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Hui Qi Loo December 3, 2021

Intraspecific diversity among *Staphylococcus aureus* isolated from cystic fibrosis patients

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Abstract

Intraspecific diversity among *Staphylococcus aureus* isolated from cystic fibrosis patients
By Hui Qi Loo

Staphylococcus aureus was recently recognized as the most common pathogen found in the airway of patients with cystic fibrosis (CF). Current medical microbiology tests in CF are limited in their ability to predict antimicrobial treatment responses in patients. We hypothesize that this is in part impacted by intrahost genotypic diversity and microbial interactions that are yet to be elucidated. Previous studies have shown that *S. aureus* isolated from CF lungs are diverse phylogenetically and functionally. Here, we present evidence for within-host heterogeneity in the antibiotic resistance gene profile of *S. aureus* isolated from CF patients. We then examined the effect of interactions between different *S. aureus* isolates purified from a single CF patient on their growth dynamic and antibiotic-resistance-related phenotypes. Specifically, we studied interactions between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates obtained from a single sputum sample of an individual with CF. By fluorescently labeling the respective *S. aureus* isolates, we assessed bacterial growth in cocultures and the impact of antibiotic exposure on their growth dynamics. We also experimentally evolved cocultures of the MRSA and MSSA isolates in increasing concentrations of antibiotics to assess the rate of resistance evolution. Our data suggest that *S. aureus* coisolated from the same host shows no evidence of adaptation for coexistence *in vitro*, but exposure to vancomycin helps to stabilize the coexistence. We also showed that coexistence of these isolates does not contribute to a higher rate of antibiotic resistance evolution, suggesting a trade-off between drug resistance and coexistence. Our results highlight the interplay between intraspecific diversity and antibiotic resistance among *S. aureus* and have implications on efforts to improve the clinical outcome of these infections in CF patients.

Intraspecific diversity among *Staphylococcus aureus* isolated from cystic fibrosis patients

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Human subject samples and bacterial isolates were provided by the CF Biospecimen Repository (CFBR) at the Children's Healthcare of Atlanta and Emory University CF Discovery Core. I thank Dr. Arlene Stecenko and Katy Clemmer for their assistance in providing metadata on relevant bacterial isolates. I thank Dr. Timothy Read's lab for providing bacterial strains for vancomycin controls. I thank Lauren Askew (Emory School of Medicine) for her previous work on the phenotypic characterization part of this project during her lab rotation in the Goldberg lab. This work was supported by grants from the Cystic Fibrosis Foundation (GOLDBE19P0) and the NIH (R21-AI48847) to Joanna B. Goldberg.

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disorder that affects more than 70,000 individuals worldwide [1]. The leading cause of mortality in CF patients is lung disease, characterized by frequent and persistent pulmonary infection and inflammation [2]. Bacterial colonization in CF lungs is common, with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* being some of the most commonly found pathogens [2]. Notably, *S. aureus* was recently recognized by the Cystic Fibrosis Foundation as the pathogen with highest prevalence in the airway of CF patients [3]. Other than being prevalent in CF infections, *S. aureus* is the leading cause of healthcare-associated infections in the United States and can cause serious infection in people with chronic conditions such as diabetes and cancer. In particular, methicillin-resistant *S. aureus* (MRSA), which is resistant to beta-lactam antibiotics, is one of the most important antibiotic resistant bacteria actively tracked by the CDC's Emerging Infections Program [4].

Co-infections of multiple bacteria species is common in CF patients, and these trends can change over the course of disease progression [3]. Among the most prevalent co-infections and well-studied interactions is that between *P. aeruginosa* and *S. aureus* [5]. In addition to interspecific diversity, intraspecies heterogeneity within these bacterial populations is also evident, which may result from multiple inoculation events or within-host evolution [6, 7]. Extensive studies have shown that intrahost antimicrobial diversity is evident in CF infections with *P. aeruginosa* [8-11]. Other related studies have shown this for the *B. cepacia* complex [12] and *B. dolosa* [13]. Previous studies in our lab observed intraspecific diversity of *S. aureus* isolates from CF patients in their virulence factors and interaction with *P. aeruginosa* [14]. Genomic diversity and intrahost adaptation of *S. aureus* has also been observed in patients with chronic MRSA infection, including variations in antibiotic resistance [15].

Antibiotic treatment is a crucial aspect of lung health management for individuals with CF, mainly to suppress bacterial populations and alleviate symptoms of infection. Current antimicrobial susceptibility testing (AST) methods, however, are limited in their ability to accurately predict the effective *in vivo* antibiotic susceptibility status and thus clinical responses in CF lung infections [16]. One reason for this limitation is the phenotypic and genotypic diversity of bacterial populations within the CF lung. Microbial interactions are known to influence a pathogen's viability, virulence, and tolerance to antibiotics [17]. It is widely accepted that *inter*specific interactions in polymicrobial infections can alter responses to antibiotics by means of collective resistance, collective tolerance, and exposure protection [18-20]. However, less is known of the impact of *intra*specific interactions on antibiotic resistance. AST methods limited to a small subset of the sampled bacterial population within a patient's sputum may underrepresent both the interspecific and intraspecific diversity, thus failing to reflect the true antimicrobial resistance profile of the lung infection.

To address this gap in our understanding, especially in CF related *S. aureus* infections, I built upon previous work in the Goldberg lab by Lauren Askew and Ashley Alexander, whereby they performed phenotypic analysis, whole genome sequencing and bioinformatics analyses on corresponding single colony isolates and populations of *S. aureus* purified from sputum samples collected from three CF patients. I specifically assessed the diversity of antimicrobial resistance related genes within hosts and then investigated the growth dynamics of coisolated MRSA and MSSA *S. aureus in vitro* in the presence and absence of antibiotics. Finally, I examined the effect of intraspecific interactions on the rate of antibiotic resistance evolution among these isolates. I therefore am able to interrogate the phenotypic and genotypic diversity of *S. aureus*

within patients, intraspecies interactions between coisolates and the interplay between coexistence and antibiotic resistance.

RESULTS

Within-host genotypic and phenotypic diversity among *S. aureus* isolated from CF patients

We obtained single colony isolates and pooled isolates from sputum samples of three CF patients. The isolates were processed and analyzed as outlined in **Fig. 1**. Isolates from Patient 1 (CFBR Patient ID: 623) and Patient 2 (CFBR Patient ID: 196) were homogenous within the host in terms of sequence type (ST), colony morphology, hemolysis capability, and polysaccharide production (**Table 1, Fig. S1**). In contrast, there was intrahost heterogeneity in isolates taken from Patient 3 (CFBR Patient ID: 311). In Patient 3, there were mainly two distinct groups of single colony isolates that differed in ST, hemolysis capability and polysaccharide production (**Table 1, Fig. S1**). Specifically, two isolates from Patient 3 (Sa117 and Sa118) were ST8 while others were ST5.

Within-host diversity of antimicrobial resistance related gene profile

We analyzed the antimicrobial resistant related genes found in the genomic sequences of all isolates using the NCBI AMRFinderPlus output through the Bactopia pipeline [21]. The same set of resistance genes was detected in all isolates from Patient 1; these genes were expected to confer resistance to aminoglycosides, beta-lactams, macrolides, fosfomycin, and tetracyclines (**Fig. 2**). Similar results were seen in isolates from Patient 2, with the presence of genes known to confer resistance to beta-lactams, fosfomycin, and tetracyclines (**Fig. 2**).

Similar to the observations of heterogeneity in ST and phenotypes, intrahost heterogeneity of the antimicrobial resistance profile was observed in Patient 3. The larger group of isolates (Sa111-116) has aminoglycosides, beta-lactams, bleomycin, macrolides, fosfomycin and tetracyclines resistance genes, while the minor group of isolates (Sa117-118) only has genes that encode

resistance to beta-lactams, fosfomycin, and tetracyclines. Of note, the larger group of isolates (Sa111-116) has *mec* genes that likely confer resistance to methicillin, making them MRSA isolates (**Fig. 2**). Isolates Sa112 and Sa117 were randomly chosen to represent each subpopulation in subsequent experiments.

Relative fitness of MRSA and MSSA isolates cocultured in normal growth conditions

We hypothesized that distantly related *S. aureus*, which in this case happened to be a MRSA isolate and a MSSA isolate, purified from the same chronic infection can coexist under normal growth conditions. To test this, fluorescently labeled isolates of interest (MRSA Sa112 and MSSA Sa117) were grown in monoculture and in coculture with either an unlabeled coisolate (Sa112, Sa117) or non-coisolate (Sa92, Sa106, JE2). The coculture setup for this experiment is summarized in **Fig. S2**. The growth of these *S. aureus* isolates was compared by estimating the area under curve (AUC) of growth curves based on fluorescence intensity. The relative fitness of these isolates when in coculture was evaluated by comparison to their growth in coculture with their respective unlabeled wildtype (WT) isolate.

The fluorescently labeled isolates usually suffered a fitness cost, indicated by a relative fitness that was less than 0.5 when grown in a 1:1 coculture with the unlabeled WT isolate compared to their monoculture across four biological replicates (**Fig. S3A**). These fitness costs were consistently seen in separately transformed isolates, but the level of fitness cost varies between different isolates and between different colonies of the same isolate. This variability was particularly noticeable in Sa117 isolates whereby the Sa117::*dsRed* isolate suffered a large fitness cost in three replicates and the Sa117::*gfp* isolate suffered large fitness cost in the second and third replicates. This suggests an inherent but variable fitness cost associated with the *dsRed*

and *gfp* plasmids in these isolates. Therefore, by calculating relative AUC using the growth of a labeled isolate in coculture with its unlabeled WT in coculture as the comparison control, we took into account the experimental variability affecting fitness cost associated with these isolates when expressing the fluorescent genes.

Our results suggested that both MRSA (Sa112) and MSSA (Sa117) isolates are able to grow for at least a 24-hour period, whether in coculture with their respective coisolates or non-related isolates, as shown in a representative biological replicate (**Fig. 3**). The MRSA (Sa112) isolate suffered a slight fitness cost when grown with its MSSA coisolate (Sa117) as indicated by a lower relative AUC, whereas the MSSA (Sa117) isolate seemed to benefit from the coexistence. Both isolates, when cocultured with other non-coisolates, showed a large range of fitness cost or benefit. For example, both isolates grow better in coculture with Sa106 isolated from Patient 2. Overall, by simply comparing the relative AUC between growth with coisolates versus non-coisolates, our data showed no evidence of adaptation for coexistence between coisolates, at least *in vitro*.

In each biological replicate, no significant difference was observed between isolates labeled with *dsRed* and *gfp*, suggesting that the general patterns of relative fitness in each experimental run are mainly driven by the isolates in the coculture and not the fluorescent protein being expressed.We suspect, however, that relative AUC seen across coculture with different isolates are influenced, in part, by the extent to which the specific colony of labeled isolates express the fluorescent proteins. It is possible that some colonies lose the fluorescent gene plasmid or express lower levels of fluorescent protein to achieve greater growth potential in stressful environments. If this happens more so when the isolates are in coculture compared to monoculture, their relative growth in coculture may be overestimated. For this reason, we

presented data of several biological replicates separately (**Fig. S3**) to highlight the nuances in each experimental run.

Relative fitness of MRSA and MSSA isolates cocultured in the presence of antibiotics

Given that antibiotic treatments are common for bacterial infections in CF patients, we then examined the growth of the coisolates from Patient 3 (Sa112 and Sa117) in the presence of antibiotics. Specifically, we tested (1) whether coexistence affects the minimum inhibitory concentration (MIC) of each *S. aureus* isolate within the heterogenous population, and (2) whether antibiotic exposure affects their growth dynamic in the heterogenous population.

Our results show that in the presence of antibiotics, the isolate with a corresponding resistance gene dominates over the isolate without the relevant resistance gene in a mixed population. In the case of growth in the presence of oxacillin, which is a β-lactam antibiotic, the MRSA isolate (Sa112) dominated the population in an antibiotic-concentration-dependent manner (**Fig. 4A**). The MSSA isolate (Sa117) was able to persist in the coculture environment up to the same concentration of oxacillin it could survive in monoculture. Thus, the presence of the MRSA co-isolate (Sa112) does not provide greater protection against oxacillin to the MSSA isolate (Sa117), at least in a 24-hour growth period. In experiments with erythromycin, the MRSA isolate (Sa112) which has *erm(A)* gene excludes the MSSA isolate (Sa117) from growing in all concentrations of erythromycin tested (**Fig. 4B**).

Interestingly, in experiments with vancomycin, the MSSA isolate (Sa117) had a higher vancomycin MIC than the MRSA isolate (Sa112). The WT isolates of the corresponding MRSA (Sa112) and MSSA (Sa117) isolates have a vancomycin MIC of 2μg/mL (vancomycin-sensitive) and 4μg/mL (vancomycin-intermediate), respectively, when evaluated with the CLSI microbroth

dilution method for antibiotic susceptibility testing (data not shown). In cocultures, the MSSA dominates and outcompetes the MRSA isolate as the concentration of vancomycin increases (**Fig. 4C**). This result suggests that exposure to vancomycin could be a possible selective pressure that may allow for the persistence of the MSSA isolate in the presence of the MRSA isolate within a patient.

Intraspecies interaction and antibiotic resistance evolution

Next, we investigated the impact of coexistence on the rate of antibiotic resistance evolution. We hypothesized that susceptible *S. aureus* subpopulations will benefit from coexisting with more resistant subpopulations through the sharing of genetic material, quorum sensing or other cooperative mechanisms, allowing the population to evolve resistance at a faster rate than in a single-isolate environment.

Through experimental evolution of the MRSA and MSSA isolates along a concentration gradient of oxacillin or vancomycin, our results suggest that, whether the isolates are grown in monoculture or coculture, there is no significant difference between the rate of resistance of antibiotic resistance (**Fig. 5**). In the case of the MSSA isolate grown in coculture with oxacillin, the MIC of the isolate appears to decrease over time as it is driven to extinction after several transfers. When exposed to vancomycin, the MSSA isolate dominates over the MRSA isolate in coculture as seen in **Fig. 4** experiments. However, both isolates do not evolve to very high resistance to vancomycin in these evolution experiments, plateauing at MICs of approximately 2-4μg/mL.

While intraspecific interaction does not seem to significantly affect the evolution of resistance, the isolates generally have a lower MIC throughout the 6-day period when in coculture compared

to when they are grown in monoculture under the same conditions. Again, this suggested that there is fitness cost to the level of drug resistance when these isolates have to compete with a genetically distinct isolate.

DISCUSSION

Within-host heterogeneity of *S. aureus* antimicrobial resistance gene profile and clinical relevance

Many studies had shown that within-host diversity is observed in bacterial species in the CF lung, therefore it is not surprising that this was also the case for *S. aureus* in this study. Results from this study provide evidence for intrahost diversity in *S. aureus* isolates in terms of ST, phenotypes, and more importantly, in their antibiotic resistance profile. The two distinct population of *S. aureus* in Patient 3 must have resulted from two separate infection events, as they are different in sequence type and have an overall average nucleotide identity (ANI) of 0.98995. Notably, the two populations not only differ in sequence type, but also in hemolysis capability (hemolysis negative for MRSA subpopulation; hemolysis positive for MSSA), which reflects the virulence of *S. aureus*, and polysaccharide production, which is a characteristic of the mucoid phenotype.

In a typical clinical microbiological setting, the laboratory personnel take a potentially infected region of a sputum sample produced by CF patients and isolate microbes for microbiological identification and AST, which then guides treatment plans. However, this isolation method may not represent the full antibiotic resistance profile of *S. aureus* present in a patient's lung, therefore this selection could lead to misinterpretation and unproductive treatment of the lung infection. As such, our results suggest that more sampling and multiple isolations may be required to increase the accuracy of AST done in medical laboratory.

We also noted inconsistencies in the antimicrobial resistance genotypes based on sequencing data when compared to clinical AST data, which is based on MIC interpretation (**Table 2**).

Specifically, we observed that *S. aureus* isolate from Patient 1 showed susceptibility to beta-lactam and tetracycline classes of antibiotics, despite having the corresponding antimicrobial resistant genes *BlaI, blaZ* and *tet38* (**Fig. 2**). Inconsistency with tetracycline susceptibility is also seen with Patient 2. These inconsistencies may have been due to underexpression of those resistant genes in the culture environment. It should also be noted that genes of the beta lactamase (*bla*) gene family do not confer resistance to many types of beta lactam antibiotics [22]. Overall, this study highlights the deviation between antibiotic resistance profile inferred from genotypic data and that from AST methods. However, we do not have relevant data to conclude whether the genotypic data or clinical lab data more accurately reflects the patient's response to antibiotics treatment.

Interplay between intraspecific diversity and antibiotic resistance

In addition to the clinical relevance of a heterogenous bacterial population, this study also highlights the intraspecies interactions among *S. aureus* in a CF lung, selective pressure that maintains intraspecies diversity, and the potential trade-off between antibiotic resistance and coexistence.

Data from our coculture experiments in laboratory growth conditions *in vitro* suggest that *S. aureus* coisolated from the same host shows no evidence of adaptation or stable coexistence. This is shown through comparisons of relative fitness whereby the isolates cocultured with their coisolates do not show significantly greater growth compared to when they were cocultured with non-coisolates. However, many confounding factors may influence the preferences for coexistence, including but not limited to genetic similarities, ST, resource availability, infection environment and time, which are beyond the scope of this study.

In our antibiotic resistance evolution experiments, we noticed that the coisolates from the same patient do not coexist well *in vitro* over longer time period (5 or 6 days), even when they were not exposed to antibiotics. The MRSA isolate slowly outcompetes the MSSA isolate in the control wells without antibiotics (data not shown) after several transfers. One possible explanation is that the isolates were sampled during a short period of transient coinfection in which the coexistence of the two lineages does not persist. Due to the lack of longitudinal data, we are currently unable to eliminate this possibility, but pursuing further sampling of this patient may be of interest in future work. Another possibility is that the isolates form spatially structured colonization regions in the lung environment that supports coexistence but is not well replicated in our liquid culture assays.

We also showed that the dynamics of the two populations play out differently in the presence of antibiotics in an antibiotic-specific manner. The MRSA isolates dominates over the MSSA isolate in exposures to oxacillin and erythromycin, consistent with the resistance profile of the MRSA population. This dominance is antibiotic concentration dependent in the case of oxacillin, but concentration independent for erythromycin. The oxacillin concentration dependent pattern of *S. aureus* growth may be explained the resistance mechanisms of MRSA which is mediated by the altered penicillin-binding protein (PBP2a) encoded by the *mecA* gene [23]. This variant protein binds β-lactam antibiotics with lower avidity, thus conferring resistance. Since there is a limitation on the amount of PBP2a the bacterial cell can synthesize, the resistance may be overcome as oxacillin concentration increases. On the other hand, the dominance of the MRSA isolates is independent of erythromycin concentration. This is likely due to the ribosomal binding site modification encoded by *erm* genes, which entirely blocks the erythromycin's protein-

synthesis-inhibiting-activity by preventing the binding of erythromycin to the 23S ribosomal RNA molecule in the 50S subunit of the bacteria [24].

Interestingly, this dominance of MRSA is reversed when the isolates are cocultured in the presence of vancomycin. While no genes associated with vancomycin resistance were found in either isolate, in part due to vancomycin resistance being less characterized, the MSSA isolate is found to be more resistant to vancomycin than the MRSA isolate. Our experimental data with vancomycin suggest that vancomycin exposure, which is commonly used in clinical settings to treat MRSA, may be a selective pressure that stabilizes the coexistence of the two populations of *S. aureus*. This argument is consistent with metadata about Patient 3 obtained from Cystic Fibrosis Biospecimen Repository (CFBR): Patient 3 was hospitalized and treated with vancomycin for a 2-week period, about one month before the sputum sample for this study was obtained. More broadly, this may be an interesting explanation to consider for why MSSA strains continue to persist in human populations or CF patients in particular.

In addition, our experiments with antibiotics suggest that coexistence of *S. aureus* isolates with varying antibiotic susceptibility do not contribute to greater drug resistance or higher rate of resistance evolution, which conflicts with theories of collective resistance [18]. This suggests that the cost of intraspecific competition may outweigh the possible benefits of shared drug tolerance, at least in this sampled community.

Taken together, this study demonstrates the importance of considering ecological context in microbial communities when studying antibiotic resistance and their evolution. The results of this study have implications on guiding sputum sampling and isolation techniques in the clinical setting to improve treatment plan. We also presented a potential conflicting interpretation of antibiotics resistance profile based on sequencing method and clinical microbiology laboratory

testing. Furthermore, we showed that coexistence of diverse *S. aureus* isolates is common but does not readily persist and is influenced by antibiotic exposure. This highlights the interplay between intraspecies diversity and antibiotics that is often overlooked in CF infections.

METHODS

Isolation of Sputum Samples and Whole Genome Sequencing

Sputum samples were collected at the Emory CF clinic and spread onto Mannitol Salt Agar by collaborators at Georgia Tech. Either 10ul or 100ul of resuspended sputum was plated on the day of sample collection. Three sputum samples from three different patients were collected and processed. From each agar plate, 8 single colony isolates were collected and re-streaked on *Staphylococcus* isolation agar (SIA) before being made into frozen stocks in the Goldberg lab. Pool samples were also collected by scraping all remaining colonies and re-streaking onto SIA before being made into frozen stocks. Genomic DNA was extracted from the single colony isolates and pool samples and subsequently sequenced with Illumina MiSeq through the Microbial Genome Sequencing Center (MiGS). Genomics sequences of isolates from all three patients were submitted to NCBI as accession PRJNA742745 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA742745).

Population Structure and Phenotypic Characterization

A phylogeny of the isolates was built using pheatmap package in *R* [25]. Hemolysis assays and phenotypic characterization of polysaccharide production were carried out for the CF clinical isolates with methods described previously [14]. Briefly, hemolysis capability was measured by the presence or absence of clear hemolysis on blood agar plates, while polysaccharide production was assessed through the Congo Red Assay.

Antimicrobial Resistance Gene Profile Analysis

Bacterial genome data was processed using the Bactopia pipeline [21]. Through this pipeline, the antimicrobial resistance gene output from NCBI's AMRFinderPlus was used. Post-Bactopia analyses were conducted in *R* [25].

Bacteria isolates and transformation

Bacteria isolates used in this study are listed in **Table 3**. Sa112 was selected to represents the MRSA subpopulation in Patient 3, whereas Sa117 represents the MSSA subpopulation in Patient 3. Sa112 and Sa117 were fluorescently labelled by transforming them with multicopy plasmids containing constitutively expressed fluorescent genes: *gfp* or *dsRed* with protocols as described in [26]. Sa92 from Patient 1, Sa106 from Patient 2 and laboratory *S. aureus* strain JE2 were selected as non-coisolate controls for relative fitness experiments.

Growth dynamic in normal growth condition

Isolates were streaked onto Trypticase soy agar (TSA) plates from freezer stocks and incubated for approximately 24 hours at 37°C. A single colony was then picked from TSA plates for overnight culture in 3mL of lysogeny broth (LB) at 37°C in a rotating incubator. Overnight cultures were diluted to an OD_{600nm} of 0.01 in 1.5mL microcentrifuge tubes. For cocultures, isolates were mixed in a 1:1 ratio that maintained an overall bacterial cell density of 0.01 at OD_{600nm}. 200µL of diluted cultures were added into each well of a black, flat bottom 96-well plate with closed lid. 200µL of undiluted overnight culture of labeled isolates were used as optics gain scaling reference. 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. *gfp* fluorescence was excited at 485 nm and emission was measured at 515 nm. *dsRed* fluorescence was excited at 554 nm and emission was measured at 586 nm. Growth data was analyzed using *R* [25]. The *R* package *Growthcurver* was used to fit optical density and fluorescence data into standard logistic curves and determine the area under curve (AUC).

Growth dynamic in presence of antibiotics

Isolates were streaked onto Trypticase soy agar (TSA) plates from freezer stocks and incubated for approximately 24 hours at 37°C. A single colony was then picked from TSA plates for overnight culture in 3mL of cation-adjusted Mueller-Hinton broth (CAMH), or CAMHB + 2% NaCl for growth with oxacillin, at 37°C in a rotating incubator. Isolate cultures were diluted and added to 96-well plates such that the resulting starting density for growth curve assay is 0.05 at OD_{600nm} and resulting antibiotic gradient is 0.25μg/mL to 128μg/mL with two-fold increases across the plate with antibiotic-free control wells. For cocultures, isolates were mixed in a 1:1 ratio that maintained an overall bacterial cell density of 0.05. The plate was incubated in a microplate reader for 24 hours at 37°C and measurements were taken as described in the previous section.

Experimental evolution of *S. aureus* isolates in presence of antibiotics

Initial bacterial cultures were prepared as described in the previous section. Protocols for experimental evolution in antibiotics was adapted from Adamowicz *et al.* [27], schematics of the experiment setup is shown in **Fig. S4**. Isolate cultures were diluted and distributed to 96-well plates such that the resulting starting density for growth curve assay is 0.1 at OD_{600nm} and resulting antibiotic gradient is 0.25μg/mL to 128μg/mL with two-fold increases across the plate with antibiotic-free control wells. For cocultures, isolates were mixed in a 1:1 ratio that

maintained an overall bacterial cell density of 0.05. Plates were incubated for 24 hours at 37°C with shaking at 450 rpm.

After each growth phase, cells were transferred to a new 96-well plate with fresh media. In total, 2µl of bacterial cells were added into 196µl of the appropriate monoculture medium with 2µL of antibiotic stock that would confer the correct gradient concentration in the new plate. The bacteria cells were transferred such that 1µL culture from wells of each antibiotic concentration was transferred to a new well with the same antibiotic concentration, and 1µL cells to a new well that was one step higher on the antibiotic gradient. Transfer was carried out whether or not there is visible growth. This was repeated for 6 days. When populations evolved to the upper concentration limit, the upper end of the gradient was increased, and the lowest concentration was removed.

OD600 and fluorescence measurements of the plate were taken at the end of each growth phase. The 90% minimum inhibitory concentration (MIC90) for each isolate was calculated. Growth at a given antibiotic concentration is confirmed if the well OD600 was above 10% of the OD600 of the antibiotic-free control well for a given replicate.

MAIN FIGURES

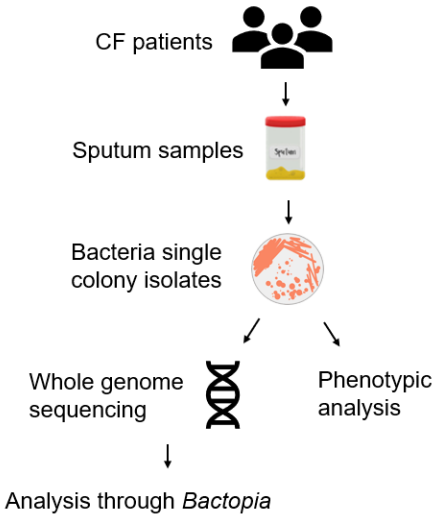

Figure 1. Sampling and analysis methodology. Sputum samples were collected from three CF patients. From each sample, single colony isolates were purified. Genomic DNA was extracted from these single colony isolates and subsequently sequenced with Illumina MiSeq. Phenotypic analysis was also carried on each isolate. Genomes sequences were analyzed through the Bactopia pipeline.

Table 1. Compilation of genotypes and phenotypes of CF clinical isolates of *S. aureus*[a]

Patient	Isolate ID	Isolate Type	Sequence Type	Morphology	Hemolysis	Polysaccharide Production
1 (CFBR ID: 623)	Sa90	Pool	72	White	+	Overproducer
	Sa91	Pool	72	White	+	Overproducer
	Sa92	Single colony	72	White	+	Overproducer
	Sa93	Single colony	72	White	+	Overproducer
	Sa94	Single colony	72	White	+	Overproducer
	Sa95	Single colony	72	White	+	Overproducer
	Sa96	Single colony	72	White	+	Overproducer
	Sa97	Single colony	72	White	+	Overproducer
2 (CFBR ID: 196)	Sa100	Pool	5* 6/7	Yellow	+	Overproducer
	Sa101	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa102	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa103	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa104	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa105	Pool	5* 6/7	Yellow	+	Overproducer
	Sa106	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa107	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa108	Single colony	5* 6/7	Yellow	+	Overproducer
	Sa109	Single colony	5* 6/7	Yellow	+	Overproducer
3 (CFBR ID: 311)	Sa110	Pool	5* 4/7	Multicolored	+	Overproducer
	Sa111	Single colony	5	Yellow	-	Nonproducer
	Sa112	Single colony	5	Yellow	-	Nonproducer
	Sa113	Single colony	5	Yellow	-	Nonproducer
	Sa114	Single colony	5	Yellow	-	Nonproducer
	Sa115	Single colony	5	Yellow	-	Nonproducer
	Sa116	Single colony	5	Yellow	-	Nonproducer
	Sa117	Single colony	8	Yellow	+	Overproducer
	Sa118	Single colony	8	Yellow	+	Overproducer

[a]*, 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.

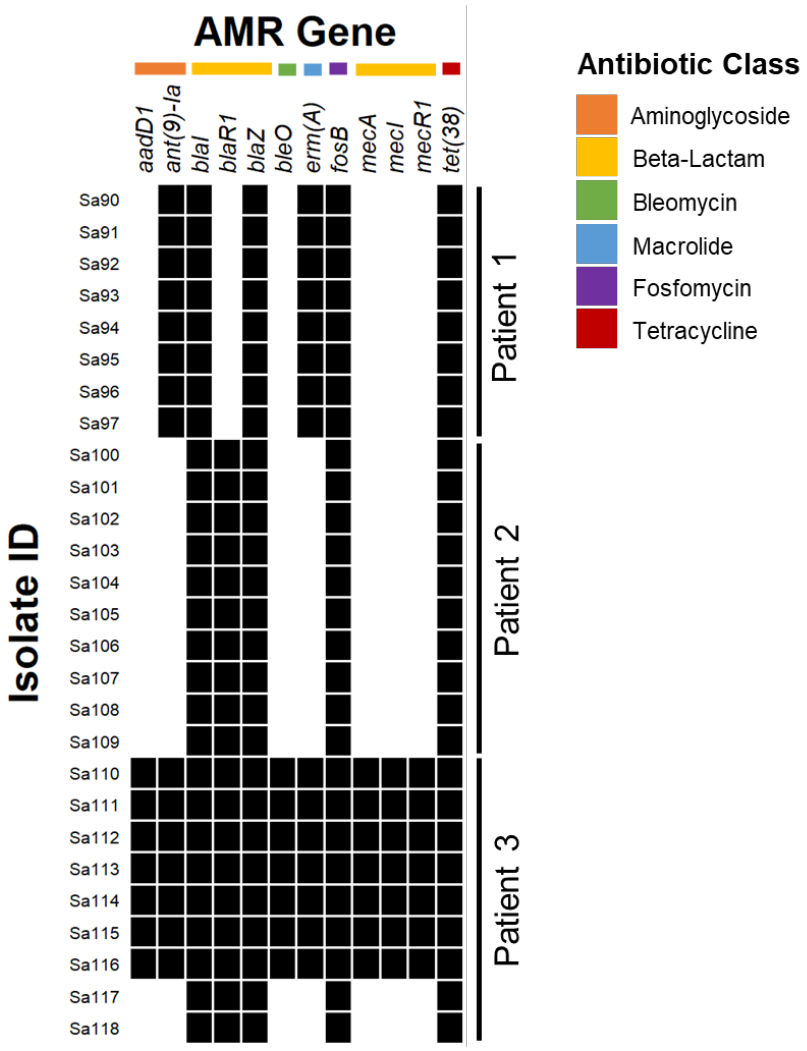

Figure 2. Antimicrobial resistant genes profile of *S. aureus* clinical isolates from three CF patients. The black box indicates presence of AMR gene in each isolate. Color indicates class of antibiotic.

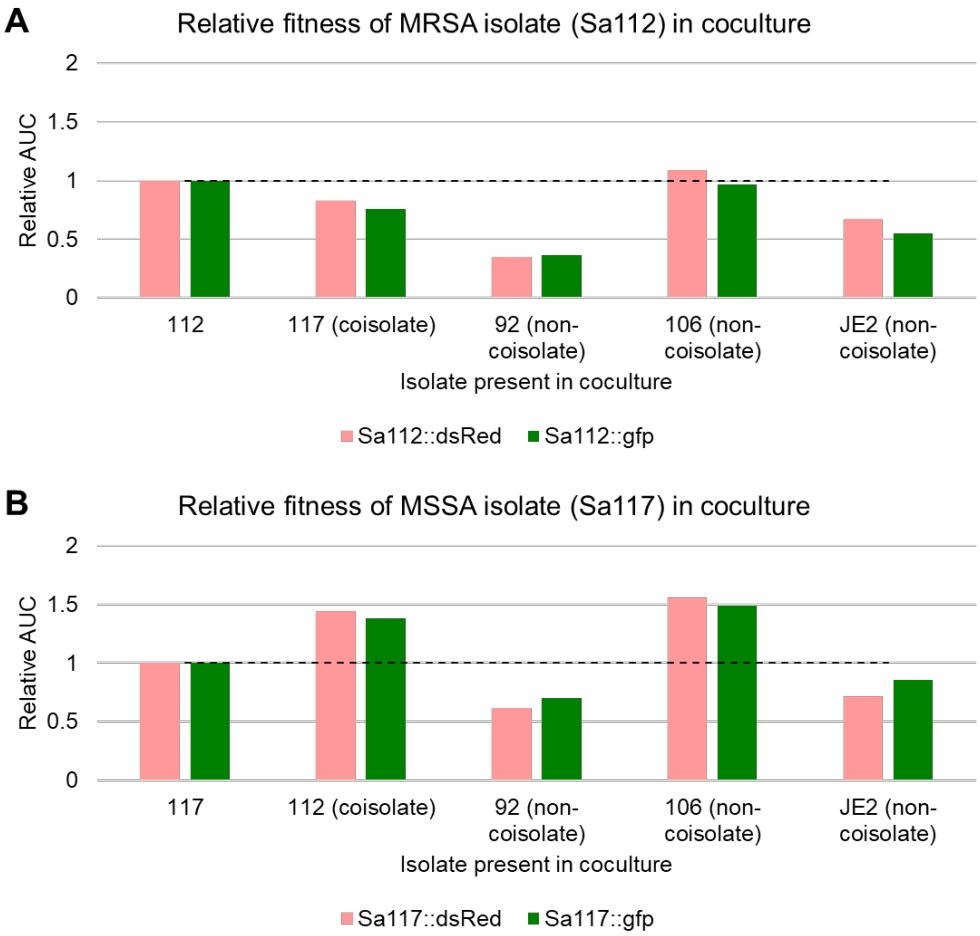

Figure 3. Representative relative fitness of fluorescently labeled *S. aureus* isolates in coculture with an unlabeled isolate. Fitness is derived from AUC of growth curves based on fluorescence intensity. Relative fitness is inferred from AUC relative to growth of the labeled isolates in coculture with its WT unlabeled isolate. Color indicates the expressed fluorescent protein: pink for *dsRed* and green for *gfp*. Panel **(A)** shows the relative fitness of isolate Sa112 from the MRSA subpopulation; **(B)** shows the relative fitness of isolate Sa117 from the MSSA subpopulation. Isolate Sa92, Sa106 and JE2 serves as a non-coisolate controls. Each bars show average of two technical replicates.

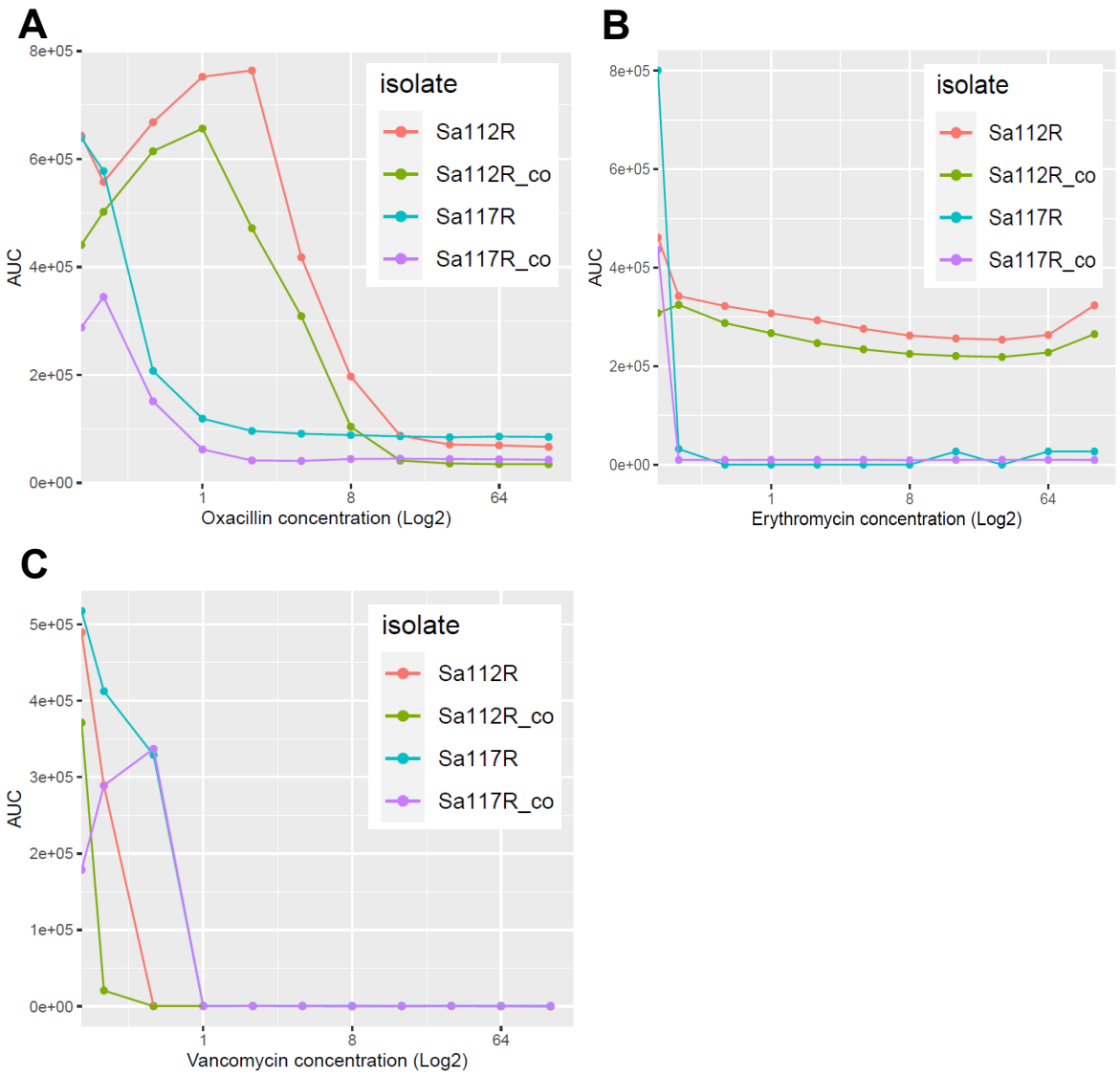

Figure 4. Growth potential of *S. aureus* isolates in monoculture and cocultures in the presence of antibiotics. Data shows growth potential of *dsRed* labeled *S. aureus* isolates inferred from AUC of 24-hour growth curves based on red fluorescent intensity, grown in monoculture and coculture with a coisolate in two-fold increasing concentrations of **(A)** oxacillin, **(B)** erythromycin, and **(C)** vancomycin. Sa112 isolate was detected with *mec* family genes and *erm(A)* gene, but not Sa117 isolate. Neither isolate was detected with vancomycin resistance related genes. Data shows one representative replicate.

TABLE 2 | Antibiotic resistance report of *S. aureus* isolated from the corresponding CF patients from medical microbiology laboratory[a]

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	Glycopeptide	Susceptible	Susceptible	*NA*

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

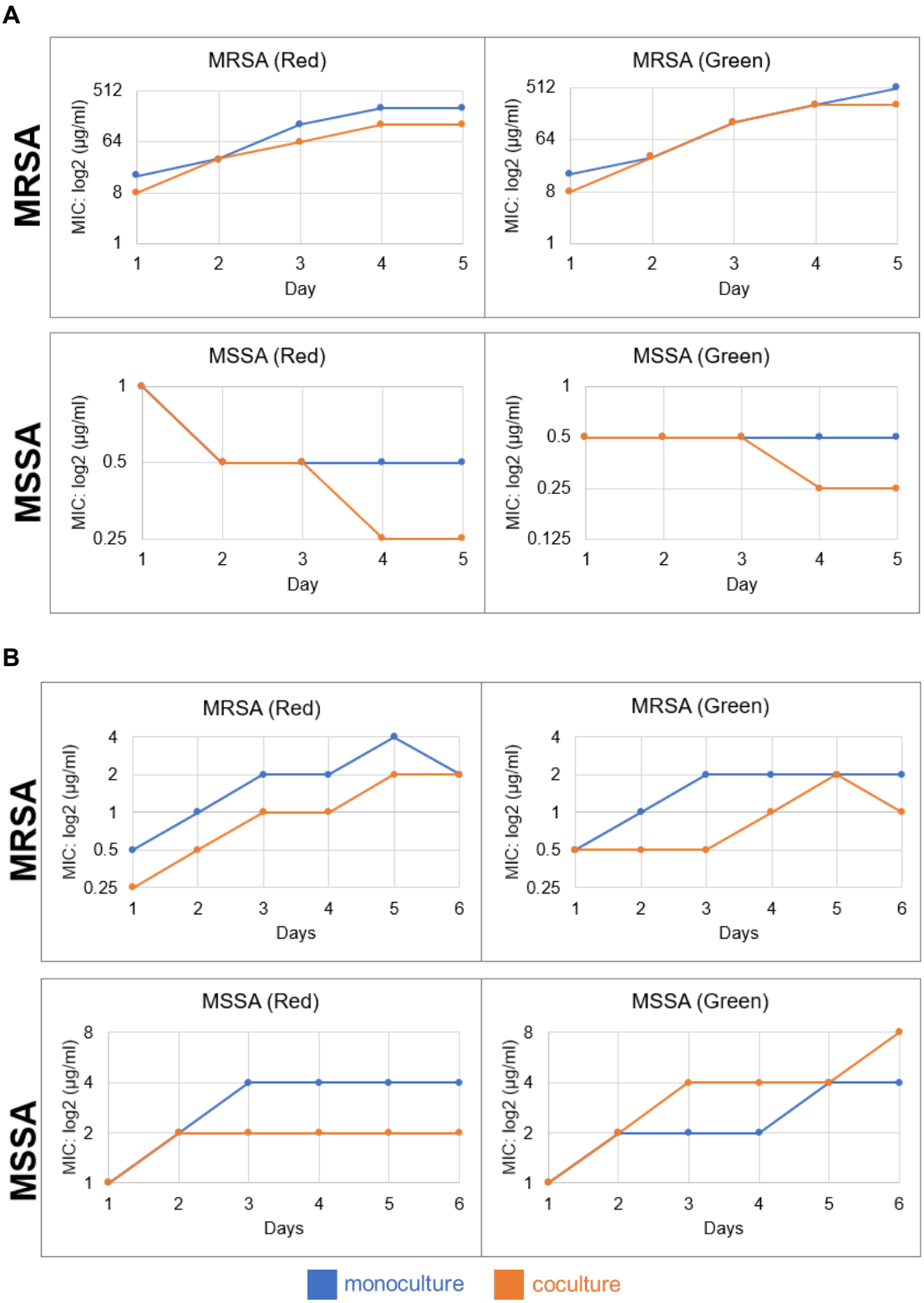

Figure 5. Evolution of antibiotic resistance of MRSA and MSSA isolates to antibiotics. (A) shows evolution in oxacillin and **(B)** shows evolution in vancomycin based on MIC over six days. Color indicates growth in monoculture or coculture. Data shows one replicate.

Table 3 | Isolates used in this study

Strain	Short name	Feature	Reference
Staphylococcus aureus USA300 JE2	JE2	MRSA control	Diep et al. 2006
Staphylococcus aureus RN4220	RN4220	MSSA control	Nair et al. 2011
Staphylococcus aureus CA26	CA26	Vancomycin control	Satola et al. 2011
Staphylococcus aureus CFBR_EB_Sa92	Sa92	Clinical isolate (Patient 1)	This study
Staphylococcus aureus CFBR_EB_Sa106	Sa106	Clinical isolate (Patient 2)	This study
Staphylococcus aureus CFBR_EB_Sa112	Sa112	Clinical isolate (Patient 3)	This study
Staphylococcus aureus CFBR_EB_Sa117	Sa117	Clinical isolate (Patient 3)	This study

SUPPLEMENTAL FIGURES

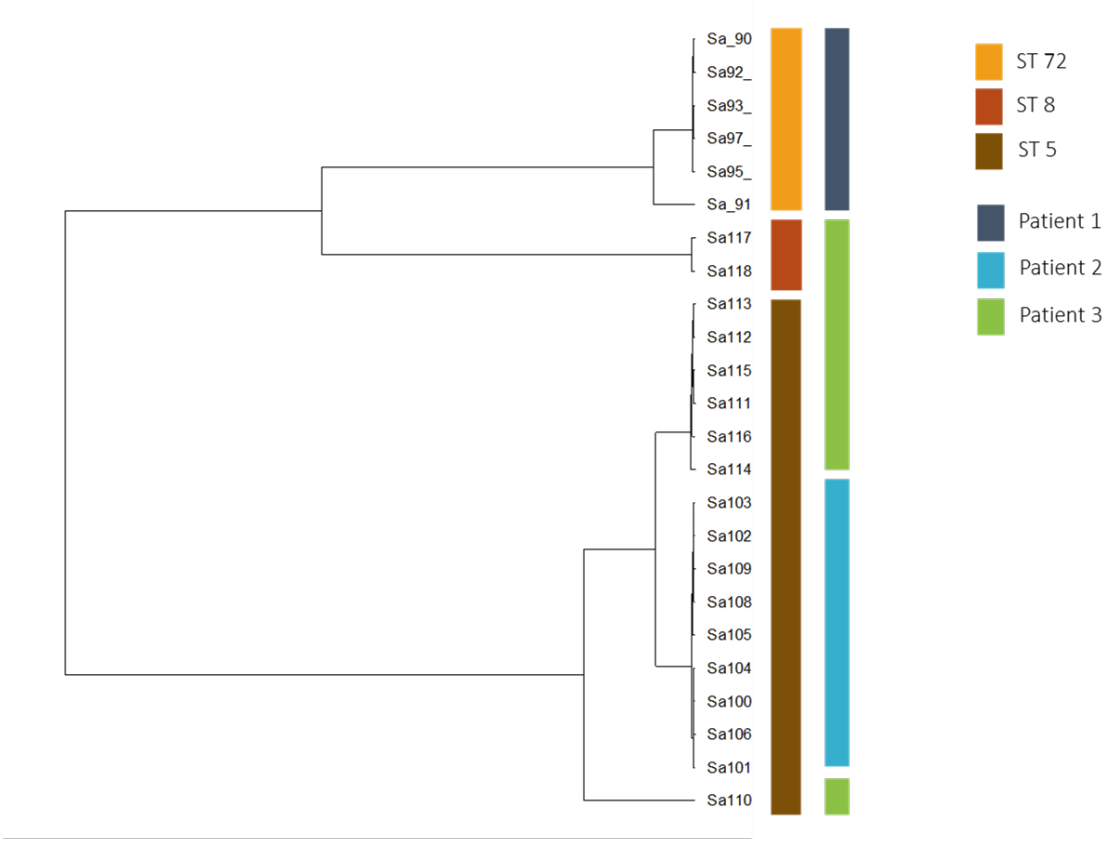

Figure S1. Phylogeny of S. aureus clinical isolates based on ST. Color indicates sequence types and patient which the isolate is purified from. Sa90, Sa 100, and Sa110 are pooled isolates.

		MSSA ST72	MSSA ST5*6/7	MRSA ST8	MRSA ST5	MSSA ST8
	Isolates	Sa92	Sa106	JE2	Sa112 WT	Sa117 WT
MRSA ST5	Sa112R	Non-coisolate			Unlabeled "self"	Coisolate
	Sa112G					
MSSA ST8	Sa117R				Coisolate	Unlabeled "self"
	Sa117G					

Figure S2. Coculture setups to assess relative fitness. R and G indicates expression of *dsRed* and *gfp* fluorescent protein respectively. WT indicates wildtype isolated without transformation with fluorescent proteins.

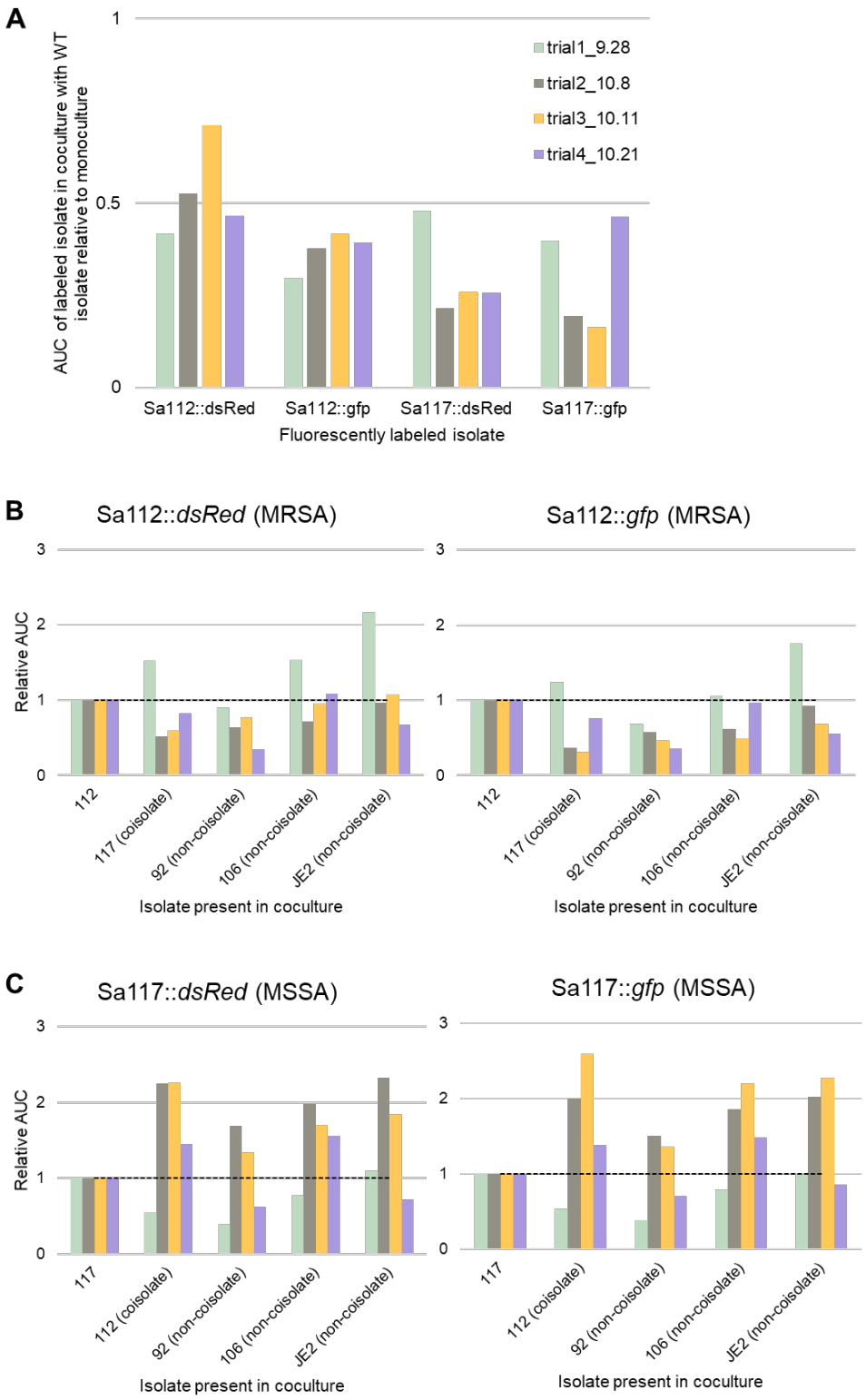

Figure S3. Relative fitness of fluorescently labeled *S. aureus* isolates in coculture with an unlabeled isolate across 4 biological replicates.

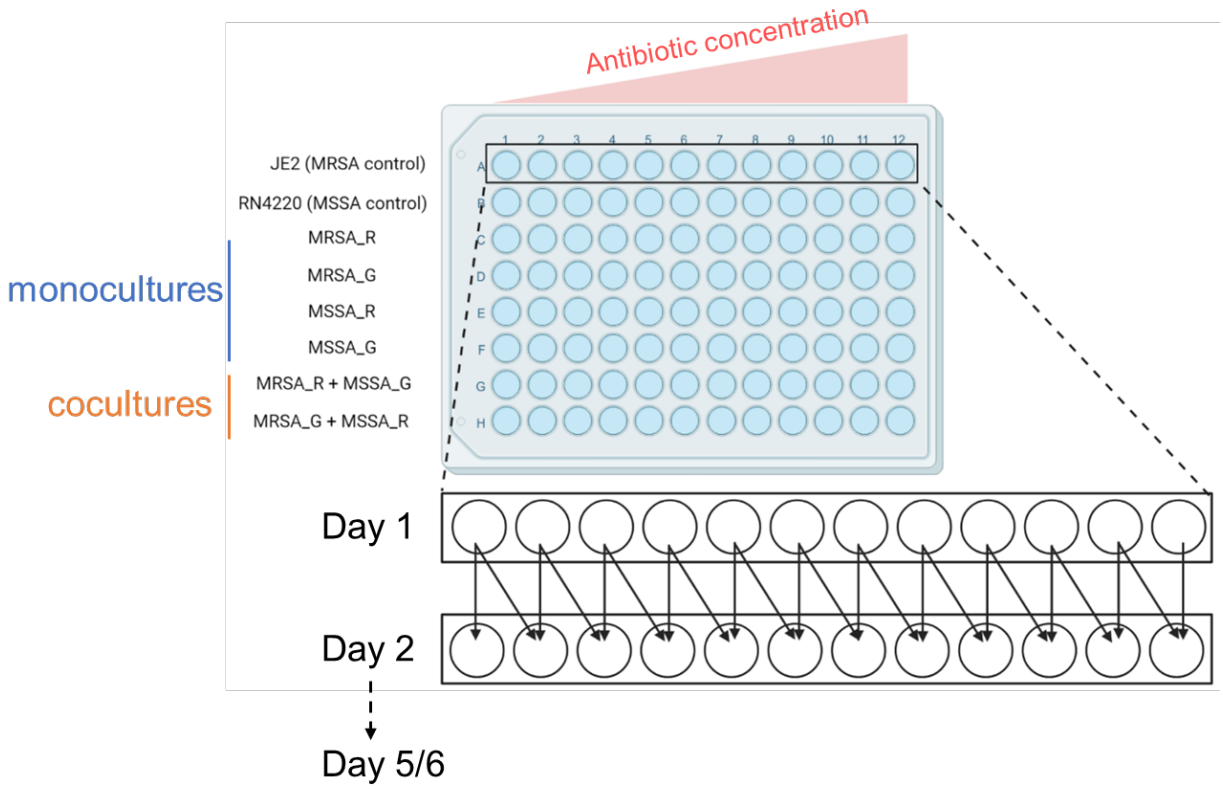

Figure S4. Experimental evolution setup in increasing concentrations of antibiotics.

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