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Adaptive modulation of antibiotic resistance through intragenomic coevolution

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Abstract

Bacteria gain antibiotic resistance genes by horizontal acquisition of mobile genetic elements (MGE) from other lineages. Newly acquired MGEs are often poorly adapted causing intragenomic conflicts, resolved by compensatory adaptation of the chromosome, 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 multi-drug resistant (MDR) 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 comprised of 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.

Horizontal gene transfer (HGT) is a fundamental process in bacterial evolution that accelerates adaptation by sharing ecologically important accessory traits between lineages¹. These accessory traits are themselves frequently located on semi-autonomous mobile genetic elements (MGE), such as conjugative plasmids, that encode genes for their own replication, partition and horizontal transfer^{2,3}. Conjugative multidrug resistance (MDR) plasmids,

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Contributions M.A.B. and A.J.W. supervised the project. M.J.B. performed the experiments and analysed the data. All authors contributed towards the design of the study and wrote the manuscript.

Competing interests The authors declare no competing financial interests.

Data Availability

The sequence data supporting the findings of this study are available at the European Nucleotide Archive, accession: PRJEB20735. All other data in this study is available at Figshare data depository (<https://doi.org/10.6084/m9.figshare.5092225.v1>). Custom code used to map possible IS elements are available online (https://github.com/mbottery/Co_Evo_IS_Analysis).

encoding antibiotic resistance genes (ARG) against multiple classes of antibiotics, are of particular clinical concern since they allow instantaneous acquisition of MDR phenotypes and thus potentiate the rapid emergence of MDR bacterial pathogens^{4,5}. Newly acquired conjugative plasmids are often costly since the plasmid is unlikely to be well adapted to the new genetic background, causing intragenomic conflict⁶. The cost of plasmid carriage is likely to arise due to the metabolic burden of maintaining, transcribing and translating plasmid genes⁷, as well as via disruption of cellular homeostasis caused by gene regulatory interference between chromosomal and plasmid regulators^{8,9}, and cytotoxic effects of plasmid gene products⁶.

An important route to resolving this intragenomic conflict is compensatory evolution to ameliorate the cost of plasmid acquisition¹⁰. Experimental evolution suggests that compensatory evolution can arise via mutations located on either the chromosome or the plasmid, or via intragenomic coevolution involving both plasmid and chromosome mutations^{8,9,11,12}. Comparative genomics suggests a key role for compensatory evolution in natural bacterial populations, potentially stabilising MDR plasmids within lineages and thus allowing the evolutionary emergence by HGT of MDR pathogens¹³. A key outstanding question is how the mode of compensatory evolution following MDR plasmid acquisition varies with antibiotic treatment. Here we experimentally evolved *Escherichia coli* MG1655 carrying the MDR plasmid RK2 (encoding tetracycline and ampicillin resistance genes) under a range of antibiotic treatment regimes including no antibiotic, mono- and combination-therapies of tetracycline and ampicillin. Following 530 generations of selection we quantified evolved changes in antibiotic resistance and fitness, and used genome sequencing to determine the genetic bases of the observed adaptation.

Results

Thirty independent isogenic populations of *E. coli* MG1655 carrying the MDR plasmid RK214, which encodes resistances to tetracycline (TET) and ampicillin (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 hour cycling between AMP and TET (A/T) (see methods). Plasmids remained at high frequency in all populations for the duration of the selection experiment. Plasmid-free segregants were only observed 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 four-fold 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 tetracycline 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 to the concentration of TET15. 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 treatments (ANOVA, $F_{5,30} = 24.25$, $p < 0.001$); with CML MIC increasing 8-, 4-, and 2-fold in T, AT, 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 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 (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 excluding hypermutators $F_{5,26} = 1.8617$, $p = 0.1358$), the loci affected by non-synonymous mutations did vary between treatments (Permutational ANOVA, permutation test: $F_{5,26} = 2.5231$, $p < 0.01$, Bonferroni corrected). Clones that had evolved under TET selection (T, AT, A/T) had significantly different sets of non-synonymous mutations compared to evolved clones from the other treatments (C, N, A) (permutation test: $F_{1,30} = 6.9463$, $p < 0.01$, 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 suggest 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 *yehH* (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 porin16, were all predicted loss-of-function mutations, including the insertion of IS elements, frameshifts or premature stop codons. 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 antibiotics¹⁷ including TET and CML^{18,19}. Deletion of *ompF* (*E. coli* K-12 *ompF* JW091220) significantly increased resistance to TET without

the RK2 ($t_{9,09} = 4.2836$, $p < 0.01$), and further increased TET resistance when carrying RK2 (Two-way ANOVA Interaction $F_{1,20} = 14.724$, $p < 0.01$; 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^{21,22}, but deletion of *ychH* (*E. coli* K-12 *ychH* JW119620) 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 SNP in the ethanol dehydrogenase domain²³. The phenotypic significance of these mutations is unclear due to the multiple roles assigned to this protein, including multiple metabolic pathways²⁴, but intriguingly the AdhE protein is known to exhibit binding activity to the 30S ribosome²⁵, the primary TET target. The *acrR* gene encodes a repressor of AcrAB multidrug efflux pump²⁶, 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^{27,28}. Deletion of *acrR* (*E. coli* K-12 *acrR* JW045320) alone did not significantly increase resistance to TET ($t_{9,32} = -0.591$, $p = 0.339$), but when combined with the RK2 plasmid did allow significantly increased growth in TET ($t_{6,4} = 3.665$, $p < 0.01$, 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 to the T and AT treatments where TET selection was constant. Interestingly, stronger TET selection appeared to constrain evolution at chromosomal loci not involved in resistance. For example, we observed highly parallel loss of function 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 media²⁹ 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 resistance³⁰. Evolved strains carrying putative chromosomal resistance mutations displayed increased TET (ANOVA, $F_{5,30} = 42.63$, $p < 0.001$), AMP (ANOVA, $F_{5,30} = 12.55$, $p < 0.001$) and CML (ANOVA, $F_{5,30} = 35.88$, $p < 0.001$) resistance (Fig. 1b). Across all tested antibiotics, evolved clones carrying both *ompF* and *acrR* mutations had significantly increased resistance compared to the ancestral MG1655 (Post-hoc Tukey Tests, all $p < 0.05$), whereas cured evolved strains without either of these mutations (i.e. 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 tetracycline-specific efflux pump. The expression *tetA* is tightly regulated by the repressor *tetR* in the absence of tetracycline^{31,32}. Mutations in *tetA* were dispersed throughout the gene, affecting the protein's transmembrane, periplasmic and cytoplasmic domains³³. Three of the five mutations observed in *tetR* are in direct contact with or in close proximity to the tetracycline binding pocket³⁴, while the other two mutations are located in the central scaffolding 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 to ancestral RK2 (Fig. 1d, ANOVA, $F_{5,30} = 4.586$, $p < 0.01$). 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, $F_{5,30} = 71.86$, $p < 0.001$, 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 $\mu\text{g/ml}$ TET (i.e., the concentration used in our selection experiment). The evolved chromosomal background carrying resistance mutations displayed a significantly shortened lag phase compared to the ancestral chromosomal background, irrespective of the plasmid genotype (Supplementary Fig. 6; ANOVA, $F_{3,56} = 76.92$, $p < 0.001$; 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 to ancestral plasmids (Supplementary Fig. 6; Max OD, $W = 93$, $p < 0.01$). 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 appear 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, $t_{25.71} = -2.287$, $p < 0.05$). 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 tetracycline efflux pump³⁵. Taken together with the growth data, this suggests that although mutations to *tetA/tetR* reduce growth under tetracycline 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 tetracycline efflux pump. To test this, for one population (AT2) we tracked the frequency over time of an observed IS-insertion in *ompF* by PCR and then determined by sequencing when these genotypes acquired mutations in the *tetA/tetR* genes. Consistent with the hypothesised 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 figure 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* mutations¹. However, recent population genomic data suggesting that lineages independently acquire and then subsequently coevolve with MDR plasmids^{13,36,37} imply a more dynamic evolutionary process. Consistent with this, here we show here 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 reducing membrane permeability and enhancing efflux of TET and providing cross-resistance to other antibiotics, shortening lag phase in the presence of TET. These mutations also reduced the need for a fully operational plasmid-encoded tetracycline efflux pump, expression of which is highly costly³⁵, 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 comprised of 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 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 lab (University of York). The RK2 plasmid was introduced to the strains through conjugation from *E. coli* MV10 provided by the Thomas lab (University of Birmingham). All cultures were grown in Oxiod® Nutrient Broth (NB) at 37°C 5 ml in 50 ml microcosms shaken at 180 rpm. Independent selection lines were founded by 30 independent single colonies of *E. coli* MG1655-GFP harbouring RK2. These were grown overnight in non-selective conditions and split into the 5 antibiotic treatments, no antibiotic selection, 100 µg/ml ampicillin, 10 µg/ml tetracycline, 100 µg/ml ampicillin plus 10 µg/ml tetracycline, and 24 hour cycling between 100 µg/ml ampicillin and 10 µg/ml tetracycline, with 6 replicate populations per treatment. In parallel, 6 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 5ml of selective media. These populations were maintained through transfer of 1% of the population into fresh media and antibiotics every 24 hours for 80 transfers, resulting in ~6.64 generations per day, totalling ~530 bacterial generations. For the cycling treatment 3 populations were initiated with 100µg/ml ampicillin and 3 populations were initiated with 10µg/ml tetracycline. Culture density (OD₆₀₀) was recorded every 24 hours. Plasmid prevalence was measure 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 (Fw: ctcacatgtcaacgccgc, Rv: aaccggctatgtcgtgct), β-lactamase (Fw: ataactacgatacgggagggc, Rv: acattccgtgtcgcctta), and tetracycline efflux pump (Fw: tgggttctctatcgggcg, Rv: 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 hours 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 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 5ml of non-selective NB media. The relative fitness of the evolved strains was calculated by gaining exact viable cell counts at 0 hours and 24 hours, strains were distinguished through detection of fluorescent markers using Zeiss Stereo Lumar v12 microscope. The relative fitness of plasmid-bearing bacteria was calculated as a ratio of Malthusian parameters³⁸:

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

Fitness effects due 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 difference from 1 ($t_5=0.015584$, $p=0.9882$) 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 same method as above, with eighteen replicate strains per competition, but grown in 100 μ l cultures in a 96 well plate, 37°C shaken at 600 rpm, 3 mm orbital radius, inoculated to an initial dilution of 1:500. Again no fitness effect of markers was observed ($t_5=-0.2795$, $p=0.791$).

Curing RK2 from evolved strains

Evolved strains were cured using the pCURE curing system³⁰. The anti-incP-1 cassette (RK2 *oriV*, *parD*, *korA*, and *incC* genes) from pCURE11 was ligated into the pLAZ2 chloramphenicol resistant vector that contains the *sacB* gene allowing counter selection for plasmid free segregants. The resultant plasmid was transformed into chemically competent evolved strains and selected for using Cml 12.5 μ g/ml. Single colony transformants were re-streaked on to Cml 12.5 μ g/ml plates and Cml 12.5 μ g/ml + 5% sucrose. Sucrose sensitive colonies were checked by PCR for the presence of the curing plasmid (Fw: aagtttgggtgactgcgctc, Rv: caaagacgatgtgtagccg) and absence of RK2 β -lactamase and *tetA* (primers as above). Successfully cured clones were cultured for 24 hours in non-selective media to allow segregation of the curing plasmid; sergeants were selected on antibiotic free, 5% sucrose plates. To confirm loss of both plasmids sucrose resistant colonies were check for sensitivity to chloramphenicol, ampicillin, and tetracycline, as well as PCR using primers mentioned above. Both the ancestral strain harbouring RK2 and ancestral plasmid free strains under went the curing process and were used as a comparison to cured evolved strains to control for curing process. Ancestral RK2 was introduced into the cured evolved strains, and evolved RK2 was introduced into the plasmid free ancestor though conjugation. Again, to control for the curing and conjugation steps, ancestral RK2 was conjugated into cured ancestral strains and used for comparison. Saturated overnight cultures of donor plasmid containing strains and recipient plasmid free strains were mixed 1:1, and 50 μ l was used to inoculate 5ml NB. The mixed cultures were grown for 24 hours and plated out on to 100 μ g/ml ampicillin to select for transconjugants. Transconjugants were confirmed by fluoresces and PCR screening for RK2 plasmid.

MIC

To measure minimal inhibitory concentrations, six replicate cultures per-treatment were grown overnight until stationary phase in 5 ml NB, the saturated cultures were then sub-cultured 50 μ l into 5 ml fresh NB and grown to an OD₆₀₀ of 0.5. These were then diluted

into 96-well plates containing a log₂ serial dilution of antibiotic (AMP, TET or CML) to an initial density of 5×10⁵ CFU/ml. 100 µl cultures were grown for 24 hours 37°C shaken at 600 rpm, 3 mm orbital radius. OD₆₀₀ was measured after 24 hours.

Growth Curves

Six replicate saturated overnight cultures per-treatment were sub-cultured to an OD₆₀₀ of 0.5, and used to inoculate 100 µl NB supplemented with 10 µg/ml TET per well in 96-well plates at a final dilution of 1:1000. Plates were grown at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, OD₆₀₀ was measured every 16 minutes by Tecan infinite M200 Pro plate reader. Growth rates were calculated as the maximum slope of log₂ transformed OD₆₀₀ covering four time points (~1 hour 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 BBSRC (grant number BB/L024209/1), using Illumina MiSeq. Reads were mapped to *E. coli* MG1655 K-12 genome (GenBank accession U00096.3) and RK2 (GenBank accession BN000925.1) reference using BWA-MEM39. Single nucleotide variants and small indel events were detected using GATK UnifiedGenotyper40 and SnpEff41, insertion sequences were identified using custom scripts and Integrative Genomics Viewer42, and large genome-wide structural variants were detected using BreakDancer43. 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 insertion sequences within *ompF*, and mutations in the tetracycline 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. Insertion sequences within *ompF* were identified within whole populations of T4, AT2, AT3 and AT5 by PCR of the *ompF* gene (Fw: ACTTCAGACCAGTAGCCCAC, Rv: GCGCAATATTCTGGCAGTGA). A short product of 716 bp indicated no insertion sequence, a long product of 1484 bp indicated IS1 and a long product of 1911 bp indicated IS5. Whole population PCR indicated that *ompF*:IS mutants had swept into the population by transfer 40 for populations T4, AT2 and AT5, and transfer 48 in population AT3. Frequency of *ompF* insertion sequences were calculated by PCR of 20 clones from transfers 8, 16, 24, 32, and 40. Tetracycline resistance genes (*tetA* and *tetR*) from clones containing *ompF*:IS mutations from population AT2, transfers 8, 16, 24, and 32 were then Sanger sequenced to determine if *ompF* mutations arise before *tetAR* mutations (*tetA*: Fw: GGCTGCAACTTTGTCATG, Rv: TTCCAACCGCACTCCTAG, Internal1: ACAGCGCCTTTCTTTG, Internal2: AAGGCAAGCAGGATGTAG; *tetR*: Fw: TCTGACGCGGTGGAAAG, Rv: ACGCGCGGATTCTTTATC, Internal1: GAGCCTGTTCAACGGTG, Internal2: TCTGACGACACGCAAAC).

Statistical analysis

To test if the mutations observed within each treatment had significantly different variances a multivariate homogeneity of groups variances test was conducted⁴⁴. 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* {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 groups⁴⁵. Permutational Multivariate Analysis of Variance was used to calculate whether different evolutionary treatments resulted in different sets of mutations^{45,46}. Using the Euclidean distance matrix with hypermutators removed, the significance of within- and between-group distances was calculated using *adonis2* {vegan 2.4-0}. The data was partitioned into different groups, multiple testing was corrected for using Bonferroni 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-package* {ape 4.0}. Significant difference between two related samples was calculated using two sided, two-sample t-test. 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 treatments growth under antibiotic selection were calculated by ANOVA of the integral of the resistance profiles, with subsequent Tukey multiple comparison of means. All statistical analysis was conducted in R (version 3.2.3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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plasmid plots (c) show ancestral MG1655 and ancestral MG1655(RK2) after curing process. Dashed lines in ancestral host evolved plasmid plots (d) show ancestral MG1655 and ancestral MG1655(RK2) which had under gone curing with ancestral RK2 subsequently reintroduced. Vertical dashed lines in AMP and TET resistance profiles show the concentrations of AMP (100 $\mu\text{g/ml}$) or TET (10 $\mu\text{g/ml}$) used in the selection experiment.

