1   **Positive selection inhibits plasmid coexistence in bacterial genomes**

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12

13   **Abstract**

14   Plasmids play an important role in bacterial evolution by transferring niche adaptive

15   functional genes between lineages, thus driving genomic diversification. Bacterial genomes

16   commonly contain multiple, coexisting plasmid replicons (i.e., plasmid coinfection), which

17   could fuel adaptation by increasing the range of gene functions available to selection and

18   allowing their recombination. However, plasmid coinfection is difficult to explain because the

19   acquisition of plasmids typically incurs high fitness costs for the host cell. Here, we show that

20   plasmid coinfection was stably maintained without positive selection for plasmid-encoded

21   gene functions and was associated with compensatory evolution to reduce fitness costs. By

22   contrast, with positive selection, plasmid coinfection was unstable despite compensatory

23   evolution. Positive selection discriminated between differential fitness benefits of functionally

24   redundant plasmid replicons, retaining only the more beneficial plasmid. These data suggest

25   that while the efficiency of negative selection against plasmid fitness costs declines over

26   time due to compensatory evolution, positive selection to maximise plasmid-derived fitness

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27 benefits remains efficient. Our findings help to explain the forces structuring bacterial

28 genomes: Coexistence of multiple plasmids in a genome is likely to require either rare

29 positive selection in nature or non-redundancy of accessory gene functions among

30 coinfecting plasmids.

31

Introduction

33 Plasmids play an important role in the evolution of bacterial genomes, promoting

34 evolutionary divergence by transferring niche-adaptive accessory gene functions between

35 lineages[1-3]. Bioinformatic analyses suggest that carriage by single bacterial host cells of

36 multiple, coexisting plasmid replicons—i.e. plasmid coinfection—is more commonly

37 observed than would be expected by chance[4]. Accumulation of multiple, coexisting

38 plasmid replicons thus drives genome expansion and can lead to the evolution of multipartite

39 genomes [5-7]. Moreover, this process could potentially fuel evolutionary innovation through

40 reassortment of new combinations of accessory functions and the formation of new mobile

41 genetic elements through plasmid-plasmid recombination[8, 9]. Nevertheless, the observed

42 high rates of plasmid coinfection are surprising given that plasmid acquisition usually

43 disrupts normal cellular function and is often associated with large fitness costs for the host

44 cell[10-14].

45

46 One explanation for abundant plasmid coinfection is that the fitness costs of acquiring

47 multiple plasmids could be less than additive. Positive epistasis between plasmid costs

48 could permit the accumulation of multiple plasmids by reducing the cost for plasmid-bearers

49 of acquiring additional plasmids[4, 15, 16], however, positive epistatic interactions among

50 plasmid costs are not universal[4]. Moreover, as we will show in this study, the methods by

51 which positive epistasis has been previously estimated (i.e., competition of plasmid-carriers

52 against plasmid-free cells[4]) may not measure the actual cost of plasmid coinfection.

53 Compensatory evolution to ameliorate the cost of plasmids has been described in a wide

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54   range of plasmid systems and prevents plasmid loss by weakening negative selection

55   against the plasmid backbone over time[17-20]. If the fitness costs of multiple plasmids have

56   common mechanistic causes, it is possible that the same compensatory mechanisms could

57   simultaneously ameliorate the costs of multiple plasmids which may in turn then promote

58   stable plasmid coinfection[15, 19]. The role of positive selection for beneficial plasmid-

59   encoded accessory gene functions in plasmid coinfection is less well studied. Positive

60   selection could promote plasmid coinfection if the fitness benefits of carrying multiple

61   plasmids outweighed the accumulative fitness costs, or, alternatively, may inhibit coinfection

62   by selecting for consolidation of beneficial functions onto fewer replicons, accompanied by

63   the loss of redundant, costly plasmid backbone(s)[13, 14]. Understanding the roles of these

64   various mechanisms in plasmid coinfection requires experimental tests, but while the fitness

65   costs of single- and co-infection by plasmids have been estimated, studies tracking the

66   longer-term dynamics of plasmid coinfection in bacterial populations are lacking.

67

68   Here, we consider the experimental coinfection dynamics for two distantly-related, naturally

69   co-occurring conjugative mercury-resistance plasmids—pQBR103 and pQBR57—originally

70   isolated from a field-site in the UK[21, 22]. Each of the plasmids individually causes a

71   substantial fitness cost in the host bacterium *Pseudomonas fluorescens* SBW25[23]. Both

72   plasmids encode a near identical copy of the *mer* mercury-resistance operon encoded on a

73   Tn5042 transposon [23, 24], which allows plasmid-bearers to reduce toxic Hg(II) to Hg,

74   providing a fitness benefit to plasmid-carriers at increased Hg(II) concentrations[23].

75   Although SBW25(pQBR57) outcompetes SBW25(pQBR103) in the absence of mercury, this

76   competitive hierarchy is reversed in mercury-containing environments[23]. This suggests

77   that while pQBR57 imposes a lower fitness cost on SBW25, it also provides less of a fitness

78   benefit in the presence of mercury, relative to pQBR103. Both plasmids are maintained in

79   single-infected bacterial populations and, while this appears in each case to be linked to

80   compensatory evolution [18, 25], unlike pQBR103, pQBR57 is capable of a high rate of

81   conjugative transfer, which contributes to its survival and spread particularly in the absence

82   of mercury[26, 27]. Multiple mechanisms of compensatory evolution have previously been

83   described for the pQBR plasmids. Specifically, chromosomal compensatory mutations that

84   occur either in *gacA*/*gacS* encoding a two-component global regulatory system or in

85   *PFLU4242* encoding a hypothetical protein with two domains of unknown function, reduce

86   the cost of these and other pQBR plasmids individually[17, 18].

87

88   Replicate laboratory populations of SBW25 that were originally either singly-infected or

89   coinfected with the plasmids pQBR103 and pQBR57 were propagated by serial transfer with

90   or without positive selection (i.e. addition of mercury (II) chloride) for approximately ~265

91   bacterial generations. We tracked bacterial population densities and the dynamics of

92   mercury resistance over time and used multiplex-PCR to determine the plasmid-carriage

93   status of mercury resistant clones. We show that plasmid coinfection was stable in

94   populations without positive selection, whereas positive selection drove the loss of pQBR57

95   and the dominance of SBW25 carrying pQBR103-only. Loss of plasmid coinfection occurred

96   despite compensatory evolution to ameliorate plasmid fitness costs and was caused by

97   positive selection discriminating between the differential fitness benefits of the plasmids,

98   retaining only the more beneficial plasmid.

99

100   **Material and Methods**

101   *Bacterial Strains and Culture Conditions*

102   Bacterial populations were grown in liquid Kings B (KB)[28] broth microcosms (6 ml of KB

103   broth in a 30ml glass universal vial). These were incubated at 28° C and shaken at 180 rpm.

104   To generate positive selection for mercury resistance, microcosms were supplemented with

105   40 μM Hg (II) chloride, as required. Bacterial colonies were obtained by plating serial

106   dilutions onto KB agar. To select particular bacterial strains (described below), agar plates

107   were supplemented with gentamycin (30 μg/ml), streptomycin (250 μg/ml), Hg (II) chloride

108   (20 μM for plating and 100 μM for replica plating), kanamycin (25 μg/ml) or X-gal (75 μg/ml),

109   as required.

110

111   Two isogenic *P. fluorescens* SBW25 strains[21] with chromosomal resistance markers

112   [either gentamycin resistance (Gm^R) or a streptomycin resistance with LacZ (Sm^R*lacZ*)] were

113   used to enable creation of transconjugants[23, 29, 30]. Derived *P. fluorescens* SBW25

114   deletion mutants for the *gacS* gene [SBW25-Gm^R-ΔgacS; [18]] or the *PFLU4242* gene

115   [SBW25-Gm^R-ΔPFLU4242; [17]] were used to enable measurement of the effects of these

116   genes on plasmid costs in competition experiments. An isogenic plasmid-free *P. fluorescens*

117   SBW25 Gm^R strain with a chromosomal Tn5042 mercury resistance transposon derived

118   from the pQBR103 plasmid [SBW25(Tn5042)] was used to enable measurement of the

119   fitness cost of the plasmid backbones in competition experiments[27].

120

121   Two mercury resistance plasmids were used in this study that had been previously isolated

122   from agricultural soil in Oxfordshire, UK: pQBR57 [21] and pQBR103 [22]. An isogenic

123   variant of pQBR103 marked with an mCherry fluorescent protein gene and a kanamycin

124   resistance gene (mCherryKm^R) was used to enable selection of coinfected bacterial

125   cells[31]. Plasmids were introduced to bacterial strains by conjugation using standard

126   protocols[23, 32]. Briefly, transconjugants were selected by plating onto KB agar plates

127   supplemented with 20 μM Hg (II) chloride or 25 μg/ml kanamycin and the relevant antibiotic

128   [either gentamycin (30 μg/ml) or streptomycin (250 μg/ml)] as appropriate to select the

129   recipient strain. Plasmid status of transconjugant colonies was determined by PCR, as

130   previously described[18, 26].

131

132   *Selection experiment*

133   Six replicate populations each of SBW25-Sm^R*lacZ*(pQBR57+pQBR103-Km^R), SBW25-

134   Sm^R*lacZ*(pQBR57), SBW25-Sm^R*lacZ*(pQBR103-Km^R) were propagated with or without

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135   positive selection (i.e., supplementation with 40 µM Hg (II) chloride or 0 µM Hg (II) chloride,

136   respectively), and six replicate populations of the SBW25-Sm^R*lacZ* plasmid-free control were

137   propagated without Hg (II) chloride. (Note: plasmid-free populations cannot survive in 40 µM

138   Hg (II) chloride.) Each replicate was founded by 60 µl of an overnight liquid culture initiated

139   from a single independent colony previously streaked on KB agar. One percent of each

140   population was serially transferred to fresh media every 48h for 40 transfers, resulting in

141   approximately 265 bacterial generations. Every 10 transfers serial dilutions of each

142   population were plated onto KB agar and incubated at 28° C to enumerate bacterial

143   densities. These plates were replica plated onto KB agar supplemented with Hg (II) chloride

144   100 µM/ml to determine the frequency of mercury resistance (Hg^R). Twenty-four mercury

145   resistant colonies per population per time-point were chosen at random to determine the

146   presence of each plasmid and Tn5042 by multiplex PCR. We used three set of primers to

147   target the Mer-Tn5042 transposon [F-TGCAAGACACCCCCTATTGGAC, R-

148   TTCGGCGACCAGCTTGATGAAC], the pQBR103-plasmid specific origin of replication *oriV*

149   [F- TGCCTAATCGTGTGTAATGTC, R-ACTCTGGCCTGCAAGTTTC] and the pQBR57-

150   plasmid specific *uvrD* gene [F-CTTCGAAGCACACCTGATG, R-

151   TGAAGGTATTGGCTGAAAGG] [18, 26]. Briefly, a mixture of 1x GoTaq Green (Promega,

152   WI USA) with 0.71 µM of each primer to detect pQBR103, 0.89 µM of each primer to detect

153   pQBR57 and 0.36 µM of each primer to detect Tn5042 was used with the following

154   thermocycle program: 95 °C 5′, 30 x (95 °C 30′, 58 °C 30′, 72 °C 1′), 72 °C 5′ [26].

155

156   *Competition experiments*

157   Competition experiments were used to measure the fitness costs associated with carrying

158   plasmid(s) against a range of competitor strains. In all cases, overnight cultures of

159   competitors were mixed in a 1:1 ratio and diluted 100-fold into KB microcosms ± 40 µM Hg

160   (II) chloride and incubated for 48h at 28°C with shaking at 180rpm. Starting and final

161   densities of each marked strain were determined by plating onto KB agar supplemented with

162   X-gal, and relative fitness (*w*) was calculated as previously described[18].

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163

164   To measure the fitness cost of the plasmid backbones per se, that is once the fitness effect

165   of the Tn5042 is accounted for, the plasmid-bearing strains [SBW25-Sm^R*lacZ*(pQBR103-

166   Km^R) or SBW25-Sm^R*lacZ*(pQBR57) or SBW25-Sm^R*lacZ*(pQBR57+pQBR103-Km^R)] were

167   competed against SBW25-Gm^R(Tn5042) both with and without Hg (II) chloride. Six

168   replicates were performed per comparison.

169

170   To   measure   the   fitness   cost   of   plasmid   coinfection   we   competed   SBW25-

171   Sm^R*lacZ*(pQBR57+pQBR103-Km^R) against either SBW25-Gm^R(pQBR103-Km^R) or SBW25-

172   Gm^R (pQBR57) or SBW25-Gm^R(pQBR57+pQBR103-Km^R) as a control, both with and

173   without Hg (II) chloride. Twelve replicates were performed per comparison. Normalised

174   fitness was calculated by subtracting the mean of the control competition [i.e., SBW25-

175   Sm^{R}LacZ(pQBR57+pQBR103-Km^R) versus SBW25-Gm^R(pQBR57+pQBR103-Km^R)].

176

177   To measure the fitness effect of putative compensatory mutations on plasmid carriage we

178   competed SBW25-Gm^R or SBW25-Gm^R-ΔgacS or SBW25-Gm^R-ΔPFLU4242 carrying

179   plasmid(s) (pQBR57 or pQBR103-Km^R or pQBR57+pQBR103-Km^R) against plasmid-free

180   SBW25-Sm^R*lacZ*. Six replicates were performed per comparison.

181

182   *Genomic analysis*

183   The whole genome sequence for at least one randomly chosen clone per population was

184   obtained at the end of the selection experiment. For populations containing multiple mercury

185   resistant genotype subpopulations—i.e., clones without mercury resistance or Tn5042-only

186   or pQBR103-only or pQBR57-only or both pQBR57+pQBR103—and where these

187   genotype(s) comprised at least 10% of the population, we obtained the whole genome

188   sequence for one randomly chosen clone per subpopulation. Whole-genome sequencing

189   was performed by MicrobesNG using a 250bp paired-end protocol on the Illumina HiSeq

190   platform. Paired reads were aligned to the annotated ancestral genome sequence using

7

191 Burrows-Wheeler Aligner[33] and duplicate reads were removed using picard

192 (https://broadinstitute.github.io/picard/). Variants were called using GATK Haplotype

193 Caller[34] and annotated using SnpEff[35]. Called variants were then filtered to remove low

194 quality calls with either low coverage (< 12 reads per bp), low quality (scores <200) or low

195 frequency of the alternative allele (< 90% of reads with alternative). In addition, as a

196 complementary and confirmatory approach, variants were also called against the ancestral

197 reference genome using the Breseq computational pipeline using the standard default

198 settings [36]. All variants not called by both methods were validated visually using the

199 alignment viewer IGV[37, 38]. All sequencing data are available on the Short Read Archive

200 under accessions PRJEB38218 / ERP121615.

201

202 *Statistical analysis*

203 The integral of mercury resistance frequency over time was calculated as the area under the

204 curve using the AUC function of the 'flux' package in R and compared between treatments

205 using Welch's ANOVA due to unequal variances. Posthoc pairwise comparisons were

206 performed using the Dunnetts T3 test. The integral of plasmid coinfection frequency over

207 time was calculated as the area under the curve using the AUC function of the 'flux' package

208 in R and compared between treatments using a Mann Whitney U test due to unequal

209 variances. Relative fitness data from competition experiments was analysed using ANOVA

210 and posthoc pairwise comparisons were performed using Tukey tests. Analyses were

211 performed in R 3.6.1 [39] or Prism v8.1.2.

212

213 **Results**

214 *Temporal dynamics of mercury resistance and plasmid carriage*

215 To study the effect of positive selection on the dynamics of plasmid coinfection we

216 propagated replicate populations of SBW25 carrying either both plasmids or pQBR103-alone

217 or pQBR57-alone, both with and without selective levels of Hg(II) chloride by serial transfer

218 for approximately 265 bacterial generations. We also propagated replicate plasmid-free

8

219 control SBW25 populations without Hg(II) chloride. Mercury resistance (Hg^R) was maintained

220 near fixation under positive selection for all plasmid treatments, but without positive selection

221 its frequency varied according to plasmid treatment (Figure S1; comparison of cumulative

222 Hg^R frequency between plasmid treatments without Hg(II) selection: Welch's ANOVA;

223 $W_{2,6.685}$ = 18.32, P = 0.0019). Without positive selection, whereas Hg^R remained at high

224 frequency in the both-plasmids treatment, Hg^R declined in the single-plasmid treatments,

225 and significantly so in the pQBR103-alone treatment (Dunnett's T3 test for pairwise

226 comparisons of plasmid treatments without Hg(II) selection: pQBR103:both P = 0.0044;

227 pQBR57:both P = 0.4038; pQBR103:pQBR57 only P = 0.3157). The Hg^R phenotype

228 indicates the presence of the Tn5042-encoded *mer* operon within the cell, which could be

229 explained by the maintenance of one or both plasmids or by the relocation of the Tn5042 to

230 the chromosome accompanied by plasmid loss[18, 26, 30]. We therefore used multiplex-

231 PCR to determine the presence of the Tn5042 and of each plasmid. In the single-plasmid

232 treatments, whereas plasmid-encoded Hg^R predominated in populations propagated without

233 positive selection, we observed the invasion of plasmid-free cells carrying a chromosomal

234 Tn5042 in some replicates with positive selection (Figure 1; Figure S2; 2 / 6 replicates of the

235 pQBR57-alone treatment; 1 / 6 replicates of the pQBR103-alone treatment). Plasmid

236 dynamics also varied with positive selection in the both-plasmids treatments. Plasmid

237 coinfection was maintained at higher frequency in populations without positive selection

238 compared to those propagated with positive selection (Figure 1; comparison of cumulative

239 coinfection frequency: Mann Whitney U test, U = 0, P = 0.0022). This was driven by the loss

240 of pQBR57 from initially coinfected cells under positive selection, such that plasmid-

241 coinfected cells were replaced by cells carrying pQBR103-only in 4 out of 6 replicates

242 (Figure S2). Taken together, these data suggest that positive selection inhibited plasmid

243 coinfection and, furthermore, that the pQBR57-backbone was selected against more

244 strongly than the pQBR103-backbone under positive selection for mercury resistance.

245

246 *Fitness effects of plasmid carriage*

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247   To understand the effect of positive selection on the relative fitness of the various Hg^R

248   genotypes observed here—i.e., chromosomal Tn5042, either plasmid alone or both plasmids

249   together—we performed a series of competition experiments. First, we tested how positive

250   selection affected the cost of carrying the plasmid backbone(s) by competing SBW25

251   plasmid-bearers against an isogenic ancestral SBW25 encoding a chromosomal copy of the

252   Tn5042 [SBW25(Tn5042)]. Here, in each pairwise competition both competitors are resistant

253   to mercury, but the plasmid bearers must pay the additional fitness costs of maintaining the

254   plasmid backbone(s). Plasmids bearers varied in fitness relative to SBW25(Tn5042)

255   according to their plasmid complement: pQBR103 had a higher fitness cost than either

256   pQBR57 or both plasmids together (Figure 2; ANOVA; plasmid main effect, $F_{2,28} = 7.813$ P =

257   0.002; Tukey pairwise contrasts: pQBR103:pQBR57 P = 0.0217; pQBR103:both P = 0.0019;

258   pQBR57:both P = 0.6399). Moreover, the addition of Hg(II) chloride increased the fitness

259   cost of plasmid carriage (mercury main effect, $F_{1,28} = 31.298$ P = 5.48×10^{-6}), suggesting that

260   once the benefit of mercury resistance is negated, Hg(II) chloride increased the costs of the

261   plasmid backbones *per se*. Together, these data confirm previous studies reporting a higher

262   cost of the pQBR103 backbone relative to the pQBR57 backbone[23] and, moreover,

263   explain the loss of redundant plasmid replicons under positive selection seen here and in

264   other studies[26, 31].

265

266   Nevertheless, these data cannot explain the preferential loss of the pQBR57 backbone from

267   coinfected cells that we observed. To further explore this, we next directly competed SBW25

268   coinfected with both plasmids against SBW25 carrying either of the plasmids alone, both

269   with and without positive selection. We found that the relative fitness of coinfected SBW25

270   was lower when competed against SBW25(pQBR103) than against SBW25(pQBR57)

271   (Figure 3; ANOVA; plasmid main effect, $F_{1,40} = 7.438$ P = 0.009435) and was reduced by the

272   presence of Hg(II) in the media (mercury main effect, $F_{1,40} = 5.727$ P = 0.02149) while the

273   interaction of these factors was marginally non-significant (plasmid:mercury interaction, $F_{1,40}$

274   = 3.616 P = 0.06443). Post-hoc pairwise comparisons were consistent with positive selection

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275   favouring the loss of pQBR57 from coinfected cells as we observed in our serial transfer

276   experiment: The addition of Hg(II) chloride further reduced the fitness of coinfected

277   SBW25(pQBR57+pQBR103) when competed against SBW25(pQBR103) (Tukey pairwise

278   comparison; with versus without Hg(II) chloride, P = 0.0203) but not when competed against

279   SBW25(pQBR57) (Tukey pairwise comparison; with versus without Hg(II) chloride, P =

280   0.9967), such that the fitness of coinfected cells was higher against SBW25(pQBR57) than

281   against SBW25(pQBR103) in the presence of Hg(II) chloride (Tukey pairwise comparison; P

282   = 0.0113). Taken together and considering previous findings [23], these data suggest that

283   although the pQBR103 backbone is costlier than the pQBR57 backbone, pQBR103 is more

284   beneficial in mercury-containing environments than pQBR57. Thus, the preferential loss of

285   pQBR57 from coinfected cells under positive selection is better explained by selection

286   favouring the more beneficial plasmid rather than selection against the more costly plasmid

287   backbone.

288

289   *Compensatory mutations and their fitness effects*

290   A potential alternative explanation for the contrasting patterns of plasmid maintenance

291   among treatments would be if there existed differences in the propensity for compensatory

292   evolution to occur according to plasmid content or positive selection. To test this, we

293   obtained the whole genome sequences of one randomly chosen evolved clone per Hg^R

294   genotype present at greater than 10% frequency per population at the end of the serial

295   transfer experiment. There was no evidence for differential compensatory evolution

296   according to treatment: all sequenced plasmid-bearing evolved clones carried a mutation in

297   a known compensatory locus. Specifically, we observed mutations in *gacA, gacS* or

298   *PFLU4242*, or in regions immediately upstream of compensatory loci (Figure 4; data for all

299   sequenced clones provided in Figure S3-S9). By contrast, mutations at these loci were

300   never observed in evolved clones from the plasmid-free control populations. To confirm that

301   loss of either *gacS* or *PFLU4242* was sufficient to ameliorate the cost of plasmid co-

302   infection, we compared the effect of compensatory mutations on the fitness of plasmid

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303 bearers carrying either one or both plasmids relative to the plasmid-free ancestor using

304 competition experiments. Consistent with a role for these genes in compensatory evolution,

305 deletion of either *gacS* or *PFLU4242* reduced the fitness cost of carrying plasmids (Figure

306 5). However, while deletion of either gene completely ameliorated the cost of carrying either

307 both plasmids or pQBR103 alone, only deletion of *PFLU4242* completely ameliorated the

308 cost of carrying pQBR57 alone, whereas deletion of *gacS* offered only partial amelioration

309 (ANOVA; strain by plasmid interaction, $F_{4, 39}$ = 2.628, P = 0.049). Thus, compensatory

310 evolution occurred in all treatments and was effective at ameliorating the additional cost of

311 carrying multiple plasmids. While this may explain the stability of plasmid coinfection without

312 positive selection, it cannot explain the decline in coinfection due to loss of pQBR57 with

313 positive selection, which instead appears to have been driven by the differential benefits of

314 the plasmids in the presence of Hg(II) chloride.

315

316 **Discussion**

317 Plasmid coinfection is more commonly observed in bacterial genomes than is expected by

318 chance[4] which is surprising given the high fitness costs usually associated with plasmid

319 acquisition[1, 2, 11, 13]. Here, we show that coinfection by plasmids encoding the same

320 functional trait, namely mercury resistance, was inhibited by positive selection for this trait.

321 With positive selection, coinfection declined due to loss of pQBR57 from originally coinfected

322 cells, whereas without positive selection, plasmid coinfection was stably maintained. This

323 difference could not be explained by the fitness costs of plasmid carriage, firstly because

324 coinfection was maintained in environments where the net cost of plasmid carriage was

325 highest (i.e. without Hg (II) chloride), and secondly because compensatory mutations to

326 ameliorate these costs occurred across all treatments. Instead, the loss of pQBR57 from

327 originally coinfected cells under positive selection could be explained by the differential

328 fitness benefits provided by the two plasmids in the presence of Hg (II) chloride. Addition of

329 Hg (II) chloride reduced the fitness of coinfected cells when competing against pQBR103-

330 carriers but not when competing against pQBR57-carriers. Consistent with previous data

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331   from singly-infected cells[23], we show that this occurs because although the pQBR103

332   backbone is costlier, it provides a greater benefit in the presence of Hg (II) chloride. Thus,

333   where multiple plasmids encode the same functional trait, under positive selection this

334   redundancy can drive loss of the less beneficial plasmid.

335

336   Loss of a redundant plasmid replicon by coinfected cells under positive selection is

337   conceptually similar to the replacement of single-infected plasmid bearers by the

338   chromosomal Tn5042, which was also only observed under positive selection. In both cases,

339   redundancy allows for loss of one of the replicons encoding resistance. It may be the case

340   that if our experiment was run for even longer, the initially coinfected populations would also

341   eventually consist only of cells with a chromosomal Tn5042. Indeed, our relative fitness data

342   show that SBW25(Tn5042) outcompeted both single- and double-infected plasmid bearers

343   in mercury-containing environments. This outcome may have been delayed (or prevented)

344   because the loss of both plasmids by segregation is likely to occur with low probability

345   and/or because the fitness difference between chromosomal and plasmid-encoded mercury

346   resistance was reduced by compensatory evolution. The key difference between

347   environments with versus without positive selection, is that, in the former, both the benefit

348   and the cost of the plasmids contribute to their fitness effect, whereas in the latter only their

349   costs do. This appears to increase the fitness difference between coinfected cells and those

350   carrying only the more beneficial plasmid. Selection for plasmid benefits may therefore be

351   more efficient than negative selection against plasmid costs, which becomes less efficient

352   over time due to compensatory evolution to ameliorate the plasmid fitness cost.

353

354   Previous studies suggest that the positive epistasis of plasmid fitness costs enables plasmid

355   coinfection[4]. Our data provide contradictory evidence both for and against this idea. When

356   competing against a plasmid-free genotype [SBW25(Tn5042)], SBW25 coinfected with both

357   plasmids showed a lower fitness cost than expected from the individual fitness costs of each

358   plasmid alone. This is consistent with positive epistasis. However, when coinfected SBW25

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359   was directly competed against SBW25 singly-infected with either of the plasmids, we

360   measured an appreciable additional cost of plasmid coinfection. This is not consistent with

361   positive epistasis. It seems likely that direct competition of coinfected versus singly-infected

362   cells offers the most accurate method to measure the fitness effect of carrying a second

363   plasmid replicon. Whereas, competing singly-infected and coinfected cells separately

364   against a plasmid-free competitor seems likely to underestimate the actual cost of carrying a

365   second plasmid. Importantly, the study by San Millan and colleagues (2014) used a set-up

366   similar to our first set of experiments, namely separate competition against a common

367   plasmid-free strain[4], a method that our data suggest is likely to underestimate the actual

368   fitness cost of coinfection. Our data when taken together do not, therefore, support a role for

369   positive epistasis in explaining plasmid coinfection and urge caution when interpreting

370   epistasis from relative fitness data obtained against plasmid-free competitors.

371

372   At present, it is unclear why the two plasmids differ in the benefit they provide to SBW25

373   under mercury positive selection. Both plasmids possess one copy of the Tn5042

374   transposon encoding the Mer operon, which provides mercury resistance. These Tn5042

375   sequences are identical except for a single base pair difference in *merR*, a repressor

376   controlling expression of the Mer operon[23]. Low concentrations of Hg(II) are bound by

377   MerR, causing a conformational change of the protein which relieves repression, allowing

378   expression of the Mer operon, which imports Hg(II) into the cell and reduces it to Hg[40, 41].

379   It is possible that the single base pair difference in the *merR* sequence between the

380   pQBR103 and pQBR57 copies of the Tn5042 could change the sensitivity of the MerR

381   repressor leading to altered expression of the Mer operon. For example, by detoxifying the

382   environment through reduction of Hg(II) to Hg, mercury resistance benefits all neighbouring

383   cells [42], thus a reduced affinity for Hg(II) could accrue fitness benefits when in competition

384   with strains encoding MerR with a higher affinity and thus earlier expression of the Mer

385   operon. Alternatively, the plasmids differ extensively in their gene content, and it is possible

386   that epistatic interactions between these variable genes and the Mer operon could underlie

14

387   the observed differential fitness benefits. Resolving this is beyond the scope of this paper

388   but identifying the causes of differences in plasmid benefit will be a focus of future work.

389

390   We observed mutations in three loci previously associated with compensatory evolution for

391   pQBR plasmids. Specifically, mutations to either of the genes encoding the GacAS two-

392   component global regulatory system[17, 18], or the gene *PFLU4242* of unknown

393   function[17]*,* were sufficient to ameliorate the fitness costs associated with coinfection by

394   both pQBR103 and pQBR57. Similarly, deletion of either locus could completely ameliorate

395   the cost of pQBR103, whereas amelioration of pQBR57 was more complete via deletion of

396   *PFLU4242* than *gacS*. Together this suggests that these plasmids, despite being genetically

397   divergent, cause their associated fitness costs through a similar (or a shared) mechanism.

398   Shared targets of compensatory evolution among divergent plasmids have been reported in

399   other plasmid-host systems[15, 19], for example compensatory mutations in chromosomal

400   helicases[19]. Consistent with previous work on other host-plasmid systems[15, 19], our

401   findings suggest that compensatory evolution could indeed promote plasmid coinfection by

402   reducing the cost of multiple plasmids simultaneously. This has the effect of reducing the

403   efficiency of negative selection against plasmid costs over time. It is notable, however, that

404   although compensatory evolution ameliorating the fitness costs of plasmid coinfection

405   occurred in both the mercury-containing and mercury-free environments, plasmid coinfection

406   was only stable in the mercury-free environment (at least for the duration of this study). This

407   occurs because positive selection remains efficient even after compensatory evolution

408   occurs, allowing discrimination of differential fitness benefits among functionally redundant

409   plasmid replicons.

410

411   These data reveal that coinfecting plasmids are unlikely to be maintained under positive

412   selection if they encode redundant functions and there are differences in the fitness benefits

413   these accessory genes provide. Moreover, while compensatory evolution can promote

414   plasmid coinfection in the absence of positive selection, it does not do so in the presence of

15

415 positive selection where not only the cost but also the benefit of the plasmid is subject to

416 selection. These findings suggest therefore that widespread plasmid coinfection is likely to

417 be explained either by positive selection for accessory gene functions being rare in nature,

418 or by coinfecting plasmids encoding non-redundant accessory gene functions. These

419 findings have implications for understanding the forces structuring bacterial genomes[1, 5-7],

420 and suggest a process whereby recurrent phases of genome expansion and contraction are

421 driven by variable positive selection: Multiple redundant replicons can be acquired, and their

422 costs ameliorated by compensatory evolution thus allowing genome expansion between

423 bouts of periodic positive selection, which, when they subsequently occur, then select

424 reduced genomes containing only the highest benefit replicon(s).

425

426

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434

435 **Competing Interests Statement**

436 The authors declare no competing interests.

437

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546 **Figure Legends**

547 **Figure 1 | Population and mercury resistance genotype dynamics over time.** Plots are

548 facetted by treatment, horizontally by mercury treatment (0 µM or 40 µM Hg (II) chloride) and

549 vertically by plasmid treatment (pQBR103-alone or pQBR57-alone or both

550 pQBR103+pQBR57). Solid black lines show the mean (n = 6) ± standard error (grey shaded

551 area) bacterial population density estimated from colony counts. Coloured dotted lines show

552 the mean ± standard error (correspondingly coloured shaded area) population density of

553 pQBR103-only carriers (red), pQBR57-only carriers (blue), coinfected pQBR103+pQBR57-

554 carriers (purple), and chromosomal Tn5042 carrying plasmid-free cells (gold) inferred from

555 their genotype frequencies. Plots for the population and genotype dynamics of individual

556 replicates are shown in Figure S2. Raw data is provided in DataS1.

557 **Figure 2 | The fitness of plasmid carriers relative to SBW25(Tn5042) competed with or**

558 **without Hg (II) chloride.** Black symbols show mean ± standard error relative fitness of

559 SBW25(pQBR103) (circles), SBW25(pQBR57) (triangles) or SBW25(pQBR103+pQBR57)

560 (squares) against SBW25(Tn5042). Grey symbols in corresponding shapes show the

561 individual replicate values (n = 5 or 6). Points are jittered to prevent over-plotting. All

562 competition assays were six-fold replicated except for SBW25(pQBR103) versus

563 SBW25(Tn5042) in 0 µM Hg (II) chloride and SBW25(pQBR57) versus SBW25(Tn5042) in 0

564 µM Hg (II) chloride, where one replicate each was lost due to contamination. Raw data is

565 provided in DataS1.

566 **Figure 3 | The fitness of coinfected plasmid carriers against singly-infected plasmid**

567 **carriers competed with or without Hg (II) chloride.** Black symbols show mean ± standard

568 error normalised relative fitness of SBW25(pQBR103+pQBR57) against SBW25(pQBR103)

569 (circles) or SBW25(pQBR57) (triangles). Grey symbols in corresponding shapes show the

570 individual replicate values (n = 10 or 12). Points are jittered to prevent over-plotting.

571 Competition assays against SBW25(pQBR103) were twelve-fold replicated, whereas

21

572   competition assays against SBW25(pQBR57) were ten-fold replicated due to the loss of

573   replicates to contamination. Raw data is provided in DataS1.

574   **Figure 4 | Parallel evolution plot summarising the mutations arising in evolved clones**

575   **per treatment.** Rings represent the SBW25 chromosome plotted separately for each

576   treatment as indicated by the colour of the ring (see legend). Dots indicate genetic loci

577   where mutations (excluding hypermutator clones) were observed in that treatment; the size

578   of the dot corresponds to the number of replicate populations where a mutation at that locus

579   occurred per treatment. Loci previously associated with compensatory mutations are

580   highlighted in bold. In populations where more than one plasmid-bearing clone was

581   sequenced, only mutations present in the dominant genotype are shown here. Hyper-

582   mutator clones are not shown to avoid over-plotting. Plots for all sequenced clones are

583   provided in Figure S3. The full table of called sequence variants is provided in DataS2.

584   **Figure 5 | Compensatory mutations ameliorate the fitness costs of plasmid carriage.**

585   Plots are facetted horizontally by the plasmid status of the plasmid-carrying competitor.

586   Open symbols show the mean (n = 6) ± standard error of fitness for plasmid carrying

587   genotypes relative to plasmid-free SBW25. Coloured filled dots denote individual replicate

588   values for SBW25 (maroon), SBW25-ΔgacS (green) or SBW25-ΔPFLU4242 (orange)

589   genotypes. Outliers (grey) were excluded from the statistical analysis. Raw data is provided

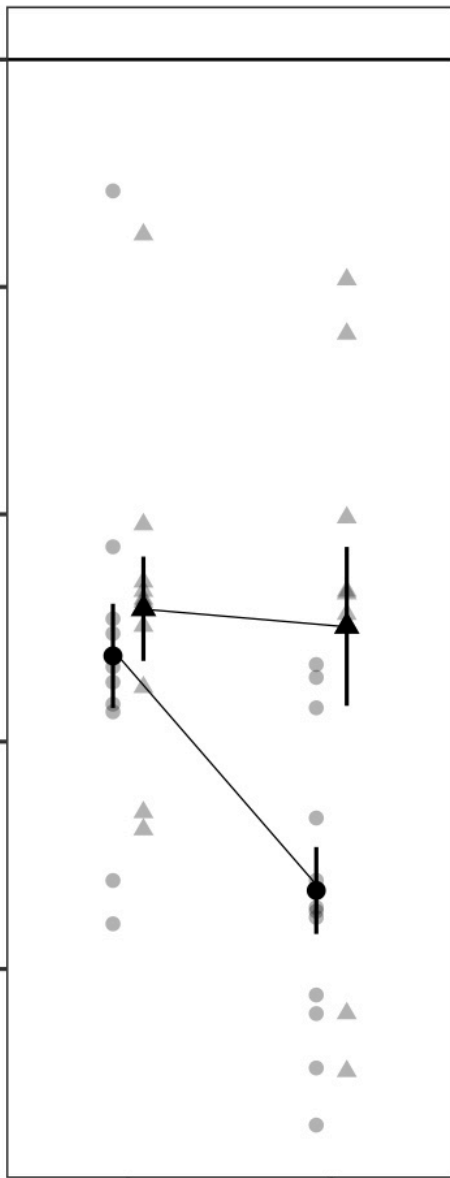590   in DataS1.

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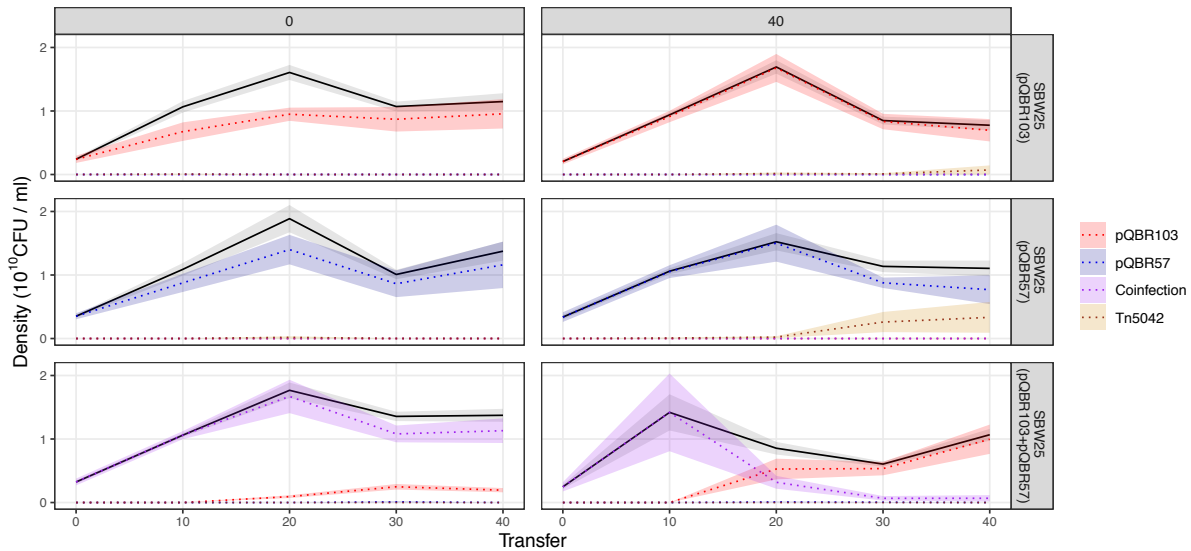
Relative fitness of plasmid carriers
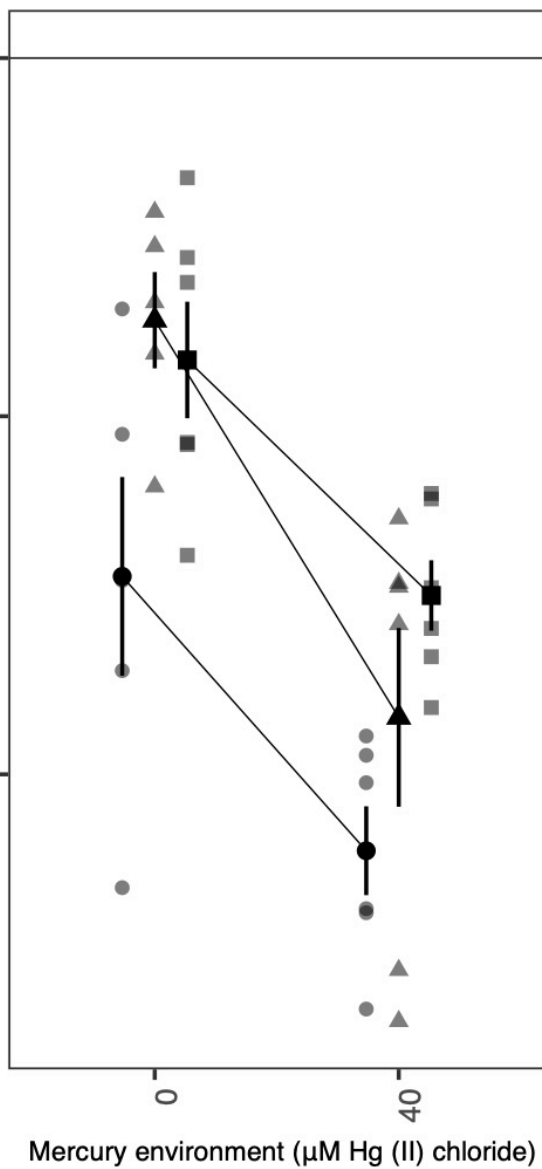
Mercury environment (μM Hg (II) chloride)

Mercury environment (μM Hg (II) chloride)

Legend:
- No plasmid
- SBW25(pQBR103) no Hg
- SBW25(pQBR103) with Hg
- SBW25(pQBR57) no Hg
- SBW25(pQBR57) with Hg
- SBW25(pQBR103&pQBR57) no Hg
- SBW25(pQBR103&pQBR57) with Hg

Labels around circle:
- PFLU_5899–rho
- PFLU_0116–PFLU_0117
- PFLU_0174, PFLU_0185
- wss B,C,E
- PFLU_0795/nadC
- PFLU_0956–PFLU_0957
- PFLU_0957
- glpD–glpR
- wsp F,A,R
- ppc
- fadB
- PFLU_1654
- PFLU_1701
- PFLU_4748
- PFLU_4498
- [PFLU_4508]–[PFLU_4511]
- PFLU_4443, PFLU_4453, PFLU_4498
- PFLU_4414, PFLU_4428
- PFLU_4242
- PFLU_4198
- PFLU_3808
- gacS
- PFLU_3502
- PFLU_3478
- PFLU_3216
- PFLU_2938, PFLU_3000
- PFLU_2639, PFLU_2704
- PFLU_2614–PFLU_2615
- PFLU_2288
- gacA

Scale markers: 0, 1Mb, 2Mb, 3Mb, 4Mb, 5Mb, 6Mb