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Conflicting selection alters the trajectory of molecular evolution in a tripartite bacteria-plasmid-phage interaction

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Running title : Mobile elements shape bacterial evolution

Abstract

Bacteria engage in a complex network of ecological interactions, which includes mobile genetic elements (MGEs) such as phages and plasmids. These elements play a key role in microbial communities as vectors of horizontal gene transfer but can also be important sources of selection for their bacterial hosts. In natural communities bacteria are likely to encounter

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multiple MGEs simultaneously and conflicting selection among MGEs could alter the bacterial evolutionary response to each MGE. Here we test the effect of interactions with multiple MGEs on bacterial molecular evolution in the tripartite interaction between the bacterium, *Pseudomonas fluorescens*, the lytic bacteriophage SBW25φ2 and conjugative plasmid, pQBR103, using genome sequencing of experimentally evolved bacteria. We show that, individually, both plasmids and phages impose selection leading to bacterial evolutionary responses that are distinct from bacterial populations evolving without MGEs, but that together, plasmids and phages impose conflicting selection on bacteria, constraining the evolutionary responses observed in pairwise interactions. Our findings highlight the likely difficulties of predicting evolutionary responses to multiple selective pressures from the observed evolutionary responses to each selective pressure alone. Understanding evolution in complex microbial communities comprising many species and MGEs will require that we go beyond studies of pairwise interactions.

Main text

Bacteria engage in complex networks of ecological interactions both with other species and also with a wide diversity of mobile genetic elements (MGEs), including phages, plasmids and transposons. These MGEs play a central role in bacterial evolution as vectors of horizontal gene transfer (Jain et al 2003), but also act as important sources of selection on bacterial populations. Pairwise co-culture studies have demonstrated that both phages and plasmids can be important drivers of bacterial evolution. Phages are a major cause of bacterial mortality (Bouvier and del Giorgio 2007) and antagonistic coevolution between bacteria and phage generates strong, reciprocal selection for adaptations in phage infectivity and bacterial resistance traits (Buckling and Rainey 2002) resulting in rapid genomic evolution of both phage (Paterson et al 2010) and bacteria (Scanlan et al 2015). Plasmids meanwhile, often carry large cargos of accessory genes as well as bacterial regulatory elements (Smillie et al 2010, Tett et al 2007), and so can place a considerable fitness cost on the host cell. The additional genetic material can impose a physiological burden by expressing and translating plasmid genes, whereas regulatory elements can cause disruption of bacterial intracellular regulatory networks (Baltrus 2013). Co-culture experiments suggest that plasmid carriage selects for compensatory mutations, either on the

chromosome or the plasmid, which ameliorate the cost of plasmid carriage (Harrison et al 2015a, San Millan et al 2015, Yano et al 2016). For the majority of bacteria-plasmid interactions, because rates of conjugative transfer are typically too low to counteract purifying selection against costly plasmids (Bergstrom et al 2000, Levin 1993), compensatory evolution is thought to be important for the survival of plasmids in bacterial populations (Harrison and Brockhurst 2012). While pairwise co-culture experiments reveal the impact of MGE-mediated selection on bacterial evolution, studies to date typically consider the effect of each MGE in isolation. However, in nature bacteria are likely to interact with multiple MGEs simultaneously and therefore will experience concurrent selective pressures. An important question is to understand how these constituent pairwise interactions between bacterial hosts and MGEs combine in more complex communities to determine the overall effect on bacterial genomic evolution.

The effect of multiple, concurrent selective pressures on bacterial evolution will depend upon the relative strengths of selection and the genetic correlations between the traits under selection. An overview of these potential effects is given in table S1. In general, it is likely that stronger selective pressures will outweigh weaker ones due to clonal interference (Gerrish and Lenski 1998, Rozen et al 2002), for example selection arising from antagonistic coevolution weakens the evolutionary response to abiotic selection in phages (Zhang and Buckling 2011). Furthermore, changes to host demography can alter the strength of selection from interacting partners. For instance, reductions in bacterial density due to protist predation lead to reduced encounter rates between bacteria and phages, and consequently weaker bacterial evolutionary responses to phage attack (Friman and Buckling 2013). Genetic correlations between the traits under selection can range from positive to negative, and can arise via a range of mechanisms including pleiotropy (Bohannan and Lenski 2000, Örmälä-Odegrip et al 2015), epistasis (Buckling et al 2006, Scanlan et al 2015) or linkage. Positive genetic correlations, e.g. where a mutation conferring resistance against one enemy pleiotropically enhances resistance to a second enemy, favours the evolution of generalist prey defenses in multi-enemy communities (Örmälä-Odegrip et al 2015). By contrast, negative genetic correlations are likely to constrain evolution to one or both selective pressures. For example, negative epistasis between mutations conferring phage resistance and mutations conferring environmental adaptation constrained bacterial abiotic adaptation in populations coevolving with phages (Scanlan et al 2015). However, because

relatively few studies have identified the causative mutations we have only a limited understanding of how multiple selective pressures shape bacterial molecular evolution.

In this study we used genome sequencing to contrast the evolutionary responses of bacteria to abiotic selection, phage selection, plasmid selection and combined phage-plasmid selection. Our experimental system comprised a conjugative mercury resistance plasmid, pQBR103; a lytic phage, SBW25φ2; and their shared bacterial host, *Pseudomonas fluorescens* SBW25. In a previous study we experimentally evolved *P. fluorescens* populations under four treatments — MGE-free, phage-only, plasmid-only, or phage-plasmid together — and reported the effects of plasmid carriage on bacteria-phage coevolution at the phenotypic level. In populations coevolving with phages, plasmids constrained bacterial evolution of resistance to phage infection relative to plasmid-free bacteria. This reduction in the strength of phage resistance was unlikely to have been driven by demographic effects as plasmid carriage was not associated with reduced bacterial or phage population density. However, plasmids did select for the evolution of higher frequencies of mucoidy in bacterial populations in response to phage attack. Mucoidy is caused by overproduction of exopolysaccharides (Hay et al 2014) and confers partial resistance to phage infection (Scanlan and Buckling 2012). Thus plasmids altered the phenotypic evolutionary responses of bacteria to phage selection. Here we additionally characterize the effect that phage selection had on bacterial evolutionary responses to plasmid selection, in particular compensatory evolution. We determine how bacterial molecular evolution varied between the four treatments allowing us to compare evolution between bacterial hosts and MGEs under pairwise versus tripartite interactions.

Methods

For this study we sequenced single bacterial clones from the experiment described in Harrison et al. (2015b). In brief, 6 replicate populations of the bacteria *P. fluorescens* SBW25, each initiated from a single bacterial colony, were allowed to evolve either with or without the mercury resistance plasmid pQBR103 (Tett et al 2007), and in the presence and absence of the lytic phage SBW25φ2 (Buckling and Rainey 2002). Populations were grown in King's B (KB) with

shaking at 28°C, and propagated by 1% serial transfer for 20 transfers corresponding to approximately 150 bacterial generations. Populations were evolved in sub-MIC levels of Hg(II) (8µM $HgCl_2$) (Harrison et al 2015b). These conditions provided sufficient positive selection on plasmid-borne mercury resistance genes to ensure plasmid maintenance but are permissible for plasmid-free bacteria that do not carry the *mer* operon (Harrison et al 2015b). For this study we randomly selected a single clone, representative of the majority phenotype, from each of the 24 populations from the final time point. Clones were sequenced on the illumina MiSeq platform and reads aligned using BWA. Small variants (SNPs and small indels) were identified using GATK HaplotypeCaller and SNPeff and structural rearrangements identified using BreakDancer. A list of all mutational targets can be found in table S2.

To investigate the dynamics of compensatory mutations associated with plasmid amelioration we screened 20 clones per population at 4 transfer intervals (transfers 0, 4, 8, 12, 16 and 20) for exoprotease production. Both this and previous experiments have revealed extensive parallel evolution of the bacterial GacAS system, which controls a large suite of secreted proteins, including exoproteases (Cheng et al 2013). GacAS function was detected by spotting ~1 µl of overnight culture onto skimmed milk agar (10% milk powder in nutrient broth agar) alongside GacAS positive and negative controls (Harrison et al 2015a). Colonies were grown at 28°C for 24 hrs and then at room temperature for 24 hrs. Clones able to produce exoproteases can be identified by a halo around the colony, due to digestion of casein in the media (Cheng et al 2013).

Results

Rates of bacterial genome evolution

Evolved clones contained between 1 and 6 mutations, the vast majority of which were predicted to have strong phenotypic effects, with only 2 synonymous mutations identified among the 24 sequenced clones. Average pairwise genetic distance from the ancestor varied between treatments ($ANOVA_{effect\ of\ treatment}$: $F_{3,20} = 5.49$, $p = 0.0064$), this effect was largely driven by phage selection accelerating bacterial evolution relative to the MGE-free controls (average no.

mutations under 'phage-only' 4 ± 0.58SE vs 'MGE-free' 1.17 ± 0.17SE: post hoc comparison, t = 3.97, p = 0.0039). In contrast, phage selection did not lead to accelerated evolution in plasmid-carrying bacteria (no. mutations under 'phage-plasmid' 2.17 ±0.60SE vs 'MGE-free': post hoc comparison, t = 1.403, p = 0.512). Thus the plasmid appears to have constrained the bacterial response to phage selection.

Targets of selection varied between treatments

Loci targeted by mutations varied significantly between treatments. Bacterial clones were more likely to share mutational targets with those from replicate populations from the same treatment than with those from different treatments (Permutational MANOVA (Scanlan et al 2015), N=1000, F=3.05, p=0.001). Notably, clones that had evolved without MGEs shared no mutational targets with clones from the MGE treatments (Fig. 1a). Mutations identified in the 6 clones from the MGE-free treatment affected just 2 loci: PFLU0956, which was mutated in 1/6 clones from this treatment and is known to be associated with lab adaptation (Lind et al 2015) and PFLU4382, which was mutated in all 6 clones (one clone had a mutation affecting the promotor region while all others had mutations disrupting the coding sequence). PFLU4382 is predicted to be a thiol-disulfide interchange protein associated with protein folding (Silby et al 2009). Mutations at this locus are likely to be involved in ameliorating mercury toxicity, since mercury binds to thiol groups and interferes with the formation of disulfide bonds (Sharma et al 2008). Given this role, it is perhaps unsurprising that these mutations were not observed in clones carrying the plasmid which can efficiently detoxify mercury via the mercury resistance operon (*mer*). However it is notable that in the phage-only treatment, where increased mercury tolerance would likely have been beneficial, PFLU4382 evolution was not observed, suggesting that phage selection impeded environmental adaptation, as has been observed previously (Scanlan et al 2015).

Plasmid carriage alters the rate and trajectory of the bacterial evolutionary response to phages

Mutational targets were more variable among bacterial clones from the MGE treatments. The majority of clones (13/18), in particular in the phage-only treatment (6/6), carried at least one singleton mutation (i.e. mutational targets unique to one clone). However, overall, mutations were not randomly distributed, as even amongst the MGE treatments mutational targets were

still more likely to be shared within than between treatments (Fig. 1a; Permutational MANOVA excluding the MGE free treatment, N=1000, F=1.69, p=0.026).

Excluding genes of unknown function, a large proportion of the mutational targets in the phage-only and phage-plasmid selection treatments were in lipopolysaccharide (LPS) biosynthesis associated loci, which have been linked to evolved phage resistance (Scanlan et al 2015). By contrast, no LPS mutations were identified in clones evolved without phage selection. Consistent with the reduced rate of phage resistance evolution observed in plasmid-carrying bacterial populations (Harrison et al 2015b), we observed a higher frequency of mutations in LPS associated loci in clones from the phage-only treatment (on average 1.76 mutations/clone) compared to those from the phage-plasmid treatment (on average 0.83 mutations/clone). Moreover, the targets of selection differed somewhat between the phage-only and the phage-plasmid treatments (Fig. 1b). In total 7 LPS-associated loci were targeted in the 12 clones coevolved with phages. Five of these evolved in parallel (i.e. in > 1 clone), and of these loci, 3 were treatment-specific. Two loci (PFLU0478 and PFLU1653) were unique to the phage-only treatment, each targeted in 2 clones, and have been previously associated with evolved phage resistance in SBW25 (Scanlan et al 2015). One locus, *fnl1*, was unique to the phage-plasmid treatment and was found in 2 clones, both of which had evolved the mucoid colony phenotype. *fnl1* is a homolog of *fnlA*, which has been associated with capsule production in *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mulrooney et al 2005). Although the genetics which underlie the mucoid phenotype are not well understood (Scanlan and Buckling 2012) it is likely that the mutations in *fnl1* contribute to mucoidy as the phenotype is caused by overproduction of alginate, a polysaccharide involved in bacterial capsule formation.

Phage selection impedes compensatory evolution in plasmid-carriers

Comparison of the plasmid-only and phage-plasmid treatments reveals a clear difference in the loci targeted by mutations between these treatments. Notably, 5/6 bacterial clones from the plasmid-only treatment carried mutations in the *gacA/gacS* two-component global regulatory system, whereas mutations in these loci were observed in only 1/6 clones from the phage-plasmid treatment (Fig. 1b, clone level mutations shown in Fig. S1). The lower frequency of mutations in *gacA/gacS* under phage selection suggests that coevolving with phages

constrained the potential for bacteria to ameliorate the cost of plasmid carriage. Loss of function mutations in *gacA* or *gacS* have previously been shown to be an important mechanism of compensatory evolution in this bacteria-plasmid association, occurring with a high degree of parallelism across varied selective environments and completely ameliorating the cost of plasmid carriage (Harrison et al 2015a). It is possible that compensatory evolution may have been achieved through alternative mechanisms in the presence of phages. Five out of six sequenced clones from the phage-plasmid treatment contained mutations whose functional effects are currently unclear. However, it should be noted that unlike the highly parallel evolution of *gacA* and *gacS* mutations across evolutionary replicates, the majority of other mutations observed in the phage-plasmid treatment occurred in only a single strain suggesting that they are unlikely to be under strong selection.

To further understand the dynamics of *gacA/gacS* compensatory mutations, we tracked the frequency dynamics of exoprotease production, which is positively regulated by GacAS and therefore an indicator of GacAS function (Cheng et al 2013) (Fig. 2). Loss of exoprotease production occurred early and swept to high frequency in all 6 populations of the plasmid-only selection treatment, including the population where the sequenced clone did not contain a *gacA* or *gacS* mutation. In agreement with the sequencing, this particular clone was positive for exoprotease production but was in the minority (2/20 clones tested) within the population. By contrast under phage-plasmid selection, loss of exoprotease function, although detectable, only reached high frequency in 1/6 populations, consistent with the sequencing data. Intriguingly, this population was also distinct from the other replicates within the phage-plasmid treatment in that it was the only population in which the mucoid colony morphology did not sweep to high frequency (Fig. 2); moreover it displayed a high level of complete resistance against its phage population (Fig. S2) (Harrison et al 2015b). Notably, the clone sequenced from this population carried a mutation in LPS-associated loci PFLU0479 which was also targeted by a clone from the phage-only treatment. This suggests a potential link between GacAS function and the evolution of mucoid-associated phage resistance.

Discussion

We show that individually plasmids and phages impose selection leading to bacterial evolutionary responses that are distinct from bacterial populations evolving without MGEs, but that together plasmids and phages impose conflicting selection on bacteria, constraining the evolutionary responses observed in pairwise interactions. Without MGE selection, bacteria adapted to the low concentration of Hg(II) present in the growth medium via parallel mutations to PFLU4382, a thiol-disulfide interchange protein involved in protein-folding. Phage-only selection led to high rates of bacterial evolution, in particular at LPS-associated loci linked to phage resistance (Scanlan et al 2015), but bacterial evolution in response to phages was constrained in the presence of plasmids and in some cases targeted different genes. Plasmid-only selection led to the evolution of parallel compensatory mutations at *gacA/gacS*, which have previously been shown to completely ameliorate the cost of plasmid carriage (Harrison et al 2015a), an evolutionary trajectory that was impeded when coevolving with phages in the phage-plasmid treatment.

Constraints on evolutionary responses to conflicting selective pressures are predicted where there are negative genetic correlations between the traits under selection. This mechanism may explain the lower frequency of *gacA/gacS* compensatory mutations observed in the phage-plasmid treatment compared to the plasmid-only treatment. GacAS is a global regulator that positively controls biosynthesis of a large suite of secreted molecules (Cheng et al 2013), including capsular polysaccharides such as alginate (Cheng et al 2013, Hay et al 2014), the overproduction of which causes mucoidy (Hay et al 2014). Loss of function mutations in *gacA* or *gacS* to ameliorate the cost of plasmid carriage would have likely prevented the evolution of the mucoid phenotype, the form of partial phage resistance which evolved to high frequency in the phage-plasmid treatment (Harrison et al 2015b). Phage-imposed selection for mucoidy could therefore have prevented compensatory evolution through loss of GacAS function. This inference is further supported by the genetic and phenotypic data from the single replicate population from the phage-plasmid treatment that did not evolve the mucoid phenotype: Here, loss of GacAS function swept to high frequency, suggesting that this evolutionary trajectory is mutually exclusive from the evolution of mucoidy. Compensatory evolution is thought to be a key factor stabilizing plasmids in bacterial populations and we have previously demonstrated that mutations in *gacA* or *gacS* are extremely efficient at ameliorating the cost of pQBR103 in

this bacterial host (Harrison et al 2015a). By impeding this important route to compensatory evolution, phage selection could limit the conditions allowing the survival of plasmids, and in so doing restrict the potential for future horizontal gene transfer within the bacterial community.

Nevertheless, the observed evolution of high frequencies of mucoidy in the phage-plasmid treatment is somewhat counter intuitive, because it not only limits the available mechanisms for compensatory evolution but also only confers weak partial resistance to phage infection when other forms of complete resistance via modification of LPS phage binding sites were readily available. Why, then, was mucoidy favoured in the presence of plasmids? A possible explanation is that acquisition of large plasmids, such as pQBR103, can have major effects on the bacterial phenotype, beyond those of the accessory gene cargo itself, through large-scale remodeling of bacterial gene regulatory networks (Dougherty et al 2014). We have previously shown that acquisition of the pQBR103 plasmid causes ~17% of SBW25 chromosomal genes to be upregulated, including a large proportion of the alginate biosynthesis pathway (7 positive regulators, 3 negative regulators and the biosynthesis locus PFLU5986, Harrison et al 2015a). Thus plasmid-mediated alterations to bacterial gene expression could have primed plasmid-carrying cells for the evolution of mucoid-based resistance against phages. Subsequent mutations, for example those occurring in the *fnl1* loci, may have led to genetic assimilation (Pigliucci et al 2006) of these regulatory effects of plasmid carriage. Moreover, the emergence of mucoid-based resistance in the phage-plasmid treatment may in turn have weakened selection for mutations conferring complete resistance. Thus plasmid-mediated alterations to the intracellular environment of bacteria, especially through regulatory disruption, could have important evolutionary consequences, altering the trajectory of subsequent bacterial evolution.

These data show that multiple MGEs can impose conflicting selective pressure on bacterial populations, leading to divergent outcomes for bacterial molecular evolution. Differences in evolutionary trajectory can have important ecological implications in microbial communities (Beaume et al 2017, McClean et al 2015). The evolution of the mucoid phenotype under co-selection by phage and plasmids observed here is likely to alter the local environment, increasing viscosity and leading to formation of biofilms that are resilient to environmental stressors (Hentzer et al 2001). In addition, mucoidy has also been associated with reduced

receptiveness to plasmid conjugation (Pérez-Mendoza & de la Cruz 2009) potentially limiting rates of horizontal gene transfer. Moreover, by limiting the bacterial response to phage-mediated selection, plasmids could stabilise bacteria-phage coexistence (Wright et al 2016) which can be destabilised due to asymmetries in the evolutionary potential for phage infectivity compared to bacterial resistance (Buckling and Brockhurst 2012). However, given the role of phages as drivers of bacterial diversity (Koskella & Brockhurst 2014) changes to, and perhaps weakening of, these coevolutionary interactions are likely to have important implications for natural microbial communities. Intriguingly, conflicting selection appears to arise due to the negative pleiotropic effects of the mutations that compensate for the cost of plasmid carriage: Whereas loss of the global regulator GacAS allows bacteria to ameliorate the cost of plasmid carriage, it concomitantly prevents them from evolving mucoid-based resistance against phage infection. While the molecular details underpinning genetic correlations will vary between systems, these findings highlight the likely difficulties of predicting evolutionary responses to multiple selective pressures from the evolutionary responses observed to each selective pressure alone. Understanding evolution in complex microbial communities comprising many species and MGEs will require that we go beyond studies of pairwise interactions.

Conflict of Interest statement

The authors declare that this research was conducted in the absence of any commercial or financial relationships that may cause a potential conflict of interest.

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Data Availability

Sequencing data is available via the European Nucleotide Archive (PRJEB19606) and phenotypic data and variant call data are available on Dryad (doi:10.5061/dryad.3hk0v).

Figure legends

Fig. 1. Bacterial genome evolution in the presence and absence of symbionts. a. Venn diagram comparing different loci targetted across 6 replicate clones within each treatment. b. Summary of mutations identified across 6 seqeuenced clones for each treatment. Circles represent a summaried bacterial genome for each MGE treatment, with dots representing loci targetted by mutations across the 6 replicate clones per treatment. Dots are scaled by the number of times mutations appear across replicates. Expanded sections are shown for clarity. Loci highlighted in the text are named, with LPS-associated loci indicted by *.

Fig. 2. The population dynamics of mucoid and GacAS deficient genotypes in replicate populations. Twenty-four colonies collected from evolving populations at 4 transfer intervals were screened for exoprotease production, a phenotype associated with GacAS function. The proportion of exoprotease-negative mutants is shown for each replicate population through time (blue), overlaid with the proportion of mucoid mutants (yellow) in the same population (data from Harrison et al 2015b).