ABSTRACTS

http://pseudomonasconference.com

SESSION I: GENOMICS AND GENE REGULATION

PLENARY

Joseph Bondy-Denomy, PhD

Joe Bondy-Denomy is an Associate Professor in the Department of Microbiology & Immunology at the University of California, San Francisco. The lab is currently focused on studying the roles of CRISPR-Cas immune systems in their native host microbes and understanding how bacterial viruses “fight back” with anti-CRISPR proteins and “phage nucleus” compartments. More recently, the lab has also begun to characterize the mechanisms of new immune systems in bacteria that inhibit bacterial viruses. This work is supported by funding from the NIH, DARPA, the Searle Scholars Program, the Vallee Foundation, and the Innovative Genomics Institute. Joe is also the co-founder of Acrigen Biosciences, a company focused on using anti-CRISPR proteins to disable Cas nucleases after on-target editing is complete. Prior to coming to UCSF, Joe was a PhD student with Alan Davidson at the University of Toronto and received his undergraduate degree in Biology from the University of Waterloo.

PA-2022-001

How *Pseudomonas aeruginosa* bacteriophages evade CRISPR-mediated demise

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From humans to bacteria, the threat of viral attack is all too real. Bacteria protect themselves from viruses, called bacteriophages, with immune systems like restriction-modification, CRISPR-Cas, and a plethora of others that have been recently discovered. Our group focuses on the discovery and characterization of mechanisms that phages use to counter immune processes in *Pseudomonas aeruginosa*. Recent screening efforts have unveiled three advances in this area, which I will summarize in my lecture. First, the discovery of anti-CRISPR enzymes that covalently modify CRISPR-Cas proteins or degrade the Cas protein effectors. Second, the observation that some obligately lytic phages evade CRISPR-Cas and restriction enzymes in novel ways, including shielding their DNA with a “phage nucleus”, as opposed to inhibiting CRISPR-Cas. Third, there has been recent expansion in the list of known bacterial anti-phage immune systems, some homologous to human innate immune processes. Many of these systems are found in the *P. aeruginosa* pangenome, which I will argue makes it the premier model organism for “bacterial immunology”. We have developed new

bioinformatic and genomic tools to discover anti-phage immune systems in this organism, identified genomic “defense hot spots” and launched experimental approaches to determine how they target phages and how phages fight back. Early progress on the CBASS anti-phage system (homologous to human cGAS-STING) will be discussed as an example. Together, we hope that studies of strategies deployed by lytic and temperate phages will provide a comprehensive understanding of phage-immune interactions in bacteria and fortify phage therapeutics.

PA-2022-002

O-antigen structure and surface hydrophobicity determines aggregate assembly type in *Pseudomonas aeruginosa*

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Bacteria live in spatially organized aggregates during chronic infections, where they adapt to the host environment, evade immune responses and resist therapeutic interventions. Although it is known that environmental factors such as polymers influence bacterial aggregation, it is not clear how bacterial adaptation during chronic infection impacts the formation and spatial organization of aggregates in the presence of polymers. Here we show that in an *in vitro* model of cystic fibrosis (CF) containing the polymers eDNA and mucin, O-antigen is a major factor in determining the formation of two distinct aggregate assembly types of *Pseudomonas aeruginosa* due to alterations in cell surface hydrophobicity. Our findings suggest that during chronic infection, interplay between cell surface properties and polymers in the environment may influence the formation and structure of bacterial aggregates, which would shed new light on the fitness costs and benefits of O-antigen production in environments such as CF lungs.

PA PA**PA-2022-003**

A novel small RNA governs disseminated infection of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa (*Pa*) is a prominent causative agent of a wide variety of acute and chronic infections. Understanding how *Pa* alters its gene expression and adapts to distinct infection lifestyles within human hosts is critical for designing effective treatment and prevention strategies. In recent studies, the Whiteley lab has used transcriptomic approaches to directly probe *Pa* gene expression within human-derived samples. The *Pa* gene *PA1414* was often found as the most highly expressed *Pa* gene in different types of human infections and, in some cases, *PA1414* reads constituted over 20% of the total mRNA reads in *Pa* transcriptomes. To date, this gene has not been functionally characterized, likely due to its poor expression and presumably negligible function during *Pa* growth under standard laboratory conditions. In this study, we investigate the roles of *PA1414* and specifically how it may regulate *Pa* behavior during infection. Here we show that the gene *PA1414* encodes a novel small RNA (sRNA) and is highly expressed in response to host-associated environmental cues such as oxygen depletion. Using a mouse chronic wound model where *PA1414* is expressed at a similar level as observed in humans, we show that the high abundance of *PA1414* sRNA contributes to localized non-disseminated infection, whereas lacking this sRNA leads to higher rates in dissemination and lethal sepsis. Using multi-omics and genetic approaches, we find that the *PA1414* sRNA primarily targets ubiquinone biosynthesis in *Pa*. Specifically, *PA1414* sRNA induces ubiquinone biosynthesis and in turn allows *Pa* to undergo anaerobic denitrification, which is important for the biofilm lifestyle of *Pa*. Taken together, we suggest that *PA1414* sRNA may act as a molecular lever that governs the transition between localized persistence and systemic dissemination of *Pa* infection.

PA-2002-004

How does *P. syringae* manage stress through AlgU when living in *Arabidopsis*

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Pseudomonas syringae pv. *tomato* DC3000 (*Pto*) is a plant pathogen that can travel through different natural environments and plant host niches. To survive different niches, *Pto* needs to adjust its cellular function according to the corresponding signals. The plant host niche is associated with signals of specific nutrients, immune responses, and the presence of different tissue-specific microbiota. How *P. syringae* responds to these niche signals, and what cues are sensed by *Pto* in the host-pathogen relationship remains an open question. Previous studies have shown that AlgU may play a role in plant niche signal sensing. AlgU is a conserved extracytoplasmic function sigma factor that can sense various stressors and regulates hundreds of genes. Deletion of *algU* negatively affects *Pto* growth in plants. However, it is not known which *Pto* genes and pathways are AlgU regulated when inside a plant niche. Here we present results from a transcriptome study using RNA-seq. Our result shows that approximately 250 genes are induced by AlgU, while another 150 genes are suppressed by AlgU in the host plant *Arabidopsis*. AlgU induced genes related to stress response and pathogenicity, while AlgU suppressed genes in the flagellar assembly and chemotaxis pathways. We had previously observed that plant immunity may interfere with AlgU signaling. Here we show that in the immune-induced environment, half of AlgU regulons have lost induction/suppression in contrast to naïve plants. Our result provides a detailed map of genes involved in the host-pathogen relationship that are regulated by AlgU, and allows us to better understand the plant-pathogen battleground.

PA-2002-005

Characterization of PemB, a novel effector of *Pseudomonas aeruginosa* type III secretion system

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Pseudomonas aeruginosa is able to colonize various habitats and utilize a broad range of nutrients. To establish an acute infection, *P. aeruginosa* employs an extensive arsenal of virulence determinants, including the type III secretion system (T3SS). T3SS is a multi-protein complex with a needle-like structure, delivering effector proteins from the bacteria's cytosol directly into the host's cytosol. Despite the versatility of hosts, environments and large genome, only six known effectors were identified in this

pathogen thus far. This sets up the basis for our hypothesis that more undiscovered T3SS effectors are encoded in the *P. aeruginosa* genome. A machine-learning algorithm constructed a top-ranked putative effectors list, using several genomes of *P. aeruginosa* strains representing both clinical isolates and laboratory strains. We identified six novel effectors, PemA-F, and assessed their effect on the multicellular organism model — *Caenorhabditis elegans*. Using a lifespan assay, we established that T3SS plays an important role in *P. aeruginosa* infection of *C. elegans*. We found that PemB showed a toxic effect on *C. elegans*, via the Programmed Cell Death pathway. Using a microfluidic device, we revealed that PemB is localized in the *C. elegans* intestine. Hence, we examined whether *P. aeruginosa* has a physiological effect on *C. elegans* intestine using the intestine staining approach. We found that PemB causes leakage in *C. elegans* intestine, impairing its integrity. Our next goal is to unravel the molecular pathway and protein interactions of PemB in the nematode. The findings of this research constitute an important step towards a better understanding of virulence and host evasion mechanisms adopted by *P. aeruginosa*.

SESSION II: ANTIMICROBIAL TOLERANCE

KEYNOTE

Paul Turner, PhD

Dr. Paul Turner is the Rachel Carson Professor of Ecology and Evolutionary Biology at Yale University, and Microbiology faculty member at Yale School of Medicine. He obtained a BA in Biology (1988) from University of Rochester, a PhD in Microbial Evolution (1995) from Michigan State University, and did postdocs at National Institutes of Health, University of Valencia in Spain, and University of Maryland-College Park, before joining Yale in 2001. He serves as Director of the Center for Phage Biology and Therapy at Yale, and also directs Yale's Quantitative Biology Institute. Dr. Turner studies evolutionary genetics of viruses, particularly phages (bacteria-specific viruses) that infect bacterial pathogens and RNA viruses transmitted by mosquitoes, and researches the use of phages to treat antibiotic-resistant bacterial diseases. He is very active in science-communication outreach to the general public, and is involved in programs where faculty collaborate with K-12 teachers to improve STEMM education in underserved public schools. Dr. Turner's current service includes the National Science Foundation's Bio Advisory Committee and President-elect of the International Society for Evolution, Medicine and Public Health. His honors include Fellowships in the National Academy of Sciences, American Academy of Arts & Sciences, and American Academy of Microbiology.

PA-2022-006

Phage therapy to combat infections by antibiotic-resistant *Pseudomonas aeruginosa*

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One possible strategy to combat the antibiotic resistance crisis is a renewed approach to 'phage therapy', which acknowledges that target bacteria should be expected to evolve resistance against therapeutic phages. Our innovation is to administer viruses that not only kill the target bacteria, but also predictably select for evolved phage resistance in bacteria that reduces virulence and/or increases antibiotic sensitivity (evolutionary trade-offs). By utilizing virulence factors and/or drug-resistance mechanisms as receptor binding sites, the phages exert selection for bacteria to evolve

phage resistance by modifying (or losing) the structure, potentially reducing (or eliminating) bacterial pathogenicity. We present examples of newly-discovered lytic phages of *Pseudomonas aeruginosa* that kill broadly across clinical isolates of these bacteria, while also selecting for evolved phage-resistance in remaining bacteria which constitute trade-offs useful in the clinic (decreased virulence; re-sensitivity to antibiotics). We present successful case examples for emergency inhaled-phage-therapy, administered against multi- and pan-drug-resistant pulmonary infections in individuals with cystic fibrosis and bronchiectasis. These *in vitro* results are compared to phenotypic, genetic and metagenomics analyses of microbes isolated longitudinally from patient samples before, during and after emergency phage therapy treatments. Our data suggest that phenotypic and genomic effects of evolutionary trade-offs in the target *P. aeruginosa* population are more accurately predicted when therapeutic phages are delivered singly or sequentially, compared to therapies where phages are combined together in cocktails. We discuss the relevance of our findings for ongoing development of lytic phages in personalized treatment of *P. aeruginosa* in human patients, as stand-alone therapy or in combination therapy with chemical antibiotics.

PA-2022-007

Molecular mechanisms of cefiderocol resistance in *Pseudomonas aeruginosa*

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Cefiderocol (CFDC), a novel siderophore cephalosporin, represents a potential candidate for the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections. However, the specific mutations underlying CFDC resistance phenotypes remain unknown. Here, we used experimental evolution and whole-genome sequencing to identify chromosomal mutations underlying CFDC resistance in planktonic and biofilm *P. aeruginosa* cultures, and we determined the fitness consequences of these mutations. Populations were evolved using synthetic cystic fibrosis (CF) sputum medium (SCFM2) and synthetic human urine (SHU), which recapitulates host environments. All evolved populations were genetically diverse, exhibiting hundreds of mutations at intermediate frequencies, and fixed mutations were rare. Genome sequencing showed that CFDC-resistance arises through different mutations in populations evolved in SHU and SCFM2. we narrowed our focus on non-synonymous mutations detected in more than one replicate population because mutations in the same gene found in independently derived lineages provide strong evidence for adaptive evolution. CFDC-resistant populations evolved in SCFM2 accumulated mutations in *cpxS, vqsM, pvdT* and *pchF*., while *nalD* and PA2550 were recurrently mutated in populations in SHU media. To confirm the

functional consequence of candidate resistance mutations, we tested transposon mutants in PA2550, *pvdT, cpxS, pchF* and *vqsM* genes and found that mutation of these genes led to a 2- to 5-fold increase in CFDC MIC compared to PAO1-WT strains. Analyses of competitive fitness and fitness in the presence of other anitbiotics showed that the CFDC-evolved populations outcompeted the ancestral population in competition assays during CFDC treatment and exhibited cross resistance against structurally related cephalosporins, ceftazidime and cefepime. Overall, this study provides an initial understanding of how *P. aeruginosa* evolves CFDC resistance in synthetic host environments. Finally, this study provides valuable information that might help researchers and clinicians to improve the usage of cefiderocol, aiming to delay the spread of CFDC resistance.

PA-2022-008

Activity-independent evaluation of antibiotic permeability barriers in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a challenging pathogen that causes a variety of infections in humans and animals. Two-membrane cell envelopes of *P. aeruginosa* act as selective permeability barriers, protecting cells against the antibacterial action of antibiotics and other therapeutic agents. We hypothesize that understanding how small molecules permeate these barriers will facilitate development of novel therapeutics effective against *P. aeruginosa*. In this study, we developed a liquid chromatography mass spectrometry (LC-MS) approach to analyze the accumulation of compounds in *P. aeruginosa*. The approach uses four strains of *P. aeruginosa* with varied permeability barriers and does not rely on high-affinity intracellular binding or inhibition of cell growth. We assembled a library of compounds with properties representative of large drug-like libraries. These compounds vary in molecular weight between 200 and 750 Da and have cLogD7.4 values between 5.0 and -3.5. The accumulation of 83 compounds was analyzed at several extracellular concentrations and two time points in four *P. aeruginosa* strains that differ in efflux proficiency and permeability properties of the outer membrane. We found that the contribution of active efflux varied broadly between compounds, and it was not the major contributor to the intracellular accumulation. In contrast, permeation across the outer membrane was the dominating factor for intracellular accumulation of the analyzed compounds. We further identified “good” permeators and analyzed their

properties. In conclusions, we developed an experimental approach that can be applied to analyses of intracellular accumulation of a broad range of structurally unrelated compounds including those lacking affinities to intracellular targets.

PA-2022-009

Phenotypic selection following antibiotic treatment of heterogeneous *Pseudomonas aeruginosa* in Cystic Fibrosis patients

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Pseudomonas aeruginosa (*Pa*) is an apex pathogen associated with persistent bacterial infection of airway mucus in individuals with cystic fibrosis (CF). Despite the routine use of high-dose inhaled antibiotics, failure to eradicate infection is the norm. *Pa* populations show high phenotypic diversity within individual patients which may contribute to treatment failure due to antibiotic tolerant subpopulations. The mechanisms by which bacterial diversity contributes to treatment failure and therefore to persistent airways infections is poorly understood. This study aims to identify the impact of antibiotic treatment on in-patient *Pa* population dynamics. Fresh sputum samples, obtained from individuals with CF, were treated with antibiotics or vehicle control. After treatment, *Pa* burden was quantified, and individual colonies arrayed into isoclonal populations. We assayed these arrayed populations for various bacterial phenotypes including biofilm formation, antibiotic minimal inhibitory concentration (MIC), quorum sensing (QS), and antibiotic tolerance as potential phenotypic contributors to antibiotic treatment failure. Our results demonstrate that a single round of antibiotic treatment of *ex vivo* sputum selects for a phenotypic shift in the patient evolved *Pa* populations, including the enrichment of antibiotic tolerant subpopulations. Using these patients derived *Pa* populations, we also demonstrate that an *in vitro* synthetic sputum medium containing hyper-concentrated mucin recapitulates the antibiotic tolerance and post antibiotic phenotypic dysbiosis observed in *ex vivo* CF sputum. These results provide an initial understanding of the characteristics of treatment failure in persistent *Pseudomonas* airways infection and demonstrate that antibiotic pressure under biologically relevant conditions selects for increased tolerance and altered phenotypic patterns that may influence bacterial virulence, persistence, and disease severity.

Mapping and tackling diversity in antibiotic resistance of sputum isolates in cystic fibrosis

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Conventional antibiotic susceptibility tests (AST) poorly predict antibiotic efficacy against *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients. The previously reported AST diversity of *P. aeruginosa* isolates within one sputum sample may contribute to the low predictive value of AST, especially since only one isolate per sputum sample is included. A possible solution is to pool isolates, but a comprehensive study on that approach is currently lacking. The present study aims to characterize the intrapatient diversity in antibiotic susceptibility of *P. aeruginosa* for five antibiotics commonly used in CF patients, and to determine the minimal number of isolates that needs to be pooled to obtain an AST result that is representative of the phenotypically diverse *P. aeruginosa* population. Sputum samples were collected from 5 CF patients chronically colonized with *P. aeruginosa*, and 30 *P. aeruginosa* isolates were randomly selected per patient. The intrapatient diversity of the *P. aeruginosa* population was assessed by determining the minimal inhibitory concentration. Next, the influence of pooling randomly selected isolates on AST diversity was assessed by pooling an increasing number of isolates to determine the minimal number of isolates per sample needed to obtain a representative AST. We observed AST diversity of *P. aeruginosa* isolates recovered from a single patient for aztreonam, ceftazidime and tobramycin, but not for colistin and ciprofloxacin. In diverse populations, pooling isolates lowered diversity and for each population we obtained a representative AST after pooling 3-9 isolates, depending on the patient. Interestingly, a correlation was found between the initial diversity in antibiotic susceptibility and the number of isolates needed to be pooled to obtain a representative AST. We are currently confirming our findings with additional patient samples. In conclusion, our results suggest that a comprehensive study.

SESSION III: HOST-PATHOGEN INTERACTIONS

KEYNOTE

Katharina Ribbeck, PhD

Prof. Ribbeck obtained her Bachelor's degree and her PhD in Biology from the University of Heidelberg, Germany. She continued her postdoctoral research at the European Molecular Biology Laboratory, Heidelberg, Germany, and the Department of Systems Biology, Harvard Medical School. Katharina Ribbeck established her independent research group as a Bauer Fellow at the FAS Center for Systems Biology, Harvard University in 2007, and joined the Department of Biological Engineering at MIT as an Assistant Professor in 2010. The Laboratory for Biological Hydrogels' focus is on basic mechanisms by which mucus barriers exclude, or allow passage of different molecules and pathogens, and the mechanisms pathogens have evolved to penetrate mucus barriers. It hopes to provide the foundation for a theoretical framework that captures general principles governing selectivity in mucus, and likely other biological hydrogels such as the extracellular matrix, and bacterial biofilms. The Lab's work may also be the basis for the reconstitution of synthetic gels that mimic the basic selective properties of biological gels.

PA-2022-011

Partners in Slime: How mucus regulates microbial virulence

Mucus is a biological gel that lines all wet epithelia in the body, including the mouth, lungs, and digestive tracts, and has evolved to protect us from pathogenic invasion. Microbial pathogenesis in these mucosal systems, however, is often studied in mucus-free environments, which lack the geometric constraints and microbial interactions that are found in natural, three- dimensional mucus gels. To bridge this gap, my laboratory has developed model test systems based on purified mucin polymers, the major gel-forming constituents of the mucus barrier, and their glycans. We use this model to understand how the mucus barrier influences microbial virulence, and moreover, to elucidate strategies used by microbes to overcome the normal protective mucus barrier. I will discuss data showing that the mucin polymers, and specifically their associated glycans, have a significant impact on the physiological behavior of microbes, including surface attachment, quorum sensing, the expression of virulence genes, and biofilm formation. The picture is emerging that mucin glycans are key host players in the regulation of microbial virulence and underscores the untapped therapeutic opportunities found in these host-derived molecules.

Filamentous Bacteriophages produced by *Pseudomonas aeruginosa* prevent wound healing

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Pseudomonas aeruginosa (*Pa*) biofilms inhibit wound healing but the underlying mechanisms are unclear. We have identified a novel role for filamentous bacteriophage in the delayed healing associated with chronic *Pa* wound infections. Pf phages produced by *Pa* prevented re-epithelialization and delayed wound healing in both mouse and pig models of chronic *Pa*- infected wounds. These effects were mediated by structural contributions of filamentous phages to biofilm formation. Pf phages organize polymers present in human wound fluid and bacterial biofilms into highly adherent biofilms with higher order, liquid crystalline organization. These biofilms physically disrupt interactions between keratinocytes and the underlying wound matrix and prevent integrin-mediated adhesion and migration. Pf phages organize wound fluid into films and inhibit wound healing even in the absence of *Pa* infection. In a prospective cohort study of 37 human patients with Pa infected wounds, we find that Pf phage was present in all wounds (8/8; 100%) that increased in size over time. Together, these data implicate Pf in the delayed

wound healing associated with *Pa* infection. We propose that Pf phages may have potential as a biomarker and therapeutic target for delayed wound healing.

PA-2022-013

Single-cell resolution of plant response to bacterial infection

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Plant responses to pathogen infection vary by tissue type and even in the same tissue, yet heterogeneity in pathogen response among cells is not well resolved. Here, we exposed Arabidopsis leaf tissue to *Pseudomonas syringae* pv. tomato DC3000 (or a mock treatment) at an early infection stage (24 hours), and quantified the response of over ten thousand individual cells using single-cell RNA sequencing. Graph-based unsupervised clustering and differential expression analyses identified 18 cell clusters comprising the major cell types within a leaf, with a large proportion of the cell population corresponding to mesophyll identity. Integrative analysis of cell populations from *Pst* DC3000- and mock-inoculated leaves revealed five distinct clusters enriched for cells from bacterial inoculated leaves. These clusters represent a gradient of responses, from susceptibility to immunity. Of the pathogen-associated cell clusters, we identified two immune response clusters, indicating that Arabidopsis perceives and attempts to mount a defense response to virulent *Pst* DC3000. The largest pathogen-associated cell cluster, comprising over 1,000 cells, is enriched with transcripts manipulated by *Pst* DC3000 to promote disease including biological processes related to water transport and response to jasmonic acid. We hypothesize that cells within the largest pathogen-associated cluster are directly targeted by *P. syringae*, while clusters enriched with immune-related transcripts are proximal to directly targeted cells. Current experiments are focused on generating promoter:reporter lines for genes representing pathogen-associated clusters to validate spatial dynamics of plant cell responses during infection. Overall, our work uncovers cellular heterogeneity within an infected leaf and provides a unique insight into plant differential responses to infection at single-cell level.

Regional evolution of *Pseudomonas aeruginosa* in the human host

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Pseudomonas aeruginosa (*Pa*) diversifies genetically and phenotypically during cystic fibrosis (CF) lung infections, but the drivers of diversification are poorly understood. One potential mechanism is geographic isolation of *Pa* subpopulations in different lung regions. Isolation promotes diversification by sheltering specialists from competition, and by enhancing drift and local selection pressures. Regional isolation has been shown to contribute to evolution of diversity during HIV infections. Here we tested the hypothesis that *Pa* infecting CF patients evolve in regional isolation early in disease. We collected washings from 5 lung regions in 9 chronically *Pa*-infected CF subjects with mild disease using bronchoscopy and sequenced ~95 *Pa* isolates per region. We then tested for regionally isolated evolution with the Slatkin-Maddison test, which examines the association between isolates' location and phylogeny. Analysis of 3 subjects revealed that regional *Pa* subpopulations show strong signals of isolated evolution ($p < 0.00001$ in all cases), but some region-to-region mixing of isolates does occur. Drift and selection can both promote evolutionary divergence of isolated subpopulations. Selection is more likely to be a driver if regional conditions differ in important ways. We measured lung injury by CT scans, neutrophil elastase levels, and *Pa* density, and found most subjects had ~1000-fold variations in regional *Pa* density and ~10-fold differences in regional neutrophil elastase levels. Together these data suggest that at early stages of disease, CF *Pa* evolve in isolation in lung regions that differ markedly in conditions, but locally-evolved isolates sometimes move to new areas. Isolated evolution early in disease likely contributes to the extensive genetic diversity of CF lineages. It could also produce regional *Pa* that differ in resistance to host defenses and antibiotics and vary in injury potential. The limited mixing we observed could enable adaptive and pathogenic traits to spread, as locally-evolved specialists colonize new lung regions.

PA-2022-015

l-Undecene odor from *Pseudomonas aeruginosais* a pathogen associated molecular pattern for *Caenorhabditis elegans*

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Animals utilize pattern recognition receptors (PRRs) such as *tol* family, *nod* family and cGAS receptors to recognize conserved molecules in microbes such as lipopolysaccharides and other cell wall components, flagellin, dsRNA etc. It is not clear how animals lacking canonical PRRs recognize and distinguish amongst microbes. We utilized *C. elegans* as a genetically tractable host to understand recognition mechanisms for *Pseudomonas aeruginosa* resulting in a pathogen specific flight and fight response. Specifically, we ask whether bacterial volatiles constitute microbe-associated molecular patterns. Using gas chromatography–mass spectrometry, we identify six prominent volatiles released by the bacterium *Pseudomonas aeruginosa*. We show that a specific volatile, 1-undecene, activates nematode odor sensory neurons inducing both flight and fight responses in worms. Using behavioral assays, we show that worms are repelled by 1-undecene and that this aversion response is driven by the detection of this volatile through AWB odor sensory neurons. Furthermore, we find that 1-undecene odor can induce immune effectors specific to *P. aeruginosa* via AWB neurons and that brief pre-exposure of worms to the odor enhances their survival upon subsequent bacterial infection. These results show that 1-undecene derived from *P. aeruginosa* serves as a pathogen-associated molecular pattern for the induction of protective responses in *C. elegans*.

PA-2022-016

Pseudomonas aeruginosa PA14 produces R-bodies, extendable protein polymers with roles in host colonization and virulence

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Refractile bodies ("R-bodies") are intracellular protein ribbons that are produced as coils and extend into long rods when exposed to acidic pH. They have primarily been studied in parasitic and symbiotic bacteria, where they have been shown to be toxic to host organisms. Genomic analyses have revealed that a subset of pseudomonads contain genes with the potential to confer production of R-bodies (i.e., a "*reb* cluster"). Among isolates of *Pseudomonas aeruginosa*, the presence of the *reb* cluster shows a correlation with virulence; e.g. *P. aeruginosa* PA14 contains a *reb* cluster, while the less virulent strain PAO1 does not. We hypothesized that PA14 produces R-bodies and that these structures contribute to virulence. Using scanning electron microscopy and mass spectrometry, we observed R-bodies in SDS-insoluble preparations from PA14 and identified R-body-associated proteins. Using reporter strains, we found that R-body structural genes are expressed stochastically during independent growth and during host association. Super-resolution microscopy revealed the formation of intracellular coiled structures and also confirmed the co-localization of a novel R-body-associated protein, encoded by another gene in the *reb* cluster, to the R-body structure. Genes in the *reb* cluster also code for regulators--one of which is the enigmatic sigma factor FecI2, which is present in PA14 but not PAO1--that are required for expression of the structural genes. R-body production contributed to colonization of *Arabidopsis thaliana* seedlings and to virulence in a *Caenorhabditis elegans* slow-kill assay. Feeding with various PA14 knock-out mutants indicated that products of the *reb* cluster harm the nematode host via a mechanism involving ribosome cleavage and translational inhibition. These observations identify R-bodies as *P. aeruginosa* virulence factors and provide insight into the consequences of *reb* cluster gene expression during infection.

SESSION IV: METABOLISM AND PHYSIOLOGY

KEYNOTE

Gregg Beckham, PhD

Gregg T. Beckham is a Senior Research Fellow and Group Leader at NREL. He received his PhD in Chemical Engineering at MIT. He currently leads and works with an interdisciplinary team at NREL on the development of green processes and products in the areas of biomass conversion and plastics upcycling, and his favorite microbe is *Pseudomonas putida* KT2440.

PA-2022-017

Lignin degradation via outer membrane vesicles in *Pseudomonas putida*

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Lignin valorization is essential for the viability of lignocellulosic biorefineries, but the heterogeneity of lignin presents a substantial challenge for the production of chemicals. The concept of biological funneling, or the conversion of a heterogeneous substrate to a single target compound, has gained popularity in the last decade as a means to valorize lignin, especially enabled by the robustness and catabolic capacity of *Pseudomonas putida* KT2440. This talk will cover our team's investigations into how *P. putida* is able to convert lignin-derived compounds to central metabolism via the use of outer membrane vesicles, which we recently reported are able to harbor aromatic-catabolic enzymes that are functional for the turnover of aromatic compounds via the beta-ketoadipate pathway.

PA-2022-018

Role of ribosome hibernation in the physiological heterogeneity of *Pseudomonas aeruginosa* biofilms

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Pseudomonas aeruginosa causes acute and chronic infections that are often associated with biofilms. Biofilms are difficult to treat with antibiotics, in part, because the cells within biofilms are in a variety of physiological states. Some cells deep within biofilms, with limited access to nutrients and oxygen, enter a dormant, antibiotic-tolerant state. In previous work, we showed that hibernation promoting factor (HPF) protects ribosomes from degradation during nutrient-limiting conditions and is necessary for long-term survival of *P. aeruginosa* during starvation. Here, we quantified the effect of HPF on the survival of individual *P. aeruginosa* cells isolated from biofilms. Starting from single cells, we assayed the ability of *P. aeruginosa* to regrow from biofilms that were starved for up to eight days. The results showed that the Δ*hpf* mutant had extended lag times with greater heterogeneity during regrowth compared to the wild-type strain and to the stringent response (Δ*relA/*Δ*spoT)* mutant. Regrowth of the wild-type strain was inhibited by ciprofloxacin, but some single cells of the Δ*hpf* and Δ*relA/*Δ*spoT* biofilms regrew in the presence of ciprofloxacin. We also used fluorescence *in situ* hybridization (FISH) to assay the spatial distribution of rRNA in wild-type, Δ*hpf*, and Δ*relA/*Δ*spoT* biofilms. The FISH results showed vertical heterogeneity in rRNA content of cells in all biofilms. Following starvation, Δ*hpf* biofilm cells had decreased total rRNA abundance compared to wild-type and to the Δ*relA/*Δ*spoT* biofilm cells. Overall, the results demonstrate that there is spatial heterogeneity in ribosome content of *P. aeruginosa* biofilms, with cells deeper in the biofilms having reduced ribosome abundance. However, the wild-type cells maintain low but sufficient ribosome levels for uniform resuscitation of individual cells when regrowth conditions become favorable. In the absence of HPF, individual cells within starved biofilms are impaired in their ability to maintain ribosome abundances necessary for cell resuscitation.

PA-2022-019

A temporally-resolved, multi-omic analysis of *Pseudomonas putida* reveals a complex regulatory architecture underlying glucose metabolism

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Microbial metabolism involves a vast number of molecules that interact at several layers of regulation, tightly controlled in a spatiotemporal fashion. Metabolite dynamics, protein abundance(s) as well as metabolic flux distribution through central pathways are critical components of a multidimensional physiological representation of microorganisms. Glucose metabolism studies in *Pseudomonas putida* usually consider one regulatory layer at a time in a narrow temporal window, and conclusions on intracellular metabolism are then build on qualitative interpretations of such results. These traditional approaches are not sufficient to tackle more complex questions, e.g. how environmental bacteria cope with the niches they thrive in or which potential capabilities can be exploited for bioproduction purposes. In this study, we describe a temporally-resolved quantitative physiology analysis of *Pseudomonas putida* (wild-type and glycolytic mutants) to understand how the proteome, metabolome, and fluxome are reshaped when consuming glucose. Intracellular and extracellular metabolites along with protein availability were measured by using HPLC/LC-MS and intracellular reaction rates were estimated by metabolic flux analysis. Endo-metabolomics showed significant changes in metabolite intermediates of the oxidative and pentose phosphate pathway during the different growth stages. Time-resolved proteomic analysis revealed upregulation of enzymes of the peripheral sugar oxidation reactions while those involved in phosphorylative reactions were downregulated. Finally, fluxomics analysis showed that fluxes were distributed around three metabolic nodes: 1) glucose/gluconate/gluconate-6-phosphogluconate, further processed via the EDEMP cycle, 2) glucose/glucose-6-phosphate/6-phosphogluconate or 3) glucose/gluconate/2-keto-gluconate/6-phosphogluconate node depending the strain in study. By combining these analytical approaches, we built a map integrating the omic data that reflects the robustness of the metabolic network of *P. putida*, not only providing key information about network topology but also guiding engineering efforts.

PA-2022-020

Pseudomonas putida and its response to metabolic burden: trade-off between transcription and translation during heterologous protein production

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Pseudomonas putida is a promising workhorse in synthetic biology and industrial biotechnology due to its highly versatile metabolism and resilience to toxic compounds. In order to harness its full potential, it is pivotal to address phenomena not fully

understood, such as metabolic burden, and unravel fundamental mechanisms that affect cell productivity and resource allocation. When cellular resources, normally used by the host organism for cell growth and maintenance, are heavily drained toward heterologous gene expression, additional stress, such as reduced cell growth and increased mutation rates, is inflected on the host organism. We constructed a *P. putida* strain that produces the stress-triggering fusion protein MBPeGFP and a plasmid library with varying genetic elements to inspect resource allocation and heterologous protein production under metabolic burden. By varying inducer concentration, introducing a mRNA stem-loop upstream of the RBS, and by quantifying mRNA via qPCR, we analyzed the cell's response to metabolic burden from a transcriptional perspective. By varying RBS strength, we also inspected the cell's response on a translational level. Strains were grown in a microplate reader while optical density and fluorescence were measured *in vivo*. Red fluorescence reflected the cell's native expression via a genomically integrated mCherry, while green fluorescence reflected the plasmid-based heterologous expression of MBPeGFP. *P. putida* showed a MBPeGFP production rate of 491.17 ± 5 [RLU/(OD*h), an increased lag phase of 13 h, a decreased growth rate of 0.2 h^{-1}, and had a reduced resource capacity by 2.3-fold. Transcription experiments resulted in an inducer-Km of 16 μM and a 107-fold increase in mRNA transcript due to the stem-loop presence. Translation experiments revealed a threshold in RBS strength. These tools give us insight into cellular resource distribution and bottlenecks during metabolic burden and the means to tune heterologous gene expression in the future platform organism *P. putida*.

PA-2022-021

Dynamics of biofilm matrix assembly in the model organism *Pseudomonas aeruginosa*

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Bacteria form multicellular aggregates called biofilms. A crucial component of these aggregates is a protective matrix that holds the community together. Biofilm matrix composition varies depending upon bacterial species but typically includes exopolysaccharides (EPS), proteins, and extracellular DNA (eDNA). *Pseudomonas aeruginosa,* which is clinically important and also one of the model biofilm organisms, assembles aggregates using the structurally distinct EPS Psl and Pel, the matrix protein

CdrA, and eDNA. Interestingly, both Psl and Pel have been observed to localize to the periphery of aggregates, forming a “shell” around the enclosed bacteria. To examine how this specific peripheral localization occurs, we applied confocal laser scanning microscopy (CLSM) of live biofilms along with complementary biochemical and microbiological approaches to investigate three key variables: matrix degradation, production, and retention. We observed that biofilms retain matrix degrading enzymes, resulting in matrix components that are partially degraded without loss of biofilm biomass. These results suggest that matrix turnover is a normal aspect of biofilm physiology and does not necessarily precede biofilm aggregate disassembly.

SESSION V: HARNESSING THE POTENTIAL OF *PSEUDOMONAS* SPECIES

KEYNOTE

Wei Huang, PhD

Wei Huang is Professor of Biological Engineering at Department of Engineering, University of Oxford. He is a fellow of St Edmund Hall College. He was an EPSRC fellow on synthetic biology and a lecturer and senior lecturer at University of Sheffield. His research interests are synthetic biology and single cell biotechnology. He developed SimCells (simple cells) as a novel platform for synthetic biology, which can be used for biomanufacturing, vaccines, and medical therapy. He has developed various biosensors, including novel COVID-19 test, which have been applied to medical diagnosis in many countries. He is one of pioneers who use Raman micro-spectroscopy technology to the study of physiology and metabolism at the single cell level. He developed various Raman activated cell sorting techniques including optical tweezers, microfluidic device and laser ejection to isolate single cells of microbes. He has published >100 journal papers. He is inventor for 16 patents. He is an associate editor of two academic journals: Environmental Microbiology and Microbial Biotechnology. He has trained many successful scientists

PA-2022-022

Chromosome-free SimCells

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SimCells (Simple Cells) are chromosome-free bacteria controlled by designed DNA for novel tasks. They are genetically reprogrammable platforms, ready to read and express gene circuits and mini-genomes, designed from scratch. The design of SimCells involves sketching a cellular “hardware” blueprint (e.g. transcription/translation, membrane proteins and structure) and encoding DNA “software” that defines the ‘hardware’ and controls the performance of a bacterial cell. SimCells are non-replicating and highly controllable, taking advantages of live and non-live systems. We built SimCells from bacteria and used designed gene-circuits to hijack their cellular machinery for the applications in medicine, biomanufacturing and sustainable engineering.

Transcriptomic characterisation of *Pseudomonas putida* KT2440 for the sustainable production of monoaromatics for the plastics industry

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The plastics industry is almost exclusively reliant on petrochemical feedstocks for the synthesis of large-volume commodity chemicals. The petrochemically derived monoaromatic hydrocarbon styrene is used in the production of a number of polymers of industrial relevance including polystyrene and styrene-acrylonitrile resin. Styrene is produced from ethylbenzene via catalytic dehydrogenation, with ethylbenzene produced from the elementary petrochemical benzene. In order to improve the sustainability and reduce the environmental impact of this sector, we are investigating the application of a model *Pseudomonas* for the biosynthesis of such compounds. Both ethylbenzene and styrene are highly toxic to bacteria, limiting the choice of chassis organism: *Pseudomonas putida* KT2440 has recently drawn interest for the biocatalytic production of large-volume commodity chemicals due to its metabolic flexibility, tolerance to oxidative stress and its amenability to genetic modification. *P. putida* crucially, is also naturally styrene and ethylbenzene tolerant, making it ideally suited as a bacterial host for the sustainable production of ethylbenzene or styrene. Despite the structural similarity of ethylbenzene and styrene, we have found that *P. putida* is substantially more tolerant of ethylbenzene than styrene. By sequencing the global transcriptional responses of *P. putida* to exogenous ethylbenzene and styrene, we investigated the observed disparity in tolerance and the suitability of KT2440 as a chassis for their synthesis. Despite the near identical structures, we have identified distinct transcriptional responses to ethylbenzene and styrene, with different efflux systems upregulated to different degrees in response to each compound. This approach has identified a number of candidate systems to exploit in order to both improve the intrinsic tolerance of *P. putida* KT2440 to ethylbenzene and styrene and to facilitate the sustainable production or bioremediation of these compounds.

Tuning outer membrane vesicle formation to increase production of natural compounds in *Pseudomonas putida* KT2440

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Pseudomonads have become promising cell factories because of their inherent robustness for the production of natural compounds. However, an efficient high-level microbial production is still challenging due to chemical stress caused by high product and substrate concentrations, respectively. Although the bacteria have naturally evolved several strategies to deal with toxic chemicals, biotechnological applications benefit from the construction of new *chassis* organisms with specifically adapted tolerance features. Here, we assess outer membrane vesicle (OMV) formation as a tolerance trait for the production of different natural compounds in *Pseudomonas putida* KT2440. First, we found that vesiculation in a previously established strain for the recombinant production of the antibiotic tripyrrolic compound prodigiosin was increased 10- to 15-fold compared to a *P. putida* non-producer determined using protein- and lipid-based quantification assays as well as dynamic light scattering. In addition, triggering vesiculation by the addition of alkanols or PQS was shown to lead not only to increased OMV formation, but also to a 1.5-fold increase in prodigiosin production. Next, we tried to transfer these observations to the production of other natural compounds like the indolcarbazole arcyriaflavin A. To this end, we constructed an arcyriaflavin A production strain by integrating the respective biosynthetic operon from *Lentzea aerocolonigens* into the chromosome of *P. putida* KT2440. Again, the investigations showed a correlation between arcyriaflavin A production and OMV formation and, again, triggered vesiculation leads to increased production levels. Consequently, our findings indicate that recombinant natural compound production influences vesiculation in *P. putida* KT2440 and, more interestingly for the construction of robust production strains, that increased vesiculation leads to a higher production of the respective compound. We now assess options to specifically control vesiculation by genetic cell envelope manipulation of *P. putida* KT2440, which appears to be a powerful tool for significantly improving hitherto limited biotechnological applications.

PA-2022-025

Increased methyl ketone production through central carbon pathway rerouting in *Pseudomonas putida* KT2440 and fermentation process optimization

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Plant Biomass represents a valuable renewable source of carbon for bio-based chemicals such as biofuels. Pseudomonas putida KT2440 has been shown to be an organism capable of tolerating the toxic environment of processed biomass and be able to convert parts of its products into valuable products such as methyl ketones. In particular, KT2440 with knockouts of beta oxidation *fadA, fadB,* and PHA synthesis *phaC1, phaZ, phaC2,* as well as heterologous plasmid expression of *'tesA, fadB, Mlut_11700,* and *fadM* had proven to be one of the better platforms for this production. However, further understanding of its metabolic process is necessary as the carbon utilization can be further optimized and its pathway proved to be not robust enough as small different process condition variations reduced the overall titers. Through the rerouting of the TCA cycle we were able to further increase the titer of methyl ketone in pseudomonas putida from sorghum treated with cholinium hydroxide or cholinium lysenate. Knocking out *aceA* increased titers by 20% while knocking out *crc* decreased most methyl ketone production. Titers of 350 (mg/L) in 10 mL fermentations, and 2.5 (g/L) in AMBR 150 mL fermentations were achieved through further fermentation optimization where media nitrogen composition and induction timing proved to be the most sensitive variables. Furthermore, we were able to maintain these higher titers in the AMBR fermentation as well as maintain cholinium concentrations through the knockout of *betAB PP_0308-13 PP_0322-26* genes involved in cholinium catabolism.

PA-2022-026

Towards the development of strains from the unexplored *Pseudomonas pertucinogena* lineage to robust *chassis* platforms

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The species of the *Pseudomonas pertucinogena* lineage form a unique branch within the phylogenetic tree of the *Pseudomonas* genus. Most of them have only been described in the last decade. These strains share a rather small genome indicating a limited metabolic flexibility, nevertheless they can grow in a wide temperature range (4 – 41 °C) and, even more interestingly, within a salinity range of 0 – 15% NaCl. These properties as well as their harsh natural habitats (like oil- or metal-contaminated sites or the deep sea) suggest a high intrinsic potential to resist physical and chemical stresses and therefore *P. pertucinogena* strains as promising candidates for biotechnological applications. However, an application as microbial cell factories is currently hampered by lacking of microbiological and molecular genetic methodology, as protocols for other Pseudomonads can often not readily be transferred to these unique species. Thus, we selected four particularly interesting strains and first compared them with regard to their capabilities. We identified suitable cultivation media with exchangeable carbon and nitrogen sources for *P. aestusnigri* VGXO14*, P. bauzanensis* BZ93*, P. litoralis* 2SM5, and *P. oceani* KX20 leading to higher growth rates. Besides, we started to analyse their response to osmotic stress. Here, HPLC analysis showed that all selected strains produce the highly valuable compatible solute ectoine, which is not a common feature of *Pseudomonas sp.*. Moreover, *P. bauzanensis* and *P. litoralis* convert ectoine to the more powerful osmolyte hydroxyectoine. Furthermore, aiming to harness the strains' potential, we focused on genetic access. This was achieved for the first time by developing transfer protocols and testing of vectors with different oriV and promotor systems, confirmed by successful heterologous expression of target genes in all selected strains. Consequently, our findings corroborate the potential of these strains and lay ground for the construction of robust *chassis* platforms for biotechnological applications.

PA-2022-027

Powering Efflux: Deciphering the energetics of bacterial tolerance to platform chemicals of the plastics industry to enhance sustainable manufacturing

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Increasing global demand for high-clarity and high tensile strength plastic has led to industrial interest in a fermentation process for the production of monomers from non-

petrochemical sources. The main roadblock in a monomer fermentation is the toxicity of the solvent product to the production organism. *Pseudomonas spp.* are promising candidates as production organisms as their intrinsic levels of solvent tolerance have been widely demonstrated. Here, there is particular focus on the role of efflux in solvent tolerance. *Pseudomonas aeruginosa* PA14 transposon insertion mutants of RND pumps and transporters associated with various efflux systems show increased sensitivity to industrial solvents compared to the wild type. We have identified a group of membrane bound metalloproteins and an associated maturation system we believe drive RND efflux activity by maintaining a proton gradient within the cell. We demonstrate the purification of a variety of efflux pumps, associated metalloproteins and their maturation system that we believe are essential for solvent tolerance in PA14. The functional relationship between these systems is investigated using solid supported membrane electrophysiology (SSME). Using SSME, minute translocation of substrate can be measured through efflux systems while altering a range of conditions, providing an *in vitro* assay for transport measurement. We also demonstrate growth analysis of transposon mutants on proxy substrates to provide further insight into the role of these proteins in solvent tolerance. We are conducting a variety of chemical sensitivity assays to solvents such as styrene and the transcriptomic analyses of disrupting these systems are being determined using RNA sequencing. We therefore demonstrate biochemical, physiological and genetic approaches for the detection of tolerance conferring systems in *Pseudomonas spp*. Understanding the relationship between these systems will not only aid in the development of solvent production strains but may also provide a new drug target for resistant *Pseudomonas* strains.

SESSION VI: EVOLUTION AND COEVOLUTION

KEYNOTE

Jenna Gallie, PhD

Jenna Gallie is an evolutionary microbiologist. She completed her PhD in New Zealand in 2010, and then held two postdoc positions at the University of Washington and ETH Zurich. Since 2016, she has been running a research group at the Max Planck Institute for Evolutionary Biology in Ploen, Germany. Her group investigates fundamental evolutionary principles using microbes – mainly *Pseudomonas fluorescens.* Currently her work focuses on the evolution of genome content, with emphasis on tRNA gene sets and protein synthesis. She is also interested in genetic and molecular routes to biofilm formation in pseudomonads.

PA-2022-028

Adaptive flexibility in bacterial tRNA gene sets via large-scale duplications in *Pseudomonas fluorescens* SBW25

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Large-scale duplications are a highly dynamic class of mutation. They arise and are subsequently lost at rates far exceeding those typically observed for SNPs. The transient nature of large duplications means that their contribution to evolutionary processes is often overlooked. Our group is following the dynamics of adaptive, large-scale duplications in evolving populations of the model bacterium *Pseudomonas fluorescens* SBW25. We have passaged replicate lineages of two slow-growing SBW25 mutants – both lacking one or more transfer RNA genes – through 100 days (~750 generations) of evolution. We show that adaptation initially occurs via large-scale duplications, with each duplication fragment containing up to 16% of the wild-type chromosome and affecting the copy number of hundreds of genes (including some tRNA genes). As expected, these large-scale duplication fragments are highly unstable and, despite providing a significant growth advantage, are lost at high rates. Our ongoing analyses indicate that in each evolving population multiple duplication fragments arise and compete, with progressively shorter (and hence more stable) duplication fragments dominating over time. Together, our results demonstrate that large-scale duplications generate an unexpected degree of flexibility in genome (and tRNA gene) content at the population level, with the potential to influence evolutionary outcomes.

Pseudomonas aeruginosa adapts and evolves toward host restriction in the sinuses of people with cystic fibrosis

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Pseudomonas aeruginosa (*Pa*) undergoes a predictable route of adaptation to establish lifelong respiratory infections in people with cystic fibrosis (CF), yet little is known regarding how the size and structure of these microbial populations impacts their ongoing evolution. Genomes of host-restricted bacterial pathogens or symbionts are degraded relative to closely related free-living bacteria because their small population sizes support the increased effect of drift relative to selection. Three genomic changes that occur upon host restriction are: (1) reduced coding density due to increased pseudogene formation, (2) proliferation of IS elements, and (3) evolution of nutritional auxotrophies as genomes degrade and the host-restricted bacterium relies on supplementation by the nutrient-rich host environment. Examining longitudinal *Pa* isolated from the sinuses of 6 CF adults, we used genomics, phenotyping, and MiPACT-HCR imaging of *Pa* populations associated with explanted sinus tissue to test the hypothesis that the size and structure of *Pa* populations drives the mechanisms of genome evolution at play. We found that the most phenotypically host-adapted *Pa* lineages, all of which harbored mutator isolates, displayed the most genomic signatures of recent host restriction. In contrast, the least host-adapted lineage displayed the fewest, as did select laboratory strains originally isolated from acute infections. MiPACT-

HCR revealed that population size tracks with level of early genome degradation, with the most host-adapted, early genome-degraded *Pa* (the mutators) residing in small aggregates. These genomic changes strongly suggest that, following an initial period of adaptive evolution in response to strong selective pressures in the host, *Pa* persists in small, fragmented populations that are subject to stronger effects of genetic drift. Mutators are enriched under these conditions and lead to early stages of degenerative genome evolution. Our findings underscore the importance of infection site biogeography to pathogen evolution and the relevance of the sinuses to overall CF respiratory health.

PA-2022-030

Observation of social cheating in *Pseudomonas aeruginosa* biofilms

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Many bacteria employ a chemical communication system known as quorum sensing (QS), through which cells produce and sense diffusible signals that coordinate group behaviors in a cell density-dependent manner. Some QS-regulated secreted products benefit the entire bacterial community, and bacteria that produce these are cooperators. Individuals in the community that take advantage of communal resources without contributing to their production can gain a fitness advantage by avoiding the cost of synthesis. This behavior is known as social cheating, and it reliably evolves in *Pseudomonas aeruginosa* cultured in well-mixed laboratory conditions that require QS for growth. Cheating by *P. aeruginosa* growing in spatially structured aggregates (biofilms) is much less understood, despite aggregate growth being more characteristic of wild *P. aeruginosa* communities than planktonic growth. We have developed a continuous flow culture system that permits sequential imaging of growing *P. aeruginosa* aggregates and used it to characterize cooperator-cheater dynamics in individual aggregates tracked over time. This culture system offers advantages over conventional flow cell designs during prolonged growth at 37 °C and permits high-resolution confocal imaging using water-dipping optics that minimize spherical aberration. Using this method, we observed the formation and development of aggregates cultured in media requiring the production of elastase, a QS-regulated extracellular protease. In cultures inoculated with a mix of cheating and cooperating strains, aggregates that formed during the first few days of culture were dominated by cooperator cells, and aggregates formed later were dominated by cheater cells. Furthermore, cooperator-dominated aggregates

often developed strong spatial segregation of cooperators and cheaters as growth progressed. These findings indicate that cooperator-cheater interactions respond to spatial heterogeneity in the biofilm environment. Our ongoing work investigates how local heterogeneity in QS signal response and elastase abundance might lead to the community structures observed in this study.

PA-2022-031

Loss of flagellar motility enhances aggregation and drives antibiotic tolerance in *Pseudomonas aeruginosa*

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Muco-obstructive airway diseases (MADs) are a collection of diseases characterized by accumulation of mucus within the airways and heightened presence of the immune system, with neutrophils being among the most abundant cell type. Chronic bacterial infections, in which *Pseudomonas aeruginosa* (*Pa*) is the dominant pathogen, are common in people with MADs. Through unknown mechanisms, chronic *Pa* infections exhibit high rates of antibiotic treatment failure. Neutrophils contribute to lung pathology through degranulation and discharge of neutrophil extracellular traps (NETs), in which the serine protease, neutrophil elastase (NE), is the primary effector. During exacerbations of MADs, the presence of NE in the lung dramatically increases, although it is unknown what effect NE has on *Pa* treatment outcomes. We utilize a well characterized synthetic sputum medium (SSM) that recapitulates disease relevant concentrations of metabolites and mucus to study *Pa* biology. In SSM, we show that the addition of NE increases antibiotic tolerance to tobramycin. Addition of other NET effectors myeloperoxidase or lactoferrin had no effect on antibiotic tolerance. Previous work has shown that NE cleaves *Pa* flagellin. We show that deletion of *fliC*, which encodes the major flagellin subunit, also increases tolerance to tobramycin. This result sheds light on the observation that many chronically adapted clinical isolates of *Pa* exhibit loss of motility. Further investigation into how flagellar deficiencies increase antibiotic tolerance revealed that flagellar mutants of *Pa* exhibit significantly increased aggregation in SSM compared to wildtype. In agreement with this observation, the addition of NE to SSM also lead to an increase in aggregation. These results reveal a previously unknown role of the immune system in driving antibiotic tolerance of chronic *Pa* infection.

Transcription factor evolution is constrained by pre-existing gene regulatory network architecture

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Evolutionary change to transcription factors and gene regulatory networks (GRNs) frequently underscores adaptation to novel environments. Promiscuous transcription factor activity (or crosstalk) in a locally adapted setting is costly, however such crosstalk can facilitate adaptation by revealing new regulatory connections that, if beneficial, can be strengthened by selection. As such, conflict exists between the need for precise cognate gene regulation, which optimizes phenotype to an existing environment, and for promiscuous non-cognate interactions, which facilitates adaptation to a new environment. The driving factors determining when and where such promiscuity emerges within GRNs remains largely unknown, but essential to resolving this conflict. Here we show that a transcription factor's position within its GRN is key in creating and constraining opportunities for promiscuity and evolutionary innovation. Utilizing an elegant experimental model, we investigate the reliable and repeatable emergence of promiscuous transcription factor activity in the bacterium *Pseudomonas fluorescens* SBW25. In this model, the nitrogen regulatory protein NtrC is found to become promiscuous and rescue flagellar gene expression when an immotile strain lacking the flagellar regulator FleQ is placed under selection to swim. NtrC's homology to FleQ permits this promiscuity, but curiously only NtrC becomes promiscuous to the exclusion of the other 21 FleQ homologs in *P. fluorescens*. We utilize a Δ*fleQ* Δ*ntrC* knockout to unmask an additional novel regulator capable of rescuing motility via promiscuous activity and explore what biases the system towards NtrC promiscuity. Commonalities between these alternative evolutionary routes revealed that mutations remodeling global or local network structure facilitated the emergence of promiscuity in coopted regulators. This indicates pre-existing GRN structure determines which regulators are 'primed' for promiscuity and advances our understanding of the viability of a particular regulator for innovation beyond simple structural homology, with broad implications for understanding fundamental bacterial adaptation to novel environments.

SESSION VII: ECOLOGY

KEYNOTE

Elijah Mehlferber

Elijah obtained his Bachelor of Science in Biology in 2017 from the University of Georgia and is currently a PhD. Candidate in the Koskella lab at University of California Berkeley in the Integrative Biology Department. His doctoral work has focused on understanding the role of bacterial communities in providing functions for their host organism (primarily disease resistance), with a special focus on the contributions of individual bacterial traits to community composition and those associated community functions. He will be pursuing a postdoc this coming Fall in the Brown lab at Georgia Tech where his research will focus on understanding the traits that allow certain strains of *Pseudomonas aeruginosa* to succeed at invading novel environments.

PA-2022-033

Unraveling the role of the microbiome in resistance to *Pseudomonas syringae*

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The bacteria residing in the phyllosphere (the aboveground portion of the plant) can enhance resistance to invasion and infection by the foliar pathogen *Pseudomonas syringae*. However, the mechanisms underlying this resistance are complex, and can be mediated by both direct (competition between bacterial species) and indirect (influenced through the host immune system) inhibition of pathogen development. This talk covers three projects that our lab has undertaken in our work to disentangle these interactions. Firstly, focusing on the roles of bacterial community density and diversity, as well as host nutrient status on *P. syringae* resistance in tomato plants. Secondly, describing a consumer-resource modeling approach to understand the contribution of individual bacterial carbohydrate preferences to competition with and exclusion of *P. syringae*. And finally, outlining the shifting role that the phyllosphere community plays in *P. syringae* resistance across ploidy level in Arabidopsis, where diploids depend on their microbiota for inducing pathogen resistance while tetraploids are constitutively resistant regardless of their bacterial exposure.

PA-2022-034

The impact of a *Pseudomonas aeruginosa* phage on microbial community dynamics

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Bacteria possess a range of distinct immune mechanisms that provide protection against bacteriophages, including the mutation or complete loss of the phage receptor, and CRISPR-Cas adaptive immunity. Previously, we demonstrated that *P. aeruginosa* evolves increased levels of CRISPR-based immunity in the presence of an artificial community consisting of *Staphylococcus aureus, Acinetobacter baumannii*, and *Burkholderia cenocepacia*, all of which are opportunistic pathogens that cause severe infection. Yet little is known about how phages and the evolution of different phage resistance mechanisms affect the wider microbial community in which they are embedded. Here, we carry out a fully factorial 10-day *in vitro* evolution experiment using *Pseudomonas aeruginosa* PA14 and its lytic phage DMS3vir in the presence or absence of competitor bacterial species (*S. aureus*, *A. baumannii*, and *B. cenocepacia*) to measure the effect of phage on microbial community dynamics. Our results show that the microbial community structure is drastically altered by the addition of phage, with *A. baumannii* becoming the dominant species and *P. aeruginosa* being driven nearly extinct, whereas *P. aeruginosa* rapidly outcompetes the other species in the absence of phage. Moreover, we find that a *P. aeruginosa* strain with the ability to evolve CRISPR-based resistance generally does better when in the presence of *A. baumannii*, but that this benefit is largely lost over time as phage is driven extinct. Our data highlight how competitive release by phage may drastically alter community structure, facilitate secondary infection by other pathogenic species, and underline the importance of mapping community composition before therapeutically applying phage.

PA-2022-035

Genomic barcoding of *Pseudomonas aeruginosa* is a high-throughput technique for studying evolutionary dynamics with single-cell resolution

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To understand the rules that govern early evolution dynamics, better tools are needed for tracking the fates of individual cells. Toward that goal, we have developed an innovative genomic barcoding technique that has been successfully implemented in *Pseudomonas aeruginosa*, allowing for single-cell resolution of intraspecies population demography over time. Our method is high-throughput, only requires a small sample volume comprised of whole cells, supports frequent sampling of continuous cultures, and allows many samples to be multiplexed into a single next-generation sequencing run, saving time and money while allowing for single-cell lineage tracking. Currently, we are using it to compare the early dynamics of planktonic and biofilm-based cultures, with a special focus on the factors that govern the rate of lineage loss in the first few hours and days of experimental evolution. Our Tn7-based barcoding method is readily adapted for use in other microbial species, and this technology therefore holds enormous promise for hosting complex competition assays that seamlessly incorporate intraspecies and interspecies diversity in the same test tube, bringing us one step closer to recapitulating and examining the complexities of microbial communities. Our chromosomal barcoding method is a versatile and practical platform for understanding early evolution dynamics, and this work furnishes the field with a valuable new tool for the study of microbial diversity and demography.

PA-2022-036

Prevalence and conditions for emergence of Small Colony Variants (SCVs) among *Pseudomonas aeruginosa* strains

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An alternative phenotype of *Pseudomonas aeruginosa*, the Small Colony Variant (SCV) is often isolated from colonized individuals. SCVs possess distinctive features such as higher adherence, efficient biofilm formation and reduced motility. However, conditions promoting the emergence of SCVs are unknown. We have found that oxygen limitation promotes the conversion of the prototypical strain PA14 to a SCV morphotype under controlled laboratory conditions. Oxygen limitation is notably found in microenvironments colonized during chronic infections, such as the lungs of people with cystic fibrosis. More

precisely, we observed that a shortage in electron acceptors rather than a specific oxygen limitation is responsible for the emergence of SCVs. Absence of electron acceptors results in increased concentrations of NADH and an imbalance in the intracellular redox state. The emergence of SCVs coincides with a rebalancing of the redox state. We also investigated the emergence of SCVs in strains from environments other than clinical such as soil, water, food products, and built environments. The ability to produce a SCV morphotype was observed for all the *P. aeruginosa* isolates from the various environments, when cultivated under appropriate oxygen-limiting conditions. These results demonstrate that the ability of *P. aeruginosa* to form SCVs is naturally widespread, and not restricted to the clinical context. Interestingly, sequencing of the genomes of SCVs from three distinct isolates from different environments revealed strain-specific mutations in genes implicated in the metabolism of the second messenger c-di-GMP. All together, these results indicate that the SCV morphotype is widely adopted by the *P. aeruginosa* species, and likely promoted by an imbalance in the redox state resulting from a low availability of electron acceptors, such as conditions present in biofilms. The emergence of the SCV morphotype in diverse environments could participate in the versatility of *P. aeruginosa* by allowing the bacteria to rapidly adapt and persist.

PA-2022-037

Plant-responsive bacterial signaling systems in *Pseudomonas*

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We are interested in the fundamental question of how bacteria sense plant-released chemicals. In our studies of the root microbiome of cottonwood tree we identified an effector sensed by an endophytic isolate of *Pseudomonas.* This compound, *N*-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide (HEHEAA), is derived from ethanolamine. The receptor for HEHEAA belongs to a plant-responsive PipR family of transcription factors that is present in many plant-associated proteobacteria. PipR mediates gene regulation in our isolate *Pseudomonas* GM79, and the PipR-controlled regulon includes genes important for peptidase activity and anaerobic growth with nitrate as the terminal electron acceptor. Import and delivery of HEHEAA to PipR requires an

ATP-binding cassette (ABC)-type transporter and the genes encoding this transporter are adjacent to *pipR*. We determined crystal structures of the periplasmic substrate-binding protein (SBP) component of the transporter in both the HEHEAA-bound and unbound states. We discovered that a closely related SBP from the plant pathogen *Pseudomonas syringae* pv tomato DC3000 does not recognize HEHEAA. However, a single site-directed amino acid substitution in the *P. syringae* SBP converted it to a weak HEHEAA-binding protein. We reason that the *P. syringae* effector is similar, but not identical, to HEHEAA. PipR-like systems are widespread amongst and limited to plant-associated Proteobacteria. Understanding the molecular details of these plant-responsive systems could identify a means of controlling plant colonization.

PA-2022-038

Polyamines released by lysed bacteria induce *Pseudomonas aeruginosa* phage tolerance via the Gac/Rsm pathway

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In response to continued pressure from phage infection, bacteria have evolved diverse phage-defense mechanisms. We hypothesized that lysed bacteria release a danger signal that induces phage defense mechanisms in neighboring bacterial cells. To test this hypothesis, cell-free lysates were prepared from *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Burkholderia* spp. *P. aeruginosa* grown on LB agar plates prepared from bacterial lysates were tolerant to several species of phage compared to standard LB agar plates. The active cell lysate fraction that induced phage tolerance was water soluble, small (<3 kDa), and survived autoclaving. Previous work demonstrates that an uncharacterized danger signal present in *P. aeruginosa* lysates signals through the Gac/Rsm regulatory system to activate type VI secretion-mediated killing of non-kin bacteria. We find that disabling the Gac/Rsm pathway (Δ*gacS*, Δ*rsmY*, or Δ*rsmZ*) eliminated the phage tolerance phenotype induced by the active lysate fraction. RNA-seq transcriptional analyses of *P. aeruginosa* exposed to the active lysate fraction revealed the upregulation of several genes related to polyamine transport and metabolism. Polyamines are small polycations that are well conserved across all cellular life. In Bacteria, polyamines can be present at high intracellular concentrations (~1-10 mM). Addition of mM levels of the polyamines putrescine, spermidine, or spermine to agar plates promoted phage tolerance in wild-type *P. aeruginosa*, but not in bacteria where Gac/Rsm signaling was disabled. Collectively, our results suggest polyamines released by lysed bacteria promote transient phage tolerance in *P. aeruginosa* through

the Gac/Rsm pathway. Transient tolerance to phage infection has implications for phage therapy. For example, phage mediated lysis could produce phage tolerant bacteria that give rise to isolates with fixed phage resistance mutations.

SESSION VIII: MULTI-SPECIES INTERACTIONS

KEYNOTE

Jessica Scoffield, PhD

Jessica Anne Scoffield, PhD, is an Assistant Professor of Microbiology at the University of Alabama at Birmingham (UAB) School of Medicine. Dr. Scoffield received her Bachelor's (2002) and Master's (2004) degrees in Biology from Tuskegee University and her doctoral degree in Microbiology from Auburn University in 2012. Her dissertation research explored the role of glycerol metabolism on *Pseudomonas aeruginosa* adaptation in chronic infection. Dr. Scoffield completed her postdoctoral training in the laboratory of Dr. Hui Wu in the Department of Pediatric Dentistry at UAB where she developed expertise in oral microbiology. Following the completion of her postdoctoral training, Dr. Scoffield accepted a primary appointment in the Department of Microbiology at UAB in 2018 as an Assistant Professor. Dr. Scoffield also holds secondary faculty appointments in the Division of Pulmonary, Allergy, and Critical Care Medicine, Pediatric Dentistry, and the Gregory Fleming James Cystic Fibrosis Research Center at UAB. Dr. Scoffield's laboratory studies the role of oral commensal bacteria in polymicrobial lung and oral infections. Her research studies have led to the discovery of novel antimicrobial mechanisms used by commensal bacteria that inhibit the major cystic fibrosis lung pathogen *Pseudomonas aeruginosa* and the oral pathogen *Streptococcus mutans*. Dr. Scoffield's laboratory is funded by a R00 from the National Institute of Dental and Craniofacial Research and a R35 from the National Institute of General Medical Sciences.

PA-2022-039

Interactions between *Pseudomonas aeruginosa* and Commensal Streptococci

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Chronic lung infections with *Pseudomonas aeruginosa* remain the major cause of lung decline and mortality in individuals with cystic fibrosis (CF). CF is a genetic, multi-system disorder caused by mutations in the transmembrane conductance regulator (CFTR) gene. Defects in the CFTR protein inhibit proper mucociliary clearance of microbes from the lung, and as a result, multiple species of bacteria colonize the airway and establish decade-long polymicrobial infections. Recent findings obtained from microbiome studies report that oral commensal streptococci are prevalent in the CF lung and their existence correlates with improved lung function. *Streptococcus salivarius* is the most abundant

oral commensal associated with beneficial health outcomes in CF lung disease. However, little is known about how *S. salivarius* incorporates into the polymicrobial CF airway and interacts with the major CF pathogen *P. aeruginosa* to potentially improve lung function. Using two-species *in vitro* and *in vivo* biofilm models, we have discovered that the *P. aeruginosa* exopolysaccharide Psl promotes *S. salivarius* biofilm formation. Further, we identified a *S. salivarius* maltose-binding protein (MalE) that is required for commensal promotion of biofilm formation both *in vitro* and in a *Drosophila melanogaster* co-infection model. Finally, we demonstrate that promotion of dual biofilm formation with *S. salivarius* is common among environmental and clinical *P. aeruginosa* isolates. Overall, our data support a model in which *S. salivarius* uses a sugar-binding protein to interact with *P. aeruginosa* exopolysaccharide, which may be a strategy by which *S. salivarius* establishes itself within the CF airway microbial community.

PA-2022-040

Commensal bacteria of the lung microbiota inhibit the production of *Pseudomonas aeruginosa* virulence factors

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It is increasingly recognized that interspecies interactions may modulate the pathogenicity of *Pseudomonas aeruginosa* during respiratory infections. Nevertheless, most studies have focused on the interaction between *P. aeruginosa* and a small number of pathogenic microorganisms co-infecting the airways of people with chronic lung disease. Little is known about the influence of other members of the lung microbiota on the infection process of *P. aeruginosa*. The present study aims at identifying beneficial bacteria in the lung microbiota that are able to interfere with the virulence of *P. aeruginosa*. A comprehensive collection of bacteria was cultured from 15 sputum samples of cystic fibrosis patients chronically colonized with *P. aeruginosa*. High-throughput screening of these isolates for their ability to downregulate the expression of relevant virulence-related genes was performed using a series of GFP-based

transcriptional reporters of *P. aeruginosa* genes. Virulence gene expression (fluorescence) and growth (optical density) of the reporter strains in the presence of cell-free spent media (CFSM) obtained from stationary-phase cultures of individual microbiota members were simultaneously monitored. Among 32 bacterial species tested, we found that the CFSM from *Prevotella intermedia* and *Prevotella nigrescens* significantly decreased the expression of *lasB* (elastase), *rhlA* (rhamnolipids), *phzA1* (phenazines) and *hcnA* (hydrogen cyanide) by *P. aeruginosa*, with no impact on growth. This correlated with the ability of the CFSM to inhibit protease production by *P. aeruginosa*, as determined with an azocasein assay. Comparable anti-virulence activity was observed in all tested CF isolates of *P. intermedia* and *P. nigrescens*, indicating that this effect is not isolate-specific. Interestingly, several reports indicate that the abundance of anaerobes such as *Prevotella* sp. In the lung microbiota is associated with positive health outcomes in people with chronic lung disease. Our results suggest that commensal bacteria with anti-virulence properties in the lung may downregulate the pathogenicity of *P. aeruginosa*.

PA-2022-041

A semi-synthetic microbial community resists *Pseudomonas*-invasion by community-level resistance towards *Pseudomonas*-produced secondary metabolites

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The role of antagonistic secondary metabolites produced by the model biocontrol species, *Pseudomonas protegens*, in suppression of soil-borne phytopathogens has been clearly documented, whereas their contribution to the ability of *P. protegens* to invade natural soil and rhizosphere microbiomes remains less clear. In the present study we used a four-species synthetic community of naturally co-existing bacteria cultivated *in vitro* in an artificial soil to determine the effects of *Pseudomonas*-produced metabolites on the ability of *P. protegens* to invade the community. Secondly, we used the system to identify community traits that affect the secondary metabolism of *P. protegens*. Initially, we showed that *P. protegens* readily invaded and altered the community composition, and that invasion into the community led to altered production of key antimicrobial metabolites (DAPG, pyoluteorin and orfamide A) in *P. protegens*. Unexpectedly, mutants unable to produce these antibiotics invaded the community as

efficiently as the wildtype did. We found that the *Pseudomonas*-produced antibiotics pyoluteorin and orfamide A could reach levels that were toxic to individual community members, suggesting the existence of community-level resistance mechanisms towards the toxic metabolites. For the cyclic lipopeptide, orfamide A, we identified the underlying resistance mechanism by which the Gram-positive *Rhodococcus globerulus* D757 enzymatically inactivated the antibiotic. Furthermore, we observed degradation of the inactivated orfamide A by one or more additional community members, which could indicate a novel community-level catabolism of an antimicrobial secondary metabolite. Altogether, the demonstration that the synthetic community had mechanisms for constraining or dampening invasion by means of detoxifying an antibiotic might provide a mechanistic explanation to inconsistencies in biocontrol effectiveness *in situ*.

PA-2022-042

Leveraging the soil-clinic axis to develop novel therapeutics for chronic infections

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Pseudomonas aeruginosa survives in chronic infections when challenged with antibiotics by forming robust biofilms. Redox-active small molecules known as phenazines are synthesized by *P. aeruginosa* and are critical to its ability to develop a biofilm. One such phenazine, pyocyanin (PYO), is blue in color and can be found in clinical samples from chronic infections. *P. aeruginosa* strains that cannot synthesize PYO fail to form biofilms and have a lower fitness *in vivo*. We hypothesize that depleting this small molecule from wounds presents a new method of treating *P. aeruginosa* infections. To test this hypothesis, we searched for phenazine degraders in the soil and isolated an organism (*Mycobacterium fortuitum*) that could degrade phenazines. We identified a PYO-demethylase enzyme, PodA, that demethylated PYO and generated 1-hydroxyphenazine, which blocked biofilm development. Our ability to study the therapeutic potential of PodA was initially hindered by low purification yields. With the Fleishman lab at the Weizmann Institute of Science, we used protein design to increase PodA yield by 20-fold, which allowed us to study the effects of a PodA/tobramycin mixture on *P. aeruginosa* biofilms. We applied PodA and tobramycin, separately and in combination, to *P. aeruginosa* mature biofilms and found that combined PodA and

tobramycin treatment reduced cell survival by 10-fold compared to tobramycin or PodA alone, a classic indication of synergy. We performed these treatments on biofilms grown in either a standard rich medium (Lysogeny Broth) or a medium that mimics chronic lung infections (synthetic cystic fibrosis medium) and observed similar results. Further analysis of designed PodA stability showed no change in activity with pH (pH 5-9), salt (10-500 mM), or viscosity (1-20%, v/v) and PodA remained 100% active after 30 minutes of boiling. This enzyme's robustness is promising for application in a complex environment, such as a chronic infection.

PA-2022-043

Predicting lung health from cystic fibrosis lung microbiome data

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Microbiome sequencing has highlighted the polymicrobial context of chronic infections. *Pseudomonas aeruginosa* is the most dominant pathogen found in cystic fibrosis (CF) lung microbiomes. Here, we investigate the relationships between microbiome structure, pathogen abundance and overall host health. We collected sputum samples and clinical information for a cohort of 77 children and adults with CF during a period of clinical stability. We used 16S sequencing to determine sputum microbiome profiles and trained linear models with ElasticNet regularization to identify microbial predictors of lung function. Our models identified key pathogens as negative health predictors (*Pseudomonas, Achromobacter*) as well as non-pathogenic taxa independently predictive of lung health. These results support the consideration of non-pathogenic taxa in the clinical assessment of CF patient health. For future work, we will evaluate models predicting CF pathogen abundance, providing insight into how *Pseudomonas* presence alters the composition of non-pathogenic taxa in the infection microbiome.

SESSION A: POSTER PRESENTATIONS

PA-2022-A001

Impact of RND efflux pumps deletion on *Pseudomonas aeruginosa* physiology

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Pseudomonas aeruginosa is one of the major opportunistic pathogens and a leading cause of nosocomial infections that are difficult to treat because of its intrinsic and acquired antibiotic resistance. Synergy between low permeability barrier of the outer membrane and transporters belonging to the resistance-nodulation-division superfamily (RND) of proteins cause the intrinsic multidrug resistance and thus reduce the intracellular accumulation of compounds and the efficacy of antimicrobial agents but also limit the choices of therapy against *P. aeruginosa*. To characterize physiological changes induced by inactivation of efflux pumps we applied transcriptomics, metabolomics, genetic, and analytical approaches. We focused on *P. aeruginosa* PAO1 wild type, and its efflux deficient PΔ6 strain lacking six major, and best described RND efflux pumps (*mexAB-oprM*, *mexCD-oprJ*, *mexJK*, *mexEF-oprN*, *mexXY* and *triABC*). Loss of active efflux generated specific physiological and transcriptomic response that helped to adapt and protect *P. aeruginosa* cells in the absence of active efflux. It included changes in transport activities across the cell envelope, cell-to-cell communication and environmental responses. PΔ6 cells accumulated increased amounts of Pseudomonas Quinolone Signal (PQS), were stimulated to activate specific iron acquisition pathways, and secreted increased amounts of siderophores like pyochelin and pyoverdine. In conclusion, RND-type efflux transporters have a major effect on the physiology of *Pseudomonas aeruginosa.*

PA-2022-A002

Synthetic serotype switching from O4 to O12 reveals serotype specific adhesion and biofilm phenotypes

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Evolution of the highly successful and multidrug resistant clone type ST111 in *P. aeruginosa* has previously been shown to include the acquisition of the serotype O12 biosynthetic gene cluster, replacing the native serotype O4 BGC. The serotype of a strain relates to the structure of the O-specific Antigen, which is a constituent of the outer leaflet of gram-negative bacteria, also known as the lipopolysaccharide. While the different facets of the lipopolysaccharide has been studied for well over 60 years, the role of the 20 known O-specific Antigen variants has been little explored. The objective of this project is to determine how and why serotype O12 has become more successful than the ancestral O4 lineage, and if it is related to the O-specific Antigen structure.
In this study, we present the cloning and expression of serotypes O4 and O12 in isogenic strain derivates of PAO1, PA14 and ST111. We use recombineering for capturing and transferring the large serotype BGCs to genomic tagging plasmids that can facilitate synthetic serotype switching. We then characterized these strains using clinically relevant phenotypic tests to assay antibiotic susceptibility, adherence to surfaces, biofilm quantity, and more, in order to determine the impact of a serotype switch. We find that serotype O12 isolates has different abilities for surface adherence and biofilm formation compared to O4 isolates. In addition, serotype O12 isolates form higher quantities of biofilm in a centrifugal microfluidic system. Based on these observations, we believe that serotype O12 could have an advantage in terms of persistence in a clinical setting, which explains the clonal success of serotype O12 ST111.

PA-2022-A003

On the path to elucidation of cell-wall degradome of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic pathogen that is the most prevalent in the hospital setting. Approximately 51,000 *P. aeruginosa* infections are reported in the U.S. alone each year, and more than 13% of these infections have been identified as multi-drug-resistant strains, according to the CDC. Cell wall in pseudomonas is turned over by as much as 50% during homeostatic growth. Furthermore, it undergoes repair after exposure to cell-wall-active antibiotics. The health of the organism depends on maintenance of a functional cell wall, a process governed by a complex set of events involving dozens of enzymes. Lytic transglycosylases (LTs) are enzymes that facilitate cell-wall degradation and recycling by catalyzing the non-hydrolytic cleavage of the cell-wall peptidoglycan. It is understood that these periplasmic enzymes work in concert with many other proteins, an assembly that we refer to as the cell-wall degradome. Rare lipoprotein A (RlpA), one of the 11 LTs of *P. aeruginosa*, is involved in the process of septation during bacterial growth. We have identified the binding partners of RlpA using a pull-down strategy, followed by proteomics analysis. We have cloned and purified the putative recombinant partner proteins detected in our mass-spectrometry assays and have explored binary and ternary interactions with RlpA using Microscale Thermophoresis (MST) to obtain dissociation constants (K_D). Additionally, we have assessed the rate constants of association (k_{on}) and of dissociation (k_{off}) for binary complexes between RlpA and the binding partners by Surface Plasmon Resonance (SPR). These analyses reveal a minimum of 20 partners for RlpA, whose physiological roles are under investigation presently.

PA-2022-A004

Mechanisms regulating the cellular level of the chromosome segregation protein ParB in *Pseudomonas aeruginosa*

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The *Pseudomonas aeruginosa* (Pa) ParB protein is a component of the bacterial chromosome segregation system (ParAB-*parS*). While recent works, concerning ParBs from different bacteria, are focused on additional biological roles of ParBs and the molecular basis of ParBs functioning, little is known about the mechanisms controlling ParB level in bacterial cells. In this study, we present the results on Pa PAO1161 ParB turnover. Using the anti-ParB antibodies we have demonstrated that the number of ParB monomers/c.f.u. decreases during culture growth. It suggests that ParB level is affected

by transcriptional regulation and/or ParB degradation. In Pa chromosome, *parA* and *parB* are preceded by *gidA* and *gidB* (glucose-inhibited division) genes forming the *gidA-gidB-parA-parB* operon what has been confirmed by deletion analysis and RT-PCR. Two promoters of this operon have been identified: p1 located upstream of *gidA* in close proximity of DnaA boxes, and internal promoter p2, located in the *gidB*. The role of DnaA in the regulation of expression of *the gidA-gidB-parA-parB* operon has been analyzed. ParB is also clearly degraded in the late stationary phase by serine protease. To identify protease(s) responsible for ParB degradation a library of PAO1161 deletion mutants in the defined and putative proteases encoding genes was constructed. Their analysis pointed out two proteases, ClpP1X complex and Lon1 as those involved in *Pa* ParB cell cycle-dependent degradation. In this work, we identified the crucial elements regulating the level of ParB. Further investigations will be conducted to precisely determine their roles in the ParB turnover. This work was financed by the Polish National Science Centre, grant no 2018/29/B/NZ2/01745.

PA-2022-A005

The role of calcium leak channel, CalC, in transcriptional regulation of virulence and antibiotic resistance of *Pseudomonas aeruginosa* in response to calcium

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Calcium (Ca) is an important signaling ion regulating many biological functions, both in eukaryotic and prokaryotic cells. Disruptions in cellular Ca homeostasis has been shown to be the cause or the outcome of diseases in humans. One such disease, cystic fibrosis (CF), results in increased levels of Ca in nasal and pulmonary fluids in those afflicted. In previous studies, our group showed that *Pseudomonas aeruginosa* increases the production of virulence factors when grown at clinically relevant levels of Ca. Such virulence enhancement along with biofilm induction may contribute to why *P. aeruginosa* is the predominate cause of death in CF patients. Previously we have identified a calcium leak channel, named CalC, in *P. aeruginosa* and showed that the channel plays a key role in the influx of Ca into the cytoplasm of the organism. By using the R-GECO1 fluorescent probe, we are currently studying the impact of pH on CalC-dependent translocation of Ca. Genome-wide RNA-Seq analysis of wild type PAO1 and *calC* deletion mutant grown at elevated Ca revealed that CalC is involved in regulation of 73% of Ca-regulated genes, which in turn represent ~25% of the genome. The most

represented functional categories include phosphorus metabolism and transport, stress responses, transport, antibiotic resistance, and secreted factors. So far, by using RT-qPCR, we have validated that Ca-induction of phospholipases requires CalC. We also showed that Ca-dependent increase in tobramycin resistance requires CalC, which likely depends on the expression of *mexAB* RND transporter. Finally, by using *Galleria melonella* as an animal model, we showed that the Ca-regulated enhancement in virulence also requires CalC. Overall, this study establishes the regulatory role of the intracellular Ca in bacteria that requires CalC and shows the role of the channel in the Ca regulation of *P. aeruginosa* virulence and antibiotic resistance.

PA-2022-A006

Iron-dependent differential regulation of T6SS in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that causes chronic lung infection in immunocompromised hosts. *P. aeruginosa* requires iron to cause infection, and induces numerous virulence genes in response to iron deprivation. Recently, our lab showed that some virulence factors are differentially regulated by iron in shaking versus static growth conditions, the latter of which more closely mimic growth in the chronic-infected lung. One of these factors is the HS2-type six secretion system (T6SS), a sub-class of T6SS that is induced during chronic infection. HS2-T6SS is upregulated by iron depletion in static, but not in shaking cultures. This regulation is partially dependent on the iron-responsive PrrF sRNAs and the PQS quorum sensing pathway. In the current study, we analysed the impact of PqsA, PrrF, and static growth on the RsmY and RsmZ sRNAs that sequester RsmA, a global regulator that acts as a translation inhibitor of T6SS. We show that iron starvation upregulates the RsmY and RsmZ sRNAs but only under static conditions. This regulatory effect is interrupted in *pqsA* mutant background in which PQS is not produced. Consistent with the impact of PrrF sRNAs on HS2-T6SS, we also show that iron regulation of RsmY and RsmZ is partially dependent on PrrF sRNAs. We are currently exploring the impact of this newly identified regulatory pathway on T6SS expression through the use of transcriptional and translational gene expression reporters. We propose that this differential gene expression leads to phenotypic diversion in *P. aeruginosa* which promotes their adaptation to the lung environment during chronic infections.

In vitro analysis of colistin and ciprofloxacin antagonism of phage infection activities against antibiotic-sensitive *Pseudomonas aeruginosa*

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Phage therapy is a century-old technique employing viruses (phages) to treat bacterial infections. In the clinic, phage therapy often is used in combination with antibiotics, including against *Pseudomonas aeruginosa* infections. Antibiotics, however, interfere with critical bacterial activities, such as DNA and protein synthesis, which also are required for phage infection processes. Antibiotic antagonism of phage infection activities nevertheless is not commonly determined in phage therapy studies using standard, planktonic approaches. Here we assess the impact of two antibiotics, colistin and ciprofloxacin, on the bactericidal, bacteriolytic, and new virion production activities of *P. aeruginosa* phages using an optical density-based 'lysis profile' assay. This involved combination treatment of colistin- and ciprofloxacin-sensitive strains PAO1 and a primary wound isolate, in Mueller-Hinton broth. Though phage-antibiotic combinations were more potent in reducing *Pseudomonas* counts than phages or antibiotics alone, colistin substantially interfered with phage PEV2 bacteriolytic and virion-production activities even at minimum inhibitory concentration (MIC). Ciprofloxacin, by contrast, had no such impact at 1× MIC or 3× MIC. We corroborated these lysis profile results by more traditional measurements (colony forming units, plaque forming units, one-step growth experiments) and using two other phages. At higher but still clinically relevant ciprofloxacin concentrations (9× MIC), burst sizes were still significant (~30 phages/infected bacterium rather than 135 without antibiotic present). To our knowledge this is the first study in which detailed antibiotic impact on *P. aeruginosa* phage infection activities has been determined under conditions similar to those used to determine antibiotic MICs and could point especially to ciprofloxacin as a minimally antagonistic phage therapy co-treatment of *P. aeruginosa* infections. More generally, our results point to a utility to routine testing of antibiotic antagonism on phage treatment activities under simplified laboratory conditions as antibiotics may interfere with the activity of treatment phages including to unexpected degrees (colistin).

Using airway models to profile the spatial and temporal dynamics of *Pseudomonas aeruginosa* infection

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Cystic fibrosis (CF) and chronic obstructive pulmonary disease are progressive, life-shortening lung diseases, characterized by production of a thick layer of viscous mucus in the lungs. The mucus build up in the lungs offer favourable colonization environments for several opportunistic pathogens, including *Pseudomonas aeruginosa* (*Pa*), which is associated with poor prognosis and increased mortality rates in both patient groups. This project aims to analyse individual host-pathogen interaction profiles, which may shed light on the dynamic interplay between the human host and *Pa* and clarify the establishment and progression of persistent bacterial infections. By collecting pluripotent basal cells from the airways, we have generated differentiated cell cultures; organoids and Air-Liquid interface, mimicking the pseudostratified epithelia of the airways. Using the *Pa* reference strain, we have determined a baseline for the colonization process and host response measuring epithelial permeability, bacterial growth, tissue damage and activation of the innate immune response. Using confocal microscopy, we have visualized bacterial attachment, colony formation and biofilm establishment. Previous work from our group have shown that clinical strains of *Pa* collected from patients with CF show high prevalence for mutations in the efflux pumps. These strains do not show phenotypic increase in antibiotic resistance, which would otherwise explain this prevalence. To study fitness advantages associated with mutations in the efflux pumps we generated a MexXY pump overexpression mutant. Using our infection models we have found that strains overexpressing the MexXY pump show an altered colonization progress compared to the wild type strain. We find that the mexXY mutant mainly form colonies within the epithelial layer, whereas the wild type are colonizing the apical surface. This may explain the prevalence of efflux pump mutations seen in clinical *Pa* isolates, since the intra epithelial localization will provide a fitness advantage due to the protective environment and reduced antibiotic exposure.

The NAD(P)H quinone oxidoreductase NQO increases the virulence of carbapenem resistant OprD-defective *Pseudomonas aeruginosa* in human airway epithelium

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Pseudomonas aeruginosa (PA) is an opportunistic pathogen causing chronic lung infections in mechanically ventilated patients, people with cystic fibrosis and chronic obstructive pulmonary diseases. Recently, it has been shown that a strain of PA with a mutated OprD porin (*oprD* mutant) had increased fitness and virulence in mouse models of intestinal colonization and acute pneumonia. As carbapenems use the OprD porin to enter the bacterial cytoplasm, mutations leading to loss of the OprD porin confer carbapenem resistance, one of the treatments of last resort for antibiotic-resistant PA. The aim of our study was to assess the virulence of a PA *oprD* mutant on Human Airway Epithelia (HAE). Fully differentiated epithelia were obtained from primary cells cultured at the air-liquid interface that were infected with carbapenem-susceptible WT and a carbapenem-resistant *oprD* mutant PA strain. The dynamics of epithelial destruction was studied using time-lapse microscopy. Epithelia were damaged by the *oprD* mutant PA strain after 40.9 ± 2.7 h of exposure vs 54.7 ± 3.9 h for the WT PA strain (P=0.019), indicating a higher virulence of the *oprD* mutant strain. This higher virulence was due to enhanced multiplication of the *oprD* mutant during infection. RNA sequencing and transcriptomic analyses to compare the gene expression of the 2 strains grown on the HAE revealed an overexpression in the *oprD* mutant of the NAD(P)H quinone oxidoreductase NQO (EDGE test, fold change > 2, $P < 0.05$), a key player in the response of *P. aeruginosa* to antibacterial oxidative stress. Thus, our findings showed that a PA *oprD* carbapenem-resistant mutant is more destructive to the HAE owing to a better response to oxidative stress. This study will extend our knowledge of PA pathogenesis and may form the basis for designing effective interventions to reduce epithelial cell destruction in chronic PA infection.

PA-2022-A010

Pseudomonas aeruginosa RpoS and RpoN influence biofilm metabolism and antibiotic tolerance via differential effects on phenazine synthesis

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Pseudomonas aeruginosa is a biofilm-forming opportunistic pathogen that establishes chronic infections in immunocompromised individuals. Within biofilms, the formation of chemical gradients leads to physiological differentiation that is associated with enhanced antibiotic tolerance. Previous studies have shown that the production of phenazines, endogenous pigments that act as alternate electron acceptors, promotes metabolic activity in hypoxic biofilm subzones and increases *P. aeruginosa* antibiotic tolerance. The *P. aeruginosa* phenazine biosynthetic pathway is well-understood and involves a branchpoint in which phenazine-1-carboxylic acid is either aminated (to produce phenazine-1-carboxamide (PCN)) or methylated (to produce 5-methyl-PCA (5-Me-PCA) and pyocyanin (PYO)). The goals of this project have been to elucidate mechanisms regulating the decision to aminate or methylate PCA and to investigate how this decision affects biofilm physiology. Because phenazines influence biofilm metabolism, we reasoned that metabolic regulators might control PCA modification and sought to test whether specific phenazine derivatives, with unique chemical properties, have differential effects on biofilm physiology. Here, we show genetically that two major sigma factors, RpoS and RpoN, antagonistically regulate the carbon catabolite repressor protein (Crc) to control the methylation of PCA. Further, utilizing biofilm thin-sectioning and stimulated Raman spectroscopy, we show that the production of methylated phenazines specifically enables increased metabolic activity in microaerobic or anaerobic zones within colony biofilms. Finally, we present results indicating that the amination of PCA to produce PCN is biofilm-specific and increases the tolerance of biofilm cells to the antibiotic ciprofloxacin. Our results demonstrate that the decision to modify PCA by two competing branches of phenazine biosynthesis is antagonistically regulated by RpoS and RpoN and that this has dramatic consequences for biofilm metabolism and antibiotic tolerance.

PA-2022_A011

Integrating multi-omics data with genome scale metabolic modeling for the analysis of *Pseudomonas aeruginosa* persister cells

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Pseudomonas aeruginosa is a Gram-negative multi-drug resistant bacterial pathogen, capable of forming environmentally-resistant subpopulations known as persister cells. These cells are transient phenotypic variants that are able to tolerate antimicrobial treatment and have been implicated in the recalcitrant nature of chronic infections and in the resistance to disinfection. While persister cells are classically associated with reduced metabolic activity, the characteristics of their metabolism are not well understood. In this work, we performed an experimental and computational systems-level analysis to characterize the metabolic state of persister cells. To accomplish this, we deeply profiled both wildtype and persister samples of *P. aeruginosa* with transcriptomic sequencing and metabolomic analyses. This revealed a distinct metabolic repertoire in persister cells, marked by an increase in central metabolism activity. To aid in the analysis, integration of both the transcriptomic and metabolomic datasets with a *P. aeruginosa* genome-scale metabolic network reconstruction (GENRE) provided condition-specific models of the persister and untreated states. We then used the model of persister cell metabolism to hypothesize single-gene targets of persister cell viability. Experimental testing of model predictions suggested that persister cells are robust to single-gene deletions and that combinatorial targeting strategies may be necessary to completely inhibit the persister phenotype. Using this approach, we gained insight into *P. aeruginosa* persister cell metabolism and highlighted possible combinatorial gene targeting strategies to inhibit the development of the persister cell phenotype.

PA-2022-A012

Kinetic and bioinformatic characterization of D-2-Hydroxyglutarate dehydrogenase: a therapeutic target for *Pseudomonas aeruginosa* PAO1

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Pseudomonas aeruginosa causes diseases in human, animals, and plants. Nosocomial infections of *Pseudomonas* in humans are primarily controlled with antibiotics but developed multidrug resistance of bacteria results in >35000 recorded death each year in the US. With >600 complete genomes in the *Pseudomonas* databank, new essential biochemical pathways can be identified to combat *P. aeruginosa* infections through the development of novel inhibitors. L-Serine is a precursor for the biosynthesis of glycine, cysteine, tryptophan, and phospholipids, and is indispensable for the survival of *P. aeruginosa*. In the first step of L-serine biosynthesis the thermodynamic unfavorable oxidation of D-3-phosphoglycerate to 3-phosphooxopyruvate catalyzed by D-3-phospohoglycerate dehydrogenase is driven by the reduction of 2-ketoglutarate to D-2-hydrohyglutarate. The depletion of the Krebs cycle intermediate 2-ketoglutarate is then avoided by D-2-hydroxylglutarate dehydrogenase (D2HGDH), which regenerates 2-ketoglutarate form D-2-hydroxyglutarate. *Pseudomonas* has no compensatory activity for D2HGDH, as D2HGDH gene knockouts stop growth of *P. aeruginosa* and *P. stutzeri*. To develop structure-function informed inhibitors of D2HGDH, we have identified in the genome of *P. aeruginosa* strain PAO1 a gene (PA0317) that may encode D2HGDH. Recombinant PA0317 was cloned, expressed and purified, and shown to be a D2HGDH through biochemical and bioinformatic approaches, including steady-state kinetics, mass spectrometry, fluorescence, sequence-function relationships, phylogenetic analysis, and homology modeling. The enzyme contains FAD and displays a k_{cat} value of 11 s^{-1} and a K_m value of 60 μM with D-2-hydroxyglutarate at pH 7.4, it can react with D-malate, but cannot use O_2 as an electron acceptor. Homologues of the enzyme have been grouped in the VAO/PMCH family of flavoproteins, with which D2HGDH shares a flavin-binding domain but not a substrate-binding domain. A Sequence Similarity Network analysis shows that bacterial D2HGDH may have unique functional properties from the human enzyme, making D2HGDH a target for new therapeutics against *P. aeruginosa*.

PA-2022-A013

Building reusable phage and antibiotic treatments via exploitation of bacteria-phage coevolutionary dynamics.

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Phage therapy has the potential to treat currently intractable infections, yet multiple challenges remain over initial efficacy and also over the rapidity of failure due to the

evolution of resistance. People with chronic (long-lasting) infections face the problem that treatment options diminish in time as the pathogen evolves increasing resistance. To address this challenge, we investigate phage therapy's ability to be a robust therapeutic. We exploit phage and bacterial co-evolution, producing dynamic selection pressures that can return the pathogen to a state of susceptibility to the initial (regulator-approved) therapy. We show that phage OMKO1 alone triggers Arms Race Dynamic (ARD) co-evolution with the pathogen *Pseudomonas aeruginosa*, leading to generalized phage resistance and crucially – failure at reuse. In contrast, co-administration of the phage with antibiotics triggers Fluctuating Selection Dynamics (FSD) co-evolution, allowing for effective reuse after 20 days of treatment. We pursue medical relevance in our experiments with the use of clinically important pathogens, antibiotics, phage, and a benchmarked synthetic sputum medium. Phenotypic and genomic characterization of evolved isolates demonstrates that efflux-targeting phage OMKO1 exerts continued selection for antibiotic susceptibility regardless of co-evolutionary dynamic or antibiotic co-treatment, opening the door for evolutionary robust phage therapy.

PA-2022-A014

Mucoid *Pseudomonas aeruginosa* can produce calcium-gelled biofilms independent of the matrix components Psl and CdrA

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Biofilms are aggregates of microorganisms embedded in an extracellular matrix comprised largely of exopolysaccharides (EPS), nucleic acids, and proteins. *Pseudomonas aeruginosa* is an opportunistic human pathogen that is also a model organism for studying biofilms in the laboratory. We define here a novel program of biofilm development used by mucoid (alginate-overproducing) *P. aeruginosa* in the presence of elevated calcium. Calcium cations cross-link negatively charged alginate polymers resulting in individual cells suspended in an alginate gel. The formation of this

type of structurally distinct biofilm is not reliant on the canonical biofilm EPS components Psl and Pel, or the matrix protein CdrA. We also observed that mucoid *P. aeruginosa* biofilm cells do not have the typical elevated levels of secondary messenger cyclic di-GMP (c-di-GMP) as expected of biofilm cells, nor does the overproduction of alginate rely on high c-di-GMP. This contrasts with non-mucoid biofilms in which the production of matrix components Psl, Pel, and CdrA are positively regulated by elevated c-di-GMP. We further demonstrate that calcium-gelled alginate biofilms impede penetration of the antibiotic tobramycin, thus protecting the biofilm community from antibiotic-mediated killing. Finally, we show that bacterial aggregates with disperse cell arrangement like laboratory-grown calcium-alginate biofilm structures are present in explanted CF lung samples. Our findings illustrate the diverse nature of biofilm formation and structure in *P. aeruginosa*.

PA-2022-A015

Calcium and putative phytase, CarP, regulate metabolism in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, is notorious for its ability to adapt to different environments, such as the lungs of individuals with Cystic Fibrosis (CF), where it resides, causing infections. CF is associated with a dysregulation in ion levels, including the increase in Ca^{2+} concentrations in the lungs and nasal liquids. Previously we have shown that *P. aeruginosa* responds to elevated extracellular Ca^{2+} via a two-component system, CarSR, leading to an alteration in gene transcription and attenuation in virulence. One of the known regulatory targets of CarSR, *carP*, encodes a predicted 5-bladed β-propellor with phytase-like activity. We have shown that CarP plays a role in regulating intracellular Ca^{2+} levels, contributes to the regulation of pyocyanin production and antibiotic resistance, and is involved in the oxidative stress response. To identify the function of CarP in response to elevated Ca^{2+}, a global untargeted metabolomics analysis was performed, for which PAO1 and Δ*carP* were grown to mid-log and stationary phases at 0 or 10 mM Ca^{2+}. The supernatants and cellular components were extracted and analyzed using GC-MS. Out of the 187 metabolites that were detected, 88 were dysregulated by Ca^{2+} in mid-log, 73% of them required CarP. In stationary phase, 127 metabolites were dysregulated by calcium, of which 48% were CarP-dependent. In addition, the production of 45 Ca^{2+}-independent

metabolites in PAO1 either required CarP or were significantly altered in the mutant. These metabolites represented the major metabolic pathways including those of amino acids, nucleotides, fatty acids, and carbohydrates. Taken together, these findings suggest that CarP plays a major role in the regulation of the metabolism of *P. aeruginosa.* The Ca^{2+}-dependent changes are likely due to the ability of CarP to control intracellular Ca^{2+} homeostasis, and others due to the potential enzymatic activity of CarP, or its interactions with other proteins.

PA-2022-A016

An updated genome-scale metabolic network reconstruction of *Pseudomonas aeruginosa* PA14 to characterize mucin-driven shifts in bacterial metabolism

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The mucosal barrier is a hydrated mucus gel that lines wet epithelial cells throughout the body and serves to protect against pathogens. Mucins, a key component of mucus, impair bacterial virulence mechanisms such as attachment and biofilm formation. However, some bacteria such as *P. aeruginosa* can reside within the mucus layer and cause infection. Insights into the metabolic responses of *P. aeruginosa* to mucins may enable the development of protective approaches against infection. Such insights can be gained through the application of metabolic network reconstructions and associated metabolic models, which allow for the mechanistic study of cellular metabolism. Here, we present *i*Pau21, an updated genome-scale metabolic network reconstruction of *P. aeruginosa* strain UCBPP-PA14 that we integrate with transcriptomic data to characterize the metabolic shifts of *P. aeruginosa* upon exposure to mucins. We updated the metabolic network reconstruction through metabolic coverage expansion, format update, extensive annotation addition, and literature/database-based curation to produce *i*Pau21. We then validated *i*Pau21 through growth rate, substrate utilization, and gene essentiality testing to demonstrate its improved quality and predictive capabilities. Published transcriptomic data was then integrated with the network using RIPTiDe to

produce context-specific models that are more consistent with a given biological state. These models were analyzed using flux balance analysis-based methods to gain insights into their metabolic states. The contextualized models showed shifts in network utilization and structure in response to mucins, recapitulated known phenotypes of unaltered growth and differential utilization of fumarate metabolism, and provided a novel insight of increased propionate metabolism upon MUC5B exposure. This work serves to validate *i*Pau21 and apply it to the investigation of metabolic shifts of the pathogen in response to mucins. Our analysis recapitulated known phenotypes, provided novel insights into changes in pathogen metabolism following mucin exposure, and identified potential therapeutic strategies that could be further explored.

PA-2022-A017

Immune response to vaccination with the *Pseudomonas aeruginosa* Pel polysaccharide

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Pseudomonas aeruginosa is an opportunistic pathogen that causes severe and potentially life-threatening infections, including ventilator associated pneumonia, pulmonary infections in cystic fibrosis patients, and chronic wounds. The rise in multidrug resistant strains highlights the need for a vaccine to prevent or treat these deadly infections. One challenge in vaccine design is to identify a target that is immunogenic and sufficiently conserved to be broadly protective. We previously identified the structure of an extracellular polysaccharide produced by *P. aeruginosa* called Pel that promotes bacterial aggregation and antibiotic tolerance. Pel is highly conserved, serotype-independent, and as demonstrated by our recent work (Jennings et al., *Cell Reports*, 2021), prevalent at sites of infection. We hypothesize that immunizing against Pel will produce durable, broad protective immunity against *P. aeruginosa*. Here we elucidate the immunological response to Pel vaccination. To determine if Pel is immunogenic, C57BL/6 mice were immunized by intramuscular injection with purified Pel polysaccharide. A quantitative ELISA was developed for detecting serum antibodies against Pel. We found that the anti-Pel total IgG antibody response at 14 days post-primary increased in a Pel dose-dependent manner with the highest observed titers at doses of 100 µg Pel. At 14 days post-secondary, both doses of Pel elicited significantly higher total serum IgG than the vehicle control group. Collectively, these data

demonstrate that vaccination with Pel elicits a robust immune response in mice and may be a potentially promising strategy to combat *P. aeruginosa* infections.

PA-2022-A018

Pyoverdine-dependent virulence and its inhibition in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a multidrug-resistant pathogen that causes acute, life-threatening infections in immunocompromised patients and debilitating chronic infections in those with cystic fibrosis. One key virulence factor in this pathogen is the siderophore pyoverdine, which not only provides the bacterium with iron during infection, but also regulates the production of several secreted toxins. We recently demonstrated that in addition to these previously characterized roles in pathogenesis, pyoverdine can also directly exert virulence against *Caenorhabditis elegans* and murine macrophages by translocating into host cells, disrupting iron and mitochondrial homeostasis (e.g.: mitochondrial morphology, redox metabolism). Due to a combination of these functions, pyoverdine production is necessary for *P. aeruginosa* virulence during murine lung infection. Importantly, we have used various panels of *P. aeruginosa* clinical isolates to show that pyoverdine production correlates to pathogen virulence against *C. elegans*, murine macrophages, and mice, demonstrating that pyoverdine is a clinically important target for new drug development. One such therapeutic is the biosynthetic inhibitor 5-fluorocytosine (5-FC). 5-FC attenuates pyoverdine-dependent virulence against a variety of hosts without overtly affecting bacterial titer, consistent with antivirulent mechanism of action. However, 5-FC can synergize with the antipseudomonal agent gallium nitrate to inhibit bacterial growth. This is likely due to pyoverdine's ability to sequester the metal, preventing it from reaching cytoplasmic targets. Interestingly, even in the presence of gallium, 5-FC largely functioned as an antivirulent. Spontaneously resistant mutants that emerged in the presence of both drugs were resistant to gallium but remained sensitive to 5-FC, carrying mutations in the HitAB ferric iron transporter as well as a putative ATP-binding protein in this system. We expect these drug-adapted populations to remain less virulent during Ga-5-FC treatment due to pyoverdine inhibition. These results suggest that Ga-5-FC may be a promising drug combination to target both pathogen virulence and growth during multidrug-resistant pseudomonal infections.

PA-2022-A019

Analysis of *Pseudomonas aeruginosa* partitioning protein ParB protein-protein and protein-DNA interaction network suggests its role beyond DNA segregation

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In the majority of bacterial species, including pseudomonads, the tripartite ParAB-*parS* system, composed of an ATPase (ParA), a DNA-binding protein (ParB), and its target *parS* sequence(s), facilitates the initial steps of chromosome partitioning. Generally ParB forms large nucleoprotein complexes at *parS*(s), located in the vicinity of *oriC,* which after replication are subsequently relocated by ParA to polar positions. Remarkably, ParAB-*parS* systems participate not only in the chromosome segregation but also in other cellular functions in a species-specific manner. In this work we provide an overview of *P*. *aeruginosa* ParB interactions with DNA, its cognate partner ParA and other newly identified partners. Using proteome and genome-wide techniques as the pull-down, co-immunoprecipitation and screening of a genomic library in bacterial two-hybrid system as well as the targeted approaches, we have identified ParB's partners and mapped their interaction domains. Among new partners there are the putative ATPases PA5028 and PA3481 displaying polar localization in the cells. Concomitantly, a genome-wide identification of ParB bound sites using ChIPseq confirmed ParB binding to *parS* sites, but also revealed thousands of the secondary ParB binding sites containing one arm of *parS* palindrome. We show that these half-*parS* sequences are the targets for ParB binding under various growth conditions and analyze the role of ParB binding to there sites in regulation of gene expression. Our data indicate that ParB is part of an intricate network linking chromosome segregation with other cell cycle related processes.
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The evolution of extreme aminoglycoside resistance *in vitro* and *in vivo*

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Patients with chronic *Pseudomonas aeruginosa* (Pa) infections are repeatedly exposed to antibiotics, and infecting isolates can develop extreme resistance. Chronic infection isolates generally develop resistance via chromosomal mutations rather than by acquiring resistance-producing genetic elements. Experimental evolution has expanded understanding of resistance mutations; however, *in vivo* conditions and drug exposure dynamics could affect mechanisms. We are investigating differences between *in vitro* and *in vivo* tobramycin resistance evolution using 12 PAO1 lineages passaged in tobramycin *in vitro,* and longitudinal isolates from tobramycin-treated cystic fibrosis patients. The Pa lineages evolved *in vitro* developed extreme tobramycin resistance with MICs >512 ug/ml (some as high as 65,536 ug/ml), and resistance of some isolates rapidly diminished after growth in the absence of drug. Clinical isolates from our initial study subject also exhibited extreme and unstable resistance. Genomic comparisons between resistant isolates from the 12 *in vitro*-passaged PAO1 lineages and the sensitive parent identified mutations in 43 genes (mean of 6.3 genes per isolate, range of 2-14) not mutated in isolates passaged in drug-free conditions. Genome comparisons between 10 CF Pa isolates that evolved extreme resistance *in vivo* and a clonally-related sensitive isolate collected 5-11 years earlier found only 4/43 genes mutated during *in vitro* evolution were also mutated in similarly-resistant clinical isolates (and not present in co-existing sensitive isolates). Moreover, only one mutant allele (in the *fusA1* gene) was common to extremely resistant *in vitro* and *in vivo* lineages. Experimental evolution experiments underway utilizing the “early” sensitive clinical isolate will help determine the relative contributions of strain differences and *in vivo* conditions on the evolved resistance mechanisms. Additionally, sequencing and phenotyping studies will examine mechanisms of unstable resistance and fitness cost differences inherent to *in vitro* and *in vivo* resistance. A better understanding of these evolutionary processes could improve treatment and combat antibiotic resistance.

PA-2022-A021

Pseudomonas aeruginosa **establishes distinct intracellular niches in vacuoles and the cytoplasm that differ in antibiotic susceptibility**

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Pseudomonas aeruginosa can survive and replicate in a variety of host cell types. Previously, we showed that it can colonize the cytoplasm requiring the type three secretion system (T3SS) and can spread intracellularly utilizing twitching motility. Here, we report discovery of a separate intracellular niche that does not require the T3SS. Human corneal epithelial cells were infected with *P. aeruginosa* strain PAO1 or T3SS (*exsA*) mutants transformed with reporters for T3SS (pJNE05-GFP), c-di-GMP (RFP) or CdrA (GFP) gene expression. Extracellular bacteria were eliminated with amikacin at 3 h, and intracellular bacteria studied from 4 to 12 h using time-lapse imaging with and without ofloxacin, a cell permeable antibiotic. Intracellular bacteria were quantified by reporter immunofluorescence at 6 h, and anti-PAO1 antibody to label all intracellular bacteria after cell permeabilization. Imaging revealed viable c-di-GMP-positive and CdrA-positive (but T3SS-negative) bacteria localized to vacuoles not the cytoplasm. Of 520 corneal epithelial cells analyzed at 6 h, 129 (24.8 %) contained CdrA-positive bacteria in vacuoles and 105 (20.2 %) contained CdrA-negative bacteria in the cytoplasm; 33.7 % of invaded cells contained both phenotypes simultaneously. Cytoplasmic (T3SS+) but not vacuolar (CdrA+) bacteria were killed by 1 µg/mL ofloxacin (Two way-ANOVA $p < 0.05$, 6 h; $p < 0.001$ 12 h for cytoplasmic bacteria; vacuolar bacteria NS at both time points). Moreover, 10 % of invaded cells maintained replicating vacuolar bacteria at 4 µg/mL ofloxacin (16X MIC). Mutants in biofilm-associated genes (Δ*cdrA*, Δ*pel*, Δ*psl*) also contained vacuolar/*cdrA*-reporter+ bacteria that tolerated ofloxacin (8X MIC). This study shows that *P. aeruginosa* can diversify within host cells to express the T3SS in the cytoplasm and c-di-GMP/CdrA in vacuoles, the latter associated with enhanced antibiotic resistance. Mechanisms for vacuolar persistence in the absence of CdrA, Pel and Psl and relevance to treatment failure *in vivo* remain to be determined.

PA-2022-A022

Cadaverine is a switch in the lysine degradation pathway in *Pseudomonas aeruginosa* biofilm identified by untargeted metabolomics

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P. aeruginosa readily forms biofilms in many chronic infections that are difficult to eradicate due to their high antibiotic-tolerance and evasion of immune defenses. Therefore, there is a critical need for new approaches to prevent and treat biofilms. Biofilm development is a highly complex and regulated process resulting in multiple phenotypic changes, requiring changes in gene expression and metabolic pathways. However, the underlying biological mechanisms that allow the transition from the planktonic to biofilm growth modes are not fully understood. Metabolomics provides a global analysis of metabolites giving an unbiased view of cellular activity. We hypothesize that the metabolic profiles of cells in the planktonic and biofilm states will reveal differences in pathways linked to coordinating the changes in phenotype, which could help identify diagnostic biomarkers for biofilm, increase the understanding of mechanisms required for biofilm formation, and point to pathways that could be targeted to slow, prevent, or reverse biofilm formation. We utilized multidimensional NMR to simultaneously detect, quantify, and reveal molecular structural information of all abundant known and unknown metabolites in a single set of measurements. Statistical analysis was completed using our COLMAR web server. We validated the role of our metabolite changes on biofilm growth using crystal violet staining assays and microscopy. We identified statistically significant differences in 52 metabolites between planktonic and agar biofilm phenotypes. The metabolites of the cadaverine branch of the lysine degradation pathway were systematically decreased in biofilm. Exogenous supplementation of cadaverine significantly increased planktonic growth, decreased biofilm accumulation and altered biofilm morphology. Cadaverine has been associated with combatting cellular stress, stimulating protein synthesis, increasing cellular respiration, and altering adhesion protein expression. Our findings show how metabolic pathway differences directly affect the growth mode in *P. aeruginosa* and could support interventional strategies to control biofilm formation.

A quantitative framework for microbiological model improvement

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Laboratory models are a key component of both basic science and translational microbiology research. Models serve multiple purposes. Minimally, they provide a tractable system to test hypotheses and validate results in multiple laboratories. Maximally, they provide a versatile, biologically relevant system for studying inaccessible environments of interest. Most model systems have been designed based on intuition or *ad hoc* rationalizations, and their relevance to the environment they are meant to mimic is not known. We recently developed a quantitative framework for measuring the accuracy of microbial models based on gene expression, and we applied this approach to demonstrate that an *in vitro* synthetic CF sputum medium model (SCFM2) and an epithelial cell model were both highly accurate models of *Pseudomonas aeruginosa* transcription in the cystic fibrosis (CF) lung, capturing the expression of over 80% of *P. aeruginosa* genes. Here, we present two approaches for improving model systems. First, we aimed to improve a model through informed modification of specific functions. Using hundreds of *P. aeruginosa in vitro* transcriptomes, we identified zinc limitation as a key cue present in the CF lung and absent in SCFM2. Addition of the transition metal binding protein calprotectin to SCFM2 induced the *P. aeruginosa* zinc limitation response, improving the accuracy of SCFM2 to human infection for approximately 250 genes. Second, we combined the two highly accurate models together: SCFM2 and the epithelial cell model. This approach improved the accuracy of approximately 150 genes

relative to SCFM2 and approximately 450 genes relative to the epithelial cell model. Importantly, different functions were captured in these two approaches, allowing for each improved model to meet different experimental needs. Together, these data provide a validated, grounded framework for microbiological model improvement that can be applied to any model system of interest.

PA-2022-A024

Investigating the temporal dynamics of R-pyocin regulation and release

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Bacteriocins are proteinaceous antimicrobials produced by bacteria, which are active against other strains of the same species. R-type pyocins are phage tail-like bacteriocins produced by *Pseudomonas aeruginosa,* a Gram-negative opportunistic pathogen, problematic in chronic infections. Due to their anti-pseudomonal activity, R-pyocins have potential as therapeutics in infection, however little is understood regarding the specific timing of R-pyocin induction and regulation. Similar to prophages, R-pyocins are located on the chromosome and are induced by the SOS response via DNA-damaging agents. Following SOS activation, R-pyocins are produced and then released into the environment by lysis. While several DNA-damaging agents are known to induce R-pyocin activity – including mitomycin C, ciprofloxacin, and hydrogen peroxide – it is unknown if all of three agents induce R-pyocin production to the same magnitude or along the same temporal regime. The induction of R-pyocins by the SOS response in *P. aeruginosa* also suggests that there may be regulatory ties between pyocins and chromosomal prophages - also induced under the same response. However, the regulation of R-pyocins and prophage have yet to be disentangled. There are two more lytic systems in *P. aeruginosa*: the Alp and the Cid systems, which have previously been ignored in much of the R-pyocin work thus far. Using chromosomal transcriptional reporters, we have generated a number of strains to (i) quantify the regulatory activity of various R-pyocin genes, (ii) assess differential gene expression by disparate induction agents, (iii) establish a timeline of R-pyocin regulation, from induction to lysis, and (vi) disentangle the involvement of prophage and other lytic systems in the regulation of pyocins in *P. aeruginosa.* Overall, our work provides a more cohesive picture of R-pyocin regulation in *P. aeruginosa.*

PA-2022-A025

In-vitro evolution reveals CbrAB-dependent metabolism drives *lasR* mutant selection

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A striking fraction of *Pseudomonas aeruginosa* isolates harbor loss-of-function mutations in the gene encoding the central quorum-sensing regulator LasR. LasR positively regulates the production of an array of secreted factors in response to its cognate autoinducer. These loss-of-function mutants (i.e. LasR- strains) are associated with worse disease prognosis in acute and chronic infections but are not unique to an infection environment. They are identified from environmental samples and *in vitro* evolution. Relative to wildtype, LasR- strains have distinguishable intra- and inter-species interactions, and prior work suggests that LasR- strains arise to due to increased frequency-dependent fitness, commonly referred to as "social cheating", wherein LasR- strains reap the benefits of social goods secreted by neighboring wild-type cells without incurring the production costs. However, wild-type independent fitness advantages have also been reported including enhanced microoxic fitness and resistance to lysis. Our data suggests that a major contributor to the selection for LasR- cells in *P. aeruginosa* populations relates to changes in catabolism mediated through the CrbAB two component system. Through *in vitro* evolution of wild type and *cbr* mutants in both laboratory strain and clinical isolate backgrounds, we showed that CbrAB was necessary for the rise of LasR- strains in diverse media. As predicted by this model, the addition of a CbrAB repressive substrate (i.e. succinate) suppressed the rise of *lasR* mutants in LB, and overexpression of CbrB or the small RNA it regulates, *crcZ,* were sufficient to recapitulate some of the observed growth advantages. Mathematical models constructed with mono-culture growth parameters accurately predicted LasR- strain abundance in evolution experiments and wild type co-culture assays. Overall, our data support an alternate model of *lasR* mutant fitness, provide new insights into environments in which LasR- strains will thrive, and unravel connections between quorum sensing and metabolism that may alter *P. aeruginosa* interactions.

PA-2022-A026

The role of spatial structure on *Pseudomonas aeruginosa* quorum sensing dynamics

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Pseudomonas aeruginosa uses quorum sensing (QS) to establish infection, evade the host immune response and survive stressors. During chronic infection, *P. aeruginosa* undergoes adaptive radiation where QS genes are frequently mutated. Most of the work to date has studied QS dynamics in planktonic liquid cultures, but during chronic infection, such as in wounds and cystic fibrosis lungs, *P. aeruginosa* grows in a spatially structured environment. It is therefore important to study how spatial structure impacts on *P. aeruginosa* QS dynamics. In structured environments, we have found that *P. aeruginosa* forms two types of aggregates (clumping and stacking aggregates), the nature of which is dependent on the lipopolysaccharide (LPS) structure on the surface of cells. Wildtype *P. aeruginosa* cells form stacking aggregates in a medium structured with eDNA, while LPS core mutants form clumping aggregates. Using fluorescent confocal microscopy and a *lasB* QS-regulated reporter, we show LPS core mutants form clumped aggregates which activate QS heterogeneously in small patches early in growth. However, wildtype cells form stacked aggregates which activate QS homogenously late in growth, with complete activation of the stacking aggregate population when cells reach late log phase. The aggregate type also determines how the different strains interact. Stacking aggregates form by intermingling kin and non-kin strains, while clumping aggregates form with only one strain type. Our work highlights the role spatial structure plays in QS signaling and microbial social behaviors in general.

PA-2022-A027

Cross-species induction of outer membrane vesicle production in bacterial co-culture

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Outer membrane vesicles (OMVs) are derived from the outer membrane of Gram-negative bacteria. They are integral to the transport of a wide variety of cargo to target cells and serve as versatile tools for interaction with the environment. While much is known about their functions, especially their roles in virulence and cell-cell interaction, there is currently no unifying model explaining how OMVs are produced. Previous work from our group and others has shown that the Pseudomonas Quinolone Signal (PQS) is

capable of inducing OMV formation not only in *P. aeruginosa* (the producing organism), but also in other Gram-negative recipient bacteria such as *E. coli*, *K. pneumoniae*, and *P. mirabilis*. Our team has shown that spent supernatants from these other species are also capable of stimulating OMV formation in the *P. aeruginosa* Δ*pqsA* mutant, which cannot produce PQS. This suggests that many species secrete OMV-inducing factors that can act on the producer and on other species. The current study has extended the list of PQS-responding organisms to include medically relevant species that are often found with *P. aeruginosa* at infection sites. We further tested whether different species pairs could mutually stimulate OMV production when actually grown together. We report that co-culture growth of *P. aeruginosa* with several other species results in increased OMV production per cell *vs.* what would be expected if production was simply additive. This result supports the hypothesis of cross-species induction of OMV biogenesis and provides a model to help understand pathogen synergy at multi-species infection sites.

PA-2022-A028

The master regulator FleQ post-transcriptionally regulates the production of RTX adhesins in *Pseudomonas fluorescens*

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Biofilm formation by the bacterium *Pseudomonas fluorescens* (*Pf*) relies on the secretion and retention of the repeat-in-toxin (RTX) adhesins LapA and MapA at the cell surface. While previous studies have determined that retention of the adhesins on the surface is post-translationally regulated by the cyclic-di-GMP sensing protein LapD and the protease LapG, little is known about the mechanisms that regulate adhesin production. Here, we identify the master regulator FleQ as a protein that post-transcriptionally regulates the production of LapA and MapA. To determine the effects of FleQ on biofilm formation, we made a markerless deletion of the gene encoding this protein and assessed the ability of this *fleQ* mutant strain to form a biofilm both in static assays with 96-well polyvinyl chloride plates and in microfluidic devices. To assess the impact of the loss of FleQ, expression of the *lapA* and *mapA* genes was determined by qRT-PCR and production of these adhesins was determined by whole cell lysate western blot analysis. Cell surface localization of these adhesins was also determined by dot blot analysis. Our biofilm formation studies demonstrate that a strain lacking FleQ forms a poor biofilm in static assays, and only forms a monolayer of attached cells in microfluidic devices. Additionally, our qRT-PCR experiments reveal that the loss of FleQ leads to modest

increases in *lapA* and *mapA* expression while our whole cell lysate westerns blots and dot blots show that the loss of FleQ leads to a marked reduction in the production of these adhesins and loss of localization to the cell surface. Together, our data suggest a post-transcriptional mechanism by which FleQ regulates biofilm formation through the modulation of LapA and MapA production.

PA-2022-A029

A *Pseudomonas* bacteriophage modulates neutrophil migration and function in response to bacterial infection

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Pseudomonas aeruginosa (Pa) is a major human pathogen, causing a large proportion of hospital-acquired infections such as ventilator-associated pneumonia and UTIs. The mammalian immune response to Pa infection is exquisitely dependent on neutrophils: neutropenic patients are frequently colonized by Pa, and mice deficient in neutrophil chemoattractants reach 50% mortality (EC50) at a 10^5x lower dose of Pa than WT mice. Pa also forms tenacious biofilms in the airways of cystic fibrosis patients, establishing chronic infections that eventually necessitate lung transplant. We have previously shown that Pf4, a temperate filamentous bacteriophage produced by Pa, alters TNF cytokine production by mouse dendritic cells in response to Pa. We report now that LPS-stimulated human macrophages incubated with purified Pf4 preparation exhibit significant downregulation of potent neutrophil chemoattractants and activators, including CXCL1 and CXCL5. This downregulation is TLR3- and IFNAR-dependent, suggesting that viral recognition of Pf4 drives impaired immune responses to bacterial infection. These changes in chemokine production are functionally relevant: conditioned media from Pf4 and LPS-treated cells are significantly less effective at inducing neutrophil migration *in vitro* than media from LPS-treated cells, and phagocytosis of Pf4-producing Pa by human neutrophils is impaired. Furthermore, incubation of human primary neutrophils with Pf4 phage almost completely abrogates activation in response to LPS, as measured by CD11b surface expression. In a Pa acute pneumonia model, mice infected with Pf4-overproducing Pa show significantly less neutrophil infiltration and neutrophil chemokines in BAL fluid than mice infected with low-Pf4-producing Pa. Correspondingly, CF patients colonized with Pf4-infected Pa have significantly lower levels of CXCL5 in sputum, indicating that Pf4 may play a role in the innate immune response to Pa in these patients.

Antisense molecules show synergy in *Pseudomonas aeruginosa*

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Growing antibiotic resistance in *Pseudomonas aeruginosa* necessitates the need for new therapeutic approaches. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) are novel antibiotics that inhibit translation of bacterial mRNA representing a potential solution to this problem. Our group has previously tested PPMOs targeting the essential genes *acpP, lpxC,* and *rpsJ* in *P. aeruginosa* and has demonstrated inhibition *in vitro* and *in vivo* when used by themselves or in combination with conventional antibiotics. However, it remains unclear whether combining PPMOs against different gene targets could provide a synergistic response. PPMOs targeted to essential *P. aeruginosa* genes *acpP, lpxC,* and *rpsJ* were combined in antimicrobial synergy assays to elucidate any relevant interactions, as measured by the fractional inhibitory concentration (FIC). Among the PPMOs tested, the combination of PPMOs targeted to *acpP* and *lpxC* showed potent synergy, with almost all pairings resulting in additive or synergistic effects (average FIC= 0.437, range 0.25 to 1.01). Combinations of *acpP* and *rpsJ* also demonstrated FIC indices that ranged from 0.5 to 1.00. The combination of *lpxC* and *rpsJ* displayed few synergistic relationships (average FIC= 0.682; range 0.26 to 1.03). These *in vitro* synergy studies set the stage for similar studies *in vivo*. Here we demonstrate that multiple delayed therapeutic dosing with combinations of PPMOs reduced mortality up to 5 days post infection using a murine model of acute pneumonia compared to treatments with a single compound. These data suggest that PPMOs can be combined in vivo and represent a promising therapeutic option for combating *P. aeruginosa* infections. Developing narrow-spectrum pathogen-specific therapeutics, such as PPMOs, is an attractive approach enabling drug stewardship while maintaining efficacy. PPMOs have already shown great effectiveness against clinical multidrug resistance *P. aeruginosa* and could play even a bigger role in replacing

conventional antibiotics and combating antibiotic resistance than just serving as new antimicrobials.

PA-2022-A031

Identification of host-pathogen interactions associated with *P. aeruginosa* chronic respiratory infections

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Understanding host-pathogen interactions is critical for the development of new therapies against highly adaptive pathogens such as *P. aeruginosa*, that causes chronic respiratory infections in people with cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease (COPD). Elucidation of host receptors for *P. aeruginosa* colonisation is crucial for the development of effective treatments. In order to investigate bacterial colonisation, we first evaluated the adhesion of two panels of sequential CF *P. aeruginosa* isolates to 16HBE14o- and CFBE41o$^-$ lung epithelial cells. The late isolate AMT0060-1 showed higher capacity for attachment to both cell lines, relative to its early isolate AMT0060-3, while no significant differences were found in the other series of isolates (AA2, AA43 and AA44). To evaluate the effect of *P. aeruginosa* interactions, host responses elicited against the sequentially isolated *P. aeruginosa* strains were analysed showing a decrease in the stimulation of interleukin (IL)-8 between the early isolate AA2 and late isolates AA43 and AA44 ($p<0.0007$), suggesting changes in the interactions throughout the course of infection. To further explore the human receptors involved in *P. aeruginosa* adhesion in chronic infections, we applied a novel non-biased proteomic approach, and probed 16HBE14o- cell membrane proteins (resolved by 2-D electrophoresis) with the late infection *P. aeruginosa* CF isolate AA43. Among the proteins identified by mass spectrometry were Annexin A2, a known *P. aeruginosa* host receptor, validating our novel technique. Other interesting receptor candidates identified are disulfide isomerases and the cell surface receptor HLA Class 1 histocompatibility antigen. Disulfide isomerases are also important in the attachment of *Burkholderia cenocepacia* to 9HTEo- cells. Overall, our proteomic approach may represent an interesting tool for the identification of novel host receptors for *P. aeruginosa* colonisation facilitating the discovery of new antibiotic treatments against this challenging pathogen.

PA-2022-A032

Pseudomonas aeruginosa type IV pili-mediated directional motility enhances microbial competition

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Pseudomonas aeruginosa (Pa) and *Staphylococcus aureus (Sa)* are two of the most common and difficult to eradicate bacterial pathogens infecting the lungs of people with cystic fibrosis (CF). Critically, coisolation of *Pa* and *Sa* is associated with poor patient outcomes and recalcitrance to treatment. Therefore, understanding how interspecies interactions influence bacterial survival during CF airway infections is important to increase treatment opportunities. Through live-imaging of *Pa* and *Sa* at the single-cell level, we previously demonstrated that *Pa* responds to *Sa* by increasing type IV pili (TFP)-mediated motility and moving towards *Sa*. Here we studied the consequences of this attraction. Live single-cell confocal imaging was performed to visualize and measure the survival and distribution of Sa in coculture with Pa in a spatially structured environment. In the presence of wild type (WT) *Pa*, *Sa* initially multiplied and formed microcolonies, but as *Pa* approached, individual WT *Pa* cells invaded and dispersed the edges of *Sa* colonies, reducing colony size. We hypothesized that invasion of *Sa* colonies provides *Pa* a competitive advantage during coculture. Accordingly, a non-motile, TFP-deficient *Pa* mutant (Δ*pilA*), was unable to invade and was significantly less competitive. Surprisingly, *Sa* colonies exhibited higher cell packing and colony edge thickness during coculture with Δ*pilA* than in monoculture. To determine if Pa uses a combination of motility and secreted antimicrobials for effective competition against Sa, we imaged *Pa* mutants unable to produce HQNO (Δ*pqsL*), pyoverdine (Δ*pvdA*), pyochelin (Δ*pchE*), or LasA (Δ*lasA*), or combinations thereof. While loss of *Pa* antimicrobials improved *Sa* survival, *Pa* remained proficient at invasion and colony dispersal. We propose that *Pa* attraction towards, invasion, and dispersal of *Sa* colonies allows *Pa* to locally concentrate antimicrobials to maximize interspecies competition. Furthermore, these data reinforce accumulating observations in the field that spatial structuring is an essential determinant of community resilience during infection.

PA-2022-A033

Letting Go: New insights into outer membrane vesicle release in *Pseudomonas aeruginosa*

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Bacterial Outer Membrane Vesicles (OMVs) contribute to virulence, competition, immune avoidance and communication. This has led to great interest in how they are formed. To date, investigation has focused almost exclusively on what controls the *initiation* of OMV biogenesis. Work in different organisms has implicated several factors (membrane stress, cell wall turnover, lipid composition, small molecule interactions) in this process. Regardless of the mechanism of initiation, all species face a similar challenge before an OMV can be released: *How does the OM become detached from the underlying cell wall in regions that will ultimately bulge and then vesiculate?* Our analysis led us to investigate the OmpA family of OM proteins (OprF in *P. aeruginosa*). This widely conserved family is unusually abundant in OMVs across species, considering their major role in peptidoglycan (PG) attachment. Members also have the interesting ability to adopt two conformations: one with a PG-interacting periplasmic domain, and one unbound from PG and fully inserted into the OM. Using targeted deletion of the PG-binding domain we showed that loss of cell wall association, and not general membrane destabilization, is responsible for hypervesiculation in OprF-modified strains. We therefore propose that OmpA family members play the role of 'latch' proteins capable of releasing PG in regions destined to become OMVs. To test this hypothesis, we developed a protocol to assess OprF conformation in live cells and purified OMVs. While >90% of OprF proteins exist in the PG-bound conformation in the OM of cells, we show that the majority of OprF in OMVs is present in the PG-unbound conformation. With this work we have taken some of the first steps in characterizing late-stage mechanisms of OMV biogenesis and have identified a family of proteins whose critical role can be explained by their unique ability to fold into two distinct conformations.

PA-2022-A034

The *Pseudomonas aeruginosa* AlgZ (FimS) histidine kinase controls transcription in response to Redox

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Pseudomonas aeruginosa is an opportunistic pathogen that causes many types of infections that can have a broad range of duration from acute to chronic. One of the two component systems utilized by the organism during both pathogenic scenarios is AlgZ(FImS) AlgR. The AlgR regulon has been characterized by ChIP-seq to include as many as 155 genes but the physiological signal that induces the AlgR regulon is currently unknown. There is evidence that AlgR responds to oxidative stress as it induces alginate production and transcription of ribonucleotide reductases and catalase. However, the AlgZR regulon also includes numerous iron dependent genes, therefore the hypothesis that AlgZ responds to redox activity by direct control of iron acquisition was tested. The *algZ-S-tag* and *algZ-GFP* genes were placed onto the PAO1 chromosome and the tagged AlgZ proteins were followed during planktonic and sessile growth and after exposure to low iron and hypoxic conditions by fluorescence microscopy and Phos-tag western blots. The AlgZ phosphorylation state increased as cells reached stationary phase, under low iron and hypoxic conditions and formed protein complexes under low iron and oxygen. AlgZ-GFP localized to the poles *in vivo* under the same conditions and addition of iron or oxygen dispersed AlgZ-GFP throughout the membrane. RNA-seq data from stationary phase LB, PIA and from murine lungs comparing PAO1 to PAO1 *algZ* H175A (encoding inactivated AlgZ) revealed differential transcription of 133 genes, most of the genes (46/133, 35%) were associated with phenazine, pyoverdine, pyochelin and heme production or acquisition. Taken together, these results strongly indicate that AlgZ phosphorylation responded to decreased cellular redox state by localizing to the poles where it appears to interact with the respirasome, the siderosome and type IV pili to control downstream iron acquisition, likely through AlgR.

PA-2022-A035

Ethanol is a foraging signal for swarming motility in *P. aeruginosa* PA14

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The opportunistic human pathogen *Pseudomonas aeruginosa* is known for exhibiting diverse forms of collective behaviors like swarming motility and biofilm formation. Swarming in *P. aeruginosa* is a collective movement of the bacterial population over a

semisolid surface, but specific swarming signals are not well understood. We hypothesize that specific environmental signals induce swarming in *P. aeruginosa*. We show that under nutrient-limiting conditions, low concentration of ethanol provides a strong ecological motivation for swarming in *P. aeruginosa* strain PA14. Ethanol serves as a signal and not a source of carbon under these conditions. Moreover, ethanol-driven swarming relies on the ability of the bacteria to metabolize ethanol to acetaldehyde using a periplasmic quinoprotein alcohol dehydrogenase. We find that ErdR, an orphan response regulator linked to ethanol oxidation, is necessary for the transcriptional regulation of a cluster of seventeen genes, including ethanol dehydrogenase, during swarm lag. Further, we show that *P. aeruginosa* displays characteristic foraging motility on a lawn of an ethanol producing yeast, in a manner dependent on the ErdR, ethanol dehydrogenase and on rhamnolipids. Finally, we show that ethanol, as a volatile, could induce swarming in *P. aeruginosa* at a distance suggesting long-range spatial effects of ethanol as a signaling molecule.

PA-2022-A036

Pseudomonas aeruginosa strains with a non-canonical quorum sensing-response: The case of strain ATCC9027

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Several *P. aeruginosa* virulence factors, like elastase, rhamnolipids and pyocyanin, are regulated at the level of transcription by a complex regulatory network called quorum-sensing (QS) that has been mainly characterized in the PAO1 and PA14 type-strains. QS is comprised by three systems composed by a transcriptional regulator and an autoinducer (AI) that forms a complex with and activates the regulatory protein. These three systems are: LasR/3-oxo-dodecanoyl homoserine lactone (3O-C12-HSL), RhlR/butanoyl homoserine lactone (C4-HSL) and PqsR/2-heptyl-3,4-dihydroxyquinoline (PQS) or its precursor 4-hydroxy-2-heptylquinoline (HHQ). They are arranged hierarchically with the LasR/3O-C12-HSL system at the top of the regulatory cascade activating the expression of RhlR/C4-HSL and PqsR/PQS systems. In turn, PqsR/PQS is required for the RhlR-dependent production of pyocyanin, because it activates the

expression of the *pqsABCDE* operon and PqsE is indispensable for its production. Even though the QS response is very much conserved among *P. aeruginosa* strains, *lasR* mutants that are still able to produce virulence factors are frequently isolated. Strain ATCC9027, belonging to the genetically diverse PA7 clade, is avirulent in the mice model, so we hypothesized that it presents a defective QS response. To determine which QS system was non-functional, we used different experimental approaches, like the complementation for virulence factors production of PAO1 mutants with ATCC9027 genes and the use of gene-fusions, among others. Our results showed that ATCC9027 LasR protein was defective and that its *pqsR* is inactivated by a frameshift mutation, but PqsE was still expressed in some culture conditions enabling its pyocyanin production. The defective LasR protein is particular to strain ATCC9027, but the analysis of PqsR amino acid sequence among different strains belonging to the PA7 clade showed that this QS-system shares functional characteristics among them. Thus, we conclude that QS regulatory network varies among different *P. aeruginosa* isolates or even among different phylogroups.

PA-2022-A037

Data-driven identification of the impacts of antibiotic exposure on *Pseudomonas aeruginosa* growth

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Antibiotic treatment of microbial infections presents a number of challenges, including the evolution of resistance and ecological enrichment of previously rare drug-resistant pathogens (competitive release). Infections occur in complex and dynamic spatial environments, and microbes experience spatiotemporal heterogeneity in antibiotics as a result of periodic temporal dosing and diffusive movement into organs and tissues. In contrast, commonly used math models of microbial dynamics tend to make strong assumptions on the constancy and homogeneity of growth rates, with antibiotic treatment leading to decreased growth rate (bacteriostatic) or active killing in the form of an additional loss term (bactericidal). We aim to take a data-led approach to identify the dynamics of pathogen growth, without assuming constant rate processes or

homogeneity across cells. We begin with fine-scale data on the population dynamics of the pathogen *Pseudomonas aeruginosa* under a range of antibiotic exposures, and apply nonlinear dynamic identification algorithms to build a data-informed model. Our analyses show that standard math models (e.g. Lotka-Volterra) fail to capture higher order nonlinearities, which can similarly describe time series trajectories, but have a greater number of additional qualitative outcomes. This suggests that while standard models may adequately capture bacterial growth in well controlled environments, largely different dynamics may result in a more realistic and variable setting. Because diverse mathematics can produce similar patterns in both space and time, a concrete data-driven foundation is vital for the predictive and explanatory value of our analysis. We then numerically simulate our data-led model in a spatially extended setting, to assess the impact of environmental heterogeneities on the dynamics of bacterial clearance under antibiotic exposure. Future investigation will pursue novel treatment strategies that are more targeted in space and time, helping to mitigate competitive release and emergent antimicrobial resistance and prevent uncontrolled infection.

PA-2022-A038

Riboswitch-based reporters to characterize selective permeability and active efflux in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa exhibits a remarkably high resistance to several types of antibiotics, partly due to the restrictive nature of its outer membrane and the action of its numerous efflux systems. However, the dynamics of compound uptake, retention and efflux in this bacterium remain poorly understood. We propose to take advantage of the sensor capabilities of bacterial riboswitches to describe the physicochemical and structural properties of compounds that cross the bacterial envelope and remain inside. We employed riboswitch-based sensors to generate reporter strains detecting alterations in essential biological pathways. As a proof of concept, we implemented in *P. aeruginosa* a reporter system that consists in a fusion of the *Pectobacterium carovotorum* ZTP riboswitch sequence to the *lacZ* gene. This riboswitch senses

perturbations in the folate cycle and in the *de novo* purine synthesis pathway. Our data show that the reporter is responsive to antifolate drug-induced folate cycle disruptions in *P. aeruginosa*. Notably, the concentrations of the antifolate drug trimethoprim that are required to activate the ZTP riboswitch reporter in an efflux-deficient strain were significantly lower than the ones required in a wild-type strain, thus highlighting the relevance of active efflux in antimicrobial resistance. Finally, we developed a method using the ZTP riboswitch reporter-carrying strains to screen for small molecules with the potential of altering *Pseudomonas aeruginosa* metabolism. This procedure can be readily adapted to high throughput screening assays and was validated by screening a curated library of structurally diverse compounds that included antifolate drugs. By screening for increased reporter activity in an efflux-deficient background, compounds entering the cell and disrupting the folate pathway were identified. Rescreening of these initial hits in an efflux-proficient background allowed us to separate these compounds into two categories: efflux substrates and non-efflux substrates. We aim to apply this approach using different classes of riboswitches in an effort to provide comprehensive knowledge for the design of novel, more efficient antimicrobial compounds.

PA-2022-A039

Determining the accuracy of antimicrobial susceptibility testing in heterogeneous populations of *Pseudomonas aeruginosa* sourced from cystic fibrosis airways

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Pseudomonas aeruginosa (*Pa*) is the predominant pathogen in chronic lung infection of adults with cystic fibrosis (CF), contributing significantly both to morbidity and mortality. It is proposed that individuals with CF are infected at a young age by a single strain of *Pa*, which then evolves and diversifies extensively over time, leading to a genetically and phenotypically heterogeneous bacterial community. Despite this, modern antimicrobial susceptibility testing (AST) in clinics is usually conducted on only one or two isolates from each CF sputum sample, overlooking the diversity in antimicrobial resistance (AMR) phenotypes within a patient and potentially leading to treatment complications. To assess the accuracy of individual isolate-level susceptibility testing in regards to population-level susceptibility, we compared the results of AST for whole *Pa* populations versus 75 individual isolates sourced from the same population for four independent CF patient sputum samples, using three different methodologies: (i) standard broth dilution;

(ii) VITEK® 2 automated instrumentation; and (iii) agar disc diffusion, per CSLI guidelines. Our testing showed significant within-patient heterogeneity in antimicrobial susceptibility profiles for all four patients. We additionally found that the three methodologies often disagreed with each other, and that testing of individual isolates frequently underestimated resistance to numerous antimicrobials in comparison to whole population testing. Furthermore, a number of isolates from one patient sputum sample were fastidious or entirely unable to grow in Mueller-Hinton medium. Our findings suggest that current AST methods should be re-evaluated for the development of novel methods to accurately assess AMR levels in complex chronic infections such as CF.

PA-2022-A040

Use of alternative gelling agents highlights the role of rhamnolipids in *Pseudomonas aeruginosa* surface motility

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Pseudomonas aeruginosa is a versatile bacterium able to colonize various environmental niches using its vast genetic toolset. One such feature is the social surface behaviour known as swarming motility. Swarming relies on flagella as the driving force and the production of wetting agents to alleviate surface tension and ease the spread on the surface. *P. aeruginosa* possesses a single polar flagellum and produces mono- and di-rhamnolipids—and their lipidic precursors—as its wetting agents. Rhamnolipids are synthesized using three enzymes: RhlA produces the dilipidic rhamnolipid precursor while RhlB and RhlC add the first and second rhamnose moieties, respectively. What is known on swarming comes from assays conducted using standard agar-based media. It is relatively well known, although generally dismissed, that different agar brands will impact the observed swarming phenotype. We pushed this idea further and assessed surface motility capabilities of the prototypical strain PA14 on semi-solid media solidified with alternative gelling agents gellan gum and carrageenan. Interestingly, on these alternative surfaces, the spreading pattern of *P. aeruginosa* is drastically altered. One striking feature, however, is the loss of dependence on rhamnolipids to spread effectively on plates solidified with these alternative gelling agents. While the wildtype strain spreads in its characteristic dendritic pattern, a *rhlA*-null mutant unable to produce the wetting agents still spreads effectively, albeit in a circular shape. Furthermore, *rhlB* and *rhlC* mutants display altered motility phenotypes compared to the wildtype, cementing a role for rhamnolipids in the control of the

spreading pattern of *P. aeruginosa*. Our data indicate that rhamnolipids do not have such a crucial role in achieving surface colonization of non-agar plates, suggesting a strong dependence on the physical properties of the tested surface. The use of alternating gelling agent provides new means to reveal unknown features of the surface behaviour of bacteria.

PA-2022-A041

iATLANTIS: Using Cas3 to make *Pseudomonas* immune islands disappear

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Bacterial immune systems that antagonize bacteriophages cluster together in “immune islands”, which has enabled their discovery via bioinformatics. However, experimental methods to uncover immune islands in their native host are lacking. Cascade-Cas3 is a programmable genome engineering tool that generates large deletions in bacterial genomes at specified regions, but with non-specific boundaries. Here, we harnessed the variability of Cas3-generated deletions to develop iATLANTIS (immune Ablation via Targeting of Large Nucleotide Islands) to delete anti-phage immune systems and their neighboring regions. In this regard, our main objective is to utilize iATLANTIS to discover new anti-phage immune systems using native model systems. Large deletions were generated in 5 *Pseudomonas aeruginosa* strains by targeting the *hsdR* gene in Type I restriction-modification (R-M) systems because they are commonly enriched in immune islands. After challenging deletion strains with a diverse phage panel, one strain showed three unique phenotypic outcomes, based on the emergence of phage plaques only on deletion strains: (i) phages that are likely targeted by the Type I R-M system that was deleted (i.e. all clones derived from one crRNA targeting event became sensitized), (ii) phages that are likely targeted by a system adjacent to the targeted Type I R-M system (i.e. some clones from a single targeting event were sensitized while others maintained resistance), and (iii) phages that grew only when multiple deletions were applied (i.e. targeting of two different Type I R-M systems in the same strain). Notably, a strain with two large deletions was sensitized to 11 phages from 2 families, which the strain previously resisted. These data suggest that multiple immune systems reside in these two loci. In conclusion, island removal with iATLANTIS has the potential to uncover new anti-phage bacterial immune systems as an experimental complement to bioinformatic predictions. Our next steps include genome sequencing of the deletion strains and clean knockouts of candidate systems. Altogether, genomic deletions with Cas3 serves as a powerful tool for basic research and discovery of anti-phage immunity.

Systematic identification of molecular mediators underlying sensing of *Staphylococcus aureus* by *Pseudomonas aeruginosa*

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Bacteria typically exist in dynamic, multispecies communities where polymicrobial interactions influence fitness. Elucidating the molecular mechanisms underlying these interactions is critical for understanding and modulating bacterial behavior in natural environments. While bacterial responses to foreign species are frequently characterized at the molecular and phenotypic level, the exogenous molecules that elicit these responses are understudied. Here we outline a systematic strategy based on transcriptomics combined with genetic and biochemical screens of promoter-reporters to identify the molecules from one species that are sensed by another. We utilized this method to study interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* that are frequently found in co-infections. Upon exposure to *S. aureus* cell-free supernatant, *P. aeruginosa* increases the expression of genes involved in zinc and iron starvation responses and intermediate metabolite uptake and catabolism. To identify the *S. aureus* molecules that induce these pathways, we designed promoter-reporters in *P. aeruginosa* based on upregulated genes in each pathway and used the reporters to screen a *S. aureus* mutant library for loss of induction. From our screen, we discovered that *P. aeruginosa* senses diverse staphylococcal exoproducts including the metallophore staphylopine, intermediate metabolites citrate and acetoin, and multiple molecules that modulate its iron starvation response, and responds by upregulating its own metallophores, pseudopaline and pyoverdine. We demonstrate that staphylopine inhibits biofilm formation and that *P. aeruginosa* can utilize citrate and acetoin for growth, revealing that these interactions have both antagonistic and beneficial effects. Further, a combination of these identified *S. aureus* products recapitulates a majority of the transcriptional response of *P. aeruginosa* to *S. aureus* supernatant, validating our screening strategy. Our study thus identified multiple *S. aureus* secreted molecules that are sensed by *P. aeruginosa* and affect its physiology, demonstrating the efficacy of our

approach, and yielding new insight into the molecular basis of interactions between these species.

PA-2022-A043

Filamentous Pf4 bacteriophage modulate *Pseudomonas aeruginosa* pigment production and virulence

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Many *Pseudomonas aeruginosa* isolates are infected by temperate filamentous Pf bacteriophages. Pf phages aid *P. aeruginosa* in evading the eukaryotic host immune response, contribute to the biofilm lifecycle, and enhance virulence potential. However, how Pf phages affect *P. aeruginosa* virulence phenotypes remains unclear. In *P. aeruginosa,* many virulence phenotypes are controlled by quorum sensing. In this study, we find that the production of pyocyanin, a quorum-regulated virulence factor, was suppressed by Pf phage replication and pyocyanin production was enhanced when the Pf prophage was deleted from the *P. aeruginosa* chromosome. We hypothesized that Pf phages encode proteins that suppress *P. aeruginosa* quorum sensing systems, reducing pyocyanin production, and ultimately altering the virulence potential of *P. aeruginosa*. To test this, we over-expressed each Pf gene in the core Pf phage genome. We found that expression of the phage protein PfsE suppresses pyocyanin production by binding to PqsA, which is required to produce the *Pseudomonas* quinolone signal PQS. In a *Caenorhabditis elegans* infection model, we discovered that deleting the Pf prophage reduces virulence compared to wild-type *P. aeruginosa*, even though the Pf prophage mutant produces more pyocyanin. We observed that enhanced pigment production by the Pf prophage mutant is detected by the eukaryotic aryl-hydrocarbon receptor, which detects bacterial pigments such as pyocyanin, modulating the *C. elegans* immune response.

PA-2022-A044

A single genomic locus in *Pseudomonas aeruginosa* is an anti-phage immune system hotspot

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Bacteria use a diverse arsenal of immune systems to defend against phage infections, including CRISPR-Cas and restriction enzymes. These systems can be found as gene neighbors, in so-called “defense islands”. We developed a computational tool that can identify published immune systems, which was applied to more than 180,000 genomes available on NCBI. From these data, we determined that *P. aeruginosa* has the most diverse defense arsenal of any bacterium with >200 sequenced genome and will likely be a source of novel anti-phage immune mechanisms. Network analysis of defense islands in *P. aeruginosa* led to the unearthing of a single locus, core to *P. aeruginosa*, that possesses a hypervariable set of genes devoted to anti-phage immunity, which we term a defense hotspot (DHS). Across more than 1,000 *P. aeruginosa* strains, we find 10 known immune systems including RM systems, BREX, DISARM, CBASS, and other recently discovered systems at this locus. This site, which we call DHS1, is defined by a hyper-conserved $tRNA_{Pro}$, bears resemblance to a mobile element with flanking transposases and low GC% spanning the DHS. Most genes in this region have an unknown function but contain domains that are commonly found in anti-phage systems. We hypothesize that these regions likely encode new defense systems. To this end, we experimentally validated a novel system found in the DHS1 of PA14 as anti-phage and conferring broad immunity. We believe this approach for immune system searching may extend to other bacterial phyla. A benefit of our approach is that discovery is not necessitated by the identification of known immune systems (traditional method), but instead, relies on core marker genes which expands the ability to find systems that are not found near any other known systems.

SESSION B: POSTER PRESENTATIONS

PA-2022-B001

Coordination between chromosome and condensin dynamics and cell division in *P. aeruginosa*

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Pseudomonas aeruginosa is an opportunistic human pathogen highly fit to grow in diverse environments. This fitness can be attributed in part to the presence of two condensins: a canonical SMC-ScpAB and MksBEF. These two proteins play an important role in global organization and segregation of the chromosomes. In this study, we explore a hypothesis that condensins are also involved in cell cycle progression in *P. aeruginosa* and further delineate activities of SMC and MksBEF. To this end, we employ fluorescence microscopy and observe the dynamics of select chromosomal loci using Fluorescence Repressor Operator System (FROS) and parallel it with the dynamics of fluorescently tagged SMC, MksBEF and FtsZ, which defines the location of the cell division site. We then constructed mutant strains of condensins to test the causative relationship between localization patterns of condensins and cell division proteins. We found that *oriC* duplication occurs at midcell followed by their relocation to opposite cell quarters. SMC always colocalized with *oriC* except briefly after *oriC* duplication. MksB clusters on the other hand remained at midcell but relocated towards the cell quarters shortly before the second round of *oriC* duplication, suggesting a cause-and-effect relationship between the two events. Curiously, the sites of condensin localization are also the sites of Z-ring formation, suggesting that chromosome dynamics might be also linked to cell division. In support of this view, we found Z-ring formation was affected by condensin mutations and always took place after the relocation of MksB cluster from midcell to the cell quarters. These data indicate that condensins are integrated into the chromosome duplication and cell division cycles in *P. aeruginosa* and suggest that the bacterial life cycle begins prior to the birth of the cell, at the onset of chromosome replication.

PA-2022-B002

Systematic characterization of *Pseudomonas aeruginosa* strains isolated from non-cystic fibrosis bronchiectasis patients

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Non-cystic fibrosis bronchiectasis (referred to as bronchiectasis) is a chronic and progressive lung disease frequently associated with significant symptom burden requiring intensive treatment. In patients with bronchiectasis, bacteria provoke an inflammatory response that can further drive colonization, infection and structural damage. *Pseudomonas aeruginosa* is the leading cause of chronic infections in bronchiectasis patients. Despite its importance, the adaptation mechanism of *P. aeruginosa* during bronchiectasis infections is largely unknown, with only few studies showing that bacterial progression during this disease is similar, although not entirely the same, to that during cystic fibrosis (CF). Thus, this work aims at exploring the distinctive molecular basis for the adaptation of persistent *P. aeruginosa* clonal lineages to the bronchiectasis lung. For this purpose, fifteen clonally distinctive bronchiectasis isolates were selected based on a microarray multi-marker genotyping method, allowing us to cover a broad spectrum of globally distributed clinical types. The strains were subjected to a battery of phenotypic assays, including growth, motility, biofilm formation, virulence and production of quorum sensing molecules. As expected, the strain collection displayed very different phenotypic traits among each other and did not disclose any specific pattern that differentiates them from other P. aeruginosa isolates. Next, to get systematic, more detailed insights into possible distinctive pathogenicity strategies in bronchiectasis, transcriptomic and metabolomic analyses were carried out simultaneously in our strain collection and in isolates obtained from other clinical sources, such as CF-, chronic obstructive pulmonary disease (COPD) and urinary tract infections. Preliminary results show a remarkable similarity in the metabolic fingerprint for all clinical strains, regardless of the isolation source and irrespective of the wide genetic background covered in our study. Although preliminary, these results highlight the metabolic robustness of P. aeruginosa, which stem (among other features) from the expression of a plethora of transcription factors, sigma factors and redundant genes.

Quorum sensing regulation by the nitrogen-phosphotransferase system in *Pseudomonas aeruginosa*

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Nitrogen phosphotransferase systems (PTS^{Ntr}) are well conserved in many bacteria and thought to be important for balancing metabolic changes in response to carbon and nitrogen availability. In *Pseudomonas aeruginosa,* the PTS^{Ntr} system is critically important for virulence and is also known to regulate the LasR-I quorum-sensing system. These systems derive phosphate from phosphoenolpyruvate and transfers it through three different enzymes, PtsP, which begins the phosphotransfer cascade through autophosphorylation, and the two other enzymes PtsO and PtsN. Here, we explore the mechanism of PTS^{Ntr} regulation of LasR activity. We use a GFP fusion to the LasR-controlled *lasI* promoter (P*lasI-gfp*) to show that deleting *ptsP* increases P*lasI-gfp* reporter activity by about ~3-fold, similar to previous studies. Previous reports implicated that the QS anti-activator, QscR may be involved in PtsP-dependent regulation of the LasR-I system. Our results show that QscR is dispensable for PtsP-dependent suppression of the P*lasI-gfp* reporter, suggesting QscR is not the PTS^{Ntr} effector on the LasR-I system as previously proposed. In other bacteria, glutamine binds to the GAF domain in PtsP and subsequently regulates autophosphorylation and phosphotransfer to the other PTS^{Ntr} proteins. Our genetic studies demonstrated that the *P. aeruginosa* PtsP-GAF domain is dispensable for PtsP-dependent regulation of the P*lasI-gfp* reporter. We further tested P*lasI-gfp* activation in *ptsN* and *ptsO* mutants singly and in combination with the *ptsP* mutant. Our results support a model whereby *ptsP* deletion blocks phosphate flow through the PTS^{Ntr} system and lead to accumulation of unphosphorylated PtsO and PtsN proteins, which are both needed to fully activate the P*lasI-gfp* reporter. Results of our studies provide new insights into the *P. aeruginosa* PTS^{Ntr} system and how it regulates *quorum sensing.* This information may be important for developing novel anti-*P. aeruginosa* therapeutics that target the PTS^{Ntr} system.

Development of a modular T7 RNA polymerase based genetic platform for the efficient production of heterologous proteins in *Pseudomonas putida*

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The T7 RNA polymerase has proven itself to be one of the most powerful tools for heterologous gene expression. The exploitation of this tool in many prospective biotechnological hosts is, however, still very scarce. To this intent, a modular T7 RNA polymerase-based system for stable heterologous protein production in *Pseudomonas putida* was established and characterized. In this system, the T7 RNA polymerase gene was introduced into the genome whilst the target gene was located downstream of the T7 promotor on a plasmid. The model protein eGFP was employed as an easy quantifiable reporter, facilitating *in vivo* tracking of the systems behaviour during microbial cultivations. The initial genetic set-up of the system suffered from slow growth and low protein production rates. After confirmation that the limitations of our system were caused by transcriptional problems, the role of the terminator sequence downstream of the eGFP gene was evaluated. Upon performing *in vitro* transcription assays, it became apparent that the T7-phage inherent TΦ terminator displayed low termination efficiency and allowed read-trough transcription of the T7 RNA polymerase. Next, the TΦ terminator downstream of the heterologous gene was replaced with the synthetic tZ terminator, which drastically improved the performance of the system. Additionally, a T7 RNA polymerase ribosome binding site library was constructed to tune heterologous protein production by varying the amount of T7 RNA polymerase. Experiments with this library led to the identification of a T7 RNA polymerase saturation in the system. Therefore, the choice of ribosome binding site in front of the T7 RNA polymerase gene is a key factor in the optimization of the system. It should be chosen in a way that the system operates at the verge of saturation, enabling maximum eGFP production rate and growth, without wasting cellular resources on surplus T7 RNA polymerase production and mRNA synthesis.

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Kinetic RNA Seq analysis reveals the role of calcium sensor, EfhP, in regulating iron uptake and biofilm formation in *Pseudomonas aeruginosa* as a response to Ca^{2+}

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The human pathogen *Pseudomonas aeruginosa* poses a major risk for severe infections in immunocompromised individuals, particularly those suffering from cystic fibrosis (CF). Increased levels of calcium (Ca^{2+}), as in pulmonary fluids of CF patients, have previously been shown to increase production of secreted virulence factors in *P. aeruginosa*. A Ca^{2+}-binding EF-hand protein, EfhP (PA4107), was demonstrated to be critical for the Ca^{2+}-regulated virulence in *P. aeruginosa* strain PAO1. Our current work seeks to characterize EfhP's regulation of the PAO1 transcriptional response to Ca^{2+} and to identify its protein partners which may be involved in signal transduction. Here we describe the results obtained from RNA sequencing of an *efhP* deletion mutant and PAO1 after rapid (10 min, 60 min), and adaptive (12 h) exposure to elevated Ca^{2+}. Consistent with previous results, deletion of *efhP* significantly hinders Ca^{2+} induction of several *P. aeruginosa* virulence factors. This includes genes necessary for the production and uptake of the iron-sequestering siderophores, pyoverdine and pyochelin, as well as genes involved in ferric-enterobactin and heme uptake. RNA seq data suggests that EfhP is responsible for regulating both rapid and adaptive inductions of all these iron uptake pathways. Additionally, EfhP is required for signaling a rapid upregulation of *pel* and *psl* genes associated with the pathogen's biofilm formation. Pull-down assays using EfhP expressed in *P. aeruginosa* periplasm identified the pyoverdine recycling pump PvdR, anti-σ factor SbrR, and tyrosine phosphatase TpbA as potential interacting partners of EfhP, whose validation using protein-fragment complementation assay is ongoing. These proteins may mediate the role of EfhP in the Ca^{2+}-regulated iron uptake and biofilm formation. Overall, the results suggest a regulatory interconnectedness between Ca^{2+}, iron, and biofilm formation signaling systems integrating multiple host signals and controlling *P. aeruginosa* virulence.

PA-2022-B006

Induction of a novel aggregation phenotype by citrate: differential responses in LasR- and wild-type *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa contains extensive regulatory networks which allow the bacterium to establish chronic infections in the lungs of individuals with cystic fibrosis (CF). Loss of function mutations in the gene encoding the LasR transcription factor are ubiquitous in long-term infections and associated with worse lung function. LasR- strains

differ from wild-type in many ways including metabolism. We have found that citrate activates the Rhl quorum sensing system in LasR- strains, but not in wild-type *P. aeruginosa.* Citrate is present in the lungs and secreted by *P. aeruginosa*. In the presence of citrate, LasR- strains form unique aggregates. Using fluorescent microscopy, we can observe LasR- cells adhering to each other rather than an abiotic surface, unlike wild-type cells which prioritize surface association. These aggregates are characterized by tight adherence to agar. LasR- strains are also known to have higher activity of the oxygen sensing transcription factor, Anr. Anr is required for the expression of *cupA* which has homology to type I fimbriae. The role of *cupA* in *P. aeruginosa* has been unclear. *cupA* expression and CupA protein levels are higher in *P. aeruginosa* LasR- strains than in their wild-type counterparts. LasR- aggregation and agar adherence are dependent on CupA. Genetic screens and follow up assays found that production of PQS and other uncharacterized proteins are also required. In contrast, this phenotype is not dependent on canonical biofilm genes such as *pilA, flgK,* or *pelA.* Citrate-induced agar adherence has been observed in multiple clinical isolates including LasR- and LasR+ strains. Screening of the PA14 non-redundant transposon insertion library also identified several mutants which are adherent in the absence of citrate. Here we demonstrate that the ubiquitous metabolite citrate induces a novel aggregation phenotype in some strains of *P. aeruginosa*. This work reveals the potential for diverse biofilm strategies across *P. aeruginosa* genotypes.

PA-2022-B007

Moving towards breath biomarkers: Translating *Pseudomonas aeruginosa* genotype and phenotype to the volatile metabolome

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In individuals with cystic fibrosis (CF), *Pseudomonas aeruginosa* chronic lung infections significantly reduce quality of life and increase morbidity and mortality. Tracking these infections is critical for monitoring patient health and informing treatments. We are working toward the development of novel breath-based biomarkers to track chronic *P. aeruginosa* lung infections *in situ* via its volatile metabolome. We hypothesize that clinically important genetic and phenotypic trends (*e.g.* mutations in the Las and Rhl quorum sensing systems) will be reflected in the volatile metabolome of *P. aeruginosa*; identifying these volatile features is an important step in this pursuit for diagnostic

biomarkers. Using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOFMS), we characterized the *in vitro* volatile metabolomes of a set of *P. aeruginosa* chronic infection isolates collected from individuals with CF. We identified differences in the relative abundances of volatile compounds that correlate with an early- or late-chronic infection phenotype, as well as mutations in *lasRI* and *rhlRI* genes. These results indicate that it may be feasible to track *P. aeruginosa* chronic lung infections by measuring changes to the infection volatilome, and lays the groundwork for exploring the translatability of this approach to direct measurement using patient breath.

PA-2022-B008

A Clash of Kingdoms: Illuminating *Pseudomonas aeruginosa* and *Aspergillus fumigatus* interactions in the CF lung environment

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Pseudomonas aeruginosa (Pa) is the most prevalent and persistent microorganism isolated from cystic fibrosis (CF) sputum and is a leading cause of mortality in CF patients. Approximately 15% of people with CF are infected with *Aspergillus fumigatus* (Af), a devastating human fungal pathogen. Af can accelerate lung function decline in people with CF, and some of these Af infected patients will develop allergic bronchio-pulmonary aspergilliosis. Clinical evidence suggests that the co-isolation of both Pa and Af indicates a poorer prognosis, but the exact reason for this is unclear. This work aims to understand how the behavior of both Pa and Af change in co-culture when compared to monoculture, and to dissect the specific cues which drive these physiological changes. These pathogens were cultured in media which is representative of the CF respiratory tract - synthetic CF-sputum media (SCFM2), where Pa and Af co-exist. Comparative omic analysis (RNA-seq, TMT-proteomics and temporal untargeted metabolomics) and spatiotemporal imaging was carried out on Pa and Af in SCFM2 monoculture and co-culture. These data revealed that co-culture has a drastic effect on Pa and Af, with both pathogens activating distinct nutrient scavenging mechanisms, natural product biosynthesis, metabolic rewiring, and complementary stress responses. Remarkably, most of the Pa and Af co-culture omic signatures could be recapitulated through targeted modification of the SCFM2 base composition and supplementation of SCFM2 with key Af and Pa metabolites. In summary, the co-culture of Pa and Af in SCFM2 stimulates the expression of Pa and Af systems with established roles during infection – micronutrient

scavenging, toxin biosynthesis, allergen expression, protease production, and central metabolic nodes. The activation of these systems during coinfection may partly explain the synergy of these pathogens during respiratory infection. These data will be used to develop a clear model of Pa-Af interactions in CF, catalyzing biomarker development efforts.

PA-2022-B009

Investigating polymicrobial interactions in the cystic fibrosis lung

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A characteristic complication associated with cystic fibrosis (CF) is chronic respiratory infections due to polymicrobial communities that reside within the airways of persons with CF, accelerating the decline in their lung function. Culture-independent studies that leveraged 16S rRNA gene sequencing and metabolic modelling predicted that *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus sanguinis*, and *Prevotella melaninogenica*, among the most abundant and prevalent bacterial species within the CF lung, serve as a representative community in pwCF. Modeling studies predicted that multiple metabolites were cross-fed among these microbes. Interestingly, we observed that *Prevotella* could not be recovered as a monoculture in artificial sputum medium (ASM) under anaerobic conditions. However, its recovery was consistently enhanced in the presence of *Pseudomonas*, as compared to the other organisms in the 4-species CF polymicrobial community model. Therefore, this study aims to investigate the mechanisms that govern the interactions between *Pseudomonas* and *Prevotella*. We conducted a pilot screen of the *P. aeruginosa* PA14 mutant library and identified mutations in genes that encode enzymes involved in malonate and propionate metabolism as potential mediators implicated in the interaction between these two organisms. Upon testing deletion mutants, we demonstrated that PA14 *ΔmdcC* and *ΔprpB* could not significantly support the growth of *Prevotella*. Complementing the mutants or supplementing ASM with acetate or succinate, the respective metabolic end-products of malonate and propionate, rescued the growth phenotype of *Prevotella*. Additionally, we observed that the omission of mucin from the ASM formula no longer allowed for the recovery of *Prevotella* in co-culture with *Pseudomonas*. Since malonate and propionate are not components of ASM and anaerobic organisms such as *Prevotella* can ferment mucin. We conclude that there is a cross-feeding mechanism in which *Prevotella* ferments mucin into malonate and propionate that *Pseudomonas* then

metabolizes into acetate and succinate, which *Prevotella* in turn utilizes to support its growth.

PA-2022-B010

Antibiotic-driven competitive release in the respiratory microbiota: using the micro-ecological context of infection to inform treatment strategies

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Lungs contain communities of bacteria that include oral flora and opportunistic pathogens. Antibiotic treatment of respiratory infections generally focuses on a single pathogen of interest without considering the other bacterial taxa present in the lungs. Ignoring the micro-ecological context of infection creates the potential for the enrichment of previously rare pathogens (competitive release) in response to antibiotics, rather than the successful treatment of infection. In our work, we combine clinical descriptive studies with ex-vivo and in-vitro experimentation to investigate the ecological relationships between antibiotics, pathogens, and the lung microbiota. We use this multi-pronged approach to answer questions in both chronic infections (cystic fibrosis) and acute infections (pneumonia in the pediatric ICU). In a chronic cystic fibrosis (CF) lung infection model, we describe the respiratory flora of people with CF via 16s rDNA sequencing of sputum. We then use this information to construct a synthetic CF lung microbiota model of the top ten taxa in artificial CF sputum medium. We show that, in the absence of antibiotics, the microbiome structure in a synthetic sputum medium is highly repeatable and dominated by oral commensals. In contrast, challenge with physiologically relevant antibiotic doses leads to substantial community perturbation characterized by multiple alternate pathogen-dominant states and enrichment of drug-resistant species. These results provide evidence that antibiotics can drive the competitive release of previously rare opportunistic pathogens and offer a path towards microbiome-informed conditional treatment strategies. Using this same framework, we are developing ex-vivo and in-vitro models of the lung microbiota in intubated pediatric ICU patients.

Redundant lactate utilization genes in *Pseudomonas aeruginosa* exhibit differential regulation and adaptation to unique environments

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The maintenance of duplicate genes can confer fitness advantages, especially if the genes are expressed under different conditions. The opportunistic pathogen *Pseudomonas aeruginosa* contains redundant genes for lactate utilization, and their expression is differentially sensitive to distinct isoforms of lactate. The D- isoform of lactate is produced by many bacteria, including *P. aeruginosa*. The L- isoform of lactate is a prevalent metabolic intermediate in eukaryotes, found within tumors and in the cystic fibrosis lung environment, which is commonly colonized by *P. aeruginosa*. *P. aeruginosa* possesses two L-lactate dehydrogenase genes, *lldD* (which is co-transcribed with an adjacent D-lactate dehydrogenase gene, *lldE*) and *lldA*. My goals are to elucidate the mechanisms controlling *lldD* and *lldA* expression and to characterize the contributions of these genes to biofilm physiology and infection. Expression of *lldDE* is controlled by the repressor LldR and I have shown that this operon can become de-repressed under micromolar concentrations of *either* D- or L-lactate. Conversely, I have demonstrated that *lldA* expression is activated by a LysR-family regulator, specific to L-lactate in the millimolar concentration range. I postulate that the differential regulation of these loci helps *P. aeruginosa* adapt to distinct environments: the *lldDE* operon functions primarily in monospecies biofilms, where D-lactate is produced, whereas *lldA* functions in the host environment, where L-lactate is at much higher concentrations. Metabolic labelling paired with stimulated Raman scattering microscopy shows that the ability to utilize endogenously produced lactate enhances metabolic activity in the oxic zone of glucose-grown biofilms. Finally, ongoing work using multicellular tumor spheroids is investigating the contributions of *lldA* during colonization of a hypoxic host environment. Together, these observations inform our model for the roles of *P. aeruginosa*'s redundant lactate utilization genes during adaptation to distinct lifestyles and/or host environments.

Adaptation of a *Pseudomonas aeruginosa* biofilm deficient mutant during chronic infection

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Chronic infections persist despite extensive treatment strategies. These infections are commonly caused by bacterial biofilms. *Pseudomonas aeruginosa* is often implicated in chronic infections, especially in immune-compromised individuals. Further complicating these infections are the ability of bacteria to adapt, usually by evolving variants that are more fit and persistent. Using a porcine chronic wound model, we previously demonstrated that *P. aeruginosa* rugose small-colony variants (RSCVs), with mutations in the *wsp* pathway, are selected during infection. These variants had elevated levels of c-di-GMP, and a hyperbiofilm phenotype that is dependent on the overproduction of exopolysaccharides, Psl and Pel. We therefore wanted to determine if there are other adaptive benefits of high c-di-GMP levels, independent of exopolysaccharide production, and if other pathways are under selection that promote persistence. To test this, porcine wounds were inoculated with a Δ*wspF*Δ*pslBCD*Δ*pelA* triple mutant. This mutant retains high c-di-GMP levels due to the *wspF* deletion, however is unable to produce Psl and Pel exopolysaccharides, and as such has a reduced biofilm phenotype. The wound bacterial burden was examined at days 7, 14 and 35 post infection. At each time point small-colony variants (SCVs) were isolated at a frequency of 5% of the total *P. aeruginosa* population. Whole genome sequencing revealed that these SCVs had acquired mutations in either lipopolysaccharide (LPS) or type IV pili (T4P). These mutations led to an increase in both c-di-GMP levels and biofilm formation relative to the Δ*wspF*Δ*pslBCD*Δ*pelA* parent. Together these results indicate that SCVs can evolve independently to the overproduction Psl and Pel. We predict that LPS and T4P variants experience selection during infection due to reduced immune recognition and increased adhesion by these variants. Finally, these data suggest that there may be a hierarchy of mutations under selection during infection (*wsp* > T4P > LPS).

The influence of growth medium on CF lung pathogen volatilomes

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Pseudomonas aeruginosa and *Staphylococcus aureus* are the predominant opportunistic lung pathogens for persons with CF and are leading causes of respiratory failure and mortality. Currently, sputum culture remains the standard of care for lung infection detection, but sputum production is on the decline due to improvements in CF therapies. To fill this diagnostic gap, we are working toward the development of breath tests for lung infections by characterizing the volatile metabolome (or "volatilome") of *P. aeruginosa* and *S. aureus*. In this study, we explored the influence of growth medium on the volatilomes of two strains of *P. aeruginosa* (PAO1 and PA14) and *S. aureus*, as well as two other species from the same genera, *S. epidermidis* and *P. chlororaphis*. We hypothesized that the volatilomes would be influenced by the growth medium, but that biological differences between these species and strains would dominate the volatilomes and facilitate identification. *P. aeruginosa* strains PAO1 and PA14, *P. chlororaphis, S. aureus*, and *S. epidermidis* were grown aerobically for 24 h at 37°C in biological triplicates in lysogeny broth Lennox (LB), brain heart infusion (BHI), Mueller Hinton broth (MHB), and tryptic soy broth (TSB). The cell-free culture supernatants were sampled by head space solid phase microextraction (HS-SPME) and analyzed by comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS). Hierarchal clustering analysis (HCA) and principal components analysis (PCA) were performed to observe the clustering of the samples based on their volatile metabolites. We observed that the PA14 volatilome was not significantly impacted by the growth medium, whereas the PAO1 volatilome was medium-dependent. However, these influences of media were weaker at the genus or species level, thus enabling samples to cluster according to taxonomic hierarchy in PCA and HCA analyses. The results indicate *P. aeruginosa* and *S. aureus* can be differentiated by their volatilomes independent of nutrient availability and differences in strains.

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Investigating the role of *Pseudomonas aeruginosa* lipid A deacylase PagL in cystic fibrosis airway infection

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Pseudomonas aeruginosa (*Pa*) is an opportunistic pathogen that causes chronic severe airway infection in patients with cystic fibrosis (CF). During infection, *Pa* undergoes functional and structural alterations to adapt to the unique airway environment. Lipid A, the membrane-bound component of lipopolysaccharide and TLR4/MD-2 complex ligand, can be modified in response to airway environmental pressures. This influences signaling through TLR4, impacting treatment and clinical outcomes. The *Pa* PagL enzyme removes a 3-OH C10 acyl chain from lipid A, resulting in a hexa-acylated structure found in many CF isolates. However, using MALDI-TOF MS we identified *Pa* isolates with hepta-acylated lipid A structures that arise and persist throughout longitudinal sample collection. Whole genome sequencing revealed loss-of-function mutations in *pagL*, resulting in retention of the 3-OH C10 acyl chain. To expand upon the role of *pagL* during infection, *Scnn1b*-transgenic BALB/c mice were infected with CF *Pa* isolates from the same patient: an early isolate (obtained at 8 years of age) with a hexa-acylated lipid A phenotype or a late isolate (at 16 years of age) with a hepta-acylated lipid A phenotype. To evaluate the role of growth phase, isolates were grown under planktonic or biofilm growth conditions prior to infection. At 48 hours post-intranasal infection, with inocula grown planktonically, there were elevated levels of *Pa* in the hexa-acylated infected mice, whereas *Pa* in the hepta-acylated infected animals was not culturable. However, when the inocula were grown as biofilms, there were no differences seen between the hexa- and hepta-acylated infected animals. Our data indicate a complex role for *Pa* PagL in CF airway infection. Selection and persistence of *pagL* mutations in *Pa* isolates over time may reveal a beneficial adaptation for survival in the CF lung; however, future work characterizing the interaction between *pagL*-deficient *Pa* and innate immune cells in the lung is needed.

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Rapid genetic characterization of metabolisms in *Pseudomonas putida*

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Pseudomonas putida is a robust growing microbe with diverse metabolisms and mechanisms of resistance. Efforts to categorize gene function in-silico are hampered by the number of gene paralogs across the *P. putida* genome. Functional genomics methods like transposon sequencing (TnSeq) provide a route to rapidly characterize gene function. Using random barcode transposon sequencing (RB-TnSeq), fitness phenotypes for hundreds of conditionally essential genes involved in *P. putida's* response to diverse carbon and nitrogen sources were identified. Informed by these data, a variety of genes and enzymes have been repurposed for use as biosensors or in engineered metabolic pathways. Beyond the applications for these data, more fundamental information can be gained as well. Genes involved in resistance, metabolism, and global regulation have been identified. The protein domains of unknown function (DUF), DUF1302 and DUF1329, have strong fitness defects in the presence of diverse aliphatic molecules as carbon sources. We hypothesize that these proteins could be providing resistance against or transport for these molecules. The central regulators, GacS/GacA, also showed diverse phenotypes across the conditions tested. These results point to a particular metabolite playing a role in the regulation of the GacS/GacA system. The diverse data provided from these functional assays deepen our understanding of this polyphagous microbe, and they enable us to make informed genetic mutations to promote desirable phenotypes.

PA-2022-B016

Modeling polymicrobial interactions in the cystic fibrosis lung

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Cystic fibrosis (CF) is a multiorgan genetic disease where the accumulation of thick mucus in the airways of persons with CF (pwCF) is an excellent nutritional environment for pathogens such as *Pseudomonas*, *Staphylococcus*, *Streptococcus,* and anaerobes growing as polymicrobial biofilm-like communities to thrive and cause disease. These communities are difficult to eradicate, at least in part, because they exhibit both resistance and tolerance (collectively called “recalcitrance”) to antimicrobial agents. The current challenge for treating such polymicrobial, biofilm-based infections is supported by numerous observations indicating that interactions among microorganisms can lead to unexpected changes in antimicrobial sensitivity and poor clinical outcomes. Here, we leveraged published large microbiome 16S rRNA gene amplicon datasets to identify polymicrobial community clusters that encompass most of the microbial diversity existing in the CF airway. We then experimentally modeled a mixed-species polymicrobial biofilm including *Pseudomonas, Staphylococcus, Streptococcus and Prevotella* species by cultivating these microbes in artificial sputum medium (ASM). Using this new model, we tested the hypothesis that inactivation of the *lasR* gene in *Pseudomonas aeruginosa*, known to be prevalent in the airway of pwCF and associated with worsened clinical outcomes, could impact tobramycin sensitivity in the context of a polymicrobial versus mono-species biofilms. We observed that, while wild-type *P. aeruginosa* is further sensitized to the presence of this aminoglycoside in a mixed-species community versus its monoculture counterpart, the inactivation of the *lasR* gene results in the inability of tobramycin to eradicate *P. aeruginosa* in the community context, and our work implicates an MvfR-regulated factor in this LasR-dependent, community-specific recalcitrance to this front-line antibiotic. Overall, our work shows that, through the utilization of complex polymicrobial communities, we can identify unexpected clinically important traits that would not readily be observed by using pure culture conditions.

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Identification of novel *Pseudomonas aeruginosa* vaccine antigens using an innovative proteomic approach

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Pseudomonas aeruginosa is an opportunistic pathogen with great adaptability to both non-clinical and clinical settings. It causes severe infections for immunocompromised individuals and patients with cystic fibrosis (CF), bronchiectasis or chronic obstructive pulmonary disease (COPD), usually establishing chronic infections that lead to poor prognosis and lung function decline. Due to its multiple antimicrobial resistance (AMR) mechanisms, drug therapy is often ineffective, so *P. aeruginosa* infections are a common cause of high morbidity and mortality in those people. Therefore, a vaccine that prevents infection would improve susceptible patients' quality of life and reduce both mortality and the spread of AMR. Because bacterial adhesins have shown good efficacy as vaccine antigens, we aim to identify proteins involved in *P. aeruginosa* attachment to human lung epithelial cells (16HBE14o-), which may be potential effective antigens for a prophylactic vaccine. The ability of six clinical *P. aeruginosa* isolates from different sources of infection to attach to 16HBE14o- cells was shown to be strain-dependent by two different methods (colony counting and confocal microscopy). A CF transmissible strain (LES 431) showed the lowest level of attachment, followed by a burn isolate (Mi 162). Strains from COPD and CF patients (57P31PA, AA2, ATM 0060-3) showed moderate attachment, while the community acquired pneumonia isolate (A5803) showed the highest attachment. Based on these results, we selected four strains with different attachment (LES 431, AA2, 57P31PA, A5803) to identify adhesins. Using our innovative 2-dimensional cell-blot method and mass spectrometry, we identified seven novel proteins involved in attachment, common to at least two strains. Bioinformatic tools and literature showed that they are surface-exposed, predicted to be immunogenic, and have low homologies with human, mouse or *Escherichia coli* proteins. Consequently, these novel candidates have the potential to be effective vaccine antigens, and reduce morbidity and mortality due to *P. aeruginosa* infections.

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Using computational analyses to establish a SCFM2-airway epithelial cell co-culture model for chronic *Pseudomonas aeruginosa* infections in cystic fibrosis

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Pseudomonas aeruginosa (*Pa*) is an opportunistic pathogen associated with chronic infections in the respiratory tract of people with cystic fibrosis (pwCF). Existing animal models do not accurately replicate the transcriptional program of *Pa* in the CF respiratory tract, so we have endeavored to improve the models used to study *Pa* in the CF airway. We previously developed a computational approach to assign accuracy scores to various models of *Pa* infection, based on their ability to recapitulate the gene expression observed in CF sputum. We reported that synthetic CF sputum (SCFM2) and airway epithelial cell (AEC) co-culture models performed well in capturing *Pa* gene expression in CF sputum (Cornforth, et al. mBio, 2020). Interestingly, the models missed 783 and 896 genes, respectively, of *Pa* gene expression in CF sputum transcriptomes; however, only 363 genes were not captured by both models when computationally combined. These results suggest that when SCFM2 and AEC co-culture models are integrated, a more accurate representation of *Pa* physiology in CF sputum could be achieved. We developed a reproducible, combined SCFM2-AEC co-culture model of *Pa* growing in sputum in the luminal space above the AEC cultures, using laboratory strain (PAO1) and CF clinical isolates. Confocal microscopy revealed *Pa* aggregate formation in the sputum layer, as well as in association with regions of the epithelium and reduced host cell cytotoxicity. Both are features of *Pa* aggregates in the CF respiratory tract. Current analysis is focused on using dual-species RNA sequencing to examine the accuracy scores of the combined SCFM2-AEC co-culture model and identifying differences in *Pa* and host gene expression vs CF sputum transcriptomes. The data provided herein endorses computational analyses to improve model systems for studying chronic *Pa* infections in the CF respiratory tract, with the long-term goal of using these models for antimicrobial drug development in CF.

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Inhibition of *Pseudomonas aeruginosa* alginate synthesis by ebselen oxide and its analogues

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that is frequently found in the airways of cystic fibrosis (CF) patients. During this life-long chronic infection, *P. aeruginosa* cells accumulate mutations that lead to inactivation of the *mucA* gene that results in the constitutive expression of *algD-algA* operon and the production of alginate exopolysaccharide. The viscous alginate polysaccharide further occludes the airways of CF patients and serves as a protective matrix to shield *P. aeruginosa* from host immune cells and antibiotic therapy. Development of inhibitors of alginate production by *P. aeruginosa* would reduce the negative impact from this viscous polysaccharide. In addition to transcriptional regulation, alginate biosynthesis requires allosteric activation by bis (3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) binding to an Alg44 protein. Previously, we found that ebselen (Eb) and ebselen oxide (EbO) inhibited diguanylate cyclase from synthesizing c-di-GMP. In this study, we show that EbO, Eb, ebsulfur (EbS), and their analogues inhibit alginate production. Typically, EbO was less toxic to mammalian cell and had a higher selective index (SI) compared to Eb and EbS. The mechanism whereby Eb inhibits alginate production remains unknown. Eb, EbO and EbS can covalently modify the cysteine 98 (C98) residue of Alg44 and prevent its ability to bind c-di-GMP. However, *P. aeruginosa* with Alg44 C98 substituted with alanine or serine was still inhibited for alginate production by Eb and EbS, suggesting that there is another target of inhibition. Our results indicate that EbO, Eb, and EbS are lead compounds for reducing alginate production by *P. aeruginosa*. Future development of these inhibitors could provide a potential treatment for CF patients infected with mucoid *P. aeruginosa*.

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Interlinking carbon metabolism, growth, and biofilm formation in *Pseudomonas aeruginosa*

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With its highly versatile metabolism, *Pseudomonas aeruginosa* is able to grow under various environmental conditions and to colonize different host tissues causing life-threatening acute and chronic infections. However, little is known about how specific metabolic properties relate to growth and survival under changing environments and in the host. Here, we analysed the role and regulation of three paralogous glyceraldehyde

3 phosphate dehydrogenases (GAPDH; GapA, GapB, and GapC), key enzymes of the central carbon metabolism. Monitoring growth of *gap* mutants in liquid cultures and in microfluidic devices demonstrated that GapA and GapB catalyse glycolytic reactions, while GapC primarily serves gluconeogenesis. Nevertheless, GapB supports mainly growth during the lag and logarithmic phase whereas GapA becomes relevant in stationary phase conditions. Specific Gap protein function is mirrored by the expression of *gap* genes under different nutritional conditions, providing tools to monitor directionality of the central carbon metabolism in individual cells and under host-like conditions. We found that when facing starvation conditions, *P. aeruginosa* cultures display heterogeneous *gap* gene expression patterns, indicating metabolic bet-hedging to optimize fitness. A mutant lacking *gapC* was not able to colonize a simple, non-vertebrate infection model, indicating that gluconeogenesis is of key importance in the host. Surprisingly, GapC specifically binds the global bacterial second messenger c-di-GMP, indicating that the activity of this protein is tightly controlled during growth and biofilm formation. We could demonstrate that metabolic activity translates directly into biofilm formation. While the role of c-di-GMP in bacterial behaviour and virulence control is well established, its interference with bacterial metabolism is largely unexplored. Ongoing studies probe the physiological role of GapC and its control by c-di-GMP during *P. aeruginosa* growth in surrogate host systems. Our long-term goal is to use this information to assess important metabolic processes of *P. aeruginosa* in the human patient.

PA-2022-B021

Multi-scale computational model of *P. aeruginosa* biofilm dispersal in lung mucin MUC5AC

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Mucus is a critical barrier against bacterial lung infections. Nevertheless, drug-resistant *P. aeruginosa* infections occur in the healthy and diseased lung mucus state. Previous studies demonstrated the lung mucin MUC5AC, which is a gel-forming glycoprotein that constitutes lung mucus, dispersed *P. aeruginosa* biofilms, with a 50% biomass reduction compared to growth medium alone. Increased biofilm dispersal may improve *P.*

aeruginosa antibiotic susceptibility. However, the mechanisms controlling the undispersed *P. aeruginosa* biofilm subpopulations in MUC5AC are unknown. Mechanistic computational models are powerful tools to simulate host-pathogen interactions at multiple spatial and temporal scales for *in silico* hypothesis testing of regulatory mechanisms and treatment strategies. In this work, we constructed a multi-scale computational model to investigate the hypothesized physiological microenvironments controlling the undispersed biofilm regions in 0 – 1wt% MUC5AC concentrations, spanning the healthy and diseased mucin state. To link intracellular *P. aeruginosa* metabolism to the extracellular mucin environment, the model coupled a genome-scale metabolic network model (GENRE), agent-based model (ABM), and metabolite diffusion model. Experimentally derived rules and parameters predicted biofilm dispersal dynamics and heterogeneous dispersal patterns. Model parameters for c-di-GMP, an intracellular molecule that regulates *P. aeruginosa* biofilm dispersal, production and degradation were determined from swim plate assays in variable oxygen and MUC5AC concentrations. The model identified *P. aeruginosa* subpopulations with heterogenous dispersal susceptibility regulated by MUC5AC concentration, oxygen gradients, intracellular c-di-GMP signaling, flagella synthesis, and extracellular biofilm polysaccharides. Biofilm microscopy with fluorescently-labeled polysaccharides and FISH imaging was performed for model validation of emergent biofilm microenvironments before and after mucin exposure. An unsupervised machine-learning pattern recognition algorithm achieved high-throughput feature extraction and classification of simulated and experimental biofilm dispersal patterns. Achieved by the multi-scale model and established microfluidic device, future work aims to investigate *P. aeruginosa* biofilm dispersal in controlled MUC5AC gradients representative of the heterogeneous lung mucus environment.

PA-2022-B022

PqsE mainly acts through RhlR to modulate and expand the *Pseudomonas aeruginosa* quorum sensing regulon

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Pseudomonas aeruginosa employs a sophisticated quorum sensing (QS) network, based on three main interwoven signaling systems, *las, rhl* and *pqs,* to finely modulate the expression of virulence traits. A particularly interesting interplay has emerged

between the effector protein PqsE and the transcriptional regulator RhlR, both required for full production of key virulence factors, including pyocyanin, rhamnolipids, and hydrogen cyanide, and for biofilm formation. Different studies focused on the characterization of the PqsE and RhlR regulons in genetic backgrounds in which PqsE can modulate RhlR activity, and in which RhlR controls *pqsE* expression, thus hampering the identification of genes specifically regulated by PqsE or RhlR. In this study, a *P. aeruginosa* PAO1 mutant strain with deletion of multiple QS elements and inducible expression of PqsE and/or RhlR has been generated and validated. Transcriptomic analyses performed in this genetic background allowed to unequivocally decipher the regulons controlled by PqsE and RhlR when expressed alone or in combination. These results were validated via qRT-PCR and transcriptional fusions, and unveiled that PqsE i) mainly impacts on *P. aeruginosa* transcriptome via an RhlR-dependent pathway, ii) expands the RhlR regulon, and iii) splits the RhlR-regulon in PqsE-dependent and PqsE-independent subregulons. Overall, these findings contribute to untangling the unique regulatory link between the *pqs* and the *rhl* QS systems mediated by PqsE and RhlR, and further confirm PqsE as a promising target for the development of anti-virulence drugs reducing *P. aeruginosa* pathogenicity.

PA-2022-B023

Pseudomonas aeruginosa exopolysaccharide Psl antagonizes *Staphylococcus aureus* growth

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Pseudomonas aeruginosa and *Staphylococcus aureus* are the top two pathogens present in the lungs of people with Cystic Fibrosis (CF), indicating they can co-exist *in vivo.* However, *P. aeruginosa* has been found to utilize various mechanisms to antagonize the growth of *S. aureus in vitro*. Our objective is to address this conundrum by studying the interactions between these two pathogens. Psl is one of the exopolysaccharides produced by *P. aeruginosa*. It is important for biofilm formation and exists in both a cell-associated and cell-free form. In this study, we test the hypothesis that Psl can antagonize *S. aureus* growth. By quantifying *S. aureus* survival co-cultured with planktonically grown *P. aeruginosa*, we found that a *P. aeruginosa* mutant lacking Psl production was less competitive towards *S. aureus* than wildtype. This is independent of known *S. aureus* killing mechanisms, including PQS (*Pseudomonas* quinolone signal) and pyoverdine. We found that cell-free Psl was critical for antagonizing *S. aureus* growth. Purified cell-free Psl demonstrated direct *S. aureus*

antimicrobial activity. Transmission Electron Microscopy of *S. aureus* treated with Psl revealed disrupted cell envelopes, suggesting that Psl causes *S. aureus* cell lysis. Further genetic experiments revealed that Psl-mediated killing appears to be dependent on *S. aureus* autolysis. We also found that *P. aeruginosa* CF clinical isolates with reduced or no Psl production showed reduced killing towards *S. aureus*. This could be a result of *P. aeruginosa* co-evolution with *S. aureus* in CF lungs and therefore promoting co-existence of both pathogens. In conclusion, this study defines a novel role for *P. aeruginosa* Psl in antagonizing *S. aureus* growth and impacting co-existence of the two pathogens *in vivo*.

PA-2022-B024

Inactivation of Entner Doudoroff pathway facilitates *Pseudomonas aeruginosa* pathogenesis during Catheter-associated Urinary Tract Infection

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Pseudomonas aeruginosa is one of the most common organisms responsible for catheter-associated urinary tract infection (CAUTI) and other nosocomial infections. Using a murine model of CAUTI, we previously showed that urea within urine represses *P. aeruginosa* quorum sensing. To understand the transcriptional response of *P. aeruginosa* to urine/urea, we performed RNAseq of PA14 WT strain and showed that the transcript levels of Entner Doudoroff (ED) pathway genes (glucose-6-phosphate 1-dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), keto-hydroxyglutarate-aldolase/keto-deoxy- phosphogluconate aldolase (*eda*) and phosphogluconate dehydratase (*edd*) genes) were significantly up-regulated with transcript levels ranging between 7.3 and 12.8 fold higher in LB supplemented with urea as compared to plain LB. *P. aeruginosa* uses ED pathway as an alternative to glycolysis due to lack of phosphorfructo-kinase-1 that converts fructose 6-phosphate to fructose 1,6 bi-phosphate in the classical glycolysis pathway. Based on the RNA sequencing data, we hypothesized that a functional ED pathway is necessary for *P. aeruginosa* pathogenesis. To address this, we generated in-frame deletion mutants lacking *eda*, *edd*, *pgl* or *zwf* genes and showed that Δ*eda* and Δ*edd* failed to grow in minimal media supplemented with glucose or gluconate as the sole carbon source, while *Δpgl* or *Δzwf* had attenuated growth as compared to wild-type highlighting different genetic requirements for utilization of these carbon sources. Our in vivo data showed that mice infected with *Δeda* had a significantly higher bacterial burden while Δ*edd*, Δ*pgl* and Δ*zwf* behaved similar to the

parental strain. Interestingly, the *Δeda, Δedd* or *Δpgl* increased the frequency of ascending kidney infection in mice during CAUTI. Taken together, our data suggest that the individual genes of the ED pathway act separately, rather than in concert in *Pseudomonas* pathogenesis during CAUTI model of mice.

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Probing the accessory genome of *Pseudomonas aeruginosa* for persistent infection determinants

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Pseudomonas aeruginosa is found chronically in the airways of 70% of Cystic Fibrosis (CF) patients, with infections decreasing the life quality and expectancy of patients. The mechanisms by which *P. aeruginosa* is able to escape treatment and generate persistent infections within the CF lung have not been fully elucidated. Mostly, the success of *P. aeruginosa* relies on its adaptability, which derives from its large genome (5.5-7 Mb) carrying genes for survival in a wide spectrum of environments. Most of these genes are not conserved among strains, composing the accessory genome, built up through horizontal gene transfer. Accessory genes involved in virulence and resistance have been described, yet the importance of this variable part of the genome has not been assessed for persistent infections. Genomic comparison of DK01 isolates, longitudinally obtained over six years, versus the laboratory strains PAO1 and PA14 revealed a pool of 324 strain-specific genes. Over one third of them have no predicted function. To investigate the relevance of these genes during infections, we have adapted a CRISPR interference (CRISPRi) method for tight transcriptional regulation of *P. aeruginosa.* With this technique, we can generate knock-down libraries comprising the whole accessory genome of the isolated of interest, which can be phenotypically tested for growth deficiencies. As a proof of concept, we generated an initial library targeting 541 genes in PA14, to test under planktonic and infection conditions. For the latter, we have developed a lung infection model with primary cells obtained from the airways of CF patients. These air-liquid interface cultures provide a biologically relevant scenario to test the function of the accessory genes, by infection with the CRISPRi libraries. Overall, this model allows to uncover novel mechanisms of persistent infections relying on accessory genes, and to identify novel markers and targets to improve treatment.

PA-2022-B026

Pseudomonas aeruginosa Wsp system responds to periplasmic stress

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Bacterial species use a variety of mechanisms to perceive a surface. *Pseudomonas aeruginosa* uses the Wsp chemosensory-like signal transduction pathway to sense surfaces and promote biofilm formation. The methyl-accepting chemotaxis protein WspA recognizes an unknown surface-associated signal and initiates a phosphorylation cascade that activates the diguanylate cyclase WspR. Here we show that the Wsp system is activated by different aspects of cell envelope stress. We screened a chemical library and found that chemicals that alter the cell envelope induce Wsp signaling, increase intracellular c-di-GMP levels, and can promote surface attachment. Furthermore, we heterologously expressed the Wsp system in *E. coli* and found it sufficient for sensing environmental changes and surfaces. Using well-characterized reporters for *E. coli* cell envelope stress response, we determined that Wsp sensitivity overlaps with the sensitivity of the *E. coli* cell envelope stress response system RCS, which response to LPS damage, osmotic shock, and peptidoglycan damage. Using CRISPRi and mutagenesis, we determined that loss of periplasmic chaperones, like DsbA, activates the Wsp system. Finally, treating cells with Wsp-inducing chemicals also resulted in unfolded periplasmic proteins or misregulation of inner membrane permeability. Our results suggest that cell envelope perturbations represent an important feature of surface sensing in *P. aeruginosa.*

PA-2022-B027

Unraveling the protein machinery and protein-interaction dynamics essential for vesicle formation of *Pseudomonas aeruginosa*

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The opportunistic bacterial pathogen *Pseudomonas aeruginosa* forms vesicles during infection of its host via one or more unknown molecular processes. Since these membrane-enclosed structures contain specific cargo molecules, they fulfill a variety of important tasks during host-pathogen interaction, immune cell interaction or intracellular trafficking. This organism is known to produce extracellular outer membrane vesicles (OMVs), solely composed of the outer membrane of the bacterium, as well as outer inner membrane vesicles (OIMV); and intracellular cytoplasmic membrane vesicles (CMV). However, data shows that two proteins that belong to the LemA family are responsible for re-structuring the inner (LemA1) and the outer membrane (LemA2) during vesicle production. Overproduction of the integral inner membrane protein LemA1 strongly induced the formation of intracellular vesicles, while overproduction of the LemA2 protein forced OMV production. It was hypothesized that these two proteins coordinate a dynamic protein super-complex network localized in the inner membrane–periplasm-outer membrane space. At first the isolation and basic characterization of OMVs from *P. aeruginosa* PA14 was established, using density gradient centrifugation approaches, filter-based isolation methods or nanoparticle tracking analysis (NTA) for size determination. Proteomic determination of the vesicle content, as well as immuno-gold labelling of each LemA-like protein was performed to disclose their localization within the vesicles. The outer membrane protein LemA2 was shown to be secreted from the cell within the vesicles, whereas the integral membrane protein LemA1 remains in the cell. For elucidation of the protein machinery associated to LemA1 and LemA2, involved interacting proteins were identified to show a strong connection to several membrane associated mechanisms like membrane transport and peptidoglycan recycling but predominantly cell division. Highly abundant interaction partners like ZipA, FtsE, FtsK, FtsI, FtsH and the freshly annotated ZapG lead to the assumption of membrane vesicle formation being strongly connected to the cell division apparatus.

PA-2022-B028

An updated genome-scale metabolic network reconstruction of *Pseudomonas aeruginosa* PA14 to characterize mucin-driven shifts in bacterial metabolism

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The mucosal barrier is a hydrated mucus gel that lines wet epithelial cells throughout the body and serves to protect against pathogens. Mucins, a key component of mucus, impair bacterial virulence mechanisms such as attachment and biofilm formation. However, some bacteria such as *P. aeruginosa* can reside within the mucus layer and cause infection. Insights into the metabolic responses of *P. aeruginosa* to mucins may enable the development of protective approaches against infection. Such insights can be gained through the application of metabolic network reconstructions and associated metabolic models, which allow for the mechanistic study of cellular metabolism. Here, we present *i*Pau21, an updated genome-scale metabolic network reconstruction of *P. aeruginosa* strain UCBPP-PA14 that we integrate with transcriptomic data to characterize the metabolic shifts of *P. aeruginosa* upon exposure to mucins. We updated the metabolic network reconstruction through metabolic coverage expansion, format update, extensive annotation addition, and literature/database-based curation to produce *i*Pau21. We then validated *i*Pau21 through growth rate, substrate utilization, and gene essentiality testing to demonstrate its improved quality and predictive capabilities. Published transcriptomic data was then integrated with the network using RIPTiDe to produce context-specific models that are more consistent with a given biological state. These models were analyzed using flux balance analysis-based methods to gain insights into their metabolic states. The contextualized models showed shifts in network utilization and structure in response to mucins, recapitulated known phenotypes of unaltered growth and differential utilization of fumarate metabolism, and provided a novel insight of increased propionate metabolism upon MUC5B exposure. This work serves to validate *i*Pau21 and apply it to the investigation of metabolic shifts of the pathogen in response to mucins. Our analysis recapitulated known phenotypes, provided novel insights into changes in pathogen metabolism following mucin exposure, and identified potential therapeutic strategies that could be further explored.

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Molecular mechanism of *Pseudomonas aeruginosa* induced cardiac dysfunction

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Pneumonia in ICU patients carry a bleak prognosis with high risk for cardiac dysfunction, largely due to cardiac infection and inflammation with subsequent tissue remodeling and fibrosis. *Pseudomonas aeruginosa* (*P.a.*) infection accounts for up to 20% of all cases of hospital acquired pneumonia with mortality rate of more than 30%. In the United States, it is estimated *P.a.* is associated with 51,000 cases of infection per year, and these infections are often difficult to treat due to multidrug resistance against many commonly used antibiotics. During bloodstream infection, *P. aeruginosa* induces a robust inflammatory response (TNFα, IL-1β, IL-6, MIP-2, and MCP-1), which promotes bacterial clearance. Unfortunately, an excessive inflammatory response can also have negative effects on the host, and unchecked inflammation may result in cardiac dysfunction. It remains unclear whether *P. a.* virulence factors or host inflammatory mediators are the contributing factors for cardiac dysfunction. To investigate this, we used a murine pneumonia model of *P.a.* infection, which often results in subsequent bacteremia. Our results shows that this *P.a.* infection results in severe cardiac electrical dysfunction and left ventricular dysfunction (increased ejection fraction, fractional shortening and decreased cardiac output). Surprisingly, there is no evidence of *P.a.* colonization in the heart. Mechanistically, we found that neutrophil recruitment and release of S100A8/9 in the lungs activate the TLR4/miR155 signaling pathways, which enhances systemic inflammation and subsequent cardiac dysfunction. Furthermore, we found that *P.a.* infection increases the accumulation inflammatory myeloid cells in the heart and alters the cardiac resident macrophage phenotype and function. Notably, we found that S100A8/9 deficiency aggravates cardiac dysfunction and increased mouse mortality, due to uncontrolled bacterial growth in the lungs and subsequent dissemination of bacteria into the heart. Our results indicate that *P.a.* induced release of S100A8/9 is double-edged, providing increased risk for cardiac dysfunction yet limiting *P.a.* growth.

PA-2022-B030

Inflammasomes expressed by corneal surface epithelial cells targeted by *P. aeruginosa*

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Pseudomonas aeruginosa is a leading cause of blinding corneal infections. One outcome of *P. aeruginosa* encountering a corneal epithelial cell is that bacteria can invade the cell and replicate inside of it, which may contribute to its ability to cause disease even when the protective corneal epithelium is intact, such as during soft contact lens wear. Previous research identified that Exotoxin S from *P. aeruginosa* was required for intracellular survival and replication in corneal epithelial cells, but precisely how ExoS contributes to these phenomena remains unknown. Using live imaging and immortalized corneal epithelial cells grown in culture, we discovered that in the absence of exotoxins, bacteria invade corneal epithelial cells but elicit rapid pyroptotic cell death, dependent on the caspase-4 inflammasome. When ExoS is present, pyroptosis of invaded cells is significantly delayed by an average of 4.3 +/- 2.7 hours, extending time for bacteria to replicate inside intact corneal epithelial cells. Extension of host cell survival time requires the ADP ribosyltransferase activity of ExoS. In addition to providing an intracellular niche for bacteria, ExoS also significantly also reduces the secretion of pro-inflammatory cytokines including IL-1β, suggesting involvement of an additional canonical inflammasome pathway. These data support a model in which corneal epithelial cells can limit colonization of invasive microbes through the caspase-4 inflammasome pathway, however *P. aeruginosa* is uniquely equipped to delay the lytic endpoint of inflammasome activation and persist within intact corneal surface cells.

PA-2022-B031

A novel role of Calcium in *Pseudomonas aeruginosa* resistance to Polymyxin B

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The well-known nosocomial pathogen *Pseudomonas aeruginosa* causes life-threatening infections especially in patients suffering from cystic fibrosis (CF). Its ability to rapidly develop resistance to many antibiotics including the "last resort" Polymyxin B (PMB) has resulted in extremely limited choices of therapeutic options. Despite all efforts, multifactorial PMB resistance mechanisms of *P. aeruginosa* are inadequately understood. This study examines the role of calcium (Ca) at the levels commonly present in the CF airways in *P. aeruginosa* PMB resistance. We observed that the PMB resistance in *P. aeruginosa* PAO1 increased twelve-fold at elevated Ca. Susceptibility testing of the corresponding mutants and genome-wide RNA-seq analyses of PAO1 grown at elevated Ca showed that the known PMB resistance mechanisms have no role in Ca-induced PMB resistance. Through random chemical mutagenesis followed by susceptibility testing and sequencing, we identified three genes contributing to the Ca^{2+}-dependent resistance: PA2803, PA3237, and PA5317. To decipher the role of PA2803 encoding a putative phosphonatase, we purified the recombinant protein and, in agreement with the sequence-based predictions, confirmed that PA2803 has no phosphonatase activity. Using a pull-down assay with the *P. aeruginosa* produced PA2803 as a bait, we identified several putative binding partners, the validation of which is underway. To study the Ca-dependent alterations in *P. aeruginosa* membranes, we performed the NPN and PI uptake assays. The results showed that both the outer and the inner membranes' permeability increased during growth at elevated Ca. This effect does not involve the aforesaid genes. The membrane proteomics and transcriptomics analyses showed that the membrane make-up is substantially altered in response to Ca. Together, these results suggest novel Ca-dependent alterations in *P. aeruginosa* membrane biogenesis leading to the increased PMB resistance. The ongoing studies aim to provide the molecular details of the membrane reconstitutions to help designing affective antimicrobial strategies in the future.

PA-2022-B032

Antibiotic tolerance of a human pathogen generated by self-induced toxin-mediated interference with NAD metabolism

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Owing to the gradual development of antibiotic resistance and tolerance, chronic infections are difficult to eradicate. We have recently identified mutations in a novel toxin-antitoxin (TA) system of *P. aeruginosa* as drivers for multi-drug tolerance. Follow-up

biochemical and structural analyses revealed that the NatT RES domain endotoxin forms a complex with its cognate repressor NatR, in which a central NatT toxin dimer is flanked by two tightly associated dimers of the NatR antitoxin. We showed that NatT activation depletes the intracellular NAD^+ and $NADP^+$ pools and leads to severe oxidative stress and growth arrest of a subpopulation of bacteria. Single cell reporters, FACS sorting and drug treatment of isolated subpopulations demonstrated a strict correlation between oxidative stress, growth limitation and (p)ppGpp-dependent drug tolerance. Expression of the *natRT* TA module is repressed by the antitoxin NatR and by nicotinamide, a precursor for NAD biosynthesis. This, and the observation that supplementation of the growth medium with nicotinamide effectively restores antibiotic susceptibility of NatT-mediated hyper-tolerant variants, implies a bidirectional control of the NatT endotoxin and redox metabolism. The isolation of activating mutations in the *natT* gene of *P. aeruginosa* isolates from CF airways argues that NatT contributes to antibiotic resilience during chronic infections. The identification a novel toxin that induces beneficial self–poisoning and survival by targeting a central metabolite of *P. aeruginosa* provides an entry point into defining the molecular basis of antibiotic tolerance in chronic human infections.

PA-2022-B033

Exploiting the potential of *Pseudomonas putida* as a host for engineered type I polyketide synthases

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The biotechnological production of drugs and commodity chemicals is essential to create a renewable economy. Yet despite great progress in metabolic engineering, many industrial chemicals still require synthesis from petroleum-derived precursors. Type I polyketide synthases (PKSs) have the potential to play an important role in addressing this problem. Due to their highly modular fashion, PKSs can be rationally designed to produce a wide range of molecules. These engineered PKSs can then be leveraged to valorize renewable feedstocks when expressed in appropriate microbial hosts. This study aims to develop the bacterium *Pseudomonas putida* KT2440 for the production of polyketides through rational host engineering. After assessing *P. putida*'s native ability to support PKS expression and function, we chose an engineered type I PKS to produce industrially relevant 3-hydroxy acids. Both malonyl-CoA and the acyl-CoAs derived from branched chain amino acid (BCAA) degradation were identified as limiting factors in product formation. The application of a barcoded mutant library and quantification of the DNA barcodes in a competitive growth assay (RB-TnSeq), allowed us to rapidly validate the identities of the BCAA catabolic genes. As a final step, we deleted parts of this catabolism to promote accumulation of precursor acyl-CoAs and increased polyketide titers. This work is the first example of successfully expressing an engineered PKS in *P. putida*. In addition, the strategy we developed is a universal approach that could be applied to establish other microorganisms as non-native PKS hosts.

PA-2022-B034

Rise of *lasR* quorum sensing mutants in keratitis isolates in the United States and the role of LasR in corneal infections

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Introduction: a previous report described numerous *Pseudomonas aeruginosa* isolates with *lasR* mutations among keratitis patients collected in India between 2006-2010

(21.8%, n=101). Of concern, patients infected with *lasR* mutants had worse visual outcomes than those infected with LasR positive strains. **Hypothesis:** *lasR* mutants will be found among keratitis isolates worldwide and these will cause more severe keratitis. **Objectives**: The first goal of this study was to determine whether *lasR* mutants were represented among keratitis isolates collected at our hospital in the North Eastern USA over the past 25 years. A second goal was to evaluate protease phenotypes of the sheen isolates in vitro and the importance of LasR in a rabbit keratitis model. **Methods**: the *lasR* alleles of several sheen positive isolates were cloned, sequenced, and evaluated in a *lasR* mutant for the ability to complement a protease deficient phenotype. **Results** indicate a significant ($p<0.01$) increase in *lasR* isolates isolated among keratitis patients ranging from 0% of isolates from 1993-1997 (n=57) to 25.7% from isolates from 2013-2017 (n=101). The majority of *lasR* alleles isolated from the sheen positive isolates had different mutations, and generally failed to restore protease activity of a Δ*lasR* mutant strain. In vivo, a PA14 Δ*lasR* isolate was significantly less virulent than the wild type with respect to bacterial burden and clinical signs of inflammation. Strikingly, the PA14 strain caused corneal perforations in 59% of eyes, whereas 0% of Δ*lasR* infected eyes perforated. **Discussion:** A caveat of the infection model is that the ocular surface defenses are breached and further studies regarding the role of LasR on the ocular surface are underway. **Conclusions:** these data suggest a concerning increase in *lasR* isolates among keratitis patients in the USA; however, experiments suggest a positive role for LasR in promoting pathogenesis in corneal infections.

PA-2022-B035

Adaptive evolution of the quorum sensing regulon in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa has become a model for studies of cell-cell communication and coordination of cooperative activities and uses quorum sensing (QS) to activate expression of a relatively large number of genes compared to many other organisms. We hypothesized that long-term growth of bacteria under conditions where only limited QS-activated functions were required would result in a reduction in the size of the QS-controlled regulon. To test this hypothesis, we grew *P. aeruginosa* PAO1 for about 1000 generations in a minimal medium with casein and adenosine as energy sources, a condition in which expression of QS-activated genes is required for growth. We

compared the QS regulons of populations after 30 generations to those after about 1000 generations in two independent lineages by using quorum quenching and RNA-Seq technology. In one evolved lineage the number of QS-activated genes identified was reduced by about 70% and in the other by about 45%. There were many genes lost from the QS regulons of both lineages. We describe diverse pathways to elimination of genes from the QS regulon. Our results indicate that variations in the number of QS-activated genes reported for different *P. aeruginosa* strains may reflect their environmental histories, and we suggest that modifications to the QS circuitry are one of the many adaptive strategies that can be employed by *P. aeruginosa* as it encounters new environments.

PA-2022-B036

The LasR quorum sensing master regulator becomes globally dispensable in low phosphate conditions

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Pseudomonas aeruginosa is major opportunistic pathogen, its ability to cause infections relies on the production of multiple virulence factors, most of them regulated via Quorum Sensing (QS). The QS systems promote the activation of several genes when high bacterial cell densities are reached. *P. aeruginosa* harbors three QS systems (Las, Rhl and Pqs), which are arranged as a hierarchy, being the Las system on the top. In the last years, strains that lack a functional Las system have been isolated, and some of them still have the ability to produce virulence factors and infect animal models, so it has been suggested that the hierarchy is flexible under some conditions like very late growth times (days) or with atypical strains. Here, we analyze the PAO1 type strain and its *lasR*-derived mutant and report, for the first time, a growth condition (phosphate limitation) where LasR absence has no effect either on virulence factor production but also on the gene expression profile, in contrast to a condition of phosphate repletion where the LasR hierarchy is maintained. This work provides evidence on how the QS hierarchy in PAO1 type strain can change from being a strictly LasR-dependent to a LasR-independent RhlR-based hierarchy in the same points in the stationary phase, only by changing the phosphate availability. This Las dispensability, support the fact that in nature Las system mutants are commonly selected. Also, it is important to highlight that the low phosphate availability is common in human tissues, and it is even lower in immunosuppressed

patients, so describing the QS regulation in these conditions is very important in order to design better QS based treatments.

PA-2022-B037

The anti-sigma factor MucA is required for viability in *Pseudomonas aeruginosa*

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During the decades-long infections in the cystic fibrosis (CF) airway, *Pseudomonas aeruginosa* undergoes selection. One bacterial genetic adaptation often observed in CF isolates is *mucA* mutations. MucA inhibits the sigma factor AlgU. Mutations in *mucA* lead to AlgU misregulation, resulting in a mucoid phenotype that is associated with poor CF disease outcomes. Due to its ability to be mutated, *mucA* is assumed to be dispensable for bacterial viability. Here we show that, paradoxically, a portion of *mucA* is essential and depletion of MucA is lethal in *P. aeruginosa*. We demonstrate that *mucA* is no longer required in a strain lacking *algU*, that *mucA* alleles encoding for proteins that do not bind to AlgU are insufficient for viability, and that *mucA* is no longer essential in mutant strains containing AlgU variants with reduced sigma factor activity. Furthermore, we found that overexpression of *algU* prevents cell growth in the absence of MucA. Our data suggests that the toxicity associated with AlgU overproduction is due to overexpression of its own regulon, as overproduction of an AlgU DNA-binding mutant lead to a less severe growth defect. Together, these results suggest that in the absence of MucA, the inability to regulate AlgU activity results in the loss of bacterial viability and that the truncated MucA produced in mucoid isolates is vital for keeping AlgU activity under control. Finally, we speculate that essentiality of anti-sigma factors that regulate envelope function may be a widespread phenomenon in bacteria and that this essentiality can be exploited to either kill bacteria or select for those with reduced envelope stress response.

Virulence, Fitness and Antibiotic Resistance: the multiple faces of a single mutation in *Pseudomonas aeruginosa* during macrophage infection

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During chronic cystic fibrosis (CF) lung infections, *P. aeruginosa* (PA) is thought to adapt to the lung environment by acquiring antibiotic resistance and decreasing production of virulence factors. However, during a 5-year clinical observational study, aztreonam resistance was associated with a higher incidence of pulmonary exacerbations. In line with these clinical data, we previously showed that mutations in PA *nalD* increased virulence *in vivo* in addition to conferring resistance to aztreonam and other antibiotics commonly used in CF. The objective of this study was to determine the mechanisms involved in PA *nalD* mutant hypervirulence. To investigate this, bone marrow-derived macrophages (BMDMs) were infected with WT or *nalD* mutant PAO1. The *nalD* mutant burden grew more and was more cytotoxic to BMDMs compared to WT. Because hypervirulence was not attenuated by deletion of MexAB-OprM efflux pump, the only target known to be regulated by *nalD*, we performed RNA-sequencing to identify other virulence factors that could explain the *nalD* mutant hypervirulence. RNA-seq showed that the *nalD* mutant significantly overexpressed >100 genes involved in siderophore synthesis and transport, type III secretion (T3S), denitrification and metabolism during BMDM infection. Using GO Enrichment Analysis, we showed that increases in siderophore and T3S genes expression occurred prior to denitrification and metabolic pathways, highlighting a crucial role for virulence and iron uptake in bacterial fitness during macrophage infection. We hypothesized that iron overload could disrupt iron homeostasis and cause ferroptosis in macrophages. Indeed, during the *nalD* infections the iron content was significantly greater in BMDMs, as well as reactive oxygen species, lipid peroxidation, and cell death compared to WT infections. Finally, gallium nitrate (III) abrogated bacterial-induced cytotoxicity in BMDMs and inhibited both WT and *nalD* mutant growth by 10^2-10^3-fold. These data suggest that *nalD* mutant increase virulence and fitness during BMDM infection by upregulating siderophore, T3S, denitrification and metabolism genes. Finally, gallium represents a promising treatment for this “superbug” in CF patients.

Investigating the effect of microbial zinc competition on *Pseudomonas aeruginosa* zinc metalloprotease-mediated virulence

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Pseudomonas aeruginosa secretes virulence-associated zinc metalloproteases that accumulate during infection of the lungs of people with cystic fibrosis (CF). These proteases are activated by one another as part of a proteolytic cascade upon secretion and are subsequently involved in several processes including immune modulation and microbial competition. The zinc necessary for proteolytic activity is also needed by host cells and co-colonizing pathogens. The objective of this study was to investigate the effect of co-culturing *P. aeruginosa* with other common CF pathogens, namely *Staphylococcus aureus* and *Candida albicans*, on *P. aeruginosa* zinc metalloprotease-mediated virulence. *P. aeruginosa* strains were grown on a lawn of *S. aureus* or *C. albicans* on artificial sputum medium. Agar plugs or filters were then disrupted in water and suspended cells were used to plate for colony forming units (CFUs), to measure LasB and LasA enzymatic activities by colorimetric assays, and to measure P_{rhlI}-*lacZ-gfp* (quorum sensing) and P_{rpmJ2}-*lacZ-gfp* (zinc starvation) via β-galactosidase assay or flow cytometry. Co-culture with *S. aureus* did not affect *P. aeruginosa* proteolytic activities, quorum sensing activation, nor induce zinc starvation likely due to *P. aeruginosa*'s ability to kill *S. aureus*. Co-culture with *C. albicans* reduced quorum sensing corresponding to a reduction in *P. aeruginosa* cells and the induction of a zinc-starvation response. This, in turn, corresponded to a reduction in LasB activity and a disproportionate inhibition of LasA activity. Further, *C. albicans* induced a greater zinc-starvation response in a *P. aeruginosa* zincophore mutant suggesting that *P. aeruginosa* requires its zincophore to compete with *C. albicans* for zinc. Overall, the data show how competition for zinc can shape the microbial composition of CF lung infections as well as the virulence of *P. aeruginosa*. Ongoing studies will investigate how clinical strains and zincophore mutants grow and compete with one another to affect virulence-associated zinc metalloprotease activity.

Prevalence and genetic variation of the *Pseudomonas aeruginosa* elastase gene in clinical isolates from Cystic Fibrosis patients

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The *Pseudomonas aeruginosa* LasB elastase is the predominant protease in the *P. aeruginosa* secretome. It is a 33kDA non-specific metalloprotease which has been shown to exhibit hydrolytic activity against a wide range of targets, both host and pathogen specific, including degradation of host proteins involved in anti-microbial defence. LasB has been demonstrated to have effects on protease activation, immune evasion, extracellular matrix degradation and cleavage of the CFTR protein, leading to the consensus that it plays an important role in establishment of early infections in the Cystic Fibrosis lung. With the challenges of anti-microbial resistance and biofilm-mediated tolerance, adjunctive therapies including anti-virulence approaches are being proposed as a strategy to treat *P. aeruginosa* infections, with LasB being one such target. This study has investigated the natural variation of the *lasB* gene; documented in publicly available genomic databases and within a CF patient cohort clinical study, conducted at University Hospital Southampton (UHS), in which 106 *P. aeruginosa* isolates were isolated from 49 patients and their *lasB* gene sequenced. Key variants identified were cloned and expressed in a chromosomal *lasB* gene deletion mutant of PAO1 utilising a single promoter construct in a low copy number vector allowing assessment of the role of these variants in the organism's phenotype. This study identified unique LasB variants from the UHS cohort, not previously recorded in databases. Additionally, it is demonstrated that particular variants exhibit reduced elastase activity which may be relevant to evolutionary adaptation of *P. aeruginosa,* leading to reduced virulence in the context of establishment of chronic infections in the CF lung. Improved understanding and profiling of variant LasB proteins will facilitate development of specific LasB inhibitor drugs, which may be beneficial in the treatment of early *P. aeruginosa* infections in CF patients.

A quantitative inventory of flagellar motility genes in *Pseudomonas syringae* pv. tomato DC3000

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Motility is crucial for bacterial dispersal and survival. In plant pathogenic bacteria like *Pseudomonas syringae* pv. tomato DC3000 (*Pto* DC3000), flagellar motility assists cells throughout the infection process. Swimming and swarming motility enable cells to move over leaf surfaces and find entrance into leaf tissue. Once inside, flagellar synthesis and motility are suppressed to help cells hide from detection by host immunity. Here we aim to help advance understanding of the signal transduction pathways and structural proteins underlying swimming motility in plant pathogenic bacteria by building a complete inventory of motility related genes in *Pto* DC3000. We used an in vitro selection scheme and transposon insertion sequencing (TnSeq) to identify genes that alter swimming motility. We conducted two series of swimming assays in which a transposon insertion library of *Pto* DC3000 was used as the starting population. To enrich for mutants with insertions in genes necessary for motility, we collected cells that failed to move from the inoculation point after 48 hours and then used these cells to inoculate fresh swimming medium. As a control we sampled the entire swimming colony for reinoculation on fresh medium. This isolation and reinoculation was repeated for a total of 6 passages. The enrichment effect was confirmed by sampling motility phenotypes of individual mutants in both resulting populations. We then used next-generation sequencing to determine the number of transposon insertions in each gene for the enriched and control populations. We quantified each gene's contribution to motility by tracking the insertion frequencies for each gene as the populations evolved in response to passaging. Besides chemotaxis and flagellar synthesis components, we found several new genes with strong motility altering phenotypes, including those encoding c-di-GMP enzymes, an uncharacterized hypothetical protein and an anti-sigma factor that has not previously been associated with motility.

Correlation of mRNA and protein in *Pseudomonas aeruginosa*

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A major challenge in studying bacterial pathogens such as *Pseudomonas aeruginosa* (*PA*) is to understand their physiology during infections. Recently, RNA-seq has been used as a proxy to interpret bacterial functions from human specimens. This approach directly measures messenger RNA (mRNA) levels, while the protein levels which ultimately determine bacterial functions are impacted by the coupled processes of transcription, translation and degradation of mRNAs and proteins. In addition, it remains unclear how growth rates impact bacterial gene expression globally by modulating abundance of RNA polymerases and ribosomes. Here, we aim to evaluate if mRNA level is a good proxy to predict protein abundance in model organism *PA* across a range of growth rates which cover *in situ* growth rates during chronic infections. We cultured *PA* under highly-controlled growth conditions using a chemostat and quantified mRNA and protein abundances using RNA-seq and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), respectively. The correlation of mRNA and protein abundance across diverse growth rates were modest (~0.45-0.65), and mRNA abundance alone cannot precisely predict protein abundance. In addition to overall correlations, we also studied the relationships among mRNA and protein levels across distinct gene subsets 1) highly- and lowly- expressed genes and 2) different functional categories. These studies provide a quantitative analysis of the cellular abundance of mRNA and protein across a range of growth rates relevant to chronic infections. Further, understanding the global effects of how growth rate impacts bacterial gene expression is fundamental and necessary to gain knowledge on underlying principles of gene expression control.

Pseudomonas quorum sensing molecules antagonize *Prevotella* in an in vitro model of the CF lung

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Quorum sensing (QS) is a density-dependent mechanism by which bacteria can coordinate the expression of various traits. *Pseudomonas aeruginosa*, a prevalent cystic fibrosis (CF) pathogen, possesses three QS systems, including the unique Pseudomonas Quinolone Signal (PQS) system. The PQS system controls factors including biofilm formation, pyocyanin, and elastase production through the signalling molecule PQS and its precursor HHQ. It has also been reported that PQS and HHQ can regulate interspecies behaviour, altering motility and inhibiting growth in many species through unknown mechanisms. Within the CF lung, *P. aeruginosa* exists as part of abundant, diverse, and dynamic microbial communities. These communities consist of canonical CF pathogens and many other species, including anaerobes. While reports have indicated that anaerobic species like *Prevotella* can passively protect *P. aeruginosa* from antimicrobials, a significant knowledge-gap remains in our understanding of how *P. aeruginosa* and anaerobes interact and ultimately impact CF lung disease. Recent evidence has shown that *P. aeruginosa*-produced HHQ is negatively correlated with the abundance of anaerobes like *Prevotella* in CF sputum. Using an *in vitro* synthetic microbiome model of the CF lung containing *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Prevotella*, we tested the hypothesis that the PQS-regulated molecule HHQ could alter *Prevotella*'s physiology. Using a genetic approach, we demonstrated that *Pseudomonas*-produced HHQ decreases the abundance of *Prevotella* in an oxygen-dependent fashion. These results indicate that *P. aeruginosa* QS metabolites can influence community structure in the CF lung, specifically through depletion of *Prevotella* and that our model can recapitulate *in vivo* observations. These findings are supported by evidence from marine and CF lung microbial communities showing that HHQ is negatively correlated with anaerobic species. Through this work, we seek to understand the mechanism by which PQS-regulated molecules can negatively regulate anaerobes, and how this depletion may shape CF lung microbial communities over time and oxygen gradients.

SESSION C: POSTER PRESENTATIONS

PA-2022-C001

Two-component regulatory systems PmrAB and PhoPQ independently activate colistin resistance in *Pseudomonas aeruginosa* in a magnesium concentration dependent manner

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Colistin is a cationic antimicrobial peptide (CAP) used as a last resort antibiotic to treat multidrug-resistant bacterial infections such as those caused by *Pseudomonas aeruginosa* in cystic fibrosis (CF) lungs. CAP resistance is conferred by the *arn* operon, which is regulated by the two-component systems (TCS) PmrAB and PhoPQ. In *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella typhimurium* PmrAB and PhoPQ work in concert to regulate *arn* expression. In contrast, PmrAB and PhoPQ appear to independently activate *arn* expression in *P. aeruginosa,* creating a redundancy. We hypothesized that if the two TCS indeed work independently, then PmrAB and PhoPQ will be differentially active dependent on the growth conditions or cues. Low concentrations of magnesium, which is found in CF lungs, but not calcium activate *arn* expression. We next determined the phosphorylation state of the response regulators PmrA and PhoP in a growth mode and magnesium concentration dependent manner. The phosphorylation state was then correlated to activity, by determining colistin susceptibility and transcript abundance of the *arn* operon and PmrA- and PhoP-regulated genes in strains inactivated in *pmrA* and *phoP.* The data indicated that PmrA and PhoP were phosphorylated in a reciprocal manner, with PhoP phosphorylation being greatest at normal magnesium and PmrA at limiting magnesium concentrations. Susceptibility assays indicated planktonic and biofilm cells to be more susceptible to colistin at low magnesium concentrations upon inactivation of *pmrA* and *phoP*. Inactivating *pmrA* and *phoP* resulted in down regulation of *arn* in low magnesium conditions, as well as PmrA- and PhoP-regulated genes, respectively. Together, this suggests that PmrAB is more active and phosphorylated at low magnesium while PhoPQ is more active at reduced phosphorylation levels at low magnesium conditions. Our findings suggest that low magnesium conditions present in the CF lung are not conducive for colistin to be fully effective against *P. aeruginosa.*

PA-2022-C002

A core regulon for the *Pseudomonas aeruginosa* quorum sensing receptor RhlR

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Chronic bacterial infections involving the opportunistic pathogen *Pseudomonas aeruginosa* present a major medical challenge. *P. aeruginosa* virulence is partly regulated through a cell-cell signaling mechanism termed quorum sensing (QS). In one type of *P. aeruginosa* QS, two acyl-homoserine lactone (AHL) signals are released and received by a hierarchically arranged series of cognate receptors, called LasR and RhlR, that are activated sequentially as cell density increases. LasR controls RhlR and downstream gene expression in laboratory strains. Paradoxically, *P. aeruginosa* isolates with mutations in LasR are frequently isolated from chronic infections. Recent evidence has revealed that many of these isolates still employ AHL QS, but with the Rhl system in control of virulence gene expression. We hypothesized that RhlR controls a core regulon of QS-controlled genes important to chronic infection. We engineered RhlR deletion alleles into 5 infection isolates with natural inactivating mutations in LasR, allowing characterization of QS activation and RhlR-regulated gene expression without the confounding effects of LasR control. As expected, deletion of RhlR abrogated pyocyanin production and extracellular proteolysis. RNA-seq transcriptomics of each isolate yielded a core RhlR regulon of 20 genes, including genes coding for known virulence factors, as well as the Rhl QS machinery and a large biosynthetic cluster encoding an azetidomonamide biosynthetic operon. The RhlR panregulon includes 106 genes not previously reported as QS-regulated, including 26 genes not present in laboratory strains, and a number of metabolic genes that likely reflect adaptation to the chronic infection niche. Our results provide a view of chronic infection adaptation where QS under RhlR may be streamlined, yet RhlR appears to maintain tight control of several virulence factors important to chronic infection in the absence of functional LasR. Our results lay the groundwork for understanding QS beyond the paradigms of laboratory strains.

PA-2022-C003

Functional characterization of TetR-like transcriptional regulator PA3973 from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, a human opportunistic pathogen, is a common cause of nosocomial infections. Its ability to survive under different conditions relies on a complex regulatory network engaging transcriptional regulators controlling metabolic pathways and capabilities to efficiently use the available resources. *P. aeruginosa PA3973* encodes a putative TetR family transcriptional regulator, with a helix-turn-helix motif involved in DNA binding. In this study, we applied phenotype analyses, as well as transcriptome profiling (RNA-seq), and genome-wide identification of binding sites using ChIP-seq to unravel the biological role of PA3973. *PA3973* was not essential for *P. aeruginosa* PAO1161 strain under laboratory conditions. The chromosomal mutant did not show significant differences in the growth rate, ability to metabolize different carbon sources, growth under stress conditions, motility, and biofilm formation in comparison to the reference strain. Transcriptional profiling of *P. aeruginosa* PAO1161 overexpressing PA3973 showed changes in the mRNA level of 648 genes; among them, 374 were down-regulated. Concomitantly, ChIP-seq analysis identified 487 PA3973 binding sites in *P. aeruginosa* genome, among them 287 were located in intergenic regions and 75 in promoters of PA3973 regulated genes. The PA3973 regulon encompasses genes involved in stress response, including the *PA3970-PA3973* operon. This operon encodes a probable acyl-CoA dehydrogenase, AidB homolog (PA3972), thioesterase (PA3971), and AMP nucleosidase, Amn (PA3970). *In vitro* analysis confirmed PA3973 interactions with *PA3973*p (a 14 bp palindromic sequence is the putative binding site of PA3973). Lack of PA3973 in Δ*PA3973* mutant in comparison with WT strain allowed increased expression of *PA3972* and *PA3971*. Overall our results showed that TetR-type transcriptional regulator PA3973 has multiple binding sites in *P. aeruginosa* genome and is involved in gene expression regulation. It acts as a repressor of *PA3972-PA3971* genes, encoding proteins putatively engaged in stress response. This work was supported by the National Science Centre in Poland (grant 2015/18/E/NZ2/00675).

PA-2022-C004

Pseudomonas aeruginosa PAO1 is attracted to bovine bile in a Cystic fibrosis-respiratory human bronchial epithelial cell model

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Cystic fibrosis (CF) is a life-threatening, inherited, multi-organ disease characterized by a universal thickening of a person's mucosal layers. Throughout their lives, CF patients are susceptible to chronic and ultimately, deteriorating, multi-year pulmonary infections, which account for over 90% of the morbidity and mortality associated with the disease. Those infections are dominated in adulthood by the opportunistic pathogen, *Pseudomonas aeruginosa* (*Pa*). CF patients, like many others with advancing respiratory diseases, also often suffer from gastroesophageal reflux disease (GERD), including bile aspiration into the lung. GERD is a major co-morbidity factor in CF patients, having a prevalence of 25%-81% in adults and children. Typically, bile is considered bactericidal, however, its association with the early acquisition of *Pa* in CF patients is well known. Moreover, i*n vitro* studies show that bile causes *Pa* to adapt a chronic lifestyle and enhances its biofilm formation, type VI secretion, swarming, and quorum sensing. We hypothesized that *Pa* is attracted to bile in the lung environment. Testing this hypothesis, we developed a novel chemotaxis experimental system using human bronchial epithelial (HBE) cells that mimic the lung environment which allowed for the evaluation of *Pa* (strain PAO1) chemotaxis in a physiological scenario superior to the standard *in vitro* system. To study the chemotactic behavior of *Pa* in response to bile, we performed qualitative and quantitative chemotaxis tests using HBE cell lines and microcapillary assays. Our findings show that bovine bile is a chemoattractant for *Pa* which is positively correlated with bile concentration. These results suggest that the compounds present in bile are likely implicated in the accumulation of *Pa* in the lung, which is associated with pathogenesis and chronic lung infections. Increased reflux burden may predispose patients to *Pa* infection and worse lung function, as under certain conditions, pathogenesis is seeded or enhanced by bacterial chemotaxis.

PA-2022-C005

Novel regulators of the phosphoethanolamine transferase gene *eptA* involved in colistin resistance in *Pseudomonas aeruginosa*

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Colistin represents one of the last-line treatment options for many multi-drug resistant Gram-negative pathogens. The aminoarabinosylation of the lipid A moiety of LPS is the main resistance mechanism observed in colistin-resistant *Pseudomonas aeruginosa* isolates. Accordingly, several studies have demonstrated that *P. aeruginosa* mutants in the *arn* operon, which are impaired in aminoarabinose biosynthesis, are unable to acquire colistin resistance in *in vitro* evolution experiments. However, *P. aeruginosa* has also an endogenous phosphoethanolamine transferase gene (*eptA*), and we recently demonstrated that EptA overexpression and the consequent lipid A phosphoethanolamination confer colistin resistance levels similar to those provided by lipid A aminoarabinosylation or by exogenous phosphoethanolamine transferases, such as MCR-1. To investigate if phosphoethanolaminated lipid A could also lead to the acquisition of high-level colistin resistance, we overexpressed the *eptA* or *mcr-1* gene in an aminoarabinose-deficient *P. aeruginosa* mutant and performed *in vitro* evolution experiments. Our results indicate that lipid A phosphoethanolamination can support the evolution towards high levels of colistin resistance in *P. aeruginosa*. Since little is known about the expression pattern of *eptA* and the regulatory pathways that control its transcription, we carried out transposon mutagenesis experiments in a bioluminescent reporter strain carrying chromosomal *lux* genes under the control of the *eptA* promoter (P*eptA*). The screening of over ten thousand mutants led us to identify P*eptA* inducing transposon insertions in genes involved in some metabolic or global regulatory pathways. Deletion mutagenesis and/or complementation experiments are in progress to confirm the involvement of these pathways in the regulation of *eptA* gene expression and in the acquisition of phosphoethanolamine-mediated colistin resistance in *P. aeruginosa*.

PA-2022-C006

The crystal structure of *P. aeruginosa* chitin binding Protein D

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P. aeruginosa secretes diverse proteins via its Type II Secretion System, including a 39 KDa Chitin-Binding Protein. CbpD was shown by Askarian *et al.* to be a lytic polysaccharide monooxygenase and to contribute substantially to virulence. CbpD has

three domains, a copper-containing active site, at least two sugar binding interfaces, and flexible interdomain linkers. The three CbpD domains are related to the CAZy Auxiliary Activity Family 10 domain, PFAM GbpA2 domain and CAZy Carbohydrate Binding Module 73 family, respectively. The first two are represented structurally in the PDB in *Vibrio cholerae* GbpA, while CBM73 is found in a NMR structure as a standalone domain from *Cellvibrio japonicus* CjLPMO10A. A 3-dimensional structure of CbpD would further our investigations into structural signal(s) that recruit T2SS effectors into the secretion pathway. Moreover, it will provide the structure and orientation of the third domain of CbpD, which is implicated in chitin binding. We therefore initiated a crystallographic study of CbpD. We expressed, purified, and crystallized full-length CbpD and collected a 3.0 Å resolution dataset at the Soleil Synchrotron. Using the deep learning structure prediction approach RoseTTAFold from the on-line Robetta server, models of CbpD domains were constructed. The first two were individually placed *via* molecular replacement. Their structure was refined with intermediate R value of 22%/R_{free} 27%. Several possible placements for the third domain were evaluated. As expected, the first two domains are structurally similar to the AA10 and GbpA2 folds, respectively. While CbpD does not display the extensive 3,120 $Å^2$ dimerization interface observed in GbpA, an intriguing continuation of the beta sheet from one CbpD GbpA2 domain into its symmetry mate buries 990 $Å^2$. This structure will serve as the basis for future work analyzing interactions of specific residues in CbpD with the T2SS proteins *in vivo*.

PA-2022-C007

Experimental evolution of *Pseudomonas aeruginosa* readily selects for mutants with impaired quorum sensing regulation

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Pseudomonas aeruginosa is an opportunistic pathogen that poses a significant threat to human health. The expression of several of its virulence determinants is regulated by an intricate circuitry of three quorum sensing (QS) systems. The Las system is generally considered at top of the QS hierarchy and activates the Rhl and Pqs systems. It is composed of the transcriptional regulator LasR and the autoinducer synthase LasI, which produces the ligand of LasR. The transcriptional regulator RhlR is activated by the signaling molecule produced by RhlI. The third QS system is composed of *pqsABCDE* activated by the regulator MvfR (PqsR). We have observed the emergence of LasR-deficient mutants in an experimental evolution consisting of passages of the wildtype

strain PA14 in rich culture media. The emergence of these mutants happened in earlier passages and was notably more frequent in strains already impaired in QS (mutants affecting the Rhl or Pqs systems). QS-regulated phenotypes, such as the production of the blue phenazine pyocyanin, depends on the combined effect of the QS systems. However, unexpectedly, we have isolated an evolved Δ*rhlI* with a defective Las system still able to produce pyocyanin on solid media, but not in liquid cultures. We have confirmed the inactivation of both Rhl and Las systems by measuring the signals produced by this isolate by liquid chromatography/mass spectrometry. In liquid culture, none of the signaling molecules that mediate the QS system are produced by this strain, as expected in a Δ*rhlI lasR*-null background. We have linked the pyocyanin induction upon surface-sensing to the expression of a unique mutated allele of *lasR*. The molecular mechanism underlying the activation of the QS-regulated phenotypes in response to surface-sensing is currently under investigation. Understanding the intricacies of QS regulation is a step towards controlling the virulence of this pathogen.

PA-2022-C008

Transcriptional thermoregulation of *prpL*

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Pseudomonas aeruginosa is the leading cause of chronic life-threatening lung infections in people living with cystic fibrosis and also keratitis resulting in blindness. In both lung and corneal infections, the secreted protease PrpL (also known as protease IV) degrades connective tissues, transferrin, and factors important for the innate immune response. Published transcriptomic studies show that more *prpL* mRNA is detected at 25°C than 37°C. However, how *prpL* is thermoregulated is unknown. We hypothesize that *prpL* is thermoregulated at the level of transcription. *P. aeruginosa* PAO1 was grown at 25°C and 37°C, total RNA was extracted, and *prpL* transcript levels were measured by RT-qPCR with *rpoD* as an internal, temperature insensitive control. At mid-exponential growth, *prpL* was transcribed about ten times more at 25°C than at 37°C. A greater amount of PrpL was also detected at 25°C than 37°C by immunoblotting for protein in supernatants from cells. Reporter fusions of regions upstream of *prpL* and a promoterless *lacZ* were constructed and inserted in single copy in the chromosome to

measure promoter activity. Fusions of both 500 and 200 base pairs upstream of *prpL* displayed about ten times more LacZ enzymatic activity at 25°C than 37°C. This indicated that *prpL* thermoregulation occurs at the level of transcription, promoter elements required for this thermoregulation are contained within 200bp upstream of *prpL*, and that thermoregulation can occur outside of the native *prpL* locus. When strains of *P. aeruginosa* isolated from corneal infections were surveyed for *prpL* thermoregulation, we found that isolates with *lasR* mutations lacked *prpL* thermoregulation. Currently strains with mutations in known regulators of *prpL*, including LasR, PvdS, MvaT, and MvaU, are being examined for their specific role in *prpL* thermoregulation. A better understanding of *prpL* thermoregulation will enhance our knowledge of how *P. aeruginosa* adapts to the human host during an infection.

PA-2022-C009

Pseudomonas aeruginosa modulates the response of primary bronchial epithelial cells to rhinovirus infection *in vitro*

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Pseudomonas aeruginosa (PA) can modify host immunity. We investigated how PA modulates antiviral responses in airway epithelia. Primary bronchial epithelial cells, differentiated at air-liquid-interface, were infected with a clinical PA isolate for 16 days and then infected with human rhinovirus (HRV). Whole proteome analysis was combined with N-terminal enrichment by tandem-mass-tag terminal amine isotopic labeling of substrates. Cytokines and viral RNA were quantified by cytometric bead array and qPCR. To assess proteolysis, recombinant IL-6 was added to conditioned cell culture media from infection experiments. Almost 6500 host, viral and bacterial proteins were detected. HRV infection led to increase in abundance of 50 proteins in cystic fibrosis (CF) and 44 proteins in healthy control (HC) cells, mainly interferon-induced and antigen-

presenting proteins. Compared to virus infection, coinfection with PA and HRV increased abundance of 25 host proteins in HC and lowered levels of 5 proteins. In CF cells, additional proteins were detected with differential abundance. Host proteins with increased abundance included proteins linked to IL-1ß production (CARD8, ZFP91) and pathogen internalization (CAV1, NDRG1), while decreased levels were detected of proteins with functions in immunity (HLA-B, TLR2), epithelial repair (DDR1) and ciliary movement (CFAP70). Compared to HRV alone, coinfection with PA drastically decreased IL-6 protein (mean±SD 51.7±60.4 pg/ml vs. 2301±1873) but not mRNA, and increased IL-1ß concentrations (33.3±13.3 vs. 3.1±4.5 pg/ml). Neither IL-8, IP-10, TNF-α protein, IFN-ß / -λ1 mRNA, nor viral load differed significantly between HRV-infected and coinfected cells. Recombinant IL-6 was degraded when added to conditioned medium from coinfections, but not from single infections. IL-6 degradation was inhibited by the serine protease inhibitor Tosyl-L-lysine-chloromethylketone. Our analysis highlights differences in host and bacterial responses during coinfections, distinct from changes induced by single pathogens. Understanding these interactions between PA, viruses and the host could improve management of viral infections in chronically PA-infected patients.

PA-2022-C010

The role of extracellular DNA in mucoid *Pseudomonas aeruginosa* biofilms

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Pseudomonas aeruginosa (PA) is one of the most common biofilm-forming pathogens responsible for lung infections of immunocompromised individuals, such as people with cystic fibrosis (CF). In these infections, PA gains enhanced tolerance to antimicrobials in the biofilm state and is difficult to treat. Production of extracellular polymeric substances (EPS) is one of the main factors that characterize biofilms, allowing adherence to abiotic and biotic surfaces, and antimicrobial evasion. In chronic CF lung infections, selective pressures cause variants with enhanced biofilm capabilities to emerge. Mucoid variants of PA are frequently isolated from sputum samples and are associated with worsening patient outcomes. Mucoid variants overproduce the exopolysaccharide alginate, which contributes to evasion by impeding antimicrobials and host defenses. Here, we investigate the mechanism behind how alginate, and other EPS components such as extracellular DNA (eDNA), contribute to mucoid biofilm structure and antimicrobial tolerance. eDNA is a major structural component for nonmucoid

biofilms, but its role in mucoid biofilms remains unclear. We predict that eDNA, like in nonmucoid PA, plays a role in mucoid biofilm structure and physiology. Therefore, we hypothesize that by enzymatically degrading eDNA, the mucoid biofilm structure and integrity can be disrupted. To test this, we isolated and quantified biofilm eDNA to elucidate the abundance in mucoid biofilms, compared to nonmucoid. We also used rheological methods and microscopy to investigate changes in biofilm structure and phenotypes upon DNaseI treatment. Our studies show that eDNA in mucoid biofilms is generally lower than in nonmucoid, although biofilm eDNA abundance is diverse across several mucoid strains. We also show that degradation of eDNA increases stiffness of mucoid biofilms, therefore impacting viscoelastic properties and the integrity of the biofilm. These findings can lead to new approaches on targeting biofilms by exploiting the functions of EPS components, to increase antimicrobial effectiveness in infected patients.

PA-2022-C011

A variant of IscS suppresses growth defects of a *Pseudomonas aeruginosa ridA* mutant

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Members of the RidA subfamily of the larger Rid (YjgF/YER057c/UK114) protein superfamily prevent damage to pyridoxal 5'-phosphate (PLP)-dependent enzymes by deaminating the reactive metabolite 2-aminoacrylate (2AA). RidA (PA5339) is required for full growth, motility and biofilm formation of *P. aeruginosa*. The understanding of RidA integration into the metabolic network of *P. aeruginosa* is at an early stage with analysis largely guided by the well-established RidA paradigm from *Salmonella enterica. P. aeruginosa* lacking a functional RidA exhibit a growth defect in minimal glucose medium. Exogenous serine exacerbates these defects and isoleucine eliminates them. These data demonstrate the primary generator of 2AA in *P. aeruginosa* is threonine/serine dehydratase (IlvA), as it is in *S. enterica*. Nutritional studies indicate that the critical (i.e., phenotype determining) target(s) of 2AA in *P. aeruginosa* is different than the one defined in *S. enterica*, serine hydroxymethyl transferase. This study was initiated to identify key targets of 2AA and thus further characterize the integration of RidA in the metabolic network of *P. aeruginosa.* Spontaneous mutations that restored growth to a *P. aeruginosa ridA* mutant were isolated. Among these mutations was variant of *iscS*, a PLP-dependent cysteine desulfurase. This IscS variant restored growth to a *P.*

aeruginosa ridA mutant by a previously uncharacterized mechanism, as it was the first to suppress a *ridA* phenotype without lowering 2AA levels. Our working model suggests this variant is no longer sensitive to 2AA and further, that IscS is the critical target of 2AA in *P. aeruginosa.* This work contributes to understanding the integration of RidA into the metabolic network of *P. aeruginosa* and identifies an important role for IscS under conditions of 2AA stress.

PA-2022-C012

Nitric oxide produced during *Pseudomonas aeruginosa* denitrification increases antibiotic killing of tolerant cells.

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Muco-obstructive airway diseases (MADs), a collection of diseases characterized by dehydration, stagnation, and thickening of airway mucus, provide a nutrient-rich scaffold in the lungs for bacterial pathogens to thrive. *Pseudomonas aeruginosa* (*Pa*) is the predominant bacterial pathogen in people with MADs. Chronic *Pa* lung infections are characterized by high rates of antibiotic treatment failure, often not linked to resistance. Current hypotheses propose that anaerobiosis increases antibiotic treatment failure due to a lack of metabolic respiration. In *Pa*, denitrification catalyzes the reduction of nitrate (NO_3^-) → nitrite (NO_2^-) → nitric oxide ($NO^\cdot$) → nitrous oxide (N_2O) → nitrogen gas (N_2). However, only the reduction of nitrate by the Nar enzyme complex is demonstrated to increase respiration. Consequently, groups have shown that under anaerobic conditions activation of respiration through Nar-dependent denitrification activates cells to respire and increases antibiotic killing. Utilizing a well-characterized *in vitro* synthetic sputum medium (SSM), we are able to investigate aspects of *Pa* biology that contribute to and overcome antibiotic tolerance in physiologically relevant conditions. Our preliminary data indicate that Nar-dependent respiration does not exclusively account for increased antibiotic killing. We have observed increased antibiotic killing through the addition of downstream metabolites in the denitrification pathway (nitrate and nitric oxide). These data suggest that other elements of the denitrification pathway contribute to antibiotic killing. Termination of the denitrification pathway prior to the production of nitric oxide ablates the observed antibiotic sensitization. In the same way, detoxification of nitric oxide eliminated the increase in antibiotic killing. Our model proposes that while the addition of nitrate contributes toward an increase in cellular respiration, the mechanism that leads to killing is dependent on the downstream production of nitric oxide.

Understanding how intrinsic nitric oxide production correlates with antibiotic efficacy can improve antibiotic treatment outcomes in people chronically infected with *Pa*.

PA-2022-C013

Shallow shotgun metagenomic sequencing for microbiome analysis of respiratory tract samples

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Respiratory bacterial infections are the most significant cause of morbidity and mortality in people with cystic fibrosis (CF). Through the use of traditional culture methods, we have known for decades that there are a small number of important opportunistic pathogens that dominate the CF respiratory tract microflora. The most widely recognized bacterial pathogens in CF lung disease are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia* complex. However, these are not the only organisms present in the CF respiratory microbiome. Research into other organisms present in the CF respiratory tract has been largely limited to studies performed using 16S rRNA sequencing which suffers from shortfalls associated with reference-based amplicon taxonomic assignment. 16S rRNA sequencing does not allow for the accurate identification of microbes to species level and cannot detect genera that are not present within the reference database used during analysis. Additionally, sequencing bias can be introduced as a result of inter-species variation between bacterial 16S rRNA gene copy numbers. Shotgun metagenomic sequencing allows for construction of *de novo* genomes for species present in the sample and verification of genome identity through gene calling and predicted open reading frame mapping against protein databases. Additionally, genomes assembled from short reads can be interrogated to identify key virulence factors and antimicrobial resistance genes present in important and dominant pathogens. Here, we present methodology for accurate identification of bacterial species from mock bacterial communities, healthy human samples, and airway samples from people with CF through the use of shallow shotgun metagenomic sequencing and offer this as an improved alternative to 16S rRNA sequencing for affordable and straightforward microbiome studies.

PA-2022-C014

Systematic identification of novel genetic targets for fungal-bacterial antagonism

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Fungi and bacteria often occupy the same environmental niches, where they compete against each other. The outcomes of fungal-bacterial competitions have broad implications for human health and agriculture, but our understanding of such inter-kingdom interactions is very limited. We are interested in competition between the fungus Candida albicans and the bacterium Pseudomonas aeruginosa. These are two opportunistic pathogens that frequently co-colonize and compete within infected tissues in human. We hypothesize that the antagonism between C. albicans and P. aeruginosa results in an “arms race” in which virulence of either organism increases as a consequence of the evolutionary pressure created by the other. Using these two microbes as an experimental model, we sought to identify genes that determine outcomes in this fungal-bacterial antagonism. We established a co-culture system in which C. albicans suppresses P. aeruginosa viability and growth. To ask which bacterial genes are involved in this suppression, we screened P. aeruginosa transposon mutants in the presence and absence of C. albicans. We identified 13 bacterial genes required to defend against C. albicans. Three of these genes were involved in bacterial magnesium transport, suggesting that competition for magnesium underlies one of antagonistic interactions between these two species. Furthermore, we also identified 23 genes that were dispensable in co-culture with C. albicans, indicating that they function as mediators or direct targets of fungal antagonism. Many of these dispensable genes regulate essential bacterial physiology, like cell division and translation, while other genes remain functionally uncharacterized. These results are consistent with the idea that the antagonism between these two organisms can affect virulence phenotypes. Overall, our work highlights the power of functional genomics to discover multiple and novel antagonistic interactions across microbial kingdoms.

PA-2022-C015

Phenome-genome co-clustering in *Pseudomonas aeruginosa* clinical isolates

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Pseudomonas aeruginosa is a leading cause of nosocomial infection and infection in patients with cystic fibrosis, which can be complicated by a variety of antimicrobial resistance and virulence mechanisms. However, the mechanisms of the specific modulating effects during Pseudomonas infection, as well as the metabolic functions that are modulated across diverse phenotypes, are poorly understood. We hypothesize that metabolic modulations in P. aeruginosa are dependent on a complex combination of host and pathogen-specific factors, which can be delineated using a combination of omic analyses and the experimental characterization of unique metabolic traits in different clinical isolates. With that goal, between 2019 and 2020, we obtained 971 clinical isolates of P. aeruginosa from 590 patients from the UVA Health System Clinical Microbiology Laboratory, which collects samples from a range of outpatient and inpatient settings in the region. For each isolate, we recorded associated patient metadata, bacterial morphological phenotypes, and antimicrobial susceptibility profiles. We selected a set of 25 phenotypically representative isolates from the entire isolate collection through stratified random sampling while guaranteeing the robustness of original phenotypic characteristics during repeat cultures. These 25 isolates were then cultured for whole genome sequencing. The genome sequence data was used for comparative genomic analysis using the PA14 strain as the reference genome. A dissimilarity matrix was enumerated from the output of multiple local alignment searches and was used to cluster the isolates according to their sequence similarities. The genotypic clustering was contrasted with the phenotypic clustering generated from a multi-parametric analysis to assess the genotype-phenotype correlation. Overall, this study demonstrates the value in surveying clinical data in terms of pathogen isolate sources, patient metadata, morphological phenotypes, and correlating that data with genome sequence data to answer biologically relevant questions about genotype-phenotype relationships.

PA-2022-C016

Diversity in T3SS expression and cooperation during *Pseudomonas aeruginosa* traversal of the corneal epithelium

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Pseudomonas aeruginosa can cause vision-threatening corneal infection. Previously, we showed the mouse corneal epithelium becomes susceptible to *P. aeruginosa*

traversal if tissue-paper blotted to induce superficial-injury then EGTA treated. We subsequently used this model to demonstrate requirement for ExsA, the transcriptional activator of the Type 3 Secretion System (T3SS), for invasive (ExoS-encoding) *P. aeruginosa* to traverse. Separately, we found T3SS expression bistability influences *P. aeruginosa* interactions with cultured epithelial cells. Here, a T3SS-GFP reporter pJNE05 was used to study T3SS expression during corneal epithelial traversal in situ using the murine model. C57BL/6 mouse corneas were Kimwipe-blotted, then EGTA-treated (100 mM in PBS) for 1 h. They were then inoculated with *P. aeruginosa* PAO1 expressing the T3SS-GFP reporter and constitutive d-Tomato (~10^{11} CFU/ml bacteria in DMEM) for 6 h, or with mutants in *exsA* (PAO1Δ*exsA*, T3SS "Locked-Off") or *exsD* (PAO1Δ*exsD*, T3SS "Locked-On"). Corneas were imaged using confocal microscopy and Imaris software to quantify distance from basement membrane for individual bacteria. Surprisingly, the $T3SS^{ON}$ sub-population of wild-type traversed significantly less than the $T3SS^{OFF}$ sub-population ($P < 0.0001$, Kruskal-Wallis test, $n = 3$). As previously shown, *exsA* mutants alone did not traverse. To determine if cooperation between individual $T3SS^{ON}$ and $T3SS^{OFF}$ bacteria promoted traversal, co-inoculation experiments were performed using *exsA* and *exsD* mutants in the same eyes (50 % each). Results showed little or no traversal by either mutant, with no difference observed when traversal of an *exsA* mutant alone was compared with *exsA* and *exsD* mutants combined ($P > 0.05$, Kruskal-Wallis test, $n = 2$). Accordingly, the *exsD* mutant (T3SS "Locked-On") did not traverse when used alone. These data suggest that while *P. aeruginosa* requires its T3SS to traverse the corneal epithelium, bistability giving rise to variable T3SS expression within the infecting population is required for this critical step in infection pathogenesis.

PA-2022-C017

A network of previously uncharacterized conserved proteins contributes to biofilm formation and drug tolerance in two gram-negative pathogens

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Transcriptomic studies have revealed many uncharacterized genes that are differentially expressed in biofilms, which may be important in regulating biofilm phenotypes such as resistance to antimicrobial agents. To identify biofilm genes of unknown function in *P. aeruginosa*, we made use of RNA-seq. We selected 4 biofilm-induced genes, PA0918, PA2146, PA2184, and PA3915, as homologs of these genes are conserved in ɣ-

proteobacteria including *Klebsiella pneumoniae* and *Escherichia coli*. Increased expression was confirmed in biofilms formed by *P. aeruginosa* PAO1 and clinical isolates using qRT-PCR. Biofilms formed by respective mutants were subsequently analyzed for two biofilm characteristics: biofilm architecture and drug susceptibility. PA0918, PA2146, and PA3915, were found to contribute to biofilm formation, while PA0918, PA2146, PA2184, and PA3915 contributed to biofilm drug tolerance. We also determined whether homologs of the respective genes play similar roles in *E. coli*. Genes of interest demonstrated increased transcript abundance in E. coli biofilms compared to planktonic cells. Inactivation of PA0918, PA2146, PA2184, and PA3915 homologs in *E. coli* had similar effects on biofilm formation and drug tolerance*,* and heterologous expression of *E. coli* homologs rescued the biofilm phenotypes in *P. aeruginosa* mutants to wild-type levels, suggesting that *E. coli* homologs can substitute for, and play a similar role as, the respective *P. aeruginosa* genes. Given the similarity in function, we next determined whether the corresponding *P. aeruginosa* proteins interact, using bacterial two-hybrid and pulldown assays. The four proteins were found to form an extensive interaction network, with the interactome extending to SagS, which has been previously reported to contribute to both biofilm formation and the heightened tolerance of *P. aeruginosa* biofilms. Together, our findings identified four previously uncharacterized proteins that contribute to biofilm formation and drug tolerance by both *P. aeruginosa* and *E. coli.*

PA-2022-C018

Antibiotic substrate selectivity in the *Pseudomonas aeruginosa* MexB and MexY efflux transporters is determined by a Goldilocks Affinity

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Resistance-**N**odulation-**D**ivision (RND) efflux pumps are tripartite complexes composed of an inner membrane transporter protein, a periplasmic adaptor protein, and an outer membrane protein. The serious threat pathogen *Pseudomonas aeruginosa* has four RND efflux pumps with overlapping but distinct antibiotic substrate specificities (e.g., MexAB-OprM effluxes β-lactams and MexXY-OprM effluxes aminoglycosides) and they contribute extensively to multidrug resistance. Using phylogenetic evolution analyses of 700 representative RND transporter protein sequences, we identified potential substrate discriminatory residues in the transporter MexY. To provide a structural framework to

these residues, we used the published structures of *E. coli* AcrAB-TolC and *P. aeruginosa* MexAB-OprM to build a computational model for MexXY-OprM. To predict differential substrate binding we used flexible computational docking of aminoglycosides and β-lactams to MexB and MexY. Using the MexY structural model for comparison with MexB, distinct properties were identified in the proximal binding pocket (PBP) and distal binding pocket (DBP). The PBP of MexB has a network of negatively charged residues while MexY is enriched in polar/positively charged residues. The MexY DBP contained small/flexible residues and more aliphatic hydrophobic residues compared to MexB. We found that non-substrates have higher predicted binding affinities in the DBP during docking to transporters (e.g., β-lactams bind tighter to MexY than MexB). We propose that substrates must bind the DBP with an affinity that allows them to be both taken up by the transporter and moved through the pump by the peristaltic movement of the protomer conformational states. For efficient efflux, substrate should possess a 'Goldilocks' affinity: not too weak (no interaction) and not too strong (impede translocation). These analyses set the scene for detailed studies of substrate selection by the RND systems in *P. aeruginosa*. A deeper understanding of these mechanisms also holds promise to support design of novel efflux pump inhibitors to counter efflux-mediated antibiotic resistance.

PA-2022-C019

Microscale measurements of surface O_2 gradients surrounding *Pseudomonas aeruginosa* biofilms

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Individual bacterial cells modulate chemical gradients in their environment on the micron scale which collectively shapes chemical interactions within a community. Molecular oxygen (O_2) is a key metabolite that affects virtually all bacterial cells and modulates cell physiology – including tolerance to antimicrobials. While bulk measurements of O_2 have provided valuable insights, including O_2 consumption rates and gradients formed throughout biofilms, a micron-scale view of the O_2 gradient surrounding biofilms has not been extensively studied. The Whiteley lab recently developed electrochemical methodology based on Scanning Electrochemical Microscopy (SECM) techniques for measuring O_2 above biofilms, in real time, and over extended periods of time. After development of a modified micron-sized tip electrode, we measured O_2 gradients hundreds of microns above metabolically active biofilms. Within minutes it was observed

that relatively nascent biofilms are capable of creating local hypoxic regions that extended a few hundred microns from the biofilm surface. The O_2 consumption rate was estimated using digital simulation and found to be maximum for the *P. aeruginosa* biofilm (5.9×10^9 molecules of O_2 consumed per second by each bacterium on the biofilm surface). We next treated the biofilm with 400 times the MIC of ciprofloxacin. Although it was observed that 99% of cells died, the O_2 consumption at the surface of ciprofloxacin-treated biofilms remained at a maximum rate. Together, we find *P. aeruginosa* forms hypoxic regions within several minutes above biofilms that recapitulate cells numbers found in human infections and O_2 gradients surrounding biofilms persist in the presence of antibiotics over two hours after treatment. Ultimately, the methodology developed in this work allows for further exploration of O_2 when studying micron-scale microbial interactions.

PA-2022-C020

Deciphering the lifestyle of the human pathogen *Pseudomonas aeruginosa* grown on various carbon sources using a novel ^{13}C metabolic flux approach

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Metabolic flux analysis using ^{13}C carbon tracers is the only method available to estimate *in vivo* reaction rates within living cells. Here, we present a novel approach for accurate, precise and convenient analysis of metabolic fluxes in *Pseudomonas aeruginosa* PAO1. The microbe possesses a unique pathway architecture and operates a periplasmic glucose oxidation and a cyclic upper hexose metabolism, recently coined the EDEMP cycle. The cyclic architecture cannot be resolved by common workflows, which rely on GC-MS-based labelling analysis of proteinogenic amino acids. Computational simulations revealed that this limitation can be overcome by parallel labelling experiments on specific tracers, extended by the additional consideration of ^{13}C patterns from glucose and glucosamine. These compounds display building blocks for glycogen, peptidoglycan and lipopolysaccharides, reflecting the pools of glucose 6-phosphate and fructose 6-phosphate. The developed approach enabled high-resolution flux analysis in the opportunistic human pathogen PAO1. The strain oxidized ~90% of the assimilated glucose directly into gluconate via the periplasmic route, whereas a minor fraction was phosphorylated and catabolized via glucokinase. Further studies supplementing carbon sources present in cystic fibrosis airways like glycerol, short-chain fatty acids and organic

acids revealed an impressive flexibility and overall reorganization of *P. aeruginosa*s' central metabolic pathways depending on the entry point of carbon into metabolism. Surprisingly, the oxidative pentose phosphate pathway enzyme 6-phosphogluconate dehydrogenase was found to be completely inactive under all conditions, underpinning the essentiality of the Entner-Doudoroff pathway as main catabolic route and triose recycling into anabolic precursors which has strong implications on the cells' physiology and its resistance against oxidative stress imposed by the infected host.

PA-2022-C021

Vancomycin treatment promotes gastro-intestinal carriage of *Pseudomonas aeruginosa* in a murine model

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A growing number of reports indicate gastrointestinal (GI) carriage of *P. aeruginosa* (PA) may play an essential role in the later development of infections by this pathogen. While PA is rarely part of the normal human microbiome, carriage is quite frequent in hospitalized patients (~40% of those admitted to the intensive care unit). Thus far, only a limited number of factors have been identified as necessary for GI carriage. Additionally, current murine models of PA GI carriage either rely on extended exposure to the bacterium or the use of immunocompromised mice. **We aimed to develop a novel animal model of PA GI carriage** that better mimics how hospitalized patients typically acquire this pathogen. We administered daily intraperitoneal injections of vancomycin (370mg/kg) and a single dose of PA through oral gavage to C57BL/6 mice. Subsequent GI carriage was assessed through quantification of fecal bacterial load. While stopping vancomycin treatment prior to bacterial inoculation did not support long-term GI carriage, extending the daily vancomycin injections from four days prior the oral gavage to include two days post-gavage supported GI carriage of PA for at least two weeks. With inocula of ~$10^{5.6}$ CFU, GI carriage reached 10^6-10^8 CFU/g of feces during the first week post-oral-gavage and decreased to 10^3-10^6 CFU/g of feces 14 days post-oral-gavage. Reproducible GI carriage was obtained in both female and male mice and with multiple PA clinical strains. This model has the advantage of using one of the most highly utilized antibiotics in US hospitals and of providing a single precisely controlled inoculum of PA to better mimic how hospitalized patients potentially become carriers of PA. This model

will be a useful tool for understanding the mechanisms used by PA to facilitate carriage in the GI tract of human patients.

PA-2022-C022

Social Cheating in the *Pseudomonas aeruginosa* clinical isolate E94

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Pseudomonas aeruginosa quorum sensing (QS), a form of intercellular communication that allows bacteria to measure cell density and regulate gene expression, has been used as a model to study cooperation in populations. *P. aeruginosa* QS is mediated in part by two *N*-acyl homoserine lactone (AHL) responsive transcription factors: LasR and RhlR. In the lab strain PAO1, LasR directly activates the gene encoding RhlR, creating a “hierarchy” of QS; however, we and others have described several strains and isolates in which LasR is inactivated and RhlR is the sole AHL QS regulator. Among the gene products controlled by both LasR and RhlR are extracellular proteins that may function as “public goods” which are shared among the population. In laboratory conditions where QS is required for growth, *lasR* mutants reproducibly arise. These mutants no longer respond to QS signals, but still benefit from public goods, and thus are social cheaters. Interestingly, mutations in *rhlR* have not been observed during evolution studies and rarely are found in environmental isolates. We studied clinical isolate E94 in which RhlR is the sole AHL QS transcription factor and found that social cheaters emerged in a condition that required QS for growth. These cheats constituted a majority of the population within 3-5 days of passage, implying a greater fitness advantage over the parent strain. Additionally, we found social cheaters can revert their phenotype after passage in nutrient rich media. We sequenced and analyzed the genome of E94 and discovered that it contains many transposable elements. These transposable elements appear to allow for rapid, reversible acquisition of new phenotypes, suggesting a mechanism for this and other *P. aeruginosa* strains to sample genetic variation.

PA-2022-C023

Genetic adaptation to tobramycin alters the antibiotic susceptibility of quorum sensing (LasR)-null mutants in *Pseudomonas aeruginosa*

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The LasR-I quorum-sensing system contributes to *Pseudomonas aeruginosa* virulence and has also been shown to increase resistance to the antibiotic tobramycin. Paradoxically, *lasR*-null mutations are commonly isolated from chronic infections of patients treated over sustained periods with tobramycin, suggesting there may be some mechanism allowing the *lasR* mutants to persist under antibiotic selection. Here, we explore the hypothesis that adaptive mutations conferring tobramycin resistance change the role of *lasR* in tobramycin resistance. We show that a single-nucleotide mutation in the translation elongation factor gene f*usA1* G61A (FusA1^{A21T}) confers a specific advantage to *lasR* mutants under tobramycin selection, a phenomenon known as sign epistasis. Under tobramycin selection, a *lasR*-null mutation decreases fitness in the ancestral PA14 parent but this same mutation increases fitness in a *fusA1* G61A mutant background. *fusA1* mutations are common in infection isolates and are known to increase tobramycin resistance through activation of an aminoglycoside-specific efflux pump called the MexXY pump. We demonstrate that in *fusA1* G61A mutants, *lasR*-dependent changes in antibiotic resistance were dependent on MexXY and the *mexXY* regulator ArmZ. MexXY was not needed for differences in PA14 and a PA14 *lasR* mutant. Overall, our results highlight the importance of adaptation on the evolutionary trajectory of quorum sensing and may provide a potential explanation for how quorum sensing-null mutants persist in antibiotic-treated patients. Future experiments are focused on understanding the mechanism of *fusA1*-dependent LasR sign epistasis and how other adaptive mutations cause constraints on the evolution of quorum sensing.

PA-2022-C024

Elucidation of the c-di-GMP Binding Site of the *Pseudomonas aeruginosa* Transcriptional Regulator BrlR

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Biofilms are surface-associated communities of bacteria encased in an extracellular matrix which often exhibit high levels of tolerance to antimicrobial agents. In the case of *Pseudomonas aeruginosa* biofilms, this tolerance is attributed to the presence of the transcriptional regulator BrlR. BrlR confers drug tolerance by activating the expression of genes encoding multi-drug efflux pumps and ABC transport systems. BrlR activity requires the binding of the coactivator molecule c-di-GMP. However, the precise binding location of this molecule has yet to be confirmed for *P. aeruginosa* BrlR. The goal of this study was to elucidate residues involved in c-di-GMP binding by *P. aeruginosa* produced BrlR. Sequence analysis revealed the presence of 5 potential c-di-GMP binding sites comprised of the following motifs: RxxR, RxxxR and RxxD. The conserved amino acid residues were subjected to alanine substitution, yielding 10 variant BrlR proteins. The resulting variant proteins were then evaluated for their ability to bind c-di-GMP, oligomerize, and contribute to drug tolerance. Variant BrlR proteins harboring single alanine substitutions at R67, R70, R152, and R156 (RxxR and RxxxR respectively) remained capable of dimerization in the presence of c-di-GMP but demonstrated reduced c-di-GMP binding relative to wild-type protein, apparent by decreased maximal binding and/or increased Kd. Reduced c-di-GMP binding correlated with mutant biofilms producing BrlR variants being more susceptible to killing by tobramycin compared to wild-type biofilms and demonstrating reduced BrlR target gene expression. The findings suggest the proposed RxxR and RxxxR c-di-GMP binding motifs to play a role in c-di-GMP binding by BrlR. Considering that BrlR function requires c-di-GMP binding, our findings indicate that it is possible to interfere with biofilm drug tolerance by manipulating BrlR function through impairment of the indicated binding motifs.

PA-2022-C025

Characterization of the hypothetical protein PA14_69090

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Cystic fibrosis (CF) is one of the most common, potentially lethal autosomal-recessive diseases of Caucasians, caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator protein. The protein malfunction causes mucus dehydration, which generates thickened mucus with steep hypoxic gradients onto lung epithelial surfaces, creating favourable conditions for the proliferation of microbial pathogens.

Pseudomonas aeruginosa is one of the most important and abundant pathogens in the lung of CF-patients during the chronic infection phase. Its versatile metabolism allows this bacterium to proliferate and persist within the thick mucus of the CF-lung, encompassing denitrification and oxidative phosphorylation to adapt to the different oxygen gradients. Despite its importance, the function of 35-40% of *P.aeruginosa* genes is unknown to date. Many of these genes are upregulated during infections, unravelling their importance in developing infection processes. In this work, we focus on the role of one of these hypothetical proteins encoded in the locus PA14_69090 in an operon with PA14_69060 and PA14_69070. This protein was found to be expressed in anaerobic conditions under the control of the anaerobic regulator Anr. Moreover, interactomics experiments revealed that PA14_69090 interacts with UspK, an important regulator for survival under pyruvate fermentation. To gain deeper insight into its function, PA14_69090 and the coexpressing genes within its operon were deleted, and their roles were investigated. Secretome analysis under microaerophilic conditions revealed that mutants secrete NirS and KatA, suggesting a possible role in resistance to reactive oxygen species. This was confirmed by growing the strains in the presence of H_2O_2. Additionally, the gene deletion leads to dysregulation of Quorum Sensing molecules, whereas the ΔPA14_69090 shows overproduction of biofilm matrix. Further investigation of these mutants is being currently analyzed by transcriptomics. These analyses will help to shed more light into the colonization and infection mechanisms exerted by this operon during anaerobic/microaerophilic conditions.

PA-2022-C026

FleQ and its role in Antimicrobial Tolerance of *Pseudomonas aeruginosa* Biofilms

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In *Pseudomonas aeruginosa*, the transcriptional regulator FleQ reciprocally regulates the expression of flagellar and pel exopolysaccharide biosynthesis genes in a c-di-GMP dependent manner. However, little is known about the role of FleQ in biofilms. Recent findings in our lab suggest that FleQ not only contributes to biofilm formation but also the drug tolerance of *P. aeruginosa* biofilms. This is supported by the finding of Δ*fleQ* mutant biofilms being susceptible to a variety of antimicrobial agents including tobramycin and

the cationic antimicrobial peptide colistin. Additionally, FleQ was found to interact with the two-component sensor SagS. Given the link between FleQ, SagS and antimicrobial susceptibility, we asked whether FleQ contributes to the activation of biofilm tolerance regulator BrlR. Δ*fleQ* mutant biofilms were found to produce BrlR at wild-type levels, with Δ*fleQ*-produced BrlR capable of binding to its own promoter, as determined using EMSAs. Analysis of the transcript abundance of BrlR-activated genes by qRT-PCR, however, indicated FleQ to affect the expression of a subset of BrlR-target genes. While inactivation of *fleQ* correlated with reduced transcript abundance of PA1874 belonging to an operon of genes encoding an ABC transport system that is required for tobramycin drug tolerance, *arn* genes contributing to the LPS modification system and thus, colistin resistance, were unaffected. We furthermore explored the role of the FleQ-SagS interaction in BrlR-target gene expression. To do so, we interrupted the interaction by FleQ-SagS in wild-type biofilms using interfering peptides and evaluated gene expression of BrlR downstream target (PA1874) by qRT-PCR. Production of interfering peptides P1 and P5 suppressed the expression of PA1874 and biofilm formation in a manner similar to *fleQ* inactivation. Overall, our findings suggest that FleQ is required for BrlR-mediated biofilm tolerance to tobramycin in a manner dependent on its interaction with SagS.

PA-2022-C027

Manganese acts as an environmental inhibitor of the *Pseudomonas aeruginosa* biofilm development by modulating c-di-GMP and exopolysaccharide production via RbdA

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The opportunistic human pathogen *Pseudomonas aeruginosa* causes chronic infections that involve multicellular aggregates called biofilms. Biofilm formation is modulated by the host environment and the presence of cues and/or signals, likely affecting the pool of the bacterial second messenger cyclic diguanylate monophosphate (c-di-GMP). Manganese ion (Mn^{2+}) is a divalent metal cation that is essential for pathogenic bacterial survival and replication during the infection in a host organism. In this study, we investigated how the manganese ions alter *P. aeruginosa* biofilm formation via the regulation of c-di-GMP. Exposure to manganese ions was found to temporally enhance attachment, but to impair subsequent biofilm development, apparent by reduced biofilm biomass accumulation and lack of microcolony formation. Moreover, exposure to

manganese coincided with reduced production of the exopolysaccharides Psl and Pel, decreased transcriptional abundance of *pel* and *psl*, and decreased levels of c-di-GMP. To determine whether the effect of manganese ions on biofilm formation, polysaccharides, and c-di-GMP was linked to the activation of phosphodiesterases (PDEs), we screened several PDE mutants for manganese-dependent phenotypes (attachment, polysaccharide production) as well as PDE activity. The screen revealed that the PDE RbdA is activated by manganese, and to be responsible for manganese-dependent attachment and inhibition of Psl production. Taken together, our findings suggest manganese to be an environmental inhibitor of *P. aeruginosa* biofilm development that acts through the PDE RbdA to modulate c-di-GMP levels, thereby impeding polysaccharide production and biofilm formation.

PA-2022-C028

Examining lactam degradation and its regulation in *Pseudomonas putida* KT2440

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With applications ranging from manufacturing solvents to precursors for plastics and pharmaceuticals, lactams are an industrially relevant class of chemicals. Because they are often produced from petrochemical precursors, biological production of lactams and

their derivatives has been the subject of much research. *Pseudomonas putida* KT2440 has become a popular host for metabolic engineering because of its diverse carbon metabolisms and its ability to make value-added products from lignin monomers. However, *P. putida* can degrade many lactams and the ω-amino acids corresponding to their linear form, impeding its viability as a host for lactam production. We previously identified and knocked out the lactam hydrolase responsible for the degradation of valerolactam and caprolactam, OplBA, and developed its transcriptional regulator, OplR, into a highly sensitive biosensor, with a limit of detection < 12 nM in *E. coli*. Using Random Barcode Transposon Sequencing (RB-TnSeq), we identified two more lactam hydrolases and their respective regulators, and examined their substrate specificity. It appears that their native substrates are butyrolactam and pyroglutamate. We hypothesize that *P. putida* has evolved these three lactam hydrolases to combat the natural cyclization of ω-amino acid metabolites. From the RB-TnSeq data, we also identified the aminotransferases responsible for degrading these ω-amino acids, which are precursors to the lactams in some production pathways. An understanding of *P. putida's* lactam hydrolases, aminotransferases, and lactam-sensing regulators is essential for engineering *P. putida* as a host for lactam production. From our data, we have constructed a lactam production strain, with the relevant degradation genes knocked out and an integrated valerolactam/caprolactam biosensor. Moreover, *P. putida's* lactam regulators have the potential to be developed as biosensors for use in other bacteria, exemplifying *P. putida's* utility as both a host and source of tools for metabolic engineering.

PA-2022-C029

Bacterial quorum sensing allows graded and bimodal cellular responses to variations in population density

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Quorum sensing (QS) is a mechanism of cell-to-cell communication via diffusible signal molecules that controls multiple secreted factors including virulence factors in bacterial

pathogens. While the standard view is that QS functions as a density-sensing mechanism, the functional and evolutionary context of QS continues to be disputed. A critical step in assessing the various adaptive hypotheses is establishing the functional capacities and limits of QS. Current functional studies largely focus on a dichotomy of QS on/off (or, quorate / sub-quorate) states, overlooking the potential for intermediate, graded responses and heterogeneity on a cellular scale. In this work we explore the functional capacity of QS to resolve finely graded environmental densities and introduce the use of reaction norms as a way to holistically characterize QS response. We show that *Pseudomonas aeruginosa* can deliver a graded response to variation in environmental population density on both the population and individual scales. We further resolve the linear population response to be the product of two component cellular reaction norms: the likelihood of being responsive and the intensity of response. Overall, this work reveals that there is no critical cell mass or 'quorum', at which QS is activated on either the individual cell or population scale.

PA-2022-C030

Colistin efficacy against *Pseudomonas aeruginosa* is enhanced and colistin resistance overcome using novel combination therapies

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Multi-drug resistant *Pseudomonas aeruginosa* infections are often treated with the polymyxin antibiotic colistin as a last-resort salvage therapy. Unfortunately, colistin therapy against *P. aeruginosa* is very ineffective, with a high rate of treatment failure (up to 50%), and colistin resistance is rapidly emerging as a growing threat. Efforts to address this and improve the effectiveness of colistin are hampered by a lack of understanding about colistin's mode of action and the mechanism by which colistin resistance is mediated. Here, we show that colistin kills *P. aeruginosa* by targeting lipopolysaccharide (LPS) in the cytoplasmic membrane (CM). We then exploit this information to design and test a new combination treatment strategy – using the LPS transport inhibitor murepavadin to accumulate LPS in the CM – and show that this dual therapy is extremely potent at enhancing colistin's activity against clinical isolates (including cystic fibrosis strains) and in an *in vivo* murine model. We also show that colistin resistance in *P. aeruginosa* is mediated by LPS modifications at the CM, with the polymyxin drug still able to permeabilise the outer membrane (OM) of colistin non-susceptible clinical strains. Finally, we take advantage of this fact by demonstrating that

exposing colistin-resistant *P. aeruginosa* to the polymyxin antibiotic sensitises the bacteria to rifampicin – a hydrophobic compound that normally cannot cross the cellular OM. Our findings provide for the first time a complete model of how colistin kills *P. aeruginosa* and how colistin resistance is conferred. This laid the foundations for the development of two novel combination therapies that can dramatically increase colistin efficacy and even surmount the danger of colistin non-susceptibility. It is anticipated that this work could lead to urgently-needed improved clinical outcomes in patients undergoing treatment with this crucial last-resort antibiotic.

PA-2022-C031

Specific cell-surface glycans on phagocytes mediate binding of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a Gram-negative bacterium that, as an opportunistic pathogen, substantially contributes to high morbidity and mortality rates in susceptible individuals such as those with cystic fibrosis or neutropenia. Our studies have identified that the downregulation or loss of bacterial flagellar motility, typically observed within chronic infections, enables bacteria to evade interactions with phagocytic cells that would result in phagocytic uptake. Our previous work demonstrated that exogenous addition of a negatively charged lipid, phosphatidylinositol-(3,4,5)-triphosphate (PIP_3), induces binding and phagocytosis of non-motile strains of *P. aeruginosa*. Based on this work, we hypothesized that the engagement of *P. aeruginosa* by host innate cells, and subsequent phagocytosis, is mediated by motility-dependent interactions with cell-surface polyanions. We now report that endogenous polyanionic N-linked glycans and heparan sulfate mediate bacterial binding of *P. aeruginosa* by human monocytic cells. These specific cell-surface interactions result in *P. aeruginosa* phagocytosis, bacterial type 3 secretion system (T3SS)-mediated cellular intoxication and the IL-1β (beta) inflammatory response of the host innate immune cells. Concomitantly, inhibition of host cell N-glycan synthesis reduces T3SS-mediated cytotoxicity and the IL-1β (beta) response induced by this bacterium. Importantly, the bacterial interactions with the glycans were motility-dependent and could be recapitulated with purified, immobilized glycans. Therefore, this work describes novel interactions of *P. aeruginosa* with specific

phagocyte cell-surface glycans that modulate relevant host innate immune responses to the bacteria, including phagocytosis, inflammation and cytotoxicity.

PA-2022-C032

Complexome profiling of the *Pseudomonas aeruginosa* PAO1 inner membrane with focus on c-di-GMP modulating proteins

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The molecule c-di-GMP is a bacterial second messenger that controls various processes such as motility or biofilm formation in bacteria. Synthesis and degradation of c-di-GMP is mediated by two types of enzymes, the diguanylate cyclases (DGC) containing a GGDEF domain and the phosphodiesterases (PDE) containing either an EAL domain or HD-GYP domain. *Pseudomonas aeruginosa* PAO1, a model organism for the investigation of biofilm formation and dispersion, encodes 18 GGDEF, 5 EAL, 16 GGDEF / EAL, and 3 HD-GYP domain-containing proteins. In total, 22 of these proteins are predicted to be membrane bound. In our work, *Pseudomonas* membranes are analyzed by mass spectrometry and complexome profiling. With this method we aim to identify large membrane complexes and interacting regulatory modules of c-di-GMP regulating proteins. In first experiments 15 out of 22 membrane bound c-di-GMP modulating proteins in complexes were detected from planktonically grown cells. One example is the phosphodiesterase NbdA with a large N-terminal membrane domain, predicted to be a sensory domain, and a cytoplasmic GGDEF and EAL domain. Using confocal laser scanning microscopy, a polar localization of NbdA was observed, pointing towards a local function in the polar region of the *P. aeruginosa* cell. In detail colocalization studies and interaction analyses are in progress.

PA-2022-C033

Elucidation of the mechanism of phage-derived, lytic proteins attenuating key virulence factors of *P. aeruginosa*

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Chemical compounds arresting essential, cellular processes are commonly used a strategy against *Pseudomonas aeruginosa* infections. However, these antibiotics place a burden on the bacterial cell, which might result in the development of resistance against the corresponding factor. Therefore, novel approaches to combat this critical pathogen are gaining attention. Bacteriophages have co-evolved for millions of years with their bacterial host and can share both predatory and mutualistic relationships with them. Moreover, it is known that temperate phages drive the bacterial virulence and some of those features have already been exploited for biotechnological purposes. In this research, we performed multiple high-throughput screens with a library of individually expressed, lytic phage proteins to identify potential effectors of virulence factors of *P. aeruginosa*. We discovered four different phage ORFans that significantly attenuate key virulence factors of the pathogen, including the Type IV pili, protease IV (a virulence factor secreted by the T2SS) as well as ExoS (a T3SS product). Subsequently, the bacterial interaction partners of the aforementioned phage proteins are being unravelled in order to grasp their working mechanisms. To our knowledge, we are the first to prove that lytic bacteriophages are not only of interest for phage therapy or discovery and exploitation of novel antibacterial targets, but also for their virulence attenuating proteins. The identified phage-encoded virulence attenuators expand the diversity of regulatory mechanisms encoded by phages to impact the bacterial physiology and may serve as a source for the development of phage-inspired anti-virulence drugs or diverse biotechnological applications.

PA-2022-C034

PrrT/A a *Pseudomonas aeruginosa* bacterial encoded toxin-antitoxin system involved in prophage regulation and biofilm formation

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Toxin-antitoxin (TA) systems are highly distributed among bacterial chromosomes. The functions attributed to TA systems in bacteria are controversial and remain largely unknown. Here, we identified and characterized a novel type II TA system *prrT/A* encoded in the chromosome of the clinical isolate 39016 of the opportunistic pathogen *Pseudomonas aeruginosa*. We have shown that the *prrT/A* system exhibits classical type II TA characteristics and novel regulatory properties. We also found that the system is involved in biofilm and motility regulation as the *prrA* antitoxin deletion resulted in increased biofilm formation and reduced motility. Importantly, we found that the *prrA* deletion significantly reduced prophage induction and bacteriophage production in 39016 strain. Our study suggests new insights into the potential functions of bacterial TA systems as we reveal that a chromosome-encoded TA system can regulate prophages genes expression and infectious phage particles production

PA-2022-C035

Antiactivator-dependent self-sensing in *Pseudomonas aeruginosa* quorum sensing

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Quorum sensing is described as a widespread cell density-dependent signaling mechanism in bacteria. Groups of cells coordinate gene expression by secreting and responding to diffusible signal molecules. Theory however predicts that individual cells may short-circuit this mechanism by directly responding to the signals they produce irrespective of cell density. In this study, we characterize this self-sensing effect in the acyl-homoserine lactone quorum sensing system of *Pseudomonas aeruginosa*, and we show that antiactivators, a set of proteins known to affect signal sensitivity, are involved in it. Measuring quorum-sensing gene expression in individual cells at very low densities, we find that successive deletion of antiactivator genes *qteE* and *qslA* produces a bimodal response pattern, in which increasing proportions of constitutively induced cells co-exist

with uninduced cells. Comparing responses of signal-proficient and deficient cells in co-cultures, we find that signal-proficient cells show a much higher response in the antiactivator mutant background but not in the wild-type background. Our results experimentally demonstrate the antiactivator-dependent transition from group to self-sensing in the quorum-sensing circuitry of *P. aeruginosa*. They broaden our understanding of the functional capacity of quorum sensing, provide a mechanistic basis for the quorum-sensing response heterogeneity observed in other bacterial species, and have implications for the design of cell-cell signaling circuits in synthetic biology.

PA-2022-C036

Elucidating the mechanism by which an oxygen-binding hemerythrin protein in *Pseudomonas aeruginosa* is necessary for microoxic fitness

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Pseudomonas aeruginosa persists and often thrives in microoxic environments including sites of infection, multispecies communities, and biofilms where available oxygen ranges from low micromolar to nanomolar concentrations. Once established, *Pa* infections are difficult to treat leading to massive healthcare costs and high mortality rates. We have shown that the oxygen-sensing transcription factor, Anr is necessary for this microoxic fitness. In addition, our lab discovered that Anr regulates a hemerythrin-like protein (Mhr) in *Pa* that reversibly binds oxygen with a K_d relevant to microoxic conditions. Using competition assays, we determined that Mhr plays a significant role in *Pa* microoxic fitness though, the mechanism remains unclear. Compared to other bacterial hemerythrins, Mhr has a 23-amino acid extension on its C-terminus that we have shown is necessary for membrane localization. In addition, truncated Mhr cannot compliment fitness of a Δ*mhr* mutant in microoxia like full-length Mhr can. Mhr is tightly co-regulated with a membrane-bound, high-affinity Cbb_3-type oxidase that catalyzes the final step of respiration by shuttling electrons onto oxygen and is particularly important for respiration in microoxia. Interestingly, overexpression of Mhr in a Δ*anr* background can partially compliment fitness in microoxia so we hypothesize that its contribution to microoxic fitness is both Anr-dependent and Anr-independent. *Pa* has two high-affinity oxidases, one is Anr regulated while the other is constitutively expressed, and epistasis

experiments show that Mhr has an epistatic relationship with both Cbb_3-type oxidases. We hypothesize Mhr reversibly binds oxygen and delivers it to both high-affinity oxidases at the membrane via C-term mediated localization allowing for more efficient respiration in microoxia. Understanding how Mhr contributes to microoxic fitness can reveal a dynamic mechanism by which *Pa* strategically manages oxygen utilization in ways that allow it to establish chronic infections in low-oxygen environments which could reveal potential drug targets for more effective treatment.

PA-2022-C037

Lung tissue-like culture system to study colonization and virulence of *P. aeruginosa* at the single cell level

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P. aeruginosa surface colonization entails complex behaviour including adherence, virulence induction and dissemination. Infection studies are hampered by the lack of experimental models that faithfully recapitulate the physiology of human tissue and, at the same time, offer the experimental power to investigate the infection process with high temporal and spatial resolution. Here, we establish an *in vitro* 3D lung infection model from human stem cells, with air-liquid interface in a Transwell. Immunocytochemistry- and histology staining confirmed that the architecture and cellular composition of the tissue closely resembled the human bronchial epithelium. Using live cell microscopy, we demonstrate that the tissue recapitulates lung functions such as production of mucus and cilia beating. We next established standard infection conditions for *Pseudomonas aeruginosa* and documented its efficient tissue colonization. Lung cell viability and integrity of the epithelial barrier were monitored during infection using fluorescence-activated cell sorting and trans-epithelial electrical resistance measurements, respectively. We also developed an inverted (upside-down) Transwell model to image *Pseudomonas aeruginosa* infection and the decline of tissue integrity in real time. Using this model, we analysed early stages of infection and tissue spreading

of *P. aeruginosa* wild type and virulence mutants. Our results establish lung Transwells as versatile *in-vitro* model to study bacterial infections and drug response in a human-like environment.

PA-2022-C038

Assessing virulence factors and resistance in *Pseudomonas aeruginosa* after gene inactivation

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Pseudomonas aeruginosa is a gram-negative bacterium that is naturally resistant to a wide range of antibiotics and is associated with nosocomial infections. Though many mechanisms of resistance and virulence are well understood, treatment of *P. aeruginosa* infections are exceedingly difficult. One approach to potentially alleviate resistance or virulence is through manipulation of *P. aeruginosa* genes. Based on transcriptome analysis of *P. aeruginosa* persister cells, we have selected a handful of genes (*PA5159, PA1283, PA2898, PA4571, PA2897 and PA5157*) to identify what role they may play in virulence and resistance. We hypothesized that the mutant strains would have decreased virulence and increased antibiotic sensitivity compared to the wild type PAO1 strain. We obtained transposon mutants for each of these genes to assess the function of the associated proteins in *P. aeruginosa*. We have performed pyocyanin production, persister cell formation, antibiotic sensitivity, quorum sensing, and motility assays for each of these strains. We found that one mutant strain (*PA5159*) has significantly increased persister cell formation and two other separate mutants had increased twitching motility (*PA2898* and *PA1283*). We found that all the mutant strains exhibited decreased sensitivity to rifampin when compared to the wild type. Seven of the mutants showed increased sensitivity to kanamycin while seven other mutants had decreased sensitivity to erythromycin. With doxycycline treatment, four mutants had increased sensitivity while the remaining seven had decreased sensitivity. In order to further characterize the role of these genes, we plan to run sequence analysis of the mutant strains and compare them to the wild type. By understanding which proteins are contributing to *P. aeruginosa* antibiotic resistance and virulence, it may be possible to design novel therapies for treatment of these dangerous infections.

Chewing the fat: characterization of fatty acyl-CoA dehydrogenases from *P. aeruginosa*

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Lung surfactant-derived long chain fatty acid (LCFA) is the primary carbon source for *Pseudomonas aeruginosa* (PA) when the organism infects the airways of people with cystic fibrosis (CF). However, our understanding of how fatty acids are catabolized in PA remains limited, although we do know that this is a lot more complicated than the biochemistry in *Escherichia coli*. To investigate this further, we have used quantitative TMT-proteomics to analyze the proteome of a clinical isolate, PA10348, grown on three different fatty acids (octanoate, palmitate and oleate). This revealed that two fatty acyl-CoA dehydrogenases (encoded by PA0506 and PA0508) are likely to be the dominant enzymes in the first step of LCFA-related β-oxidation in PA. However, growth of the corresponding mutants on a range of fatty acids with different chain lengths suggests that PA0506 is mainly responsible for LCFA degradation whereas PA0508 plays a more important role in medium chain fatty acid (MCFA) catabolism. We have purified each enzyme and determined its kinetic parameters, which confirm the distinct substrate specificity profiles. To further investigate the molecular basis of the substrate specificity in each enzyme, we have solved their X-ray crystal structure, and we are using these data to identify potential inhibitors. Current efforts are aimed at investigating the identity and function of the transcriptional regulators controlling PA0506 and PA0508 expression, and the possible function of the intervening gene, PA0507, which encodes another fatty acyl-CoA dehydrogenase.

The CoIR/S two component system is a conserved determinant of host association across *Pseudomonas* species

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Bacteria in the genus *Pseudomonas* are commonly associated with eukaryotic hosts. These range from the human opportunistic pathogen *Pseudomonas aeruginosa* to the plant beneficial strains of *Pseudomonas fluorescens*. To survive in association with a eukaryote, bacteria must be able adapt to this potentially hostile environment. We show that the two-component regulatory system ColR/S is required for colonization of the Arabidopsis rhizosphere in both *P. fluorescens* WCS365 and in *P. aeruginosa* PAO1. Since *P. aeruginosa* can also cause disease in animals, we tested the requirement of *colR* in a mouse subcutaneous abscess model and found that *P. aeruginosa colR* was required for virulence in mice. Using transcriptional profiling we identified genes upregulated in the rhizosphere relative to minimal media in both species. We identified GO terms likely involved in tolerance to pH or metal stress that were enriched in our RNAeq experiment in the rhizosphere. We found that *colR* is required for tolerance to high concentrations of iron, zinc, and manganese, and low pH, and that this is exacerbated in the presence of both high metals and low pH, suggesting that ColR may be required to protect against metal stress and low pH in the rhizosphere. We identified three ColR-dependent genes in *P. aeruginosa* PAO1 which are required for rhizosphere colonization. However, we saw that only one gene, *tpbA*, is required for pH and metal tolerance, suggesting that low pH and high metals aren't the only rhizosphere component inhibiting *colR* mutant growth. We further identified that all three genes required for *P. aeruginosa* PAO1 rhizosphere colonization are also required for virulence in a mouse abscess. Our work has identified novel colonization factors which allow distantly related species of *Pseudomonas* to associate with both plants and animals, suggesting that there are be conserved host association mechanisms across the genus *Pseudomonas*.

PA-2022-C041

Pseudomonas aeruginosa **senses secreted interspecies signals via a c-di-GMP and cAMP-controlled signaling module**

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Bacteria often exist in complex communities composed of cohabitating and competing species. Species interact through physical contact and secreted factors, which ultimately steer the function and health of the community and surrounding environment. For example, pathogens *Pseudomonas aeruginosa (Pa)* and *Staphylococcus aureus (Sa)* are frequently coisolated from patients with chronic airway or wound infections, and are deleterious to the host. Through live-imaging *Pa–Sa* cocultures, we previously reported that *Pa* can sense and move towards *Sa* secreted factors from a distance using type IV pili (TFP)-mediated motility (TM). To unravel *Pa* interspecies sensing pathways, we systematically analyzed mutants in genes necessary for chemotaxis, motility, and biofilm formation to identify mutants that retain TM, but have reduced movement towards *Sa.* Candidates were analyzed by live-imaging and single-cell tracking in coculture with *Sa,* which revealed two required *Pa* pathways: 1) Pil-Chp, a putative TFP chemotaxis (TFPC) system, and 2) the second messengers cyclic adenosine monophosphate (cAMP) and cyclic diguanosine monophosphate (c-di-GMP). While loss of the predicted Pil-Chp chemoreceptor, PilJ, rendered the cells non-motile, cells lacking the predicted PilJ adaptation proteins remained motile, but unable to bias motility towards *Sa,* suggesting PilJ is important for initial sensing. To dissect the involvement of cAMP and c-di-GMP, levels of each were monitored during TFPC towards *Sa*. While cAMP increases as *Pa* approaches *Sa*, population levels of c-di-GMP remain unchanged. However, we find a subset of four of the 40 c-di-GMP regulation enzymes (SiaD, MorA, BifA, and PA2870) and two previously uncharacterized c-di-GMP effector proteins (PA0012 and PA2989) are each critical for TFPC, suggesting a local c-di-GMP signaling network modulates TFPC. We predict this network coordinates with Pil-Chp to fine-tune TFPC. Our work unravels how *Pa* senses and responds to interspecies signals and may provide an opportunity to block harmful interactions before they begin.

PA-2022-C042

Condensins are integrated into lifestyle and virulence programs in *Pseudomonas aeruginosa*

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Condensins play key roles in global chromosome packing and organization. In *Pseudomonas aeruginosa*, this function requires two condensins, SMC-ScpAB and MksBEF. Intriguingly, inactivation of SMC or MksB induces, respectively, either a sessile or planktonic state in the bacterium and renders them avirulent in mouse models of lung and corneal infections. We tested here a hypothesis that this physiological transition is

mediated by regulatory activities of condensins. To this end, we constructed individual and double in-frame deletion mutants of *P. aeruginosa* condensins and analyzed their gene expression patterns using RNA-seq. The predictions of the analysis were corroborated by a phenotypic and secretomic survey of the mutants and an inspection of genetic interactions between condensins and various lifestyle regulators. The overall transcriptional signatures of the *smc* and *mksB* mutants were broad, largely dissimilar and non-additive. Smc mutants mildly activated several host-directed virulence mechanisms, including T6SS, pyocins, hemolysin, hemagglutinin, α2-macroglobulin, iron and sulfur assimilation, and denitrification, almost completely downregulated T3SS. In contrast, MksB deficient cells downregulated surface adhesion regulon Pel/Psl, but upregulating T3SS. These regulatory patterns are reminiscent of cells during an acute and chronic stages of infection. Specifically, the extracellular secretion pattern mirrors transcriptional profiles, whereas T3SS proteins declined and three pyocins increase in smc mutants. Expression changes of many regulons paralleled those of their primary regulators. The greatest resemblance of condensin phenotypes was found to the c-di-GMP signaling pathway. We conclude that condensins, whose primary function is to control the chromosome structure, are also involved in regulation of gene expression. This regulation triggers the intracellular signaling network and affects both the lifestyle of *P. aeruginosa* and its production of numerous virulence factors. In all, we conclude that condensins are an important determinant of the lifestyle and virulence behavior of *P. aeruginosa* and as such are an attractive therapeutic target.

PA-2022-C043

Obligate anaerobic bacterial species induces growth arrest of *P. aeruginosa* via Reactive Nitrogen Species.

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It is increasingly apparent that the human microbiota impacts virtually every facet of health, including the clinical outcome for certain diseases. These effects are often mediated by interactions between pathogens and commensal microbes. In Cystic Fibrosis (CF), where patients are commonly afflicted with chronic bacterial infections, most notably due to *Pseudomonas aeruginosa*, the role of resident anaerobes in disease outcome is poorly studied. However, microbiota-based studies have revealed that some anaerobic lung commensals may impair *P. aeruginosa* colonization. We have identified one anaerobic bacterial species, *Porphyromonas catoniae*, that negatively impacts *P.*

aeruginosa viability. Co-culture assays and incubation in *P. catoniae* cell-free conditioned medium (CFCM) results in total growth inhibition of both *P. aeruginosa* PA14 as well as at least five clinical isolates. Transcriptional profiling revealed that *P. aeruginosa* genes involved in response to nitrogen containing compounds, including reactive nitrogen species (RNS), are highly upregulated in *P. catoniae* CFCM, while virulence-related genes are downregulated. RNS, such as nitric oxide, are important intermediates of anaerobic respiration and can be toxic depending on oxygen concentration. Therefore, we hypothesized a *P. catoniae*-derived RNS may be partly responsible for the reduction in *P. aeruginosa* viability. We detected high levels of nitric oxide in *P. catoniae* CFCM when compared to controls. A combination of flow cytometry and plate reader assays demonstrated that nitric oxide was detected intracellularly in *P. aeruginosa* cells exposed to *P. catoniae* CFCM. Interestingly, addition of RNS scavengers rescued *P. aeruginosa* growth in *P. catoniae* CFCM. Combined, this data suggests that *P. catoniae* produced RNS, potentially nitric oxide, likely impairs the growth of *P. aeruginosa* under anaerobic and aerobic conditions. Future efforts will seek to identify the *P. catoniae* inhibitory molecule(s), as well as *P. aeruginosa* gene(s) that may be involved in the response and resistance to the *P. catoniae*- antagonism.

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