Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature: DocuSigned by: Ashley Alexander 044ED5B6700E42A...

Ashley Alexander
Name

10/4/2023 | 6:38 PM EDT
Date

Title Inter- and Intra-specific Interactions of Staphylococcus aureus Modeling Cystic Fibrosis Infection

Author Ashley Alexander

Degree Doctor of Philosophy

Program Biological and Biomedical Sciences
Population Biology, Ecology, and Evolution

Approved by the Committee

DocuSigned by:
Joanna Goldberg
001C9394541C4B8...
Joanna Goldberg
Advisor

DocuSigned by:
Timothy Read
2A4D4A4362BB4E0...
Timothy Read
Advisor

DocuSigned by:
Levi Morran
10531AD88C174AE...
Levi Morran
Committee Member

DocuSigned by:
Chris LaRock
9052981654E0425...
Chris LaRock
Committee Member

DocuSigned by:
Nicole Gerardo
1ED7AF2AB9724BF...
Nicole Gerardo
Committee Member

DocuSigned by:
Steve Diggle
385295F23A4B449...
Steve Diggle
Committee Member

Accepted by the Laney Graduate School:

_____
Kimberly Jacob Arriola, Ph.D, MPH
Dean, James T. Laney Graduate School

_____
Date

Inter- and Intra-specific Interactions of *Staphylococcus aureus* Modeling Cystic Fibrosis Infection

By

Ashley M. Alexander
BS, Georgia Institute of Technology, 2018

Advisors:

Joanna B. Goldberg, PhD

Timothy D. Read, PhD

An Abstract of

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Population Biology, Ecology, and Evolution

2023

Abstract

The diversity that exists among and between species is a captivating wonder of the living world. At the microbial scale, competition, mutualism, and commensalism between populations drives the evolution of traits like pathogenesis, cross-feeding, and bactericidal warfare. In the case of opportunistic pathogens like *Staphylococcus aureus* inter- and intra-specific interactions can have major impacts on the emergence of pathogenic traits. For individuals with the genetic disease cystic fibrosis (CF), complex communities of microbes can cause severe and long-term disease that is difficult to treat. In the last several decades *S. aureus* has become the prominent pathogen in CF related respiratory infections. The research presented in this thesis explores how coexisting populations influence the evolution or emergence of traits that are important factors in cystic fibrosis associated infections like the ability to coexist with *P. aeruginosa* and the ability to evolve and maintain antimicrobial resistance. In the introduction chapter 1, I frame this work in the context of previous findings on interactions between bacteria and research on cystic fibrosis associated pathogens. I also expand on the utility of experimental evolution approaches in studying complex traits within and between populations. In chapter 2 I present the findings of an evolution experiment and the resulting identification of a genetic signature that had not previously been observed in *S. aureus – P. aeruginosa* interactions. I demonstrated how the disruption of the amino acid transporter, *gltT* enables *S. aureus* to outcompete wild-type *S. aureus* under specific nutrient conditions and how that relates to its survival in the presence of *P. aeruginosa*. In chapter 3, I summarize findings about a set of clinical isolates taken from fresh CF sputum. I discuss the intraspecific diversity among single colonies and pool samples and the observed interactions between co-isolated strains. Altogether, the research presented here adds important insight into the role that diversity plays in the evolution of pathogenic traits in the important opportunistic pathogen *S. aureus.*

Inter- and Intra-specific Interactions of *Staphylococcus aureus* Modeling Cystic Fibrosis Infection

Ashley M. Alexander

BS, Georgia Institute of Technology, 2018

Advisors:

Joanna B. Goldberg, PhD

Timothy D. Read, PhD

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Population Biology, Ecology, and Evolution

2023

Acknowledgements

I am an incredibly fortunate young researcher who has benefited from the support of many. I am first and foremost grateful for my family, especially my parents, who fostered in me a love for the natural world and science from a very early age and who have lovingly supported me throughout my education. I'm also grateful for Sam and his idea to do a PhD in solidarity, and so much more.

I have benefited from the mentorship of many incredible teachers and researchers including Dr. Ron Hardy who offered me my first position working in a research lab. Dr. Frank Rosenzweig opened my eyes to the wonders of microbial evolution and eloquent scientific communication and continues to enthusiastically support me in all my endeavors. I thank all the members of the Rosenzweig lab who treated me as one of their own from day one, especially Dr. Dong Dong Yang who taught me the beauty of microbial genetics and determination. I have been fortunate to find the same comradery in the Goldberg and Read labs. Many members of both labs have contributed to this work and freely offered their own skills and brilliance, I could not have done this work without their support. I would like to thank all the members of my committee for their perspectives and enthusiasm for my work. Members of the PBEE program, especially my cohort, have all made this experience incredibly rich and memorable.

I could not have asked for better mentors than Drs. Joanna Goldberg and Tim Read. They have been constant in their support and patient guidance, allowing me to develop my own ideas and helping me communicate my findings from my own unique perspective. Their expertise has been invaluable, but their compassionate mentorship and collaborative nature are what I will strive to emulate most.

Table of Contents

Chapter 1 – **Introduction**

What microbes have taught us about ecology and evolution

When Darwin contemplated the complexity of an entangled bank (Darwin, 1859), he could not envision just how broad and deep ecological diversity went beyond what he could see. Smaller than 'worms crawling through the damp earth' and more consequential for the life beyond a single habitat, microbes not only expanded on that diversity of life but also enabled it. Despite the fact that evolutionary biologists today are equipped with knowledge about microbes and their impact, there are still many fundamental questions about diversity within microbial communities to be answered. For instance, the reason that diversity exists at all is not readily apparent. When considering the forces of evolution alone in a simple environment, diversity within and between species is unlikely to persist because selection and competition should generally reduce a community to a singular fittest lineage. When it comes to evolution within a single species, this theoretical process is referred to as clonal interference (**Figure 1**) where new genotypes emerge and either quickly go extinct or sweep the community entirely based on their relative fitness. While clonal interference has been observed in some *in vitro* experiments such as the long-term evolution experiment with serially transferred *E. coli* (Maddamsetti et al., 2015), what happens in nature, however, is quite different. We can readily observe that even in the simplest of environments complex communities with many species and variants within species thrive and persist over time.

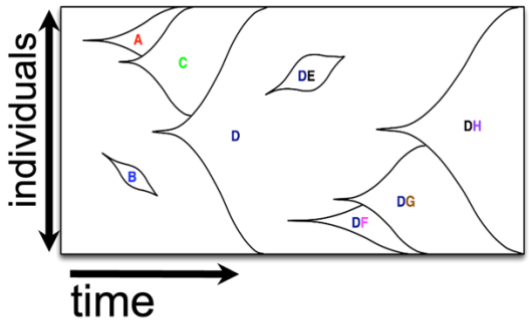


Figure 1. Clonal interference and its impact on population structure over time. Adapted from Desai & Fisher (2007). Schematic depicting the emergence of new genotypes in a community where new more fit mutations successively sweep the population and prevent the maintenance of multiple variants.

One major research topic in microbial ecology and evolution research is investigating the environmental, genetic, and even stochastic factors lead to community stability or disruption. Microbes are ideal subjects for these kinds of investigations as they grow rapidly, maintain large population sizes, and have the ability to adapt to a wide variety of environmental and ecological challenges. In microbial evolution, we often equate fitness to growth rate as models such as Lotka Voltera tell us that the timing of when both species reach their carrying capacities predicts whether or not they will coexist in those conditions (Fort, 2020). When coexistence does occur, lineages may coexist commensally (neither enabling or inhibiting the other) or they may mutually benefit from each other as is often the case in cross-feeding or nutrient exchange (Adamowicz et al., 2020). When one species or variant outcompetes another, it can do so directly through interference competition like contact dependent killing or the release of bacteriocins (Ho et al., 2014). Competition can also happen indirectly through exploitation by competing over a shared resource or as apparent competition where both species share a predator like a host immune system (Howden et al., 2023). These dynamics play out within shared hosts in polymicrobial infections, in endemic microbiomes, and in environmental communities and studying these dynamics will lead to useful insights about health, agriculture, and environmental nutrient exchange. Additionally, using microbial populations and communities to better understand ecological patterns like competition and reinforcement will also add to fundamental knowledge about how diverse ecosystems evolve and persist across all biomes. In order to design studies and experiments with ecology and evolution in mind we must first consider how populations and communities respond to changes in their environment and how adaptations and responses to those changes influence variant fitness and evolution in the context of their environments.

Using ecology and evolution to gain a pathogen's perspective – beyond Koch's postulates

For more than 100 years, the prevailing approach to studying infectious disease has been to define the relationship between a pathogen and its host (Méthot & Alizon, 2014). However, bacterial populations rarely exist in isolation, especially in host environments, where pathogens interact with other present microbial species and even other variants of the same species. Interactions with competing populations in a shared environment generate selective pressures that change how populations grow and adapt. Highly adaptable microbes like *Staphylococcus aureus* and *Pseudomonas aeruginosa* are particularly influenced by other populations of microbes because of the multitudes of ways they can alter their metabolism, cellular defense mechanisms, and behaviors (Briaud et al., 2019; Giulia & O'Toole, 2017; Niggli et al., 2022; Rezzoagli et al., 2020). Identifying patterns in population interactions using principles from ecology and evolution, allows for the identification of key features in phenotypic and genomic traits related to virulence and persistence within a host. We may even be able to predict the acquisition of virulence related adaptations when we are able to identify selective pressures exerted not only by the host environment but also by coexisting microbial populations.

The prevailing approach to studying infectious disease as single pathogenic populations may be due to the way microbiologists have historically defined what it means to be a 'pathogen'. Shortly after germ theory was presented by Louis Pasteur, Robert Koch presented his postulates, dictating a method for studying pathogenesis through the isolation of infecting microbes from sick individuals and studying them in pure culture (Koch, 1884; Pasteur, 1857). While useful for studying obligate pathogens like *Bacillus anthracis,* Koch recognized that his postulates could not account for diseases like cholera, an infection caused by the bacteria *Vibrio cholerae*, which is asymptomatic in as many as 80% of cases (Harris et al., 2012). Stanley Falkow attempted to resolve some of these discrepancies by linking pathogenesis to groups of

genes which were later termed ‘pathogenicity islands’ (Falkow, 1997). In 1988 Falkow adapted Koch’s postulates to fit a molecular definition of pathogenesis, identifying an infective agent based on specific genes they possess (Falkow, 1988).

Koch’s Postulates (Walker et al., 2006)*:*

1. The organism must be shown to be invariably present in characteristic form and arrangement in the diseased tissue.

2. The organism, which from its relationship to the diseased tissue appears to be responsible for the disease, must be isolated and grown in pure culture.

3. The pure culture must be shown to induce the disease experimentally.

4. The organism should be re-isolated from the experimentally infected subject

Falkow’s Molecular Koch’s Postulates (Falkow, 1988)*:*

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.

2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence.

3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

Both Koch and Falkow’s methods of defining what makes a pathogen contributed greatly to the study of infectious disease, but their approach to studying pathogenesis continued to leave out the importance of diversity within an infection community. By not considering the role that interspecific and intraspecific interactions play in the manifestation of disease, it becomes easy to forget that pathogenesis is an adaptive strategy adopted by populations vying for niche space in a shared environment. To gain a deeper understanding of key virulence factors like antibiotic resistance, immune evasion, and tissue damage, we must identify sources of selective pressure that drive populations toward more pathogenic states.

When we take the perspective of the pathogen in polymicrobial infections, factors that push a population toward cooperation or competition with the host or other microbes become clearer. For instance, in an iron limited environment like the respiratory tract, *Staphylococcus aureus* is forced to upregulate its iron sequestration pathways and use excreted toxins to lyse tissue and gain access to host hemoglobin (Reid et al., 2009). Other neighboring microbes may utilize *S. aureus'* hemolysis and subsequent release of public goods (Mashburn et al., 2005). Tissue lysis, however, triggers an immune response which then pressures *S. aureus* to reduce its quorum sensing activity and related virulence factors. Pressures from the host immune system and competing microbes like *Pseudomonas aeruginosa* then select for *S. aureus* that transitions to a persisting phenotype of slower fermentative growth and reduced quorum sensing activity (Filkins et al., 2015). This phenotype often presents morphologically as small colony variants (SCVs) that are often seen in chronic or persistent infections. SCVs and lineages with reduced quorum sensing activity are unlikely to be transmitted to another host, however they may continue evolving within their host for many years and could serve as reservoirs for antimicrobial resistance genes for new invading lineages of *S. aureus* (Kahl, 2014). In another example, pathogens like *Vibrio cholerae* can utilize contact-dependent killing mechanisms to outcompete other resident microbes. However, in one study using a zebrafish host model, researchers found that while *Vibrio cholerae* can directly inhibit the commensal species *Aeromonas veronii* with its type VI secretion system, it was much more efficient for *V. cholerae* to use the same system indirectly on host epithelial cells to induce increased intestinal motility which effectively cleared *A. veronii* entirely (Logan et al., 2018). By considering how ecological forces shape the adaptation of pathogens and how those acquired traits impact population fitness, we may be able to better predict how pathogens will respond based on the introduction of new populations or treatments.

Cystic fibrosis – an important disease that lends fundamental observations about microbial ecology and evolution

Cystic fibrosis (CF) is a genetic disease that in 2021 affected 32,100 diagnosed individuals according to the Cystic Fibrosis Foundation (2021). CF is an autosomal recessive disease that can be caused by approximately 700 documented mutations that lead to a malfunctioning cystic fibrosis transmembrane conductance regulator (CFTR) protein (Ong & Ramsey, 2023; Veit et al., 2016). CFTR mutations are grouped into 5 classes based on their impact on CFTR function from entirely inhibiting protein production (Class I and II) to the production of malfunctioning proteins (Class III and IV) and mutations that lower the expression or production of functional CFTR proteins (Class V) (Veit et al., 2016). Each class of mutations and combination of alleles leads to slightly different manifestations of a disease that is largely characterized by an ionic imbalance across the cell-membrane. In the respiratory system, this imbalance leads to a buildup of respiratory sputum in the lung tissue and improper fluid circulation. Sputum buildup in the lungs fosters the maintenance of long-term and complex bacterial infections that are a significant contributor to morbidity and mortality among individuals with CF (Lyczak et al., 2002).

A wide range of bacterial species can be the cause of long-term and impactful CF associated infections, and many individuals with CF harbor multiple pathogens in coinfections. Coinfections have been observed to be more detrimental to lung function and associated with higher rates of pulmonary exacerbations (Limoli et al., 2016). Additionally, coinfections pose the challenge of identifying a course of antibiotic treatment that will address multiple potentially drug resistant pathogens like methicillin resistant *S. aureus* and naturally drug-resistant gram-negative pathogens like *P. aeruginosa* and members of the *Burkholderia cenocepacia* complex.

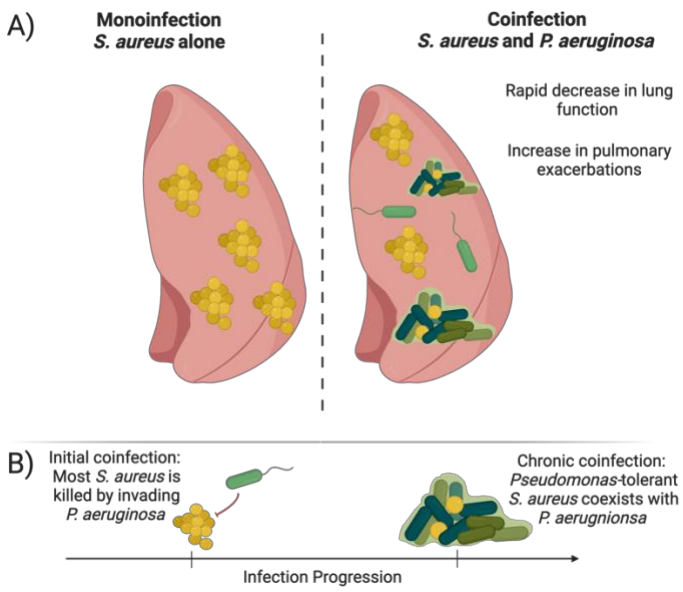


Figure 2. Model of Monoinfections and Coinfections with *S. aureus* and *P. aeruginosa* in CF lungs. A) Comparison of monoinfection and coinfection impacts on CF lung health. B) Schematic representing the different phases of coinfection development with initial invasion occurring early on in disease and developing into a chronic coinfection as disease progresses.

Because CF infections often harbor multiple coinfecting populations, these complex long-term infections pose important questions to microbiologists and microbial ecologists alike (Fischer et al., 2020; Harrison et al., 2008; Limoli et al., 2019). CF pathogens are likely to evolve within their host alongside a diverse commensal and pathogenic microbial community over years and potentially decades. CF associated infections therefore bring interspecific and intraspecific interactions to the forefront of research questions and innovations. Investigations of CF associated pathogens have the potential to not only illuminate a greater understanding of this important CF complication, but also more broadly contribute foundational insights to infection-related microbial ecology.

Staphylococcus aureus

General history and physiology

Alexander Ogston was likely the first person to view *Staphylococcus* under a microscope and coined the genus term which is a mashup of the Greek words staphyle – meaning bunch of grapes, and kokkos – meaning berry (Ogston, 1882). Anton J. Rosenbach further differentiated *Staphylococcus* isolates into the species *aureus* and *albus* (now *epidermidis*) based on their colors when cultured (gold and white) (Rosenbach, 1884). Since its discovery, *S. aureus* has been a focus of microbiology research both for its role as an important human pathogen and its ability to thrive in a wide variety of host environments.

S. aureus is a gram-positive cocci bacterium that is characterized by its golden color on trypticase soy agar. It is taxonomically grouped among other gram positives with low guanine and cytosine content like *Bacillus, Lactobacillus* and *Streptococcus,* however *Staphylococci* species can be distinguished microscopically by their cell-division across two planes, which gives rise to ‘grape-like’ clusters of cells rather than chains as seen with *Streptococcus* species

(Foster, 1996). *S. aureus* is unique among opportunistic pathogens as it is capable of infecting nearly every tissue in the human body and while it can infect other animals it is mainly considered to be a largely human associated pathogen (Howden et al., 2023). Equipped with adept immune evasion techniques and human-specific toxins, as well as its ability to persist commensally in an estimated 30% of the human population – *S. aureus* is a sophisticated opportunistic pathogen that poses unique research questions and challenges (Krismer et al., 2017).

Key traits and adaptive strategies

One set of significant features of *S. aureus* that made it difficult to study for many decades are its effective restriction enzyme modification systems that prohibit the transfer of foreign DNA. In 1983 a strain was engineered through mutagenesis that could accept *Escherichia coli* shuttle plasmids known as strain RN4220 (Kreiswirth et al., 1983). It was later confirmed that RN4220 accepts foreign DNA due to mutations that disrupt both its Type I and Type IV restriction modification systems (Monk & Foster, 2012). The development of this strain along with methylation negative *E. coli* strains like DH10B have allowed researchers to genetically manipulate a diverse range of *S. aureus* isolates and study their virulence factors (Monk & Foster, 2012). Improvements in genetic engineering techniques and resources like the USA300 Nebraska Transposon Mutant Library have greatly expanded the kinds of empirical genetic investigations that can be conducted, allowing for new major insights into *S. aureus'* molecular physiology and key traits (Fey et al., 2013a).

Many *S. aureus* virulence factors are regulated by its quorum sensing locus, *agr*. *S. aureus* isolates can all be categorized into one of four *agr* groups each with a unique auto inducing peptide. The strict constraint of these four *agr* groups across all known *S. aureus* genomes raises interesting evolutionary questions about how diversity arises and how distantly related

lineages interact when they co-occur (Raghuram et al., 2022). The *agr* locus regulates some of *S. aureus'* most severe virulence factors like alpha-hemolysin, beta-hemolysin, proteins involved with capsule production and toxic shock syndrome toxin-1 (Tan et al., 2018). *Agr* is also involved in biofilm production regulation, an important phenotype in the transition from acute to chronic infection. Interestingly, despite their key role in establishing an infection, dysfunctional *agr* loci are associated with established long-term and recurrent chronic or latent infection isolates (Altman et al., 2018). Disruptions of the *agr*-quorum sensing locus are predicted to enable a population to evade cheating from co-occurring lineages by eliminating 'share-able' goods like toxins. Reduced *agr* activity has also been associated with complex and recurrent bacteremia infections as an important mechanism for evading host immune defenses (Kwiecinski & Horswill, 2020). Isolates with defective *agr*-loci may be less likely to outcompete other microbes when transmitted to new hosts and therefore could be considered evolutionary 'dead-ends', however this adaptation signifies one of *S. aureus'* effective strategies for persisting in a host environment and navigating intraspecific interactions.

One of *S. aureus'* most utilized adaptive strategies is to alter its metabolism. Metabolic states have been shown to be a major factor in immune evasion and infection recurrence as well as the commonly observed phenotypic shift to small colony variants (SCVs) (Howden et al., 2023). SCVs are not only characterized by their small colony morphology but also signficanlty impaired metabolism by altering the expression level of key regulators like *sigB* and *agr* (Kahl, 2014). SCVs have been observed to be associated with CF related infections, higher rates of antimicrobial resistance and are generally considered to be a phenotype associated with persistence (Long et al., 2020a). Despite some of their phenotypic advantages SCVs are usually observed as part of a polyclonal population of infecting *S. aureus* and not a phenotype shared among all isolates observed in a chronic infection. This heterogeneity within an infection

population and reliance on major shifts in metabolism signify just how plastic *S. aureus* is when it comes to adapting to a host and the microbial community within an infection environment.

Nutrient landscapes in general can dictate *S. aureus* virulence and metabolism in a host environment. In glucose rich environments *S. aureus* toxin production can be altered, in the case of TSS-1 in vaginal infections, high glucose concentrations can reduce toxin production activity (Karine et al., 2022). In glucose limited environments *S. aureus* is able to turn to available amino acids like glutamate to continue growing at high rates even when carbohydrates are limited (Halsey et al., 2017). *S. aureus* utilizes nutrient detection to direct its adaptive trajectories whether it is infecting a new host or migrating to new body sites, its metabolic and virulence plasticity make it able to evade its host and readily outcompete other resident microbial populations.

Patterns in interspecific interactions with *S. aureus*

The kinds of species that *S. aureus* encounters within a host are largely determined by the site of infection as most colonizing microbes are associated with particular body sites. In the nasal passageways where *S. aureus* is often a commensal colonizer, it commonly co-occurs with other genera like *Corynebacterium, Propionibacterium* and coagulase negative *Staphylococcal* species like *S. epidermidis. S. lugdunensis* is another common commensal microbe in the nasal passageway that interestingly is quite effective at inhibiting *S. aureus* with the production of its bacteriocin lugdunin (Krismer et al., 2017). Even in the absence of species-specific bacteriocins, *S. aureus* must invest heavily in adhesion and iron acquisition in order to successfully establish in this environment (Krismer et al., 2017). This contrasts with dry skin environments, another place that *S. aureus* can establish as a pathogen or a commensal – here *Staphylococcal* species tend to dominate, and immune evasion and the production of antimicrobial peptides and proteases become key adaptive traits (Burian et al., 2021). When it comes to sepsis and

bloodstream infections, *S. aureus* tends to reduce *agr* activity in order to evade the host immune system, however coinfecting microbes like *Candida albicans* have been observed to co-opt *S. aureus* quorum sensing and associated virulence factors (Todd et al., 2019).

Population dynamics in acute and recurrent soft tissue infections greatly emphasize the role of adhesion and establishment among competing commensals and other pathogens. In contrast, chronic or recurrent infections capitalize on *S. aureus'* ability to persist in changing environmental conditions, evade the host immune system, and outcompete coexisting species and lineages. *S. aureus* can be found causing chronic infections in respiratory systems and burn wounds alongside gram negatives like *Pseudomonas aeruginosa, Burkholderia cepacia* and *Acinitobacter baumanii* (Magalhães et al., 2022; Moriano et al., 2021; Smith et al., 2021). As an adept glucose scavenger *S. aureus* is able to competitively grow alongside coinfecting microbes at initial introduction and once established, long-term coexistence can occur (Fischer et al., 2020). Some studies have observed that in these long-term interactions *S. aureus* can serve as an important resource for other species like *P. aeruginosa* – sharing nutrients like iron and secondary metabolites like lactate (Filkins et al., 2015; Mashburn et al., 2005a). In order to maintain large populations in the presence of microbes like *P. aeruginosa* however, *S. aureus* must first make important adaptive changes that allow it to evade bacteriocins and contact-dependent secretion systems (Filkins et al., 2015; Long et al., 2020a). *S .aureus* is capable of evading direct competition from *P. aeruginosa* generally by altering its metabolism to a more fermentative state or making major changes in cellular respiration pathways (Filkins et al., 2015; Lalitha et al., 2006). In general, while *S. aureus* may be initially vulnerable to being outcompeted by other co-occurring pathogens, it is able to readily alter its metabolism and related phenotypes to persist despite the presence of formidable coinfecting pathogens. Despite lacking motility, chemotaxis, and contact dependent secretion systems, *S. aureus* is truly a sophisticated and

adaptable microbe, capable of establishing and persisting in a wide variety of environments alone or in concert with other members of a complex microbial community.

Pseudomonas aeruginosa

A useful foe

P. aeruginosa is a well-studied gram-negative opportunistic pathogen that has received much research attention as it is one of the most common and detrimental pathogens that can infect people with CF. Relatives to *P. aeruginosa* like *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens* are plant-associated pathogens and can often be isolated from soil environments. With this genetic ancestry it is unsurprising that *P. aeruginosa* is particularly adept at surviving in a wide array of environments from surfaces to catheters and even in nutrient deplete-saline eyedrops (Velcani et al., 2023). *P. aeruginosa* is also adept at colonizing host tissues and readily replacing resident microbes by eliminating them with excreted toxins, contact dependent secretion systems like its membrane piercing type VI secretion system, or simply by consuming shareable nutrients more efficiently than most other populations (Rezzoagli et al., 2020). *P. aeruginosa* is therefore a useful microbe to use for studying how other species respond and adapt to the presence and selective pressures exerted by the presence of other microbes in a shared environment. Other studies have used *P. aeruginosa* to study secretion systems in gram-negatives like *Vibrio cholerae* (MacIntyre et al., 2010). It has also been used to study adaptive responses from gram-positives that it often encounters in oral and respiratory systems like *Streptococcus parasanguinis* (Baty et al., 2022). Its impact on co-occurring specie's antimicrobial resistance profiles like those of *Acinetobacter baumannii,* has also been observed (Potron et al., 2015).

One aspect of *P. aeruginosa* that can make it a challenging microbe to study is the immense variability that exists between isolates within this species. *P. aeruginosa's* genome is quite large at about 6 mega base pairs and average nucleotide identities between strains dipping below 94% (Quiroz-Morales et al., 2022). With this amount of intraspecific diversity, it can be difficult to know if results from studies using individual lab strains can be extrapolated to the entire species and dynamics that may actually occur within a host environment. Researchers using *P. aeruginosa* as a useful foe for studying interspecific interactions and coinfection virulence must be mindful of this aspect of this species when drawing general conclusions based on their findings.

S. aureus and *P. aeruginosa* – a model system for interspecific interactions in infections

S. aureus and *P. aeruginosa* coinfections have been associated with higher rates of pulmonary exacerbations and a more rapid decrease of lung function in individuals with CF (Limoli et al., 2016). Both species are important pathogens in their own right with decades of research on their individual pathogenicity and genetics. This combined with the clinical relevance of coinfections with both species has made their interspecific interactions a popular study system. However, the factors that enable their coexistence and competition have proven to be complex. Researchers across many disciplines have worked to isolate factors and identify patterns of interaction. Some trends that have arisen including *P. aeruginosa's* ability to inhibit *S. aureus* growth by producing quorum sensing regulated molecules like 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) and pyocyanin that disrupt *S. aureus'* electron transport chain and oxidative respiration (Hoffman et al., 2006; Hotterbeekx et al., 2017; Yung et al., 2021). There is also evidence that in a host environment *S. aureus* and *P. aeruginosa* may interact commensally or even mutualistically collaborating to access key nutrients (Camus et al., 2020; Mashburn et al., 2005a). It has also been observed that clinical isolates of *S. aureus* and *P. aeruginosa* from CF

respiratory infections can interact in a variety of ways. Isolates of *S. aureus* range from highly sensitive to fairly tolerant to the lab strain of *P. aeruginosa* PAO1 (Bernardy et al., 2020). Paired isolates also range from being highly competitive with *P. aeruginosa* effectively inhibiting its co-isolated *S. aureus,* to co-isolated pairs coexisting sustainably when cocultured in the lab (Bernardy et al., 2022). What has yet to be investigated are the potential pathways of adaptation that *S. aureus* can take when it encounters *P. aeruginosa* in its environment. With such an immense amount of diversity within both species it is difficult to parse out key genetic and phenotypic traits among clinical isolates that enable commensal or competitive interactions. Using approaches like experimental evolution we can observe the process of one species adapting to the other in real time and isolate key adaptive traits.

Experimental evolution as a tool for studying complex traits

Increased accessibility of sequencing technology over the last several decades has led to a wave of studies analyzing large sample sets of clinical isolates. These studies have led to important findings and identification of patterns among colonizing and pathogenic populations. However, due to the immense diversity among bacterial species, the identification of key traits and common adaptive strategies is difficult to untangle from the diversity of genotypes and phenotypes of clinical isolates. Experimental or directed evolution offers a useful way to study what adaptations are possible for important pathogens in the context of individual selective pressures like antibiotics and co-occurring microbial populations.

One major limitation of *in vitro* experimental evolution that is important to keep in mind when designing or analyzing experimental results, is that replicating natural environments like a CF lung are generally not feasible even with current animal models. Therefore, findings from

experimental evolution must always be considered within the context of the selective pressures of the *in vitro* environment. This is generally true for most *in vitro* empirical findings. For instance, in the previously described experiment of *Vibrio cholerae* interactions with commensal *Aeromonas* in a zebrafish model, *in vitro* experiments alone may have led to the conclusion that *Vibrio cholerae* mainly uses its type VI secretion system to directly inhibit competing microbes, leaving out the important indirect competition that was observed in the model host. Similarly, it has been documented that *S. aureus* gene expression when cultured in most lab conditions is highly divergent from the genetic expression profile in the CF lung (Ibberson & Whiteley, 2019). Before we can extend findings from *in vitro* experiments including experimental evolution we can first look to species level genomics and identify whether patterns observed in the lab are found among naturally evolving isolates. Whatever the findings of this additional analysis indicate, it doesn't necessarily invalidate the importance of the empirical observations, rather it indicates that other selective forces that exist in nature make the adaptations observed in the lab highly unlikely. Conclusions like this outline a need for future studies to identify balancing selective forces that exist in natural environments. Importantly, not all evolution experiments seek to replicate evolution that happens in nature. In many cases, experimental evolution has been used to study fundamental principles of biology like the evolution of sex and the evolution of symbiotic relationships (Blount et al., 2012; Hoang et al., 2016; Morran et al., 2009; Rosenzweig et al., 1994). There is so much we can learn from observing how organisms respond to changes and selective challenges in their environment that will improve our understanding of the evolution of life and interactions between species, including pathogenesis.

Experimental evolution is a useful tool for revealing patterns in adaptation and evolution and therefore it not only has utility in researching organisms and their traits but also as a tool for teaching genetics, evolution, and experimental design. Evolution experiments are inherently exploratory and open-ended they can be carried out on short scales to study known changes in

gene expression (Cooper et al., 2019) or carried out for decades to observe evolution on extensive time-scales (Maddamsetti et al., 2015). In the classroom, where the topics of genetics and evolution can be abstract and often taught with an emphasis on memorization, evolution experiments have the potential to be used as demonstrations of exerting pressures on populations and observing responses. EvolvingSTEM is a learning module designed by microbial evolutionary biologist Dr. Vaughn Cooper and colleagues that attempts to make experimental evolution an accessible learning tool for high school and early college students (Cooper et al., 2019). They provide protocols and materials for the short-term evolution experiment with the non-pathogenic bacteria *P. fluorescens,* using plastic beads to select for increased biofilm production and providing an easy method for serial transfer evolution experiments. Other groups like the Dunham lab at the University of Washington have developed experimental evolution kits that can be conducted safely in a conventional kitchen with the safe and accessible *Saccharomyces cerevisiae* (Moresi et al., 2023). The opportunity to learn how to conduct a long-term experiment with the ability for students to ask their own questions or implement their own design, makes experimental evolution an excellent tool for teaching topics like genetics and evolution that can otherwise be difficult to teach or superficially grasped without hands-on experiments and individual ownership of a project.

Goals of this thesis

In this thesis I aim to present in-depth investigations of interspecific and intraspecific interactions of *Staphylococcus aureus* in the context of chronic CF-associated infections. These studies will be clinically relevant with their use of clinical isolates and metadata for influencing experimental design and will incorporate tenant principles of ecology and evolution.

In chapter II we investigate the question of how *S. aureus* evolves in the presence of *P. aeruginosa* outside of a host. Findings from genomic sequencing led us to investigate the role that amino acid transport plays in interspecific interactions between these two pathogens – a mechanism that was previously unknown. While this adaptation was found to be uncommon among publicly available *S. aureus* genomic data, the role of aspartate transporter, *gltT* was found to be an definitive way for *S. aureus* strain JE2 to improve its survival in the presence of *P. aeruginosa* strain PAO1. With few fitness trade-offs associated with disrupting this core gene we further investigated the role that *gltT* activity had on *S. aureus* fitness. Experimental evolution led us to this otherwise unlikely finding and illuminates previously unknown aspects of *S. aureus* adaptation in the presence of *P. aeruginosa.*

In chapter III we summarize the findings of an exploratory study that quantified the intraspecific diversity of *S. aureus* isolated from three individual fresh sputum samples from three different patients. By sampling single colonies and pooled colonies, we were able to capture clinically relevant diversity within the three sampled populations including one sample which had two numerically significant populations of MRSA and MSSA. Diving deeper into how these coexisting lineages impacted each other's antibiotic susceptibility and evolution in the presence of antibiotics, we found that despite their large phenotypic and genotypic differences, multiple lineages of *S. aureus* may be able to coexist over long periods of time, altering population level antibiotic susceptibilities and infection progression.

Overall, the aims of this thesis are to answer the following questions: 1) How does *S. aureus* evolve in the presence of *P. aeruginosa* outside of a host environment? 2) How do mutations acquired in *in vitro* evolution experiments impact strain competitive fitness *in vitro* and do those mutations occur in natural populations? 3) Can intraspecific diversity be captured from individual sputum samples with a simple sampling approach and 4) how do co-occurring lineages of *S.*

aureus influence each other's antibiotic resistance and ability to evolve further resistance. *S. aureus* in the context of cystic fibrosis associated infections brings together opportunities to ask these fundamental and clinically applicable questions in a framework of well-developed empirical and analytical tools. Findings presented in this thesis offer novel insights and invite exciting new areas of investigation in microbial ecology and evolution in the context of chronic infections.

REFERENCES

Adamowicz, E. M., Muza, M., Chacón, J. M., & Harcombe, W. R. (2020). Cross-feeding modulates the rate and mechanism of antibiotic resistance evolution in a model microbial community of Escherichia coli and Salmonella enterica. *PLOS Pathogens*, *16*(7), e1008700-. https://doi.org/10.1371/journal.ppat.1008700

Altman, D. R., Sullivan, M. J., Chacko, K. I., Divya, B., Pak, T. R., Sause, W. E., Krishan, K., Robert, S., Gintaras, D., Oliver, A., Hannah, R., Martha, L., Yi, F., Ali, B., Andrew, K., Schadt, E. E., Richardson, A. R., Torres, V. J., Bo, S., & Harm, van B. (2018). Genome Plasticity of agr-Defective Staphylococcus aureus during Clinical Infection. *Infection and Immunity*, *86*(10), 10.1128/iai.00331-18. https://doi.org/10.1128/iai.00331-18

Baty, J. J., Huffines, J. T., Stoner, S. N., & Scoffield, J. A. (2022). A Commensal Streptococcus Dysregulates the Pseudomonas aeruginosa Nitrosative Stress Response. *Frontiers in Cellular and Infection Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fcimb.2022.817336

Bernardy, E. E., Petit, R. A., Raghuram, V., Alexander, A. M., Read, T. D., & Goldberg, J. B. (2020). Genotypic and Phenotypic Diversity of Staphylococcus aureus Isolates from Cystic Fibrosis Patient Lung Infections and Their Interactions with Pseudomonas aeruginosa. *MBio*, *11*(3). https://doi.org/10.1128/mbio.00735-20

Bernardy, E. E., Vishnu, R., & Goldberg, J. B. (2022). Staphylococcus aureus and Pseudomonas aeruginosa Isolates from the Same Cystic Fibrosis Respiratory Sample Coexist in Coculture. *Microbiology Spectrum*, *10*(4), e00976-22. https://doi.org/10.1128/spectrum.00976-22

Blount, Z. D., Barrick, J. E., Davidson, C. J., & Lenski, R. E. (2012). Genomic analysis of a key innovation in an experimental Escherichia coli population. *Nature*, *489*(7417), 513–518. https://doi.org/10.1038/nature11514

Briaud, P., Camus, L., Bastien, S., Doléans-Jordheim, A., Vandenesch, F., & Moreau, K. (2019). Coexistence with Pseudomonas aeruginosa alters Staphylococcus aureus transcriptome, antibiotic resistance and internalization into epithelial cells. *Scientific Reports*, *9*(1), 16564. https://doi.org/10.1038/s41598-019-52975-z

Burian, M., Plange, J., Schmitt, L., Kaschke, A., Marquardt, Y., Huth, L., Baron, J. M., Hornef, M. W., Wolz, C., & Yazdi, A. S. (2021). Adaptation of Staphylococcus aureus to the Human Skin Environment Identified Using an ex vivo Tissue Model. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.728989

Camus, L., Briaud, P., Bastien, S., Elsen, S., Doléans-Jordheim, A., Vandenesch, F., & Moreau, K. (2020). Trophic cooperation promotes bacterial survival of Staphylococcus aureus and Pseudomonas aeruginosa. *The ISME Journal*, *14*(12), 3093–3105. https://doi.org/10.1038/s41396-020-00741-9

Darwin, C. (1859). *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life* (1st ed.). Murray.

Cooper, V. S., Warren, T. M., Matela, A. M., Handwork, M., & Scarponi, S. (2019). EvolvingSTEM: a microbial evolution-in-action curriculum that enhances learning of

evolutionary biology and biotechnology. *Evolution: Education and Outreach*, *12*(1), 12. https://doi.org/10.1186/s12052-019-0103-4

Cystic Fibrosis Foundation. (2021). *Cystic Fibrosis Foundation patient registry 2021 annual data report*.

Desai, M. M., & Fisher, D. S. (2007). Beneficial Mutation–Selection Balance and the Effect of Linkage on Positive Selection. *Genetics*, *176*(3), 1759–1798. https://doi.org/10.1534/genetics.106.067678

Falkow, S. (1988). Molecular Koch's Postulates Applied to Microbial Pathogenicity. *Reviews of Infectious Diseases*, *10*, S274–S276. www.jstor.org/stable/4454582

Falkow, S. (1997). What is a pathogen? *ASM News*, *63*, 359–365.

Fey, P. D., Endres, J. L., Yajjala, V. K., Widhelm, T. J., Boissy, R. J., Bose, J. L., & Bayles, K. W. (2013). A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. *MBio*, *4*(1), e00537-12-e00537. https://doi.org/10.1128/mbio.00537-12

Filkins, L. M., Graber, J. A., Olson, D. G., Dolben, E. L., Lynd, L. R., Bhuju, S., & O'Toole, G. A. (2015). Coculture of Staphylococcus aureus with Pseudomonas aeruginosa Drives S. aureus towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *Journal of Bacteriology*, *197*(14), 2252–2264. https://doi.org/10.1128/jb.00059-15

Fischer, A. J., Singh, S. B., LaMarche, M. M., Maakestad, L. J., Kienenberger, Z. E., Peña, T. A., Stoltz, D. A., & Limoli, D. H. (2020). Sustained Coinfections with Staphylococcus aureus and Pseudomonas aeruginosa in Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *203*(3), 328–338. https://doi.org/10.1164/rccm.202004-1322OC

Fort, H. (2020). From growth equations for a single species to Lotka–Volterra equations for two interacting species. In *Ecological Modelling and Ecophysics* (pp. 1–1 to 1–41). IOP Publishing. https://doi.org/10.1088/978-0-7503-2432-8ch1

Foster, T. (1996). Staphylococcus. In S. Baron (Ed.), *Medical Microbiology* (4th Edition).

Giulia, O., & O'Toole, G. A. (2017). Pseudomonas aeruginosa Alters Staphylococcus aureus Sensitivity to Vancomycin in a Biofilm Model of Cystic Fibrosis Infection. *MBio*, *8*(4), 10.1128/mbio.00873-17. https://doi.org/10.1128/mbio.00873-17

Halsey, C., Shulei, L., K, W. J., K, L. M., S, N. A., Laurey, S., Marat, S., Robert, P., & D, F. P. (2017). Amino Acid Catabolism in Staphylococcus aureus and the Function of Carbon Catabolite Repression. *MBio*, *8*(1), e01434-16. https://doi.org/10.1128/mBio.01434-16

Harris, J. B., LaRocque, R. C., Qadri, F., Ryan, E. T., & Calderwood, S. B. (2012). Cholera. *The Lancet*, *379*(9835), 2466–2476. https://doi.org/10.1016/S0140-6736(12)60436-X

Harrison, F., Paul, J., Massey, R. C., & Buckling, A. (2008). Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. *The ISME Journal*, *2*(1), 49–55. https://doi.org/10.1038/ismej.2007.96

Ho, B. T., Dong, T. G., & Mekalanos, J. J. (2014). A View to a Kill: The Bacterial Type VI Secretion System. *Cell Host & Microbe*, *15*(1), 9–21. https://doi.org/10.1016/j.chom.2013.11.008

Hoang, K. L., Morran, L. T., & Gerardo, N. M. (2016). Experimental Evolution as an Underutilized Tool for Studying Beneficial Animal–Microbe Interactions. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.01444

Hoffman, L. R., Déziel, E., D'Argenio, D. A., Lépine, F., Emerson, J., McNamara, S., Gibson, R. L., Ramsey, B. W., & Miller, S. I. (2006). Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences*, *103*(52), 19890–19895. https://doi.org/10.1073/pnas.0606756104

Hotterbeekx, A., Kumar-Singh, S., Goossens, H., & Malhotra-Kumar, S. (2017). In vivo and In vitro Interactions between Pseudomonas aeruginosa and Staphylococcus spp. *Frontiers in Cellular and Infection Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fcimb.2017.00106

Howden, B. P., Giulieri, S. G., Wong Fok Lung, T., Baines, S. L., Sharkey, L. K., Lee, J. Y. H., Hachani, A., Monk, I. R., & Stinear, T. P. (2023). Staphylococcus aureus host interactions and adaptation. *Nature Reviews Microbiology*, *21*(6), 380–395. https://doi.org/10.1038/s41579-023-00852-y

Ibberson, C. B., & Whiteley, M. (2019). The Staphylococcus aureus Transcriptome during Cystic Fibrosis Lung Infection. *MBio*, *10*(6), 10.1128/mbio.02774-19. https://doi.org/10.1128/mbio.02774-19

Jens, K., Antje, M., Jens, N., Davenport, C. F., Frauke, S., Larbig, K. D., Stephan, H., Ulrike, S., Pohl, T. M., Lutz, W., & Burkhard, T. (2010). Genome Diversity of Pseudomonas aeruginosa PAO1 Laboratory Strains. *Journal of Bacteriology*, *192*(4), 1113–1121. https://doi.org/10.1128/jb.01515-09

Kahl, B. C. (2014). Small colony variants (SCVs) of Staphylococcus aureus – A bacterial survival strategy. *Infection, Genetics and Evolution*, *21*, 515–522. https://doi.org/https://doi.org/10.1016/j.meegid.2013.05.016

Karine, D., Podskalniy, V. A., Herfst, C. A., Lovell, G. F. M., Lee, I. S., DeJong, E. N., McCormick, J. K., & Tuffs, S. W. (2022). Glucose Mediates Niche-Specific Repression of Staphylococcus aureus Toxic Shock Syndrome Toxin-1 through the Activity of CcpA in the Vaginal Environment. *Journal of Bacteriology*, *204*(10), e00269-22. https://doi.org/10.1128/jb.00269-22

Koch, R. (1884). Die Aetiologie der Tuberculose. *Mitt. Kaiser. Gesundh*.

Kreiswirth, B. N., Löfdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature*, *305*(5936), 709–712. https://doi.org/10.1038/305709a0

Krismer, B., Weidenmaier, C., Zipperer, A., & Peschel, A. (2017). The commensal lifestyle of Staphylococcus aureus and its interactions with the nasal microbiota. *Nature Reviews Microbiology*, *15*(11), 675–687. https://doi.org/10.1038/nrmicro.2017.104

Kwiecinski, J. M., & Horswill, A. R. (2020). Staphylococcus aureus bloodstream infections: pathogenesis and regulatory mechanisms. *Current Opinion in Microbiology*, *53*, 51–60. https://doi.org/https://doi.org/10.1016/j.mib.2020.02.005

Lalitha, V., Steffen, S., Raja, B., Ralf, R., Christian, R., & Friedrich, G. (2006). Microevolution of Cytochrome bd Oxidase in Staphylococci and Its Implication in Resistance to Respiratory Toxins Released by Pseudomonas. *Journal of Bacteriology*, *188*(23), 8079–8086. https://doi.org/10.1128/jb.00858-06

Limoli, D. H., Warren, E. A., Yarrington, K. D., Donegan, N. P., Cheung, A. L., & O'Toole, G. A. (2019). Interspecies interactions induce exploratory motility in Pseudomonas aeruginosa. *ELife*, *8*. https://doi.org/10.7554/elife.47365

Limoli, D. H., Yang, J., Khansaheb, M. K., Helfman, B., Peng, L., Stecenko, A. A., & Goldberg, J. B. (2016). Staphylococcus aureus and Pseudomonas aeruginosa co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. *European Journal of Clinical Microbiology & Infectious Diseases*, *35*(6), 947–953. https://doi.org/10.1007/s10096-016-2621-0

Logan, S. L., Thomas, J., Yan, J., Baker, R. P., Shields, D. S., Xavier, J. B., Hammer, B. K., & Parthasarathy, R. (2018). The Vibrio cholerae type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. *Proceedings of the National Academy of Sciences*, *115*(16), E3779–E3787. https://doi.org/10.1073/pnas.1720133115

Long, D. R., Wolter, D. J., Lee, M., Precit, M., McLean, K., Holmes, E., Penewit, K., Waalkes, A., Hoffman, L. R., & Salipante, S. J. (2020). Polyclonality, Shared Strains, and Convergent Evolution in Chronic Cystic Fibrosis Staphylococcus aureus Airway Infection. *American Journal of Respiratory and Critical Care Medicine*, *203*(9), 1127–1137. https://doi.org/10.1164/rccm.202003-0735OC

Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2002). Lung Infections Associated with Cystic Fibrosis. *Clinical Microbiology Reviews*, *15*(2), 194–222. https://doi.org/10.1128/cmr.15.2.194-222.2002

MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The Vibrio cholerae type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences*, *107*(45), 19520–19524. https://doi.org/10.1073/pnas.1012931107

Maddamsetti, R., Lenski, R. E., & Barrick, J. E. (2015). Adaptation, Clonal Interference, and Frequency-Dependent Interactions in a Long-Term Evolution Experiment with Escherichia coli. *Genetics*, *200*(2), 619–631. https://doi.org/10.1534/genetics.115.176677

Magalhães, A. P., França, A., Pereira, M. O., & Cerca, N. (2022). Unveiling Co-Infection in Cystic Fibrosis Airways: Transcriptomic Analysis of Pseudomonas aeruginosa and Staphylococcus aureus Dual-Species Biofilms. *Frontiers in Genetics*, *13*. https://www.frontiersin.org/articles/10.3389/fgene.2022.883199

Mashburn, L. M., Jett, A. M., Akins, D. R., & Whiteley, M. (2005). Staphylococcus aureus Serves as an Iron Source for Pseudomonas aeruginosa during In Vivo Coculture. *Journal of Bacteriology*, *187*(2), 554–566. https://doi.org/10.1128/jb.187.2.554-566.2005

Méthot, P.-O., & Alizon, S. (2014). What is a pathogen? Toward a process view of host-parasite interactions. *Virulence*, *5*(8), 775–785. https://doi.org/10.4161/21505594.2014.960726

Monk, I., & Foster, T. (2012). Genetic manipulation of Staphylococci – Breaking through the barrier. *Frontiers in Cellular and Infection Microbiology*, *2*. https://www.frontiersin.org/articles/10.3389/fcimb.2012.00049

Moresi, N. G., Geck, R. C., Skophammer, R., Godin, D., Students, yEvo, Taylor, M. B., & Dunham, M. J. (2023). Caffeine-tolerant mutations selected through an at-home yeast experimental evolution teaching lab. *MicroPublication Biology*, *2023*. https://doi.org/10.17912/micropub.biology.000749

Moriano, A., Serra, D. O., Hoard, A., Montaña, S., Degrossi, J., Bonomo, R. A., Papp-Wallace, K. M., & Ramirez, M. S. (2021). Staphylococcus aureus Potentiates the Hemolytic Activity of Burkholderia cepacia Complex (Bcc) Bacteria. *Current Microbiology*, *78*(5), 1864–1870. https://doi.org/10.1007/s00284-021-02458-0

Morran, L. T., Parmenter, M. D., & Phillips, P. C. (2009). Mutation load and rapid adaptation favour outcrossing over self-fertilization. *Nature*, *462*(7271), 350–352. https://doi.org/10.1038/nature08496

Niggli, S., Schwyter, L., Poveda, L., Grossmann, J., & Kümmerli, R. (2022). Rapid and strain-specific resistance evolution of Staphylococcus aureus against inhibitory molecules secreted by Pseudomonas aeruginosa *BioRxiv*, 2021.08.10.455797. https://doi.org/10.1101/2021.08.10.455797

Ogston, A. (1882). Micrococcus Poisoning. *Journal of Anatomy and Physiology*, *17*(Pt 1), 24–58.

Ong, T., & Ramsey, B. W. (2023). Cystic Fibrosis: A Review. *JAMA*, *329*(21), 1859–1871. https://doi.org/10.1001/jama.2023.8120

Pasteur, L. (1857). Mémoire sur la fermentation appelée lactique (Extrait par l'auteur). *Molecular Medicine*, *1*(6), 599.

Potron, A., Poirel, L., & Nordmann, P. (2015). Emerging broad-spectrum resistance in Pseudomonas aeruginosa and Acinetobacter baumannii: Mechanisms and epidemiology. *International Journal of Antimicrobial Agents*, *45*(6), 568–585. https://doi.org/https://doi.org/10.1016/j.ijantimicag.2015.03.001

Raghuram, V., M, A. A., Qi, L. H., A, P. R., B, G. J., & D, R. T. (2022). Species-Wide Phylogenomics of the Staphylococcus aureus Agr Operon Revealed Convergent Evolution of Frameshift Mutations. *Microbiology Spectrum*, *10*(1), e01334-21. https://doi.org/10.1128/spectrum.01334-21

Reid, D. W., Anderson, G. J., & Lamont, I. L. (2009). Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *297*(5), L795–L802. https://doi.org/10.1152/ajplung.00132.2009

Rezzoagli, C., Granato, E. T., & Kümmerli, R. (2020). Harnessing bacterial interactions to manage infections: a review on the opportunistic pathogen Pseudomonas aeruginosa as a case example. *Journal of Medical Microbiology*, *69*(2), 147–161. https://doi.org/https://doi.org/10.1099/jmm.0.001134

Rosenbach, A. (1884). Mikro-Qrganismen bei den Wund-Infections-Krankheiten des Menschen. *Wiesbaden: J.F. Bergmann*, 18.

Rosenzweig, R. F., Sharp, R. R., Treves, D. S., & Adams, J. (1994). Microbial evolution in a simple unstructured environment: genetic differentiation in Escherichia coli. *Genetics*, *137*(4), 903–917. https://doi.org/10.1093/genetics/137.4.903

Smith, N. M., Alexa, A., Fanny, T., Katelyn, M., Sarah, J., Jasleen, S., & Lenhard, J. R. (2021). Interaction of Staphylococcus aureus and Acinetobacter baumannii during In Vitro β-Lactam Exposure. *Antimicrobial Agents and Chemotherapy*, *65*(4), 10.1128/aac.02414-20. https://doi.org/10.1128/aac.02414-20

Tan, L., Li, S. R., Jiang, B., Hu, X. M., & Li, S. (2018). Therapeutic Targeting of the Staphylococcus aureus Accessory Gene Regulator (agr) System. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/articles/10.3389/fmicb.2018.00055

Todd, O. A., Fidel, P. L., Harro, J. M., Hilliard, J. J., Tkaczyk, C., Sellman, B. R., Noverr, M. C., & Peters, B. M. (2019). Candida albicans Augments Staphylococcus aureus Virulence by Engaging the Staphylococcal agr Quorum Sensing System. *MBio*, *10*(3), e00910-19. https://doi.org/10.1128/mBio.00910-19

Veit, G., Avramescu, R. G., Chiang, A. N., Houck, S. A., Cai, Z., Peters, K. W., Hong, J. S., Pollard, H. B., Guggino, W. B., Balch, W. E., Skach, W. R., Cutting, G. R., Frizzell, R. A., Sheppard, D. N., Cyr, D. M., Sorscher, E. J., Brodsky, J. L., & Lukacs, G. L. (2016). From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Molecular Biology of the Cell*, *27*(3), 424–433. https://doi.org/10.1091/mbc.e14-04-0935

Velcani, F., Kuo, I. C., Shanks, R. M. Q., Chodosh, J., Garg, P., Amescua, G., & Zegans, M. E. (2023). Association of Artificial Tears with Ocular and Systemic Infection: Carbapenem-resistant Pseudomonas aeruginosa (VIM-GES-CRPA) Outbreak. *Ophthalmology*. https://doi.org/10.1016/j.ophtha.2023.06.003

Walker, L., LeVine, H., & Jucker, M. (2006). Koch's postulates and infectious proteins. *Acta Neuropathologica*, *112*(1), 1. https://doi.org/10.1007/s00401-006-0072-x

Yung, D. B. Y., Sircombe, K. J., & Pletzer, D. (2021). Friends or enemies? The complicated relationship between Pseudomonas aeruginosa and Staphylococcus aureus. *Molecular Microbiology*, *116*(1), 1–15. https://doi.org/https://doi.org/10.1111/mmi.14699

Chapter II - **Experimentally Evolved *Staphylococcus aureus* Survives in the Presence of *Pseudomonas aeruginosa* by Acquiring Mutations in the Amino Acid Transporter, GltT**

Ashley M. Alexander [a,c,d], Justin M. Luu [b,d], Vishnu Raghuram [b,c,d], Giulia Bottacin [e], Simon van Vliet [e,f], Timothy D. Read [c], Joanna B. Goldberg [d]

[a] Population Biology, Ecology, and Evolution Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia, USA

[b] Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia, USA

[c] Division of Infectious Diseases and Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USA

[d] Department of Pediatrics, Division of Pulmonology, Asthma, Cystic Fibrosis, and Sleep, Emory University School of Medicine, Atlanta, Georgia, USA

[e] Biozentrum, University of Basel, Spitalstrasse 41,4056 Basel, Switzerland

[f] Department of Fundamental Microbiology, University of Lausanne, Quartier Unil-Sorge, 1015 Lausanne, Switzerland

Author contributions

AMA - conceived of and designed experimental evolution and oversaw or conducted the collection of all empirical data collection, analysis, methodology, data visualization, as well as writing and editing.

JML – conducted all murine model experiments, wrote the methodology for these experiments and designed the data visualization for **Fig 6**

VR – wrote the program LIVID and used it to screen *S. aureus* genomes for *gltT*, they also analyzed this data and designed the summary of it in **sup table 2**.

GB – designed and conducted microscopy and collected and analyzed the corresponding data used in **Fig 4**

SvV – wrote the code used to analyze microscopy data, conceived of data visualization in **Fig 4**, supervised GB and assisted with writing and editing

TDR – supervised AMA and VR and assisted with study design, analysis, writing and editing

JBG – supervised AMA, JML and VR and assisted with study design, funding analysis, writing and editing

Abstract

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common bacterial pathogens isolated from cystic fibrosis (CF) related lung infections. When both of these opportunistic pathogens are found in a coinfection, CF patients tend to have higher rates of pulmonary exacerbations and experience a more rapid decrease in lung function. When cultured together under standard laboratory conditions, it is often observed that *P. aeruginosa* effectively inhibits *S. aureus* growth. Previous work from our group revealed that *S. aureus* from CF infections have isolate-specific survival capabilities when cocultured with *P. aeruginosa*. In this study, we designed a serial transfer evolution experiment to identify mutations that allow *S. aureus* to adapt to the presence of *P. aeruginosa*. Using *S. aureus* USA300 JE2 as our ancestral strain, populations of *S. aureus* were repeatedly cocultured with fresh *P. aeruginosa* strain, PAO1. After 8 coculture periods, *S. aureus* populations that survived better in the presence of PAO1 were observed. We found two independent mutations in the highly conserved *S. aureus* aspartate transporter, *gltT*, that were unique to evolved *P. aeruginosa*-tolerant isolates. Subsequent phenotypic testing demonstrated that *gltT* mutants have reduced uptake of glutamate and outcompete wild-type *S. aureus* when glutamate is absent from chemically defined media. These findings together demonstrate that the presence of *P. aeruginosa* exerts selective pressure on *S. aureus* to alter its uptake and metabolism of key amino acids when the two bacteria are cultured together.

Importance

Staphylococcus aureus and *Pseudomonas aeruginosa* are the two most common bacterial pathogens that infect people with the genetic disease, cystic fibrosis (CF). They are often found together in CF-associated polymicrobial infections that are associated with worse patient prognosis. Understanding how these very different opportunistic pathogens influence each other in a shared environment is pertinent to improving the treatment of polymicrobial infections.

While much attention has been brought to the interspecific interactions between *S. aureus* and *P. aeruginosa,* few studies have used experimental evolution methods to identify determinants of their competition and coexistence. Here, we use a serial transfer experimental evolution approach and identified a single genetic change associated with improved survival of *S. aureus* in the presence of *P. aeruginosa.* Our findings implicate metabolism of shared resources as an important factor in *S. aureus's* ability to survive in the presence of *P. aeruginosa*.

Introduction

Often, a community of microbes contributes to the shaping of the infection environment through metabolic interactions, altering adaptive trajectories, or changing the antibiotic susceptibilities of interacting strains (Brown et al., 2008, 2012; Dalton et al., 2011; Stacy et al., 2016; Wadsworth et al., 2018). In the case of chronic infections, multiple opportunistic pathogens can coexist in their shared host environment for many generations, exerting their own respective selective pressures on each other (Hamelin et al., 2019). Pairwise interactions between coexisting opportunistic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* have become a topic of great interest to microbiologists both for the importance of the interaction to the course of the genetic disease cystic fibrosis (CF) and as model system for pathogen coevolution (Barraza & Whiteley, 2021; Camus et al., 2020).

In 2021, 32,100 people were documented as living with CF in the United States, a genetic disease that impacts multiple organ systems and greatly reduces life-expectancy and requires lifelong treatment (Cystic Fibrosis Foundation, 2021). One of the major complications of this disease is an increased risk for developing chronic respiratory infections that are exacerbated by the buildup of respiratory sputum. Over the last decade, *S. aureus* has displaced *P. aeruginosa* as the most common infective agent responsible for respiratory infections in people

with CF and is detected in as many as 70% of CF associated lung infections. *S. aureus* is the predominant pathogen infecting young people with CF; older patients are more likely to be infected with *P. aeruginosa* and many individuals maintain both pathogens in coinfections (Cystic Fibrosis Foundation, 2021). Coinfections with *P. aeruginosa* and *S. aureus* may persist in the same patient for many years and even decades (Bernardy et al., 2020; Camus et al., 2020; Fischer et al., 2020). Chart reviews of more than 200 patients have revealed that coinfected patients experience significantly more pulmonary exacerbations and a more rapid decline in lung function compared to those with mono-infections of *S. aureus* or *P. aeruginosa* (Limoli et al., 2016).

When *P. aeruginosa* and *S. aureus* are cultured together outside of a host, there are a range of outcomes that may be dependent on strain identity or environmental conditions (Bernardy et al., 2022; Filkins et al., 2015; Limoli et al., 2017; Mashburn et al., 2005). Previously, our group observed that *S. aureus* isolated from CF patients range from highly sensitive to tolerant in their ability to coexist with the lab-adapted strain of *P. aeruginosa,* PAO1. Sensitive clinical isolates experienced as much as a 6-fold decrease in recovered colony-forming units (CFUs) after coculture, while others maintained most of their population, experiencing less than a 2-fold decrease in population size (Bernardy et al., 2020). We have also observed that *S. aureus* is able to adapt to the presence of *P. aeruginosa* in its environment by showing that co-isolated *S. aureus* and *P. aeruginosa* strains grow better compared to non-concurrent isolates (Bernardy et al., 2022). Additionally, other groups have found that fermentative metabolism, polysaccharide production, and toxin excretion are all important phenotypes for *S. aureus* in its coexistence with *P. aeruginosa* (Filkins et al., 2015; Wieneke et al., 2021). It is also known that *S. aureus* can adapt to *P. aeruginosa* bactericidal compounds such as 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and pyocyanin and that such adaptations may impact antibiotic resistance profiles of

either species (Filkins et al., 2015; Limoli et al., 2016; Nguyen et al., 2014; Orazi & O'Toole, 2017).

In this study we sought to gain a greater understanding for how *S. aureus* adapts to the presence of *P. aeruginosa* and which *S. aureus* genotypes/phenotypes are under strong selective pressure in their shared environment. We showed that *S. aureus* adapted to the negative selective pressures presented by *P. aeruginosa* in a serial transfer evolution experiment. We found that rather than adapting to secreted toxins or contact dependent killing, *S. aureus* reduced its uptake of aspartate by disrupting its singular aspartate transporter, *gltT* (Potter et al., 2020). We hypothesize that loss of function of this membrane transporter results in *S. aureus* becoming more resilient to fluctuations in nutrient availability caused by the presence of *P. aeruginosa* in its environment. These results are surprising given that *P. aeruginosa* has other well-characterized mechanisms for directly inhibiting *S. aureus* in its environment, however, our findings suggest that optimizing amino acid metabolism is a potential pathway for adaptation for *S. aureus* that co-occurs with *P. aeruginosa.*

Methods

Bacterial Strains

Staphylococcus aureus USA 300 strain JE2 (Kennedy et al., 2008, 2010) was used as the ancestral strain for experimental evolution with *Pseudomonas aeruginosa* strain PAO1. Using the Nebraska Transposon Mutant Library (NTML) (Fey et al., 2013b)an isogenic JE2 *gltT* mutant (*SAUSA300_2329)* was obtained and transduced into our own JE2 background. To do this we amplified the *gltT* locus with the transposon from *SAUSA300_2329*. The *gltT* gene with the inserted transposon was then confirmed and amplified by PCR and then transduced into our parental JE2 strain using phage φ11 (Iandolo et al., 2002) to generate strain JE2 *gltT*::Tn, as described below.

In brief, transduction was carried out by first preparing fresh φ11 lysate first with *S. aureus* strain RN4220. Collected lysate was then inoculated with NTML isolate *SAUSA300_2329* and titers were measured at $3x10^8$ pfu/mL. Transduction was then carried out with a multiplicity of infection of 0.1 according to methods in Krausz & Bose (2016). Transduced colonies were isolated on Trypticase Soy Agar (TSA) plates with 25µg/mL erythromycin and confirmed by PCR, identifying the presence of the complete transposon at the correct site on the chromosome. Primer sequences used to confirm transposon by amplicon size – 5' AAAATTAGCCTACCTATGCAAGTTGT 3' and 5' TTTTGCTTTGTCATATACGTTTTCC 3'. We also used transposon specific primers to amplify from within the transposon and the gene itself using primers (negative strand) 5' GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC 3' and (positive strand) 5' CTCGATTCTATTAACAAGGG 3', as described by Fey et al. (2013).

Fluorescently labeled strains were generated by transforming multicopy plasmids obtained from Dr. Marvin Whiteley's lab (Georgia Institute of Technology), pCM29 (Pang et al., 2010) and pHC48 (Ibberson et al., 2016) into both JE2 and JE2 *gltT*::Tn via electroporation (Grosser & Richardson, 2016). This gave us the fluorescently labeled set of JE2.GFP, JE2.DsRed and JE2 *gltT*::Tn.GFP and JE2 *gltT*::Tn.DsRed (**Table 1**)**.**

Strain	Description	Plasmid	*gltT* Genotype	Reference
JE2	USA 300, background for NTML	None	wild type *gltT*	Fey et. al 2013
PAO1	*P. aeruginosa* strain			
EV2	Evolved JE2, *P. aeruginosa* tolerant isolate from EE1	None	1150 4bp deletion	This study
EV3	Evolved JE2, *P. aeruginosa* tolerant isolate from EE1	None	951 G -> A early stop	This study
JE2 *gltT*::Tn	NTML transposon mutant NE566 transduced into JE2 background	None	*gltT*::Tn	Fey et. al 2013
JE2 *gltT*::Tn (pgltT)	JE2 *gltT* transposon mutant complemented with pgltT construct	pgltT	complemented *gltT*	This study
EV2 (pgltT)	Evolved isolate EV2 complemented with pgltT construct	pgltT	complemented *gltT*	This study
JE2.GFP	JE2 fluorescently labeled with GFP	pCM29	wild type *gltT*	Pang et al., 2010
JE2.DsRed	JE2 fluorescently labeled with RFP	pHC48	wild type *gltT*	Ibberson et al., 2016
JE2 *gltT*::Tn. GFP	JE2 *gltT*::Tn fluorescently labeled with GFP	pCM29	*gltT*::Tn	Pang et al., 2010
JE2 *gltT*::Tn.DsRed	JE2 *gltT*::Tn fluorescently labeled with RFP	pHC48	*gltT*::Tn	Ibberson et al., 2016

Table 1. List of *Staphylococcus aureus* strains used in this study. Plasmid pgltT was made as part of this study from the pOS1.plgT vector using methods described in Potter et al. (2020).

Media

Cocultures for evolution experiments and phenotyping were conducted on TSA. After each coculture period each species was isolated on their respective isolation agar, Pseudomonas Isolation agar (PIA; BD Difco) and Staphylococcus isolation agar (SIA; BD Difco TSA with 7.5% NaCl). For liquid cultures, bacteria were cultured in lysogeny broth (LB; Teknova) which was supplemented with erythromycin (25 µg/mL) to select for transposon mutants and/or chloramphenicol (10 µg/mL) to maintain fluorescent plasmids. Chemically defined media with glucose (CDMG) was made according to Hussain et al. (1991), with varying levels of aspartate (1.1 mM or 2.2 mM) and glutamate (1.0 mM or 2.0 mM), as needed. CDMG batches were always used within 5 days and stored at room temperature, in the dark. Depleted Trypticase Soy Broth (TSB) medium, used for single-cell microscopy, was prepared by diluting an overnight culture of PAO1 1:100 into 10 mL TSB and growing the culture for either 3 or 16 hours before filter sterilizing (0.2 µm filter, Sarstedt) to remove cells from the supernatant.

Experimental evolution

Before being cultured with *P. aeruginosa* PAO1, 4 single colony isolates of JE2 were picked and grown overnight in 3 mL of LB media in a test tube at 37°C in a rolling incubator. 10 µl of an overnight culture was then inoculated on 0.45 µm filters (MF-Millipore® Membrane Filter) on TSA and cultured at 37°C for 24 hours. Each filter was collected, and adhering cells were resuspended in 1.5 mL of LB media by vortexing for 30 seconds. To prepare *P. aeruginosa* for coculturing, a single colony of PAO1 was incubated in 3 mL of LB media overnight at 37°C in a rolling incubator. The optical density 600nm (OD_{600}) of the resuspended *S. aureus* as well as the overnight PAO1 culture was measured. Cultures were normalized to the same OD by diluting the PAO1 overnight culture in LB before inoculating a coculture at a ratio of 30:1 (*S. aureus:P. aeruginosa).* A 30:1 inoculum ratio was determined through preliminary experiments to exert optimal amount selective pressure on *S. aureus* at without risking a population level extinction

(approximately a 4-fold decrease in population size after coculture). 10 µl of coculture mixture was inoculated onto 0.45 µm filters on TSA plates and incubated at 37°C for 48 hours. To account for any adaptation to culture conditions, control populations of JE2 were passaged alongside experimental populations under identical conditions, but never cocultured with *P. aeruginosa.*

Each subsequent coculture was carried out by recovering filters and vortexing them in 1.5 mL of LB media for 30 seconds to collect adhering cells. Resuspended cell mixtures were serially diluted and plated for CFUs on SIA and PIA. 50 µl of resuspended coculture was also plated on SIA to be used to inoculate the next coculture (**Figure 1A**). After at least 24 hours of incubation at 37°C, isolated *S. aureus* was then collected off SIA plates with an inoculation loop and resuspended in LB media. This suspension was used to inoculate the next coculture period as well as to create a glycerol stock of the recovered population of *S. aureus*. PAO1 liquid cultures were incubated overnight at 37°C and then measured at OD_{600} and diluted to the same OD_{600} of the resuspended culture of *S. aureus* before being mixed at a ratio of 30:1. Inoculum densities fluctuated throughout the experiment based on the amount of *S. aureus* that could be recovered from the previous coculture period. Total inoculum densities ranged between 10^8-10^{10} CFUs. We designed the experiment with an initial large population of *S. aureus* (10^8-10^{10} CFUs) to mimic the event of *P. aeruginosa* invading an established *S. aureus* population as is often the case in CF-associated respiratory infections (Cystic Fibrosis Foundation, 2021).

Whole genome sequencing

Single colonies were isolated from evolved *S. aureus* populations and control populations and were used to inoculate overnight cultures and subsequent glycerol freezer stocks. Isolates were later struck out on SIA plates and incubated overnight at 37°C. Cells were collected off plates with an inoculation loop the next day and resuspended in 480 µl of EDTA. *S. aureus* cells were

then lysed by adding 20 µl of 10 mg/mL lysozyme and 20 µl of 5 mg/mL lysostaphin to the resuspended cell mixture. This mixture was then incubated at 37°C for one hour before proceeding with the rest of the protocol outlined for the Promega Wizard genomic DNA purification kit (Silberstein et al., 2018). Genomic DNA was sequenced using the Illumina NextSeq 2000 platform at the Microbial Genome Sequencing Center (Pittsburgh, PA). Whole genome sequences were evaluated for quality using the program FASTQC (Wingett & Andrews, 2018) and adapter sequences were removed using Trimmomatic (Bolger et al., 2014). Sequences were then screened for variants using Snippy with JE2 NCBI NC_007793.1 sequence as a reference (Seeman, 2015).

Complementation of *gltT*

The *gltT* gene was cloned using the multicopy pOS1 shuttle vector with the constitutive plgT promoter (Bubeck Wardenburg et al., 2006; Schneewind et al., 1992). The ancestral gene was amplified from wild-type JE2 using primers:

Grosser & Richardson, 2016)5'- AGAGCTCGAGATGGCTCTATTCAAGAG-3' and 5'-AGATGGATCCTTAAATTGATTTTAAATATTCTTGAC-3' and cloned downstream of the plgT promoter, as described in Potter et al. (2020). The resulting construct was confirmed by whole plasmid sequencing through Plasmidsaurus (Eugene, OR) and will be referred to as pgltT here (**Supplementary Figure 1**). The confirmed pgltT construct was transformed through electroporation into JE2 *gltT*::Tn, as well as the evolved isolate, EV2. All plasmids were transformed using electroporation (Grosser & Richardson, 2016)

Analysis of variation of the *S. aureus gltT* gene

To detect the variation of the *gltT* gene, we assessed a dataset of *S. aureus* genomes that combined 380 assemblies from the Staphopia non-redundant diversity set (Petit III & Read,

2018) and 64 CF isolates (Bernardy et al., 2020) to create a dataset of 444 *S. aureus* genome assemblies. Then, we extracted and calculated the number of mutations in the genes *gltT, gltS, alsT, rpoD,* and *agrC,* using a custom software, LIVID (https://github.com/VishnuRaghuram94/LIVID), which performs *in-silico* PCR to extract nucleotide regions of interest from genome assemblies and compares the extracted sequence with a user-specified reference region to report mutations. For *gltT, gltS, alsT,* and *rpoD*, we used the corresponding gene sequences from the *S. aureus* strain JE2 (NCBI RefSeq accession GCF_002993865.1) as a reference. To account for different *agr* groups requiring a different reference sequence, we used the software tool AgrVATE (Raghuram et al., 2022) to calculate the number of mutations in *agrC*. Both LIVID and AgrVATE use Snippy v4.6 for identifying mutations (Seeman, 2015). LIVID was run with the parameters -x 1000 (minimum product size) -y 2000 (maximum product size) -d 5 (maximum allowed primer mismatch bases). AgrVATE was run with default parameters, as described in Raghuram et al. (2022) (**Supplementary Table 1**). Mutations labelled as 'synonymous' were single or multi-nucleotide substitutions that did not affect the amino acid sequence. Mutations labelled as 'AA-sequence altering' were single /multi-nucleotide substitutions in-frame insertions/deletions that cause local changes in the amino acid sequence. Putative 'Loss of function' variants include frameshift mutations, start-codon variants and early stops caused by non-synonymous mutations (**Supplementary Table 1**).

Phenotypic testing for *P. aeruginosa* tolerance

S. aureus tolerance to *P. aeruginosa* strain PAO1, was determined by coculturing *S. aureus* and PAO1 at high initial densities (>10^8 CFUs) at a 1:1 ratio for 24 hours and measuring recovered CFUs by serially diluting resuspended cultures and plating on SIA and PIA medias to select for *S. aureus* and *P. aeruginosa,* respectively (Bernardy et al., 2020). Phenotyping assays were carried out in 5 separate experiments with 2 biological replicates per strain.

Murine acute pneumonia model

The impact of *gltT* activity on *S. aureus* colonization was determined in a murine acute pneumonia model. All animal procedures were conducted in accordance with the guidelines of the Emory University Institutional Animal Care and Use Committee (IACUC), under approved protocol number PROTO201700441. 8 to 10-week-old C57BL/6 female mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with a 0.2 mL mixture of ketamine (6.7 mg/mL) and xylazine (1.3 mg/mL) administered through intraperitoneal injection. All mice were euthanized 24 hours post-infection.

S. aureus strains JE2 and JE2 *gltT*::Tn were grown on SIA for 18 to 24 hours at 37°C and resuspended in phosphate buffered saline (PBS) to an OD_{600} of 8, corresponding to ~2 x 10^9 CFU/mL. Anesthetized mice were infected with 50 µl (~1 x 10^8 CFU) of *S. aureus* through intranasal administration (25 µL per nostril). Following euthanasia, whole lung and nasal wash were collected aseptically. The lungs were weighed and homogenized in 1 mL of PBS (Bullet Blender Storm 5). Homogenized lungs and nasal wash were serially diluted and plated on SIA to determine CFUs. For the acute pneumonia murine competition infection, JE2 and JE2 *gltT*::Tn were grown on SIA for 18 to 24 hours at 37°C and suspended in PBS to an-OD_{600} of 14, followed by a 1:2 dilution corresponding to ~4.8 x 10^9 CFU/mL. Anesthetized mice were infected with 12.5 µl of culture of each *S. aureus* strain (~6 x 10^7 CFU) administered sequentially and single-strain control mice were infected with either 25 µl of JE2 or JE2 *gltT*::Tn (~1 x 10^8 CFU). Following euthanasia, whole lung and nasal wash were collected and processed following the procedures stated above. Serial dilutions were plated on both SIA and LA supplemented with erythromycin (25 µg/mL) to determine CFU, for both strains and JE2 *gltT*::Tn, respectively. Results were analyzed using one-way analysis of variance (ANOVA) corrected with Šidák in GraphPad Prism 9 (**Figure 4**).

Competitive fitness assay

Fluorescently labeled JE2 and JE2 *gltT*::Tn were grown individually and together in complete CDMG (1.1 mM asp and 1.0 mM glu) (+A/+G) and CDMG with additional asp (2.2 mM asp) and no glu added (++A/0G). Cultures were inoculated at initial densities of OD_{600} 0.01 in flasks with 25 mL of media and incubated at 37°C with continuous shaking for 24 hours. CFUs were determined at inoculation, early growth (4 - 8 hours after inoculation) and endpoint (22 - 24 hours after inoculation). Both versions of each strain (JE2.GFP and JE2.DsRed and JE2 *gltT*::Tn.GFP and JE2 *gltT*::Tn.DsRed) were tested in these conditions in replicate experiments (**Figure 3**).

Amino acid utilization

Amplite™ Fluorimetric L-Aspartate (Aspartic Acid) Assay Kit, and Amplite™ Fluorimetric Glutamic Acid Assay Kit *Red Fluorescence* (AAT Bioquest) were used to measure the concentration of aspartate and glutamate, respectively. Cell-free spent media was collected by filtering resuspended cocultures that were grown for 24 hours on TSA plates (as was done for phenotypic testing for *P. aeruginosa* tolerance) through 0.22 µm syringe filters. Spent media was collected from *S. aureus* monocultures and cocultures, as well as PAO1 monocultures and controls. Control conditions were made by inoculating sterile filters with 10 µl of LB or PBS and incubating for 24 hours. Measurements were taken across 3 separate experiments, each with 2 replicates for each culture condition.

Single cell imaging

Batch cultures were grown in TSB media supplemented with chloramphenicol 10 µg/mL to maintain fluorescent plasmids. Overnight cultures were diluted 1:100 into 3 mL fresh TSB media and grown to mid-exponential phase in a test tube at 37°C in a shaking incubator (OD_{600} between 0.5-0.8). Subsequently, cells were washed 3 times with PBS to remove antibiotics and diluted to an OD_{600} of 0.1. Finally, a 1:1:1 coculture was prepared consisting of either

JE2.DsRed + JE2 *gltT*::Tn.GFP + PAO1 or JE2.GFP + JE2 *gltT*::Tn.DsRed + PAO1. Agarose media was prepared by adding 1.5% agarose to either fresh or depleted TSB media.

A 1 mL droplet of agarose media was suspended between two coverslips and dried at room temperature for 30 minutes to create a ~3 mm thick slap, which was then cut into 5x5 mm pads. 1 µL of the coculture was added to the pad and dried until the liquid was absorbed. Afterwards the pad was carefully inverted and placed in a glass-bottomed dish (WillCo Wells). 6 pads were added to the same dish with the following media conditions: fresh TSB, depleted TSB from a 3-hour culture, and depleted TSB from a 16-hour culture. Each media condition was inoculated with one of the two strain mixtures. A small piece of water-soaked tissue was added to preserve humidity and the dish was sealed with parafilm. The experiment was repeated four times using different biological replicates on two separate days. We conducted 4 replicate experiments with each labeled version (GFP or DsRed) of each strain being tested twice. Imaging of the samples began 1 hour after the agar pads were inoculated.

The pads were imaged using a Nikon Ti2 inverted microscope with perfect focus system, equipped with a Hamamatsu ORCA-Flash4.0 V3 Digital CMOS camera, a Nikon NA1.42 60X Plan Apochromat phase contrast oil objective, a SPECTRA-X LED fluorescent light source, and Chroma filter sets. Images were taken every 3 minutes in the phase, GFP, and RFP channels. Cells were kept at 37°C while imaging using a climate-controlled incubator (Oko-lab).

Image analysis

To analyze colony growth, we segmented and tracked colonies using a custom-build pipeline (code is available at: https://github.com/simonvanvliet/PA-SA_Agarpads). The time-lapse movies were manually trimmed to remove later time points where cells overlapped in 3D or where excessive cell movement of *P. aeruginosa* was observed. Subsequently, images were registered using the phase_cross_correlation method of scikit-image (van der Walt et al., 2014).

Segmentation of colony outlines was done using the Ilastik supervised pixel classification workflow (Berg et al., 2019). Pixels in the multi-channel image (phase, GFP, RFP) were classified in four classes (GFP / DsRed labeled *S. aureus*, *P. aeruginosa*, and background). The classification probabilities were post-processed using custom written python code to extract individual masks for each colony. In short, the probabilities were smoothed with a gaussian kernel and thresholded using a fixed threshold value of 0.5. The masks were then post-processed using a binary closing operation to fill-in gaps between neighboring cells. Finally, small objects were removed, and holes closed.

Colonies were tracked using a custom written tracking algorithm that matched colonies across subsequent frames based on the minimal center-to-center distance. Tracks were stopped when colonies merged. An automated filtering procedure was used to trim tracks whenever an unexpectedly large change in colony area was observed (indicative of missed colony merger and/or segmentation error).

Colony growth rate *r* was calculated as: $r = \frac{1}{\Delta t}\log\frac{A(T)}{A(0)}$, where *A*(0) and *A(T)* are the area (in pixels squared) of the colony at the start of the movie and after *T*=1 hour, and where Δt=3 minutes is the imaging interval. To quantify the spatial arrangement, we calculated the minimal distance reached between the edge of the focal *S. aureus* colony to the closest pixel occupied by *P. aeruginosa*. Colonies within 300 pixels of the image frame were excluded from the analysis, as we could not accurately quantify their spatial arrangement.

Results

gltT truncation in *S. aureus* is an adaptation to *P. aeruginosa* tolerance

Serial transfer experimental evolution generated multiple populations of *S. aureus* with improved survival in the presence of *Pseudomonas aeruginosa* strain PAO1 (referred to as PAO1). At the

completion of 8 serial transfers, two out of four experimental populations had significantly increased their relative survival compared to the JE2 ancestral strain. EE1 and EE3 both increased in the number of recovered CFUs by more than 3 orders of magnitude, with about 10^5 CFUs being initially recovered to about 10^8 CFUs recovered after 8 serial transfers (**Figure 1B**). Similar results were observed in replicate experiments. Single individual colonies from evolved *P. aeruginosa*-tolerant populations maintained this phenotype when cultured at a 1:1 ratio with PAO1 for 24 hours (**Supplementary Figure 2a**).

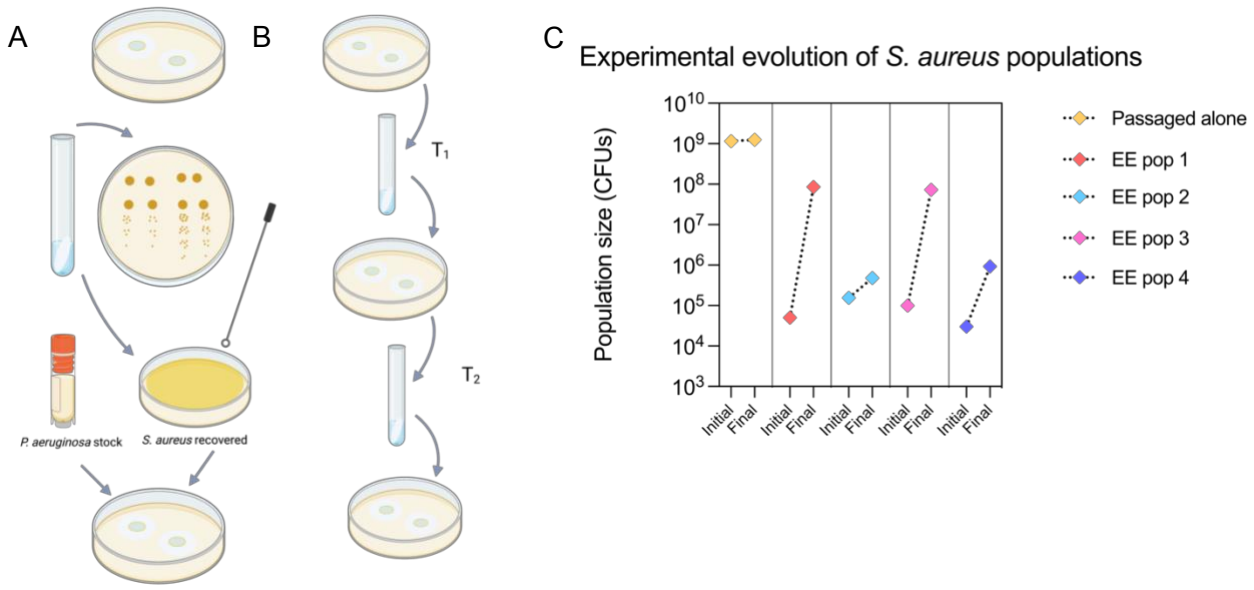
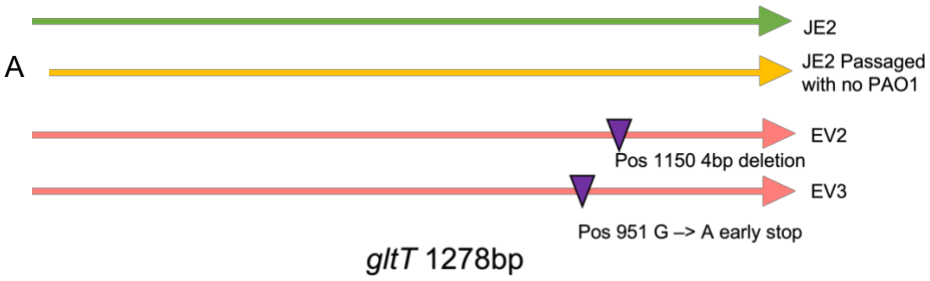


Figure 1. Experimental evolution with *Staphylococcus aureus* USA300 JE2 generates populations that are tolerant to *Pseudomonas aeruginosa* in coculture. *S. aureus* and *P. aeruginosa* were cocultured for 48 hours at an inoculation ratio of 30:1 (*S. aureus*:*P. aeruginosa*). A) Transfer procedure - cocultures are inoculated on solid TSA agar with 0.45 µm filter paper used to contain and recover the culture. After the coculture period, filters are resuspended in liquid media and serial dilutions of the resuspension are spot plated on selective agar. After 24 hours of growth, CFUs are counted for both species on their respective selective agar SIA and PIA. Schematic created with BioRender. B) Serial transfer method - a new transfer occurs when *S. aureus* is isolated from the resuspension by plating 50 µl on SIA. After overnight incubation, *S. aureus* was inoculated with *P. aeruginosa* from a fresh overnight culture that was diluted to the same OD_{600}. Control populations are repeatedly transferred under the same conditions but never exposed to *P. aeruginosa.* C) Experimental evolution results: four populations (pop 1-4) were evolved in the presence of PAO1 for 8 coculture periods; control population (passage alone) never exposed to *P. aeruginosa* also shown. CFU counts from the first coculture period (initial) and 8th coculture period (final) are shown.

We sequenced the genomes of two single colonies from an evolved tolerant population EE1 and compared those sequences to those of colonies from an evolved but still sensitive population EE4 and colonies from control populations which were transferred in parallel with experimental populations but never cocultured with PAO1. Each isolate was screened for its survivability in coculture with PAO1 before being sequenced and the pattern of tolerance observed during the evolution experiment was confirmed. In the two colonies sequenced from population EE1, only one mutation site was unique to them, not appearing in any other compared sequence. Each evolved tolerant isolate (EV2 and EV3) had an independent putative loss of function mutation in the gene encoding for the *S. aureus* amino acid transporter, *gltT.* GltT has been previously described as being the sole aspartate transporter in *S. aureus* that also interacts with glutamate (Potter et al., 2020; Zeden et al., 2020; Zhao et al., 2018). In isolate EV3, a single nucleotide base substitution G → A introduced an early stop; in isolate EV2, a 4-base-pair deletion resulted in a frameshift mutation (**Figure 2A**). Both mutations occur between 800-1200bp downstream of the start codon and were predicted to truncate the protein by disrupting the 3' portion of the coding region. Both single colony isolates displayed a *P. aeruginosa*-tolerant phenotype compared to their common ancestor, JE2 (**Supplementary Figure 2b**).

To confirm the linkage between *gltT* disruption and *P. aeruginosa*-tolerant phenotype, we retrieved the *gltT* mariner transposon knockout mutant, *SAUSA300_2329* from the Nebraska Transposon Mutant Library (NTML) (Fey et al., 2013b). After transducing the mutation to the ancestral JE2 background, we validated the strain by PCR and confirmed that this strain, which we now refer to as JE2 *gltT*::Tn, had improved CFU recovery after coculture with PAO1 compared to its parent (**Figure 2B**). These results confirmed that the *gltT* disruption was responsible for the enhanced fitness of *S. aureus* in coculture with PAO1. When the mutant *gltT* strains were complemented in *trans*, the PAO1 sensitivity phenotype was restored for both the JE2 *gltT*::Tn and evolved isolate EV2 **(Figure 2B).**



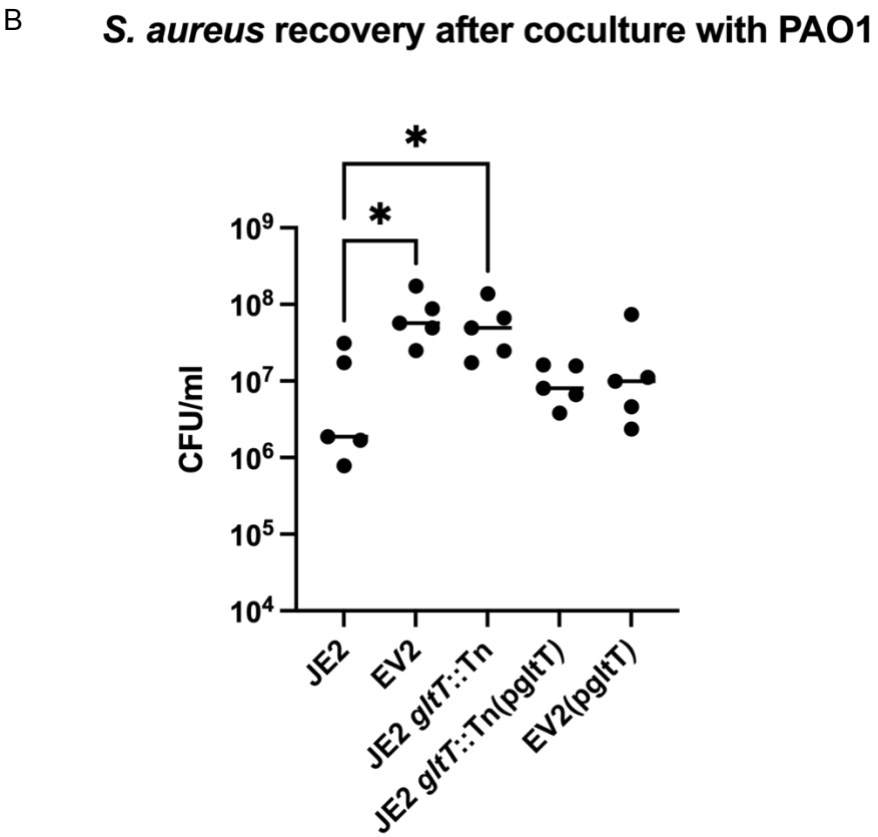


Figure 2. *gltT* truncation enhances *S. aureus* recovery after coculture with PAO1. A) Whole genome sequencing reveals two independent truncations of the aspartate transporter, *gltT*, in sequences of two single colony isolates EV2 and EV3 taken from the same evolved *P. aeruginosa*-tolerant population - EE pop 1 (Fig 1C). B) Evolved phenotype of *P. aeruginosa* tolerance is observed in the *gltT* transposon mutant JE2 *gltT*::Tn and evolved isolate EV2. Wild-type *P. aeruginosa* sensitivity is restored in the complemented transposon mutant JE2 *gltT*::Tn(pgltT) and complemented evolved isolate EV2(pgltT). Friedman's test for multiple comparisons yielded p-values of 0.0127 and 0.0205 (*) when comparing JE2 CFUs to EV2 and JE2 *gltT*::Tn, respectively.

JE2 *gltT*::Tn outcompetes wild-type *S. aureus* in CDMG without glutamate

Growing strains individually in CDMG (**Supplementary Table 2**) or rich LB (**Supplementary Figure 3**) media yielded no insights into fitness differences associated with *gltT* disruption. However, we hypothesized that *gltT* mutants may be able to outcompete wild-type *S. aureus* under certain conditions. To test this, we conducted competition experiments with mutant and wild type strains fluorescently labeled with either GFP or DsRed multicopy plasmids. Labeled strains were inoculated in complete CDMG with 1.1 mM asp and 1.0 mM glu (+A/+G) and CDMG with double the amount of aspartate (2.2 mM asp) and no glutamate added (++A/0G). CFU counts from three replicate experiments showed that JE2 and JE2 *gltT*::Tn were equally fit when grown in complete CDMG, with each strain making up about half of the total culture density. Total CFUs were greater than 10^8 CFUs across all conditions and replicates. However, in the (++A/0G) condition, JE2 *gltT*::Tn outcompeted wild-type JE2 which only made up about 5% of all CFUs recovered from this growth condition (**Figure 3**).

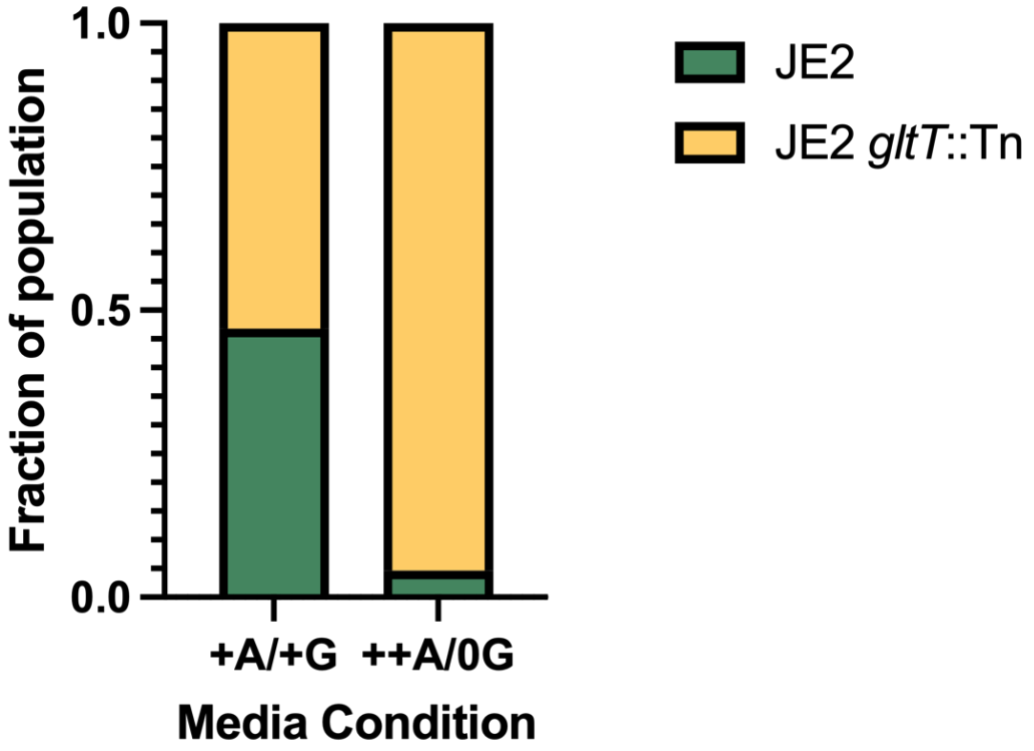


Figure 3. JE2 *gltT*::Tn outcompetes wild-type JE2 in CDMG when glutamate is limiting and aspartate is in excess. JE2.GFP, JE2.DsRed and JE2 *gltT*::Tn.GFP and JE2 *gltT*::Tn.DsRed strains labeled with fluorescent plasmids were used to test for competitive fitness in CDMG conditions. One GFP labeled strain and one ds.Red labeled strain were cultured for 24 hours in CDMG media alone and in coculture. Incubated cultures were then serially diluted and incubated overnight at 37°C before CFUs were counted and number of red and green colonies recorded. Each version of each strain was tested at least once across 3 biological replicates. JE2 *gltT*::Tn made up the majority of CFUs recovered after 24 hours when aspartate (A) was in excess (++), and glutamate (G) was limited (0G) (++A/0G) Chi-square p-value< 0.0001.

Growth rate differences, as measured by single cell microscopy, are not responsible for the *P. aeruginosa*-tolerant phenotype

Based on results from CDMG assays, we hypothesized that glutamate depletion by PAO1 would reduce the growth rate in nearby JE2 cells, but not in JE2 *gltT*::Tn cells. To test this hypothesis, we conducted single cell microscopy with wild-type JE2 and JE2 *gltT*::Tn and measured fitness by CFU counts after a 24 hour coculture with PAO1 . We conducted single cell image analysis on cocultures with equal starting ratios of JE2, JE2 *gltT*::Tn and PAO1 using agar pads and GFP and DsRed fluorescently labeled strains. We hypothesized that growth rate differences may only be apparent in depleted media conditions as CFU differences were most obvious between JE2 and JE2 *gltT*::Tn when cocultures are inoculated at high initial densities. However, even in depleted TSB collected from a 16 hour culture of PAO1, JE2 *gltT*::Tn did not have an observable difference in growth rate compared to wild-type JE2 (**Figure 4**). Moreover, we did not find a dependence of *S. aureus* growth rates for either wild-type JE2 or JE2 *gltT*::Tn based on their proximity to PAO1 colonies (**Supplementary Figure 4**). In fact, the only growth difference observed was a slight advantage for wild-type JE2 in depleted TSB collected from a 3-hour culture of PAO1. Both strains had very low growth rates in the most depleted media condition, TSB collected from a 16-hour culture of PAO1. Morphology of microcolonies were indistinguishable between *S. aureus* strains and no evidence of small colony variants were observed. These results suggest that the growth advantage of JE2 *gltT:*::Tn is a population-level trait that is not explained by growth rate differences between it and its isogenic wild-type counterpart.

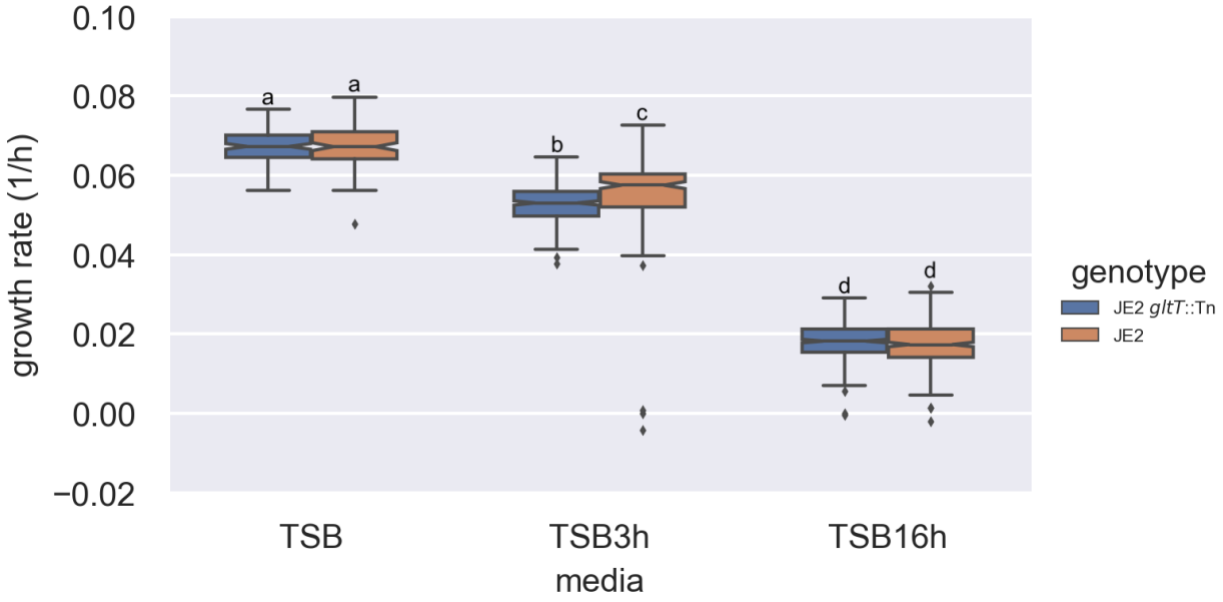


Figure 4. Growth rates of wild-type JE2 and JE2 *gltT*::Tn in coculture with PAO1 in rich and depleted media . Fluorescently labeled strains - JE2.GFP or JE2.DsRed and JE2 *gltT*::Tn.GFP or JE2 *gltT*::Tn.DsRed were cultured together with PAO1 on agar pads made from rich Trypticase Soy Broth (TSB) and depleted cell-free TSB collected from 3 hour and 16-hour PAO1 monocultures. Growth rates of micro colonies were measured using single cell microscopy image analysis. Wild-type JE2 is observed to have a slight growth advantage over mutant JE2 *gltT*::Tn in slightly depleted TSB from a 3-hour culture, however strains grow generally at the same rate in all other conditions. Data was collected over four replicates for each of the two strain combinations. We did not observe consistent differences between the different DsRed or GFP strain combinations and their data was thus pooled. PAO1 also carried a GFP label and was easily distinguished based on cell shape. The two-way ANOVA revealed significant effects of media ($F(2, 18) = 11800.29$, $p < 0.001$) and genotype ($F(1, 18) = 4.37$, $p = 0.036$), as well as a significant interaction between media and genotype ($F(2, 18) = 17.55$, $p < 0.001$) on growth. Post-hoc Tukey's HSD test indicated significant differences between different media conditions ($p < 0.05$), suggesting that the growth varied significantly depending on the media used. Additionally, significant differences were observed between genotypes in the TSB3h media ($p < 0.05$).

gltT disruption alters amino acid uptake in *S. aureus* strains

We measured concentrations of aspartate and glutamate in cell-free spent media that was collected from 24-hour TSA cultures (monocultures and cocultures with PAO1) of the following *S. aureus* strains: JE2, JE2 *gltT*::Tn, EV2 and complemented strains JE2 *gltT*::Tn (pgltT) and EV2 (pgltT). These data revealed that aspartate concentration remained highest in all culture conditions where PAO1 was not present (**Figure 5**). In the case of glutamate, there was very little of the amino acid remaining in any culture condition with wild-type or *gltT* complemented *S. aureus* strains even when PAO1 was not present. Both amino acids were present at higher concentrations in the spent media of *S. aureus* monocultures compared that of cocultures or PAO1 monocultures. This suggested that PAO1 was more efficient at metabolizing both aspartate and glutamate than *S. aureus.* Additionally, the levels of remaining glutamate were higher in *S. aureus* cultures of the *gltT* mutant, suggesting that a functional *gltT* locus was essential to utilizing most of the available glutamate (**Figure 5**).

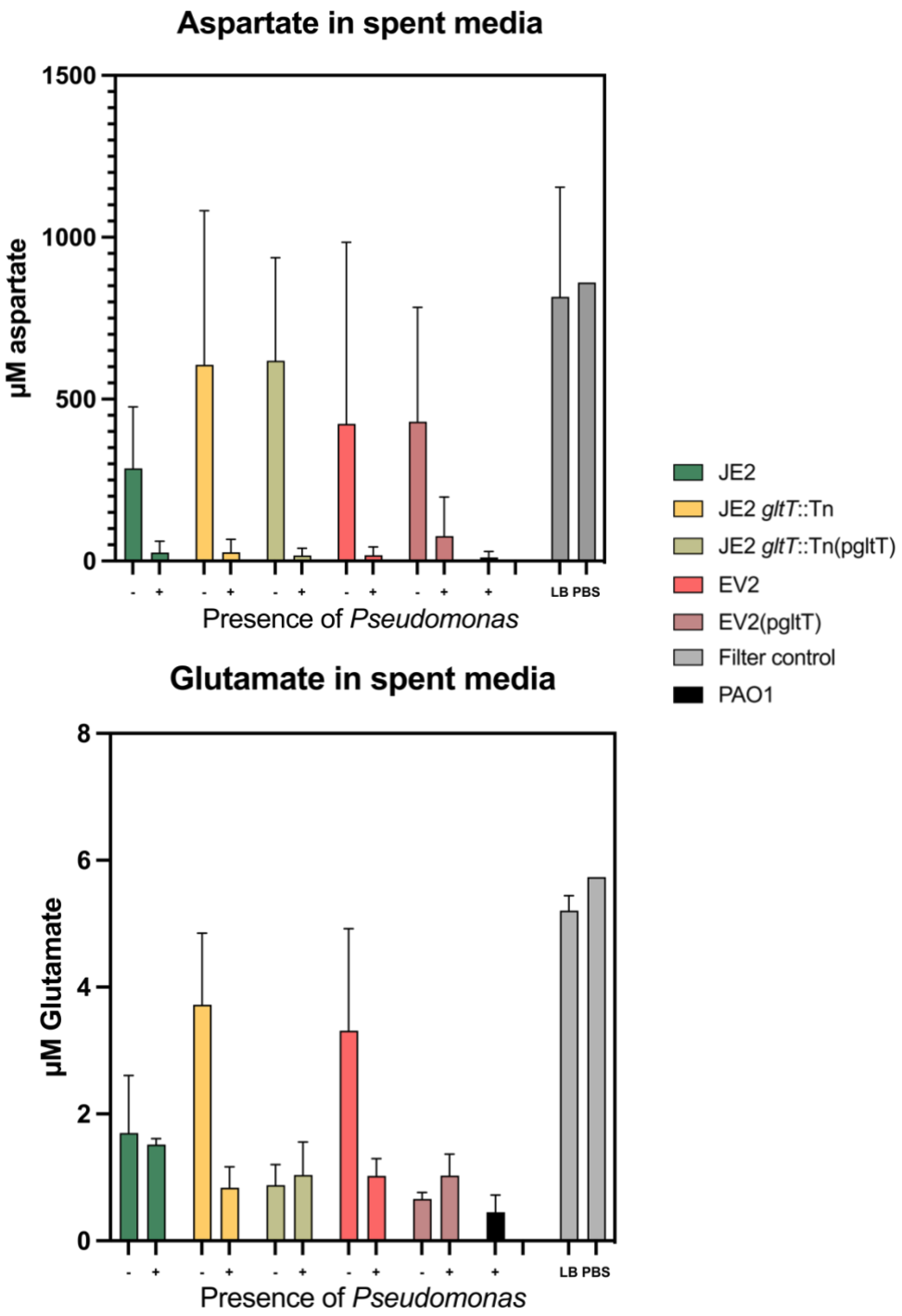


Figure 5. *gltT* is required for *S. aureus* glutamate uptake and aspartate and glutamate are greatly reduced when PAO1 is present. Remaining aspartate and glutamate levels after 24-hours of culturing on 0.45µM filters on TSA plates, were measured using Amplite Fluorimetric kits. Both cocultures with PAO1 (+) and *S. aureus* monocultures (-) were tested. Control conditions were tested by inoculating filters with 10µL of LB media or 10µL of PBS.

gltT disruption does not impact *S. aureus* host colonization

To gain an understanding of the impact of *gltT* disruption in a host environment, we carried out experiments using an acute murine pneumonia model system where mice were infected with JE2 *gltT*::Tn*,* wild type JE2, or both strains in a coinfection. There were similar population sizes of the ancestral JE2 strain and JE2 *gltT*::Tn recovered from both the nasal wash and lung tissue for both single strain cultures and cocultures of the two strains **(Figure 6).** Therefore, we concluded that *gltT* disruption did not greatly impact *S. aureus'* ability to colonize host respiratory tissues.

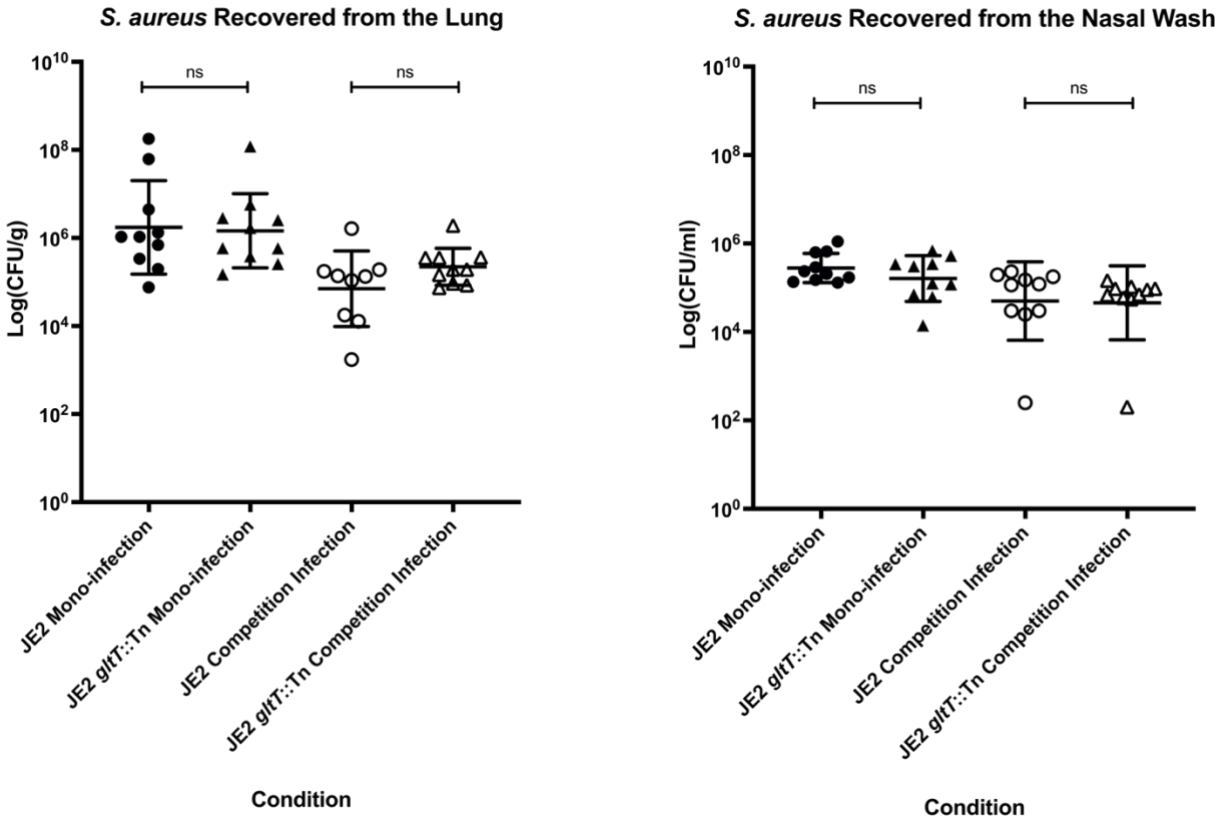


Figure 6. Colonization ability of *S. aureus* is not impacted by *gltT* genotype. Similar amounts of *S. aureus* are recovered from both lung (left) and nasal wash (right) when JE2 or JE2 *gltT*::Tn are cultured in a mouse alone or in coculture. All mice were euthanized 24 hours post infection. All statistics were performed using GraphPad Prism 9 using one-way analysis of variance (ANOVA) with Šidák correction. Similar levels of colonization in the lungs and upper respiratory tract (nasopharynx) was observed between JE2 and JE2 *gltT*::Tn in 8- to 10-week-old C57BL/6 female mice. For mono-infections, $1x10^8$ CFU of JE2 and JE2 *gltT*::Tn was administered intranasally. For the competition infections, $6x10^7$ CFU of both JE2 and JE2 *gltT*::Tn was sequentially administered intranasally. After 24-hours post-infection, all mice were euthanized and CFUs from the nasal wash and lungs were recovered either on SIA (mono-infection) or both LB + erythromycin (25 µg/ml) and SIA (competition infection). All statistics were performed using GraphPad Prism 9 using one-way analysis of variance (ANOVA) with Šidák correction.

gltT disruption was rare in diverse *S. aureus* genomes

Findings from the evolution experiment and *gltT* mutant phenotyping tests indicated that *gltT* could be disrupted without severe impacts on strain fitness. We therefore, sought to estimate the variability of *gltT* across diverse *S. aureus* lineages, including CF-associated isolates. Previous work had shown that *gltT* is a core gene (Petit III & Read, 2018) and so we were able to look at *gltT* variability by screening a diverse dataset of 444 *S. aureus* genomes representing 380 MLST sequence types. We did not find mutations identical to the ones we saw during our experimental evolution. Furthermore, we identified only one mutation that caused an early stop, at position 1255, truncating the protein by 8 amino acids. This was the only putative non-functional mutant we observed in *gltT* and it was present in only one sample. This putative loss of function mutant was not isolated from a CF-associated infection.

We did not observe a significant enrichment of mutations in *gltT* when compared to other amino acid transporters in our dataset. We observed 115 occurrences of non-synonymous mutations in *gltT*, with 26 distinct mutations, 25 of which were found in < 10 strains. One mutation - a glutamate → aspartate change at position 891, was found in in 71 strains. Overall, these results suggested that *gltT* disruption was rare in *S. aureus*, even compared to other core genes encoding amino acid transporters (**Supplementary Table 1**). This was also true for CF associated isolates which, in our screen, did not have elevated rates of mutation in *gltT* compared to non-CF isolates.

Discussion

Impact of inactivation of *S. aureus gltT* gene in *S. aureus-P. aeruginosa* interactions

Interactions between *S. aureus* and *P. aeruginosa* have proven to be complex, and dependent on environment and strain background (Bernardy et al., 2020, 2022). Previous studies have

implicated factors such as the *P. aeruginosa* mucoidy phenotype, compounds or toxins excreted by *P. aeruginosa*, and *S. aureus* metabolic pathways such as the production of acetoin as important factors in the interspecific interactions between *S. aureus* and *P. aeruginosa* (Barraza & Whiteley, 2021; Bernardy et al., 2020, 2022; Camus et al., 2020; Lasse et al., 2022; Zarrella & Khare, 2021). Surprisingly, despite the wealth of research on the coexistence and competition of these species, in our experimental system, we observed mutation of a highly conserved gene, *gltT*, that had not previously been linked to *S. aureus-P. aeruginosa* co-occurrence. Our findings indicated that *S. aureus* JE2 and *P. aeruginosa* PAO1 directly compete over limiting glutamate, particularly when grown at high densities on TSA plates. Our evolved isolates appear to have gained a phenotypic advantage over their JE2 ancestor by disrupting the *gltT* locus – limiting import of aspartate and glutamate and likely relying on alternative metabolic pathways. Under the conditions of the evolution experiment we designed here, there is apparently significant selective pressure for *S. aureus* to optimize its amino acid metabolism for a glutamate-limited environment. The *S. aureus gltT* gene was also found to be disrupted in osteomyelitis studies that revealed how excess glutamate competitively inhibits aspartate transport through *gltT* (Potter et al., 2020). While the osteomyelitis study of Potter et al. (2020)presents the inverse of the nutrient landscape *S. aureus* is adapting to in our experiment – a challenge of excess glutamate rather than it being a limiting nutrient - it also demonstrates the importance of exogenous amino acids in *S. aureus* competitive fitness and the importance of altering metabolic pathways as an adaptive strategy in changing environments. Our findings suggest that we still do not understand enough about the interaction between *S. aureus* and *P. aeruginosa* to predict the genes that give adaptive advantages in any given combination of strains and environmental conditions.

We acknowledge some limitations of our experimental evolution approach. For instance, while fresh PAO1 was introduced to each coculture period we found that a minority population of *P.*

aeruginosa were able to survive on SIA agar even though they did not form colonies. We suspect that some *P. aeruginosa* cells may have been transferred between coculture periods and could have coevolved with the JE2 populations. However, fresh PAO1 was introduced at each coculture period, and the evolved *P. aeruginosa*-tolerant phenotypes of *S. aureus* were maintained after populations were fully isolated from any retained PAO1. Therefore, any effect of carryover *P. aeruginosa* appeared to have little effect compared to the larger population of fresh introduced ancestral PAO1. Additionally, while the population sizes in our evolution experiment are likely much denser than what occurs during a CF lung infection, populations of *S. aureus* and *P. aeruginosa* have both been observed at levels as high as 10^8 CFUs/mL in CF sputum samples collected from coinfected patients (Fischer et al., 2020)

The role of aspartate and glutamate in *P. aeruginosa* tolerance

Previous analysis of *S. aureus* metabolism has shown that glutamate derivatives are required for *S. aureus* to metabolize aspartate into oxaloacetate, a secondary metabolite required in the citric acid cycle (Halsey et al., 2017). Therefore, the absence of extracellular glutamate may lead to reduced activity of the TCA cycle. This could explain why all tested *S. aureus* strains showed a reduced growth-rate when glutamate was not present in CDMG compared to complete CDMG (**Supplementary Table 2**). However, we did not observe significant growth rate differences between wild type JE2 *S. aureus* and JE2 *gltT*::Tn in our CDMG monoculture assays or by single-cell microscopy. These data suggest that despite its important role in amino acid metabolism there were few apparent fitness trade-offs associated with disrupting *gltT* when *P. aeruginosa* is not present in standard laboratory conditions (Zhao et al., 2018). In addition, the finding that *gltT* mutations are extremely rare in non-laboratory adapted stains reinforces the key metabolic role of this core gene. We postulate that continuing to import aspartate in the wild-type strain in the absence of glutamate may lead to a buildup of aspartate intracellularly and a corresponding reduced competitive fitness. This hypothesis is supported by our finding

that along with its increased *P. aeruginosa*-tolerance, JE2 *gltT*::Tn was able to outcompete wildtype JE2 in the CDMG condition where additional aspartate was added and no glutamate was added (++A/0G) **(Figure 3)**.

Further experimentation is needed to better understand how amino acid metabolism facilitates *S. aureus-P. aeruginosa* interactions; however, we conclude here that in our evolution experiment, *S. aureus* is primarily adapting to the limitation of glutamate in its environment by disrupting its aspartate transporter and relying on alternative metabolic pathways to carry out the TCA cycle. In short, the major source of negative selective pressure that *S. aureus* experienced when grown in the presence of *P. aeruginosa* was competition over exogenous amino acids.

Experimental evolution as a useful tool for studying pathogens

Experimental or directed evolution experiments carried out in laboratory conditions can be a powerful way to reduce complex adaptive phenotypes in important pathogens like *S. aureus* and *P. aeruginosa* to single genetic determinants. The lab environments used for experimental evolution studies are removed from the conditions we study, such as the cystic fibrosis lung environment or the host environment in general. Despite this however, there is still a lot to be gained from evolution experiments conducted in laboratory conditions with lab-adapted strains. Even if such experiments identify genes that are highly conserved (and thus unlikely to be important for adaptation in the setting of human infection) as we have found in this study, these findings reveal potential adaptive trajectories that may lead to possible treatment targets as well as a greater understanding of pathogen biology and physiology. For instance, our findings here lay important groundwork in the development of coinfection disruption therapy by highlighting the importance of the nutrient landscape in the facilitation of *S. aureus-P. aeruginosa* coexistence in the cystic fibrosis lung or other chronic infections. The link between *gltT* and *P.*

aeruginosa tolerance likely could not have been identified by screening clinical isolates because the gene is so highly conserved. Our *in vitro* experiments suggest that *gltT* mutants can colonize lung tissue just as well as wild-type strains and would be more likely to coexist with *Pseudomonas* in a coinfection. Therefore, even though likely rare, this genotype could be important to screen for when treating coinfections and is certainly important to consider in the development of new therapies to treat polymicrobial infections.

Acknowledgements

This work was supported in part with grants for the National Institutes of Health (R21 AI148847) and the US Cystic Fibrosis Foundation (GOLDBE19I0) to JBG. Research reported in this publication was supported in part by National Institute of Allergy and Infectious Diseases of the National Institutes of Health (T32AI138952), and Emory University and the Infectious Disease Across Scales Training Program (IDASTP) (AMA). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, Emory University or IDASTP.

GB and SVV were supported by an Ambizione grant from the Swiss National Science Foundation (grant no. PZ00P3_202186) and by the University of Basel.

We would like to thank Rachel Done for her assistance with cloning and strain preparation. We would also like to thank members of the Goldberg and Read labs for their support and review of the manuscript.

REFERENCES

Barraza, J. P., & Whiteley, M. (2021). A Pseudomonas aeruginosa Antimicrobial Affects the Biogeography but Not Fitness of Staphylococcus aureus during Coculture. *MBio*, *12*(2), e00047-21. https://doi.org/10.1128/mBio.00047-21

Berg, S., Kutra, D., Kroeger, T., Straehle, C. N., Kausler, B. X., Haubold, C., Schiegg, M., Ales, J., Beier, T., Rudy, M., Eren, K., Cervantes, J. I., Xu, B., Beuttenmueller, F., Wolny, A., Zhang, C., Koethe, U., Hamprecht, F. A., & Kreshuk, A. (2019). ilastik: interactive machine learning for (bio)image analysis. *Nature Methods*, *16*(12), 1226–1232. https://doi.org/10.1038/s41592-019-0582-9

Bernardy, E. E., Petit, R. A., Raghuram, V., Alexander, A. M., Read, T. D., & Goldberg, J. B. (2020). Genotypic and Phenotypic Diversity of Staphylococcus aureus Isolates from Cystic Fibrosis Patient Lung Infections and Their Interactions with Pseudomonas aeruginosa. *MBio*, *11*(3). https://doi.org/10.1128/mbio.00735-20

Bernardy, E. E., Vishnu, R., & Goldberg, J. B. (2022). Staphylococcus aureus and Pseudomonas aeruginosa Isolates from the Same Cystic Fibrosis Respiratory Sample Coexist in Coculture. *Microbiology Spectrum*, *10*(4), e00976-22. https://doi.org/10.1128/spectrum.00976-22

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)*, *30*(15), 2114–2120. https://www.ncbi.nlm.nih.gov/pubmed/24695404

Brown, S. P., Cornforth, D. M., & Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends in Microbiology*, *20*(7), 336–342. https://doi.org/10.1016/j.tim.2012.04.005

Brown, S. P., Le Chat, L., & Taddei, F. (2008). Evolution of virulence: triggering host inflammation allows invading pathogens to exclude competitors. *Ecology Letters*, *11*(1), 44–51. https://doi.org/10.1111/j.1461-0248.2007.01125.x

Bubeck Wardenburg, J., Williams, W. A., & Missiakas, D. (2006). Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. *Proceedings of the National Academy of Sciences*, *103*(37), 13831–13836. https://doi.org/10.1073/pnas.0603072103

Camus, L., Briaud, P., Bastien, S., Elsen, S., Doléans-Jordheim, A., Vandenesch, F., & Moreau, K. (2020). Trophic cooperation promotes bacterial survival of Staphylococcus aureus and Pseudomonas aeruginosa. *The ISME Journal*, *14*(12), 3093–3105. https://doi.org/10.1038/s41396-020-00741-9

Chen, J., Yoong, P., Ram, G., Torres, V. J., & Novick, R. P. (2014). Single-copy vectors for integration at the SaPI1 attachment site for Staphylococcus aureus. *Plasmid*, *76*, 1–7. https://doi.org/https://doi.org/10.1016/j.plasmid.2014.08.001

Cystic Fibrosis Foundation. (2021). *Cystic Fibrosis Foundation patient registry 2021 annual data report*.

Dalton, T., Dowd, S. E., Wolcott, R. D., Sun, Y., Watters, C., Griswold, J. A., & Rumbaugh, K. P. (2011). An In Vivo Polymicrobial Biofilm Wound Infection Model to Study Interspecies Interactions. *PLOS ONE*, *6*(11), e27317-. https://doi.org/10.1371/journal.pone.0027317

Fey, P. D., Endres, J. L., Yajjala, V. K., Widhelm, T. J., Boissy, R. J., Bose, J. L., & Bayles, K. W. (2013). A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. *MBio*, *4*(1), e00537-12-e00537. https://doi.org/10.1128/mbio.00537-12

Filkins, L. M., Graber, J. A., Olson, D. G., Dolben, E. L., Lynd, L. R., Bhuju, S., & O'Toole, G. A. (2015). Coculture of Staphylococcus aureus with Pseudomonas aeruginosa Drives S. aureus towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *Journal of Bacteriology*, *197*(14), 2252–2264. https://doi.org/10.1128/jb.00059-15

Fischer, A. J., Singh, S. B., LaMarche, M. M., Maakestad, L. J., Kienenberger, Z. E., Peña, T. A., Stoltz, D. A., & Limoli, D. H. (2020). Sustained Coinfections with Staphylococcus aureus and Pseudomonas aeruginosa in Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *203*(3), 328–338. https://doi.org/10.1164/rccm.202004-1322OC

Grosser, M. R., & Richardson, A. R. (2016). Method for Preparation and Electroporation of S. aureus and S. epidermidis. In J. L. Bose (Ed.), *The Genetic Manipulation of Staphylococci: Methods and Protocols* (pp. 51–57). Springer New York. https://doi.org/10.1007/7651_2014_183

Halsey, C., Shulei, L., K, W. J., K, L. M., S, N. A., Laurey, S., Marat, S., Robert, P., & D, F. P. (2017). Amino Acid Catabolism in Staphylococcus aureus and the Function of Carbon Catabolite Repression. *MBio*, *8*(1), e01434-16. https://doi.org/10.1128/mBio.01434-16

Hamelin, F. M., Allen, L. J. S., Bokil, V. A., Gross, L. J., Hilker, F. M., Jeger, M. J., Manore, C. A., Power, A. G., Rúa, M. A., & Cunniffe, N. J. (2019). Coinfections by noninteracting pathogens are not independent and require new tests of interaction. *PLOS Biology*, *17*(12), e3000551-. https://doi.org/10.1371/journal.pbio.3000551

Iandolo, J. J., Worrell, V., Groicher, K. H., Qian, Y., Tian, R., Kenton, S., Dorman, A., Ji, H., Lin, S., Loh, P., Qi, S., Zhu, H., & Roe, B. A. (2002). Comparative analysis of the genomes of the temperate bacteriophages φ11, φ12 and φ13 of Staphylococcus aureus 8325. *Gene*, *289*(1), 109–118. https://doi.org/https://doi.org/10.1016/S0378-1119(02)00481-X

Ibberson, C. B., Parlet, C. P., Kwiecinski, J., Crosby, H. A., Meyerholz, D. K., & Horswill, A. R. (2016). Hyaluronan Modulation Impacts Staphylococcus aureus Biofilm Infection. *Infection and Immunity*, *84*(6), 1917–1929. https://doi.org/10.1128/IAI.01418-15

Kennedy, A. D., Otto, M., Braughton, K. R., Whitney, A. R., Chen, L., Mathema, B., Mediavilla, J. R., Byrne, K. A., Parkins, L. D., Tenover, F. C., Kreiswirth, B. N., Musser, J. M., & DeLeo, F. R. (2008). Epidemic community-associated methicillin-resistant Staphylococcus aureus: Recent clonal expansion and diversification. *Proceedings of the National Academy of Sciences*, *105*(4), 1327–1332. https://doi.org/10.1073/pnas.0710217105

Kennedy, A. D., Porcella, S. F., Martens, C., Whitney, A. R., Braughton, K. R., Chen, L., Craig, C. T., Tenover, F. C., Kreiswirth, B. N., Musser, J. M., & DeLeo, F. R. (2010). Complete Nucleotide Sequence Analysis of Plasmids in Strains of Staphylococcus aureus Clone

USA300 Reveals a High Level of Identity among Isolates with Closely Related Core Genome Sequences. *Journal of Clinical Microbiology*, *48*(12), 4504–4511. https://doi.org/10.1128/jcm.01050-10

Krausz, K. L., & Bose, J. L. (2016). Bacteriophage Transduction in Staphylococcus aureus: Broth-Based Method. In J. L. Bose (Ed.), *The Genetic Manipulation of Staphylococci: Methods and Protocols* (pp. 63–68). Springer New York. https://doi.org/10.1007/7651_2014_185

Lasse, K., Stephanie, C., H, C. M., Rita, L., Morten, A., Maria, A., Dan, S., & Thomas, B. (2022). Investigation of the Mechanism and Chemistry Underlying Staphylococcus aureus' Ability to Inhibit Pseudomonas aeruginosa Growth In Vitro. *Journal of Bacteriology*, *204*(11), e00174-22. https://doi.org/10.1128/jb.00174-22

Limoli, D. H., Whitfield, G. B., Kitao, T., Ivey, M. L., Davis, M. R., Grahl, N., Hogan, D. A., Rahme, L. G., Howell, P. L., O'Toole, G. A., & Goldberg, J. B. (2017). Pseudomonas aeruginosa Alginate Overproduction Promotes Coexistence with Staphylococcus aureus in a Model of Cystic Fibrosis Respiratory Infection. *MBio*, *8*(2), e00186-17. https://doi.org/10.1128/mBio.00186-17

Limoli, D. H., Yang, J., Khansaheb, M. K., Helfman, B., Peng, L., Stecenko, A. A., & Goldberg, J. B. (2016). Staphylococcus aureus and Pseudomonas aeruginosa co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. *European Journal of Clinical Microbiology & Infectious Diseases*, *35*(6), 947–953. https://doi.org/10.1007/s10096-016-2621-0

Mashburn, L. M., Jett, A. M., Akins, D. R., & Whiteley, M. (2005). Staphylococcus aureus Serves as an Iron Source for Pseudomonas aeruginosa during In Vivo Coculture. *Journal of Bacteriology*, *187*(2), 554–566. https://doi.org/10.1128/jb.187.2.554-566.2005

Nguyen, A. T., O'Neill, M. J., Watts, A. M., Robson, C. L., Lamont, I. L., Angela, W., & Oglesby-Sherrouse, A. G. (2014). Adaptation of Iron Homeostasis Pathways by a Pseudomonas aeruginosa Pyoverdine Mutant in the Cystic Fibrosis Lung. *Journal of Bacteriology*, *196*(12), 2265–2276. https://doi.org/10.1128/JB.01491-14

Orazi, G., & O'Toole, G. A. (2017). Pseudomonas aeruginosa Alters Staphylococcus aureus Sensitivity to Vancomycin in a Biofilm Model of Cystic Fibrosis Infection. *MBio*, *8*(4), e00873-17. https://doi.org/10.1128/mBio.00873-17

Pang, Y. Y., Schwartz, J., Thoendel, M., Ackermann, L. W., Horswill, A. R., & Nauseef, W. M. (2010). agr-Dependent Interactions of Staphylococcus aureus USA300 with Human Polymorphonuclear Neutrophils. *Journal of Innate Immunity*, *2*(6), 546–559. https://doi.org/10.1159/000319855

Petit III, R. A., & Read, T. D. (2018). Staphylococcus aureus viewed from the perspective of 40,000+ genomes. *PeerJ*, *6*, e5261. https://doi.org/10.7717/peerj.5261

Potter, A. D., Butrico, C. E., Ford, C. A., Curry, J. M., Trenary, I. A., Tummarakota, S. S., Hendrix, A. S., Young, J. D., & Cassat, J. E. (2020). Host nutrient milieu drives an essential role for aspartate biosynthesis during invasive Staphylococcus aureus infection.

Proceedings of the National Academy of Sciences, *117*(22), 12394–12401. https://doi.org/10.1073/pnas.1922211117

Raghuram, V., M, A. A., Qi, L. H., A, P. R., B, G. J., & D, R. T. (2022). Species-Wide Phylogenomics of the Staphylococcus aureus Agr Operon Revealed Convergent Evolution of Frameshift Mutations. *Microbiology Spectrum*, *10*(1), e01334-21. https://doi.org/10.1128/spectrum.01334-21

Schneewind, O., Model, P., & Fischetti, V. A. (1992). Sorting of protein a to the staphylococcal cell wall. *Cell*, *70*(2), 267–281. https://doi.org/10.1016/0092-8674(92)90101-H

Seeman, T. (2015). *snippy: fast bacterial variant calling from NGS reads*. https://github.com/tseemann/snippy

Silberstein, E., Serna, C., Fragoso, S. P., Nagarkatti, R., & Debrabant, A. (2018). A novel nanoluciferase-based system to monitor Trypanosoma cruzi infection in mice by bioluminescence imaging. *PLOS ONE*, *13*(4), e0195879-. https://doi.org/10.1371/journal.pone.0195879

Stacy, A., Fleming, D., Lamont, R. J., Rumbaugh, K. P., & Whiteley, M. (2016). A Commensal Bacterium Promotes Virulence of an Opportunistic Pathogen via Cross-Respiration. *MBio*, *7*(3), e00782-16. https://doi.org/10.1128/mBio.00782-16

van der Walt, S., Schönberger, J. L., Nunez-Iglesias, J., Boulogne, F., Warner, J. D., Yager, N., Gouillart, E., Yu, T., & contributors, the scikit-image. (2014). scikit-image: image processing in Python. *PeerJ*, *2*, e453. https://doi.org/10.7717/peerj.453

Wadsworth, C. B., Arnold, B. J., Abdul, S. M. R., & Grad, Y. H. (2018). Azithromycin Resistance through Interspecific Acquisition of an Epistasis-Dependent Efflux Pump Component and Transcriptional Regulator in Neisseria gonorrhoeae. *MBio*, *9*(4), 10.1128/mbio.01419-18. https://doi.org/10.1128/mbio.01419-18

Wieneke, M. K., Felix, D., Claudia, N., Dennis, G., Lena, K., Theo, T., Angelika, D., Christina, K., Jörg, G.-O., Peter, K., Holger, S., Bianca, S., Johannes, R., Jerzy-Roch, N., Janina, T., Julia, P., Marie, B. J., Mariele, S., Miriam, A., … Kahl, B. C. (2021). Association of Diverse Staphylococcus aureus Populations with Pseudomonas aeruginosa Coinfection and Inflammation in Cystic Fibrosis Airway Infection. *MSphere*, *6*(3), e00358-21. https://doi.org/10.1128/mSphere.00358-21

Wingett, S. W., & Andrews, S. (2018). FastQ Screen: A tool for multi-genome mapping and quality control [version 2; peer review: 4 approved]. *F1000Research*, *7*(1338). https://doi.org/10.12688/f1000research.15931.2

Zarrella, T. M., & Khare, A. (2021). Systematic identification of molecular mediators underlying sensing of Staphylococcus aureus by Pseudomonas aeruginosa *BioRxiv*, 2021.10.24.465352. https://doi.org/10.1101/2021.10.24.465352

Zeden, M. S., Kviatkovski, I., Schuster, C. F., Thomas, V. C., Fey, P. D., & Gründling, A. (2020). Identification of the main glutamine and glutamate transporters in Staphylococcus aureus

and their impact on c-di-AMP production. *Molecular Microbiology*, *113*(6), 1085–1100. https://doi.org/https://doi.org/10.1111/mmi.14479

Zhao, H., Roistacher, D. M., & Helmann, J. D. (2018). Aspartate deficiency limits peptidoglycan synthesis and sensitizes cells to antibiotics targeting cell wall synthesis in Bacillus subtilis. *Molecular Microbiology*, *109*(6), 826–844. https://doi.org/https://doi.org/10.1111/mmi.14078

Supplementary Methods

Growth in chemically defined media

To test for the nutrient requirements of mutant and wild-type *S. aureus,* strains were grown in CDMG according to Hussain et al. (1991) with varying levels of aspartate and glutamate across six different conditions. The tested conditions were: 1) No asp or glu added (0A/0G), 2) 1.1 mM asp with no glu added (+A/0G), 3) 2.2 mM asp with no glu (++A/0G), 4) 1.1 mM asp with 1.0 mM glu (+A/+G), 5) 2.2 mM asp with 1.0 mM glu (++A/+G), 6) no asp with 1.0 mM glu (0A/+G). This array of nutrient conditions was used to test the growth of the evolved isolate (EV2), JE2 *gltT*::Tn, and ancestral JE2 Growth rate (r - $hour^{-1}$) and area under the curve integral (AUC – OD_{600}xhour) were calculated using the program growthcurver (https://github.com/cran/growthcurver)(Sprouffske & Wagner, 2016). Growth assays were conducted in 96 well microtiter plates for 24 hours at 37°C with continual shaking. Growth was measured via OD_{600} measurements taken every 20 minutes (**Supplementary Table 2**).

Supplementary Results

gltT disruption does not alter *S. aureus* growth in chemically defined media

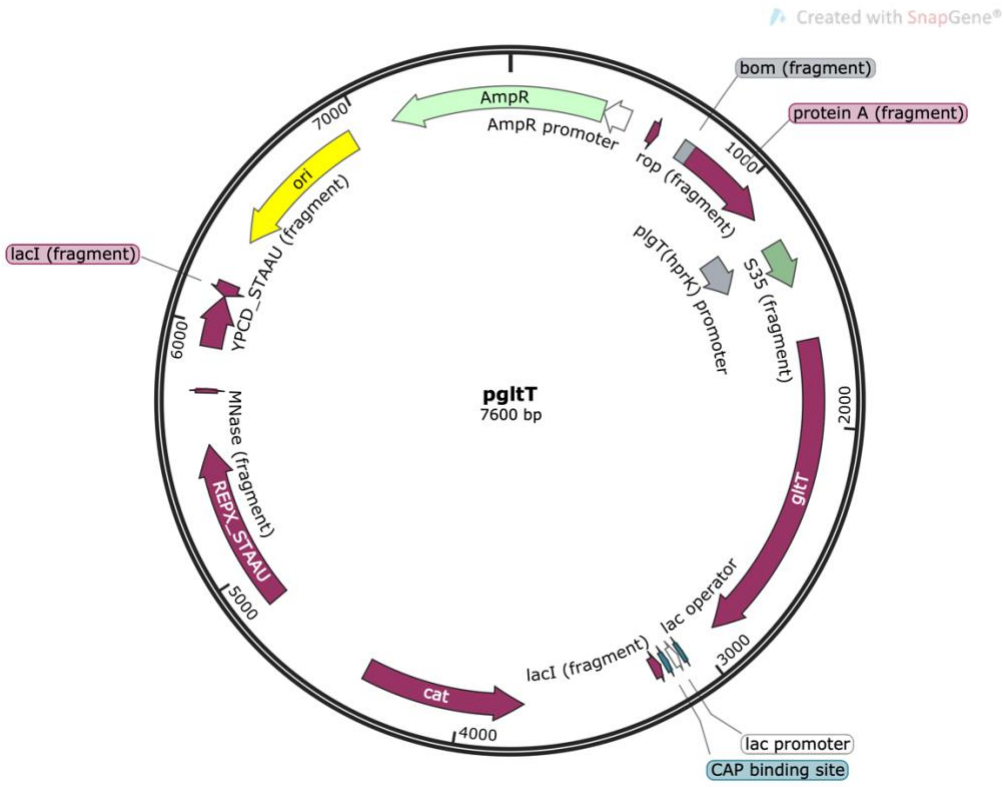
When grown alone in nutrient rich LB media *gltT* mutants, JE2 *gltT*::Tn and EV2 were not observed to have significant fitness differences compared to the parental JE2 (**Supplementary Figure 3**). We did not find statistically significant growth rate differences for any CDMG condition tested between wild-type JE2, JE2 *gltT*::Tn or *gltT* complemented strain JE2 *gltT*::Tn(pglT). We noted that all strains grew slower in the absence of glutamate (**Supplementary Table 2**). Additionally, complemented strain JE2 *gltT*::Tn(pgltT) had a significantly lower AUC value compared to JE2 *gltT*::Tn in the conditions with aspartate and no glutamate, (+A/0G) and (++A/0G).

Gene	Gene length (bp)	Number of mutations					
		Absolute number of mutations			1kb normalized number of mutations		
		Putative loss of function	AA sequence altering	Synonymous	Putative loss of function	AA sequence altering	Synonymous
gltT	1278	1	114	2341	0.78	89.2	1831.77
gltS	1209	4	344	5539	3.31	284.53	4416.05
alsT	1461	0	450	4457	0	308.01	3050.65
rpoD	1107	0	12	1019	0	10.84	920.51
agrC	1245	14	1504	5196	11.24	1208.03	4173.49

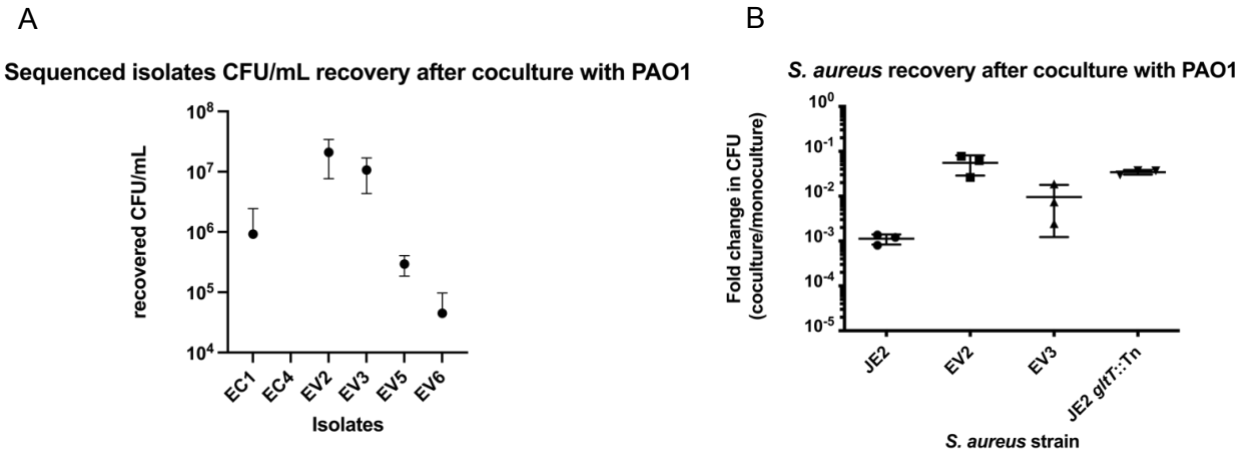
Supplementary Table 1. Summary of genes screened for variability across 444 diverse *S. aureus* genomes. The custom tool LIVID was used for genes, *gltT, gltS, alsT,* and *rpoD.* AGRVATE was used to screen variability of *agrC* with default parameters (Raghuram et al., 2022).

Strain	Chemically defined media condition					
	0A/0G	**+A/0G**	**++A/0G**	**+A/+G**	**++A/+G**	**0A/+G**
JE2 (r)	0.46 ± 0.15	0.52 ± 0.19	0.53 ± 0.14	1.12 ± 0.08	1.03 ± 0.09	0.90 ± 0.07
AUC	11.01 ± 4.90	12.35 ± 2.08	11.30 ± 2.52	15.72 ± 1.23	16.33 ± 1.97	15.48 ± 0.50
JE2 *gltT*::Tn (r)	0.59 ± 0.21	0.66 ± 0.05	0.65 ± 0.12	1.03 ± 0.11	1.11 ± 0.20	0.99 ± 0.11
AUC	13.23 ± 4.33	15.11 ± 1.21	14.53 ± 2.48	14.98 ± 1.89	14.95 ± 2.59	15.42 ± 2.01
JE2 *gltT*::Tn (pgltT) (r)	0.59 ± 0.13	0.42 ± 0.10	0.59 ± 0.09	1.24 ± 0.11	1.09 ± 0.30	0.84 ± 0.34
AUC	12.1 ± 3.10	9.79 ± 3.27	9.64 ± 2.70	15.95 ± 0.87	17.46 ± 2.15	16.69 ± 0.65

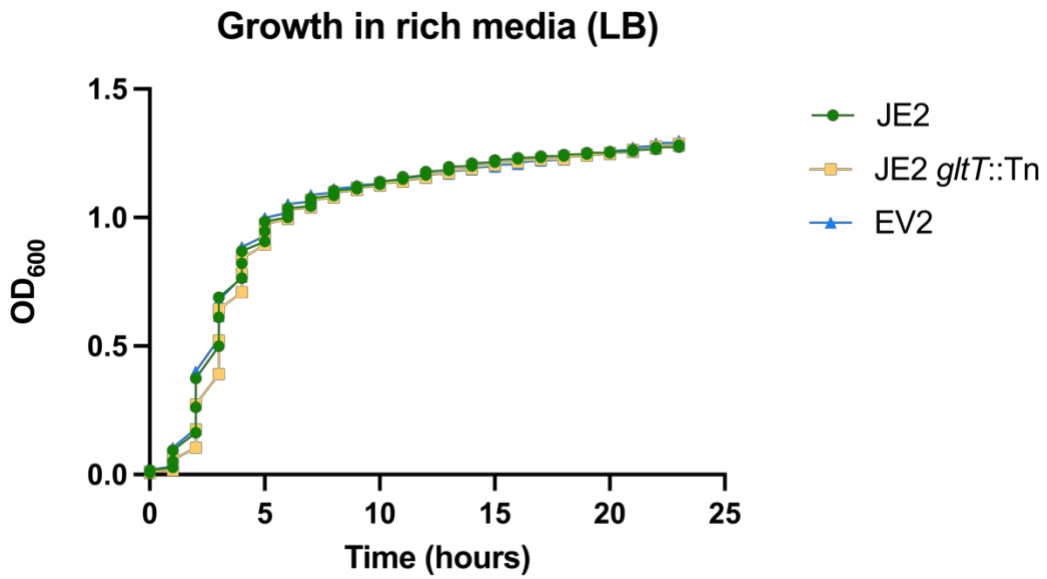
Supplementary Table 2. Wild-type JE2, JE2 *gltT*::Tn, and complemented strain JE2 *gltT*::Tn (pgltT) were tested in six different chemically defined media conditions with varying levels of glutamate and aspartate to determine the effects that aspartate and glutamate had together and individually on mutant *gltT* fitness. None of the conditions tested yielded statistically significant growth rate differences between any of the three strains when tested with nonparametric Kruskal-Wallis test with Dunn's correction. JE2 *gltT*::Tn (pgltT) had significantly lower values for AUC when compared to JE2 *gltT*::Tn in the +A/0G and ++A/0G conditions (p-values p=0.0144, and p= 0.0267 respectively - nonparametric Kruskal-Wallis test with Dunn's correction). Statistical analysis performed using GraphPad Prism 9.



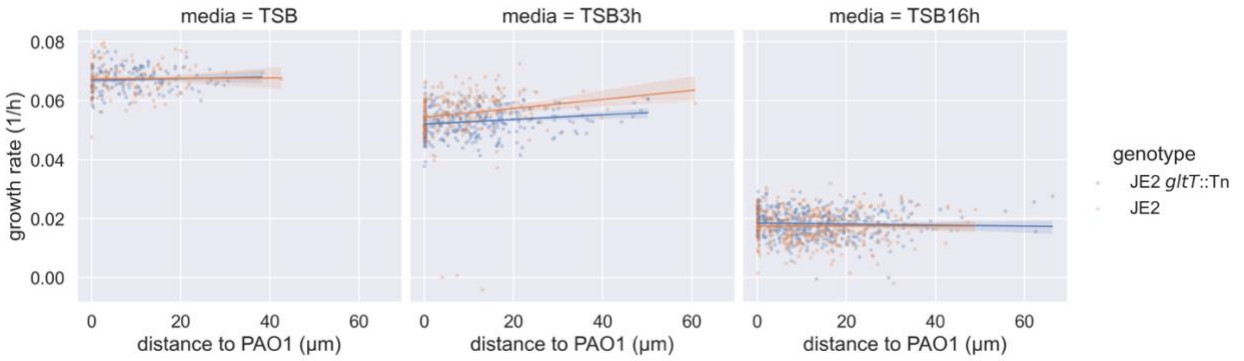
Supplementary Figure 1. Map of pgltT complementation vector made using the program Snapgene®. Construct was created by cloning the JE2 wild-type *gltT* coding sequence into the multiple cloning site of the pOS1.plgT *E. coli – S. aureus* shuttle vector. plgT promoter was found by matching plasmid sequence to the region 275 bp upstream of the *hprK* operon which includes the *lgT* gene as described in Bubeck Wardenburg et al. (2006). Primers used for amplifying *gltT* from wild-type JE2 were 5'-AGAGCTCGAGATGGCTCTATTCAAGAG-3' and 5'-AGATGGATCCTTAAATTGATTTTAAATATTCTTGAC-3' as described in Potter et al. (2020).



Supplementary Figure 2. A) Recovered CFUs/mL of all sequenced isolates after a 24-hour coculture period with PAO1 at a 1:1 initial ratio and initial OD_{600} of 0.01. Isolates represent single colony isolates taken from experimental or control populations that were selected for whole genome sequencing. EC1 and EC4 are control isolates from populations passaged in parallel but never cocultured with PAO1 during the evolution experiment. There were no *S. aureus* colonies recovered from EC4 when cocultured with PAO1 so therefore, there is no value plotted for this isolate. EV2 and EV3 isolates were isolated from experimental population EE1 where high CFU/mL recovery is indicative of their evolved *P. aeruginosa* tolerant phenotype. EV5 and EV6 were isolated from experimental population EE4 and their lower CFU/mL recovery is representative of the population's sensitivity to PAO1's presence even after 8 serial transfers. B) Fold change in CFU/mL recovered from strains JE2, EV2, EV3, and JE2 *gltT*::Tn. Fold change is used here to control for any growth differences among strains by dividing the CFUs/mL recovered after coculture by those recovered from corresponding *S. aureus* monocultures. JE2 *gltT*::Tn shares the evolved *P. aeruginosa* tolerant phenotype with evolved isolates EV2 and EV3.



Supplementary Figure 3. Growth of *gltT* variants in rich LB media. Strains JE2, JE2 *gltT*::Tn and evolved isolate EV2 grow very similarly under nutrient rich conditions.



Supplementary Figure 4. Microcolony growth rates of wild-type JE2 and JE2 *gltT*::Tn plotted against distance to nearest PAO1 cell in rich media. Fluorescently labeled strains - JE2.GFP or JE2.RFP and JE2 *gltT*::Tn.GFP or JE2 *gltT*::Tn.RFP were cultured together with PAO1 on agar pads made from rich Trypticase Soy Broth (TSB) and depleted cell-free TSB collected from 3-hour and 16-hour PAO1 monocultures. Growth rates of micro colonies were measured using single cell microscopy image analysis. Wild-type JE2 growth advantage over mutant JE2 *gltT*::Tn slightly increases with distance to PAO1 in depleted TSB from a 3-hour culture. However, strains grow generally at the same rate in all other conditions. Data was collected over four replicates for each of the two strain combinations.

SUPPLEMENTARY REFERENCES

Bubeck Wardenburg, J., Williams, W. A., & Missiakas, D. (2006). Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. *Proceedings of the National Academy of Sciences*, *103*(37), 13831–13836. https://doi.org/10.1073/pnas.0603072103

Hussain, M., Hastings, J. G. M., & White, P. J. (1991). A chemically defined medium for slime production by coagulase-negative staphylococci. *Journal of Medical Microbiology*, *34*(3), 143–147. https://doi.org/https://doi.org/10.1099/00222615-34-3-143

Potter, A. D., Butrico, C. E., Ford, C. A., Curry, J. M., Trenary, I. A., Tummarakota, S. S., Hendrix, A. S., Young, J. D., & Cassat, J. E. (2020). Host nutrient milieu drives an essential role for aspartate biosynthesis during invasive Staphylococcus aureus infection. *Proceedings of the National Academy of Sciences*, *117*(22), 12394–12401. https://doi.org/10.1073/pnas.1922211117

Raghuram, V., M, A. A., Qi, L. H., A, P. R., B, G. J., & D, R. T. (2022). Species-Wide Phylogenomics of the Staphylococcus aureus Agr Operon Revealed Convergent Evolution of Frameshift Mutations. *Microbiology Spectrum*, *10*(1), e01334-21. https://doi.org/10.1128/spectrum.01334-21

Sprouffske, K., & Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics*, *17*(1), 172. https://doi.org/10.1186/s12859-016-1016-7

Chapter III- **Intraspecific Diversity and Dynamics of *Staphylococcus aureus* Populations Isolated from Cystic Fibrosis Respiratory Infections**

Ashley Alexander [a,d,e], Hui Qi Loo [b,e], Lauren Askew [c,e], Vishnu Raghuram [c,d,e], Timothy Read [d], Joanna Goldberg [e]

[a] Population Biology, Ecology, and Evolution Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia, USA

[b] Department of Biology, Emory University, Atlanta, Georgia, USA

[c] Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia, USA

[d] Division of Infectious Diseases and Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USA

[e] Department of Pediatrics, Division of Pulmonology, Asthma, Cystic Fibrosis, and Sleep, Emory University School of Medicine, Atlanta, Georgia, USA

Author contributions

AMA – designed and supervised empirical data collection.

HQL - compiled AMR data and visualized in **Figure 3**. She also conducted experiments analyzing the dynamics between co-occurring MRSA and MSSA lineages and designed **Figures 5 and 6**. She also designed the original layouts for **Tables 1 and 2**.

LA – assisted with phenotyping all patient samples and collecting data used in **Table 1**.

VR – created the agrVATE tool used to identify agr types among samples and assisted with the development of these conclusions.

TDR – Supervised AA, HQL and VR. Resources from his group were used to run sequencing analysis.

JBG – Supervised AA, HQL, VR and LA. Conceived of the project itself and secured resources and funding.

Abstract

Multispecies bacterial infections have the potential to harbor significant intraspecific diversity. Traditional methods for sampling infections generally involve isolating one or two single colonies from a given sample, which limits the amount of diversity that can be observed by clinicians and researchers. In this study we sought to investigate the intraspecific diversity among *Staphylococcus aureus* isolated sputum samples from three different patients with cystic fibrosis. We were able to observe diversity across phenotypic and genomic scales. Additionally, we conducted an in-depth investigation of two co-isolated lineages from a single patient and observed their population dynamics with and without antibiotics over both a 24-hour growth period and over a longer time scale with a stepwise evolution experiment. Altogether, our observations here demonstrate the potentially clinically relevant diversity that can be missed with general sampling methods. More broadly, this work illustrates the intraspecific dynamics that can occur within a long-term infection and the potential we have to observe those dynamics by taking an ecologically minded approach to sampling.

Introduction

Staphylococcus aureus became the most common bacteria infecting individuals with the genetic disease, cystic fibrosis (CF), in the early 2000s, surpassing *Pseudomonas aeruginosa* and other prevalent gram-negative pathogens isolated from expectorated sputum (Cystic Fibrosis Foundation, 2021). Like many CF-associated pathogens, *S. aureus* is a species with immense diversity across lineages (Petit III & Read, 2018) and it has been observed that mixed populations of unrelated *S. aureus* strains can occur within an individual with CF (Long et al., 2020). However, current clinical surveillance techniques do not allow for the detection of multiple lineages of *S. aureus* within a single sample as only one or two colonies are selected (Long et al., 2020b).

In this study we sought to take an in-depth look at the diversity within populations of *S. aureus* isolated from fresh sputum collected from three patients by sampling both single colonies and whole-population pool samples. In one patient, we were able to isolate two unrelated lineages that made up significant portions of the *S. aureus* population in the sampled sputum. We further investigated the dynamics of these co-occurring populations, estimating their relative fitness in rich media, growth dynamics in the presence of antibiotics, and their influence on each other's ability to increase antimicrobial resistance. Altogether, and in the context of the patient's treatment history, our findings show that intraspecific diversity can have significant clinical implications when it comes to traits related to disease severity and treatment potential like antimicrobial resistance, hemolysis, and quorum sensing activity. In the future, methods that account for and quantify intraspecific diversity may assist in the design of more targeted treatment plans for individuals with chronic infections and would inform future research on population dynamics in long-term infections.

Methods

Sample collection

The labs of Dr Stephen P. Diggle and Sr. Samuel P. Brown at the Georgia Institute of Technology generously shared *S. aureus* populations isolated from fresh CF sputum collected at the Emory CF clinic (Vanderwoude et al., 2023). Sputum isolated from patients on the same day had been sampled by diluting collected sputum and spreading on Mannitol Salt Agar (MSA). Plates were then shared with our group and after 24-48 hours of growth at 37°C 8 single colonies were picked and inoculated into 3 mL of lysogeny broth (LB; Teknova). Liquid cultures were incubated overnight at 37°C in a rotating platform before being restruck on *Staphylococcus* Isolation Agar (SIA; BD Difco TSA with 7.5% NaCl) and made into 25% glycerol freezer stocks after incubating overnight at 37°C. All remaining colonies were scraped together into a 'pool'

sample which was resuspended in LB media and 25% glycerol and frozen directly. Samples Sa_92-Sa_97 are single colony isolates from patient 1 and Samples Sa_90*P* and Sa_91*P* were pool samples from the same patient (CFBR ID 623). Samples Sa_101-Sa_104 and Sa_106-Sa_109 are single colony isolates from patient 2 and its pools from this patient are Sa_100*P* and Sa_105*P* (CFBR ID 196) and samples Sa_111-Sa_118 were single colonies isolated from patient 3 and Sa_110*P* is this patient's pool sample (CFBR ID 311).

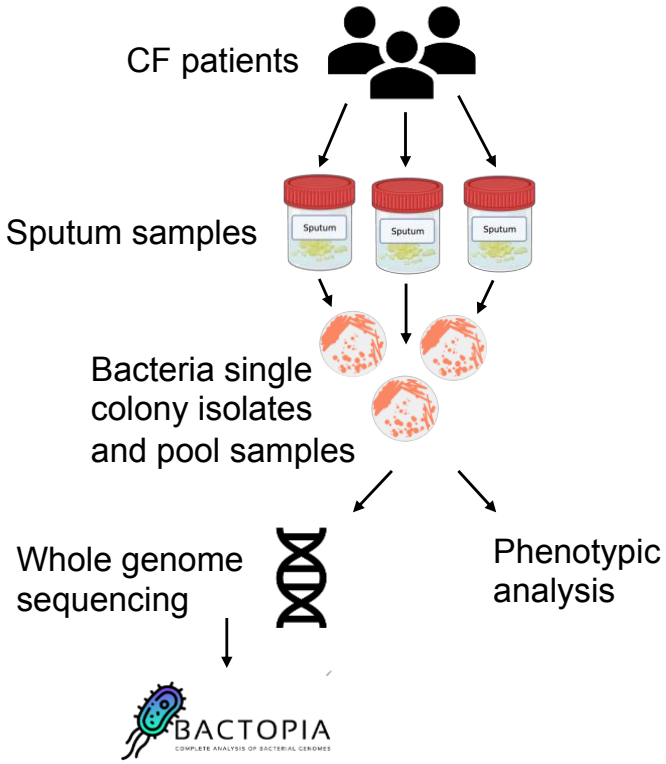


Figure 1. Sampling methods. Method for which isolates were processed from three patients' fresh sputum samples. 6-8 single colony isolates were archived per patient sample and 1-2 pool samples were taken for each sputum sample by scraping all remaining colonies. Isolates were then phenotypically and genotypically analyzed for their diversity of traits as described in the rest of this study.

Phenotyping

A qualitative analyses of colony morphology was conducted on all isolates including the appearance of colonies from pool samples. While no significant differences in size or texture were observed or recorded, color did vary across samples (white or yellow) and was recorded. Alpha toxin production was observed by noting clear hemolysis on sheep blood agar plates. Presence or absence of capsule was determined for all isolates by observing colony texture on Congo Red agar (CRA) plates (Bernardy et al., 2020; Schwartbeck et al., 2016). CRA was prepared as described in Freeman et al. (1989). MRSA status was determined for all isolates by streaking each isolate and pool sample on ChromAgar™ MRSA plates and observing whether or not purple pigmented colonies appear, indicating methicillin resistance (Samra et al., 1998).

Table 1. Compilation of genotypes and phenotypes of CF clinical isolates

Patient	Isolate ID	Isolate Type	Sequence Type	*agr* group	MRSA status by ChromAgar™	Colony Color	Alpha toxin Hemolysis	Polysaccharide production on Congo Red
1 (CFBR ID: 623)	Sa90 *P*	Pool	72	1	S	White	+	Red/Matte (overproducer)
	Sa91 *P*	Pool	72	1	S	White	+	Red/Matte (overproducer)
	Sa92	Single colony	72	1	S	White	+	Red/Matte (overproducer)
	Sa93	Single colony	72	1	S	White	+	Red/Matte (overproducer)
	Sa94	Single colony	72	**	S	White	+	Red/Matte (overproducer)
	Sa95	Single colony	72	1	S	White	+	Red/Matte (overproducer)
	Sa96	Single colony	72	**	S	White	+	Red/Matte (overproducer)
	Sa97	Single colony	72	1	S	White	+	Red/Matte (overproducer)
2 (CFBR ID: 196)	Sa100 *P*	Pool	5* 6/7	2	R	Yellow	+	Red/Matte (overproducer)
	Sa101	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa102	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa103	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa104	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa105 *P*	Pool	5* 6/7	2	R	Yellow	+	Red/Matte (overproducer)
	Sa106	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa107	Single colony	5* 6/7	**	S	Yellow	+	Red/Matte (overproducer)
	Sa108	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
	Sa109	Single colony	5* 6/7	2	S	Yellow	+	Red/Matte (overproducer)
3 (CFBR ID: 311)	Sa110 *P*	Pool	5* 4/7	2(m)	R	Multicolored	+	Red/Mixed shiny and matte (normal/nonproducer)
	Sa111	Single colony	5	2(f)	R	Yellow	-	Red/Shiny (nonproducer)
	Sa112	Single colony	5	2(f)	R	Yellow	-	Red/Shiny (nonproducer)
	Sa113	Single colony	5	2(f)	R	Yellow	-	Dark/Matte colonies (normal)
	Sa114	Single colony	5	2(f)	R	Yellow	-	Red/Matte and shiny (normal/overproducer)
	Sa115	Single colony	5	2(f)	R	Yellow	-	Red/Shiny (nonproducer)
	Sa116	Single colony	5	2(f)	R	Yellow	-	Red/Shiny (nonproducer)
	Sa117	Single colony	8	1	S	Yellow	+	Red/Matte (overproducer)
	Sa118	Single colony	8	1	S	Yellow	+	Red/Matte (overproducer)

**, not a complete match; fraction after * indicates the number of loci that map to that ST out of the 7 total loci used to assign ST; +, clear hemolysis; -, no hemolysis. ** indicates that genome assembly failed for that sequence. (m) indicates that multiple agr groups were detected. (f) indicates that a frameshift mutation was identified in the *agrC* locus. MRSA status is denoted as resistant (R) and sensitive (S).

Whole genome sequencing

To isolate genomic DNA for sequencing, all isolates and pool samples were streaked on Staphylococcus isolation agar (SIA; BD Difco TSA with 7.5% NaCl) and incubated overnight at 37°C. The next day, cells were collected off plates with an inoculation loop (about a full-loop) and resuspended in 480 µl of EDTA. 20 µl of 10 mg/mL lysozyme and 20 µl of 5 mg/mL lysostaphin were then added to the resuspension and mixtures were incubated at 37°C to lyse cells. After incubation we proceeded with the rest of the protocol outlined for the Promega Wizard genomic DNA purification kit (Silberstein et al., 2018). Sequencing was conducted using the Illumina NextSeq 2000 platform at the Microbial Genome Sequencing Center (Pittsburgh, PA). Received genome sequences were evaluated for quality using the program FASTQC (Wingett & Andrews, 2018). Genome sequences were made publicly available at the NCBI accession PRJNA742745. Samples listed in this BioProject will have the prefix CFBR_EB_ before their sample number Sa###, numbers in this BioProject correspond to the same sample number in this chapter.

Analysis of Genomic Diversity

Raw fastq files were run directly through the Bactopia genome analysis pipeline (Petit III & Read, 2018). ARIBA outputs were used to determine MLSTs for all samples (Hunt et al., 2017). NCBI AMRFinderPlus was used to evaluate antimicrobial resistance associated genotypes (Michael et al., 2019) (**Figure 3**). All samples were run through agrVATE (https://github.com/VishnuRaghuram94/AgrVATE) separately from Bactopia to determine agr types for all isolates (Raghuram et al., 2022).

Population-level genomic analysis was conducted by processing fasta files with cutadapt to remove adapter sequences and spADES to generate assemblies (Martin, 2011; Prjibelski et al., 2020). The Python program pyani was used to calculate average nucleotide identity (ANI) within

patient samples and across all samples (Pritchard et al., 2016). The R-package pheatmap was used to generate a more detailed heatmap of sample ANI values as well as a dendrogram (Kolde, 2012) (**Figure 2**). The program Snippy (https://github.com/tseemann/snippy) was used to estimate the number of single nucleotide polymorphisms between a previous isolate taken from patient 2 in 2012 and the isolates taken in 2020 (Seeman, 2015).

Relative fitness of co-isolates

Two representative isolates were chosen from the co-isolated lineages from patient 3 (CFBR ID 311). Sa_112 (MRSA) and Sa_117 (MSSA) is how they will be referred to throughout the rest of this chapter. Plasmids containing the fluorescent proteins, DsRed, pHC48 (Ibberson et al., 2016), and GFP, pCM29 (Pang et al., 2010), were transformed into each strain, using methods outlined in Grosser & Richardson (2016).

Relative fitness was approximated by measuring strain dynamics in coculture and monoculture in lysogeny broth over 24 hours. Isolates were prepared by streaking on Trypticase soy agar and incubating overnight at 37°C. Single colonies were picked and cultured overnight and rotating in 3 mL of LB media at 37°C. Overnight cultures were diluted to an optical density 600 nm (OD_{600}) of 0.01. For cocultures, isolates were mixed in a 1:1 ratio that maintained an overall bacterial density of 0.01. 200µL of each diluted culture or coculture was added in triplicate to a black flat-bottom 96-well microtiter plate with lid. Undiluted overnight cultures were used as optics gain scaling references for DsRed and GFP fluorescence measurements. Plates were incubated for 24 hours in a Synergy H1 Hybrid Multi-Mode microplate reader at 37°C with continuous double orbital shaking at 280 cpm. Readings of optical density at 600 nm and fluorescence were measured in bottom optic mode every 20 minutes. Fluorescence measurements were taken for GFP (excitation 485 nm/emission 515 nm) and DsRed (excitation at 554 nm/emission 586 nm). Area under the curve (AUC) and growth rate (r) were calculated

for each well using the R-package growthcurver (Sprouffske & Wagner, 2016). Each colored version of each isolate was cultured in triplicate in coculture and monoculture during each experiment and this experimental setup was repeated in 3 independent experiments.

Co-isolated strain growth in the presence of antibiotics

Isolates were streaked on TSA for single colonies, as described above. Selected colonies were picked and cultured in a rolling incubator overnight at 37°C in 3 mL of cation-adjusted Mueller-Hinton broth (CAMHB), or CAMHB + 2% NaCl for growth with oxacillin (CLSI, 2018). Overnight cultures were then diluted to an initial OD_{600} of 0.05 before being inoculated in 96-well plates with antibiotic gradients from 0.25 µg/mL to 128 µg/mL for all antibiotics tested with two-fold increases across the plate and antibiotic-free control wells. Base-media for all antibiotics tested was CAMH or CAMHB + 2% NaCl for oxacillin tests. Strains Sa_112 (MRSA) and Sa_117 (MSSA) were tested in monoculture and coculture with antibiotics: oxacillin, erythromycin, and vancomycin. Cocultures were inoculated in a 1:1 ratio with a total initial OD_{600} of 0.05. All plates were incubated for 24 hours with continual shaking at 37°C in a Synergy H1 Hybrid Multi-Mode and measurements were taken for OD_{600}, GFP and DsRed signals as described above.

Experimental evolution of co-isolated strains in the presence of antibiotics

A stepwise serial transfer evolution experiment was designed and modeled after methods outlined in Adamowicz et al. (2020). Bacterial cultures were initially prepared from isolates Sa_112 (MRSA) and Sa_117 (MSSA) as was done for the analysis of relative fitness of co-isolates, streaking on TSA for single colonies and culturing overnight in 3 mL of LB media. Isolate cultures were diluted to an initial OD_{600} culture density of 0.1. Antibiotic conditions were setup as described above with gradients from 0.25 µg/mL to 128 µg/mL with two-fold increases across plates and antibiotic-free wells used as controls. Cocultures were inoculated at an initial

ratio of 1:1 with a final initial OD_{600} of 0.05 for each strain. Plates were incubated for 24 hours at 37 °C with shaking at 450 rpm per growth period.

After each growth period, cells were transferred to a new plate with fresh media and antibiotics. 2 µL of culture from the previous growth period was added into 196 µL of media with 2 µL of antibiotic stock solution which would confer the correct gradient concentration in the new plate. Culture mixtures were transferred such that 1 µL of culture from wells of each antibiotic concentration was inoculated into a new well of the same antibiotic concentration, and 1 µL of this same culture mixture was added to a new well with 2-fold higher antibiotic concentration, resulting in a stepwise serial transfer method. Transfers were carried out whether or not populations had appeared to have gone extinct. This was repeated for 5-6 growth periods. When populations evolved to the upper concentration limit, the upper end of the gradient was increased, and the lowest concentration was removed. The transfer process is outlined in **Figure 6c**.

OD_{600} and fluorescence readings were taken at the end of each growth period and the 90% minimum inhibitory concentration (MIC_{90}) was calculated for each isolate. Growth was defined as an increase in OD_{600} measurement was at least 10% of the antibiotic free control well for a given growth period.

Clinical data collection

Clinical laboratory testing results for patients 1 and 2 (CFBR ID 623 and CFBR ID 196, respectively) were made available to us from the Cystic Fibrosis Biospecimen Repository (CFBR) and are outlined in **Table 2.** Additional metadata and treatment history of patient 3 (CFBR ID 311) was also made available by the CFBR.

Results

Phenotypic analysis of all isolates

To assess the phenotypic diversity of all isolates, within and between patients, we first started by assessing the colony morphology of all samples on TSA plates. At initial processing, two small colonies were selected from Patient 1's MSA plate, however, upon re-streaking on SIA these isolates were found to be non-viable. All other isolates from all three patients appeared very similar to each other in colony size and texture. Notably though, colony color or pigment varied across isolates. Patient 1's isolates were all white in appearance on SIA plates while all of Patient 2's isolates were yellow. Patient 3's single colony isolates were all yellow except the pool sample Sa_110 had both white and yellow colonies (**Table 1**).

All isolates, pool and single colonies from patients 1 and 2 were positive for alpha toxin hemolysis when cultured on sheep blood agar. In patient 3 only the pool sample and isolates Sa_117 and Sa_118 were positive for alpha hemolysis (**Table 1**).

All isolates, pools and single colonies, from patients 1 and 2 were determined to be overproducers of polysaccharide based on their rough and matte appearance on Congo Red Agar (Bernardy et al., 2020). Isolates Sa_110*P*-116 from patient 3 presented with some variation on CRA with isolates Sa_110*P* and Sa_114 having both textured and shiny red colonies and therefore classified as mixtures of normal and overproducing cells. Isolate Sa_113 appeared as dark shiny colonies classifying it as a normal producer based on the darker color. Isolates Sa_115 and Sa_116 were bright red and shiny on plates classifying them as clear nonproducers. Finally, isolates Sa_117 and Sa_118 from patient 3 were determined to be overproducers based on their red and matte appearance (**Table 1**).

ChromAgar™ plates were used as an additional test of MRSA status alongside genomic analysis for all isolates. Colony growth and pigmentation on ChromAgar™ can be reliably used to detect methicillin resistance in *S. aureus* as described in Samra et al. (1998). All isolates from patient 1 showed no growth on ChromAgar™ plates and therefore were determined to be MSSA. All single colony isolates from patient 2 were determined to be MSSA, however both pool samples showed some pigmented colonies on ChromAgar™ plates. Samples Sa_110*P*-Sa_116 from patient 3 including the pool sample Sa_110*P* were determined to be MRSA, however samples Sa_117 and Sa_118 from the same patient were determined to be MSSA based on their lack of growth on ChromAgar™ (**Table 1**).

Genotypic analysis

Whole genome sequence data was successfully obtained for all isolates. The first analysis we ran on the genomic dataset was for average nucleotide identity (ANI). ANI was determined for all sequences that were able to be assembled into contigs. Isolates Sa_94, Sa_96 (patient 1) and Sa_107 (patient 2) failed to be assembled and were not included in the ANI analysis. Across all samples ANI values ranged from 99.99982% (Sa_112 vs Sa_115) to 98.97548% (Sa_118 vs Sa_101) similar. ANI distances confirmed that isolates from patient 1 were generally homogenous with pool sample Sa_91*P* being slightly more similar to other isolates not taken from patient 1 (**Figure 2B**). Isolates from patient 2 were all genotypically identical according to ANI and pool samples were indistinguishable from single colony isolates. When comparing ANI across isolates from patient 3 samples Sa_117 and Sa_118 were distant from other patient 3 isolates and distant from other isolates across the sample set. This was further confirmed when looking at the multi-locus sequence type (MLST) identities of samples denoted in **Figure 2A** as sequence type (ST). Samples Sa_117 and Sa_118 were identified as belonging to the ST 8 lineage while all other isolates from patient 3 belong to the ST 5 lineage. The pool sample

Sa_110*P* from patient 3 is similar to other isolates from the same patient based on ANI, however, it is not placed in the same clade as other patient 3 ST 5 isolates.

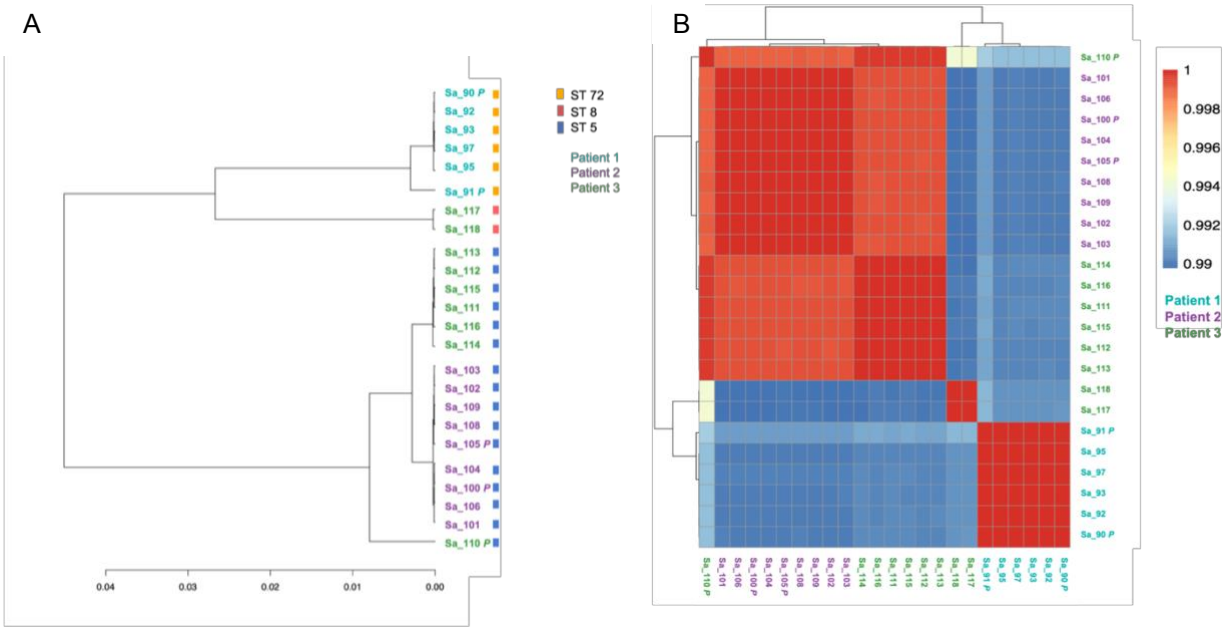


Figure 2. Genetic distances across all patient isolates. A) Dendrogram generated with the R-package pheatmap based on average nucleotide identity values calculated using the python program pyANI. ST denotes the multi locus sequence type identity which was determined for each isolate in separate analysis using the program NCBI AMRFinderPlus built into the Bactopia sequence analysis. Isolates from patient 2 are denoted as ST 5, however only 6 out of 7 sites matched the ST 5 MLST signature. Sa_110*P* is denoted as ST 5 as well however only 4 out of 7 sites matched the ST 5 MLST signature. Scale is based on ANI difference (0.00 = 100% identical sequences). B) Heatmap visualizing the ANI distances between all patient samples. Note, samples Sa_94, Sa_96 and Sa_107 failed to be assembled and were not included in the ANI analysis. *P* denotes pool samples.

Genes associated with antibiotic resistance were identified in the genomic data for all isolates using the NCBI AMRFinderPlus program as part of the Bactopia genomic analysis pipeline (Michael et al., 2019; Petit & Read, 2020). Patient 1 and 2's isolates all shared the same antibiotic resistance gene profiles within patient samples. All isolates from these patients had alleles associated with resistance to fosfomycin and tetracycline. While no isolates from patients 1 and 2 had methicillin resistance associated genotypes, *mecA*, *mec1* or *mecR1* isolates from patient 1 and 2 had variants of *blaI,* and *blaZ* associated with beta-lactam resistance. Additionally, all isolates from patient 1 had alleles *aadD1* and *erm(A)* associated with resistance to aminoglycosides and erythromycin. Patient 2 isolates also had the genotype in *blaR1* associated with beta-lactam resistance, a signature not present in any patient 1 isolates. All alleles associated with antibiotic resistance in the NCBI AMRFinderPlus database were identified as present in the genomes of isolates Sa_110*P*-Sa_116 from patient 3, including all *mec* alleles associated with methicillin resistance. Isolates Sa_117 and Sa_118 however, only had genetic signatures associated with fosfomycin, tetracycline and the three *bla* beta-lactam resistance alleles in *blaI, blaR1,* and *blaZ.*

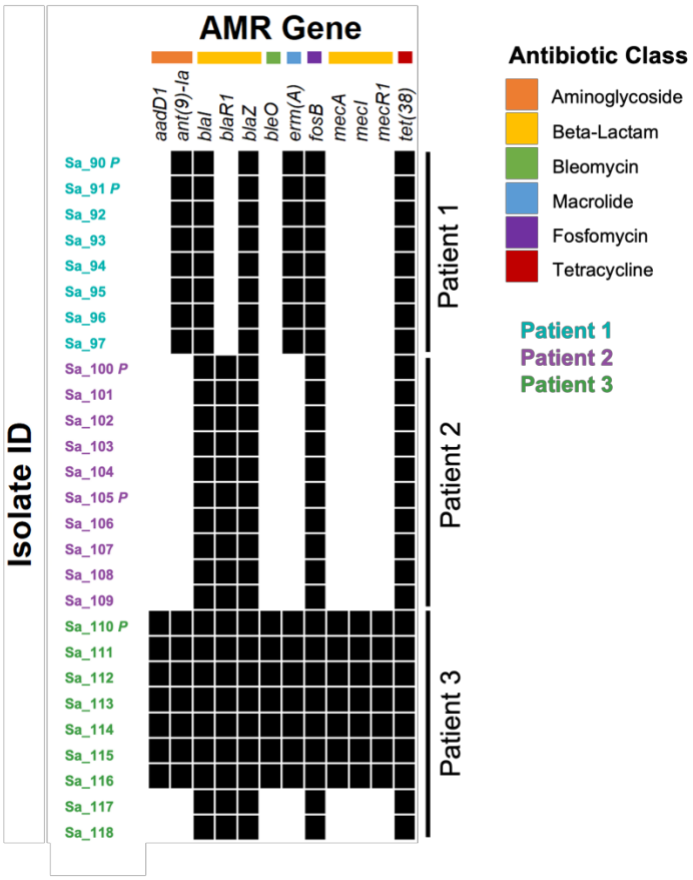


Figure 3. Antimicrobial resistance genetic signatures. Black boxes indicate presence of allele associated with antimicrobial resistance. Colors across the top of the figure group genes by the antibiotic class their resistance is associated with. *P* next to isolate ID denotes pool samples.

All genome assemblies were run through the program agrVATE to determine their *agr* type and nature of the *agrC* locus (Raghuram et al., 2022). All assembled genomes from patient 1 were identified as *agr* group 1 with no frameshifts in the *agrC* locus. All assembled genomes from patient 2 were identified as group 2 with no frameshifts in the *agrC* locus. Isolates Sa_111-Sa_116 from patient 3 were classified as *agr* group 2. AgrVATE was able to identify multiple *agr* types present in isolate Sa_110*P*'s coding sequence – the pool sample for patient 3, it was ultimately classified as *agr* group 2. Additionally, isolates Sa_111-Sa_116 were identified as having frameshifts present in their *agrC* reading frame. Isolates Sa_117 and Sa_118 from patient 3 were identified as *agr* group 1 with no frameshifts in their *agrC* coding sequence (**Table 1**).

Clinical testing and metadata

When sputum was collected from patients 1 and 2 the Emory CF clinic also processed *S. aureus* isolates from the same collected sputum. The clinical lab shared with us results from those patient's antibiotic susceptibility testing. Patient 1's clinically isolated *S. aureus* was identified as being resistant to clindamycin and susceptible to oxacillin, tetracycline, trimethoprim and sulfamethoxazole and vancomycin. Clinically isolated *S. aureus* was identified as being resistant to clindamycin as well as oxacillin and susceptible to daptomycin, linezolid, tetracycline, trimethoprim and sulfamethoxazole and vancomycin (**Table 2**).

Table 2. Clinical Antibiotic Resistance report of S. aureus isolates

Drug	Antibiotic Class	Patient		
		1	2	3
Clindamycin	Lincosamide	Resistant	Resistant	*NA*
Daptomycin	Lipopeptide	*NA*	Susceptible	*NA*
Linezolid	Oxazolidinone	*NA*	Susceptible	*NA*
Oxacillin	Beta-Lactam	Susceptible	Resistant	*NA*
Tetracycline	Tetracycline	Susceptible	Susceptible	*NA*
Trimethoprim/Sulfamethoxazole	Sulfonamide	Susceptible	Susceptible	*NA*
Vancomycin	Gyclopeptide	Susceptible	Susceptible	*NA*

[a]*NA:* susceptibility test was not performed by the clinical microbiology laboratory

Patient 3's sputum sample was taken shortly before the clinic was closed due to Covid-19, therefore likely the reason they were unable to process this sample for antibiotic susceptibilities. Patient 3 (CFBRID 311) had also completed a month-long intensive antibiotic treatment with vancomycin one month before the sample was taken (IV vancomycin treatment); source: CFBR.

Our group had previously processed a sample taken from patient 2 in February of 2012 (CFBR ID 196) which was identified as ST 225 and MRSA (Bernardy et al., 2020). Using the program snippy we were able to estimate the number of SNPs between the 2012 isolate and an isolate from the 2020 sample set, Sa_102. Snippy identified 958 variants between the two genomes (https://github.com/tseemann/snippy) (Seeman, 2015).

Relative fitness of MRSA and MSSA co-isolates

We sought to gain further understanding of the population dynamics of the two co-isolated lineages from patient 3. To do this we selected representative isolates: Sa_112 (MRSA) and Sa_117 (MSSA) to represent the co-occurring MRSA and MSSA populations, respectively. We then transformed plasmids containing GFP and DsRed markers into each isolate in order to be able to distinguish them in coculture. When isolates were grown together in coculture in rich media we noted that throughout a 24-hour growth period we were able to measure fluorescent signals from all labeled isolates suggesting that events of extinction potentially did not occur when these isolates were cocultured. We quantified total fluorescent signal for each isolate during a 24-hour growth period by computing AUC for DsRed and GFP measurements. We saw that when area under the curve (AUC) was normalized against each strain's signal in coculture with its unlabeled self, there were no significant differences in fluorescent protein output for either isolate (Sa_112 (MRSA) and Sa_117 (MSSA)) (**Figure 4A**). Notably, relative AUC values tended to be near or above 1, indicating that total fluorescent output was higher in cocultures than monocultures for both Sa_112 (MRSA) and SA_117 (MSSA). However, when

we measured each strains growth alone with OD_{600} measurements we saw that Sa_117 (MSSA) had a significantly higher growth rate than Sa_112 (MRSA) (**Figure 4B**).

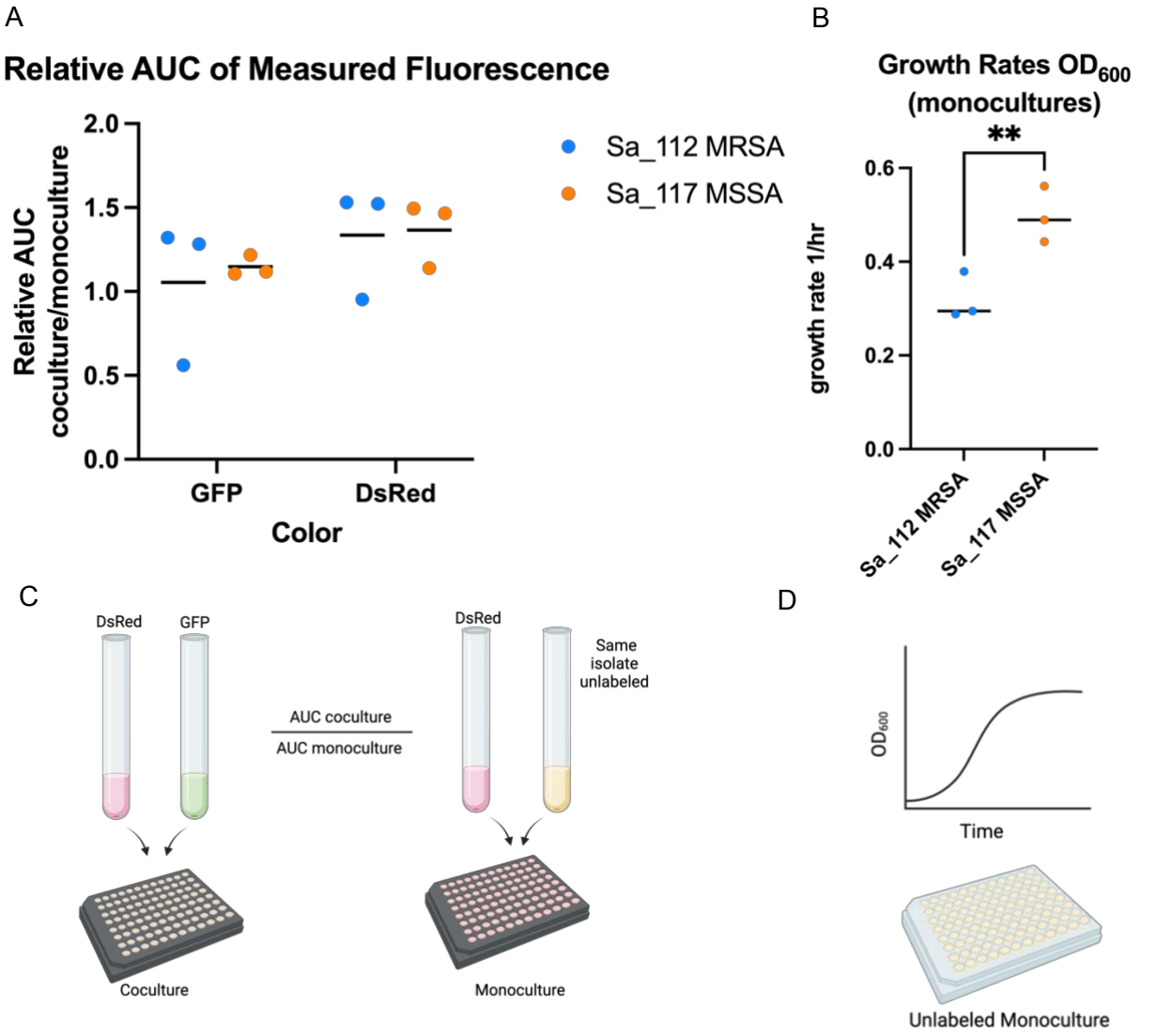


Figure 4. Relative fitness of co-isolated MRSA and MSSA. A) Growth of representative isolates Sa_112 (MRSA) and Sa_117 (MSSA) was quantified by calculating area under the curve (AUC) for fluorescent measurements of both DsRed and GFP versions of each isolate in coculture with the other. AUC measurements were normalized for each experiment by dividing coculture AUC values over fluorescent readings taken from monocultures with each isolate's labeled and unlabeled version grown under the same conditions. AUC was calculated using the R-package growthcurver. Lines indicate mean. B) Growth rates for each isolate when grown alone based on OD_{600} measurements. Total growth rate was calculated for each isolate using the R-package growthcurver. * Indicates p-value for paired t-test ($p<0.01$). C) Experimental design for data visualized in panel A with cocultured isolates being labeled with DsRed or GFP and their corresponding monoculture AUC values being collected from 1:1 cultures of the fluorescently labeled isolate and its unlabeled self. D) Experimental design for data visualized in panel B. Schematic panels C and D were generated using BioRender.

Growth in antibiotics of co-isolated MRSA and MSSA

We sought to measure the influence that the co-isolated MRSA and MSSA populations isolated from patient 3 had on each other in the presence of antibiotics. We quantified growth in coculture and monocultures by calculating AUC of DsRed readouts, where the isolate of interest was always labeled with the DsRed containing plasmid. In the presence of oxacillin, representative isolate Sa_112 (MRSA) achieved a much higher AUC than Sa_117 (MSSA) and a slightly lower AUC when it was in coculture with Sa_117 (MSSA). Sa_112 (MRSA) was able to maintain high levels of fluorescence output at oxacillin levels below 8 μg/mL after which it became significantly inhibited (**Figure 5A**). When grown in the presence of vancomycin however Sa_117 (MSSA) maintained the higher AUC at vancomycin concentrations below 1 μg/mL whereas Sa_112 (MRSA) was significantly inhibited at all vancomycin concentrations (**Figure 5b**). When grown in the presence of erythromycin Sa_112 (MRSA) maintained a consistent AUC value across all concentrations of erythromycin whereas Sa_117 (MSSA) was completely inhibited at all concentrations of erythromycin (**Figure 5C**).

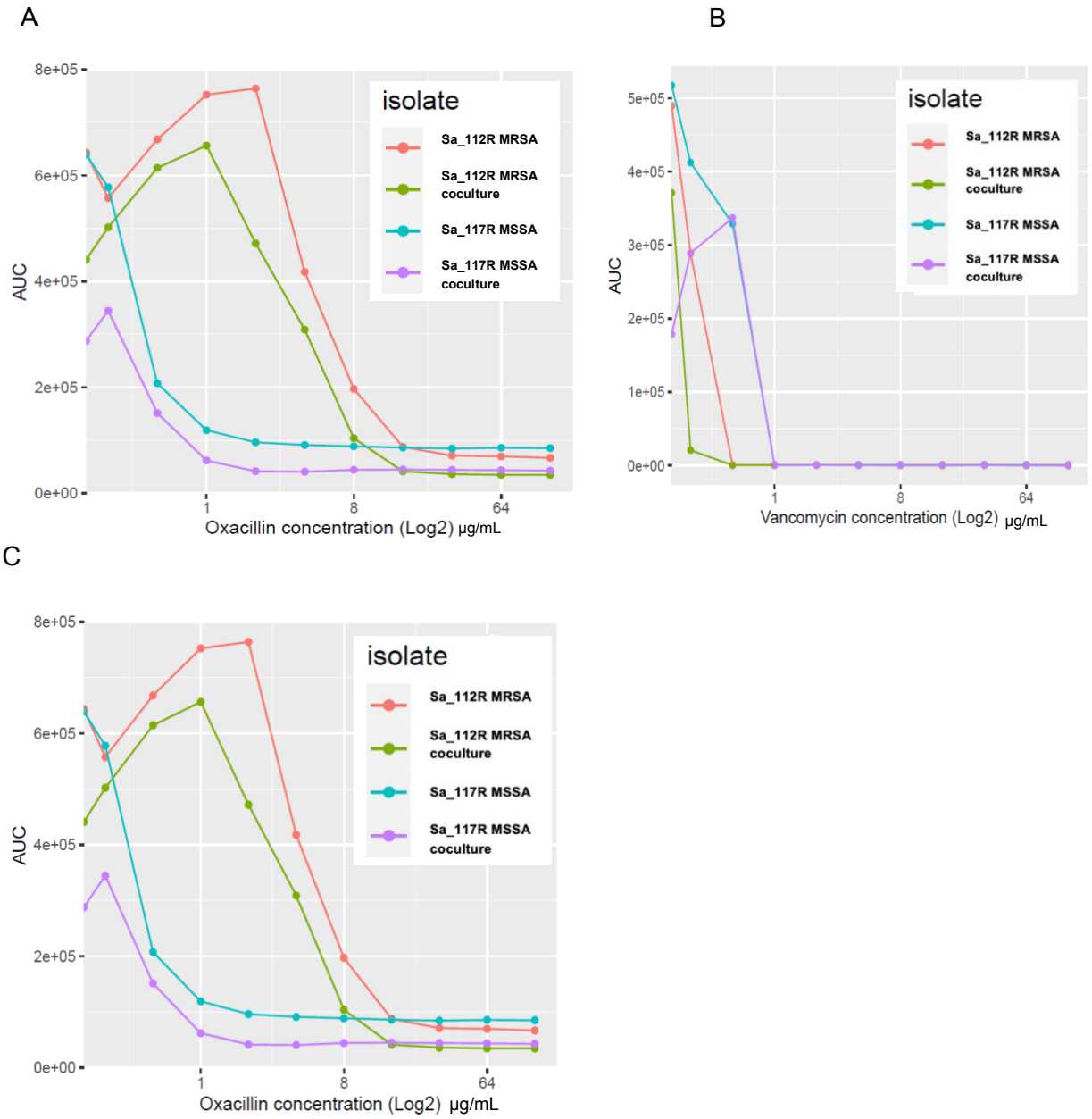


Figure 5. Growth of co-isolated MRSA and MSSA populations with antibiotics. Growth for each isolate was quantified by calculating the AUC of DsRed fluorescent measurements taken over a 24-hour growth period. Concentrations of antibiotics are expressed in units of µg/mL and increase 2-fold across a 96 well plate. A) growth in the presence of oxacillin B) growth in the presence of vancomycin C) growth in the presence of erythromycin. All plots are of single representative experiments.

Experimental evolution of co-isolates in the presence of antibiotics

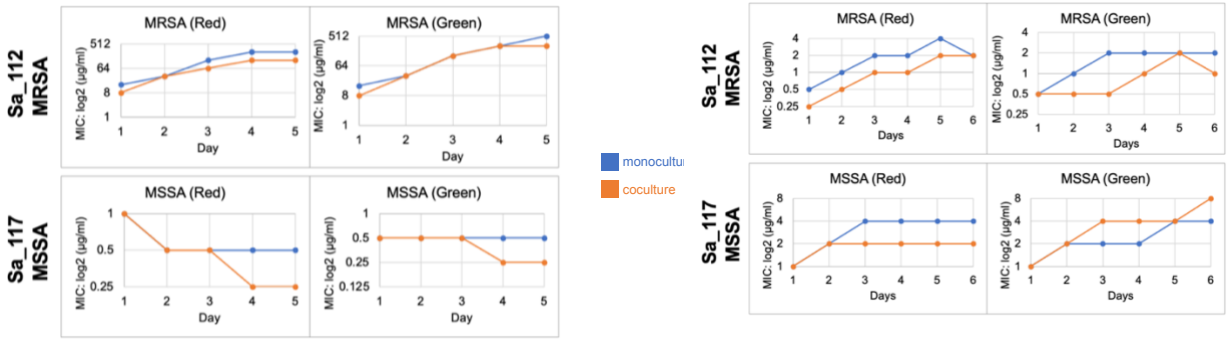
We hypothesized that co-isolated populations may benefit each other's ability to evolve and persist in the presence of oxacillin and vancomycin. To test this, we conducted a stepwise serial transfer evolution experiment where populations were exposed to increasingly higher concentrations of antibiotics over the course of 5 growth periods. Fluorescence signal was used to identify isolate growth and both color versions (DsRed and GFP) of each isolate was tested in the presence of both antibiotics in monocultures and cocultures.

In the case of oxacillin Sa_117 (MSSA) did not increase its antibiotic tolerance and it was not benefited from evolving in the presence of Sa_112 (MRSA). While Sa_117 (MSSA) was maintained in the coculture population with no antibiotics present it did appear to be lost from the coculture population at all levels of antibiotics after the third growth period. Sa_112 (MRSA) initially had an oxacillin MIC_{90} of 16 µg/mL when cultured alone and at the end of the 5 growth periods had reached an MIC_{90} of more than 256 µg/mL. Like Sa_117 (MSSA), Sa_112's (MRSA) ability to evolve increased antibiotic resistance was not greatly altered by the presence of its MSSA co-isolate Sa_117 (MSSA).

When evolved with vancomycin, Sa_117 (MSSA) began the experiment with an MIC_{90} of 1 µg/mL and achieved an MIC_{90} of at least 4 µg/mL after 6 growth periods. Again, evolving in coculture with its co-isolate Sa_112 (MRSA) did not greatly alter Sa_117's (MSSA) ability to evolve greater resistance to vancomycin. Despite starting at a lower initial MIC_{90} Sa_112 (MRSA) was also able to greatly increase its tolerance to vancomycin over the course of 6 growth periods. Unlike Sa_117 (MSSA) in oxacillin, Sa_112 (MRSA) persisted in coculture with its more-vancomycin-tolerant co-isolate; however, its increase in vancomycin tolerance was slower when in coculture.

A
Evolution in the presence of Oxacillin

B
Evolution in the presence of Vancomycin



C
Experimental evolution transfer process

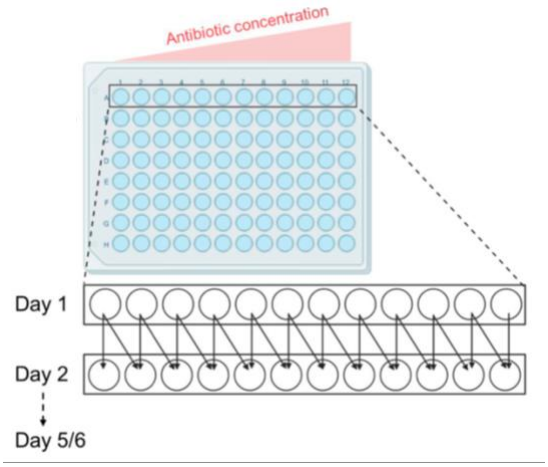


Figure 6. Stepwise experimental evolution of co-isolates alone and cocultured with oxacillin and vancomycin. A) Co-isolates Sa_112 (MRSA) and Sa_117 (MSSA) experimentally evolved in monoculture and coculture in the presence of increasing concentrations of oxacillin. Blue lines denote monoculture MIC_{90} values over 6 growth periods and orange lines denote MIC_{90} values of the indicated isolate when in coculture with its co-isolate. Antibiotic gradient increased at 2-fold increments and each fluorescent version of each isolate (DsRed or GFP) was tested.

Discussion

Observed diversity of *S. aureus* patient isolates

At initial phenotypic analysis we had concluded that the *S. aureus* isolates from patients 1 and 2 were nearly identical and that the only within-patient diversity captured in our sample set were the two unique strains identified in patient 3. However, after collecting more data on these samples, a more complex picture unfolded. In addition to the two identified strains, in patient 3 we suspect that there is another unsampled unique population based on the presence of both white and yellow colonies in the pool sample Sa_110*P* from this patient – no sample single colonies present as white on SIA (**Table 1**). It was interesting to see that the heterogeneity in patient 3's samples appeared in the genotypic analysis of Sa_110*P* as well with multiple *agr* types having been identified by the *in-silico* PCR program agrVATE (**Table 1**). ANI analysis also allows us to distinguish the two lineages sampled from patient 3 as truly distinct strains based on the definition outlined in Rodriguez-R et al. (2023), as the ANI values between isolates Sa_111-Sa_116 are all at most 99.00% similar to isolates Sa_117 or Sa_118. Additionally, we hypothesized that an unsampled lineage existed within patient 2 as both pool samples, Sa_100*P* and Sa_105*P* showed methicillin resistance by ChromAgar™ testing while all single colony isolates were MSSA (**Table 1**). Methicillin resistance was also noted by the CFBR for this sputum sample from patient 2 by the Emory CFBR (**Table 2**). A previous sample from patient 2 from 2012 analyzed by our group had also been identified as MRSA. Based on this previous isolate's ST (225) and the number of variants between it and one isolate from our 2020 sample set, we concluded that it is not part of the same lineage as the more recent isolates. It is possible that the previously sampled MRSA lineage, while no longer numerically dominant, persists in this patient. It is also possible that a new MRSA lineage was introduced more recently than 2012 and is maintained as a subpopulation. Upon ANI analysis of isolates, even patient 1 had some indications of heterogeneity in its pool sample Sa_91*P* with its slightly lower

ANI values when compared to rest of its co-isolates (**Figure 2**). We were surprised at how much diversity we were able to capture with our limited patient sample set and simple isolate collecting methods. The amount of clinically relevant diversity identified in our survey highlights the importance of bringing in ecologically minded sampling practices to patient sample surveys especially in the case of long-term infections like those associated with CF.

Diving deeper into the population dynamics and intraspecific interactions between two co-isolated populations we found that while each lineage exhibited significant differences in their traits and fitness that these unrelated populations could likely coexist especially in a fluctuating environment. Of particular note is the observation that despite the significant differences in their growth rates Sa_112 (MRSA) and Sa_117 (MSSA) are able to persist together in coculture for 24 hours (**Figure 4**). Sa_112 (MRSA) should not only be at a competitive disadvantage due to its slower growth but also having a non-functional *agrC* locus as predicted by our agrVATE analysis would limit its ability to quorum sense and activate key virulence factors such as its observed lack of production of alpha toxin and corresponding hemolysis (Raghuram et al., 2022). Antibiotics, however, turn the tables. Patient 3's MRSA isolates had every antimicrobial resistance associated allele in the NCBI AMRfinderPlus search function (Michael et al., 2019) (**Figure 3**). When tested for its growth in the presence of oxacillin and erythromycin it clearly outcompeted representative isolate Sa_117 (**Figure 5**). However, in the case of vancomycin Sa_117 had the upper hand with a higher initial vancomycin MIC it was able to maintain a larger proportion of the coculture at vancomycin concentrations below 1µg/mL (**Figure 5**).

Based on their ability to coexist during a 24-hour growth period and the potential for shared goods in the case of oxacillin, we expected there to be a benefit for either or both representative isolates Sa_112 (MRSA) and Sa_117 (MSSA) when evolving in the presence of oxacillin and vancomycin. This hypothesis was not upheld by our observations and instead it became clear

that initial MIC_{90} was a critical factor in an isolates ability to evolve increasing levels of resistance to a given antibiotic. We also observed that growing in coculture with a co-isolate did not appear to have any significant effect on an isolates ability to evolve greater resistance to oxacillin or vancomycin (**Figure 6**).

Taking into consideration the known treatment history of patient 3 (CFBR ID 311), we postulate that the lineages present in the patient's sample are representative of a period of fluctuation and that unless further treatment was administered, that the MSSA lineage likely overtook the MRSA lineage based on its higher growth rate and functional *agrC* locus. It is also likely that it was this MSSA population that caused more severe symptoms in the patient, raising the need for treatment with vancomycin. Additionally, the dysfunctional *agrC* locus in the MRSA population could signify its long-term presence in the host as reduced *agr* activity is associated with persistence in chronic infections and immune evasion (Kwiecinski & Horswill, 2020). With this in mind, it is possible that antibiotic treatment combined with immune activity would eliminate the faster growing MSSA population and the chronic-type MRSA population would reassume its previous state. It is interesting to have captured this dynamic period within a single patient's infection. However, more broadly our sampling and analysis of these lineages show that an ecologically minded sampling method could yield important information about the heterogeneity within a given sample. Ongoing work with our group and collaborators indicates that sampling pools and single colonies may be an effective way to capture diversity in a simple and high-throughput manner.

Future directions for this work include further testing and applying the sampling approach described here to a larger amount of patient samples as well as longitudinal patient samples. Studies analyzing intraspecific diversity across samples like this could answer the lingering questions of how *S. aureus* populations change over time within a host and in response to

clinical treatment. This initial study provides an exploratory but in-depth analysis of diversity across a small sample set. Importantly, we did not seek to replicate the host environment in our analysis of intraspecific population dynamics, and it is very likely that factors from the host environment and immune system would play a major role in changing the outcomes of intraspecific interactions. While many of our findings cannot be applied to within-host *S. aureus* intraspecific diversity on a broad scale, they do lay a foundation for a useful workflow and sampling approach that could be applied to a more extensive and targeted sample set and aid in answering highly pertinent questions to the clinical treatment of chronic CF infections and microbial ecology and evolution.

Acknowledgements

This work was supported in part with grants for the National Institutes of Health (R21 AI148847) and the US Cystic Fibrosis Foundation (GOLDBE19I0) to JBG. Research reported in this publication was supported in part by National Institute of Allergy and Infectious Diseases of the National Institutes of Health (T32AI138952), and Emory University and the Infectious Disease Across Scales Training Program (IDASTP) (AMA). Bacterial isolates were obtained in partnership with the Cystic Fibrosis Biospecimen Registry, which is supported in part by the CF Discovery Core of the CF@LANTA RDP Center, and by the Center for Cystic Fibrosis and Airways Disease Research, components of the Children's CF Center of Excellence at Emory University, and Children's Healthcare of Atlanta. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, Emory University, IDASTP, or the Cystic Fibrosis Biospecimen Registry and its supporters.

REFERENCES

Adamowicz, E. M., Muza, M., Chacón, J. M., & Harcombe, W. R. (2020). Cross-feeding modulates the rate and mechanism of antibiotic resistance evolution in a model microbial community of Escherichia coli and Salmonella enterica. *PLOS Pathogens*, *16*(7), e1008700-. https://doi.org/10.1371/journal.ppat.1008700

Bernardy, E. E., Petit, R. A., Raghuram, V., Alexander, A. M., Read, T. D., & Goldberg, J. B. (2020). Genotypic and Phenotypic Diversity of Staphylococcus aureus Isolates from Cystic Fibrosis Patient Lung Infections and Their Interactions with Pseudomonas aeruginosa. *MBio*, *11*(3). https://doi.org/10.1128/mbio.00735-20

CLSI. (2018). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. In *CLSI: Vol. CLSI standard M07* (11th ed.). Clinical and Laboratory Standards Institute.

Cystic Fibrosis Foundation. (2021). *Cystic Fibrosis Foundation patient registry 2021 annual data report*.

Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology*, *42*(8), 872. https://doi.org/10.1136/jcp.42.8.872

Grosser, M. R., & Richardson, A. R. (2016). Method for Preparation and Electroporation of S. aureus and S. epidermidis. In J. L. Bose (Ed.), *The Genetic Manipulation of Staphylococci: Methods and Protocols* (pp. 51–57). Springer New York. https://doi.org/10.1007/7651_2014_183

Hunt, M., Mather, A. E., Sánchez-Busó, L., Page, A. J., Parkhill, J., Keane, J. A., & Harris, S. R. (2017). ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microbial Genomics*, *3*(10). https://doi.org/https://doi.org/10.1099/mgen.0.000131

Ibberson, C. B., Parlet, C. P., Kwiecinski, J., Crosby, H. A., Meyerholz, D. K., & Horswill, A. R. (2016). Hyaluronan Modulation Impacts Staphylococcus aureus Biofilm Infection. *Infection and Immunity*, *84*(6), 1917–1929. https://doi.org/10.1128/IAI.01418-15

Kolde, R. (2012). Pheatmap: pretty heatmaps. *R Package Version*, *1*(2), 726.

Kwiecinski, J. M., & Horswill, A. R. (2020). Staphylococcus aureus bloodstream infections: pathogenesis and regulatory mechanisms. *Current Opinion in Microbiology*, *53*, 51–60. https://doi.org/https://doi.org/10.1016/j.mib.2020.02.005

Langhanki, L., Berger, P., Treffon, J., Catania, F., Kahl, B. C., & Mellmann, A. (2018). In vivo competition and horizontal gene transfer among distinct Staphylococcus aureus lineages as major drivers for adaptational changes during long-term persistence in humans. *BMC Microbiology*, *18*(1), 152. https://doi.org/10.1186/s12866-018-1308-3

Long, D. R., Wolter, D. J., Lee, M., Precit, M., McLean, K., Holmes, E., Penewit, K., Waalkes, A., Hoffman, L. R., & Salipante, S. J. (2020). Polyclonality, Shared Strains, and Convergent Evolution in Chronic Cystic Fibrosis Staphylococcus aureus Airway Infection.

American Journal of Respiratory and Critical Care Medicine, *203*(9), 1127–1137. https://doi.org/10.1164/rccm.202003-0735OC

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Michael, F., Vyacheslav, B., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., Tyson, G. H., Shaohua, Z., Chih-Hao, H., McDermott, P. F., Tadesse, D. A., Cesar, M., Mustafa, S., Glenn, T., Jamie, W., Folster, J. P., & William, K. (2019). Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrobial Agents and Chemotherapy*, *63*(11), 10.1128/aac.00483-19. https://doi.org/10.1128/aac.00483-19

Pang, Y. Y., Schwartz, J., Thoendel, M., Ackermann, L. W., Horswill, A. R., & Nauseef, W. M. (2010). agr-Dependent Interactions of Staphylococcus aureus USA300 with Human Polymorphonuclear Neutrophils. *Journal of Innate Immunity*, *2*(6), 546–559. https://doi.org/10.1159/000319855

Petit III, R. A., & Read, T. D. (2018). Staphylococcus aureus viewed from the perspective of 40,000+ genomes. *PeerJ*, *6*, e5261. https://doi.org/10.7717/peerj.5261

Petit, R. A., & Read, T. D. (2020). Bactopia: a Flexible Pipeline for Complete Analysis of Bacterial Genomes. *MSystems*, *5*(4), 10.1128/msystems.00190-20. https://doi.org/10.1128/msystems.00190-20

Pritchard, L., Glover, R. H., Humphris, S., Elphinstone, J. G., & Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Analytical Methods*, *8*(1), 12–24. https://doi.org/10.1039/C5AY02550H

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics*, *70*(1), e102. https://doi.org/https://doi.org/10.1002/cpbi.102

Raghuram, V., M, A. A., Qi, L. H., A, P. R., B, G. J., & D, R. T. (2022). Species-Wide Phylogenomics of the Staphylococcus aureus Agr Operon Revealed Convergent Evolution of Frameshift Mutations. *Microbiology Spectrum*, *10*(1), e01334-21. https://doi.org/10.1128/spectrum.01334-21

Rodriguez-R, L. M., Conrad, R. E., Viver, T., Feistel, D. J., Lindner, B. G., Venter, F., Orellana, L., Amann, R., Rossello-Mora, R., & Konstantinidis, K. T. (2023). An ANI gap within bacterial species that advances the definitions of intra-species units. *BioRxiv*, 2022.06.27.497766. https://doi.org/10.1101/2022.06.27.497766

Samra, Z., Heifetz, M., Talmor, J., Bain, E., & Bahar, J. (1998). Evaluation of Use of a New Chromogenic Agar in Detection of Urinary Tract Pathogens. *Journal of Clinical Microbiology*, *36*(4), 990–994. https://doi.org/10.1128/jcm.36.4.990-994.1998

Schwartbeck, B., Birtel, J., Treffon, J., Langhanki, L., Mellmann, A., Kale, D., Kahl, J., Hirschhausen, N., Neumann, C., Lee, J. C., Götz, F., Rohde, H., Henke, H., Küster, P., Peters, G., & Kahl, B. C. (2016). Dynamic in vivo mutations within the ica operon during

persistence of Staphylococcus aureus in the airways of cystic fibrosis patients. *PLOS Pathogens*, *12*(11), e1006024-. https://doi.org/10.1371/journal.ppat.1006024

Seeman, T. (2015). *snippy: fast bacterial variant calling from NGS reads*. https://github.com/tseemann/snippy

Silberstein, E., Serna, C., Fragoso, S. P., Nagarkatti, R., & Debrabant, A. (2018). A novel nanoluciferase-based system to monitor Trypanosoma cruzi infection in mice by bioluminescence imaging. *PLOS ONE*, *13*(4), e0195879-. https://doi.org/10.1371/journal.pone.0195879

Sprouffske, K., & Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics*, *17*(1), 172. https://doi.org/10.1186/s12859-016-1016-7

Vanderwoude, J., Azimi, S., Read, T. D., Read, T. D., & Diggle, S. P. (2023). Evolutionary Drivers of Antimicrobial Resistance Diversity of Pseudomonas aeruginosa in Cystic Fibrosis Lung Infection. *BioRxiv*, 2023.06.14.544983. https://doi.org/10.1101/2023.06.14.544983

Wingett, S. W., & Andrews, S. (2018). FastQ Screen: A tool for multi-genome mapping and quality control [version 2; peer review: 4 approved]. *F1000Research*, *7*(1338). https://doi.org/10.12688/f1000research.15931.2

Chapter IV - **Discussion and Conclusions**

Interactions between populations are often a source of strong natural selection. In the study of opportunistic pathogens, like *Staphylococcus aureus,* identifying how populations of other microbial species or other variants of the same species influence the acquisition or loss of traits is important to understanding how pathogenesis emerges and changes over time. This was the major focus of my dissertation research. In this thesis I have presented how I explored the influence that *Pseudomonas aeruginosa* has on the evolution of *S. aureus* using experimental evolution. I also investigated how intraspecific diversity impacts population-level traits in clinically isolated populations of *S. aureus*. In this final chapter of my thesis, I will reiterate the conclusions made in each research chapter as well as place them in the context of concepts emphasized in the introduction of this dissertation and lastly, I will outline potential future directions of this work.

In chapter II, I sought to replicate the scenario of *P. aeruginosa* invading an established *S. aureus* infection, as often is the case for *S. aureus* and *P. aeruginosa* coinfections in cystic fibrosis (CF) (Fischer et al., 2020). To do this, I designed an evolution experiment that allowed me to observe *S. aureus* populations and their adaptation to the presence of *P. aeruginosa* over time. After conducting whole genome sequence analysis, I concluded that experimentally evolved *S. aureus* isolates survive better than wild-type *S. aureus* in the presence of *P. aeruginosa* because of their disrupted aspartate transporter *gltT.* Eliminating the function of this gene likely aids *gltT* mutants by allowing them to evade fitness costs incurred by growing in media that had been depleted of key amino acids by *P. aeruginosa*. Altogether, this project demonstrated that experimental evolution is an effective tool for studying how populations adapt to individual selective pressures, and in this case, we brought a previously unknown factor in competitive interactions of *S. aureus* and *P. aeruginosa* to light.

In chapter III I turned my focus to the occurrence of intraspecific diversity in *S. aureus* populations collected from CF sputum and the impact that diversity can have on clinically relevant phenotypes like antibiotic resistance. With the aid of an undergraduate mentee, Hui Qi Loo, we showed that coexisting lineages can be isolated from individual sputum samples and that that intraspecific diversity may alter population-level phenotypes like antibiotic resistance and hemolysis. Upon finding coexisting MRSA and MSSA lineages in one patient we investigated their relative fitness and their influence on each other's growth and evolution in the presence of antibiotics. We found that while the impact each species had on the other in the presence of antibiotics was limited, each strain's phenotype reflected key factors about their relationship with their shared host. We observed that the MRSA population had a disrupted quorum sensing locus and reduced hemolysis which are phenotypes often associated with chronic infections, whereas that the MSSA population had a functional quorum sensing locus and hemolysis activity as well as intermediate tolerance to vancomycin. These findings were made even more interesting when we learned that the patient these isolates came from had recently been administered vancomycin antibiotic treatment. Altogether, findings from this study led us to conclude that clinically relevant intraspecific diversity can be captured by isolating both single colonies and pooled samples, and that knowledge collected from analyzing such diversity could inform more holistic approaches to research and treatment.

Experimental evolution – an effective tool with important limitations

In chapter II I used experimental evolution to investigate *S. aureus'* ability to adapt to the presence of *P. aeruginosa.* My design of serial coculture periods with repeated and consistent selective pressure from *P. aeruginosa,* yielded populations whose survival in the presence of *P. aeruginosa* significantly improved in about 50% of experimental populations. Additionally, the

design of the evolution experiment allowed me to compare evolved tolerant genomes to that of the ancestor as well as the genomes of control population isolates and isolates which were evolved in the presence of *P. aeruginosa* but didn't improve their survival. With this set of comparative genome sequences, we were able to effectively filter identified variants down to a single gene. Additionally, identifying two isolates from one population that each had unique mutations in this identified gene, *gltT*, was indicative of this gene's importance to the phenotype as well as the effectiveness of this methodological approach. Experimental evolution proved to be more efficient at identifying single genes involved with *S. aureus's* ability to survive in the presence of *P. aeruginosa* in comparison to screening clinical isolates for genotypes associated with coinfections (Bernardy et al., 2020). However, when we looked to species level *S. aureus* genomic data to determine the variability of *gltT*, we found that this gene is rarely mutated even compared to similarly functioning amino acid transporters. This was also true for the clinical *S. aureus* isolate genomes that were screened in this analysis. This finding that *gltT* disruption was a very effective method for *S. aureus* to evolve tolerance to *P. aeruginosa* in the lab but was not an adaptation that occurred in nature highlighted an important limitation of evolution experiments. While some evolution experiments can be designed to replicate specific host or environmental factors, the selective pressures that microbes are subjected to in the lab will always differ on some account from the forces the encounter in the environment (Ibberson & Whiteley, 2019). That doesn't mean that experimental evolution can't be effectively used to study phenotypes that occur in nature. In fact, experimental evolution is an effective method for observing singular selective pressures, in our case, the presence of a competing microbe. When adaptations occur under laboratory conditions but are not found in nature, we can then initiate studies to identify balancing selective pressures that make such an adaptation observed in the lab not beneficial or unlikely to occur in nature. Additionally, studying selective pressures in isolation under conditions that do not replicate nature can reveal the potential magnitude of a singular selective pressure and systems that are involved that would be otherwise difficult to

identify in complex natural environments. *In vitro* evolution experiments have the capacity to improve our understanding of how microbes are evolving and adapting in complex environments even if the results do not replicate exactly what we observe in nature.

In chapter III we used experimental evolution in a very different application. Rather than seeking to link an evolved genotype to a phenotype of interest, we hypothesized that co-occurring lineages may influence each other's ability to increase their tolerance to oxacillin or vancomycin. Using a step-wise experimental evolution design, we grew monocultures and cocultures with both isolates in increasingly higher concentrations of each antibiotic. We were able to measure the productivity of each isolate in coculture by labeling each isolate with fluorescent protein containing plasmids. Interestingly, we found that evolving with a co-isolate neither inhibited or improved a strain's ability to increase their tolerance of either oxacillin or vancomycin. However, complete extinction events for either strain were very rare, occurring only once in the case of the red-labeled MSSA isolate evolving in the presence of oxacillin (**Chapter 3 Figure 6A**). This observation that even under repeated exposure to increasing concentrations of antibiotics both isolates could coexist for a long period of time, added nuance to our understanding of what we had captured with our sampling methods. Rather than capturing a transitory period of coexistence between two distinct strains, these co-isolates may have the capacity to coexist for long periods within their shared host, despite antibiotic treatment. Experimental evolution allowed us to analyze strain dynamics in a changing environment and even though our findings did not support our hypothesis, we did learn that the coexistence of these different lineages may be relatively stable even in the presence of antibiotics.

As a teaching tool, experimental evolution also has immense utility and flexibility. Using the teaching framework of the learning module EvolvingSTEM developed by Dr. Vaughn Cooper and colleagues, I had the opportunity to introduce three different classrooms of high school

students to experimental evolution as a way to interactively learn about microbes and evolution (Cooper et al., 2019). Giving students the opportunity to adapt the given protocol and design to their own questions and ideas was an effective way to give them agency over their projects and allowed for a more engaging experience with the scientific method. Evolution experiments consisted of regular transfers of *Pseudomonas fluorescens,* using polystyrene beads to select for biofilm production. Students were able to visually observe changes in evolving populations as increased biofilm formation leads to wrinkly colony morphology in *P. fluorescens.* The ability to observe changes in phenotypes in real time, combined with the exploratory nature of evolution experiments makes it a particularly effective teaching tool. Allowing students to generate their own scientific questions, identify appropriate controls, and consider the implications of their findings makes the learning the experience during this project much more impactful. The flexibility in experimental design is a feature not often found in traditional laboratory exercises. In my outreach experiences, I noticed that students that implemented more of their own independent ideas into the project had the most agency over their work and asked more questions than students who followed the EvolvingSTEM protocol more closely. These observations solidified for me that exploratory analyses are effective and important tools for students that are learning how to implement the scientific method and that experimental evolution is a particularly adaptable and effective method for students to study how microbes interact with their environment and change over time.

Infections as evolving ecosystems

Findings from the studies presented in this thesis demonstrate that diversity in infections, whether it is interspecific or intraspecific, can influence important pathogenic outcomes. However, identifying universal patterns and trends is not readily apparent based on our conclusions. In the case of *P. aeruginosa* influencing *S. aureus* populations, I presented results

from four evolving populations and of those four populations only two had significantly increased their ability to maintain large populations in coculture with *P. aeruginosa.* This observation indicates that acquiring the phenotype of tolerance to *P. aeruginosa* is costly for *S. aureus* and that mutations that lead to such a phenotype rarely occur or rarely become dominant in a population. Additionally, when we looked to the patterns of mutations we identified in our *in vitro* experiments among more than 500 *S. aureus* genomes, we found that genes that were readily mutated in our experiments rarely vary in natural populations. However, previous work showed that among clinical isolates, that *S. aureus* isolates range from being highly sensitive to *P. aeruginosa* to quite tolerant indicating that alternate mechanisms of *Pseudomonas* tolerance exist in nature (Bernardy et al., 2020). Altogether, these findings demonstrate how both strain background and environmental factors greatly influence the potential pathways that species can potentially take as they evolve and adapt to a given selective pressure. This is important to consider as we attempt to better understand how populations of microbes change over time in response to their host, treatments, or disturbances in the surrounding community.

When we investigated the role that intraspecific diversity plays in shaping *S. aureus* populations present in infections we found that when populations maintain multiple lineages at relatively high abundances we can detect that level of diversity by sampling single colonies. However, when populations are largely dominated by a single clone with a few persisting lineages present at low abundance, we are unlikely to detect diversity by sampling individual colonies. Both highly abundant and minimally abundant lineages however, may alter population level phenotypes like antimicrobial resistance or hemolysis activity. We also found that while coexisting lineages may have very different phenotypes, growth rates, and antibiotic resistance profiles, they may be able to coexist over long periods of time, even in the presence of low amounts of antibiotics. Intraspecific diversity gives *S. aureus* populations a greater chance of persisting through environmental changes or treatments because the number of genotypes and phenotypes

available to the entire infecting population increases as more unique variants are added to and maintained in the population (Boles et al., 2004; Loreau & Hector, 2001). Knowing whether or not a variant exists that is likely to have a high fitness in the presence of a treatment or change in disease is important when trying to treat chronic and complex infections like those common among individuals with CF.

In the introduction of this thesis, I proposed that historically we have studied microbes and pathogenesis through the lens of Koch's postulates and Falkow's molecular Koch's postulates both of which reduce pathogenesis to individual strains and genes respectively. These approaches simplify the complex nature of what makes a microbe a pathogen into useful patterns, but they also limit our view, excluding the community of microbes that comprise an infection and how they respond to changes in their shared environment over time. Findings from the investigations presented in this thesis support the assertion that while patterns in the role that diversity plays in infections are difficult to synthesize into a handful of postulates, interactions between species and strains heavily influence which variants and traits will become abundant and what populations will persist after a major change or treatment. Understanding how members of complex chronic infections change over time is a key factor in learning how to treat and prevent them. By expanding our research approaches and assumptions beyond Koch and Falkow's postulates and considering infections as the evolving ecosystems they are with all the complexity, stochasticity, and variability of a forest, we may be able to identify more effective treatments and predict how populations will change in response to perturbations. For individuals with CF, this is an important line of study to improve their lifespans and quality of life, and in the face of growing antimicrobial resistance, many previously mundane infections may become longer lasting, more complex, and more difficult to treat. Furthermore, studying pathogens and all microbes in the context of their communities may lead to a more fundamental understanding of how communities, populations and individuals change over time.

Future Directions

When we evolved *S. aureus* under repeated exposure to *P. aeruginosa,* we discovered a previously undescribed mechanism for these two species' coexistence. We revealed that changes in amino acid uptake, not previously known to play a role in their interactions, may be a determining factor in whether or not *S. aureus* will persist in the presence of invading *P. aeruginosa*. However, this genetic adaptation appears to be specific to the *in vitro* environment we created in the lab. Which begs the question – why did this gene mutate multiple times independently in a single evolving population in the lab but is highly conserved in natural populations? Our investigations of this gene's role in maintaining fitness in wild-type *S. aureus* did not yield obvious answers. Among all our tests for growth and fitness differences in different nutrient landscapes, including direct competition in rich media, we did not find whether there were any conditions where *gltT* mutants suffered significant fitness costs. One potentially fruitful future direction of this work is to find what forces exist outside the laboratory that keep *gltT* so highly conserved during infections, especially in the CF lung specifically. Identifying these factors may illuminate why disrupting genes like *gltT* can be so seemingly harmless in lab environments and yet are so rare in nature. Additionally bringing in environmental factors that more closely resemble environments where *S. aureus* and *P. aeruginosa* frequently co-occur like using synthetic sputum media or animal models to see if the adaptations *S. aureus* makes are different or similar to what we've observed in this study. Using clinical isolates from both single species infections and coinfections in future evolution experiments may also be an effective way to identify adaptive strategies that are dependent on strain backgrounds or are dependent on host-associated factors.

Our investigation, while thorough, also did not manage to fully illustrate the mechanism of how *gltT* disruption enables *S. aureus* survival in coculture with *P. aeruginosa.* One future experiment that could shed more light on this would be to use radio-labeled nutrients and track their absorption by *S. aureus* and *P. aeruginosa* in monocultures and cocultures. The results from this test could confirm whether or not our hypothesized scenario of intracellular aspartate poisoning by *S. aureus* and glutamate scavenging by *P. aeruginosa* is correct. Additionally, testing how the fitness of *gltT* mutants change under increasingly limited glucose could demonstrate how effective this adaptation is in later stages of growth where shared resources are limited. Arguably, working out the entirety of this mechanism may not be entirely necessary. While it would be beneficial for our understanding of *S. aureus* physiology, a fully defined mechanism of this adaptation may not lead to useful insights on how to better treat CF coinfections or inform how *S. aureus* adapts to interspecific competition in nature as our findings indicate that disrupting *gltT* may only be beneficial in lab-generated conditions.

Our work on the intraspecific diversity that exists in CF sputum samples revealed that by sampling single colonies and pools we can detect and estimate the amount of intraspecific diversity that exists in a patient. While the data presented here makes important conclusions about how diversity can impact the virulence and treatability of an infection, many new questions arise such as how diversity changes after treatment and over time. Collecting longitudinal samples from CF patients including before and after antibiotic treatment and using the method of sampling both single colonies and pooled populations could be a fruitful line of research. Studies like this could reveal how much populations in CF change over time and how different treatments alter diversity and corresponding population level traits like hemolysis and antibiotic resistance are altered after disturbances and treatments. Layering insights from both research studies one could also ask how populations of *S. aureus* change before and after new species are detected in CF infections. Developing accurate animal model for chronic respiratory

infections is also an ongoing area of research and if others working on this topic identify a host that can maintain multiple species and variants over long periods of time would be a fantastic tool for studying how inter- and intraspecific interactions impact the outcomes and features of complex infections.

As research in the fields of microbial ecology and evolution expand our understanding of how co-occurring populations influence each other in shared environments, we will be able to better anticipate and address how pathogens change over time. More generally, we improve our understanding of how diversity influences the evolution of populations in complex environments. Methods and findings such as our experimental evolution approach and population level sampling of clinical isolates, lay a foundation for future studies that address important concepts and questions about microbial ecology, evolution, and pathogenesis.

REFERENCES

Bernardy, E. E., Petit, R. A., Raghuram, V., Alexander, A. M., Read, T. D., & Goldberg, J. B. (2020). Genotypic and Phenotypic Diversity of Staphylococcus aureus Isolates from Cystic Fibrosis Patient Lung Infections and Their Interactions with Pseudomonas aeruginosa. *MBio*, *11*(3). https://doi.org/10.1128/mbio.00735-20

Boles, B. R., Thoendel, M., & Singh, P. K. (2004). Self-generated diversity produces “insurance effects” in biofilm communities. *Proceedings of the National Academy of Sciences*, *101*(47), 16630–16635. https://doi.org/10.1073/pnas.0407460101

Cooper, V. S., Warren, T. M., Matela, A. M., Handwork, M., & Scarponi, S. (2019). EvolvingSTEM: a microbial evolution-in-action curriculum that enhances learning of evolutionary biology and biotechnology. *Evolution: Education and Outreach*, *12*(1), 12. https://doi.org/10.1186/s12052-019-0103-4

Fischer, A. J., Singh, S. B., LaMarche, M. M., Maakestad, L. J., Kienenberger, Z. E., Peña, T. A., Stoltz, D. A., & Limoli, D. H. (2020). Sustained Coinfections with Staphylococcus aureus and Pseudomonas aeruginosa in Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *203*(3), 328–338. https://doi.org/10.1164/rccm.202004-1322OC

Ibberson, C. B., & Whiteley, M. (2019). The Staphylococcus aureus Transcriptome during Cystic Fibrosis Lung Infection. *MBio*, *10*(6), 10.1128/mbio.02774-19. https://doi.org/10.1128/mbio.02774-19

Loreau, M., & Hector, A. (2001). Partitioning selection and complementarity in biodiversity experiments. *Nature*, *412*(6842), 72–76. https://doi.org/10.1038/35083573