Title:

Biofilms preserve transmissibility of a multi-drug resistance plasmid

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ABSTRACT:

Self-transmissible multidrug resistance (MDR) plasmids are a major health concern because they can spread antibiotic resistance to pathogens. Even though most pathogens form biofilms, little is known about how MDR plasmids persist and evolve in biofilms. We hypothesize that (i) biofilms act as refugia of MDR plasmids by retaining them in the absence of antibiotics longer than well-mixed planktonic populations, and that (ii) the evolutionary trajectories that account for the improvement of plasmid persistence over time differ between biofilms and planktonic populations. In this study, we evolved *Acinetobacter baumannii* with an MDR plasmid in biofilm and planktonic populations with and without antibiotic selection. In the absence of selection biofilm populations were better able to maintain the MDR plasmid than planktonic populations. In planktonic populations plasmid persistence improved rapidly but was accompanied by a loss of genes required for the horizonal transfer of plasmids. In contrast, in biofilms most plasmids retained their transfer genes, but on average plasmid persistence improved less over time. Our results showed that biofilms can act as refugia of MDR plasmids and favor the horizontal mode of plasmid transfer, which has important implications for the spread of MDR.

INTRODUCTION

Antibiotic resistance is widely recognized as one of the most serious problems facing healthcare today. Resistance to antibiotics, including those of last resort, often results from the acquisition of resistance genes by horizontal gene transfer (HGT) [1,2]. When present as part of mobile genetic elements such as plasmids, resistance genes can rapidly spread to various species or strains of bacteria [3,4]. This makes multidrug resistance (MDR) plasmids, particularly those with a broad host range, a major problem in fighting the spread of antibiotic resistance.

Understanding how these plasmids evolve in the presence and absence of antibiotics is critically important. In the presence of antibiotics, MDR plasmids are maintained in bacterial populations via positive selection [5], but when antibiotics are removed the fitness cost of plasmid carriage [6–8] is no longer offset by the benefit of antibiotic resistance [9]. Therefore, plasmids are expected to be lost from bacterial populations via purifying selection unless (i) partitioning and post-segregational mechanisms limit the formation of plasmid-free cells, (ii) reacquisition of plasmids counteracts plasmid loss and cost, (iii) co-residing plasmids and other bacteria in the community can prevent plasmid loss [10,11], or (iv) the cost of plasmid carriage is reduced, eliminated or reversed by evolution [12]. The rate at which plasmids are lost from a population probably depends on a combination of these factors and is inversely proportional to 'plasmid persistence' – the ability of a plasmid to maintain itself in the absence of known positive selection for the plasmid. Previous studies from our and other research groups have repeatedly shown that plasmid persistence can improve during growth in the presence or absence of antibiotics (for example, [13–24]). However, almost all these studies used planktonic populations. Much less is known about how plasmid persistence evolves in spatially structured populations such as biofilms, even though they represent the most common form of bacterial growth and are the causes of many recalcitrant infections [25–28].

Due to spatial structure, bacteria that grow in biofilms only compete locally with their neighbors, which protracts selective sweeps and leads to increased genotypic and phenotypic variation within a population [29–39]. Additionally, the gradients of nutrients and electron acceptors cause habitat heterogeneity that results in local adaptation of subpopulations. These biofilm features allow bacteria to access more fitness peaks in rugged adaptive landscapes. This can result in evolutionary outcomes that may not be observed in planktonic populations that routinely experience strong selective sweeps [40,41]. Previously we showed that under antibiotic selection, evolution in biofilms results in a higher diversity of plasmid persistence phenotypes as compared to planktonic

70 populations [42]. In a separate study we have found that biofilms of plasmid-bearing cells maintain a
71 greater diversity of mutations than planktonic populations. Among these diverse biofilm genotypes
72 were clones that better retained their plasmid than any clone evolved in planktonic populations [43].
73 These seminal studies showed how biofilm growth can affect the evolution of plasmids persistence.
74 Because many bacterial pathogens that can acquire resistance genes via plasmid transfer [44,45] form
75 biofilms, we need to study the evolution of their resistance plasmids under these growth conditions.

76 The biofilm-forming bacterium *Acinetobacter baumannii* is an emerging threat in the United
77 States and worldwide because it causes wound infections, ventilator-associated pneumonia, and
78 sepsis [46,47]. Further, it is known to rapidly acquire new forms of antibiotic resistance by HGT [48–50]
79 and pan-drug resistant strains have been documented [51]. Despite the medical importance of *A.*
80 *baumannii* and the role of HGT in its acquisition of antibiotics resistance, little information is
81 available on how this organism and recently acquired MDR plasmids co-evolve.

82 In our previous study we found that the persistence of a broad-host range MDR plasmids
83 pB10 in *A. baumannii* increased over time in both biofilm and planktonic populations treated with
84 antibiotics, but this phenotype was more diverse after evolution in biofilms [42]. Building on this
85 previous study we sought to determine the effect of biofilm growth (i) on plasmid persistence in the
86 absence of antibiotics, and (ii) on the evolutionary trajectories of this plasmid in the presence and
87 absence of antibiotics. Our findings can be summarized as follows. First, in the absence of selection,
88 biofilm populations maintained the MDR plasmid longer than planktonic populations. Second,
89 biofilms hampered the evolution of plasmid persistence in the absence of antibiotic selection. Third,
90 large deletions of the plasmid conjugative transfer regions were a common evolutionary outcome in
91 planktonic populations but less so in biofilms, indicating that growth in biofilms conserved the
92 plasmid's ability to transfer horizontally. Finally, plasmid adaptation to one bacterial species can
93 either promote or decrease its persistence in other bacteria, including other opportunistic pathogens.

94 **RESULTS**

95 *Experimental set-up of biofilm and planktonic populations.*

96 To investigate the effects of biofilm growth and the presence of a plasmid-selective
97 antibiotic on the persistence and evolution of MDR plasmid pB10 in *A. baumannii,* we set up an
98 experiment with a full factorial design with two growth environments and two treatments. As shown
99 in Fig. 1, the growth environment was either a biofilm flow cell or serial batch culture; the
100 treatments were the presence (Tet+) or absence (Tet-) of tetracycline in the growth medium. In
101 order to initiate all four environment/treatment combinations with equivalent populations, replicate
102 biofilm populations were first established in flow cells for a period of four days ($t_{-4} – t_0$). To avoid
103 plasmid loss during this initial phase, the medium was supplemented with Tet for four days,
104 regardless of the subsequent treatment (Tet+ or Tet-). After this biofilm establishment phase at t_0,
105 triplicate flow cells were harvested and used to inoculate triplicate serial batch cultures per
106 treatment. From here on we refer to these serial batch cultures as planktonic populations. The
107 remainder of the growth and sampling scheme is depicted in Fig. 1 (see Materials and Methods for
108 more details).

109 *Biofilms retain the plasmid in the absence of selection.*

110 To assess the ability of bacterial biofilms to retain an MDR plasmid in the absence of
111 antibiotics, we compared the proportion of plasmid-containing cells present in biofilm and
112 planktonic populations at t_0 and after 14 days of growth in Tet-free media (t_{14}) (Table 1). This was
113 done by plating samples of these populations on selective and non-selective media. The plasmid
114 was lost more slowly from biofilms than from planktonic populations. After 14 days 12.3 (± 3.5)%
115 of the viable cells from the biofilms still carried the plasmid. In contrast, plasmid-bearing cells
116 could no longer be detected in two of the three planktonic populations (i.e., their frequency in these
117 populations was $<10^{-8}$), and were present in only 0.016% of the third. This observation
118 demonstrates the importance of biofilms in the persistence of plasmid-mediated antibiotic
119 resistance.

120 *Biofilms protracted the evolution of plasmid persistence in the absence of selection.*

121 We previously showed that after 28 days of evolution in the presence of Tet, bacteria
122 isolated from biofilms showed a higher diversity in plasmid persistence dynamics than their
123 counterparts evolved in planktonic populations, and on average, a smaller increase in plasmid

124 persistence [42]. In the current study we compared the evolution of plasmid persistence in clones
125 evolved over 14 days in the presence and absence of Tet (Tet+ and Tet- populations). Because the
126 plasmid was rapidly lost in Tet- planktonic populations, we ended the entire Tet- experiment on day
127 14. In one of these three Tet- populations, plasmid-containing clones could no longer be detected
128 after day 6, and in another after day 10. Therefore the plasmid-containing clones from Tet-
129 planktonic populations were isolated from samples archived on t_6, t_{10}, and t_{14}, whereas all the clones
130 from Tet- biofilm populations were isolated at t_{14}. For each of the triplicate growth
131 environment/treatment combinations plasmid persistence was tested for six clones. First, we
132 verified that clones isolated at t_0 showed the same plasmid persistence profile as the ancestor (Fig.
133 2A). Based on linear regression there was no significant change in the rate of plasmid loss, meaning
134 the plasmid persistence in the populations used to start the experiment did not change during the 4-
135 day biofilm establishment phase (for t_0, Tet+ $p = 0.65$; for t_0, Tet- $p = 0.55$). Therefore, the t_0 clones
136 were used for the comparisons of plasmid persistence described below.

137 After 6–14 days of evolution in the absence of antibiotics, plasmid pB10 showed improved
138 persistence in *A. baumannii* clones from planktonic populations but not in clones from biofilms
139 (Fig. 2B; $p = 0.0324$ and $p = 0.2107$, respectively). These results are in contrast to the clones
140 evolved in the same conditions but in the presence of Tet, where plasmid persistence improved in
141 clones isolated from both biofilm and planktonic populations after 14 days (Fig. 2C; $p = 0.0053$ and
142 $p < 0.0001$, respectively). The latter result is consistent with our previously published findings after
143 28 days of biofilm and planktonic growth [42]. Thus on average, plasmid persistence always improved
144 in planktonic populations regardless of antibiotic treatment, but only improved in biofilms grown
145 with Tet.

146 *Plasmid transfer genes were mostly retained in biofilms but completely lost in planktonic*
147 *populations.*

148 To determine the genotypic changes that occurred during the experiment, we sequenced all
149 18 clones per environment/treatment at each time point (6 per triplicate population, Fig. 1), for a
150 total of 144 strains. Here we focus on genetic changes in the plasmid pB10. We identified three
151 types of mutations, as shown by a visual summary in Fig. 3 and a list in Table S1. By far the most
152 common genetic changes were large deletions in the plasmid regions encoding conjugative transfer
153 (*tra*), mating pair formation (*trb*), and an intervening class 1 integron containing sulfonamide and
154 amoxicillin resistance genes. These deletions also often included a few genes thought to be involved

155 in plasmid maintenance and central control, adjacent to the *tra* region. We also identified nine
156 deletions smaller than 1,000 bp located in maintenance/control genes *kfrA*, *klcB*, or one of the *trb*
157 genes. Finally, there were four single nucleotide polymorphisms (SNPs): two in *kfrA*, one in a *trb*
158 gene, and one in an intergenic region near the *tetR* gene. In summary, large deletions that remove
159 virtually all the genes required for horizontal transfer were by far the most common genetic
160 changes.

161 The size of the large deleted plasmid regions varied drastically between clones, ranging
162 from 21,755 bp to 34,594 bp. The number of genes deleted per plasmids ranged from 23 to 35, but a
163 common set of 19 genes (from *trbF* through *traC*) were deleted or truncated in every deletion
164 variant (Fig. 3, Table S1). As plasmid pB10 has 65 complete coding sequences (CDS) in addition to
165 a few truncated transposases [52], all large deletions represented more than a third of the gene content
166 of the plasmid. Whereas the boundaries of these deletions were all different, most contained short
167 flanking direct repeats (typically 8-12 bp, with one being only 6 bp, data not shown). One of these
168 repeats was always eliminated during deletion of the intervening region. Deletions between these
169 direct repeats are indicative of recombination events [53].

170 There were striking differences in the kinds of plasmid genotypes observed in the biofilm
171 versus planktonic populations (Fig. 3, Table S1). Most of the clones from planktonic populations
172 showed the large deletions described above. Only 2 of the 54 clones had retained the ancestral pB10
173 sequence, both from the Tet- population sampled at t_6. In five of the six Tet+ planktonic populations
174 sampled at either 14 or 28 days, all six clones contained plasmids with identical large deletions.
175 Moreover, two of the three planktonic populations showed the same large deletion at both time
176 points. These results indicate strong selective sweeps of large deletion mutants in planktonic
177 populations under antibiotic selection for the plasmid. Even in the absence of Tet, all six clones
178 from the population that still retained plasmids by day 14 showed an identical large deletion. In
179 stark contrast, all clones from the three Tet+ biofilms at t_{14} still contained the ancestral plasmid
180 genotype. Even two weeks later, one of the three Tet+ biofilm populations still showed intact
181 plasmids in 100% of the clones, and in the other two populations around half the clones still had
182 intact plasmids. In the Tet- biofilms the deletions were more frequent at t_{14} but still only found in
183 half or less than half of the clones per population (Table S1 and Fig. 3). Moreover, of the 54
184 evolved biofilm clones only two pairs showed an identical deletion. These results clearly show that
185 there were no sweeps of large deletion mutants in the biofilms. In conclusion, biofilms still

186 contained a large proportion of transmissible plasmids after 14 or 28 days, whereas transmissible
187 plasmids were no longer detected at these time points in planktonic populations.

188 *Loss of plasmid transfer genes improved plasmid persistence.*

189 To determine the effect of the large plasmid sequence deletions, we compared the
190 persistence of truncated and full-length plasmids in two ways. First, we compared the persistence of
191 these plasmids in their co-evolved hosts. On average, clones with truncated plasmids showed a
192 significantly higher plasmid persistence than clones with full-length plasmids (Fig. 4A, $p < 0.001$).
193 However, a few biofilm clones with full-length plasmids identical in sequence to ancestral pB10,
194 also demonstrated high plasmid persistence (Fig. 4A), suggesting this change was caused by
195 chromosomal mutations. Next, to ensure that the observed improvement in persistence of the
196 truncated plasmids could entirely be explained by the plasmid deletions and not chromosomal
197 changes, we transformed the ancestral host with one full-length pB10 and several truncated
198 plasmids from t_{28} of the Tet^+ treatments. Plasmid persistence was higher for the truncated plasmids
199 than for the full-length plasmid ($p < 0.0001$) or ancestral pB10 (Fig. 4B; $p = 0.015$). Thus, the large
200 deletions in plasmid pB10 were a major driver of improved plasmid persistence in *A. baumannii*.

201 *Persistence of evolved pB10 in other naive hosts.*

202 To understand the broader effects of the large deletions in pB10 on plasmid persistence, we
203 tested one evolved truncated plasmid from a t_{28} clone in three bacterial strains: *Pseudomonas*
204 *moraviensis* R28, *Pseudomonas sp. nov.* H2, and *Stenotrophomonas maltophilia* P21 (see footnote 3
205 in Table S1). All three strains were previously shown to rapidly lose pB10 [54]. The evolved plasmid
206 was even less persistent than ancestral pB10 in the two *Pseudomonas* hosts, but it was more
207 persistent in *S. maltophilia* P21 (Fig. 5). Our data demonstrate that even though deletions that
208 eliminate conjugative transfer of the plasmid may be beneficial in some species, that benefit may
209 not extend to all.

210 *Some chromosomal mutations are also responsible for improved plasmid persistence.*

211 While the mutations found in the plasmids explained most of the increased persistence in the
212 isolated clones, we identified a few clones where chromosomal mutations must be responsible for
213 the improved persistence (Figure 4). In the genome sequences of the 144 clones, we identified many
214 chromosomal mutations when compared to the ancestral sequence (Table S2). However, none of
215 these mutations were in genes previously shown to be involved in plasmid persistence. Due to the

216 high number of unique mutations, mutation reconstruction in the ancestor would be required to test
217 their effects, which is outside the scope of this study.

218

219

220 **DISCUSSION**

221 We showed that growth in biofilms can promote the retention of MDR plasmids in the
222 absence of selection, and better preserve the horizontal mode of plasmid transmission than
223 planktonic populations. These findings have several important implications. First, they demonstrate
224 the positive effect of biofilm growth on MDR plasmid persistence in the absence of antibiotics. This
225 is significant given most bacteria in clinical and environmental habitats grow in biofilms [25]. Our
226 findings are consistent with previous studies that showed that the deeper layers of biofilms can act
227 as refugia for plasmids even in the absence of selection for the plasmid [55,56]. A plausible explanation
228 is that bacteria in biofilm layers farthest away from the bulk medium grow slowly or not at all. This
229 allows plasmids to be better retained in these subpopulations as plasmid loss requires cells to divide.
230 Our results thus suggest that reduced use of antibiotics may help lower the relative abundance of
231 resistance genes in biofilms but does not necessarily eliminate these genes when they are plasmid
232 encoded. Second, evolution in biofilms conserved the horizontal mode of transmission of a MDR
233 plasmid, while growth in well-mixed systems resulted in sweeps of much smaller, non-self-
234 transmissible plasmids that persist better through vertical transmission. Finally, this study cautions
235 against indiscriminately extrapolating results from experimental evolution in planktonic cultures to
236 more complex environments such as bacterial biofilms.

237 Perhaps the most important finding of our study was the striking difference in evolutionary
238 trajectories between the biofilm and planktonic populations. Independent of the presence of
239 selection for the plasmid, virtually every tetracycline resistant *A. baumannii* clone analyzed from
240 planktonic populations had lost a very large segment of plasmid pB10 that contained horizontal
241 transfer and a few antibiotic resistance genes. The loss of these genes resulted in the inability of the
242 plasmid to transfer by conjugation (data not shown). These findings are consistent with previous
243 studies that observed loss of plasmid transmissibility due to deletion or mutations of transfer genes
244 during bacteria-plasmid evolution in planktonic populations [21,43,57,58]. In contrast, the majority of
245 plasmids in evolved biofilm clones remained intact and thus self-transmissible. In only one of the
246 nine evolved biofilm populations did plasmid genotypes with deletions in transfer genes outnumber

247 the ancestral genotype (a Tet+ biofilm at t_{28}). Biofilms thus protract the emergence of non-
248 transmissible plasmid mutants.

249 There are a few possible explanations for these contrasting results between biofilms and
250 planktonic populations. The most parsimonious one is that the spatial structure of biofilms
251 protracted selective sweeps, which is in line with previous studies [29–33,43]. Although we did not
252 compare plasmid fitness cost, the higher persistence of the much smaller evolved plasmids can
253 almost certainly be explained by a reduced fitness cost on their host. First, loss of the two transfer
254 regions would be expected to reduce plasmid cost as horizontal transfer (conjugation) is known to
255 be costly [21,57,58]. Moreover, in the class 1 integron region that was always deleted together with the
256 transfer regions, the integrase gene could have added to the cost of the plasmid [59,60]. Since this gene
257 is not repressed by LexA in *Acinetobacter* spp., a highly active integrase expressed from pB10
258 could have had a toxic effect on *Acinetobacter* spp. [61]. Regardless of which genes conferred the
259 highest cost, losing these large plasmid segments allowed the hosts of truncated plasmids to rapidly
260 sweep through the planktonic Tet resistant populations. In contrast, due to the absence of global
261 competition in biofilms, clones with wild-type plasmids were not as readily outcompeted by clones
262 with deletion variants. An argument could be made that the bacteria in biofilms possibly underwent
263 fewer doublings than those in planktonic populations [62,63], and that this is what slowed evolution.
264 Unfortunately, while the number of doublings in the planktonic populations is largely homogenous
265 and can be estimated (140 after 14 days), the number of doublings in biofilm populations varies
266 according to depth and is impossible to estimate accurately. Table 1 shows an increase in biofilm
267 cell density over time, but it is unknown what fraction of the population was washed from the flow
268 cells. Even though the number of generations differed between the two environments, what matters
269 is that in most infections pathogens grow in biofilms, where we showed evolution is protracted
270 during the course of antibiotic treatment. Our data suggest that during such an antibiotic course
271 MDR plasmids lose their ability to transfer less rapidly than expected based on results from well-
272 mixed systems.

273 An additional hypothesis that can explain the increased retention of plasmid transfer genes
274 in bacteria evolving in biofilms is that a tradeoff exists between horizontal and vertical transmission
275 [57]. It is known that in biofilms close cell-to-cell contact and stabilized mating pair formation can
276 improve the efficiency of conjugative transfer to neighboring cells relative to planktonic
277 populations [64–67]. This makes horizontal transfer a more important mechanism of plasmid 'survival'

278 in biofilms [68]. It would then follow that selection on plasmid transferability may be stronger in
279 biofilms than in axenic planktonic populations, where it would present a cost rather than a benefit.
280 This is especially true for IncP-1 plasmids like pB10, which do not transfer efficiently in shaken
281 liquid cultures due to their short rigid sex pili [69,70]. In such a well-mixed environment, smaller non-
282 self-transmissible plasmids with a lower fitness cost could thus outcompete transmissible ones and
283 propagate through vertical transmission only.

284 Another plausible explanation for higher persistence of self-transmissible plasmids in
285 biofilms is the positive selection for retention of the sex pilus genes in this environment, which are
286 part of the transfer gene region *trb* that was deleted in the planktonic clones. Previous studies have
287 shown that conjugative plasmids can stabilize biofilms during the early stages of biofilm formation
288 due to the formation of sex pili [64,71]. If pilus expression was favorable for biofilm formation in our
289 flow cells, loss of the pilus could have been selected against in this environment.

290 Overall, our results suggest that in planktonic populations, the cost of maintaining the
291 transfer regions and integron is apparently higher than the benefit of retaining the plasmid through
292 horizontal transfer. The stark difference in plasmid evolutionary trajectories between planktonic and
293 biofilm populations and the higher diversity in plasmid persistence phenotypes reported by us
294 previously [42,43] suggests that it is critical to study bacteria-plasmid coevolution in biofilms to fully
295 understand plasmid population biology.

296 The deletions of the large plasmid segments in *A. baumannii* may result from homology-
297 facilitated illegitimate recombination due to short direct repeats that flanked the boundaries[53].
298 Illegitimate recombination due to short repeats is well understood in *E. coli,* and has also been
299 reported in *Acinetobacter baylyi* [72]. The same plasmid pB10 was previously shown by us to lose its
300 tetracycline resistance operon in *E. coli* through recombination between much larger direct repeats
301 [73]. Interestingly Porse *et al.* (2016) [21] identified a similar pattern of large deletions of a plasmid's
302 conjugative transfer region in an IncN plasmid from *Klebsiella pneumoniae* that was evolved in
303 strains of *Escherichia coli*. In that study the observed deletions were very similar to one another and
304 corresponded to the location of IS26, whereas the pB10 deletions in our study were not adjacent to
305 any IS element. Given the very short length of the flanking repeats on plasmid pB10 and the very
306 large deleted fragments, it would be interesting in future studies to determine the rate at which these
307 deletions occur in this *A. baumannii* strain.

308 In our previous studies we never observed loss of the entire plasmid transfer regions of pB10
309 or other plasmids of the same IncP-1 group [13,17,18]. Therefore we examined the persistence of one of
310 our pB10 deletion variants in three other bacterial species. The deletion caused a large increase in
311 plasmid persistence in *S. maltophilia*, but not in two environmental *Pseudomonas* isolates. *S.*
312 *maltophilia* is increasingly found to be an important nosocomial pathogen[74]. Our findings thus show
313 that deletion of plasmid transfer genes could also contribute to the persistence of MDR plasmids in
314 distantly related bacterial pathogens.

315 The persistence of plasmids that remained in the planktonic populations had always
316 improved within 14 days (140 generations) or less, regardless of the presence or absence of Tet.
317 This finding is consistent with previous work that showed a reduction in plasmid cost or
318 improvement of persistence over time in the absence of plasmid-selective antibiotics[15,19,75]. Thus
319 even when MDR plasmids are in bacteria that are not exposed to antibiotics, they can undergo
320 genetic changes that rapidly improve their persistence. Upon later exposure to antibiotics, these
321 deletion variants would sweep through the population, as they can outcompete ancestral or plasmid-
322 free clones. In contrast, in biofilms the average plasmid persistence only improved significantly in
323 the Tet+ biofilms. The observed discrepancy between biofilm and planktonic populations may be
324 due to the same variety of factors described above.

325 Our findings suggest that bacterial biofilms play an important role in the maintenance and
326 spread of MDR plasmids in natural bacterial populations in several ways. First, growth in biofilms
327 can increase MDR plasmid persistence in the absence of antibiotics. Second, evolution in biofilms
328 may facilitate the preservation of plasmid transferability. So far this has not been considered as a
329 possible positive effect of biofilm growth on the spread of MDR plasmids. These findings point out
330 that while experimental evolution of bacteria in planktonic populations have addressed important
331 basic evolutionary questions, these growth conditions are far removed from those of naturally
332 occurring bacterial populations. It is thus vital that future research on the ecology and evolution of
333 MDR plasmids includes studies on bacterial biofilms.

334

335

336 **MATERIALS AND METHODS**

337 *Bacteria, plasmid, and growth media.*

338 The ancestor used in our study was derived from *Acinetobacter baumannii* strain ATCC 17978
339 (Accession #CP000521) and is the same as used in our previous study [42]. Here we refer to this strain
340 simply as *A. baumannii*. The plasmid used was pB10, a 64.5-kbp broad-host-range IncP-1 plasmid
341 from a German wastewater treatment plant [52] that is poorly maintained in naïve *A. baumannii*. It
342 encodes resistance to tetracycline, streptomycin, amoxicillin, sulfonamides, and $HgCl_2$. Upon
343 whole-genome sequencing of the ancestral strain after electroporation with pB10 we observed the
344 loss of a large DNA segment (~140-kbp). This region was previously thought to be a chromosomal
345 island [76] but more recently shown to be part of a 150-kbp plasmid pAB3 in this strain [77]. We
346 determined that upon introduction of plasmid pB10 in ATCC 17978 by electroporation, our
347 ancestor lost pAB3 and is thus a derivative of ATCC 17978 without that mobile genetic element. In
348 addition to pB10, our ancestor retained the two small native plasmids, pAB1 and pAB2. Other
349 strains used in this study were *Pseudomonas* sp. nov. H2 [78], *Pseudomonas moraviensis* R28 [79], and
350 *Stenotrophomonas maltophilia* P21 [54].

351 For information regarding the construction of the ancestral host, the MBMS medium with
352 and without tetracycline (Tet, 10 mg/l), and the culture conditions, we refer to Ridenhour et al. [42].

353 *Experimental evolution protocol.*

354 A timeline of our experimental plan is shown in Fig. 1. The evolution experiment consisted
355 of a full factorial design with two growth environments and two treatments. The growth
356 environment was either a biofilm flow cell or serial batch culture. In one treatment (Tet+) the
357 plasmid-selective antibiotic Tet was added during the entire length of the experiment, whereas in
358 the Tet- treatment Tet was only present during the 4-day biofilm establishment period ($t_{-4} – t_0$). This
359 antibiotic selection phase was necessary to avoid establishing biofilms that would consist of a
360 mixture of plasmid-containing and plasmid-free cells. In all treatments, time point t_0 represents the
361 end of the 'biofilm establishment phase', which occurred four days after the flow of MBMS-Tet
362 through the flow cells was initiated following strain inoculation. As the set-up and growth
363 conditions of the Tet+ treatments have been previously described in detail [42], we focus here on
364 conditions that differed between treatments. The ancestral strain, grown overnight in MBMS-Tet,
365 was inoculated in multiples of three flow cells, as biofilm sampling was destructive, and triplicate
366 populations were harvested at each time point. This harvesting was done at t_0 and after 14 and 28

367 days (t_{14}, t_{28}) for the Tet+ treatment, and at t_0 and t_{14} for the Tet- treatment. The Tet- treatment was
368 ended at t_{14} instead of t_{28}, due to rapid plasmid loss in the parallel Tet- planktonic populations. The
369 harvesting procedures are detailed in Ridenhour *et* al. [42].

370 The Tet+ and Tet- planktonic populations were initiated by inoculating three replicate test
371 tubes containing five ml of MBMS-Tet or MBMS with cell suspensions from the triplicate t_0
372 biofilms of the Tet+ and Tet- treatments, respectively (Fig. 1). Each of these planktonic populations
373 were then grown in parallel to the remaining biofilm populations. We used this inoculation
374 approach to control for the level of diversity already present in the biofilms at t_0, due to evolution
375 that had taken place during the biofilm establishment phase. Following inoculation, the planktonic
376 populations were serially passaged every 24 (± 1) hours to obtain approximately 10 generations per
377 day, as described previously [42]. For the Tet+ treatment, the planktonic populations were archived at
378 t_{14} and t_{28}. For the Tet- treatment, the planktonic populations were archived daily because we did not
379 know how long plasmid-containing (Tet-resistant) cells would be maintained in these populations.
380 Appropriate dilutions were plated at t_5, t_{10}, and t_{14} on lysogeny broth agar (LBA) and LBA
381 supplemented with tetracycline (10 mg/l) (LBA_{Tet}). When plasmid-containing cells were no longer
382 detected on LBA_{Tet}, the populations archived at previous time points were plated until we could
383 identify the last time point at which the population still contained plasmid-bearing cells.

384 For both biofilm and planktonic populations at each sampling point (see Fig. 1), serial
385 dilutions of cell suspensions were plated on LBA_{Tet} and plates were incubated overnight. To isolate
386 evolved clones for plasmid persistence tests and whole-genome sequencing, six resistant clones per
387 replicate population were purified by restreaking twice and archived. The procedures used for
388 archiving clones and for plasmid extractions were described by us previously [42].

389 We also transferred plasmid DNA extracted from five resistant clones obtained from t_{28} of
390 the Tet+ treatment into the ancestral *A. baumannii* host to determine the effect of large plasmid
391 deletions on plasmid persistence. This was done by electroporation, essentially as described
392 previously, except for washing the cells in cold deionized water instead of glycerol or sucrose [80].

393 *Plasmid fate in biofilms versus planktonic populations in the absence of antibiotics.*

394 To determine the loss of the plasmid in the Tet- biofilms and planktonic populations
395 between t_0 and t_{14}, cell suspensions were diluted and plated on LBA and LBA_{Tet}. From these
396 colony-forming unit (cfu) counts, we determined the total number of culturable cells and the
397 proportion of plasmid-containing cells. In addition to the number of culturable (=viable) cells, we

398 also determined the total number of cells in the Tet- biofilms using phase-contrast microscopy and a
399 Petroff-Hausser counting chamber.

400 *Plasmid persistence assays.*

401 To determine plasmid persistence, we performed triplicate plasmid persistence assays on six
402 clones per time point per treatment, as described in Ridenhour *et al.* [42]. Briefly, each clone was
403 grown overnight in MBMS-Tet and used to inoculate three test tubes with MBMS, which were
404 incubated for 24 hours (+/- 1 hour) before passage into new media. This was repeated for a total of
405 8 days. To estimate the fraction of plasmid-bearing cells on days 5 and 8 by real-time quantitative
406 PCR (qPCR), cell pellets obtained after centrifugation of population samples were frozen at -20°C.
407 DNA extractions and qPCR done to determine the copy number of pB10 relative to the number of
408 16S rRNA copies, and qPCR-based estimates of the plasmid:chromosome ratio were used as a
409 measure of the fraction of plasmid-bearing cells. As described by us previously [42], we used the log-
410 linear model to estimate the rate of plasmid loss over time.

411 *Plasmid persistence in other hosts.*

412 We extracted ancestral pB10 and one evolved truncated pB10 variant from a clone obtained
413 from a t_{28} biofilm (Table S1) and used electroporation to insert each into three additional bacterial
414 strains. Plasmid persistence assays for these clones were performed as described above, but for a
415 total of seven days. They were sampled at days 0, 4, 7, and plasmid presence was determined using
416 serial dilutions and plate counting on LBA and LBA_{Tet} instead of qPCR.

417 *Sequencing, and sequence analysis.*

418 For DNA sequencing, total genomic DNA from each clone was extracted from 2mL of an
419 overnight culture using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) and the quality
420 and integrity of the DNA was assessed on a 1% agarose gel and the concentrations were determined
421 fluorometrically using Quant-iT™ PicoGreen® dsDNA Reagent (ThermoFisher Scientific,
422 Waltham, MA, USA) with the TBS-380 Mini-Fluorometer (Turner BioSystems) (Molecular
423 Devices, Sunnyvale, CA, USA). Samples were submitted to the University of Idaho IBEST
424 Genomic Resources Core facility for library preparation and whole-genome sequencing using
425 Illumina MiSeq (Illumina, San Diego, CA, USA) and associated chemistry.

426 Following sequencing, the sequence data were screened to remove low-quality reads,
427 sequencing adapters, and duplicate read pairs using the software package htstream
428 (https://s4hts.github.io/HTStream/). To identify mutations in the genome, cleaned reads were

429 mapped against the reference sequence for pB10 (AJ564903.1) and *A. baumannii* ATCC 17978
430 (GCA_000015425.1) using *breseq* v0.35.7 [81]. Prior mapping discrepancy between the reference
431 sequence of *A. baumannii* ATCC 17978 and our ancestral seed stock were corrected using the
432 gdtools APPLY command from *breseq*. The command was applied iteratively until no more
433 mutations were detected between the ancestor reads and the reference sequence. Mutations between
434 strains were compared using the gdtools COMPARE from *breseq* and the presence of undetected
435 deletions was manually screened by searching for Missing Coverage Evidence and the presence of
436 corresponding New-Junction Evidence, indicating reads spanning the area where no reads were
437 mapped.

438 *Statistical analysis.*

439 Statistical analysis of the qPCR data was done using a linear mixed-effect model approach
440 in R with the nlme package. The log plasmid:chromosome ratio of each group was predicted using
441 the origin of the clones (i.e., t_0, biofilm, or planktonic population) as fixed effect and the clone
442 replicate measure as random effect. Each parameter was nested within the day of the experiment
443 (Days 0, 5, 8) as a continuous variable.

444

445 **DATA AVAILABILITY**

446 All sequencing data pertaining to this project will be made available at the National Center for
447 Biotechnology Information upon publication. All other data that support the findings of this study
448 will be made available on open-source platforms.

449

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461

462 **AUTHORS CONTRIBUTION**

463 E.M.T. and L.J. F., conceived the project; G.A.M., E.M.T., L.J. F., B.J.R., and M.L.S., designed the
464 study; G.A.M., M.F., K.G., and J.M. performed experiments and collected the data; G.A.M. and
465 T.S. performed the genomic analysis; G.A.M. and B.J.R. performed the statistical analyses; G.A.M.,
466 T.S. and E.M.T. wrote the manuscript; All authors helped revising the manuscript.

467

468 **COMPETING INTERESTS**

469 The authors declare no competing interests.

470

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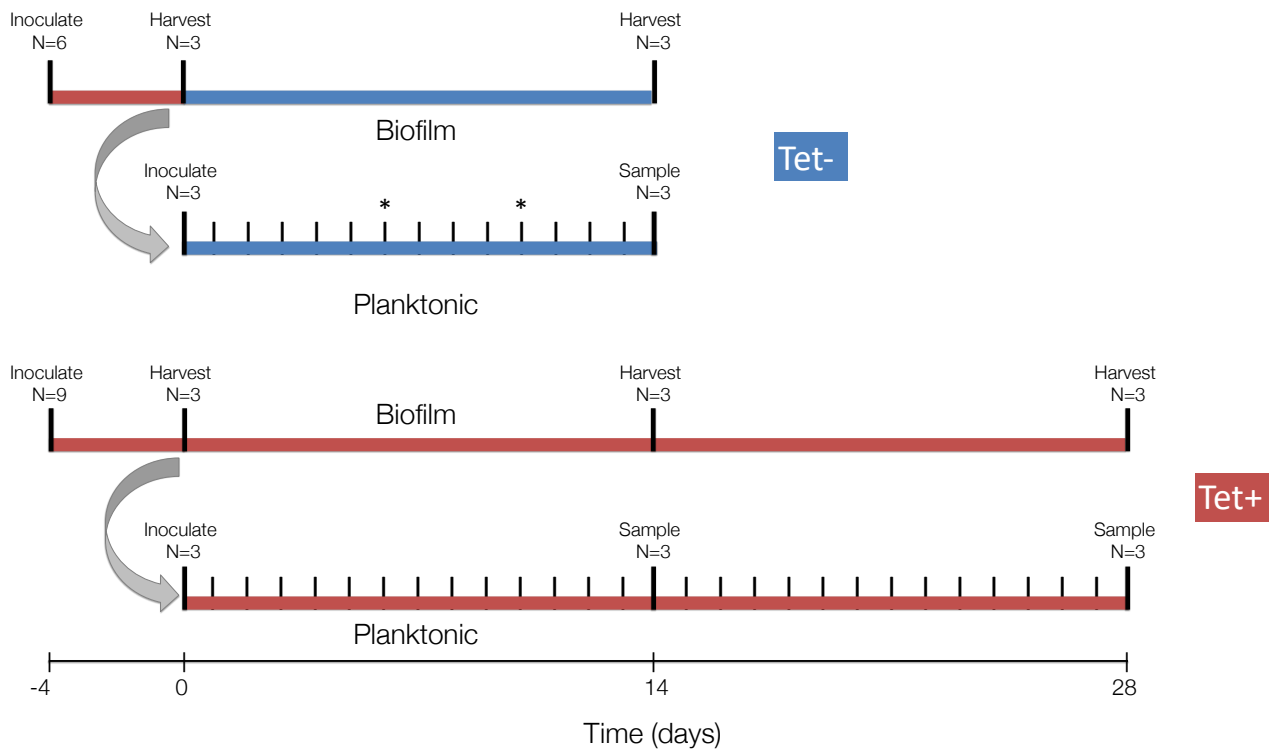
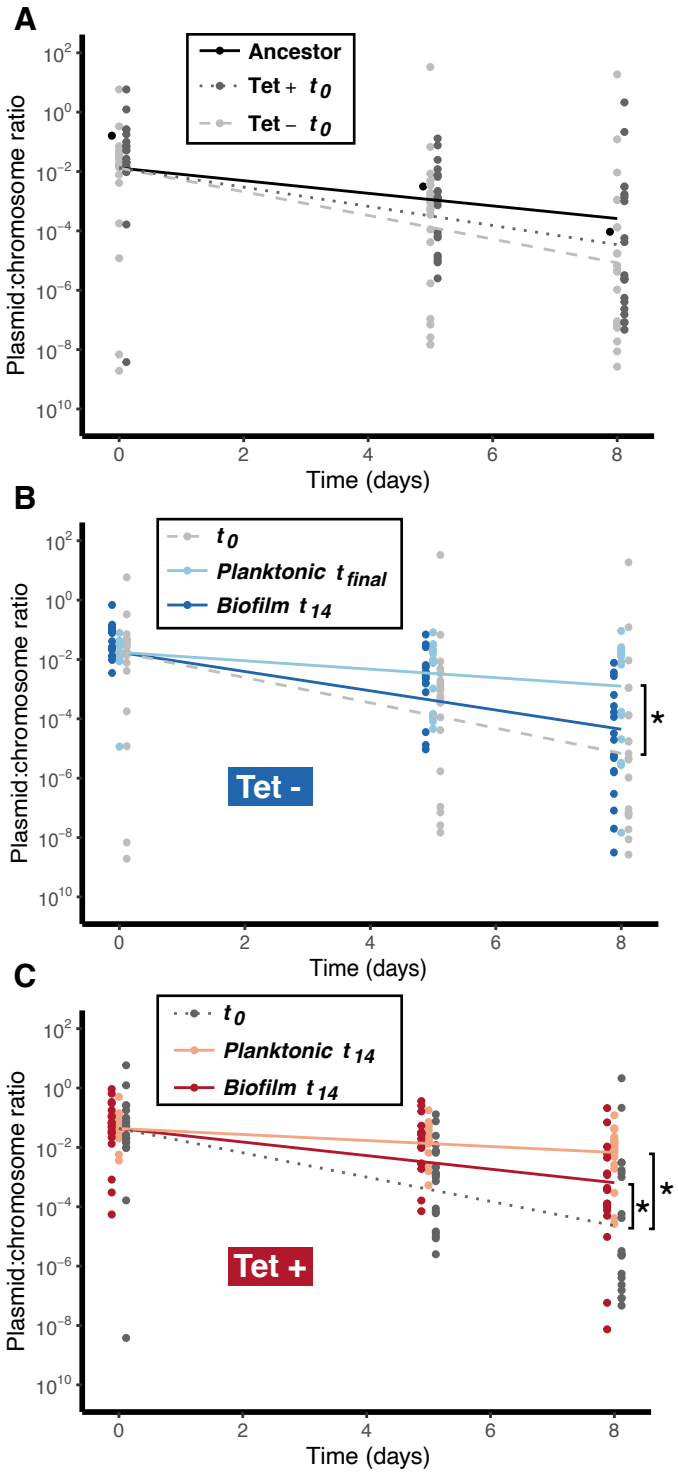
669 **FIGURES AND TABLES**



670

671 Figure 1: Timeline of the evolution experiments. Red represents time periods when media contained
672 tetracycline (Tet+ treatment); blue represents the absence of antibiotics (Tet- treatment). Large ticks
673 represent inoculation and harvest/sampling events. Four days prior to initiation of the evolution
674 experiments (-4) the biofilm flow cells were inoculated with the ancestral strain. Four days later, at
675 day 0 (t_0), the first set of randomly selected flow cells (n=3) were harvested. A subsample of the cell
676 suspensions from each of these replicates was used to inoculate each of the three planktonic
677 populations (grey curved arrow). Small tick marks indicate the daily serial passage of planktonic
678 populations. Asterisks on days 6 and 10 in the timeline of the Tet- planktonic populations indicate
679 the last day at which plasmid-containing cells were detected in two of the three populations due to
680 rapid plasmid loss; these time points were the final sampling points for these two populations (t_6
681 and t_{10}). The Tet- experiment was terminated after 14 days and the Tet+ experiment after 28. For
682 more details on the actual protocol, see Materials and Methods and Ridenhour *et al.* [42].



683

684 Figure 2: Plasmid persistence shown as the estimated ratio of *trfA*/16S rRNA genes over a period of
685 eight days in liquid serial batch cultures in the absence of selection; (A) the ancestor used to
686 inoculate the flow cells at t_{-4} compared to clones isolated from all the biofilms harvested after four
687 days of growth with tetracycline, t_0, showing similar plasmid persistence dynamics (Tet+ and Tet-:

688 populations treated with and without tetracycline after t_0); (B) clones isolated from the Tet-
689 populations, showing a drastic increase in plasmid persistence after evolution in planktonic but not
690 in biofilm populations (note that two of the three planktonic populations no longer contained
691 detectable plasmids after day 6 and 10, respectively, requiring analysis on these days instead of t_{14} –
692 the final sampling times are therefore referred to as t_{final}); (C) clones isolated at t_0 and t_{14} of the Tet+
693 evolution experiment, showing an increase in plasmid persistence over time for all populations.
694 Lines are the output of the log-linear model for each group. * denotes a significant difference
695 between the groups. The plasmid persistence data from t_0 in the Tet+ experiment were reported by
696 us previously [42].

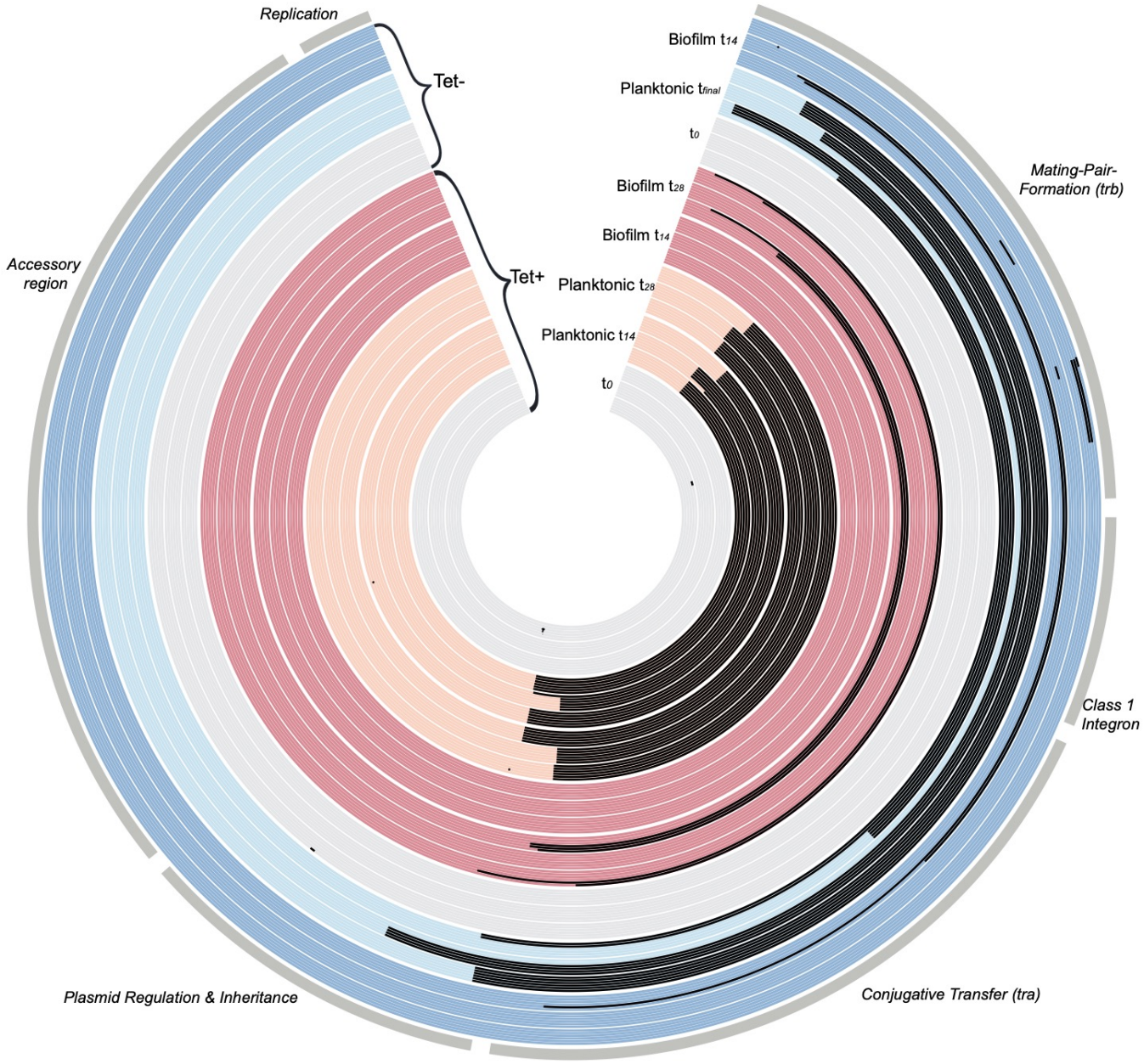


Figure 3: Maps of evolved plasmids with major segment functions identified, showing all plasmid mutations observed in each sequenced clone evolved under different treatments (Tet+ and Tet-) in different environments (biofilm and planktonic populations). Evolved clones are grouped by treatment, environment, and time point, as shown by different colors and shades and identified in the open wedge of the circle. The plasmid genome of each clone is shown as a single band – at each time point, 18 clones were analyzed per treatment and environment (six clones from each triplicate population). Tet+,

Tet-: evolved with and without Tet. Black areas in the plasmid maps represent deletions (note that small indels are shown as small spots) and the dots represent SNPs; see Table S1 for details. Note that the tetracycline resistance operon is located in the 'accessory region'.

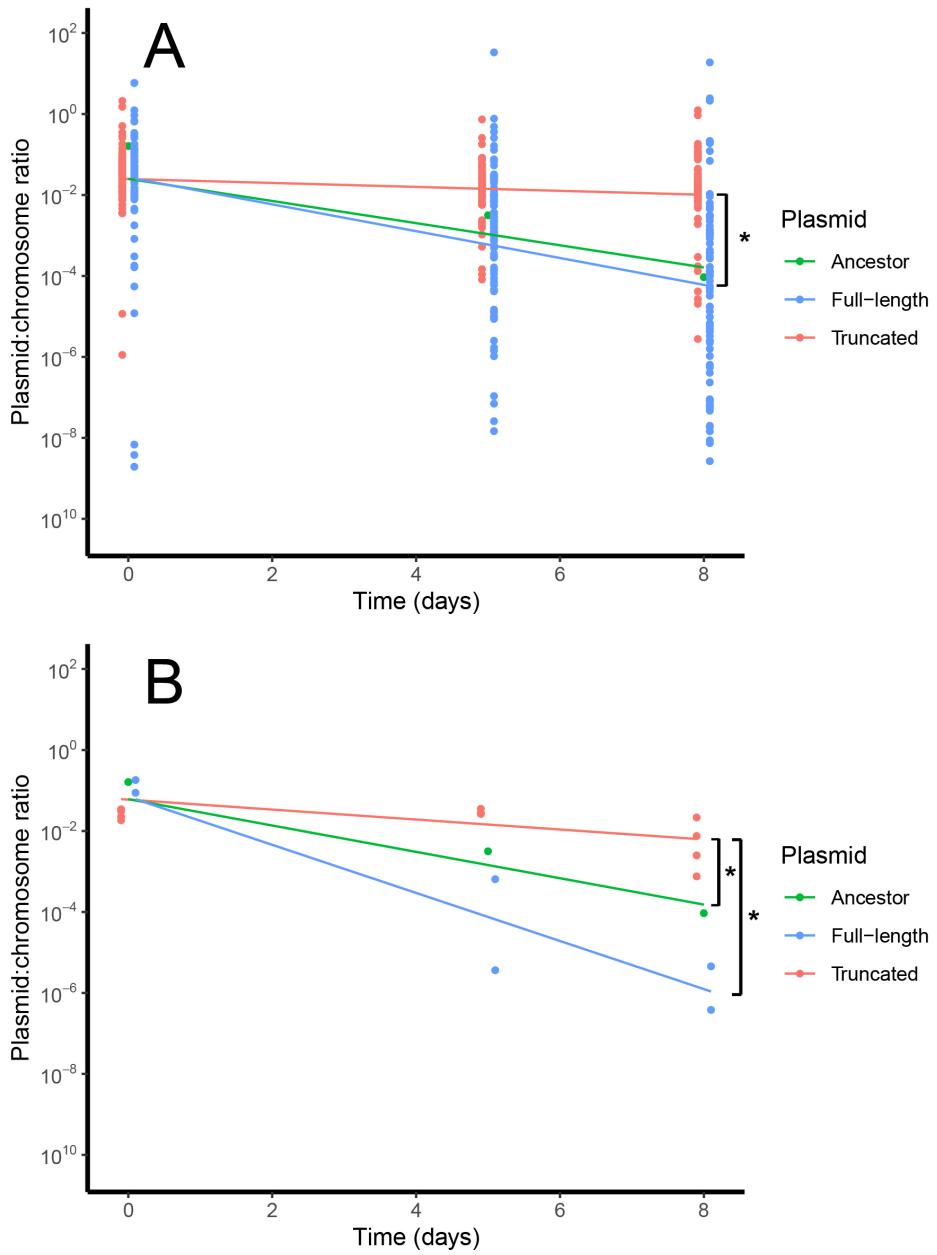


Figure 4: Persistence of plasmids evolved in Tet+ biofilms and planktonic populations tested in (A) their co-evolved hosts (92 full-length and 49 truncated plasmids), and (B) in the ancestral host (2 full-length and 4 truncated plasmids). In both panels the ancestral plasmid was tested in the ancestral host. Plasmid persistence is shown as the estimated ratio of *trfA*/16S rRNA genes over time. Lines are the results of the log-linear model for each group.

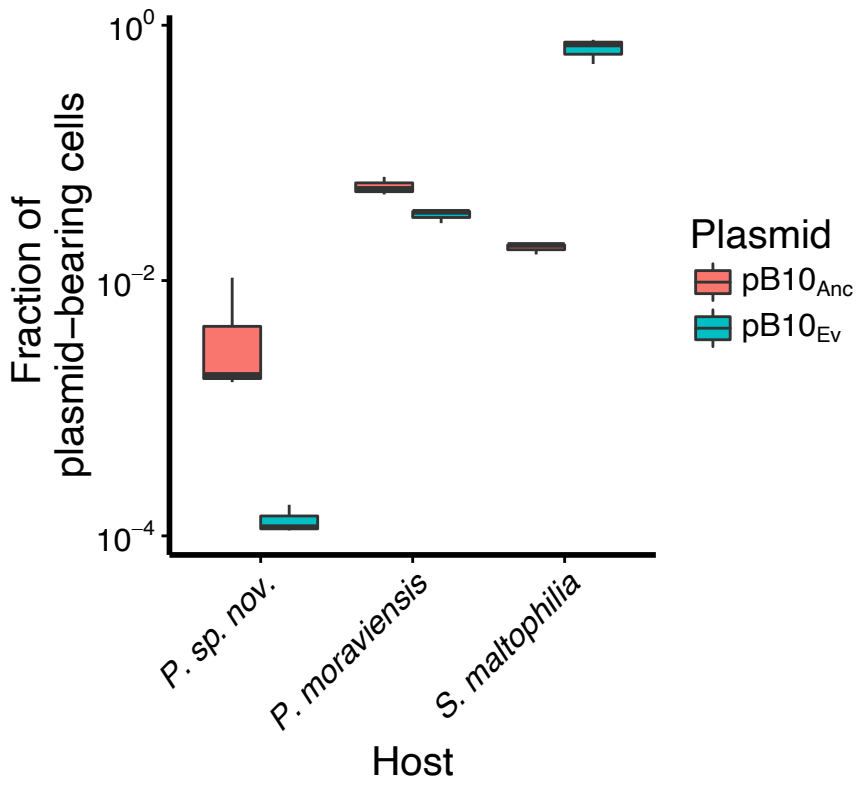


Figure 5: Plasmid persistence of ancestral plasmid $pB10_{Anc}$ and truncated plasmid $pB10_{ev}$ in three additional hosts, as determined by plate counting after 8 days, showing that the plasmid deletion was only beneficial to persistence in *Stenotrophomonas maltophilia*. (P= *Pseudomonas*)

Table 1: Total cell counts (microscopy), viable cell counts (colony forming units, cfu), and absolute counts and fraction of plasmid-containing (P+) cells for each replicate biofilm or planktonic population of the Tet- treatment. P+ fractions were calculated as the ratio of Tet^R over total cfu counts, obtained by plating on selective and non-selective media. Note that total cell counts estimated by microscopy were only determined for biofilms. n/a: count not performed.

	Total cells (per mL)	Total viable cells (per mL)	P+ cells (per mL)	Fraction P+ cells
***t* 0**				
Rep 1	9.6×10^9	6.1×10^6	5.9×10^6	0.98
Rep 2	3.9×10^9	4.7×10^6	3.8×10^6	0.82
Rep 3	3.5×10^9	3.4×10^6	3.4×10^6	1.00
t0 Avg	*5.6×10^9*	*4.7×10^6*	*4.4×10^6*	*0.93*
***t* 14 Biofilm**				
Rep 1	1.3×10^{11}	2.9×10^6	5.5×10^5	0.19
Rep 2	1.3×10^{10}	3.1×10^6	3.6×10^5	0.11
Rep 3	1.1×10^{10}	1.8×10^6	1.3×10^5	0.07
Biofilm Avg	*5.1×10^{10}*	*2.6×10^6*	*3.5×10^5*	*0.12*
***t* 14 Planktonic**				
Rep 1	n/a	2.0×10^8	3.2×10^4	0.00016
Rep 2	n/a	1.6×10^8	<1	0
Rep 3	n/a	2.0×10^8	<1	0
Planktonic Avg	*n/a*	*1.9×10^8*	*1.1×10^4*	*0.00005*

Table S1: Summary of evolved plasmid genotypes with their affected genes and gene functions, found in all 144 sequenced clones.

Table S2: List of all sequenced strains with their predicted mutations