Biofilm-dependent evolutionary pathways to antibiotic resistance

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Abstract

Many opportunistic pathogens live in surface-attached communities called biofilms that generate ecological structure and can increase stress tolerance. Theory suggests that bacterial populations evolving in biofilms may harbor greater genetic diversity and become resistant to antibiotics by different pathways than in well-mixed environments. We used experimental evolution and whole genome sequencing to test how the mode of growth influences dynamics and mechanisms of antibiotic resistance in *Acinetobacter baumannii* populations. Biofilm and planktonic populations were propagated under conditions lacking antibiotics, under constant sub-inhibitory concentrations of ciprofloxacin, or under steadily increasing concentrations of this drug. As predicted, both the evolutionary dynamics and the identities of selected mutations differed between treatments and lifestyle. Planktonic populations exposed to ciprofloxacin underwent sequential selective sweeps of single mutations including the primary drug targets, *gyrA* and *parC*. In contrast, biofilm-adapted populations diversified by multiple contending mutations in regulators of efflux pumps. Mutants isolated from both lifestyles exhibited a trade-off between fitness and resistance level, wherein biofilm-adapted clones were less resistant but more fit in the absence of drug. Further, biofilm-adapted populations evolved collateral sensitivity to cephalosporins whereas the planktonic populations displayed cross-resistance with several classes of antibiotics. This study demonstrates that growth in biofilms, arguably the predominant bacterial lifestyle, may substantially alter the routes, dynamics, and consequences of the evolution of antibiotic resistance and is therefore an important consideration when treating infections.

Introduction

Antimicrobial resistance (AMR) is one of the main challenges facing modern medicine. The emergence and rapid dissemination of resistant bacteria is decreasing the effectiveness of antibiotics and it is estimated that 700,000 people die per year due to AMR-related problems (1). AMR, like all phenotypes, is an evolved property, either the ancient product of living amidst other microbial producers of antimicrobials (2), or the recent product of strong selection by human activities for novel resistance-generating mutations (3). AMR can be acquired via two principal routes: horizontally, when AMR genes are acquired from other bacteria; or vertically, where the resistance mechanism originates by *de novo* mutation and is inherited by the progeny. These vertically inherited, novel evolved resistance mechanisms are the focus of this study. Here, we study the evolutionary dynamics and effects of new resistance mutations in the opportunistic nosocomial pathogen *Acinetobacter baumannii*, which is often intrinsically resistant to antibiotics or has been reported to rapidly evolve resistance to them (4). This pathogen is categorized as one of the highest threats to patient safety (5), partly due to its ability to live on inanimate surfaces in biofilms, which are surface-attached communities protected from external stresses by secreted polymers (6).

The biofilm lifestyle is central to AMR (7-9). Microbes that are surface-bound and encased in secreted polymers are less susceptible to compounds that fail to penetrate the matrix or can persist through the lower metabolic rate of biofilm residents (7, 8, 10). But what is less understood or appreciated is that the physical and ecological heterogeneity of biofilms can influence the evolutionary dynamics of microbial populations in multiple ways. First, the protective effect of biofilms could reduce the strength of selection for resistance by reducing the effective antibiotic concentration. This would modify the rate of adaptation to a particular drug since it has been observed that subinhibitory

63 concentrations of antibiotic may select for multiple mutations with low fitness costs (11,
64 12). Second, the spatial structure of subpopulations within biofilms may facilitate greater
65 genetic diversity (13-16), either by the effect of structure alone allowing independent
66 lineages of varied fitness to coexist, or by adaptive diversification to inhabit discrete
67 niches in the heterogeneous biofilm environment (17). For example, bacteria or yeasts
68 evolving in biofilms became more diverse than in planktonic cultures (15, 18) which
69 could improve the capacity of these populations to respond to new selective forces (19).
70 Third, life in biofilms may align selective forces on traits that were formerly opposed and
71 increase the efficiency of adaptation (20, 21). For example, bacteria growing in a
72 planktonic environment may experience selection for rapid growth, and this can make
73 these cells more susceptible to antimicrobials that are more effective on metabolically
74 active cells. However, bacteria dwelling in biofilms experience selection for life in
75 crowded, resource-limited conditions, and adaptation to slow growth may actually also
76 generate antimicrobial resistance by limiting the availability of molecular drug targets.
77 Therefore, in biofilms, growth and resistance may become aligned traits that can jointly
78 improve, and the transition from planktonic growth to biofilms can theoretically change
79 the form of pleiotropy between resistance and fitness (22).
80 The potential for different evolutionary dynamics in biofilms is clinically significant.
81 Biofilm-associated bacteria are particularly challenging in chronic infections (23, 24),
82 where biofilm adaptations increase virulence and resistance (6, 25). Yet with few
83 exceptions (9, 16, 26) most of the research on the evolution of AMR has been conducted
84 in well-mixed populations [see review (27)] or on agar plates (28), conditions that cannot
85 simulate the effects of biofilms on the evolution of AMR.
86 We experimentally propagated populations of *A. baumannii* exposed either to
87 subinhibitory or increasing concentrations of ciprofloxacin (CIP) over 12 days in biofilm

88 or planktonic conditions to ascertain whether these lifestyles select for different
89 mechanisms of AMR. Rather than focusing on the genotypes of single isolates, which can
90 limit the scope of an analysis, we conducted whole-population genomic sequencing over
91 time to define the dynamics of adaptation and the fitness of certain resistance alleles
92 compared to others in the experiment. We then identified clones with specific genotypes
93 that we linked to fitness and resistance phenotypes. This approach sheds new light on the
94 ways that pathogens adapt to antibiotics while growing in biofilms and has implications
95 for treatment decisions.
96
97 **Results and Discussion**
98 **1. Experimental evolution**
99 Replicate cultures of the susceptible *A. baumannii* strain ATCC 17978 (29, 30) were
100 established under planktonic or biofilm conditions in one of three treatments: i) no
101 antibiotics, ii) sub-inhibitory concentration of the antibiotic ciprofloxacin (CIP) and iii)
102 evolutionary rescue (31) in which CIP concentrations were increased every 72 hours from
103 subinhibitory concentrations to four times the minimum inhibitory concentration (MIC)
104 (Figure 1A). CIP was chosen because of its clinical importance in treating *A. baumannii*
105 (4, 32, 33), its ability to penetrate the biofilm matrix (34) allowing similar efficacy in well
106 mixed and structured populations (35), and because it is not known to stimulate biofilm
107 formation in *A. baumannii* (36). Planktonic populations were serially passaged by daily
108 1:100 dilution while biofilm populations were propagated using a bead model simulating
109 the biofilm life cycle (13, 17, 37). This model selects for bacteria that attach to a 7mm
110 polystyrene bead, form a biofilm, and then disperse to colonize a new bead each day. The
111 transfer population size in biofilm and in planktonic cultures was set to be nearly
112 equivalent at the beginning of the experiment (approximately $1x10^7$ CFU/ml), because

113 population size influences mutation availability and the response to selection (38, 39).
114 The mutational dynamics of three lineages from each treatment were tracked by whole-
115 population genomic sequencing (Figure 1A). We also sequenced 49 single clones isolated
116 from 22 populations at the end of the 12-day experiment to determine mutation linkage.

117 **2. Evolution of CIP resistance**

118 Passaging bacterial populations in the presence of antibiotics will select for fitter, more
119 resistant mutants if the populations do not go extinct. The rate and extent of evolved
120 resistance depends on the strength of antibiotic selection (12, 40), the distribution of
121 fitness effects of mutations that increase resistance to the drug (41), and the population
122 size of replicating bacteria (38, 39). The mode of bacterial growth may alter each of these
123 three variables, in theory generating different dynamics and magnitudes of AMR. In the
124 populations exposed to the increasing concentrations of CIP (the evolutionary rescue),
125 the magnitude of evolved CIP resistance differed substantially between planktonic and
126 biofilm populations. Planktonic populations became approximately 160x more resistant
127 on average than the ancestral clone while the biofilm populations became only 6x more
128 resistant (Figure 1B and Table S1). Planktonic populations also evolved resistance much
129 more rapidly, becoming 10x more resistant after only 24 hours of growth in sub-inhibitory
130 CIP. This level of resistance would have been sufficient for surviving the remainder of
131 the experiment, but MICs continued to increase at each sampling (Figure 1B). The
132 evolution of resistance far beyond the selective requirement indicates that mutations
133 conferring higher resistance also increased fitness in planktonic populations exposed to
134 CIP.
135 In contrast, biofilm-evolved populations evolved under the evolutionary rescue regime
136 acquired much lower levels of resistance (*ca.* 3– 7x the ancestral MIC) and primarily in
137 a single step between days 3 and 4 (Figure 1B). In one notable exception, the MIC of

biofilm population B2 increased ~50x after 3 days of selection in subinhibitory concentrations of CIP (Figure 1B), but then the resistance of this population declined to only 6x higher than the ancestral strain. This dynamic suggested that a mutant conferring high-level resistance rose to intermediate frequency but was replaced by a more fit, yet less resistant, mutant (this possibility is evaluated below).

Lower levels of resistance were observed in populations selected at subinhibitory concentrations of CIP. Biofilm populations were 4x more resistant than the ancestor and planktonic populations were 20x more resistant (Table S1). We can infer that biofilm growth does not select for the high-level resistance seen in planktonic populations, instead favoring mutants that would barely survive at their selected CIP concentration if not grown in biofilms. It is important to note that these MIC measurements were made in planktonic conditions according to the clinical standards (42) and that these values increased greater than 100x when measured in biofilm (Table S2). Our results correspond with studies of clinical isolates in which those producing more biofilm (and likely having adapted in biofilm conditions) were less resistant than non-biofilm-forming isolates (43). However, growth and context-dependent MIC measurements are important (35, 44, 45) and the biofilm environment at least partially protects cells from antibiotic exposure (Table S2). Furthermore, these results demonstrate that exposing bacteria to low levels of antibiotic risks selection for high levels of resistance that can make future treatment more difficult (11).

3. Evolutionary dynamics under CIP treatment

In large bacterial populations (>10^5 cells) growing under strong selection, adaptive mutations conferring beneficial traits (such as antibiotic resistance) will dominate population dynamics (38, 46). Therefore, if a single mutation renders the antibiotic ineffective and provides the highest fitness gain, it would be expected to outcompete all

163 other less fit mutations. Further, the stronger the selection for resistance, the greater the
164 probability of genetic parallelism – or the repeated evolution of the same gene, domain,
165 or even residue providing the best adaptation – among replicate populations (47). Under
166 the conditions of these experiments, approximately 10^6 mutations occur in the first growth
167 cycle and roughly 10^7 mutations arise over the 12 days of selection, leading to a
168 probability of 0.98 that every site in the 4Mbp *A. baumannii* genome experiences a
169 mutation at least once over the course of the 12 day experiment (see Table S3 for details
170 of these calculations). The dramatic differences in the evolved resistance levels of
171 planktonic and biofilm populations suggested distinct genetic causes of resistance
172 resulting from different selective forces in these treatments. We also predicted to observe
173 greater genetic diversity in the biofilm treatments than in the planktonic cultures. Whereas
174 in well-mixed populations one or few genotypes outcompetes the other genotypes and
175 quickly achieves fixation (48), in structured environments like biofilms, multiple
176 beneficial mutations may arise to high frequency and persist, with potentially none fixing
177 owing to spatial structure and niche differentiation (13).
178
179 We conducted whole-population genomic sequencing of three replicates per treatment to
180 identify all contending mutations above a detection threshold of 5% (see Methods). The
181 spectrum of mutations from CIP-treated populations are consistent with expectations
182 from strong positive selection on altered or disrupted coding sequences (see Table 1 for
183 day-12 results and Table S4 for dynamics across the experiment). High nonsynonymous
184 to synonymous mutation ratios were observed in both lifestyles (8.5 in planktonic and 9.7
185 in biofilm). 43% of the total mutations in planktonic and 34% in biofilm were insertions
186 or deletions, which is vastly enriched over typical mutation rates of ~10 SNPs/indel under
187 neutral conditions (49, 50). Roughly 30% of the mutations in CIP-treated populations of

188 either lifestyle occurred in intergenic regions (30% in planktonic-propagated populations
189 and 32% in biofilm ones). Of the intergenic mutations, 72% of the planktonic mutations
190 and 18% of the biofilm mutations occurred in promoters, 5' untranslated regions, or in
191 putative terminators (51).
192
193 As expected from theory, in CIP-selected planktonic populations one or two mutations
194 rapidly outcompeted others and fixed (Figure 2). Selection in biofilms, however,
195 produced fewer selective sweeps and maintained more contending mutations, especially
196 at lower antibiotic concentrations. Overall, across all treatments and timepoints, biofilm-
197 adapted populations were significantly more diverse than the planktonic-adapted
198 populations (Shannon index; Kruskal Wallis, chi-squared = 7.723, p = 0.005), particularly
199 at subinhibitory concentrations of CIP (Figure S1A). Notably, increasing drug
200 concentrations eliminated the differences in diversity between treatments (Figure S1B).
201 Consistent with theory, the spatial structure in biofilms generated more initial standing
202 diversity for selection to act upon. This higher standing diversity is important when
203 considering dosing and when antibiotic exposure may be low (*e.g.* in the external
204 environment or when bound to tissues) (52, 53) because biofilms with more allelic
205 diversity have a greater chance of survival to drug and immune attack (19).
206 In contrast with the data observed in the populations evolving under CIP pressure, drug-
207 free control populations contained no mutations that achieved high frequency during the
208 experiment (Figures 2C and 2D). These results suggest that the ancestral starting clone
209 was already well-adapted to our experimental conditions, perhaps because we had
210 previously propagated the *A. baumannii* ATCC 17978 clone under identical drug-free
211 conditions for 10 days. This preadaptation phase led to the fixation of mutations in three
212 genes (Table S5).

213 **4. Lifestyle determines the selected mechanisms of resistance**

214 *A. baumannii* clinical samples acquire resistance to CIP by two principal mechanisms:
215 modification of the direct antibiotic targets — gyrase A or B and topoisomerase IV — or
216 by the overexpression of efflux pumps reducing the intracellular concentrations of the
217 antibiotic (4). To directly associate genotypes with resistance phenotypes, we sequenced
218 49 clones isolated at the end of the experiment, the majority of which were selected to
219 delineate genotypes in the evolutionary rescue populations (Figures 2F and S2).

220

221 Both the genetic targets and mutational dynamics of selection in planktonic and biofilm
222 environments differed. Mutations disrupting three negative regulators of efflux pumps
223 evolved in parallel across populations exposed to CIP, but mutations in two of these (*adeL*
224 and *adeS*) were nearly exclusive to biofilm clones (Figure 2F). The most common and
225 highest frequency mutations observed in the biofilm populations were in the repressor
226 gene *adeL* (Figures 2F, S2, and Table S6), which regulates AdeFGH, one of three
227 resistance-nodulation-division (RND) efflux pump systems in *A. baumannii* (54-56). In
228 the planktonic lines, the predominant mutations were found in *adeN,* which is a negative
229 regulator of AdeIJK and were mainly IS701 insertions that disrupted the gene (57).

230

231 In biofilm lines, different contending *adeL* mutations were detected in each replicate after
232 24 hours then eventually fixed as CIP concentrations increased (green lines in Figure 2B),
233 sometimes along with a secondary *adeL* mutation. This pattern suggests that altering
234 efflux via *adeL* generates adaptations to the combination of CIP and biofilm. Further,
235 mutants with higher resistance than necessary were evidently maladaptive in the biofilm
236 treatment. For example, *adeN* (found more often in planktonic culture) and *adeS*
237 mutations found simultaneously on day 3 in population B2 (Figure 2) led to a spike in

238 resistance at that timepoint (Figure 1), but these alleles were subsequently outcompeted
239 by *adeL* mutants that were evidently more fit despite lower resistance.
240
241 In contrast to the biofilm populations, all planktonic populations with increasing
242 concentrations of CIP eventually acquired a single high frequency mutation in *gyrA*
243 (S81L), the canonical ciprofloxacin-resistant mutation in DNA gyrase. These *gyrA*
244 mutations evolved in the genetic background containing either an *adeN* mutant or a *pgpB*
245 mutant. *pgpB* is gene that encodes a putative membrane associated lipid phosphatase that
246 is co-regulated by *adeN* (58). Other mutations associated with high levels of resistance
247 affected *parC,* encoding topoisomerase IV, and regulatory regions of two putative
248 transporters, ACX60_RS15145 and ACX60_RS1613, the latter being co-transcribed with
249 the multidrug efflux pump *abeM* (59). Few other mutations exceeded 10% of the total
250 population in the planktonic lines. The rapid fixation of *adeN* and *adeN*-regulated alleles
251 in the planktonic CIP-exposed populations indicate that *adeN* conferred higher fitness
252 than other CIP-resistant mutations at low drug concentrations, as the population size is
253 sufficiently large that these other mutations occurred in the populations (38, 60) and their
254 absence implies they were less fit. Subsequently, at increased concentrations of CIP, on-
255 target mutations in *gyrA* were favored.
256
257 Together, our results demonstrate that bacterial lifestyle influences the evolutionary
258 dynamics and targets of selection of AMR. Loss-of-function mutations in regulators of
259 the *adeFGH* and *adeABC* RND efflux pumps that increased CIP resistance ~4-fold in
260 biofilm populations treated with CIP. Adaptation by planktonic populations exposed to
261 CIP proceeded first by altering the *adeN*-controlled *adeIJK* efflux pump and then by

262 directly altering the targets of the fluoroquinolone, *gyrA* and *parC*, leading to much higher
263 levels of resistance.
264
265 **5. Evolutionary consequences of acquiring resistance**
266 The large population sizes (10^7 – 10^9 cells) and number of generations (~100) in all
267 evolved lines mean that similar mutations very likely arose in each replicate regardless of
268 treatment, meaning that the success of some mutations over others reflects their greater
269 fitness in that condition (Table S3) (38). Yet *de novo* acquired antibiotic resistance is
270 often associated with a fitness cost in the absence of antibiotics (61). The extent of this
271 cost and the ability to compensate for it by secondary mutations (compensatory evolution)
272 is a key attribute determining the spread and maintenance of the resistance mechanism
273 (41, 61-63). A negative correlation between CIP resistance and fitness of resistant
274 genotypes in the absence of antibiotics has been previously described in *Escherichia coli,*
275 suggesting a trade-off between these traits (64-66).
276
277 To determine the relationship between resistance and fitness in the absence of antibiotics
278 in our experiment, we chose 10 clones (5 each from biofilm and planktonic populations,
279 Figures 2F and S2) with different genotypes and putative resistance mechanisms and
280 measured their resistance and fitness phenotypes in both planktonic and biofilm
281 conditions (Figure 3). As expected from the populations (Figure 1B), the biofilm clones
282 much were less resistant in planktonic conditions than those evolved planktonically [MIC
283 = 0.58 mg/L (SEM = 0.13) vs. MIC = 8.53 mg/L (SEM = 1.96), two-tailed t-test: $p < 0.05$,
284 $t = 4.048$, $df = 80$]. However, biofilm-evolved clones were more fit relative to the
285 ancestral strain than the planktonic-evolved clones in the absence of antibiotic (two-tailed
286 t-test: $p = 0.008$, $t = 2.984$ $df = 18$) (Figure 3). Importantly, these fitness measurements

were made in both planktonic and biofilm conditions, demonstrating that even in the lifestyle selection environment they evolved in, the planktonic selected clones were less fit as a result of antibiotic resistance trade-offs. However, one planktonic-evolved clone with mutations in both *gyrA* and *parC* exhibited no significant fitness cost and high levels of resistance. This suggests that, as in *Pseudomonas aeruginosa*, the *parC* mutation may compensate for the cost imposed by *gyrA* mutation (67), an example of sign epistasis (68). Overall, mutants selected in biofilm-evolved populations were less resistant than mutants selected in planktonic populations (Figure 1B) but produced more biofilm (Figure S3) and paid little or no fitness cost in the absence of antibiotics (Figures 3). This cost-free resistance implies that these subpopulations could persist in the absence of drug, limiting the treatment options and demanding new approaches to treat high fitness, resistant pathogens (69).

6. Evolutionary interactions with other antibiotics

When a bacterium acquires resistance to one antibiotic, the mechanism of resistance can also confer resistance to other antibiotics (cross-resistance) or increase the susceptibility to other antibiotics (collateral sensitivity) (70). We tested the MIC of the evolved populations to 23 different antibiotics. We observed changes in susceptibilities to 13 of the 23 antibiotics tested, and these changes were growth mode dependent (Figure 4). For example, planktonic populations exhibited cross resistance to cefpodoxime and ceftazidime but biofilm populations evolved collateral sensitivity to these cephalosporins. Cross-resistance was associated genetically with *adeN, adeS, gyrA* or *pgpB* mutations, and collateral sensitivity was associated with *adeL* mutations. Selection in these environments evidently favors the activation of different efflux pumps or modified targets that have different pleiotropic consequences for multidrug resistance (71).

312 The mechanisms leading to collateral sensitivity are still poorly understood but they
313 depend on the genetic background of the strain, the nature of the resistance mechanisms
314 (72, 73), and the specific physiological context of the cells (74). In *A. baumannii*, each
315 RND efflux pump is specific for certain classes of antibiotics (74-76). Similar to our
316 results (Figure 4), Yoon and collaborators demonstrated that efflux pumps AdeABC and
317 AdeIJK, regulated by *adeS* and *adeN* respectively, increased the resistance level to some
318 beta-lactams when overexpressed (77). However, production of AdeFGH, the efflux
319 pump regulated by *adeL,* decreased resistance to some beta-lactams and other families of
320 antibiotics or detergents by an unknown mechanism (74, 77). Even with the previously
321 demonstrated relationship between active proton pumps and the increase of sensitivity to
322 beta-lactams in *P. aeruginosa* (78), a clearer understanding of the physiological basis of
323 collateral sensitivity is important for using these features to control AMR evolution.
324 Nevertheless, treatments that exploit collateral sensitivity have been proposed to
325 counteract the evolution of resistant populations both in bacteria (79-81) and in cancer
326 (82). Here, we show that bacterial lifestyle influences these selective pressures and that
327 biofilm growth, commonly thought to broaden resistance, may actually generate collateral
328 sensitivity during treatment with CIP and potentially other fluoroquinolones.
329

330 **7. Clinical relevance**

331 Our results demonstrate that the mode of growth determines both the mechanism of
332 evolved resistance and the spectrum of sensitivity to other families of antibiotics.
333 Additionally, we argue that the mutations selected in our experimental conditions also
334 play an important role in clinical samples, as fluoroquinolone resistance mediated by
335 plasmids in *A. baumannii* appears to be rare (83). The mutations S81L in *gyrA* and S80L
336 *in parC* acquired by the sensitive ATCC 17978 strain used in this study have been

337 reported worldwide as the primary mechanism conferring high levels of resistance to
338 fluorquinolones in clinical isolates (84-86).
339
340 In addition to the on-target mechanisms of resistance through gyrase or topoisomerase
341 mutations, *A. baumannii* isolates acquire comparatively moderate levels of
342 fluoroquinolone resistance by modifications in the RND efflux pumps. These RND efflux
343 pumps have overlapping yet differing substrate profiles and may act synergistically in
344 increasing the resistance level (Table S7) (54, 55, 87, 88). In our experiment, all biofilm
345 and planktonic populations and nearly all clones isolated had acquired mutations in at
346 least one of the three regulators of the RND efflux pumps (*adeL, adeS, adeN*) or in a gene
347 regulated by one of these regulators (*pgpB*). Mutations in *adeL* upregulate the expression
348 of the RND efflux pump AdeFGH (Figure 2, Table S7), leading to a multidrug resistant
349 phenotype in clinical isolates (54-56, 74). Further, AdeL-AdeFGH has been previously
350 described as a clinically-relevant resistance factor and the genes are often highly
351 expressed in clinical isolates, indicating possible adaptation to the biofilm lifestyle (54,
352 55). Relevant to the selective conditions in our biofilm treatment, overexpression of *adeG*
353 is predicted to enhance transport of acylated homoserine lactones, including quorum-
354 sensing autoinducers, increasing both drug resistance and biofilm formation (89, 90).
355 However, in clinical isolates, overexpression of the AdeFGH pump is less common than
356 the AdeIJK efflux pump that is regulated by *adeN* (77, 88). Interestingly, AdeIJK
357 contributes to resistance to biocides, hospital disinfectants, and to both intrinsic and
358 acquired antibiotic resistance in *A. baumannii* (87, 88) and may decrease biofilm
359 formation (77). Perhaps more importantly, this study demonstrates that the
360 overexpression of RND efflux pumps may produce little fitness cost in *A. baumannii*, as

361 has previously been demonstrated in both *P. aeruginosa* and *Neisseria gonorrhoeae* (91,
362 92).
363
364 **Conclusions**
365 We used experimental evolution of the opportunistic pathogen *A. baumannii* in both well-
366 mixed and biofilm conditions to examine how lifestyle influences the dynamics, genetic
367 mechanisms, and direct and pleiotropic effects of resistance to a common antibiotic.
368 Experimental evolution is a powerful method of screening naturally arising genetic
369 variation for mutants that are the best fit in any condition (38, 93, 94). When population
370 sizes are large and reproductive rates are rapid, as they were here, the probability that all
371 possible single-step mutations that can increase both resistance and fitness occurred in
372 each population becomes nearly certain. The enrichment of the few mutations reported
373 here as well as their ordering with increasing CIP concentrations demonstrates that these
374 are the best such mutations in this *A. baumannii* strain and set of environmental
375 conditions, and the prevalence of some of these mutations in clinical samples illustrates
376 that they too may have been exposed to selection in biofilms. Likewise, the absence of
377 other mutations reported in shotgun mutant screens of resistance in *A. baumannii* (95)
378 means that these missing mutants engendered less resistance, fitness, or both.
379 Experimental evolution can be used in a wide variety of conditions, including in
380 susceptible hosts, to identify the most probable mutants that would be selected for under
381 antibiotic treatment and ultimately to enable forecasting of treatment outcomes, including
382 the diversification of the pathogen population and the likelihood of collateral sensitivity
383 or cross-resistance. Furthermore, knowledge of the prevailing lifestyle of the pathogen
384 population may be critically important for treatment design. Most infections are likely
385 caused by surface-attached populations (23, 24), and some treatments include cycling

386 antibiotics that promote biofilm as a primary response. For example, tobramycin is used
387 for treating *P. aeruginosa* in cystic fibrosis patients (96) and promotes biofilm formation
388 (97, 98), wherein the evolution of antibiotic resistance without a detectable fitness cost
389 may arise during treatment. But the more diverse, biofilm-adapted lineages in our
390 experiments revealed a striking vulnerability to cephalosporins, which could provide a
391 new strategy for treatment. Broader still, conventional wisdom has long held that the
392 relationship between resistance and fitness is antagonistic, and that the efficacy of many
393 antimicrobials is aided by a severe fitness cost of resistance (27, 61, 69). This study
394 demonstrates that the form of the relationship between fitness and resistance can be
395 altered by the mode of growth, whereby biofilms can align resistance and fitness traits.
396 Therefore, a clearer picture of how the fitness landscape of various resistance pathways
397 depends on the environment, including both drug type and growth mode, and could be a
398 valuable forecasting tool in our armament to stem the rising AMR tide.
399
400 **Methods**
401 **Experimental evolution**
402 Before the start of the antibiotic evolution experiment, we planktonically propagated one
403 clone of the susceptible *A. baumannii* strain ATCC 17978-mff (29, 30) in a modified M9
404 medium (referred to as $M9^{+}$) containing 0.37 mM $CaCl_2$, 8.7 mM $MgSO_4$, 42.2 mM
405 Na_2HPO_4, 22 mM KH2PO4, 21.7mM NaCl, 18.7 mM NH_4Cl and 0.2 g/L glucose and
406 supplemented with 20 mL/L MEM essential amino acids (Gibco 11130051), 10 mL/L
407 MEM nonessential amino acids (Gibco 11140050), and 10 mL each of trace mineral
408 solutions A, B, and C (Corning 25021-3Cl). This preadaptation phase was conducted in
409 the absence of antibiotics for 10 days (*ca.* 66 generations) with a dilution factor of 100
410 per day.

411 After the ten days of preadaptation to $M9^+$ medium, we selected a single clone and
412 propagated for 24 hours in $M9^+$ in the absence of antibiotic. We then subcultured this
413 population into twenty replicate populations. Ten of the populations (5 planktonic and 5
414 biofilm) were propagated every 24 hours in constant subinhibitory concentrations of CIP,
415 0.0625 mg/L, which corresponds to 0.5x the minimum inhibitory concentration (MIC).
416 After 72 hours under subinhibitory concentrations of CIP, the populations were exposed
417 to two different antibiotic regimes for 9 more days, either constant subinhibitory
418 concentrations of CIP or increasing concentrations of CIP (called the evolutionary
419 rescue). For the latter, we doubled the CIP concentrations every 72 hours until 4x MIC.
420 As a control, the 20 remaining populations were propagated in the absence of CIP (Figure
421 1).
422
423 We propagated the populations into fresh media every 24 hours as described by Turner *et*
424 *al.* 2018 (37). For planktonic populations, we transferred a 1:100 (50 µl into 5 ml of $M9^+$)
425 dilution, which corresponded to 6.64 generations per day. For biofilm populations, we
426 transferred a polystyrene bead (Polysciences, Inc., Warrington. PA) to fresh media
427 containing three sterile beads. We rinsed each bead in PBS before the transfer, therefore
428 reducing the transfer of planktonic cells. Each day we alternated between black and white
429 marked beads, ensuring that the bacteria were growing on the bead for 24 hours, which
430 corresponds to approximately 6 to 7.5 generations/day (13, 37). For the experiment with
431 increasing concentrations of antibiotics, we froze a sample of each bacterial population
432 on days 1, 3, 4, 6, 7, 9, 10 and 12. In the experiment with constant exposure to
433 subinhibitory concentrations of antibiotics, we froze the populations on days 1, 3, 4, 9,
434 and 12. We froze the control populations at days 1, 4, 9, and 12. For planktonic
435 populations, we froze 1 mL of culture with 9% of DMSO. For freezing the biofilm

436 populations, we sonicated the beads in 1 mL of PBS with a probe sonicator and
437 subsequently froze with 9% DMSO.
438
439 **Phenotypic characterization: antimicrobial susceptibility and biofilm formation**
440 We determined the MIC of CIP of the whole population by broth microdilution in $M9^{+}$
441 according to the Clinical and Laboratory Standards Institute guidelines (42), in which
442 each bacterial sample was tested to 2-fold-increasing concentration of CIP from 0.0625
443 to 64 mg/L. To obtain a general picture of the resistance profiles we determined the MIC
444 to 23 antibiotics (amikacin, ampicillin, ampicillin/sulbactam, aztreonam, cefazolin,
445 cefepime, cephalothin, meropenem, ertapenem, cefuroxime, gentamicin, CIP,
446 piperacillin/tazobactam, cefoxitin, trimethoprim/sulfamethoxazole, cefpodoxime,
447 ceftazidime, tobramycin, tigecycline, ticarcillin/clavulanic acid, ceftriaxone and
448 tetracycline) by broth microdilution in commercial microtiter plates following the
449 instructions provided by the manufacturers (Sensititre GN3F, Trek Diagnostics Inc.,
450 Westlake, OH). We tested the MIC at days 1, 3, 4, 6, 7, 9, 10 and 12 for the populations
451 propagated under increasing concentrations of antibiotic, and at days 1 and 12 for the
452 subinhibitory and non-antibiotic treatments. For the CIP-MICs, we used *Pseudomonas*
453 *aeruginosa* PAO1 in Mueller Hinton broth as a control. No differences in the MICs were
454 found between Mueller Hinton and $M9^{+}$ or if measuring the MIC in 96 well-plate or in 5
455 ml tubes, which are the experimental conditions. Each MIC was performed in triplicate.
456 The CIP was provided by Alfa Aesar (Alfa Aesar, Wardhill, MA).
457
458 We estimated the biofilm formation of the selected clones using a modification of the
459 previously described protocol (99). We resurrected each clone in 5 mL of $M9^{+}$ containing
460 0.5 mg/L of CIP and grew them for 24 hours. For each strain, we transferred 50 µl into

15 ml of $M9^+$. We tested 200 µl of the previous dilution of each clone to 4 different subinhibitory CIP concentrations (0 mg/L, 0.01 mg/L, 0.03 mg/L and 0.0625 mg/L). After 24 hours of growing at 37ºC, we measured population sizes by optical density (OD) at 590nm ($OD_{Populations}$). Then, we added 250 µl of 0.1% crystal violet and incubated at room temperature for 15 minutes. After washing the wells and drying for 24 hours, we added 250 µl 95% EtOH solution (95% EtOH, 4.95% dH2O, 0.05% Triton X-100) to each well and incubated for 15 minutes and biofilm formation was measured by the OD at 590nm ($OD_{Biofilm}$). Biofilm formation was corrected by population sizes ($OD_{Biofilm}/OD_{Population}$). Results are the average of three experiments (Figure S3).

Fitness measurement

We selected 5 biofilm and 5 planktonic clones at the end of the evolutionary rescue experiment (Figure 2) and determined the fitness by directly competing the ancestral strain and the evolved clone variants both in planktonic and in biofilm conditions (Figure 3) (37). We revived each clone from a freezer stock in $M9^+$ for 24 hours. We maintained the same evolutionary conditions to revive the clones, adding 3 beads and/or CIP to the broth when required. After 24 hours, we added equal volume of the clones and the ancestors in $M9^+$ in the absence of antibiotics. For planktonic populations, we mixed 25 µl of each competitor in 5 ml of $M9^+$. For biofilm competitions, we sonicated one bead per competitor in 1 ml of PBS and mixed in 5 ml of $M9^+$ containing 3 beads. The mix was cultured at 37ºC for 24 hours. We plated at time zero and after 24 hours. For each competition, we plated aliquots onto nonselective tryptic soy agar and tryptic soy agar containing CIP. Selection rate (r) was calculated as the difference of the Malthusian parameters for the two competitors: $r = (\ln(\text{CIP resistant}_{d=1}/\text{CIP resistant}_{d=0}))/(\ln(\text{CIP susceptible}_{d=1}/\text{CIP susceptible}_{d=0}))/\text{day}$ (100). Susceptible populations were calculated as

the difference between the total populations (number of colonies/mL growing on the nonselective plates) and the resistant fraction (number of colonies/mL growing on the plates containing CIP). As a control for calculating the correct ratio of susceptible vs. resistant populations, we replica plated 50 to 100 colonies from the nonselective plates onto plates containing CIP as previously described (101). Results are the average of three to five independent experiments.

Genome sequencing

We sequenced whole populations of three evolving replicates per treatment. We sequenced the populations at days 1, 3, 4, 6, 7, 9, 10, and 12 of the populations under increasing concentrations of CIP (hereafter populations P1, P2, P3 and B1, B2, B3 for planktonic and biofilm populations) and at days 1, 4, 9, and 12 of the populations under subinhibitory concentration and no antibiotic treatments. In addition, we selected 49 clones for sequencing at the end of the experiment (Figure 2F). 12 of the clones were recovered from the populations propagated in the absence of the antibiotic, 12 clones from the subinhibitory concentrations of CIP treatment and 25 were isolated from the increasing concentrations of antibiotic. We revived each population or clone from a freezer stock in the growth conditions under which they were isolated (*i.e.* the same CIP concentration which they were exposed to during the experiment) and grew for 24 hours. DNA was extracted using the Qiagen DNAeasy Blood and Tissue kit (Qiagen, Hiden, Germany). The sequencing library was prepared as described by Turner and colleagues (37) according to the protocol of Baym *et al.* (102), using the Illumina Nextera kit (Illumina Inc., San Diego, CA) and sequenced using an Illumina NextSeq500 at the Microbial Genome Sequencing center (http://micropopbio.org/sequencing.html).

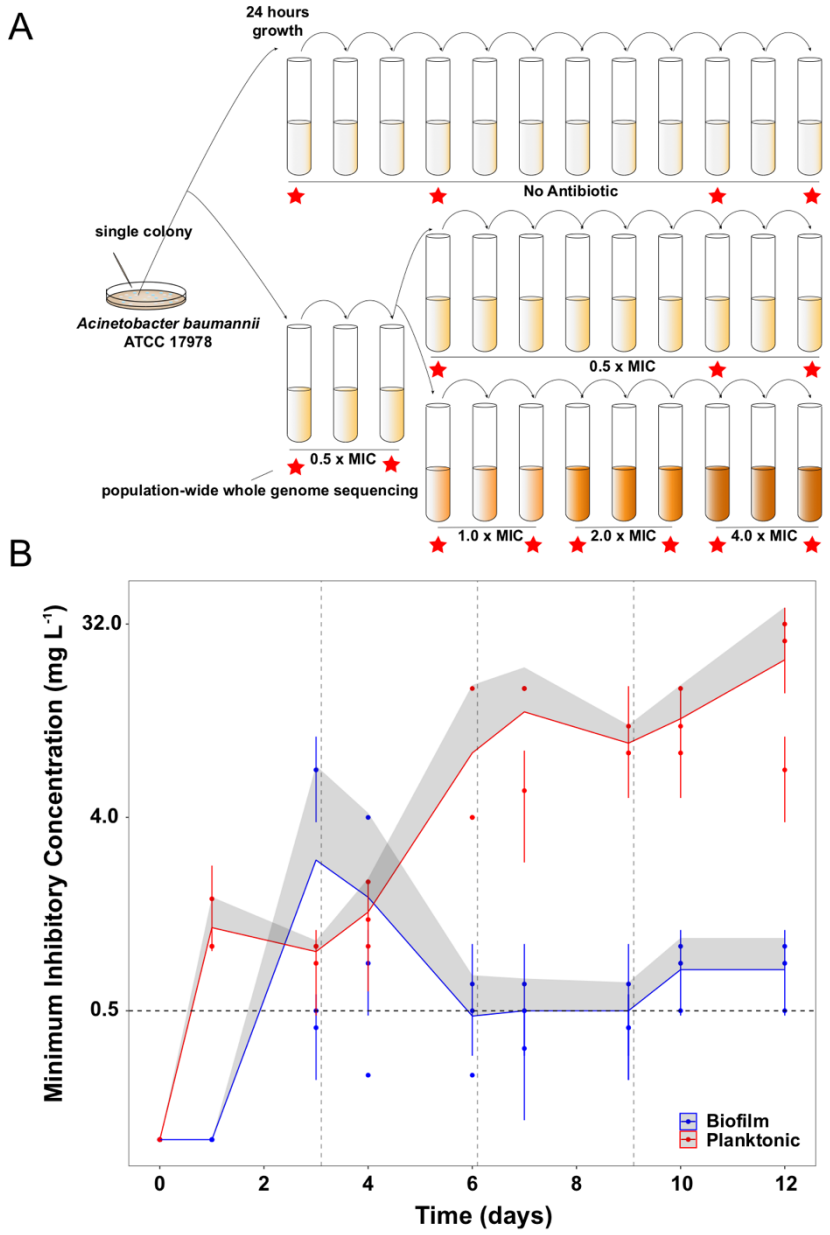
511 **Data processing**

512 All sequences were first quality filtered and trimmed with the Trimmomatic software
513 v0.36 (103) using the criteria: LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20
514 MINLEN:70. Variants were called with the breseq software v0.31.0 (104) using the
515 default parameters and the -p flag when required for identifying polymorphisms in
516 populations. The reference genome used for variant calling was downloaded from the
517 NCBI RefSeq database using the 17-Mar-2017 version of *A. baumannii* ATCC 17978-
518 mff complete genome (GCF_001077675.1). In addition to the chromosome
519 NZ_CP012004 and plasmid NZ_CP012005 sequences, we added two additional plasmid
520 sequences to the reference genome that are known to be present in our working strain of
521 *A. baumannii* ATCC 17978-mff: NC009083, NC_009084. Mutations were then manually
522 curated and filtered to remove false positives. Mutations were filtered if the gene was
523 found to contain a mutation when the ancestor sequence was compared to the reference
524 genome or if a mutation never reached a cumulative frequency of 10% across all replicate
525 populations. Diversity measurements were made in R using the Shannon index
526 considering the presence, absence, and frequency of alleles. Significant differences
527 between biofilm and planktonic populations were determined by the Kruskal Wallis test.
528 Filtering, mutational dynamics, and plotting were done in R v3.4.4 (www.r-project.org)
529 with the packages ggplot2 v2.2.1 (https://CRAN.R-project.org/package=ggplot2), dplyr
530 v0.7.4 (https://CRAN.R-project.org/package=dplyr), and vegan v2.5-1
531 (https://github.com/vegandevs/vegan).

532

533 **Data Availability**

534 R code for filtering and data processing can be found here:
535 https://github.com/sirmicrobe/U01_allele_freq_code. All sequences were deposited into
536 NCBI under the Biosample accession numbers SAMN09783599-SAMN09783682.
537
538 **Acknowledgments**
539 We thank Caroline B. Turner for helpful discussions and proofreading of the paper and
540 Allison L. Welp for laboratory assistance. This research was supported by NIH
541 U01AI124302-01.



542

543 **Figure 1. Experimental design (A) and dynamics of evolved resistance levels**

544 **during the evolutionary rescue experiment (B).**

545 A) A single clone of *A. baumannii* ATCC 17978 was propagated both in biofilm and

546 planktonic conditions for 12 days under no antibiotics (top), subinhibitory concentrations

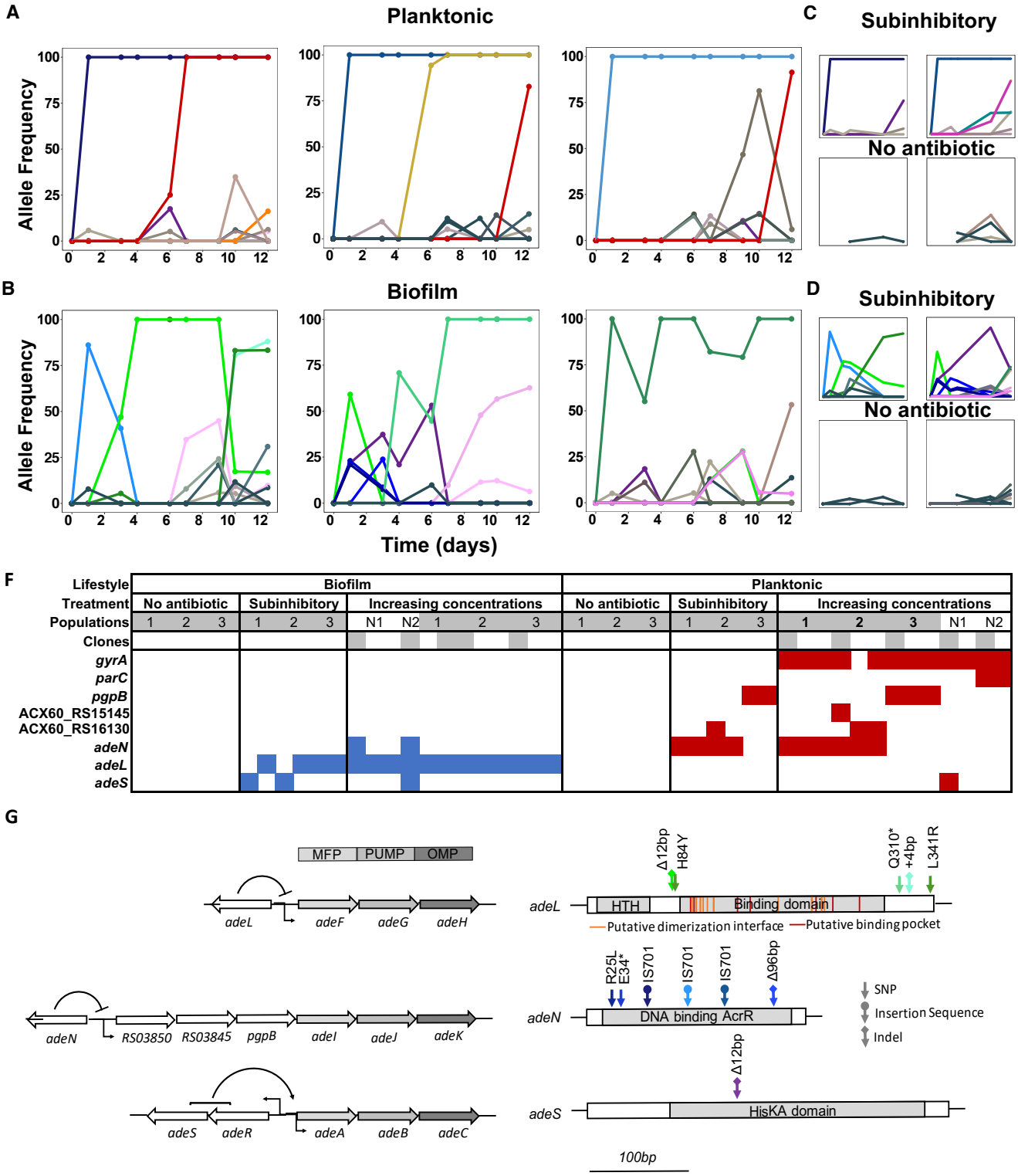
547 of CIP (0.0625 mg/L = 0.5x MIC) (middle) or in increasing concentrations of CIP

548 (bottom). For the latter, termed evolutionary rescue, the concentration of CIP was doubled

549 from 0.5 x MIC to 4.0 x MIC every 72 hours. As a control, five populations of *A.*

550 *baumannii* ATCC 17978 were propagated in biofilm and five in planktonic in the absence

551 of antibiotics. We estimated the MICs to CIP and froze the populations for sequencing
552 before and after doubling the antibiotic concentrations (red stars). B) MICs (mg/L) of CIP
553 were measured for replicate populations during the evolutionary rescue. The red and blue
554 points represent the MICs of three populations propagated in planktonic or biofilm,
555 respectively, with the 95% CI represented by the error bars. The red and blue lines
556 represent the grand mean of the three planktonic and biofilm populations, respectively,
557 with the upper 95% CI indicated by the grey shaded area. Horizontal dashed line indicates
558 the highest CIP exposure during the experiment (4x MIC) and vertical lines indicate time
559 when CIP concentration was doubled.



560

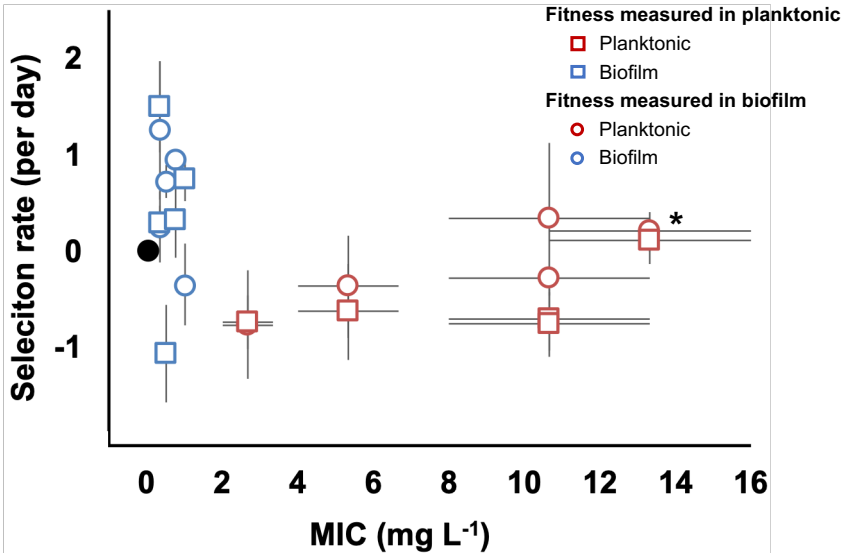
561 **Figure 2. Lifestyle-dependent mutations and dynamics under increasing CIP**

562 **selection.**

563 Mutation frequencies in planktonic (A and C) and biofilm populations (B and D) over

564 time as determined by population-wide whole genome sequencing. A) and B) show the

565 mutation frequencies obtained under increasing concentrations of CIP. From left to right:
566 P1, P2 and P3 in A) and B1, B2 and B3 in B). C) and D) show the mutation frequencies
567 obtained under the subinhibitory (top) and no antibiotic (bottom) treatments. Mutations
568 in the same gene share a color. Blue: *adeN* or genes regulated by *adeN*; green: *adeL*; gold:
569 MFS putative transporter ACX60_RS15145; purple: *adeS*; pink: *sohB*; red: *gyrA;* and
570 orange: *parC*. Grey and brown colors indicate genes potentially unrelated to adaptation
571 to CIP. F) Mutated genes in the sequenced clones. Each column represents one clone.
572 Grey shading of populations indicates whole population sequencing and N1 and N2
573 indicate populations where only clones were sequenced. Grey shaded clones were used
574 for MIC and fitness estimations. Blue and red indicate SNPs in biofilm and planktonic
575 growing populations respectively. For all SNPs identified in the 49 clones, see Figure S2
576 and Table S6. G) The genetic organization of the RND efflux pumps is shown on the left.
577 MFP and OMP denote membrane fusion protein and outer membrane protein
578 respectively. All mutations found in the RND regulators are shown on the right.

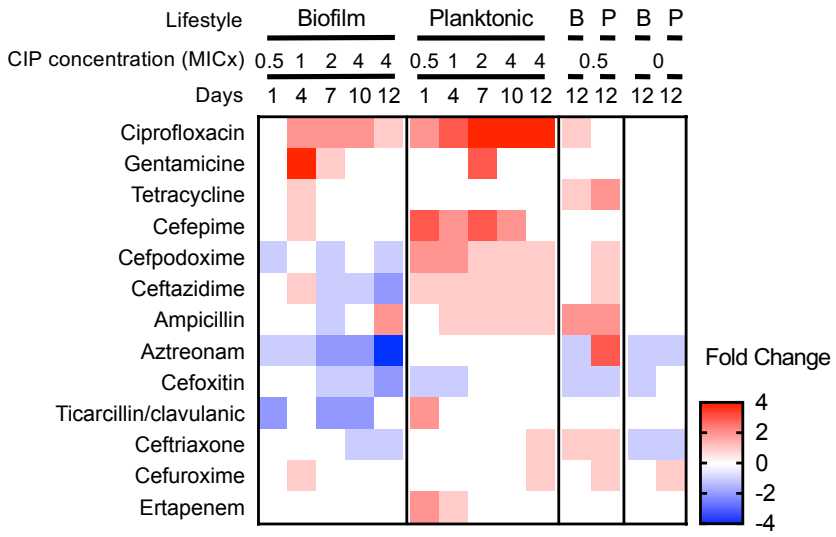


579

580 **Figure 3. Evolved trade-off between resistance level and fitness.**

581 Relative fitness (average ± SEM) of 10 evolved clones from the evolutionary rescue
582 experiment compared to the ancestor and their MICs (mg/L) to CIP. Fitness was measured
583 in both planktonic (squares) and biofilm (circles) conditions. Biofilm-evolved mutants
584 were more fit than the ancestor in the absence of CIP, either in planktonic (two-tailed t-
585 test: $p = 0.1675$, $t = 1.518$ $df = 8$) or in biofilm conditions (two-tailed t-test: $p = 0.1674$, t
586 $= 1.519$ $df = 8$). MICs were estimated in planktonic conditions. Black dot represents the
587 ancestral clone. *Denotes the clone with *gyrA* and *parC* mutations.

588



589

590 **Figure 4. Collateral sensitivities and cross resistances to various antibiotics.**

591 Heat map showing the relative changes in antimicrobial susceptibility to 13 of the 23
592 antibiotics tested in the evolved populations (those not shown had no changes). Results
593 shown are the median values of the fold change in the evolved populations compared to
594 the ancestral strain. For subinhibitory and no-antibiotic treatments, only day 12 is shown.

	Increasing concentrations		**Subinhibitory concentrations**	
	Planktonic	**Biofilm**	**Planktonic**	**Biofilm**
Total mutations	30	40	6	16
Nonsynonymous/Synonymous[b]	8.5	9.67	2/0	6
Intergenic	8	11	0	4
Nonsynonymous	9	13	2	6
Percent intergenic mutations[b]	0.32	0.29	0	0.25

595

596 **Table 1. Mutation spectrum of different selective environments.** Attributes of the
597 contending mutations during the 12 days of the evolution experiment. [a]Results from the
598 last day of the experimental evolution. [b]Accounting for all unique mutations detected after
599 filtering (see methods). For mutation dynamics over time, see Table S3.

References

1. O´Neill J (2016) TACKLING DRUG-RESISTANT INFECTIONS GLOBALLY: FINAL REPORT AND RECOMMENDATIONS. *Review on Antimicrobal Resistance*.
2. Martínez JL (2008) Antibiotics and Antibiotic Resistance Genes in Natural Environments. *Science* 321(5887):365-367.
3. Ventola CL (2015) The antibiotic resistance crisis: part 1: causes and threats. *P T* 40(4):277-283.
4. Doi Y, Murray GL, & Peleg AY (2015) Acinetobacter baumannii: evolution of antimicrobial resistance-treatment options. *Semin Respir Crit Care Med* 36(1):85-98.
5. Asif M, Alvi IA, & Rehman SU (2018) Insight into Acinetobacter baumannii: pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. *Infect Drug Resist* 11:1249-1260.
6. Eze EC, Chenia HY, & El Zowalaty ME (2018) Acinetobacter baumannii biofilms: effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. *Infect Drug Resist* 11:2277-2299.
7. Hoiby N, Bjarnsholt T, Givskov M, Molin S, & Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35(4):322-332.
8. Olsen I (2015) Biofilm-specific antibiotic tolerance and resistance. *Eur J Clin Microbiol Infect Dis* 34(5):877-886.
9. Ahmed MN, Porse A, Sommer MOA, Hoiby N, & Ciofu O (2018) Evolution of antibiotic resistance in biofilm and planktonic P. aeruginosa populations exposed to sub-inhibitory levels of ciprofloxacin. *Antimicrob Agents Chemother*.
10. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, & Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47(1):317-323.
11. Wistrand-Yuen E*, et al.* (2018) Evolution of high-level resistance during low-level antibiotic exposure. *Nat Commun* 9(1):1599.
12. Andersson DI & Hughes D (2014) Microbiological effects of sublethal levels of antibiotics. *Nat Rev Microbiol* 12(7):465-478.
13. Traverse CC, Mayo-Smith LM, Poltak SR, & Cooper VS (2013) Tangled bank of experimentally evolved Burkholderia biofilms reflects selection during chronic infections. *Proc Natl Acad Sci U S A* 110(3):E250-259.
14. Cooper VS, Staples RK, Traverse CC, & Ellis CN (2014) Parallel evolution of small colony variants in Burkholderia cenocepacia biofilms. *Genomics* 104(6 Pt A):447-452.
15. Ellis CN, Traverse CC, Mayo-Smith L, Buskirk SW, & Cooper VS (2015) Character displacement and the evolution of niche complementarity in a model biofilm community. *Evolution* 69(2):283-293.
16. France MT, Cornea A, Kehlet-Delgado H, & Forney LJ (2019) Spatial structure facilitates the accumulation and persistence of antibiotic-resistant mutants in biofilms. *Evolutionary Applications* 12(3):498-507.
17. Poltak SR & Cooper VS (2011) Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *ISME J* 5(3):369-378.
18. Frenkel EM*, et al.* (2015) Crowded growth leads to the spontaneous evolution of semistable coexistence in laboratory yeast populations. *Proc Natl Acad Sci U S A* 112(36):11306-11311.
19. Fux CA, Costerton JW, Stewart PS, & Stoodley P (2005) Survival strategies of infectious biofilms. *Trends Microbiol* 13(1):34-40.
20. Arnold SJ (1992) Constraints on phenotypic evolution. *Am Nat* 140 Suppl 1:S85-107.
21. Pavličev M & Cheverud JM (2015) Constraints Evolve: Context Dependency of Gene Effects Allows Evolution of Pleiotropy. *Annual Review of Ecology, Evolution, and Systematics* 46(1):413-434.
22. Saltz JB, Hessel FC, & Kelly MW (2017) Trait Correlations in the Genomics Era. *Trends Ecol Evol* 32(4):279-290.
23. Wolcott RD (2017) Biofilms cause chronic infections. *J Wound Care* 26(8):423-425.

24. Wolcott RD, *et al.* (2010) Chronic wounds and the medical biofilm paradigm. *J Wound Care* 19(2):45-46, 48-50, 52-43.
25. Geisinger E & Isberg RR (2015) Antibiotic modulation of capsular exopolysaccharide and virulence in Acinetobacter baumannii. *PLoS Pathog* 11(2):e1004691.
26. Ridenhour BJ, *et al.* (2017) Persistence of antibiotic resistance plasmids in bacterial biofilms. *Evol Appl* 10(6):640-647.
27. Hughes D & Andersson DI (2017) Evolutionary Trajectories to Antibiotic Resistance. *Annu Rev Microbiol*.
28. Baym M, *et al.* (2016) Spatiotemporal microbial evolution on antibiotic landscapes. *Science* 353(6304):1147-1151.
29. Piechaud M & Second L (1951) [Studies of 26 strains of Moraxella Iwoffi]. *Annales de l'Institut Pasteur* 80(1):97-99.
30. Baumann P, Doudoroff M, & Stanier RY (1968) A study of the Moraxella group. II. Oxidative-negative species (genus Acinetobacter). *J Bacteriol* 95(5):1520-1541.
31. Bell G & Gonzalez A (2009) Evolutionary rescue can prevent extinction following environmental change. *Ecol Lett* 12(9):942-948.
32. Lopes BS & Amyes SG (2013) Insertion sequence disruption of adeR and ciprofloxacin resistance caused by efflux pumps and gyrA and parC mutations in Acinetobacter baumannii. *Int J Antimicrob Agents* 41(2):117-121.
33. Ardebili A, Lari AR, & Talebi M (2014) Correlation of ciprofloxacin resistance with the AdeABC efflux system in Acinetobacter baumannii clinical isolates. *Annals of laboratory medicine* 34(6):433-438.
34. Tseng BS, *et al.* (2013) The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environ Microbiol* 15(10):2865-2878.
35. Kirby AE, Garner K, & Levin BR (2012) The relative contributions of physical structure and cell density to the antibiotic susceptibility of bacteria in biofilms. *Antimicrob Agents Chemother* 56(6):2967-2975.
36. Aka ST & Haji SH (2015) Sub-MIC of antibiotics induced biofilm formation of Pseudomonas aeruginosa in the presence of chlorhexidine. *Braz J Microbiol* 46(1):149-154.
37. Turner CB, Marshall CW, & Cooper VS (2018) Parallel genetic adaptation across environments differing in mode of growth or resource availability. *Evolution Letters* 2(4):355-367.
38. Cooper VS (2018) Experimental Evolution as a High-Throughput Screen for Genetic Adaptations. *mSphere* 3(3).
39. Salverda MLM, Koomen J, Koopmanschap B, Zwart MP, & de Visser J (2017) Adaptive benefits from small mutation supplies in an antibiotic resistance enzyme. *Proc Natl Acad Sci U S A* 114(48):12773-12778.
40. Oz T, *et al.* (2014) Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. *Mol Biol Evol* 31(9):2387-2401.
41. Maclean RC, Hall AR, Perron GG, & Buckling A (2010) The evolution of antibiotic resistance: insight into the roles of molecular mechanisms of resistance and treatment context. *Discov Med* 10(51):112-118.
42. CLSI (2017) CLSI. Clinical and Laboratory Standars Institute. Performance Standards for Antimicrobial Susceptibility Testing-Seventeenth edition: Approved Standar M100-S17. (CLSI, Wayne, PA, USA).
43. Wang Y-C, *et al.* (2018) Biofilm formation is not associated with worse outcome in Acinetobacter baumannii bacteraemic pneumonia. *Scientific Reports* 8(1):7289.
44. Borriello G, *et al.* (2004) Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms. *Antimicrob Agents Chemother* 48(7):2659-2664.
45. Hill D, *et al.* (2005) Antibiotic Susceptibilities of Pseudomonas aeruginosa Isolates Derived from Patients with Cystic Fibrosis under Aerobic, Anaerobic, and Biofilm Conditions. *Journal of Clinical Microbiology* 43(10):5085-5090.

46. Barrick JE & Lenski RE (2013) Genome dynamics during experimental evolution. *Nature reviews. Genetics* 14:827-839.
47. Bolnick DI, Barrett RDH, Oke KB, Rennison DJ, & Stuart YE (2018) (Non)Parallel Evolution. *Annual Review of Ecology, Evolution, and Systematics* 49(1):303-330.
48. Barrick JE*, et al.* (2009) Genome evolution and adaptation in a long-term experiment with Escherichia coli. *Nature* 461:1243-1247.
49. Dillon MM, Sung W, Sebra R, Lynch M, & Cooper VS (2017) Genome-Wide Biases in the Rate and Molecular Spectrum of Spontaneous Mutations in Vibrio cholerae and Vibrio fischeri. *Mol Biol Evol* 34(1):93-109.
50. Lynch M*, et al.* (2016) Genetic drift, selection and the evolution of the mutation rate. *Nature Reviews Genetics* 17:704.
51. Kröger C*, et al.* (2018) The primary transcriptome, small RNAs and regulation of antimicrobial resistance in Acinetobacter baumannii ATCC 17978. *Nucleic Acids Res* 46(18):9684-9698.
52. Khan GA, Berglund B, Khan KM, Lindgren PE, & Fick J (2013) Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities--a study in Pakistan. *PLoS One* 8(6):e62712.
53. Baquero F, Negri MC, Morosini MI, & Blazquez J (1998) Antibiotic-selective environments. *Clin Infect Dis* 27 Suppl 1:S5-11.
54. Coyne S, Rosenfeld N, Lambert T, Courvalin P, & Perichon B (2010) Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in Acinetobacter baumannii. *Antimicrob Agents Chemother* 54(10):4389-4393.
55. Fernando D, Zhanel G, & Kumar A (2013) Antibiotic resistance and expression of resistance-nodulation-division pump- and outer membrane porin-encoding genes in Acinetobacter species isolated from Canadian hospitals. *Can J Infect Dis Med Microbiol* 24(1):17-21.
56. Pournaras S, Koumaki V, Gennimata V, Kouskouni E, & Tsakris A (2016) In Vitro Activity of Tigecycline Against Acinetobacter baumannii: Global Epidemiology and Resistance Mechanisms. *Adv Exp Med Biol* 897:1-14.
57. Li X-Z, Elkins CA, & Zgurskaya HI (2016) *Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications* (Springer).
58. Hua X, Chen Q, Li X, & Yu Y (2014) Global transcriptional response of Acinetobacter baumannii to a subinhibitory concentration of tigecycline. *Int J Antimicrob Agents* 44(4):337-344.
59. Su XZ, Chen J, Mizushima T, Kuroda T, & Tsuchiya T (2005) AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother* 49(10):4362-4364.
60. Desai MM & Fisher DS (2007) Beneficial mutation selection balance and the effect of linkage on positive selection. *Genetics* 176(3):1759-1798.
61. Vogwill T & MacLean RC (2015) The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl* 8(3):284-295.
62. Moore FB, Rozen DE, & Lenski RE (2000) Pervasive compensatory adaptation in Escherichia coli. *Proc Biol Sci* 267(1442):515-522.
63. Zhao X & Drlica K (2002) Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window. *J Infect Dis* 185(4):561-565.
64. Marcusson LL, Frimodt-Moller N, & Hughes D (2009) Interplay in the selection of fluoroquinolone resistance and bacterial fitness. *PLoS Pathog* 5(8):e1000541.
65. Huseby DL*, et al.* (2017) Mutation Supply and Relative Fitness Shape the Genotypes of Ciprofloxacin-Resistant Escherichia coli. *Mol Biol Evol* 34(5):1029-1039.
66. Basra P*, et al.* (2018) Fitness tradeoffs of antibiotic resistance in extra-intestinal pathogenic Escherichia coli. *Genome Biol Evol*.
67. Kugelberg E, Lofmark S, Wretlind B, & Andersson DI (2005) Reduction of the fitness burden of quinolone resistance in Pseudomonas aeruginosa. *J Antimicrob Chemother* 55(1):22-30.

68. Sackman AM & Rokyta DR (2018) Additive Phenotypes Underlie Epistasis of Fitness Effects. *Genetics* 208(1):339-348.
69. Baym M, Stone LK, & Kishony R (2016) Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 351(6268):aad3292.
70. Pal C, Papp B, & Lazar V (2015) Collateral sensitivity of antibiotic-resistant microbes. *Trends Microbiol* 23(7):401-407.
71. Podnecky NL, *et al.* (2018) Conserved collateral antibiotic susceptibility networks in diverse clinical strains of Escherichia coli. *Nature Communications* 9(1):3673.
72. Yen P & Papin JA (2017) History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLoS Biol* 15(8):e2001586.
73. Barbosa C, *et al.* (2017) Alternative Evolutionary Paths to Bacterial Antibiotic Resistance Cause Distinct Collateral Effects. *Mol Biol Evol* 34(9):2229-2244.
74. Leus IV, *et al.* (2018) Substrate specificities and efflux efficiencies of RND efflux pumps of Acinetobacter baumannii. *J Bacteriol*.
75. Li XZ, Elkins CA, & Zgurskaya HI (2016) *Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications* (Springer International Publishing).
76. Coyne S, Courvalin P, & Perichon B (2011) Efflux-mediated antibiotic resistance in Acinetobacter spp. *Antimicrob Agents Chemother* 55(3):947-953.
77. Yoon EJ, *et al.* (2015) Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in Acinetobacter baumannii. *MBio* 6(2).
78. Azimi L & Rastegar Lari A (2017) Collateral sensitivity between aminoglycosides and beta-lactam antibiotics depends on active proton pumps. *Microb Pathog* 112:122-125.
79. Kim S, Lieberman TD, & Kishony R (2014) Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proc Natl Acad Sci U S A* 111(40):14494-14499.
80. Imamovic L & Sommer MO (2013) Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Science translational medicine* 5(204):204ra132.
81. Nichol D, *et al.* (2019) Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nature Communications* 10(1):334.
82. Dhawan A, *et al.* (2017) Collateral sensitivity networks reveal evolutionary instability and novel treatment strategies in ALK mutated non-small cell lung cancer. *Sci Rep* 7(1):1232.
83. Yang H, Hu L, Liu Y, Ye Y, & Li J (2016) Detection of the plasmid-mediated quinolone resistance determinants in clinical isolates of Acinetobacter baumannii in China. *J Chemother* 28(5):443-445.
84. Adams-Haduch JM, *et al.* (2008) Genetic basis of multidrug resistance in Acinetobacter baumannii clinical isolates at a tertiary medical center in Pennsylvania. *Antimicrob Agents Chemother* 52(11):3837-3843.
85. Warner WA, *et al.* (2016) Molecular characterization and antimicrobial susceptibility of Acinetobacter baumannii isolates obtained from two hospital outbreaks in Los Angeles County, California, USA. *BMC Infect Dis* 16:194.
86. Dahdouh E, *et al.* (2017) Clonality, virulence determinants, and profiles of resistance of clinical Acinetobacter baumannii isolates obtained from a Spanish hospital. *PLoS One* 12(4):e0176824.
87. Damier-Piolle L, Magnet S, Bremont S, Lambert T, & Courvalin P (2008) AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. *Antimicrob Agents Chemother* 52(2):557-562.
88. Rosenfeld N, Bouchier C, Courvalin P, & Perichon B (2012) Expression of the resistance-nodulation-cell division pump AdeIJK in Acinetobacter baumannii is regulated by AdeN, a TetR-type regulator. *Antimicrob Agents Chemother* 56(5):2504-2510.

89. He X, *et al.* (2015) Biofilm Formation Caused by Clinical Acinetobacter baumannii Isolates Is Associated with Overexpression of the AdeFGH Efflux Pump. *Antimicrob Agents Chemother* 59(8):4817-4825.
90. Alav I, Sutton JM, & Rahman KM (2018) Role of bacterial efflux pumps in biofilm formation. *J Antimicrob Chemother* 73(8):2003-2020.
91. Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M, & Martínez JL (2017) Metabolic Compensation of Fitness Costs Is a General Outcome for Antibiotic-Resistant Pseudomonas aeruginosa Mutants Overexpressing Efflux Pumps. *mBio* 8(4):e00500-00517.
92. Warner DM, Folster JP, Shafer WM, & Jerse AE (2007) Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. *J Infect Dis* 196(12):1804-1812.
93. Elena SF & Lenski RE (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews Genetics* 4:457.
94. Van den Bergh B, Swings T, Fauvart M, & Michiels J (2018) Experimental Design, Population Dynamics, and Diversity in Microbial Experimental Evolution. *Microbiology and Molecular Biology Reviews* 82(3):e00008-00018.
95. Geisinger E, *et al.* (2018) The landscape of intrinsic and evolved fluoroquinolone resistance in Acinetobacter baumannii includes suppression of drug-induced prophage replication. *bioRxiv*:442681.
96. Hamed K & Debonnett L (2017) Tobramycin inhalation powder for the treatment of pulmonary Pseudomonas aeruginosa infection in patients with cystic fibrosis: a review based on clinical evidence. *Therapeutic advances in respiratory disease* 11(5):193-209.
97. Linares JF, Gustafsson I, Baquero F, & Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A* 103(51):19484-19489.
98. Hoffman LR, *et al.* (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436(7054):1171-1175.
99. O'Toole GA & Kolter R (1998) Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 28(3):449-461.
100. Lenski RE (1991) Quantifying fitness and gene stability in microorganisms. *Biotechnology* 15:173-192.
101. Santos-Lopez A, *et al.* (2017) A Naturally Occurring Single Nucleotide Polymorphism in a Multicopy Plasmid Produces a Reversible Increase in Antibiotic Resistance. *Antimicrob Agents Chemother* 61(2).
102. Baym M, *et al.* (2015) Inexpensive multiplexed library preparation for megabase-sized genomes. *PLoS One* 10(5):e0128036.
103. Bolger AM, Lohse M, & Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114-2120.
104. Deatherage DE & Barrick JE (2014) Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol Biol* 1151:165-188.