Genomic Background Governs Opposing Responses to Nalidixic Acid Upon Megaplasmid Acquisition in *Pseudomonas*

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Abstract

Horizontal gene transfer is a significant driver of evolutionary dynamics across microbial populations. Although the benefits of the acquisition of new genetic material are often quite clear, experiments across systems have demonstrated that gene transfer events can cause significant phenotypic changes and entail fitness costs in a way that is dependent on the genomic and environmental context. Here we test for the generality of one previously identified cost, sensitization of cells to the antibiotic nalidixic acid after acquisition of a ~1Mb megaplasmid, across Pseudomonas strains and species. Overall, we find that the presence of this megaplasmid sensitizes many different Pseudomonas strains to nalidixic acid, but that this same horizontal gene transfer event increases resistance of *Pseudomonas putida* KT2440 to nalidixic acid across assays as well as to ciprofloxacin under competitive conditions. These phenotypic results are not easily explained away as secondary consequences of overall fitness effects and appear to occur independently of another cost associated with this megaplasmid, sensitization to higher temperatures. Lastly, we draw parallels between these reported results and the phenomenon of sign epistasis for *de novo* mutations and explore how context dependence of effects of plasmid acquisition could impact overall evolutionary dynamics and the evolution of antimicrobial resistance.

42 **Importance**

43

44 Numerous studies have demonstrated that gene transfer events (e.g. plasmid
45 acquisition) can entail a variety of costs that arise as byproducts of the incorporation of foreign
46 DNA into established physiological and genetic systems. These costs can be ameliorated
47 through evolutionary time by the occurrence of compensatory mutations, which stabilize
48 presence of a horizontally transferred region within the genome but which also may skew future
49 adaptive possibilities for these lineages. Here we demonstrate another possible outcome, that
50 phenotypic changes arising as a consequence of the same horizontal gene transfer event are
51 costly to some strains but may actually be beneficial in other genomic backgrounds under the
52 right conditions. These results provide new a new viewpoint for considering conditions that
53 promote plasmid maintenance and highlight the influence of genomic and environmental
54 contexts when considering amelioration of fitness costs after HGT events.

55 **Introduction**

56

57 Horizontal Gene Transfer (HGT), a catch-all term for the transfer of DNA between
58 individuals, is one of the main drivers of evolutionary dynamics across microbial populations (1–
59 3). Although there are countless examples where HGT has been shown to increase microbial
60 fitness and/or enable colonization of new niches, it is increasingly appreciated that HGT brings
61 with it a variety of costs that arise as byproducts of the incorporation of foreign DNA into
62 established physiological and genetic systems (4–6). Recent efforts have begun to categorize
63 and characterize phenotypic effects of HGT across systems, and have clearly demonstrated
64 that acquisition of the same genes can lead to different outcomes across genomic backgrounds
65 (7, 8). Such differences in phenotypic outcomes following HGT are analogous if not identical to
66 the concept of sign epistasis in the context of de novo mutations, whereby the fitness effects of
67 a mutation change sign (e.g. from positive to negative) under specific genetic combinations (9–
68 11). Much like sign epistasis, we currently lack a general understanding of the underlying
69 causes of differences in phenotypic outcomes following HGT, and as a consequence, there is
70 no clear model for predicting if and when acquisition of DNA could be detrimental in some
71 genomic backgrounds but beneficial in others.

72

73 We have previously reported numerous phenotypic changes that occur as a
74 consequence of acquisition of a ~1Mb megplasmid, pMPPla107, by *Pseudomonas* strains (12,
75 13). In addition to fitness costs correlated with slower growth, acquisition of pMPPla107 alters a
76 variety of other phenotypes in some strains including: increased motility, decreased biofilm
77 formation, decreased thermal tolerance, increased sensitivity to an unidentified compound
78 produced by Pseudomonads, and increased sensitivity to quinolone antibiotics like nalidixic acid
79 and ciprofloxacin. In the particular context of antibiotic sensitivity, it is important to note that
80 current annotations of megaplasmid pMPPla107 contain no known antibiotic resistance genes

81 (14) and that, at present, the mechanistic basis and overall strain specificity of these phenotypic
82 changes remains unknown.
83
84 Here, we test for how frequently sensitivity to nalidixic acid is observed across
85 Pseudomonas strains after acquisition of megaplasmid pMPPla107. We show that, although the
86 presence of this megaplasmid increases sensitivity to nalidixic acid in many strain backgrounds,
87 one strain in particular - *Pseudomonas putida* KT2440 - displays increased resistance to
88 nalidixic acid and ciprofloxacin after this HGT event. Therefore, our report provides an additional
89 example whereby the precise phenotypic effects of HGT are dependent on strain background,
90 but also suggests that that antibiotic resistance can be increased by megaplasmid pMPPla107
91 even in the absence of identifiable resistance genes in the region acquired by HGT. These
92 results, and specifically strain-dependent changes in sign of phenotypic effects of plasmid
93 acquisition, provide a new viewpoint for considering conditions that promote plasmid
94 maintenance and highlight the influence of genomic and environmental contexts when
95 considering amelioration of fitness costs after HGT events.
96
97

98 **Methods**

99

100 Bacterial strains and culture conditions

101 All strains used in this study are listed in Table 1. We note that one of the main *P.*
102 *stutzeri* strains for previous megaplasmid papers from our group is originally named 28a24
103 (15), but that in some previous papers from our group we have incorrectly referenced this
104 as strain 23a24 (12, 13). We also note that DBL332 and DBL386 are actually derived from
105 two independent rifampicin resistant mutants selected from strain 28a24 and frozen
106 independently as two different stocks.

107 Strain propagation of all cultures largely took place at 27°C in King's B medium
108 supplemented with rifampicin unless otherwise specified. Antibiotics were supplemented
109 into media at the following concentrations when appropriate and unless otherwise specified:
110 50 µg/mL rifampicin, 10 µg/mL tetracycline, and 20 µg/mL kanamycin. 5-bromo-4-chloro-3-
111 indolyl-β-D-galactopyranoside (Xgal) was supplemented at 40 µg/mL. For competition
112 assays, cultures were supplemented with nalidixic acid at 20µg/mL and 40µg/mL and with
113 ciprofloxacin at 0.5µg/mL. Antibiotic discs used in the diffusion assays contained antibiotics
114 at concentrations of 30 µg/mL for nalidixic acid and 5 µg/mL for ciprofloxacin.

115

116 Kirby-Bauer Disc Diffusion Assays

117

118 Strains of interest for each assay were streaked from frozen stocks and incubated on
119 KB media at 27°C for 2 to 3 days. At this point a small clump of cells was picked to liquid
120 KB medium and grown for 24 hours at 27°C with shaking. Cells from each culture were then
121 pelleted in a centrifuge, the supernatant was poured out, and pelleted cells were
122 resuspended in 10Mm $MgCl_2$ for measurement of OD_{600}. Each culture was then diluted to

123 0.01 OD_{600} in 10Mm $MgCl_2$ and 300uL of each culture was added to 3mL of 0.4% water
124 agar (which was previously melted and allowed to cool until warm to the touch). This
125 bacteria/agar mixture was then poured onto a plain KB agar plate (1.5% agar) and allowed
126 to solidify. Lastly, a disc containing the antibiotic of interest was added to the center of the
127 plate and each was placed face up in an incubator at 27°C. Pairs of megaplasmid +/-
128 strains were always evaluated together within the same assay block.
129 After 3 days of growth, each plate was taken out of the incubator and scanned. This
130 image was imported into ImageJ (16), and the area of the inhibition zone was measured. An
131 average area was calculated for the megaplasmid- strain within each assay, and individual
132 data points for each pair of megaplasmid + and - strains in each assay were divided by this
133 value in order to normalize the data. Therefore, the average inhibition zone for each
134 megaplasmid- strain within each assay was normalized to a value of 1. Each strain pairing
135 was tested in at least (but usually more than) two independent assays.
136
137 Competitive Fitness Assays
138 In each assay, *lacZ*+ strains (either DBL1604 or DBL1620, - and + megaplasmid
139 derivatives of strain KT2442, respectively) were competed against the common *lacZ*- strain
140 DBL305 (strain KT2440). Strains of interest for each assay were streaked from frozen
141 stocks for 2 to 3 days (on media containing kanamycin for megaplasmid+ strains) at which
142 point a small clump of cells was picked to liquid KB medium and grown for 24 hours at 27°C
143 with shaking. Cells from each culture were then pelleted in a centrifuge, the supernatant
144 was poured out, and pelleted cells were resuspended in 10Mm $MgCl_2$ for measurement of
145 OD_{600}. Each culture was then diluted to 1.0 OD_{600} in 10Mm $MgCl_2$.
146 A 10mL master culture was prepared for each set of comparative fitness assays and
147 for each different treatment. To prepare this master culture, 5uL of each 1.0 OD_{600} dilution of

148 the two comparison strains within each assay was added to the 10mL master culture. At this
149 point, 2mL samples of this master culture were added to 4 different test tubes, and the test
150 tubes were placed in a shaking incubator and grown for 24 hours at 27°C. For day 0
151 measurements, the master mix culture from the control (no antibiotic supplementation)
152 competitions was diluted 1:100 by adding 10μL into 990μL10Mm MgCl2, which was
153 followed by an additional 1:10 dilution. 3 replicate dilution series were made from each
154 master mix culture, and 150μL of each 1:100/1:1000 dilution was plated on LB media
155 containing 40μg/mL Xgal and placed 27°C. After 24 hours of growth each of the 4 replicate
156 cultures was diluted 1:10,000,000 and 1:100,000,000, with 150μL of these dilutions plated
157 on LB media containing 40μg/mL Xgal and placed at 27°C.
158 White and blue colonies were independently counted for each of the plated day 0
159 dilutions, these 3 values were averaged together, and the $\log_{10}$ value of this average taken
160 as the day 0 colony forming unit count. White and blue colonies were independently
161 counted for each of the day 1 replicate cultures and $\log_{10}$ transformed. Relative fitness was
162 calculated as the ratio of the difference between $\log_{10}$ transformed white and blue colony
163 counts for each replicate culture at day 1 over the difference of the $\log_{10}$ transformed
164 average white and blue colony counts at day 0. Depending on the assay, either two or three
165 assays were carried out with each pair of strains (DBL305/DBL1604 and
166 DBL305/DBL1620), with four replicate cultures per assay.
167 All competitive fitness assays were carried out in the same manner as described
168 above, with one exception. Since cultures grew more slowly in the presence of 40 μg/mL
169 nalidixic acid, cultures from the nalidixic acid competitions as well as the control
170 competitions were allowed to grow in liquid media at 27°C for 48 hours.
171
172 Statistical Methods

173

174 All statistical tests were performed in R version 3.3.0 (17). Scripts for these tests and all

175 underlying data can be found at **https:/doi.org/10.5281/zenodo.4409341**

176

177 **Results**

178

179 A Modified Kirby-Bauer Disc Diffusion Assay Replicates Previous Results For Nalidixic Acid

180 Sensitivity Upon Megaplasmid Acquisition

181

182 Our previously published experiments thoroughly explored how acquisition of

183 pMPPla107 increases nalidixic acid sensitivity in *Pseudomonas stutzeri* 28a84 (13), and here

184 we demonstrate that this effect is replicated by a simple modified assay based on Kirby-Bauer

185 disc diffusion assays ((18), Figure 1A). Since antibiotics diffuse out from the disc into the media

186 forming a concentration gradient, size of the inhibition halo is positively correlated with

187 sensitivity to nalidixic acid. We screened for nalidixic acid sensitivity in two different

188 backgrounds of *P. stutzeri* strain 28a24, DBL332 and DBL386. Derivative strains of these

189 (DBL453 and DBL408, respectively, which contain megaplasmid pMPPla107) have a larger halo

190 of inhibition around the antibiotic disc containing nalidixic acid than their immediate progenitor

191 strains which lack megaplasmid pMPPla107 (Figure 1B). There is an overall significant effect of

192 megaplasmid on nalidixic acid sensitivity ($F_{1,36}$=132.143, $p<0.0001$), and there are clear

193 statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+

194 strains measured separately (DBL332/DBL453, t-test $p<0.001$; DBL386/DBL408, t-test

195 $p<0.001$). Therefore, the acquisition of this megaplasmid sensitizes these strains to this

196 antibiotic.

197

198 Acquisition of pMPPla107 Sensitizes Most, But Not All, Strains to Nalidixic Acid

199

200 Extending the Kirby-Bauer diffusion assay design, we tested for sensitivity to nalidixic
201 acid across a variety of other *Pseudomonas stutzeri* strains as well as a *P. syringae* pv.
202 *lachrymans* 8003 and *P. fluorescens* Pf0-1. The *P. syringae* strains are relatively closely related
203 to the strain in which this megaplasmid was originally found, *P. syringae* pv. *lachrymans* 107
204 (19), and acquisition of the megaplasmid has been previously shown to affect growth of this
205 strain (12). Overall, we find that megaplasmid acquisition increases sensitivity to nalidixic acid
206 across all of these strains as reflected by increased halo size in these Kirby-Bauer assays
207 (Figure 2). However, we also find that the magnitude of this difference appears to be smaller in
208 *P. syringae* than across all of the *P. stutzeri* strains and even smaller for the *P.fluorescens* Pf0-1
209 comparisons. All comparisons between megaplasmid- and megaplasmid+ strains are
210 significantly different (t-test, $p<0.001$ for each with the exception of $p<0.05$ for the Pf0-1
211 comparison). We have also included representative scans from each of these comparisons in
212 Supplemental Figure 1 to demonstrate relative levels of antibiotic sensitivity (found at
213 https://doi.org/10.6084/m9.figshare.10257704.v2)

214

215 Acquisition of Megaplasmid pMPPla107 by *P. putida* Increases Resistance to Nalidixic Acid

216

217 Using the same diffusion assays as above, we find that acquisition of megaplasmid
218 pMPPla107 by *Pseudomonas putida* KT2440 repeatedly increased resistance of this strain to
219 nalidixic acid (as reflected by a smaller halo in megaplasmid+ strains in Figure 3). This effect
220 was replicated in an independently created pair of *P. putida* strains originally derived from *P.*
221 *putida* KT2442, where megaplasmid pMPPla107 was tagged with a different antibiotic
222 resistance marker (Figure 3A). *P. putida* KT2442 is a spontaneous rifampicin resistant isolate of
223 strain KT2440 (20). Across both strain pairs, presence of megaplasmid pMPPla107 increases

224 resistance of *P. putida* strains to nalidixic acid ($F_{1,85}$=147.57, p<0.0001) and there are clear
225 statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+
226 strains (DBL305/DBL759, t-test p<.001; DBL1604/DBL1620, t-test p<0.001). However the
227 magnitude of this effect does appear to be somewhat strain specific (Figure 3A, $F_{2,83}$=58.36,
228 p<0.001)).
229 Nalidixic acid and ciprofloxacin are both quinolone antibiotics that have the same
230 mechanism of action and cause bacterial cell death by inhibiting the ability of gyrase or
231 topoisomerase IV to covalently connect DNA strands after unwinding. Using the same Kirby-
232 Bauer diffusion assays as above, but instead replacing nalidixic acid discs with ciprofloxacin
233 discs, we find that acquisition of megaplasmid pMPPla107 does not clearly increase resistance
234 to this antibiotic (Figure 3B). Although the results are subtle, strains appear to be overall
235 sensitized by the megaplasmid to ciprofloxacin ($F_{1,80}$=12.99, p<0.001). However, only one of the
236 megaplasmid+/- strain pairs is significantly different when evaluated individually
237 (DBL305/DBL759 t-test, p<0.001; DBL1604/DBL1620 t-test, p=0.29).
238
239 A General Cost of Acquisition of Megaplasmid pMPPla107 by *P. putida*
240
241 We have previously shown that acquisition of pMPPla107 by *P. stutzeri* 28a84 leads to
242 dramatic fitness costs when measured by growth rate and by competitive fitness assay, and
243 have demonstrated that growth in the presence of nalidixic acid increases this fitness cost (12,
244 13). We therefore carried out competitive fitness assays of differentially marked *P. putida* strains
245 in order to test whether megaplasmid acquisition also affected competitive growth in this strain
246 background. As one can see in Fig. 4A, there is no measurable difference in competitive fitness
247 between the *lacZ*+ background (KT2442) and the wild type background (KT2440) (t-test
248 compared to value of 1, p=0.14). However, in media without any antibiotic supplementation,
249 megaplasmid acquisition leads to a ~10% loss in competitive fitness over one growth cycle

250 (Figure 4A), and this difference is significantly different from the control comparison ($F_{1,20}$=600.3
251 $p<0.0001$). Therefore, increased nalidixic acid resistance as seen in the assays above occurs
252 even though megaplasmid acquisition significantly lowers strain growth rates in the absence of
253 antibiotics.
254
255 Supplementation with either Nalidixic Acid or Ciprofloxacin Shifts The Results of Competitive
256 Fitness Assays In *P. putida*
257
258 Since megaplasmid acquisition by *P. putida* appears to increase nalidixic acid resistance
259 according to Kirby-Bauer diffusion assays, we tested whether supplementation of media with
260 nalidixic acid could shift the outcomes of competitive fitness assays between wild type and a
261 megaplasmid containing strain. In the absence of any antibiotic supplementation, we are able to
262 independently recapitulate the fitness costs of megaplasmid acquisition reported above, and
263 again find a significant cost of megaplasmid acquisition when measured by competitive fitness
264 (Figure 4B). However, fitness relationships between strain pairs that lack or contain the
265 megaplasmid shift when nalidixic acid is supplemented into media ($F_{2,17}$=264.82 $p<0.0001$). At
266 20µg/mL, a ~10% fitness cost of the megaplasmid changes to a slight benefit for carriage of the
267 plasmid. Moreover, we see increasing fitness according to these competitive assays for the
268 megaplasmid strain at even higher levels of nalidixic acid (40µg/mL), although the exact
269 magnitude of this effect appears to be quite variable (Figure 4B). Competitive fitness
270 measurements are significantly different between each concentration of antibiotics
271 (Tukey'sHSD, $p<0.05$). Therefore, megaplasmid acquisition by *P. putida* significantly increases
272 resistance to nalidixic acid as measured by both Kirby-Bauer diffusion assays as well as by
273 competitive fitness assays. Despite somewhat somewhat confounding results from disc diffusion
274 assays from these same strains with ciprofloxacin, but consistent with the results of competitive
275 assays supplemented with nalidixic acid, we also find that acquisition of megaplasmid

276 pMPPla107 by *P. putida* increases the competitive ability of this strain background in the
277 presence of 0.5ng/uL ciprofloxacin (Figure 4C; $F_{1,18}$=178.89, p<0.0001).
278
279 Megaplasmid Dependent Resistance to Nalidixic Acid in P. putida Is Not Positively Correlated
280 with Temperature Sensitivity
281
282 Acquisition of megaplasmid pMPPla107 by *P. stutzeri* also renders this strain more
283 sensitive to relatively higher temperatures (35-37°C, (12)). To test for this effect in *P. putida,* we
284 carried out competitive fitness assays as above at both 27 and 37°C. Much like our previous
285 results in *P. stuzeri*, the cost of megaplasmid acquisition in *P. putida* is greater when strains
286 undergo competitive fitness assays at 37°C compared to 27°C ($F_{1,18}$=319.38 p<0.0001).
287
288 **Discussion**
289
290 There is a growing interest in predicting the evolutionary dynamics within microbial
291 populations, as a means to drive evolution within populations and communities towards
292 beneficial outcomes as well as a way to control or limit change within extant populations (21).
293 Horizontal Gene Transfer (HGT) is well recognized as a powerful driver of evolutionary
294 dynamics within microbial communities (1–3), and as such, should be incorporated into
295 scenarios for predicting future evolutionary dynamics. Plasmid conjugation and acquisition is a
296 major contributor to HGT in bacterial populations and communities, and plasmids are well
297 known to provide large-scale evolutionary benefits like enabling antibiotic resistance (2, 3, 22).
298 However, plasmid carriage can also entail a variety of fitness costs, which can be manifest as
299 lowered fitness or other phenotypic changes for plasmid bearing strains, in specific
300 environments (4–6). The presence of costs associated with plasmid carriage has led to
301 speculation about the conditions that enable plasmid maintenance within populations through

302 time, with one likely scenario being that costs associated with plasmid carriage are ameliorated
303 over time through subsequent and potentially rapid compensatory adaptation (23, 24). Although
304 not all HGT events entail fitness costs, rapid amelioration of fitness costs associated with
305 plasmid acquisition could lead to genetic and phenotypic tradeoffs that fundamentally shift future
306 evolutionary trajectories for the affected populations. As such, and with a lofty goal of ultimately
307 being able to predict evolution across bacterial populations, it is therefore critical to understand
308 how costs of plasmid carriage are affected by genetic, genomic, and environmental contexts.
309 Here we demonstrate that the direction of one previously observed phenotype
310 associated with acquisition of megaplasmid pMPPla107, sensitivity to nalidixic acid in
311 *Pseudomonas (13)*, depends upon the interactions between this megaplasmid and genomic
312 background of the strain of interest. Across almost all surveyed strains, acquisition of
313 pMPPla107 sensitizes cells to nalidixic acid, but in *P. putida* KT2440 this same horizontal
314 transfer event increases resistance to this antibiotic. These contrasting phenotypic effects occur
315 in the absence of identifiable megaplasmid genes which could be hypothesized to influence
316 antibiotic sensitivity and resistance. Thus, this is not simply a scenario where a common
317 antibiotic resistance gene found on the megaplasmid is active in one background compared to
318 the others, but rather a situation where the phenotypic effects of genes present on a
319 megaplasmid are polar opposites under certain environments in ways that depend on the
320 chromosomal background. Also important, these same phenotypic benefits are seen when
321 megaplasmid + and - strains of *P. putida* are competed together in fitness assays. We also
322 demonstrate that these same competitive fitness effects can be seen when megaplasmid + and
323 - strains of *P. putida* are competed in the presence of ciprofloxaxin, an antibiotic which has the
324 same mechanism of action as nalidixic acid. These results suggest that the mechanism
325 underlying changes in sensitivity to nalidixic acid and/or ciprofloxacin could be due to alterations
326 in the activity or amounts of gyrase and topoisomerase activity within *P. putida* cells after
327 acquisition of an additional 1Mb of DNA.

328 Although we did not see a clear and similar benefit to megaplasmid acquisition by *P.*
329 *putida* using disc assays for ciprofloxacin, it is possible that discrepancies could arise because
330 of differences in the concentration of ciprofloxacin across both types of assay. In disc assays,
331 ciprofloxacin diffuses out into the plate from a disc seeded with 5μg/mL of this antibiotic
332 whereas competitive fitness assays took place at 0.5ng/mL concentrations. We also observed
333 that there was a sharp gradient at ciprofloxacin concentrations in competitive fitness assays
334 whereby above 0.5ng/mL all strains were inhibited in growth (data not shown). It is possible that
335 there is a small window to dial in the benefits of megaplasmid pMPPla107 across
336 concentrations of ciprofloxacin and that the concentrations in the disc assay do not enable the
337 resolution to pick apart clear differences in strains. It is also possible that the benefits of
338 acquisition of pMPPla107 for ciprofloxacin resistance by *P. putida* are subtly dependent on the
339 precise type of assay and potentially on the exact strain background being assayed. For
340 instance, only one of the two strain pairings used for *P. putida* (DBL305 and DBL759) was
341 significantly different in the ciprofloxacin disc assays and it's possible that secondary mutations
342 that occurred during construction of these strains are responsible for this result. Ultimately, and
343 regardless of confounding results between multiple types of assays for ciprofloxacin, the
344 consistency of the nalidixic acid results across assays and support from competitive fitness
345 assays, clearly demonstrate that the phenotypic consequences of acquisition of pMPPla107 by
346 *P. putida* are fundamentally different than that in *P. stutzeri.*
347 Quinolone antibiotics have been used as a means of curing a variety of plasmids from
348 many different bacterial strains, given that they inhibit the functions of topoisomerase IV as well
349 as gyrase (25), which implies that plasmid driven sensitivity to quinolones could be a
350 widespread phenomenon (26). These previously reported effects are thought to be mediated by
351 stress at the level of gyrase and topoisomerase function and should therefore likely be affected
352 by either nalidixic acid or ciprofloxacin (26). At present there are no clear mechanistic
353 explanations for differing phenotypic responses of *Pseudomonas* strains to acquisition of

354 pMPPla107 and any potential scenario must involve interactions between genes on both the
355 chromosome and megaplasmid with one outcome of this interaction being differential sensitivity
356 to nalidixic acid. While annotations of pMPPla107 do not point to any genes directly implicated
357 in nalidixic acid resistance (14), this megaplasmid does contain two genes that together are
358 annotated as *parC* and *parE* (locus tags PLA107_031430 and PLA107_031415 encoding
359 topoisomerase IV subunits A and B, respectively). Interestingly, blastP comparisons of all
360 complete Pseudomonas genomes through Pseudomonas.com (27), suggests that the
361 megaplasmid *parC* is closely related to chromosomal orthologs from *P. putida* (~75% amino
362 acid similarity) while *parE* is closely related to chromosomal orthologs in *P. stutzeri* (~82%
363 amino acid similarity). Moreover, quinolones are known to bind to topoisomerase subunit A to
364 disrupt its activity with resistance mutations often occurring in this subunit across different
365 bacteria (25). It is certainly possible that negative and positive interactions between
366 megaplasmid encoded topoisomerase IV subunits with their chromosomal orthologs in each
367 strain background could mediate the differences reported here in response to quinolone
368 antibiotics and this will be a focus of future studies.
369 Gene annotations also suggest that the megaplasmid contains multiple efflux pumps.
370 While the substrates for these pumps are currently unknown, it is plausible that one could
371 specifically transport quinolone antibiotics outside of the cell in *P. putida*. Indeed, there is
372 precedence for efflux pumps in *Pseudomonas aeruginosa* and other bacteria to differentially
373 transport quinolone antibiotics (28, 29). Following this line of thought, it would then be possible
374 that either the particular regulation or activity of this pump differs based on chromosomal
375 background. There may also be a mechanism to differentially detoxify nalidixic acid and
376 ciprofloxacin that resides on the chromosome (whether through efflux pumps or other means),
377 but where the activity of which is differentially affected by megaplasmid encoded pathways.
378 Perhaps there is a global regulator which is similarly affected by megaplasmid presence, but
379 where the downstream phenotypic effects differ across strain backgrounds. For example,

380 megaplasmid presence could uniformly lead to upregulation of AmrZ across Pseudomonads,
381 but upregulation of AmrZ may trigger opposing phenotypic effects in the context of quinolone
382 resistance in different strain backgrounds (30). It is also possible that phenotypic outcomes of
383 increased sensitivity and resistance to quinolone in the presence of pMPPla107 are due to
384 mechanistically different effects. Under this case, the default condition might be that plasmid
385 acquisition causes cells to become more sensitive to certain antibiotics, but that in some
386 backgrounds (in this case *P. putida*) there is an additional pathway or efflux pump that is
387 differentially affected by megaplasmid presence and which enables cells to overcome
388 sensitization found in other *Pseudomonas* species.

389 As we have demonstrated previously in *P. stutzeri,* we show that acquisition of
390 megaplasmid pMPPla107 is associated with a large (~10%) fitness cost in *P. putida* in
391 conditions without antibiotic supplementation. Therefore, differing responses to nalidixic acid
392 cannot simply be explained as a secondary consequence of overall growth defects due to
393 acquisition of pMPPla107 and there must be some other inherent systems level effect that
394 creates opposing phenotypic responses across strain backgrounds upon acquisition of this
395 megaplasmid. We further demonstrate that acquisition of pMPPla107 by *P. putida* also
396 increases sensitivity of this strain background to relatively higher temperatures. These
397 contrasting results of megaplasmid acquisition on fitness across multiple environments in *P.*
398 *putida* suggest that the multiple previously observed fitness costs associated with megaplasmid
399 pMPPla107 (sensitization to antibiotics and higher temperatures) have independent underlying
400 mechanistic bases, at least in *P. putida.*

401 Specific evolutionary responses to selective pressures are driven by the availability of
402 adaptive mutations within the context of an adaptive landscape (31). Costs associated with
403 plasmid acquisition can rapidly be ameliorated by new mutations that occur on either the
404 plasmid or throughout the rest of the genome, with the speed of compensation correlated with
405 selection pressures acting on the plasmid bearing population (23, 24). Extrapolation from work

406 focused on the molecular and theoretical basis of adaptation predicts that the target size or
407 number of potential compensatory mutations should increase with the cost of plasmid carriage
408 (32). Given that results presented here as well as those reported in other recent papers strongly
409 suggest that costs of plasmid carriage change depending on the environment (33), it is therefore
410 likely that both the speed and molecular basis of amelioration of plasmid costs will greatly differ
411 across environments. Likewise, since the costs and phenotypic consequences of plasmid
412 carriage differ dramatically across genomic backgrounds, there will likely be substantial variation
413 in both the evolutionary paths and molecular basis of compensatory mutations for the exact
414 same plasmid within different strains. The phenotypic effects reported here suggest that
415 plasmids could be differentially maintained or lost across bacterial populations in ways that
416 depend on genotype by environment interactions rather than on simply based on the presence
417 or absence of beneficial genes. Results presented here therefore also strongly bear on our
418 understanding and predicting the evolutionary forces that enable plasmid sharing across
419 microbial communities.
420
421 The phenomenon reported here suggests that plasmid acquisition could fundamentally
422 change evolutionary dynamics of antibiotic resistance in a strain dependent way. For instance,
423 there will likely be a different suite of resistance mutations available towards nalidixic acid in *P.*
424 *putida* KT2440 depending on whether this genomic background contains pMPPla107 or not.
425 This is very much in the vein of sign epistasis when discussing phenotypic effects of *de novo*
426 mutation and evolutionary pathways of adaptation (9, 10, 34, 35). It is also currently unknown
427 how phenotypic effects of particular resistance mutations are altered in the presence of this
428 megaplasmid, but we note that the values evaluated here (20-40ug/mL for Nalidixic acid and 0.5
429 ug/mL for ciprofloxacin) are relatively close to relevant minimum inhibitory concentrations of
430 hospital isolates of *P. putida* (36). Given that there will likely be tradeoffs in gyrase activity with
431 mutations towards quinolone resistance, one could envision scenarios where acquisition of this

432 megaplasmid simply adds to the level of resistance to nalidixic acid, has no phenotypic effect in
433 a background that already contains nalidixic acid resistance mutations, or even sensitizes cells
434 to nalidixic acid. For example, if a mutation arises in *P. putida* KT2440 that provides resistance
435 to nalidixic acid at 100ng/uL, it is possible that further acquisition of pMPPla107 may either
436 provide no benefit (Minimum Inhibitory Concentration (MIC)+pMPPla107=100ng/uL), an additive
437 increase to resistance (MIC+pMPPla107>100ng/uL), or sensitizes this strain
438 (MIC+pMPPla107<100ng/uL). Future experiments will be necessary to differentiate between
439 these possibilities.
440 In conclusion, we report here that acquisition of megaplasmid pMPPla107 leads to
441 opposing phenotypic effects of strains in the presence of nalidixic acid but not ciprofloxacin. In
442 most *Pseudomonas* strains evaluated here (which represent a relatively diverse slice from the
443 broader *Pseudomonas* phylogeny (37)), there is a cost to acquisition of pMPPla107 in that cells
444 containing this megaplasmid are sensitized to this antibiotic. However, at least in *P. putida,*
445 acquisition of pMPPla107 increases resistance to nalidixic acid in the absence of identifiable
446 genes involved in antimicrobial resistance. We highlight that these results of HGT are analogous
447 to the phenomenon of sign epistasis when discussing phenotypic effects of *de novo* mutations
448 within a genome. Therefore, as with sign epistasis, it is therefore likely that some HGT events
449 will lead to fundamental shifts in the adaptive landscape in ways that depend on both the
450 genomic background and environment. Depending on how widespread such results are, context
451 dependence of phenotypic effects will potentially increase uncertainty in models and predictions
452 for the evolutionary dynamics of microbial adaptation after horizontal gene transfer events as
453 well as antimicrobial resistance.
454
455 **Data Availability**
456

Scripts for recreating all figures and statistical tests using R, as well as all underlying data for these scripts, can be found at **https:/doi.org/10.5281/zenodo.4409341.** Additionally, supplemental Figure 1 can be found at https://doi.org/10.6084/m9.figshare.10257704.v2.

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Table 1: Strains used in this study

Strain Number	Strain Description	Citation
DAB282	a RifR (50ng/µL) isolate of *P. stutzeri* strain JM300	This Paper
DAB462	a RifR (50ng/µL) isolate of *P. syringae* pv. *lachrymans* 8003	(12)
DAB837	DAB462 containing Tn7 transposon providing Kanamycin resistance	(12)
DAB885	a RifR (50ng/µL) isolate of *P. syringae* pv. *lachrymans* 107 containing tetR tagged version of megaplasmid pMPPla107	(12)
DAB895	DAB835 containing tetR tagged version of megaplasmid pMPPla107	(12)
DBL163	*P. fluorescens* strain Pf0-1 acquired from Jeff Chang	(38)
DBL187	DAB282 containing a KanR megaplasmid from DAB885	This Paper
DBL305	*P. putida* KT2440 acquired from ATCC, strain ATCC47054	(20)
DBL332	a RifR (50ng/µL) isolate of *P. stutzeri* strain 28a24	(12)
DBL386	DBL332 containing Tn7 transposon providing Kanamycin resistance	(12)
DBL408	DBL386 containing the megaplasmid from DAB885	(12)
DBL453	DBL332 containing an unmarked version of megaplasmid pMPPla107	(12)
DBL494	A Nalidixic acid (100ng/µL) and Gentamycin (10ng/µL) resistant isolate of *P. stutzeri* 28a24, with a mariner transposon insertion in megaplasmid pMPPla107	(39)
DBL717	DBL453 containing a Tn5 tagged version of megaplasmid pMPPla107	This Paper
DBL740	a RifS isolate of *P. syringae* pv. *lachrymans* 107 containing tetR tagged version of megaplasmid pMPPla107	This Paper
DBL759	DBL305 containing the megaplasmid from DBL717	This Paper
DBL877	DBL173 containing the megaplasmid from DBL494	This Paper
DBL880	a RifR (50ng/µL) isolate of *P. stutzeri* strain 28a69	This Paper
DBL883	a RifR (50ng/µL) isolate of *P. stutzeri* strain 28a72	This Paper
DBL885	a RifR (50ng/µL) isolate of *P. stutzeri* strain 28a63	This Paper
DBL907	DBL880 containing megaplasmid from DBL740	This Paper
DBL910	DBL883 containing megaplasmid from DBL740	This Paper
DBL912	DBL885 containing megaplasmid from DBL740	This Paper
DBL1604	*P. putida* KT2442 LacZ+ isolate acquired from V. di Lorenzo	(40)
DBL1620	DBL1604 containing the megaplasmid from DBL1715	This Paper
DBL1715	DBL332 containing a second Tn5 tagged version of megaplasmid pMPPla107	This Paper

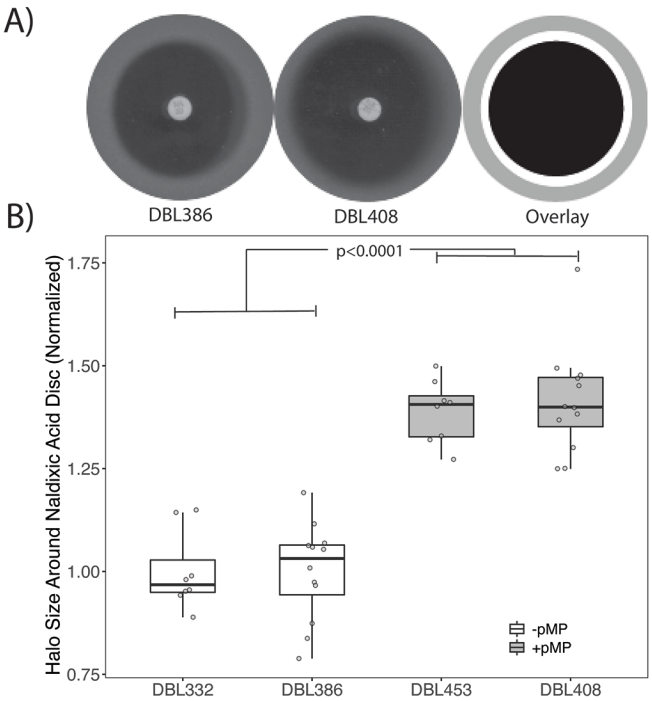


Figure 1. Kirby-Bauer Diffusion Assays Recapitulate A Nalidixic Acid Sensitivity Phenotypes in *Pseudomonas stutzeri*. We have previously demonstrated that acquisition of megaplasmid pMPPla107 increases sensitivity to nalidixic acid in two independent rifampicin resistant derivatives of *P. stutzeri* 28a24, DBL332 and DBL386. Here we show that assays based on diffusion of nalidixic acid out of a disc recapitulate this phenotype. A) Representative scans from a disc diffusion assay from using (from left to right) a strain that either lack (DBL386) or contain (DBL408) megaplasmid pMPPla107. Image areas covered by each of these two representative pictures (grey), the sensitivity halo around the disc containing nalidixic acid and strain DBL386 (black) or strain DBL408 (white) are overlaid onto each other at the far right of this image. The larger the inhibition halo, the greater the sensitivity to nalidixic acid. B) Data from disc diffusion overlay assays comparing *P. stutzeri* strains DBL332 and DBL386 with derivatives which have acquired megaplasmid pMPPla107. Each strain was assayed at least three independent times, with at least 2 (but usually 6) replicates per strain per assay. Halo size was normalized to the relevant "wild type" strain within each assay (DBL332 for DBL453, DBL386 for DBL408) and plotted on the Y-axis. Individual data points are shown for all assays, with box hinges corresponding to first and third quartiles and mean plotted as horizontal lines in the center of the boxes. Strains containing the megaplasmid are plotted in the grey boxes and are more sensitive to inhibition by nalidixic acid ($F_{1,36}$=132.143, $p<0.0001$) than paired parental strains lacking the megaplasmid (plotted in white). Additionally, there are clear statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+ strains (DBL332/DBL453, ttest $p<.001$; DBL386/DBL408, ttest $p<0.001$)

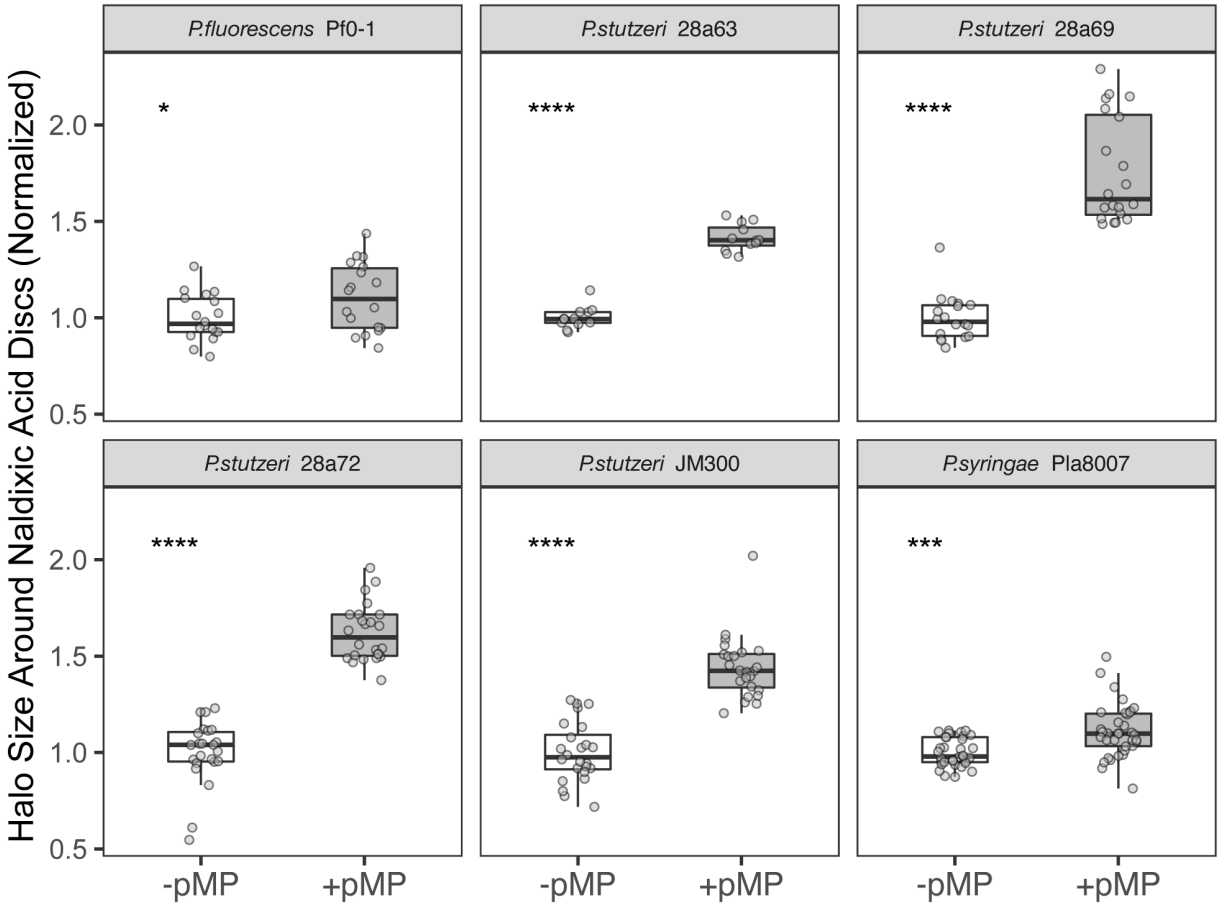


Figure 2. Acquisition of Megaplasmid pMPPla107 Sensitizes Numerous Pseudomonas strains to Nalidixic Acid. Data is shown for disc diffusion overlay assays comparing *P. stutzeri* strains DBL880, DAB282, DBL883, and DBL885 with derivatives which have acquired megaplasmid pMPPla107 (DBL907, DBL287, DBL910, DBL912 respectively). Data is also shown for derivatives of *P. syringae* pv. *lachrymans* strain 8003 and *P. fluorescens* Pf0-1 which either lack (DBL462 and DBL163, respectively) or contain (DBL895 and DBL877, respectively) megaplasmid pMPPla107. Strain names of the original isolates of genomic backgrounds lacking the megaplasmid are shown in grey headers. Data for "wt" strains are plotted in white boxes, while data for strains containing the megaplasmid are plotted in grey boxes. Each strain was assayed at least three independent times, with at least 4 (but usually 6) replicates per strain per assay. Two additional assays were performed for *P. syringae* strains. Halo size was normalized to the relevant "wild type" strain within each assay and plotted on the Y-axis. Individual data points are shown for all assays, with box hinges corresponding to first and third quartiles and mean plotted as horizontal lines in the center of the boxes. In each case, presence of megaplasmid pMPPla107 sensitizes strains to nalidixic acid as shown by increased halos of inhibition. Asterisks indicate statistical significance by t-test: >0.05=ns, *=<0.05, **=<0.01, ***=<0.001, ****=<0.0001. All intrastrain comparisons between parent strains that lack the megaplasmid and derivative strains containing the megaplasmid are significantly different ($p<0.001$) by t-test with the exception of the Pf0-1 comparison which was $p<0.05$ by t-test.

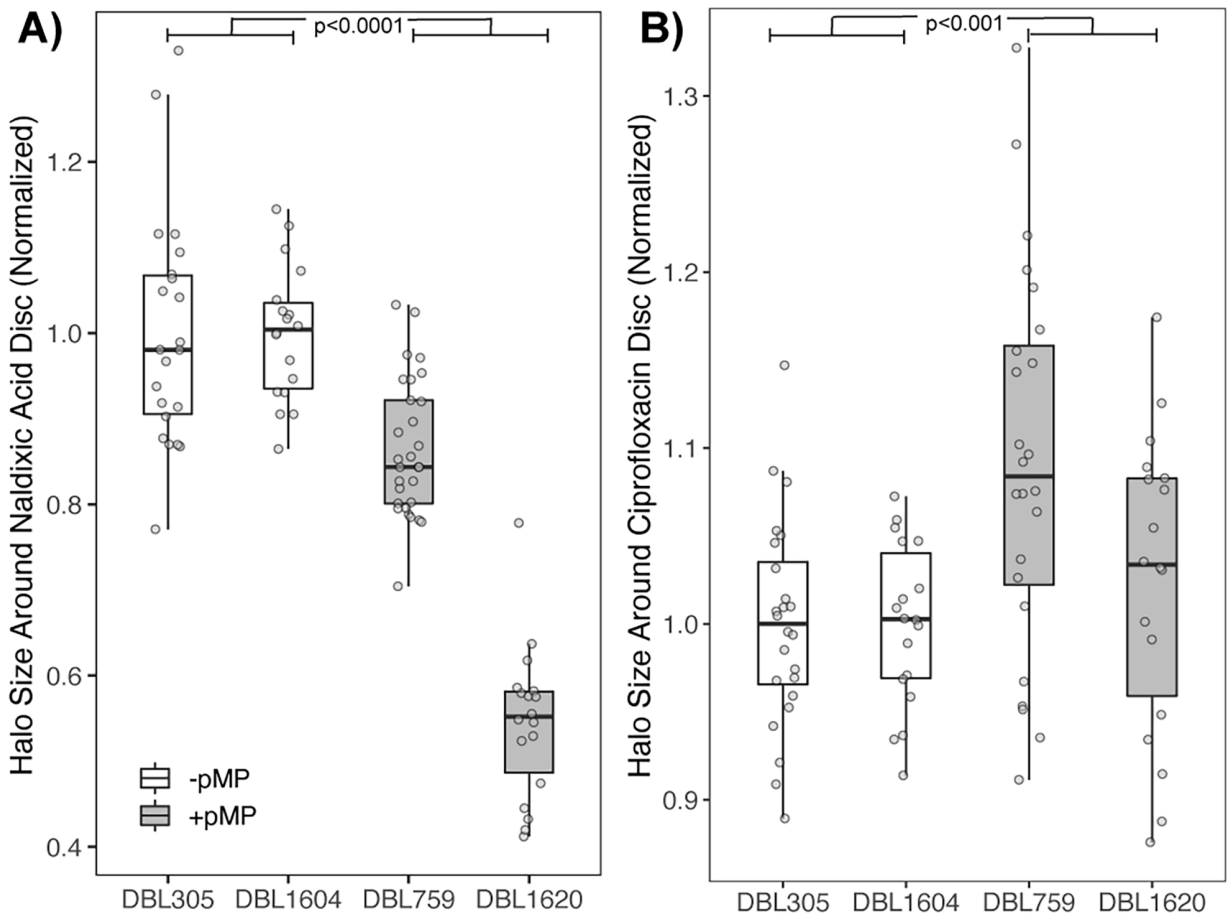
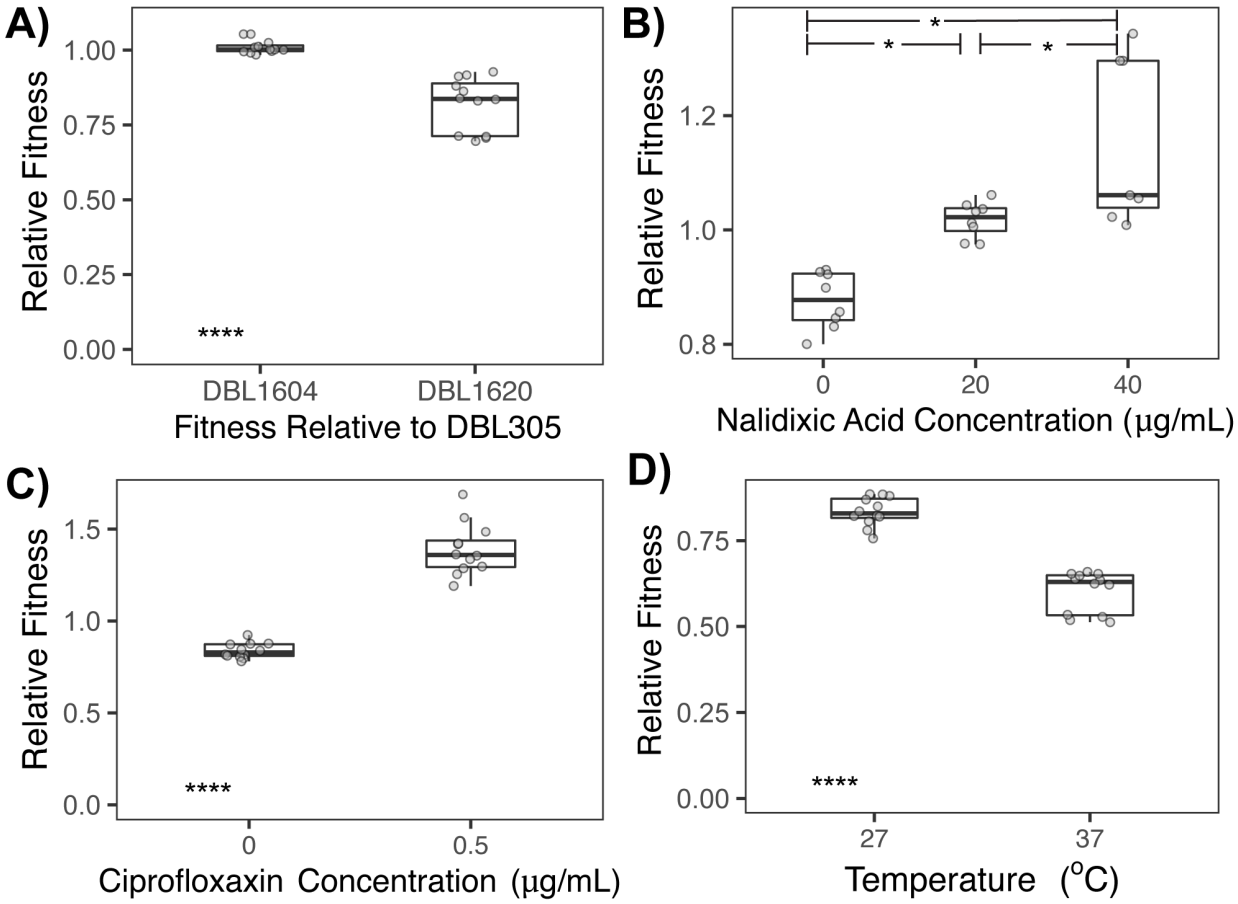


Figure 3. Acquisition of Megaplasmid pMPPla107 by *P. putida* Increases Resistance to Nalidixic Acid but Not Ciprofloxacin in Disc Diffusion Assays. Data is shown for disc diffusion overlay assays comparing *P. putida* strains DBL305 (KT2440) and DBL1604 (KT2442) with derivatives which have acquired megaplasmid pMPPla107 (DBL759 and DBL1620 respectively). Each strain was assayed at least three independent times for each antibiotic, with 6 replicates per strain per assay. Halo size was normalized to the relevant "wild type" strain within each assay and plotted on the Y-axis. Individual data points are shown for all assays, with box hinges corresponding to first and third quartiles and mean plotted as horizontal lines in the center of the boxes. Data for "wt" strains are plotted in white boxes, while data for strains containing the megaplasmid are plotted in grey boxes. A) Presence of megaplasmid pMPPla107 increases resistance of *P. putida* strains to nalidixic acid as shown by decreased halos of inhibition ($F_{1,85}$=147.57, p<0.0001). Moreover, there are clear statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+ strains (DBL305/DBL759, t-test p<.001; DBL1604/DBL1620, t-test p<0.001) B) Presence of megaplasmid pMPPla107 appears to sensitize *P. putida* strains to ciprofloxacin as shown by increased halos of inhibition ($F_{1,80}$=12.99, p<0.001). However, although ciprofloxacin halo inhibition areas statistically differ between strains DBL305 and DBL759 (ttest, p<0.001), there is no clear difference in the inhibition halos between strains DBL1604 and DBL1620 (ttest, p=0.29).



530

531 **Figure 4. Contrasting Effects of Acquisition of Megaplasmid pMPPla107 in *P. putida***
532 **Across Different Environments as Measured by Competitive Fitness Assays** Competitive
533 fitness assays in liquid KB media were carried out between strain DBL305 (*P. putida* KT2440)
534 and *lacZ*+ strains that either lack (DBL1604) or contain (DBL1620) megaplasmid pMPPla107. At
535 least two competitive fitness assays were carried out for each pairing with 4 replicates per assay
536 in all but one case (one assay at Nal40 only had 3 replicates). Individual data points are shown
537 for all assays, with box hinges corresponding to first and third quartiles and mean plotted as
538 horizontal lines in the center of the boxes. Asterisks indicate statistical significance by t-test:
539 >0.05=ns, *=<0.05, **=<0.01, ***=<0.001, ****=<0.0001. A) Although there is no measurable
540 difference in competitive fitness due to the *lacZ*+ marker (assays between DBL305 and
541 DBL1604), presence of the megaplasmid leads to a ~10% fitness cost during growth in KB
542 medium ($F_{1,20}$=600.3 p<0.0001). B) Although the ~10% fitness cost of the megaplasmid is again
543 apparent during competition between DBL305 and DBL1620 in unsupplemented KB medium,
544 strain 1620 (containing megaplasmid pMPPla107) displays increasingly higher relative fitness
545 compared to strain DBL305 when media is supplemented with nalidixic acid at either 20 or 40
546 ng/uL (($F_{2,17}$=264.82 p<0.0001), all comparisons significant at p<0.05 by Tukey's HSD) or with
547 C) ciprofloxacin at 0.5µg/mL ($F_{1,18}$=178.89 p<0.0001). D) The fitness cost of megaplasmid
548 carriage greatly increases, to >50%, when competitions are carried out at higher temperatures
549 of 37°C ($F_{1,18}$=319.38 p<0.0001).

1. Soucy SM, Huang J, Gogarten JP. 2015. Horizontal gene transfer: building the web of life. Nat Rev Genet 16:472–482.

2. Hall JPJ, Brockhurst MA, Harrison E. 2017. Sampling the mobile gene pool: innovation via horizontal gene transfer in bacteria. Philos Trans R Soc Lond B Biol Sci 372.

3. Brockhurst MA, Harrison E, Hall JPJ, Richards T, McNally A, MacLean C. 2019. The Ecology and Evolution of Pangenomes. Current Biology.

4. Baltrus DA. 2013. Exploring the costs of horizontal gene transfer. Trends Ecol Evol 28:489–495.

5. Carroll AC, Wong A. 2018. Plasmid persistence: costs, benefits, and the plasmid paradox. Can J Microbiol 64:293–304.

6. San Millan A, MacLean RC. 2017. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. Microbiol Spectr 5.

7. Kottara A, Hall JPJ, Harrison E, Brockhurst MA. 2018. Variable plasmid fitness effects and mobile genetic element dynamics across Pseudomonas species. FEMS Microbiol Ecol 94.

8. Alonso-del Valle A, León-Sampedro R. 2020. The distribution of plasmid fitness effects explains plasmid persistence in bacterial communities. bioRxiv.

9. Silva RF, Mendonça SCM, Carvalho LM, Reis AM, Gordo I, Trindade S, Dionisio F. 2011. Pervasive sign epistasis between conjugative plasmids and drug-resistance chromosomal mutations. PLoS Genet 7:e1002181.

10. Weinreich DM, Watson RA, Chao L. 2005. Perspective: Sign epistasis and genetic

572 constraint on evolutionary trajectories. Evolution 59:1165–1174.

573 11. de Visser JAGM, Cooper TF, Elena SF. 2011. The causes of epistasis. Proc Biol Sci
574 278:3617–3624.

575 12. Romanchuk A, Jones CD, Karkare K, Moore A, Smith BA, Jones C, Dougherty K, Baltrus
576 DA. 2014. Bigger is not always better: transmission and fitness burden of 1 MB
577 Pseudomonas syringae megaplasmid pMPPla107. Plasmid 73:16–25.

578 13. Dougherty K, Smith BA, Moore AF, Maitland S, Fanger C, Murillo R, Baltrus DA. 2014.
579 Multiple Phenotypic Changes Associated with Large-Scale Horizontal Gene Transfer. PLoS
580 ONE.

581 14. Smith BA, Leligdon C, Baltrus D. 2018. Just the Two of Us? A Family of Pseudomonas
582 Megaplasmids Offers a Rare Glimpse Into the Evolution of Large Mobile Elements. bioRxiv.

583 15. Sikorski J, Teschner N, Wackernagel W. 2002. Highly different levels of natural
584 transformation are associated with genomic subgroups within a local population of
585 Pseudomonas stutzeri from soil. Appl Environ Microbiol 68:865–873.

586 16. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image
587 analysis. Nat Methods 9:671–675.

588 17. R Core Team. 2016. R: A language and environment for statistical. R Foundation for
589 Statistical Computing, Viena, Austria, Vienna, Austria.

590 18. Bauer AW, Kirby WM, Sherris JC, Turck M. 1966. Antibiotic susceptibility testing by a
591 standardized single disk method. Am J Clin Pathol 45:493–496.

592 19. Baltrus DA, Nishimura MT, Romanchuk A, Chang JH, Mukhtar MS, Cherkis K, Roach J,
593 Grant SR, Jones CD, Dangl JL. 2011. Dynamic evolution of pathogenicity revealed by

594 sequencing and comparative genomics of 19 Pseudomonas syringae isolates. PLoS
595 Pathog 7:e1002132.

596 20. Bagdasarian M, Lurz R, Rückert B, Franklin FCH, Bagdasarian MM, Frey J, Timmis KN.
597 1981. Specific-purpose plasmid cloning vectors II. Broad host range, high copy number,
598 RSF 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas.
599 Gene 16:237–247.

600 21. Lässig M, Mustonen V, Walczak AM. 2017. Predicting evolution. Nat Ecol Evol 1:77.

601 22. Heuer H, Smalla K. 2012. Plasmids foster diversification and adaptation of bacterial
602 populations in soil. FEMS Microbiol Rev 36:1083–1104.

603 23. Zwanzig M, Harrison E, Brockhurst MA, Hall JPJ, Berendonk TU, Berger U. 2019. Mobile
604 Compensatory Mutations Promote Plasmid Survival. mSystems 4.

605 24. Hall JPJ, Wright RCT, Guymer D, Harrison E, Brockhurst MA. 2019. Extremely fast
606 amelioration of plasmid fitness costs by multiple functionally diverse pathways.
607 Microbiology https://doi.org/10.1099/mic.0.000862.

608 25. Hooper DC, Jacoby GA. 2016. Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of
609 Action and Resistance. Cold Spring Harb Perspect Med 6.

610 26. Buckner MMC, Ciusa ML, Piddock LJV. 2018. Strategies to combat antimicrobial
611 resistance: anti-plasmid and plasmid curing. FEMS Microbiol Rev 42:781–804.

612 27. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FSL. 2016. Enhanced
613 annotations and features for comparing thousands of Pseudomonas genomes in the
614 Pseudomonas genome database. Nucleic Acids Res 44:D646–53.

615 28. Piddock LJV, Jin YF, Webber MA, Everett MJ. 2002. Novel ciprofloxacin-resistant, nalidixic

616 acid-susceptible mutant of Staphylococcus aureus. Antimicrob Agents Chemother
617 46:2276–2278.

618 29. Köhler T, Michea-Hamzehpour M, Plesiat P, Kahr AL, Pechere JC. 1997. Differential
619 selection of multidrug efflux systems by quinolones in Pseudomonas aeruginosa.
620 Antimicrob Agents Chemother 41:2540–2543.

621 30. Baltrus DA, Dougherty K, Diaz B, Murillo R. 2018. Evolutionary Plasticity of AmrZ
622 Regulation in Pseudomonas. mSphere 3.

623 31. Orr HA. 2005. The genetic theory of adaptation: a brief history. Nat Rev Genet 6:119–127.

624 32. Orr HA. 2010. The population genetics of beneficial mutations. Philos Trans R Soc Lond B
625 Biol Sci 365:1195–1201.

626 33. San Millan A, Toll-Riera M, Qi Q, Betts A, Hopkinson RJ, McCullagh J, MacLean RC. 2018.
627 Integrative analysis of fitness and metabolic effects of plasmids in Pseudomonas
628 aeruginosa PAO1. ISME J 12:3014–3024.

629 34. Chiotti KE, Kvitek DJ, Schmidt KH, Koniges G, Schwartz K, Donckels EA, Rosenzweig F,
630 Sherlock G. 2014. The Valley-of-Death: reciprocal sign epistasis constrains adaptive
631 trajectories in a constant, nutrient limiting environment. Genomics 104:431–437.

632 35. Wu NC, Dai L, Olson CA, Lloyd-Smith JO, Sun R. 2016. Adaptation in protein fitness
633 landscapes is facilitated by indirect paths. Elife 5.

634 36. Molina L, Udaondo Z, Duque E, Fernández M, Molina-Santiago C, Roca A, Porcel M, de la
635 Torre J, Segura A, Plesiat P, Jeannot K, Ramos J-L. 2014. Antibiotic resistance
636 determinants in a Pseudomonas putida strain isolated from a hospital. PLoS One 9:e81604.

637 37. Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E. 2015. Phylogenomics and

638 systematics in Pseudomonas. Front Microbiol 6:214.

639 38. Thomas WJ, Thireault CA, Kimbrel JA, Chang JH. 2009. Recombineering and stable
640 integration of the Pseudomonas syringae pv. syringaeâ61 hrp/hrc cluster into the genome
641 of the soil bacterium Pseudomonas fluorescens Pf0-1. The Plant Journal.

642 39. Baltrus DA, Medlen J, Clark M. 2019. Identifying transposon insertions in bacterial
643 genomes through nanopore sequencing. Cold Spring Harbor Laboratory.

644 40. Silva-Rocha R, de Lorenzo V. 2011. A composite feed-forward loop I4-FFL involving IHF
645 and Crc stabilizes expression of the XylR regulator of Pseudomonas putida mt-2 from
646 growth phase perturbations. Mol Biosyst 7:2982–2990.

647