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Title: Extremely fast amelioration of plasmid fitness costs by multiple functionally-diverse pathways

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Abstract

The acquisition of plasmids is often accompanied by fitness costs such that compensatory evolution is required to allow plasmid survival, but it is unclear whether compensatory evolution can be extensive or rapid enough to maintain plasmids when they are very costly. The mercury-resistance plasmid pQBR55 drastically reduced the growth of its host, *Pseudomonas fluorescens* SBW25, immediately after acquisition, causing a small colony phenotype. However, within 48 hours of growth on agar plates we observed restoration of the ancestral large colony morphology, suggesting that compensatory mutations had occurred. Relative fitness of these evolved strains, in lab media and in soil microcosms, varied between replicates, indicating different mutational mechanisms. Using genome sequencing we identified that restoration was associated with chromosomal mutations in either a hypothetical DNA-binding protein PFLU4242, RNA polymerase, or the GacA/S two-component system. Targeted deletions in *PFLU4242*, *gacA*, or *gacS* recapitulated the ameliorated phenotype upon plasmid acquisition, indicating three distinct mutational pathways to compensation. Our data shows that plasmid compensatory evolution is fast enough to allow survival of a plasmid despite it imposing very high fitness costs upon its host, and indeed may regularly occur during the process of isolating and selecting individual plasmid-containing clones.

Introduction

Plasmids are important vehicles for horizontal gene transfer (HGT), allowing bacteria to rapidly adapt to new environments by transferring niche-adaptive traits (1). Plasmid acquisition can, however, disrupt normal cellular function (2). The consequent fitness costs can limit plasmid survival because plasmid-bearers will be outcompeted by plasmid-free cells that do not suffer the cost (2). Beneficial genes carried by the plasmid cannot ensure its long-term persistence, as these genes can recombine onto the chromosome (3,4). An important mechanism allowing plasmid survival is compensatory evolution whereby mutations in chromosomal and/or plasmid genes ameliorate fitness costs (e.g. (5–7)). Where transmission rates are too low to maintain plasmids by infectious transfer, plasmid survival effectively becomes a race between the rate of compensatory evolution and the rate at which plasmid-bearers are outcompeted (8).

Plasmid compensation is often explored using experimental evolution. In general, these experiments involve the introduction of an initially costly plasmid to a strain, prolonged culture of plasmid bearers (often through dozens of serial transfers), followed by assays on evolved plasmid-bearing strains and often re-sequencing to identify underlying mutations. These studies have highlighted targets of loss-of-function compensatory mutation, varying with the bacterial-plasmid pairing. Chromosomal accessory helicases have been implicated with *Pseudomonas aeruginosa* and the small plasmid pNUK73 (7), and with *Pseudomonas* sp. H2 and plasmid RP4 (6). Chromosomal global regulators have been identified: the *fur* gene in *Shewanella oneidensis* MR-1 with pBP136 (9), and the *gacA/gacS* genes in *P. fluorescens* SBW25 with pQBR103 (5). Compensatory mutations can also occur on plasmids, targeting replication genes (10) or conjugation machinery (11–13). However, for very costly plasmids compensatory evolution may be insufficient or too slow to enable persistence. For example, the mercury resistance plasmid pQBR103 was lost from all populations of *P. aeruginosa* PAO1 even under mercury selection (3), and plasmid pMS0506 was either lost or suffered large deletions when grown in *Acinetobacter baumannii* ATCC19606 under selective (kanamycin) conditions (10).

In the current work, we show that several different routes of compensatory mutation, emerging during the process of transconjugant colony growth, can overcome the heavy costs imposed when *P. fluorescens* SBW25 newly acquires the conjugative plasmid pQBR55. pQBR55 is a member of the pQBR plasmid collection, a set of relatively large (>130 kb) conjugative mercury resistance elements exogenously isolated in the 1990s from Wytham Farm, Oxford. The sequenced pQBR plasmids, pQBR55, pQBR57, and pQBR103, fall into different 'groups' based on RFLP fingerprint (14) (all of the sequenced pQBR plasmids fall outwith the Enterobacteriaceae incompatibility typing scheme (15)) but DNA sequencing indicated that pQBR57 and pQBR103 are distantly related to one another, and both are distantly related to a family of IncP-2-related *Pseudomonas* megaplasmids (16,17). Besides mercury resistance, the sequenced pQBR plasmids have few identifiable accessory traits, and no antibiotic resistance genes. Our results indicate that compensatory evolution can rapidly and effectively facilitate the maintenance of newly acquired mobile genetic elements.

Materials and Methods

Strains and standard culture

Pseudomonas fluorescens SBW25 was tagged with the mini-Tn7 streptomycin resistance (Sm^R) or gentamicin resistance (Gm^R) cassette (5). For the 20 replicates used in the evolution experiment a *lacZ*-expressing strain (18) was used, generating *P. fluorescens* SBW25 Sm^R-*lacZ* to aid identification of small colonies by addition of X-gal (50 µg/ml) to solid media. Strains were cultured in King's B media at 28°C, with addition of 1.2% w/v agar where appropriate. The pQBR55 donor strain, *P. putida* UWC1(pQBR55), was a gift from Andy Lilley (KCL) via Andrew Spiers (University of Abertay).

Establishing experimental lines

Each replicate was established from an independent recipient colony. Overnight cultures of *P. fluorescens* SBW25 Sm^R-*lacZ* and *P. putida* UWC1(pQBR55) were mixed in equal ratios and diluted 1:100 into 6 ml KB broth which was incubated for 24 h at 180 rpm. Samples were spread on selective plates (250 µg/ml

streptomycin, 20 mM $HgCl_2$, with 50 µg/ml X-gal) and colonies allowed to grow for 48 hours before photographing (Figure S1). For each replicate, a colony was randomly selected as the one closest to a line drawn down the centre of the plate, and was re-streaked twice onto selective media. A random colony was again photographed, resuspended in KB + 20% w/v glycerol, and maintained at -80°C. Colonies were tested for pQBR55 carriage by PCR using 1x GoTaq Green (Promega, WI USA) and 0.4 µM each of primers pQBR55_0051_R1, pQBR55_0050_F1, merA_F, merA_R and thermocycling for 95°C 5', 30 x (95°C 30", 58°C 30", 72°C 1'), 72°C 1' and in all cases were found to carry the plasmid. pQBR55_0050–pQBR55_0051 adjoin the experimentally determined origin of pQBR55 replication, and thus their presence is consistent with an independently-replicating plasmid (19). Primer sequences are in Table S3. Glycerol stocks were used to establish subsequent experiments. Images were analysed with Imagemagick (ImageMagick Studio LLC) and ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997-2018). pQBR55 was conjugated into knockout strains from UWC1(pQBR55) as described above, except selective plates included 30 µg/ml gentamicin and 20 mM $HgCl_2$.

Assays on experimental lines

Phenotypic assays were performed largely according to Cheng et al. 2013 (20) on inocula from overnight cultures. Production of extracellular protease was tested on nutrient agar supplemented with 5% w/v powdered milk. Spots of 1:100 dilutions of culture (2.5 µl) were added and zones of clearing assessed after 24 h incubation at room temperature. Viscosin production was assessed by drop collapse assay. A sample (2 µl) of culture supernatant was spotted onto parafilm. The 'beading up' of the droplet indicated a lack of viscosin. Flagellum-mediated motility was assessed by spotting cultures onto 'swim plates' consisting of 5 g NaCl and 10 g tryptone per litre supplemented with 0.3% w/v agar, and assessing the distance travelled after 24 h. Pectinase activity was assessed on slices of potato, which had been peeled, soaked in 10% v/v bleach solution, and washed twice in deionised water (dH_2O). Approximately 8 mm sections were placed in a petri dish and a depression cut into the surface, into which 50 µl culture was

pipetted. Potato slices were scored for soft rot after 4 days. Assays were repeated at least two times for each evolved strain. All strains were tested alongside a *P. fluorescens* SBW25 wild-type positive control and Δ*gacA* and Δ*gacS* negative controls.

Growth curves were carried out by subculturing overnight cultures and growing to OD600 ~ 0.4. Samples of each culture were diluted 1:100 in KB and 5 µl dilution used to inoculate 150 µl KB in a 96-well microtitre plate. Cultures were grown at 28°C, 180 rpm shaking in a Tecan M200 plate reader with measurements taken every 15 minutes for 48 hours. Maximum growth rate across 3 timepoints was estimated using Magellan (Tecan, Switzerland). Replica plating samples of endpoint culture onto 100 mM $HgCl_2$ and testing by PCR showed no evidence of plasmid loss during the course of the experiment. Rifampicin resistance of sequenced clones was assessed in a similar manner, except samples were added to varying concentrations of KB + rifampicin (twofold dilutions from 0.2 to 200 µg/ml). The threshold for growth was set at OD600 > 0.03 after 48 h.

Phenotypic assays and growth curves were conducted on all 20 lineages, but six experimental lineages either had a small colony morphology and/or slow or inconsistent growth, suggesting that they had not ameliorated pQBR55 carriage. Competitions were therefore conducted only on the 14 remaining lineages. Competitions were performed as described previously (21). Briefly, overnight cultures were washed in M9 buffer (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 19 mM NH_4Cl, pH 7.4, (22)), mixed with plasmid-free *P. fluorescens* SBW25 Gm^R in approximately equal numbers, and samples serially diluted and spread on KB + X-gal (50 µg/ml) to enumerate starting cfus. Mixtures were diluted 1:100 into 6 ml fresh KB media in a 30 ml glass universal and grown for 48 hours, or added 1:100 v/w to potting soil microcosms pre-wetted with 900 µl dH2O and grown for 96 hours. Potting soil microcosms designed to represent a more natural growth substrate of *P. fluorescens* consisted of 10 g unseived twice-autoclaved John Innes #2 (manufactured by J. Arthur Bower, supplied by Vertigrow Ltd., UK) with ~25% w/v water content as described previously (4,21). At the endpoint, samples of culture or soil wash were serially diluted and spread on KB + X-gal and endpoint cfus were calculated. Relative fitness was calculated as

the ratio of Malthusian parameters (24). Endpoint colonies were replicated onto 100 mM $HgCl_2$ which showed maintenance of mercury resistance in pQBR55-starting clones in all cases, and PCR analysis of colonies from each sample showed no evidence of plasmid loss.

Photographs of transconjugant colonies were analysed with ImageJ using the Watershed and AnalyseParticles tools. Measurements ±2 standard deviations from the mean colony area for each image were discarded as doublets or other errors, and the mean of the remaining measurements ($n \geq 8$for each sample) was analysed. To estimate the number of generations occurring during colony growth, three 48 h old colonies were measured using ImageJ before each was dispersed in KB broth, serially diluted, and plated to calculate cfu/colony. Ancestral plasmid-free colonies were calculated to contain 4.9×10^8 cells with a standard error of 1.3×10^8 ($2.5 \pm 0.5 \times 10^7$ cells/mm^2), whereas small colonies contained $2.2 \pm 0.8 \times 10^6$ cells ($3.3 \pm 1.1 \times 10^6$ cells/mm^2). We therefore estimate an average of $\log2(2.2 \times 10^6) = 21$ generations per small colony and $\log2(4.9 \times 10^8) = 29$ generations per large colony. Assuming that each lineage underwent two cycles of small colony and one of large colony morphology, we calculate ~70 generations.

Sequencing

Lineages were picked for genome resequencing based on gac and fitness phenotypes. Resequencing was performed using Illumina technology (MiSeq/HiSeq) by MicrobesNG (https://microbesng.uk) which is supported by the Biotechnology and Biological Sciences Research Council (BBSRC; grant number BB/L024209/1), and reads are available at the ENA Short Read Archive with project accession number PRJEB32206. Targeted sequencing of *gacA/S* and *PFLU4242* was performed by PCR amplifying the loci with Phusion HF polymerase (NEB, MA USA) using 1x High-Fidelity buffer, 0.2 mM dNTPs, 0.5 µM each primer (see Table S3), and 1 µl of glycerol stock as a template, with the following program: 98°C 30“, 30 x (98°C 20”, 63°C 30“, 72°C 30”), 72°C 5’. Samples were run on agarose gels to ensure a single product and amplicons purified using the QIAGEN PCR purification kit before sending for Sanger

sequencing. In the case of lineage 04, it was not possible to generate amplicons with any of six different combinations of *PFLU4242* primers and thus this lineage was considered a *PFLU4242* deletion.

Analysis of short-read sequencing

Reads were mapped onto the *P. fluorescens* SBW25 chromosome (EMBL accession AM181176) and pQBR55 (LN713927) using bwa mem version 0.7.17 (25) and variants called using gatk HaplotypeCaller version 4.0.11.0 (26). Additionally, analysis with breseq version 0.33.0 (27) was carried out using the default parameters. For each approach, predicted variants were compared between sequences and with the plasmid-free ancestor to exclude those common to all sequenced strains. Repetitive regions prone to spurious calling (28) were likewise masked from the analysis. The remaining predicted mutations were examined manually for depth of coverage and consistency between reads using IGV (29), using a threshold of mapping quality ≥ 60 and depth ≥ 10. *PFLU4242* from lineages 19 and 20 was additionally Sanger sequenced to verify this approach and confirm mutation presence.

To analyse plasmid copy number, coverage for plasmid and chromosome over 1 kb windows was calculated. Windows with coverage ±2 SD from the mean, due largely to poor mapping quality in repetitive regions, were removed, and the mean across remaining windows was calculated. The ratio of plasmid/chromosomal coverage in sequenced lineages ranged from 2.86 to 3.90 with a mean of 3.4, suggesting modal pQBR55 copy number for all lineages of 3/cell. For pQBR57 the ratio in the ancestral strain was 1.18 and for pQBR103 it was 1.36, suggesting modal copy number for these plasmids is 1/cell.

Allelic replacement to generate knockout strains

Strains lacking *gacA* or *gacS* were previously described by Harrison et al. (2015)(5). The *PFLU4242* knockout was generated in a similar manner using a two-step process with the suicide vector pUIC3. Both PCR and whole genome resequencing indicated that the gene of interest had been knocked out. The Δ*gacS* mutant and the Δ*PFLU4242* mutant had no evidence of second-site mutations, however Δ*gacA* had a single A3084294>G transition resulting in a F155L substitution in the putative integral membrane

protein PFLU2795, which was confirmed by Sanger sequencing. The consistent phenotypes between the two different *gac* mutants and the fact that the Δ*gacA* mutant recapitulates the phenotype of lineage 13 suggests that this second-site mutation does not have a significant impact on our findings.

Statistical analysis

Fitness data for plasmid-bearers was analysed using a linear model with lineage and media as fixed effects. Replicates where fitness could not be estimated due to lack of plasmid-bearer growth were excluded. We did not detect an effect of marker (plasmid-free fitness no different from 1, KB $t[3] = 0.485$, $p = 0.66$, soil $t[3] = 0.815$, $p = 0.475$), so to test whether plasmid-bearing strains were statistically indistinguishible from plasmid-free we ran one-sample t-tests with $\mu = 1$. Logistic regression was used to test association between gac activity and fitness, and non-parametric (Kruskal-Wallis) analysis was used to test for the effect of different knockouts on colony size, due to heteroscedasticity. Analysis was performed in R (R Core Team, Vienna).

Results and discussion

Acquisition of the plasmid pQBR55 caused a small-colony phenotype in *P. fluorescens* SBW25 transconjugants, a phenomenon not observed following acquisition of unrelated sympatric plasmids pQBR57 or pQBR103 (Figure 1A) (21). The small colony phenotype was, however, transient: a further 48 h growth in liquid media or on agar plates (approx. 21 generations, Figures 1A, S1; Movie S1) restored the ancestral (large) colony morphology. Re-emergence of large colony morphologies was not due to plasmid loss as colonies remained mercury resistant and pQBR55-positive by PCR testing for *oriV*. Small colony phenotypes in *P. aeruginosa* and *Staphylococcus aureus* reflect adaptations enabling prolonged infection of humans and animals (30,31). However, as the pQBR55 small colony phenotype was associated with plasmid acquisition and was rapidly lost, we hypothesized that it represented a maladaptive response consistent with exceptionally high plasmid cost, and that restoration of the large colony morphology was due to extremely fast compensatory evolution.

Unlike previous studies (e.g. (5–7,32)) it was not possible to conduct assays on transconjugants that had just received the plasmid, as putatively compensated large colony variants emerged during assay preparation. We therefore compared transconjugants that had restored the large morphology with one another and with the ancestral plasmid-free strain. Twenty fresh independent *P. fluorescens* SBW25(pQBR55) transconjugants were twice re-streaked on selective KB agar (~70 generations growth in total). All remained pQBR55-positive, and 14/20 evolved a clear compensated (large colony) morphology (Figures 1B, S1, S3). These 14 lineages varied in their fitness relative to the plasmid-free ancestor when measured in nutrient-rich KB broth and in soil microcosms designed to approximate the natural substrate of *P. fluorescens* (21) (Figures 1B, S2, Table S4). While most showed amelioration such that fitness was similar to plasmid-free, five lineages still displayed low relative fitness, particularly in soil (linear model, effect of lineage:media $F(10,74) = 11.94$, $p < 0.001$), where in some cases we failed to detect competitive growth altogether. The fitness differences between evolved lineages suggested occurrence of different compensatory mechanisms, as found previously (7,9), which could enhance plasmid survival by increasing the supply of compensatory mutations.

To identify targets of compensatory mutation we chose 6 representative lineages (Figure 1B) and performed whole genome resequencing. A single non-synonymous chromosomal mutation was detected in each clone (Table S1), suggesting that the rapid emergence of compensation was not associated with plasmid-induced hypermutation. No pQBR55 mutations were detected in any lineage. Lineages with the least amelioration, 03 and 16, had mutations affecting RNA polymerase: lineage 03 had a 7 bp deletion in the P26 partial terminator upstream of *rpoB* (*PFLU5534*) while lineage 16 had acquired a missense mutation Pro584Ser in *rpoC* (*PFLU5533*). Lineages with intermediate amelioration, 13 and 18, had mutations in *gacS* (*PFLU3777*) and *gacA* (*PFLU2189*), respectively (lineage 13 had an additional synonymous C>T transition in *PFLU3410*). Lineage 19, which showed the most complete amelioration, and 20, had mutations in *PFLU4242*, which encodes a hypothetical 527 amino acid protein with two domains of unknown function (DUF262, DUF2081). The remaining evolved lineages phenotypically

resembled lineage 19 and targeted sequencing revealed each also had a mutation in *PFLU4242* (Figure 1B, Table S6). To test the ameliorative roles of *gacA/S* and *PFLU4242* mutations we generated pQBR55 transconjugants in *P. fluorescens* SBW25 Δ*gacA*, Δ*gacS*, and Δ*PFLU4242*. Unlike wild-type, deletion-mutant strains immediately and exclusively produced large-sized transconjugant colonies with fitness similar to the evolved lineages (Figure 2, Figure S4, Table S7), suggesting that disrupting any of these genes rendered *P. fluorescens* 'pre-ameliorated' for pQBR55 acquisition, and implicating these genes in the high fitness costs of pQBR55.

GacA/GacS signaling positively regulates a suite of stationary phase phenotypes including flagella-mediated motility and secreted products, suggesting that mutations affecting these genes have major pleiotropic effects (20,33). Correspondingly, only strains with *gacA* or *gacS* mutations (13 and 18) had lost the ability to produce extracellular protease, pectinase, surfactant (viscosin) and to swim (Figure 1B), potentially restricting amelioration via this pathway in complex habitats like soil (34). Indeed, lineages with the highest level of fitness in soil tended to retain GacA/GacS-regulated phenotypes (logistic regression, effect of fitness in soil on protease result LRT Chisq(1) = 15.8, p = 6.9e-5). Mutation of *gacA/gacS* was previously implicated in ameliorating the unrelated plasmid pQBR103, through widespread effects on transcription (5). In *P. aeruginosa*, GacA/S controls small colony variants generation through the activity of RsmA and the second messenger cyclic-di-GMP (31). Interestingly, pQBR55 carries an RsmA homologue (PQBR55_0192), which might directly affect GacA/S or cyclic-di-GMP signalling to cause small colonies in *P. fluorescens* (35).

Mutations to RNA polymerase are a common response to cellular stress, e.g. in rifampicin resistance (36) and elevated temperature (37), and have previously been implicated in plasmid adaptation (6). Of the two mutations detected here, one (RpoC Pro584Ser) probably affects specificity, whereas the other (deletion of an attenuator upstream of *rpoB*) likely increases expression (36). These mutations may be adaptations to modulate expression of specific disruptive genes, or to generally compensate for the transcriptional burden of plasmid gene expression (38), and seem functionally distinct from those conferring rifampicin

258 resistance as we did not detect an increased rifampicin minimum inhibitory concentration (MIC) for
259 lineages 03 and 16 (Table S2).

260 The most complete amelioration occurred through *PFLU4242* mutation (Figure 1, Figure S4). *PFLU4242*
261 was likely acquired by *P. fluorescens* SBW25 via recent HGT as related strains *P. protegens* Pf-5 and *P.*
262 *fluorescens* Pf0-1 do not encode homologues whereas similar genes are found in distantly related genera
263 e.g. *Burkholderia* (WP_059533732.1, 80.5% amino acid identity), *Fischerella* (WP_016860471.1,
264 60.4%), and *Streptomyces* (WP_086730045.1, 50.8%). The GC-content of *PFLU4242* was 43.5%,
265 compared with 60.5% across the whole genome, also consistent with recent horizontal acquisition,
266 however we could not identify any neighbouring genes associated with mobility (e.g. transposases or
267 integrases), and the genes neighbouring *PFLU4242* homologues in these other species are not
268 homologous to those in *P. fluorescens* SBW25. The function of PFLU4242 remains mysterious, but its
269 principal domain, DUF262, is a member of the ParB superfamily that includes nucleases as well as
270 partitioning systems (39). We did not detect any gross phenotypic effects of *PFLU4242* knockout on
271 colony morphology or growth in the absence of pQBR55. Further work is required to elucidate the
272 mechanistic basis of PFLU4242 function, but our finding is consistent with a growing body of evidence
273 implicating horizontally-transferred DNA-binding proteins as key determinants of plasmid cost (6,7).

274 Re-examination of genome-resequencing data from previous experiments with *P. fluorescens* SBW25
275 implicates *PFLU4242* disruption in ameliorating unrelated plasmids pQBR103 (5) and pQBR57 (40),
276 suggesting a general mechanism behind plasmid costs in this host. Though *P. fluorescens* SBW25
277 isolated from the same site as the pQBR plasmids, it was originally plasmid-free (21), indicating it may
278 be a naive host, potentially vulnerable to conflicts between resident and newly-acquired genes.

279 Our observed rates of amelioration, occurring during the process of isolating individual plasmid-
280 containing clones, exceed those of previous studies and suggest a prominent role for compensation in
281 bacterial evolution. Similar mutations emerging during the preparation of plasmid-containing strains may
282 also influence the interpretation of experimental assays, leading, for example, to an underestimation of

plasmid carriage costs. In natural communities, rapid compensatory mutations could overcome the oftentimes heavy metabolic and regulatory costs of plasmid-mediated horizontal gene transfer, and, in combination with stability functions that expand the window of opportunity for such mutations to arise, are likely to be a major factor promoting plasmid carriage and thus the maintenance and spread of adaptive traits.

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Competing interests

The authors have no competing financial interests to declare.

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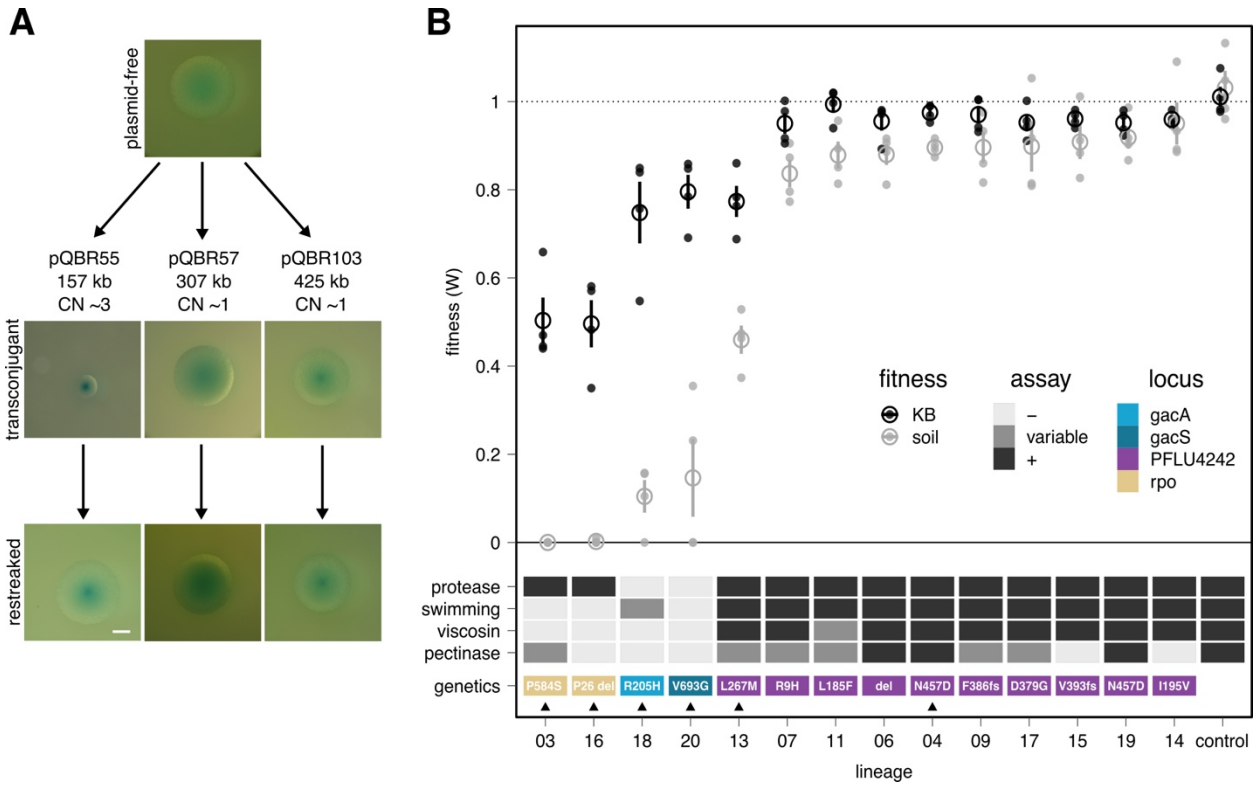
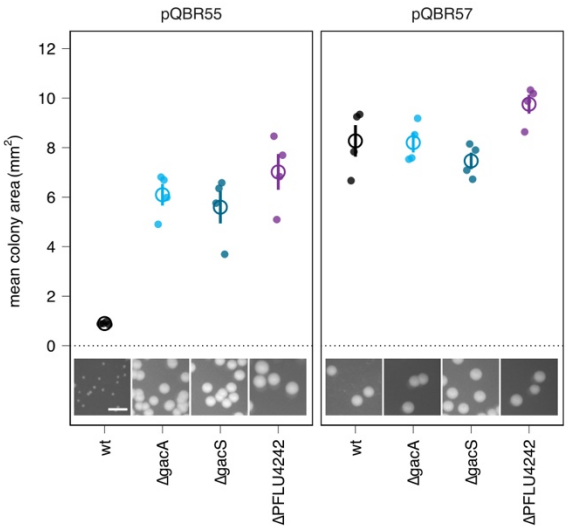


Figure 1. pQBR55-bearers rapidly ameliorate plasmid cost. **(A)** Transconjugants of pQBR55, but not sympatric mercury resistance plasmids, consistently produce a small colony phenotype which is rapidly resolved on restreaking. Scale bar indicates 1 mm. Lineage 20 is shown, photographs of all 20 lineages are in Figure S1. Plasmid size and approximate modal copy number (CN) are provided for reference. **(B)** Evolved lineages. Top: fitness relative to plasmid-free in KB (black) or in soil (grey). Four independent replicates are shown, with mean and standard error overlayed. Where fitness could not be estimated (no growth) it was set to zero. Control refers to competition between plasmid-free strains to assess any fitness effects of the antibiotic markers. Middle: Gac phenotype assay results. Bottom: evolved loci. Colour indicates target of locus-level parallel mutation, and the mutation is overlaid. del = deletion; P26 refers to a partial attenuator upstream of rpoB (36). Strains subject to whole genome resequencing are marked with triangles, the remaining *PFLU4242* sequences were determined by Sanger sequencing of amplicons.



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312 **Figure 2.** pQBR55 transconjugant strains lacking in *gacA*, *gacS*, or *PFLU4242* do not display a small
313 colony morphology. Transconjugants of pQBR55 and pQBR57 were photographed and mean colony size
314 after 48 hours growth was measured. Four independent replicates are shown with mean and standard error
315 overlaid. Scale bar indicates 5 mm. We detected a significant effect of recipient on pQBR55 colony size
316 (Kruskal-Wallis p = 0.016) driven by the wild-type recipient (planned contrast Wilcoxon Test p = 0.001),
317 but not on pQBR57 (K-W p = 0.061; Wilcoxon p = 0.8615).

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Supplementary information for 'Extremely fast amelioration of plasmid fitness costs by multiple functionally-diverse pathways'

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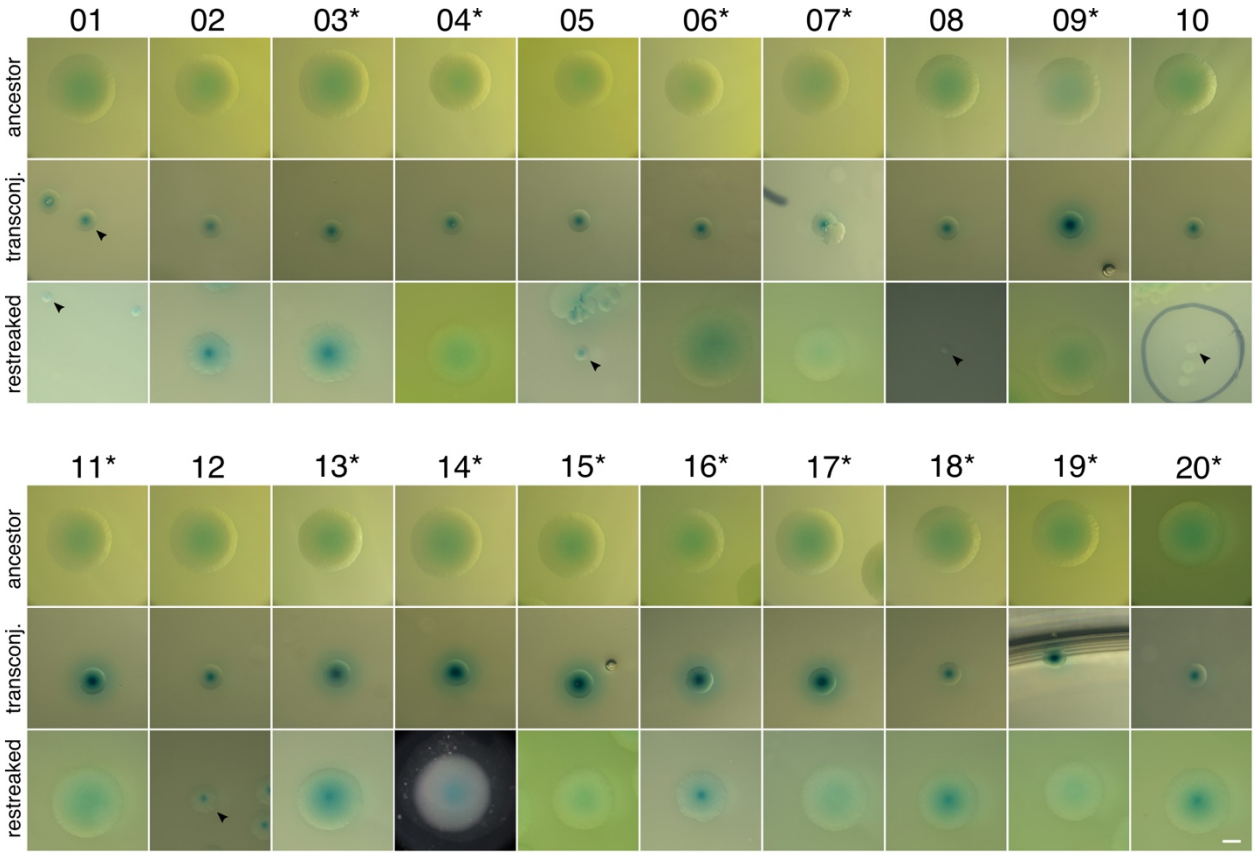
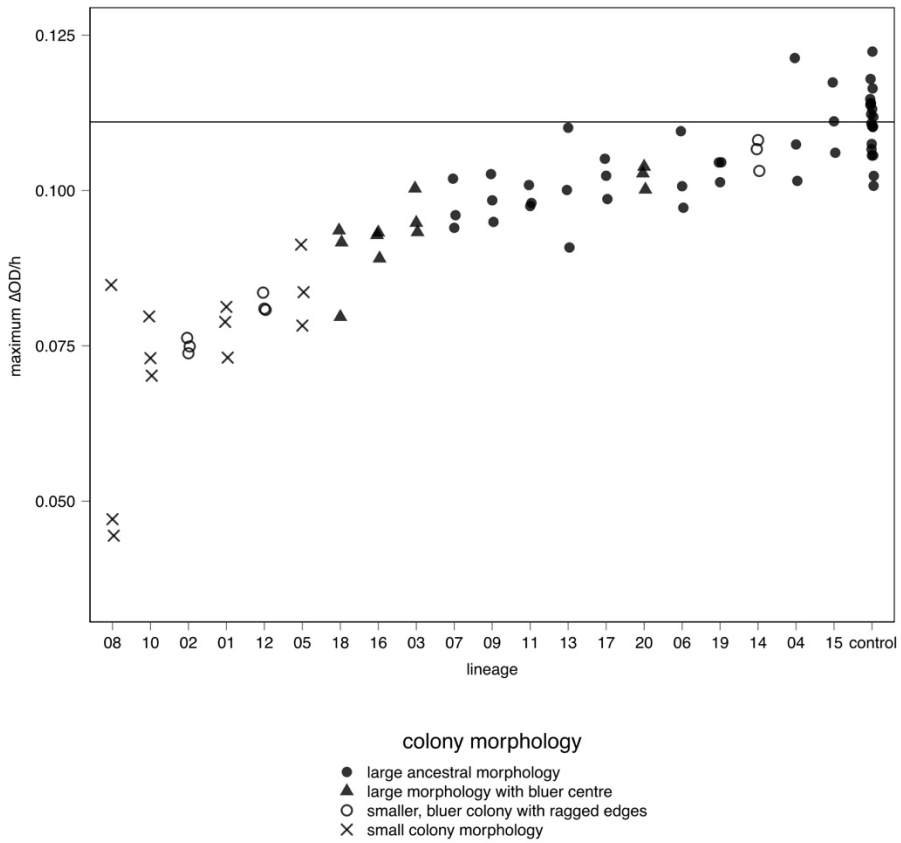
Data accessibility statement: .csv tables of data supporting the results are in Supplementary Tables S1-S9, at doi:10.17638/datacat.liverpool.ac.uk/953, and short read sequences are on the Short Read Archive, accession PRJEB32206.



433

434 **Figure S1.** Colony morphologies of the 20 lineages. Where multiple colonies are visible, arrows mark the
435 one which was selected. Lineages marked with an asterisk were deemed to have recovered ancestral
436 phenotype based on growth curves and colony morphology.

437

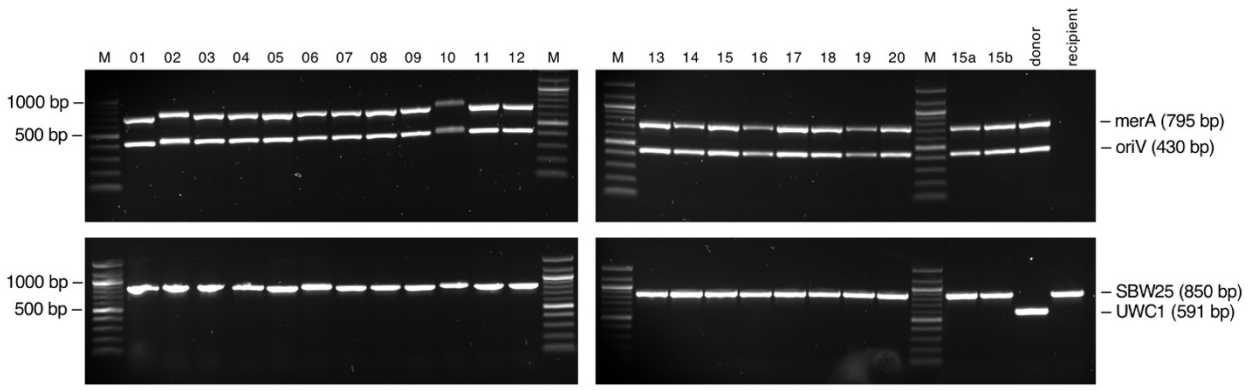


438

439 **Figure S2.** Maximum growth rate of the 20 lineages. Three independent replicates were performed for

440 each lineage. The ancestral plasmid-free clone for each lineage was also measured (n = 20, 'control').

441



442

443 **Figure S3.** The 20 lineages carry pQBR55. PCR for the *merA* resistance gene and the pQBR55 origin of
444 replication was performed on the stocked colony for each lineage. PCR using primers specific for *P.*
445 *fluorescens* SBW25 and *P. putida* KT2440 (from which the donor strain UWC1 was derived) were used
446 to ensure strains were transconjugants. 15a and 15b are additional colonies retrieved from lineage 15. M =
447 NEB 100 bp ladder.

448

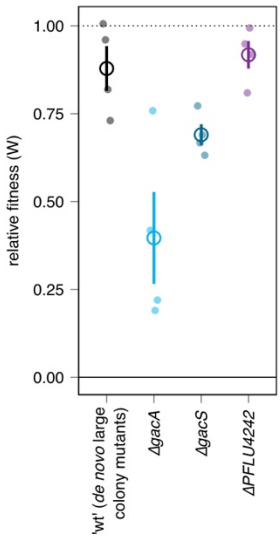


Figure S4. Knockout transconjugants have relative fitness similar to evolved lines. Transconjugant colonies from Figure 2 were restreaked twice to isolate a single clone and competed against a plasmid-free competitor in KB. During this process, the wild-type small colony morphologies reverted to the large colony phenotype, and thus should be considered de novo mutants. We detected a significant effect of recipient (ANOVA $F_{3,12} = 9.6$, $p = 0.002$), driven largely by reduced amelioration by the Δ*gacA* mutant.

sample	chromosome	position	reference	alternative	depth	mapping quality	quality	name	locus_tag	nucleotide mutation	amino acid mutation	GATK
ΔgacA	AM181176	3084294	A	G	21	60	786	PFLU_2795	PFLU_2795	c.463T>C	p.Phe155Leu	1:0,21:21:99:816,0
lineage 03	AM181176	6062127	ATCAGCCT	A	165	60	7242	rpoB-rplL	PFLU_5534-PFLU_5535	n.6062128_6062134delTCAGCCT		1:0,163:163:99:7281,0
lineage 13	AM181176	4174597	T	G	94	60	3164	PFLU_3777	PFLU_3777	c.2078T>G	p.Val693Gly	1:0,94:94:99:3194,0
lineage 16	AM181176	6056066	G	A	204	60	7317	rpoC	PFLU_5533	c.1750C>T	p.Pro584Ser	1:0,204:204:99:7347,0
lineage 18	AM181176	2373295	G	A	72	60	2252	gacA	PFLU_2189	c.614G>A	p.Arg205His	1:0,72:72:99:2282,0
lineage 18	AM181176	3773830	C	T	73	60	2246	PFLU_3410	PFLU_3410	c.618G>A	p.Ser206Ser	1:0,73:73:99:2276,0
lineage 19	AM181176	4684561	T	C	63	60	2042	PFLU_4242	PFLU_4242	c.1369A>G	p.Asn457Asp	1:0,63:63:99:2072,0
lineage 20	AM181176	4685131	A	T	83	60	2820	PFLU_4242	PFLU_4242	c.799T>A	p.Leu267Met	1:0,83:83:99:2850,0

456

457 **Table S1.** Mutations detected in whole genome resequenced lineages. Results are shown from the

458 bwa/GATK pipeline, similar results were obtained using breseq.

459

lineage	replicate		
	A	B	C
13	12.5	12.5	12.5
16	6.25	6.25	12.5
18	12.5	12.5	12.5
19	12.5	12.5	12.5
20	12.5	12.5	12.5
03	12.5	12.5	12.5
plasmid-free	12.5	25	12.5

460

461 **Table S2.** Rifampicin minimum inhibitory concentrations (MIC) for sequenced evolved lineages. Values

462 are in μg/ml.

463

Primer name	Sequence	Purpose
pQBR55_0051_R1	ATGGCTGCCCTAGACCTGG	Detecting pQBR55 origin of replication
pQBR55_0050_F1	CAACGCCCGAACAAACGC	
merA_F	TGCAAGACACCCCCTATTGGAC	Detecting merA presence
merA_R	TTCGGCGACCAGCTTGATGAAC	
ΔPFLU4242_UF	TATAGGATCCACTAGTATGTTCCGTGGCTCCTC	Generating the PFLU4242 knockout
ΔPFLU4242_UR	GAAATTATTTAACGACTCTTCCCTACTCGCATT	
ΔPFLU4242_DF	GGAAGAGTCGTTAAATAATTTCGCTTTAATACCGTTAT	
ΔPFLU4242_DR	TATAGAATTCACTAGTGATTTTTGGCCTTAAAGCAAAGC	
ΔPFLU4242_TESTF	GTCTCTACTACGATCAAGTATTGACAC	
ΔPFLU4242_TESTR	CTTGGGCCAAAAGCGGAC	
PFLU4242_FAMP	GAACTCGAGTACATTGGCGC	Amplifying and sequencing PFLU4242
PFLU4242_RAMP	CGGGGCCTTTTACATAACGG	
PFLU4242_FINT	ACCTGGAGCGATGACTTTGA	
PFLU4242_RINT	TTCCAGAAAAGCACGTACGC	
PFLU4242_intF_3	TTGCTGTGCGAAGGAATTTG	
PFLU4242_intR_5	TCCCAAACAAAGCCACGC	
PFLU4242_FSEQ	AGGGAAGAGTCGTATGGAGT	
gacA_F	CAGCAAAATAGAGCCGTCCGCCTC	Amplifying and sequencing gacA
gacA_R	CCAGCGCAGCTGTCTTGG	
gacA_400F	CGCCACGACCAAGTTGTTG	
gacA_600R	GGCTGGAAAGACTTGATGGC	
gacS_F	CCAATCGCCGCCGGAC	Amplifying and sequencing gacS
gacS_R	CAGGCCTGGCGGCAG	
gacS_600F	CTTGCTGCCGGTGTTTGG	
gacS_750R	GAGTTGATCGTACGGCTGATG	
gacS_1500F	AGGACGAGCAGGAAGACAG	
gacS_1650R	GATTTCACCGCCCATCTGC	
gacS_2350F	TCGACTTGGTGCTGATGGAC	
gacS_2550R	GGTCAGGTAATCGTCCATGC	
SBW25_F	ACTGCATTCAAAACTGACTGA	Distingishing strains
SBW25_R	AATCACACCGTGGTAACCG	
KT2440_F	ATGGCAATGTCCGCAATCC	
KT2440_R	CGGAAGCCTCTGAACACG	

Table S3. Sequences of primers used in this study.

Tables S4-S9 are provided as .csv files at doi:10.17638/datacat.liverpool.ac.uk/953

Table S4. Relative fitness plotted in Figure 1B.

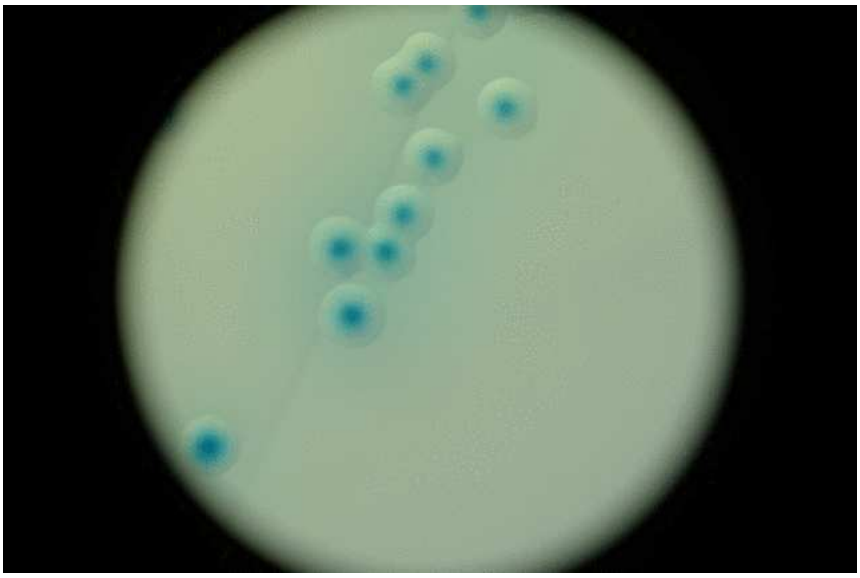
Table S5. Gac assay results plotted in Figure 1B.

Table S6. Summary of genetics information plotted in Figure 1B.

Table S7. Colony size measurements plotted in Figure 2.

Table S8. Growth curve data plotted in Figure S2. Time is in seconds and rate is in ΔOD/s.

Table S9. Relative fitness of knockout strains plotted in Figure S4. Two replicate experiments were performed, data from experiment 1 was plotted.



Movie S1. Breakout of compensated mutants from a small morphology colony. Small colonies restreaked from lineage 15 were maintained at room temperature and photographed every 30 minutes. Multiple independent mutants can be seen emerging from each colony. This movie is provided through the journal website and at doi:10.17638/datacat.liverpool.ac.uk/953.