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The evolution and determination of plasmid transfer rate and subsequent effect on competition

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Abstract

Horizontal gene transfer by conjugation is one of the processes that determines the persistence, prevalence and transmission of antibiotic resistance genes that can be found on bacterial plasmids. In order to appropriately tackle the spread of antibiotic resistance we must therefore understand how plasmid dynamics function in complex microbial communities. Various aspects of plasmid dynamics and how they contribute to the spread of antibiotic resistance are unclear and require attention. For example, plasmid transfer rates vary widely, but the ways in which environmental, plasmid and host factors explain this variation and the relative importance of each factor is unclear. In addition, the evolutionary forces that differentially affect plasmids and hosts to determine specific transfer rates have not been fully explored; in particular, the effects of host-plasmid conflicts in non-selective conditions and the impact of the relationship between plasmid cost on host growth and plasmid transfer rate. A theoretical understanding of transfer rates must then be placed within the context of the other parameters that affect plasmid dynamics (e.g. plasmid cost, loss etc.) to make assertions on plasmid persistence and prevalence, and theoretical results must be compared with experimental data in increasing microbial complexity. Experiments are rarely conducted using multiple species and the impacts and interactions of plasmid presence on a community have yet to be explored fully in the lab.

The first data chapter of this thesis (chapter 2) seeks to address the question of how transfer

rate variation can be attributed to various environmental variables in addition to the effects of plasmid, donor and recipient identities. A meta-analysis of published transfer rates was therefore conducted and the variation assessed by applying series of multivariate linear models to the data. Over three quarters of the variation from the meta-analysis could be explained, with plasmid repression and media type explaining the most variation. The results also identify the recipient identity as an important variable that explains up to 34% of the variation.

Given the variation in transfer rates, the next chapter (chapter 3) asks how the various selection pressures on host and plasmid may interact to determine specific rates of transfer. In particular, it asks how the costs of plasmid transfer impact transfer rates, and how host-plasmid conflicts in transfer rate may subsequently affect plasmid prevalence. Adaptive dynamics and invasion analyses were applied to simple conjugation models under selective and non-selective conditions, and using different plasmid transfer-cost relationships. The findings were then combined to model the effects that host-plasmid conflicts in non-selective conditions may have on transfer rates and plasmid prevalence. The results of separate analyses demonstrate the role of the recipient in controlling transfer rates, and show that plasmid-controlled transfer rate can be predicted with only three parameters (host growth rate, plasmid loss rate and the cost of plasmid transfer on growth). Low frequency genetic variation in transfer rate is predicted to accumulate, which can facilitate rapid adaptation to changing conditions. Further modelling showed that in order to substantially affect plasmid prevalence (and corresponding cumulative costs a plasmid has on a population in non-selective conditions) a host may need to decrease the transfer rate by several orders of magnitude, indicating that hosts must have strong control mechanisms to be valuable.

In the final data chapter (chapter 4) I ask if and how plasmid dynamics focusing on the interaction of plasmid presence and inter-species competition in simple microbial communities can be predicted using independently measured parameters. In particular, how does the rate of plasmid transfer impact species competitive advantage and the outcomes of competition? A series of experiments were conducted to estimate parameters for two plasmids and two bacterial species for use in a simplified two-species bacterial conjugation model to

make predictions of competitive advantage. These predictions were then compared with a series of corresponding competition assays. The effects of the plasmid distribution on competition and the presence of multiple species on plasmid stability in the community were also noted and described. The model accurately predicted many of the experimental results, but deviated from those results where specific parameters were over or underestimated. The results emphasise the importance of appropriate parameter measurement. Plasmid presence reduced the competitive ability of each host and incurred higher costs from the plasmid with a higher transfer rate. These effects were limited or exacerbated dependent on whether the plasmid was able to successfully invade the other species where it incurred similar costs. These results demonstrate the complex effects of plasmid transfer, cost and host interactions on plasmid dynamics in a microbial community and the competitive dynamics of that community.

These results show that transfer rates are highly variable according to environmental conditions and that, while the majority of the variation can be assigned to some variables, additional work is required to evaluate the effects of particular variables, such as temperature and the effects of plasmid-host coevolution. While this work demonstrates how selective pressures act on transfer rates, more work is also required to link particular observed transfer rates to the conditions in which they evolve. The results highlight the importance of the variable and potentially conflicting selection pressures on host and plasmid that combine to determine the rate of transfer, emphasising the sometimes neglected role of the recipient. The relationship between plasmid cost and plasmid transfer rate is identified as a key part of transfer rate evolution and also requires future attention to describe this relationship in order to fully understand how plasmid transfer rates are constructed. These results increase our understanding of the factors that affect plasmid dynamics, have implications for the way we consider and handle the spread of antibiotic resistance, and provide direction for future research opportunities.

Preface

Dedication

Stuck between carols in a Christmas choir in December 2018, I had an epiphany moment while looking out over the congregation. The thought was: "oh, I think my PhD is worthless". I had been struggling for a long time worrying about whether my work was interesting and useful, and whether any of it mattered. Some researchers I had spoken to had expressed reservations about some of my research projects, and a man had been rather rude about a poster I had presented at a conference. I was feeling quite insecure and uncertain about my work. Interestingly, to me at least, my epiphany did not come from a place of panic. It wasn't an: "oh no, my PhD is worthless and everything is terrible". It was an: "oh, I think my PhD is worthless... and I am one with the world". Somehow I felt a lot of peace with that thought. I think it was an acceptance or an embrace of the fact that my contribution is likely to be small and that that is OK. This moment was quite liberating to me and allowed me to let go of many of the worries I had felt up until that point. I had been preoccupied with what I perceived others to be thinking about my work, rather than simply doing what I thought and felt was a useful thing to do. Since then I have repeatedly asked myself this question: "based on what I know now, what am I going to do next?". I don't expect myself to know everything and/or do everything perfectly. Instead, I choose to open myself to being able to learn as I go, and try to make good notes as I do so. A year after my epiphany-moment my first paper was published, based on the ideas that had been doubted by the aforementioned researchers, with a second paper submitted to another journal. I

have been contacted several times by researchers asking after my dataset, and the published paper continues to provide more opportunities for communication and collaboration with experts in the field.

Acknowledgements

With this dedication in mind, I would like to thank my supervisors, Tim Barraclough and Vincent Jansen, for all their time and effort in working through problems, helping me shape my thoughts and ideas, pushing me when needed and allowing me space to expand my work where appropriate, and for being genuinely good people to work with. I would also like to thank James Rosindell and Tom Bell for sitting on my progress review panel; their positive feedback helped me to gain more confidence in my work. Andrew Lilley was an extremely useful contact as an expert in the field giving useful initial constructive and positive feedback. Everyone I worked with, across several lab groups: Reuben Nowell, Chris Wilson, Damian Rivett, Emma Ransome, Shorok Mombrikotb, Tom Vogwill, Tom Smith, Nilita Mukjang, Tim Russell, Bethany Nichols, Leanne Massie, Chris Adams, Cara Patel, Penelope Blyth, Richard Dearden and Alice Beddis. I would also like to thank Jisoo Jean for being my write-up buddy, for being an ear for my complaints and helping me get through it all, and the rest of my emotional support animals: Camie Rasmussen, Melissa Regan, Ben Schmidt, Elizabeth Neilsen, Emma Parkin, Thomas McCormack, Michie Takeuchi, Sulochana Omwenga and Amira Hassan. I'd like to thank my parents for encouraging me to pursue higher education, and thank Samraat Pawar for supervising my undergraduate project that ultimately led me to this point.

Statement of originality

The work is original and my own unless explicitly stated. An undergraduate student (Alice E. Beddis) collected part of the data used in the meta-analysis in chapter 2. A paper has also

been published covering the material in chapter 2, and a second paper has been submitted to a journal covering the material in chapter 3.

Copyright declaration

Publications

A modified version of chapter two was published here:

Sheppard RJ, Beddis AE, Barraclough TG. The role of hosts, plasmids and environment in determining plasmid transfer rates: A meta-analysis. Plasmid. 2020 Mar [cited 2020 Feb 19];108:102489. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31926878

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Chapter 1

Introduction

Antibiotic resistance increasingly presents a major problem to public health in our ability to treat bacterial disease (O'Neill, 2016). Resistance can spread through horizontal gene transfer (HGT) including the transfer of extrachromosomal elements, such as plasmids, through conjugation (Trevors, 1999). This thesis looks at the factors and evolutionary pressures that determine rates of plasmid transfer and the interaction between plasmid presence and competition in a simple community.

In this introduction, I first review the discovery, history and development of antibiotics (Gould, 2016), and detail how their use, misuse and overuse has led to the dangerous spread of antibiotic resistance, resulting in increasingly serious global public health issues (O'Neill, 2016). Second, I describe mechanisms by which antibiotic resistance spreads through HGT and how these HGT processes interact (Trevors, 1999). Finally, I look at plasmids (vectors of conjugation), the factors that determine plasmid persistence and the methods used to study them.

1.1 Antibiotic use and resistance

While antibiotics were used to treat infections anciently, only during the renaissance and enlightenment did our understanding of microbiology and bacterial infections improve enough

to pave the way for the formal discovery of antibiotics (Gould, 2016). Since 1928, when Alexander Fleming famously identified penicillin, more and more antibiotics have been discovered and developed, leading to what is sometimes described as the golden age of antibiotics during the mid-1900s. Antibiotics have transformed medicine by curing previously untreatable fatal illnesses and permitting operations and transplantation to occur without fear of subsequent bacterial infection (Cantas et al., 2013; Watkins and Bonomo, 2016a).

Coupled closely with antibiotic discovery and use is the emergence of antibiotic resistance (Cantas et al., 2013; Clatworthy et al., 2007; Watkins and Bonomo, 2016a, Figure 1.1). As the successful use of antibiotics in medicine and animal agriculture increased there has been a complementary increase in the prevalence of antibiotic resistance. While both antibiotics and antibiotic resistance are found naturally (Cantas et al., 2013; Davies, 1997; Olivares et al., 2013; Walsh, 2013), resistance can and does evolve de novo, and frequently within a short space of time (Watkins and Bonomo, 2016a). Once evolved, resistance can spread rapidly, stimulated by the strong selection pressures from the use of antibiotics (Heinemann, 1999). This has decreased the efficacy of antibiotics, rendering many of them essentially useless against some bacterial strains. Many pathogenic strains are now resistant to multiple antibiotics, sometimes described as superbugs (Alpert, 2016). It is estimated that 700,000 deaths can currently be attributed to antibiotic resistant infections each year, and this figure is expected to rise to 10 million by 2050 unless action is taken (O'Neill, 2016). This is a serious issue that needs to be addressed. We must understand how resistance works, how it spreads, and what we can do about it.

1.1.1 Antibiotic mechanisms

Antibiotics function by exploiting the differences between eukaryotic and bacterial cells (e.g. synthesis of DNA, RNA or protein, cell membrane or cell wall, Figure 1.2, Kapoor et al., 2017) to target and either kill (bacteriocidal) or prevent the growth of bacteria (bacteriostatic). Both kinds can be used to tackle bacterial infections, where an effective immune

[1]Reprinted from Nat Chem Biol, Volume 3, Clatworthy et al., Targeting virulence: a new paradigm for antimicrobial therapy, 541548, ©2007, with permission from Springer Nature Limited.

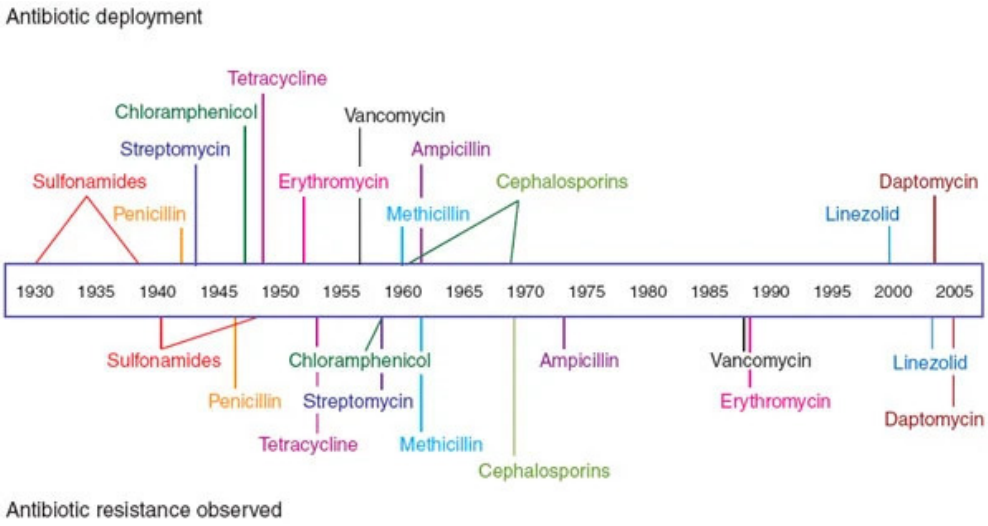

Figure 1.1: 'Timeline of antibiotic deployment and the evolution of antibiotic resistance.'[1]

system can clear the remaining static pathogenic cells (Nemeth et al., 2014). The range of bacterial strains and species each antibiotic is effective against varies, and antibiotics are classified as having a broad or narrow spectrum of activity, although these terms are poorly defined (Acar, 1997). Some antibiotics are effective against gram-positive or gram-negative bacterial strains (van Saene et al., 1998) and vary in how effective they are, according to the antibiotic dose and the level of resistance that exists.

1.1.2 Antibiotic resistance mechanisms

Antibiotic resistance mechanisms primarily work in three ways (Edson and Kwon, 2014; Kapoor et al., 2017, Figure 1.2). The first is simply for the cells to remove the antibiotic from the cell through efflux pumps (Li and Mehrotra, 2016), of which there are five identified efflux protein families. The second is through the modification or inactivation of antibiotics through hydrolysis (Davies, 1994), group transfer (acylation, phosphorylation, thiolation, glycosylation, nucleotidylation, ribosylation) or through oxidation or reduction (Edson and Kwon, 2014). Beta-lactams, for example, can be inactivated through hydrolysis. Finally, the cell can prevent the action of the antibiotic through the mutation or modification of the target site (Munita and Arias, 2016).

[2]Reprinted from Journal of Controlled Release, Volume 189, Edson and Kwon, RNAi for silencing drug resistance in microbes toward development of nanoantibiotics, 150-157, ©2014, with permission from Else-

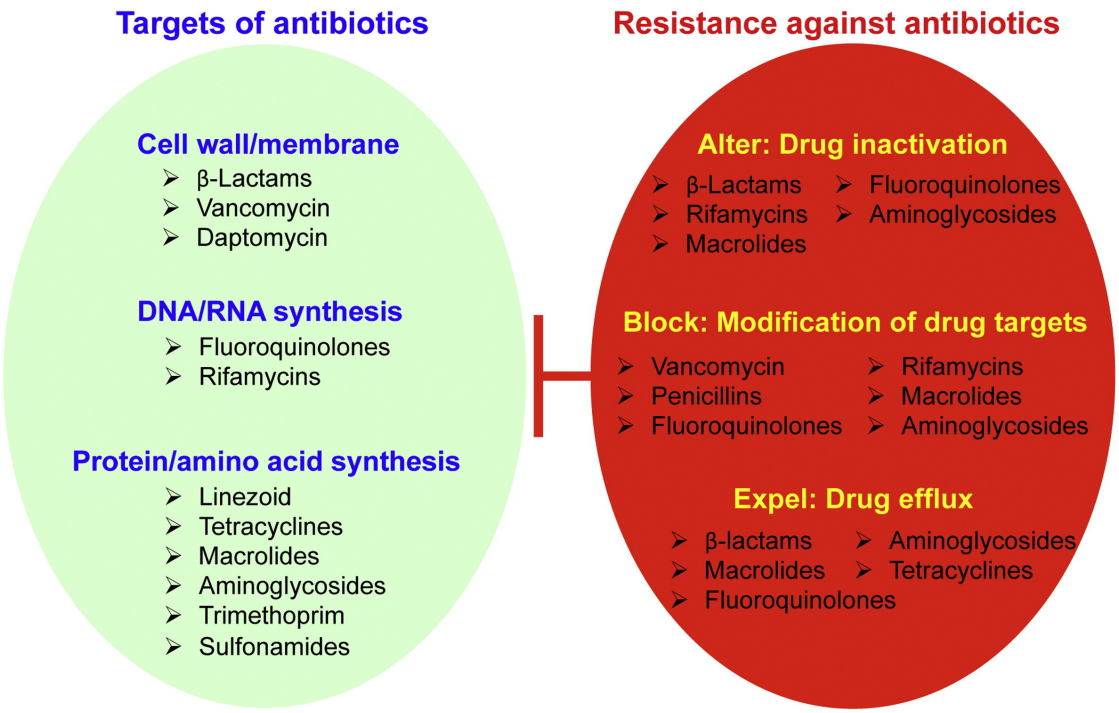

Figure 1.2: 'Resistance developed against common antibiotics.'[2]

1.1.3 Antibiotic use

Antibiotics have revolutionised medical treatments (Cantas et al., 2013) and their use has steadily increased worldwide (Klein et al., 2018; Van Boeckel et al., 2014), although this use has come with consequences (i.e. antibiotic resistance). Antibiotics are often incorrectly and ineffectively (e.g. in viral or fungal infections, Shiley et al., 2010) prescribed as cure-alls when the source of the illness is unknown (Smieszek et al., 2018; Turnidge et al., 2016) and without sufficient thought to the resistance they stimulate (Aslam et al., 2018) or the harm they could incur to the patient (Azevedo et al., 2015). Antibiotics have also been far overused, not only in medicine and veterinary medicine (Prescott and Boerlin, 2016; Seiffert et al., 2013), but in animal agriculture and aquaculture (Jechalke et al., 2014) where there are fewer regulatory limitations (Cabello, 2006). Antibiotics can and have been used prophylactically to reduce infection and as animal growth promoters, stimulating resistance in the environment (Economou and Gousia, 2015; Jechalke et al., 2014; Watkins and Bonomo, 2016a, Figure 1.3).

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[3]Reprinted from Environmental Health and Preventive Medicine, Volume 20, Fletcher, Understanding the contribution of environmental factors in the spread of antimicrobial resistance, 243252, ©2015, with

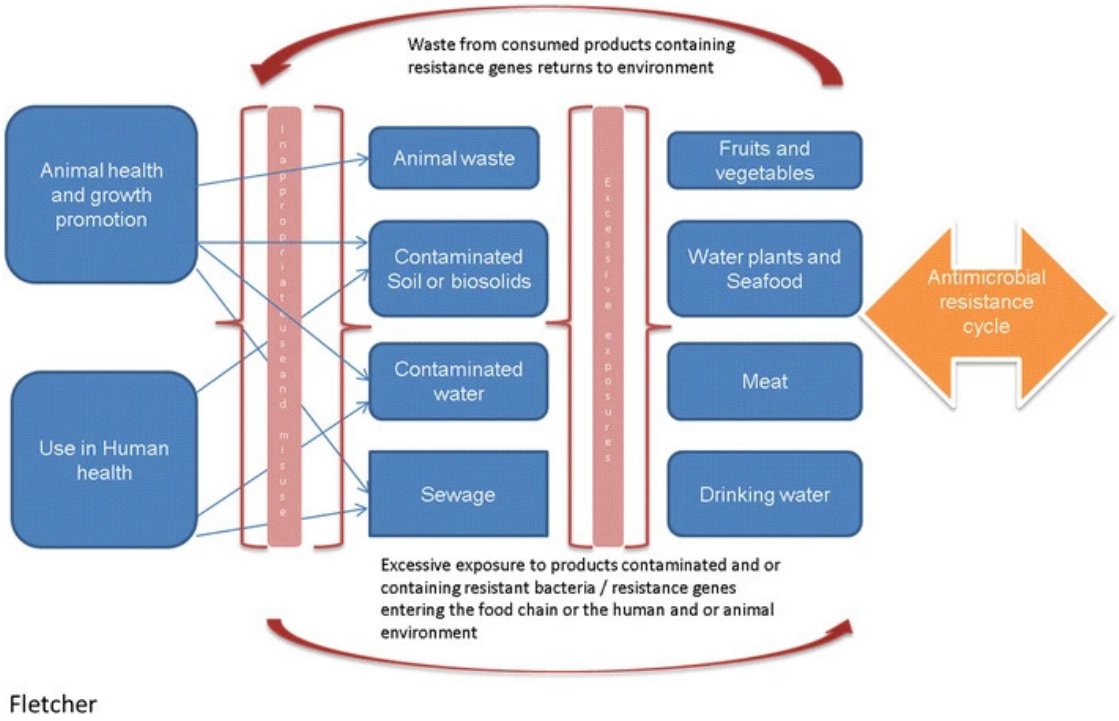

Figure 1.3: 'Complex interactions amongst environmental- and health-related factors that contribute to the spread of antimicrobial resistance. The figure summarises how various often interlinked factors contribute to contamination of the environment; with the transfer of antibiotic resistance genes and antibiotic-resistant bacteria going in both directions, perpetuating the cycle of antimicrobial resistance.'[3]

1.1.4 Antibiotic resistance prevalence

Wastewater from hospitals, agriculture and aquaculture containing antibiotics stimulate measurable antibiotic resistance in soils and rivers across the world (Biao et al., 2015; Chen et al., 2016), but the extent to which resistance persists and causes a problem for human health remains unclear (Garcillán-Barcia et al., 2011). Antibiotic resistance genes in pathogenic bacteria have been detected in wastewater from hospitals across the world, in both developed and developing nations (Abdel Rahim et al., 2015; Asfaw et al., 2017; Lyimo et al., 2016; Noordin et al., 2016; Walia et al., 2016), and even in places where waste is treated to target and remove antibiotics (Rodriguez-Mozaz et al., 2015). The high level of antibiotics and resistance genes can make hospitals and their waste reservoirs for antibiotic resistant pathogens (Weingarten et al., 2018), and increase the risks of infection by resistant pathogenic strains, particularly for patients who visit hospitals regularly.

[3] requested permission from Springer Nature.

Antibiotic use in agriculture and aquaculture can lead to resistant pathogenic infections in livestock that increases the potential for human infection through uncooked meat or dairy (Ibrahim et al., 2016; Osman et al., 2014; Phillips et al., 2004; Randall et al., 2014). The frequent use of agricultural waste as fertiliser provides opportunity for these resistant strains to migrate to and be found on the resulting produce (Wang et al., 2015). Pathogenic bacteria have been found on raw foods and in places where those foods are stored (Ongeng et al., 2014), and are places where antibiotic resistance can reside (Fischer et al., 2014). Aquaculture finds similarly high levels of antibiotic resistance, up to four times higher than in controls (Huang et al., 2017).

1.1.5 Antibiotic development

While antibiotic resistance quickly becomes widespread following antibiotic use, the problems caused by resistant strains have, to date, been mitigated by the discovery and development of new antibiotics (Gould, 2016). Unfortunately, within recent years, new antibiotics have become more difficult to identify, as the "low-hanging fruit" of antibiotic research becomes exhausted (Payne et al., 2007). Recent advances in antibiotic development have largely only been within antibiotic classes, with no discovery of new antibiotic classes and a 90% decrease in the development of new antibiotics between the 1980s and 2000s (Luepke et al., 2017). Antibiotic discovery investment is disincentivised by the long and expensive process of development and testing (Katz et al., 2006) combined with a low return on investment and limited use of antibiotics due to the rapid spread of resistance (Watkins and Bonomo, 2016b). Some government schemes have been initiated to encourage investment in antibiotic discovery and development (Eichberg, 2015; Watkins and Bonomo, 2016b).

Without appropriate investment into the development of new antibiotics or alternative strategies for tackling pathogens, we could soon enter a post-antibiotic era (Kåhrström, 2013). It is vital that more antibiotics are developed, and that long-term sustainable strategies are developed to replace them (Allen et al., 2014). Current antibiotics must be used effectively to kill pathogens and in a way that minimises the stimulation of antibiotic resis-

tance, and new therapies must be developed and implemented to replace antibiotics (Renwick et al., 2016).

1.1.6 Using antibiotics effectively

Antibiotics can be used more appropriately by reducing their use in medicine and agriculture (Lenski, 1998; Levin et al., 2014), limiting their use to cases where they are effective. This requires the development of sophisticated diagnostics (O'Neill, 2015), and a shift in public expectation of antibiotic use (O'Neill, 2016). Antibiotic doses and courses must also be tailored to maximise effectiveness while minimising resistance stimulation (Ambrose et al., 2016; Gehring et al., 2010). Governing bodies around the world are taking measures to implement these recommendations in varying degrees (Centers for Disease Control and Prevention, 2020; Department of Health and Department for Environment Food and Rural Affairs, 2016; European Parliament, 2018). Unfortunately, the dependence of agriculture on antibiotics mean that these restrictions will negatively affect yields, and have consequences for the price of meat products (National Research Council (US) Committee on Drug Use in Food Animals, 1999). However, this comes at a time when there is a notable trend towards plant-based diets in the west that could reduce the burden on animal agriculture, in addition to the benefits of reducing meat production to tackle climate change (Hever, 2016).

Greater care can also be taken to prevent antibiotics and resistance genes from entering the environment, particularly as wastewater treatment facilities have been flagged as reservoirs that encourage the spread of resistance (Asfaw et al., 2017). Furthermore, identifying the dissemination routes of antibiotics and resistance genes to reservoirs (Fischer et al., 2014; Remus-Emsermann et al., 2018) and the pathways by which these pathogens infect individuals can aid us in reducing the chance of infections through limiting exposure (Garcillán-Barcia et al., 2011; Larsson, 2014).

There are many promising alternative therapies to antibiotics, the majority of which are still in development, that may be used therapeutically in the future. These include antimicrobial peptides (De Oca, 2013; Snyder and Worobo, 2014), predatory bacteria (Kadouri

et al., 2013), phages (Ojala et al., 2013) and vaccines (Czaplewski et al., 2016). These could reduce or eliminate the need for antibiotics (Jansen et al., 2018; O'Neill, 2016). Many of these therapies face similar developmental and regulatory hurdles as antibiotics. Ultimately, it may require research and development into many of these methods, in addition to comprehensive changes in human behaviour to reduce our dependence on antibiotics (O'Neill, 2016).

While these methods are broadly believed to be able to reduce the prevalence of antibiotic resistant infections (Lenski, 1998), some studies suggest that the damage already done may be difficult to reverse (De Gelder et al., 2004; Heinemann et al., 2000; Slater et al., 2008b). Also, due to the effects of the global spread of antibiotic resistance genes, action must be taken consistently across the world to limit its damage (Shallcross and Davies, 2014; World Health Organization, 2015), which may be difficult in less economically developed nations that have other, more urgent, health care priorities (Klein et al., 2018; Li et al., 2016). Economically secure nations must work together with other countries to develop sustainable methods for dealing with these issues without compromising on the health or livelihood of individuals (O'Neill, 2016; Shallcross and Davies, 2014; World Health Organization, 2015).

1.2 Horizontal gene transfer

In addition to the spread of antibiotic resistance driven by selection and antibiotic use, the prevalence of resistance genes has been exacerbated by HGT (Davies, 1994). HGT comprises a group of extremely important processes in microbial evolution that enable genetic recombination between individual microbes (Frost et al., 2005; Tazzyman and Bonhoeffer, 2015; Trevors, 1999). While multicellular organisms undergo sexual recombination, microbes primarily reproduce asexually through binary fission and would only evolve through mutation, selection and drift, were it not for the recombination made possible by these varied processes (Lawrence, 1999). These processes prevent the accumulation of deleterious mutations within species (Koonin, 2016), enhance the speed of adaptation and have ultimately

been essential in the evolution of all multicellular life (Hall and Harrison, 2016). They also give bacterial populations and communities a wide pool of shared genetic resources to draw upon (Jain et al., 2003). Bacteria have large populations, high density and fast turnover that, in addition to the variety of genetic transmission modes, makes them highly adaptable in relatively short time frames (Hall and Harrison, 2016). Increasing our knowledge and understanding of these HGT processes will aid our understanding of current and historical microbial evolution, including the transition from single-cellular to multicellular life and our own microbial evolutionary origins (Markov, 2014). The three main HGT processes in bacteria are well known in the literature to be transformation, conjugation and transduction (Trevors, 1999).

1.2.1 Transformation

Transformation was first discovered in 1928 and is the process by which cells take up DNA from the environment (Veal et al., 1992). For transformation to occur cells must be competent (capable of taking in DNA). This frequently occurs when cells are deprived of nutrients, at high cell density (or at stationary phase), or when they are under stress (e.g. exposure to UV light) (Blokesch, 2016). During transformation, DNA fragments bind to cell surface receptors and are transported into the cell and to the chromosome where the DNA can be incorporated into the genome through homologous recombination (Trevors, 1999). Transformation can occur for loose strands of DNA and for entire plasmids (circles of extrachromosomal DNA, Paul et al., 1991).

The conditions that induce bacterial transformation are conflicting and variable. Transformation is believed to have partly evolved in bacteria to utilise DNA as a nutrient source, consistent with the increase in transformation competence at low nutrient concentrations (Redfield, 2001), although evidence for this relationship is not always found (Johnston et al., 2014). Alternatively, transformation of DNA from closely related cells followed by homologous recombination can reduce the effects of negative mutations (Redfield et al., 1997), and may explain the increase in competence in stressful environments (Claverys et al., 2006) such

as exposure to U.V. light (Charpentier et al., 2011). Transformation primarily occurs within closely-related cells due to proximity and the induction of competence at high cell density (Solomon and Grossman, 1996; Veening and Blokesch, 2017), but can also occurs between strains. Some species undergo programmed cell death (autolysis) to facilitate within species gene-sharing, releasing their DNA into the environment and facilitating the transmission of its genes with closely related cells (Johnston et al., 2014). As such, transformation favours unity over genetic diversity. Restriction enzymes that target foreign DNA can further limit the uptake of DNA to that of genetically similar strains (Thomas and Nielsen, 2005).

1.2.2 Transduction

Transduction has been observed since 1952 (Griffiths et al., 2000b) and occurs when phages (bacterial viruses) adopt portions of bacterial DNA from their cell hosts into the DNA packed into their protein capsids (Haaber et al., 2016). This DNA is then injected with the remaining DNA into a recipient cell during the normal phage replication cycle. Phages undergo lytic (virulent) and lysogenic (temperate) life cycles (Griffiths et al., 2000b). In the lytic cycle the phage commandeers the host machinery to replicate resulting in the death of the cell and the spread of more phages. While lysogenic phages can also cause cell death, their primary mode of replication is through incorporation into the host genome where they reproduce with the cell cycle of the host (Paul and Jiang, 2001). The DNA that phages transport can contain antibiotic resistance genes, facilitating the spread of resistance (Trevors, 1999). Although some phages can infect multiple bacterial species, most phages have a narrow host range that limits the potential spread of genes (Loc-Carrillo and Abedon, 2011).

1.2.3 Conjugation

Conjugation was discovered in 1946 (Griffiths et al., 2000a) and occurs when plasmids (circles of extra-chromosomal DNA found in microbes) are copied and inserted ("transferred") into

neighbouring cells through pili (Curtiss, 1969). Plasmids frequently replicate and transfer autonomously (Von Wintersdorff et al., 2016) and can contain whole sets of complementary genes (Loftie-Eaton et al., 2016), including resistance genes for multiple antibiotics. Antibiotic resistance genes encoded on plasmids include genes for efflux pumps and antibiotic modification or deactivation (Shutter and Akhondi, 2020). Also, due to a frequently high copy number (Boros et al., 1984), plasmids can be a source of great individual evolutionary potential (Hall and Harrison, 2016; Millan et al., 2016). For these reasons (autonomous replication and transfer, multiple resistance genes), conjugation is believed to be particularly important in the rise and spread of antibiotic resistance, and its study is therefore of primary importance to inform our response (Von Wintersdorff et al., 2016). Thus far, substantial progress has been made in understanding the biochemistry of conjugation, plasmids, and the genes they carry (Cabezón et al., 2015). As plasmids are the main focus of this thesis, I expand on their relevant biology in greater depth in section 1.3.

1.2.4 Conflicting transfer mechanisms

HGT processes do not occur in isolation and there are numerous examples of ways that they interact to facilitate and limit interactions between them (Croucher et al., 2016). For example, transduction can promote transformation through cell lysis that provides genetic material for uptake into surrounding cells (Keen et al., 2017). Transformation, however, can limit the effects of mobile genetic elements (MGEs) by replacing genetic changes made during transfer with sequences from the surrounding isogenic cells (Croucher et al., 2016). Cells producing conjugative pili are vulnerable to attack by phages and can increase the burden of plasmid-carriage, limiting the conditions where plasmids are able to persist (Harrison et al., 2015). The use of phages has thus been suggested as a potential strategy for tackling plasmid-based antibiotic resistance (Ojala et al., 2013) and has the capacity to drive some plasmids extinct (Jalasvuori et al., 2011). At the same time, phages can transfer partial or whole plasmids through transduction under some circumstances, blurring the boundaries of these processes (Jacob and Hobbs, 1974).

1.2.5 Additional transfer mechanisms

These three processes are not a comprehensive list of the ways that DNA can be transmitted horizontally. Beyond these canonical HGT mechanisms, there is a wide variety of MGEs and other mechanisms of transmission that contribute to a larger picture of horizontal genetic transmission (García-Aljaro et al., 2017). Integrative conjugative elements (ICEs, or conjugative transposons) are genetic elements that are capable of not only conjugative transfer, but also integration into and excision from the host chromosome (Johnson and Grossman, 2015). When excised they behave similarly to conjugative plasmids and transfer using the mechanisms already established, thus blurring the lines between plasmids and ICEs. Gene transfer agents are similar to phages but only contain chromosomal DNA (Lang et al., 2012). They do not contain genes for the production of phage proteins, meaning that they do not require a previous infection or propagate infectious phages following infection. Genomic islands can also be mobilised by ICEs, conjugative plasmids and phages (Bellanger et al., 2014b). Integrative mobilisable elements are equivalent to mobilisable plasmids but which are able to integrate into the chromosome, similar to ICEs. They require outside machinery from ICEs or conjugative plasmids for transfer and excision but frequently contain genes for integration following transmission (Bellanger et al., 2014b). Similarly, phage-inducible mobile elements are genomic islands that can be excised and transported in a capsid (García-Aljaro et al., 2017). Membrane vesicles are capable of transferring genetic material, proteins, ions, metabolites, and signalling molecules when released from the cell membrane (García-Aljaro et al., 2017). Nanotubes are similar in function to pili, connecting donor and recipient cells, but facilitate the transfer of other cytoplasmic molecules in addition to plasmid DNA. Some cell to cell transfer of plasmids can be stimulated by pheromones and occurs in the absence of pili (Etchuuya et al., 2011; Matsumoto et al., 2016). Endosymbiosis is another example of HGT, when considered broadly (Keeling and Palmer, 2008), particularly in the historical movement and incorporation of plastid DNA into the chromosome.

1.2.6 The communal gene pool

HGT is not limited to recombination occurring within species and allows microbes to access the wide scope of genetic variation found across their communities (Frost et al., 2005; Mc Ginty et al., 2013). Some even describe microbes as having a pangenome or a communal gene pool (Medini et al., 2005; Norman et al., 2009). These diverse genetic resources greatly enhance microbes' ability to adapt and evolve to changing environments, including the contribution of HGT to the rapid spread of antimicrobial resistance (AMR) in response to the use of antibiotics. While questions remain about how gene-sharing can evolve between competing strains, gene-sharing is generally thought to facilitate mutualism and cooperation between different strains in a community (Shapiro and Turner, 2014), particularly through genes that code for social goods (Fullmer et al., 2015). These social goods genes can benefit the whole community and have impacts on gene and group-level selection (Dimitriu, 2014; Mc Ginty et al., 2013).

1.2.7 Phylogenetic studies

HGT is often identified or inferred retrospectively using phylogenetic and parametric methods to compare microbial genotypes, identifying portions of DNA that are likely to be the result of transfer (Doolittle and Brunet, 2016; Ravenhall et al., 2015). The identification of HGT is complicated by gene loss (Poptsova and Gogarten, 2007), particularly prevalent among parasitic species (Moran and Wernegreen, 2000), although various methods are employed to reduce false-positives (Poptsova, 2009). While estimates vary, large portions (up to 20%, Philippe and Douady, 2003) of some bacterial species genotypes are found to have been transferred from other species (Groussin et al., 2016), although these may be overestimated. Ancient transfer events are more difficult to identify due to genetic noise created by mutations and more recent transfer events that overwrite historic ones (Philippe and Douady, 2003). While the amount of transferred genetic content varies, an average of 6% of bacterial genomes are believed to be composed of transferred material, suggesting that transfer events occur infrequently (Kurland et al., 2003).

The amount of transferred genes creates problems for the resolution of bacterial phylogenies and a universal tree of life becomes impossible to construct due to the differing histories of individual transferred genes (Snel et al., 1999, Figure 1.4); even 16S genes, commonly used in phylogeny construction (Hug et al., 2016), are transferable (Yokoyama et al., 2003). HGT also substantially blurs species boundaries through gene sharing (Andam et al., 2010; Doolittle and Brunet, 2016; Gogarten and Townsend, 2005; Groussin et al., 2016), making phylogenetics and identification of common bacterial ancestors even more difficult (Fournier et al., 2015). The universal tree of life for single-celled organisms frequently gets rejected in favour of other structures (Forterre, 2015) including nets (Hilario and Gogarten, 1993), mosaics (Martin, 1999), rings (Rivera and Lake, 2004) and cobwebs (Ge et al., 2005).

While the phylogenetic results are interesting and useful in identifying historical HGT, they only demonstrate successful transfer events and give little indication of active rates of transfer, nor do they track the mechanisms by which the genes have moved (Kurland et al., 2003; Lal et al., 2008). Active experimental work is required to address these gaps to estimate the parameters that determine the amount of transfer that occurs and has been under investigation for several decades. Experimental results indicate a discrepancy between the regularly high active rates of transfer and the rarer resulting observable genetic evidence of HGT (Levin and Bergstrom, 2000). There does not appear to be as much permanent gene flow as might be expected from the measured rates of transfer and that transfer is limited by genomic regions of high and low transferability (Kurland et al., 2003; Philippe and Douady, 2003). Regions of high transferability are called the mobilome, containing plasmids, transposons, and phage vectors, and tends to correspond with relatively small functional changes (Levin and Bergstrom, 2000). Individual genes (including antibiotic resistance found on plasmids) in these regions are highly transferable and can spread rapidly in response to selection. The remaining intra-species HGT may therefore primarily assist populations in retaining species integrity through recombination, rather than as a source of new diversity. While HGT is infrequent outside of the mobilome, historical gene transfer events involving these areas tend to correspond with major, functionally important changes (Koonin and Wolf, 2008).

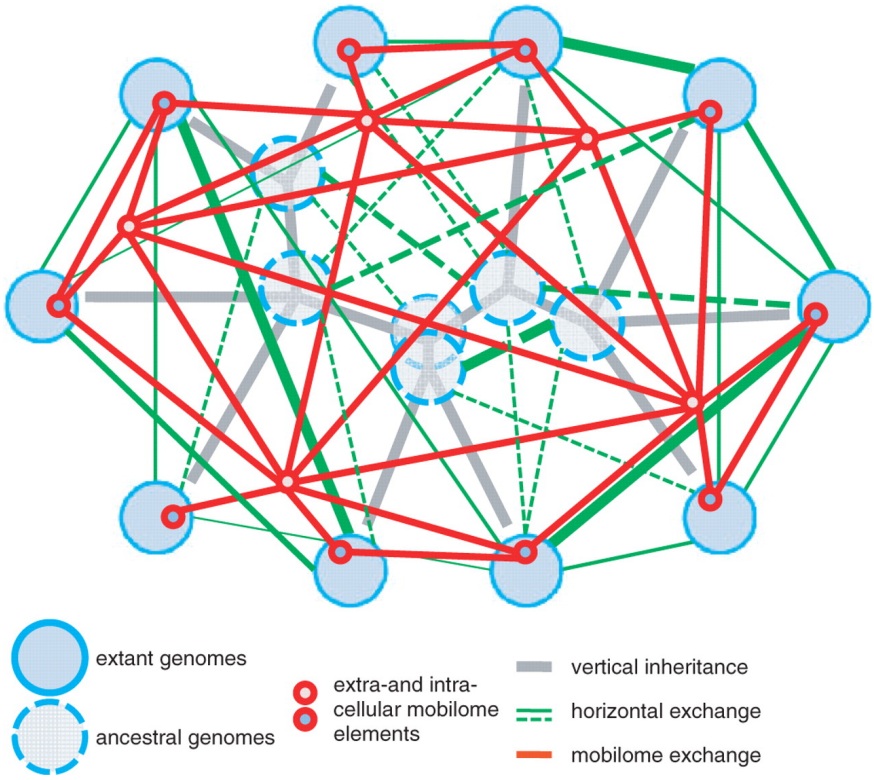

Figure 1.4: 'The dynamic view of the prokaryotic world. The figure is a conceptual schematic representation that is not based on specific data. The larger blue circles denote extant (solid lines) or ancestral (dashed lines) archaeal and bacterial genomes. The small red circles denote mobilome components such as plasmids or phages. Gray lines denote vertical inheritance of genes; green lines denote recent (solid) or ancient (dashed) HGT; red lines denote the permanent ongoing process of the exchange of genetic material between mobilome elements. The thickness of connecting lines reflects the intensity of gene transfer between the respective genetic elements.'[4]

1.3 Plasmids

As established, HGT propagates the spread of antibiotic resistance and pathogenicity genes. In this thesis, I have chosen to focus primarily on conjugative plasmids due to their particular role in the spread of AMR (Baker et al., 2016). Plasmids are extra-chromosomal genetic elements that are predominantly circular, although can be linear (Meinhardt et al., 1997), and vary greatly in size (Slater et al., 2008a). Large plasmids can be larger than 30 kb, tend to be conjugative and contain all the genetic machinery required for transfer (Simonsen, 1991). Some megaplasmids can be as large as 2.09 Mb (Salanoubat et al., 2002).

[4]Reprinted from Nucleic Acids Research, Volume 36, Issue 21, Koonin and Wolf, Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world, 66886719, ©2020, with permission from Oxford University Press.

Small plasmids may only be mobilisable, or non-conjugative (Smillie et al., 2010), with or without mobility regions encoding the machinery required to exploit the conjugation of other plasmids (Simonsen, 1991). Key plasmid characteristics include automonous replication, transferability and dispensibilty (in that the genes they carry are not necessary for the normal functioning of the cell, Slater et al., 2008a).

Plasmids that cannot be stably maintained together in the same bacterial host cell due to similarities in replication or partition genes are often categorised into incompatibility groups (Kittell and Helinski, 1993). While plasmids of the same incompatibility group can vary substantially, which limits the usefulness of the classification system (Novick, 1987), incompatibility groups broadly correlate with sequence similarity and taxonomies have been proposed in line with these categorisations (Fernandez-Lopez et al., 2017). Taxonomies of plasmids based on mobility genes have also been proposed (Garcillán-Barcia et al., 2011) and plasmids may have a relatively stable backbone of these core genes from which phylogenies can also be sensibly constructed (Brown et al., 2013). Plasmids are also frequently described as having broad (Brown et al., 2013) and narrow host ranges (Pukall et al., 1996) based on the bacterial species they can invade (De Gelder et al., 2007).

1.3.1 Plasmid genes and selection

The genes found on plasmids are largely qualitatively different from genes found on bacterial chromosomes (Rankin et al., 2011; Tazzyman and Bonhoeffer, 2015). Chromosomes tend to contain core housekeeping genes that are necessary for the general upkeep of the bacterial cell (genes for structural proteins, metabolic functions, protein-protein interaction genes) and would be vulnerable if plasmid-based due to the potential loss of the plasmid from the cell (Tazzyman and Bonhoeffer, 2014). For this reason, aside from plasmid housekeeping genes (replication, transfer or mobilisation regions and other maintenance genes, Cottell et al., 2014), plasmids tend to only contain accessory genes (Frost et al., 2005). Plasmids are good locations for genes that have transient value depending on the environmental conditions, including antibiotic resistance genes (of which a plasmid may carry multiple resistance genes,

Condit and Levin, 1990; Ramirez et al., 2015), and tolerance to heavy, toxic metals (Norberg et al., 2014) and bacteriocins (Rankin et al., 2011). Other plasmid-based genes include catabolic and virulence genes (Bahl et al., 2009), and genes that enable survival in anaerobic conditions. While phylogenetic methods imply that functionally substantial transfer events are rare, these mentioned genes are part of the mobilome and can spread rapidly following stimuli from selective pressures. Many plasmid genes have public benefits (Dimitriu et al., 2018; Mc Ginty et al., 2013) which create interesting and potentially cooperative population and community dynamics (Dimitriu et al., 2014, 2016).

1.3.2 Plasmid cost

In the absence of selection pressures, plasmids often confer a cost to the growth of the bacterial host (Baltrus, 2013; Kottara et al., 2018; Millan et al., 2015) that can be as high as 40% (De Gelder et al., 2007; Heuer et al., 2007; Subbiah et al., 2011). These costs can be measured by comparing the growth rates of donor and recipient populations and are reported using relative or absolute values, although measurements can sometimes be overestimated due to the presence of plasmid-free cells. Costs are specific to the combination of plasmid and host (Kottara et al., 2018; Yano et al., 2013) and are multi-faceted. Plasmid costs can broadly be divided into costs of plasmid presence and costs of plasmid conjugation (San Millan and MacLean, 2017). Costs of plasmid presence include the costs of replication and equal segregation, which occur within the life-cycle and division of the cell (Tazzyman and Bonhoeffer, 2015), and the costs of plasmid metabolic activity and interference caused by plasmid genes (Diaz Ricci and Hernández, 2000). Conjugative costs are incurred through the production of pili and mating complexes (Ilangovan et al., 2015) and compounded by the costs of increased vulnerability to phages that target pili (Dionisio et al., 2005; Harrison et al., 2015). Plasmid costs can also change depending on the environment, increasing when cells are exposed to U.V. light, for example (Zhang et al., 2019). Unintuitively, the relationship between size and plasmid cost is obscure, where plasmid size is predicted to be linked more to environmental selection pressures, rather than plasmid-host fitness (Ledda

and Ferretti, 2014; Slater et al., 2008a). Plasmids also vary in copy number (Futcher and Cox, 1984): the number of copies of the plasmid within each cell. Cells can carry hundreds of copies of a single plasmid and multiple kinds of plasmids that all contribute to the total plasmid burden. *E. coli* cells carry an average of four plasmids (Simonsen, 1991). Additional costs can be incurred through the dosage effects of sub-optimal plasmid copy numbers, although these effects are not explicitly additive (Morton et al., 2014). Models and knowledge of plasmid replication show that plasmid copy number is often tightly regulated (Paulsson, 2002) and closely tied to the host replication cycle to limit unnecessary costs (Uchiumi et al., 2019). However, a high copy number can be beneficial through the increase in mutation supply rate and the corresponding chances of adaptation that increases fitness (Hülter et al., 2017).

Due to the costs of plasmid presence and activity, some strains of bacteria use restriction enzymes to neutralise foreign DNA, including plasmids and phages, to limit their impact on the host (Gandon and Vale, 2014; Roer et al., 2015). When plasmids are not directly removed, evolution acts on the host and plasmid to minimise the costs of the plasmid and has been demonstrated theoretically and experimentally (Harrison et al., 2016; Loftie-Eaton et al., 2017). Costly genes (including plasmid transfer genes (Dahlberg and Chao, 2003; Haft et al., 2009; Raz and Tannenbaum, 2014; Turner et al., 1998) and genes that interfere with cell functioning (Yano et al., 2016)) are deactivated or optimised, corresponding with an increase in the host growth rate (Hong et al., 2014). In some cases, plasmid cost can be almost completely ameliorated (Fischer et al., 2014), greatly increasing the ability of the plasmid to persist in the absence of selection (Porse et al., 2016) that may increase the prospective host range of the plasmid (De Gelder et al., 2008; Sota et al., 2010). While mutations reducing the costs of plasmid-host interactions can be found on both the host and the plasmid (Stalder et al., 2017), the amelioration of cost is particularly effective when these genes are plasmid-based (Zwanzig et al., 2019). Plasmids with lower costs can be carried to other cells of the same strain, whereas host-based cost-reductions are not transferable. Adaptation to a specific strain (Sherley et al., 2003), however, can increase the plasmid's cost in other species, so that there are pros and cons to both broad and narrow

plasmid host range strategies (Martins, 2013). This is important to consider in the context of complex microbial communities, where plasmids may be able to persist in a few species but not all species in the community (Kottara et al., 2018; Yano et al., 2013). Species in which plasmids can persist may become reservoirs of genes (including antibiotic resistance genes) for all the species in the community through source-sink dynamics (Hall et al., 2016). Exposure to selective agents increases the benefits of plasmid carriage and allows plasmid persistence in species where persistence in non-selective conditions may not be possible, and which can facilitate the evolution of cost reduction (Stevenson et al., 2018).

The long term stability of plasmid-based genes is considered to be somewhat paradoxical (MacLean and SanMillan, 2015). If plasmid genes are consistently beneficial, they should integrate into the host chromosome where the cost is lower (Kottara et al., 2018). If the plasmid is costly in an environment where it confers no benefit the donor cells should be less competitive than recipient cells, and the plasmid will go extinct. Plasmid genes should therefore either integrate into the host chromosome or be lost, and the mechanisms that allow plasmids to persist have not been satisfactorily established (Carroll and Wong, 2018). Transient and variable selection pressures including pulses of selection for plasmid genes (Stevenson et al., 2018), high transfer rates, coadaptation, plasmid hitchhiking, selective sweeps and cross-ecotype transfer (Bergstrom et al., 2000) may all contribute to longer-term plasmid persistence. Alternatively, gene-plasmid combinations may themselves be transient and the majority of plasmid-based genes could be ephemeral. Furthermore, not all plasmids contain beneficial genes, and the persistence of these plasmids is particularly confusing and remains an unanswered question (Brown et al., 2013). These plasmids may either contain unidentified beneficial genes or persist through other mechanisms such as high transfer rates, low cost and low rates of loss (MacLean and SanMillan, 2015; San Millan et al., 2014).

1.3.3 Plasmid transfer

Plasmid transfer occurs when a donor cell containing a plasmid produces conjugative pili that attach to a recipient cell (Cabezón et al., 2015). The plasmid is then nicked and a single strand of the plasmid is transferred to the recipient cell where each single-stranded half of the plasmid synthesises a complementary strand. Although many plasmids carry all the genes necessary for autonomous transfer, other plasmids are non-conjugative and can either be mobilised by conjugative plasmids (Klümper et al., 2014) or are non-transmissible (San Millan et al., 2014). About a quarter of plasmids are each mobilisable and conjugative, while half are non-transmissible (Smillie et al., 2010), although these plasmids may have transfer rates so low that they are below detection limits (Peña-Miller et al., 2015). Some plasmids can also transfer from recipient to donor through the donor pili during conjugation in a process named retrotransfer (Heinemann and Ankenbauer, 1993; Sia et al., 1996; Top et al., 1992).

Lab experiments can be used to estimate transfer rates (Bradley et al., 1980; Wan et al., 2011), isolating specific donor-recipient combinations and plasmids of interest (Dionisio et al., 2002; Gordon, 1992). While a reasonable amount of conjugation rate estimations have been published in the literature, the methods and metrics used to estimate transfer rate are variable and lack consistency that make them difficult to compare (Huisman et al., 2020; O'Keefe et al., 2006). Many experiments report transfer efficiencies and ratios, such as transconjugants per donor (TD^{-1}, Piper and Farrand, 1999), rather than measuring the transfer rate per se (Simonsen et al., 1990; Zhong et al., 2012). These measurements are particularly susceptible to differences caused by experimental conditions, such as initial density and donor to recipient ratios (Simonsen et al., 1990). Others report transfer rates using model-based metrics (e.g. endpoints, Freter et al., 1983; Simonsen et al., 1990) that are expected to be more reliable and comparable across studies, although are still (to a lesser extent) susceptible to some of the same problems (Huisman et al., 2020). These methods normally rely on drop-plating and thereby frequently give results that lack in precision and reliability (Bradley et al., 1980). These issues make it more difficult to estimate the "true"

rate of transfer (Simonsen et al., 1990; Zhong et al., 2012).

In contrast to lab experiments, field experiments (e.g. conducted in soil (Hill and M. Top, 1998; Neilson et al., 1994), agricultural and other waste (Baker et al., 2016; Gealt et al., 1985), hospital environments (Hardiman et al., 2016) or the animal gut (Fischer et al., 2019; Rang et al., 1996)) give more realistic and applicable results (Kruse and Sørum, 1994) but are difficult to perform in controlled ways due to the complexity and variability of microbial communities (Bellanger et al., 2014a). Plasmid transfer can also be difficult to detect in some of these environments (Freter et al., 1983; Simonsen, 1991). While this is ultimately the direction in which we need to move, most of the currently available data from field studies in the literature consist primarily of plasmid prevalence in different locations (Biao et al., 2015).

Plasmid transfer is the express focus of two of the chapters of this thesis. These chapters focus respectively on the factors that determine the variability in transfer rate and the selection pressures that shape the evolution and control of transfer rate. These areas are given more specific and comprehensive attention in the remainder of this section.

Environmental and biotic variability in transfer rate

Observed transfer rates can be highly variable within measurement methods and metrics. Transfer efficiencies (TD^{-1}, transconjugants per recipient (TR^{-1}), or transconjugants per donor per hour ($TD^{-1}h^{-1}$)) tend to vary within the region of 10^{-5} to 1 (Bradley and Whelan, 1985; Lampkowska et al., 2008; Piper and Farrand, 1999), while endpoint estimates tend to be from 10^{-16} to 10^{-8} ml cell^{-1} h^{-1} (Freter et al., 1983; Hall et al., 2015; Lilley and Bailey, 2002) although cases of unobserved transfer may have simply been below the experimental limits of detection (Peña-Miller et al., 2015). Often rates of transfer are described in the literature as “high” (Futcher et al., 1988; Neilson et al., 1994; Smets et al., 1993) or “low” (Christensen et al., 1998; Delavat et al., 2016; Gordon, 1992) without an explicit frame of reference for what those terms mean. Furthermore, the specific rate of transfer does not provide enough information to assess plasmid persistence and must be considered with other

plasmid dynamic parameters and the environmental context and community to establish this (Simonsen, 1991). Rates of plasmid mobilisation (Bradley, 1985) and retrotransfer can also be measured, where rates of retrotransfer can be higher than rates of plasmid mobilisation (Beaudoin et al., 1998a), although this is likely to vary.

Transfer rates are also highly specific to plasmid, donor (Hardiman et al., 2016) and recipient combinations (Sansonetti et al., 1980) and vary according to the biotic and environmental conditions (Gordon, 1992). The response of transfer rate to some abiotic factors is sometimes summarised using the framework of the plasmid-host stress response (Atsmon-Raz et al., 2015; Beaber et al., 2004), where the transfer rate increases to facilitate adaptation to a stressful environment, although this view is often too simplistic. For example, the response of plasmid transfer rate to increasing media concentrations is variable (Simonsen, 1991). Plasmid transfer rate can increase as cells become starved of nutrition (Freter et al., 1983; Smith, 1977), potentially enabling them to gain additional metabolic capabilities, facilitating survival in new environments or conditions (Bahl et al., 2009). However, transfer also requires a base-level of substrate concentration to occur and as the substrate concentration decreases the rate of transfer can also correspondingly decrease (MacDonald et al., 1992; Smets et al., 1995; Zahrl et al., 2006). Other stressors, such as high temperature (Simonsen et al., 1990) or antibiotic exposure (Atsmon-Raz et al., 2015; Liu et al., 2019) are thought to increase transfer rate, although these decrease the transfer rate as the stressor burden on the host becomes too intense (Bradley and Whelan, 1985; Fernandez-Astorga et al., 1992; Forns et al., 2005). Also, the increase of transfer rates in the presence of antibiotics has been questioned, where changes in plasmid prevalence are driven by selection itself, rather than through a change in transfer rate (Lopatkin et al., 2016). pH is also anticipated to affect plasmid transfer rates (Stalder and Top, 2016).

The physical environment type can also affect the rate of transfer (Molin and Tolker-Nielsen, 2003), is again plasmid-host specific, and has a particular interaction with pilus-type. Pili are frequently described as thick or thin (Bradley, 1984), flexible or rigid (Simonsen, 1991), and these differences are likely to be adaptations to specific environments. For example, transfer rates of plasmids that form rigid pili are far lower in liquid media than on solid

surfaces or as part of a matrix (Bradley and Williams, 1982), while flexible pili enable plasmids to transfer at a higher rate in liquid media (Bradley, 1983, some plasmids can produce both kinds (Bradley, 1985)). Expression of pilus genes and the number of pili are broadly correlated to the rate of transfer, where transfer rate is higher when pili are produced constitutively (Bradley, 1984). The surface type can also affect the mobility of bacterial cells and the subsequent interactions they have (Fox et al., 2008; Krone et al., 2007; Simonsen, 1990; Zhong et al., 2012), and are particularly relevant in environments that feature a combination of surface types, such as soil (Slater et al., 2008a; Smiles, 1988). The majority of conjugation models assume homogeneity of cell mixing when, in reality, natural cell cultures are heterogeneous due to the close proximity of isogenic cells following cell division. This particularly occurs on solids, but also occurs in liquids when cells aggregate and form biofilms (Costerton et al., 1987; Simonsen, 1991). While close contact of cells increases the probability of the occurrence of transfer events, the opportunities for donor contact with new recipients decreases when the cells have reduced motility (as in a biofilm or on a solid surface, Christensen et al., 1998). These factors affect plasmid transfer rates, change through time, and must be accounted for in transfer rate estimations. Some models explore these issues using individual-based modelling and examine the estimation of transfer rates on surfaces (del Campo et al., 2012; Krone et al., 2007), finding fundamental differences in the way we need to estimate rates of plasmid transfer in liquids and on solid surfaces (Fox et al., 2008).

Cell density and the ratio of donors to recipients can affect plasmid transfer rate, although these effects are not always clear (Simonsen et al., 1990). High cell density increases the likelihood of cell interactions including transfer events that can be facilitated by biofilms and other structures (Dimitriu et al., 2016), although many of the observed differences can be explained quantitatively, rather than biologically (Freter et al., 1983). Environments with high substrate concentrations (rhizosphere, phyllosphere (Remus-Emsermann et al., 2018), decaying matter, agricultural waste) facilitate high cell density and can become hotspots for transfer (van Elsas and Bailey, 2002). There is substantial potential for HGT in wastewater and wastewater treatment facilities (Jacquiod et al., 2017). High recipient density can

increase the impact of per donor transfer events (Turner et al., 1998), while high donor density can limit donor exposure to recipient cells required for transfer. Transfer rates are thus expected change in response to donor and recipient prevalences (Lipsitch et al., 1996). While cell structures can encourage individual transfer events, plasmids can spread further when migration allows cells to travel large distances (Christensen et al., 1998; Hooper et al., 2008; Niehus et al., 2015). General migration into a community with high plasmid prevalence can also increase the presence of recipients, affecting the selection pressures on transfer rates and the absolute number of transfer events that can occur (Turner et al., 1998). The migration of cells must be considered when tackling the spread of plasmids and antibiotic resistance genes. The phase of cell growth can also affect the rate of plasmid transfer where transfer rate is thought to increase during exponential phase and decrease during stationary phase (Freter et al., 1983; Seoane et al., 2011; Smets et al., 1993). Finally, the specific growth rate of some bacterial strains may influence the conjugation rate (Smets et al., 1995) but this effect is not seen consistently (Beaudoin et al., 1998a; Simonsen et al., 1990). It can be difficult to tease apart the impacts of growth rate, growth phase and substrate concentration. For example, although cells in a biofilm have close proximity, transfer rates can be lower due to the low growth of cells in a biofilm (Merkey et al., 2011). By clarifying the effects of each we can gain a more fundamental understanding of the factors that affect plasmid transfer rate.

Some studies have explored the synthesised effects of coresident plasmids on individual transfer rates, as many cells are known to carry multiple plasmids (Gama et al., 2017a, 2018). Coresidence allows shared utilisation of both plasmids' conjugative machinery, and tends to increase the rate of transfer of the plasmid with the lower transfer rate to a level comparable to the higher transfer rate plasmid. Through coresidency, plasmids can also gain access to pili that affect the rates of transfer according to surface type (Bradley, 1983). In other plasmid combinations, coresidency can reduce transfer rates through repression systems that impact both plasmids (Gama et al., 2017b). Plasmids cannot, therefore, be considered in isolation when applying these principles to natural environments. Bacteria exist in complex communities that must also be considered when attempting to understand

plasmid prevalence, persistence and transmission (Christensen et al., 1998; Martins, 2013). The dynamics previously established are unique for each species, and their interactions will have eco-evolutionary effects on the populations and plasmids (Barraclough, 2015; Scheuerl et al., 2020). When plasmids can persist in a single species in a community the rest of that community may gain access to that genetic material (Hall et al., 2016). Sharing of genes may also facilitate cooperative interactions through public goods genes (Mc Ginty et al., 2011). Alternatively, cell predation to ingest foreign DNA can encourage conflict (e.g. in *Acinetobacter*, Cooper et al., 2017). Our theoretical and experimental understanding of community plasmid dynamics is weak and requires increased methodological sophistication.

While estimations of transfer rate can be accurate in the lab contexts in which they are measured, they may have little practical application when attempting to apply them more broadly to natural environments (Aminov, 2011; Freter et al., 1983; Mach and Grimes, 1982; O'Keefe et al., 2006). Transfer rates measured in the lab often vary from those estimated in natural environments (Fischer et al., 2014; Hardiman et al., 2016; Neilson et al., 1994). Furthermore, all the parameters are subject to adaptive and evolutionary pressure at multiple levels. While various factors have been shown to affect transfer rates in different ways and under different circumstances, the relative contributions of these factors in the determination of transfer rate has not been sufficiently explored or explained (Dimitriu et al., 2019a). It is therefore difficult to make broad assertions on the parameter space of transfer rates and other plasmid dynamic parameters and on the implications of observed transfer rates (Ashbolt et al., 2013; Baker et al., 2016).

Evolution and control of plasmid transfer rate

Due to the costs of plasmids on cell growth, and particularly the costs of plasmid transfer (pilus production, vulnerability to phages, Dionisio et al., 2005), it is common for plasmids and hosts to evolve mechanisms that control the rate of plasmid transfer (Raz and Tannenbaum, 2014). While it has been suggested that cost reduction is a better strategy for persistence than a high transfer rate due to the high costs of plasmid transfer (Dim-

itriu et al., 2016; Duncan et al., 1995; Hall et al., 2017), the control of plasmid transfer directly affects plasmid persistence and dynamics, and it is essential that we develop our understanding of the processes that affect it. Some plasmid transfer is managed through genetic switches (Refardt and Rainey, 2010) that use quorum sensing mechanisms such as biomolecules (Kozlowicz et al., 2006; McAnulla et al., 2007). The default setting for transfer tends to be "off" and only turns "on" when conditions for transfer success are favourable, such as high recipient density (Singh and Meijer, 2014). In other cases, only a subset of donors become competent for transfer, reducing the total population burden of plasmid transfer (Koraimann and Wagner, 2014). Plasmid transfer is often reported to be higher within bacterial strains and species than between species (Dionisio et al., 2002; Gordon, 1992), indicating the presence of donor discrimination (Dimitriu et al., 2019a; Lilley and Bailey, 2002). Although plasmid transfer is costly, the cells can receive indirect fitness benefits when the plasmid is beneficial or when HGT facilitates genetic recombination (Dimitriu et al., 2016). Recipient cells can also play a role in controlling plasmid transfer by initiating plasmid transfer through biomolecules (Chatterjee et al., 2013; Pérez-Mendoza and de la Cruz, 2009), although this kind of mechanism would only evolve with substantial host-plasmid relationships.

Some of the models investigating plasmid transfer rate optimisation demonstrate the simultaneous persistence of two incompatible plasmids in a single host population under some parameter ranges (Lipsitch et al., 1996; Turner et al., 2014). These plasmids must have different transmission strategies for co-persistence to occur: high transfer, high virulence, and low transfer, low virulence. It is possible that these two plasmids could be of the same "strain", cooperating to reduce the total burden on the host population whilst providing the benefits the plasmid confers (Van Den Bosch et al., 2010). A single plasmid may, therefore, have two specialised variants that maximise transfer while minimising cost. Other plasmids are described as having repressed and derepressed rates of transfer (often correlated with the expression of constitutive pili, Bradley, 1984), where a plasmid has genes that repress its own transfer (Lundquist and Levin, 1986; Raz and Tannenbaum, 2014). Transitory derepression occurs following the transfer of a plasmid into a new cell, and allows an increase of

the transfer rate by several orders of magnitude that lasts until the plasmid transfer genes become repressed (Freter et al., 1983; Lundquist and Levin, 1986; Simonsen et al., 1990). Other plasmids repression/derepression systems may be triggered by environmental signals (Christensen et al., 1998). Again, this allows plasmids to be highly transmissible and maintain a high prevalence while minimising the costs on the host population incurred through high transfer rates, including vulnerability to phages (Simonsen, 1991).

The cell growth rate is also believed to impact evolutionary pressures on the rate of plasmid transfer and is classically studied in parasite-host dynamics (Kaltz and Koella, 2003) that illustrate the conflict between vertical and horizontal transmission (Lipsitch et al., 1995, 1996). A high growth rate means that plasmids have more to gain from vertical transmission (through cell division), while plasmids in cells with a low growth rate have more to gain from horizontal transfer. Plasmids are expected to adapt to these pressures (Turner et al., 1998), further compounded by growth phase (Seoane et al., 2011), substrate concentration (Zahrl et al., 2006), cell density (Al-Masaudi et al., 1991) and donor to recipient ratios. The control mechanisms that host cells maintain are not necessarily aligned with the interests of the plasmids and can create host-plasmid conflict (Werren, 2011). Plasmid-host interactions can range from parasitic (Eberhard, 1990) to commensal to mutualistic (Shapiro and Turner, 2014) depending on the plasmid genes and the environment. An outstanding question is: to what extent do plasmids and hosts control and determine the rate of transfer?

1.3.4 Plasmid loss

Plasmid loss occurs during host cell division when there is unequal segregation of plasmids into daughter cells (Bahl et al., 2009), and plasmids frequently carry genes that reduce the rates of their loss through several mechanisms. The first is through segregative partition systems (par genes) that facilitate equal segregation of plasmid copies into each daughter cell during cell division (Ebersbach and Gerdes, 2005; Salje et al., 2010). The second is through post segregational killing (PSK) systems/addiction complexes (Cooper and Heinemann, 2000; Gerdes et al., 1986). These plasmids create toxins and anti-toxins that kill

the cell if the plasmid is lost, and are an example of the conflict between plasmid and host where the plasmid behaves spitefully rather than in the interests of the host (Mongold, 1992). PSK systems are also believed to be an adaptation to inter-plasmid competition (Rankin et al., 2012). To improve segregation, multimer resolution systems separate plasmids that have become joined together (Krause and Guiney, 1991). Finally, plasmids with a high copy number have a greater chance of being in both daughter cells (Paulsson and Ehrenberg, 2000), regardless of the presence of partition systems (Jahn et al., 2016). These mechanisms are particularly valuable to non-conjugative plasmids in non-selective conditions where reduction of loss is the primary persistence strategy.

While mechanisms exist to limit plasmid loss, unequal segregation may occur that results in a plasmid-free recipient bacterial cell (Friehs, 2004; Tal and Paulsson, 2012). Plasmid loss can also be induced in certain strains by chemicals such as acridine orange (Bahl et al., 2009; Riva et al., 1973). Due to the aforementioned costs of plasmid carriage under non-selective conditions, this frequently leads to growth rate differences between donors and recipients, where donors are selected against and recipients are favoured until they reach dominance in the population (Carroll and Wong, 2018). Plasmid loss can be difficult to detect and measure due to the need to identify plasmid absence rather than presence, the difficulty of distinguishing plasmid loss from recipient cell growth (Boe, 1996) and the re-transfer of plasmids into recipient cells. Measured rates of plasmid loss are often believed to be both over and underestimated (Bahl et al., 2009; Tolker-Nielsen and Boe, 1994) and while technical methods attempting to identify individual incidences of plasmid loss have been developed to address this, they are manually intensive (Lau et al., 2013).

1.3.5 Plasmid persistence

To understand how plasmids mediate and spread antibiotic resistance genes, we must first understand how plasmids are maintained in natural environments and the dynamics that affect them (Martínez and Baquero, 2014). Some studies use genomic techniques to identify plasmid prevalence in natural environments (Fan et al., 2019; Lilley and Bailey, 1997;

Remus-Emsermann et al., 2018; Silvester et al., 2015). These results are limited by the complexity of microbial communities, the detection limits for low density plasmids and by providing only a snapshot of plasmid presence without inferring the mechanisms that allow plasmids persistence. Plasmid dynamics can be tracked much more easily in lab experiments (Kottara et al., 2018; Lilley and Bailey, 2002; Lopatkin et al., 2017), although these results can become too abstracted from natural environments to provide enough realism for application (Stevenson et al., 2018). Some techniques use fluorescence to observe plasmid dynamics in real-time, (Andersen et al., 1998; Christensen et al., 1996; Dahlberg et al., 1998), although the scale at which this can be measured is limited, and the question remains of how representative the resulting data is. Finally, plasmid dynamics can be assessed using theoretical, mathematical and computational modelling techniques (Leclerc et al., 2019; Smets and Lardon, 2009). These enable the isolation and exploration of specific effects and interactions and can give some idea of what happens in non-observable sections of time. Then, these results can be compared with real experimental data to validate or invalidate the findings (Levin et al., 1979; Popov et al., 2011; Simonsen et al., 1990). A lot of progress has been made over the years by pulling these methods together where each field complements and enhances the findings of the others. However, there are still areas that require more investigation, particularly as the level of complexity is increased.

1.3.6 Plasmid modelling

Plasmid dynamics have been modelled since the 1970s (Anderson and Lustbader, 1975; Levin and Stewart, 1977). Stewart and Levin (1977) designed and analysed a foundational conjugative plasmid model in one bacterial species, on which many subsequent models have been based. Their models used differential equations for donor and recipient host populations and substrate in continuous flow and serial transfer environments to investigate the conditions that enabled plasmid persistence. They included the key processes that affect plasmid dynamics: plasmid transfer (using mass-action kinetics), plasmid loss, plasmid cost, cell growth through substrate consumption and dilution (in the continuous flow model).

The primary finding was that costly plasmids are able to overcome selective pressures and plasmid loss, persisting when transfer rates are high enough, with a broad range of conditions where this is possible. They described a persistence equation based on these results:

$$\gamma N > \alpha D + \tau \tag{1.1}$$

where the plasmid can persist when the rate of transfer (γ) factored by the population density (N) exceeds the cost of the plasmid (α) factored with the dilution rate (D) and the rate of plasmid loss (τ). These models were later qualitatively validated experimentally (Levin et al., 1979) and have been used to describe plasmid dynamics (e.g. Smets et al., 1993, 1994).

While the Stewart and Levin (1977) model included the basic factors that determine plasmid dynamics, there are other aspects of plasmid biology that can have an effect, many of which have since been explored. Levin and Stewart (1980) looked at the mobilisation of non-conjugative plasmids via conjugative plasmids by adding equations for hosts containing a mobilisable plasmid and both types of plasmid. They found that conditions for the persistence of mobilisable plasmids are more stringent, in comparison to conjugative plasmids, later providing experimental validation (Levin and Rice, 1980). Lundquist and Levin (1986) used another extension of the model to identify experimentally observed transitory derepression in the transconjugants of some plasmids. Condit et al. (1988) applied the model to gene transposition between plasmid and chromosome, showing how genes can spread from one host chromosome to another strain through excision and plasmid conjugation, finding that transposons are unlikely to exist solely as parasites. Condit and Levin (1990) looked at gene transposition between plasmids, finding that genes are likely to migrate to the same plasmid in order to reduce the total plasmid cost. Their complementary experimental work, however, showed a variety of results, including the migration of an antibiotic resistance gene to the chromosome and the persistence of a plasmid containing the other resistance gene.

Simonsen et al. (1990) used the Stewart and Levin (1977) model to provide a relatively

simple calculation and method for estimating the rate of plasmid transfer in well-mixed liquid culture. This model was adapted for batch cultures and a Monod function was added to the transfer rate the reflect the reduction of transfer in the absence of cell growth (Levin et al., 1979). The method overcomes some of the problems of metrics previously used to communicate transfer rates (i.e. transfer efficiencies, ratios) by being less susceptible to differences in initial cell density, donor to recipient ratios and experiment time (Toda et al., 1990). Simonsen et al. (1990) were able to demonstrate the robustness of the method theoretically and experimentally. Many studies have since used these methods to estimate plasmid transfer rates (Hall et al., 2015; Licht et al., 1999; Lilley and Bailey, 2002) and have built on these methods (Huisman et al., 2020; Zhong et al., 2010, 2012). Simonsen (1991) went on to review some of the previous plasmid modelling in a (then) current biologically meaningful context and then expanded the Levin and Stewart (1980) model to investigate donor superinfection. They found that, based on the observed rates of transfer, plasmids are unlikely to be able to persist solely as parasitic elements, citing the roles of "unstable selection pressures, migration, periodic selection or changes in population structure" in plasmid persistence. The review also identified an absence of modelling outside of liquid environments and advocated for the expansion of experimental data using plasmids from more diverse incompatability groups. Much later, Bergstrom et al. (2000) extended the Stewart and Levin (1977) model to show that plasmids should integrate into the host chromosome (where they are less costly) when beneficial, and should be selected against and lost when the plasmid is costly: the plasmid paradox. They suggested that plasmids may persist due to transfer to new hosts (later modelled by Niehus et al., 2015), variable selection pressures (later modelled by Willms et al., 2006) and a reduction of plasmid cost through evolution. The unlikeliness of plasmid persistence through transfer has since been questioned by Lili et al. (2007) who found a broader range of conditions where plasmids can persist through transfer alone.

Various other plasmid processes have also been modelled to investigate their impacts on plasmid dynamics. Sýkora et al. (1989) modelled the effects of plasmid-loss inducing (curing) drugs with experimental application using acridine orange. Some models investigated

the regulation of plasmid copy number and the role the regulation in plasmid maintenance (Ataai and Shuler, 1986; Ehrenberg, 1996; Paulsson, 2002; Paulsson and Ehrenberg, 1998, 2000; Paulsson et al., 1998). Others looked at post-segregational killing/toxin-antitoxin systems as mechanisms to reduce plasmid loss (Hsu and Waltman, 1997; Lauffenburger, 1985; Mongold, 1992; Rankin et al., 2012; Sardonini and DiBiasio, 1987). Macken et al. (1994) modelled mortality as a function of population size, finding multiple stable states and results that indicate the need for sufficient plasmid density to enable establishment. Top et al. (1992) used experimental data to show that retrotransfer can be modelled as a one-step process. MacDonald et al. (1992) applied models estimating conjugation parameters, showing increased transfer rates with increased substrate concentration. Novozhilov et al. (2005) investigated the effects of mutational inactivation in a stochastic HGT model. Ponciano et al. (2007) further emphasised the importance of stochasticity to explain outcomes when modelling plasmid dynamics, using a hidden Markov approach, and identified key differences in the experimental outcomes based on host differences. Massoudieh et al. (2007) demonstrated the need for a transfer lag function due to donor recovery time following transfer and the time taken for transconjugant cells to become transfer competent. Other papers have looked at the impacts of phages on plasmid existence conditions in a chemostat using stochastic simulations (Dionisio, 2005), applying models to quantitative PCR data (Wan and Goddard, 2012) and by combining experimental data with individual-based modelling (Harrison et al., 2015). Tazzyman and Bonhoeffer (2014) showed that plasmid mutations can be limited in transmission when the population has high prevalence of incompatible plasmids and, later, that essential genes can be maintained more stably on the chromosome (Tazzyman and Bonhoeffer, 2015). Ledda and Ferretti (2014) modelled costs of a plasmid based on their sequence length. Niehus et al. (2015) showed through modelling that immigration of new cells can facilitate horizontal transmission above vertical transmission. Gama et al. (2017a) modelled the probability of co-transfer of co-resident plasmids, finding that derepression of one plasmid impacts other resident plasmids. Some models examined plasmid competition in chemostats but did not include plasmid transfer (De Gelder et al., 2004; Ganusov and Brilkov, 2002; Hsu et al., 1995, 1994; Luo and Hsu, 1995; Stephanopoulos and

Lapudis, 1988).

A subset of plasmid models use greater complexity of mathematical analyses. Some of these studies look at the effect of plasmid presence on competition within the population under different transmission constructions (Tremblay and Rose, 1985) and the regions of donor and recipient coexistence using bifurcation analyses (Parulekar et al., 1987). Other papers conduct global analyses (Hsu et al., 1994; Luo and Hsu, 1995) or spectral analyses Stadler (2019), and their content can be difficult for non-mathematicians to grasp (Champagnat et al., 2019). These studies require greater mathematical knowledge but had similar findings to contemporary papers, and highlight the importance of effective communication to open the results to a wider, less mathematically savvy audience.

A number of papers focused on model application to specific environments. Freter et al. (1983) modelled plasmid dynamics using ODEs with complementary experimentation in gnotobiotic mice and in a mouse microfloral continuous flow system. They used the model to estimate parameters, including repressed and derepressed rates of plasmid transfer. They then used these results to show that plasmid transfer was not inhibited in the gut and that transfer rate could be affected by the other plasmids contained in the donor and recipient strains. Seo and Bailey (1985) and Srienc et al. (1986) applied conjugation ODEs to a bioreactor with mathematical analysis, first looking at the the effects of copy number and plasmid loss on product synthesis, then including the effects of plasmid selection with a similar model. Models have been developed and applied to phyllospheres and rhizopheres (Knudsen et al., 1988), soil microcosms (Clewlow et al., 1990), seed and root surfaces (Sudarshana and Knudsen, 2006), rivers (Hellweger et al., 2011), broilers (Fischer et al., 2014) and agricultural waste (Baker et al., 2016), adequately predicting many of the complementary experimental results. Others have explored the effects of antibiotic treatments on plasmid dynamics (Garber, 1987; Svara and Rankin, 2011; Webb et al., 2005; Willms et al., 2006). Massoudieh et al. (2010) modelled plasmids in granular porous media, including bacterial transport through the media, surface attachment processes and lags in transfer rate (Massoudieh et al., 2007).

Licht et al. (1999) demonstrated that chemostat models cannot appropriately be applied to some environments, such as the animal intestine, due to non-homogeneous cell mixing and the formation of biofilms. Following initial work by Lagido et al. (2003) and Beaudoin et al. (1998b), Krone et al. (2007) considered spatial structure using an individual based lattice model with corresponding data to validate their findings. They demonstrated that plasmid dynamics on surfaces and biofilms require specific modelling different from the Stewart and Levin (1977) model. Fox et al. (2008) then extended the model and showed that spatial structure can facilitate plasmid persistence where plasmids would not be able to invade in a well-mixed liquid culture using theoretical and experimental methods. Individual based modelling has been applied to plasmid incompatibility in selection (Gregory et al., 2008), TOL plasmid systems showing transitory derepression of transconjugants (Seoane et al., 2011) and theoretically to explain the lack of transfer in biofilms due to a dependence of transfer on active cell growth (Merkey et al., 2011). Connelly et al. (2011) built on the work by Krone et al. (2007), increasing interactions and modelling the evolution of transfer based on plasmid transfer costs. They found that transfer rates increased as the plasmid benefit increased, but did not include vertical transmission of the plasmid or use biologically meaningful rates of plasmid transfer.

The relationship between plasmid transfer rate and cost is key to explaining plasmid dynamics. Van der Hoeven (1984, 1986) found that two incompatible plasmids can coexistence in chemostat and feast-famine models when one plasmid has a high transfer rate and a high cost and the other has a low transfer rate and low cost. While focusing on parasites, Lipsitch et al. (1995, 1996) modelled the effects of selection on vertical and horizontal parasite transmission - with applications for plasmids (which are parasitic in non-selective conditions). They used density-dependent ODEs similar to Stewart and Levin (1977), finding that high plasmid prevalence reduces the opportunity for horizontal transmission and that low-transferring plasmids can protect against more virulent high-transferring ones, again, leading to coexistence of multiple incompatible plasmids in the absence of donor superinfection. Turner et al. (1998) modelled the effects of recipient density on transfer rates where a trade-off was assumed between transfer and cost, finding increased rates of transfer

when recipients were maintained at high levels. Their complementary experiments, while confirming the trade-off between vertical and horizontal plasmid transmission, did not validate the impact of recipient density on transfer rate over the course of the experiment. Haft et al. (2009) showed theoretically (using the Levin et al. (1979) model adapted to serial-transfer experiments) and experimentally that competition between cells can select for plasmid transfer rate repression/fertility inhibition in order to decrease plasmid costs. They brought together and estimated parameters for both repressed and derepressed plasmid mutants. Although not applied primarily to plasmids, Ferdy (2009) used adaptive dynamics on a spatially structured metapopulation model finding that spatial structure increased parasite virulence when vertically transmitted. Raz and Tannenbaum (2010, 2014) showed how conjugation reduces the mean fitness of a population and Misevic et al. (2013) used *Aevol*, a digital evolution system applied to plasmid evolution, to show that donor and recipient ability/control of transfer is typically plasmid-based, based on the assumptions of that model.

Over the last several years the evolution of plasmid cost has been investigated through modelling and experimentation, the results of which consistently show that plasmid cost should be ameliorated. San Millan et al. (2014) and Porse et al. (2016) showed that non-transmissible and low transfer rate plasmids can be stabilised by adaptation through evolutionary modelling and experimental evolution (although they suggest that "non-transmissible" plasmids may maintain a low transfer rate to persist (Peña-Miller et al., 2015)). Loftie-Eaton et al. (2016) applied a model based on De Gelder et al. (2004) and Ponciano et al. (2007) to experimental data, showing that plasmid cost reduction can allow plasmids to increase their host range. While Loftie-Eaton et al. (2017) found that host-based adaptations can increase plasmid stability, Zwanzig et al. (2019) showed that plasmid-based mutations can be more effective at promoting plasmid persistance. Hall et al. (2017) used individual-based modelling to investigate the interaction between plasmid cost amelioration and transfer as strategies to improve plasmid persistence, finding that cost amelioration is a more reliable strategy due to the inherent costs of transfer, although both strategies may be present.

Models have also been used to explore the interactions between relatedness on plasmid

dynamics within populations and communities. The presence and transfer of plasmids are believed to be correlated with cooperative behaviours caused by the increased relatedness due to gene sharing (Nogueira et al., 2009). Smith (2001) modelled the invasion of cheaters in public-good producing plasmids, demonstrating that donors can punish cheaters through high rates of retransfer into cheater cells. Mc Ginty et al. (2011) showed that this strategy is susceptible to individual-goods (in this context, cheater) plasmids fulfilling the same function can invade these populations, but later showed how relatedness due to plasmid mobility can stabilise public goods genes (Mc Ginty et al., 2013). Dimitriu et al. (2014) showed how plasmid transfer can promote cooperation through increasing relatedness, particularly in structured environments that facilitate assortment of alleles, and that relatedness (via structural assortment or donor discrimination) can allow donor cells to overcome the costs of transfer through indirect fitness benefits (Dimitriu et al., 2016; Shapiro and Turner, 2014). The interaction between plasmids with public goods genes, relatedness and cooperation also impact selection pressures on transfer rates, where transfer is increased in systems of closely related bacteria (Dimitriu et al., 2018, 2019a).

Other models of community effects demonstrate source-sink dynamics (Hall et al., 2016) where a plasmid may persist in a single species, granting access of the plasmid genes to other species through plasmid transfer. After exploring dynamics in a single population using a stochastic model, Landis et al. (2000) used a model analagous to meta-population models to look at plasmid dynamics in multiple (up to three) populations, where simulations demonstrated how plasmids can infect multiple species. Miki et al. (2007) expanded the Stewart and Levin (1977) model to look at long-term batch-culture plasmid dynamics when a donor population is introduced to an indigenous population. While the introduced population went extinct from the system the plasmid was able to persist in the resident population due to a high rate of intrageneric transfer. Martins (2013) looked at the effects of broad and narrow host range plasmids in a two species community, finding that the plasmids can enhance or reduce the persistence of one another, depending on plasmid compatibility, costs and transfer rates. Grover and Wang (2019) were able to identify conditions of coexistence between two species in the presence of non-conjugative, toxin-producing plasmids.

Leclerc et al. (2019) recently conducted a systematic review identifying 43 papers modelling horizontal gene transfer. They found that the majority were models of conjugation focusing only on plasmids with a single gene in *E. coli* in culture, leaving gaps in the literature for transformation, transduction and noted a need for clearer public health implications from modelling.

1.3.7 Competition and cooperation in microbial communities

Bacterial communities are extremely complex and their interactions with plasmids can affect plasmid dynamics, community structure and inter-strain community interactions (Gorter et al., 2020; Hall et al., 2020). Community interactions can be competitive, commensal or cooperative (Freilich et al., 2011). Competition can occur when species consume a shared resource, and where strains do not need to be very different in order to be competitors (Bauer et al., 2018). The theory states that competitive exclusion occurs when two species use the same resource (e.g. space, light, nutrition, Freilich et al., 2011). Coexistence of multiple species in a community can be enabled through species divergence that reduces the overlap of resource sharing. More direct forms of competition (e.g. interference) in microbes can occur through the production of bacteriocins or antibiotics that can be directly inserted into competitors (Bauer et al., 2018). Interference can also occur when bacteria change the environmental conditions to favour themselves or limit the other (Coyte and Rakoff-Nahoum, 2019), by producing chemicals that are toxic, that change the pH or that inhibit quorum sensing of their competitor (Bauer et al., 2018). Predation (e.g. by predatory bacteria) and parasitism (by phages) are other form of direct competition (Coyte and Rakoff-Nahoum, 2019).

Plasmids can impact these kinds of competitive interactions by incurring benefits or costs (depending on the environment) that can give their hosts competitive advantages or disadvantages compared with their competitors (Carroll and Wong, 2018). In non-selective environments, plasmids tend to confer a cost and, if they are stably maintained at a high prevalence, can substantially affect the overall growth and competitive ability of the popu-

lation within the community (Joshi et al., 2009). Stable plasmids may transfer throughout the community, where they experience unique host-plasmid relationships and play a role in determining the growth rates of competing species (Hall et al., 2015). Even when plasmids are not stably maintained in a host, they may still have presence there due to source-sink dynamics (Hall et al., 2016). Hosts can have various combinations of multiple plasmids from different incompatibility groups that can cumulatively affect their growth patterns (Harrison et al., 2012). The distribution and prevalence of plasmids within individual populations therefore can affect community structure and subsequent functioning in non-selective conditions.

Transfer into multiple species gives a plasmid less dependence on its relationship with a single host species (Bergstrom et al., 2000), and by invading multiple ecotypes it can increase its survival in the community. Community interactions can then affect plasmid dynamics and evolution by minimising plasmid costs to host growth (Porse et al., 2016; San Millan et al., 2014), including reductions in transfer rate (Haft et al., 2009). Plasmids show variable dynamics between host species, and even between strains of the same species (De Gelder et al., 2007). Host-plasmid dynamics are variable through time and evolution can expand host range (Heuer et al., 2007) although plasmids may not be able to adequately reduce costs in all hosts simultaneously (Martins, 2013).

Plasmid persistence with low costs to a host may provide opportunity for spiteful plasmid transfer into less well-adapted hosts where a higher plasmid cost is incurred (Dimitriu et al., 2016). However, if plasmid transfer is strongly related to cost this may disadvantage the host organism more than the target. Plasmids sometimes show transitory derepression (Lundquist and Levin, 1986), and an invading host-plasmid strain could hypothetically transfer a plasmid into a new population where it proliferates incurring a high cost to the resident population while enabling the incoming strain enough of a competitive advantage to establish itself in a population. The expectation here is that the invading host had plasmid control mechanisms (e.g. transfer repression) not found in the resident population/community.

When a plasmid is beneficial it can improve the competitive ability of the host population in which it resides. This again can affect community structure. In these circumstances the plasmid and host may have differing selective pressures. While positive selection favours plasmid replication and transfer, selection on the host would not favour the transfer of beneficial genes to its competitors. Transfer tends to be biased towards kin (Dimitriu et al., 2019a) but this may only be caused by recipient mechanisms such as restriction modification, rather than active donor discrimination. If any transfer occurs, however, this means that the plasmid may still invade other species, dependent on the plasmid dynamics in the new host, such that discrimination would only be effective if no inter-species transfer occurs. Exposure to selection can then dramatically affect the community structure according to the plasmid distribution in the community.

Positive species interactions include cross feeding and can be commensal when a species produces a substrate that benefits another strain or cooperative where production and benefits are reciprocal (Coyte and Rakoff-Nahoum, 2019; Freilich et al., 2011). These kinds of interactions can affect the benefits and costs of gene sharing. Cooperative species may show the opposite plasmid selection pressures from those in competition (i.e. increased transfer of beneficial plasmids, reduced transfer of costly plasmids), but experimental work in this area is yet to be conducted. Furthermore, cells can cooperate through quorum sensing that can occur between species in addition to within species (Abisado et al., 2018). Bacteria often use quorum sensing to regulate the production of public goods, which facilitates cooperation but can be vulnerable to cheaters. Public goods genes are frequently plasmid-based and their transfer increases relatedness and facilitates cooperation (Dimitriu et al., 2014; Nogueira et al., 2009).

Until recently, there have been relatively few models that investigate the dynamics of plasmids in even two species (Grover and Wang, 2019; Martins, 2013), and it is important that we understand how community interactions affect plasmid dynamics. In my Masters' project (Sheppard, 2016), which I completed in September 2016, I extended a simple single-species conjugation model (Stewart and Levin, 1977) to look at dynamics between two species and a single plasmid. I discovered a range of parameter values in the two-species model that

allow coexistence between the species where only one would normally be maintained due to competitive exclusion. Coexistence has been independently identified from other models more recently (Grover and Wang, 2019), although for a non-conjugative, toxin-producing plasmid. I also frequently found cyclical interactions between the different elements of the model that have also been found in similar models (Yurtsev et al., 2016). These findings suggest that competitive systems can be impacted by plasmid presence and density.

1.4 Aims

This thesis focuses on three areas related to plasmid transfer and persistence, using data, mathematical modelling and a combination of lab work with complementary modelling.

Chapter 2: The role of hosts, plasmids and environment in determining plasmid transfer rates: a meta-analysis

Plasmid transfer rates have been measured and reported for over 30 years, but it is still difficult to assess the parameter space of transfer rates (Martínez and Baquero, 2014; Simonsen et al., 1990). These data vary in experimental conditions and reporting methods, and largely only focus on one or two treatment factors, which make them difficult to compare and assess collectively (Huisman et al., 2020). Key questions include: what are the main factors that determine and explain the variation in plasmid transfer rate, and what are the relative roles of hosts, plasmids and environmental factors in that determination (Dimitriu et al., 2019a)? In chapter 2 I conduct a meta-analysis of plasmid transfer rates published in the literature to describe and explain the variation. I explore the relative importance of donors, recipients and plasmids, in addition to plasmid differences (such as pilus type) and environmental conditions in the determination of plasmid transfer rate. The results identify the factors that explain the majority of the variation in plasmid transfer rate and improve the ease of access to relevant data for use in modelling (Ashbolt et al., 2013), and have implications for the outcomes (persistence and prevalence) of plasmid-host dynamics.

Chapter 3: The evolution of plasmid transfer rate in bacteria and its effect on plasmid persistence

Modelling has been used to investigate selective pressures that affect some of the parameters that determine plasmid persistence and prevalence (Leclerc et al., 2019; Slater et al., 2008a; Smets and Lardon, 2009). The evolution of transfer rate has received some attention, but primarily in the context of parasite-host modelling, which assumptions do not always apply to plasmids (Haft et al., 2009; Lipsitch et al., 1995, 1996). The question of how plasmids, hosts and their environment interact to determine observed plasmid transfer rates remains. In chapter 3 I formulate and explore a series of adaptive dynamic models to demonstrate the primary ways in which plasmid transfer rate is affected by selection. I explore selective and non-selective conditions and focus on the potential conflicts between plasmid and host in the determination of transfer rate, and the subsequent effect on plasmid prevalence.

Chapter 4: The interactions between competition and plasmid presence and the application of a two-species conjugation model to a simple bacterial community

The majority of plasmid modelling has explored plasmid-host dynamics under varying conditions and focuses on specific aspects of plasmid biology (Leclerc et al., 2019; Smets and Lardon, 2009). Much of the complexity that exists is yet to be investigated through models, notably the effects of community interactions on plasmid dynamics (Barraclough, 2015; Slater et al., 2008a; Veal et al., 1992). Models tend to use only one or two species and do not accurately reflect the complexity of microbial communities (Blokesch, 2016). Furthermore, the comparison of these models to experimental systems for validation of the findings is necessary for the correct application of the model results (Leclerc et al., 2019), and there is a particular lack of data demonstrating the variability of plasmid behaviour across species (Kottara et al., 2018). My masters' project (Sheppard, 2016) and more recent work (Grover and Wang, 2019) look at the interactions between plasmid distribution and species competition. Chapter 4 seeks to address the question of the extent to which these findings be seen in experimental systems. I adapt the model formulated in my Masters'

project and apply it to a series of experiments that vary plasmid distribution, and noting the interactions between plasmid presence and interspecies competition. The experimental results are compared with the complementary results from the model and the limitations of the model fitting are assessed.

Table 1.1: Models of plasmid conjugation.

Authors	Year	Model Description
Preliminary models		
Anderson and Lustbader	1975	Population genetic model showing plasmid loss through segregation (no transfer).
Levin and Stewart	1977	Plasmid establishment probability, informed with data.
Cullum et al.	1978	Population genetic model showing plasmid loss through segregation (no transfer).
Levin plasmid conjugation models (and application)		
Stewart and Levin	1977	Original conjugation model (batch and chemostat); establishment of plasmids.
Levin et al.	1979	Experimental validation of Stewart and Levin (1977) model; parameters estimated.
Levin and Stewart	1980	Stewart and Levin (1977) model extended for mobilisable plasmids.
Levin and Rice	1980	Experimental validation of mobilisable plasmid model (Levin and Stewart, 1980), parameters estimated.
Lundquist and Levin	1986	Stewart and Levin (1977) model extended for transitory derepression, validated experimentally.
Condit et al.	1988	Stewart and Levin (1977) model extended for transposition between plasmid and chromosome.
Condit and Levin	1990	Stewart and Levin (1977) model extended for transposition between two plasmids and chromosome; partial experimental validation.
Simonsen et al.	1990	Stewart and Levin (1977) model used to derive estimation method for transfer rate using endpoint bacterial densities.
Simonsen	1991	Examined and reviewed modelling assumptions; extended Levin and Stewart (1980) model to include superinfection.

Table 1.1 – *Continued*

Authors	Year	Model Description
Smets et al.	1993	Stewart and Levin (1977) applied to investigate kinetics for TOL plasmid.
Smets et al.	1994	Stewart and Levin (1977) applied to investigate kinetics for TOL plasmid in a tranconjugant strain (see Smets et al., 1993).
Bergstrom et al.	2000	Stewart and Levin (1977) model extended to demonstrate plasmid paradox; plasmid-chromosome integration and plasmid loss.
Lili et al.	2007	Bergstrom et al. (2000) model expanded to explicitly include carrying capacity; found broader conditions for plamisd persistence.
Huisman et al.	2020	Simonsen et al. (1990) model extensions and evaluations using simulations.
Additional plasmid processes		
Sýkora et al.	1989	Kinetic model for plasmid curing.
Top et al.	1991	Modelled retrotransfer with experimental data.
MacDonald et al.	1992	Model estimated parameters; transfer increases with substrate concentration.
Macken et al.	1994	Modelled host mortality as function of population size.
Novozhilov et al.	2005	Stochastic model including mutational genetic inactivation in HGT.
Ponciano et al.	2007	Stochasticity (using hidden Markov approach); modelled plasmid transfer based on enzyme kinetics.
Philipsen et al.	2010	Stochastic model applied to data; expression for substrate dependent transfer.
Tazzyman and Bonhoeffer	2014	Stochastic model; evolution of plasmid-based resistance can limit rescue.
Tazzyman and Bonhoeffer	2015	Essential genes stably inherited on the chromosome.
Ledda and Ferretti	2014	Modelling plasmid cost based on plasmid length.
Niehus et al.	2015	Evo-evolutionary model featuring migration.
Billiard et al.	2016	Stochastic, competition; density or frequency dependent transfer.
Knopoff and Sánchez Sansó	2017	Kinetic model including mutation, transfer and antibiotic resistance.

Table 1.1 – *Continued*

Authors	Year	Model Description
Lopatkin et al.	2017	Application of general plasmid model; plasmid transfer rates can be high enough for persistence.
Gama et al.	2017b	Modelled interactions of co-resident plasmids to affect transfer rate.
Copy number		
Ataai and Shuler	1986	Simulated plasmid replication; accurately predicted plasmid copy number.
Ehrenberg	1996	Regulation of plasmid replication initiation and inhibition decreases plasmid loss.
Paulsson et al.	1998	Copy number regulation via inhibition.
Paulsson and Ehrenberg	1998	Trade-off between metabolic burden and plasmid stability through copy number.
Paulsson and Ehrenberg	2000	Synchrony of plasmid replication with host replication decreases plasmid loss.
Paulsson	2002	Multi-level selection on plasmid copy number and replication.
PSK genes		
Lauffenburger	1985	Modelling bacteriocin plasmids, compared with experimental results.
Sardonini and DiBiasio	1987	Model where plasmid produces public good used enabling plasmid-free cell persistence despite selection.
Mongold	1992	PSK genes, plasmid competition in a chemostat.
Hsu and Waltman	1997	Identified chemostat model solutions for two kinds of plasmid selection: essential metabolite production model expansion (Sardonini and DiBiasio, 1987), toxin-antitoxin plasmid model (adapted from Chao and Levin, 1981).
Rankin et al.	2012	Toxin-antitoxin plasmids, addiction complexes.
Phages		
Dionisio	2005	Chemostat model, presence of plasmid-specific phages.
Wan and Goddard	2012	Quantification and modelling of plasmid-phage dynamics with application to data.
Harrison et al.	2015	Lili et al. (2007) model adapted to include phage dynamics, with individual based simulations and experimental data.

Table 1.1 – *Continued*

Authors	Year	Model Description
Antibiotic treatments on plasmid dynamics		
Garber	1987	Variable bacterial sensitivity to antibiotics.
Willms et al.	2006	Stewart and Levin (1977) model extension to look at cyclical selection bacteriostatic antibiotic pressures.
Webb et al.	2005	Two-level (bacterial and patient) model and spread of resistance within a hospital.
Gehring et al.	2010	Combined with pharmacokinetic model to optimise antimicrobial use and minimise resistance.
Svara and Rankin	2011	Plasmid dynamics in response to antibiotic treatment regimes (dosage, interval).
Non-conjugative plasmid models		
Müller et al.	1982	Stochastic Markov chain model, plasmid loss, copy number.
Stephanopoulos and Lapudis	1988	Chemostat plasmid dynamics, no plasmid transfer.
Lenski and Bouma	1987	Analysis of modelled non-conjugative plasmid; segregation and selection, no transfer.
Bentley and Quiroga	1993	Discrete segregated model for plasmid stability, with experimental data.
Hsu et al.	1994	Global solution for plasmid competition in Stephanopoulos and Lapudis (1988) chemostat model, no plasmid transfer.
Hsu et al.	1995	Plasmid competition in a chemostat with an inhibitor, no transfer.
Luo and Hsu	1995	Global solutions for competition in Hsu et al. (1995) chemostat with an inhibitor, no transfer.
Ganusov and Brilkov	2002	Using a chemostat model to estimate plasmid cost and loss parameters, no transfer.
De Gelder et al.	2004	Loss of plasmid-based antibiotic resistance, mutation and selection, no transfer; fitting a model to experimental data.
Müller et al.	2017	Discrete model of plasmid segregation and copy number, using hyperbolic partial differential equations.
Stadler	2019	Eigensolutions and spectral analysis, no transfer.

Table 1.1 – *Continued*

Authors	Year	Model Description
Complex mathematical analyses		
Tremblay and Rose	1985	General model of gene transfer, focusing on competition and transmission.
Parulekar et al.	1987	Bifurcation analyses; coexistence of donor-recipient steady states.
Champagnat et al.	2019	Stochastic Markov pure jump process evolutionary model; cyclic behaviour and evolutionary suicide are two outcomes.
Natural/industrial environment models		
Freter et al.	1983	Mouse gut and microfloral continuous flow systems, with experimental data.
Seo and Bailey	1985	Modelling of a bioreactor: copy number, plasmid loss and product synthesis.
Srienc et al.	1986	Seo and Bailey (1985) model extended to include selection for plasmids.
Chang and Lim	1987	Bioreactor model with antibiotic selection (Monod death rate), bifurcation, coexistence (donor-recipient) steady states.
Knudsen et al.	1988	Modelling long term plasmid dynamics in phyllosphere and rhizophere, with experimental data.
Clewlow et al.	1990	Modelling long term plasmid dynamics in soil microcosms, with experimental data.
Sudarshana and Knudsen	2006	Transfer of non-conjugative plasmids on seeds and roots.
Massoudieh et al.	2010	Modelled plasmid dynamics in granular porous media, included attachment kinetics and time lags (see Massoudieh et al., 2007).
Hellweger et al.	2011	Model of resistance in aquatic environment, with application.
Fischer et al.	2014	Conjugation dynamics within broilers.
Baker et al.	2016	Modelling spread of resistance in agricultural waste.

Table 1.1 – *Continued*

Authors	Year	Model Description
Spatial structure models, including biofilms		
Beaudoin et al.	1998b	Mobilisation of plasmid in two species biofilm, with comparable data (Beaudoin et al., 1998a).
Lagido et al.	2003	Plasmid dynamics on a solid surface.
Krone et al.	2007	Plasmid dynamics using an individual based lattice model, with experimental data.
Fox et al.	2008	Krone et al. (2007) extended to include 3D aspects, with experimental data.
Gregory et al.	2008	Rule-based individual based model; selection, incompatability.
Seoane et al.	2011	Application of individual based model; transitory derepression.
Merkey et al.	2011	Individual based model; low transfer in biofilms due to dependence of transfer on growth.
Connelly et al.	2011	Population structure, costly plasmid transfer, no vertical transmission. Inclusion of population spatial structure.
Plasmid transmission modes, transfer/cost tradeoff		
Van der Hoeven	1984	Coexistence of two plasmids (based on tradeoffs between plasmid transfer and cost) in a chemostat.
Van der Hoeven	1986	Coexistence of two plasmids in a feast-famine regime.
Lipsitch et al.	1995	Parasite prevalence due to vertical and horizontal parasite transmission.
Lipsitch et al.	1996	Lipsitch et al. (1995) extended to look at the impact of recipient density on vertical and horizontal parasite transmission.
Turner et al.	1998	Tradeoff between vertical and horizontal plasmid transmission; did not validate recipient density effect on transfer rate.
Ferdy	2009	Applied adaptive dynamics to parasite metapopulation model with spatial structure.
Raz and Tannenbaum	2010	Mutation selection model; conjugation negatively affects mean fitness of a population.
Misevic et al.	2013	Application of *Aevol*, digital evolution system, to plasmids.

Table 1.1 – *Continued*

Authors	Year	Model Description
Raz and Tannenbaum	2014	Raz and Tannenbaum (2010) model extended to include transitory derepression.
Plasmid cost evolution and amelioration		
San Millan et al.	2014	Plasmid cost amelioration in non-transmissible plasmid; application of a model to data.
Peña-Miller et al.	2015	Analysis of San Millan et al. (2014) model; non-transmissible plasmid persistence may require some transfer.
Loftie-Eaton et al.	2016	Based on De Gelder et al. (2004); Ponciano et al. (2007); plasmid cost amelioration expands host-range.
Porse et al.	2016	Application of model based on Proctor (1994) to experimental data, no transfer.
Loftie-Eaton et al.	2017	Loftie-Eaton et al. (see 2016); Compensatory mutations in the host increase plasmid permissiveness.
Hall et al.	2017	Plasmid stability through vertical and horizontal transmission.
Zwanzig et al.	2019	Plasmid-based compensatory mutations improves persistence more than host-based mutations.

Table 1.1 – *Continued*

Authors	Year	Model Description
Public goods genes, cooperation, cheaters and community		
Landis et al.	2000	Stochastic model simulations, including single and multiple host models.
Smith	2001	Modelling cheaters of public goods plasmids and punishment through retransfer.
Miki et al.	2007	Extension of Stewart and Levin (1977) model for plasmid transfer from introduced donor population to indigenous population.
Nogueira et al.	2009	Gene mobility increases relatedness and facilitates cooperation.
Mc Ginty et al.	2011	Modelling public and individual goods plasmids; cooperation and cheaters.
Mc Ginty et al.	2013	Evolution of plasmid public goods production.
Martins	2013	Deterministic and stochastic (individual-based) conjugation in two species community; broad and narrow range plasmids.
Dimitriu et al.	2014	Plasmid metapopulation model; plasmid transfer favours cooperation, facilitated by structured environment due to allele assortment.
Shapiro and Turner	2014	Modified susceptible-infected model; symbiont transfer can be beneficial due to relatedness.
Dimitriu et al.	2016	Based on Dimitriu et al. (2014); indirect fitness benefits through relatedness can overcome costs of transfer.
Hall et al.	2016	Source-sink dynamics in a two-species community.
Dimitriu et al.	2018	Carriage of public goods plasmids and indirect selection effects.
Grover and Wang	2019	Non-conjugative toxin producing plasmid dynamics in two-species showing coexistence.
Review papers		
Smets and Lardon	2009	Summary of mass-action models.
Rankin et al.	2011	Summary of social goods and cooperation models.
Leclerc et al.	2019	HGT modelling systematic review; 43 studies, primarily single gene conjugation in *E. coli* culture.

Chapter 2

The role of hosts, plasmids and environment in determining plasmid transfer rates: a meta-analysis

2.1 Introduction

Various theoretical models have been constructed over the last 40 years to investigate microbe-plasmid dynamics (Krone et al., 2007; Levin and Stewart, 1980; Merkey et al., 2011; Stewart and Levin, 1977; Volkova et al., 2013) and validated with experimental data (Levin et al., 1979; Lopatkin et al., 2017). These models show that plasmid persistence and prevalence is primarily determined by the interaction of a number of key parameters, originally described by Stewart and Levin (1977). These parameters are plasmid transfer (γ) and loss rates (τ), the cost (α) or benefit of plasmid carriage on the host (Carroll and Wong, 2018), population size (N) and the dilution rate of media into and out of the system (D). In theory, a plasmid can persist when its transfer rate is greater than the combination of the rate of loss and the cost of the plasmid, with a large population size and low dilution rate further enhancing persistence; that is, when

$$\gamma N > \alpha D + \tau \tag{2.1}$$

This theory has proved useful in predicting plasmid dynamics and persistence in controlled experiments (Levin et al., 1979; Lopatkin et al., 2017), but its application to natural environmental and medical systems has been more difficult. Parameters can be measured experimentally but vary widely among studies, making it hard to generalise observations and predictions. Additional research is needed to understand how and why these parameters vary.

Of particular interest are the relative roles of host and plasmid, and the effect of coevolution between them in determining transfer rate. Plasmids are often described as self-transmissible, containing all the genes required for transfer, but other studies have emphasised the effect of the donor and recipient strains in affecting transfer rates (Bradley and Williams, 1982; Gordon, 1992; Koraimann and Wagner, 2014). Transfer is a costly process due to the production of conjugation pili and increased vulnerability to phages (Hall et al., 2017). In addition, the metabolic and replicative costs incurred by plasmid presence can be detrimental in environments where the plasmid genes are not beneficial. This can lead to the development of host-based transfer control mechanisms which affect natural rates of transfer and cause host-plasmid conflicts (Bañuelos-Vazquez et al., 2017; Chatterjee et al., 2013; Koraimann and Wagner, 2014).

These coevolutionary processes might explain why transfer rates vary so much over several orders of magnitude (Zhong et al., 2012). Low transfer rates might evolve to reduce the costs of plasmid transfer. For example, plasmid genes that repress transfer can spread by enhancing host replication and vertical plasmid transmission (Haft et al., 2009). In contrast, high transfer rates might evolve when indirect benefits of transferring beneficial plasmids to closely related cells outweigh the inherent costs of transfer to the donor (Dimitriu et al., 2016).

Here, I perform a meta-analysis of plasmid transfer rates reported from laboratory experiments. The chapter investigates how rates depend on donor, recipient and plasmid identities, and on additional environmental conditions. The results summarise key aspects of transfer to provide a more robust foundation for modelling conjugative processes, and increase our understanding of the determinants of plasmid dynamics.

2.2 Methods

2.2.1 Literature search

A literature search was conducted using Scopus, Web of Science and Google, to identify papers that measured and reported rates of conjugative plasmid transfer, using search terms "plasmid", "transfer", "conjugation", and "rate OR rates OR efficiency OR dynamics". Additional results were included from papers identified while conducting the primary literature review. Rates that were reported graphically were extracted using plot digitisation software (Ankit, 2019). In total, 1224 transfer rates were collected from 33 papers, about half of which were collected by an undergraduate student (Alice E. Beddis). The dataset was filtered to remove non-conjugative plasmids, data where the measurement type could not be ascertained, data from a study reporting a range of transfer rates for a group of plasmids rather than individual values, and data with an unexplained discrepancy between measurements reported in the paper (see Table A.1). Results with multiple measurements from experiments were averaged by experiment on a $\log_{10}$ scale to avoid pseudoreplication.

2.2.2 Measures of transfer rate

Transfer rate is measured in mating assays in which donor and recipient cells are cultured together and the number of recipient cells that have taken up the plasmid (transconjugants) at the end is measured. There is wide variation, however, in how results are reported. Each study reported some but not all of the following: cell densities, various efficiencies and ratios of donors, recipients and transconjugants, and more sophisticated model-based methods that take into account growth of bacteria during the assay. For example, Simonsen's endpoint (1990) uses batch culture growth equations with plasmid transfer to calculate a mass-action transfer rate (γ) taking into account the growth and re-transfer of transconjugants, dependent on substrate availability

$$\gamma = r \ln(1 + \frac{T}{R}\frac{N}{D}) \cdot \frac{1}{N - N_0} \tag{2.2}$$

$$r = \frac{\ln\left(\mathrm{OD}_b/\mathrm{OD}_a\right)}{t_b - t_a}, \tag{2.3}$$

where T, R, D and N are transconjugant, recipient, donor and total cell densities at the endpoint, respectively, and N_0 is the initial total cell density. r is the species exponential growth rate where OD_a, OD_b and t_a, t_b are the optical density and the time at two different timepoints (a and b). It assumes that growth rates are equal across donors and recipients regardless of strain differences, or plasmid cost on growth, both for simplicity and because data are usually lacking to quantify these separately. Endpoint transfer rate units are of ml cell^{-1} h^{-1}, the number of recipient cells a single donor can infect in a milliliter of cell culture over an hour.

Endpoint measurements are less susceptible than other measurement types to the effects of initial cell densities, donor to recipient ratios, re-transfer by transconjugants, and cell growth, and I use them by preference here. However, endpoints measurements are still vulnerable to plasmid segregation, and differences between donor, recipient and transconjugant growth. The endpoint model also assumes that growth and transfer are affected equally by substrate availability, which may not be correct in all circumstances.

Freter et al. (1983) used an extension of Stewart and Levin's original plasmid conjugation model (1977) to estimate transfer rates using least-squares, optimising the fit of the model when applied to data from control flow experiments. Freter's transfer rate estimations and Simonsen's endpoint estimations are based on mass-action kinetics and are therefore on a similar scale. These rates can thus be analysed together, and are hereafter described as and included with endpoints. Although only 276 transfer rates were reported as endpoints, it was possible to convert additional densities, ratios and efficiencies to endpoints where enough data was reported. Some papers gave the required initial and end cell densities. A number of papers reported only initial or final donor and recipient densities, but by using species growth rates found in the literature (Table A.2), the appropriate missing densities were estimated using the standard bacterial exponential growth formula. This conversion is expected to be appropriate where cells are growing exponentially and was only used to estimate cell densities in conjugation experiments of short mating times (less than 12 hours).

$$N_t = N_0 e^{rt} \tag{2.4}$$

Transconjugant densities could be estimated from reported measures of efficiencies or ratios and total cell densities. These densities were then combined to estimate endpoint transfer rates using Simonsen's transfer rate calculation (Simonsen et al., 1990), resulting in an additional 411 endpoint estimates. Where initial densities were not reported, $TD^{-1}R^{-1}$ has been shown to be a good proxy of Simonsen's endpoint (Zhong et al., 2012), and was used instead. This gave a further 7 estimates, and a total of 716 data points. Most of these measurements took place in short mating experiments, meeting the criteria from Zhong et al. (2012): well mixed initial populations, high initial density, short mating times. It was assumed that bacterial densities did not change after reaching steady state, allowing conversion and inclusion of data from longer mating time experiments. This is not expected to be a confounding factor due to the correlation of plasmid transfer with bacterial growth phase (Simonsen, 1990). Experiment time was included as a covariate in the analysis to test whether it affected conclusions.

A final complication is that the endpoint model assumes a well-mixed liquid population, whereas many experiments conduct matings on solid surfaces. Simonsen compared rates of transfer on slides with liquid matings and found similar estimates when initial density was high and assuming short experiment times so that differences due to lack of mixing are limited (Simonsen, 1990). To calculate rates that are comparable to liquid matings, I therefore used their conversion to convert cell density estimates per cm^2 to ml (namely 9.375 cm^2 is equivalent to 1 ml). Densities of cells from filter mating experiments were converted to ml^{-1} based on the dimensions of the filter. Some filter mating experiments used low initial bacterial densities, which do not give accurate conversions to endpoints. Data with reported final total cell density below 10^6 ml^{-1} (10 data points) were removed from the set.

Note that only a few papers included confidence intervals or measures of uncertainty of their estimates, and so I was unable to adopt formal meta-analytic methods. Instead I treat each estimate as an independent data point and look for correlations across those estimates.

2.2.3 Explanatory variables

Donor and recipient strain and species were recorded and the strain was used to represent their identity groups. Donor and recipient strains were grouped by origin (Table A.3): a binary variable of related or unrelated, determined on whether donor and recipient strains belonged to the same origin grouping or not. Plasmids were grouped into sets with similar "plasmid backbones" which included small variations in derived plasmids, where the plasmid backbone was used as its identity group. In addition, plasmids RP1, RP4, RP68 and RK2 were identified as the same plasmid, and grouped as such (Burkardt et al., 1979). A categorical variable of native or non-native was used to record if the plasmid was native to the host used in the experiment or not (Table A.4). Further plasmid attributes that were recorded were: size in number of base pairs (sizes reported in mass, Daltons, were converted to base pairs); incompatibility group (which categorises whether plasmids are sometimes found in the same host cell simultaneously or not); pilus type (the appendage used for plasmid transfer, being rigid, flexible or expressing both); and repression status (repressed or derepressed).

Repression status presented some complications in interpretation. A number of papers compared plasmid variants described as "repressed" and "derepressed" for either pilus synthesis or transfer. Plasmids often carry regulatory regions that repress transfer rate but that are temporarily switched off following transfer into a new host (transitory derepression (Freter et al., 1983; Haft et al., 2009; Lundquist and Levin, 1986)). In addition, repression systems can be subverted by mutations, permanently derepressing transfer rates and allowing the derepressed rate to be measured. Papers varied in the way they measured and reported repression and derepression. While the majority associated high numbers of pili with derepression, others identified derepression by phage susceptibility. Some inferred repression status from high transfer rates themselves, a rather circular argument. In order to include as much information as possible on this trait, I grouped together all descriptions of pilus or transfer repression/derepression together. Where repression was unspecified, the plasmid was assumed to be repressed, except for two plasmids (Sa, RP1) which had distributions more consistent with derepressed plasmids. Cases where pili were repressed, but transfer derepressed or the reverse were determined to be derepressed. Repression status of donors with co-resident plasmids were assessed in conjunction with pilus type, media type and

the effect of the co-resident plasmid on transfer rate. When the co-resident plasmid either increased or decreased plasmid transfer substantially from the focal plasmid baseline the repression status was changed accordingly.

Experimental conditions that were recorded were temperature, media type, media richness and mating experiment duration. Media type included liquid, plate, filter plate, soil, biofilm, or in vivo mating experiments. Media richness was divided into high (LB, BHI, TS, TSB, TSBA, PY, NB, KB, GM17, MRS, plate count, or animal gut media) or low richness (minimal media, AB, MSB-G, DM and natural environments where low substrate richness is expected). Duration of the mating experiment was treated as a categorical variable (where 10 hours was the threshold between long and short mating times). Where endpoint studies did not clearly specify the mating time, a long mating long was assumed. Where temperature and media type were unspecified, room temperature and high medium richness were assumed, respectively.

2.2.4 Statistical analysis

The final dataset used in the analyses contained 612 data points, following filtering of data with missing variable information and the removal of transfer rates equal to 0. ANOVAs were used to model the $\log_{10}$ rates of plasmid transfer in R (R Core Team, 2018). Mixed effects models including experiment source as random effects were not appropriate for the analysis of this dataset due to overparameterisation of the model. Type 1 ANOVAs were selected because type 2 and 3 ANOVAs obscured some of the potential results due to the large number of variables used in the analysis. Two sets of models were constructed in order to first investigate the variation that can be explained by variables which can be generally applied, before donor, recipient and plasmid identity differences were included. The explanatory variables in the initial set of models were media type, pilus type, repression status, temperature, relatedness, media richness, plasmid size, plasmid nativity, mating time and all two way interactions. In the subsequent set of models, donor, recipient and plasmid identities with all two way interactions were additionally included. Because type 1 ANOVA are affected by the order of variables, these were selected in the order which minimised Akaike information criterion (AIC) or the Bayesian information criterion (BIC, Vrieze, 2012) when added sequentially in the absence of two-way interactions (Table A.5). AIC and BIC are

used for model comparison, mediating between goodness of fit through log-likelihood of the model ($\hat{L}$) and model overfitting by penalising the number of estimated parameters (k). The BIC additionally factors the penalisation by the number of data points (n), resulting in greater penalisation for additional parameters when compared with AIC and when n is greater than about 8.

$$\text{AIC} = 2k - 2\ln(\hat{L}) \qquad \text{BIC} = k\ln(n) - 2\ln(\hat{L}) \tag{2.5}$$

These models were then simplified using the stepwise function from the "RcmdrMisc" package (Fox, 2018), using all combinations of the AIC or BIC with "forward/backward" or "backward/forward" simplification methods to gain a greater understanding of the variation between potential models. This gave a total of eight models: four in each set. I report the AIC and "backward/forward" model results, and then draw attention to any discrepancy among the results using alternative simplification methods.

2.3 Results

Transfer rates varied over 13 orders of magnitude, ranging from 1.6×10^{-20} to 4.8×10^{-7} (Figure. 2.1). The lower bound is lower than previously reported, and these lower rates result from the conversion process used here to calculate endpoint measures. These lower rates may not have been experimentally observed due to the limits of detection.

The first set of linear models included pilus type, media type, media richness, pilus repression, temperature, donor and recipient relatedness, plasmid size, mating time and all two-way interactions (Table 2.1, A.6-A.9). Repression (27.5%), media type (10.3%), relatedness (6.8%) and media richness (1.3%) explained the most variation consistently across the four variants of the model. While temperature consistently explained variation, this varied according to final variable order (0.8-1.1%). Plasmid nativity was only significant in three of the four model variants (0.3%). Two interaction terms were significant in all four of the models: media type and media richness (2-2.5%) and relatedness and temperature (1-1.7%), while three interaction terms were significant in three of the four models: derepression and pilus type (4.3-4.5%), media type and pilus type (1.5-1.8%) and derepression

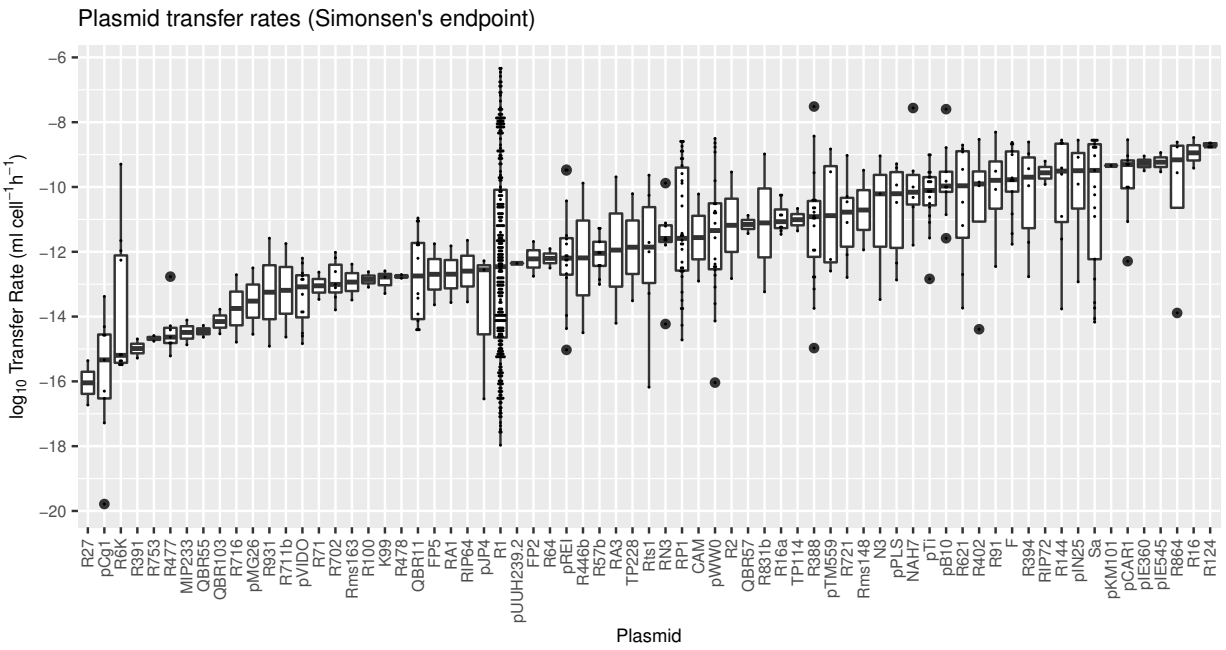

Figure 2.1: Variation in plasmid transfer rate, grouped by plasmid and sorted by median value per plasmid. Dots show the distribution of data points. Boxes show the interquartile range (IQR) and whiskers show the lowest and highest values within 1.5xIQR of the first and third quartiles respectively.

and relatedness (1.2%).

The top three variables and their interaction terms were independently removed from the model to assess the unique variation explained by each, independently of other variables (Table A.14). The removal of repression resulted in a 18-21% decrease in the total variation explained, while media type and relatedness decreased the explained variation by 6-10% and 4-8% respectively across the four versions of the model.

Plotting revealed the effects and interactions of the most important variables (Figure 2.2, Table A.15). Derepressed transfer on agar plates and in liquid mating experiments was about 10^{-10} ml cell^{-1} h^{-1} for plasmids with flexible pili only. This was reduced slightly in filter mating experiments ($10^{-10.7}$ ml cell^{-1} h^{-1}). Repression reduced transfer rate for all three media types, with the greatest change found in liquid (10^{-14} ml cell^{-1} h^{-1}), a difference of about four orders of magnitude, while repression reduced plate and filter mating transfer rates by only two orders of magnitude ($10^{-12.1}$ and $10^{-12.8}$ ml cell^{-1} h^{-1}, respectively). Derepressed plasmids with rigid pili transferred at a higher rate than flexible pili on plate and filter matings (10^{-9} and $10^{-9.8}$ ml cell^{-1} h^{-1}, respectively), but far lower in liquid matings ($10^{-12.5}$ ml cell^{-1} h^{-1}). Rigid pili are expected to be adapted to solid environments (plate

Source	DF	SS	MS	F	p		TV(%)
Repression	1	1123.54	1123.54	422.24	<0.001	***	27.5
Media Type	2	420.68	210.34	79.05	<0.001	***	10.3
Relatedness	1	279.31	279.31	104.97	<0.001	***	6.84
Media Richness	1	54.1	54.1	20.33	<0.001	***	1.32
Plasmid Size	1	18.55	18.55	6.97	<0.01	**	0.45
Plasmid Nativity	1	13.24	13.24	4.98	<0.05	*	0.32
Temperature	1	34.06	34.06	12.8	<0.001	***	0.83
Mating Time	1	11.21	11.21	4.21	<0.05	*	0.27
Pilus Type	2	6.89	3.45	1.3	0.2747		0.17
Repression : Media Richness	1	1.05	1.05	0.4	0.5293		0.03
Repression : Temperature	1	1.38	1.38	0.52	0.4718		0.03
Repression : Mating Time	1	55.07	55.07	20.69	<0.001	***	1.35
Repression : Pilus Type	2	183.75	91.87	34.53	<0.001	***	4.5
Media Type : Media Richness	2	98.58	49.29	18.52	<0.001	***	2.41
Media Type : Plasmid Size	2	32.9	16.45	6.18	<0.01	**	0.81
Media Type : Plasmid Nativity	2	2.23	1.12	0.42	0.6575		0.05
Media Type : Pilus Type	3	70.29	23.43	8.81	<0.001	***	1.72
Relatedness : Media Richness	1	0.04	0.04	0.01	0.907		0
Relatedness : Plasmid Size	1	0.42	0.42	0.16	0.6911		0.01
Relatedness : Temperature	1	46.22	46.22	17.37	<0.001	***	1.13
Relatedness : Pilus Type	2	3.22	1.61	0.6	0.5465		0.08
Media Richness : Plasmid Size	1	27.8	27.8	10.45	<0.01	**	0.68
Plasmid Size : Temperature	1	2.07	2.07	0.78	0.3787		0.05
Plasmid Size : Mating Time	1	0.2	0.2	0.07	0.7857		0
Plasmid Size : Pilus Type	2	37.93	18.96	7.13	<0.001	***	0.93
Plasmid Nativity : Temperature	1	31.09	31.09	11.68	<0.001	***	0.76
Error	575	1530.02	2.66	NA	NA		37.45
Total	611	4085.81	2118.51	NA	NA		100

Table 2.1: Results of the simplified Model 1 (AIC, b/f) ANOVA. Original variables input are media type, pilus type, repression, temperature, media richness, donor-recipient relatedness and plasmid size, plasmid nativity, mating time and all two-way interactions. Degrees of freedom (DF), sum of squares (SS), mean sum of squares (MS), F-statistic (F), p-value (p), Total variation explained (TV).

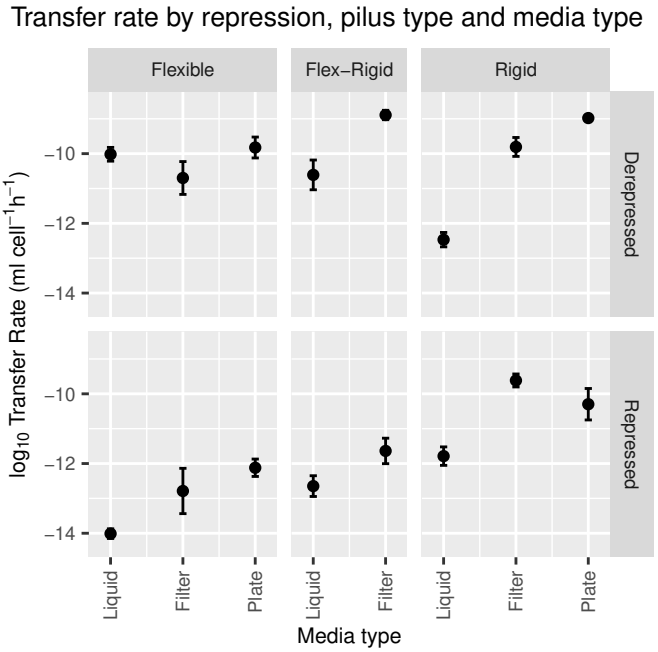

Figure 2.2: Rates or transfer (± standard errors) - grouped by repression, pilus type and media type. Data is separated according to pilus type (Flexible, Rigid and the presence of both rigid and flexible (Flex-Rigid)).

and filter surfaces) where pilus flexibility is unnecessary due to limited cell mobility. Cells have greater mobility in liquid environments, greatly reducing the effectiveness of rigid pili in plasmid transfer. Flexible pili, by contrast, accommodate greater cell mobility and enable transfer to occur at reasonably high rates when cells are more mobile in liquid environments. Repression had a relatively small effect on plasmids with rigid pili, without notable differences between repressed and derepressed transfer rates in liquid and filter mating experiments. Rigid pili plate matings showed a decrease of about one order of magnitude in ml cell^{-1} h^{-1} when repressed ($10^{-10.3}$ ml cell^{-1} h^{-1}). Derepressed plasmid transfer where both kinds of pili were present had rates of plate transfer consistent with plasmids with only rigid pili ($10^{-8.9}$ ml cell^{-1} h^{-1}), while transfer in liquid showed rates more consistent with plasmids with flexible pili only ($10^{-10.6}$ ml cell^{-1} h^{-1}). When repressed, these rates were each between the repressed rates for flexible and rigid only: $10^{-12.6}$ ml cell^{-1} h^{-1} for liquid, and $10^{-11.6}$ ml cell^{-1} h^{-1} for plate matings. Transfer between closely related strains was higher than between unrelated strains, and high media richness showed lower transfer than low media richness, although this was only consistently found in liquid matings. Plasmid transfer increased at higher temperatures, although this pattern is more likely to be unimodal, with an optimal temperature.

The second set of models included the same variables as the first set, but with donor, recipient and plasmid identities added (Table 2.2, A.10-A.13). The results of the four variants of these models differed from each other. Recipient identity was significant in 3 out of 4 simplified models, and explained more variation than either donor or plasmid identity, although these also explained significant variation in the models simplified using AIC, where both were significant. Recipient identity was the primary explanatory variable in the AIC variants (34.3%), followed by repression (12.9%), donor identity (10.1%), media type (3%), plasmid nativity (2.3%) and temperature (2%). Only the interaction between host nativity and medium richness was significant in both variants, but explained little variation (0.4-0.6%). When BIC was used as the comparison metric, however, donor, recipient and plasmid identities were heavily penalised in the model, due to the high number of coefficients estimated. Of the identities, only the recipient was significant, and only in the "backward/forward" model variant. In this variant the recipient identity explained only 12.8% of the variation, with the variation it previously explained in the AIC model variants now attributed primarily to repression (27.5%). In addition, relatedness regained significance in this model variant, where host and plasmid identities had reduced or lost significance, indicating that these variables might explain similar variation. The BIC "forward/backward" variant collapsed to the BIC "forward/backward" model variant in the first set of models. Neither donor or plasmid identities were significant in the second set of BIC model variants.

No interactions were consistently significant across the second set of model variants, although some significant interactions were found that were also noted in the first set of model variants. The interaction between media type and media richness was significant in both BIC variants (2.2-3.2%), while the interaction between media type and pilus type was significant in both "backward/forward" variants (1.4-1.8%). Repression and pilus type (1.9%) and repression and relatedness (1.2%) were both only significant in one model variant.

The top three variables in each model variant with their interaction terms were independently removed to assess the variation that could not be attributed to other variables in the model (Table A.14). These variables were not consistent across model variants, and each term was removed from all variants if it was one of the top three explanatory variables in any of the model variants. The removal of recipient identity resulted in a 5-6% decrease in variation in the AIC models, but a decrease of about 11% in the BIC model where it

Source	DF	SS	MS	F	p		TV(%)
Recipient identity	22	1399.82	63.63	38.3	<0.001	***	34.26
Repression	1	527.31	527.31	317.39	<0.001	***	12.91
Donor Identity	19	412.65	21.72	13.07	<0.001	***	10.1
Media Type	2	123.93	61.97	37.3	<0.001	***	3.03
Plasmid Identity	46	354.97	7.72	4.64	<0.001	***	8.69
Plasmid Nativity	1	92.27	92.27	55.54	<0.001	***	2.26
Temperature	1	82.2	82.2	49.48	<0.001	***	2.01
Mating Time	1	5.36	5.36	3.23	0.073		0.13
Pilus Type	2	5.65	2.82	1.7	0.1839		0.14
Plasmid Size	1	0.03	0.03	0.02	0.8925		0
Media Richness	1	0.43	0.43	0.26	0.6128		0.01
Relatedness	1	3.39	3.39	2.04	0.154		0.08
Recipient Identity : Plasmid Nativity	1	5.81	5.81	3.49	0.0622		0.14
Repression : Mating Time	1	10.16	10.16	6.11	<0.05	*	0.25
Repression : Plasmid Size	1	14.07	14.07	8.47	<0.01	**	0.34
Media Type : Temperature	2	16.04	8.02	4.83	<0.01	**	0.39
Media Type : Pilus Type	3	73.06	24.35	14.66	<0.001	***	1.79
Plasmid Identity : Pilus Type	10	37.68	3.77	2.27	<0.05	*	0.92
Plasmid Nativity : Media Richness	1	24.34	24.34	14.65	<0.001	***	0.6
Temperature : Mating Time	1	0.06	0.06	0.04	0.8507		0
Temperature : Plasmid Size	1	6.36	6.36	3.83	0.051		0.16
Media Type : Media Richness	2	76.17	38.08	22.92	<0.001	***	1.86
Error	490	814.07	1.66	NA	NA		19.92
Total	611	4085.81	1005.51	NA	NA		100
Plasmid Size : Pilus Type	2	37.93	18.96	7.13	<0.001	***	0.93
Plasmid Nativity : Temperature	1	31.09	31.09	11.68	<0.001	***	0.76
Error	575	1530.02	2.66	NA	NA		37.45
Total	611	4085.81	2118.51	NA	NA		100

Table 2.2: Results of the simplified Model 2 (AIC, b/f) ANOVA. Original variables input are media type, pilus type, repression, temperature, media richness, donor-recipient relatedness and plasmid size, plasmid nativity, mating time and all two-way interactions. Degrees of freedom (DF), sum of squares (SS), mean sum of squares (MS), F-statistic (F), p-value (p), total variation explained (TV).

was present. This shows that although recipient identity explains a considerable variation in the AIC simplified models (34%), that variation overlaps with and can be explained by other variables, such as repression, when recipient is absent. The removal of the donor term decreased the explained variation by 4-5% where present. Repression and surface type decreased variation by 5-10% and 8-10% respectively. Medium richness was the third variable in the BIC "backward/forward" variant and decreased the explained variation by 2%.

Although the host nativity only explained a small amount of variation, it did so consistently, except in the BIC "forward/backward" variants (Figure 2.3). While most of the plasmid mating experiments occurred in non-native donors, there were a few examples of plasmid transfer from their native donors. Plasmids in native hosts had either higher and lower rates of transfer when compared with their respective non-native plasmid-host combinations. In most cases plasmids transferred at a lower rate in the native host (pPLS, pREI, pVIDO, R1). Notably, the derepressed rates of pWW0 in its native host were higher than other plasmid-host combinations with the same repression and host-nativity. Derepressed transfer rates in non-native hosts were not available for comparison, so the cause of this is unclear.

2.4 Discussion

The rate of plasmid transfer is one of the primary determinants of plasmid persistence and prevalence (Stewart and Levin, 1977), but the way that specific transfer rates are determined is unclear, particularly in the relative roles of the interacting variables which can affect them. Exploring these roles can increase understanding and enable better predictions of transfer rates and plasmid presence in natural communities. A meta-analysis was therefore conducted on plasmid transfer rates in the literature to assess the relative role of plasmid, donor and recipient identities, in addition to experimental variables, in determining the rate of transfer. Over three quarters of the variation in plasmid transfer rate could be explained, with plasmid repression and media type being the most important when host and plasmid identity were excluded. Relatedness of donor and recipient, media richness, temperature, and plasmid nativity were also significant, although to a lesser extent. Interactions between media type and media richness, relatedness and temperature or plasmid repression status were regularly significant. Pilus type was only significant in interaction with media type

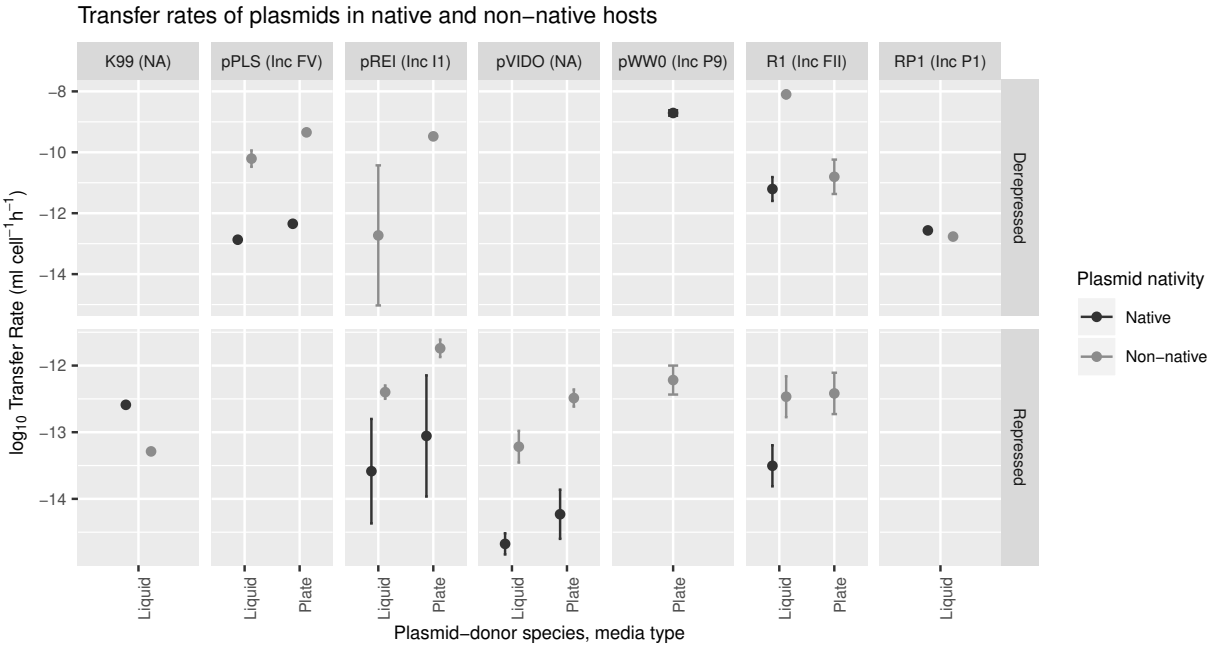

Figure 2.3: Transfer rates (± standard errors) comparing transfer of plasmids in native and non-native hosts. Grouped by plasmid repression and media type. K99 (Native donor: *E. coli* (B41), non-native donor: *E. coli* (JE2571), recipient: *E. coli* (JE2571)), pPLS (Native donor: *E. coli* (263), non-native donor: *E. coli* (JE2571), recipient: *E. coli* (JE2571)), pREI (Native donor: *E. coli* (CG140), non-native donor: *E. coli* (JE2571), recipient: *E. coli* (JE2571)), pVIDO (Native donor: *E. coli* (V374), non-native donor: *E. coli* (JE2571), recipient: *E. coli* (JE2571)), pWW0 (Native donor: *P. putida* (PaW1, PaW226), recipient from native donor: *P. putida* (PaW340), non-native donor: *P. putida* (PpS388), non-native recipient: *P. putida* (PpS388)), R1 (Native donor: *E. coli* (K-12, J53), non-native donor: *E. coli* (K-12), recipient: *E. coli* (K-12, ECOR strains)), RP1 (Native donor: *P. nov* (H2 - evolved), non-native donor: *P. nov* (H2 - unevolved), recipient: *P. nov* (H2)).

or plasmid repression status. Donor, recipient and plasmid identities explained further variation when included, with recipient identity the strongest predictor.

The repression status of plasmid transfer was consistently the major explanatory factor of the variance in transfer rate. Repression is an interesting concept in plasmid transfer, because it subverts the selection pressures on plasmids as parasitic agents to increase transmission rate. It has been suggested that high transfer rates are suppressed due to the costs of transfer (pilus production and vulnerability to phages, Haft et al., 2009). In this case, high transfer rates will only occur when the plasmid is in a new host, prior to the expression of repressors, or rarely when there are mutations in the plasmid repression genes, disabling repression. This allows plasmids to take advantage of high transfer rates when transfer is advantageous, but also reduces the costs of transfer when recipients are few, limiting costs while maximising spread. Rather than causing host-plasmid conflicts, the cost of plasmid transfer may serve to align the interests of host and plasmid. It is possible that a low rate of plasmid transfer becomes optimal due to the cost of plasmid transfer but only if the cost is great enough to affect the fitness of the plasmid. The cost required to limit transfer and the way it relates to the reported rates of derepressed and repressed transfer in the literature is currently unclear, and it would be worth exploring the costs of plasmid transfer (as opposed to presence) in both theoretical models and experiments. A non-zero rate of repressed transfer enables a residual level of plasmid motility, increasing the likelihood of persistence. Note that not all plasmids in the study had distinct repressed and derepressed rates described in their original studies (only 12 of the 50 plasmids included in the analysis had data points for both; 11 and 27 plasmids have data for derepressed and repressed pilus production respectively). This may be due to a deficiency in the data rather than the absence of multiple repression states.

Among the environmental variables, media type was consistently significant, with transfer on plates higher than in liquids. Due to the absence of the consideration of spatial configuration in the endpoint method, it is not clear whether the higher plate transfer rates represent a higher intrinsic transfer rate, or are superficial observations of "bulk" transfer rates due to cell population structure (Zhong et al., 2012). This distinction may not matter when our main consideration is plasmid propagation and persistence: transfer is more efficient on plates. Cells on solid surfaces have greater probability of forming mating complexes due to

close proximity and reduced mobility, increasing rates of transfer. While rates are higher on surfaces, the limited cell mobility reduces mixing, thereby limiting the total transfer that may be able to occur in time when a population is able to thoroughly mix. The further exploration of these ideas is needed to improve comparisons of transfer rate between surface types.

Relatedness was found to be significant in most of the model variants, where transfer within strains was broadly higher than transfer between strains, although the amount of variance it explained differed according to its position in the model. This has been previously reported (Dimitriu et al., 2016), and could either be a consequence of chance incompatability of transfer mechanisms or evidence of donor discrimination to share genes with cells of its own type to increase indirect fitness benefits (Dimitriu et al., 2016). These indirect fitness effects are further supported by the interaction between relatedness and repression, where repression was less substantial between closely related strains. It is interesting to note that although transfer is reduced between strains or species, this transfer rate is frequently non-zero. This suggests that the reduction in transfer might not be in order to prevent beneficial genes from being shared with competitors, but rather to reduce the costs of transfer. Studies that report no transfer may be the result of the experimental limits of detection.

Media richness was consistently significant in most of the model variants: low media richness resulted in a higher transfer rate in liquid experiments. This is consistent with previous results which show a higher transfer rate when substrate is scarce. Conjugation is sometimes increased as a response to stressful environments (Pearce et al., 2000), enabling the cells access to greater genetic diversity that might enable them to survive (e.g. metabolic genes). Temperature increased transfer rate consistently and was significant across all models, explaining up to 3.4% of the variation.

Pilus type and its interaction with media type were also significant in many model variants, although it only explained less than 2% of the variation. This emphasises the documented inability of plasmids without flexible pili to transfer in liquid media (Bradley et al., 1980). Flexible pili, unlike rigid pili, enable higher rates of transfer to occur when cells are in liquid media because they are able to adapt to greater cell mobility in liquids. Interestingly, in some of the models, the interaction between repression and pilus type was also significant. If this result is correct, it could speculatively be due to increased costs of flexible pili production,

with a corresponding decrease in rate when repressed to reduce this larger cost.

While the meta-analysis data is largely from non-native plasmids (non-adapted), there are several examples which show plasmid transfer rates in native hosts, with comparable non-native host transfer rates. Overall, transfer rates were higher when plasmids were in non-native hosts, consistent with some studies in the literature (Haft et al., 2009; Koraimann and Wagner, 2014). Of the seven examples in the meta-analysis, four showed a marked increase in transfer in the non-native host. These increases are expected to be examples of the absence of host control mechanisms evolved in non-selective conditions to limit transfer rate. Two cases showed little change in transfer rate. One plasmid (pWW0) showed particularly high derepressed rates of transfer in its native donor strains (PaW1, PaW226) when compared with other derepressed plasmids in their native hosts. In all other examples, coevolution between plasmid and host resulted in a decrease in transfer rate. While this difference could simply be a result of the differences in plasmid repression status, one might expect host evolution to reduce the rate of transfer to a similar level as those within that group. I hypothesise that these rates suggest that the donor comes from an environment where the plasmid traits were beneficial, enabling selection for the plasmid and complementary high transfer rates.

The effect of evolution again emphasises the role of the host in the control of plasmid transfer. As mentioned previously, while plasmids frequently contain the genes required for pilus synthesis and transmission, and are often described as being "self-transmissible" (Frost and Koraimann, 2010; Koraimann and Wagner, 2014; Silva et al., 2011; Smit et al., 1998), transfer initiation and subsequent rates are determined by both the plasmid and the host.

Of the host and plasmid identities, the recipient identity was the most important and, when the primary explanatory variable, explained over 30% of the total variation - more than any other in any model. The role of the host - and in particular the recipient - in determining transfer rate may have been understated in the past. Plasmids are often described as infectious, self-transmissible agents, although coevolution is noted to play a role in repressing transfer rate to reduce transfer costs. Some studies have shown how recipients can limit transfer through restriction systems (Roer et al., 2015), and in some cases can stimulate transfer through the use of pheromones and other signaling molecules (Chatterjee et al.,

2013; Clewell, 2011; Dunny, 2007; Koraimann and Wagner, 2014; Singh and Meijer, 2014). Although the majority of the results here are unlikely to have evolved these kinds of mechanisms, they strongly highlight the importance of the recipient in determining successful transfer. It is, however, important to note that the effects of the recipient might derive from donor discrimination against some recipients, rather than a mechanism of control by the recipient.

In conclusion, while plasmids are able to transfer at high rates, these are not necessarily selected for by the plasmid. The cost of plasmid transfer (Haft et al., 2009) might limit the maximum rates that would otherwise be found, encouraging sleeper strategies (Hall et al., 2017), minimising plasmid cost and reducing plasmid loss through segregation (Haft et al., 2009; Harrison and Brockhurst, 2012; Koraimann and Wagner, 2014; Loftie-Eaton et al., 2016; Turner et al., 1998). High transfer rates are only found in selective conditions, or during transitory derepression or when repression genes are disabled (Lundquist and Levin, 1986), limiting transfer costs while maximising spread. While rates may be low, transfer is still likely to occur at a non-zero rate into some, if not all of the species in natural microbial communities (Hall et al., 2016). These factors together increase the likelihood of persistence, even where prevalence may not be maintained at a high level and explains why the reduction of antibiotic use will not directly lead to the extinction of antibiotic resistance in the short term. Quantifying the range of transfer rates and their predictors are useful for future modelling, where transfer rate can be more accurately chosen from an expected range with a given confidence interval.

Chapter 3

The evolution of plasmid transfer rate in bacteria and its effect on plasmid persistence

3.1 Introduction

Although the work of Stewart and Levin (1977) identified the key parameters that shape plasmid dynamics, a key open question is what determines the value of fundamental traits such as transfer rate in the first place. In particular, for traits that are encoded by plasmid and/or host genetics, what causes particular values to evolve? Recent models have explored the evolution of plasmid cost and demonstrated its amelioration in theory and practice (Harrison et al., 2016; Loftie-Eaton et al., 2016, 2017; Porse et al., 2016; Zwanzig et al., 2019). Other traits require the same degree of attention, in particular transfer rate.

The evolution of transfer rates was first explored in the context of host-parasite modelling (Cressler et al., 2016; Kribs-Zaleta, 2014; Lipsitch and Levin, 1997; Lipsitch et al., 1996; Misevic et al., 2013) which is appropriate when plasmids are viewed as infectious replicators, exerting a cost on their host cells. These models identified conditions favouring vertical versus horizontal transmission (e.g. low transfer rate plasmids are favoured in high growth rate cells) but lacked detailed application to plasmid systems, especially the mutual benefits that arise in selective conditions. While later evolutionary models focused more explicitly

on plasmid transfer (Atsmon-Raz et al., 2015; Dimitriu et al., 2016; Haft et al., 2009; Hall et al., 2017; Porse et al., 2016; Raz and Tannenbaum, 2014; Turner et al., 1998), they did not provide a mathematical analysis of plasmid transfer rate parameter evolution with results for plasmid transfer rate following selection. Similarly, empirical studies report variation in transfer rates over many orders of magnitude (10^{-20}-10^{-6}), and identify statistical correlates of high versus low transfer rate (e.g. derepression, media type, host differences), but mechanistic explanations for why transfer rates vary remain scarce (Chapter 2, Sheppard et al., 2020).

Theoretical explorations into the evolutionary forces affecting plasmid transfer are challenging due to the aforementioned plasmid-host mutualisms (Carroll and Wong, 2018; Dimitriu et al., 2016) and conflicts in selective and non-selective conditions (Kottara et al., 2018). These forces can separately affect transfer genes found on the plasmid, donor and recipient that collectively determine the observed transfer rates (e.g. repression/derepression genes, Kozlowicz et al., 2006; McAnulla et al., 2007). In particular, there is empirical evidence that cells receiving the plasmids affect the rate of transfer (i.e. different recipient strains cause different transfer rates, Reniero et al., 1992; Sansonetti et al., 1980), but this has rarely been explored theoretically.

Analysis of transfer rate evolution also needs to evaluate all possible sources of costs on the host. Traditionally, models have considered costs of carrying a plasmid that were independent of the transfer rate (Harrison et al., 2016), but there are multiple ways that plasmids can incur a cost on the host cells. For example, plasmids can confer metabolic costs to the host due to plasmid replication and gene expression, including the expression of plasmid transfer and pilus production genes, and additional costs through greater susceptibility to infection by phage (Dimitriu et al., 2019b; Jalasvuori et al., 2011; Porse et al., 2016; Reinhard et al., 2013; San Millan and MacLean, 2017; Turner et al., 1998). Some plasmids, however, do not demonstrate the same negative fitness effects often caused by high rates of transfer (Shapiro and Turner, 2014; Turner et al., 2014). For investigating the control of transfer rate, the key theoretical distinction is whether plasmid cost depends on the transfer rate or not if it does, this could provide a check on the evolution of high transfer rates.

Here, I present new theory on the evolution of transfer rates that considers the perspective of the different partners in the system and a wider range of cost types that can arise. We

explore how evolution shapes and determines plasmid transfer rates using adaptive dynamics theory, focussing on the costs of plasmid presence, transfer and host-plasmid conflicts. I also investigate how the selected rates of transfer and host-plasmid conflicts affect plasmid prevalence in bacterial populations. Published data and parameter estimates from the well-studied R1 plasmid in *E. coli* (Haft et al., 2009) are used as a case study to place model predictions in realistic parameter space and to estimate new parameters proposed here. I aimed to keep my model as simple as possible by building on earlier chemostat models, while including some variants to explore further complexity (e.g. superinfection, the ability of a plasmid-bearing donor toinfect other donor cells, Smith, 2011). The potential impact of the model simplifications in relation to the incredible complexity of plasmid biology in nature are considered in the discussion.

3.2 Models and analyses

The model, based on the bacterial conjugation model by Stewart and Levin (1977), specifies the growth of an evenly mixed, homogeneous, single-species bacterial population with plasmid-free (density, N) and plasmid-bearing (density, N_P) cells (variables and parameters listed in table 3.1 and a schematic diagram given in Appendix B.1, Fig B.1).

$$\frac{dN}{dt} = rNS - DN - \gamma_N N_P N + \tau r_P N_P S \tag{3.1}$$

$$\frac{dN_P}{dt} = (1-\tau) r_P N_P S - DN_P + \gamma_N N_P N \tag{3.2}$$

$$\frac{dS}{dt} = D(S_0 - S) - yr(N + N_P)S \tag{3.3}$$

Cells grow utilising a single substrate (concentration, S), which flows into the chemostat at a dilution rate (D), from a source with a constant concentration (S_0). Chemostat models assume a well mixed population and continuous inflow of resources/outflow of cells/resources, and are used here for ease of analysis, consistent with previous studies of bacterial dynamics

(Stewart and Levin, 1977). I discuss the likely effects of alternative growth conditions on our results below. Plasmid free and plasmid bearing cells grow at rates of r and r_P, respectively, which differ by the net cost or benefit given by the plasmid. I model the difference in two ways: as a flat cost/benefit of plasmid presence, due to metabolic activity, or a cost proportionate to the rate of transfer due to the costs of transfer (San Millan and MacLean, 2017, metabolic and transfer related costs are considered together in Appendix B.9). Cell growth is directly proportionate to substrate concentration for simplicity (Monod kinetics are considered in Appendix B.9 and the addition does not make qualitative differences to the results) scaled by a yield coefficient (y). Cells and substrate are lost from the chemostat through dilution at rate D. Plasmids are lost from donor cells through unequal segregation during cell division at a constant rate (τ), which is accordingly proportional to the growth of N_P. Plasmid transfer (γ_N) occurs at a rate proportional to the mass action product of the interaction of N and N_P, with a coefficient assumed to be constant. This model deviates from Stewart and Levin (1977) in that donors and recipients have the same resource consumption rate (r), despite the different growth rates, and it is assumed that resources are reallocated away from growth to plasmid maintenance and transfer. I also model plasmid loss as a proportion of donor cell growth rather than as a proportion of donor cells. These changes are made to provide greater biological realism, emphasising the energy requirements for plasmid gene expression (San Millan and MacLean, 2017) and the mechanism of plasmid loss during cell division (Haft et al., 2009). Additional parameters and processes are further described in the specific models where they appear.

Using this population model to define my system, I then calculate the fitness of an entity with a mutant trait in terms of its ability to invade the equilibrated resident population when initially rare. I use the rate at which rare mutants invade the resident population as the measure for fitness (Geritz et al., 1998; Metz et al., 1992), described mathematically by the sign of the differential equation showing the change in mutant population size with time

$$\frac{dM}{dt} > 0 \tag{3.4}$$

where M represents a mutant. Specifically, I consider the fitness of invading mutant plasmids (N_M) and hosts (M), in turn, that alter the transfer rate (γ_M). Plasmids and hosts receive different selection pressures that can impact and determine the evolution of plasmid transfer

rate. Establishing the effects of selection on each can enable us to understand the relative role of the host and plasmid, particularly when the pressures on transfer rate are in conflict. Two versions of the model were considered: in model 1, the plasmid has a constant cost or benefit to the host growth rate that is independent of the plasmid transfer rate (i.e. metabolic and replicative effects); whereas in model 2, the cost or benefit of the plasmid is proportional to plasmid transfer. When considering the invasion of a mutant host I also draw out the roles of the donor and recipient in the invasion. For each invasion scenario, the models were analysed mathematically by hand and using Mathematica (Wolfram Research Inc., 2020) to identify the parameter conditions that permit the invasion of the mutant from rare. I then bring together the results of the two models to investigate the impacts of host-plasmid conflicts on plasmid prevalence and persistance.

Adaptive dynamics makes a number of assumptions including clonal reproduction, rare mutations, incremental phenotypic effects of mutations and time-scale separation of ecology and evolution Geritz et al. (1998). The results are therefore correct when these principles apply, although the results are still likely to be informative in terms of the general patterns that they demonstrate even if not all assumptions are met. Bacteria are clonal and have fast reproduction times, facilitating replacement of a resident population by a fitter mutant. Transfer rates have been measured at regular intervals on a continuous scale (Sheppard et al., 2020, Chapter 2), indicating that small differences in transfer rate may be possible through mutation, although other mutations (such as permanent plasmid derepression) can be exceptionally large. These kinds of mutations may require further analysis.

The text is kept mathematically abstract for applicability to any plasmid system, while results are plotted with estimated parameter values of the R1 conjugative plasmid in *E. coli* measured in lab experiments and reported in Haft et al. (2009), with recipient growth rate: $r = 1.459$ h^{-1}, loss rate: $\tau = 10^{-4}$, substrate concentration: $S_0 = 200$ μg ml^{-1}, and yield coefficient: $y = 8 \cdot 10^{-8}$ μg cell^{-1}. The data from this study were chosen because they include the majority of the required parameters for a single plasmid, including repressed and derepressed growth and transfer rates that enable the calculation and of metabolic and transfer costs. Dilution rate (D) was not available and was set to 0.001 ml h^{-1}. This allowed us to visualise functions in a potentially realistic parameter space, increasing the application of the results. In a few graphs some parameters needed to be adjusted to show the shape

Table 3.1: Model variables, key parameters and metrics.
Values taken from Haft et al. (2009).

		Units	Description
Variables			
S		μg ml^{-1}	Substrate concentration
N, N_P, N_M		cells ml^{-1}	Wild-type recipient and donor (wild-type plasmid: P, mutant plasmid: M) cell densities
M, M_P		cells ml^{-1}	Mutant recipient and donor (wild-type plasmid: P) cell densities
Key parameters			
S_0	200	μg ml^{-1}	Substrate stock concentration
D	0.001	ml h^{-1}	Dilution rate
r	1.459	h^{-1}	Recipient growth rate
r_P	1.405	h^{-1}	Donor growth rate (repressed)
y	$8{\cdot}10^{-8}$	μg cell^{-1}	Yield coefficient
τ	10^{-4}		Plasmid loss rate
γ_N, γ_{NN}	$4.4{\cdot}10^{-12}$	ml cell^{-1} h^{-1}	Wild-type plasmid transfer rate (repressed)
r_M		h^{-1}	Mutant donor growth rate
γ_M, γ_{MM}		ml cell^{-1} h^{-1}	Mutant plasmid transfer rate
γ_{NM}		ml cell^{-1} h^{-1}	Transfer rate from wild-type to mutant
γ_{MN}		ml cell^{-1} h^{-1}	Transfer rate from mutant to wild-type
b	$4.61{\cdot}10^{7}$	cell ml^{-1}	Transfer rate cost coefficient
Metrics			
γ_{sel}		ml cell^{-1} h^{-1}	Selected transfer rate
γ_{min}		ml cell^{-1} h^{-1}	Minimum transfer rate for plasmid persistence
γ_{90}		ml cell^{-1} h^{-1}	90% plasmid prevalence transfer rate
P_{Prev}		%	Plasmid prevalence

of the curves clearly as the parameters change, detailed in the figure legends.

3.2.1 Model 1: Plasmid costs independent of transfer rate

Mutant plasmid invasion (N_M)

I assume that each cell can only contain one plasmid, either the wild-type (at cell density N_P) or the mutant (at cell density N_M). Cells bearing the mutant plasmid are identical to cells that carry the wildtype, except in the rate of plasmid transfer (γ_M) and change density as follows:

$$\frac{dN_M}{dt} = (1-\tau)r_P N_M S^* - DN_M + \gamma_M N_M N^* \tag{3.5}$$

where S^* and N^* are the steady state equilibria of equations 3.1-3.3. The fitness of the mutant plasmid (W_{N_M}) is equal to the per capita change in density N_M from rare when the system (Eq. 3.1-3.3) has reached equilibrium. Rearranging (see Appendix B.2) gives the invasion success (fitness) of the plasmid:

$$W_{N_M} = \frac{1}{N_M}\frac{dN_M}{dt} = N^*(\gamma_M - \gamma_N) \tag{3.6}$$

A mutant plasmid can invade therefore when $W_{N_M} > 0$, which for positive solutions requires $N^* > 0$ and $\gamma_M > \gamma_N$. The first condition indicates that in order for a mutant plasmid to invade the population must contain recipients. Plasmid transfer cannot occur in the absence of recipients, and therefore selective pressures do not affect the rate of transfer. The second condition states that the mutant can only invade when its transfer rate is higher than the wild-type. Evolutionary pressures on the plasmid will, therefore, select only for an increase in the rate of transfer, regardless of other circumstances.

Mutant host cell invasion (M, M_P)

Mutant host cells can be plasmid-free (density M) or plasmid-bearing (density M_P), and their dynamics are identical to N and N_P respectively, except for the rate of plasmid transfer (γ_M, where the rate of transfer is now defined by the host, not the plasmid as in the previous analysis). The equations are also updated to emphasise the roles of donors and recipients in determining transfer (see Appendix B.3), where the first and second subscripts are the donor and recipient respectively ($\gamma_N = \gamma_{NN}$, $\gamma_M = \gamma_{MM}$), and transfer from wild-type to mutant is included (γ_{NM}). Transfer from mutant to wild-type does not affect the mutant invasion under these assumptions and is not included. The resulting mutant growth equations are:

$$\frac{dM}{dt} = rMS - DM - \gamma_{MM}M_PM - \gamma_{NM}N_PM + \tau r_PM_PS \tag{3.7}$$

$$\frac{dM_P}{dt} = (1-\tau)r_PM_PS - DM_P + \gamma_{MM}M_PM + \gamma_{NM}N_PM \tag{3.8}$$

These mutant cells invade the original bacterial population at steady state (Eq. 3.1-3.3). The rarity of mutant interactions allows simplification and linearization of the system of

equations allowing the calculation of the fitness equation (see Appendix B.3):

$$W_M = (\gamma_{NM} - \gamma_{NN})N_P^*(r_P S^* - D) \tag{3.9}$$

where S^* and N_P^* are the steady state equilibria of equations 3.1-3.3. A mutant host cell can invade when two conditions are met: 1. $N_P^* > 0$, and 2. $(\gamma_{NM} - \gamma_{NN})(r_P S^* - D) > 0$. The first condition requires the presence of plasmids for mutant host cells to invade, necessary for the evolution of plasmid transfer rate. Without plasmids, the mutant is indistinguishable from the wild-type, and the plasmid transfer rate is meaningless. The second condition requires that the product of two terms be positive, which can be achieved in two ways: 1. when $\gamma_{NM} - \gamma_{NN} > 0$ and $r_P S^* - D > 0$, and 2. when $\gamma_{NM} - \gamma_{NN} < 0$ and $r_P S^* - D < 0$.

The first term describes the relationship between the original transfer rate (γ_{NN}) and the rate of transfer from the wild-type to the mutant (γ_{NM}). γ_{NM} is determined by the genotype of N as a donor and M as a recipient, and mutants with higher or lower transfer rate can invade depending on whether $r_P S^* - D$ is positive or negative. Further analysis of the sign of the term $r_P S^* - D$ reveals identity with the sign of the effect (positive or negative) of the plasmid on the hosts growth (Appendix B.3, Fig. B.2).

$$sign(r_P S^* - D) = sign(r_P - r) \tag{3.10}$$

This indicates that mutant cells with a transfer rate lower than the wild-type can invade when $r_P S^* - D$ is negative: when the plasmid is costly ($r_P < r$, Fig. 3.1A). In contrast, mutant cells with a transfer rate higher than the wild-type can invade when $r_P S^* - D$ is positive: when the plasmid is beneficial ($r_P > r$, Fig. 3.1B). In summary, when the plasmid is beneficial the selection drives an increase in plasmid transfer rate, and when the plasmid is costly selection drives a decrease in the transfer rate by selecting for host cells with a higher/lower propensity of transfer.

Importantly, the change in transfer rate comes as a result of a mutation in γ_{NM}, not γ_{MM} or γ_{MN}, i.e. where the mutant host cell is a recipient, not a donor. This makes biological sense: it will be unlikely for a rare mutant host cell to encounter a fellow mutant host cell in a well mixed environment. The vast majority of the mutant host encounters will be with

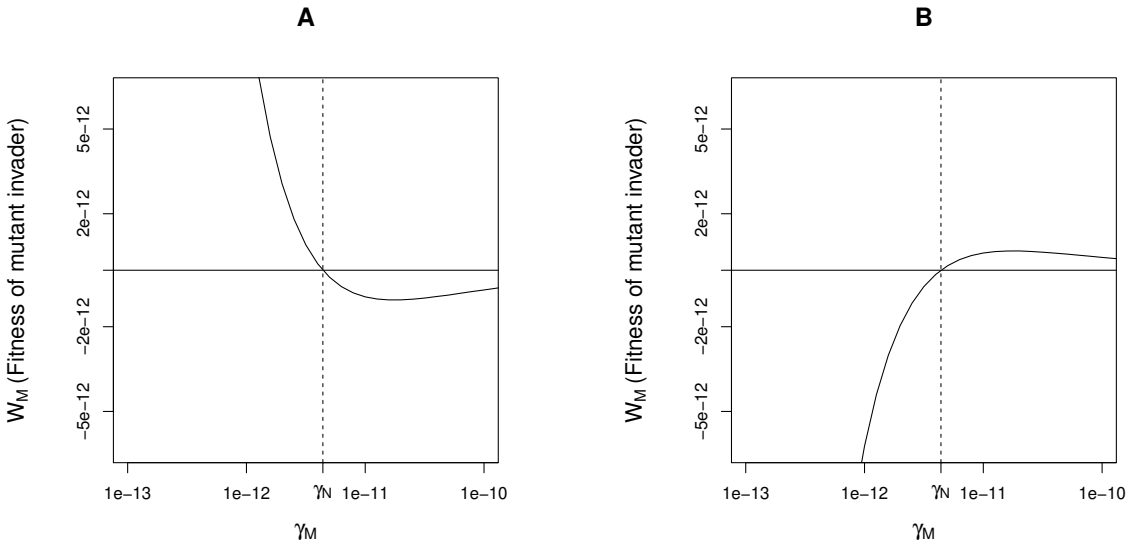

Figure 3.1: Mutant fitness (W_M) as mutant transfer rate changes (γ_M), where the plasmid is costly (A, $r_P = 1.4$ h^{-1}) and beneficial (B, $r_P = 0.15$ h^{-1}). Parameter assignments as default.

wild type cells. From the point of view of a mutant host, the only interactions that matter here are the ones where a plasmid is received, because the plasmid costs and benefits are conferred in reception of a plasmid, not in donation. According to these model assumptions, plasmid donation does not affect the host fitness. Therefore the fitness is determined by $\gamma_{NM} - \gamma_{NN}$: the difference between plasmid receipt in a rare mutant host compared to receipt in a wild type host in that same population. This indicates that the trait under selection is the reception propensity of the mutant recipient, and in this model is the mode by which evolution of transfer rate occurs. This is contrary to studies that describe plasmid transfer as infectious and driven or affected by the donor rather than the recipient (De Gelder et al., 2005; Frost and Koraimann, 2010), although recipient control can be difficult to distinguish from donor discrimination. In other respects, the results of model 1 conform with the idea that high transfer rates are always favourable for the plasmid, irrespective of conditions, whereas a high transfer rate is only selected in hosts when plasmids are beneficial and plasmid-free recipients are present in the population. While the assumption that transfer is not costly for the donor cell is unrealistic (San Millan and MacLean, 2017), and is explored in model 2, these results reveal selection pressures affecting recipient cells that can lead to recipient-led control of plasmid transfer.

3.2.2 Model 2: Plasmid costs depend on transfer rate

While donor and recipient growth rates were previously independent of other parameters, the growth rate is now assumed to decline with increasing transfer rate in proportion to a transfer-cost coefficient b (Eq. 3.11). The precise relationship between cost and transfer is unknown, except that transfer rates and costs are related. This means that plasmid cost is only determined through plasmid transfer and that plasmids cannot be beneficial under these assumptions as this would require a negative transfer rate. Because host and plasmid interests only differ when plasmids are costly, we focus on this scenario from now on. We do not explicitly model selection on the host transfer rate using model 2 because the result is too complex and does not simplify enough to be intuitive using these methods, but use an alternative way to explore host-plasmid conflict in the next section.

$$r_P = r - b\gamma_N \tag{3.11}$$

An empirical estimate of plasmid cost coefficient (b) was calculated using the transfer and growth parameters of repressed (γ_N, r_P) and derepressed (γ_{N_D}, r_{P_D}) plasmids reported in Haft et al. (2009), assuming a linear relationship between donor growth rates and plasmid transfer rates. Again, the estimation of this parameter is speculative, but we make these assumptions based on the greater costs of higher transfer rates in the absence of adequate quantitative data. This estimate is used along with the other Haft et al. (2009) parameters to draw visual representations of the results in figures, and to provide a speculative parameter space to put those results into a meaningful context.

$$b = \frac{r_P - r_{P_D}}{\gamma_{N_D} - \gamma_N} = \frac{1.405 - 1.23}{3.8 \cdot 10^{-9} - 4.4 \cdot 10^{-12}} = 4.61 \cdot 10^7 \,\mathrm{cell\,ml^{-1}} \tag{3.12}$$

Analysis of the system of equations (3.1-3.3 where 3.11) reveals only one real, non trivial steady-state solution that is used in the following sections, not given here due to its length and complexity (see Appendix B.4 for details).

Mutant plasmid invasion (N_M)

The equation for the invading mutant plasmid differing only in transfer rate (γ_M, repeated here, and once again defined by the plasmid):

$$\frac{dN_M}{dt} = (1-\tau)r_M N_M S^* - DN_M + \gamma_M N_M N^* \tag{3.13}$$

and modified as follows to capture the transfer-cost relationship:

$$r_M = r - b\gamma_M \tag{3.14}$$

The described transfer-cost relationship is based on the relationship between increased transfer rate and cost to the host. While the specific relationship is unclear, there is a positive correlation between the two and this analysis assumes that transfer is the only cost to host growth (although additional replicative/metabolic costs are considered in appendix B.9). Rearranging the equations (see Appendix B.5) gives the per capita fitness equation:

$$W_{N_M} = (\gamma_M - \gamma_N)\frac{rN^* - bD}{r_P} \tag{3.15}$$

Mutant plasmids cannot invade when $W_M \leqslant 0$. This means that a mutant with a higher or lower transfer rate can invade when $rN^* - bD$ is positive or negative, respectively (Fig. 3.2). r_P must always be positive for donor viability and biological realism. Selection therefore acts on transfer rate towards the point where the equilibrium density satisfies:

$$N^* = \frac{bD}{r} \tag{3.16}$$

(see Fig. 3.2).

We name the transfer rate at which this occurs γ_{sel}. When this condition (Eq. 3.2.2) is used in conjunction with the real, non trivial solution previously established (Appendix B.4) we find:

$$\gamma_{sel} = \frac{r\tau}{b}\frac{rS_0(1-\tau) - D}{rS_0(1-\tau) - D(1+yb)} \tag{3.17}$$

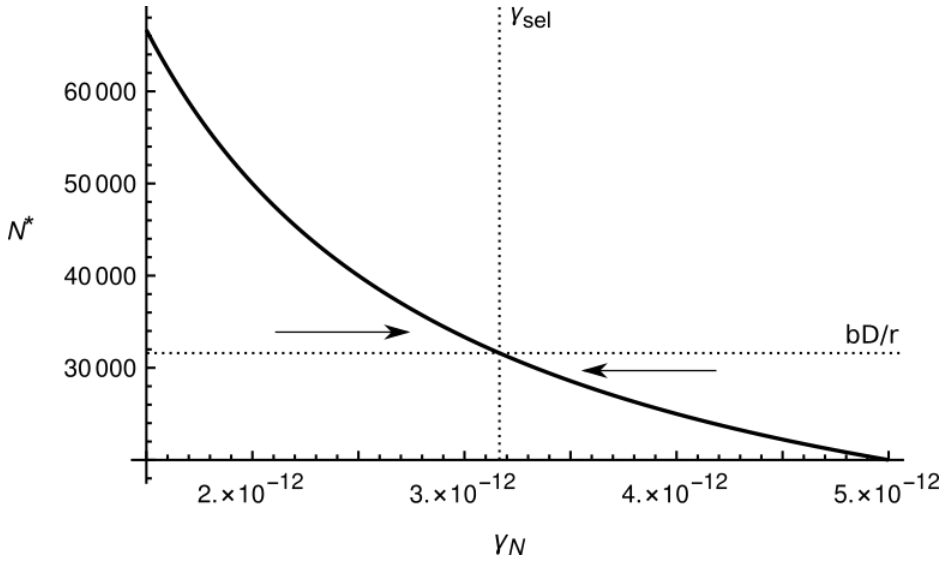

Figure 3.2: Equilibrium density of recipients, N^*, as a function of transfer rate, γ_N, demonstrating the effects of selection on γ_N. The figure identifies bD/r (the value of the equilibrium for N^* above which there is selection for larger γ_N, and below which for smaller γ_N). Selection leads to γ_{sel}, with arrows indicating the direction of selection.

Using the methods described by Geritz et al. (1998, see Appendix B.6) we find that γ_{sel} is always convergence stable, meaning that selection drives the transfer rate towards γ_{sel}, and has neutral evolutionary stability. For the linear trade-off that we have used (Eq. 3.14), this means that once the plasmid population has attained transfer rate γ_{sel}, mutants with different transfer rates will be able to reach appreciable frequencies by drift leading to the accumulation of diversity in the transfer rate. This is because selection pressures acting on plasmid transfer rate become weaker when the recipient density is in balance with the other parameters (when N^* approaches bD/r, W_{N_M} approaches 0). When mutant plasmids persist and increase in density through drift the recipient density changes (either increasing or decreasing) increasing the strength of selection on the plasmid population again. These results have implications for measuring transfer rates, as mutants with a wide range of transfer rates would be present at low frequency within the population and can constitute a reservoir of standing variation on which selection could quickly act in changing conditions.

The effect of each parameter on γ_{sel} is not immediately apparent due to the complexity of the equation (3.17). By varying the parameters over a realistic parameter space, around the Haft et al. (2009) data, we were able to identify two kind of behaviour (Appendix B.7, Fig. B.5, B.6). The first behaviour is when $D(1 + yb)$ is small compared with $rS_0(1 - \tau)$ that

enables the simplification of γ_{sel} to

$$\gamma_{sel} \approx \frac{r\tau}{b} \tag{3.18}$$

This simplification occurs when using the parameters given by Haft et al. (2009) and a low dilution rate as inputs. γ_{sel} therefore increases as the growth and loss rates increase and decrease as the transfer cost coefficient increases (Appendix B.7, Fig. B.5). With the empirical estimates for τ, r and b from Haft et al. (2009) the predicted value for γ_{sel} is $3.165 \cdot 10^{-12}$, which is remarkably close to the experimental estimate for repressed plasmid transfer rate of $4.4 \cdot 10^{-12}$.

The second behaviour occurs when the simplification cannot be made (i.e. when D, y, and b are high in reference to r, S_0 and τ, Appendix B.7, Fig. B.6). In these conditions γ_{sel} increases exponentially as each parameter becomes less conducive to plasmid persistence, until the denominator approaches 0: $rS_0(1-\tau) = D(1+yb)$. For some parameters, this reverses the relationship so that increasing b and decreasing r begin to increase γ_{sel} rather than decreasing γ_{sel} as found previously (compare Appendix B.7, Fig. B.5 with B.6, row 1, r and b).

When costs depend on transfer rate, the selection of transfer rate no longer drives it infinitely higher, but instead towards an intermediate value that balances transmission with the costs incurred on the host. We also see that the equation for this intermediate value simplifies under conditions of low dilution rate, among others.

3.2.3 The effects of γ_N on plasmid prevalence

We now investigate the effect of host-plasmid conflicts on plasmid prevalence in non-selective conditions. We previously found that selection acting on the host drives transfer rate down in non-selective conditions until the plasmid goes extinct (model 1), while selection acting on the plasmid drives the plasmid transfer rate to γ_{sel} when the cost is dependent on transfer rate (model 2). Assuming that both these selection pressures are at play, it is reasonable to expect that the combined selection pressures result in a value of γ_N that is between the transfer rate resulting in plasmid extinction (i.e. the value that should be optimum from the host's perspective, which we name γ_{min}) and γ_{sel} due to host-plasmid conflict. We therefore investigate the effects of transfer rates on plasmid prevalence in this range. Specifically, we

ask how much would the host need to reduce transfer rate in order to impact the plasmid prevalence in the population?

We start by using model 2 as a foundation for calculating plasmid prevalence (P_{Prev}, calculated in Mathematica, Wolfram Research Inc., 2020) and γ_{min} (see Appendix B.8 for details):

$$P_{Prev} = \frac{N_P^*}{N^* + N_P^*} = 1 - \frac{bDy}{rS_0(1-\tau) - D} \qquad \gamma_{min} = \frac{rDy\tau}{rS_0 - D(yb(1-\tau)+1))} \tag{3.19}$$

Similarly to γ_{sel}, these equations exhibit two kinds of behaviour as parameters vary (Appendix B.7, Fig. B.5, B.6). The first behaviour occurs when some parameters (D, y, b) are low compared with others (r, S_0) allowing simplification to:

$$P_{Prev} \approx 1 \qquad \gamma_{min} \approx \frac{Dy\tau}{S_0} \tag{3.20}$$

We immediately see that plasmid prevalence is likely to be at saturation (≈ 1) in these simplifying conditions (i.e. when bDy is small compared with $rS_0(1-\tau) - D$). Also, we see the simplified γ_{min} increase as yield coefficient, dilution and loss rate increase, and as substrate concentration decreases (Appendix B.7, Fig. B.5). Although there is a slight variation between the points at which these three metrics (γ_{sel}, P_{Prev} and γ_{min}) simplify, the main factors which allow these simplifications are low dilution, yield and plasmid cost, relative to higher bacterial growth and substrate concentration. Under appropriate conditions (e.g. low dilution rate, chemostat environment) these simplifications may provide a quick and easy way to estimate the expected plasmid transfer rate when the plasmid is in control of transfer, and by how much that exceeds the minimum rate required for plasmid persistence.

When the simplification cannot be made γ_{min} increases exponentially, similarly to γ_{sel}, although reaching a slightly different limit: $rS_0 = D(yb(1-\tau)+1)$. The difference between the limits of each metric mean that the values of γ_{sel} and γ_{min} diverge as the system moves into the exponential part where they may have previously been converging (Appendix B.7, Fig. B.6). A small change in any parameter can result in a large difference in all three

metrics when in this exponential part, limiting the precision of any applications of the model results. The exponential increase is where we also observe substantial decrease in plasmid prevalence over a relatively small parameter space (see Appendix B.7, Fig. B.6, row 4: τ, b, D, y), indicating a narrow region where sub-saturation levels of plasmid prevalence can be found when the system is at equilibrium.

To investigate the region where plasmid prevalence undergoes this sharp decreases, we now introduce a fourth metric: γ_{90} to indicate the transfer rate at 90% plasmid prevalence and which can be compared with the other metrics. First, γ_{90}, γ_{sel} and γ_{min} were plotted over an increasing dilution rate to map the change from simple to complex exponential behaviour (Fig. 3.3). γ_{90} was about an order of magnitude higher than γ_{min}, and the two metrics increased proportionately with each other for the majority of the parameter space investigated. γ_{sel} was largely unaffected by changes in D (as expected from Eq. 3.18), which means that the difference between γ_{sel} and γ_{90} can potentially be several orders of magnitude dependent on the specific parameter values. This prospective difference between γ_{sel} and γ_{90} indicates that the host may need to reduce transfer rate considerably to affect plasmid prevalence substantially. When the metrics reach the exponentially increasing part of their curves, γ_{90} increases above γ_{sel}, indicating that a transfer rate of γ_{sel} produces an intermediate plasmid prevalence ($< 90\%$), suggesting that intermediate plasmid prevalence is only achieved when the transfer rate is within an order of magnitude of γ_{min}.

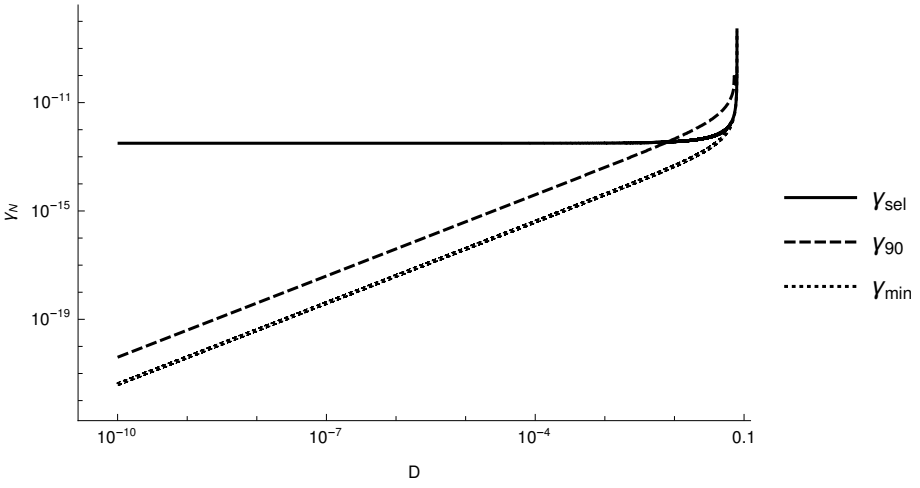

Figure 3.3: Variation in the plasmid selected transfer rate (γ_{sel}), the host selected transfer rate (γ_{min}), and the transfer rate resulting in 90% prevalence for the plasmid (γ_{90}) as dilution rate increases. Parameter assignments as default except $b = 4.61 \cdot 10^8$ cell ml^{-1}, $y = 8 \cdot 10^{-6}$ μg cell^{-1}.

We then compared γ_{90} with γ_{sel} and γ_{min} across a range of reasonable parameter combinations (see Table 3.2 legend for details). The dilution rate was set at 10^{-6} ml h^{-1} to enable metric simplifications to occur across the majority of the parameter space. Results which did not include a positive γ_{sel} or where $\gamma_{sel} < \gamma_{min}$, and results where the algorithm used to estimate γ_{90} failed due to numerical rounding errors were filtered out.

Regardless of parameter combinations, there was consistently a difference of about one order of magnitude between γ_{min} and γ_{90}, contrasting with the several orders of magnitude between γ_{90} and γ_{sel} (Table 3.2). The consistent difference suggests that, similarly to γ_{min}, γ_{90} is approximately proportional to $\tau Dy/S_0$, adjusted by a factor of 10. The relatively small difference further means that when there is a large difference between γ_{sel} and γ_{min} the host would need to reduce the rate of transfer by several orders of magnitude before plasmid prevalence is substantially affected. For some parameter combinations γ_{sel} was lower than γ_{90}, indicating intermediate plasmid prevalence at γ_{sel} due to the difference of less than an order of magnitude between γ_{sel} and γ_{min}. In summary, the results show that a decrease in transfer rate due to host-plasmid conflicts will result in little change in plasmid prevalence until the rate of transfer is within an order of magnitude of the minimum transfer rate. In many cases, the host would need to decrease the rate of transfer substantially before plasmid prevalence is affected.

Table 3.2: The average differences between the plasmid selected rate of transfer (γ_{sel}) and the transfer rate that determines 90% plasmid prevalence (γ_{90}), and between γ_{90} and the host selected rate of transfer (γ_{min}) with standard deviations over a range of parameters at increments of an order of magnitude unless otherwise stated: growth rate (r: 2×10^{-4} to 2), plasmid loss rate (τ, 10^{-7} to 10^{-1}, increments of 2 orders of magnitude), plasmid cost coefficient (b, 10^5 to 10^9), stock substrate concentration (S_0, 0.2 to 200, increments of half an order of magnitude), yield coefficient (y, 10^{-10} to 10^{-6}). Dilution rate (D) set to 10^{-6} to enable simplifications to occur.

	Mean	SD	Min	Max
γ_{sel}-γ_{90}	5.186	2.570	-1.127	12.601
γ_{90}-γ_{min}	1.001	0.007	0.995	1.232

3.2.4 Variations on the model results

Three further variants of the model were used to investigate plasmid metabolic and transfer costs simultaneously, and the effect of a more complex (Monod) growth function (Appendix

B.9). The plasmid metabolic cost (a) was estimated at 0.054 h^{-1} using the Haft et al. (2009) data, smaller than the costs incurred from derepressed rates of transfer (estimated at 0.175 h^{-1}), and far higher than transfer costs at repressed transfer rates (0.0002 h^{-1}). These variations did not qualitatively alter the results. In addition, a version of the model was created in which a plasmid can transfer into a cell already containing a plasmid (superinfection). Although this made some changes to the equation, with constant plasmid costs these changes act to cancel each other out and so do not affect the broad results (Appendix B.10).

3.3 Discussion

To investigate the evolutionary pressures that affect plasmid transfer rate we constructed two mathematical models with different cost functions, that were analysed using adaptive dynamics and invasion analyses. The models gave very different results, highlighting the importance of the plasmid cost function for realistic evolutionary outcomes. Model 1 has a fixed cost of plasmid replication and metabolism. It predicts that plasmid-controlled transfer always selects for a higher transfer rate, as long as recipients are present, whereas host-controlled transfer can select for either increasing or decreasing transfer rate, leading to host-plasmid conflicts when the plasmid gives a net cost. Analysis showed that selection on host-controlled transfer rate acts on recipient ability to receive plasmids, rather than in donor ability. These findings are limited by the assumptions of the model that plasmid cost is not linked with transfer rate, and thus conflict with the results of model 2 that include the costs of plasmid donation. Analyses of model 2 show that plasmid-controlled transfer rate converges to an equilibrium that is convergence stable but has neutral ESS-stability, enabling standing variation in transfer rates at low frequencies. Transfer rates between this selected rate of transfer and transfer rates that cause plasmid extinction were explored to investigate the effects of host-plasmid conflicts in non-selective conditions and showed that the host must evolve mechanisms that reduce the transfer rate substantially in order to reduce plasmid prevalence. We now discuss the implications of these results in turn for interpreting plasmid dynamics in nature.

3.3.1 Predicting transfer rates assuming plasmid control

The prediction of the first model that selection always drives a higher plasmid transfer rate when the plasmid is in control is inconsistent with the literature, where low plasmid transfer rates are frequently observed experimentally (Sheppard et al., 2020) and plasmids often contain transfer-repression genes (Fernandez-Lopez et al., 2014; Haft et al., 2009; Lundquist and Levin, 1986). This inconsistency is due to the absence of transfer-dependent plasmid cost in the initial model. The addition of variable plasmid costs connected to the transfer rate (shown in model 2) demonstrate that selection on plasmid-controlled transfer rate finds an equilibrium. Equilibria balancing transmission and cost are well-known in parasite-host modelling (Cressler et al., 2016; Gibson et al., 2015; Lipsitch et al., 1996; Magalon et al., 2010; Turner et al., 1998), and the results of our models demonstrate the importance of accurate representation of plasmid cost relationships when modelling plasmid transfer.

An interesting result from model 2 is that the selected equilibrium transfer rate has neutral ESS stability, enabling standing variation in transfer rates to persist at low frequency. If true, this variation would provide a resource for selection to act on under changing conditions, increasing the potential for rapid plasmid adaptation. The variation in transfer rates might also complicate experiments attempting to measure plasmid transfer rate, where a single clone may not be representative of the dominant behaviour. Other models have identified conditions where multiple plasmids with different transfer rates may invade and coexist based on the trade-offs in transfer rate and plasmid cost (when a low transfer, low cost plasmid prevents invasion of an incompatible high-transfer, high-cost plasmid in some cells, Lipsitch et al., 1996; Van Den Bosch et al., 2010; Van der Hoeven, 1984, 1986). These models describe the invasion conditions where coexistence is possible, but do not use adaptive dynamics to look at how the rate of transfer evolves based on the relationship between plasmid cost and transfer rate. Coexistence conditions do not appear to occur when the transfer rate is convergence stable.

The simplified equation for the selected transfer rate (γ_{sel}) in model 2 shows that plasmid-controlled transfer rate could be predictable from only three parameters (bacterial growth rate, plasmid loss rate, plasmid transfer cost coefficient), approximately predicting the experimentally measured repressed transfer rate from the Haft et al. (2009) data. While the equation could provide a useful and simple metric to predict the plasmid-controlled selected

rate of transfer, the conditions where this metric would be directly applicable are extremely limited, requiring the coevolution of host and plasmid in a chemostat system where the plasmid has complete control over its transfer. Chemostat models assume that there is constant inflow of resources and outflow of resources/waste and cells and therefore only apply directly to industrial systems where this is true. These conditions also favour fast cell growth at low substrate concentrations and keep cells in active growth phase, contrasting with batch-transfer models that assume periodic arrival of resources followed by dilution, selecting for cells that can survive lean periods and dispersal. While direct applications of the model results may be limited, the principles may be broadly applied to similar natural systems with flowing resources (e.g. rivers, the animal gut), although the extent to which this can be done is unknown and requires further investigation, including appropriate parameter sets and results. While plenty of data exists for some of the parameters (e.g. bacterial growth rate, transfer rate) there are few data for others such as transfer cost coefficient or plasmid loss rate, and even fewer examples where all required parameters are found for a single plasmid in an environment (Sheppard et al., 2020).

Considering each parameter in the equation for γ_{sel} in turn, bacterial growth rate is proposed to increase the selected rate of transfer. This is contrary to some parasite-host models which find that high growth rates promote lower transfer and a reduction in virulence (Dusi et al., 2015; Magalon et al., 2010). These models emphasise that an increase in growth rate leads to greater benefits from vertical parasite transmission. The parasite must mediate between the potential benefits and costs of its transfer, which vary between parasite type. In our model, however, constant plasmid costs (and corresponding transfer rates) are proportionately higher for strains with a low growth rate and mean that selection pressures on the plasmid drive the transfer rate down as growth decreases to facilitate survival. In addition, an increase in overall growth rate also increases the growth rate of donor cells, permitting the maintenance of plasmids with higher costs and corresponding transfer rates without substantially affecting plasmid densities. Based on the adaptive dynamic modelling here, low transfer rate strategies may be susceptible to invasion by plasmids with higher transfer rates, resulting in rates higher than those that may actually be optimal, although some plasmid mechanisms do exist to address the high costs that come with high transfer, such as transitory derepression (Lundquist and Levin, 1986). Bacterial growth rates are well described in the literature and relatively easy to measure, but vary considerably and are

dependent on environmental conditions (e.g. substrate type, concentration, temperature, pH). While many lab strains have a high growth rate, the doubling time of other strains in harsher environments can be as long as hundreds of hours in rivers (Hendricks, 1972), and hundreds of days in soil (Gibson et al., 2018; Harris and Paul, 1994). The full range of these environmental conditions are of interest in order to manage antibiotic disposal and resistance in relation to plasmid prevalence in hospital and agricultural waste, for example.

The next parameter is the rate of loss of plasmid during cell division. While loss rates are expected to vary over several orders of magnitude due to various mechanisms plasmids employ to improve fidelity (e.g. partition or post-segregational killing genes, Bahl et al., 2009), they are notoriously difficult to measure (Lau et al., 2013). While plasmid presence is easily observed through selective markers on the plasmid, it is much more difficult to detect the absence of the selective marker as the plasmid is lost. Also, as plasmids are lost, loss rate itself can be difficult to separate from differences in donor and recipient growth rate, and further confounded by retransfer. Some methods have been designed to overcome these problems, but tend to be labour intensive and may still over or underestimate rates of loss (Lau et al., 2013). It is also appropriate to consider the effects of plasmid copy number on the results, not included in the scope of this study. Increased copy number, while increasing the metabolic burden on the host, decreases the risk of plasmid loss and co-resident plasmid interactions may also affect transfer rates (Gama et al., 2017b), and these factors must be included in future models to investigate these effects comprehensively. Reliability of these methods and measurements have yet to be adequately resolved, and potentially limit the use of plasmid loss rate in the estimation of selected transfer rate.

The cost of transfer emerges in our model as a key parameter, but it has been less well studied than other parameters. This work may be the first time that the cost of plasmid transfer has been estimated from real data, but it is integral to understanding transfer rate evolution. The coefficient is particularly difficult to measure because it requires multiple transfer rates per plasmid to estimate the effect of transfer on plasmid cost and subsequent growth. The plasmid data used in this study included transfer and growth rates from repressed and derepressed strains of the same plasmid, which enabled direct comparison (Haft et al., 2009), but this may not be as easy to establish in other plasmids. It is extremely unclear how the coefficient varies among plasmids, and between hosts, and any assumptions

are at this point speculative, including the linear relationship we assumed between cost and transfer.

The other parameters (yield coefficient, dilution rate and substrate concentration) are also important to consider when the simplified prediction of plasmid-controlled transfer rate does not apply. The yield coefficient is likely to be high in the majority of cases, assuming that the Haft et al. (2009) estimations are representative. Substrate concentration and dilution are likely to be far more variable and environment-specific. For example, hospital and agricultural waste are likely to have high substrate concentration but could vary in dilution depending on how the waste is treated. Dilution rate can also vary in water and soil systems depending on the speed of the flow and soil composition. Fast-flowing rivers or waste treatment plants may facilitate selection for high rates of plasmid transfer, although a high enough dilution rate would completely prevent plasmid maintenance. In many static environments dilution does not occur, and dilution can be interpreted as a death rate, although cell death does not necessarily mean that the genetic material leaves the system. Genetic material can remain in the system following cell death, and may be adopted by surrounding cells in transformation, contributing to horizontal gene transfer, and complicating our results. Our simplified theory might help to predict the consequences of different management actions of conditions on plasmid prevalence, although the complexity of systems not considered in our models make specific applications less likely.

Our models are based on single infections. We explored the effect of multiple infections in a variant of our model (Appendix B.10) and found that our results are robust under superinfection. Superinfection of donor cells leads to within host competition and through this to selection for large transfer rates, if the trait is controlled by the plasmid. When the trait is controlled by the host, superinfection makes no difference. This is in agreement with experimental results which in which superinfection increases within host competition (Smith, 2011). We did not explore the effect of co-infection which is much more challenging to model correctly (Alizon, 2013) and beyond the scope of this study. Coinfection can lead to an increased copy number and inclusive fitness effects. This would be an interesting avenue for subsequent studies that build on these results.

Estimating parameter values in both laboratory and field settings remains a challenging area. In practice, plasmids are frequently transferred into naïve strains preceding transfer

experiments in the laboratory (Sheppard et al., 2020), limiting the adaptation of plasmid and host to each other and to the environment. Observed transfer rates may additionally be affected by exposure to selective agents that occurs in the construction of donor strains prior to mating experiments, or during the experiments themselves, potentially increasing the transfer rate in some plasmids (although this is not found consistently, Lopatkin et al., 2016). Many transfer rate estimations also assume even mixing of cells, which can be unrealistic due to cell aggregation that can alter outcomes. These aspects must be considered when comparing theoretical and empirical values.

3.3.2 Consequences of host-plasmid conflicts

The calculated rate of plasmid transfer (γ_{sel}) assumes that plasmids adapt to their environment and control the rate of plasmid transfer without the influence of host-control mechanisms, and could serve as a base-rate for quantification of host control effects in non-selective conditions. If the observed rate matches the calculated rate of plasmid-controlled transfer it would indicate plasmid-control, whereas a lower observed rate would support adaptation of the host to reduce the rate of transfer.

Our model only considers the evolution of transfer rate. Several studies show that coadaptation of plasmid and host results in a reduction of cost of the plasmid on the host growth rate (Loftie-Eaton et al., 2016; San Millan, 2018; Zwanzig et al., 2019). An extension to model 2 (Appendix B.9) separates two kinds of plasmid cost upon which selection could act and estimates the effect of each from real data - namely costs that are independent or dependent on transfer rate in turn. The parameters we estimated show that the independent costs are low (0.054 h^{-1}) compared with the costs caused by derepressed rates of transfer, indicating that the majority of plasmid cost of high transferring plasmids can be ameliorated through the reduction of transfer rate. If these parameter estimates are representative across different plasmids, the reduction of transfer may therefore be the primary source of plasmid cost amelioration. Experimental studies showing an evolving transfer rate yield conflicting results. For example, some studies show a decrease in plasmid costs with little change in transfer rate, citing optimisation of the plasmid to its hosts and the resolution of inefficiencies as causes for cost reduction (Dahlberg and Chao, 2003; Loftie-Eaton et al., 2016). Other plasmids do not have a measurable cost (Fischer et al., 2014; Wein et al.,

2019), and do not necessarily maintain themselves primarily through transfer, opting for strategies which improve vertical transmission above horizontal (Hall et al., 2017).

One interesting finding from model 1 was that the evolution of host control occurs in the plasmid reception ability of the recipient. The literature tends to focus on plasmids or donors as infectious agents in control of plasmid transfer (Bergstrom et al., 2000; Hall et al., 2017; McAnulla et al., 2007), as they frequently contain genes for initiation and control of plasmid transfer (Frost and Koraimann, 2010; Smit et al., 1998). Our results demonstrate the selection pressures on recipients that could lead to the evolution of recipient control mechanisms. While some studies show that recipients affect plasmid transfer rate (Reniero et al., 1992), there are few examples in the literature that have established mechanisms of recipient control (Chatterjee et al., 2013; Sansonetti et al., 1980). Evolution of reception rate may be more difficult to observe due to the stronger impact of other pressures (e.g. reduction of donor ability due to the costs of transfer) and the difficulties in the evolution of recipient entry exclusion mechanisms (Pérez-Mendoza and de la Cruz, 2009). The results also highlight that the presence of recipients is necessary for the evolution of transfer rate, at least in cases where plasmid transfer does not occur between cells already carrying a plasmid. While this is an important consideration due to selection against recipients in the presence of antibiotics, for instance, the complete removal of recipients is likely to be uncommon in natural environments. Many plasmids confer social goods (e.g. environmental detoxification) which enable heterogenous donor-recipient populations (Rankin et al., 2011). The presence of biofilms can also protect recipient cells from the effects of selective agents (Penesyan et al., 2015). Environments also tend not to exhibit selection homogeneously and antibiotics can be only partially pervasive (Bahl et al., 2009). Donor cells may also be able to infect other donors (superinfection, Smith, 2011), which provides an opportunity for transfer and subsequent selection on transfer rates (Gandon et al., 2002), although with little benefit to either donor or recipient (Smith, 2012) and can be prevented by entry exclusion mechanisms (Hülter et al., 2017).

3.3.3 Predicting plasmid prevalence

Under the majority of conditions that we considered, plasmid prevalence was found to be high when plasmids are in control of transfer. Reductions in transfer rate due to host-control

are unlikely to reduce plasmid prevalence until the transfer rate is close to the minimum transfer rate for plasmid persistence, which may be several orders of magnitude below the plasmid selected transfer rate. A host may therefore need to reduce the transfer rate by several orders of magnitude to have a substantial effect on plasmid prevalence, and a corresponding effect of the burden of the plasmid on the population. Furthermore, there is only a very narrow window of parameters within which values of prevalence between extinction and near-saturation occur. This prompts the question of why intermediate plasmid prevalence is frequently observed experimentally (Fan et al., 2019; Fox et al., 2008; Kottara et al., 2018; Lilley and Bailey, 1997, 2002). Some intermediate plasmid prevalences may be the result of the absence of adaptation in newly formed host-plasmid combinations, and further studies investigating the evolution of these parameters in controlled and natural environments would be valuable.

The models make several assumptions which limit their application in favour of simplicity. Many of these missing aspects can be considered in future models to improve realism. For example, biofilms shape plasmid-host dynamics through the population structures they facilitate, and specific modelling is needed to assess selection on plasmid transfer rate in these environments (Beaudoin et al., 1998a; Merkey et al., 2011). Phages confer an additional facet of cost, because cells are particularly vulnerable to some phages during pilus production for transfer, and which could be explored in conjunction with the costs previous described (Dionisio et al., 2005; Harrison et al., 2015; Wan and Goddard, 2012). The evolution of rates of transfer in plasmid repression/derepression systems is particularly interesting and can lead to very divergent repressed and derepressed transfer rates several orders of magnitude apart (Haft et al., 2009). Other interesting aspects of transfer rate control include competence switches and specialisation. Some plasmids only become transfer competent when conditions are optimal (e.g. high density of recipients) while in other populations only a fraction of cells in a population become transfer competent. These mechanisms use quorum sensing to reduce the costs of transfer while maximising benefits (Koraimann and Wagner, 2014; McAnulla et al., 2007; Refardt and Rainey, 2010). The model could be extended in future to include and investigate these factors and their impact on the results.

Chapter 4

The interactions between competition and plasmid presence and the application of a two-species conjugation model to a simple bacterial community

4.1 Introduction

Plasmids are bacterial vectors of genetic material, including genes for antibiotic resistance. It is therefore vitally important that we understand plasmid dynamics in bacterial communities in order to manage and control the spread of antibiotic resistance. Many theoretical and experimental studies investigate plasmid-host dynamics in single populations. These studies demonstrate that plasmids can persist in host populations in non-selective conditions when the rate of plasmid transfer is high enough to overcome the costs of plasmid maintenance and transfer (Leclerc et al., 2019; Smets and Lardon, 2009; Stewart and Levin, 1977). In addition, plasmids often contain genes that reduce the probability of loss through segregation (Lauffenburger, 1985; Mongold, 1992; Seo and Bailey, 1985; Srienc et al., 1985) including partition (par) and post-segregational killing (PSK)/toxin-antitoxin genes, or by having a

large copy number (Paulsson and Ehrenberg, 1998; Uchiumi et al., 2019).

While population dynamics in a single species are well studied and broadly understood, bacteria exist in complex communities within which plasmids can transfer and be maintained. It is currently unclear how and to what extent plasmids affect the outcome of competitive interactions between bacteria, and how the species interactions, in turn, affect plasmid dynamics and stability. There have been few theoretical and experimental studies that look at plasmid dynamics in multiple species at a time (Leclerc et al., 2019). Some studies feature multiple species, but do not focus on plasmid transmission (Christensen et al., 1998) or the interaction between plasmid prevalence and competition (Beaudoin et al., 1998a; Hall et al., 2016). One doctoral thesis (Martins, 2013) modelled the outcomes of scenarios comparing narrow and broad-host-range plasmids and their effects on species and plasmid survival (with complementary experimentation), but did not look at the differences between competing species (with differing growth rates, for example).

My masters' thesis (Sheppard, 2016, box 4.1) investigated the interaction between plasmid prevalence and competition in two species using modelling, finding that stable maintenance of costly plasmids by bacterial populations negatively affects their competitive ability, which can have unusual effects in some scenarios. Of particular interest was the identification of species coexistence due to plasmid presence under a narrow range of parameters where exclusion would otherwise occur. In this scenario, costly plasmids are maintained by high transfer rates, while the difference between donor and recipient growth rates provides a niche that the second species can exploit if its growth rate is within that range. Coexistence has recently been independently demonstrated through modelling using non-conjugative toxin-prodicing plasmids (Grover and Wang, 2019). Other results are also possible, and there are some parameter combinations where plasmid presence can enhance the competitive dynamics between the two species if the brunt of the plasmid costs are taken by the non-dominant species. To date, there is no experimental evidence of these findings and the extent to which they apply to real biological systems is unclear. For example, do plasmids increase or decrease the propensity for competitive exclusion, and under what conditions does this change with different combinations of plasmid cost and transfer rate? While these areas can be partially studied theoretically, they require practical experimentation for validation of any theoretical findings.

To address this I performed a series of experiments to test whether plasmid presence affects the coexistence and plasmid persistence in a system with two species and two plasmids and complementary simulations to see if and how well a model captures and predicts the actual dynamics of the system. The model parameters (based on the model used in my Masters' project: box 4.1) were independently estimated and then used to predict outcomes of competition by varying initial plasmid presence and prevalence. Complementary competition experiments were performed to verify those results and used to assess the reliability and challenges when applying specific model findings to real data. These results assist in unpicking some of the complex relationships between plasmids and their hosts in communities, and provide a greater diversity of data which aids our understanding of the breadth of conditions in which plasmids persist.

Box 4.1. Masters Thesis summary (Sheppard, 2016)

A two-species conjugative plasmid model was constructed based on principles from Stewart and Levin (1977) to explore plasmid-host dynamics in a two species continuous-flow system. Some of the parameters have been adjusted for consistency with the literature and the rest of this thesis. Two bacterial species recipient strain densities N_1 and N_2 and donor (plasmid-bearing) strain densities N_{1P} and N_{2P} consume and grow on a substrate S described with Monod kinetics, with unique growth rates (r) and Monod constants (K_M) of recipients (1 and 2) and donors ($1P$ and $2P$). Cells are removed from the system by a constant dilution rate (D). Plasmids transfer at constant rates (γ) based on the unique donor, plasmid and recipient combination (where first and second subscripts here refer to the donor and recipient strains respectively), and are lost at a constant rate (τ, where the subscript again refers to the strain) and as a proportion of donor growth.

$$\frac{dN_1}{dt} = \frac{r_1 N_1 S}{K_M + S} - DN_1 - \gamma_{11} N_{1P} N_1 + \tau_1 \frac{r_{1P} N_{1P} S}{K_M + S} - \gamma_{21} N_{2P} N_1 \tag{4.1}$$

$$\frac{dN_{1P}}{dt} = (1 - \tau_1)\frac{r_{1P} N_{1P} S}{K_M + S} - DN_{1P} + \gamma_{11} N_{1P} N_1 + \gamma_{21} N_{2P} N_1 \tag{4.2}$$

$$\frac{dN_2}{dt} = \frac{r_2 N_2 S}{K_M + S} - DN_2 - \gamma_{22} N_{2P} N_2 + \tau_2 \frac{r_{2P} N_{2P} S}{K_M + S} - \gamma_{12} N_{1P} N_2 \tag{4.3}$$

Box 1 - *continued*

$$\frac{dN_{2P}}{dt} = (1-\tau_2)\frac{r_{2P}N_{2P}S}{K_M+S} - DN_{2P} + \gamma_{22}N_{2P}N_2 + \gamma_{12}N_{1P}N_2 \tag{4.4}$$

$$\frac{dS}{dt} = D(S_0 - S) - (r_1N + r_{1P}N_{1P} + r_2N_2 + r_{2P}N_{2P})\frac{S}{K_M+S} \tag{4.5}$$

The results demonstrated that plasmid presence and activity decrease growth rate, which can affect the outcomes of competition between species, depending on the combination of parameters. This can sometimes result in coexistence (the persistence of donors of recipients) of the two species where competitive exclusion would normally occur (Figure 4.1, graphs A and C).

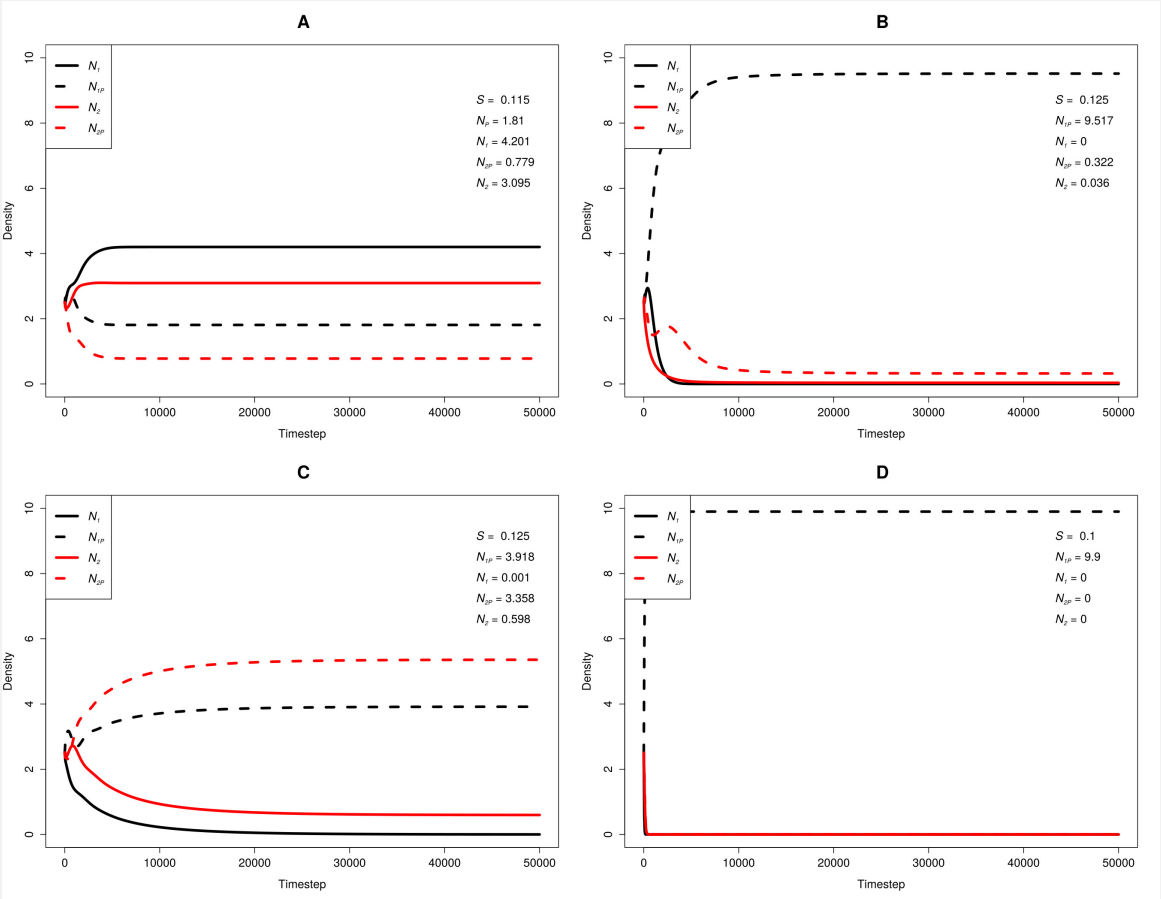

Figure 4.1: Coexistence of two species (as seen primarily in graphs A and C) can occur under some parameter ranges (Sheppard (2016): Figure 4). Coexistence here is defined as the persistence of the two species as donors, recipients or both. $\gamma_{12} = 5 \times 10^{-4}$ (A, C, D) or $\gamma_{12} = 0.001$ (B), $\gamma_{21} = 5 \times 10^{-4}$ (A, B) or $\gamma_{21} = 0.001$ (C, D). Final

Box 1 - *continued*

strain densities and substrate concentrations given in each plot. Other parameters assignments: $r_1 = 1$, $r_{1P} = 0.9$, $r_2 = 0.99$, $r_{2P} = 0.89$, $\gamma_{11} = 0.0015$, $\gamma_{22} = 0.0015$, $\tau_1 = 10^{-7}$, $\tau_2 = 10^{-7}$, $D = 0.1$, $K_M = 1$.

4.2 Methods

4.2.1 Adapted two-species bacterial conjugation model

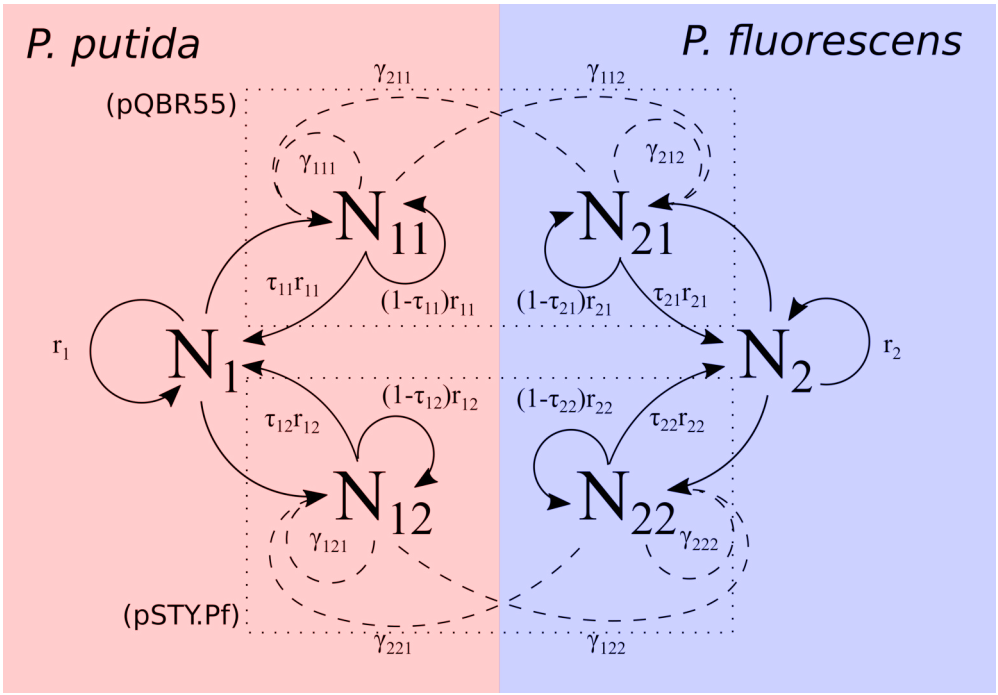

Figure 4.2: Two-species community plasmid conjugation model representation (Equations 4.6-4.8). Solid lined arrows show transformation of individuals from one sub-population to another. Dashed arrows represent the effect of donors on the transformation of recipients to donors through transfer, where the donor individuals are not transformed and sub-population sizes do not change.

The initial bacterial conjugation model (Sheppard, 2016, box 4.1) was adapted into a serial dilution model to maintain consistency with experimental procedures (Equation 4.6-4.8, Figure 4.2), which were serially transferred due to the difficulties of maintaining sterility in chemostats. The model was also standardised for consistency with other models previously described in the thesis. Each recipient and donor strain is represented by N_i and N_{gh}, where

g and i represent the donor and recipient strains ($g = 1, 2; i = 1, 2$ for each of the species), and h represents the plasmid strain ($h = 1, 2$ for each of the two plasmids that are used in these simulations and experiments, see Table 4.1). "Donor" and "recipient" are here used to denote plasmid-bearing and plasmid-free cells.

	Recipient	pQBR55	pSTY.Pf
P. putida	N_1	N_{11}	N_{12}
P. fluorescens	N_2	N_{21}	N_{22}

Table 4.1: Bacterial recipients and donors as found in the model (Figure 4.2).

Per capita growth rates are calculated using Monod kinetics, where each donor or recipient strain has a unique set of parameters: r (maximum growth rate, V_{max} in Lineweaver-Burk plots or ψ in Simonsen's endpoint), K_M (substrate concentration at half the maximum growth rate, comparable to the Michaelis-Menten or Monod constant) and y (yield coefficient, which allows conversion between substrate concentration and cell density). Plasmid metabolic and replicative costs and costs incurred due to plasmid transfer are captured in the differences between donor and recipient growth rates, such that $r_{gh} < r_i$ (where $g = i$: the donor and recipient are of the same strain, albeit with presence or absence of a plasmid). Transfer within and between species, γ, is modelled using mass-action kinetics using three subscripts (to represent the donor: g, plasmid: h and recipient: i). Lag times preceding exponential growth phase were consistently observed in *P. fluorescens* (see Appendix C.1, Figure C.1) and a lag function, $\frac{t^n}{\lambda^n + t^n}$ (Baranyi and Roberts, 1994; Baty and Delignette-Muller, 2004), was added to the model to take this into account. The dilution term (used in chemostat models) is no longer required in the adjusted model and therefore removed. Plasmid loss is also removed from the model due to the relatively small effect of loss compared with growth rate costs (see Appendix C.3) and to reduce model complexity.

$$\frac{dN_i}{dt} = \frac{t^n}{\lambda_i^n + t^n}\left(\frac{r_i N_i S}{K_{M_i} + S} - N_i \sum_{g,h} \gamma_{ghi} N_{gh}\right) \tag{4.6}$$

$$\frac{dN_{gh}}{dt} = \frac{t^n}{\lambda_{gh}^n + t^n}\left(\frac{r_{gh} N_{gh} S}{K_{M_{gh}} + S} + N_i \sum_{h} \gamma_{ghi} N_{gh}\right) \tag{4.7}$$

$$\frac{dS}{dt} = -\sum_{i} y_i r_i N_i - \sum_{gh} y_{gh} r_{gh} N_{gh} \tag{4.8}$$

The parameters that require estimation are therefore growth parameters (r, K_M), yield coefficients (y), lag parameters (λ and n) and plasmid transfer rates (γ). Parameter estimations were taken from a series of experiments, full details of which are given in Appendix C.4.

4.2.2 Strains and plasmids

Pseudomonas putida (UWC1-BRGT, KT2440) and *Pseudomonas fluorescens* (376N)[1] strains (Table 4.2) were obtained from Imperial College, Silwood Park freezer archives maintained by Damien Rivett, originally collected by Lilley et al. (1996). These strains are well-characterised and frequently contain large mercury resistance plasmids and chromosomal antibiotic resistance genes (e.g. rifampicin resistance, naladixic acid resistance) which evolve through simple point mutations, and which can be used as genetic markers for strain identification. A rifampicin-resistant recipient strain of 376N was created to be used as a recipient in within-species *P. fluorescens* transfer experiments by plating cells on plate count agar (PCA) with increasing concentrations of rifampicin (0.025, 0.05, 0.1gl^{-1}), and labelled 376NR.

Two plasmids were selected for experimental use: pQBR55 (Hall et al., 2015) and pSTY.Pf (Köhler et al., 2013). UWC1-pQBR55 is a 150 kbp, group 3 conjugative plasmid, containing a mercury resistance transposon (Tn5042[2]). This plasmid has been reported to confer a high cost on host growth, a low rate of loss during cell division (due to partition genes), and a low copy number. These factors increase the ease of model application to the experimental conditions (low copy number, low loss rates) and reflect the conditions required for coexistence (e.g. high cost, Sheppard, 2016). pSTY.Pf was identified during the competition experiments in this study, and distinguished from pQBR55 due to its high transfer rate and

[1]Previously characterised as *Pseudomonas marginalis* (Anzai et al., 2000; Lilley et al., 1996)

[2]Containing merA (mercuric ion reductase), merC (mercuric transport protein), merP (periplasmid mercury binding protein), merT (mercuric transport protein) and merR (mercuric resistance operon regulatory protein)

cost on host growth rate cost compared with pQBR55 under the same conditions. Sequencing and bioinformatic work showed that the plasmid shows strong similarity to pSTY,[3] a 322 kbp conjugative mega-plasmid with unknown incompatibility group, that also confers mercury resistance through the Tn5042 transposon (Köhler et al., 2013). While the original source of the plasmid was unclear, pSTY.Pf provided a valuable contrast to the behaviour of pQBR55, and was used in this study to demonstrate the effects of different plasmid persistence strategies (Hall et al., 2017).

All additional plasmid-host combinations were created as follows. 376N-pQBR55 was constructed in a simple 12-hour mating experiment in 1gl^{-1} glucose media where UWC1-pQBR55 and 376N cultures were mixed and grown together, followed by plating which selected for transconjugants (nalidixic acid and mercury, Table 4.2), and where the plasmid was then confirmed using primers (Table 4.3, Figure 4.3). UWC1-pSTY.Pf and 376N-pSTY.Pf were both obtained directly from competition experiments. These processes resulted in four recipient and four donor strains (Table 4.2),

Species	**Strain**	**Source**	**Selective marker(s) (Conc.)**
Recipients:			
P. putida	UWC1-BRGT	McClure et al. (1989)	Rif. (0.1gl^{-1})
	KT2440	Bagdasarian et al. (1981)	Nal. (0.3gl^{-1})
P. fluorescens	376N	Lilley et al. (1994)	Nal. (0.3gl^{-1})
	376NR	This study	Nal. (0.3gl^{-1}); Rif. (0.1gl^{-1})
Donors:			
P. putida	UWC1-pQBR55	Lilley et al. (1996)	Rif. (0.1gl^{-1}); Mer^{R} (0.1mM)
	UWC1-pSTY.Pf	This study	Rif. (0.1gl^{-1}); Mer^{R} (0.1mM)
P. fluorescens	376N-pQBR55	This study	Nal. (0.3gl^{-1}); Mer^{R} (0.1mM)
	376N-pSTY.Pf	This study	Nal. (0.3gl^{-1}); Mer^{R} (0.1mM)

Table 4.2: Bacterial strains (recipients and donors) with corresponding resistance markers and selective concentrations. Rifampicin (Rif.), naladixic acid (Nal.), mercury (Mer^{R}).

[3]MicrobesNG Illumina sequencing with next era protocols from strain. Sequences mega-blasted against the "nr" database on Geneious (including GenBank, EMBL, DDBJ, PDB and RefSeq) where the relevant contigs showed highest similarity with *Pseudomonas* sp. VLB120 plasmid pSTY (Köhler et al., 2013) (UWC1-pSTY.Pf: 97.4% pairwise identity, 58.8% ref-seq coverage; 376N-pSTY.Pf: 98.1% pairwise identity, 67.8% ref-seq coverage).

Plasmid	Gene		Sequence (5'-3')	Temp. (°C)	Size (bp)
pQBR55	dnaB	Forward	TGGATCGAGGGTTACTGGCT	65	1400
		Reverse	ATCTGGTTGCAGTCGACGAT		
pSTY.Pf	DinB1	Forward	CGCATACCCCAATCTGGTGT	65	500
		Reverse	ATGGACCTGGAGCAATCTGC		

Table 4.3: Primers used for identification of plasmids.

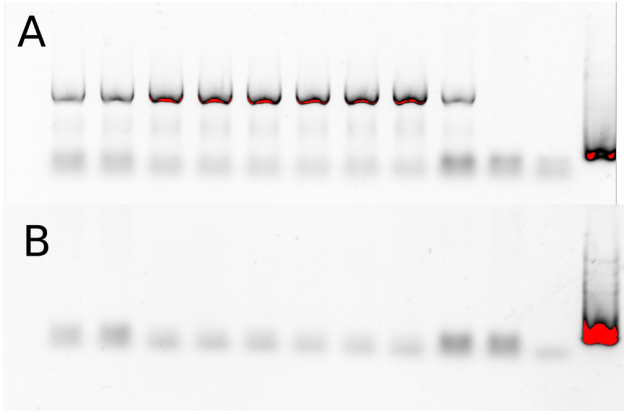

Figure 4.3: Plasmid identification following transfer experiments. Red highlighting shows high fluoresence, indicating successful PCR of the relevant DNA section. Lanes 1-9 show 9 sampled replicate transconjugants, 2 blanks and a DNA ladder in the final lane. In all cases the primers identified pQBR55 in the transconjugants (A) using the dnaB primers and the absence of pSTY.Pf (B) using the DinB1 primers.

4.2.3 Parameter estimation

Growth parameters and yield coefficient (r, K_M, y)

Donor strains (UWC1-pQBR55, UWC1-pSTY.Pf, 376N-pQBR55, 376N-pSTY.Pf) were grown on selective agar plates (Table 4.2) to remove recipients cells. A single donor colony was selected and transferred into 1gl^{-1} glucose solution which were grown overnight along with recipient strains (UWC1, 376N) sampled from freezer stocks, also in 1gl^{-1} glucose. Following growth, the cultures were diluted to approximately 0.05 $OD_{600\text{-est.}}$ and 10μl diluted culture was used to inoculate 90μl of glucose solution at a range of concentrations (0.1, 0.25, 0.5, 1, 1.5, 2, 3, 4gl^{-1})[4] and grown for 12 hours to allow acclimation to occur and to maintain the cells in their exponential growth phase. Following acclimation, 10μl cell culture was transferred into 90μl fresh media of the corresponding concentration of glucose in a 96-well plate (16 replicates, at each concentration), and grown until stationary phase had been reached. Growth assays were shaken for 10 seconds on a medium setting every 30 minutes during growth to homogenise the culture and discourage cell aggregation, following which the OD_{600} was measured to estimate cell density. Initial and final cell densities and final plasmid prevalence of donor growth cultures were estimated using serial dilution and drop plating on PCA containing the the appropriate antibiotics (Table 4.2).

The resulting growth curves were taken and converted to approximate cell density using a linear model derived from a small dataset (n = 399) of OD_{600} readings and colony forming units (CFU) measurements through serial dilution of cell cultures collected throughout the course of this PhD. Data points with $OD_{600} < 0.05$ and $OD_{600} > 0.4$ were filtered due to the limits of detection at low density (minimum observable $OD_{600} \approx 0.04$) and deviation from the linear relationship at high densities (Widdel, 2010). In addition, the minimum observable OD_{600} (0.04) was deducted from OD_{600} readings to make 0.04 equivalent to the absence of bacterial cells to improve the log transformation (Equation 4.9, Figure 4.4, Table 4.4).

$$\ln(CFU) = \beta_1 + \ln(OD_{600} - 0.04)\beta_2 \tag{4.9}$$

[4]Except for 376N-pQBR55, grown at 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 4gl^{-1} glucose

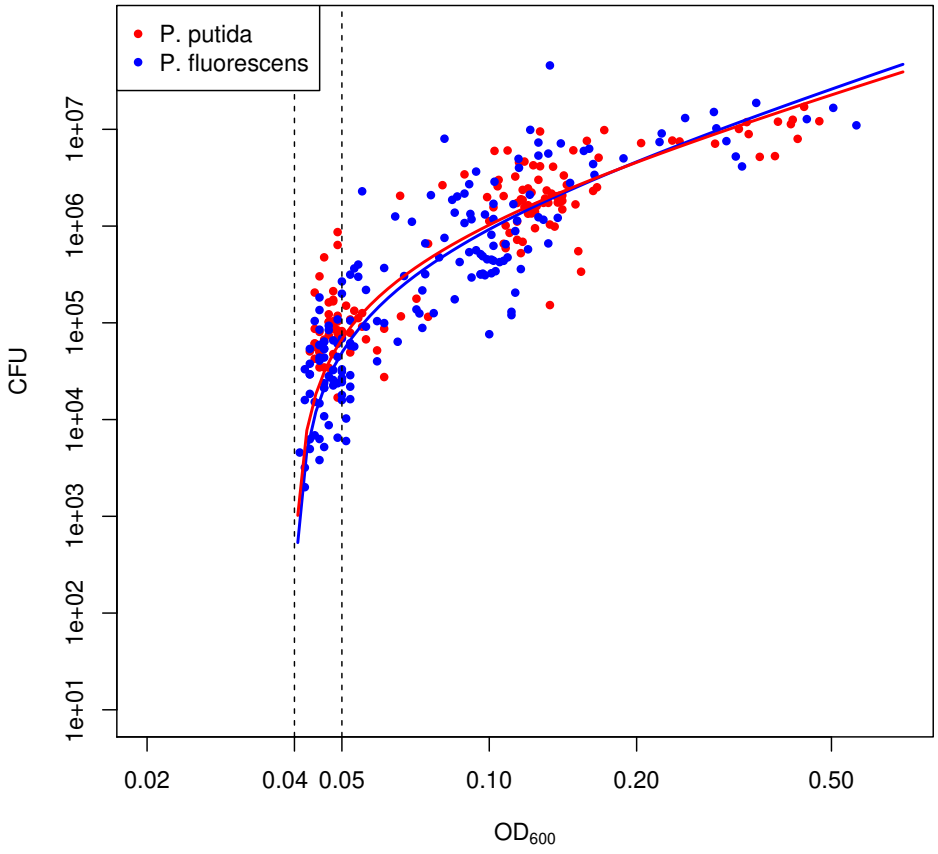

Figure 4.4: Predicting CFU from OD_{600} with linear models (Equation 4.9) for *P. putida* (red) and *P. fluorescens* (blue).

Strain	$\beta_1(\pm S.E.)$	$\beta_2(\pm S.E.)$	F	R^2	**p-value**
P. putida	18.12 (±0.30)	1.52 (±0.11)	F(1,91) = 194.9	0.68	< 0.001
P. fluorescens	18.35 (±0.42)	1.64 (±0.14)	F(1,105) = 146.8	0.58	< 0.001

Table 4.4: Linear model results, where CFU is a function of OD_{600} (Equation 4.9). Values rounded to two decimal places.

The exponential growth rate of each replicate at each glucose concentration was estimated using the "growthrates" package (function = fit_easylinear, quota = 0.95, width of window = 15 (*P. putida*) or = 10 (*P. fluorescens*) matching the length of the exponential growth phase) in R (R Core Team, 2018) and averaged across replicates. OD_{600} values below 0.055 OD (approx. 125,000 CFU, see graphs in Appendix C.2) were not used due to the poor reliability of values within this range following conversion from OD to CFU, and which would otherwise exaggerate the steepness of the growth curves. Lineweaver-Burk (LB) plots can normally be used to estimate the maximum exponential growth rate (r) and the substrate concentration at which growth rate is half maximum (K_M), where r is the inverse of the y-axis intercept and the slope is K_M/r (Figure 4.5). However, it was not possible to appropriately fit a line through all the estimated growth rates at each concentration due the the absence of data linearity (Figures 4.6 and 4.7). Non-linear least squares (NLLS) fitting of a model featuring all growth parameters was attempted as an alternative but abandoned because the model did not adequately identify the exponential parts of the curves as the maximum growth rates.[5] The parameter estimation methods were therefore adjusted as follows. The growth rates of *P. fluorescens* strains estimated using the "growthrates" package clearly reached saturation at a relatively low glucose concentration (≈0.5gl^{-1}, Figure 4.6) and r values were estimated as the highest growth rate of each *P. fluorescens* strain.[6] The corresponding standard errors of the growth rates at those substrate concentrations are reported with the r values. In contrast to *P. fluorescens*, the growth rates of *P. putida* did not reach saturation at the concentrations measured, nor did the plotted data reveal a linear relation across all concentrations as expected in LB plots (Figure 4.7). The deviation from this expectation is likely due to poor estimation of growth rates at low substrate concentrations caused by low accuracy of CFU estimation at low density and the cell growth that occurs from previously consumed media. These measurement issues at low substrate concentrations obscure the

[5]Initial growth from prior substrate consumption, biphasic growth and poor estimation at low cell density prevented this from occurring.

[6]376N: 1.5gl^{-1}; 376N-pQBR55 and 376N-pSTY.Pf: 2gl^{-1}, Figure 4.6

growth and result in the overestimation of growth rates at these concentrations. Growth rates at higher glucose substrate concentrations ($\geq$1gl^{-1}), however, followed the expected LB relationship and were therefore used to estimate r values for *P. putida* strains. Standard error for r^{-1} for *P. putida* is reported, based on the described points, along with an estimated error for the r based on the inverse standard error.[7]

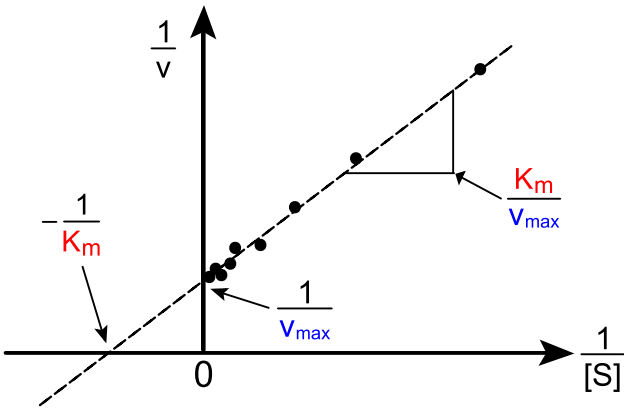

Figure 4.5: Example LB plot describing the estimation of r (V_{max}) and K_M (Diberri, 2007).

Non-linear least squares (NLLS) was then used with the growth assay data to estimate K_M and y, setting r as a constant using the values previously estimated. The growth model (Eq. 4.11-4.12) was fitted to growth data of each strain at each substrate concentration using the "optim" function in R. Initial parameters were taken from Haft et al. (2009) with $K_M = 0.2$mg L^{-1}, $y = 8 \cdot 10^{-8} \mu$g CFU^{-1}. The initial cell density (N_0) was allowed to vary and data below 0.065 OD (approx 250,000 cells) were not used to avoid distorting the fit of the model at low cell density. N_0 was initially set at 1000 and scaling values for K_M, y and N_0 were 0.1, 10^{-9} and 10^3, respectively. All parameters were constrained to be positive.

$$\frac{dN_i}{dt} = \frac{r_i N_i S}{K_{M_i} + S} \tag{4.11}$$

$$\frac{dS}{dt} = -\frac{y_i r_i N_i S}{K_{M_i} + S} \tag{4.12}$$

[7]Standard error of r for *P. putida* was calculated as the difference between r and the inverted difference between r^{-1} and the standard error of the fitted model (Equation 4.10).

$$s.e.(r) = \frac{1}{(r^{-1} - s.e(r^{-1}))} - r \tag{4.10}$$

Lag parameters (λ, n)

A further NLLS optimisation process was then used to estimate lag parameters in *P. fluorescens* strains (λ and n) with "optim" in R, fixing the previously estimated parameters (r, K_M, y), where N_0 was allowed to vary and the lag was reinitialised following each transfer. This model (Eqs. 4.13-4.14) was fitted to data from a preliminary serial transfer experiment (CompEx1, see Appendix C.4) that lasted 60 hours with 5 12-hour growth periods and 1:20 dilutions each transfer in 1 gL^{-1} glucose, using the methods described in the competition experiment methods section (see section 4.2.4). Data from two replicate assays of each recipient strain were used to estimate lag parameters for those strains and their corresponding donor strains. Initial parameters were $\lambda = 0.05$, $n = 2$ and $N_0 = 1000$. Scaling values for λ, n and N_0 were 0.01, 1 and 10^2 respectively.

$$\frac{dN_i}{dt} = \frac{t^n}{\lambda_i^n + t^n} \frac{r_i N_i S}{K_{M_i} + S} \tag{4.13}$$

$$\frac{dS}{dt} = -\frac{t^n}{\lambda_i^n + t^n} \frac{y_i r_i N_i S}{K_{M_i} + S} \tag{4.14}$$

Plasmid transfer rate estimation (γ)

Donor and recipient strains were plated on PCA containing the appropriate antibiotic for the strain with mercury for donor strains (Table 4.2). Following cell growth colonies were sampled and grown in $1gl^{-1}$ glucose overnight. The resulting cultures were diluted to approximately 0.05 $OD_{600\text{-est}}$, of which $5\mu l$ of donor and recipient cultures (each $\approx 50,000$ cells) were combined and used to inoculate $90\mu l$ of $1gl^{-1}$ glucose solution. OD_{600} was measured every 30 minutes, and plates shaken for 10 seconds to homogenise the cultures and discourage cell aggregation. Plasmid transfer was allowed to occur for 12 hours before final cell densities were measured. Initial and final CFU and plasmid prevalence in donor and recipient populations were estimated by serial dilution and drop plating on PCA containing the appropriate antibiotic to select for the strain and with or without mercury to measure

plasmid prevalence within each strain. UWC1 and 376N were used as donors for pQBR55 and pSTY.Pf, and the corresponding recipients for between species transfer, while KT2440 and 376NR were used for within species *P. putida*, and *P. fluorescens* transfer respectively. Some experiments did not yield any transconjugants (376N-pQBR55 to 376N and 376N-pQBR55 to UWC1). In these cases, the experiments were repeated with an increased initial cell density. In all cases, Simonsen's endpoint method (1990) was used to estimate the specific rates of transfer (γ).

$$\gamma = r \ln(1 + \frac{T_{End}}{R_{End}} \frac{N_{End}}{D_{PEnd}}) \frac{1}{N_{End} - N_0} \tag{4.15}$$

$$r = \frac{ln(OD_b/OD_a)}{t_b - t_a} \tag{4.16}$$

N_0[8] N_E,[9] D_E, R_{End} and T_E are the initial total and final total, plasmid-bearing donor, plasmid-free recipient, and transconjugant cell densities respectively. OD and t are the optical density and time at times a and b (where a and b are the final 5 hours of growth), which are used to estimate per capita growth rate (r). CFU estimation in one replicate experiment resulted in $N_{end} < N_0$, due to an unrealistically low final recipient density and which led to a negative transfer rate. This replicate was omitted from the reported results and transfer rate estimation. Replicate numbers used are reported. In addition, due to low initial experimental cell densities, the OD measurements used to calculate the culture growth rates (r) underestimated the growth rates, and therefore the transfer rates, when compared to the growth rates previously estimated (see results section). To compensate for the underestimation of growth rate, these values were replaced by growth rates that were the average of the maximum growth rates of the donor and recipient strains (r_{est}). This is expected to be a more accurate estimation of growth, and therefore transfer rates, despite the lag seen in *P. fluorescens* curves.

[8] $N_0 = D_0 + R_0$
[9] $N_E = D_E + R_E + T_E$

4.2.4 Competition experiments

Experimental cultures were prepared by growing strains[10] on PCA plates containing the appropriate antibiotic (rifampicin or naladixic acid) with mercury for donor strains (Table 4.2), from which single colonies were sampled and grown in $1gl^{-1}$ glucose solution overnight.

The behaviour of each strain in isolation and combinations of *P. putida* strains with *P. fluorescens* strains were observed with varying presence and distribution of the two plasmids (Table 4.5). Each assay was assigned a number (1-13) for ease of reference. Initial cell density was standardised by dilution to $OD_{600\text{-est}} = 0.05$ and initial cell density and plasmid prevalence was estimated through plating on PCA. Assays were initialised by inoculating 10μl of cell culture into 90μl glucose solution, or 5μl of each strain in each competition assays, into either 2 or 4 wells of a 96 well plate.[11]

Cell density and plasmid prevalence of each cell type in each assay was tracked for 120 hours in 10 12-hour growth phases with intermittent serial transfers. Serial transfers were of a 20-fold dilution: 5μl of cell culture was transferred into 95μl fresh media. Following serial transfer cell densities of each strain and plasmid prevalence within each strain was estimated from the remaining culture through serial dilution and drop plating on PCA containing each antibiotic with and without mercury. Plasmid-free cell densities of mixed donor-recipient strains were estimated by subtracting the donor density from the total strain density. Competition assay plates were shaken on a medium setting for 10 seconds every 30 minutes throughout the experiment to limit cell aggregation.

		P. fluorescens			
	Strain	x	376N	376N-pSTY.Pf	376N-pQBR55
P. putida	x	x	1	2	3
	UWC1	4	5	6	7
	UWC1-pQBR55	8	9	10	x
	UWC1-pSTY.Pf	11	12	x	13

Table 4.5: Strain combinations used in competition experiments.

[10] UWC1, UWC1-pQBR55, UWC1-pSTY.Pf, 376N, 376N-pQBR55 and 376N-pSTY.Pf

[11] 2 replicates: UWC1, 376N, 4 replicates: UWC1-pQBR55, UWC1-pSTY.Pf, 376N-pQBR55, 376N-pSTY.Pf

4.2.5 Simulations

Simulations were run using the full competition model (Eqs. 4.6-4.8) and estimated parameter values for 1200 hours, including a 20-fold dilution into fresh 1gL^{-1} glucose media every 12 hours to reflect the experimental competition assays. Initial total cell density was set to 50,000 cells ml^{-1} for single strain assays, and 25,000 cells ml^{-1} for each strain in competition assays. Due to the absence of plasmid loss in the model, the simulations were run with an additional single plasmid free recipient cell for each donor strain at the start). From these simulations, qualitative predictions were made regarding the competitive ability of strains. These predictions were then compared with the experimental results.

4.3 Results

4.3.1 Parameter estimation

Growth parameters and yield coefficient: (r, K_M, y)

LB plots for each strain-plasmid combination are presented with standard axes for *P. fluorescens* strains (Figure 4.6) and inverted axes for all strains, (Figures 4.6-4.7). These graphs demonstrate the estimation of the values for r that are reported in table 4.6. pSTY.Pf is more costly to growth than pQBR55 in both species, which is still costly when compared with the grwoth rate of the recipient strain. In addition, the three *P. fluorescens* strains have higher growth rates than the growth rates of the corresponding *P. putida* strains.

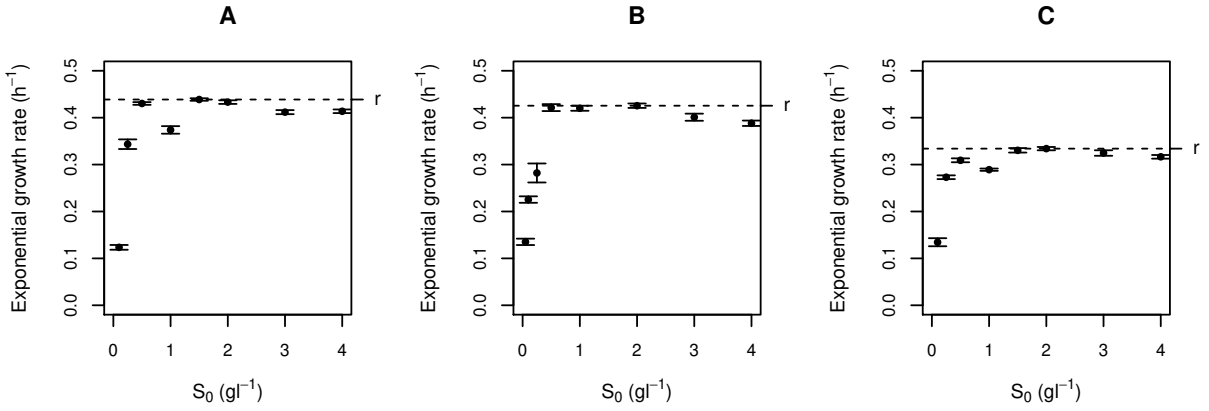

Figure 4.6: Inverted LB plots for *P. fluorescens* strains: recipient (A), pQBR55 donor (B) and pSTY-Pf donor (C) with standard error bars shown. Dotted lines show estimates for the maximum growth rate (r).

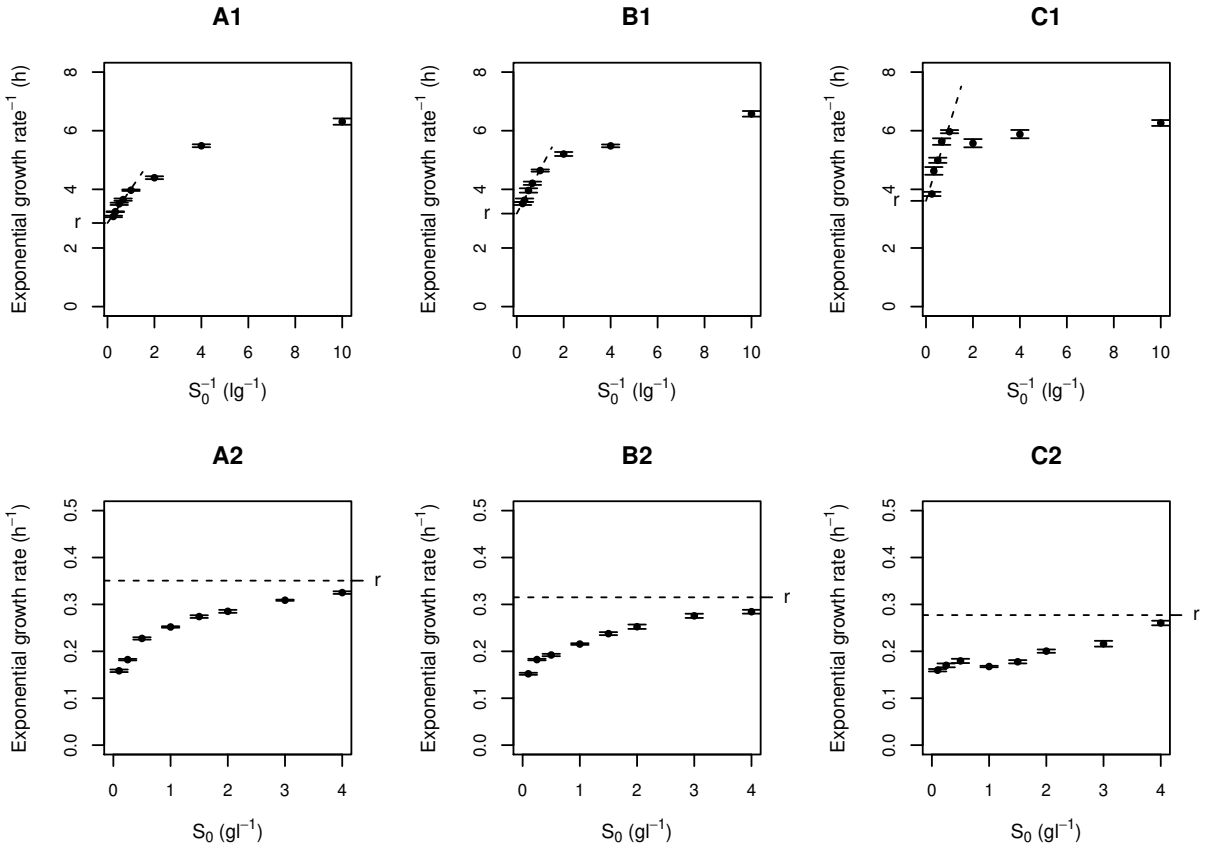

Figure 4.7: LB (A1-C1) and Inverted LB plots (A2-C2) for *P. putida* strains: recipient (A), pQBR55 donor (B) and pSTY-Pf donor (C) with standard error bars shown. Dotted lines show the fit of the linear model in graphs A1-C1 to the points at substrate concentrations greater than or equal to 1 gL^{-1} glucose or estimates for the maximum growth rate (r) in graphs A2-C2

Strain	r^{-1}	($\pm S.E.$)	t	p-value	F	R^2	r	($\pm S.E.$)
Pp	2.852	($\pm$0.032)	90.0	< 0.001	F(1,78)=502.6	0.866	**0.351**	($\pm$0.004)
Pp-pQ	3.173	($\pm$0.057)	55.8	< 0.001	F(1,78)=262.1	0.771	**0.315**	($\pm$0.006)
Pp-pS	3.607	($\pm$0.119)	30.3	< 0.001	F(1,78)=178.6	0.696	**0.277**	($\pm$0.009)
Pf	-	-	-	-	-	-	**0.439**	($\pm$0.003)
Pf-pQ	-	-	-	-	-	-	**0.426**	($\pm$0.005)
Pf-pS	-	-	-	-	-	-	**0.334**	($\pm$0.004)

Table 4.6: Maximum exponential growth rates (r) of *P. putida* (*Pp*) and *P. fluorescens* (*Pf*) recipients and donors (pQBR55: pQ, pSTY-Pf: pS). r values for *P. putida* were estimated from LB plots while values for *P. fluorescens* were estimated as the highest growth rate across substrate concentrations.

Values for K_M and y were estimated using NLLS fitting (see Appendix C.2) and are reported in table 4.7. K_M values for the *P putida* strains are lower than for *P. fluorescens* strains. The value of K_M for the *P. fluorescens* recipient strain is much higher than its corresponding donor strains. Values for y were all within the same order or magnitude ($\approx 8 \times 10^{-8}$).

Strain	K_M	y
Pp	0.035	8.06×10^{-8}
Pp-pQ	0.032	8.05×10^{-8}
Pp-pS	0.028	8.09×10^{-8}
Pf	0.163	9.19×10^{-8}
Pf-pQ	0.043	7.82×10^{-8}
Pf-pS	0.067	8.07×10^{-8}

Table 4.7: Michaelis-Menten constant (K_M) and yield coefficient (y) estimates from NLLS fitting. *P. putida* (*Pp*), *P. fluorescens* (*Pf*), pQBR55 (pQ), pSTY.Pf (pS).

Lag parameter (λ, n)

The fit of the model including the lag parameters to serial transfer data for the *P. fluorescens* recipient strain is shown in Figure 4.8. Lag parameters were estimated as $\lambda = 5.67 \times 10^{-3}$ and $n = 4.4$ (for *P. putida*) and $\lambda = 1.24 \times 10^{-4}$ and $n = 1.01 \times 10^{-1}$ (for *P. fluorescens*).

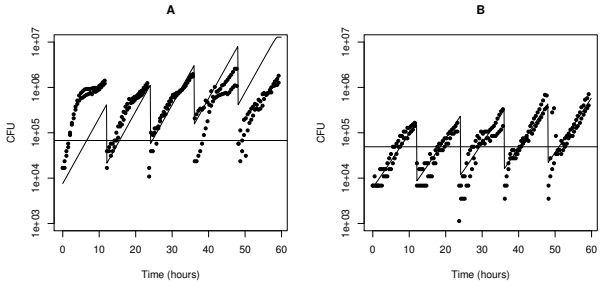

Figure 4.8: NLLS fitting of model with lag function to *P. fluorescens* serial transfer data to estimate lag parameters (λ and n).

Plasmid Transfer Rate

Transfer rate measurements were collected across a series of experiments (see Appendix C.4, Tables 4.8-4.9). Transfer from *P. putida*-pQBR55 is fairly consistent (≈E-10), and transfers at a higher rate within *P. putida* strains, than from *P. putida* to *P. fluorescens*. Transfer from *P. fluorescens*-pQBR55 was undetectable under the same conditions. This could be because *P. fluorescens* has stronger control mechanisms, or has incompatabilities with pQBR55 which would reduce transfer. Transconjugants were detected when these two mating assays were rerun with higher initial donor and recipient densities (indicated by * in Tables 4.8-4.9), but these high densities may distort the assumptions of the model, resulting in an overestimation of the rate of transfer. For the purposes of use in the simulations, transfer of pQBR55 from *P. fluorescens* was assumed to be less than the limits of detection, and thus set to 0.

Rates of pSTY.Pf transfer are more variable, and are highest when transferred from *P. putida* to *P. fluorescens*, although this may be overestimated due to experimental abnormalities (e.g. the low final cell density compared with initial cell density, Table 4.9). Overall, these rates are clearly higher than pQBR55. The two plasmids show differences in the relationships between within-species and between-species transfer, where pQBR55 transfers at a higher

rate within species, but pSTY.Pf transfers at a higher rate between species than within species.

There are some incongruities in some of the results which require examination. First, some experimental results show a higher plasmid-bearing donor density than total donor strain (plasmid-bearing and plasmid free) density (both *P. putida* donors, within species transfer: TrEx1 (C.4), data not shown). These are clearly incorrect CFU estimates, and demonstrate the potential for error in cell plating. The plasmid-bearing donor densities are more consistent with the corresponding OD_{600}, and are therefore expected to be more reliable and are used to estimate of transfer rate. Second, some results show the reverse problem: substantially lower donor strain plasmid prevalence compared with total donor density (pSTY.Pf: between species transfer). This is likely the result of plasmid loss and decrease in prevalence due to growth rate differences caused by costs incurred by pSTY.Pf. These experiments will have a higher density of cells not used in transfer (plasmid-free donor cells) and which may limit successful collisions of plasmid-bearing donor and recipient cells. Third, some experiments have a substantial difference between plasmid-bearing donor density and recipient density (D_0 and R_0, pSTY.Pf: within species from *P. putida*, and between species), which may also bias transfer dynamics and results. Fourth, the results may be skewed more generally due to differences in growth patterns between the two species and between donors and recipients, due to differences in growth rate and lag times of *P. fluorescens* strains. Fifth, one experiment (pSTY.Pf: *P. putida* within species) shows only a small increase in recipient density over the course of the mating assay, where a larger increase was expected due to growth. While this may be another example of cell plating errors, this growth may again have been limited by the lag of *P. fluorescens* and the plasmid costs incurred as the recipients became transconjugants.

While the transfer rate estimates may be distorted by these incongruities, they broadly give an idea of the approximate transfer rates and show that transfer rate of pSTY.Pf is multiple magnitudes higher than pQBR55, but which rates vary as donor and recipient strains change.

Exp.	n	t_a	t_b	OD_a	OD_b	r	r_{est}
Pp-pQ to *Pp*	4	6.5	11.5	0.060	0.088	0.078	0.333
Pp-pQ to *Pf*	4	7	12	0.067	0.097	0.074	0.377
**Pf*-pQ to *Pf*	2	7	12	0.138	0.242	0.112	0.433
**Pf*-pQ to *Pp*	2	7	12	0.148	0.223	0.083	0.389
Pp-pS to *Pp*	4	6.5	11.5	0.058	0.077	0.058	0.314
Pp-pS to *Pf*	3	7	12	0.061	0.100	0.100	0.358
Pf-pS to *Pf*	4	7	12	0.050	0.063	0.047	0.387
Pf-pS to *Pp*	4	7	12	0.078	0.112	0.071	0.343

Table 4.8: Growth rate (r) estimation for use in transfer rate calculation (Simonsen et al., 1990) for pQBR55 (pQ) and pSTY.Pf (pS) in *P. putida* (*Pp*) and *P. fluorescens* (*Pf*), within and between species. Growth rates calculated with OD are given for comparison with growth rates estimated from the average of maximum growth rates previously estimated (r_{est}). * indicates assays with excessively high initial cell density.

Exp.	r_{est}	N_0	N_E	D_E	R_E	T_E	$\log_{10}(\gamma)$	(±S.E.)	γ
Pp-pQ to *Pp*	0.333	46,833	462,083	123,125	338,948	10	-10.00	(±0.209)	8.80×10^{-11}
Pp-pQ to *Pf*	0.377	34,813	388,646	183,958	204,680	8	-10.09	(±0.264)	8.25×10^{-11}
**Pf*-pQ to *Pf*	0.433	4,837,500	7,450,000	2,620,833	4,825,304	3,863	-9.41	(±0.221)	3.76×10^{-10}
**Pf*-pQ to *Pp*	0.389	4,070,833	7,408,333	2,837,500	4,570,195	638	-10.56	(±0.096)	4.24×10^{-11}
Pp-pS to *Pp*	0.314	32,354	447,271	13,000	433,888	382	-7.57	(±0.235)	2.26×10^{-8}
Pp-pS to *Pf*	0.358	30,329	52,634	23,153	28,505	977	-6.74	(±0.644)	1.20×10^{-6}
Pf-pS to *Pf*	0.387	82,417	170,655	124,989	45,655	11	-8.82	(±0.119)	1.50×10^{-9}
Pf-pS to *Pp*	0.343	33,571	807,385	22,177	784,966	242	-8.32	(±0.112)	4.95×10^{-9}

Table 4.9: Plasmid transfer rate calculations (Simonsen et al., 1990) for pQBR55 (pQ) and pSTY.Pf (pS) in *P. putida* (*Pp*) and *P. fluorescens* (*Pf*), within and between species. * indicates assays with excessively high initial cell density. Reported standard errors (S.E.) are of the $\log_{10}$ values.

4.3.2 Simulation results

Strain	r	K_M	y	γ_{X_PX}	γ_{X_PY}	λ	n
Pp	0.351	0.035	8.06E-8			5.67E-3	4.4
Pp-pQ	0.315	0.032	8.05E-8	8.80E-11	8.25E-11	5.67E-3	4.4
Pp-pS	0.277	0.028	8.09E-8	2.26E-8	1.20E-6	5.67E-3	4.4
Pf	0.439	0.163	9.19E-8			1.24E-4	0.101
Pf-pQ	0.426	0.043	7.82E-8	0	0	1.24E-4	0.101
Pf-pS	0.334	0.067	8.07E-8	1.50E-9	4.95E-9	1.24E-4	0.101

Table 4.10: Experimental parameter estimate results.

The results of the simulations with experimentally determined parameters (Table 4.10, table 4.5) are shown in figure 4.9. These results enable a number of qualitative predictions to be made. Firstly, one can see that both recipient strains are able to persist in isolation (Figure 4.9: 1 and 4). All donor strains show an initial positive trajectory which indicates persistence in isolation (Figure 4.9: 3, 8 and 11) except for *P. fluorescens*-pSTY.Pf which decreases in density from the start (Figure 4.9: 2). *P. fluorescens*-pSTY.Pf appears unable to persist due to the lag and high cost of the plasmid on cell growth without maintaining a high enough transfer rate to allow persistence. *P. putida*-pQBR55 and *P. fluorescens*-pSTY.Pf donors strains have a competitive disadvantage compared with their corresponding plasmid-free strains (shown through the increase and persistence of recipient cells, Figure 4.9: 2 and 8) while *P. fluorescens*-pQBR55 and *P. putida*-pSTY.Pf have competitive advantages over their corresponding recipient strains (Figure 4.9: 3 and 11). The advantage in *P. fluorescens*-pQBR55 is due to the higher estimated K_M value in the *P. fluorescens* recipient that makes the donor more competitive at lower substrate concentrations. The same competitive advantage is not seen in the *P. fluorescens*-pSTY.Pf donor because the plasmid cost is too great to allow persistence. In contrast, *P. putida*-pSTY.Pf maintains an advantage due to its high rate of transfer that allows it to dominate the population, despite its high cost.

The *P. putida* recipient has a competitive advantage over all *P. fluorescens* strains (Figure 4.9: 5, 6 and 7), but can be invaded by the *P. putida*-pSTY.Pf donor when the plasmid begins in *P. fluorescens* (Figure 4.9: 6). Both *P. putida* donors (pQBR55 and pSTY.Pf) have a competitive advantage compared with all *P. fluorescens* strains (Figure 4.9: 9, 10, 12) except for *P. putida*-pSTY.Pf that has an initial disadvantage compared with *P. fluorescens*-pQBR55 due to the high and low costs of pSTY.Pf and pQBR55 respectively (Figure 4.9:

13). Plasmid transfer can be seen from all of the initial donor strains into their recipient competitor strains (Figure 4.9: 6, 9 and 12) except from *P. fluorescens*-pQBR55 into *P. putida* (Figure 4.9: 7).

Figure 4.9, panels 10 and 13, show interesting cyclical plasmid-host dynamics, including species coexistence. In panel 10 the *P. putida*-pQBR55 donor is invaded by its recipient, but then re-invaded by a donor strain. Upon further inspection (see Figure 4.10), it is the pSTY.Pf donor that invades the recipient (due to its high transfer rate), which can then be invaded by the pQBR55 donor due to its low cost compared with the pSTY.Pf donor, essentially inoculating the recipient from infection by pSTY.Pf when superinfection is not permitted. In this simulation (Figure 4.10: 13), *P. putida*-pSTY.Pf density dips below 1 cell before persisting. This highlights how initial cell densities can potentially impact outcomes. *P. fluorecens*-pQBR55 initially dominates due to the low cost of pQBR55, but is invaded by the *P. putida* recipient due to its higher growth rate. The *P. putida* recipient is then invaded as in panel 10, which is again invaded by *P. fluorescens*-pSTY.Pf. If pQBR55 could transfer into *P. putida* it is likely that it would also be able to persist as seen in panel 10.

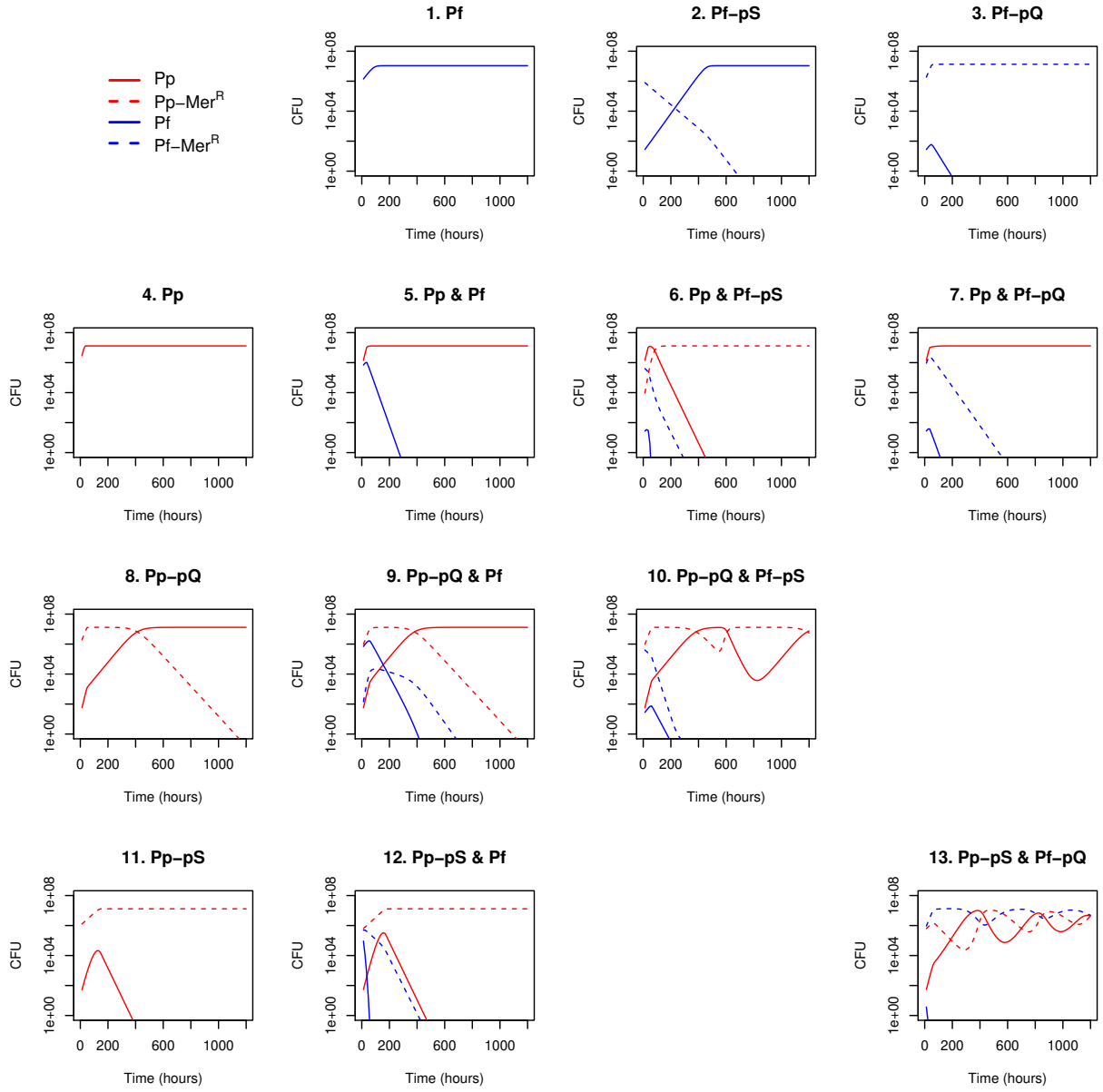

Figure 4.9: Simulations using experimentally estimated parameters (Table 4.10). Red and blue lines indicate *P. putida* (*Pp*) and *P. fluorescens* (*Pf*), respectively, while solid and dashed lines indicate plasmid-free and total mercury resistant (Mer^R) plasmid bearing strans (pQBR55 (pQ) and pSTY.Pf (pS)), respectively. Simulations were run in 1 gL^{-1} glucose with a 1:20 dilution of the culture into fresh media every 12 hours. Strains grown in isolation (1, 2, 3, 4, 8, 11) were initialised with 50,000 cells mL^{-1} while competition strains were initialised with 25,000 cell mL^{-1} for each strain. A single additional recipient cell was added to each donor strain to observe the effects of plasmid loss.

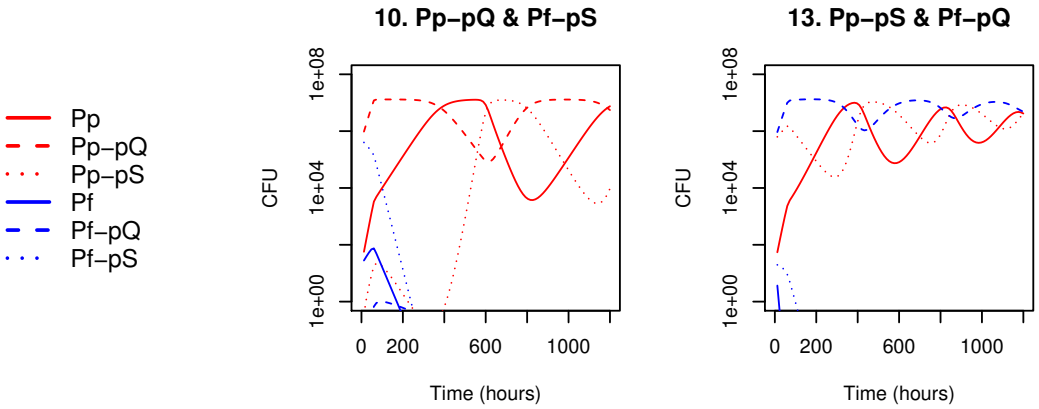

Figure 4.10: Simulations using experimentally estimated parameters (Table 4.10, panels 10 and 13). Red and blue lines indicate *P. putida* (*Pp*) and *P. fluorescens* (*Pf*), respectively, while solid, dashed and dotted lines indicate plasmid-free, pQBR55 (pQ) donors and pSTY.Pf (pS) donor strans, respectively. Simulations were run in 1 gL^{-1} glucose with a 1:20 dilution of the culture into fresh media every 12 hours. Strains were initialised with 25,000 cell mL^{-1} for each strain. A single additional recipient cell was added to each donor strain to observe the effects of plasmid loss.

4.3.3 Competition experiment results

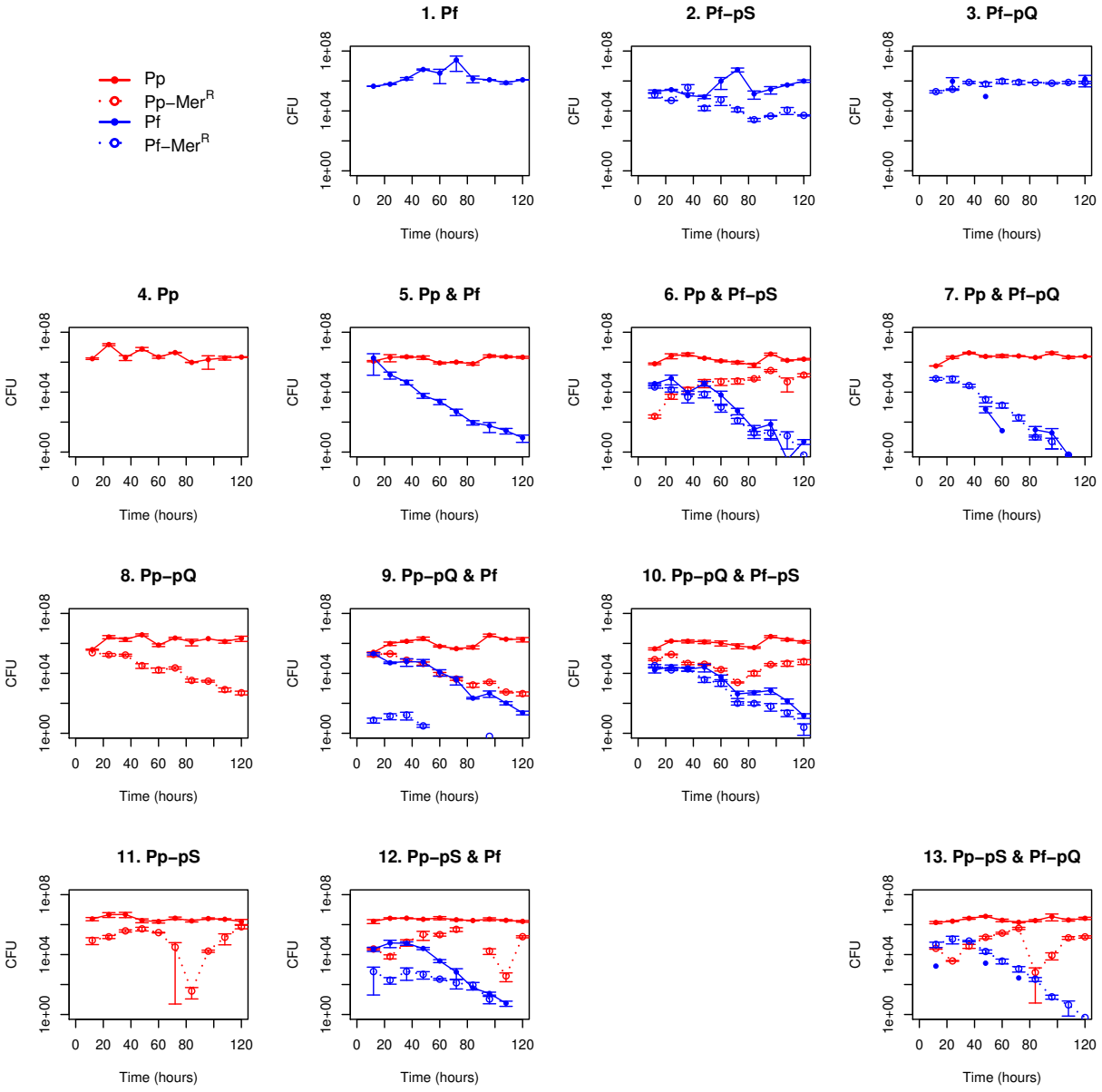

Figure 4.11: Competition experiment results. Red and blue lines indicate *P. putida* (*Pp*) and *P. fluorescens* (*Pf*), respectively, while solid and dashed lines indicate plasmid-free and total mercury resistant (Mer^R) plasmid bearing strans (pQBR55 (pQ) and pSTY.Pf (pS)), respectively.

Despite a few technical experimental setbacks,[12] the competition experiment results show a reflection of the majority of the qualitative simulation findings (Figure 4.11), albeit over shorter timescales. Both *P. fluorescens* and *P. putida* recipient strains are able to persist

[12]Experimental errors: OD_{600} reader stopped working between 60 and 72 hours in CompEx2 which meant that regular shaking did not occur. This is likely to have caused some cell aggregation and the transfer of non-homogeneous samples (the results show a lower-than-expected density following this transfer (Figure 4.11: 1, 2 and 4), but which does not largely affect the patterns).

independently (Figure 4.11: 1 and 4). pQBR55 decreases in prevalence through time when in *P. putida* and would likely go extinct from the system given more time (Figure 4.11: 8), while it persists in *P. fluorescens* without any indication of recipient invasion (Figure 4.11: 3). This may be due to a high plasmid fidelity (from par genes), a competitive advantage of the donor strain over the recipient strain (potentially through a higher K_M value as seen in the simulations) or a high transfer rate of pQBR55 in *P. fluorescens* (less likely, given the low estimated transfer rate). pSTY.Pf donor strains show clear persistence in *P. putida* (fig 4.11: 6, 10, 11, 12 and 13), although not at saturation as seen in the simulations. Models show a narrow parameter range where intermediate plasmid prevalence can occur (see Chapter 3) despite this being seen more frequently experimentally (Fan et al., 2019; Fox et al., 2008), and this inconsistency is yet to be adequately resolved. pSTY.Pf also shows potential persistence in *P. fluorescens* (Figure 4.11: 2), although this is not completely clear given the length of the experiment. Transfer at a higher rate than estimated experimentally, enabling the plasmid to persist despite the costs it incurs. A sudden drop in plasmid density is also seen in *P. putida*-pSTY.Pf at around 80 hours (Figure 4.11: 11, 12 and 13), the cause of which is unknown, although the pattern is repeated across replicates from that experiment. It is speculatively possible that this is evidence of parameter evolution, that a lower transfer rate evolves leading to decreased prevalence, followed by either a higher transfer rate, or a reduction in cost which enables the plasmid to increase in prevalence again.

All competition assays resulted in the dominance of *P. putida* and the decrease of *P. fluorescens* donor and recipient densities through time, leading to extinction in some assays. Some transconjugants were detected in *P. fluorescens* from *P. putida*-pQBR55 (Figure 4.11: 9) while transconjugants were not seen in *P. putida* from *P. fluorescens*-pQBR55 (Figure 4.11: 7), consistent with the simulations. Where *P. putida*-pQBR55 and *P. fluorescens*-pSTY.Pf are in competition (Figure 4.11: 10), we see a decrease in *P. putida* plasmid prevalence as pQBR55 is not maintained, then increase as it is replaced by pSTY.Pf due to its high transfer rate, which can be seen in the simulation (Figure 4.9: 10). No further oscillations are detected as were seen in the simulation.

Coexistence between strains (donors or recipients) of the two species was not observed in the competition experiments, even though pSTY.Pf was able to persist in *P. putida*, meeting one

of the requirements of coexistence (Box 4.1). Despite this, the effects of plasmid presence and prevalence on competition can be observed through comparison of *P. fluorescens* strain density through time. Initial pQBR55 distribution now clearly (compared with the simulation results) affects the competitive ability of *P. fluorescens* when comparing *P. fluorescens* cell density in the 9^{th} serial transfer (108 hours, figure 4.12). High pQBR55 prevalence in *P. putida* decreases its competitive advantage resulting in a higher *P. fluorescens* density (Figure 4.11: 9) compared with its density in the recipient competition assay (Figure 4.11: 5). Similarly, a high pQBR55 prevalence increases the competitive disadvantage of *P. fluorescens* resulting in a lower *P. fluorescens* density (Figure 4.11: 7) compared with the recipient competition assay. A Kruskal-Wallis test was used to statistically establish the significance of the effect of initial prevalence and distribution of pQBR55 in assays 5, 7 and 9 ($H(2)=7.5426$, $p<0.05$). A Dunn test subsequently identified that the significant difference ($p<0.05$) was between assays 7 (*P. putida* & *P. fluorescens*-pQBR55) and 9 (*P. putida*-pQBR55 & *P. fluorescens*). The observed effects of pQBR55 are contrary to the simulations where *P. fluorescens*-pQBR55 has a greater competitive advantage than the recipient. The experimental methods may overestimate the K_M value for *P. fluorescens*-pQBR55 due to the limitations of its estimation process caused by diauxic growth (Figure C.6). pSTY.Pf is costlier than pQBR55 in both *P. putida* and *P. fluorescens*, but the effects of its cost are less clear than the costs of pQBR55. Both assays featuring pSTY.Pf (Figure 4.11: 6 and 12) show that *P. fluorescens* goes extinct faster than in the recipient competition assay. This is because pSTY.Pf is able to transfer at a high enough rate to gain high prevalence in *P. fluorescens*, regardless of the initial source of the plasmid, decreasing its competitive ability. A Kruskal-Wallis test was unable to show a significant difference between the pSTY.Pf competition assay results dependent on the initial prevalence and distribution of pSTY.Pf ($H(2)=2.0284$, $p=0.3625$).

Depending on the community composition, plasmid persistence can be variable. Over the length of this experiment, pQBR55 was only able to persist when in *P. fluorescens*, and only in the absence of *P. putida* (Figure 4.11: 3). pQBR55 could not persist in *P. putida* or when *P. fluorescens*-pQBR55 was in competition with *P. putida* (Figure 4.11: 7 and 13). In contrast, pSTY.Pf was able to persist in *P. putida* due to its high rate of transfer.

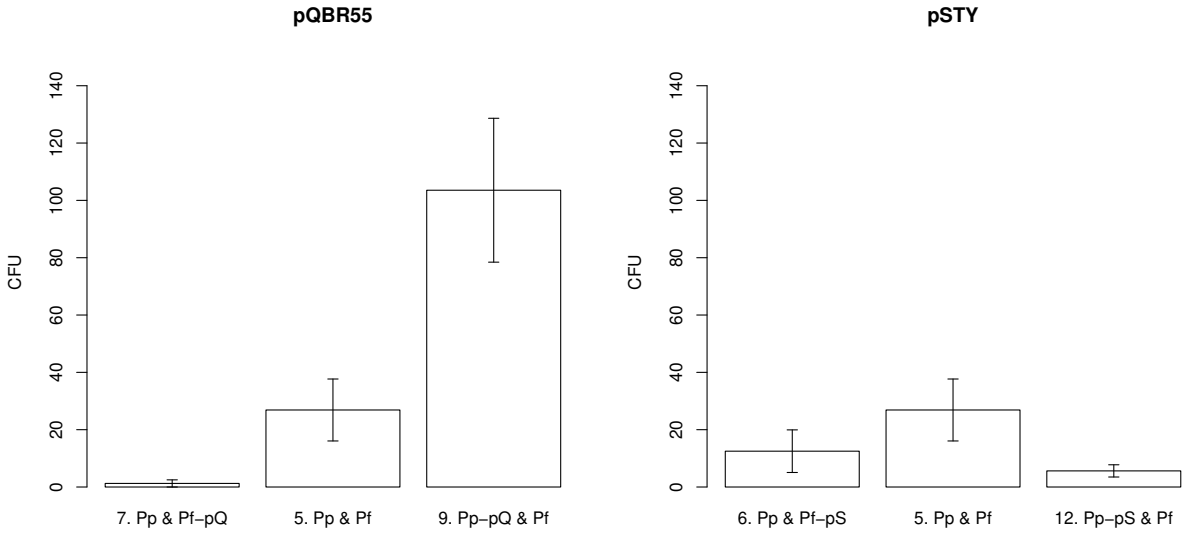

Figure 4.12: Cell density (CFU) of *P. flourescens* (*Pf*) at 108 hours as initial plasmid distribution (pQBR55 (pQ): left and pSTY.Pf (pS): right) changes. *P. putida* (*Pp*).

4.4 Discussion and future experiments

Competition experiments were performed between *P. putida* and *P. fluorescens* where initial plasmid (pQBR55 and pSTY.Pf) presence was varied. Simulation results from a model using independently estimated parameters was compared with the competition results. The results provide case studies for plasmid-host combination dynamics in simple communities and show some impacts of interactions between plasmid prevalence and competition.

4.4.1 Costly plasmid prevalence on competition

Plasmids are well-known to have a negative effect on growth due to the costs incurred in transfer, and the additional replicative and metabolic costs (San Millan and MacLean, 2017). This reduction in growth rate can impact the results of competition, increasing the potential for species coexistence (Grover and Wang, 2019). While the results of these experiments did not demonstrate a scenario where the species were able to coexist, the effect of plasmid presence on competition was clearly observed. High plasmid prevalence in a species reduces the growth of the species when the plasmid is costly. This can either reduce or increase the disparity between growth rates of competing species resulting in increased or decreased likelihood of coexistence, depending on the plasmid prevalence in either species. However, these effects are mitigated when the plasmid has a high rate of transfer (e.g. pSTY.Pf). In these circumstances the initial source of a high transfer rate plasmid is less important to the outcome, due to the rapid spread of the plasmid throughout the community, regardless of the original host. This has the greatest effect on the population which has the highest plasmid burden (combined prevalence and cost to growth) and will tend to have a stronger effect on the non-dominant species. Maintenance of a high transfer rate plasmid in the dominant species in these experiments has the appearance of "spiteful" transfer (Dimitriu et al., 2016), where the higher plasmid burden in the non-dominant species decreases the time it takes for the species to reach extinction. It is, however, unlikely to be an evolved competitive strategy.

These results have broader implications for species interaction dynamics in more complex communities. The presence of a plasmid, its within-species prevalence and pervasiveness in the community may affect community structure. In addition, if plasmid persistence even

occurs in a single species, then it is possible that the plasmid can be maintained at low prevalence in species across the community including in species where the plasmid is not able to persist when grown in isolation (Hall et al., 2016).

4.4.2 Competition on plasmid persistence

The effects of competition on plasmid persistence correspond with the behaviour of the plasmid. Competition prohibits the persistence of both *P. fluorescens* and pQBR55, where both were able to persist at high prevalence in the absence of competition. In contrast, pQBR55 was not able to persist in *P. putida* in any assay. pSTY.Pf was able to persist in all assays where it occurred, regardless of initial host and community composition despite its high cost on growth. In the examples demonstrated, the high cost and high transfer rate of pSTY.Pf was a better strategy, when in a competitive environment, than the low transfer, low cost, high fidelity strategy of pQBR55.

Assays featuring both plasmids saw the replacement of pQBR55 with pSTY.Pf in *P. putida*, which carries mercury detoxification genes, fulfilling the same function, albeit with a greater cost. These plasmids in the two hosts show an interesting reflection of how different plasmid strategies can result in perhaps counter-intuitive outcomes. The literature suggests that plasmids evolve towards a vertical persistence strategy of low transfer, low cost, high fidelity, and while this may be more useful when a species is in isolation, it clearly doesn't allow for plasmid persistence in all scenarios. According to these results, plasmid persistence in a community (albeit a small one, and one undergoing continuous exponential growth) is likely to be better correlated with a higher transfer rate, despite the higher costs, than a high fidelity, low transfer, low cost plasmid.

4.4.3 Issues with model simulation predictions

The model was able to successfully predict many of the qualitative outcomes of the competition experiments through the use of independently estimated parameters, differing where specific parameters were over or underestimated. The effects of plasmids on growth and their persistence through transfer was not always adequately captured, resulting in some

donors having a greater competitive advantage over their corresponding recipients (*P. fluorescens*-pQBR55). The *P. fluorescens* value for K_M is likely to have been overestimated due to the complexities of its growth that were not captured by the model (e.g. diauxic growth). The model simulations did not show intermediate prevalence of the persisting plasmid, that can only be seen in a narrow parameter space (Chapter 3), and that was observed experimentally. Finally, while the model was able to show coexistence of the two bacterial species through oscillatory behaviour, this was not seen experimentally. The absence may have been due to incorrect parameter estimations, or due to assumptions of the model (such as plasmid incompatibility and no superinfection) that enable coexistence and oscillations to occur theoretically.

4.4.4 Future work

The plasmids used in these experiments are only two examples of the various behaviours plasmids exhibit. While these increase the breadth of examples of plasmid behaviour in the literature, many more need to be demonstrated in order to construct a comprehensive picture of general plasmid dynamics in communities. In addition, due to the nature of these assays, natural plasmid environments vary widely from the dramatically simplified conditions used here. Further experiments mimicking realistic natural environments (e.g. agricultural waste, hospital effluent, rivers, soils, animal guts), and with greater microbial complexity by increasing the number of species, can be constructed. Interesting further experiments can be constructed to show how and in which species plamsids are able to persist, how the plasmid affects the community structure and dynamics, and how those things could change through exposure to selective agents. Additional experiments could be conducted to show the adaptation of more plasmids to various hosts (e.g. the adaptation of a costly, high transfer plasmid such as pSTY.Pf).

Some aspects of bacterial growth, transfer and competition could be better captured by the model, including the initial growth following serial transfer, non-logistic growth of *P. putida* and diauxic growth of strains that deviate from the expected exponential growth. Better estimation of CFU at low cell density and inter-cellular competitive interactions could also be considered. In addition, these models tend to result in plasmid extinction or saturation except over a narrow parameter range, something seen reasonably frequently in

experiments (Fan et al., 2019; Fox et al., 2008; Kottara et al., 2018) including in *P. putida*-pSTY.Pf (shown in this chapter) that is yet to find resolution. Furthermore, the model assumed plasmid incompatibility without plasmid superinfection, and this may affect the outcome of simulations.

Chapter 5

Discussion

This thesis aimed to investigate aspects of plasmid dynamics in three ways. First, it looked at the role of environmental and biotic determinants of plasmid transfer rates to quantify the relative importance of these factors. Transfer rates had a distribution of over ten orders of magnitude: between 10^{-20} and 10^{-6}, with the primary determinants being plasmid repression/derepression and media type. Next, it looked at some of the evolutionary forces that affect and determine plasmid transfer rates, the role of plasmid-host conflicts and recipients under selective and non-selective conditions, and the subsequent effects on plasmid prevalence. The use of adaptive dynamic modelling identified the importance of the recipient cell and a simple equation that can estimate the evolutionarily selected transfer rate from only a few parameters. Host mechanisms that reduce the transfer rate in non-selective conditions require a substantial decrease before plasmid prevalence is affected under the majority of tested realistic parameter combinations. Finally, the thesis attempted to experimentally demonstrate the theoretically established potential for the coexistence of two species due to plasmid transfer and costs on cellular growth rate (Grover and Wang, 2019; Sheppard, 2016). While species coexistence did not occur experimentally, the effects of plasmid presence and prevalence on competition were evident. High plasmid prevalence reduced the growth of each species and affected the competitive dynamics accordingly. Other parameters such as plasmid fidelity and invasion into competing species also played a role in the stability of the plasmid in the community. The model results predicted many of the experimental outcomes, although there were some differences due to limitations in parameter estimation and modelling assumptions.

5.1 Identifying the sources of variation in transfer rate

In the first two chapters, I attempted to identify some of the specific causes of plasmid transfer rate differences. The results of the meta-analysis focus on the primary sources of variation in transfer rate due to environmental and biotic factors, while the modelling results highlight how transfer rates are subject to evolution due to specific parameters: particularly the relationship of transfer rate with plasmid cost. While these results were valuable, much of the variation and its link with selective pressures remains unexplained. Many of the factors have unclear or inconsistent effects on transfer rate, which remain unresolved, and the effects of evolution on these rates are unidentified. Although many studies have attempted to identify the effects of environmental, biotic and evolutionary factors, the experimental methods have not been reductive or systematic enough to get to the bottom of these differences. There is still much to do to disambiguate the sources of this variation.

Ultimately, these differences will only be fully explained by going to the genetic level and quantifying the effects of specific plasmid and host genes on transfer rate and plasmid cost. This quantification would enable comprehensive assessments to occur that would allow predictions of the plasmid transfer rates and corresponding growth rate costs of sequenced plasmids. Many genomes of plasmids and hosts are sequenced and annotated sufficiently to be able to identify and compile a list of the genes affecting plasmid transfer and the effects of those genes on the growth rate of the host. By systematically constructing and manipulating simple plasmids and hosts to insert and knock out combinations of these genes, it may be possible to measure the individual and cumulative effects of each gene on plasmid transfer rate and cost. From this point, the hosts can be varied more broadly, including different recipients for inter-strain and inter-species transfer to see how this affects plasmid transfer rates and costs and establish the source of these differences. Only with this secure foundation, variable abiotic conditions can then be sensibly introduced, such as temperature and substrate type and concentration. Temperature (Bradley and Whelan, 1985; Fernandez-Astorga et al., 1992; Simonsen et al., 1990) and substrate concentration (Freter et al., 1983; MacDonald et al., 1992; Smets et al., 1995; Smith, 1977; Zahrl et al., 2006) are both factors that have variable effects on different plasmid-host combinations. An understanding of the genetic components can help us identify the source of these variable

effects.

The quantification of individual genes on plasmid transfer rates and costs may enable us to understand the relationship between plasmid transfer rate and plasmid costs on host growth, including the plasmid transfer rate cost coefficient (a key parameter in the transfer rate prediction equation). The composite and additive effects of these discrete genes make it unlikely that the relationship between transfer rate and plasmid cost is linear, although there may still be a model that makes a decent approximation of this relationship for use in modelling and the estimation of plasmid selected transfer rate. With this basis, these results can be linked with evolutionary experiments under variable abiotic conditions to see if and how transfer rates and plasmid costs evolve in line with the model expectations. If the observed transfer rates deviate from the model's predictions, it may be possible to identify the causes of these deviations through the locations of genes that affect transfer and are likely to be due to host control mechanisms driven by host-plasmid conflicts.

At each stage in this process, the effects of plasmid transfer rate and plasmid cost on plasmid prevalence in the population can also be simulated, measured and compared with the findings of the persistence criteria of Stewart and Levin (1977). Furthermore, there will be almost unending ways of exploring the additionally complex effects of other genes and systems such as genes affecting plasmid loss, transitory-derepression, quorum sensing and competence switches, the effects of which could also be quantified. This kind of thorough exploration could enable a comprehensive understanding of plasmid dynamics in populations and communities, including the results found in the experimental work chapter.

5.2 Plasmid transfer rate predictions by the model

One of the most exciting findings of the thesis is the result that demonstrates the effects of selection on transfer rate, where the analysis results in a simple equation that predicts the expected transfer rate when the plasmid is in control. Depending on the parameter variability, this equation can potentially be applied to evaluate and make inferences on observed rates of plasmid transfer, identifying likely examples of host-evolved control mechanisms that reduce transfer rates in non-selective conditions. Before this is possible, however, there are some follow-up questions and extensions that require exploration. First, does it work?

How reliable are the equation estimates, and can they be used in practice? While the compiled plasmid transfer rates from the meta-analysis contained over one thousand plasmid transfer rates, the other necessary equation parameters were not available for the same plasmids. Only one plasmid had estimates for all the required parameters (Haft et al., 2009). While the equation predicted the rate of repressed transfer of this plasmid to a reasonable degree of accuracy using those parameter estimations, a comprehensive dataset needs to be collected to establish the validity of the equation. A stepping stone to reach this point is being able to accurately model the relationship between transfer rate and cost, one of the essential parameters for the equation. As previously mentioned, the costs incurred in transfer are likely to be synthesised through a combination of genes and needs to be comprehensively unpicked. Being able to show how the measured rates of plasmid transfer are affected by costs to the host would be extremely rewarding. Connecting these rates with the evolutionary history of the host and plasmid is also essential for understanding the determination of transfer rate. Transfer rates in the meta-analysis were mostly not from coadapted plasmids and hosts, so the observed rates of transfer may not correspond with the plasmid parameters in their natural environments. Furthermore, coevolution does not guarantee that the plasmid alone determines the plasmid transfer rate. Plasmid-host conflicts lead to the evolution of host control mechanisms in non-selective conditions that play a role in determining the transfer rate. It may be possible to identify plasmid and host-based control genes and their corresponding effects on transfer rate and plasmid cost by using knock-out experiments. Also, there may be limitations in applying the model results to real environments and their corresponding data due to the model assumptions of cell growth and homogeneity in the liquid media. Other models incorporating elements of spatial and heterogeneous environments can look at the selection pressures in these circumstances.

Secondly, if the equation is correct, albeit only under certain circumstances, what are its potential inferences? Comparing the expected transfer rate using the model with observed rates of transfer could give insight into the control of transfer. If the observed transfer rate is lower than expected, it can be inferred that there are host control mechanisms in place and that the plasmid is not in complete control. Also, if any of the parameters have a relatively small variance (such as the growth rate or the plasmid transfer cost coefficient), then it would be possible to simplify the model further, and model the expected transfer rate based on fewer parameters. If the growth rates and the cost coefficient each have a

narrow distribution, there would be a more direct relationship between the expected transfer rate and the remaining parameter (loss rate) and would be a useful finding. It may also be possible to make inferences about growth, loss or the transfer rate cost coefficient based on the observed transfer rate. The equation can be applied to the results from the experimental chapter to determine if the plasmids are transferring at the expected rates, if evolution is required for the transfer rate to reach this value, or if host control mechanisms have evolved to alter the transfer rate. Further evolutionary experiments could assess the ways that transfer rates and the other parameters adapt to the environment and community. The simplified model equation only applies when the environment has a low dilution rate but does the model still predict what happens in environments with high dilution? Namely, the transfer rate required for plasmid stability in the population increases until the costs become too great to permit plasmid persistence. Does this have an impact on the persistence of plasmid-based genes? Does this increase the selection pressure for those genes to migrate to the host where there are reduced costs? If so, is this an improvement or a danger to the further spread of antibiotic resistance genes? If not, can we utilise high dilution of a system to control the plasmid spread in water-based antibiotic resistance reservoirs?

5.3 The importance of the recipient in determining plasmid transfer rates

Both the meta-analysis and the model chapter results identified the recipient as playing a role in determining plasmid transfer rates, although in different ways. The meta-analysis identified the recipient strain as one of the primary determinants of transfer rate, explaining more variation than the donor and plasmid strains. The model identified the recipient as the element capable of evolving host control mechanisms. The results show that the recipient is a primary factor in determining transfer rates, and there are examples of evolved recipient control mechanisms (Chatterjee et al., 2013). The recipient is often neglected in the literature in favour of the plasmid and donor when considering the initiation and control of plasmid transfer rates, and its importance should retain proper credence. While these results of each chapter show consistency and the findings are valid, there are limitations in each. The role of the recipient in plasmid transfer is not often likely to be due to specific

evolutionary pressures and are probably the result of stochastic differences in recipients: the presence of restriction enzymes (Thomas and Nielsen, 2005), for example. Also, it is difficult to distinguish between recipient differences and donor discrimination as the source of transfer rate differences (Dimitriu et al., 2016). These differences could be established by identifying discrimination genes and mechanisms and removing them to see how this changes the observed rates. Evolution to control transfer rate in the recipient is somewhat convoluted, and various other selection pressures target the plasmid and donor directly. For example, the costs of pilus production and transfer initiation to the donor were not included and can be modelled. The experimental results demonstrate that, in a community, the recipient can also be a donor (as it becomes infected with the plasmid), and the transfer rate must be considered with the other plasmid parameters to describe the plasmid dynamics comprehensively.

5.4 Plasmid persistence

Transfer rate alone is less relevant to plasmid stability than the combined effect of all parameters that determine plasmid prevalence in a population or community. While the meta-analysis collected transfer rates that can give some indication to plasmid persistence, plasmid costs and loss must also be taken into account. Modelling has shown how plasmids, in theory, will largely saturate a population or go extinct, with only a limited range of values where intermediate plasmid prevalence occurs and also illustrated how the costs of plasmid transfer impact persistence. The plasmid persistence criteria (Stewart and Levin, 1977) separates plasmid transfer and plasmid cost. It may, therefore, be useful to rewrite this criterion based on the relationship between plasmid transfer and plasmid cost.

The experimental results demonstrated two examples of how plasmids can persist. The high transfer rate enabled pSTY to be maintained in the competition assays, although it is unclear how the plasmid might evolve given time to adapt. The plasmid might not be able to persist in non-selective conditions if selective pressures on the plasmid and the evolution of host control mechanisms lead to a reduction in plasmid transfer. pQBR55 was only able to persist by maintaining high fidelity in a population that did not go extinct, enabled by its lower cost. Plasmid persistence strategies in a community are more complex than considering

high transfer and low-cost methods and must also consider the species composition of the environment. There are so many intricate aspects of community dynamics that need to be explored in the future to show how plasmids persist in communities, such as cooperative interactions and how plasmid presence affects the community dynamics. It may be useful to develop metrics that describe plasmid persistence in individual species populations within a community. Taken together, these might paint a picture of the plasmid behaviour in a community.

Plasmid persistence in populations and communities can inform our understanding of the spread of antibiotic resistance genes. Ultimately, the rates of plasmid transfer are less relevant than the fact that any transfer can enable antibiotic resistance genes to cross species boundaries. Following exposure to antibiotics, these genes can rise to high prevalence, further increasing their spread. The results of this thesis indicate that many plasmids have the potential to persist indefinitely, even in the absence of selective pressures. Reducing antibiotic use as much as possible will be vital to limit the stimulation of antibiotic resistance. These include diversification of alternatives to antibiotics, but more must be done to tackle routes of infection (Garcillán-Barcia et al., 2011; Larsson, 2014). Waste from places where antibiotic use is prevalent needs review: particularly in hospitals and agriculture. Trends towards plant-based diets reduce the need for antibiotics in agriculture and will likely result in the reduction of use (Losasso et al., 2018).

5.5 The need for development of improved parameter estimation methods and metrics

All three of the data chapters rely on having reliable and usable plasmid transfer rate metrics and estimations. The parameter estimations in the third chapter showed that estimations of some of the parameters can be variable depending on estimation method, which lead to questions about how accurate the measurements are. Many biological features are not captured accurately in the model: converting density to cell count, fitting a growth curve to the data (including diauxic growth and serial transfer events), and bacterial competition. These issues compound to increase the potential for error in the final results and mean that although individual estimates may be approximately correct, their broad application is

limited. Improved modelling can tackle these problems: better models predicting cell density from optical density and a better model of cell competition through substrate consumption. The Michaelis-Menten model has some limitations where the growth rates can be difficult to estimate at various concentrations, and the data do not always match the expectations of the standard LB plot. Plasmid transfer rate could also be modelled more accurately by taking into account rate changes during different growth phases (Freter et al., 1983; Seoane et al., 2011; Smets et al., 1993). This relationship remains unclear and is likely to vary according to plasmid and host strains, and therefore requires attention. Different bacterial species have different growth patterns (e.g. diauxic growth), such that it may be appropriate to use different models for different species to reflect this.

Some of the experimental methods resulted in estimates with low accuracy, particularly cell density estimates through serial dilutions and drop-plating and the transfer of a representative sample of cells in serial transfers. Methods that are more automated and reliable can reduce cell clumping and get a better accuracy of cell density estimates. Alternative methods attempt this using flow cytometers and fluorescence (Andersen et al., 1998; Christensen et al., 1996; Dahlberg et al., 1998). These methods are manually intensive and still face some of the same problems as the less technical methods used in this thesis.

Although Simonsen's endpoint method to estimate plasmid transfer rate is more robust than others, the method initially underestimated the transfer rates due to low resolution of cell count at low OD. Some studies have explored the effects of cell density and donor-recipient ratios on this endpoint (Simonsen et al., 1990). Recently a standardised plasmid transfer rate protocol was published and is welcomed, but still has limitations (Huisman et al., 2020). Precise ranges of cell densities and ratios for where the endpoint estimates are functional and experiment replicate numbers would be useful in linking the models with experimental results.

These results and improvements to these metrics will have impacts on the results from the first two chapters. The model results are dependent on the models of bacterial growth and plasmid transfer, and adjusting the model may change the results. Any improvements in the model are going to improve the outcomes, though, and are welcome.

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Appendix A

Chapter 2 appendices

A.1 Data sources

Table A.1: Data sources.

No.	Authors	Year	Title	Notes
1	Bradley	1983	Specification of the Conjugative Pili and Surface Mating Systems of Pseudomonas Plasmids	
2	Bradley	1984	Characteristics and Function of Thick and Thin Conjugative Pili Determined by Transfer-derepressed Plasmids of Incompatibility Groups I1, I2, I5, B, K and Z	
3	Bradley	1985	Transfer systems of K88 and K99 plasmids	Mobilisable plasmid transfer rates removed
4	Bradley et al.	1980	Specification of Surface Mating Systems Among Conjugative Drug Resistance Plasmids in Escherichia coli K-12	Data averaged to avoid pseudoreplication
5	Bradley and Whelan	1985	Conjugation Systems of IncT Plasmids	
6	Bradley and Williams	1982	The TOL Plasmid is Naturally Derepressed for Transfer	
7	del Campo et al.	2012	Determination of conjugation rates on solid surfaces	
8	Dahlberg and Chao	2003	Amelioration of the Cost of Conjugative Plasmid Carriage in Escherichia coli K12	
9	Dennison and Baumberg	1975	Conjugational behavior of N plasmids in Escherichia coli K12	Mobilisable plasmid transfer rates removed
10	Dionisio et al.	2002	Plasmids Spread Very Fast in Heterogeneous Bacterial Communities	Unable to convert to endpoints.

Table A.1 – *Continued*

No.	Authors	Year	Title	Notes
11	Ehlers	1997	RP4 Plasmid Transfer Among Strains of Pseudomonas in a Biofilm	Data averaged to avoid pseudoreplication
12	Freter et al.	1983	Experimental and Mathematical Models of Escherichia coli Plasmid Transfer In Vitro and In Vivo	Mobilisable plasmid transfer removed
13	Froehlich et al.	2004	Horizontal Transfer of CS1 Pilin Genes of Enterotoxigenic Escherichia coli	Unable to convert to endpoints
14	Gama et al. Gama et al.	2017b	Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of otherplasmids but repress co-colonizing plasmids	
15	Genthner et al.	1988	Capacity of Aquatic Bacteria To Act as Recipients of Plasmid DNA	Unable to convert to endpoints
16	Gordon	1992	Rate of plasmid transfer among Escherichia coli strains isolated from natural populations	
17	Hall et al.	2015	Environmentally co-occurring mercury resistance plasmids are genetically and phenotypically diverse and confer variable context-dependent fitness effects	
18	Hennequin et al.	2012	Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate	Dimensions for plate mating transfer rates not included, removed

Table A.1 – *Continued*

No.	Authors	Year	Title	Notes
19	Jergensen and Stenderup	1982	An INCN Plasmid Exhibiting a High Frequency of Transfer in Broth Media	Not used: unknown measurement type (no access)
20	Lampkowska et al.	2008	A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species	Unable to convert to endpoints
21	Licht et al.	1999	Plasmid transfer in the animal intestine and other dynamic bacterial populations: The role of community structure and environment	
22	Lilley and Bailey	2002	The transfer dynamics of Pseudomonas sp. plasmid pQBR11 in biofilms	
23	Lilley et al.	1994	In situ transfer of an exogenously isolated plasmid between Pseudomonas spp. in sugar beet rhizosphere	Data averaged to avoid pseudoreplication, unable to convert to endpoints.
24	Lilley et al.	1996	Diversity of mercury resistance plasmids obtained by exogenous isolation from the bacteria of sugar beet in three successive years	One data point removed: covering several plasmids and reporting a range of rates, unable to convert to endpoints.
25	Loftie-Eaton et al.	2017	Compensatory mutations improve general permissiveness to antibiotic resistance plasmids	
26	Neilson et al.	1994	Frequency of Horizontal Gene Transfer of a Large Catabolic Plasmid (pJP4) in Soil	

Table A.1 – *Continued*

No.	Authors	Year	Title	Notes
27	Park et al.	2003	Identification and Characterization of the Conjugal Transfer Region of the pCg1 plasmid from Naphthalene-Degrading Pseudomonas putida Cg1	Removed long mating times prior to conversion
28	Piper and Farrand	1999	Conjugal Transfer but not Quorum-Dependent tra Gene Induction of pTiC58 Requires a Solid Surface	
29	Rang et al.	1996	Transfer of the plasmid RP1 in vivo in germ free mice and in vitro in gut extracts and laboratory media	Not used: unresolved discrepancies between measurements
30	Reniero et al.	1992	High frequency of conjugation in Lactobacillus mediated by an aggregation-promoting factor	Unable to convert to endpoints
31	Savage et al.	2013	Staphylococcus aureus Biofilms Promote Horizontal Transfer of Antibiotic Resistance	Unable to convert to endpoints
32	Simonsen	1990	Dynamics of plasmid transfer on surfaces	
33	Yanagida et al.	2016	Comparisons of the transferability of plasmids pCAR1, pB10, R388, and NAH7 among Pseudomonas putida at different cell densities	Removed points with too low density

A.2 Estimated growth rates with references

Table A.2: Estimated growth rates with references.

Species combination	Growth rates conditions and refs	Growth rate
1. Donor: *Alcaligenes eutrophus*, Recipient: *Variovorax paradoxus*		0.34
	Experimental Conditions: Media: PY agar Temperatures: 4, 23, 27 ***Alcaligenes*:** (Repaske and Mayer, 1976) Doubling time: 2 hours (0.34) at 31 degrees ***Variovorax*** No information found for *Variovorax*	
2. *Agrobacterium tumefaciens*		0.27
	Experimental Conditions: Media: AB mannitol minimal ***A. tumefaciens* (C58C1 pTFS40):** (Leth and McDonald, 2017) Max. growth rate: 0.46 (LB media) Max. growth rate: 0.43 (YEP media) Max. growth rate: 0.27 (Defined media (AB))	

Table A.2 – *Continued*

Species combination	Growth rates conditions and refs	Growth rate
3. *Agrobacterium tumefaciens*		1.06 (27°C) 1.34 (30°C) 1.75 (37°C)
	Experimental Conditions: Media: BHI, DM Temperatures: 27, 30, 37°C ***E. coli* (O'Mahony and Papkovsky, 2006)**: Doubling time: 42.74 mins (0.98) at 25°C Doubling time: 31 mins (1.34) at 30°C Doubling time: 24.84 mins (1.70) at 35°C Doubling time: 23.81 mins (1.75) at 37°C Doubling time: 20.41 mins (2.04) at 40°C Estimated 1.06 at 27°C	
4. Donor: *Klebsiella pneumoniae*, Recipient: *Escherichia coli*		1.08
	Experimental Conditions: Media: LB Temperature: 37°C ***K. pneumoniae*:** (Baranowski et al., 1985) Generation time: 4.57 hours (0.15) at 10°C Generation time: 0.79 hours (0.88) at 25°C Generation time: 0.64 hours (1.08) at 37°C	

Table A.2 – *Continued*

Species combination	**Growth rates conditions and refs**	**Growth rate**
5. *Pseudomonas aeruginosa*		0.76
	Experimental conditions: Media: BHI Temperature: 30, 37°C ***P. aeruginosa* (Gibson et al., 2018)**: Max doubling time: 0.91 hours (0.76) on glucose	
6. Donor: *Pseudomonas fluorescens*, Recipient: *Pseudomonas putida*		0.65
	Experimental Conditions: Media: LB, Minimal media, low and high acetate Temperature: 22°C ***P. fluorescens*:** (O'Mahony and Papkovsky, 2006) Doubling time: 58.29 minutes (0.71) at 25 °C Doubling time: 51 minutes (0.81) at 30°C Estimated 0.65 at 22°C	

Table A.2 – *Continued*

Species combination	Growth rates conditions and refs	Growth rate
7. *Pseudomonas putida*		0.71
	Experimental Conditions: Media: LB, MSB-G, MSB-2, Nutrient Broth Temperature: 30°C ***P. putida:*** (Wang and Nomura, 2010) Doubling time: 1.63 (0.42) at 30°C on citrate Doubling times at 30°C (Nikel et al., 2015): 100 mins (0.416) on 2-ketoglutarate 75 mins (0.55) on glucose 65 mins (0.64) on gluconate 55 mins (0.71) on succinate TOL plasmid donor (Smets et al., 1993) Growth rate: 0.343 on minimal media Max selected: 0.71	

A.3 Bacterial strain groups

Species	Source	Strain
E. coli	K-12	BW86 CD100 CD148 CSH50 J53 J62 JE2571 K12 MG1555 50N
E. coli	1665	1665 1665rs 1666 1776
P. fluorescens	SBW25	SWB25 SBW25ETC
P. nov	H2	H2 H2 following 600 generations
P. putida	KT2440	SM1443

Table A.3: Bacterial strain groups.

Additional strains:

A. tumefacciens: C58C1RS

A. eutrophus: JMP134

E. coli: 263, B41, BJ4, BM21, C25, CG140, ECOR, NG624, QC774, V374

K. pneumoniae

P. aeruginosa: PAO1150

P. fluorescens: Pf-5G, Pf-5S, RP4

P. putida: Cg1, Mes300, PaW1, PaW226, PaW340, PpS388

V. paradoxus: unknown 1

A.4 Determination of plasmid nativity

Table A.4: Determination of plasmid nativity.

Plasmid	Native Strain	Non-native Strains	Notes
CAM	-	P. aeruginosa: PAO1150	Plasmid introduced into strain
F	-	E. coli: BM21, K12	Plasmid introduced into strains
FP2	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
K99	E. coli: B41	E. coli: K-12	B41 is native strain
MIP233	-	E. coli: K-12	Plasmid introduced into strains
N3	-	E. coli: K-12	Plasmid introduced into strains
NAH7	-	P. putida: KT2440	Transferred from E. coli
pB10	-	P. putida: KT2440	Not explicitly from P. putida
pCAR1	-	P. putida: KT2440	Native species is P. resinovans
pCg1	P. putida: Cg1	-	Plasmid-host combination obtained from different study
pIN25	-	E. coli: K-12	Likely plasmid introduced into strain
pKM101	-	E. coli: K-12	Original strain unclear
pMG26	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
pPLS	E. coli: CG140	E. coli: K-12	CG140 is native strain
pREI	E. coli: 263	E. coli: K-12	263 is native strain
pTi	A. tumefaciens	-	Plasmid native to Agrobacterium
pVIDO	E. coli: V374	E. coli: K-12	V374 is native strain
pWW0	P. putida: PaW1, PaW226	P. aeruginosa: PAO1150 P. fluorescens: Pf-5S P. putida: PpS388	pWW0 native to arvilla strains
R1	E. coli: K-12 (J53)	E. coli: K-12, 1665, C25, BJ4, ECOR strains	E. coli: K-12 (J53) was considered a native host for R1
R100	-	E. coli: K-12	Plasmid introduced into strains
R124	-	E. coli: K-12	Plasmid introduced into strains
R144	-	E. coli: K-12	Plasmid introduced into strains
R2		P. aeruginosa: PAO1150	Plasmid introduced into strains
R7	-	E. coli: K-12	Plasmid introduced into strains
R388		P. putida: KT2440 E. coli: K-12	Plasmid introduced into strains
R391	-	E. coli: K-12	Plasmid introduced into strains
R394	-	E. coli: K-12	Plasmid introduced into strains
R402	-	E. coli: K-12	Likely plasmid introduced into strain
R446b	-	E. coli: K-12	Plasmid introduced into strains
R478	-	E. coli: K-12	Plasmid introduced into strains
R621	-	E. coli: K-12	Likely plasmid introduced into strain
R64	-	E. coli: K-12	Plasmid introduced into strains
R6K	-	E. coli: K-12	Plasmid introduced into strains
R702	-	E. coli: K-12	Plasmid introduced into strains
R721	-	E. coli: K-12	Likely plasmid introduced into strain

Table A.4 – *Continued*

Plasmid	**Native Strain**	**Non-native Strains**	**Notes**
R753	-	E. coli: K-12	Plasmid introduced into strains
R831b	-	E. coli: K-12	Plasmid introduced into strains
R91	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
R931	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
RA1	-	E. coli: K-12	Plasmid introduced into strains
RA3	-	E. coli: K-12	Plasmid introduced into strains
RIP64	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
Rms148	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
Rms163	-	P. aeruginosa: PAO1150	Plasmid introduced into strains
RN3	-	E. coli: K-12	Plasmid introduced into strains
RP1	P. fluorescens: RP4 P. nov: H2(evolved.)	E. coli: K-12 P. aeruginosa: PAO1150 P. nov: H2	P. fluorescens recipient strain needed to be cured of the plasmid. Evolved strain considered native.
Rts1	-	E. coli: K-12	Plasmid introduced into strains
Sa	-	E. coli: K-12	Plasmid introduced into strains
TP114	-	E. coli: K-12	Plasmid introduced into strains
TP228	-	E. coli: K-12	Plasmid introduced into strains

A.5 Model variants variable orders

	AIC	BIC
Model 1	Repression status	Repression status
	Media type	Media type
	Relatedness	Relatedness
	Medium richness	Medium richness
	Plasmid size	Plasmid size
	Plasmid nativity	Plasmid nativity
	Temperature	Temperature
	Mating time	Mating time
	Pilus type	Pilus type
Model 2	Recipient identity	Repression status
	Repression status	Media type
	Donor identity	Relatedness
	Media type	Medium richness
	Plasmid identity	Plasmid size
	Plasmid nativity	Plasmid nativity
	Temperature	Temperature
	Relatedness	Mating time
	Mating time	Recipient identity
	Pilus type	Pilus type
	Plasmid size	Donor identity
	Medium richness	Plasmid identity

Table A.5: Model variants variable order.

A.6 All model variant results

R^2: 0.63, R^2-adj: 0.6	df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	422.24	0	27.5
Media type	2	420.68	210.34	79.05	0	10.3
Relatedness	1	279.31	279.31	104.97	0	6.84
Medium richness	1	54.1	54.1	20.33	0	1.32
Plasmid size	1	18.55	18.55	6.97	0.0085	0.45
Plasmid nativity	1	13.24	13.24	4.98	0.0261	0.32
Temperature	1	34.06	34.06	12.8	0.0004	0.83
Mating time	1	11.21	11.21	4.21	0.0406	0.27
Pilus type	2	6.89	3.45	1.3	0.2747	0.17
Repression status : Medium richness	1	1.05	1.05	0.4	0.5293	0.03
Repression status : Temperature	1	1.38	1.38	0.52	0.4718	0.03
Repression status : Mating time	1	55.07	55.07	20.69	0	1.35
Repression status : Pilus type	2	183.75	91.87	34.53	0	4.5
Media type : Medium richness	2	98.58	49.29	18.52	0	2.41
Media type : Plasmid size	2	32.9	16.45	6.18	0.0022	0.81
Media type : Plasmid nativity	2	2.23	1.12	0.42	0.6575	0.05
Media type : Pilus type	3	70.29	23.43	8.81	0	1.72
Relatedness : Medium richness	1	0.04	0.04	0.01	0.907	0
Relatedness : Plasmid size	1	0.42	0.42	0.16	0.6911	0.01
Relatedness : Temperature	1	46.22	46.22	17.37	0	1.13
Relatedness : Pilus type	2	3.22	1.61	0.6	0.5465	0.08
Medium richness : Plasmid size	1	27.8	27.8	10.45	0.0013	0.68
Plasmid size : Temperature	1	2.07	2.07	0.78	0.3787	0.05
Plasmid size : Mating time	1	0.2	0.2	0.07	0.7857	0
Plasmid size : Pilus type	2	37.93	18.96	7.13	0.0009	0.93
Plasmid nativity : Temperature	1	31.09	31.09	11.68	0.0007	0.76
Residuals	575	1530.02	2.66	NA	NA	37.45
Total	611	4085.81	2118.51	NA	NA	100

Table A.6: Model 1, AIC, backward/forward.

R^2: 0.61, R^2-adj: 0.59	Df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	410.25	0	27.5
Media type	2	420.68	210.34	76.8	0	10.3
Relatedness	1	279.31	279.31	101.99	0	6.84
Medium richness	1	54.1	54.1	19.76	0	1.32
Plasmid nativity	1	10.61	10.61	3.87	0.0495	0.26
Temperature	1	44.15	44.15	16.12	0.0001	1.08
Pilus type	2	11.64	5.82	2.12	0.1204	0.28
Media type : Medium richness	2	82.74	41.37	15.11	0	2.02
Repression status : Relatedness	1	50.49	50.49	18.43	0	1.24
Relatedness : Temperature	1	50.62	50.62	18.48	0	1.24
Relatedness : Plasmid nativity	1	68.41	68.41	24.98	0	1.67
Media type : Temperature	2	22.11	11.06	4.04	0.0181	0.54
Repression status : Pilus type	2	173.87	86.94	31.74	0	4.26
Media type : Pilus type	3	59.93	19.98	7.29	0.0001	1.47
Medium richness : Pilus type	1	20.54	20.54	7.5	0.0064	0.5
Residuals	589	1613.08	2.74	NA	NA	39.48
Total	611	4085.81	2080	NA	NA	100

Table A.7: Model 1, AIC, forward/backward.

R^2: 0.61, R^2-adj: 0.59	Df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	414.33	0	27.5
Media type	2	420.68	210.34	77.57	0	10.3
Relatedness	1	279.31	279.31	103	0	6.84
Medium richness	1	54.1	54.1	19.95	0	1.32
Plasmid size	1	18.55	18.55	6.84	0.0091	0.45
Plasmid nativity	1	13.24	13.24	4.88	0.0275	0.32
Temperature	1	34.06	34.06	12.56	0.0004	0.83
Pilus type	2	14.67	7.33	2.7	0.0677	0.36
Repression status : Relatedness	1	47.38	47.38	17.47	0	1.16
Repression status : Pilus type	2	183.29	91.65	33.8	0	4.49
Media type : Medium richness	2	97.89	48.94	18.05	0	2.4
Media type : Pilus type	3	73.29	24.43	9.01	0	1.79
Relatedness : Plasmid nativity	1	5.11	5.11	1.88	0.1705	0.12
Relatedness : Temperature	1	73.57	73.57	27.13	0	1.8
Medium richness : Plasmid size	1	16.34	16.34	6.03	0.0144	0.4
Plasmid size : Pilus type	2	36.34	18.17	6.7	0.0013	0.89
Residuals	588	1594.47	2.71	NA	NA	39.02
Total	611	4085.81	2068.77	NA	NA	100

Table A.8: Model 1, BIC, backward/forward.

R^2: 0.54, R^2-adj: 0.53	Df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	358.88	0	27.5
Media type	2	420.68	210.34	67.19	0	10.3
Relatedness	1	279.31	279.31	89.22	0	6.84
Medium richness	1	54.1	54.1	17.28	0	1.32
Plasmid nativity	1	10.61	10.61	3.39	0.0661	0.26
Temperature	1	44.15	44.15	14.1	0.0002	1.08
Media type : Medium richness	2	88.38	44.19	14.11	0	2.16
Repression status : Relatedness	1	50.47	50.47	16.12	0.0001	1.24
Relatedness : Temperature	1	40.9	40.9	13.07	0.0003	1
Relatedness : Plasmid nativity	1	73.71	73.71	23.54	0	1.8
Relatedness : Medium richness	1	27.84	27.84	8.89	0.003	0.68
Residuals	598	1872.13	3.13	NA	NA	45.82
Total	611	4085.81	1962.29	NA	NA	100

Table A.9: Model 1, AIC, forward/backward.

R^2: 0.80, R^2-adj: 0.75	Df	Sum Sq	Mean Sq	F value	p	% expl.
Recipient identity	22	1399.82	63.63	38.3	0	34.26
Repression status	1	527.31	527.31	317.39	0	12.91
Donor identity	19	412.65	21.72	13.07	0	10.1
Media type	2	123.93	61.97	37.3	0	3.03
Plasmid identity	46	354.97	7.72	4.64	0	8.69
Plasmid nativity	1	92.27	92.27	55.54	0	2.26
Temperature	1	82.2	82.2	49.48	0	2.01
Mating time	1	5.36	5.36	3.23	0.073	0.13
Pilus type	2	5.65	2.82	1.7	0.1839	0.14
Plasmid size	1	0.03	0.03	0.02	0.8925	0
Medium richness	1	0.43	0.43	0.26	0.6128	0.01
Relatedness	1	3.39	3.39	2.04	0.154	0.08
Recipient identity : Plasmid nativity	1	5.81	5.81	3.49	0.0622	0.14
Repression status : Mating time	1	10.16	10.16	6.11	0.0138	0.25
Repression status : Plasmid size	1	14.07	14.07	8.47	0.0038	0.34
Media type : Temperature	2	16.04	8.02	4.83	0.0084	0.39
Media type : Pilus type	3	73.06	24.35	14.66	0	1.79
Plasmid identity : Pilus type	10	37.68	3.77	2.27	0.0134	0.92
Plasmid nativity : Medium richness	1	24.34	24.34	14.65	0.0001	0.6
Temperature : Mating time	1	0.06	0.06	0.04	0.8507	0
Temperature : Plasmid size	1	6.36	6.36	3.83	0.051	0.16
Media type : Medium richness	2	76.17	38.08	22.92	0	1.86
Residuals	490	814.07	1.66	NA	NA	19.92
Total	611	4085.81	1005.51	NA	NA	100

Table A.10: Model 2, AIC, backward/forward.

R^2: 0.80, R^2-adj: 0.73	Df	Sum Sq	Mean Sq	F value	p	% expl.
Recipient identity	22	1399.82	63.63	35.38	0	34.26
Repression status	1	527.31	527.31	293.17	0	12.91
Donor identity	19	412.65	21.72	12.08	0	10.1
Media type	2	123.93	61.97	34.45	0	3.03
Plasmid identity	46	354.97	7.72	4.29	0	8.69
Plasmid nativity	1	92.27	92.27	51.3	0	2.26
Temperature	1	82.2	82.2	45.7	0	2.01
Relatedness	1	5.59	5.59	3.11	0.0786	0.14
Medium richness	1	1.22	1.22	0.68	0.4098	0.03
Plasmid size	1	1.83	1.83	1.02	0.3137	0.04
Media type : Plasmid identity	46	196.24	4.27	2.37	0	4.8
Repression status : Media type	1	7.98	7.98	4.44	0.0357	0.2
Recipient identity : Plasmid nativity	1	5.78	5.78	3.21	0.0736	0.14
Plasmid nativity : Medium richness	1	14.99	14.99	8.33	0.0041	0.37
Repression status : Medium richness	1	8.39	8.39	4.66	0.0313	0.21
Plasmid identity : Medium richness	1	6.43	6.43	3.57	0.0593	0.16
Repression status : Plasmid nativity	1	4.86	4.86	2.7	0.1008	0.12
Repression status : Plasmid size	1	4.15	4.15	2.31	0.1296	0.1
Temperature : Plasmid size	1	4.23	4.23	2.35	0.1259	0.1
Residuals	462	830.96	1.8	NA	NA	20.34
	611	4085.81	928.33	NA	NA	100

Table A.11: Model 2, AIC, forward/backward.

R^2: 0.70, R^2-adj: 0.68	Df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	528.39	0	27.5
Media type	2	420.68	210.34	98.92	0	10.3
Medium richness	1	55.69	55.69	26.19	0	1.36
Plasmid nativity	1	47.59	47.59	22.38	0	1.16
Temperature	1	139.08	139.08	65.41	0	3.4
Mating time	1	87	87	40.92	0	2.13
Recipient identity	22	524.28	23.83	11.21	0	12.83
Pilus type	2	14.85	7.43	3.49	0.0311	0.36
Relatedness	1	66.68	66.68	31.36	0	1.63
Repression status : Pilus type	2	76.25	38.12	17.93	0	1.87
Media type : Medium richness	2	130.47	65.23	30.68	0	3.19
Media type : Pilus type	3	56.72	18.91	8.89	0	1.39
Plasmid nativity : Temperature	1	64.35	64.35	30.26	0	1.57
Temperature : Relatedness	1	29.6	29.6	13.92	0.0002	0.72
Medium richness : Plasmid nativity	1	16.08	16.08	7.56	0.0061	0.39
Media type : Temperature	2	27.3	13.65	6.42	0.0017	0.67
Residuals	567	1205.65	2.13	NA	NA	29.51
	611	4085.81	2009.25	NA	NA	100

Table A.12: Model 2, BIC, backward/forward.

R^2: 0.54, R-adj: 0.53	Df	Sum Sq	Mean Sq	F value	p	% expl.
Repression status	1	1123.54	1123.54	358.88	0	27.5
Media type	2	420.68	210.34	67.19	0	10.3
Relatedness	1	279.31	279.31	89.22	0	6.84
Medium richness	1	54.1	54.1	17.28	0	1.32
Plasmid nativity	1	10.61	10.61	3.39	0.0661	0.26
Temperature	1	44.15	44.15	14.1	0.0002	1.08
Media type : Medium richness	2	88.38	44.19	14.11	0	2.16
Repression status : Relatedness	1	50.47	50.47	16.12	0.0001	1.24
Relatedness : Temperature	1	40.9	40.9	13.07	0.0003	1
Relatedness : Plasmid nativity	1	73.71	73.71	23.54	0	1.8
Relatedness : Medium richness	1	27.84	27.84	8.89	0.003	0.68
Residuals	598	1872.13	3.13	NA	NA	45.82
	611	4085.81	1962.29	NA	NA	100

Table A.13: Model 2, BIC, forward/backward.

A.7 Removal of specific factors from full models

	R^2	% expl.	Adj-R^2	% expl.
Model 1 (AIC, b/f)	0.63	NA	0.6	NA
-repression	0.44	0.19	0.41	0.19
-media type	0.54	0.09	0.52	0.08
-relatedness	0.58	0.05	0.56	0.04
Model 1 (AIC, f/b)	0.61	NA	0.59	NA
-repression	0.4	0.21	0.38	0.21
-media type	0.51	0.1	0.5	0.09
-relatedness	0.55	0.06	0.53	0.06
Model 1 (BIC, b/f)	0.61	NA	0.59	NA
-repression	0.4	0.21	0.38	0.21
-media type	0.52	0.09	0.51	0.08
-relatedness	0.56	0.05	0.55	0.04
Model 1 (BIC, f/b)	0.54	NA	0.53	NA
-repression	0.36	0.18	0.35	0.18
-media type	0.48	0.06	0.47	0.06
-relatedness	0.46	0.08	0.45	0.08
Model 2 (AIC, b/f)	0.8	NA	0.75	NA
-recipient	0.75	0.05	0.7	0.05
-repression	0.75	0.05	0.68	0.07
-donor	0.76	0.04	0.71	0.04
-media type	0.71	0.09	0.65	0.1
-medium richness	0.78	0.02	0.72	0.03
-relatedness	0.8	0	0.75	0
Model 2 (AIC, f/b)	0.8	NA	0.73	NA
-recipient	0.74	0.06	0.67	0.06
-repression	0.75	0.05	0.67	0.06
-donor	0.75	0.05	0.68	0.05
-media type	0.7	0.1	0.65	0.08
-relatedness	0.79	0.01	0.73	0
-medium richness	0.79	0.01	0.72	0.01
Model 2 (BIC, b/f)	0.7	NA	0.68	NA
-repression	0.6	0.1	0.58	0.1
-media type	0.62	0.08	0.6	0.08
-medium richness	0.68	0.02	0.66	0.02
-recipient	0.59	0.11	0.58	0.1
-relatedness	0.66	0.04	0.64	0.04
-donor	0.7	0	0.68	0

Table A.14: Removal of specific factors from full models.

	R^2	% expl.	Adj-R^2	% expl.
Model 2 (BIC, f/b)	0.54	NA	0.53	NA
-repression	0.36	0.18	0.35	0.18
-media type	0.48	0.06	0.47	0.06
-relatedness	0.46	0.08	0.45	0.08
-medium richness	0.48	0.06	0.48	0.05
-recipient	0.54	0	0.53	0
-donor	0.54	0	0.53	0

Table A.14 – *Continued*

A.8 Transfer rates by key categories

Pilus derepression	**Pilus type**	Media type	Mean	S.E.
Derepressed	Flexible	Filter	-10.70	0.47
Derepressed	Flexible	Liquid	-10.02	0.20
Derepressed	Flexible	Plate	-9.82	0.30
Derepressed	Flex-Rigid	Liquid	-10.61	0.43
Derepressed	Flex-Rigid	Plate	-8.89	0.13
Derepressed	Rigid	Filter	-9.81	0.27
Derepressed	Rigid	Liquid	-12.47	0.21
Derepressed	Rigid	Plate	-8.98	0.08
Repressed	Flexible	Filter	-12.79	0.65
Repressed	Flexible	Liquid	-14.01	0.14
Repressed	Flexible	Plate	-12.12	0.25
Repressed	Flex-Rigid	Liquid	-12.65	0.30
Repressed	Flex-Rigid	Plate	-11.64	0.37
Repressed	Rigid	Filter	-9.62	0.19
Repressed	Rigid	Liquid	-11.79	0.26
Repressed	Rigid	Plate	-10.30	0.45

Table A.15: Transfer rates by key categories.

Appendix B

Chapter 3 appendices

B.1 Schematic model diagram

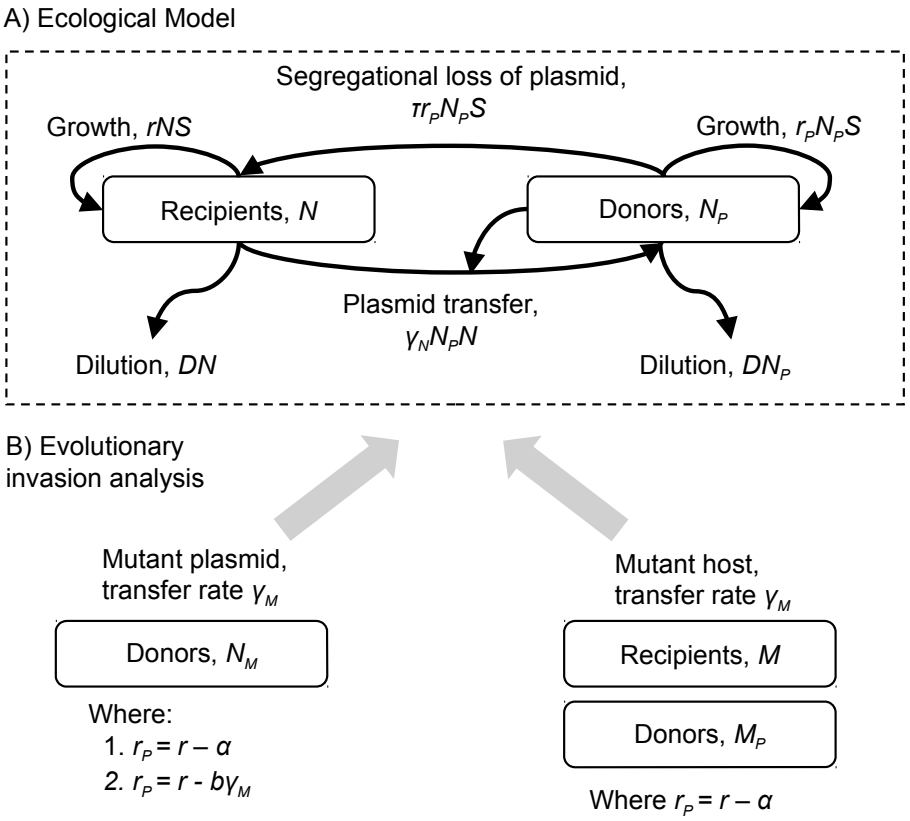

Figure B.1: Schematic diagram of the base model (A, Eq. 3.1-3.3), and main invasion analysed (B, mutant plasmid (N_M) with constant and transfer-dependent costs, and mutant host (M, M_P) with constant cost).

B.2 Calculating W_{N_M} (Model 1: plasmid control)

Rearranging equation 3.2 at equilibrium provides an equality:

$$\frac{dN_P}{dt} = (1-\tau)r_P N_P S - DN_P + \gamma_N N_P N = 0 \tag{B.1}$$

$$(1-\tau)r_P S^* - D = -\gamma_N N^* \tag{B.2}$$

which allows simplification of invasion success (fitness) of the mutant plasmid:

$$\frac{dN_M}{dt} = (1-\tau)r_P N_M S^* - DN_M + \gamma_M N_M N^* \tag{B.3}$$

$$\frac{1}{N_M}\frac{dN_M}{dt} = (1-\tau)r_P S^* - D + \gamma_M N^* \tag{B.4}$$

$$W_{N_M} = \frac{1}{N_M}\frac{dN_M}{dt} = -\gamma_N N^* + \gamma_M N^* \tag{B.5}$$

$$W_{N_M} = \frac{1}{N_M}\frac{dN_M}{dt} = N^*(\gamma_M - \gamma_N) \tag{B.6}$$

B.3 Calculating W_M (Model 1: host control)

We assume that host controlled plasmid transfer is determined by two separate traits: a trait determining the donation propensity (μ) and a trait determining the reception propensity (ν). Consequently, transfer rate is a function of these: $\gamma_{ij} = \Gamma(\mu_i, \nu_j)$, where i and j are the donor and recipient, respectively. The function that describes the relationship between donation, reception and transfer rate is currently unclear, and for simplicity we assume here that the rate of transfer between strains is the geometric mean of the donation and reception propensities:

$$\begin{aligned} \gamma_{NN} = \Gamma(\mu_N, \nu_N) = \sqrt{\mu_N \nu_N}, \qquad \gamma_{MM} = \Gamma(\mu_M, \nu_M) = \sqrt{\mu_M \nu_M}, \\ \gamma_{NM} = \Gamma(\mu_N, \nu_M) = \sqrt{\mu_N \nu_M}, \qquad \gamma_{MN} = \Gamma(\mu_M, \nu_N) = \sqrt{\mu_M \nu_N} \end{aligned} \tag{B.7}$$

The rarity of the mutant (M, M_P, Eq. 3.7-3.8) as it invades limits the probability of interactions between mutant cells, which means that terms involving mass action kinetic interactions between mutant cells can be ignored. This includes transfer between mutant cells ($\gamma_{MM} M_P M$) and linearises the system of equations, summarised in matrix form as:

$$\frac{d}{dt}\begin{pmatrix} M \\ M_P \end{pmatrix} = A \begin{pmatrix} M \\ M_P \end{pmatrix} \tag{B.8}$$

where

$$A = \begin{bmatrix} rS^* - D - \gamma_{NM} N_P^* & \tau r_P S^* \\ \gamma_{NM} N_P^* & (1-\tau) r_P S^* - D \end{bmatrix}. \tag{B.9}$$

The only transfer rate found in the linearised matrix is, therefore, the rate of transfer from wild-type donors to mutant recipients ($\gamma_{NM} N_P M$). The invasion condition for the mutant is given by the sign of the dominant eigenvalue of the matrix, which can be evaluated by looking at the determinant of the matrix. A mutant can invade when one of the eigenvalues is positive, but cannot when both are negative. One of the eigenvalues is always negative, and therefore the sign of the determinant predicts the invasion criteria and fitness of the mutant (W_M). Mathematical analysis can be used to calculate and simplify the determinant. Rearranging gives a usable fitness equation:

First, equations 3.1 and 3.2 are rearranged at equilibria to give

$$-rN^*S^* + DN^* + \gamma_{NN} N^* N_P^* = \tau r_P N_P^* S^* \tag{B.10}$$

$$(1-\tau) r_P N_P^* S^* - D N_P^* = -\gamma_{NN} N^* N_P^* \tag{B.11}$$

Equations B.10 and B.11 are then multiplied together

$$(-rN^*S^* + DN^* + \gamma_{NN} N^* N_P^*)((1-\tau) r_P N_P^* S^* - D N_P^*) = \tau r_P N_P^* S^* (-\gamma_{NN} N^* N_P^*) \tag{B.12}$$

and rearranged to give

$$(rS^* - D - \gamma_{NN}N_P^*)((1-\tau)r_P S^* - D) - \gamma_{NN}N_P^*(\tau r_P S^*) = 0 \tag{B.13}$$

Because the resulting equation is equal to 0, the resulting equation can be added to the determinant of the system:

$$\det(A) = (rS^* - D - \gamma_{NM}N_P^*)((1-\tau)r_P - D) - \tau_P S^* \gamma_{NM} N_P^* \tag{B.14}$$

$$W_M = -\det(A) + (rS^* - D - \gamma_{NN}N_P^*)((1-\tau)r_P S^* - D) - \gamma_{NN}N_P^*(\tau r_P S^*) \tag{B.15}$$

and simplified, giving the usable fitness equation

$$W_M = (\gamma_{NM} - \gamma_{NN})N_P^*(r_P S^* - D) \tag{B.16}$$

Regardless of the relative contributions of donation and reception to plasmid transfer rate, plasmid reception in the trait that is under selection.

B.3.1 Supplementary figures for mutant host invasion

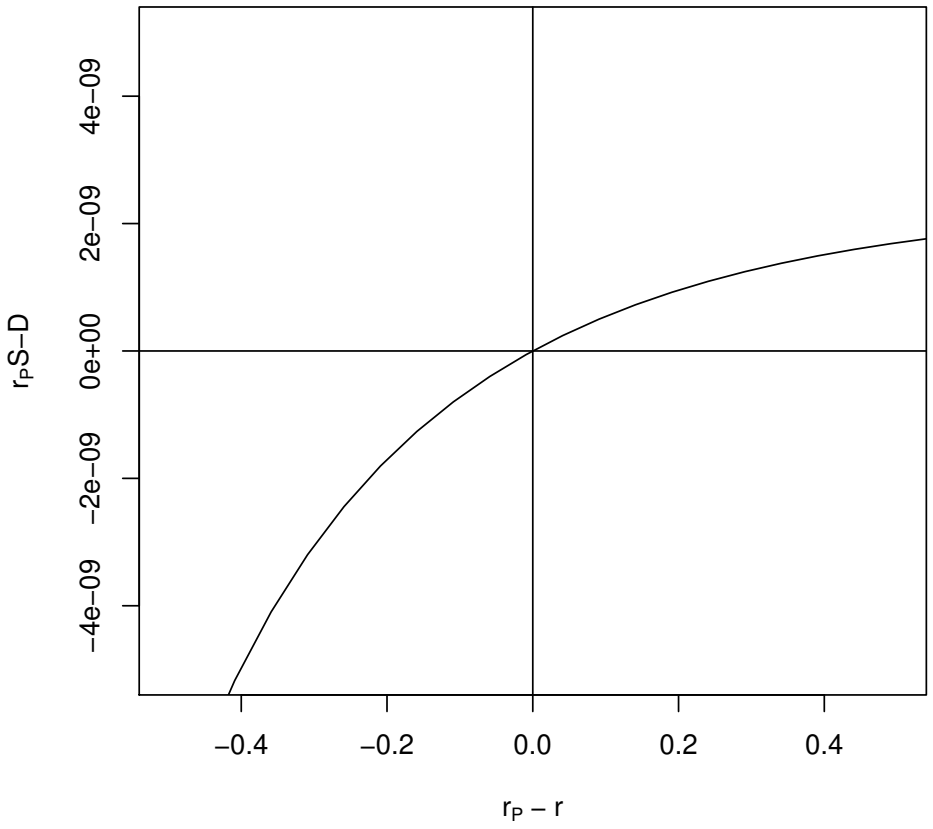

Figure B.2: Mutant host invasion (represented by $r_P S^* - D$) as plasmid cost/benefit varies ($r_P - r$) over a range of donor growth rates (r_P) based on the mutant host fitness equation ($W_M = (\gamma_{NM} - \gamma_{NN}) N_P^* (r_P S^* - D)$, Eq. 3.9). Parameter assignments as default.

B.4 Model solutions when transfer is costly

Solving the system of equations (3.1-3.3) with the transfer cost condition (Eq. 3.11) in Mathematica yielded five equilibria, two of which are trivial (Solution 1: $N^* = 0$ and $N_P^* = 0$, Solution 2: $N_P^* = 0$). The other three solutions are more complex and too long to present here. Two of these solutions (Solutions 3 and 4) are complex and conjugate, which indicates that they are not real solutions. The final solution (Solution 5) is therefore the only true solution. Solutions 3-5 are plotted using the established parameter set to confirm this (Fig. B.3). Solution 5 is the only solution which contains all positive components in the region where $\gamma_N < r/b = 3.16 \cdot 10^{-8}$ (which is required for $r_P > 0$), and is confirmed as the real, non-trivial solution we are looking for. There is a region in which Solution 4 contains all positive components, but this is where $r_P < 0$, which is unrealistic.

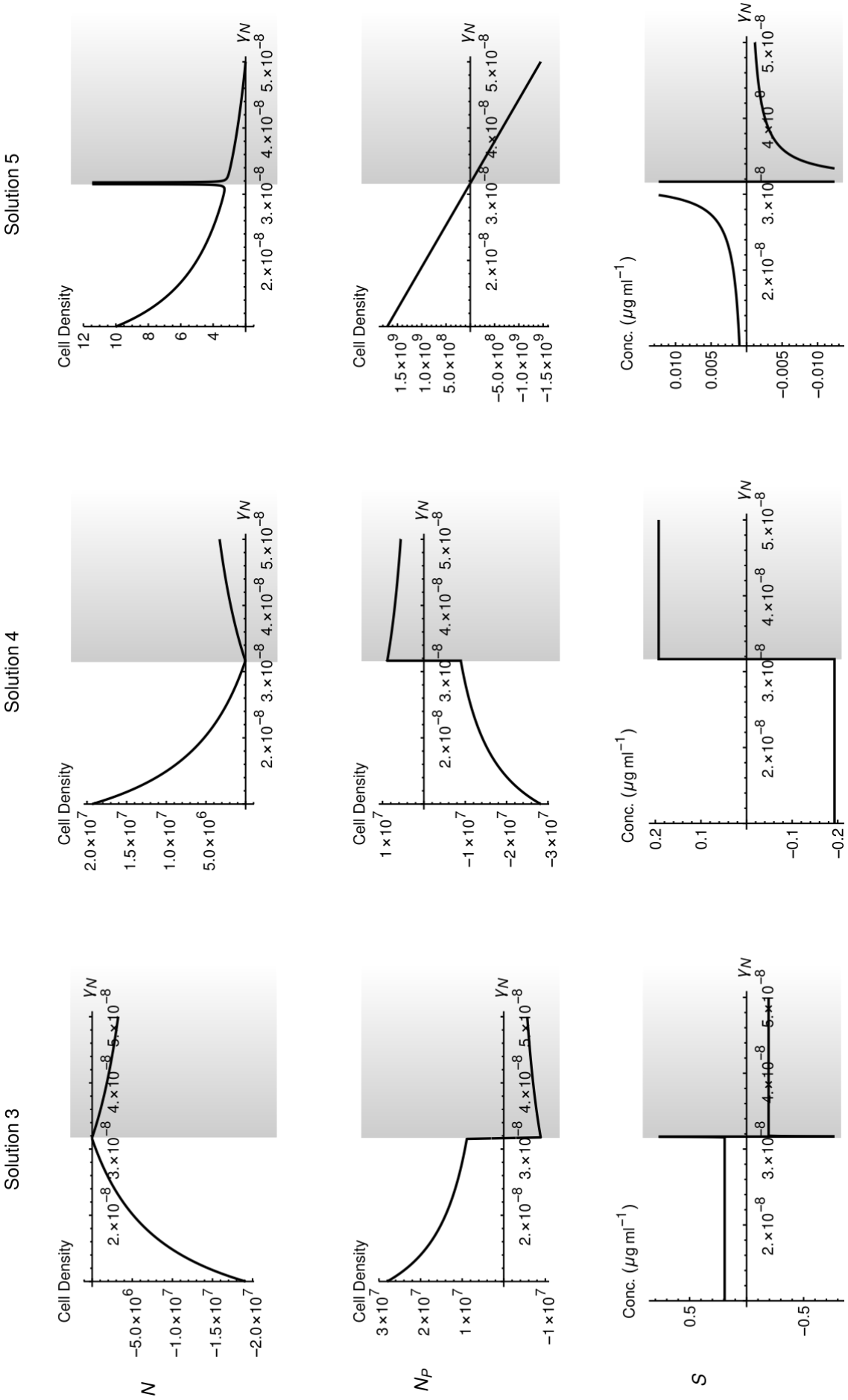

Figure B.3: Mathematical solutions (3-5) to model 2 (equilibrium values of recipients (N, top), donors (N_P, middle) and substrate (S, bottom) as transfer rate changes (γ_N, ml cell^{-1} h^{-1})). Shaded area represents $r_P < 0$, which is not permitted. Solution 5 has all positive components in the permitted range, and is therefore a real, non-trivial solution. Parameter assignments as default.

B.5 Calculating W_{N_P} (Model 2: plasmid control)

Equation 3.2 is set to equilibrium and rearranged:

$$\frac{dN_P}{dt} = (1-\tau)r_P N_P S - DN_P + \gamma_N N_P N = 0 \tag{B.17}$$

$$(1-\tau)S^* = \frac{D - \gamma_N N^*}{r_P} \tag{B.18}$$

and then used to simplify the invasion criteria expressed as per capita change in density of the mutant plasmid:

$$\frac{dN_M}{dt} = (1-\tau)r_M N_M S^* - DN_M + \gamma_M N_M N^* \tag{B.19}$$

$$\frac{1}{N_M}\frac{dN_M}{dt} = r_M(\frac{D - \gamma_N N^*}{r_P}) - D + \gamma_M N^* \tag{B.20}$$

$$W_M = r_M \frac{D - \gamma_N N^*}{r_P} - r_P \frac{D + \gamma_M N^*}{r_P} \tag{B.21}$$

$$W_M = \frac{(r - \gamma_M)(D - \gamma_N N^*) - (r - \gamma_N)(D + \gamma_M N^*)}{r_P} \tag{B.22}$$

$$W_M = (\gamma_M - \gamma_N)\frac{rN^* - bD}{r_P} \tag{B.23}$$

B.6 Assessing the model system stability at equilibrium

Geritz et al. (1998) described methods for calculating the stability of equilibria using the second derivatives of the fitness equation with regard to the resident and invading mutant phenotypes. We evaluate two aspects of stability. ESS-stability is defined as resistance to invasion by any rare mutant and is indicated by the criterion

$$\frac{\partial^2 W_M}{\partial \gamma_M^2} < 0 \tag{B.24}$$

Convergence stability is defined as an equilibrium that evolution will approach by a sequence of mutant strategies when starting in its close neighbourhood, and is indicated by the criterion

$$\frac{\partial^2 W_M}{\partial \gamma_N^2} - \frac{\partial^2 W_M}{\partial \gamma_M^2} < 0 \tag{B.25}$$

When applied to the fitness equation (Eq. 3.15) the results show that the equilibrium has neutral ESS-stability and convergence stability.

$$\frac{\partial^2 W_M}{\partial \gamma_M^2} = 0 \tag{B.26}$$

$$\frac{\partial^2 W_M}{\partial \gamma_N^2} - \frac{\partial^2 W_M}{\partial \gamma_M^2} = -2\frac{\partial}{\partial \gamma_N} f(\gamma_N) + (\gamma_M - \gamma_N)\frac{\partial^2}{\partial \gamma_N^2} f(\gamma_N) \tag{B.27}$$

where

$$f(\gamma_N) = \frac{rN^* - bD}{r_P} = N^* - b(1-\tau)S^* \tag{B.28}$$

Stability is demonstrated in the pairwise invasibility plot (PIP, Fig. B.4), showing convergence to γ_{sel}, with neutral ESS-stability indicated by the vertical line along which mutant plasmids with any value of transfer rate have equal fitness to the resident plasmid.

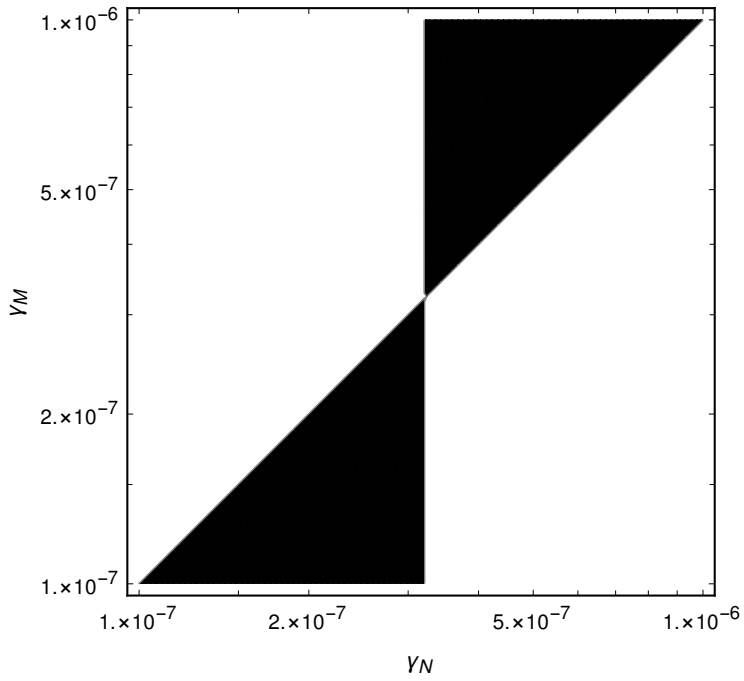

Figure B.4: Pairwise invasion plot for model 2, showing the invasion of mutant plasmids (γ_M, ml cell^{-1} h^{-1}). White: positive fitness, Black: negative fitness. Parameter assignments as default except $b = 4.61 \cdot 10^4$ cell ml^{-1}, $\tau = 10^{-2}$, $y = 8 \cdot 10^{-2}$ μg cell^{-1} (altered to improve plot resolution which is unclear at low γ_N).

B.7 Graphs of γ_{sel}, γ_{min} and P_{Prev} as parameters change

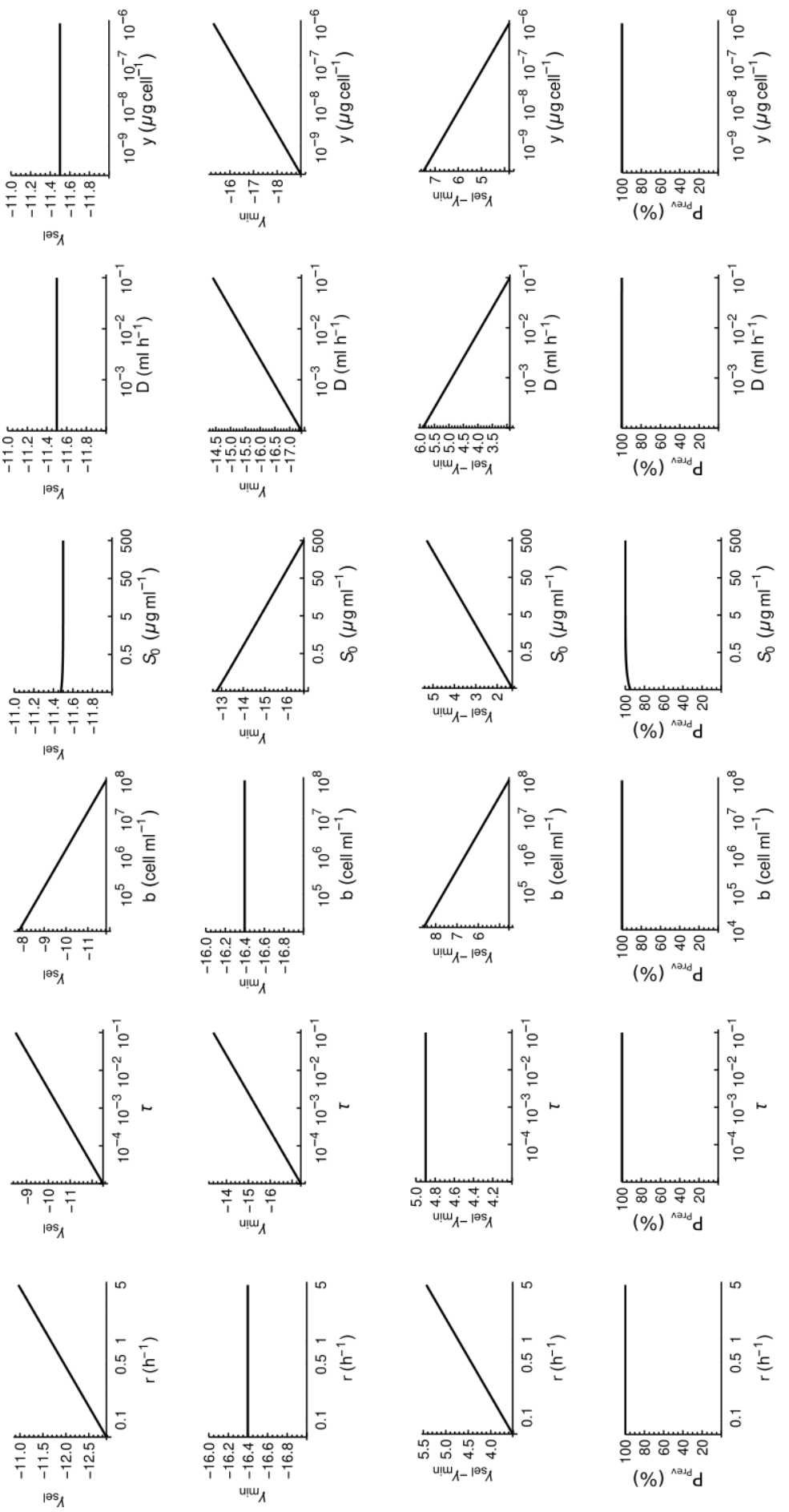

Figure B.5: Plasmid selected transfer rate (γ_{sel}, ml cell^{-1} h^{-1}), host selected transfer rate (γ_{min}, ml cell^{-1} h^{-1}), the difference between plasmid and host selected transfer rates ($\gamma_{sel} - \gamma_{min}$, ml cell^{-1} h^{-1}) and plasmid prevalence (P_{Prev}) as parameters are varied in the parameter space where the simplifications are valid (D is low). Parameter assignments as default.

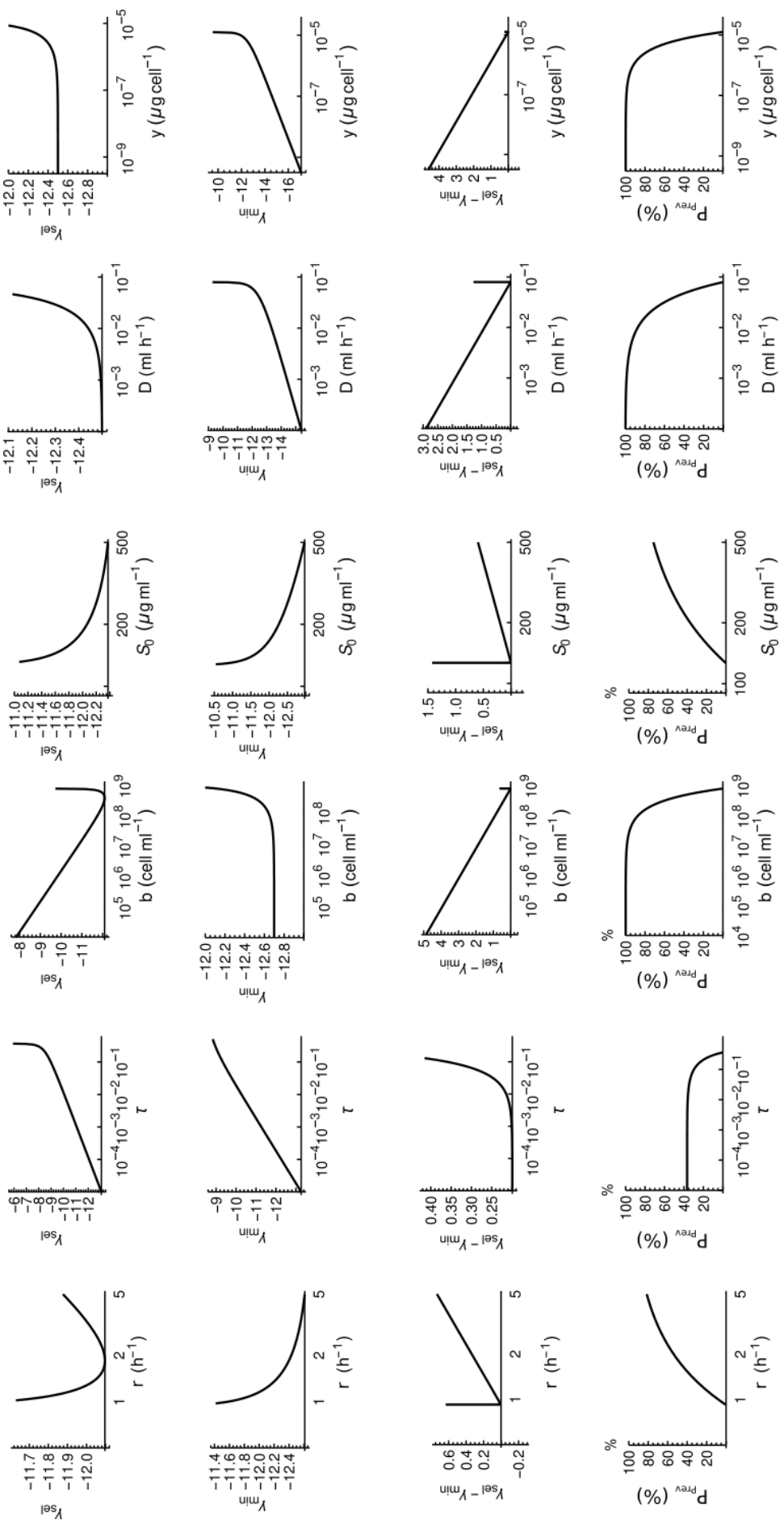

Figure B.6: Plasmid selected transfer rate (γ_{sel}, ml cell^{-1} h^{-1}), host selected transfer rate (γ_{min}, ml cell^{-1} h^{-1}), the difference between plasmid and host selected transfer rates ($\gamma_{sel} - \gamma_{min}$, ml cell^{-1} h^{-1}) and plasmid prevalence (P_{Prev}) as parameters are varied in the parameter space where dilution rate is high ($D = 0.05$) and the simplifications are not valid. Parameter assignments as default except $b = 4.61 \cdot 10^8$, $y = 8 \cdot 10^{-6}$.

B.8 Calculation of γ_{min}, (model 2)

Allow equations 3.1 and 3.3 to equilibrate in the absence of N_P, solving for N and S:

$$S^* = D/r \qquad N^* = \frac{rS_0 - D}{ry} \tag{B.29}$$

Solve equation 3.2 for γ_N at the point of invasion (where $\frac{dN_P}{dt} = 0$):

$$\gamma_N = \frac{rS(1-\tau)}{bS(1-\tau) - N} \tag{B.30}$$

Use S^* and N^* in the equation for γ_N:

$$\gamma_{min} = \frac{dry\tau}{rS_0 - D(yb(1-\tau) + 1)} \tag{B.31}$$

B.9 Analysis of model variants

Three further variants of the model were used to investigate another scenario of plasmid costs, and the effect of considering a more complex growth function. First, the metabolic and replicative costs of plasmid presence were denoted by a and included in addition to the coefficient of transfer cost, b (Eq. B.32).

$$r_P = r - a - b\gamma_N \tag{B.32}$$

Then the model was modified to specify Monod growth kinetics by substituting r for $r/(S + K_M)$ in the dynamic equations, where K_M is the Monod constant.

The four model variants are therefore:

1. Type I growth, $r_P = r - b\gamma$ (previously demonstrated)
2. Type I growth, $r_P = r - a - b\gamma$
3. Type II growth, $r_P = r - b\gamma$
4. Type II growth, $r_P = r - a - b\gamma$

Each variant resulted in one real solution, given appropriate parameter ranges, each with a single equilibrium. These equilibria were found to have convergence stability, and on the cusp of evolutionary stability, consistent with the original results. The addition of K_M in variant 3 complicated the equilibrium equations for γ_{min}, γ_{sel} and P_{Prev}, but had little effect on changing the resulting metrics. In addition, each equation simplified to the same simplified results found from the original model. The addition of a in variant 2 also increased the complexity of each metric, and while the equations simplify, a has an impact on the simplifications.

$$\gamma_{sel} \approx \frac{(r-a)\tau}{b} \qquad \gamma_{min} \approx \frac{Dy(a + r\tau)}{rS_0} \qquad P_{Prev} \approx 1 \tag{B.33}$$

The effect of a on each metric is complicated. In brief, because a reduces the maximum growth rate of donor cells, a greater cost decreases γ_{sel}, but it only has an impact when a is within an order of magnitude of r. When a is small compared with r, γ_{min} simplifies again to $Dy\tau/S_0$, but when a is large enough to have an effect it increases γ_{min}. Increasing a also decreases plasmid prevalence P_{Prev}, and decreases the difference between γ_{90} and γ_{min} to a very narrow range, much smaller than an order of magnitude. This suggests that intermediate plasmid prevalences are even less likely to be found when plasmid have a large metabolic and replicative cost.

While the text is kept mathematically pure, plots use values under a realistic parameter range where possible, taken from Haft et al. (2009). New parameters include $K_M = 0.2$ μg ml^{-1} (from Haft et al. (2009)), a (calculated using eq. B.32, using inputs from from Haft et al. (2009) (Table. 3.1) and the calculated b).

$$a = r - r_P - b\gamma_N = 1.459 - 1.23 - 4.61 \cdot 10^7 \times 3.8 \cdot 10^{-9} = 0.054 \tag{B.34}$$

The estimated plasmid metabolic cost $a = 0.054$ is less than a third of the value of the costs incurred by transfer at derepressed rates of transfer ($b\gamma_{N_D} = 4.61 \cdot 10^7 \times 3.8 \cdot 10^{-9} = 0.175$ h^{-1}). This indicates that when transfer rate is derepressed the majority of plasmid costs can be reduced by decreasing the transfer rate. In contrast when transfer rate is repressed the costs of transfer are less than the metabolic costs $b\gamma_N = 4.61 \cdot 10^7 \times 4.4 \cdot 10^{-12} = 0.0002$ h^{-1}.

Solutions of variant 2 were similar to the original model (five solutions, two trivial, two complex, and one realistic). In contrast, the Monod kinetic model variants (3 and 4) each have 6 solutions (of which two are trivial and two are complex). Of the remaining two solutions only solution 5 gives all positive components (Fig. B.7), and is therefore realistic.

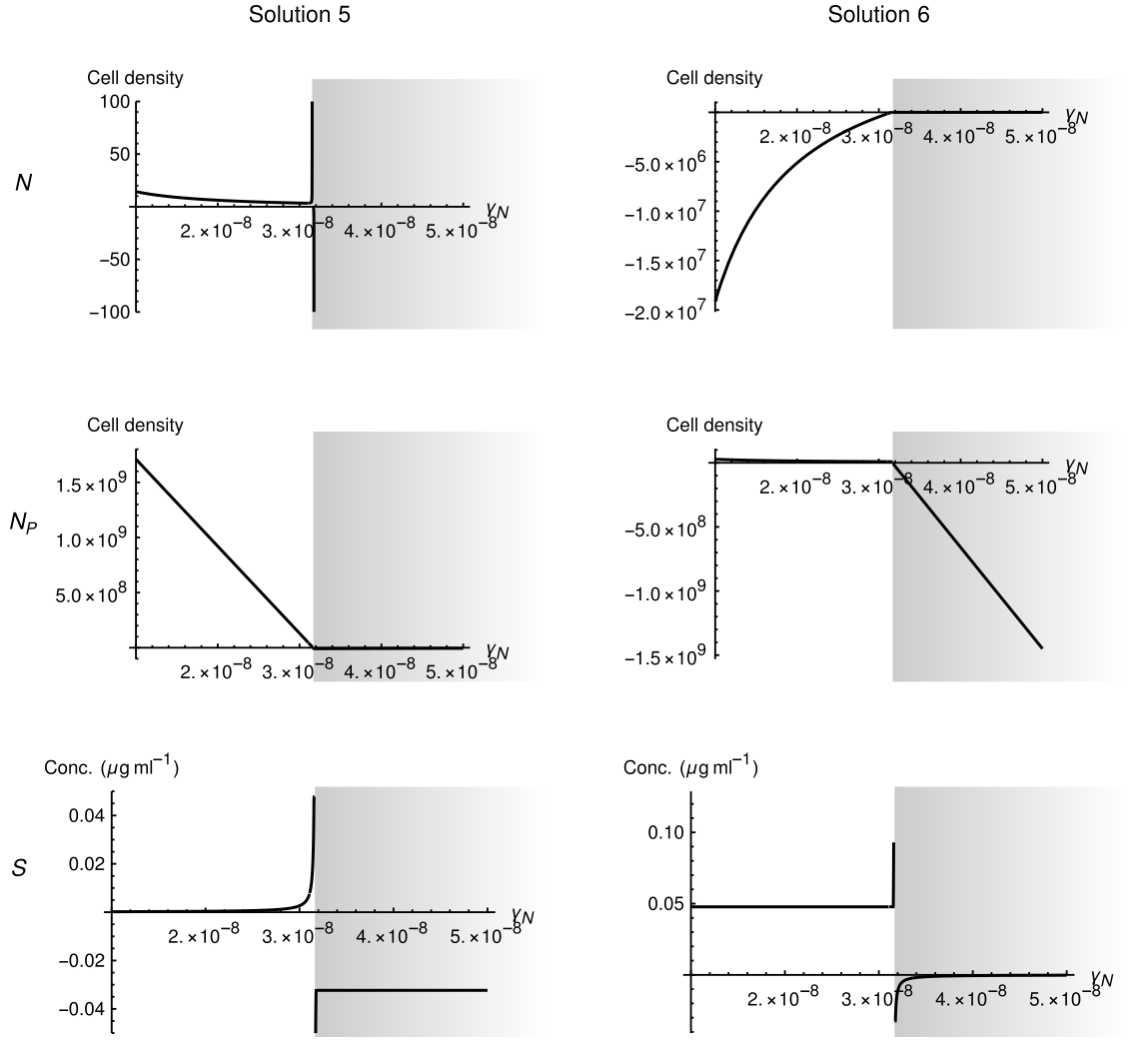

Figure B.7: Solutions 5 and 6 of model 2, variant 3 (Monod growth kinetics). Equilibrium values of recipients (N), donors (N_P) and substrate concentration (S) as transfer rate (γ_N) changes. Shaded area represents $r_P < 0$, which is not permitted. Parameter assignments as default.

Each model variant was used to find the conditions where a mutant invader differing only in transfer rate can invade. Mutant plasmids have plasmid cost relationship and growth dynamics which correspond with the resident plasmid (Eq. B.35-B.37).

Variant 2:

$$\frac{dN_M}{dt} = (1-\tau) r_M N_M S^* - D N_M + \gamma_M N_M N^* \qquad r_M = r - a - b\gamma_M \tag{B.35}$$

Variant 3:

$$\frac{dN_M}{dt} = (1-\tau) \frac{r_M N_M S^*}{S^* + K_M} - D N_M + \gamma_M N_M N^* \qquad r_M = r - b\gamma_M \tag{B.36}$$

Variant 4:

$$\frac{dN_M}{dt} = (1-\tau) \frac{r_M N_M S^*}{S^* + K_M} - D N_M + \gamma_M N_M N^* \qquad r_M = r - a - b\gamma_M \tag{B.37}$$

The equations plasmid invasion equations were used in conjunction with their corresponding host system of equations at equilibrium to calculate fitness equations for the invading plasmids with transfer rate (γ_M). For the Monod growth variants, a corresponding simplifying equation (Eq. B.39) was derived from the differential equation for the donor strain (Eq. B.38).

$$\frac{dN_P}{dt} = (1-\tau) \frac{r_P N_N S^*}{S^* + K_M} - D N_N + \gamma_N N_N N^* \qquad r_P = r - b\gamma_N \tag{B.38}$$

$$(1-\tau) \frac{S^*}{S^* + K_M} = \frac{D - \gamma_N N^*}{r_P} \tag{B.39}$$

The fitness equations for each variant were calculated (Eq. B.40-B.42), indicating equilibria at $(r-a)N = bD$ (for variants 2 and 4), and $rN = bD$ (for variant 3). The addition of Michaelis-Menten kinetics did not change the fitness equations.

Variant 2:

$$W_M = \frac{1}{N_M} \frac{dN_M}{dt} = (\gamma_M - \gamma_N) \frac{(r-a)N^* - bD}{r_P} \tag{B.40}$$

Variant 3:

$$W_M = \frac{1}{N_M} \frac{dN_M}{dt} = (\gamma_M - \gamma_N) \frac{rN^* - bD}{r_P} \tag{B.41}$$

Variant 4:

$$W_M = \frac{1}{N_M} \frac{dN_M}{dt} = (\gamma_M - \gamma_N) \frac{(r-a)N^* - bD}{r_P} \tag{B.42}$$

Fixing N^* at equilibrium enables calculation of N_P^*, S^* and γ_{sel} for each model variant. There are three solutions for the second variant, and four solutions for each of the third and fourth variants, corresponding to the non-trivial solutions previously identified (where a pair of each set of solutions is complex and conjugate). In each case only one solution is realistic , and all the other solutions result in either $\gamma_{sel} = r/b$ (for model variant 3) or $\gamma_{sel} = (r-a)/b$ for model variants 2 and 4, which are unrealistic. Realistic solutions of N_P^* and γ_{sel} for the model variants are reported (Eq. B.43-B.48).

Variant 2:

$$N_P^* = \frac{r^2 S_0(1-\tau) - r(1+yb)D - a(2rS_0(1-\tau) - D) + a^2 S_0(1-\tau)}{yr(r-a)} \tag{B.43}$$

$$\gamma_{sel} = \frac{(r-a)^2\tau((r-a)S_0 + D) - (r-a)^3 S_0\tau^2 - abDry}{b((r-a)^2 S_0(1-\tau) - D(r(1+yb) - a))} \tag{B.44}$$

Variant 3:

$$N_P^* = \frac{(rS_0(1-\tau) - D(K_M + S_0))(1-\tau)}{ry(1-\tau) - D} - \frac{bD}{r} \tag{B.45}$$

$$\gamma_{sel} = \frac{r\tau}{b}\frac{r(1-\tau)(rS_0(1-\tau) - D(K_M + S_0))}{r(1-\tau)(rS_0(1-\tau) - D(K_M + S_0)) - bDy(r(1-\tau) - D)} \tag{B.46}$$

Variant 4:

$$N_P^* = \frac{(r-a)(1-\tau)(S_0(r-a)(1-\tau) - D(K_M + S_0))}{ry((r-a)(1-\tau) - D)} - \frac{bD}{r-a} \tag{B.47}$$

$$
\begin{aligned}
\gamma_{sel} = \Bigg(& r^3(1-\tau)\tau\Big(D(K_M+S_0) - rS_0(1-\tau)\Big) \\
& - a^3(1-\tau)\tau\Big(D(K_M+S_0) - 4rS_0(1-\tau)\Big) \\
& + a^4 S_0(1-\tau)^2\tau \\
& + a^2 r(1-\tau)\Big(bDy + 3D(K_M+S_0)\tau - 6rS_0(1-\tau)\tau\Big) \\
& + ar\Bigg(bDy\Big(D - r(1-\tau)\Big) \\
& \qquad - r(1-\tau)\tau\Big(3D(K_M+S_0) - 4rS_0(1-\tau)\Big)\Bigg)\Bigg) \Bigg/ \\
\Bigg(b\Bigg(& - r\Big(bDy\big(D - r(1-\tau)\big) \\
& \qquad - r(1-\tau)\big(D(K_M+S_0) - rS_0(1-\tau)\big)\Big) \\
& + a^2(1-\tau)\big(D(K_M+S_0) - 3rS_0(1-\tau)\big) \\
& - ar(1-\tau)\big(bDy + 2D(K_M+S_0) - 3rS_0(1-\tau)\big) - a^3 S_0(1-\tau)^2\Bigg)\Bigg)
\end{aligned}
\tag{B.48}
$$

The stability of each equilibria is determined by calculating $\frac{\partial^2 W_M}{\partial \gamma_M^2}$ and $\frac{\partial^2 W_M}{\partial \gamma_N^2}$ (shown in Eq. B.24-B.25). Each variant has neutral ESS-stability at γ_{sel}, as found in the initial model, but differs in the second derivative with regard to γ_N (Eq. B.49-B.52), although all show convergence to γ_{sel}. PIPs were constructed for each variant, and demonstrate convergence to a point of neutral stability.

$$\frac{\partial^2 W_M}{\partial \gamma_M^2} = 0 \tag{B.49}$$

$$\frac{\partial^2 W_M}{\partial \gamma_N^2} - \frac{\partial^2 W_M}{\partial \gamma_M^2} = -2\frac{\partial W_M}{\partial \gamma_N} f(\gamma_N) + (\gamma_M - \gamma_N)\frac{\partial^2 W_M}{\partial \gamma_N^2} f(\gamma_N) \tag{B.50}$$

where

Variant(s 1 and) 3:

$$f(\gamma_N) = \frac{rN^* - bD}{rp} \tag{B.51}$$

Variants 2 and 4:

$$f(\gamma_N) = \frac{(r-a)N^* - bD}{rp} \tag{B.52}$$

The invasion criteria and plasmid prevalence equations were calculated for each model variant (Eq. B.53-B.58).

Variant 2:

$$\gamma_{min} = \frac{yD(a(1-\tau)+r\tau)}{rS_0 - D(1+yb(1-\tau))} \tag{B.53}$$

$$P_{Prev} = 1 - \frac{bDyr}{(r-a)((r-a)S_0(1-\tau)-D)} \tag{B.54}$$

Variant 3:

$$\gamma_{min} = \frac{D(r-D)r\tau y}{r(rS_0 - D(K_M+S_0)) - bDy(r-D)(1-\tau)} \tag{B.55}$$

$$P_{Prev} = 1 - \frac{bDy(r(1-\tau)-D)}{r(1-\tau)(rS_0(1-\tau) - D(K_M+S_0))} \tag{B.56}$$

Variant 4:

$$\gamma_{min} = \frac{Dy(r-D)(a-a\tau+r\tau)}{r(rS_0 - D(K_M+S_0)) - bDy(r-D)(1-\tau)} \tag{B.57}$$

$$P_{Prev} = 1 - \frac{bDry((r-a)(1-\tau)-D)}{(r-a)^2((r-a)S_0(1-\tau) - D(K_M+S_0))(1-\tau)} \tag{B.58}$$

In each case, γ_{sel}, γ_{90} and γ_{min} can be simplified when D, b and y are low compared with r and S_0 (Eq. B.59-B.60). The simplifications including both a and b plasmid costs are more complex, particularly the invasion criteria. D, S_0 and y broadly affect the invasion criteria in the same ways as previously described, and when a is low compared with $r\tau$, γ_{min} simplifies to $D\tau y/S_0$. When τ and r are low compared with a it simplifies to Dya/rS_0, both a and r have a substantial effect on the invasion criteria, and these factors mitigate each other. a generally increases γ_{min} while decreasing γ_{sel}, but only when large enough to have an effect on r where the growth rate does not already put the curve into the exponential phase. Changing K_M in model variants 2 and 4 has very little effect on any of the simplified metrics (Fig. B.8). When the dilution rate and proportional plasmid cost (b) are high γ_{sel} and γ_{min} both move into the exponential phases previously described.

Variant(s 1 and) 3:

$$\gamma_{sel} \approx \frac{r\tau}{b} \qquad \gamma_{min} \approx \frac{D\tau y}{S_0} \qquad P_{Prev} \approx 1 \tag{B.59}$$

Variants 2 and 4:

$$\gamma_{sel} \approx \frac{(r-a)\tau}{b} \qquad \gamma_{min} \approx \frac{Dy(a+r\tau)}{rS_0} \qquad P_{Prev} \approx 1 \tag{B.60}$$

Variant 2 γ_{90} shows that the addition of complex plasmid cost (a in addition to b) increases the complexity in its relationship to γ_{min}. γ_{sel}, γ_{90} and γ_{min} were plotted against a (Fig. B.9), and shows that when a is small, it has little effect on the metrics, but as it increases it

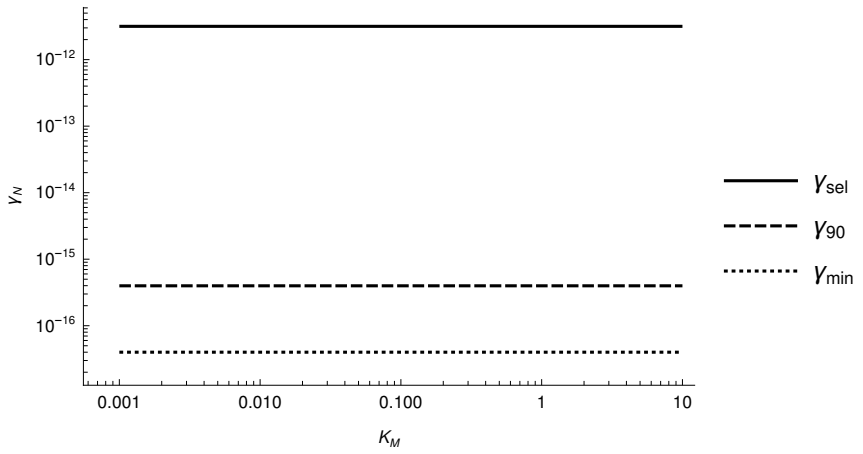

Figure B.8: Plasmid selected transfer rate (γ_{sel}), transfer rate determining 90% plasmid prevalence (γ_{90}) and host selected transfer rate (γ_{min}) as the Monod constant (K_M) increases (model variant 3). Parameter assignments as default.

affects all three, increasing them at different values of a. γ_{min} increases first as a increases until it approaches γ_{90}, where they increase in parallel with very little difference between them. γ_{90} and γ_{min} continue to increase together as a increases until they approach γ_{sel}, where γ_{90} instead begins to increase in line with γ_{sel} as it increases exponentially until the point where the cost of the plasmid loses viability, $a = r$. Above this point, plasmids are unable to persist because selection acts against them, even though the invasion criteria has not gone into its exponential phase. The value of $a = 0.054$ calculated using the data from Haft et al. (2009) puts the difference between γ_{min} and γ_{90} into the region where the difference between γ_{min} and γ_{90} is very low.

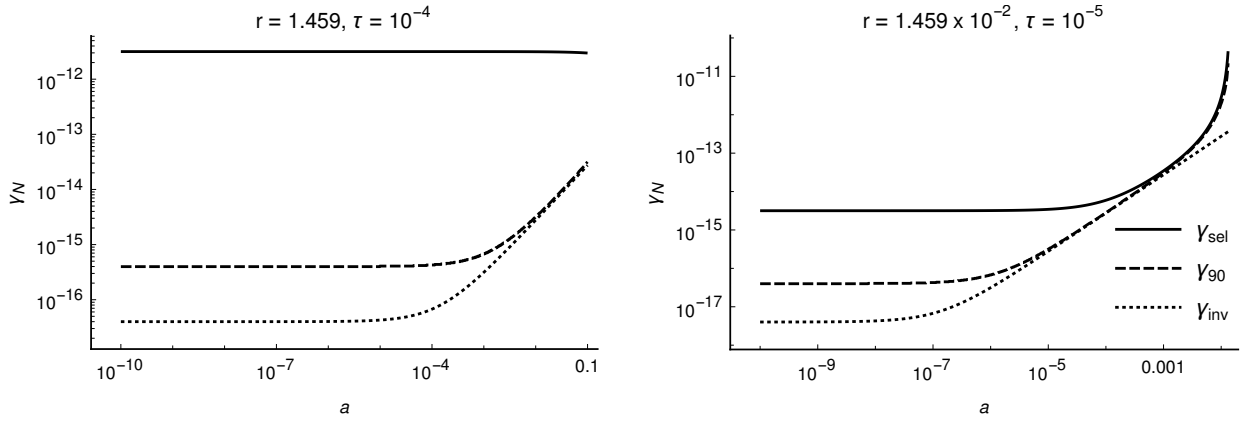

Figure B.9: Plasmid selected transfer rate (γ_{sel}), transfer rate determining 90% plasmid prevalence (γ_{90}) and host selected transfer rate (γ_{min}) as metabolic/replicative plasmid costs (a, h^{-1}) increase (model variant 2). Parameter assignments as default.

Due to the numerical calculation of γ_{90} it is difficult to explicitly define the parameter conditions under which γ_{90} becomes closely aligned with γ_{min}. Through investigation of the effect of changing individual parameters, the key parameters which mediate this relationship

are τ and r. $\log_{10}(\gamma_{90}/\gamma_{min})$ was plotted against a for over a range of τ and r (Fig. B.10). Decreasing r by an order of magnitude shifts the whole region to the left by an order of magnitude, while decreasing τ by an order of magnitude decreases only the lower value where this region begins, increasing the size of the region. Overall, the difference between γ_{90} and γ_{sel} remains small for the majority of parameter sets, within an order of magnitude, except for where a is within an order of magnitude of r, in which the difference increases exponentially.

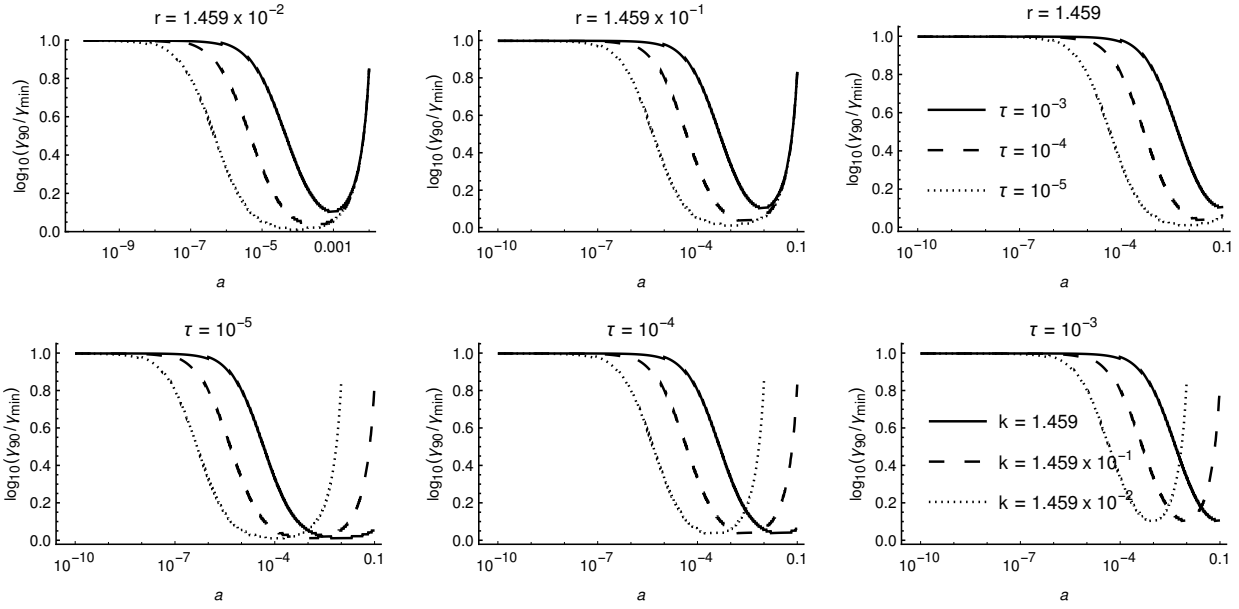

Figure B.10: The difference between transfer rate at 90% plasmid prevalence and the host selected transfer rate ($\log 10(\gamma_{90}/\gamma_{min})$), as metabolic-replicative costs (a, h^{-1}) change over a range of growth rates (r, h^{-1}) and loss rate (τ) combinations (model variant 2). Other parameter assignments as default.

B.10 Superinfection

Mutant plasmid invasion (N_M)

As before, we assume that each cell can only contain one plasmid, either the wild-type (at density N_P) or the mutant (at density N_M). Following (Gandon et al., 2001, 2002) we assume that host cells can get superinfected, in which case there is a probability σ that the superinfecting plasmid replaces the original plasmid. The original resident equations do not change because superinfection replaces the resident plasmid with another resident pladmid, and the additional superinfection terms are cancelled out:

$$\frac{dN_P}{dt} = (1-\tau)r_P N_P S - DN_P + \gamma_N N_P N + \gamma_N \sigma N_P N_P - \gamma_N \sigma N_P N_P \tag{B.61}$$

$$\frac{dN_P}{dt} = (1-\tau)r_P N_P S - DN_P + \gamma_N N_P N \tag{B.62}$$

Cells bearing the mutant plasmid are identical to cells that carry the wild type, except in the rate of plasmid transfer (γ_M) and change density as follows:

$$\frac{dN_M}{dt} = (1-\tau)r_P N_M S^* - DN_M + \gamma_M N_M N^* - \gamma_N \sigma N_M N_P^* + \gamma_M \sigma N_M N_P^* \tag{B.63}$$

For model 1, where the cost of plasmid carriage is independent of the transfer rate, the fitness of the mutant plasmid (W_{N_M}) is, after rearranging:

$$W_{N_M} = \frac{1}{N_M}\frac{dN_M}{dt} = (\gamma_M - \gamma_N)(N^* + \sigma N_P^*). \tag{B.64}$$

A mutant plasmid with a larger transfer rate can therefore invade if $N^* + \sigma N_p^* > 0$. Superinfection increases the fitness of plasmid mutants with a larger mutation rate but, in this model variant, does not alter our results qualitatively: evolution selects the plasmid with the largest transfer rates.

For model 2, where the plasmid cost depends on transfer rate the fitness of the mutant plasmid (W_{N_M}) is, after rearranging:

$$W_{N_M} = \frac{1}{N_M}\frac{dN_M}{dt} = (\gamma_M - \gamma_N)\frac{rN^* - bD + \sigma r_P N_P^*}{r_P}. \tag{B.65}$$

A mutant plasmid with a larger transfer rate can invade if $rN^* - bD + \sigma r_P N_P^* > 0$. Because the N^* decreases with increasing transfer rate, the transfer rate, γ_{sel}, for which $rN^* - bD + \sigma r_P N_P^* = 0$ increases with the rate of successful superinfection, indicating an equilibrium when $rN^* - bD + \sigma r_P N_P^* = 0$.

Mutant host cell invasion (M, M_P) with superinfection

Under superinfection the plasmid bearing, mutant host will be superinfected with rate $\gamma_{MM}\sigma M_P^2 + \gamma_{NM}\sigma M_p N_p$. However, a superinfected host cell was plasmid bearing and remains plasmid bearing and therefore these rates are added to and subtracted from the same equation. The net result is that superinfection does not change the host's growth equations for this model. Superinfection therefore has no impact on the host's fitness in this model when plasmid transfer does not confer a cost (as in model 1), and the results are consistent with the previous results. We have not explored the model when plasmid transfer is costly (as in model 2), and although there are many ways in which this might be done, it is work for a future study.

Appendix C

Chapter 4 appendices

C.1 Lag, serial transfer and diauxic growth

Lag times preceding exponential growth phase were consistently observed in *P. fluorescens* (Fig. C.1). A lag function (Baranyi and Roberts, 1994; Baty and Delignette-Muller, 2004) was added to the model to take this into account (Eq. C.1).

$$\frac{t^n}{\lambda^n + t^n} \tag{C.1}$$

In the example shown, a small amount of cell growth can be seen before the true exponential phase. This growth is likely continued growth from substrate consumed from the previous serial transfer assay (the same patterns could also be seen when the culture was transferred into media containing no substrate). When estimating the growth parameters, this initial growth was ignored.

In contrast, *P. putida* grows quickly from the beginning. These growth curves, however, contain idiosyncrasies at higher densities. There is a clear shift in the observed growth rates, likely due to the multi-stage digestion of glucose which can lead to diauxic growth patterns. While every idiosyncrasy cannot be taken into account when modelling, approximating the growth curve using r and K_M is enough to appropriately simplify and represent the growth of the bacterial strains.

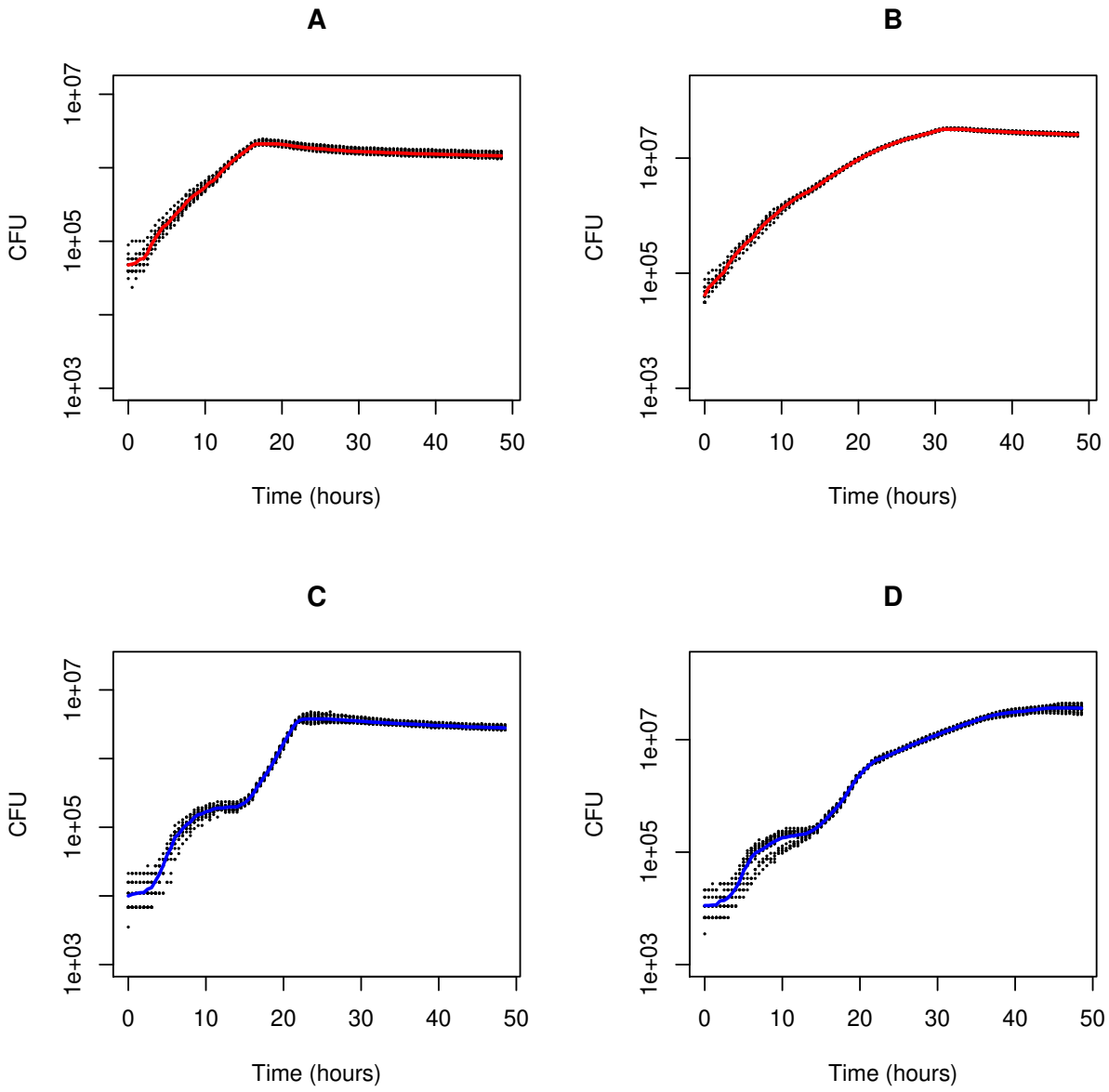

Figure C.1: Growth curves of *P. putida* (A, B) and *P. fluorescens* (C, D) in $0.25gl^{-1}$ (left: A, C) and $3gl^{-1}$ (right: B, D) glucose concentrations. Each graph shows all the data from 16 replicates, with a smoothed average curve using runmed function in R (k = 11). *P. fluorescens* shows a lag before exponential growth, across all concentrations.

C.2 Results of NLLS fitting of Michaelis-Menten constant (K_M) and yield coefficient (y) parameters to growth curves.

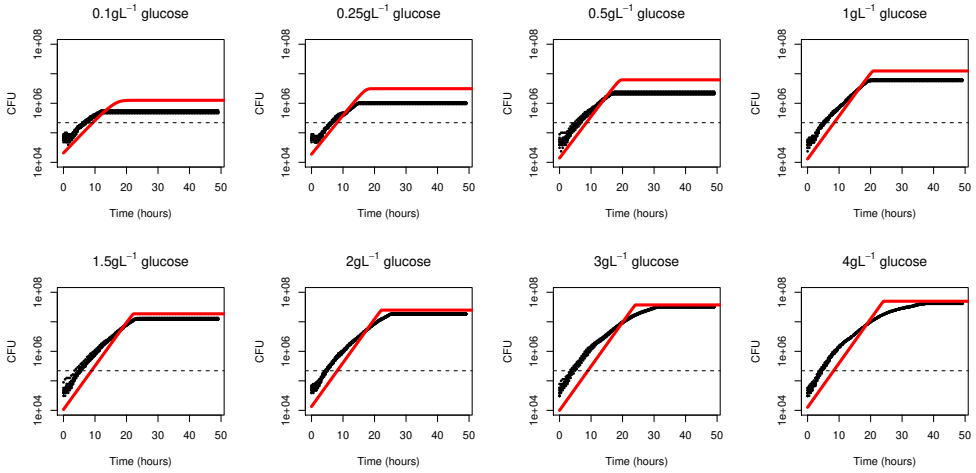

Figure C.2: P. putida recipient strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

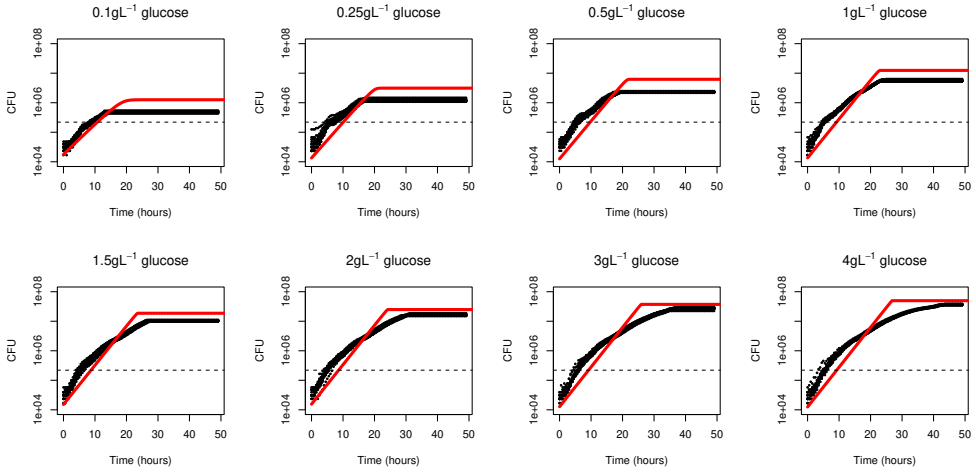

Figure C.3: P. putida pQBR55 donor strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

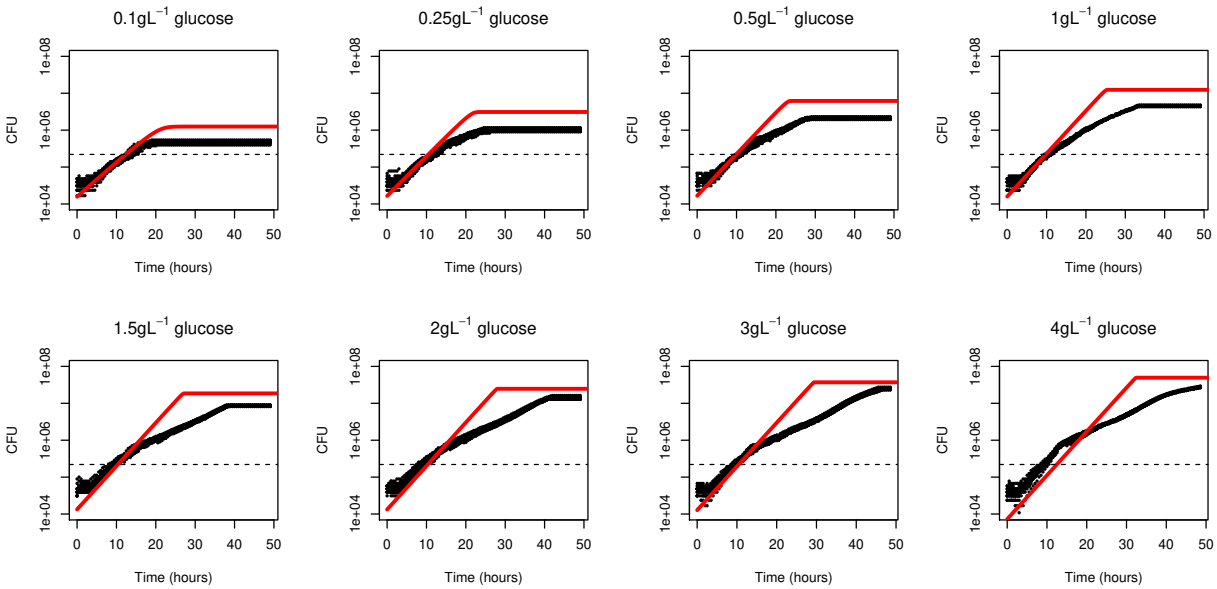

Figure C.4: P. putida pSTY-Pf donor strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

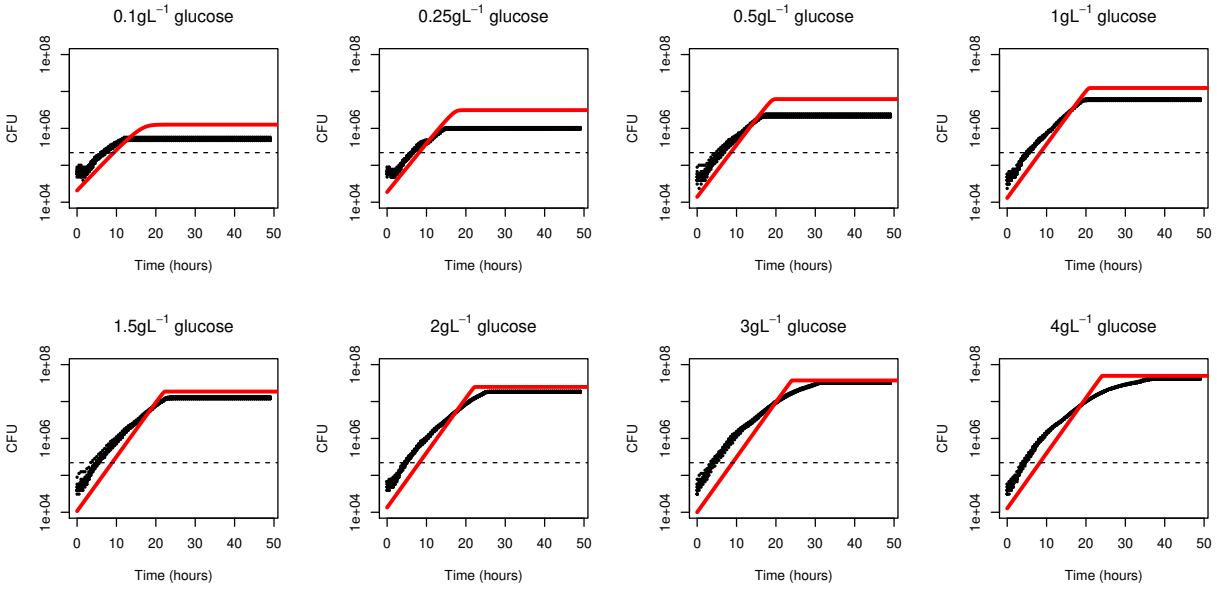

Figure C.5: P. fluorescens recipient strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

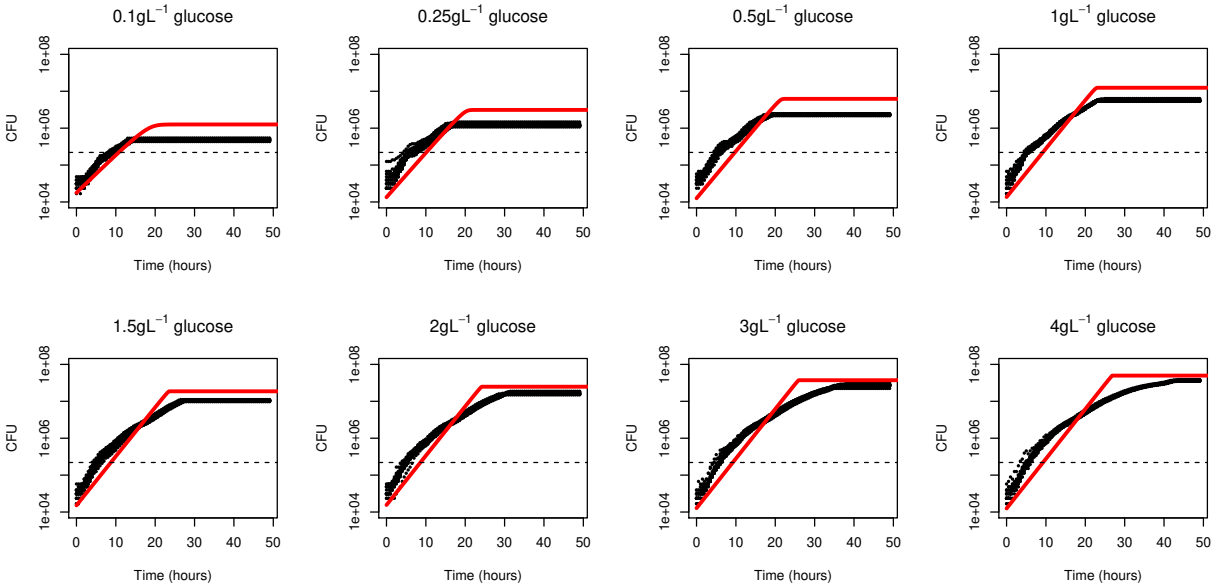

Figure C.6: P. fluorescens pQBR55 donor strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

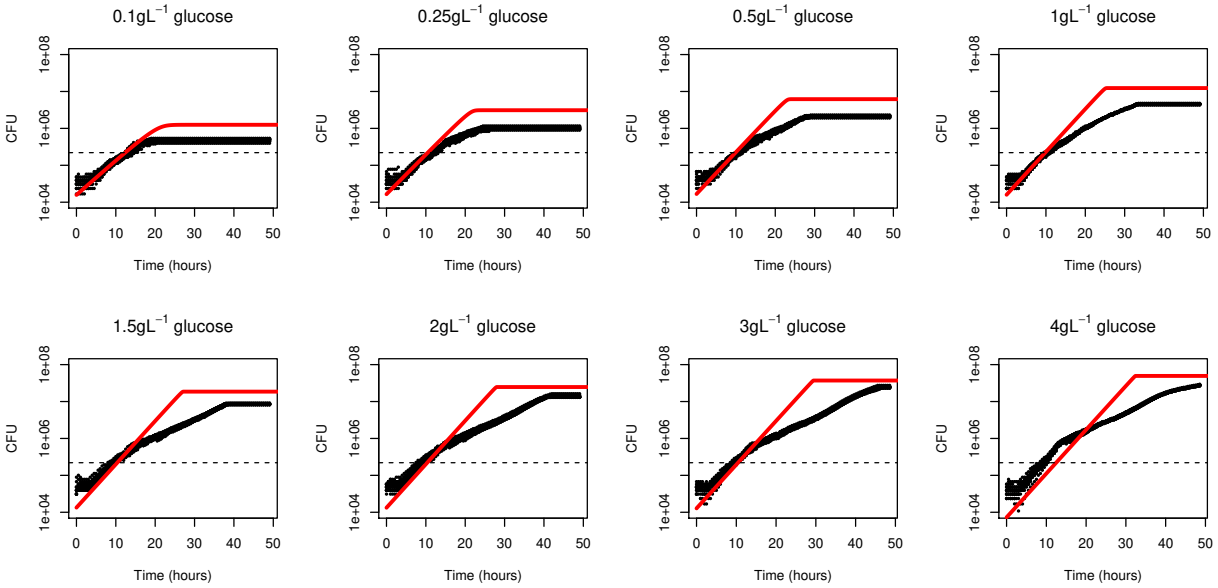

Figure C.7: P. fluorescens pSTY-Pf donor strain growth curves across concentrations. The black points show data from all replicates while the red line shows the fit of the model. Data points under the dotted line were not included in the model fitting.

C.3 Plasmid loss rates

Unless great care is taken, some of the parameters can be difficult to separate from each other when attempting to estimate them. For example, distinguishing differences in donor and recipient growth rates from plasmid loss is tricky, particularly when the plasmid is costly. Similarly, it is also difficult to separate the effects of transfer and loss from each other, and further growth differences between donors and recipients. These difficulties can be mitigated when plasmid loss rates are low enough to maintain high plasmid-host fidelity.

pQBR55 is reported to have a low loss rate, likely containing partition genes (*parA*, *parB*, (Hall et al., 2015)) which promote plasmid fidelity during cell division and post-segregational killing genes which can remove plasmid-free cells. Similarly, pSTY.Pf contains multiple parB-like genes (parB-like partition protein and parB-like nuclease), both of which are found in the pSTY.Pf plasmid identified in this study. Plasmid loss under these conditions is believed to have a negligible effect when compared to growth differences caused by the costs of plasmid carriage and transfer. Furthermore, loss does not tend to be the primary driver of change to plasmid prevalence (Freter et al., 1983).

Assays were conducted to observe plasmid fidelity and prevalence in donors over five days to determine if plasmid loss rate was rare enough to remove from the model. Donor strains were grown on selective media to remove recipients, and a single colony was selected and grown in 1 gl^{-1} glucose. Serial transfers occurred every 12 hours, where replicates were plated on PCA, preceding transfer of 5 μl into fresh media. Cell strains were shaken even 30 minutes. UWC1-pQBR55 and 376N data are from CompEx3 (see appendix C.4) where larger volumes of glucose (0.95 ml) were used and serial transfers of 50 μL, rather than the standard 95 μL and 5 μl serial transfers. Data for UWC1-pSTY.Pf and 376N-pQBR55 were taken from CompEx4 (see appendix C.4).

There is no loss of either pQBR55 or pSTY.Pf from *P. fluorescens* over the course of 5 days (Fig. C.8: B and D). There is some loss of pQBR55 from *P. putida* (A), but this only becomes substantial after 36 hours, likely caused by differences between donor and recipient growth rate. In each 12 hour timestep, we can assume that loss is negligible, relative to the effects of other parameters. The only dataset for pSTY.Pf in *P. putida* comes from the final competition experiment I conducted, in which plasmid prevalence unfortunately did not begin at 100% (C). While there is no clear data for plasmid fidelity for this plasmid-host combination, we can assume that the substantive changes in plasmid prevalence occur due growth rate differences, consistent with previous modelling work (Freter et al., 1983). This enables justification of the removal of loss parameters from the model, and replacement with mixed populations when appropriate.

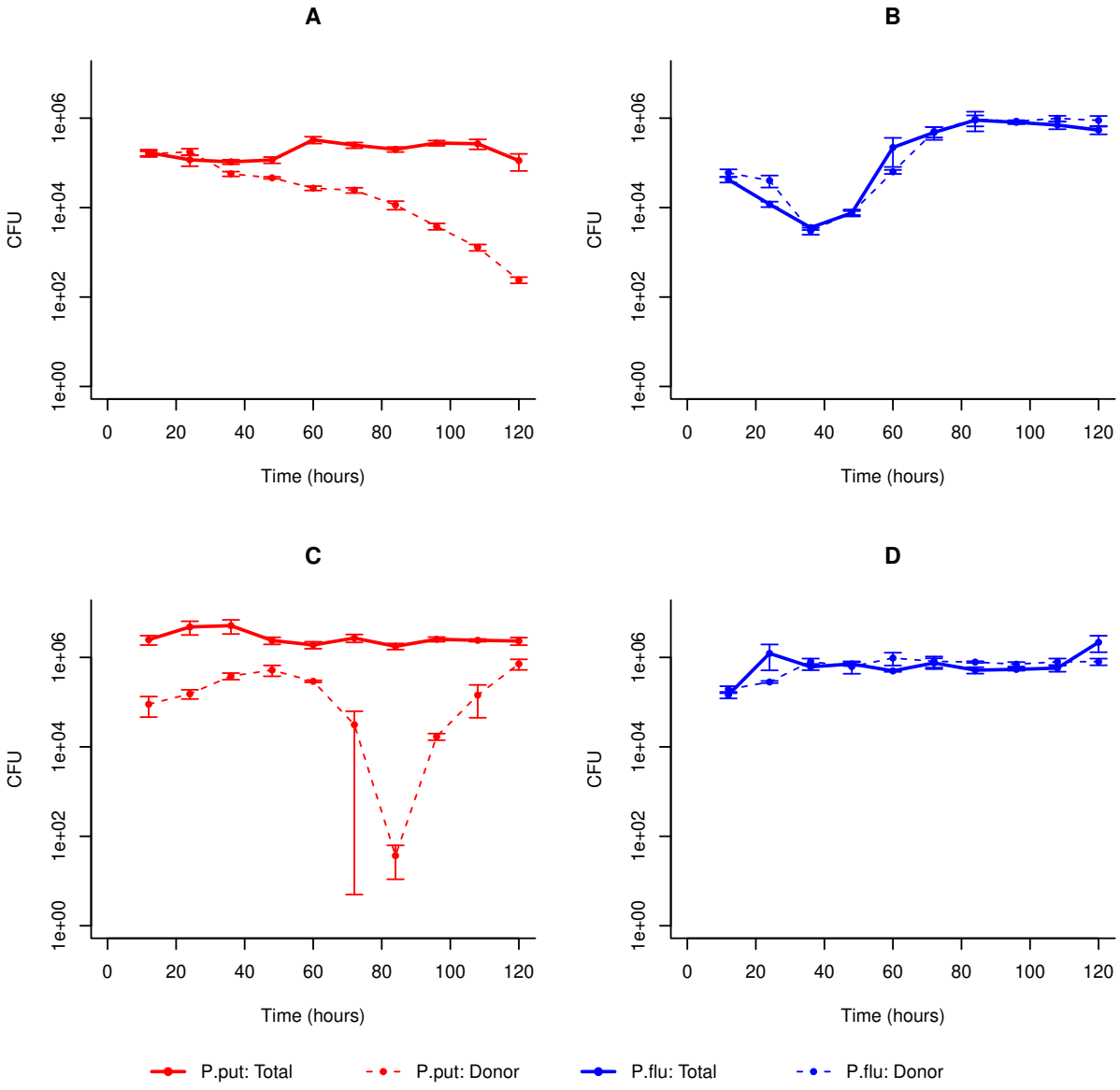

Figure C.8: pQBR55 (A, B) and pSTY.Pf (C, D) loss in *P. putida* (A, C) and *P. fluorescens* (B, D) over 5 days (10 transfers).

C.4 Experiment summary

GroEx1. Growth experiment 1: (r, K_M, y)

1. UCW1, 2. UWC1-pQBR55, 3. UWC1-pSTY.Pf, 4. 376N, 5. 376N-pSTY.Pf

GroEx2. Growth experiment 2: (r, K_M, y)

1. 376N-pQBR55

TraEx1. Transfer experiment 1: (γ)

1. UWC1-pQBR55 to KT2440

2. UWC1-pSTY.Pf to KT2440

TraEx2. Transfer experiment 2:(γ)

1. 376N-pSTY.Pf to 376NR

TraEx3. Transfer experiment 3: (γ)

1. 376N-pQBR55 to 376NR (no transconjugants detected)

TraEx4. Transfer experiment 4: (γ, higher density, unreliable)

1. 376N-pQBR55 to 376NR

2. 376N-pQBR55 to UWC1

TraEx3 results not presented: no transconjugants detected. TraEx4 results presented, although unreliable.

CompEx1. Competition experiment 1: 60 hours, recipients only (**Lag:** λ, n)

1. UWC1 (OD growth curves in optimisation)

2. 376N (OD growth curves in optimisation)

3. UWC1 x 376N

CompEx2. Competition experiment 2: UWC1-pQBR55, 376N-pSTY.Pf

1. UWC1

2. UWC1-pQBR55 (Loss, τ)

(3. UWC1-pQBR55, mixed donor-recipient populations, unused)

4. 376N

5. 376N-pSTY.Pf (Loss, τ)

(6. 376N-pSTY.Pf, mixed donor-recipient populations, unused)

7. UWC1 x 376N

8. UWC1-pQBR55 x 376N **(Transfer, γ)**

9. UWC1 x 376N-pSTY.Pf **(Transfer, γ)**

10. UWC1-pQBR55 x 376N-pSTY.Pf

(11. UWC1-pQBR55 x 376N-pSTY.Pf, mixed donor-recipient populations, unused)

CompEx3. Competition experiment 3: Supernatant experiment

1. (UWC1, unused)

2. UWC1-pQBR55 **(Loss, τ)**

3. (376N, unused)

4. 376N-pSTY.Pf **(Loss, τ)**

5. (UWC1 x 376N, unused)

6. (UWC1-pSTY.Pf x 376N-pQBR55, unused)

CompEx4. Competition experiment 4: UWC1-pSTY.Pf, 376N-pQBR55

1. UWC1-pSTY.Pf **(Loss, τ)**

2. 376N-pQBR55 **(Loss, τ)**

3. UWC1 x 376N-pQBR55 **(Transfer, γ)**

4. UWC1-pSTY.Pf x 376N **(Transfer, γ)**

5. UWC1-pSTY.Pf x 376N-pQBR55

CompEx1 used in growth parameter optimisation to OD curves. Partial results from CompEx2 (1, 2, 4, 5, 7, 8, 9, 10) and full results from CompEx4 presented (Competition). Donor prevalence through time, indicating plasmid fidelity and loss presented CompEx3 (2, 4), and CompEx4 (1, 2). CompEx4 (3, 4) data used to generate transfer rates from first timestep.

Appendix D

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Table D.1: Permissions.

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24	figure	Fig 1. Resistance developed against common antibiotics.	RNAi for silencing drug resistance in microbes toward development of nanoantibiotics. Vol. 189, Journal of Controlled Release. 2014. p. 1507.	©2020 Elsevier B.V. permissionshelpdesk@elsevier.com	25.04.15	yes	Written permission
25	figure	Fig. 1. Complex interactions amongst environmental-and health-related factors that contribute to the spread of antimicrobial resistance ...	Understanding the contribution of environmental factors in the spread of antimicrobial resistance. Environmental health and preventive medicine vol. 20,4 (2015): 243-52.	©The Japanese Society for Hygiene 2015 journalpermissions@springernature.com	25.04.15	not yet	Requested on 25.04.15

Table D.1 – *Continued*

Page No.	**Type of work**	**Name of work**	**Source of work**	**Copyright holder and contact**	**Permission requested on**	**I have permission yes/no**	**Permission note**
35	figure	Fig. 17. The dynamic view of the prokaryotic world ...	Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. Nucleic Acids Research, Volume 36, Issue 21, 1 December 2008, Pages 66886719.	©2020 Oxford University Press journals.permissions@oup.com	3.09.2020	yes	Written permission

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