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.

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. 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 multiple and concurrent selective pressures as drivers of pervasive pathogen traits.

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) exist 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 can decline over time due to metabolism and barriers to absorption, and sub-MIC levels can arise as a result of pathogens attaining increased drug resistance [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. 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 differs drastically in nutrient availability than rich 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]. 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 causes a variety of diseases, including skin and bone infection, food poisoning, and bacteremia across hospital and community settings [29]. In 2017, more than 119,000 bloodstream infections caused by *S. aureus* occurred in the United States with a mortality rate of 18% [30]. While methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* both produce similar levels of toxins, MRSA populations have maintained virulence while becoming increasingly multi-drug resistant [31]. Treatment of MRSA with beta-lactam antibiotics

can generate adverse responses from the immune system [32]. Furthermore, sub-MIC concentrations of these antibiotics can affect *S. aureus* virulence by increasing production of toxins and adhesion molecules *in vitro* [33]. Thus, interactions between antibiotics and host factors have the potential to drive *S. aureus* virulence evolution.

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 [10,18,28,34]. Conversely, *in vivo* studies have focused on transmitting pathogens through individual hosts for a single to a handful of passages [26,35,36]. Experimental evolution in hosts nonetheless remains severely underutilized but has considerable potential to address key questions regarding the impact of host populations on the evolution of virulence and antibiotic resistance [16]. 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 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 [37]. Virulence screens in *C. elegans* using *S. aureus* transposon libraries also showed overlapping results to other infection models [38,39], with the degree of *S. aureus* virulence in *C. elegans* reflecting disease severity of human infections [40,41]. 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.

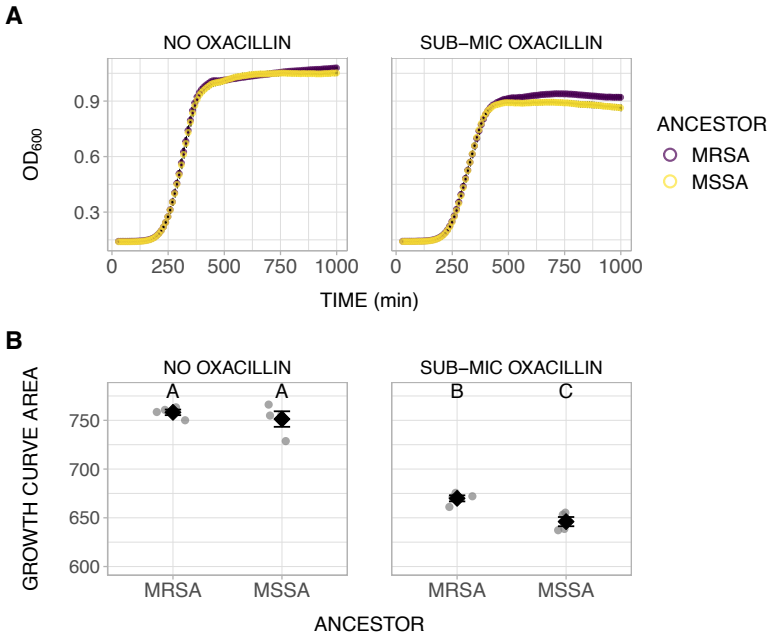
Here, we directly test the impact of host and sub-MIC antibiotic exposure on pathogen evolution. Selection exerted by two concurrent forces may impede the pathogen's response to one or both forces [6]. Adaptation may require resources to be expended toward either virulence or antibiotic resistance, leading to a trade-off between these traits [42]. 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 [43]. 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 then quantified the ability of evolved pathogens to kill hosts as the metric for virulence. We also ascertained 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 identify mutations potentially underlying evolved traits.

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).



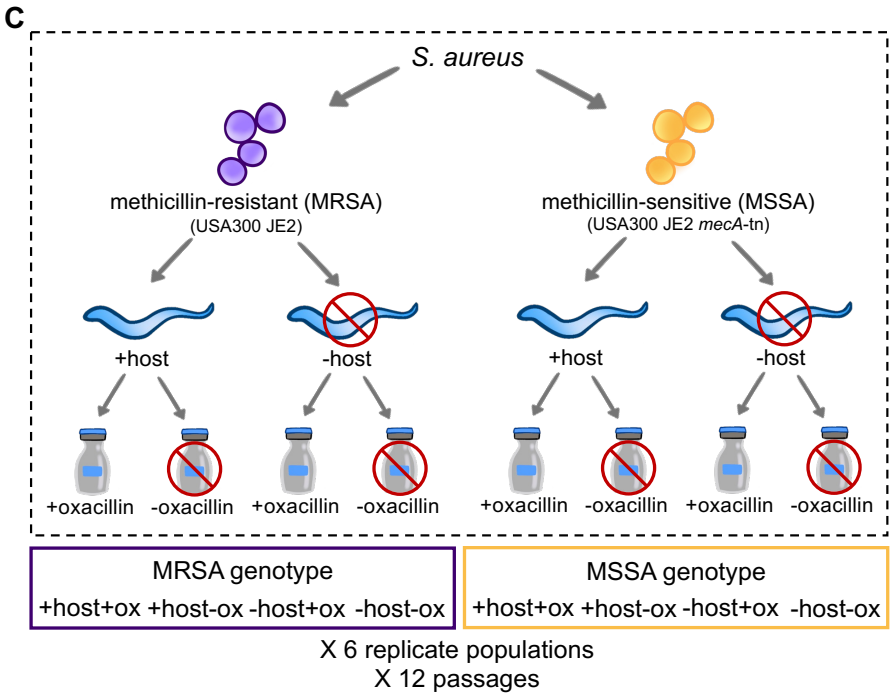


Figure 1. Experimental evolution design. A) Growth curves and B) total growth of ancestral MRSA and MSSA populations *in vitro* with and without sub-MIC oxacillin. Different letters indicate significant differences. C) MRSA and MSSA were passaged 12 times with or without hosts, in the presence or absence 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 [45]. 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.

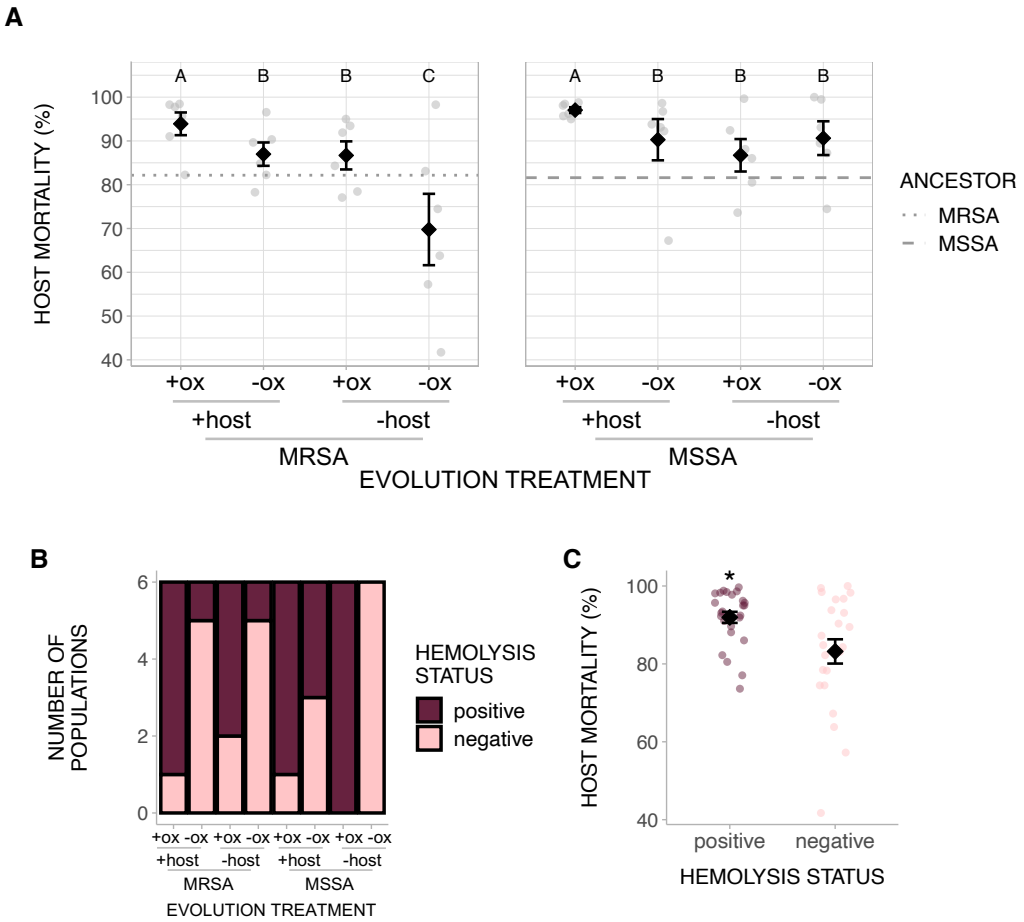


Figure 2. Evolution of virulence is facilitated by exposure to both host and sub-MIC antibiotic. A) Virulence in terms of *C. elegans* mortality. Dashed lines indicate respective ancestral virulence. Different letters indicate significant differences. B) Virulence in terms of the ability to hemolyze sheep's blood. The y-axis indicates the number of evolved populations for each category. C). Host mortality from A) grouped by hemolysis status in B). 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. 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 [46].

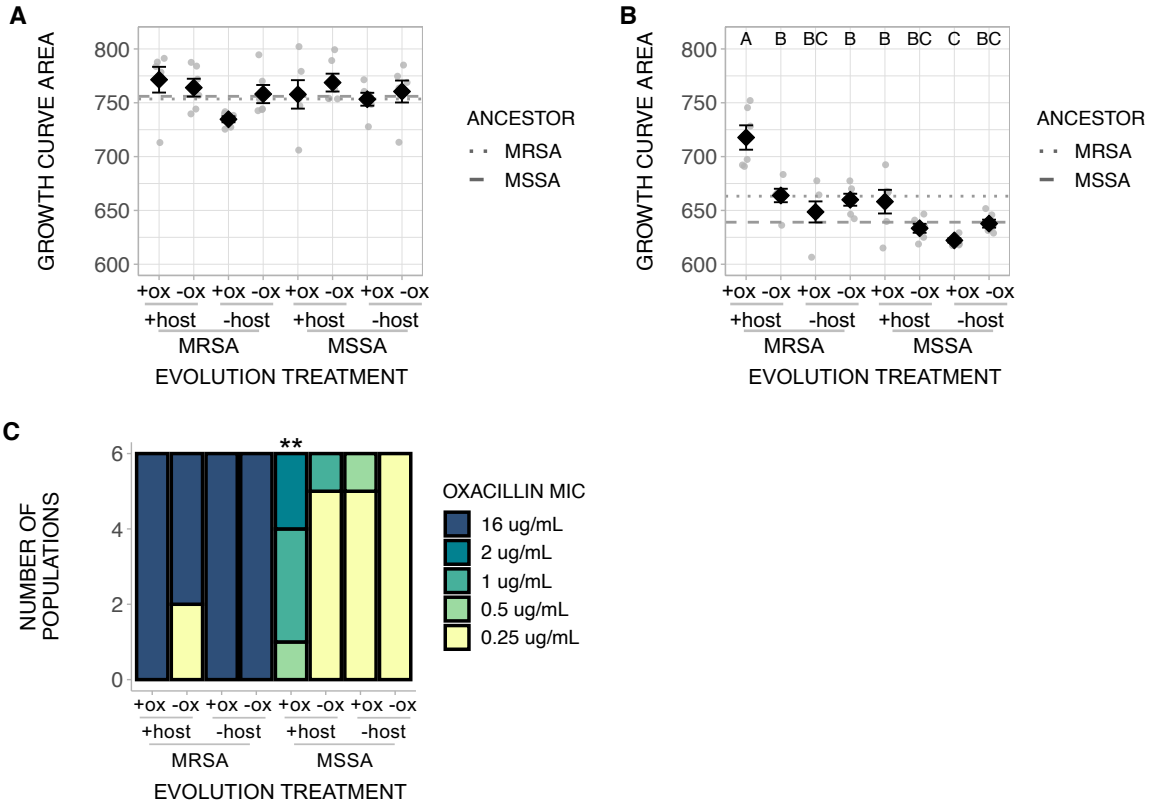


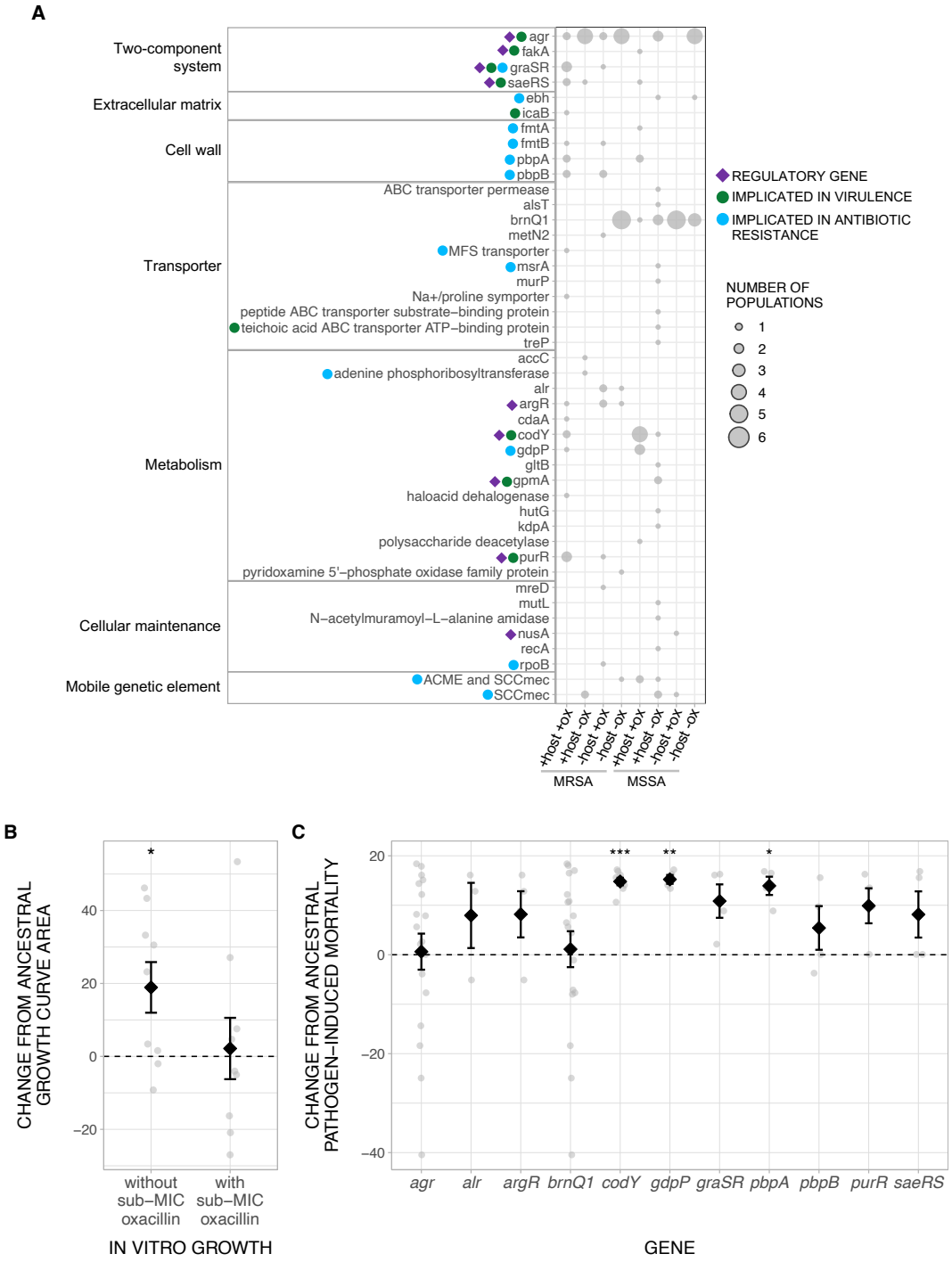
Figure 3. **Host and sub-MIC antibiotic selection facilitated pathogen growth in antibiotics**. Pathogen *in vitro* growth A) without oxacillin and B) in sub-MIC oxacillin. Different letters indicate significant differences. C). Oxacillin MIC of evolved populations. The y-axis indicates the number of 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); Figures S1 and S2 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 S3; χ_1^2 = 5.28, P = 0.022). 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

263 mutations in virulence regulators *codY* [47] and *saeRS* [48] suggesting some shared pathways to
264 heightened virulence among the two treatments, but mutations in these genes did not occur
265 across all replicate populations. These treatments also had mutations in *gdpP* and *pbpA*, which
266 have been implicated in resistance to beta-lactam antibiotics [49,50], with *gdpP* playing a role in
267 MSSA resistance to oxacillin in particular [51]. Five out of six populations evolved from the MSSA
268 ancestor had a mutation in either gene, which may have contributed to the reduced oxacillin
269 sensitivity in these populations.
270
271 Regulatory genes were enriched with mutations (Figure 4A; one-sample Poisson test $P < 0.001$
272 for MRSA and MSSA genotypes). These included *agr*, *saeRS,* and *codY*. *agr* is a quorum sensor
273 that regulates the expression of virulence factors [52]. Out of the 22 mutations in *agr* (15 in *agrA*
274 and seven in *agrC*), 14 arose from the MRSA ancestor, 18 arose in the absence of oxacillin
275 selection, and 10 arose under host selection. All 22 mutations were found in populations that have
276 lost the ability to hemolyze red blood cells. Three of four mutations in *saeRS*—another known
277 virulence regulator—were found in populations evolved from the MRSA ancestor. By contrast, of
278 the eight *codY* mutations, all were from host-associated populations, six were in populations
279 arisen from MSSA, and seven were from populations under oxacillin selection. In contrast to the
280 other two regulators, *codY* is a transcriptional repressor found in gram-positive bacteria that
281 controls virulence by monitoring nutrient levels in the environment [47].
282
283 More mutations were fixed in genes associated with resistance to beta-lactam antibiotics (*pbpA,*
284 *pbpB, graSR, gdpP*) [49,53] in populations evolved from MRSA (10 mutations) than those from
285 MSSA (5 mutations). Mutations in these genes only occurred in populations exposed to oxacillin.
286 Nine populations experienced large scale deletions that included the entire SCC*mec* cassette.
287 Five deletions only encompassed SCC*mec* (23.7 kb), and four deletions were much larger (54.7
288 kb) and included the Arginine Catabolic Mobile Element (ACME) [44]. Out of these nine
289 populations, six evolved from the MSSA ancestor, and seven are from host-associated
290 populations. *arcA*, part of the arginine-deiminase system encoded by ACME, also had a 99-base
291 pair deletion in one population evolved from the MRSA ancestor. Deletion of ACME has been
292 shown to enhance the competitive fitness of *S. aureus* USA300 *in vivo* [54]. The two MRSA
293 populations that lost their resistance (Figure 3C) had SCC*mec* deletions, suggesting that
294 resistance could be more costly when evolving in a host. In general, the loss of SCC*mec* and
295 ACME was associated with an increase in total growth from the ancestor outside the host (Figure
296 4B; one-sample t-test $t = 2.73$, $df = 8$, $P = 0.026$).
297
298 Mutations in other metabolic genes included those in *brnQ1, purR,* and *gpmA*. Sixteen of the 20
299 *brnQ1* mutations were most common in populations that evolved in the absence of host selection,
300 and thus potentially resulting from adaptation to the environment outside the host. The other
301 mutations, however, were found in the pathogens under host selection and have well-known roles
302 in virulence [55,56]. Three of the four *purR* mutations were found in populations evolved from the
303 MRSA ancestor exposed to sub-MIC oxacillin, and both *gpmA* mutations were found in
304 populations evolved from the MSSA ancestor in the absence of oxacillin.
305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Two-component system
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graSR
saeRS
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ebh
icaB
Cell wall
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fmtB
pbpA
pbpB
Transporter
ABC transporter permease
alsT
brnQ1
metN2
MFS transporter
msrA
murP
Na+/proline symporter
peptide ABC transporter substrate−binding protein
teichoic acid ABC transporter ATP−binding protein
treP
Metabolism
accC
adenine phosphoribosyltransferase
alr
argR
cdaA
codY
gdpP
gltB
gpmA
haloacid dehalogenase
hutG
kdpA
polysaccharide deacetylase
purR
pyridoxamine 5'−phosphate oxidase family protein
Cellular maintenance
mreD
mutL
N−acetylmuramoyl−L−alanine amidase
nusA
recA
rpoB
Mobile genetic element
ACME and SCCmec
SCCmec
+host +ox
+host -ox
-host +ox
-host -ox
MRSA
MSSA
REGULATORY GENE
IMPLICATED IN VIRULENCE
IMPLICATED IN ANTIBIOTIC RESISTANCE
NUMBER OF POPULATIONS
1
2
3
4
5
6
B
CHANGE FROM ANCESTRAL GROWTH CURVE AREA
40
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without sub−MIC oxacillin
with sub−MIC oxacillin
IN VITRO GROWTH
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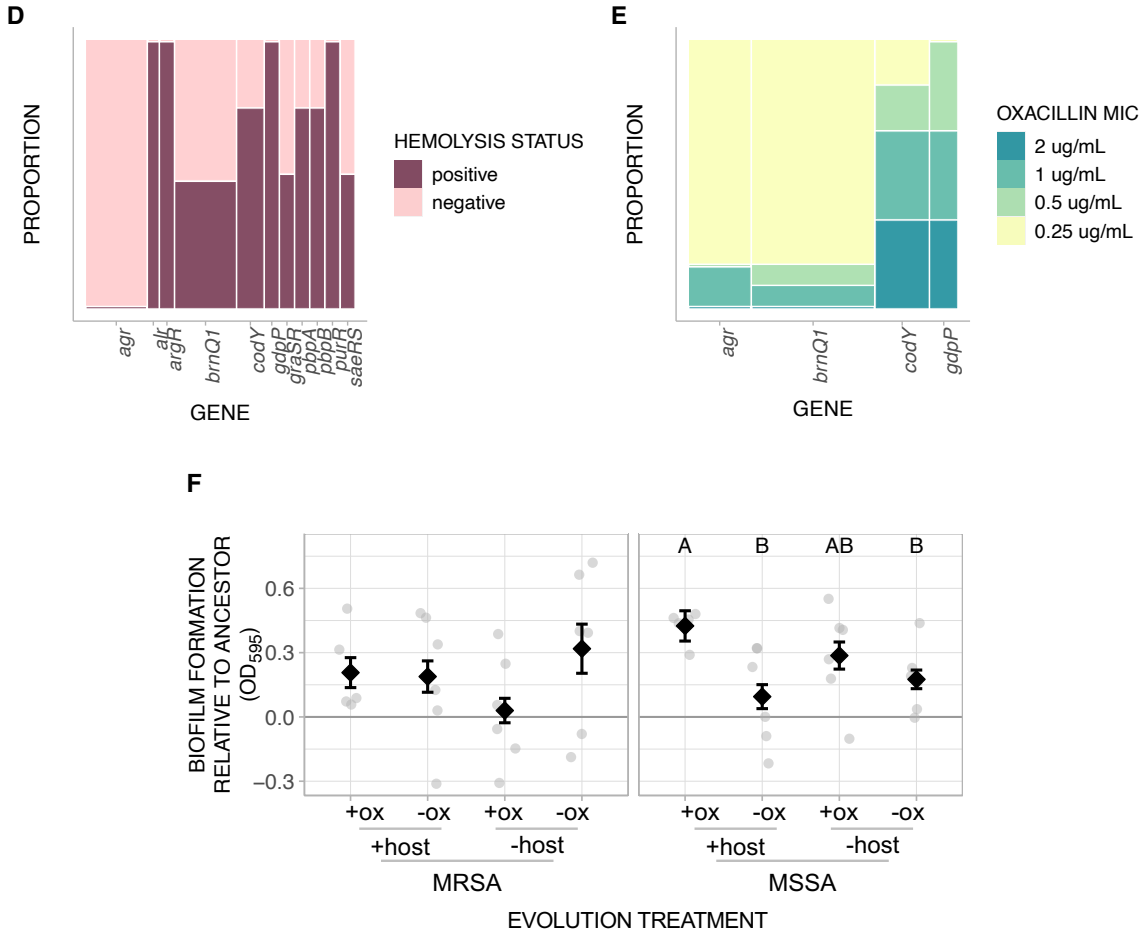

Figure 4. Regulatory genes likely played an important role in pathogen adaptation. A) Mutations swept to fixation, excluding intergenic and synonymous mutations, grouped by general function. The size of each point indicates how many populations had acquired at least one mutation in the gene. Colored shapes next to genes indicate whether these genes are regulatory or have been implicated in virulence or antibiotic resistance in the literature. 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 correlated with *agr* mutations arising from 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.

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 [34]. In *C. elegans*, *S. aureus* biofilm enhances virulence and protects the pathogen from host innate immune defenses [57]. Both sub-MIC level of beta-lactam antibiotics and null-mutants of *codY* induce robust biofilm formation and hemolysis activity [14,58,59]. 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_3^2 = 7.24, P = 0.06). By contrast, biofilm production differed between treatments for the MSSA genotype (Figure 4F; χ_3^2 = 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).

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 5A and 5B). We found a significant effect of treatment for the MRSA genotype (PERMANOVA $F_{(3,23)}$ = 8.40, 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 = 0.012 for all pairwise comparisons). The MSSA genotype had a marginally non-significant effect of treatment (PERMANOVA $F_{(3,23)}$ = 2.14, r^2 = 0.243, P = 0.062). 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 5C 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 [60,61]. There was no significant difference between treatments for MRSA populations in terms of genetic distance to the ancestor (Figure S4A; $F_{(3,20)}$ = 2.39, P: 0.099), suggesting that populations generally exhibited similar evolutionary rates. By contrast, MSSA populations had a significant effect of treatment (Figure S4B; $F_{(3,19)}$ = 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).

Populations within each treatment tended to exhibit more genetic similarity for MSSA than for MRSA (Figures 5C and 5D). For MRSA, treatments varied in how divergent populations were (Figure S4C; Kruskal-Wallis χ^2_3= 21.09, P < 0.001), where populations exposed to both selective pressures diverged more than populations in the other three treatments (Dunn's post-hoc test P < 0.05 for all pairwise comparisons). Likewise, populations within MSSA treatments also differed in how much they varied genetically (Figure S4D; $F_{(3,51)}$ = 54.20, P < 0.001). Populations under solely host selection had greater divergence 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 from each other (P < 0.05 for all pairwise comparisons). These findings altogether suggest that even concentrations well below the MIC differentially impacts sensitive and resistant pathogens.

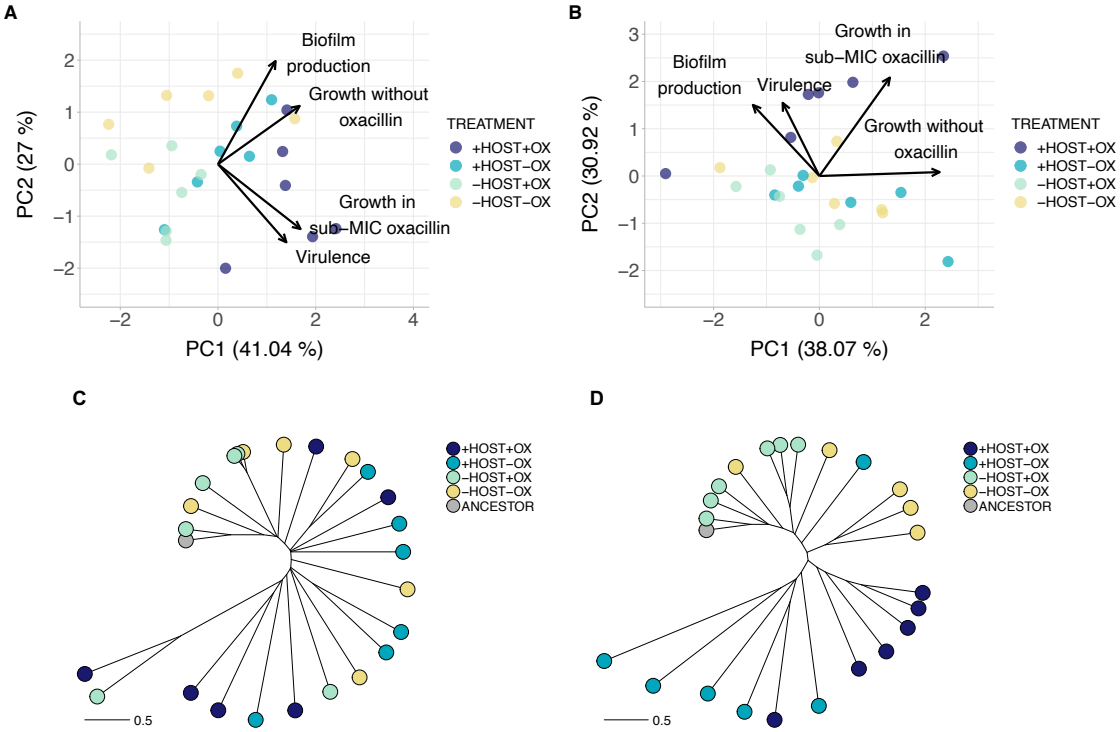

Figure 5. **MRSA and MSSA underwent distinct evolutionary trajectories.** Principal component analysis of traits evolved from A) MRSA and B) MSSA ancestors. Phylogenetic tree constructed from frequencies of mutations in populations evolving from C) MRSA and D) MSSA ancestors. Scale bar indicates Euclidean distance.

DISCUSSION

While antibiotics have existed for millennia, the mass production and overuse of antibiotics by humans in the last century have exerted unprecedented selection on pathogens unlike anything they have experienced in nature [62]. In addition to antibiotics, pathogens face numerous selective pressures such as host defenses and competitors [2,63,64]. 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. 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 (e.g., media) [15,19]. At the cellular level, sub-MIC antibiotics can affect *S. aureus* by directly binding to virulence factors or modulating virulence regulators [14]. Exposure to sub-MIC can thus mediate traits involved in host infection such as virulence. 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 [65,66] and antibiotic resistance [67]. For example, mutations in chromosomal- and plasmid-encoded metabolic genes are widely prevalent among *E. coli* strains and protect them against antibiotic challenges [68,69]. Furthermore, antibiotics can directly select for reduced metabolic rates to dampen the deleterious effects of drugs [69], and increased resistance is more likely to evolve at high nutrient concentrations [70]. Similarly, metabolic efficiency can alter virulence [65], while resource limitations can hinder the evolution of virulence [71]. 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 [47]. 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.

463 Regulatory genes in general acquired more mutations than by chance. This finding supported
464 previous work showing that regulatory elements were more likely to acquire phenotype-altering
465 mutations, especially in negative regulators (e.g., *codY*) [72]. Pleiotropic genes like regulators
466 may increase the capacity to adapt to multiple environments. Because *C. elegans* is a novel host
467 to *S. aureus*, selection is more likely to act on genes that have large initial gains in the fitness
468 landscape, such as those that have pleiotropic effects [43]. Indeed, mutations in several metabolic
469 regulators (*codY, purR, gpmA*) and virulence regulators (*saeRS*) were mainly associated with
470 host infection. The resulting phenotypes of these mutations, such as increased biofilm production,
471 can then confer multiple benefits like increased virulence and antibiotic resistance. While host
472 and sub-MIC oxacillin selection favored robust biofilms, we also found that oxacillin by itself also
473 increased biofilm formation, suggesting low antibiotic concentrations contributed significantly to
474 the evolution of drug-sensitive populations. The loci underlying biofilm formation may be different
475 in these two treatments, since mutations in *codY* did not appear in populations under solely
476 oxacillin selection (biofilm is a polygenic trait in *S. aureus* [73]). Changes in genes of large effects
477 like global regulators can thus act as mediators between different traits that altogether allow the
478 pathogen to rapidly adapt to new and stressful environments.
479
480 Antibiotic resistance did not impede the evolution of virulence. Antibiotic resistance tends to be
481 costly in the absence of antibiotics; this cost can subsequently constrain evolution of traits
482 requiring cellular resources, such as virulence [42]. However, mutations conferring resistance can
483 have little to no costs, depending on the pathogen species and drugs, and costs can be offset by
484 compensatory mutations [74]. Sub-MIC levels favor low-cost mutations [18], therefore virulence
485 and antibiotic resistance may not trade-off during disease emergence [8]. Virulence may
486 alternatively be costly in the presence of antibiotic resistance—populations evolved from the
487 MRSA ancestor without either selective pressure exhibited the lowest virulence. The
488 corresponding MSSA populations did not have a similar decline in virulence, suggesting that
489 relaxed selection attenuated virulence for populations with existing resistance.
490
491 Recent work has shown that diverse genotypes of one pathogen species converge on similar
492 phenotypes when facing similar selective pressures [75]. Here, we showed that antibiotic
493 resistance status can lead otherwise isogenic pathogens to use different adaptive strategies.
494 Initially susceptible pathogens, facing novel selection from host and antibiotics, exhibited reduced
495 phenotypic and genomic variation over evolutionary time and thus may have had a limited number
496 of pathways to adapt to new environments. By contrast, resistant populations had greater
497 variance and thus may have had more routes to utilize. Overall, the potential for low-cost [8,18]
498 and pleiotropic mutations [43] may have created ideal conditions for a novel pathogen to establish
499 in host populations regardless of its susceptibility to antibiotics. Our work demonstrated that
500 responding to multiple pressures can result in rapid adaptation to new hosts and even evolution
501 of traits not under selection, emphasizing the importance of non-canonical pathways (e.g.,
502 metabolism) in shaping these traits. Integrating multiple traits, such as virulence, antibiotic
503 resistance, and fitness may be critical in identifying the factors that facilitate host shifts and
504 persistence of drug-resistant pathogens.

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767

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 [76].

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 [2,60]. Bacterial extraction methods were adapted from [77]. 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 [78] 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.

Mortality assay

Nematodes were age-synchronized as in [79] and grown on *E.coli* OP50 until L4 larval stage (48 hours at 20˚C). Nematodes were subsequently washed off, counted, and approximately 200 individuals were plated on *S. aureus* lawns on BHI plates. Another set of nematodes were plated

819 on *E. coli* OP50 to estimate the total number of nematodes. Plates of *S. aureus* were prepared
820 24 hours prior by plating 200μL of an overnight population culture on BHI agar and grown at 28˚C.
821 After two days at 20˚C, plates were scored by counting live nematodes. Host mortality data were
822 analyzed using a generalized linear model with a binomial distribution and followed by Tukey
823 multiple-comparison tests to determine pairwise differences. To compare variances across
824 treatments, we conducted Levene's test for homogeneity of variance. All statistical analyses,
825 including those below, were conducted in R (version 4.2.0).
826
827 **Hemolysis phenotype assay**
828 Populations of *S. aureus* were streaked onto Trypticase Soy Agar II with 5% sheep's blood plates
829 and incubated overnight at 37˚C. Bacteria were assessed as either hemolysis positive or negative
830 depending on whether there was a complete clearing around the colonies. We did not cross-
831 streak with RN4220, which produces beta-hemolysin, and allows for detection of delta-hemolysin.
832 Therefore, our assay mainly detects alpha-toxin and phenol-soluble modulins. Hemolysis data
833 were analyzed using a generalized linear model with a binomial distribution. To determine whether
834 there was an association between hemolysis status and host mortality, we compared the host
835 mortality means of hemolysis-positive vs. hemolysis-negative pathogens using a Kruskal–Wallis
836 test.
837
838 ***In vitro* growth assay**
839 Overnight cultures of each evolved population was diluted 1:1000 in BHI broth, then 200μl of each
840 dilution was added to 96-well plates, covered with a Breathe-Easy sealing membrane (Sigma) to
841 minimize evaporation. The optical density at 600nm at 37˚C with shaking at 282 cycles per minute
842 was recorded every 10 minutes for 16 hours (1000 minutes) using a BioTek Synergy HTX
843 Multimode Reader. We randomly assigned each of the 48 evolved populations to the inner wells
844 of the 96-well plates, with at least three technical replicates per population. Each plate also
845 included technical replicates of each ancestor. Each ancestral strain was assayed with four
846 technical replicates per plate across four plates. These steps were repeated for media with
847 0.03125μg/mL oxacillin. We used the R package *gcplyr* [80] to calculate the area under the curve
848 of density, a metric that quantifies the overall bacterial growth. We compared the growth of
849 populations across treatments using the mean area under the curve of the technical replicates for
850 each population. We used a linear model followed by Tukey multiple-comparison tests to
851 determine pairwise differences between the ancestors, and linear mixed models for evolved
852 populations grown with or without oxacillin.
853
854 **Antimicrobial susceptibility assay**
855 Antimicrobial susceptibility testing for oxacillin was done according to Clinical & Laboratory
856 Standards Institute (CLSI) standard protocols for broth microdilution [81]. In brief, populations
857 were streaked from frozen stock and re-streaked the next day. From the second day culture,
858 populations were resuspended in normal saline to a 0.5 McFarland standard. Then, these cultures
859 were diluted 1:20 in normal saline before 10μL was added to 90μL of CAMHB + 2% NaCl with the
860 appropriate concentration of oxacillin. Plates were incubated at 35˚C without shaking and read at
861 24 hours. For statistical analysis of populations evolved from the MSSA ancestor, we set a
862 threshold such that anything above the ancestral susceptibility level (0.25ug/ml) was considered
863 a decrease in oxacillin sensitivity. We then compared the proportion of populations exhibiting a
864 decrease in oxacillin sensitivity with those that had not using Fisher's exact test with false
865 discovery rate-adjusted (FDR) p-values.
866
867 **Biofilm assay**
868 Overnight cultures of each population were diluted 1:40 in BHI broth containing 0.5% glucose,
869 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). 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 [82]. Mutations were called based on comparison to the reference USA300 JE2 genome (BioProject: PRJNA224116, BioSample: SAMN06677988, Assembly: GCF_002085525.1) using breseq [83]. The presence of large-scale deletions and other genetic changes were confirmed by analysis of de novo assembled genomes performed by the SKESA assembler software [84]. The ancestral populations were almost identical to the reference genome used except for three mutations that were not found in any evolved populations. Another three mutations in *bioD1, cls* and *ccdC* were present in all populations derived from the MSSA ancestor and likely occurred in the first passage from the frozen culture before experimental evolution was performed. We therefore removed these six mutations from the analysis.

The number of mutations (excluding synonymous or intergenic regions) across treatments were analyzed using a generalized linear model with a Poisson distribution. For mutation enrichment analysis, we followed the steps as described previously [85]. Briefly, we calculated the “mutation rate” for each ancestor by dividing the total number of mutations by the reference genome size. We multiplied the mutation rate by the total length of regulatory genes [86] with mutations in our study to generate the expected number of mutations for these genes. We then conducted single-tailed Poisson tests to determine whether the actual number of mutations in regulatory genes was greater than expected.

For phenotypes *vs.* mutations, we considered only mutations occurring at least twice throughout the experiment. For host mortality *vs.* mutations and growth rate *vs.* SCC*mec* and ACME, we conducted a one sample t-test for each evolved pathogen against its respective ancestor. P-values were adjusted using the FDR. For hemolysis status *vs.* mutations and oxacillin MIC *vs.* mutations, we conducted Fisher’s exact tests with Bonferroni corrections.

Principal component analysis

We conducted a principal component analysis on the phenotypes quantified in evolved populations using the prcomp function in R for each *S. aureus* genotype. We performed permutational analysis of variances (PERMANOVA, 999 permutations) to test for differences between treatments using adonis2 of the *vegan* package [87] and pairwiseAdonis [88].

921
922 **Genetic distance**
923 We generated a matrix of Euclidian genetic distances (the square root of pairwise differences)
924 using the frequencies of mutations that arose for each *S. aureus* genotype, from which we
925 constructed phylogenies [60,61]. We used pairwise differences between the ancestor and each
926 evolved populations to compare rates of evolution across treatments, and pairwise differences
927 between replicate populations within each treatment to compare the degree of divergence across
928 treatments. We compared rates of evolution for MRSA and MSSA treatments, and degree of
929 divergence for MSSA treatments, using linear models followed by Tukey multiple-comparison
930 tests. We compared the degree of divergence for MRSA treatments using a Kruskal–Wallis test
931 followed by Dunn's post-hoc test.

SUPPLEMENTAL FIGURES

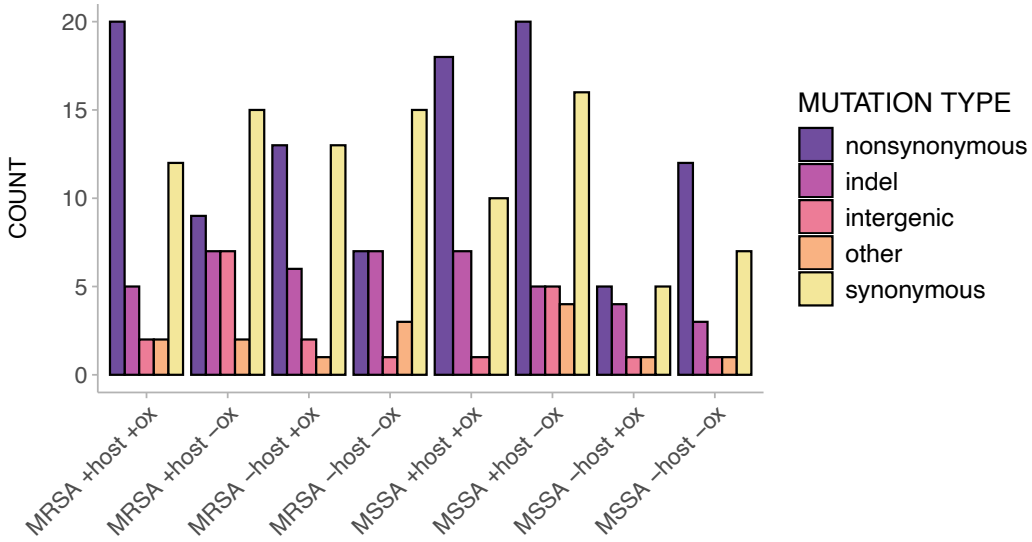

Figure S1. Count of all mutations (from 10-100% frequency) arisen in evolved populations.

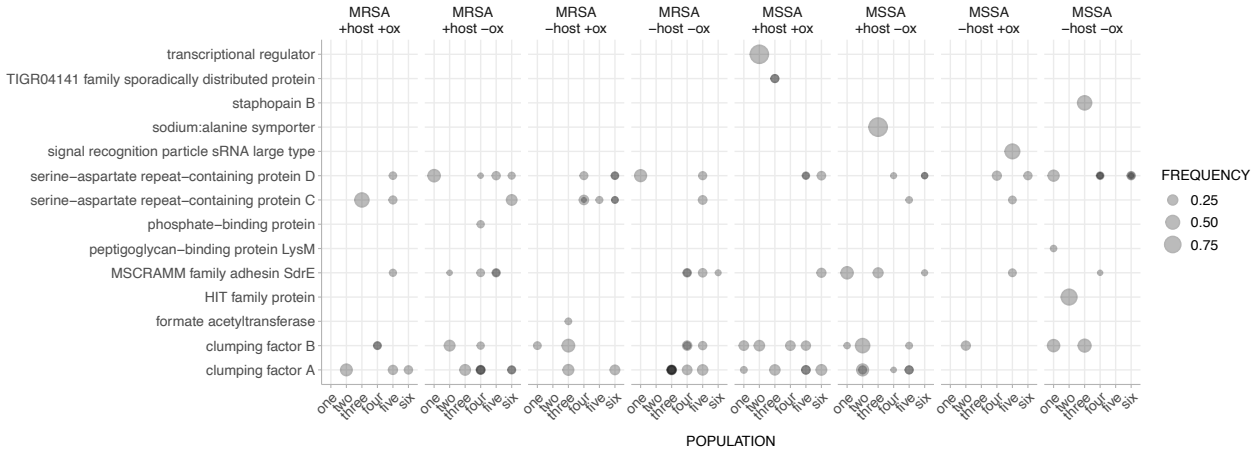

Figure S2. Mutations at frequencies between 0.1 and < 1, excluding synonymous or intergenic mutations, in each evolved population. Points with darker shades indicate more than one mutation present. All mutations in MRSA +host +ox populations between 0.1 and < 1 are all either synonymous or intergenic.

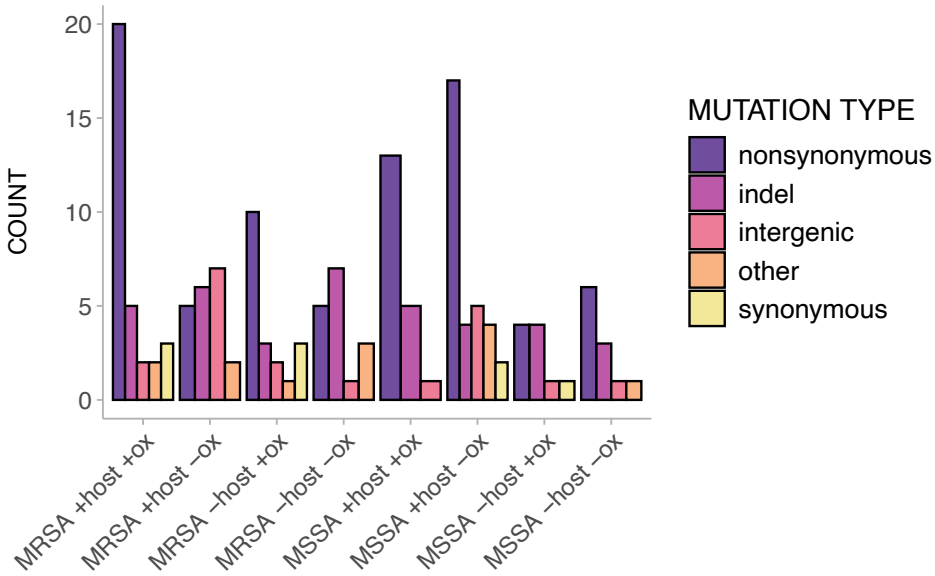

Figure S3. Count of mutations swept to fixation in evolved populations.

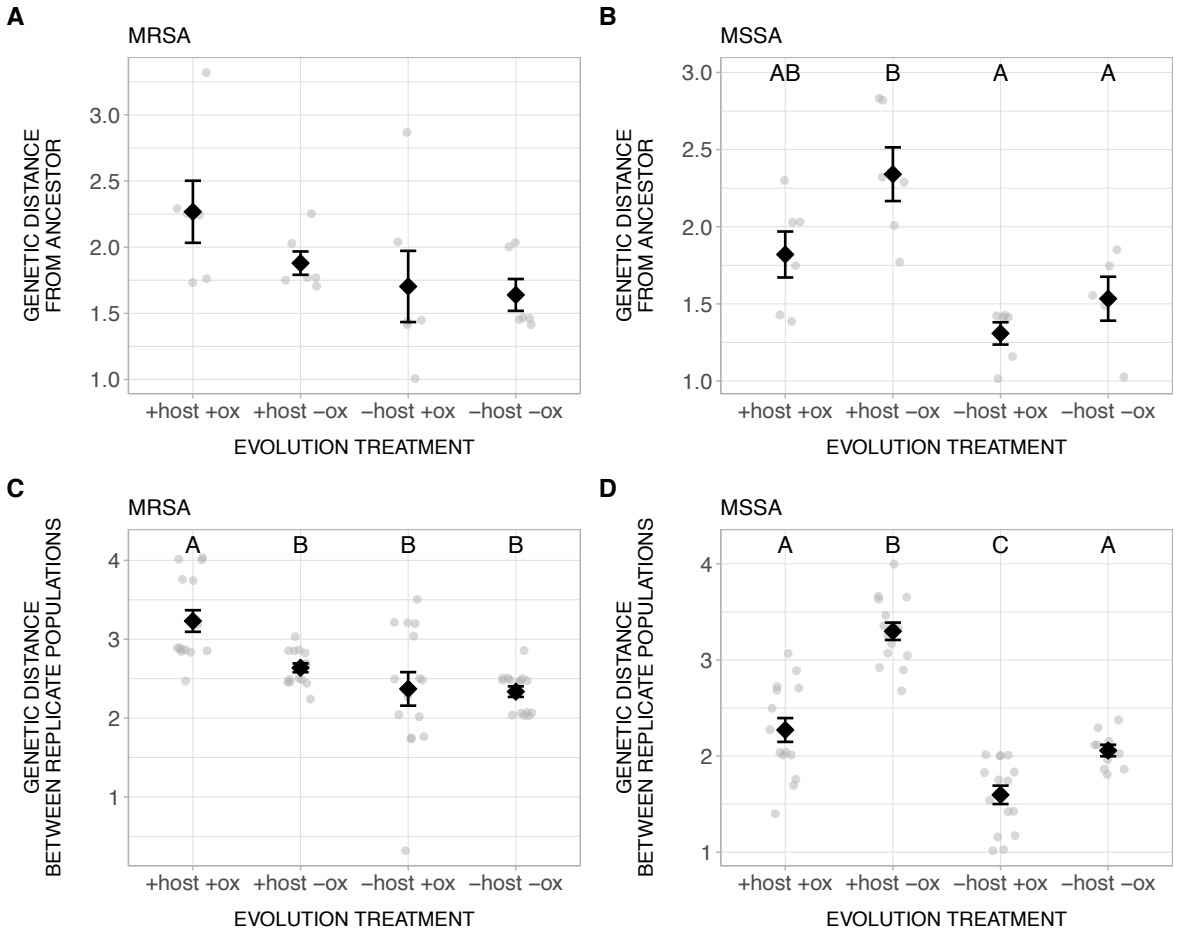

Figure S4. Genetic distance between the ancestor and evolved populations for A) MRSA and B) MSSA genotypes. Genetic distance between replicate populations within each treatment for C) MRSA and D) MSSA genotypes. Different letters indicate significant differences. Error bars indicate standard errors.