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.

41 **Introduction**

42

43 Horizontal Gene Transfer (HGT), a catch-all term for the transfer of DNA between
44 individuals, is one of the main drivers of evolutionary dynamics across microbial populations (1–
45 3). Although there are countless examples where HGT has been shown to increase microbial
46 fitness and/or enable colonization of new niches, it is increasingly appreciated that HGT brings
47 with it a variety of costs that arise as byproducts of the incorporation of foreign DNA into
48 established physiological and genetic systems (4–6). Recent efforts have begun to categorize
49 and characterize phenotypic effects of HGT across systems, and have clearly demonstrated
50 that acquisition of the same genes can lead to different outcomes across genomic backgrounds
51 (7, 8). Such differences in phenotypic outcomes following HGT are analogous if not identical to
52 the concept of sign epistasis in the context of de novo mutations, whereby the fitness effects of
53 a mutation change sign (e.g. from positive to negative) under specific genetic combinations (9–
54 11). Much like sign epistasis, we currently lack a general understanding of the underlying
55 causes of differences in phenotypic outcomes following HGT, and as a consequence, there is
56 no clear model for predicting if and when acquisition of DNA could be detrimental in some
57 genomic backgrounds but beneficial in others.

58

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

67 (14) and that, at present, the mechanistic basis and overall strain specificity of these phenotypic
68 changes remains unknown.
69
70 Here, we test for how frequently sensitivity to nalidixic acid is observed across
71 Pseudomonas strains after acquisition of megaplasmid pMPPla107. We show that, although the
72 presence of this megaplasmid increases sensitivity to nalidixic acid in many strain backgrounds,
73 one strain in particular - *Pseudomonas putida* KT2440 - displays increased resistance to
74 nalidixic acid and ciprofloxacin after this HGT event. Therefore, our report provides an additional
75 example whereby the precise phenotypic effects of HGT are dependent on strain background,
76 but also suggests that that antibiotic resistance can be increased by megaplasmid pMPPla107
77 even in the absence of identifiable resistance genes in the region acquired by HGT. These
78 results, and specifically strain-dependent changes in sign of phenotypic effects of plasmid
79 acquisition, provide a new viewpoint for considering conditions that promote plasmid
80 maintenance and highlight the influence of genomic and environmental contexts when
81 considering amelioration of fitness costs after HGT events.
82
83

84 **Methods**

85

86 Bacterial strains and culture conditions

87 All strains and plasmids used in the study are listed in Table 1. Strain propagation of
88 all cultures largely took place at 27°C in King's B medium supplemented with rifampicin
89 unless otherwise specified. Antibiotics were supplemented into media at the following
90 concentrations when appropriate and unless otherwise specified: 50 µg/ml rifampicin, 10
91 µg/ml tetracycline, and 20 µg/ml kanamycin. 5-bromo-4-chloro-3-indolyl-β-D-
92 galactopyranoside (Xgal) was supplemented at 40 µg/ml. For competition assays, cultures
93 were supplemented with nalidixic acid at 20µg/mL and 40µg/mL and with ciprofloxacin at
94 0.5µg/mL. Antibiotic discs used in the diffusion assays contained antibiotics at
95 concentrations of 30 µg/ml for nalidixic acid and 5 µg/ml for ciprofloxacin.

96

97 Kirby-Bauer Disc Diffusion Assays

98

99 Strains of interest for each assay were streaked from frozen stocks and incubated on
100 KB media at 27°C for 2 to 3 days. At this point a small clump of cells was picked to liquid
101 KB medium and grown for 24 hours at 27°C with shaking. Cells from each culture were then
102 pelleted in a centrifuge, the supernatant was poured out, and pelleted cells were
103 resuspended in 10Mm $MgCl_2$ for measurement of OD_{600}. Each culture was then diluted to
104 0.01 OD_{600} in 10Mm $MgCl_2$ and 300uL of each culture was added to 3mL of 0.4% water
105 agar (which was previously melted and allowed to cool until warm to the touch). This
106 bacteria/agar mixture was then poured onto a plain KB agar plate (1.5% agar) and allowed
107 to solidify. Lastly, a disc containing the antibiotic of interest was added to the center of the

108 plate and each was placed face up in an incubator at 27°C. Pairs of megaplasmid +/-
109 strains were always evaluated together within the same assay block.
110 After 3 days of growth, each plate was taken out of the incubator and scanned. This
111 image was imported into ImageJ (15), and the area of the inhibition zone was measured. An
112 average area was calculated for the megaplasmid- strain within each assay, and individual
113 data points for each pair of megaplasmid + and - strains in each assay were divided by this
114 value in order to normalize the data, Therefore, the average inhibition zone for each
115 megaplasmid- strain within each assay was normalized to a value of 1. Each strain pairing
116 was tested in at least (but usually more than) two independent assays.
117
118 Competitive Fitness Assays
119 Strains of interest for each assay were streaked from frozen stocks for 2 to 3 days
120 (on media containing kanamycin for megaplasmid+ strains) at which point a small clump of
121 cells was picked to liquid KB medium and grown for 24 hours at 27°C with shaking. Cells
122 from each culture were then pelleted in a centrifuge, the supernatant was poured out, and
123 pelleted cells were resuspended in 10Mm $MgCl_2$ for measurement of OD_{600}. Each culture
124 was then diluted to 1.0 OD_{600} in 10Mm $MgCl_2$.
125 A 10mL master culture was prepared for each set of comparative fitness assays and
126 for each different treatment. To prepare this master culture, 5uL of each 1.0 OD_{600} dilution of
127 the two comparison strains within each assay was added to the 10mL master culture. At this
128 point, 2mL samples of this master culture were added to 4 different test tubes, and the test
129 tubes were placed in a shaking incubator and grown for 24 hours at 27°C. For day 0
130 measurements, the master mix culture from the control (no antibiotic supplementation)
131 competitions was diluted 1:100 by adding 10µL into 990µL10Mm MgCl2, which was
132 followed by an additional 1:10 dilution. 3 replicate dilution series were made from each

133 master mix culture, and 150μL of each 1:100/1:1000 dilution was plated on LB media
134 containing 40μg/ml Xgal and placed 27°C. After 24 hours of growth each of the 4 replicate
135 cultures was diluted 1:10,000,000 and 1:100,000,000, with 150μL of these dilutions plated
136 on LB media containing 40μg/ml Xgal and placed at 27°C.
137 White and blue colonies were independently counted for each of the plated day 0
138 dilutions, these 3 values were averaged together, and the $\log_{10}$ value of this average taken
139 as the day 0 colony forming unit count. White and blue colonies were independently
140 counted for each of the day 1 replicate cultures and $\log_{10}$ transformed. Relative fitness was
141 calculated as the ratio of the difference between $\log_{10}$ transformed white and blue colony
142 counts for each replicate culture at day 1 over the difference of the $\log_{10}$ transformed
143 average white and blue colony counts at day 0. Depending on the assay, either two or three
144 assays were carried out with each pair of strains (DBL305/DBL1604 and
145 DBL305/DBL1620), with four replicate cultures per assay.
146 All competitive fitness assays were carried out in the same manner as described
147 above, with one exception. Since cultures grew more slowly in the presence of nalidixic
148 acid, cultures from the nalidixic acid competitions as well as the control competitions were
149 allowed to grow in liquid media at 27°C for 48 hours.
150
151 Statistical Methods
152
153 All statistical tests were performed in R version 3.3.0 (16). Scripts for these tests and all
154 underlying data can be found at **https:/doi.org/10.5281/zenodo.4409341**
155
156 **Results**
157

158 A Modified Kirby-Bauer Disc Diffusion Assay Replicates Previous Results For Nalidixic Acid
159 Sensitivity Upon Megaplasmid Acquisition
160
161 Our previously published experiments thoroughly explored how acquisition of
162 pMPPla107 increases nalidixic acid sensitivity in *Pseudomonas stutzeri* 28a84 (13), and here
163 we demonstrate that this effect is replicated by a simple modified assay based on Kirby-Bauer
164 disc diffusion assays ((17), Figure 1A). Since antibiotics diffuse out from the disc into the media
165 forming a concentration gradient, size of the inhibition halo is positively correlated with
166 sensitivity to nalidixic acid. We screened for nalidixic acid sensitivity in two different
167 backgrounds of *P. stutzeri* strain 28a24, DBL332 and DBL386. Derivative strains of these
168 (DBL453 and DBL408, respectively, which contain megaplasmid pMPPla107) have a larger halo
169 of inhibition around the antibiotic disc containing nalidixic acid than their immediate progenitor
170 strains which lack megaplasmid pMPPla107 (Figure 1B). There is an overall significant effect of
171 megaplasmid on nalidixic acid sensitivity ($F_{1,36}$=132.143, $p<0.0001$), and there are clear
172 statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+
173 strains measured separately (DBL332/DBL453, t-test $p<0.001$; DBL386/DBL408, t-test
174 $p<0.001$). Therefore the acquisition of this megaplasmid sensitizes these strains to this
175 antibiotic.
176
177 Acquisition of pMPPla107 Sensitizes Most, But Not All, Strains to Nalidixic Acid
178
179 Extending the Kirby-Bauer diffusion assay design, we tested for sensitivity to nalidixic
180 acid across a variety of other *Pseudomonas stutzeri* strains as well as a *P. syringae* pv.
181 *lachrymans* 8003 and *P. fluorescens* Pf0-1. The *P. syringae* strains are relatively closely related
182 to the strain in which this megaplasmid was originally found, *P. syringae* pv. *lachrymans* 107
183 (18), and acquisition of the megaplasmid has been previously shown to affect growth of this

184 strain (12). Overall, we find that megaplasmid acquisition increases sensitivity to nalidixic acid
185 across all of these strains as reflected by increased halo size in these Kirby-Bauer assays
186 (Figure 2). However, we also find that the magnitude of this difference appears to be smaller in
187 *P. syringae* than across all of the *P. stutzeri* strains and even smaller for the *P.fluorescens* Pf0-1
188 comparisons. All comparisons between megaplasmid- and megaplasmid+ strains are
189 significantly different (t-test, $p<0.001$ for each with the exception of $p<0.05$ for the Pf0-1
190 comparison). We have also included representative scans from each of these comparisons in
191 Supplemental Figure 1 (found at https://doi.org/10.6084/m9.figshare.10257704.v2)
192
193 Acquisition of Megaplasmid pMPPla107 by *P. putida* Increases Resistance to Nalidixic Acid
194
195 Using the same diffusion assays as above, we find that acquisition of megaplasmid
196 pMPPla107 by *Pseudomonas putida* KT2440 repeatedly increased resistance of this strain to
197 nalidixic acid (as reflected by a smaller halo in megaplasmid+ strains in Figure 3). This effect
198 was replicated in an independently created pair of *P. putida* strains originally derived from *P.*
199 *putida* KT2442, where megaplasmid pMPPla107 was tagged with a different antibiotic
200 resistance marker (Figure 3A). Across both strain pairs, presence of megaplasmid pMPPla107
201 increases resistance of *P. putida* strains to nalidixic acid ($F_{1,85}=147.57$, $p<0.0001$) and there are
202 clear statistical differences between the areas of the inhibition halos for each pair of
203 megaplasmid -/+ strains (DBL305/DBL759, t-test $p<.001$; DBL1604/DBL1620, t-test $p<0.001$).
204 However the magnitude of this effect does appear to be strain specific as the DBL1604
205 background is to be more sensitive to nalidixic acid than the DBL305 background (Figure 3A).
206 Nalidixic acid and ciprofloxacin are both quinolone antibiotics that have the same
207 mechanism of action and cause bacterial cell death by inhibiting the ability of gyrase to
208 covalently connect DNA strands after unwinding. Using the same Kirby-Bauer diffusion assays
209 as above, but instead replacing nalidixic acid discs with ciprofloxacin discs, we find that

210 acquisition of megaplasmid pMPPla107 does not clearly increase resistance to this antibiotic
211 (Figure 3B). Although the results are subtle, strains appear to be overall sensitized by the
212 megaplasmid to ciprofloxacin ($F_{1,80}$=12.99, p<0.001). However, only one of the megaplasmid+/-
213 strain pairs is significantly different when evaluated individually (DBL305/DBL759 t-test,
214 p<0.001; DBL1604/DBL1620 t-test, p=0.29).
215
216 A General Cost of Acquisition of Megaplasmid pMPPla107 by *P. putida*
217
218 We have previously shown that acquisition of pMPPla107 by *P. stutzeri* 28a84 leads to
219 dramatic fitness costs when measured by growth rate and by competitive fitness assay, and
220 have demonstrated that growth in the presence of nalidixic acid increases this fitness cost (12,
221 13). We therefore carried out competitive fitness assays of differentially marked *P. putida* strains
222 in order to test whether megaplasmid acquisition also affected competitive growth in this strain
223 background, and likewise how competitive fitness might change in the presence of nalidixic
224 acid. As one can see in Fig. 4A, there is no measurable difference in competitive fitness
225 between the *lacZ*+ background (KT2442) and the wild type background (KT2440) (t-test
226 compared to value of 1, p=0.14). However, in media without any antibiotic supplementation,
227 megaplasmid acquisition leads to a ~10% loss in competitive fitness over one growth cycle
228 (Figure 4A), and this difference is significantly different from the control comparison ($F_{1,20}$=600.3
229 p<0.0001). Therefore, increased nalidixic acid resistance as seen in the assays above occurs
230 even though megaplasmid acquisition significantly lowers strain growth rates in the absence of
231 antibiotics.
232
233 Supplementation with either Nalidixic Acid or Ciprofloxacin Shifts The Results of Competitive
234 Fitness Assays In *P. putida*
235

236 Since megaplasmid acquisition by *P. putida* appears to increase nalidixic acid resistance
237 according to Kirby-Bauer diffusion assays, we tested whether supplementation of media with
238 nalidixic acid could shift the outcomes of competitive fitness assays between wild type and a
239 megaplasmid containing strain. In the absence of any antibiotic supplementation, we are able to
240 independently recapitulate the fitness costs of megaplasmid acquisition reported above, and
241 again find a significant cost of megaplasmid acquisition when measured by competitive fitness
242 (Figure 4B). However, fitness relationships between strain pairs that lack or contain the
243 megaplasmid shift when nalidixic acid is supplemented into media ($F_{2,17}$=264.82 $p<0.0001$). At
244 20µg/ml, a ~10% fitness cost of the megaplasmid changes to a slight benefit for carriage of the
245 plasmid. Moreover, we see increasing fitness according to these competitive assays for the
246 megaplasmid strain at even higher levels of nalidixic acid (40µg/ml), although the exact
247 magnitude of this effect appears to be quite variable (Figure 4B). Competitive Fitness
248 measurements are significantly different between each concentration of antibiotics
249 (Tukey'sHSD, $p<0.05$). Therefore, megaplasmid acquisition by *P. putida* significantly increases
250 resistance to nalidixic acid as measured by both Kirby-Bauer diffusion assays as well as by
251 competitive fitness assays. Despite somewhat somewhat confounding results from disc diffusion
252 assays from these same strains with ciprofloxacin, but consistent with the results of competitive
253 assays supplemented with nalidixic acid, we find that acquisition of megaplasmid pMPPla107 by
254 *P. putida* increases the competitive ability of this strain background in the presence of 0.5ng/uL
255 ciprofloxacin (Figure 4C; $F_{1,18}$=178.89, $p<0.0001$).
256
257 <u>Megaplasmid Dependent Resistance to Nalidixic Acid in P. putida Is Not Positively Correlated</u>
258 <u>with Temperature Sensitivity</u>
259
260 Acquisition of megaplasmid pMPPla107 by *P. stutzeri* also renders this strain more
261 sensitive to relatively higher temperatures (35-37°C). To test for this effect in *P. putida,* we

262 carried out competitive fitness assays as above at both 27 and 37°C. Much like our previous
263 results in *P. stuzeri*, the cost of megaplasmid acquisition in *P. putida* is greater when strains
264 undergo competitive fitness assays at 37°C compared to 27°C ($F_{1,18}$=319.38 $p<0.0001$).
265
266 **Discussion**
267
268 There is a growing interest in predicting the evolutionary dynamics within microbial
269 populations, as a means to drive evolution within populations and communities towards
270 beneficial outcomes as well as a way to control or limit change within extant populations (19).
271 Horizontal Gene Transfer (HGT) is well recognized as a powerful driver of evolutionary
272 dynamics within microbial communities (1–3), and as such, should be incorporated into
273 scenarios for predicting future evolutionary dynamics. Plasmid conjugation and acquisition is a
274 major contributor to HGT in bacterial populations and communities, and plasmids are well
275 known to provide large-scale evolutionary benefits like enabling antibiotic resistance (2, 3, 20).
276 However, plasmid carriage can also entail a variety of fitness costs, which can be manifest as
277 lowered fitness or other phenotypic changes for plasmid bearing strains, in specific
278 environments (4–6). The presence of costs associated with plasmid carriage has led to
279 speculation about the conditions that enable plasmid maintenance within populations through
280 time, with one likely scenario being that costs associated with plasmid carriage are ameliorated
281 over time through subsequent and potentially rapid compensatory adaptation (21, 22). Although
282 not all HGT events entail fitness costs, rapid amelioration of fitness costs associated with
283 plasmid acquisition could lead to genetic and phenotypic tradeoffs that fundamentally shift future
284 evolutionary trajectories for the affected populations. As such, and with a lofty goal of ultimately
285 being able to predict evolution across bacterial populations, it is therefore critical to understand
286 how costs of plasmid carriage are affected by genetic, genomic, and environmental contexts.

287 Here we demonstrate that the direction of one previously observed phenotype
288 associated with acquisition of megaplasmid pMPPla107, sensitivity to nalidixic acid in
289 *Pseudomonas (13)*, depends upon the interactions between this megaplasmid and genomic
290 background of the strain of interest. Across almost all surveyed strains, acquisition of
291 pMPPla107 sensitizes cells to nalidixic acid, but in *P. putida* KT2440 this same horizontal
292 transfer event increases resistance to this antibiotic. These contrasting phenotypic effects occur
293 in the absence of identifiable megaplasmid genes which could be hypothesized to influence
294 antibiotic sensitivity and resistance. Thus, this is not simply a scenario where a common
295 antibiotic resistance gene found on the megaplasmid is active in one background compared to
296 the others, but rather a situation where the phenotypic effects of genes present on a
297 megaplasmid are polar opposites under certain environments in ways that depend on the
298 chromosomal background. Also important, these same phenotypic benefits are seen when
299 megaplasmid + and - strains of *P. putida* are competed together in fitness assays. We also
300 demonstrate that these same competitive fitness effects can be seen when megaplasmid + and
301 - strains of *P. putida* are competed in the presence of ciprofloxaxin, an antibiotic which has the
302 same mechanism of action as nalidixic acid. These results suggest that the mechanism
303 underlying changes in sensitivity to nalidixic acid and/or ciprofloxacin could be due to alterations
304 in the activity or amounts of gyrase and topoisomerase activity within *P. putida* cells after
305 acquisision of an additional 1Mb of DNA.
306 Although we did not see a clear and similar benefit to megaplasmid acquisition by *P.*
307 *putida* using disc assays for ciprofloxacin, it is possible that discrepancies could arise because
308 of differences in the concentration of ciprofloxacin across both types of assay. In disc assays,
309 ciprofloxacin diffuses out into the plate from a disc seeded with 5ug/mL of this antibiotic
310 whereas competitive fitness assays took place at 0.5ng/mL concentrations. We also observed
311 that there was a sharp gradient at ciprofloxacin concentrations in competitive fitness assays
312 whereby above 0.5ng/mL strains were inhibited in growth (data not shown). It is possible that

313 there is a small window to dial in the benefits of megaplasmid pMPPla107 across
314 concentrations of ciprofloxacin and that the concentrations in the disc assay do not enable the
315 resolution to pick apart clear differences in strains. It is also possible that the benefits of
316 acquisition of pMPPla107 for ciprofloxacin resistance by *P. putida* are subtly dependent on the
317 precise type of assay and potentially on the exact strain background being assayed. For
318 instance, only one of the two strain pairings used for *P. putida* (DBL305 and DBL759) was
319 significantly different in the ciprofloxacin disc assays and it's possible that secondary mutations
320 occurred during construction of strain DBL759 are responsible for this result. Ultimately, and
321 regardless of confounding results between multiple types of assays for ciprofloxacin, the
322 consistency of the nalidixic acid results across assays and support from competitive fitness
323 assays, clearly demonstrate that the phenotypic consequences of acquisition of pMPPla107 by
324 *P. putida* are fundamentally different than that in *P. stutzeri.*
325 Quinolone antibiotics have been used as a means of curing a variety of plasmids from
326 many different bacterial strains, which implies that plasmid driven sensitivity to quinolones could
327 be a widespread phenomenon (23). These previously reported effects are thought to be
328 mediated by stress at the level of gyrase and topoisomerase function and should therefore likely
329 be affected by either nalidixic acid or ciprofloxacin (23). At present there are no clear
330 mechanistic explanations for differing phenotypic responses of *Pseudomonas* strains to
331 acquisition of pMPPla107 and any potential scenario must involve interactions between genes
332 on both the chromosome and megaplasmid with one outcome of this interaction being
333 differential sensitivity to nalidixic acid. While annotations of pMPPla107 do not point to any
334 genes directly implicated in nalidixic acid resistance (14), these same annotations do suggest
335 that the megaplasmid contains multiple efflux pumps. While the substrates for these pumps are
336 currently unknown, it is plausible that one could specifically transport quinolone antibiotics
337 outside of the cell in *P. putida*. Indeed, there is precedence for efflux pumps in *Pseudomonas*
338 *aeruginosa* and other bacteria to differentially transport quinolone antibiotics (24, 25). Following

339 this line of thought, it would then be possible that either the particular regulation or activity of this
340 pump differs based on chromosomal background. There may also be a mechanism to
341 differentially detoxify nalidixic acid and ciprofloxacin that resides on the chromosome (whether
342 through efflux pumps or other means), but where the activity of which is differentially affected by
343 megaplasmid encoded pathways. Perhaps there is a global regulator which is similarly affected
344 by megaplasmid presence, but where the downstream phenotypic effects differ across strain
345 backgrounds. For example, megaplasmid presence could uniformly lead to upregulation of
346 AmrZ across Pseudomonads, but upregulation of AmrZ may trigger opposing phenotypic effects
347 in the context of quinolone resistance in different strain backgrounds (26). It is also possible that
348 phenotypic outcomes of increased sensitivity and resistance to quinolone in the presence of
349 pMPPla107 are due to mechanistically different effects. Under this case, the default condition
350 might be that plasmid acquisition causes cells to become more sensitive to certain antibiotics,
351 but that in some backgrounds (in this case *P. putida*) there is an additional pathway or efflux
352 pump that is differentially affected by megaplasmid presence and which enables cells to
353 overcome sensitization found in other *Pseudomonas* species.
354 As we have demonstrated previously in *P. stutzeri,* we show that acquisition of
355 megaplasmid pMPPla107 is associated with a large (~10%) fitness cost in *P. putida* in
356 conditions without antibiotic supplementation. Therefore, differing responses to nalidixic acid
357 cannot simply be explained as a secondary consequence of overall growth defects due to
358 acquisition of pMPPla107 and there must be some other inherent systems level effect that
359 creates opposing phenotypic responses across strain backgrounds upon acquisition of this
360 megaplasmid. We further demonstrate that acquisition of pMPPla107 by *P. putida* also
361 increases sensitivity of this strain background to relatively higher temperatures. These
362 contrasting results of megaplasmid acquisition on fitness across multiple environments in *P.*
363 *putida* suggest that the multiple previously observed fitness costs associated with megaplasmid

364 pMPPla107 (sensitization to antibiotics and higher temperatures) have independent underlying
365 mechanistic bases, at least in *P. putida.*
366 Specific evolutionary responses to selective pressures are driven by the availability of
367 adaptive mutations within the context of an adaptive landscape (27). Costs associated with
368 plasmid acquisition can rapidly be ameliorated by new mutations that occur on either the
369 plasmid or throughout the rest of the genome, with the speed of compensation correlated with
370 selection pressures acting on the plasmid bearing population (21, 22). Extrapolation from work
371 focused on the molecular and theoretical basis of adaptation predicts that the target size or
372 number of potential compensatory mutations should increase with the cost of plasmid carriage
373 (28). Given that results presented here as well as those reported in other recent papers strongly
374 suggest that costs of plasmid carriage change depending on the environment (e.g. 29), it is
375 therefore likely that both the speed and molecular basis of amelioration of plasmid costs will
376 greatly differ across environments. Likewise, since the costs and phenotypic consequences of
377 plasmid carriage differ dramatically across genomic backgrounds, there will likely be substantial
378 variation in both the evolutionary paths and molecular basis of compensatory mutations for the
379 exact same plasmid within different strains. The phenotypic effects reported here suggest that
380 plasmids could be differentially maintained or lost across bacterial populations in ways that
381 depend on genotype by environment interactions rather than on simply based on the presence
382 or absence of beneficial genes. Results presented here therefore also strongly bear on our
383 understanding and predicting the evolutionary forces that enable plasmid sharing across
384 microbial communities.
385
386 The phenomenon reported here suggests that plasmid acquisition could fundamentally
387 change evolutionary dynamics of antibiotic resistance in a strain dependent way. For instance,
388 there will likely be a different suite of resistance mutations available towards nalidixic acid in *P.*
389 *putida* KT2440 depending on whether this genomic background contains pMPPla107 or not.

390 This is very much in the vein of sign epistasis when discussing phenotypic effects of *de novo*
391 mutation and evolutionary pathways of adaptation (9, 10, 30, 31). It is also currently unknown
392 how phenotypic effects of particular resistance mutations are altered in the presence of this
393 megaplasmid. Given that there will likely be tradeoffs in gyrase activity with mutations towards
394 quinolone resistance, one could envision scenarios where acquisition of this megaplasmid
395 simply adds to the level of resistance to nalidixic acid, has no phenotypic effect in a background
396 that already contains nalidixic acid resistance mutations, or even sensitizes cells to nalidixic
397 acid. For example, if a mutation arises in *P. putida* KT2440 that provides resistance to nalidixic
398 acid at 100ng/uL, it is possible that further acquisition of pMPPla107 may either provide no
399 benefit (Minimum Inhibitory Concentration (MIC)+pMPPla107=100ng/uL), an additive increase
400 to resistance (MIC+pMPPla107>100ng/uL), or sensitizes this strain
401 (MIC+pMPPla107<100ng/uL). Future experiments will be necessary to differentiate between
402 these possibilities.
403 In conclusion, we report here that acquisition of megaplasmid pMPPla107 leads to
404 opposing phenotypic effects of strains in the presence of nalidixic acid but not ciprofloxacin. In
405 most *Pseudomonas* strains, there is a cost to acquisition of pMPPla107 in that cells containing
406 this megaplasmid are sensitized to this antibiotic. However, at least in *P. putida,* acquisition of
407 pMPPla107 increases resistance to nalidixic acid in the absence of identifiable genes involved
408 in antimicrobial resistance. We highlight that these results of HGT are analogous to the
409 phenomenon of sign epistasis when discussing phenotypic effects of *de novo* mutations within a
410 genome. Therefore, as with sign epistasis, it is therefore likely that some HGT events will lead to
411 fundamental shifts in the adaptive landscape in ways that depend on both the genomic
412 background and environment. Depending on how widespread such results are, context
413 dependence of phenotypic effects will potentially increase uncertainty in models and predictions
414 for the evolutionary dynamics of microbial adaptation after horizontal gene transfer events as
415 well as antimicrobial resistance.

Data Availability

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.

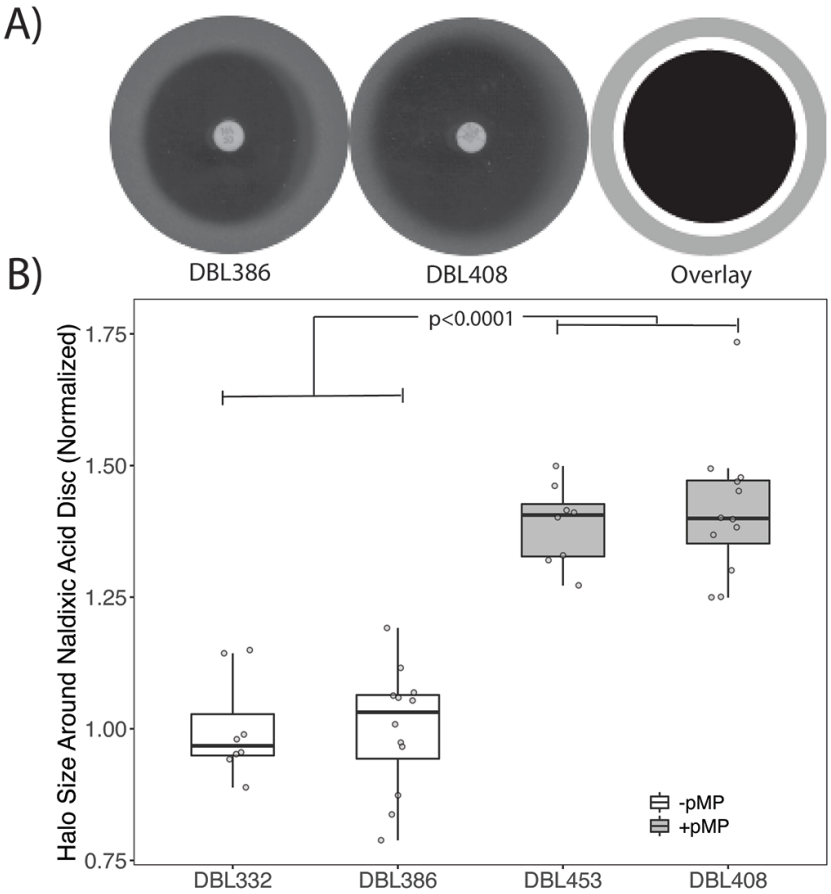

Figure 1. Kirby-Bauer Diffusion Assays Recapitulate A Nalidixic Acid Sensitivity Phenotypes in *Pseudomonas stutzeri*. We have previously demonstrated that acquisition of megaplasmid pMPPla107 increases sensitivity to nalidixic acid in derivatives of *P. stutzeri* 28a24. Here we show that assays based 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)

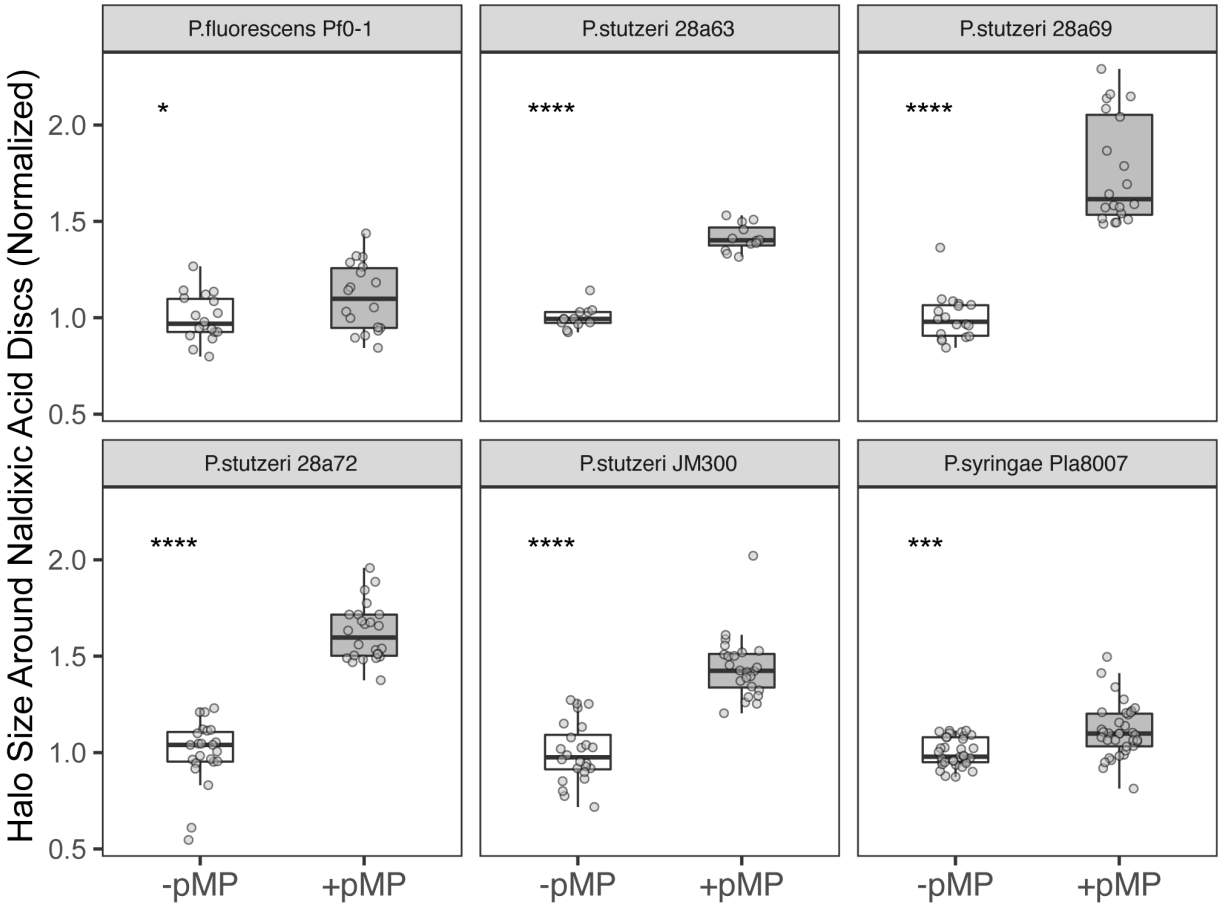

Figure 2. Acquisition of Megaplasmid pMPPla107 Sensitizes Numerous Pseudomonas strains to Nalidixic Acid. Data is shown for disc diffusion overlay assays comparing *P. stutzeri* strains DBL880, DAB282, DBL883, and DBL885 with derivatives which have acquired megaplasmid pMPPla107 (DBL907, DBL287, DBL910, 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 those lacking the megaplasmid are shown in grey boxes. 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 $p<0.045$ by t-test.

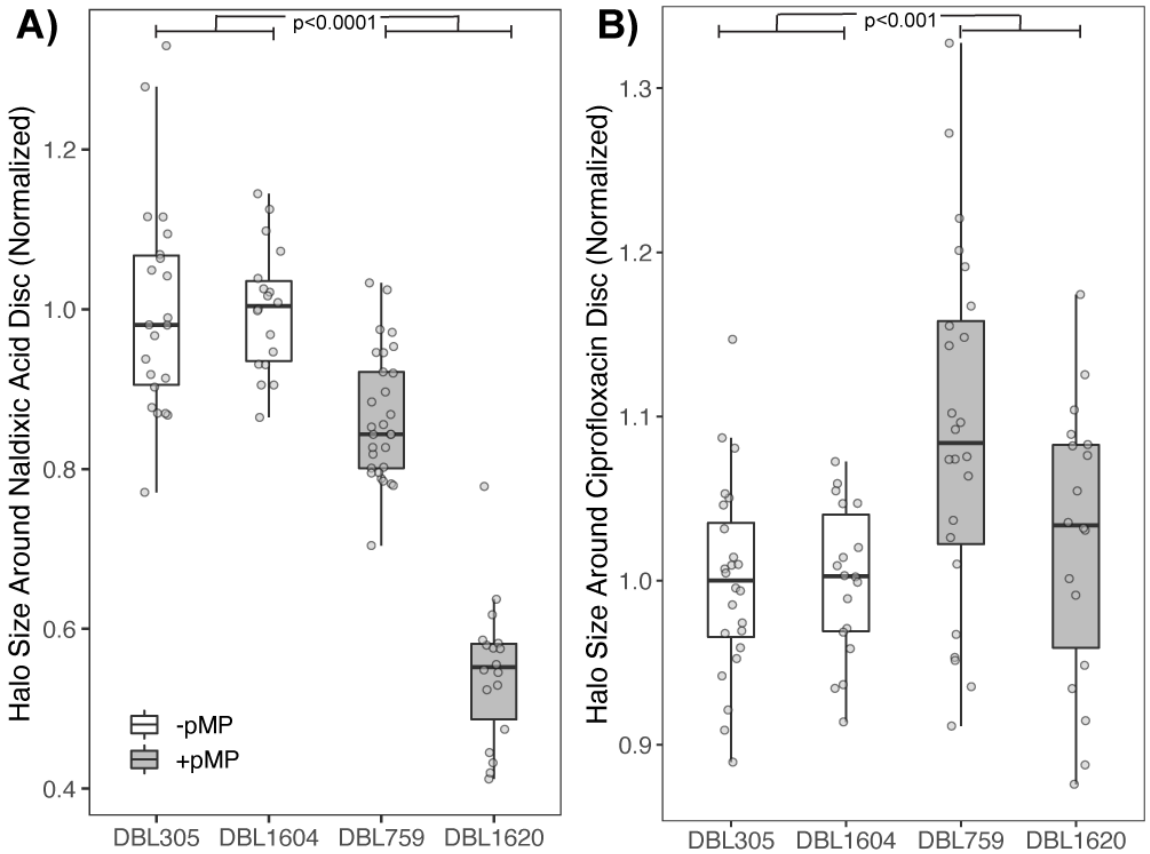

Figure 3. Acquisition of Megaplasmid pMPPla107 by *P. putida* Increases Resistance to Nalidixic Acid but Not Ciprofloxacin in Disc Diffusion Assays. Data is shown for disc diffusion overlay assays comparing *P. putida* strains DBL305 (KT2440) and DBL1604 (KT2442) with derivatives which have acquired 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).

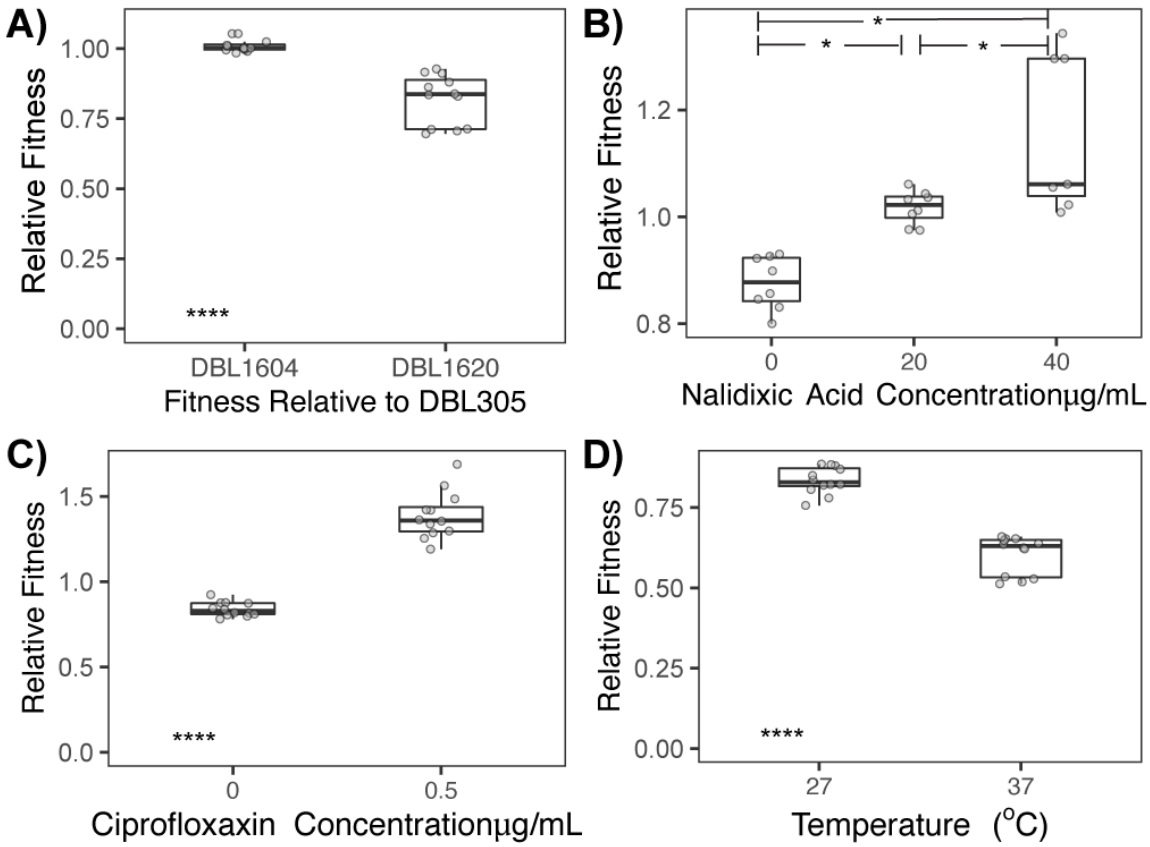

Figure 4. Contrasting Effects of Acquisition of Megaplasmid pMPPla107 in *P. putida* Across Different Environments as Measured by Competitive Fitness Assays Competitive fitness assays in liquid KB media were carried out between strain DBL305 (*P. putida* KT2440) and *lacZ*+ strains that either lack (DBL1604)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

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