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Research Article

Compensatory mutations reducing the fitness cost of plasmid carriage occur in plant rhizosphere communities

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Abstract

Plasmids drive bacterial evolutionary innovation by transferring ecologically important functions between lineages, but acquiring a plasmid often comes at a fitness cost to the host cell. Compensatory mutations, which ameliorate the cost of plasmid carriage, promote plasmid maintenance in simplified laboratory media across diverse plasmid–host associations. Whether such compensatory evolution can occur in more complex communities inhabiting natural environmental niches where evolutionary paths may be more constrained is, however, unclear. Here, we show a substantial fitness cost of carrying the large conjugative plasmid pQBR103 in *Pseudomonas fluorescens* SBW25 in the plant rhizosphere. This plasmid fitness cost could be ameliorated by compensatory mutations affecting the chromosomal global regulatory system *gacA/gacS*, which arose rapidly in plant rhizosphere communities and were exclusive to plasmid carriers. These findings expand our understanding of the importance of compensatory evolution in plasmid dynamics beyond simplified lab media. Compensatory mutations contribute to plasmid survival in bacterial populations living within complex microbial communities in their environmental niche.

Keywords: compensatory evolution, experimental evolution, fitness cost, horizontal gene transfer, plant rhizosphere, plasmid

Background

Plasmids play an important role in bacterial evolution by transferring ecological functions between lineages, and thus driving genomic divergence (Thomas and Nielsen 2005). Acquiring a new conjugative plasmid is, however, often costly for the host cell (San Millan and MacLean 2017). Such fitness costs are caused by a variety of mechanisms including genetic conflicts, regulatory disruption, cytotoxicity, codon use mismatches, or the metabolic burden of maintaining the plasmid (Baltrus 2013, San Millan and MacLean 2017). Due to these fitness costs and the potential for segregational loss of the plasmid at cell division, plasmids are expected to be lost from bacterial populations inhabiting environments where plasmid-encoded functions are not beneficial (Bergstrom et al. 2000). Moreover, even in environments where plasmid-encoded functions are beneficial, these genes can often transfer to the chromosome, enabling loss of the plasmid (Bergstrom et al. 2000). Nonetheless, plasmids are common features of bacterial genomes, a situation termed the plasmid paradox (Bergstrom et al. 2000, Harrison and Brockhurst 2012, Brockhurst and Harrison 2022).

Laboratory evolution experiments have shown that compensatory mutations that reduce the fitness costs associated with plasmid carriage can stabilize plasmids across diverse plasmid–host pairs (reviewed in Brockhurst and Harrison 2022). Compensatory mutations can affect genes resident on the chromosome (Loftie-Eaton et al. 2017), the plasmid (Sota et al. 2010, Porse et al. 2016, Hall et al. 2021), or both (Loftie-Eaton et al. 2016) replicons. They target a wide range of gene functions across plasmid–host pairs, including regulators (Harrison et al. 2015, Hall et al. 2020, Kloos et al. 2021), RNA polymerase (Hall et al. 2020), helicases (Loftie-Eaton et al. 2017), other resident mobile genetic elements (San Millan et al. 2015), or hypothetical genes without known functions (Hall et al. 2021). Although commonly observed in simplified laboratory media, the importance of compensatory evolution for plasmid stability in more realistic, natural environments is far less well-studied.

In this study, we ask whether compensatory mutations arise in response to plasmid carriage in bacterial communities living on plants. *Pseudomonas fluorescens* SBW25 is a plant commensal bacterium that was isolated from sugar beet in the 1990s at Wytham Woods, Oxfordshire, UK (Rainey and Bailey 1996). Around the

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same time, a large collection of conjugative mercury resistance plasmids was isolated by exogenous capture from the same field site (Lilley and Bailey 1997a), including the 425 kb conjugative megaplasmid pQBR103 (Tett et al. 2007). SBW25 carrying pQBR103 can persist on plants both in the greenhouse and in the field (Lilley and Bailey 1997b), displaying an intriguing temporal dynamic: plasmid carrying populations initially decline in abundance compared to plasmid-free populations, but later recover to roughly equal abundances (~100 days after sowing).

The initial decline in plasmid-carrying SBW25 abundance relative to the plasmid-free population on plants (Lilley and Bailey 1997b) suggests that pQBR103 imposed a fitness cost. Subsequent experimental analysis showed pQBR103 acquisition imposes a large ~50% fitness cost upon SBW25 both in nutrient broth and in potting soil (Hall et al. 2015), however, whether pQBR103 is costly on plants (i.e. SBW25's and pQBR103's natural ecological niche) is unknown. The recovery of plasmid-carrier abundance over time is also currently unexplained. The authors of the original study suggested that this dynamic may have been due to an unknown fitness benefit of the plasmid, i.e. apparent only on more mature plants (Lilley and Bailey 1997b). However, it is also possible that plasmid dynamics vary over time due to compensatory evolution to reduce the fitness cost of plasmid carriage. In nutrient broth, the cost of pQBR103 carriage is rapidly negated by compensatory mutations that target either the two-component global regulatory system, *gacA/gacS*, or the gene of unknown function *PFLU4242*, which itself is positively regulated by *gacA/gacS* (Harrison et al. 2015, Hall et al. 2021). Single mutations affecting either of these genes completely ameliorate the fitness cost of pQBR103 carriage in nutrient broth (Hall et al. 2021). Whether these compensatory mutations arise or can ameliorate plasmid fitness costs on plants is unknown.

The GacA/GacS two component system works together with Rsm proteins to post-transcriptionally regulate hundreds of SBW25 chromosomal genes (Cheng et al. 2013). The SBW25 Gac–Rsm regulon includes a variety of traits that may affect colonization and competitiveness on plants, including secreted secondary metabolites, motility, and biofilm formation (Cheng et al. 2013), suggesting that loss of this system may be detrimental in complex natural environments such as plants. Interestingly, the pQBR103 sequence encodes an Rsm homologue, *PQBR443* (named *rsmQ*) (Tett et al. 2007, Hall et al. 2015, Thompson et al. 2023). RsmQ interacts with Gac–Rsm signalling within SBW25 cells to alter the expression of chemotaxis, motility, and metabolic phenotypes (Thompson et al. 2023) and could thus alter the fitness effect of pQBR103 and the potential for compensatory mutation in plant rhizosphere communities.

To test if pQBR103 carriage imposes a fitness cost upon SBW25 living on plants and whether this cost can be ameliorated by known compensatory mutations, we competed plasmid carriers against plasmid-free SBW25 in the rhizosphere of wheat for 2 weeks. Specifically, we tested the effect of plasmid carriage with or without *rsmQ* on competitive fitness of wild-type SBW25 and of SBW25 with a deletion of either the *gacS* gene or the *PFLU4242* gene. Next, we tracked the abundance of SBW25 with or without a plasmid for 4 weeks within bacterial communities inhabiting the rhizospheres of wheat plants. We quantified plasmid maintenance over time in SBW25 and used whole genome sequencing of clones isolated after 4 weeks on plants to determine if compensatory mutations had arisen in plasmid carrying bacteria. Our competition data showed that pQBR103 did indeed impose a large fitness cost on SBW25 in the wheat rhizosphere and that this cost was ameliorated by mutation of either of the known compensatory loci we tested. Both the fitness cost and the efficiency of amelioration by compensatory mutations were unaffected by the presence of *rsmQ*. Over 4 weeks, plasmid carriers typically reached lower abundance than plasmid-free SBW25 in the wheat rhizosphere community but stably maintained the plasmid at appreciable frequencies. Genome sequencing of one randomly chosen clone per plant from the end of the experiment revealed that compensatory mutations affecting the *gacA/gacS* two component global regulatory system had arisen in multiple plasmid-carrying clones. Conversely, these genes were never mutated in plasmid-free controls. Further genome sequencing of earlier sampled clones revealed that compensatory mutations affecting *gacA/gacS* could be detected even after just 1 week on plants. These findings extend our understanding of the maintenance of costly plasmids from the lab into a more natural environment, showing that compensatory mutations rapidly and repeatedly occur in the plant rhizosphere. This suggests that evolution to compensate plasmid-imposed fitness costs may indeed promote the survival of plasmids in natural communities.

Materials and methods

Plant varieties and culture conditions

Wheat plants (Skyfall variety, RATG) were grown in 50 ml Falcon tubes containing 40 cm^3 of washed autoclaved vermiculite. A volume of 10 ml 1x Jensens nutrient solution was added to each microcosm and reautoclaved. Wheat seeds were sterilized by agitating 5 g in 30 ml 30% bleach/0.01% TritronX solution for 10 min at room temperature. Bleach solution was removed by washing 10 times in 50 ml sterile water. Seeds were stratified overnight at 4°C and then spread onto sterile filter paper to germinate in the dark for 48 h. Single seedlings were placed into sterile vermiculite microcosms and allowed to establish for 48 h in sterile conditions (lid on tube) prior to inoculation with bacterial populations. Plants were grown in growth chambers (Conviron) at 250 $\mu mol/m^2/s^1$, 16:8 h light:dark cycle, 22°C day/18°C night and 60% relative humidity.

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are given in Table 1. Plasmids were introduced into bacterial strains by conjugation and confirmed by PCR as previously described (Hall et al. 2015). Overnight cultures were grown in 6 ml KB liquid medium in 30 ml glass universals shaken at 28°C. Colony counts were performed on selective KB agar plates supplemented with streptomycin (250 μg/ml) or gentamicin (30 μg/ml) plus 50 μg/ml X-gal to distinguish Sm^RlacZ- and Gm^R-labelled *P. fluorescens* strains or with Kanamycin (50 μg/ml) to select for plasmid carriers. Plates were incubated at 28°C for 48 h. To detect protease producing *P. fluorescens* colonies—i.e. those with a functional *gacAS* system—KB agar was supplemented with skimmed milk powder (250 μg/ml) (Stevenson et al. 2018).

An inoculum of the culturable fraction of the wheat rhizosphere community was generated from 6-week-old wheat plants. Specifically, to isolate this microbial community plants were grown in 2 l pots containing John Innes Number 2 compost (Arthur Bower) for 6 weeks in a Conviron growth chamber at 250 $\mu mol/m^2/s^1$, 16:8 h light:dark cycle, 22°C day/18°C night and 60% relative humidity. The roots were destructively harvested and washed in 100 mM Tris-phosphate buffer (pH 5.4) plus 1.8 mg/ml lipase (Sigma), 100 μg/ml β-galactosidase (Merck), and 4 μg/ml

Table 1. Bacterial strains and plasmids.

Bacterial strain or plasmid	Additional information; reference
P. fluorescens SBW25	Environmental isolate collected from a sugar beet leaf in Oxfordshire UK (Rainey and Bailey 1996)
P. fluorescens SBW25-Gm^R	Gentamicin-resistant-marked strain (Hall et al. 2015)
P. fluorescens SBW25-Sm^RlacZ	Streptomycin-resistant-marked strain (Hall et al. 2015)
P. fluorescens SBW25-Gm^RΔ*gacS*	Gentamicin-resistant-labelled strain with a deletion of the *gacS* locus (Harrison et al. 2015)
P. fluorescens SBW25-Gm^RΔ*PFLU4242*	Gentamicin-resistant-labelled strain with a deletion of the PFLU4242 locus (Hall et al. 2020)
pQBR103	Mercury resistance plasmid isolated from soil in Oxfordshire UK (Lilley and Bailey 1997a)
pQBR103-Kan^R	Kanamycin-resistant-labelled plasmid (Carrilero et al. 2021)
pQBR103-Kan^RΔ*rsmQ*	Kanamycin-resistant-labelled plasmid with a deletion of the *rsmQ* locus (Thompson et al. 2023)

α-glucosidase (Merck) and incubated at 37°C for 30 min, then vortexed with glass beads for 5 min at room temperature. The root wash was diluted with an equal volume of sterile water, mixed and allowed to settle on ice for 10 min. The wash was then centrifuged at 1000 *g* for 30 s and the supernatant carefully removed. The total bacterial rhizosphere community was quantified by plating out serial dilutions onto 0.1x NB agar plates supplemented with cycloheximide (1 mg/ml) to suppress fungal growth and 50 μg/ml X-gal and incubated at 28°C for 48 h. Frozen aliquots of the rhizosphere community were stored in 10% glycerol at −80°C for subsequent use in plant rhizosphere dynamics experiments.

Plant rhizosphere competition assays

Overnight KB cultures of the relevant *P. fluorescens* strains were subcultured into M9 pyruvate minimal liquid medium and preconditioned overnight shaken at 28°C. These cultures were then centrifuged for 10 min at 3500 *g* at 4°C and cell pellets resuspended in M9 salts solution. Competing strains were mixed at 1:1 ratios and plated onto X-gal to distinguish between strains. A total of six replicate gnotobiotic wheat seedling microcosms were inoculated per competition with 1 ml of each 1:1 strain mix (total inoculated bacterial cells = ~1×10^4 colony forming units). Plants were propagated in a Conviron growth chamber and destructively harvested after 14 days. To recover the bacterial populations, plant roots were separated from aerial tissue and transferred to Falcon tubes containing 10 ml of sterile 10 mM MgSO4 0.1% Tween (root wash solution) and 2 cm^3 of sterile glass beads. The roots were vortexed for 3 min at room temperature, left for 30 min on ice and then vortexed again for 3 min. The root wash supernatant was removed and serial dilutions plated onto KB agar plates supplemented with 50 μg/ml X-gal to distinguish the competing strains and incubated for 48 h at 28°C. The relative fitness of a focal strain was calculated as υ given by $\upsilon = x_2\ (1–x_1) / x_1\ (1–x_2)$, where x_1 is the starting proportion of the focal strain and x_2 is the final proportion of the focal strain (Ross-Gillespie et al. 2009). The relative fitness metric υ was used here, instead of more standard relative fitness metrics (e.g. *w*), because it was not possible to accurately estimate starting population densities from the inoculated seeds whereas it was straightforward to quantify the frequency of competing genotypes in the inoculum.

Plant rhizosphere dynamics experiment

Seedling microcosms were inoculated with 1 ml 6×10^6 CFU/ml of either SBW25, SBW25(pQBR103), or SBW25(pQBR103Δ*rsmQ*) grown overnight from an independent colony per replicate or an equal volume of sterile water and allowed to establish for 5 days. Plants were then inoculated with ~10.5×10^6 colony forming units of the standardized rhizosphere community. The initial 5-day establishment phase was necessary to enable SBW25 to persist in the rhizosphere community long-term. On days 5, 8, 15, 21, and 29, six to eight plant microcosms were destructively harvested per treatment. Roots were processed as described above. Serial dilutions were plated onto selective agar plates to enumerate the total bacterial community (0.1x NB agar), the total *P. fluorescens* population and the frequency of protease producers (KB + streptomycin + skimmed milk), and the frequency of plasmid carriers (KB + streptomycin + kanamycin). Agar plates were incubated at 28°C for 48 h prior to colony forming unit counts. On average ~100 colonies were counted per plate such that the detection limit for observing phenotypically Gac-negative mutants was ~1/100.

Genomic analysis

One random *P. fluorescens* colony per plant sampled on day 29 and several colonies of protease-negative *P. fluorescens* sampled from plants across the experiment were chosen for whole genome sequencing. Whole-genome sequencing was performed by MicrobesNG using a 250 bp paired-end protocol on the Illumina HiSeq platform. Variants were called against the ancestral reference genome using the Breseq computational pipeline using standard default settings (Deatherage and Barrick 2014). All variants were validated visually using the alignment viewer IGV (Robinson et al. 2011). All sequencing data are available on the Sequencing Read Archive under accession PRJNA839377.

Statistical analysis

All analysis was performed in R statistical software. Competition data was analysed using a linear model (lm(), base R) and further interrogated using Tukey test multiple comparisons (glht(), multcomp). As comparisons showed no effect of the plasmid type on fitness a further analysis was performed grouping plasmid treatments to reduce the number of multiple comparisons. For the plant rhizosphere dynamics data, whole community and SBW25 population density were analysed using a linear model (lm(), base R) and where appropriate treatments were compared using Tukey's pairwise comparisons (lsmeans(), library{emmeans}) Proportion data was analysed from counts using generalized linear model (glm(), base R) with a quasi-binomial variance structure.

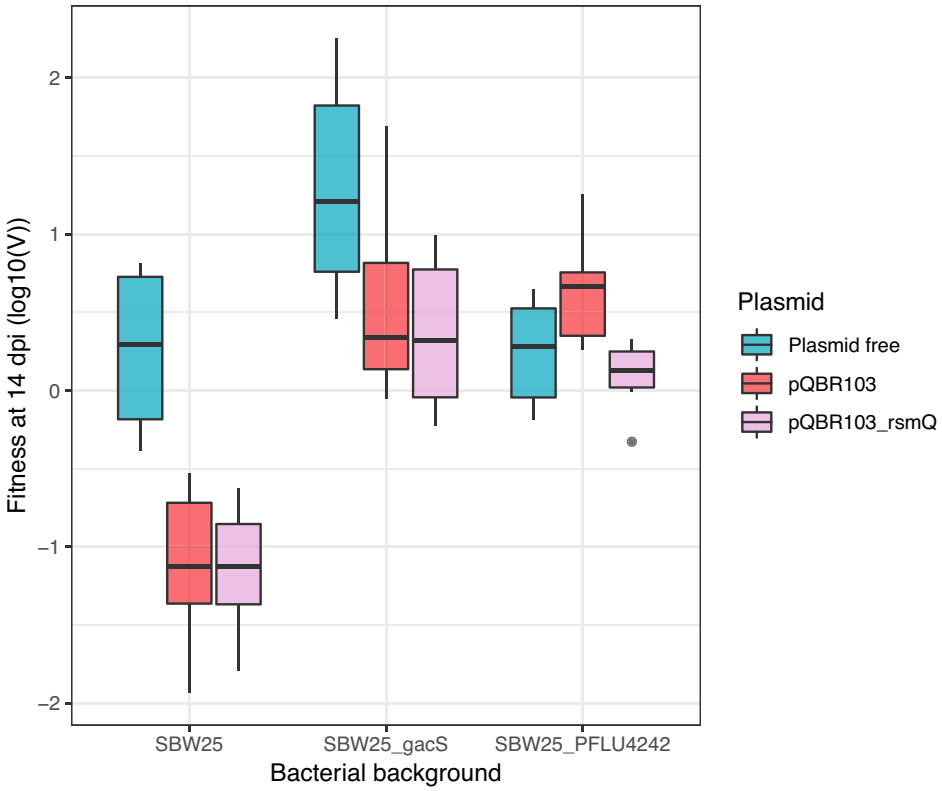

Figure 1. Fitness effects of pQBR103 carriage upon SBW25 with or without compensatory mutations in the wheat rhizosphere. Relative fitness (v) of strains from three bacterial backgrounds (SBW25, SBW25gacS, and SBW25PFLU4242) either carrying no plasmid (blue), a wild-type plasmid (red), or a *rsmQ* knockout plasmid (pink) was measured in direct competition with plasmid-free wild-type SBW25 in the wheat rhizosphere after 14 days. Boxplots show mean and interquartile range with replicate values shown as points in black ($n = 6$).

Data access statement

All experimental data sets are provided in the Supplementary Information of this article. All genome sequence data is freely available from the Sequence Read Archive accession PRJNA839377.

Results

To quantify if acquisition of pQBR103 by SBW25 is costly on plants, and whether known compensatory mutations ameliorate fitness costs, we competed plasmid carriers against isogenic plasmid-free SBW25 in the wheat rhizosphere. We used three bacterial genetic backgrounds as plasmid carriers, wild-type SBW25 or mutants carrying deletions of either *gacS* or *PFLU4242*, enabling us to test the fitness effect of these known compensatory mutations on plants. In addition, we used two pQBR103 genotypes: wild-type pQBR103 or a mutant carrying a deletion of *rsmQ* (pQBR103rsmQ). The inclusion of this mutant enables us to additionally test if interactions between RsmQ and the SBW25 Gac–Rsm system contributed to fitness costs on plants. Consistent with our previous experiments using nutrient broth or potting soil, we observed a large fitness cost of plasmid carriage in SBW25 in the wheat rhizosphere (Fig. 1; SBW25(pQBR103) vs. SBW25: $t = -4.868$, $P < .001$; SBW25(pQBR103rsmQ) vs. SBW25: $t = -4.883$, $P < .001$). Moreover, the plasmid fitness cost in SBW25 was unaffected by deletion of *rsmQ* (pQBR103 vs. pQBR103rsmQ: $t = -0.015$, $P = 1$), and therefore, subsequent analyses combined these plasmid treatments. These data show that pQBR103 does indeed cause a substantial fitness cost in SBW25 on plants, confirming that this plasmid is costly in an environment similar to that from which it was isolated (i.e. the plant rhizosphere). Deletion of either *gacS* or *PFLU4242* ameliorated the fitness cost of plasmid carriage (SBW25gacS(pQBR103) vs. SBW25: $t = 0.808$, $P = .964$; SBW25PFLU4242(pQBR103) vs. SBW25: $t = 0.437$, $P = .998$). These data show that both of the SBW25 compensatory mechanisms previously observed to evolve in nutrient broth function equivalently to negate fitness costs in the more natural environment of the plant rhizosphere. Intriguingly, although deletion of *PFLU4242* had no fitness effect in the absence of plasmids, deletion of *gacS* was beneficial in the absence of plasmids in competition with SBW25 (SBW25gacS vs. SBW25: $t = 3.559$, $P = .0102$). In line with previous studies of plant-associated *Pseudomonas* (Ferreiro and Gallegos 2021), this suggests that the Gac regulon is expressed by SBW25 on plants, that this expression is costly, but that nonresponsive *gacS* mutants growing alongside wild-type cells may benefit from their Gac-regulated products.

By screening for transconjugants in the originally plasmid-free SBW25 competitor populations at 14 days, we found evidence for conjugation of both pQBR103 and pQBR103rsmQ plasmids in the rhizosphere in all replicates. Transconjugant frequencies reached up to ~1.5% of the originally plasmid free SBW25 competitor, and were highest from donors that carried compensatory mutations (Figure S1, Supporting Information; SBW25gacS vs. SBW25: $t = 4.830$, $P < .001$, SBW25PFLU4242 vs. SBW25: $t = 8.579$, $P < .001$) consistent with their higher fitness ($t = 4.454$, $P < .0001$, $R^2 = 0.349$). There was no effect of *rsmQ* on transconjugant frequency ($F = 0.0251$, $P = .875$). These data confirm that pQBR103 transfers by conjugation in the rhizosphere and further show that spread of the plasmid is promoted by host compensatory mutations.

We next tested if plasmid carriage altered the ecological dynamics of SBW25 when in a bacterial community inhabiting the wheat rhizosphere. Wheat seedlings were colonized with

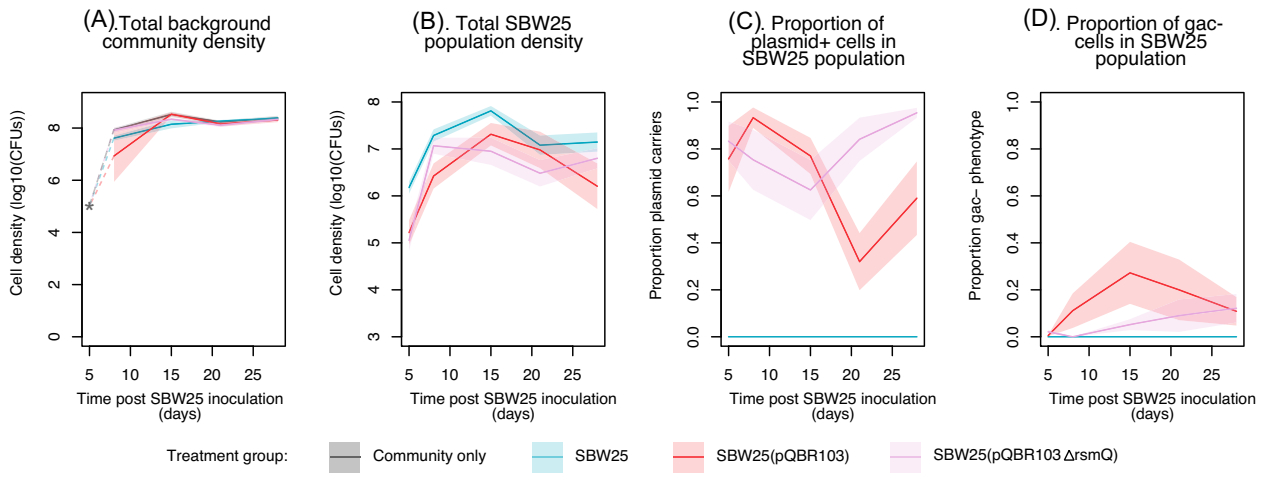

Figure 2. Dynamics of the SBW25 populations and background community in the wheat rhizosphere over time. (A). Total background community density, (B). Total SBW25 population density. (C). Proportion of plasmid carriers within the SBW25 population. (D). Proportion of Gac-negative phenotypes in the SBW25 population. Lines denote means for each treatment group with standard error shown as the area around the mean. SBW25 populations were founded from clones carrying either no plasmid (blue), pQBR103 (red) or pQBR103rsmQ (pink). In panel (A), the value shown at timepoint 5 is based on inoculum introduced and was not included in analysis. Note that plants were destructively sampled and as such at each timepoint we sampled different individual plants.

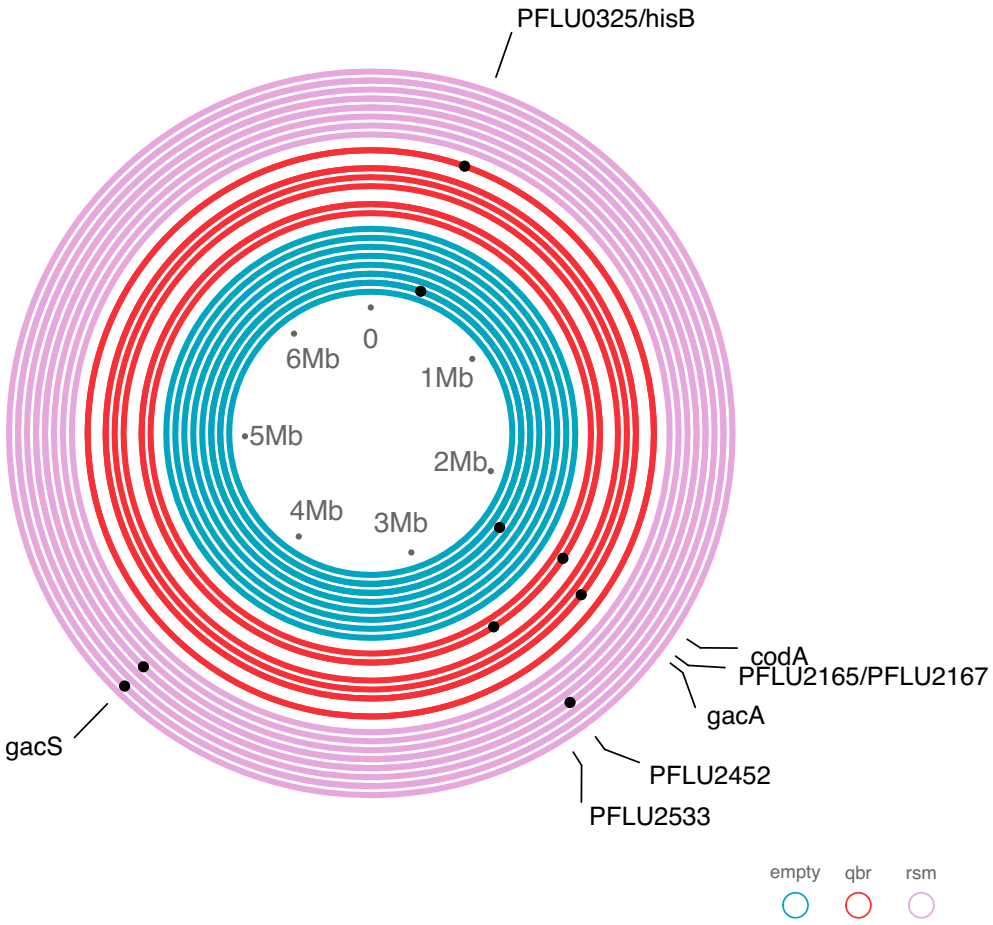

Figure 3. Genome plots showing the chromosomal mutations observed in evolved clones sampled on day 28 of the plant experiment. Concentric rings represent the chromosome of individual sequenced clones colour-coded by treatment—SBW25 (blue), SBW25(pQBR103) (red), and SBW25(pQBR103*rsmQ*) (pink). Black dots denote the location of mutations with gene targets shown on the outer labels.

SBW25, SBW25(pQBR103), or SBW25(pQBR103*rsmQ*) alongside a background microbial community previously plated onto nutrient agar from root washes of mature wheat plants. A SBW25-free control treatment containing only background community was also included. Plants were destructively sampled weekly for 4 weeks and rhizosphere communities were plated onto selective agar to distinguish SBW25 populations from the background microbial community and plasmid carriers from plasmid-free cells. SBW25 reached lower abundance in the wheat rhizosphere in the plasmid treatments (Fig. 2B; SBW25(pQBR103) vs. SBW25: $t = 3.22$, $P = .005$; SBW25(pQBR103*rsmQ*) vs. SBW25: $t = 3.17$, $P = .006$) with no effect of plasmid genotype (SBW25(pQBR103) vs. SBW25(pQBR103*rsmQ*): $t = 0.005$, $P = .999$). The presence of SBW25 with or without plasmids had no effect on the abundance of the background microbial community inhabiting the wheat rhizosphere (Fig. 2A: $F = 2.219$, $P = .0894$). Both plasmids remained at appreciable frequencies in the SBW25 population for the duration of the experiment (Fig. 2C), although pQBR103 declined to lower prevalence than pQBR103*rsmQ* during the later stages of the experiment ($t = 4.061$, $P = .0001$). These data suggest, consistent with our relative fitness estimates, that plasmid carriage is costly in the plant rhizosphere, but that plasmids are stably maintained nonetheless.

At the final sampling point, we picked one random SBW25 colony per plant for whole genome sequencing to determine the genetic response to selection and how this varied among treatments. We observed nine mutations across the 22 sequenced genomes, with individual genomes containing between 0 and 2 mutations (Fig. 3). All observed mutations affected chromosomal loci and no mutations were observed on plasmids. Parallel mutations arising independently in multiple genomes are a signal of natural selection having acted upon these mutations, and we focus here on those that were exclusive to plasmid carrying clones suggesting they evolved in response to plasmid-mediated selection. This subset of mutations included two chromosomal loci, *gacA* and *gacS*, together encoding the Gac two-component regulatory system. Specifically, *gacA* was mutated in one evolved clone carrying pQBR103 whilst *gacS* was mutated in two evolved clones carrying pQBR103rsmQ (Fig. 3; observed mutations are detailed in Table S1, Supporting Information). Because the SBW25-selective agar plates had been supplemented with skimmed milk powder we were able to detect the activity of a secreted protease, i.e. positively regulated by *gacA/gacS*, enabling us to track the emergence of phenotypically Gac-negative SBW25 mutants over time (Stevenson et al. 2018). These data reveal that Gac-negative SBW25 colonies were detected only in the presence of plasmids, but never in their absence, and arose rapidly (within 1–2 weeks; Fig. 2D) (effect of time; $t = 5.113$, $P < .001$) reaching intermediate frequencies in SBW25 populations containing either plasmid genotype. Colonies with a Gac-negative phenotype were marginally more common in populations containing pQBR103 compared to pQBR103rsmQ ($t = -2.159$, $P = .0349$; Fig. 2D). Genome sequencing of some of these plasmid-carrying phenotypically Gac-negative SBW25 colonies confirmed that the majority (8 out of 10 colonies) contained mutations affecting either *gacA* ($n = 2$) or *gacS* ($n = 6$) (Figure S2, Supporting Information; observed mutations are detailed in Table S2, Supporting Information). In addition, two of the pQBR103rsmQ carrying evolved clones with *gacS* mutations also had mutations in *PFLU2452*, a hypothetical gene encoding a putative asparagine synthase. Mutation of this locus was also observed in one of the endpoint pQBR103rsmQ carrying evolved clones, which did not also contain a *gac* mutation (Fig. 3). These data show that mutation of *gacA/gacS* was exclusively associated with plasmid carriage and suggest, in combination with our relative fitness data, that such mutations arose to compensate for the fitness cost of carrying the plasmid in the plant rhizosphere. Moreover, these data are suggestive of a previously unknown compensatory mechanism involving mutation of *PFLU2452*, encoding a putative asparagine synthase, which was only observed in evolved clones carrying the pQBR103rsmQ plasmid.

Discussion

Laboratory evolution experiments suggest that compensatory evolution to reduce the cost of plasmid carriage plays an important role enabling the persistence of plasmids in bacterial populations (Brockhurst and Harrison 2022). However, whether similar evolutionary processes operate in more complex natural settings, such as plant rhizosphere communities, is poorly understood. Indeed, the genomic flexibility afforded by rich lab media might be expected to be far more constrained in natural environments, potentially limiting the accessibility of compensatory mutations. Here we show, using the plant commensal bacterium *P. fluorescens* SBW25 and the environmental mercury resistance plasmid pQBR103, that although plasmid carriage is costly in the plant rhizosphere, such fitness costs can be ameliorated by compensatory mutations. Specifically, mutations affecting *gacA/gacS* arose rapidly and exclusively in plasmid carrying SBW25 populations, where they completely ameliorated the fitness cost of carrying pQBR103 in the plant rhizosphere.

Using competition experiments with defined mutants, we show that loss of either the two-component system *gacA/gacS* or the hypothetical gene *PFLU4242* is sufficient to reduce the fitness cost of carrying pQBR103 on plants. Our previous studies have shown that the pQBR103 fitness cost arises from a specific genetic conflict between *PFLU4242* and genes on pQBR103, inducing upregulation of chromosomal mobile genetic elements including prophages that are thought to cause cell damage (Hall et al. 2021). Surprisingly, we did not observe mutation of *PFLU4242* in evolved plasmid carrying clones, despite mutation of this gene offering complete amelioration on plants. Instead, *de novo* compensatory mutations targeted *gacA/gacS*. Loss of the *gacA/gacS* two component system likely reduces plasmid fitness cost by downregulating *PFLU4242*, providing an indirect regulatory route to amelioration (Hall et al. 2021). The predominance of *gacA/gacS* mutations is consistent with *in vitro* studies using this system where, although *PFLU4242* mutations were observed, they were much less common than mutations affecting *gacA/gacS* (Harrison et al. 2015, Hall et al. 2021). It is possible that *gacA/gacS* merely represents a larger mutational target than PFLU4242, making mutations at these loci more likely to arise. Similarly, it has been argued that *gacA/gacS* may represent contingency loci (Moxon et al. 1994, van den Broek et al. 2005a, b) with elevated mutation rates (van den Broek et al. 2005a, b), and thus would be predicted to acquire mutations more frequently than other regions of the genome.

Gac-negative mutants have been reported in natural *Pseudomonas* populations isolated from both soil and plants (reviewed in van den Broek et al. 2005), where they may gain a fitness benefit from not paying the metabolic costs of expressing Gac-regulated genes, whilst benefiting from the secreted metabolites produced by neighbouring wild-type cells (Jousset et al. 2009, Song et al. 2016) [although cf. Driscoll et al. (2011) for an alternative view]. Indeed, our competition experiments suggested that in addition to ameliorating the fitness cost of pQBR103, loss of *gacA/gacS* was also beneficial in the absence of plasmids. However, *de novo* Gac-negative mutants were only observed in plasmid carriers, and

never in plasmid-free SBW25 populations in the presence of a plant rhizosphere community. As such, *gac* mutations appear to have been insufficiently beneficial to drive loss of Gac signalling by SBW25 in rhizosphere communities in the absence of plasmids. Compensatory evolution in response to plasmid carriage may, therefore, help to explain the variability of Gac phenotypes observed within rhizosphere communities (van den Broek et al. 2005, Driscoll et al. 2011). Moreover, it is possible that Gac-negative compensated plasmid-carrying genotypes may be at a fitness disadvantage in the long-term in rhizosphere communities if they subsequently lose the plasmid.

Although the plasmid genotypes did not differ in their fitness costs, we observed subtle differences in their dynamics and compensatory evolution. pQBR103rsmQ was maintained at higher frequency in SBW25 over time compared to pQBR103, whereas Gac-negative mutants were marginally more common in pQBR103 than pQBR103rsmQ containing SBW25 populations. Moreover, whereas a mixture of *gacA* and *gacS* mutations occurred in pQBR103 carriers, we only ever observed *gacS* mutations in pQBR103rsmQ carriers. In addition, we observed mutations in *PFLU2452* encoding a putative asparagine synthase exclusively in evolved pQBR103rsmQ carriers; two such mutations co-occurred alongside *gacS* mutations whereas one did not. Intriguingly, our previous work shows that acquisition of pQBR103 decreases SBW25 growth on asparagine as a sole carbon source, but that this growth defect is negated by deletion of *rsmQ* (Thompson et al. 2023), suggesting that *rsmQ* itself alters regulation of asparagine metabolism in SBW25. These results are consistent with our earlier findings that RsmQ interferes with the host Rsm pathway (Thompson et al. 2023), which is itself induced by *gac* loss-of-function mutations. Furthermore, *rsmQ* deletion recovers certain plasmid-induced phenotypes in SBW25 (Thompson et al. 2023), which may slightly reduce the fitness cost of plasmid carriage in some environments, but in turn may reveal other traits to selection, as seen here for asparagine metabolism.

Our data suggest an additional potential explanation for the previously reported seasonal dynamics of SBW25(pQBR103) on plants in the field, wherein plasmid carriers declined after sowing before rebounding later in the growing season on sugar beets (Lilley and Bailey 1997b). The large competitive fitness cost of the plasmid that we measure in the rhizosphere is consistent with the reduced abundance of plasmid carriers compared to plasmid-free SBW25 we observed in rhizosphere communities, and as such this fitness cost seems the most likely reason for the initial decline of SBW25(pQBR103) observed on plants in the field (Lilley and Bailey 1997b). Compensatory mutations that completely ameliorate the fitness cost of the plasmid arise and spread to appreciable frequencies within weeks in plasmid carrier populations of SBW25 within rhizosphere communities. Such large fitness effects and rapid timescales of compensatory mutations in the rhizosphere is consistent with the reinvasion of SBW25(pQBR103) in the field after 100 days being driven, at least in part, by compensatory evolution. By reducing the fitness cost of plasmid carriage, compensatory evolution could augment the contribution of other plasmid genes providing a delayed benefit of pQBR103 later in the growing season as proposed in the original study (Lilley and Bailey 1997b).

This study expands our understanding of the importance of compensatory evolution in plasmid dynamics from simplified lab media to a more complex and plant-associated environment. Rapid compensatory evolution to reduce the fitness costs of plasmid carriage is likely to enable the stable persistence of costly plasmids in plant rhizosphere communities as well as in a range of other natural microbial communities (DelaFuente et al. 2022).

Authors' contributions

Susannah M Bird (Data curation, Investigation, Methodology, Project administration, Writing – review & editing), Samuel Ford (Investigation), Catriona M. A. Thompson (Resources, Writing – review & editing), Richard Little (Methodology, Resources), James P. J. Hall (Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing), Robert W Jackson (Conceptualization, Funding acquisition, Methodology, Writing – review & editing), Jacob Malone (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), Ellie Harrison (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing), and Michael A Brockhurst (Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing)

Supplementary data

Supplementary data is available at *FEMSEC* online.

Conflicts of interest statement. None declared.

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