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# Parallel Compensatory Evolution Stabilizes Plasmids across the Parasitism-Mutualism Continuum

## Highlights

- Compensatory evolution stabilized plasmids across a parasitism-mutualism continuum
- Selection repeatedly targeted the same genes in independently evolving populations
- Mutations in a global regulatory system, gacA/gacS, ameliorated the plasmid cost
- Evolved genotypes had reduced the translational demand caused by the plasmid

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## In Brief

Harrison et al. show that parallel regulatory mutations of large effect in bacteria rapidly compensated for the cost of plasmid carriage to stabilize both parasitic and mutualistic resistance plasmids. The findings help to explain the widespread occurrence of resistance plasmids and their role in horizontal gene transfer.

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# Parallel Compensatory Evolution Stabilizes Plasmids across the Parasitism-Mutualism Continuum

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## SUMMARY

**Plasmids drive genomic diversity in bacteria via horizontal gene transfer [1, 2]; nevertheless, explaining their survival in bacterial populations is challenging [3]. Theory predicts that irrespective of their net fitness effects, plasmids should be lost: when parasitic (costs outweigh benefits), plasmids should decline due to purifying selection [4–6], yet under mutualism (benefits outweigh costs), selection favors the capture of beneficial accessory genes by the chromosome and loss of the costly plasmid backbone [4]. While compensatory evolution can enhance plasmid stability within populations [7–15], the propensity for this to occur across the parasitism-mutualism continuum is unknown. We experimentally evolved *Pseudomonas fluorescens* and its mercury resistance mega-plasmid, pQBR103 [16], across an environment-mediated parasitism-mutualism continuum. Compensatory evolution stabilized plasmids by rapidly ameliorating the cost of plasmid carriage in all environments. Genomic analysis revealed that, in both parasitic and mutualistic treatments, evolution repeatedly targeted the *gacA/gacS* bacterial two-component global regulatory system while leaving the plasmid sequence intact. Deletion of either *gacA* or *gacS* was sufficient to completely ameliorate the cost of plasmid carriage. Mutation of *gacA/gacS* downregulated the expression of ~17% of chromosomal and plasmid genes and appears to have relieved the translational demand imposed by the plasmid. Chromosomal capture of mercury resistance accompanied by plasmid loss occurred throughout the experiment but very rarely invaded to high frequency, suggesting that rapid compensatory evolution can limit this process. Compensatory evolution can explain the widespread occurrence of plasmids and allows bacteria to retain horizontally acquired plasmids even in environments where their accessory genes are not immediately useful.**

## RESULTS AND DISCUSSION

We combined experimental evolution, whole-genome sequencing, and gene expression analysis to examine the dynamics and mechanisms of bacteria-plasmid adaptation along a parasitism-mutualism continuum in the environmental bacterium *Pseudomonas fluorescens* SBW25 [17] and a naturally associated 425-kb mega-plasmid, pQBR103 [18]. pQBR103 carries a Tn5042 transposon containing a mercury resistance operon, *mer*, which allows the bacterial host to detoxify mercury-contaminated environments by reducing toxic mercuric ions to elemental mercury [16]. In the absence of mercury, pQBR103 imposes a large fitness cost on *P. fluorescens* SBW25 when measured in lab media, in soil or in planta [18, 19]. This combined with an appreciable rate of segregational loss and a comparatively low rate of conjugation [19] means that pQBR103 cannot counteract the action of purifying selection and should theoretically go extinct in mercury-free environments [20]. We established 36 populations of SBW25-pQBR103 that we propagated by serial transfer for c.450 bacterial generations under six mercury treatments (i.e., six replicates per treatment) spanning the parasitism-mutualism continuum (0, 8, 16, 24, 32, and 40 μM $HgCl_2$; Figure S1). Plasmids remained at high prevalence in all treatments containing >8 μM $HgCl_2$. In 0 μM $HgCl_2$ where there was no benefit to plasmid carriage, prevalence was highly variable, but we only observed complete loss of the plasmid in one case. In the five populations that retained the plasmid, prevalence was on average 40.2% by the end of the experiment (Figure S2). We observed frequent transposition of Tn5042 to the bacterial chromosome accompanied by loss of the plasmid across all treatments, confirming that such genetic rearrangements were possible in our experiment (Figure S2). However, the appearance of these genotypes was often transient, and in only a single population, at 8 μM $HgCl_2$, did a chromosomal copy of Tn5042 invade to appreciable frequency and coexist in the population with plasmid-bearing strains. To determine the fitness response to selection, at the end of the experiment, we directly competed three randomly selected bacteria-plasmid clones from each population against the ancestral plasmid-containing strain in their selection environments (Figure 1A). Large increases in fitness (between 10.7% ± 15.2% SE and 120% ± 62.4% SE) were observed in all treatments, confirming that substantial evolutionary adaptation had occurred across the parasitism-mutualism continuum. Conjugation rates of

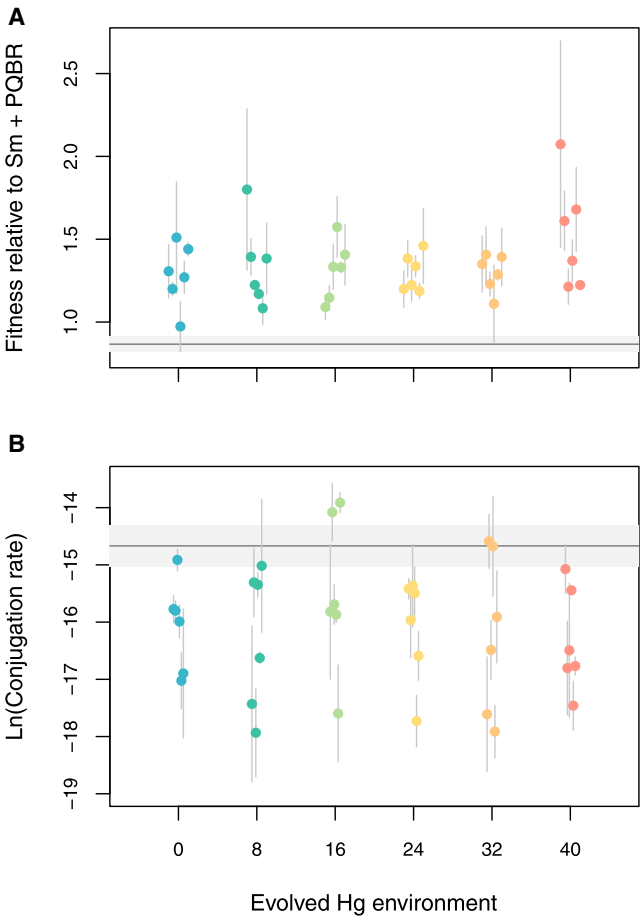


**Figure 1. Phenotypic Evolution of Fitness and Conjugation Rate**

(A) Fitness of evolved plasmid-containing clones from each mercury environment relative to the plasmid-containing ancestor assayed in their evolved mercury environment. Points represent means for three randomly selected clones for each population with error bars showing SE. The gray line shows the fitness of the ancestral strain, with the gray shading showing SE (n = 6). Fitness increased in all treatments relative to the ancestor (pairwise contrasts: all $p < 0.0163$), although no significant difference was observed between treatments ($t_{71,34} = 1.481$, $p = 0.148$).

(B) Conjugation rates of evolved plasmids from evolved clones into ancestral bacteria. Points represent means for three randomly selected clones for each population with error bars showing SE. The gray line shows the conjugation rate of the ancestral strain, with the gray shading showing SE (n = 6). On average, conjugation rates decreased by approximately 10-fold, but not significantly so (pairwise contrasts: all $p > 0.131$), and no significant difference was observed between treatments ($t_{71,34} = -0.498$, $p = 0.622$).

the evolved bacteria-plasmid clones remained low or declined in all treatments (Figure 1B), suggesting that plasmids were not persisting through conjugation in this experiment.

To determine the genetic bases of the observed adaptation, we obtained the whole-genome sequence for one randomly selected clone from each population (i.e., six independent evolved clones per treatment). Four evolved clones were found to have acquired hypermutability, carrying single-nucleotide polymorphisms (SNPs) in the *mutL* or *mutS* mismatch repair loci, and between 70 and 189 mutations in total. Excluding these hypermutators, evolved clones contained between one and six mutations, and these mutations were further analyzed. Of these, 78.1% were non-synonymous, and 13.5% were in non-coding regions. All except one of the observed mutations occurred on the bacterial chromosome; the sole exception was a duplication of the Tn5042 transposon, containing the *mer* operon, on the plasmid, but, surprisingly, this occurred in a clone evolved in the 0 μM $HgCl_2$ treatment. No significant associations were observed between either the total number (chi-square $test_{5,7} = 8.83$, $p = 0.12$) or the type (i.e., non-synonymous, synonymous, or intergenic; chi-square $test_{10,19} = 8.74$, $p = 0.56$) of mutations and mercury treatment. These data indicate that evolutionary change of the bacterium alone was sufficient for adaptation across the parasitism-mutualism continuum.

We observed strikingly parallel evolution at the operon, locus, and nucleotide levels both within and between treatments (Figure 2A and Table S1). Of the 95 non-synonymous mutations identified, we observed 74 cases of operon-level parallelism, 68 cases of locus-level parallelism, and 14 cases of nucleotide-level parallelism (i.e., precisely the same mutational change occurred at the same nucleotide position in multiple independently evolved clones). Nucleotide-level parallelism was identified in five loci, comprising three open reading frames (ORFs) and two intergenic regions. Three loci in particular displayed exceptionally high rates of parallel evolution: PFLU1661 (18 out of 32 clones locus parallelism; 4 out of 32 clones nucleotide parallelism at two sites), *gacA* (PFLU2189; 5 out of 32 clones locus parallelism; 2 out of 32 clones nucleotide parallelism), and *gacS* (PFLU3777; 20 out of 32 clones locus parallelism; 3 out of 32 clones nucleotide parallelism). PFLU1661 is a hypothetical structural gene believed to contribute to cell-wall biogenesis and sits within the *wsw* operon [21] (wrinkly spreader cell-wall biogenesis), whereas the sensor kinase *gacS* and its cognate response regulator *gacA* form a two-component regulatory system that controls the biosynthesis of a wide range of secondary metabolites [22, 23]. Further sequencing, both of evolved clones that had naturally lost the plasmid during the experiment and evolved clones from plasmid-free control lines, revealed that while mutations in PFLU1661 were observed in both the plasmid-free and the plasmid-containing clones, we only ever observed mutations in *gacA* or *gacS* in the plasmid-containing clones. This suggests that mutations in this regulatory system may have played a causal role in the adaptation of the bacterium to the plasmid. To examine the dynamics of evolution at *gacA*/*gacS*, we tracked the allele frequency dynamics at these loci by FreqSeq [24] in two subsets of our experimental populations: first, in the populations containing each of the nucleotide-level parallel mutations at these loci since such highly parallel evolution is likely to reflect adaptive substitutions; and second, in the populations from the 0 μM $HgCl_2$ treatment in which the plasmid persisted, where we hypothesized that compensatory evolution at *gacA*/*gacS* may have contributed to the observed plasmid dynamics. Consistent with strong selection for rapid compensatory evolution operating on the nucleotide parallel mutations, these arose early in the experiment and, in four out of five cases, rapidly swept to high frequency among the plasmid-bearing population (Figure 2B). Among populations from the 0 μM $HgCl_2$ treatment that retained the plasmid, the frequencies of mutations in *gacS* closely tracked the plasmid dynamics in three out of five populations. This strongly suggests an important

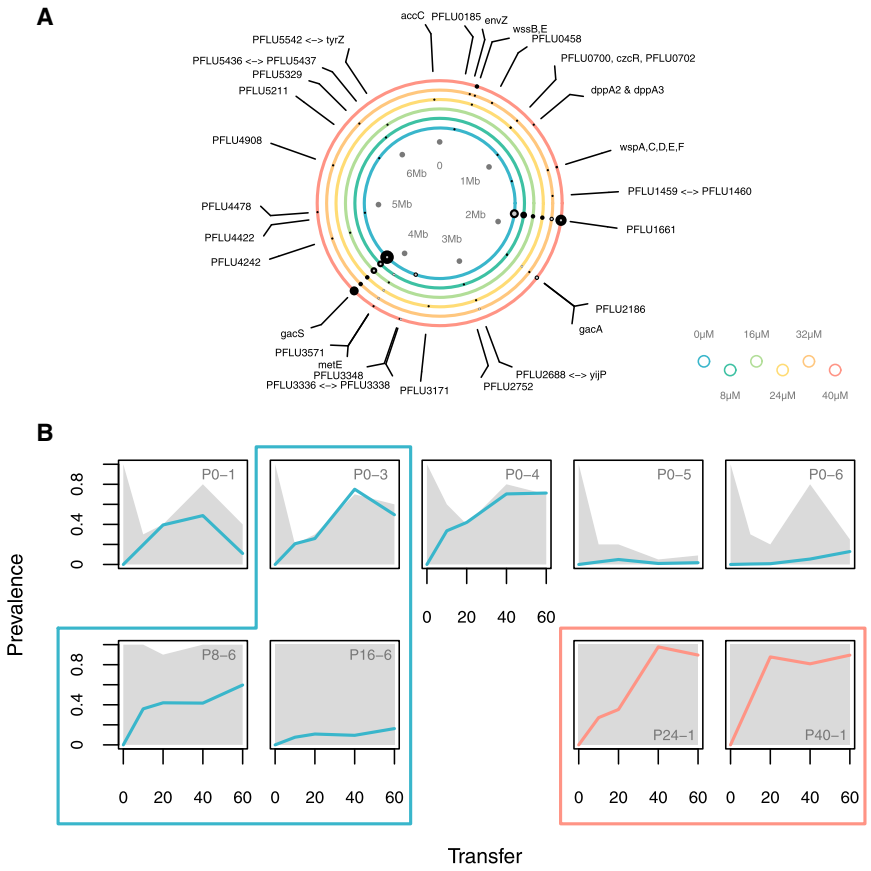


**Figure 2. Genomic Evidence for Parallel Compensatory Evolution**

(A) Summary of mutations identified in evolved clones (excluding hypermutator clones) across treatments. Rings represent the SBW25 bacterial genome, with each ring representing an evolved mercury treatment. Mutations are shown as dots, with the size of the dot representing the number of times mutations appear at each locus across replicate lines. Gray dots denote nucleotide level parallelism (NLP). Plots of individual replicates are shown in Figure S3.

(B) Allele frequency dynamics of *gacS* and *gacA* mutations in plasmid-retaining populations from the 0 μM $HgCl_2$ treatment and in populations exhibiting NLP. Plots show individual populations, with plasmid prevalence shown in gray and lines showing the frequency of *gacS* (blue) and *gacA* (red) mutations. Boxes denote NLP mutations shared between populations.

role for compensatory evolution at *gacS* in preventing extinction of the plasmid in these populations. However, it is possible that plasmid hitchhiking on other beneficial chromosomal mutations could also have contributed to the observed plasmid dynamics in this treatment [4, 20].

Twenty-five unique mutations were observed in *gacA*/*gacS* located across all predicted domains of the two proteins (Figure 3A), with the exception of the C-terminal DNA-binding domain of *gacA*. All but 3 of the 25 unique mutations were predicted [25] to have deleterious effects on protein function, including three nonsense mutations and one deletion spanning over half of the *gacA* gene. The three remaining mutations not predicted to alter function were located within the HAMP, HPT, and extracellular sensory domains of GacS, with one SNP in the HAMP domain arising in parallel three times in evolved clones (plus a fourth time in one of the hypermutator clones). Such nucleotide-level parallelism is strongly suggestive of adaptation and furthermore suggests that these mutations were in fact likely to have altered protein function. Together these data suggest that the observed mutations would have caused loss of function of the GacA/GacS two-component regulatory system.

To directly test whether loss of GacA/GacS function plays a role in ameliorating the cost of plasmid carriage, we constructed gene deletion knockout mutants of *gacA, gacS*, and *gacA*/*gacS* in the ancestral background. We measured the competitive fitness relative to SBW25 of the knockout mutants and the wild-type, both with and without the plasmid. In the competitions conducted without the plasmid, the knockout mutants showed equal or reduced relative fitness compared to the wild-type, indicating that mutations in these genes are not involved in general adaptation to the lab environment. Crucially, however, in the competitions conducted with the plasmid, the knockout mutants were significantly fitter than the wild-type (Figure 3B): whereas the plasmid imposed a 27% fitness cost upon the wild-type ($t_{2.42}$ = 12.45, p = 0.0025), we observed no appreciable cost of plasmid carriage in the knockout mutants (p > 0.168 for all three mutants). This confirms that loss of function of the GacA/GacS two-component regulator is sufficient to completely ameliorate the cost of plasmid carriage.

To further understand the gene-regulatory consequences of plasmid acquisition that may have contributed to the cost of the plasmid in the ancestor and the effects of subsequent compensatory evolution, we performed a microarray analysis of gene expression in ancestral and evolved strains. We extracted RNA from mid-log phase cultures of ancestral SBW25, ancestral SBW25-pQBR103, and six randomly selected evolved clones from the 0 and 40 μM $HgCl_2$ treatments, each of which carried a mutation in the *gacA* or *gacS* genes. In the ancestral genetic background, plasmid carriage was associated with widespread upregulation of chromosomal genes (Figure 4A). Out of 6,018 bacterial genes, 1,005 genes or 16.7% of the genome were significantly upregulated in SBW25-pQBR103 compared to plasmid-free SBW25, whereas only six were significantly downregulated (five proteins of unknown function, PFLU2260, PFLU2594, PFLU2776, PFLU3162, and PFLU3545, and one TetR family transcriptional regulator, PFLU3214). Functional analysis of the upregulated gene set revealed significant enrichment of ten gene ontology (GO) categories, all of which are involved in protein production. Indeed, over 90% of all the chromosomal genes associated by annotation with translation, protein folding, tRNA aminoacylation, rRNA binding, and ribosomes were within the significantly upregulated gene set. The most highly upregulated genes were those encoding structural components of the

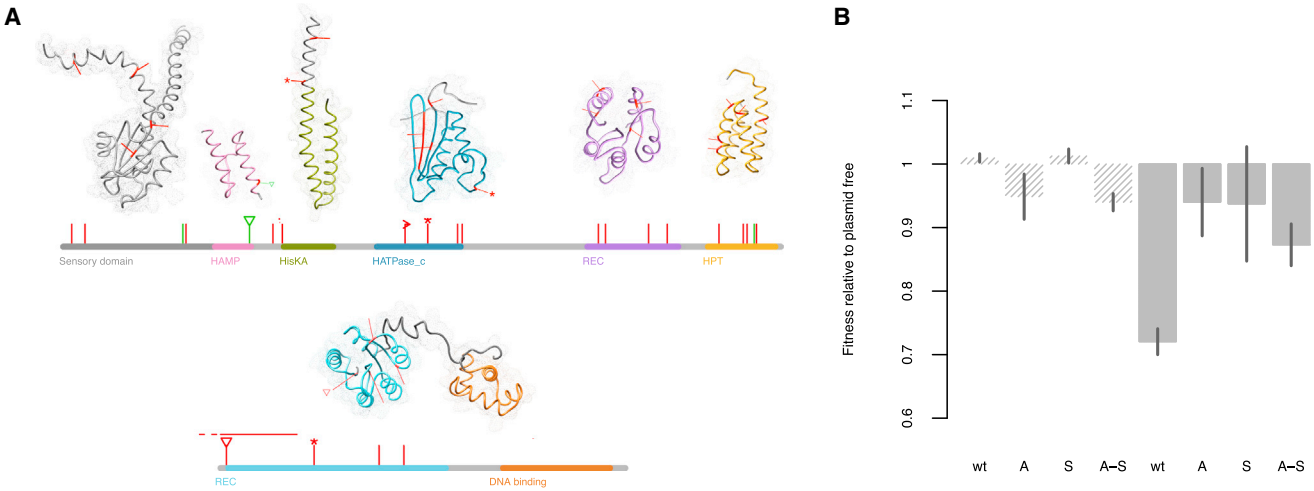


**Figure 3. Functional Consequences of the *gacA* and *gacS* Mutations**

(A) Representation of the protein sequences of GacS and GacA, including predicted domains. Unique amino acid changes are marked with asterisks denoting nonsense mutations, triangles showing parallel mutations appearing in more than one evolved clone, and arrows showing deletions. Mutations predicted as having negative effects on protein function are shown in red, with those predicted as being neutral in green. We had low confidence in the full-length GacS structural prediction; therefore, structural analysis was performed by domain.

(B) Fitness effects of *ΔgacA/gacS* knockout strains in the absence (hatched) and presence (filled) of the pQBR103 plasmid. Fitness of the ancestor, *ΔgacA, ΔgacS* and *ΔgacA ΔgacS* double knockout was measured relative to a labeled plasmid-free ancestor. Bars show the means of four replicates, with error bars showing SE.

ribosome; the relative expression of these genes was on average 13.7-fold that of the plasmid-free ancestor (Figure 4A). In contrast, these changes appear to be reversed in the evolved clones. Bacterial gene expression profiles of clones from both selection environments were highly similar to those of the plasmid-free ancestor (Figures 4B and 4C) and correspondingly showed significant downregulation when compared to the plasmid-containing ancestor (Figures 4D and 4E). Evolved changes in expression of plasmid genes followed a similar pattern to that of the chromosomal genes, with ~17.1% of plasmid genes downregulated in evolved compared to ancestral bacteria-plasmid clones with no significant differences between 0 μM and 40 μM $HgCl_2$ treatments (Figures 4D and 4E). No enriched functions could be identified among this plasmid gene set due to sparse functional annotation of pQBR103 genes. Taken together, these data suggest that the plasmid caused increased translational demand during exponential growth but that this negative effect of plasmid carriage was ameliorated by compensatory mutations in the evolved clones. Other than *gacA/gacS*, no other mutational targets were shared between the six evolved clones tested (highlighted in Table S1). Therefore, both the observed reversion in evolved clones to ancestral levels of chromosomal gene expression and the downregulation of plasmid genes were likely associated with loss of GacA/GacS function.

The *gacA/gacS* two-component regulatory system is highly conserved among gamma-proteobacteria [26, 27] and positively regulates the production of many extracellular proteins in response to environmental stimuli [28]. This regulation is mediated through several small RNAs that bind to and inhibit the RNA-binding proteins RsmE and RsmA [27], relieving post-transcriptional repression [29]. The loss of GacA/GacS function leaves in place post-transcriptional repression by RsmE and RsmA and is likely therefore to reduce overall translational demand within the cell. Moreover, it appears that loss of GacA/GacS function also resulted in downregulation of a large set of plasmid genes. As newly acquired plasmid genes are likely to be poorly adapted to the host cell, it is possible that some of these plasmid gene products may have had cytotoxic effects on the bacterial cell [30], which were alleviated by downregulation of their expression. Further research will be required to determine the precise mechanism of the cost of plasmid carriage in this system and specifically whether any of the downregulated plasmid gene products were cytotoxic [30]. Loss-of-function mutants in *gacA/gacS* have been isolated both in nature [31] and in the laboratory [22]. Indeed, elevated mutation rates and frequent reversion in *gacA/gacS* have been reported in *Pseudomonas* sp. PCL1171 [32] and associated with Xer site-specific recombinase activity in *P. fluorescens* F113 in the rhizosphere [33]. The *gacA/gacS* two-component system may therefore represent contingency loci, where an elevated mutation rate is adaptive, allowing rapid phenotypic switching in response to environmental unpredictability [34]. In natural communities, where bacteria are likely to frequently encounter novel mobile genetic elements that impose increased translational demand upon the cell, the high evolvability of *gacA/gacS* may be advantageous, offering an evolutionary solution to the immediate costs of plasmid carriage.

We demonstrate that rapid compensatory evolution can stabilize plasmids by reducing the cost of plasmid carriage. We observed exceptionally parallel evolution, including multiple instances of nucleotide-level parallelism, due to the requirement for just a single bacterial regulatory mutation of large effect to completely ameliorate a 425-kb plasmid. This extends the importance of parallel compensatory evolution in understanding plasmid stability [8, 14] by showing that this evolutionary process can occur in parallel across a wide gradient of environmental conditions comprising the entire parasitism-mutualism continuum.

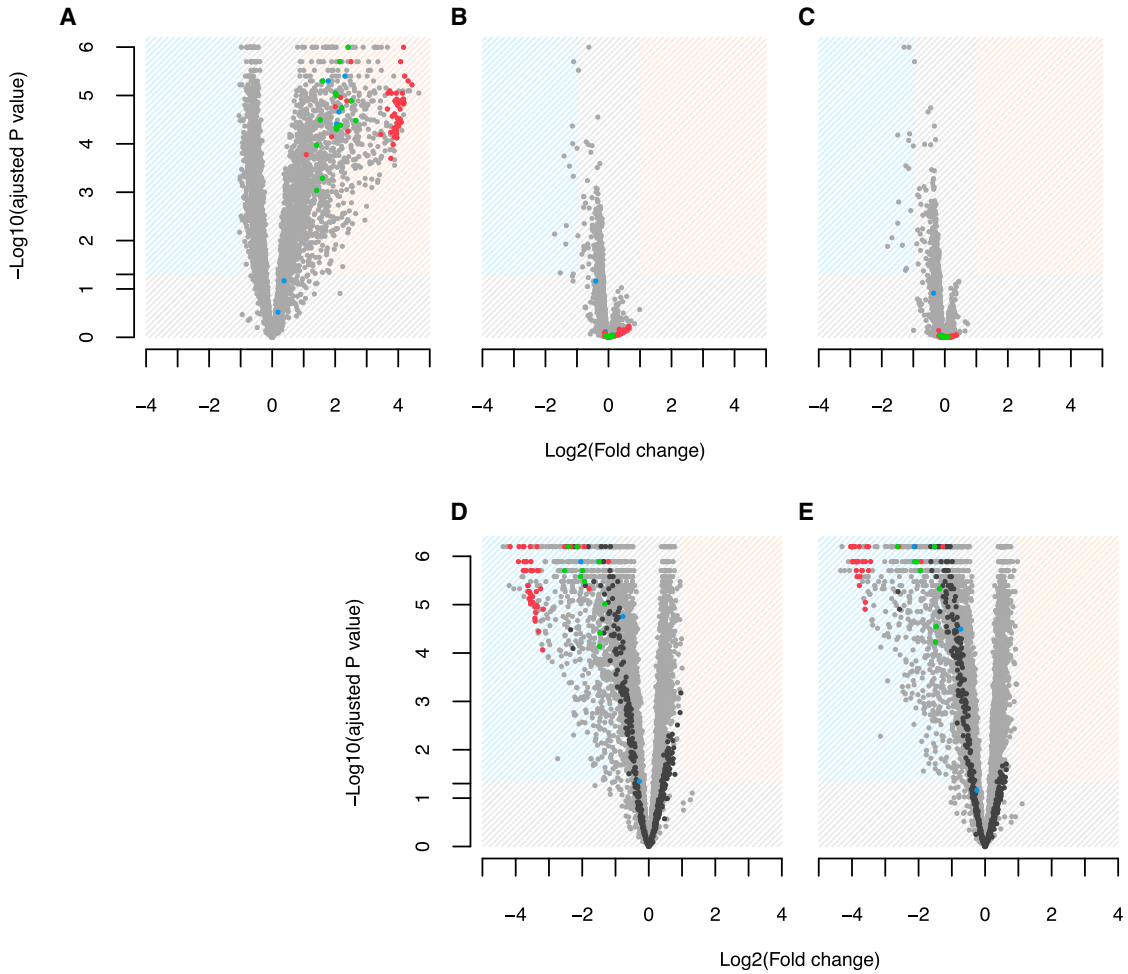


**Figure 4. Transcriptional Consequences of Plasmid Carriage and Compensatory Evolution**

(A–E) Top panels show gene expression relative to the plasmid-free ancestor for the plasmid-containing ancestor (A) and clones evolved in 0 μM $HgCL_2$ (B) and in 40 μM $HgCL_2$ (C). Lower panels show expression of clones evolved in 0 μM $HgCL_2$ (D) and 40 μM $HgCL_2$ (E) relative to the plasmid-containing ancestor. The colored areas define regions of significant (*$p < 0.05$) differential expression (>2-fold change). Points represent individual genes on the chromosome (light gray) and plasmid (dark gray). Chromosomal genes annotated as the enriched GO functions "ribosome" (red), "aminoacyl-tRNA ligase activity" (blue), and "rRNA binding" (green) are highlighted.

Contrary to theory [4], although capture of the Tn5042 by the chromosome and accompanying loss of the plasmid occurred throughout the experiment, these genotypes very rarely invaded to high frequency, suggesting that rapid compensatory evolution may limit this process and should be accounted for in theoretical models (cf. [8]). Therefore, horizontally acquired plasmids can quickly be stabilized and retained by bacteria even in environments where their accessory genes are not immediately useful, helping to explain the widespread occurrence of plasmids in natural bacterial communities.

## EXPERIMENTAL PROCEDURES

Populations were initiated from single colonies of SBW25-pQBR103. Six replicate populations were established for each of six mercury treatments (0, 8, 16, 24, 32, and 40 μM $HgCl_2$) and propagated by 1% serial transfer for 60 transfers in 30-ml glass microcosms containing 6 ml King's B (KB) liquid media. At the end of the experiment (or at the last transfer where bacteria-plasmid clones were observed), three bacteria-plasmid clones were isolated at random from each population. Competitive fitness and conjugation rate were estimated for three clones per population. One bacteria-plasmid clone per population was genome sequenced using Illumina MiSeq. Reads were aligned using Bowtie and variants identified using GATK, SNPeff, and Breakdancer. Functional characterization of SNPs was determined using PROVEAN, and protein structures were determined in Phyre2. Additionally, one plasmid-free bacterial clone from each 0 μM $HgCl_2$ population was genome sequenced and for six clones evolved without plasmids for 60 transfers in 0 μM $HgCl_2$, the *gacA, gacS*, and PFLU1661 genes were sequenced by Sanger sequencing. Allele frequencies of selected *gacA* or *gacS* mutations were analyzed using the Freq-Seq amplicon sequencing strategy at transfers 20, 40, and 60. Gene expression was assayed using microarrays to compare three randomly chosen clones from the 0 and 40 μM $HgCl_2$ treatments, the plasmid-free ancestor, and the plasmid-containing ancestor. Statistical and functional analyses were performed in R. Full methods are given in the Supplemental Information.

## ACCESSION NUMBERS

The accession number for the sequencing data reported in this paper is European Nucleotide Archive: PRJEB8710. The accession number for the microarray data reported in this paper is ArrayExpress: A-MTAB-554.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.06.024.

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