Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria

Alvaro San Millan [1,2,*], Jose Antonio Escudero [3,4Ψ], Danna R Gifford [1,Ψ], Didier Mazel [3,4], R Craig MacLean [1].

[1] Department of Zoology, University of Oxford. OX1 3PS, Oxford, United Kingdom.

[2] Department of Microbiology, Hospital Universitario Ramon y Cajal (IRYCIS). 28034, Madrid, Spain.

[3] Institut Pasteur, Unité de Plasticité du Génome Bactérien, Département Génomes et Génétique, 28 Rue du Dr. Roux, 75015, Paris, France.

[4] CNRS, UMR3525, 28 Rue du Dr. Roux, 75015, Paris, France.

[Ψ] These authors contributed equally to this study.

* Correspondence: alvaro.sanmillan@hrc.es

Abstract

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 *E. coli* carrying bla_{TEM-1} β-lactamase gene on either the chromosome or a multicopy plasmid (19/cell) with increasing concentrations of ceftazidime. The plasmid accelerated resistance evolution by increasing the rate of appearance of novel TEM-1 mutations conferring resistance to ceftazidime, and then by amplifying the effect of TEM-1 mutations due to 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 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 remains challenging to understand how plasmids persist in bacterial populations. Classical theoretical models predict that horizontal transfer 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 produced by plasmids [8-10], allowing plasmids to stably

persist for hundreds of generations, even in the absence of horizontal transfer [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 lacking 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 to 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 number existing 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 host bacteria to new selective pressures.

In this paper, 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 antibiotic resistance evolution in particular. We inserted bla_{TEM-1} in the bacterial chromosome to generate *E. coli* MG1655::bla_{TEM-1} (MG::bla_{TEM-1}, see methods). In parallel we constructed a non-transmissible multicopy plasmid carrying bla_{TEM-1} with an identical promoter (Figure 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, SD=3.8, n=6), which is similar to the copy number of other natural small multicopy plasmids [26,27]. This experimental system allowed us to evaluate the effects of plasmid versus chromosomal location of bla_{TEM-1} in isogenic strains.

Characterization of plasmid and chromosome bla_{TEM-1}-mediated resistance

Previous studies have shown that increasing the dosage of the bla_{TEM-1} gene 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 and it increased to 512 mg/L in MG::bla_{TEM-1} and to 8,192 mg/L in MG/pBGT.

Therefore, even if *bla*$_{TEM-1}$ conferred high-level ampicillin resistance both in the chromosomal and plasmid location, the multicopy plasmid bearing strain has a 16-fold increase in MIC compared to 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 (Figure 1b). The presence of *bla*$_{TEM-1}$ in the chromosome produced no cost in MG in the absence of antibiotics (w= 0.997, Two-sample *t*-test, *P*= 0.343, t= 1, df= 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 (Figure 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, df= 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 the benefits of carrying pBGT exceeded its costs, and pBGT carriage became highly beneficial at 256 mg/L. 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 beta-lactam antibiotics. For example, the MIC of ceftazidime, a 3rd generation cephalosporin, was similar in the parental MG strain (0.25 mg/L) and in strains carrying TEM-1 (MG::*bla*$_{TEM-1}$ 0.25mg/L; MG/pBGT 0.5-1mg/L). 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 (Figure 2).

We propagated 48 independent populations of each strain (MG, MG1::bla_{TEM-1} and MG/pBGT) in LB medium with increasing concentrations of ceftazidime. The experiment started with ¼ of the MIC of ceftazidime for each parental strain (0.06 mg/L for MG and MG::bla_{TEM-1} and 0.25 mg/L for MG/pBGT) and 1% of each population was transferred to fresh medium with doubling concentration of ceftazidime every day. We measured the number of surviving populations over time in all the treatments and, for MG/pBGT, we also measured plasmid stability and copy number in a subset of 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 ceftazidime). 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, χ^2= 0.8, df=1, Figure 2). On the other hand, populations of MG/pBGT showed a very different trend compared to the control (viable populations over concentrations, log-rank test, P< 0.001, χ^2= 41.7, df=2). Although there was also an important initial decrease in surviving populations, a subset of 7 populations was able to survive up to very high dose of ceftazidime: >4000 mg/L (Figure 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 when 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 to 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 nonsynonymous mutations and indels in the transcriptional repressors of active drug efflux systems such as *marR* and *acrR* and *baeRS*, and also in the regulator system *envZ-ompR*, which activates the expression of 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 nonsynonymous mutations in *envZ-ompR*, or in *ompF.* However, unlike MG::bla_{TEM-1}, 4 out of 6 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 been previously reported as 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 driving high-level resistance in MG/pBGT populations, we sequenced a time series of samples from the 7 populations that survived up to 4,096 mg/L of ceftazidime (days 0, 4, 8, 12 and 16, Supplementary Data 1). Selective sweeps of bla_{TEM-1} mutations occurred in all 7 populations, demonstrating a link between modification in the β-lactamase and high-level ceftazidime resistance (Figure 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 7 populations that reached high-level ceftazidime resistance: R164S (5/7 populations) or R164H (2/7 populations) [32] (Figure 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 evolved for 16 days in 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).

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 copy number increased approximately 10 fold (Figure 3a). Analysis of pBGT sequences revealed that the increase in copy number was

196 associated with the rise of mutations near the origin of replication (*oriV*) of the
197 plasmid at days 12 and 16 (Figure 3). These mutations were located in a strategic
198 region, which codes for the small RNAs responsible for the control of replication and
199 copy number of ColE1 plasmids: RNAI and RNAII [40]. Briefly, RNAII acts as the primer
200 precursor for plasmid replication and RNAI binds to RNAII blocking the initiation of
201 replication. These RNAs are coded in opposite strands of the same DNA region and
202 they present a secondary structure of three consecutive hairpins (Figure 3b),
203 recognizing each other by complementarity [41]. The two most frequent mutations in
204 the *oriV* of pBGT were placed in the loop of the central hairpin of RNAI, which is the
205 first binding site between RNAI and RNAII [42]. Both mutations produced a G to U
206 change in one of two contiguous residues at positions 54 and 55 in the RNAI
207 molecule (Figure 3b). Interestingly, G/C to A/U mutations in residues in the loop of
208 the central hairpin of RNAI in ColE1-type plasmids are known to be responsible for a
209 reduction in the binding rate constant between RNAI and RNAII due to a lower
210 strength of the initial recognition between the loops [41]. This lower affinity between the
211 two RNA molecules leads to an increase in the initiation of replication and a
212 subsequent increase in plasmid copy number [43]. The remaining mutations in RNAI
213 were present in the stem region of the three different hairpins (Figure 3). These
214 mutations probably affected the secondary structure of RNAI, leading to a de-
215 repression of plasmid replication [43].

216 *Reconstruction of ceftazidime resistance and high plasmid copy number mutations*

217 Our sequencing results suggested that plasmid carriage of TEM-1 increases the
218 ability of populations to evolve high levels of ceftazidime resistance by facilitating the
219 evolution of novel TEM-1 variants that are capable of hydrolysing ceftazidime more
220 effectively. To test this idea, we reconstructed the most commonly observed TEM-1
221 substitution (R164S) into both the MG::$bla_{\text{TEM-1}}$ and the MG/pBGT ancestral strains
222 (Figure 4a). Inserting this mutation into the chromosomal copy of $bla_{\text{TEM-1}}$ resulted in

223 a modest 2-fold increase in ceftazidime resistance compared to MG::*bla*$_{TEM-1}$ (and to
224 MG, Table 1). In contrast, adding mutation R164S into the pBGT plasmid increased
225 ceftazidime resistance 128 fold compared to MG/pBGT (MIC=64 mg/L). Importantly,
226 this increase in resistance easily surpasses the clinical breakpoint for ceftazidime
227 resistance (8 mg/L)[44] (Figure 4b, Table 1). This difference in MIC between plasmid
228 and chromosomal location of the β-lactamase mutation is due to a gene dosage
229 effect previously observed for these enzymes [28,29].

230 Pleiotropic costs could potentially reduce the fitness benefit associated with
231 resistance mutations in high copy number plasmids. For example, R164S
232 substitutions in TEM-1 decrease ampicillin resistance as a result of antagonistic
233 pleiotropiy (Table 1, Supplementary Figure 1) [45]. However, we did not find any fitness
234 cost in the absence of antibiotics associated with introducing substitution R164S into
235 either the chromosomal (Two sample t-test, *P*= 0.654, t= -0.47, df= 5.9) or plasmid-
236 carried TEM-1 (Two sample t-test, *P*= 0.282, t= -1.12, df= 14.9) (Figure 4d, Table 1).
237 Collectively, these results demonstrate that plasmid pBGT accelerates the evolution
238 of ceftazidime resistance by massively amplifying the increase in resistance
239 associated with TEM-1 mutations. Importantly, this effect is sufficient to explain how
240 plasmid carriage allows populations to evolve levels of ceftazidime resistance that
241 are above the clinical breakpoint.

242 A second key insight of our sequencing results is that high level ceftazidime
243 resistance repeatedly evolves by a process of TEM-1 modification followed by an
244 increase in the copy number of pBGT associated to mutations in the RNAI. To check
245 if the fitness benefits associated with an increased plasmid copy number were
246 dependent on the previous modification of TEM-1, we reconstructed the two most
247 common mutations in RNAI (G54U and G55U) into strains MG/pBGT and MG/pBGT
248 R164S (Figure 4a). Introducing mutations into RNAI led to large increases in plasmid
249 copy number, ranging from 40 to 130 copies per cell, confirming the role of these

mutations in increased pBGT copy number (Figure 4c, Table 1). In contrast to the massive increases in resistance associated with TEM-1 substitutions, increased copy number had subtle effects on ceftazidime resistance, leading to up to only 2 fold increases in MIC (Figure 4b, Table 1). Increased copy number was associated with profound fitness costs in the absence of antibiotics (Figure 4d, Table 1); fitness declined at a rate of approximately 0.4% per plasmid copy (Figure 4e, Pearson's test, t= -8.56, df = 4, p-value = 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 horizontal gene transfer. Over 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 ß-lactam resistance. Over the long term, plasmid pBGT acts as 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/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

277 breakpoint when encoded in the plasmid, has very little effect when cloned as a
278 single copy in the chromosome (Figure 4).

279 Gene amplification and modification are thought to play key roles in the evolution of
280 novel bacterial phenotypes [38,39,46-48]. We argue that pBGT is such an effective
281 evolutionary catalysts because it provides a vehicle for the simultaneous modification
282 of bla_{TEM-1} and expansion in the frequency of the mutated allele. This platform
283 provides an enhanced "gene duplication-amplification" system for adaptive evolution
284 [48], which aligns with the "amplification-mutagenesis" model proposed by Hendrickson
285 et al [19]. Although it is easy to understand how multicopy plasmids accelerate
286 evolution by increased mutation supply using classical population genetics, it is more
287 challenging to understand the amplification of plasmid-carried mutations. An
288 important consequence of the partitioning of small plasmids at cell division is that
289 copies of plasmids carrying beneficial mutations are randomly distributed between
290 daughter cells. When combined with plasmid replication, this random partitioning
291 ensures that a substantial fraction of daughter cells have an increased number of
292 copies of beneficial mutations relative to their mother cells (Supplementary Figure 2).
293 Crucially, this amplification of beneficial mutations by random segregation is
294 expected to occur at a much greater rate than by spontaneous duplication of genes
295 carrying beneficial mutations. Selection will subsequently fuel the spread of the cells
296 carrying higher frequency of plasmid-born mutated alleles, driving the rapid increase
297 in frequency of the mutation at both the cellular and population level in a few
298 generations (Supplementary Figure 2). However, the dynamics of this process are
299 potentially very complex, and we are currently developing deterministic population
300 dynamics models and stochastic agent-based simulations to better understand this
301 process.

302 One important feature of our experimental design is that we used a rapid ramp-up of
303 antibiotics, which may have reduced the accessibility to alternative evolutionary

trajectories to high level ß-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 of 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} (Figure 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 [28, 12], and they frequently carry antibiotic resistance genes [20-22,26], but their role in the ecology and evolution of antibiotic resistance has been generally overlooked. Our work shows that these genetic elements could accelerate 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 available in supplementary tables 1 to 3. Bacterial strains were cultured in LB broth at 37°C in 96-well plates with continuous shaking (225 rpm) and on LB agar plates at 37° C (Fisher Scientific, USA). 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

Escherichia coli MG1655 strain was used for all our constructions. Chromosomal insertion of bla_{TEM-1} was designed to take place in the integration site of 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 to produce homology-based allelic exchanges between the chromosome and PCR products [56]. Selection for carbenicillin resistance at 37ºC 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, and tested for *gfp* induction with L-arabinose. Sequence of bla_{TEM-1} was verified. pBGT was transformed into the parental MG1655 producing the strain MG/pBGT.

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

355 the selection on carbenicillin, chloramphenicol and L-arabinose (to induce GFP
356 expression) allowed confirming the functionality of all plasmid components.

357 *Competitive fitness assays*

358 The fitness of each clone was determined basically as described in San Millan *et al*
359 2014 [9]. We performed competitions between a plasmid-bearing clone and a plasmid-
360 free clone in each case (MG/pBGT and MG::*bla*$_{TEM-1}$ were the control strains to
361 compete against). Four biological replicates of the competition (of four technical
362 replicates each) were performed for each clone. Pre-cultures of the clones were
363 incubated at 37ºC with 225 RPM shaking overnight in 96-well plates carrying 200 µl
364 of LB broth per well (Fisher Scientific, NJ, USA). Pre-cultures were diluted 400-fold in
365 200 µl of fresh LB and mixed at a ratio of approximately 50% of each clone. The
366 exact initial proportions were confirmed via flow cytometry using an Accuri C6 Flow
367 Cytometer Instrument (BD Accuri, San Jose, CA, USA) with the following
368 parameters: flow rate: 66 µL min-1, core size: 22 µm, events recorded per sample:
369 10,000. To measure this proportions we incubated an aliquot of the mix in LB with L-
370 arabinose 0.25% for two hours to induce GFP expression from pBGT. Mixtures were
371 competed for 24 hours in LB at 37°C with 225 RPM shaking (~8 generations). Again,
372 the final proportion was measured by flow cytometry as described above. The fitness
373 of the strain carrying the plasmid relative to the plasmid-free strain was determined
374 using the formula:

$$W_{p+} = ln(Nfinal_{,p+}/Ninitial_{,p+})/ln(Nfinal_{,p-}/Ninitial_{,p-})$$

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

Experimental evolution

We cultured strains (MG, MG/pBGT and MG::bla_{TEM-1}) on LB agar plates at 37ºC 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 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 into 198 µl of LB with ¼ 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 overnight OD_{600} value higher than 0.1. We kept a frozen stock of all the populations from day 0, 4, 8, 12 and 16. On those days we also (i) checked plasmid stability by flow cytometry (using L-arabinose induction, as explained in the previous section) and (ii) performed DNA extraction to quantify plasmid copy number in the population (see section bellow), from four populations belonging to MG/pBGT. We also measured 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, USA). DNA extraction, quantification and digestion (with *Bam*HI-HF, New England Biolabs, USA) 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=

406 0.99) and we used a previously described qPCR for the *dxs* chromosomal monocopy
407 gene (dxs-F: CGAGAAACTGGCGATCCTTA, dxs-R: CTTCATCAAGCGGTTTCACA,
408 amplicon size: 113 bp, efficiency: 95.52%, R^2= 0.99)[58] to compare the ratio of
409 plasmid and chromosomal DNA. Efficiency of the reactions was calculated from the
410 standard curve generated by performing qPCR with four 8-fold dilutions of template
411 DNAs in triplicate (~5 ng/µl to 12 pg/µl working range of DNA concentration). qPCRs
412 were performed using ABI SYBR Select Master Mix (Life Technologies, USA) at a
413 final DNA concentration of 0.1 ng/µl and following manufacturers' instructions. The
414 amplification conditions were: initial denaturation for 2 min at 95°C, followed by 40
415 cycles of denaturation for 15 s at 95°C, annealing and extension for 1 min at 60°C.
416 After the amplification was complete and to control for the specificity of the reaction,
417 a melting curve analysis was performed by cooling the reaction to 60°C and then
418 heating slowly to 95°C. Inter-run calibration samples were used to normalize the
419 results from different plates of each qPCR. Copy number was calculated using the
420 $\Delta\Delta C_T$ method as previously described, given that the amplification efficiencies of the
421 target and reference genes were approximately equal [58]. Average plasmid copy
422 number was corrected using the plasmid stability frequencies, which were measured
423 from the cultures used to obtain the DNA samples.

424 *Genome sequencing and bioinformatic analysis*

425 We sequenced six random populations from each strain from the experimental
426 evolution assay on the last day when at least 50% of the populations were still viable.
427 We also sequenced a time series of samples from the 7 MG/pBGT populations that
428 survived up to 4,096 mg/L of ceftazidime (days 0, 4, 8, 12 and 16) and nine
429 MG/pBGT clones from three populations with increased plasmid copy number.
430 Finally, we also sequenced six control populations of each strain evolved for 16 days
431 in LB medium with no antibiotics (Supplementary Data 1). DNA extractions were
432 performed from 2 ml of LB broth (Fisher Scientific, NJ, USA) cultures incubated at

37ºC with 225 RPM shaking overnight using the Promega Wizard Genomic 4 DNA Purification Kit (Promega, UK). DNA was quantified using the QuantiFluor dsDNA system (Promega, Madison, WI, USA), 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 IS-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 data set. Further, mutations present in the ancestral clones relative to the reference sequence were excluded, leaving only mutations which 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 number (3 clones/populations from 3 different populations: 9 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 other from MG::$bla_{\text{TEM-1}}$, 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 to 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 done 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 generalised linear effects model, with time and treatments (and their interaction) as factors and assuming Gaussian error distribution.

References

1 Ochman, H., Lawrence, J. G. & Groisman, E. A. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299-304, doi:10.1038/35012500 (2000).

2 Wiedenbeck, J. & Cohan, F. M. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol Rev* 35, 957-976, doi:10.1111/j.1574-6976.2011.00292.x (2011).

3 Baltrus, D. A. Exploring the costs of horizontal gene transfer. *Trends Ecol Evol* 28, 489-495, doi:10.1016/j.tree.2013.04.002 (2013).

4 Vogwill, T. & MacLean, R. C. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl* 8, 284-295, doi: 10.1111/eva.12202 (2014).

5 Stewart, F. M. & Levin, B. R. The Population Biology of Bacterial Plasmids: A PRIORI Conditions for the Existence of Conjugationally Transmitted Factors. *Genetics* 87, 209-228 (1977).

6 Levin, B. R. & Stewart, F. M. The population biology of bacterial plasmids: a priori conditions for the existence of mobilizable nonconjugative factors. *Genetics* 94, 425-443 (1980).

7 Bergstrom, C. T., Lipsitch, M. & Levin, B. R. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. *Genetics* 155, 1505-1519 (2000).

8 Harrison, E. & Brockhurst, M. A. Plasmid-mediated horizontal gene transfer is a coevolutionary process. *Trends Microbiol* 20, 262-267, doi:S0966-842X (2012).

9 San Millan, A. *et al.* Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. N*at Commun* 5, 5208, doi:10.1038/ncomms6208 (2014).

10 Harrison, E., Guymer, D., Spiers, A. J., Paterson, S. & Brockhurst, M. A. Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum. C*urr Biol* 25, 2034-2039, doi:10.1016/j.cub.2015.06.024 (2015).

11 Peña-Miller, R., Rodríguez-González, R., MacLean, R. C. & San Millan, A. Evaluating the effect of horizontal transmission on the stability of plasmids under different selection regimes. Mobile Genetic Elements 22, 1-5 (2015).

12 Modi, R. I., Castilla, L. H., Puskas-Rozsa, S., Helling, R. B. & Adams, J. Genetic changes accompanying increased fitness in evolving populations of Escherichia coli. G*enetics* 130, 241-249 (1992).

13 Stoesser, N. *et al.* Evolutionary History of the Global Emergence of the Escherichia coli Epidemic Clone ST131. M*Bio* 7, doi:10.1128/mBio.02162-15 (2016).

14 Latorre, A., Gil, R., Silva, F. J. & Moya, A. Chromosomal stasis versus plasmid plasticity in aphid endosymbiont Buchnera aphidicola. H*eredity (Edinb)* 95, 339-347, doi:10.1038/sj.hdy.6800716 (2005).

15 Zhang, Z., Qian, W. & Zhang, J. Positive selection for elevated gene expression noise in yeast. M*ol Syst Biol* 5, 299, doi:10.1038/msb.2009.58 (2009).

16 Martinez, J. L. & Baquero, F. Mutation frequencies and antibiotic resistance. A*ntimicrob Agents Chemother* 44, 1771-1777 (2000).

17 Couce, A., Rodriguez-Rojas, A. & Blazquez, J. Bypass of genetic constraints during mutator evolution to antibiotic resistance. P*roc Biol Sci* 282, 20142698, doi:10.1098/rspb.2014.2698 (2015).
18 Sano, E., Maisnier-Patin, S., Aboubechara, J. P., Quinones-Soto, S. & Roth, J. R. Plasmid copy number underlies adaptive mutability in bacteria. G*enetics* 198, 919-933, doi:10.1534/genetics.114.170068 (2014).
19 Hendrickson, H., Slechta, E. S., Bergthorsson, U., Andersson, D. I. & Roth, J. R. Amplification-mutagenesis: evidence that "directed" adaptive mutation and general hypermutability result from growth with a selected gene amplification. P*roc Natl Acad Sci U S A* 99, 2164-2169, doi:10.1073/pnas.032680899 (2002).
20 Sarno, R., McGillivary, G., Sherratt, D. J., Actis, L. A. & Tolmasky, M. E. Complete nucleotide sequence of Klebsiella pneumoniae multiresistance plasmid pJHCMW1. A*ntimicrob Agents Chemother* 46, 3422-3427 (2002).
21 Søndergaard, A. *et al.* Molecular Organization of Small Plasmids Bearing blaTEM-1 and Conferring Resistance to beta-lactams in Haemophilus influenzae. A*ntimicrob Agents Chemother,* doi:AAC.00408-12 (2012).
22 Vignoli, R. e*t al. N*ew TEM-derived extended-spectrum beta-lactamase and its genomic context in plasmids from Salmonella enterica serovar derby isolates from Uruguay. An*timicrob Agents Chemother 5*0, 781-784, doi:10.1128/AAC.50.2.781-784.2006 (2006).
23 Bershtein, S., Segal, M., Bekerman, R., Tokuriki, N. & Tawfik, D. S. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. Na*ture 4*44, 929-932, doi:10.1038/nature05385 (2006).
24 Stiffler, M. A., Hekstra, D. R. & Ranganathan, R. Evolvability as a function of purifying selection in TEM-1 beta-lactamase. Ce*ll 1*60, 882-892, doi:10.1016/j.cell.2015.01.035 (2015).
25 Schenk, M. F., Szendro, I. G., Krug, J. & de Visser, J. A. Quantifying the adaptive potential of an antibiotic resistance enzyme. PL*oS Genet 8*, e1002783, doi:10.1371/journal.pgen.1002783 (2012).
26 San Millan, A. e*t al. S*mall-plasmid-mediated antibiotic resistance is enhanced by increases in plasmid copy number and bacterial fitness. An*timicrob Agents Chemother 5*9, 3335-3341, doi:10.1128/AAC.00235-15 (2015).
27 San Millan, A., Heilbron, K. & MacLean, R. C. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. IS*ME J 8*, 601-612, doi:10.1038/ismej.2013.182 (2014).
28 Reguera, J. A., Baquero, F., Perez-Diaz, J. C. & Martinez, J. L. Synergistic effect of dosage and bacterial inoculum in TEM-1 mediated antibiotic resistance. Eu*r J Clin Microbiol Infect Dis 7*, 778-779 (1988).
29 Martinez, J. L. e*t al. R*esistance to beta-lactam/clavulanate. La*ncet 2*, 1473 (1987).
30 Artemova, T., Gerardin, Y., Dudley, C., Vega, N. M. & Gore, J. Isolated cell behavior drives the evolution of antibiotic resistance. Mo*l Syst Biol 1*1, 822, doi:10.15252/msb.20145888 (2015).
31 Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L. & Andersson, D. I. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MB*io 5*, e01918-01914, doi:10.1128/mBio.01918-14 (2014).
32 Salverda, M. L., De Visser, J. A. & Barlow, M. Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance. FE*MS Microbiol Rev 3*4, 1015-1036, doi:10.1111/j.1574-6976.2010.00222.x (2010).
33 Tavio, M. M., Aquili, V. D., Vila, J. & Poveda, J. B. Resistance to ceftazidime in Escherichia coli associated with AcrR, MarR and PBP3 mutations and overexpression of sdiA. J *Med Microbiol 6*3, 56-65, doi:10.1099/jmm.0.063727-0 (2014).
34 Hirakawa, H., Nishino, K., Yamada, J., Hirata, T. & Yamaguchi, A. Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in Escherichia coli. J *Antimicrob Chemother 5*2, 576-582, doi:10.1093/jac/dkg406 (2003).
35 Cai, S. J. & Inouye, M. EnvZ-OmpR interaction and osmoregulation in Escherichia coli. J *Biol Chem 2*77, 24155-24161, doi:10.1074/jbc.M110715200 (2002).

36 Jacquier, H. *et al.* In vivo selection of a complex mutant TEM (CMT) from an inhibitor-resistant TEM (IRT) during ceftazidime therapy. J *Antimicrob Chemother* 68, 2792-2796, doi:10.1093/jac/dkt278 (2013).

37 Negri, M. C., Lipsitch, M., Blazquez, J., Levin, B. R. & Baquero, F. Concentration-dependent selection of small phenotypic differences in TEM beta-lactamase-mediated antibiotic resistance. An*timicrob Agents Chemother* 44, 2485-2491 (2000).

38 Nasvall, J., Sun, L., Roth, J. R. & Andersson, D. I. Real-time evolution of new genes by innovation, amplification, and divergence. Sc*ience* 338, 384-387, doi:10.1126/science.1226521 (2012).

39 Sandegren, L. & Andersson, D. I. Bacterial gene amplification: implications for the evolution of antibiotic resistance. Na*t Rev Microbiol* 7, 578-588, doi:10.1038/nrmicro2174 (2009).

40 Tomizawa, J., Itoh, T., Selzer, G. & Som, T. Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. Pr*oc Natl Acad Sci U S A* 78, 1421-1425 (1981).

41 Tomizawa, J. Control of ColE1 plasmid replication: the process of binding of RNA I to the primer transcript. Ce*ll* 38, 861-870, doi:0092-8674(84)90281-2 [pii] (1984).

42 Lacatena, R. M. & Cesareni, G. Interaction between RNA1 and the primer precursor in the regulation of Co1E1 replication. J *Mol Biol* 170, 635-650 (1983).

43 Lacatena, R. M. & Cesareni, G. Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. Na*ture* 294, 623-626 (1981).

44 The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. http://www.eucast.org. (2016).

45 Wang, X., Minasov, G. & Shoichet, B. K. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J *Mol Biol* 320, 85-95, doi:10.1016/S0022-2836(02)00400-X (2002).

46 Ohno, S. Ev*olution by gene duplication.* (Springer Science & Business Media, 1970).

47 Toll-Riera, M., San Millan, A., Wagner, A. & MacLean, R. C. The Genomic Basis of Evolutionary Innovation in Pseudomonas aeruginosa. PL*oS Genet* 12, e1006005, doi:10.1371/journal.pgen.1006005 (2016).

48 Andersson, D. I. & Hughes, D. Gene amplification and adaptive evolution in bacteria. An*nu Rev Genet* 43, 167-195, doi:10.1146/annurev-genet-102108-134805 (2009).

49 Lindsey, H. A., Gallie, J., Taylor, S. & Kerr, B. Evolutionary rescue from extinction is contingent on a lower rate of environmental change. Na*ture* 494, 463-467, doi:10.1038/nature11879 (2013).

50 Baquero, F. & Negri, M. C. Selective compartments for resistant microorganisms in antibiotic gradients. Bi*oessays* 19, 731-736, doi:10.1002/bies.950190814 (1997).

51 Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 19th ed. Approved standard M100-S19. CLSI, Wayne, PA, USA, (2009).

52 Escudero, J. A. *et al.* Unmasking the ancestral activity of integron integrases reveals a smooth evolutionary transition during functional innovation. Na*t Commun* 7, 10937, doi:10.1038/ncomms10937 (2016).

53 Demarre, G., Frumerie, C., Gopaul, D. N. & Mazel, D. Identification of key structural determinants of the IntI1 integron integrase that influence attC x attI1 recombination efficiency. Nu*cleic Acids Res* 35, 6475-6489, doi:10.1093/nar/gkm709 (2007).

54 Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. Na*t Methods* 6, 343-345, doi:10.1038/nmeth.1318 (2009).

55 Chaveroche, M. K., Ghigo, J. M. & d'Enfert, C. A rapid method for efficient gene replacement in the filamentous fungus Aspergillus nidulans. Nu*cleic Acids Res* 28, E97 (2000).

56 Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Pr*oc Natl Acad Sci U S A* 97, 6640-6645, doi:10.1073/pnas.120163297 (2000).

57 Le Roux, F., Binesse, J., Saulnier, D. & Mazel, D. Construction of a Vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Ap*pl Environ Microbiol* 73, 777-784, doi:10.1128/AEM.02147-06 (2007).

58 Lee, C., Kim, J., Shin, S. G. & Hwang, S. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. J *Biotechnol 1*23, 273-280, doi:10.1016/j.jbiotec.2005.11.014 (2006).
59 Deatherage, D. E. & Barrick, J. E. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. M*ethods Mol Biol 1*151, 165-188, doi:10.1007/978-1-4939-0554-6_12 (2014).
60 Barrick, J. E. e*t al. I*dentifying structural variation in haploid microbial genomes from short-read resequencing data using breseq. BM*C Genomics 1*5, 1039, doi:10.1186/1471-2164-15-1039 (2014).
61 Deatherage, D. E., Traverse, C. C., Wolf, L. N. & Barrick, J. E. Detecting rare structural variation in evolving microbial populations from new sequence junctions using breseq. Fr*ont Genet 5*, 468, doi:10.3389/fgene.2014.00468 (2014).
62 Lorenz, R. e*t al. V*iennaRNA Package 2.0. A*lgorithms Mol Biol 6*, 26, doi:10.1186/1748-7188-6-26 (2011).

639 Correspondence and requests for materials should be addressed to Alvaro San
640 Millan: alvaro.sanmillan@hrc.es

641 **Acknowledgments**

642 A.S.M. thanks E. Frago for his help with the statistical analyses. This work was
643 supported by funding from the European Research Council under the European
644 Union's Seventh Framework Programme (FP7/2007-2013)/ERC grant (StG-2011-
645 281591). A.S.M. is supported by a Miguel Servet fellowship from the *Instituto de*
646 *Salud Carlos III* (MS15/00012) co-financed by the European Social Fund and The
647 European Development Regional Fund "A way to achieve Europe" (ERDF). J.A.E. is
648 supported by a Marie Curie Intra-European Fellowship for Career Development (FP-
649 7-PEOPLE-2011-IEF, ICADIGE). Work in the Mazel lab was supported by the *Institut*
650 *Pasteur*, the *Centre National de la Recherche Scientifique* (CNRS-UMR3525) and
651 the European Union Seventh Framework Programme (FP7-HEALTH- 2011-single-
652 stage), the "Evolution and Transfer of Antibiotic Resistance" (EvoTAR, FP7-
653 HEALTH-282004). The funders had no role in study design, data collection and
654 analysis, decision to publish, or preparation of the manuscript.

655 We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for
656 Human Genetics funded by Wellcome Trust grant reference 090532/Z/09/Z and
657 Medical Research Council Hub grant G0900747 91070 for generation of the high-
658 throughput sequencing data.

659 **Author Contributions**

660 Conceptualization, A.S.M. and R.C.M.; Methodology, A.S.M., J.A.E., and D.R.G.;
661 Formal analysis, D.R.G.; Investigation, A.S.M. and J.A.E.; Writing – Original Draft,
662 A.S.M. and R.C.M; Writing – Review & Editing, A.S.M and R.C.M.; Funding
663 Acquisition, R.C.M. and D.M.; Resources, D.M.; Supervision, R.C.M. and D.M.
664 Project Administration, A.S.M.

Competing financial interests

We have no competing interests to declare

Figure legends

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 arrows, with the direction of transcription indicated by the arrowhead. (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 standard error of the mean (SEM, 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 compared to on the chromosome.

Figure 2. Survival curves under increasing concentrations of ceftazidime.

Plasmid-carrying populations are able to survive up to very high level of ceftazidime concentration. Number of viable populations belonging to strains MG1655, MG1655::bla_{TEM-1}, and MG1655/pBGT over time. 48 populations of each strain were propagated under increasing concentrations of ceftazidime (CAZ), starting with ¼ of the MIC (Day 1; 0.06 mg/L for MG and MG::bla_{TEM-1} and 0.25 mg/L 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 7 populations viable at 4,096 mg/L 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.

Figure 3. pBGT mutations in the ceftazidime resistant populations.

691 Plasmid-carrying populations acquire mutations in bla_{TEM-1} followed by mutations in
692 the RNAI of pBGT. (A) Average copy number of plasmid pBGT per cell in populations
693 of MG1655/pBGT over time. Black circles represent plasmid copy number in the
694 control populations propagated in LB in the absence of antibiotics and blue circles
695 represent plasmid copy number in populations evolved in the presence of increasing
696 concentrations of ceftazidime. Plasmid copy number of MG/pBGT populations
697 evolving with ceftazidime at day 12 and 16 showed a significant increase compared
698 to the one in the control MG/pBGT populations propagated with no antibiotics (t-test,
699 day 12, $P< 0.001$, $t= 4.89$, day 16 $P< 0.01$, $t= 5.18$, $df= 1$). (B) Secondary structure of
700 RNAI predicted using RNAfold software (see methods). Residues highlighted in red
701 are those where mutations were found during the experimental evolution. The
702 changes produced by those mutations are also indicated with blue letters. (C)
703 Frequency of the mutations in TEM-1 and RNAI in the seven populations reaching
704 very high-level ceftazidime resistance over time. Black curves represent mutations in
705 TEM-1 and red curves represent mutations in RNAI.

706 **Figure 4.** Characterization of the mutations in bla_{TEM-1} and RNAI.

707 Effect of the most common mutations observed during the experimental evolution in
708 TEM-1 (R164S) and the RNAI (G54U, G55U) reconstructed in the parental MG1655
709 strain. (A) Schematic representation of the different strains constructed from *E. coli*
710 MG1655 (MG). (B) Level of ceftazidime (CAZ) resistance of each strain represented
711 in $\log_2$ scale. The strains carrying bla_{TEM-1} on the chromosome are represented in red
712 boxes and the strains carrying bla_{TEM-1} on plasmid pBGT are represented in green
713 boxes. Note that bla_{TEM-1} does not confer almost any resistance to CAZ. R164S
714 mutation in TEM-1 increases resistance to CAZ but only confer resistance levels
715 above the MIC (8 mg/L, red dotted line) when is encoded on the plasmid. Also note
716 that these MIC values of these clones are lower than the final concentration of
717 ceftazidime that were tolerated during the selection experiment. We argue that this

718 difference was caused by two factors: first, the evolving MG/pBGT populations
719 carried additional chromosomal mutations related to ceftazidime resistance, which
720 probably contributed to the increased resistance level (Supplementary Data 1). And
721 second, it is known that ß-lactamase-mediated resistance increases with cell density
722 (inoculum effect)[30], and the standard MIC techniques use initial cell densities 20
723 times lower than those used during the selection experiment. The estimates of MIC
724 should therefore be considered as relative, rather than absolute, measures of
725 resistance. (C) Average copy number of pBGT per cell (± SEM, n= 3). (D) Fitness
726 relative to MG::bla_{TEM-1} (± SEM, n= 4) of the different constructs. (E) Comparison of
727 the relative fitness of the different plasmid-bearing constructs and their plasmid copy
728 number (± SEM). Black points indicate pBGT-carrying TEM-1 and red points indicate
729 pBGT carrying the mutant version TEM-1 R164S. Note that the plasmid copy number
730 strongly correlates with the reduction in fitness.

Tables

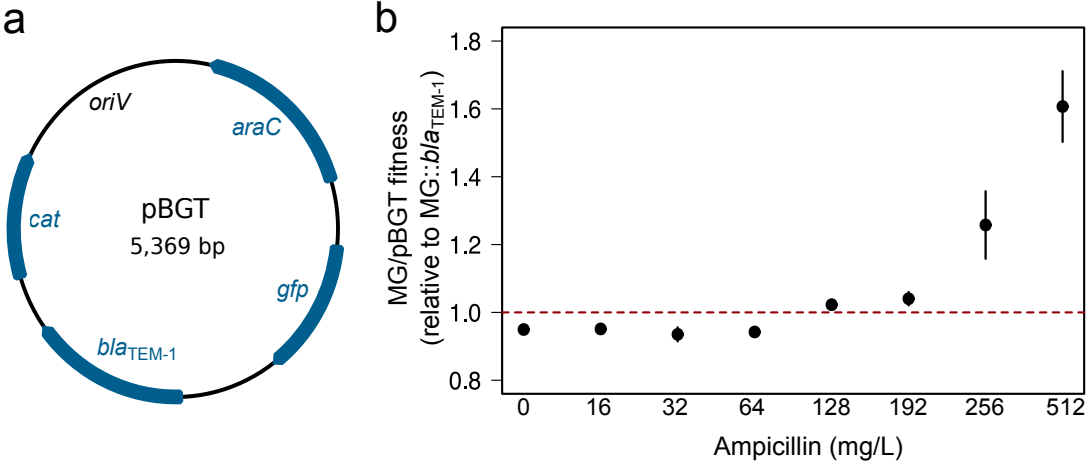
Table 1. Characteristics of the strains used in this study.

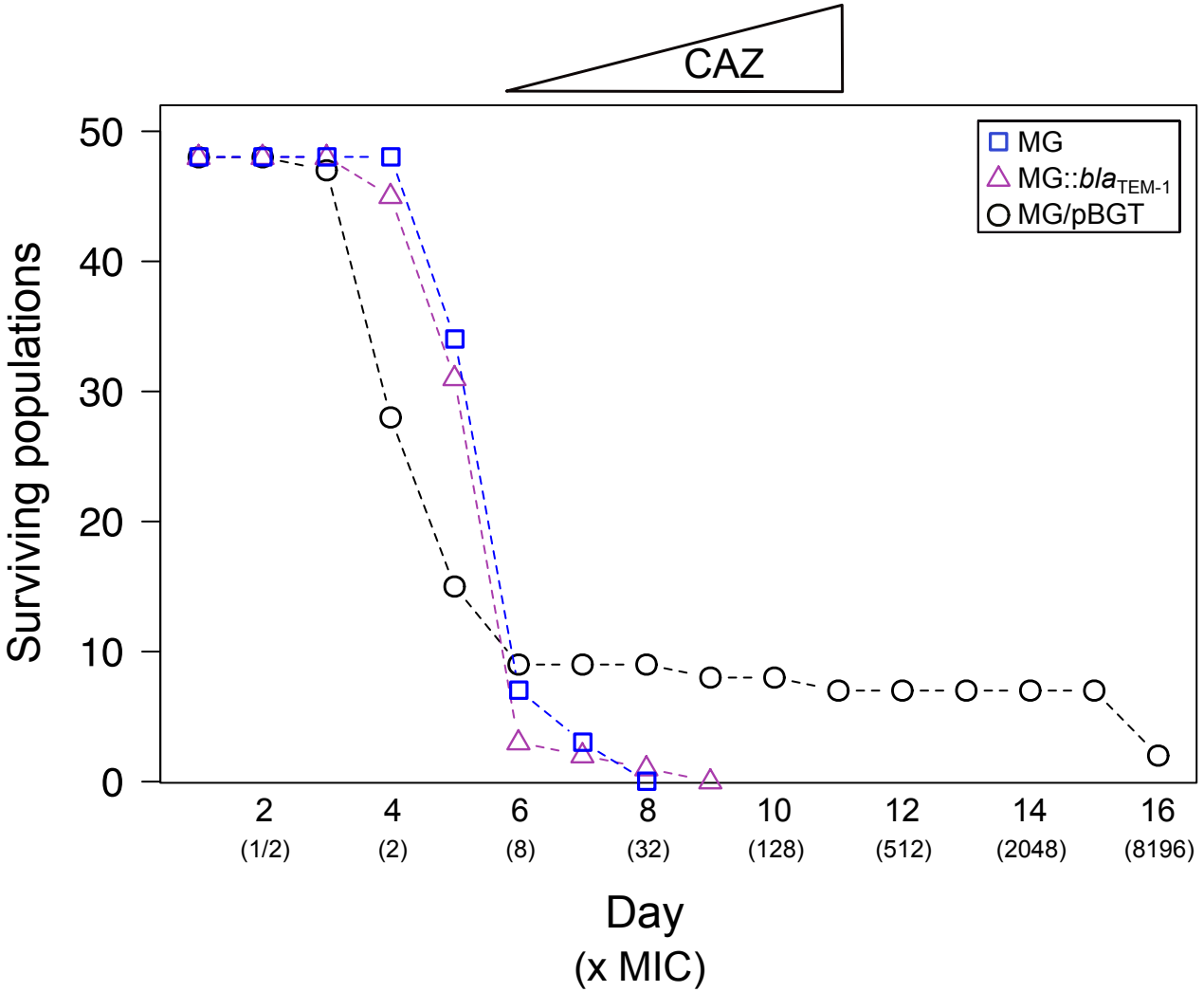
Strain name	Fitness [1]	PCN [2]	CMI CAZ (mg/L)	CMI AMP (mg/L)
MG	1.003 ± 0.011	NA	0.25	4
MG::*bla*$_{TEM-1}$	NA	NA	0.25	512
MG/pBGT	0.943 ± 0.013	19.12 ± 1.56	0.5	8192
MG::*bla*$_{TEM-1}$ R146	1.008 ± 0.011	NA	1	128
MG/pBGT R164S	0.943 ± 0.003	21.10 ± 0.85	64	4096
MG/pBGT G54U	0.793 ± 0.019	44.50 ± 3.81	0.5	32768
MG/pBGT G55U	0.557 ± 0.116	88.93 ± 15.65	1	32768
MG/pBGT R164S G54U	0.762 ± 0.016	52.30 ± 2.19	128	4096
MG/pBGT R164S G55U	0.509 ± 0.082	127.29 ± 4.58	128	8192

1 Fitness relative to MG::*bla*$_{TEM-1}$ ± S.E.M, n= 4.

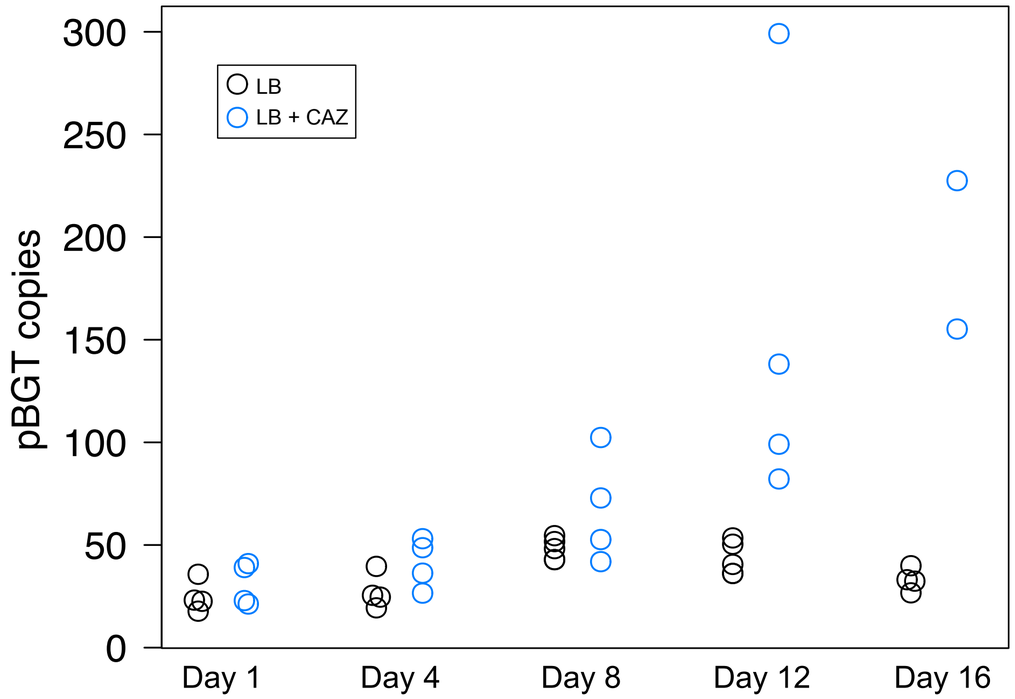
2 Plasmid copy number ± S.E.M, n= 3.

NA, not applicable.

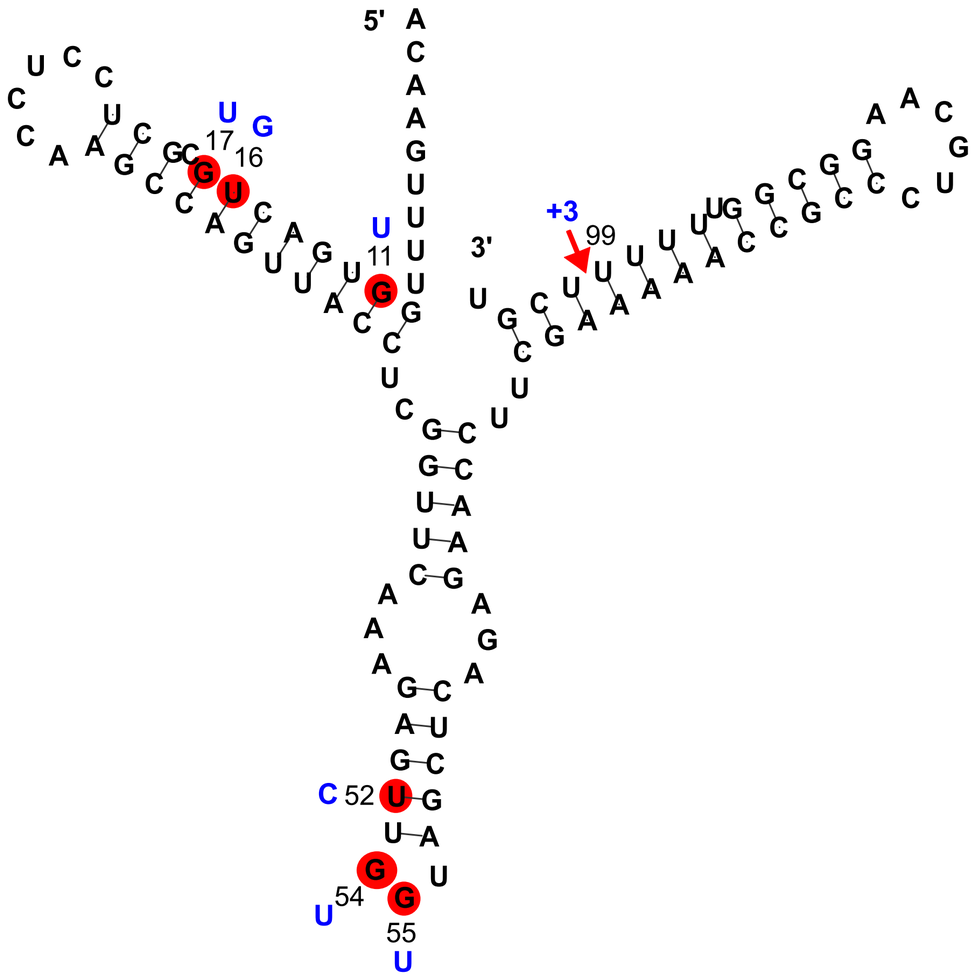

a
oriV
araC
cat
pBGT
5,369 bp
gfp
bla TEM-1
b
MG/pBGT fitness
(relative to MG::bla TEM-1)
1.8
1.6
1.4
1.2
1.0
0.8
0
16
32
64
128
192
256
512
Ampicillin (mg/L)


CAZ
MG
MG::bla TEM-1
MG/pBGT
Surviving populations
0
10
20
30
40
50
2
(1/2)
4
(2)
6
(8)
8
(32)
10
(128)
12
(512)
14
(2048)
16
(8196)
Day
(x MIC)

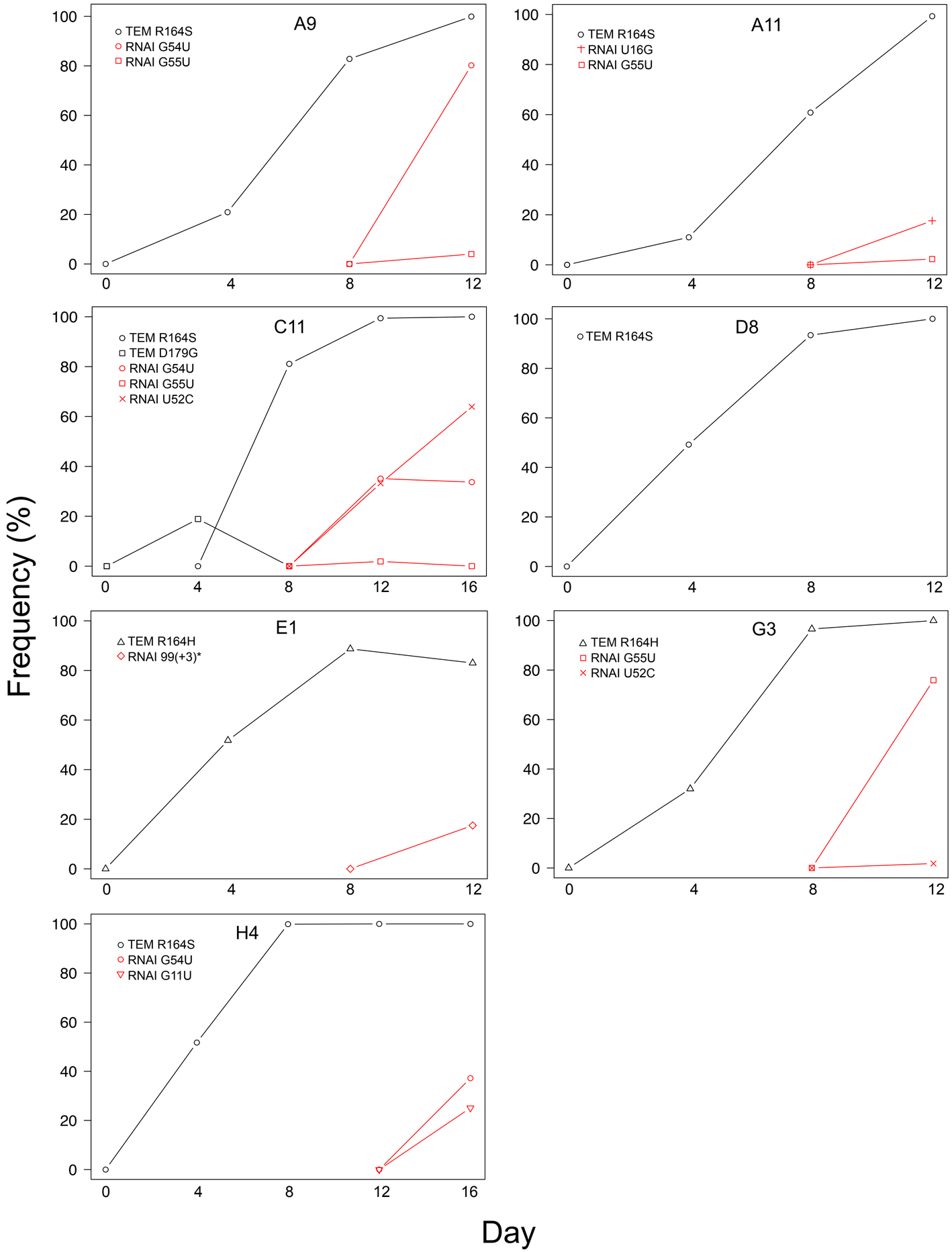
a

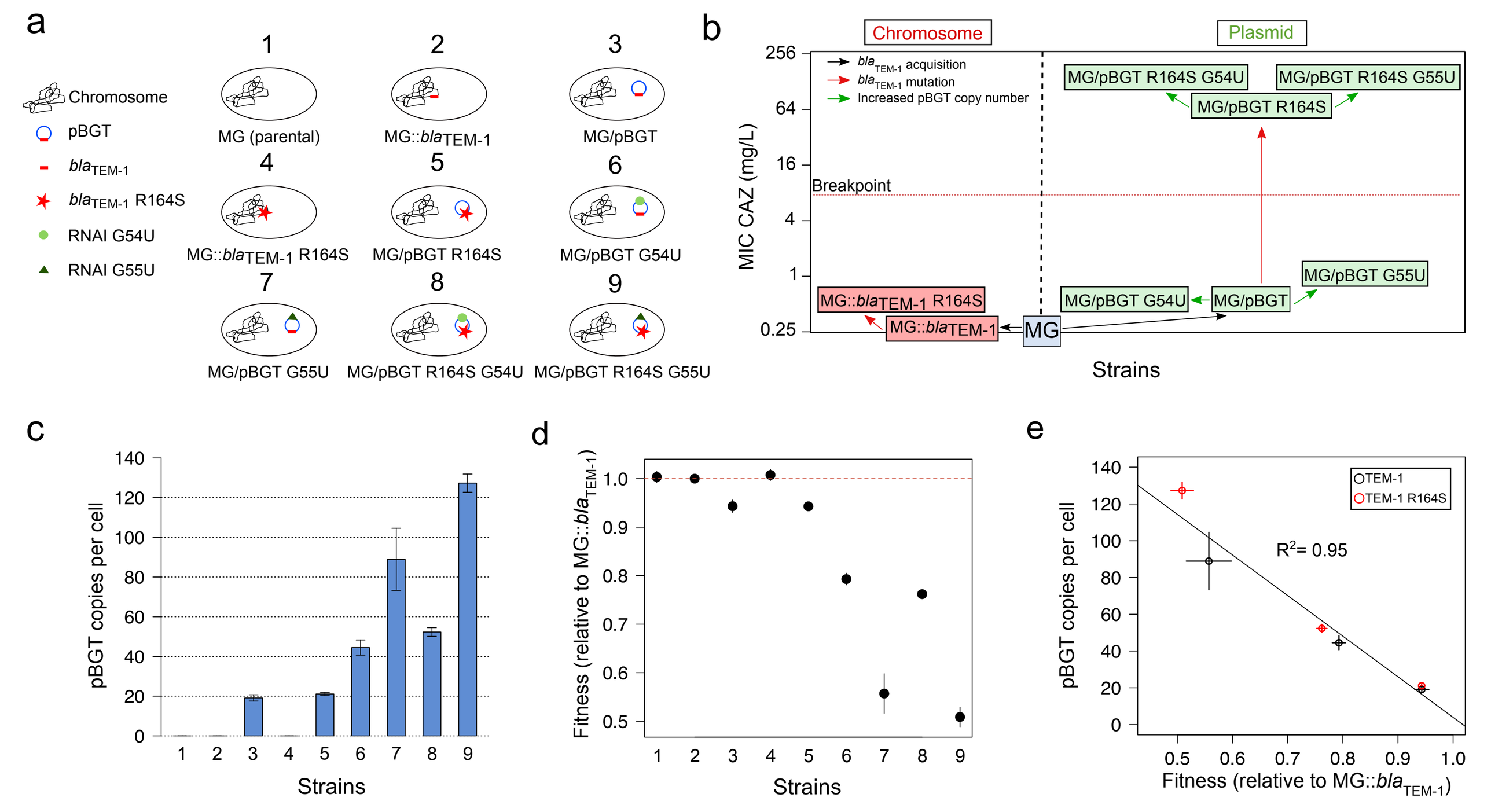

LB
LB + CAZ
pBGT copies
0
50
100
150
200
250
300
Day 1
Day 4
Day 8
Day 12
Day 16

b


5'
3'
+3
99
17
16
11
52
54
55

c


A9
TEM R164S
RNAI G54U
RNAI G55U
A11
TEM R164S
RNAI U16G
RNAI G55U
C11
TEM R164S
TEM D179G
RNAI G54U
RNAI G55U
RNAI U52C
D8
TEM R164S
E1
TEM R164H
RNAI 99(+3)*
G3
TEM R164H
RNAI G55U
RNAI U52C
H4
TEM R164S
RNAI G54U
RNAI G11U
Frequency (%)
Day


a
Chromosome
pBGT
bla TEM-1
bla TEM-1 R164S
RNAI G54U
RNAI G55U
1
MG (parental)
2
MG::bla TEM-1
3
MG/pBGT
4
MG::bla TEM-1 R164S
5
MG/pBGT R164S
6
MG/pBGT G54U
7
MG/pBGT G55U
8
MG/pBGT R164S G54U
9
MG/pBGT R164S G55U
b
Chromosome
Plasmid
bla TEM-1 acquisition
bla TEM-1 mutation
Increased pBGT copy number
MIC CAZ (mg/L)
256
64
16
4
1
0.25
Breakpoint
MG/pBGT R164S G54U
MG/pBGT R164S G55U
MG/pBGT R164S
MG/pBGT G55U
MG::bla TEM-1 R164S
MG/pBGT G54U
MG/pBGT
MG::bla TEM-1
MG
Strains
c
pBGT copies per cell
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
Strains
d
Fitness (relative to MG::bla TEM-1)
1.0
0.9
0.8
0.7
0.6
0.5
1 2 3 4 5 6 7 8 9
Strains
e
pBGT copies per cell
140
120
100
80
60
40
20
0
TEM-1
TEM-1 R164S
R2= 0.95
0.5 0.6 0.7 0.8 0.9 1.0
Fitness (relative to MG::bla TEM-1)