Adaptive modulation of antibiotic resistance through intragenomic coevolution

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Bacteria gain antibiotic resistance genes by horizontal acquisition of mobile genetic elements (MGEs) from other lineages. Newly acquired MGEs are often poorly adapted causing intragenomic conflicts; these are resolved by either compensatory adaptation—or the chromosome or the MGE—or reciprocal coadaptation. The footprints of such intragenomic coevolution are present in bacterial genomes, suggesting an important role promoting genomic integration of horizontally acquired genes, but direct experimental evidence of the process is limited. Here we show adaptive modulation of tetracycline resistance via intragenomic coevolution between *Escherichia coli* and the multidrug resistant plasmid RK2. Tetracycline treatments, including monotherapy or combination therapies with ampicillin, favoured de novo chromosomal resistance mutations coupled with mutations on RK2 impairing the plasmid-encoded tetracycline efflux pump. These mutations together provided increased tetracycline resistance at reduced cost. Additionally, the chromosomal resistance mutations conferred cross-resistance to chloramphenicol. Reciprocal coadaptation was not observed under ampicillin-only or no antibiotic selection. Intragenomic coevolution can create genomes comprising multiple replicons that together provide high-level, low-cost resistance, but the resulting co-dependence may limit the spread of coadapted MGEs to other lineages.

**Results**

Thirty independent isogenic populations of *E. coli* MG1655 carrying the MDR plasmid RK2\(^1\), which encodes resistances to TET and AMP, were experimentally evolved for ~530 generations (80 days), under five antibiotic treatments (six independently evolving lines per treatment): no antibiotic (N); AMP (A); TET (T); AMP plus TET (AT); and 24 h cycling between AMP and TET (A/T) (see Methods). Six control populations of plasmid-free MG1655 were similarly experimentally evolved with no antibiotic (C). Plasmids remained at high frequency in all populations for the duration of the selection experiment. Plasmid-free segregants were observed only at very low frequency in two of the six populations from treatment N (Supplementary Fig. 1), whereas transposition of resistance genes from RK2 onto the host’s chromosome was never observed. To test for changes in antibiotic resistance profiles following evolution, we first determined the minimum inhibitory concentration (MIC) of evolved lineages to TET and AMP. The susceptibility of the evolved strains to antibiotics differed between treatments (Fig. 1a). We observed a fourfold increase in TET MIC in evolved strains from the T and AT treatments, and a small increase in lineages that had evolved under the cycling A/T treatment compared with the ancestral MG1655 with ancestral RK2 (Anc-RK2), whereas evolved strains from treatments N and A showed no change in TET MIC (ANOVA: $F_{5,30}=6.103$, $P<0.001$; post hoc Tukey tests: Anc-RK2:T $P<0.001$, Anc-RK2:AT $P<0.01$, Anc-RK2:N $P=0.525$, Anc-RK2:A $P=0.783$). By contrast, we observed no change in resistance to AMP in any treatment (ANOVA: $F_{5,30}=1.212$, $P=0.327$), possibly due to a lower relative selection pressure imposed by the concentration of AMP used in the experiment compared with the concentration of TET\(^3\). Interestingly, TET selection led to the evolution of bacteria that were cross-resistant to chloramphenicol (CML), although the extent of the evolved cross-resistance varied between

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treatments (ANOVA: $F_{(5,30)}=24.25, P<0.001$); with CML MIC increasing eight-, four- and twofold in T, AT and A/T treatments, respectively. Consistent with CML cross-resistance being a correlated response to TET selection, evolved strains from both the N and A treatments remained equally sensitive to CML as the ancestral MG1655(RK2) (post hoc Tukey tests: Anc-RK2:N $P=0.975$, Anc-RK2:A $P=0.993$). Thus, whereas T and AT treatments, and to a lesser extent the cycling A/T treatment, led to the evolution of increased TET resistance and cross-resistance to CML, evolved lineages from the N and A treatments showed no change in their resistance profile.

To examine the genetic bases of evolved changes in resistance, we next obtained whole genome sequences for one randomly selected clone per population. Excluding hypermutators, evolved clones had acquired between 2 and 11 mutations, located exclusively on the chromosome in non-TET treatments (C, N, A), and on both the chromosome and plasmid in the treatments including TET (T, AT, A/T) (Supplementary Fig. 2, Supplementary Table 1). Of all the observed mutations, 13.2% were synonymous and 19% were intergenic, the remaining non-synonymous mutations (67.8%) comprised missense mutations (42.8%), frameshifts (10.6%), insertion sequences (ISs; 5.6%) and gene...
deletions (5.4%), and these were analysed further. While the variance in the number of non-synonymous mutations did not differ between treatments (analysis of multivariate homogeneity of group variances including hypermutators: $F_{3,56} = 1.8617, P = 0.1358$), the loci affected by non-synonymous mutations did vary between treatments (permutational ANOVA, permutation test: $F_{3,56} = 2.5231, P = 0.01$, Bonferroni corrected). Clones that had evolved under TET selection (T, AT, A/T) had significantly different sets of non-synonymous mutations compared with evolved clones from the other treatments (C, N, A) (permutation test: $F_{3,56} = 6.9463, P = 0.01$, Bonferroni corrected), with a larger genetic distance between TET and non-TET treatments than within these treatment groups (Fig. 2a). Thus, TET-selected lineages followed an evolutionary trajectory distinct from non-TET-selected lineages, leading to mutations on both the chromosome and the plasmid, which suggests that TET selection favoured bacteria–plasmid coadaptation.

Strikingly parallel mutations were observed between independent replicate populations both within and between TET-containing treatments (Fig. 2b). Highly parallel mutations are likely to represent adaptive evolution at these loci, and because mutations at these loci were not observed in the populations from the N and A treatments, these mutations were likely to be TET-specific adaptations. Mutations in the chromosomal genes $ompF$ (16 out of 18 clones) and $ychH$ (16 out of 18 clones) showed strong locus-level parallelism within all three TET-containing treatments. Mutations in $ompF$, encoding a major non-specific diffusion porin $t$, were all predicted loss-of-function mutations, including the insertion of IS elements and frameshifts observed in evolved strains. The loss of $ompF$ in $E. coli$ reduces membrane permeability, including to antibiotics, and consequently is known to increase resistance to a wide spectrum of antibiotics, including TET and CML. Deletion of $ompF$ ($E. coli$ K-12 $\Delta$ompF JW0912) significantly increased resistance to TET without the RK2 ($t_{M1.92} = 4.2836, P < 0.01$), and further increased TET resistance when carrying RK2 (two-way ANOVA interaction:

$$F_{1,20} = 14.724, P < 0.01; \text{Supplementary Fig. 4a}$$

Parallel loss of function mutations (IS elements and frameshifts) in $ychH$ were observed across all the TET treatments. $ychH$ is a hypothetical stress-induced inner membrane protein $t$, but deletion of $ychH$ ($E. coli$ K-12 $\Delta$ $ychH$ JW1196$^{15}$) did not significantly increase the resistance to TET with or without the plasmid (Supplementary Fig. 4b), suggesting that this general stress response may not be required under TET selection and is consequently selected against.

Mutations in several loci observed in the T and AT treatments were not present in the cycling A/T treatment. These included mutations in both $acrR$ (10 out of 12 clones) and $adhE$ (9 out of 12 clones). Mutations in $adhE$ were extensively parallel at the nucleotide level, with 8 clones from independent populations all having the same missense single-nucleotide polymorphism in the ethanol dehydrogenase domain $t$. The phenotypic significance of these mutations is unclear due to the numerous roles assigned to this protein, including multiple metabolic pathways $t$, but intriguingly the AdhE protein is known to exhibit binding activity to the 30 S ribosome $t$, the primary TET target. The $acrR$ gene encodes a repressor of AcrAB multidrug efflux pump $t$; the majority of mutations in $acrR$ are predicted loss of function mutations, with IS elements and frameshifts observed in evolved strains. The deletion of $acrR$ results in the overexpression of $acrAB$ leading to MDR phenotypes $t$. Deletion of $acrR$ ($E. coli$ K-12 $\Delta$ $acrR$ JW0453) alone did not significantly increase resistance to TET ($t_{M1.92} = 0.591, P = 0.339$), but when combined with the RK2 plasmid did allow significantly increased growth in TET ($t_{M1.92} = 3.665, P < 0.01$; Supplementary Fig. 4c). These findings are consistent with the higher TET resistance of evolved clones from the T and AT treatments versus the A/T treatment (Fig. 1a), and reflect overall weaker TET selection under the A/T cycling compared with the T and AT treatments where TET selection was constant. Interestingly, stronger TET selection seemed to constrain evolution at chromosomal loci not involved in resistance. For example, we observed highly parallel loss of function

![Fig. 2 | Mutations show treatment-specific parallelism. a. An unrooted neighbour joining tree of end-point evolved clones. The distance matrix was constructed from the binary presence or absence of variants at each gene relative to the ancestral strain. Hypermutators were excluded from the analysis. Scale bar represents number of gene variants; percentage bootstrap support is shown at the branches, bootstraps = 1,000, values below 30 are omitted. Blue branches represent clones isolated from TET treatments. Branch labels denote replicate population identity and selection treatment where C is No Ab / No RK2, N is No Ab, A is AMP, T is TET, AT is AMP plus TET, and A/T is cycling AMP and TET. b. Mutations observed in evolved clones (excluding hypermutators) across treatment. Rings represent $E. coli$ chromosomes or RK2 plasmids. Dots represent mutations, the size of the dots represents the number of mutations at the same loci across independent replicate populations. Plots of individual treatments are in Supplementary Fig. 3.](image-url)
mutation in the flagellum operon in the A, N and A/T treatments, but only rarely observed mutations at these loci in T and AT treatments. Loss of the flagellar motility is a commonly observed adaptation of E. coli to growth in liquid media and this may have been impeded by clonal interference or negative epistasis with chromosomal resistance mutations in populations under strong TET selection. Consistent with this, whereas evolved clones from the N and A treatments increased in fitness relative to the plasmid-free ancestor in antibiotic-free media, such fitness gains were not observed in evolved clones from the TET-containing treatments (Supplementary Fig. 5).

To confirm that TET selection had led to the evolution of chromosomal resistance, we next cured evolved strains of their plasmids and quantified resistance. Evolved strains carrying putative chromosomal resistance mutations displayed increased TET (ANOVA: $F_{1,80} = 42.63, P < 0.001$, AMP (ANOVA: $F_{1,80} = 12.55, P < 0.001$) and CML (ANOVA: $F_{1,80} = 35.88, P < 0.001$) resistance (Fig. 1b). Across all tested antibiotics, evolved clones carrying both $ompF$ and $acrR$ mutations had significantly increased resistance compared with the ancestral MG1655 (post hoc Tukey tests: all $P < 0.05$), whereas cured evolved strains without either of these mutations (that is, from the N and A treatments) did not (post hoc Tukey tests: all $P > 0.05$).

Interestingly, cured evolved clones from the cycling A/T treatment that carried only mutations in $ompF$ but not in $acrR$ showed marginally increased resistance to both TET and CML, but no detectable increase in AMP resistance, relative to MG1655. Thus TET selection favoured the de novo evolution of chromosomal resistance despite pre-existing plasmid-encoded TET resistance, and these chromosomal resistance mutations are responsible for the observed cross-resistance to CML.

We observed parallel mutations on the plasmid exclusively in evolved clones from the TET-containing treatments (T, AT and A/T). These mutations occurred in $tetA/tetR$ (18 out of 18 clones; $tetA$: 13, $tetR$: 2, both: 3; Fig. 2b), which encode the TET-specific efflux pump. The expression of $tetA$ is tightly regulated by the repressor $tetR$ in the absence of TET. Mutations in $tetA$ were dispersed throughout the genome, affecting the protein’s transmembrane, periplasmic and cytoplasmic domains. Three of the five mutations observed in $tetR$ are in direct contact with or close proximity to the TET binding pocket, while the other two mutations are located in the central scaffold of the protein, suggesting that they are likely to interfere with activity of the $tetR$ repressor. Evolved plasmids carrying mutations in $tetA$ or $tetR$ displayed reduced resistance to TET in the ancestral MG1655 background compared with ancestral RK2 (Fig. 1d; ANOVA: $F_{1,80} = 4.586, P < 0.01$). Consistent with reduced efficacy of plasmid-encoded resistance in evolved lineages with $tetA/tetR$ mutations, when we replaced the evolved plasmid with ancestral RK2, this led to increased TET resistance (ANOVA: $F_{1,80} = 71.86, P < 0.001$; Anc-RK2/T, AT, A/T: all $P < 0.05$).

Our data suggest that evolved strains from TET-containing treatments adapted their resistance to TET by acquisition of weak chromosomal resistance mutations in combination with mutations that reduced the efficacy of the plasmid-encoded TET efflux pump. To understand the evolutionary benefits of this counterintuitive dual resistance strategy, we first compared the effect of chromosomal background (evolved or ancestral) and plasmid genotype (evolved or ancestral) on growth in the presence of 10 μg ml$^{-1}$ TET (that is, the concentration used in our selection experiment). The evolved chromosomal background carrying resistance mutations displayed a significantly shortened lag phase compared with the ancestral chromosomal background, irrespective of the plasmid genotype (Supplementary Fig. 6; ANOVA: $F_{1,60} = 76.92, P < 0.001$; post hoc Tukey tests: evolved host/ancestral host all $P < 0.001$). This suggests that chromosomal resistances reducing membrane permeability to antibiotics allowed evolved strains to start growing faster in the presence of TET.

Whereas evolved bacteria grew equally well with evolved or ancestral plasmids, ancestral bacteria displayed impaired growth with evolved compared with ancestral plasmids (Supplementary Fig. 6; maximum optical density: Wilcoxon test: $W = 93, P < 0.01$). This is consistent with the mutations in $tetA/tetR$ reducing resistance, but importantly confirms that this reduction is not evident when in combination with the chromosomal resistance mutations, which seem to compensate for the reduced efficacy of the plasmid-encoded efflux pump.

We next competed evolved bacteria with either the evolved or ancestral plasmid against the ancestral MG1665 (RK2) to compare the costs of carrying each plasmid genotype. The ancestral plasmid displayed a significantly higher cost than the evolved plasmid in the evolved chromosomal background (Supplementary Fig. 7; $t_{5,71} = -2.287, P < 0.05$). This suggests that the mutations to $tetR/tetA$ ameliorate the cost of plasmid carriage but at the price of reduced efficacy of TET efflux. This is consistent with previous studies showing a high cost of expressing the specific TET efflux pump. Taken together with the growth data, this suggests that although mutations to $tetA/tetR$ reduce growth under TET in the ancestral chromosomal background, they have minimal effect on resistance in the evolved chromosomal background due to the reduced membrane permeability and additional efflux systems expressed in the evolved chromosomal background carrying mutations in $ompF$ and $acrR$, leading to high resistance and a lowered cost of plasmid carriage. This suggests that the chromosomal resistance mutations must have been gained prior to the mutations in the plasmid-encoded TET efflux pump. To test this, for one population (AT2) we tracked the frequency over time of an observed IS insertion in $ompF$ by polymerase chain reaction (PCR) and then determined by sequencing when these genotypes acquired mutations in the $tetA/tetR$ genes. Consistent with the hypothesized order of mutations, the IS insertion in $ompF$ was first detected at transfer 8 and had swept to fixation by transfer 32, whereas mutations in $tetA/tetR$ were not observed in this $ompF$:IS background until transfer 32 (Supplementary Fig. 8).

Discussion

Our current model of bacterial evolution suggests that horizontal acquisition of ARGs accelerates resistance evolution by providing bacteria with ready-made resistance mechanisms, bypassing the requirement for rare de novo mutations. However, recent population genomic data suggesting that lineages independently acquire and then subsequently coevolve with MDR plasmids imply a more dynamic evolutionary process. Consistent with this, here we show that gaining an ARG can be just the starting point in the evolution of resistance and, due to the costs of expressing horizontally acquired ARGs, does not preclude subsequent de novo evolution of chromosomal resistance. Evolved strains from TET-containing treatments gained chromosomal resistance mutations that reduced membrane permeability, enhanced antibiotic efflux, provided cross-resistance to other antibiotics, and shortened lag phase in the presence of TET. These mutations also reduced the need for a fully operational plasmid-encoded TET efflux pump, expression of which is highly costly, allowing plasmid mutations in the TET efflux pump and its regulator, which reduced the cost of plasmid-encoded resistance. A consequence of this intragenomic coevolution is that the increased TET resistance of evolved strains from T, AT and A/T treatments required the action of both the chromosomal- and plasmid-encoded resistances, which together acted multiplicatively. Thus intragenomic coevolution can lead to the evolution of bacterial genomes comprising co-dependent replicons, limiting the potential for onward transmission of the plasmid due to the weaker resistance it now encodes in other lineages.

Methods

Strains, culture conditions and evolution experiment. E. coli MG1655 chromosomally labelled with green fluorescent protein (GFP) at the attB lambda attachment site was used in the evolution experiments. Isogenic E. coli MG1655::mCherry was used as a reference strain in competition and conjugation rate experiments. Both E. coli strains were provided by the Van Der Woude laboratory (University of York). The RK2 plasmid was introduced to the strains through
conjugation from E. coli MV10 provided by the Thomas laboratory (University of Birmingham). All cultures were grown in Oxoid Nutrient Broth (NB) at 37 °C, 5 ml in 50 ml microcups and shaken at 180 r.p.m. Individual selection lines were founded by 30 independent single colonies of MG1655-GFP harbouring RK2. These were grown overnight in non-selective conditions and split into the five antibiotic treatments, no antibiotic selection, 100 μg ml⁻¹ AMP, 10 μg ml⁻¹ TET, 100 μg ml⁻¹ AMP plus 10 μg ml⁻¹ TET, and 24 h cycling between 100 μg ml⁻¹ AMP and 10 μg ml⁻¹ TET, with six replicate populations per treatment. In parallel, six independent E. coli MG1655-GFP colonies were picked for control treatments and grown under no selection. Selection lines were established by transferring 50 μl of saturated overnight culture into 5 ml of selective media. These populations were maintained through transfer of 1% of the population into fresh media and antibiotics every 24 h for 80 transfers, resulting in ~6.64 generations per day, totalling ~530 bacterial generations. For the cycling treatment, 3 populations were initiated with 100 μl of AMP and 3 populations with 10 μg ml⁻¹ TET. Culture density (OD₆00) was recorded every 24 h. Plasmid prevalence was measured at the start and end of the selection experiment by screening 20 randomly picked colonies from each population using multiplex primers specific to RK2 replication origin (forward: ctcatctgtcaacgccgc, reverse: tgggcgagtgaatgcagaat). These primers allowed for the detection of plasmid loss and transposition of resistances onto the chromosome. One end-point clone was randomly selected from each population for phenotypic typing, curing, calculation of MICs and sequencing. Every eight transfers throughout the experiment, 500 μl samples of whole populations were collected and stored in 25% glycerol at −80 °C. Whole populations were also plated out on non-selective media, 20 individual clones were then randomly selected, sub-cultured for a further 24 h in non-selective media and stored in 25% glycerol in 96-well plates.

**Relative fitness.** The relative fitness of the evolved plasmid-bearing versus ancestral plasmid-free strain (Wₑv) was estimated by direct competition, with six replicate strains per treatment. The competitions were initiated with 50 μl of 1:1 mixtures of plasmid-bearing evolved strain and plasmid-bearing ancestral strain marked with mCherry from overnight cultures in 5 ml of non-selective NB media. The relative fitness of the evolved strains was calculated by gaining exact viable cell counts at 0 h and 24 h; strains were distinguished through detection of fluorescent markers using a Zeiss Stereo Lunar v12 microscope. The relative fitness of plasmid-bearing bacteria was calculated as a ratio of Malthusian parameters:\(^3\):

\[
Wₑv = \frac{\ln Nₐₚ₂₄}{\ln Nₐₚ₇₂₄}
\]

Fitness effect owing to different markers was determined by competing plasmid-free MG1655-GFP with plasmid-free MG1655-mCherry; the relative fitness of MG1655-GFP was not significantly different from 1 (t₀.015584, P = 0.9882), showing that there is no significant difference between the two marker strains.

Relative fitness of evolved strains harbouring evolved plasmid or evolved strains harbouring ancestral plasmid versus ancestral plasmid-bearing cells was estimated using the method as above, with 18 replicate strains per competition, but grown in 100 μl cultures in a 96-well plate at 37 °C, shaken at 600 r.p.m. with a 3 mm orbital radius and inoculated to an initial dilution of 1:500. Again, no fitness effect of markers was observed (t₀ = −0.2795, P = 0.791).

**Curing RK2 from evolved strains.** Evolved strains were cured using the pCURE curing system. The anti-incP1 cassette (RK2 orfV, parD, korA, and incC genes) from pCURE11 was digested into the pLAZ2 CML-resistant vector that contains the sacB gene allowing counter selection of plasmid-free segregants. The resultant plasmid was transformed into chemically competent evolved strains and selected for using CML 12.5 μg ml⁻¹. Single-colony transformants were re-streaked onto CML 12.5 μg ml⁻¹ plates and CML 12.5 μg ml⁻¹ + 5% sucrose. Sucrose-sensitive colonies were checked by PCR for the presence of the curing plasmid (forward: aaggttgtcgagcgtg, reverse: cacagctgacctagtg) and absence of RK2 β-lactamase and tetA (primers as above). Successfully cured clones were cultured for 24 h in non-selective media to allow segregation of the curing plasmid; segregants were selected on antibiotic-free, 5% sucrose plates. To confirm loss of both plasmids, sucrose-resistant colonies were checked for sensitivity to CML, AMP, and TET, as well as PCR using the primers mentioned above. Both the ancestral strains harbouring RK2 and the ancestral plasmid-free strains underwent the curing process and were used as a comparison for cured evolved strains to control for the curing process. Ancestral RK2 was introduced into the cured evolved strains, and evolved RK2 was introduced into the plasmid-free ancestor through conjugation. Again, to control for the curing and conjugation steps, ancestral RK2 was conjugated into ancestral plasmid-free strains and used for the experiment. Saturated overnight cultures of donor plasmid containing strains and recipient plasmid-free strains were mixed 1:1, and 50 μl was used to inoculate 5 ml NB. The mixed cultures were grown for 24 h and plated out on to 100 μg ml⁻¹ AMP to select for transconjugants. Transconjugants were confirmed by fluoroscence and PCR screening for RK2 plasmid.

**MIC.** To measure MICs, six replicate cultures per treatment were grown overnight until stationary phase in 5 ml NB. The saturated cultures were then sub-cultured 50 μl into 5 ml fresh NB and grown to an OD₆00 of 0.5. These were then diluted into 96-well plates containing a log, serial dilution of antibiotic (AMP, TET or CML) to an initial density of 5 × 10⁶ CFU ml⁻¹. Cultures of 100 μl were grown for 24 h at 37 °C, with shaking at 600 r.p.m. with a 3 mm orbital radius. OD₆00 was measured after 24 h.

**Growth curves.** Six replicate saturated overnight cultures per treatment were cultured to an OD₆00 of 0.5 and used to inoculate 100 μl NB supplemented with 100 μg ml⁻¹ TET per well in 96-well plates at a final dilution of 1:1000. Plates were grown at 37 °C with shaking at 300 r.p.m. with a 3 mm orbital radius for 24 h. OD₆00 was measured every 16 min at 37 °C. A logistic model was fitted to the growth phase data using a non-linear growth model. Growth rates were calculated as the maximum slope of log, transformed OD₆00 covering four time points (~1 h of growth), lag phase was calculated to end when growth rate reached 10% of the maximum achieved growth rate.

**Genome sequencing and analysis.** Whole genomes were extracted from each evolved population’s clone as well as the ancestral strain and ancestral strain harbouring the RK2 plasmid using the DNeasy Blood and Tissue extraction kit (Qiagen). The total DNA was sequenced by MicrobesNG (http://www.microbesng.uk), which is supported by the Biotechnology and Biological Sciences Research Council (BBSRC; grant number BB/L024209/1), using Illumina MiSeq. Reads were mapped to E. coli MG1655 (Genbank accession CP003654) and RK2 (GenBank accession BAA00925.1) reference using BWA-MEM⁷. Single-nucleotide variants and small indel events were detected using GATK UnifiedGenotyper® and SnpEff⁸, IRRs were identified using custom scripts and Integrative Genomics Viewer⁹, and large genome-wide structural variants were detected using BreakDancer®. Mutations that were present in the ancestral clones were excluded, resulting in a set of mutations that were acquired during the selection experiment.

**Tracking mutations.** Populations that did not show a hypermutator phenotype, had IRRs within omfp and mutations in the TET resistance genes on the plasmid from the constant TET treatments (T and AT treatments) were selected for further analysis to gain an understanding of the mutational timeline during the selection experiment. IRRs within omfp were identified within whole populations of T4, AT2, AT3 and AT5 by PCR of the omfp gene (forward: ACGCGCGTCGAGTACCAC, reverse: GGCGAAATTCGCGAGT). A short product of 716 base pairs (bp) indicated no IRR, a long product of 1,484 bp indicated IRR and a long product of 1,911 bp indicated IRR. Whole population PCR indicated that omfp:IS mutants had swept into the population by transfer 40 for populations T4, AT2 and AT5, and transfer 48 in population AT3. Frequency of omfp IRRs were calculated by PCR of 20 clones from transfers 8, 16, 24, 32 and 40. TET resistance genes (tetA and tetR) from clones containing omfp:IS mutations from population AT2 transfers, 16, 24, 32 and 32, were then Sanger sequenced to determine if omfp:IS mutants had swept before tetA or tetR mutations (tetA: forward: GGCGACCTTCTTTGTACAT, reverse: TCTACGCCAGCAGCCCAAC, tetR: forward: ATCGCGACCTTCTTTGTACAT, reverse: ACGCGCGTCGAGTACCAC). A short product of 716 base pairs (bp) indicated no IRR, a long product of 1,484 bp indicated IRR and a long product of 1,911 bp indicated IRR. The variances between treatments were significantly different, with hypermutators not being assumed, a non-parametric Wilcoxon signed rank test was used. Differences among treatment growth under antibiotic selection were calculated by ANOVA of means. All statistical analysis was conducted in R (version 3.2.3).

**Statistical analysis.** To test if the mutations observed within each treatment had significantly different variances, a multivariate homogeneity of groups variances test was conducted¹⁰. The binary presence or absence of a variant at each allele was used to calculate a Euclidean distance matrix between each population. This was used to test for homogeneity of variances between treatments using ‘betadisper’ in vegan 2.4-0. The variances between treatments were significantly different, with hypermutators significantly affecting within-group variation. These clones were removed from further analysis as significant differences in within-group variance can lead to falsely significant results when testing for differences between groups⁵. Permutational multivariate analysis of variance was used to calculate whether different evolutionaries treatments resulted in different sets of mutations⁶. Using the Euclidean distance matrix with hypermutators removed, the significance of within- and between-group distances was calculated using ‘adonis2’ in vegan 2.4-0. The data were partitioned into different groups and multiple testing was corrected for using Benferroni correction. Neighbour joining phylogeny was constructed using the binary presence or absence table with hypermutators removed. Tree estimation and bootstrap support was conducted using ape 4.0, Suffix package in ape 4.0. Phylogenetic trees among two related samples were calculated using a two-sided, two-sample t-test. A Shapiro–Wilk test was conducted to check for normality; when normality could not be assumed, a non-parametric Wilcoxon signed rank test was used. Differences among treatment growth under antibiotic selection were calculated by ANOVA of the integral of the resistance profiles, with subsequent Tukey multiple comparison of mean. All statistical analyses were conducted in R (version 3.2.3).

**Data availability.** The sequence data supporting the findings of this study are available at the European Nucleotide Archive, accession: PRJEB20785. All other
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