The University Of Sheffield.

The ecology and evolution of horizontal gene transfer in bacteria

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A thesis submitted for the degree of Doctor of Philosophy

University of Sheffield

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September 2019

Abstract

Horizontal gene transfer plays a vital role in bacterial evolution, primarily by spreading adaptive genes between cells both within and between species. Genes capable of HGT are generally encoded on mobile genetic elements such as plasmids, and therefore have a potential advantage over those encoded exclusively on the chromosome, as mobile genes can be spread both vertically and horizontally. This thesis investigates how gene mobility affects the spread of resistance genes in bacterial populations and communities, and how this process is affected by the environmental and community context. First, I demonstrate that being encoded on a mobile plasmid widened the range of parameter space in which resistance genes could spread, allowing resistance to reach fixation in the absence of positive selection, through horizontal transmission of the plasmid. Secondly, I show that high frequencies of pulsed positive selection increased plasmid stability in the population, but that under low frequencies of pulsed positive selection, horizontal transmission played a greater role in plasmid stability. Thirdly, I showed that the presence of lytic bacteriophages limited the selective conditions under which mobile plasmid-encoded resistance could persist in a population, but that sufficiently high rates of horizontal transmission could overcome this cost. Finally, I showed that mobile plasmid-encoded resistance genes were able to horizontally transfer into a natural soil community, enabling the survival of a more diverse portion of the community in response to lethal environmental change. Overall, this thesis demonstrates how environmental context favours the spread of mobile versus non-mobile resistance genes, and provides insight into the consequences this may have for the evolutionary potential of the wider bacterial community.

Acknowledgements

This thesis would not have been possible without the support of my supervisors (Mike Brockhurst and Jamie Wood), the lab group, my family and friends, and funding from the Natural Environment Research Council.

In particular, I would like to thank members of the Brockhurst Lab past and present, for what has been a wonderful four years. I could not have wished for a better work environment. I would like to thank my supervisor, Michael Brockhurst, as without his clear vision, and invaluable advice, this PhD would not be what it is today. A huge thank you to Ellie Harrison and Jamie Hall for having endless time and patience, you taught me everything I know in the lab and I am so grateful to you both. As a lab group, you have become some of my closest friends, and I wish you all the very best of luck in the future.

Author's Declaration

I declare that this thesis is a presentation of original work and that I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged in the Bibliography.

The following publications have arisen from this thesis:

- C Stevenson, JPJ Hall, E Harrison, AJ Wood, MA Brockhurst 2017. Gene mobility promotes the spread of resistance in bacterial populations. *ISME J*. 11: 1930-1932
- C Stevenson, JPJ Hall, MA Brockhurst, E Harrison 2018. Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Pro. R. Soc. B*. 285: 20172497

Chapter 2 presents published data from Stevenson et al (2017). I performed the experiments and analysed the data under the supervision of M.A.B, J.P.J.H, E.H and A.J.W. All authors contributed towards the design of the experiment, interpretation of results and writing of the manuscript.

Chapter 3 presents the published version of Stevenson et al (2018). I performed the experiments and analysed the data under the supervision of M.A.B, J.P.J.H and E.H. All authors contributed towards the design of the experiment, interpretation of results and writing of the manuscript.

Chapter 4 presents data collected in collaboration with Professor Calvin Dytham at the University of York. I performed the lab experiments and carried out the data analysis. C.D carried out the IBM simulations, and I performed the data visualisation from the model output.

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Abbreviations

AMR	**A**nti-**M**icrobial **R**esistance
BHR	**B**road **H**ost **R**ange
bp	**b**ase **p**air
CFU	**C**olony **F**orming **U**nits
CR	**C**ommunity **R**escue
DNA	**D**eoxyribo**n**ucleic **A**cid
ER	**E**volutionary **R**escue
Gm	**G**entamicin
HGT	**H**orizontal **G**ene **T**ransfer
kb	**k**ilo**b**ase
KB	**K**ing's **B** medium
MGE	**M**obile **G**enetic **E**lements
MIC	**M**inimum **I**nhibitory **C**oncentration
NHR	**N**arrow **H**ost **R**ange
OD	**O**ptical **D**ensity
OTU	**O**perational **T**axonomic **U**nit
PCR	**P**olymerase **C**hain **R**eaction
PCA	**P**rincipal **C**omponent **A**nalysis
Sm	**S**treptomycin

Chapter 1

Introduction

1.1 Mechanisms of evolutionary innovation in bacterial populations

Natural microbial communities are highly diverse and contain a vast amount of genetic variation (Fraser et al., 2009), with prokaryote genomes ranging in content from just over 100 genes to almost 10,000 (McCutcheon et al., 2009; Schneiker et al., 2007). Sequencing data has revealed large amounts of genomic variation even among the genomes within a single bacterial species (Perna et al., 2001). Bacterial genomes can be characterised into 'core' and 'accessory' genomes (Young et al., 2006). The 'core' genome refers to the set of conserved genes, found in all members of a species, which includes those encoding genes required for fundamental cellular processes. In contrast, the 'accessory' genome refers to 'dispensable' genes, found only in a subset of the species, and often encoding environment-specific functions, such as toxin degradation (Lilley et al., 1996), antibiotic resistance (Tettelin et al., 2005) or the ability to metabolise novel carbon sources (Norman et al., 2009). The 'pangenome' comprises both the 'core' and 'accessory' genome and contains all the genes found in the species (Ku et al., 2015). The size of the pangenome and the proportion of core genes varies widely between different species, ranging from large 'open' pangenomes with a low proportion of core genes, to smaller 'closed' pangenomes with a high proportion of core genes (McInerney et al., 2017). There exists, therefore, a negative correlation between the proportion of core genes and the pangenome size of a species, and where a species sits upon this spectrum appears to be linked to its lifestyle (McInerney et al., 2017). For example, at one end of the spectrum, the obligate intracellular pathogen *Chlamydia trachomatis* has a small pangenome and a core genome of 84% of its pangenome whereas the free-living *Prochlorococcus marinus* has a large pangenome and core genome of approximately 18% of its pangenome (McInerney et al., 2017). One hypothesised driver of pangenome size is the effective population size (*Ne*) of the species. Host-associated microbes (i.e. intra-cellular symbionts and pathogens) typically have smaller *Ne* compared to free-living microbes (Bobay and Ochman, 2018; Funk et al., 2001). Where *Ne* is small, the effects of genetic drift are likely to be exacerbated, driving the decay/loss of non-essential genes by drift (Moran, 2002). Furthermore, populations with small a *Ne* typically possess lower genetic

diversity (Andreani et al., 2017). In addition to *Ne*, other aspects of bacterial lifestyle could impact upon the composition of MGE's within a population. For example, intracellular bacteria which undergo strict vertical transmission are likely to experience reduced access to MGEs than those with higher rates of horizontal transmission between hosts or free-living environmental life-stages (Bordenstein and Reznikoff, 2005). Whether adaptive or neutral processes drive the evolution of the bacterial pangenome has recently come under debate (McInerney et al., 2017; Shapiro, 2017; Vos and Eyre-Walker, 2017), although it is widely acknowledged that horizontal gene transfer (HGT) will have an important role in shaping the pangenome (Shapiro, 2017).

In microbial populations, new genes may arise through two main sources. One source is intra-chromosomal gene duplication (Alm et al., 2006; Serres et al., 2009). Here, a section of the bacterial chromosome duplicates, and the multiple gene copies then provides the opportunity for adaptive mutations to occur on these copied sections of the chromosome, resulting in evolutionary divergence between the gene duplicates (Andersson and Hughes, 2009; Francino, 2005; Kugelberg et al., 2006). The second source of new genes is acquisition through HGT (Gogarten et al., 2002; Ochman et al., 2000). These two processes are likely to occur at different speeds: HGT is likely to result in faster gene acquisition than chromosomal gene duplication and subsequent divergence, as it enables the instantaneous acquisition of new genetic information, rather than waiting for divergence of pre-existing genes (Gogarten et al., 2002; Ochman et al., 2000). This is supported by comparative analyses, which showed that horizontal transfer, and not gene duplication, drove the expansion of microbial protein families (Treangen and Rocha, 2011). HGT has been suggested as a critical driving force of bacterial evolution, spreading important ecological traits across bacterial lineages (Aminov, 2011; Frost et al., 2005; Thomas and Nielsen, 2005). Thus HGT is thought to play a key role in promoting adaptation to new ecological conditions in both clinical and environmental contexts (Cordero et al., 2012; Ojala et al., 2014), and it has been suggested that the dominant source of genome content variability comes from HGT events (Treangen and Rocha, 2011). The majority of HGT relies on mobile genetic elements (MGEs) including plasmids, bacteriophages and transposable elements (Jain et al., 2003; Ochman et al., 2000; Stokes and Gillings, 2011). MGEs act as vectors and transmit DNA between cells allowing the dissemination of traits between potentially distant phylogenetic relatives (Gogarten et al., 2002).

HGT offers an additional advantage for gene spread relative to gene duplication: horizontally transmissible genes can replicate both vertically and horizontally whereas chromosomal gene duplications can only replicate via vertical transmission through clonal expansion (Cordero and Polz, 2014; Maynard Smith et al., 1993; Shapiro, 2016). The balance of vertical and horizontal

transmission of genes encoded on MGEs is not constant in space or time (Cordero and Polz, 2014). Variation in this balance is likely to depend on the level of environmental selection for the encoded trait: vertical transmission is favoured by positive selection which increases clonality within populations via genome-wide selective sweeps (Wiedenbeck and Cohan, 2011), whereas horizontal transmission is favoured in the absence of positive selection (Stevenson et al., 2017; Tazzyman and Bonhoeffer, 2014a). Genome-wide selective sweeps appear to be less common than gene-level selective sweeps in natural populations (Cordero and Polz, 2014; Shapiro et al., 2014). Instead adaptive genes appear to spread independently of the rest of the genome supporting the idea that HGT plays a significant role in bacterial evolution (Shapiro, 2016). Comparisons of related genomes suggests that MGEs are transient through time, and frequently supplanted by newcomers (Lukjancenko et al., 2010), suggesting that HGT events are often costly to the host cell, and therefore that the MGEs themselves may frequently be subjected to purifying selection. The following thesis focuses on the transmission dynamics of MGEs and the traits they encode and how these dynamics are affected by environmental context including: varying regimes of positive selection, presence of lytic phages and the presence of a diverse bacterial community.

1.2 Plasmids and their role in bacterial evolution

1.2.1 The costs and benefits of plasmid carriage

The majority of HGT relies on the transmission of MGEs between bacterial cells (Ochman et al., 2000; Stokes and Gillings, 2011). Conjugative plasmids are considered the primary agent of HGT in bacteria (Norman et al., 2009), enabling the spread of genes across both species and genus boundaries (Frost et al., 2005; Thomas and Nielsen, 2005). Plasmid sequences are typically modular in structure, containing clusters of genes that can be separated into those that encode plasmid functions essential for maintenance (i.e. those involved in plasmid replication, maintenance and transfer), and those that encode non-essential 'accessory' functions of potential use to the bacterial host cell (e.g., those involved in toxin resistance, novel metabolism, pathogenesis etc.) (Cazares et al., 2019).

Conjugative plasmids can be considered as selfish genetic elements, whose fitness interests may not necessarily be aligned to those of the host cell. Indeed, plasmid acquisition often leads to large fitness costs for the bacterial host cell (Baltrus, 2013) (i.e. plasmid-free cells have a significant growth advantage compared to plasmid-bearers). Plasmid fitness costs can arise from a range of effects on the host cell (Baltrus, 2013). Direct costs of plasmid carriage stem from the transfer

process (Koraimann and Wagner, 2014), the disruption of the host genome by the integration of foreign DNA (Canchaya et al., 2004), and metabolic costs associated with the replication and expression of the plasmid genes (Bragg et al., 2009; Shachrai et al., 2010). Indirect costs of plasmid carriage come from deleterious interactions between proteins encoded by the MGE and the host (Bagdasarian et al., 1986), which can lead to the disturbance of gene regulatory networks within the host (Bagdasarian et al., 1986), and cytotoxic effects including those from mis-folded proteins (Geiler-Samerotte et al., 2011).

The fitness costs of plasmids may be offset in some environments by the fitness benefits of the accessory genes they encode (Hall et al., 2015). These encompass a wide variety of functions including: resistance to antibiotics (Clewell et al., 1975; Livermore, 1995; Svara and Rankin, 2011), heavy metal resistance (Hall et al., 2015; Tett et al., 2007), genes involved in virulence and host colonisation (Boucher et al., 1986) and the ability to metabolise novel carbon sources (Norman et al., 2009). These horizontally acquired genes are potentially beneficial for their host bacterial cell, allowing them to exploit new ecological niches or survive in toxic environments (i.e. when antibiotics are present) (Gogarten and Townsend, 2005). The net fitness effects of plasmids are therefore context dependent, and exist on a continuum between mutualism and parasitism across environmental gradients (Bergstrom et al., 2000; Harrison and Brockhurst, 2012; Levin, 1993). Mutualism occurs when the fitness benefits of plasmid-carriage outweigh the costs (e.g. carrying an antibiotic resistant plasmid in the presence of antibiotics), whereas parasitism occurs when the costs outweigh the benefits (e.g. carrying a costly resistance plasmid in the absence of antibiotics).

1.2.2 The plasmid paradox

Despite the widespread occurrence of plasmids in bacterial communities in natural environments (Brown et al., 2013; Heuer and Smalla, 2012; Kav et al., 2012; Lilley et al., 1996), due to their fitness costs it remains challenging to explain the long term persistence of plasmids, a problem which has been termed the 'plasmid paradox' (Harrison and Brockhurst, 2012). The paradox arises because under both parasitism and mutualism, plasmids are expected to be lost from the population. Under parasitism, this occurs due to the combined effects of segregational loss (i.e. imperfect vertical transmission) and the net cost of carriage, plasmids should decline in frequency through purifying selection (Bergstrom et al., 2000; Lili et al., 2007; Stewart and Levin, 1977). In contrast, under mutualism, although plasmids may be maintained by positive selection in the short term, because their benefit outweighs the cost of carriage, in the longer term, capture of beneficial accessory genes by the bacterial host chromosome would make the plasmid redundant

and costly to the cell, leading to its loss by purifying selection (Bergstrom et al., 2000). Given that accessory genes are frequently located on other, nested mobile elements such as transposons, chromosomal capture is likely to occur at an appreciable rate (Bergstrom et al., 2000). Nevertheless, it has consistently been found that costly plasmids are not easily lost from experimental bacterial populations (Dahlberg and Chao, 2003; Hall et al., 2016; Modi and Adams, 1991; Porse et al., 2016; Ridenhour et al., 2017; San Millan et al., 2014a) but see also (De Gelder et al., 2007; Kottara et al., 2018).

1.2.3 Resolving the paradox

There is increasing experimental evidence that suggests plasmid costs can be ameliorated through rapid compensatory evolution (Harrison et al., 2015a; Loftie-Eaton et al., 2016; Porse et al., 2016; San Millan et al., 2014b; Yano et al., 2016). The amelioration of plasmid fitness costs relieves the purifying selection against the plasmid, enabling plasmid persistence (Harrison et al., 2015a). Several evolutionary trajectories may lead to successful amelioration: compensatory evolution may arise through plasmid evolution (Modi and Adams, 1991), host evolution (Harrison et al., 2015a; San Millan et al., 2014a) or by plasmid-host coadaptation (Bottery et al., 2017; Dahlberg and Chao, 2003; Loftie-Eaton et al., 2016; Modi and Adams, 1991). Several mechanisms of compensatory evolution have been described: plasmids may decrease their conjugation rate (Turner et al., 1998), alter their replication machinery (Sota et al., 2010) or undergo large-scale deletions of the plasmid backbone (Porse et al., 2016). Chromosomal compensatory mutations have been shown to target regulatory genes, helicases, and genes encoding enzymes involved in DNA and RNA metabolism including DNA repair, recombination, transcription and translation (Harrison et al., 2015a; Loftie-Eaton et al., 2017; San Millan et al., 2014a). Transcriptomic analysis has revealed that while plasmid acquisition often causes increased transcription of large numbers of chromosomal genes, this is relieved by compensatory evolution. For example, acquisition of the mega-plasmid pQBR103 by *Pseudomonas fluorescens* led to upregulation of 18% of chromosomal genes including many involved in translation, but wild-type expression levels were restored by compensatory mutations in the regulatory genes *gacA/gacS* (Harrison et al., 2015a). Similar patterns were described for *Pseudomonas aeruginosa* upregulation of chromosomal gene expression caused by the plasmid PNUK73 was relieved by compensatory mutations in host-encoded helicases (Millan et al., 2015).

In addition to compensatory evolution, a sufficiently high rate of horizontal transmission via conjugative transfer may be adequate to allow plasmid persistence (Lundquist and Levin, 1986), whereby high rates of conjugation enable the plasmid to escape purifying selection and exist as

an infectious agent. There has been long-running debate surrounding whether such high conjugation rates exist in nature (Bergstrom et al., 2000; Levin, 1993), but recent research suggests that high transfer rates can sustain plasmids at least over ecological timescales (Bahl et al., 2007; Fox et al., 2008; Lopatkin et al., 2016; Stevenson et al., 2017). Furthermore, in multi-species communities stable maintenance of a plasmid in a permissive donor species can enable it to persist in less permissive recipient host species by inter-specific conjugation (Hall et al., 2016).

1.3 The impact of lytic phages on HGT

In nature, bacteria are subjected to numerous selection pressures potentially affecting not only their own population dynamics but also those of the MGEs they harbour. One key selective pressure affecting bacterial populations are lytic bacteriophages, obligate viral parasites of bacteria that infect the bacterial host and hijack the bacterial cellular machinery to replicate and kill the host cell via cellular lysis to release phage progeny (Weinbauer, 2004). Phages are abundant, outnumbering bacteria by as much as 100-to-1 in a range of natural environments (Weinbauer, 2004) and are a major cause of bacterial mortality (Bouvier and del Giorgio, 2007; Proctor and Fuhrman, 1990). Lytic phages have been shown to affect plasmid dynamics in several ways: First, some lytic phages target the plasmid conjugative pilus and thus can directly select against the plasmid by killing only those host cells harbouring the plasmid (Jalasvuori et al., 2011; Ojala et al., 2013). Second, by reducing bacterial density, lytic phage can reduce opportunities for plasmid conjugation, which is likely to be host-cell density dependent, and thus limit plasmid persistence (Harrison et al., 2015b). Third, phage-mediated killing is likely to select for bacterial mutations providing resistance against phage infection, driving selective sweeps in the bacterial population that can affect plasmid dynamics through hitch-hiking (Harrison et al., 2015b). Additionally, bacterial phage resistance mutations could further impact plasmid dynamics if there exist epistatic interactions between phage-resistance mutations and compensatory mutations to ameliorate the cost of plasmid carriage. Previous experimental work using *P. fluorescens*, pQBR103 and the lytic phage phi-2, has shown that plasmid bearers became resistant against phage infection by evolving mucoidy (Harrison et al., 2015c), wherein over-expression of alginate exopolysaccharides forms a physical barrier against phage infection (Hay et al., 2014; Scanlan and Buckling, 2012). This in turn prevented these resistant plasmid-bearing genotypes from gaining compensatory mutations in the *gacA/gacS* regulatory system (Harrison et al., 2017), since expression of mucoidy is positively regulated by GacA/GacS (Hay et al., 2014). This demonstrates that presence of plasmids and phages can impose conflicting selection pressures on bacterial populations, with phages likely to limit the persistence of plasmids over time.

1.4 The role of HGT in evolutionary rescue

Deleterious environmental change can necessitate that bacterial species undergo rapid adaptive evolution to avoid extinction, a process termed evolutionary rescue (ER) (Gomulkiewicz and Holt, 1995; Gonzalez et al., 2013). A classic example of ER in bacteria is the evolution of antibiotic resistance in bacterial populations treated with antibiotics (Levin et al., 1997; Tazzyman and Bonhoeffer, 2014b). Populations are more likely to undergo successful evolutionary rescue if they possess a sufficiently large population size (Samani and Bell, 2010), if the population contains pre-existing standing adaptive genetic variation (Agashe et al., 2011), an elevated mutation supply rate (Anciaux et al., 2019; Eliopoulos and Blazquez, 2003), or if it has experienced prior exposure to the specific stress (Bell and Gonzalez, 2011; Gonzalez and Bell, 2012).

Horizontal acquisition of adaptive genes through HGT could increase the probability of ER, reducing the necessity for populations to wait for spontaneous beneficial mutations to arise (Jain et al., 2003; Tazzyman and Bonhoeffer, 2014b). HGT may be particularly important in cases where ER requires complex adaptive traits as plasmids are able to provide multi-gene adaptations instantaneously that might be less likely to arise by spontaneous mutation alone (Hülter et al., 2017). Conjugative plasmid transfer may also be an important factor in ER. Where conjugation rates are sufficiently high, plasmids can persist in a population through horizontal transmission even if their encoded traits are not currently beneficial, enabling traits that are currently costly but that may potentially become beneficial under future conditions to be maintained within a population without the need for sustained positive selection (Hall et al., 2017a; Stevenson et al., 2017). In addition, maintenance within a permissive host capable of acting as a community-wide hub for HGT may enable ER of other sensitive species within the community (Hall et al., 2016). Some plasmids have been shown to have broad host ranges (Klümper et al., 2015), allowing them to infect phylogenetically diverse bacterial species (Norman et al., 2009). Therefore, plasmid-mediated HGT of adaptive functions could enable ER of diverse bacterial communities, although this possibility remains untested. However, given that diversity enhances the productivity (Bell et al., 2005), stability (Awasthi et al., 2014; Girvan et al., 2005) and ability of communities to resist invasion (van Elsas et al., 2012), community ER via HGT could have important long-term consequences for the maintenance of community function post-environmental change.

1.5 Thesis Outline

Chapter 2: Gene mobility promotes the spread of resistance in bacterial populations

The main objective of this chapter was to determine how the strength of positive selection affected the dynamics of mobile and non-mobile resistance genes. I tracked the dynamics of chromosomal (i.e. non-mobile) and pQBR57 plasmid-encoded (i.e. mobile) mercury-resistance in experimental populations of *P. fluorescens*. I found that when mercury-resistance was mobile, compared to non-mobile, the selective conditions under which it could spread were expanded, with resistance reaching fixation in both the presence and absence of positive mercury-selection. Tracking the resistance dynamics through time revealed that while strong positive selection favoured vertical transmission of both mobile and non-mobile resistance, in the absence of positive selection, mobile but not non-mobile resistance could spread to fixation by horizontal transmission.

This chapter has been adapted from the following article published in a peer-reviewed journal:
C Stevenson, JPJ Hall, E Harrison, AJ Wood, MA Brockhurst 2017. Gene mobility promotes the spread of resistance in bacterial populations. *ISME J*. 11: 1930-1932 (Appendix A)

Chapter 3: Plasmid stability is enhanced by higher-frequency pulses of positive selection

The main objective of this chapter was to investigate how the frequency of pulsed positive selection affected plasmid stability in bacterial populations. I tracked the dynamics of the mercury-resistance plasmid pQBR103 in experimental populations of *P. fluorescens* under varying regimes of pulsed positive mercury selection. Plasmid stability increased with increasing frequency of pulsed positive selection. Compensatory evolution to ameliorate the costs of plasmid carriage occurred under all positive selection regimes, but conjugation played a greater role in plasmid maintenance at lower frequencies of pulsed positive selection.

This chapter has been published in a peer-reviewed journal:
C Stevenson, JPJ Hall, MA Brockhurst, E Harrison 2018. Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Pro. R. Soc. B*. 285: 20172497 (Appendix B)

Chapter 4: Bacteriophages alter the dynamics of mobile- and non-mobile resistance genes in populations of *Pseudomonas fluorescens*

The main objective of this chapter was to investigate how the lytic bacteriophage phi-2 affected the spread of mobile and non-mobile resistance genes in the absence of positive selection. I tracked the dynamics of chromosomal (i.e. non-mobile), pQBR103 (i.e. mobile), and pQBR57 (i.e. highly mobile) mercury-resistance in experimental populations of *P. fluorescens*. The data showed that phages constrained the spread of mercury-resistance: both pQBR103 and pQBR57 were lost in the majority of populations in the presence of phages. However, unlike pQBR103, the more highly conjugative plasmid, pQBR57, was able to maintain itself in some replicates. Individual-based simulations were used to generalise these results and showed that phages limit the conditions under which resistance genes can persist, requiring higher rates of horizontal transmission and lower costs than are necessary in the absence of phages.

Chapter 5: Community evolutionary rescue via horizontal gene transfer

The main objective of this chapter was to investigate how the mobility of resistance genes affected the potential for evolutionary rescue of soil bacterial communities experiencing a lethal environmental deterioration. I tested how the mobility or mercury resistance genes affected the ER of a two-species (*P. fluorescens* + *P. putida*) and a multi-species (*P. fluorescens* + soil community) bacterial community following exposure to lethal mercury contamination. I found that mobile mercury-resistance enabled the maintenance of higher community diversity owing to the horizontal transfer of resistance genes into *P. putida* and the natural soil community, from *P. fluorescens*. In multi-species communities this process improved the survival of rare taxa.

Chapter Two

Gene mobility promotes the spread of resistance in bacterial populations[1]

2.1 Introduction

Microbial populations reproduce clonally by vertical descent but can also exchange genes by horizontal gene transfer (HGT). Where genes are encoded on the bacterial chromosome, they are only able to replicate by vertical transmission (i.e. from mother to daughter cell). By contrast, genes encoded on mobile genetic elements (MGEs) (Frost et al., 2005) such as conjugative plasmids, integrative conjugative elements or temperate phages, can replicate by both vertical, and horizontal transmission (i.e. by transfer from donor to recipient cell). HGT is an important process in bacterial evolution, accelerating adaptation by allowing the spread of ecologically and clinically relevant traits between lineages (Frost et al., 2005; Thomas and Nielsen, 2005). This appears particularly important for the spread of antibiotic resistance genes: analysis of *Escherichia* and *Shigella* strains demonstrated that a larger proportion of plasmid genes encoded for antibiotic resistance than chromosomal genes (Svara and Rankin, 2011). There is increasing evidence that there are distinctions between those genes found on the chromosome, and those found on MGEs (Rankin et al., 2011). Therefore, the balance of vertical versus horizontal inheritance is expected to have important effects on bacterial evolution and thus function (Cordero and Polz, 2014; Maynard Smith et al., 1993; Shapiro, 2016). Comparative genomics has revealed that bacterial species may undergo shifts in the balance of vertical versus horizontal inheritance over time (Cordero and Polz, 2014). Pathogenic bacterial populations have been shown to switch between panmictic (i.e. high rates of HGT from a common gene pool) and clonal lifestyles (Cui et al., 2015; Rosen et al., 2015; Takeuchi et al., 2015). For example, the marine bacteria *Vibrio cholerae* generally lives in a state of panmixis, resulting in diverse populations which share common genes (Boucher et al., 2011). However, under certain ecological conditions, pathogenic

[1] C Stevenson, JPJ Hall, E Harrison, AJ Wood, MA Brockhurst 2017. Gene mobility promotes the spread of resistance in bacterial populations. *ISME J*. 11: 1930-1932

strains have been shown to emerge from these populations and undergo rapid clonal expansion (Mutreja et al., 2011).

A number of factors may influence the level of vertical and horizontal inheritance within a bacterial population (Shapiro, 2016). Generally, it is assumed that positive selection will favour vertical inheritance resulting in the clonal expansion of cells harbouring the adaptive genes (Shapiro, 2016). Metagenomic studies tracking bacterial populations through time have found evidence for clonal expansion (or genome-wide selective sweeps) in nature: the freshwater bacteria *Chlorobium*-111 was seen to undergo an almost complete genome-wide selective sweep across a 9-year period (Bendall et al., 2016). Despite this, examples of genome-wide selective sweeps remain relatively rare (Cordero and Polz, 2014; Shapiro et al., 2014). Instead, adaptive genes appear to often spread by gene-specific selective sweeps, where the gene of interest spreads through the population, unlinked from any particular genomic background (Takeuchi et al., 2015). Gene-specific sweeps are indicative of high rates of horizontal inheritance over vertical inheritance, as these sweeps demonstrate the spread of a gene into diverse host backgrounds. In addition, because horizontally inherited genes can spread in the absence of positive selection (Bahl et al., 2007; Hall et al., 2017a), this will likely expand the selective conditions under which genes may spread. For example, whereas antimicrobial resistance genes encoded by the chromosome are expected to spread only under positive selection (i.e. in the presence of the antimicrobial substance), resistance encoded by an MGE can in theory spread both with or without positive selection given a sufficiently high rate of horizontal transmission (Tazzyman and Bonhoeffer, 2014b).

The balance of vertical and horizontal transmission may be further affected by the costs of vertically or horizontally inherited genes. For a gene encoded on the chromosome, the fitness interests of the gene are generally aligned to that of the host, with any costly non-adaptive genes that arise tending to be rapidly lost through their selective disadvantage. Here, whether the gene is maintained will depend on the costs and benefits it exerts on its host. On the other hand, when a gene is encoded on an MGE, the costs of the gene are coupled to that of any costs the MGE exhibits on the host cell. These costs arise because as MGEs replicate and transmit independently of their host cell, they can exert heavy costs without necessarily affecting their rates of overall transmission, particularly if reduced vertical replication is traded-off against higher rates of horizontal transmission. In the case of conjugative plasmids, the primary agent of HGT in bacteria (Norman et al., 2009), their introduction into the host often results in large scale fitness costs arising from a number of factors including: the introduction of new genetic material into the cell (Baltrus, 2013), production of plasmid proteins (Rozkov et al., 2004) and the occupation of

cellular machinery (Glick, 1995). Taken together, this suggests that, for a given functional gene, the host cell will usually pay a lower cost for a chromosomal copy compared to an MGE-encoded copy, the chromosomal version will therefore have a higher rate of vertical transmission irrespective of positive selection whilst forgoing the opportunity for horizontal transmission in the absence of positive selection.

Using experimental evolution, we investigated how strength of positive selection affected the persistence of mobile and non-mobile mercury-resistance (Hg^R) in populations of *Pseudomonas fluorescens* SBW25 (Rainey and Bailey, 1996). Hg^R was provided by the mercury-resistance operon *mer* encoded either chromosomally or carried on the conjugative plasmid pQBR57 (Lilley and Bailey, 1997). The density of bacterial populations and the frequency of Hg^R genes were tracked over time in populations progated in one of three mercury environments representing a gradient in the strength of positive selection for the Hg^R genes.

2.2 Materials and Methods

2.2.1 Strains and culture conditions

Experimental populations were founded using *P. fluorescens* SBW25 (Rainey and Bailey, 1996). Initially, isogenic strains were constructed with mobile or non-mobile Hg^R. An Hg^R strain, harbouring the resistance transposon Tn5042 on the bacterial chromosome, was used to construct a Hg^S control strain using protocols for site directed mutagenesis (Heckman and Pease, 2007). Following this the plasmid pQBR57 (Lilley and Bailey, 1997) was then conjugated into this strain using standard protocols (Hall et al., 2015; Simonsen et al., 1990). Hg^R and Hg^S populations were labelled with mini-Tn7 gentamicin resistance (Gm^R) and streptomycin resistance lacZ (Sm^RlacZ) cassettes (Hall et al., 2015; Lambertsen et al., 2004) in order to distinguish between strains in mixed culture when plated onto Kings Medium B agar (King, Ward and Raney, 1954) (10 g glycerol, 20g proteose peptone no. 3, 1.5g K2HPO4•3H2O, 1.5g MgSO4•7H2O, per litre) supplemented with X-gal (50 μg ml^{-1}). All experiments were conducted in 6ml KB broth in 30ml microcosms shaking at 180rpm and incubated at 28°C.

2.2.2 Selection experiment

36 independent overnight cultures of Hg^R and Hg^S strains were mixed at a 1:1 ratio and 60μl (~10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Of the Hg^R populations, 18 encoded Hg^R

on their chromosome (i.e. non-horizontally transferable) and 18 Hg^R on the plasmid pQBR57 (i.e. horizontally transferable). Six replicate populations were established for each of the three mercury treatments (0, 20 and 40 μM $HgCl_2$) and propagated by 1% serial transfer every 24 hours for a total of 8 transfers. These mercury treatments represent a selective gradient wherein wherein plasmid-encoded mercury resistance is under, respectively, strong negative selection, weak positive selection and strong positive selection, due to the balance between the cost of plasmid carriage and the benefits of mercury resistance (Supplementary Information, Figure 2.3) (Hall et al., 2015). Because pQBR57 is maintained at low copy number (Hall et al., 2015), the chromosomal and plasmid-encoded Hg^R genes provide equivalent levels of resistance (Supplementary Information, Figure 2.4). Population numbers were determined by diluting and plating onto KB agar + 50ug ml^{-1} X-gal every two transfers. Plasmid prevalence was determined by replica plating agar plates onto KB agar + 100μM $HgCl_2$ + 50ug ml^{-1} X-gal which allowed tracking of the plasmid. Furthermore, as our donors and recipients were differentially marked we were able to track the frequency of pQBR57 in both donor and recipient populations.

2.2.3 Competitive fitness assays

18 independent overnight cultures of mercury resistant (Hg^R) and mercury sensitive (Hg^S) strains were mixed at a 1:1 ratio and 60μl (~10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Six replicate populations were established for each of the three mercury treatments (0, 20 and 40 μM $HgCl_2$); 3 replicates used Gm^R as the reference marker and 3 used the Sm^RlacZ marker as the reference to control for marker effects. Population numbers were determined by diluting and plating onto KB agar + 50ug/ml X-gal at 0 and 24 hours. Fitness was estimated from these counts as the ratio of Malthusian parameters ($W = (\ln(test_{end}/test_{start})/ (\ln(reference_{end}/reference_{start}))$ (Lenski et al., 1991). Based on known estimates of the conjugation rate of pQBR57 in *P. fluorescens* (Hall et al., 2015) conjugation of the plasmid into recipient cells is likely to be minimal across the 24 hours (transconjugants make up ~ 5% of the total population) and thus conjugation has little impact on the fitness estimates.

2.2.4 MIC measurements

To measure the minimum inhibitory concentration (MIC) of $HgCl_2$ cultures were grown over night until stationary phase in 6ml KB broth. The saturated cultures were then diluted into 96 well plates to an initial density of ~10^3 cells ml^{-1}. Cultures were grown for 24 hours with OD_{600} measured at the end point. The MIC was defined as the lowest concentration which completely inhibited bacterial growth.

2.2.5 Statistical analysis

All analyses were conducted in R statistical package version 3.3.2 (R Foundation for Statistical Computing).To assess the effect of gene mobility on the maintenance of Hg^R, the endpoint proportion of Hg^R was analysed using a generalized linear model with gene mobility as a fixed effect. A quasibinomial distribution was used to account for over-dispersion within the data. The model was constructed using only populations subjected to 0 μM $HgCl_2$ as populations which had been subjected to the 20 and 40 μM $HgCl_2$ treatments did not display adequate variance (i.e. Hg^R was fixed at 1 across the majority of populations) within the data and therefore were not suitable for analysis using a GLM. To assess the effect of mercury on conjugative plasmid transfer, the endpoint proportion of transconjugants was analysed using mercury treatment as a fixed effect. Again, a quasibinomial distribution was used to account for over-dispersion within the data. For the fitness assays the competitive fitness of plasmid bearers compared to plasmid-frees was analysed using an ANOVA with mercury treatment as a fixed effect.

2.3 Results

The endpoint proportion of mercury-resistant (Hg^R) cells in the population was significantly affected by the horizontal transmissibility of Hg^R (Figure 2.1; MAIN EFFECT OF MOBILITY AT 0 μM HgCl2: $F_{1,10} = 74.34$, $p < 0.001$). Where Hg^R was encoded on the chromosome, positive selection was required to drive the spread of resistance: Hg^R rapidly became fixed within the population in the 20 and 40 μM $HgCl_2$ environments, whereas in the 0 μM $HgCl_2$ environment chromosomal Hg^R remained at ~50% prevalence. In contrast, when Hg^R was encoded on the conjugative plasmid pQBR57, and thus horizontally transferable, Hg^R reached high frequencies across all mercury environments (i.e. 0, 20 and 40 μM $HgCl_2$). Thus, the opportunity for horizontal transfer expanded the selective conditions allowing the fixation of Hg^R such that this occurred both with and without positive selection for resistance.

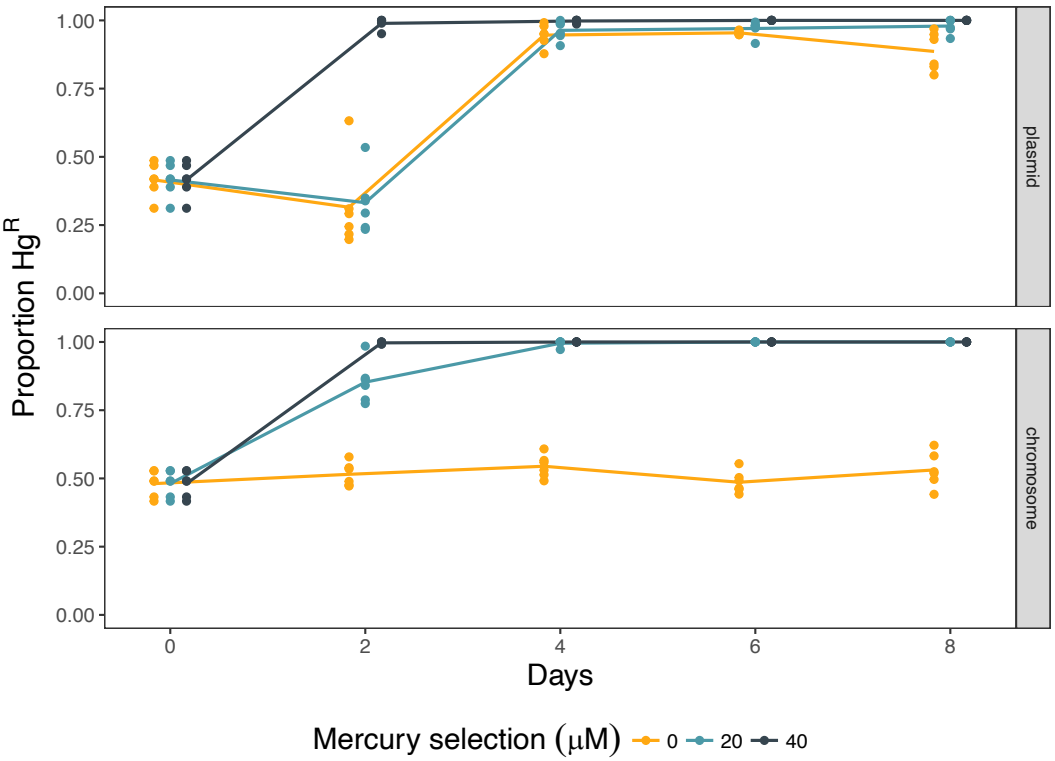

Figure 2.1 Horizontal transmission had a significant impact on the proportion of Hg^R. The proportion of chromosome- and plasmid- encoded Hg^R was determined over time across the three mercury treatments (0, 20 and 40 μM $HgCl_2$). Points represent replicate populations and are slightly offset by treatment on the x-axis to prevent over-plotting. Lines represent means (n = 6).

Tracking plasmid dynamics over time revealed that the strength of positive selection determined the balance of horizontal versus vertical inheritance of plasmid-encoded Hg^R in bacterial populations. HGT played a significantly greater role as the strength of selection decreased (Figure 2.2; MAIN EFFECT OF MERCURY: $F_{1,16} = 392.72$, $p < 0.001$). Under strong positive selection (i.e. 40 μM $HgCl_2$), Hg^R swept through the population by clonal expansion of the original Hg^R donor population. This was presumably due to the high toxicity of the environment strongly selecting against plasmid-free recipients, limiting the opportunity for HGT via plasmid conjugation as a consequence. The contribution of vertical inheritance to the spread of Hg^R reduced with weakening positive selection. Under weak positive selection (i.e. 20 μM $HgCl_2$), Hg^R spread through the population by a mixture of vertical clonal expansion of donor cells and horizontal transmission of the plasmid into the recipient subpopulation. Under negative selection (i.e. 0 μM $HgCl_2$) Hg^R spread by conjugative plasmid transfer into available plasmid-free recipient cells. Therefore, whilst strong positive selection favoured vertical inheritance, the contribution of horizontal transfer to the spread of resistance genes increased as positive selection weakened.

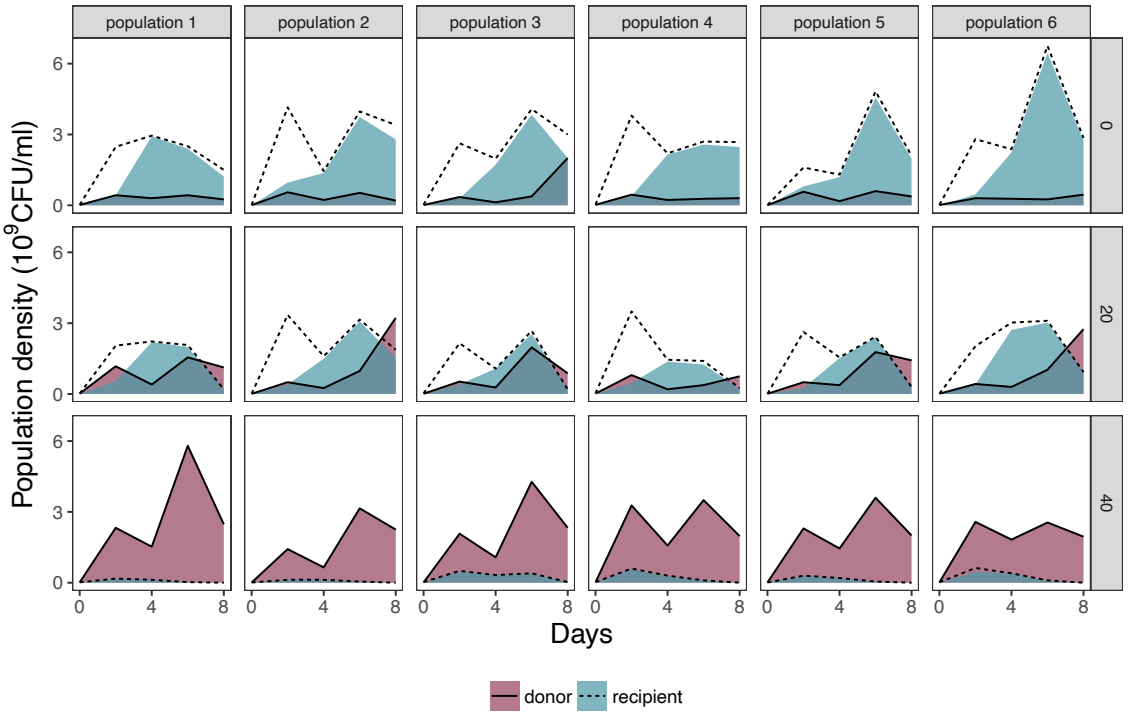

Figure 2.2 Selection determines the balance of horizontal vs. vertical inheritance of plasmid-encoded Hg^R. Plasmid transfer in each of six replicate populations was tracked over time across the three mercury treatments (0, 20 and 40 μM $HgCl_2$). Dotted lines indicate densities of recipient populations; solid lines indicate densities of donor populations. For each population, shaded regions represent plasmid prevalence within donor (purple) and recipient (blue) sub-populations.

2.4 Discussion

Here, we show that HGT expanded the selective conditions under which Hg^R could spread. Chromosomal Hg^R only increased in frequency under positive selection whereas plasmid-encoded Hg^R reached fixation with or without positive selection. Tracking plasmid dynamics over time revealed that the mode of Hg^R inheritance varied across the levels of mercury selection. Under high mercury selection, the spread of Hg^R was driven primarily by clonal expansion, whilst in the absence of mercury, Hg^R dynamics were dominated by infectious transfer. Thus, HGT is most likely to drive the spread of resistance genes in environments where resistance is useless.

Our data confirm previous theory that under strong positive selection, traits primarily spread through vertical inheritance where a genome-wide sweep of the adaptive trait results in a largely clonal population of bacteria (Shapiro, 2016; Wiedenbeck and Cohan, 2011). On the other hand,

where selection for a trait is weak, zero or negative, horizontal inheritance of genes may be more likely, allowing the focal gene to spread into diverse genomic backgrounds whereupon subsequent positive selection would appear as a gene-level selective sweep. Our results indicated that under intermediate levels of positive selection, a mixture of clonal expansion and HGT resulted in the maintenance of Hg^R. Under 20 μM $HgCl_2$, we observed a lag in the spread of plasmid-encoded Hg^R compared to chromosomal Hg^R in the first 2 transfers, which appeared to be driven by rapid outcompetition of plasmid-bearers by plasmid-free cells (Figure 2.2). This likely occurred because pQBR57 imposed a significant fitness cost on the host cell (Figure 2.3), making plasmid-bearers less fit than their plasmid-free counterparts. This lag demonstrates that under intermediate selection, in direct competition chromosomal Hg^R may have the advantage over plasmid-encoded Hg^R in that it can more rapidly spread by vertical inheritance by not paying the costs of plasmid carriage, but that over longer timescales this can be overcome by plasmid horizontal transmission .

Our results indicate a prevalent role of HGT in the absence of positive selection. This confirms previous results that positive antibiotic selection can reduce the fraction of transconjugants within the population (Lopatkin et al., 2016) because only conjugation promotes the spread of plasmids in the absence of positive selection (Lopatkin et al., 2017). This outcome is dependent upon the survival of sensitive cells enabling them to act as potential recipients, which requires either zero or weak antibiotic selection. Alongside the strength of positive selection, other parameters may affect the likelihood of HGT. For example, plasmid stability, driven by the balance of plasmid cost and conjugation, has been shown to vary with different host species (Kottara et al., 2018). Despite this, conjugation appears to play a prevalent role in the maintenance of plasmids from a diverse range of incompatibility groups, suggesting a crucial role of conjugation in natural plasmid populations (Lopatkin et al., 2017). This may explain how such a wealth of plasmids are maintained in natural communities where there is apparently little positive selective pressure for them: alongside pQBR57 a number of other mercury resistance plasmids were isolated from the same field site in Oxfordshire despite low levels of mercury within the soil environment (Boyd and Barkay, 2012; Hall et al., 2015). Together these findings suggest conjugation is a major factor maintaining plasmids in the absence of positive selection, allowing the spread of traits through HGT. Microbial populations often live in interconnected populations with webs of gene exchange (Slater et al., 2008), with access to communal gene pools (Norman et al., 2009). Therefore, our results indicate that in the absence of positive selection, we may in fact expect greater plasmid exchange with the wider community, providing community members with access to the mobile gene pool. This is confirmed by previous work which showed that in the absence of positive

selection, plasmid transfer enabled the mobilisation and transmission of genes between two species (Hall et al., 2017a).

These findings have implications for understanding the maintenance and spread of resistance genes in nature. Antibiotic resistance genes are commonly isolated from pristine uncontaminated sites (Van Goethem et al., 2018): these data suggest that this is likely to be at least in part a consequence of the infectious horizontal transmission of MGEs encoding these genes. Under such conditions, plasmids are likely to disseminate resistance genes into a broad diversity of genetic backgrounds. Thereafter, periods of antibiotic selection will enrich for these taxa by driving their clonal expansion, and potentially purge sensitive genotypes. It is perhaps unsurprising therefore that hospital plumbing, where periodic positive selection is likely to occur, has been shown to sustain diverse communities of plasmids encoding a wealth of antibiotic resistance genes (Weingarten et al., 2018). Given the role of HGT in the dissemination of resistance genes in the absence of selection, interventions which focus on attempting to inhibit conjugative transfer (Lopatkin et al., 2017), rather than simply limiting antibiotic use, may provide a promising route to limiting the spread of resistance genes.

2.5 Supplementary Information

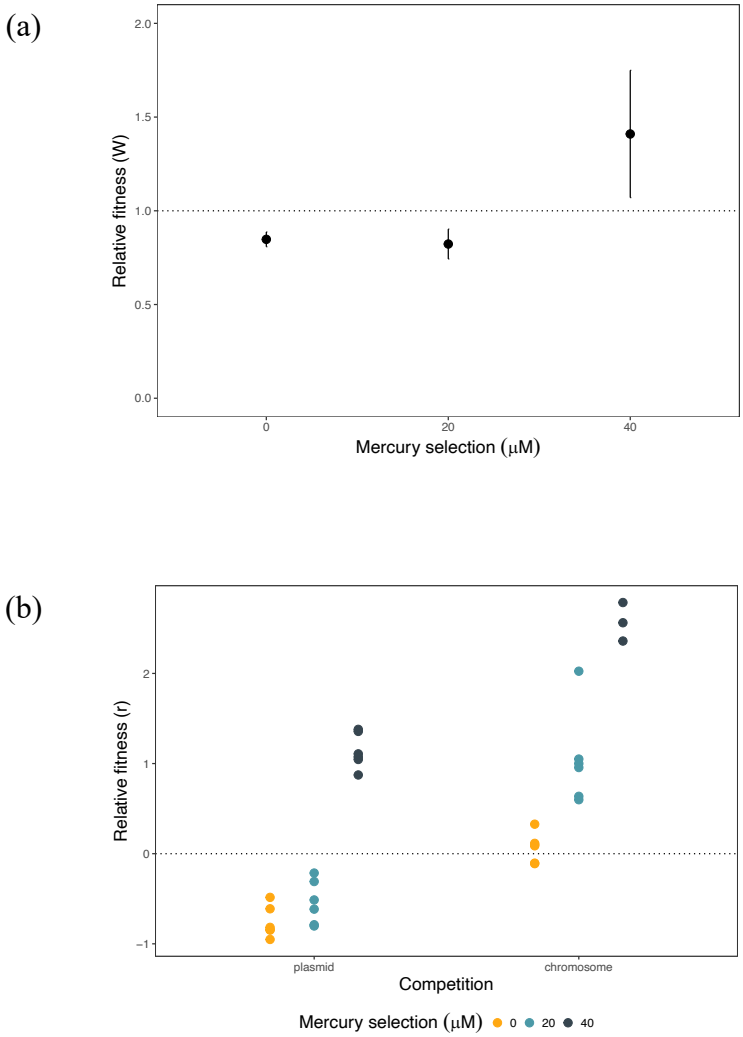

Figure 2.3 The fitness of plasmid-bearing cells significantly increases with mercury selection. a: The relative fitness (W) of Hg^R plasmid-bearers relative to Hg^S plasmid-frees was determined across the three mercury treatments (0, 20 and 40 μM $HgCl_2$). Points represent means ± SE. A value of 1 indicates equal competitive fitness. **b.** Relative fitness extracted from data presented in Figure 2.1. Fitness calculated as selection rates between 0 and 48 hours ($r=(\ln(test_{end}/test_{start})- \ln(reference_{end}/reference_{start}))/day$) (Lenski et al., 1991). Points represent replicate populations (n = 6). A value of 0 indicates equal competitive fitness. Both chromosome and plasmid-encoded *mer* show increased relative fitness with increasing mercury selection.

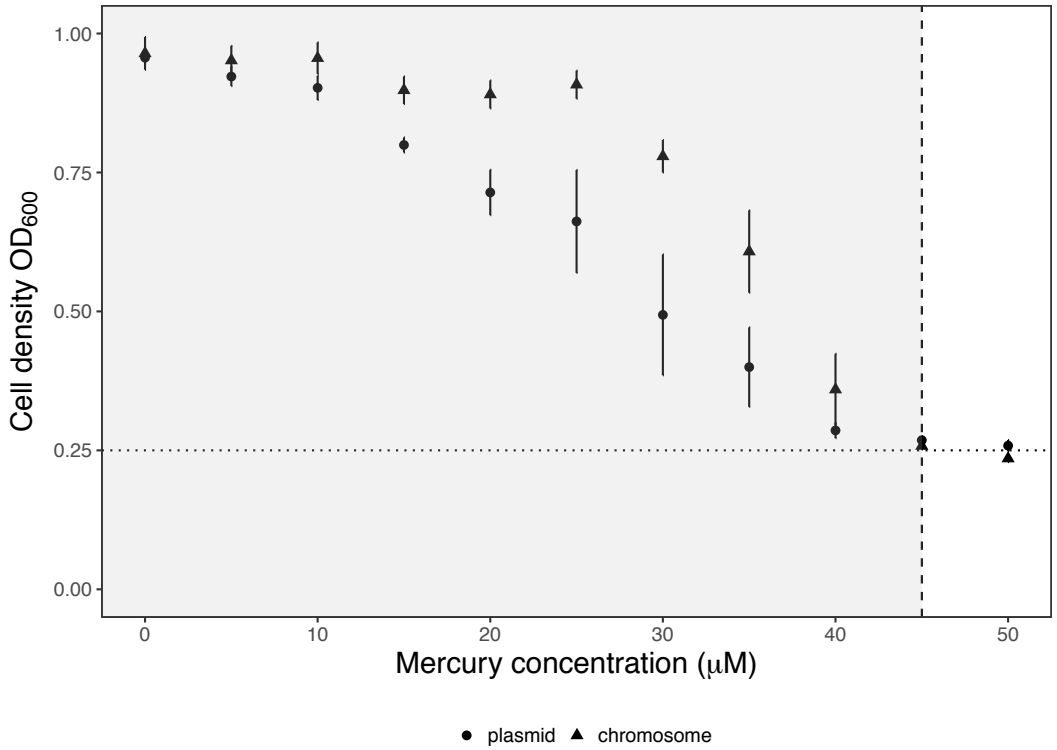

Figure 2.4 Chromosomal and plasmid-encoded Hg^R genes provide equivalent levels of resistance. Cell density (OD_{600}) of SBW25 with chromosomal or plasmid-encoded *mer* as a function of mercury concentration after 24 hours growth in monoculture. Points represent means ± SE of four replicate populations (triangles and circles represent chromosome and plasmid encoded *mer* respectively. Area shaded in grey shows the sub-MIC selective window.

Chapter Three

Plasmid stability is enhanced by higher-frequency pulses of positive selection[2]

3.1. Introduction

Conjugative plasmids are extrachromosomal genetic elements that, alongside the genes required for their own replication, maintenance and transfer (Norman et al., 2009), carry cargos of accessory genes encoding functional traits. Common plasmid-encoded accessory traits include resistance to toxins, virulence factors, and metabolic capabilities (Frost et al., 2005). By transferring ecologically important functional traits within and between bacterial lineages and species, plasmids can accelerate bacterial adaptation (Ochman et al., 2000). Therefore, the dynamics and stability of conjugative plasmids in bacterial populations have potentially important implications for understanding bacterial evolution (Jain et al., 2003; Thomas and Nielsen, 2005). Nevertheless, it remains challenging to explain the long-term stability of plasmids. This is because plasmid maintenance is frequently costly for the bacterial host cell (Baltrus, 2013). Although such costs may be outweighed by the benefits of plasmid-encoded functional traits in some environments (Hall et al., 2015), theory predicts that plasmids should be evolutionarily unstable whether parasitic (i.e. costs outweigh benefits) or mutualistic (i.e. benefits outweigh costs) (Bergstrom et al., 2000; Lili et al., 2007; Macken et al., 1994; Stewart and Levin, 1977). In the short term, parasitic plasmids are expected to decline in frequency due to negative selection, since observed rates of horizontal transmission appear too low to counteract this process (Bergstrom et al., 2000; Simonsen, 1991). While mutualistic plasmids can be temporarily favoured by positive selection for accessory gene functions, they are expected to decline in frequency over longer evolutionary timescales. This is because the useful accessory genes can be integrated into the chromosome, rendering the plasmid backbone dispensable. Thus, consistent positive selection for accessory genes should favour plasmid-free cells with the accessory traits on their chromosome,

[2] C Stevenson, JPJ Hall, MA Brockhurst, E Harrison 2018. Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Pro. R. Soc. B*. 285: 20172497

which outcompete plasmid-bearers who still pay the cost of plasmid carriage (Bergstrom et al., 2000; Hall et al., 2016; Svara and Rankin, 2011).

In both natural and clinical environments, plasmids are likely to experience temporally variable selection, resulting in fluctuating positive selection for the accessory genes they carry (Coutu et al., 2013; Marti et al., 2014; Schlüter, 2000). Recent theory suggests that temporally heterogeneous environments where plasmids experience pulsed positive selection may favour their maintenance through two non-mutually exclusive mechanisms (San Millan et al., 2014a; Svara and Rankin, 2011). Firstly, rare pulses of strong positive selection can theoretically promote the maintenance of conjugative plasmids carrying accessory gene functions. This occurs because plasmid-free cells outcompete both plasmid-bearers and cells with chromosomal accessory genes between bouts of positive selection, but only the plasmid-encoded copies of the accessory genes can conjugate into these plasmid-free cells. These plasmid-bearing transconjugant cells can then sweep to high frequency upon the next pulse of positive selection (Svara and Rankin, 2011). In contrast, where pulses of positive selection are frequent, the frequency of plasmid-free cells and thus the benefits of conjugation, are reduced. Therefore, under constant or high frequency pulses of positive selection, cells with chromosomal accessory genes are favoured at the expense of accessory genes encoded on the conjugative plasmid. Secondly, pulses of positive selection have been shown to promote compensatory evolution to ameliorate the cost of plasmid carriage thereby weakening negative selection against the plasmid-backbone. This occurs because positive selection temporarily increases the population size of plasmid-bearing cells thus increasing the probability that they will gain compensatory mutations (San Millan et al., 2014a). Compensatory evolution appears to be a fairly general mechanism by which plasmid survival is ensured, it has been observed in a range of bacteria-plasmid interactions (Harrison et al., 2015a; San Millan et al., 2014a; Yano et al., 2016) and across environments where the fitness effect of plasmid acquisition ranges from parasitic to mutualistic (Harrison et al., 2015a).

Here, we tested how the frequency of pulsed positive selection affected plasmid stability (i.e. the stable maintenance of the plasmid in the bacterial population). We experimentally evolved populations of *Pseudomonas fluorescens* SBW25 with the mercury resistance (Hg^R) plasmid pQBR103 (Harrison et al., 2015a) across a range of treatments varying in the frequency of exposure to toxic concentrations of mercuric ions (Hg^{2+}). Mercuric ions are normally lethal to the bacterial cell, binding to protein sulfhydryl groups and causing major cellular disruption (Boyd and Barkay, 2012). However, in this bacteria-plasmid system, pQBR103 encodes a Tn5042 transposon which in turn harbours a mercury-resistance operon, *mer*, that catalyses reduction of Hg^{2+} to a less toxic form Hg^0. Thus, while in the absence of Hg^{2+}, pQBR103 imposes a large

fitness cost on SBW25, at higher Hg^{2+} concentrations this fitness cost is offset by benefit of Hg^R (Hall et al., 2015; Harrison et al., 2015a). Populations were propagated under one of six treatments: in the absence of mercury, under constant mercury selection or pulsed mercury selection at varying time intervals (i.e. every 2, 4, 8 or 16 transfers). After 16 transfers of these selection regimes, all populations were propagated for a further 16 transfers in the absence of Hg^{2+} to test the effect of prior evolution under the varying frequencies of pulsed positive selection on longer-term plasmid stability. Throughout the experiment we tracked plasmid prevalence and the frequency of phenotypes associated with a previously described mechanism of compensatory evolution in this bacteria-plasmid interaction.

3.2 Materials and methods

3.2.1 Strains and culture conditions

Experiments used *P. fluorescens* SBW25 (Rainey and Bailey, 1996) differentially marked with either gentamicin resistance (Gm^R) or streptomycin resistance + lacZ (Sm^RlacZ) cassettes (Hall et al., 2015; Lambertsen et al., 2004) allowing them to be distinguished on selective agar plates as previously described (Hall et al., 2015; Harrison et al., 2015a). pQBR103 was conjugated into the Gm^R background using standard methods (Harrison et al., 2015a; Simonsen et al., 1990). All experiments were conducted in 6ml KB broth in 30ml microcosms shaking at 180rpm and incubated at 28°C. The carrying capacity of KB microcosms is approximately 1 x 10^{10} CFU/ml, Supplementary Information, Figure 3.4.

3.2.2 Selection experiment

Independent overnight cultures of plasmid-bearing, mercury resistant (Hg^R) and plasmid-free, mercury sensitive (Hg^S) strains were mixed at a 1:1 ratio and 60µl (~10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Six replicate populations were established for each mercury treatment. Populations were propagated by 1% serial transfer every 48 hours for a total of 32 transfers. Two 'constant' treatments were established with either 0 or 40 µM $HgCl_2$ added at each transfer. In the four pulsed treatments, populations were grown without mercury except for 40 µM $HgCl_2$ added every 2, 4, 8 or 16 transfers. After 16 transfers addition of $HgCl_2$ was stopped and all populations were propagated in 0 µM $HgCl_2$ for a further 16 transfers to measure plasmid stability in the absence of selection. Every two transfers population densities of each marker background were determined by diluting and plating onto KB agar supplemented with 50 µg/ml

X-gal and 5% powdered milk solution. In addition, frequency of the Hg^R phenotype was determined by selective plating onto KB agar supplemented with 40 µM $HgCl_2$ and 50 µg/ml X-gal and 5% milk. The addition of milk powder allowed us to determine the frequency of *gacA/gacS* mutants (Gac^-) in the populations. Previously it was shown that loss of function mutation to the *gacA/gacS* bacterial regulatory system is the main mechanism of compensatory evolution in this system ameliorating the cost of pQBR103 carriage in *P. fluorescens* SBW25 (Harrison et al., 2015a). The gacA/gacS system positively regulates expression of an extracellular protease allowing colonies of wild-type Gac^+ SBW25 to digest a halo zone of clearing around the colony on milk plates (Cheng et al., 2013), allowing Gac^+ phenotypes to be easily distinguished from Gac^- mutants, which cannot form the halo. The frequency of transconjugants was determined by scoring Sm^RlacZ marked cells that grew on Hg^{2+} plates, forming a blue colony. To check that Hg^R colonies were unlikely to have arisen by mutation, we quantified the frequency of spontaneous Hg^R mutations against 40 µM Hg^{2+}, using the fluctuation test assay protocol described in ref. (MacLean and Buckling, 2009). We never detected any spontaneous Hg^R mutants strongly suggesting mercury resistance requires the *mer* operon, and could not have evolved *de novo* in our experiments.

At the end of the experiment 24 Hg^R clones from each population were isolated and colony PCR was used to test whether the plasmid was still present or whether it was lost following chromosomal acquisition of the resistance genes. PCRs targeted *oriV* (for: 5'-TGCCTAATCGTGTGTAATGTC -3' and rev: 5'- ACTCTGGCCTGCAAGTTTC -3') to determine presence of the plasmid backbone and *merA* (for: 5'-TGAAAGACACCCCCTATTGGAC – 3') and rev: 3'- TTCGGCGACCAGCTTGATGAAC-3') to determine presence of the *mer* operon.

3.2.3 Statistical analysis

All analyses were conducted in R statistical package version 3.1.3 (R Development Core Team, 2016). Packages used were 'nlme' and 'userfriendlyscience'. For all analyses of Hg^R plasmid dynamics the mercury-free treatment was removed so that mercury treatments were compared to one another. Comparisons across the mercury pulsed treatments looking at average prevalence of Hg^R, average proportion of transconjugants, proportion of Gac^- phenotypes at T_{16}, time to first Gac^- mutant, and average Gac^- frequency over time were analysed using Welch's ANOVA with mercury treatment as a fixed effect to adjust for non-homogenous variance across treatments. Comparisons of Gac^- dynamics across plasmid-bearing and plasmid-free populations were analysed using Welch's ANOVA with presence of plasmid as a fixed effect. Maintenance of Hg^R

over time between T_{16} and T_{32} was analysed using linear mixed effects models with mercury treatment and time as fixed effects, and random effects of population on intercept and slope to account for repeated sampling of populations through time. Fixed effects were assessed using Likelihood Ratio Tests on nested models.

3.3 Results

3.3.1 Hg^R plasmid dynamics varied between mercury treatments.

Populations were propagated for 16 transfers either without mercury, with mercury addition every transfer (constant mercury) or in pulsed treatments where mercury exposure occurred at varying time intervals (i.e. every 2, 4, 8 or 16 transfers), and the frequency of Hg^R was measured every second transfer. In all treatments where Hg^R was detected, PCR analysis on endpoint clones revealed that Hg^R remained associated with the plasmid (i.e. we did not detect any mutants which had acquired chromosomal *mer* and lost the plasmid backbone). In the mercury-free treatment, Hg^R cells harbouring pQBR103 were rapidly outcompeted by plasmid-free Hg^S cells, as expected based on the known fitness cost associated with carrying pQBR103 (Harrison et al., 2015a) (Figure 3.1). By contrast, under constant mercury selection Hg^R was maintained at high prevalence in all populations. During the first 16 transfers Hg^R prevalence varied across pulsed treatments, such that mean prevalence averaged over time was significantly higher under more frequent pulses (Supplementary Information, Figure 3.5; $_{\text{EFFECT OF MERCURY TREATMENT:}}$ $F_{4,25} = 55.77$, $p < 0.001$).

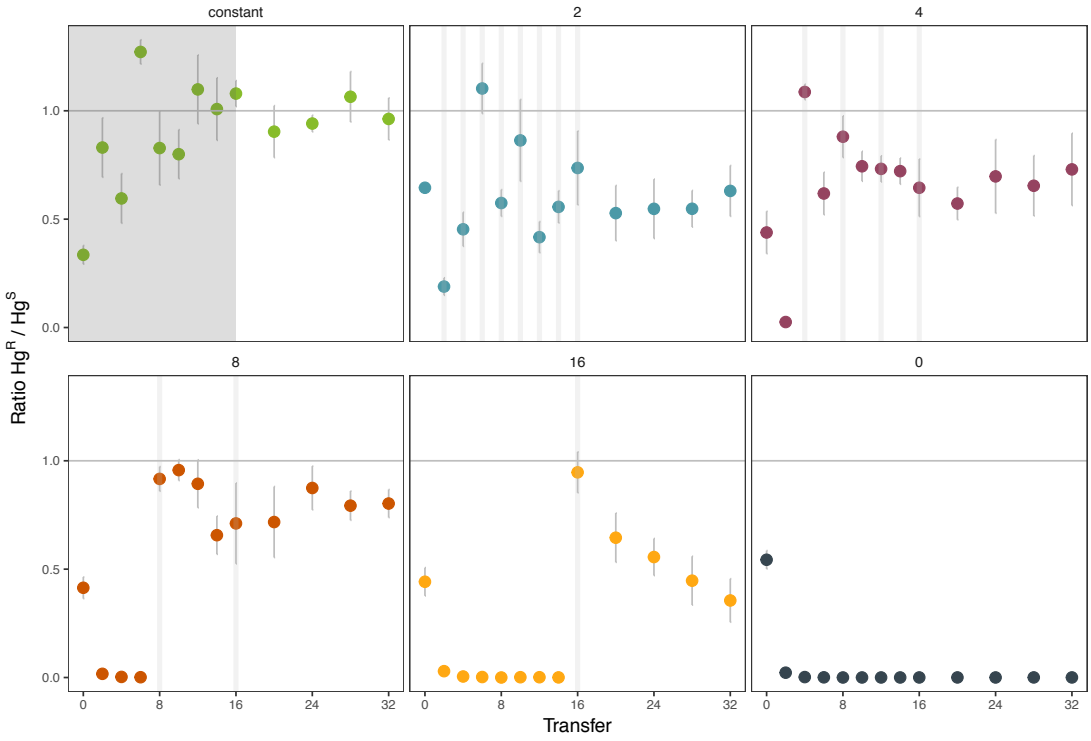

Figure 3.1 Pulses of mercury selection maintain pQBR103. The proportion of Hg^R (ratio of Hg^R counts over Hg^S counts) was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Points represent means ± standard errors of six replicate populations. Colours represent each pulsed mercury treatment.

In all pulsed mercury treatments, plasmid prevalence declined prior to the initial mercury pulse. However, in all cases, a single mercury pulse was sufficient to sweep Hg^R to high frequencies, such that by transfer 16, by which time every pulsed treatment had experienced at least 1 mercury pulse, Hg^R was at high frequency in all populations and did not differ significantly between pulsed treatments ($_{EFFECT\ OF\ MERCURY\ TREATMENT;}$ $F_{4,25} = 1.77$, $p = 0.166$). The increase in Hg^R frequency was particularly striking in populations from the treatment with the lowest frequency of mercury pulse (i.e. single pulse at T_{16}) where, prior to the pulse, Hg^R was virtually undetectable (Figure 3.1). Together these results demonstrate across the first 16 transfers, that higher frequency pulses of positive selection favoured high plasmid prevalence but also that even rare positive selection events could boost plasmid persistence, at least in the short term.

3.3.2 Compensatory evolution occurred across all mercury treatments.

We screened the Hg^R fraction of each population to determine the presence of phenotypes associated with compensatory evolution. In this bacteria-plasmid interaction we have previously described a mechanism of compensatory evolution associated with the loss of function in the bacterial *gacA/gacS* two-component regulator (Harrison et al., 2015a). The *gacA/gacS* system is encoded by the bacterial chromosome and controls the expression of genes involved in a broad range of biological functions including secondary metabolism, virulence and motility (Bull et al., 2001; Cheng et al., 2013). Addition of milk powder to agar plates allowed us to screen for Gac^- phenotypes: cells carrying *gacA/gacS* compensatory mutations were unable to produce the extracellular proteases capable of digesting milk. We therefore used this phenotype to compare the frequency of Gac^- phenotypes between treatments. Gac^- phenotypes arose in both plasmid-bearing and plasmid-free cells (shown in Figure 3.2 and Supplementary Information, Figure 3.6, respectively). This is not necessarily surprising given that *gacA/gacS* loci are known to have an elevated mutation rate relative to the genome as a whole (van den Broek et al., 2005). Among the plasmid-bearers we found that Gac^- phenotypes appeared rapidly in all mercury treatments and were maintained for the duration of the experiment (Figure 3.2). This was not observed in plasmid-free control populations (Supplementary Information, Figure 3.6), where Gac^- phenotypes appeared later ($_{\text{PLASMID-BEARING VS. PLASMID-FREE:}}$ $F_{1,10} = 62.8$, $P < 0.001$), and remained at significantly lower frequency ($_{\text{PLASMID-BEARING VS. PLASMID-FREE:}}$ $F_{1,10} = 17.06$, $P = 0.002$). This is consistent with our previous data showing that deletion of *gacA/gacS* was only beneficial in cells with the pQBR103 plasmid, but had no significant fitness effects in plasmid-free SBW25 (Harrison et al., 2015a). Within plasmid-containing treatments there was no significant effect of mercury treatment on Gac^- frequency in the plasmid-bearing population over the selective period of the experiment (i.e. averaged over transfer 1-16) ($_{\text{EFFECT OF MERCURY TREATMENT:}}$ $F_{5,30} = 1.99$, $p = 0.108$) or the proportion Gac^- mutants at T_{16} ($_{\text{EFFECT OF MERCURY TREATMENT:}}$ $F_{4,25} = 0.99$, $P = 0.433$) suggesting that amelioration of the plasmid cost was strongly favoured across all conditions regardless of mercury exposure (Harrison et al., 2015a). Furthermore there was no significant effect of mercury treatment on time taken for Gac^- mutants to arise: Gac^- phenotypes arose rapidly across all the plasmid-bearing populations ($_{\text{EFFECT OF MERCURY:}}$ $F_{5,30} = 0.74$, $p = 0.598$).

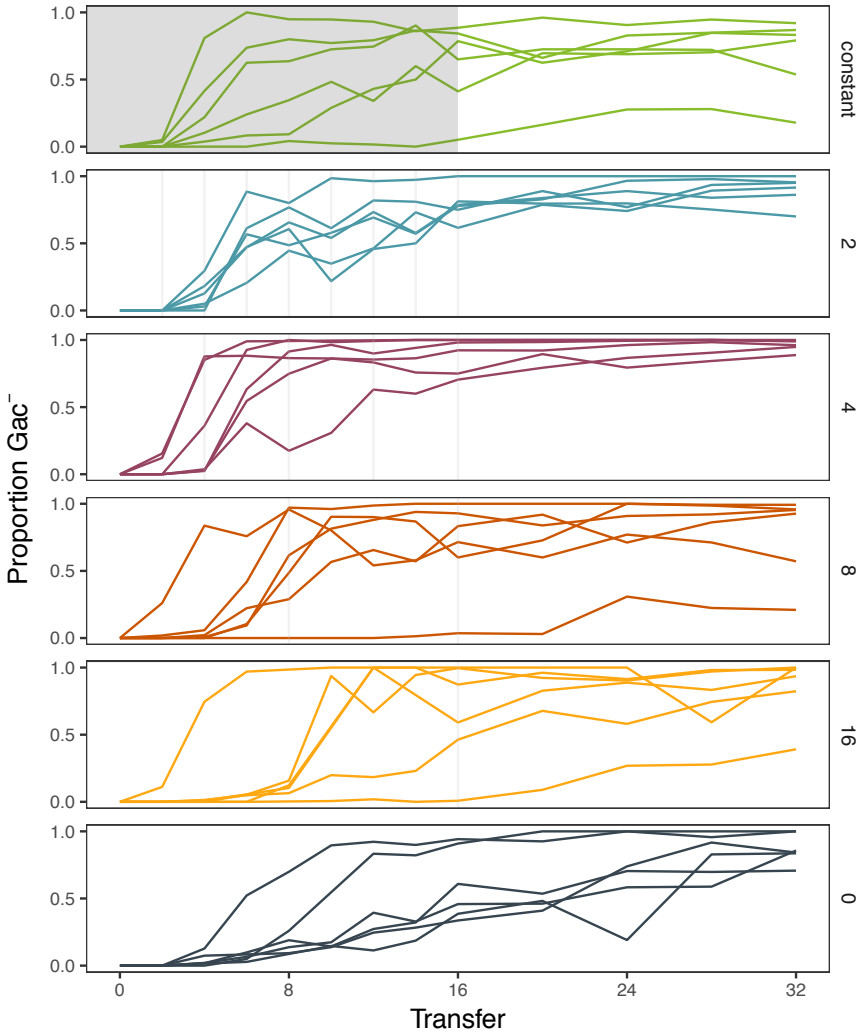

Figure 3.2 Gac mutations sweep through all Hg^R populations regardless of selective regime. The proportion of Gac^- phenotypes within the Hg^R population was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Lines represent the six replicate populations. Colours represent each pulsed mercury treatment.

3.3.3 Infrequent pulses promoted plasmid transfer into Hg^S recipients.

Theory suggests that longer intervals between pulses of selection may favour conjugative plasmid transfer (Svara and Rankin, 2011). This occurs by allowing the survival and propagation of plasmid-free Hg^S bacteria which can then act as recipient hosts for the plasmid (Stevenson et al., 2017). The frequency of transconjugants across each population revealed that the level of conjugative plasmid transfer significantly increased with decreasing frequency of pulsed mercury selection (Figure 3.3; data for individual replicate populations shown in Supplementary Information, Figure 3.8; EFFECT OF MERCURY TREATMENT: $F_{4,25} = 7.19$, $p = 0.001$). This is likely to have

been driven by frequent mercury pulses reducing the frequency of plasmid-free cells (Supplementary Information, Figure 3.4), whereas less frequent mercury pulses allowed plasmid-free cells to rise to high frequency, allowing greater opportunity for conjugation from the remaining plasmid-bearing cells. Therefore, in treatments with rare pulses of positive selection, conjugation indeed appears to play a larger role in the persistence of Hg^R within populations.

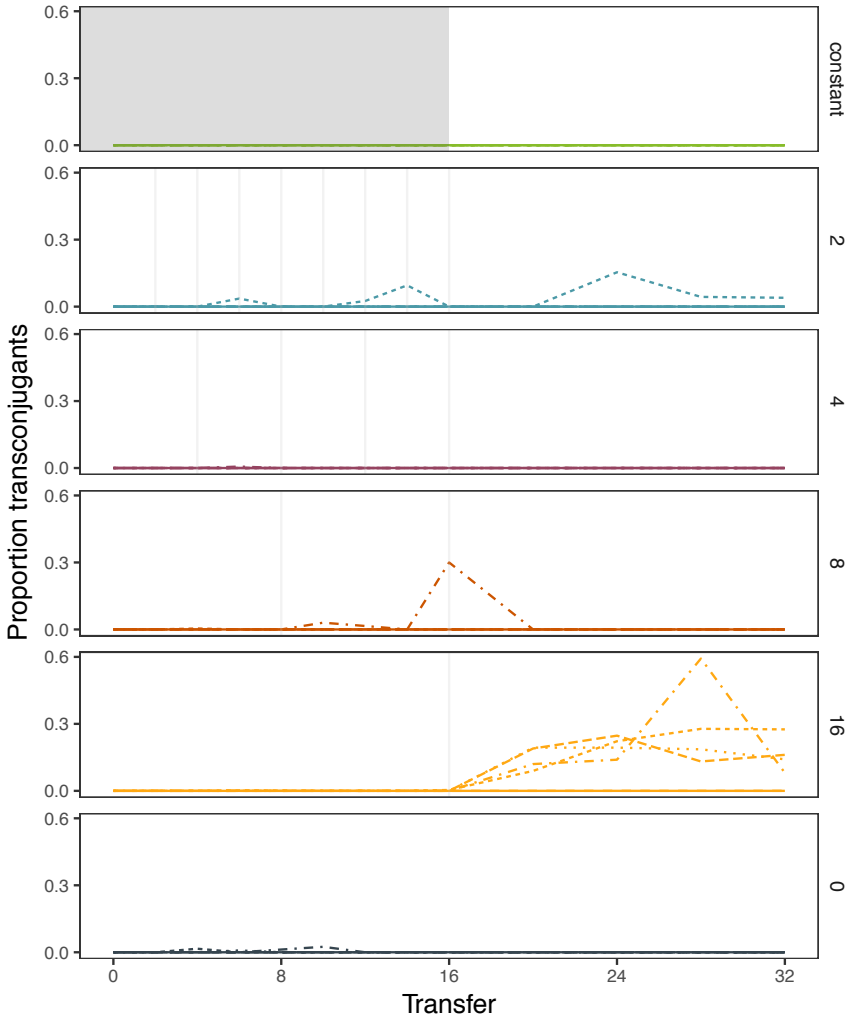

Figure 3.3 Infrequent pulses promote plasmid transfer into Hg^S recipients. The proportion of transconjugants within the Hg^R population was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Lines represent the six replicate populations. Colours represent each pulsed mercury treatment.

3.3.4 High frequency pulses stabilised Hg^R plasmids over the longer term.

After T_{16}, all populations were propagated without mercury, to test how adaptation to the various selection regimes had affected plasmid stability in the absence of positive selection. Hg^R stability varied according to the past frequency of pulsed positive selection (Figure 3.1; TIME X MERCURY TREATMENT: χ^2 (4) = 13.92, P = 0.0076). Comparisons revealed that this effect was largely driven by the populations subjected to a single mercury pulse at T_{16} (b = -0.0327, t (114) = -2.63, p = 0.0096) where Hg^R steadily declined over time in the absence of mercury selection, whereas Hg^R was stable in populations from all of the other pulsed mercury treatments.

3.4 Discussion

Understanding the conditions that favour the stability of conjugative plasmids is important for understanding bacterial evolution (Bergstrom et al., 2000; Harrison and Brockhurst, 2012; Lili et al., 2007; Stewart and Levin, 1977; Svara and Rankin, 2011). Most experimental studies of plasmid stability have used constant environmental conditions, yet in nature, bacteria inhabit environments that are likely to be temporally variable with pulses of positive selection for plasmid-borne traits (Coutu et al., 2013; Marti et al., 2014; Schlüter et al., 1993). While there have been theoretical studies of the impact of pulsed positive selection on conjugative plasmid stability (Svara and Rankin, 2011), there have been few experimental tests (however see (San Millan et al., 2014a) and (Starikova et al., 2012) for studies on non-conjugative plasmids and integrases respectively) . Here, we show short-term and longer-term effects of the frequency of pulsed positive selection on the stability of a mercury resistance plasmid. In the short-term, constant or frequent pulses of positive selection allowed plasmids to be maintained at higher prevalence, but even in treatments where the plasmid had declined to undetectable levels, the first pulse of positive selection was sufficient to sweep the plasmid to high prevalence. Surprisingly, the high plasmid prevalence observed under frequent pulses did not appear to affect the rate of compensatory evolution via loss of function mutations to the *gacA/gacS* pathway (Harrison et al., 2015a), which arose in all mercury environments. In the longer term however, plasmids that only experienced a single pulse of positive selection did appear to be at a disadvantage: following the removal of positive selection, plasmids evolved under high frequency or constant positive selection remained at high prevalence, whereas plasmids evolved under the lowest frequency of positive selection declined.

Previous theoretical analysis of plasmid stability predicted that horizontally-transferable, plasmid-encoded resistance would be favoured over chromosomally-encoded resistance by rare pulses of strong positive selection (Svara and Rankin, 2011). This is predicted to occur because plasmid-free cells, which pay no cost of carrying the resistance gene, can outcompete both plasmid-encoded and chromosomally-encoded resistant genotypes in the intervals between pulses of positive selection. While this leads to the loss of chromosomal resistance, plasmid-encoded resistance can transfer by conjugation into the population of plasmid-free cells, and these transconjugants may then sweep to high frequency following the next pulse of positive selection (Svara and Rankin, 2011). Although we did not observe the emergence of chromosomally-encoded resistance in our study, even though this outcome is possible in our experimental system (Harrison et al., 2015a), we did observe the out-competition of plasmid-bearers by plasmid-free cells during long intervals between infrequent pulses of positive selection. Moreover, consistent with the prediction of the model (Svara and Rankin, 2011), under the lowest frequency of pulsed positive selection we observed a significantly higher proportion of transconjugant cells during the experiment, suggesting that conjugation played a more important role in the persistence of the plasmid where positive selection was rarest. This is consistent with previous work which demonstrated that conjugation played a larger role in the maintenance of the Hg^R plasmid pQBR57 in the absence, rather than presence, of positive mercury selection (Stevenson et al., 2017). The balance of vertical versus horizontal transmission of genes determines population genomic structure and thus the evolutionary potential of populations to changing environmental conditions (Shapiro, 2016). As plasmids can spread to a wide range of hosts (Klümper et al., 2015), our finding that infrequent pulses of positive selection favoured horizontal transfer via conjugation suggests that we may expect to observe functional genes in a broader range of bacterial species when positive selection is a rare event (Stevenson et al., 2017; Wiedenbeck and Cohan, 2011).

Contrary to our prediction, based on recent theory and experimental data (San Millan et al., 2014a), we did not observe higher rates of compensatory evolution (via loss of function mutation to the *gacA/gacS* pathway) under higher-frequency pulsed positive selection even though such environments did support higher plasmid prevalence. By contrast we observed that compensatory phenotypes evolved rapidly and rose to high frequency among plasmid-bearers across all our mercury environments. Compensatory evolution in this bacteria-plasmid interaction is associated with loss of function in the *gacA/gacS* two-component regulatory system (Harrison et al., 2015a), which activates the expression of a wide range of secondary metabolism and secreted products (Bull et al., 2001; Cheng et al., 2013). Consistent with our findings here, it was previously found that Gac^- mutants arose in parallel across a wide range of mercury concentrations, suggesting that

neither the strength or the frequency of positive selection has a major effect on the process of compensatory evolution in this system (Harrison et al., 2015a). A potential explanation for this widespread prevalence of compensatory evolution across the range of positive selective environments, is that *gacA/gacS* appear to be contingency loci in *P. fluorescens* (Moxon et al., 1994), i.e. loci with an elevated mutation rate relative to the rest of the genome (van den Broek et al., 2005). Consequently the abundant supply of compensatory mutations in this system may obscure any effect of the frequency or strength of positive selection. It is likely that loss of *gacA/gacS* function may be detrimental in more complex, natural environments, where the suite of genes activated within the gac regulon perform important functions, notably associated with host colonisation and interspecific competition including the production of toxins and antibiotics (Bull et al., 2001; Cheng et al., 2013). Under such conditions, where expression of the Gac regulon is advantageous, the bacteria-plasmid assemblage would be forced to find alternative mechanisms of amelioration, and the frequency of pulsed positive selection may have a stronger effect on the rate of compensatory evolution.

Interestingly, we observed contrasting longer-term effects of the history of positive selection on the fate of plasmids following removal of positive selection. Unlike plasmids evolved under high frequency pulses of positive selection, plasmids evolved under the lowest frequency of pulsed positive selection, declined in prevalence in mercury-free environments. This cannot be explained by a lack of compensatory evolution (via loss of function mutation to the *gacA/gacS* pathway), since we observed compensatory phenotypes at high frequency among plasmid-bearers in all mercury selection environments. At present we do not know the evolutionary mechanism driving this effect. However, one possibility is that where plasmids have very recently swept from very low (in some cases undetectable) frequency, these lineages may be poorly adapted compared to the plasmid-free cells. This could arise because, until the recent pulse of mercury selection, the plasmid-free lineage had been at far higher population density than the plasmid-bearers and therefore had access to a higher mutational supply allowing them greater opportunity to adapt to the abiotic environment (Arjan et al., 1999).

Pulsed positive selection is likely to be a common feature of both environmental contamination and clinical antibiotic treatments, such that positive selection for plasmid-encoded traits is likely to be temporally heterogeneous (Coutu et al., 2013; Marti et al., 2014; Schlüter, 2000). Our findings suggest that this is likely to have both short-term and longer-term effects on plasmid stability. High-frequency pulsed positive selection increases plasmid prevalence and promotes the longer-term survival of plasmids in bacterial populations in the absence of positive selection, whereas low frequency pulsed positive selection increases the importance of horizontal gene

transfer and may lead to plasmid-encoded functional genes spreading into, and subsequently being selected in, a greater diversity of bacterial hosts. Crucially we show how even very rare periods of positive selection can be sufficient to sweep plasmids from undetectable levels to high frequency. Thus plasmids need not be present at high frequency to have an impact on bacterial evolution in temporally heterogeneous environments, because even vanishingly rare plasmids can enhance the responsiveness of bacterial populations to changing and uncertain conditions (Heuer and Smalla, 2012).

3.5 Supplementary Information

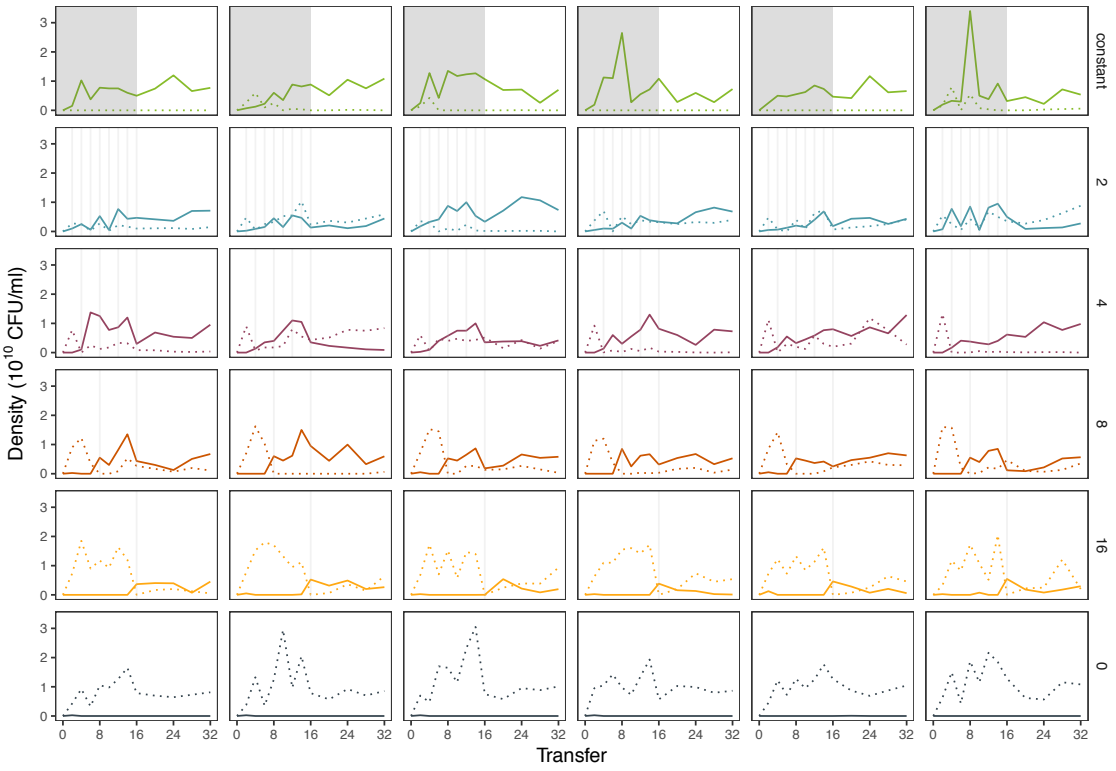

Figure 3.4 Donors and recipients across replicate populations. The densities of plasmid-bearing and plasmid- free cells were plotted for each replicate population across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Solid lines indicate donors, dotted lines indicate recipients. Colours represent each pulsed mercury treatment.

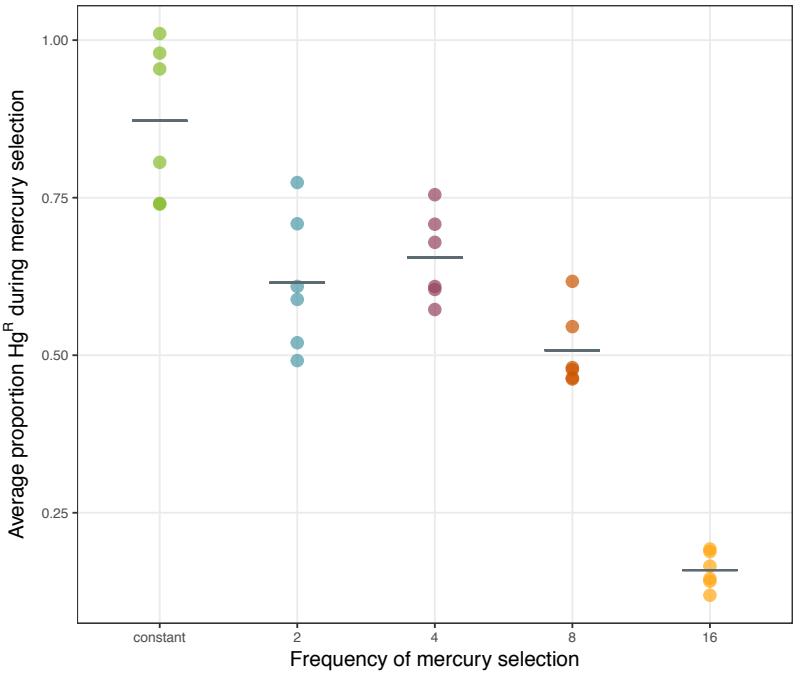

Figure 3.5 Hg^R abundance varies across mercury treatments. Average proportion of Hg^R (ratio of Hg^R counts over Hg^S counts) across the mercury selective period (i.e. first 16 transfers) was plotted for each mercury treatment. Points represent each replicate population and the grey line represents the mean (n = 6). Colours represent each pulsed mercury treatment. Post-hoc pairwise comparisons revealed that Hg^R abundance was affected by the frequency of pulsed mercury with constant pulsing resulting in significantly higher abundance than the less frequently pulsed treatments ($p = 0.011$ and $p = 0.001$ for comparisons with treatments 8 and 16 respectively) and infrequent pulsing (i.e. singular pulse at T_{16}) resulting in significantly lower abundance than the other treatments ($p = 0.003$, $p < 0.01$, $p < 0.01$ for comparisons with 2, 4 and 8 respectively.

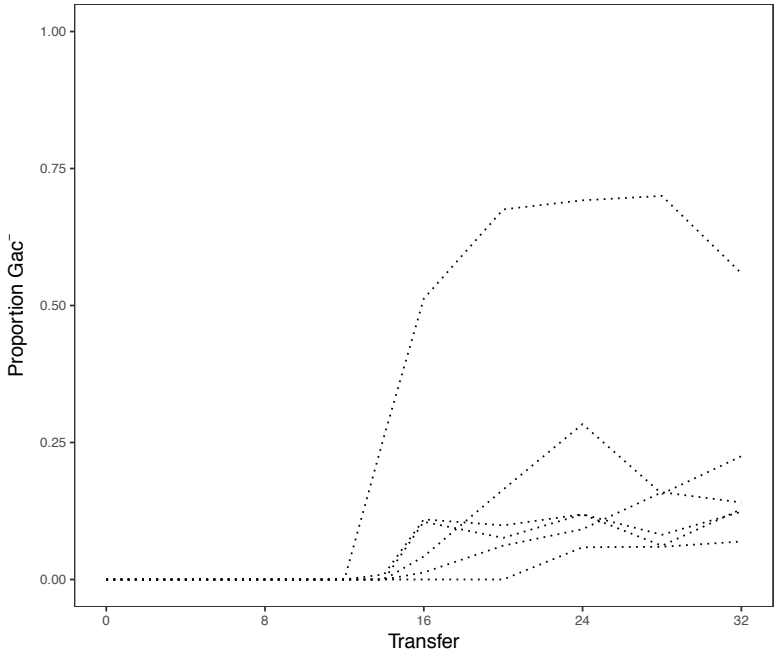

Figure 3.6 Gac- phenotypes arise in plasmid- free control populations. The proportion of Gac- phenotypes was plotted for each replicate population within the plasmid-free control populations (n = 6).

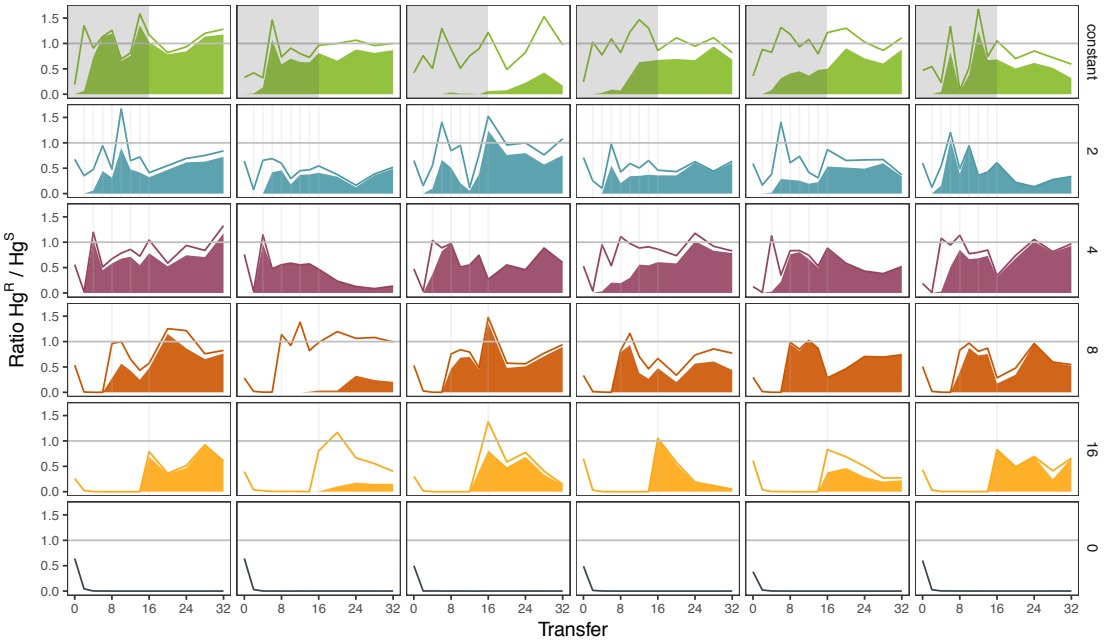

Figure 3.7 Gac mutations are prevalent in Hg^R populations across all selective regimes. The proportion of Hg^R (ratio of Hg^R counts over Hg^S counts) was plotted for each replicate population across the six selection treatments (line). Shaded region represents prevalence of Gac^- phenotypes within each Hg^R population. Grey bars indicate transfers where mercury was applied. Colours represent each pulsed mercury treatment.

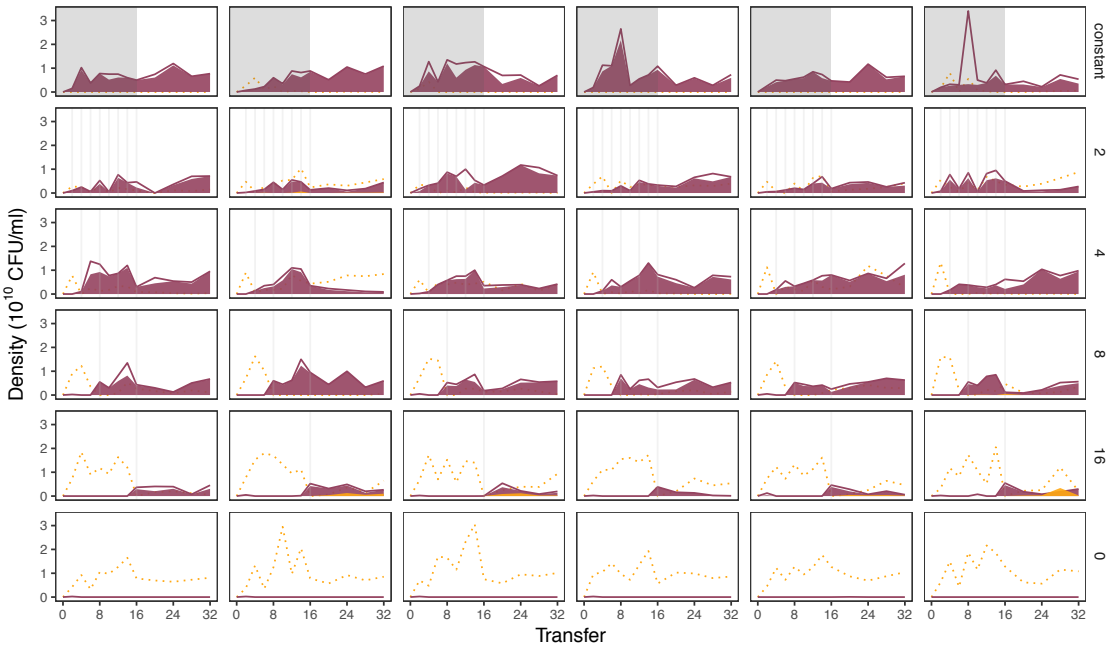

Figure 3.8 Plasmid transfer into donor and recipient populations. The densities of plasmid-bearing and plasmid-free cells was plotted for each replicate population across the six selection treatments (solid lines indicate donors, and dotted lines indicate recipients). The shaded region represents presence of Hg^R within each donor and recipient population (coloured with purple and yellow, respectively).

Chapter 4

Bacteriophages alter the persistence of mobile- and non-mobile resistance genes in the absence of positive selection

4.1 Introduction

Horizontal gene transfer (HGT) is a key process in bacterial evolution, driving the spread of ecologically important traits in bacterial populations (Frost et al., 2005). As agents of HGT, conjugative plasmids are particularly important as they can transmit genes across both species and genus boundaries (Frost et al., 2005; Norman et al., 2009; Thomas and Nielsen, 2005). The mobility of genes via horizontal gene transfer is expected to expand the selective conditions under which genes can spread in bacterial populations. Whereas non-mobile genes replicate by vertical inheritance alone (i.e. by clonal expansion), mobile genes, such as those encoded on conjugative plasmids, can also replicate by horizontal transmission (Shapiro, 2016). Unlike non-mobile genes, this allows mobile genes to spread even in the absence of positive selection (Hall et al., 2017a; Lopatkin et al., 2017; Stevenson et al., 2017). Because plasmids often encode 'accessory' genes which are of potential use to their bacterial host (Rankin et al., 2011), plasmid dynamics have important consequences for the ecology and evolution of bacteria. The transmission of plasmids depends on the costs and benefits of plasmid-carriage and the rates of plasmid loss and conjugation (Bergstrom et al., 2000; Harrison and Brockhurst, 2012). In environments where the benefits outweigh the costs, plasmids are expected to persist at near fixation due to positive selection unless the useful genes are transferred to the host chromosome, making the plasmid itself surplus to requirements, and resulting in its subsequent loss from the population (Dionisio et al., 2005; Heuer et al., 2007). On the other hand, in the absence of positive selection, plasmids are expected to be purged by purifying selection due to their fitness costs (Subbiah et al., 2011), unless plasmid decline is counteracted by sufficient conjugative horizontal transmission (Lundquist and Levin, 1986; Stevenson et al., 2017) or through the amelioration of plasmid cost (Harrison and Brockhurst, 2012; Harrison et al., 2015a; San Millan et al., 2014a).

A key selective force acting on bacteria in natural communities is their predation by lytic phages (Weinbauer, 2004). Phages are highly abundant in microbial populations (Clokie et al., 2011), outnumbering bacteria by as much as 100-to-1 (Weinbauer, 2004). Phages are a major source of bacterial mortality and therefore impose strong selection on bacterial populations (Koskella, 2013; Koskella and Brockhurst, 2014; Vos et al., 2009), driving selective sweeps of phage resistance mutations (Buckling and Rainey, 2002). Through their impact on bacterial populations, it is likely therefore that phages may impact the dynamics of the plasmids carried by bacteria. In the absence of positive selection, phages can limit the persistence of plasmids by reducing bacterial population size thereby reducing the opportunity for plasmid conjugation (Harrison et al., 2015b). In addition, selective sweeps for phage resistance mutations can purge bacterial populations of genetic diversity leading to the loss of plasmids if resistance mutations happen to occur in the plasmid-free class (Harrison et al., 2015b).

Using experimental evolution, we investigated how the lytic bacteriophage phi-2 (Buckling and Rainey, 2002) affected the dynamics of mobile or non-mobile mercury-resistance genes in the absence of positive selection in populations of the soil bacterium *Pseudomonas fluorescens* SBW25 (Rainey and Bailey, 1996). Mercury-resistance genes were either encoded on the chromosome (non-mobile) (Stevenson et al., 2017), or were encoded on one of two mega-plasmids that varied in their rate of conjugative horizontal transmission: pQBR103 has a relatively low conjugation rate whereas pQBR57 has a relatively high conjugation rate (Hall et al., 2015). Experimentally, the frequency of the mercury-resistance (Hg^R) was tracked over time in populations propagated with or without phages. We then used an individual-based model (IBM) simulation to explore how phage presence affected the parameter space in which plasmids could persist by exploring a range of costs and conjugation rates.

4.2 Materials and methods

4.2.1 Strains and culture conditions

Experiments were conducted using *Pseudomonas fluorescens* SBW25 (Rainey and Bailey, 1996) differentially marked with either gentamicin resistance (Gm^R) or streptomycin resistance + lacZ (Sm^RlacZ) cassettes (Hall et al., 2015; Lambertsen et al., 2004) allowing them to be distinguished on selective agar plates as previously described (Hall et al., 2015; Harrison et al., 2015a). Plasmids pQBR103 and pQBR57 were conjugated into the Gm^R background using standard methods (Harrison et al., 2015a; Simonsen et al., 1990). All experiments were conducted in 6ml KB broth

in 30ml microcosms shaking at 180rpm and incubated at 28°C. The carrying capacity of KB microcosms is approximately 1 x 10^{10} CFU/ml (Stevenson et al., 2018).

4.2.2 Selection experiment

We had a fully factorial experimental design with 4 levels of Hg^R mobility (the Hg^R transposon encoded on the SBW25-GM^R chromosome, on plasmid pQBR103, plasmid pQBR57 or absent) and two phage treatments (either present or absent). Independent overnight cultures of mercury-resistant (Hg^R) and mercury-sensitive (HgS) strains were mixed at a 1:1 ratio and 60µl of the bacteria culture (~10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Six replicate populations were established for each of the eight treatments. Phage-containing treatments were inoculated with a 6µl of a 10^{11} phage/ml stock solution at the beginning of the experiment. Populations were propagated by 1% serial transfer every 48 hours for a total of 12 transfers. Every 4 transfers population densities of each marker background were determined by diluting and plating onto KB agar supplemented with 50 µg/ml X-gal. In addition, frequency of the Hg^R phenotype (either plasmid or chromosomally-encoded) was determined by replica plating the agar plates onto KB agar supplemented with 100µM $HgCl_2$ and 50 µg/ml X-gal. The frequency of trans-conjugants was determined by scoring Sm^RlacZ marked cells that grew on the Hg^{2+} replica plates, forming a blue colony.

4.2.3 Competitive fitness assays

Independent overnight cultures of Gm^R-Hg^R and Sm^R-Hg^S were mixed at a 1:1 ratio and 60µl (~10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Three replicate populations were established for each of the four Hg^R ancestors (chromosomal, pQBR103, pQBR57 and Hg^S) along with 12 replicate populations for the Sm^R competitor. Population numbers were determined by diluting and plating onto KB agar + 50µg ml^{-1} X-gal at 0 and 24 hours. Fitness was estimated from these counts as the ratio of Malthusian parameters ($W = (\ln(test_{end}/test_{start})/(\ln(reference_{end}/reference_{start}))$)(Lenski et al., 1991). Based on known estimates of the conjugation rate of pQBR57 in *P. fluorescens* (Hall et al., 2015) conjugation of the plasmid into recipient cells is likely to be minimal across the 24 hours (transconjugants make up ~5% of the total population) and thus conjugation has little impact on the fitness estimates.

4.2.4 Conjugation rates

Measurement of conjugation rate (y) was according to (Simonsen et al., 1990): $\gamma = \Psi \ln(1 + (T/R)(N/D))(1/(N - N_0)$, where Ψ is the population growth rate (h^{-1}), N_0 the starting cell density (ml^{-1}) and T, R, D and N the end point densities (ml^{-1}) of transconjugants, recipients, donors and the total population respectively. Conjugations were started with a 1:1 mixture of plasmid-bearing *P. fluorescens* SBW25-Gm^Rdonor and plasmid-free *P. fluorescens* SBW25-Sm^R *lacZ* recipient and grown under conditions similar to the competitive fitness assays. KB agar containing appropriate antibiotics and Hg(II) (20 μM) were used to select plasmid-bearing recipients.

4.2.5 Individual-based model simulations

The IBM simulations were performed by our collaborator Professor Calvin Dytham at the University of York, and I performed the data visualisation from the model output. C.D modified an existing individual-based model (Harrison et al., 2015b, 2016) to simulate the evolutionary dynamics for this bacteria-plasmid-phage system allowing us to explore a wider parameter space for cost and conjugation rate than in the original model. The IBM was designed to test how the spread of Hg^R was affected by the presence and absence of phage across a range of conjugation and cost parameter space. The simulations were parameterised with empirical data where possible with parameters taken from (Harrison et al., 2015b) (see Supplementary Information Table 4.1). As described in Harrison *et al*. (2015b), populations are modelled in continuous time (Allen and Dytham, 2009), with individuals randomly chosen and subjected to one of the following four possible events, chosen at random: death, conjugation and cell division, with or without an additional event of phage encounter. After each event, time was moved forward by a short incremental step (for n bacteria, time increments are drawn from a negative exponential distribution with a mean of $1/4n$). Where an event does not occur, time was still moved forward incrementally. When the time increments summed to 1 (known as a timestep), data was collected and recorded. The probability of cell death was 0.1 per event, resulting in a 0.1 probability of death (per timestep) if the cell, on average, was called for one death event in a timestep. On conjugation, a second bacterium was selected at random. If one bacterium carried the plasmid and the other did not there was a probability (varying between 0 – 0.4) that the plasmid-free cell would gain the plasmid. Fission occurred using a logistic probability of n/K for plasmid-free cells and $x(n/K)$ for plasmid-bearing cells with x varying between 0.4 – 1 to represent the range of plasmid costs. The derived cell will retain the plasmid status of their parent, but there is a 0.0001 probability of segregation leading to loss of the plasmid. Encounters with phage occurred at a variable probability. In these simulations K is fixed at 1000000. To simulate experimental transfers, every 8 timesteps the population experiences a 1% bottleneck, where 99% of the

population drawn at random, dies. After '12 timesteps' the output from one realisation of the model was recorded across the entire cost/conjugation parameter space, and proportion Hg^R in the population was calculated from this realisation.

4.2.6 Statistical analysis

All analyses were conducted in R statistical package version 3.4.3 (R Development Core Team, 2016). HgR dynamics were analysed using a linear mixed effects model (LME) using R package 'nlme' with phage presence, transfer and Hg^R mobility as fixed effects, with random effects of population on intercept and slope to account for repeated sampling of populations through time. Endpoint differences in proportion Hg^R were analysed using an ANOVA with phage presence and Hg^R mobility as fixed effects. For post-hoc comparisons of endpoint proportion, the R package 'emmeans' was used to obtain estimated marginal means on the ANOVA model.

4.3 Results

4.3.1 Bacteriophages affect the dynamics of mobile and non-mobile mercury-resistance genes.

Using experimental evolution, we investigated the effect of phage predation on the dynamics of horizontally mobile (plasmid-encoded) and non-mobile (chromosomally-encoded) mercury-resistance (Hg^R) genes in populations of the soil bacterium *Pseudomonas fluorescens*. Specifically, we tracked the dynamics of Hg^R, harboured on either the bacterial host chromosome (non-mobile), or on mega-plasmids pQBR103 (low mobility), or pQBR57 (high mobility) with or without the bacteriophage phi-2 in the absence of positive selection for the plasmid.

The impact of phi-2 on the spread of mercury-resistance (Hg^R) differed through time depending on the level of Hg^R gene mobility (Figure 4.1: $_{\text{HGR TYPE x PHAGE x TRANSFER;}}$ $F_{2, 102} = 7.82$, $p = 0.0007$). Post-hoc comparisons revealed that at transfer 12, in the absence of phages, there was a significant difference in the proportion of pQBR57-encoded Hg^R compared to both chromosomal ($_{\text{PQBR57 VERSUS CHROMOSOME;}}$ $p = 0.004$) and pQBR103-encoded Hg^R ($_{\text{PQBR57 VERSUS PQBR103;}}$ $p < 0.0001$). This was driven by the fact that in the absence of phages, pQBR57 swept to fixation, whereas the less conjugative and more costly plasmid pQBR103 was lost from the population, and chromosomal Hg^R, which was neither costly nor beneficial under these experimental conditions (Supplementary Information, Figure 4.3, (Stevenson et al., 2017), was maintained at an intermediate level (Figure 4.1). In the presence of phages, at least half the replicate populations declined in each Hg^R

treatment demonstrating the negative effect of phi-2 on the maintenance of Hg^R. Post-hoc comparisons revealed that at transfer 12, in the presence of phages, chromosomal Hg^R was significantly greater than pQBR103 ($_{CHROMOSOME\ VS\ PQBR103:}$ $p = 0.0209$), due to the maintenance of chromosomal-Hg^R in three replicates, but the decline of pQBR103-Hg^R in all six replicates. There was no significant difference between chromosomal and pQBR57-Hg^R ($_{PQBR57\ VERSUS\ CHROMOSOME;}$ $p = 0.7323$) as both modes of Hg^R exhibited variable frequencies of Hg^R among replicates: Hg^R was lost in half chromosomal and pQBR57-Hg^R replicate populations (3/6), while persisting at intermediate Hg^R frequencies in the other 3 replicates per treatment. There was no difference between the frequency of Hg^R in populations with pQBR57 or pQBR103 ($_{PQBR57\ VERSUS\ PQBR103;}$ $p = 0.293$).

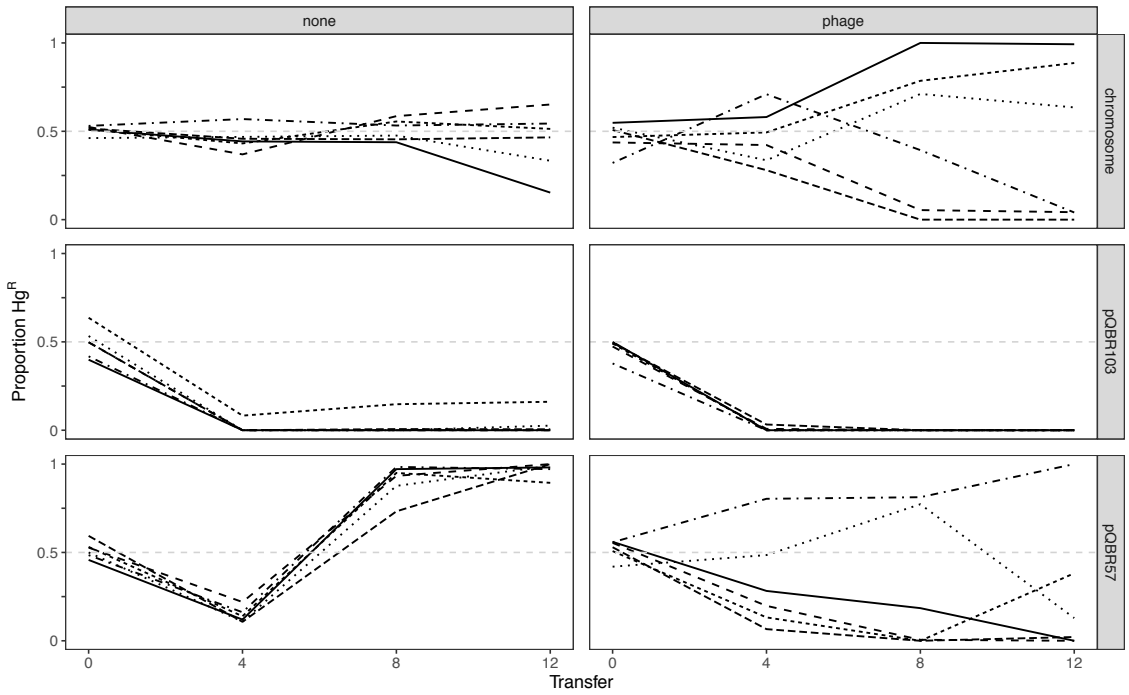

Figure 4.1 Presence of phi-2 altered the spread of Hg^R in populations of *P. fluorescens*. The proportion of chromosome and plasmid-encoded Hg^R cells were determined over time in the presence and absence of phi-2. Lines represent each replicate population (n = 6).

4.3.2 Phages alter the parameter space where Hg^R can persist in bacterial populations

We hypothesised that the different effects of phages on Hg^R dynamics between the modes of Hg^R were driven by differences in their cost and mobility. To explore this, and generalise our results we modified an existing IBM (Harrison et al., 2015b) to test how cost and mobility interacted to determine Hg^R frequency, and how this was affected by phage predation. Table 4.1 lists the

parameters in the model. Relative growth rates (determined using competitive fitness assay data) (Supplementary Information, Figure 4.3), and conjugation rates for the two plasmids (Supplementary Information, Figure 4.4) were determined from this data. The points in parameter space corresponding to the various modes of Hg^R are as follows: chromosomal Hg^R, conjugation = 0, cost = 0; pQBR103, conjugation = 0.0008, cost = 0.55; pQB57, conjugation = 0.4, cost = 0.14.

Figure 4.2 shows Hg^R prevalence from the IBM simulations after an iteration period of approximately 12 transfers, corresponding to the duration of the experiments. In both the absence and presence of phages Hg^R prevalence increased with increasing conjugation rate, and decreasing fitness cost (Figure 4.2, filled squares). Phages decreased the parameter space in which Hg^R could persist: in the presence of phages higher conjugation rates and lower fitness costs were required to allow plasmid persistence than were necessary in the absence of phages. For pQBR103, which has low conjugation (estimated at 0.0008), and high cost (estimated at 0.55), the plasmid sits well within the model parameter space where no Hg^R can persist, both in the absence and presence of phages (Figure 4.2, empty squares), and therefore we predict its loss under both conditions. This confirmed experimental data, which showed loss across almost all replicate populations both with and without phages. For pQBR57 which has high conjugation (estimated at 0.4) and low cost (estimated at 0.14), the addition of phages moved the plasmid from occupying parameter space where plasmid maintenance was greatly expected (Figure 4.2, filled squares depicting 50% Hg^R frequency), towards occupying parameter space where plasmid loss was more likely (i.e. Figure 4.2, filled squares depicting 10% Hg^R frequency). This confirmed experimental data where the sweeps of pQBR57 to near-fixation that occurred in the absence of phages, were severely limited in the presence of phages, with the plasmid persisting at low frequencies in the majority of replicate populations. Finally, for chromosomal Hg^R with no conjugation, and undetectable cost, phages again moved the parameter space from a high likelihood of Hg^R maintenance (50% Hg^R frequency) towards where Hg^R loss was more likely (10% Hg^R frequency), confirming experimental data which demonstrated maintenance of chromosomal-Hg^R in the absence of phages, but significant loss in the presence of phages. Generally, these findings suggest that phages limit the parameter space where mobile genes can persist, but that increased levels of conjugation, or lower costs can increase the likelihood of a traits survival.

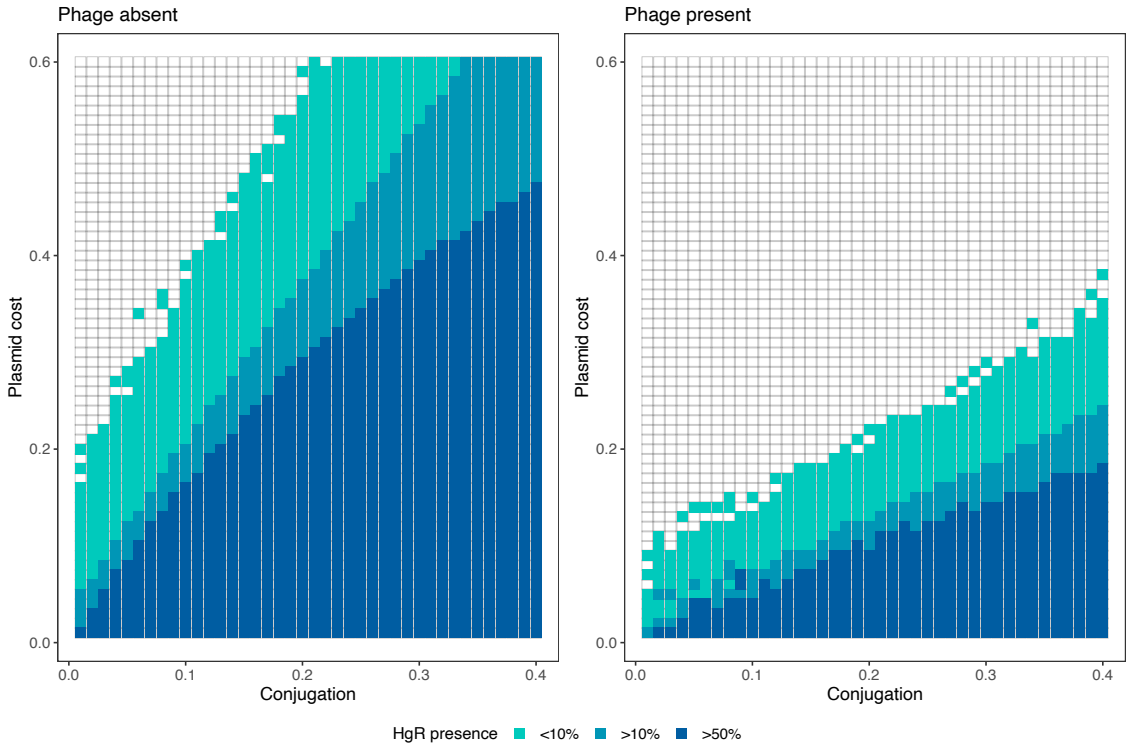

Figure 4.2 The dynamics of Hg^R taken from the IBM simulation. Each squares depicts the output from one realisation of the model after 12 'timesteps' (i.e. corresponding to 12 experimental transfers) across the cost/conjugation parameter space. Fill squares highlight where Hg^R was detected in <10% (light-blue), >10% (mid-blue), >50% (dark-blue) of the population. Plots (a) and (b) correspond to without phage pressure and with phage pressure respectively. The points in parameter space corresponding to the various modes of Hg^R are as follows: chromosomal Hg^R, conjugation = 0, cost = 0; pQBR103, conjugation = 0.0008, cost = 0.55; pQB57, conjugation = 0.4, cost = 0.14.

4.4 Discussion

Previous work showed that phages limited the existence conditions for conjugative plasmids (Harrison et al., 2015b). Here, we extend this to look at the persistence of Hg^R across three levels of gene mobility (non-mobile, mobile, and highly mobile). Using a combination of experiments and modelling we confirm that phages limited the persistence of Hg^R in a bacteria population compared to the non-phage treatments. In the presence of phages, plasmid-encoded Hg^R was only able to survive when rates of conjugation were sufficiently high and costs sufficiently low. This suggests that in natural environments, where phages and bacteria co-exist, we might expect selection for plasmids to evolve increasingly lower costs and higher conjugation rates.

A prediction from our findings is that under phage pressure, plasmids should evolve high conjugation rates to ensure their stable maintenance. Plasmids have been shown to have high conjugation rates (Dahlberg et al., 1998; Hall et al., 2015; Lopatkin et al., 2017). However, plasmid conjugation rates vary in natural communities (Dionisio et al., 2002; Gordon, 1991), with many plasmids displaying low conjugation rates, or foregoing the ability to conjugate completely (San Millan et al., 2014b; Smillie et al., 2010). This is perhaps surprising given the ubiquity of phages in natural communities (Clokie et al., 2011). One factor to consider is that conjugation itself imposes a cost on the host cell as the host has to invest resources into the formation of pili and replication of the plasmid (Turner et al., 1998). Indeed, experimentally it has been shown that plasmids that evolve high conjugation rates impose a greater fitness cost on their host (Turner et al., 1998). Therefore, given that phages reduce the cost parameter space where plasmids can persist, these increased costs of high conjugation may in fact limit the evolution of greater conjugation rates.

A second prediction from our findings is that the host or plasmid should attempt to reduce the costs of plasmid carriage. One mechanism to do this is for the host or plasmid to undergo compensatory evolution to alleviate the costs of plasmid carriage, allowing for plasmid persistence in bacterial populations (Harrison and Brockhurst, 2012). Mechanisms for compensation have been shown to target genes on the host (Harrison et al., 2015a; San Millan et al., 2014a), and the plasmid (Yano et al., 2016), suggesting that compensation may be a common strategy to promote plasmid stability. Therefore in communities with phages present, we might expect compensation to be more widespread. Where compensation is incorporated into this model, we might predict that plasmids would persist under a greater range of conjugation rates, alleviating the need for such high rates of conjugative transfer to evolve. Previous modelling work has looked at the interplay between compensatory evolution and conjugation rates on plasmid stability within a population (Hall et al., 2017b). Here, they found that once the population had ameliorated the costs of the plasmid, the need for high conjugation rates was reduced, and plasmid conjugation deceased over time (Hall et al., 2017b). However, previous experimental work has demonstrated that phages may limit the trajectory of compensatory evolution within the host (Harrison et al., 2017): here the method for compensation, was curtailed as bacteria evolved mucoidy in response to phage, which then limited the mutations which could arise in the *gacA/gacS* global regulator (Harrison et al., 2015a). This suggests that in systems where phage resistance mutations and compensatory mutations interact, increased conjugation may be the preferred strategy. A further way to alleviate plasmid associated costs completely is for the trait to move onto the host chromosome, allowing the disposal of the plasmid backbone entirely. Although we were able to show that Hg^R persisted at high frequency in half the replicate

populations bearing chromosomal-Hg^R, the fact that it was reduced to very low frequencies in the other half, suggests that this was perhaps not a stable mechanism for maintenance of the trait.

Plasmid host range may be a further factor affecting survival in the presence of phages. Phages appear to be specialists, able to infect only a small subset of bacterial species (Flores et al., 2011; Koskella and Meaden, 2013; Weinbauer, 2004). This is likely to be because of the costs associated with infecting a broader range of bacterial hosts (Poullain et al., 2007). Under these circumstances, plasmids with a broader host range, may be better equipped to survive in communities harbouring phages. This is because a broad host range allows the plasmid to move into diverse hosts, which are less likely to succumb to phage attack. Experimentally, plasmids have been shown to increase their host range (Loftie-Eaton et al., 2016), enabling their survival within a wider community. Within the natural world, broad host range (BHR) plasmids have been isolated from numerous environments including: soil (Heuer and Smalla, 2012; Klümper et al., 2015), freshwater (Brown et al., 2013) and wastewater (Schlüter et al., 2007). Despite this, plasmid spread may be limited as plasmids may be more likely to transfer into kin (Dimitriu et al., 2019). In addition, plasmid persistence within a wider community may be linked to its persistence in a key donor species (Hall et al., 2016). If this key species is susceptible to phages, this will increase the likelihood of plasmid loss from the entire community.

Previous work has shown that plasmid-encoded traits expand the selective conditions under which a gene may spread, allowing functional traits to spread in the absence of positive selection (Stevenson et al., 2017). Here we show that the presence of lytic phages limits this spread, resulting in plasmid loss in the majority of the populations. These findings suggest that even highly conjugative plasmids may not be able to sustain themselves through infectious transmission in bacterial communities subject to phage pressure in the absence of positive selection. In these cases, intermittent positive selection (Stevenson et al., 2018) for plasmid-borne traits may be the missing component to ultimately ensure the long-term survival of conjugative plasmids.

4.5 Supplementary Information

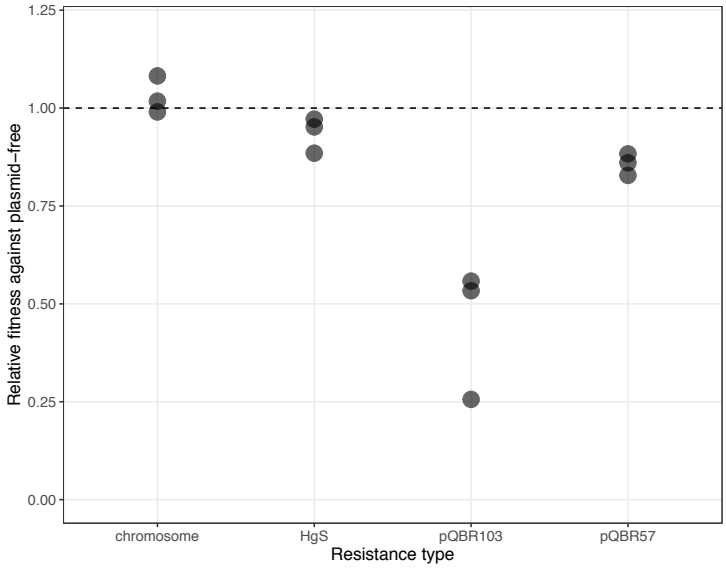

Figure 4.3 Relative fitness measures. The relative fitness of the four Hg^R ancestors (chromosomal, pQBR103, pQBR57 and Hg^S) was determined against a plasmid-free ancestor. A relative fitness of 1 indicates equal competitive fitness.

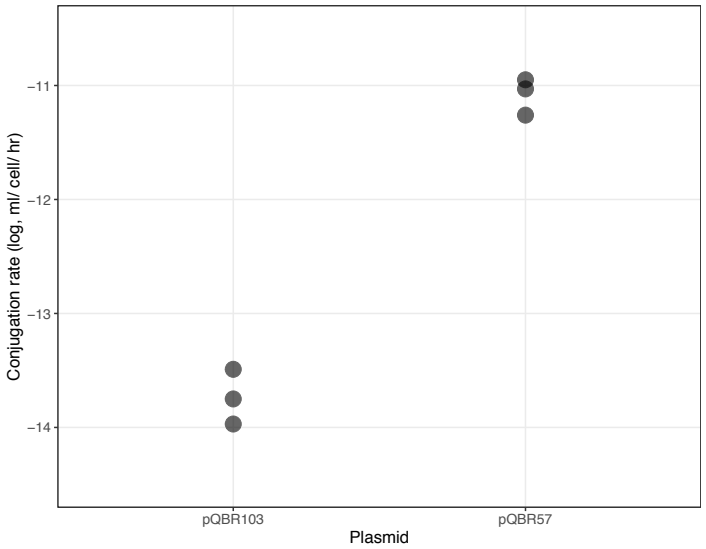

Figure 4.4 Conjugation rates. The conjugation rate (log, ml/ cell/ hour) was determined for plasmids pQBR57 and pQBR103 for three independent replicate populations.

Parameter	Variable measured	Value	Source
α	Growth rate of a plasmid-free cell	1 h^{-1}	Relative growth rates representing the cost of the plasmid in the plasmid-containing clones (see materials and methods)
β	Growth rate of a plasmid-carrying cell with pQBR57	0.86 h^{-1}	Estimated from this system using competitve fitness assays (see materials and methods)
$\beta 1$	Growth rate of a plasmid-carrying cell with pQBR103	0.45 h^{-1}	Estimated from this system using competitve fitness assays (see materials and methods)
$\beta 2$	Growth rate of a chromosomal-mer carrying cell	1 h^{-1}	Estimated from this system using competitve fitness assays (see materials and methods)
$[1 - (\mu_{bg}/\alpha)]\kappa$	Population carrying capacity	5×10^6 cells/ml	
γ	Conjugation rate of the pQBR103	2×10^{-14} $cell^{-1}h^{-1}$	Estimated within this system following the standard methods of Simonsen (1990) (see materials and methods)
$\gamma 1$	Conjugation rate of pQBR57	1×10^{-11} $cell^{-1}h^{-1}$	Estimated within this system following the standard methods of Simonsen (1990) (see materials and methods)
μ_{bg}	Background mortality rate	0.1 h^{-1}	Taken from (Harrison 2015b)
δ	Segregation rate	10^{-4} h^{-1}	Taken from (Harrison 2015b)
μ	Mortality due to phage, plus background mortality	0.7 h^{-1}	Taken from (Harrison 2015b)
η	Mortality due to mercury	0.3 h^{-1}	Taken from (Harrison 2015b)

Table 4.1 Parameter values for the Individual Based Model.

Chapter Five

Community evolutionary rescue via horizontal gene transfer

5.1 Introduction

Deleterious environmental change could threaten species survival unless populations can rapidly adapt to avoid extinction (Maynard Smith, 1989). Cases of successful adaptive evolution to survive lethal environmental change have been defined as evolutionary rescue (ER) events (Gomulkiewicz and Holt, 1995; Gonzalez et al., 2013). One of the most widespread examples of ER in bacteria is the repeated evolution of antibiotic resistance in bacterial populations exposed to the widespread use of antibiotics both in clinical and agricultural practice (Andersson and Hughes, 2012; Levin et al., 1997; Orr and Unckless, 2008; Ramsayer et al., 2013). Population genetic theory predicts that species are more likely to undergo successful ER where populations are sufficiently large (Samani and Bell, 2010), have elevated mutational supply or contain adaptive standing genetic variation (Agashe et al., 2011). Each of these factors increase the amount of genetic variation available to selection, thereby increasing the likelihood ER. In addition, prior exposure to the relevant environmental stressor(s) is predicted to enable ER, by increasing the likelihood that the population will contain adaptive genetic variants (Bell and Gonzalez, 2011; Gonzalez and Bell, 2012). Finally, the likelihood of ER is also expected to depend on the costs, benefits and availability of adaptive mutations (Kawecki, 2008; Willi et al., 2006). ER is more likely where adaptation can be achieved through one or a few mutations, but less likely if it requires complex adaptations or relies upon very costly mutations that are unlikely to persist without positive selection.

In bacterial populations, adaptive genetic variation can arise either through spontaneous mutation of existing genes (zur Wiesch et al., 2011) or through the acquisition of new genes encoded by horizontal gene transfer, a process facilitated by mobile genetic elements, such as conjugative plasmids (Halary et al., 2010; Norman et al., 2009). HGT is likely to have several important consequences for the ER of bacterial populations (Cordero and Polz, 2014; Maynard Smith et al., 1993; Shapiro, 2016). First, HGT is likely to increase the supply of genetic variation available to

selection compared to that available through spontaneous mutation alone. Moreover, acquisition of genetic variation through HGT is likely to occur more rapidly than waiting for rare spontaneous mutations to arise, potentially accelerating adaptation (Jain et al., 2003) and thereby increasing the probability that adaptation will occur fast enough to out-pace environmental change. Second, HGT can provide bacteria with ready-made complex multi-gene adaptations instantaneously, which are unlikely to arise rapidly though spontaneous mutation alone (Hülter et al., 2017). Previously, studies have shown ER by HGT in single species bacterial populations exposed to lethal levels of an antibiotic (Ojala et al., 2014): Antibiotic-resistance genes were horizontally transferred from a resistant donor to a sensitive recipient enabling their survival in high concentrations of antibiotic.

Many plasmids have broad host ranges, extending across diverse bacterial groups (Klümper et al., 2015), and permitting HGT of resistance genes across species and genus boundaries (Norman et al., 2009). Unlike ER via spontaneous chromosomal mutation(s), it is possible therefore that plasmid-mediated HGT could enable the ER of multiple host species in a diverse bacterial community, allowing the maintenance of diversity. Community evolutionary rescue (CR) occurs when a community comprising of multiple species, exhibits a rapid eco-evolutionary response to environmental stress that would have been lethal to the original population, resulting in the recovery of a viable community (Fussmann and Gonzalez, 2013; Low-Décarie et al., 2015). The resultant community may then differ from the original with changes to species diversity and abundance. Experimental work has shown that community rescue was promoted by species diversity, prior exposure to the stress and dispersal (Low-Décarie et al., 2015), but the consequences of HGT on CR remain under-explored.

We predicted that HGT would enable CR upon a lethal environmental deterioration by allowing the spread of adaptive genes into a wider range of the bacterial community. Using experimental evolution, we first tested the effect of gene mobility on ER of a simple two-species soil bacterial community: Populations of *Pseudomonas putida* KT2440 were co-cultured with *Pseudomonas fluorescens* SBW25 harbouring non-mobile (i.e. chromosomal) or mobile (i.e. plasmid-encoded) mercury-resistance (Hg^R), with or without weak Hg^{2+} selection in soil; after 10 serial transfers all experimental communities were exposed to a lethal pulse of high concentration Hg^{2+}. We then tested the effect of gene mobility on CR of a diverse bacterial community containing 96 soil bacterial isolates following a similar experimental regime. Bacterial density and the frequency of Hg^R was tracked over time, and the composition and diversity of bacterial communities were quantified prior to, during and after the mercury-pulse using either selective plating for the two-species communities or amplicon sequencing of the 16S rRNA gene taxonomic marker for diverse

communities. Consistent with our predictions we found that mobile resistance genes enabled community evolutionary rescue by horizontal gene transfer.

5.2 Materials and Methods

5.2.1 Strains and culture conditions

For the two-species bacterial community, experimental populations were founded using *P. fluorescens* SBW25 (detailed in (Stevenson et al., 2017)) and *P. putida* KT2440 (detailed in (Hall et al., 2016)) labelled with gentamicin or streptomycin resistance markers using the mini-Tn7 transposon system (Hall et al., 2016; Lambertsen et al., 2004; Stevenson et al., 2017). This allowed us to distinguish between strains in mixed culture when plated onto Kings Medium B agar (King et al., 1954) (10 g glycerol, 20g proteose peptone no. 3, 1.5g K2HPO4•3H2O, 1.5g MgSO4•7H2O, per litre) containing species-specific antibiotics. *P. fluorescens* harboured Hg^R either on the chromosome, or on the conjugative plasmid pQBR57 (Hall et al., 2015; Stevenson et al., 2017). Individual colonies of each strain (one for each replicate) were isolated on selective KB agar and grown overnight at 28 degrees in 6ml KB broth in a shaking incubator. Samples were re-suspended in M9 buffer and mixed in equal volumes, diluted 1:10 with M9 buffer, and 100µl used to initiate the selection experiment. Populations were cultured in soil microcosms consisting of 10 grams twice autoclaved John Innes No.2 potting soil supplemented with 900µl of sterile H_2O, 900 µl of $HgCl_2$ solution (giving a final concentration of 16 or 64 µg/g) and maintained at 28 degrees at 80% relative humidity. Every 4 days, 10ml of M9 buffer and 20 glass beads (5-mm diameter) were added to each population and mixed by vortexing for 1 min, and 100 µl (1%) soil wash was transferred to fresh media. For the multi-species community, experimental populations were founded using *P. fluorescens* SBW25 as detailed above with an additional treatment harbouring plasmid pQBR103 (Hall et al., 2015). The soil community was generated by plating supernatant from unautoclaved John Innes No.2 potting soil onto nutrient agar. Plates were grown for 48 hours in a 28 degree centigrade incubator and 96 colonies were randomly picked. Colonies were screened against Hg^{2+} and Gm to make sure no phenotypic resistance to either of these toxins was pre-existing within the community population. This soil community was grown in overnight culture and each member mixed at an equal volume to make a 96-member community. An aliquot of the community and an equal volume of an overnight culture of *P. fluorescens* were re-suspended in M9 buffer, diluted 1:10 with M9 buffer, and 100µl used to initiate each experimental replicate. Populations were cultured by serial transfer as described above.

5.2.2 Evolution experiment

Populations were transferred every 4 days into fresh microcosms, that had either been pre-treated with selective levels of mercuric chloride (16 µg/g $HgCl_2$, or 64 µg/g $HgCl_2$) or with an equal volume of water (0 µg/g $HgCl_2$). Six replicate populations were established for each mercury treatment (0, 16 and 64). Populations were propagated by 1% serial transfer every 4 days for a total of 12 transfers. For the first 2 transfers, populations were propagated in the absence of mercury to allow populations to establish with mercury treatments commenced at transfer 3. After 10 transfers addition of $HgCl_2$ was stopped and all populations, and all populations were subjected to a mercury shock of 128 µg/g $HgCl_2$. Populations were then propagated in 0 µg/g $HgCl_2$ for the remaining two transfers. Throughout the experiment population densities of *P. putida* and *P. fluorescens* were determined by diluting and plating onto KB agar supplemented with species specific antibiotics (50 µg/ml streptomycin to screen for *P. putida* and 6 µg/ml gentamicin to screen for *P. fluorescens*). In addition, frequency of the Hg^R phenotype was determined plating the populations onto the species specific antibiotics specified above as well as 20µM $HgCl_2$. At the end of the experiment 24 Hg^R clones from each population where mercury resistance was plasmid encoded were isolated and colony PCR was used to test whether the plasmid was still present or whether it was lost following chromosomal acquisition of the resistance genes. PCRs targeted *oriV* (for: 5'- TGCCTAATCGTGTGTAATGTC -3' and rev: 5'- ACTCTGGCCTGCAAGTTTC -3') to determine presence of the plasmid backbone and *merA* (for: 5'- TGACCACACTGCGAATCCA -3' and rev: 3'- GGAGATTCCCTCCAGAGCATCT-3') to determine presence of the *mer* operon. To determine the extent to which populations grown in the absence of Hg^{2+} selection underwent population bottlenecks after the Hg^{2+} pulse, populations BA – BF isolated at transfer 10 (i.e. prior to receiving the Hg^{2+} pulse) were grown in KB media (3 replicate populations for each) for 30 hours and were then plated onto KB agar supplemented with species specific antibiotics ± 20µM $HgCl_2$ to test for the frequency of Hg^R in *P. fluorescens* and *P. putida*. For the community experiment populations were propagated by serial transfer every 4 days into fresh soil microcosms that were pre-treated with selective levels of mercuric chloride (16 µg/g $HgCl_2$) or with an equal volume of water (0 µg/g $HgCl_2$). Throughout the experiment population densities of *P. fluorescens* were determined by diluting and plating onto KB agar supplemented with 6 µg/ml gentamicin. In addition, frequency of the Hg^R phenotype was determined plating the populations onto nutrient agar + 20µM $HgCl_2$. In order to capture population diversity before and after the pulse, populations were frozen at T_{10}, T_{11} and T_{12} to in order to run downstream DNA extraction and sequence analysis.

5.2.3 16S rRNA gene sequencing and analysis

DNA was extracted from thawed stocks using the QIAGEN DNeasy PowerSoil kit according to the manufacturer's instructions with the exception that stocks were initially spun down and re-suspended in 1x M9 in order to remove glycerol. DNA concentrations were assessed using Qubit fluorometer 3.0 (Thermo Fisher Scientific) and diluted to 20ng μl^{-1} where possible before samples were sent for downstream PCR amplification of the v4 region of 16s rRNA gene and sequencing on the illumina MiSeq platform. The raw forward and reverse reads were merged and processed using QIIME1 (Caporaso et al., 2010). Reads were stripped of their primer and barcoding sequences using Cutadapt (Martin, 2011) and untrimmed reads were discarded. Reads were truncated at 254 bp (size of the amplicon). Reads were then dereplicated using Vsearch (Rognes et al., 2016) and clustered into operational taxonomic units (OTUs) using Usearch (Edgar, 2010) with 97% similarity. OTUs were then filtered based on OTUs which appeared in the positive control (14 OTUs in total). Putative taxonomic identification of the 14 OTU's was performed using BLAST (Altschul et al., 1990) to align the OTU sequence data to the NCBI nucleotide database, listed in Table 5.1.

OTU_ID	Species
OTU_3	Pseudomonas sp.
OTU_107	Pseudomonas fluorescens
OTU_167	Pseudomonas stuzeri
OTU_14	Pseudomonas umsongensis
OTU_9	Bacillus megaterium
OTU_19	Bacillus simplex
OTU_174	Pseudomonas sp.
OTU_131	Pseudomonas sp.
OTU_103	Pseudomonas sp.
OTU_148	Actinobacterium
OTU_309	Pseudomonas sp.
OTU_187	Pseudomonas sp.
OTU_166	Methylobacterium
OTU_239	Pseudomonas lini

Table 5.1 List of the 14 OTU's identified in the positive control. Putative taxonomic identification was performed using BLAST with the highest scoring hit for each OTU sequence listed here.

5.2.4 Statistical analysis

All analyses were conducted in R statistical package version 3.4.3 (R Development Core Team, 2016). Diversity in the two-species community was analysed at T_{11} using an ANOVA with mercury selection and Hg^R mobility as fixed effects. Diversity in the multi-species community was analysed using a linear mixed effects model (LME) using R package 'nlme' with mercury selection, transfer and Hg^R mobility as fixed effects, and random effects of population on intercept and slope to account for repeated sampling of populations through time. For both selection experiments, population densities during the selection period were analysed by sub-setting the data between T_3 and T_{10}, and running LME models with fixed and random effects as stated above. For all post-hoc comparisons the R package 'emmeans' was used to obtain estimated marginal means for the LME models. Principal component analysis on the OTU abundance was conducted using the 'prcomp' function in R. To test whether community structure varied significantly between the different populations, we conducted permutational multivariate analysis of variance using the 'adonis' function in the R package 'vegan' with mercury selection, transfer and Hg^R mobility as fixed effects.

5.3 Results

5.3.1: Evolutionary rescue in a two-species bacterial community

We first determined the effect of gene mobility on evolutionary rescue in a simple two-species community. Specifically, the Hg^{2+}-sensitive bacterium KT2240 was propagated alongside SBW25 encoding either non-mobile (i.e. chromosomal) or mobile (i.e. plasmid-encoded) Hg^R genes. Communities were evolved with or without prior Hg^{2+} selection for ten transfers before being subjected to a lethal Hg^{2+} pulse. Following the lethal pulse, diversity was significantly higher when Hg^R was mobile (Figure 5.1: $_{\text{MERCURY X HGR MOBILITY:}}$ $F_{2,30} = 53.79$, $p < 0.001$). This high diversity was driven by plasmid-mediated transfer of Hg^R from SBW25 into KT2440 (Figure 5.2), whereas KT2440 populations without access to the Hg^R plasmid could not evolve mercury resistance either through acquisition of the *mer* operon or *de novo* mutation (Figure 5.2). Mercury-selection increased the prevalence of Hg^R in SBW25 populations prior to receiving the lethal pulse at transfer 10 (Figure 5.2; $_{\text{MERCURY X MOBILITY x TRANSFER:}}$ $F_{4, 369} = 3.68$, $p = 0.0059$). This was also the case for KT2240 populations propagated alongside plasmid-bearing SBW25 (Figure 5.2; $_{\text{MERCURY X MOBILITY x TRANSFER:}}$ $F_{4, 369} = 2.76$, $p = 0.0275$). Without prior mercury-selection, plasmid-encoded Hg^R declined to ~10% prevalence in SBW25, and was rarely detectable in KT2440 (Figure 5.2). Surprisingly, however, despite almost undetectably low levels of plasmid carriage in the KT2440 population prior to the lethal Hg^{2+} pulse (~0.02%, Figure 5.3), this was still sufficient to allow subsequent recovery of the KT2440 population. Together these data confirm that HGT of Hg^R allowed the evolutionary rescue of a two-species bacterial community, and that this was facilitated by prior positive selection for Hg^R.

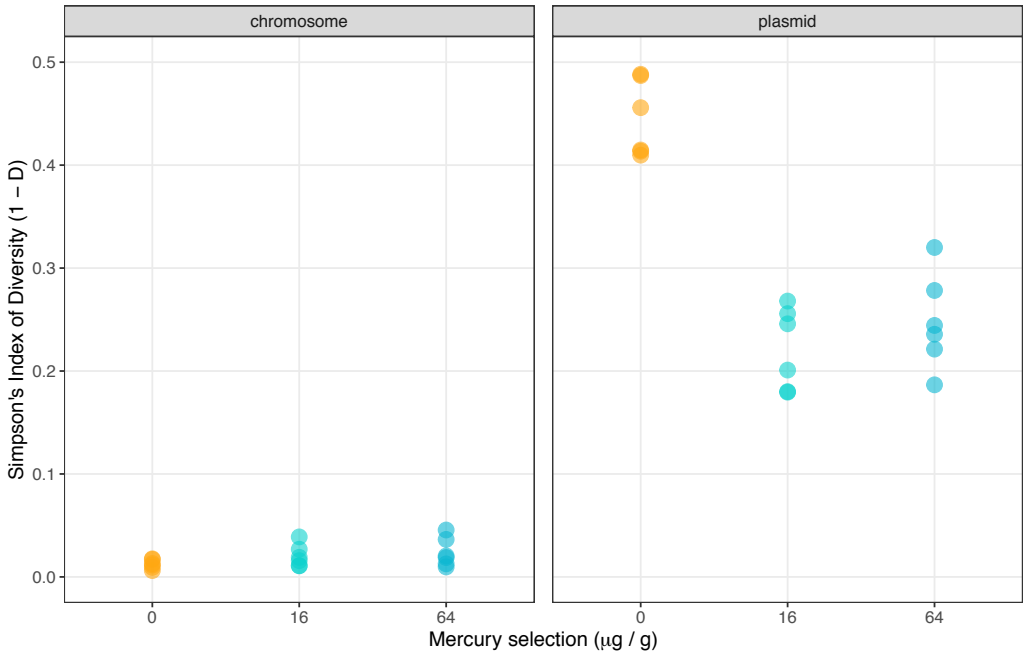

Figure 5.1 Community diversity post-pulse is higher in populations that contain plasmid-encoded Hg^R. Simpson's Index of Diversity (1-D) was calculated for communities at T_{11} after receiving the Hg^{2+} shock. Panels represent chromosomal and plasmid-encoded Hg^R respectively. Each panel shows, the diversity value for each of the six replicate populations across the three levels of mercury selection (0, 16 and 64 μg/g Hg^{2+}).

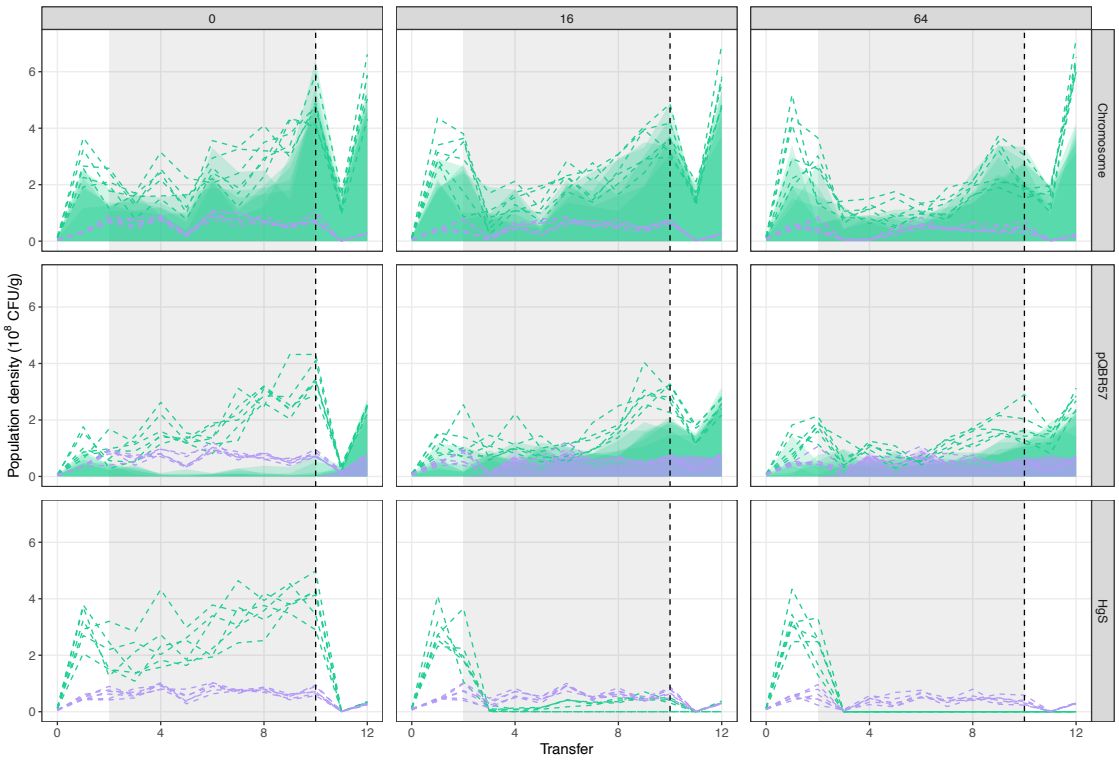

Figure 5.2 Selection maintains mobile Hg^R and allows horizontal transfer into recipient *P. putida*. Population densities of *P. fluorescens* and *P. putida* in co-culture were tracked over time. The upper row of subpanels shows where Hg^R was encoded on the chromosome of *P. fluorescens*. The middle row of subpanels shows where Hg^R was encoded on the plasmid pQBR57. The lower row of subpanels shows where there was no Hg^R present in the population. Each panel shows, for an individual population, the density of the *P. fluorescens* population (solid line), density of the *P. putida* population (dotted line) and the density of Hg^R within each respective population (shaded area under curve: blue and pink for *P. fluorescens* and *P. putida* respectively). Vertical panels represent the three mercury treatments (0, 16 and 64 µg/g Hg^{2+})

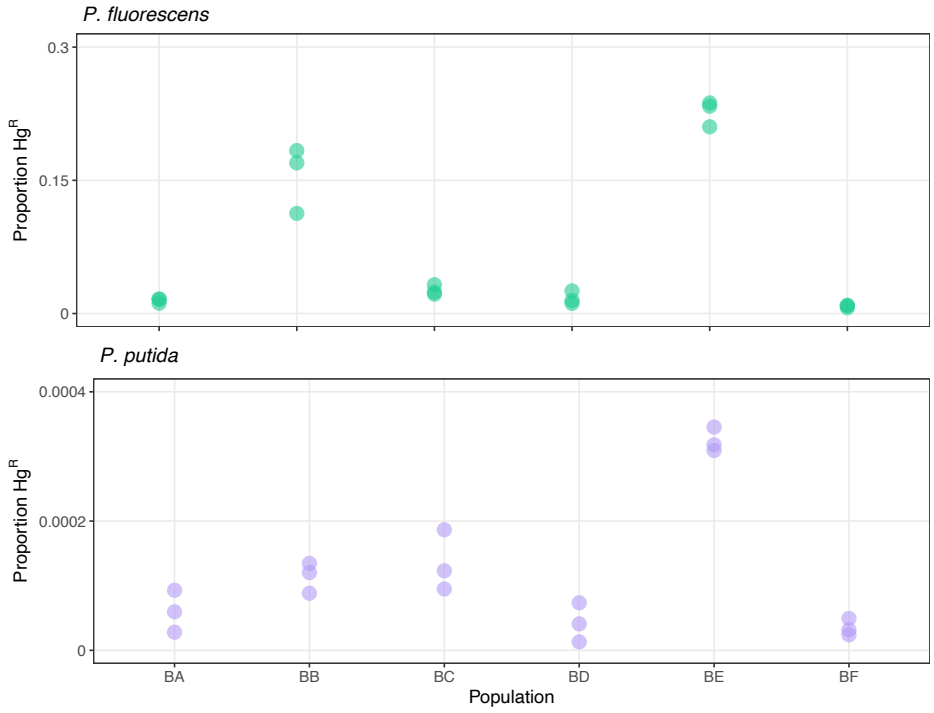

Figure 5.3 In the absence of mercury selection Hg^R reached very low prevalence in *P. putida*. The proportion of plasmid- encoded Hg^R in the *P. fluorescens* (panel A) and *P. putida* (panel B) population was determined at T_{10} (i.e. prior to populations receiving the mercury shock). Proportion Hg^R was plotted for each replicate population across the six populations BA - BF (n = 3).

5.3.2 Evolutionary rescue of a multi-species bacterial community

We next tested whether the positive effect of gene mobility on the evolutionary rescue of coexisting species also occurred in more diverse communities. Experimental communities were assembled from 96 bacterial isolates randomly chosen from a soil wash plated onto nutrient agar and contained ~14 genetically distinct OTUs (determined by 16S rRNA gene sequences; see materials and methods). This community was then propagated together with SBW25 encoding either mobile or non-mobile Hg^R, with or without prior mercury-selection for ten transfers before being subjected to a lethal Hg^{2+} pulse. We measured community diversity before, during and after the lethal mercury-pulse (Figure 5.4) using amplicon sequencing of the 16S rRNA gene. The response of community diversity to the lethal Hg^{2+} pulse varied depending on whether the communities had access to mobile Hg^R and the level of prior Hg^{2+} selection (Figure 5.4: TRANSFER X HGR MOBILITY X MERCURY: $F_{6,100} = 40.917$, $p < 0.0001$). Without prior Hg^{2+} selection, following the lethal Hg^{2+} pulse diversity was significantly higher in communities with access to mobile Hg^R compared to those with non-mobile Hg^R (PQBR103 (T11) – CHROMOSOME (T11): $p < 0.0001$, PQBR57 (T11) –

CHROMOSOME (T11): $p < 0.0001$), or communities without any Hg^R genes (PQBR103 (T11) – HGS (T11): $p < 0.0001$, PQBR57 (T11) – HGS (T11): $p < 0.0001$) suggesting that mobile Hg^R allowed for the maintenance of a more diverse community following the lethal Hg^{2+} pulse. Unlike communities containing mobile Hg^R, those without mobile Hg^R genes suffered sharp declines in diversity following the lethal Hg^{2+} pulse (CHROMOSOME (T10) – CHROMOSOME (T11): $p < 0.0001$; HGS (T10) – HGS (T11): $p < 0.0001$). Immediately following the lethal pulse (i.e. T_{11}), we observed high levels of Hg^R in the other members of the community besides SBW25 where Hg^R was mobile (Figure 5.4: PQBR103 (T11) – CHROMOSOME (T11): $p < 0.0001$; PQBR57(T11) - CHROMOSOME (T11): $p < 0.0001$). Interestingly, whereas, in communities containing SBW25 encoding non-mobile chromosomal Hg^R, diversity subsequently recovered by transfer 12, this recovery was not observed in communities lacking Hg^R genes. This suggests a longer-term community-wide benefit of Hg^R irrespective of mobility, allowing more species to survive the lethal Hg^{2+} pulse albeit at very low relative abundance, which may be linked to the environmental detoxification performed by the *mer* operon. Prior Hg^{2+} selection dampened the effect of the lethal Hg^{2+} pulse on diversity in all Hg^R treatments, such that while these treatments varied in the level of community diversity present prior to the lethal Hg^{2+} pulse we observed no change in diversity during or after the lethal Hg^{2+} pulse (CHROMOSOMAL – PQBR103: $p = 0.255$, CHROMOSOMAL - PQBR57: $p = 0.136$). Communities containing SBW25 encoding Hg^R contained higher frequencies of Hg^R genes prior to the lethal Hg^{2+} pulse when they had been subjected to prior Hg^{2+} selection, and where Hg^R was mobile (Figure 5.5: TRANSFER X HGR X MERCURY: $F_{3, 322} = 10.24$, $P < 0.0001$). Due to higher frequencies of Hg^R, these communities may have been better able to detoxify Hg^{2+} allowing survival of a wider range of taxa.

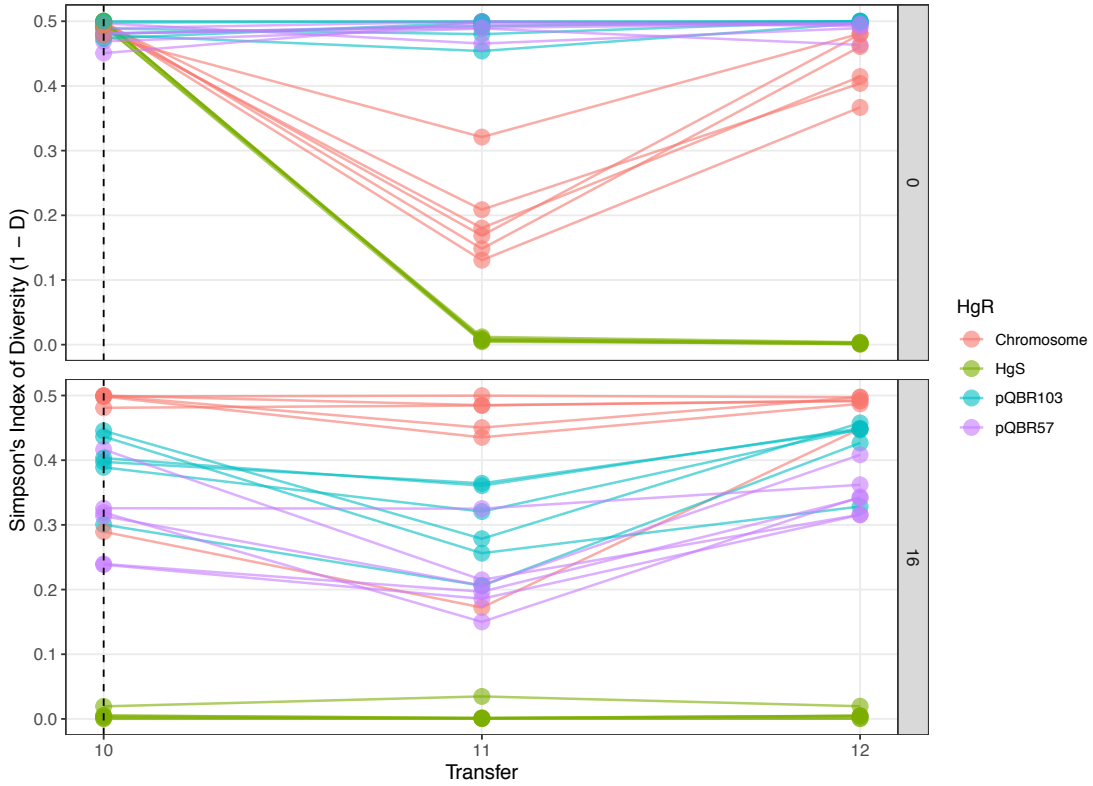

Figure 5.4 Community diversity before, during and after the Hg^{2+} toxic pulse. Simpson's Index of Diversity (1-D) was calculated for communities at T_{10}, T_{11} and T_{12} before and after receiving the Hg^{2+} pulse. Panels represent the two levels of prior mercury exposure (0 and 16 µg/g Hg^{2+}). Colours represent the four modes of Hg^R transmission (chromosomal, pQBR103, pQBR57 and none).

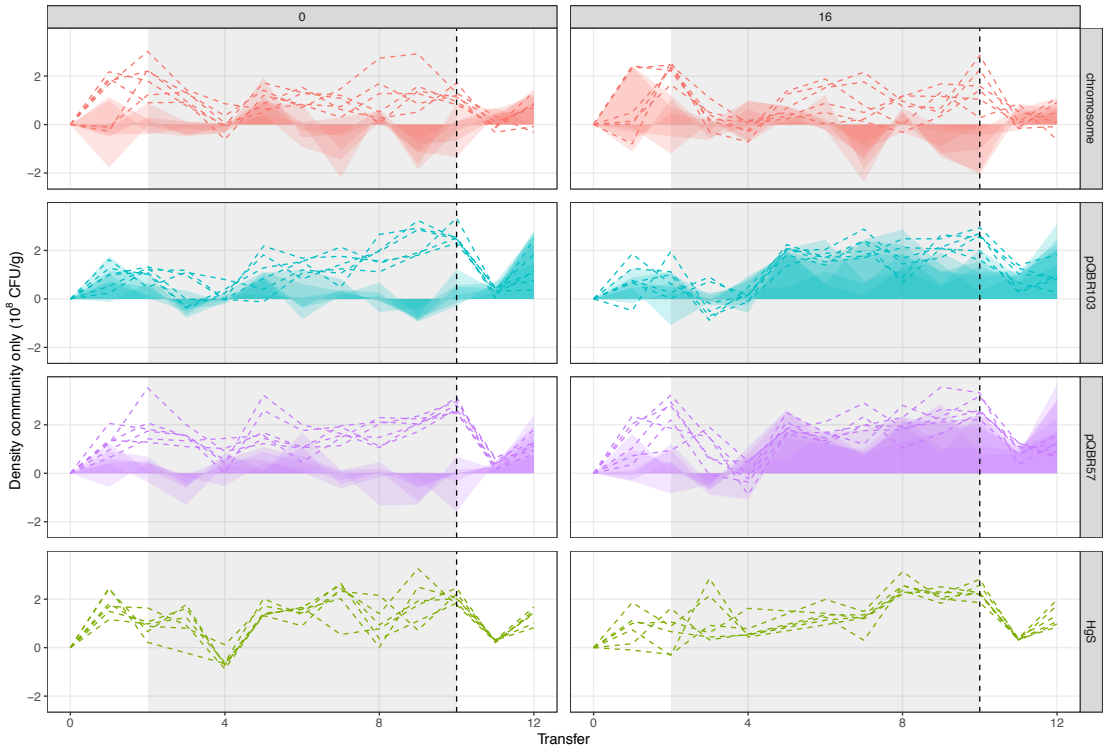

Figure 5.5 Dynamics of Hg^R in the soil community. Population densities of the soil community were calculated by subtracting density counts on nutrient agar + gentamicin from density counts on nutrient agar. The upper row of subpanels shows where Hg^R was encoded on the chromosome of *P. fluorescens*. The second row of subpanels shows where Hg^R was encoded on the plasmid pQBR103. The third row of subpanels shows where Hg^R was encoded on the plasmid pQBR57. The last row of subpanels shows where there was no Hg^R present in the population. Each panel shows, for an individual population, the density of the *P. fluorescens* population and the density of Hg^R within each respective population (shaded area under curve). Vertical panels represent the two mercury treatments (0 and 16 µg/g Hg^{2+}).

The Simpson Index is affected by both the richness and evenness of OTUs, yet rare taxa in bacterial communities are known to perform important functions (Rivett and Bell, 2018). Therefore, we tested the effects of gene mobility and Hg^{2+} selection on OTU richness *per se* and community composition. OTU richness was higher in communities without prior Hg^{2+} selection (Figure 5.6; TRANSFER X MERCURY: $F_{2, 115} = 20.973$, $p < 0.0001$) and was reduced by the lethal Hg^{2+} pulse, demonstrating the negative effect of Hg^{2+} toxicity on community richness. Overall, communities with access to mobile Hg^R had higher OTU richness than those containing chromosomally-encoded or no Hg^R genes (MAIN EFFECT OF HGR: $F_{3, 20} = 10.914$, $p = 0.0002$). This suggests that mobile Hg^R facilitates the maintenance of community diversity, particularly by enabling the recovery of rarer taxa following the lethal Hg^{2+} pulse. We used principal component

analysis to visualise the changes in community composition in response to the Hg^{2+} pulse (Figure 5.7). Communities with access to mobile Hg^R responded differently to prior Hg^{2+} selection than those containing either chromosomal or no Hg^R ($_{HGR\ MODE\ X\ MERCURY:}$ $F_{3, 143} = p = 0.015$). Moreover, Hg^R type affected the response to the lethal Hg^{2+} pulse: Before the Hg^{2+} pulse at transfer 10, communities clustered according to prior mercury-selection treatment, whereas, afterwards at transfer 12, communities were clustered more strongly by Hg^R type (Figure 5.7: $_{MERCURY\ X\ TRANSFER:}$ $F_{2, 143} = 4.33$, $p = 0.001$; $_{HGR\ MODE\ X\ TRANSFER:}$ $F_{6, 143} = 1.56$, $p = 0.004$). Taken together these data suggest that Hg^R mobility played a key role in community responses to a lethal environmental change, allowing greater survival of rare taxa and mediating community structure.

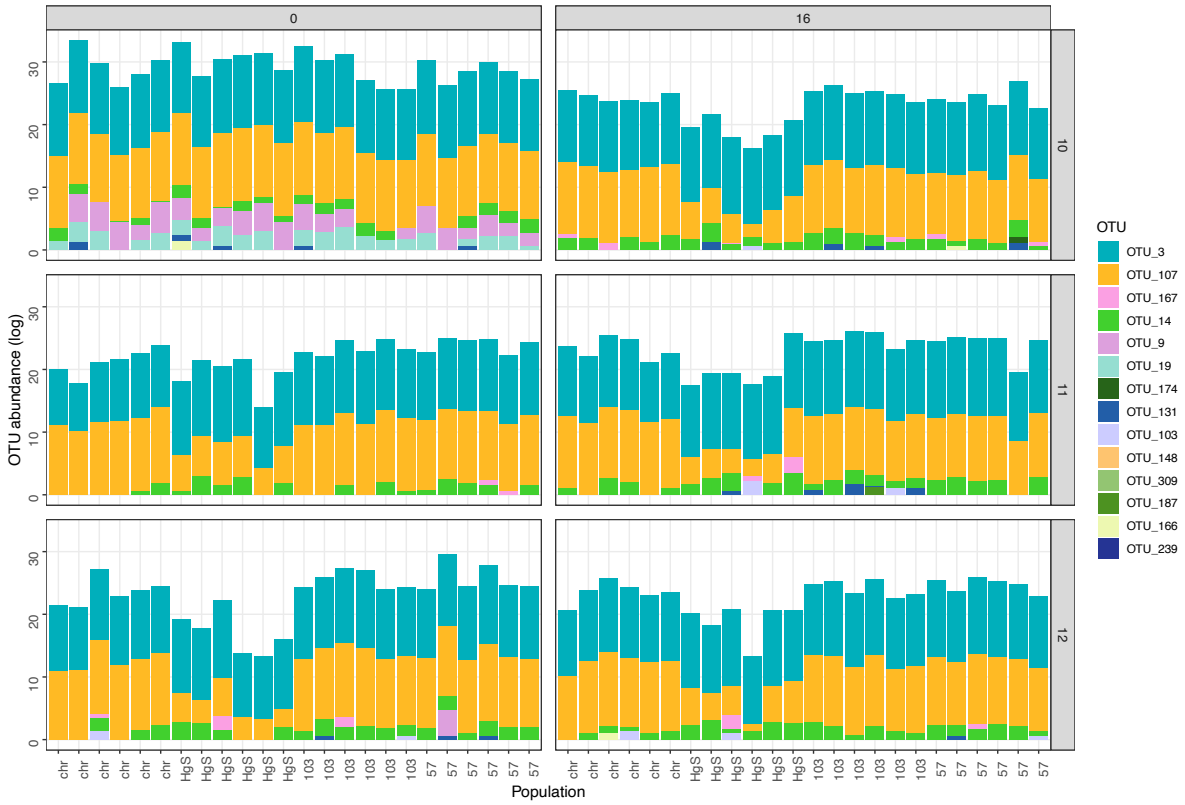

Figure 5.6 OTU abundance across all populations before, during and after the mercury pulse. OTU abundance was tracked before, during and after the mercury pulse. Vertical panels represent the two prior levels of mercury-selection (0 and 16 μg/g Hg^{2+}). Horizontal panels represent the before (transfer 10), during (transfer 11) and after (transfer 12) pulse transfers. Each bar represents one replicate population for the four modes of Hg^R transmission (Chromosomal, Hg^S (mercury-sensitive), pQBR103 and pQBR57.

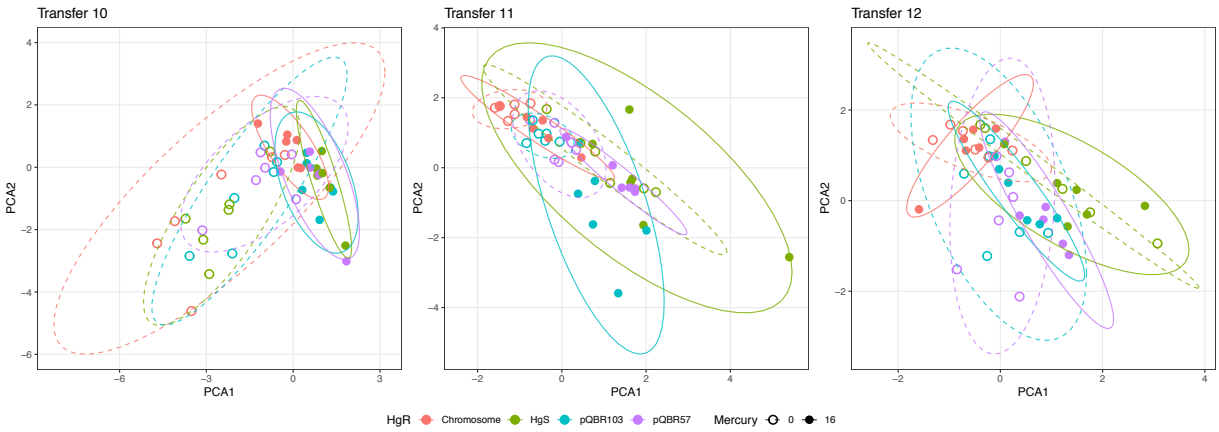

Figure 5.7 Principal component analysis on communities before, during and after the mercury pulse. Principal component analysis was performed on OTU abundance for each of the communities. Here, principal component 1 is plotted against principal component 2.

5.4 Discussion

Microbial communities underpin many important ecosystem functions (Konopka, 2009; Soliveres et al., 2016), but these could be compromised if the diversity of bacterial communities is threatened by extinctions caused by rapid environmental change (Cavicchioli et al., 2019). While ER by spontaneous mutation can permit the survival of individual species (Ojala et al., 2014; Tazzyman and Bonhoeffer, 2014b), HGT has the potential to enable the survival both of the focal species and of other neighbouring species inhabiting diverse communities following environmental deterioration. Experimentally, we showed that in a two-species community, horizontal transfer of plasmid-encoded Hg^R from donor *P. fluorescens* rescued populations of sensitive *P. putida*, whereas non-mobile chromosomal Hg^R primarily benefited the Hg^R *P. fluorescens* population. We then expanded this to look at the role of HGT in a multi-species natural soil community. We found that mobile Hg^R (i.e. encoded on pQBR57 or pQBR103) underwent horizontal transmission into the natural community, allowing for community evolutionary rescue following the Hg^{2+} pulse, compared to communities which had access to chromosomal Hg^R only. Following the lethal pulse, the composition of communities with access to mobile Hg^R differed significantly from those with access to only chromosomal-Hg^R, as mobile Hg^R communities retained a greater number of the rare OTU's. Together, these results suggest that the mobility of the resistance genes played an important role in how the community responded to the lethal environmental change as mobile Hg^R was able to transfer into the wider soil community, thus enabling its rescue.

The relative importance of HGT versus spontaneous mutation in bacterial ER is likely to depend on the nature of the environmental change. Whereas some environmental changes can be survived by acquiring point mutations (e.g. resistance to some antibiotics) others require acquisition of entire operons, and will be more likely to arise by HGT. In this study, mercury resistance is provided by the *mer* operon encoding genes involved in the detection, import and reduction of ionic mercury into its elemental form (Barkay et al., 2003; Boyd and Barkay, 2012). Conversely, adaptation to some environmental stressors, such as high temperature, may involve multiple mutations in many different core genes, and thus will likely be unobtainable by HGT. Similarly, the likelihood that HGT will play a role in ER will vary among bacteria-plasmid associations: these vary extensively in their costs of plasmid carriage and the rate of conjugation, with consequent effects on plasmid stability (De Gelder et al., 2007; Hall et al., 2015; Kottara et al., 2018). ER by HGT is more likely in associations where plasmid carriage incurs low fitness costs or where these can be rapidly ameliorated by compensatory mutation, and more likely when the required traits are encoded upon plasmids with higher conjugation rates, all of which will promote plasmid maintenance in the absence of positive selection.

The importance of plasmids in enabling CR is likely to also be a function of their host range. Generally, plasmids can be classified depending on their host range [defined as the number of host species a plasmid can conjugate into and replicate in (Jain and Srivastava, 2013)]: Broad host range (BHR) plasmids can transmit between and be stably maintained across phylogenetically diverse hosts, whereas narrow host range plasmids (NHR) and more phylogenetically restricted in their range of hosts. BHR plasmids are likely to play a more significant role in bacterial CR. BHR plasmids have been recovered from a range of natural environments including soils (Heuer and Smalla, 2012; Klümper et al., 2015), freshwater (Brown et al., 2013) and wastewater (Schlüter et al., 2007), suggesting that CR by HGT could be common. Although, evolutionary theory suggests that the evolution of ecological generalists, such as BHR plasmids, is likely to be constrained by fitness trade-offs (Egas et al., 2004; Kassen, 2002; McPeek, 1996), when co-cultured with multiple hosts, plasmids can evolve to expand their host range (De Gelder et al., 2008), and reduce their fitness costs in the multiple host backgrounds (Kottara et al., 2016). Therefore, determining the extent to which BHR plasmids can invade a natural community may provide a clearer idea of the likelihood of CR within different environments (Klümper et al., 2015).

Plasmid stability can vary dramatically among host species (Hall et al., 2015; Kottara et al., 2018), and CR is likely to be enhanced by the presence of permissive plasmid hosts in the community to act as a hub for HGT, disseminating the required adaptive trait. For example, *P. fluorescens* has

been previously shown to act as a plasmid source for a non-permissive host in a two-species bacterial community, maintaining access to the plasmid in the non-permissive host species via source-sink transfer dynamics (Hall et al., 2016). Determining the characteristics that make bacterial species permissive plasmid hosts could help promote CR, by allowing identification of taxa that could act as HGT hubs and promote the wide dissemination of adaptive genes to natural bacterial communities.

Compensatory evolution to alleviate cost of plasmid carriage is thought to commonly permit plasmid stability and may occur through mutations occurring on either the bacterial chromosome (Harrison et al., 2015a, 2016) or the plasmid itself (Dionisio et al., 2005; Heuer et al., 2007). Which form of compensatory mutation occurs (either host or plasmid) could affect the potential for CR. Where host compensation occurs, this will primarily favour only this host taxon, and lead to high fitness costs in plasmid recipients, thus potentially limiting the stability of plasmids in the wider community and resulting in a lower likelihood of CR. Plasmid-encoded compensation on the other hand may alleviate the plasmid fitness cost across multiple host taxa, and has been theoretically shown to promote plasmid persistence (Zwanzig et al., 2019). Under this mode of compensation, plasmids are more likely to be stable across a wider range of the community, increasing the likelihood that CR will maintain a broad array of ecological functions. It should also be noted that although plasmid acquisition may offer many benefits in the short-term, it may be detrimental to long-term evolutionary potential if acquisition of one plasmid prevents acquisition of further plasmids from the same incompatibility group that encode different accessory genes (Novick, 1987). Theoretically, this has been demonstrated where a plasmid harbouring resistance genes was unable to transmit throughout a population when there was a resident incompatible plasmid already present (Tazzyman and Bonhoeffer, 2014b). In this case, plasmid segregation rate will play an important role in determining the likelihood of CR, as any plasmid-free cells that arise will be able to be recipients for the plasmid.

The nature of functional trait required for CR may also alter the probability of CR. For example, some microbial functions are ‘public goods’ where not only the cell expressing the trait but also its neighbours benefit (Smith and Schuster, 2019). For example, enzymatic inactivation of certain antibiotics and heavy metal ions (including Hg^{2+}) allows sensitive cells to survive otherwise lethal concentrations of the antibiotic if they are growing alongside resistant cells (Dugatkin et al., 2005; Yurtsev et al., 2013). This social effect of Hg^R may explain why we saw some maintenance of diversity in communities that harboured only chromosomally-encoded Hg^R, and particularly with prior mercury selection. Here, detoxification of Hg^{2+} to Hg by *SBW25* encoding chromosomal Hg^R may have protected, at least to some extent, the sensitive taxa and prior mercury selection

would have maintained resistance at a higher frequency and level of expression. In contrast, where functional traits provide only self-directed fitness benefits, for example resistance provided by an efflux pump (Bottery et al., 2016), this will benefit only the cell harbouring the resistance gene, and therefore in these cases, HGT of resistance genes would be crucial to CR.

Soils play a vital role in the provision of ecosystem services, making the preservation of soil health a priority (Barrios, 2007). Where soils are contaminated, bioremediation (i.e. the use of microbes to degrade the contaminants), may offer a way to restore soil health (Ojuederie and Babalola, 2017). Certain microbes can be inoculated into the soil to enhance the rate of contaminant degradation (Garbisu et al., 2017), and the use of plasmids has been proposed as a promising strategy for the dissemination of the required functional genes throughout the wider soil community (Venkata Mohan et al., 2009). Because plasmids allow the transfer of the genes into the native soil community, this may be a more feasible remediation solution, as native species will likely be well adapted to the prevailing environment, and thus more competitive. For example, transfer of a DDT-remediation plasmid from an introduced *Escherichia coli* donor to the native soil bacteria enabled the breakdown of ~50% of the DDT (Gao et al., 2015). Our findings suggest that infectious plasmids harbouring the required functional genes may provide protection against future environmental deterioration. Seeding natural communities with such plasmids, will allow the dissemination of these genes throughout the community, enabling the maintenance of community diversity upon environmental deterioration and potentially more effective bioremediation.

Chapter Six

Discussion

Horizontal gene transfer plays a vital role in bacterial evolution, primarily by spreading adaptive genes between cells both within and between species (Frost et al., 2005). Genes capable of HGT, by virtue of being encoded on mobile genetic element for example, have a potential advantage over those exclusively encoded on the chromosome because mobile genes can replicate both vertically and horizontally. Using a series of selection experiments, I investigated how gene mobility affected the spread of resistance genes in bacterial populations and communities, and how this process is affected by the environmental and community context. I showed first that being encoded on a mobile plasmid widened the range of parameter space in which resistance genes could spread, permitting resistance to reach fixation even in the absence of positive selection through infectious horizontal transmission (Chapter 2). I next showed that plasmid stability was enhanced by higher-frequency pulses of positive selection, but that horizontal plasmid transfer played a more important role in plasmid stability with lower frequencies of pulsed positive selection (Chapter 3). The presence of lytic phages limited the persistence of plasmids in bacterial populations, but higher rates of horizontal transmission were associated with the survival of plasmids in some replicates (Chapter 4). Finally, where resistance genes were mobile, as opposed to non-mobile, they enable a greater diversity of a bacterial soil community to survive a lethal environmental change by disseminating resistance genes into the other taxa (Chapter 5). These results suggest that the horizontal mobility of genes is crucial to their persistence in the absence of positive selection, thus maintaining the evolutionary potential of populations and communities to future challenges. Understanding the environmental factors which drive the dynamics of mobile and non-mobile traits will ultimately enable us to predict the spread of traits, and potentially manipulate bacterial communities to be more resilient to environmental change.

Theoretically, because vertical transmission is linked to host reproduction, vertically transmitted elements should evolve over time to minimise their costs to the host (Lipsitch et al., 1996). Here, we show that vertical transmission of plasmid-encoded resistance was favoured when positive selection was strong (Chapter 2) or frequent (Chapter 3). Therefore, in such conditions over time, we might expect either the capture of beneficial plasmid borne traits to the bacterial chromosome, or the domestication of the plasmid. Chromosomal capture of beneficial traits would allow

bacteria to retain the benefits of gene carriage, whilst eliminating the costly plasmid backbone through purifying selection (Bergstrom et al., 2000; Stewart and Levin, 1977). Our results indicate that beneficial genes encoded on the chromosome are able to transmit more rapidly through vertical selection, than plasmid-borne genes (Chapter 2), suggesting fitness benefits for the genes encoding the trait of moving to the chromosome. Alternatively, plasmid domestication is likely to reduce the costs of plasmid maintenance and may often lead to the reduction, or complete loss of, the plasmid's ability to conjugate (Dahlberg and Chao, 2003; Turner et al., 1998), limiting its future mobility. It is likely that over time exclusively vertically transmitted plasmid backbones may undergo decay or evolve into secondary chromosomes. For example, the secondary chromosome of *Vibrio cholerae* contains a plasmid-like origin of replication (Egan and Waldor, 2003), and replicates independently at a different point in the cell cycle from chromosome 1 (Rasmussen et al., 2007), suggesting a plasmid origin. Unlike the immediate loss of the plasmid backbone by purifying selection following chromosomal capture, domestication of the plasmid would provide the host cell with additional genetic material of potential use for future adaptation.

On the other hand, in the absence of positive selection, plasmids are selfish genetic elements and must counteract negative selection with sufficiently high rates of horizontal transfer (Lundquist and Levin, 1986). We showed that when positive selection was absent (Chapter 2) horizontal transmission of resistance genes was the primary mode of spread of resistance genes. Therefore, in environments where there are infrequent or low levels of positive selection, we might expect plasmids to evolve higher rates of horizontal transmission. This could be associated with increased fitness costs to the host, described by the virulence/transmission trade-off whereby infectious elements that invest more in horizontal transmission tend to achieve this at the price of higher virulence and thus reduced vertical transmission (Turner et al., 1998). Under sustained infectious transmission, we might expect plasmids to streamline their genomes, allowing them to dispense with unnecessary accessory traits. Indeed, pathogenic bacteria appear to have smaller genomes than their non-pathogenic counterparts (Moran, 2002; Reuter et al., 2014; Weinert et al., 2015), suggesting that a pathogenic lifestyle selects for reductive genome evolution. For example, clinical isolates of *Streptococcus suis*, a bacteria that causes respiratory tract infections, contained significantly fewer genes when compared to non-clinical isolates (Weinert et al., 2015). Support for a streamlined, parasitic lifestyle comes from the finding that genes coding for host essential proteins are nearly always absent from small plasmids (Smillie et al., 2010). Indeed, experimental evolution has shown that plasmids may acquire deletions to costly regions of their plasmid backbone, improving its stability in a novel host (Porse et al., 2016). In addition, numerous 'cyptic' plasmids, those devoid of any identifiable accessory gene regions, have been isolated from natural environments (Brown et al., 2013) and clinical environments (Burian et al., 1997).

However, rather than existing at either end of this continuum, the majority of plasmids in nature are likely to lie somewhere in between, exhibiting a range of differing sizes, costs and rates of conjugation. Sizes of plasmid are highly variable with plasmids ranging from as small as 1kb to >1000 kb (Smillie et al., 2010). In addition, conjugation rates are highly variable (Dionisio et al., 2002; Gordon, 1991), with only about 50% of plasmids encoding specific conjugation machinery (Smillie et al., 2010). These findings suggest that a high level of life-history strategy diversity exists in natural plasmid communities. One possible explanation for this diversity is that natural environment are highly variable such that no single plasmid strategy is optimal under all conditions. Soils, for example, are highly heterogenous, imposing variable selection pressures on the bacteria which inhabit them at micrometre spatial scales (Vos et al., 2013). Therefore, these plasmids are not likely to be subject to one specific level of selection, but instead are a product of the huge complexity of the natural environment. We showed that under infrequent positive selection (Chapter 3) conjugative transfer maintained plasmid-encoded resistance genes, suggesting that heterogenous selection may have an important role in maintaining plasmid conjugative ability. In addition, we showed that within spatially structured soil microcosms, both of the plasmids studied here were able to persist and infect the natural soil community (Chapter 5). Given that spatial structure is likely to be prevalent in most natural communities, particularly as most microbes live in biofilms (Hall-Stoodley et al., 2004), the effects of spatial structure and spatial heterogeneity in selection pressures on plasmid persistence in a bacterial communities are likely to be non-trivial. Although evidence exists that biofilms promote conjugation initiation by providing increased cell to cell contact, the subsequent spread of plasmids through a population may be limited to sub-populations, such that spatial structure may slow their spread throughout the entire population (Stalder and Top, 2016).

The two plasmids used in experiments in this thesis exhibited varying costs and conjugation rates within the focal host *Pseudomonas fluorescens*. On the one hand, pQBR57 exhibited lower costs, and a higher conjugation rate, such that it spread even in the absence of positive selection. Whereas pQBR103, displayed higher fitness costs, and a lower conjugation rate, and was therefore maintained in the population only when there was some degree of positive selection (Chapter 3), with compensatory evolution of the host promoting its stability over longer time periods (Chapter 3). Interestingly, these two plasmids were isolated from the same soil site in Oxfordshire, along with a number of other pQBR mega-plasmids which themselves display a variety of sizes, accessory genome content and cost (Hall et al., 2015; Lilley and Bailey, 1997; Lilley et al., 1994, 1996). Previous work has shown that the fitness costs and benefits of these two specific plasmids vary with environmental context: under mercury selection pQBR103

outcompetes pQBR57 whereas in the absence of mercury, pQBR57 outcompetes pQBR103 (Hall et al., 2015). This suggests that although pQBR103 is costlier, it is also more beneficial in mercury environments. Thus, despite both plasmids co-existing within the same natural environment, the levels of micro-selection that exist within soil, will likely favour one plasmid over the other. In addition, migration has been shown to enable the spread of plasmids from patches where they are under positive selection, to populations where they are not, maintaining their stability in the patches without positive selection (Harrison et al., 2018) .

Both pQBR57 and pQBR103 were stably maintained and able to rescue a natural soil community from lethal environmental change (Chapter 5), which may have important consequences for the survival of natural communities in contaminated environments. Soils play a vital role in the provision of ecosystem services and are one of the most biodiverse habitats on Earth (Bardgett and van der Putten, 2014; Barrios, 2007). Consequently, reduced diversity of soil communities in degraded soils is likely to have widespread negative effect on soil health. One particular source of soil degradation comes from industrialisation and urbanisation, which have both led to large areas of contaminated land (Liu et al., 2014). High levels of contamination have had a negative impact on the soil microbial communities, resulting in altered activity, biomass and diversity (Burges et al., 2015; Wang et al., 2017). The inoculation of beneficial microbes into contaminated sites has been suggested as a way of bioremediating these sites (Dixit et al., 2015). For example, the mercury-resistance operon *mer* described in this thesis, allows for the uptake and reduction of toxic mercuric ions to elemental mercury which is then volatilised from the soil environment (Barkay et al., 2003). However, bioremediation may not be effective if microbes inoculated for bioremediation are outcompeted by the native soil microbes present in the soil, which are likely to be locally adapted to the prevailing environment. Plasmids may offer an alternative solution, whereby detoxification genes encoded on a plasmid can be used to infect the natural community, enabling this local community to carry out the bioremediation itself (Garbisu et al., 2017). In soils, metagenomic analysis has shown that a core community fraction dominates gene transfer (Klümper et al., 2015). Here these key community members, act as nodes within a highly connected network of horizontal gene transfer (Popa et al., 2011). Determining which hosts are more permissive to plasmids and therefore more likely to act as nodes in HGT networks will provide important knowledge for predicting plasmid dynamics in microbial communities, allowing us to select the best donor species for plasmid transmission into the wider community.

Antibiotic resistance is a widespread problem (O'Neill, 2014), and plasmids are an important vector for the dissemination of antibiotic resistance genes (Bennett, 2008). It is generally considered that resistance genes will be eliminated in environments where they are not positively

selected, however generally this approach has proved unsuccessful (Salyers and Amábile-Cuevas, 1997). One possible explanation for the long-term persistence of antibiotic resistance genes in antibiotic free environments is that plasmids are able to spread resistance genes in the absence of positive selection through conjugative transfer (Chapter 2). In addition, we showed that plasmids, persisting even at vanishingly low frequencies by infectious transmission, could rapidly sweep to fixation once positive selection was applied (Chapter 5). This suggests that in the absence of positive selection, conjugative plasmids will transfer into the wider microbial community, infecting a broad range of species within the environment (Klümper et al., 2015). Subsequent bouts of positive selection will then enrich for these species, driving their clonal expansion. Combining efforts to inhibit conjugative transfer within the population (Lopatkin et al., 2017), in addition to limiting positive antibiotic selection may be therefore required to limit the spread of antibiotic resistance in natural communities and host-associated microbiomes.

Appendices

Appendix A

C Stevenson, JPJ Hall, E Harrison, AJ Wood, MA Brockhurst 2017. Gene mobility promotes the spread of resistance in bacterial populations. *ISME J*. 11: 1930-1932

The ISME Journal (2017) 11, 1930–1932
www.nature.com/ismej

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SHORT COMMUNICATION

Gene mobility promotes the spread of resistance in bacterial populations

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Theory predicts that horizontal gene transfer (HGT) expands the selective conditions under which genes spread in bacterial populations. Whereas vertically inherited genes can only spread by positively selected clonal expansion, mobile genetic elements can drive fixation of genes by infectious HGT. We tested this using populations of *Pseudomonas fluorescens* and the conjugative mercury resistance (Hg^R) plasmid pQBR57. HGT expanded the selective conditions allowing the spread of Hg^R: Chromosomal Hg^R only increased in frequency under positive selection, whereas plasmid-encoded Hg^R reached fixation with or without positive selection. Tracking plasmid dynamics over time revealed that the mode of Hg^R inheritance varied across mercury environments. Under mercury selection, the spread of Hg^R was driven primarily by clonal expansion while in the absence of mercury Hg^R dynamics were dominated by infectious transfer. Thus, HGT is most likely to drive the spread of resistance genes in environments where resistance is useless.
The ISME Journal (2017) **11,** 1930–1932; doi:10.1038/ismej.2017.42; published online 31 March 2017

Microbial populations reproduce clonally by vertical descent but can also exchange genes by horizontal gene transfer (HGT). HGT is an important process in bacterial evolution, accelerating adaptation by allowing the spread of ecologically and clinically relevant traits between lineages (Frost *et al.*, 2005; Thomas and Nielsen, 2005). Therefore, the balance of vertical versus horizontal inheritance is expected to have important effects on bacterial evolution and thus function (Smith *et al.*, 1993; Cordero and Polz, 2014; Shapiro, 2016). Comparative genomics has revealed that bacterial species undergo dramatic shifts in the balance of vertical versus horizontal inheritance over time (Cordero and Polz, 2014). These shifts may be due to changes in selection on inherited traits, as theory predicts that vertical inheritance is favoured by strong positive selection, increasing clonality via genome-wide selective sweeps (clonal expansion), whereas horizontally inherited genes can spread even in the absence of positive selection (Tazzyman and Bonhoeffer, 2014), maintaining population genomic diversity. However, experimental data addressing this issue are lacking.

To test how selection alters the balance of vertical versus horizontal transmission of bacterial genes, we quantified the dynamics of mercury resistance (Hg^R) in populations of the soil bacterium *Pseudomonas fluorescens* SBW25 (Rainey and Bailey, 1996) with the Hg^R operon *mer* encoded either chromosomally or carried on an Hg^R plasmid pQBR57 (Lilley and Bailey, 1997). We established 36 replicate populations of SBW25, 18 with Hg^R encoded on their chromosome (non-horizontally transferable), 18 with Hg^R encoded on pQBR57 (horizontally transferable). Each population was mixed 50:50 with a mercury-sensitive differentially marked SBW25 strain and then propagated by serial transfer every 24 h for 8 days. Populations were grown in one of three mercury environments, 0, 20 and 40 μM $HgCl_2$ (six replicates per treatment); this represents a selective gradient wherein plasmid-encoded Hg^R is under, respectively, strong negative selection, weak positive selection and strong positive selection, due to the balance between the cost of plasmid carriage and the benefits of Hg^R (Supplementary Figure S1; Hall *et al.*, 2015). Because pQBR57 is maintained at low copy number (Hall *et al.*, 2015), the chromosomal and plasmid-encoded Hg^R genes provide equivalent levels of resistance (Supplementary Figure S2). Every 2 days we determined the proportion of Hg^R cells within each population by plate counts. Furthermore, as our donors and recipients were differentially marked we were able to track the frequency of pQBR57 in both donor and recipient populations (for full methods see Supplementary Information).

The end point proportion of mercury-resistant (Hg^R) cells in the population was significantly affected by the horizontal transmissibility of Hg^R (Figure 1; main effect of mobility at 0 μM $HgCl_2$: $F_{1,10} = 74.34$, $P < 0.001$). Where Hg^R was encoded on the chromosome, positive selection was required to drive the spread of resistance: Hg^R

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Received 15 November 2016; revised 2 February 2017; accepted 10 February 2017; published online 31 March 2017

rapidly became fixed within the population in the 20 and 40 μM $HgCl_2$ environments, whereas in the 0 μM $HgCl_2$ environment chromosomal Hg^R remained at ~50% prevalence. In contrast, when Hg^R was encoded on the conjugative plasmid pQBR57, and thus horizontally transferable, Hg^R reached high frequencies across

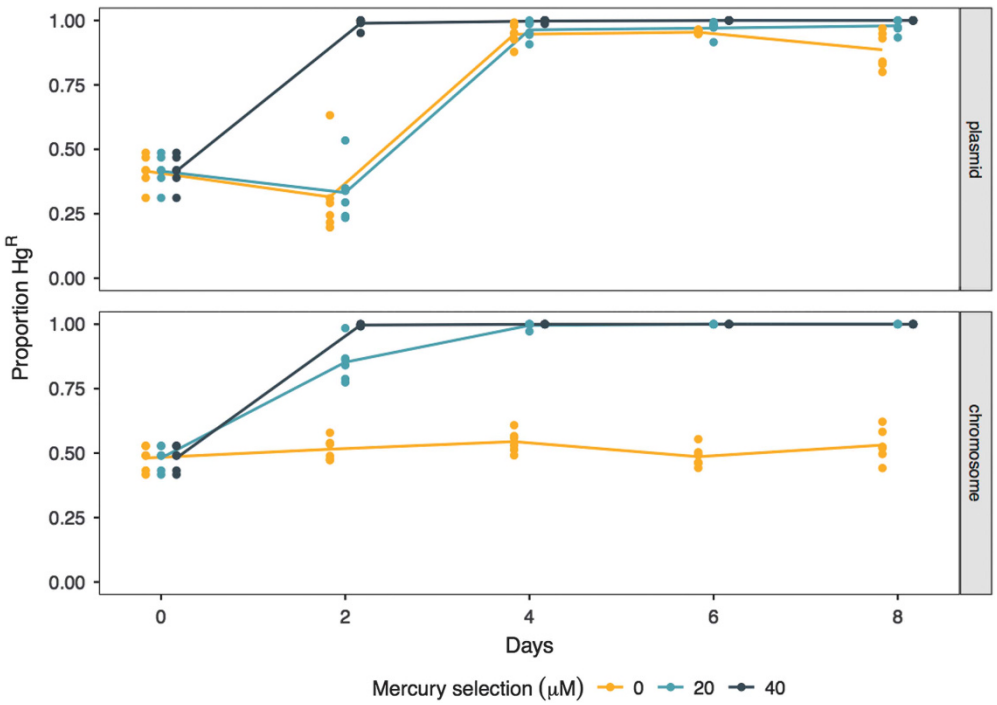

Figure 1 Horizontal transmission had a significant impact on the proportion of Hg^R. The proportion of chromosome- and plasmid-encoded Hg^R was determined over time across the three mercury treatments (0, 20 and 40 μM $HgCl_2$). Points represent replicate populations and are slightly offset by treatment on the *x* axis to prevent over plotting. Lines represent means ($n = 6$).

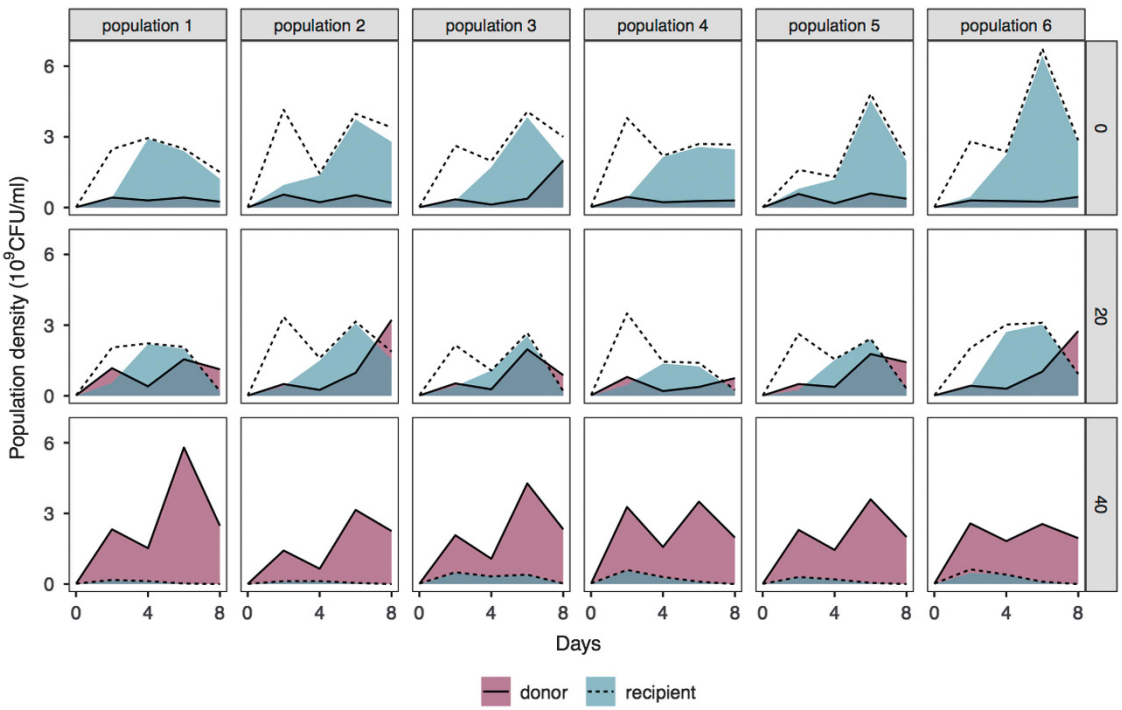

Figure 2 Selection determines the balance of horizontal versus vertical inheritance of plasmid-encoded Hg^R. Plasmid transfer in each of six replicate populations was tracked over time across the three mercury treatments (0, 20 and 40 μM $HgCl_2$). Dotted lines indicate densities of recipient populations; solid lines indicate densities of donor populations. For each population, shaded regions represent plasmid prevalence within donor (purple) and recipient (blue) subpopulations.

all mercury environments (0, 20 and 40 μM $HgCl_2$). Thus, the opportunity for horizontal transfer expanded the selective conditions allowing the fixation of Hg^R such that this occurred both with and without positive selection for resistance.

Tracking plasmid dynamics over time revealed that the strength of positive selection determined the balance of horizontal versus vertical inheritance of plasmid-encoded Hg^R in bacterial populations. HGT played a significantly greater role as the strength of selection decreased (Figure 2; Main effect of mercury: $F_{1,16} = 392.72$, $P < 0.001$). Under strong positive selection (40 μM $HgCl_2$), Hg^R swept through the population by clonal expansion of the original Hg^R donor population. This was presumably due to the high toxicity of the environment strongly selecting against plasmid-free recipients, limiting the opportunity for HGT via plasmid conjugation as a consequence. The contribution of vertical inheritance to the spread of Hg^R reduced with weakening positive selection. Under weak positive selection (20 μM $HgCl_2$), Hg^R spread through the population by a mixture of vertical clonal expansion of donor cells and horizontal transmission of the plasmid into the recipient subpopulation. Under negative selection (0 μM $HgCl_2$) Hg^R spread by conjugative plasmid transfer into available plasmid-free recipient cells. Therefore, while strong positive selection favoured vertical inheritance, the contribution of horizontal transfer to the spread of resistance genes increased as positive selection weakened.

Our data are consistent with theory that HGT can overcome selective barriers to drive the spread of resistance genes in the absence of positive selection, whereas resistance genes spread through vertical transmission only under positive selection. Thus, whereas positive selection for resistance would purge genomic diversity via genome-wide sweeps of resistance (Wiedenbeck and Cohan, 2011), negative selection against resistance coupled with infectious HGT of resistance genes can spread resistance genes into diverse genomic backgrounds. Consequently, the sharing of resistance genes between lineages is most likely to occur in environments without positive selection, and therefore where resistance genes have little use.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank D Guymer for technical support, and V Friman and J Pitchford for valuable comments. This work was supported by an ERC Starting Grant from the European Research Council awarded to MAB (StG-2012-311490-COEVOCON), a Leverhulme Prize from the Leverhulme Trust awarded to MAB (PLP-2014-242), the University of York and a NERC studentship to CS supervised by MAB and AJW.

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

Appendix B

C Stevenson, JPJ Hall, MA Brockhurst, E Harrison 2018. Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Pro. R. Soc. B*. 285: 20172497

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Cite this article: Stevenson C, Hall JPJ, Brockhurst MA, Harrison E. 2018 Plasmid stability is enhanced by higher-frequency pulses of positive selection. *Proc. R. Soc. B* **285**: 20172497.
http://dx.doi.org/10.1098/rspb.2017.2497

Received: 8 November 2017
Accepted: 1 December 2017

Subject Category:
Evolution

Subject Areas:
evolution, ecology, microbiology

Keywords:
experimental evolution, fluctuating selection, compensatory evolution, horizontal gene transfer, plasmid, mercury resistance

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3954094.

THE ROYAL SOCIETY PUBLISHING

Plasmid stability is enhanced by higher-frequency pulses of positive selection

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Plasmids accelerate bacterial adaptation by sharing ecologically important traits between lineages. However, explaining plasmid stability in bacterial populations is challenging owing to their associated costs. Previous theoretical and experimental studies suggest that pulsed positive selection may explain plasmid stability by favouring gene mobility and promoting compensatory evolution to ameliorate plasmid cost. Here we test how the frequency of pulsed positive selection affected the dynamics of a mercury-resistance plasmid, pQBR103, in experimental populations of *Pseudomonas fluorescens* SBW25. Plasmid dynamics varied according to the frequency of Hg^{2+} positive selection: in the absence of Hg^{2+} plasmids declined to low frequency, whereas pulses of Hg^{2+} selection allowed plasmids to sweep to high prevalence. Compensatory evolution to ameliorate the cost of plasmid carriage was widespread across the entire range of Hg^{2+} selection regimes, including both constant and pulsed Hg^{2+} selection. Consistent with theoretical predictions, gene mobility via conjugation appeared to play a greater role in promoting plasmid stability under low-frequency pulses of Hg^{2+} selection. However, upon removal of Hg^{2+} selection, plasmids which had evolved under low-frequency pulse selective regimes declined over time. Our findings suggest that temporally variable selection environments, such as those created during antibiotic treatments, may help to explain the stability of mobile plasmid-encoded resistance.

1. Introduction

Conjugative plasmids are extrachromosomal genetic elements that, alongside the genes required for their own replication, maintenance and transfer [1], carry cargos of accessory genes encoding functional traits. Common plasmid-encoded accessory traits include resistance to toxins, virulence factors and metabolic capabilities [2]. By transferring ecologically important functional traits within and between bacterial lineages and species, plasmids can accelerate bacterial adaptation [3]. Therefore, the dynamics and stability of conjugative plasmids in bacterial populations have potentially important implications for understanding bacterial evolution [4,5]. Nevertheless, it remains challenging to explain the long-term stability of plasmids. This is because plasmid maintenance is frequently costly for the bacterial host cell [6]. Although such costs may be outweighed by the benefits of plasmid-encoded functional traits in some environments [7], theory predicts that plasmids should be evolutionarily unstable whether parasitic (i.e. costs outweigh benefits) or mutualistic (i.e. benefits outweigh costs) [8–11]. In the short term, parasitic plasmids are expected to decline in frequency owing to negative selection, because observed rates of horizontal transmission appear too low to counteract this process [10,12]. While mutualistic plasmids can be temporarily favoured by positive selection for accessory gene functions, they are expected to decline in frequency over longer evolutionary timescales. This is because the useful accessory genes can be integrated into the chromosome, rendering the plasmid-backbone dispensable. Thus, consistent positive selection

for accessory genes should favour plasmid-free cells with the accessory traits on their chromosome, which outcompete plasmid-bearers who still pay the cost of plasmid carriage [10,13,14].

In both natural and clinical environments, plasmids are likely to experience temporally variable selection, resulting in fluctuating positive selection for the accessory genes they carry [15–17]. Recent theory suggests that temporally heterogeneous environments where plasmids experience pulsed positive selection may favour their maintenance through two non-mutually exclusive mechanisms [13,18]. Firstly, rare pulses of strong positive selection can theoretically promote the maintenance of conjugative plasmids carrying accessory gene functions. This occurs because plasmid-free cells outcompete both plasmid-bearers and cells with chromosomal accessory genes between bouts of positive selection, but only the plasmid-encoded copies of the accessory genes can conjugate into these plasmid-free cells. These plasmid-bearing transconjugant cells can then sweep to high frequency upon the next pulse of positive selection [13]. By contrast, where pulses of positive selection are frequent, the frequency of plasmid-free cells and thus the benefits of conjugation are reduced. Therefore, under constant or high-frequency pulses of positive selection, cells with chromosomal accessory genes are favoured at the expense of accessory genes encoded on the conjugative plasmid. Secondly, pulses of positive selection have been shown to promote compensatory evolution to ameliorate the cost of plasmid carriage thereby weakening negative selection against the plasmid-backbone. This occurs because positive selection temporarily increases the population size of plasmid-bearing cells thus increasing the probability that they will gain compensatory mutations [18]. Compensatory evolution appears to be a fairly general mechanism by which plasmid survival is ensured, it has been observed in a range of bacteria–plasmid interactions [18–20] and across environments where the fitness effect of plasmid acquisition ranges from parasitic to mutualistic [19].

Here, we tested how the frequency of pulsed positive selection affected plasmid stability (i.e. the stable maintenance of the plasmid in the bacterial population). We experimentally evolved populations of *Pseudomonas fluorescens* SBW25 with the mercury-resistance (Hg^R) plasmid pQBR103 [19] across a range of treatments varying in the frequency of exposure to toxic concentrations of mercuric ions (Hg^{2+}). Mercuric ions are normally lethal to the bacterial cell, binding to protein sulfhydryl groups and causing major cellular disruption [21]. However, in this bacteria–plasmid system, pQBR103 encodes a Tn5042 transposon which in turn harbours a mercury-resistance operon, *mer*, that catalyses reduction of Hg^{2+} to a less toxic form Hg^0. Thus, while in the absence of Hg^{2+}, pQBR103 imposes a large fitness cost on SBW25, at higher Hg^{2+} concentrations this fitness cost is offset by benefit of Hg^R [7,19]. Populations were propagated under one of six treatments: in the absence of mercury, under constant mercury selection or pulsed mercury selection at varying time intervals (i.e. every 2, 4, 8 or 16 transfers). After 16 transfers of these selection regimes, all populations were propagated for a further 16 transfers in the absence of Hg^{2+} to test the effect of prior evolution under the varying frequencies of pulsed positive selection on longer-term plasmid stability. Throughout the experiment, we tracked plasmid prevalence and the frequency of phenotypes associated with a previously described mechanism of compensatory evolution in this bacteria–plasmid interaction.

2. Material and methods

(a) Strains and culture conditions

Experiments used *P. fluorescens* SBW25 [22] differentially marked with either gentamicin resistance (Gm^R) or streptomycin resistance + lacZ (Sm^RlacZ) cassettes [7,23] allowing them to be distinguished on selective agar plates as previously described [7,19]. pQBR103 was conjugated into the Gm^R background using standard methods [19,24]. All experiments were conducted in 6 ml King's B (KB) broth in 30 ml microcosms shaking at 180 r.p.m. and incubated at 28°C. The carrying capacity of KB microcosms is approximately 1×10^{10} colony forming units ml^{-1}; electronic supplementary material, figure S1.

(b) Selection experiment

Independent overnight cultures of plasmid-bearing, mercury resistant (Hg^R) and plasmid-free, mercury sensitive (Hg^S) strains were mixed at a 1 : 1 ratio and 60 µl (approx. 10^9 cells ml^{-1}) were used to inoculate treatment microcosms. Six replicate populations were established for each mercury treatment. Populations were propagated by 1% serial transfer every 48 h for a total of 32 transfers. Two 'constant' treatments were established with either 0 or 40 µM $HgCl_2$ added at each transfer. In the four pulsed treatments, populations were grown without mercury except for 40 µM $HgCl_2$ added every 2, 4, 8 or 16 transfers. After 16 transfers addition of $HgCl_2$ was stopped and all populations were propagated in 0 µM $HgCl_2$ for a further 16 transfers to measure plasmid stability in the absence of selection. Every two transfers population densities of each marker background were determined by diluting and plating onto KB agar supplemented with 50 µg ml^{-1} X-gal and 5% powdered milk solution. In addition, frequency of the Hg^R phenotype was determined by selective plating onto KB agar supplemented with 40 µM $HgCl_2$ and 50 µg ml^{-1} X-gal and 5% milk. The addition of milk powder allowed us to determine the frequency of *gacA*/*gacS* mutants (Gac^-) in the populations. Previously, it was shown that loss of function mutation to the *gacA*/*gacS* bacterial regulatory system is the main mechanism of compensatory evolution in this system ameliorating the cost of pQBR103 carriage in *P. fluorescens* SBW25 [19]. The gacA/gacS system positively regulates expression of an extracellular protease allowing colonies of wild-type Gac^+ SBW25 to digest a halo zone of clearing around the colony on milk plates [25], allowing Gac^+ phenotypes to be easily distinguished from Gac^- mutants, which cannot form the halo. The frequency of transconjugants was determined by scoring Sm^RlacZ marked cells that grew on Hg^{2+} plates, forming a blue colony. To check that Hg^R colonies were unlikely to have arisen by mutation, we quantified the frequency of spontaneous Hg^R mutations against 40 µM Hg^{2+}, using the fluctuation test assay protocol described in [26]. We never detected any spontaneous Hg^R mutants, strongly suggesting mercury resistance requires the *mer* operon and could not have evolved *de novo* in our experiments.

At the end of the experiment 24 Hg^R clones from each population were isolated and colony polymerase chain reaction (PCR) was used to test whether the plasmid was still present or whether it was lost following chromosomal acquisition of the resistance genes. PCRs targeted *oriV* (for: 5′-TGCCTAATCGTGTGTAATGTC-3′ and rev: 5′-ACT CTGGCCTGCAAGTTTC-3′) to determine the presence of the plasmid-backbone and *merA* (for: 5′-TGAAA GACACCCCCTA TTGGAC-3′) and rev: 3′-TTCGGCGACCA GCTTGATGAAC-3′) to determine the presence of the *mer* operon.

(c) Statistical analysis

All analyses were conducted in R statistical package v. 3.1.3 [27]. Packages used were 'nlme' and 'userfriendlyscience'. For all analyses of Hg^R plasmid dynamics the mercury-free treatment was removed so that mercury treatments were compared to one

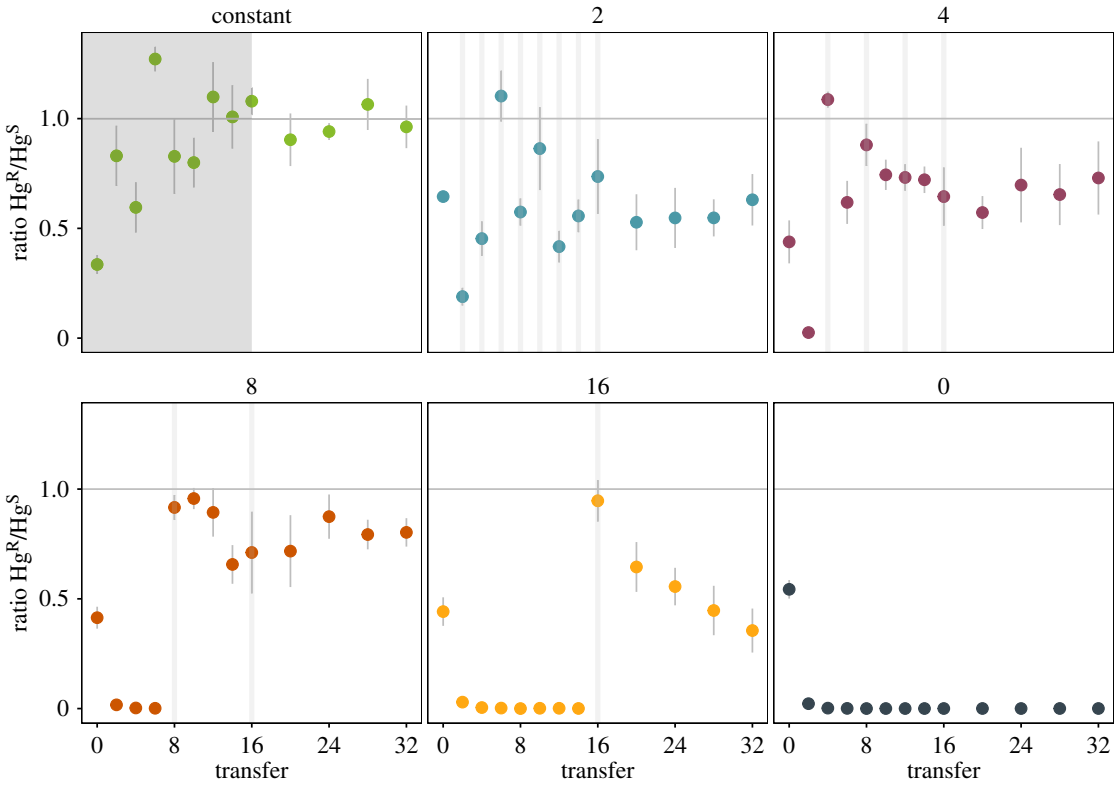

Figure 1. Pulses of mercury selection maintain pQBR103. The proportion of Hg^R (ratio of Hg^R counts over Hg^S counts) was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Points represent means ± standard errors of six replicate populations. Colours represent each pulsed mercury treatment.

another. Comparisons across the mercury pulsed treatments looking at average prevalence of Hg^R, average proportion of transconjugants, proportion of Gac^- phenotypes at T_{16}, time to first Gac^- mutant and average Gac^- frequency over time were analysed using Welch's ANOVA with mercury treatment as a fixed effect to adjust for non-homogeneous variance across treatments. Comparisons of Gac^- dynamics across plasmid-bearing and plasmid-free populations were analysed using Welch's ANOVA with the presence of plasmid as a fixed effect. Maintenance of Hg^R over time between T_{16} and T_{32} was analysed using linear mixed effects models with mercury treatment and time as fixed effects, and random effects of population on intercept and slope to account for repeated sampling of populations through time. Fixed effects were assessed using likelihood ratio tests on nested models.

3. Results

(a) Hg^R plasmid dynamics varied between mercury treatments

Populations were propagated for 16 transfers either without mercury, with mercury addition every transfer (constant mercury) or in pulsed treatments where mercury exposure occurred at varying time intervals (i.e. every 2, 4, 8 or 16 transfers), and the frequency of Hg^R was measured every second transfer. In all treatments where Hg^R was detected, PCR analysis on endpoint clones revealed that Hg^R remained associated with the plasmid (i.e. we did not detect any mutants which had acquired chromosomal *mer* and lost the plasmid-backbone). In the mercury-free treatment, Hg^R cells harbouring pQBR103 were rapidly outcompeted by plasmid-free Hg^S cells, as expected based on the known fitness cost associated with carrying pQBR103 [19] (figure 1). By contrast, under constant mercury selection Hg^R was maintained at high prevalence in all populations. During the first 16 transfers Hg^R prevalence varied across pulsed treatments, such that mean prevalence averaged over time was significantly higher under more frequent pulses (electronic supplementary material, figure S2; effect of mercury treatment: $F_{4,25} = 55.77$, $p < 0.001$).

In all pulsed mercury treatments, plasmid prevalence declined prior to the initial mercury pulse. However, in all cases, a single mercury pulse was sufficient to sweep Hg^R to high frequencies, such that by transfer 16, by which time every pulsed treatment had experienced at least 1 mercury pulse, Hg^R was at high frequency in all populations and did not differ significantly between pulsed treatments (effect of mercury treatment: $F_{4,25} = 1.77$, $p = 0.166$). The increase in Hg^R frequency was particularly striking in populations from the treatment with the lowest frequency of mercury pulse (i.e. single pulse at T_{16}) where, prior to the pulse, Hg^R was virtually undetectable (figure 1). Together these results demonstrate across the first 16 transfers, that higher-frequency pulses of positive selection favoured high plasmid prevalence and also that even rare positive selection events could boost plasmid persistence, at least in the short term.

(b) Compensatory evolution occurred across all mercury treatments

We screened the Hg^R fraction of each population to determine the presence of phenotypes associated with compensatory evolution. In this bacteria–plasmid interaction, we have previously described a mechanism of compensatory evolution associated with the loss of function in the bacterial *gacA*/*gacS*

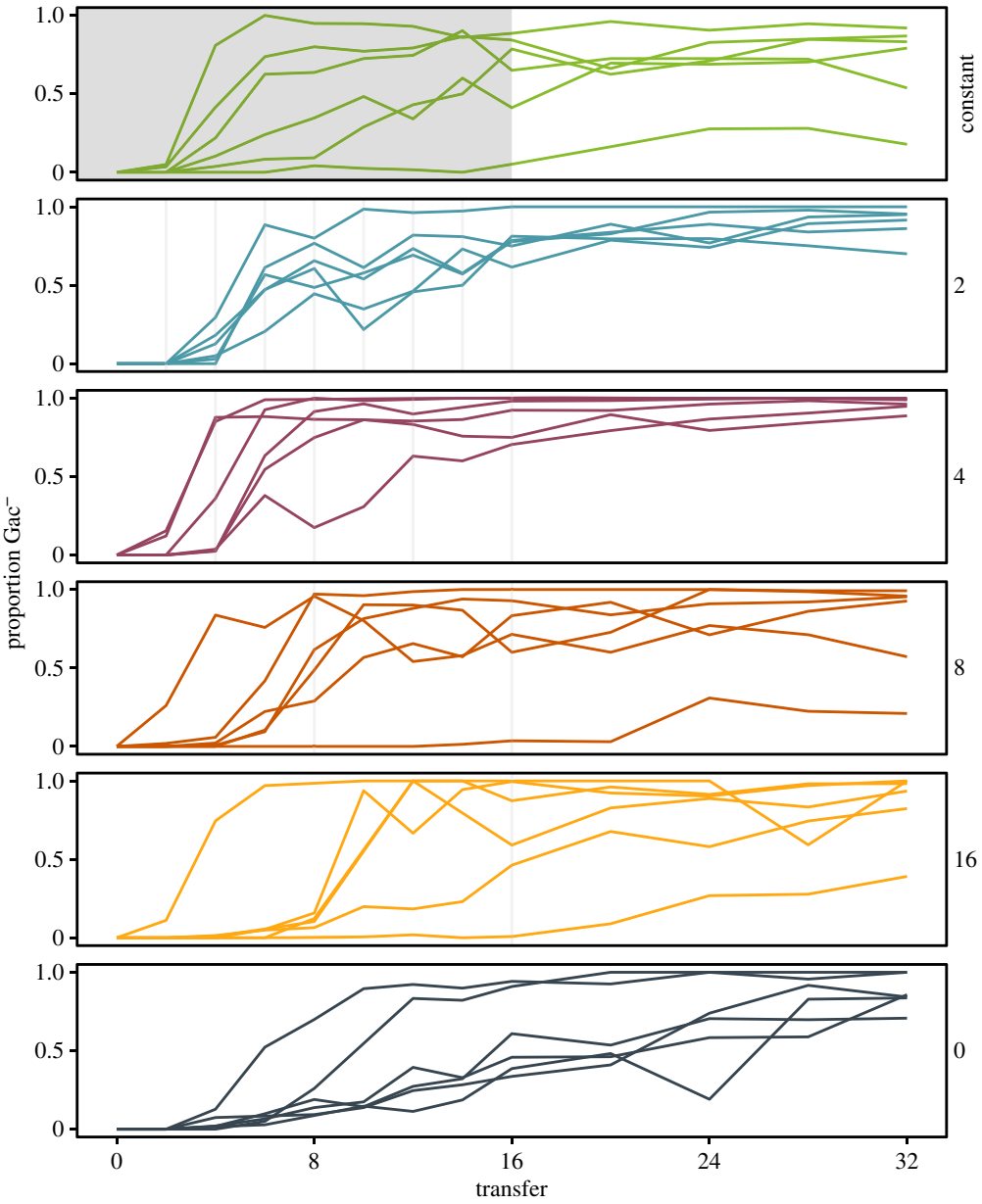

Figure 2. Gac mutations sweep through all Hg^R populations regardless of selective regime. The proportion of Gac^- phenotypes within the Hg^R population was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Lines represent the six replicate populations. Colours represent each pulsed mercury treatment.

two-component regulator [19]. The *gacA*/*gacS* system is encoded by the bacterial chromosome and controls the expression of genes involved in a broad range of biological functions including secondary metabolism, virulence and motility [25,28]. Addition of milk powder to agar plates allowed us to screen for Gac^- phenotypes: cells carrying *gacA*/*gacS* compensatory mutations were unable to produce the extracellular proteases capable of digesting milk. We, therefore, used this phenotype to compare the frequency of Gac^- phenotypes between treatments. Gac^- phenotypes arose in both plasmid-bearing and plasmid-free cells (shown in figure 2 and electronic supplementary material, figure S3, respectively). This is not necessarily surprising given that *gacA*/*gacS* loci are known to have an elevated mutation rate relative to the genome as a whole [29]. Among the plasmid-bearers we found that Gac^- phenotypes appeared rapidly in all mercury treatments and were maintained for the duration of the experiment (figure 2). This was not observed in plasmid-free control populations (electronic supplementary material, figure S3), where Gac^- phenotypes appeared later (plasmid-bearing versus plasmid-free: $F_{1,10} = 62.8$, $p < 0.001$), and remained at significantly lower frequency (plasmid-bearing versus plasmid-free: $F_{1,10} = 17.06$, $p = 0.002$). This is consistent with our previous data showing that deletion of *gacA*/*gacS* was only beneficial in cells with the pQBR103 plasmid, but had no significant fitness effects in plasmid-free SBW25 [19]. Within plasmid-containing treatments there was no significant effect of mercury treatment on Gac^- frequency in the plasmid-bearing population over the selective period of the experiment (i.e. averaged over transfer 1–16) (effect of mercury treatment: $F_{5,30} = 1.99$, $p = 0.108$) or the proportion Gac^- mutants at T_{16} (effect of mercury treatment: $F_{4,25} = 0.99$, $p = 0.433$), suggesting that amelioration of the plasmid cost was strongly favoured across all conditions regardless of mercury exposure [19]. Furthermore, there was no significant effect of mercury treatment on time taken for Gac^- mutants to arise: Gac^- phenotypes arose rapidly across all the plasmid-bearing populations (effect of mercury: $F_{5,30} = 0.74$, $p = 0.598$).

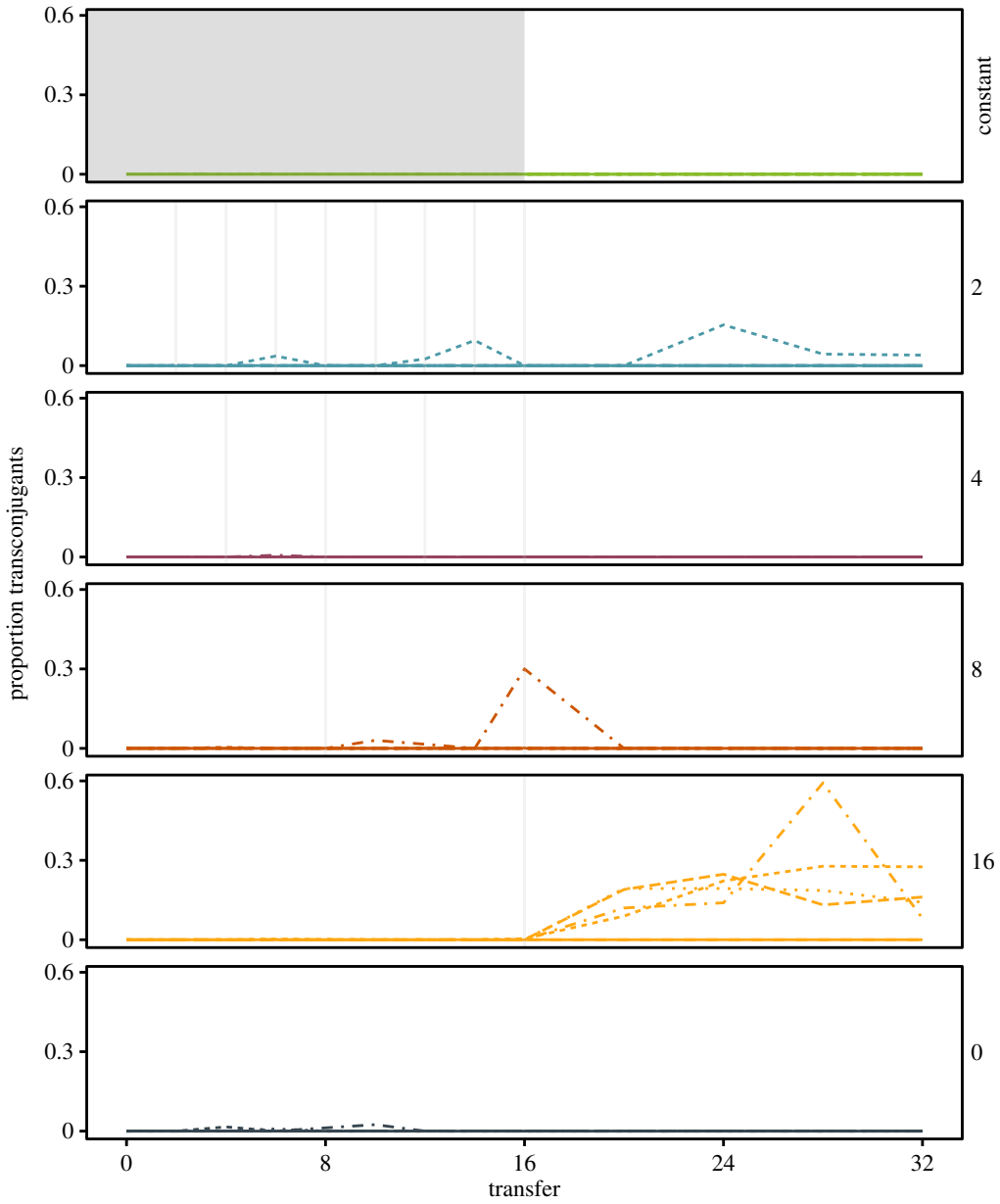

Figure 3. Infrequent pulses promote plasmid transfer into Hg^S recipients. The proportion of transconjugants within the Hg^R population was determined over time across the six selection treatments (constant mercury, mercury pulsed every 2, 4, 8 and 16, and absence of mercury). Grey bars indicate transfers where mercury was applied. Lines represent the six replicate populations. Colours represent each pulsed mercury treatment.

(c) Infrequent pulses promoted plasmid transfer into Hg^S recipients

Theory suggests that longer intervals between pulses of selection may favour conjugative plasmid transfer [13]. This occurs by allowing the survival and propagation of plasmid-free Hg^S bacteria which can then act as recipient hosts for the plasmid [30]. The frequency of transconjugants across each population revealed that the level of conjugative plasmid transfer significantly increased with decreasing frequency of pulsed mercury selection (figure 3; data for individual replicate populations shown in the electronic supplementary material, figure S5; effect of mercury treatment: $F_{4,25} = 7.19$, $p = 0.001$). This is likely to have been driven by frequent mercury pulses reducing the frequency of plasmid-free cells (electronic supplementary material, figure S1), whereas less frequent mercury pulses allowed plasmid-free cells to rise to high frequency, allowing greater opportunity for conjugation from the remaining plasmid-bearing cells. Therefore, in treatments with rare pulses of positive selection, conjugation indeed appears to play a larger role in the persistence of Hg^R within populations.

(d) High-frequency pulses stabilized Hg^R plasmids over the longer term

After T_{16}, all populations were propagated without mercury, to test how adaptation to the various selection regimes had affected plasmid stability in the absence of positive selection. Hg^R stability varied according to the past frequency of pulsed positive selection (figure 1; time × mercury treatment: $\chi^2_4 = 13.92$, $p = 0.0076$). Comparisons revealed that this effect was largely driven by the populations subjected to a single mercury pulse at T_{16} ($b = -0.0327$, $t_{114} = -2.63$, $p = 0.0096$), where Hg^R steadily declined over time in the absence of mercury selection, whereas Hg^R was stable in populations from all the other pulsed mercury treatments.

4. Discussion

Understanding the conditions that favour the stability of conjugative plasmids is important for understanding bacterial evolution [8,10,11,13,31]. Most experimental studies of plasmid stability have used constant environmental conditions, yet in nature, bacteria inhabit environments that are likely to be temporally variable with pulses of positive selection for plasmid-borne traits [16,17,32]. While there have been theoretical studies of the impact of pulsed positive selection on conjugative plasmid stability [13], there have been few experimental tests (however, see [18] and [33] for studies on non-conjugative plasmids and integrases, respectively). Here, we show short- and longer-term effects of the frequency of pulsed positive selection on the stability of a mercury-resistance plasmid. In the short term, constant or frequent pulses of positive selection allowed plasmids to be maintained at higher prevalence, but even in treatments where the plasmid had declined to undetectable levels, the first pulse of positive selection was sufficient to sweep the plasmid to high prevalence. Surprisingly, the high plasmid prevalence observed under frequent pulses did not appear to affect the rate of compensatory evolution via loss of function mutations to the *gacA*/*gacS* pathway [19], which arose in all mercury environments. In the longer term, however, plasmids that only experienced a single pulse of positive selection did appear to be at a disadvantage: following the removal of positive selection, plasmids evolved under high frequency or constant positive selection remained at high prevalence, whereas plasmids evolved under the lowest frequency of positive selection declined.

Previous theoretical analysis of plasmid stability predicted that horizontally transferable, plasmid-encoded resistance would be favoured over chromosomally encoded resistance by rare pulses of strong positive selection [13]. This is predicted to occur because plasmid-free cells, which pay no cost of carrying the resistance gene, can outcompete both plasmid-encoded and chromosomally encoded resistant genotypes in the intervals between pulses of positive selection. While this leads to the loss of chromosomal resistance, plasmid-encoded resistance can transfer by conjugation into the population of plasmid-free cells, and these transconjugants may then sweep to high frequency following the next pulse of positive selection [13]. Although we did not observe the emergence of chromosomally encoded resistance in our study, even though this outcome is possible in our experimental system [19], we did observe the out-competition of plasmid-bearers by plasmid-free cells during long intervals between infrequent pulses of positive selection. Moreover, consistent with the prediction of the model [13], under the lowest frequency of pulsed positive selection we observed a significantly higher proportion of transconjugant cells during the experiment, suggesting that conjugation played a more important role in the persistence of the plasmid where positive selection was rarest. This is consistent with previous work which demonstrated that conjugation played a larger role in the maintenance of the Hg^R plasmid pQBR57 in the absence, rather than presence, of positive mercury selection [30]. The balance of vertical versus horizontal transmission of genes determines population genomic structure and thus the evolutionary potential of populations to changing environmental conditions [34]. As plasmids can spread to a wide range of hosts [35], our finding that infrequent pulses of positive selection favoured horizontal transfer via conjugation suggests that we may expect to observe functional genes in a broader range of bacterial species when positive selection is a rare event [30,36].

Contrary to our prediction, based on recent theory and experimental data [18], we did not observe higher rates of compensatory evolution (via loss of function mutation to the *gacA*/*gacS* pathway) under higher-frequency pulsed positive selection even though such environments did support higher plasmid prevalence. By contrast, we observed that compensatory phenotypes evolved rapidly and rose to high frequency among plasmid-bearers across all our mercury environments. Compensatory evolution in this bacteria–plasmid interaction is associated with loss of function in the *gacA*/*gacS* two-component regulatory system [19], which activates the expression of a wide range of secondary metabolism and secreted products [25,28]. Consistent with our findings here, it was previously found that Gac^- mutants arose in parallel across a wide range of mercury concentrations, suggesting that neither the strength nor the frequency of positive selection has a major effect on the process of compensatory evolution in this system [19]. A potential explanation for this widespread prevalence of compensatory evolution across the range of positive selective environments, is that *gacA*/*gacS* appear to be contingency loci in *P. fluorescens* [37], i.e. loci with an elevated mutation rate relative to the rest of the genome [29]. Consequently, the abundant supply of compensatory mutations in this system may obscure any effect of the frequency or strength of positive selection. It is likely that loss of *gacA*/*gacS* function may be detrimental in more complex, natural environments, where the suite of genes activated within the gac regulon perform important functions, notably associated with host colonization and interspecific competition including the production of toxins and antibiotics [25,28]. Under such conditions, where expression of the Gac regulon is advantageous, the bacteria–plasmid assemblage would be forced to find alternative mechanisms of amelioration, and the frequency of pulsed positive selection may have a stronger effect on the rate of compensatory evolution.

Interestingly, we observed contrasting longer-term effects of the history of positive selection on the fate of plasmids following removal of positive selection. Unlike plasmids evolved under high-frequency pulses of positive selection, plasmids evolved under the lowest frequency of pulsed positive selection declined in prevalence in mercury-free environments. This cannot be explained by a lack of compensatory evolution (via loss of function mutation to the *gacA*/*gacS* pathway), because we observed compensatory phenotypes at high frequency among plasmid-bearers in all mercury selection environments. At present we do not know the evolutionary mechanism driving this effect. However, one possibility is that where plasmids have very recently swept from very low (in some cases undetectable) frequency, these lineages may be poorly adapted compared with the plasmid-free cells. This could arise because, until the recent pulse of mercury selection, the plasmid-free lineage had been at far higher population density than the plasmid-bearers and therefore had access to a higher mutational supply allowing them greater opportunity to adapt to the abiotic environment [38].

Pulsed positive selection is likely to be a common feature of both environmental contamination and clinical antibiotic treatments, such that positive selection for plasmid-encoded traits is likely to be temporally heterogeneous [15–17]. Our findings suggest that this is likely to have both short- and longer-term effects on plasmid stability. High-frequency pulsed positive

selection increases plasmid prevalence and promotes the longer-term survival of plasmids in bacterial populations in the absence of positive selection, whereas low-frequency pulsed positive selection increases the importance of horizontal gene transfer and may lead to plasmid-encoded functional genes spreading into, and subsequently being selected in, a greater diversity of bacterial hosts. Crucially, we show how even very rare periods of positive selection can be sufficient to sweep plasmids from undetectable levels to high frequency. Thus plasmids need not be present at high frequency to have an impact on bacterial evolution in temporally heterogeneous environments, because even vanishingly rare plasmids can enhance the responsiveness of bacterial populations to changing and uncertain conditions [39].

Data accessibility. Data deposited to Dryad (http://dx.doi.org/10.5061/dryad.1cd62) [40].

Authors' contributions. C.S., J.P.J.H., E.H. and M.A.B. conceived the study and designed the experiments; C.S. performed the experiments and analysed the data; J.P.J.H., E.H. and M.A.B. supervised the experimental work; C.S., J.P.J.H., E.H. and M.A.B. drafted the manuscript.

Competing interests. The authors declare no competing interests.

Funding. This work was supported by funding from the European Research Council awarded to M.A.B. (StG-2012-311490-COEVOCON), a Leverhulme Prize from the Leverhulme Trust awarded to M.A.B. (PLP-2014-242), an NERC Research Fellowship awarded to E.H. (NE/P017584/1) and an NERC ACCE DTP studentship awarded to C.S. (NE/L002450/1) supervised by M.A.B.

Acknowledgement. We thank A. J. Wood, V. Friman and J. Pitchford for their valuable comments.

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