Project Science Areas: 4 IDI; 6 MCB

Project Description

Shortly after the introduction of antibiotics to modern medicine, bacteria that were refractory to these life-saving compounds started to emerge, challenging our ability to effectively treat infectious diseases[1]. In addition to acquiring mutations and genes that confer antibiotic resistance, individual bacteria in a culture can reversibly reprogram their gene expression and metabolism, enabling them to enter into an antibiotic tolerant state[2]. These bacterial persisters can survive lethal doses of antibiotics that kill their genetically clonal kin and contribute to infection relapse once treatment terminates[2]. To preserve our ability to cure bacterial infections, we urgently need to achieve a better understanding of the diverse repertoire of strategies that bacteria depend on to overcome adverse conditions and antimicrobial agents at infection sites. *Staphylococcus aureus*, which can cause relapsing skin and soft tissue infections in as many as 70% of patients after initial successful treatment, highlights the burden of recurrent infections[3]. *S. aureus* routinely colonizes and infects a range of host sites, where they can establish polymicrobial infections with other pathogens as well as with the host's native flora[4-6]. **Our long-term objective is to map key molecular interactions between *S. aureus* and infection site-colonizing microbes that impact *S. aureus*'s persistence toward different classes of antibiotics.**

This proposal outlines our plan to develop a novel large-scale screening pipeline to investigate *S. aureus* antibiotic persistence in the context of polymicrobial interactions and growth conditions that recapitulate two host sites of frequent staphylococcal infection. I will build upon my investigation of persister phenotypes by implementing a combination of single-cell, transcriptomic, and metabolomic techniques to investigate how microbial interactions modulate *S. aureus*'s phenotypic heterogeneity and antibiotic persistence (Fig. 1). The New Innovator Award will give me the opportunity to focus on developing these methods and analytical strategies. We will balance the risks of this extensive effort with well-controlled experiments and logical alternative strategies. Findings from this work will contribute fundamental knowledge of the impact of pathogen-microbiome crosstalk on persistence, which can potentially lead to the discovery of innovative treatment strategies to tackle chronic infections.

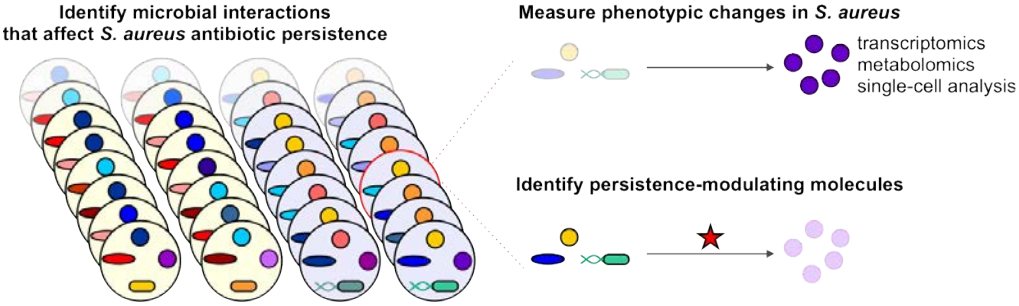

Fig. 1. Effect of polymicrobial interactions on antibiotic persistence. We will develop a large-scale screen to deduce polymicrobial interactions that impact the persistence of *S. aureus* toward different classes of antibiotics. We will then use a combination of multi-omic and single-cell strategies to investigate the molecular bases of interactions that modulate *S. aureus* persistence.

Individual cells within genetically homogeneous cultures can display heterogeneous phenotypes[7, 8]. This ensures that at least some members of the population will remain viable under adverse conditions, such as those brought on by antibiotic treatment. Consequently, when a clonal bacterial population is treated with a lethal dose of a bactericidal antibiotic, a biphasic killing kinetic is routinely observed, revealing the presence of bacterial persisters that are killed at a slower rate compared with their susceptible brethren[2] (Fig. 2). Unlike antibiotic resistant mutants, bacterial persisters cannot replicate in the presence of the drug[2]. Once the antibiotic is removed, persisters resume growth and give rise to progenies with comparable frequencies of persisters and susceptible cells as the ancestral population[2]. These observations indicate that antibiotic persistence is attributed to reversible phenotypic changes rather than acquisition of heritable mutations. Antibiotic treatment failure associated with bacterial persistence is especially discouraging, as the infection is genetically susceptible yet phenotypically tolerant to the therapeutic agent, which can lead to infection relapse when treatment terminates.

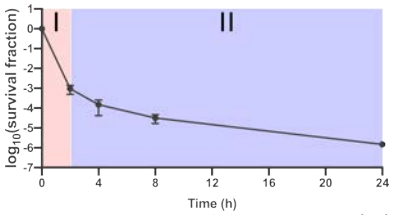

Fig. 2. Biphasic killing of fluoroquinolone (FQ)-treated *S. aureus*. Survival of exponentially growing bacteria treated with 5 μg/mL of levofloxacin, which is 20-fold above the minimum inhibitory concentration (MIC) of this antibiotic against this strain (0.25 μg/mL). The rapid killing regime (I) of the kill curve represents the death of normal cells, whereas the slower killing phase (II) represents colonies arising from persisters. Our preliminary data, n=2.

Bacterial persisters that remain after antimicrobial therapy can promote the horizontal transfer of resistance-conferring genetic determinants and the fixation of chromosomal mutations that lead to enhanced persistence, as indicated by higher survival fraction in phase II of biphasic kill curves, or antibiotic resistance[9, 10]. It was recently reported that in methicillin-resistant *S. aureus* (MRSA) isolates from patients undergoing

combination Vancomycin/ Daptomycin and Rifampicin therapy, the emergence of mutants with enhanced persistence toward Vancomycin/Daptomycin can facilitate the establishment mutants that are highly resistant to Rifampicin[11]. We have also observed that *E. coli* fluoroquinolone (FQ) persisters originating from non-/ slow-growing cultures suffer DNA damage similar to their dead and dying kin, but these persisters will recover after treatment and can activate the DNA repair regulon (Fig. 3A)[12]. This can lead to the induction of error-prone polymerases and a significant enhancement in the frequency of mutants resistant to multiple classes of antibiotics after only one round of treatment (Fig. 3B). These findings highlight the challenges that persisters pose for the treatment of bacterial infections and the threat they impose on our dwindling antimicrobial arsenal.

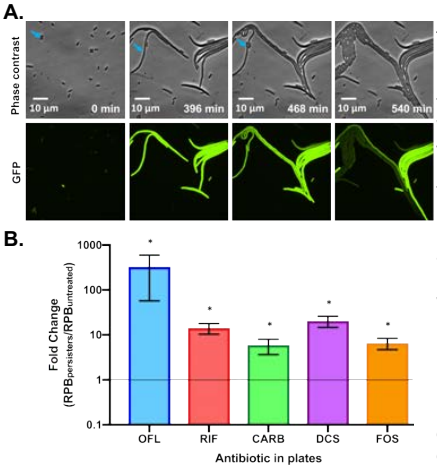

Fig. 3. Fluoroquinolone persisters depend on DNA repair for survival. (A) Representative time-lapse microscopy images of *E. coli* bearing a fluorescent *recA* expression reporter recovering on nutrient-rich media following FQ treatment. These images capture a persister (blue arrow), which divides and gives rise to a colony of daughter cells by 468 min. The persister filaments and mounts an indistinguishable DNA damage response as other cells that fail to divide. Fluorescence decreased considerably in the persister progeny observed at 540 min, suggesting reduction in *recA* expression following damage repair. These images are representative of six biological replicates. **(B)** After recovering FQ-treated cells in a nutrient-rich environment, we observed significant enhancement in antibiotic resistant mutants per billion persister-derived cells compared with untreated controls (RPB: resistant mutants per billion). Persister-derived populations exhibited increases in mutants resistant to ofloxacin (OFL, an FQ), rifampicin (RIF, targets RNA polymerase), as well as carbenicillin (CARB), D-cycloserine (DCS), and fosfomycin (FOS), all of which target cell wall biosynthesis. n≥3. Data from our publication[12].

Advances in single-cell techniques that enable exploration of heterogeneous phenotypes within clonal populations have reinvigorated research on antibiotic persistence, leading to the discovery of persister survival strategies[12-16]. In many cases, antibiotic persistence is a triggered phenomenon, where exposure of a bacterial population to environmental signals and stresses— including nutrient limitation, high cell density, host immunity, and even antibiotics themselves— has been shown to increase persister frequencies[2]. A number of environmental triggers modulate cellular metabolism. In fact, we can frame antibiotic persistence as a metabolic program, where coordinated metabolic shutdown stalls growth or specific cellular processes, ushering persisters into an antibiotic tolerant state[17, 18]. During stasis, a subset of metabolic pathways must remain active to ensure that persisters retain sufficient ATP to remain viable[19, 20]. Once treatment terminates, coordinated metabolic activation mediates persister reawakening[21]. A long-standing paradigm in the field argues that persisters originate from metabolically dormant bacteria, where the activities of primary antibiotic targets are reduced and remain undamaged throughout the course of treatment. While this is applicable to persisters that survive certain classes of antibiotics, such as cell wall-targeting β-lactams, persisters that withstand DNA topoisomerase-inhibiting FQs challenge this conjecture, as they do not escape unscathed from treatment[13]. We recently discovered that post-FQ treatment environmental cues can modulate the timing of DNA repair and the resumption of growth-related processes[21]. When FQ-treated cells were starved following antibiotic removal, more cells had adequate time to repair damaged DNA before *de novo* DNA synthesis resumed, resulting in a significant increase in persisters (Fig. 4). We and others have shown that we can target the metabolic capacity and processes critical to post-antibiotic treatment repair in persisters to sensitize these cells to existing antibiotics[19, 20]. **A better understanding of how persisters respond to environmental triggers and orchestrate their metabolic programs can inspire innovative anti-persister strategies.**

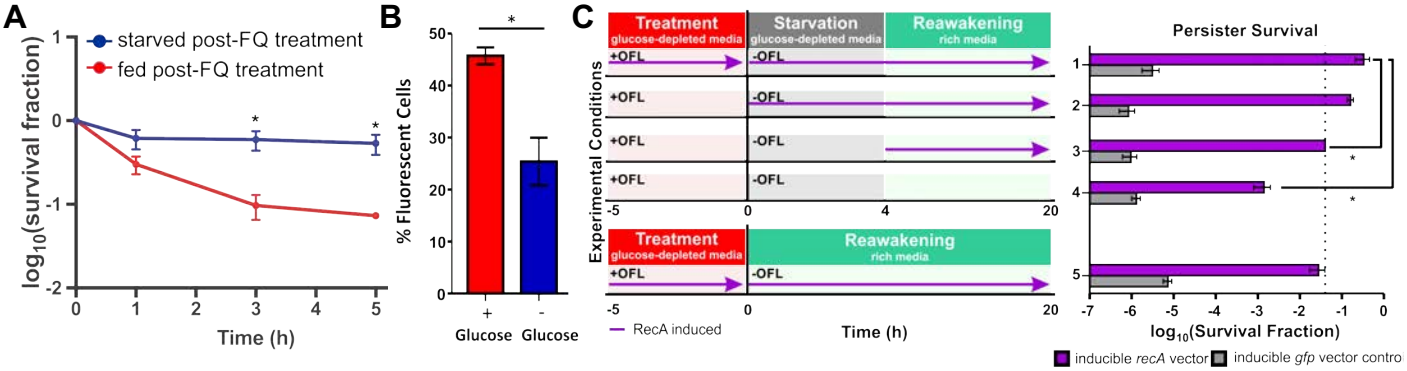

Fig. 4. Starvation following FQ treatment boosts persistence. (A) Glucose-starved *E. coli* was treated with FQ at 0 h and survivors were enumerated at designated times. Cells were either immediately exposed to a nutrient rich environment (red line) or starved for 4 h before being fed (blue line). **(B)** Using a fluorophore to label a thymidine analog incorporated into newly synthesized DNA, we found that glucose deprivation post-FQ treatment reduced *de* novo DNA synthesis. **(C)** With an *E. coli* strain bearing an inducible copy of *recA,* we showed that when *recA* expression and DNA repair were delayed after FQ treatment, starvation no longer enhanced persistence (3 compared with 1 and 2). These data demonstrate that increase in FQ persisters following post-antibiotic treatment starvation hinged on the timing of DNA repair and *de novo* DNA synthesis. Data from our publication[21], n≥3, and * indicates that the data points being compared are significantly different (p≤0.05 calculated using t-tests).

Antibiotic susceptibility is traditionally assessed in mono-species cultures grown in standard laboratory culture media. Yet, at infection sites, bacteria often coexist in diverse host niches with other pathogens, host microbiome constituents, and nutrient sources[4, 6]. In these polymicrobial communities, bacteria can establish

cross-feeding networks[22], exchange quorum sensing signals[23], and secrete exoproducts that allow them to compete or coexist with the other microbial species in a contact-independent manner[24]. Some of these interactions can potentially modulate bacterial growth and persister metabolic programs. Indeed, many bacterial pathogens have been found to be more refractory to antibiotics in mixed-species communities compared with mono-species cultures; however, the impact of microbial interactions on antibiotic persistence is largely unexplored[25]. **We propose that knowledge of antibiotic persistence under conditions that recapitulate microbial interactions and nutrient availability at infection sites can inspire novel, clinically-relevant treatment strategies and bolster our ability to cure chronic infections.**

A main focus of my research program is on antibiotic persistence in *S. aureus* infections— a leading cause of community- and hospital-acquired infections[26]. It is estimated that 50% of the global population are perpetual or transient *S. aureus* carriers[5, 27]. *S. aureus* colonization can increase the risk of developing an invasive infection affecting a range of sites, including the bones, heart, skin and soft tissues, and implantable medical devices[27]. In certain infections, such as diabetic foot ulcers, the presence of *S. aureus* is associated with delayed healing and poor outcomes[4]. To exacerbate the problem, *S. aureus* infections are often refractory to antibiotics, with persisters dwelling in biofilms implicated in infection chronicity and recurrence[28]. Chronic *S. aureus* infections often involve polymicrobial biofilms. For instance, *S. aureus* can co-infect burn wounds and cystic fibrosis lungs with *Pseudomonas aeruginosa*[29]. In chronic diabetic foot wounds, *S. aureus* exists amongst microbiomes that are rich with species that are considered to be skin commensals and mixed anaerobes[4]. Our overarching goal is to uncover molecular interactions between *S. aureus* and bacterial species prevalent at sites of colonization and infection that impact *S. aureus's* persistence toward different classes of antibiotics. **In this proposal, we will develop a novel platform to systematically map interactions between *S. aureus* and microbiome constituents that affect antibiotic persistence; we will implement a combination of single-cell and multi-omic approaches to elucidate the molecular bases of these interactions under conditions that mimic distinct host niches.**

To comply with the funding opportunity announcement, we are not providing a detailed experimental plan and extensive preliminary data. We will, however, describe how we will develop a large-scale screening platform and experimental plan to address the following questions:
(1) How do microbial constituents in polymicrobial communities modulate antibiotic persistence in S. aureus?
(2) How do the active microbiome constituents reprogram S. aureus persisters?
(3) What are the molecular determinants of these interactions?

(1) How do microbial constituents in polymicrobial communities modulate antibiotic persistence in S. aureus?

To map microbial interactions that impact antibiotic persistence, we will focus on microbial communities comprised of *S. aureus* and species prevalent in two niches: diabetic foot ulcers (DFU) and nares of adults colonized with *S. aureus*. In both of these niches, *S. aureus* was found to be the dominant species, as identified via whole shotgun metagenomic analysis of DFU isolates[4] and culture-independent 16S rRNA analysis of human nasal isolates[6]. An overall reduction in biodiversity and diminished abundance of other bacterial species were also observed with *S. aureus* blooms. In DFUs, *Pseudomonas aeruginosa*, *Corynebacterium striatum*, and *Alcaligenes faecalis* were amongst the most abundant species, whereas *Staphylococcus epidermidis, Cutibacterium acnes,* and *Corynebacterium accolens* were abundant in the nares of individuals with *S. aureus* colonization. We will systematically investigate the impact of these two three-species communities on antibiotic persistence in *S. aureus.* Previous studies on microbial interactions have shown that species identity is often insufficient to explain their observations, as the activities can be strain-specific[30]. To ensure that we account for potential strain-to-strain variation in activity, we will perform our experiments with two methicillin-sensitive *S. aureus* strains*,* two methicillin-resistant *S. aureus* (MRSA) strains*,* and at least three strains of each interacting species. These strains will be obtained from ATCC, BEI Resources, or the UConn Health Clinical Microbiology Lab with the help of Dr. Maroun Sfeir. Where possible, we will use strains isolated from wounds or nares and we will verify the identities of these isolates using 16S rRNA sequencing.

We aim to develop a novel platform to quantify *S. aureus* persistence with the following design criteria: the platform will (1) capture the nutrient environment at sites of *S. aureus* colonization and infection; (2) allow *S. aureus* to grow as surface-attached biofilms, a more clinically relevant lifestyle; (3) enable microbial crosstalk; and (4) facilitate segregation of *S. aureus* from other community members for multi-omic and single-cell analyses. Rather than using conventional—often nutrient-rich— laboratory media that do not accurately recapitulate the nutrient environment in the host, we will culture our bacterial communities in media prepared with simulated nasal[31] and wound[32] fluids. Such media have previously been used to assess bacterial physiology and gene expression. For example, we will use a synthetic nasal medium that was formulated based on metabolomic

analysis of human nasal secretions and triggered similar *S. aureus* gene expression changes compared with the nasal habitat[31].

To establish a platform that enables contact-independent microbial interactions while allowing *S. aureus* to grow as a biofilm that can be easily separated from the other three species in the community, we will embed 10^6 cells of each communal species together in 0.5% agarose prepared with synthetic wound or nasal media in individual wells of a 24-well plate. We will ultimately test 108 different community compositions for each niche by permuting the three strains of each of the three communal species against the four strains of *S. aureus*. Agarose pads embedded with 3 X 10^6 *S. aureus* cells will serve as negative (monoculture) controls in our experiment. Prior to antibiotic treatment, we will culture *S. aureus* cells on nutrient-permeable, but bacteria impermeable membranes atop agarose pads embedded with the microbiome constituents. Considering that *S. aureus* is significantly more abundant than other bacterial species in DFUs and the nares of *S. aureus* carriers, we will inoculate 10^7 cells on each membrane. We will incubate these communities for 6 hours (to capture interactions in young biofilms) or 24 hours (to capture interactions in older biofilms). Recently, we used bacteria harbouring inducible fluorescent proteins to demonstrate that this design enables diffusion of small molecules without transferring the agar-embedded strain onto the membrane (Fig. 5). This satisfies our design criteria of separable *S. aureus* cultures that can freely crosstalk with microbiome constituents.

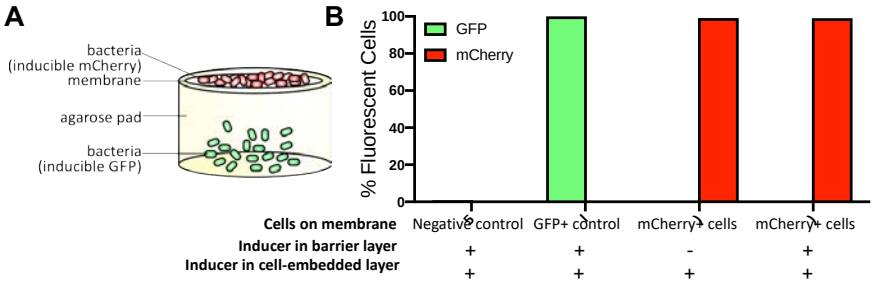

Fig. 5. Testing the set-up of the co-culturing phenotypic screen. (A) Set-up of agarose pads used for our screen. One strain (bacteria with inducible GFP here; microbiome constituents in our large-scale screen) is embedded in the pad, whereas a second strain (bacteria with inducible mCherry here; *S. aureus* in our large-scale screen) is cultured on a cell-impermeable but nutrient-permeable membrane overlaid on the pad. (B) Our preliminary flow cytometry data shows that we can collect the mCherry-expressing cells cultured on the membrane without picking up GFP-expressing cells in the cell-embedded layer. Further, an inducer added in the agarose pad can diffuse through the membrane, leading to mCherry expression.

The persister phenotype is heterogeneous and bacteria can use unique strategies to survive classes of antibiotics targeting distinct cellular processes[33]. As such, we will investigate the impact of interactions between *S. aureus* and microbial communities on persistence toward four classes of bactericidal drugs routinely administered for infections involving *S. aureus*[34]. These include antifolates (*e.g.*, trimethoprim/ sulfamethoxazole); glycopeptides (*e.g.,* vancomycin, which blocks cell wall synthesis); FQs (*e.g.,* delafloxacin, which targets DNA topoisomerases); and tRNA synthetase inhibitors (*e.g.,* mupirocin/ Bactroban).

In our laboratory, we have found that secreted products from stationary-phase cultures of a panel of *Staphylococcus epidermidis* strains can potentiate the activity of FQs against *S. aureus* 43300, a MRSA strain (Fig. 6). A previous study also showed that certain strains of *P. aeruginosa* can secrete secondary metabolites that alter the antibiotic susceptibility of *S. aureus*[24]. We will embed *P. aeruginosa* and *S. epidermidis* strains in our agarose pads as controls. We will use the output from these controls and from a treatment-free (negative) control to assess the dynamic range and limit-of-detection of our screen[35], and we will optimize parameters such as culture density and duration of interaction prior to antibiotic treatment, as needed. In our screens, we will treat each *S. aureus* biofilm with antibiotics administered at 10- to 100-fold above the minimum inhibitory concentration for 24 hours. This will kill all of the susceptible cells in the population, leaving persisters as the only culturable cells. Following antibiotic treatment, the semi-permeable membranes will be transferred to sterile conical tubes with saline and vortexed to dislodge the S. aureus cells from the biofilms. Formation of *S. aureus* colonies on nutritive media following antibiotic removal will serve as an activity read-out. Survival of each of these co-culture samples will be calculated using colony counts from the antibiotic treatment-free control. We will perform each screen with three biological replicates. We will identify bacterial communities that can significantly potentiate or antagonize *S. aureus* killing compared with the monoculture control, where 3 X 10^6 cells of the *S. aureus* strain inoculated on the membrane is embedded in the agarose and biofilms are treated under the same conditions, using two-tailed t-tests.

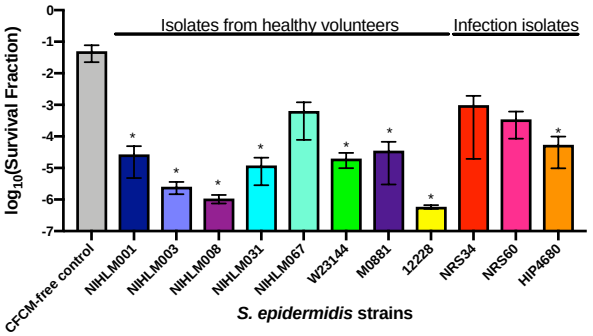

Fig. 6. Effect of *S. epidermidis* secreted products on *S. aureus* persistence. Cell-free conditioned media (CFCM) from eleven *S. epidermidis* strains was added to exponentially growing MRSA (*S. aureus* 43300) prior to levofloxacin treatment. Addition of 25% (v/v) CFCM from eight of these strains significantly enhanced MRSA killing. Our preliminary data, n≥3, * denotes significant difference (p≤0.05) between survival of the control, where CFCM was not added, and each sample.

Upon identifying microbial communities that increase or decrease *S. aureus* antibiotic persistence under simulated wound and nasal fluid niches, we will test each strain individually to determine whether the activity we

observed is attributed to a specific strain or if it is dependent on the community as a whole. We will follow up with the active communities and enumerate persisters that remain at different times throughout the course of treatment. If biphasic killing is still observed in the presence of a microbial community, but the overall persister level observed in phase II of the kill curve is higher or lower compared with the monoculture control, this would inform us that the community of interest affects persistence. We will also investigate if there is a correlation between the magnitude of activity and the duration of interaction. For strains that antagonize antibiotics, we will recover persister progenies of *S. aureus* that survived antibiotic treatment in the presence of these strains, and we will quantify the minimum inhibitory concentration of the antibiotic against this population to assess whether resistance has developed. We will archive these samples for future studies. To further develop our methodology, we will focus on interactions that impact the persister phenotype; we will rank microbial communities and specific strains according to the magnitude of their effect on *S. aureus*'s persistence toward a given antibiotic.

(2) How do the active microbiome constituents reprogram S. aureus persisters?

After we identify microbial communities that impact *S. aureus* antibiotic persistence, we will use a multi-omics approach to elucidate how these interactions modulate the pathogen's phenotype on the population level. Specifically, we will quantify their transcriptomic and metabolomic responses before and during antibiotic exposure. To test the feasibility of this approach, we will first select five highly ranked interactions from our prioritized list, encompassing those that involve microbial communities in both niches and affect persistence toward all four classes of drugs. We will also aim to cover both antibiotic-potentiating and antibiotic-antagonizing interactions if possible. We will mirror the set-up of our persistence screen, and assess phenotypic changes in *S. aureus* grown in biofilms atop semi-permeable membranes on agarose pads embedded with select microbial communities at different times before and during antibiotic treatment. This will enable us to capture phenotypic changes prior to and in response to antibiotic treatment at times before the majority of the population starts to die (death will be evaluated using BacLight Live/Dead stains). Cells from biofilms grown on top of agarose pads embedded with the same *S. aureus* strain (monoculture control) will be subjected to the same antibiotic treatment and collected at the same time points. We will carry out these experiments in biological triplicates.

For metabolomic analyses, we need to rapidly quench metabolism at designated sample collection times to ensure that our data reflect changes stemming from microbial interactions and response to antibiotics. At these times, we will quickly transfer the semi-permeable membranes containing *S. aureus* biofilms into sterile conical tubes with ice-cold extraction solvent (*e.g.,* acetonitrile: methanol: water, 40:40:20, all HPLC-grade) and dislodge *S. aureus* from the biofilms using a vortex[36]. We will then lyse cells using a bead beater. After removing the solvent and cell debris, we will reconstitute the metabolites in water and analyze the extracted metabolites using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Dr. Dennis Wright, a medicinal chemist at UConn's School of Pharmacy, has agreed to work with us on these experiments. We will identify metabolites by matching retention time and ion identification of pure chemical standards, and their optical density-normalized relative abundance will be determined. For each *S. aureus*-microbial community pair, we will identify and cluster metabolites whose relative abundance is significantly different from the monoculture control at each time point, and we will map pathways enriched in these metabolites using MetaboAnalyst[37].

Similarly, we will collect cells into TRIzol for RNAseq and lyse them via bead beating for RNA extraction and purification. UConn's Center for Genome Innovation will work with us to deplete ribosomal RNAs prior to cDNA library construction, and RNA will be sequenced on an Illumina NextSeq 550. Using software such as EdgePro and DESeq2[38], we will align reads and identify differentially-expressed genes from *S. aureus* cultured and treated with antibiotics in monocultures and in co-cultures, where genes with adjusted p-values <0.01 will be considered differentially expressed. We will use tools such as the Database for Annotation, Visualization, and Integrated Discovery (DAVID) for functional annotation of differentially expressed genes[39]. We will deposit complete RNAseq datasets in NCBI's Gene Expression Omnibus following publication. These metabolomic and transcriptomic data can illuminate how microbial interactions modulate the *S. aureus* metabolic program over time, altering their metabolic homeostasis and antibiotic persistence. We will confirm the roles of differentially expressed genes and genes encoding affected metabolites with *S. aureus* knock-down and knock-out mutants generated using CRISPR[40], and we will measure antibiotic persistence in these mutants in the presence of microbial communities of interest.

As antibiotic persistence is attributed to phenotypic heterogeneity[41], we will elucidate whether microbial interactions that affect *S. aureus* persistence are triggering phenotypic responses in individual cells. To assess single-cell responses in the population, we will engineer fluorescent gene expression reporters, which contain the promoters of the genes of interest cloned upstream of *gfp*[42]. We will construct reporters for the top 10 differentially expressed genes in each of the five microbial communities selected for transcriptomic analysis. We will culture *S. aureus* bearing these reporters in biofilms on semi-permeable membranes on top of agarose with

each microbial community, and we will transfer a biofilm into sterile conical tubes with saline at different times during co-culturing and antibiotic treatment. After dislodging cells into the saline using a vortex, we will assess gene expression in individual cells by flow cytometry. Of the genes that are heterogeneously expressed, we will select the three from each *S. aureus*-microbial community combination that exhibit the broadest distribution and collect *S. aureus* from these niches before antibiotic administration or shortly after treatment, depending on when heterogeneous expression is observed. Using fluorescence-activated cell sorting (FACS), we will sort 10% of cells with the highest and lowest expression, continue treating these subpopulations with antibiotics for up to 24 hours, and quantify antibiotic persistence of each subpopulation after sorting. We will also verify expression levels of these genes in the subpopulations with reverse transcription-quantitative PCR. Furthermore, using fluorescent light-sheet microscopy at UConn Health's Microscopy Facility, we will investigate whether the level of expression of these genes is related to the location of a given cell in the *S. aureus* biofilm.

Using fluorescent reporters can potentially illuminate the impact of microbial interactions on *S. aureus* persisters that are reawakening following antibiotic treatment. As dying cells and viable but non-culturable cells (VBNCs) overwhelmingly exceed persisters in a culture following antibiotic treatment[43], using population-wide measurements, including metabolomics and transcriptomics, in evaluating the impact of these interactions on the persister recovery is not feasible. The use of fluorescent gene expression reporters enables us to quantify these changes at the single-cell level. We can gain insight on how microbial interactions perturb the recovery processes and alter the expression (both in terms of magnitude and temporal dynamics) of genes involved in repair and reawakening (*e.g.,* those involved in DNA repair, heat shock, and metabolism). We will first focus on five of the highest ranked microbial community-antibiotic combinations identified in our persistence screen before expanding to other conditions. We will also test reporters of 10 genes encompassing those involved in repair, stress response, and metabolism. After 24 hours of antibiotic treatment in co-cultures, we will dislodge *S. aureus* bearing reporters from biofilms and wash the cells to reduce the antibiotic concentration by 100-fold, which is below its MIC. We will inoculate ~3 x 10^5 *S. aureus* cells (in a 2 µL spot) on a 1% agarose pad prepared with host niche-mimicking media and embedded with a microbial community of interest. Agarose pads embedded with *S. aureus* will serve as a control. The agarose pads will be transferred to a Lab-Tek Chambered Coverglass, which will allow for *S. aureus* imaging, and sealed with a cover slip and VALAP (Vaseline-Lanolin-Paraffin). Using time-lapse fluorescence microscopy, we can track gene expression in *S. aureus* persisters as they recover and reawaken after treatment with different classes of antibiotics in monoculture and in the presence of microbial communities prevalent at two sites of *S. aureus* colonization and infection. These findings can potentially inspire new antibiotic adjuvants or the use of certain bacterial strains as bacteriotherapy to potentiate the activities of existing drugs.

(3) What are the molecular determinants of these interactions?

To decipher the interactions between host microbiome constituents and *S. aureus* that enhance or diminish antibiotic persistence, we need to listen to both sides of the conversation. In addition to investigating phenotypic changes in *S. aureus*, we will decipher the triggers that the active strains exert. In our persistence phenotypic screens, we will assess the impact of three strains of a given host-colonizing species. If the genomic sequences of these strains are not already available, we will perform whole genome sequencing of strains of interest using the Illumina NextSeq 550 at UConn's Center for Genome Innovation and perform *de novo* assembly of these genomes using software, such as Assembler in MacVector. Comparing the genomes of strains that have different effects on *S. aureus* can potentially reveal gene clusters encoding the active, persistence-modulating exoproduct.

To complement these efforts, we will implement an analytical approach and systematically elucidate the exoproduct(s) responsible for the activity. In a pilot experiment, we will test the utility of this approach with one of the active strains/ communities (chosen based on magnitude of activity and/or ability to alter the response of *S. aureus* toward more than one class of drugs) before testing additional cohorts. Using our co-culture set-up, we will culture *S. aureus* biofilms on semi-permeable membranes overlaid on 0.5% agarose prepared with synthetic nasal media or wound exudate, depending on the microbial community chosen in our persistence screen. We will co-culture *S. aureus* biofilms with the selected community for the same duration as we established in our screen. We will then transfer the agarose to a sterile culture tube with 5 mL of synthetic nasal or wound media and crush the agarose with a clean scalpel. We will vortex the sample and remove the solid material along with cells by centrifugation and sterile filtration. Some bacterial strains, such as *P. aeruginosa* PA14, secrete a known repertoire of secondary metabolites in dense cultures, including quinolones and siderophores[24, 44]. As a proof of concept, we will embed and culture PA14 in our soft agarose and determine if our method enables effective secondary metabolite extraction. Alternatively, we can chemically extract microbial exoproducts in solvents (*e.g.,* acetonitrile or methanol) instead of culture media.

We will first determine if the active, persistence-modulating exoproducts in the extracts are proteins by assessing whether the extracts retain their activities following exposure to heat, proteases, and alkaline conditions. This information will guide us in selecting the appropriate column for fractionating the consortium of molecules using high-pressure liquid chromatography (HPLC). For example, if the exoproduct is hypothesized to be a small molecule or peptide rather than a protein, we will fractionate the molecules using ion exchange followed by reverse-phase HPLC. We will then identify the active fraction(s) that retain persister-modulating activity by applying the fraction to *S. aureus* strains before antibiotic treatment, and we will characterize the exoproducts from these fractions using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and/or nuclear magnetic resonance (NMR). My team will work with Dr. Dennis Wright and Dr. Mark Maciejewski at our NMR facility on these experiments. For protein or peptide MS data, we will use MaxQuant, MASCOT, and Scaffold for spectral count quantification and analyte identification; ProgenesisQI (Waters Corp.) will be used to analyze MS data of small molecules. These software packages are available through UConn's Proteomics and Metabolomics Facility. Upon establishing a methodology that enables us to identify exoproducts responsible for modulating *S. aureus* persistence in our pilot study, we will implement a similar approach to identify the molecular determinants that mediate additional top *S. aureus*-microbiome constituent interactions identified from our screen.

Focusing on six of the most abundant microbial species in wounds and nares of patients colonized with *S. aureus*, we will gain insight on the impact of microbial interactions on antibiotic persistence of this important opportunistic pathogen. The methods that we develop to quantify the effect of microbial communities on *S. aureus* persistence, measure perturbations to *S. aureus*'s phenotypic responses, and identify molecules that mediate microbial cross-talk will be applied in future studies, where we investigate interactions in more complex real-world microbiome isolates. Findings from our four-species screens will serve as a roadmap to guide the discovery of additional nodes and edges that affect *S. aureus* persistence in these complex "interactomes."

Innovativeness

Bacterial physiology and antibiotic susceptibility are routinely interrogated in a mono-species context using cultures grown in standard laboratory media. While this approach has yielded a wealth of information, it leaves a major gap in our knowledge of how bacteria behave and respond to antibiotics in an infection environment. At the same time, the impact of microbial interactions on antibiotic persistence in polymicrobial infections— which are often more difficult to treat—remains largely uncharted. One innovative aspect of our proposed research is the development of a screening pipeline that can capture microbial interactions affecting antibiotic responses under distinct nutrient niches in the host. This platform will enable us to (1) better recapitulate the nutrient and chemical environment at sites of *S. aureus* colonization and infection; (2) treat *S. aureus* populations as biofilms, a more clinically relevant lifestyle; (3) co-culture the pathogen and microbiome constituents; and (4) easily separate the two populations to specifically quantify *S. aureus* persistence phenotypes. Our preliminary data demonstrate that our prototype meets these design criteria. We have also identified bacterial strains that affect *S. aureus* persistence, which can serve as controls in our screen. Our experimental set-up will enable us to easily collect *S. aureus* for multi-omic (*e.g.,* genomic, transcriptomic, and metabolomic) and single-cell (*e.g.,* fluorescent gene expression reporters and FACS) measurements. Using single-cell measurements that are omitted from traditional antibiotic susceptibility assays (*e.g.,* MIC measurements), we can decipher the impact of microbial interactions on a phenotypically heterogeneous community. This effort can illuminate novel bacterial survival strategies in response to different classes of antibiotics, potentially leading to the discovery of new drug targets. This relatively cost-effective and high-throughput set-up can be broadly applied to investigate antibiotic persistence in other biofilm-forming pathogens that establish polymicrobial infections, including but not limited to *P. aeruginosa*, uropathogenic *E. coli*, and *Streptococcus mutans*. This approach can vastly improve our ability to assess antibiotic susceptibility of clinical isolates compared with conventional assays done with planktonic mono-species cultures grown in nutrient-rich laboratory media.

Recent discoveries, including those stemming from our research[12, 21], have challenged the long-standing paradigm that persisters originate from cells that are dormant at the time of antibiotic administration and are therefore undamaged from treatment[45]. We found that in some persisters, the timing of molecular events following antibiotic removal can dictate survival. As such, a deeper understanding of persister survival strategies—including activation of repair pathways and metabolic reprogramming—during this critical (yet overlooked) period shortly after antimicrobial therapy termination can point to strategies to fully eradicate an infection. To our knowledge, the project proposed here would be the first to deduce microbial interactions that impact post-antibiotic treatment molecular events critical to *S. aureus* survival. Mapping these interactions can potentially lead to the discovery of antibiotic adjuvants that target post-antibiotic treatment repair programs. As interactions between microbial species are often strain-specific, we will execute our screen with multiple strains of *S. aureus* (both MRSA and MSSA) and various host-colonizing species to identify active strains that can

potentiate or antagonize *S. aureus* antibiotic killing. With the advent of the age of precision and personalized medicine, we envision that the microbiomes of individuals who are prone to chronic infections (*e.g.,* immunocompromised patients or those with diabetic foot ulcers) can be sequenced and catalogued economically. A map of infection site-specific pathogen-microbiome constituent interactions that impact antibiotic treatment success can guide the design of more effective therapeutic strategies, including those involving the use of probiotics as adjuvants.

Investigator Qualifications

My doctoral and postdoctoral training took place in multidisciplinary labs, where I learned that approaching a scientific problem from different angles could lead to creative solutions. My doctoral advisor, Prof. Yingfu Li, pursued questions at the interface of biology and chemistry. My thesis focused on exploring the sequence-structure-function relationship of bacterial toxin-antitoxin systems and I also had the opportunity to take on projects on engineering nucleic acid-based enzymes and biosensors[46]. My postdoctoral advisor, Prof. Mark Brynildsen, brought together an eclectic group of chemical engineers, biochemists, medical students, and mathematicians who shared the goal of combatting antibiotic treatment failure. During this period, I learned to apply engineering approaches to investigate antibiotic persistence. Specifically, we framed persistence as a metabolic program and worked toward elucidating metabolic perturbations that contribute to the survival of these phenotypic variants during antibiotic therapy, an emergent concept in the field at the time. In my lab here at UConn Health, I collaborate with microbiologists (*e.g.*, Dr. Peter Setlow), medicinal chemists (*e.g.,* Dr. Dennis Wright), clinicians (*e.g.,* Dr. Tom Murray at Yale), and biophysicists in my department. Being on the faculty of an umbrella graduate program, I have the opportunity to build a team comprised of trainees with diverse and unique backgrounds (https://health.uconn.edu/mok-lab/).

Thus far in my career, I have drawn inspiration from methodologies used in diverse fields of research to develop tools to solve specific problems. During my Ph.D., I conducted a genetic screen to identify IbsC-SibC, a novel toxin-antitoxin (T-A) system comprised of a small toxic peptide and a regulatory RNA that antagonizes the toxin's expression[47]. To enhance our understanding of the sequence-structure-function relationship of small peptide toxins, which are abundant across bacterial genomes, I developed a high-throughput screening platform to deduce functional sequence determinants of the toxin[48]. I conceptualized this screen based on the use of oligonucleotide-generated sequence libraries that the Li Lab uses to identify deoxyribozyme and aptamer candidates via Systematic Evolution of Ligands by Exponential Enrichment (SELEX), along with high-throughput screening platforms that my departmental colleagues used to identify drug leads. Despite being well-conserved in Proteobacteria, I discovered that the functional sequence space for this toxin is in fact quite large, and both the toxin and its active variants are membrane-active. I further demonstrated that the active variants of the toxin could be developed into molecular tools, such as ones that improve cloning efficiency. Knowledge of the sequence space of small peptide toxins can be used to guide their discovery across microbial genomes.

During my postdoctoral training, I used T-A systems as tools to investigate persister metabolic programs. As persisters are rare phenotypic variants, the lack of robust methodology to segregate persisters from other cell types for direct quantitative analysis hindered investigations into their metabolic capacity. Existing methodologies that were implemented to "isolate" persisters enriched for these cells, but they also captured dead cell debris, normal cells, and an abundance of VBNCs that fail to resume growth after antibiotic removal[16]. To overcome this isolation challenge, I engineered a library of bacterial strains with orthogonally inducible toxins and antitoxins from modules that targeted different cellular components, as many T-A systems have been implicated in persistence. I found that strains accumulating distinct toxins produced populations that were tolerant to different classes of antibiotics, suggesting that we can gain insight on heterogeneous paths toward antibiotic persistence using these model systems. For example, our metabolomic analyses revealed that one of these model persisters retained metabolic capacity and engaged in a transcription-transcript degradation futile cycle, sensitizing itself to aminoglycoside antibiotics. I also discovered that the survival of these model persisters following FQ treatment hinged on the timing of molecular events after antibiotic removal[21]. With this insight, I demonstrated that the survival of wild-type FQ persisters originating from stationary phase cultures also depended on the coordination of DNA repair and resumption of growth-related processes during the critical recovery period. This work was largely funded by a fellowship from the Charles H. Revson Sr. Foundation. Additionally, I was involved in a project where we used time-lapse fluorescence microscopy to show that FQs induced an indistinguishable DNA damage response in persisters compared with cells that succumbed to treatment. We further found that activation of error-prone DNA repair enzymes during the post-FQ treatment recovery period can lead to increased frequency of multidrug-resistant mutants in the persister progeny. My training has prepared me to lead my lab in discovering novel persister survival tactics and anti-persister strategies using rigorous and multi-faceted experimental methodologies.

Currently, my team and I are engineering probiotic persisters to inhibit the recovery of pathogenic persisters. Supported by UConn's Microbiome Seed Grant and funding from the Charles H. Hood Foundation, we are investigating the impact of environmental cues stemming from microbial interactions on antibiotic persistence and microbial adaptation during treatment. Building on this work and on my background, we will use a combination of single-cell (*e.g.,* FACS and time-lapse microscopy) and multi-omic (*e.g.,* transcriptomic, genomic, and metabolomic) strategies to investigate phenotypic reprogramming in persisters in response to in infection site microbiomes, as described in this proposal. In collaboration with Dr. Dennis Wright, we are developing analytical techniques to elucidate microbial exoproducts that modulate persistence, which can potentially lead to the discovery of novel antimicrobial adjuvants. We aim to leverage the knowledge we gain from our work to devise more effective, personalized antibiotics regimens and to successfully eradiate chronic infections.

Suitability for the New Innovator Program

The novel and large-scale pipeline that we are developing in this proposal will enable us to gain a breadth of information on antibiotic persistence in the context of polymicrobial communities and under nutrient conditions that recapitulate two distinct host niches. The New Innovator Award will give me the opportunity to establish these methods in my laboratory. Findings from this work— including those on the responses of *S. aureus* to the microbiota at the site of infection and antibiotics, as well as the molecular determinants that mediate these interactions— will lay the foundation for proposals suitable for traditional, hypothesis-driven funding mechanisms. Furthermore, the experimental pipeline that we develop here can be implemented to explore antibiotic persistence in other pathogens in diverse polymicrobial communities and ecological niches, enabling us to assemble a more comprehensive understanding of these clinically-relevant phenotypic variants.

Statement of Research Effort Commitment

As a tenure-track faculty at UConn Health, 80% of my supported time is dedicated to research, and I will be able to commit 55% of my research efforts to the activities supported by this program each year.

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