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Pseudomonas aeruginosa rhamnolipids facilitate avoidance of Staphylococcus aureus and Pseudomonas aeruginosa stressed by bacteriophage and antibiotics

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Pseudomonas aeruginosa rhamnolipids facilitate avoidance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* stressed by bacteriophage and antibiotics

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Jean-Louis Bru

Dissertation Committee:
Associate Professor Albert Siryaporn, Chair
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2022

DEDICATION

I dedicate this dissertation to science. Here is the summary of my most significant data on *Pseudomonas aeruginosa* swarming. I basically did all these experiments, so you don't have to. You're welcome.

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It is likely impossible to cite every single individual that has helped me become the successful person I am today. To those I have not mention, thank you.

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P U B L I C A T I O N S

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Bru, J.-L., Høyland-Kroghsbod, N.M., Siryaporn, A. 2020. Time-lapse Imaging of Bacterial Swarms and the Collective Stress Response. *JoVE.* https://doi.org/10.3791/60915

Bru, J.-L., Rawson, B., Trinh, C., Whiteson, K., Høyland-Kroghsbod, N.M., Siryaporn, A. 2019. PQS produced by the *Pseudomonas aeruginosa* stress response repels swarms away from bacteriophage and antibiotics. *J. Bacteriol.* https://doi.org/10.1128/jb.00383-19

Kim, J., Chen, B., **Bru, J.-L.**, Huynh, E., Momen, M., Aswad, D.W. 2018. New findings on SNP variants of human protein L-isoaspartyl methyltransferase that affect catalytic activity, thermal stability, and aggregation. *PLOS ONE.* https://doi.org/10.1371/journal.pone.0198266

Juang, C., Chen, B., **Bru, J.-L.**, Nguyen, K., Huynh, E., Momen, M., Kim, J., Aswad, D.W. 2017. Polymorphic Variants of Human Protein l-Isoaspartyl Methyltransferase Affect Catalytic Activity, Aggregation, and Thermal Stability. *J. Biol. Chem.* https://dx.doi.org/10.1074%2Fjbc.M116.765222

AWARDS AND HONORS

Graduate Assistance in Areas of National Need January 2022
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Edward Steinhaus Teaching Award June 2020
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Annual award presented to outstanding graduate students with promising future as educator

Graduate Research Fellowships Program May 2019
National Science Foundation
Received Honorable Mention out of 12,000 applications and 2,000 award offers
Program supports outstanding graduate students who pursue research-based doctoral degrees

ABSTRACT OF THE DISSERTATION

Pseudomonas aeruginosa rhamnolipids facilitate avoidance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* stressed by bacteriophage and antibiotics

By

Jean-Louis Bru

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2022

Professor Albert Siryaporn, Chair

Pseudomonas aeruginosa is an opportunistic pathogen that can move collectively on semi-solid surfaces through swarming motility, which gives rise to biofilms. The impact of diverse environmental factors on the organization of swarms is not well understood. We demonstrate that healthy *P. aeruginosa* swarming populations are re-directed by the *Pseudomonas* Quinolone Signal (PQS) quorum sensing molecule, which is over-produced by stressed *P. aeruginosa* infected with bacteriophage or treated with antibiotics. PQS has multiple functions, including serving as a quorum-sensing molecule, activating an oxidative stress response, and regulating the release of virulence and host-modifying factors. These mechanisms have the overall effect of limiting the threat of danger to a subpopulation, which promotes the survival of the overall population. *Staphylococcus aureus*, which is a natural competitor of *P. aeruginosa*, shows similar phenotype as stressed *P. aeruginosa* populations and repels *P. aeruginosa* swarms. This causes both bacterial species to remain spatially segregated and unmixed. The repulsion by *S. aureus* requires production of the small peptide phenol-soluble modulin (PSM), which is an amphipathic peptide that has surfactant properties owing to its large hydrophobic chains and hydrophilic side chains. We show that PSM fibrils produced by *S. aureus* mediate the repulsion of *P. aeruginosa*

swarms. We determine that several long-chain amphipathic molecules with surfactant properties also repel *P. aeruginosa* swarms. Our results suggest a model in which surfactants can disrupt the surfactant layer produced by *P. aeruginosa*, causing reorganization of the swarming population. The segregation of *P. aeruginosa* and *S. aureus* via surfactant interactions promotes the survival of both species. This represents a general mechanism in which the organization of bacterial populations can be described by the interaction of the surfactants produced by the bacterial species. This observation has important implications for the formation and maintenance of bacterial populations in environments containing multiple species and on the outcomes of pathogenesis.

CHAPTER 1: Introduction and Background of *Pseudomonas aeruginosa*

1.1 Impact of *P. aeruginosa* on Healthcare and the Environment

Pseudomonas aeruginosa is a ubiquitous and opportunistic bacterial pathogen that is recognized as one of the most significant Gram-negative pathogens that produce chronic colonization and infection in human. It is largely responsible for common nosocomial diseases including hospital-acquired airway diseases, surgical site infections, and burn wound colonization (1). Due to the range of survival mechanisms and resistance to antibiotics, infections by *P. aeruginosa* can be life-threatening and it is progressively emerging as a serious public health concern. Organizations like the Centers for Disease Control and Prevention (CDC) categorized multidrug-resistant *P. aeruginosa* as serious threats to the United States and estimated 32,600 cases of patients hospitalized with *P. aeruginosa* infections in 2017 which resulted in 2,700 deaths from health-related complications such as severe pneumonia that resulted in death from pulmonary failure (2). Since then, the threats of *P. aeruginosa* has continued growing and the potential risks to die from their infections have increased.

When patients are infected with *P. aeruginosa*, the progressive growth of the bacterial populations weakens the host's defenses mechanism and results in significant deleterious effects. The symptoms of bacterial infections can range from mild headache, shortness of breath, and cough to fever, chills, and vomiting. Since this bacterial species prospers in moist environment, *P. aeruginosa* is therefore commonly found in the respiratory tract and is a major cause of chronic airway inflammation especially for patients with asthma, obstructive pulmonary disease, cystic fibrosis, or bronchiectasis (3). These issues have significantly contributed to lower life expectancy for those infected with *P. aeruginosa* (4).

1.2 Introduction of the Swarming Behavior in *P. aeruginosa*

Swarming in *P. aeruginosa* involves the entire cell population to communicate through cell-cell signaling molecules in order to coordinate a collective movement over a defined surface. In the laboratory settings, swarming is commonly observed in conditions that carefully control nutrients and surface viscosity compositions (5–7). As the cell population grows, they continuously secrete signaling molecules but do not act as a group yet. Only after the molecules reach a certain threshold concentration, individual cells sense the signaling molecules and act simultaneously with the rest of the population (8, 9). This behavior is known as quorum sensing and allows the overall population to respond collectively to their environment such as forming swarming tendrils on a semisolid surface (10, 11). In *P. aeruginosa*, the quorum sensing system has a direct control over the production of rhamnolipids which play an essential role in collective swarming behavior by lowering surface tension on semi solid surfaces and directing tendrils organization (12, 13). On an individual level, each cell goes through hyperflagellation activity to navigate on the surface (14, 15).

P. aeruginosa swarming motility can therefore be defined as a form of migration induced by rhamnolipids production and hyperactivity of the flagella on semi-solid surfaces such as the mucosal membranes found in the lungs (16). Swarming in *P. aeruginosa* allow the bacterial cell population to spread throughout the host and contributes to the resistance against antibiotics by forming biofilms (17). From the initial inoculation point, *P. aeruginosa* swarms by forming distinct tendrils that migrate in a coordinated manner. These tendrils are capable of sensing and responding to their environment and to various bacterial species (18).

1.3 *P. aeruginosa* Pathway to Produce Rhamnolipids

In *P. aeruginosa*, the overall rhamnolipids production is dependent on the quorum sensing system that involves transcriptional regulators and N-acylhomoserine lactone (AHL) signaling molecules (19). By regulating surfactant production, *P. aeruginosa* populations control swarming and tendrils formation on semi solid surfaces. Specifically, the *rhlAB* genes that form rhamnolipids are mediated by the transcriptional regulator RhlR which belongs to the rhl quorum sensing system and by N-butanoyl-L-homoserine lactone (C4-HSL) (Figure 1A) (20, 21). The complex RhlR and C4-HSL dimerizes and activates a positive feedback loop to produce rhamnolipids (22, 23). Additionally, the las and pqs quorum sensing system produces N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* Quinolone Signal, PQS) that bind respectively to LasR and PqsR, which then regulate RhlR and rhamnolipids production (24). Lastly, the iqs system produces 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing Signal, IQS) that binds to IqsR which then controls indirectly the rhl system by regulating the pqs system (25). Overall, the quorum sensing system in *P. aeruginosa* is an interconnected and co-regulated complex system that influences swarming organization by primarily regulating rhamnolipids production.

Specifically, swarming in *P. aeruginosa* is controlled by the secretion of rhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAAs), which are synthesized by the rhl quorum sensing system, particularly the *rhlAB* operon and *rhlC* (26, 27). RhlA contributes to the production of HAAs, which are then converted to mono-rhamnolipids by rhlB and di-rhamnolipids by rhlC (28). Rhamnolipids lower surface tension so that *P. aeruginosa* swarm on semi-solid surfaces and HAAs maintain spatial organization to avoid tendrils overlaps (Figure 1.1B).

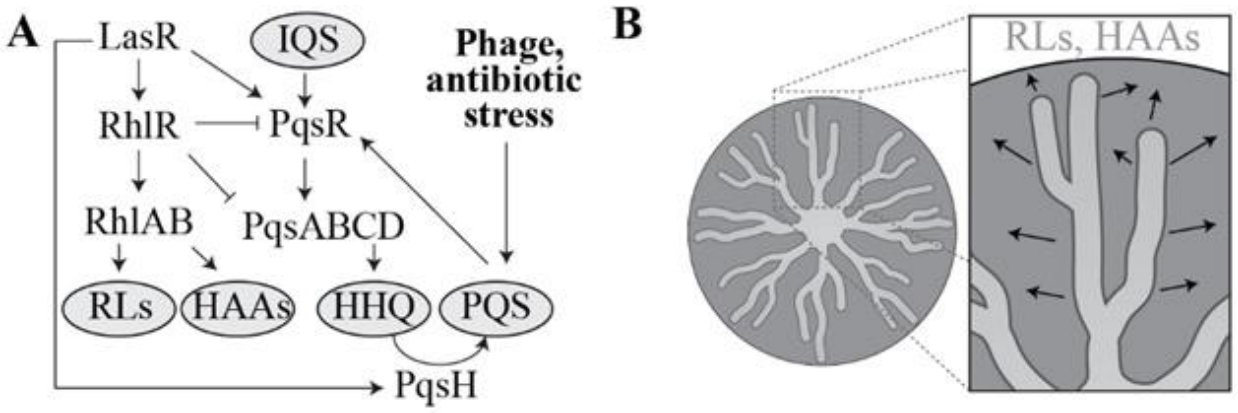

Figure 1.1. Environmental Stress Inhibits Swarming and Increases PQS Production. (A) Pathway representing the regulation of rhamnolipids, HAAs, HHQ, and PQS by stress and quorum sensing. (A) *P. aeruginosa* releases rhamnolipids (RLs) and HAAs (arrows) during swarming.

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1.4 *Pseudomonas Quinolone Signal* (PQS) Quorum Sensing System in *P. aeruginosa*

P. aeruginosa regulates many collective group behaviors among the cell population and the expression of various virulence genes through an interconnected quorum sensing network (29). The *Pseudomonas* quinolone signal (PQS), otherwise known as 2-heptyl-3-hydroxy-4-quinolone, is a molecule secreted by *P. aeruginosa* that has multiple varied roles such as coordinating cell-to-cell interactions through quorum sensing signals, regulating the expression of virulence factors, iron intake, inducing both oxidative stress and an antioxidative response, and modulating host immune responses (30, 31). PQS is commonly found in the lungs of cystic fibrosis patients, demonstrating that the signaling molecule has an essential role in long-term persistence of *P. aeruginosa* infection (32). The synthesis of PQS is regulated by a convoluted network of quorum sensing regulators, including the Las, Rhl, and IQS quorum sensing systems (Figure 1.1A) (24, 25). These systems are also enhanced in response to various environmental conditions such as nutrient starvation, antibiotics, and phage infection (5, 25, 33, 34).

To focus on the PQS quorum sensing system, PQS is synthesized by pqsH through hydroxylation of 4-hydroxy-2-heptylquinoline (HHQ), which is first synthesized by *pqsABCD* from anthranilic acid (29, 30). Both PQS and HHQ acts as co-inducing ligands of pqsR, the transcriptional regulator of *pqsABCD* (37). The multifunctional role of the molecule PQS in signaling and stress responses such as stress in response to bacteriophage infections or antibiotic treatments suggests that it coordinates diverse functions in the collective behavior of swarming (Figure 1.1B).

1.5 From Individual Cells to Collective Communities

1.5.1 Organizing of a Swarming Community with Rhamnolipids

P. aeruginosa are commonly viewed as unicellular organisms that use their flagella to move across a planktonic environment. However, these bacterial communities are commonly found in multicellular colonies such as biofilms that give them extended protection against dangerous environmental conditions and help them survive various circumstances (38, 39). They often rely on chemical signaling to communicate within their population in a process known as quorum sensing to coordinate colony-wide behavior (9, 40, 41). Bacterial swarming uses quorum sensing signals to organize the cells spatially by creating a multilayer swarm throughout its colony and a monolayer of cells at the edge of the swarm (42). This organization results in physical properties that effectively enhance rapid surface swarming, enabling efficient expansion that promote survivability of the overall colony.

Although individual *P. aeruginosa* cells frequently have cell-cell interaction within a population, it is not yet determined how these interactions impact the outcome of the collective population. It is also unknown how rhamnolipids which creates a surfactant layer around the *P. aeruginosa* colonies promote a competitive advantage. By cooperating with their neighboring cells and producing significant quantities of rhamnolipids, the bacterial population is likely not limited to resources found within its colony and can search for nutrients in their environment by swarming (43). Without rhamnolipids production, *P. aeruginosa* cannot swarm and grow beyond its initial spot which may result in overcrowding and nutrient deficiency for the overall population. Rhamnolipids are especially important for changing the viscoelastic properties of their colonies to

coordinate their swarming pattern and move collectively towards environments with rich nutrients (44, 45). Overall, rhamnolipids create an essential surfactant layer for swarming that promotes the survival of the population.

1.5.2 From Swarming Motility to Biofilms Formation

P. aeruginosa swarming is considered a form of rapid migration on a nutrient-rich semi solid surface that involves growth, motility, and quorum sensing across a bacterial population (10, 14, 46). This complex multicellular behavior often precedes the formation of biofilm which is known to rapidly grow on a surface and is important for bacterial survival (12, 47). In *P. aeruginosa*, cyclic di-GMP (c-di-GMP) plays an important role in driving the mechanism for switching motile bacterial cells to biofilm forming units (48). C-di-GMP is involved in the biofilm formation by promoting the free planktonic cells to attach themselves on a surface (48, 49). The bacterial cells then grow to form a microcolony and eventually use the complex quorum sensing systems to mature into an established biofilm (50). The *P. aeruginsoa* population continues to colonize their environment by regulating the level of quorum sensing molecules and especially c-di-GMP, which can promote the detachment of bacterial cells from the biofilm and induce the cells to swim toward available surfaces in order to form additional biofilms (51, 52). Although this cycle commonly occurs in a planktonic environment, the solid or semi solid environment represents a challenge for bacteria to colonize due to the motility restriction of the habitat surrounding the bacterial populations. Therefore, the swarming behavior in *P. aeruginosa* represents an alternative mechanism to swimming in which populations can migrate on surfaces and form biofilms through the regulation of c-di-GMP and quorum sensing molecules.

1.5.3 The Importance of Single Cell Behavior to Maintain Collective Communities

Swarming in *P. aeruginosa* first requires the bacterial population to grow on a semi solid surface and reach log growth phase before they begin producing a significant concentration of surfactant for swarming. *P. aeruginosa* rhamnolipids play the surfactant role by lowering the colony surface tension allowing the bacterial cells to start swarming on semi solid surfaces (14, 53). Without rhamnolipids, *P. aeruginosa* is unable to overcome the surface tension which results in the bacterial population inability to swarm. Solid surfaces such as surfaces with over 2% agar plates also prevent *P. aeruginosa* from swarming because rhamnolipids produced by *P. aeruginosa* is insufficient to overcome the high surface tension (54). On the other hand, this bacterial behavior on soft surfaces such as surfaces with less than 0.3% agar is considered as swimming, not swarming. Here, the surfactant has limited to no impact on bacterial motility since surface tension is close to or completely nonexistent. Overall, *P. aeruginosa* swarming requires exponential growth and the production of rhamnolipids on a semi solid surface.

Although the production of surfactant is essential in swarming motility, *P. aeruginosa* also requires significant flagella activity for swarming to occur. In preparation for swarming, *P. aeruginosa* is known to increase its count of flagella through hyperflagellation (14, 55). By growing exponentially and rapidly moving their flagella, *P. aeruginosa* bacterial cells build up pressure and creates a pressure-driven flow that pushes cells forward while rhamnolipids pull the population ahead through the Marangoni flow effect (56–58). Without flagella, the individual bacterial cell cannot create enough force to progress forward even in the presence of significant concentration of rhamnolipids. The combination of flagella push and rhamnolipids pull are therefore essential for swarming; the lack of either force results in a non-swarming population.

1.6 The Rise of Antibiotic Resistance in *P. aeruginosa*

1.6.1 The Current State of Antibiotic Resistance with *P. aeruginosa*

P. aeruginosa is an opportunistic pathogen that contributes to severe infections in patients with cystic fibrosis or body immune system and is estimated to cause for more than 10% of bacterial infections (59). Strains of *P. aeruginosa* have progressively acquired resistance to multiple categories of antibiotics and is estimated to cost more than 70% for patients compared to non-resistant strains (60, 61). Strains of *P. aeruginosa* that are resistant to most antibiotics have recently emerged in healthcare settings (62–67). Preventing the emergence of these resistant microorganisms is essential to limit the socio-economy impact on patients and reduce the costs for insurances and hospitals. In addition to cost, antimicrobial resistance strains of bacteria are estimated to globally cause 10 million death per year by 2050 (68, 69). Bacterial infections are therefore an increasing public health and economic problem that has been stimulated by the dramatic increase in antibiotic resistance.

Bacterial strains that are multi-drug or extensively drug-resistant have emerged in the healthcare settings. This causes almost if not all antimicrobial drugs to become less effective at treating patients with infections. This is a serious concern as *P. aeruginosa* is a gram-negative bacterial species that is already one of the most difficult to treat due to its intrinsic resistance profile and remarkable ability to develop antibiotic resistance through spontaneous chromosomal mutations and acquisition of resistance genes from multi-drug resistance bacteria (70). Agencies such as the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) therefore insist that the rapid rise of antibiotic resistance in *P. aeruginosa* is becoming a

serious threat to our society when antibiotics are slowly declining in efficacy (2, 69). These agencies also predict that we are quickly entering an era without effective antibiotics, which will result in a larger number of deaths due to bacterial infections (62, 71, 72). Additionally, the recent emergence of high-risk *P. aeruginosa* strains that are multi-drug or extensively-drug resistant has been found across the world suggesting that a large portion of the population can be exposed to these dangerous strains (73, 74). The increase in antibiotic resistant *P. aeruginosa* seriously compromises the ability of the modern society to fight infections. Acting now to restrict the growth of resistant bacteria is therefore essential to prevent future death.

There is an urgent need to develop new therapeutic approaches to combat the rise of antibiotic resistance *P. aeruginosa*. Strategies to address these healthcare issues include the development of novel antimicrobial agents and bacteriophage therapies (75–78). However, the fundamental ways that *P. aeruginosa* respond to these treatments in host environments have not been determined. For new therapeutic agents to be highly effective against the growing infection of antibiotic resistance *P. aeruginosa*, there is a critical need to determine how these bacterial populations respond to antibiotic and bacteriophage in host environments.

1.6.2 The Serious Concern of Antibiotic Resistant *P. aeruginosa* due to Biofilm Formation

In the natural environment, most bacterial cells can attach themselves to different surfaces and collectively form biofilms (79). Biofilm formation starts when free-floating cells encounter a surface and begin colonizing the area by encasing in a self-generated matrix of extracellular polymeric substances (EPS) (80). The EPS is a network of polysaccharides, proteins, and

extracellular nucleic acid such as DNA, which enable the biofilm to form a scaffold for the population to adhere together and on surfaces. The matrix facilitates cell-cell communication within the biofilm and can even provide essential nutrients, enzymes, and proteins for the bacterial community (81). This complex network is one of the key strategies to increase the species survival rate under harsh living conditions such as limited nutrient availability or temperature fluctuation (80). Due to the thickness of biofilms, harmful substances that normally fight bacterial cells have difficulty in penetrating the inner layers of the biofilms (82). This biofilm defense mechanism dilutes the antimicrobial treatments to the point they are no longer effective and leaves a few cells unaffected by the treatments. This gives the bacterial population the possibility to grow back the biofilms. Biofilms therefore provide a protective barrier that is resistant to the host immune mechanism and to antimicrobial treatments up to 1000 times more than planktonic cells (83).

P. aeruginosa is well-known to have the ability to form biofilm and is effective at colonizing a variety of surfaces including medical materials, industrial food equipment, water facilities (82). In addition to the increased antibiotic resistance in *P. aeruginosa*, the ability for this bacterial species to form biofilms in various environments can result in serious infection for the patients. Therefore, a greater understanding of the molecular composition, complex structure, and mechanisms inducing the formation of the biofilm is essential for the development of effective treatments that prevent and eliminate biofilm-associated infections. Since swarming behavior often precedes biofilm formation in *P. aeruginosa,* it is evident that one option to fight these bacterial infections is to tackle the swarming population. This solution targets a bacterial population that is more vulnerable to treatments and prevents the formation of highly resistant biofilm. One focus of this work is to understand how *P. aeruginosa* swarming behavior functions to potentially design antimicrobial treatments that are effective against *P. aeruginosa* invasion.

1.7 References

1. Bodey GP, Bolivar R, Fainstein V, Jadeja L. 1983. Infections caused by Pseudomonas aeruginosa. Rev Infect Dis 5:279–313.

2. Centers for Disease Control and Prevention (U.S.). 2019. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention (U.S.).

3. Faure E, Kwong K, Nguyen D. 2018. Pseudomonas aeruginosa in Chronic Lung Infections: How to Adapt Within the Host? Front Immunol 9:2416.

4. Moore JE, Mastoridis P. 2017. Clinical implications of Pseudomonas aeruginosa location in the lungs of patients with cystic fibrosis. J Clin Pharm Ther 42:259–267.

5. Morales-Soto N, Anyan ME, Mattingly AE, Madukoma CS, Harvey CW, Alber M, Déziel E, Kearns DB, Shrout JD. 2015. Preparation, imaging, and quantification of bacterial surface motility assays. J Vis Exp https://doi.org/10.3791/52338.

6. Schneider WR, Doetsch RN. 1974. Effect of viscosity on bacterial motility. J Bacteriol 117:696–701.

7. Berg HC, Turner L. 1979. Movement of microorganisms in viscous environments. Nature 278:349–351.

8. Ding F, Oinuma K-I, Smalley NE, Schaefer AL, Hamwy O, Greenberg EP, Dandekar AA. 2018. The Pseudomonas aeruginosa Orphan Quorum Sensing Signal Receptor QscR Regulates Global Quorum Sensing Gene Expression by Activating a Single Linked Operon. mBio 9:e01274-18.

9. Rutherford ST, Bassler BL. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2:a012427.

10. Daniels R, Vanderleyden J, Michiels J. 2004. Quorum sensing and swarming migration in bacteria. FEMS Microbiol Rev 28:261–289.

11. Mukherjee S, Bassler BL. 2019. Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol 17:371–382.

12. Thi MTT, Wibowo D, Rehm BHA. 2020. Pseudomonas aeruginosa Biofilms. Int J Mol Sci 21:E8671.

13. Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J Bacteriol 190:2671–2679.

14. Köhler T, Curty LK, Barja F, van Delden C, Pechère J-C. 2000. Swarming of *Pseudomonas aeruginosa* Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. J Bacteriol 182:5990–5996.

15. Anyan ME, Amiri A, Harvey CW, Tierra G, Morales-Soto N, Driscoll CM, Alber MS, Shrout JD. 2014. Type IV pili interactions promote intercellular association and moderate swarming of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 111:18013–18018.

16. Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas aeruginosa Is a Complex Adaptation Leading to Increased Production of Virulence Factors and Antibiotic Resistance. Journal of Bacteriology 190:2671–2679.

17. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/18793317. Retrieved 4 July 2019.

18. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids Modulate Swarming Motility Patterns of Pseudomonas aeruginosa. Journal of Bacteriology 187:7351–7361.

19. Soberón-Chávez G, Lépine F, Déziel E. 2005. Production of rhamnolipids by Pseudomonas aeruginosa. Appl Microbiol Biotechnol 68:718–725.

20. Déziel E, Lépine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology (Reading) 149:2005–2013.

21. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

22. Ventre I, Ledgham F, Prima V, Lazdunski A, Foglino M, Sturgis JN. 2003. Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy. Mol Microbiol 48:187–198.

23. Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW. 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 92:9427–9431.

24. Lee J, Zhang L. 2015. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell 6:26–41.

25. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, Zhang L-H. 2013. A cell-cell communication signal integrates quorum sensing and stress response. Nat Chem Biol 9:339–343.

26. Déziel E, Lépine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149:2005–2013.

27. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

28. Wood TL, Gong T, Zhu L, Miller J, Miller DS, Yin B, Wood TK. 2018. Rhamnolipids from Pseudomonas aeruginosa disperse the biofilms of sulfate-reducing bacteria. npj Biofilms and Microbiomes 4:22.

29. Papenfort K, Bassler BL. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nat Rev Microbiol 14:576–588.

30. Lin J, Cheng J, Wang Y, Shen X. 2018. The Pseudomonas Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. Front Cell Infect Microbiol 8.

31. Häussler S, Becker T. 2008. The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations. PLoS Pathog 4:e1000166.

32. Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC. 2002. A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol Lett 215:41–46.

33. Schafhauser J, Lepine F, McKay G, Ahlgren HG, Khakimova M, Nguyen D. 2014. The Stringent Response Modulates 4-Hydroxy-2-Alkylquinoline Biosynthesis and Quorum-Sensing Hierarchy in Pseudomonas aeruginosa. J Bacteriol 196:1641–1650.

34. Blasdel BG, Ceyssens P-J, Chevallereau A, Debarbieux L, Lavigne R. 2018. Comparative transcriptomics reveals a conserved Bacterial Adaptive Phage Response (BAPR) to viral predation. preprint. Microbiology.

35. Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG. 2004. Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. PNAS 101:1339–1344.

36. Dulcey CE, Dekimpe V, Fauvelle D-A, Milot S, Groleau M-C, Doucet N, Rahme LG, Lépine F, Déziel E. 2013. The End of an Old Hypothesis: The Pseudomonas Signaling Molecules 4-Hydroxy-2-Alkylquinolines Derive from Fatty Acids, Not 3-Ketofatty Acids. Chemistry & Biology 20:1481–1491.

37. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. 2012. The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa. Microbiol Mol Biol Rev 76:46–65.

38. Mann EE, Wozniak DJ. 2012. Pseudomonas biofilm matrix composition and niche biology. FEMS Microbiol Rev 36:893–916.

39. Stubbendieck RM, Vargas-Bautista C, Straight PD. 2016. Bacterial Communities: Interactions to Scale. Front Microbiol 7:1234.

40. Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199.

41. Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319–346.

42. Be’er A, Ariel G. 2019. A statistical physics view of swarming bacteria. Mov Ecol 7:9.

43. Harshey RM. 2003. Bacterial motility on a surface: many ways to a common goal. Annu Rev Microbiol 57:249–273.

44. Czaplicka N, Mania S, Konopacka-Łyskawa D. 2021. Influence of Rhamnolipids and Ionic Cross-Linking Conditions on the Mechanical Properties of Alginate Hydrogels as a Model Bacterial Biofilm. Int J Mol Sci 22:6840.

45. Stoppel WL, White JC, Horava SD, Bhatia SR, Roberts SC. 2011. Transport of biological molecules in surfactant-alginate composite hydrogels. Acta Biomater 7:3988–3998.

46. Tremblay J, Déziel E. 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. J Basic Microbiol 48:509–515.

47. Grobas I, Bazzoli DG, Asally M. 2020. Biofilm and swarming emergent behaviours controlled through the aid of biophysical understanding and tools. Biochem Soc Trans 48:2903–2913.

48. Valentini M, Filloux A. 2016. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from Pseudomonas aeruginosa and Other Bacteria. J Biol Chem 291:12547–12555.

49. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. 2010. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol Microbiol 75:827–842.

50. de Kievit TR. 2009. Quorum sensing in Pseudomonas aeruginosa biofilms. Environ Microbiol 11:279–288.

51. Andersen JB, Kragh KN, Hultqvist LD, Rybtke M, Nilsson M, Jakobsen TH, Givskov M, Tolker-Nielsen T. 2021. Induction of Native c-di-GMP Phosphodiesterases Leads to Dispersal of Pseudomonas aeruginosa Biofilms. Antimicrob Agents Chemother 65:e02431-20.

52. Kahl LJ, Price-Whelan A, Dietrich LEP. 2020. Light-Mediated Decreases in Cyclic di-GMP Levels Inhibit Structure Formation in Pseudomonas aeruginosa Biofilms. J Bacteriol 202:e00117-20.

53. Yang A, Tang WS, Si T, Tang JX. 2017. Influence of Physical Effects on the Swarming Motility of Pseudomonas aeruginosa. Biophys J 112:1462–1471.

54. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 187:7351–7361.

55. Doyle TB, Hawkins AC, McCarter LL. 2004. The complex flagellar torque generator of Pseudomonas aeruginosa. J Bacteriol 186:6341–6350.

56. Still T, Yunker PJ, Yodh AG. 2012. Surfactant-induced Marangoni eddies alter the coffee-rings of evaporating colloidal drops. Langmuir 28:4984–4988.

57. Azevedo MA, Cerqueira MA, Fuciños P, Silva BFB, Teixeira JA, Pastrana L. 2021. Rhamnolipids-based nanostructured lipid carriers: Effect of lipid phase on physicochemical properties and stability. Food Chem 344:128670.

58. Nguyen VX, Stebe KJ. 2002. Patterning of small particles by a surfactant-enhanced Marangoni-Bénard instability. Phys Rev Lett 88:164501.

59. A report from the NNIS System. 2004. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. American Journal of Infection Control 32:470–485.

60. Lin DM, Koskella B, Lin HC. 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. WJGPT 8:162.

61. Morales E, Cots F, Sala M, Comas M, Belvis F, Riu M, Salvadó M, Grau S, Horcajada JP, Montero MM, Castells X. 2012. Hospital costs of nosocomial multi-drug resistant Pseudomonas aeruginosa acquisition. BMC Health Serv Res 12:122.

62. Ventola CL. 2015. The antibiotic resistance crisis: part 1: causes and threats. P T 40:277–283.

63. Rossolini GM, Arena F, Pecile P, Pollini S. 2014. Update on the antibiotic resistance crisis. Current Opinion in Pharmacology 18:56–60.

64. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, Aboderin AO, Al-Abri SS, Awang Jalil N, Benzonana N, Bhattacharya S, Brink AJ, Burkert FR, Cars O, Cornaglia G, Dyar OJ, Friedrich AW, Gales AC, Gandra S, Giske CG, Goff DA, Goossens H, Gottlieb T, Guzman Blanco M, Hryniewicz W, Kattula D, Jinks T, Kanj SS, Kerr L, Kieny M-P, Kim YS, Kozlov RS, Labarca J, Laxminarayan R, Leder K, Leibovici L, Levy-Hara G, Littman J, Malhotra-Kumar S, Manchanda V, Moja L, Ndoye B, Pan A, Paterson DL, Paul M, Qiu H, Ramon-Pardo P, Rodríguez-Baño J, Sanguinetti M, Sengupta S, Sharland M, Si-Mehand M, Silver LL, Song W, Steinbakk M, Thomsen J, Thwaites GE, van der Meer JW, Van Kinh N, Vega S, Villegas MV, Wechsler-Fördös A, Wertheim HFL, Wesangula E, Woodford N, Yilmaz FO, Zorzet A. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases 18:318–327.

65. Golkar Z, Bagasra O, Pace DG. 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. The Journal of Infection in Developing Countries 8.

66. Klevens RM. 2007. Invasive Methicillin-Resistant Staphylococcus aureus Infections in the United States. JAMA 298:1763.

67. Lushniak BD. 2014. Antibiotic resistance: a public health crisis. Public Health Rep 129:314–316.

68. Chokshi A, Sifri Z, Cennimo D, Horng H. 2019. Global Contributors to Antibiotic Resistance. J Glob Infect Dis 11:36–42.

69. Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, Greko C, So AD, Bigdeli M, Tomson G, Woodhouse W, Ombaka E, Peralta AQ, Qamar FN, Mir F, Kariuki S, Bhutta ZA, Coates A, Bergstrom R, Wright GD, Brown ED, Cars O. 2013. Antibiotic resistance—the need for global solutions. The Lancet Infectious Diseases 13:1057–1098.

70. Horcajada JP, Montero M, Oliver A, Sorlí L, Luque S, Gómez-Zorrilla S, Benito N, Grau S. 2019. Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-Resistant Pseudomonas aeruginosa Infections. Clin Microbiol Rev 32:e00031-19.

71. Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJV, Projan S, Thomas CM, Tomasz A, Tulkens PM, Walsh TR, Watson JD, Witkowski J, Witte W, Wright G, Yeh P, Zgurskaya HI. 2011. Tackling antibiotic resistance. Nature Reviews Microbiology 9:894–896.

72. Bald D, Koul A. 2013. Advances and strategies in discovery of new antibacterials for combating metabolically resting bacteria. Drug Discovery Today 18:250–255.

73. Cabot G, Ocampo-Sosa AA, Domínguez MA, Gago JF, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Oliver A, Spanish Network for Research in Infectious Diseases (REIPI). 2012. Genetic markers of widespread extensively drug-resistant Pseudomonas aeruginosa high-risk clones. Antimicrob Agents Chemother 56:6349–6357.

74. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736–755.

75. Lin DM, Koskella B, Lin HC. 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. WJGPT 8:162.

76. Servick K. 2016. Beleaguered phage therapy trial presses on. Science 352:1506–1506.

77. Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, Foster S, Gilmore BF, Hancock REW, Harper D, Henderson IR, Hilpert K, Jones BV, Kadioglu A, Knowles D, Ólafsdóttir S, Payne D, Projan S, Shaunak S, Silverman J, Thomas CM, Trust TJ, Warn P, Rex JH. 2016. Alternatives to antibiotics—a pipeline portfolio review. The Lancet Infectious Diseases 16:239–251.

78. Labrie SJ, Samson JE, Moineau S. 2010. Bacteriophage resistance mechanisms. Nature Reviews Microbiology 8:317–327.

79. Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890.

80. Rollet C, Gal L, Guzzo J. 2009. Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: a comparative study of adhesion and physiological characteristics in Pseudomonas aeruginosa. FEMS Microbiol Lett 290:135–142.

81. Ryder C, Byrd M, Wozniak DJ. 2007. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr Opin Microbiol 10:644–648.

82. Ghafoor A, Hay ID, Rehm BHA. 2011. Role of exopolysaccharides in Pseudomonas aeruginosa biofilm formation and architecture. Appl Environ Microbiol 77:5238–5246.

83. Lewis K. 2001. Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007.

CHAPTER 2: Swarming Motility of *Pseudomonas aeruginosa* Through the Lens of Biophysics

2.1 Swarming Pattern Formation

2.1.1 The Role of Quorum Sensing in Swarming Coordination

Quorum sensing is the basis of bacterial cell-to-cell communication and coordinates swarming in *P. aeruginosa* by synchronizing the production of 3-(3- hydroxyalkanoyloxy) alkanoic acids (HAAs) and rhamnolipids (1–4). The expression of the *rhlAB* and *rhlC* genes, which synthesize HAAs and rhamnolipids (5–7), are controlled by a complex network of quorum sensing regulators, which are summarized here. This system ensures that HAAs and rhamnolipids are produced only when *P. aeruginosa* reaches high cell density(8). LasR and RhlR are the key quorum sensing regulators in this network (9–11). The expression of *rhlAB* and *rhlC* is controlled directly by the transcriptional regulator RhlR (5, 7, 9, 12). The activity of RhlR is regulated by the autoinducer signaling molecule N-butanoyl-L-homoserine lactone (C4-HSL). The binding of C4-HSL to RhlR activates transcription of *rhlAB* and *rhlC*, thereby activating the production of rhamnolipids (7, 9, 13, 14). The autoinducer C4-HSL is produced by the lactone synthase RhlI, which is controlled by a few small RNAs, including RsmA, RsmY, and RhlS (15–17). The activity of RhlR is also regulated by the alternative ligand thioesterase PqsE (18, 19). The expression of RhlR itself is regulated by the LasR regulator (20), which is also only activated at high cell density (7, 9, 11, 21, 22). Additional regulation of RhlR has been proposed through the IQS system (23, 24). Together, this complex quorum sensing network ensures that rhamnolipids and HAAs are

produced when *P. aeruginosa* reach high cell density. Both compounds have been proposed to have critical roles in swarm tendril formation and swarm expansion.

2.1.2 The Role of Rhamnolipids in Swarming Coordination

2.1.2.1 Wetting agents facilitate swarming

Rhamnolipids and HAAs are surfactants – they decrease the surface tension of the liquid-containing *P. aeruginosa* population with the surface. The hydrophobic component of these surfactants interacts with the surface while the hydrophilic component interacts with the *P. aeruginosa* population (25–28). Rhamnolipids and HAAs also function as wetting agents, which decrease the angle that the *P. aeruginosa* population makes with the surface (contact angle) (5, 29–31). Strains that are deficient in HAAs and rhamnolipids do not expand on surfaces (29, 32). On the other hand, strains that are deficient in rhamnolipid production but produce HAAs expand slower on surfaces and have altered tendril morphologies (29). The production of wetting agents alone is not sufficient to explain how a *P. aeruginosa* swarm expands or how tendrils are formed. While wetting agents decrease the energetic barrier for a swarm to expand, a missing element to the interpretation is identifying what drives a swarm to expand in the first place. Bacterial growth itself is typically implied as the source of swarm expansion. However, this interpretation is drawn into question by fluid mechanical models that will be discussed below.

2.1.2.2 Rhamnolipid and HAAs sensing in tendril formation

How do HAAs and rhamnolipids impact the formation of swarming tendrils? Rhamnolipids have been proposed to coordinate tendril formation due their concentration

gradients (33). In this model, the lowest concentrations of rhamnolipids result in the linear movement of tendrils and higher concentrations result in movement inhibition. This model explains why tendrils move forward to areas devoid of rhamnolipids and are repelled from neighboring tendrils. Further support of this model is offered by the *sadB* mutant (29). Tendrils of this mutant do not change direction in response to approaching tendrils and move through rhamnolipid zones. This has led to the proposal that *sadB* has a role in rhamnolipid sensing. In such a model, tendrils move forward linearly due to the sensation of lower concentrations of rhamnolipids at the tendril tips and are inhibited from lateral movements at the side due to sensation of high concentrations of rhamnolipids from neighboring tendrils. To date, a molecular sensor of rhamnolipids has not been identified.

An additional model posits that di-rhamnolipids and HAAs have opposing roles: di-rhamnolipids attract swarming cells and promote tendril formation whereas HAAs are strong repellants and inhibit tendril formation (12, 34). The tendril pattern is thus a result of competition between opposing forces. A more recent report demonstrated that rhamnolipids at concentrations above 2.5 mM attract swarming tendrils but repel tendrils below this concentration (33). This observation suggests the concentration-dependent properties of surfactants may need to be accounted for in an opposing force model.

Additional molecules have been observed to modulate or disrupt tendril patterns. Tendrils are repelled by the quorum sensing molecule 2-heptyl-3-hydroxy-4-quinolone (PQS), which is produced by *P. aeruginosa* colonies that are treated with antibiotics or bacteriophage (33) and by phenol soluble modulin fibers that are produced by *S. aureus* (the results are demonstrated in chapter 6). The molecule 2-heptyl-4-quinolone, which is a precursor to the synthesis of PQS, also repelled tendrils (33). In addition, tendrils are repelled by abiotic compounds including

polydimethylsiloxane and oleic acid (the results are demonstrated in chapter 6). It is unclear how a sensory system detects the broad range of molecules and modulates swarm patterns in response. The ability of *P. aeruginosa* swarms to respond to biotic and abiotic alike raises the issue of the extent that tendril formation is guided by a sensory system. It is possible that tendril formation has a sensory response mechanism for select molecules but is guided by a mechanism that does not require direct detection for others. An additional alternative is that tendril formation does not require a sensory system.

2.1.3 Nutrient and Rhamnolipids Sensing

The movement of tendrils towards areas of greater nutrient availability has suggested that swarming expansion is a chemotactic response that promotes the search for nutrients. Indeed, iron and phosphate limitation and the presence of ethanol promote swarming in *P. aeruginosa* (35–38) and the chemotaxis system is required for swarming in *E. coli* (39). Simulations of nutrient gradients using reaction-diffusion equations and chemotaxis can reproduce many of the tendril features observed in experiments (40–42). However, extensive nutrient depletion is required to create these gradients, which has drawn into question whether such models represent experimental conditions (42). Additional experimental evidence has demonstrated that while the chemotaxis system is needed, the chemosensor itself is not required for swarming in *E. coli* (39, 43, 44). This supports the critical role of flagella function in swarming but suggests that direct nutrient sensing does not drive the outwards expansion of tendrils. Rather, the chemosensory system is reprogrammed during swarming to increase runs, resulting in Lévy walk trajectories that have the

potential to maximize search of space (45). In this model, the chemotaxis system operates on a 'locked on' mode that is insensitive to chemical gradients.

2.1.4 The Role of Fluid Mechanics

To what extent are swarm patterns the result of biological mechanisms? To what extent are patterns due to fluid mechanical mechanisms? Here, fluid mechanical mechanisms are considered. Multiple physical mechanisms may contribute to tendril formation that do not rely on biological sensing. Tendril formation is observed in thin films of abiotic fluids that spread on solid surfaces. For example, tendrils are formed by a process referred to as viscous fingering and Saffman-Taylor instabilities (46–50). Here, a fluid that is driven by pressure displaces another fluid of higher viscosity, forming tendrils or fingers in the process. In Hele-Shaw devices, pressure that is exerted from a central point forms tendrils that move radially outwards. The shape and dynamics of tendrils formed in Hele-Shaw devices have remarkable resemblance to *P. aeruginosa* tendrils observed in swarms (Figure 2.1). Critical tendril properties, including finger number and length, and number of branch points, can be altered in abiotic fluids to resemble patterns observed in swarming patterns produced by diverse bacteria (51–53). The formation of tendrils by abiotic fluids that resemble bacterial swarms has raised the possibility that fluid mechanics could have an important role in the formation of swarming patterns. Two fluidic processes have been discussed: Marangoni-driven flow and pressure-driven flow. Both models treat bacterial swarms as thin liquid films and recapitulate the formation of tendrils that are observed during swarming. However, the mechanisms in each model that drive swarming and form tendrils are markedly distinct.

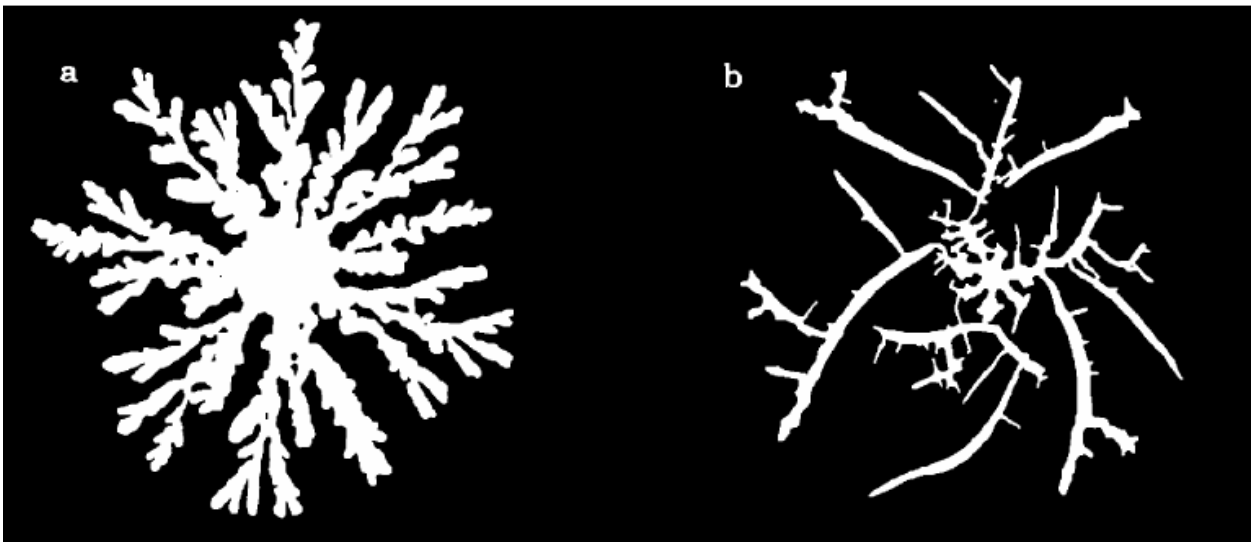

Figure 2.1. Fractal Structures that Resemble *P. aeruginosa* Swarming. Fingering patterns obtained after injection of water in a (A) thin liquid paste or (B) thick liquid paste (54).

2.1.4.1 Marangoni Flow

The production of surfactants is required for swarming by *P. aeruginosa*. The involvement of surfactants has raised the possibility that surface tension has a major role in driving swarms and forming swarming patterns. Understanding the role of surface tension and surfactants requires a deeper understanding of the physicochemical properties of the swarm environment. Bacteria in swarms are embedded in a liquid medium. At the front edge of the swarm boundary is an air-liquid interface. The cohesive forces in the liquid medium at the interface are responsible for producing surface tension, which is a force that opposes the movement of the bacteria-containing liquid beyond the interface (Figure 2.2A). Surfactants such as rhamnolipids can modify surface tension within the swarm, which could impact its movement.

In particular, the presence of a surface tension gradient in the swarm could give rise to Marangoni-driven flow (55, 56). Marangoni flows run cyclically within the liquid medium of a colony and can have a significant impact on colony morphology, causing cells to move to the edge or the center of a colony (Figure 2.2A) (57). The flows arise when a gradient of surface tension is present. Liquids flow along surface tension gradients from the direction of low surface tension to higher surface tensions. The expression of rhamnolipid-producing genes decreases from the center towards the swarming front (56, 58), suggesting that a gradient of surface tension is present. Fauvart et al. proposed that a gradient in rhamnolipid concentration would produce a surface tension gradient, with the least surface tension in the center of a swarm and the greatest surface tension at the swarming front (56). This condition would produce a Marangoni flow in which the bacteria-containing liquid flows from the center of the swarm towards the front edge of the swarm, thus driving the swarm outwards (Figure 2.2A). In this model, the formation of swarming tendrils can result from the non-linearity of Marangoni flows and the multiple parameters (i.e.,

concentration gradients, surface tension, surface diffusion, dynamic surface tension, viscosity) that impact their dynamics. Small perturbations at the front edge can be amplified, resulting in large tendrils.

Du et al. showed through simulations that tendril formation could be explained by Marangoni forces that arise from the production of rhamnolipids (59, 60). They simulated swarming as a thin viscous liquid film and found that densely-pack *P. aeruginosa* increases the viscosity of the liquid film, thereby reducing the film's spreading speed. Rhamnolipids create a surface tension gradient that pushes the liquid film and *P. aeruginosa* outward. Small perturbations at the swarming boundary are amplified by Marangoni forces and result in tendril formation. Trinschek et al. demonstrated the emergence of swarming tendrils by implementing a thin liquid film model that incorporates the effects of surface wettability and Marangoni flows due to surfactants (61). This model incorporates a passive thin film layer that is driven by a growth law and production of surfactant, to produce a diverse range of growth phenotypes including swarms without distinct tendrils and pronounced tendrils. Modification of this model to incorporate chemo-sensing and motility demonstrated that swarming tendrils could detect and be repelled by antibiotics (62), a feature that was observed in recent experiments (33, 63). While these reports have demonstrated that swarming tendrils and the advancement of the swarming front can be explained by Marangoni flows, it is unclear whether this is the primary mechanism that drives swarming or tendril formation.

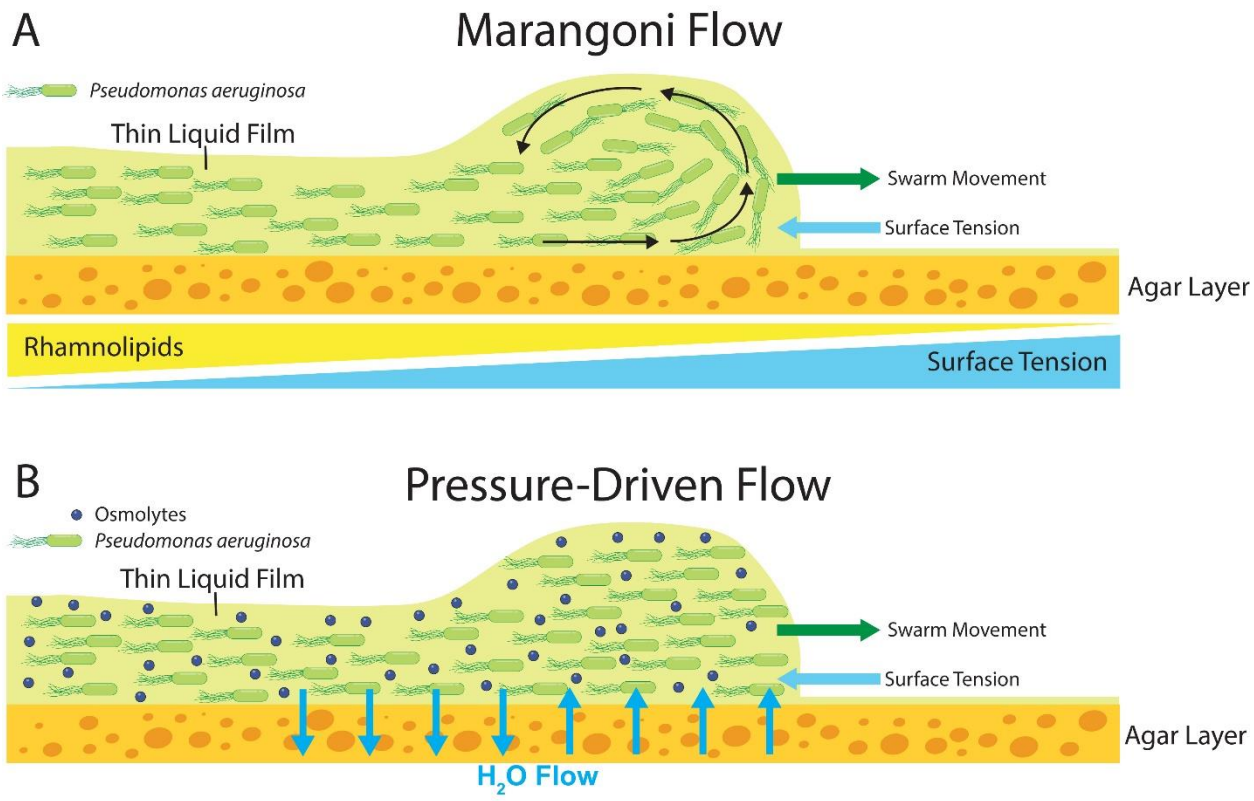

Figure 2.2. Representation of Marangoni Flow and Pressure-Driven Flow. (A) Cyclical flow of cells at the edge of the tendril. (B) Fluid influx from the agar layer into the swarming layer caused by osmolytes.

2.1.4.2 Pressure-Driven Flow

In a Hele-Shaw device, viscous fingering produces tendrils in abiotic fluids that resemble the tendrils of bacterial swarms (Figure 2.1A). The formation of tendrils by viscous fingering is governed by Darcy's law, which describes the movement of a fluid that is driven by pressure. In the Hele-Shaw cell, a fluid expansion is driven by externally-supplied pressure (Figure 2.1A). Giverso et al. investigated the impact of pressure-driven flow on tendril formation by bacterial swarms (64, 65). In their model, the bacterial swarm is treated as a thin liquid film that is surrounded by 'lubricant' fluid that is present on the petri dish. Giverso et al. treat the swarm as a thin film of viscous fluid that is driven forward by a pressure. Here, Darcy's law describes the expansion of the swarm as a thin liquid film by an internal pressure. The critical issue that arises is how an internal pressure could arise in a swarm. They consider two models that are described by mass balance equations: volumetric expansion and chemotactic growth. In the volumetric expansion model, an abundant nutrient supply facilitates bacterial growth, which creates a pressure in a swarm that drives the population expansion outwards. The swarming population displaces the lubricant fluid and forms tendrils in the process. This tendril formation has similarities to the formation of viscous fingers in a Hele-Shaw cell. While the model is not developed specifically for *P. aeruginosa*, the model could describe swarming of this species, with the rhamnolipids function as the lubricant fluid. In the alternative chemotactic growth model, pressure in a swarm is created by the chemotactic motility of bacteria towards an attractant. Simulations of both models yield tendrils that are qualitatively similar to experimental observations but have quantitative differences such as the translation of center of mass of swarms. In light of experimental evidence suggesting that chemosensing is dispensable for swarming (39, 45), the volumetric expansion model may be more relevant for tendril formation in *P. aeruginosa.*

Wu et al. examined the role of fluid influx from the agar layer into the swarm layer as a driver of swarm expansion (66). The authors tracked the fluid flow in *E. coli* swarms using microbubbles. They identified the presence of fluid reservoirs near the leading edge of swarms and proposed a model in which water is drawn up from the agar layer to the swarm layer at the leading edge and pumped outward by flagella, resulting in swarm expansion. The authors suggest that by-products of metabolism function as osmolytes that drive the water flux from the agar layer to the swarm layer. Ping et al. measured spatial changes in osmolarity in an *E. coli* swarm using liposomes and confirmed a significant peak in osmolarity at the leading edge of the swarm. The authors suggest that the osmolyte is relatively high molecular weight and could be lipopolysaccharide. Srinivasan et al. performed simulations based on the experimental work of Wu et al. and Ping et al., referring to the model as steady state swarming, based on the assumption that bacteria produce osmolytes (67). Here, Darcy's law appears again but describes the flow of fluid between the agar and swarm layers. This presence of osmolytes induces fluid to flow from the agar layer and is driven into the swarm layer by capillary and viscous stresses. This flow occurs near the swarming edge; in locations distant from the swarming edge, some of the fluid is eventually returned into the agar layer. Here, the agar layer and the swarm layer function as a circulatory system that results in overall swarm expansion.

The entry of water into the swarm layer described by these models causes volumetric expansion of the swarm, like the volumetric expansion model proposed by Giverso et al. The notable difference is that Wu et al., Ping et al., and Srinivasan et al. attributed the swarm expansion to the flow of fluid from the agar layer into the swarm layer, whereas Giverso et al. attributed the swarm expansion to bacterial growth. It is notable that the studies focus on *E. coli*, which does not

produce surfactants. The studies also describe swarm expansion and do not explain tendril formation.

Yang et al. investigated the role of fluid flux between the agar and swarm layers in swarming and tendril formation in *P. aeruginosa* (68). The study posited that osmolytes such as lipopolysaccharides that are produced by *P. aeruginosa* create osmotic pressure that draws of fluid from the agar layer into the swarm layer. They propose that the flow of fluid here is governed by Darcy's law. Their experiments showed that increasing osmolarity in the agar layer decreased swarming motility, which can be understood by reduced osmolyte differential between the swarm and agar layers. This results in decreased flow to the swarm layer and concomitantly, decreased expansion of the swarm. These findings support an important role for fluid influx from the agar layer into the swarming layer and a role for pressure-driven flow in *P. aeruginosa* swarming (Figure 2.2B).

It is noted that Darcy's law, which describes flow that is induced by pressure, is used to describe multiple mechanisms relevant to swarming. Giverso et al. describe pressure within the swarm itself that drives the expansion of the swarm and can result in the formation of tendrils (65). Srinivasan et al., based on the analyses of Wu et al. and Ping et al., arrive at a Darcy law-like form to describe the influx of fluid into the swarm layer of *E. coli* from the agar layer that is driven by osmotic pressure (66, 67, 69). Similarly, Yang et al. describe the influx of fluid into the *P. aeruginosa* swarm layer using Darcy's law (68). While the influx of fluid into the swarm layer describes swarm expansion, the mechanism does not explain tendril formation. Indeed, tendrils are not a prominent feature of *E. coli* swarms. How the same mechanism may explain swarm expansion in *E. coli* but in addition, give rise to tendrils in *P. aeruginosa*, is not understood. However, the different outcomes could be attributed to the production of surfactants in the latter

species. The displacement of rhamnolipids by the swarm is suggestive of a scenario proposed by Giverso et al., in which the swarm displaces a lubricant fluid(65). Swarming in *P. aeruginosa* could involve coupling of two processes that are described by Darcy's law: fluid transfer between the agar and swarm layers and volumetric expansion due to the influx of water. Such a mechanism could in principle give rise to tendril formation, though no such model has been investigated.

To what extent can swarming be attributed to pressure-driven flow and to Marangoni flow? Yang et al. added the surfactant Triton X-100 to the agar (68), which is expected to minimize the surface tension gradient that drives Marangoni flows. A decrease in this gradient is expected to decrease Marangoni flows, and thus decrease swarm growth and tendril formation. However, the opposite was observed, with the addition of the surfactant enhancing swarm growth and tendril formation. The authors rationalize that enhancement of the swarm growth is the expected outcome in a system that is driven by pressure. In this model, surface tension acts as a force that opposes the growth of the swarm. Ke et al. found similarly that surface tension restricts swarm growth in *B. subtilis* (70). Decreasing surface tension relieves this opposing force and results in enhanced swarm growth. Importantly, the results suggest that the surface tension gradient is dispensable for swarming, which would imply that Marangoni flows are not the primary drivers of swarm growth or tendril formation. The relative contribution between Marangoni flows and pressure-driven flows in *P. aeruginosa* swarms remains to be determined.

2.2 Conclusion

2.2.1 The Optimal Conditions for Swarming

Swarming in *P. aeruginosa* occurs when bacterial populations are in a tightly controlled environment (71, 72). *P. aeruginosa* swarming happens when the colonies are on a plate with an agar content between 0.3% to 1% (71, 73, 74). Plate dryness also affects swarming patterns and drying the surface over an extended period of time in a laminar flow cabinet (71). By retaining water on the surface, bacterial cells easily overcome the surface tension and begin swarming. The velocity of swarming is defined by the speed at which bacterial populations swarm across a surface and could be determined by the percentage of water present on the surface at a given time (68).

Additionally, nutrients are essential for bacterial growth which drive swarming behavior. Under low nutrient conditions, a limited number of cells are capable of dividing and producing enough rhamnolipids for swarming. This likely causes the internal forces of the swarming populations to be weakened, thus lowering the pushing forces that drive swarming behavior. Consequently, the velocity of swarming is reduced significantly (59, 75). Although previous observations suggest that optimal conditions for *P. aeruginosa* swarming involve a soft agar surface with agar concentration between 0.3% and 1.5% with high water and nutrient content, it is unclear how these conditions are optimal for swarming behavior (71).

2.2.2 The Relevance of *P. aeruginosa* Swarming Behavior

P. aeruginosa swarming behavior has been observed and studied primarily in the laboratory under controlled conditions as a single species. However, it remains undetermined how the bacterial population swarms in their natural environment. *P. aeruginosa* is generally found in soil, plants, and water and can form biofilms to sustain harsh environments (76–78). Although studies have shown that swarming often precedes biofilm formations, it is unclear if swarming specifically happens in these environments (79–82). The conditions for *P. aeruginosa* to swarm require a soft surface in a high humidity environment with abundant nutrients and only a few habitats such as the lungs meet the requirements for enabling swarming behavior (83).

P. aeruginosa is commonly found in the lungs and many studies suggest that swarming occurs on mucosal lung tissues similarly to soft agar surfaces (83–85). The lungs offer a relatively soft surface with a moist environment that could promote the swarming behavior in *P. aeruginosa* during infection (83). However, it is important to note that studies have not shown clear swarming pattern formation on lung tissue models. Most observations were made on soft agar plates that mimicked lung conditions (1). Therefore, it is essential to determine swarming behavior in the lung environment.

Most studies show *P. aeruginosa* swarming on a soft agar plate that only contains one species of bacterium. However, in their natural environment, *P. aeruginosa* cohabits with a multitude of organisms that likely interact with the bacterial population (86–89). These interactions can impact swarming behavior by disrupting the rhamnolipids layer or by directly preventing *P. aeruginosa* growth (33). Therefore, understanding the interaction between *P. aeruginosa*

swarming and its environment is essential to determine the relevance of the swarming on bacterial growth and pathogenesis.

Although researchers have observed *P. aeruginosa* swarming in artificial environments such as soft agar surfaces with high humidity conditions and nutrients content, the swarming behavior remains relevant to study. This species puts extensive energy and resources into developing this form of behavior which likely promotes increased survivability in certain environments (58). This suggests that it is critical for the bacterial species to swarm. Additionally, swarming often precedes biofilms formation which is a leading cause of infections in hospital settings (90, 91). By understanding swarming behavior in *P. aeruginosa*, we can expand our knowledge on therapeutic options to help us combat these infections.

2.3 References

1. Köhler T, Curty LK, Barja F, van Delden C, Pechère J-C. 2000. Swarming of *Pseudomonas aeruginosa* Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. J Bacteriol 182:5990–5996.

2. Daniels R, Vanderleyden J, Michiels J. 2004. Quorum sensing and swarming migration in bacteria. FEMS Microbiol Rev 28:261–289.

3. Mukherjee S, Bassler BL. 2019. Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol 17:371–382.

4. Schuster M, Greenberg EP. 2006. A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa. Int J Med Microbiol 296:73–81.

5. Déziel E, Lépine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology (Reading) 149:2005–2013.

6. Ochsner UA, Fiechter A, Reiser J. 1994. Isolation, characterization, and expression in Escherichia coli of the Pseudomonas aeruginosa rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 269:19787–19795.

7. Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, Soberón-Chávez G. 2001. Cloning and functional characterization of the Pseudomonas aeruginosa rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 40:708–718.

8. Henkel M, Schmidberger A, Vogelbacher M, Kühnert C, Beuker J, Bernard T, Schwartz T, Syldatk C, Hausmann R. 2014. Kinetic modeling of rhamnolipid production by Pseudomonas aeruginosa PAO1 including cell density-dependent regulation. Appl Microbiol Biotechnol 98:7013–7025.

9. Medina G, Juárez K, Valderrama B, Soberón-Chávez G. 2003. Mechanism of Pseudomonas aeruginosa RhlR transcriptional regulation of the rhlAB promoter. J Bacteriol 185:5976–5983.

10. Pearson JP, Pesci EC, Iglewski BH. 1997. Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol 179:5756–5767.

11. Pesci EC, Pearson JP, Seed PC, Iglewski BH. 1997. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. J Bacteriol 179:3127–3132.

12. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

13. Ventre I, Ledgham F, Prima V, Lazdunski A, Foglino M, Sturgis JN. 2003. Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy. Mol Microbiol 48:187–198.

14. Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, Bally M, Chapon V, Salmond GP, Bycroft BW. 1995. Multiple N-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 92:9427–9431.

15. Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Cámara M, Haas D, Williams P. 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in Pseudomonas aeruginosa. J Bacteriol 183:6676–6683.

16. Sonnleitner E, Schuster M, Sorger-Domenigg T, Greenberg EP, Bläsi U. 2006. Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in Pseudomonas aeruginosa. Mol Microbiol 59:1542–1558.

17. Thomason MK, Voichek M, Dar D, Addis V, Fitzgerald D, Gottesman S, Sorek R, Greenberg EP. 2019. A rhlI 5' UTR-Derived sRNA Regulates RhlR-Dependent Quorum Sensing in Pseudomonas aeruginosa. mBio 10:e02253-19.

18. Déziel E, Gopalan S, Tampakaki AP, Lépine F, Padfield KE, Saucier M, Xiao G, Rahme LG. 2005. The contribution of MvfR to Pseudomonas aeruginosa pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-L-homoserine lactones. Mol Microbiol 55:998–1014.

19. Mukherjee S, Moustafa DA, Stergioula V, Smith CD, Goldberg JB, Bassler BL. 2018. The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 115:E9411–E9418.

20. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. 2003. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185:2080–2095.

21. Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. 1996. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol 21:1137–1146.

22. Rutherford ST, Bassler BL. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2:a012427.

23. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, Zhang L-H. 2013. A cell-cell communication signal integrates quorum sensing and stress response. Nat Chem Biol 9:339–343.

24. Lee J, Zhang L. 2015. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell 6:26–41.

25. Srinivas G, Nielsen SO, Moore PB, Klein ML. 2006. Molecular dynamics simulations of surfactant self-organization at a solid-liquid interface. J Am Chem Soc 128:848–853.

26. Paria S, Khilar KC. 2004. A review on experimental studies of surfactant adsorption at the hydrophilic solid-water interface. Adv Colloid Interface Sci 110:75–95.

27. Boles BR, Thoendel M, Singh PK. 2005. Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. Mol Microbiol 57:1210–1223.

28. Kim LH, Jung Y, Yu H-W, Chae K-J, Kim IS. 2015. Physicochemical interactions between rhamnolipids and Pseudomonas aeruginosa biofilm layers. Environ Sci Technol 49:3718–3726.

29. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 187:7351–7361.

30. Ozdemir G, Malayoglu U. 2004. Wetting characteristics of aqueous rhamnolipids solutions. Colloids Surf B Biointerfaces 39:1–7.

31. Costa SGVAO, de Souza SR, Nitschke M, Franchetti SMM, Jafelicci M, Lovaglio RB, Contiero J. 2009. Wettability of Aqueous Rhamnolipids Solutions Produced by Pseudomonas aeruginosa LBI. J Surfact Deterg 12:125–130.

32. Ghorbal SKB, Chourabi K, Maalej L, Ammar AB, Ouzari H-I, Hassen A, Jaafoura H, Chatti A. 2019. Pseudomonas aeruginosa Swarmer Cells Adaptation Toward UVc Radiations. Front Microbiol 10:556.

33. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the Pseudomonas aeruginosa Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201:e00383-19.

34. Rütschlin S, Böttcher T. 2020. Inhibitors of Bacterial Swarming Behavior. Chemistry 26:964–979.

35. Badal D, Jayarani AV, Kollaran MA, Prakash D, P M, Singh V. 2021. Foraging Signals Promote Swarming in Starving Pseudomonas aeruginosa. mBio 12:e0203321.

36. Bains M, Fernández L, Hancock REW. 2012. Phosphate starvation promotes swarming motility and cytotoxicity of Pseudomonas aeruginosa. Appl Environ Microbiol 78:6762–6768.

37. Boyle KE, Monaco H, van Ditmarsch D, Deforet M, Xavier JB. 2015. Integration of Metabolic and Quorum Sensing Signals Governing the Decision to Cooperate in a Bacterial Social Trait. PLoS Comput Biol 11:e1004279.

38. Kollaran AM, Joge S, Kotian HS, Badal D, Prakash D, Mishra A, Varma M, Singh V. 2019. Context-Specific Requirement of Forty-Four Two-Component Loci in Pseudomonas aeruginosa Swarming. iScience 13:305–317.

39. Burkart M, Toguchi A, Harshey RM. 1998. The chemotaxis system, but not chemotaxis, is essential for swarming motility in Escherichia coli. Proc Natl Acad Sci U S A 95:2568–2573.

40. Kozlovsky Y, Cohen I, Golding I, Ben-Jacob E. 1999. Lubricating bacteria model for branching growth of bacterial colonies. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics 59:7025–7035.

41. Luo N, Wang S, Lu J, Ouyang X, You L. 2021. Collective colony growth is optimized by branching pattern formation in Pseudomonas aeruginosa. Mol Syst Biol 17:e10089.

42. Marrocco A, Henry H, Holland IB, Plapp M, Séror SJ, Perthame B. 2010. Models of Self-Organizing Bacterial Communities and Comparisons with Experimental Observations. Mathematical Modelling of Natural Phenomena 5:148–162.

43. Wang Q, Suzuki A, Mariconda S, Porwollik S, Harshey RM. 2005. Sensing wetness: a new role for the bacterial flagellum. EMBO J 24:2034–2042.

44. Mariconda S, Wang Q, Harshey RM. 2006. A mechanical role for the chemotaxis system in swarming motility. Mol Microbiol 60:1590–1602.

45. Partridge JD, Nhu NTQ, Dufour YS, Harshey RM. 2019. Escherichia coli Remodels the Chemotaxis Pathway for Swarming. mBio 10:e00316-19.

46. 1958. The penetration of a fluid into a porous medium or Hele-Shaw cell containing a more viscous liquid. Proc R Soc Lond A 245:312–329.

47. Homsy GM. 1987. Viscous Fingering in Porous Media. Annu Rev Fluid Mech 19:271–311.

48. Coussot P. 1999. Saffman–Taylor instability in yield-stress fluids. J Fluid Mech 380:363–376.

49. DiCarlo DA. 2013. Stability of gravity-driven multiphase flow in porous media: 40 Years of advancements: Stability of Multiphase Gravity-Driven Flow. Water Resour Res 49:4531–4544.

50. Tabeling P, Zocchi G, Libchaber A. 1987. An experimental study of the Saffman-Taylor instability. J Fluid Mech 177:67–82.

51. Ristroph L, Thrasher M, Mineev-Weinstein MB, Swinney HL. 2006. Fjords in viscous fingering: selection of width and opening angle. Phys Rev E Stat Nonlin Soft Matter Phys 74:015201.

52. Bischofberger I, Ramachandran R, Nagel SR. 2014. Fingering versus stability in the limit of zero interfacial tension. Nat Commun 5:5265.

53. Cheng X, Xu L, Patterson A, Jaeger HM, Nagel SR. 2008. Towards the zero-surface-tension limit in granular fingering instability. 3. Nature Phys 4:234–237.

54. Tzschichholz F, Wangen M. 2001. Modellization of hydraulic fracturing of porous materials. arXiv:cond-mat/0101369. arXiv.

55. Daniels R, Reynaert S, Hoekstra H, Verreth C, Janssens J, Braeken K, Fauvart M, Beullens S, Heusdens C, Lambrichts I, De Vos DE, Vanderleyden J, Vermant J, Michiels J. 2006. Quorum signal molecules as biosurfactants affecting swarming in Rhizobium etli. Proc Natl Acad Sci U S A 103:14965–14970.

56. Fauvart M, Phillips P, Bachaspatimayum D, Verstraeten N, Fransaer J, Michiels J, Vermant J. 2011. Surface tension gradient control of bacterial swarming in colonies of Pseudomonas aeruginosa. Soft Matter 8:70–76.

57. Sempels W, De Dier R, Mizuno H, Hofkens J, Vermant J. 2013. Auto-production of biosurfactants reverses the coffee ring effect in a bacterial system. Nat Commun 4:1757.

58. Tremblay J, Déziel E. 2010. Gene expression in Pseudomonas aeruginosa swarming motility. BMC Genomics 11:587.

59. Du H, Xu Z, Anyan M, Kim O, Leevy WM, Shrout JD, Alber M. 2012. High density waves of the bacterium Pseudomonas aeruginosa in propagating swarms result in efficient colonization of surfaces. Biophys J 103:601–609.

60. Du H, Xu Z, Shrout JD, Alber M. 2011. MULTISCALE MODELING OF PSEUDOMONAS AERUGINOSA SWARMING. Math Models Methods Appl Sci 21 Suppl 1:939–954.

61. Li A, Schertzer JW, Yong X. 2018. Molecular dynamics modeling of *Pseudomonas aeruginosa* outer membranes. Physical Chemistry Chemical Physics 20:23635–23648.

62. Kotian HS, Abdulla AZ, Hithysini KN, Harkar S, Joge S, Mishra A, Singh V, Varma MM. 2020. Active modulation of surfactant-driven flow instabilities by swarming bacteria. Phys Rev E 101:012407.

63. Morales-Soto N, Dunham SJB, Baig NF, Ellis JF, Madukoma CS, Bohn PW, Sweedler JV, Shrout JD. 2018. Spatially dependent alkyl quinolone signaling responses to antibiotics in Pseudomonas aeruginosa swarms. J Biol Chem 293:9544–9552.

64. Giverso C, Verani M, Ciarletta P. 2015. Branching instability in expanding bacterial colonies. J R Soc Interface 12:20141290.

65. Giverso C, Verani M, Ciarletta P. 2016. Emerging morphologies in round bacterial colonies: comparing volumetric versus chemotactic expansion. Biomech Model Mechanobiol 15:643–661.

66. Wu Y, Berg HC. 2012. Water reservoir maintained by cell growth fuels the spreading of a bacterial swarm. Proc Natl Acad Sci U S A 109:4128–4133.

67. Srinivasan S, Kaplan CN, Mahadevan L. 2019. A multiphase theory for spreading microbial swarms and films. Elife 8:e42697.

68. Yang A, Tang WS, Si T, Tang JX. 2017. Influence of Physical Effects on the Swarming Motility of Pseudomonas aeruginosa. Biophys J 112:1462–1471.

69. Ping L, Wu Y, Hosu BG, Tang JX, Berg HC. 2014. Osmotic pressure in a bacterial swarm. Biophys J 107:871–878.

70. Ke W-J, Hsueh Y-H, Cheng Y-C, Wu C-C, Liu S-T. 2015. Water surface tension modulates the swarming mechanics of Bacillus subtilis. Front Microbiol 6:1017.

71. Tremblay J, Déziel E. 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. J Basic Microbiol 48:509–515.

72. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. 2006. The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. Mol Microbiol 62:1264–1277.

73. Rashid MH, Kornberg A. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97:4885–4890.

74. Kohler T, Curty LK, Barja F, van Delden C, Pechere J-C. 2000. Swarming of Pseudomonas aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. Journal of Bacteriology 182:5990–5996.

75. Be'er A, Ariel G. 2019. A statistical physics view of swarming bacteria. Mov Ecol 7:9.

76. Kerr KG, Snelling AM. 2009. Pseudomonas aeruginosa: a formidable and ever-present adversary. J Hosp Infect 73:338–344.

77. Moradali MF, Ghods S, Rehm BHA. 2017. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front Cell Infect Microbiol 7:39.

78. Mulcahy LR, Isabella VM, Lewis K. 2014. Pseudomonas aeruginosa biofilms in disease. Microb Ecol 68:1–12.

79. Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J. 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol 16:496–506.

80. Grobas I, Bazzoli DG, Asally M. 2020. Biofilm and swarming emergent behaviours controlled through the aid of biophysical understanding and tools. Biochem Soc Trans 48:2903–2913.

81. Worlitzer VM, Jose A, Grinberg I, Bär M, Heidenreich S, Eldar A, Ariel G, Be'er A. 2022. Biophysical aspects underlying the swarm to biofilm transition. Sci Adv 8:eabn8152.

82. Murray TS, Ledizet M, Kazmierczak BI. 2010. Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of Pseudomonas aeruginosa clinical isolates. J Med Microbiol 59:511–520.

83. Hutchison ML, Govan JR. 1999. Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect 1:1005–1014.

84. Hancock REW, Speert DP. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat 3:247–255.

85. Rajan S, Saiman L. 2002. Pulmonary infections in patients with cystic fibrosis. Semin Respir Infect 17:47–56.

86. Alves PM, Al-Badi E, Withycombe C, Jones PM, Purdy KJ, Maddocks SE. 2018. Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. Pathog Dis 76.

87. Woods PW, Haynes ZM, Mina EG, Marques CNH. 2018. Maintenance of S. aureus in Co-culture With P. aeruginosa While Growing as Biofilms. Front Microbiol 9:3291.

88. Monteiro R, Magalhães AP, Pereira MO, Sousa AM. 2021. Long-term coexistence of Pseudomonas aeruginosa and Staphylococcus aureus using an in vitro cystic fibrosis model. Future Microbiol 16:879–893.

89. Limoli DH, Warren EA, Yarrington KD, Donegan NP, Cheung AL, O'Toole GA. 2019. Interspecies interactions induce exploratory motility in Pseudomonas aeruginosa. Elife 8:e47365.

90. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, Ali M, Rafiq M, Kamil MA. 2018. Bacterial biofilm and associated infections. J Chin Med Assoc 81:7–11.

91. Percival SL, Suleman L, Vuotto C, Donelli G. 2015. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. J Med Microbiol 64:323–334.

92. Yeung ATY, Torfs ECW, Jamshidi F, Bains M, Wiegand I, Hancock REW, Overhage J. 2009. Swarming of Pseudomonas aeruginosa is controlled by a broad spectrum of transcriptional regulators, including MetR. J Bacteriol 191:5592–5602.

93. Girod S, Zahm JM, Plotkowski C, Beck G, Puchelle E. 1992. Role of the physiochemical properties of mucus in the protection of the respiratory epithelium. Eur Respir J 5:477–487.

94. Swiecicki J-M, Sliusarenko O, Weibel DB. 2013. From swimming to swarming: Escherichia coli cell motility in two-dimensions. Integr Biol (Camb) 5:1490–1494.

95. Belas R, Schneider R, Melch M. 1998. Characterization of Proteus mirabilis precocious swarming mutants: identification of rsbA, encoding a regulator of swarming behavior. J Bacteriol 180:6126–6139.

96. Kim W, Surette MG. 2005. Prevalence of surface swarming behavior in Salmonella. J Bacteriol 187:6580–6583.

97. Jaques S, McCarter LL. 2006. Three new regulators of swarming in Vibrio parahaemolyticus. J Bacteriol 188:2625–2635.

CHAPTER 3: Spatial Organization of Pseudomonas aeruginosa by Stress Response

3.1 Current Status

Bacteria use physical and chemical cues to adjust their metabolism in response to environmental changes and can employ stress responses to trigger defense mechanisms against threats. Two prominent dangers that bacteria encounter are bacteriophages (phages), which infect, replicate within, and kill bacteria, as well as antimicrobial compounds. Recent work has significantly advanced our knowledge of how bacteria respond to such threats in homogeneous environments. However, natural and host settings are typically heterogeneous. Understanding how phage infections and antimicrobial compounds propagate and affect the spatial organization of bacterial biofilms is necessary to fully understand how bacteria increase their defense mechanism against threats in natural multi-species environments.

Bacteria have evolved anti-phage defenses such as blocking infection, degrading phage genetic material, with some bacteria committing altruistic suicide to prevent the spread of phage progeny to neighboring cells. Since phages require a host to proliferate, the risk of phage infection correlates with bacterial cell density. Bacteria can monitor population density through quorum sensing; otherwise known as cell-cell communication processes. Quorum sensing is used both to measure overall cell-density dependent risk of phage attack and is also used to produce stress signals that activate phage defenses. Specifically, the spatial release of the quorum sensing and stress signal such as *Pseudomonas* Quinolones Signals (PQS) promotes the survival of the bacterial population by redirecting the movement of the healthy population away from stress induced by phages and antibiotics (Figure 3.1) (1). Such signaling strategy coupled with spatial reorganization

could improve bacterial survival in complex host environments. In fact, a recent article reported that bacteria hiding in the mucosal layers of the gut are protected from phages present in the lumen, and that seeding of these bacteria into the lumen allows phage-host persistence in this niche (2). Moreover, bacteria that are resistant to phage can protect fellow phage sensitive bacteria from phage predation when grown together in a spatially structured colony, but not in a liquid planktonic culture, again highlighting the importance of spatial structure in phage defense (3).

In the healthcare settings, stress molecules from bacterial populations could induce resistance or evasion against phage therapy and antibiotic treatment, making these strategies to fight bacterial infections potentially ineffective. Understanding the impact of stress responses on the spatial organization of biofilms and microbial communities is therefore critical for the development of more effective treatments against pathogenic bacterial species.

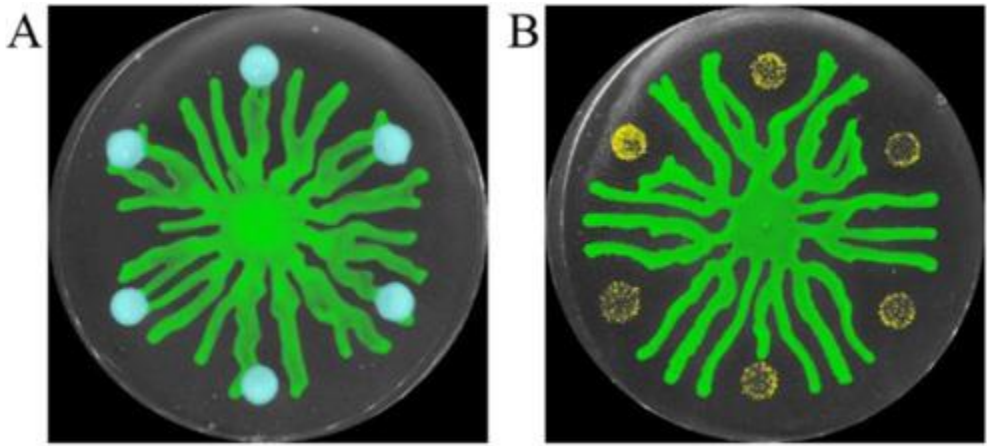

Figure 3.1. Spatial organization by a stress response in bacterial populations. (A) *P. aeruginosa* swarms (green) merge with unstressed sub-populations (blue). (B) *P. aeruginosa* swarms (green) are re-directed away from sub-populations that are infected by phage (yellow), which release the cell–cell signaling molecule PQS. Images are shown with a filter to improve visibility of the bacterial colonies and swarming cells.

3.2 Ongoing and Future Challenges

Several critical challenges need to be addressed to investigate the spatial impact of stress responses on bacterial biofilms, including recreating multispecies biofilm communities in host and natural environments and determining the spatial organization of bacterial metabolism and stress-induced signaling molecules. Recent studies on the cellular activity of biofilms revealed that they are spatially organized such that different areas of biofilms have distinct metabolic, transcriptional, and translational activities (4). For example, bacteria that are located at the periphery of a biofilm are exposed to different stresses and molecules, such as phages and antimicrobial compounds, compared to those that are insulated deep within the biofilm core and are starved of oxygen and nutrients (5, 6). The ability to measure the spatial distribution of cellular activity has typically relied on fluorescent reporters and dyes. However, for long-term monitoring of cellular activity, there is a risk of photobleaching reporters, phototoxicity to the cells, and incomplete staining of biofilm with dyes, which do not diffuse into biofilm cores. Although optogenetics enables precise regulation and monitoring of specific activity of mammalian cells, this technique is not well-established for imaging individual cells within a biofilm (7). Thus, the ability to spatially resolve metabolic activity at the single cell level as well as signaling molecules and metabolites within a biofilm remains a challenge.

While stress responses are investigated in laboratory settings, the relevance of these studies to natural and host environments can at times be unclear. A critical challenge is the ability to imitate the growth conditions of these environments under well-controlled conditions in the laboratory. This includes mimicking the physical properties of tissue, mucus layers, immune responses and gradients in nutrients and oxygen. These challenges make it difficult to study the

dynamics of spatially structured multi-species communities in a laboratory setting. The agonistic and antagonistic interactions between different species greatly affect the overall outcome of bacterial encounters with stresses. Therefore, the establishment of structured biofilm models as multi-species communities of bacteria, phages, and other microbes is central to understand fundamental interactions across kingdoms.

3.3 Advances in Science and Technology to Meet Challenges

Advances in label-free imaging and tissue culturing technologies have the potential to address many of the current challenges to studying stress responses in microbial communities with minimal impact on cell physiology. Advances in confocal Raman spectroscopy have enabled label-free studies that map the spatial distribution of individual molecules such as the QS molecule PQS (8) and has the potential to be used with many other signaling molecules and metabolites. Mass spectroscopy imaging is another label-free method (9) that enables the mapping of individual molecular species in different locations within a biofilm (8). Coupling an optical visualization method with molecular identification has the potential to monitor the spatial distribution and concentration of different molecular species in response to stresses. Fluorescence lifetime imaging microscopy (FLIM) is another label-free technique that measures the spatial organization of metabolic activity with sub-cellular resolution (10). Due to the non-invasive nature of the method, it has the potential to track both metabolic activities spatially and temporally.

Recent advances in cryogenic electron microscopy (cryo-EM) have enabled the study of complex biological structures such as biofilms at high resolution. Cryo-EM imaging can reveal the spatial organization of the population and differences in cell morphology and subcellular structures throughout sections of a biofilm. Of interest is to analyze the spatial effects of stresses including nutrient and oxygen limitation within the core of the biofilm and external stresses such as phage predation and antibiotic stress in the outer layer of biofilms.

Advances in organoid and organ-on-a-chip technologies have the promise to imitate more accurately the conditions of the host, including restoring tissue and cellular function, producing mucus layers, and providing representative nutrient environment and gradients. The technology

has been extended to produce many tissues including lung, skin, and gut (11). Biofilms can be cultured on such devices or may even be 3D printed into complex structures (12) to observe spatial organization of the bacterial cells using the label-free imaging methods described here.

3.4 Conclusion

Stress responses help with bacterial survival and resistance to environmental threats from phage infection and antibiotic treatments in part through the re-arrangement of the spatial organization of their physical environments. However, significant challenges in imaging and analysis have hampered the ability to investigate the spatial component of stress responses in high resolution. Recent developments in label-free imaging through optical, electron, and mass spectrometry imaging have the potential to address these challenges. Coupling recent advances in imitating host environments through organ-on-chip devices and organoids will enable the study of bacterial stress responses that are relevant in hosts as well as providing a path to investigating stress response in multi-species communities in higher detail. Uncovering how bacteria organize structurally to avoid dangers such as phages and antibiotics in natural- and host environments may lead to development of drugs, which can inhibit such mechanisms. In turn, this may render populations of pathogens more vulnerable to treatments with antimicrobials.

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3.5 References

1. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the Pseudomonas aeruginosa Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201.

2. Lourenço M, Chaffringeon L, Lamy-Besnier Q, Campagne P, Eberl C, Bérard M, Stecher B, Debarbieux L, De Sordi L. 2019. The spatial heterogeneity of the gut limits bacteriophage predation leading to the coexistence of antagonist populations of bacteria and their viruses. preprint. Microbiology.

3. Testa S, Berger S, Piccardi P, Oechslin F, Resch G, Mitri S. 2019. Spatial structure affects phage efficacy in infecting dual-strain biofilms of Pseudomonas aeruginosa. Commun Biol 2:405.

4. Díaz-Pascual F, Hartmann R, Lempp M, Vidakovic L, Song B, Jeckel H, Thormann KM, Yildiz FH, Dunkel J, Link H, Nadell CD, Drescher K. 2019. Breakdown of Vibrio cholerae biofilm architecture induced by antibiotics disrupts community barrier function. Nat Microbiol https://doi.org/10.1038/s41564-019-0579-2.

5. Miller C, Kong J, Tran TT, Arias CA, Saxer G, Shamoo Y. 2013. Adaptation of Enterococcus faecalis to daptomycin reveals an ordered progression to resistance. Antimicrob Agents Chemother 57:5373–5383.

6. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317–323.

7. Liu Z, Zhang J, Jin J, Geng Z, Qi Q, Liang Q. 2018. Programming Bacteria With Light-Sensors and Applications in Synthetic Biology. Front Microbiol 9:2692.

8. Morales-Soto N, Dunham SJB, Baig NF, Ellis JF, Madukoma CS, Bohn PW, Sweedler JV, Shrout JD. 2018. Spatially dependent alkyl quinolone signaling responses to antibiotics in Pseudomonas aeruginosa swarms. J Biol Chem 293:9544–9552.

9. Buchberger AR, DeLaney K, Johnson J, Li L. 2018. Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights. Anal Chem 90:240–265.

10. Stringari C, Cinquin A, Cinquin O, Digman MA, Donovan PJ, Gratton E. 2011. Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in a live tissue. Proceedings of the National Academy of Sciences 108:13582–13587.

11. Huh D, Hamilton GA, Ingber DE. 2011. From 3D cell culture to organs-on-chips. Trends Cell Biol 21:745–754.

12. Connell JL, Ritschdorff ET, Whiteley M, Shear JB. 2013. 3D printing of microscopic bacterial communities. Proc Natl Acad Sci USA 110:18380–18385.

CHAPTER 4: PQS Produced by *Pseudomonas aeruginosa* Stressed with Bacteriophage or Antibiotics Repels Healthy Swarming Populations

4.1 Introduction to the Stress Response of *P. aeruginosa*

4.1.1 Overview of the *P. aeruginosa* Stress Response

Stress responses help individual bacterial cells adapt to environmental stresses such as low pH, ion concentrations, and nutrient availability (1–4). At the community level, bacterial stress responses promote survival of the overall population. Bacteria often communicate from cell to cell using signals, which is a mechanism known as quorum sensing (QS). This communication system has the ability to activate bacteriophage defense mechanisms at high cell density when bacteriophages could otherwise rapidly spread throughout the bacterial community (5–8). Under antibiotic stress, quorum sensing can also warn the overall bacterial population of incoming danger and help the community preparing for it. These defense mechanisms promote the survival of the bacterial species. In addition, the emergence of phenotypic heterogeneity in bacterial populations gives rise to persisters, otherwise known as antibiotic-tolerant cells, that are transiently resistant to antibiotics (9–11). In the natural and host environments, bacterial populations are spatially heterogeneous, resulting in multiple sub-colonies of the same species (12, 13). It is therefore common to find coordinated spatial stress responses between sub-colonies which has the potential to increase the species chance to survive.

4.1.2 The Composition of the Stress Response in *P. aeruginosa*

Swarming is a collective form of bacterial behavior driven by rhamnolipids production and flagella to induce antibiotic resistance and pathogenesis in humans and animals (14–19). High cell density bacterial populations swarm on soft agar surfaces, which have physical features like those of mucosal membranes (20). The spatial organization and dynamic motion of *P. aeruginosa* swarms are controlled by the secretion of 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAAs), mono-rhamnolipids, and di-rhamnolipids, which modulate the repulsion and attraction between different swarming tendrils (Figure 4.1A) (21, 22). Whether and how these signals are coordinated in response to stress is not fully determined yet. However, studies have suggested that *P. aeruginosa* swarming populations are directed by sub-population signals (23). In particular, threats to the bacterial communities in the form of bacteriophage invasion and antibiotic treatments could have a significant impact on the *P. aeruginosa* group behaviors.

P. aeruginosa regulates virulence genes and community behaviors through quorum sensing molecules (24). The *Pseudomonas* quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS) is secreted by *P. aeruginosa* to coordinate specific cell-to-cell coordination such as virulence factor expression, iron acquisition, and stress response (25, 26). PQS is commonly found in the lungs of patients with underlying lungs conditions, suggesting the importance of this molecule in *P. aeruginosa* infection (27). LasR, RhlR, and IQS quorum sensing systems are known to regulate the synthesis of PQS and can be upregulated under environmental stress (28, 29). PQS is synthesized from anthranilate by enzymes encoded by the *pqsABCD* operon and *pqsH* (30–33). The multi-functional role of PQS in cell-to-cell communication and stress responses strongly

implies that this quorum sensing molecule could also coordinate diverse functions among a collective bacterial community of *P. aeruginosa*.

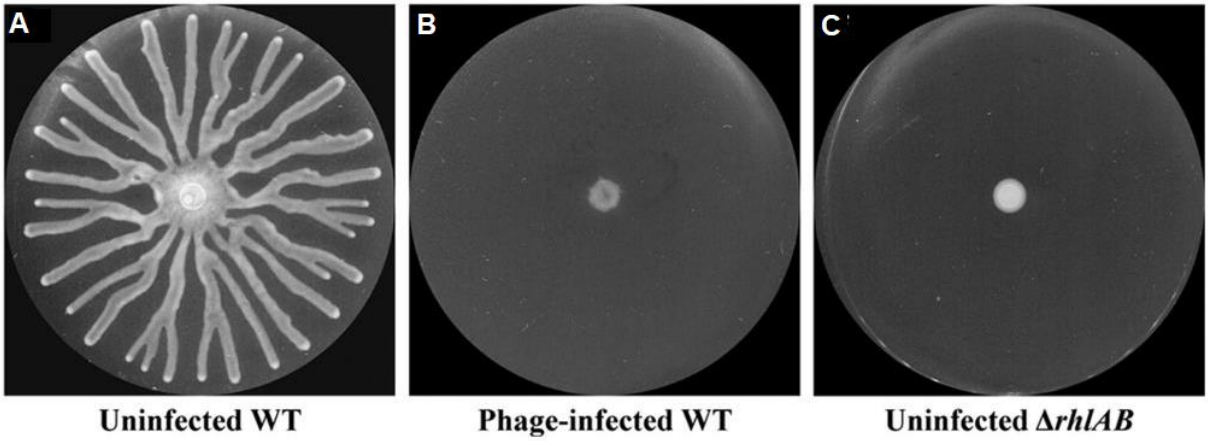

Figure 4.1. Infection of *P. aeruginosa* by Bacteriophage Inhibits Swarming Motility. Wild-type (WT) *P. aeruginosa* strain PA14 without phage (A) or with phage (B) or the Δ*rhlAB* strain without phage (C) was spotted at the center.

4.2 PQS Produced by Infected *P. aeruginosa* Repel Uninfected Swarming Populations

4.2.1 Bacteriophage-Infected *P. aeruginosa* Repel Uninfected Swarming Populations

We used the bacteriophage DMS3vir, which is an engineered lytic form of the DMS3 bacteriophage that was previously isolated from clinical human samples, on *P. aeruginosa* UCBPP-PA14 swarms to characterize the effect of virulent bacteriophage infection (34). Overnight stationary phase *P. aeruginosa* cultures were mixed with DMS3vir and spotted on semi solid agar swarming plates. The plates were incubated for 16-18 hours at 37 °C. Bacteriophage were prepared by diluting the stock lysate 1:100 to establish a concentration of phage (10^{12} PFU/ml) that did not cause the complete lysis of the *P. aeruginosa* population. Infection of the *P. aeruginosa* population by bacteriophage inhibited swarming motility (Figure 4.1B), which is consistent with a previous study in which DMS3 lysogenization of *P. aeruginosa* suppressed swarming through a CRISPR-Cas dependent mechanism (35). The inhibition of swarming motility resembles the Δ*rhlAB* strain which does not produce rhamnolipids and HAAs, which are normally required for swarming.

We also investigated the bacteriophage infection ability to spread to neighboring swarms by comparing two uninfected spots on a plate (Figure 4.2A *i*), both uninfected/infected *P. aeruginosa* colonies (Figure 4.2A *ii*), and an uninfected spot on one side and the Δ*rhlAB* strain on the other side on the same plate (Figure 4.2A *iii*). A six-way swarming assays were performed for reproducibility and consisted of uninfected *P. aeruginosa* at the center of the swarming plate and *P. aeruginosa* test cultures at satellite positions surrounding the center (Figure 4.2B). Uninfected *P. aeruginosa* spotted at the satellite positions without phage exhibited swarming motility and

repulsed the tendrils of the center swarm (Figure 4.2B *i*). This phenotype is consistent with the ability of HAAs to repel swarming populations (36). Swarming motility was inhibited for bacteriophage-infected satellite colonies and the bacterial population repulsed uninfected swarms from the vicinity of the infection (Figure 4.1B, 4.2A *ii* and 4.2B *ii*). The inhibition of swarming motility in phage-infected wild-type cells was comparable to the Δ*rhlAB* strain, which is defective in swarming motility due to the lack of HAA and rhamnolipid production (Figure 4.2A *iii*). As expected, in the absence of rhamnolipids and HAAs to repel swarming population, wild-type cells swarm through the Δ*rhlAB* colonies (Figure 4.2B *iii*).

This repulsion was not due to factors present in the cell-free phage lysate or the concentration of these factors was insufficient to trigger the repulsion, as spotting cell-free phage lysates at the satellite positions alone did not induce repulsion of the uninfected swarms (Figure 2.3). These results indicate that phage infection inhibits swarming motility, and the infected colonies repel uninfected swarms.

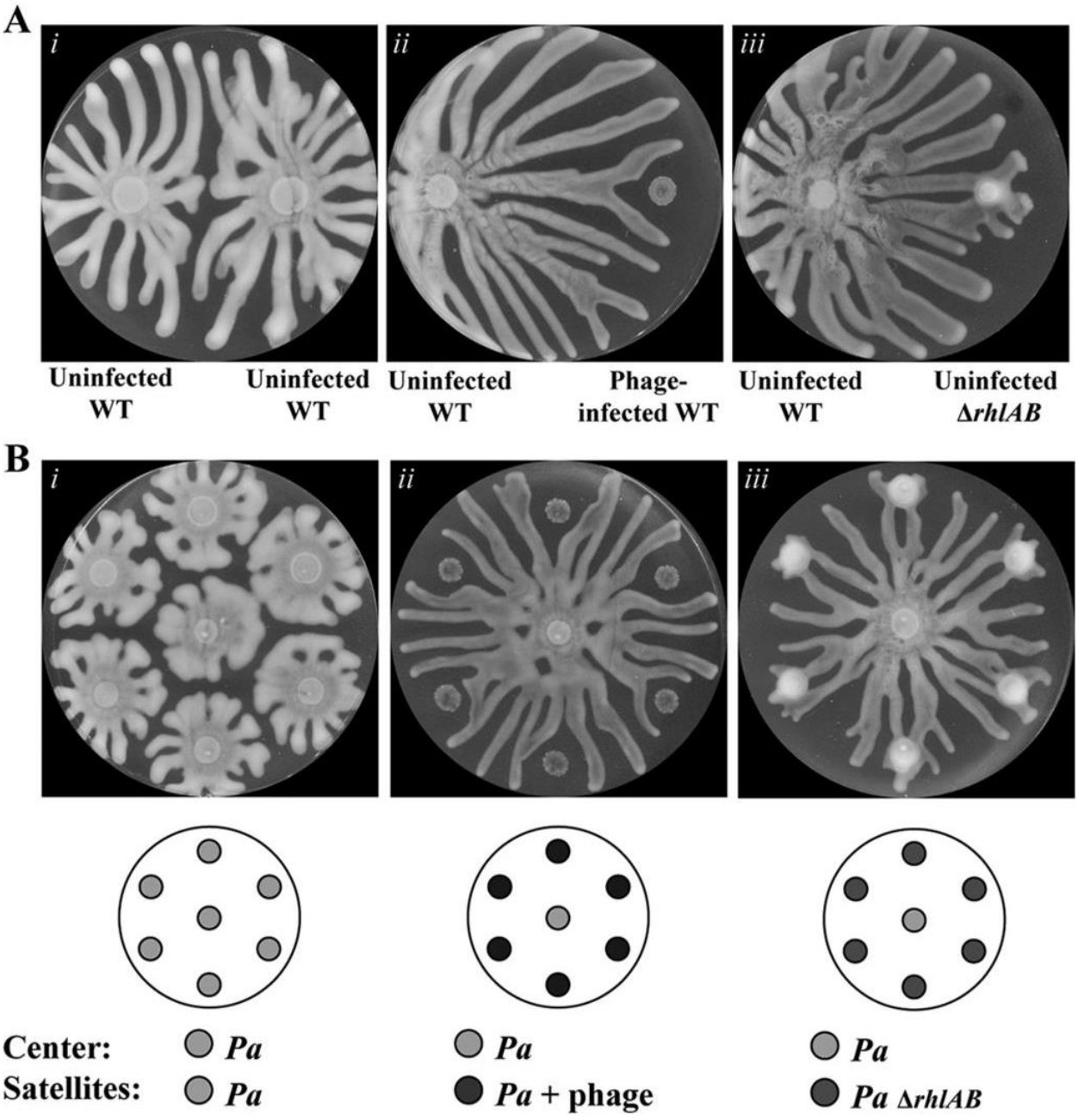

Figure 4.2. Infection of *P. aeruginosa* by Bacteriophage Inhibits Motility and Induces Repulsion of Swarming Populations. Wild-type (WT) *P. aeruginosa* strain PA14 was spotted on the right (A) or at the centner (B) while WT without phage (*i*) or with phage (*ii*) or the Δ*rhlAB* strain without phage (iii) was spotted on the right (A), or at concentric satellite positions surrounding the center (B). Graphics below the images indicate the initial spot positions and corresponding cultures. Swarm images were taken after 16 to 18 h of growth at 37°C. Petri dishes are 9 cm in diameter.

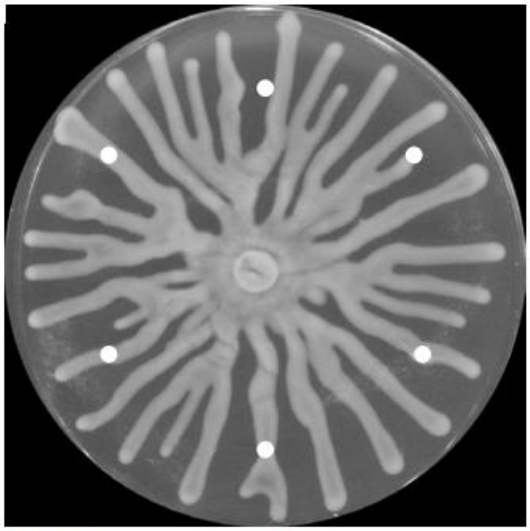

Figure 4.3. *Pseudomonas aeruginosa* Swarming Through Cell-Free Bacteriophage Lysate. Wild-type *P. aeruginosa* at the center and (A) phage only at satellite positions, imaged after 16 hours of growth.

4.2.2 *P. aeruginosa* Surviving Phage Infection Are Heterogeneous

To determine whether the repulsion phenotype was inherited in descendants of phage-infected *P. aeruginosa*, individual colonies were isolated from phage-infected satellite colonies and tested for the ability to be re-infected using the cross-streaking method, in which cells are streaked past a line of phage. The ability of bacteria to grow beyond the phage line indicates that the strains are not susceptible to phage killing. We observed that two out of the four colonies that were isolated from the phage-infected satellite colonies were not susceptible to phage killing (Figure 4.4A). Phage-resistant strains that were mixed again with phage were not inhibited for swarming motility (Figure 4.4B), suggesting that these isolates are spontaneous surface mutants and/or have acquired CRISPR-Cas-dependent adaptive immunity against the phage. In contrast, reinfection of phage-sensitive strains inhibited motility and caused repulsion of the uninfected swarm (Figure 4.4C), which was phenotypically identical to the *P. aeruginosa* organisms that were initially infected (Figure 4.2B, 4.3A *ii*, 4.3B *ii*). These results show that the dual phenotype of swarming inhibition and repulsion of uninfected swarms requires phage to actively infect. We expected most *P. aeruginosa* organisms that survive phage infection would be resistant to phage. However, half of the surviving *P. aeruginosa* isolates were phage resistant (Figure 4.4A). This observation indicates that a transient phage defense mechanism is associated with the swarming deficiency and repulsion phenotypes.

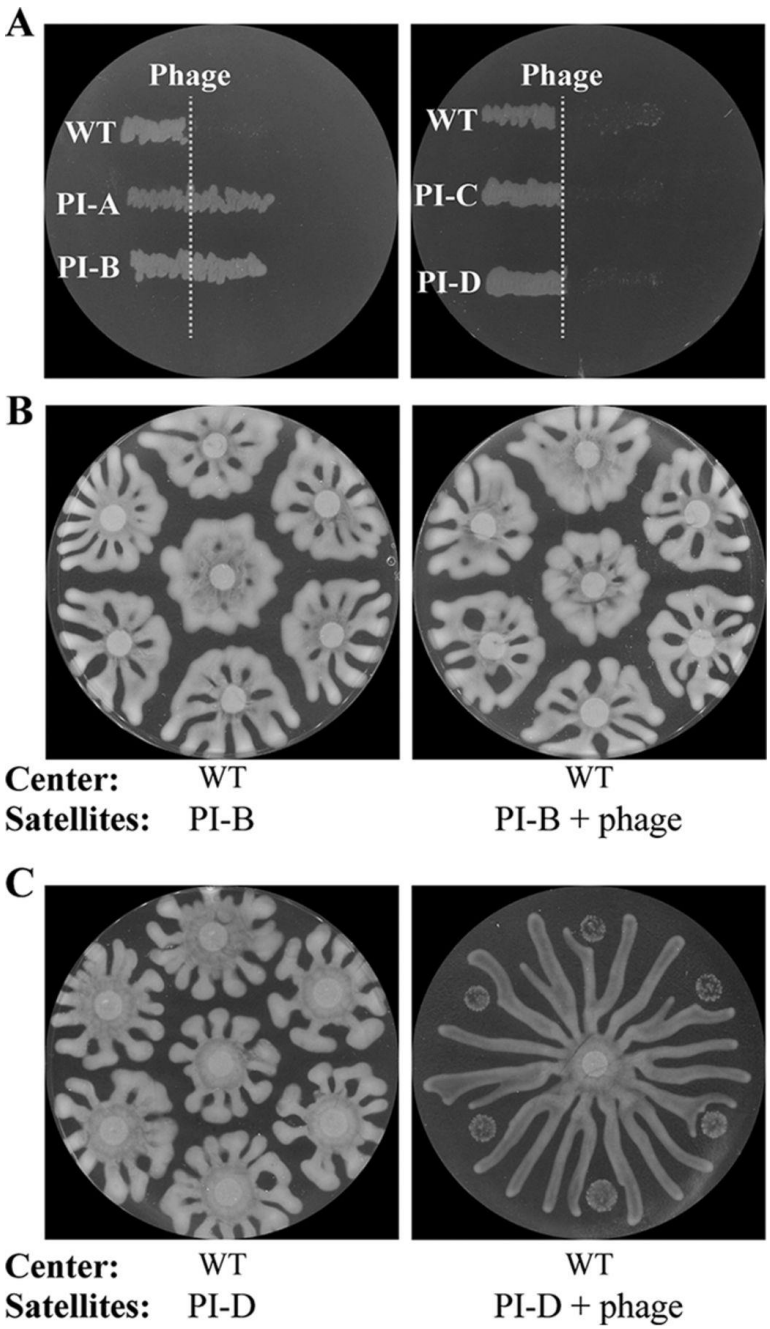

Figure 4.4. Phage Resistance, Inhibition of Motility, and Repulsion in Isolates that Survive Treatment of Bacteriophage. (A) Cross-streak assay in which phage (dashed line) is applied along a line on the dish and phage-infected isolates (PI) of *P. aeruginosa* from satellite colonies are streaked from left to right across the phage line. Growth beyond the phage line is observed in PI-A and PI-B, indicating resistance to phage, whereas no growth is observed in PI-C and PI-D, indicating sensitivity. (B and C) PI-B (B) or PI-D (C) on swarm agar petri dishes in the absence or presence of phage at the satellite positions. Uninfected wild-type *P. aeruginosa* is spotted at the center. Inhibition of motility and repulsion are observed in PI-D, which is phage sensitive, in the presence of phage.

4.2.3 Bacteriophage-Infected *P. aeruginosa* Repels Swarming Populations Independently of Rhamnolipids

Rhamnolipids and HAAs are required for swarming motility and modulate the repulsion and attraction of swarming tendrils (21, 37). The secretion of rhamnolipids and HAAs by *P. aeruginosa* into the vicinity of phage-infected cells was the most likely explanation for the observed repulsion (Figure 4.2A *ii* and 4.2B *ii*). Indeed, when purified rhamnolipids were spotted at the satellite positions, they induced repulsion of approaching swarms at intermediate concentrations (Figure 4.5A). However, higher concentrations of rhamnolipid attracted the swarms (Figure 4.5A), indicating that the effects of attraction and repulsion by rhamnolipids depend on the rhamnolipids concentration. To show that phage infection causes repulsion through the production of rhamnolipids and HAAs by *P. aeruginosa*, Δ*rhlAB* mutant cells, which do not produce rhamnolipids and HAAs (38), were spotted at the satellite positions. Swarming motility was inhibited in the Δ*rhlAB* satellite colonies, consistent with previous studies showing that rhamnolipids and HAAs are required for swarming motility (21). Additionally, uninfected swarming *P. aeruginosa* population that approached the uninfected Δ*rhlAB* satellite colonies were not repulsed (Figure 4.2A *iii*, B *iii*, 4.5B, 4.8B), consistent with the role of HAAs as a repellant (38). However, phage-infected Δ*rhlAB* satellite colonies repelled the uninfected center swarming cells (Figure 4.5B, 4.8B), demonstrating that rhamnolipids and HAAs were not responsible for the phage-induced repulsion.

Overall, these results indicate that the repulsion mechanism observed during phage infection (Figure 4.2A *ii*, 4.2B *ii*) is distinct from the repulsion by uninfected wild-type cells (Figure 4.2A *i* and 4.2B *i*), the latter of which relies on the production of HAAs. Furthermore, the phage infection of strains defective in the quorum sensing master regulator LasR, the quorum

sensing regulator RhlR, the combination of both, and the CRISPR-Cas system caused repulsion of the center swarm (Figure 4.6A-D). These results suggest that phage infection induces the secretion of a molecule that repulses approaching uninfected swarms using a pathway that is activated independently of the master regulators of quorum sensing and CRISPR-Cas.

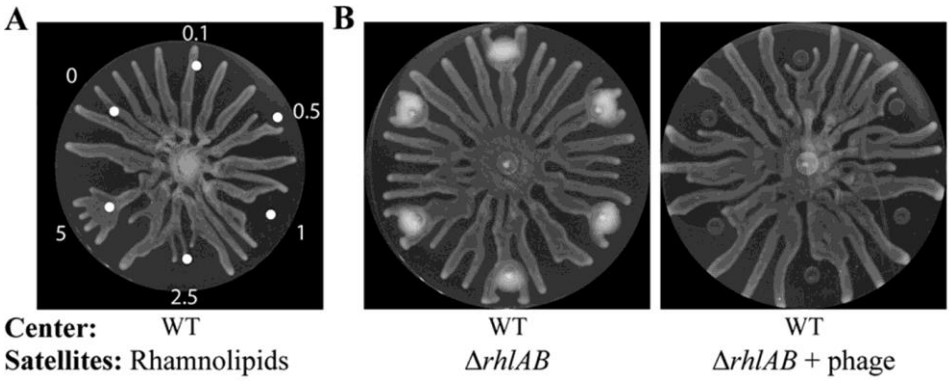

Figure 4.5. *P. aeruginosa* Deficient in Rhamnolipids and HAAs Production Causes Repulsion under Bacteriophage Infection. (A) Swarming assay in which increasing concentrations (millimolar) of rhamnolipids are spotted at the satellite positions. (B) Spotting of the Δ*rhlAB* strain at the satellite positions without phage (left) or with phage (right). Uninfected wild-type *P. aeruginosa* is spotted at the center for all assays.

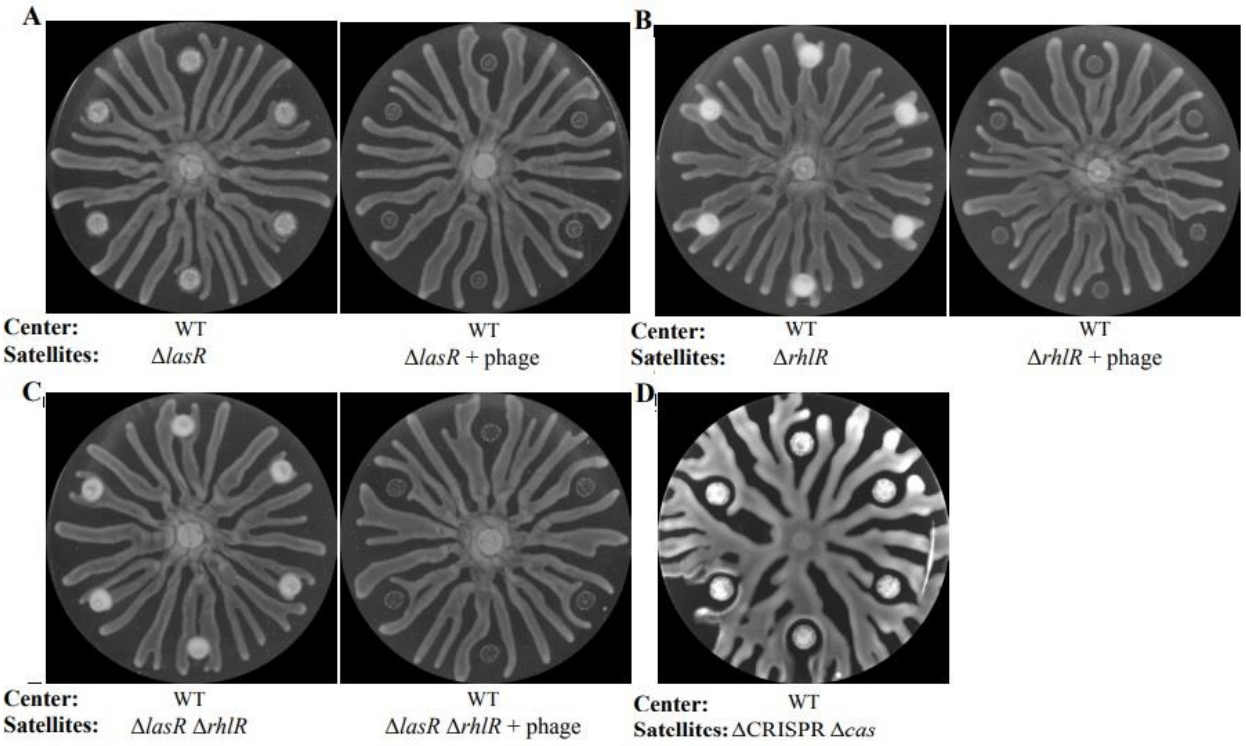

Figure 4.6. Infection of *P. aeruginosa* Quorum Sensing Mutants Causes Swarm Repulsion. Swarming assays of wild-type *P. aeruginosa* at the center and (A) Δ*lasR*, (B) ΔrhlR, and (C) Δ*lasR* Δ*rhlR* spotted at the satellite positions (left images) without phage or (right images) with phage. (D) Swarming assays of wild-type *P. aeruginosa* at the center and ΔCRISPR Δ*cas* at the satellite positions.

4.2.4 Bacteriophage Infection Upregulates PQS Quorum Sensing System

A previous study suggested that genes required for producing the quorum sensing molecule (PQS) are upregulated in response to bacteriophage infection (39). We verified that the genes expression responsible to produce PQS, *pqsA* and *pqsB* (30–32), was significantly increased in bacteriophage-infected satellite colonies compared to the uninfected center swarm ($P < 0.05$) (Figure 4.7A), suggesting that PQS is involved in the repulsion mechanism. The relative transcript levels of the rhamnolipid production genes *rhlA* and *rhlB* were slightly decreased in cells infected with bacteriophage, but the decrease was not statistically significant ($P > 0.1$).

We examined whether PQS itself induces repulsion of *P. aeruginosa* swarming populations. Spotting increasing concentrations of PQS over the same range of rhamnolipids at the satellite positions resulted in greater repulsion radius (Figure 4.7B and C). Additionally, spotting of 2-heptyl-4-quinolone (HHQ), the precursor molecule to PQS, at the satellite positions caused swarm repulsion to a lesser extent (Figure 4.7B and C), showing that PQS can repel *P. aeruginosa* swarms greater than HHQ.

Given the ability of PQS to repulse the center swarm tendrils, we hypothesized that PQS is responsible for the repulsion observed during bacteriophage infection. Deletion of the *pqsA* gene, which encodes the synthase that produces the early precursor to PQS, abolished swarming of uninfected satellite colonies (Figure 4.7D and 4.8B). The lack of swarming is consistent with the requirement that PQS production is necessary for swarming (40, 41). The Δ*pqsA* strain retained the capacity to repel the wild-type center swarm (Figure 4.7D and 4.8B), but this phenotype is likely due to the production of rhamnolipids and HAAs in this strain. Infection of the Δ*pqsA* strain decreased the repulsion of the wild-type center swarm (Figure 4.7D and 4.8B), which suggests that

bacteriophage infection decreases the production of non-PQS repulsion molecules. The repulsion of the wild-type center swarm was significantly decreased in the phage-infected Δ*rhlAB* Δ*pqsA* strain (Figure 4.8A and B) which does not produce rhamnolipids, HAAs, or PQS (32). We observed that the center swarm collided with four out of six of the phage-infected Δ*rhlAB* Δ*pqsA* satellite colonies, which induced a change in the optical properties of the newly infected tendril population, consistent with phage-mediated lysis (Figure 4.8A). The minimal repulsion by the infected Δ*rhlAB* Δ*pqsA* strain may be due to the infection of the approaching swarm by bacteriophage that diffuse out of the satellite colonies, which triggers repulsion itself, or due to unidentified secreted factors.

PQS is produced during stationary-phase growth (42). However, the repulsion effects observed here are not due to PQS in the inoculum, as the sterile-filtered supernatant from the Δ*rhlAB* inoculum did not cause repulsion of the wild-type center swarm (Figure 4.8C). Additionally, the effect is not due to PQS contained within cells in the inoculum, as the sonication and subsequent sterile filtration of the Δ*rhlAB* inoculum did not cause repulsion (Figure 4.8C). Together, these observations suggest that bacteriophage infection upregulates PQS production, which is mainly responsible for the repulsion of uninfected swarms.

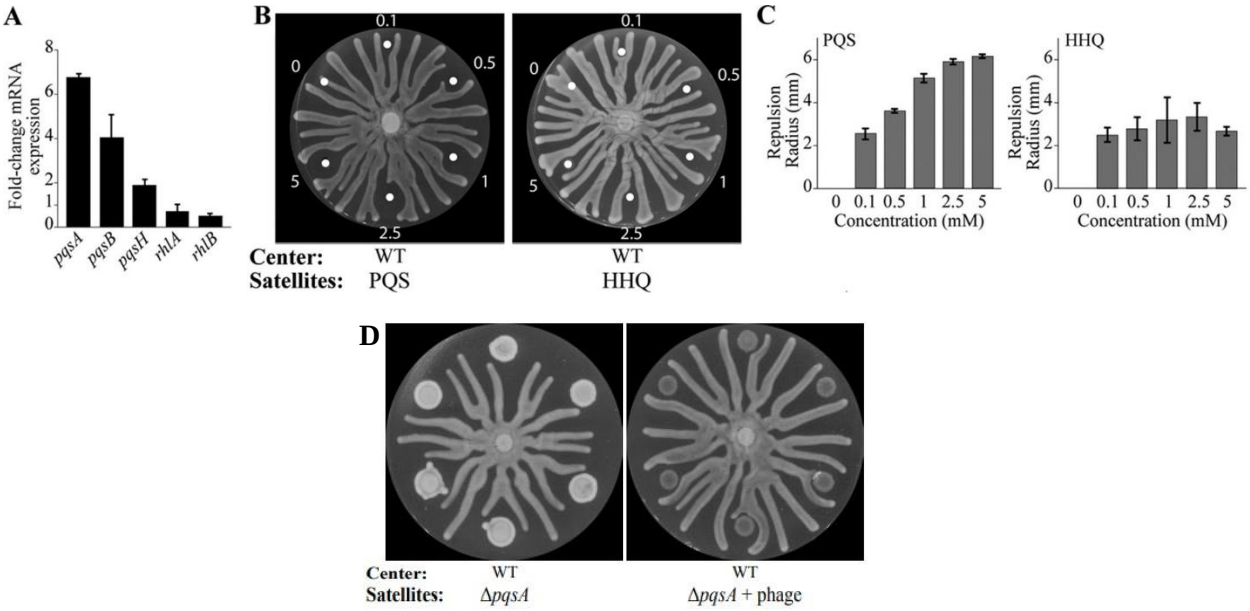

Figure 4.7. PQS Causes Repulsion of *P. aeruginosa* Swarms. (A) Fold change in relative mRNA transcript levels of *pqsA*, *pqsB*, *pqsH*, *rhlA*, and *rhlB*, normalized by 5S expression, in bacteriophage-infected wild-type *P. aeruginosa* at the satellite positions and compared to levels for uninfected cells at the center of the swarm dish, determined through qRT-PCR. (B and C) Swarming assays, and quantification thereof, in which dimethyl sulfoxide (DMSO) and increasing concentrations (millimolar) of PQS and HHQ are spotted at the satellite positions. (D) Swarming assay of wild-type *P. aeruginosa* at the center and the Δ*pqsA* strain (left) without phage or (right) with phage. Uninfected wild-type *P. aeruginosa* is spotted at the center of all assays. White dots indicate the centers of positions where rhamnolipids, PQS, or HHQ was spotted. Bars in panel A are the averages from at least 2 independent experiments (n = 2). Bars in panel C for PQS and HHQ are the averages from 2 or 4 satellite colonies (n = 2 or 4), respectively.

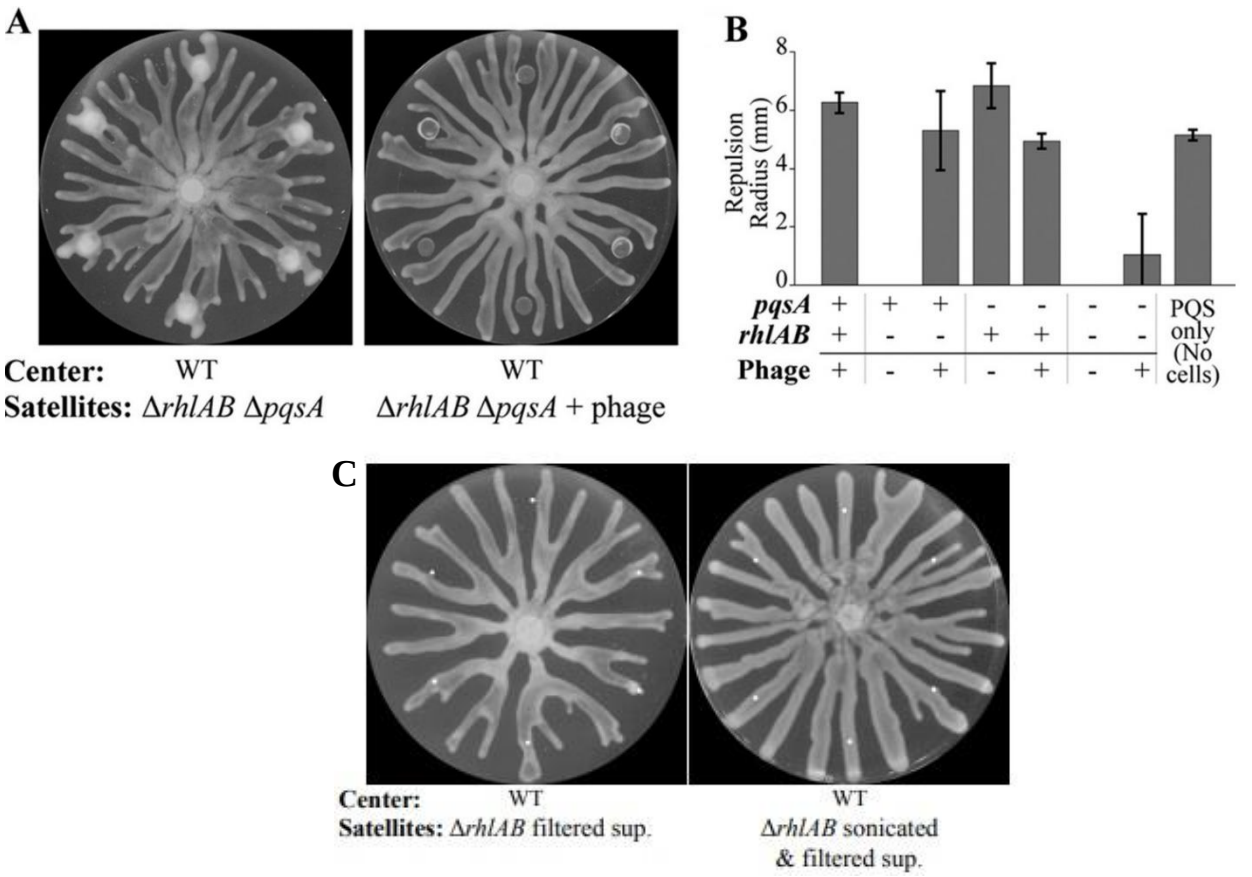

Figure 4.8. PQS from Bacteriophage-Infected *P. aeruginosa* Causes Swarming Repulsion. (A) Spotting of the Δ*rhlAB* Δ*pqsA* strain at the satellite positions without (left) or with (right) phage. Uninfected wild-type *P. aeruginosa* is spotted at the center. (B) Quantification of the repulsion radii at the satellite positions for different strain backgrounds and with or without phage. The repulsion induced by spotting 1 mM PQS is included as a reference. Bars in panel B are the averages for at least 6 satellite colonies (n = 6). Error bars indicate standard deviations. (C) Swarming assay of wild-type *P. aeruginosa* at the center and (left) filtered overnight culture or (right) sonicated and filtered overnight culture at the satellite positions. White dots at the satellite positions indicate the precise centers of positions where the cultures were spotted.

4.3 PQS Produced by Antibiotic-Treated *P. aeruginosa* Repel Uninfected Swarming Populations

4.3.1 Gentamicin Treatments Induce Swarms Repulsion by Promoting PQS Production

PQS promotes the survival of the overall bacterial cells within well-mixed *P. aeruginosa* communities by sensitizing cells to oxidative stress and other stresses (26). From previous data, we reasoned that the PQS-mediated repulsion of healthy swarming cells promotes the bacterial population survival by directing the community spatial organization. Therefore, the application of cell stress, such as antibiotic treatment, could also direct the spatial organization of the population to promote the overall survival. To test this hypothesis, wild-type *P. aeruginosa* was mixed with gentamicin, which inhibits growth through stalling ribosomal tRNA translocation (43, 44), and together the mixture was immediately spotted at satellite positions. Minimal growth of *P. aeruginosa* was observed at the satellite positions (Figure 4.9A), indicating that the effective concentration of gentamicin was below the MIC due to the diffusion of the antibiotic through the agar. The slow growth of antibiotic-treated *P. aeruginosa* (Figure 4.9A) was phenotypically comparable to that due to the bacteriophage infection (Figure 4.1B).

Gentamicin treatment inhibited specifically swarming motility at the satellite positions and caused repulsion of untreated center swarm tendrils (Figure 4.9A and B). These results were comparable to that observed in bacteriophage-infected *P. aeruginosa* (Figure 4.2A *ii* and 4.2B *ii*). The swarm repulsion was not caused by the presence of the gentamicin alone since untreated swarms swarmed through the *P. aeruginosa*-free gentamicin spots (Figure 4.9A and B).

Gentamicin-induced swarm repulsion was observed in the Δ*rhlAB* strain but was significantly reduced in the Δ*pqsA* strain and was not observed in the Δ*rhlAB* Δ*pqsA* strain (Figure 4.9A and B). The repulsion effect was abolished in the strain deleted for the gene *pqsH*, which encodes the synthase that converts HHQ into PQS, and in the Δ*rhlAB* Δ*pqsH* strain (Figure 4.10C). This suggests that PQS is the dominant molecule responsible for inducing repulsion in healthy swarming cells. Together, these data show that PQS production is required for antibiotic-induced repulsion.

4.3.2 PQS Quorum Sensing System Upregulation Under Various Antibiotic Treatments

The inhibition of motility and induction of repulsion were observed with the treatment of *P. aeruginosa* at satellite positions using the antibiotics kanamycin (Figure 4.9B and 4.10A), which blocks tRNA translocation similarly to gentamicin (43, 44), and fosfomycin (Figure 4.9B-D and 4.10B), which inhibits the synthesis of the cell wall component peptidoglycan (45). The repulsion radius at satellite positions increased with increasing concentrations of antibiotics (Figure 4.9C and C, and Figure 4.10B, E, and F). We notice that the repulsion of *P. aeruginosa* is triggered sharply by a small change in fosfomycin concentration between 25 and 30 mg/ml and is concomitant with the inhibition of growth of the satellite colonies. In contrast, continuously increasing repulsion is observed using synthetic PQS (Figure 4.7B and C). These results demonstrate that PQS activation in response to antibiotic stress is switch-like rather than graded.

The effects of various antibiotics such as carbenicillin, cefsulodin, ciprofloxacin, and tetracycline were assessed on repulsing center swarms. However, these antibiotics alone (without

P. aeruginosa) inhibited the motility of center swarms at lower concentrations than required to inhibit growth of the satellite colonies (Figure 4.11A-D). We therefore could not assess whether these antibiotics cause *P. aeruginosa* to repel swarming populations.

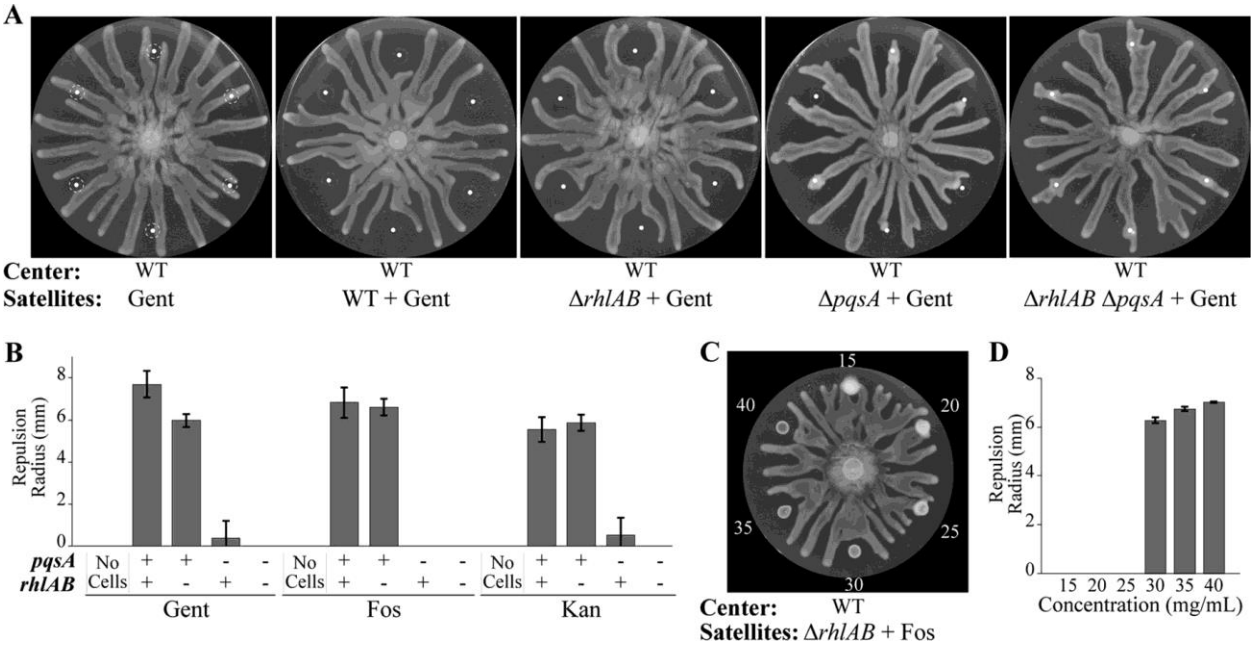

Figure 4.9. Antibiotics Inhibit *P. aeruginosa* Motility and Promote Repulsion Response. (A) Swarming assays in which satellite positions are spotted with 0.5 mg/ml gentamicin (Gent) and wild type PA14, Δ*rhlAB*, Δ*pqsA*, and Δ*rhlAB* Δ*pqsA* strains of *P. aeruginosa*. The dashed lines indicate the boundaries of the initial spots. (B) Repulsion radii of wild-type and mutant *P. aeruginosa* strains that were spotted with 0.5 mg/ml gentamicin (Gent), 40 mg/ml fosfomycin (Fos), or 25 mg/ml kanamycin (Kan). (C and D) Swarming assay (C) and corresponding repulsion radii (D) in which the initial culture of the Δ*rhlAB* strain was spotted with fosfomycin (concentrations in mg/ml). White dots indicate the centers of positions where antibiotics, cells, or the combination of antibiotics and cells was spotted. Bars in panels B and D indicate averages from at least 6 or 2 independent experiments (n = 6 or 2), respectively. Error bars indicate standard deviations. The swarming assays for the kanamycin and fosfomycin treatments depicted in panel B are shown in Figure 4.10A and B. Overnight cultures were mixed with antibiotics to the indicated concentrations, and 6-µl aliquots of the mixtures were spotted at satellite positions on antibiotic-free swarming media.

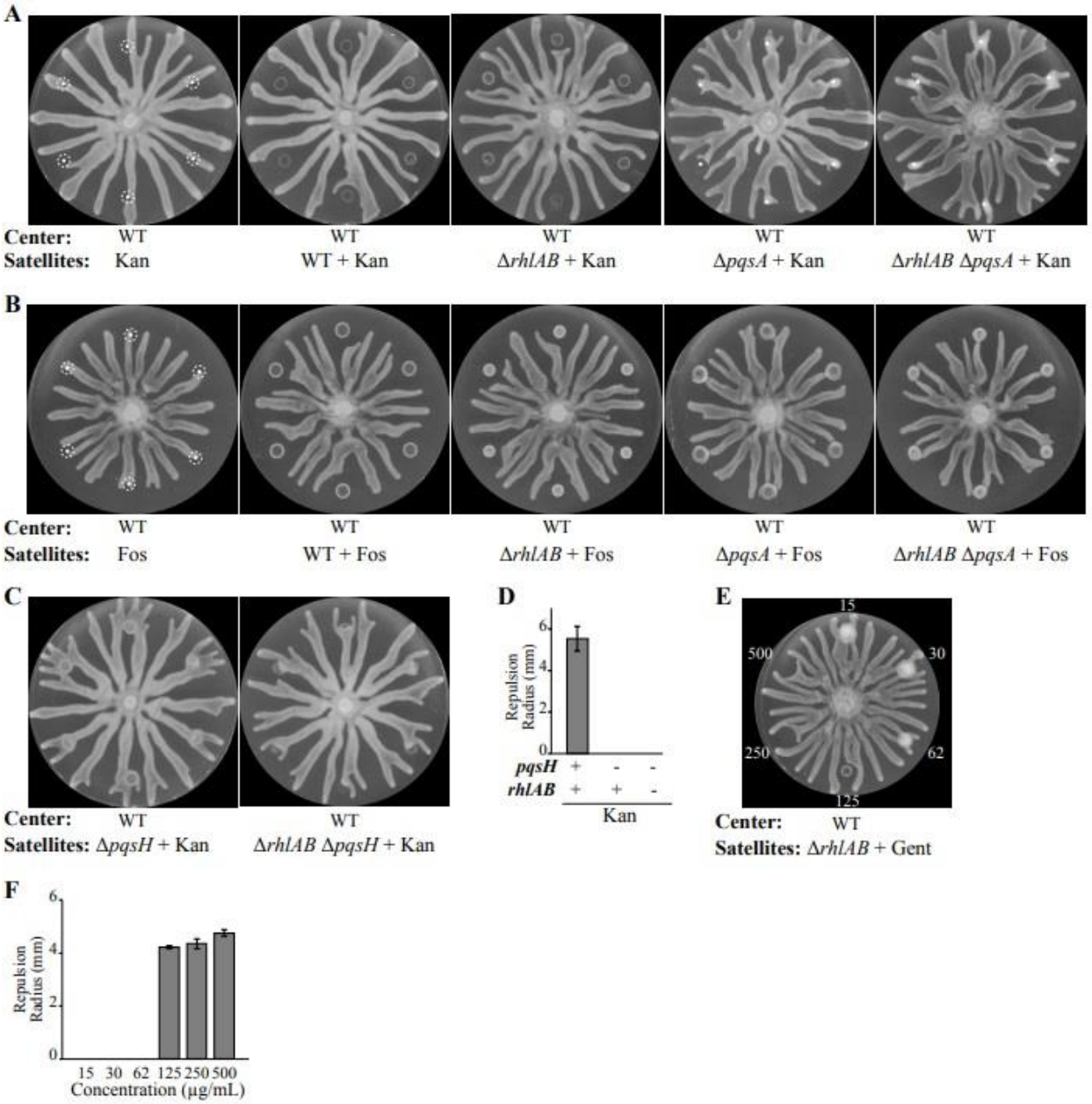

Figure 4.10. Swarming Assay of *P. aeruginosa* with Kanamycin and Fosfomycin. Wild type *P. aeruginosa* was spotted at the center and 6 μL of (A) 25 mg / mL kanamycin or (B) 40 mg / mL fosfomycin was spotted alone or with wild-type, Δ*rhlAB*, Δ*pqsA*, or Δ*rhlAB* Δ*pqsA P. aeruginosa* at the satellite positions on antibiotic-free plates. (C) Swarming assay with 6 μL of 25 mg / mL kanamycin spotted with the Δ*pqsH* or Δ*rhlAB* Δ*pqsH* strains at the satellite positions and (D) the corresponding repulsion radii at the satellite positions. (E) Swarming assay and (F) corresponding repulsion radii in which 6 μL of the initial culture of Δ*rhlAB* is spotted with gentamycin (concentrations in μg / mL) at the satellite positions. White dots on the satellite positions indicate the precise centers of positions where the cultures or antibiotics were spotted. The dashed lines indicate the boundaries of the initial spots. Bars are the average of at least 6 positions and error bars indicate standard deviation.

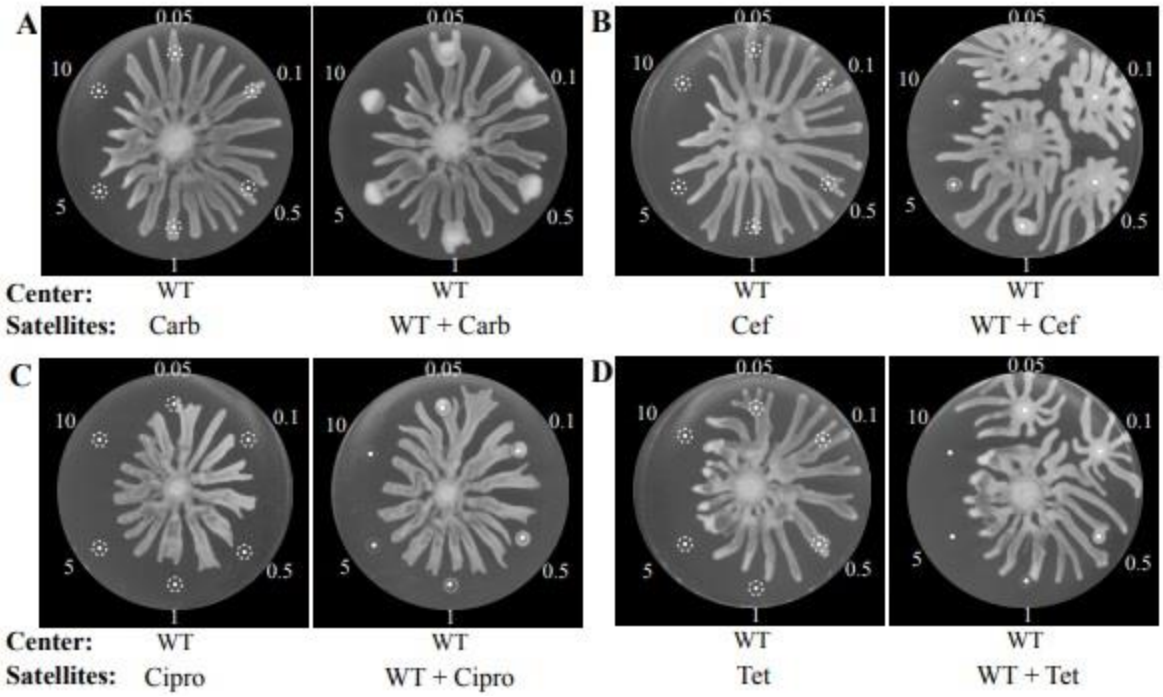

Figure 4.11. Swarming Assay of *P. aeruginosa* with Various Antibiotics. Swarming assay with (A) carbenicillin, (B) cefsulodin, (C) ciprofloxacin, or (D) tetracycline (concentrations in mg / mL) spotted alone or with wild-type *P. aeruginosa* PA14 at the satellite positions on antibiotic-free plates. White dots at the satellite positions indicate the precise centers of positions where the cultures or antibiotics were spotted. The dashed lines indicate the boundaries of the initial spots.

4.4 Virulent *P. aeruginosa* Strains Repulse Healthy Swarming Cells

PQS importance in host pathogenesis suggests a role for the stress response observed here during human infection (25–27). We investigated the potential role of the PQS-mediated repulsion response in the Liverpool epidemic strain LESB58, a hypervirulent isolate of *P. aeruginosa* (46). The strain was spotted without antibiotics or bacteriophage at the satellite positions, while wild-type PA14 was spotted in the center. LESB58 grew slowly, did not swarm, repulsed PA14 swarms (Figure 4.12C), and produced elevated levels of PQS in the surrounding agar (Figure 4.13B). Thus, the hypervirulent LESB58 strain showed a phenotype that is consistent with a constitutively active cell stress response, even when no external stress was present. Additionally, the repulsion response was characterized in the mucoid *P. aeruginosa* cystic fibrosis isolate P2m (47) and in the strain MPAO1 (48). Center swarm tendrils contacted untreated satellite colonies but were repulsed by antibiotic-treated colonies (Figure 4.12C and 4.13A and B). Together, these data indicate that the stress response is also found in clinical isolates and demonstrate a role for the stress response in pathogenesis and treatment tolerance.

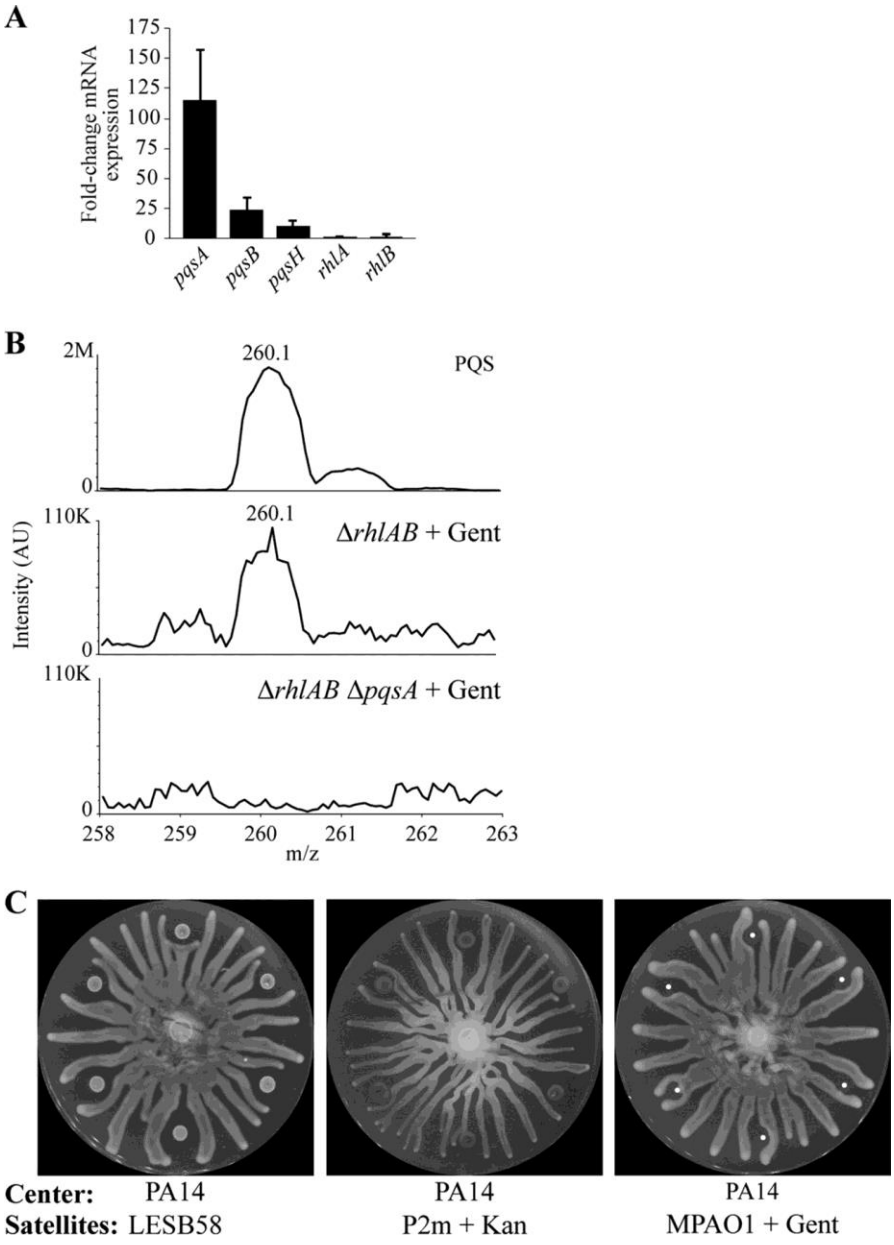

Figure 4.12. Detecting PQS Production due to Antibiotic Treatments. (A) Fold change in mRNA transcripts, as determined by qRT-PCR, of gentamicin-treated wild-type *P. aeruginosa* on swarming dishes compared to that of untreated cells at the center of the swarming dish. (B) Liquid chromatography-mass spectrometry of 10 μM PQS and agar extracts of the zone of repulsion surrounding Δ*rhlAB* and Δ*rhlAB* Δ*pqsA* strains of *P. aeruginosa* that were treated with 0.5 mg/ml gentamicin. The area containing cells is excluded. AU, arbitrary units. (C) Swarming assay in which the untreated hypervirulent *P. aeruginosa* strain LESB58, the mucoid *P. aeruginosa* cystic fibrosis isolate P2m treated with 25 mg/ml of kanamycin, or MPAO1 treated with 0.5 mg/ml of gentamicin was spotted at the satellite positions. Bars in panel A indicate averages from at least 2 independent experiments. Error bars indicate standard deviations. Overnight cultures were mixed with antibiotics to the indicated concentrations, and 6-μl aliquots of the mixtures were spotted on antibiotic-free swarming media.

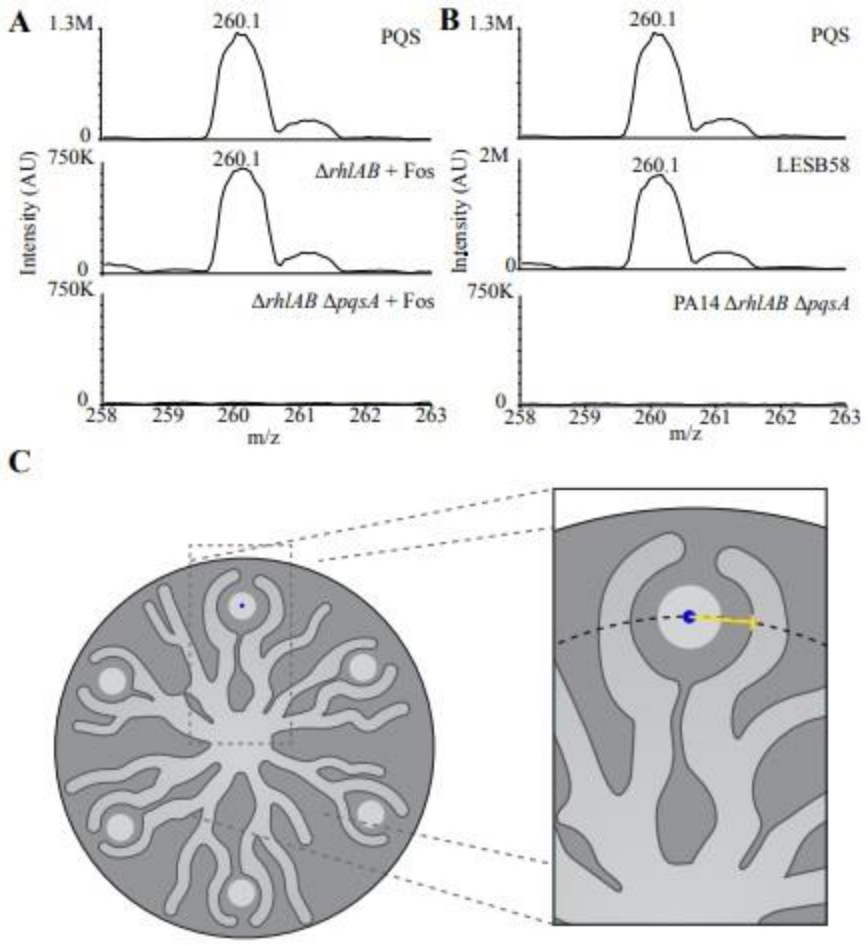

Figure 4.13. PQS Production and Measurements of PA14 Strains on Swarming Agar Plate. (A) LC-MS analysis of agar extracts for PA14 Δ*rhlAB* or PA14 Δ*rhlAB* Δ*pqsA* in which cultures are mixed with 40 mg / mL of fosfomycin. 6 μL of the mixture is spotted onto antibiotic-free swarming medium, incubated for 16-18 hours, and the area surrounding the colony is extracted. Pure 10 μM PQS, which is not spotted onto agar, is provided as a reference. (B) LC-MS analysis of agar extracts surrounding the LESB58 or PA14 Δ*rhlAB* Δ*pqsA* strains. (C) Schematic indicating the arc (dashed line) along which the radius of repulsion is measured (yellow). The blue dot indicates the center of the satellite colony

4.5 Conclusion

Stress responses help bacteria respond appropriately to stimuli that threaten their survival. We show that the detection of stress by a *P. aeruginosa* subpopulation increases the production of PQS, which repulses approaching swarming population far beyond the area containing the stress and thereby serves as a long-range signal (Figure 4.14). This long-range stress response complements the short-range kin lysis stress system previously reported in *P. aeruginosa* (49). PQS activates heightened stress responses in *P. aeruginosa*, including the formation of outer membrane vesicles, which interfere with cytokine production and deliver toxins to target host cells (50–52). Through activation of the PqsR receptor, PQS also induces *pqsE* expression, which is known to synthesize a quorum sensing molecule that activates RhlR (33). It is is responsible for regulation of key virulence factors that kill plants and animals, including hydrogen cyanide and elastase (53, 54). Our data suggest that PQS functions additionally as a coordinator of spatial organization during swarming. This work shows that PQS repulses healthy swarming populations from the area containing stress. Given the ability of a stress response to impact swarming, which is a collective behavior, we refer to this response as the collective stress response.

The collective stress response is activated by diverse types of stress and could provide protection from stress to the overall population. In the context of bacteriophage infection, the stress response physically quarantines the infected population by repelling healthy (uninfected) swarms. This response may serve as a warning defense mechanism to direct the healthy population to stay clear of dangerous zones. The overall effect is that the healthy *P. aeruginosa* population is protected from infection by population infected with bacteriophages. Therefore, the repulsion of uninfected *P. aeruginosa* swarms by the infected subpopulation reduces the potential spread of the

infection to additional parts of the population. In the Δ*rhlAB* Δ*pqsA* strain, which is defective in the repulsion response, uninfected wild-type *P. aeruginosa* swarms approached and contacted infected populations, which enabled the infection to spread to the healthy population (Figure 4.5B).

In natural environments, the stress response could limit the spread of bacteriophage infection throughout a population and serve as a bacterial defense mechanism against bacteriophage infection to complement defenses like the CRISPR-Cas system (55). Additionally, membrane vesicles function as phage decoys, and their production is upregulated in response to PQS in *P. aeruginosa* (51, 52, 56). Therefore, PQS and membrane vesicles production in response to bacteriophage infection could function as a defense mechanism to increase transient immunity to bacteriophage. We note that bacteria that survive bacteriophage infection are typically resistant to further infection. However, only half of the surviving bacteriophage-infected *P. aeruginosa* organisms were resistant (Figure 4.4), which suggests that *P. aeruginosa* has a protection mechanism that enables survival against bacteriophage infection without acquiring heritable bacteriophage resistance. This mechanism is consistent with the upregulation of membrane vesicle production by PQS in response to bacteriophage infection.

The collective stress response to antibiotics induces *P. aeruginosa* populations to secrete higher concentration of PQS into the surrounding environment. Healthy swarming populations are repulsed from the area of cells treated with antibiotics altogether, directing the swarming tendril toward areas free of antibiotics. In natural environments, this mechanism could promote the evasion of *P. aeruginosa* from competing microbes that produce antibiotics, enabling the greater population to avoid a close encounter with potentially dangerous microbes. In the context of antibiotic use to treat human disease, the collective stress response could have a role in lowering the efficacy of antibiotic treatment by guiding the bacterial population away from areas of high

local concentrations of antibiotics. Together, our results show that the collective stress response repels *P. aeruginosa* populations from environments containing bacteriophage or antibiotics, which promotes the survival of the overall population.

The ability of rhamnolipids/HAAs and PQS to repulse swarms raises the question of why PQS is produced in higher quantity under stressful conditions. It is possible that PQS is produced as an alternative repulsive molecule because rhamnolipids/HAAs cannot be produced by stressed cells or because rhamnolipids/HAAs do not guarantee repulsion, as high concentrations of rhamnolipids attract swarms (Figure 4.4A). Additionally, the production of PQS could provide functions that help *P. aeruginosa* such as signaling the presence of stress (26), the production of an oxidative environment, to close *P. aeruginosa* populations. This response could protect against invasion by harmful microbes, and the production of membrane vesicles, which could kill invading host cells, serve as phage decoys (51, 57).

Future work will address the mechanisms by which antibiotic treatment and bacteriophage infection activate PQS production and the impacts of the stress signal on other *P. aeruginosa* organisms, microbes, and host cells in the vicinity. Our results suggest that the regulation of PQS in response to stress is not achieved through the canonical quorum-sensing regulators LasR and RhlR. Additionally, stress-mediated PQS production of outer membrane vesicles could have a significant role in bacterium-host interactions and impact the severity of bacterial infections.

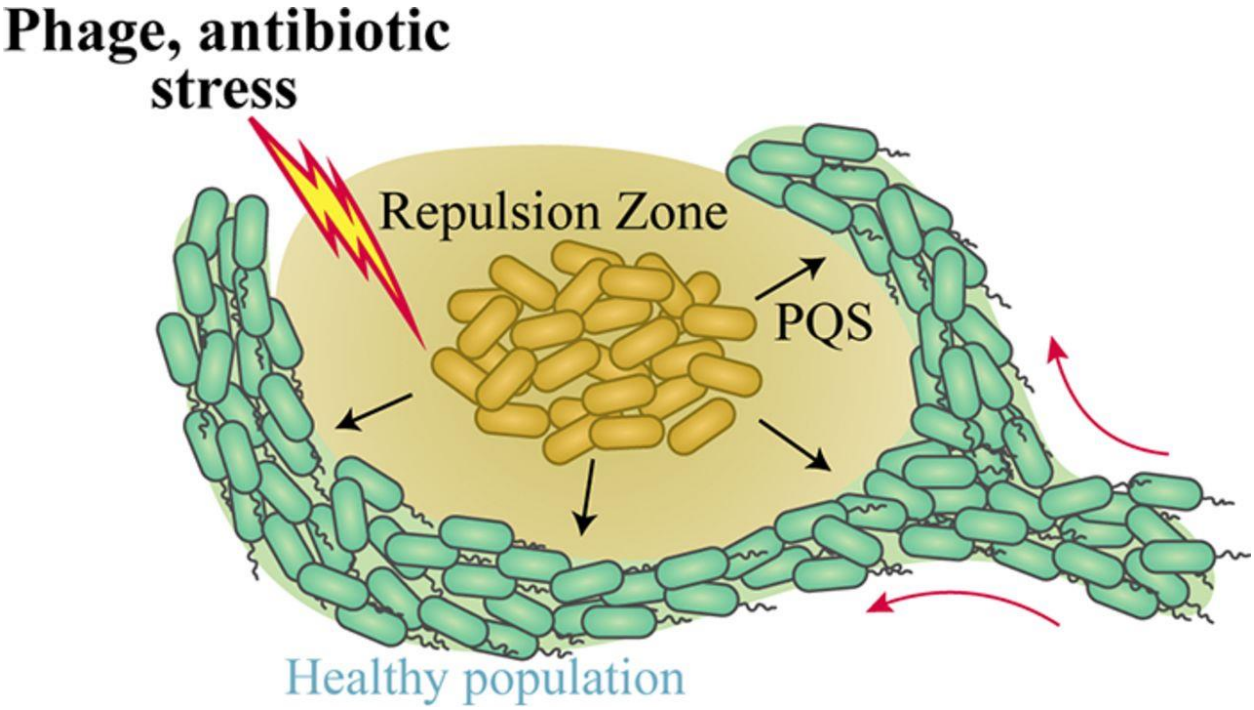

Figure 4.14. Schematic of the Collective Stress Response. Antibiotics and bacteriophage inhibit swarming motility and promote the production and release of PQS. The release of PQS repels swarms such that untreated bacterial population do not approach the infected/treated area. The stress response potentially reduces the spread of phage and the exposure of *P. aeruginosa* to antibiotics.

4.6 Materials and Methods

4.6.1 Bacterial Strains Growth Conditions

Strains were streaked from frozen stocks, which were maintained at –80°C, onto LB broth, Miller (Becton, Dickinson, Franklin Lakes, NJ)-supplemented petri dishes containing 1.5 to 2% Bacto agar (Becton, Dickinson) and grown at 37°C overnight. Single colonies were inoculated into sterilized LB medium and grown to saturation for 16 to 18 h in a shaker at 225 rpm or in a roller drum. For strain construction and plasmid maintenance, gentamicin and carbenicillin were used at 30 μg/ml and 200 μg/ml, respectively. DMS3vir lysate was prepared using standard phage preparation procedures (58) using the ΔCRISPR Δ*cas* strain as a host. Phage were diluted into LB to a concentration of 10^{12} PFU/ml and stored at 4°C. All strains and plasmids used in this study are described in Table S1 in the supplemental material. All *P. aeruginosa* PA14 strains used in this study were derived from PA14 UCBPP-PA14, obtained from the O'Toole laboratory.

4.6.2 Strain Construction

A markerless strain of PA14 that constitutively expresses mCherry was constructed by flipping out the *aacC1* gene, which is responsible for gentamicin resistance, by transforming AFS27E with pFLP2, yielding AFS27E.1.

The Δ*rhlAB* strain was constructed through lambda red recombineering using the procedure described previously (59). All primers used for strain construction are given in Table S2. The upstream region of *rhlA* (*rhlA′*) and the downstream region of *rhlB* (*′rhlB*) were amplified

using the primer pairs rhlA-lred-u1/rhlA-lred-l1 and rhlB-lred-u3/rhlB-lred-l3, respectively. The region containing FRT-*aacC1*-FRT was amplified from pAS03 using the primers rhlA-lred-u2 and rhlB-lred-l2. The *rhlA'*, FRT-*aacC1*-FRT, and '*rhlB* products were combined through isothermal assembly, amplified using rhlA-lred-u1 and rhlB-lred-l3, transformed into PA14/pUCP18-RedS, selected for gentamicin resistance, and cured of pUCP18-RedS by growing on sucrose, resulting in a PA14 Δ*rhlAB*::*aacC1* strain. The resulting strain was transformed with pFLP2 to flip out the gentamicin resistance, yielding BR04.1.

The Δ*pqsA* strain was constructed by amplifying the upstream region of *pqsA* (*pqsA'*) by using the primers pqsA-lred-u1 and pqsA-lred-l1. The downstream region of *pqsA* (*'pqsA*) was amplified using the primers pqsA-lred-u3 and pqsA-lred-l3. The region containing FRT-*aacC1*-FRT was amplified from pAS03 using the primers pqsA-lred-u2 and pqsA-lred-l2. The *pqsA'*, FRT-*aacC1*-FRT, and *'pqsA* products were combined through isothermal assembly, amplified using pqsA-lred-u1 and pqsA-lred-l3, transformed into AFS27E.1/pUCP18-RedS, selected for gentamicin resistance, and cured of pUCP18-RedS by growing on sucrose, resulting in an AFS27E.1 Δ*pqsA*::*aacC1* strain. The resulting strain was transformed with pFLP2 to flip out the gentamicin resistance, yielding AFS79.1. The Δ*rhlAB* Δ*pqsA* strain was constructed by transforming the *pqsA'*-FRT-*aacC1*-FRT-*'pqsA* product used in the construction of AFS79.1 into BR04.1/pUCP18-RedS, selecting for gentamicin resistance, and curing of pUCP18-RedS by growing on sucrose, yielding AFS82.1.

The *pqsH* strain was constructed by amplifying the *pqsH*::Mar2xT7 allele from the *P. aeruginosa* ordered transposon library (60) using the primers pqsH-u1 and pqsH-l1, transforming the product into AFS27E.1/pUCP18-RedS, selecting for gentamicin resistance, and curing of pUCP18-RedS by growing on sucrose, yielding AFS77. The Δ*rhlAB* Δ*pqsH* strain was constructed

by transforming the product into BR04.1/pUCP18-RedS, selecting for gentamicin resistance, and curing of pUCP18-RedS by growing on sucrose, yielding BR07.

4.6.3 Cross-Streak Assay

A straight line of phage was introduced into LB petri dishes containing 1.5% agar by pressing on the agar with a sterilized straight edge and pipetting 25 µl of 10^{12} PFU/ml DMS3vir phage lysate down the line. Plates were dried until the line was no longer visibly wet. Single colonies were streaked across the plate perpendicular to the line of phage. Plates were incubated overnight at 37°C.

4.6.4 Swarming Assay

Swarming petri dishes (100 mm by 15 mm) contained 20 ml of M8 minimum medium supplemented with 1 mM $MgSO_4$, 0.2% glucose, 0.5% Casamino Acids (Becton, Dickinson), and 0.5% agar (61). Petri dishes were dried in a single stack for 1 h on the bench and for an additional 30 to 60 min at room temperature with the petri dish lids off in a laminar flow hood at 300 cubic ft/min with approximately 40 to 50% ambient humidity. *P. aeruginosa* was cultured overnight (16 to 18 h) from single colonies to saturation in LB in a roller drum or shaker at 225 rpm at 37°C. Five microliters of culture was spotted in the center or at 6 equidistant satellite positions on a 5.8-cm-radius concentric circle around the center of the dish. Plates were incubated overnight at 37°C in a humidified chamber with a modified petri dish lid on an Epson photo scanner (Epson, Long

Beach, CA). Images were acquired at 30-min intervals for 16 to 18 h and processed using ImageJ (NIH, Bethesda, MD).

For phage infection assays, 1 μl of 10^{12} PFU/ml of DMS3vir was mixed with 5 μl of overnight *P. aeruginosa* culture, and 6 μl of the resulting mixture was spotted at satellite positions. For the phage-only experiment, 1 μl containing 10^{12} PFU/ml was mixed with 5 μl water and spotted. For antibiotic treatments, overnight culture or water was mixed with antibiotics to final concentrations of 500 μg/ml of gentamicin (Sigma-Aldrich, St. Louis, MO), 25 mg/ml of kanamycin (Sigma-Aldrich), 40 mg/ml of fosfomycin (Tokyo Chemical Industry, Portland, OR), 0.05 mg/ml to 10 mg/ml of carbenicillin (Teknova, Hollister, CA), cefsulodin (Research Product International, Mount Prospect, IL), ciprofloxacin (Fisher Scientific, Hampton, NH), or tetracycline (Sigma-Aldrich), and 6 μl of the resulting mixture was spotted at satellite positions. For compound repulsion/attraction assays, 6 μl of rhamnolipids (R90-1G; AGAE Technologies, Corvallis, OR), PQS (94398-10MG; Sigma-Aldrich), or HHQ (SML0747-10MG; Sigma-Aldrich) was spotted at the satellite positions. For sonicated culture assays, strains were cultured overnight for 16 to 18 h to saturation and sonicated at 75% power using 15-s on/5-s off intervals for 1 min using a Sonic Dismembrator 500 sonicator (Fisher Scientific) and centrifuged at 21,000 × *g* for 5 min. The supernatant was filtered using a 0.2-μm filter and then filtered additionally using a 0.02-μm filter. Six microliters of the supernatant were spotted at satellite positions. For filtered culture assays, the sonication step was skipped.

4.6.5 Measurement of Repulsion Radius

The radius of repulsion by each satellite colony was determined by identifying the concentric circle centered at the middle of the petri dish that passed through the center of the satellite colony (Figure 4.13C). The distance from the center of each satellite colony to the two nearest swarming tendrils along a line tangent to the concentric circle was measured and averaged (Figure 4.13C). If the tendril visibly contacted the initial zone of inoculation, which was determined by the first time-lapse image, or the distance between the center of the satellite colony to a tendril was less than or equal to the average radius of an initial spot (determined to be 1.99 mm with a standard deviation of 0.05 mm measured from 72 satellite colonies), the radius of repulsion was set to 0. Repulsion radii were averaged over at least six satellite colonies.

4.6.6 qRT-PCR

P. aeruginosa cells were harvested from the center of the swarming petri dish or from satellite colonies after 18 to 20 h at 37°C. The agar was cut, placed in a separate petri dish, and washed with water. Cells were bound to a 0.22-μm filter membrane and resuspended in total lysis solution (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 1% SDS, pH 8.0). RNA was harvested using hot phenol extraction as performed previously (62) and digested with DNase (Life Technologies, Carlsbad, CA). cDNA was synthesized using a reverse transcriptase kit (Applied Biosystems, Foster City, CA) and quantified using SsoAdvanced Universal SYBR green (Bio-Rad, Hercules, CA) on a CFX96 Touch real-time PCR detection system (Bio-Rad) quantitative PCR machine. All primers used for quantitative PCR are given in Table S2. The transcript

abundance for each sample was normalized by 5S rRNA abundance, which was determined using the 5S_qPCR primer (8). The fold change in transcript abundance due to phage or antibiotic treatment was computed by dividing the average of the normalized transcript abundances in the satellite colonies by that of cells from the center swarm and using the resulting value (n) as the exponent in 2^{-n}.

4.6.7 Mass Spectrometry

Agar was harvested from the zone of repulsion at satellite positions (outside the cell growth region) from swarming plates after 20 h of incubation at 37°C. Samples were prepared by excising the agar using the wide end of a 1-ml pipet tip (diameter, 7.5 mm), placing the agar in a microcentrifuge tube, adding ethyl acetate, vortexing for 30 s, and incubating for 10 min. The top layer was transferred and dried for 10 min in a speed vacuum at 50°C, resuspended in acetonitrile, and analyzed using an Acquity UPLC, Acquity QDa single-quadrupole detector (Waters, Milford, MA) with acetonitrile and 0.1% formic acid as column solvents. The peak at 1 to 2 s was used for analysis. For the LESB58 strain, supernatant from an overnight culture in LB was pelleted and extracted using ethyl acetate. Ten micromolars PQS in acetonitrile was used as a reference.

4.6.8 Statistical Analysis

Bars indicate the means, and error bars indicate the standard deviations unless otherwise noted. The statistical significance of a change between two data sets was determined using two-tailed heteroscedastic Student's t test comparisons. Changes were deemed significant if the

probability of the null hypothesis that two data sets originated from the same distribution was less than 0.05.

4.7 References

1. Andrews SC, Robinson AK, Rodríguez-Quiñones F. 2003. Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237.

2. Schaible UE, Kaufmann SHE. 2004. Iron and microbial infection. Nat Rev Microbiol 2:946–953.

3. Groisman EA, Hollands K, Kriner MA, Lee E-J, Park S-Y, Pontes MH. 2013. Bacterial Mg2+ homeostasis, transport, and virulence. Annu Rev Genet 47:625–646.

4. Storz, G., Hengge, R. 2011. Bacterial Stress Responses, Second Edition. American Society of Microbiology. http://www.asmscience.org/content/book/10.1128/9781555816841. Retrieved 13 February 2019.

5. Patterson AG, Jackson SA, Taylor C, Evans GB, Salmond GPC, Przybilski R, Staals RHJ, Fineran PC. 2016. Quorum Sensing Controls Adaptive Immunity through the Regulation of Multiple CRISPR-Cas Systems. Mol Cell 64:1102–1108.

6. Tan D, Svenningsen SL, Middelboe M. 2015. Quorum Sensing Determines the Choice of Antiphage Defense Strategy in Vibrio anguillarum. mBio 6:e00627.

7. Høyland-Kroghsbo NM, Mærkedahl RB, Svenningsen SL. 2013. A Quorum-Sensing-Induced Bacteriophage Defense Mechanism. mBio 4:e00362-12.

8. Høyland-Kroghsbo NM, Paczkowski J, Mukherjee S, Broniewski J, Westra E, Bondy-Denomy J, Bassler BL. 2017. Quorum sensing controls the Pseudomonas aeruginosa CRISPR-Cas adaptive immune system. Proc Natl Acad Sci USA 114:131–135.

9. Vega NM, Gore J. 2014. Collective antibiotic resistance: mechanisms and implications. Curr Opin Microbiol 21:28–34.

10. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. 2004. Bacterial Persistence as a Phenotypic Switch. Science 305:1622–1625.

11. Allison KR, Brynildsen MP, Collins JJ. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216–220.

12. O'Brien S, Williams D, Fothergill JL, Paterson S, Winstanley C, Brockhurst MA. 2017. High virulence sub-populations in Pseudomonas aeruginosa long-term cystic fibrosis airway infections. BMC Microbiol 17:30.

13. Xavier JB. 2011. Social interaction in synthetic and natural microbial communities. Mol Syst Biol 7:483.

14. Pezzlo M, Valter PJ, Burns MJ. 1979. Wound infection associated with Vibrio alginolyticus. Am J Clin Pathol 71:476–478.

15. Coker C, Poore CA, Li X, Mobley HL. 2000. Pathogenesis of Proteus mirabilis urinary tract infection. Microbes Infect 2:1497–1505.

16. Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J Bacteriol 190:2671–2679.

17. Lai S, Tremblay J, Déziel E. 2009. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. Environ Microbiol 11:126–136.

18. Kim W, Killam T, Sood V, Surette MG. 2003. Swarm-cell differentiation in Salmonella enterica serovar typhimurium results in elevated resistance to multiple antibiotics. J Bacteriol 185:3111–3117.

19. Butler MT, Wang Q, Harshey RM. 2010. Cell density and mobility protect swarming bacteria against antibiotics. Proc Natl Acad Sci USA 107:3776–3781.

20. Tremblay J, Déziel E. 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. J Basic Microbiol 48:509–515.

21. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 187:7351–7361.

22. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

23. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the Pseudomonas aeruginosa Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201.

24. Papenfort K, Bassler BL. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. Nat Rev Microbiol 14:576–588.

25. Lin J, Cheng J, Wang Y, Shen X. 2018. The Pseudomonas Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. Front Cell Infect Microbiol 8:230.

26. Häussler S, Becker T. 2008. The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations. PLoS Pathog 4:e1000166.

27. Collier DN, Anderson L, McKnight SL, Noah TL, Knowles M, Boucher R, Schwab U, Gilligan P, Pesci EC. 2002. A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol Lett 215:41–46.

28. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, Zhang L-H. 2013. A cell-cell communication signal integrates quorum sensing and stress response. Nat Chem Biol 9:339–343.

29. Lee J, Zhang L. 2015. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein & Cell 6:26–41.

30. Dulcey CE, Dekimpe V, Fauvelle D-A, Milot S, Groleau M-C, Doucet N, Rahme LG, Lépine F, Déziel E. 2013. The end of an old hypothesis: the pseudomonas signaling molecules 4-hydroxy-2-alkylquinolines derive from fatty acids, not 3-ketofatty acids. Chem Biol 20:1481–1491.

31. Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG. 2004. Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc Natl Acad Sci USA 101:1339–1344.

32. Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C. 2002. Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J Bacteriol 184:6472–6480.

33. Mukherjee S, Moustafa DA, Stergioula V, Smith CD, Goldberg JB, Bassler BL. 2018. The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in Pseudomonas aeruginosa. Proc Natl Acad Sci USA 115:E9411–E9418.

34. Cady KC, Bondy-Denomy J, Heussler GE, Davidson AR, O'Toole GA. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J Bacteriol 194:5728–5738.

35. Zegans ME, Wagner JC, Cady KC, Murphy DM, Hammond JH, O'Toole GA. 2009. Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of Pseudomonas aeruginosa. J Bacteriol 191:210–219.

36. Deziel E. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149:2005–2013.

37. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

38. Déziel E, Lépine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology (Reading, Engl) 149:2005–2013.

39. Blasdel BG, Ceyssens P-J, Chevallereau A, Debarbieux L, Lavigne R. 2018. Comparative transcriptomics reveals a conserved Bacterial Adaptive Phage Response (BAPR) to viral predation. bioRxiv https://doi.org/10.1101/248849.

40. Ha D-G, Merritt JH, Hampton TH, Hodgkinson JT, Janecek M, Spring DR, Welch M, O'Toole GA. 2011. 2-Heptyl-4-quinolone, a precursor of the Pseudomonas quinolone signal molecule, modulates swarming motility in Pseudomonas aeruginosa. J Bacteriol 193:6770–6780.

41. Brouwer S, Pustelny C, Ritter C, Klinkert B, Narberhaus F, Häussler S. 2014. The PqsR and RhlR transcriptional regulators determine the level of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa by producing two different pqsABCDE mRNA isoforms. J Bacteriol 196:4163–4171.

42. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P. 2003. The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol Microbiol 50:29–43.

43. Cabañas MJ, Vázquez D, Modolell J. 1978. Inhibition of ribosomal translocation by aminoglycoside antibiotics. Biochem Biophys Res Commun 83:991–997.

44. Feldman MB, Terry DS, Altman RB, Blanchard SC. 2010. Aminoglycoside activity observed on single pre-translocation ribosome complexes. Nat Chem Biol 6:244.

45. Kahan FM, Kahan JS, Cassidy PJ, Kropp H. 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann N Y Acad Sci 235:364–386.

46. Cheng K, Smyth RL, Govan JR, Doherty C, Winstanley C, Denning N, Heaf DP, van Saene H, Hart CA. 1996. Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. Lancet 348:639–642.

47. Quinn RA, Phelan VV, Whiteson KL, Garg N, Bailey BA, Lim YW, Conrad DJ, Dorrestein PC, Rohwer FL. 2016. Microbial, host and xenobiotic diversity in the cystic fibrosis sputum metabolome. ISME J 10:1483–1498.

48. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C. 2003. Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc Natl Acad Sci USA 100:14339–14344.

49. LeRoux M, Kirkpatrick RL, Montauti EI, Tran BQ, Peterson SB, Harding BN, Whitney JC, Russell AB, Traxler B, Goo YA, Goodlett DR, Wiggins PA, Mougous JD. 2015. Kin cell lysis is a danger signal that activates antibacterial pathways of Pseudomonas aeruginosa. Elife 4.

50. Bomberger JM, Maceachran DP, Coutermarsh BA, Ye S, O'Toole GA, Stanton BA. 2009. Long-distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles. PLoS Pathog 5:e1000382.

51. Florez C, Raab JE, Cooke AC, Schertzer JW. 2017. Membrane Distribution of the Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in Pseudomonas aeruginosa. mBio 8:e01034-17.

52. Reyes-Robles T, Dillard RS, Cairns LS, Silva-Valenzuela CA, Housman M, Ali A, Wright ER, Camilli A. 2018. Vibrio cholerae Outer Membrane Vesicles Inhibit Bacteriophage Infection. J Bacteriol 200:e00792-17.

53. Hazan R, He J, Xiao G, Dekimpe V, Apidianakis Y, Lesic B, Astrakas C, Déziel E, Lépine F, Rahme LG. 2010. Homeostatic interplay between bacterial cell-cell signaling and iron in virulence. PLoS Pathog 6:e1000810.

54. Rampioni G, Pustelny C, Fletcher MP, Wright VJ, Bruce M, Rumbaugh KP, Heeb S, Cámara M, Williams P. 2010. Transcriptomic analysis reveals a global alkyl-quinolone-independent regulatory role for PqsE in facilitating the environmental adaptation of Pseudomonas aeruginosa to plant and animal hosts. Environ Microbiol 12:1659–1673.

55. Horvath P, Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. Science 327:167–170.

56. Manning AJ, Kuehn MJ. 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol 11:258.

57. Mashburn LM, Whiteley M. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437:422–425.

58. Malke H. 1993. Jeffrey H. Miller, A Short Course in Bacterial Genetics – A Laboratory Manual and Handbook forEscherichia coli and Related Bacteria. Cold Spring Harbor 1992. Cold Spring Harbor Laboratory Press. ISBN: 0–87969-349–5. Journal of Basic Microbiology 33:278–278.

59. Shen Y, Siryaporn A, Lecuyer S, Gitai Z, Stone HA. 2012. Flow directs surface-attached bacteria to twitch upstream. Biophys J 103:146–151.

60. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proc Natl Acad Sci USA 103:2833–2838.

61. Ha D-G, Kuchma SL, O'Toole GA. 2014. Plate-based assay for swarming motility in Pseudomonas aeruginosa. Methods Mol Biol 1149:67–72.

62. Siryaporn A, Kuchma SL, O'Toole GA, Gitai Z. 2014. Surface attachment induces Pseudomonas aeruginosa virulence. Proc Natl Acad Sci USA 111:16860–16865.

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CHAPTER 5: Extensive Protocol on Time-Lapse Imaging of Bacterial Swarms and the Collective Stress Response

5.1 Background on Swarming and Collective Stress Response

Swarming is a collective form of coordinated bacterial motility that has been shown to increase antibiotic resistance and virulence factors production in the host (1–3). This multicellular behavior happens on semi solid surfaces that resemble those of the mucous layers covering epithelial membranes in the lungs (4, 5). Biosurfactants are commonly produced by swarming populations to overcome the surface tension on surfaces and the production of these surfacants is regulated by quorum sensing, which are complex cell-cell signaling systems (6–8). Many bacterial species are capable of swarming, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* (9–12). The patterns of swarming created by bacterial organisms are diverse and are affected by the physical and chemical properties of the surface layer such as nutrient composition, porosity, and moisture (13, 14). In addition to surface properties, growth temperature and ambient humidity affect several aspects of swarming dynamics, including swarming rate and patterns (12–15). The growth variables that affect swarming create challenges that impact experimental reproducibility and the ability to interpret results. In this chapter, we describe a simple standardized method to monitor the dynamics of bacterial swarms through time-lapse imaging. The method describes how to control critical growth conditions that significantly affect the progression of swarming. Compared to traditional methods of swarm analysis, this time-lapse imaging method enables tracking the motility of multiple swarms concurrently during extended periods of time and with high resolution. These aspects improve the depth of data that can be gained from monitoring swarms and facilitate the identification of factors that affect swarming.

P. aeruginosa swarming is facilitated through the production and release of rhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acids into the surrounding area (6, 16). The introduction of stress from sublethal concentrations of antibiotics or infection by bacteriophage impacts the swarms organization. These stresses induce *P. aeruginosa* to release the quorum sensing molecule *Pseudomonas* quinolone signal (PQS), also known as 2-heptyl-3-hydroxy-4-quinolone (17, 18). In swarm assays that contain two swarming populations, PQS produced by the stress induced population to repel untreated swarms from entering the area containing the stress (Figure 5.1). This collective stress response constitutes a danger communication signaling system that warns *P. aeruginosa* about nearby threats (18, 19). The stress effect on *P. aeruginosa*, the activation of the collective stress response, and the repulsion of swarms can be visualized using the time-lapse imaging method described here. The protocol described here explains how to: (1) prepare agar plates for swarming, (2) culture *P. aeruginosa* for two types of assays (traditional swarming assays or collective stress response assays) (Figure 5.1), (3) acquire time-lapse images, and (4) use ImageJ to compile and analyze the images.

Briefly, *P. aeruginosa* from an overnight inoculum is spotted in the middle of a swarming agar plate while *P. aeruginosa* that are infected with bacteriophage or treated with antibiotics are spotted at the satellite positions. The *P. aeruginosa* swarming progression is monitored on a consumer document flatbed scanner that is placed in a humidity-regulated 37 °C incubator. The scanner is controlled by an automatic software that scans the plates at regular intervals over the swarm growth period, usually between 16 to 20 hours. This technique yields time-lapse videos of up to six swarming plates. The images are compiled into movies and swarm repulsion by stress-induced populations is quantified by using freely available ImageJ software. Special consideration is given to ensure consistency and reproducibility between different swarming experiments.

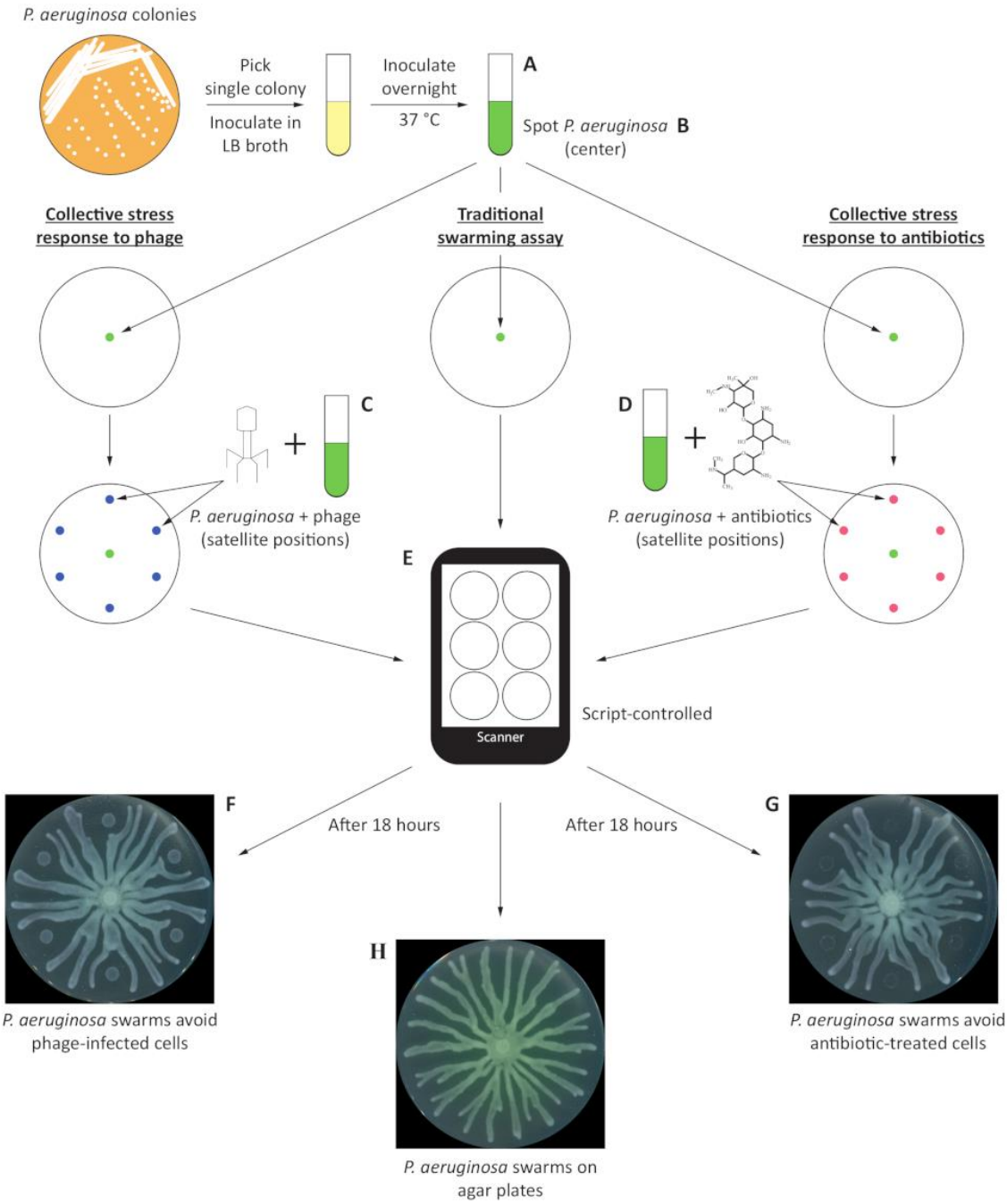

Figure 5.1 Schematic of the *P. aeruginosa* swarming assay and collective stress response. (A) *P. aeruginosa* cells are grown overnight (16–18 h to OD_{600} of approximately 1.5) in LB broth at 37 °C and (B) spotted in the middle of the swarming agar plate. Overnight cultures are mixed with (C) phages or (D) antibiotics and spotted at the satellite positions for collective stress response assays. (E) Up to 6 plates are imaged on a scanner at 30 min intervals for 16–18 h at 37 °C. After 18 h, *P. aeruginosa* swarming populations avoid (F) cells infected with phage or (G) cells treated with antibiotics (gentamicin). (H) *P. aeruginosa* populations swarm across the swarming agar plate.

5.2 Preparing M8 Agar Plates for Time-Lapse Imaging of *P. aeruginosa* Swarms

1. Prepare 1 L of 5x M8 minimum media in a glass bottle by adding 64 g of Na2HPO4•7H2O, 15 g of KH2PO4, and 2.5 g of NaCl in 500 mL double-distilled water (ddH_2O). Adjust the final volume to 1 L with additional ddH2O. Autoclave to sterilize and store liquid media at room temperature.
2. Prepare 100 mL of 1 M MgSO4 (magnesium sulfate) in a glass bottle by adding 24.6 g of MgSO4•7H2O in 50 mL ddH2O. Adjust the final volume to 100 mL with additional ddH_2O. Autoclave to sterilize. Store at room temperature.
3. Prepare 100 mL of 20% casamino acids in a glass bottle by adding 20 g of casamino acids in 50 mL ddH2O. Adjust the final volume to 100 mL with additional ddH_2O. Autoclave to sterilize. Store at room temperature.
4. Prepare 100 mL of 20% glucose in a glass bottle by adding 20 g of glucose in 50 mL ddH2O. Adjust the final volume to 100 mL with additional ddH_2O. Sterilize by filtration with 0.22 μm filter. Store at room temperature.
5. To make 10 swarming agar plates, add 1 g of agar in 100 mL of ddH_2O and adjust the final volume to 160 mL with additional ddH_2O in a 250 mL Erlenmeyer flask. Sterilize by autoclaving.
    a. Immediately after autoclaving, place the agar solution in a 55 °C water bath for 15 min.
    b. Remove the agar solution from the water bath and add 40 mL of 5x M8 minimum media, 200 μL of 1 M MgSO4, 2 mL of 20% glucose, and 5 mL of 20% casamino acids15. Proceed to step 1.6 immediately after mixing. NOTE: The final concentrations are 0.5% agar, 1 mM MgSO4, 0.2% glucose, and 0.5% casamino acids.

6. Using a 25 mL pipette for consistent volume, add 20 mL of the swarming agar solution per 10 cm diameter Petri dish. NOTE: A fixed volume of agar solution is important, as the volume affects the drying time and moisture content of the agar. Avoid bubbles when making the swarming agar plates.
7. Allow the agar to solidify by placing the swarming agar plates in a single stack with lids on for 1 h on the bench at room temperature. Turn on the dehumidifier to decrease relative humidity of the room to 40–50% 1 h prior to the next step.
8. Dry the swarming agar plates for an additional 30 min with the lids off in a laminar flow hood at 300 ft3/min with 40–50% relative humidity at room temperature. Dry the interior of the lids by placing them face up in the laminar flow hood. Store swarming agar plates at 4 °C for up to 24 h.
9. Prepare black 10 cm Petri dish lids for imaging by smoothing the inside of the lid with sandpaper. Put the lids inside a packaging box and place the packaging box under a chemical hood. Spray inside the lids using black spray paint. Allow the lids to dry. NOTE: Black lids may be re-used for additional experiments. It is important that the lids are painted so that they do not reflect light during scanning.

5.3 Growing *P. aeruginosa* and Plating Colonies on M8 Agar Plates

1. Prepare 400 mL of lysogeny broth (LB) by adding 10 g of LB-Miller powder mix into 400 mL ddH2O. For 2% LB-agar Petri dishes, add an additional 8 g of agar. Autoclave to sterilize.
2. Pour 20 mL of molten LB-agar medium into 10 cm diameter Petri dishes and allow them to solidify at room temperature overnight. Store liquid media at room temperature and agar plates at 4 °C.
3. Streak *P. aeruginosa* on an LB-agar Petri dish from a frozen stock stored at -80 °C using sterile loops or wooden sticks. Incubate the Petri dish upside-down overnight at 37 °C. Store LB-agar plate at 4 °C for up to 1 week.
4. Pick a single colony from the Petri dish with a sterile loop or wooden stick, inoculate it into 2 mL LB medium, and incubate the culture to saturation overnight (16–18 h) at 37 °C in a roller drum set at 100 rpm.
5. Pipet 5 µL of overnight culture from step 2.4 using a P20 pipet and spot at the center of the swarming agar plate by approaching the pipet tip at an angle (10–45°) 2.5 cm above the spotting area, pipetting down to the first stop, and touching the agar with only the liquid drop (Figure 5.1B).
    a. Avoid touching the agar with the pipet tip as it damages the agar. Use a template to position the spot consistently across different swarming agar plates (Figure 5.2).
    b. For traditional swarming assays, use only the center spot and skip to step 2.8. For collective stress response assays continue to step 2.6 (for phage infection) or step 2.7 (for antibiotic stress).

6. For phage infection, mix 30 μL of overnight culture of *P. aeruginosa* from step 2.4 with 6 μL of 1 x 10^{12} pfu/mL phage DMS3vir (20). Proceed immediately to the next step.
    a. Pipet 6 μL of the *P. aeruginosa*-phage mixture from step 2.6 using a P20 pipet and spot at 6 equidistant satellite positions on a 2.8 cm radius concentric circle that is centered at the Petri dish by approaching the pipet tip at an angle (10 to 45°) 2.5 cm above the spotting area, pipetting down to the first stop, and touching the agar with only the liquid drop (Figure 5.1C).
    b. Avoid touching the agar with the pipet tip as it damages the agar. Use a plating template for consistency (Figure 5.2). Proceed to step 2.8.
7. For antibiotic treatments, mix 30 μL overnight culture *P. aeruginosa* from step 2.4 with 6 μL of 3 mg/mL gentamycin, 10 μL of 100 mg/mL kanamycin, or 7.5 μL of 100 mg/mL fosfomycin. Proceed immediately to the next step.
    a. Pipet 6 μL of antibiotic treated *P. aeruginosa* from step 2.7 using a P20 pipette and spot at 6 equidistant satellite positions on a 2.8 cm radius concentric circle about the center of the dish by approaching the pipet tip at an angle (10 to 45°) 2.5 cm above the spotting area, pipetting down to the first stop, and touching the agar with only the liquid drop (Figure 5.1D).
    b. Avoid touching the agar with the pipet tip as it damages the agar. Use a plating template for consistency (Figure 5.2). Proceed to step 2.8.
8. Replace the clear Petri dish lids with black lids made in step 1.9 (Figure 5.3A).
9. Place the swarming agar plates on a scanner in an incubator set at 37 °C with a 10 L water bath to maintain humidity at 75% (Figure 5.1E and 5.3B). CAUTION: Do not disturb spotted cells on the swarming agar plates. Keep plates always facing up.

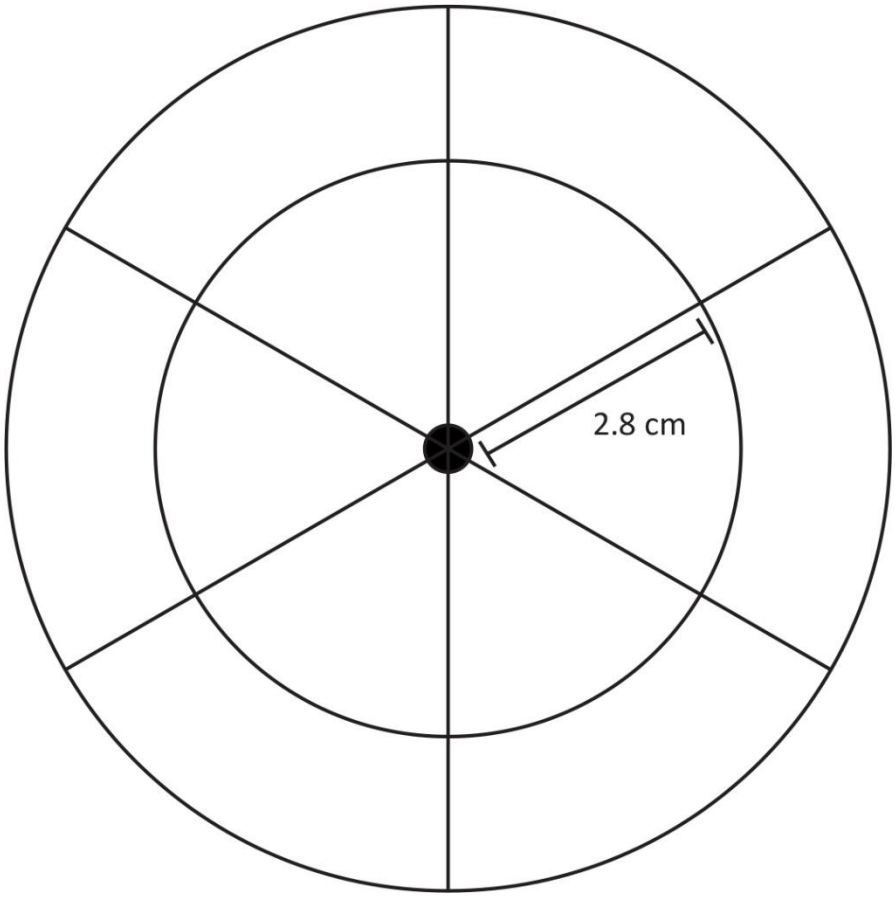

Figure 5.2. Plating template for spotting *P. aeruginosa* cells. The middle black dot represents the spotting area of 5 μL overnight *P. aeruginosa* culture. The radius of the inner circle is 2.8 cm away from the center of the plate. The intersection between the inner circle and the straight line across the outer circle indicates the spotting area of 6 μL of stressed *P. aeruginosa*, phage infected or antibiotics treated cells. The outer circle represents the circumference of 10 cm Petri dish.

Figure 5.3 Scanner Setup Inside the Incubator. (A) Black Petri dish lids constructed in section 1. These lids are used during scanning to reduce light reflections and replace clear Petri dish lids. (B) The flatbed document scanner is placed in an incubator set at 37 °C. Six plates with black lids are placed on the scanner (left image). Black matte fabric is attached to the rack 60 cm above the scanner to further reduce reflections and stray light (right image).

5.4 Using Document Scanner to Capture Swarming of *P. aeruginosa*

1. Decrease the ambient lighting of the Petri dishes by attaching black matte fabric to a rack 40–60 cm above the flatbed document scanner. Secure it using zip ties (Figure 5.2B).
2. The scanner will be controlled using a scanning software and an automatic scripting software.
    a. In the scanning software, select **Home Mode** (Figure 5.4A). Capture images in color by selecting **Color** under **Image Type**. To set the image quality, select **Other** under **Destination** and adjust the **Resolution** to 300 dpi. Keep the standard size for the images by selecting **Original** for **Target Size**. Leave all options under **Image Adjustments** unchecked for standard image quality. NOTE: Target Size is set to Original by default. To select other options for Target Size, click on **Preview** first.
3. Set the saving path of images by clicking on the folder icon to the right of Scan to open File Save Settings (Figure 5A).
    a. Select the folder destination for saving images by selecting **Other** under **Location** and click on **Browse**. Choose a folder to save the images.
    b. Name the images in the **Prefix** text box. Set **Start Number** 001 to begin naming sequence for the images. Set the file format to JPEG by choosing JPEG (*.jpg) for **Type** under **Image Format** and click on **Options** to adjust for **Details**. Set the image format quality by adjusting **Compression Level** to 16, **Encoding** to Standard, and check **Embed ICC Profile**. Click **OK** to close the window (Figure 5.4B).
    c. Leave the first option unchecked ("Overwrite any files with the same name") and check the 3 next options ("Show this dialog box before next scan", "Open image folder after scanning", and "Show Add Page dialog after scanning"). Click **OK** to close the window

d. Check the image quality by clicking on **Preview**. The preview window appears, and the **Scan** icon becomes functional (Figure 5.4C).

4. Use the scripting software to automate the image acquisition. The provided script clicks on Scan in the Scan window and OK in the File Save Settings window at 30 min intervals.

   a. Import the script by clicking on **Task | Import** and select both **Single_scan.tsk** and **Idle_scanning.tsk**. See Figure 5.4D. NOTE: *Single_scan.tsk* clicks on the **Scan** button in the Scan window and **OK** in the File Save Settings window. *Idle_scanning.tsk* activates *Single_scan.tsk* every 30 min. One may change the scan frequency by changing the activation of *Idle_scanning.tsk*.

   b. Enable automatic scanning at 30 min intervals by selecting both **Idle scanning (imported)** and **Single scan (imported)**, right clicking on **Idle scanning (imported)**, and left clicking on **Enabled** (Figure 5.4D and 5.5). NOTE: Automatic scanning runs until the user manually stops the script. To stop the script, select **Idle scanning (imported)**, right click **Idle scanning (imported)**, and left click on **Enabled**. The check mark will be removed.

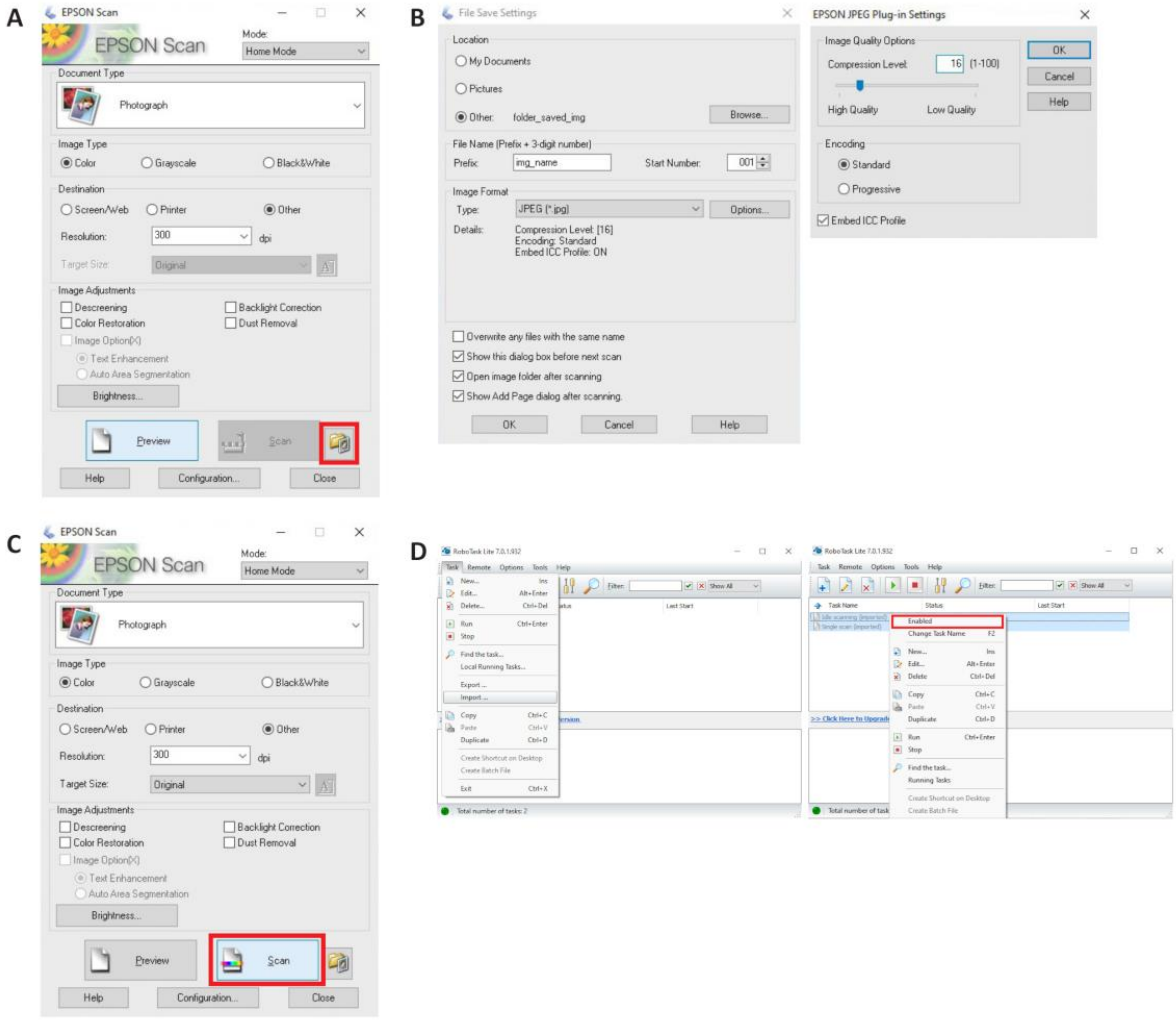

Figure 5.4 Automated Image Acquisition from Flatbed Document Scanner using Scanning and Automatic Scripting Software. (A) Screenshot of main Scan window. Selection of Image type (Color) and Resolution (300 dpi). The red square indicates the folder icon to open File Save Settings window. Note the Preview button can be pressed but the Scan button is disabled. (B) Screenshot of File Save Settings window to set folder destination for saving images, naming the images, and choosing the format of the images (left). The Plug-In Settings window is used to set the image format quality (right). (C) Screenshot of Scan window after clicking on Preview. The Scan button is clickable after a preview has been acquired. The program can now be automated using the scripting software. (D) Screenshot of the scripting software windows indicating the Import button used to import the automation scripts (left). Once Single_scan.tsk and Idle_scanning.tsk are imported, these appear as tasks in the main window (right). After selecting both tasks and right clicking them, the Enabled button appears. Left clicking Enable starts the scripts to automatically scan at 30 min intervals (right).

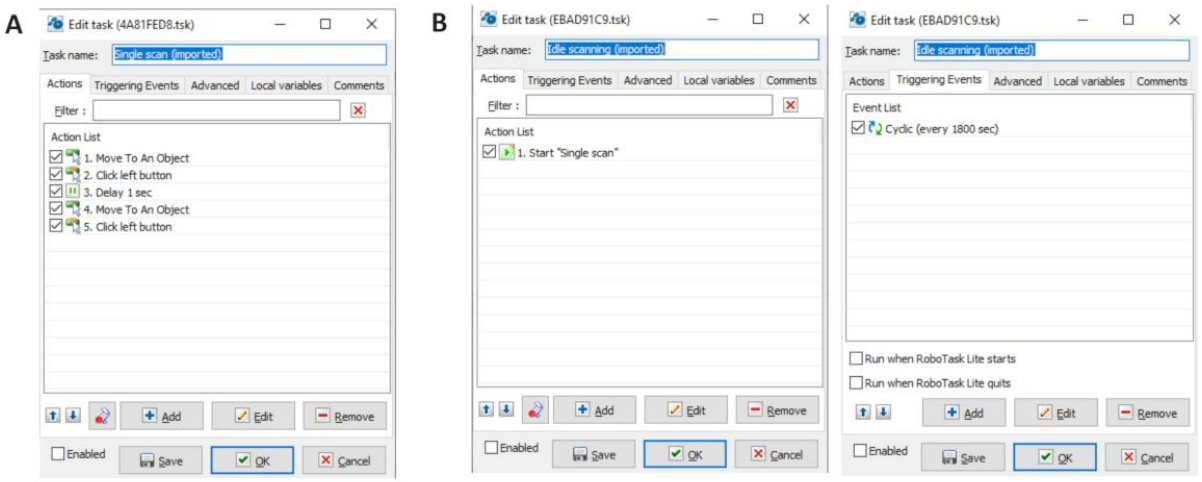

Figure 5.5. Scripting Software to Activate Macro Commands that Periodically Start Scanning. (**A**) The macro commands in Single_scan.tsk moves the cursor to Scan in Scan window, clicks on Scan, moves to OK in File Save Settings window, and clicks on OK. (**B**) Commands to scan in 30 min intervals. The task Idle_scanning.tsk starts Single_scan.tsk and is set to activate in 30 min intervals.

5.5 Assembling Time-Lapse Images

1. Perform movie editing and image analysis using ImageJ.
2. Import all the scanned images to ImageJ by clicking on **File | Import | Image Sequence** and select the images. In the **Sequence Options** window, check **Convert to RGB** to keep images in color. **Number of images** indicates the number of images selected.
3. Keep **Starting image** at 1 to start from the first picture in the folder and **Scale images** at 100% to conserve original size of the images. Leave **Use virtual stack** unchecked. Click **OK** and wait for images to load (Figure 5.6A).
4. Set the video compression level to 100 by clicking on **Edit | Options | Input/Output…** and adjust **JPEG quality** to 100.
5. Save the file as an .avi by clicking on **File | Save As | AVI**. Adjust **Compression** to JPEG and **Frame Rate** to 5 fps (Figure 5.6B). Save the .avi time-lapse in the desired folder.

5.6 Measuring Swarm Repulsion

1. To quantify swarm repulsion distances, open an image near the end of the swarming period in ImageJ. Click on **File | Open** and select the image. Adjust the scale by clicking on **Analyze | Set Scale** and setting **Distance in pixels** to 118, **Known distance** to 1, **Pixel aspect ratio** to 1.0, and **Unit of length** to cm (Figure 5.6C). Leave **Global** unchecked. Click **OK** to close the window.
2. Click on the **Straight icon** and measure from the center of the colony at the satellite position to the edge of the swarming population. Select **Analyze | Measure** to make a new window appear with the measurements (Length) (Figure 5.6D). NOTE: Use "+" to zoom in closer and "-" to zoom out.

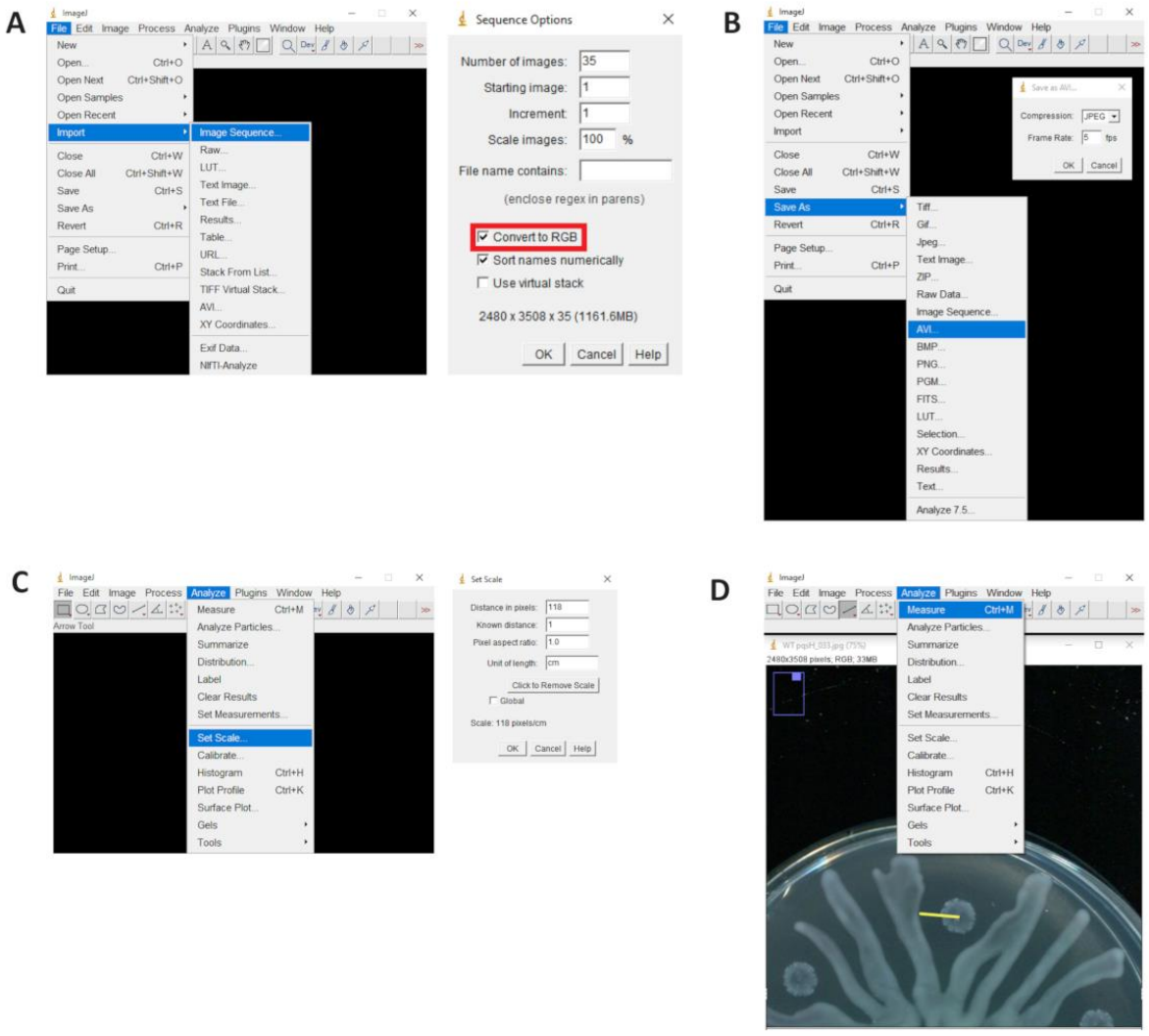

Figure 5.6. Image Analysis of *P. aeruginosa* Swarming Avoidance using ImageJ. (A) Steps to import an image sequence from the time-lapse scanner images. Clicking on File | Import | Image Sequence in the main ImageJ window (left) brings up the Sequence Options window (right) and opens all the scanned images. The red square indicates the checked option to load images in RGB format. All other options are left as default. (B) Steps to save the time-lapse video in AVI format. Selecting File | Save As | AVI brings up the Save as AVI window. Compression is set to JPEG and Frame Rate to 5 fps. (C) Setting the scale units for images. Selecting Analyze | Set Scale bring up the Set Scale window. For 300 dpi images, the appropriate scale is 118 pixels/cm. (D) Measurement of avoidance from swarming populations. A yellow line is drawn from the center of the stressed colonies to the edge of the tendrils. Selecting Analyze | Measure reports the length of the line in a new window labeled Results. Ctrl + M is a keyboard shortcut that performs the measurement without selecting the menu items.

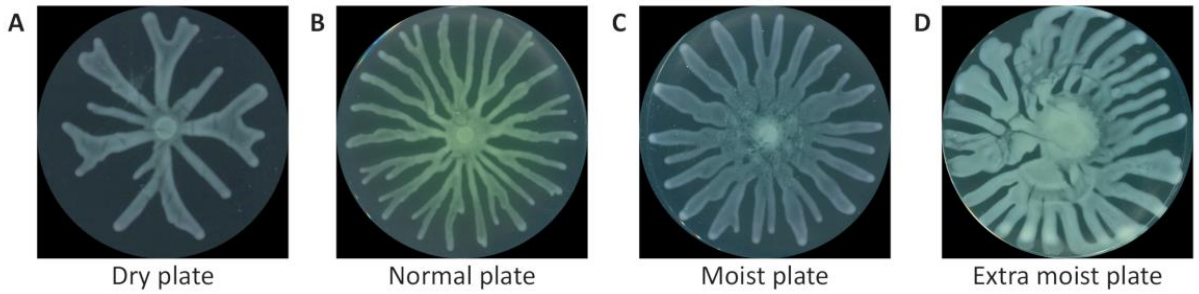

Figure 5.7. Representation of *P. aeruginosa* Swarms. *P. aeruginosa* swarming populations on swarming agar plates that are (**A**) dry, (**B**) normal, (**C**) moist, and (**D**) extra moist. Dry swarming agar plates inhibit the swarm rate of *P. aeruginosa* and reduce the number of tendrils. Moist swarming agar plates cause formation of large tendrils. Under extra moist conditions, tendrils form unevenly throughout the swarming agar plates. Drying times in the laminar flow hood and ambient humidity have significant effects on swarming plate moisture content. The dishes are 10 cm Petri dishes.

5.7 Conclusion

This procedure aims on minimizing the variability in swarming agar plates and providing a simple and low-cost technique to acquire time-lapse images of *P. aeruginosa* swarming and responding to stress. This protocol can be extended to image other bacterial systems by adapting the media composition and growth conditions of the plates. For *P. aeruginosa*, although M9 or FAB minimal medium could be used to induce swarming (16, 21), the protocol presented here uses M8 medium with casamino acids, glucose, and magnesium sulfate (6). Swarming in *P. aeruginosa* is sensitive to medium composition such as iron availability and nutrient sources including amino acids (22–24). Therefore, the selection of media for swarming agar plates demonstrates an important aspect of assaying swarming motility.

Controlling the humidity and temperature in an open laboratory area is one of the largest challenges for consistency of swarming assays. Seasonal changes contribute to variability in the swarming agar plates moisture, which can significantly impact swarming patterns. Therefore, constant control of the relative humidity is required to ensure optimal plate quality. Starting the dehumidifier one hour prior to drying the swarming agar plates under the laminar flow hood will help control the relative humidity to a constant 45%, which keeps drying time to 30 min. If ambient moisture cannot be controlled, increasing the drying time is a potential simple solution to compensate for humid environments. During swarming, relative humidity should stay at 70% in the 37 °C incubator to avoid dying out the agar plates. An opened bin of water in the incubator can serve as a water reservoir to help with the humidity. Dry swarming agar plates slow down the progression of swarming populations and reduce the number of tendrils while moist plates cause broad tendril structure (Figure 5.7A-C). Extra moist swarming agar plates prevent clear tendril

formation and cause the tendrils to spread in an uneven pattern (Figure 5.7D). The method described here can be used to maintain a constant humid environment that will ensure consistency of swarming on plates (Figure 5.7B). Additionally, plate size and agar thickness play a role in retaining moisture in the plate. We have used 10 cm diameter Petri dishes and added 20 mL of swarming agar solution per plate to ensure consistency. Pouring plates without measuring volumes is not recommended. Due to the many variables that affect the swarming assay, we recommend optimizing the assay to local laboratory conditions and we emphasize the importance of performing multiple biological replicates on separate batches of plates to observe consistent and comparable swarming patterns.

The advantage of the time-lapse imaging method to record swarming motility is the ability to observe the progression of motility without disturbing the swarms. Our method conveniently creates time-lapses of 6 plates concurrently under the same conditions, which provides both a controlled environment for the simultaneous assessment of multiple strains, multiple experimental conditions, or biological replicates. Using six satellite positions on each plate facilitates statistical analysis and using ImageJ enables the quantification of swarming repulsion. The protocol described here is a simple technique to show the interaction between sub-populations of *P. aeruginosa*: a healthy swarming population and stressed cells. Beyond DMS3vir and gentamicin, additional types of bacteriophages, antibiotics, and competing bacteria or fungi can be used to study stress signaling. Although this protocol focuses on *P. aeruginosa* swarming motility, other bacterial species such as *S. aureus* and *E. coli* also exhibit swarming patterns, but they require adapted media to swarm (10, 11). By optimizing media compositions and plate conditions, this technique is applicable toward analyzing swarming behavior such as swarming interactions between bacterial strains and stress responses.

5.8 References

1. Butler MT, Wang Q, Harshey RM. 2010. Cell density and mobility protect swarming bacteria against antibiotics. Proc Natl Acad Sci USA 107:3776–3781.

2. Lai S, Tremblay J, Déziel E. 2009. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. Environmental Microbiology 11:126–136.

3. Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas aeruginosa Is a Complex Adaptation Leading to Increased Production of Virulence Factors and Antibiotic Resistance. Journal of Bacteriology 190:2671–2679.

4. Yeung ATY, Torfs ECW, Jamshidi F, Bains M, Wiegand I, Hancock REW, Overhage J. 2009. Swarming of Pseudomonas aeruginosa is controlled by a broad spectrum of transcriptional regulators, including MetR. J Bacteriol 191:5592–5602.

5. Girod S, Zahm JM, Plotkowski C, Beck G, Puchelle E. 1992. Role of the physiochemical properties of mucus in the protection of the respiratory epithelium. Eur Respir J 5:477–487.

6. Caiazza NC, Shanks RMQ, O'Toole GA. 2005. Rhamnolipids Modulate Swarming Motility Patterns of Pseudomonas aeruginosa. Journal of Bacteriology 187:7351–7361.

7. Déziel E, Lépine F, Milot S, Villemur R. 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology 149:2005–2013.

8. Dusane DH, Zinjarde SS, Venugopalan VP, McLean RJC, Weber MM, Rahman PKSM. 2010. Quorum sensing: implications on rhamnolipid biosurfactant production. Biotechnol Genet Eng Rev 27:159–184.

9. Kohler T, Curty LK, Barja F, van Delden C, Pechere J-C. 2000. Swarming of Pseudomonas aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. Journal of Bacteriology 182:5990–5996.

10. Pollitt EJG, Crusz SA, Diggle SP. 2015. Staphylococcus aureus forms spreading dendrites that have characteristics of active motility. 1. Scientific Reports 5:17698.

11. Burkart M, Toguchi A, Harshey RM. 1998. The chemotaxis system, but not chemotaxis, is essential for swarming motility in Escherichia coli. Proc Natl Acad Sci USA 95:2568–2573.

12. Kearns DB. 2010. A field guide to bacterial swarming motility. Nat Rev Microbiol 8:634–644.

13. Tremblay J, Déziel E. 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. J Basic Microbiol 48:509–515.

14. Morales-Soto N, Anyan ME, Mattingly AE, Madukoma CS, Harvey CW, Alber M, Déziel E, Kearns DB, Shrout JD. 2015. Preparation, imaging, and quantification of bacterial surface motility assays. J Vis Exp https://doi.org/10.3791/52338.

15. Ha D-G, Kuchma SL, O'Toole GA. 2014. Plate-based assay for swarming motility in Pseudomonas aeruginosa. Methods Mol Biol 1149:67–72.

16. Tremblay J, Richardson A-P, Lépine F, Déziel E. 2007. Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming motility behaviour. Environ Microbiol 9:2622–2630.

17. Morales-Soto N, Dunham SJB, Baig NF, Ellis JF, Madukoma CS, Bohn PW, Sweedler JV, Shrout JD. 2018. Spatially dependent alkyl quinolone signaling responses to antibiotics in Pseudomonas aeruginosa swarms. J Biol Chem 293:9544–9552.

18. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the *Pseudomonas aeruginosa* Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201:e00383-19, /jb/201/23/JB.00383-19.atom.

19. van Kessel JC. 2019. PQS signaling for more than a quorum: the collective stress response protects healthy Pseudomonas aeruginosa populations. J Bacteriol https://doi.org/10.1128/JB.00568-19.

20. Zegans ME, Wagner JC, Cady KC, Murphy DM, Hammond JH, O'Toole GA. 2009. Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of Pseudomonas aeruginosa. J Bacteriol 191:210–219.

21. Kamatkar NG, Shrout JD. 2011. Surface hardness impairment of quorum sensing and swarming for Pseudomonas aeruginosa. PLoS One 6:e20888.

22. Mattingly AE, Kamatkar NG, Morales-Soto N, Borlee BR, Shrout JD. 2018. Multiple Environmental Factors Influence the Importance of the Phosphodiesterase DipA upon Pseudomonas aeruginosa Swarming. Appl Environ Microbiol 84.

23. Boyle KE, Monaco H, van Ditmarsch D, Deforet M, Xavier JB. 2015. Integration of Metabolic and Quorum Sensing Signals Governing the Decision to Cooperate in a Bacterial Social Trait. PLoS Comput Biol 11:e1004279.

24. Bernier SP, Ha D-G, Khan W, Merritt JH, O'Toole GA. 2011. Modulation of Pseudomonas aeruginosa surface-associated group behaviors by individual amino acids through c-di-GMP signaling. Res Microbiol 162:680–688.

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CHAPTER 6: *Pseudomonas aeruginosa* Swarming Rearrangement by *Staphylococcus aureus* and Synthetic Hydrophobic Molecules

6.1 The Impact of *S. aureus* on Health and the Environment

Staphylococcus aureus is a ubiquitous and opportunistic bacterial pathogen that is identified as a facultative anaerobic Gram-positive bacterium. This microorganism is frequently found in the respiratory tract and on the skin (1, 2) which has major implications for human health as it can result in chronic infections with sometimes fatal consequences in the long-term (3). This bacterial strain has developed significant survival mechanisms and resistance toward antibiotics which has been contributing to infections that are increasingly difficult to treat (4). Therefore, health institutions including the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have categorized the methicillin-resistance *S. aureus* (MRSA) as a serious threat and one of twelve priority pathogens that endanger human healthcare (5, 6). Specifically in 2017, the CDC estimated 323,700 cases of *S. aureus* infection in hospitalized patients in 2007 and 10,600 deaths from health-related complications overall resulting in $1.7 billion in healthcare costs (5). It is predicted that the cases will continue to increase worldwide making this bacterial strain an important concern for public health.

S. aureus infections frequently occur when patients stay at the hospital, especially in the ICUs for an extended period of time as this pathogen takes advantage of compromised or weaken hosts (7, 8). This is primarily caused by an extensive resistance toward conventional antibiotics and the ability to form biofilms. Biofilms are an organized group of cells forming extracellular polymeric matrix that help *S. aureus* adhere to surfaces and provide increased protection against environmental stress (9, 10). The ability of *S. aureus* to resist antibiotic resistance through multiple

defense mechanism originates from a selective environment at the hospital in which a strong selective pressure is driving resistant bacterial strains. It is prevalent that *S. aureus* are often responsible for nosocomial infections which contribute to lower life expectancy for those especially infected with multi-drug resistance *S. aureus* (11). Therefore, studying *S. aureus* defense mechanism and its ability to colonize a variety of environment is critical for finding effective treatments against this bacterial strain infection.

One of the reasons why *S. aureus* can effectively invade the hosts, cause chronic infections, and induce health complications is due to its virulence factors that often overwhelmed the host immune system (12). The expression of these factors is tightly regulated by quorum sensing molecules produced from the overall *S. aureus* population. Within this quorum sensing cell-cell communication process, the *agr* system plays a central role in modulating virulence factors (13). Particularly, the *agr* genes regulates the production of phenol soluble modulins (PSMs) which has emerged as a toxin that defines the virulence potential of aggressive *S. aureus* isolates (14). Here, we focus on how *S. aureus* population uses PSMs to interact with their environment and its effect on *P. aeruginosa*, which commonly cohabit with *S. aureus*.

6.2 Phenol Soluble Modulins (PSMs) in *S. aureus*

6.2.1 Structure of PSMs

PSMs produced by *S. aureus* are a family of small amphipathic peptides with a hydrophobic side and a hydrophilic side that can form short α-helices and assemble into fibrils (14, 15). The genes encoding the PSMs peptides are highly conserved across *S. aureus* strains and three types of peptides exist for PSMs: PSMα, PSMβ, and PSMγ, with PSMγ previously described as δ-toxin in *S. epidermidis*. PSMs are subdivided into two main groups: PSMα and PSMβ. While most of the α-type PSMs have neutral or positive net charge and they are between 20 and 25 amino acids in length, the β-type PSMs commonly have negative net charge and measure between 43 and 45 amino acids in length (16). All PSMs can form amphipathic α-helices, giving them surfactant properties (Figure 6.1A). Cheung *et al*. has predicted the PSMs structure with primary and secondary bioinformatics analysis and found that the α-helices stretch over the entire length of PSMα and form the C-terminal of PSMβ (16). The PSMγ is encoded by the gene *hld* located within RNAIII in the Agr system and resembles the structure of PSMα. However, its structure has been shown to be more stable, resisting heat better than alpha and beta PSMs (17).

6.2.2 Regulation of PSMs

In liquid culture, the PSMα1-4 and PSMβ1-2 are known to be tightly regulated by the accessory gene regulator (agr) quorum sensing system in *S. aureus* (18). The *agr* operon is organized around two divergent promoters P2 and P3 which generate two primary transcripts,

RNAII and RNAIII, respectively (Figure 6.1B) (19). AgrA binds directly to the recognition sites of RNAII promoter domain to regulate *agrBDCA* and RNAIII promoter to regulate hld. Beyond RNAII and RNAIII, AgrA also upregulates PSMα and PSMβ production by binding to their promoter domains (20). Since *psmα*1-4 *psmβ*1-2 genes sequences are grouped under their respective promoter as the sequences are short with less than 150 base pairs.

6.2.3 Functions of PSMs

PSMs are commonly produced by *S. aureus* under various conditions including on a semi solid surface (21), which has been shown to promote formation of spreading dendrites similar to swarming tendrils from *P. aeruginosa*. Since PSMs are amphipathic and have cytolytic properties that can even disintegrate cell membranes like a detergent, they are considered biosurfactant. By producing abundant amounts of PSMs and secreting them around their colonies, *S. aureus* populations have the opportunity to slide beyond their colony boundaries (21). PSMs are also observed on a semi solid agar plate under a specific light angle similarly to *P. aeruginosa* producing rhamnolipids. This causes them to form dendrites that have characteristics of active motility.

Additionally, PSMs have been shown to be responsible for forming biofilm and maintaining its structure (22). Aggregated peptides in the form of functional amyloids fortify the biofilm matrix which ultimately increases resistance toward environmental stresses and degrading enzymes (23). Despite knowing PSMs form fibrils, very little is understood about the self-assembly mechanisms and the extended role of PSMs in *S. aureus*.

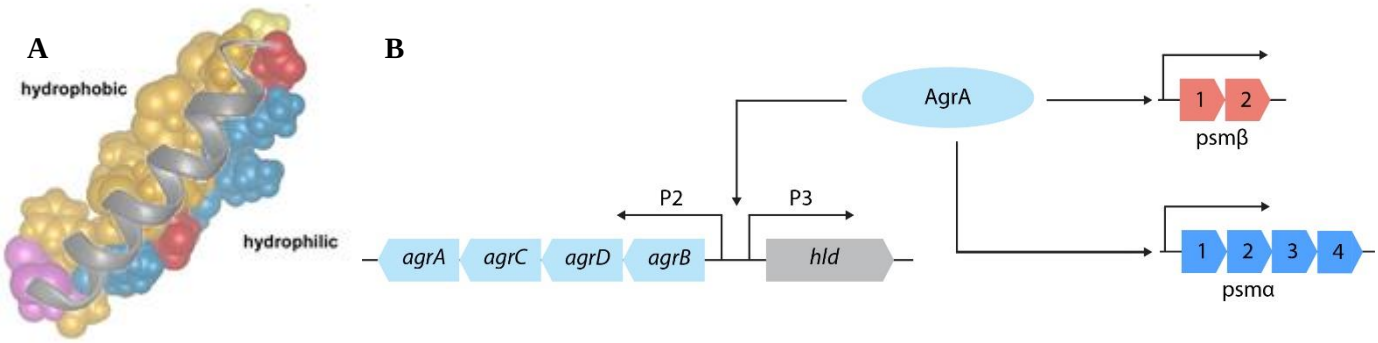

Figure 6.1. PSM Structure and Regulation by the Agr System in *S. aureus*. (A) PSMs form amphipathic α-helices with a hydrophobic side and a hydrophilic side (16). (B) AgrA binds to P2 and P3 to regulate *agrBDCA* and *hld*, respectively. AgrA also upregulates PSMα1-4 and PSMβ1-2 by binding to their promoter domains.

6.3 The Interactions Between *P. aeruginosa* and *S. aureus*

Staphylococcus aureus and *Pseudomonas aeruginosa* are opportunistic pathogens that co-colonize diverse environments such as human lungs and cause disease. Although each species is responsible for many severe pathological diseases, dual infections from both *P. aeruginosa* and *S. aureus* result in worse outcomes for the patients (24). Since both bacterial species are found in similar environments, it is common to see them battle for resources and survival. On semi-solid surfaces, *P. aeruginosa* moves collectively through swarming motility. This motion involves the rotation of flagella and the production of rhamnolipids, which decreases surface tension ahead of the advancing swarming population (25). *P. aeruginosa* swarms can be disrupted by the presence of other molecules, such as the quorum sensing molecule 2-heptyl-3-hydroxy-4-quinolone (PQS), which is produced by *P. aeruginosa* that are infected with bacteriophage or treated with antibiotics (26).

Here, we show that *S. aureus* disrupts *P. aeruginosa* swarming through the production of the small peptide phenol-soluble modulin (PSM) on semi solid surfaces. To understand the interaction between *P. aeruginosa* rhamnolipids and *S. aureus* PSMs, we develop a physical model that could explain the mechanism of repulsion. PSM, which has large hydrophobic side chains and a few hydrophilic side chains forms a layer of water between PSM and rhamnolipids produced from *P. aeruginosa*. The three-layer rhamnolipid-water-PSM interface creates a cell-free zone of repulsion that prevents physical contact between *S. aureus* and *P. aeruginosa* populations. The production of PSM thus protects the *S. aureus* population from *P. aeruginosa* by redirecting rhamnolipids and causing *P. aeruginosa* to swarm away from *S. aureus* colonies. Therefore, the *S. aureus* defense mechanism promotes the survival of bacterial populations by staying out of

danger from *P. aeruginosa* invasion. These results demonstrate that competing bacteria pathogens remain segregated on semi-solid surfaces through the production of liquid-liquid interfaces, which has major implications for the cohabitation and co-colonization of these species during pathogenesis.

6.3.1 *P. aeruginosa* Swarming Interaction with *P. aeruginosa* Clinical Strain Isolates

We previously showed that *P. aeruginosa* produces PQS under stress from antibiotics, including gentamicin, kanamycin, and fosfomycin, causing repulsion of healthy swarming population (26). Here, we expended to include tobramycin, which target bacterial 30S ribosome and block tRNA translocation, as a stress for *P. aeruginosa* (27, 28). Overnight culture of *P. aeruginosa* UCBPP-PA14 was spotted in the middle of all plates. Water or tobramycin was spotted at the satellite positions and showed no avoidance indicating that tobramycin itself does not cause repulsion (Figure 6.2A *i* and B *i*). When wild-type *P. aeruginosa* was spotted at the satellite, the repulsion is caused by rhamnolipids production which has previously been demonstrated (Figure 6.2 *ii*) (26). The Δ*rhlAB* mutant which does not produce rhamnolipids caused no repulsion similarly to observations made with the *P. aeruginosa* clinical isolates (PAnmFLR01) (Figure 6.2A *iii* and *iv*). Additionally, wild-type *P. aeruginosa*, Δ*rhlAB*, or PAnmFLR01 was mixed with 0.5 mg/mL as the final concentration of tobramycin and immediately spotted at the satellite positions (Figure 6.2B *ii*, *iii*, and *iv*). Minimal growth of *P. aeruginosa* was observed at the satellite position for all strains, indicating that the effective concentration of fosfomycin was below the MIC due to the antibiotic diffusion on the agar. Tobramycin-treated cells caused repulsion of untreated center swarms (Figure 6.2B *ii*, *iii*, *iv* and C). Additionally, when we tested the effect of

tobramycin on clinical isolates of *P. aeruginosa*, we observed that they also cause repulsion of wild-type *P. aeruginosa* swarms indicating that the PQS mechanism to repulse healthy swarms is widespread among laboratory and clinical isolates (Figure 6.2D). Two clinical isolates did not show avoidance likely due to low PQS production or tobramycin resistance.

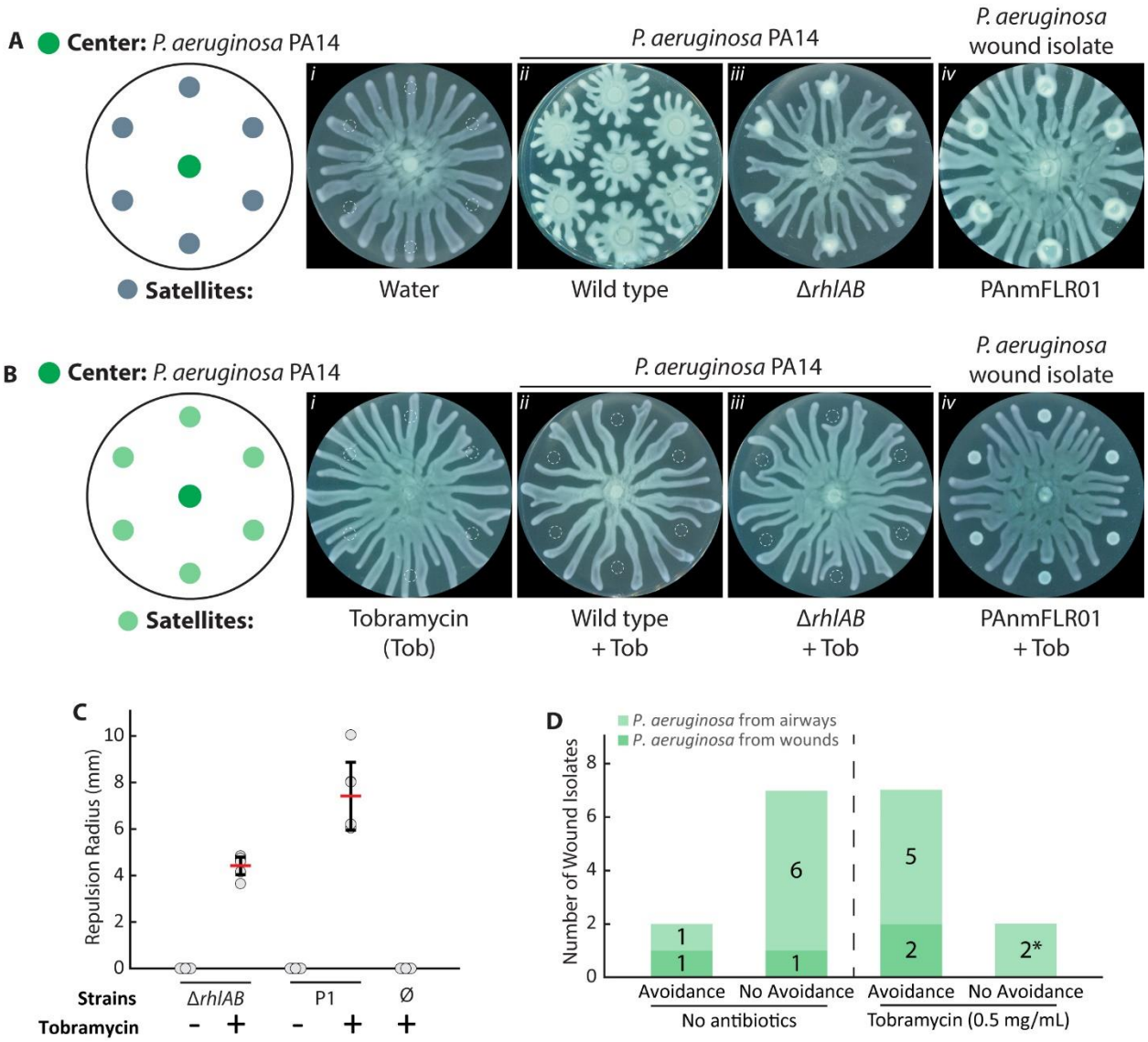

Figure 6.2. ***Pseudomonas aeruginosa*** **Treated with Tobramycin Induces Swarming Repulsion.** Graphic indicates the initial spot positions of corresponding culture conditions (A) without tobramycin and (B) with tobramycin. Wild-type (WT) *P. aeruginosa* strain PA14 was spotted at the center and (*i*) water, (*ii*) WT PA14, (*iii*) PA14 Δ*rhlAB*, or (*iv*) *P. aeruginosa* wound isolate PAnmFLR01 were spotted (A) without tobramycin or (B) with tobramycin at concentric satellite positions surrounding the center. Swarm images were taken after 16 to 18 h of growth at 37°C. The dashed lines indicate the boundaries of the initial spots. (C) Quantification of the repulsion radii at the satellite positions for previous culture conditions. Red line indicates averages from 6 independent experiments (n = 6). Error bars indicate standard deviations. (D) Number of bacterial strain isolates of *P. aeruginosa* from airways or wounds non-treated or treated with tobramycin were spotted on M8 media with 0.5% agar showing avoidance or no avoidance after 16 to 18 hours at 37°C. The swarming assays for the airway or wound bacterial isolates are shown in Figure 6.3.

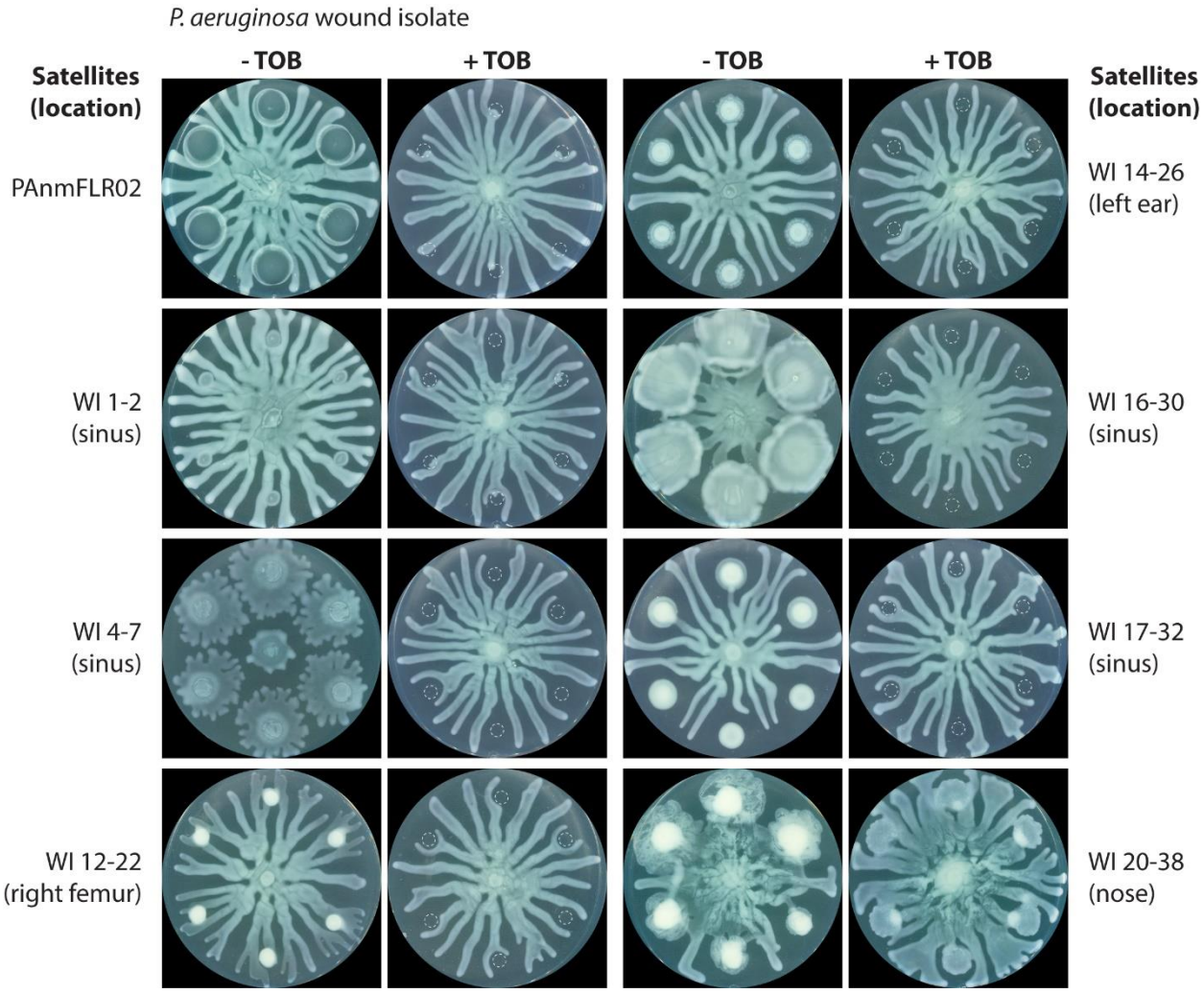

Figure 6.3. Clinical Isolates of *Pseudomonas aeruginosa* Treated with Tobramycin. Wild-type (WT) *P. aeruginosa* strain PA14 was spotted at the center for all conditions and *P. aeruginosa* clinical isolates were spotted without tobramycin (left images) or with tobramycin (right images) at concentric satellite positions surrounding the center. Swarm images were taken after 16 to 18 h of growth at 37°C.

6.3.2 *P. aeruginosa* Swarming Interaction with *S. aureus* Clinical Strain Isolates

Since PQS from stressed *P. aeruginosa* promotes the survival of the bacterial population by redirecting the spatial organization, we reasoned that *S. aureus*, which frequently coexist with *P. aeruginosa*, can have similar mechanisms to *P. aeruginosa* under stress. We therefore spotted wild-type swarming *P. aeruginosa* in the middle and wild-type *S. aureus* at the satellite position with and without tobramycin (Figure 6.4A-B). The *S. aureus* without tobramycin treatment caused repulsion of *P. aeruginosa* swarms similarly to antibiotic-treated *P. aeruginosa* (Figure 6.4A and C). This suggests that *S. aureus* produces a compound that causes swarms repulsion like the mechanism that promotes PQS secretion under cell stress. On the other hand, the tobramycin-treated *S. aureus* did not show repulsion of swarms (Figure 6.4B and C). Minimal to no growth was observed for all strains at the satellite positions indicating that growth is necessary for promoting the repulsion phenotype. Moreover, we tested the effect of tobramycin on clinical isolates of *S. aureus* and found that non-treated cells caused swarms repulsion while the cells treated with tobramycin did not (Figure 6.4C and 6.5). Therefore, the repulsion phenotype by *S. aureus* is commonly found in laboratory and clinical strains. One *S. aureus* isolate from the right femur did not show avoidance without antibiotic treatment and one sinus isolate still repulse *P. aeruginosa* swarms even under tobramycin treatment. Since the secretion of quorum sensing molecule PQS by *P. aeruginosa* promotes repulsion of swarming populations, it was hypothesized that *S. aureus* also produces a compound within its quorum sensing system to repulse *P. aeruginosa* swarms.

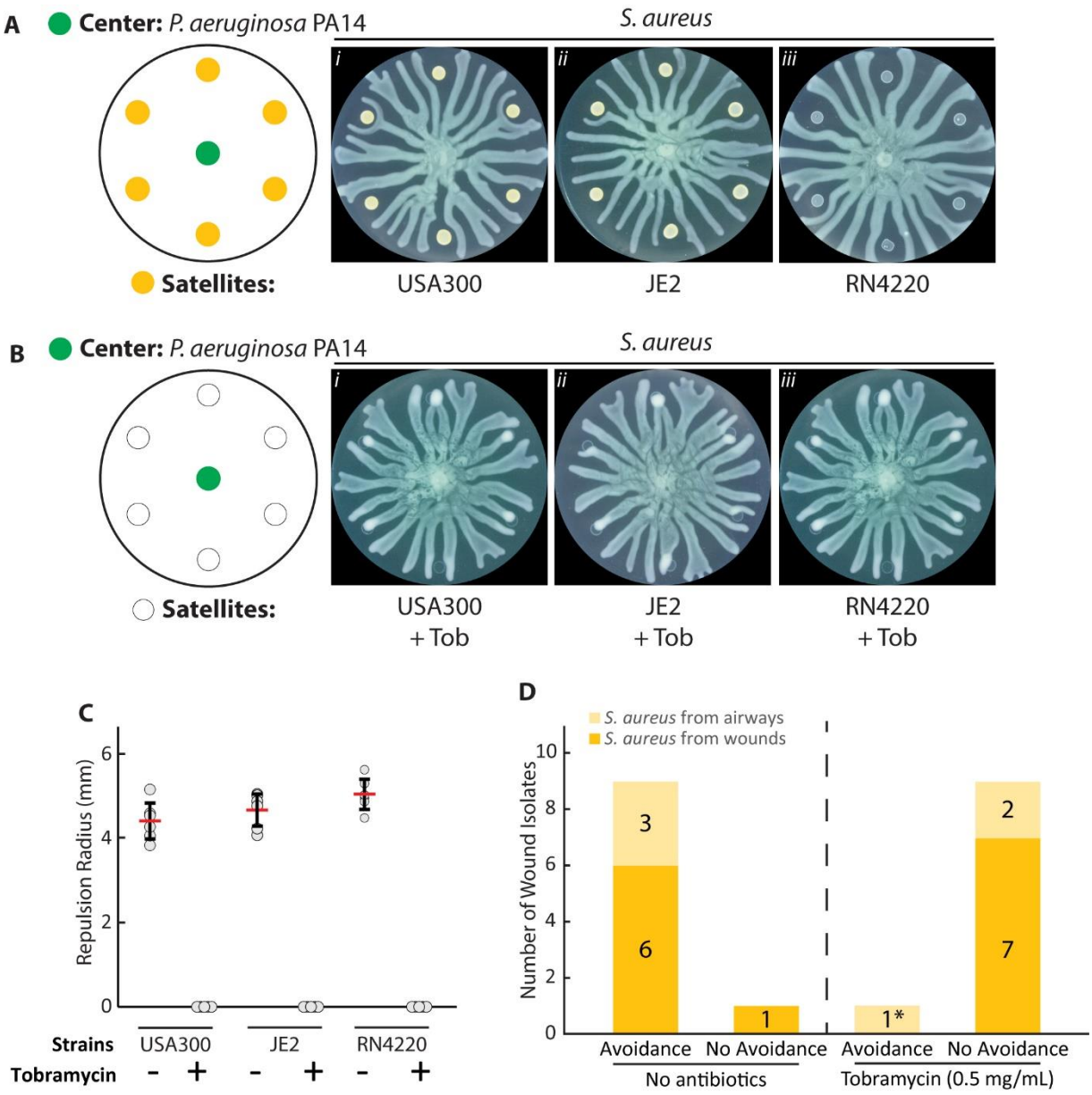

Figure 6.4. *Staphylococcus aureus* Causes Repulsion of *Pseudomonas aeruginosa* Swarms. Graphic indicates the initial spot positions of corresponding culture conditions (A) without tobramycin and (B) with tobramycin. Wild-type *P. aeruginosa* PA14 was spotted at the center and wild-type (WT) *S. aureus* strain (*i*) USA300, (*ii*) WT JE2, (*iii*) WT RN4220 were spotted (A) without tobramycin or (B) with tobramycin at the satellite positions. Swarm images were taken after 16 to 18 h of growth at 37°C. (C) Quantification of the repulsion radii at the satellite positions for previous culture conditions. Red line indicates averages from 6 independent experiments (n = 6). Error bars indicate standard deviations. (D) Number of bacterial strain isolates of *S. aureus* from airways or wounds non-treated or treated with tobramycin were spotted on M8 media with 0.5% agar showing avoidance or no avoidance after 16 to 18 hours at 37°C. The swarming assays for the airway or wound bacterial isolates are shown in Figure 6.5.

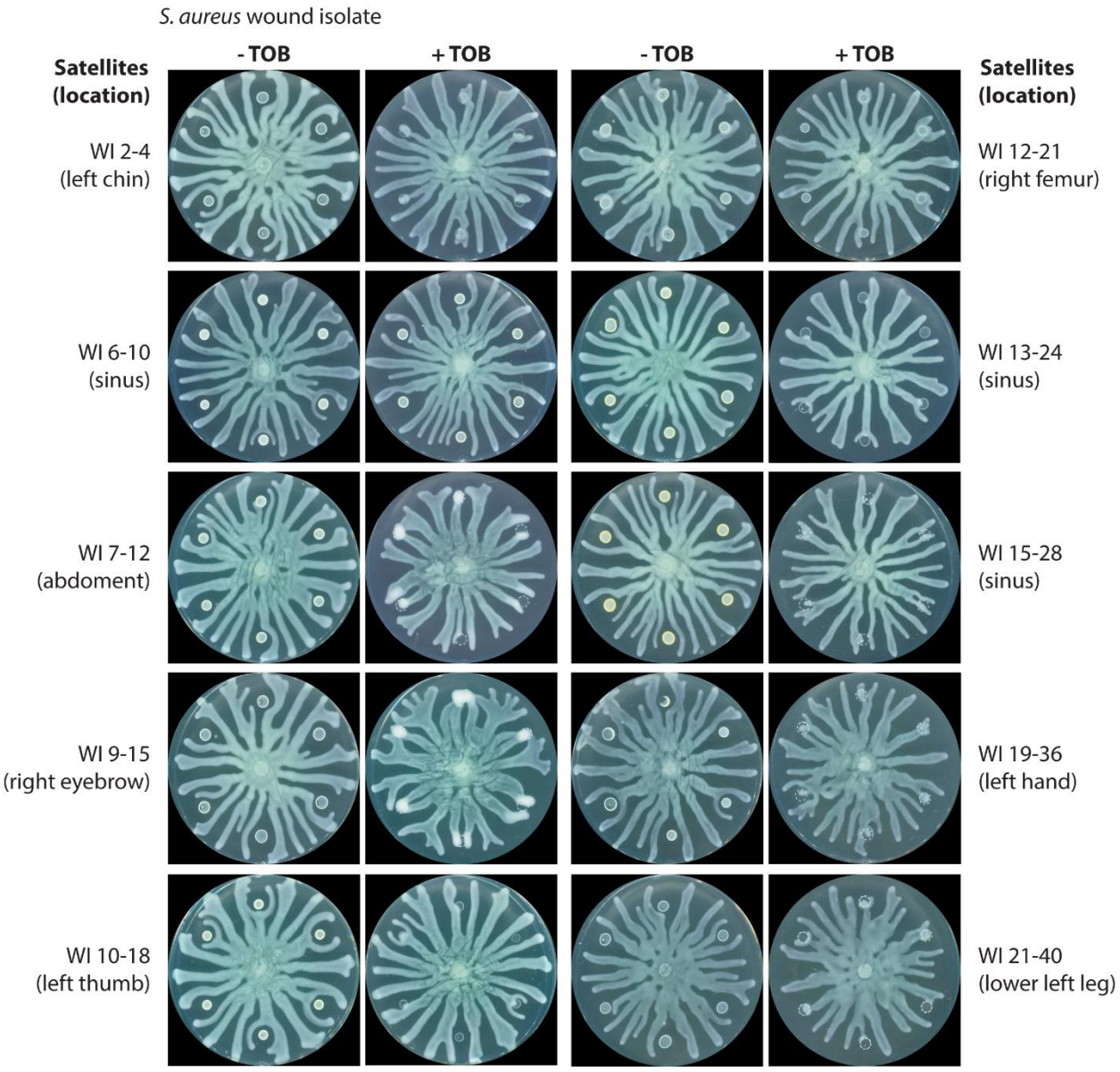

Figure 6.5. Clinical Isolates of *Staphylococcus aureus* Treated with Tobramycin. Wild-type (WT) *P. aeruginosa* strain PA14 was spotted at the center for all conditions and *P. aeruginosa* clinical isolates were spotted without tobramycin (left images) or with tobramycin (right images) at concentric satellite positions surrounding the center. Swarm images were taken after 16 to 18 h of growth at 37°C.

6.4 Hydrophobic Molecules Cause *P. aeruginosa* Swarming Rearrangement

6.4.1 PSMs Produced by *S. aureus* Repulse *P. aeruginosa* Swarms

After observing that wild-type *S. aureus* causes *P. aeruginosa* swarm repulsion, our goal was to find the molecule responsible for this phenotype. The quorum sensing molecule PQS produced by stressed *P. aeruginosa* has been shown to repulse swarming cells (26). Therefore, we hypothesized that *S. aureus* also secretes a molecule within its quorum sensing system to repulse *P. aeruginosa* swarms. We observed that the *S. aureus* Δ*psmα* Δ*hld*, which cannot produce PSMα and PSMγ, does not cause repulsion as the swarming *P. aeruginosa* go through the *S. aureus* colonies (Figure 6.6A-B). The Δ*psmα* mutant was enough to remove the repulsion mechanism of *S. aureus* (Figure 6.6A-B). To observe the rhamnolipids layer produced by *P. aeruginosa*, we used the IRIS technique to image *P. aeruginosa* swarms being repulsed by wild-type *S. aureus* but not PSM-deficient strains. We showed that the rhamnolipids layer goes around the wild-type *S. aureus* and the tendrils followed the path directed by the *P. aeruginosa* surfactants causing swarm repulsion (Figure 6.6C). On the other hand, rhamnolipids overtook Δ*psmα* and Δ*psmα* Δ*hld* colonies giving the *P. aeruginosa* swarming population the ability to swarm over the *S. aureus* mutants. This highlighted that rhamnolipids are repulsed by PSMs since removing PSM production resulted in no swarm repulsion.

To confirm if PSMs were produced around the WT *S. aureus* colonies, we put a copper grid next to each colony and observed deposited compounds with a transmission electron microscope (TEM). We showed that WT *S. aureus* produced PSMs fibrils up to 3 mm away from the colony, while the Δ*psmα* Δ*hld* produces PSM fibrils only near the colony.

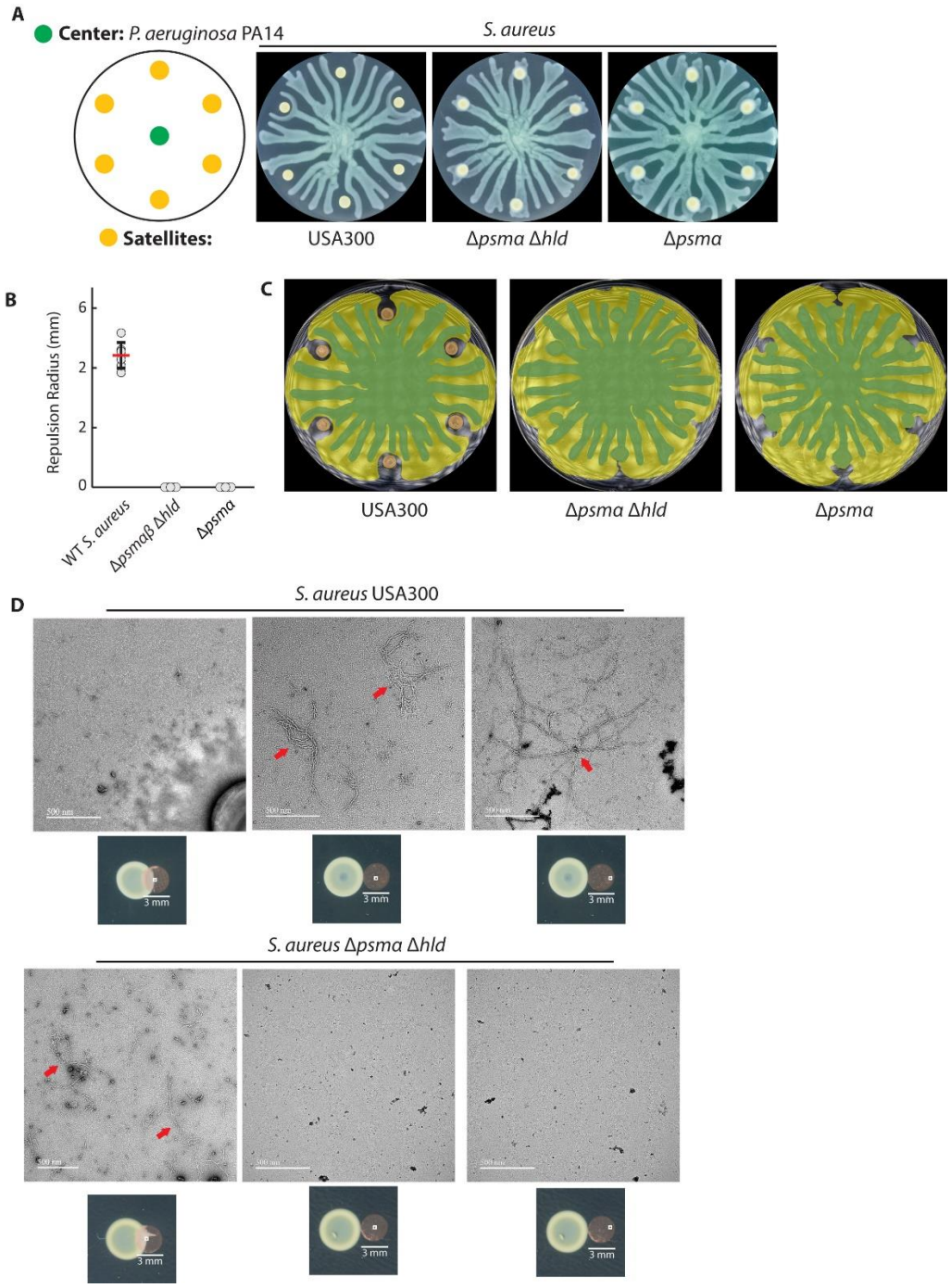

Figure 6.6. *S. aureus* Produces PSMs to Repulse *P. aeruginosa* Swarms. (A) Wild-type *P. aeruginosa* was spotted at the center of each plate and *S. aureus* strains, USA300, Δ*psmα* Δ*hld*, and *Δpsmα*, were each spotted at the satellite position. (B) Quantification of the repulsion radii at the satellite positions for previous culture conditions. Red line indicates averages from 6 independent experiments (n = 6). Error bars indicate standard deviations. (C) Images taken using the IRIS technique which shows the rhamnolipids layer in yellow, *P. aeruginosa* swarms in green, and *S. aureus* colonies in orange. (D) TEM images of tendrils from USA300 and *S. aureus* mutant Δ*psmα* Δ*hld*. Swarm images were taken after 16 to 18 h of growth at 37°C.

6.4.2 *P. aeruginosa* Swarms Avoid Polydimethylsiloxane (PDMS) and Synthetic Surfactants

Our next goal was to determine the mechanism responsible for rhamnolipids repulsion. Since PSMs have surfactant properties meaning that they have a hydrophobic and hydrophilic side and can also form fibrils, we hypothesized that similar compounds like PDMS can also cause rhamnolipids repulsion. We therefore took an overnight culture of wild-type *P. aeruginosa* and spotted it in the middle of the plate while PDMS at 200 cSt* and 1000 cSt* are spotted at the satellite positions. We noticed that the *P. aeruginosa* populations swarmed around the PDMS droplets which resembles the observation made with *S. aureus* colonies (Figure 6.7A). When PDMS 200 cSt is spotted at the satellite position, *P. aeruginosa* swarming population avoids at a greater distance than when PDMS 1000 cSt is spotted at the same position (Figure 6.7A). This means that lower viscosity causes a larger zone of repulsion than higher viscosity for PDMS.

While hydrophobicity is essential for the molecules at the satellite position to push rhamnolipids aside, the hydrophobicity scale also needs to be considered. Contact angles determine the hydrophobicity level of a liquid. To measure the liquid hydrophobicity, 1 µl of liquid was dropped onto a surface that have both hydrophobic and hydrophilic proprieties. The contact angle is measured at the edge of the drop where the liquid, solid, and vapor phase interact. Water is taken as a reference with a contact angle close to 90°. Contact angles lower than 90° indicates that the solution is more hydrophobic. The results show that the PDMS 200 cSt and PDMS 1000 cSt have contact angles of 45° and 44°, respectively. PDMS can also form long hydrophobic chains and the viscosity depends on the length of these chains. This suggests that swarming avoidance is caused by highly hydrophobic molecules that can form long hydrophobic chains.

Additional hydrophobic compounds also showed this phenotype including oleic acid and linoleic acid with contact angle of 51° and 45°, respectively (Figure 6.7). However, it is critical to note that only hydrophobic compounds with contact angles less than 53° showed swarms avoidance (Figure 6.7B-C). This can be explained by rhamnolipids avoiding the droplets of hydrophobic compounds like PDMS (Figure 6.8). Overall, the rhamnolipids layer plays a role in directing and organizing swarming populations of *P. aeruginosa*.

*NOTE: cSt is the unit for viscosity. For comparison, water has a viscosity of 1 cSt at 20°C.

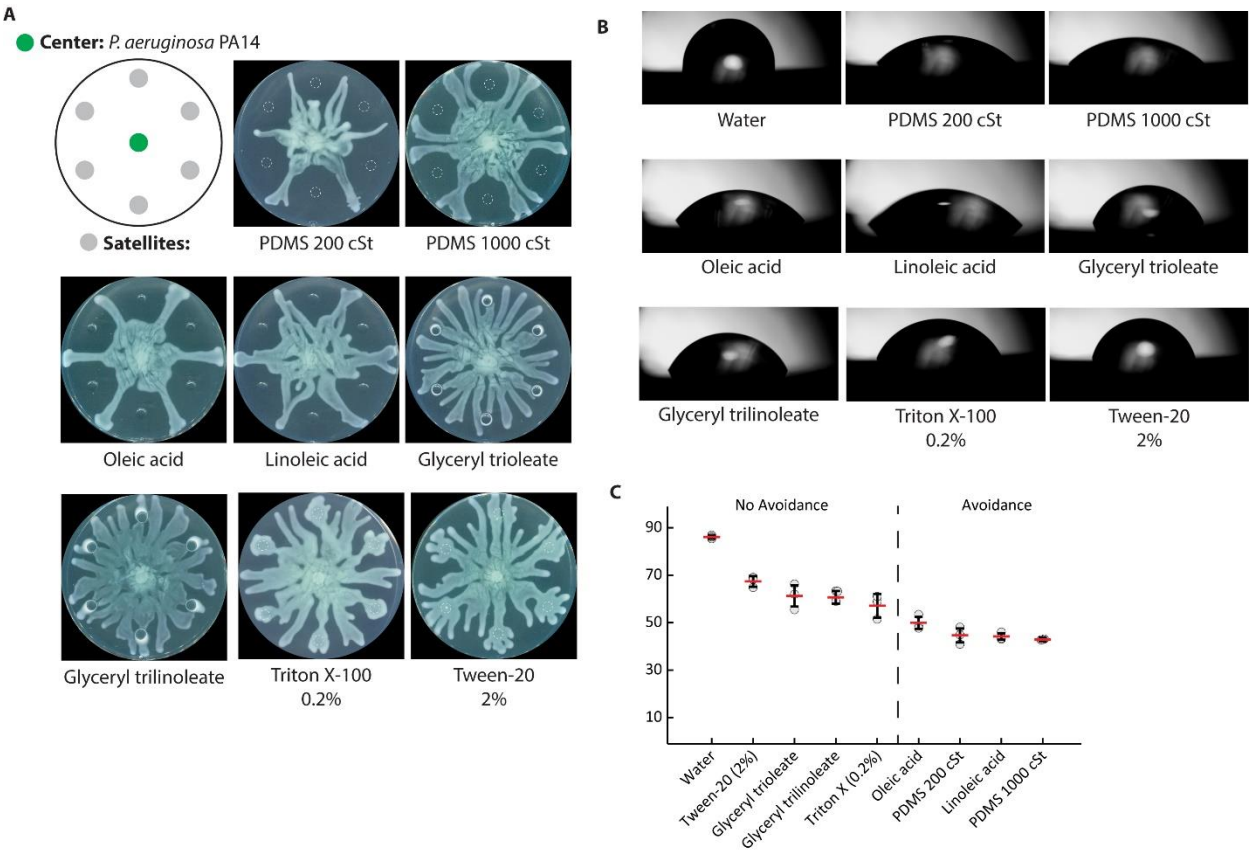

Figure 6.7. *P. aeruginosa* Swarms Around Hydrophobic Molecules. PDMS (200 cSt and 1000 cSt), oleic acid, linoleic acid, glyceryl trioleate, glyceryl trilinoleate, triton X-100, and tween-20 were (A) spotted on separate plates at the satellite positions or (B) imaged with a tensiometer (C) to measure the contact angle. Contact angle measurement is indicated by red lines and is the average of 3 independent experiments (n = 6). Error bars indicate standard deviations. Overnight culture of wild-type *P. aeruginosa* was spotted at the center of each plate. Swarm images were taken after 16 to 18 h of growth at 37°C.

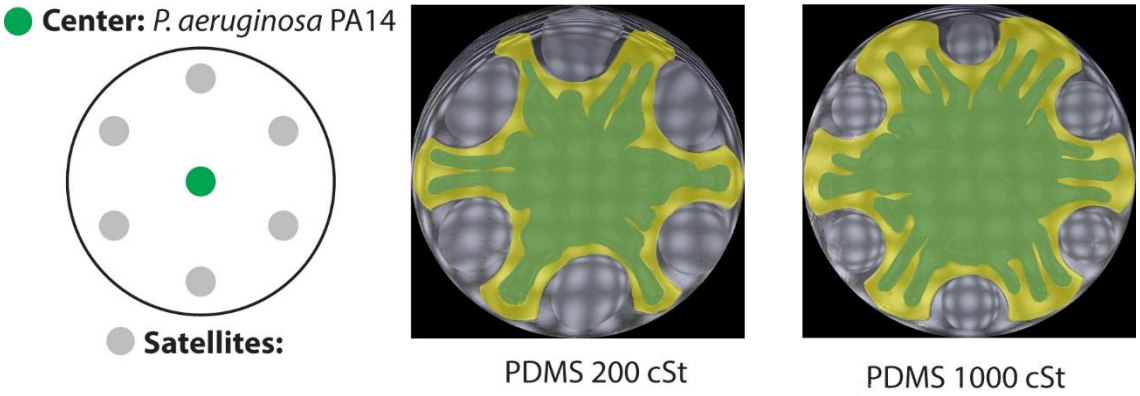

Figure 6.8. Rhamnolipids Direct Swarms Organization in *P. aeruginosa*. Wild-type *P. aeruginosa* was spotted at the center of each plate and PDMS at 200 cSt and 1000 cSt were spotted at the satellite positions. Images taken using the IRIS technique which shows the rhamnolipids layer in yellow, *P. aeruginosa* swarms in green, and *S. aureus* colonies in orange. Swarm images were taken after 16 to 18 h of growth at 37°C.

6.5 Conclusions

Although each species is primarily studied individually *in vitro*, both *P. aeruginosa* and *S. aureus* are commonly found together on skin infections or in cystic fibrosis patients (29). Here, we examined the interspecies competition between wild-type *P. aeruginosa* and clinical samples of *S. aureus* and *P. aeruginosa*, which were isolated pathogens from skin wounds or upper respiratory tract infections. We confirm previous study from Bru et al. that subpopulation of *P. aeruginosa* that is stressed with tobramycin repulses approaching swarms far beyond the area containing the antibiotic treatment. This serves as a warning mechanism for approaching swarms to avoid the danger. Our data also suggests that PQS functions as a coordinator of spatial organization during swarming to ensure that cells navigate toward safe area. Overall, the PQS response to stress is commonly found across the tested strains here which suggest that most *P. aeruginosa* strains found in the laboratory or as isolates also have this defense mechanism. The implications of such response to antibiotics can impact the severity of bacterial infections and treatments against those infections.

The ability to repulse swarming cells is not just reserved to stressed *P. aeruginosa* secreting PQS since non-stressed *S. aureus* can also reorganize *P. aeruginosa* swarms by producing PSMs. Under collective cohabitation, both *S. aureus* and *P. aeruginosa* share the same environmental space. In this context, it is therefore beneficial for *S. aureus* to avoid interacting with *P. aeruginosa*. This response by *S. aureus* may serve as a defense mechanism to avoid invasion from *P. aeruginosa*. The overall effect is that *P. aeruginosa* is forced to swarm away from *S. aureus* colonies. In the *S. aureus* Δ*psmα* Δ*hld* or Δ*psmα* strains, which are defective in the repulsion

response, wild-type *P. aeruginosa* swarms approached and invaded *S. aureus* populations. This emphasizes that the importance of PSMs to promote heterogeneity of two bacterial populations.

The ability of PSMs to repulse swarms raises the question of how PSMs interact with rhamnolipids. As hydrophobic peptides, PSMs redirect rhamnolipids while hydrophobic molecules such as triton X-100 and tween-20 do not. Since rhamnolipids and PSMs are both hydrophobic, it is expected that they merge instead of repulsing each other. Here, we propose that a three-layer rhamnolipid-water-PSM interface creates a cell-free zone of repulsion that prevents physical contact between *S. aureus* and *P. aeruginosa* populations (Figure 6.9). by having that layer of water in between both hydrophobic compounds, it is possible to create a barrier that redirect rhamnolipids and causing swarm avoidance. Future work will address the mechanisms by which PSMs interact with rhamnolipids to repulse swarming populations and the implications of heterogeneity of both bacterial strains.

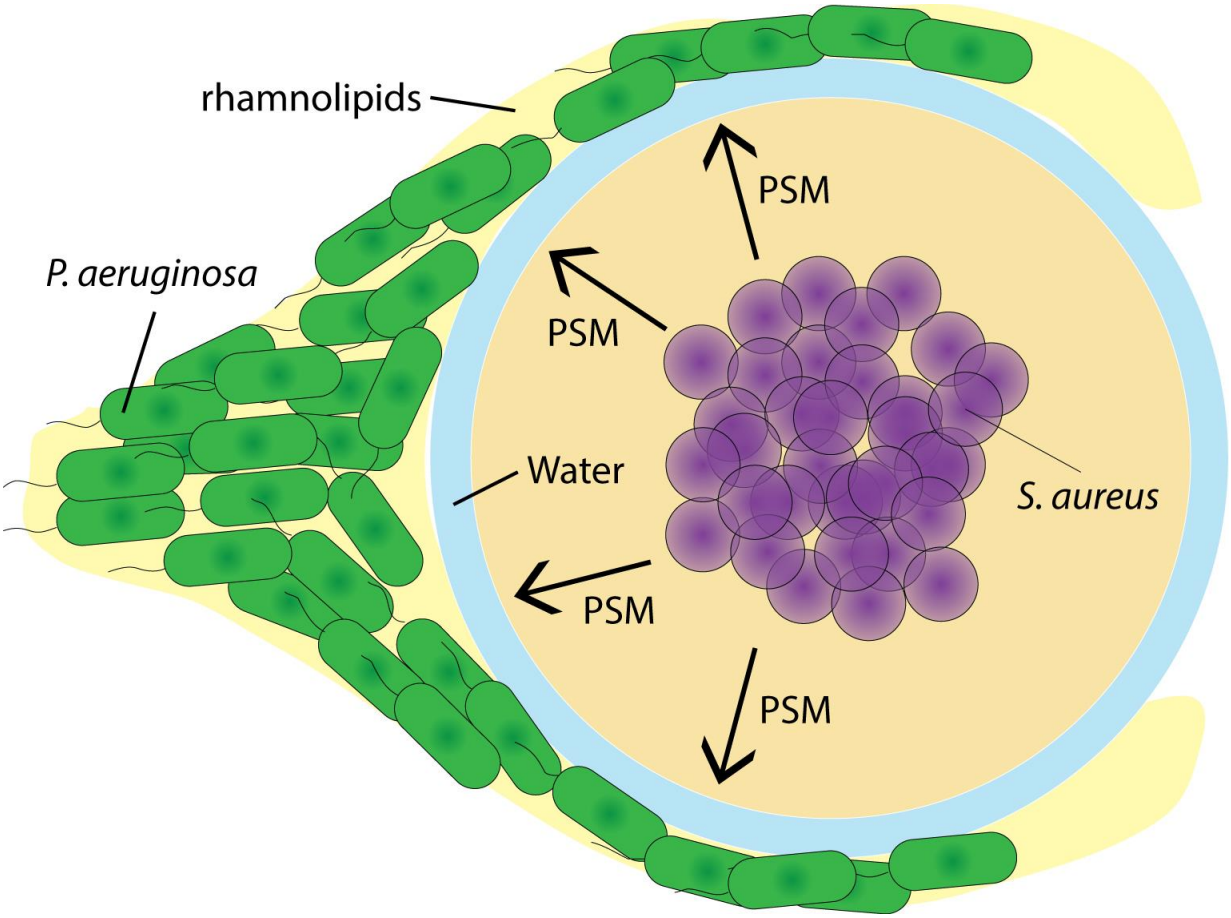

Figure 6.9. Schematic of the PSM Repulsing Rhamnolipids. *S. aureus* produces PSM to repel *P. aeruginosa* swarming population. This defense mechanism by *S. aureus* reduces the chance of invasion by *P. aeruginosa*.

6.6 References

1. McCaig LF, McDonald LC, Mandal S, Jernigan DB. 2006. Staphylococcus aureus-associated skin and soft tissue infections in ambulatory care. Emerg Infect Dis 12:1715–1723.

2. Parker D, Prince A. 2012. Immunopathogenesis of Staphylococcus aureus pulmonary infection. Semin Immunopathol 34:281–297.

3. Conlon BP. 2014. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. Bioessays 36:991–996.

4. Chambers HF, Deleo FR. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol 7:629–641.

5. Centers for Disease Control and Prevention (U.S.). 2019. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention (U.S.).

6. Craft KM, Nguyen JM, Berg LJ, Townsend SD. 2019. Methicillin-resistant Staphylococcus aureus (MRSA): antibiotic-resistance and the biofilm phenotype. Medchemcomm 10:1231–1241.

7. Vincent J-L, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y, Reinhart K, EPIC II Group of Investigators. 2009. International study of the prevalence and outcomes of infection in intensive care units. JAMA 302:2323–2329.

8. Oztoprak N, Cevik MA, Akinci E, Korkmaz M, Erbay A, Eren SS, Balaban N, Bodur H. 2006. Risk factors for ICU-acquired methicillin-resistant Staphylococcus aureus infections. Am J Infect Control 34:1–5.

9. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322.

10. Anderson GG, O'Toole GA. 2008. Innate and induced resistance mechanisms of bacterial biofilms. Curr Top Microbiol Immunol 322:85–105.

11. van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB. 2012. Predictors of mortality in Staphylococcus aureus Bacteremia. Clin Microbiol Rev 25:362–386.

12. Rooijakkers SHM, van Kessel KPM, van Strijp JAG. 2005. Staphylococcal innate immune evasion. Trends Microbiol 13:596–601.

13. Novick RP, Geisinger E. 2008. Quorum sensing in staphylococci. Annu Rev Genet 42:541–564.

14. Peschel A, Otto M. 2013. Phenol-soluble modulins and staphylococcal infection. Nat Rev Microbiol 11:667–673.

15. Zheng Y, Joo H-S, Nair V, Le KY, Otto M. 2018. Do amyloid structures formed by Staphylococcus aureus phenol-soluble modulins have a biological function? Int J Med Microbiol 308:675–682.

16. Cheung GYC, Joo H-S, Chatterjee SS, Otto M. 2014. Phenol-soluble modulins – critical determinants of staphylococcal virulence. FEMS Microbiol Rev 38:698–719.

17. Thelestam M, Möllby R, Wadström T. 1973. Effects of staphylococcal alpha-, beta-, delta-, and gamma-hemolysins on human diploid fibroblasts and HeLa cells: evaluation of a new quantitative as say for measuring cell damage. Infect Immun 8:938–946.

18. Queck SY, Jameson-Lee M, Villaruz AE, Bach T-HL, Khan BA, Sturdevant DE, Ricklefs SM, Li M, Otto M. 2008. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. Mol Cell 32:150–158.

19. Ji G, Beavis RC, Novick RP. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci U S A 92:12055–12059.

20. Thoendel M, Kavanaugh JS, Flack CE, Horswill AR. 2011. Peptide signaling in the Staphylococci. Chem Rev 111:117–151.

21. Pollitt EJG, Crusz SA, Diggle SP. 2015. Staphylococcus aureus forms spreading dendrites that have characteristics of active motility. 1. Scientific Reports 5:17698.

22. Schwartz K, Syed AK, Stephenson RE, Rickard AH, Boles BR. 2012. Functional amyloids composed of phenol soluble modulins stabilize Staphylococcus aureus biofilms. PLoS Pathog 8:e1002744.

23. Bleem A, Francisco R, Bryers JD, Daggett V. 2017. Designed α-sheet peptides suppress amyloid formation in Staphylococcus aureus biofilms. NPJ Biofilms Microbiomes 3:16.

24. Fazli M, Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, Givskov M, Tolker-Nielsen T. 2009. Nonrandom Distribution of Pseudomonas aeruginosa and Staphylococcus aureus in Chronic Wounds. J Clin Microbiol 47:4084–4089.

25. Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas aeruginosa Is a Complex Adaptation Leading to Increased Production of Virulence Factors and Antibiotic Resistance. Journal of Bacteriology 190:2671–2679.

26. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the Pseudomonas aeruginosa Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201.

27. Ying L, Zhu H, Shoji S, Fredrick K. 2019. Roles of specific aminoglycoside-ribosome interactions in the inhibition of translation. RNA 25:247–254.

28. Doi Y, de Oliveira Garcia D, Adams J, Paterson DL. 2007. Coproduction of novel 16S rRNA methylase RmtD and metallo-beta-lactamase SPM-1 in a panresistant Pseudomonas aeruginosa isolate from Brazil. Antimicrob Agents Chemother 51:852–856.

29. Salsgiver EL, Fink AK, Knapp EA, LiPuma JJ, Olivier KN, Marshall BC, Saiman L. 2016. Changing Epidemiology of the Respiratory Bacteriology of Patients With Cystic Fibrosis. Chest 149:390–400.

CHAPTER 7: Conclusions

7.1 The Nuance of Swarming Motility in *P. aeruginosa*

Several assessments of *P. aeruginosa* swarming have been based on observations of tendrils that grow from colonies on Petri dishes. Such assessments of swarming differ from early metrics of swarming, which were based instead of micromorphological features that require singe-cell resolution of bacteria at the swarming edges (1, 2). In particular, Henrichsen precisely defined a number of bacterial translocation modes, including swimming, swarming, and sliding (1). Swimming and swarming were defined as surface translocation that are driven by flagella. Swarming differs from swimming because individual cells aggregate in bundles during swarming, whereas swimming cells do not have an obvious micromorphological organization. Sliding was defined as surface translocation due to expansion of the culture by growth, in combination with reduced friction between the cell and substrate. Sliding differs from both swarming and swimming because it does not require the activity of flagella. The micromorphological feature of sliding is that cells move together as a single uniform sheet of cells.

Swimming has been widely observed in *P. aeruginosa* due to the robust activity of its flagella. However, the distinction between swarming and sliding motilities in *P. aeruginosa* have been less clear. Murray et al. observed sliding motility in strains that are deficient in pili and flagella (*fliC pilA*) (3). The colony expansion was reduced by a *rhlA* mutation, which disables the production of rhamnolipids. The ability of *P. aeruginosa* to expand without flagella and with the assistance of rhamnolipids is consistent with the Henrichsen's description of sliding motility, though no micromorphological features were reported in the study. An early report concluded that multiflagellated mutants of *P. aeruginosa* exhibited swarming (4). The assessment that the mutants

swarmed was based on the increased diameter of colonies on Petri dishes. However, the micromorphological features of cell alignment and aggregates were not assessed. Two reports, from Köhler et al. and Rashid and Koernberg, described first observations of swarming by *P. aeruginosa* based on colony morphology and the dependence of the phenotypes on flagella (5, 6). While neither report investigated the micromorphologies of cells at the leading edge of the colony, the strong dependence of colony expansion and tendril formation on flagella are suggestive of a swarming phenotype. Köhler et al. additionally described the dependence of the phenotype on pili(5). However, the tendrils have since been observed in strains of *P. aeruginosa* that lack pili (7, 8).

More recently, Madukokma et al. investigated micromorphologies and dynamics of *P. aeruginosa* at the edge of expanding colonies in swarming conditions (9). The authors identify 4 phases of colony expansion that are marked by distinct morphologies and kinematics. Phases I is marked by uniform community expansion and low single-cell velocities. Phase II has the highest single-cell velocities and phase III has the highest community expansion. Phase IV, which marks tendril formation, is characterized by lowered community expansion and the decreased single-cell velocities. The presence of multiple phases suggests that multiple motility mechanisms may be at work in *P. aeruginosa* swarms. In particular, community expansion during phases I, III, and IV are reminiscent of sliding motility described by Henrichsen (1) whereas the high motility during phase II is consistent with flagellar activity. A notable observation is the lack of organized aggregates, which are a defining feature of swarming (1). It does not appear that swarming by *P. aeruginosa* conforms to the precise description of swarming laid out by Henrichsen (1). It is possible that *P. aeruginosa* swarms reflect the convolution of multiple motility mechanisms. *P. aeruginosa* swarms have characteristics of both swarming and sliding. The requirement for flagella

suggests a swarming-like mechanism whereas the requirement for surfactant production suggests a sliding-like mechanism. The interplay between both swarming-like and sliding-like mechanisms may be responsible for the resulting growth patterns that emerge.

7.2 The Role of Flagella in Swarming

Flagella have a critical role in promoting the expansion of swarms and forming tendrils. *P. aeruginosa* that are deficient in flagella produce colonies that are significantly reduced in size and do not form tendrils that are characteristic of swarms in this species. *P. aeruginosa* that are hyperflagellated produce enlarged swarms (4, 10–12). However, there is a critical knowledge gap about how flagella promote swarming. In the Henrichsen definition, cells move together as organized aggregates that are driven by flagella. However, it is unclear how such a mechanism would cause the expansion of a bacterial swarm. At the heart of the issue is that flagellar motility could cause the surface translocation of bacterial cells across the surface, thereby expanding the area of a swarm across a surface. Flagellar reversals can help cells escape confined environments or increase the outflow of cells across the edge of swarms (13–17). However, this motility alone would not result in volumetric expansion. To achieve this, additional biomass is needed, which would require an influx of nutrients and liquid from the agar into the swarm.

How could the activity of flagella cause the influx of nutrients and liquid? Much of insight into this question has come from models of *S. enterica* serovar Typhimurium, where it has been proposed that flagella sense and generate wetness (18–20). In this model, the activity of flagella removes lipopolysaccharide (LPS) from the outer surface of bacteria, creating an osmolyte that is suspended in the swarming medium. Consistent with this model, strains that are defective in the production of LPS are deficient in swarming (20). Other potential osmolytes that are increased by flagellar activity include enterobacterial common antigen and colonic acid (21). In *P. aeruginosa*, mutations that give rise to a greater rate of flagellar reversals give rise to hyperswarming (22–25). It is possible that flagellar reversals could increase concentration of an osmolyte such as LPS in *P.*

aeruginosa swarms. Ping et al. propose that an osmolyte that alters liquid flux from agar should be high molecular weight and have a small diffusion coefficient (26). Substances of low molecular weight such as salts or trehalose would be insufficient to have this effect due to their relatively large diffusion coefficient. To date, however, an osmolyte that is increased by flagellar reversals has not been identified. In addition, the dependence of osmolyte concentration on flagellar reversals has not been measured.

If the effect of flagella is to increase liquid flux from the agar into a bacterial layer, it is unclear if such a mechanism would be considered sliding or swarming as defined by Henrichsen. On one hand, the volumetric expansion of the swarm would be due to liquid influx, which would suggest a sliding-like mechanism. The fluidic models proposed by Giverso et al., Ping et al., and Yang et al. are essentially volumetric expansion models that give rise to sliding-like motility. On the other hand, the volumetric expansion is dependent on the activity of flagella, which would suggest a swarming-like mechanism. This reasoning additionally suggests that *P. aeruginosa* swarms may need to be considered a combination of sliding and swarming mechanisms and that the Henrichsen definitions may need to be expanded.

P. aeruginosa swarming focus on rhamnolipids production over flagella activity which demonstrates that very little is still known on the role of flagella. Previous studies have suggested that flagella could promote volumetric expansion of the swarms by increasing the intake of water, osmolytes, and nutrients into the swarm (14, 26, 27). However, it remains unclear how flagella activity could increase the intake of nutrients and liquid from the agar to the swarming population. Furthermore, swarming has been defined as surface translocation that is driven by flagella activity that results in cell aggregates (1). Yet, models generally describe the importance of flagella for swarming motility and frequently omit the importance of cell aggregation as a feature of swarming.

Although flagella are used for single-cell swimming in a liquid, it is not understood why these same flagella are not enough to promote swarming on a semi solid surface since swarming motility requires an entire community of cell aggregates.

7.3 The Environmental Relevance of Swarming

Swarming in *P. aeruginosa* has been observed in laboratory settings when bacterial populations are in a tightly humidity-controlled environment and on a semi solid surface with agar content between 0.3% and 1% (6, 28–30). Additionally, a lack of specific nutrient is required to promote outward growth and expansion (31, 32). Since *P. aeruginosa* swarms only happens under distinct environmental conditions, it is unclear which types of habitats is best suited for this type of behavior. *P. aeruginosa* is generally found in soil, plants, and water and can form biofilms to sustain harsh environments (33–35). Although studies have shown that swarming often precedes biofilm formations, it is unclear if swarming specifically happens in these environments (36–39). The conditions for *P. aeruginosa* to swarm require a soft surface in a high humidity environment with specific nutrient needs and only a few habitats such as the lungs meet the requirements for enabling swarming behavior (40). *P. aeruginosa* is commonly found in the lungs and many studies suggest that swarming could occur on mucosal lung tissues similarly to soft agar surfaces (40–42). The lungs offer a relatively soft surface with a moist environment that could promote the swarming behavior in *P. aeruginosa* during infection (40). However, it is important to note that studies have not shown clear swarming pattern formation on lung tissue models. Most observations were made on soft agar plates that mimicked lung conditions (5). Therefore, determining swarming behavior in the lung environment remains a challenge.

Although researchers have mainly observed *P. aeruginosa* swarming in artificial environments such as soft agar surfaces with high humidity conditions and specific nutrients content, the swarming motility remains relevant to study. This species puts extensive energy and resources into developing this form of behavior which likely promotes increased survivability in

certain habitats (43). This suggests that it is critical for the bacterial species to swarm. Additionally, swarming often precedes biofilms formation which is a leading cause of infections in hospital settings (44, 45). By understanding swarming behavior in *P. aeruginosa*, we can expand our knowledge on therapeutic options to help us combat these infections.

7.4 The Interaction of *P. aeruginosa* Swarms

Additionally, *P. aeruginosa* swarming has been observed and studied primarily in the laboratory under controlled conditions as a single species. However, in their natural environment, *P. aeruginosa* coexists with a multitude of microorganisms that likely interact with this bacterial population (46–49). For example, *S. aureus* is a bacterial species that frequently interacts with *P. aeruginosa* as they are both commonly found in similar environment (46). These interactions can impact swarming behavior by disrupting the rhamnolipids layer or by directly preventing *P. aeruginosa* growth (50). Therefore, understanding the interaction between *P. aeruginosa* swarming population and various bacterial species is essential to determine the relevance of the swarming on bacterial growth and pathogenesis.

7.5 Closing the Knowledge Gap of Swarming

Swarming is a common bacterial behavior that remains unresolved, and many questions continue to be raised when studying this motility. Although this review focused on *P. aeruginosa* swarming, this behavior is also observed in other bacterial species such as *Escherichia*, *Proteus*, *Salmonella*, *Serratia*, or *Vibrio* (51–54)**.** Due to the natural differences between gram-positive and gram-negative bacteria, it is difficult to have a common definition of swarming that encompasses all the bacteria. Even by focusing on *P. aeruginosa* swarming, it remains a challenge to understand the role of flagella, surface sensing, surface condition, and rhamnolipids layer. It is therefore evident that additional studies need to be done on swarming to understand this bacterial behavior and its importance on pathogenesis.

7.6 References

1. Henrichsen J. 1972. Bacterial surface translocation: a survey and a classification. Bacteriol Rev 36:478–503.

2. Kearns DB. 2010. A field guide to bacterial swarming motility. Nat Rev Microbiol 8:634–644.

3. Murray TS, Kazmierczak BI. 2008. Pseudomonas aeruginosa exhibits sliding motility in the absence of type IV pili and flagella. J Bacteriol 190:2700–2708.

4. Suzuki T, Iino T. 1980. Isolation and characterization of multiflagellate mutants of Pseudomonas aeruginosa. J Bacteriol 143:1471–1479.

5. Köhler T, Curty LK, Barja F, van Delden C, Pechère J-C. 2000. Swarming of *Pseudomonas aeruginosa* Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. J Bacteriol 182:5990–5996.

6. Rashid MH, Kornberg A. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97:4885–4890.

7. Anyan ME, Amiri A, Harvey CW, Tierra G, Morales-Soto N, Driscoll CM, Alber MS, Shrout JD. 2014. Type IV pili interactions promote intercellular association and moderate swarming of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 111:18013–18018.

8. Yang A, Tang WS, Si T, Tang JX. 2017. Influence of Physical Effects on the Swarming Motility of Pseudomonas aeruginosa. Biophys J 112:1462–1471.

9. Madukoma CS, Liang P, Dimkovikj A, Chen J, Lee SW, Chen DZ, Shrout JD. 2019. Single Cells Exhibit Differing Behavioral Phases during Early Stages of Pseudomonas aeruginosa Swarming. J Bacteriol 201:e00184-19.

10. Harshey RM, Matsuyama T. 1994. Dimorphic transition in Escherichia coli and Salmonella typhimurium: surface-induced differentiation into hyperflagellate swarmer cells. Proc Natl Acad Sci U S A 91:8631–8635.

11. Kearns DB. 2013. You get what you select for: better swarming through more flagella. Trends Microbiol 21:508–509.

12. van Ditmarsch D, Boyle KE, Sakhtah H, Oyler JE, Nadell CD, Déziel É, Dietrich LEP, Xavier JB. 2013. Convergent evolution of hyperswarming leads to impaired biofilm formation in pathogenic bacteria. Cell Rep 4:697–708.

13. Cisneros L, Dombrowski C, Goldstein RE, Kessler JO. 2006. Reversal of bacterial locomotion at an obstacle. Phys Rev E Stat Nonlin Soft Matter Phys 73:030901.

14. Wu Y, Kaiser AD, Jiang Y, Alber MS. 2009. Periodic reversal of direction allows Myxobacteria to swarm. Proc Natl Acad Sci U S A 106:1222–1227.

15. Männik J, Driessen R, Galajda P, Keymer JE, Dekker C. 2009. Bacterial growth and motility in sub-micron constrictions. Proc Natl Acad Sci U S A 106:14861–14866.

16. Turner L, Zhang R, Darnton NC, Berg HC. 2010. Visualization of Flagella during bacterial Swarming. J Bacteriol 192:3259–3267.

17. Wu Z, He R, Zhang R, Yuan J. 2020. Swarming Motility Without Flagellar Motor Switching by Reversal of Swimming Direction in E. coli. Front Microbiol 11:1042.

18. Wang Q, Suzuki A, Mariconda S, Porwollik S, Harshey RM. 2005. Sensing wetness: a new role for the bacterial flagellum. EMBO J 24:2034–2042.

19. Mariconda S, Wang Q, Harshey RM. 2006. A mechanical role for the chemotaxis system in swarming motility. Mol Microbiol 60:1590–1602.

20. Toguchi A, Siano M, Burkart M, Harshey RM. 2000. Genetics of swarming motility in Salmonella enterica serovar typhimurium: critical role for lipopolysaccharide. J Bacteriol 182:6308–6321.

21. Partridge JD, Harshey RM. 2013. Swarming: flexible roaming plans. J Bacteriol 195:909–918.

22. Merritt JH, Brothers KM, Kuchma SL, O'Toole GA. 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J Bacteriol 189:8154–8164.

23. Caiazza NC, Merritt JH, Brothers KM, O'Toole GA. 2007. Inverse regulation of biofilm formation and swarming motility by Pseudomonas aeruginosa PA14. J Bacteriol 189:3603–3612.

24. Caiazza NC, O'Toole GA. 2004. SadB is required for the transition from reversible to irreversible attachment during biofilm formation by Pseudomonas aeruginosa PA14. J Bacteriol 186:4476–4485.

25. Kuchma SL, Delalez NJ, Filkins LM, Snavely EA, Armitage JP, O'Toole GA. 2015. Cyclic Di-GMP-Mediated Repression of Swarming Motility by Pseudomonas aeruginosa PA14 Requires the MotAB Stator. Journal of Bacteriology 197:420–430.

26. Ping L, Wu Y, Hosu BG, Tang JX, Berg HC. 2014. Osmotic pressure in a bacterial swarm. Biophys J 107:871–878.

27. Srinivasan S, Kaplan CN, Mahadevan L. 2019. A multiphase theory for spreading microbial swarms and films. Elife 8:e42697.

28. Tremblay J, Déziel E. 2008. Improving the reproducibility of Pseudomonas aeruginosa swarming motility assays. J Basic Microbiol 48:509–515.

29. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. 2006. The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. Mol Microbiol 62:1264–1277.

30. Kohler T, Curty LK, Barja F, van Delden C, Pechere J-C. 2000. Swarming of Pseudomonas aeruginosa Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. Journal of Bacteriology 182:5990–5996.

31. Kollaran AM, Joge S, Kotian HS, Badal D, Prakash D, Mishra A, Varma M, Singh V. 2019. Context-Specific Requirement of Forty-Four Two-Component Loci in Pseudomonas aeruginosa Swarming. iScience 13:305–317.

32. Burkart M, Toguchi A, Harshey RM. 1998. The chemotaxis system, but not chemotaxis, is essential for swarming motility in Escherichia coli. Proc Natl Acad Sci U S A 95:2568–2573.

33. Kerr KG, Snelling AM. 2009. Pseudomonas aeruginosa: a formidable and ever-present adversary. J Hosp Infect 73:338–344.

34. Moradali MF, Ghods S, Rehm BHA. 2017. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front Cell Infect Microbiol 7:39.

35. Mulcahy LR, Isabella VM, Lewis K. 2014. Pseudomonas aeruginosa biofilms in disease. Microb Ecol 68:1–12.

36. Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J. 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol 16:496–506.

37. Grobas I, Bazzoli DG, Asally M. 2020. Biofilm and swarming emergent behaviours controlled through the aid of biophysical understanding and tools. Biochem Soc Trans 48:2903–2913.

38. Worlitzer VM, Jose A, Grinberg I, Bär M, Heidenreich S, Eldar A, Ariel G, Be'er A. 2022. Biophysical aspects underlying the swarm to biofilm transition. Sci Adv 8:eabn8152.

39. Murray TS, Ledizet M, Kazmierczak BI. 2010. Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of Pseudomonas aeruginosa clinical isolates. J Med Microbiol 59:511–520.

40. Hutchison ML, Govan JR. 1999. Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect 1:1005–1014.

41. Hancock REW, Speert DP. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat 3:247–255.

42. Rajan S, Saiman L. 2002. Pulmonary infections in patients with cystic fibrosis. Semin Respir Infect 17:47–56.

43. Tremblay J, Déziel E. 2010. Gene expression in Pseudomonas aeruginosa swarming motility. BMC Genomics 11:587.

44. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, Ali M, Rafiq M, Kamil MA. 2018. Bacterial biofilm and associated infections. J Chin Med Assoc 81:7–11.

45. Percival SL, Suleman L, Vuotto C, Donelli G. 2015. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. J Med Microbiol 64:323–334.

46. Alves PM, Al-Badi E, Withycombe C, Jones PM, Purdy KJ, Maddocks SE. 2018. Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. Pathog Dis 76.

47. Woods PW, Haynes ZM, Mina EG, Marques CNH. 2018. Maintenance of S. aureus in Co-culture With P. aeruginosa While Growing as Biofilms. Front Microbiol 9:3291.

48. Monteiro R, Magalhães AP, Pereira MO, Sousa AM. 2021. Long-term coexistence of Pseudomonas aeruginosa and Staphylococcus aureus using an in vitro cystic fibrosis model. Future Microbiol 16:879–893.

49. Limoli DH, Warren EA, Yarrington KD, Donegan NP, Cheung AL, O'Toole GA. 2019. Interspecies interactions induce exploratory motility in Pseudomonas aeruginosa. Elife 8:e47365.

50. Bru J-L, Rawson B, Trinh C, Whiteson K, Høyland-Kroghsbo NM, Siryaporn A. 2019. PQS Produced by the Pseudomonas aeruginosa Stress Response Repels Swarms Away from Bacteriophage and Antibiotics. J Bacteriol 201:e00383-19.

51. Swiecicki J-M, Sliusarenko O, Weibel DB. 2013. From swimming to swarming: Escherichia coli cell motility in two-dimensions. Integr Biol (Camb) 5:1490–1494.

52. Belas R, Schneider R, Melch M. 1998. Characterization of Proteus mirabilis precocious swarming mutants: identification of rsbA, encoding a regulator of swarming behavior. J Bacteriol 180:6126–6139.

53. Kim W, Surette MG. 2005. Prevalence of surface swarming behavior in Salmonella. J Bacteriol 187:6580–6583.

54. Jaques S, McCarter LL. 2006. Three new regulators of swarming in Vibrio parahaemolyticus. J Bacteriol 188:2625–2635.