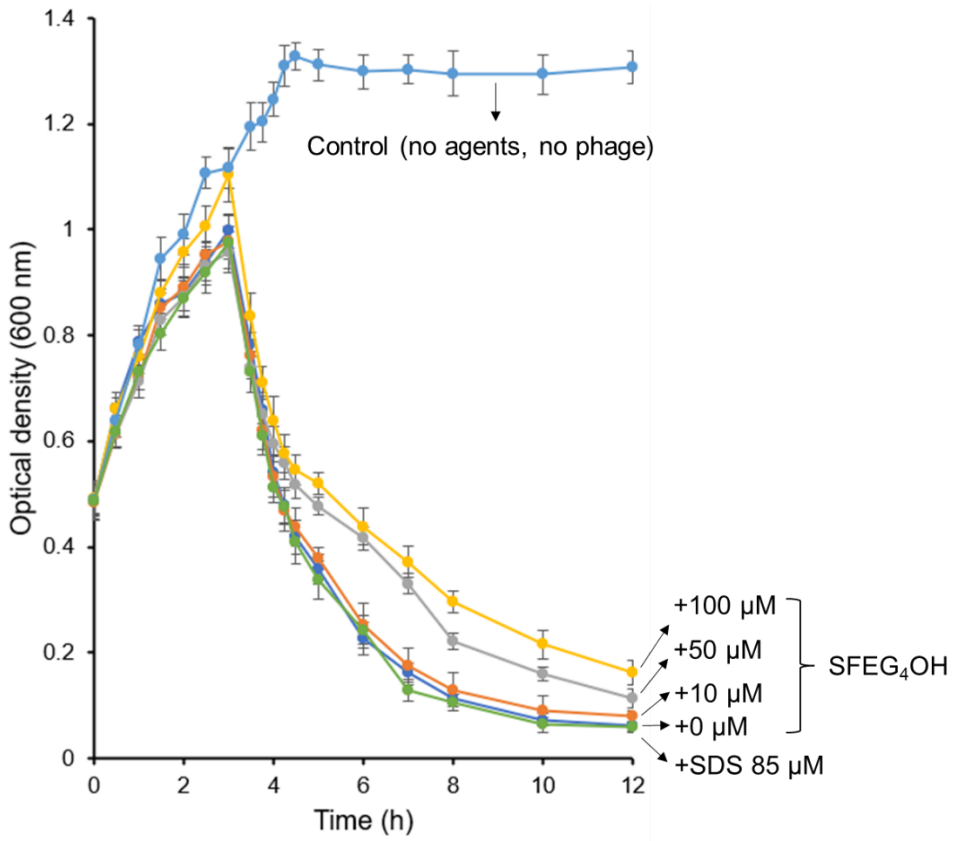
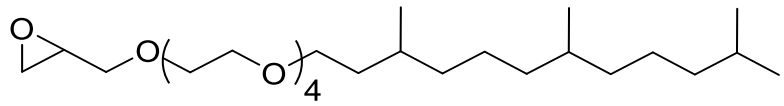


Figure 4.7 PAO1k growth (initial OD 0.5) with phage in the presence of different concentrations of $SFEG_4OH$ (0, 10, 50 and 100 μM) in planktonic media at 37°C with shaking at 250 rpm. The PAO1k culturing with sodium dodecyl sulfate (SDS) was included as comparison. Error bars were obtained from three experimental replicates.

4.2.4 $SFEG_4OH$ binding to pili cause pili retraction (summary of other people' work)

The inhibition of bacteriophage adsorption on PAO1k and *pilT::Tn* has suggested that $SFEG_4OH$ may bind to pili to cause retraction. However, more evidence is needed to make conclusions. Dr. Hewen Zheng from Luk's lab studied the ligand-receptor binding between saturated farnesol derivatives and pilin protein that was expressed by PA1224N3, a clinical isolate that is hyper-piliated.[237] The purified pilin protein showed the same mass on SDS-PAGE gel as reported. The binding of saturated farnesol derivatives to purified pilin protein was conducted by MALDI-MS. Pilin has molecule mass of 16313.0508 g/mol, and the molecule $SFEG_4O$-epoxy has a molecule mass of 460 g/mol, the reaction involved SN2 attack resulting in a ring opening without loss of any mass, and 16772.8398 g adduct mass was observed. The results suggested that pili were covalently modified by $SFEG_4O$-epoxy.



$SFEG_4O$-epoxy

In addition to study the effect of saturated farnesol derivatives on pili, Hewen also performed the alkaline buffer extraction experiment. Alkaline extraction has been wildly used to strip surface proteins off cell membranes without affecting the disposition of integral components.[238] In the experiment, the wild type PAO1 was cultured with saturated farnesol derivative followed by centrifugation and resuspension the cell in PBS, Tris and 0.1 M Na_2CO_3 buffer to extract the surface proteins from bacterial membrane. The planktonic bacteria were lysed and the unlysed cells were removed by centrifugation.

The extracted surface protein on membrane was precipitated by adding HCl and pelleted for SDS-PAGE analysis. The result showed that PAO1 culturing with saturated farnesol derivative resulted in decrease in protein expression that had a mass of ~15 kDa, which is consistent with the reported mass of pili protein. The supernatant from saturated farnesol derivative treated PAO1 culture showed no pili protein, indicating that pili were not sheared off from bacterial membrane into supernatant. This result demonstrated that saturated farnesol derivative may cause the pili retraction, leading to the less amount of pili protein on bacterial surface. Another possibility is that saturated farnesol derivative caused the pili assembly fall off from the bacterial surface and further disintegrate into pilin monomers. Thus, less pili proteins were collected by alkaline extraction.

Together with the bacteriophage adsorption results done in this chapter, we think $SFEG_4OH$ binding to pili cause pili retraction (Figure 4.8). However, the binding site on pili is still unknown. Small molecules could either bind to tip or side of pili. The C-terminal region of pilin is exposed at the tip of the pilus, and it is believed to be important for binding interactions. However, the observation of bacteriophage adsorption on side of pili suggested that there may be more binding sites available on the stem of pili.

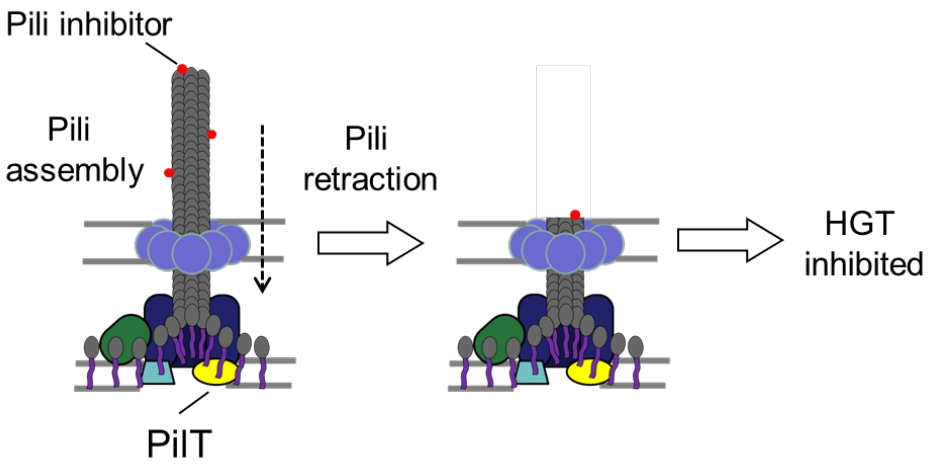


Figure 4.8 modified pili dynamics figure to show ligand binding induced retraction.

4.3 Conclusion

In this chapter, we studied pili binding and inhibition by bacteriophage adsorption. The results suggested that inhibit pili-mediated bacteriophage adsorption on PAO1k. However, $SFEG_4OH$ did not inhibit bacteriophage adsorption on pili-retraction defective strain *pilT::Tn*, indicating that $SFEG_4OH$ may bind and cause the retraction of pili to inhibit its functionality. The delayed planktonic killing by $SFEG_4OH$ also supported that the pili extension and dynamic process may cause the $SFEG_4OH$ non-permanent inhibition of bacteriophage adsorption on pili. Together with other studies in Dr. Luk's lab that did not provide a whole mechanistic picture at the time, these results collectively suggest that ligand binding causes pili retraction. This retraction is likely the mechanism for a broad range of chemical inhibition of pili's and related function, including swarming and twitching motility, horizontal gene transfer and production of virulence factors associated with low bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG) .[239, 240] To further study the pili inhibition effects, we could expand the HGT inhibition studies in biofilm, as well as the HGT inhibition between different bacterial strains.

4.4 Materials and Methods

4.4.1 Bacteriophage (φKMV) adsorption

Bacteriophage adsorption assay was adopted as described previously.[132] To prepare the bacteriophage sensitive culture, an overnight culture (100 µL) of PAO1k (φKMV sensitive strain) was diluted with 10 mL of LB supplemented with 10 mM MgSO4 (LB-Mg^{2+}) and with or without $SFEG_4OH$ (0, 10, 20, 50, 60, 80 and 100 µM). The mixture was sub-cultured to OD_{600} around 0.6 at 37°C with shaking at 250 rpm.

Sub-cultured bacteria were centrifuged (4185 g, 5 min) and washed with 5 mL LB-Mg^{2+}, following by resuspending in the same medium to an OD_{600} of 0.6. $SFEG_4OH$ (stock: 10 mM) was added into the culture to obtain different concentrations of $SFEG_4OH$ (0, 10, 20, 50, 60, 80 and 100 µM). Further culturing the bacteria with $SFEG_4OH$ for 1 h with shaking (250 rpm) at 37°C before mixing with phage solution.

To prepare the double-layer agar plate (0.7% and 1.5% LB agar), 3 mL of warm top agar solution (7 g/L agar, 0.5 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract in sterile water) was mixed with 200 µL of PAO1k culture (OD 0.6), and spread on 1.5% “hard” agar plate (LB-Miller, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl ,15 g/L agar, 10-cm in diameter), and cooled for 15 min in biosafety level-2 hood to solidify the top gel at ambient temperature. The top agar plates were incubated at 37 °C for 3 h statically.

After couple hours culturing at 37℃, the titers (the clear zone) are observed on agar indicating the plaque-forming units (PFU) of bacteriophage

For the bacteriophage adsorption experiment, the bacteria suspension (100 µL) was mixed with 900 µL of LB-Mg^{2+} containing 10^6 PFU/mL φKMV phage (PAO1 specific lytic phage). As a negative control, LB-Mg^{2+} (100 µL) without bacteria was mixed with the

same 900 µL phage solution. Following incubation (10 min at 37 °C with 100 rpm shaking), bacteria were removed by centrifugation (4185 g, 5 min at 4°C) followed by 0.22 µm sterile filtration, and 900 µL of the supernatant was transferred to a new Eppendorf tube. The supernatant (10 µL) was inoculated on the

To quantify the bacteriophage adsorption, the plaque-forming titer of the supernatant was determined by the top agar overlay method with PAO1 as the host indicator. The percentage of phage bound to bacteria was calculated as [(titer of added phage − titer in supernatant)/(titer of added phage)] × 100.

Chapter 5: Conclusions and future work

5.1 Conclusion

5.1.1 Identification of filamentous bacteria in antibiotic-induced biofilm

Antibiotics were reported to induce biofilm. However, the mechanisms were still unclear. In this thesis, we found that antibiotics induced bacteria to grow to filamentous form in planktonic medium and to attach on surface. We identified the filamentous bacteria in antibiotic-induced biofilm, and we revealed that the growth of antibiotic-induced filamentous bacteria is surface-mediated.

5.1.2 Saturated farnesol derivatives inhibit pili-mediated horizontal gene transfer

Three mechanisms of horizontal gene transfer were studied in this thesis, including transformation, transduction and conjugation. Saturated farnesol derivatives showed inhibition effect on tetracycline-enhanced plasmid transformation, ciprofloxacin-enhances PAPI-1 transduction, and PAPI-1 conjugation. Further study of serial passage assay suggested that saturated farnesol derivative inhibited the development of resistance to ciprofloxacin in *P. aeruginosa*.

5.1.3 Binding of Saturated farnesol derivatives causes pili retraction

The pili-mediated bacteriophage adsorption was conducted to demonstrate that saturated farnesol derivative could cause pili retraction, leading to inhibition of bacteriophage adsorption. More examinations done by other col leagues in Dr. Luk's lab supported that saturated farnesol derivatives may covalently binds to pili. The alkaline buffer extraction experiment done by Dr. Hewen Zheeng demonstrated that PAO1

culturing with saturated farnesol derivative resulted in decrease in pili protein expression. Together, saturated farnesol derivative binding to pili caused pili retraction.

5.2 Future work

5.2.1 "Farming" of filamentous bacteria for membrane protein expression

The results in Chapter 2 provide a strong foundation for future work in application of filamentous bacteria for protein expression. One possibility is to express the membrane protein proteorhodopsin in filamentous bacteria. Proteorhodopsin, a light-driven proton pump, could convert light into energy for bacterial growth. Proteorhodopsin can be applied for different aspect research. For example, proteorhodopsin optical proton sensor (PROPS) was developed to monitor membrane voltage changes in bacteria by means of changes in fluorescence, serving as an alternative approach of green fluorescent protein (GFP) family. Proteorhodopsin was also considered to produce biohydrogen in *Escherichia coli*. In Chapter 2, we found that cell wall related antibiotics could cause the significant growth of filamentous bacteria in medium rapidly in the presence of surfaces. It is possible to engineer a container with introduced large surface area to promote the induction of filamentous bacteria. One advantage of using filamentous bacteria is that the larger cell size of filamentous bacteria may give a higher production efficiency in protein biosynthesis. Another advantage is that filamentous bacteria form aggregate in medium, making it easier to clean out the filamentous bacteria and possibly to reuse the filamentous bacteria.

5.2.2 Synthesis of all four stereoisomers

The four stereoisomers of $SFEG_4OH$ will be further modified with disugars and tetra(ethylene glycol) and other molecules; these syntheses are established in Luk lab. These molecules can be studied for a wide range of bioactivities, including the inhibition of horizontal gene transfer mechanisms, bacteriophage adsorption and the development of drug tolerance, persistence, and resistance.

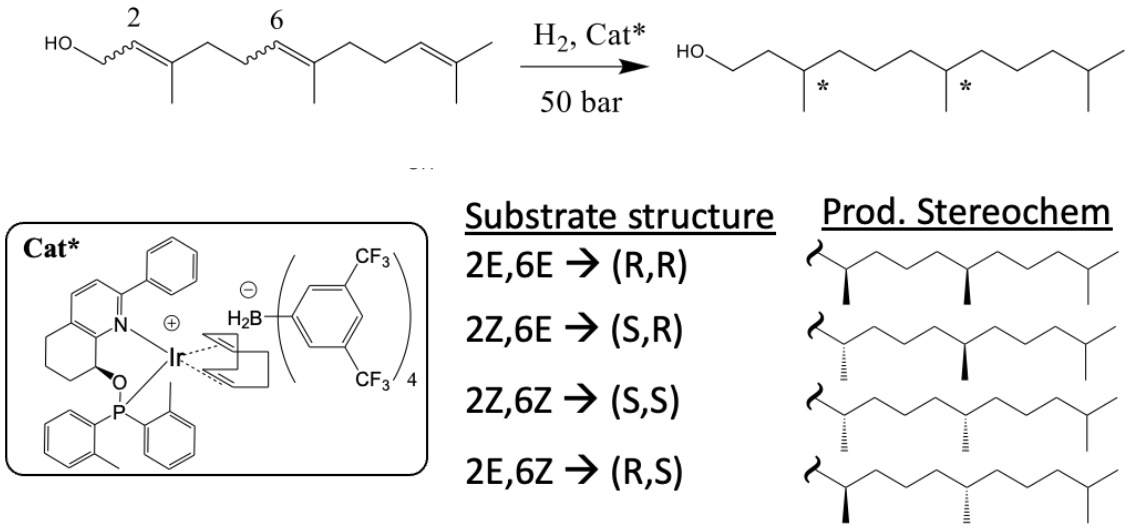


Figure 5.1 Synthesis of all four stereoisomers by using Iridium chiral catalytic with different alkene isomers starting materials; the diastereomeric selectivity ranges from 91-96%, the enantiomeric selectivities are all above 99% (Citation: *STEPHEN J. ROSEBLADE AND ANDREAS PFALTZ, Iridium-Catalyzed Asymmetric Hydrogenation of Olefins, Acc. Chem. Res. 2007, 40, 1402–1411*).

5.2.3 Mechanism study and inhibition of horizontal gene transfer among different bacterial species

In Chapter 3, we studied horizontal gene transfer between bacteria in planktonic environment. However, biofilm may promote horizontal gene transfer since it provides an "shelter", in which bacteria develop tolerance for antibiotics. The physical structure of biofilm also creates a high density of bacterial environment that may increase the horizontal gene transfer frequency. The inhibition of horizontal gene transfer in biofilm may be more important and relevant in clinical treatment. Bacteria transform into biofilm living style in the presence of antibiotic. Importantly, antibiotics were reported to promote the biofilm formation and horizontal gene transfer. The mechanism study of horizontal gene transfer in biofilm is needed and the development of small molecule inhibitors for horizontal gene transfer in biofilm have significance for bacterial infectious disease treatment.

Extensive studies have shown that pili are important for bacterial HGT, although many details are still not clear.[99, 103] The studies in chapter 3 demonstrated that Type IV pili mediated the HGT in *P. aeruginosa*, a Gram-negative bacterium. However, the pili system varies for different bacterial species. For example, there are more than four types of pili found in *E. coli*, and the type I pili mediate adhesion to surfaces.[241, 242] P-associated-pilus (pap) and F pilus on *E. coli* are reported to mediate horizontal gene transfer.[243, 244] Those pili with different structures may facility HGT with different mechanisms. The question here is: are saturated farnesol derivatives, or the same principle of using small molecules to inhibit pili, effective on inhibiting HGT in Gram-negative bacteria, such as *E. coli* with different pili systems?

Pili in Gram-positive bacteria differs in assembly than in Gram-negative bacteria. Pili in Gram-negative bacteria are formed by non-covalent interactions between pilin subunits, whereases the pili in Gram-positive pathogens are formed by covalent polymerization of pilin subunits.[245] Even though the Type IV pili are also widespread among Gram-positive bacteria, the structure of the pili is different than the pili in Gram-negative bacteria. The pili assembly process in Gram-positive bacteria is mediated by sortase catalyzing transpeptidase reactions.[245, 246] In Gram-positive bacteria, the C-terminus of ComEA is identified as the DNA-binding domain that has no sequence specificity.[99] However, the DNA-binding site in Gram-negative bacteria is still unclear. DNA uptake sequences (DUS), a specific sequence found in Gram-negative bacteria such as *Neisseria* and *Haemophilus influenzae*, is required for DNA update.[99] DUS-specific binding of DNA to bacteria is pilin dependent, even though the DNA receptor is not identified yet on bacterial surface.[99] The studies of pili inhibition in this thesis suggested that our small molecules may bind to pili. However, the specific binding sites are believed to be at the C-Terminus, the disulfide loop region.[229] The binding of saturated farnesol derivatives to type IV pili appears to be strong,[247] and exhibit some level of specificity (only nonspecific adsorption is observed for generic proteins such as BSA), but because the selectivity from other bacterial proteins is not clear. Those unrevealed details bring up another question: could saturated farnesol derivatives also inhibit HGT in Gram-positive bacteria, and which protein do these small molecules target, ComEA or pili? If they are not active, can one redesign new small molecule structures to inhibit the HGT of other bacteria?

Furthermore, the contribution of drug-resistant and drug-tolerant bacteria on HGT is still unknown in current assay. It is also not clear that if drug-resistant and drug- tolerant bacteria are effective at conducting horizontal gene transfer.

5.2.4 Confirmation of tetracycline-enhanced plasmid transformation

In Chapter 3, we observed more colonies growth in the presence of tetracycline (Figure 3.5). To find out if those colonies were from promoted plasmids transformation, or from promoted spontaneous mutation, we proposal an additional study: comparing the growth rate of the recipient strain with and without 20 μg/mL of tetracycline by OD measurement over 24 h. If the bacteria grow slower in the presence of tetracycline, likely the tolerant strains are developed. Then we will screen the bacteria on hard agar containing 250 μg/mL of tetracycline to examine if there are more colonies from culturing with 20 μg/mL of tetracycline than without. Because 20 μg/mL of tetracycline does not kill bacteria, this experiment will explore if 20 μg/mL of tetracycline will cause more spontaneous mutation for tetracycline resistance.

5.2.5 Mechanism study of controlling antibiotic resistance by inhibiting horizontal gene transfer

There are numerous papers using serial passage assay to study antibiotic resistance.[248-250] Most of those papers that observed development of antibiotic resistance in the assay mentions merely that spontaneous mutation was the mechanism.[248-250] However, there was no specific study or experiment conducted to support this statement. Understandably, it is challenging to identify and distinguish the different mechanisms of antibiotic resistance development in both planktonic culture and in biofilm.

In Chapter 3, we adopted serial passage assay to demonstrate that $SFEG_4OH$ could inhibit the development of antibiotic resistance in *P. aeruginosa* during repeated application of antibiotics. We thought that horizontal gene transfer was likely a significant contributor to the development of drug resistance in the process of serial passage culturing. Without $SFEG_4OH$, the resistance to ciprofloxacin started to develop after 5 passages, and the MIC value increased significantly in the next days. In the presence of $SFEG_4OH$ (100 μM), the resistance started to show after 11 passages, and the MIC value increased slowly (Figure 3. 14). The antibiotic resistance was delayed by 6 days and the development rate was significantly reduced in the presence of $SFEG_4OH$. Spontaneous mutants usually grow extremely slower than regular bacteria and their activities are downregulated in the presence of antibiotic. The fast promotion of MIC (Figure 3. 14) after 5 passages does not seem to be due to the spontaneous mutation only. Instead, the spread of resistance gene among the bacterial community could explain the abnormal resistance development. We know the MIC is measuring the lowest concentration of a drug that prevents the growth of a bacterial population. The high level resistance of a bacterium in the population does not cause the high MIC measurement. Also, the process of horizontal gene transfer of drug-resistance genes does not cause the immediate increase of MIC in the population. However, horizontal gene transfer spreads the resistance gene to surrounding bacteria and increases the proportion of the resistant bacteria in the population. Those resistant bacteria have a higher probability to mutate to be more resistant, leading to a higher MIC. Thus, horizontal gene transfer increases the probability of the bacterial population to mutate to be more drug resistant.

Further studies to understand the effect of HGT inhibition on the development of antibiotic resistance could be explored in different bacterial species. Also, the other HGT inhibitors that with different structures may also have effect on inhibiting the development of antibiotic resistance. For example, SFβM and SFβC that has similar structure to $SFEG_4OH$ may also show effect in serial passage assay. However, the condition of the experiment may need to be optimized to provide with a window for different inhibitors to show effects.

Another interesting work is to explore if $SFEG_4OH$ inhibits single-step spontaneous mutation. Multi-step spontaneous mutation that was developed in serial passage culturing, was believed to be the accumulation of several mutations.[251, 252] The individual mutations occur with antibiotic treatment, leading to drug-resistance development, such as in serial passage culturing. However, the single-step spontaneous mutation could result in high drug resistance immediately, such as in the presence of high concentration of antibiotics.[251, 252] We revealed that $SFEG_4OH$ could inhibit the drug-resistance development in serial passage culturing. Likely, this inhibition caused the delay of multi-step spontaneous mutation. It raised another question: Does $SFEG_4OH$ inhibit single-step spontaneous mutation?

We could also study the HGT inhibition by adapting the Griffith's Experiment conditions as shown below (Figure 5.1). The nonvirulent *P. aeruginosa* strain would not kill the mouse, whereas the virulent *P. aeruginosa* kill the mouse. We sonicate the nonvirulent *P. aeruginosa* culture to release the virulent gene (maybe plasmids) into the supernatant followed by 0.2 μm filtration to remove any bacteria. This cell-free supernatant is expected to be nonlethal for mouse. However, the mixture of nonvirulent

and the plasmid-containing supernatant may cause the death of the mouse. Based on Griffith's explanation, the virulent gene may be transferred into the nonvirulent strains, leading to the mutation of nonvirulent to virulent. Our hypothesis is that $SFEG_4OH$ may inhibit this gene transfer. Thus, the strain keeps nonvirulent. The detailed experimental condition may need to be explored, such as the choose of nonvirulent and virulent *P. aeruginosa* strains, the culturing time period, the treatment time period by $SFEG_4OH$ before injecting into mouse. All those conditions may affect the results. An optimized experimental condition and time window is needed to conduct this study.

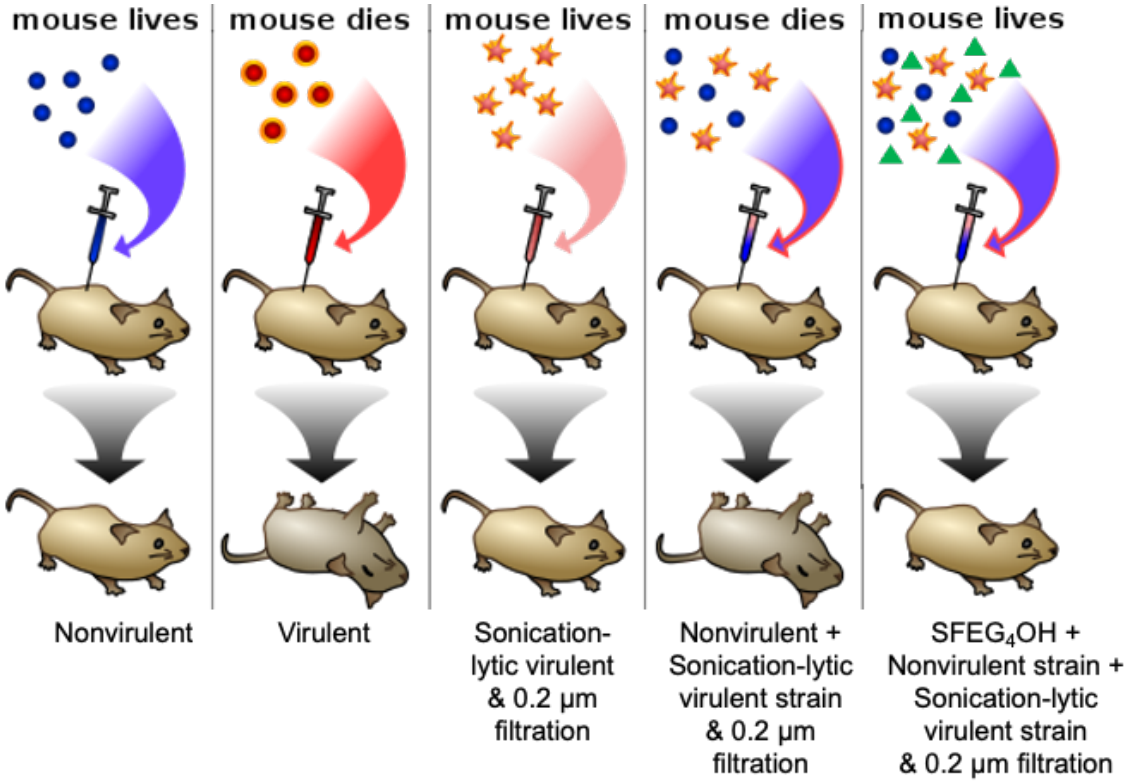


Figure 5.2 Adapted Griffith's Experiment to study $SFEG_4OH$ inhibiting HGT in animal models.

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Curriculum Vitae

Yuchen Jin

17005 Silver Crest Dr., San Diego, CA 92127 | jycsu1991@gmail.com | (315) 913-5787

PROFESSIONAL OVERVIEW

Biochemist with 9+ years of research experience in Microbiology including 5+ years in small molecule drug discovery. Over 1 year experience in pharmaceutical industry for formulation development. Proficiency in analytical methods, including HPLC analysis, particle size distribution, microscope imaging (TEM, Confocal), pH solubility, dissolution, GC, lyophilization stability, Gamma radiation feasibility. Extensive skills in development of microplate-based assays for drug candidates screening and potency evaluation of biologics. Wide range of experience in protein expression/purification, ELISA, BLI binding assays.

EXPERTISE & SKILLS

- **Analytical chemistry:** HPLC, GC, NMR, IR, Mass spectrometry, particle size distribution, microscope imaging, osmolality, RNA quantification.
- **Bioanalytical assay development:** BSL-2 bacteria operation, microplate-based assays, drug candidates screening, protein expression and purification, antibiotic resistance evaluation, microscopy (confocal, TEM, SEM, polarized microscopy), biolayer interferometry (BLI), ELISA, bacterial swarming/twitching, bacteriophage adsorption, biofilm measurement/inhibition, horizontal gene transfer inhibition, dissection of mouse.
- **Small molecule synthesis:** Farnesol modification.
- **Software:** Agilent ChemStation and OpenLab, Excel, PowerPoint, Word, EndNote, Minitab, ChemDraw, SciFinder PubMed etc.

EDUCATION

Ph.D. Syracuse University, Chemical Biology 8/2016 – Present

M.S. SUNY College of Environmental Science and Forestry, Bioprocess Engineering 5/2016

B.S. Hefei Normal University (China), Biotechnology 6/2014

PUBLICATIONS

- **Jin**, **Y**.; Zheng, H.; Ibanez, A.C.; Patil, P.D., Lv, S., Luo, M., Duncan, T.M., Luk, Y.Y. "Cell-Wall-Targeting Antibiotics Cause Lag-Phase Bacteria to Form Surface-Mediated Filaments Promoting the Formation of Biofilms and Aggregates". *ChemBioChem,* **2020,** *21*(6), 825-835.

- Patil, P.D., **Jin, Y**. and Luk, Y.Y., 2022. Chemical control over Asialo-GM1: A Dual Ligand for Pili and Lectin A that Activates Swarming Motility and Facilitates Adherence of *Pseudomonas aeruginosa*. *Colloids and Surfaces B: Biointerfaces*, p.112478.
- Patil, P.D., Zheng, H., Burns, F.N., Ibanez, A.C., **Jin, Y.,** and Luk, Y.Y. "Chimeric Ligands of Pili and Lectin A Inhibit Tolerance, Persistence, and Virulence Factors of Pseudomonas aeruginosa over a Wide Range of Phenotypes". *ACS Infectious Diseases*, 2022.
- Zheng, H.; Singh, N.; Shetye. G.S.; **Jin**, **Y**.; Li, D.; Luk, Y.Y. "Synthetic analogs of rhamnolipids modulate structured biofilms formed by rhamnolipid-nonproducing mutant of *Pseudomonas aeruginosa*". *Bioorganic & Medicinal Chemistry*. **2017**, *25*(6), 1830-8.
- **Jin**, **Y**.; Patil, P.D.; Luk, Y.Y. "Inhibition of Transformation, Conjugation, and Transduction by Ligand-Binding Induced Retraction of Pili Appendages ". *in preparation.*

WORK EXPERIENCE

Senior Scientist (LATITUDE Pharmaceuticals Inc., LLC. San Diego, CA) 9/2021 – Present

- Led projects of formulation development for mRNA vaccines. Developed a novel manufacturing process for making mRNA nanoparticles. Developed the analytical method for RNA quantification in nanoparticles.
- Led projects of ball-milling formulations for IV, oral and subcutaneous nanosuspension.
- Led projects of formulation development for intramuscular injection, emulsion, capsule and topical.
- Trained new employees for formulation and analytical methods, including HPLC, Zetasizer, Ball milling, Microfluidization, UV-Vis spectroscopy, GC and Particulate Matter by USP <788>.
- Troubleshooting the analytical and instrument issues.
- Tracked and reported project metrics to the clients. Managed the meetings with clients about project milestones.

Research Scientist (LifeUnit, Inc. Syracuse, NY) 5/2019 – 8/2019; 5/2017 – 8/2017

- Screened small molecule drug candidates targeting cystic fibrosis (CF). Performed biological assays to evaluate the potency of the molecules.
- Synthetically modified the structure of a drug candidate for better potency and reduction of drug resistance.
- Developed and standardized biofilm assay protocols for future use.

Teaching Assistant (Syracuse University) 8/2016 – 12/2020

- Instructed Organic Chemistry, General Chemistry and Forensic Science.
- Tutored students with lab techniques and background sciences.

Research Assistant (Anhui Hui-King Food Co., Ltd. China) 6/2013 – 5/2014

- Led the research of antioxidant identification and evaluation in blueberry leaf. Extracted and isolated active ingredients followed by measurement of their antioxidant activities.
- Provided technical support and quality control of the subsequent production.
- Conducted market research with marketing team through customer surveys and interviews.
- Co-authored patent application about the two different blueberry leaf extraction methods.

PRESENTATIONS

- 08/2020 ACS Fall 2020 National Meeting & Exposition in San Francisco, California, USA

CERTIFICATIONS

- Certificate of Advanced Food Inspection (Ministry of Human Resources and Social Security of the People's Republic of China)