TITLE

Host and antibiotic jointly select for greater virulence in *Staphylococcus aureus*

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AUTHOR CONTRIBUTIONS

MS, LTM, and TDR conceptualized the project. MS, KLH, MP, MHD, and JDG conducted the experiments. KLH and MS analyzed the data and drafted the manuscript. All authors contributed to manuscript revision.

DATA AVAILABILITY

Raw sequences have been deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA1162848, and phenotypic data deposited in the Figshare Repository (10.6084/m9.figshare.28745558).

CONFLICTS OF INTERESTS

The authors declare no conflicts of interests.

ABSTRACT

Widespread antibiotic usage has resulted in the rapid evolution of drug-resistant bacterial pathogens and poses significant threats to public health. Resolving how pathogens respond to antibiotics under different contexts is critical for understanding disease emergence and evolution going forward. The impact of antibiotics has been demonstrated most directly through *in vitro* pathogen passaging experiments. Independent from antibiotic selection, interactions with hosts have also altered the evolutionary trajectories and fitness landscapes of pathogens, shaping infectious disease outcomes. However, it is unclear how interactions between hosts and antibiotics impact the evolution of pathogen virulence. Here, we evolved and re-sequenced *Staphylococcus aureus,* a major bacterial pathogen, varying exposure to host and antibiotics to tease apart the contributions of these selective pressures on pathogen adaptation. After 12 passages, *S. aureus* evolving in *Caenorhabditis elegans* nematodes exposed to a sub-minimum inhibitory concentration of antibiotic (oxacillin) became highly virulent, regardless of whether the ancestral pathogen was methicillin-resistant (MRSA) or methicillin-sensitive (MSSA). Host and antibiotic exposure selected for reduced drug susceptibility in MSSA lineages while increasing MRSA total growth outside hosts. We identified mutations in genes involved in complex regulatory networks linking virulence and metabolism, including *codY*, *agr*, and *gdpP*, suggesting that rapid adaptation to infect hosts may have pleiotropic effects. In particular, MSSA populations under selection from host and antibiotic accumulated mutations in the global regulator gene *codY*, which controls biofilm formation in *S. aureus.* These populations had indeed evolved more robust biofilms—a trait linked to both virulence and antibiotic resistance—suggesting evolution of one trait can confer multiple adaptive benefits. Mutations that arose in these genes were also enriched in clinical isolates associated with systemic infections in humans. Despite evolving in similar environments, MRSA and MSSA populations proceeded on divergent evolutionary paths, with MSSA populations exhibiting more similarities across replicate populations. Our results underscore the importance of considering the host context as a critical driver of pathogen traits like virulence and antibiotic resistance.

KEYWORDS

Antibiotic resistance, virulence evolution, experimental evolution, *Staphylococcus aureus*, pathogen adaptation

INTRODUCTION

Antibiotic resistance is a global crisis [1]. Pathogens that evolve resistance and are able to survive antibiotic therapy can go on to cause disease and transmit to other hosts; hence antibiotic resistance and virulence are intertwined. Selective pressures like antibiotic usage and host defenses have independently been shown to alter the evolution of pathogen traits [1–5]. However, pathogens face multiple selective pressures in their environment, and selection by concurrent forces can interact synergistically to alter evolutionary rates and trajectories relative to individual pressures alone [6,7]. It is unclear how virulence evolves in the face of simultaneous selective pressures and whether this trait is constrained or facilitated by antibiotic resistance. These dynamics are even less understood in the early stages of disease emergence, where pathogens face a different suite of selection dynamics than pathogens having reached equilibrium [8,9]. Identifying conditions under which antibiotic resistance and virulence evolve in novel host populations will shed light on how diseases emerge and potential mitigation measures.

The strength of selection impacts pathogen evolution [10–13]. While antibiotic therapy administers concentrations that should kill all pathogen cells, low concentrations of antibiotics (sub-minimum inhibitory concentration, or sub-MIC) are pervasive in a variety of settings, including in natural environments (e.g., water bodies and soil) due to pollution and biological waste [1]. In the host, antibiotic concentrations will gradually decline after administration due to metabolism and excretion [14–16]. Sub-MICs allow susceptible populations to continue dividing, affording opportunities for mutations conferring greater resistance to emerge [15]. Ultimately, selection by sub-MICs can lead to highly resistant pathogens [10,17], which tend to incur less fitness costs compared to those under selection by high antibiotic concentrations [18]. Importantly, mutations not related to antibiotic resistance can arise, such as those involved in adaptation to the growth environment [19]. Low concentrations of antibiotics can also alter expression of virulence factors *in vitro* across a broad range of pathogens [20–23], suggesting that they can affect virulence during infection. Sub-MIC antibiotics therefore have the potential to alter the early stages of disease emergence in host populations, particularly for those pathogens that can spend parts of their life history outside the host. Taken together, exposure to low antibiotic concentrations likely alters how pathogens interact with their hosts, shaping evolution of both virulence and antibiotic resistance. However, there is a knowledge gap in the interaction between antibiotics and pathogen evolution *in vivo* [16].

The host can exert strong selection on pathogens, especially during the period when a new organism is infected (e.g., a zoonotic transition). Infection of new host individuals tends to bottleneck pathogen populations [24,25]. These bottlenecks can confer a fitness advantage to resistant bacteria in the face of low antibiotic concentrations [26]. In addition to defenses like the immune system, the host environment differs drastically in nutrient availability compared to rich laboratory media [16]. For example, *Staphylococcus aureus* passaged through macrophage cell lines had increased pathogen survival as well as resistance to antibiotics; these traits were lost when the pathogen was exposed to nutrient-rich media [27]. Similarly, the human defensive peptide b-defensin 3 can maintain reduced susceptibility of *S. aureus* to the antibiotic vancomycin [28]. Adapting to different niches within a host can also bring about drastic genomic changes. *Salmonella enterica* transitioning from an intestinal to a systemic lifestyle exhibited genome degradation in genes no longer necessary for inhabiting the gastrointestinal tract [24]. While sub-MIC antibiotic exposure and host factors have been shown to independently shape pathogen evolution, it is unclear how the collective actions of both selective pressures affect the evolution of virulence and antibiotic resistance.

Staphylococcus aureus is an opportunistic pathogen that commonly colonizes nares and skin in humans but can invade internal organs and blood, causing systemic infections and bacteremia

[29]. In 2017, more than 119,000 bactemia infections caused by *S. aureus* occurred in the United States with a mortality rate of 18% [30]. *S. aureus* colonizing host surfaces exhibit distinct genomic signatures against those isolated during systemic infection, indicating that the pathogen employs different strategies to adapt to different host sites [31]. For example, hemolytic strains tend to be more abundant than non-hemolytic ones in murine systemic infection models, whereas the opposite occurs for wound models [32]. Once inside the host, *S. aureus* evades detection by the immune system by hindering or lysing immune cells [33,34]. Sub-MIC concentrations of beta-lactam antibiotics—which disrupts bacterial cell wall synthesis—modify expression of virulence factors in *S. aureus*, increasing the expression of alpha-hemolysin [35]. Treatment with beta-lactam antibiotics systemically exposes *S. aureus* to low antibiotic levels at non-target host tissues [36]. The pathogen can also survive for extended periods of time outside of hosts and have been isolated from environments such as veterinary clinics and schools, as well as natural and constructed settings such as wastewater and beaches [37–40]. Combined with the increasing prevalence and concentrations of antibiotics in the environment, these factors likely increase opportunities for exposure of *S. aureus* to sub-MIC antibiotics, subsequently affecting its interaction with the host during infection.

Experimental evolution has been used to elucidate how different selective pressures impact pathogen evolution. *In vitro* studies have yielded insights into the phenotypes and genetic loci generated by longer-term antibiotic selection through passaging experiments lasting hundreds of generations [10,18,28,41]. Conversely, *in vivo* studies have focused on transmitting pathogens through individual hosts for a single to a handful of passages [26,42,43]. However, few systems have been suitable to examine how antibiotics affect pathogen adaptation within a host context. In this study, we take advantage of the suitability of a multicellular host, *Caenorhabditis elegans* nematodes, to study designs of high replication across an appreciable temporal scale. The *S. aureus* used here was originally isolated from a skin and soft tissue infection of a human inmate [44] and thus is a novel pathogen to *C. elegans*. Nonetheless, *S. aureus* can kill nematodes by colonizing the host intestine and lysing cells, inducing expression of defense genes in nematodes with roles conserved in humans [45,46]. Virulence screens in *C. elegans* using *S. aureus* transposon libraries also showed overlapping results to other infection models [47,48], with the degree of *S. aureus* virulence in *C. elegans* reflecting disease severity of human infections [49,50]. Experimentally evolving *S. aureus* in *C. elegans* thus allows us to track the early stages of virulence and antibiotic resistance evolution in novel host populations with the potential to identify clinically-relevant genomic regions underlying evolved traits.

Here, we directly test the impact of host and sub-MIC antibiotic exposure on pathogen evolution. Selection exerted by two concurrent forces may impede the pathogen's response to one or both forces [6]. Adaptation may require resources to be expended toward either virulence or antibiotic resistance, leading to a trade-off between these traits [51]. Alternatively, weaker selection from sub-MIC antibiotics may interact synergistically with hosts and facilitate the evolution or maintenance of high virulence and antibiotic resistance. Sub-MIC antibiotics can favor no-cost mutations [18], wherein pathogens can rapidly adapt without affecting other traits [8]. Because emerging pathogens tend to be far from the optimum of the fitness landscape, we expect pleiotropic or large-effect mutations to play a role [52]. We took advantage of the tractability of the system to determine how virulence, fitness, and antibiotic resistance are connected and how multiple selective pressures shape the evolutionary trajectory of two *S. aureus* isolates differing only in their antibiotic susceptibility.

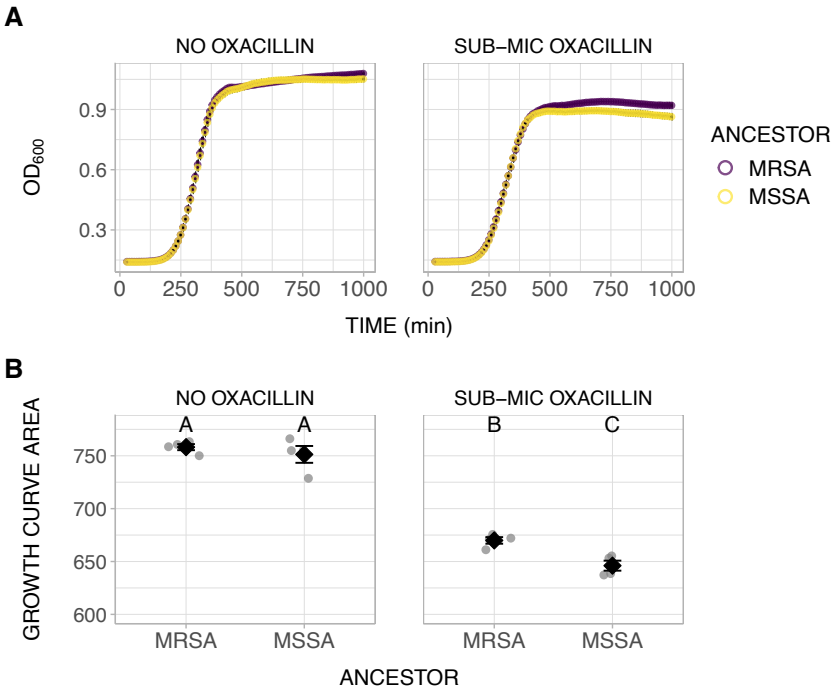
Carriage of Staphylococcal cassette chromosome *mec* (SCC*mec*), which encodes *mecA* and provides resistance against beta-lactam antibiotics, is a major mechanism for existing antibiotic resistance in *S. aureus*. We passaged two *S. aureus* isogenic strains, one with existing resistance

(*mecA*+, MRSA) and one with a transposon insertion in *mecA* (*mecA*-, MSSA) under selection by *C. elegans* and a sub-MIC level of oxacillin, a beta-lactam antibiotic that has replaced methicillin as a therapy for Staphylococcal infections. We selected for virulence in both strains to determine whether antibiotic resistance can hinder the evolution of greater virulence. We then quantified the ability of evolved pathogens to kill hosts as the metric for virulence. We also assessed whether populations maintained their ability to hemolyze red blood cells—an indicator of virulence expression in both humans and nematodes. Because growth outside the host is important for transmission of opportunistic pathogens, we quantified the *in vitro* growth and MIC of evolved pathogens. Finally, we identified mutations potentially underlying evolved traits and compared them against a database of over 80,000 *S. aureus* genomes to ascertained whether these mutations may be associated with adaptation to different human host sites.

RESULTS

Host and sub-MIC antibiotic exposure selected for the greatest virulence in both MRSA and MSSA.

We experimentally evolved two *S. aureus* isogenic variants of USA300 JE2 [44], MRSA and MSSA, with or without a host and sub-MIC antibiotic exposure. The ancestral MRSA and MSSA isolates did not differ in terms of total growth without oxacillin, but exhibited a slight decline in sub-MIC oxacillin (Figures 1A and 1B; χ_1^2 = 6.39, P = 0.01). For each ancestor, we passaged six independently evolving populations under each condition 12 times, for a total of 48 evolved *S. aureus* populations (Figure 1C).



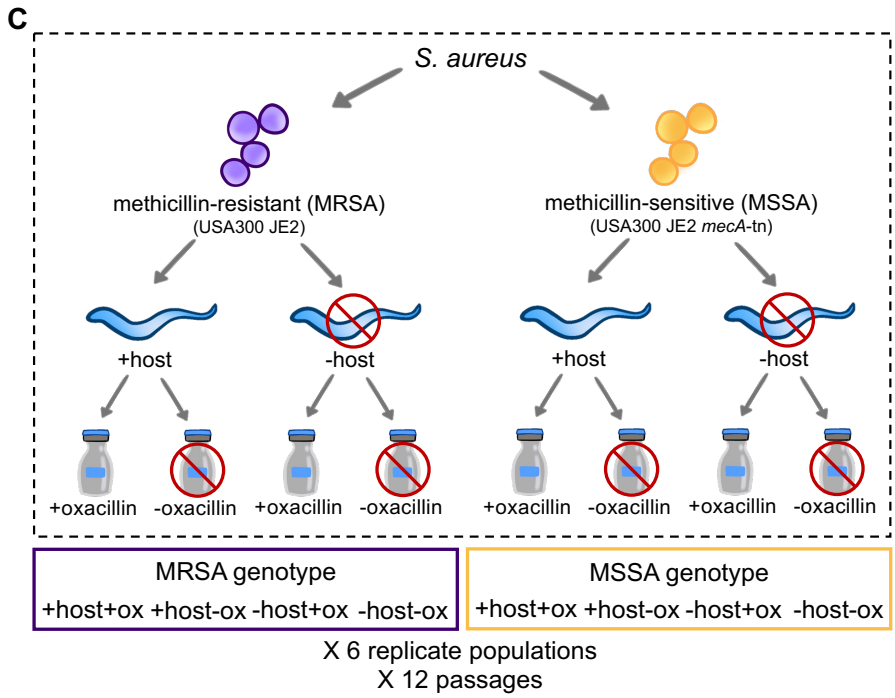


Figure 1. Experimental evolution design. A) Growth curves and B) total growth of ancestral MRSA and MSSA populations *in vitro* with and without sub-MIC oxacillin. Different letters indicate significant differences. C) MRSA and MSSA were passaged 12 times with or without hosts, in the presence or absence of a sub-MIC of the antibiotic oxacillin. Each treatment consisted of six independently evolving replicate populations. Experimental evolution treatment abbreviations are indicated in the purple and yellow boxes. Error bars indicate standard errors.

To assess the changes in virulence of evolved *S. aureus*, we measured the mortality of *C. elegans* infected with evolved pathogens. While the MRSA and MSSA ancestors caused similar levels of host mortality (dotted and dashed lines in Figure 2A), there was a significant treatment effect for evolved pathogens (Figure 2A; χ^2_7 = 460.43, P < 0.001). For the MRSA genotype, host and oxacillin exposure selected for the greatest virulence (P < 0.001 for all Tukey's post-hoc tests of +host+ox *vs*. other treatments). Conversely, pathogens evolved in the absence of either pressure exhibited attenuated virulence (-host-ox *vs.* +host-ox: P < 0.001; -host-ox *vs.* -host+ox: P < 0.001). These populations also had significantly greater variance (Levene's test for homogeneity of variance $F_{3,20}$ = 4.06, P = 0.021) compared to those under selection from host and oxacillin (Tukey's post-hoc test P = 0.026), and those under solely host selection (P = 0.045). For MSSA, host and oxacillin exposure similarly favored greater virulence over the other three conditions (P < 0.001 for all Tukey's post-hoc tests of +host+ox *vs*. other treatments). There was no difference between variances (Levene's test for homogeneity of variance $F_{3,20}$ = 0.99, P = 0.417).

We also characterized the hemolytic activity of evolved populations, which correlates with the ability to secrete extracellular toxins and virulence [53]. At least one population from most treatments had lost the ancestral ability to hemolyze sheep's blood (Figure 2B). Sub-MIC oxacillin maintained hemolytic activity (χ^2_1 = 5.82, P = 0.016), suggesting that constant exposure to low concentrations of oxacillin favored retention of extracellular toxicity. While hemolysis was not necessary for increased host-killing, this ability was correlated with greater nematode mortality (Figure 2C; Kruskal-Wallis χ^2_1 = 3.97, P = 0.046). As virulence was still heightened in pathogens unable to destroy red blood cells (e.g., MSSA pathogens evolving without host or oxacillin caused

greater than 90% mean mortality despite none being hemolysis-positive), other virulence factors may be compensating for the absence of hemolysis.

Natural populations of *S. aureus* exhibit variation in hemolytic ability [54], even within an individual host [55]. We thus hypothesized that a significant selective pressure like oxacillin would favor little variation of this trait within a population. We determined the hemolysis status of isolates from each evolved population (10 isolates x 48 populations)—most populations were in consensus (Figure S1). Furthermore, oxacillin still has a significant effect, where hemolysis was maintained when under sub-MIC exposure selection (Figure 2D; χ_1^2 = 23.30, P < 0.001). Oxacillin favored less hemolytic diversity in MSSA populations (Levene's test for homogeneity of variance $F_{1,22}$ = 26.06, P < 0.001), further demonstrating the interaction between antibiotics and virulence, and that antibiotics can shape variation in virulence traits.

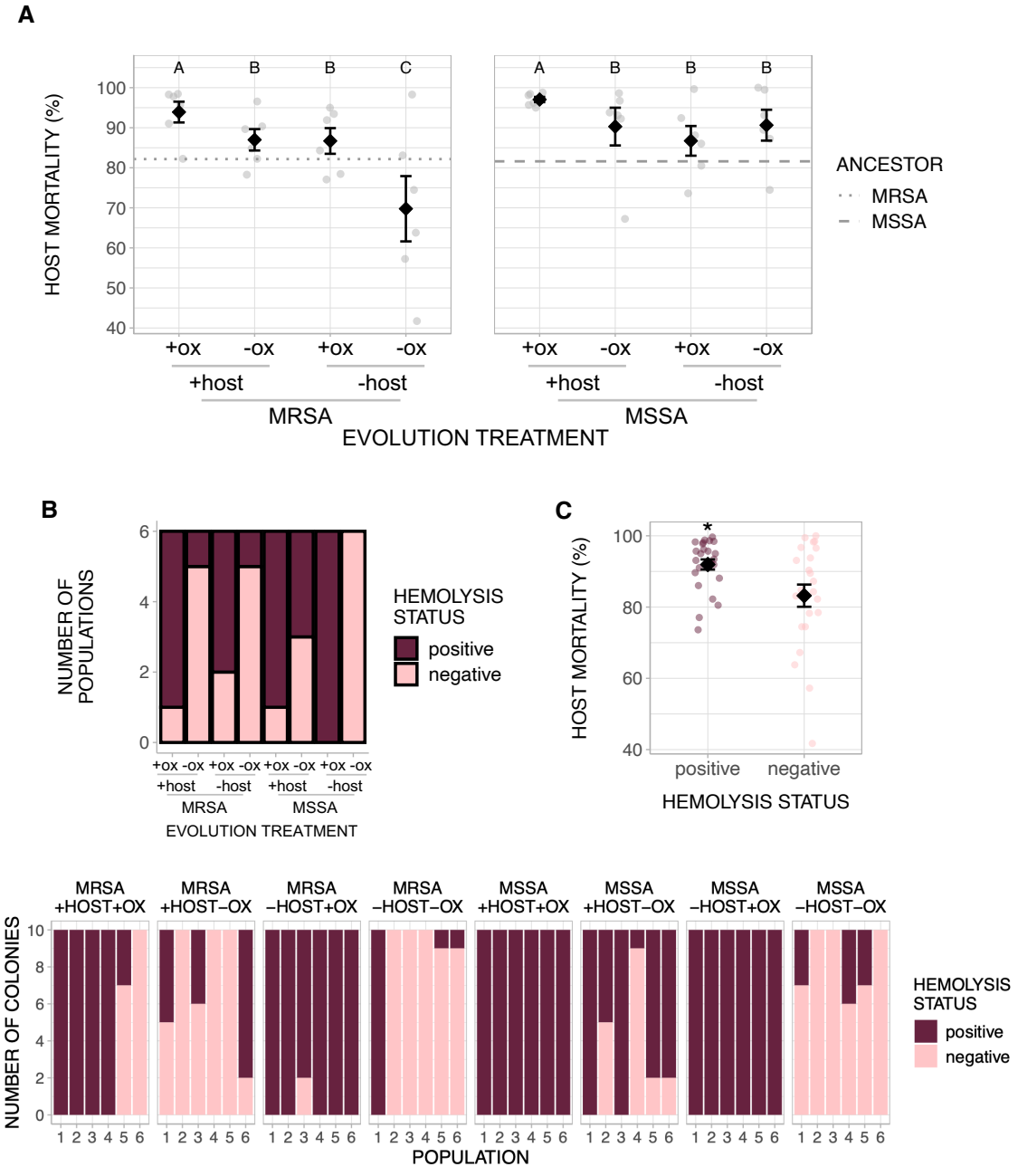


Figure 2. Evolution of virulence is facilitated by exposure to both host and sub-MIC antibiotic. A) Virulence in terms of *C. elegans* mortality. Dashed lines indicate respective ancestral virulence. Different letters indicate significant differences. B) Virulence in terms of the ability to hemolyze sheep's blood, assayed at the population level. The y-axis indicates the number of evolved populations for each category. C). Host mortality from A) grouped by hemolysis status in B). D) The proportion of colonies sampled from each evolved population that are able to hemolyze sheep's blood. Error bars indicate standard errors. *P < 0.05

Host and sub-MIC antibiotic promoted growth of MRSA outside hosts and reduced drug susceptibility in MSSA.

We measured the *in vitro* growth of evolved populations to evaluate how pathogen fitness outside the host had been impacted. We used rich media to replicate the conditions under which *S. aureus* evolved during the experiment. Importantly, rich media prevents introduction of additional selective pressures than those being tested. In media without oxacillin, there were no significant differences in total growth between treatments (Figure 3A; χ^2_7 = 9.24, P = 0.24). However, in sub-MIC oxacillin, MRSA populations under selection from both pressures exhibited the greatest growth (Figure 3B; χ^2_7 = 106.16, P < 0.001), with some achieving more growth than those in the absence of oxacillin. Similarly, MSSA populations exposed to host and sub-MIC oxacillin exhibited a moderate increase in growth compared to other combinations of selective pressures.

We then determined the MICs of evolved populations to ascertain how host and sub-MIC exposure affected the evolution and maintenance of antibiotic resistance. For the MRSA genotype, all but two populations retained the ancestral level of oxacillin resistance (16ug/mL oxacillin; Figure 3C). During evolution in hosts without antibiotic selection, two populations lost their resistance and exhibited similar susceptibility as the MSSA ancestor (0.25ug/mL). For the MSSA genotype, host and oxacillin exposure selected for decreased antibiotic sensitivity (Fisher's exact test P < 0.001; pairwise post-hoc P < 0.05 for +host+ox *vs.* all other treatments), up to 8-fold the ancestral MIC. These results suggested sub-MIC exposure combined with host factors potentiated the increase in antibiotic resistance in MSSA. One population under selection from solely the host also had an increased MIC, supporting previous evidence showing non-antibiotic selective pressures, such as a host in our study, can select for reduced antibiotic susceptibility [56]. There was no variation in antibiotic resistance within MRSA populations and little variation within MSSA populations across the most genetically diverse populations (Figure S2).

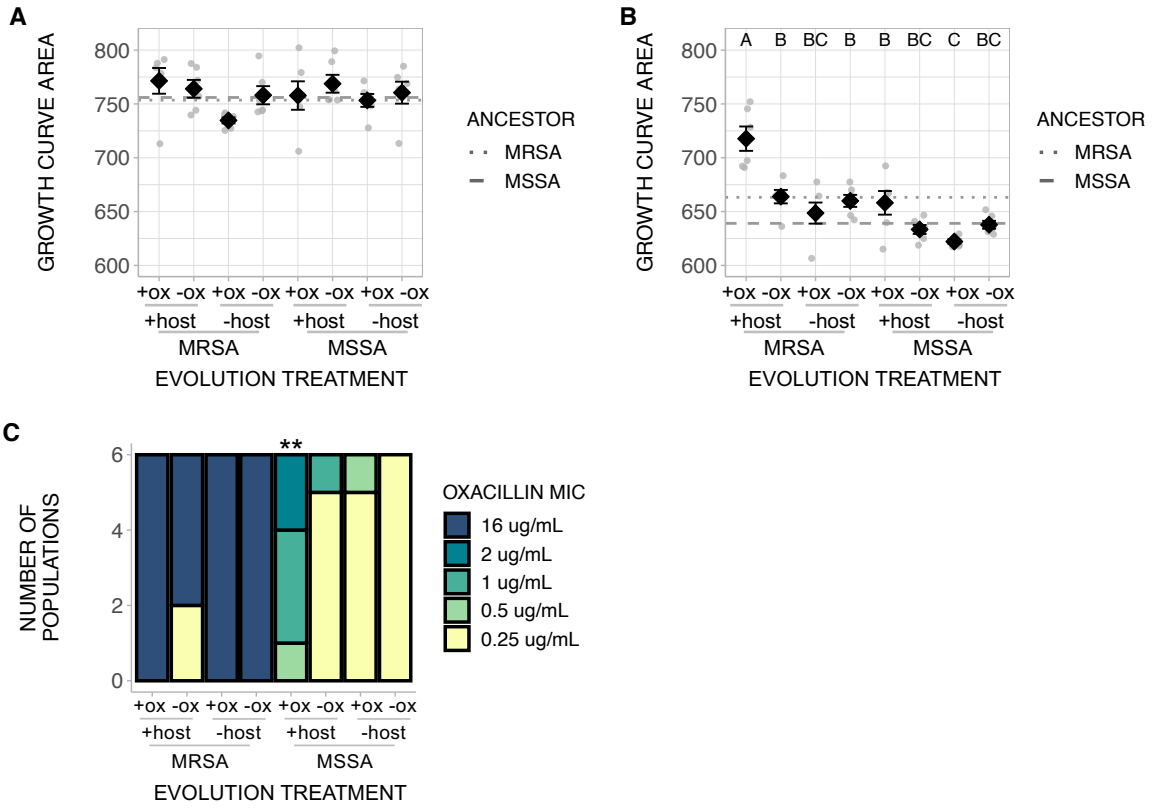


Figure 3. **Host and sub-MIC antibiotic selection facilitated pathogen growth in antibiotics**. Pathogen *in vitro* growth A) without oxacillin and B) in sub-MIC oxacillin. Different letters indicate significant differences. C). Oxacillin MIC of evolved populations. The y-axis indicates the number of evolved populations for each category. Error bars indicate standard errors. **P < 0.01

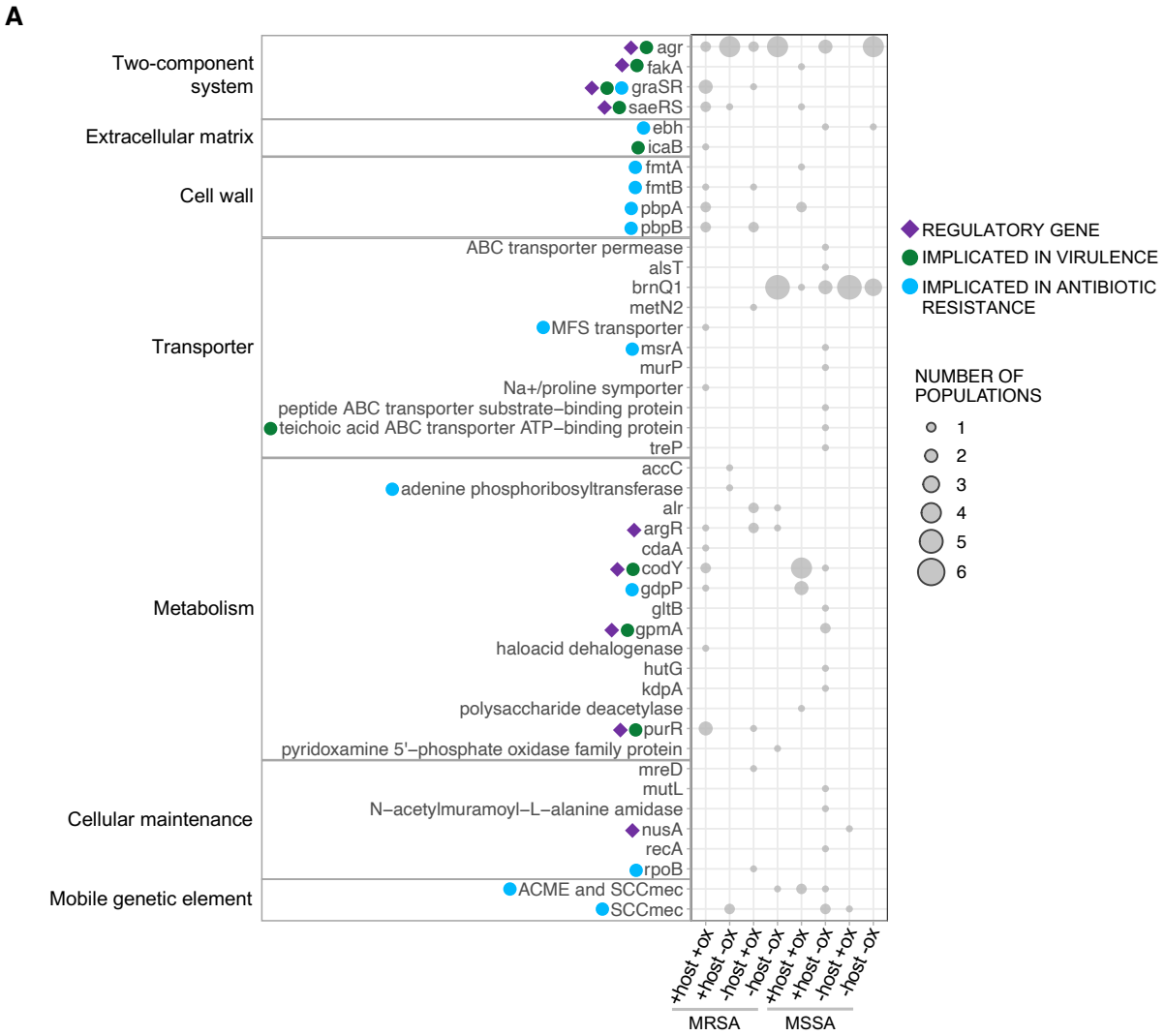
Parallel evolution of regulators of virulence and antibiotic resistance across evolved populations.

We conducted whole-genome sequencing of populations to identify mutations arisen from host and antibiotic selection. Below we focus on mutations that had swept to fixation (Figure 4A, Table S1); Figures S3 and S4 summarize mutations that were below 100% frequency in each population. Populations evolved from the MSSA ancestor had significantly fewer mutations (excluding those intergenic or synonymous) than MRSA populations (Figure S5; χ_1^2 = 5.28, P = 0.022), potentially due to the slightly reduced growth of MSSA in the presence of oxacillin (Figure 1B). Sub-MIC oxacillin selection also resulted in more mutations (χ_1^2 = 5.92, P = 0.015).

Between pathogens with the highest virulence—those under selection from both selective pressures—populations arisen from MRSA had mutations in 25 genes and intergenic regions, while those from MSSA had mutations in 12 genes and intergenic regions. Both treatments had mutations in virulence regulators *codY* [57] and *saeRS* [58] suggesting some shared pathways to heightened virulence among the two treatments, but mutations in these genes did not occur across all replicate populations. These treatments also had mutations in *gdpP* and *pbpA*, which have been implicated in resistance to beta-lactam antibiotics [59,60], with *gdpP* playing a role in MSSA resistance to oxacillin in particular [61]. Five out of six populations evolved from the MSSA ancestor had a mutation in either gene, which may have contributed to the reduced oxacillin sensitivity in these populations.

Regulatory genes were enriched with mutations (Figure 4A; one-sample Poisson test P < 0.001 for MRSA and MSSA genotypes). These included *agr*, *saeRS,* and *codY*. *agr* is a quorum sensor that regulates the expression of virulence factors [62]. All 22 mutations in *agr* were found in populations that have lost the ability to hemolyze red blood cells. In contrast to the other two regulators, *codY* is a transcriptional repressor found in gram-positive bacteria that controls virulence by monitoring nutrient levels in the environment [57].

Nine populations experienced large scale deletions that included the entire SCC*mec* cassette. Deletion of ACME has been shown to enhance the competitive fitness of *S. aureus* USA300 *in vivo* [63]. The two MRSA populations that lost their resistance (Figure 3C) had SCC*mec* deletions, suggesting that resistance could be more costly when evolving in a host. The loss of SCC*mec* and ACME was associated with an increase in total growth from the ancestor outside the host (Figure 4B; one-sample t-test t = 2.73, df = 8, P = 0.026).



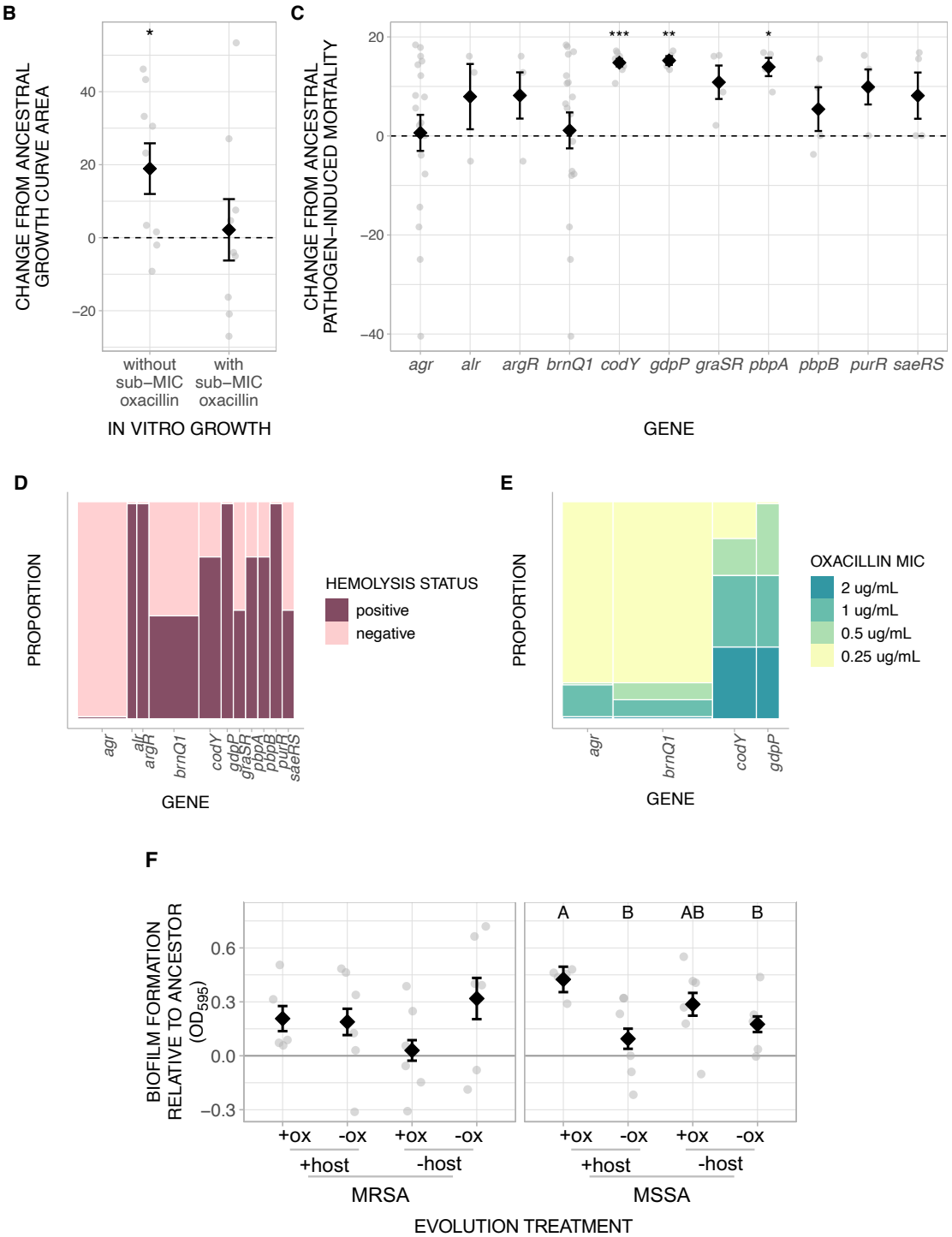


Figure 4. Regulatory genes likely played an important role in pathogen adaptation. A) Mutations swept to fixation, excluding intergenic and synonymous mutations, grouped by general function. The size of each point indicates how many populations had acquired at least one mutation in the gene. Colored shapes next to genes indicate whether these genes are regulatory or have been implicated in virulence or antibiotic resistance in the literature (see Table S1). B) *In vitro* growth of populations with SCC*mec* and ACME deletions with or without sub-MIC oxacillin. C) Change from ancestral pathogen-induced mortality *vs*. mutations. D)

Hemolysis status *vs*. mutations. E) Oxacillin MIC *vs*. mutations. F) Biofilm production of evolved populations. Different letters indicate significant differences. The column widths in D and E corresponds to the number of mutations. Error bars indicate standard errors. All evolved populations were sequenced except for one population from the -host-ox treatment. *P < 0.05, **P < 0.01

Mutations in antibiotic resistance genes are associated with heightened virulence.
We correlated mutations arising in specific genes with measured traits to identify loci potentially underlying evolved phenotypes. We focused on genes where two or more mutations (excluding intergenic or synonymous mutations) had fixed during the experiment regardless of treatment (Figure 4C). Mutations in three genes were associated with significant increases in virulence from the ancestor: *codY* (one-sample t-test = 19.56, df = 7, P < 0.001), *gdpP* (one-sample t-test = 17.02, df = 3, P = 0.002), and *pbpA* (one-sample t-test = 7.54, df = 3, P = 0.012). Mutations in *agr* in general were not associated with changes in overall virulence, but greater host mortality was associated with *agr* mutations arising from the MSSA ancestor compared to the MRSA ancestor (Wilcoxon rank sum exact test P = 0.045).

Mutations in specific genes had a significant association with the ability to hemolyze red blood cells (Figure 4D; Fisher's exact test P < 0.001). There was a greater proportion of populations with *agr* mutations unable to hemolyze red blood cells compared to *alr* (P = 0.036), *argR* (P = 0.006), *codY* (P = 0.005), *gdpP* (P = 0.006), and *purR* (P = 0.006). There were also significant differences between the mutations associated with oxacillin resistance in populations evolved from the MSSA ancestor (Figure 4E; Fisher's exact test P = 0.0015), where a greater proportion of populations with mutations in *codY* had reduced oxacillin susceptibility compared to *brnQ1* (P = 0.043). Exposure to oxacillin maintained hemolysis in evolved populations (Figure 2B), suggesting low concentrations of oxacillin exerted negative selection on the *agr* locus. By contrast, mutations in *agr* were associated with the loss of hemolytic activity, consistent with previous findings [32].

Because mutations in *codY* appeared to be important for both virulence (Figure 4C) and antibiotic resistance (Figure 4E), we hypothesized that traits controlled by *codY* were responsible for the traits observed in MSSA populations under selection from both pressures. A potential mechanism underlying changes in virulence and antibiotic sensitivity in MSSA may involve biofilm formation. Biofilm is implicated in *S. aureus* pathogenesis as well as in dampening the efficacy of antibiotic treatment [41]. In *C. elegans*, *S. aureus* biofilm enhances virulence and protects the pathogen from host innate immune defenses [64]. Both sub-MIC level of beta-lactam antibiotics and null-mutants of *codY* induce robust biofilm formation and hemolysis activity [14,65,66]. Taken together, exposure to sub-MIC level of oxacillin and acquisition of potentially deleterious mutations in *codY* may underlie both increased virulence and reduced antibiotic susceptibility. Our findings partially supported this hypothesis: biofilm formation did not significantly differ for the MRSA genotype (χ^2_3 = 7.24, P = 0.06). By contrast, biofilm production differed between treatments for the MSSA genotype (Figure 4F; χ^2_3 = 17.91, P < 0.001), with populations exposed to both selective pressures forming more biofilm than those evolved without host (Tukey's post-hoc test P < 0.001) or either pressure (P = 0.014), but not those evolved in solely sub-MIC oxacillin (P = 0.095). While host and sub-MIC oxacillin selection favored robust biofilms in MSSA, oxacillin by itself also increased biofilm formation, suggesting low antibiotic concentrations contributed significantly to the evolution of drug-sensitive populations. The loci underlying biofilm formation may be different in these two treatments, since mutations in *codY* did not appear in populations under solely oxacillin selection (biofilm is a polygenic trait in *S. aureus* [67]).

Mutations that arose during experimental evolution are associated with human systemic infections.

We then determined whether mutations that arose in our experiment exist in natural *S. aureus* isolates. We conducted BLAST searches against a dataset of 83,383 high quality public *S. aureus* whole genome assemblies [68] (Figure 5A) and found matches against the majority of our mutations. We hypothesized that these mutations may be associated with *S. aureus* adaptation to different environments. Indeed, for many genes implemented in virulence and antibiotic resistance, a greater proportion of natural isolates containing our mutations were found in blood and systemic infections compared to skin/nose/throat colonization than expected (Figure 5, statistics shown in Table S2). Mutations arisen in MRSA populations under selection from both host and sub-MIC oxacillin had the most matches to natural isolates (Figure 5B), further highlighting the importance of the interaction between these two selective forces. Overall, our results demonstrate that evolution experiments in an invertebrate model can give rise to clinically-relevant mutations in a major bacterial pathogen.

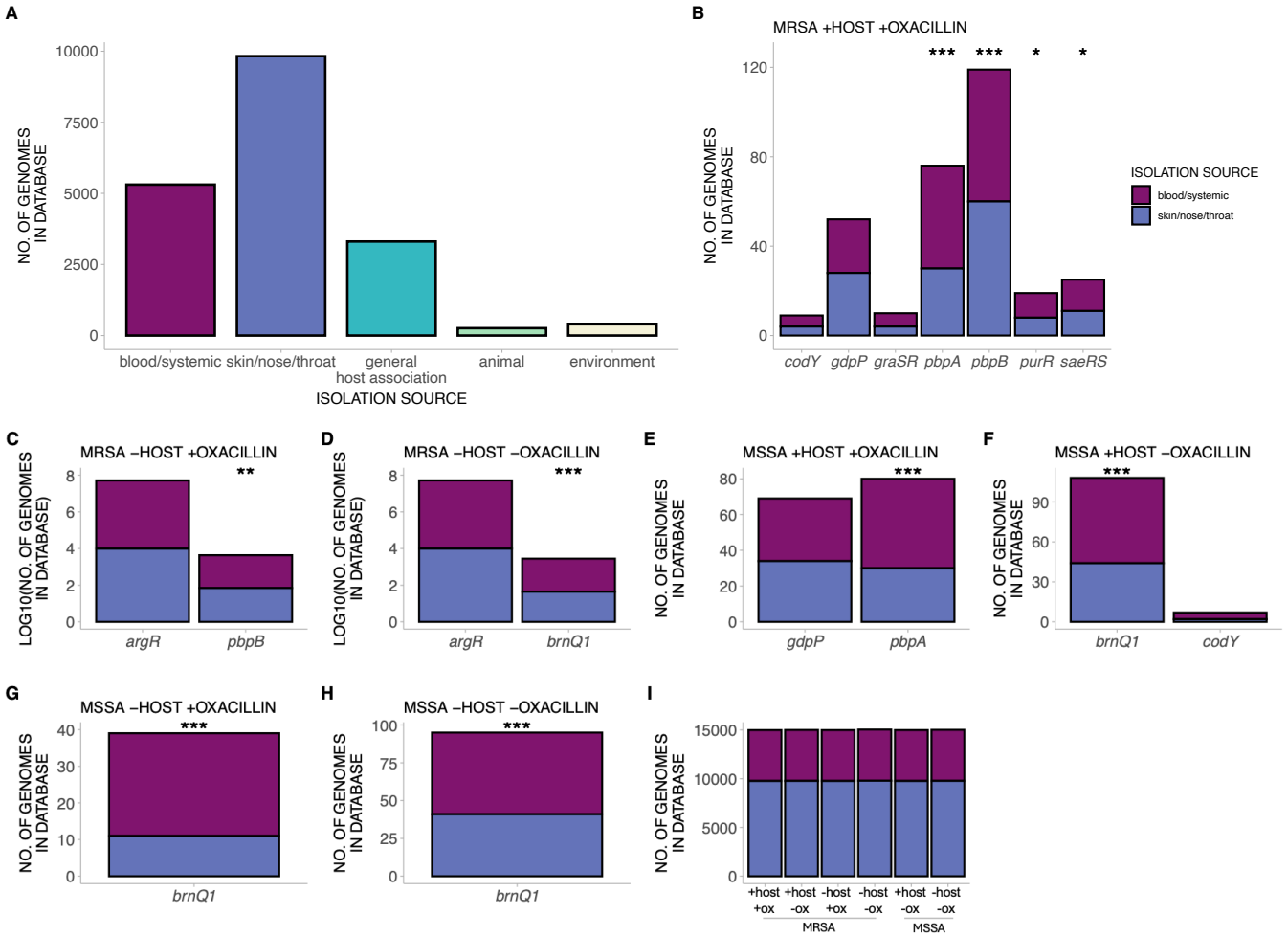


Figure 5. **Mutations that arose during experiment are enriched in isolates associated with systemic infection in humans**. A) Number of genomes in our public *S. aureus* genome dataset (see Methods) grouped by isolation source. General host-association refers to descriptions not specific enough to assign to other categories. We excluded samples that were ambiguously specified or missing information. B) to H) Number of genomes in the database containing mutations in the respective gene. I) Number of genomes in the dataset containing the

mutations arisen in *agr*. This gene is separate from the others to facilitate ease of visualization due to the magnitude of the y-axis. Asterisks indicate significant difference in the proportion of blood/systemic-associated genomes compared to the expected distribution in the dataset. *P < 0.05, **P < 0.01, ***P < 0.001

MRSA and MSSA exhibited divergent phenotypic and genomic evolution during adaptation

We conducted principal component analysis on the evolved traits (virulence, growth in media with or without sub-MIC oxacillin, and biofilm production) in MRSA and MSSA populations to determine the impact of selective pressures on overall pathogen phenotypic evolution (Figures 6A and 6B). We found a significant effect of treatment for the MRSA genotype (PERMANOVA $F_{(3,23)} = 8.38$, $r^2 = 0.557$, $P = 0.001$), where populations exposed to host and sub-MIC oxacillin formed a distinct cluster from all other treatments ($P < 0.05$ for all pairwise comparisons). The MSSA genotype had a marginally non-significant effect of treatment (PERMANOVA $F_{(3,23)} = 2.20$, $r^2 = 0.248$, $P = 0.064$). Populations evolved from the MRSA ancestor had similar trajectories for growth in sub-MIC oxacillin and virulence, whereas biofilm production and virulence were more correlated for populations evolved from the MSSA ancestor.

To determine how host and sub-MIC antibiotic shaped pathogen genomic evolution, we built phylogenies using mutations that arose from the MRSA and MSSA ancestors (Figures 6C and D). We compared Euclidean distances calculated from the frequency of each mutation to determine the genetic distance from the ancestor—how fast each population was evolving—and the genetic distance between populations within each treatment—how much each population had diverged from one another [69,70]. There was no significant difference between treatments for MRSA populations in terms of genetic distance to the ancestor (Figure S6A; $F_{(3,20)} = 2.39$, P: 0.099), suggesting that populations generally exhibited similar evolutionary rates. By contrast, MSSA populations had a significant effect of treatment (Figure S6B; $F_{(3,19)} = 10.44$, $P < 0.001$), where populations under selection from solely the host had greater genetic distance from the ancestor than populations under selection from solely oxacillin (Tukey's post-hoc test $P < 0.001$) or under neither selective pressure ($P = 0.004$).

For the MSSA group, populations within each treatment tended to exhibit more genetic similarity (Figures 6C and 6D). For MRSA, treatments varied in how divergent populations were (Figure S6C; Kruskal-Wallis $\chi^2_3 = 21.09$, $P < 0.001$), where populations exposed to both selective pressures diverged more from each other than populations in the other three treatments (Dunn's post-hoc test $P < 0.05$ for all pairwise comparisons). Likewise, MSSA populations also differed in how much they varied (Figure S6D; $F_{(3,51)} = 54.20$, $P < 0.001$). Populations under solely host selection had greater divergence from each other than all other treatments (Tukey's post-hoc test $P < 0.001$ for all pairwise comparisons). By contrast, populations under selection solely from sub-MIC oxacillin diverged the least ($P < 0.05$ for all pairwise comparisons). These findings altogether suggest that even concentrations well below the MIC differentially impacts sensitive and resistant pathogens.

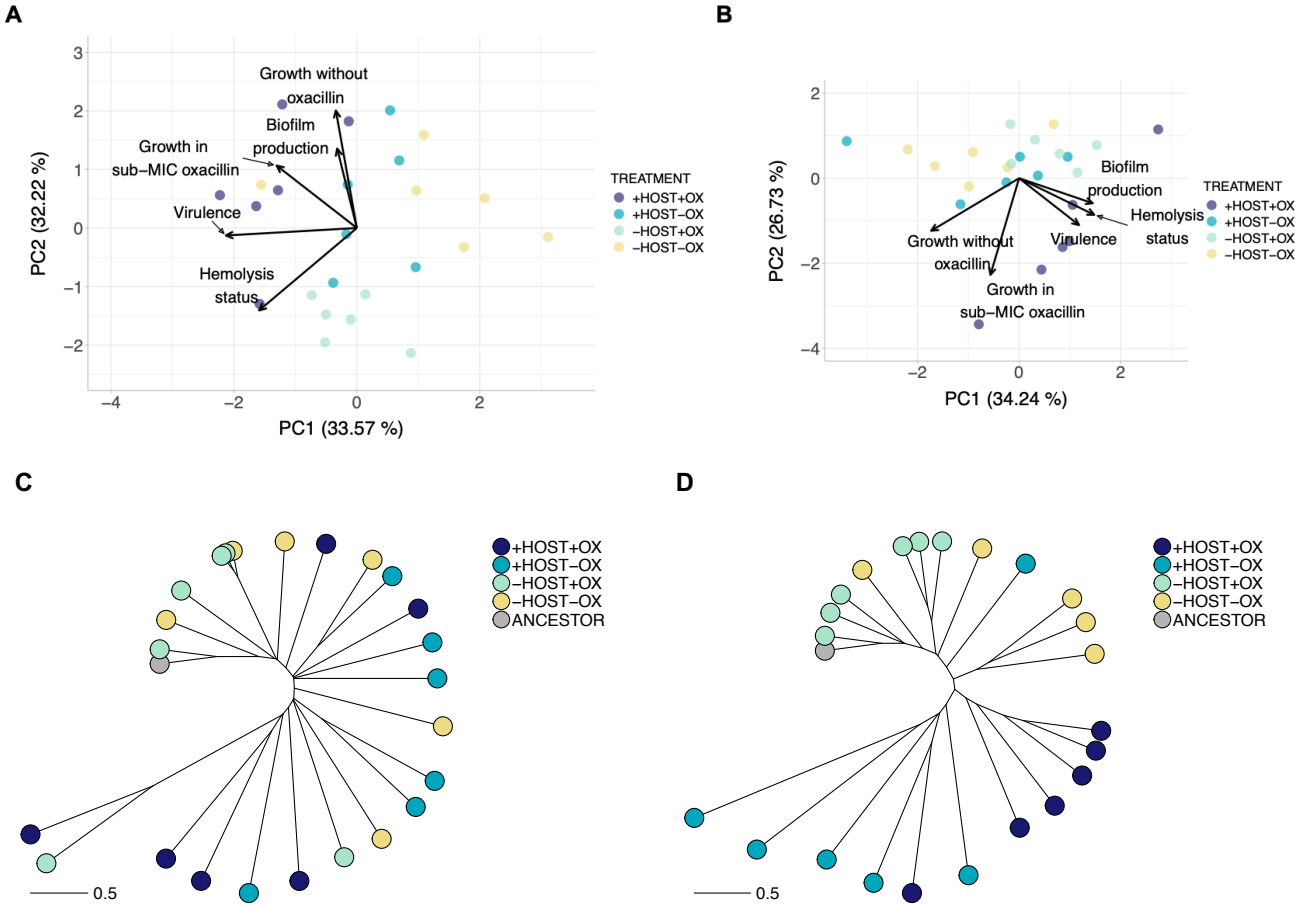


Figure 6. **MRSA and MSSA underwent distinct evolutionary trajectories.** Principal component analysis of traits evolved from A) MRSA and B) MSSA ancestors. Phylogenetic tree constructed from frequencies of mutations in populations evolving from C) MRSA and D) MSSA ancestors. Scale bar indicates Euclidean distance.

DISCUSSION

While antibiotics have existed for millions of years, the mass production and overuse of antibiotics by humans in the last century have exerted an unprecedented level of selection on pathogens [71,72]. In addition to antibiotics, pathogens face numerous selective pressures such as host defenses and competitors [2,73,74]. Opportunistic pathogens are especially likely to encounter diverse selective pressures due to their ability to proliferate outside a host. In this study, we evolved *S. aureus* with or without pre-existing antibiotic resistance under different combinations of host and sub-MIC antibiotics to explore the evolution of virulence and antibiotic resistance. We determined whether increased virulence trades-off with antibiotic resistance by directly selecting for virulence across all host-associated treatments. Nonetheless, we observed differing evolutionary trajectories, where exposure to oxacillin resulted in pathogens causing the greatest host mortality. Our experimental design allowed us to tease apart the contributions of the host, sub-MIC antibiotics, and their interaction to demonstrate that adaptation to novel hosts did not require trade-offs in pathogen traits. Indeed, selection from both host and antibiotics actually resulted in rapid pathogen adaptation, potentially creating the conditions under which a pathogen may emerge in new hosts.

Simultaneous selection from the host and sub-MIC antibiotic favored the greatest virulence and growth in antibiotics, indicating that interactions between these pressures can shape both traits. Sub-MIC antibiotics can facilitate adaptation to high drug concentrations and to the growth environment [15,19]. Frequent exposure to low concentrations of antibiotics in *S. aureus* may occur due to its ability to grow and survive in non-host environments [37–40], in addition to infection of antibiotic-treated hosts, where there is uneven distribution of drugs across tissues [36]. At the cellular level, sub-MIC antibiotics can affect *S. aureus* by directly binding to virulence factors, modulating virulence regulators, and inducing the expression of toxins like hemolysin [14,35]. Prior exposure to sub-MIC inside and outside hosts can thus mediate traits involved in virulence upon host encounter. Our results further provided evidence for potential links between responses to antibiotic selection and virulence. Mutations genes involved in resistance to antibiotics were correlated with increased virulence, suggesting that adaptation to antibiotics may allow for virulence to evolve. Furthermore, in the absence of oxacillin selection, populations lost their ability to hemolyze red blood cells, supporting previous findings that antibiotics can influence expression of virulence factors [14,15].

Likewise, host factors contributed to antibiotic resistance. All populations initially sensitive to oxacillin exhibited increased MICs when under selection from both host and sub-MIC oxacillin. One population evolving under selection from either solely sub-MIC antibiotics or solely the host exhibited decreased oxacillin sensitivity, which is consistent with previous studies examining hosts [26] and sub-MIC [10,19] exposure separately. The pathogens in these studies evolved for much longer than our study, suggesting that having a host accelerated the evolution of antibiotic resistance, potentially due to bottlenecks that favor resistant isolates [26]. Evolution in hosts also contributed to pathogen growth outside hosts, an important trait for opportunistic pathogens as persistence in the environment increases the likelihood of contact with new hosts. Ultimately, exposure to hosts and antibiotics together could create a feedback loop where both evolutionary pressures select for better adaptation in the other, resulting in highly virulent and resistant pathogens that can persist outside hosts.

Several mutations arose in genes with primary roles in metabolism, such as *codY*. It is becoming clear that pathogen metabolism plays a central role in its virulence [75,76] and antibiotic resistance [77]. For example, mutations in chromosomal- and plasmid-encoded metabolic genes are widely prevalent among *E. coli* strains and protect them against antibiotic challenges [78,79]. Furthermore, antibiotics can directly select for reduced metabolic rates to dampen the deleterious effects of drugs [79], and increased resistance is more likely to evolve at high nutrient concentrations [80]. Similarly, metabolic efficiency can alter virulence [75], while resource limitations can hinder the evolution of virulence [81]. In *S. aureus*, CodY de-represses expression of virulence factors under resource-depleted conditions in order to acquire nutrients through the lysing of host cells [57]. Our genomic data were consistent with the observation that *codY* may be mediating virulence through control of *agr*: MSSA populations under selection from both host and sub-MIC oxacillin exhibited the greatest virulence and was one of the two treatments without mutations in *agr*. These results suggested that the *codY* mutations in these populations may be deleterious, rendering CodY less efficient in repressing *agr*-mediated virulence as a result. As resources likely differ in abundance and type between hosts and media, our findings illustrated the importance of taking into consideration the environment in which pathogens evolve when evaluating traits of interest.

Regulatory genes in general acquired more mutations than expected by chance alone. This finding supported previous work showing that regulatory elements were more likely to acquire phenotype-altering mutations, especially in negative regulators (e.g., *codY*) [82]. Enrichment of mutations in regulatory genes has also been found in *E. coli* under antibiotic selection [83].

522 Pleiotropic genes like regulators may increase the capacity to adapt to multiple environments.
523 Because *C. elegans* is a novel host to *S. aureus*, selection is more likely to act on genes that have
524 large initial gains in the fitness landscape, such as those that have pleiotropic effects [52]. Indeed,
525 mutations in several metabolic regulators (*codY, purR, gpmA*) and virulence regulators (*saeRS*)
526 were mainly associated with host infection. The resulting phenotypes of these mutations, such as
527 increased biofilm production, can then confer multiple benefits like increased virulence and
528 antibiotic resistance. Our experimental results revealed similar patterns to a comparative study
529 analyzing over 2000 *S. aureus* genomes, where mutations in genes with pleiotropic effects, such
530 as *purR*, may have underly pathogen adaptation during systemic infection [31]. Thus, changes in
531 genes of large effects like global regulators appear to be critical mediators between different traits
532 that altogether allow bacteria to rapidly adapt to new and stressful environments.
533
534 Antibiotic resistance did not impede the evolution of virulence. Antibiotic resistance tends to be
535 costly in the absence of antibiotics; this cost can subsequently constrain evolution of traits
536 requiring cellular resources, such as virulence [51]. However, mutations conferring resistance can
537 have little to no costs, depending on the pathogen species and drugs, and costs can be offset by
538 compensatory mutations [84]. Sub-MIC levels favor low-cost mutations [18], therefore virulence
539 and antibiotic resistance may not trade-off during disease emergence [8]. Virulence may
540 alternatively be costly in the presence of antibiotic resistance—populations evolved from the
541 MRSA ancestor without either selective pressure exhibited the lowest virulence. The
542 corresponding MSSA populations did not have a similar decline in virulence, suggesting that
543 relaxed selection attenuated virulence for populations with existing resistance. Genomic
544 differences between evolved MRSA and MSSA further demonstrate diverging evolutionary
545 pathways in antibiotic-resistant *versus* antibiotic-sensitive populations. Oxacillin-exposed MSSA
546 had no mutations in *agr* and retained their hemolytic ability, while some MRSA populations gained
547 *agr* mutations and lost the trait, suggesting that other pathways are utilized to express virulence.
548 Lastly, mutations in MRSA under selection from both host and antibiotics were significantly
549 enriched in blood and systemic *S. aureus* isolated from humans, suggesting that antibiotics may
550 facilitate MRSA invasive infection. Acquiring mutations in genes that impact antibiotic resistance,
551 such as *pbpA* and *pbpB*, may allow *S. aureus* to persist longer in hosts.
552
553 Recent work has shown that diverse genotypes of one pathogen species converge on similar
554 phenotypes when facing similar selective pressures [85]. Here, we showed that antibiotic
555 resistance status can lead otherwise isogenic pathogens to use different adaptive strategies.
556 Initially susceptible pathogens, facing novel selection from host and antibiotics, exhibited reduced
557 phenotypic and genomic variation over evolutionary time and thus may have had a limited number
558 of pathways to adapt to new environments. By contrast, resistant populations had greater
559 variance and thus may have had more routes to utilize. Overall, the potential for low-cost [8,18]
560 and pleiotropic mutations [52] may have created ideal conditions for a novel pathogen to establish
561 in host populations regardless of its susceptibility to antibiotics. Our work demonstrated that
562 responding to multiple pressures can result in rapid adaptation to new hosts and even evolution
563 of traits not under selection, emphasizing the importance of non-canonical pathways (e.g.,
564 metabolism) in shaping these traits. Pathogen evolution in a tractable animal model recapitulated
565 phenotypic and genomic findings from human studies, highlighting the utility of evolution
566 experiments to identify potential ecological and genetic mechanisms that give rise to pathogen
567 traits of interest. Our findings ultimately emphasize the importance of considering the host context
568 in the evolution of antibiotic resistance. Integrating multiple traits, such as virulence, antibiotic
569 resistance, and fitness may be critical in identifying the factors that facilitate host shifts and
570 persistence of drug-resistant pathogens.

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MATERIALS AND METHODS

Bacteria and nematode strains

Staphylococcus aureus USA300 JE2 (NR-46543) and USA300 JE2 *mecA*-tn (Transposon Mutant NE1868) from the Nebraska Transposon Mutant Library were acquired from BEI resources (https://www.beiresources.org/). To ensure true isogenic ancestors, the *mecA*-tn was transduced into the USA300 JE2 background, selecting for the erythromycin resistance marker on the Mariner transposon, and the insertion site was confirmed by polymerase chain reaction. Transposon maintenance was confirmed by testing for resistance to erythromycin (5µg/mL) or by genome sequencing when available. In cases where transposon loss was verified by genome sequencing, strains were tested and shown to maintain sensitivity to oxacillin. As the primary function of the transposon was to knock out *mecA* function, loss of the transposon should not confound subsequent analyses as antibiotic sensitivity was maintained.

Caenorhabditis elegans strain N2 Bristol and *Escherichia coli* strain OP50 were provided by the Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Nematodes were maintained on Nematode Growth Medium Lite (US Biological, Swampscott, MA) according to WormBook protocols [86].

Experimental evolution

Selection for increased virulence of *S. aureus* (USA300 JE2 or USA300 JE2 *mecA-tn*) in *C. elegans* was performed by passaging *S. aureus* from dead hosts 24 hours post-infection. Half of the *S. aureus* populations were additionally subjected to antibiotic pressure during the passages before and after *C. elegans* selection with sub-MIC oxacillin exposure (0.03125µg/mL). This concentration was tested against both ancestral strains and did not substantially inhibit growth of the methicillin-sensitive USA300 JE2 *mecA*-tn strain.

For each passage of experimental evolution, *S. aureus* was grown in Brain Heart Infusion (BHI) broth with 4µg/mL colistin (to prevent *E. coli* contamination from the nematode intestine) ± 0.03125µg/mL oxacillin and incubated at 37˚C overnight, shaking at 250rpm. The next day, 200µL of the overnight *S. aureus* culture was plated onto BHI agar and incubated at 28˚C overnight. Approximately 1500 *C. elegans* were plated and allowed to consume *S. aureus* for 24 hours before 30 dead nematodes were picked from each plate. Nematodes were identified as dead by a lack of response to tapping by a platinum wire [2,69]. Bacterial extraction methods were adapted from [87]. In brief, picked dead nematodes were washed once with M9 buffer supplemented with 0.1% Triton X-100, once with M9 buffer, then subjected to a 1:1000 bleach-M9 buffer solution for 15 minutes. Nematodes were then washed twice with M9 buffer and ground with a motorized pestle to extract *S. aureus* from the host intestine. *S aureus* populations evolving without hosts were obtained from nematode-free bacterial lawns with a loop. Populations of *S. aureus* were grown as previously before freezing and storage at -80˚C. Twenty-five percent of each frozen culture was used as inoculum for the next round of experimental evolution. Preliminary experiments determined colony-forming units per milliliter dropped by approximately 50% after freezing. To maintain population diversity, the inoculum amount (25%), adjusted for freezing, was chosen to be within the dilution ratio presented in [88] that minimizes the chance that rare beneficial mutations are lost. Twelve rounds of passage were completed for 2 *S. aureus* genotypes x 2 host treatments x 2 antibiotic treatments x 6 replicates = 48 populations. Whole populations were frozen for subsequent analyses. We conducted all assays to evaluate changes in evolved *S. aureus* at the population level instead of single isolates to represent the context under which *S. aureus* evolved in our experiment and generally how pathogens exist in nature and in hosts [55,89–92]. Population level assays also allow for potential interactions between genotypes that give rise to phenotypes like virulence [93] and antibiotic resistance [89,94] that would otherwise not be captured in single-isolate assays.

940
941 **Mortality assay**
942 Nematodes were age-synchronized as in [95] and grown on *E.coli* OP50 until L4 larval stage (48
943 hours at 20˚C). Nematodes were subsequently washed off, counted, and approximately 200
944 individuals were plated on *S. aureus* lawns on BHI plates. Another set of nematodes were plated
945 on *E. coli* OP50 to estimate the total number of nematodes. Plates of *S. aureus* were prepared
946 24 hours prior by plating 200μL of an overnight population culture on BHI agar and grown at 28˚C.
947 After two days at 20˚C, plates were scored by counting live nematodes. Host mortality data were
948 analyzed using a generalized linear model with a binomial distribution and followed by Tukey
949 multiple-comparison tests to determine pairwise differences. To compare variances across
950 treatments, we conducted Levene's test for homogeneity of variance. All statistical analyses,
951 including those below, were conducted in R (version 4.2.0).
952
953 **Hemolysis phenotype assay**
954 Populations of *S. aureus* were streaked onto Trypticase Soy Agar II with 5% sheep's blood plates
955 and incubated overnight at 37˚C. Bacteria were assessed as either hemolysis positive or negative
956 depending on whether there was a complete clearing around the colonies. We did not cross-
957 streak with RN4220, which produces beta-hemolysin, and allows for detection of delta-hemolysin.
958 Therefore, our assay mainly detects alpha-toxin and phenol-soluble modulins. Hemolysis data
959 were analyzed using a generalized linear model with a binomial distribution. To determine whether
960 there was an association between hemolysis status and host mortality, we compared the host
961 mortality means of hemolysis-positive vs. hemolysis-negative pathogens using a Kruskal–Wallis
962 test.
963
964 To determine whether there was variation in hemolysis ability within populations, we streaked 10
965 colonies from each of the 48 evolved populations onto Trypticase Soy Agar II with 5% sheep's
966 blood plates and incubated overnight at 37˚C. To compare the number of isolates able to
967 hemolyze, we conducted a generalized linear model with a binomial distribution. To compare
968 variances, we conducted Levene's test for homogeneity of variance.
969
970 ***In vitro* growth assay**
971 We measured growth in BHI to simulate conditions under which pathogens were grown in our
972 experiment. Importantly, rich media allowed us to determine growth when conditions are optimal
973 for evolved pathogens. Measuring growth in minimal media, while being more similar to conditions
974 that *S. aureus* may face in its environment, would introduce another selective pressure. Overnight
975 cultures of each evolved population was diluted 1:1000 in BHI broth, then 200μl of each dilution
976 was added to 96-well plates, covered with a Breathe-Easy sealing membrane (Sigma) to minimize
977 evaporation. The optical density at 600nm at 37˚C with shaking at 282 cycles per minute was
978 recorded every 10 minutes for 16 hours (1000 minutes) using a BioTek Synergy HTX Multimode
979 Reader. We randomly assigned each of the 48 evolved populations to the inner wells of the 96-
980 well plates, with at least three technical replicates per population. Each plate also included
981 technical replicates of each ancestor. Each ancestral strain was assayed with four technical
982 replicates per plate across four plates. These steps were repeated for media with 0.03125μg/mL
983 oxacillin. We used the R package *gcplyr* [96] to calculate the area under the curve of density, a
984 metric that quantifies the overall bacterial growth. We compared the growth of populations across
985 treatments using the mean area under the curve of the technical replicates for each population.
986 We used a linear model followed by Tukey multiple-comparison tests to determine pairwise
987 differences between the ancestors, and linear mixed models for evolved populations grown with
988 or without oxacillin.
989

Antimicrobial susceptibility assay

Antimicrobial susceptibility testing for oxacillin was done according to Clinical & Laboratory Standards Institute (CLSI) standard protocols for broth microdilution [97]. In brief, populations were streaked from frozen stock and re-streaked the next day. From the second day culture, populations were resuspended in normal saline to a 0.5 McFarland standard. Then, these cultures were diluted 1:20 in normal saline before 10μL was added to 90μL of CAMHB + 2% NaCl with the appropriate concentration of oxacillin. Plates were incubated at 35˚C without shaking and read at 24 hours. For statistical analysis of populations evolved from the MSSA ancestor, we set a threshold such that anything above the ancestral susceptibility level (0.25ug/ml) was considered a decrease in oxacillin sensitivity. We then compared the proportion of populations exhibiting a decrease in oxacillin sensitivity with those that had not using Fisher's exact test with false discovery rate-adjusted (FDR) p-values.

To determine whether there was variation in antibiotic susceptibility within evolved populations, we sampled 10 colonies from each of the four populations with the most number of mutations (two from MRSA and two from MSSA), and two additional randomly selected populations. We argued that variation would be the most observable in populations with the most genetic diversity. We then followed the standard protocol for a Kirby-Bauer disk diffusion susceptibility test [98] using oxacillin. Briefly, we inoculated Mueller-Hinton agar plates (Hardy Diagnostics) of overnight cultures with standardized OD_{600} and placed an oxacillin-impregnated disk (HardyDisk AST Oxacillin, OX1) onto each plate. We measured the diameter of the zone of inhibition after incubation at 37˚C overnight.

Biofilm assay

Overnight cultures of each population were diluted 1:40 in BHI broth containing 0.5% glucose, then 100μl of each dilution was added to the inner wells of 96-well plates (NEST Biotechnology, flat-bottom, tissue culture-treated). Plates were incubated for 24 hours at 20˚C, the temperature that nematodes experience during infection. Each well was washed three times with 200μl of phosphate-buffered saline (PBS) to remove non-adherent cells. We then stained cells with 125μl of 0.1% crystal violet for 20 minutes, followed by washing each well twice with 200μl PBS. We then resuspended the crystal violet in 125μl of 30% acetic acid. After incubation for 10 minutes, we measured the optical density at 595nm using a BioTek Synergy HTX Multimode Reader. We randomly assigned each of the 48 evolved populations to the inner wells of the 96-well plates, with at least three technical replicates per population. Each plate also included technical replicates of each ancestor. We standardized the mean of technical replicates for each population against the respective ancestor within each plate to control for variation across plates. We used mixed linear models followed by Tukey multiple-comparison tests to assess pairwise differences between treatments.

Whole-genome shotgun sequencing and variant annotation

Bacterial genome DNA was extracted from whole populations using Wizard Genomic DNA Purification Kit (Promega). Population sequencing allows us to capture the genetic variation within populations, which is critical to understanding how pathogens evolve [2,12,69,94,99,100]. Genome sequencing of these DNA samples was performed at the M-Gen center using a paired-end library preparation method based on Illumina Nextera and sequenced on the NextSeq 550.

The raw data was quality trimmed and adapters removed using illumina-cleanup (https://github.com/rpetit3/illumina-cleanup) or with bactopia [101]. Mutations were called based on comparison to the reference USA300 JE2 genome (BioProject: PRJNA224116, BioSample: SAMN06677988, Assembly: GCF_002085525.1) using breseq [102]. The presence of large-scale deletions and other genetic changes were confirmed by analysis of de novo assembled genomes

1041 performed by the SKESA assembler software [103]. The ancestral populations were almost
1042 identical to the reference genome used except for three mutations that were not found in any
1043 evolved populations. Another three mutations in *bioD1, cls* and *ccdC* were present in all
1044 populations derived from the MSSA ancestor and likely occurred in the first passage from the
1045 frozen culture before experimental evolution was performed. We therefore removed these six
1046 mutations from the analysis.
1047
1048 The number of mutations (excluding synonymous or intergenic regions) across treatments were
1049 analyzed using a generalized linear model with a Poisson distribution. For mutation enrichment
1050 analysis, we followed the steps as described previously [104]. Briefly, we calculated the "mutation
1051 rate" for each ancestor by dividing the total number of mutations by the reference genome size.
1052 We multiplied the mutation rate by the total length of regulatory genes [105] with mutations in our
1053 study to generate the expected number of mutations for these genes. We then conducted single-
1054 tailed Poisson tests to determine whether the actual number of mutations in regulatory genes was
1055 greater than expected.
1056
1057 For phenotypes *vs.* mutations, we considered only mutations occurring at least twice throughout
1058 the experiment. For host mortality *vs.* mutations and growth rate *vs.* SCC*mec* and ACME, we
1059 conducted a one sample t-test for each evolved pathogen against its respective ancestor. P-
1060 values were adjusted using the FDR. For hemolysis status *vs.* mutations and oxacillin MIC *vs.*
1061 mutations, we conducted Fisher's exact tests with Bonferroni corrections.
1062
1063 **Comparing mutations in experiment against publicly available genomes**
1064 For each non-synonymous mutation in each gene, we created the FASTA file of the USA300 JE2
1065 protein product and a cognate file of the mutant protein with the amino acid substitution edited
1066 manually. We ran tBLASTn (version 2.12.0) searches of both proteins against 83,383 high quality
1067 *S. aureus* whole genome assemblies [68]. We filtered out matches to genomes with less than
1068 95% identity. We screened for cases where mutants had a better match to a genome than the
1069 ancestral protein (higher bitscore).
1070
1071 We collected meta-data for BioSamples accession linked to each genome from NCBI Entrez using
1072 a Python script from Jason Stajich (https://github.com/stajichlab/biosample_metadata), then
1073 manually curated the "isolation_source" column to either blood/systemic infection,
1074 skin/nose/throat, general host-association, animal, environment, others, and missing (see Table
1075 S3 for specific terms). For BioSamples matching mutants within each treatment, we compared
1076 the proportion of blood/systemic infection *vs.* skin/nose/throat against the expected proportion (all
1077 BioSamples in the dataset) using a chi-square goodness-of-fit test.
1078
1079 **Principal component analysis**
1080 We conducted a principal component analysis on the phenotypes quantified in evolved
1081 populations using the prcomp function in R for each *S. aureus* genotype. We performed
1082 permutational analysis of variances (PERMANOVA, 999 permutations) to test for differences
1083 between treatments using adonis2 of the *vegan* package [106] and pairwiseAdonis [107].
1084
1085 **Genetic distance**
1086 We generated a matrix of Euclidian genetic distances (the square root of pairwise differences)
1087 using the frequencies of mutations that arose for each *S. aureus* genotype, from which we
1088 constructed phylogenies [69,70]. We used pairwise differences between the ancestor and each
1089 evolved populations to compare rates of evolution across treatments, and pairwise differences
1090 between replicate populations within each treatment to compare the degree of divergence across
1091 treatments. We compared rates of evolution for MRSA and MSSA treatments, and degree of

1092 divergence for MSSA treatments, using linear models followed by Tukey multiple-comparison
1093 tests. We compared the degree of divergence for MRSA treatments using a Kruskal–Wallis test
1094 followed by Dunn's post-hoc test.