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14. ABSTRACT

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as of 09-Aug-2023

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Proposal Number: 72532BBREP

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Report Date: 30-Aug-2023

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Final Report for Period Beginning 01-Oct-2018 and Ending 30-May-2023

Title: Using Periodic Spatial Disturbance to Manipulate Cooperation in Bacteria

Begin Performance Period: 01-Oct-2018

End Performance Period: 30-May-2023

Report Term: 0-Other

Submitted By: Ph.D. Robert Smith

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Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees: 17

STEM Participants: 20

Major Goals: The overarching goal of the proposed work was to examine how periodic disturbance to spatial structure of bacterial populations affects cell-cell communication mediated by the exchange of small molecules. Our central hypothesis is that intermediate frequencies of periodic spatial disturbance will alter cooperative cell-cell interactions mediated by the exchange of small molecules. To address this central hypothesis, we proposed two Aims:

Aim 1: Determine how periodic spatial disturbance affects cooperation in *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The objective of Aim 1 was to develop an understanding of how periodic spatial disturbance affects cooperation in biofilms composed of either *P. aeruginosa* or *S. aureus*. We proposed to accomplish this by using a combination of experiments, mathematical modeling using both ordinary differential equations and network logic modeling.

Aim 2: : Investigate how periodic spatial disturbance affects interspecies interactions in a mixed species biofilm. The objective of Aim 2 was to understand how periodic spatial disturbance affects the interactions between *S. aureus* and *P. aeruginosa*. We proposed to accomplish this using experiments and agent based modeling.

Accomplishments: Finding 1: Periodically disturbing biofilms of *P. aeruginosa* can alter pyoverdine synthesis. Using experimentation (microplate reader assays, RT-qPCR) and mathematical modeling (ordinary differential equations), we found that the ability of periodic disturbance to affect pyoverdine synthesis is dependent upon biofilm density and pyoverdine synthesis rate. This was tested in over 20 strains of *P. aeruginosa*, the majority of which were isolated from individuals in the military who had infections (acquired from BEI Resources, originally isolated at Walter Reed). For some these strains, periodic disturbance at an intermediate frequency could decrease the expression of pyoverdine, which would make an infection less virulent. These findings are detailed in Quinn et.

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al. (2021), mSystems.

Finding 2: *P. aeruginosa* does not evolve resistance to the pyoverdine reducing effect of periodic disturbance over 250 generations. We evolved *P. aeruginosa* strain PA14 over 14 days (or > 250 generations) in TBS medium containing six different carbon sources and either in an undisturbed control condition (0/hr) or at a disturbance frequency of 6/hr. We chose different carbon sources so that our findings were relevant over multiple different growth environments. We measured the amount of pyoverdine produced every 24 hours whereupon we bottlenecked the population by transferring the biofilms to fresh medium. After 14 days, we harvested the cells and placed them at -80°C. We then tested the resulting population for their ability to form biofilms and the ability of disturbance to reduced pyoverdine production. In both the undisturbed and disturbed conditions, we found that *P. aeruginosa* evolved to form biofilms that were more dense as compared to their unevolved counterpart; this likely reflects adaptation to the presence of each carbon source. Interestingly, we found that bacteria evolved in either the undisturbed or disturbed conditions remained susceptibility to disturbance. There was significantly less pyoverdine produced when evolved strains were disturbed at 6/hr as compared to evolved strains grown in the undisturbed control condition. These experiments provide support for one hypothesis proposed in our original proposal; that because disturbance will target multiple aspects of bacterial physiology, it will be challenging for bacteria to adapt to any reductions in virulence factor production during disturbance. This work is important as it suggests that disturbing the spatial structure of biofilms may represent a sustainable strategy in the military clinic to reduce the virulence and severity of infections, thus increasing healing time.

Finding 3: Periodically disturbing biofilms of *P. aeruginosa* can reduce expression of multiple quorum sensing regulated virulence. Using experimentation (RT-qPCR) and mathematical modeling (network logic modeling), we found that disturbing biofilms composed of *P. aeruginosa* can reduce virulence factor expression in the *las*, *rhl*, and *pqs* quorum sensing networks. However, this is both disturbance frequency and amplitude dependent. At a disturbance amplitude of 0.3 mm, only disturbance at a frequency of 12 disturbance events/hr reduced expression of virulence factors. However, the magnitude of this reduction could be increased by decreasing amplitude (0.2 mm) and increasing frequency (15/hr). Our network logic modeling suggests that perturbing activity the *pqsR* receptor is responsible for this reduction; this prediction is supported by mutagenesis analysis that revealed that removing *pqsR* replicates the effects of disturbance. Interestingly, removing the *lasR* receptor results in a significant increase in the fold reduction in the expression of multiple virulence factors as a result of disturbance. This suggests that *lasR*, or a node that interactions with *lasR*, buffers the system against disturbance. This opens the door to explore interactions between physical sensors and those that interpret chemical signals (i.e., quorum sensing) in *P. aeruginosa*. These findings are published in García-Diéguez et. al. (2023) iScience.

Finding 4: Periodic disturbance can reduce expression of some, but not all, quorum sensing regulated virulence factors in *S. aureus* in a growth environment dependent manner. Using a combination of experimentation (RT-PCR) and mathematical modeling (ordinary differential equations), we found that interactions between the ability to sense AIP and bacterial metabolism determine the effect of periodic disturbance on the expression of virulence factors. In nutrient poor environment, periodically disturbing biofilms can enhance the expression of quorum sensing regulated virulence factors. This is owing to increase access to AIP (as measured through the expression of *agrC*) and a reduction in cellular metabolism (as measured through the expression of *saeR*). Owing to the architecture of the quorum sensing network in *S. aureus*, increased access to AIP increases the expression of exotoxins and proteases whereas decreased metabolism augments expression of surface attachment proteins. In nutrient rich environments, periodic disturbance can reduce the expression of virulence factors in a disturbance amplitude dependent manner. At a disturbance amplitude of 0.3 mm, a reduction in the expression of surface attachment was observed concurrent with an increase in bacterial metabolism. Only a modest increase in access to AIP resulted in insignificant changes to the expression of exotoxins and proteases. Increasing or decreasing disturbance amplitude increase the expression of exotoxins and proteases. This research demonstrates that differences in the growth environment may impact the effect of disturbance on the expression of quorum sensing regulated virulence factors. These findings are detailed in Barraza et al., (2023) Applied and Environmental Microbiology.

Finding 5: Combinations of disturbance, growth and metabolism can determine the population composition of a community composed of *P. aeruginosa* and *S. aureus*. Key to determining the population composition are virulence factors produced by *P. aeruginosa* that reduce growth/metabolism in *S. aureus*. Using a combination of experimentation, mathematical modeling (ordinary differential equation) and agent based modeling, we found that in an undisturbed or continually disturbed environment, the ratio of bacterial ATP/growth rate determines population composition in multiple growth environments, including those designed to mimic the human growth environment. If this ratio of ATP/growth between *P. aeruginosa* and *S. aureus* is sufficiently high, *P. aeruginosa* will dominate the

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co-culture. Otherwise, low ratios favor *S. aureus* allowing it to outcompete *P. aeruginosa*. Both modeling and mutagenesis analysis suggest that this is due to the production of HQNO, which reduces growth and metabolism in *S. aureus*. If metabolism and growth in *S. aureus* is sufficiently high owing to the composition of the growth environment, *S. aureus* can buffer against the effects of HQNO. The ratio of ATP/growth rate also determines population tipping points in an undisturbed or continually disturbed environment. Thus, for a given growth environment and initial population composition, the final population composition can be accurately predicted. Finally, we found that periodically disturbing the population disrupts the relationship between ATP/growth rate and population composition (Figure 5). Here, *S. aureus* dominates the culture across a range of growth environments. Agent based modeling suggests that this is because periodically disturbing the population disrupts the spatial positioning of *S. aureus* and HQNO produced by *P. aeruginosa*; this allows *S. aureus* to effectively sense less HNQO allowing it to outcompete *P. aeruginosa*. Our results reveal a simple metric that may be able to predict population composition in additional microbial consortia. These findings are detailed in Pajon et al (2023) eLife.

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Training Opportunities: Across the entire funding period, we trained 20 different students. Each student was involved in experimentation and/or modeling, and writing of manuscripts and/or theses. Below we list all students involved in the work, their specific accomplishments, and their current career goals.

Graduate students (8 total)

Rebecca Quinn

- Graduated from Masters in Biological Science Program in 2020
- One first author publication in mSystems
- Thesis entitled: Disruption of Pyoverdine Synthesis in *Pseudomonas Aeruginosa* Through Periodic Disturbance of Biofilm Structure
- Co-authored publication in International Review of Cell and Molecular Biology
- 3 presentations including an oral poster presentation at ASM Microbe
- Currently a Research Associate at Pfizer

Ivana Barraza

- Graduated from Masters in Biological Science Program in 2021
- One first author publication in Applied and Environmental Microbiology
- Co-authored publications in mSystems, International Review of Cell and Molecular Biology, and iScience
- Thesis entitled: Increasing the Frequency of Periodic Spatial Disturbance Decreases Surface Attachment Protein Expression in *Staphylococcus Aureus*
- Five presentations at conferences
- 2nd place oral presentation at NSU Research Jamboree
- Currently a PhD Candidate at Duke University
- Has already won multiple awards as a PhD candidate

Laura García-Diéguez

- Graduated from Masters in Biological Science Program in 2022
- One first author publication in iScience
- Co-authored publications in mSystems and Applied and Environmental Microbiology
- 3 conference presentations
- Thesis entitled: Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in *Pseudomonas Aeruginosa*
- Currently a Research Specialist at TCXpress and the University of Pittsburgh

Gabriela Diaz-Tang

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publications in iScience, Applied and Environmental Microbiology and eLife
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Beginning PhD program at Duke University in Fall 2023
- Won prestigious/highly competitive fellowship at Duke University

Estefania Marin Meneses

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publications in iScience, Applied and Environmental Microbiology and eLife
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Currently applying for MD/PhD programs

Ariane Kalifa

- Currently enrolled in the Masters of Biological Sciences Program
- Co-authored publication in eLife
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Plan on pursuing PhD program in infectious disease

Elinor Sevy

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publication in eLife
- Received Future Leaders Mentoring Program award from the American Society of Microbiology

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- Starting as a Systems Analyst at Duke University in September 2023

Fatima Abu Rumman

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publication in Applied and Environmental Microbiology
- One publication in preparation
- Thesis entitled: The Growth Environment Affect Virulence Factor Expression and Biofilm Formation in *Pseudomonas aeruginosa* in an Unstable Environment
- Two conference presentations
- Currently a grant officer in the Office of Sponsored programs at NSU. Pursuing a PhD in the future.

Undergraduate students (12 students)

Camryn Pajon

- Graduated from the Bachelor of Science, Biology Program in 2022
- One first author publication in eLife
- Co-authored publications in Applied and Environmental Microbiology and mSystems
- Five conference presentations
- Multiple awards including travel grants (ASM Microbe), attendance grants (ASM/FESEB Joint Conference), 1st place presentation at FLASM, 1st place presentation at NSU Research Jamboree
- Currently a PhD candidate at the University of North Carolina, Chapel Hill

Marla Fortoul

- Graduated from the Bachelor of Science, Biology Program in 2020
- One first author publication in eLife
- One co-authored publication in International Review of Cell and Molecular Biology
- Two conference presentations
- Currently a third year medical student at NSU MD

Brandon Toscan

- Graduated from the Bachelor of Science, Biology Program in 2022
- Completed an Honor's in Major Project with the research
- One co-authored publication in eLife.
- Applying to PhD programs.

Taniya Mariah

- Graduated from the Bachelor of Science, Biology Program in 2021
- Completed an Honor's in Major Project with the research
- One co-authored publication in eLife.
- Currently attending medical school

Maili Marcelin

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in eLife.
- Currently applying to podiatry school

Daniella Hernandez

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in eLife.
- Currently applying to medical school and working in the lab with NIH support

Melissa Marzouk

- Currently attending the Bachelor of Science, Biology Program
- One co-authored publication in eLife.
- Currently applying to medical school and working in the lab with NIH support

Kerollos Ibrahim

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in mSystems

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- Currently attending medical school at Florida State University

Laura Enzinna

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in mSystems
- Currently attending medical school at the Idaho College of Osteopathic Medicine

Morgan Thorn

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in mSystems
- Currently attending medical school at the Alabama College of Osteopathic Medicine

Vanessa Cruise

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in iScience.
- Currently applying to medical school

Marina Handel

- Graduated from the Bachelor of Science, Biology Program in 2020
- Currently attending medical school at NSU (Osteopathic Medicine)

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Results Dissemination: We published the follow papers during the final reporting period.

Barraza, I., Pajon, C., Diaz-Tang, G., Marin Meneses, E., Abu-Rumman, F., García-Diéguez, L., Castro, V., Lopatkin, A.J. and Smith, R.P., 2023. Disturbing the spatial organization of biofilm communities affects expression of agr-regulated virulence factors in *Staphylococcus aureus*. *Applied and environmental microbiology*, 89(2), pp. e01932-22.

García-Diéguez, L., Diaz-Tang, G., Meneses, E.M., Cruise, V., Barraza, I., Craddock, T.J. and Smith, R.P., 2023. Periodically disturbing biofilms reduces expression of quorum sensing-regulated virulence factors in *Pseudomonas aeruginosa*. *iScience*, 26(6).

Pajon, C., Fortoul, M.C., Diaz-Tang, G., Meneses, E.M., Kalifa, A.R., Sevy, E., Mariah, T., Toscan, B., Marcelin, M., Hernandez, D.M. and Marzouk, M.M., Lopatkin, A.J., Eldakar, O.T., Smith, R.P. 2023. Interactions between metabolism and growth can determine the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Elife*, 12, p.e83664.

The following manuscripts were previously published with the entire funding period:

Smith, R.P., Barraza, I., Quinn, R.J. and Fortoul, M.C., 2020. The mechanisms and cell signaling pathways of programmed cell death in the bacterial world. *International Review of Cell and Molecular Biology*, 352, pp.1-53.

Quinn, R.J., Barraza, I., García-Diéguez, L., Pajon, C., Krausfeldt, L.E., Ibrahim, K., Enzinna, L.A., Thorn, M.E., Eldakar, O.T., Craddock, T.J. and Smith, R.P., 2021. Periodically disturbing the spatial structure of biofilms can affect the production of an essential virulence factor in *Pseudomonas aeruginosa*. *Msystems*, 6(5), pp.e00961-21.

Conference presentations for the entire funding period:

Presentations associated with Aim 1

1. Barraza, I., Cruise, V., Smith, R.P. (2019). Periodic Spatial Disturbance Alters the Expression of Virulence Factors in *Staphylococcus aureus*. *ASM Microbe*, San Francisco
2. Quinn, R., Fortoul, M.C., Smith, R.P. (2019) Manipulation of Quorum Sensing Genes and Virulence Factors Using Spatial Disturbance in *Pseudomonas aeruginosa*. (2019) *ASM Microbe*, San Francisco
3. Barraza, I., Cruise, V., Smith, R.P. (2020). Periodic Spatial Disturbance Alters the Expression of Virulence Factors in *Staphylococcus aureus*. *NSU Research Jamboree*. Ft. Lauderdale, FL
4. Quinn, R., Fortoul, M.C., Smith, R.P. (2020) Manipulation of Quorum Sensing Genes and Virulence Factors Using Spatial Disturbance in *Pseudomonas aeruginosa*. *NSU Research Jamboree*. Ft. Lauderdale, FL
5. Barraza, I., and Smith, R.P. (2020) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. *ASM Microbe*. Abstract accepted by cancelled due to COVID-19 pandemic.
6. Quinn, R., and Smith, R.P. (2020) The Impact of Physical Disturbance and Biofilm Robustness on Cooperative Pyoverdine Production in *Pseudomonas aeruginosa*. *ASM Microbe*. Abstract accepted but cancelled due to COVID-19 pandemic.
7. Barraza, I., Smith, R.P. (2021) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. *FLASM/SEB ASM joint meeting*. Online.
8. García-Diéguez, L., Smith, R.P. (2021). Periodic spatial disturbance of biofilms modulates expression of quorum sensing virulence genes in *Pseudomonas aeruginosa*. *FLASM/SEB ASM joint meeting*. Online.
9. Barraza, I., Smith, R.P. (2021) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. *Congress Joint American Society of Microbiology and the Federation for European Microbiological Society*. Online.
10. García-Diéguez, L., Smith, R.P. (2021). Disrupting the Spatial Structure of Biofilms Modulates the Expression of Quorum Sensing Virulence Genes in *Pseudomonas aeruginosa*. *Congress Joint American Society of Microbiology and the Federation for European Microbiological Society*. Online.
11. García-Diéguez, L., Smith, R.P. (2022) Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in *Pseudomonas aeruginosa*. *HCAS Ocean Science Research Symposium*, Ft. Lauderdale, FL.
12. García-Diéguez, L., Smith, R.P. (2022). Changes in Frequency and Amplitude of Spatial Disturbances Modulate the Expression of Quorum Sensing-Mediated Virulence Effectors in *Pseudomonas aeruginosa*. *ASM Florida Branch Meeting*. St. Augustine, FL.

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13. Abu-Rumman, F. Smith, R.P. (2022) How Does Growth Environment Affect the Stability of Virulence Factor Expression in Pseudomonas Aeruginosa Biofilms in an Unstable Environment. ASM Microbe. Washington, DC
14. García-Diéguez, L., Smith, R.P. (2022). Periodic Disturbances Modulate Expression of Quorum Sensing-Mediated Virulence Effectors in Pseudomonas aeruginosa. ASM Microbe

Presentations associated with Aim 2

1. Fortoul, M.C., Eldakar, O.T., Smith, R.P. (2020) Periodically disturbing the spatial structure of a microbial community composed of Pseudomonas aeruginosa and Staphylococcus aureus determines its composition. ASM Microbe. Abstract accepted by cancelled due to COVID-19 pandemic.
2. Pajon, C., Smith, R.P. (2021) Using periodic spatial disturbance to manipulate cooperation in bacteria. Congress Joint American Society of Microbiology and the Federation for European Microbiological Society. Online.
3. Pajon, C., Mariah, T., Patel, V., Smith, R.P. (2021) (Periodically disturbing the spatial structure of a microbial community composed of Pseudomonas aeruginosa and Staphylococcus aureus determines its composition) Undergraduate student symposium Ft. Lauderdale, FL.
4. Pajon, C., Smith, R.P. (2021) (Carbon source driven metabolic buffering determines the co-existence of Staphylococcus aureus and Pseudomonas aeruginosa.) FLASM/SEB ASM joint meeting. Online.
5. Pajon, C., Smith, R.P. (2022) Carbon source driven metabolic buffering determines the co-existence of Staphylococcus aureus and Pseudomonas aeruginosa. ASM Florida Branch Meeting. St. Augustine, FL.
6. Pajon, C., Smith, R.P. (2022) Carbon source driven metabolic buffering determines the co-existence of Staphylococcus aureus and Pseudomonas aeruginosa. NSU Ocean Science Research Symposium. Ft. Lauderdale, FL.
7. Smith et al. (2023) Interactions between metabolism and growth can determine the co-existence of Staphylococcus aureus and Pseudomonas aeruginosa. GRC Microbial Populations. Andover, NH.

Honors and Awards: Awards for this reporting period:

Gabriela Diaz Tang, Elinor Sevy, Estefania Marin Meneses and Ariane Kalifa were awarded American Society of Microbiology Future Leader Mentoring Fellowships.

Gabriela Diaz-Tang was awarded a prestigious scholarship to attend her PhD at Duke University.

All award across the entire funding period:

Ivana Barraza won:

1. 2nd place oral presentation at the NSU Research Jamboree

Camryn Pajon won:

1. a travel grant to ASM Microbe,
2. an attendance grant to the ASM/FESEB joint conference,
3. a 1st place oral presentation at the Florida Branch American Society of Microbiology meeting, and
4. a 1st place oral presentation at the NSU Research Jamboree.

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI

Participant: Robert P Smith

Person Months Worked: 8.00

Project Contribution:

National Academy Member: N

Funding Support:

Participant Type: Co PD/PI

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Participant: Travis JA Craddock
Person Months Worked: 1.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Co PD/PI
Participant: Omar T Eldakar
Person Months Worked: 1.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Graduate Student (research assistant)
Participant: Gabriela Diaz-Tang
Person Months Worked: 8.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Graduate Student (research assistant)
Participant: Estefania Marin Menese
Person Months Worked: 4.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Undergraduate Student
Participant: Daniela Hernandez
Person Months Worked: 1.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Graduate Student (research assistant)
Participant: Ariane Kalifa
Person Months Worked: 1.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Graduate Student (research assistant)
Participant: Elinor Sevy
Person Months Worked: 1.00
Project Contribution:
National Academy Member: N

Funding Support:

Participant Type: Undergraduate Student
Participant: Melissa Marzouk
Person Months Worked: 1.00

Funding Support:

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Project Contribution:
National Academy Member: N

Participant Type: Undergraduate Student

Participant: Maili Marcelin

Person Months Worked: 1.00

Funding Support:

Project Contribution:

National Academy Member: N

ARTICLES:

Publication Type: Journal Article

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Publication Status: 1-Published

Journal: mSystems

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Volume:

Issue:

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Date Submitted: 9/28/21 12:00AM

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Publication Location:

Article Title: Periodically disturbing the spatial structure of biofilms can affect the production of an essential virulence factor.

Authors: Rebecca J. Quinn, Ivana Barraza, Laura García-Diéguez, Camryn Pajon, Lauren E. Krausfeldt, Kerollos

Keywords: Pseudomonas aeruginosa, virulence factor, pyoverdine, spatial disturbance, cooperation, mathematical model, physical force, fluctuations

Abstract: Understanding the environmental factors that affect the production of virulence factors has major implications in evolution and medicine. While spatial structure is important in virulence factor production, observations of this relationship have occurred in undisturbed or continuously disturbed environments. However, natural environments are subject to periodic fluctuations, including changes in physical forces, which could alter the structure of bacterial populations and impact virulence factor production. Using *Pseudomonas aeruginosa* PA14, we periodically applied a physical force to biofilms and examined production of pyoverdine. Intermediate frequencies of disturbance reduced the amount of pyoverdine produced compared to undisturbed or frequently disturbed conditions. To explore the generality of this finding, we examined how an intermediate disturbance frequency affected pyoverdine production in 21 different strains of *P. aeruginosa*.

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Journal: Applied and Environmental Science

Publication Identifier Type: Publication Identifier:

Volume: Issue: First Page #:

Date Submitted: 1/27/23 12:00AM Date Published:

Publication Location:

Article Title: Disturbing the Spatial Organization of Biofilm Communities Affects Expression of agr-Regulated Virulence Factors in *Staphylococcus aureus*

Authors: Ivana Barraza, Camryn Pajon, Gabriela Diaz-Tang, Estefania Marin Meneses, Fatima Abu-Rumman, La

Keywords: quorum sensing, physical disturbance, *Staphylococcus aureus*, virulence factors, pathogenesis, metabolism, autoinducer

Abstract: Cooperation can facilitate the expression of virulence factors in bacteria. While biofilm formation can increase cooperation, it also promotes competition. Accordingly, bacteria must balance the interplay between cooperation and competition. We show that perturbing the spatial structure of *Staphylococcus aureus* affects the expression of agr regulated virulence factors by altering the balance between cooperation and competition. In nutrient poor environments, disturbing spatial structure increased the expression of agr regulated virulence factors as a result of increased cooperation and competition. Conversely, disturbance into a nutrient rich environment enhanced cooperation, but not competition; this led to a decrease in some, but not all, agr regulated virulence factors. Interestingly, this trend was dependent upon the amplitude of the force leading to the disturbance.

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Journal: eLife

Publication Identifier Type: Publication Identifier:

Volume: Issue: First Page #:

Date Submitted: 5/23/23 12:00AM Date Published:

Publication Location:

Article Title: Interactions between metabolism and growth can determine the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Authors: Camryn Pajon, Marla C. Fortoul, Gabriela Diaz-Tang, Estefania Marin Meneses, Taniya Mariah, Brando

Keywords: spatial disturbance, metabolism, polymicrobial community, ATP, growth rate, co-culture

Abstract: Polymicrobial infections, including those cause by *Pseudomonas aeruginosa* and *Staphylococcus aureus*, constitute a significant source of morbidity and mortality in the clinic. When co-cultured, virulence factors secreted by *P. aeruginosa* reduce metabolism and growth in *S. aureus*; this augments virulence and antibiotic resistance. We found that changing the carbon source in growth medium affects the absolute growth, which we define as the ratio of ATP to growth rate. By manipulating absolute growth, we could predictably alter the final population composition. We also demonstrate that the relationship between absolute growth and final population composition can be perturbed by altering spatial structure in the community. Our results may offer a novel mechanism to rationally manipulate polymicrobial populations.

Distribution Statement: 2-Distribution Limited to U.S. Government agencies only; report contains proprietary info
Acknowledged Federal Support: Y

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Journal: iScience

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Publication Location:

Article Title: Periodically disturbing biofilms can reduce the expression of quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*.

Authors: Laura García-Diéguez, Gabriela Diaz-Tang, Estefania Marin Meneses, Vanessa Cruise, Ivana Barraza,

Keywords: physical disturbance, biofilms, spatial structure, quorum sensing, virulence factor, gene expression, network, autoinducer

Abstract: Quorum sensing regulates the production of many virulence factors in *P. aeruginosa*. We found that periodically disturbing biofilm communities of *P. aeruginosa* using a physical force can reduce the expression of multiple virulence factors that are regulated by quorum sensing. Our computational and experimental analyses suggest that disturbance primarily affects the activity of the pqsR receptor, which is implicated in regulating the production of ~ 60 small molecules and virulence factors that are essential in pathogenesis and in the recalcitrant nature of *P. aeruginosa* infections. Moreover, we found that removing the lasR receptor increases the magnitude of the reduction in the expression of virulence factors owing to disturbance. Together, our results highlight the effect disturbance on the expression of virulence factors in *P. aeruginosa*, identify a potential novel role for lasR, and may pave the way for approaches to reduce virulence of *P. aeruginosa* using physical forces.

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Title: Disruption of pyoverdine synthesis in *Pseudomonas aeruginosa* through periodic disturbance of biofilm structure.

Authors: Rebecca,Quinn

Acknowledged Federal Support: Y

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Title: Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*

Authors: Ivanna Barraza

Acknowledged Federal Support: Y

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Title: Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in *Pseudomonas aeruginosa*

Authors: LAURA GARCIA-DIEGUEZ

Acknowledged Federal Support: Y

RPPR Final Report
as of 09-Aug-2023

Partners

,

I certify that the information in the report is complete and accurate:

Signature: Robert P Smith

Signature Date: 8/4/23 9:35AM

FINAL REPORT

In the final year of our funding, we completed all of the work outlined in our original proposal. In this final technical report, we outline all of the major accomplishments across all funding years. Overall, we found that periodically disturbing the spatial structure of populations can reduce the expression of virulence factors in both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. We also found that such disturbances can alter the interactions between both of these pathogens when grown in co-culture; this impacts the community composition. Our findings have been shared with the community in five manuscripts with a six manuscript in preparation. In completing our work, we engaged 20 students many of whom won awards as outlined below. Importantly, all students are, or are planning on, continuing careers in science and/or medicine. Finally, members of the research team presented their research at multiple venues. Overall, we are excited to report that we met all of our goals as outlined in our original proposal.

SCIENTIFIC ACCOMPLISHMENTS

Aim 1: Determine how periodic spatial disturbance affects cooperation in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

The objective of Aim 1 was to develop an understanding of how periodic spatial disturbance affects cooperation in biofilms composed of either *P. aeruginosa* or *S. aureus*. We proposed to accomplish this by using a combination of experiments, mathematical modeling using both ordinary differential equations and network logic modeling.

Student participants

- a) Graduate students
 - a. Rebecca Quinn
 - b. Laura García-Diéguez
 - c. Ivana Barraza
 - d. Estefania Marin Meneses
 - e. Fatima Abu Rumman
- b) Undergraduate students
 - a. Camryn Pajon
 - b. Kerollos Ibrahim
 - c. Laura Enzinna
 - d. Morgan Thorn
 - e. Vanessa Cruise
 - f. Marina Handel
- c) Faculty and senior investigators
 - a. Travis Craddock
 - b. Omar Eldakar
 - c. Victor Castro
 - d. Lauren Krausfeldt (post doc)
 - e. Allison Lopatkin

Publications

Quinn, R.J., Barraza, I., García-Diéguez, L., Pajon, C., Krausfeldt, L.E., Ibrahim, K., Enzinna, L.A., Thorn, M.E., Eldakar, O.T., Craddock, T.J. and Smith, R.P., 2021. Periodically disturbing the spatial structure of biofilms can affect the production of an essential virulence factor in *Pseudomonas aeruginosa*. *Msystems*, 6(5), pp.e00961-21.

Barraza, I., Pajon, C., Diaz-Tang, G., Marin Meneses, E., Abu-Rumman, F., García-Diéguez, L., Castro, V., Lopatkin, A.J. and Smith, R.P., 2023. Disturbing the spatial organization of biofilm communities affects expression of agr-regulated virulence factors in *Staphylococcus aureus*. *Applied and environmental microbiology*, 89(2), pp.e01932-22.

García-Diéguez, L., Diaz-Tang, G., Meneses, E.M., Cruise, V., Barraza, I., Craddock, T.J. and Smith, R.P., 2023. Periodically disturbing biofilms reduces expression of quorum sensing-regulated virulence factors in *Pseudomonas aeruginosa*. *iScience*, 26(6).

Manuscripts in preparation

Abu-Rumman, F., Diaz-Tang, G., and Smith, R.P. Long term periodic spatial disturbance consistently reduces pyoverdine production in *Pseudomonas aeruginosa* biofilms.

Conference Presentations

1. Barraza, I., Cruise, V., Smith, R.P. (2019). Periodic Spatial Disturbance Alters the Expression of Virulence Factors in *Staphylococcus aureus*. ASM Microbe, San Francisco
2. Quinn, R., Fortoul, M.C., Smith, R.P. (2019) Manipulation of Quorum Sensing Genes and Virulence Factors Using Spatial Disturbance in *Pseudomonas aeruginosa*. (2019) ASM Microbe, San Francisco
3. Barraza, I., Cruise, V., Smith, R.P. (2020). Periodic Spatial Disturbance Alters the Expression of Virulence Factors in *Staphylococcus aureus*. NSU Research Jamboree. Ft. Lauderdale, FL
4. Quinn, R., Fortoul, M.C., Smith, R.P. (2020) Manipulation of Quorum Sensing Genes and Virulence Factors Using Spatial Disturbance in *Pseudomonas aeruginosa*. NSU Research Jamboree. Ft. Lauderdale, FL
5. Barraza, I., and Smith, R.P. (2020) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. ASM Microbe. Abstract accepted by cancelled due to COVID-19 pandemic.
6. Quinn, R., and Smith, R.P. (2020) The Impact of Physical Disturbance and Biofilm Robustness on Cooperative Pyoverdine Production in *Pseudomonas aeruginosa*. ASM Microbe. Abstract accepted but cancelled due to COVID-19 pandemic.
7. Barraza, I., Smith, R.P. (2021) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. FLASM/SEB ASM joint meeting. Online.
8. García-Diéguez, L., Smith, R.P. (2021). Periodic spatial disturbance of biofilms modulates expression of quorum sensing virulence genes in *Pseudomonas aeruginosa*. FLASM/SEB ASM joint meeting. Online.
9. Barraza, I., Smith, R.P. (2021) Increasing the frequency of periodic spatial disturbance decreases surface attachment protein expression in *Staphylococcus aureus*. Congress Joint American Society of Microbiology and the Federation for European Microbiological Society. Online.
10. García-Diéguez, L., Smith, R.P. (2021). Disrupting the Spatial Structure of Biofilms Modulates the Expression of Quorum Sensing Virulence Genes in *Pseudomonas aeruginosa*. Congress Joint American Society of Microbiology and the Federation for European Microbiological Society. Online.
11. García-Diéguez, L., Smith, R.P. (2022) Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in *Pseudomonas aeruginosa*. HCAS Ocean Science Research Symposium, Ft. Lauderdale, FL.
12. García-Diéguez, L., Smith, R.P. (2022). Changes in Frequency and Amplitude of Spatial Disturbances Modulate the Expression of Quorum Sensing-Mediated Virulence Effectors in *Pseudomonas aeruginosa*. ASM Florida Branch Meeting. St. Augustine, FL.

13. Abu-Rumman, F. Smith, R.P. (2022) How Does Growth Environment Affect the Stability of Virulence Factor Expression in *Pseudomonas Aeruginosa* Biofilms in an Unstable Environment. ASM Microbe. Washington, DC
14. García-Diéguez, L., Smith, R.P. (2022). Periodic Disturbances Modulate Expression of Quorum Sensing-Mediated Virulence Effectors in *Pseudomonas aeruginosa*.ASM Microbe

Major findings

Background: *Staphylococcus aureus* and *Pseudomonas aeruginosa* represent two of the most challenging ESKAPE pathogen to treat in the military clinic. Both have complex virulence factors regulatory systems that are largely governed by the cooperative behavior of quorum sensing. The production and secretion of small diffusible molecules or peptides called autoinducers allows each population to act as a collective. Once autoinducers reach a sufficiently high density, they are bound by receptors and alter the expression of virulence factors in both species. Since autoinducers are diffusible, bacteria must be at the right place and the right time to sense them. Decoupling the spatial position of both autoinducers and bacteria by periodically disturbing the spatial structure of these bacterial communities could reduce the expression of quorum sensing regulated virulence factors. However, prior to our work, this had yet to be investigated. Our hypothesis was: *intermediate periodic spatial disturbance will change the expression of virulence factors driven by small diffusible molecules*. To address this hypothesis, we proposed to use an approach consisting of microplate reader assays, gene expression assays and mathematical modeling.

P. aeruginosa

Periodically disturbing biofilms of *P. aeruginosa* can alter pyoverdine synthesis. Using experimentation (microplate reader assays, RT-qPCR) and mathematical modeling (ordinary differential equations), we found that the ability of periodic disturbance to affect pyoverdine synthesis is dependent upon biofilm density and pyoverdine synthesis rate. This was tested in over 20 strains of *P. aeruginosa*, the majority of which were isolated from individuals in the military who had infections (acquired from BEI Resources, originally isolated at Walter Reed, Figure 1). For some these strains, periodic disturbance at an intermediate frequency could decrease the expression of pyoverdine, which would make an infection less virulent. These findings are detailed in Quinn et. al. (2021), *mSystems*.

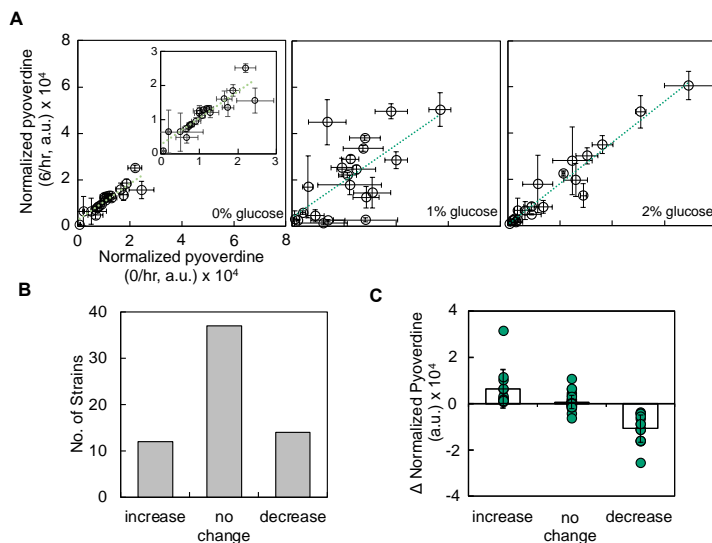


Figure 1: Strains of *P. aeruginosa* vary in their susceptibility to periodic disturbance at 6/hr.

A. The amount of normalized pyoverdine produced by 21 different strains of *P. aeruginosa* in the undisturbed (0/hr) and 6/hr conditions when the growth media contained 0% (left, inset – reduced axes), 1% (center) and 2% (right) glucose. Pyoverdine normalized by cell density (OD₆₀₀). Standard deviation from a minimum of six biological replicates. Linear line drawn as a guide; deviations from the line indicated differences in pyoverdine between the undisturbed and disturbed conditions.

B. Number of strains that had an increase, no change, or a decrease in the amount of normalized pyoverdine produced at 6/hr relative to 0/hr. Significance was assessed using a two tailed t-test with a Benjamini-Hochberg correction.

C. The change in normalized pyoverdine grouped by strains that had an increase, no change, or a decrease at 6/hr as compared to 0/hr. $P < 0.0001$ using a Wilcoxon/Kruskal-Wallis test ($P < 0.0001$, Shapiro-Wilk test). Image and captions from Quinn et al.

P. aeruginosa does not evolve resistance to the pyoverdine reducing effect of periodic disturbance over 250 generations. We evolved *P. aeruginosa* strain PA14 over 14 days (or > 250 generations) in TBS medium containing six different carbon sources and either in an undisturbed control condition (0/hr) or at a disturbance frequency of 6/hr. We chose different carbon sources so that our findings were relevant over multiple different growth environments. We measured the amount of pyoverdine produced every 24 hours whereupon we bottlenecked the population by transferring the biofilms to fresh medium (Figure 2A). After 14 days, we harvested the cells and placed them at -80°C. We then tested the resulting population for their ability to form biofilms and the ability of disturbance to reduced pyoverdine production. In both the undisturbed and disturbed conditions,

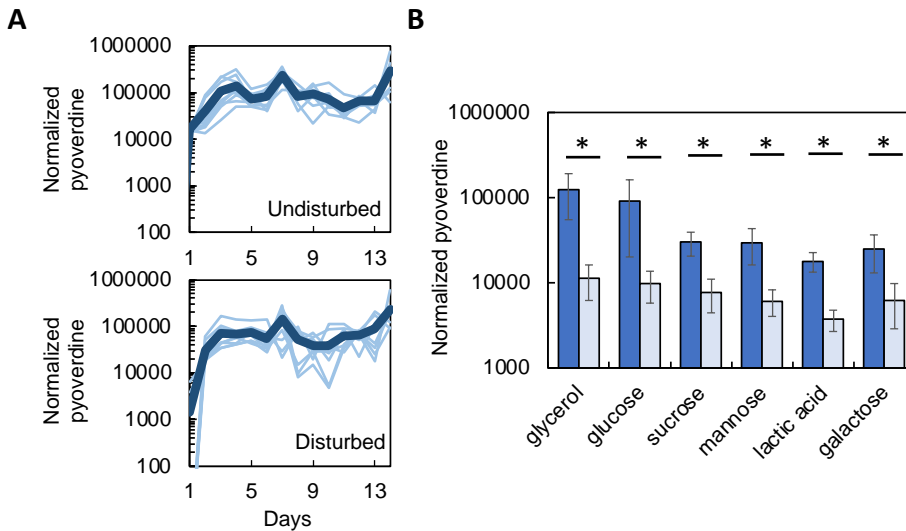


Figure 2: *P. aeruginosa* does not evolve resistance to spatial disturbance over > 250 generations. A) The amount of pyoverdine produced by evolving populations every 24 hours. Dark lines indicate population average (min 6 bio reps), lighter lines are individual population traces. In these panels, the bacteria were grown in TBS medium containing glycerol. B) The average amount of pyoverdine produced after 24 hours of growth in undisturbed (dark bars) or disturbed (6/hr, light bars). * indicates significant difference in the amount of pyoverdine as determined by a 2-way Wilcoxon ($P < 0.0004$). Error bars are standard deviation. In both panels, pyoverdine was measured using fluorescence in a microplate reader; this value was then normalized by cell density measured at OD600.

we found that *P. aeruginosa* evolved to form biofilms that were more dense as compared to their unevolved counterpart; this likely reflects adaptation to the presence of each carbon source. Interestingly, we found that bacteria evolved in either the undisturbed or disturbed conditions remained susceptible to disturbance. There was significantly less pyoverdine produced when evolved strains were disturbed at 6/hr as compared to evolved strains grown in the undisturbed control condition (Figure 2B). These experiments provide support for one hypothesis proposed in our original proposal; that because disturbance will target multiple aspects of bacterial physiology, it will be challenging for bacteria to adapt to any reductions in virulence factor production during disturbance. This work is important as it suggests that disturbing the spatial structure of biofilms may represent a sustainable strategy in the military clinic to reduce the virulence and severity of infections, thus increasing healing time.

Periodically disturbing biofilms of *P. aeruginosa* can reduce expression of multiple quorum sensing regulated virulence. Using experimentation (RT-qPCR) and mathematical modeling (network logic modeling), we found that disturbing biofilms composed of *P. aeruginosa* can reduce virulence factor expression in the *las*, *rhl*, and *pqs* quorum sensing networks. However, this is both disturbance frequency and amplitude dependent. At a disturbance amplitude of 0.3 mm, only

disturbance at a frequency of 12 disturbance events/hr reduced expression of virulence factors (Figure 3). However, the magnitude of this reduction could be increased by decreasing amplitude (0.2 mm) and increasing frequency (15/hr). Our network logic modeling suggests that perturbing activity the *pqsR* receptor is responsible for this reduction; this prediction is supported by mutagenesis analysis that revealed that removing *pqsR* replicates the effects of disturbance. Interestingly, removing the *lasR* receptor results in a significant increase in the fold reduction in the expression of multiple virulence factors as a result of disturbance. This suggests that *lasR*, or a node that interactions with *lasR*, buffers the system against disturbance. This opens the door to explore interactions between physical sensors and those that interpret chemical signals (i.e., quorum sensing) in *P. aeruginosa*. These findings are published in García-Diéguez et. al. (2023) *iScience*.

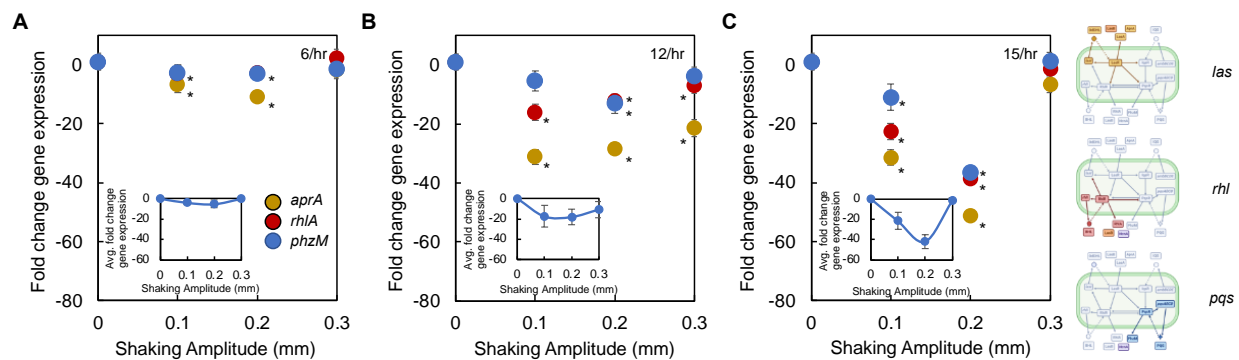


Figure 3: Frequency and amplitude dependent reductions in the expression of quorum sensing regulated virulence factors in *P. aeruginosa*.

A. The effect of disturbance amplitude (frequency = 6/hr) on the expression of virulence factors from the *las*, *rhl* and *pqs* quorum sensing systems. Expression of *aprA* ($P < 0.035$, two-tailed t-test, compared to 0/hr) and *rhIA* ($P < 0.044$, data points behind *phzM* at 0.1 mm and 0.2 mm) were significantly (*) reduced at amplitudes of 0.1 mm and 0.2 mm. In panels A-C, average and standard deviation from ≥ 4 bio. reps. Inset: Average fold change in expression for all virulence factors measured.

B. The effect of disturbance amplitude (frequency = 12/hr) on the expression of virulence factors from the *las*, *rhl* and *pqs* regulons. Expression of *aprA* ($P < 0.01$, Mann-Whitney (Shapiro-Wilk; $P = 0.0086$), compared to 0/hr) and *rhIA* ($P < 0.041$, two-tailed t-test) were significantly (*) reduced at all amplitudes tested. Expression of *phzM* was significantly reduced at an amplitude of 0.2 mm ($P = 0.014$, Mann-Whitney (Shapiro-Wilk; $P = 0.0009$)).

C. Left panel: The effect of disturbance amplitude (frequency = 15/hr) on the expression of virulence factors from the *las*, *rhl* and *pqs* regulons. Expression of *aprA* ($P < 0.002$, two-tailed t-test, compared to 0/hr), *rhIA* ($P < 0.007$, Mann-Whitney (Shapiro-Wilk; $P = 0.0305$) and *phzM* ($P < 0.012$, two-tailed t-test) was significantly (*) reduced at amplitudes of 0.1 mm and 0.2 mm. Right panel: Quorum sensing networks in *P. aeruginosa*. *las* regulon = yellow; *rhl* regulon = red; *pqs* regulon = blue. Caption and figure adapted from García-Diéguez et. al. (2023) *iScience*.

S. aureus

Periodic disturbance can reduce expression of some, but not all, quorum sensing regulated virulence factors in *S. aureus* in a growth environment dependent manner. Using a combination of experimentation (RT-PCR) and mathematical modeling (ordinary differential equations), we found that interactions between the ability to sense AIP and bacterial metabolism determine the effect of periodic disturbance on the expression of virulence factors. In nutrient poor environment, periodically disturbing biofilms can enhance the expression of quorum sensing regulated virulence factors (Figure 4). This is owing to increase access to AIP (as measured through the expression of *agrC*) and a reduction in cellular metabolism (as measured through the expression of *saeR*). Owing to the architecture of the quorum sensing network in *S. aureus*, increased access to AIP increases the expression of exotoxins and proteases whereas decreased metabolism augments

expression of surface attachment proteins. In nutrient rich environments, periodic disturbance can reduce the expression of virulence factors in a disturbance amplitude dependent manner. At a disturbance amplitude of 0.3 mm, a reduction in the expression of surface attachment was observed concurrent with an increase in bacterial metabolism. Only a modest increase in access to AIP resulted in insignificant changes to the expression of exotoxins and proteases. Increasing or decreasing disturbance amplitude increase the expression of exotoxins and proteases. This research demonstrates that differences in the growth environment may impact the effect of disturbance on the expression of quorum sensing regulated virulence factors. These findings are detailed in Barraza et al., (2023) *Applied and Environmental Microbiology*.

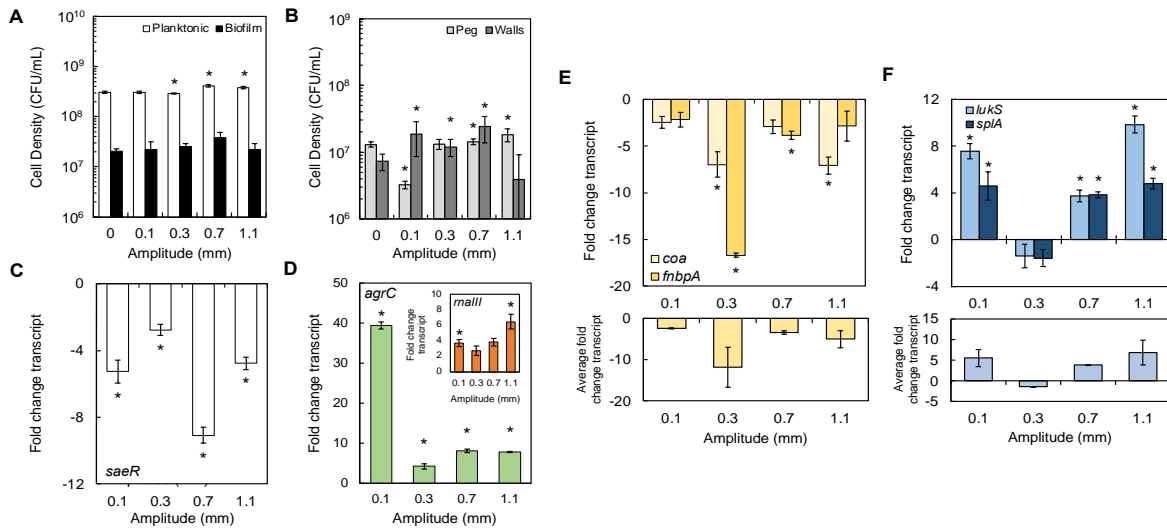


Figure 4: Amplitude-dependent effects of periodic disturbance (15/hr) on the expression of *agr* regulated virulence factors in a nutrient rich environment.

A) Density of bacteria in the biofilm and planktonic states following 24 hours of disturbance at the amplitude indicated. * = different from undisturbed control ($P < 0.036$, Wilcoxon ($P < 0.0001$, Shapiro-Wilk)). Standard deviation from > 7 biol. reps.

B) Density of bacteria in the biofilm state on either the peg (in the MBEC inoculator device) or the walls of the well. * = significant difference from undisturbed control ($P < 0.041$, Wilcoxon ($P < 0.0001$, Shapiro-Wilk)). Standard deviation from > 7 biol. reps.

C) Effects of increasing disturbance amplitude on the expression of *saeR*, which reports on bacterial metabolism (increasing *saeR* indicates decreasing metabolism). * = significantly different from undisturbed control ($P \leq 0.0066$, two-tailed t-test). Standard deviation from ≥ 3 biol. reps.

D) Effects of increasing shaking amplitude on the expression of *agrC*, which reports on access to the autoinducer AIP (increasing *agrC* indicates increased AIP sensed). * = significantly different from undisturbed control ($P \leq 0.0006$ two-tailed t-test). The inset shows the effects of disturbance amplitude on expression of *mallI*. Standard deviation from ≥ 3 biol. reps.

E) Top panel: Effects of increasing amplitude on the expression of the surface attachment proteins, *coa* and *fnbA*. * = significantly different from undisturbed control ($P \leq 0.0166$ two-tailed t-test). Bottom panel: Average fold change of genes encoding surface attachment proteins. Data from top panel. Standard deviation from ≥ 3 biol. reps.

F) Top panel: Effects of increasing disturbance amplitude (shaking frequency = 15/hr) on the expression of exotoxins/proteases *lukS* and *splA*. * = significantly different from undisturbed control ($P \leq 0.0375$, two-tailed t-test). Bottom panel: Average fold change of genes encoding exotoxins and proteases. Data from top panel. Standard deviation from ≥ 3 biol. reps. Figure and caption adapted from Barraza et al., 2023

Aim 2: : Investigate how periodic spatial disturbance affects interspecies interactions in a mixed species biofilm.

The objective of Aim 2 was to understand how periodic spatial disturbance affects the interactions between *S. aureus* and *P. aeruginosa*. We proposed to accomplish this using experiments and agent based modeling.

Student participants

- a) Graduate students
 - a. Rebecca Quinn
 - b. Ivana Barraza
 - c. Gabriela Diaz-Tang
 - d. Estefania Marin Meneses
 - e. Ariane Kalifa
 - f. Elinor Sevy
- b) Undergraduate students
 - a. Camryn Pajon
 - b. Marla Fortoul
 - c. Taniya Mariah
 - d. Brandon Toscan
 - e. Maili Marcelin
 - f. Daniella Hernandez
 - g. Melissa Marzouk
- c) Faculty
 - a. Omar Eldakar
 - b. Allison Lopatkin

Publications

Smith, R.P., Barraza, I., Quinn, R.J. and Fortoul, M.C., 2020. The mechanisms and cell signaling pathways of programmed cell death in the bacterial world. *International Review of Cell and Molecular Biology*, 352, pp.1-53.

Pajon, C., Fortoul, M.C., Diaz-Tang, G., Meneses, E.M., Kalifa, A.R., Sevy, E., Mariah, T., Toscan, B., Marcelin, M., Hernandez, D.M. and Marzouk, M.M., Lopatkin, A.J., Eldakar, O.T., Smith, R.P. 2023. Interactions between metabolism and growth can determine the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Elife*, 12, p.e83664.

Conference Presentations

1. Fortoul, M.C., Eldakar, O.T., Smith, R.P. (2020) Periodically disturbing the spatial structure of a microbial community composed of *Pseudomonas aeruginosa* and *Staphylococcus aureus* determines its composition. ASM Microbe. Abstract accepted by cancelled due to COVID-19 pandemic.

2. Pajon, C., Smith, R.P. (2021) Using periodic spatial disturbance to manipulate cooperation in bacteria. Congress Joint American Society of Microbiology and the Federation for European Microbiological Society. Online.
3. Pajon, C., Mariah, T., Patel, V., Smith, R.P. (2021) (Periodically disturbing the spatial structure of a microbial community composed of *Pseudomonas aeruginosa* and *Staphylococcus aureus* determines its composition) Undergraduate student symposium Ft. Lauderdale, FL.
4. Pajon, C., Smith, R.P. (2021) (Carbon source driven metabolic buffering determines the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.) FLASM/SEB ASM joint meeting. Online.
5. Pajon, C., **Smith, R.P.** (2022) Carbon source driven metabolic buffering determines the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. ASM Florida Branch Meeting. St. Augustine, FL.
6. Pajon, C., **Smith, R.P.** (2022) Carbon source driven metabolic buffering determines the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. NSU Ocean Science Research Symposium. Ft. Lauderdale, FL.
7. Smith et al. (2023) Interactions between metabolism and growth can determine the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. GRC Microbial Populations. Andover, NH.

Major findings

Background: Infections are often composed of a mixture of bacterial species that interact. These interactions can enhance virulence, prolong infection time and severity, and increase resistance to antibiotics. Two species of bacteria that are commonly found cohabiting chronic wounds are *P. aeruginosa* and *S. aureus*. Indeed, their presence in a multitude of different infection environments leads to worse patient outcomes. The interactions between these two species have been well-documented and involve diffusible molecules; N-acetylglucosamine shed from *S. aureus* results in increased expression of virulence factors in *P. aeruginosa*. Furthermore, the production of 2-HQNO and pyoverdine by *P. aeruginosa* reduced growth and metabolism in *S. aureus*, which enhances antibiotic resistance and alters population composition. Spatial structure is critical in facilitating these interactions as co-cultures grown in well-mixed states exhibit reduced virulence and antibiotic resistance; this is likely owing to the diffusible nature of the small molecules that mediate the interactions between *P. aeruginosa* and *S. aureus*.

Prior to our work, it had yet to be established how periodic spatial disturbance affects the interactions between *P. aeruginosa* and *S. aureus* when grown together. Our hypothesis was: *periodic spatial disturbance performed at intermediate frequencies will alter small molecule mediated cooperative and competitive interactions in a polymicrobial community*. To address this hypothesis, we proposed to use experimentation, agent based modeling, and mathematical modeling.

Significant findings:

We found that combinations of disturbance, growth and metabolism can determine the population composition of a community composed of *P. aeruginosa* and *S. aureus*. Key to determining the population composition are virulence factors produced by *P. aeruginosa* that reduce growth/metabolism in *S. aureus*. Using a combination of experimentation, mathematical modeling (ordinary differential equation) and agent based modeling, we found that in an undisturbed or continually disturbed environment, the ratio of bacterial ATP/growth rate determines population composition in multiple growth environments, including those designed to mimic the human growth environment. If this ratio of ATP/growth between *P. aeruginosa* and *S. aureus* is sufficiently high, *P. aeruginosa* will dominate the co-culture. Otherwise, low ratios favor *S. aureus* allowing it to outcompete *P. aeruginosa*. Both modeling and mutagenesis analysis suggest that this is due to the production of HQNO (and to a lesser extent pyoverdine and pyochelin), which reduces growth and metabolism in *S. aureus*. However, if metabolism and growth in *S. aureus* is sufficiently high owing to the composition of the growth environment, *S. aureus* can buffer against the effects of HQNO. The ratio of ATP/growth rate also determines population tipping points in an undisturbed or continually disturbed environment. Thus, for a given growth environment and initial population composition, the final population composition can be accurately predicted. Finally, we found that periodically disturbing the population disrupts the relationship between ATP/growth rate and population composition (Figure 5). Here, *S. aureus* dominates the culture across a range of growth environments. Agent based modeling suggests that this is because periodically disturbing the population disrupts the spatial positioning of *S. aureus* and HQNO produced by *P. aeruginosa*; this allows *S. aureus* to effectively sense less HNQO allowing it to outcompete *P.*

aeruginosa. Our results reveal a simple metric that may be able to predict population composition in additional microbial communities. It also demonstrates that disturbance can shift the population composition of a polymicrobial community. All of the findings detailed above have been published in Pajon et al. (2023) *eLife*.

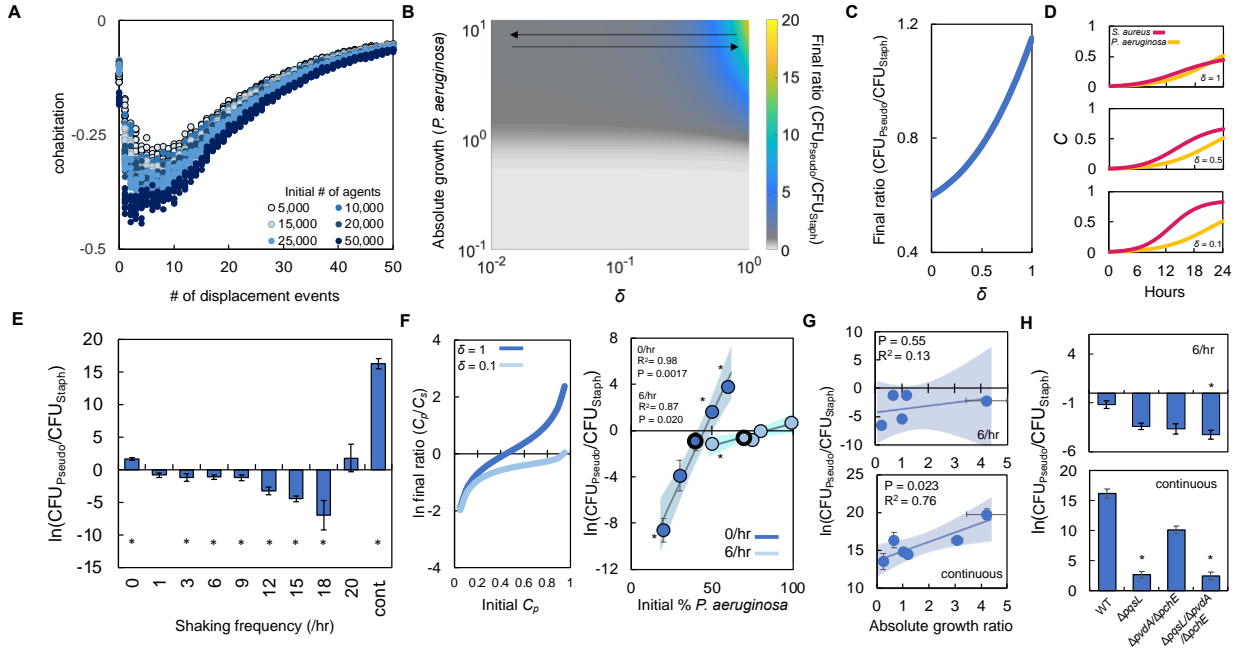


Figure 5: Periodically disturbing co-cultured populations of *P. aeruginosa* and *S. aureus* determines their co-existence.

- A)** Simulation using agent based modeling showing the 'cohabitation' variable (which described the degree of overlap between *P. aeruginosa* and *S. aureus* agents) as a function of the number of disturbance events. Each data point represents an outcome from a single simulation (10 simulations total).
- B)** Heat map showing the effect of δ and absolute growth of *P. aeruginosa* (fixed absolute growth of *S. aureus*) on the final ratio of *P. aeruginosa* and *S. aureus*. Total simulation time = 24 hours.
- C)** Simulations showing the effect of changing δ on the final ratio of *P. aeruginosa* and *S. aureus* for an absolute growth value of 1. Total simulation time = 24 hours.
- D)** Simulations showing the temporal changes in the density of *P. aeruginosa* (C_p) and *S. aureus* (C_s) at different values of δ . Total simulation time = 24 hours.
- E)** The ratio of *P. aeruginosa* to *S. aureus* as a function of the number of disturbance events. * = significant difference in the final density of both populations ($P \leq 0.0202$ using a Mann-Whitney, Shapiro-Wilk < 0.0001). SEM from a minimum of three biological replicates.
- F)** Simulations showing the effect of changing δ on the final population density when the initial density of *P. aeruginosa* (C_p) is varied. Total simulation time = 24 hours. SEM from a minimum of three biological replicates. R^2 and P values shown on plot are from a linear regression. * = significant difference in the final density of *P. aeruginosa* and *S. aureus* (Mann-Whitney; $P \leq 0.0202$, Shapiro-Wilk; $P = 0.0190$). Shaded regions = 95% confidence interval.
- G)** The ratio of *P. aeruginosa* to *S. aureus* after 24 hours of growth and with 6 disturbance events per hour (top) or in a continuously disturbed environment (bottom). Difference between all data points: 6/hr: $P = 0.0023$ (Kruskal-Wallis); continuous: $P < 0.0366$, (Kruskal-Wallis). Shaded regions = 95% confidence interval.
- H)** The effect of 6 disturbance events per hour and continuous disturbance on the final density ratio of *P. aeruginosa* knockout strains and *S. aureus* wild type strain. * = final density ratio was significantly different from co-culture with the wildtype strain ($P < 0.0129$, Dunn test for joint ranks using wildtype as the control group). SEM from a minimum of five biological replicates. Figure legends and figure adapted from Pajon et al. 2023

STUDENT AND TRAINING ACCOMPLISHMENTS

In addition to the scientific goals of our award, we also proposed to engage multiple graduate and undergraduate students from backgrounds that are underrepresented in STEM. The majority of the students below indeed are from backgrounds that are underrepresented in STEM, and have been very successful owing the funding. Below, we highlight the accomplishment of each student supported by the award (either through salary, supplies, or both). The majority of students involved in the work are listed on at least one published manuscript. Many students won awards for their work. All students have remained in careers that involve science within a range of areas (academia, industry, and medicine). Accordingly, we have met (if not exceeded) the goals of the original proposal.

Graduate students (8 total)

Rebecca Quinn

- Graduated from Masters in Biological Science Program in 2020
- One first author publication in *mSystems*
- Thesis entitled: Disruption of Pyoverdine Synthesis in *Pseudomonas Aeruginosa* Through Periodic Disturbance of Biofilm Structure
- Co-authored publication in *International Review of Cell and Molecular Biology*
- 3 presentations including an oral poster presentation at ASM Microbe
- Currently a Research Associate at Pfizer

Ivana Barraza

- Graduated from Masters in Biological Science Program in 2021
- One first author publication in *Applied and Environmental Microbiology*
- Co-authored publications in *mSystems*, *International Review of Cell and Molecular Biology*, and *iScience*
- Thesis entitled: Increasing the Frequency of Periodic Spatial Disturbance Decreases Surface Attachment Protein Expression in *Staphylococcus Aureus*
- Five presentations at conferences
 - 2nd place oral presentation at NSU Research Jamboree
- Currently a PhD Candidate at Duke University
 - Has already won multiple awards as a PhD candidate

Laura García-Diéguez

- Graduated from Masters in Biological Science Program in 2022
- One first author publication in *iScience*
- Co-authored publications in *mSystems* and *Applied and Environmental Microbiology*
- 3 conference presentations
- Thesis entitled: Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in *Pseudomonas Aeruginosa*
- Currently a Research Specialist at TCXpress and the University of Pittsburgh

Gabriela Diaz-Tang

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publications in *iScience*, *Applied and Environmental Microbiology* and *eLife*
- Received Future Leaders Mentoring Program award from the American Society of Microbiology

- Beginning PhD program at Duke University in Fall 2023
 - Won prestigious/highly competitive fellowship at Duke University

Estefania Marin Meneses

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publications in *iScience*, *Applied and Environmental Microbiology* and *eLife*
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Currently applying for MD/PhD programs

Ariane Kalifa

- Currently enrolled in the Masters of Biological Sciences Program
- Co-authored publication in *eLife*
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Plan on pursuing PhD program in infectious disease

Elinor Sevy

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publication in *eLife*
- Received Future Leaders Mentoring Program award from the American Society of Microbiology
- Starting as a Systems Analyst at Duke University in September 2023

Fatima Abu Rumman

- Graduated from Masters of Biological Sciences Program in 2023
- Co-authored publication in *Applied and Environmental Microbiology*
- One publication in preparation
- Thesis entitled: The Growth Environment Affect Virulence Factor Expression and Biofilm Formation in *Pseudomonas aeruginosa* in an Unstable Environment
- Two conference presentations
- Currently a grant officer in the Office of Sponsored programs at NSU. Pursuing a PhD in the future.

Undergraduate students (12 students)

Camryn Pajon

- Graduated from the Bachelor of Science, Biology Program in 2022
- One first author publication in *eLife*
- Co-authored publications in *Applied and Environmental Microbiology* and *mSystems*
- Five conference presentations
 - Multiple awards including travel grants (ASM Microbe), attendance grants (ASM/FESEB Joint Conference), 1st place presentation at FLASM, 1st place presentation at NSU Research Jamboree
- Currently a PhD candidate at the University of North Carolina, Chapel Hill

Marla Fortoul

- Graduated from the Bachelor of Science, Biology Program in 2020
- One first author publication in *eLife*
- One co-authored publication in *International Review of Cell and Molecular Biology*

- Two conference presentations
- Currently a third year medical student at NSU MD

Brandon Toscan

- Graduated from the Bachelor of Science, Biology Program in 2022
- Completed an Honor's in Major Project with the research
- One co-authored publication in *eLife*.
- Applying to PhD programs.

Taniya Mariah

- Graduated from the Bachelor of Science, Biology Program in 2021
- Completed an Honor's in Major Project with the research
- One co-authored publication in *eLife*.
- Currently attending medical school

Mali Marcelin

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in *eLife*.
- Currently applying to podiatry school

Daniella Hernandez

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in *eLife*.
- Currently applying to medical school and working in the lab with NIH support

Melissa Marzouk

- Currently attending the Bachelor of Science, Biology Program
- One co-authored publication in *eLife*.
- Currently applying to medical school and working in the lab with NIH support

Kerollos Ibrahim

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in *mSystems*
- Currently attending medical school at Florida State University

Laura Enzinna

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in *mSystems*
- Currently attending medical school at the Idaho College of Osteopathic Medicine

Morgan Thorn

- Graduated from the Bachelor of Science, Biology Program in 2019
- One co-authored publication in *mSystems*
- Currently attending medical school at the Alabama College of Osteopathic Medicine

Vanessa Cruise

- Graduated from the Bachelor of Science, Biology Program in 2020
- One co-authored publication in *iScience*.
- Currently applying to medical school

Marina Handel

- Graduated from the Bachelor of Science, Biology Program in 2020
- Currently attending medical school at NSU (Osteopathic Medicine)