

1 Biofilm-dependent evolutionary pathways to antibiotic resistance

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15 **Abstract**

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

37

## 38 **Introduction**

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

55 The biofilm lifestyle is central to AMR (7-9). Microbes that are surface-bound and  
56 encased in secreted polymers are less susceptible to compounds that fail to penetrate the  
57 matrix or can persist through the lower metabolic rate of biofilm residents (7, 8, 10). But  
58 what is less understood or appreciated is that the physical and ecological heterogeneity of  
59 biofilms can influence the evolutionary dynamics of microbial populations in multiple  
60 ways. First, the protective effect of biofilms could reduce the strength of selection for  
61 resistance by reducing the effective antibiotic concentration. This would modify the rate  
62 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  $1 \times 10^7$  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

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

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

### 158 **3. Evolutionary dynamics under CIP treatment**

159 In large bacterial populations ( $>10^5$  cells) growing under strong selection, adaptive  
160 mutations conferring beneficial traits (such as antibiotic resistance) will dominate  
161 population dynamics (38, 46). Therefore, if a single mutation renders the antibiotic  
162 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  $\sim 10$  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 ( $\sim 100$ ) 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

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

299

## 300 **6. Evolutionary interactions with other antibiotics**

301 When a bacterium acquires resistance to one antibiotic, the mechanism of resistance can  
302 also confer resistance to other antibiotics (cross-resistance) or increase the susceptibility  
303 to other antibiotics (collateral sensitivity) (70). We tested the MIC of the evolved  
304 populations to 23 different antibiotics. We observed changes in susceptibilities to 13 of  
305 the 23 antibiotics tested, and these changes were growth mode dependent (Figure 4). For  
306 example, planktonic populations exhibited cross resistance to cefpodoxime and  
307 ceftazidime but biofilm populations evolved collateral sensitivity to these cephalosporins.  
308 Cross-resistance was associated genetically with *adeN*, *adeS*, *gyrA* or *pgpB* mutations,  
309 and collateral sensitivity was associated with *adeL* mutations. Selection in these  
310 environments evidently favors the activation of different efflux pumps or modified targets  
311 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<sup>+</sup>) containing 0.37 mM CaCl<sub>2</sub>, 8.7 mM MgSO<sub>4</sub>, 42.2 mM  
405 Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 21.7mM NaCl, 18.7 mM NH<sub>4</sub>Cl 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<sup>+</sup> medium, we selected a single clone and  
412 propagated for 24 hours in M9<sup>+</sup> 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  $\mu$ l into 5 ml of M9<sup>+</sup>)  
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<sup>+</sup>  
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<sup>+</sup> 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<sup>+</sup> containing  
460 0.5 mg/L of CIP and grew them for 24 hours. For each strain, we transferred 50 µl into

461 15 ml of M9<sup>+</sup>. We tested 200  $\mu$ l of the previous dilution of each clone to 4 different  
462 subinhibitory CIP concentrations (0 mg/L, 0.01 mg/L, 0.03 mg/L and 0.0625 mg/L). After  
463 24 hours of growing at 37°C, we measured population sizes by optical density (OD) at  
464 590nm (OD<sub>Populations</sub>). Then, we added 250  $\mu$ l of 0.1% crystal violet and incubated at room  
465 temperature for 15 minutes. After washing the wells and drying for 24 hours, we added  
466 250  $\mu$ l 95% EtOH solution (95% EtOH, 4.95% dH<sub>2</sub>O, 0.05% Triton X-100) to each well  
467 and incubated for 15 minutes and biofilm formation was measured by the OD at 590nm  
468 (OD<sub>Biofilm</sub>). Biofilm formation was corrected by population sizes (OD<sub>Biofilm</sub>/OD<sub>Population</sub>).  
469 Results are the average of three experiments (Figure S3).

470

#### 471 **Fitness measurement**

472 We selected 5 biofilm and 5 planktonic clones at the end of the evolutionary rescue  
473 experiment (Figure 2) and determined the fitness by directly competing the ancestral  
474 strain and the evolved clone variants both in planktonic and in biofilm conditions (Figure  
475 3) (37). We revived each clone from a freezer stock in M9<sup>+</sup> for 24 hours. We maintained  
476 the same evolutionary conditions to revive the clones, adding 3 beads and/or CIP to the  
477 broth when required. After 24 hours, we added equal volume of the clones and the  
478 ancestors in M9<sup>+</sup> in the absence of antibiotics. For planktonic populations, we mixed 25  
479  $\mu$ l of each competitor in 5 ml of M9<sup>+</sup>. For biofilm competitions, we sonicated one bead  
480 per competitor in 1 ml of PBS and mixed in 5 ml of M9<sup>+</sup> containing 3 beads. The mix  
481 was cultured at 37°C for 24 hours. We plated at time zero and after 24 hours. For each  
482 competition, we plated aliquots onto nonselective tryptic soy agar and tryptic soy agar  
483 containing CIP. Selection rate ( $r$ ) was calculated as the difference of the Malthusian  
484 parameters for the two competitors:  $r = (\ln(\text{CIP resistant}_{d=1}/\text{CIP resistant}_{d=0})) / (\ln(\text{CIP}$   
485  $\text{susceptible}_{d=1}/\text{CIP susceptible}_{d=0})) / \text{day}$  (100). Susceptible populations were calculated as

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

492

### 493 **Genome sequencing**

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

510

## 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](http://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](https://github.com/sirmicrobe/U01_allele_freq_code). All sequences were deposited into

536 NCBI under the Biosample accession numbers SAMN09783599-SAMN09783682.

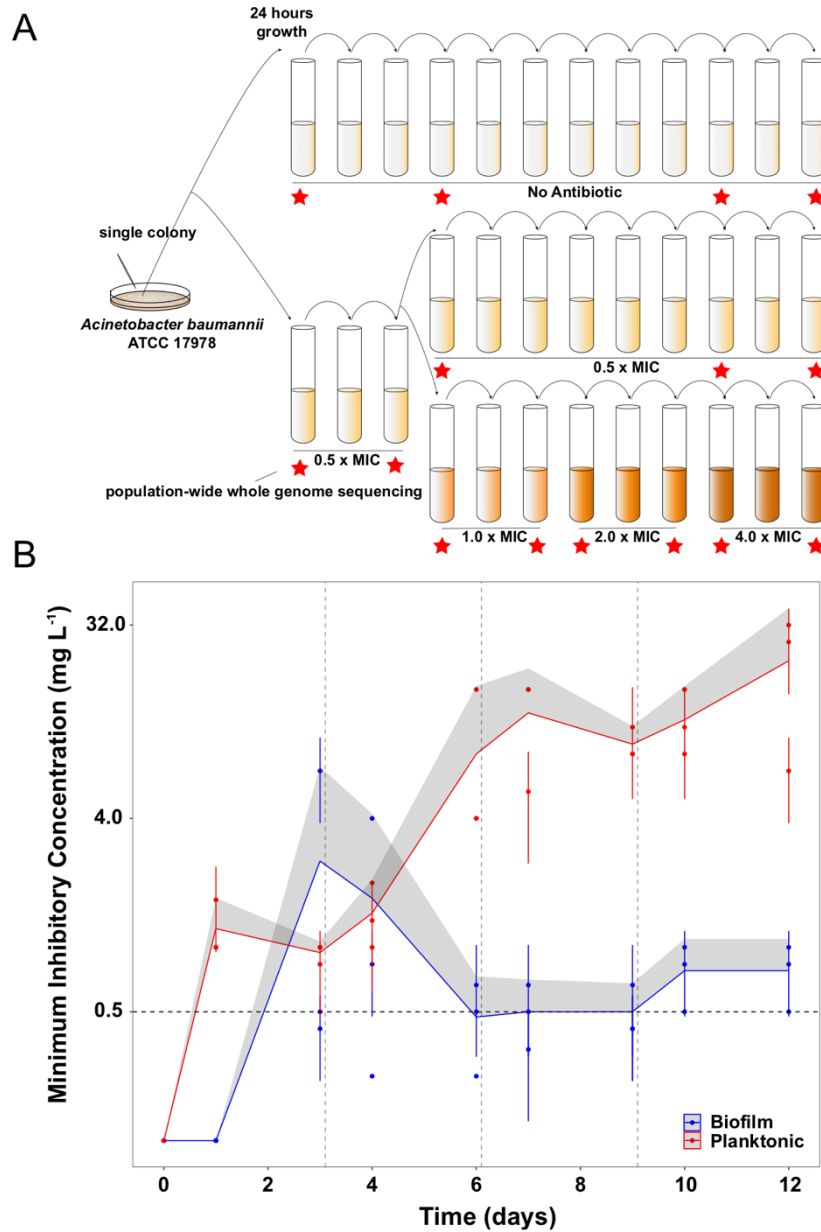
537

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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

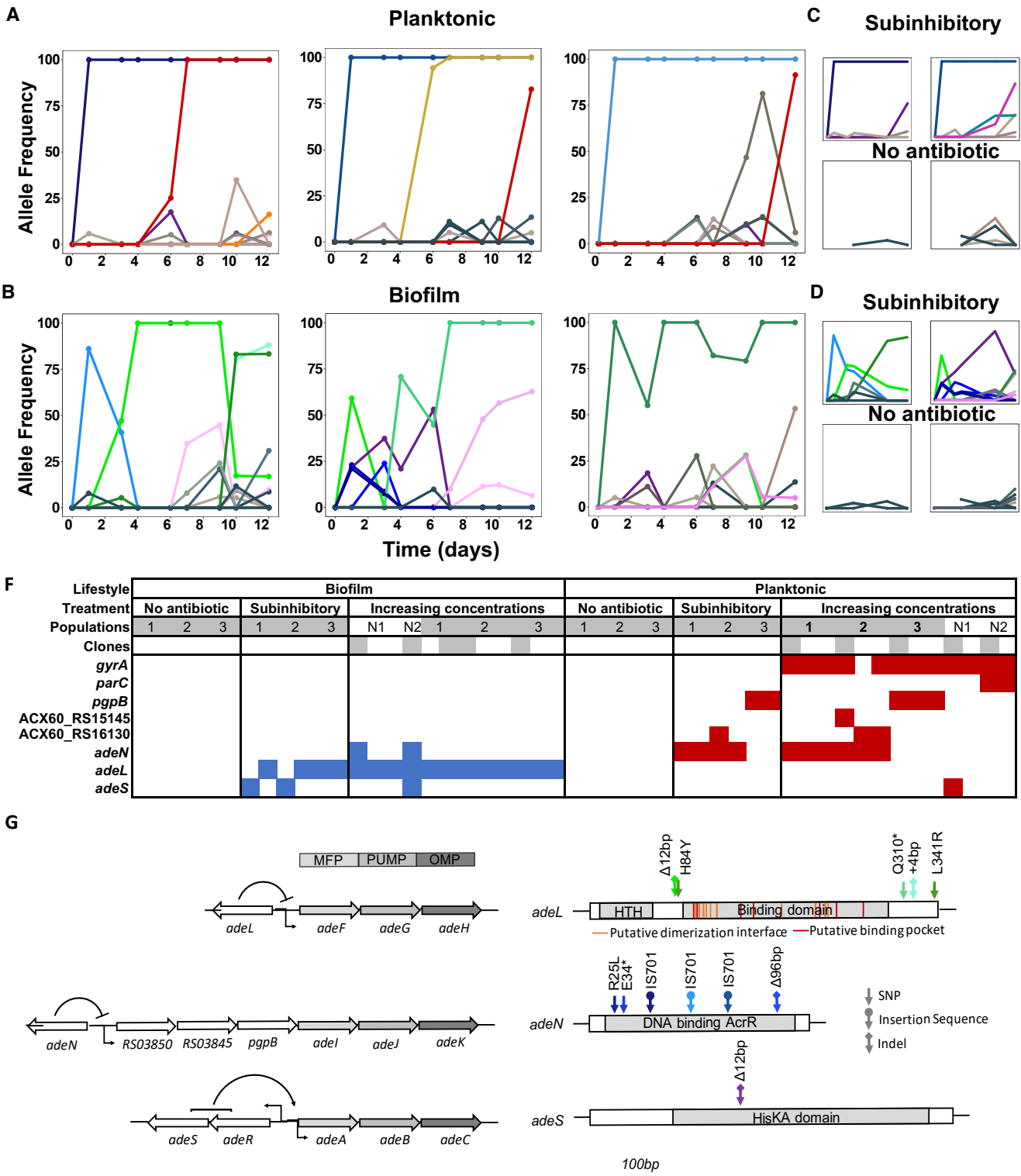
548 (bottom). For the latter, termed evolutionary rescue, the concentration of CIP was doubled

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

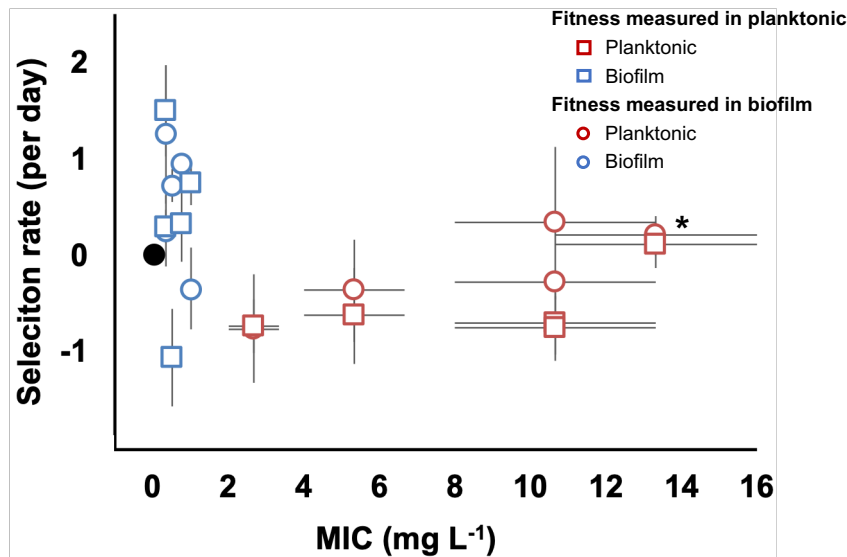
561 **Figure 2. Lifestyle-dependent mutations and dynamics under increasing CIP**

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.

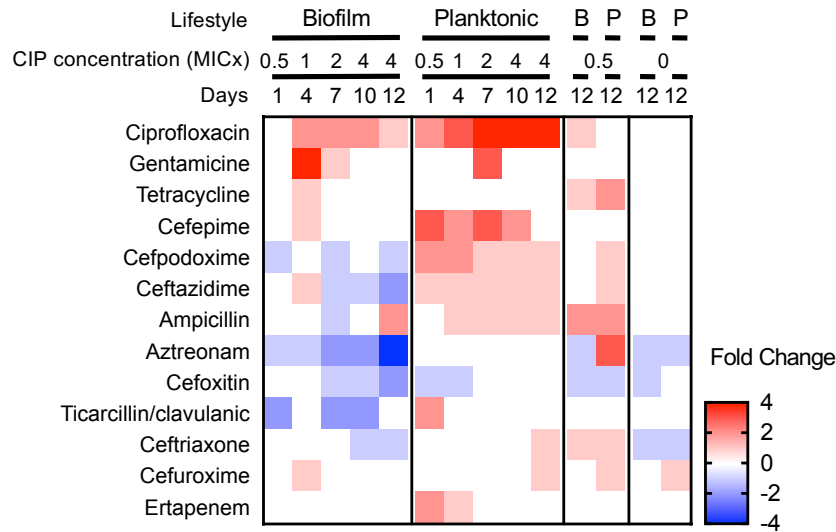


579

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

581 Relative fitness (average  $\pm$  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.

	<b>Increasing concentrations</b>		<b>Subinhibitory concentrations</b>	
	<b>Planktonic</b>	<b>Biofilm</b>	<b>Planktonic</b>	<b>Biofilm</b>
Total mutations	30	40	6	16
Nonsynonymous/Synonymous <sup>b</sup>	8.5	9.67	2/0	6
Intergenic	8	11	0	4
Nonsynonymous	9	13	2	6
Percent intergenic mutations <sup>b</sup>	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. <sup>a</sup>Results from the  
598 last day of the experimental evolution. <sup>b</sup>Accounting for all unique mutations detected after  
599 filtering (see methods). For mutation dynamics over time, see Table S3.

647 **References**

- 648 1. O'Neill J (2016) TACKLING DRUG-RESISTANT INFECTIONS GLOBALLY:  
649 FINAL REPORT AND RECOMMENDATIONS. *Review on Antimicrobial Resistance*.  
650 2. Martínez JL (2008) Antibiotics and Antibiotic Resistance Genes in Natural  
651 Environments. *Science* 321(5887):365-367.  
652 3. Ventola CL (2015) The antibiotic resistance crisis: part 1: causes and threats. *P T*  
653 40(4):277-283.  
654 4. Doi Y, Murray GL, & Peleg AY (2015) Acinetobacter baumannii: evolution of  
655 antimicrobial resistance-treatment options. *Semin Respir Crit Care Med* 36(1):85-98.  
656 5. Asif M, Alvi IA, & Rehman SU (2018) Insight into Acinetobacter baumannii:  
657 pathogenesis, global resistance, mechanisms of resistance, treatment options, and  
658 alternative modalities. *Infect Drug Resist* 11:1249-1260.  
659 6. Eze EC, Chenia HY, & El Zowalaty ME (2018) Acinetobacter baumannii biofilms:  
660 effects of physicochemical factors, virulence, antibiotic resistance determinants, gene  
661 regulation, and future antimicrobial treatments. *Infect Drug Resist* 11:2277-2299.  
662 7. Hoiby N, Bjarnsholt T, Givskov M, Molin S, & Ciofu O (2010) Antibiotic resistance of  
663 bacterial biofilms. *Int J Antimicrob Agents* 35(4):322-332.  
664 8. Olsen I (2015) Biofilm-specific antibiotic tolerance and resistance. *Eur J Clin Microbiol*  
665 *Infect Dis* 34(5):877-886.  
666 9. Ahmed MN, Porse A, Sommer MOA, Hoiby N, & Ciofu O (2018) Evolution of  
667 antibiotic resistance in biofilm and planktonic P. aeruginosa populations exposed to  
668 sub-inhibitory levels of ciprofloxacin. *Antimicrob Agents Chemother*.  
669 10. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, & Stewart PS (2003)  
670 Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to  
671 tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.  
672 *Antimicrob Agents Chemother* 47(1):317-323.  
673 11. Wistrand-Yuen E, et al. (2018) Evolution of high-level resistance during low-level  
674 antibiotic exposure. *Nat Commun* 9(1):1599.  
675 12. Andersson DI & Hughes D (2014) Microbiological effects of sublethal levels of  
676 antibiotics. *Nat Rev Microbiol* 12(7):465-478.  
677 13. Traverse CC, Mayo-Smith LM, Poltak SR, & Cooper VS (2013) Tangled bank of  
678 experimentally evolved Burkholderia biofilms reflects selection during chronic  
679 infections. *Proc Natl Acad Sci U S A* 110(3):E250-259.  
680 14. Cooper VS, Staples RK, Traverse CC, & Ellis CN (2014) Parallel evolution of small  
681 colony variants in Burkholderia cenocepacia biofilms. *Genomics* 104(6 Pt A):447-452.  
682 15. Ellis CN, Traverse CC, Mayo-Smith L, Buskirk SW, & Cooper VS (2015) Character  
683 displacement and the evolution of niche complementarity in a model biofilm  
684 community. *Evolution* 69(2):283-293.  
685 16. France MT, Cornea A, Kehlet-Delgado H, & Forney LJ (2019) Spatial structure  
686 facilitates the accumulation and persistence of antibiotic-resistant mutants in biofilms.  
687 *Evolutionary Applications* 12(3):498-507.  
688 17. Poltak SR & Cooper VS (2011) Ecological succession in long-term experimentally  
689 evolved biofilms produces synergistic communities. *ISME J* 5(3):369-378.  
690 18. Frenkel EM, et al. (2015) Crowded growth leads to the spontaneous evolution of  
691 semistable coexistence in laboratory yeast populations. *Proc Natl Acad Sci U S A*  
692 112(36):11306-11311.  
693 19. Fux CA, Costerton JW, Stewart PS, & Stoodley P (2005) Survival strategies of  
694 infectious biofilms. *Trends Microbiol* 13(1):34-40.  
695 20. Arnold SJ (1992) Constraints on phenotypic evolution. *Am Nat* 140 Suppl 1:S85-107.  
696 21. Pavličev M & Chaverud JM (2015) Constraints Evolve: Context Dependency of Gene  
697 Effects Allows Evolution of Pleiotropy. *Annual Review of Ecology, Evolution, and*  
698 *Systematics* 46(1):413-434.  
699 22. Saltz JB, Hessel FC, & Kelly MW (2017) Trait Correlations in the Genomics Era.  
700 *Trends Ecol Evol* 32(4):279-290.  
701 23. Wolcott RD (2017) Biofilms cause chronic infections. *J Wound Care* 26(8):423-425.

- 702 24. Wolcott RD, *et al.* (2010) Chronic wounds and the medical biofilm paradigm. *J Wound*  
703 *Care* 19(2):45-46, 48-50, 52-43.
- 704 25. Geisinger E & Isberg RR (2015) Antibiotic modulation of capsular exopolysaccharide  
705 and virulence in *Acinetobacter baumannii*. *PLoS Pathog* 11(2):e1004691.
- 706 26. Ridenhour BJ, *et al.* (2017) Persistence of antibiotic resistance plasmids in bacterial  
707 biofilms. *Evol Appl* 10(6):640-647.
- 708 27. Hughes D & Andersson DI (2017) Evolutionary Trajectories to Antibiotic Resistance.  
709 *Annu Rev Microbiol*.
- 710 28. Baym M, *et al.* (2016) Spatiotemporal microbial evolution on antibiotic landscapes.  
711 *Science* 353(6304):1147-1151.
- 712 29. Piechaud M & Second L (1951) [Studies of 26 strains of *Moraxella Iwoffii*]. *Annales de*  
713 *l'Institut Pasteur* 80(1):97-99.
- 714 30. Baumann P, Doudoroff M, & Stanier RY (1968) A study of the *Moraxella* group. II.  
715 Oxidative-negative species (genus *Acinetobacter*). *J Bacteriol* 95(5):1520-1541.
- 716 31. Bell G & Gonzalez A (2009) Evolutionary rescue can prevent extinction following  
717 environmental change. *Ecol Lett* 12(9):942-948.
- 718 32. Lopes BS & Amyes SG (2013) Insertion sequence disruption of *adeR* and ciprofloxacin  
719 resistance caused by efflux pumps and *gyrA* and *parC* mutations in *Acinetobacter*  
720 *baumannii*. *Int J Antimicrob Agents* 41(2):117-121.
- 721 33. Ardebili A, Lari AR, & Talebi M (2014) Correlation of ciprofloxacin resistance with  
722 the AdeABC efflux system in *Acinetobacter baumannii* clinical isolates. *Annals of*  
723 *laboratory medicine* 34(6):433-438.
- 724 34. Tseng BS, *et al.* (2013) The extracellular matrix protects *Pseudomonas aeruginosa*  
725 biofilms by limiting the penetration of tobramycin. *Environ Microbiol* 15(10):2865-  
726 2878.
- 727 35. Kirby AE, Garner K, & Levin BR (2012) The relative contributions of physical  
728 structure and cell density to the antibiotic susceptibility of bacteria in biofilms.  
729 *Antimicrob Agents Chemother* 56(6):2967-2975.
- 730 36. Aka ST & Haji SH (2015) Sub-MIC of antibiotics induced biofilm formation of  
731 *Pseudomonas aeruginosa* in the presence of chlorhexidine. *Braz J Microbiol* 46(1):149-  
732 154.
- 733 37. Turner CB, Marshall CW, & Cooper VS (2018) Parallel genetic adaptation across  
734 environments differing in mode of growth or resource availability. *Evolution Letters*  
735 2(4):355-367.
- 736 38. Cooper VS (2018) Experimental Evolution as a High-Throughput Screen for Genetic  
737 Adaptations. *mSphere* 3(3).
- 738 39. Salverda MLM, Koomen J, Koopmanschap B, Zwart MP, & de Visser J (2017)  
739 Adaptive benefits from small mutation supplies in an antibiotic resistance enzyme. *Proc*  
740 *Natl Acad Sci U S A* 114(48):12773-12778.
- 741 40. Oz T, *et al.* (2014) Strength of selection pressure is an important parameter contributing  
742 to the complexity of antibiotic resistance evolution. *Mol Biol Evol* 31(9):2387-2401.
- 743 41. Maclean RC, Hall AR, Perron GG, & Buckling A (2010) The evolution of antibiotic  
744 resistance: insight into the roles of molecular mechanisms of resistance and treatment  
745 context. *Discov Med* 10(51):112-118.
- 746 42. CLSI (2017) CLSI. Clinical and Laboratory Standards Institute. Performance Standards  
747 for Antimicrobial Susceptibility Testing-Seventeenth edition: Approved Standar M100-  
748 S17. (CLSI, Wayne, PA, USA).
- 749 43. Wang Y-C, *et al.* (2018) Biofilm formation is not associated with worse outcome in  
750 *Acinetobacter baumannii* bacteraemic pneumonia. *Scientific Reports* 8(1):7289.
- 751 44. Borriello G, *et al.* (2004) Oxygen limitation contributes to antibiotic tolerance of  
752 *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* 48(7):2659-2664.
- 753 45. Hill D, *et al.* (2005) Antibiotic Susceptibilities of *Pseudomonas aeruginosa*  
754 Isolates Derived from Patients with Cystic Fibrosis under Aerobic, Anaerobic, and  
755 Biofilm Conditions. *Journal of Clinical Microbiology* 43(10):5085-5090.



- 756 46. Barrick JE & Lenski RE (2013) Genome dynamics during experimental evolution.  
757 *Nature reviews. Genetics* 14:827-839.
- 758 47. Bolnick DI, Barrett RDH, Oke KB, Rennison DJ, & Stuart YE (2018) (Non)Parallel  
759 Evolution. *Annual Review of Ecology, Evolution, and Systematics* 49(1):303-330.
- 760 48. Barrick JE, *et al.* (2009) Genome evolution and adaptation in a long-term experiment  
761 with *Escherichia coli*. *Nature* 461:1243-1247.
- 762 49. Dillon MM, Sung W, Sebra R, Lynch M, & Cooper VS (2017) Genome-Wide Biases in  
763 the Rate and Molecular Spectrum of Spontaneous Mutations in *Vibrio cholerae* and  
764 *Vibrio fischeri*. *Mol Biol Evol* 34(1):93-109.
- 765 50. Lynch M, *et al.* (2016) Genetic drift, selection and the evolution of the mutation rate.  
766 *Nature Reviews Genetics* 17:704.
- 767 51. Kröger C, *et al.* (2018) The primary transcriptome, small RNAs and regulation of  
768 antimicrobial resistance in *Acinetobacter baumannii* ATCC 17978. *Nucleic Acids Res*  
769 46(18):9684-9698.
- 770 52. Khan GA, Berglund B, Khan KM, Lindgren PE, & Fick J (2013) Occurrence and  
771 abundance of antibiotics and resistance genes in rivers, canal and near drug formulation  
772 facilities--a study in Pakistan. *PLoS One* 8(6):e62712.
- 773 53. Baquero F, Negri MC, Morosini MI, & Blazquez J (1998) Antibiotic-selective  
774 environments. *Clin Infect Dis* 27 Suppl 1:S5-11.
- 775 54. Coyne S, Rosenfeld N, Lambert T, Courvalin P, & Perichon B (2010) Overexpression  
776 of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in  
777 *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 54(10):4389-4393.
- 778 55. Fernando D, Zhanel G, & Kumar A (2013) Antibiotic resistance and expression of  
779 resistance-nodulation-division pump- and outer membrane porin-encoding genes in  
780 *Acinetobacter* species isolated from Canadian hospitals. *Can J Infect Dis Med*  
781 *Microbiol* 24(1):17-21.
- 782 56. Pournaras S, Koumaki V, Gennimata V, Kouskouni E, & Tsakris A (2016) In Vitro  
783 Activity of Tigecycline Against *Acinetobacter baumannii*: Global Epidemiology and  
784 Resistance Mechanisms. *Adv Exp Med Biol* 897:1-14.
- 785 57. Li X-Z, Elkins CA, & Zgurskaya HI (2016) *Efflux-Mediated Antimicrobial Resistance*  
786 *in Bacteria: Mechanisms, Regulation and Clinical Implications* (Springer).
- 787 58. Hua X, Chen Q, Li X, & Yu Y (2014) Global transcriptional response of *Acinetobacter*  
788 *baumannii* to a subinhibitory concentration of tigecycline. *Int J Antimicrob Agents*  
789 44(4):337-344.
- 790 59. Su XZ, Chen J, Mizushima T, Kuroda T, & Tsuchiya T (2005) AbeM, an H<sup>+</sup>-coupled  
791 *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of  
792 transporters. *Antimicrob Agents Chemother* 49(10):4362-4364.
- 793 60. Desai MM & Fisher DS (2007) Beneficial mutation selection balance and the effect of  
794 linkage on positive selection. *Genetics* 176(3):1759-1798.
- 795 61. Vogwill T & MacLean RC (2015) The genetic basis of the fitness costs of antimicrobial  
796 resistance: a meta-analysis approach. *Evol Appl* 8(3):284-295.
- 797 62. Moore FB, Rozen DE, & Lenski RE (2000) Pervasive compensatory adaptation in  
798 *Escherichia coli*. *Proc Biol Sci* 267(1442):515-522.
- 799 63. Zhao X & Drlica K (2002) Restricting the selection of antibiotic-resistant mutant  
800 bacteria: measurement and potential use of the mutant selection window. *J Infect Dis*  
801 185(4):561-565.
- 802 64. Marcusson LL, Fridomt-Moller N, & Hughes D (2009) Interplay in the selection of  
803 fluoroquinolone resistance and bacterial fitness. *PLoS Pathog* 5(8):e1000541.
- 804 65. Huseby DL, *et al.* (2017) Mutation Supply and Relative Fitness Shape the Genotypes of  
805 Ciprofloxacin-Resistant *Escherichia coli*. *Mol Biol Evol* 34(5):1029-1039.
- 806 66. Basra P, *et al.* (2018) Fitness tradeoffs of antibiotic resistance in extra-intestinal  
807 pathogenic *Escherichia coli*. *Genome Biol Evol*.
- 808 67. Kugelberg E, Lofmark S, Wretling B, & Andersson DI (2005) Reduction of the fitness  
809 burden of quinolone resistance in *Pseudomonas aeruginosa*. *J Antimicrob Chemother*  
810 55(1):22-30.

- 811 68. Sackman AM & Rokyta DR (2018) Additive Phenotypes Underlie Epistasis of Fitness  
812 Effects. *Genetics* 208(1):339-348.
- 813 69. Baym M, Stone LK, & Kishony R (2016) Multidrug evolutionary strategies to reverse  
814 antibiotic resistance. *Science* 351(6268):aad3292.
- 815 70. Pal C, Papp B, & Lazar V (2015) Collateral sensitivity of antibiotic-resistant microbes.  
816 *Trends Microbiol* 23(7):401-407.
- 817 71. Podnecky NL, *et al.* (2018) Conserved collateral antibiotic susceptibility networks in  
818 diverse clinical strains of *Escherichia coli*. *Nature Communications* 9(1):3673.
- 819 72. Yen P & Papin JA (2017) History of antibiotic adaptation influences microbial  
820 evolutionary dynamics during subsequent treatment. *PLoS Biol* 15(8):e2001586.
- 821 73. Barbosa C, *et al.* (2017) Alternative Evolutionary Paths to Bacterial Antibiotic  
822 Resistance Cause Distinct Collateral Effects. *Mol Biol Evol* 34(9):2229-2244.
- 823 74. Leus IV, *et al.* (2018) Substrate specificities and efflux efficiencies of RND efflux  
824 pumps of *Acinetobacter baumannii*. *J Bacteriol*.
- 825 75. Li XZ, Elkins CA, & Zgurskaya HI (2016) *Efflux-Mediated Antimicrobial Resistance in*  
826 *Bacteria: Mechanisms, Regulation and Clinical Implications* (Springer International  
827 Publishing).
- 828 76. Coyne S, Courvalin P, & Perichon B (2011) Efflux-mediated antibiotic resistance in  
829 *Acinetobacter* spp. *Antimicrob Agents Chemother* 55(3):947-953.
- 830 77. Yoon EJ, *et al.* (2015) Contribution of resistance-nodulation-cell division efflux  
831 systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*.  
832 *MBio* 6(2).
- 833 78. Azimi L & Rastegar Lari A (2017) Collateral sensitivity between aminoglycosides and  
834 beta-lactam antibiotics depends on active proton pumps. *Microb Pathog* 112:122-125.
- 835 79. Kim S, Lieberman TD, & Kishony R (2014) Alternating antibiotic treatments constrain  
836 evolutionary paths to multidrug resistance. *Proc Natl Acad Sci U S A* 111(40):14494-  
837 14499.
- 838 80. Imamovic L & Sommer MO (2013) Use of collateral sensitivity networks to design  
839 drug cycling protocols that avoid resistance development. *Science translational*  
840 *medicine* 5(204):204ra132.
- 841 81. Nichol D, *et al.* (2019) Antibiotic collateral sensitivity is contingent on the repeatability  
842 of evolution. *Nature Communications* 10(1):334.
- 843 82. Dhawan A, *et al.* (2017) Collateral sensitivity networks reveal evolutionary instability  
844 and novel treatment strategies in ALK mutated non-small cell lung cancer. *Sci Rep*  
845 7(1):1232.
- 846 83. Yang H, Hu L, Liu Y, Ye Y, & Li J (2016) Detection of the plasmid-mediated  
847 quinolone resistance determinants in clinical isolates of *Acinetobacter baumannii* in  
848 China. *J Chemother* 28(5):443-445.
- 849 84. Adams-Haduch JM, *et al.* (2008) Genetic basis of multidrug resistance in *Acinetobacter*  
850 *baumannii* clinical isolates at a tertiary medical center in Pennsylvania. *Antimicrob*  
851 *Agents Chemother* 52(11):3837-3843.
- 852 85. Warner WA, *et al.* (2016) Molecular characterization and antimicrobial susceptibility of  
853 *Acinetobacter baumannii* isolates obtained from two hospital outbreaks in Los Angeles  
854 County, California, USA. *BMC Infect Dis* 16:194.
- 855 86. Dahdouh E, *et al.* (2017) Clonality, virulence determinants, and profiles of resistance of  
856 clinical *Acinetobacter baumannii* isolates obtained from a Spanish hospital. *PLoS One*  
857 12(4):e0176824.
- 858 87. Damier-Piolle L, Magnet S, Bremont S, Lambert T, & Courvalin P (2008) AdeIJK, a  
859 resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter*  
860 *baumannii*. *Antimicrob Agents Chemother* 52(2):557-562.
- 861 88. Rosenfeld N, Bouchier C, Courvalin P, & Perichon B (2012) Expression of the  
862 resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is  
863 regulated by AdeN, a TetR-type regulator. *Antimicrob Agents Chemother* 56(5):2504-  
864 2510.

- 865 89. He X, *et al.* (2015) Biofilm Formation Caused by Clinical *Acinetobacter baumannii*  
866 Isolates Is Associated with Overexpression of the AdeFGH Efflux Pump. *Antimicrob*  
867 *Agents Chemother* 59(8):4817-4825.
- 868 90. Alav I, Sutton JM, & Rahman KM (2018) Role of bacterial efflux pumps in biofilm  
869 formation. *J Antimicrob Chemother* 73(8):2003-2020.
- 870 91. Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M, & Martínez JL (2017)  
871 Metabolic Compensation of Fitness Costs Is a General Outcome for Antibiotic-  
872 Resistant *Pseudomonas aeruginosa* Mutants Overexpressing Efflux Pumps.  
873 *mBio* 8(4):e00500-00517.
- 874 92. Warner DM, Folster JP, Shafer WM, & Jerse AE (2007) Regulation of the MtrC-MtrD-  
875 MtrE efflux-pump system modulates the in vivo fitness of *Neisseria gonorrhoeae*. *J*  
876 *Infect Dis* 196(12):1804-1812.
- 877 93. Elena SF & Lenski RE (2003) Evolution experiments with microorganisms: the  
878 dynamics and genetic bases of adaptation. *Nature Reviews Genetics* 4:457.
- 879 94. Van den Bergh B, Swings T, Fauvart M, & Michiels J (2018) Experimental Design,  
880 Population Dynamics, and Diversity in Microbial Experimental Evolution.  
881 *Microbiology and Molecular Biology Reviews* 82(3):e00008-00018.
- 882 95. Geisinger E, *et al.* (2018) The landscape of intrinsic and evolved fluoroquinolone  
883 resistance in *Acinetobacter baumannii* includes suppression of drug-induced prophage  
884 replication. *bioRxiv*:442681.
- 885 96. Hamed K & Debonnett L (2017) Tobramycin inhalation powder for the treatment of  
886 pulmonary *Pseudomonas aeruginosa* infection in patients with cystic fibrosis: a review  
887 based on clinical evidence. *Therapeutic advances in respiratory disease* 11(5):193-209.
- 888 97. Linares JF, Gustafsson I, Baquero F, & Martinez JL (2006) Antibiotics as  
889 intermicrobial signaling agents instead of weapons. *Proc Natl Acad Sci U S A*  
890 103(51):19484-19489.
- 891 98. Hoffman LR, *et al.* (2005) Aminoglycoside antibiotics induce bacterial biofilm  
892 formation. *Nature* 436(7054):1171-1175.
- 893 99. O'Toole GA & Kolter R (1998) Initiation of biofilm formation in *Pseudomonas*  
894 *fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic  
895 analysis. *Mol Microbiol* 28(3):449-461.
- 896 100. Lenski RE (1991) Quantifying fitness and gene stability in microorganisms.  
897 *Biotechnology* 15:173-192.
- 898 101. Santos-Lopez A, *et al.* (2017) A Naturally Occurring Single Nucleotide Polymorphism  
899 in a Multicopy Plasmid Produces a Reversible Increase in Antibiotic Resistance.  
900 *Antimicrob Agents Chemother* 61(2).
- 901 102. Baym M, *et al.* (2015) Inexpensive multiplexed library preparation for megabase-sized  
902 genomes. *PLoS One* 10(5):e0128036.
- 903 103. Bolger AM, Lohse M, & Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina  
904 sequence data. *Bioinformatics* 30(15):2114-2120.
- 905 104. Deatherage DE & Barrick JE (2014) Identification of mutations in laboratory-evolved  
906 microbes from next-generation sequencing data using breseq. *Methods Mol Biol*  
907 1151:165-188.