This is a repository copy of *Adaptive modulation of antibiotic resistance through intragenomic coevolution*.

1 **Adaptive modulation of antibiotic resistance through intragenomic**

2 **coevolution**

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11

12   **Bacteria gain antibiotic resistance genes by horizontal acquisition of**

13   **mobile genetic elements (MGE) from other lineages. Newly acquired**

14   **MGEs are often poorly adapted causing intragenomic conflicts, resolved**

15   **by compensatory adaptation of the chromosome, the MGE or reciprocal**

16   **coadaptation. The footprints of such intragenomic coevolution are**

17   **present in bacterial genomes, suggesting an important role promoting**

18   **genomic integration of horizontally acquired genes, but direct**

19   **experimental evidence of the process is limited. Here we show adaptive**

20   **modulation of tetracycline resistance via intragenomic coevolution**

21   **between *Escherichia coli* and the multi-drug resistant (MDR) plasmid**

22   **RK2. Tetracycline treatments, including monotherapy or combination**

23   **therapies with ampicillin, favoured *de novo* chromosomal resistance**

24   **mutations coupled with mutations on RK2 impairing the plasmid-**

25   **encoded tetracycline efflux-pump. These mutations together provided**

26   **increased tetracycline resistance at reduced cost. Additionally, the**

27   **chromosomal resistance mutations conferred cross-resistance to**

28   **chloramphenicol. Reciprocal coadaptation was not observed under**

29   **ampicillin-only or no antibiotic selection. Intragenomic coevolution can**

30   **create genomes comprised of multiple replicons that together provide**

31   **high-level, low-cost resistance, but the resulting co-dependence may**

32   **limit the spread of coadapted MGEs to other lineages.**

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34

35 Horizontal gene transfer (HGT) is a fundamental process in bacterial evolution

36 that accelerates adaptation by sharing ecologically important accessory traits

37 between lineages[1]. These accessory traits are themselves frequently located

38 on semi-autonomous mobile genetic elements (MGE), such as conjugative

39 plasmids, that encode genes for their own replication, partition and horizontal

40 transfer[2,3]. Conjugative multidrug resistance (MDR) plasmids, encoding

41 antibiotic resistance genes (ARG) against multiple classes of antibiotics, are

42 of particular clinical concern since they allow instantaneous acquisition of

43 MDR phenotypes and thus potentiate the rapid emergence of MDR bacterial

44 pathogens[4,5]. Newly acquired conjugative plasmids are often costly since the

45 plasmid is unlikely to be well adapted to the new genetic background, causing

46 intragenomic conflict[6]. The cost of plasmid carriage is likely to arise due to the

47 metabolic burden of maintaining, transcribing and translating plasmid genes[7],

48 as well as via disruption of cellular homeostasis caused by gene regulatory

49 interference between chromosomal and plasmid regulators[8,9], and cytotoxic

50 effects of plasmid gene products[6].

51

52 An important route to resolving this intragenomic conflict is compensatory

53 evolution to ameliorate the cost of plasmid acquisition[10]. Experimental

54 evolution suggests that compensatory evolution can arise via mutations

55 located on either the chromosome or the plasmid, or via intragenomic

56 coevolution involving both plasmid and chromosome mutations[8,9,11,12].

57 Comparative genomics suggests a key role for compensatory evolution in

58 natural bacterial populations, potentially stabilising MDR plasmids within

59 lineages and thus allowing the evolutionary emergence by HGT of MDR

60   pathogens[13]. A key outstanding question is how the mode of compensatory

61   evolution following MDR plasmid acquisition varies with antibiotic treatment.

62   Here we experimentally evolved *Escherichia coli* MG1655 carrying the MDR

63   plasmid RK2 (encoding tetracycline and ampicillin resistance genes) under a

64   range of antibiotic treatment regimes including no antibiotic, mono- and

65   combination-therapies of tetracycline and ampicillin. Following 530

66   generations of selection we quantified evolved changes in antibiotic

67   resistance and fitness, and used genome sequencing to determine the

68   genetic bases of the observed adaptation.

69

70   **Results**

71   Thirty independent isogenic populations of *E. coli* MG1655 carrying the MDR

72   plasmid RK2[14], which encodes resistances to tetracycline (TET) and ampicillin

73   (AMP), were experimentally evolved for ~530 generations (80 days), under

74   five antibiotic treatments (six independently evolving lines per treatment): no

75   antibiotic (N), AMP (A), TET (T), AMP plus TET (AT), and 24 hour cycling

76   between AMP and TET (A/T) (see methods). Plasmids remained at high

77   frequency in all populations for the duration of the selection experiment.

78   Plasmid-free segregants were only observed at very low frequency in two of

79   the six populations from treatment N (Supplementary Fig. 1), whereas

80   transposition of resistance genes from RK2 onto the host's chromosome was

81   never observed.

82

83   To test for changes in antibiotic resistance profiles following evolution we first

84   determined the minimum inhibitory concentration (MIC) of evolved lineages to

85   TET and AMP. The susceptibility of the evolved strains to antibiotics differed

86   between treatments (Fig. 1a). We observed a four-fold increase in TET MIC in

87   evolved strains from the T and AT treatments and a small increase in lineages

88   that had evolved under the cycling A/T treatment compared with the ancestral

89   MG1655 with ancestral RK2 (Anc-RK2), whereas evolved strains from

90   treatments N and A showed no change in tetracycline MIC (ANOVA, $F_{5,30}$ =

91   6.103, p < 0.001; Post-hoc Tukey Tests, Anc-RK2:T p < 0.001, Anc-RK2:AT p

92   < 0.01, Anc-RK2:N p = 0.525, Anc-RK2:A p = 0.783). By contrast, we

93   observed no change in resistance to AMP in any treatment (ANOVA, $F_{5,30}$ =

94   1.212, p = 0.327), possibly due to a lower relative selection pressure imposed

95   by the concentration of AMP used in the experiment compared to the

96   concentration of TET[15]. Interestingly, TET selection led to the evolution of

97   bacteria that were cross-resistant to chloramphenicol (CML), although the

98   extent of the evolved cross-resistance varied between treatments (ANOVA,

99   $F_{5,30}$ = 24.25, p < 0.001); with CML MIC increasing 8-, 4-, and 2-fold in T, AT,

100   A/T treatments, respectively. Consistent with CML cross-resistance being a

101   correlated response to TET selection, evolved strains from both the N and A

102   treatments remained equally sensitive to CML as the ancestral MG1655(RK2)

103   (Post-hoc Tukey Tests, Anc-RK2:N p = 0.975, Anc-RK2:A p = 0.993). Thus

104   whereas T and AT treatments, and to a lesser extent the cycling A/T

105   treatment, led to the evolution increased TET resistance and cross-resistance

106   to CML, evolved lineages from the N and A treatments showed no change in

107   their resistance profile.

108

109   To examine the genetic bases of evolved changes in resistance we next

110   obtained whole genome sequences for one randomly selected clone per

111   population. Excluding hypermutators, evolved clones had acquired between 2

112   and 11 mutations, located exclusively on the chromosome in non-TET

113   treatments (C, N, A), and on both the chromosome and plasmid in the

114   treatments including TET (T, AT, A/T) (Supplementary Fig. 2, Supplementary

115   Table 1). Of all the observed mutations 13.2% were synonymous and 19%

116   were intergenic, the remaining non-synonymous mutations (67.8%) comprised

117   missense mutations (42.8%), frameshifts (10.6%), insertion sequences (5.6%)

118   and gene deletions (5.4%), and these were analysed further. While the

119   variance in the number of non-synonymous mutations did not differ between

120   treatments (Analysis of multivariate homogeneity of group variances excluding

121   hypermutators $F_{5,26}$ = 1.8617, p = 0.1358), the loci affected by non-

122   synonymous mutations did vary between treatments (Permutational ANOVA,

123   permutation test: $F_{5,26}$ = 2.5231, p < 0.01, Bonferroni corrected). Clones that

124   had evolved under TET selection (T, AT, A/T) had significantly different sets

125   of non-synonymous mutations compared to evolved clones from the other

126   treatments (C, N, A) (permutation test: $F_{1,30}$ = 6.9463, p < 0.01, Bonferroni

127   corrected), with a larger genetic distance between TET and non-TET

128   treatments than within these treatment groups (Fig. 2a). Thus TET-selected

129   lineages followed an evolutionary trajectory distinct from non-TET-selected

130   lineages, leading to mutations on both the chromosome and the plasmid,

131   which suggest that TET selection favoured bacteria-plasmid coadaptation.

132

133   Strikingly parallel mutations were observed between independent replicate

134   populations both within and between TET-containing treatments (Fig. 2b).

135   Highly parallel mutations are likely to represent adaptive evolution at these

136   loci, and because mutations at these loci were not observed in the

137   populations from the N and A treatments, these mutations were likely to be

138   TET-specific adaptations. Mutations in the chromosomal genes *ompF* (16 out

139   of 18 clones), and *ychH* (16 out of 18 clones) showed strong locus-level

140   parallelism within all three TET-containing treatments. Mutations in *ompF,*

141   encoding a major non-specific diffusion porin[16], were all predicted loss-of-

142   function mutations, including the insertion of IS elements, frameshifts or

143   premature stop codons. The loss of OmpF in *E. coli* reduces membrane

144   permeability, including to antibiotics, and consequently is known to increase

145   resistance to a wide spectrum of antibiotics[17] including TET and CML[18,19].

146   Deletion of *ompF* (*E. coli* K-12 Δ*ompF* JW0912[20]) significantly increased

147   resistance to TET without the RK2 ($t_{9.09}$ = 4.2836, $p < 0.01$), and further

148   increased TET resistance when carrying RK2 (Two-way ANOVA Interaction

149    $F_{1,20}$ = 14.724, $p < 0.01$; Supplementary Fig. 4a). Parallel loss of function

150   mutations (IS elements and frameshifts) in *ychH* were observed across all the

151   TET treatments. YchH is a hypothetical stress-induced inner membrane

152   protein[21,22], but deletion of *ychH* (*E. coli* K-12 Δ*ychH* JW1196[20]) did not

153   significantly increase the resistance to TET with or without the plasmid

154   (Supplementary Fig. 4b), suggesting that this general stress response may

155   not be required under TET selection and is consequently selected against.

156

157   Mutations in several loci observed in the T and AT treatments were not

158   present in the cycling A/T treatment. These included mutations in both *acrR*

159   (10 out of 12 clones) and *adhE* (9 out of 12 clones). Mutations in *adhE* were

160   extensively parallel at the nucleotide level, with 8 clones from independent

161   populations all having the same missense SNP in the ethanol dehydrogenase

162   domain[23]. The phenotypic significance of these mutations is unclear due to

163   the multiple roles assigned to this protein, including multiple metabolic

164   pathways[24], but intriguingly the AdhE protein is known to exhibit binding

165   activity to the 30S ribosome[25], the primary TET target. The *acrR* gene

166   encodes a repressor of AcrAB multidrug efflux pump[26], the majority of

167   mutations in *acrR* are predicted loss of function mutations, with IS elements

168   and frameshifts observed in evolved strains. The deletion of *acrR* results in

169   the overexpression of *acrAB* leading to MDR phenotypes[27,28]. Deletion of *acrR*

170   (*E. coli* K-12 Δ*acrR* JW0453[20]) alone did not significantly increase resistance

171   to TET ($t_{9.32}$ = -0.591, p = 0.339), but when combined with the RK2 plasmid

172   did allow significantly increased growth in TET ($t_{6.4}$ = 3.665, p < 0.01,

173   Supplementary Fig. 4c). These findings are consistent with the higher TET

174   resistance of evolved clones from the T and AT treatments versus the A/T

175   treatment (Fig. 1a) and reflect overall weaker TET selection under the A/T

176   cycling compared to the T and AT treatments where TET selection was

177   constant. Interestingly, stronger TET selection appeared to constrain evolution

178   at chromosomal loci not involved in resistance. For example, we observed

179   highly parallel loss of function mutation in the flagellum operon in the A, N and

180   A/T treatments, but only rarely observed mutations at these loci in T and AT

181   treatments. Loss of the flagellar motility is a commonly observed adaptation of

8

182   *E. coli* to growth in liquid media[29] and this may have been impeded by clonal

183   interference or negative epistasis with chromosomal resistance mutations in

184   populations under strong TET selection. Consistent with this, whereas

185   evolved clones from the N and A treatments increased in fitness relative to the

186   plasmid-free ancestor in antibiotic-free media, such fitness gains were not

187   observed in evolved clones from the TET-containing treatments

188   (Supplementary Fig. 5).

189

190   To confirm that TET selection had led to the evolution of chromosomal

191   resistance we next cured evolved strains of their plasmids and quantified

192   resistance[30]. Evolved strains carrying putative chromosomal resistance

193   mutations displayed increased TET (ANOVA, $F_{5,30}$ = 42.63, p < 0.001), AMP

194   (ANOVA, $F_{5,30}$ = 12.55, p < 0.001) and CML (ANOVA, $F_{5,30}$ = 35.88, p <

195   0.001) resistance (Fig. 1b). Across all tested antibiotics, evolved clones

196   carrying both *ompF* and *acrR* mutations had significantly increased resistance

197   compared to the ancestral MG1655 (Post-hoc Tukey Tests, all p < 0.05),

198   whereas cured evolved strains without either of these mutations (i.e. from the

199   N and A treatments) did not (Post-hoc Tukey Tests, all p > 0.05). Interestingly,

200   cured evolved clones from the cycling A/T treatment that carried only

201   mutations in *ompF* but not in *acrR* showed marginally increased resistance to

202   both TET and CML, but no detectable increase in AMP resistance, relative to

203   MG1655. Thus TET selection favoured the *de novo* evolution of chromosomal

204   resistance despite pre-existing plasmid-encoded TET resistance, and these

205   chromosomal resistance mutations are responsible for the observed cross-

206   resistance to CML.

207

208   We observed parallel mutations on the plasmid exclusively in evolved clones

209   from the TET-containing treatments (T, AT, and A/T). These mutations

210   occurred in *tetA/tetR* (18 out of 18 clones; *tetA*: 13, *tetR*: 2, both: 3, Fig. 2b)

211   which encode the tetracycline-specific efflux pump. The expression *tetA* is

212   tightly regulated by the repressor *tetR* in the absence of tetracycline[31,32].

213   Mutations in *tetA* were dispersed throughout the gene, affecting the protein's

214   transmembrane, periplasmic and cytoplasmic domains[33]. Three of the five

215   mutations observed in *tetR* are in direct contact with or in close proximity to

216   the tetracycline binding pocket[34], while the other two mutations are located in

217   the central scaffolding of the protein, suggesting that they are likely to

218   interfere with activity of the *tetR* repressor. Evolved plasmids carrying

219   mutations in *tetA* or *tetR* displayed reduced resistance to TET in the ancestral

220   MG1655 background compared to ancestral RK2 (Fig. 1d, ANOVA, $F_{5,30}$ =

221   4.586, $p < 0.01$). Consistent with reduced efficacy of plasmid-encoded

222   resistance in evolved lineages with *tetA/tetR* mutations, when we replaced the

223   evolved plasmid with ancestral RK2, this led to increased TET resistance

224   (ANOVA, $F_{5,30}$ = 71.86, $p < 0.001$, Anc-RK2:T,AT,A/T all $p < 0.05$).

225

226   Our data suggest that evolved strains from TET-containing treatments

227   adapted their resistance to TET by acquisition of weak chromosomal

228   resistance mutations in combination with mutations that reduced the efficacy

229   of the plasmid-encoded TET efflux pump. To understand the evolutionary

230   benefits of this counterintuitive dual resistance strategy we first compared the

231   effect of chromosomal background (evolved or ancestral) and plasmid

232   genotype (evolved or ancestral) on growth in the presence of 10 µg/ml TET

233   (i.e., the concentration used in our selection experiment). The evolved

234   chromosomal background carrying resistance mutations displayed a

235   significantly shortened lag phase compared to the ancestral chromosomal

236   background, irrespective of the plasmid genotype (Supplementary Fig. 6;

237   ANOVA, $F_{3,56}$ = 76.92, p < 0.001; Post-hoc Tukey Tests, Evolved

238   Host:Ancestral Host all p < 0.001). This suggests that chromosomal

239   resistances reducing membrane permeability to antibiotics allowed evolved

240   strains to start growing faster in the presence of TET.

241

242   Whereas evolved bacteria grew equally well with evolved or ancestral

243   plasmids, ancestral bacteria displayed impaired growth with evolved

244   compared to ancestral plasmids (Supplementary Fig. 6; Max OD, W = 93, p <

245   0.01). This is consistent with the mutations in *tetA/tetR* reducing resistance

246   but importantly confirms that this reduction is not evident when in combination

247   with the chromosomal resistance mutations, which appear to compensate for

248   the reduced efficacy of the plasmid-encoded efflux pump.

249

250   We next competed evolved bacteria with either the evolved or ancestral

251   plasmid against the ancestral MG1665(RK2) to compare the costs of carrying

252   each plasmid genotype. The ancestral plasmid displayed a significantly higher

253   cost than the evolved plasmid in the evolved chromosomal background

254   (Supplementary Fig. 7, $t_{25.71}$ = -2.287, p < 0.05). This suggests that the

255   mutations to *tetR/tetA* ameliorate the cost of plasmid carriage but at the price

256   of reduced efficacy of TET efflux. This is consistent with previous studies

257 showing a high cost of expressing the specific tetracycline efflux pump[35].

258 Taken together with the growth data, this suggests that although mutations to

259 *tetA/tetR* reduce growth under tetracycline in the ancestral chromosomal

260 background, they have minimal effect on resistance in the evolved

261 chromosomal background due to the reduced membrane permeability and

262 additional efflux systems expressed in the evolved chromosomal background

263 carrying mutations in *ompF* and *acrR*, leading to high resistance and a

264 lowered cost of plasmid carriage. This suggests that the chromosomal

265 resistance mutations must have been gained prior to the mutations in the

266 plasmid-encoded tetracycline efflux pump. To test this, for one population

267 (AT2) we tracked the frequency over time of an observed IS-insertion in *ompF*

268 by PCR and then determined by sequencing when these genotypes acquired

269 mutations in the *tetA/tetR* genes. Consistent with the hypothesised order of

270 mutations, the IS-insertion in *ompF* was first detected at transfer 8 and had

271 swept to fixation by transfer 32, whereas mutations in *tetA/tetR* were not

272 observed in this *ompF*::IS background until transfer 32 (supplementary figure

273 8).

274

275 **Discussion**

276 Our current model of bacterial evolution suggests that horizontal acquisition of

277 ARGs accelerates resistance evolution by providing bacteria with ready-made

278 resistance mechanisms, bypassing the requirement for rare *de-novo*

279 mutations[1]. However, recent population genomic data suggesting that

280 lineages independently acquire and then subsequently coevolve with MDR

281 plasmids[13,36,37] imply a more dynamic evolutionary process. Consistent with

12

282 this, here we show here that gaining an ARG can be just the starting point in

283 the evolution of resistance and, due to the costs of expressing horizontally

284 acquired ARGs, does not preclude subsequent *de novo* evolution of

285 chromosomal resistance. Evolved strains from TET-containing treatments

286 gained chromosomal resistance mutations reducing membrane permeability

287 and enhancing efflux of TET and providing cross-resistance to other

288 antibiotics, shortening lag phase in the presence of TET. These mutations

289 also reduced the need for a fully operational plasmid-encoded tetracycline

290 efflux pump, expression of which is highly costly[35], allowing plasmid mutations

291 in the TET efflux pump and its regulator which reduced the cost of plasmid-

292 encoded resistance. A consequence of this intragenomic coevolution is that

293 the increased TET resistance of evolved strains from T, AT and A/T

294 treatments required the action of both the chromosomal- and plasmid-

295 encoded resistances, which together acted multiplicatively. Thus intragenomic

296 coevolution can lead to the evolution of bacterial genomes comprised of co-

297 dependent replicons, limiting the potential for onward transmission of the

298 plasmid due to the weaker resistance it now encodes in other lineages.

299

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435

436

437

438   **Methods**

439   **Strains, culture conditions and evolution experiment**

440   *E. coli* MG1655 chromosomally labelled with GFP at the *attB* lambda

441   attachment site was used in the evolution experiments. Isogenic *E. coli*

442   MG1655-mCherry was used as a reference strain in competition and

443   conjugation rate experiments. Both *E.* coli strains were provided by the Van

444   Der Woude lab (University of York). The RK2 plasmid was introduced to the

445   strains through conjugation from *E. coli* MV10 provided by the Thomas lab

446   (University of Birmingham). All cultures were grown in Oxiod® Nutrient Broth

447   (NB) at 37°C 5 ml in 50 ml microcosms shaken at 180 rpm. Independent

448   selection lines were founded by 30 independent single colonies of *E. coli*

449   MG1655-GFP harbouring RK2. These were grown overnight in non-selective

450   conditions and split into the 5 antibiotic treatments, no antibiotic selection, 100

451   µg/ml ampicillin, 10 µg/ml tetracycline, 100 µg/ml ampicillin plus 10 µg/ml

452   tetracycline, and 24 hour cycling between 100 µg/ml ampicillin and 10 µg/ml

453   tetracycline, with 6 replicate populations per treatment. In parallel, 6

454   independent *E. coli* MG1655-GFP colonies were picked for control treatments

455   and grown under no selection. Selection lines were established by transferring

456   50 µl of saturated overnight culture into 5ml of selective media. These

457   populations were maintained through transfer of 1% of the population into

458   fresh media and antibiotics every 24 hours for 80 transfers, resulting in ~6.64

459   generations per day, totalling ~530 bacterial generations. For the cycling

460   treatment 3 populations were initiated with 100µg/ml ampicillin and 3

461   populations were initiated with 10µg/ml tetracycline. Culture density (OD_{600})

462   was recorded every 24 hours. Plasmid prevalence was measure at the start

463   and end of the selection experiment by screening 20 randomly picked

464   colonies from each population using multiplex primers specific to RK2

465   replication origin (Fw: ctcatctgtcaacgccgc, Rv: aaccggctatgtcgtgct), β-

466   lactamase (Fw: ataactacgatacgggagggc, Rv: acatttccgtgtcgccctta), and

467   tetracycline efflux pump (Fw: tgggttctctatatcgggcg,Rv: tgggcgagtgaatgcagaat).

468   These primers allowed for the detection of plasmid loss and transposition of

469   resistances onto the chromosome. One end point clone was randomly

470   selected from each population for phenotypic typing, curing, calculation of

471   MICs and sequencing. Every eight transfers throughout the experiment 500 µl

472   samples of whole populations were collected and stored in 25% glycerol at -

473   80°C. Whole populations were also plated out on non-selective media, 20

474   individual clones were then randomly selected, sub-cultured for a further 24

475   hours in non-selective media, and stored in 25% glycerol in 96 well plates.

476

477   **Relative Fitness**

478   The relative fitness of the evolved plasmid bearing versus ancestral plasmid

479   free strain was estimated by direct competition, with six replicate strains per-

480   treatment. The competitions were initiated with 50 µl of 1:1 mixtures of

481   plasmid-bearing evolved strain and plasmid-bearing ancestral strain marked

482   with mCherry from overnight cultures in 5ml of non-selective NB media. The

483   relative fitness of the evolved strains was calculated by gaining exact viable

484   cell counts at 0 hours and 24 hours, strains were distinguished through

485   detection of fluorescent markers using Zeis Stereo Lumar v12 microscope.

486   The relative fitness of plasmid-bearing bacteria was calculated as a ratio of

487   Malthusian parameters[38]:

$$W_{evo} = \frac{\ln\left(\frac{N_{final,evo}}{N_{inital,evo}}\right)}{\ln\left(\frac{N_{final,anc}}{N_{inital,anc}}\right)}$$

488

489   Fitness effects due to different markers was determined by competing plasmid

490   free MG1655-GFP with plasmid free MG1655-mCherry, the relative fitness of

491   MG1655-GFP was not significantly difference from 1 (t_5=0.015584, p=0.9882)

492   showing that there is no significant difference between the two marker strains.

493

494   Relative fitness of evolved strains harbouring evolved plasmid or evolved

495   strains harbouring ancestral plasmid versus ancestral plasmid bearing cells

496   was estimated using the same method as above, with eighteen replicate

497   strains per competition, but grown in 100 µl cultures in a 96 well plate, 37°C

498   shaken at 600 rpm, 3 mm orbital radius, inoculated to an initial dilution of

499   1:500. Again no fitness effect of markers was observed (t_5=-0.2795, p=0.791).

500

Curing RK2 from evolved strains

501 **Curing RK2 from evolved strains**

502 Evolved strains were cured using the pCURE curing system[30]. The anti-incP-1

503 cassette (RK2 *oriV*, *parD*, *korA*, and *incC* genes) from pCURE11 was ligated

504 into the pLAZ2 chloramphenicol resistant vector that contains the *sacB* gene

505 allowing counter selection for plasmid free segregants. The resultant plasmid

506 was transformed into chemically competent evolved strains and selected for

507 using Cml 12.5 µg/ml. Single colony transformants were re-streaked on to

508 Cml 12.5 µg/ml plates and Cml 12.5 µg/ml + 5% sucrose. Sucrose sensitive

509 colonies were checked by PCR for the presence of the curing plasmid (Fw:

510 aagttttggtgactgcgctc, Rv: caaagacgatgtggtagccg) and absence of RK2 β-

511 lactamase and *tetA* (primers as above). Successfully cured clones were

512 cultured for 24 hours in non-selective media to allow segregation of the curing

513 plasmid; sergeants were selected on antibiotic free, 5% sucrose plates. To

514 confirm loss of both plasmids sucrose resistant colonies were check for

515 sensitivity to chloramphenicol, ampicillin, and tetracycline, as well as PCR

516 using primers mentioned above. Both the ancestral strain harbouring RK2 and

517 ancestral plasmid free strains under went the curing process and were used

518 as a comparison to cured evolved strains to control for curing process.

519 Ancestral RK2 was introduced into the cured evolved strains, and evolved

520 RK2 was introduced into the plasmid free ancestor though conjugation. Again,

521 to control for the curing and conjugation steps, ancestral RK2 was conjugated

522 into cured ancestral strains and used for comparison. Saturated overnight

523 cultures of donor plasmid containing strains and recipient plasmid free strains

524 were mixed 1:1, and 50 µl was used to inoculate 5ml NB. The mixed cultures

525 were grown for 24 hours and plated out on to 100 µg/ml ampicillin to select for

526   transconjugants. Transconjugants were confirmed by fluoresces and PCR

527   screening for RK2 plasmid.

528

529   **MIC**

530   To measure minimal inhibitory concentrations, six replicate cultures per-

531   treatment were grown overnight until stationary phase in 5 ml NB, the

532   saturated cultures were then sub-cultured 50 µl into 5 ml fresh NB and grown

533   to an OD_{600} of 0.5. These were then diluted into 96-well plates containing a

534   log_2 serial dilution of antibiotic (AMP, TET or CML) to an initial density of

535   5×10^5 CFU/ml. 100 µl cultures were grown for 24 hours 37°C shaken at 600

536   rpm, 3 mm orbital radius. OD_{600} was measured after 24 hours.

537

538   **Growth Curves**

539   Six replicate saturated overnight cultures per-treatment were sub-cultured to

540   an OD_{600} of 0.5, and used to inoculate 100 µl NB supplemented with 10 µg/ml

541   TET per well in 96-well plates at a final dilution of 1:1000. Plates were grown

542   at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, OD_{600} was

543   measured every 16 minutes by Tecan infinite M200 Pro plate reader. Growth

544   rates were calculated as the maximum slope of log_2 transformed OD_{600}

545   covering four time points (~1 hour of growth), lag phase was calculated to end

546   when growth rate reached 10% of the maximum achieved growth rate.

547

548   **Genome sequencing and analysis**

549   Whole genomes were extracted from each evolved population's clone as well

550   as the ancestral strain and ancestral strain harbouring the RK2 plasmid using

551 the DNeasy Blood and Tissue extraction kit (Qiagen). The total DNA was

552 sequenced by MicrobesNG (http://www.microbesng.uk), which is supported

553 by the BBSRC (grant number BB/L024209/1), using Illumnia MiSeq. Reads

554 were mapped to *E. coli* MG1655 K-12 genome (GenBank accession

555 U00096.3) and RK2 (GenBank accession BN000925.1) reference using BWA-

556 MEM[39]. Single nucleotide variants and small indel events were detected using

557 GATK UnifiedGenotyper[40] and SnpEff[41], insertion sequences were identified

558 using custom scripts and Integrative Genomics Viewer[42], and large genome-

559 wide structural variants were detected using BreakDancer[43]. Mutations that

560 were present in the ancestral clones were excluded, resulting in a set of

561 mutations that were acquired during the selection experiment.

562

563 **Tracking mutations**

564 Populations that did not show a hypermutator phenotype, had insertion

565 sequences within *ompF,* and mutations in the tetracycline resistance genes

566 on the plasmid, from the constant TET treatments (T and AT treatments) were

567 selected for further analysis to gain an understanding of the mutational

568 timeline during the selection experiment. Insertion sequences within *ompF*

569 were identified within whole populations of T4, AT2, AT3 and AT5 by PCR of

570 the *ompF* gene (Fw: ACTTCAGACCAGTAGCCCAC, Rv:

571 GCGCAATATTCTGGCAGTGA). A short product of 716 bp indicated no

572 insertion sequence, a long product of 1484 bp indicted IS1 and a long product

573 of 1911 bp indicted IS5. Whole population PCR indicated that *ompF*::IS

574 mutants had swept into the population by transfer 40 for populations T4, AT2

575 and AT5, and transfer 48 in population AT3. Frequency of *ompF* insertion

576   sequences were calculated by PCR of 20 clones from transfers 8, 16, 24, 32,

577   and 40. Tetracycline resistance genes (*tetA* and *tetR*) from clones containing

578   *ompF*::IS mutations from population AT2, transfers 8, 16, 24, and 32 were

579   then Sanger sequenced to determine if *ompF* mutations arise before *tetAR*

580   mutations (*tetA*: Fw: GGCTGCAACTTTGTCATG,Rv:

581   TTCCAACCGCACTCCTAG, Internal1: ACAGCGCCTTTCCTTTG, Interal2:

582   AAGGCAAGCAGGATGTAG; *tetR*: Fw: TCTGACGCGGTGGAAAG, Rv:

583   ACGCGCGGATTCTTTATC, Internal1: GAGCCTGTTCAACGGTG, Internal2:

584   TCTGACGACACGCAAAC).

585

586   **Statistical analysis**

587   To test if the mutations observed within each treatment had significantly

588   different variances a multivariate homogeneity of groups variances test was

589   conducted[44]. The binary presence or absence of a variant at each allele was

590   use to calculate a Euclidean distance matrix between each population. This

591   was used to test for homogeneity of variances between treatments using

592   *betadisper* {vegan 2.4-0}. The variances between treatments were

593   significantly different, with hypermutators significantly affecting within-group

594   variation. These clones were removed from further analysis as significant

595   differences in within-group variance can lead to falsely significant results

596   when testing for differences between groups[45]. Permutational Multivariate

597   Analysis of Variance was used to calculate whether different evolutionary

598   treatments resulted in different sets of mutations[45,46]. Using the Euclidean

599   distance matrix with hypermutators removed, the significance of within- and

600   between-group distances was calculated using *adonis2* {vegan 2.4-0}. The

601   data was partitioned into different groups, multiple testing was corrected for

602   using Bonferroni correction. Neighbour Joining phylogeny was constructed

603   using the binary presence or absence table with hypermutators removed.

604   Tree estimation and bootstrap support was conducted using *ape-package*

605   {ape 4.0}. Significant difference between two related samples was calculated

606   using two sided, two-sample t-test. Shapiro-Wilk test was conducted to check

607   for normality, when normality could not be assumed a non-parametric

608   Wilcoxon signed-rank test was used. Differences among treatments growth

609   under antibiotic selection were calculated by ANOVA of the integral of the

610   resistance profiles, with subsequent Tukey multiple comparison of means. All

611   statistical analysis was conducted in R (version 3.2.3).

612

613   **Data Availability**

614   The sequence data supporting the findings of this study are available at the

615   European Nucleotide Archive, accession: PRJEB20735. All other data in this

616   study is available at Figshare data depository

617   (https://doi.org/10.6084/m9.figshare.5092225.v1). Custom code used to map

618   possible IS elements are available online

619   (https://github.com/mbottery/Co_Evo_IS_Analysis).

620

621

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631

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633 the experiments and analysed the data. All authors contributed towards the

634 design of the study and wrote the manuscript.

635

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637

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640

641 **Figure 1 I Resistance profiles of evolved plasmids and hosts.** Growth of

642 **a**, evolved MG1655 strains with evolved RK2 plasmids **b**, evolved MG1655

643 strains cured of evolved RK2 plasmids **c**, evolved MG1655 strains with

644 ancestral RK2 plasmid and **d**, ancestral MG1655 clones with evolved RK2

645 plasmids in the presence of tetracycline, ampicillin or chloramphenicol in

646 comparison to ancestral MG1655. Points represent means of one clone from

647 each of the six independent treatment populations, with SEM error bars.

648 Dashed grey and black lines show the resistance profiles of plasmid free and

649 plasmid containing ancestral strains respectively. Dashed lines in evolved

650 host cured of plasmid plots (**c**) show ancestral MG1655 and ancestral

27

651   MG1655(RK2) after curing process. Dashed lines in ancestral host evolved

652   plasmid plots (**d**) show ancestral MG1655 and ancestral MG1655(RK2) which

653   had under gone curing with ancestral RK2 subsequently reintroduced.

654   Vertical dashed lines in AMP and TET resistance profiles show the

655   concentrations of AMP (100 µg/ml) or TET (10 µg/ml) used in the selection

656   experiment.

657

658   **Figure 2 I Mutations show treatment specific parallelism. a**, An unrooted

659   neighbour joining phylogeny of end-point evolved clones. The distance matrix

660   was constructed from the binary presence or absence of variants at each

661   gene relative to the ancestral strain; hypermutators were excluded from the

662   analysis. Scale bar represents number of gene variants; percentage bootstrap

663   support is shown at the branches, B=1000, values below 0.3 are omitted. Blue

664   branches represent clone isolated from TET treatments. **b**, Mutations

665   observed in evolved clones (excluding hypermutators) across treatment.

666   Rings represent *E. coli* chromosomes or RK2 plasmids. Dots represent

667   mutations, the size of the dots represent the number of mutations at the same

668   loci across independent replicate populations. Plots of individual treatments

669   are in Supplementary Fig. 3.

a Evolve Host Evolved Plasmid

b Evolve Host Cured of Plasmid

c Evolve Host Ancestral Plasmid

d Ancestral Host Evolved Plasmid

Ancestor
- MG1655
- MG1655 (RK2)

Evolved
- No Ab
- No Ab/No RK2
- Amp
- Amp + Tet
- Tet
- Cycling

Tetracycline (μg/ml)
Ampicillin (μg/ml)
Chloramphenicol (μg/ml)

OD$_{600}$

a

b