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, it has also been demonstrated across various systems that gene transfer events can cause significant phenotypic changes and entail fitness costs depending on the environmental context. Here we test for the generality of one of these costs, 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 strains to nalidixic acid, but that this same horizontal gene transfer event actually increases resistance of *Pseudomonas putida* KT2440 to this antibiotic. These results are not easily explained away as secondary consequences of overall fitness effects, and the effects do not appear to be correlated with sensitization to ciprofloxacin. 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.

Introduction

Horizontal Gene Transfer (HGT), a catch all term for the transfer of DNA between individuals, is one of the main drivers of evolutionary dynamics across microbial populations (1–3). Although there are countless examples where HGT has been shown to increase microbial fitness or enable colonization of new niches, it is increasingly appreciated that HGT brings with it a variety of costs that arise as byproducts of the incorporation of foreign DNA into established physiological and genetic systems (4–6). Recent efforts have begun to categorize and characterize phenotypic effects of HGT across systems, and have clearly demonstrated that acquisition of the same genes can lead to different outcomes across genomic backgrounds (7). Such differences in phenotypic outcomes following HGT are highly related to the concept of sign epistasis in the context of de novo mutations, whereby the fitness effects of a mutation change sign (e.g. from positive to negative) under specific genetic combinations (8–10). Much like sign epistasis, we currently lack a general understanding of the underlying causes of differences in phenotypic outcomes following HGT, and as a consequence, there is no clear model for predicting if and when acquisition of DNA could be detrimental in some genomic backgrounds but beneficial in others.

We have previously reported on numerous phenotypic changes that occur as a consequence of acquisition of a ~1Mb megplasmid, pMPPla107, by *Pseudomonas* strains (11, 12). In addition to fitness costs correlated with slower growth, acquisition of pMPPla107 alters a variety of other phenotypes in some strains including: increased motility, decreased biofilm formation, decreased thermal tolerance, increased sensitivity to an unidentified compound

62 produced by Pseudomonads, and increased sensitivity to quinolone antibiotics like nalidixic acid
63 and ciprofloxacin. In the particular context of antibiotic sensitivity, it is important to note that
64 current annotations of megaplasmid pMPPla107 contain no known antibiotic resistance genes
65 (13) and that, at present, the mechanistic basis and overall strain specificity of these phenotypic
66 changes remains unknown.
67
68 Here, we test for how frequently sensitivity to nalidixic acid is observed across
69 Pseudomonas strains after acquisition of megaplasmid pMPPla107. We show that, although the
70 presence of this megaplasmid increases sensitivity to nalidixic acid in many strain backgrounds,
71 one strain in particular - *Pseudomonas putida* KT2440 - displays increased resistance to nalidixic
72 acid after this HGT event. We further demonstrate that resistance to nalidixic acid upon
73 megaplasmid acquisition is not correlated with increased resistance to ciprofloxacin, even though
74 both antibiotics affect the same cellular target. Therefore, our report provides an additional
75 example whereby the precise phenotypic effects of HGT are dependent on strain background, but
76 also suggests that that antibiotic resistance can be increased by megaplasmid pMPPla107 even in
77 the absence of identifiable resistance genes in the region acquired by HGT. These results, and
78 specifically strain-dependent changes in sign of phenotypic effects of plasmid acquisition,
79 provide new a new viewpoint for considering conditions that promote plasmid maintenance and
80 highlight the influence of genomic and environmental contexts when considering amelioration of
81 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 all
88 cultures largely took place at 27°C in King's B medium supplemented with rifampicin unless
89 otherwise specified. Antibiotics were supplemented into media at the following concentrations when
90 appropriate and unless otherwise specified: 50 µg/ml rifampin, 10 µg/ml tetracycline, and 20 µg/ml
91 kanamycin. Xgal was supplemented at 40 µg/ml. Antibiotic discs used in the diffusion assays
92 contained antibiotics at concentrations of 30 µg/ml for nalidixic acid and 5 µg/ml for ciprofloxacin.

93

94 Kirby-Bauer Disc Diffusion Assays

95

96 _____Strains of interest for each assay were streaked from frozen stocks and incubated on KB
97 media at 27°C for 2 or 3 days. At this point a small clump of cells was picked to liquid KB medium
98 and grown for 48 hours at 27°C with shaking. Cells from each culture where then pelleted in a
99 centrifuge, the supernatant was poured out, and pelleted cells were resuspended in 10Mm $MgCl_2$ for
100 measurement of OD_{600}. Each culture was then diluted to 0.01 OD_{600} in 10Mm $MgCl_2$ and 300uL of
101 each culture was added to 3mL of 0.4% water agar (which was previously melted and allowed to
102 briefly cool so that it was warm to the touch). This bacteria/agar mixture was then poured onto a
103 plain KB agar plate (1.5% agar) and allowed to solidify. Lastly, a disc containing the antibiotic of
104 interest was added to the center of the plate and each was placed in an incubator at 27°C. Pairs of
105 megaplasmid +/- strains were always evaluated together within the same assay.

106 After 3 days of growth, each plate was taken out of the incubator and scanned. This scanned
107 image was imported into ImageJ (14), and the area of the inhibition zone was measured. An average

108 area was calculated for the megaplasmid- strain within each assay, and individual data points for
109 each pair of megaplasmid + and - strains in each assay were divided by this value in order to
110 normalize the data, Therefore, the average inhibition zone for each megaplasmid- strain within each
111 assay was normalized to a value of 1.
112
113 Competitive Fitness Assays
114 Strains of interest for each assay were streaked from frozen stocks for 2 or 3 days (on media
115 containing kanamycin for megaplasmid+ strains) after which point a small clump of cells was picked
116 to liquid KB medium and grown for 24 hours at 27°C with shaking. Cells from each culture where
117 then pelleted in a centrifuge, the supernatant was poured out, and pelleted cells were resuspended in
118 10Mm $MgCl_2$ for measurement of OD_{600}. Each culture was then diluted to 1.0 OD_{600} in 10Mm
119 $MgCl_2$.
120 A 10mL master culture was prepared for each set of comparative fitness assays. To prepare
121 this master culture, 5uL of each 1.0 OD_{600} dilution of the two comparison strains within each assay
122 was added to the 10mL master culture. At this point, 2mL samples of this master culture were added
123 to 4 different test tubes, and the test tubes were placed in a shaking incubator and grown for 24 hours
124 at 27°C. For day 0 measurements the master mix culture was diluted 1:100 by adding 10µL into
125 990uL10Mm MgCl2, which was followed by an additional 1:10 dilution. 3 replicate dilution series
126 were made from each master mix culture, and 150µL of each 1:100/1:1000 dilution was plated on LB
127 media containing 40µg/ml Xgal and placed 27°C. After 24 hours of growth each of the 4 replicate
128 cultures was diluted 1:10,000,000 and 1:100,000,000, with 150µL of these dilutions plated on LB
129 media containing 40µg/ml Xgal and placed at 27°C.
130 White and blue colonies were independently counted for each of the plated day 0 dilutions,
131 the 3 values were averaged together, and the log10 value of this average taken as the day 0 colony
132 forming unit count. White and blue colonies were independently counted for each of the day 1

133 replicate cultures and $\log_{10}$ transformed. Relative fitness was calculated as the ratio of the difference
134 between $\log_{10}$ transformed white and blue colony counts for each replicate culture at day 1 over the
135 difference of the $\log_{10}$ transformed average white and blue colony counts at day 0. Two assays were
136 carried out with each pair of strains (DBL305/DBL1604 and DBL305/DBL1620), with four replicate
137 cultures per assay.

138 Relative fitness was measured in the same way in cultures supplemented with nalidixic acid,
139 except that 5µL samples of each strain were independently added to separate master mix cultures for
140 each antibiotic concentration. Additionally, given that cultures grew more slowly in the presence of
141 nalidixic acid, cultures were allowed to grow in liquid media at 27°C for 48 hours.

142

143 Statistical Methods

144 All statistical tests were performed in R version 3.3.0 (15). Scripts for these tests and all underlying
145
146 data can be found at **https://doi.org/10.6084/m9.figshare.10257704.v1**
147

148

149 **Results**

150

151 A Modified Kirby-Bauer Disc Diffusion Assay Replicates Previous Results For Nalidixic Acid
152 Sensitivity Upon Megaplasmid Acquisition

153

154 Our previously published experiments thoroughly explored how acquisition of
155 pMPPla107 increases nalidixic acid sensitivity in *Pseudomonas stutzeri* 28a84 (12), and here we
156 demonstrate that this effect is replicated by a simple modified assay based on Kirby-Bauer disc
157 diffusion assays ((16), Figure 1A). Since antibiotics diffuse out from the disc into the media
158 forming a concentration gradient, size of the inhibition halo is positively correlated with

159 sensitivity to nalidixic acid. We screened for nalidixic acid sensitivity in two different
160 backgrounds of *P. stutzeri* strain 28a24, DBL332 and DBL386. Derivative strains of these
161 (DBL453 and DBL408, respectively, which contain megaplasmid pMPPla107) have a larger
162 halo of inhibition around the antibiotic disc containing nalidixic acid than their immediate
163 progenitor strains which lack megaplasmid pMPPla107 (Figure 1B). There is an overall
164 significant effect of megaplasmid on nalidixic acid sensitivity ($F_{1,36}$=132.143, $p<0.0001$), and
165 there are clear statistical differences between the areas of the inhibition halos for each pair of
166 megaplasmid -/+ strains measured separately (DBL332/DBL453, t-test $p<0.001$;
167 DBL386/DBL408, t-test $p<0.001$). Therefore the acquisition of this megaplasmid sensitizes these
168 strains to this antibiotic.
169
170 Acquisition of pMPPla107 Sensitizes Most, But Not All, Strains to Nalidixic Acid
171
172 Extending the Kirby-Bauer diffusion assay design, we tested for sensitivity to nalidixic
173 acid across a variety of other *Pseudomonas stutzeri* strains as well as a *P. syringae* pv.
174 *lachrymans* 8003. The *P. syringae* strains are relatively closely related to the strain in which this
175 megaplasmid was originally found, *P. syringae* pv. *lachrymans* 107 (17). Overall, we find that
176 megaplasmid acquisition increases sensitivity to nalidixic acid across all of these strains as
177 reflected by increased halo size in these Kirby-Bauer assays (Figure 2). However, we also find
178 that the magnitude of this difference appears to be smaller in *P. syringae* than across all of the *P.*
179 *stutzeri* strains. All comparisons between megaplasmid- and megaplasmid+ strains are
180 significantly different (t-test, $p<0.001$ for each).
181

182 Phenotypic Responses to Nalidixic acid and Ciprofloxacin Differ in P. putida in the Presence of
183 Megaplasmid pMPPla107
184
185 Using the same diffusion assays as above, we find that acquisition of megaplasmid
186 pMPPla107 by *Pseudomonas putida* KT2440 repeatedly increased resistance of this strain to
187 nalidixic acid (as reflected by a smaller halo in megaplasmid+ strains in Figure 3). This effect
188 was replicated in an independently created pair of *P. putida* strains originally derived from *P.*
189 *putida* KT2442, where megaplasmid pMPPla107 was tagged with a different marker (Figure 3).
190 Nalidixic acid and ciprofloxacin are both quinolone antibiotics that have the same
191 mechanism of action and cause bacterial cell death by inhibiting the ability of gyrase to
192 covalently connect DNA strands after unwinding. Using the same Kirby-Bauer diffusion assays
193 as above, but instead replacing nalidixic acid discs with ciprofloxacin discs, we find that
194 acquisition of megaplasmid pMPPla107 does not increase resistance to this antibiotic (Figure 3).
195 Although the results are subtle, strains are overall sensitized by the megaplasmid to ciprofloxacin
196 ($F_{1,80}$=12.99, $p<0.001$). However, only one of the megaplasmid+/- strain pairs is significantly
197 different when evaluated individually (DBL305/DBL759 t-test, $p<0.001$; DBL1604/DBL1620 t-
198 test, $p=0.29$).
199
200 Megaplasmid Dependent Resistance to Nalidixic Acid in P. putida Is Not Positively Correlated
201 with Other Fitness Costs
202
203 We have previously shown that acquisition of pMPPla107 by *P. stutzeri* 28a84 leads to
204 dramatic fitness costs when measured by growth rate and by competitive fitness assay, and have

205 demonstrated that growth in the presence of nalidixic acid increases this fitness cost (11, 12). We
206 therefore carried out competitive fitness assays of differentially marked *P. putida* strains in order
207 to test whether megaplasmid acquisition also affected competitive growth in this strain
208 background, and likewise how competitive fitness might change in the presence of nalidixic acid.
209 As one can see in Fig. 4A, there is no measurable difference in competitive fitness between the
210 *lacZ*+ background (KT2442) and the wild type background (KT2440) (t-test compared to value
211 of 1, p=0.14). However, in media without any antibiotic supplementation, megaplasmid
212 acquisition leads to a ~10% loss in competitive fitness over one growth cycle (Figure 4A), and
213 this difference is significantly different from the control comparison ($F_{2,17}$=264.82, p<0.0001).
214 Therefore, increased nalidixic acid resistance as seen in the assays above occurs even though
215 megaplasmid acquisition significantly lowers strain growth rates in the absence of antibiotic.
216
217 Supplementation of Nalidixic Acid Shifts The Results of Competitive Fitness Assays In *P.*
218 *putida*
219
220 Since megaplasmid acquisition by *P. putida* appears to increase nalidixic acid resistance
221 according to Kirby-Bauer diffusion assays, we tested whether supplementation of media with
222 nalidixic acid could shift the outcomes of competitive fitness assays between wild type and a
223 megaplasmid containing strain. In the absence of any antibiotic supplementation, we are able to
224 independently recapitulate the fitness costs of megaplasmid acquisition reported above, and
225 again find a significant cost of megaplasmid acquisition when measured by competitive fitness
226 (Figure 4B). However, fitness relationships between strain pairs that lack or contain the
227 megaplasmid shift when nalidixic acid is supplemented into media. At 20µg/ml, a ~10% fitness

228 cost of the megaplasmid changes to a slight benefit for carriage of the plasmid. Moreover, we see
229 increasing fitness according to these competitive assays for the megaplasmid strain at even
230 higher levels of nalidixic acid (40μg/ml), although the exact magnitude of this effect appears to
231 be quite variable (Figure 4B). Competitive Fitness measurements are significantly different
232 between each concentration of antibiotics (TukeysHSD, $p<0.02$). Therefore, megaplasmid
233 acquisition by *P. putida* significantly increases resistance to nalidixic acid as measured by both
234 Kirby-Bauer diffusion assays as well as by competitive fitness assays.
235
236 **Discussion**
237
238 There is a growing interest in predicting the evolutionary dynamics within microbial
239 populations, as a means to drive evolution within populations and communities towards
240 beneficial outcomes as well as a way to control or limit change within extant populations (18).
241 Horizontal Gene Transfer (HGT) is well recognized as a powerful driver of evolutionary
242 dynamics within microbial communities (1–3), and as such, should be incorporated into
243 scenarios for predicting future evolutionary dynamics in natural communities. Plasmid
244 conjugation and acquisition is a major contributor to HGT in bacterial populations and
245 communities, and plasmids are well known to provide large-scale evolutionary benefits like
246 enabling antibiotic resistance (2, 3, 19). However, plasmid carriage can also entail a variety of
247 fitness costs, which can be manifest as lowered fitness or other phenotypic changes for plasmid
248 bearing strains, in specific environments (4–6). The presence of costs associated with plasmid
249 carriage has led to speculation about the conditions that enable plasmid maintenance within
250 populations through time, with one likely scenario being that costs associated with plasmid

251 carriage are ameliorated over time through subsequent and potentially rapid compensatory
252 adaptation (20, 21). Although not all HGT events entail fitness costs, rapid amelioration of
253 fitness costs associated with plasmid acquisition could lead to genetic and phenotypic tradeoffs
254 that fundamentally shift future evolutionary trajectories for the affected populations. As such,
255 and with a lofty goal of ultimately being able to predict evolution across bacterial populations, it
256 is therefore critical to understand how costs of plasmid carriage are affected by genetic, genomic,
257 and environmental contexts.
258
259 Here we demonstrate that the direction of one previously observed phenotype associated
260 with acquisition of megaplasmid pMPPla107, sensitivity to nalidixic acid in *Pseudomonas (12)*,
261 depends upon the interactions between this megaplasmid and genomic background of the strain
262 of interest. Across almost all surveyed strains, acquisition of pMPPla107 sensitizes cells to
263 nalidixic acid but in *P. putida* KT2440 this same horizontal transfer event increases resistance to
264 this antibiotic. These contrasting phenotypic effects occur in the absence on this megaplasmid of
265 identifiable genes known to influence antibiotic sensitivity and resistance. Thus, this is not
266 simply a scenario where a common antibiotic resistance gene found on the megaplasmid is active
267 in one background compared to the others, but rather a situation where the phenotypic effects of
268 genes present on a megaplasmid are polar opposites under certain environments in ways that
269 depend on the chromosomal background. Also important, these same phenotypic effects are not
270 seen when strains are challenged with ciprofloxacin, an antibiotic that is structurally different
271 than but which has the same mechanism of action as nalidixic acid as *P. putida* KT2440 displays
272 either no change or a slight sensitivity to ciprofloxacin after acquisition of megaplasmid
273 pMPPla107. Therefore, these results imply that the mechanism underlying changes in sensitivity

274 to nalidixic acid and/or ciprofloxacin are not simply due to alterations in gyrase and
275 topoisomerase activity within a cell due to the presence of an additional 1Mb of DNA. As we
276 have demonstrated previously in *P. stutzeri,* we further show that acquisition of megaplasmid
277 pMPPla107 is associated with a large (~10%) fitness cost in *P. putida* in conditions without
278 antibiotic supplementation. Therefore, differing responses to nalidixic acid cannot simply be
279 explained as a secondary consequence of overall growth defects due to acquisition of
280 pMPPla107 and there must be some other inherent systems level effect that creates opposing
281 phenotypic responses across strain backgrounds upon acquisition of this megaplasmid.
282
283 Quinolone antibiotics have been used as a means of curing a variety of plasmids from
284 many different bacterial strains, which implies that plasmid driven sensitivity to quinolones
285 could be a widespread phenomenon (22). These previously reported effects are thought to be
286 mediated by stress at the level of gyrase and topoisomerase function and should therefore likely
287 be affected by either nalidixic acid or ciprofloxacin (22). At present there are no clear
288 mechanistic explanations for differing phenotypic responses of *Pseudomonas* strains to
289 acquisition of pMPPla107 and any potential scenario must involve interactions between genes on
290 both the chromosome and megaplasmid with one outcome of this interaction being differential
291 sensitivity to nalidixic acid but not ciprofloxacin. While annotations of pMPPla107 do not point
292 to any genes directly implicated in nalidixic acid resistance (13), these same annotations do
293 suggest that the megaplasmid contains multiple efflux pumps. While the substrates for these
294 pumps are currently unknown, it is plausible that one could differentially transport nalidixic acid
295 and not ciprofloxacin outside of the cell. Indeed, there is precedence for efflux pumps in
296 *Pseudomonas aeruginosa* and other bacteria to differentially transport quinolone antibiotics (23,

297 24). Following this line of thought, it would then be possible that either the particular regulation
298 or activity of this pump for nalidixic acid differs based on chromosomal background. There may
299 also be a mechanism to differentially detoxify nalidixic acid that resides on the chromosome
300 (whether through efflux pumps or other means), but where the activity of which is differentially
301 affected by megaplasmid encoded pathways. Perhaps there is a global regulator which is
302 similarly affected by megaplasmid presence, but where the downstream phenotypic effects differ
303 across strain backgrounds. For example, megaplasmid presence could uniformly lead to
304 upregulation of AmrZ across Pseudomonads, but upregulation of AmrZ may trigger opposing
305 phenotypic effects in the context of nalidixic acid resistance in different strain backgrounds (25).
306 It is also possible that phenotypic outcomes of increased sensitivity and resistance to nalidixic
307 acid in the presence of pMPPla107 are due to mechanistically different effects. Under this case,
308 the default condition might be that plasmid acquisition causes cells to become more sensitive to
309 certain antibiotics, but that in some backgrounds (in this case *P. putida*) there is an additional
310 pathway or efflux pump that is differentially affected by megaplasmid presence and which
311 enables cells to overcome sensitization found in other *Pseudomonas* species.
312
313 Specific evolutionary responses to selective pressures are driven by the availability of
314 adaptive mutations within the context of an adaptive landscape (26). Costs associated with
315 plasmid acquisition can rapidly be ameliorated by new mutations that occur on either the plasmid
316 or throughout the rest of the genome, with the speed of compensation correlated with selection
317 pressures acting on the plasmid bearing population (20, 21). Extrapolation from work focused on
318 the molecular and theoretical basis of adaptation predicts that the target size or number of
319 potential compensatory mutations should increase with the cost of plasmid carriage (27). Given

320 that results presented here as well as those reported in other recent papers strongly suggest that
321 costs of plasmid carriage change depending on the environment, it is therefore likely that both
322 the speed and molecular basis of amelioration of plasmid costs will greatly differ across
323 environments. Likewise, since the costs and phenotypic consequences of plasmid carriage differ
324 dramatically across genomic backgrounds, there will likely be substantial variation in both the
325 evolutionary paths and molecular basis of compensatory mutations for the exact same plasmid
326 within different strains. Likewise, the phenotypic effects reported here suggest that plasmids
327 could be differentially maintained or lost across bacterial populations in ways that depend on
328 genotype by environment interactions rather than on simply based on the presence or absence of
329 beneficial genes. Results presented here therefore also strongly bear on our understanding and
330 predicting the evolutionary forces that enable plasmid sharing across microbial communities.
331
332 The phenomenon reported here also suggests that plasmid acquisition could
333 fundamentally change evolutionary dynamics of antibiotic resistance in a strain dependent way.
334 For instance, there will likely be a different suite of resistance mutations available towards
335 nalidixic acid in *P. putida* KT2440 depending on whether this genomic background contains
336 pMPPla107 or not. This is very much in the vein of sign epistasis when discussing phenotypic
337 effects of *de novo* mutation and evolutionary pathways of adaptation (8, 9, 28, 29). It is also
338 currently unknown how phenotypic effects of particular resistance mutations are altered in the
339 presence of this megaplasmid. Given that there will likely be tradeoffs in gyrase activity with
340 mutations towards quinolone resistance, one could envision scenarios where acquisition of this
341 megaplasmid simply adds to the level of resistance to nalidixic acid, has no phenotypic effect in
342 a background that already contains nalidixic acid resistance mutations, or even sensitizes cells to

343 nalidixic acid. For example, if a mutation arises in *P. putida* KT2440 that provides resistance to
344 nalidixic acid at 100ng/uL, it is possible that further acquisition of pMPPla107 may either
345 provide no benefit (MIC+pMPPla107=100ng/uL), an additive increase to resistance
346 (MIC+pMPPla107>100ng/uL), or sensitizes this strain (MIC+pMPPla107<100ng/uL). Future
347 experiments will be necessary to differentiate between these possibilities.
348
349 In conclusion, we report here that acquisition of megaplasmid pMPPla107 leads to
350 opposing phenotypic effects of strains in the presence of nalidixic acid but not ciprofloxacin. In
351 most *Pseudomonas* strains, there is a cost to acquisition of pMPPla107 in that cells containing
352 this megaplasmid are sensitized to this antibiotic. However, at least in *P. putida,* acquisition of
353 pMPPla107 increases resistance to nalidixic acid in the absence of identifiable genes involved in
354 antimicrobial resistance. We highlight that these results of HGT are analogous to the
355 phenomenon of sign epistasis when discussing phenotypic effects of *de novo* mutations within a
356 genome. Therefore, as with sign epistasis, it is therefore likely that some HGT events will lead to
357 fundamental shifts in the adaptive landscape in ways that depend on both the genomic
358 background and environment. Depending on how widespread such results are, context
359 dependence of phenotypic effects will potentially increase uncertainty in models and predictions
360 for the evolutionary dynamics of microbial adaptation after horizontal gene transfer events as
361 well as antimicrobial resistance.

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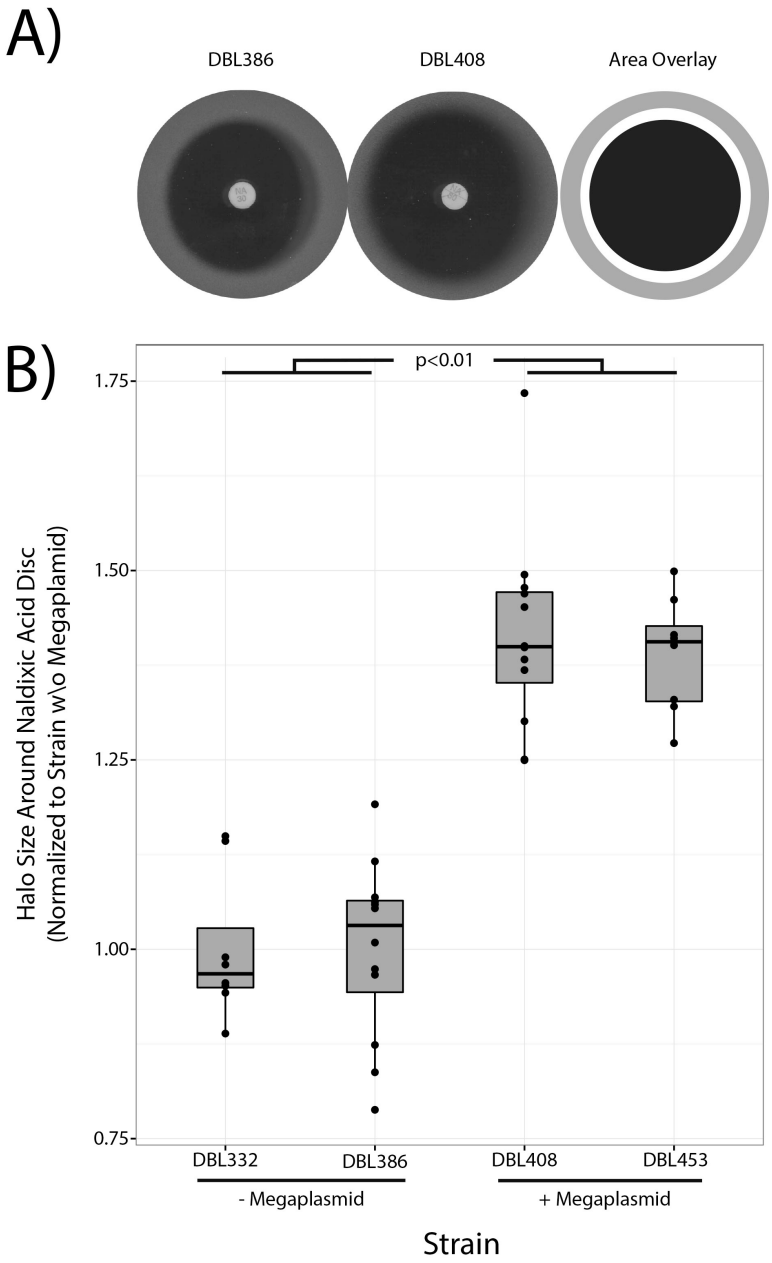

Figure 1. Kirby-Bauer Diffusion Assays Recapitulate A Nalidixic Acid Sensitivity Phenotypes in *Pseudomonas stutzeri*. A) Representative scans from a disc diffusion assay from using (from left to right) a strain that either lack (DBL386) or contain (DBL408) megaplasmid pMPPla107. Image areas covered 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. 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 more sensitive to inhibition by nalidixic acid ($F_{1,36}$=132.143, $p<0.0001$). Additionally, there are clear statistical differences between the areas of the inhibition halos for each pair of megaplasmid -/+ strains (DBL332/ DBL453, t-test $p<.001$; DBL386/DBL408, t-test $p<0.001$).

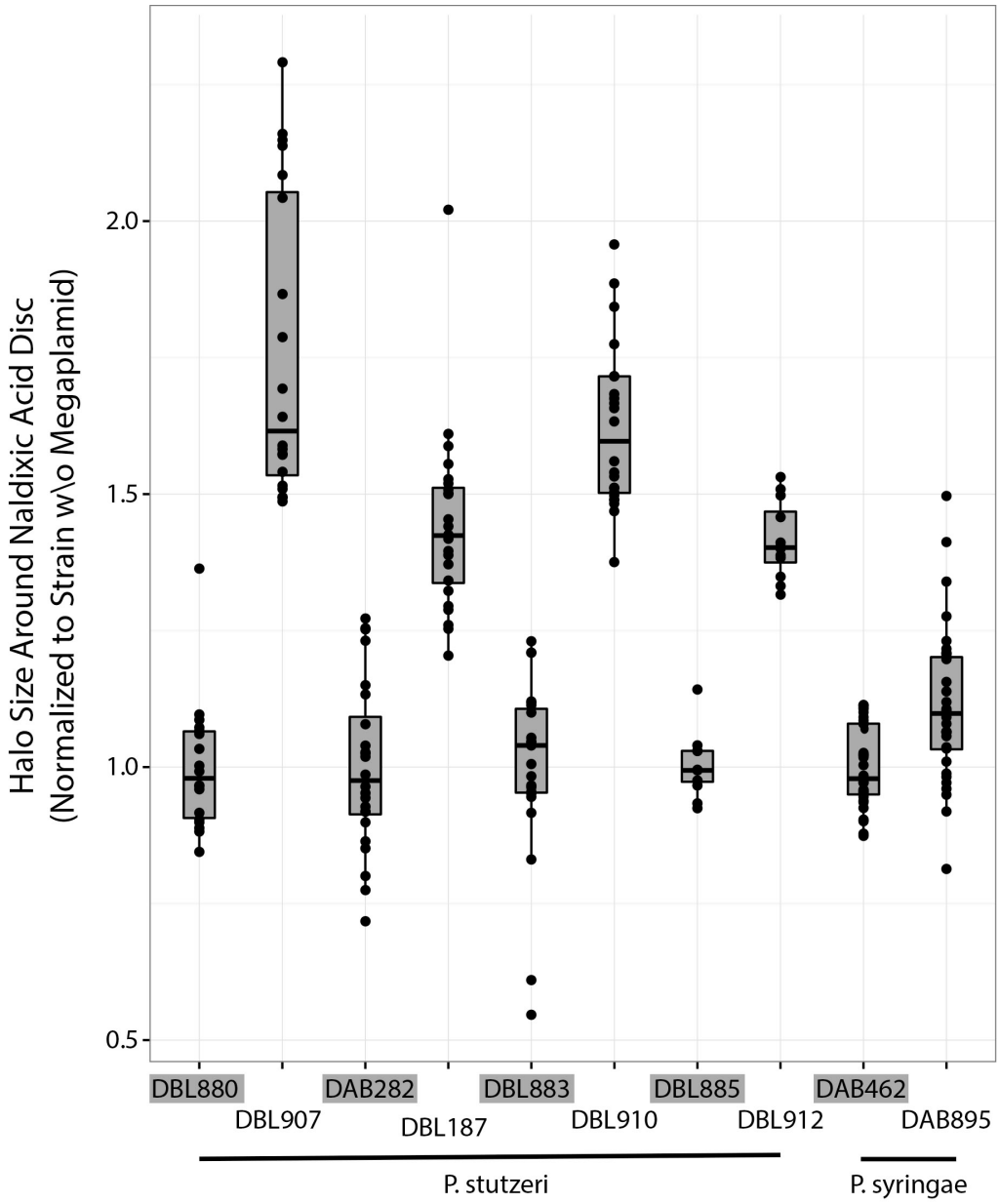

Figure 2. Acquisition of Megaplasmid pMPPla107 Sensitizes Numerous Pseudomonas strains to Nalidixic Acid. Data is shown for disc diffusion overlay assays comparing *P. stutzeri* strains DBL880, DAB282, DBL883, and DBL885 with derivatives which have acquired megaplasmid pMPPla107 (DBL907, DBL287, DBL910, DBL912 respectively). Data is also shown for derivatives of *P. syringae* pv. *lachrymans* strain 8003 which either lack (DBL462) or contain (DBL895) megaplasmid pMPPla107. Strain names of those lacking the megaplasmid are shown 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. 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.

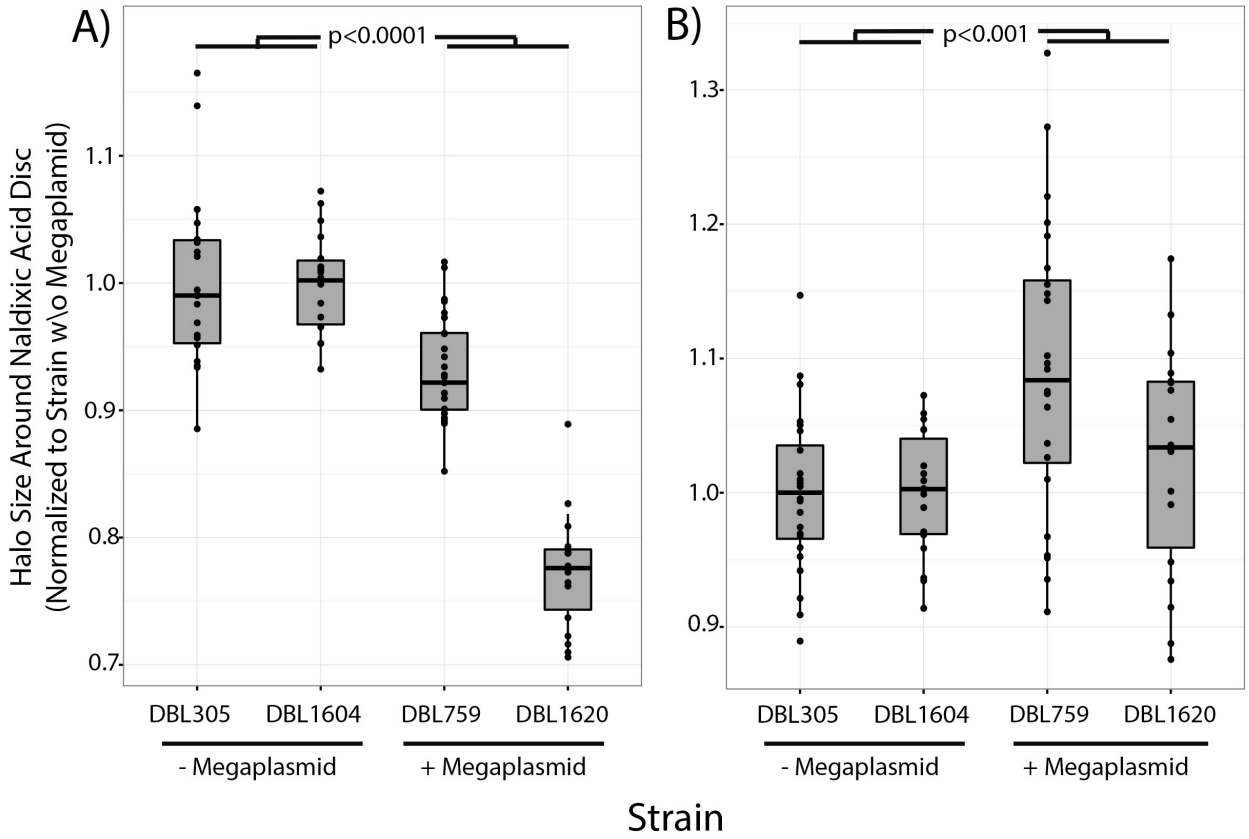

Figure 3. Acquisition of Megaplasmid pMPPla107 by *P. putida* Increases Resistance to Nalidixic Acid but Not Ciprofloxacin. Data is shown for disc diffusion overlay assays comparing *P. putida* strains DBL305 (KT2440) and DBL1604 (KT2442) with derivatives which have acquired megaplasmid pMPPla107 (DBL759 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. 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, ttest p<.001; DBL1604/DBL1620, ttest 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).

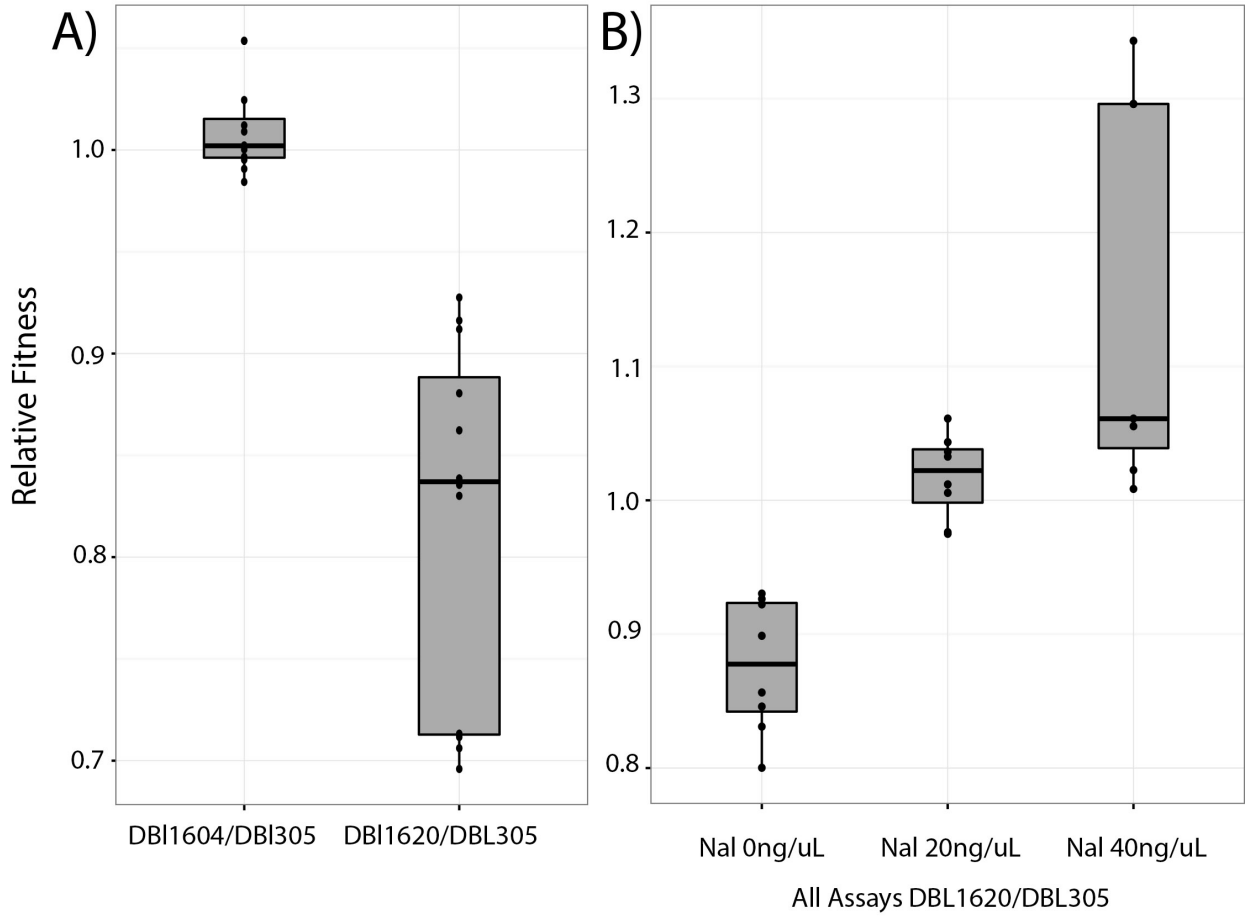

Figure 4. Acquisition of Megaplasmid pMPPla107 in *P. putida* Increases Relative Fitness in the Presence of Nalidixic Acid. Competitive fitness assays in liquid KB media were carried out between strain DBL305 (*P. putida* KT2440) and *lacZ*+ strains that either lack (DBL1604) or contain (DBL1620) megaplasmid 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. 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. B) Although the ~10% fitness cost of the megaplasmid again apparent during competition between DBL305 and DBL1620 in unsupplemented KB medium, strain 1620 (containing megaplasmid pMPPla107) display increasingly higher relative fitness compared to strain DBL305 when media is supplemented with nalidixic acid at either 20 or 40 ng/uL. Comparisons between all strains within each sub-figure are significantly different according to Tukey's HSD at $p<0.05$.