Resolving the plasmid paradox: costs and benefits of horizontal gene transfer in a community context

by

Anastasia Kottara

A thesis submitted for the degree of
Doctor of Philosophy

in the
University of Sheffield
Department of Animal and Plant Sciences

June 2018

Abstract

Horizontal gene transfer (HGT) is a central evolutionary process enhancing genome diversification and rapid adaptation of species to new environmental conditions. Mobile genetic elements (MGE) facilitate genetic exchange between species through HGT by carrying accessory gene cargos encoding beneficial traits such as resistance to metals and antibiotics. MGE-mediated transfer of antibiotic resistance genes between species in natural microbial communities has contributed to the global spread of antibiotic resistance. It is therefore essential to understand the ecological drivers of the maintenance and transmission of MGEs in bacterial communities. Here I use conjugative plasmids as an example MGE to study the ecological and evolutionary dynamics of plasmids in bacterial populations and communities across a range of environments. First, I demonstrate that plasmids selected in a single-host environment evolved host specialism due to fitness trade-offs, whereas plasmids evolved in a multi-host environment could overcome this trade-off to evolve host-generalism. Secondly, I show that the costs and benefits of plasmid carriage and the long-term dynamics of the plasmid and the mercury resistance transposon it encodes varied extensively between diverse species of *Pseudomonas*. I next show that plasmid maintenance was facilitated by compensatory evolution to ameliorate the cost of plasmid carriage. Compensatory loci varied between species, with parallel mutations targeting different regulatory and biosynthetic pathways in each species. Lastly, I examine the effect of community structure on plasmid dynamics in simple bacterial communities. When plasmids were carried by proficient plasmid-donor species this led to higher plasmid abundance at the community-level, while in diverse communities, plasmid transmission could be impeded through the dilution effect, limiting plasmid spread. This thesis demonstrates that plasmid dynamics in bacterial communities are determined by the combination of ecological and evolutionary processes, depending on the selective environment, the structure of the bacterial community and variation among species in their proficiency to host plasmids and to undergo compensatory evolution to ameliorate their costs. These data highlight the importance of studying plasmid dynamics in a community-context.

Acknowledgements

I would have never been able to complete my PhD without the guidance of my supervisors in Sheffield and York, along with the help and support of my friends and family.

Firstly, I would like to share my deep appreciation of my supervisor Michael Brockhurst for giving me the opportunity to work on this project but also for his assistance, support and time. I am also grateful to Maggie Smith for her support as a co-supervisor at the University of York and her prompt help whenever it was needed.

I would also like to thank Daniela Barillá and Ville-Petri Friman for their valuable comments and suggestions during the first two years of my PhD at the University of York.

Furthermore, I owe special thanks to Ellie Harrison and James Hall for their time and generous help. I would also like to thank all members of Brockhurst lab because we have shared the best moments over the last three years. Working together has been my pleasure, and provided an amazing opportunity to grow both personally and professionally.

Last, but not least, I would like to express my deepest thanks to my family, friends and Francisco for being the driving force behind the work I accomplished. Their love inspired me to persevere through the tough times, and I owe them far more than I can express.

Anastasia Kottara

4th June, 2018

Contents

Abstract **i**

Acknowledgements **ii**

List of Figures **vi**

List of Tables **viii**

Abbreviations **ix**

1 Introduction **1**
1.1 Conjugative plasmids 2
1.2 Plasmid fitness costs and benefits 6
1.3 Solving the plasmid-paradox 8
1.3.1 Compensatory evolution 8
1.3.2 Interspecific conjugation 10
1.4 Aim and research questions 11
1.5 Thesis outline 11

2 Multi-host environments select for host-generalist conjugative plasmids **14**
2.1 Introduction 14
2.2 Materials and Methods 15
2.2.1 Bacterial and plasmid strains 15
2.2.2 Selection experiment 16
2.2.3 Conjugation assay 17
2.2.4 Competitive fitness assay 17
2.2.5 Statistical analyses 17
2.3 Results and Discussion 18
2.4 Conclusions 22

3 Variation in mobile genetic element dynamics across diverse *Pseudomonas* host species **23**

3.1 Introduction . . . 23
3.2 Materials and Methods . . . 25
3.2.1 Bacterial strains and culture conditions . . . 25
3.2.2 Competitive fitness assay . . . 27
3.2.3 Selection experiment . . . 27
3.2.4 Statistical analyses . . . 28
3.3 Results . . . 29
3.3.1 Plasmid fitness costs varied between *Pseudomonas* species . 29
3.3.2 Variable dynamics of mercury resistance over time . . . 30
3.3.3 Variation in plasmid and transposon dynamics over time . . 32
3.4 Discussion . . . 33
3.5 Conclusions . . . 37
3.6 Supplementary Information . . . 38

4 Diverse compensatory evolution across *Pseudomonas* species following acquisition of a large conjugative plasmid 39
4.1 Introduction . . . 39
4.2 Materials and Methods . . . 41
4.2.1 Bacterial strains and culture conditions . . . 41
4.2.2 Competitive fitness assay . . . 42
4.2.3 Minimum inhibitory concentration assay . . . 42
4.2.4 Genome analysis . . . 43
4.2.5 Statistical analyses . . . 43
4.3 Results . . . 44
4.3.1 Relative fitness of evolved clones . . . 44
4.3.2 Resistance phenotypes of evolved clones . . . 45
4.3.3 Parallel evolution in chromosomal global regulatory and metabolic systems . . . 47
4.4 Discussion . . . 51
4.5 Conclusions . . . 53
4.6 Supplementary Information . . . 55

5 Community-level plasmid dynamics are governed by the proficiency of the plasmid-donor species 61
5.1 Introduction . . . 61
5.2 Materials and Methods . . . 63
5.2.1 Bacterial strains and plasmid . . . 63
5.2.2 Selection experiment . . . 64
5.2.3 Statistics . . . 65
5.3 Results . . . 66
5.3.1 Donor species identity affects community-level plasmid abundance . . . 66
5.3.2 Species-level plasmid dynamics within communities . . . 67
5.3.3 Diversity within the plasmid-donor communities . . . 72
5.4 Discussion . . . 73

5.5 Conclusions 75

6 The dilution effect limits plasmid co-infection in diverse bacterial communities 76
6.1 Introduction 76
6.2 Materials and Methods 78
6.2.1 Bacterial strains and plasmid 78
6.2.2 Selection experiment 78
6.2.3 Competitive fitness assay 80
6.2.4 Conjugation assay 81
6.2.5 Statistics 81
6.3 Results 82
6.3.1 Plasmid co-infection limited in community 82
6.4 Discussion 86
6.5 Conclusions 88

7 Discussion 89

A Appendix 94

Bibliography 95

List of Figures

1.1 Organisation of conjugative plasmids, pQBR103 and pQBR57 that were used in the thesis 5

2.1 Conjugation rate over time for plasmids in the single-host and multi-host treatments 19
2.2 Selection rate of *P. fluorescens* or *P. putida* carrying evolved plasmids from the single and multi-host treatments 20

3.1 Phylogenetic tree of *Pseudomonas* species used in this experiment . 26
3.2 Fitness of *Pseudomonas* species carrying the plasmid measured as selection rate in a mercury regime ranging from 0-60 μM mercury(II)chloride 30
3.3 Frequency of mercury resistance in the parallel evolving clonal populations in absence and presence of mercury throughout the selection experiment 31
3.4 Population density and tracked mercury resistance transposon, Tn5042 and plasmid density in the evolving *Pseudomonas* species . 33
3.5 Growth of the ancestral plasmid-free *Pseudomonas* species in mercury environment ranging from 0-60 μM mercury(II)chloride . . 38
3.6 Growth of the ancestral plasmid-bearing *Pseudomonas* species in mercury environment ranging from 0-135 μM mercury(II)chloride . 38

4.1 Fitness of the ancestral and evolved, plasmid-free and plasmid-bearing *Pseudomonas* species 45
4.2 Growth of the ancestral and evolved plasmid-bearing *Pseudomonas* species in mercury environment ranging from 0-135 μM mercury(II)chloride 46
4.3 Growth of the ancestral and evolved plasmid-free *Pseudomonas* species in mercury environment ranging from 0-60 μM mercury(II)chloride 46
4.4 Summary of mutations identified in the evolved plasmid-bearing *Pseudomonas* species in absence and presence of mercury 48
4.5 *Pseudomonas* species with ortholog genes of the parallel mutated genes across the plasmid-treatments 50
4.6 Number and type of mutations identified in each sequenced *Pseudomonas* species 55
4.7 Mutations identified in the evolved pQBR103 plasmid 55

4.8 Mutations identified in the evolved *P. fluorescens* SBW25 56
4.9 Mutations identified in the evolved *P. savastanoi pv. phaseolicola* 1448A 57
4.10 Mutations identified in the evolved *P. stutzeri* JM300 (DSM 10701) 57

5.1 Total plasmid density in the community throughout the selection experiment 67
5.2 Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. fluorescens* 69
5.3 Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. stutzeri* 70
5.4 Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. putida* 71
5.5 Diversity of plasmid-bearer species in each plasmid-donor community 72

6.1 Plasmid ratio and plasmid profile in *P. fluorescens* SBW25 83
6.2 Relative fitness of *P. fluorescens* (pQBR103:pQBR57) in absence and presence of the five-species community 84
6.3 Plasmid density of the background community at the end of the experiment 85
6.4 Conjugation rate of *P. fluorescens* (pQBR103:pQBR57), *P. fluorescens* (pQBR103) and *P. fluorescens* (pQBR57) in high, medium and low resource media 86

List of Tables

3.1 Primers used for the PCR screening of the mercury resistance genes and the plasmid pQBR103. 28

4.1 Description of the genes where mutations identified in the evolved *P. fluorescens* SBW25 58
4.2 Description of the genes where mutations identified in the evolved *P. savastanoi pv. phaseolicola* 1448A 59
4.3 Description of the genes where mutations identified in the evolved *P. stutzeri* JM300 (DSM 10701) 60

6.1 Primers used for the PCR screening of the mercury resistance genes and the plasmids pQBR57 and pQBR103. 80

A.1 Overview of the *Pseudomonas* species used in this thesis 94

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
DNA	**D**eoxyribo **N**ucleic **A**cid
Gm	**G**entamicin
GTA	**G**enetic **T**ransfer **A**gents
HGT	**H**orizontal **G**ene **T**ransfer
ICE	**I**ntegrative **C**onjugative **E**lements
IS	**I**nsertion **S**equence
kb	**k**ilobase
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
PCR	**P**olymerase **C**hain **R**eaction
SEM	**S**tandard **E**rror **M**ean
Sm	**S**treptomycin
SNP	**S**ingle **N**ucleotide **P**olymorphism

Chapter 1

Introduction

Natural microbial communities typically contain a huge diversity of bacterial species representing an enormous pool of genetic information [Fraser et al., 2009]. This genetic information can be vertically transmitted from parent to offspring cells via binary fission, or horizontally transmitted from donor to recipient cells via horizontal gene transfer (HGT) [Lawrence and Hendrickson, 2003]. HGT enhances evolutionary potential through genetic recombination and sharing of important ecological traits, such as antibiotic and metal resistance, between lineages [Aminov, 2011]. While HGT can occur solely via the action of the bacteria themselves through transformation–acquiring and integrating naked DNA from the environment–the majority of HGT is believed to rely on mobile genetic elements (MGE) to act as vectors transmitting DNA between cells [Ochman et al., 2000; Stokes and Gillings, 2011]. MGEs–including, conjugative plasmids and elements [Norman et al., 2009; Wozniak and Waldor, 2010], temperate bacteriophages [Canchaya et al., 2003a], transposons and other phage-like elements such as the genetic transfer agents (GTA) [Lang et al., 2012]–are common feature of bacterial genomes and widespread across a range of natural environments [Frost et al., 2005]. MGEs allow HGT to occur between phylogenetically distantly related bacterial species [Frost et al., 2005], thus promoting genetic diversification of species [Gogarten et al., 2002] and accelerating their evolutionary innovation to allow colonisation of new

environments with important ecological implications [Wiedenbeck and Cohan, 2011]. An example of the applied importance of HGT is the spread of antibiotic resistance genes in microbial populations, which is currently of major clinical concern [von Wintersdorff et al., 2016].

Two major modes of HGT rely on MGE vectors to transmit DNA between cells, namely transduction by temperate phages and conjugation by plasmids or other conjugative elements [Thomas and Nielsen, 2005]. Cell-to-cell contact is not required for transduction because phages can survive autonomously between infections in the environment, whereas during conjugation the donor and recipient cells are connected by a conjugative pilus through which the conjugative plasmid or element and its accessory gene cargo is transmitted [Thomas and Nielsen, 2005]. Temperate phages often encode accessory genes that can be beneficial for the growth and virulence of their host cell [Roossinck, 2011]. However, compared with conjugative plasmids, temperate phages are limited in their genome size as they can only carry genomes that can be packaged in the phage capsid, limiting the size of typical temperate phage to <150 kb [Canchaya et al., 2003b]. By contrast conjugative plasmids can encode a larger number and greater diversity of accessory genes [Sen et al., 2011]. In this thesis I have used conjugative plasmids as a model to study MGE population dynamics in single and multi-host environments, thus the following sections are focused on plasmid biology.

1.1 Conjugative plasmids

Plasmids are extrachromosomal DNA molecules commonly found in prokaryotic genomes, ranging in size from a few kilobase pairs (kb) to over a megabase (Mb) [Norman et al., 2009]. Plasmids can be grouped based on their mobility into non-transmissible, mobilisable and conjugative plasmids [Smillie et al., 2010]. Whereas non-transmissible plasmids can only be vertically transmitted during cell division, mobilisable and conjugative plasmids can be both vertically and

horizontally transmitted between cells [Smillie et al., 2010]. Conjugative plasmids can be autonomously horizontally transmitted via conjugation, whereas mobilisable plasmids lack the propagation system of conjugative plasmids thus rely on hitchhiking on other conjugative elements for their horizontal transmission [Smillie et al., 2010]. Conjugative plasmids are usually larger than non-transmissible plasmids (R338 is the smallest known conjugative plasmid ∼34 kb) [Norman et al., 2009]. Conjugative plasmids are organised with a backbone that encodes genes for the plasmid's own replication, propagation and stability; in addition, most plasmids also encode suites of accessory genes of potential ecological benefit to the host cell [Norman et al., 2009, Figure 1.1].

Conjugative plasmid backbones typically encode replication mechanisms (*rep* and *cop* region) controlling replication and copy number in the host cell, stability and partitioning mechanisms (*mrs* and *par* region) that ensure their vertical transmission and segregational fidelity, and propagation mechanisms (*pil* region) allowing horizontal transmission [Norman et al., 2009]. During plasmid replication, the replication initiation proteins (Rep proteins) which are encoded by the plasmid (*rep* region), identify unique sequences at the origin of replication (*ori* region) where they bind and cleave one stand of the plasmid DNA while they recruit the host's DNA helicases and polymerases [Del Solar et al., 1998]. To control the plasmid replication, plasmids encode Cop proteins (*cop* region) which can repress the expression of the Rep proteins and restrict the replication mechanism in order to maintain a stable plasmid copy number [Norman et al., 2009]. However, large conjugative plasmids are often found in low copy number [Norman et al., 2009], thus plasmids also carry partitioning and segregational fidelity mechanisms to prevent their loss during cell division. The plasmid-encoded Par proteins (*par* region) are the modulator proteins of the plasmid active partitioning system that interact with the plasmid DNA to stabilise and position the plasmids in the offspring cells after the plasmid replication and cell division [Hayes and Barillà, 2006]. To further control the plasmid segregation, plasmids also encode site-specific recombination systems (multimer resolution systems, *mrs* region) that stabilise the plasmids by

resolving the excess of plasmid multimers [Summers et al., 1993]. Moreover, in order to control their horizontal transmission, conjugative plasmids carry mating pair formation systems (Mpf system-*pil* region) [Schröder and Lanka, 2005]. The Mpf system facilitates the horizontal transfer of plasmids by forming a bridge that consists of a protein complex and the conjugative pilus to bring in physical contact two bacterial cells during conjugation [Schröder and Lanka, 2005]. These mechanisms are believed to play an important role in determining the plasmid stability and host range [Norman et al., 2009].

Conjugative plasmids can be further categorised based on their host range. Their host range is defined by the number of host species that a plasmid can conjugate and replicate in [Jain and Srivastava, 2013]. For instance, plasmids such as IncP-1 and IncQ plasmids are capable of surviving in a broad range of hosts (broad host range; BHR) whereas plasmids such as IncFI and IncI1 only invade a narrow range of hosts (narrow host range; NHR) [Pukall et al., 1996]. This natural diversity in the breadth of plasmid host range is partly explained by the plasmid replication system and particularly the efficiency of plasmid Rep proteins to recruit host helicases, which determines establishment efficiency, while the partitioning and segregational mechanisms determine retention efficiency [Thomas and Nielsen, 2005]. In addition, BHR plasmids often encode multiple origins of replication, enabling their replication in a broader diversity of hosts [Jain and Srivastava, 2013].

In this thesis I focus on the pQBR group of plasmids, in particular, the conjugative plasmids pQBR57 and pQBR103. The pQBR plasmids are a group of 136 plasmids that were isolated during a long-term field experiment in Oxfordshire, UK, and represent an ecologically cohesive sample of the co-occurring plasmid community [Lilley et al., 1994, 1996; Lilley and Bailey, 1997]. pQBR plasmids are self-transmissible, conjugative, Group-I plasmids that were isolated by their ability to confer mercury resistance (Hg^R) from the bacterial population inhabiting the sugar beet rhizosphere and phyllosphere [Lilley et al., 1996]. Specifically, pQBR103 and pQBR57 were acquired by conjugation into labelled strains of *P. fluorescens* SBW25 and *P. putida* UWC1

that were introduced into the naturally occurring bacterial community colonising the sugar beet rhizosphere [Lilley et al., 1996]. These early experiments showed that different *Pseudomonas* species could act as hosts to the pQBR plasmids. Therefore, in this thesis, the population dynamics of pQBR57 and pQBR103 plasmids were examined by using diverse bacterial strains from across the *Pseudomonas* phylogeny as plasmid hosts (Table A.1- Appendix).

In common with other conjugative plasmids, pQBR plasmids are organised with a backbone carrying the essential genetic information for the plasmids' replication (*rep* and *oriV* region), propagation (*pil* and *tra* region) and stability (*par* region) and a range of accessory genes, including mercury resistance (*mer* operon), UV resistance (*uvr* genes), chemotaxis (*che* operon) and the radical SAM enzymes (*sam* operon) [Hall et al., 2015, Figure 1.1].

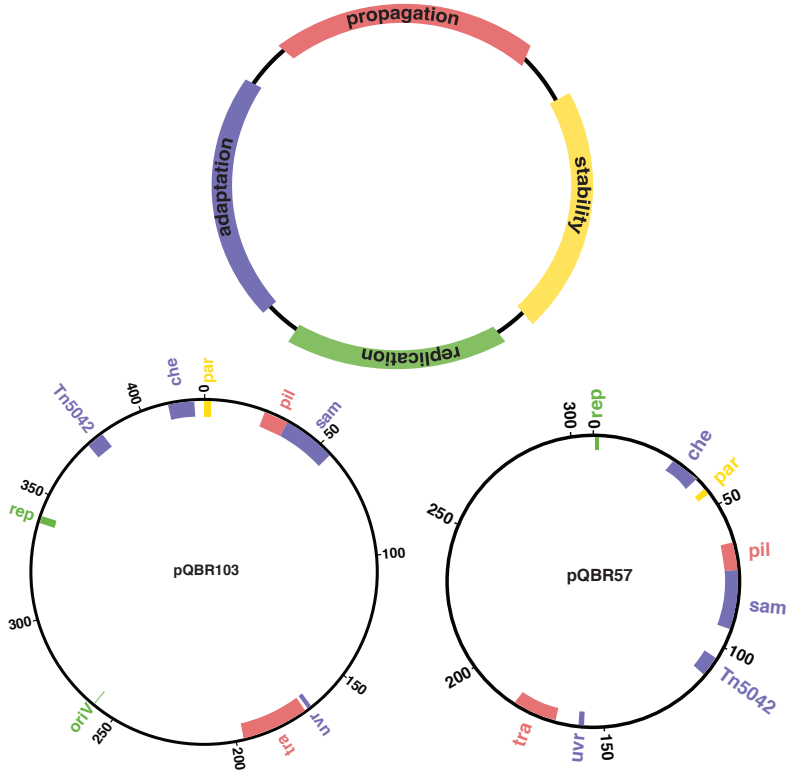

FIGURE 1.1: Organisation of conjugative plasmids, pQBR103 and pQBR57 that were used in the thesis-adapted from Norman et al. [2009] and Hall et al. [2015]. **Green (replication)**: *rep* region, *oriV*- origin of replication; **Yellow (stability)**: *par* region; **Red (propagation)**: *pil* region- conjugation, *tra* region-transfer; **Purple (adaptation)**; *uvr*- UV resistance genes, Tn5042 transposon- *mer* operon- mercury resistance, *sam*- radical SAM enzymes, *che*- chemotaxis operon.

Sequence analysis of pQBR103 (∼425 kb), the largest of the pQBR plasmids, showed that 80% of the predicted coding sequences were not associated with any known function and only a small percentage of the other 20% with predicted functions was associated with well-defined phenotypes, such as Hg and UV resistance [Tett et al., 2007; Slater et al., 2008]. Although pQBR plasmids have been found to vary in size, structure, conjugative ability and fitness effect to their hosts, sequence analysis revealed that pQBR55 and pQBR57 plasmids carry similar sets of accessory genes as pQBR103 [Hall et al., 2015]. As an example, *mer* operon is carried in Tn5042 transposon that has been found in all the sequenced pQBR plasmids although in different locations suggesting its horizontal acquisition [Hall et al., 2015]. These plasmids also share distant similarities with other megaplasmids isolated from pseudomonads such as the *P. aeruginosa* pOZ176 megaplasmid [Xiong et al., 2013; Hall et al., 2015].

1.2 Plasmid fitness costs and benefits

Plasmids are often considered to be 'selfish elements' as they have evolved to infect host cells to ensure their replication and existence [Bahl et al., 2009]. Consistent with this parasitic side to their lifestyle, maintaining a plasmid can have a negative effect on the fitness of the host organisms due to the physiological and energetic cost that plasmids impose on the host cell [Baltrus, 2013]. This cost can be related to different stages of the plasmid lifecycle [San Millan and MacLean, 2017]. Acquisition of a plasmid by conjugation typically induces the SOS response and increases transcriptional activity due to the increase in intracellular single-stranded DNA [Baharoglu et al., 2010; Fernandez-Lopez et al., 2014]. Once conjugative plasmids are established in the host cell, they can disrupt the host cell's homeostasis through the expression of plasmid-encoded genes and proteins for plasmid replication, conjugation and maintenance in the host cell [Sorek et al., 2007; Tuller et al., 2011], and interference with host gene regulation by plasmid-encoded regulatory genes

[Harrison et al., 2016], and potentially via integration of part or all of the plasmid into the host chromosome [Hall et al., 2017].

However, plasmids often confer beneficial traits to their hosts such as resistance to antibiotics [Svara and Rankin, 2011] and metals [Tett et al., 2007; Hall et al., 2015] and catabolic functions to degrade xenobiotics [Top and Springael, 2003] that can potentially provide fitness benefits under certain environmental conditions. Thus, the net fitness effects of plasmids on bacterial cells are dependent upon the environmental context, and this can form a continuum between mutualism and parasitism across environmental gradients [Levin, 1993; Bergstrom et al., 2000]. Mutualism occurs where the fitness benefit of accessory traits outweighs the cost of plasmid carriage, i.e. there is a net fitness benefit to the bacterium of carrying the plasmid, which results in positive selection for the beneficial traits that are carried by the plasmid [Dionisio et al., 2012]. However, mutualism does not guarantee plasmid survival because over longer evolutionary timescales it is expected that integration of the beneficial genes into the host chromosome will be favoured, allowing host cell to jettison the costly plasmid backbone entirely [Bergstrom et al., 2000]. Parasitism occurs in environments where the costs of plasmid carriage outweigh the fitness benefits of accessory traits, i.e. there is a net fitness cost to the bacterium of carrying the plasmid. Here, plasmids are selected against by purifying selection but may be able to be maintained as infectious elements if the rates of loss by segregation and purifying selection are overcome by the plasmid conjugation rate [Dionisio et al., 2002; Hall et al., 2016; Lopatkin et al., 2017; Stevenson et al., 2017], although whether most plasmids can achieve sufficiently high conjugation rates in nature has been questioned [Levin et al., 1979; Simonsen, 1991; Gordon, 1992; Lilley and Bailey, 2002]. It is thus difficult to understand how conjugative plasmids survive given that (1) plasmid maintenance is costly; (2) plasmids can be lost by segregation; (3) even if plasmids do carry beneficial genes, those genes can be captured by transfer to the chromosome, so that the plasmid is no longer required [Harrison and Brockhurst, 2012]. Plasmids, however, are abundant in bacterial communities independent of the selective environment

[Heuer and Smalla, 2012; Smalla et al., 2015] giving rise to a situation termed the 'plasmid paradox' [Harrison and Brockhurst, 2012].

1.3 Solving the plasmid-paradox

Experimental studies in a wide range of bacteria-plasmid interactions have tracked the dynamics of plasmids in bacterial populations. In such experiments, plasmids are often more stable than would be expected from simple ecological models, and two main mechanisms have been proposed allowing plasmid stability. First, compensatory evolution can reduce the cost of plasmid carriage, weakening purifying selection against the plasmid and allowing its maintenance [Dahlberg and Chao, 2003; Sota et al., 2010; San Millan et al., 2014; Harrison et al., 2015a; San Millan et al., 2015; Porse et al., 2016; Bottery et al., 2017; Loftie-Eaton et al., 2017]. Second, in bacterial communities, plasmids may be maintained through conjugation and in particular horizontal transmission of the plasmid between species [Dionisio et al., 2002; Hall et al., 2016]. In the following section I expand upon the evidence for these two mechanisms of plasmid stability.

1.3.1 Compensatory evolution

Over evolutionary time scales, the cost of plasmid acquisition can be ameliorated through compensatory evolution. Host compensatory evolution permitting plasmid persistence in absence of selection was first observed by Bouma and Lenski [1988]. Recent studies combining experimental evolution and a broad scale genomic analysis of the host and plasmid have determined the genetic basis of compensatory evolution. These studies reveal that compensatory evolution can occur via mutation of the chromosomal genes, plasmid genes or through co-evolution with mutations occurring on both the chromosome and the plasmid. Host compensatory evolution is associated in several studies with large-scale

changes in gene expression [Harrison et al., 2015a; San Millan et al., 2015]. For example, stable maintenance of pQBR103 by *P. fluorescens* SBW25 over 450 bacterial generations was permitted by loss of function mutations in the GacS/GacA global regulator system, which led to downregulation of ∼17% of chromosomal and plasmid genes [Harrison et al., 2015a]. *P. aeruginosa* PAO1 ameliorated the cost of carrying the non-conjugative plasmid pNUK37 through mutations inactivating one putative helicase (PA1372) and two protein kinases (PA4673.15 and PA4673.16) leading to down-regulation of the plasmid replication protein gene *rep* and 13% of the chromosomal genes [San Millan et al., 2014, 2015]. Similarly, Loftie-Eaton et al. [2017] showed that *Pseudomonas* sp. H2 ameliorated the cost of carrying the plasmid RP4 through mutations that inactivated two accessory helicases and the RNA polymerase β-subunit. Intriguingly, these compensatory mutations each affected loci encoding important factors for the intracellular metabolism and homeostasis.

Alternatively, amelioration of the plasmid cost can occur through evolution of the plasmid itself. Sota et al. [2010] described that the IncP-1β mini plasmid replicon was maintained in *S. oneidensis* MR-1 by ameliorating its cost of carriage via the inactivation of its replication initiation protein TrfA. While, more recently, Porse et al. [2016] reported that deletions on the IS26 region of the plasmid pKP33 could compensate the cost for the plasmid maintenance in both *E. coli* and *K. pneumonia*.

Finally, co-evolved mutations occurring on both the plasmid and the chromosome can be required to compensate for the plasmid-cost in some cases [Dahlberg and Chao, 2003; Loftie-Eaton et al., 2015; Bottery et al., 2017]. Dahlberg and Chao [2003] first described the amelioration of the plasmid-cost via plasmid-host co-adaptation following a long-term experiment wherein *E. coli* hosts were co-cultured with either plasmid R1 or plasmid RP4. Co-adaptation of the plasmid pMS0506 and *P. moraviensis* resulted in host chromosomal mutations and the transposition of the Tn6231 transposon encoding a toxin-antitoxin (TA) system from the native plasmid, pR28, to pMS0506, which also facilitated expansion of the pMS0506 host range [Loftie-Eaton et al., 2015]. Most recently,

Bottery et al. [2017] showed that both chromosomal and plasmid mutations were required to allow the evolution of increased tetracycline resistance at a reduced cost. Together these studies demonstrate the key role that compensatory evolution plays in promoting the stability of plasmids in bacterial populations.

1.3.2 Interspecific conjugation

An alternative mechanism for plasmid stability is infectious transmission, whereby the rate of conjugative transfer exceeds the rate of loss to miss-segregation and purifying selection. Persistence through infectious transmission can be particularly effective if it allows plasmids to jump into new genetic backgrounds, allowing plasmids to hitchhike beneficial mutations or ecotypes favoured by the prevailing environment [Bergstrom et al., 2000]. Thus infectious transmission is thought to be an especially important mechanism for plasmid stability in diverse bacterial communities [Dionisio et al., 2002; Bahl et al., 2009]. An example of this was demonstrated by Hall et al. [2016], where the highly conjugative plasmid, pQBR57 was sustained in a community through interspecific conjugation. Specifically, whereas *P. putida* KT2440 could not maintain pQBR57 when in monoculture, the plasmid could be maintained in the *P. putida* population when growing alongside *P. fluorescens* SBW25. Thus, interspecific conjugation of pQBR57 from a source species, *P. fluorescens*, to a sink species, *P. putida*, allowed sustained access to the plasmid across the community [Hall et al., 2016]. More recent studies have further highlighted the role of conjugation in plasmid transfer, with examples of infectious transmission allowing plasmid maintenance within bacterial populations without positive selection [Lopatkin et al., 2017; Stevenson et al., 2017]. Therefore, although, the rate of conjugation was originally thought to be too low for the plasmids to persist as infectious elements in microbial communities [Levin et al., 1979; Simonsen et al., 1990; Gordon, 1992], these recent studies clearly indicate that conjugation can be a successful mechanism for the plasmid transmission and maintenance among diverse microbial communities.

1.4 Aim and research questions

The aim of this thesis is to understand the ecological and evolutionary dynamics of plasmid-host interactions in multi-species communities. Specifically, I investigated the following research questions:

1. How does a plasmid adapt to a multi-host environment?
2. Do the costs, benefits and dynamics of a plasmid vary between different host species?
3. Do the mechanisms of compensatory evolution to ameliorate the plasmid cost vary between host species?
4. How do community-level plasmid dynamics vary according to the donor host species?
5. How do the dynamics of multi-plasmid communities vary according to the host community structure?

To achieve this, I used experimental evolution of model communities comprising various combinations of two large conjugative plasmids (pQBR57 and pQBR103) and seven *Pseudomonas* species (Table A.1- Appendix).

1.5 Thesis outline

Chapter 2: *Multi-host environments select for host-generalist conjugative plasmids*

The main objective of this chapter was to investigate the role of bacterial host species heterogeneity on plasmid evolution. I experimentally evolved the environmental mercury resistance conjugative plasmid pQBR57 either in a single host–*P. fluorescens* or *P. putida*–or an alternating host environment with both

P. fluorescens and *P. putida*. I found that plasmids evolved in *P. fluorescens* only became host-specialists due to a fitness trade-off, but that this trade-off could be circumvented in the alternating multi-host environment, allowing the evolution of host-generalist plasmids.

This work has been published in a peer-reviewed journal:
Kottara, A., Hall, J. P., Harrison, E. and Brockhurst, M. A. [2016]. Multi-host environments select for host-generalist conjugative plasmids, *BMC evolutionary biology* **16**(1): 70.

Chapter 3: *Variation in mobile genetic element dynamics across diverse Pseudomonas host species*

The main objective of this chapter was to compare the costs, benefits and ecological dynamics of plasmid carriage in diverse *Pseudomonas* host species. The conjugative mercury resistance plasmid pQBR103 was propagated in each of five *Pseudomonas* species–*P. fluorescens* SBW25, *P. putida* KT2440, *P. savastanoi pv. phaseolicola* 1448A, *P. aeruginosa* PAO1, and *P. stutzeri* DSM10701–both with and without mercury selection for ∼400 generations. The plasmid dynamics as well as its costs and benefits varied extensively between the *Pseudomonas* species.

This work has been published in a peer-reviewed journal:
Kottara, A., Hall, J. P., Harrison, E. and Brockhurst, M. A. [2017]. Variable plasmid fitness effects and mobile genetic element dynamics across *Pseudomonas* species, *FEMS microbiology ecology* **94**(1): fix172.

Chapter 4: *Diverse compensatory evolution across Pseudomonas species following acquisition of a large conjugative plasmid*

Building on the previous chapter, the main objective of this chapter was to investigate evolutionary changes in the plasmid-host relationships following experimental evolution. I estimated the relative fitness and mercury resistance of

the evolved clones and obtained whole genome sequences. All of the 3 *Pseudomonas* species tested (*P. fluorescens*, *P. stutzeri* and *P. savastanoi*) had ameliorated the plasmid-cost while *P. fluorescens* and *P. savastanoi* also showed increased mercury resistance. Putative compensatory mutations were identified in different regulatory and biosynthetic pathways in each host species.

Chapter 5: *Community-level plasmid dynamics are governed by the proficiency of the plasmid-donor species*

The main objective of this chapter was to investigate how plasmid dynamics at the community-level varied according to the identity of the donor species. To test this, I constructed 3 species communities containing *P. fluorescens*, *P. stutzeri* or *P. putida* and varied which species was the plasmid donor. Total plasmid abundance at the community level was higher in communities with a more proficient plasmid-donor species and stronger mercury selection.

Chapter 6: *Plasmid abundance is constrained by the species-richness of a community*

In this chapter, the main objective was to determine how the dynamics of a simple plasmid community were affected by the bacterial community structure. To test this, I tracked the dynamics of pQBR57 and pQBR103 in *P. fluorescens* either with or without a background community of five other *Pseudomonas* species. The presence of the background community limited plasmid co-infection of *P. fluorescens* due to a dilution effect which limited the efficiency of plasmid transmission within the *P. fluorescens* population.

Chapter 2

Multi-host environments select for host-generalist conjugative plasmids

2.1 Introduction

Conjugative plasmids mediate genetic exchange in bacterial communities promoting bacterial adaptation and diversification [Ochman et al., 2000]. Broad host range (BHR) conjugative plasmids, which can transmit between and be stably maintained across phylogenetically diverse hosts, play a particularly important role because they traffic ecologically important accessory genes between species [Thomas and Nielsen, 2005; Norman et al., 2009]. While broad host ranges benefit plasmids by increasing available hosts, evolutionary theory suggests that the evolution of ecological generalists, such as BHR plasmids, is likely to be constrained by fitness trade-offs [McPeek, 1996; Kassen, 2002; Egas et al., 2004]. Thus single-host environments are expected to select for specialist plasmids, whereas generalist plasmids are expected to evolve in environments where they regularly encounter multiple host bacterial species [De Gelder et al., 2008].

Previous studies have reported evolutionary changes in the effects of plasmid carriage across different host species following experimental evolution in single or multi-host environments [Dahlberg and Chao, 2003; Dionisio et al., 2005; Heuer et al., 2007; De Gelder et al., 2008; Haft et al., 2009; Sota et al., 2010]. Specifically, the BHR plasmid pB10 adapted to the originally unfavourable host *P. putida* H2 in a single-host environment [Heuer et al., 2007], whereas in a multi-host environment, *S. maltophilia* P21 and *P. putida* H2, adaptation of pB10 to either host species was impeded [De Gelder et al., 2008]. A key limitation of previous studies however is that they allow extended periods of bacterium-plasmid co-adaptation, which makes it difficult to disambiguate plasmid adaptation from host adaptation to understand how the plasmids themselves adapt to their hosts.

To overcome this limitation here the bacterial hosts were held in evolutionary stasis while allowing only the plasmid to evolve by conjugating the evolving plasmid population into the ancestral bacterial host genotype(s) every 24h. Specifically, to investigate the role of bacterial host species heterogeneity on plasmid evolution we experimentally evolved the environmental mercury resistance plasmid pQBR57 [Hall et al., 2015] under single host *Pseudomonas* species, *P. fluorescens* or *P. putida*, or multi-host, both *P. fluorescens* and *P. putida*, treatments. We observed evidence for a fitness trade-off in plasmids adapted to the single-host *P. fluorescens* treatment, but that exposure to *P. putida* in the multi-host treatment allowed plasmids to circumvent this trade-off.

2.2 Materials and Methods

2.2.1 Bacterial and plasmid strains

P. fluorescens SBW25 is a plasmid-free soil bacterium isolated from sugar beets grown at a field site in Oxford [Rainey et al., 1994; Bailey et al., 1995] whereas *P. putida* KT2440 is a soil bacterium derived from toluene-degrading *P. putida*

strain mt-2 [Bagdasarian et al., 1981]. Both strains were chromosomally modified by directed insertion of an antibiotic marker gene coding the resistance in streptomycin (Sm) or gentamicin (Gm) by using the mini-Tn7 transposon system [Lambertsen et al., 2004]. pQBR57 is a 307 kb conjugative mercury resistance plasmid isolated by mercury resistance selection from the bacterial population inhabiting the sugar beet rhizosphere and phyllosphere of sugar beets [Lilley et al., 1996]. Briefly, a marked *P. putida* UWC1 host was released and allowed to acquire plasmids from the natural bacterial community by conjugation. Plasmid-containing isolates were then recaptured by selecting for mercury resistance [Lilley et al., 1996]. As the primary host of the plasmid was not recovered the plasmid's host-range in nature is still unknown.

2.2.2 Selection experiment

In each treatment, plasmid pQBR57 was forced to conjugated between either: differentially marked lines of *P. fluorescens* SBW25; differentially marked lines of *P. putida* KT2440; or between *P. fluorescens* SBW25 and *P. putida* KT2440. Each treatment consisted of 6 replication lines. Donor bacteria carrying the plasmid were incubated with the plasmid-free, differentially marked recipient bacteria in King's medium B (KB) for 24 h at 28°C with shaking (180 rpm), after which time a sample of the mixture was diluted and spread on solid media that contained mercury(II)chloride (20 μM) and antibiotics (10 μg/ml gentamicin or 200 μg/ml streptomycin) to select for transconjugant colonies. Twenty-four hours later, 25 transconjugant colonies were selected randomly and used as donors to conjugate with overnight cultures of plasmid-free recipient bacteria revived from frozen stocks. The antibiotic resistances of the bacterial strains were used to ensure the conjugative transfer of the plasmid from one host to the other at each transfer step.

2.2.3 Conjugation assay

Plasmid conjugation rate was measured through-out the selection experiment. Saturated cultures of plasmid-free recipients and plasmid-carrying donors were mixed in 1:1 ratio, diluted 100-fold in fresh KB media and incubated at 28°C for 24 h. Densities of donors and recipients at the start and end of conjugation were estimated by diluting and spreading on KB agar containing either 10 μg/ml gentamicin or 200 μg/ml streptomycin. The density of transconjugants following conjugation were estimated by plating onto KB agar containing 20 μM mercury(II)chloride plus antibiotics to select for transconjugants. Conjugation rate (γ) was calculated using the end-point method [Simonsen et al., 1990].

2.2.4 Competitive fitness assay

Following 36 conjugative transfers one plasmid-containing bacterial clone from each population was used as a donor for conjugation into *P. fluorescens* and *P. putida* bacterial host backgrounds. Relative fitness was measured by mixing differentially labeled test (containing evolved plasmid) and reference (containing ancestral plasmid) in 1:1 ratio, diluted 100-fold and incubated at 28°C for 24 h. Samples were plated on selective KB agar plates at the beginning and end of the competition and relative fitness was calculated as the selection rate (r) [Lenski et al., 1991]. To remove marker effects the selection rate of the test strain was normalized to the fitness of the focal marked strain carrying the ancestral plasmid when competed against the opposite marker labelled strain carrying the ancestral plasmid.

2.2.5 Statistical analyses

The statistical analysis was carried out using the software RStudio, version 3.1.0 [R Core Team, 2015]. A repeated measures mixed-effect linear model was fitted to the longitudinal data of the conjugation rate using the lme4 package [Bates et al.,

2014] testing the effect of treatment and transfer number on conjugation rate, with 'population' as a random effect to account for repeated measures. A linear model was used to analyse normalized selection rate of bacteria carrying the evolved plasmids, fitting test species, treatment, and their interactions as fixed effects. Welch's t-test was used to compare selection rate between the test species within each treatment.

2.3 Results and Discussion

The conjugation rate of pQBR57 varied between selection treatments (main effect of selection treatment, chi-square test, χ^2(2, N=432)=30.49, p=2.39e-07), owing to a lower conjugation rate in *P. putida* than *P. fluorescens*, but increased over time in all treatments (main effect of time, chi-square test, χ^2(1, N=432)=18.24, p=1.94e-05) (Figure 2.1). This suggests that pQBR57 adapted to the selection regimes by increasing its conjugation rate. In this experimental set-up, which involved both horizontal and vertical plasmid replication, conjugation is an essential part of the plasmid life-cycle; thus increasing conjugation rate is equivalent to increasing replication rate and therefore perhaps a predictable response to selection. However, increases in conjugation rate can be linked to increased costs of plasmid carriage [Turner et al., 1998; Dahlberg and Chao, 2003], which would impair the plasmid's spread by vertical transmission (i.e. growth of transconjugants).

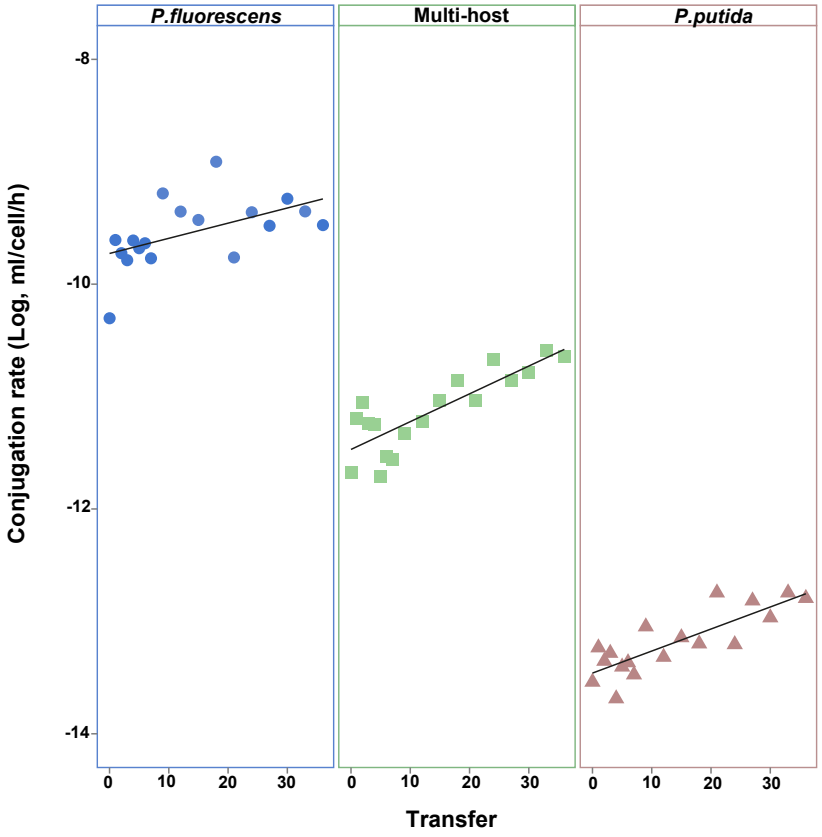

FIGURE 2.1: Conjugation rate over time for plasmids in the single-host and multi-host treatments (Solid circle: Conjugation in *P. fluorescens*; Solid square: Conjugation between *P. fluorescens* and *P. putida*; Solid triangle: Conjugation in *P. putida*; Black line: linear regression).

To estimate the fitness effects of carrying the evolved plasmids for host bacteria, bacteria carrying evolved plasmids were competed against bacteria carrying the ancestral plasmid, in both host species backgrounds. The fitness effect of evolved plasmids depended on the combination of selection treatment and the test host species background (Figure 2.2; effect of species background and selection treatment interaction, factorial ANOVA, $F_{2,36}$=4.50, p=0.017). Particularly, plasmids from the single-host *P. fluorescens* treatment evolved lower costs in *P. fluorescens*, but this adaptation was accompanied by an increased cost in *P. putida* relative to the ancestral plasmid (Welch's t-test, $t_{6.81}$=2.592, p=0.036) (Figure 2.2). Contrastingly, although plasmids from the single-host *P. putida* treatment evolved marginally lower costs in *P. putida*, no change to the cost of

carriage in *P. fluorescens* was observed (Welch's t-test, $t_{9.88}$=-0.618, p=0.55) (Figure 2.2).

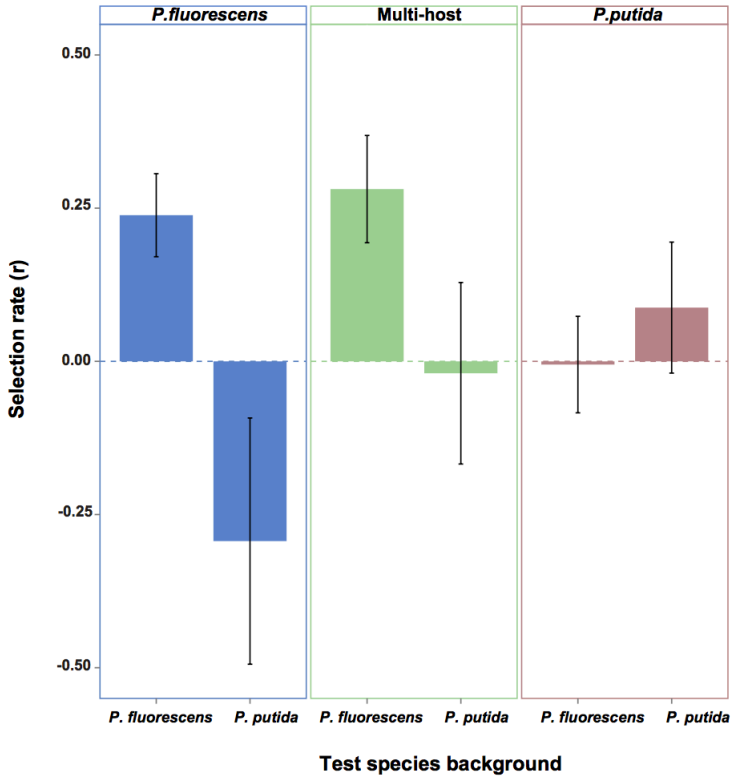

FIGURE 2.2: Selection rate of *P. fluorescens* or *P. putida* carrying evolved plasmids from the single and multi-host treatments relative to isogenic strains carrying the ancestral plasmid. Selection rate of 0 indicates no difference between test and reference strains (dotted line), error bars: SEM).

Together this suggests an asymmetric trade-off, whereby pQBR57 adapted to *P. fluorescens* suffers a fitness trade-off in *P. putida*, but that there is not a corresponding fitness trade-off associated with adaptation to *P. putida*. Although the exact mechanism underlying the fitness trade-off in this study is still unknown, previous work suggests that costs of plasmid carriage can arise from a range of mechanisms, including: the metabolic burden, expression of plasmid genes, copy number variation, and interference between plasmid and host cell regulatory systems [Harrison and Brockhurst, 2012; Baltrus, 2013]. It is tempting to speculate that the last of these, regulatory interference, might be the most host-specific and thus more likely to generate the observed fitness trade-off [Perez and Groisman, 2009].

Interestingly, evolved plasmids from the multi-host treatment evolved reduced cost-of-carriage in *P. fluorescens* but without increasing their cost-of-carriage in *P. putida* (Figure 2.2). This suggests that adaptation in a multi-host environment allowed pQBR57 to circumvent the fitness trade-off associated with adaptation to *P. fluorescens* in the single-host treatment. Due to time constraints, the specific mutations involved in plasmid adaptation in this experiment were not investigated but the contrasting responses to selection between treatments suggests different genetic mechanisms. In particular, it seems likely that the different responses to selection in the *P. fluorescens* single-host treatment versus the multi-host treatment are due either to the fixation of different mutations, or the fixation of additional mutation(s) in the multi-host treatment to ameliorate the cost in *P. putida* of plasmid adaptation in *P. fluorescens*.

Environmental heterogeneity is thought to play a key role in the evolution of generalism and specialism in a wide variety of species [Van Tienderen, 1991]. Heterogeneous environments are predicted to select for generalist genotypes whereas homogeneous environments select for specialist genotypes [Kassen, 2002]. For example, evolution experiments with algae adapting to light and dark show that algae adapted to light have lower fitness in dark environments and vice versa, whereas algae exposed to both environments evolve to be generalists [Reboud and Bell, 1997]. The results of this evolution experiment show that this evolutionary principle also applies to the evolution of mobile genetic elements in different hosts, in this case a conjugative plasmid. A fitness trade-off was associated with adaptation to a single host environment. The appearance of a fitness trade-off can be due, at the genetic level, to antagonistic pleiotropy or mutation accumulation [Reboud and Bell, 1997; Kassen, 2002]. It seems more likely that the pattern observed here is the result of antagonistic pleiotropy, since there was equal opportunity for mutation accumulation in all treatments, but the trade-off was asymmetric affecting only the plasmids evolving in one of the species (*P. fluorescens*). Interestingly, exposure to both host species in the multi-host treatment did not constrain adaptation. This suggests that fitness

trade-offs can be circumvented if plasmids are exposed to alternative hosts. Diverse bacterial communities are likely therefore to select for broad host range plasmids and consequently promote interspecific horizontal gene transfer, with implications for understanding the spread of important plasmid-borne traits like antibiotic resistance.

2.4 Conclusions

This study shows that evolution in a single-host environment selected for host-specialist plasmids due to a fitness trade-off, but this trade-off could be circumvented in the multi-host environment, leading to the evolution of host-generalist plasmids. Thus, the plasmid persistence and fate in the environment is regulated by the selection of the beneficial traits that carry in their accessory genes and fitness trade-offs.

Chapter 3

Variation in mobile genetic element dynamics across diverse *Pseudomonas* host species

3.1 Introduction

Bacterial evolutionary innovation and adaptation is often dependent upon acquisition of novel accessory genes carried on mobile genetic elements [Frost et al., 2005]. This fundamental evolutionary process is termed horizontal gene transfer (HGT) [Thomas and Nielsen, 2005]. Conjugative plasmids are important vectors of HGT as they can be both inherited vertically during cell division and transmitted horizontally by conjugation within and between bacterial species [Norman et al., 2009]. In addition to genes for their own replication, propagation and stability, many plasmids also encode a complement of accessory genes: bacterial genes that do not benefit the plasmid directly but can be beneficial for the bacterial host under specific environmental conditions, e.g. traits such as antibiotic and metal resistance [Eberhard, 1990]. Understanding the maintenance and spread of accessory genes is a pressing concern for microbiologists,

particularly because of the grave threat that plasmid-borne antibiotic resistance poses in opportunistic infection [zur Wiesch et al., 2011; Holmes et al., 2016].

The fate of a plasmid within a host is determined by several key factors: the cost of plasmid carriage, the conjugation rate and segregation rate of the plasmid, and the strength of positive selection on plasmid-borne accessory genes which will vary according to the environment [Simonsen, 1991; Bergstrom et al., 2000; Slater et al., 2008]. In environments where the benefits of accessory genes outweigh the costs of carrying the plasmid (i.e. where the plasmid-bacteria interaction is mutualistic) plasmids may be maintained at high frequency through positive selection [San Millan et al., 2014; Harrison et al., 2015a]. However, over longer evolutionary timescales it is likely that positive selection will favour integration of the beneficial accessory genes into the host chromosome [Bergstrom et al., 2000], a process facilitated by accessory genes often being located on transposons or other integrative elements [Osborn and Böltner, 2002]. By contrast, in environments where the cost to the host of carrying the plasmid outweighs the benefit of the plasmid-borne accessory genes (i.e. when the bacteria-plasmid interaction is parasitic), plasmids will be lost due to purifying selection unless the rate of loss is counteracted by a sufficiently high rate of conjugation such that they are maintained by infectious transmission [Bergstrom et al., 2000; Hall et al., 2016]. These key parameters affecting plasmid dynamics are likely to vary, leading to differences in the dynamics of plasmids and their constituent MGEs among host species.

Variation in plasmid population dynamics between host species has typically been considered in terms of plasmid host range, i.e. the subset of host species wherein the plasmid can be stably maintained [Bahl et al., 2009]. However, the ability to infect and replicate may not reflect the long-term stability of a plasmid in a host population, because of high rates of segregation and/or plasmid costs [Turner et al., 1998]. Previous studies which have demonstrated variation in long-term plasmid population dynamics in different hosts [De Gelder et al., 2007; Sota et al., 2010; Porse et al., 2016] have tended to focus on one selective environment and do not assess the role of chromosomal capture of accessory genes.

Here we quantify for a range of bacterial host species the fitness effects of plasmid acquisition, and variation in the plasmid dynamics, under both negative selection (plasmid is parasitic) and positive selection (plasmid is mutualistic). The experimental system consisted of a conjugative plasmid, pQBR103, conferring mercury resistance via a *mer* operon encoded on a Tn5042 transposon, and five *Pseudomonas* species representing *P. fluorescens*, *P. putida*, *P. savastanoi*, *P. aeruginosa*, and *P. stutzeri*. Populations were propagated with and without mercury and the dynamics of the mercury resistance phenotype, the pQBR103 plasmid and the Tn5042 transposon were tracked over time. We report that the fitness effects of plasmid carriage and mobile genetic element dynamics varied extensively between the different *Pseudomonas* species, indicating that plasmid-host dynamics are governed by species-specific interactions between plasmids and the host chromosome.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

Five phylogenetically diverse *Pseudomonas* species (Figure 3.1) isolated from a range of environments were utilised: *P. fluorescens* SBW25 was isolated from the leaf surface of the sugar beet plant [Rainey et al., 1994]; *P. putida* KT2440 is a derivative of the toluene degrading and soil isolate *P. putida* mt-2 [Nakazawa and Yokota, 1973]; *P. savastanoi pv. phaseolicola* 1448A is a plant-associated isolate and pathogen of the common bean [Arnold et al., 2011]; *P. stutzeri* JM300 (DSM10701) is a denitrifying soil isolate [Busquets et al., 2012]; *P. aeruginosa* PAO1 is a derivative of the original Australian PAO that was isolated from a wound in Alfred hospital in Melbourne [Holloway, 1955]. Furthermore, *P. savastanoi pv. phaseolicola* carries two native plasmids (131 kb and 51 kb) [Joardar et al., 2005], while the other aforementioned *Pseudomonas* species are plasmid-free isolates.

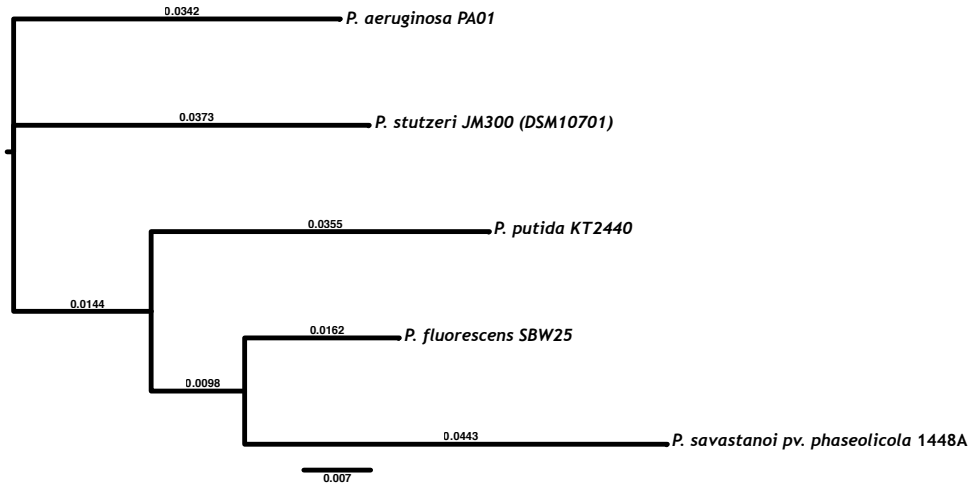

FIGURE 3.1: Phylogenetic tree of *Pseudomonas* species used in this experiment. The tree was build using the whole genome sequence data in the online pipeline REALPHY 1.10 [Bertels et al., 2014]. The genome sequence of *P. fluorescens* SBW25 was used as a reference genome where the other *Pseudomonas* genomes were aligned by using bowtie2 [Langmead and Salzberg, 2012]. PhyML was used to build the maximum likelihood tree; the tree topologies were obtained based on the nearest-neighbor interchanges (NINs) [Guindon et al., 2010]. FigTree v1.4.3 was used to display and draw the tree (http://tree.bio.ed.ac.uk/software/figtree/). Branch labels: relative distances along the branches.

The plasmid used in this study, pQBR103, was isolated from the natural bacterial community colonizing the rhizosphere and phyllosphere of sugar beets [Lilley et al., 1996; Tett et al., 2007]. *Pseudomonas* species were labelled by directed insertion of a gentamicin resistance (Gm^R) marker using the mini-Tn7 transposon system [Lambertsen et al., 2004]. Plasmid-carrying Gm^R strains were obtained by incubating streptomycin resistant (Sm^R) *P. fluorescens* SBW25 stocks that were carrying pQBR103 plasmid, with the plasmid-free Gm^R strains for 48 h and spreading on King's B (KB) agar plates containing 10 μg/ml gentamicin and 20 μM of mercury(II)chloride to select for transconjugant colonies [Simonsen et al., 1990]. All experiments were conducted in 6 ml KB growth medium in 30 ml universal vials ('microcosms') at 28°C in shaking conditions (180 rpm).

3.2.2 Competitive fitness assay

Six individual colonies of each *Pseudomonas* species containing the ancestral plasmid were grown overnight in microcosms and later each was competed against the relevant isogenic plasmid-free strain across a range of mercury(II)chloride concentrations from 0-60 μM. Relative fitness was measured by mixing differentially labelled test (plasmid-bearer, labelled gentamicin) and reference (plasmid-free, wild-type) in 1:1 ratio, diluted 100-fold in KB microcosms containing the relevant mercury concentration and incubated at 28°C for 48 h. Samples were plated on KB agar plates at the beginning and end of the competition and replica plated onto KB agar plates supplemented with selective concentration of gentamicin to estimate the density of plasmid-bearers. The relative fitness was calculated as the selection rate (r) [Lenski et al., 1991] and normalised for the marker effects by subtracting the selection rate of gentamicin labelled, plasmid-free strains over the plasmid-free, wild-type strains.

3.2.3 Selection experiment

Prior to the evolution experiment 12 individual colonies of each *Pseudomonas* species carrying the ancestral plasmid were reconditioned from frozen stocks overnight in KB 6 ml microcosms at 28°C with shaking (180 rpm) after which time 1% of each population was transferred to grow for 24 h in fresh KB microcosms containing 50 μM of mercury(II)chloride at same temperature and shaking conditions. This step was necessary to ensure high starting frequencies of plasmid carriage across all the tested bacterial strains due to the high segregation rate of the plasmid in *P. putida* KT2440. For the evolution experiment, six clonal populations of each bacterial strain were grown in absence of mercury and six populations were grown in presence of mercury selection (50 μM of mercury(II)chloride) in KB microcosms at 28°C with shaking (180 rpm). Every 48 h 1% of each population was transferred into a fresh microcosm for 60 transfers [$\sim$400 generations]. The density of each bacterial population was

monitored every 4 transfers by plating a sample onto KB agar plates incubated at 28°C for 48 h. Each plate was then replica plated onto KB agar plates supplemented with 100 μM of mercury(II)chloride to assess the frequency of mercury resistance. Subsequently, 24 bacterial colonies were randomly selected from each mercury replica plate. The prevalence of the mercury resistance transposon, Tn5042, and the plasmid, pQBR103, was estimated at transfers 2, 4 and 8 and then at every 12 transfers by PCR screening of the 24 bacterial colonies randomly selected from each clonal population. The PCR screening was designed as previously described [Harrison et al., 2015a] with 2 sets of primers (Table 3.1), one targeted to *mer* operon on Tn5042 transposon and the other to origin of replication of the plasmid. Detection limits were estimated by Poisson calculations.

Table 3.1: Primers used for the PCR screening of the mercury resistance genes and the plasmid pQBR103.

mer operon	F-TGCAAGACACCCCCTATTGGAC R-TTCGGCGACCAGCTTGATGAAC
oriV	F-TGCCTAATCGTGTGTAATGTC R-ACTCTGGCCTGCAAGTTTC

3.2.4 Statistical analyses

Statistical analyses were performed using RStudio version 3.2.3 [R Core Team, 2015]. We used a linear model to analyse the variation in the plasmid cost between the *Pseudomonas* species and mercury selection environments where mercury was fitted as a quadratic term for each species [r $\sim$ species background x mercury x mercury^2]. Mercury was fitted as a quadratic term as 4/5 species showed non-linear fitness responses to the increase of mercury. We further used model comparisons to test the linearity of genotype by environment interactions. To analyse the end-point frequency of mercury resistance and plasmid prevalence of the evolution experiment, a linear model was fitted to the different

Pseudomonas species across the mercury selection environments (0 μM and 50 μM mercury(II)chloride). Plasmid population dynamics in the *P. stutzeri* parallel evolving populations were further investigated by comparing plasmid prevalence across mercury conditions using a oneway-ANOVA. Plasmid prevalence was estimated as the area under the curve using the function *auc* of the package 'flux' [Jurasinski et al., 2012].

3.3 Results

3.3.1 Plasmid fitness costs varied between *Pseudomonas* species

The fitness effect of plasmid carriage on bacterial hosts was first quantified using competition experiments across a wide range of mercury environments, ranging from no mercury where the plasmid confers no benefit (0 μM mercury(II)chloride) to high levels of mercury contamination where the plasmid and its mercury transposon are essential (60 μM mercury(II)chloride). Although, in general, the plasmid was costly to host species in the absence of mercury, the magnitude of the cost and the form of the fitness response with increasing mercury concentration varied between species (effect of species background x mercury x mercury^2 interaction, ANOVA $F_{4,159}$=9.616, p=5.451e-07; Figure 3.2). For instance, in the absence of mercury the plasmid was highly costly in *P. aeruginosa* whereas it imposed a far lower fitness cost in *P. fluorescens* and *P. savastanoi*. Moreover, whereas *P. fluorescens* showed a positive and linear fitness response with increasing mercury concentration (effect of mercury (*P. fluorescens* fitness data), ANOVA $F_{1,32}$=72.829, p=9.425e-10), the fitness of the other plasmid bearing *Pseudomonas* species increased rapidly at low concentrations of mercury (>/=7.5 μM mercury(II)chloride) (species background by mercury^2 interaction, ANOVA $F_{4,159}$=10.34, p=1.809e-07). Model comparison revealed

that the fitness response to mercury concentration was non-linear in 4/5 of the species (ANOVA F=19.058, p<2.2e-16).

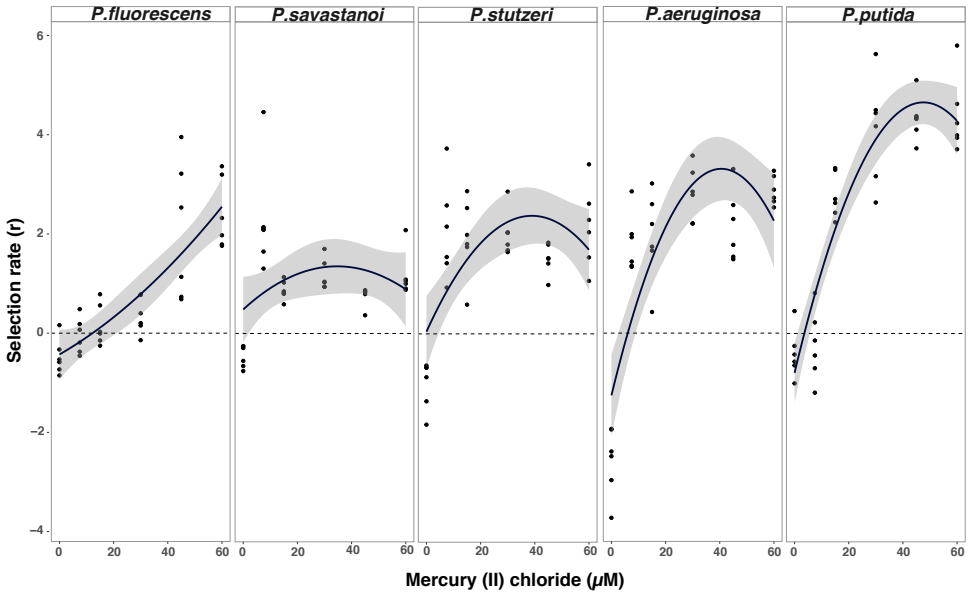

FIGURE 3.2: Fitness of *Pseudomonas* species carrying the plasmid measured as selection rate in a mercury regime ranging from 0-60 μM mercury(II)chloride. Solid circle: each clonal population carrying the plasmid and competing with the isogenic wild-type strain; solid line: curve fitting mercury as a quadratic term for each species; dashed line: selection rate 0 indicates no difference between test and reference strains; shaded area: confidence interval of fitting curve.

Furthermore, minimum inhibitory concentration assays showed that species varied both in their inherent susceptibility to mercury (Figure 3.5; Supplementary Information) and in the level of mercury resistance conferred by pQBR103 (Figure 3.6; Supplementary Information). These data demonstrate extensive variation in the fitness effect of plasmid acquisition across the *Pseudomonas* phylogeny, suggesting that even in relatively closely related bacteria (Figure 3.1), plasmids and their accessory genes can have markedly different fitness effects.

3.3.2 Variable dynamics of mercury resistance over time

To examine the consequences of these fitness effects on long-term population dynamics, we tracked the mercury resistance in populations evolved for ∼400

generations either with or without mercury selection. Mercury resistance was assessed by replica plating populations every 4 transfers onto mercury selective media. At the end of the experiment, we found that the level of mercury resistance varied between *Pseudomonas* host species and with mercury environment (species by mercury interaction, ANOVA $F_{4,50}$=8.808, p=1.83e-05). As expected, mercury selection promoted the maintenance of mercury resistance in all host species. Without mercury selection, the maintenance of mercury resistance was highly dependent on host species (Figure 3.3). In the absence of mercury, resistance was maintained throughout the experiment at high frequency in *P. fluorescens* and *P. savastanoi*, but lost rapidly from *P. putida* and *P. stutzeri*. Specifically, mercury resistance was not detected in 5/6 and 4/6 replicates of *P. putida* and *P. stutzeri* respectively at the end of the experiment (>95% probability of detecting Hg^R if present at frequencies of $\geq$ 9.1%). In *P. aeruginosa*, resistance dynamics varied across replicate populations, with final frequencies ranging from 4 - 80% of the population evolving in mercury-free environment (>95% probability of detecting Hg^R if present at frequencies of $\geq$ 3.2%).

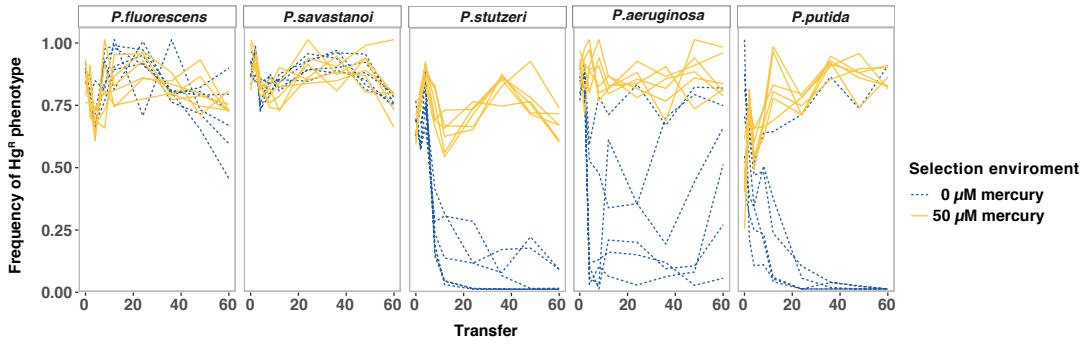

FIGURE 3.3: Frequency of mercury resistance in the parallel evolving clonal populations in absence (blue dashed lines) and presence (50 μM) of mercury (yellow lines) throughout the selection experiment. Each line represents a clonal population evolving during the evolution experiment.

3.3.3 Variation in plasmid and transposon dynamics over time

Plasmid-borne accessory genes can transfer to the chromosome, allowing loss of a redundant plasmid but retention of the resistance gene(s) [Bergstrom et al., 2000; Hall et al., 2016]. Therefore, we used PCR to test whether mercury resistant clones isolated during the experiment still carried the plasmid, or whether it had been lost following acquisition of chromosomal mercury resistance. Plasmid maintenance varied between the different *Pseudomonas* host species (effect of species background, ANOVA $F_{4,50}$=158.33, p<2e-16; Figure 3.4). The plasmid was maintained in *P. fluorescens* and *P. savastanoi* in both mercury environments: although pQBR103-free mercury resistant clones arose in several populations, they did not invade over the course of the experiment. *P. stutzeri* maintained the plasmid at intermediate levels, but only under mercury selection, and even then the plasmid was lost in 3/6 populations due to invasion of plasmid-free mercury resistant clones (plasmid prevalence by mercury environment in *P. stutzeri*, ANOVA $F_{1,10}$=12.86, p=0.004). By contrast, we observed rapid, complete loss of the plasmid in *P. putida* and *P. aeruginosa* regardless of mercury selection (>95% probability of detecting pQBR103 if present at frequencies ≥2.1%), and where mercury resistance was observed in these hosts this resulted from chromosomal capture of the resistance genes (Figure 3.4). These findings indicate clear variation in plasmid stability between hosts dependent upon the environment, and, moreover, variation between host species in the propensity to replace plasmid-borne resistance with chromosomal resistance via capture of Tn5042.

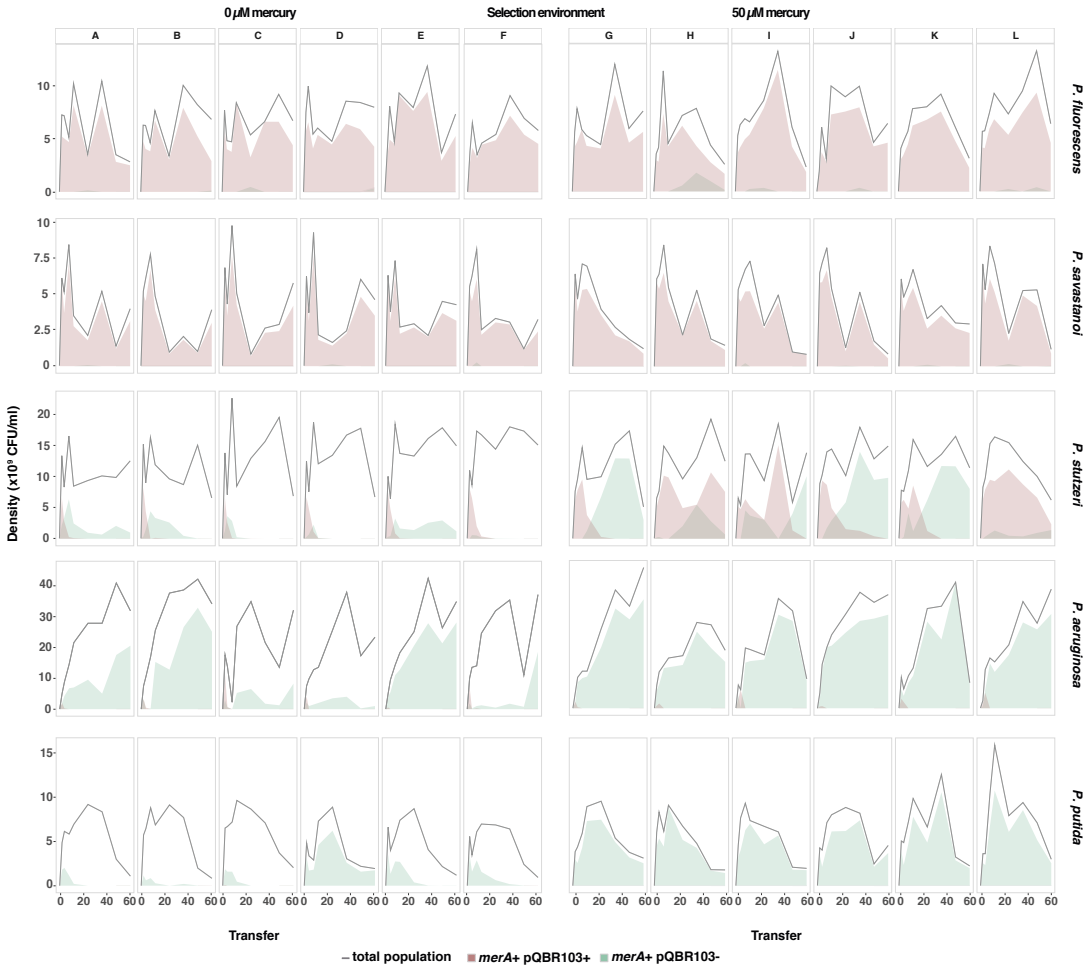

FIGURE 3.4: Population density and tracked mercury resistance transposon, Tn5042 and plasmid density. A-F clonal populations evolving in absence of mercury(II)chloride; G-L clonal population evolving in presence of 50 μM mercury(II)chloride. Total population density (grey line); tracked mercury transposon in presence of the plasmid (brown density plot area); tracked mercury transposon when the plasmid was not detected (green density plot area).

3.4 Discussion

It is clear that the relationship between a plasmid and its host is highly context dependent [De Gelder et al., 2007; Humphrey et al., 2012]. Here, the same plasmid can levy different costs on different hosts: we found that the cost of plasmid carriage was 2.5-fold higher in *P. aeruginosa* compared with other *Pseudomonas* species. The environment can convert the relationship from parasitism to mutualism by selection for plasmid-borne genes: in this case

application of 7.5 μM mercury was sufficient for pQBR103 to go from a significant parasite (inhibiting the growth of *P. aeruginosa* by 13% compared with plasmid-free) to a clear mutualist (enhancing the relative fitness of its host by 71%), similar to previous work [Gullberg et al., 2014; Hall et al., 2015]. Furthermore, the effect of environmental selection varies between hosts, implying that the addition of mercury to the environment does not benefit plasmid bearers of all species equally. This is clearest in the case of *P. fluorescens*, which, compared with the other species, required higher levels of mercury selection for the plasmid to be beneficial. A potential limitation of our fitness measurements is that the rapid segregational loss of the plasmid observed in some of the host species, most notably *P. putida*, may have led to underestimation of plasmid costs in these hosts. Nevertheless, the fact that the emergence and success of such segregants varies between species is consistent with the main finding of this study. Previously, we showed that different mercury resistance plasmids imposed varying costs, which varied for each plasmid depending on the selective environment, despite the fact that the plasmids shared the same resistance genes [Hall et al., 2015]. Together, these data paint an increasingly complex picture whereby plasmids, hosts, and the environment interact to determine whether plasmid-bearers suffer from the burden of carriage or profit from the accessory genes that plasmids often provide. This is consistent with a recent meta-analysis of variation in plasmid costs, which showed that the variation in costs for a given plasmid across different host genotypes can be as large as the variation for different plasmids in a given host [Vogwill and MacLean, 2015].

Consistent with the short-term measurements of fitness, we observed divergent long-term dynamics between the different species. *P. fluorescens* and *P. savastanoi* maintained the plasmid, while *P. aeruginosa* and *P. putida* lost the plasmid, regardless of mercury selection. *P. stutzeri* plasmid maintenance required mercury selection. The extinction of pQBR103 in *P. aeruginosa* populations can be readily explained by the high cost the plasmid levies on this host (in the absence of mercury, average plasmid prevalence was decreased 42% within the first 2 transfers and 96% from transfer 2 to 4), but measurements of

fitness can only partly explain the long-term dynamics. For example, long-term plasmid dynamics in *P. fluorescens* and *P. putida* were widely divergent despite the fact that costs-of-carriage were similar. Rates of segregation and/or conjugation, which are known to vary between species [Hall et al., 2016], may help explain longer-term maintenance. Alternatively, species may vary in their ability to accommodate an incoming plasmid through compensatory mutation. Plasmid cost is likely to come primarily from specific interactions between plasmid and host [Baltrus, 2013; San Millan et al., 2014]: for some hosts, modulating such interactions may be readily achieved through mutation, for others it may be easier to simply lose the plasmid. The cost pQBR103 carriage by *P. fluorescens*, for example, has been shown to be associated with rapid compensatory evolution, facilitated by mutations targeting the GacS/A system [Harrison et al., 2015a]. Though the GacS/A system is a conserved global regulatory system, it responds to different signals and controls different processes in each species, and may be tightly associated with niche occupation [Lapouge et al., 2008]. In other hosts, mutations to GacS/A might not alleviate plasmid costs, may impose excessively negative pleiotropic effects, or might not occur readily enough, all of which would limit plasmid survival [Harrison et al., 2016].

Theory predicts that under selection, beneficial plasmid accessory genes are captured by the chromosome and the plasmid is lost [Bergstrom et al., 2000]. In all of the tested species, we tested we detected mutants which had lost the plasmid but maintained mercury resistance, presumably through chromosomal acquisition of the mercuric reductase MerA. Acquisition of MerA most likely occurred by transposition of the Tn5042 mercury resistance transposon from the plasmid onto the chromosome: Tn5042 is wide-spread in the environment and across pQBR plasmids [Mindlin et al., 2005; Hall et al., 2015], and is known to mobilise to the chromosome (at least in *P. fluorescens*; [Harrison et al., 2015a]). Studies have extensively reported that resistance genes are frequently located on transposons, increasing the propagation and persistence of the resistance genes in the environment [Frost et al., 2005; Partridge et al., 2009]. Interestingly, we found that genotypes carrying chromosomal mercury resistance did not only

invade lineages treated with mercury, but also formed a substantial fraction of the *P. aeruginosa* and *P. putida* populations evolved without mercury. These data suggest that the spread of resistance could be a species-specific characteristic, and that for some species even transient plasmid carriage is sufficient for successful mobilisation of resistance genes to the chromosome and subsequent spread. Species which are poor plasmid hosts need not be excluded from the mobile gene pool, provided they can transfer plasmid-borne accessory genes to their chromosome rapidly enough.

This work is shown that long-term plasmid-host dynamics vary with environmental selection and host genotype. Laboratory experiments such as these are revealing a complex and contingent partnership, but are necessarily simple, stripping away many of the details of the natural environment. Natural environments have complex and diverse structure that acts both antagonistically and synergistically to HGT between species; antagonistically when the structural environments prohibit HGT due to the fewer encounter opportunities between species and synergistically when the formation of biofilms promotes the maintenance of species' communities [Heuer and Smalla, 2012]. Therefore, in the wild, plasmids and their hosts do not exist in a dyadic relationship — microbial communities contain many species [Lozupone and Knight, 2007] and many different mobile elements [Norman et al., 2009] and a small subset of hosts able to maintain plasmids could act as hubs of exchange, spreading genes throughout the community [Hall et al., 2016]. In this study, we found that 2 out of 5 of the Pseudomonads tested maintained the plasmid, indicating that hubs may be fairly common in this genus, although further work is required to identify the genetic basis of plasmid maintenance across the species tested. In addition, this data suggests that selection for plasmid-borne genes may not be a widespread mechanism for maintenance, since plasmid fate was only enhanced by mercury treatment for one of the species we tested (*P. stutzeri*). If maintenance and spread of plasmids is governed more by species presence than by selection for plasmid-borne genes, this has important implications for the control of resistance elements: without an understanding of the species involved, attempts to limit

the spread of resistance by limiting antibiotic use (for example) may prove to be unsuccessful.

3.5 Conclusions

This work shows that *Pseudomonas* species varied greatly both in their ability to stably maintain the plasmid and in their propensity to integrate the mercury resistance transposon onto the chromosome, which occurred both in absence and presence of mercury selection. The observed variation in MGE dynamics between *Pseudomonas* species confirms that plasmid stability is the product of the host-plasmid interaction, rather than a property of the plasmid alone, and suggests that species vary in their propensity to successfully capture horizontally acquired genes to their chromosomes. These results suggest that in natural communities: (a) a subset of potential host species is likely to be responsible for the long-term survival of conjugative plasmids and (b) the persistence of resistance genes would be mainly promoted under selection but highly dependent on the species background.

3.6 Supplementary Information

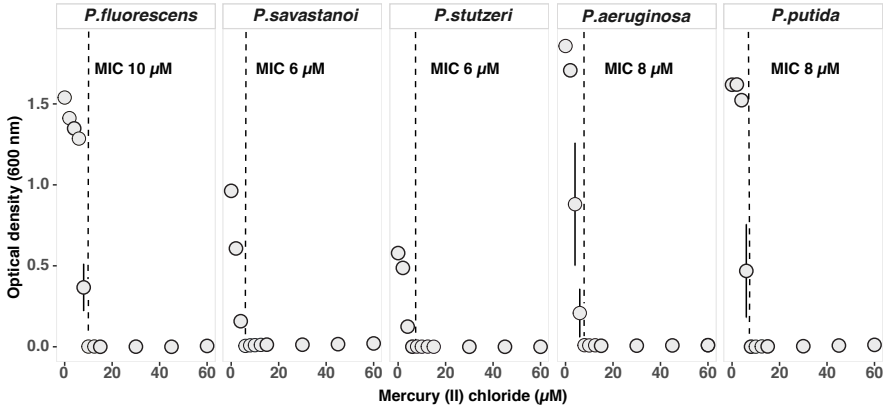

FIGURE 3.5: Growth of the ancestral plasmid-free *Pseudomonas* species in mercury environment ranging from 0-60 μM mercury(II)chloride at 48 h. Four individual colonies of each *Pseudomonas* species were grown at the exponential phase (optical density at 600 nm: $\sim$0.4) and later each diluted 1000-fold in KB containing the relevant mercury concentration and incubated at 28°C for 48 h. Points represent the mean growth measured as optical density at 48 h, based on the absorbance at 600 nm. Error bars represent SEM of four clonal replicates.

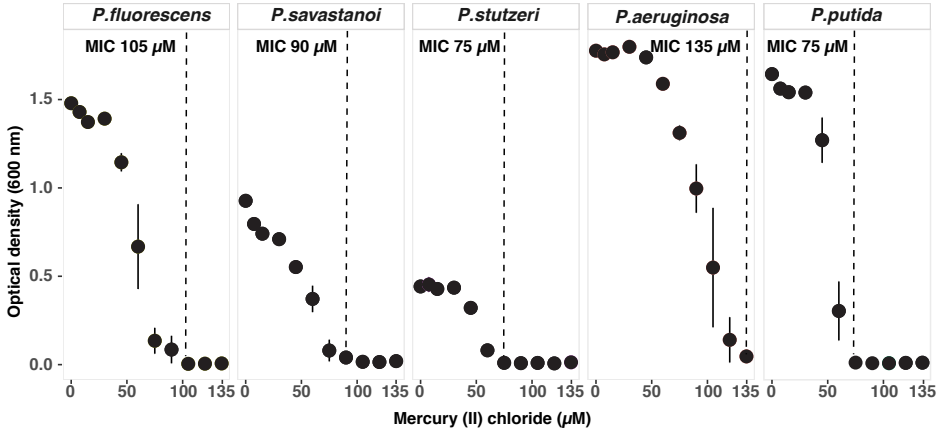

FIGURE 3.6: Growth of the ancestral plasmid-bearing *Pseudomonas* species in mercury environment ranging from 0-135 μM mercury(II)chloride at 48 h. Four individual colonies of each *Pseudomonas* species containing the ancestral plasmid were grown at the exponential phase (optical density at 600 nm: $\sim$0.4) and later each diluted 1000-fold in KB containing the relevant mercury concentration and incubated at 28°C for 48 h. Points represent the mean growth measured as optical density at 48 h, based on the absorbance at 600 nm. Error bars represent SEM of four clonal replicates.

Chapter 4

Diverse compensatory evolution across *Pseudomonas* species following acquisition of a large conjugative plasmid

4.1 Introduction

Conjugative plasmids are key facilitators of microbial evolution, promoting genomic diversification through horizontal gene transfer (HGT) and expanding their hosts' adaptability to novel, changing environments [Frost et al., 2005; Thomas and Nielsen, 2005]. However, although the plasmids may encode traits of potential benefit to their hosts (e.g. antibiotic and metal resistance), they also frequently impose a cost of carriage upon the host cell [Baltrus, 2013]. This cost has been associated to different stages of the plasmid lifestyle (i.e insertion, integration, replication and conjugation), including the metabolic burden of replicating the plasmid and expressing plasmid genes, disruption of cellular homeostasis, expression of cytotoxic gene products, crosstalk between plasmid and chromosomal gene regulation and interactions of mobile genetic elements

(MGE) [San Millan and MacLean, 2017]. Theory predicts that costly plasmids should be lost whether or not they encode host-beneficial traits: in the absence of fitness benefits plasmids should be lost to purifying selection, whereas where the benefits of plasmid-encoded genes outweigh the costs, these genes should be transferred to the chromosome and the redundant plasmid lost [Harrison and Brockhurst, 2012]. However, a number of recent studies suggest that compensatory evolution to ameliorate the cost of plasmid carriage is an important mechanism allowing the long-term survival of plasmids in bacterial populations [San Millan et al., 2014; Harrison et al., 2015a; San Millan et al., 2015; Porse et al., 2016; Bottery et al., 2017; Loftie-Eaton et al., 2017; Santos-Lopez et al., 2017].

Compensatory evolution often occurs via loss-of-function mutations in genes in either the chromosome or the plasmid. Examples of compensatory mutations include mutations in genes coding putative helicases and protein kinases of *P. aeruginosa* [San Millan et al., 2014, 2015], the GacS/A two-component regulatory system of *P. fluorescens* [Harrison et al., 2015a], and accessory helicases and the RNA polymerase β-subunit of *Pseudomonas* sp. H2 [Loftie-Eaton et al., 2017]. Other studies have reported compensatory mutations occurring in both chromosomal and plasmid genes, suggesting a process of bacteria-plasmid co-evolution [Porse et al., 2016; Bottery et al., 2017]. Most of the previous studies however, have focused on characterising compensatory evolution following plasmid acquisition in a single host species. An important outstanding question is the extent to which compensatory evolution mechanisms for a given plasmid are shared across multiple host species. Here, we examine this question using genome sequencing of three *Pseudomonas* host species previously experimentally evolved along with a large conjugative mercury resistance plasmid. In the previous study, five phylogenetically diverse host species (*P. fluorescens* SBW25, *P. putida* KT2440, *P. stutzeri* JM300 (DSM 10701), *P. savastanoi pv. phaseolicola* 1448A, *P. aeruginosa* PAO1) were experimentally evolved with the large conjugative plasmid pQBR103 for ∼400 generations. The host species varied in their ability to maintain pQBR103:

pQBR103 was stably maintained in *P. fluorescens* and *P. savastanoi* with and without mercury selection, whereas it was only maintained in 4/6 replicate populations of *P. stutzeri* that evolved with mercury, while the plasmid was rapidly lost in all *P. putida* and *P. aeruginosa* populations [Kottara et al., 2017]. In this study, for the three host species that retained plasmids at the end of the selection experiment, we quantify the relative fitness of the evolved populations, measured their mercury resistance and genome sequenced the evolved clones to identify possible compensatory mutations that occurred during the selection experiment.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

Three *Pseudomonas* species [*P. fluorescens* SBW25 [Rainey et al., 1994], *P. savastanoi pv. phaseolicola* 1448A [Arnold et al., 2011], *P. stutzeri* JM300 (DSM 10701) [Busquets et al., 2012] carrying the pQBR103 plasmid were used in this study [Kottara et al., 2017]. pQBR103 is a large conjugative plasmid that confers mercury resistance via a *mer* operon encoded on a Tn5042 transposon [Lilley et al., 1996; Tett et al., 2007]. All experiments were conducted in 6 ml King's B growth medium in 30 ml universal vials ('microcosms') at 28°C in shaking conditions (180 rpm). During the selection experiment, six replicate plasmid-bearing populations of each bacterial species were grown in microcosms without mercury and six replicate populations were grown in microcosms supplemented with mercury (50 μM of mercury(II)chloride). Populations were serially transferred to fresh microcosms every 48 h for 60 transfers (approx. $\sim$400 generations) [Kottara et al., 2017]. To control for adaptation of the species to the growth conditions, we also experimentally evolved six replicate populations of each species without the plasmid for $\sim$400 generations in KB microcosms.

4.2.2 Competitive fitness assay

Four individual colonies of each ancestral and evolved clonal replicate of the plasmid-bearing *Pseudomonas* species [*P. fluorescens* SBW25, *P. savastanoi pv. phaseolicola* 1448A, *P. stutzeri* JM300 (DSM 10701)] were grown overnight in microcosms and later each was competed against the relevant isogenic plasmid-free, wild-type strain without mercury selection. To control for the species' adaptation to the growth medium, four individual colonies of each ancestral and evolved plasmid-free *Pseudomonas* species were competed against the relevant isogenic plasmid-free, wild-type strain. Relative fitness was measured by mixing differentially labelled test (plasmid-bearer or plasmid-free, labelled gentamicin) and reference (plasmid-free, wild-type) in ∼1:1 ratio, diluted 100-fold in KB microcosms and incubated at 28°C for 48 h. Samples were plated on KB agar plates at the beginning and end of the competition and replica plated onto KB agar plates supplemented with selective concentration of gentamicin to estimate the density of plasmid-bearers. The relative fitness was calculated as the selection rate (r) [Lenski et al., 1991].

4.2.3 Minimum inhibitory concentration assay

Growth of the ancestral and evolved, plasmid-free and plasmid-bearing *Pseudomonas* species [*P. fluorescens* SBW25, *P. savastanoi pv. phaseolicola* 1448A, *P. stutzeri* JM300 (DSM 10701)] was estimated in mercury concentrations ranging from 0-60 μM mercury(II)chloride for the plasmid-free strains and 0-135 μM mercury(II)chloride for the plasmid-bearing strains. Four individual colonies of each ancestral *Pseudomonas* species and four individual colonies of each evolved population of *Pseudomonas* species were grown at the exponential phase (optical density at 600 nm: ∼0.4) and later each diluted 1000-fold in KB containing the relevant mercury concentration in 96-well flat bottom cell culture microplates (Corning^{TM} Costar^{TM}) and incubated at 28°C for 48 h. Points represent the mean growth measured as optical density at 48 h

by the microplate reader Spark®(Tecan), based on the absorbance at 600 nm. The data of each clone was normalised with their growth at 0 μM mercury(II)chloride.

4.2.4 Genome analysis

One randomly chosen clone of each replicate evolved population that carried the plasmid until the end of the selection experiment was genome sequenced (12 evolved clones of *P. fluorescens*, 12 clones of *P. savastanoi* and 4 clones of *P. stutzeri*), plus the ancestral plasmid-free and plasmid-bearing *P. fluorescens*, *P. savastanoi* and *P. stutzeri*. Furthermore, one randomly chosen clone from each of three evolved plasmid-free control populations was also genome sequenced to distinguish mutations associated with adaptation to laboratory conditions. The total DNA of each bacterial clone was extracted by using the DNeasy Blood & Tissue Kit [Qiagen]. The whole-genome sequencing was performed by Microbes NG using the Illumina platform [`https://microbesng.uk`]. Reads were processed by SAMtools [Li et al., 2009] and aligned to the reference genome with the Burrows-Wheeler Alignment tool (BWA-MEM algorithm) [Li and Durbin, 2009; Li, 2013]. Duplicate reads were identified and removed by Picard Tools (GATK) [McKenna et al., 2010; DePristo et al., 2011]. Call Variants (GATK) [McKenna et al., 2010; DePristo et al., 2011] was used to identify the variants and SnpEff was used to annotate and predict the effect of the variants [Cingolani et al., 2012]; structural variations such as insertions, deletions, inversions and translocations, were predicted by BreakDancer [Chen et al., 2009].

4.2.5 Statistical analyses

Statistical analyses were performed using RStudio version 3.2.3 [R Core Team, 2015]. To analyse the relative fitness of the evolved against the ancestral strains, since the data was normal distributed, we performed a two-way ANOVA fitting X, Y and their interaction. Two sample Welch's t-tests were used to compare fitness

gains of evolved plasmid-bearing *P. fluorescens* and *P. savastanoi* that had evolved with or without mercury. Analysis of minimum inhibitory concentration curves for each species was conducted by estimating the area under the curve using the function *auc* of the package 'flux' [Jurasinski et al., 2012] which was analysed using the Kruskal-Wallis test because the data was not normally distributed. Welch's t-test was used to compare the growth of the evolved versus ancestral plasmid-bearing *P. fluorescens* at 105 μM and 120 μM mercury(II)chloride.

4.3 Results

4.3.1 Relative fitness of evolved clones

To determine the fitness response to selection in each species, we competed each of the evolved populations against their plasmid-free ancestor. We first confirmed that the ancestral plasmid was indeed costly in each of the ancestral *Pseudomonas* genotypes, and consistent with previous findings found that the magnitude of the cost varied between species [Kottara et al., 2017]. Evolved plasmid-bearing genotypes had increased in fitness relative to their ancestor in all species (effect of evolved background; ANOVA $F_{2,118}$=7.381, p<0.001), and for evolved *P. fluorescens* and *P. savastanoi* equivalent fitness gains were observed in both mercury environments (Welch's t-test, $t_{90.204}$=0.177, p=ns) (Figure 4.1). By contrast, plasmid-free controls of the different species varied in the extent of their adaptation to the selection environment; whereas evolved *P. fluorescens* increased in fitness relative to the ancestor, *P. stutzeri* and *P. savastanoi* showed no gain in fitness compared to their ancestor (effect of evolved background; ANOVA $F_{2,80}$=3.394, p=0.038; Figure 4.1). These data suggest that evolved hosts compensated for the cost of pQBR103 irrespective of whether they evolved in 0 μM mercury(II)chloride or in 50 μM mercury(II)chloride.

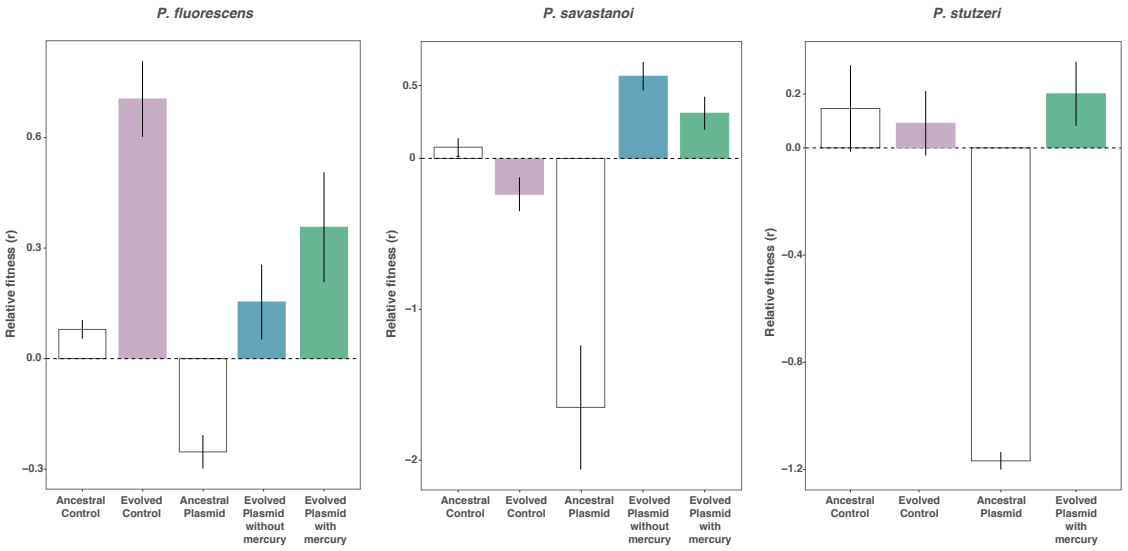

FIGURE 4.1: Fitness of the ancestral and evolved, plasmid-free and plasmid-bearing *Pseudomonas* species measured as relative fitness (r) between the test strain and the reference strain. Relative fitness 0 indicates no difference between test and reference strains; Error bars represent the SEM: SEM of four clonal replicates of the ancestral-plasmid-free (ancestral control) and ancestral-plasmid-bearing (ancestral plasmid) strain; SEM of four randomly selected clones of each evolved clonal population (evolved plasmid-free, evolved control; evolved plasmid in 0μM of mercury; evolved plasmid in 50μM of mercury).

4.3.2 Resistance phenotypes of evolved clones

To examine evolved changes in mercury resistance, the growth of the evolved plasmid-bearing and plasmid-free clones of *P. fluorescens*, *P. savastanoi* and *P. stutzeri* was compared to the ancestral genotypes across a gradient of mercury concentrations (Figure 4.2, Figure 4.3). The evolved plasmid-bearing strains of *P. fluorescens* and *P. savastanoi* were less susceptible to mercury than their plasmid-bearing ancestor. The MIC increased, in *P. fluorescens*, from 105 μM to 120 μM mercury(II)chloride (Welch's t-test, $t_{47.009}$=-3.052, p=0.003; Figure 4.2), and in *P. savastanoi*, from 75 μM to 90 μM mercury(II)chloride in strains that evolved without mercury and 105 μM mercury(II)chloride in strains that evolved with mercury (effect of evolved background; χ^2(2, N=52)=17.937, p<0.001; Figure 4.2). In contrast, *P. stutzeri* did not evolve greater resistance to mercury (effect of evolved background; χ^2(1, N=20)=1.508, p=ns), and, surprisingly, the evolved clones reached lower density than the ancestral strain in sub-inhibitory

concentrations of mercury (effect of evolved background from 0-60 μM mercury; $\chi^2(1, N=20)=4.723$, p=0.029) (Figure 4.2).

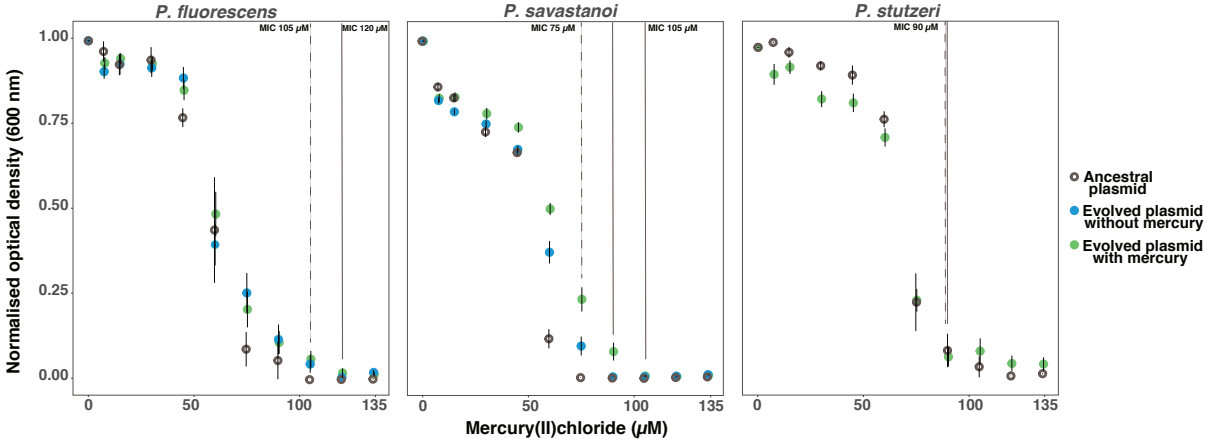

FIGURE 4.2: Growth of the ancestral and evolved plasmid-bearing *Pseudomonas* species in mercury environment ranging from 0-135 μM mercury(II)chloride. Points represent the mean growth measured as optical density at 48 h, based on the absorbance at 600 nm. Error bars represent the SEM: SEM of four clonal replicates of the ancestral-plasmid (ancestral plasmid); SEM of four randomly selected clones of each evolved plasmid-bearing clonal population in 0 μM and 50 μM of mercury(II)chloride.

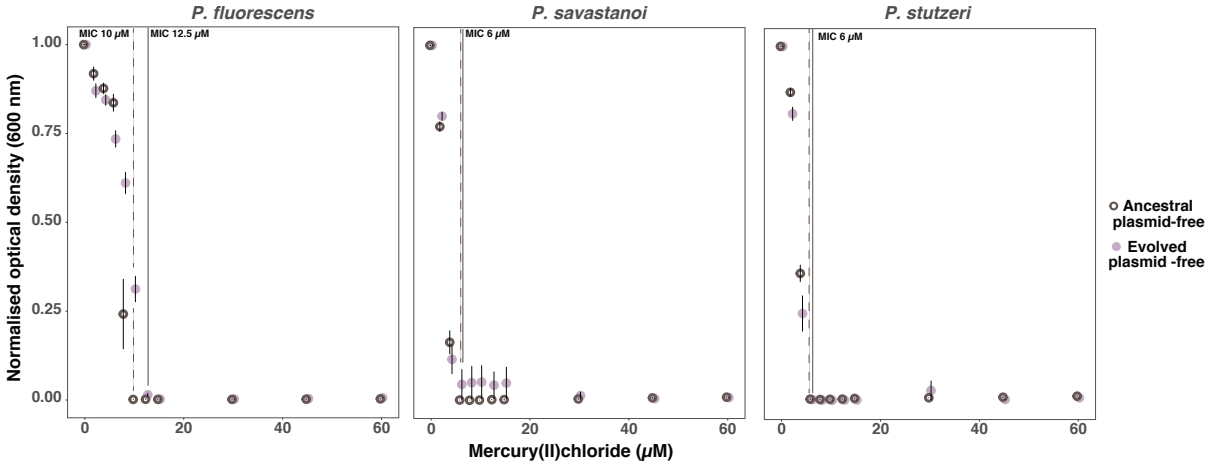

FIGURE 4.3: Growth of the ancestral and evolved plasmid-free *Pseudomonas* species in mercury environment ranging from 0-60 μM mercury(II)chloride. Points represent the mean growth measured as optical density at 48 h, based on the absorbance at 600 nm. Error bars represent the SEM: SEM of four clonal replicates of the ancestral-plasmid-free (ancestral plasmid-free) strain; SEM of four randomly selected clones of each evolved plasmid-free clonal population.

Plasmid-free evolved clones in general did not change in their mercury resistance, with the exception of a small increase in the resistance of *P. fluorescens* (effect of evolved background; $\chi^2(1, N=28)=4.982$, p=0.025; Figure 4.3). These data

suggest that increases in relative fitness were accompanied by increased mercury resistance in plasmid-bearing *P. fluorescens* and *P. savastanoi*, but not in *P. stutzeri*, potentially suggesting contrasting evolutionary trajectories in these species.

4.3.3 Parallel evolution in chromosomal global regulatory and metabolic systems

To identify the genetic response to selection, the evolved plasmid-bearing clones of *P. fluorescens*, *P. savastanoi* and *P. stutzeri* were genome sequenced. The number and the type of synonymous and non-synonymous mutations, mutations in coding and intergenic regions and frameshift mutants were variable between the different species (Figure 4.6-Supplementary Information). We found in total 37 mutations with 45.7% being non-synonymous in *P. fluorescens* (not including hypermutators, which increase the number of the detected SNPs by 5.8 times); 52 mutations with 50.2% being non-synonymous in *P. savastanoi*; and 32 mutations with 28.1% being non-synonymous in *P. stutzeri* (detailed descriptions of the evolved SNPs can be found in Table 4.1-4.3-Supplementary Information). Most of the identified mutations were observed in chromosomal genes while only a small subset of SNPs (3.3% of the total number of SNPs) observed in the plasmid pQBR103 (Figure 4.7-Supplementary Information). Major genomic rearrangements (translocations, inversions etc.) were not observed in any of the evolved clones.

Parallel evolution–i.e.where genetic locus acquires mutations in multiple independently evolving replicate populations–is strong evidence for positive selection acting at these sites. Possible compensatory mutations can then be identified as parallel evolving loci which were mutated in the plasmid-bearing treatments but not (or rarely) in the plasmid-free controls. In *P. fluorescens*, highly parallel evolution exclusive to plasmid-containing treatments was observed at *gacS* (5/6 clones from 0 μM mercury(II)chloride treatment, 2/6 clones from the 50 μM mercury(II)chloride treatment), while *gacA* was mutated in one plasmid-bearing hypermutator clone (Figure 4.4, Figure 4.8-Supplementary

Information). This confirms a previous study implicating *gacS*/*gacA* mutations in compensatory evolution following pQBR103 acquisition in *P. fluorescens* [Harrison et al., 2015a].

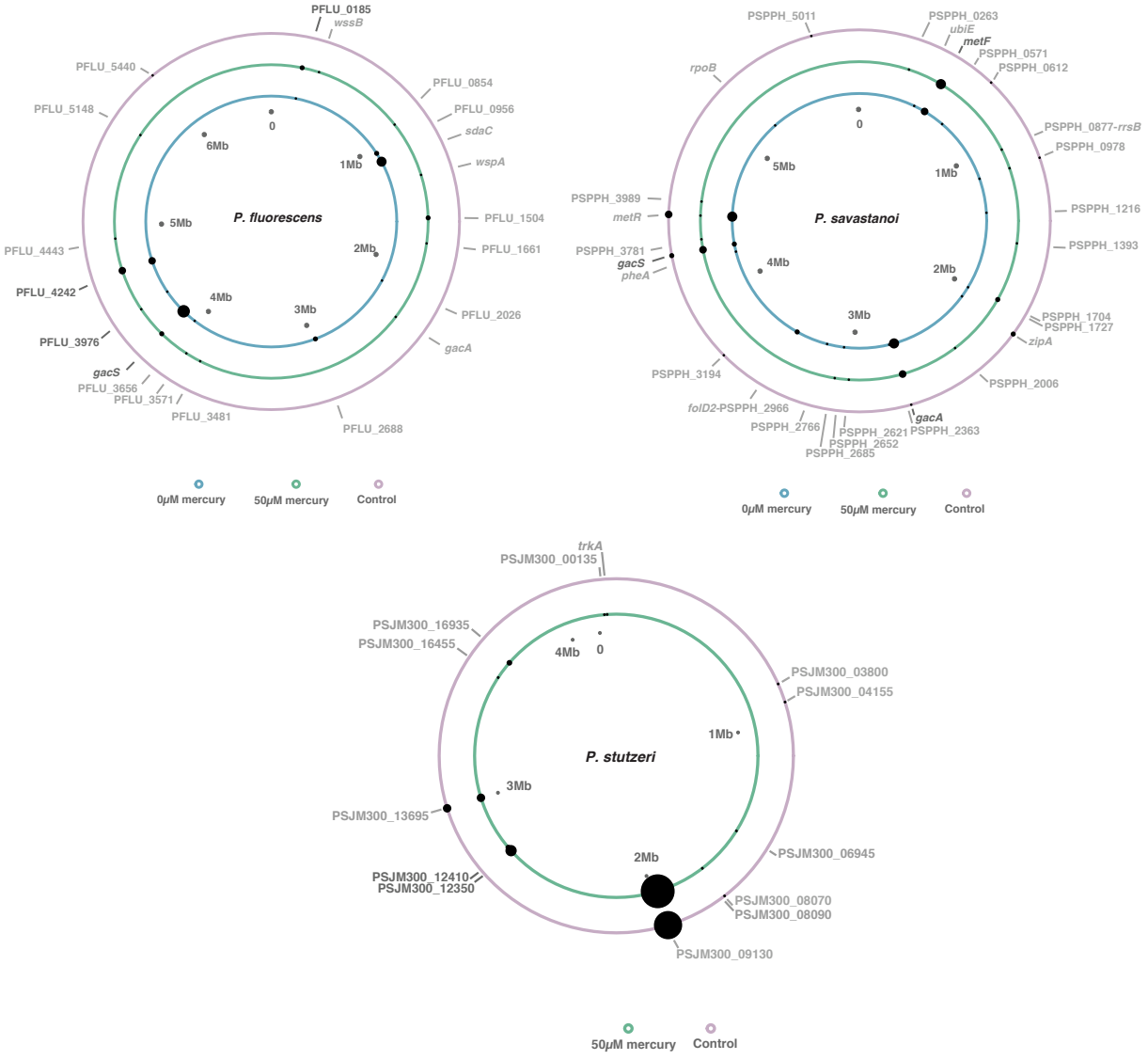

FIGURE 4.4: Summary of mutations identified in the evolved plasmid-bearing *Pseudomonas* species in absence and presence of mercury. Each circle represents the bacterial genome (purple: genome of the evolved plasmid-free *Pseudomonas*-control treatment, green: genome of the evolved in mercury plasmid-bearing *Pseudomonas*-mercury, plasmid treatment, blue: genome of the evolved in absence of mercury plasmid-bearing *Pseudomonas*-no mercury, plasmid treatment); the dots represent the single nucleotide polymorphisms (SNPs) where the size of the dots represents the number of times mutations identified at each locus across the clonal replicates (excluding the mutations of the hypermutators). Gene names in dark grey show the genes where mutations were parallel observed across the plasmid treatments; Gene names in light grey show the genes where either the mutations were found in the control treatment or the mutations were not parallel observed across the plasmid-treatments.

Interestingly, mutations in *gacS* or *gacA* were also observed in multiple evolved plasmid-bearing clones of *P. savastanoi*, however, unlike in *P. fluorescens*, in *P. savastanoi* mutations in *gacS*/*gacA* were also observed in evolved plasmid-free control clones. In addition to mutations in *gacS*/*gacA*, parallel mutations in *P. savastanoi* that were exclusive to the plasmid-containing treatment were observed at *metF* (3/6 clones from 0 μM mercury(II)chloride treatment, 4/6 clones from the 50 μM mercury(II)chloride treatment; Figure 4.4, Figure 4.9-Supplementary Information).

In *P. stutzeri*, all the evolved plasmid-bearing clones had acquired mutations in PSJM300_12350/PSJM300_12410 (these loci are related to RelA/SpoT system) and mutations at these loci were never found in the plasmid-free controls (Figure 4.4, Figure 4.10-Supplementary Information). Parallel evolution of plasmid loci was not observed in any host species (Figure 4.7-Supplementary Information). Possible compensatory evolution therefore was associated with mutations in chromosomal loci encoding either regulatory systems (*gacS*/*gacA*, PSJM300_12350/PSJM300_12410) or biosynthetic pathways (*metF*), which varied according to host species. Interestingly, each of these loci are broadly conserved among the sequenced genomes of *Pseudomonas* species (Figure 4.5).

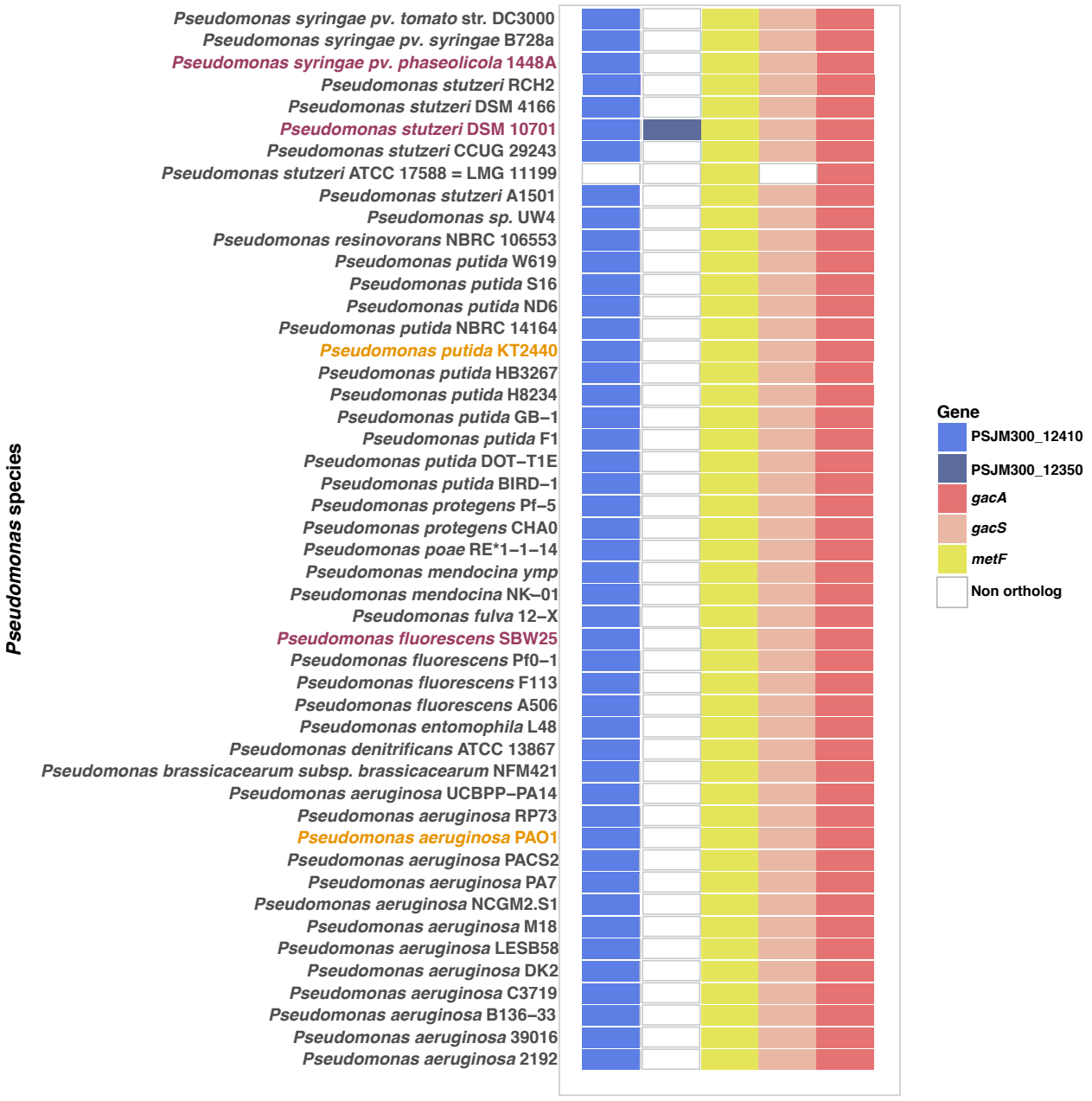

FIGURE 4.5: *Pseudomonas* species with ortholog genes of the parallel mutated genes across the plasmid-treatments. This data was sourced from the online *Pseudomonas* ortholog database. The list of putative orthologs is based on a reciprocal best-blast (RBB) search between proteins found in the genomes [`www.pseudomonas.com`]. *Pseudomonas* species that maintained the plasmid throughout the selection experiment and were re-sequenced at the end, are highlighted in red; *Pseudomonas* species that were used in the selection experiment but did not maintain the plasmid throughout the experiment, are highlighted in yellow.

4.4 Discussion

Following ∼400 generations of evolution, the plasmid-bearing clones of all three species had increased in fitness relative to their ancestor, whereas fitness gains were observed only in the *P. fluorescens* but not in the *P. stutzeri* or *P. savastanoi* plasmid-free controls. Fitness gains in plasmid-bearing clones were associated with increased mercury resistance in *P. fluorescens* and *P. savastanoi*, but not in *P. stutzeri*. Together, these data suggest that each of the three host species was able to overcome the cost of the pQBR103 carriage through compensatory evolution, and that in the case of *P. fluorescens* and *P. savastanoi* to even attain greater ecological benefits of plasmid carriage in terms of higher growth in toxic mercury environments. Genome sequencing of evolved clones revealed that, compared to plasmid-free controls, plasmid-bearing clones were enriched for mutations in the regulatory genes, *gacS*/*gacA*(*P. fluorescens*) and PSJM300_12350/PSJM300_12410 (*relA*/*spoT* orthologs in *P. stutzeri*), or in the biosynthetic gene, *metF* (*P. savastanoi*), suggesting alternative mechanisms for ameliorating the cost of pQBR103 acquisition across the different *Pseudomonas* species.

Compensatory evolution targeting regulatory systems has been previously described by a number of studies with [Harrison et al., 2015a] first demonstrating that compensatory mutations in *gacS/A* genes stabilise the pQBR103 plasmid in *P. fluorescens*. Here, mutations at the GacS/A regulatory system were observed in *P. fluorescens* and *P. savastanoi*. The GacS/A system is a two-component global regulatory system, which is present in most *Pseudomonas* species and controls the biosynthesis of a range of secondary metabolites in response to unknown environmental signals [Heeb and Haas, 2001]. GacS is the sensor kinase that is activated in response to the signals via autophosphorylation and GacA is the response regulator that is able to trigger the expression of target genes [Heeb and Haas, 2001]. Other core components of this system are the two small RNAs (sRNAs) *rsmY* and *rsmZ* that interact with the small RNA binding protein RsmA. Specifically, it has been found that GacS and GacA respond to the signals

by regulating the expression of these sRNAs which control the activity of the RsmA [Brencic et al., 2009]. RsmA is a small protein of the CsrA carbon-storage regulator family that plays an important role downstream the GacS/A pathway as an mRNA-binding post-transcriptional repressor [Haas and Défago, 2005].

Intruigingly, an ortholog of the *rsmA* gene (pQBR0443) was found in pQBR103 plasmid [Tett et al., 2007]. Based on this observation, we hypothesise that the loss-of-function mutations in the GacS/A pathway in *P. fluorescens* and *P. savastanoi* could have been arisen as a response to interference between the plasmid and the chromosomal RsmA orthologs, impairing functioning of the system. Moreover, *P. savastanoi* carries two native plasmids (131 kb and 51 kb) [Joardar et al., 2005] and the larger of theses plasmids encodes three genes (PSPPH_A0007, PSPPH_A0073, PSPPH_A0105) with homology to CsrA protein that could act in a similar way to the pQBR103-encoded RsmA. It is possible therefore that the observed mutations at the *gacS/A* genes in pQBR103-free *P. savastanoi* evolved genotypes may themselves be compensatory mutations to resolve interference between the native plasmid and chromosomal encoded carbon storage regulator-type proteins. These hypotheses remain to be tested.

Mutations in a different regulatory system, RelA/SpoT system, were observed in *P. stuzteri*. The RelA/SpoT system is a two-component regulatory system that acts as a stress response mechanism and responds in amino acid starvation by controlling the synthesis of the alarmone ppGpp, which is an activator of the 'stringent' response and regulator of cellular metabolism [Atkinson et al., 2011; Nguyen et al., 2011]. Therefore, compensatory mutations targeting the related RelA/SpoT system in *P. stutzeri* could have been induced to relieve stress responses resulting from plasmid acquisition. The factors RpoD and RpoS operate downstream of the RelA/SpoT pathway and are accumulated in the cell during the stress response, and activate expression of stress-induced (Rpo-dependent) genes [Battesti et al., 2011]. Intruigingly, pQBR103 encodes an ortholog of *rpoD* (pQBR0465), suggesting once again that direct regulatory interference may underlie the cost of pQBR103 acquisition [Tett et al., 2007].

Global regulatory systems were not the only targets of possible compensatory evolution. Mutations in *metF* gene were found in *P. savastanoi*. MetF reductase is part of the methionine biosynthesis pathway that has also been described to play a role in the induction of the type (III) secretion system (T3SS) in the related strain, *P. syringae pv. phaseolicola* B728a [Deng et al., 2009]. pQBR103 was not found to carry any part of the methionine biosynthesis pathway. At present the mechanism by which mutation at *metF* may reduce the plasmid cost is unknown.

In addition to their cargos of accessory genes encoding functional traits of potential use to their bacterial hosts, large conjugative plasmids like pQBR103 also encode regulatory genes which often have counterparts from the same or a complementary regulatory system on the host chromosome. It is possible that encoding components of the global regulatory systems could be a plasmid adaptation to increase their own fitness and spread. For example, genomic conflict between the plasmid and the chromosome could have selected plasmids to interfere with host regulation such that ribonucleotide and amino acid resources are diverted to express plasmid functions [Chen et al., 2005; Baltrus, 2013; San Millan and MacLean, 2017]. Compensatory mutations to chromosomal regulatory genes then could act to restore metabolic homeostasis to the benefit of the host cell [Yang et al., 2012].

4.5 Conclusions

This work demonstrates that *Pseudomonas* species could ameliorate the cost of plasmid carriage via compensatory evolution on regulatory and biosynthetic pathways. Loss-of-function mutations were arisen at the global regulatory systems, GacS/A and RelA/SpoT and the methionine biosynthetic pathway. Interestingly, a different combination of these mutations were detected at the different *Pseudomonas* species. *P. fluorescens* was found to carry mutations at the GacS/A system, *P. savastanoi* was found to carry mutations at the GacS/A system and the methionine biosynthetic pathway and *P. stutzeri* was found to

carry mutations at the related RelA/SpoT system. Thus, each *Pseudomonas* species used a unique compensatory mechanism to ameliorate the cost of the same plasmid. This clearly suggests that plasmids are stable in the environment via specific plasmid-host genomic interactions that potentially act to alleviate the host from the plasmid carriage.

4.6 Supplementary Information

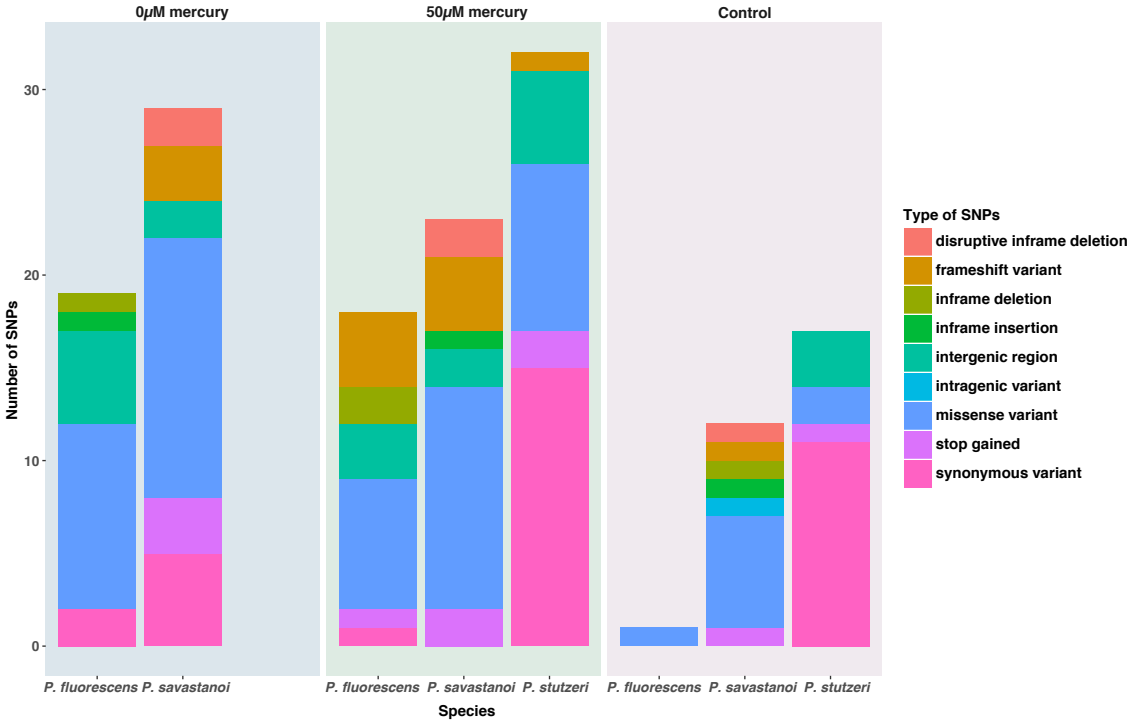

FIGURE 4.6: Number and type of mutations identified in each sequenced *Pseudomonas* species (0μM mercury – mutations found in the sequenced clones that had evolved in 0μM mercury(II)chloride; 50μM mercury – mutations found in the sequenced clones that had evolved in 50μM mercury(II)chloride; control – mutations found in the sequenced clones that had evolved without carrying the plasmid).

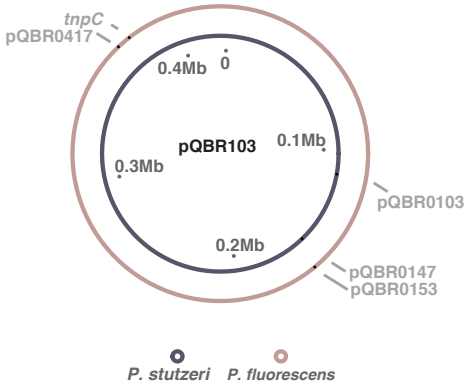

FIGURE 4.7: Mutations identified in the evolved pQBR103 plasmid. Each circle represents the plasmid pQBR103 in each *Pseudomonas* species. pQBR0103, pQBR0147, pQBR0153 and pQBR0417 genes encoding hypothetical proteins; *tnpC* gene encoding the transposase TnpC.

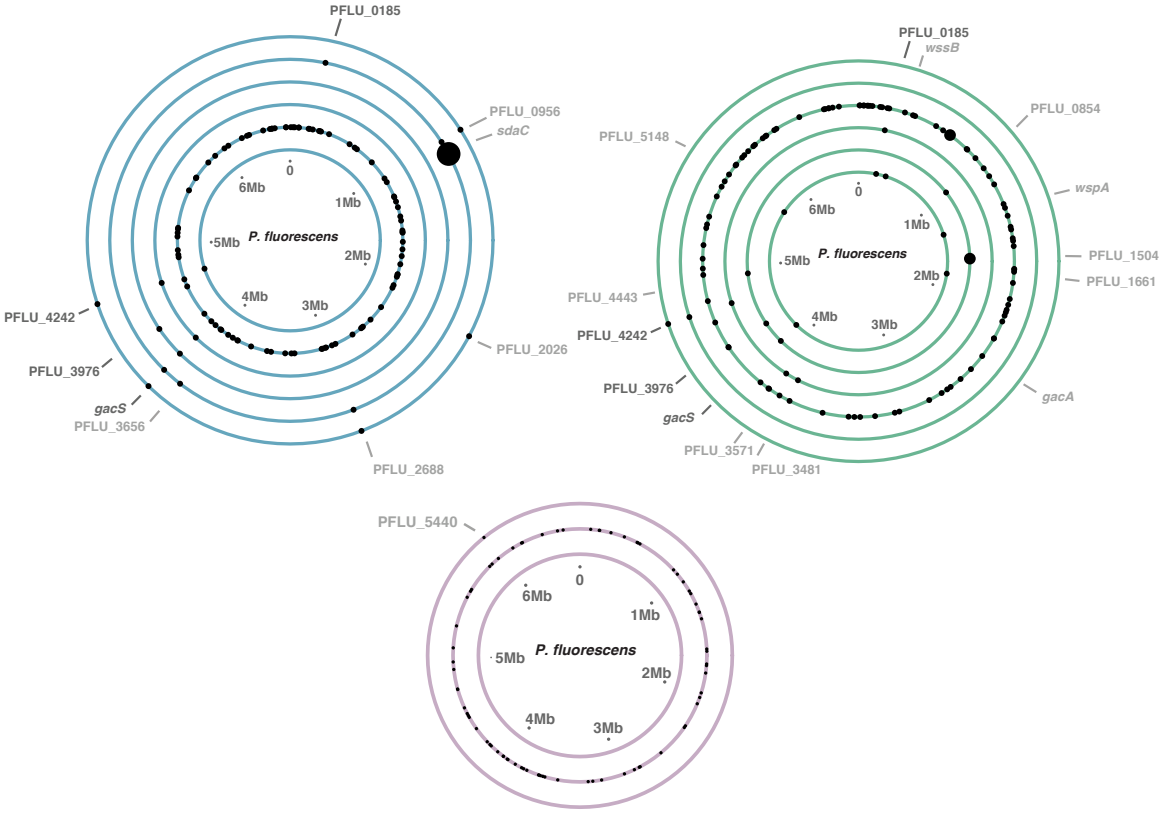

FIGURE 4.8: Mutations identified in the evolved *P. fluorescens* SBW25. Each circle represents the bacterial genome of each clone (purple: genomes of the evolved plasmid-free, green: genomes of the evolved in 50μM of mercury(II)chloride plasmid-bearing strains, blue: genomes of the evolved in 0μM of mercury(II)chloride plasmid-bearing strains; the dots represent the SNPs where the size of the dots represents the number of times mutations identified at each locus. Gene names in dark grey show the genes where mutations were parallel observed across the plasmid treatments; Gene names in light grey show the genes where either the mutations were found in the control treatment or the mutations were not parallel observed across the plasmid-treatments.

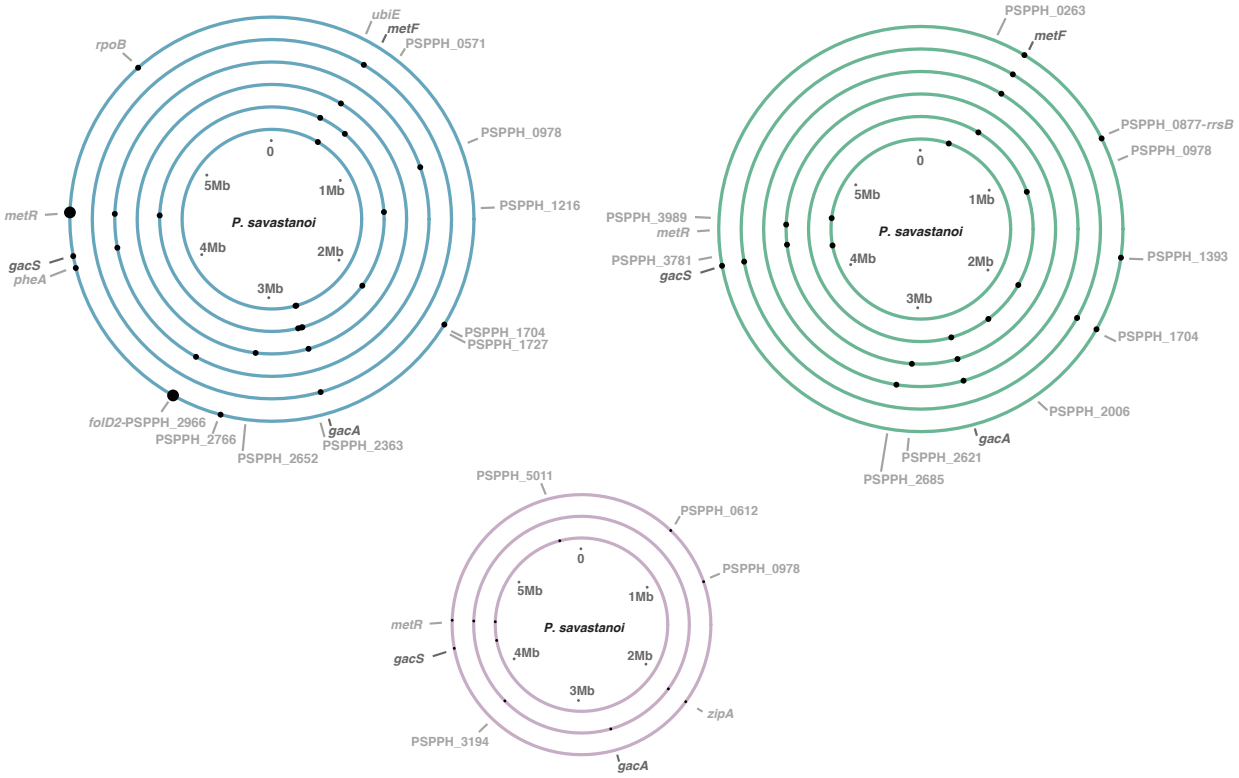

FIGURE 4.9: Mutations identified in the evolved *P. savastanoi pv. phaseolicola* 1448A. Circles and dots represent the bacterial genomes and SNPs as previously described.

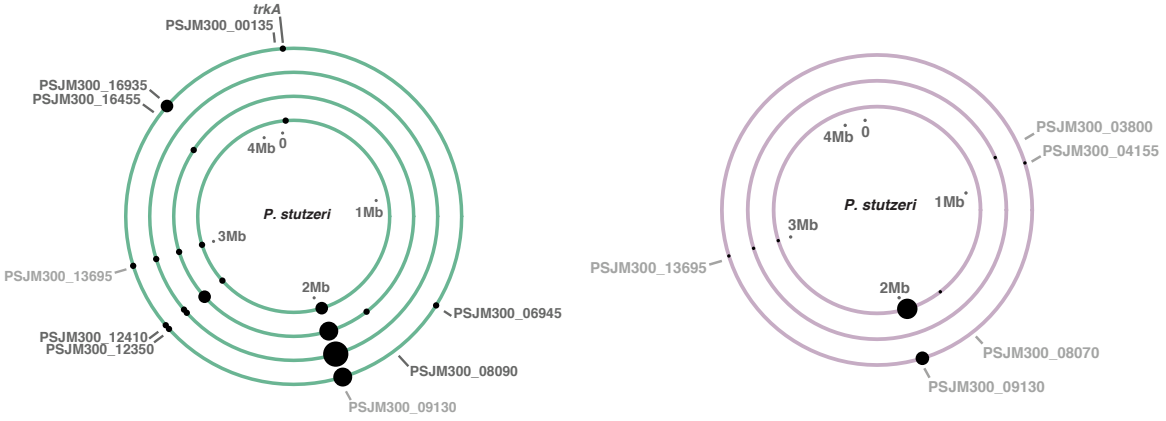

FIGURE 4.10: Mutations identified in the evolved *P. stutzeri* JM300 (DSM 10701). Circles and dots represent the bacterial genomes and SNPs as previously described.

TABLE 4.1: Description of the genes where mutations identified in the evolved *P. fluorescens* SBW25. Clones: A-F evolved in 0μM of mercury(II)chloride, G-L evolved in 50μM of mercury(II)chloride. The putative impact of variant was provided by SnpEff; The number of orthologs in other *Pseudomonas* species and the product descriptions were sourced from the online *Pseudomonas* ortholog and genome database [`www.pseudomonas.com`].

Gene name	Orthologs	Product	Cell localisation	Variant Impact	Genome location	Clones
PFLU_0185	47	Putative sensory box GGDEF/EAL domain-containing protein	Cytoplasmic Membrane	MODERATE	210540	E
				MODERATE	210952	I
				HIGH	211865	G
wssB	12	Cellulose synthase catalytic subunit	Cytoplasmic Membrane	MODERATE	329995	G
PFLU_0854	0	Hypothetical protein	Cytoplasmic Membrane	MODERATE	964625	H
PFLU_0956	32	Hypothetical protein	Cytoplasmic Membrane	MODIFIER	1062810	F
sdaC	4	Serine transporter	Cytoplasmic Membrane	MODERATE	1144650	E
				LOW	1144652	E
				MODERATE	1144653	E
				LOW	1144655	E
wspA	40	Putative methyl-accepting chemotaxis protein	Cytoplasmic Membrane	MODERATE	1353315	G
PFLU_1504	20	LysR family transcriptional regulator	Cytoplasmic	MODIFIER	1650308	H
				MODIFIER	1650311	H
PFLU_1661	0	Sugar transferase/ Part of the wrinkly spreader cell-wall biogenesis operon	Cytoplasmic Membrane	HIGH	1824310	G
PFLU_2026	32	Hypothetical protein	Unknown	MODERATE	2197856	F
gacA	48	Two-component system response regulator	Cytoplasmic	MODERATE	2372749	J
PFLU_2688	4	Putative ferric alcaligin siderophore receptor	Outer Membrane	MODIFIER	2966681	F
				MODIFIER	2966688	E
PFLU_3481	2	Hypothetical protein	Cytoplasmic	LOW	3851491	I
PFLU_3571	7	Putative GGDEF domain signaling protein	Cytoplasmic	MODERATE	3959618	I
PFLU_3656	0	Hypothetical protein	Cytoplasmic	MODERATE	4049765	E
gacS	47	Hybrid sensory histidine kinase in two-component regulatory system with UvrY	Cytoplasmic Membrane	MODERATE	4173019	C
				MODERATE	4173346	H
				MODERATE	4173817	F
				MODERATE	4174569	D
				MODERATE	4174569	E
				MODERATE	4174569	G
				MODERATE	4174756	B
PFLU_3976	35	RNA polymerase sigma factor	Cytoplasmic	MODIFIER	4391560	H
				MODIFIER	4391568	D
PFLU_4242	7	Hypothetical protein	Cytoplasmic	MODERATE	4684686	A
				MODERATE	4684686	C
				HIGH	4684768	I
				HIGH	4684768	K
				HIGH	4685238	L
				MODERATE	4685356	F
PFLU_4443	48	Putative two-component system response regulator nitrogen regulation protein NR(I)	Cytoplasmic	MODERATE	4908028	H
PFLU_5148	17	Hypothetical protein	Outer Membrane	MODERATE	5647730	G
PFLU_5440	40	Putative regulatory protein	Cytoplasmic	MODERATE	5973182	control

TABLE 4.2: Description of the genes where mutations identified in the evolved *P. savastanoi pv. phaseolicola* 1448A.

Gene name	Orthologs	Product	Cell localisation	Variant Impact	Genome location	Clones
PSPPH_0263	48	FUR family transcriptional regulator	Cytoplasmic	HIGH	305191	G
ubiE	48	ubiquinone/menaquinone biosynthesis methyltransferase	Cytoplasmic	MODERATE	426030	B
metF	48	5,10-methylenetetrahydrofolate reductase	Cytoplasmic	HIGH	512900	E
				MODERATE	512908	A
				HIGH	513178	K
				MODERATE	513223	J
				HIGH	513295	H
				MODERATE	513518	L
				HIGH	513522	C
PSPPH_0571	29	methyl-accepting chemotaxis protein	Cytoplasmic Membrane	MODERATE	672878	B
PSPPH_0612	0	Hypothetical protein	Unknown	MODERATE	723681	control
PSPPH_0877	46	sulfite oxidase subunit YedZ	Cytoplasmic Membrane	MODIFIER	1048630	L
PSPPH_0978	8	Hypothetical protein	Unknown	MODERATE	1168058	D
				MODERATE	1168058	H
				MODERATE	1168058	control
PSPPH_1216	37	glycosyl transferase family protein	Cytoplasmic Membrane	LOW	1425200	B
PSPPH_1393	9	repressor protein c2	Periplasmic	HIGH	1618303	L
PSPPH_1704	48	ABC transporter ATP-binding protein	Cytoplasmic Membrane	MODERATE	1970807	K
				MODERATE	1971766	L
PSPPH_1707	48	ABC transporter substrate-binding protein	Periplasmic	MODERATE	1974391	H
PSPPH_1727	42	methyl-accepting chemotaxis protein	Cytoplasmic Membrane	MODERATE	2000958	F
zipA	48	cell division protein ZipA	Cytoplasmic Membrane	MODERATE	2081387	control
				MODERATE	2081481	control
				MODERATE	2081526	B
PSPPH_2006	0	ISPsy19, transposase	Unknown	MODERATE	2355637	H
gacA	48	response regulator, DNA binding component GacA	Cytoplasmic	MODERATE	2703575	B
				MODERATE	2703580	I
				MODERATE	2703653	C
				MODERATE	2703745	J
				HIGH	2703798	A
				MODERATE	2703803	control
				MODERATE	2704007	E
				MODERATE	2704042	H
PSPPH_2337	14	Fis family transcriptional regulator	Cytoplasmic	MODERATE	2712009	B
PSPPH_2338	7	LacI family transcriptional regulator	Cytoplasmic	MODERATE	2712622	A
PSPPH_2363	7	ribose ABC transporter ATP-binding protein	Cytoplasmic Membrane	LOW	2740588	B
PSPPH_2621	45	esterified fatty acid cis/trans isomerase	Unknown	MODERATE	3025317	I
PSPPH_2652	47	ABC transporter ATP-binding protein	Cytoplasmic	MODIFIER	3076467	C
PSPPH_2685	9	polysaccharide deacetylase	Unknown	HIGH	3109269	J
PSPPH_2766	5	Ser/Thr protein phosphatase	Cytoplasmic Membrane	MODIFIER	3203947	F
folD2	14	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase	Cytoplasmic	LOW	3436413	D
PSPPH_2966	28	methyl-accepting chemotaxis protein	Cytoplasmic Membrane	MODERATE	3443181	F
				LOW	3443379	F
PSPPH_3194	0	Unknown	Unknown	MODIFIER	3705823	control
pheA	48	chorismate mutase	Cytoplasmic	MODERATE	4210416	F
gacS	47	response regulator, sensor histidine kinase component GacS	Cytoplasmic Membrane	HIGH	4267058	F
				HIGH	4267488	control
				MODERATE	4267554	G
				HIGH	4268071	D
				MODERATE	4268117	control
				HIGH	4268539	L
				MODERATE	4268648	K
PSPPH_3781	48	histidine triad family protein	Unknown	MODIFIER	4330813	I
metR	48	transcriptional regulator MetR	Cytoplasmic	MODERATE	4471136	I
				MODERATE	4471247	B
				HIGH	4471311	F
				LOW	4471315	F
				MODERATE	4471506	control
				HIGH	4471703	control
				MODERATE	4471712	control
				MODERATE	4471773	D
PSPPH_3989	0	amino-acid binding protein	Unknown*	MODERATE	4554276	G
rpoB	47	DNA-directed RNA polymerase subunit beta	Cytoplasmic	MODERATE	5236844	F
PSPPH_5011	30	MutT/nudix family protein	Unknown	MODERATE	5677894	control

TABLE 4.3: Description of the genes where mutations identified in the evolved *P. stutzeri* JM300 (DSM 10701).

Gene name	Orthologs	Product	Cell localisation	Variant Impact	Genome location	Clones
PSJM300_00135	6	diguanylate cyclase	Cytoplasmic	MODERATE	32080	H
trkA	48	potassium transporter peripheral membrane protein	Cytoplasmic Membrane	MODERATE	44805	L
PSJM300_03800	48	phosphotransferase enzyme IIA	Cytoplasmic	MODERATE	848968	control
PSJM300_04155	48	cell division protein FtsZ	Cytoplasmic	LOW	921292	control
PSJM300_06945	47	tRNA-dihydrouridine synthase C	Cytoplasmic	MODERATE	1493985	L
PSJM300_08070	19	UDP-N-acetyl-D-mannosamine dehydrogenase	Cytoplasmic	MODERATE	1728118	control
PSJM300_08090	0	serine acetyltransferase	Cytoplasmic	HIGH	1731324	I
PSJM300_09130	48	elongation factor G	Cytoplasmic	LOW	1966070	I
				LOW	1966607	H
				LOW	1966607	I
				LOW	1966607	control
				LOW	1966607	control
				LOW	1966619	H
				LOW	1966619	I
				LOW	1966619	control
				LOW	1966619	control
				LOW	1966733	L
				LOW	1966733	J
				LOW	1966733	control
				LOW	1966733	control
				LOW	1966751	L
				LOW	1966751	J
				LOW	1966751	control
				LOW	1966751	control
				LOW	1966817	L
				LOW	1966817	J
				LOW	1966817	control
				LOW	1966820	J
				LOW	1966820	control
PSJM300_12350	0	RelA/SpoT domain-containing protein	Cytoplasmic	MODERATE	2715054	L
				MODERATE	2715272	J
				MODERATE	2715327	I
				LOW	2715328	I
				MODIFIER	2715349	I
				MODIFIER	2715549	H
PSJM300_12410	47	GTP pyrophosphokinase	Cytoplasmic	HIGH	2734399	L
				MODERATE	2734605	J
PSJM300_13695	14	hypothetical protein	Unknown	MODIFIER	3001212	H
				MODIFIER	3001212	J
				MODIFIER	3001212	I
				MODIFIER	3001212	control
				MODIFIER	3001212	control
				MODIFIER	3001212	control
PSJM300_16455	43	Tfp pilus assembly protein PilE-like protein	Extracellular	HIGH	3586572	I
PSJM300_16935	67	elongation factor Tu	Cytoplasmic	LOW	3672981	L
				LOW	3672987	L

Chapter 5

Community-level plasmid dynamics are governed by the proficiency of the plasmid-donor species

5.1 Introduction

Mobile genetic elements (MGE) like plasmids, phages and transposons are important agents of horizontal gene transfer (HGT) driving diversification of bacterial genomes [Frost et al., 2005]. Conjugative plasmids contain genes encoding core plasmid functions—including their own propagation, replication, stability and transfer—along with accessory genes that encode traits like antibiotic and metal resistance [Norman et al., 2009]. While fulfilling the plasmid's core functions can impose a heavy burden on the host cell, the accessory genes can directly benefit the host cell by providing them with new ecological functions [Baltrus, 2013; San Millan and MacLean, 2017]. Mathematical models of plasmid population dynamics suggest that the plasmid cost, conjugation rate, segregation rate, and the strength of positive selection are

key parameters determining plasmid survival in bacterial populations [Stewart and Levin, 1977; Levin et al., 1979; Simonsen et al., 1990; Bergstrom et al., 2000].

Plasmids are expected to spread under positive selection for their encoded accessory genes [San Millan et al., 2014; Harrison et al., 2015a], however, because accessory genes can be captured by the bacterial chromosome rendering the plasmid useless, positive selection does not guarantee the long-term survival of plasmids [Bergstrom et al., 2000]. Meanwhile, in the absence of positive selection, plasmids are expected to go extinct due to purifying selection because the benefits of accessory genes do not outweigh the costs of plasmid carriage [Bergstrom et al., 2000]. Since rates of conjugation appear to often be too low for plasmids to persist as infectious elements (although see: Lopatkin et al. [2017]; Stevenson et al. [2017]), it has been argued that the widespread distribution of plasmids is paradoxical (the plasmid paradox: Harrison and Brockhurst [2012]). Yet, plasmids have been found to stably persist in natural bacterial communities in the absence of measurable positive selection, where the factors allowing plasmid stability are puzzling [Heuer and Smalla, 2012].

Most studies of plasmid dynamics focus on a single-host species, whereas, in natural bacterial communities, many potential host species co-exist, potentially broadening the range of conditions under which plasmids can survive. This limitation of current understanding is particularly interesting considering that several studies have shown that plasmids are not equally stable across host species [De Gelder et al., 2007; Kottara et al., 2017]. For example, while the mercury resistance plasmid pQBR103 was highly stable for >400 generations with or without mercury selection in *P. fluorescens* and *P. savastanoi*, it was unstable to varying degrees in *P. stutzeri* (∼100-400 generations), *P. aeruginosa* and *P. putida* (<6 generations) even with strong mercury selection [Kottara et al., 2017].

Hall et al. [2016] recently showed, by tracking the dynamics of the mercury resistance plasmid pQBR57 in a two-species soil community of *P. fluorescens* and

P. putida, that between-species transfer of the plasmid from a proficient host, *P. fluorescens*, to an unstable host, *P. putida*, allowed the plasmid to persist in *P. putida* both with and without mercury selection. This finding suggests that the dynamics of a plasmid in a bacterial community is likely to depend on the proficiency of the plasmid-donor species to stably maintain the plasmid. This leads to the prediction that, at the community-level, plasmid abundance will be higher in communities where it is carried by a proficient plasmid donor, since this species will both be able to maintain the plasmid in its own population, and then disseminate the plasmid to other species in the community.

To test this prediction, we tracked the dynamics of pQBR103 in a three-species community of *P. fluorescens*, *P. stutzeri* and *P. putida* with and without mercury selection. We varied which of the species carried the plasmid at the start of the experiment. We hypothesised that the community-level plasmid abundance would vary according to the proficiency of the plasmid-donor species to act as hosts to pQBR103, which varies hierarchically—*P. fluorescens* >*P. stutzeri* >*P. putida* [Kottara et al., 2017]. Replicate communities were propagated in potting soil microcosms, which more closely resemble natural conditions and promote the stable co-existence of the bacterial species [Gómez and Buckling, 2011; Heuer and Smalla, 2012; Hall et al., 2016].

5.2 Materials and Methods

5.2.1 Bacterial strains and plasmid

Three *Pseudomonas* species — *P. fluorescens* SBW25 [Rainey et al., 1994], *P. stutzeri* JM300 (DSM 10701) [Busquets et al., 2012] and *P. putida* KT2440 [Bagdasarian et al., 1981] — were utilised in this study. *Pseudomonas* species were labelled by directed insertion of either streptomycin (Sm^R) or gentamicin resistance (Gm^R) marker using the mini-Tn7 transposon system [Lambertsen et al., 2004]. The plasmid used in this study, pQBR103 is a large conjugative

plasmid (425 kb) that confers mercury resistance via a *mer* operon encoded on a Tn5042 transposon [Lilley et al., 1996; Tett et al., 2007]. pQBR103 plasmid was conjugated into *P. stutzeri* Gm^R, *P. putida* Sm^R and *P. fluorescens* Sm^R *lacz* from the plasmid-bearing *P. fluorescens* SBW25 Sm^R or Gm^R stocks. Plasmid conjugation was performed by mixing 1:1 each of the plasmid-free with the plasmid-bearing strains, incubating for 48 h and spreading on KB agar plates containing 5 μg/ml gentamicin or 50 μg/ml streptomycin (50 μg/ml XGal) and 20 μM of mercury(II)chloride to select for transconjugant colonies [Simonsen et al., 1990]. The conjugation assays were conducted in 6 ml King's B growth (KB) medium in 30 ml universal vials ('microcosms') at 28°C in shaking conditions (180 rpm).

5.2.2 Selection experiment

To account for the high segregation rate of the plasmid in *P. putida* KT2440 [Kottara et al., 2017] and ensure high starting frequencies of plasmid carriage across all the tested bacterial strains, single colonies of each plasmid-bearing species were reconditioned overnight and then transferred in fresh media containing mercury. Specifically, 12 colonies of each plasmid-bearing *Pseudomonas* species were incubated overnight in KB 6 ml microcosms at 28°C with shaking 180 rpm after which time 1% of each population was transferred to grow for 24 h in fresh KB microcosms containing 50 μM of mercury(II)chloride at same temperature and shaking conditions. Similarly, 24 colonies of each plasmid-free *Pseudomonas* species were also incubated overnight in KB 6 ml microcosms and transferred to grow for 24 h in fresh KB microcosms at same temperature and shaking conditions.

Bacterial communities

Three different bacterial communities differing in which species carries the plasmid at the beginning of the experiment (plasmid donor) were constructed: *P. fluorescens*(pQBR103) with *P. stutzeri* and *P. putida*; *P. fluorescens* with *P. stutzeri*(pQBR103) and *P. putida*; *P. fluorescens* with *P. stutzeri* and *P.*

putida(pQBR103). Six replicates of each community were grown without mercury and with mercury (16 μg/g Hg(II)). Each community had a starting ratio of 1:1:1 of each *Pseudomonas* species such that the starting frequency of pQBR103 in the community was approximately 30%. To remove spent media each inoculum was centrifuged for 1 min at 10,000 rpm and resuspended in 1 ml M9 salt solution (Cold Spring Harbor Protocols). 100 μl was then inoculated into soil microcosms (10 g twice-autoclaved John Innes No. 2 compost soil) and incubated at 28°C at 80% humidity [Hall et al., 2016].

Serial transfers and bacterial counts

Every 4 days, 10 ml of M9 buffer and 20 glass beads were added to each soil microcosm and mixed by vortexing for 1 min, and 100 μl of soil wash was transferred to a fresh soil microcosm as previously described by Hall et al. [2016]. Bacterial counts for each species were estimated by plating onto selective media: 50 μg/ml streptomycin–50 μg/ml XGal KB agar plates and 5 μg/ml gentamicin KB agar plates, each of which was then replica plated onto mercury KB agar plates (100 μM mercury(II)chloride). The bacterial communities were evolved for 10 transfers ($\sim$40 days).

Plasmid and mercury-transposon screening

Twenty-four mercury-resistant colonies of each *Pseudomonas* species were sampled every 2 transfers from the mercury containing plates and tested for the presence of the plasmid and mercury transposon by PCR screening. The PCR used the same sets of primers as previously described [Harrison et al., 2015a; Kottara et al., 2017].

5.2.3 Statistics

Statistical analyses were performed using RStudio version 3.2.3 [R Core Team, 2015]. Shapiro-Wilk test, normal Q-Q plots, histograms and box-plots were used to examine the normality of the data. We found that in most cases the data were not normally distributed, and in such cases used a non-parametric test.

Cumulative plasmid abundance in each community over time was estimated as the area under the curve using the function *auc* of the package 'flux' [Jurasinski et al., 2012]. Community-level plasmid abundances in the plasmid-donor treatments were compared by using the Kruskal-Wallis test. To assess the plasmid-dynamics within each species, we compared plasmid frequencies in the plasmid-recipient species population as the area under the curve. The integral estimates of the plasmid frequency in the recipient species were compared between the mercury conditions using the Kruskal-Wallis test. To assess the timing of chromosomal acquisition of the mercury transposon Tn5042 in *P. putida* differed between the plasmid-donor treatments, for each population we recorded the transfer number when we first observed plasmid-free transposon-containing genotypes of *P. putida*. We compared these values between the plasmid-donor treatments using the Kruskal-Wallis test. The species diversity of plasmid-carriers was calculated as the 1-D Simpson's Index $[D = \sum(\frac{n}{N})^2]$ where n= the end-point population density of each plasmid-bearer species in community and N=total population density of plasmid-bearer species. We compared diversities between the plasmid-donor treatments and mercury conditions by using the Kruskal-Wallis test.

5.3 Results

5.3.1 Donor species identity affects community-level plasmid abundance

The bacterial host species vary in their ability to stably maintain pQBR103 hierarchically as follows: *P. fluorescens* >*P. stutzeri* >*P. putida* [Kottara et al., 2017]. We hypothesised therefore that the identity of the plasmid donor in a community is likely to affect the dynamics of the plasmid-encoded mercury resistance at the community-level. To test this, we quantified the total plasmid abundance in each community (Figure 5.1). Mercury selection increased total

plasmid abundance (effect of mercury; $\chi^2(1, N=24)=17.28$, p=3.226e-05) and total plasmid abundance varied with plasmid-donor identity, such that both with (effect of plasmid treatment; $\chi^2(2, N=18)=11.556$, p=0.003) and without (effect of plasmid treatment; $\chi^2(2, N=18)=11.474$, p=0.003) mercury selection, the total plasmid abundance was higher when the plasmid donor was *P. fluorescens*. Together these data suggest that community-level plasmid dynamics are affected by both positive selection for plasmid-encoded traits and the identity of the plasmid-donor species, being enhanced when plasmids are beneficial and carried by a proficient plasmid donor.

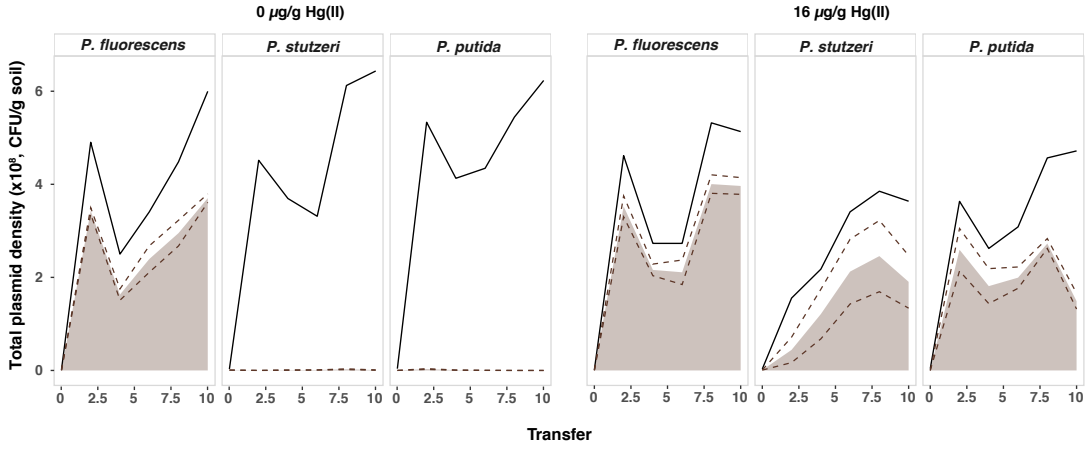

FIGURE 5.1: Total plasmid density in the community throughout the selection experiment; Each panel shows the total plasmid density in the community when the initial plasmid donor was *P. fluorescens*, *P. stutzeri* or *P. putida*. Brown area total plasmid density of the populations. Dotted lines represent the standard error of the total plasmid density from six replicates. Solid lines show the mean total community bacterial density from six replicates.

5.3.2 Species-level plasmid dynamics within communities

To understand how the variation in community-level plasmid abundance was driven by plasmid-donor identity, we next examined the species-level plasmid dynamics in each community. As predicted, when a proficient plasmid-host–*P. fluorescens*–was the plasmid donor it maintained the plasmid at high frequency within its population both with and without mercury. Plasmids were

disseminated to the other species at higher frequencies under mercury selection (effect of mercury; $\chi^2(1, N=24)=4.653$, $p=0.030$)–to *P. putida* in all replicates and to *P. stutzeri* in 2/6 replicates–but were also observed in *P. stutzeri* at low levels in some of the communities without mercury (Figure 5.2). When *P. stutzeri* was the plasmid donor, it also maintained the plasmid within its own population both with and without mercury, and disseminated plasmids to the other species at a higher rate with mercury (effect of mercury; $\chi^2(1, N=24)=11.644$, $p=0.0006$) (Figure 5.3). Variation in total plasmid abundance between replicate communities in this treatment appear to have been caused by whether or not *P. fluorescens* acquired the plasmid before it was driven extinct by toxic mercury: where transmission to *P. fluorescens* occurred, total plasmid abundances were higher (Figure 5.3). Where *P. putida* was the plasmid donor, it did not maintain the plasmid within its own population: without mercury, the plasmid was simply lost, whereas, with mercury, plasmid-bearers were replaced by mutants that had inserted the Tn5042 encoding the *mer* operon into their chromosome (Figure 5.4). Chromosomal insertions of the Tn5042 in *P. putida* were observed in the other plasmid-donor treatments, but arose much later in communities where *P. putida* had to acquire the plasmid horizontally from either *P. fluorescens* or *P. stuzeri* (effect of treatment; $\chi^2(2, N=18)=10.947$, $p= 0.004$). Although *P. putida* eventually lost the plasmid from its own population, prior to this loss it successfully disseminated the plasmid to *P. fluorescens* in 6/6 replicates and to *P. stutzeri* in 3/6 replicates with mercury selection (Figure 5.4).

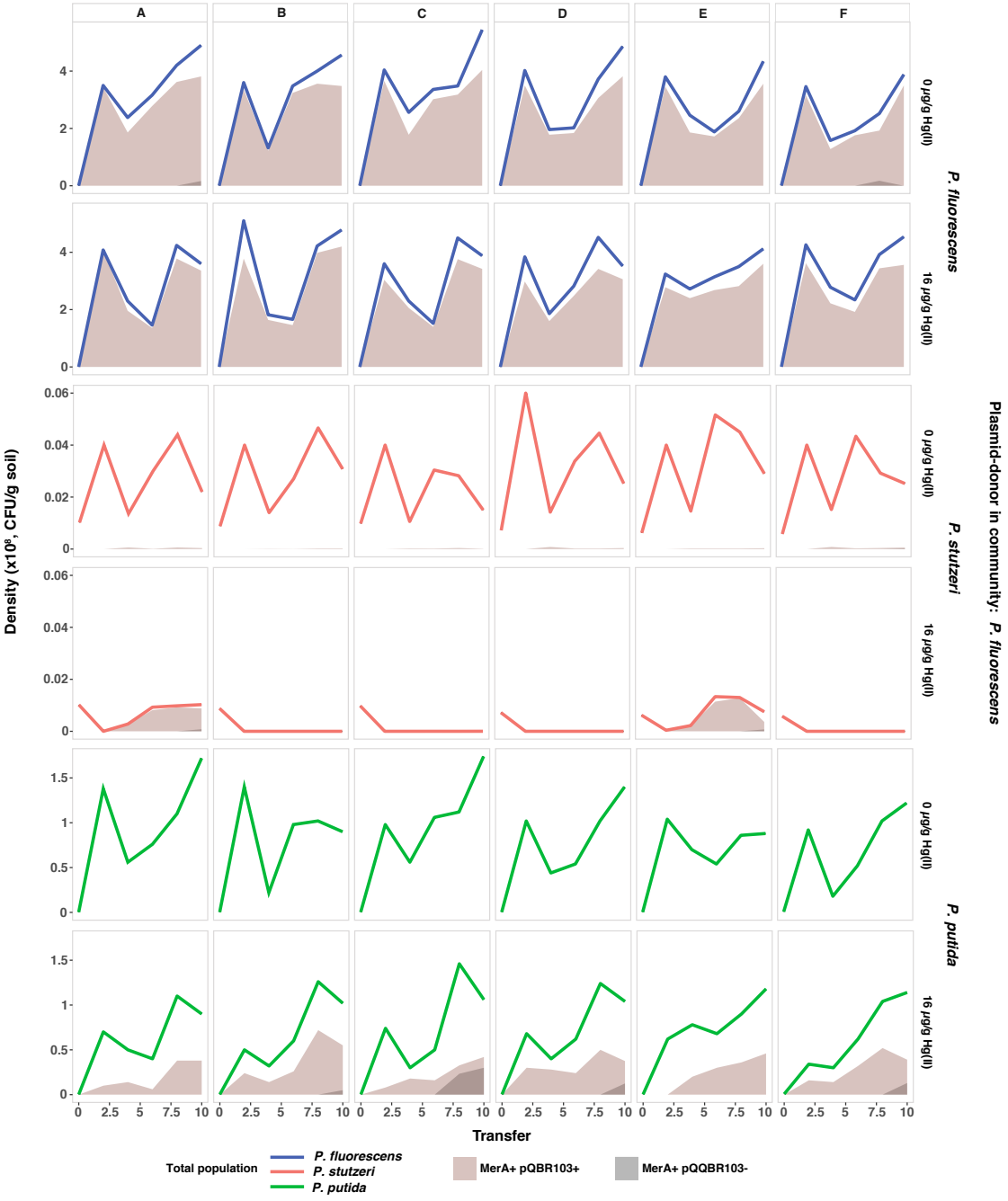

FIGURE 5.2: Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. fluorescens*. A-F clonal populations evolving with and without mercury. Lines show the population densities of *P. fluorescens* (blue); *P. stutzeri* (red); *P. putida* (green). Brown area indicates the populations that confer mercury resistance in presence of the plasmid; Grey area indicates the populations that confer mercury resistance in absence of the plasmid.

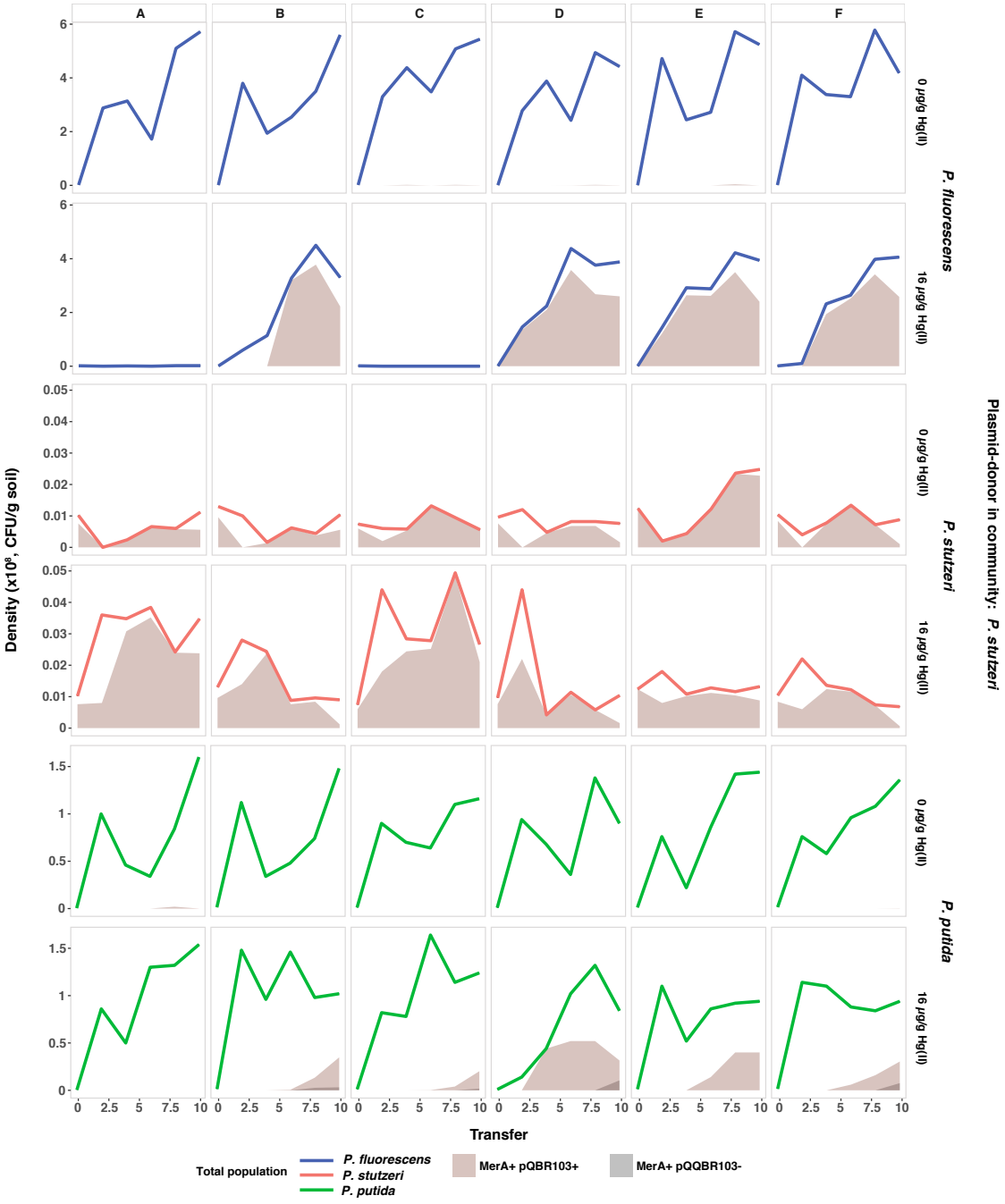

FIGURE 5.3: Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. stutzeri*. A-F clonal populations evolving with and without mercury. Lines show the population densities of *P. fluorescens* (blue); *P. stutzeri* (red); *P. putida* (green). Brown area indicates the populations that confer mercury resistance in presence of the plasmid; Grey area indicates the populations that confer mercury resistance in absence of the plasmid.

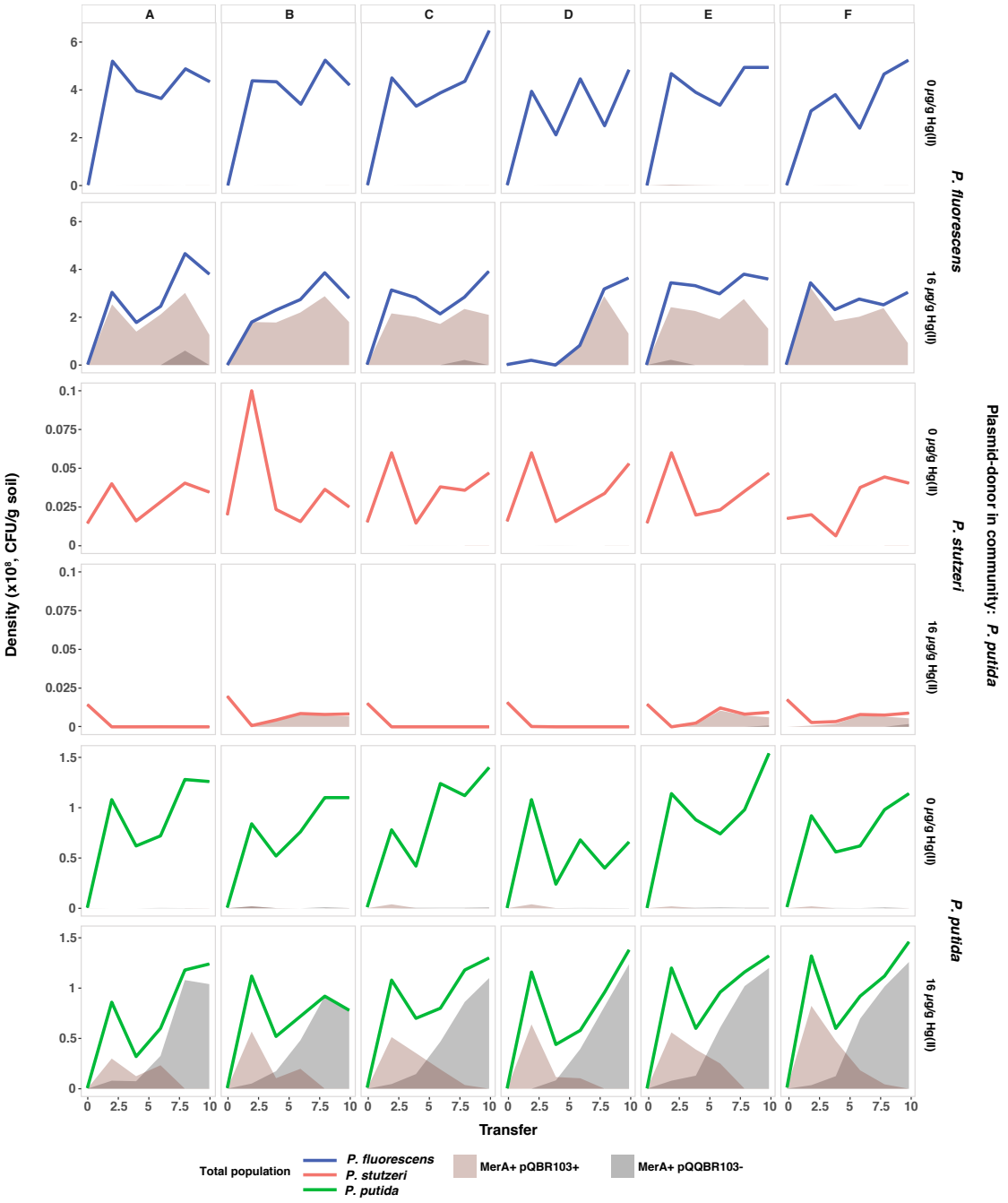

FIGURE 5.4: Population density and tracked mercury resistance transposon (Tn5042) and plasmid density in the communities with plasmid-donor *P. putida*. A-F clonal populations evolving with and without mercury. Lines show the population densities of *P. fluorescens* (blue); *P. stutzeri* (red); *P. putida* (green). Brown area indicates the populations that confer mercury resistance in presence of the plasmid; Grey area indicates the populations that confer mercury resistance in absence of the plasmid.

5.3.3 Diversity within the plasmid-donor communities

Finally, we tested how the plasmid-donor species affected the diversity of plasmid-carriers at the end of the experiment. The diversity of plasmid-carriers was affected by both the plasmid-donor species identity (effect of plasmid treatment; $\chi^2(2, N=36)=12.819$, $p=0.001$) and mercury selection (effect of mercury; $\chi^2(1, N=36)=6.082$, $p=0.013$) (Figure 5.5). Without mercury selection the diversity of plasmid-carriers was highest when *P. stutzeri* was the plasmid donor. Whereas, with mercury selection, the diversity of plasmid-carriers was higher in communities where *P. fluorescens* or *P. stutzeri* were the plasmid-donors compared to communities where *P. putida* was the plasmid donor. Consistent with our data on community-level plasmid abundance, these data show that the diversity of plasmid-carriers is likely to be higher when plasmids are beneficial and carried by proficient plasmid-donors.

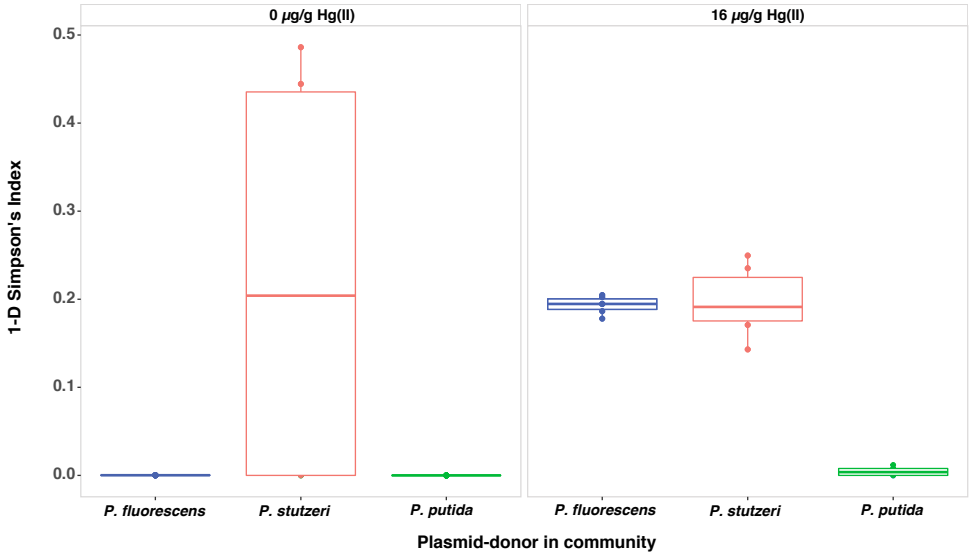

FIGURE 5.5: Diversity of plasmid-bearer species in each plasmid-donor community. Species diversity was calculated as the 1-D Simpson's Index by using the end-point population densities of the plasmid-bearer species in each plasmid-donor community.

5.4 Discussion

In natural microbial communities, broad host range plasmids are frequently transmitted to diverse host species thus highlighting the importance of plasmids in HGT and their role in the spread of resistance genes in the environment [Klümper et al., 2015]. In this study, we aimed to understand to what extend the plasmid dynamics in a microbial community are affected by the donor-species identity within that community. Our findings suggest that plasmid abundance at the community-level was driven by the plasmid-donor species. We observed that pQBR103 reached higher community-level abundance when hosted by a proficient plasmid-host, *P. fluorescens*. The importance of species identity in shaping the plasmid dynamics in a community was firstly described by Hall et al. [2016] where a proficient plasmid-host could act as a source of the plasmid for a non-proficient host species in a two-species soil community. These plasmid dynamics were explained in terms of conjugative plasmids persisting in the community as infectious agents via interspecies transfer [Bahl et al., 2007]. Here, we extend these results to a more complex three-species community, a different unrelated plasmid, and a wider range of plasmid-donor species and proficiencies.

The community-level plasmid abundance also varied according to mercury selection, such that plasmids were observed at higher frequencies in recipient species in the presence versus absence of mercury. Detecting HGT events is more likely under positive selection, because, while individual conjugation events may be rare, positively selected horizontally acquired genes will rise to high frequency due to clonal expansion. This has led to a generally accepted, but probably incorrect view, that HGT is accelerated under positive selection [Aminov, 2011; Fletcher, 2015]. By contrast, recent experimental data shows that horizontal transmission plays a more important role in plasmid stability in the absence of positive selection [Stevenson et al., 2017], leading to higher rates of gene mobilisation and transfer in these environments [Hall et al., 2017]. Mercury selection also drove the invasion of *P. putida* mutants that had lost the plasmid but captured the Tn5042 carrying the mercury resistance operon to the

chromosome, an outcome rarely observed in the other host species. This confirms our previous data that the rate and/or propensity for transposition of traits from the plasmid to the chromosome is variable among *Pseudomonas* species [Kottara et al., 2017]. We show here that the dynamics of this process are affected by the community context, specifically whether or not *P. putida* was the plasmid donor. Chromosomal capture of mercury resistance transposon in *P. putida* occurred earlier when it began the experiment with the plasmid, reflecting that transposition is random mutational event and thus more likely to occur in larger—plasmid-bearing—populations. Interestingly, however, our data also show that even poor plasmid hosts, who rapidly capture useful traits and jettison the plasmid, can act as a source of plasmids for other species in community by transferring the plasmid to more proficient host species before it is lost.

In contrast to the study of Hall et al. [2016], which used a highly conjugative plasmid, pQBR57, the plasmid used here, pQBR103, has a much lower conjugation rate [Hall et al., 2015]. While previous studies of pQBR103 have focused on the importance compensatory evolution in its longer-term stability [Harrison et al., 2015a, Chapter 4], here we show an effect of between species conjugation increasing its community-level abundance. The role for interspecific conjugation in pQBR103 stability was most notable in communities where it was initially carried by a non-proficient plasmid donor, *P. putida*. Here, while the plasmid went extinct in the *P. putida* population, it survived in the community by horizontal transmission, most commonly into *P. fluorescens*. Through interspecific conjugation, pQBR103 increased the diversity of plasmid-carriers in communities, especially under mercury selection. However, this effect depended upon the plasmid-donor identity. Surprisingly, while with mercury more proficient plasmid-donors (i.e. *P. fluorescens* and *P. stutzeri*) allowed higher diversities of plasmid-carriers, without mercury it was in communities where the intermediately proficient plasmid host, *P. stutzeri*, was plasmid donor where the highest plasmid-carrier diversity was observed. This effect is likely to have been caused by the more equitable distribution of plasmid carriage in these communities, and specifically by higher rates of plasmid carriage in *P. stutzeri*

itself compared to communities where this species had to obtain the plasmid via conjugation.

Soil microbial communities are highly diverse, which is thought to play a key role in their function [Torsvik and Øvreås, 2002] and species diversity has been suggested to play a role in the dissemination of conjugative plasmids [Dionisio et al., 2002]. Soil habitats are often characterised as hot spots for HGT [van Elsas and Bailey, 2002; Sørensen et al., 2005] possibly due to the spatially structured nature of such environments [Bahl et al., 2007; Fox et al., 2008]. Here, we show that the identity of plasmid-donor species determines the community-level abundance of conjugative plasmids in soil bacterial communities. Proficient plasmid hosts better maintain plasmids within their own population and transmit these plasmids to other species in the community. This implies that proficient plasmid-donor species could promote the robustness of communities by spreading potentially adaptive genes to diverse species, allowing their survival if the environment deteriorates.

5.5 Conclusions

We show that proficient plasmid-donor species and mercury selection increase community-level plasmid abundance and the diversity of plasmid-carriers. These findings confirm that interspecific conjugation plays an important role in determining plasmid stability in a community context, where proficient plasmid-donors are likely to act as sources of plasmids and hubs of horizontal gene transfer.

Chapter 6

The dilution effect limits plasmid co-infection in diverse bacterial communities

6.1 Introduction

Mobile genetic elements (MGE) are an abundant source of potentially beneficial ecological accessory traits for host bacteria, equipping these bacterial cells with ready-to-use novel functions and thereby allowing them to expand their environmental niche [Frost et al., 2005; Norman et al., 2009]. In particular, plasmids are common in environmental bacterial communities, and infect diverse environmental taxa [Smalla and Sobecky, 2002]. Given their abundance and diversity, multiple plasmids often co-exist in natural microbial communities [Lilley et al., 1996; Heuer and Smalla, 2012]. pQBR plasmids, as an example, were isolated from the same plant-associated soil bacterial community whilst each encoding the same ecological trait, the mercury-resistance *mer* operon [Lilley et al., 1994, 1996]. Sequence analysis of four pQBR plasmids revealed that the *mer* operon is carried on the Tn5042 transposon on each of the plasmids, suggesting that this transposon was horizontally acquired by each of the

plasmids *in situ* [Tett et al., 2007; Hall et al., 2015]. Plasmid co-existence in natural communities could result in plasmid co-infection of the same cell, which could have important implications for plasmid evolution given that plasmids are mosaic in structure and appear to frequently recombine and swap ecological accessory traits [Toussaint and Merlin, 2002].

While plasmid communities are diverse [Smillie et al., 2010], so too are the bacterial communities that they inhabit in natural environments [Gibbons and Gilbert, 2015]. Previous studies have shown that plasmids are not equally maintained across different host species [De Gelder et al., 2008; Kottara et al., 2017], while plasmid transmission dynamics are affected by bacterial community structure [Hall et al., 2016, Chapter 5]. Thus, in communities where plasmids rely on infectious transmission for their maintenance [Dionisio et al., 2002; Hall et al., 2016], plasmid dynamics could be affected by the diversity of the community, particularly if the different host species differ in their transmission rates. Studies focused on parasite transmission in host communities have shown that the transmission of multi-host parasites can be limited by species richness, which is termed the 'dilution effect' [Civitello et al., 2015; Levi et al., 2016]: A focal host species has a reduced risk of parasite infection when in a diverse community than would be expected from its intraspecific transmission rate, if transmission from other species in the community is less efficient [LoGiudice et al., 2003]. We hypothesise that the dilution effect may also apply to plasmids in communities where hosts differ in their ability to maintain and transmit plasmids.

To gain a better understanding of plasmid dynamics in complex multi-plasmid / multi-host natural communities we constructed simple bacterial soil communities under controlled laboratory conditions and tracked plasmid dynamics over-time. Specifically, communities contained two diverse conjugative plasmids, pQBR57 and pQBR103, that are known to vary in their rate of conjugation in the focal species, *P. fluorescens* SBW25 [Hall et al., 2015]. *P. fluorescens* SBW25 populations were embedded within a community of five *Pseudomonas* species, and these were compared to controls where *P. fluorescens* SBW25 was propagated in monoculture. We report that presence of the *Pseudomonas*

community reduced the rate of plasmid co-infection in *P. fluorescens* SBW25 in line with there being a dilution effect in more diverse communities.

6.2 Materials and Methods

6.2.1 Bacterial strains and plasmid

P. fluorescens SBW25 [Rainey et al., 1994] was the plasmid-donor in this study, carrying either the plasmid pQBR57 or pQBR103. *P. fluorescens* SBW25 was labelled by directed insertion of gentamicin resistance (Gm^R) as previously described [Lambertsen et al., 2004]. The plasmids used in this study, pQBR103 and pQBR57 are large conjugative plasmids (425 kb and 307 kb respectively) that confer mercury resistance via a *mer* operon encoded on a Tn5042 transposon [Lilley et al., 1996; Tett et al., 2007; Hall et al., 2015]. Both plasmids were independently conjugated into *P. fluorescens* SBW25 Gm^R from Sm^R plasmid-bearing *P. fluorescens* SBW25 by mixing 1:1 each of the plasmid-free with the plasmid-bearing strains, incubating for 48 h and spreading on KB agar plates containing 10 μg/ml gentamicin and 20 μM of mercury(II)chloride to select for transconjugant colonies [Simonsen et al., 1990]. As previously described, the conjugation assays were conducted in 6 ml King's B growth medium in 30 ml universal vials ('microcosms') at 28°C in shaking conditions (180 rpm). Background communities consisting of five different *Pseudomonas* species (*P. stutzeri* JM300 (DSM 10701) [Carlson et al., 1983], *P. putida* KT2440 [Bagdasarian et al., 1981], *P. protegens* Pf-5 [Howell and Stipanovic, 1979], *P. fluorescens* Pf0-1 [Compeau et al., 1988], *P. aeruginosa* PA01 [Holloway, 1955]) were used in some treatments.

6.2.2 Selection experiment

Twelve colonies of the plasmid-bearing *P. fluorescens* SBW25(pQBR103) and *P. fluorescens* SBW25(pQBR57) were grown overnight in KB microcosms at 28°C

with shaking 180 rpm. Six colonies of each of the plasmid-free *Pseudomonas* species (*P. stutzeri* JM300 (DSM 10701), *P. putida* KT2440, *P. protegens* Pf-5, *P. fluorescens* Pf0-1, *P. aeruginosa* PA01) were also grown overnight in KB microcosms using the same culture conditions. Six replicate populations containing equal proportions of *P. fluorescens* SBW25(pQBR103) and *P. fluorescens* SBW25(pQBR57) were propagated either with or without the background community of five *Pseudomonas* species. Populations were grown in potting soil microcosms supplemented with mercury (16 μg/g Hg(II)) by serial transfer. Each community had a starting ratio of 1:1 between *P. fluorescens* SBW25(pQBR103) and *P. fluorescens* SBW25(pQBR57) ($\sim$each 1x10^6 cfu/g) such that the starting frequencies of pQBR103 and pQBR57 were approximately 50%. The background community of *Pseudomonas* species contained each species in equal proportion ($\sim$each 4x10^5 cfu/g). To prepare the soil inoculum, the mix of each community (final volume: 100μl) was centrifuged for 1 min at 10,000 rpm and resuspended in 1 ml M9 salt solution (Cold Spring Harbor Protocols). Next, the soil microcosms (10 g twice-autoclaved John Innes No. 2 compost soil) were inoculated with 100 μl of the mix and incubated at 28°C at 80% humidity [Hall et al., 2016]. Every 4 days, 10 ml of M9 buffer and 20 glass beads were added to each soil microcosm and mixed by vortexing for 1 min, and 100 μl of soil wash was transferred to a fresh soil microcosm as previously described by Hall et al. [2016]. The communities were propagated for 6 transfers ($\sim$24 days).

At each transfer, total population counts were estimated by plating onto non-selective KB agar plates. Bacterial counts for the plasmid-bearing *P. fluorescens* SBW25 strains were estimated by plating onto selective media: 10 μg/ml gentamicin KB agar plates. Each of these plates were then replica plated onto mercury KB agar plates (100 μM mercury(II)chloride) in order to access the frequency of mercury resistance within *P. fluorescens* SBW25 and at the whole-community level. Twenty-four colonies of *P. fluorescens* SBW25 were sampled every 2 transfers from the mercury containing plates and tested for the presence of the plasmids and mercury transposon by PCR screening. Twenty-four colonies of the total community were randomly sampled at two

time-points (transfer 4, transfer 6) of the experiment from the mercury containing plates and also tested for the presence of the plasmids and mercury transposon. The PCR screening was designed to use three set of primers that targeted the *mer* operon-Tn5042 transposon , the pQBR103-plasmid specific origin of replication *oriV* and the pQBR57-plasmid specific *uvrD* gene.

TABLE 6.1: Primers used for the PCR screening of the mercury resistance genes and the plasmids pQBR57 and pQBR103.

mer operon	F-TGCAAGACACCCCCTATTGGAC R-TTCGGCGACCAGCTTGATGAAC
oriV	F-TGCCTAATCGTGTGTAATGTC R-ACTCTGGCCTGCAAGTTTC
uvrD	F-CTTCGAAGCACACCTGATG R-TGAAGGTATTGGCTGAAAGG

6.2.3 Competitive fitness assay

Four individual colonies of the ancestral *P. fluorescens* SBW25(pQBR103:pQBR57) were competed against the plasmid-free *P. fluorescens* SBW25 with and without the five-species community. The fitness assay was performed with and without mercury in soil microcosms. Relative fitness was measured by mixing differentially the plasmid-bearer (labelled gentamicin) and plasmid-free (labelled streptomycin) in 1:1 ratio. The five-species community was added in the same ratio as at the beginning of the selection experiment. The inoculum was diluted 100-fold in M9 salts before added into soil microcosms and incubated at 28°C and 80% humidity for 4 days. Samples were plated on KB agar plates supplemented with selective concentration of 10 μg/ml gentamicin and 50 μg/ml streptomycin at the beginning and end of the competition to estimate the density of plasmid-bearing and plasmid-free bacteria. The relative fitness was calculated as the selection rate (r) [Lenski et al., 1991].

6.2.4 Conjugation assay

Four individual colonies of each ancestral *P. fluorescens* SBW25 (pQBR103:pQBR57), *P. fluorescens* SBW25 (pQBR103) and *P. fluorescens* SBW25 (pQBR57) were conjugated into the isogenic plasmid-free strain. Conjugation rate of the different plasmids was measure by mixing differentially the plasmid-bearer (labelled gentamicin or streptomycin) and plasmid-free (labelled streptomycin or gentamicin respectively) in 1:1 ratio. The mix was centrifuged for 1 min at 10,000 rpm to remove spent media, resuspended in M9 salt solution, diluted 100-fold in high (KB), medium (0.1x KB) and low (0.01x KB) resource media and incubated at 28°C for 48 h. KB agar plates were supplemented with selective concentration of 10 μg/ml gentamicin or 50 μg/ml streptomycin to estimate the density of plasmid-donor and plasmid-recipient bacteria at the beginning and end of the assay. KB agar plates were supplemented with selective concentration of 10 μg/ml gentamicin and 20 μM mercury(II)chloride or 50 μg/ml streptomycin and 20 μM mercury(II)chloride to estimate the density of the transconjugant bacteria at the end of the assay. The conjugation rate was calculated with the method firstly described by Simonsen et al. [1990].

6.2.5 Statistics

Statistical analyses were performed using RStudio version 3.2.3 [R Core Team, 2015]. The prevalence of each plasmid status (pQBR103 only, pQBR57 only, or both) in *P. fluorescens* SBW25 was estimated as the area under the curve using the function *auc* of the package 'flux' [Jurasinski et al., 2012]. One-way ANOVA tests compared plasmid prevalence in *P. fluorescens* SBW25 with versus without the community. End-point densities of each plasmid in the background community was estimated by subtracting the plasmid density in *P. fluorescens* SBW25 from the plasmid density in the entire community. Thus, positive values indicated transmission of the plasmid(s) into the background community from

P. fluorescens SBW25, and we tested for a difference from zero using 1-sample Student's t-tests. Welch's t-test was used to analyse the effect of the background community on the relative fitness of *P. fluorescens* SBW25 carrying both plasmids. Kruskal-Wallis test was used to assess the differences between the conjugation rates of pQBR57 and pQBR57: pQBR103 in the different resource media as the data were not normally distributed; the conjugation rate of pQBR103 plasmid was not in detectable range in medium and low resource media thus pQBR103 was not included in this statistical analysis. Welch's t-test was used to compare the conjugation rate of pQBR57 to pQBR57:pQBR103 and pQBR103 plasmid in high resource media where the conjugation rate of each plasmid was in detectable range.

6.3 Results

6.3.1 Plasmid co-infection limited in community

While mercury resistance remained at fixation in all replicates, we observed contrasting plasmid dynamics in the *P. fluorescens* SBW25 population with versus without the background *Pseudomonas* community. In the presence of the background community, in the majority of replicates the *P. fluorescens* SBW25 population was dominated by pQBR103, such that bacteria were typically either singly-infected by pQBR103 or co-infected with both pQBR103 and pQBR57. By contrast, in the absence of the background community we observed higher rates of co-infection with both pQBR103 and pQBR57, or, in a single replicate, the fixation of pQBR57. Overall, we observed that the frequency of plasmid co-infection was higher in the absence of the background community (ANOVA $F_{1,10}$=5.569, p=0.039; Figure 6.1).

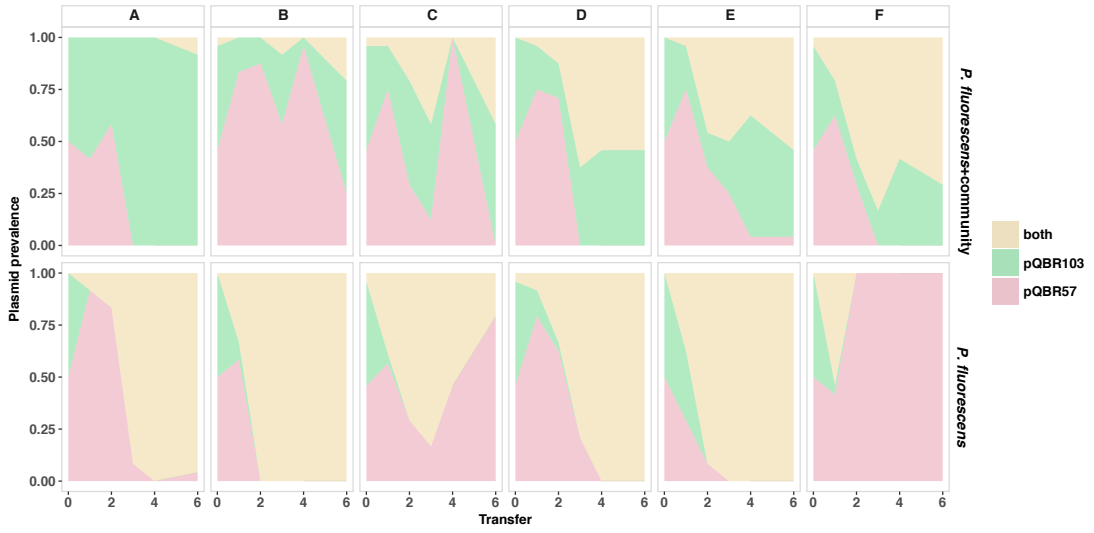

FIGURE 6.1: Plasmid ratio and plasmid profile in *P. fluorescens* SBW25. *P. fluorescens* + community panels show the plasmid prevalence in *P. fluorescens* when plasmid-bearing *P. fluorescens* species were co-cultured with the five-species community; *P. fluorescens* panels show the plasmid prevalence in *P. fluorescens* when *P. fluorescens* was cultured as single-species. Co-existence of both, pQBR57 and pQBR103 plasmids (yellow); pQBR103 plasmid (green); pQBR57 plasmid (red).

To test if this effect could be caused by higher fitness costs of co-infection in the presence versus absence of the community, perhaps due to more intense resource competition, we competed *P. fluorescens* SBW25(pQBR103:pQBR57) against plasmid-free *P. fluorescens* SBW25 with or without the background community. We found, however, that the presence of the background community had no effect on the relative fitness of *P. fluorescens* SBW25(pQBR103:pQBR57) (Welch's t-test, $t_{13.68}$=0.698, p=0.496; Figure 6.2).

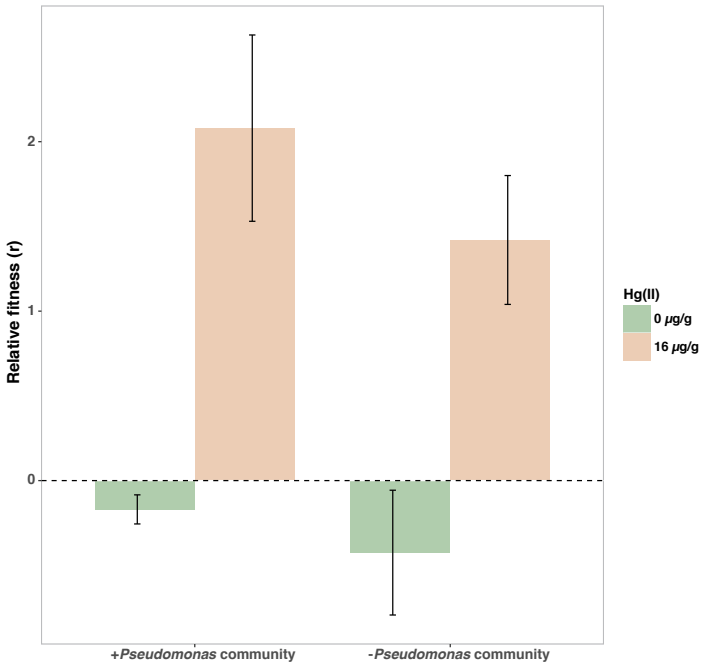

FIGURE 6.2: Relative fitness of *P. fluorescens* (pQBR103:pQBR57) in absence and presence of the five-species community. 0 μg/g Hg(II) (green), 16 μg/g Hg(II) (yellow). Error bars represent the SEM: SEM of four clonal replicates.

pQBR57 is known to have a far higher conjugation rate than pQBR103 in potting soil [Hall et al., 2015], therefore it is likely that co-infection would have often resulted from pQBR57 conjugating into cells that already carried pQBR103. This process of infectious transmission through the *P. fluorescens*SBW25 population could have been less efficient in the presence of the background community if, rather than conjugating into *P. fluorescens* SBW25(pQBR103), pQBR57 conjugated into the other *Pseudomonas* species. This is conceptually similar to the dilution effect in epidemiology whereby biodiversity reduces infection risk in a focal species [Levi et al., 2016]. Consistent with this idea, we were able to detect pQBR57, but not pQBR103, at an appreciable frequency in the background community (Student's t-test, t_5=6.198, p=0.001; Figure 6.3). This suggests that, indeed, the transmission of pQBR57 into *P. fluorescens* SBW25(pQBR103) cells was impeded by dilution by the community leading to reduced co-infection of *P. fluorescens* SBW25.

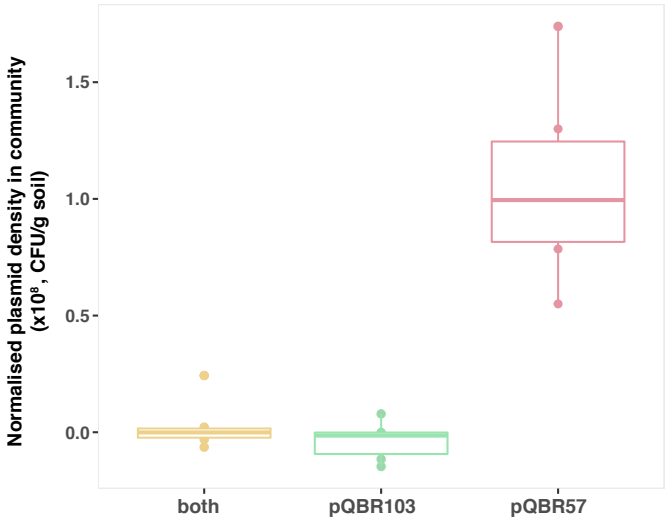

FIGURE 6.3: Plasmid density of the background community at the end of the experiment. Co-existence of both, pQBR57 and pQBR103 plasmids (yellow); pQBR103 plasmid (green); pQBR57 plasmid (red).

Finally, we tested whether the rate of conjugation to plasmid-free recipient cells varied depending on whether the donor was singly-infected or co-infected, and whether conjugation rates were affected by resource level. Conjugation rates from all backgrounds–*P. fluorescens* SBW25(pQBR103), *P. fluorescens* SBW25(pQBR57), and *P. fluorescens* SBW25(pQBR103:pQBR57)–were reduced in diluted media (effect of resource media, χ^2 (2, N=22)=16.85, p<0.001; Figure 4). Consistent with previous studies, conjugation rates from pQBR57-containing backgrounds were far higher than those from *P. fluorescens* SBW25(pQBR103) (Welch's t-test, $t_{5.581}$ = -14.973, p<0.001), but co-infected donors had a reduced conjugation rate compared to *P. fluorescens* SBW25(pQBR57) donors (Welch's t-test, $t_{5.773}$ = -5.751, p=0.001; Figure 6.4). These results suggest that co-infection itself may have reduced the rate at which pQBR57 spread in the *P. fluorescens* SBW25 population, and that greater resource competition in the presence of the background community may have reduced the rate of infectious spread of pQBR57.

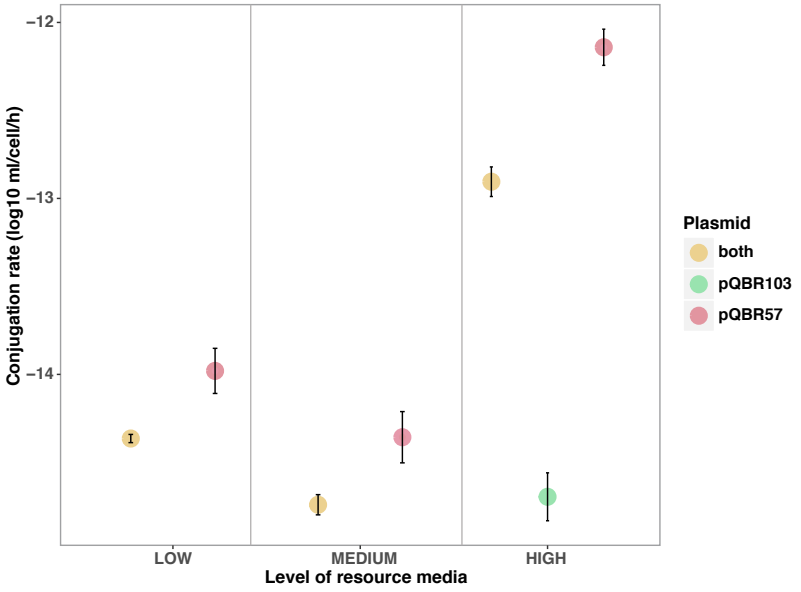

FIGURE 6.4: Conjugation rate of *P. fluorescens* (pQBR103:pQBR57), *P. fluorescens* (pQBR103) and *P. fluorescens* (pQBR57) in high, medium and low resource media. Error bars represent the SEM: SEM of four clonal replicates.

6.4 Discussion

Using simple soil bacterial communities, we show that plasmid co-infection in a focal host species was reduced in the presence of a community of other bacterial species. This was not caused by differential fitness effects of plasmid-carriage in monocultures versus communities, but rather appears to have been determined by the effect of bacterial species richness on the epidemiological spread of plasmids in the focal host population. Whereas, in monocultures, the highly conjugative plasmid pQBR57 spread into the *P. fluorescens* SBW25(pQBR103) sub-population, in communities, this spread was impeded. Detection of pQBR57 at appreciable frequencies in the background community suggests that this effect was due to a substantial fraction of conjugation events leading to the infection of non-SBW25 cells by *P. fluorescens* SBW25(pQBR57). Because the conjugation rate of pQBR57 is lower in the other *Pseudomonas* species, this interspecific conjugation is likely to have had the effect of reducing the overall conjugation

rate to *P. fluorescens* SBW25(pQBR103) cells and thus lowering the probability of plasmid co-infection.

Similar to plasmids, the transmission of parasites has often been found to be lower in species-rich communities where a focal species is diluted in the diverse community and therefore has a reduced risk of infection [Ostfeld and Keesing, 2000; LoGiudice et al., 2003; Keesing et al., 2006; Johnson et al., 2008]. The dilution effect is supported by experimental studies and epidemiological models which suggest that introducing communities of alternative hosts could help to control the transmission of vector-borne diseases caused by parasites (zooprophylaxis) [Burdon and Chilvers, 1982; Boudreau and Mundt, 1997; Elton, 2000; Dobson et al., 2006; Lane et al., 2006]. The identity of the introduced host species has important implications in preventing the parasites' transmission, as different host species are likely to vary in their susceptibility to hosting the parasite [Keesing et al., 2009]. Highly susceptible host species could amplify the disease reservoir of a parasite instead of lessening it, therefore in order to prevent the dissemination of a parasite, the enrichment of these host species should be restricted in the community [Keesing et al., 2009]. Similar dynamics could apply to plasmids, where host species are known to vary widely in their proficiency to act as plasmid hosts [Hall et al., 2016, Chapter 5].

Parasite epidemiological models also suggest that the species richness of the parasite community can affect the transmission of a focal parasite [Johnson and Hoverman, 2012; Johnson et al., 2013]. Both parasite diversity and co-infection have been found to reduce the transmission rate of parasites in a community [Johnson and Hoverman, 2012]. Similarly, here we found that the conjugation rate from the donor *P. fluorescens* SBW25(pQBR103:pQBR57) was lower compared with the *P. fluorescens* SBW25(pQBR57) donor. This suggests that plasmid co-infection itself could limit the transmission rate of highly conjugative plasmids, like pQBR57. We speculate that plasmid co-infection affected the plasmid transmission as a result of plasmid-plasmid interactions in the host cell. Co-existing plasmids could trigger a stronger cellular response in the host cell, while the increase in genetic sequence and encoded genes is likely to amplify the

physiological and metabolic cost to the host cell, moreover co-infecting plasmids are likely to compete for limited cellular resources (i.e host's replication factors; [Del Solar et al., 1998]). Indeed, we predict that intracellular competition is likely to be more intense between related plasmids, since these will have the greatest overlap in their resource requirements e.g. similar suites of tRNAs.

In nature, bacteria inhabit species-rich communities wherein they co-exist with multiple diverse plasmids [Smillie et al., 2010; Klümper et al., 2015]. The experiments reported here highlight that plasmid dynamics can be affected by both bacterial and plasmid diversity. Plasmids are currently of clinical concern as they often carry antimicrobial resistance (AMR) genes [Carattoli, 2013]. AMR genes are found in microbial communities colonizing diverse environments where microbial communities can act as resistance reservoirs [Allen et al., 2010; Wright, 2010]. Expansion of the resistance reservoirs via HGT between microbial communities is currently an increasing concern [von Wintersdorff et al., 2016]. Understanding the transmission dynamics of AMR-encoding plasmids at the community-level is therefore imperative in order to constrain the emergence of resistance in natural microbial communities. This work suggests that plasmid dissemination along with the resistance genes they encode in a focal taxon (e.g. a pathogen) could be limited in more species-rich communities, where plasmid transmission is constrained by the dilution effect.

6.5 Conclusions

This study shows that plasmid co-infection of *P. fluorescens* was limited in the presence of a five-species *Pseudomonas* community, whilst plasmid conjugation rates were reduced under co-infection. Thus plasmid epidemiology is likely often to be the product of both the bacterial and plasmid community structure, suggesting that understanding and controlling plasmid dissemination requires a community-level perspective.

Chapter 7

Discussion

Each data chapter (Chapters 2-6) includes a specific discussion section considering the findings of the chapter. The scope of this thesis discussion is therefore to synthesise the key findings of the thesis and place them into a wider context.

Conjugative plasmids are undoubtedly key genetic elements driving bacterial evolution through the horizontal transfer of genes between species [Frost et al., 2005; Norman et al., 2009]. Moreover, the role of plasmids in the dissemination of antibiotic resistance genes means that understanding plasmid ecology has important applied clinical implications [von Wintersdorff et al., 2016]. In a series of selection experiments, this thesis demonstrates a variety of ecological factors affect the evolution, stability and transmission of plasmids in single and multi-host environments. I showed that the identity of plasmid-host species and the community structure play an important role governing the fate of plasmids in bacterial communities. Specifically, I found that the costs of a plasmid varied between host species (Chapter 3) but that plasmids could adapt to multiple host species if given the ecological opportunity (Chapter 2). Plasmid dynamics varied between host species (Chapter 3) and required different mechanisms of compensatory evolution in different host species to ameliorate the plasmid cost (Chapter 4). Meanwhile, community-level plasmid abundance was determined by the proficiency of the plasmid-donor host species (Chapter 5) and by the dilution effect (Chapter 6). These results suggest that it is essential to study the ecology

of plasmids in a community-context in order to disentangle the dynamics of their persistence and spread in natural microbial communities.

Metagenomics studies suggest that natural microbial communities consist of many phylogenetically diverse host species co-existing with a plethora of genetically diverse mobile genetic elements (MGE) such as phages and plasmids [Tyson et al., 2004; Kristiansson et al., 2011; Modi et al., 2013]. Broad host range plasmids have been found in natural microbial communities colonising a range of environmental niches from soil [Heuer and Smalla, 2012] to freshwater [Brown et al., 2013] and wastewater [Schlüter et al., 2007; Moura et al., 2010]. Given the diverse microbial communities where plasmids occur and the cost and benefits associated with their acquisition (HGT) [Baltrus, 2013], it is still challenging to understand what triggers the variation among species in their permissiveness to HGT in natural conditions. Studies focusing on understanding the microbial innate immune mechanisms have found two systems; the restriction-modification and the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas system, that mediate the intracellular degradation of MGEs and therefore could constrain HGT [Horvath and Barrangou, 2010; Vasu et al., 2012; Oliveira et al., 2014]. The restriction-modification and CRISPR-Cas systems have been found extensively diverse among microbial species [Vasu and Nagaraja, 2013; Makarova et al., 2015] while HGT has been predicted to be more frequent in microbial communities where species have similar immune systems independently of their phylogenetic distance [Oliveira et al., 2016]. However, both systems are viewed as short-term defence mechanisms against HGT because these defences can be evaded by methylation of the MGEs, which makes them resistant to restriction-modification [Korona et al., 1993], or the evolution of resistance mutations to escape recognition by the CRISPR spacers that target their degradation, which rapidly arise in the population [Deveau et al., 2008; Levin et al., 2013; Sun et al., 2013]. Laanto et al. [2017] in a recent study focusing on the co-evolutionary processes between phages and their hosts in natural microbial communities have shown that as phages can evolve resistance against their hosts' immune systems so their hosts can acquire changes in their

CRISPR spacers potentiating dynamic antagonistic co-evolution, similar to previous studies [Buckling and Rainey, 2002; Paterson et al., 2010; Koskella, 2013]. This illustrates how the permissiveness of the species to HGT is not only species-specific but could also change over time, and highlights the importance of studying MGE dynamics in an eco-evolutionary framework.

Recent studies have demonstrated that plasmids are often able to transfer between phylogenetically distant host species, indicating that plasmid dissemination occurs at an appreciable rate in natural microbial communities [Klümper et al., 2015; Cairns et al., 2018]. Plasmid dynamics in multi-species communities are likely to also be affected by the local selection environment [Stevenson et al., 2018]. Frequent pulses of positive selection promotes the long-term persistence of the plasmid-borne genes in the community, while infrequent pulses promote plasmid horizontal transmission [Stevenson et al., 2018]. Indeed, the balance of vertical to horizontal transmission of plasmids has been shown to vary as a function of the strength of positive selection, with horizontal transmission becoming more important with weakening positive selection [Stevenson et al., 2017]. Given that microbial communities will often be exposed to fluctuating selective conditions in natural environments and the strength of positive selection is likely to vary among sites [Coutu et al., 2013; Marti et al., 2014], we may therefore expect that plasmids would transmit into a wider variety of host species in environments where positive selection for their encoded functions is weak of absent.

Nevertheless, it is still questioned whether the plasmids maintained in the longer-term [Harrison and Brockhurst, 2012]. Since plasmid stability varies extensively between host species (Chapter 3) the long-term survival of plasmid is thought to be associated with compensatory evolution to reduce the cost of plasmid carriage (Chapter 4). Here, I show that the same plasmid is ameliorated by mutation of different intracellular functions in the different host species (cf. *P. fluorescens* and *P. stutzeri*; Chapter 4). This suggests that compensatory evolution mechanisms may often be species-specific, and furthermore that the costs of even a single plasmid can be caused by distinct molecular mechanisms in different host backgrounds. This is further supported by the different costs of the

plasmid in the different host species (Chapter 3). In each case, however, it appears that the cost of plasmid carriage arises from interference with their hosts' cellular homeostasis [San Millan and MacLean, 2017]. Plasmids alter the intracellular fine-tuned metabolism of their hosts while expressing their own genes and proteins, interfering with their host regulatory systems [Harrison et al., 2015a, 2016], and/or integrating part or all of their genetic material into the host chromosome [Hall et al., 2017]. It is then tempting to speculate that for plasmids to adapt in a multi-species community containing a broad range of host species—each with a different cause of the cost—would be extremely challenging. This could possibly involve multiple steps of adaptation where the plasmids successfully persist in a community as infectious agents via transmission while being continuously exposed to variable host backgrounds (Chapter 2) acquiring mutations in multiple plasmid loci. However, our findings suggest that compensatory mutations often arise at chromosomal rather than plasmid loci (Chapter 4). This implies that although previous studies have shown that plasmids could evolve to ameliorate the cost of carriage to their hosts [Sota et al., 2010; Porse et al., 2016], the evolution of plasmids *per se* could be expected relatively constrained, for example due to lower mutational supply arising from their relatively smaller size compared to the chromosome. Nonetheless, the structure and species diversity of the community is likely to determine the trajectory of plasmid evolution over the longer term.

Community structure altered plasmid dynamics at the community-level by two distinct and counter-acting mechanisms: by communities containing varying species that proficiently maintained and transmitted the plasmid (Chapter 5) or by diluting these proficient hosts and impeding plasmid transmission (Chapter 6). Community structure is determined by both extrinsic (i.e., environmental conditions) and intrinsic forces (i.e., ecological interactions between species) [Lozupone and Knight, 2007; Nemergut et al., 2013]. While phylogenetically distant species are found to co-exist in natural microbial communities colonising diverse environmental niches [Torsvik et al., 2002], the species diversity of these established communities can be challenged by changing environmental conditions

[Allison and Martiny, 2008]. How communities respond to changes in the extrinsic environment, particularly its deterioration, could be dramatically altered by HGT and the presence of proficient plasmid hosts if genes preadapting cells to the new environment are encoded on the plasmid. This is because the standing variation required to adapt to the new environment are available to multiple species through HGT, potentially allowing community-level evolutionary rescue [Gonzalez et al., 2013]. This evolutionary process would be further facilitated in communities containing a high proportion of proficient plasmid hosts to disseminate the plasmid to neighbouring species (Chapter 5). Future research should address how HGT and community structure interact to determine the evolutionary responsiveness of communities to environmental change.

Multiple studies have hinted that additional environmental and ecological factors such as the spatial structuring of environments [Molin and Tolker-Nielsen, 2003] and the interactions with different MGEs like phages within the community [Harrison et al., 2015b, 2017], could further shape plasmid dynamics. Advanced genomic techniques could provide an excellent tool for future studies to focus on finding how the interplay of these ecological factors and the structure of microbial communities affect plasmid dynamics in natural microbial communities. While by using metagenomic techniques we can assess the community structure in natural microbial communities, new techniques such as epicPCR [Spencer et al., 2016] link the taxonomic identity of species to specific ecological functions at the single-cell scale. Such approaches could help to resolve which species present in natural communities are responsible for maintaining and disseminating by HGT antibiotic resistance genes with important implications for the control and elimination of antibiotic resistance [WHO, 2012; Laxminarayan et al., 2013].

Appendix A

TABLE A.1: Overview of the *Pseudomonas* species used in this thesis

***Pseudomonas* species**	**Isolation**	**Reference**	**Source**
P. fluorescens SBW25	Leaf surface of sugar beet plant (Plant)	Rainey et al., 1994	Paul Rainey
P. putida KT2440	Derivative of the toluene degrading *P. putida* mt-2 (Soil)	Bagdasarian et al., 1981	Andrew Spiers
P. savastanoi pv. phaseolicola 1448A	*P. vulgaris* (Plant)	Teverson, 1991	Rob Jackson
P. stutzeri JM300 (DSM 10701)	Soil	Carlson et al., 1983	Leibniz Institute DSMZ
P. aeruginosa PAO1	Derivative of the original Australian PAO isolate (Wound)	Holloway, 1955	Craig Winstanley
P. fluorescens Pf0-1	Soil	Compeau et al., 1988	Stuart Levy
P. protegens Pf-5	Soil	Howell and Stipanovic, 1979	Christoph Keel

Bibliography

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J. and Handelsman, J. [2010]. Call of the wild: antibiotic resistance genes in natural environments, *Nature Reviews Microbiology* **8**(4): 251.

Allison, S. D. and Martiny, J. B. [2008]. Resistance, resilience, and redundancy in microbial communities, *Proceedings of the National Academy of Sciences* **105**(Supplement 1): 11512–11519.

Aminov, R. I. [2011]. Horizontal gene exchange in environmental microbiota, *Frontiers in microbiology* **2**: 158.

Arnold, D. L., Lovell, H. C., Jackson, R. W. and Mansfield, J. W. [2011]. Pseudomonas syringae pv. phaseolicola: from 'has bean' to supermodel, *Molecular plant pathology* **12**(7): 617–627.

Atkinson, G. C., Tenson, T. and Hauryliuk, V. [2011]. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life, *PloS one* **6**(8): e23479.

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F., Bagdasarian, M., Frey, J. and Timmis, K. [1981]. Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas, *Gene* **16**(1): 237–247.

Baharoglu, Z., Bikard, D. and Mazel, D. [2010]. Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation, *PLoS genetics* **6**(10): e1001165.

Bahl, M., Hansen, L. and Sørensen, S. [2007]. Impact of conjugal transfer on the stability of IncP-1 plasmid pKJK5 in bacterial populations, *FEMS microbiology letters* **266**(2): 250–256.

Bahl, M. I., Hansen, L. H. and Sørensen, S. J. [2009]. Persistence mechanisms of conjugative plasmids, *Horizontal Gene Transfer: Genomes in Flux* pp. 73–102.

Bailey, M., Lilley, A., Thompson, I., Rainey, P. and Ellis, R. [1995]. Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet; stability and potential for marker gene transfer, *Molecular Ecology* **4**(6): 755–764.

Baltrus, D. A. [2013]. Exploring the costs of horizontal gene transfer, *Trends in ecology & evolution* **28**(8): 489–495.

Bates, D., Mächler, M., Bolker, B. and Walker, S. [2014]. Fitting linear mixed-effects models using lme4, *arXiv preprint arXiv:1406.5823* .

Battesti, A., Majdalani, N. and Gottesman, S. [2011]. The RpoS-mediated general stress response in Escherichia coli, *Annual review of microbiology* **65**: 189–213.

Bergstrom, C. T., Lipsitch, M. and Levin, B. R. [2000]. Natural selection, infectious transfer and the existence conditions for bacterial plasmids, *Genetics* **155**(4): 1505–1519.

Bertels, F., Silander, O. K., Pachkov, M., Rainey, P. B. and van Nimwegen, E. [2014]. Automated reconstruction of whole-genome phylogenies from short-sequence reads, *Molecular biology and evolution* **31**(5): 1077–1088.

Bottery, M. J., Wood, A. J. and Brockhurst, M. A. [2017]. Adaptive modulation of antibiotic resistance through intragenomic coevolution, *Nature ecology & evolution* **1**(9): 1364.

Boudreau, M. and Mundt, C. [1997]. Ecological approaches to disease control.

Bouma, J. E. and Lenski, R. E. [1988]. Evolution of a bacteria/plasmid association, *Nature* **335**(6188): 351.

Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. L. and Lory, S. [2009]. The GacS/GacA signal transduction system of Pseudomonas aeruginosa acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs, *Molecular microbiology* **73**(3): 434–445.

Brown, C. J., Sen, D., Yano, H., Bauer, M. L., Rogers, L. M., Van der Auwera, G. A. and Top, E. M. [2013]. Diverse broad-host-range plasmids from freshwater carry few accessory genes, *Applied and environmental microbiology* **79**(24): 7684–7695.

Buckling, A. and Rainey, P. B. [2002]. Antagonistic coevolution between a bacterium and a bacteriophage, *Proceedings of the Royal Society of London B: Biological Sciences* **269**(1494): 931–936.

Burdon, J. and Chilvers, G. [1982]. Host density as a factor in plant disease ecology, *Annual review of phytopathology* **20**(1): 143–166.

Busquets, A., Peña, A., Gomila, M., Bosch, R., Nogales, B., García-Valdés, E., Lalucat, J. and Bennasar, A. [2012]. Genome sequence of Pseudomonas stutzeri strain JM300 (DSM 10701), a soil isolate and model organism for natural transformation, *Journal of bacteriology* **194**(19): 5477–5478.

Cairns, J., Ruokolainen, L., Hultman, J., Tamminen, M., Virta, M. and Hiltunen, T. [2018]. Ecology determines how low antibiotic concentration impacts community composition and horizontal transfer of resistance genes, *Communications Biology* **1**(1): 35.

Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M.-L. and Brüssow, H. [2003a]. Phage as agents of lateral gene transfer, *Current opinion in microbiology* **6**(4): 417–424.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A. and Brüssow, H. [2003b]. Prophage genomics, *Microbiology and Molecular Biology Reviews* **67**(2): 238–276.

Carattoli, A. [2013]. Plasmids and the spread of resistance, *International Journal of Medical Microbiology* **303**(6-7): 298–304.

Carlson, C. A., Pierson, L. S., Rosen, J. and Ingraham, J. L. [1983]. Pseudomonas stutzeri and related species undergo natural transformation, *Journal of bacteriology* **153**(1): 93–99.

Chen, K., Wallis, J. W., McLellan, M. D., Larson, D. E., Kalicki, J. M., Pohl, C. S., McGrath, S. D., Wendl, M. C., Zhang, Q., Locke, D. P. et al. [2009]. Breakdancer: an algorithm for high-resolution mapping of genomic structural variation, *Nature methods* **6**(9): 677–681.

Chen, Y., Golding, I., Sawai, S., Guo, L. and Cox, E. C. [2005]. Population fitness and the regulation of Escherichia coli genes by bacterial viruses, *PLoS biology* **3**(7): e229.

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., Land, S. J., Lu, X. and Ruden, D. M. [2012]. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3, *Fly* **6**(2): 80–92.

Civitello, D. J., Cohen, J., Fatima, H., Halstead, N. T., Liriano, J., McMahon, T. A., Ortega, C. N., Sauer, E. L., Sehgal, T., Young, S. et al. [2015]. Biodiversity inhibits parasites: broad evidence for the dilution effect, *Proceedings of the National Academy of Sciences* **112**(28): 8667–8671.

Compeau, G., Al-Achi, B. J., Platsouka, E. and Levy, S. [1988]. Survival of rifampin-resistant mutants of Pseudomonas fluorescens and Pseudomonas putida in soil systems, *Applied and Environmental Microbiology* **54**(10): 2432–2438.

Coutu, S., Rossi, L., Barry, D. A., Rudaz, S. and Vernaz, N. [2013]. Temporal variability of antibiotics fluxes in wastewater and contribution from hospitals, *PLoS One* **8**(1): e53592.

Dahlberg, C. and Chao, L. [2003]. Amelioration of the cost of conjugative plasmid carriage in Eschericha coli K12, *Genetics* **165**(4): 1641–1649.

De Gelder, L., Ponciano, J. M., Joyce, P. and Top, E. M. [2007]. Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship, *Microbiology* **153**(2): 452–463.

De Gelder, L., Williams, J. J., Ponciano, J. M., Sota, M. and Top, E. M. [2008]. Adaptive plasmid evolution results in host-range expansion of a broad-host-range plasmid, *Genetics* **178**(4): 2179–2190.

Del Solar, G., Giraldo, R., Ruiz-Echevarría, M. J., Espinosa, M. and Díaz-Orejas, R. [1998]. Replication and control of circular bacterial plasmids, *Microbiology and molecular biology reviews* **62**(2): 434–464.

Deng, X., Xiao, Y., Lan, L., Zhou, J.-M. and Tang, X. [2009]. Pseudomonas syringae pv. phaseolicola mutants compromised for type III secretion system gene induction, *Molecular plant-microbe interactions* **22**(8): 964–976.

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., Philippakis, A. A., Del Angel, G., Rivas, M. A., Hanna, M. et al. [2011]. A framework for variation discovery and genotyping using next-generation DNA sequencing data, *Nature genetics* **43**(5): 491–498.

Deveau, H., Barrangou, R., Garneau, J. E., Labonté, J., Fremaux, C., Boyaval, P., Romero, D. A., Horvath, P. and Moineau, S. [2008]. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus, *Journal of bacteriology* **190**(4): 1390–1400.

Dionisio, F., Conceicao, I., Marques, A., Fernandes, L. and Gordo, I. [2005]. The evolution of a conjugative plasmid and its ability to increase bacterial fitness, *Biology Letters* **1**(2): 250–252.

Dionisio, F., Matic, I., Radman, M., Rodrigues, O. R. and Taddei, F. [2002]. Plasmids spread very fast in heterogeneous bacterial communities, *Genetics* **162**(4): 1525–1532.

Dionisio, F., Nogueira, T., Carvalho, L. M., Mendes-Soares, H., Mendonça, S. C., Domingues, I., Moreira, B. and Reis, A. M. [2012]. What maintains plasmids among bacteria, *Horizontal gene transfer in microorganisms. Caister Academic Press, Norwich* pp. 131–154.

Dobson, A., Cattadori, I., Holt, R. D., Ostfeld, R. S., Keesing, F., Krichbaum, K., Rohr, J. R., Perkins, S. E. and Hudson, P. J. [2006]. Sacred cows and sympathetic squirrels: the importance of biological diversity to human health, *PLoS Medicine* **3**(6): e231.

Eberhard, W. G. [1990]. Evolution in bacterial plasmids and levels of selection, *The Quarterly review of biology* **65**(1): 3–22.

Egas, M., Dieckmann, U. and Sabelis, M. W. [2004]. Evolution restricts the coexistence of specialists and generalists: the role of trade-off structure, *The American Naturalist* **163**(4): 518–531.

Elton, C. S. [2000]. *The ecology of invasions by animals and plants*, University of Chicago Press.

Fernandez-Lopez, R., del Campo, I., Revilla, C., Cuevas, A. and de la Cruz, F. [2014]. Negative feedback and transcriptional overshooting in a regulatory network for horizontal gene transfer, *PLoS genetics* **10**(2): e1004171.

Fletcher, S. [2015]. Understanding the contribution of environmental factors in the spread of antimicrobial resistance, *Environmental health and preventive medicine* **20**(4): 243.

Fox, R. E., Zhong, X., Krone, S. M. and Top, E. M. [2008]. Spatial structure and nutrients promote invasion of IncP-1 plasmids in bacterial populations, *The ISME journal* **2**(10): 1024.

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G. and Hanage, W. P. [2009]. The bacterial species challenge: making sense of genetic and ecological diversity, *Science* **323**(5915): 741–746.

Frost, L. S., Leplae, R., Summers, A. O. and Toussaint, A. [2005]. Mobile genetic elements: the agents of open source evolution, *Nature Reviews Microbiology* **3**(9): 722–732.

Gibbons, S. M. and Gilbert, J. A. [2015]. Microbial diversity—exploration of natural ecosystems and microbiomes, *Current opinion in genetics & development* **35**: 66–72.

Gogarten, J. P., Doolittle, W. F. and Lawrence, J. G. [2002]. Prokaryotic evolution in light of gene transfer, *Molecular biology and evolution* **19**(12): 2226–2238.

Gómez, P. and Buckling, A. [2011]. Bacteria-phage antagonistic coevolution in soil, *Science* **332**(6025): 106–109.

Gonzalez, A., Ronce, O., Ferriere, R. and Hochberg, M. E. [2013]. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution, *Philosophical Transactions of the Royal Society B: Biological Sciences* **368**(1610).

Gordon, D. M. [1992]. Rate of plasmid transfer among Escherichia coli strains isolated from natural populations, *Microbiology* **138**(1): 17–21.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. [2010]. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0, *Systematic biology* **59**(3): 307–321.

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L. and Andersson, D. I. [2014]. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals, *MBio* **5**(5): e01918–14.

Haas, D. and Défago, G. [2005]. Biological control of soil-borne pathogens by fluorescent pseudomonads, *Nature Reviews Microbiology* **3**(4): 307–319.

Haft, R. J., Mittler, J. E. and Traxler, B. [2009]. Competition favours reduced cost of plasmids to host bacteria, *The ISME journal* **3**(7): 761–769.

Hall, J. P., Harrison, E., Lilley, A. K., Paterson, S., Spiers, A. J. and Brockhurst, M. A. [2015]. Environmentally co-occurring mercury resistance plasmids are genetically and phenotypically diverse and confer variable context-dependent fitness effects, *Environmental microbiology* **17**(12): 5008–5022.

Hall, J. P., Williams, D., Paterson, S., Harrison, E. and Brockhurst, M. A. [2017]. Positive selection inhibits gene mobilization and transfer in soil bacterial communities, *Nature ecology & evolution* **1**(9): 1348.

Hall, J. P., Wood, A. J., Harrison, E. and Brockhurst, M. A. [2016]. Source–sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities, *Proceedings of the National Academy of Sciences* p. 201600974.

Harrison, E. and Brockhurst, M. A. [2012]. Plasmid-mediated horizontal gene transfer is a coevolutionary process, *Trends in microbiology* **20**(6): 262–267.

Harrison, E., Dytham, C., Hall, J. P., Guymer, D., Spiers, A. J., Paterson, S. and Brockhurst, M. A. [2016]. Rapid compensatory evolution promotes the survival of conjugative plasmids, *Mobile genetic elements* **6**(3): 2034–2039.

Harrison, E., Guymer, D., Spiers, A. J., Paterson, S. and Brockhurst, M. A. [2015a]. Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum, *Current Biology* **25**(15): 2034–2039.

Harrison, E., Hall, J. P., Paterson, S., Spiers, A. J. and Brockhurst, M. A. [2017]. Conflicting selection alters the trajectory of molecular evolution in a tripartite bacteria–plasmid–phage interaction, *Molecular ecology* **26**(10): 2757–2764.

Harrison, E., Wood, A. J., Dytham, C., Pitchford, J. W., Truman, J., Spiers, A., Paterson, S. and Brockhurst, M. A. [2015b]. Bacteriophages limit the existence conditions for conjugative plasmids, *MBio* **6**(3): e00586–15.

Hayes, F. and Barillà, D. [2006]. The bacterial segrosome: a dynamic nucleoprotein machine for DNA trafficking and segregation, *Nature Reviews Microbiology* **4**(2): 133.

Heeb, S. and Haas, D. [2001]. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria, *Molecular plant-microbe interactions* **14**(12): 1351–1363.

Heuer, H., Fox, R. E. and Top, E. M. [2007]. Frequent conjugative transfer accelerates adaptation of a broad-host-range plasmid to an unfavorable Pseudomonas putida host, *FEMS microbiology ecology* **59**(3): 738–748.

Heuer, H. and Smalla, K. [2012]. Plasmids foster diversification and adaptation of bacterial populations in soil, *FEMS microbiology reviews* **36**(6): 1083–1104.

Holloway, B. [1955]. Genetic recombination in Pseudomonas aeruginosa, *Microbiology* **13**(3): 572–581.

Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J. and Piddock, L. J. [2016]. Understanding the mechanisms and drivers of antimicrobial resistance, *The Lancet* **387**(10014): 176–187.

Horvath, P. and Barrangou, R. [2010]. CRISPR/Cas, the immune system of bacteria and archaea, *Science* **327**(5962): 167–170.

Howell, C. and Stipanovic, R. [1979]. Control of Rhizoctonia solani on cotton seedlings with Pseudomonas fluorescens and with an antibiotic produced by the bacterium, *Phytopathology* **69**(5): 480–482.

Humphrey, B., Thomson, N. R., Thomas, C. M., Brooks, K., Sanders, M., Delsol, A. A., Roe, J. M., Bennett, P. M. and Enne, V. I. [2012]. Fitness of Escherichia coli strains carrying expressed and partially silent IncN and IncP1 plasmids, *BMC microbiology* **12**(1): 53.

Jain, A. and Srivastava, P. [2013]. Broad host range plasmids, *FEMS microbiology letters* **348**(2): 87–96.

Joardar, V., Lindeberg, M., Jackson, R. W., Selengut, J., Dodson, R., Brinkac, L. M., Daugherty, S. C., DeBoy, R., Durkin, A. S., Giglio, M. G. et al. [2005]. Whole-genome sequence analysis of Pseudomonas syringae pv. phaseolicola

1448a reveals divergence among pathovars in genes involved in virulence and transposition, *Journal of bacteriology* **187**(18): 6488–6498.

Johnson, P. T., Hartson, R. B., Larson, D. J. and Sutherland, D. R. [2008]. Diversity and disease: community structure drives parasite transmission and host fitness, *Ecology Letters* **11**(10): 1017–1026.

Johnson, P. T. and Hoverman, J. T. [2012]. Parasite diversity and coinfection determine pathogen infection success and host fitness, *Proceedings of the National Academy of Sciences* **109**(23): 9006–9011.

Johnson, P. T., Preston, D. L., Hoverman, J. T. and LaFonte, B. E. [2013]. Host and parasite diversity jointly control disease risk in complex communities, *Proceedings of the National Academy of Sciences* **110**(42): 16916–16921.

Jurasinski, G., Koebsch, F. and Hagemann, U. [2012]. Flux rate calculation from dynamic closed chamber measurements, R package version 0.2-1.

Kassen, R. [2002]. The experimental evolution of specialists, generalists, and the maintenance of diversity, *Journal of evolutionary biology* **15**(2): 173–190.

Keesing, F., Brunner, J., Duerr, S., Killilea, M., LoGiudice, K., Schmidt, K., Vuong, H. and Ostfeld, R. [2009]. Hosts as ecological traps for the vector of Lyme disease, *Proceedings of the Royal Society of London B: Biological Sciences* **276**(1675): 3911–3919.

Keesing, F., Holt, R. D. and Ostfeld, R. S. [2006]. Effects of species diversity on disease risk, *Ecology letters* **9**(4): 485–498.

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L. H., Sørensen, S. J. and Smets, B. F. [2015]. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community, *The ISME journal* **9**(4): 934.

Korona, R., Korona, B. and Levin, B. R. [1993]. Sensitivity of naturally occurring coliphages to type I and type II restriction and modification, *Microbiology* **139**(6): 1283–1290.

Koskella, B. [2013]. Phage-mediated selection on microbiota of a long-lived host, *Current Biology* **23**(13): 1256–1260.

Kottara, A., Hall, J. P., Harrison, E. and Brockhurst, M. A. [2017]. Variable plasmid fitness effects and mobile genetic element dynamics across Pseudomonas species, *FEMS microbiology ecology* **94**(1): fix172.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H. and Larsson, D. J. [2011]. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements, *PloS one* **6**(2): e17038.

Laanto, E., Hoikkala, V., Ravantti, J. and Sundberg, L.-R. [2017]. Long-term genomic coevolution of host-parasite interaction in the natural environment, *Nature communications* **8**(1): 111.

Lambertsen, L., Sternberg, C. and Molin, S. [2004]. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins, *Environmental microbiology* **6**(7): 726–732.

Lane, R., Mun, J., Eisen, L. and Eisen, R. [2006]. Refractoriness of the western fence lizard (Sceloporus occidentalis) to the Lyme disease group spirochete Borrelia bissettii, *Journal of Parasitology* **92**(4): 691–696.

Lang, A. S., Zhaxybayeva, O. and Beatty, J. T. [2012]. Gene transfer agents: phage-like elements of genetic exchange, *Nature Reviews Microbiology* **10**(7): 472.

Langmead, B. and Salzberg, S. L. [2012]. Fast gapped-read alignment with Bowtie 2, *Nature methods* **9**(4): 357–359.

Lapouge, K., Schubert, M., Allain, F. H.-T. and Haas, D. [2008]. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour, *Molecular microbiology* **67**(2): 241–253.

Lawrence, J. G. and Hendrickson, H. [2003]. Lateral gene transfer: when will adolescence end?, *Molecular microbiology* **50**(3): 739–749.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H. et al. [2013]. Antibiotic resistance—the need for global solutions, *The Lancet infectious diseases* **13**(12): 1057–1098.

Lenski, R. E., Rose, M. R., Simpson, S. C. and Tadler, S. C. [1991]. Long-term experimental evolution in Escherichia coli.I. Adaptation and divergence during 2,000 generations, *The American Naturalist* **138**(6): 1315–1341.

Levi, T., Keesing, F., Holt, R. D., Barfield, M. and Ostfeld, R. S. [2016]. Quantifying dilution and amplification in a community of hosts for tick-borne pathogens, *Ecological applications* **26**(2): 484–498.

Levin, B. R. [1993]. The accessory genetic elements of bacteria: existence conditions and (co)evolution, *Current opinion in genetics & development* **3**(6): 849–854.

Levin, B. R., Moineau, S., Bushman, M. and Barrangou, R. [2013]. The population and evolutionary dynamics of phage and bacteria with CRISPR–mediated immunity, *PLoS genetics* **9**(3): e1003312.

Levin, B. R., Stewart, F. M. and Rice, V. A. [1979]. The kinetics of conjugative plasmid transmission: fit of a simple mass action model, *Plasmid* **2**(2): 247–260.

Li, H. [2013]. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM, *arXiv preprint arXiv:1303.3997* .

Li, H. and Durbin, R. [2009]. Fast and accurate short read alignment with Burrows–Wheeler transform, *Bioinformatics* **25**(14): 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. [2009]. The sequence alignment/map format and SAMtools, *Bioinformatics* **25**(16): 2078–2079.

Lilley, A. and Bailey, M. [2002]. The transfer dynamics of Pseudomonas sp. plasmid pQBR11 in biofilms, *FEMS microbiology ecology* **42**(2): 243–250.

Lilley, A. K. and Bailey, M. J. [1997]. The acquisition of indigenous plasmids by a genetically marked pseudomonad population colonizing the sugar beet phytosphere is related to local environmental conditions., *Applied and Environmental Microbiology* **63**(4): 1577–1583.

Lilley, A. K., Bailey, M. J., Day, M. J. and Fry, J. C. [1996]. Diversity of mercury resistance plasmids obtained by exogenous isolation from the bacteria of sugar beet in three successive years, *FEMS microbiology ecology* **20**(4): 211–227.

Lilley, A. K., Fry, J. C., Day, M. J. and Bailey, M. J. [1994]. In situ transfer of an exogenously isolated plasmid between Pseudomonas spp. in sugar beet rhizosphere, *Microbiology* **140**(1): 27–33.

Loftie-Eaton, W., Bashford, K., Quinn, H., Dong, K., Millstein, J., Hunter, S., Thomason, M. K., Merrikh, H., Ponciano, J. M. and Top, E. M. [2017]. Compensatory mutations improve general permissiveness to antibiotic resistance plasmids, *Nature ecology & evolution* **1**(9): 1354.

Loftie-Eaton, W., Yano, H., Burleigh, S., Simmons, R. S., Hughes, J. M., Rogers, L. M., Hunter, S. S., Settles, M. L., Forney, L. J., Ponciano, J. M. et al. [2015]. Evolutionary paths that expand plasmid host-range: implications for spread of antibiotic resistance, *Molecular biology and evolution* **33**(4): 885–897.

LoGiudice, K., Ostfeld, R. S., Schmidt, K. A. and Keesing, F. [2003]. The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk, *Proceedings of the National Academy of Sciences* **100**(2): 567–571.

Lopatkin, A. J., Meredith, H. R., Srimani, J. K., Pfeiffer, C., Durrett, R. and You, L. [2017]. Persistence and reversal of plasmid-mediated antibiotic resistance, *Nature communications* **8**(1): 1689.

Lozupone, C. A. and Knight, R. [2007]. Global patterns in bacterial diversity, *Proceedings of the National Academy of Sciences* **104**(27): 11436–11440.

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J., Charpentier, E., Haft, D. H. et al. [2015]. An updated evolutionary classification of CRISPR–Cas systems, *Nature Reviews Microbiology* **13**(11): 722.

Marti, E., Variatza, E. and Balcazar, J. L. [2014]. The role of aquatic ecosystems as reservoirs of antibiotic resistance, *Trends in microbiology* **22**(1): 36–41.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M. et al. [2010]. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, *Genome research* **20**(9): 1297–1303.

McPeek, M. A. [1996]. Trade-offs, food web structure, and the coexistence of habitat specialists and generalists, *The American Naturalist* **148**: S124–S138.

Mindlin, S., Minakhin, L., Petrova, M., Kholodii, G., Minakhina, S., Gorlenko, Z. and Nikiforov, V. [2005]. Present-day mercury resistance transposons are common in bacteria preserved in permafrost grounds since the upper pleistocene, *Research in microbiology* **156**(10): 994–1004.

Modi, S. R., Lee, H. H., Spina, C. S. and Collins, J. J. [2013]. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome, *Nature* **499**(7457): 219.

Molin, S. and Tolker-Nielsen, T. [2003]. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure, *Current opinion in biotechnology* **14**(3): 255–261.

Moura, A., Henriques, I., Smalla, K. and Correia, A. [2010]. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes, *Research in microbiology* **161**(1): 58–66.

Nakazawa, T. and Yokota, T. [1973]. Benzoate metabolism in Pseudomonas putida (arvilla) mt-2: demonstration of two benzoate pathways, *Journal of bacteriology* **115**(1): 262–267.

Nemergut, D. R., Schmidt, S. K., Fukami, T., O'Neill, S. P., Bilinski, T. M., Stanish, L. F., Knelman, J. E., Darcy, J. L., Lynch, R. C., Wickey, P. et al. [2013]. Patterns and processes of microbial community assembly, *Microbiology and Molecular Biology Reviews* **77**(3): 342–356.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y. et al. [2011]. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria, *Science* **334**(6058): 982–986.

Norman, A., Hansen, L. H. and Sørensen, S. J. [2009]. Conjugative plasmids: vessels of the communal gene pool, *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **364**(1527): 2275–2289.

Ochman, H., Lawrence, J. G. and Groisman, E. A. [2000]. Lateral gene transfer and the nature of bacterial innovation, *Nature* **405**(6784): 299–304.

Oliveira, P. H., Touchon, M. and Rocha, E. P. [2014]. The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts, *Nucleic acids research* **42**(16): 10618–10631.

Oliveira, P. H., Touchon, M. and Rocha, E. P. [2016]. Regulation of genetic flux between bacteria by restriction–modification systems, *Proceedings of the National Academy of Sciences* **113**(20): 5658–5663.

Osborn, A. M. and Böltner, D. [2002]. When phage, plasmids, and transposons collide: genomic islands, and conjugative-and mobilizable-transposons as a mosaic continuum, *Plasmid* **48**(3): 202–212.

Ostfeld, R. S. and Keesing, F. [2000]. Biodiversity and disease risk: the case of lyme disease, *Conservation Biology* **14**(3): 722–728.

Partridge, S. R., Tsafnat, G., Coiera, E. and Iredell, J. R. [2009]. Gene cassettes and cassette arrays in mobile resistance integrons, *FEMS microbiology reviews* **33**(4): 757–784.

Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A. J., Thomson, N. R., Quail, M., Smith, F., Walker, D., Libberton, B. et al. [2010]. Antagonistic coevolution accelerates molecular evolution, *Nature* **464**(7286): 275.

Perez, J. C. and Groisman, E. A. [2009]. Evolution of transcriptional regulatory circuits in bacteria, *Cell* **138**(2): 233–244.

Porse, A., Schønning, K., Munck, C. and Sommer, M. O. [2016]. Survival and evolution of a large multidrug resistance plasmid in new clinical bacterial hosts, *Molecular Biology and Evolution* p. msw163.

Pukall, R., Tschäpe, H. and Smalla, K. [1996]. Monitoring the spread of broad host and narrow host range plasmids in soil microcosms, *FEMS microbiology ecology* **20**(1): 53–66.

R Core Team [2015]. *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria.
URL: *http://www.R-project.org/*

Rainey, P. B., Bailey, M. J. and Thompson, I. P. [1994]. Phenotypic and genotypic diversity of fluorescent pseudomonads isolated from field-grown sugar beet, *Microbiology* **140**(9): 2315–2331.

Reboud, X. and Bell, G. [1997]. Experimental evolution in Chlamydomonas. III. Evolution of specialist and generalist types in environments that vary in space and time, *Heredity* **78**(5): 507–514.

Roossinck, M. J. [2011]. The good viruses: viral mutualistic symbioses, *Nature Reviews Microbiology* **9**(2): 99.

San Millan, A. and MacLean, R. [2017]. Fitness costs of plasmids: a limit to plasmid transmission., *Microbiology spectrum* **5**(5).

San Millan, A., Peña-Miller, R., Toll-Riera, M., Halbert, Z., McLean, A., Cooper, B. and MacLean, R. [2014]. Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids, *Nature communications* **5**.

San Millan, A., Toll-Riera, M., Qi, Q. and MacLean, R. C. [2015]. Interactions between horizontally acquired genes create a fitness cost in Pseudomonas aeruginosa, *Nature communications* **6**: 6845.

Santos-Lopez, A., Bernabe-Balas, C., San Millan, A., Ortega-Huedo, R., Hoefer, A., Ares-Arroyo, M. and Gonzalez-Zorn, B. [2017]. Compensatory evolution facilitates the acquisition of multiple plasmids in bacteria, *bioRxiv* p. 187070.

Schlüter, A., Szczepanowski, R., Pühler, A. and Top, E. M. [2007]. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool, *FEMS microbiology reviews* **31**(4): 449–477.

Schröder, G. and Lanka, E. [2005]. The mating pair formation system of conjugative plasmids—a versatile secretion machinery for transfer of proteins and DNA, *Plasmid* **54**(1): 1–25.

Sen, D., Van der Auwera, G. A., Rogers, L. M., Thomas, C. M., Brown, C. J. and Top, E. M. [2011]. Broad-host-range plasmids from agricultural soils have IncP-1 backbones with diverse accessory genes, *Applied and environmental microbiology* **77**(22): 7975–7983.

Simonsen, L. [1991]. The existence conditions for bacterial plasmids: theory and reality, *Microbial ecology* **22**(1): 187–205.

Simonsen, L., Gordon, D., Stewart, F. and Levin, B. [1990]. Estimating the rate of plasmid transfer: an end-point method, *Microbiology* **136**(11): 2319–2325.

Slater, F. R., Bailey, M. J., Tett, A. J. and Turner, S. L. [2008]. Progress towards understanding the fate of plasmids in bacterial communities, *FEMS microbiology ecology* **66**(1): 3–13.

Smalla, K., Jechalke, S. and Top, E. M. [2015]. Plasmid detection, characterization and ecology, *Microbiology spectrum* **3**(1).

Smalla, K. and Sobecky, P. A. [2002]. The prevalence and diversity of mobile genetic elements in bacterial communities of different environmental habitats: insights gained from different methodological approaches, *FEMS Microbiology ecology* **42**(2): 165–175.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. and de la Cruz, F. [2010]. Mobility of plasmids, *Microbiology and Molecular Biology Reviews* **74**(3): 434–452.

Sorek, R., Zhu, Y., Creevey, C. J., Francino, M. P., Bork, P. and Rubin, E. M. [2007]. Genome-wide experimental determination of barriers to horizontal gene transfer, *Science* **318**(5855): 1449–1452.

Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N. and Wuertz, S. [2005]. Studying plasmid horizontal transfer in situ: a critical review, *Nature Reviews Microbiology* **3**(9): 700.

Sota, M., Yano, H., Hughes, J. M., Daughdrill, G. W., Abdo, Z., Forney, L. J. and Top, E. M. [2010]. Shifts in the host range of a promiscuous plasmid through parallel evolution of its replication initiation protein, *The ISME journal* **4**(12): 1568–1580.

Spencer, S. J., Tamminen, M. V., Preheim, S. P., Guo, M. T., Briggs, A. W., Brito, I. L., Weitz, D. A., Pitkänen, L. K., Vigneault, F., Virta, M. P. et al. [2016]. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers, *The ISME journal* **10**(2): 427.

Stevenson, C., Hall, J. P., Brockhurst, M. A. and Harrison, E. [2018]. Plasmid stability is enhanced by higher-frequency pulses of positive selection, *Proc. R. Soc. B* **285**(1870): 20172497.

Stevenson, C., Hall, J. P., Harrison, E., Wood, A. and Brockhurst, M. A. [2017]. Gene mobility promotes the spread of resistance in bacterial populations, *The ISME journal* **11**(8): 1930.

Stewart, F. M. and Levin, B. R. [1977]. The population biology of bacterial plasmids: a priori conditions for the existence of conjugationally transmitted factors, *Genetics* **87**(2): 209–228.

Stokes, H. W. and Gillings, M. R. [2011]. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens, *FEMS microbiology reviews* **35**(5): 790–819.

Summers, D. K., Beton, C. W. and Withers, H. L. [1993]. Multicopy plasmid instability: the dimer catastrophe hypothesis, *Molecular microbiology* **8**(6): 1031–1038.

Sun, C. L., Barrangou, R., Thomas, B. C., Horvath, P., Fremaux, C. and Banfield, J. F. [2013]. Phage mutations in response to CRISPR diversification in a bacterial population, *Environmental microbiology* **15**(2): 463–470.

Svara, F. and Rankin, D. J. [2011]. The evolution of plasmid-carried antibiotic resistance, *BMC evolutionary biology* **11**(1): 130.

Tett, A., Spiers, A. J., Crossman, L. C., Ager, D., Ciric, L., Dow, J. M., Fry, J. C., Harris, D., Lilley, A., Oliver, A. et al. [2007]. Sequence-based analysis of pQBR103; a representative of a unique, transfer-proficient mega plasmid resident in the microbial community of sugar beet, *The ISME journal* **1**(4): 331–340.

Thomas, C. M. and Nielsen, K. M. [2005]. Mechanisms of, and barriers to, horizontal gene transfer between bacteria, *Nature reviews microbiology* **3**(9): 711–721.

Top, E. M. and Springael, D. [2003]. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds, *Current Opinion in Biotechnology* **14**(3): 262–269.

Torsvik, V. and Øvreås, L. [2002]. Microbial diversity and function in soil: from genes to ecosystems, *Current opinion in microbiology* **5**(3): 240–245.

Torsvik, V., Øvreås, L. and Thingstad, T. F. [2002]. Prokaryotic diversity–magnitude, dynamics, and controlling factors, *Science* **296**(5570): 1064–1066.

Toussaint, A. and Merlin, C. [2002]. Mobile elements as a combination of functional modules, *Plasmid* **47**(1): 26–35.

Tuller, T., Girshovich, Y., Sella, Y., Kreimer, A., Freilich, S., Kupiec, M., Gophna, U. and Ruppin, E. [2011]. Association between translation efficiency and horizontal gene transfer within microbial communities, *Nucleic acids research* **39**(11): 4743–4755.

Turner, P. E., Cooper, V. S. and Lenski, R. E. [1998]. Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids, *Evolution* pp. 315–329.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Solovyev, V. V., Rubin, E. M., Rokhsar, D. S. and Banfield, J. F. [2004]. Community structure and metabolism through reconstruction of microbial genomes from the environment, *Nature* **428**(6978): 37.

van Elsas, J. D. and Bailey, M. J. [2002]. The ecology of transfer of mobile genetic elements, *FEMS microbiology ecology* **42**(2): 187–197.

Van Tienderen, P. H. [1991]. Evolution of generalists and specialist in spatially heterogeneous environments, *Evolution* pp. 1317–1331.

Vasu, K., Nagamalleswari, E. and Nagaraja, V. [2012]. Promiscuous restriction is a cellular defense strategy that confers fitness advantage to bacteria, *Proceedings of the National Academy of Sciences* **109**(20): E1287–E1293.

Vasu, K. and Nagaraja, V. [2013]. Diverse functions of restriction-modification systems in addition to cellular defense, *Microbiology and molecular biology reviews* **77**(1): 53–72.

Vogwill, T. and MacLean, R. C. [2015]. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach, *Evolutionary applications* **8**(3): 284–295.

von Wintersdorff, C. J., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. and Wolffs, P. F. [2016]. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer, *Frontiers in microbiology* **7**: 173.

WHO [2012]. *The evolving threat of antimicrobial resistance: options for action.*, World Health Organization.

Wiedenbeck, J. and Cohan, F. M. [2011]. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches, *FEMS microbiology reviews* **35**(5): 957–976.

Wozniak, R. A. and Waldor, M. K. [2010]. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow, *Nature Reviews Microbiology* **8**(8): 552.

Wright, G. D. [2010]. Antibiotic resistance in the environment: a link to the clinic?, *Current opinion in microbiology* **13**(5): 589–594.

Xiong, J., Alexander, D. C., Ma, J. H., Déraspe, M., Low, D. E., Jamieson, F. B. and Roy, P. H. [2013]. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encoding IMP-9-mediated carbapenem resistance, from outbreak isolate Pseudomonas aeruginosa 96, *Antimicrobial agents and chemotherapy* **57**(8): 3775–3782.

Yang, J.-R., Liao, B.-Y., Zhuang, S.-M. and Zhang, J. [2012]. Protein misinteraction avoidance causes highly expressed proteins to evolve slowly, *Proceedings of the National Academy of Sciences* **109**(14): E831–E840.

zur Wiesch, P. A., Kouyos, R., Engelstädter, J., Regoes, R. R. and Bonhoeffer, S. [2011]. Population biological principles of drug-resistance evolution in infectious diseases, *The Lancet infectious diseases* **11**(3): 236–247.