

1 Population diversity jeopardizes the efficacy of antibiotic cycling

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9

10 Abstract

11 Treatment strategies that anticipate and respond to the evolution of pathogens are
12 promising tools for combating the global rise of antibiotic resistance¹⁻³. Mutations
13 conferring resistance to one drug can confer positive or negative cross-resistance to
14 other drugs⁴. The sequential use of drugs exhibiting negative cross-resistance has been
15 proposed to prevent or slow down the evolution of resistance⁵⁻⁸, although factors
16 affecting its efficacy have not been investigated. Here we show that population diversity
17 can disrupt the efficacy of negative cross-resistance-based therapies. By testing 3317
18 resistant *Staphylococcus aureus* mutants against multiple antibiotics, we show that first-
19 step mutants exhibit diverse cross-resistance profiles: even when the majority of mutants
20 show negative cross-resistance, rare positive cross-resistant mutants can appear. Using
21 a drug pair showing reciprocal negative cross-resistance, we found that selection for
22 resistance to the first drug in small populations can decrease resistance to the second
23 drug, but identical selection conditions in large populations can increase resistance to
24 the second drug through the appearance of rare positive cross-resistant mutants. We
25 further find that, even with small populations and strong bottlenecks, resistance to both
26 drugs can increase through sequential steps of negative cross-resistance cycling. Thus,
27 low diversity is necessary but not sufficient for effective cycling therapies. While
28 evolutionary interventions are promising tools for controlling antibiotic resistance, they
29 can be sensitive to population diversity and the accessibility of evolutionary paths, and
30 so must be carefully designed to avoid harmful outcomes.

31

32 Main Text

33 The use of antibiotic combinations can impede the evolution of resistance by imposing
34 evolutionary tradeoffs³. Recent attention to these phenomena has been driven by the
35 promise of developing more effective strategies to prevent treatment failure due to *de*
36 *novo* mutations in long-term infections, by exploiting the evolutionary interactions
37 between drugs to impede the development of resistance^{5-7,9-13}. In general, mutations
38 providing resistance to one drug can also either increase or decrease resistance to other

39 drugs. While increased resistance to other drugs (positive cross-resistance) is
40 common^{5,6}, for some drug pairs, resistance to one drug decreases resistance to another
41 drug (negative cross-resistance, also called collateral sensitivity⁷). Systematic studies
42 have revealed specific drug pairs with negative cross-resistance, including cases of
43 reciprocal negative interactions, whereby resistance to any of the two drugs confers
44 sensitivity to the other^{4-6,14}. Simultaneous use of drug pairs with negative cross
45 resistance has been explored as a means to slow down the evolution of resistance to
46 toxins ranging from antibiotics to pesticides^{5-7,15-18}. Alternatively, drugs with collateral
47 sensitivity can be used sequentially to slow evolution of resistance, thus avoiding toxicity
48 or incompatibility issues that complicate antibiotic mixtures in clinical settings^{5,7}.

49 It remains unknown whether the effectiveness of alternating negative cross-resistance
50 treatment is robust to the large population size often found in clinical infections, and to
51 what extent longer-term evolution can be reliably predicted by the cross-resistance
52 profiles of first-step mutants. Previous measurements on negative cross-resistance have
53 typically assayed a small number of first-step mutants, and evolution experiments were
54 conducted with small population sizes^{5-7,9,19,20}. Because the diversity of cross-resistance
55 across random mutants has not been systematically measured, it is unknown whether
56 negative cross-resistance seen in typical mutants is representative, and whether
57 negative cross-resistant treatment would be effective for larger populations with larger
58 potential for rare mutants.

59 We focus here on *Staphylococcus aureus*, a common human pathogen in which *de novo*
60 antibiotic resistance is of clinical importance²¹⁻²⁵. We developed a high-throughput assay
61 to profile the cross-resistances of 3317 resistant mutants to six antibiotics with diverse
62 mechanisms of action: oxacillin (OXA), novobiocin (NOV), ciprofloxacin (CPR),
63 gentamicin (GEN), amikacin (AMK), and doxycycline (DOX) (**Fig. 1a; Supplementary**
64 **Table 1**).

65 The frequency of surviving colonies as a function of antibiotic concentration follows a
66 characteristic shape: survival falls off when drug concentration exceeds the minimum
67 inhibitory concentration (MIC), and then plateaus around 10^{-6} - 10^{-8} until it drops below
68 detection at drug concentrations high enough to kill even the most resistant mutant
69 (Mutant Prevention Concentration, MPC)²⁶. The plateau region between the MIC and
70 MPC is termed the mutant selection window (MSW), and constitutes the range of
71 antibiotic concentrations where selection for resistance occurs. For each antibiotic, we
72 collected roughly 500 resistant mutants from plates in the MSW range (**Fig. 1a;**
73 **Supplementary Fig. 1; Methods**). We measured the level of resistance of each of
74 these mutants to each of the 6 antibiotics by growing the library on a series of agar
75 plates at increasing antibiotic concentrations (**Fig. 1b; Methods**). Positive cross-
76 resistance was detected when a mutant survived at higher drug concentrations than the
77 wild-type, while negative cross-resistance was indicated by the wild-type growing at a

78 higher drug concentration than the mutant. (**Fig. 1b**). Using a robotic pipeline, we
 79 systematically measured cross-resistance interactions in nearly 20,000 mutant-drug
 80 combinations.

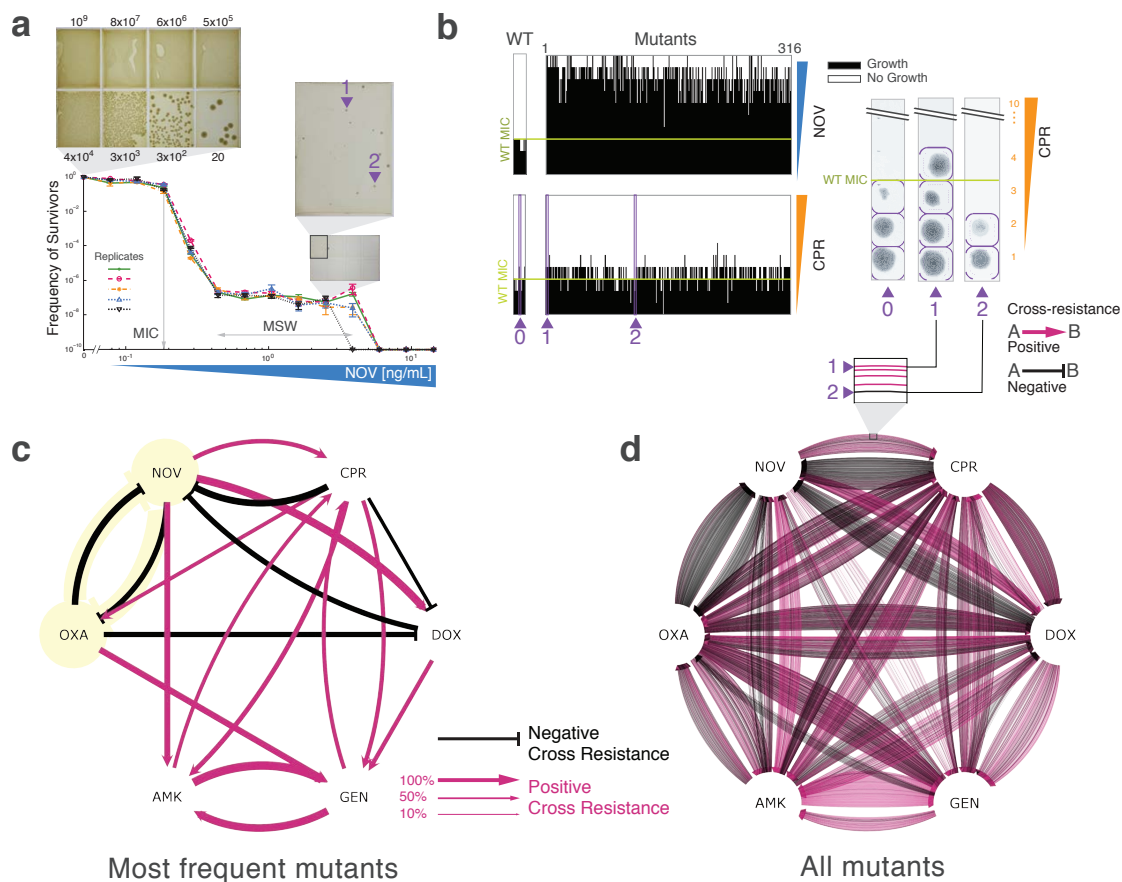


Fig. 1. High-throughput measurement of cross-resistance in 3,317 *de novo* mutants reveal considerable diversity in both the magnitude and sign of cross-resistance. a) The mutant selection window for NOV was measured by counting the frequency of survivors at 10 drug concentrations. Each line indicates an independent biological replicate. Inserts show examples from the automated imaging pipeline. b) Measured resistance to NOV and cross-resistance to CPR of mutants selected on NOV, showing a range of MICs for both drugs. Examples of the automated image pipeline are shown for two isolates (1 and 2), chosen as indicated from the inset in (a). c) The network of most frequently seen cross-resistance interactions between every drug pair in our library. The majority of pairs show positive cross-resistance. Only the most common phenotype out of three possible phenotypes (positive, negative, and neutral cross-resistance) representing a plurality (>33%) of all mutants are shown. The one reciprocal negative cross-resistant pair, OXA and NOV, is highlighted. d) The full network of cross-resistance for all observed mutants, showing heterogeneous interactions for all pairs. Here every mutant is represented as a single edge originating from the drug it was selected in.

81 To provide a high-level picture of cross-resistance interactions and identify negative-
82 cross resistance pairs, we constructed a network of the modal cross-resistance between
83 drug pairs. Out of the 30 possible ordered drug pairs, our analysis revealed 6 negative
84 and 12 positive cross-resistance interactions. The negative cross-resistance interactions
85 included the reciprocally cross-resistant pair, OXA and NOV, and the three-drug
86 negative cross-resistant cycle, OXA->DOX->NOV->OXA. In contrast to the negative
87 cross-resistance observed between aminoglycosides and other drugs in *E. coli*^{5,6}, we
88 found predominantly positive cross-resistance interactions between aminoglycosides
89 and other antibiotics (**Fig. 1c**). These observations highlight the species-specificity of
90 cross-resistance interactions, and indicate that cross-resistance-based treatment
91 strategies must be tailored for specific organisms.

92 **Cross-resistance profiles are highly heterogeneous**

93 Analysis of the distribution of cross-resistance reveals that, for all drug pairs, there is
94 considerable variation in magnitude and sign of cross-resistance among the collection of
95 resistant mutants. Even for drug pairs where mutants primarily display cross-resistance
96 of a specific sign, there are rare mutants with phenotypes opposite to the general trend
97 (**Fig. 1d; Supplementary Fig. 2a**). The MICs of the two aminoglycosides, AMK and
98 GEN, were positively correlated, however we observed no strong MIC correlations
99 between antibiotic pairs from different classes (**Supplementary Fig. 3**). Going beyond
100 the typical measurements of small numbers of resistant mutants revealed hitherto
101 unseen diversity in cross-resistance profiles (**Supplementary Fig. 3**).

102 To investigate the impact of diversity in cross-resistance, we focused on the reciprocal
103 negative cross-resistance pair OXA and NOV (**Fig. 1b**). While for the majority of OXA
104 and NOV resistant mutants, negative cross-resistance to the other drug was observed,
105 rare, positive cross-resistant mutants also appear (**Fig. 2**). Of the OXA-resistant
106 mutants, 74% have negative cross-resistance to NOV, 21% do not affect NOV
107 resistance, while 5% have positive cross-resistance. Of the NOV-resistant mutants, 62%
108 exhibit negative cross-resistance to OXA, 33.5% do not affect OXA resistance and 4.5%
109 display positive cross-resistance. These observations suggest that while small-scale
110 studies may identify OXA and NOV as a reciprocal negative cross-resistance pair, they
111 would miss the underlying fraction of rare mutants with positive cross-resistance, which
112 could in turn affect the evolution of multi-drug resistance.

113 **Rare mutants appearing in large populations reverse the outcome of cycling**

114 We next asked whether rare cross-resistant phenotypes can impact the effectiveness of
115 negative cross-resistance treatments. As rare positive cross-resistant mutants are more
116 likely to appear in larger populations, we hypothesized that the outcome of sequential
117 treatment with a reciprocal negative cross-resistance drug pair would be affected by
118 population size. We first considered treatment with OXA followed by NOV, followed by
119 the reciprocal. Based on the measured frequencies of resistance (**Supplementary Fig.**

120 1), we selected for resistant mutants at the same concentration from either a small (10^6
121 cells) inoculum, in which approximately one mutant is expected to survive, and a large
122 (10^9 cells) inoculum, in which approximately a thousand mutants are expected to survive.
123 After selection for resistance to the first drug, we expanded the two populations to the
124 same size, and measured the frequency of survivors as a function of the concentration of
125 the second drug, as compared to the ancestral wildtype (**Methods, Fig. 3a**).

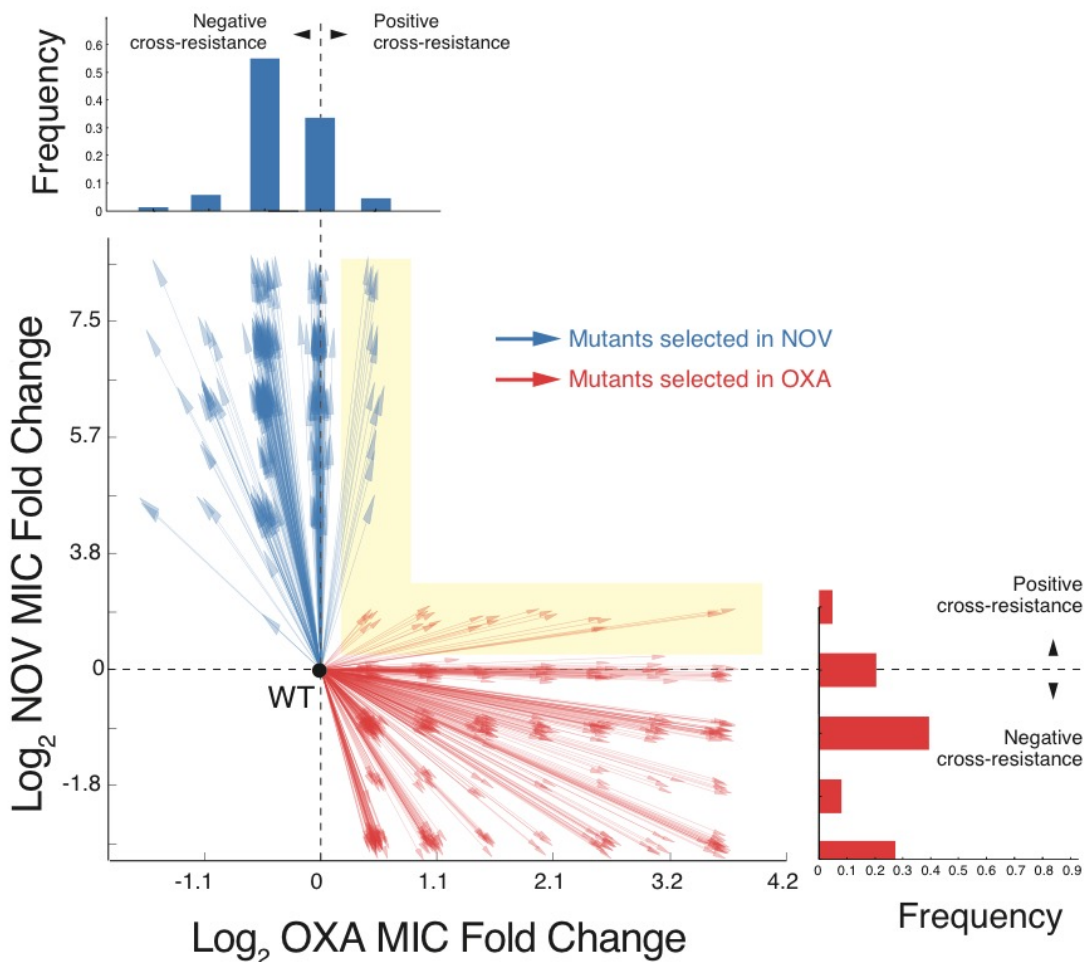


Fig. 2. Reciprocal negative cross-resistance between OXA and NOV is disrupted by rare positive cross-resistant mutants. Vectors indicate trajectories of one-step mutants on the 2D plane defined by OXA and NOV resistance. Histograms display the distribution of cross-resistance phenotypes. Red, mutants selected in OXA; blue, mutants selected in NOV. Rare, positive cross-resistant mutants are highlighted.

126 While the low-inoculum OXA-evolved population exhibited marked negative cross-
127 resistance to NOV, the high-inoculum population displayed not only a reduction but a
128 complete inversion of this negative cross-resistance (**Fig. 3b**). The MIC of the low-
129 inoculum population is reduced in comparison to the WT, demonstrating the efficacy of

130 negative cross-resistance in using one drug in order to reduce resistance to another
 131 (fewer than one in 10^5 survivors at 0.15 ng/mL NOV compared to WT 0.36 ng/mL).
 132 Beyond a reduction in MIC, this low-inoculum population also has lower potential to
 133 evolve as evident by its significantly reduced MPC (1.1 ng/mL NOV for the OXA-evolved
 134 population vs 4.0 ng/mL for the WT). In striking contrast, the population that was
 135 selected on the same exact drug concentration of OXA but with large inoculum size
 136 showed an increase rather than decrease in resistance (fewer than one in 10^5 survivors
 137 at 0.86 ng/mL). Further, unlike the low-inoculum population, the high-inoculum
 138 population has the same MPC as the wild type, showing no reduction in evolutionary
 139 potential. The reciprocal case of NOV followed by OXA resulted in similar, albeit less

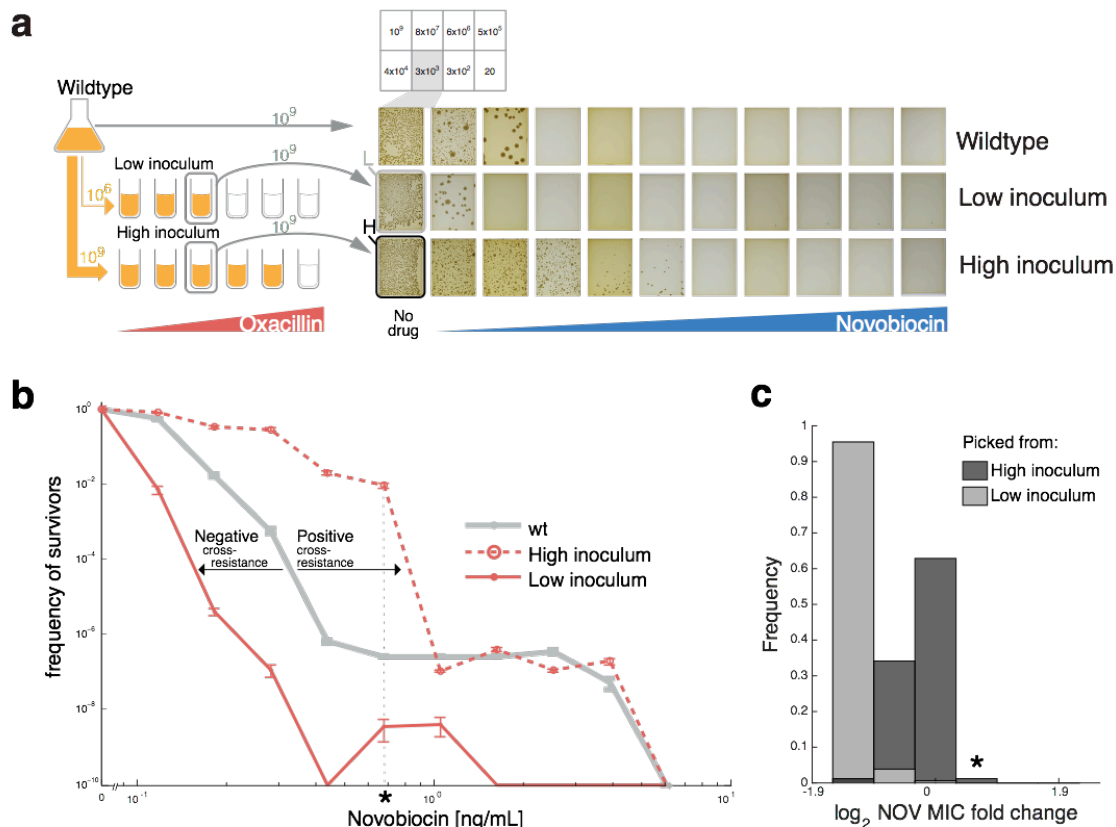


Fig. 3. Negative cross-resistance cycling selects for positive cross-resistance in large populations due to appearance of rare positive cross-resistant mutants. a) A wild type population was split into a large inoculum (10^9) or small inoculum (10^6) and selected in identical concentrations of OXA. The two populations, along with the ancestor, were then grown to the $1e9$ cells/mL before plating on NOV. b) The low diversity population (dashed red line) exhibits negative cross-resistance. However, the high diversity population (solid red line), which differs from the low diversity population only in the starting population size, exhibits positive cross resistance. c) NOV-resistance profiles of colonies were picked from the high and low inoculum populations after selection in oxacillin. The low inoculum population (L) show primarily negative cross-resistance to NOV, while the high inoculum population (H) displays a much more diverse cross-resistance profile, including rare mutants that have positive cross-resistance to NOV.

140 drastic, inversion of negative cross-resistance due to inoculum size (**Supplementary**
141 **Fig. 4a**).

142 Phenotyping mutants recovered after liquid selection, we found that the low inoculum
143 population shows homogenous negative-cross resistance phenotypes while the high
144 inoculum population is more diverse and contains rare positive cross-resistance mutants.
145 Isolation of colonies from the low diversity population after OXA selection but before
146 NOV selection shows a relatively homogenous population with negative cross-resistance
147 to NOV (**Fig. 3c, L**). In contrast, colonies from the high inoculum population before NOV
148 selection shows a wider spread of cross-resistance phenotypes (**Fig. 3c, H**). Only 1% of
149 this high inoculum population showed positive cross-resistance with 2.4-fold increase in
150 MIC over the wild type (**Fig. 3c, H**), in agreement with the sharpest drop-off in survival
151 occurring at 1 in 10^2 cells, at roughly 2.4 fold increase over the effective wild type MIC
152 (**Fig. 3b**). Together, these results show that rare positive-cross resistant mutants can
153 disrupt, and even invert the effect of negative cross-resistance cycling.

154 **Distinct mutation profiles mediate cross-resistance phenotypes**

155 To understand the genetic mechanisms behind resistance and cross-resistance, we
156 whole-genome sequenced 277 mutants that had been selected for resistance to OXA or
157 NOV (see methods). NOV mutations were clustered around known resistance hotspots
158 in *gyrB*²⁷, while OXA mutations targeted general stress response pathways through
159 binding partners of sigma factor *sigB*²⁸ ($p = 1.7 \cdot 10^{-4}$, fisher exact test, **Supplementary**
160 **table 2**). In the OXA mutants, 30 mutations occurred in *gdpP*, including 14 nonsense
161 mutations (**Supplementary table 3, supplementary Fig. S5**). *gdpP* encodes a recently
162 characterized putative c-di-AMP phosphodiesterase, and knockouts of *gdpP* have been
163 shown to increase beta-lactam resistance²⁹⁻³¹. We identified five mutations in *relA*, a
164 gene in which point mutations have been linked to Linezolid resistance by switching on
165 the stringent response in clinical isolates²⁵. We also recovered mutations in *fmt* and
166 penicillin-binding protein *pbp1*, both of which are involved in cell wall synthesis^{21,32}
167 (**supplementary table 3**). The observation that OXA mutations target different pathways
168 while NOV mutations target a single gene may explain why OXA resistance occur
169 roughly 10 times more frequently than NOV resistance.

170 For each mutant, we then tested for association with different cross-resistance
171 phenotypes (**supplementary Fig. S5**). In NOV mutants, *gyrB* R144I was associated with
172 neutral cross-resistance (enrichment, $p = 1.09 \cdot 10^{-4}$, 2-tailed fisher exact test, Bonferroni
173 adjusted) and negative cross-resistance (depletion, $p=0.01$). In OXA mutants,
174 associations were found between *gdpP* E550* and negative cross-resistance ($p = 3 \cdot 10^{-2}$);
175 *rpoB* G827D with both negative cross-resistance (depletion, $p = 1.93 \cdot 10^{-4}$) and neutral
176 cross-resistance (enrichment, $p = 2.69 \cdot 10^{-5}$); and *rpoC* V78I with both negative cross-
177 resistance (depletion, $p = 2.61 \cdot 10^{-3}$) and neutral cross-resistance (enrichment, $p =$
178 $5.83 \cdot 10^{-4}$). Due to the small number of mutants with positive cross-resistance, after

179 correcting for multiple hypothesis testing, no mutations were significantly associated with
180 positive cross-resistance. However, we recovered nonsynonymous mutations in *rsbW*,
181 the negative regulator of the stress response sigma factor *sigB*³³, only in OXA mutants
182 with positive (n=2) or neutral cross-resistance (n=1) to NOV, in concurrence with the role
183 of *rsbW* mutations in multiple drug resistance⁷. Taken together, we find that *in vitro*
184 evolution recovers clinically important mutations, and that mutation profiles differ
185 between cross-resistance phenotypes.

186 **Negative cross resistance is not preserved through sequential antibiotic selection**

187 Our results suggest that negative cross-resistance cycling may be more successful if the
188 population size was kept small at each step. To study whether maintenance of small
189 population size is sufficient for success of negative cross-resistance cycling, we
190 sequentially treated populations with OXA and NOV, transferring 10^6 or 10^7 cells at each
191 step for OXA and NOV selection, respectively, to limit diversity based on the frequency
192 of mutation (**Fig. S1**). We found that the first step consistently produces negative cross-
193 resistance. However, as mutations accumulate in subsequent steps, negative cross-
194 resistance frequently disappeared (**Fig. 4**). Thus, small population size appears
195 necessary but not sufficient for successful negative cross-resistance cycling.

196

197 **Discussion**

198 Our results show that rare positive-cross resistant mutants may circumvent negative
199 cross-resistance. Thus, disease burden and the frequency of resistance mutations
200 should be important factors in the decision to use negative cross-resistance antibiotic
201 therapy in the clinic. Further, even for small population size, antibiotic cycling may not
202 inhibit resistance: the accumulation of negative cross resistance mutations may result in
203 positive cross resistance either additively or due to epistatic interactions, or rare positive
204 cross resistant mutants may arise by chance alone. While the mechanism of negative
205 cross-resistance between OXA and NOV is beyond the scope of this study, the
206 mutations in *gyrB* and *sigB* binding factors suggest that it may be caused by an
207 imbalance between negative supercoiling in DNA and the transcriptional stress
208 response.³⁴ However, unraveling the exact mechanisms underlying the diversity of
209 cross-resistance phenotypes will require further study of these mutations in both isolation
210 and combinations.

211 It would be interesting to see how these results extend to other genetic backgrounds,
212 species, and antibiotics. Ultimately, the successful use of negative cross-resistance
213 cycling to suppress antibiotic resistance will depend on the identification of drugs or drug
214 combinations for which mutual negative cross-resistance is sufficiently frequent even in
215 large populations. Beyond the *de novo* mutations characterized in this study, mobile
216 genetic elements may have their own unique signature of negative and positive cross-

217 resistance. Future designs of sequential antibiotic therapy must take into account the full
 218 distribution of possible mutations and their cross-resistance profiles.

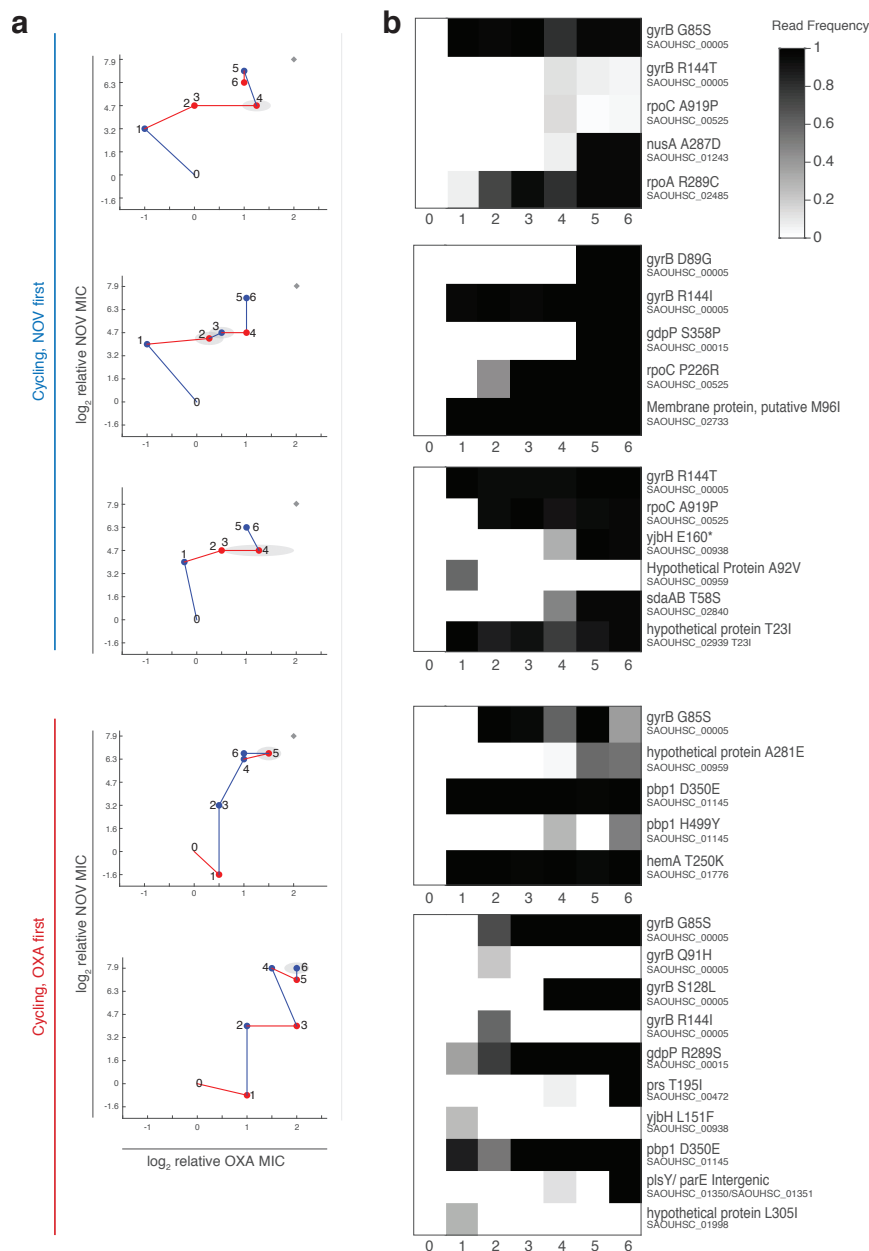


Fig. 4. Sequential treatment in OXA and NOV with a strong selective bottleneck suggest that mutations do not interact additively as they accumulate. a) Phenotypic changes of 5 replicate populations throughout OXA and NOV selective cycling, as measured by the fold-change of mutant MICs to OXA and NOV. Three replicates were selected first in NOV, and two replicates were first selected in OXA. Numbers indicate steps in the cycling process. Blue lines show a selection step in NOV, while red lines show a selection step in OXA. Gray ellipses illustrate standard deviations from three biological replicates for MIC measurements for both NOV (vertical axis) and OXA (horizontal axis). Gray diamond indicates the median end point of three control populations (cycled between OXA and TSB, or NOV and TSB). b) Genotypic change throughout cycling for each population in (a). Grayscale shading indicates the frequency of reads supporting each mutation, with white as 100%, and black as 0%. Each row represents a single mutation, labeled with the locus tag and amino acid change. Each column is a single step during the cycling process

219 **Methods**

220 **Media, strain, and antibiotics.** The ancestral strain used for all experiments is
221 *Staphylococcus aureus* RN4220, a phage-free strain derived from NCTC 8325³⁵.
222 Independent cultures of the ancestor were inoculated with single colonies, grown
223 overnight at 37°C, and stored in 16.7% (v/v) glycerol at -80°C. All experiments were
224 conducted in either trypticase soy agar (TSA, BD 211046) or trypticase soy broth (TSB,
225 BD 211771). Antibiotic solutions were prepared from powder at 10mg/ml in water and
226 stored at 4°C (unless otherwise specified): Oxacillin (Sigma 28221); Novobiocin (Sigma
227 N6160); Ciprofloxacin (Sigma 17850), in 100mM HCl; Doxycycline (Sigma 09891);
228 Gentamycin (Sigma G1264), 50mg/ml; Amikacin (Sigma A1774), 50mg/ml.

229 **Evolution of first-step mutants.** Series of 8-well plate (VWR 267062) were filled with
230 TSA containing increasing concentrations of antibiotic. A serial dilution of the ancestor at
231 1:10^{1,2} was plated in each well. This ensured that individual colonies can be seen at
232 every antibiotic concentration, and allowed accurate counting of survivors. Plates were
233 incubated at 37°C for 7 days, and imaged using a robotic imaging platform⁸. Automated
234 image segmentation and cell counting in Matlab was reviewed by eye. Frequency of
235 survivors as a function of antibiotic concentration was then calculated. Surviving colonies
236 residing in the MSW were picked using a robotic colony picker (Hudson Robotics,
237 RapidPick) into 96-well microtiter plates (Corning 3628) containing 16.7% (v/v) glycerol
238 in 150µL TSB. Each 96-well plate contains a number of empty wells as negative control
239 and ancestral cells for downstream MIC measurement, such that a combination of four
240 96-well plates into a 384-well plate will result in 322 wells containing mutants, 16 wells
241 containing the ancestor, and 46 negative controls. 96-well plates were incubated
242 overnight at 37°C, 220 rpm and stored at -80°C.

243 **High throughput measurement of cross-resistance.** Mutant libraries stored in 96-well
244 microtiter plates were replicated with a 96-well floating-pin replicator into a fresh 96-well
245 microtiter plate containing TSB and incubated overnight at 37°C, 220 rpm. Immediately
246 prior to MIC measurement, replicated plates were combined into 384-well microtiter
247 plates (VWR 82030-992) using a 96-channel benchtop pipettor (Rainin 17014207). A
248 total of 16 wildtype cultures from frozen stocks were added to empty wells in the 384-
249 well plate. A robotic liquid handler (Perkin-Elmer SciClone ALH 3000) with a 384-well
250 floating-pin replicator (V&P Scientific) was used to pin 384-well plates onto a TSA plates
251 containing increasing concentrations of antibiotics. Drug serial dilution concentrations for
252 MIC measurement were chosen with 4 steps before the MIC, and 6 steps between the
253 MIC and MPC. TSA plates were incubated at 37°C overnight, before being imaged using
254 a robotic imaging platform. Image segmentation and determination presence or absence
255 of colony was performed in Matlab, followed by manual curation. Cross-resistance
256 scores were calculated as the number of plates that a mutant grew on subtracted by the
257 median number of plates the wildtype grew on. Mutants found to not be resistant to the
258 antibiotic it was selected in were discarded for downstream analysis.

259 **Construction of directed graphs.** A directed, weighted graph was generated using the
260 python library graph-tool³⁶, with antibiotics as nodes, and the cross-resistance of each
261 mutant selected in an antibiotic as directed edges originating from that antibiotic.

262 **Measurement of the impact of population size on negative cross resistance**
263 **cycling.** For selection in OXA followed by plating on NOV, 10^6 ancestors (low inoculum)
264 were diluted in 5 mL of TSB supplemented with increasing concentrations of OXA. 10^9
265 ancestors (high inoculum) were diluted in 100mL of TSB supplemented with the same
266 gradient of OXA. To measure the OXA MIC, 10^3 ancestors were diluted into a 96-well
267 microtiter plate containing 150uL each of the same gradient used for the low and high
268 inoculum populations. All cultures were incubated overnight at 37°C, 220 rpm. Optical
269 density at 500nm (OD500) was measured with a Perkin-Elmer Victor3, through a
270 500/20nm emission filter. The OXA concentration at which both high and low inoculum
271 populations were picked was defined as the highest drug concentration in which growth
272 was observed in the 10^6 population (OD500 above 0.2). Both low and high inoculum
273 cultures were washed twice in TSB and diluted in TSB supplemented with 16.7%
274 glycerol. Aliquots of both populations were stored at -80°C. Population density was
275 measured by thawing aliquots of both populations and performing a serial dilution onto
276 petri dishes containing TSA and incubated overnight at 37°C. Population density was
277 calculated as number of observed colonies divided by the effective volume plated. To
278 measure the impact of inoculum size on population survival, aliquots of high inoculum,
279 low inoculum, and ancestor were plated onto a series of 8-well plates containing
280 increasing concentrations of NOV in TSA, as described in Evolution of first-step mutants.
281 8-well plates were imaged every day for a total of 7 days on a robotic imaging platform.
282 The time point with maximal difference in population survival was chosen. For the OXA
283 population, this occurred at 7 days. For the NOV population, this occurred at 2 days.
284 Selection in NOV followed by plating in OXA proceeded identically as described, with the
285 exception in that the low inoculum population consists of 10^7 cells to be consistent with
286 the height of the NOV MSW.

287 **Whole genome sequencing and analysis.** We picked a diverse set of mutants from
288 **Fig. 2** (E1 strains) and **Fig. 3** (E2 strains). For the E1 set, 5 strains were picked at
289 random from the MSW at each drug concentration where individual colonies were
290 visible. E2 strains were after liquid selection in OXA or NOV. Genomic DNA was isolated
291 using a PureLink 96-well DNA kit (Life Technologies). Library preparation followed³⁷. In
292 short, the DNA was prepared with an Illumina Nextera Kit at reduced volume, with
293 alternate reagents for PCR and DNA purification and custom barcodes for high-density
294 multiplexing. Alignment and mutation calling were performed using breseq³⁸. We wrote
295 a custom wrapper to collate the output of breseq (available at [github]), and performed
296 further downstream analysis in Matlab. Because cycling populations may be a
297 heterogeneous mixture of many clones, we wrote a custom wrapper based on Samtools
298 to count reads supporting all mutations called by breseq in our dataset in all cycling

299 samples, regardless of whether a mutation was originally called in that sample. We were
300 thus able to detect whether mutations called in later cycling steps existed at subclonal
301 frequencies in earlier steps.

302 ***In vitro* negative cross-resistance cycling.** Small populations of ancestor were treated
303 sequentially with OXA and NOV for a total of 6 selection steps. At each step of the cycle,
304 either 10^7 cells (NOV, one mutation expected) or 10^6 cells (OXA, one mutation expected)
305 were diluted into 5 mL TSB supplemented with the appropriate antibiotic. The highest
306 drug concentration with OD500 greater than 0.2 was propagated by first performing a
307 sterilizing dilution, and using the dilution step expected to yield either 1 cell (for NOV) or
308 10 cells (for OXA) to severely bottleneck the population size. Different starting
309 populations sizes are used for OXA and NOV to control for total generation time.

310 Controls for this experiment consisted of populations that were treated by alternating
311 rounds of NOV and TSB, or OXA and TSB. For the NOV control, 10^7 cells were
312 propagated in 5 mL NOV gradient or 10^6 cells were propagated in TSB. For OXA control,
313 10^6 cells were propagated in OXA or 10^7 cells were propagated in NOV.

314 At each step, aliquots of the propagated population were washed twice in TSB and
315 stored in TSB supplemented with 16.7% glycerol, -80°C . For MIC measurements, frozen
316 aliquots were pinned into 96-well microtiter plates and grown overnight at 37°C , 220 rpm.
317 These populations were then replica pinned into series of 384-well microtiter plates
318 containing increasing concentrations of OXA or NOV and incubated overnight at 37°C ,
319 220 rpm. Optical density at 500nm (OD500) was measured with a Perkin-Elmer Victor3,
320 through a 500/20nm emission filter.

321 **Statistical testing for enrichment *sigB* binding partners.** We performed a two-tailed
322 fisher exact test on a contingency table of all genes in the *Staphylococcus aureus*
323 genome (2892). The margins consist of 58 unique genes found in OXA-resistant mutants
324 and 15 binding partners of *sigB*²⁸. Columns indicate whether genes are *sigB* binding
325 partners, and rows indicate whether genes were mutated in our dataset. Two-tailed
326 fisher exact test gave p-value of $1.69 \cdot 10^{-4}$.

327 **Statistical testing for association of mutation with cross-resistance phenotype.** To
328 limit the number of tests, we eliminated all events that occurred fewer than 3 times in all
329 phenotypes. We then performed two-tailed fisher exact tests to detect associations
330 between each specific mutation and cross-resistance phenotype.

331

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