bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 The landscape of intrinsic and evolved fluoroquinolone resistance in *Acinetobacter baumannii*
2 includes suppression of drug-induced prophage replication
3
4
5 Running Head: Antibiotic resistance modulation of prophage induction
6
7 Edward Geisinger,[a, c] Germán Vargas-Cuebas,[a]* Nadav J. Mortman,[a] Sapna Syal,[a] Elizabeth L.
8 Wainwright,[c] David Lazinski,[a] Stephen Wood,[b] Zeyu Zhu,[b] Jon Anthony,[b] Tim van Opijnen,[b]
9 Ralph R. Isberg[a]#
10
11 [a]Department of Molecular Biology and Microbiology, Tufts University School of Medicine,
12 Boston, Massachusetts, USA
13 [b]Department of Biology, Boston College, Chestnut Hill, Massachusetts, USA
14 [c]Department of Biology, Northeastern University, Boston, Massachusetts, USA
15
16 #To whom correspondence should be addressed (ralph.isberg@tufts.edu)
17 *Present address: Department of Microbiology and Immunology, Emory University School of
18 Medicine, Atlanta, Georgia, USA
19
20
21 Abstract word count: 396 (Abstract section: 248; Importance section: 148)
22 Text word count: 5,979

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

23 **Abstract**

24 The emergence of fluoroquinolone resistance in nosocomial pathogens has restricted the clinical
25 efficacy of this antibiotic class. In *Acinetobacter baumannii*, the majority of clinical isolates
26 now show high-level resistance due to mutations in *gyrA* (DNA gyrase) and *parC* (Topo IV). To
27 investigate the molecular basis for fluoroquinolone resistance, an exhaustive mutation analysis
28 was performed in both drug sensitive and resistant strains to identify loci that alter the sensitivity
29 of the organism to ciprofloxacin. To this end, parallel fitness tests of over 60,000 unique
30 insertion mutations were performed in strains with various alleles in genes encoding the drug
31 targets. The spectrum of mutations that altered drug sensitivity was found to be similar in the
32 drug sensitive and double mutant *gyrAparC* background having resistance alleles in both genes.
33 In contrast, introduction of a single *gyrA* resistance allele, resulting in preferential poisoning of
34 Topo IV by ciprofloxacin, led to extreme alterations in the insertion mutation fitness landscape.
35 The distinguishing feature of preferential Topo IV poisoning was induction of DNA synthesis in
36 the region of two endogenous prophages, which appeared to occur *in situ*. Induction of the
37 selective DNA synthesis in the *gyrA* background was also linked to enhanced activation of SOS
38 response and heightened transcription of prophage genes relative to that observed in either the
39 WT or *gyrAparC* double mutants. Therefore, the accumulation of mutations that result in the
40 stepwise evolution of high ciprofloxacin resistance is tightly connected to suppression of
41 hyperactivation of the SOS response and endogenous prophage DNA synthesis.

42

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

43 **Importance**

44 Fluoroquinolones have been extremely successful antibiotics. Their clinical efficacy derives
45 from the ability to target multiple bacterial enzymes critical to DNA replication, the
46 topoisomerases DNA gyrase and Topo IV. Unfortunately, mutations lowering drug affinity for
47 both enzymes are now widespread, rendering these drugs ineffective for many pathogens. To
48 undermine this form of resistance, we sought to understand how bacteria with target alterations
49 differentially cope with fluoroquinolone exposures. We studied this problem in the nosocomial
50 pathogen *A. baumannii*, which causes resistant, life-threating infections. Employing genome-
51 wide approaches, we uncovered numerous pathways that could be exploited to lower
52 fluoroquinolone resistance independently of target alteration. Remarkably, fluoroquinolone
53 targeting of Topo IV in specific mutants caused dramatic prophage hyperinduction, a response
54 that was muted in strains with DNA gyrase as the primary target. This work demonstrates that
55 resistance evolution via target modification can profoundly modulate the antibiotic stress
56 response, revealing potential resistance-associated liabilities.

57
58

59 **Introduction**

60 *Acinetobacter baumannii* is a frequent cause of multidrug resistant infections in hospitals
61 and has been labeled a pathogen of critical priority for new drug development (1). This pathogen
62 class has rapidly evolved a broad array of drug resistance mechanisms, limiting the usefulness of
63 many widely-used antibiotics. A prime example is the fluoroquinolone class of antibiotics. These
64 drugs are widely used to treat infections caused by a range of Gram-negative and Gram-positive
65 bacteria, but they have been rendered obsolete against most *A. baumannii* isolates due to
66 extremely high frequencies of resistance (2-4). Understanding how *A. baumannii* and related

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

67 bacteria withstand treatment with fluoroquinolone antibiotics has the potential to lead to
68 strategies to reverse or bypass resistance.

69 Fluoroquinolones inhibit DNA replication in bacteria by targeting two enzymes essential
70 for DNA synthesis, the type II topoisomerases DNA gyrase (*gyrAB* genes) and topoisomerase IV
71 (Topo IV, *parCE*). These enzymes modulate DNA topology to maintain negative DNA
72 supercoiling (DNA gyrase) or decatenate newly replicated DNA (Topo IV), and in so doing,
73 break and religate DNA. Binding of fluoroquinolones to these enzymes traps them in an
74 intermediate state that is bound to cleaved DNA, resulting in double-strand DNA breaks, blocked
75 replication fork progression and, at high drug concentrations, cell death (5).

76 Acquired resistance to fluoroquinolones commonly arises through stepwise mutations
77 that disrupt the ability of the drug to bind its preferred target enzymes. In Gram-negative bacteria
78 including *A. baumannii*, these mutations typically arise first in *gyrA*, encoding the GyrA subunit
79 of DNA gyrase which is the more sensitive of the two enzyme targets (6). In the presence of
80 resistant GyrA, the less sensitive Topo IV (encoded by *parC*) becomes the target and the site of
81 second-step resistance mutations. In addition to target site alterations, acquisition of mutations
82 that upregulate drug efflux pumps or accessory genes that allow drug modification enable
83 bacteria to develop fluoroquinolone resistance (7). A large fraction of *A. baumannii* isolates
84 harbor target-site mutations in *gyrA* and *parC* (8, 9) and mutations causing overproduction of
85 one or more RND-class efflux systems that act on fluoroquinolone drugs (AdeABC, AdeFGH,
86 AdeIJK (10-12)).

87 Acquired resistance mechanisms generally act in combination with intrinsic resistance
88 strategies in a cumulative manner to raise the amount of fluoroquinolone antibiotic required to
89 block bacterial growth. Regulated production of native efflux pumps contributes to intrinsic

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

90 fluoroquinolone resistance in many bacteria (13). Of the RND systems in *A. baumannii*, native
91 levels of AdeIJK in wild-type (WT) strains lacking acquired mutations have been shown to
92 provide intrinsic resistance to fluoroquinolones (12). Whether regulated production of other
93 efflux systems provides intrinsic fluoroquinolone resistance is less clear.

94 Another major strategy for intrinsic fluoroquinolone resistance is activation of DNA
95 damage repair pathways (14). DNA lesions caused by fluoroquinolone intoxication are processed
96 to single-stranded DNA and subsequently induce the SOS repair response, resulting in de-
97 repression of many genes involved in DNA recombination and repair (15). Knockout mutations
98 in a variety of DNA repair genes result in increased fluoroquinolone susceptibility in several
99 species (14-24). In certain cases, the SOS response also induces mobile genetic elements that
100 carry antibiotic resistance or toxin genes, potentially influencing the spread of resistance or
101 virulence traits (25). The *A. baumannii* SOS repair response is non-canonical, lacking clear
102 orthologs of many major players in other systems (26) and is characterized by a phenotypically
103 variable response within cell populations (27). Inactivation of RecA, a central protein mediating
104 DNA recombinational repair and SOS induction, or the RecBCD Exonuclease V complex
105 responsible for double-strand break repair, greatly raises fluoroquinolone sensitivity in *A.*
106 *baumannii* (28, 29). The role of the SOS response and other DNA repair systems, however, in
107 the development of antibiotic resistance in this organism is largely unknown.

108 In this study, we present the results of a comprehensive screen for determinants of
109 intrinsic resistance to the fluoroquinolone antibiotic ciprofloxacin in *A. baumannii*. We
110 hypothesized that the ciprofloxacin resistome varies depending on the drug target (DNA gyrase
111 or Topo IV) that is preferentially poisoned, which is determined by possession of WT or resistant
112 versions of the enzymes. We therefore performed parallel screens with isogenic *A. baumannii*

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

113 strains containing sensitive or resistant *gyrA* and *parC* alleles to uncover the influence of target
114 selectivity on the intrinsic resistance landscape. This analysis led to the surprising discovery that
115 endogenous prophage activation by fluoroquinolones shows dramatic dependence on the
116 availability of a sensitive *parC* allele.
117
118
119 **Results**

120 **Identification of *Acinetobacter baumannii* loci that confer altered sensitivity to**
121 **ciprofloxacin.** As part of a largescale effort to characterize the molecular nature of intrinsic
122 resistance of *Acinetobacter baumannii* to antimicrobials, we identified the entire spectrum of
123 insertion mutations that cause altered sensitivity to the fluoroquinolone antibiotic ciprofloxacin
124 during growth in bacteriological culture. A number of studies have demonstrated that mutations
125 that cause antibiotic hypersensitization in strain backgrounds lacking demonstrable resistance
126 loci exhibit these effects independently of whether there are target site resistance mutations or
127 antibiotic-inactivating enzymes present in the strains being interrogated (20, 30). We wanted to
128 test this model by first identifying loci that confer intrinsic resistance in a strain background
129 having intact drug targets, and then comparing them with intrinsic resistance loci identified in
130 strains having drug target mutations in DNA gyrase or Topo IV. For this work, we will refer to
131 lesions resulting in lowered resistance to the antibiotic as ciprofloxacin-hypersensitizing
132 mutations in each strain background, and the genes harboring these mutations as loci of
133 hypersensitization.

134 To identify ciprofloxacin hypersensitization loci, ciprofloxacin concentrations below the
135 minimal inhibitory concentration (MIC) (Table 1) were identified that resulted in growth rates of

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

136 *A. baumannii* ATCC17978 in rich broth that were between 60-80% of that observed without
137 antibiotics (Fig. 1A). Three of these concentrations were chosen for further analysis (0.05, 0.075
138 and 0.09-0.10 μg/ml) to determine the relative fitness of insertion mutations when subjected to
139 each of the antibiotic stress conditions. Multiple independent Tn*10* insertion pools (7 pools for
140 0.05 μg/ml, and 11 pools for 0.075 and 0.09-0.10 μg/ml ciprofloxacin) having between 6,000
141 and 18,000 individual insertions (60,000 separate sites in all) were grown in broth in the
142 presence or absence of ciprofloxacin for approximately 8 generations. DNA samples taken from
143 the initial time point prior to growth (t_1) and the final timepoint after 8 generations growth (t_2)
144 were prepared from each of the pools. The insertion sites were then amplified preferentially and
145 subjected to high density sequencing, followed by determining the relative fitness of each
146 insertion mutant based on density of reads (Materials and Methods; Data Set S1). Using
147 accepted strategies, the fitness of each insertion mutant strain was calculated relative to the entire
148 pool (31). To standardized results across experiments, fitness values were normalized to
149 insertions found in 18 neutral sites located in pseudogenes or endogenous transposon-related
150 genes throughout the genome ("neutral" mutants), to allow an accurate quantitation of the
151 representation of mutants relative to control insertions predicted to have no effect on growth
152 (32). The normalized data from the individual insertion mutations were aggregated for each
153 gene to calculate a mean fitness level for the entire spectrum of mutations found within a
154 particular gene. The complete datasets were then displayed on an individual gene level as the
155 growth rate changes for mutations relative to the growth rate of the entire pool (Fig. 1B).
156 Candidate mutants were identified that showed lower (hypersensitizing loci) or higher fitness
157 levels based on the criteria that the False Discovery Rate (FDR) q value was <0.05, a change in

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

158 fitness (W_{diff}) was >10%, and fitness value was derived from at least 3 independent insertion
159 mutants ((33); Materials and Methods).

160 Small increases in the dose of ciprofloxacin greatly increased the spectrum of
161 ciprofloxacin hypersensitivity loci (Fig. 1B; black). At a dose causing approximately 20%
162 growth inhibition, mutations in only 10 genes passed the criteria for lower fitness relative to the
163 rest of the pool in the presence of the drug. These included insertions in: two genes involved in
164 double strand break repair (*recB* and *ruvA*, encoding subunits of exonuclease V and the Holiday
165 junction helicase); the major egress pump which is often found overproduced in clinical strains
166 having high level fluoroquinolone resistance (*adeIJK*); and *ctpA*, a periplasmic protease shown
167 to be a target of mutations that augment β-lactam resistance in strains lacking the *bfmRS* global
168 regulatory system (34) (Data Set S1). Increasing the drug dose had two effects on expanding the
169 spectrum of hypersensitivity loci. First, although the number of hypersensitivity loci that
170 contribute to the enzymology of DNA repair increased from 2 members to 20 in the high dose
171 regimen, this expansion largely involved hitting additional subunits of the same complexes or
172 backup systems of the enzymes identified in the low dose regimen (*recBCD, sbcCD*, *ruvABC*)
173 (Data Set S1). This emphasizes the importance of protecting against double strand breaks caused
174 by fluoroquinolone-poisoned DNA gyrase (35). Secondly, increasing dose resulted in
175 hypersensitivity loci in cell envelope integrity proteins, additional protein-processing enzymes,
176 and a MATE class proton-driven efflux pump (*abeM*) shown to export ciprofloxacin and other
177 antibiotic compounds when cloned in *E. coli* (36) (Data Set S1; Fig. 1C). Interestingly,
178 increasing the drug dose did not implicate the two other major RND efflux systems in protecting
179 from ciprofloxacin stress even though they are known to provide low-level resistance after
180 overproduction (12). This may be explained by the fact that the *adeIJK* system is the only RND

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

181 egress pump known to have a high basal level of expression in WT strains lacking acquired
182 resistance mutations, while the inducing signals for the other systems have not been identified
183 (12). Strikingly, at the higher drug doses, mutations in *adeN*, which encodes the negative
184 regulator of *adeIJK,* increased the fitness of *A. baumannii* relative to the rest of the pool (Data
185 Set S1). These data argue strongly that the primary efflux pumps involved in intrinsic protection
186 from fluoroquinolone stress are AdeIJK and AbeM.
187 In addition to hypersensitivity loci, mutations were identified at the highest drug dose that
188 resulted in increased fitness relative to the insertion pool (Fig. 1B and C, Data Set S1). The
189 mutations that most frequently increased fitness targeted nonessential components of the protein
190 translation machinery, particularly enzymes that post-translationally modify tRNA, rRNA and
191 assembly of ribosomal protein complexes. That disruption of this circuit is tightly associated
192 with increased drug resistance is consistent with studies showing that a spectrum of antibiotic
193 resistant isolates in different species evolve mutations causing slowed translation rate (37, 38).
194 The results are also consistent with a study demonstrating that lowering ribosomal synthesis
195 increases resistance to ciprofloxacin by restoring an optimal balance between protein and DNA
196 synthesis levels during DNA stress (39)). Most notable among the insertions identified were
197 those in *gidA*, which is part of a complex involved in 5-methylaminomethyl-2-thiouridine
198 (mnm^5s^2U34) modification of tRNAs (Data Set S1; (40)). We have previously identified this
199 gene as an additional target of mutations bypassing drug hypersensitivity resulting from loss of
200 *bfmRS* (34), indicating the tight connection between mutations in this gene and drug resistance.
201 **Deletion mutants have drug sensitivities predicted by Tn-seq.** Targeted deletion or
202 null mutations were isolated in nonessential genes predicted to have altered drug sensitivity in
203 the presence of ciprofloxacin. The mutations were chosen based on their fitness in the Tn-seq

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

204 analysis, the magnitude of the effects predicted, and differing functional categories (Fig. 1C). For
205 instance, mutations in the egress pump-encoding *adeIJK* showed extremely poor fitness and
206 were rarely recovered after growth in ciprofloxacin (Fig. 2A). Similarly, the insertions in *ctpA*
207 showed very low fitness. In contrast, although ciprofloxacin treatment lowered fitness for
208 mutants lacking the penicillin binding protein PBP1A, these mutations clearly had weaker effects
209 in the Tn-seq analysis. When this set of targeted mutants was analyzed further, loss of *adeIJK,*
210 *recN, ctpA* and *pbp1A* all resulted in heightened drug sensitivity (Fig. 2B). In contrast, deletion
211 of *gidA*-encoded tRNA modification enzyme resulted in enhanced fitness in the presence of
212 ciprofloxacin, with increased yields in broth cultures exposed to 0.15 µg/ml of antibiotic (Fig.
213 2B).

214 **Identification of loci that result in altered ciprofloxacin sensitivity in *A. baumannii***
215 **target site mutants.** A majority of the current clinical isolates of *A. baumannii* are resistant to
216 fluoroquinolones, and these isolates commonly have the *gyrA*(S81L) and *parC*(S84L) target site
217 mutations that lower the affinity for these antibiotics (41). To determine the spectrum of
218 insertions that cause altered sensitivity to ciprofloxacin in strains having resistance alleles,
219 *gyrA*(S81L) (hereafter referred to as *gyrA*^{R}) and *gyrA*(S81L) *parC*(S84L) (referred to as *gyrA*^{R}
220 *parC*^{R}) mutants were generated, and each strain was subjected to Tn*10* mutagenesis. Pools
221 totaling more than 70,000 insertion mutations were constructed in each background. Insertion
222 pools were challenged with ciprofloxacin, using drug concentrations below the MIC (Table 1)
223 that resulted in 30-40% growth inhibition for each strain (1.1µg/ml for *gyrA*^{R}; 13-14 µg/ml for
224 *gyrA*^{R} *parC*^{R} double mutant; Fig. 3A). The spectrum of insertions that resulted in
225 hypersensitivity to ciprofloxacin in the *gyrA*^{R} *parC*^{R} double mutant strain backgrounds was very
226 similar to the WT (Fig. 3C,D). In fact, almost every ciprofloxacin hypersensitive locus in the

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

227 double mutant background was identified previously in the WT (green circles, Fig. 3C; Data Sets
228 S1 and S3). In addition, there was a number of hypersensitivity loci identified in the WT pools
229 that did not pass the discovery criteria in the double mutant (FDR<0.05; W_{diff}>0.1). A number of
230 these below-threshold candidates in the *gyrA*^{R} *parC*^{R} double mutant strain background encoded
231 subunits of the proteins identified as ciprofloxacin-hypersensitive loci (green circles, Fig. 3C;
232 Data Set S3). These results are similar to what we had observed in our graded series of drug
233 treatments of insertion pools in the WT strain, indicating that the results from the WT strain and
234 the drug resistant double mutant are largely the same.

235 The results from the *gyrA*^{R} single mutant background, however, diverged greatly from the
236 WT and the *gyrA*^{R} *parC*^{R} double mutant (Fig. 3E). A large fraction of insertions were identified
237 that altered drug sensitivity to ciprofloxacin, with a surprising number showing increased fitness
238 during drug exposure (Fig. 3E). Over 40 of the insertions that exhibited increased fitness were
239 located in putative prophage genes (blue and yellow circles, Fig. 3E; Fig. 3F) from two of the
240 three predicted phages integrated into the bacterial chromosome (Fig. 3B; Data Set S2). No such
241 fitness changes were seen in the WT (Fig. S2, Data Set S1) or *gyrA*^{R} *parC*^{R} double mutant (Fig.
242 3C, Data Set S3). To analyze this result further, the normalized fitness of mutations in each gene
243 was plotted as a function of position on the chromosome. In the absence of antibiotic, there was
244 no clear positional effect of altered fitness levels along the length of the chromosome (Fig. 3G).
245 In contrast, in the presence of antibiotic, there was an apparent increase in fitness levels centered
246 within chromosomal locations harboring prophages P1 and P3 in the *gyrA*^{R} single mutant (Fig.
247 3G). Although some of this effect could be explained by loss of prophage gene function
248 resulting in enhanced fitness, insertions in chromosomal regions near, but outside, the prophage
249 boundaries similarly showed apparent increases in fitness relative to the rest of the chromosomal

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

250 insertions (Figs. 3G and H). As fitness levels are measured by counting the number of reads in
251 specific regions of DNA, this phenomenon is consistent with selective local amplification of
252 chromosomal material that initiates within these prophages, extending outward from the
253 integration sites into nearby DNA regions.
254 **Two prophage regions are selectively amplified in response to ciprofloxacin in the**
255 **single *gyrA*^{R} mutant**. We next tested the model that there is induction of DNA synthesis in the
256 region surrounding two of the chromosomally-located prophage clusters. Purified single colonies
257 from the WT, *gyrA*^{R}, and *gyrA*^{R}*parC*^{R} double mutant strains were grown in broth culture for 3.5
258 hours in the presence of four different concentrations of ciprofloxacin that ranged from 30-80%
259 growth inhibition and compared to bacteria grown in the absence of drug (Fig. 4A). DNA was
260 then prepared from each of the cultures and subjected to whole genome sequencing using an
261 average read length of 100 bp. The density of these individual short reads was plotted as a
262 function of the chromosomal coordinates, to identify regions of chromosomal DNA that were
263 selectively amplified in the presence of drug (Fig. 4B). Analysis of the *gyrA*^{R} single mutant
264 showed hyperamplification of prophages 1 and 3, with read density in the prophage regions
265 observed as a function of drug concentration. In contrast, there was little evidence of this
266 selective amplification in the WT strain, while the *gyrA*^{R}*parC*^{R} largely reversed these effects.
267 Consistent with the Tn-seq data, there was amplification of DNA extending beyond the
268 prophage-chromosomal DNA junction, indicating that drug-driven DNA synthesis was initiated
269 *in situ* and continued beyond the ends of the prophages into adjacent chromosomal DNA (Fig.
270 4C). We conclude that in a *gyrA*^{R} background, selective blockage of the *parC*-encoded Topo IV
271 protein resulted in DNA synthesis induction in these two prophage regions.

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

272 To determine if transcription of prophage genes is specifically amplified in the *gyrA*^{R}
273 mutant relative to the WT, the two strains were grown in triplicate cultures in two concentrations
274 of antibiotic for 3.5 hours that gave between 40-70% growth inhibition over approximately 7
275 generations (Fig. 5A). The cells were then extracted, subjected to RNAtag-seq analysis (42), and
276 the ratio of transcription for each gene in the presence/absence of ciprofloxacin was displayed as
277 a function of chromosomal map position (Fig. 5B, Data Set S4). There was preferential
278 amplification of transcription of prophage genes in the presence of antibiotic treatment in both
279 strain backgrounds (Fig. 5B). Furthermore, transcription was hyperactivated in all three
280 prophages, including prophage 2 which showed no evidence of preferential DNA amplification
281 (compare Figs 4B and 5B). Higher expression levels were observed with prophage genes in the
282 *gyrA*^{R} single mutant compared to WT (Fig. 5B), and these levels were also apparent when
283 directly comparing transcription in WT and *gyrA*^{R} after ciprofloxacin treatment (Fig. S3). In
284 *gyrA*^{R}-single mutants, enhanced expression in response to ciprofloxacin extended beyond the
285 prophage-chromosomal DNA junctions with prophage 1 and to some extent with prophage 3
286 (Fig. 5C and S3), consistent with increased DNA template availability partially contributing to
287 heightened transcription in this strain background. Hyperexpression in response to ciprofloxacin
288 terminated at the prophage-chromosomal DNA junctions with prophage 2, consistent with the
289 observation that this region experienced no DNA amplification (Fig. 5C). These results indicate
290 that preferential blockage of Topo IV in the single mutant results in hyperactivation of prophage
291 transcripts.

292 Intoxication of bacterial topoisomerase enzymes by fluoroquinolone antibiotics induces
293 DNA damage, driving an SOS response (16, 43). We investigated the extent to which
294 ciprofloxacin-induced hyperactivation of prophage gene expression coincided with SOS

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

295 response induction, and whether *gyrA* or *parC* resistance alleles influenced this response. Several
296 genes associated with the SOS response (27, 43, 44) showed heightened transcription as a
297 consequence of ciprofloxacin treatment (Fig. 6A). For several SOS genes, transcript induction
298 was significantly higher in *gyrA*^{R} compared to WT (Fig. 6A; asterisks). These included genes
299 adjacent to prophage-chromosomal DNA junctions (*umuC* and *umuD* paralogs) as well as those
300 not directly linked to prophages (*recA*, *gst*; Fig. 6B). RecA is a key component of the SOS
301 response that is induced by DNA damage in *A. baumannii* and is critical for withstanding
302 ciprofloxacin stress independent of the background resistance genotype (see Data Sets S1-S3).
303 To analyze the interplay of SOS induction by ciprofloxacin with target availability at the level of
304 single cells, we utilized a plasmid-based transcriptional fusion of the *recA* regulatory elements
305 (promoter and 5'-untranslated region) to the fluorescent reporter *mKate2* (45). WT, *gyrA*^{R}, and
306 *gyrA*^{R}*parC*^{R} strains harboring the reporter fusion were cultured in the presence of graded levels
307 of ciprofloxacin, and reporter signal was measured in individual cells by fluorescence
308 microscopy (Materials and Methods). Increasing sub-MIC doses of ciprofloxacin caused
309 increasing degrees of induction of the *recA* reporter in all strain backgrounds (Fig. 6C). Notably,
310 reporter activity was approximately 2-fold higher in the *gyrA*^{R} single mutant than in the WT or
311 double mutant at equivalent levels of growth inhibition (Fig. 6C). Varying degrees of *recA*
312 induction within populations of *gyrA*^{R} single mutant cells were observed, and this variability
313 roughly matched that observed with WT (Fig. S4). Increased signal in the *gyrA*^{R} strain was not
314 observed with a control reporter fusion to a gene that is nonresponsive to ciprofloxacin (*trpB*p-
315 UTR) ((45), Fig 6D), indicating that the SOS transcriptional response was specifically enhanced
316 as a consequence of ciprofloxacin inhibition of Topo IV.
317

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

318

319 **Discussion**

320 In this study we exploited the dual-target nature of fluoroquinolone antibiotics to uncover how
321 resistance alleles acquired in target enzymes modulate the landscape of intrinsic resistance.
322 Using Tn-seq, we performed comprehensive screens for determinants of resistance to the
323 fluoroquinolone drug ciprofloxacin in isogeneic *A. baumannii* strains in which the drug
324 preferentially targets either DNA gyrase or Topo IV. We found that the spectrum of genes
325 contributing to intrinsic resistance was similar in genetic backgrounds in which both enzymes
326 were WT or in which both enzymes had lowered drug sensitivity due to well-known acquired
327 point mutations. Intrinsic resistance determinants identified in both backgrounds included the
328 AdeIJK and AbeM efflux pumps, multiple subunits of the DNA recombination and repair
329 machinery, a periplasmic protease CtpA, the cell wall transpeptidase PBP1A, and several
330 proteins of unknown function. By contrast, interaction of ciprofloxacin with the *gyrA*^{R} single
331 mutant in which Topo IV is the sensitive target dramatically altered the profile of genes that
332 influence relative Tn-seq fitness. This altered fitness profile in *gyrA*^{R}*parC*$^{+}$ bacteria was shown
333 to directly reflect amplification of DNA in the vicinity of two endogenous prophages due to
334 preferential poisoning of Topo IV by ciprofloxacin. Prophage transcripts and the SOS pathway
335 were also hyperactivated as a consequence of this drug-genotype interaction, likely facilitating
336 the initiation of synthesis of prophage DNA in the *gyrA*^{R} strain.

337 Our data can be explained by the model shown in Fig. 7, if we assume that re-activation
338 of *A. baumannii* prophages 1 and 3 requires the function of host DNA gyrase. In WT and the
339 *gyrA*^{R} *parC*^{R} double mutant, DNA gyrase is the effective target blocked by ciprofloxacin at
340 growth-inhibitory, sub-MIC drug concentrations. Prophage DNA synthesis is blocked despite

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

341 induction of the DNA damage response and prophage gene transcription because, as postulated
342 by the model, efficient replication of the prophage genomes requires functional host gyrase (Fig.
343 7A and C). By contrast, in *gyrA*^{R} bacteria, Topo IV is the preferred target of intoxication by
344 ciprofloxacin. This interaction causes DNA lesions that robustly stimulate the SOS pathway and
345 prophage transcription; further, host DNA gyrase is available to facilitate prophage genome
346 replication because the GyrA S81L (*gyrA*^{R}) variant is resistant to the intermediate concentrations
347 of ciprofloxacin required for Topo IV poisoning (Fig. 7B). Therefore, the presence of the single
348 *gyrA*^{R} resistance generates a liability that is not observed in other strains, resulting from the
349 induction of potentially lethal prophage replication in the presence of fluoroquinolones.

350 The central assumption of the model is plausible based on analogy with several other
351 bacteriophage systems that have been shown to require host DNA gyrase for replication. Gyrase
352 inhibitors (quinolones or aminocoumarins) inhibit phage DNA replication during lytic growth
353 after infection (46-51), and disrupt induction of replicative transposition in Mu lysogens (52).
354 Moreover, host gyrase is required for propagation of replication forks within supercoiled DNA
355 substrates in reconstituted systems modeling phage lambda replication (53, 54). The importance
356 of this enzyme class for replication of *A. baumannii* strain 17978 prophages is emphasized by the
357 fact that they do not encode type II topoisomerases which are often encoded by bacteriophages to
358 bypass a requirement for the host enzymes (55, 56).

359 An alternative model is that at the sub-MIC drug doses resulting in equivalent growth
360 inhibition, gyrase poisoning results in DNA lesions that do not stimulate the SOS response above
361 the threshold required for efficient prophage induction, in contrast to lesions caused by Topo IV
362 poisoning. Arguing against this model are the observations that transcription of the SOS response
363 gene *recA* and genes from all three prophages are strongly induced (25-50 fold, Figs. 5B and 6C)

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

364 above baseline in WT cells, and Tn-seq fitness results showing the relatively similar importance
365 of DNA damage repair enzymes across all strain backgrounds (Data Sets S1-S3). We showed
366 that Topo IV intoxication stimulated the SOS pathway to a greater extent than that caused by
367 gyrase poisoning, potentially contributing to the robust activation of prophages. This is
368 consistent with previous findings that Topo IV and gyrase intoxication can be distinguished by
369 several characteristics. Topo IV lesions result in slower inhibition of DNA synthesis and are
370 thought to be more readily reversed by recombinational repair, resulting in lower cytotoxicity at
371 given drug concentrations (16). These less toxic lesions could potentially expose more numerous
372 or potent signals for the SOS response that could result in prophage induction.

373 In contrast with induction of prophages 1 and 3, ciprofloxacin-induced DNA replication
374 was not observed with prophage 2 despite activation of prophage gene transcription. One
375 possible explanation is that prophage 2 is defective for DNA replication. We consider this
376 unlikely because transposon insertions were unobtainable in a phage locus (ACX60_RS10145)
377 encoding a putative Cro/Cl family repressor (Data Sets S1-S3), indicating that this prophage has
378 the potential for lytic replication in the absence of a protein controlling lysogeny maintenance.
379 Consistent with the potential of all three prophages (including prophage 2) for replication,
380 mobilized DNA corresponding to each of the three prophages was detected in phage particles
381 resulting from treatment of WT *A. baumannii* 17978 with mitomycin C, which damages DNA
382 directly without dependence on interactions with DNA topoisomerases (44). An alternative
383 explanation for the lack of prophage 2 DNA amplification observed with fluoroquinolone
384 treatment in our study is that its replication depends on both DNA gyrase and Topo IV.

385 The findings described here have implications for the evolution of antibiotic resistance in
386 *A. baumannii* and other Gram-negative organisms. They indicate that in the trajectory toward

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

387 high-level fluoroquinolone resistance, intermediate states with moderate-level fluoroquinolone
388 resistance (exemplified by the *gyrA*^{R} single mutant) are those that possess highest potential for
389 prophage induction during growth with continued drug exposure. Depending on the outcome of
390 phage-host interactions, prophage hyperamplification within these bacteria could impose a
391 fitness burden or could result in cell death if productive lysis ensues, representing additional
392 selective pressures to influence evolution when under stress from the inducing antibiotic.
393 Acquiring the subsequent *parC* mutation would answer this pressure and result in high-level
394 fluoroquinolone resistance. It is notable that hyperamplified and hyperexpressed DNA within or
395 adjacent to induced prophages include multiple *umuCD* paralogs encoding mutagenic DNA
396 polymerases (57), whose higher levels of activity could increase mutation frequency and hasten
397 bacterial adaptation in drug-treated *gyrA*^{R} single mutants. Moreover, these findings raise the
398 possibility of enhanced horizontal transfer of phage-encoded and phage-proximal genes as a
399 consequence of fluoroquinolone-*gyrA*^{R} interactions. The relationship between stepwise
400 fluoroquinolone resistance and induction of prophages by this drug class may play out differently
401 with Gram-positive organisms in which Topo IV is typically the sensitive initial target as
402 opposed to gyrase (6). If fluoroquinolone-prophage dynamics in such bacteria have features that
403 accord with the model proposed here, WT strains with two sensitive *parC* and *gyrA* alleles may
404 represent the state with higher potential for drug-induced prophage replication than derivatives
405 that have acquired single-step target-resistance mutations.

406 In summary, we have demonstrated that in the course of stepwise selection for high drug
407 resistance, intermediate steps result in unexpected nodes of hypersensitivity that place both
408 added pressure for acquisition of additional drug resistant alleles, as well as inducing the
409 enzymatic machinery that drives acquisition of drug resistance. Future work on analysis of

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

410 proteins that modulate the survival of drug resistant mutants should uncover strategies that allow
411 these variants to be targeted therapeutically.
412
413 **Materials and Methods**
414 **Bacterial strains, growth conditions, and antibiotics.** Bacterial strains used in this work are
415 described in Table S1. *A. baumannii* strains were derivatives of ATCC 17978. Bacterial cultures
416 were grown at 37ºC in Lysogeny Broth (LB) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl)
417 in flasks with shaking or in tubes on a roller drum. Growth was monitored by measuring
418 absorbance at 600nm via a spectrophotometer. LB agar was supplemented with antibiotics
419 [ampicillin (Amp, 50-100 μg/ml), carbenicillin (Cb, 50-100 μg/ml), kanamycin (Km, 10-20
420 μg/ml), mecillinam, ciprofloxacin] or sucrose as needed (Sigma Aldrich).
421
422 **Molecular cloning and mutant construction.** Oligonucleotide primers and plasmids used in
423 this study are listed in Table S2. Single *gyrA*S81L (*gyrA*^{R}) and *parC*S84L (*parC*^{R}) point
424 mutations were generated by cloning the respective genomic fragments in pUC18, followed by
425 inverse PCR and self-ligation or amplification and substitution of a mutated gene fragment. In-
426 frame deletions of ciprofloxacin-resistance genes were generated as described (58). Constructs
427 were subcloned in pSR47S and used to isolate *A. baumannii* mutants via homologous
428 recombination with two selection steps (58). The *gyrA*^{R} *parC*^{R} double mutant was isolated by
429 selection of a derivative of the *gyrA*^{R} strain able to grow on LB agar containing 8 μg/ml
430 ciprofloxacin. A *pbp1a* mutant (N178TfsX27) was isolated as a derivative of ATCC 17978
431 selected on LB agar containing 64 μg/ml mecillinam.
432

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

433 **Antibiotic susceptibility assays.** For growth curve analysis, cultures were seeded at A_{600} =
434 0.003 in 100µl of broth in wells of a 96-well microtiter plate and growth monitored during
435 incubation at 37ºC with orbital shaking in a Tecan M200 Pro plate reader. MIC tests were
436 performed under the conditions above using serial 2-fold dilutions of drug; the MIC was the
437 lowest drug concentration preventing growth above A_{600} = 0.05 after 16 hours.
438
439 **Construction of transposon mutant libraries.** Plasmid pDL1073 was employed for transposon
440 mutagenesis. pDL1073 contains a Km^R Tn10 derivative, an altered target-specificity Tn10
441 transposase gene downstream of the phage lambda P_L promoter, a pSC101ts origin of replication,
442 and β-lactamase (Amp^R; Fig. S1). pDL1073 does not replicate in *A. baumannii* at 37°C allowing
443 efficient detection at this temperature of transposition after delivery via electroporation. *A.*
444 *baumannii* cells (50µl) were combined with 100ng pDL1073 and electroporated via a BioRad
445 Gene Pulser (0.1cm gap length cuvette; 200Ω, 25µF, and 1.8kV). Electroporated cells were
446 diluted with SOC broth and immediately spread onto membrane filters (0.45µm pore size)
447 overlaid on pre-warmed LB agar plates. After incubating 2 hours at 37ºC, the filter membranes
448 were transferred to pre-warmed LB agar plates containing 20µg/ml Km and incubated at 37ºC
449 overnight to select for transposon mutants. Bacterial colonies were lifted from the filter by
450 agitation in sterile PBS. Glycerol was added to 10% (v/v), and pooled mutant suspensions were
451 aliquoted and stored at -80ºC. 11-15 independent pools each consisting of approximately 6,000-
452 18,000 mutants were generated in each strain background.
453
454 **Tn-seq fitness measurements.** Transposon library aliquots were thawed, vortexed, diluted to
455 A_{600} = 0.1 and grown to A_{600} = 0.2 in LB. Cultures were then back-diluted to A_{600} = 0.003 in

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

456 10ml LB without drug or with graded concentrations of ciprofloxacin. Parallel cultures were
457 grown at 37˚C for approximately 8 generations to A_{600} = 0.5-1. Samples taken at the start (t_1) and
458 end (t_2) of this outgrowth were stored at -20ºC. 11 to 15 independent transposon libraries were
459 analyzed with each strain background. With WT libraries, treatments with 0.075 µg/ml and 0.09-
460 0.1 µg/ml ciprofloxacin were performed in parallel with the same untreated control.
461
462 **Tn-seq Illumina library preparation.** Genomic DNA was extracted from t_1 and t_2 samples
463 (Qiagen DNeasy Kit) and quantified by a SYBR green microtiter assay. Transposon-adjacent
464 DNA was amplified for Illumina sequencing using a modification of the Nextera^{TM} DNA Library
465 Prep method (Illumina). 30ng of genomic DNA was used as input in a 10µl tagmentation
466 reaction. Reaction conditions were 55˚C for 5min followed by inactivation at 95˚C for 0.5min.
467 Transposon-adjacent genomic DNA was amplified by adding 40µl of PCR master mix
468 containing primers olj928 and Nextera 2A-R (0.6µM final) and Q5 High-Fidelity polymerase
469 (NEB). Reaction conditions were 98˚C for 10s, 65˚C for 20s, and 72˚C for 1min (30 cycles),
470 followed by a final extension at 72˚C for 2min. A second PCR was performed using nested,
471 indexed primers. This reaction contained 0.5µL of the first PCR reaction, Left Tn10 indexing
472 primer (0.6µM), Right indexing primer (0.6µM) and Q5 polymerase in a 50µl final volume.
473 Reaction conditions were 98˚C for 10s, 65˚C for 20s, and 72˚C for 1min (12 cycles of), followed
474 by a final extension at 72˚C for 2min. A sample of the second PCR product was imaged after
475 separation on a 2% agarose/TAE gel containing SYBR Safe dye. Samples were multiplexed
476 based on signal intensity in the 250-600bp region and purified (Qiagen QIAquick). 15-20pmol of
477 DNA was used as template in a 50µl reconditioning reaction containing adapter-specific primers
478 P1 and P2 (0.6µM) and Q5 polymerase. Reaction conditions were 95˚C for 1min, 0.1˚C/sec ramp

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

479 to 64˚C, 64˚C for 20s, 72˚C for 10min. Samples were purified (Qiagen QIAquick), followed by
480 quantification and size selection (250-600bp, Pippin HT) by the Tufts University Genomics Core
481 Facility (TUCF-Genomics). Libraries were sequenced (single-end 50bp) using custom primer
482 olk115 on a HiSeq2500 with High Output V4 chemistry at TUCF-Genomics.
483
484 **Tn-seq data analysis.** Reads were demultiplexed, quality-filtered and clipped of adapters before
485 serving as input for mapping and fitness calculations (31). Reads were mapped to the *A.*
486 *baumannii* 17978-mff chromosome (NZ_CP012004) and plasmids (NC_009083, NC_009084,
487 and NZ_CP012005) using previously described parameters (59). Fitness values for each
488 transposon mutant were calculated by comparing mutant vs population-wide expansion between
489 t_1 and t_2 (31). Per-gene average fitness and SD were then computed from fitness scores for all
490 insertion mutations within a gene across multiple parallel transposon pools. Differences in
491 average gene fitness between treated and untreated conditions (W_{diff}) were considered significant
492 if they fulfilled the following 3 criteria, with minor modification from those previously described
493 (33): per-gene fitness must be calculated from at least 3 data points, the magnitude of W_{diff} must
494 be > 10%, and q value must be < 0.05 in an unpaired t-test with FDR controlled by the 2-stage
495 step-up method of Benjamini, Krieger and Yekutieli (GraphPad Prism 7). Per-insertion fitness
496 scores within a given genomic region were visualized using Integrative Genomics Viewer
497 software (60) after aggregating all scores across multiple independent transposon mutant
498 libraries using the SingleFitness Perl script (61).
499
500 **Whole-genome sequencing of individual strains subjected to ciprofloxacin.** WT, *gyrA*^{R}, or
501 *gyrA*^{R} *parC*^{R} strains were grown from single colonies to early post-exponential phase and back-

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

502 diluted to A_{600} 0.003. Parallel cultures were grown for 2.5 hours in the absence of treatment, or
503 3.5 hours in the presence of ciprofloxacin treatment. DNA was extracted (Qiagen DNeasy) and
504 Illumina sequencing libraries were amplified and sequenced as described (34). After mapping to
505 NZ_CP012004, coverage files were generated from the resulting BAM files using deepTools,
506 with reads normalized to counts per million (62).
507
508 **Transcriptional profiling.** Cultures were diluted to A_{600} 0.003 and grown for 2.5 hours
509 (untreated) or 3.5 hours (ciprofloxacin treated). Cultures were mixed with an equal volume of
510 ice-cold acetone:ethanol (1:1) and stored at -80ºC. Cells were thawed and washed with TE and
511 RNA was extracted (Qiagen RNeasy). RNA samples were diluted, combined with SUPERase-in
512 (Invitrogen), and processed via the RNAtag-seq method (42). Illumina cDNA sequencing
513 libraries were sequenced and reads processed as described (63). Differential expression was
514 calculated using DESeq2 (64).
515
516 **Fluorescence reporter assays.** Strains containing pCC1 or pCC7 were cultured in the presence
517 or absence of ciprofloxacin as in RNA-seq experiments. Cells were immobilized on agarose pads
518 and imaged on a Leica AF6000 microscope using a 100X/1.3 objective and TX2 filtercube
519 (excitation: BP 560/40, dichromatic mirror 595, emission: BP 645/75). MicrobeJ (65) was used
520 to measure background-corrected mean fluorescence intensity per cell. Median cellular
521 fluorescence intensities from populations of at least 100 bacteria were determined, and median
522 values across multiple independent experiments were averaged.
523

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

524 **Accession Number(s).** Sequencing reads analyzed in this study were deposited into SRA
525 database as: SRP157243 (Tn-seq), PRJNA495614 (RNA-seq), and PRJNA495623 (Whole
526 genome sequencing).
527
528 **Acknowledgements.**
529 This work was supported by NIAID awards U01AI124302 to RRI and TVO, R21AI128328 to
530 RRI, and F32AI098358 to EG. NSF REU Site Award #1757443 to the Northeastern University
531 Department of Biology supported the research of ELW. We thank Carly Ching and Veronica
532 Godoy for gift of pCC1 and pCC7, and Amy Tang for assistance with bioinformatics analysis.
533
534 **References**
535
536 1. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C,
537 Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M,
538 Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N,
539 Group WHOPPLW. 2018. Discovery, research, and development of new antibiotics: the
540 WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis
541 18:318-327.
542 2. Warner WA, Kuang SN, Hernandez R, Chong MC, Ewing PJ, Fleischer J, Meng J, Chu
543 S, Terashita D, English L, Chen W, Xu HH. 2016. Molecular characterization and
544 antimicrobial susceptibility of Acinetobacter baumannii isolates obtained from two
545 hospital outbreaks in Los Angeles County, California, USA. BMC Infect Dis 16:194.
546 3. Kim D, Ahn JY, Lee CH, Jang SJ, Lee H, Yong D, Jeong SH, Lee K. 2017. Increasing
547 Resistance to Extended-Spectrum Cephalosporins, Fluoroquinolone, and Carbapenem in
548 Gram-Negative Bacilli and the Emergence of Carbapenem Non-Susceptibility in
549 Klebsiella pneumoniae: Analysis of Korean Antimicrobial Resistance Monitoring System
550 (KARMS) Data From 2013 to 2015. Ann Lab Med 37:231-239.
551 4. Blanco N, Harris AD, Rock C, Johnson JK, Pineles L, Bonomo RA, Srinivasan A,
552 Pettigrew MM, Thom KA, the CDCEP. 2018. Risk Factors and Outcomes Associated
553 with Multidrug-Resistant Acinetobacter baumannii upon Intensive Care Unit Admission.
554 Antimicrob Agents Chemother 62.
555 5. Drlica K, Hiasa H, Kerns R, Malik M, Mustaev A, Zhao X. 2009. Quinolones: action and
556 resistance updated. Curr Top Med Chem 9:981-98.
557 6. Hooper DC. 1999. Mechanisms of fluoroquinolone resistance. Drug Resist Updat 2:38-
558 55.

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

559 7. Jacoby GA. 2005. Mechanisms of resistance to quinolones. Clin Infect Dis 41 Suppl
560 2:S120-6.
561 8. Hujer KM, Hujer AM, Hulten EA, Bajaksouzian S, Adams JM, Donskey CJ, Ecker DJ,
562 Massire C, Eshoo MW, Sampath R, Thomson JM, Rather PN, Craft DW, Fishbain JT,
563 Ewell AJ, Jacobs MR, Paterson DL, Bonomo RA. 2006. Analysis of antibiotic resistance
564 genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients
565 treated at the Walter Reed Army Medical Center. Antimicrob Agents Chemother
566 50:4114-23.
567 9. Valentine SC, Contreras D, Tan S, Real LJ, Chu S, Xu HH. 2008. Phenotypic and
568 molecular characterization of Acinetobacter baumannii clinical isolates from nosocomial
569 outbreaks in Los Angeles County, California. J Clin Microbiol 46:2499-507.
570 10. Fernando D, Zhanel G, Kumar A. 2013. Antibiotic resistance and expression of
571 resistance-nodulation-division pump- and outer membrane porin-encoding genes in
572 Acinetobacter species isolated from Canadian hospitals. Can J Infect Dis Med Microbiol
573 24:17-21.
574 11. Rumbo C, Gato E, Lopez M, Ruiz de Alegria C, Fernandez-Cuenca F, Martinez-Martinez
575 L, Vila J, Pachon J, Cisneros JM, Rodriguez-Bano J, Pascual A, Bou G, Tomas M,
576 Spanish Group of Nosocomial I, Mechanisms of A, Resistance to A, Spanish Society of
577 Clinical M, Infectious D, Spanish Network for Research in Infectious D. 2013.
578 Contribution of efflux pumps, porins, and beta-lactamases to multidrug resistance in
579 clinical isolates of Acinetobacter baumannii. Antimicrob Agents Chemother 57:5247-57.
580 12. Yoon EJ, Chabane YN, Goussard S, Snesrud E, Courvalin P, De E, Grillot-Courvalin C.
581 2015. Contribution of resistance-nodulation-cell division efflux systems to antibiotic
582 resistance and biofilm formation in Acinetobacter baumannii. MBio 6:e00309-15.
583 13. Poole K. 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria.
584 Antimicrob Agents Chemother 44:2233-41.
585 14. Recacha E, Machuca J, Diaz de Alba P, Ramos-Guelfo M, Docobo-Perez F, Rodriguez-
586 Beltran J, Blazquez J, Pascual A, Rodriguez-Martinez JM. 2017. Quinolone Resistance
587 Reversion by Targeting the SOS Response. MBio 8.
588 15. Cirz RT, Chin JK, Andes DR, de Crecy-Lagard V, Craig WA, Romesberg FE. 2005.
589 Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol
590 3:e176.
591 16. Khodursky AB, Cozzarelli NR. 1998. The mechanism of inhibition of topoisomerase IV
592 by quinolone antibacterials. J Biol Chem 273:27668-77.
593 17. Mo CY, Manning SA, Roggiani M, Culyba MJ, Samuels AN, Sniegowski PD, Goulian
594 M, Kohli RM. 2016. Systematically Altering Bacterial SOS Activity under Stress Reveals
595 Therapeutic Strategies for Potentiating Antibiotics. mSphere 1.
596 18. Nichols RJ, Sen S, Choo YJ, Beltrao P, Zietek M, Chaba R, Lee S, Kazmierczak KM,
597 Lee KJ, Wong A, Shales M, Lovett S, Winkler ME, Krogan NJ, Typas A, Gross CA.
598 2011. Phenotypic landscape of a bacterial cell. Cell 144:143-56.
599 19. Sutherland JH, Tse-Dinh YC. 2010. Analysis of RuvABC and RecG involvement in the
600 escherichia coli response to the covalent topoisomerase-DNA complex. J Bacteriol
601 192:4445-51.
602 20. Tamae C, Liu A, Kim K, Sitz D, Hong J, Becket E, Bui A, Solaimani P, Tran KP, Yang
603 H, Miller JH. 2008. Determination of antibiotic hypersensitivity among 4,000 single-
604 gene-knockout mutants of Escherichia coli. J Bacteriol 190:5981-8.

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

605 21. Urios A, Herrera G, Aleixandre V, Blanco M. 1990. Expression of the recA gene is
606 reduced in Escherichia coli topoisomerase I mutants. Mutat Res 243:267-72.
607 22. Brazas MD, Breidenstein EB, Overhage J, Hancock RE. 2007. Role of lon, an ATP-
608 dependent protease homolog, in resistance of Pseudomonas aeruginosa to ciprofloxacin.
609 Antimicrob Agents Chemother 51:4276-83.
610 23. Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE. 2008. Complex
611 ciprofloxacin resistome revealed by screening a Pseudomonas aeruginosa mutant library
612 for altered susceptibility. Antimicrob Agents Chemother 52:4486-91.
613 24. van Opijnen T, Camilli A. 2012. A fine scale phenotype-genotype virulence map of a
614 bacterial pathogen. Genome Res 22:2541-51.
615 25. Kelley WL. 2006. Lex marks the spot: the virulent side of SOS and a closer look at the
616 LexA regulon. Mol Microbiol 62:1228-38.
617 26. Robinson A, Brzoska AJ, Turner KM, Withers R, Harry EJ, Lewis PJ, Dixon NE. 2010.
618 Essential biological processes of an emerging pathogen: DNA replication, transcription,
619 and cell division in Acinetobacter spp. Microbiol Mol Biol Rev 74:273-97.
620 27. Macguire AE, Ching MC, Diamond BH, Kazakov A, Novichkov P, Godoy VG. 2014.
621 Activation of phenotypic subpopulations in response to ciprofloxacin treatment in
622 Acinetobacter baumannii. Mol Microbiol 92:138-52.
623 28. Aranda J, Bardina C, Beceiro A, Rumbo S, Cabral MP, Barbe J, Bou G. 2011.
624 Acinetobacter baumannii RecA protein in repair of DNA damage, antimicrobial
625 resistance, general stress response, and virulence. J Bacteriol 193:3740-7.
626 29. Saroj SD, Clemmer KM, Bonomo RA, Rather PN. 2012. Novel mechanism for
627 fluoroquinolone resistance in Acinetobacter baumannii. Antimicrob Agents Chemother
628 56:4955-7.
629 30. Gallagher LA, Lee SA, Manoil C. 2017. Importance of Core Genome Functions for an
630 Extreme Antibiotic Resistance Trait. MBio 8.
631 31. van Opijnen T, Camilli A. 2013. Transposon insertion sequencing: a new tool for
632 systems-level analysis of microorganisms. Nat Rev Microbiol 11:435-42.
633 32. van Opijnen T, Bodi KL, Camilli A. 2009. Tn-seq: high-throughput parallel sequencing
634 for fitness and genetic interaction studies in microorganisms. Nat Methods 6:767-72.
635 33. van Opijnen T, Dedrick S, Bento J. 2016. Strain Dependent Genetic Networks for
636 Antibiotic-Sensitivity in a Bacterial Pathogen with a Large Pan-Genome. PLoS Pathog
637 12:e1005869.
638 34. Geisinger E, Mortman NJ, Vargas-Cuebas G, Tai AK, Isberg RR. 2018. A global
639 regulatory system links virulence and antibiotic resistance to envelope homeostasis in
640 Acinetobacter baumannii. PLoS Pathog 14:e1007030.
641 35. Drlica K, Zhao X. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol
642 Mol Biol Rev 61:377-92.
643 36. Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. 2005. AbeM, an H+-coupled
644 Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of
645 transporters. Antimicrob Agents Chemother 49:4362-4.
646 37. Knauf GA, Cunningham AL, Kazi MI, Riddington IM, Crofts AA, Cattoir V, Trent MS,
647 Davies BW. 2018. Exploring the Antimicrobial Action of Quaternary Amines against
648 Acinetobacter baumannii. MBio 9.
649 38. Gomez JE, Kaufmann-Malaga BB, Wivagg CN, Kim PB, Silvis MR, Renedo N, Ioerger
650 TR, Ahmad R, Livny J, Fishbein S, Sacchettini JC, Carr SA, Hung DT. 2017. Ribosomal

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

651 mutations promote the evolution of antibiotic resistance in a multidrug environment. Elife
652 6.
653 39. Bollenbach T, Quan S, Chait R, Kishony R. 2009. Nonoptimal microbial response to
654 antibiotics underlies suppressive drug interactions. Cell 139:707-18.
655 40. Shippy DC, Fadl AA. 2015. RNA modification enzymes encoded by the gid operon:
656 Implications in biology and virulence of bacteria. Microb Pathog 89:100-7.
657 41. Hujer KM, Hujer AM, Endimiani A, Thomson JM, Adams MD, Goglin K, Rather PN,
658 Pennella TT, Massire C, Eshoo MW, Sampath R, Blyn LB, Ecker DJ, Bonomo RA. 2009.
659 Rapid determination of quinolone resistance in Acinetobacter spp. J Clin Microbiol
660 47:1436-42.
661 42. Shishkin AA, Giannoukos G, Kucukural A, Ciulla D, Busby M, Surka C, Chen J,
662 Bhattacharyya RP, Rudy RF, Patel MM, Novod N, Hung DT, Gnirke A, Garber M,
663 Guttman M, Livny J. 2015. Simultaneous generation of many RNA-seq libraries in a
664 single reaction. Nat Methods 12:323-5.
665 43. Simmons LA, Foti JJ, Cohen SE, Walker GC. 2008. The SOS Regulatory Network.
666 EcoSal Plus 3.
667 44. Hare JM, Ferrell JC, Witkowski TA, Grice AN. 2014. Prophage induction and
668 differential RecA and UmuDAb transcriptome regulation in the DNA damage responses
669 of Acinetobacter baumannii and Acinetobacter baylyi. PLoS One 9:e93861.
670 45. Ching C, Gozzi K, Heinemann B, Chai Y, Godoy VG. 2017. RNA-Mediated cis
671 Regulation in Acinetobacter baumannii Modulates Stress-Induced Phenotypic Variation.
672 J Bacteriol 199.
673 46. Erb ML, Kraemer JA, Coker JK, Chaikeeratisak V, Nonejuie P, Agard DA, Pogliano J.
674 2014. A bacteriophage tubulin harnesses dynamic instability to center DNA in infected
675 cells. Elife 3.
676 47. Constantinou A, Voelkel-Meiman K, Sternglanz R, McCorquodale MM, McCorquodale
677 DJ. 1986. Involvement of host DNA gyrase in growth of bacteriophage T5. J Virol
678 57:875-82.
679 48. Alonso JC, Sarachu AN, Grau O. 1981. DNA gyrase inhibitors block development of
680 Bacillus subtilis bacteriophage SP01. J Virol 39:855-60.
681 49. Alcorlo M, Salas M, Hermoso JM. 2007. In vivo DNA binding of bacteriophage GA-1
682 protein p6. J Bacteriol 189:8024-33.
683 50. Kreuzer KN, Cozzarelli NR. 1979. Escherichia coli mutants thermosensitive for
684 deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication,
685 transcription, and bacteriophage growth. J Bacteriol 140:424-35.
686 51. Itoh T, Tomizawa JI. 1977. Involvement of DNA gyrase in bacteriophage T7 DNA
687 replication. Nature 270:78-80.
688 52. Sokolsky TD, Baker TA. 2003. DNA gyrase requirements distinguish the alternate
689 pathways of Mu transposition. Mol Microbiol 47:397-409.
690 53. Dodson M, Echols H, Wickner S, Alfano C, Mensa-Wilmot K, Gomes B, LeBowitz J,
691 Roberts JD, McMacken R. 1986. Specialized nucleoprotein structures at the origin of
692 replication of bacteriophage lambda: localized unwinding of duplex DNA by a six-
693 protein reaction. Proc Natl Acad Sci U S A 83:7638-42.
694 54. Mensa-Wilmot K, Seaby R, Alfano C, Wold MC, Gomes B, McMacken R. 1989.
695 Reconstitution of a nine-protein system that initiates bacteriophage lambda DNA
696 replication. J Biol Chem 264:2853-61.

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

697 55. Kreuzer KN. 1998. Bacteriophage T4, a model system for understanding the mechanism
698 of type II topoisomerase inhibitors. Biochim Biophys Acta 1400:339-47.
699 56. Huang WM, Wei LS, Casjens S. 1985. Relationship between bacteriophage T4 and T6
700 DNA topoisomerases. T6 39-protein subunit is equivalent to the combined T4 39- and
701 60-protein subunits. J Biol Chem 260:8973-7.
702 57. Sutton MD, Smith BT, Godoy VG, Walker GC. 2000. The SOS response: recent insights
703 into umuDC-dependent mutagenesis and DNA damage tolerance. Annu Rev Genet
704 34:479-497.
705 58. Geisinger E, Isberg RR. 2015. Antibiotic modulation of capsular exopolysaccharide and
706 virulence in Acinetobacter baumannii. PLoS Pathog 11:e1004691.
707 59. Carter R, Wolf J, van Opijnen T, Muller M, Obert C, Burnham C, Mann B, Li Y, Hayden
708 RT, Pestina T, Persons D, Camilli A, Flynn PM, Tuomanen EI, Rosch JW. 2014.
709 Genomic analyses of pneumococci from children with sickle cell disease expose host-
710 specific bacterial adaptations and deficits in current interventions. Cell Host Microbe
711 15:587-599.
712 60. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov
713 JP. 2011. Integrative genomics viewer. Nat Biotechnol 29:24-6.
714 61. McCoy KM, Antonio ML, van Opijnen T. 2017. MAGenTA: a Galaxy implemented tool
715 for complete Tn-Seq analysis and data visualization. Bioinformatics 33:2781-2783.
716 62. Ramirez F, Dundar F, Diehl S, Gruning BA, Manke T. 2014. deepTools: a flexible
717 platform for exploring deep-sequencing data. Nucleic Acids Res 42:W187-91.
718 63. Jensen PA, Zhu Z, van Opijnen T. 2017. Antibiotics Disrupt Coordination between
719 Transcriptional and Phenotypic Stress Responses in Pathogenic Bacteria. Cell Rep
720 20:1705-1716.
721 64. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion
722 for RNA-seq data with DESeq2. Genome Biol 15:550.
723 65. Ducret A, Quardokus EM, Brun YV. 2016. MicrobeJ, a tool for high throughput bacterial
724 cell detection and quantitative analysis. Nat Microbiol 1:16077.
725 66. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS. 2016. PHASTER: a
726 better, faster version of the PHAST phage search tool. Nucleic Acids Res 44:W16-21.
727

728

729 **Figure Legends.**

730

731 **Fig. 1. Tn-seq quantification of genome-wide mutant fitness in *A. baumannii* (*gyrA*^{WT} and
732 *parC*^{WT}) during growth with sub-MIC ciprofloxacin.** (A) Graded concentrations of
733 ciprofloxacin cause increasing degrees of growth inhibition. Transposon mutant libraries
734 constructed in a strain background harboring WT alleles of the *gyrA* and *parC* genes were grown

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

735 with ciprofloxacin at the concentrations indicated. Growth rate relative to untreated control was
736 determined from bacterial density measurements. Data points show average ± SD ($n \geq 2$). (B)
737 Tn-seq fitness profiles during ciprofloxacin challenge. Mutant pools were grown with or without
738 the indicated ciprofloxacin concentration and average fitness for each chromosomal gene was
739 calculated. Change in fitness resulting from ciprofloxacin treatment relative to untreated controls
740 is plotted against significance score resulting from parallel t-tests. Data points shaded in black
741 indicate gene knockouts causing significant alteration in fitness during drug challenge ($W_{treated}$-
742 $W_{untreated}$ had a magnitude > 0.1 and FDR <0.05). (C) Functional categories of significant gene
743 hits determining fitness during challenge with 0.09-0.1 μg/ml ciprofloxacin. Information from
744 KEGG and UniProt functional annotations and from orthologs in well-studied reference species
745 were used to place genes into the listed broad categories.
746
747 **Fig. 2. Magnitude of growth impairment is predicted by the severity of the Tn-seq fitness**
748 **defect.** (A) Tn-seq fitness profiles of transposon mutant pools constructed in a *gyrA*^{WT}*parC*^{WT}
749 strain. Pools were grown without or with ciprofloxacin at 0.09-0.1 μg/ml. Bars show fitness
750 values of each transposon mutant at the indicated locus across all tested pools. (B) Growth of
751 pure cultures of WT or the indicated mutant in the absence or presence of ciprofloxacin (0.09
752 μg/ml for Δ*ctpA* and *pbp1A*, 0.1 μg/ml for Δ*adeIJK* and Δ*recN*, and 0.15 μg/ml for Δ*gidA*). The
753 *pbp1a* mutant tested was *pbp1a*(N178TfsX27). Data points show geometric mean ± SD (n = 3).
754 Cip, ciprofloxacin.
755
756 **Fig. 3. Acquisition of *gyrA* resistance allele dramatically alters *A. baumannii* Tn-seq profile**
757 **during ciprofloxacin challenge.** (A) Transposon pools constructed in strains harboring

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

758 resistance alleles in *gyrA* or both *gyrA* and *parC* require increasing concentrations of
759 ciprofloxacin to result in growth inhibition. Growth rate inhibition relative to untreated pools was
760 plotted as in Fig. 1A. *gyrA*^{WT} *parC*^{WT} growth data are from identical experiment shown in Fig.
761 1A and are displayed to allow comparison to behavior of drug resistant mutants. Data points
762 show average ± SD (n ≥ 2). Samples from cultures with 30-40% growth inhibition (dotted lines)
763 were processed for Tn-seq. (B) Location of prophage regions (P1-P3) on *A. baumannii* 17978-
764 mff chromosome map. Prophage positions were identified by using the PHASTER database (66).
765 (C-F) *gyrA* resistance allele influences Tn-seq fitness profiles associated with ciprofloxacin
766 stress. Mutant pools were challenged with drug concentrations that resulted in equivalent 30-
767 40% growth inhibition [*gyrA*^{R}, 1.1 µg/ml; *gyrA* ^{R}*parC*^{R}, 13-14 µg/ml]. (C, E) Tn-seq fitness
768 scores for each chromosomal gene with the indicated strain were calculated and visualized as in
769 Fig. 1B (leftmost subpanel). Middle and rightmost subpanels show the identical dataset, with
770 highlighting of loci for which knockout causes ciprofloxacin hypersensitization in the WT
771 genetic background (green), or loci within prophages (color indicated in key). (D, F) Gene hits
772 associated with significant changes in fitness during treatment were placed into functional
773 categories as in Fig. 1C. Tn-seq hits resulting from *gyrA*^{R} libraries treated with ciprofloxacin are
774 enriched in prophage genes (F). (G) Tn-seq fitness scores resulting from ciprofloxacin challenge
775 show genome positional bias that is greatly amplified in *gyrA*^{R} mutant pools. Average per-gene
776 Tn-seq fitness values are plotted in order of gene position on the chromosome or on plasmids
777 pAB1-3. Boundaries of prophage regions (P1-P3) are indicated by vertical dotted lines. Top, no
778 drug control. Bottom, ciprofloxacin was added at the concentrations indicated in panel A
779 resulting in 30-40% inhibition (WT, 0.09-0.1 µg/ml; *gyrA*^{R}, 1.1 µg/ml; *gyrA*^{R}*parC*^{R}, 13-14

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

780 μg/ml). (H) Expanded view of per-gene Tn-seq fitness scores in regions surrounding prophages

781 P1 and P3 for *gyrA*^{R} mutant treated with 1.1 μg/ml ciprofloxacin.

782

783 **Fig. 4. Ciprofloxacin-induced amplification of prophage DNA in strains harboring the**

784 ***gyrA*^{R} single-step resistance genotype.** (A) Pure cultures of strains of the indicated genotype

785 were challenged with graded ciprofloxacin doses resulting in four levels of growth inhibition

786 (Roman numerals). (B) DNA content from each culture was analyzed by deep sequencing. x-axis

787 indicates nucleotide position along the *A. baumannii* ATCC17978-mff chromosome. y-axis

788 indicates normalized read depth (0-140 counts per million). Boundaries of prophage regions P1-3

789 are indicated in red. Roman numerals indicate the level of growth inhibition caused by

790 ciprofloxacin. Data are representative of two independent experiments. (C) Expanded view of

791 300kb window showing amplification of genomic regions surrounding prophages P1 and P3. Y-

792 axis indicates normalized sequencing read depth (0-160 counts per million).

793

794 **Fig. 5. Ciprofloxacin challenge results in activation of prophage gene expression that is**

795 **heightened in *gyrA*^{R}-single mutants.** (A) Strains of the indicated genotype were challenged

796 with ciprofloxacin concentrations that resulted in two levels of growth inhibition relative to no

797 treatment (i, ~45% growth inhibition; ii, ~70% growth inhibition). Data points show average ±

798 SD (n = 3). (B). RNA-seq transcriptional profiles of panel A cultures. Fold change (log2) of each

799 gene (treated vs untreated) was plotted in order of position on the chromosome or plasmids

800 (pAB1-3). rRNA and tRNA genes were excluded from RNA-seq analysis, resulting in different

801 gene number assignments as compared to those in Fig. 3D. Boundaries of prophage regions P1-3

802 are denoted by vertical dotted lines. Roman numerals indicate growth inhibition level. (C)

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

803 Expanded views of RNA-seq log2-fold change ratios for genes surrounding P1-3 in *gyrA*^{R}-single
804 mutant (condition *ii*, ciprofloxacin 1.1µg/ml).
805
806 **Fig. 6. Enhanced SOS response induction in *gyrA*^{R}-single mutants exposed to ciprofloxacin**.
807 (A-B) SOS response genes are induced during growth with ciprofloxacin. (A) RNA-seq data
808 reveal DNA damage/SOS response induction during growth with ciprofloxacin. Bars show log2
809 fold change ± SEM (n = 3) for WT or *gyrA*^{R}-single mutant treated with ciprofloxacin
810 concentrations resulting in ~70% growth inhibition (condition *ii* from Fig. 5). *, p<0.05,
811 unpaired t test. (B) Location of DNA damage/SOS response genes induced in *gyrA*^{R}-single
812 mutant strain. x-axis indicates gene position along the *A. baumannii* chromosome, y-axis
813 indicates the log2 fold change (Cip 1.1µg/ml vs untreated, *gyrA*^{R}-single mutant) from previously
814 presented RNA-seq data. (C-D) Fluorescence reporter assays demonstrate enhanced *recA* gene
815 expression in *gyrA*^{R}-single mutant. Strains of the indicated genotype harboring (C) pCC1
816 (mKate2 fusion to *recA* promoter plus 5' untranslated region (UTR)) or (D) pCC7 (*trpB*
817 promoter replacing *recA* promoter in pCC1) were cultured as in RNA-seq experiments. Growth
818 inhibition relative to untreated control was calculated (top). Average mKate2 intensity per cell
819 within each sample was measured by fluorescence microscopy, and median fluorescence values
820 of the population were determined (bottom). Data points represent the average inhibition values
821 (top) or average of median fluorescence values (bottom) ± SD from n ≥ 2 biological replicates
822 pooled from multiple independent experiments. Dotted lines denote fluorescence intensity of
823 untreated samples.
824

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

825 **Fig. 7. Model for resistance allele-dependent prophage amplification in *A. baumannii***
826 **exposed to sub-MIC fluoroquinolone stress.** The model posits that prophage DNA replication
827 depends on host DNA gyrase activity. (A) In WT cells, both gyrase (GyrA) and topo IV (ParC)
828 are drug sensitive. Gyrase, which has higher affinity for CIP (blue triangles), is effectively
829 targeted by the drug. Ciprofloxacin-corrupted gyrase results in double-strand DNA breaks that
830 signal derepression of prophage gene expression. Prophage DNA replication cannot proceed,
831 however, because gyrase function is blocked. (B) In single *gyrA*^{R} mutant cells, topo IV/ParC has
832 higher affinity for ciprofloxacin than the resistant gyrase and is the effective drug target. Topo IV
833 corruption results in a robust DNA damage response and activation of prophage gene expression,
834 and gyrase-dependent prophage replication (prophages 1 and 3) proceeds because GyrA is not
835 drug-inhibited. (C) In double *gyrA*^{R}*parC*^{R} mutant cells growing at high drug concentrations,
836 GyrA again has relatively higher affinity for ciprofloxacin than ParC and becomes the effective
837 target despite the S81L drug binding site alteration. The resulting DNA lesions induce the SOS
838 response and prophage gene expression, but prophage replication does not proceed efficiently
839 because gyrase function is again blocked.
840
841 **Supplemental Figure Legends**
842
843 **Fig. S1. pDL1073 feature map.**
844
845 **Fig. S2. Sub-MIC ciprofloxacin treatment of *A. baumannii* with *gyrA*^{WT} and *parC*^{WT} alleles**
846 **does not significantly alter Tn-seq fitness values assigned to prophage region genes.** (A) The
847 Tn-seq dataset shown in Fig. 1B (WT background +/- treatment with ciprofloxacin 0.09-0.1

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

848 μg/ml) was reanalyzed to highlight fitness values associated with genes within prophage regions
849 P1-P3. Prophages regions are highlighted with color indicated in the key.
850
851 **Fig. S3. Comparison of transcription levels between ciprofloxacin-treated cultures of WT**
852 **and *gyrA*^{R} reveals enhanced prophage gene expression in the *gyrA*^{R} single mutant.** Strains
853 were grown in the absence or presence of ciprofloxacin at concentrations shown in Fig. 5A, and
854 RNA-seq data were analyzed such that WT and *gyrA*^{R} strains were directly compared at each
855 condition. Plots show log2 fold change (WT vs *gyrA*^{R}) of each gene in order of position on the
856 chromosome or plasmids (pAB1-3).
857
858 **Fig. S4. Fluorescence microscopy analysis of SOS response in individual cells subjected to**
859 **ciprofloxacin**. Strains harboring *recA-mKate2* were cultured and analyzed by fluorescence
860 microscopy as described in legend to Fig. 6C. (A) Phase contrast (rows 1 and 3) and fluorescence
861 (rows 2 and 4) microscopy images from one representative experiment used to quantify *recA-*
862 *mKate2* signal in Fig. 6C. Cells of the indicated genotype were treated with the noted
863 concentration of ciprofloxacin resulting in similar degrees of growth inhibition (see panel C,
864 bottom three growth inhibition data points). (B) Population fluorescence analysis from one
865 representative experiment contributing to Fig. 6C quantifying SOS response to increasing
866 ciprofloxacin dose in different strain backgrounds harboring *recA-mKate2*. In the same
867 experiment shown in panel A, 4 different ciprofloxacin concentrations were tested per strain.
868 Average mKate2 intensity per cell was measured by fluorescence microscopy. Each data point
869 represents average fluorescence intensity of a single cell (at least 100 cells per condition were

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

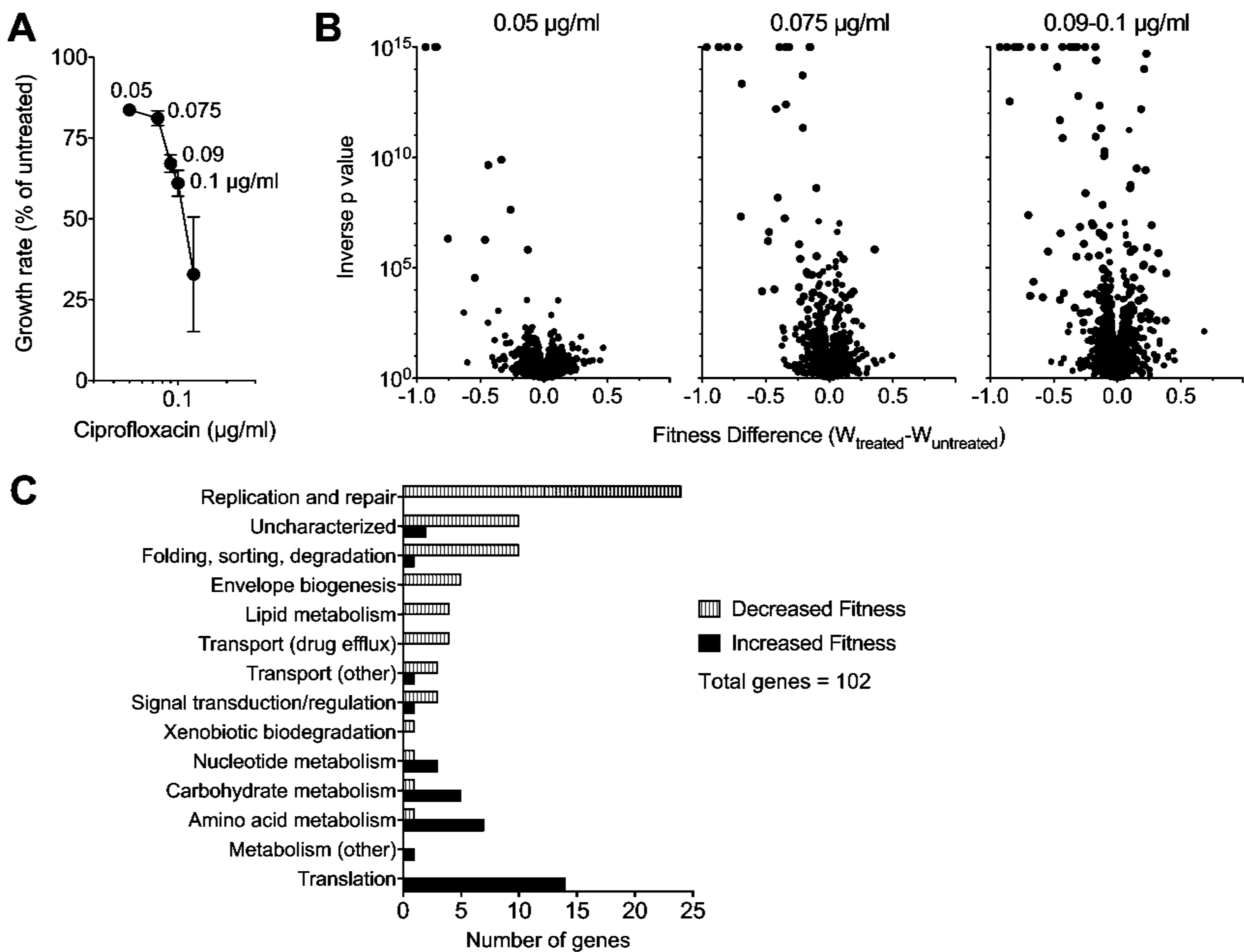
870 analyzed). Bars indicate median values. (C) Growth inhibition relative to untreated control
871 resulting from the ciprofloxacin exposures in the representative experiment shown in panel B.
872
873 **Table S1.** Bacterial strains and plasmids used in this study.
874 **Table S2.** Oligonucleotide primers used in this study.
875 **Data Set S1.** Tn-seq fitness data - WT.
876 **Data Set S2.** Tn-seq fitness data - *gyrA*^{R}.
877 **Data Set S3.** Tn-seq fitness data - *gyrA*^{R} *parC*^{R}.
878 **Data Set S4.** RNA-seq data.

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Table 1. Minimal Inhibitory Concentration (µg/ml) of Ciprofloxacin with WT and mutant *A. baumannii*.

	amino acid change:		ciprofloxacin MIC (µg/ml)
strain	GyrA	ParC	
WT	-	-	0.25
gyrA^R	S81L	-	2
parC^R	-	S84L	0.25
gyrA^R *parC*^{R}	S81L	S84L	32

bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

A

Fitness

adeK adeJ adeI

ctpA

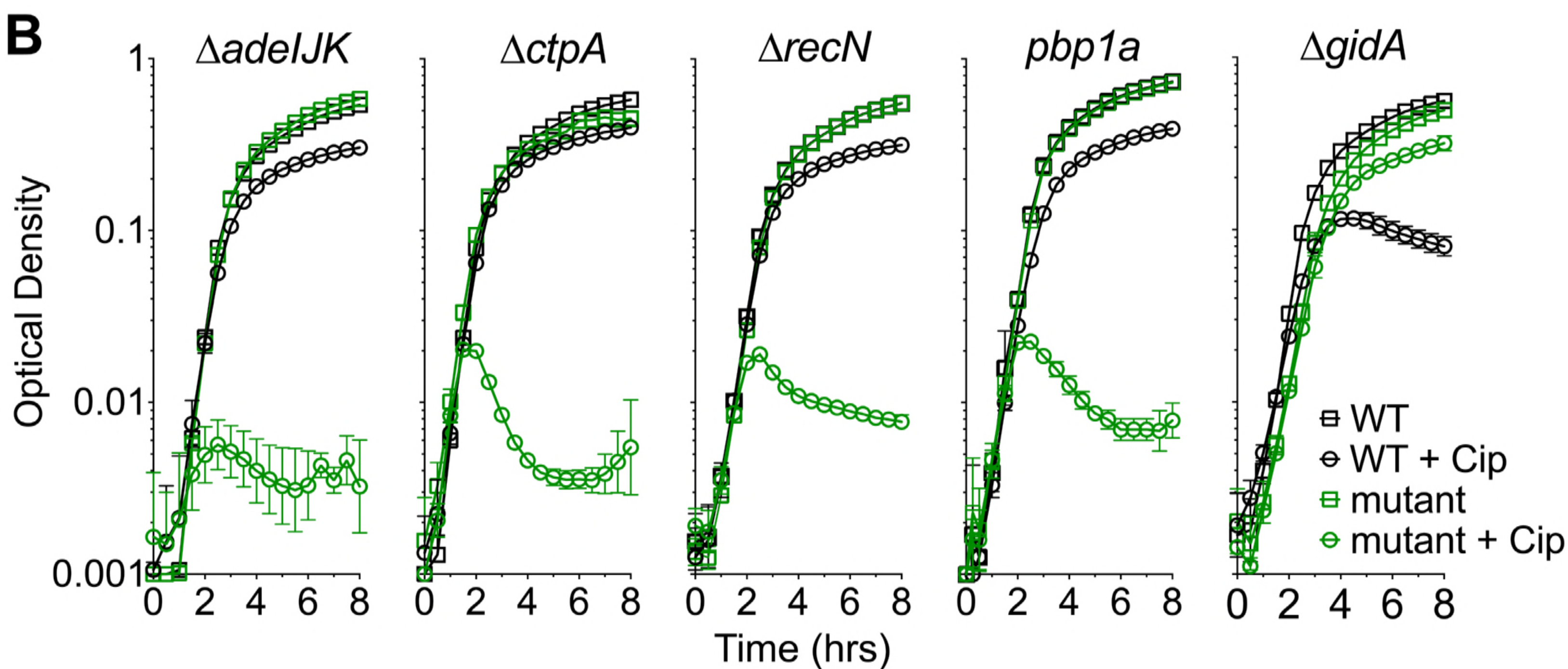
recN

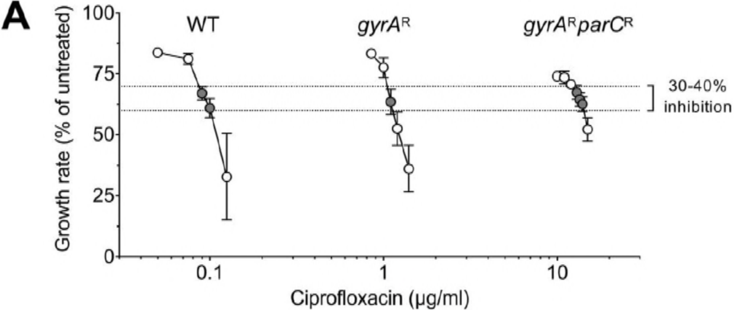
pbp1a

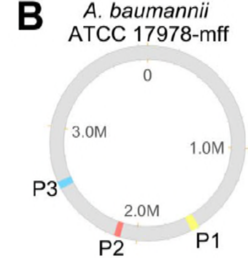
gidA

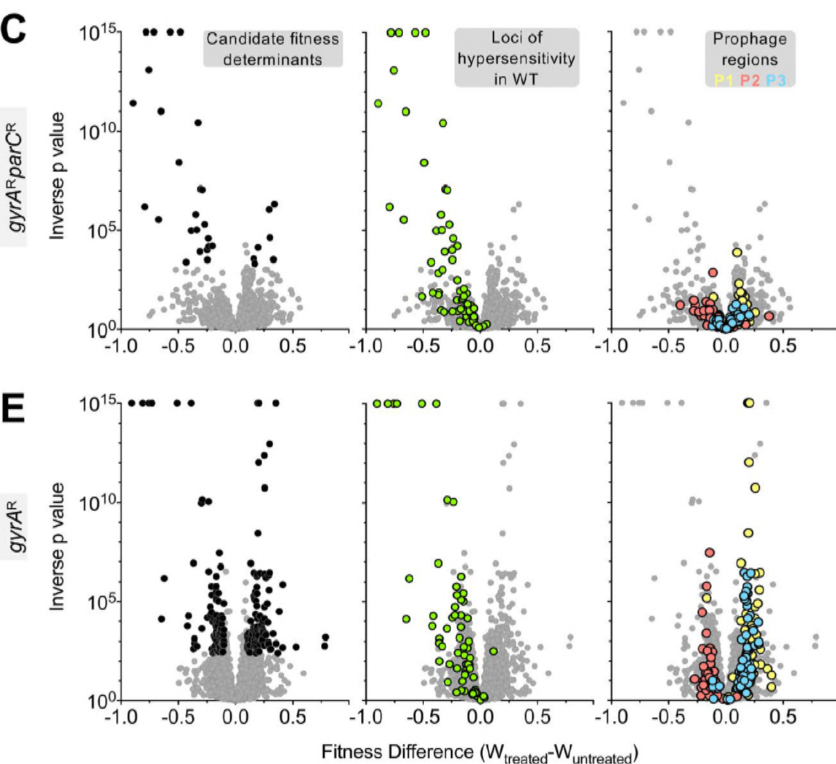
no drug

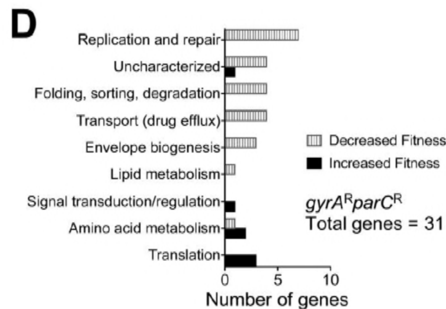
Cip 0.09-0.1μg/ml

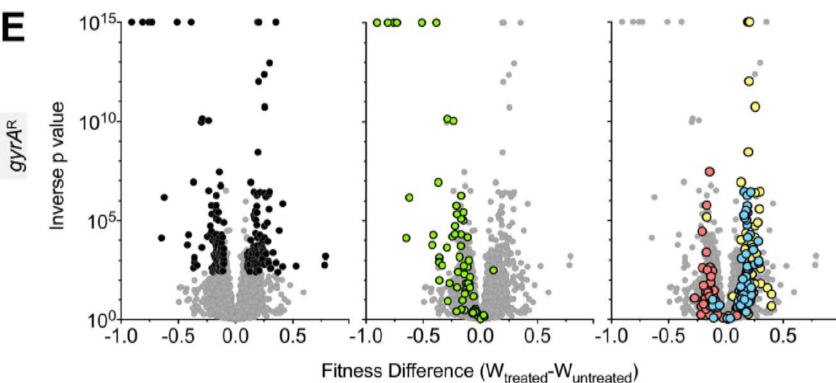


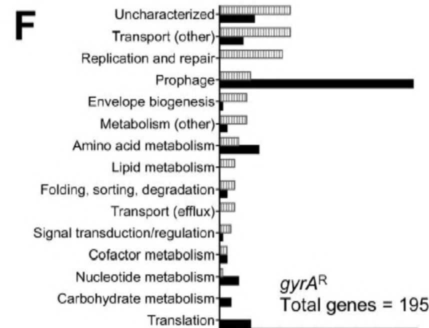

A
WT
gyrA^R
gyrA^R parC^R
30-40% inhibition
Growth rate (% of untreated)
Ciprofloxacin (µg/ml)

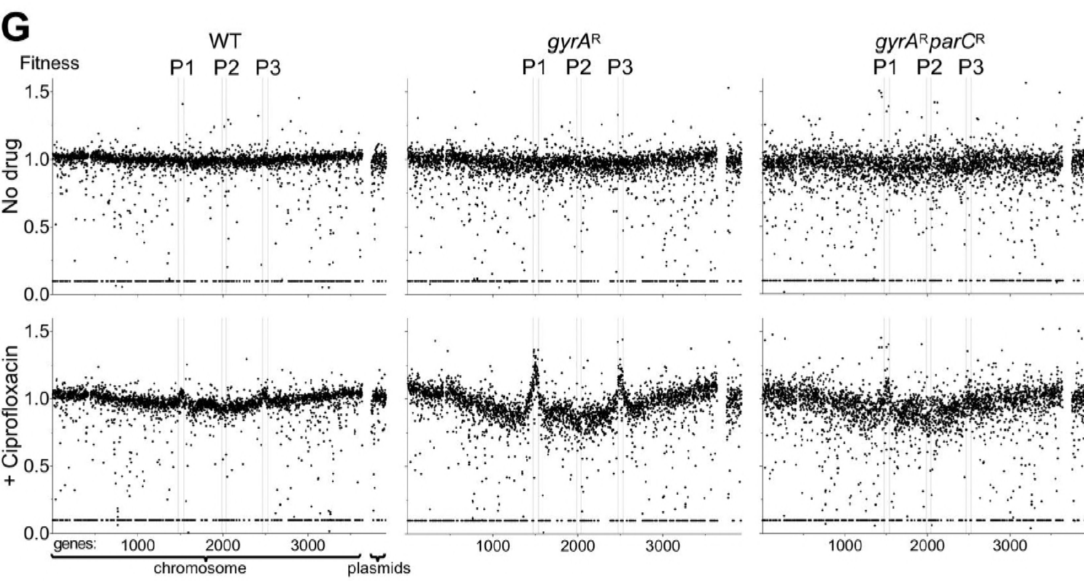

B
A. baumannii
ATCC 17978-mff
0
1.0M
2.0M
3.0M
P1
P2
P3

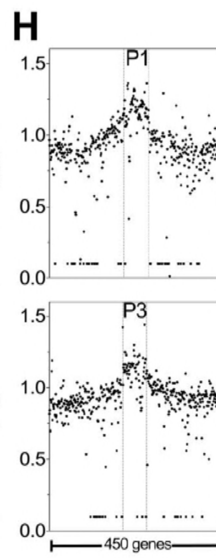

C
Candidate fitness determinants
Loci of hypersensitivity in WT
Prophage regions
P1 P2 P3
gyrA^R parC^R
Inverse p value


D
Replication and repair
Uncharacterized
Folding, sorting, degradation
Transport (drug efflux)
Envelope biogenesis
Lipid metabolism
Signal transduction/regulation
Amino acid metabolism
Translation
Decreased Fitness
Increased Fitness
gyrA^R parC^R
Total genes = 31
Number of genes

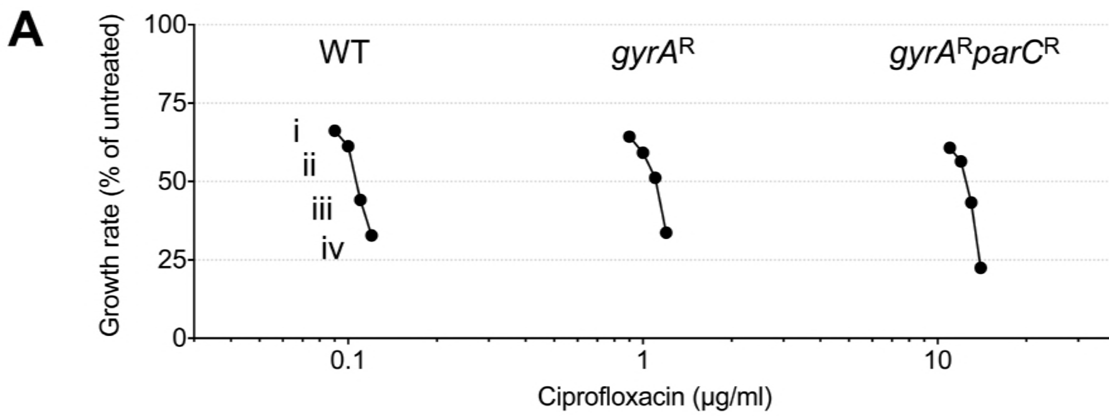

E
gyrA^R
Inverse p value
Fitness Difference (W_treated-W_untreated)

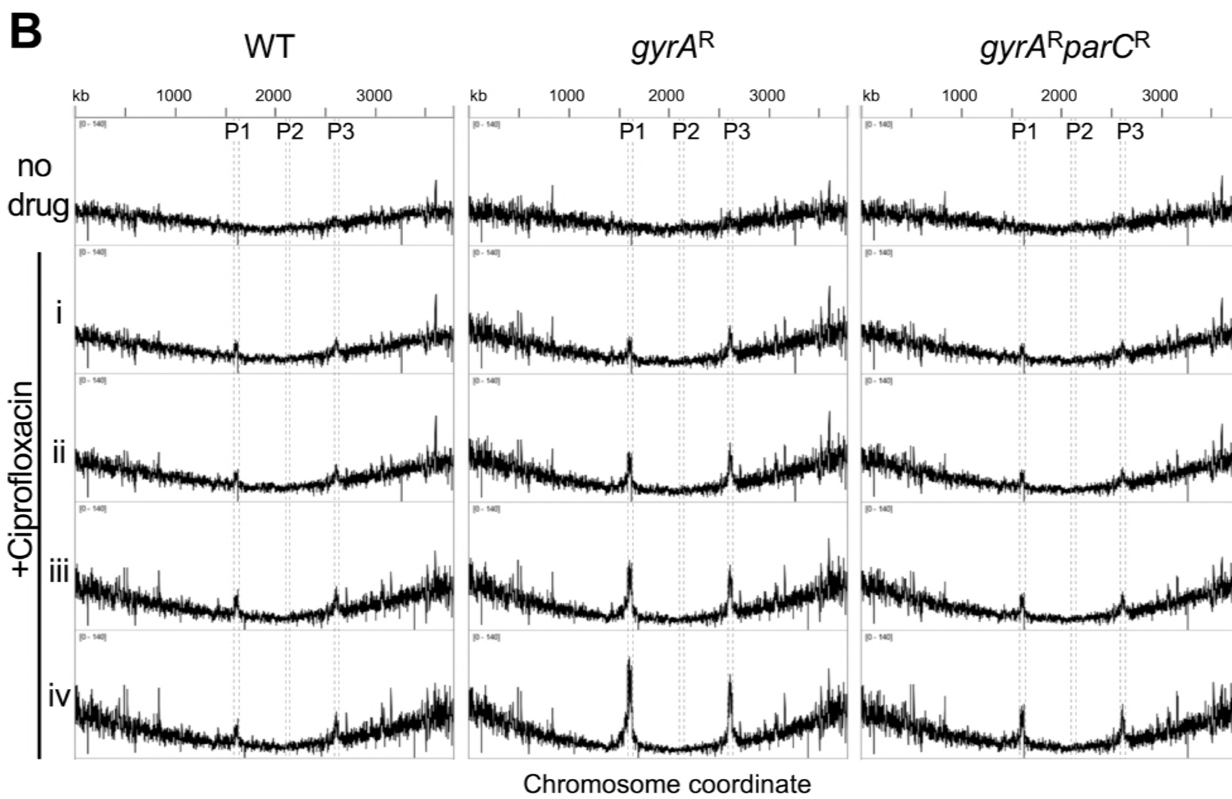

F
Uncharacterized
Transport (other)
Replication and repair
Prophage
Envelope biogenesis
Metabolism (other)
Amino acid metabolism
Lipid metabolism
Folding, sorting, degradation
Transport (efflux)
Signal transduction/regulation
Cofactor metabolism
Nucleotide metabolism
Carbohydrate metabolism
Translation
gyrA^R
Total genes = 195
Number of genes

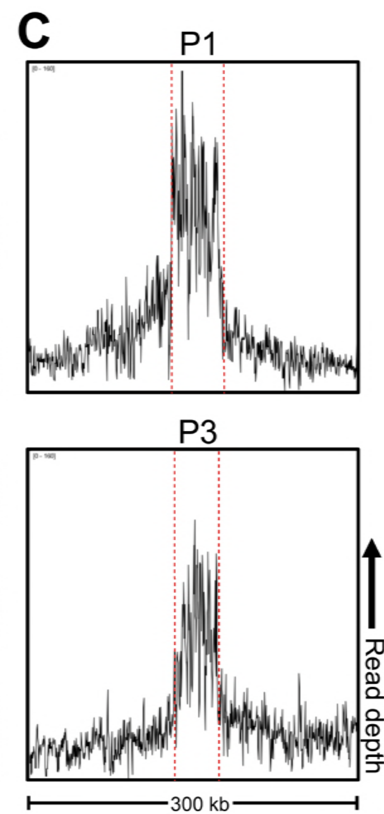

G
Fitness
WT
gyrA^R
gyrA^R parC^R
P1 P2 P3
No drug
+ Ciprofloxacin
genes:
chromosome
plasmids


H
P1
P3
450 genes

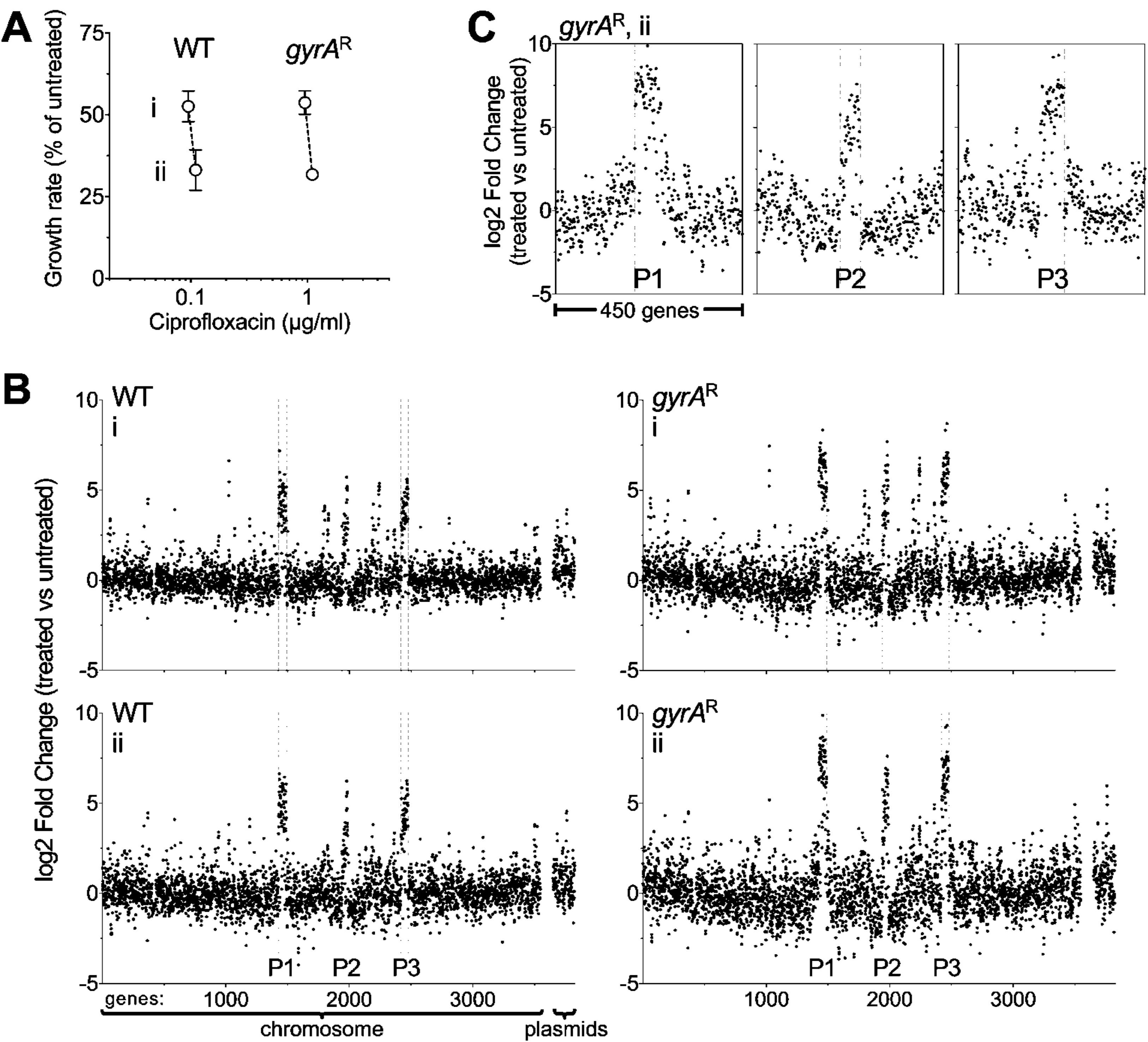
bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



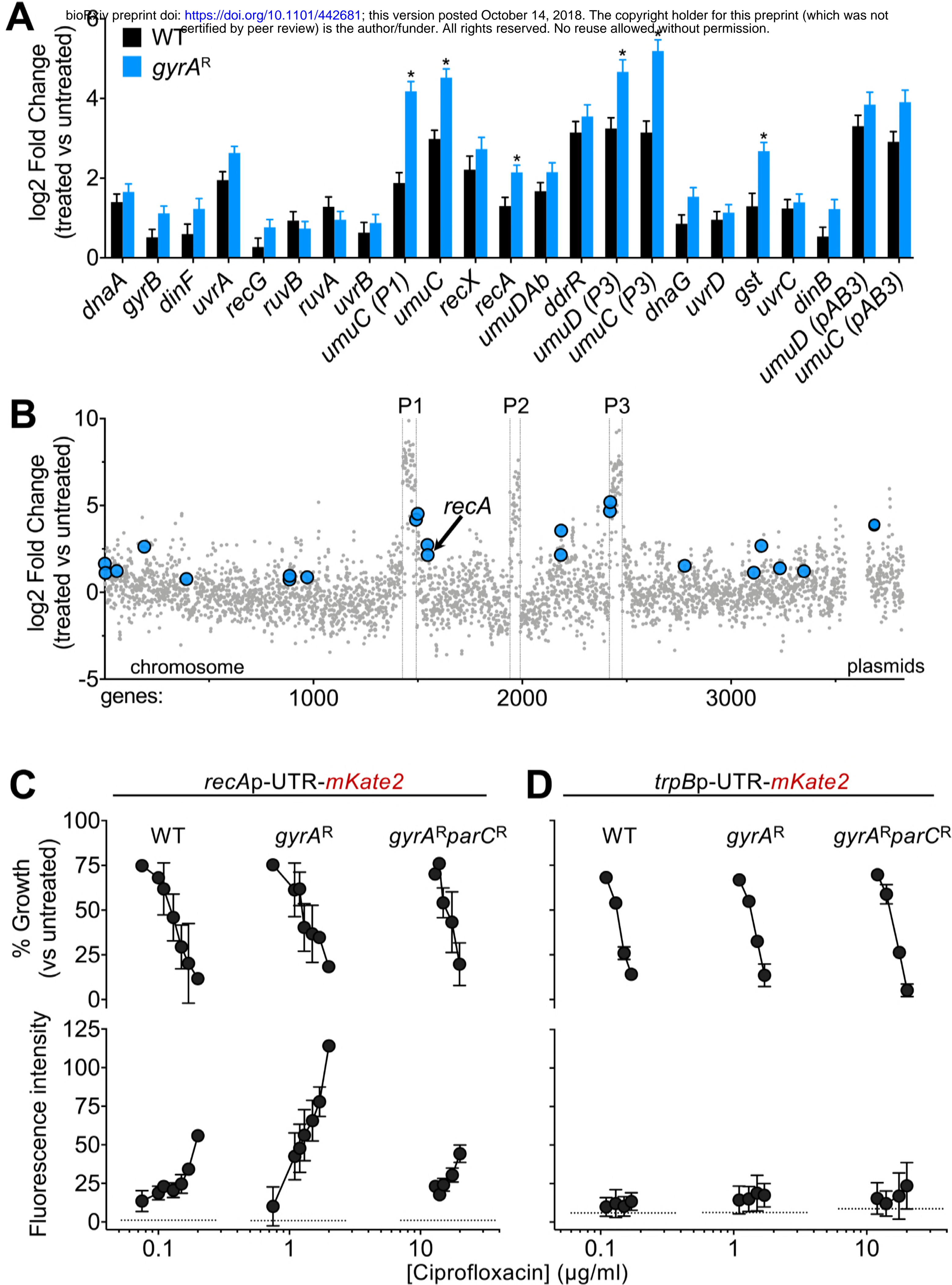




bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



bioRxiv preprint doi: https://doi.org/10.1101/442681; this version posted October 14, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.