Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria

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Plasmids are thought to play a key role in bacterial evolution by acting as vehicles for horizontal gene transfer, but the role of plasmids as catalysts of gene evolution remains unexplored. We challenged populations of *Escherichia coli* carrying the bla_{TEM-1} β-lactamase gene on either the chromosome or a multicopy plasmid (19 copies per cell) with increasing concentrations of ceftazidime. The plasmid accelerated resistance evolution by increasing the rate of appearance of novel TEM-1 mutations, thereby conferring resistance to ceftazidime, and then by amplifying the effect of TEM-1 mutations due to the increased gene dosage. Crucially, this dual effect was necessary and sufficient for the evolution of clinically relevant levels of resistance. Subsequent evolution occurred by mutations in a regulatory RNA that increased the plasmid copy number, resulting in marginal gains in ceftazidime resistance. These results uncover a role for multicopy plasmids as catalysts for the evolution of antibiotic resistance in bacteria.

Plasmids mediate the horizontal gene transfer (HGT) of accessory genes between bacteria, making them important drivers of prokaryotic evolution[1,2]. For example, the horizontal transfer of antibiotic resistance genes has played a very important role in the evolution of resistance in pathogenic bacteria. Although the benefits of HGT are obvious, it is still not understood how plasmids persist in bacterial populations. Classical theoretical models predict that HGT through conjugation is necessary for the maintenance of plasmids, given the fitness cost of plasmid carriage[3,4] and the potential for spontaneous plasmid loss during cell division[5–7]. More recent analyses demonstrate that compensatory adaptation alleviates the fitness cost due to plasmids[8–10], allowing plasmids to stably persist for hundreds of generations, even in the absence of HGT[9,11]. However, the regimes of positive selection for plasmid-encoded genes that are necessary for the long-term stability of plasmids are stringent[11]. Finally, it is possible for plasmid genes to be transferred to the bacterial chromosome, for example by transposons, allowing the bacterium to lose costly plasmids while maintaining beneficial genes[12]. Therefore, the 'plasmid paradox' of how plasmids persist in bacterial populations in the long term is not completely solved, especially for small multicopy plasmids that lack maintenance systems such as active partitioning or post-segregational killing systems. However, these small plasmids are extremely prevalent in nature and in hospitals, as has been recently shown in *E. coli*, where almost 90% of ST131 strains carry small ColE1 plasmids[13].

One possible solution for this paradox could be that certain genes confer a larger benefit to the bacteria when encoded on plasmids compared with on the chromosome. For example, carrying a gene on a plasmid with multiple copies produces higher levels of expression, and this may be beneficial for the host[14]. Another possible advantage is the variation in plasmid copy numbers that exists among single cells, which generates heterogeneity in gene expression between plasmid-bearing bacteria, leading to potentially beneficial phenotypic plasticity[15]. Finally, it is also possible that multicopy plasmids could accelerate the evolution of the genes they carry by increasing gene mutation rates due to the increase in gene copies per cell[16–19]. Under this hypothesis, multicopy plasmids could work as platforms for gene variation and evolution, facilitating the adaptation of the host bacteria to new selective pressures.

Here we investigate the evolutionary advantages associated with carrying a beneficial gene, in this case an antibiotic resistance gene, on a multicopy plasmid versus in the chromosome.

Results

Experimental system. We constructed an experimental model using *E. coli* strain MG1655 (MG) and the β-lactamase gene bla_{TEM-1}. Hundreds of variants of TEM-1 exist that provide resistance to different β-lactam antibiotics (http://www.lahey.org/studies) and they are usually encoded on plasmids, including small multicopy plasmids[20–22]. In addition, TEM-1 is one of the best-characterized systems for the study of molecular evolution in the laboratory[17,23–25]. We used TEM-1 as a model system to explore the potential of multicopy plasmids as catalysts of gene evolution in general, and of the evolution of antibiotic resistance in particular. We inserted bla_{TEM-1} in the bacterial chromosome to generate *E. coli* MG1655::bla_{TEM-1} (MG::bla_{TEM-1} hereafter, see Methods). In parallel, we constructed a non-transmissible multicopy plasmid carrying bla_{TEM-1} with an identical promoter (Fig. 1a). This plasmid also carries a *gfp* gene under the control of an inducible L-arabinose promoter, the gene coding for the repressor of this promoter *araC*, and a chloramphenicol resistance acetyltransferase gene, *cat*. We named this plasmid pBGT and transformed it into *E. coli* MG1655 generating the strain MG/pBGT. This plasmid had 19 copies per bacterium (average = 19.1, s.d. = 3.8, *n* = 6), which is similar to the copy number of other small natural multicopy plasmids[26,27]. This experimental system allowed us to evaluate the effects of a plasmid versus chromosomal location of bla_{TEM-1} in isogenic strains.

Plasmid and chromosome bla_{TEM-1}-mediated resistance. Previous studies have shown that increasing the dosage of the bla_{TEM-1} gene

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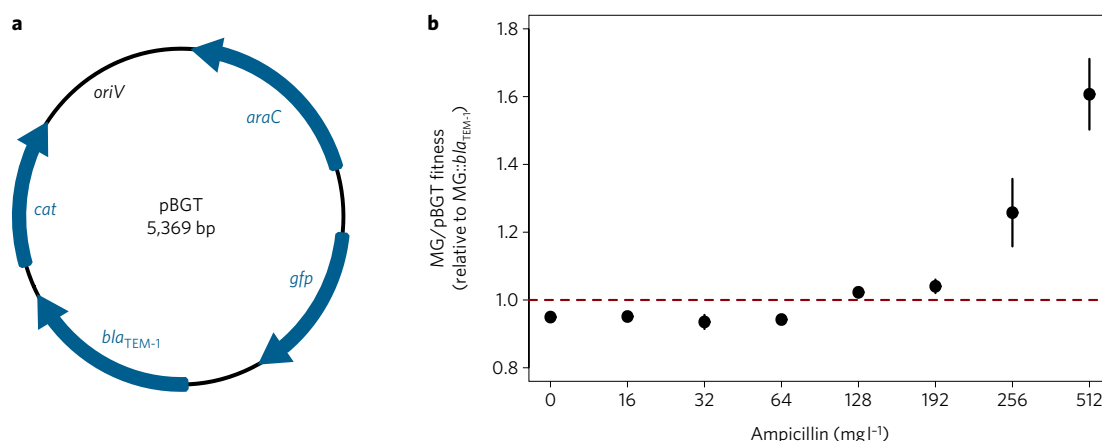
Figure 1 | Effects of plasmid pBGT on bacterial fitness. Plasmid pBGT produces variable effects on fitness depending on the ampicillin concentration. **a**, Schematic representation of plasmid pBGT carrying bla_{TEM-1}. The reading frames for genes are shown as blue arrows, with the direction of transcription indicated by the arrowheads. **b**, Relative fitness of plasmid-bearing strain MG/pBGT competing versus MG1655 coding for a chromosomal copy of bla_{TEM-1} (MG::bla_{TEM-1}) under increasing concentrations of ampicillin. Error bars represent the s.e.m. ($n = 4$). Note that the initial fitness cost produced by pBGT disappears at high concentrations of ampicillin, which is due to the higher level of resistance conferred by bla_{TEM-1} when encoded on the plasmid as opposed to the chromosome. When the error bars are not visible is because they are smaller than the data points.

produces an increase in β-lactam resistance[28–30], suggesting that carrying bla_{TEM-1} on a plasmid is likely to increase antibiotic resistance. TEM-1 confers resistance to ampicillin, so we measured the minimal inhibitory concentration (MIC) of ampicillin in the different strains (Table 1). The MIC for the susceptible parental MG strain was 4 mg l⁻¹ and it increased to 512 mg l⁻¹ in MG::bla_{TEM-1} and to 8,192 mg l⁻¹ in MG/pBGT. Therefore, even if bla_{TEM-1} conferred high-level ampicillin resistance both in the chromosomal and plasmid locations, the multicopy plasmid-bearing strain has a 16-fold increase in MIC compared with the one carrying bla_{TEM-1} in the chromosome.

Although resistance plasmids provide a fitness benefit in the presence of antibiotics, they typically impose a fitness cost in the absence of antibiotics[3,4]. We used competition experiments to measure the relative fitness of the bla_{TEM-1}-carrying strains (Fig. 1b). The presence of bla_{TEM-1} in the chromosome produced no cost in MG in the absence of antibiotics (relative fitness (W) = 0.997, two-sample t-test, $P = 0.343$, $t = 1$, d.f. = 9), suggesting that the effect of bla_{TEM-1} insertion in the chromosome was negligible. To study the fitness effects of pBGT, we competed the strains carrying bla_{TEM-1} on the chromosome (MG::bla_{TEM-1}) or on the plasmid (MG/pBGT) under a range

of ampicillin concentrations (Fig. 1b). In the absence of antibiotics, pBGT was associated with a 5.6% reduction in fitness (one-sample t-test, $P < 0.001$, $t = -9.35$, d.f. = 13), which is similar to the cost produced by natural multicopy plasmids observed in previous studies[4,26]. However, at concentrations of ampicillin above 128 mg l⁻¹ the benefits of carrying pBGT exceeded its costs, and pBGT carriage became highly beneficial at 256 mg l⁻¹. This result highlights the dynamic fitness effects of plasmids depending on the environmental conditions, producing associations with the host bacteria that range from parasitic to symbiotic[10,31].

Plasmid location of bla_{TEM-1} potentiates the evolution of ceftazidime resistance. TEM-1 produces high-level resistance to ampicillin but it has minimal activity against later β-lactam antibiotics. For example, the MIC of ceftazidime, a third-generation cephalosporin, was similar in the parental MG strain (0.25 mg l⁻¹) and in strains carrying TEM-1 (MG::bla_{TEM-1} 0.25 mg l⁻¹; MG/pBGT 0.5–1 mg l⁻¹). One possible benefit conferred by multicopy plasmids could be an increased evolvability of the genes they encode due to the higher number of gene copies per cell. In this case, it is well known that mutations in bla_{TEM-1} can expand the range of activity of TEM-1 to hydrolyse cephalosporins much more efficiently[32], suggesting that pBGT could potentiate the evolution of novel variants of TEM-1. To test this hypothesis, we compared the ability of populations of MG::bla_{TEM-1} and MG/pBGT to evolve resistance to high doses of ceftazidime relative to control populations of strain MG (Fig. 2).

We propagated 48 independent populations of each strain (MG, MG1::bla_{TEM-1} and MG/pBGT) in a lysogeny broth (LB) medium with increasing concentrations of ceftazidime. The experiment started with one-quarter of the MIC of ceftazidime for each parental strain (0.06 mg l⁻¹ for MG and MG::bla_{TEM-1} and 0.25 mg l⁻¹ for MG/pBGT) and 1% of each population was transferred to fresh medium with the concentration of ceftazidime doubled every day for the duration of the experiment. We measured the number of surviving populations over time in all of the treatments and for MG/pBGT we also measured the plasmid stability and copy number in a subset of the populations. In parallel, we propagated 16 control populations of each strain in LB medium with no antibiotics.

The parental MG strain showed a steep decrease in the number of viable populations after day 4, and all populations of this strain went extinct by day 8 of the experiment (8 mg l⁻¹ ceftazidime).

Table 1 | Characteristics of the strains used in this study.

Strain name	Fitness*	PCN†	CMI CAZ (mg l⁻¹)	CMI AMP (mg l⁻¹)
MG	1.003 ± 0.011	NA	0.25	4
MG::bla_{TEM-1}	NA	NA	0.25	512
MG/pBGT	0.943 ± 0.013	19.12 ± 1.56	0.5	8,192
MG::bla_{TEM-1} R146	1.008 ± 0.011	NA	0.5	128
MG/pBGT R164S	0.943 ± 0.003	21.10 ± 0.85	64	4,096
MG/pBGT G54U	0.793 ± 0.019	44.50 ± 3.81	0.5	32,768
MG/pBGT G55U	0.557 ± 0.116	88.93 ± 15.65	1	32,768
MG/pBGT R164S G54U	0.762 ± 0.016	52.30 ± 2.19	128	4,096
MG/pBGT R164S G55U	0.509 ± 0.082	127.29 ± 4.58	128	8,192

NA, not applicable. *Fitness relative to MG::bla_{TEM-1} ± s.e.m, $n = 4$. †Plasmid copy number ± s.e.m, $n = 3$.

The populations of the strain carrying bla_{TEM-1} in the chromosome followed the same trajectory as the wild-type MG controls, demonstrating that carrying a single copy of TEM-1 has little, if any, impact on the ability of populations to evolve elevated cephalosporin resistance (viable populations over concentrations, log-rank test, $P = 0.373$, $\chi^2 = 0.8$, d.f. = 1; Fig. 2). On the other hand, populations of MG/pBGT showed a very different trend compared with the control (viable populations over concentrations, log-rank test, $P < 0.001$, $\chi^2 = 41.7$, d.f. = 2). Although there was also an important initial decrease in surviving populations, a subset of seven populations was able to survive up to a very high dose of ceftazidime: $>4,000\,mg\,l^{-1}$ (Fig. 2). This result clearly demonstrates that carrying bla_{TEM-1} on a multicopy plasmid potentiates the ability of *E. coli* populations to evolve clinically relevant levels of cephalosporin resistance.

Molecular basis of ceftazidime resistance in MG/pBGT. We used deep DNA sequencing from samples of whole populations to investigate the genetic basis of ceftazidime resistance. Specifically, we sequenced populations on the last day that at least 50% of the populations were still viable (day 4 for MG/pBGT and day 5 for MG and MG::bla_{TEM-1}, six random populations per strain). Our rationale for this approach is that it is only possible to make meaningful comparisons across treatments by picking a common time point where all of the treatments can be adequately sampled.

Sequencing results from these populations revealed differences in the mutation profiles of MG/pBGT compared with MG::bla_{TEM-1} and wild-type MG. Populations carrying bla_{TEM-1} on the chromosome presented a very similar mutation profile to the wild-type strain MG, with mutations in chromosomal genes previously related to ceftazidime resistance but no mutations in bla_{TEM-1} (Supplementary Data 1). Changes on chromosomal genes included non-synonymous mutations and indels in the transcriptional repressors of active drug efflux systems such as *marR*, *acrR* and *baeRS*, and also in the regulator system *envZ-ompR*, which activates the expression of the outer-membrane porin proteins OmpF and OmpC[33–35]. Populations belonging to the plasmid-bearing MG/pBGT strain carried some mutations similar to the ones described above, such as non-synonymous mutations in *envZ-ompR* or in *ompF*. However, unlike MG::bla_{TEM-1}, four out of six plasmid-carrying populations also carried mutations in bla_{TEM-1}. Mutations in TEM-1 were located in residues 164 (R164S, R164C, R164H), 166 (E166K) and 179 (D179G), which have previously been reported to be responsible for ceftazidime resistance *in vivo* and *in vitro*[32,36,37]. The presence of TEM-1 mutations is consistent with the idea that plasmids accelerate evolution by increasing the supply of mutations in functionally important plasmid-carried genes.

In order to understand the specific mechanisms that drive high-level resistance in MG/pBGT populations, we sequenced a time series of samples from the seven populations that survived up to $4,096\,mg\,l^{-1}$ of ceftazidime (days 0, 4, 8, 12 and 16; Supplementary Data 1). Selective sweeps of bla_{TEM-1} mutations occurred in all seven populations, demonstrating a link between modification in the β-lactamase and high-level ceftazidime resistance (Fig. 3c). Moreover, TEM-1 mutations were already present at detectable frequencies in these populations at day 4, suggesting that early TEM-1 mutations played a key role in determining evolvability in our experiment. Two specific mutations in TEM-1 were present in the seven populations that reached high-level ceftazidime resistance: R164S (5/7 populations) or R164H (2/7 populations)[32] (Fig. 3c).

To help differentiate between mutations that are associated with increased resistance, as opposed to general adaptation to the laboratory or hypermutable regions, we also sequenced six control populations of each strain that were evolved for 16 days in the LB medium with no antibiotics. None of the six control populations propagated in LB for 16 days showed mutations in any of the chromosomal genes associated with ceftazidime resistance or in bla_{TEM-1} (Supplementary Data 1).
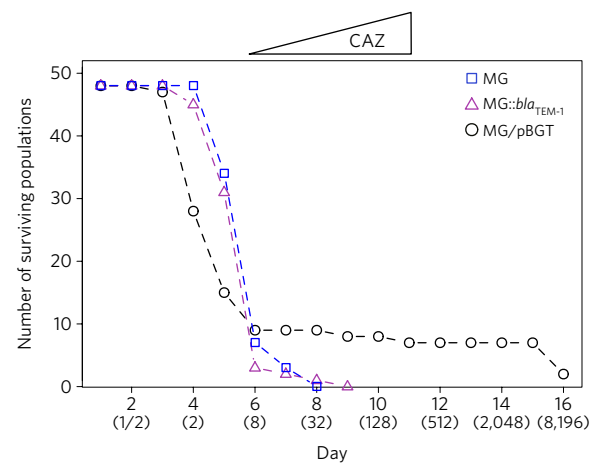
Figure 2 | Survival curves under increasing concentrations of ceftazidime. Plasmid-carrying populations are able to survive up to a very high level of ceftazidime (CAZ) concentration. The numbers of viable populations that belong to strains MG1655, MG1655::bla_{TEM-1}, and MG1655/pBGT over time are shown. Forty-eight populations of each strain were propagated under increasing concentrations of ceftazidime, starting with one-quarter of the MIC (day 1; $0.06\,mg\,l^{-1}$ for MG and MG::bla_{TEM-1} and $0.25\,mg\,l^{-1}$ for MG/pBGT) and doubling ceftazidime concentration every day. Only populations carrying plasmid pBGT are able to survive up to high-level concentrations of ceftazidime, with seven populations viable at $4,096\,mg\,l^{-1}$ of ceftazidime. Populations carrying a chromosomal copy of bla_{TEM-1} behave as the wild-type populations and go extinct before surpassing the clinical breakpoint of ceftazidime resistance. The numbers in parentheses indicate the dosage in terms of the MIC.

Increase in plasmid copy number is associated with mutations in the RNAI of pBGT. Gene amplification has been demonstrated to play an important role in bacterial evolution, including antibiotic resistance[38,39]. To test the role of gene amplification in evolution, we measured the copy number of pBGT through time. The evolution of high-level ceftazidime resistance was associated with an increase in pBGT copy number and in extreme cases the copy number increased approximately tenfold (Fig. 3a). Analysis of the pBGT sequences revealed that the increase in the copy number was associated with the rise of mutations near the origin of replication (*oriV*) of the plasmid at days 12 and 16 (Fig. 3). These mutations were located in a strategic region that codes for the small RNAs that are responsible for the control of the replication and copy number of ColE1 plasmids: RNAI and RNAII[40]. Briefly, RNAII acts as the primer precursor for plasmid replication and RNAI binds to RNAII blocking the initiation of replication. These RNAs are coded in opposite strands of the same DNA region and they present a secondary structure of three consecutive hairpins (Fig. 3b), recognizing each other by complementarity[41]. The two most frequent mutations in the *oriV* of pBGT were placed in the loop of the central hairpin of RNAI, which is the first binding site between RNAI and RNAII[42]. Both mutations produced a G to U change in one of two contiguous residues at positions 54 and 55 in the RNAI molecule (Fig. 3b). Interestingly, G/C to A/U mutations in residues in the loop of the central hairpin of RNAI in ColE1-type plasmids are known to be responsible for a reduction in the binding rate constant between RNAI and RNAII due to a lower strength of the initial recognition between the loops[41]. This lower affinity between the two RNA molecules leads to an increase in the initiation of replication and a subsequent increase in the plasmid copy number[43]. The remaining mutations in RNAI were present in the stem region of the three different hairpins (Fig. 3). These mutations probably affected the secondary structure of RNAI, leading to a de-repression of plasmid replication[43].
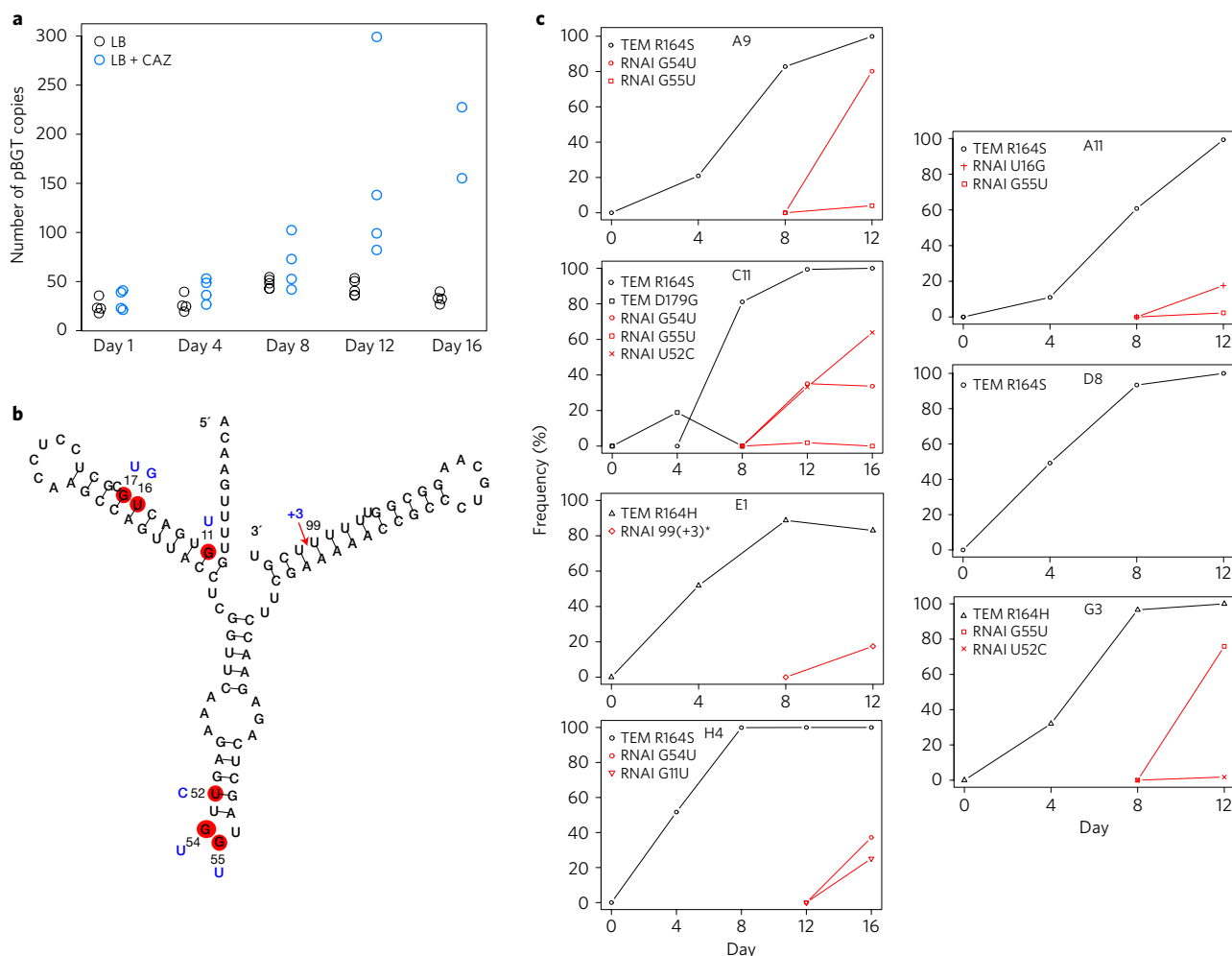
Figure 3 | pBGT mutations in the ceftazidime resistant populations. Plasmid-carrying populations acquire mutations in bla_{TEM-1} followed by mutations in the RNAI of pBGT. **a**, The average copy number of plasmid pBGT per cell in populations of MG/pBGT over time. The plasmid copy number of MG/pBGT populations evolving with ceftazidime at day 12 and 16 showed a significant increase compared with those in the control MG/pBGT populations propagated with no antibiotics (t-test, day 12, $P < 0.001$, $t = 4.89$, day 16 $P < 0.01$, $t = 5.18$, d.f. = 1). **b**, Secondary structure of RNAI predicted using RNAfold software (see Methods). Residues highlighted in red are those where mutations were found during the experimental evolution. The changes produced by those mutations are indicated by blue letters. **c**, Frequency of the mutations in TEM-1 and RNAI in the seven populations of MG/pBGT that reached very-high-level ceftazidime resistance during the experimental evolution.

Reconstruction of ceftazidime resistance and high plasmid copy number mutations. Our sequencing results suggested that plasmid carriage of TEM-1 increases the ability of populations to evolve high levels of ceftazidime resistance by facilitating the evolution of novel TEM-1 variants that are capable of hydrolysing ceftazidime more effectively. To test this idea, we reconstructed the most commonly observed TEM-1 substitution (R164S) into both the MG::bla_{TEM-1} and the MG/pBGT ancestral strains (Fig. 4a). Inserting this mutation into the chromosomal copy of bla_{TEM-1} resulted in a modest twofold increase in ceftazidime resistance compared with MG::bla_{TEM-1} (and with MG; Table 1). In contrast, adding mutation R164S into the pBGT plasmid increased ceftazidime resistance 128-fold compared with MG/pBGT (MIC = 64 mg l^{-1}). Importantly, this increase in resistance easily surpasses the clinical breakpoint for ceftazidime resistance (8 mg l^{-1})[44] (Fig. 4b, Table 1). This difference in MIC between plasmid and chromosomal location of the β-lactamase mutation is due to a gene dosage effect previously observed for these enzymes[28,29].

Pleiotropic costs could potentially reduce the fitness benefit associated with resistance mutations in high-copy-number plasmids. For example, R164S substitutions in TEM-1 decrease ampicillin resistance as a result of antagonistic pleiotropiy (Table 1, Supplementary Fig. 1)[45].

However, we did not find any fitness cost in the absence of antibiotics associated with introducing substitution R164S into either the chromosomal (two-sample t-test, $P = 0.654$, $t = -0.47$, d.f. = 5.9) or plasmid-carried TEM-1 (two-sample t-test, $P = 0.282$, $t = -1.12$, d.f. = 14.9) (Fig. 4d, Table 1). Collectively, these results demonstrate that plasmid pBGT accelerates the evolution of ceftazidime resistance by massively amplifying the increase in resistance associated with TEM-1 mutations. Importantly, this effect is sufficient to explain how plasmid carriage allows populations to evolve levels of ceftazidime resistance that are above the clinical breakpoint.

A second key insight of our sequencing results is that high-level ceftazidime resistance repeatedly evolves by a process of TEM-1 modification followed by an increase in the copy number of pBGT associated to mutations in the RNAI. To check whether the fitness benefits associated with an increased plasmid copy number were dependent on the previous modification of TEM-1, we reconstructed the two most common mutations in RNAI (G54U and G55U) into strains MG/pBGT and MG/pBGT R164S (Fig. 4a). Introducing mutations into RNAI led to large increases in the plasmid copy number, ranging from 40 to 130 copies per cell, confirming the role of these mutations in increased pBGT copy number (Fig. 4c,
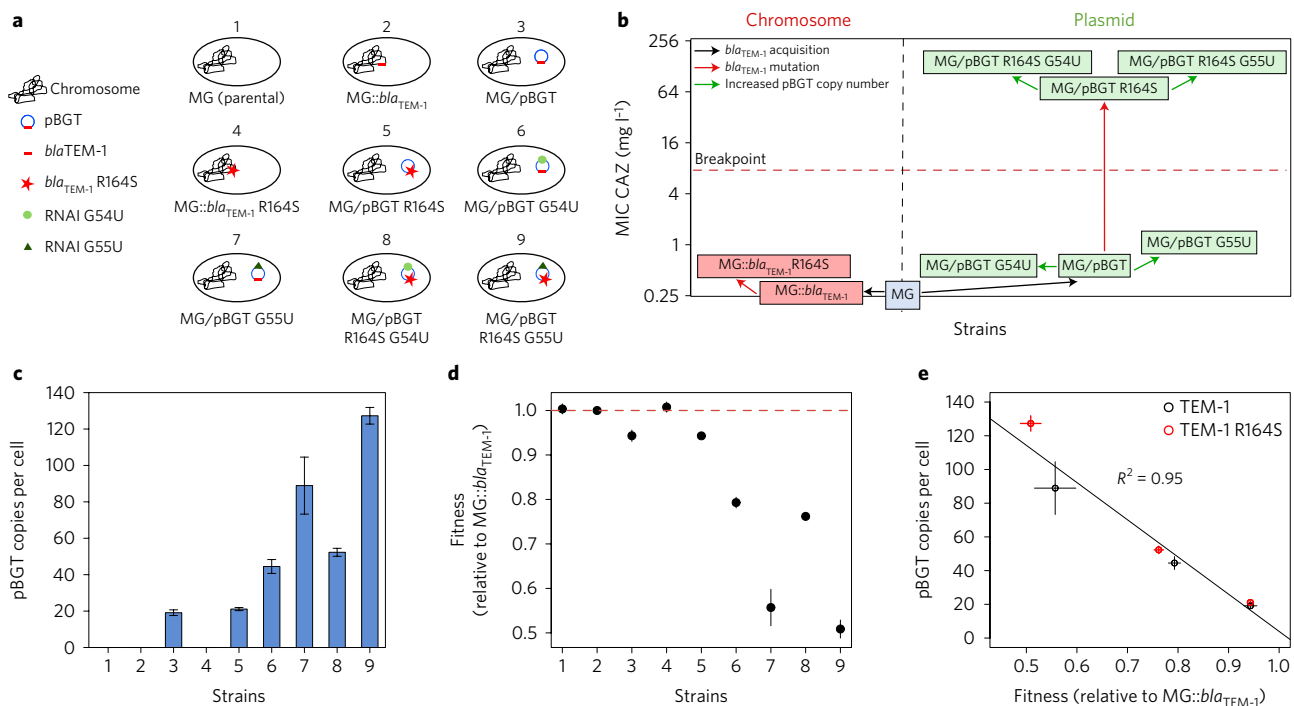
Figure 4 | Characterization of the mutations in bla_{TEM-1} and RNAI. The effects of the most common mutations observed during the experimental evolution in TEM-1 (R164S) and the RNAI (G54U, G55U) reconstructed in the parental MG1655 strain are shown. **a**, Schematic of the different strains constructed from *E. coli* MG1655 (MG). **b**, The level of ceftazidime (CAZ) resistance of each strain represented on a log scale. Note that bla_{TEM-1} does not confer much resistance to CAZ. R164S mutation in TEM-1 increases resistance to CAZ but only confers resistance level above the MIC (8 mg l⁻¹, red dotted line) when it is encoded on the plasmid. Also note that these MIC values of these clones are lower than the final concentration of ceftazidime that were tolerated during the selection experiment. We argue that this difference was caused by two factors: first, the evolving MG/pBGT populations carried additional chromosomal mutations related to ceftazidime resistance, which probably contributed to the increased resistance level (Supplementary Data 1). Second, it is known that β-lactamase-mediated resistance increases with cell density (inoculum effect)[30], and the standard MIC techniques use initial cell densities 20-times lower than those used during the selection experiment. The estimates of MIC should therefore be considered as relative, rather than absolute, measures of resistance. **c**, Average copy number of pBGT per cell (± s.e.m., $n = 3$). **d**, Fitness relative to MG::bla_{TEM-1} (± s.e.m., $n = 4$) of the different constructs. **e**, Comparison of the relative fitness of the different plasmid-bearing constructs and their plasmid copy number (± s.e.m.). Note that the plasmid copy number strongly correlates with the reduction in fitness.

Table 1). In contrast to the massive increases in resistance associated with TEM-1 substitutions, the increased copy number had subtle effects on ceftazidime resistance, leading to only twofold increases (or less) in MIC (Fig. 4b, Table 1). The increased copy number was associated with profound fitness costs in the absence of antibiotics (Fig. 4d, Table 1); fitness declined at a rate of approximately 0.4% per plasmid copy (Fig. 4e, Pearson's test, $t = -8.56$, d.f. = 4, $P = 0.001$, $R^2 = 0.95$). From these results it is clear that there is little, if any, impact of TEM-1 mutation on the fitness benefits associated with pBGT amplification. Instead, our results support the idea that TEM-1 mutations preceded pBGT amplification simply because TEM-1 mutations resulted in large increases in resistance without imposing any additional fitness burden on the cell. We argue that the very high cost associated with the elevated plasmid copy number mutants will probably reduce the likelihood of finding these genotypes in nature.

Discussion

Our results demonstrate that multicopy plasmids provide an evolutionary advantage above and beyond mediating HGT. In the short term, carrying bla_{TEM-1} on a multicopy plasmid (pBGT) is associated with an increase in fitness under conditions of strong selection for β-lactam resistance. In the long term, plasmid pBGT acts as an evolutionary catalyst that facilitates the evolution of novel variants of bla_{TEM-1} and allows bacterial populations to evolve clinically relevant levels of ceftazidime resistance. Because pBGT is a multicopy plasmid, carrying bla_{TEM-1} on the plasmid ensures an increased rate of supply

of beneficial mutations in bla_{TEM-1} with an improved rate of ceftazidime hydrolysis. The phenotypic effect of these mutations is then amplified by increased expression arising from the constitutive high copy number of the pBGT plasmid (19 copies per cell). This step of amplification of the mutation due to the multicopy nature of the plasmid is crucial, as shown by the fact that the same bla_{TEM-1} mutation that increases the MIC of ceftazidime over the clinical breakpoint when encoded in the plasmid, has very little effect when cloned as a single copy in the chromosome (Fig. 4).

Gene amplification and modification are thought to play key roles in the evolution of novel bacterial phenotypes[38,39,46–48]. We argue that pBGT is such an effective evolutionary catalyst because it provides a vehicle for the simultaneous modification of bla_{TEM-1} and expansion in the frequency of the mutated allele. This platform provides an enhanced 'gene duplication-amplification' system for adaptive evolution[48], which aligns with the 'amplification mutagenesis' model proposed by Hendrickson and colleagues[19]. Although it is easy to understand how multicopy plasmids accelerate evolution by increased mutation supply using classical population genetics, it is more challenging to understand the amplification of plasmid-carried mutations. An important consequence of the partitioning of small plasmids at cell division is that copies of plasmids carrying beneficial mutations are randomly distributed between daughter cells. When combined with plasmid replication, this random partitioning ensures that a substantial fraction of the daughter cells have an increased number of copies of beneficial mutations relative to their mother cells (Supplementary Fig. 2). Crucially, this amplification

of beneficial mutations by random segregation is expected to occur at a much greater rate than by the spontaneous duplication of genes carrying beneficial mutations. Selection will subsequently fuel the spread of the cells that carry a higher frequency of plasmid-born mutated alleles, driving the rapid increase in frequency of the mutation at both the cellular and population level in a few generations (Supplementary Fig. 2). However, the dynamics of this process are potentially very complex, and we are currently developing deterministic population dynamics models and stochastic agent-based simulations to better understand this process.

One important feature of our experimental design is that we used a rapid ramp-up of antibiotics, which may have reduced the accessibility to alternative evolutionary trajectories to high-level β-lactam resistance[49], and this may have maximized the evolutionary benefit conferred by pBGT. However, we argue that our experimental design is relevant in the context of evolution of antibiotic resistance because sharp temporal and spatial gradients in antibiotic concentrations are common in patients[50]. Another limitation of this study is the fact that pBGT carries three extra genes apart from bla_{TEM-1} (Fig. 1). However, pBGT mutations were located in bla_{TEM-1} or the RNAI, so there is no evidence indicating a possible role for the extra genes on the evolution of ceftazidime resistance.

In this work we used TEM-1 as model system, but we argue that our results have general implications for the evolution of antibiotic resistance, adaptation and innovation in bacteria. Small multicopy plasmids are very common in pathogenic bacteria[12,28] and they frequently carry antibiotic resistance genes[20–22,26], but their role in the ecology and evolution of antibiotic resistance has generally been overlooked. Our work shows that these genetic elements could accelerate the evolution of resistance *in vivo*. Further work will be required to test this possibility.

Methods

Culture conditions and antibiotic susceptibility testing. The bacterial strains, plasmids and primers used in this study are listed in Supplementary Tables 1–3. Bacterial strains were cultured in LB broth at 37 °C in 96-well plates with continuous shaking (225 r.p.m.) and on LB agar plates at 37 °C (Fisher Scientific). Minimal inhibitory concentrations (MIC) were determined in LB (as the experimental evolution assay) following Clinical and Laboratory Standards Institute guidelines[51]. To determine the different MICs, we performed six biological replicates for each strain, and we used the mode of the results as the MIC value.

Construction of the experimental system. The *E. coli* MG1655 strain was used for all of our constructions. Chromosomal insertion of bla_{TEM-1} was designed to take place in the integration site of the λ phage (*attB*)[52] as follows: the bla_{TEM-1} gene was amplified from plasmid p3938[53] with primers 3658 and 3659. We PCR-amplified the regions at both sides of the chromosomal *attB* site (primers 3652–3653 and 3654–3655) and fused them to the bla_{TEM-1} PCR product using Gibson Assembly[54]. We electroporated the fusion of these fragments into an MG1655 strain containing the pKOBEG plasmid[55]. This thermo-sensitive plasmid contains the λ Red machinery, allowing the production of homology-based allelic exchanges between the chromosome and PCR products[56]. Selection for carbenicillin resistance at 37 °C yielded clones in which bla_{TEM-1} had been integrated in *attB* and pKOBEG was lost. Verification of bla_{TEM-1} sequence and pKOBEG loss gave rise to strain MG::bla_{TEM-1}. The allele of bla_{TEM-1} coding for R164S mutation was introduced in the non-evolved strain using the same methodology.

To assess the evolvability of genes when located on plasmids, we cloned bla_{TEM-1} in p3655 (pSU18T-pBAD*gfp2*, ColE1-type origin of replication[57]) producing plasmid pBGT. To do so, we phosphorylated the PCR product of primers 4304–4305 (containing bla_{TEM-1}), and ligated it to the PCR-amplified backbone of p3655 (primers 3086–738). Ligations were transformed into *E. coli* DH5-α and selected on carbenicillin and chloramphenicol then tested for *gfp* induction with ʟ-arabinose. Sequence of bla_{TEM-1} was verified. pBGT was transformed into the parental MG1655 producing the strain MG/pBGT.

The combination of evolved replication origins with wild-type bla_{TEM-1} alleles in pBGT was performed using Gibson assembly. Briefly, replication origins were amplified using primers 837 and 296 and assembled to the backbone of pBGT amplified with primers 737 and 1701. Verification of the sequence of the *oriV* of the plasmids together with the selection on carbenicillin, chloramphenicol and ʟ-arabinose (to induce GFP expression) allowed confirmation of the functionality of all plasmid components.

Competitive fitness assays. The fitness of each clone was determined basically as described in San Millan and colleagues[9]. We performed competitions between a plasmid-bearing clone and a plasmid-free clone in each case (MG/pBGT and MG::bla_{TEM-1} were the control strains to compete against). Four biological replicates of the competition (of four technical replicates each) were performed for each clone. Pre-cultures of the clones were incubated at 37 °C with 225 r.p.m. shaking overnight in 96-well plates carrying 200 μl of LB broth per well (Fisher Scientific). Pre-cultures were diluted 400-fold in 200 μl of fresh LB and mixed at a ratio of approximately 50% of each clone. The exact initial proportions were confirmed via flow cytometry using an Accuri C6 Flow Cytometer Instrument (BD Accuri, San Jose) with the following parameters: flow rate: 66 μl min⁻¹, core size: 22 μm, events recorded per sample: 10,000. To measure these proportions we incubated an aliquot of the mix in LB with ʟ-arabinose 0.25% for 2 h to induce GFP expression from pBGT. Mixtures were competed for 24 h in LB at 37 °C with 225 r.p.m. shaking (approximately eight generations). Again, the final proportion was measured by flow cytometry as described above. The fitness of the strain carrying the plasmid relative to the plasmid-free strain was determined using the formula:

$$W_{p+} = \frac{\ln\left(\frac{N_{final,p+}}{N_{initial,p+}}\right)}{\ln\left(\frac{N_{final,p-}}{N_{initial,p-}}\right)}$$

where W_{p+} is the relative fitness of the plasmid-bearing clone, $N_{initial,p+}$ and $N_{final,p+}$ are the numbers of cells of the plasmid-carrying clone before and after the competition, and $N_{initial,p-}$ and $N_{final,p-}$ are the numbers of cells of the pBGT-free clone before and after the competition. As a control, MG/pBGT and MG::bla_{TEM-1} were competed in every experiment.

Experimental evolution. We cultured strains (MG, MG/pBGT and MG::bla_{TEM-1}) on LB agar plates at 37 °C to obtain isolated colonies. We inoculated single colonies in alternative wells of 96-well plates containing LB broth (48 populations per plate, one plate per strain). We used this checkerboard plate design of intercalating inoculated wells with bacteria-free medium to prevent and control for cross-contamination in the plates. We started the experimental evolution assay inoculating 2 μl of the initial overnight cultures in 198 μl of LB with one quarter of the MIC of ceftazidime of each strain. Every day we transferred 2 μl of the overnight culture into 198 μl of fresh medium with double the concentration of ceftazidime of the day before (approximately 6–7 generations per day). In parallel, we propagated 16 control populations of each strain in the same conditions as previously described but in the absence of ceftazidime. Populations with positive growth were defined by an optical density value (measured overnight at a wavelength of 600 nm) higher than 0.1. We kept a frozen stock of all of the populations from day 0, 4, 8, 12 and 16. On those days we also checked the plasmid stability by flow cytometry (using ʟ-arabinose induction, as explained in the previous section) and performed DNA extraction to quantify plasmid copy number in the population (see section below), from four populations belonging to MG/pBGT. We also measured the plasmid stability every day from 10 of the populations of MG/pBGT evolving in the absence of antibiotics.

Quantification of plasmid copy number. The copy number of pBGT was determined by quantitative polymerase chain reaction (qPCR) using an ABI StepOnePlus Real-Time PCR System (Life Technologies). DNA extraction, quantification and digestion (with *Bam*HI-HF, New England Biolabs) were performed as previously described[27]. We developed a specific qPCR for pBGT (pBGT-F: ACATTTCCGTGTCGCCCTT, pBGT-R: CACTCGTGCACCCAACTGA, amplicon size: 115 bp, efficiency: 94.29%, $R^2 = 0.99$) and we used a previously described qPCR for the *dxs* chromosomal monocopy gene (dxs-F: CGAGAA ACTGGCGATCCTTA, dxs-R: CTTCATCAAGCGGTTTCACA, amplicon size: 113 bp, efficiency: 95.52%, $R^2 = 0.99$)[58] to compare the ratio of plasmid and chromosomal DNA. The efficiency of the reactions was calculated from the standard curve generated by performing qPCR with four eightfold dilutions of template DNAs in triplicate (approximately 5 ng μl⁻¹ to 12 pg μl⁻¹ working range of DNA concentration). qPCRs were performed using ABI SYBR Select Master Mix (Life Technologies) at a final DNA concentration of 0.1 ng μl⁻¹ and following manufacturers' instructions. The amplification conditions were: initial denaturation for 2 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. After the amplification was complete and to control for the specificity of the reaction, a melting curve analysis was performed by cooling the reaction to 60 °C and then heating slowly to 95 °C. Inter-run calibration samples were used to normalize the results from different plates of each qPCR. The copy number was calculated using the ΔΔC$_T$ method as previously described, given that the amplification efficiencies of the target and reference genes were approximately equal[58]. The average plasmid copy number was corrected using the plasmid stability frequencies, which were measured from the cultures used to obtain the DNA samples.

Genome sequencing and bioinformatic analysis. We sequenced six random populations from each strain from the experimental evolution assay on the last day

when at least 50% of the populations were still viable. We also sequenced a time series of samples from the seven MG/pBGT populations that survived up to 4,096 mg l⁻¹ of ceftazidime (days 0, 4, 8, 12 and 16) and nine MG/pBGT clones from three populations with increased plasmid copy number. Finally, we also sequenced six control populations of each strain evolved for 16 days in LB medium with no antibiotics (Supplementary Data 1). DNA extractions were performed from 2 ml of LB broth (Fisher Scientific) cultures incubated at 37 °C with 225 r.p.m. shaking overnight using the Promega Wizard Genomic 4 DNA Purification Kit (Promega). DNA was quantified using the QuantiFluor dsDNA system (Promega), following manufacturers' instructions. All sequencing was conducted at the Wellcome Trust Centre for Human Genetics using the Illumina HiSeq2500 platform. Mutations were predicted using the breseq 0.26.1 pipeline[59,60], using polymorphism mode to estimate the frequency of mutations in populations. Mutations classes included point mutations, small (<50 bp) and large (>50 bp) indels, copy number variation and insertion-sequence-element insertions[61]. Reads were aligned to the *E. coli* MG1655 reference genome (NC_000913.3), the TEM-1 chromosomal insertion (this study), and the pBGT plasmid genome (this study). Variants that never surpassed 10% frequency in any population were filtered from the dataset. Mutations that were present in the ancestral clones relative to the reference sequence were also excluded, leaving only mutations that accumulated throughout the experiment. We sequenced the DNA from the three parental strains and from a total of 51 DNA samples obtained from evolving populations (Supplementary Data 1). In addition, we sequenced DNA samples from clones of plasmid-bearing populations showing especially high plasmid copy numbers (three clones/populations from three different populations: nine clones in total; Supplementary Data 1), to successfully confirm the presence of the mutations observed in the populations. Two out of the 63 samples were discarded due to results showing possible DNA contamination: one sample from MG and the other from MG::*bla*_TEM-1, both of them from populations at day 5.

The structure of the RNAI of plasmid pBGT was predicted using the default parameters of RNAfold software[62]. The result was compared with previous reports of the structure of this molecule[41,43] from *oriV* of almost identical ColE1 plasmids, confirming the homology.

Statistical analysis. Analyses were performed using RStudio (Version 0.99.486). Comparisons among strains were performed using two-sample *t*-tests. Survival of populations over time under increasing concentration of ceftazidime was analysed using log-rank tests. The variation of the plasmid copy number in MG/pBGT populations over time was modelled using a generalized linear effects model, with time and treatments (and their interaction) as factors and assuming Gaussian error distribution.

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Author contributions

A.S.M. and R.C.M. were responsible for the conceptualization of this study; A.S.M., J.A.E., and D.R.G. designed the methodology; formal analysis was by D.R.G.; A.S.M. and J.A.E. carried out the investigations; A.S.M. and R.C.M. prepared the original draft of the manuscript and also undertook the reviewing and editing; R.C.M. and D.M. were responsible for funding acquisition and for supervision; D.M. oversaw the resources; project administration was performed by A.S.M.

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Competing interests

The authors declare no competing financial interests.