The Sociality and Evolution of Plasmid-Mediated Antimicrobial Resistance

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Abstract

Overuse and misuse of antibiotics has led to the global spread of antimicrobial resistance, threatening our ability to treat bacterial infections. The horizontal acquisition of multidrug resistance (MDR) plasmids, from other bacterial lineages, has been instrumental in spreading resistance. Newly acquired plasmids are often poorly adapted to hosts causing intragenomic conflicts, reducing the competitiveness of plasmid-carrying strains. Costs can be overcome by positive selection for plasmid-encoded adaptive traits in the short-term, or ameliorated by compensatory evolution in the long-term. How the selection and adaptation of MDR plasmids varies with antibiotic treatment remains unclear. First, I demonstrate that the selective conditions for the maintenance of an MDR plasmid are dependent upon the sociality of resistance it encodes. Selection for efflux of antibiotics, a selfish trait, occurred at very low concentrations of antibiotic, far below the minimum inhibitory concentration of sensitive plasmid-free strain. In contrast, selection for inactivation of antibiotics, a cooperative trait, increased the amount of antibiotic required to select for the MDR plasmid, allowing sensitive plasmid-free bacteria to survive high levels of antibiotic. These selection dynamics were only accurately predicted when mathematical models included the mechanistic details of antibiotic resistance. Secondly, I show that the trajectory of evolution following MDR plasmid acquisition varies with antibiotic treatment. Tetracycline treatment favoured a distinct coevolutionary trajectory of chromosomal resistance mutations coupled with plasmid mutations impairing plasmid-borne resistance. This led to high-level, low-cost antibiotic resistance, but also produced an integrated genome of co-dependent replicons that may limit the onward spread of co-adapted MGEs to other lineages. This evolutionary trajectory was strikingly repeatable across independently evolving populations despite the emergence of multiple competing lineages within populations. The results presented here demonstrate that the interaction between positive selection and compensatory evolution can help to explain the persistence of MDR plasmids in the clinic and the environment.

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Acknowledgements

Author's Declaration

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

The following papers have been published and are presented in this thesis:

- Bottery, M.J., Wood, A.J., and Brockhurst, M.A. (2016). Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance. Antimicrob. Agents Chemother. 60, 2524-2527.

- Bottery, M.J., Wood, A.J., and Brockhurst, M.A. (2017). Adaptive modulation of antibiotic resistance through intragenomic coevolution. Nat. Ecol. Evol. 1, 1364-1369.

Chapter 2 presents the results of Bottery et al. (2016) together with additional results obtained after the publication of the paper. Chapter 3 is the accepted version of Bottery et al. (2017). Copy edited version of Bottery et al. (2016) is available at http://aac.asm.org/content/60/4/2524.full.pdf+html, and Bottery et al. (2017) is available at rdcu.be/urgl.

1. Introduction

Antibiotics are one of the greatest tools of modern medicine; their routine use as a first line therapy for bacterial infections, as well as prophylactic use during chemotherapy and invasive operations to prevent infection, has significantly reduced mortality and increase life expectancy. However, the emergence of antimicrobial resistant (AMR) bacteria, particularly those with multi-drug resistance (MDR), is a major risk to global health, as the drugs which we are dependent on diminish in effectiveness (World Health Organization, 2015). Over 2 million people are estimated to become infected with AMR infections each year in the US alone, 23,000 of whom die (CDC, 2013). In the EU 25,000 patients die each year of infections caused by AMR bacterial (EMEA and ECDC, 2009). The limited pace of novel antibiotic discovery is overshadowed by the ever increasing spread of AMR (Lewis, 2013). A recent UK review predicts 10 million deaths per-year will be attributable to AMR infections globally by 2050 if current trends in the spread of AMR are not tackled (O'Neill, 2014). The horizontal gene transfer (HGT) of resistance genes across lineages, species and families of bacteria is a key driver in the global spread of AMR (Maiden, 1998). These resistance genes often cluster upon semi-autonomous mobile genetic elements (MGEs), such as conjugative plasmids, which allow the instantaneous acquisition of multiple resistance genes in a single event, increasing the emergence of MDR bacterial pathogens (Carattoli, 2013). The spread of AMR through the exchange of plasmids remains one of the most difficult challenges in the fight against resistance. Therefore understanding the selection and evolution of plasmid borne resistance is critical in dealing with this challenge.

This thesis aims to add to our current understanding of plasmid borne AMR by examining the how selection for MDR plasmids varies with the mode of antibiotic resistance, and how plasmids and bacterial hosts evolve under different antibiotic treatments. This introductory chapter will briefly introduce the global emergence of AMR along with the core themes of the thesis, namely plasmids as vessels of AMR, the costs and sociality of antibiotic resistances, and evolution of plasmids and their bacterial hosts.

1.1 Emergence of Antimicrobial Resistance

1.1.1 *Discovery*

Alexander Fleming famously discovered the first antibiotic, penicillin, by accident in 1928 when he observed zones of clearing around a *Penicillium* fungus that was contaminating an agar plate of staphylococci. The ease with which bacteria could evolve resistance to penicillin was soon noted, and referred to in Fleming's acceptance speech for The 1945 Nobel Prize in Physiology or Medicine, awarded for the discovery of penicillin:

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them" – Alexander Fleming (1945)

However, at this time resistance was not of major concern, because the rate of antibiotic discovery for the following 40 years was sufficient to outpace the spread of resistance. This was, in part, thanks to a discovery platform introduced by Selman Waksman in the early 1940's. Waksman's screening for antimicrobial compounds from soil microbes, notably streptomycetes, led to the discovery of streptomycin, the first aminoglycoside antibiotic, and paved the way for systematic screening for novel antimicrobial compounds (Schatz et al., 1944). The cheap and efficient screening method of searching for zones of inhibition in lawns of bacteria on agar plates established by Fleming and Waksman led to an antibiotic discovery boom throughout the 50's and 60's (Figure 1.1). Approximately two thirds of naturally derived antibiotics have been isolated from soil actinomycetes (Fair and Tor, 2014). However, systematic screening for novel classes of antibiotic tailed off as the rate of discovery of new antibiotics by this method declined (Lewis, 2013).

The Antibiotic Timeline
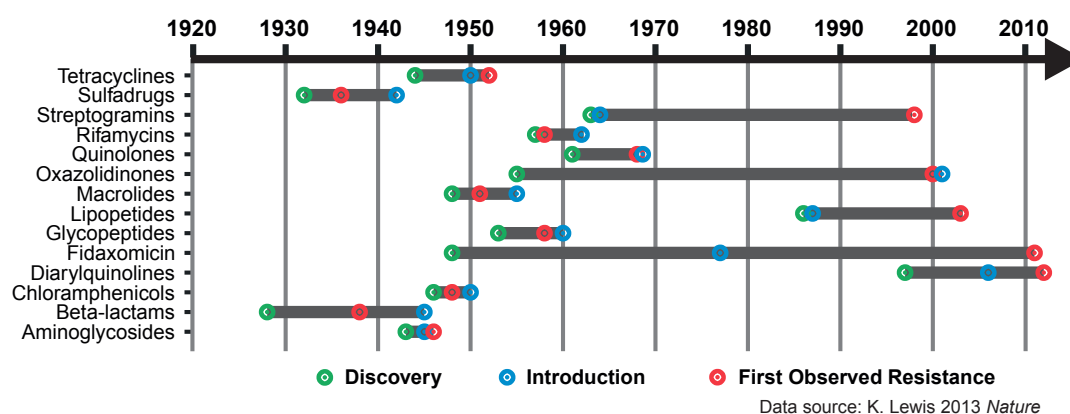
Figure 1.1 | The Antibiotic Timeline. The dates of discovery, introduction, and first observation of resistance of major classes of antibiotics. Data source: Lewis (2013).

1.1.2 *The antibiotic discovery void*

As rates of novel antibiotic discovery from natural products diminished, a greater emprises on targeted drug design and synthesis was adopted in a bid to reduce rediscovery and cross-resistance rates (Silver, 2011). Optimisation of natural products showed some success, increasing the spectrum and stability of compounds while avoiding the action of specific resistance mechanisms, with the development of semi-synthetic β-lactam's as a prime example (Elander, 2003). However, the synthetic production of antibiotics was less than fruitful (Fair and Tor, 2014), with no new classes of antibiotic being discovered between 1987 and 2015 (Ling et al., 2015; Silver, 2011). The combination of decreasing success rates of discovery and declining profitability of antibiotic production, together with difficulties in novel drug approval, led many big pharmaceutical companies to abandon their antibiotic discovery programs in the late 1990s (Projan, 2003). As a result, this has placed greater pressure on the remaining antibiotics we have at our disposal. Exacerbating this problem, global usage of antibiotics has increased by 36% from 2000 to 2010 (Boeckel et al., 2014), with use of 'last-resort' antibiotics, such as vancomycin, becoming more common. Increased use, coupled with the stagnation of antibiotic discovery, is a major threat to public heath as antibiotic resistance spreads rapidly across the globe.

This global spread of AMR is due to the evolution of resistant genotypes via *de novo* mutation or acquisition of novel resistance genes via HGT (section 1.2) which then increase in frequency due to positive selection arising from antibiotic usage. Resistant strains spread within local clinical settings from patient to patient, nationally between clinics though the movement of patients and healthcare workers, and internationally through global travel networks. For example, methicillin-resistant *Staphylococcus aureus* (MRSA), first observed in the UK in the early 1990's, rose from 2% to 40% of *S. aureus* infections within the UK within 8 years (Johnson et al., 2001). MRSA has since spread globally, being observed within Europe, Australia and Singapore (Holden et al., 2013). Similarly, the resistance enzyme New Delhi metallo-β-lactamase (NDM), first observed within a bacterial pathogen in 2008, has spread to over 40 counties due to both the spread of pandemic bacteria, and inter-species and inter-genus transmission of bla_{NDM} containing MGEs (section 1.2) (Johnson and Woodford, 2013).

1.1.3 *Appearance of resistance in bacteria*

Bacteria can be intrinsically resistant to specific classes of antibiotic due to the functional or structural characteristics of the cell, for example certain classes of antibiotic cannot permeate the gram-negative cell membrane, or specific antibiotic targets may be missing from certain bacterial taxa (Blair et al., 2015). This limits the choice of affective antibiotics but does not contribute to the spread of resistance. Alternatively, bacteria can gain resistance to antibiotics, either through *de novo* mutation or HGT of antimicrobial resistance genes (ARGs). *De novo* mutations typically enhance resistance in 3 ways: they may lead to changes in the structure of the antibiotic target to decrease the antibiotic's binding affinity (Rybkine et al., 1998), increase the expression of efflux machinery to remove antibiotic from the cell, or cause reduced membrane permeability to limit access of the antibiotic to the target (Guay et al., 1994; Phan and Ferenci, 2017; Webber and Piddock, 2001). *De novo* resistance mutations are typically vertically inherited (i.e. passed on through cell division) and therefore largely remain clonal, and are commonly observed in chronic bacterial infections (Oliver et al., 2000). By contrast, horizontally acquired ARGs can be shared among distantly related bacteria and come pre-packaged upon MGEs. Commonly horizontally acquired resistance mechanisms include new efflux systems, enzymes that modify or inactivate the antibiotic, or alternative

genes that can replace the function of the antibiotic target without being inhibited by the antibiotic (Cantón and Coque, 2006; Popowska and Krawczyk-Balska, 2013). ARGs carried on MGEs can be both vertically inherited, or transferred horizontally between cells.

Horizontally acquired resistance mechanisms are often sophisticated specialised molecules begging the question: where did these resistance mechanisms come from to begin with? The evolution of antibiotic resistance can be split into pre- and post-antibiotic eras denoting the periods before and after the clinical use of antibiotics (Aminov, 2009). Antibiotic resistance predates clinical use of antibiotics, with evidence of diversification and selection of ARGs in natural bacterial communities from 30,000 year old permafrost samples (D'Costa et al., 2011) and a four million year old cave system (Bhullar et al., 2012). Phylogenetic evidence supports this ancient evolution of ARGs, with the enzyme β-lactamase, a resistance mechanism to the most widely used antibiotics, β-lactams, predicted to have originated over two billion years ago (Hall et al., 2004). This ancient history of resistance is coupled with the fact that most of the antibiotics we use are derived from natural microbial products, produced by fungi and bacteria. In natural communities, antibiotics are excreted into the local environment by microbes to inhibit growth of neighbouring bacteria, providing a competitive advantage to the antibiotic producing population. ARGs are likely to have originally evolved to protect antibiotic producers from their own weapons or as defence mechanisms in the targeted organisms.

Interestingly, some authors have suggested that natural concentrations of antimicrobial compounds found within environments, not affected by anthropogenic contamination, are often too low to exert lethal effects on bacteria (Aminov, 2009). It has therefore been proposed that these compounds could play a role in cell-to-cell communication, signalling, and gene regulation in bacterial communities (Aminov, 2009). Sub-inhibitory concentrations of antibiotics have been shown to induce biofilm formation (Linares et al., 2006), and influence quorum sensing within bacterial populations (the process of altering gene expression in response to cell density) (Thomson et al., 2000). The role of antibiotics beyond a form of bacterial chemical warfare remains incomplete, however it is clear that these molecules have wider implications than merely inhibitory action.

The use of antibiotics over the last century can be thought of as an evolution experiment on a global scale (Andersson and Levin, 1999). The anthropogenic use of antibiotics during the post-antibiotic era has placed incredibly strong selection pressures upon bacteria to gain resistance across all ecosystems. ARGs that happen to provide resistance, which once may have been involved with other metabolic functions, have been mobilised from the gene pool of natural communities and have spread rapidly within pathogenic and commensal bacteria (Wright, 2010). ARGs can now provide a huge competitive advantage, increasing growth rates and decreasing mortality when under antibiotic selection. The selection and spread of antibiotic resistance has proceeded within a very short evolutionary time scale, less than a century, during which HGT mediated by MGEs has played a prominent role.

1.2 The Horizontal Transfer of Antimicrobial Resistance

HGT occurs extensively among prokaryotes, with large proportions of bacterial genomes consisting of horizontally acquired accessory genes (Young, 2016). The acquisition of genetic material from different lineages via the transfer of MGEs is a fundamental driver of bacterial adaptation and diversification, allowing the sharing of ecologically important genes between distantly related bacteria (Jain et al., 2003; Wiedenbeck and Cohan, 2011). These non-essential accessory genes provide traits, in addition to house-keeping functions present in the core genome, accelerating adaption to novel or fluctuating environments by bypassing the requirement for rare *de novo* mutations (Frost et al., 2005; Norman et al., 2009), and thus permitting bacteria to occupy ecological niches which were previously inaccessible. This communal gene pool of MGEs can be transferred between bacteria in three prominent ways: transformation, transduction and conjugation (Figure 1.2). Plasmids are the primary conjugative MGEs, and are the principle cause of MDR phenotypes within both clinical and commensal bacteria (Bennett, 2008).
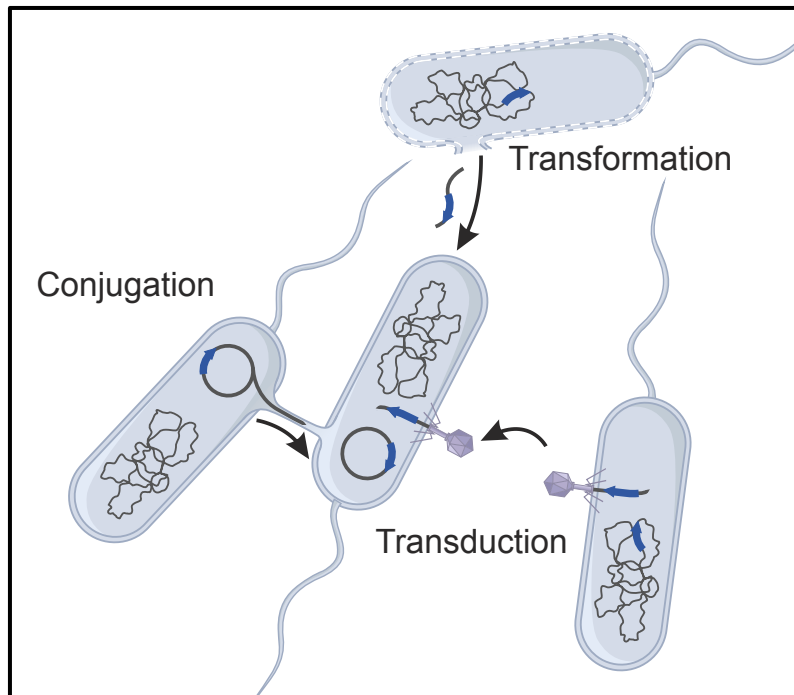
Figure 1.2 | Mechanisms of horizontal gene transfer between bacteria. Genetic material can transfer between bacteria in three main ways: 1) Transduction – bacterial DNA can be mistakenly packaged within phage (viruses that infect bacteria), and transferred into new hosts during infection. 2) Transformation – DNA from the environment, often released upon cell death, can be taken up by some species of bacteria. 3) Conjugation – the process by which mobile genetic elements are transferred from a donor bacterium to a recipient bacterium, bacteria are drawn together by a pilus produced by the donor cell to form a mating pair, at which point the mobile genetic element is copied into the new host. Extra-chromosomal plasmids and genomic integrative and conjugative elements (ICE) are often transferred in this way. Adapted from Holmes et al. (2016).

1.2.1 *Bacterial plasmids*

Conjugative plasmids are semi-autonomous MGEs that act as shuttles for the transfer of genes between bacterial lineages (Bergstrom et al., 2000). The most common structure of plasmids are closed circular double stranded DNA molecules, and the defining feature of plasmids is that their replication is independent of the host chromosome. This independence allows them to be horizontally transmitted between distantly related bacteria through conjugation, making them a major contributor to the communal gene pool (Norman et al.,

2009). Independence from the host chromosome also potentially decouples the evolutionary pressures acting upon plasmids and the host, resulting in plasmids having and pursuing their own evolutionary interests subject to distinct selective pressures. Consequently, the fitness of plasmids may not be aligned to that of their hosts, allowing for both conflict and cooperation between plasmid and host contingent upon the prevailing environment (Harrison and Brockhurst, 2012; MacLean and San Millan, 2015). This broad array of interactions generates reciprocal selection and adaptation, shaping structure and function of both plasmid and chromosomal replicons.

1.2.2 *Plasmid structure*

Plasmids are modular in structure, with genes of similar function clustered together into discrete regions of the molecule. This mosaic structure of genes and operons from different sources is the result of frequent recombination events, such as the acquisition of transposable elements (Norman et al., 2009). Plasmids can therefore be partitioned into the core 'backbone' of genes that encode plasmid-specific functions, and 'accessory' elements that are not required by the plasmid. The backbone genes can broadly be split into three functional groups controlling replication, stability, and conjugation. These plasmid-specific functions are not essential to the bacterial host; making these parts of the plasmid sequence dispensable from the viewpoint of the bacteria.

The defining feature of a plasmid is the replication (*rep*) system. This system generally requires a plasmid encoded initiator protein that binds on the plasmid's origin of replication (*oriV*) before the recruitment of the host's DNA replication machinery (DNA helicases, DNA polymerase, DNA clamp, topoisomerase etc.) (Solar et al., 1998). The plasmid's *rep* system is often coupled with modules (*cop)* that activate or inhibit the expression of the *rep* proteins, hence control the copy number of the plasmid. The *rep* system also defines the incompatibility group of a plasmid as multiple plasmids with similar *rep* systems interfere with each other's copy number, preventing similar plasmids from occupying the same host (Carattoli et al., 2005).

The host range of a plasmid is usually limited by the compatibility of the *rep* system with the hosts DNA replication machinery (Zhong et al., 2005). Some plasmids can only be stably maintained within only narrow range of hosts, such

as Inc-F or IncI group which are only found within Enterobacteriaceae (Carattoli, 2009). In contrast, broad-host-range plasmids, such as the members of the IncP-1 family, can transfer and propagate within almost all gram-negative bacteria, thanks to their control systems, multiple *rep* initiation proteins and stability systems (Fang and Helinski, 1991; Popowska and Krawczyk-Balska, 2013). These plasmids have a wide environmental distribution, present in soils, wastewater, livestock manure and river sediment (Bahl et al., 2007; Sen et al., 2011, 2013; Szczepanowski et al., 2009).

The maintenance of a stable plasmid copy number within a host is vital to a plasmid's survival. If the copy number were to raise too high, the metabolic burden of the plasmid upon the host would be unsustainable (Norman et al., 2009) whereas if copy number drops too low, the plasmids could be lost at cell division (a process called segregation). As plasmid-free segregants have the competitive advantage of not incurring the metabolic cost of plasmid maintenance (section 1.3), appreciable rates of segregational loss of a plasmid could rapidly lead to the plasmid's extinction. Small plasmids often rely on high copy numbers and diffusion to ensure plasmids are present in both mother and daughter cells (Summers and Sherratt, 1984). However, when the copy number of a plasmid drops below ~10 per cell a more active approach to maintaining plasmids over many generations is required (Thomas, 2000). This comes in the form of plasmid encoded stability modules, which ensure the plasmid is maintained in both daughters at cell division. Plasmid active partitioning systems encode actin like filaments that push the plasmids to the opposite poles of a dividing cell (Salje et al., 2010), allowing stable inheritance. Many plasmids also carry post-segregational killing (also known as addiction) systems that kill any plasmid free segregants that may arise. These generally consist of toxin-antitoxin (TA) systems comprising a stable toxin and an unstable antitoxin; upon plasmid loss the antitoxin degrades faster than the toxin, leaving sufficient toxin remaining to kill any plasmid free cells (Jensen and Gerdes, 1995).

The conjugation modules of a plasmid allow its transfer horizontally from cell-to-cell. Self-propagating conjugative plasmids encode genes for mate pair formation, the physical linkage of two cells via the formation of a conjugation pilus. A second DNA processing step is required to generate the single stranded DNA molecule, propagate its transfer via the pilus and synthesise the second strand in the donor and recipient (Norman et al., 2009). Mobilizable plasmids do

not encode the genes required for mate pair formation and only harbour the DNA processing genes, hitchhiking on the pili formed by cohabiting conjugative plasmids.

Plasmids may also carry accessory genes that impart adaptive traits on their hosts, but are not essential for the maintenance of the plasmid (Rankin et al., 2011). These accessory genes are themselves often carried upon MGEs, such as transposons, nested within the plasmid resembling a Russian doll-like architecture (Sheppard et al., 2016). The separate origin of accessory genes is also evidenced by the fact that their GC content is often different from the plasmid backbone (Norman et al., 2009). The mobility of accessory genes allows for their transposition both between plasmids and onto the chromosomes of their hosts (Hall et al., 2017a; Sheppard et al., 2016). Accessory genes encode a wide range of adaptive traits including virulence factors (Johnson and Nolan, 2009), novel metabolic functions (Sen et al., 2011; Young et al., 2006) and resistance to heavy metals or antimicrobials (Baker-Austin et al., 2006; Bennett, 2008). The benefits of accessory traits are usually ecologically contingent, only increasing bacterial fitness within specific environments. As such, these traits often allow bacteria to exploit novel niches, such as eukaryotic hosts or contaminated environments (Nogueira et al., 2009; Ramírez-Díaz et al., 2011; Schwarz and Johnson, 2016). It has been noted that many of the adaptive traits encoded by plasmids have social effects within microbial communities, with accessory gene products affecting not only the host, but the fitness of neighbouring cells as well (Rankin et al., 2011).

1.2.3 *Plasmid prevalence and the spread of AMR*

Plasmids play a prominent role in the spread of AMR, with plasmid-conferred resistance encompassing nearly all currently used antibiotics (Bennett, 2008). The dissemination of resistance plasmids has led to a number of global resistance pandemics, with plasmids promoting the spread of the CTX-M extended spectrum β-lactamases (ESBL) (Cantón and Coque, 2006; Dhanji et al., 2011), carbapenemases (Déraspe et al., 2014), and quinolone resistances (Rodríguez-Martínez et al., 2011). Of particular concern are plasmids which contain multiple ARGs providing resistance to different antibiotics, so called MDR plasmids (Popowska and Krawczyk-Balska, 2013), which offer bacteria with resistance to multiple antibiotics in a single step and thus potentiate the

rapid emergence of MDR phenotypes in bacterial pathogens (Norman et al., 2008; Wright, 2010). To understand the maintenance, enrichment, and transmission of antibiotic resistance plasmids, extensive work has been conducted exploring the fitness cost, selection and evolution of plasmid borne AMR, which will be discussed in the following sections.

1.3 The Cost of Plasmid Carriage

Although ecologically important accessory traits encoded upon plasmids can provide benefits to their bacterial hosts, plasmids often reduce the competitive fitness of bacteria in the absence of positive selection (Baltrus, 2013). These fitness costs can result in strong purifying selection against plasmid carriage, as plasmid-free bacteria may have higher growth rates, sorter lag times, or higher population densities than isogenic plasmid-containing bacteria (Andersson and Hughes, 2010). As a result, the fitness cost of plasmid carriage can impede the maintenance of plasmids within bacterial populations. Costs associated with plasmid carriage, originally coined metabolic burden in recombinant bacteria, have long been observed (Bentley et al., 1990), but recent studies utilising whole genome sequencing and transcriptomics have expanded our understanding of the basis of plasmid costs (Harrison et al., 2015; Loftie-Eaton et al., 2017; Porse et al., 2016; San Millan et al., 2015; Yano et al., 2016). The physiological costs induced by plasmids can generally be categorised as either direct metabolic costs, due to increased transcription and translation associated with the expression of plasmid-encoded genes, or indirect, through the disruption of cellular networks. The causes of fitness costs associated with plasmid carriage are not mutually exclusive; multiple interacting effects are likely to give rise to the physiological costs plasmids impose on their hosts.

1.3.1 *Metabolic costs of plasmid carriage*

The metabolic costs of plasmid carriage stem from the increase in genetic material within the cell. The maintenance and replication of extra DNA is unlikely to cause significant direct costs, as the relative quantity of DNA within the cell tends not to increase significantly upon plasmid acquisition (Baltrus, 2013; San Millan and MacLean, 2017). Rather, costs are associated with the expression of novel genes introduced by the plasmid. The transcription of the newly acquired

plasmid genes is not thought to incur a significant cost (San Millan and MacLean, 2017). However, as the levels of expression of horizontally acquired genes are generally unoptimised (Park and Zhang, 2012), high levels of expression of plasmid encoded genes increases the translational load of the cell, and is expected to be the major source of cost during gene expression (Baltrus, 2013; Glick, 1995; San Millan and MacLean, 2017). The increase in cellular mRNA places a burden upon ribosomes which are occupied during translation at the expense of translation of host encoded genes (Shachrai et al., 2010). An increase in translation can also cause costs to arise due to increased consumption of limited amino acids, starving the cells of this essential resource and slowing down growth rates (Glick, 1995; Shachrai et al., 2010). Another source of cost during translation arises due to significant differences in codon usage between the plasmid genes and the host cell. These differences lead to a reduction in translation efficiency, affecting the speed and accuracy of translation (Plotkin and Kudla, 2011). This in turn can lead to the stalling of translation, mRNA degradation before translation, errors in the protein sequence and protein misfolding, all of which are costly (Drummond and Wilke, 2008).

1.3.2 *Disruption of cellular networks*

As well as the metabolic costs of plasmid carriage, cellular networks can also be disrupted at the cost of host fitness. The increased transcriptional load induced by the acquisition of a plasmid can trigger a potentially disproportional global transcriptional response, perturbing the level of expression of large numbers of chromosomal genes, particularly those involved with translation (Harrison et al., 2015; San Millan et al., 2015). The host SOS stress response can also be triggered for a number of reasons upon acquisition of a conjugative plasmid. The SOS response is usually triggered by DNA damage and is responsible for DNA repair; however, the triggering of this complex host network also increases mutagenesis, stalls chromosomal replication and suppresses cell division, reducing the cells competitive fitness (D'Ari and Huisman, 1983; Kawai et al., 2003). Conjugation introduces single stranded plasmid DNA (ssDNA) into a novel host cell, triggering the host SOS stress response (Baharoglu et al., 2010). This potentially limits the potential success of horizontal transfer as some conjugative plasmids encode genes that act to alleviated the SOS stress response during conjugation (Althorpe et al., 1999). Plasmid *rep* systems can also trigger the SOS response by sequestering the chromosomal replication

machinery through the recruitment of host DNA replication proteins (Ingmer et al., 2001; Loftie-Eaton et al., 2017; San Millan et al., 2015).

The costs that plasmids impose on their hosts are a critical driver of the evolution of plasmid borne AMR. These fitness effects can be a major limitation to the success of both horizontal and vertical transmission of the MGEs, determining under which environments the MGE is beneficial (Bergstrom et al., 2000). Plasmid costs can also drive the adaptation of both host and chromosomal genes, as costs can lead to evolution of strains that have ameliorated the costs of plasmid carriage (Harrison and Brockhurst, 2012).

1.4 Selection of Plasmid Borne Resistance

The aim of clinical antibiotic use is to provide sufficient antibiotic so that infections are cleared while minimising the toxic side effects inherent to many classes of antibiotics. Laboratory determination of the lowest concentration at which a drug inhibits all growth of bacteria, the minimal inhibitory concentration (MIC), is a key tool in predicting clinical antibiotic dosage. The traditional view is that resistance is selected for at concentrations of antibiotic above the MIC of sensitive strains despite the costs associated with resistance (Andersson, 2006; Drlica, 2003), as high concentrations of antibiotics select for resistance at the complete exclusion of sensitive strains. However, antibiotic gradients ranging from well in excess of the MIC, to far bellow the MIC (sub-MIC) exist in humans, livestock and the environment (Berendonk et al., 2015). In fact, far more environments contain concentrations of antibiotic below the MIC of sensitive cells than above (Kümmerer, 2003). The significance of antibiotics at sub-MIC levels upon the selection and maintenance of resistance has only recently been considered (Andersson and Hughes, 2014). Sub-MIC concentrations of antibiotics still reduce the growth of sensitive strains; if the reduction in growth outweighs the cost of resistance, resistance can be selected for below the MIC (Hughes and Andersson, 2012). However, the social interactions between bacteria can alter the outcome of antibiotic treatment, confounding sub-MIC selection dynamics. Processes, such as biofilm formation and cooperative resistance mechanisms, allow a population of bacteria to survive antibiotic treatment where a single cell would not (Vega and Gore, 2014). Moreover, these

social traits are often encoded as accessories genes upon plasmids (Rankin et al., 2011). Therefore, it is likely that the selection of antibiotic resistance plasmids depends on two factors: 1) the cost of plasmid carriage, and 2) the sociality of the traits that are being selected for.

1.4.1 *Sub-MIC selection of resistance*

Direct competition between antibiotic resistant and antibiotic sensitive strains is commonly used to determine at what drug concentration the growth is equal between the two strains. At this concentration of antibiotic resistance begins to be selected for and is defined as the minimum selectable concentration (MSC, Figure 1.3) (Gullberg et al., 2011). Enrichment of pre-existing resistance at sub-MIC levels of antibiotic has been demonstrated empirically in a number of studies (Gullberg et al., 2011, 2014; Liu et al., 2011). These studies have shown that the MSC value of a resistance varies depending on the resistance under selection. Liu et al. (2011) demonstrated that the MSC for ciprofloxacin and tetracycline resistance encoded upon the *Tn*10 transposon within *Escherichia coli* was 1/5 and 1/20 of the MIC of the susceptible strain, respectively. Gullberg et al. (2011) obtained similar results; the MSC values for various resistances to tetracycline, ciprofloxacin and streptomycin were between 10-fold and 230-fold lower than the MIC of the susceptible strain in both *E. coli* and *Salmonella enterica* (Var, Typhimurium LT2). Interestingly, different mutations conferring resistance to the same antibiotic also displayed different MSC values. The MSC depended upon the cost of resistance, such that a lower cost resistance mutation had a lower MSC (Gullberg et al., 2011). The group went on to show that the concentrations of antibiotics (tetracycline, erythromycin, trimethoprim and kanamycin) and heavy metals (arsenite and copper) selecting for a costly MDR plasmid were, in all cases, lower than the MIC of the plasmid-free susceptible strain (Gullberg et al., 2014). The studies showed that very low concentrations of antibiotics that are often present in contaminated natural environments or in patients under antibiotic treatment are able to select for and maintain resistance.
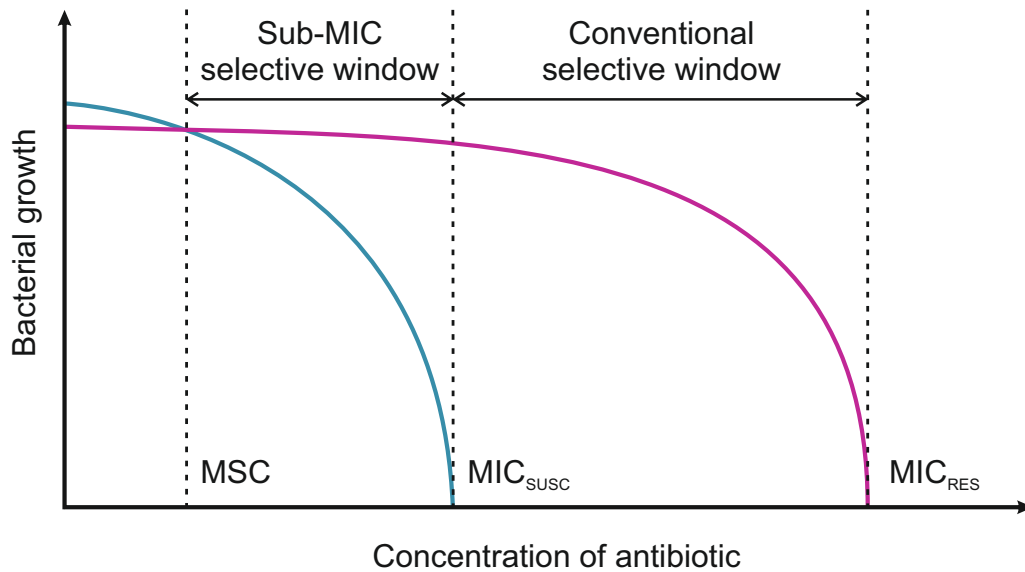
Figure 1.3 | The selective windows of antimicrobial resistance. Under no antibiotic selection or extremely low concentrations of antibiotic, the susceptible strain (blue line) outcompetes the resistant strain (purple line) due to the fitness costs associated with resistance. Conventionally resistance was thought to be selected at concentrations of antibiotic between the minimum inhibitory concentration of the susceptible strain (MIC_{SUSC}) and minimum inhibitory concentration of the resistant strain (MIC_{RES}). However, sub-inhibitory concentrations of antibiotic insufficient to kill sensitive bacteria can selected for resistance past the minimum selectable concentration (MSC), i.e. the intersect of susceptible and resistance growth. Adapted from Gullberg et al. (2011).

1.4.2 *The sociality of antimicrobial resistance*

It is becoming increasingly evident that the social dynamics imparted by qualitatively different mechanisms of antibiotic resistance can alter the outcome of antibiotic treatment (Vega and Gore, 2014; Yurtsev et al., 2013). As a consequence, determining the MSC of resistances may not simply be a function of cost, but may also be determined by the sociality of resistance. Cooperative drug resistances such as the inactivation of antibiotics, provide a benefit to surrounding bacteria regardless of their resistance phenotype, and determine the prevalence of resistance genes within a population (Yurtsev et al., 2013).

The enzymatic inactivation of antibiotics by resistant bacteria lowers the environmental concentration of antibiotics, allowing sensitive cells to survive and

compete under antibiotic treatment (Brook, 2009). Experimental systems have shown that the cooperative intracellular inactivation of chloramphenicol, via a plasmid borne acetyltransferase, allows the survival of sensitive strains through the detoxification of the environment in liquid culture, on semi-solid surfaces and in an *in vivo* mouse model (Sorg et al., 2016). The enzymatic inactivation of β-lactam antibiotics via β-lactamase also allows sensitive cells to survive initial concentrations of antibiotic that would usually kill them (Dugatkin et al., 2005; Medaney et al., 2016; Perlin et al., 2009; Yurtsev et al., 2013). When cooperative resistance carries a cost, whether due to the production of enzymes or due to resistance being encoded upon a plasmid, sensitive strains will grow faster than resistant strains once the antibiotic is cleared from the environment (Yurtsev et al., 2013). Therefore, the protection offered by cooperative resistances would be expected to push the MSC to higher values, with the precise extent of this effect dependent upon the cost of resistance.

The selection of antibiotic resistance will depend therefore both on the cost and the sociality of resistance. Sub-MIC selection of resistance mechanisms would be expected for selfish resistance mechanisms that do not impart a social benefit upon surrounding sensitive cells. In contrast, cooperative resistance should be selected for at higher relative concentrations of antibiotic (i.e. higher fraction of the sensitive MIC). Multiple mechanistically different resistances are frequently clustered together onto conjugative plasmids (Carattoli, 2013). An outstanding question is how combinatorial antibiotic usage selects for MDR plasmids, especially for combinations of antibiotics requiring qualitatively different mechanisms of drug resistance.

1.5 Amelioration of Plasmid Cost

1.5.1 *The plasmid paradox*
Conjugative plasmids are fundamental in the adaptation of bacteria, providing a source of novel genetic material, as well as acting as catalysts for evolutionary change (Frost et al., 2005). However, the existence of conjugative plasmids presents a paradox (Harrison and Brockhurst, 2012). The acquisition of a plasmid imparts a significant fitness cost upon the new host (section 1.3). Plasmid-free competitors will outcompete plasmid-containing bacteria, driving

the plasmid to extinction (Hall et al., 2016). Despite this plasmids remain widespread in bacteria.

There are evolutionary processes and plasmid encoded mechanisms that may help to explain the maintenance of plasmids within bacterial populations. When the accessory traits are under positive selection, the plasmid can be stabilised in the short-term (section 1.4). However, if the accessory trait is mobilised on to the chromosome the plasmid becomes redundant, once again exposing the host to the unneeded costs of plasmid carriage, and thus the plasmid can be lost (Harrison and Brockhurst, 2012). Segregational loss of plasmids can be minimised by plasmid encoded stability systems such as active partitioning systems and TA modules (section 1.2.2). These help to stabilise plasmids by reducing the number of plasmid-free competitors (Loftie-Eaton et al., 2016). However, active partitioning systems are not perfect and anti-toxins can be transferred on the chromosome (Ramisetty and Santhosh, 2016), leading to the emergence of plasmid-free cells that will outcompete the plasmid-harbouring population.

There are two main routes that can resolve the plasmid paradox and explain the stable existence of plasmids over the long-term – horizontal transfer and compensatory evolution. If infectious transfer rates of the plasmid between bacteria were high enough plasmids could survive within a population purely as a parasite, despite of the costs associated with them. However, the costs associated with conjugation are significant both for the host and recipient cells, the production of the conjugative pilus not only consumes host resources but opens the cell to attack from phage (Lin et al., 2011), and newly acquired ssDNA can trigger stress responses in novel hosts (section 1.3). Yet, several studies have shown that conjugative plasmids can have sufficient conjugation rates to allow them to invade and persist within bacterial populations (Hall et al., 2016; Stevenson et al., 2017). However, modelling has suggested that due to the costs associated with elevated conjugation rates, amelioration of plasmid cost is a more likely long-term solution to stabilising the maintenance of plasmids (Hall et al., 2017b).

1.5.2 *Evolution and Amelioration of Plasmid Cost*

Compensatory evolution can stabilise plasmids by reducing the cost of plasmid carriage. A number of long-term evolution experiments have demonstrated the ability of compensatory evolution to stabilise plasmids through adaptation of the host, plasmid or reciprocal coadaptation of both, reducing the fitness cost imposed by the plasmid (Dahlberg and Chao, 2003; Harrison et al., 2015; Loftie-Eaton et al., 2016, 2017; Porse et al., 2016; San Millan et al., 2015; Sota et al., 2010). The reduction in cost stabilises the plasmid within bacterial populations by reducing the purifying selection acting against it.

1.5.2.1 *Host adaptation*

Host compensatory adaptation of plasmid cost can resolve perturbed cellular homeostasis. Harrison et al. (2015) found that the acquisition of a large conjugative plasmid by *Pseudomonas fluorescens* SBW25 increased the expression of approximately 17% of the genome, including genes involved with protein synthesis, suggesting the plasmid incurred a high translational burden. A highly parallel and repeatable mutation arose within a chromosomal GacA/GacS global regulatory system, independent of whether the plasmid was under positive selection or not. The adaptation of the GacA/GacS system restored chromosomal expression to ancestral plasmid-free levels, and also down-regulated the expression of plasmid genes. These results align closely to a similar study examining the amelioration of the cost of a small, non-transmittable antibiotic resistance plasmid within *Pseudomonas aeruginosa* PAO1 (San Millan et al., 2015). Again, the acquisition of the plasmid significantly altered the expression of a large proportion of the genome. The mutational inactivation of two chromosomal genes, a UvrD-like helicase or a serine/threonine kinase, during coevolution with the plasmid reduced the expression of the plasmid encoded *rep* system, thus restored the transcriptional profile of the cells. It was likely that the *rep* system was interfering with chromosomal replication, triggering the SOS response and stalling cell division (San Millan et al., 2015). A recent study examining the amelioration of a costly MDR plasmid, RP4 within *Pseudomonas* sp. H2, also observed that mutations within chromosomal helicases compensated for the cost of the plasmid (Loftie-Eaton et al., 2017). Interestingly, these mutations also improved the persistence of other broad-host-range plasmids, suggesting that mutations within helicases that adversely

interact with plasmids may be a general mechanism for the adaptation of plasmid carriage.

1.5.2.2 *Plasmid adaptation*

The burden of plasmid carriage can also be reduced through mutation on the plasmid (Porse et al., 2016; Sota et al., 2010). Following 1000 generations of coevolution between the broad-host-range IncP-1β plasmid pMS0506 and host *Shewanella oneidensis*, Sota et al. (2010) observed that the plasmid had increase stability within both the coevolved and ancestral hosts. Parallel mutations within the plasmid replication initiation protein TrfA not only reduced the cost of the plasmid within *S. oneidensis* but also increased its stability within other naïve hosts. This provides evidence that unoptimised *rep* systems of naïve plasmids are an important source of the cost of plasmid carriage. However, other non-essential elements within plasmids can also contribute to their cost. Porse et al (2016) determined that a 25 kb region of large conjugative MDR plasmid was the major contributor to the burden it imposed upon its hosts. Compensatory evolution through *IS*26-mediated deletion of the costly region allowed stable maintenance of the plasmid.

1.5.2.3 *Coadaptation*

Co-adaptation of both the plasmid and hosts can also contribute to improved fitness (Dahlberg and Chao, 2003; Loftie-Eaton et al., 2016). Following experimental evolution of *E. coli* and one of two plasmids, R1 and RP4, reduction in cost required adaptation of both the plasmid and the host chromosome (Dahlberg and Chao, 2003). When the evolved plasmid was transferred into the ancestral host, the cost of plasmid carriage was reduced, suggestive of plasmid adaptation. Additionally, the ancestral plasmid conferred a lower cost in the evolved host, suggestive of host adaptation. A recent study determined the genetic basis of coevolution between an IncP-1β MDR plasmid and its host, *Pseudomonas moraviensis* (Loftie-Eaton et al., 2016). Evolution under antibiotic selection led to plasmid acquisition of a TA system from a native plasmid, this, coupled with host mutations, improved plasmid stability. Here, compensatory adaptation was indicative of positive epistasis because the plasmid and chromosomal mutations alone only provided a slight increase in plasmid persistence, but together they greatly increased persistence.

It is clear that compensatory adaption is an important route to ameliorate the cost of plasmid acquisition, reducing purifying selection against the plasmid, thus stabilising plasmids in bacterial populations. However, key outstanding questions remain unanswered; how does the mode of compensatory evolution following MDR plasmid acquisition vary with antibiotic treatment? What are the dynamics of coevolution between plasmid and host, and how repeatable are these coevolutionary dynamics?

1.6 RK2 Plasmid as a Model System

The work presented in this thesis experimentally tests the interaction between the broad-host-range MDR plasmid RK2, and the model organism *E. coli*, to address when the MDR plasmid is selected for and how it evolves with it's hosts. The RK2 plasmid was isolated from *Klebsiella aerogenes* following an outbreak of carbenicillin resistance in *P. aeruginosa* in the Burns Unit of the Birmingham Accident Hospital in 1969 (Ingram et al., 1973). It became evident that the RK2 plasmid was transferred from *P. aeruginosa* and maintained in *K. aerogenes*, before seeding a second outbreak of resistant *P. aeruginosa* six months later (Ingram et al., 1973). Interest in the RK2 plasmid grew due to its extreme broad-host-range and the taxonomic diversity of these host species, being able to replicate within almost all gram-negative bacteria (Schmidhauser and Helinski, 1985). RK2 is a 60-kb self-transmissible plasmid encoding ARGs against ampicillin via a TEM-1 β-lactamase, tetracycline via a *tetA/R* efflux pump, and kanamycin via a phosphotransferase (Figure 1.4) (Pansegrau et al., 1994). The replication and copy number of RK2 (approximately 5 in *E. coli*) is controlled by one of two initiator proteins, depending on its host (Durland et al., 1990; Solar et al., 1998), and stability of the plasmid is provided by active partitioning and TA systems (Pansegrau et al., 1994). The conjugation apparatus is clustered within two modules, *tra1* and *tra2*, encoding the genes required for mate pair formation and DNA transfer (Bingle et al., 2003; Zatyka et al., 1994). The RK2 plasmid provides an ideal model system to test evolutionary questions posed above due to the range of qualitatively different modes of resistances it encodes as well as the extensive knowledge of the plasmid's DNA sequence, regulation and control systems.
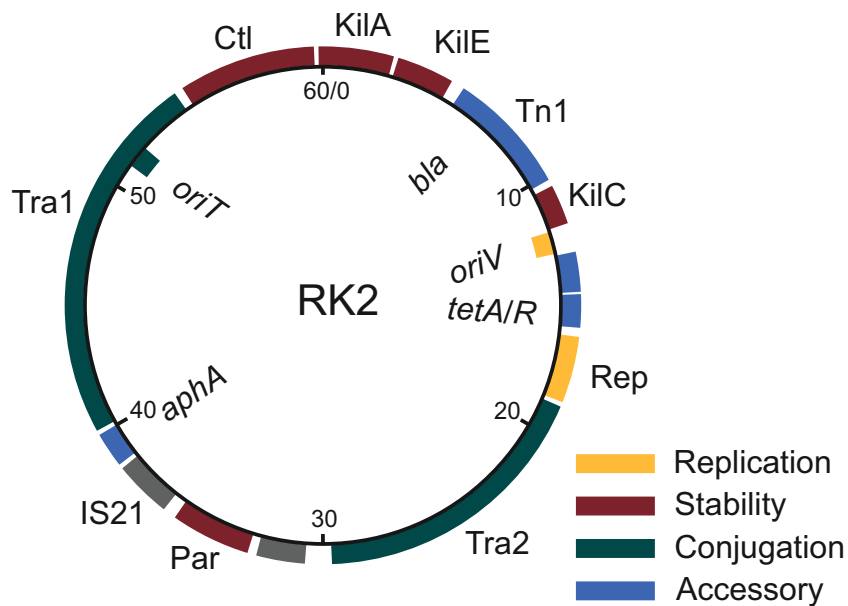
Figure 1.4 The RK2 plasmid, regions colour coded by functional groups. Scale in kb. Rep region encodes initiation factors controlling plasmid replication. Tra1 and Tra2 encode conjugation machinery. The Par region controls active partitioning, Kil regions encode genes required for host lethality controlled by the Ctl region. There are two MGEs encoded within RK2, insertion sequence IS21 and transposon Tn1 encoding β-lactamase. An efflux pump that is negative regulated in the absence of tetracycline provides tetracycline resistance, encoded by *tetA* and *tetR*. Kanamycin resistance is provided *aphA* encoding an aminoglycoside 3'-phosphotransferase. Created using the complete nucleotide sequence of RK2 produced by Pansegrau et al. (1994).

1.7 Chapter Overview

The thesis focuses on the sociality of resistance and the coevolution of plasmids and hosts when under positive selection, specifically:

Chapter 2 – Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance:

The sociality of antibiotic resistance encoded upon a MDR plasmid may alter the selective conditions required to maintain plasmids. To test this hypothesis I compared the concentrations of antibiotic required to select for two qualitatively different resistance mechanism encoded on the MDR plasmid RK2. In this

chapter I show that the antibiotic concentrations selecting for the RK2 plasmid in *E. coli* depend upon the sociality of the drug resistance: the selection for selfish drug resistance (efflux pump) occurred at very low drug concentrations, just 1.3% of the MIC of the plasmid-free antibiotic-sensitive stain, whereas selection for cooperative drug resistance (modifying enzyme) occurred at drug concentrations exceeding the MIC of the plasmid-free strain. Combining the two antibiotics at concentrations that alone do not select for resistance, selected for both resistance mechanisms. These results suggest that selfish resistances will be particularly important for the selective maintenance and spread of MDR plasmids.

Chapter 3 – Mode of antibiotic action and mechanism of resistance jointly shape selection for antibiotic resistance:

I theoretically investigate the effect of mode of antibiotic action and mechanism of resistance upon the selection for resistance. I demonstrate that the accurate prediction of the selection for resistance can only be achieved when mathematical models include whether antibiotics are bacteriostatic or bactericidal, and whether the antibiotic is inactivated by the resistance mechanism or not. I further predict that inactivation of bacteriostatic antibiotics by resistant bacteria provides greater protection to sensitive bacteria within a population than the inactivation of bactericidal antibiotics, leading to weaker selection for resistance. The chapter highlights the importance of understanding both the sociality of resistance and the antibiotic mode of action when predicting the selective conditions of resistance.

Chapter 4 – Adaptive modulation of antibiotic resistance through intragenomic coevolution:

Increasing evidence is pointing to compensatory coevolution between plasmid and host playing a key role in stabilising MDR plasmids within bacterial populations (Loftie-Eaton et al., 2016; McNally et al., 2016). In this chapter I address the outstanding question; how does the mode of compensatory adaptation vary with antibiotic treatment? I found that routes to compensatory adaptation were different between tetracycline selection and ampicillin-only or no antibiotic selection. I discovered that coevolution between the MDR plasmid RK2 and *E. coli* under tetracycline selection followed an unexpected and counterintuitive trajectory that was not observed under the other antibiotic treatments. Intragenomic coevolution ameliorating the cost of plasmid encoded

tetracycline resistance led to co-dependency between the plasmid and host chromosome; the resulting co-dependent genome provided high-level, low-cost resistance. This work demonstrates how gaining resistance plasmids can be just the start of the evolution of antibiotic resistance; gaining a MDR plasmid is a dynamic evolutionary process driven by the costs of expressing horizontally acquired ARGs.

Chapter 5 – High repeatability of bacteria-plasmid coevolution under antibiotic selection:

I extend the work presented in Chapter 4 to determine the repeatability of plasmid-host co-evolutionary dynamics. Through whole genome sequencing of clones isolated across the 530 generations of evolution from four independently evolving tetracycline populations presented in the previous chapter, I examine the temporal order of mutation acquisition. I show that the dynamics of coevolution can be strikingly parallel despite clonal interference between multiple competing lineages. The order of beneficial mutations was identical across all independent replicate populations examined. Evidence points towards mutations with the largest phenotypic effect being selected first, with mutation that induce smaller phenotypic effect being selected later in time. The study provides evidence that reciprocal coevolution between bacteria and plasmids can be highly repeatable, with mutations on the chromosome and plasmid following a specific path.

2. Selective Conditions for a Multidrug Resistance Plasmid Depend on the Sociality of Antibiotic Resistance

The following chapter is adapted from the published article: Bottery, M.J., Wood, A.J., and Brockhurst, M.A. (2016). Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance. Antimicrob. Agents Chemother. AAC. 60, 2524-2527. M.A.B. and A.J.W. supervised the project. M.J.B. performed the experiments and analysed the data. All authors contributed towards the design of the study and wrote the manuscript. Data from the paper summarized in figures 2.1 and 2.3. Additional data on frequency depend selection was acquired after the publication of the paper.

2.1 Abstract

Multiple antibiotic resistance genes (ARGs) are frequently clustered on conjugative plasmids, which are an important source of clinical resistance. It is critical therefore to understand the selective conditions promoting the spread of these multidrug resistance (MDR) plasmids. Here, we tested how the antibiotic conditions required to select for a multidrug resistant plasmid, RK2, in *Escherichia coli* depended on the mechanism of resistance, specifically whether drug resistance was selfish or cooperative. We observed highly contrasting selective conditions depending upon the sociality of resistance: a selfish drug resistance, efflux of tetracycline, was selected for at ~1% of the minimum inhibitory concentration (MIC) of the plasmid-free antibiotic-sensitive strain, whereas, a cooperative drug resistance, enzymatic inactivation of ampicillin, was only favoured at antibiotic concentrations exceeding the MIC of the plasmid-free strain. When used in combination, dual antibiotic selection was additive and thus selected for the MDR plasmid at concentrations even lower than those observed to be selective under single antibiotic treatments. Finally, we show that selection for cooperative resistance, unlike selfish resistance, is strongly favored when resistance is rare within a population. These results suggest that selfish drug resistances, such as efflux pumps, are likely to play an important role in the dynamics of MDR plasmids in the environment where they can be selected for by very low, sub-MIC concentrations of antibiotic.

2.2 Introduction

Antibiotics are critical to modern medicine, but their widespread use and misuse
have led to the evolution of strains resistant to most commonly used antibiotics
(Bush et al., 2011; Laxminarayan et al., 2013). Antibiotic resistance has become
a major threat to global health, with multidrug-resistant (MDR) bacteria observed
globally (World Health Organization, 2014). Environmental antibiotic resistance
genes (ARGs) are a major source of clinical resistance (Berendonk et al., 2015).
ARGs can be selected for at very low concentrations of antibiotics, far below the
MIC of sensitive cells (Gullberg et al., 2011, 2014), with antibiotic contamination
at sub-MICs being proposed as the main driving force behind environmental
selection for resistance (Andersson and Hughes, 2014; Liu et al., 2011;
Sandegren, 2014). However, ARGs can encode qualitatively different forms of
resistance, ranging from selfish to cooperative. Selfish drug resistances confer a
benefit only to the individual cell harboring them, for example, efflux pumps,
reduced membrane permeability, and antibiotic target alteration (Blair et al.,
2015). In contrast, cooperative antibiotic resistance benefits both the resistant
cell and surrounding cells, whether they are resistant or not. For example,
modifying enzymes, such as β-lactamases, inactivate the antibiotic through
hydrolysis, decreasing its environmental concentration. Localization of the β-
lactamase enzyme in the periplasmic space may enhance the share of the
benefit for the resistant cell, but nevertheless, the decrease in the overall
environmental concentration of antibiotic will benefit both resistant and sensitive
cells (Vega and Gore, 2014).

We hypothesized that the sociality of drug resistance alters the selective
conditions for the spread of ARGs (Conlin et al., 2014; Yurtsev et al., 2013).
Specifically, because the benefits of selfish drug resistance are directed solely
to the resistant cell, whereas the benefits of cooperative drug resistance are
shared between resistant and sensitive cells, we predicted that selfish drug
resistance should be selected at lower relative drug concentrations (i.e., % of
the sensitive MIC) than those for cooperative resistance. These contrasting
modes of resistance may also determine whether selection for resistance is
frequency dependent (Conlin et al., 2014; Dugatkin et al., 2005; Levin et al.,
1988; MacLean and Gudelj, 2006; Ross-Gillespie et al., 2007; Yurtsev et al.,
2016). We would expect that as cooperative resistance provides a public good

via the inactivation of the antibiotic, the survival of sensitive cells would depended upon the frequency of cooperators within a population. In contrast, selfish drug resistances do not alter the survival of sensitive cells and therefore selection for a selfish resistance would not be frequency dependent.

Multiple ARGs are frequently clustered together onto conjugative plasmids, including combinations of selfish and cooperative drug resistances (Carattoli, 2013). How combinatorial antibiotic usage selects for MDR plasmids is not clear, especially for combinations of antibiotics requiring qualitatively different modes of drug resistance, such as selfish or cooperative drug resistance. Here, we tested how the sociality of drug resistance and single versus combined antibiotic treatment altered the selective conditions for the MDR plasmid RK2 (Pansegrau et al., 1994) in *Escherichia coli* MG1655. RK2 carries genes encoding both cooperative ampicillin resistance, mediated by a β-lactamase, and selfish tetracycline resistance, mediated by an efflux pump. We report that the selfish drug resistance is selected for at far lower relative antibiotic concentrations than those for cooperative drug resistance and that combined antibiotic selection is additive, showing no interaction. Furthermore, we show that the selection for cooperative breakdown of ampicillin is strongly frequency dependent, whereas the selfish efflux of tetracycline is not. Therefore the selection for the cooperative RK2 plasmid can be manipulated, ranging from highly cooperative under ampicillin conditions, though non-cooperative in the absence of ampicillin, to selfish in the presence of tetracycline.

2.3 Materials and methods

2.3.1 *Strains, culture conditions*

Two isogenic *E. coli* MG1655 strains chromosomally labelled with GFP or mCherry were used for direct competition. These strains are labelled at the neutral insertion site *attB* lambda through λ red homologous recombination. Both strains were provided by the Van Der Woude lab (University of York). The RK2 multidrug resistance plasmid was introduced to the marker strains through conjugation from *E. coli* MV10(RK2) provided by the Thomas lab (University of Birmingham). Transconjugants were selected on nutrient broth agar plates supplemented with tetracycline 10 µg ml^{-1}. As there was no antibiotic resistance

selectable marker on the chromosome of the fluorescently labelled cells, transconjugants were distinguished through detection of fluorescent markers using Zeis Stereo Lumar v12 microscope. Presence of the RK2 plasmid was confirmed by PCR using RK2 specific primers amplifying the plasmids origin of replication, *oriV* (Fw: cccatcatgaccttgccaag Rv: gtaacagatgagggcaagcg). All cultures were conducted in Oxoid® Nutrient Broth (NB) at 37°C, shaken at 180 rpm, 5 ml in 50 ml microcosms unless otherwise stated.

2.3.2 *MIC measurements*

To measure minimal inhibitory concentrations (MIC), six replicate cultures of plasmid free *E. coli* MG1655 and six replicate cultures of *E. coli* MG1655(RK2) were grown overnight until stationary phase in 5 ml NB, the saturated cultures were then sub-cultured 50 µl into 5 ml fresh NB and grown to an OD_{600} of 0.5. These were then diluted into 96-well plates containing gradients of ampicillin (ranging from 0 µg ml^{-1} to 4096 µg ml^{-1}) or tetracycline (ranging from 0 µg ml^{-1} to 256 µg ml^{-1}) antibiotics in nutrient broth, to an initial density of 5×10^5 CFU ml^{-1}. 100 µl cultures were grown for 24 hours 37°C shaken at 600 rpm, 3 mm orbital radius. OD_{600} was measured after 24 hours. The MIC was defined as the lowest concentration that inhibited all bacterial growth. These conditions informed the antibiotic conditions used to in competitive fitness assays.

2.3.3 *Growth curves*

Growth curves of *E. coli* MG1655 with and without RK2 plasmid were conducted in monoculture. Three independent replicate microcosms were inoculated with individual colonies of MG1655 with and without the RK2 plasmid and grown to saturation over night. These were sub-cultured 50 µl into 5 ml NB and grown for 2 hours until OD_{600} of 0.5. The sub-cultures were used to inoculate 100 µl NB in 96-well plates to a final dilution of 1:400. The plates were incubated at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, with OD_{600} measured every 30 minutes using a Tecan infinite M200 Pro plate reader.

Growth curves of plasmid free *E.* coli MG1655 were conducted to determine the sub-MIC growth response to ampicillin and tetracycline. Three independent replicate saturated overnight cultures were sub-cultured to an OD_{600} of 0.5, and used to inoculated 100 µl NB supplemented with either 0, 2, 4, 6, 8, or

10 µg ml^{-1} ampicillin or 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1 2.4, or 2.7 µg ml^{-1} tetracycline in 96-well plates at a final dilution of 1:400. Plates were grown at 37°C with shaking at 300 rpm, 3 mm orbital radius for 48 hours, OD$_{600}$ was measured every 30 minutes by Tecan infinite M200 Pro plate reader.

2.3.4 *Relative fitness*

The relative fitness of plasmid bearing versus plasmid free cells was estimated by direct competition experiments. To control for fitness effects of the fluorescent markers, competitions were performed with the plasmid carried in the MG1655-gfp background against plasmid-free MG1655-mcherry for half of the replicates, and vice versa for the other half of the replicates. Competitions were conducted across antibiotic concentration gradients, ranging from no antibiotic to 12 µg ml^{-1} ampicillin or 250 ng ml^{-1} tetracycline. Six replicates of each antibiotic concentration were conducted. The competitions were initiated with 1:1 mixtures of plasmid-bearing against plasmid-free strains from overnight cultures at an initial density of approximately 5×10^5 CFU ml^{-1}. To gain exact viable cell counts, dilutions of culture were spread on to nutrient agar plates. Competition cultures were grown for 24 hours in 100 µl NB within 96 well plates at 37°C, 600 rpm. Final densities calculated via plate counts, strains were distinguished through detection of fluorescent markers using Zeis Stereo Lumar v12 microscope. The relative fitness of plasmid-bearing bacteria was calculated as a ratio of Malthusian parameters (Lenski et al., 1991):

$$W_{plasmid} = \frac{\ln\left(\frac{N_{final,plasmid}}{N_{initial,plasmid}}\right)}{\ln\left(\frac{N_{final,free}}{N_{initial,free}}\right)}$$

No significant difference between growth dynamics of marker strains was observed (Figure S2.1), along with no significant difference in relative fitness when plasmid free MG1655-gfp and -mcherry strains were directly competed (Figure S2.2, *t*-test difference from 1 $t_{11} = 0.6001$, p = 0.5606).

2.3.5 *Frequency dependent selection*

Frequency dependent selection was measured through direct competition between *E. coli* MG1655 and *E. coli* MG1655(RK2) at different starting frequencies. As with the relative fitness calculations, to control for fitness effects

of the differentially fluorescently marked strains, 3 replicate competitions per treatment were conducted with the RK2 plasmid in the MG1655-gfp background and 3 replicate competitions were conducted with the RK2 plasmid in the MG1655-mcherry background. Competitions were initiated with either 1:99, 1:9, 1:1, 9:1 or 99:1 mixtures of plasmid-bearing strains and with plasmid-free strains from overnight monocultures grown in 5 ml non-selective NB media. These mixtures were used to inoculate 100 µl NB containing either 0, 4, 8, or 12 µg ml^{-1} of ampicillin or 0, 13, 26 or 39 ng ml^{-1} of tetracycline at a final dilution of 1:400. The strains were competed for 24 hours at 37°C, 600 rpm in 96 well plates. To detect very low proportions of each competing strain, cell densities at the start and end of the competition were determined through flow cytometry. Saturated populations were diluted to 1:1000 in sterile 22 µm filtered PBS, and stained with 1 µg ml^{-1} DAPI to distinguish between cells and background noise. Cells were first gated from the background electrical noise using FSC and SSC, and then gated on a positive DAPI signal using 450/45 channel on a 405 nm laser. Sub-populations of plasmid containing and plasmid free cells were gated on GFP signal using 525/40 channel on a 488 nm laser (Figure S2.3). All gating was conducted using R3.3.3 {*flowCore*}. Selection rate (*r*) was calculated as the difference between the plasmid bears and plasmid free Malthusian growth parameters:

$$r_{plasmid} = \ln\left(\frac{N_{final,plasmid}}{N_{initial,plasmid}}\right) - \ln\left(\frac{N_{final,free}}{N_{initial,free}}\right)$$

Which gives the absolute difference in density over 24 hours between the two strains. This measure of fitness is preferable when comparing strains were one competitor is much less fit than the other (Travisano and Lenski, 1996).

2.3.6 *Statistics*

Significant difference between two related samples was calculated using two sided, two-sample t-test. Shapiro-Wilk test was conducted to check for normality. Interacting effects of co-treatment with ampicillin and tetracycline were calculated using multiple linear regression with antibiotics are interacting terms. The effects of frequency of resistances and concentration of antibiotic upon fitness were calculated using multiple linear regression with frequency and concentration as interacting terms. All statistical analysis was conducted in R (version 3.2.3).

2.4 Results

2.4.1 *Sub-MIC effect of antibiotics on growth*

Conventionally, ARGs are thought to be positively selected for at antibiotic concentrations exceeding the MIC of sensitive cells in monoculture (Hughes and Andersson, 2012). This is referred to as the conventional selective window (Figure 2.1), and is the concentration required to reduce to reduce the growth of a bacterial population to undetectable levels. This is the basis of calculating the dosage of antibiotics within clinical settings, with the aim to clear an infection through maintaining antibiotics concentrations above a bacteria's MIC. Due to a focus on resistance phonotypes with high levels of resistance, how antibiotic concentrations below the MIC of sensitive cells selects for resistance is often overlooked. Importantly, growth of sensitive strains is reduced when exposed to concentrations of antibiotic insufficient to kill them, which may lead to the selection for resistance (Figure 2.1).
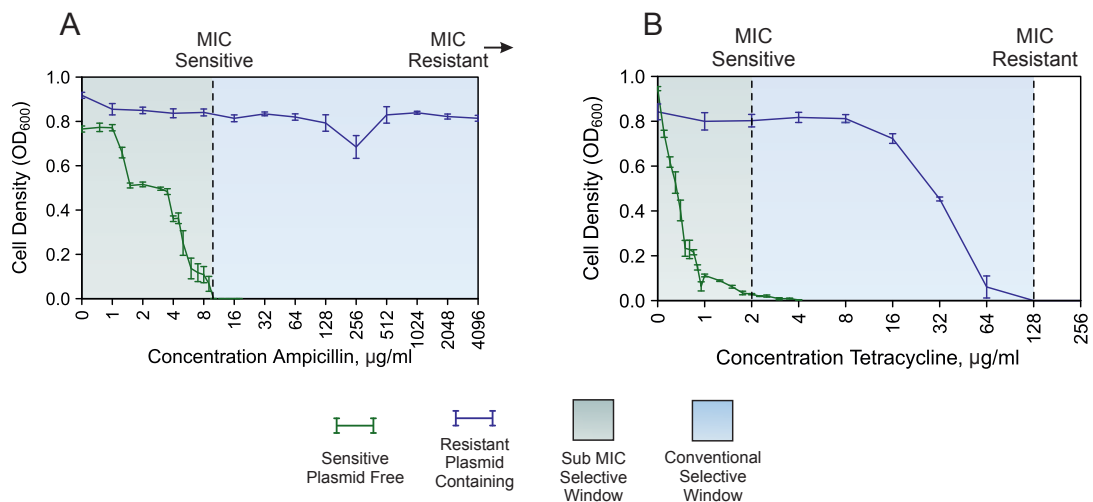
Figure 2.1 | Cell density (optical density at 600 nm [OD_{600}]) of sensitive plasmid-free bacteria (green line) and resistant plasmid containing bacteria (blue line) as a function of ampicillin concentration (A) and tetracycline concentration (B) after 24 h of growth in monoculture. The error bars show standard error of the mean (SEM) values (*n* = 6). The area shaded in green shows the sub-MIC selective window, and the area shaded in blue shows the selective window conventionally thought to select for resistance.

To determine the impact of sub-MIC concentrations of tetracycline and ampicillin we measured the growth of plasmid free MG1655 across a gradient of sub-inhibitory concentrations of antibiotic. Sub-MIC concentrations of both ampicillin and tetracycline increase the length of the lag phase and decrease the growth rate and carrying capacity of the sensitive bacterial populations (Figure 2.2). Ampicillin acts as bactericidal at concentrations exceeding the MIC, inhibiting cell wall synthesis and causing cell lysis (Waxman and Strominger, 1983), however, below the MIC ampicillin can produce bacteriostatic effects, reducing and inhibiting bacterial growth (Thonus et al., 1982). A transition between bactericidal and bacteriostatic mode of action may be evident in the sharp decrease in growth after 6 µg/ml (Figure 2.2A). This transition in mode of action may be the cause of the hill type shape of the MIC curve, whereby there is a slow reduction in growth at low concentrations of antibiotic which increases at higher concentrations (Figure 2.1A). In contrast, tetracycline is through to act predominately as a bacteriostatic (Chopra and Roberts, 2001), as a result increased concentrations of tetracycline linearly decrease growth of sensitive bacteria (Figure 2.2B).
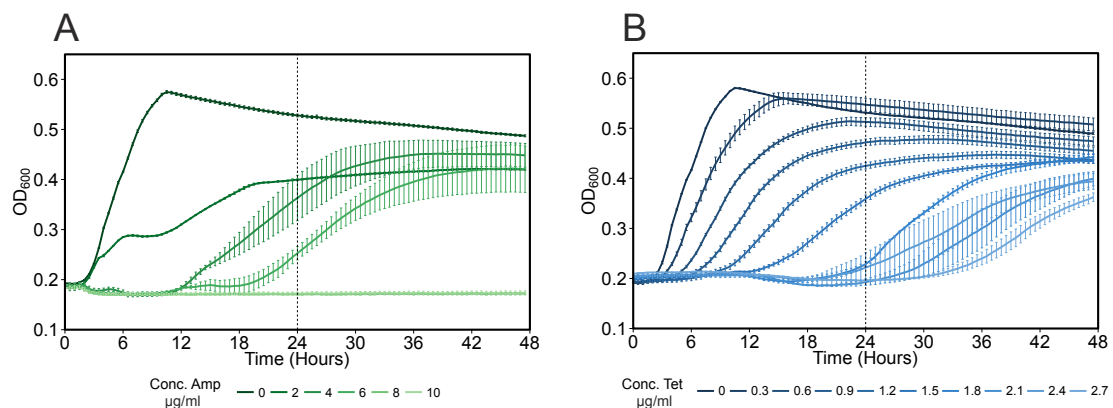
Figure 2.2 | Sub-MIC sensitivity of plasmid free *E. coli* MG1655 to ampicillin (bactericidal) and tetracycline (bacteriostatic). Growth curves (OD_{600}) of plasmid free *E. coli* MG1655 in increasing concentrations of (A) ampicillin and (B) tetracycline. Dotted vertical line represents the 24-hour mark at which competition and MIC cultures are measured for relative fitness and growth respectively. Lines show mean of three biological replicates per treatment with error bars representing SEM.

2.4.2 *Selective conditions of Multi-drug resistance*

To determine whether the sociality of resistance affected the selection window for the RK2 MDR plasmid, we estimated the relative fitness of plasmid-bearing versus isogenic plasmid-free cells by direct competition, according to standard methodology. In the absence of antibiotics, the plasmid imposed a significant cost of carriage, decreasing the fitness of *E. coli* by 19% (Figure 2.3A and B, t test, P = 0.001, t = 9.8674, df = 23). An intrinsic cost is often associated with plasmid carriage when accessory traits are not under positive selection due to cellular disruption and increased translational load (Baltrus, 2013). Cooperative ampicillin resistance was positively selected at ampicillin concentrations exceeding the MIC of plasmid-free sensitive *E. coli* strains (Figure 2.3A). Importantly, sensitive cells were able to maintain positive growth in mixed cultures at ampicillin concentrations that completely inhibited their growth in monoculture (>8 µg/ml; Figure 2.1A; see also Figure S2.4 in the supplemental material), justifying the assignment of ampicillin resistance as cooperative. Thus, cooperative resistance permits the persistence of a sensitive subpopulation beyond the sensitive MIC due to the inactivation of the antibiotic, potentially allowing reinvasion by sensitive cells once the antibiotic concentration is sufficiently reduced by the action of resistant cells.

In contrast, selfish tetracycline resistance was positively selected at tetracycline concentrations of just 1.3% of the MIC of sensitive *E. coli* (Figure 2B). Indeed, at concentrations of tetracycline that were >10% of the MIC of sensitive *E. coli*, the resistant plasmid bearers competitively excluded the plasmid-free bacteria, with no plasmid-free cells observable (see Figure S2.5). This is despite the fact that plasmid-free *E. coli* survived at these tetracycline concentrations when grown alone (Figure 2.1B). Our data suggest that selfish tetracycline resistance is positively selected in the sub-MIC selective window at very low tetracycline concentrations, similar to those observed in the natural environment (Zhang and Li, 2011).
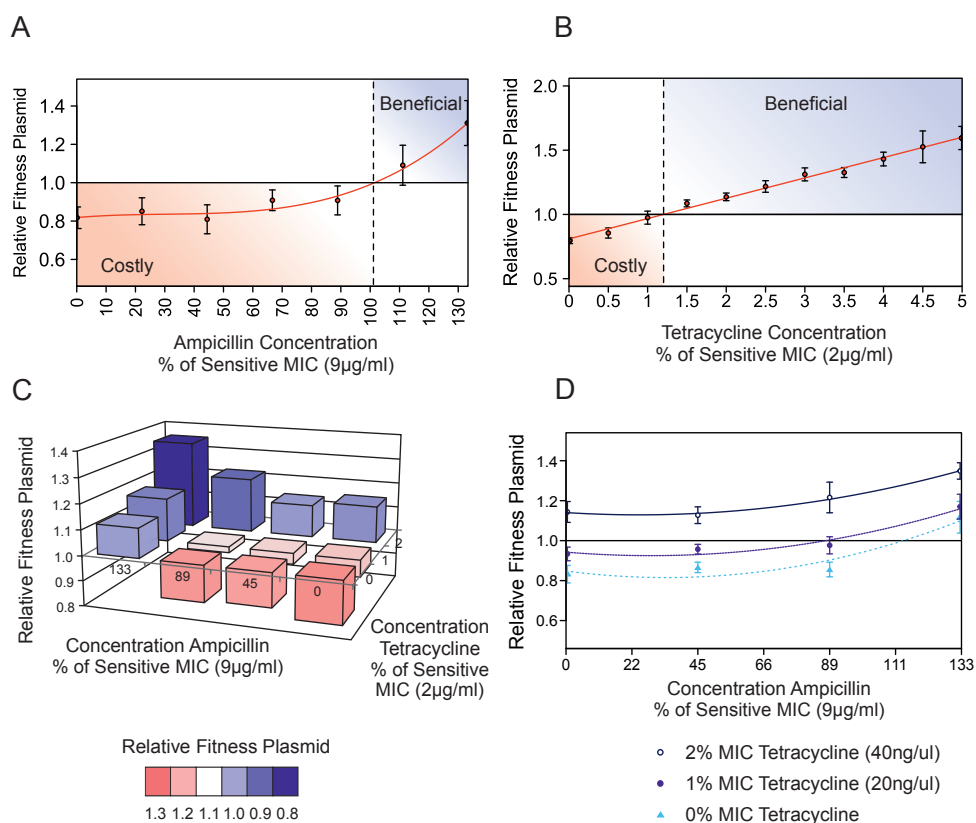
Figure 2.3 | Fitness reaction norms as a function of antibiotic concentration during competition experiments between *E. coli* harboring the RK2 plasmid and isogenic plasmid-free sensitive strains. Competition in the presence of ampicillin (A) and tetracycline (B) is shown, and the red lines show a fitted regression. Dashed lines represent antibiotic concentrations predicted to select for RK2 plasmid. (C and D) Fitness reaction norms of combination treatments with both ampicillin and tetracycline during competition experiments between *E. coli* harboring the RK2 plasmid and plasmid-free strains; these are alternative visualizations of the same experimental data. There is no significant interaction of antibiotic treatments upon the relative fitness ($F_{1,68}$ = 0.2395, P = 0.6261), indicating that treatments were non-interacting and additive. The error bars in panels A, C, and D show the SEM values (*n* = 6). Antibiotic concentrations are shown as percentages of the MIC for sensitivity.

When ampicillin and tetracycline were applied in combination, there was no significant interaction ($F_{1,68}$ = 0.2395, P = 0.6261), indicating that when these two

antibiotics were used in combination, their selective effects were independent and additive (Figure 2.3C). This means that very low concentrations of tetracycline were sufficient to completely mask the population-level effects of cooperative ampicillin resistance. With increasing tetracycline concentrations, the ampicillin concentration positively selecting for the MDR plasmid shifted to lower and lower sub-MIC levels, reducing the window of selective conditions under which sensitive cells could persist (Figure 2.3D).

Residues of multiple antibiotics are commonly found to contaminate the same environments at low concentrations (Batt et al., 2006; Zhang and Li, 2011). These combinations, and particularly the presence in the environment of antibiotics, like tetracycline, targeted by selfish efflux mediated resistance will select for the spread of MDR plasmids and competitive exclusion of sensitive cells. This is despite being present at concentrations far below the level required to positively select resistance individually. This adds further evidence that ARGs, whether chromosomal or carried by plasmids, can be positively selected at antibiotic concentrations far below the MIC of sensitive strains (Gullberg et al., 2011, 2014; Liu et al., 2011).

2.4.3 *Social resistance and frequency dependence*

Next we determined whether the starting frequency of resistance within a population altered at what concentration resistance was selected for, i.e. whether selection for the resistances were frequency dependent. Relative fitness of plasmid bearing versus plasmid free cells was estimated across a range of initial plasmid frequencies and antibiotic concentrations (Figure 2.4B, 2.4D). Both tetracycline concentration ($F_{1,116}$ = 119.28, p < 0.01) and frequency of resistance ($F_{1,116}$= 33.49, p < 0.01) have significant effects on the selection rates of the RK2 plasmid. However, both predictors did not show a significant interaction ($F_{1,116}$ = 1.186, p = 0.2784), indicating that the concentration of tetracycline and frequency of resistance in a population are independent. Therefore selection for the efflux of tetracycline is not altered by the frequency of resistance within the population, and only very low concentration of tetracycline are required to select for the selfish resistance independent of the fraction of resistance in the population.

In contrast, the starting frequency of plasmid-bearing antibiotic-resistant bacteria

within a population greatly affects the concentration of antibiotic required to select for cooperative ampicillin resistance (Figure 2.4A, 2.4C). At low starting frequencies of plasmid-bearing cells (one tenth of the total population), the concentration of ampicillin required to select for resistance is reduced to below 44% of the sensitive MIC. Where as, when the starting frequency of plasmid-bearing cells is high within a population (nine tenths of the total population), the concentration of ampicillin required to select for resistance increased to above 133% of the sensitive MIC. Both the concentration of ampicillin ($F_{1,116}$ = 103.69, $p < 0.01$) and the frequency of plasmid within the population ($F_{1,116}$ = 350.925, $p < 0.01$) are significant predictors of the selection rate of the resistant cells. But unlike the selfish tetracycline resistance, ampicillin concentration and frequency of plasmid have a significant interaction ($F_{1,116}$ = 44.914, $p < 0.01$). The interaction of selective environment and frequency of resistance results in negative frequency dependent selection for cooperative resistance, whereby selection for resistance is stronger when resistance is rare. A lower frequency of plasmid containing bacteria within a population may result in a lower average inactivation rate of antibiotic, providing little or no protection to plasmid sensitive cells within the environment. The reduced protection provided by the resistant cells results in positive selection for resistance at lower relative concentrations of antibiotic. Taken together these finding suggest that selfish resistances are important for maintaining multi-drug resistance plasmids independent of frequency within a population, but cooperative resistances are equally as important for the selective maintenance when the plasmid is rare in a population.
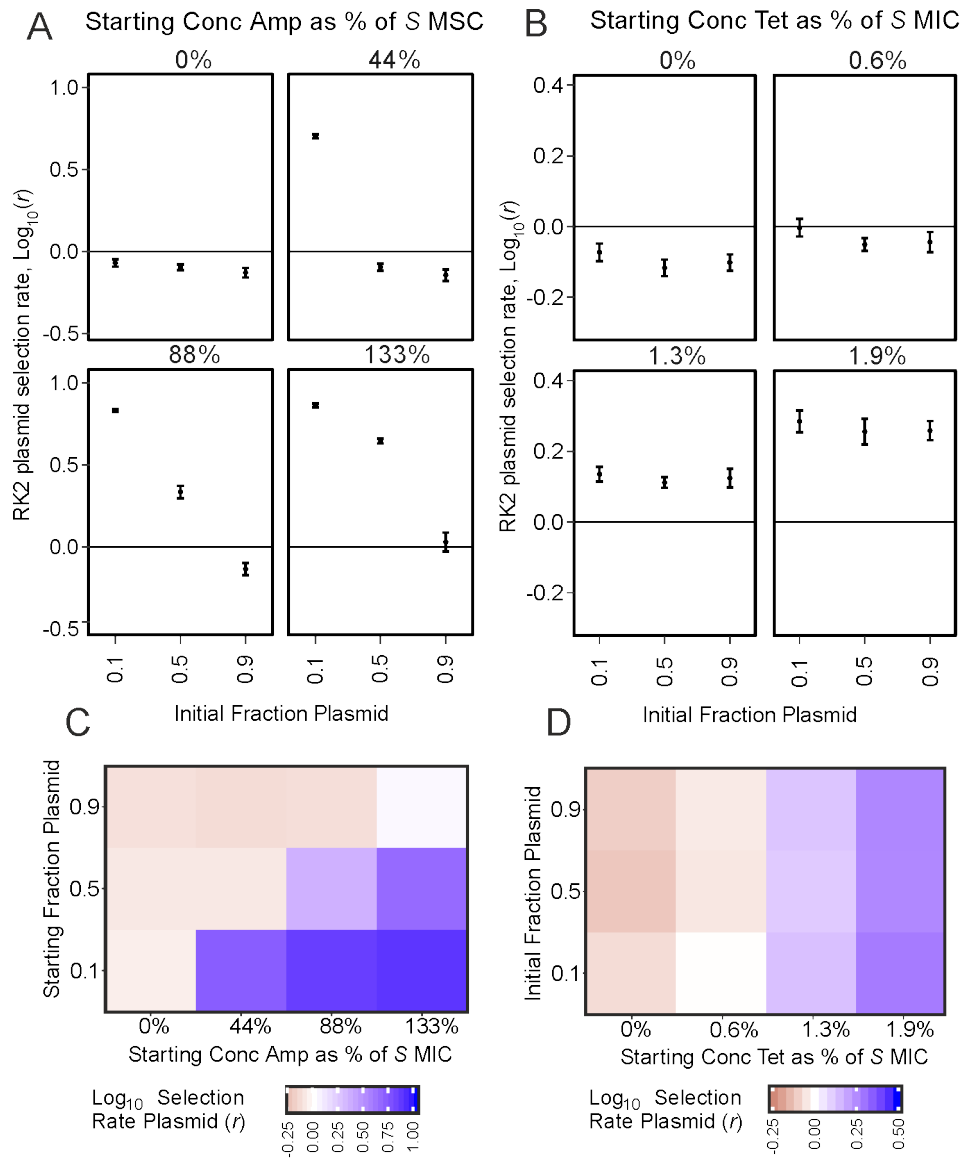
Figure 2.4 | Log_{10} selection rates (r) of plasmid bearing *E. coli* vs plasmid free *E. coli* as a function initial plasmid frequency under (A,C) ampicillin selection and (B,D) tetracycline selection. Points show means of six independent replicates; with error bars showing SEM. **C** and **D** show the same data plotted as heat maps. Selection rates were measured at frequencies of 0.01 and 0.99, these measurements have been omitted from the plot for clarity, however, the stats presented in the text include these measurements. Plots containing this additional data can be found in the supplementary information, Figure S2.6.

2.5 Discussion

Here, we show that the extent to which an ARG is positively selected at sub-MICs depends upon the sociality of the mechanism of drug resistance. Cooperative ampicillin resistance is positively selected at ampicillin concentrations exceeding the MIC, whereas selfish tetracycline resistance is positively selected at 100-fold-lower relative drug concentrations. This striking difference in the selective window for ARGs located on the same MDR plasmid probably arises because of the population-level effects of the ARGs: cooperative ampicillin resistance allowed sensitive bacteria to survive at concentrations above their MIC by reducing the ampicillin concentration and sharing the benefits of resistance, whereas selfish tetracycline resistance drove the complete competitive exclusion of sensitive cells at >10% of the MIC due to the exclusively individual benefits of efflux-mediated resistance. These contrasting mechanisms of resistance also lead to frequency dependent selection of cooperative ampicillin resistance but not selfish tetracycline resistance. Combining the two antibiotics at concentrations that would not normally select for resistance individually selects for both resistances and the spread of the MDR plasmid.

Our study has a number of possible limitations: First, it is possible that other factors in addition to sociality may have contributed to differences in the fitness reaction norms of the antibiotics, including the contrasting effects of sub-MICs on monoculture densities, and the fact that ampicillin is bactericidal while tetracycline is bacteriostatic. Second, we use exemplars of cooperative and selfish resistance, but more research will be required to test the importance of sociality on the selective conditions for other resistance mechanisms.

Understanding when antibiotic resistance is selected for within bacterial population is a central goal in studying the spread of resistance. Our findings suggest that selfish efflux-mediated drug resistances are likely to be especially important for the selective maintenance and spread of MDR plasmids independent of the frequency of resistance in the populations. However, cooperative resistances are likely to be equally as important for the selective maintenance when the plasmid is rare in a population.

# 3. Mode of Antibiotic Action and Mechanism of Resistance Jointly Shape Selection for Antibiotic Resistance

3.1 Abstract

The acquisition and spread of bacterial resistance to antibiotics threatens global public health. Mathematical models have proved to be a key tool in aiding our understanding of antibiotic resistance. Pharmacokinetic and pharmacodynamics (PK-PD) models are used to predict optimal dosage and antibiotic regimens targeted at specific pathogens. However, PK-PD models often omit important biological details that affect selection for resistant genotypes, limiting their usefulness for predicting resistance evolution. Here, we develop simple models parameterised with experimental data for two antibiotics, tetracycline and ampicillin, that differ in their modes of action and mechanisms of resistance. We show that selection for resistance can only be accurately predicted when models include whether antibiotics are bactericidal or bacteriostatic, and whether resistance involves inactivation of the antibiotic or not. We further demonstrate that inactivation of bacteriostatic antibiotics provided more protection to sensitive cells within a population than inactivation of bactericidal antibiotics, leading to weaker selection for resistance.

3.2 Introduction

The global spread of antimicrobial resistance (AMR) within bacteria is a major threat to both routine and critical care, as the drugs we rely on to cure infections diminish in effectiveness (Laxminarayan et al., 2013). Mathematical models have proven to be a valuable tool in understanding the population dynamics of bacteria under antibiotic treatment (Czock and Keller, 2007; Jacobs et al., 2016). Techniques such as pharmacokinetic-pharmacodynamic modelling (PK-PD) are used to predict optimal dosage and antibiotic regimens targeted at specific pathogens (Chung et al., 2006; Mouton et al., 1997), but can also be applied to predict the emergence and subsequent selection of resistance (Lipsitch and Levin, 1997). The minimum inhibitory concentration (MIC) of sensitive cells is commonly used in combination with these models to predict the

concentration of antibiotic required to clear infection and the probability for resistance to arise (Drlica, 2003).

Within host PK-PD models commonly assume an antibiotic effect to be bactericidal (Jacobs et al., 2016), however the mode of action of antibiotics ranges from bacteriostatic – reducing the growth rate of a cell – to bactericidal – directly killing the cells. This fundamental difference in the mode of action of antibiotics is likely to alter selection for resistance in bacterial populations simply because sensitive cells are merely outgrown rather than killed under treatment by bacteriostatic antibiotics (Pankey and Sabath, 2004). Similarly, PK-PD models often have simple approximations of resistance whereby all the benefit of resistance is directed to the resistant sub-population. While this is likely to be the case for certain mechanisms of resistance, it is not appropriate for mechanisms of resistance that inactivate the antibiotic itself. When resistance is mediated by a mechanism that does not change the environmental concentration of antibiotic – such as efflux pumps, alteration of target site or target site protection via antibiotic release (Dönhöfer et al., 2012; Rahman et al., 2017) – the benefit of resistance is purely directed towards the resistant cell. In contrast, enzymatic inactivation of an antibiotic, such as by β-lactamases or acetyltransferases, decrease the local concentration of antibiotic. This provides high levels of resistance to the producers but also lowers the environmental concentration of antibiotic and thereby allows some sensitive cells to persist in the population, providing collective resistance (Dugatkin et al., 2005; Sorg et al., 2016; Yurtsev et al., 2013). Recent experimental studies suggest that this difference in the mechanism of antibiotic resistance alters the concentration of antibiotic required to select for resistance in mixed populations containing resistant and sensitive genotypes (Bottery et al., 2016).

We develop a simple mathematical model that incorporates both bacteriostatic and bactericidal terms, as well as modes of resistance that do or do not inactivate the antibiotic. The model is parameterised against published data for sensitive and resistant strains of *Escherichia coli* where resistance is provided by the plasmid RK2 (Bottery et al., 2016). RK2 encodes resistance genes against ampicillin, a bactericidal antibiotic for which resistance inactivates the drug, and tetracycline, a bacteriostatic antibiotic for which resistance does not inactivate the drug. We report that including the mode of antibiotic action and

mechanism of resistance in the model greatly improved its power to predict selection for resistance, including whether or not selection for resistance is frequency dependent. We extended the model to allow all combinations of mode of antibiotic action and mechanism of resistance and find that these properties interact: inactivation of bacteriostatic antibiotics was predicted to provide more protection to sensitive cells within a population than inactivation of bactericidal antibiotics.

3.3 Methods

3.3.1 *Theory*

The model presented here builds upon a within-host PK-PD framework, to explore the population dynamics of a mixed population containing both antibiotic sensitive and resistant bacteria (Jacobs et al., 2016). The model incorporates two different modes of antibiotic action, bacteriostatic which reduces the growth rate of cells, and bactericidal, killing cells within the model. The model also includes terms describing two different mechanisms of antibiotic resistance, active enzymatic inactivation of the antibiotic, reducing the environmental concentration of the antibiotic, and resistance whereby the concentration of antibiotic in the environment remains unchanged. The interactions between the different components of the model are summarised in Figure 3.1.

The model system tracks two sub-populations of bacteria: antibiotic sensitive plasmid free bacteria (S) and antibiotic resistant plasmid containing bacteria (R). Both stains are inhibited to a greater or lesser degree by the concentration of antibiotic within the system (A). The bacterial population dynamics when under antibiotic treatment, are described by the following set of differential equations:

$$\frac{dS}{dt} = \left[\alpha - \left(\frac{Kmax_{TS} \cdot A^m}{(KA_{50TS})^m + A^m}\right)\right] S \left(1 - \frac{S+R}{K}\right) - \left[\left(\frac{Kmax_{CS} \cdot A^n}{(KA_{50CS})^n + A^n}\right) S\right] \quad (1)$$

$$\frac{dR}{dt} = \left[\beta - \left(\frac{Kmax_{TR} \cdot A^m}{(KA_{50TR})^m + A^m}\right)\right] R \left(1 - \frac{S+R}{K}\right) - \left[\left(\frac{Kmax_{CR} \cdot A^n}{(KA_{50CR})^n + A^n}\right) R\right] \quad (2)$$

And the change in concentration of antibiotic within the system is given by the following:

$$\frac{dA}{dt} = -R\left(\frac{V_{max} \cdot A}{k_m + A}\right) \tag{3}$$

Equations (1-3) aim to understand the population dynamics within a microcosm, where nutrient availability is limited, resulting in the bacterial population reaching stationary phase within 24 hours. We therefore implemented growth of the two sub-populations as logistic terms with a combined carrying capacity K, with growth rates for S and R as α and β respectively. As carriage of resistance plasmid is often costly (Andersson and Levin, 1999; Baltrus, 2013; Bergstrom et al., 2000) we imposed a cost c to the growth rate of resistance cells so that $\beta = (1 - c)\alpha$.
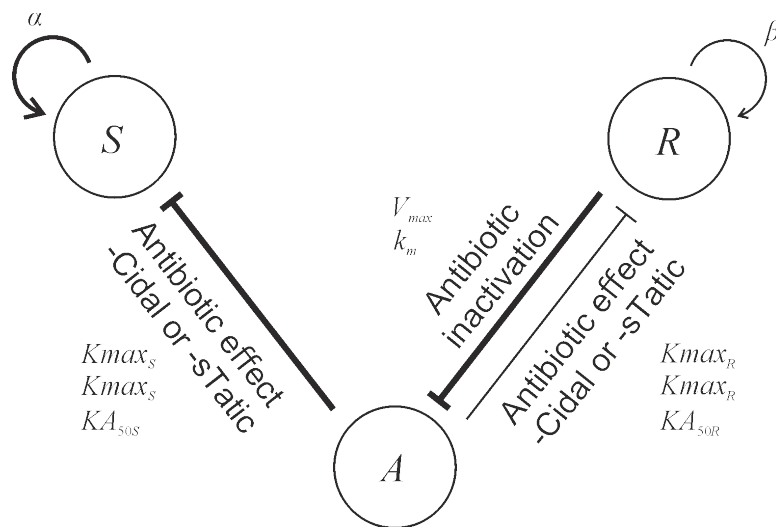
Figure 3.1 | Summary of interactions within the mass action model. Sub-populations of antibiotic-sensitive plasmid-free S and antibiotic-resistant plasmid-containing R compete, with growth limited by the combined carrying capacity K of the system. Both S and R are inhibited by the concentration of antibiotic A within the system but sensitive cells are affected to a greater extent. The antibiotic can either reduce the growth rates α and β, simulating a bacteriostatic, or directly remove cells from the model, simulating a bactericidal antibiotic. Resistance to the antibiotic is implemented through a higher KA_{50} than the sensitive population, with cooperative resistance also degrading the antibiotic within the system through a Michaelis-Menten kinetic term.

The affect of antibiotic upon bacterial growth depends upon the type of antibiotic administered. We implemented a bacteriostatic term in equations (1) and (2) as a Hill function reducing the growth rate of each bacteria type, $(Kmax_{Ti} \cdot A^m)/((KA_{50Ti})^m + A^m)$, dependent on $Kmax_{Ti}$ the maximum reduction in growth rate and KA_{50Ti} the concentration of antibiotic required to achieve 50% of $Kmax_{Ti}$ and the Hill coefficient m, for $i = S, R$. The distinguishing factor between resistance and sensitivity to the bacteriostatic antibiotic was KA_{50Ti}, we assume $KA_{50TR} > KA_{50TS}$ representing a lower probability of the antibiotic reaching its target in resistance cells. A bactericidal term was implemented as a killing rate, removing each bacteria type from the system according to the function $(Kmax_{Ci} \cdot A^n)/((KA_{50Ci})^n + A^n)i$, subject to a maximum kill rate $Kmax_{Ci}$, the concentration of antibiotic at which 50% of the maximum effect, KA_{50Ci}, and the Hill coefficient, n, for $i = S, R$. The killing rate was assumed to be equal between the sensitive and resistant populations, however the concentration of antibiotic which yielded 50% of $Kmax_{Ci}$ differed between the two strains, with $KA_{50CR} > KA_{50CS}$ (Jacobs et al., 2016).

Differentiating resistant and sensitive sub-populations purely through the parameters of the antibiotic terms as described above results in resistance that is completely privatised, i.e. the benefit of resistance is purely directed towards the resistant cells within the population. However, resistance mechanisms that inactivate the antibiotic change the local concentration of antibiotic, and therefore may alter the selection pressure upon resistant cells within a mixed population (Bottery et al., 2016; Livermore, 1997; Nikaido and Normark, 1987; Yurtsev et al., 2013). To model the inactivation antibiotic we implemented a Michaelis-Menten kinetic term with a per cell maximum achievable antibiotic degradation rate (V_{max}) and a Michaelis constant (k_m) describing the antibiotic concentration at which half of V_{max} is achieved.

The equations were solved numerically in R(3.3.3) using the LSODA method within the {*deSolve*} package (Soetaert et al., 2010). Relative fitness (W_R) of resistant population was calculated by direct comparison of the Malthusian growth parameters using the following equation (Lenski et al., 1991):

$$W_R = \frac{\ln\left(\frac{N_{Rt}}{N_{R0}}\right)}{\ln\left(\frac{N_{St}}{N_{S0}}\right)} \qquad (4)$$

Where N_{Rt} and N_{R0} are the population densities of resistance cells at time t and 0 respectively and, N_{St} and N_{S0} are the sensitive population densities at time point t and 0 respectively.

3.3.2 *Estimation of Model Parameters*

Each function within the model was parameterised independently using either *in vitro* observations, model fitting to previously acquired experimental data, or literature values. The logistic growth terms for sensitive bacteria were derived from growth experiments of *E. coli* in the absence of antibiotic, with the cost of harbouring the plasmid estimated through direct competition. The effect of each antibiotic upon the bacteria was parameterised through fitting to MIC curves. The inactivation rates of ampicillin by the RK2 plasmid encoded TEM-1 β-lactamase were predicted from literature values. Table 3.1 summarises the parameters used in the model.

The sensitive bacterial growth rate, α, was estimated from bacterial growth curves conducted in 96 well plates, 100ul cultures in Oxoid Nutrient Broth. The doubling time was estimated during logistic growth between T_0 = 120 minutes and T_t = 240 minutes post inoculation using the following formula: $g = 1/[(\log(N_t) - \log(N_0))/(\log 2 \cdot (T_t - T_0))]$ where N_0 is the optical density at T_0, N_t is the optical density at T_t. The fitness cost (c) of plasmid carriage is ~20% as measure through direct competition between isogenic plasmid free and plasmid containing bacteria in the absence of antibiotics, the growth rate β of resistant cells estimated as $(1 - c)\alpha$. The carrying capacity after 24 hours growth in 5ml nutrient broth is approximately 10^9 cells ml^{-1} when sensitive and resistant cells are grown in co-culture.

Table 3.1 | Parameter notation and estimated values used in mass action model. References: 1. Bottery et al. (2016), 2. Nikaido and Normark (1987), 3. Livermore (1997), 4. Suvorov et al. (2007).

Parameter	Description	Units		Value	Source
α	Growth rate of sensitive plasmid free bacteria	h^{-1}		1.25	This study
c	Fitness cost of resistance	-		0.2	1
β	Growth rate of resistant plasmid carrying bacteria, $(1-c)\alpha$	h^{-1}		1.0	
K	Carrying capacity	cells ml^{-1}		10^9	This study
$Kmax_{TS}$	Maximum growth reduction of sensitive bacteria, bacteriostatic	h^{-1}	Tet	1.25	This study
$Kmax_{TR}$	Maximum growth reduction of resistant bacteria, bacteriostatic	h^{-1}	Tet	1.0	This study
KA_{50TS}	Antibiotic concentration required for half reduction in growth rate of sensitive bacteria	$\mu g\ ml^{-1}$	Tet	0.08	Fitted
KA_{50TR}	Antibiotic concentration required for half reduction in growth rate of resistant bacteria	$\mu g\ ml^{-1}$	Tet	10.6	Fitted
m	Hill coefficient of antibiotic growth reduction term	-	Tet	1.0	Fitted
$Kmax_{CS}$	Maximum killing rate of sensitive bacteria, bactericidal	h^{-1}	Amp	1.5	Fitted
$Kmax_{CR}$	Maximum killing rate of resistant bacteria, bactericidal	h^{-1}	Amp	1.5	This study
KA_{50CS}	Antibiotic concentration require for half maximum death rate of sensitive bacteria	$\mu g\ ml^{-1}$	Amp	4.5	This study
KA_{50CR}	Antibiotic concentration require for half maximum death rate of resistant bacteria	$\mu g\ ml^{-1}$	Amp	3200	Fitted
n	Hill coefficient of antibiotic death term	-	Amp	1.6	Fitted
V_{max}	Maximum antibiotic inactivation rate	$\mu g\ h^{-1}\ cell^{-1}$	Amp	9.2×10^{-6}	2
			Tet	0	
k_m	Antibiotic concentration for half maximum inactivation rate	$\mu g\ ml^{-1}$	Amp	12.9	2, 3, 4
			Tet	0	

The antibiotic effect terms were parameterised against the MIC curves from Bottery et al. (2016). As ampicillin causes the lysis of cells, we assumed ampicillin to have a purely bactericidal effect in the model. Half the concentration of ampicillin required to reduce growth by half (KA_{50CS}) was estimated from the sensitive plasmid free MIC curve as 4.5 μg ml⁻¹. The maximum achievable death rate ($Kmax_{CS}$) and Hill coefficient (n) for sensitive cells exposed to ampicillin were estimated by fitting simulated death curves of sensitive cells grown in monoculture to the MIC data, while keeping the growth rate and carrying capacity fixed, along with inactivation rates at zero. As the ampicillin MIC of plasmid containing resistant cells is greater than 4 mg ml⁻¹, KA_{50CR} was parameterised as the minimum value required to achieve growth to within 10% of K at 4 mg ml⁻¹ ampicillin. Tetracycline binds to the 50S ribosomal subunit lowing the growth rate of bacteria; therefore we assumed tetracycline to have a purely bacteriostatic effect within the model. Unlike the bactericidal term, the KA_{50} within the bacteriostatic term cannot be directly estimated from the MIC curves of tetracycline, as the concentration required to reduce growth rate by half may not result in the population density reducing by half after 24 hours growth. $Kmax_{Ti}$ for each strain was assumed to be equal to the maximum growth rate of the bacteria. KA_{50Ti} and Hill coefficient (m) was estimated through fitting the simulated growth in monoculture to death curves keeping all other parameters static. Minimising RMSE and maximising R² determined best predictions for fitted values.

Parameter values for the hydrolysis of ampicillin where taken from literature estimations of plasmid encoded TEM-1 β-lactamase. The typical K_{cat} value associated with the inactivation of ampicillin by a single TEM-1 molecule is ~1000 s⁻¹ (Nikaido and Normark, 1987; Stojanoski et al., 2015; Zimmermann and Rosselet, 1977) with a k_m value of 12.9 μg ml⁻¹ (Nikaido and Normark, 1987). To estimate V_{max} per cell, an estimate of the number of β-lactamase molecules per cell is required; a previous study estimated that a high copy number plasmid with a MIC of 16 mg ml⁻¹ had ~7300 TEM-1 molecules per cell, whereas a low copy number plasmid with an MIC of 4 mg ml⁻¹ produced five times less TEM-1 molecules (Suvorov et al., 2007). The RK2 plasmid provides sufficient resistant to survive at 4 mg ml⁻¹ of ampicillin, having an MIC of approximately 9 mg ml⁻¹ (Fang and Helinski, 1991), therefore an intermediate value of ~4400 TEM molecules per cell was estimated, resulting in a V_{max} of

9.2×10^{-6} µg h^{-1} cell^{-1}. The starting conditions of simulations were chosen to reflect laboratory competition experiments, within an initial total cell density of 5×10^5 cell ml^{-1}. Models were iterated for a simulated 24 hours. Antibiotic was applied in a single dose at the start of the simulation and is simulated to be homogenously distributed throughout the environment.

Using the fitted parameter values, the concentration of antibiotic required to select for resistance was predicted. Simulations were initiated with a 50:50 co-culture of both sensitive and resistant sub-populations with a single dose of antibiotic. Mathematical solutions to the models were calculated over 24 hrs of growth, and the final density of both sub-populations were use to calculate the relative fitness of the resistant population using equation (4). This was conducted over a range of antibiotic concentrations to produce fitness reaction norms (Figure 3.2). These predicted fitness reaction norms were then compared to previously published, experimentally derived fitness reaction norms (Bottery et al., 2016). To account for the different MICs of the two antibiotics we examined the impact of antibiotic concentrations as percentage of sensitive bacteria's MIC (% of S MIC).

3.4 Results

3.4.1 *Model fitting*

The parameters of the death terms were fitted to previously published MIC data (Bottery et al., 2016). Tetracycline was modelled to have a purely bacteriostatic effect; lowering the growth rate of cells. As the sufficient quantities of tetracycline were able to reduce all growth of both resistant and sensitive cells to zero (Figure S3.1 A/B), $Kmax_{Ti}$ was parameterised as the maximum achievable growth rate for each strain. Values for KA_{50Ti} were predicted through fitting to MIC curves (Figure S3.1A/B), the fit describes the MIC of resistant monoculture well, with a R^2 value of 0.99 and RMSE of 0.03. However, the fit for sensitive cells over estimated the concentration of tetracycline required to have an effect on the population density, producing a poorer fit with a R^2 value of 0.81 and RMSE of 0.12.

The parameter fit to the ampicillin sensitive MIC curve using a death term results in a high precision fit with a R^2 value of 0.98 and RMSE of 0.04 (Figure S3.1 C). As the MIC of resistant cells was not determined it was not possible to fit the model to this curve (Figure S3.1 D), therefore the death rate was assumed to be equal to the parameterised value for sensitive cells under ampicillin treatment and the KA_{50CR} term was estimated as a value that provides growth at 4 mg ml^{-1} of ampicillin. With these values of $Kmax_{CR}$ and KA_{50CR} along with the ampicillin inactivation rate estimated form literature values, the resistant cells were predicted to have a MIC of 4800 µg ml^{-1}. This is likely an underestimate of the level of resistance provide by the RK2 plasmid (Fang and Helinski, 1991), however the value of KA_{50CR} had very little effect on the qualitative output of the mathematical model. β-lactamase accumulates within the periplasmic space of gram-negative bacteria (Bowden and Georgiou, 1990; Livermore, 1995); although some extracellular release may occur due to leakage, degradation of the antibiotic mostly occurs locally to the resistant cell and resistance is still primarily directed towards the resistant population. Therefore, when the mechanism of resistance is through the inactivation of antibiotic, we assume KA_{50CR} remains greater than KA_{50CS}, reflecting the decreased chance of the antibiotic molecule reaching its target. This acts to partly privatise the resistance to the resistant population, without adding further spatial complexity e.g. diffusion across the cell wall to the model. Without this privatisation ($KA_{50CR} = KA_{50CR}$), inactivation of ampicillin alone at the parameterised rates would provide resistance to 46 µg ml^{-1} ampicillin. Under the assumption of $KA_{50CR} = KA_{50CR}$, resistance would be completely communal, providing equal benefit to both resistant and sensitive bacterial sub-populations.

3.4.2 *Antibiotic Resistance Selection Reaction Norms*
Tetracycline resistance is modelled purely with an increased KA_{50CR} reflecting the complete privatisation of resistance encoded via an efflux pump on the RK2 plasmid. This results in the bacterial population having no influence on the concentration of antibiotic within the system. Whereas, ampicillin resistance also included an inactivation term, reducing the global concentration of antibiotic based on the population size of R. The difference in resistance mechanism resulted in different relative concentrations of antibiotic being required to select for resistance (Figure 3.2). The model accurately predicted the fitness reaction norm when the RK2 plasmid was under tetracycline selection with a R^2 fit of

0.92. Just 1% of the sensitive MIC of tetracycline (20 ng ml^{-1}) was required to select for resistance.
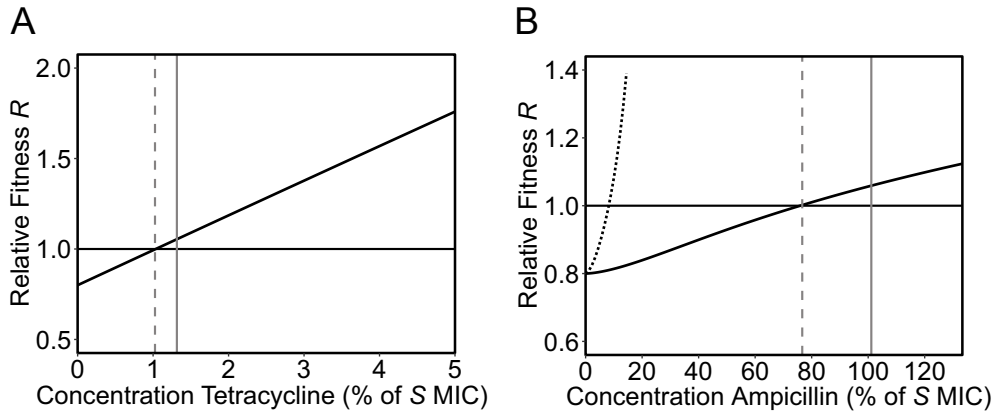

Figure 3.2 | Simulated relative fitness of plasmid carriage, calculated from the numerical solution to models (1)-(3), of resistant population (R) after 24 hr growth in competition with sensitive bacteria (S) starting at equal densities. Simulations were initiated with increasing antibiotic (A) concentrations of **A** tetracycline or **B** ampicillin. Dotted line in panel **B** represents simulated relative fitness of R when there is no cooperative inactivation of antibiotic by the R sub-population ($V_{max} = 0$). Concentration of antibiotic is labelled as a percentage of S MIC (tetracycline 2 μg ml^{-1} and ampicillin 9 μg ml^{-1}). Horizontal black line represents equal fitness between R and S (relative fitness = 1), vertical dashed lines show the models prediction of the intercept of 1, and the vertical solid line shows the experimentally determined intercept of 1.

The models fit to the ampicillin fitness reaction norm was less accurate, it did not capture the plateau in plasmid cost at low concentrations of ampicillin observed in the experimental data, and underestimated the concentration of ampicillin required to select for resistance, resulting in a R^2 of 0.67. This less accurate fit reflects the increased complexity of the ampicillin model, and may be due to inaccurate parameterisation of either the inactivation rate of ampicillin, or the parameterised death curve not reflecting the true dynamics in co-culture. An inactivation rate of 1.52 × 10^5 (approximately 7300 beta-lactamase molecules per cell, similar to high copy number TEM-1 encoding plasmids (Suvorov et al., 2007)) was required to produce the same relative fitness intercept of 1 as the experimental data, but the shape of the curve did not reflect the data, having an poorer R^2 fit of 0.58. Increasing the Hill coefficient while maintaining the same

MIC ($KA_{50CS} = 6.2$, $Kmax_{CS} = 1.6$, $n = 6$) produced an improved R^2 fit of 0.82 (Figure 3.3). However, the originally parameterised model still predicts a much higher relative concentration of ampicillin is required to select for resistance, 77% of the sensitive MIC (6.9 µg ml^{-1}), with co-culture allowing positive growth of sensitive cells beyond their MIC. When the inactivation rate is set to zero within the model, the concentration of ampicillin required to select for resistance is reduced to 8% of the sensitive MIC, confirming that the inactivation of ampicillin is providing the protection to sensitive bacteria (Figure 3.2 B).
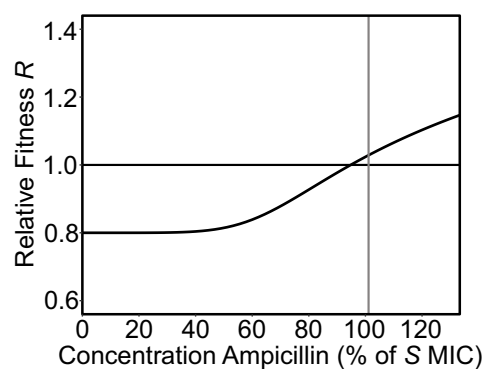

Figure 3.3 | Best simulated fit to experimental relative fitness of R when in competition with S across ampicillin concentration gradient. Horizontal black line represents equal fitness between R and S, vertical solid line shows the experimentally observed intercept of 1. Model parameters: $KA_{50CS} = 6.2$, $Kmax_{CS} = 1.6$, $n = 6$.

Treatment with tetracycline provides a constant positive selective pressure for resistance throughout logistic growth (Figure 3.4A), as the environmental concentration of antibiotic remains unchanged (Figure 3.4B). Tetracycline has no effect after the population reaches K, resulting in a linear fitness reaction norm with very low levels of antibiotic required to overcome the cost of resistance. In contrast, the population dynamics resulting from ampicillin selection are more complex. Upon inoculation there is strong selection for resistance while the concentration of antibiotic is high (Figure 3.4C). This increases the frequency of resistance within the population, but also increases the effective inactivation rate of ampicillin, resulting in a lower environmental concentration of antibiotic (Figure 3.4D). This phenomenon scales with the initial concentration of antibiotic, meaning that the higher the initial concentration of

ampicillin, the stronger the selection for resistance, resulting in faster inactivation of the antibiotic. The reduction in the environmental concentration of ampicillin allows the reinvasion of sensitive cells back into the population. However, as ampicillin is a bactericidal, reinvasion can only occur so long as the initial transient selection is not strong enough to cause the extinction of the sensitive sub-population. Reinvasion is also limited by the rate of inactivation of the antibiotic, and can only occur when ampicillin is inactivated to sufficient levels before the population reaches K.
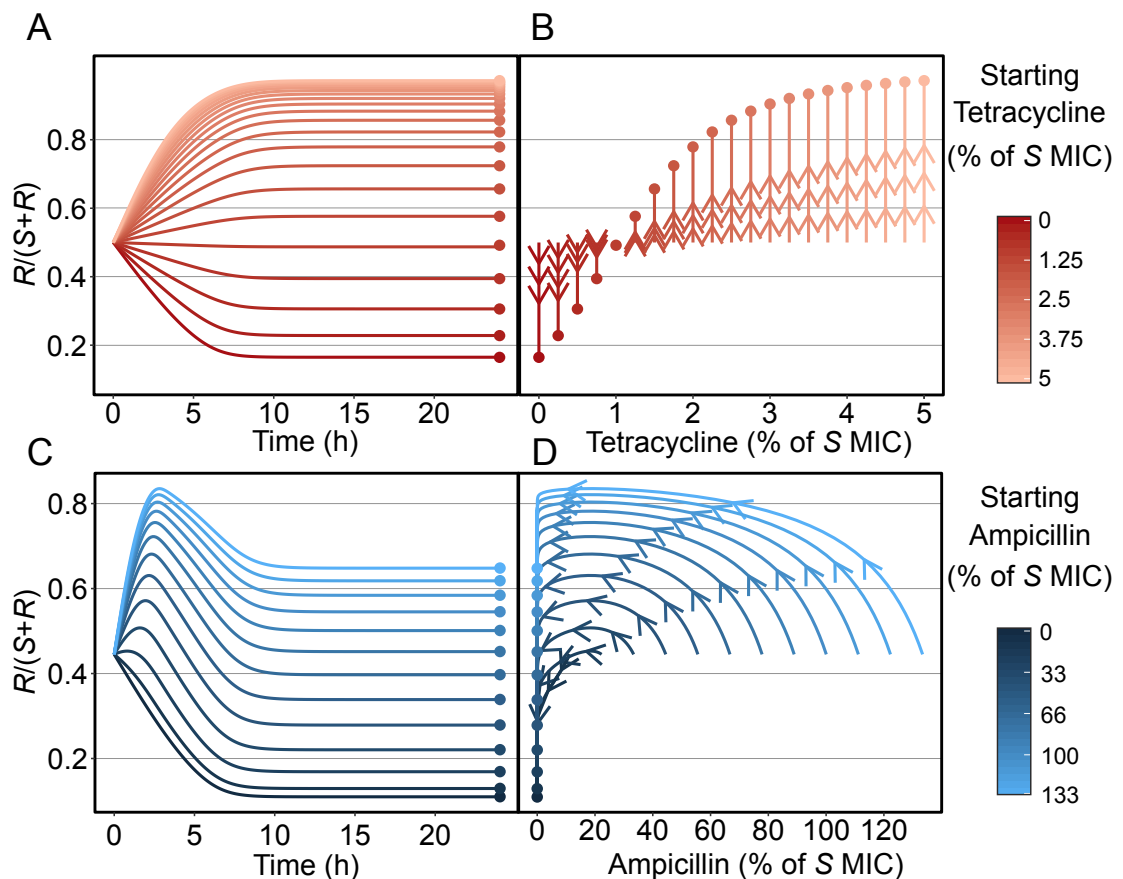
Figure 3.4 | Simulated population dynamics predicted from numerical solutions of models (1) – (3) presented in Figure 3.3. Panels **A** and **C** show change frequency of R through time in response to antibiotic selection. Panels **B** and **D** show change in frequency of R plotted against antibiotic concentration. Arrows are located every hour for the first three hours, indicating the passage of time for each simulation. Line colour represents the starting concentration of antibiotic used within each simulation. Points show the final frequency of R at t = 24 hr.

To test the predictive power of the model we went on to predict the fitness surface in response to co-treatment with both ampicillin and tetracycline. The model confirms the empirical results found by Bottery et al. (2016) showing treatment with tetracycline can mask the benefits of the social resistance mechanism, causing lower concentration of both ampicillin and tetracycline to select for resistance (Figure 3.5).
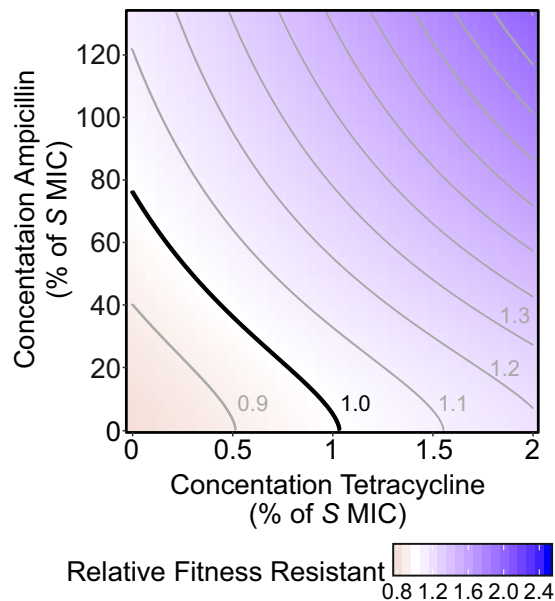
Figure 3.5 | Simulated relative fitness of R under combinatorial selection with both ampicillin and tetracycline. Black line shows intercept of 1 (equal fitness). Relative fitness of 0.9, 1.1, 1.2 and 1.3 labelled in grey.

3.4.3 *Frequency dependence*

It is expected that the relative fitness of non-cooperating cheaters within a microbial population will be greater when rare (Dugatkin et al., 2005; Ellis et al., 2007; Ross-Gillespie et al., 2007). Experimental data shows that selection for ampicillin resistance mediated by a β-lactamase is negatively frequency dependent (Chapter 2, section 2.4.3, Figure 2.4). In contrast, selection for tetracycline resistance encoded by an efflux pump is not frequency dependent (Chapter 2, section 2.4.3, Figure 2.4).

Consistent with previous findings, the model predicts that selection for tetracycline resistance via efflux is not frequency dependent (Figure 3.6B). The

concentration of tetracycline required to select for resistance remained at 1% of the sensitive MIC, independent of the initial frequency of resistant cells within the population. In contrast, the selection for ampicillin resistance showed negative frequency dependence (Figure 3.6A). When resistance is rare within a population approximately 25% of the sensitive MIC is required to select for resistance, whereas, then sensitive cells are rare over 100% of the sensitive MIC is required to select for resistance. The survival of sensitive cells is dependent upon the frequency of resistant cells within the population when under ampicillin treatment. When resistance is rare, the effective inactivation rate is low, resulting in more continuous selection for resistance. The extent that frequency alters the selection conditions may be dependent upon the localisation of the benefit produced; it is expected that frequency dependence would be favoured when the public good is preferentially directed towards the co-operators (Ross-Gillespie et al., 2007). In the case of ampicillin resistance, preferential access to resistance is provided by the localisation of the β-lactamase in the periplasm of resistant cells, is reflected in the model by partial privatisation of resistance ($KA_{50CR} > KA_{50CR}$).
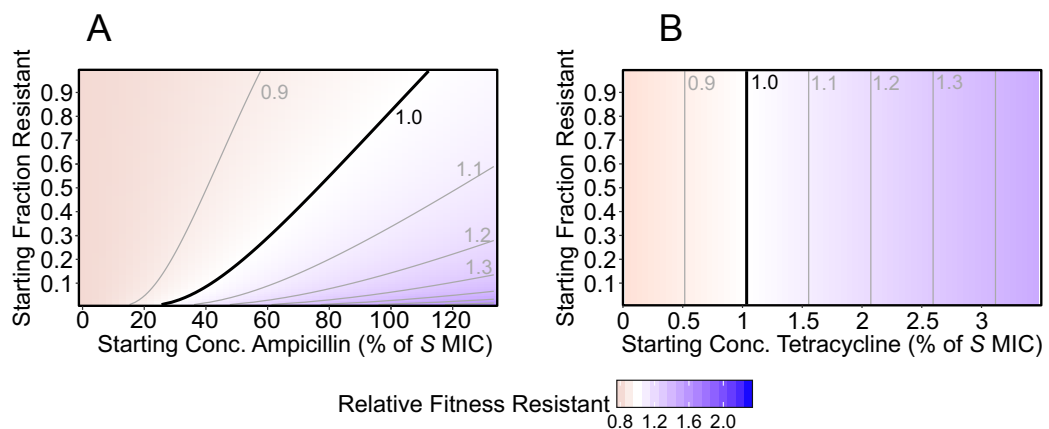

Figure 3.6 | Simulated relative fitness of R with varying antibiotic concentration (**A** ampicillin and **B** tetracycline) and starting frequency of R (starting R from 0.01 to 0.99). Black line shows intercept of 1 (equal fitness). Relative fitness of 0.9, 1.1, 1.2 and 1.3 labelled in grey.

3.4.4 *Predicting the interaction between mode of antibiotic action and mechanism of resistance*

The results show that inclusion of the mechanism of resistance, whether the antibiotic is inactivated by the resistant population or not, is required to accurately predict the selection of resistance. We now examine the potential interaction between the mechanisms of resistance and the mode of antibiotic action. By determining the fitness landscape of resistance across hypothetical inactivation rates for ampicillin and tetracycline we can predict how the mode of action of the antibiotic affects the selection of resistance. Figure 3.7 shows the effect of varying inactivation rate of ampicillin on the relative fitness of the resistance sub-population. A higher inactivation rate results in higher concentrations of ampicillin required to select for resistance, but this effect becomes saturated, with less gain as the inactivation rate increases. However, the dynamics of selection with cooperative inactivation of a bacteriostatic, such as tetracycline, differ greatly from those of the bactericidal (Figure 3.8). Increasing the inactivation rate of tetracycline provides an increasing benefit for sensitive cells. Cooperative resistance may therefore be more beneficial when the antibiotic target is a bacteriostatic rather than a bactericidal.
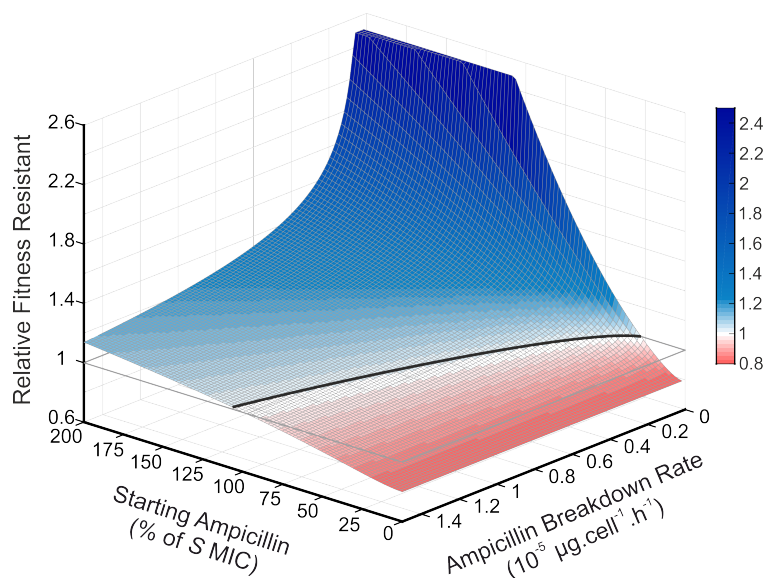

Figure 3.7 | Simulated relative fitness of R with varying ampicillin concentration and inactivation rate. $V_{max} = 0$ Is omitted from plot and is presented in Figure 3.4B. Black line shows intercept of 1 (equal fitness).
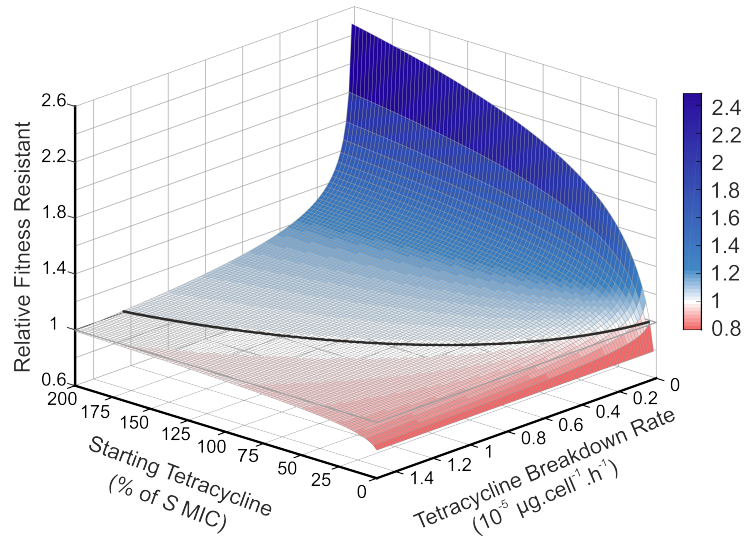
Figure 3.8 | Simulated relative fitness of R with varying tetracycline concentration and inactivation rate. $V_{max} = 0$ Is omitted from plot and is presented in Figure 3.3A. Black line shows intercept of 1 (equal fitness).

3.5 Discussion

PK-PD models are commonly used to predict the outcome of drug treatments in order to optimise the clinical effectiveness of antibiotic regimens (Chung et al., 2006; Czock and Keller, 2007; Mouton et al., 1997). These models often have simple approximations of mechanism of resistance and action of antibiotics. However, recent studies have shown that biological details, such as the mechanism of resistance and mode of action of antibiotic, can alter the selective conditions of resistance (Bottery et al., 2016; Sorg et al., 2016; Yurtsev et al., 2013), aspects which are often overlooked in PK-PD models. Here we show that, without an accurate representation of these details of antibiotic resistance, the effectiveness of PK-PD models, when used to predict the evolution of resistance, is limited.

We presented a model of bacterial population dynamics that incorporated terms describing both the mechanism of resistance and mode of action of antibiotics, allowing the distinction of bacteriostatic and bactericidal antibiotics as well as resistance mechanisms that do and don't inactivate antibiotics. Through the inclusion of these terms, our models can more accurately predict the antibiotic concentrations required to select for resistance. Firstly, the inactivation of

antibiotics by a resistant sub-population can allow sensitive cells to survive at concentrations of antibiotic alone would usually kill them. These results can only be reliably replicated through the inclusion of antibiotic inactivation terms within a model, as the rapid inactivation of antibiotic within an environment by resistant cells is the driving factor shaping the selection of resistance. Secondly, the model accurately predicts that the mechanism of resistance alters the frequency dependence of selection. The predictions of the model are consistent with experimental data showing that the selection for bacteria harbouring resistance mechanisms that do not alter the environmental concentration of antibiotic, such as efflux pumps, is independent from the frequency of resistant bacteria within the population. Whereas, resistances that inactivate antibiotics, such as β-lactamases, are negative frequency dependent; i.e. selection for resistant bacteria is stronger when they are rare.

Given the models strong predictive power of the social interaction mediated by antibiotic resistance mechanism, we sought to explore the theoretical interaction between mode of antibiotic action and mechanism of resistance. The model predicts that these properties interact; whereby, inactivation of bacteriostatic antibiotics by resistant bacteria provides greater protection to sensitive bacteria within a population than the inactivation of bactericidal antibiotics. As bacteriostatic antibiotics simply reduce the growth rate of sensitive cells, they are initially out grown by the resistant population rather than being actively killed as would be the case when under selection by a bactericidal antibiotic. This results in a more effective reinvasion of sensitive cells once the antibiotic it is cleared from the environment. Thus, the model provides a testable hypothesis that mode of action of an antibiotic influences the invasion of resistance within bacterial populations, and may help to explain the rarity of resistance mechanisms that inactivate tetracycline (Forsberg et al., 2015).

The proposed model has limitations, the effect of antibiotics upon bacteria are not purely bacteriostatic, or bactericidal, and are often weighted combinations of the two or concentration dependent. More accurate parameterisation of the weightings of a specific antibiotic towards the –static or –cidal terms can be achieved by fitting to time course data. However, data presented here suggests that fitting pure –static or –cidal terms to MIC curves provide an adequate approximation allowing the accurate prediction of social dynamics upon the selection of resistance. We use tetracycline and ampicillin ARGs as examples,

but the model in not specific to these antibiotics or resistance mechanisms, and can be applied to any antibiotic. Despite these limitations, our model shows that a systematic understanding of the nature of the action of existing antibiotics and their resistance mechanisms is needed to permit reliable mathematical modelling of the impact of AMR.

The model highlights that the mode of antibiotic action and resistance can have major implications on the evolutionary and ecological outcomes of antibiotic selection. To accurately predict the selection and maintenance of resistance within a bacterial population, whether encoded on a MDR plasmid or chromosomally, a clear understanding of the sociality of resistance and impact of antibiotic mode of action are required.

4. Adaptive Modulation of Antibiotic Resistance Through Intragenomic Coevolution

4.1 Abstract

Bacteria gain antibiotic resistance genes by horizontal acquisition of mobile genetic elements (MGE) from other lineages. Newly acquired MGEs are often poorly adapted causing intragenomic conflicts, resolved by compensatory adaptation of the chromosome, the MGE or reciprocal coadaptation. The footprints of such intragenomic coevolution are present in bacterial genomes, suggesting an important role promoting genomic integration of horizontally acquired genes, but direct experimental evidence of the process is limited. Here we show adaptive modulation of tetracycline resistance via intragenomic coevolution between *Escherichia coli* and the multi-drug resistant (MDR) plasmid RK2. Tetracycline treatments, including monotherapy or combination therapies with ampicillin, favoured *de novo* chromosomal resistance mutations coupled with mutations on RK2 impairing the plasmid-encoded tetracycline efflux-pump. These mutations together provided increased tetracycline resistance at reduced cost. Additionally, the chromosomal resistance mutations conferred cross-resistance to chloramphenicol. Reciprocal coadaptation was not observed under ampicillin-only or no antibiotic selection. Intragenomic coevolution can create genomes comprised of multiple replicons that together provide high-level, low-cost resistance, but the resulting co-dependence may limit the spread of co-adapted MGEs to other lineages.

4.2 Introduction

Horizontal gene transfer (HGT) is a fundamental process in bacterial evolution that accelerates adaptation by sharing ecologically important accessory traits

between lineages (Jain et al., 2003). These accessory traits are themselves frequently located on semi-autonomous mobile genetic elements (MGE), such as conjugative plasmids, that encode genes for their own replication, partition and horizontal transfer (Frost et al., 2005; Norman et al., 2009). Conjugative multidrug resistance (MDR) plasmids, encoding antibiotic resistance genes (ARGs) against multiple classes of antibiotics, are of particular clinical concern since they allow instantaneous acquisition of MDR phenotypes and thus potentiate the rapid emergence of MDR bacterial pathogens (Carattoli, 2013; Svara and Rankin, 2011). Newly acquired conjugative plasmids are often costly since the plasmid is unlikely to be well adapted to the new genetic background, causing intragenomic conflict (Baltrus, 2013). The cost of plasmid carriage is likely to arise due to the metabolic burden of maintaining, transcribing and translating plasmid genes (Diaz Ricci and Hernández, 2000), as well as via disruption of cellular homeostasis caused by gene regulatory interference between chromosomal and plasmid regulators (Harrison et al., 2015; San Millan et al., 2015), and cytotoxic effects of plasmid gene products (Baltrus, 2013).

An important route to resolving this intragenomic conflict is compensatory evolution to ameliorate the cost of plasmid acquisition (Harrison and Brockhurst, 2012). Experimental evolution suggests that compensatory evolution can arise via mutations located on either the chromosome or the plasmid, or via intragenomic coevolution involving both plasmid and chromosome mutations (Harrison et al., 2015; Loftie-Eaton et al., 2016; Porse et al., 2016; San Millan et al., 2015). Comparative genomics suggests a key role for compensatory evolution in natural bacterial populations, potentially stabilising MDR plasmids within lineages and thus allowing the evolutionary emergence by HGT of MDR pathogens (McNally et al., 2016). A key outstanding question is how the mode of compensatory evolution following MDR plasmid acquisition varies with antibiotic treatment. Here we experimentally evolved *Escherichia coli* MG1655 carrying the MDR plasmid RK2 (encoding tetracycline and ampicillin resistance genes) under a range of antibiotic treatment regimes including no antibiotic, mono- and combination-therapies of tetracycline and ampicillin. Following 530 generations of selection we quantified evolved changes in antibiotic resistance and fitness, and used genome sequencing to determine the genetic bases of the observed adaptation.

4.3 Methods

4.3.1 *Strains, culture conditions and evolution experiment*

E. coli MG1655 chromosomally labelled with GFP at the *attB* lambda attachment
site was used in the evolution experiments. Isogenic *E. coli* MG1655-mCherry
was used as a reference strain in competition experiments. Both *E. coli* strains
were provided by the Van Der Woude lab (University of York). The RK2 plasmid
was introduced to the strains through conjugation from *E. coli* MV10 provided by
the Thomas lab (University of Birmingham). All cultures were grown in Oxoid®
Nutrient Broth (NB) at 37°C 5 ml in 50 ml microcosms shaken at 180 rpm.
Independent selection lines were founded by 30 independent single colonies of
E. coli MG1655-GFP harbouring RK2. These were grown overnight in non-
selective conditions and split into the 5 antibiotic treatments, no antibiotic
selection, 100 µg ml^{-1} ampicillin, 10 µg ml^{-1} tetracycline, 100 µg ml^{-1} ampicillin
plus 10 µg ml^{-1} tetracycline, and 24 hour cycling between 100 µg ml^{-1} ampicillin
and 10 µg ml^{-1} tetracycline, with 6 replicate populations per treatment. In
parallel, 6 independent *E. coli* MG1655-GFP colonies were picked for control
treatments and grown under no selection. Selection lines were established by
transferring 50 µl of saturated overnight culture into 5 ml of selective media.
These populations were maintained through transfer of 1% of the population into
fresh media and antibiotics every 24 hours for 80 transfers, resulting in ~6.64
generations per day, totalling ~530 bacterial generations. For the cycling
treatment 3 populations were initiated with 100 µg ml^{-1} ampicillin and 3
populations were initiated with 10 µg ml^{-1} tetracycline. Culture density (OD$_{600}$)
was recorded every 24 hours. Plasmid prevalence was measure at the start and
end of the selection experiment by screening 20 randomly picked colonies from
each population using multiplex primers specific to RK2 replication origin (Fw:
ctcatctgtcaacgccgc, Rv: aaccggctatgtcgtgct), β-lactamase (Fw:
ataactacgatacgggagggc, Rv: acatttccgtgtcgccctta), and tetracycline efflux pump
(Fw: tgggttctctatatcgggcg, Rv: tgggcgagtgaatgcagaat). These primers allowed
for the detection of plasmid loss and transposition of resistances onto the
chromosome. One end point clone was randomly selected from each population
for phenotypic typing, curing, calculation of MICs and sequencing. Every eight
transfers throughout the experiment 500 µl samples of whole populations were
collected and stored in 25% glycerol at -80°C. Whole populations were also
plated out on non-selective media, 20 individual clones were then randomly

selected, sub-cultured for a further 24 hours in non-selective media, and stored in 25% glycerol in 96 well plates.

4.3.2 *Relative Fitness*

The relative fitness of the evolved plasmid bearing versus ancestral plasmid free strain was estimated by direct competition, with six replicate strains per-treatment. The competitions were initiated with 50 µl of 1:1 mixtures of plasmid-bearing evolved strain and plasmid-bearing ancestral strain marked with mCherry from overnight cultures in 5 ml of non-selective NB media. The relative fitness of the evolved strains was calculated by gaining exact viable cell counts at 0 hours and 24 hours, strains were distinguished through detection of fluorescent markers using Zeis Stereo Lumar v12 microscope. The relative fitness of plasmid-bearing bacteria was calculated as a ratio of Malthusian parameters (Crozat et al., 2005):

$$W_{evo} = \frac{\ln\left(\frac{N_{final,evo}}{N_{inital,evo}}\right)}{\ln\left(\frac{N_{finial,anc}}{N_{initial,anc}}\right)}$$

Fitness effects due to different markers was determined by competing plasmid free MG1655-GFP with plasmid free MG1655-mCherry, the relative fitness of MG1655-GFP was not significantly difference from 1 (t_5=0.015584, p=0.9882) showing that there is no significant difference between the two marker strains.

Relative fitness of evolved strains harbouring evolved plasmid or evolved strains harbouring ancestral plasmid versus ancestral plasmid bearing cells was estimated using the same method as above, with eighteen replicate strains per competition, but grown in 100 µl cultures in a 96 well plate, 37°C shaken at 600 rpm, 3 mm orbital radius, inoculated to an initial dilution of 1:500. Again no fitness effect of markers was observed (t_5=-0.2795, p=0.791).

4.3.3 *Curing RK2 from evolved strains*

Evolved strains were cured using the pCURE curing system (Hale et al., 2010). The anti-incP-1 cassette (RK2 *oriV*, *parD*, *korA*, and *incC* genes) from pCURE11 was ligated into the pLAZ2 chloramphenicol resistant vector that contains the *sacB* gene allowing counter selection for plasmid free segregants. The resultant plasmid was transformed into chemically competent evolved

strains and selected for using Cml 12.5 µg ml^{-1}. Single colony transformants were re-streaked on to Cml 12.5 µg ml^{-1} plates and Cml 12.5 µg ml^{-1} + 5% sucrose. Sucrose sensitive colonies were checked by PCR for the presence of the curing plasmid (Fw: aagttttggtgactgcgctc, Rv: caaagacgatgtggtagccg) and absence of RK2 β-lactamase and *tetA* (primers as above). Successfully cured clones were cultured for 24 hours in non-selective media to allow segregation of the curing plasmid; sergeants were selected on antibiotic free, 5% sucrose plates. To confirm loss of both plasmids sucrose resistant colonies were checked for sensitivity to chloramphenicol, ampicillin, and tetracycline, as well as PCR using primers mentioned above. Both the ancestral strain harbouring RK2 and ancestral plasmid free strains under went the curing process and were used as a comparison to cured evolved strains to control for curing process. Ancestral RK2 was introduced into the cured evolved strains, and evolved RK2 was introduced into the plasmid free ancestor through conjugation. Again, to control for the curing and conjugation steps, ancestral RK2 was conjugated into cured ancestral strains and used for comparison. Saturated overnight cultures of donor plasmid containing strains and recipient plasmid free strains were mixed 1:1, and 50 µl was used to inoculate 5 ml NB. The mixed cultures were grown for 24 hours and plated out on to 100 µg ml^{-1} ampicillin to select for transconjugants. Transconjugants were confirmed by fluoresces and PCR screening for RK2 plasmid.

4.3.4 *Minimum inhibitory concentration*

To measure minimal inhibitory concentrations, six replicate cultures per-treatment were grown overnight until stationary phase in 5 ml NB, the saturated cultures were then sub-cultured 50 µl into 5 ml fresh NB and grown to an OD$_{600}$ of 0.5. These were then diluted into 96-well plates containing a log$_2$ serial dilution of antibiotic (AMP, TET or CML) to an initial density of 5×10^5 CFU ml^{-1}. 100 µl cultures were grown for 24 hours 37°C shaken at 600 rpm, 3 mm orbital radius. OD$_{600}$ was measured after 24 hours.

4.3.5 *Growth Curves*

Six replicate saturated overnight cultures per-treatment were sub-cultured to an OD$_{600}$ of 0.5, and used to inoculate 100 µl NB supplemented with 10 µg ml^{-1} TET per well in 96-well plates at a final dilution of 1:1000. Plates were grown at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, OD$_{600}$ was measured

every 16 minutes by Tecan infinite M200 Pro plate reader. Growth rates were calculated as the maximum slope of $\log_2$ transformed OD_{600} covering four time points (~1 hour of growth), lag phase was calculated to end when growth rate reached 10% of the maximum achieved growth rate.

4.3.6 *Genome sequencing and analysis*

Whole genomes were extracted from each evolved population's clone as well as the ancestral strain and ancestral strain harbouring the RK2 plasmid using the DNeasy Blood and Tissue extraction kit (Qiagen). The total DNA was sequenced by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1), using Illumnia MiSeq. Reads were mapped to *E. coli* MG1655 K-12 genome (GenBank accession U00096.3) and RK2 (GenBank accession BN000925.1) reference using BWA-MEM (Li and Durbin, 2009). Single nucleotide variants and small indel events were detected using GATK UnifiedGenotyper (McKenna et al., 2010) and SnpEff (Cingolani et al., 2012), insertion sequences were identified using custom scripts and Integrative Genomics Viewer (Robinson et al., 2011), and large genome-wide structural variants were detected using BreakDancer (Chen et al., 2009). Mutations that were present in the ancestral clones were excluded, resulting in a set of mutations that were acquired during the selection experiment.

4.3.7 *Tracking mutations*

Populations that did not show a hypermutator phenotype, had insertion sequences within *ompF,* and mutations in the tetracycline resistance genes on the plasmid, from the constant TET treatments (T and AT treatments) were selected for further analysis to gain an understanding of the mutational timeline during the selection experiment. Insertion sequences within *ompF* were identified within whole populations of T4, AT2, AT3 and AT5 by PCR of the *ompF* gene (Fw: ACTTCAGACCAGTAGCCCAC, Rv: GCGCAATATTCTGGCAGTGA). A short product of 716 bp indicated no insertion sequence, a long product of 1484 bp indicted IS1 and a long product of 1911 bp indicted IS5. Whole population PCR indicated that *ompF*::IS mutants had swept into the population by transfer 40 for populations T4, AT2 and AT5, and transfer 48 in population AT3. Frequency of *ompF* insertion sequences were calculated by PCR of 20 clones from transfers 8, 16, 24, 32, and 40. Tetracycline resistance genes (*tetA* and *tetR*) from clones containing *ompF*::IS

mutations from population AT2, transfers 8, 16, 24, and 32 were then Sanger sequenced to determine if *ompF* mutations arise before *tetAR* mutations (*tetA*: Fw: GGCTGCAACTTTGTCATG, Rv: TTCCAACCGCACTCCTAG, Internal1: ACAGCGCCTTTCCTTTG, Interal2: AAGGCAAGCAGGATGTAG; *tetR*: Fw: TCTGACGCGGTGGAAAG, Rv: ACGCGCGGATTCTTTATC, Internal1: GAGCCTGTTCAACGGTG, Internal2: TCTGACGACACGCAAAC).

4.3.8 *Statistical analysis*

To test if the mutations observed within each treatment had significantly different variances a multivariate homogeneity of groups variances test was conducted (Anderson, 2006). The binary presence or absence of a variant at each allele was use to calculate a Euclidean distance matrix between each population. This was used to test for homogeneity of variances between treatments using *betadisper* {vegan 2.4-0}. The variances between treatments were significantly different, with hypermutators significantly affecting within-group variation. These clones were removed from further analysis as significant differences in within-group variance can lead to falsely significant results when testing for differences between groups (Anderson, 2001). Permutational Multivariate Analysis of Variance was used to calculate whether different evolutionary treatments resulted in different sets of mutations (Anderson, 2001; Zapala and Schork, 2006). Using the Euclidean distance matrix with hypermutators removed, the significance of within- and between-group distances was calculated using *adonis2* {vegan 2.4-0}. The data was partitioned into different groups, multiple testing was corrected for using Bonferroni correction. Neighbour Joining phylogeny was constructed using the binary presence or absence table with hypermutators removed. Tree estimation and bootstrap support was conducted using *ape-package* {ape 4.0}. Significant difference between two related samples was calculated using two sided, two-sample t-test. Shapiro-Wilk test was conducted to check for normality, when normality could not be assumed a non-parametric Wilcoxon signed-rank test was used. Differences among treatments growth under antibiotic selection were calculated by ANOVA of the integral of the resistance profiles, with subsequent Tukey multiple comparison of means. All statistical analysis was conducted in R (version 3.2.3).

4.3.9 *Data Availability*

The sequence data supporting the findings of this study are available at the European Nucleotide Archive, accession: PRJEB20735. All other data in this study is available at Figshare data depository (https://doi.org/10.6084/m9.figshare.5092225.v1). Custom code used to map possible IS elements are available online (https://github.com/mbottery/Co_Evo_IS_Analysis).

4.4 Results

Thirty independent isogenic populations of *E. coli* MG1655 carrying the MDR plasmid RK2 (Pansegrau et al., 1994), which encodes resistances to tetracycline (TET) and ampicillin (AMP), were experimentally evolved for ~530 generations (80 days), under five antibiotic treatments (six independently evolving lines per treatment): no antibiotic (N), AMP (A), TET (T), AMP plus TET (AT), and 24 hour cycling between AMP and TET (A/T) (see methods). Plasmids remained at high frequency in all populations for the duration of the selection experiment. Plasmid-free segregants were only observed at very low frequency in two of the six populations from treatment N (Figure S4.1), whereas transposition of resistance genes from RK2 onto the host's chromosome was never observed.

To test for changes in antibiotic resistance profiles following evolution we first determined the minimum inhibitory concentration (MIC) of evolved lineages to TET and AMP. The susceptibility of the evolved strains to antibiotics differed between treatments (Figure 4.1A). We observed a four-fold increase in TET MIC in evolved strains from the T and AT treatments and a small increase in lineages that had evolved under the cycling A/T treatment compared with the ancestral MG1655 with ancestral RK2 (Anc-RK2), whereas evolved strains from treatments N and A showed no change in tetracycline MIC (ANOVA, $F_{5,30}$ = 6.103, p < 0.001; Post-hoc Tukey Tests, Anc-RK2:T p < 0.001, Anc-RK2:AT p < 0.01, Anc-RK2:N p = 0.525, Anc-RK2:A p = 0.783). By contrast, we observed no change in resistance to AMP in any treatment (ANOVA, $F_{5,30}$ = 1.212, p = 0.327), possibly due to a lower relative selection pressure imposed by the concentration of AMP used in the experiment compared to the concentration of
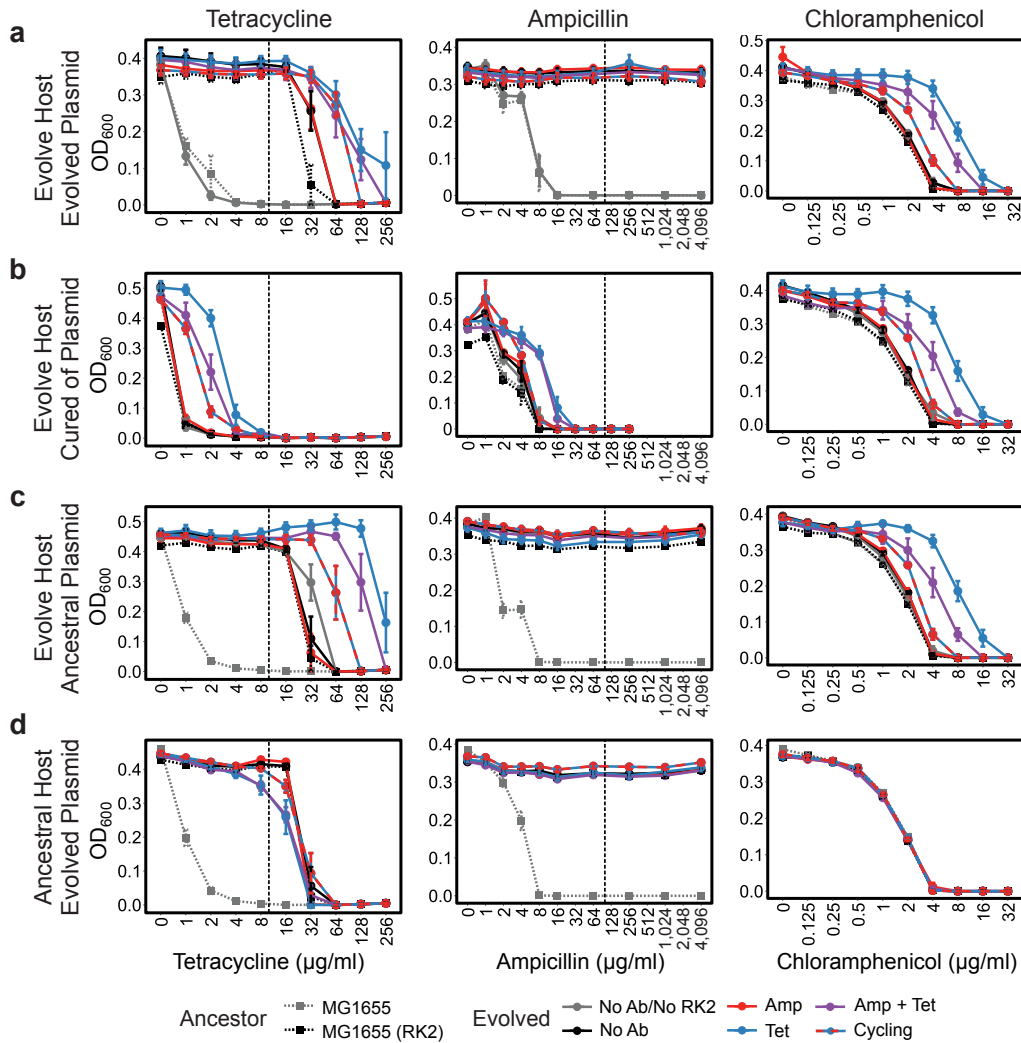
Figure 4.1 | Resistance profiles of evolved plasmids and hosts. Growth of **a**, evolved MG1655 strains with evolved RK2 plasmids **b**, evolved MG1655 strains cured of evolved RK2 plasmids **c**, evolved MG1655 strains with ancestral RK2 plasmid and **d**, ancestral MG1655 clones with evolved RK2 plasmids in the presence of tetracycline, ampicillin or chloramphenicol in comparison to ancestral MG1655. Points represent means of one clone from each of the six independent treatment populations, with SEM error bars. Dashed grey and black lines show the resistance profiles of plasmid free and plasmid containing ancestral strains respectively. Dashed lines in evolved host cured of plasmid plots (**c**) show ancestral MG1655 and ancestral MG1655(RK2) after curing process. Dashed lines in ancestral host evolved plasmid plots (**d**) show ancestral MG1655 and ancestral MG1655(RK2) which had under gone curing with ancestral RK2 subsequently reintroduced. Vertical dashed lines in AMP and TET resistance profiles show the concentrations of AMP (100 µg ml^{-1}) or TET (10 µg ml^{-1}) used in the selection experiment.

TET (Bottery et al., 2016). Interestingly, TET selection led to the evolution of bacteria that were cross-resistant to chloramphenicol (CML), although the extent of the evolved cross-resistance varied between treatments (ANOVA, $F_{5,30}$ = 24.25, p < 0.001); with CML MIC increasing 8-, 4-, and 2-fold in T, AT, A/T treatments, respectively. Consistent with CML cross-resistance being a correlated response to TET selection, evolved strains from both the N and A treatments remained equally sensitive to CML as the ancestral MG1655(RK2) (Post-hoc Tukey Tests, Anc-RK2:N p = 0.975, Anc-RK2:A p = 0.993). Thus whereas T and AT treatments, and to a lesser extent the cycling A/T treatment, led to the evolution increased TET resistance and cross-resistance to CML, evolved lineages from the N and A treatments showed no change in their resistance profile.

To examine the genetic bases of evolved changes in resistance we next obtained whole genome sequences for one randomly selected clone per population. Excluding hypermutators, evolved clones had acquired between 2 and 11 mutations, located exclusively on the chromosome in non-TET treatments (C, N, A), and on both the chromosome and plasmid in the treatments including TET (T, AT, A/T) (Figure S4.2). Of all the observed mutations 13.2% were synonymous and 19% were intergenic, the remaining non-synonymous mutations (67.8%) comprised missense mutations (42.8%), frameshifts (10.6%), insertion sequences (5.6%) and gene deletions (5.4%), and these were analysed further. While the variance in the number of non-synonymous mutations did not differ between treatments (Analysis of multivariate homogeneity of group variances excluding hypermutators $F_{5,26}$ = 1.8617, p = 0.1358), the loci affected by non-synonymous mutations did vary between treatments (Permutational ANOVA, permutation test: $F_{5,26}$ = 2.5231, p < 0.01, Bonferroni corrected). Clones that had evolved under TET selection (T, AT, A/T) had significantly different sets of non-synonymous mutations compared to evolved clones from the other treatments (C, N, A) (permutation test: $F_{1,30}$ = 6.9463, p < 0.01, Bonferroni corrected), with a larger genetic distance between TET and non-TET treatments than within these treatment groups (Figure 4.2A). Thus TET-selected lineages followed an evolutionary trajectory distinct from non-TET-selected lineages, leading to mutations on both the chromosome and the plasmid, which suggest that TET selection favoured bacteria-plasmid coadaptation.
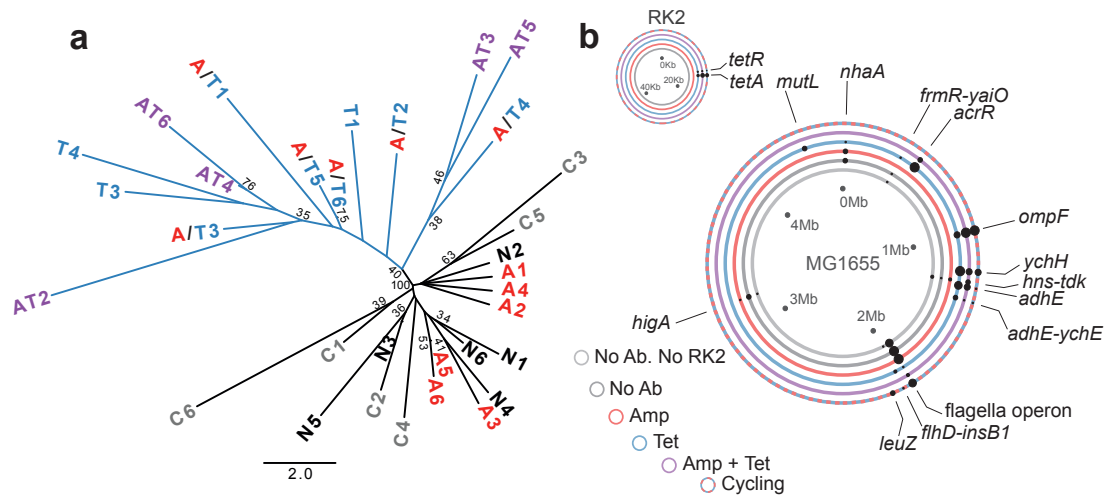
Figure 2 | Mutations show treatment specific parallelism. **a**, An unrooted neighbour joining phylogeny of end-point evolved clones. The distance matrix was constructed from the binary presence or absence of variants at each gene relative to the ancestral strain; hypermutators were excluded from the analysis. Scale bar represents number of gene variants; percentage bootstrap support is shown at the branches, B=1000, values below 0.3 are omitted. Blue branches represent clone isolated from TET treatments. **b**, Mutations observed in evolved clones (excluding hypermutators) across treatment. Rings represent *E. coli* chromosomes or RK2 plasmids. Dots represent mutations, the size of the dots represent the number of mutations at the same loci across independent replicate populations. Plots of individual treatments are in Figure S4.3.

Strikingly parallel mutations were observed between independent replicate populations both within and between TET-containing treatments (Figure 4.2B). Highly parallel mutations are likely to represent adaptive evolution at these loci, and because mutations at these loci were not observed in the populations from the N and A treatments, these mutations were likely to be TET-specific adaptations. Mutations in the chromosomal genes *ompF* (16 out of 18 clones), and *ychH* (16 out of 18 clones) showed strong locus-level parallelism within all three TET-containing treatments. Mutations in *ompF,* encoding a major non-specific diffusion porin (Cowan et al., 1992), were all predicted loss-of-function mutations, including the insertion of IS elements, frameshifts or premature stop codons. The loss of OmpF in *E. coli* reduces membrane permeability, including to antibiotics, and consequently is known to increase resistance to a wide spectrum of antibiotics (Blair et al., 2015) including TET and CML (Cohen et al.,

1989; Thanassi et al., 1995). Deletion of *ompF* (*E. coli* K-12 Δ*ompF* JW0912 (Baba et al., 2006)) significantly increased resistance to TET without the RK2 ($t_{9.09}$ = 4.2836, p < 0.01), and further increased TET resistance when carrying RK2 (Two-way ANOVA Interaction $F_{1,20}$ = 14.724, p < 0.01; Figure S4.4A). Parallel loss of function mutations (IS elements and frameshifts) in *ychH* were observed across all the TET treatments. YchH is a hypothetical stress-induced inner membrane protein (Lee et al., 2010; Mendoza-Vargas et al., 2009), but deletion of *ychH* (*E. coli* K-12 Δ*ychH* JW1196 (Baba et al., 2006)) did not significantly increase the resistance to TET with or without the plasmid (Figure S4.4B), suggesting that this general stress response may not be required under TET selection and is consequently selected against.

Mutations in several loci observed in the T and AT treatments were not present in the cycling A/T treatment. These included mutations in both *acrR* (10 out of 12 clones) and *adhE* (9 out of 12 clones). Mutations in *adhE* were extensively parallel at the nucleotide level, with 8 clones from independent populations all having the same missense SNP in the ethanol dehydrogenase domain (Membrillo-Hernández et al., 2000). The phenotypic significance of these mutations is unclear due to the multiple roles assigned to this protein, including multiple metabolic pathways (Kessler et al., 1991), but intriguingly the AdhE protein is known to exhibit binding activity to the 30S ribosome (Shasmal et al., 2016), the primary TET target. The *acrR* gene encodes a repressor of AcrAB multidrug efflux pump (Ma et al., 1996), the majority of mutations in *acrR* are predicted loss of function mutations, with IS elements and frameshifts observed in evolved strains. The deletion of *acrR* results in the overexpression of *acrAB* leading to MDR phenotypes (Okusu et al., 1996; Wang et al., 2001). Deletion of *acrR* (*E. coli* K-12 Δ*acrR* JW0453 (Baba et al., 2006)) alone did not significantly increase resistance to TET ($t_{9.32}$ = -0.591, p = 0.339), but when combined with the RK2 plasmid did allow significantly increased growth in TET ($t_{6.4}$ = 3.665, p < 0.01, Figure S4.4C). These findings are consistent with the higher TET resistance of evolved clones from the T and AT treatments versus the A/T treatment (Figure 4.1A) and reflect overall weaker TET selection under the A/T cycling compared to the T and AT treatments where TET selection was constant. Interestingly, stronger TET selection appeared to constrain evolution at chromosomal loci not involved in resistance. For example, we observed highly parallel loss of function mutation in the flagellum operon in the A, N and A/T treatments, but only rarely observed mutations at these loci in T and AT

treatments. Loss of the flagellar motility is a commonly observed adaptation of *E. coli* to growth in liquid media (Cooper et al., 2003) and this may have been impeded by clonal interference or negative epistasis with chromosomal resistance mutations in populations under strong TET selection. Consistent with this, whereas evolved clones from the N and A treatments increased in fitness relative to the plasmid-free ancestor in antibiotic-free media, such fitness gains were not observed in evolved clones from the TET-containing treatments (Figure S4.5).

To confirm that TET selection had led to the evolution of chromosomal resistance we next cured evolved strains of their plasmids and quantified resistance (Hale et al., 2010). Evolved strains carrying putative chromosomal resistance mutations displayed increased TET (ANOVA, $F_{5,30}$ = 42.63, p < 0.001), AMP (ANOVA, $F_{5,30}$ = 12.55, p < 0.001) and CML (ANOVA, $F_{5,30}$ = 35.88, p < 0.001) resistance (Figure 4.1B). Across all tested antibiotics, evolved clones carrying both *ompF* and *acrR* mutations had significantly increased resistance compared to the ancestral MG1655 (Post-hoc Tukey Tests, all p < 0.05), whereas cured evolved strains without either of these mutations (i.e. from the N and A treatments) did not (Post-hoc Tukey Tests, all p > 0.05). Interestingly, cured evolved clones from the cycling A/T treatment that carried only mutations in *ompF* but not in *acrR* showed marginally increased resistance to both TET and CML, but no detectable increase in AMP resistance, relative to MG1655. Thus TET selection favoured the *de novo* evolution of chromosomal resistance despite pre-existing plasmid-encoded TET resistance, and these chromosomal resistance mutations are responsible for the observed cross-resistance to CML.

We observed parallel mutations on the plasmid exclusively in evolved clones from the TET-containing treatments (T, AT, and A/T). These mutations occurred in *tetA/tetR* (18 out of 18 clones; *tetA*: 13, *tetR*: 2, both: 3, Figure 4.2B) which encode the tetracycline-specific efflux pump. The expression *tetA* is tightly regulated by the repressor *tetR* in the absence of tetracycline (Møller et al., 2016; Ramos et al., 2005). Mutations in *tetA* were dispersed throughout the gene, affecting the protein's transmembrane, periplasmic and cytoplasmic domains (Allard and Bertrand, 1992). Three of the five mutations observed in *tetR* are in direct contact with or in close proximity to the tetracycline binding pocket (Orth et al., 2000), while the other two mutations are located in the central scaffolding of the protein, suggesting that they are likely to interfere with

activity of the *tetR* repressor. Evolved plasmids carrying mutations in *tetA* or *tetR* displayed reduced resistance to TET in the ancestral MG1655 background compared to ancestral RK2 (Figure 4.1D, ANOVA, $F_{5,30}$ = 4.586, p < 0.01). Consistent with reduced efficacy of plasmid-encoded resistance in evolved lineages with *tetA/tetR* mutations, when we replaced the evolved plasmid with ancestral RK2, this led to increased TET resistance (ANOVA, $F_{5,30}$ = 71.86, p < 0.001, Anc-RK2:T,AT,A/T all p < 0.05).

Our data suggest that evolved strains from TET-containing treatments adapted their resistance to TET by acquisition of weak chromosomal resistance mutations in combination with mutations that reduced the efficacy of the plasmid-encoded TET efflux pump. To understand the evolutionary benefits of this counterintuitive dual resistance strategy we first compared the effect of chromosomal background (evolved or ancestral) and plasmid genotype (evolved or ancestral) on growth in the presence of 10 µg/ml TET (i.e., the concentration used in our selection experiment). The evolved chromosomal background carrying resistance mutations displayed a significantly shortened lag phase compared to the ancestral chromosomal background, irrespective of the plasmid genotype (Figure S4.6; ANOVA, $F_{3,56}$ = 76.92, p < 0.001; Post-hoc Tukey Tests, Evolved Host:Ancestral Host all p < 0.001). This suggests that chromosomal resistances reducing membrane permeability to antibiotics allowed evolved strains to start growing faster in the presence of TET.

Whereas evolved bacteria grew equally well with evolved or ancestral plasmids, ancestral bacteria displayed impaired growth with evolved compared to ancestral plasmids (Figure S4.6; Max OD, W = 93, p < 0.01). This is consistent with the mutations in *tetA/tetR* reducing resistance but importantly confirms that this reduction is not evident when in combination with the chromosomal resistance mutations, which appear to compensate for the reduced efficacy of the plasmid-encoded efflux pump.

We next competed evolved bacteria with either the evolved or ancestral plasmid against the ancestral MG1665(RK2) to compare the costs of carrying each plasmid genotype. The ancestral plasmid displayed a significantly higher cost than the evolved plasmid in the evolved chromosomal background (Figure S4.7, $t_{25.71}$ = -2.287, p < 0.05). This suggests that the mutations to *tetR/tetA* ameliorate the cost of plasmid carriage but at the price of reduced efficacy of TET efflux.

This is consistent with previous studies showing a high cost of expressing the specific tetracycline efflux pump (Nguyen et al., 1989). Taken together with the growth data, this suggests that although mutations to *tetA/tetR* reduce growth under tetracycline in the ancestral chromosomal background, they have minimal effect on resistance in the evolved chromosomal background due to the reduced membrane permeability and additional efflux systems expressed in the evolved chromosomal background carrying mutations in *ompF* and *acrR*, leading to high resistance and a lowered cost of plasmid carriage. This suggests that the chromosomal resistance mutations must have been gained prior to the mutations in the plasmid-encoded tetracycline efflux pump. To test this, for one population (AT2) we tracked the frequency over time of an observed IS-insertion in *ompF* by PCR and then determined by sequencing when these genotypes acquired mutations in the *tetA/tetR* genes. Consistent with the hypothesised order of mutations, the IS-insertion in *ompF* was first detected at transfer 8 and had swept to fixation by transfer 32, whereas mutations in *tetA/tetR* were not observed in this *ompF*::IS background until transfer 32 (Figure S4.8).

4.5 Discussion

Our current model of bacterial evolution suggests that horizontal acquisition of ARGs accelerates resistance evolution by providing bacteria with ready-made resistance mechanisms, bypassing the requirement for rare *de-novo* mutations (Jain et al., 2003). However, recent population genomic data suggesting that lineages independently acquire and then subsequently coevolve with MDR plasmids (Johnson et al., 2016; McNally et al., 2016; Stoesser et al., 2016) imply a more dynamic evolutionary process. Consistent with this, here we show that gaining an ARG can be just the starting point in the evolution of resistance and, due to the costs of expressing horizontally acquired ARGs, does not preclude subsequent *de novo* evolution of chromosomal resistance. Evolved strains from TET-containing treatments gained chromosomal resistance mutations reducing membrane permeability and enhancing efflux of TET and providing cross-resistance to other antibiotics, shortening lag phase in the presence of TET. These mutations also reduced the need for a fully operational plasmid-encoded tetracycline efflux pump, expression of which is highly costly (Nguyen et al., 1989), allowing plasmid mutations in the TET efflux pump and its regulator

which reduced the cost of plasmid-encoded resistance. A consequence of this intragenomic coevolution is that the increased TET resistance of evolved strains from T, AT and A/T treatments required the action of both the chromosomal- and plasmid-encoded resistances, which together acted multiplicatively. Thus intragenomic coevolution can lead to the evolution of bacterial genomes comprised of co-dependent replicons, limiting the potential for onward transmission of the plasmid due to the weaker resistance it now encodes in other lineages.

5. High repeatability of bacteria-plasmid coevolution under antibiotic selection

5.1 Abstract

Plasmids are important agents of horizontal gene transfer that accelerate bacterial evolution by transferring ecologically important traits between cells and species. However, gaining and expressing horizontally acquired genes can be costly leading to intragenomic conflict between the chromosome and the plasmid. These evolutionary conflicts can be resolved by compensatory evolution – of the host, the plasmid, or reciprocal coevolution of both – to reduce the cost. The dynamics of bacteria-plasmid coevolution remain largely unknown. Here we show, through temporal sequencing of multiple populations of *Escherichia coli* carrying the multidrug resistance RK2 plasmid, highly repeatable plasmid-host coevolutionary dynamics under antibiotic selection. Across all independently evolving populations the order in which beneficial mutations arose was identical despite clonal interference between multiple competing lineages within the same populations. Carrying the plasmid combined with tetracycline selection in all cases favoured chromosomal resistance mutations, reducing the length of the lag phase of the cells, followed by mutations to the plasmid, impairing the tetracycline resistance. Finally, additional resistance associated chromosomal mutations were acquired. Together these mutations provided increased resistance combined with improved growth in the presence of tetracycline. Our study provides evidence that reciprocal coevolution between bacteria and plasmids can be remarkably repeatable.

5.2 Introduction

The horizontal gene transfer (HGT) of genetic material between lineages is an important driver of bacterial evolution, facilitating the rapid adaptation and diversification of prokaryotes (Ochman et al., 2000; Wiedenbeck and Cohan, 2011). The acquisition of mobile genetic elements (MGEs) harbouring ecologically important accessory traits – as well as the genes required for their own replication, maintenance and horizontal transfer – promotes the innovation

of bacterial genomes, allowing bacteria to exploit novel niches (Nogueira et al., 2009; Ramírez-Díaz et al., 2011) or become better adapted to their current niche (Schwarz and Johnson, 2016). A worrying example is the rapid spread of antibiotic resistance within pathogenic bacteria via the HGT of conjugative multidrug resistance (MDR) plasmids (Bennett, 2008; Carattoli, 2013). However, the introduction of novel MDR plasmids often leads to conflicts between the newly acquired genes and the pre-existing genetic background (Baltrus, 2013). Direct costs are imposed through the maintenance, translation and transcription of the plasmid-encoded genes (Park and Zhang, 2012). Whereas, indirect effects reduce fitness of the host through disruption of host cellular networks (Harrison et al., 2015; San Millan et al., 2015) and cytotoxic effects (Baltrus, 2013). These costs can be ameliorated through compensatory evolution (Harrison and Brockhurst, 2012) dependent upon either the adaption of the plasmid (De Gelder et al., 2008) or host (Harrison et al., 2015; Loftie-Eaton et al., 2017; San Millan et al., 2014). Compensatory mutations can act directly to reduce the cost of the plasmid, affecting replication machinery or costly regions of the plasmid (Porse et al., 2016; Yano et al., 2016), or resolve disruption of cellular homeostasis; e.g., by limiting the bacteria's SOS response or negating global transcriptional perturbations induced by the plasmid (Harrison et al., 2015; Loftie-Eaton et al., 2017; San Millan et al., 2015; Sota et al., 2010). But there is growing experimental evidence that conflict between the plasmid and host can promote intragenomic coevolution between the independent replicons, thereby reducing the cost of the plasmid and increasing its persistence (Bottery et al., 2017; Loftie-Eaton et al., 2016). The order in which mutations are acquired by the chromosome and plasmid is likely to be important in the integration of plasmid in novel hosts. However, very little is known about the dynamics of bacteria-plasmid coevolution, or how repeatable this coevolutionary process is.

In a recent study, we investigated the compensatory adaptation between *Escherichia coli* MG1655 and the multidrug resistant plasmid RK2 (conferring resistance to ampicillin and tetracycline) when under antibiotic treatment (Bottery et al., 2017). The newly acquired plasmid produced a considerable cost in the presence of tetracycline, however, over 530 generations of coexistence, the cost of RK2 carriage was ameliorated through intragenomic coevolution. A set of beneficial mutations on the chromosome, which increased resistance and growth in the presence of tetracycline, paved the way for a reduction in the

efficiency and cost of the plasmid bourn resistance. This lead to co-dependency between the two replicons, which now provided increased resistance at a decreased cost.

In this study, we analyse four of the independently evolving populations from the previously published evolution experiment to investigate the repeatability of the coevolutionary dynamics within and between populations under tetracycline treatment. Through whole genome sequencing of clones across 530 generations of coevolution from four independent populations, we examine the temporal dynamics of the gaining of beneficial mutations. We find that coevolutionary dynamics are remarkable repeatable between independently evolving populations. Interestingly, although there is evidence of co-occurring independent lineages with genetically different but functionally similar mutations within populations, the order of the acquisition of beneficial mutations is identical between independent lineages. Plasmid carriage combined with tetracycline treatment firstly lead to the acquisition of chromosomal mutations that increased resistance to tetracycline and also decreased the cells lag phase in the presence of tetracycline. These mutations were followed by mutations on the plasmid that have previously been shown to both decrease resistance and cost of the plasmid (Bottery et al., 2017). Finally, fine-tuning of resistance followed through the acquisition of secondary and tertiary chromosomal mutations. The data shows that the trajectory of intragenomic coevolution can be highly repeatable between independent lineages.

5.3 Methods

5.3.1 *Experimental Design*

The evolution experiment, described previously (Bottery et al., 2017), coevolved *Escherichia coli* MG1655 with the multidrug resistance plasmid RK2 in various antibiotic treatments, to which the RK2 plasmid provided resistance. Cultures were grown at 37°C in 50 ml microcosms containing 5 ml of Oxoid® Nutrient Broth (NB) shaken at 180 rpm. Six independent biological replicates (populations) were initiated for each treatment, consistent with previous evolution experiments and sufficient to detect treatment specific parallel evolution (Harrison et al., 2015). Constant tetracycline treatments were

supplemented with 10 µg ml^{-1} tetracycline (populations T1-6) or 100 µg ml^{-1} ampicillin plus 10 µg ml^{-1} tetracycline (populations AT1-6). The populations were evolved for ~530 generations (80 days), through daily transfers of 50 µl of saturated culture into 5 ml of fresh media supplemented with antibiotics specific to the treatment, yielding roughly 6.6 generations per day. OD$_{600}$ of populations were measured daily. 500 µl samples from each population were frozen at -80°C on day zero and every 8 days (~53 generations) thereafter in 25% glycerol. Whole populations were also diluted and spread onto nutrient agar every 8 days, with 20 individual colonies selected at random and frozen at -80°C. A single clone from day 80 (generation 530) the end of the experiment from each population was randomly selected for sequencing, along with 3 clones from transfers 8, 16 and 40 in populations T3, AT2 and AT5, and transfers 16, 24, and 48 in population AT3. Populations under constant tetracycline treatment were selected for further analysis based on the clones' final sequenced genotype, so that the population contained insertion sequence (IS) elements within *ompF* and did not exhibit a hypermutator phenotype (no mutations within *mutL* of *mutS*). The time points chosen for sequencing were selected to provide samples prior to the observation of IS elements within the population based on whole population PCR, as well as the time point at which they were first observed and 24 transfers after first observing IS elements within the population.

5.3.2 *PCR tracking of mutations*

The frequency of IS mutation of the *ompF* genes was tracked within populations T4, AT2, AT3 and AT5 though whole population PCR. PCR primers amplifying *ompF* (Fw: ACTTCAGACCAGTAGCCCAC, Rv: GCGCAATATTCTGGCAGTGA) identified IS elements; a PCR short product of 716 bp indicated no insertion sequence, a product of 1484 bp indicted IS1 and a long product of 1911 bp indicted IS5 within *ompF*. 5 µl of frozen whole populations from every 8 transfers was mixed with 10 µl of nuclease free water, 1 µl of which was used as template for the PCR, resulting in approximately 1 × 10^{5} cells per PCR reaction. IS elements were never observed within the control population which coevolved with the RK2 plasmid in the absence of antibiotic treatment (N1). Twenty individual clones isolated from transfers 8, 16, 24, 32 and 40 from populations T4, AT2, AT3 and AT5 were subsequently checked for the presence of IS elements within *ompF* using the above primers. Standard

PCR reactions were performed using Go Taq Green Master Mix (Promega) 0.4 µM of each primer, on a program of 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2min, with a final extension of 72°C for 5 mins.

5.3.3 *Sequencing and analysis*

Three clones without IS elements in *ompF* were randomly selected from transfer 8 from populations T4, AT2, AT5 for sequencing, along with three clones from transfer 16, population AT3. Three random clones containing IS elements within *ompF* from populations T4, AT2, AT5, transfers 16 and 40, and three from population AT3 transfers 24 and 48 were also selected for sequencing. Each clone was grown for 24 hours until saturation in the same selective environment as they evolved in, before total genomic extraction using the DNeasy Blood and Tissue extraction kit (Qiagen) according to the manufactures instructions. The integrity of the DNA was assessed on a 0.75% agarose gel, and concentration estimated by Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). Total genomic DNA was sequenced by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1), using 2 × 250 base pair paired-end reads on the Illumnia MiSeq platform. Sequence adapters were trimmed using Trimmomatic (Bolger et al., 2014) and quality assessed using Samtools (Li et al., 2009), BedTools (Quinlan and Hall, 2010) and bwa-mem (Li and Durbin, 2009) by MicrobesNG. Trimmed reads were mapped to the *E. coli* MG1655 K-12 and RK2 plasmid genomes available on NCBI, accession numbers U00096.3 and BN000925.1, respectively, using the Burrows-Wheeler short read aligner (Li and Durbin, 2009). Variants were called using GATK's Unified Genotyper (McKenna et al., 2010) and annotated using SnpEff (Cingolani et al., 2012). Variants were filtered on base quality, coverage and single nucleotide polymorphisms (SNPs) around gaps. Insertion sequences were detected based on an over representation of larger inner mate gaps between mapped paired reads and structural variants were detected using Breakdancer (Chen et al., 2009). All variants were confirmed using the Integrated Genome Viewer (Robinson et al., 2011). To produce a set on mutations accumulated throughout the evolution experiment, variants that were also present in the previously sequenced ancestral strain (Bottery et al., 2017) were excluded.

5.3.4 *Phylogenetic analysis*

The phylogenetic trees were produced based on the binary presence and absence of all observed mutations within the data set for each sequenced clone. This gives each observed mutation equal weight when building the trees, which is appropriate when variants are under strong selection (Tenaillon et al., 2016). Trees were built using the neighbour joining method provided by the R package *ape* {ape 4.0} (Paradis et al., 2004) and were rooted to no observed variants, i.e. the ancestral strain harbouring RK2. The strong parallelism of mutations between populations resulted in clones from different populations clustering within the same clades when a composite tree was produced. However, as the populations evolved independently, separate trees were produced for each population and combined into a single plot.

5.3.5 *Minimum inhibitory concentrations*

To measure the minimum inhibitory concentration (MIC) of tetracycline, clones were grown over night in 5 ml of NB. The saturated cultures were sub-cultured 50 µl into 5 ml of fresh NB and allowed to grow until an OD_{600} of 0.5. These sub-cultures were then diluted 1:400, to an initial density of ~5×10^5 CFU/ml, in 96-well plates containing a $\log^2$ dilution series of tetracycline. Cultures were grown for 24 hours at 37°C, shaken at 600 rpm, 3 mm orbital radius, with OD_{600} measured at the end point. Three technical replicates were conducted per clone.

5.3.6 *Growth Curves*

Growth curves were conducted using a Tecan infinite M200 Pro plate reader in 96 well plates. Overnight cultures of clones were sub-cultured to an OD_{600} of 0.5 in fresh NB, and then diluted 1:1000 into 100 µl NB supplemented with 10 µg ml^{-1} TET. Cultures were grown at 37°C with shaking at 300 rpm, 3 mm orbital radius for 24 hours, and OD_{600} was measured every 16 minutes. Three technical replicates were conducted per clone. Growth rates were calculated as the maximum slope of $\log_2$ transformed OD_{600} covering four time points (~1 hour of growth), lag phase was calculated to end when growth rate reached 10% of the maximum achieved growth rate.

5.3.7 *Statistics*

Statistical analyses were performed in R (version 3.3.3). Linear and non-linear models were fit to the number of mutations acquired through time using Non-

linear least squares fit, quality of fit was calculated using Akaike information criterion (AIC). Comparisons among MIC curves were calculated through comparing the area under the curve, and statistical differences in length of lag phase among clones were calculated using ANOVA, with subsequent Tukey multiple comparison of means.

5.4 Results

Populations of *Escherichia coli* were previously co-evolved with the MDR plasmid RK2 under either single, combined or cycling treatment with ampicillin or tetracycline for ~530 generations (Bottery et al., 2017). Under the tetracycline-containing treatments the chromosomal and plasmid replicons co-evolved to become co-dependent, reliant upon each other to provide adequate resistance to the tetracycline concentration. Here, we explore the temporal coevolutionary dynamics of populations exposed to tetracycline treatments. Guided by previous whole genome sequencing of clones from the end-point of the selection experiment, the non-hypermutable populations T4, AT2, AT3 and AT4, which were evolved under constant tetracycline treatment (with [AT] or without [T] ampicillin), were selected for further analysis. Each population contained insertion sequence (IS) elements within *ompF*; loss of function of *ompF* was beneficial under tetracycline treatment (Bottery et al., 2017). To identify when these beneficial mutations of *ompF* occurred within the selected populations, whole population PCR of *ompF* was conducted (Figure S5.1), along with PCR of 20 individual clones from each population at transfers 8, 16, 24, 32 and 40 (Figure S5.2). IS elements were first observed at transfer 16 in population T4, transfer 8 in populations AT2 and AT5, and transfer 24 in population AT3.

5.4.1 *Acquisition of mutations*
Clones were sequenced from time-points before and after the acquisition of IS elements within *ompF* to determine the variability in genotype both within and between populations. Three clones were randomly selected from the sampled transfer before the occurrence of IS elements within the population (transfer 8 for populations T4, AT2 and AT5, transfer 16 for AT3), and then 8 and 32 transfers after this point. A single end point clone (transfer 80) from each

population that had been previously sequenced (Bottery et al., 2017) was also analysed. In total we observed 143 mutations (Figure S5.3), comprising of: 71 point mutations, 53 IS elements, 14 deletions and 5 duplications. Of these mutations 27 were intergenic variants and 3 were synonymous substitutions. The trajectories of the acquisition of mutations were similar across all populations, with the number of mutations increasing linearly over time (Figure 5.1A/B, GLM, $F_{1,28}$=181.98, P<0.01). Across all clones sequenced thirty-nine unique loci were affected by mutations, seven of which were parallel across the four populations.
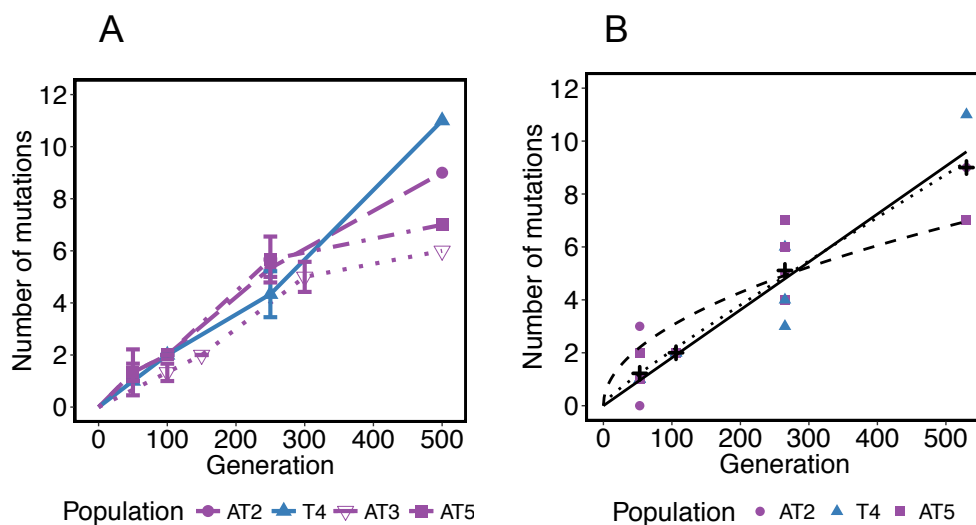

Figure 5.1 | A Average number of mutations through time, generation 530 has 1 sequenced genome, other points show mean number of mutations observed within 3 independent clones isolated from same population, with bars representing SEM. **B** Points show number of mutations in each sequenced clone, pluses show grand mean of total observed mutations across all treatments. The solid line shows the best fit to $m = at$, where a = 0.0181, R^2 = 0.931. The dashed line shows the fit to $m = b\sqrt{t}$, ΔAIC = -21.24, R^2 = 0.926. The dotted line shows the fit to the two parameter model, $m = at + b\sqrt{t}$, ΔAIC = -0.13, R^2 = 0.932, models proposed by Tenaillon *et al.* (2016). Fits to individual populations are shown in Figure S5.4.

5.4.2 *Population phylogenies*

Construction of phylogenies based on the presence or absence of mutations shows that there is considerable within population diversity across all four populations (Figure 5.2). The accumulation of short branches at the base of the trees shows early divergence, which was subsequent lost within the populations. As many of these mutations were not observed later in time, it is expected that these mutation were removed from the population through selective sweeps for more beneficial mutations.

The subsequent population trajectories differ between populations. A single lineage appears to dominate population T4, diverging early within the experiment. Likewise, in population AT2 a single lineage dominated by transfer 16, but was superseded by a second lineage that appears to have diverged earlier in time. In both of these populations the final sequenced clone came to dominate the population by half way through the experiment, suggestive of the stepwise acquisition of beneficial mutations and subsequent selective sweeps. Whereas, in populations AT3 and AT5, the phylogeny has deeper branching, showing that lineages coexisted despite strong selection for beneficial mutations. Thus, multiple different mutations with potentially similar fitness gains were emerging in parallel and being maintained within a single population. Despite identical selection conditions between the AT treatments, and parallel evolution observed to the gene loci level after 530 generations, the population dynamics leading to these parallel mutations differed between independently evolving lines. Whereas some populations were dominated by a single lineage, others had coexisted lineages over long periods of time.
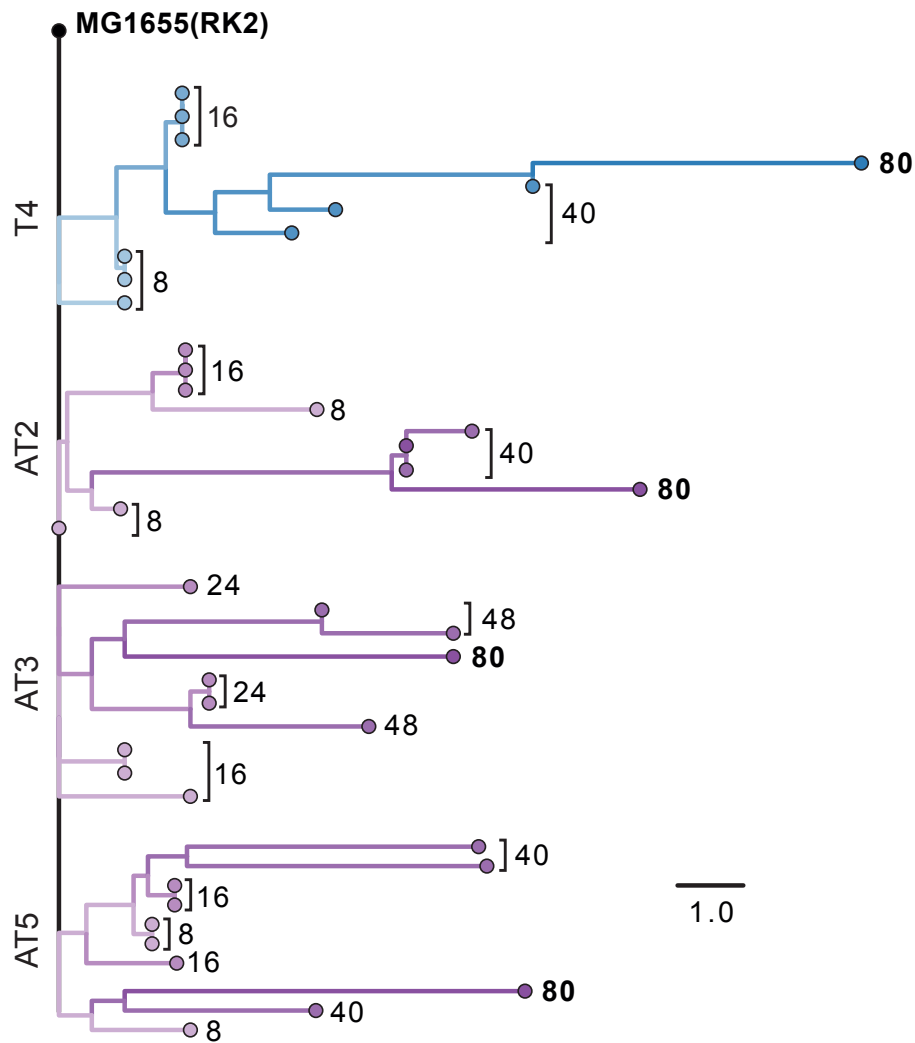
Figure 5.2 | Phylogeny of sequenced clones isolated from population T4, AT2, AT3 and AT5 rooted the ancestral MG1655(RK2). Distance matrix was constructed from the binary presence of absence of mutations, specific to the nucleotide level, relative to the ancestral strain. The scale bar represents number of mutations. Branch node labels and colour of branches show the transfer from which the clones were isolated.

5.4.3 *Parallel evolution*

Selection of MG1655(RK2) under constant tetracycline treatment led to mutated loci that were strikingly parallel across independent replicate populations, and therefore indicative of beneficial mutations under positive selection at these sites (Bottery et al., 2017). Across all four populations sequenced, the order of the acquisition of these beneficial mutations was also shared. The first parallel

mutation gained by all four populations was the lost of function of *ompF* via the acquisition of an IS element at transfers 16 or 24 (Figure 5.3). OmpF is a membrane protein that allows the passive diffusion of small molecules, including tetracycline, across the cell membrane (Phan and Ferenci, 2017). There is some genetic variability observed prior to the acquisition of *ompF* mutations, but these mutations are mostly lost from the populations upon the loss of function of *ompF*. Clones isolated from transfer 16, that had IS elements within *ompF,* had significantly increased resistance to tetracycline when compared to clones isolated from transfer 8 or the ancestral strain (Figure 5.4, Post-hoc Tukey Tests: Anc(RK2):Transfer 16 all $p > 0.05$, see Table S5.1 for ANOVA tables). By transfer 16 all clones also had a significantly reduced lag phase compared to the ancestral strain (Figure S5.8, Post-hoc Tukey Tests: Anc(RK2):Transfer 16 all $p > 0.05$, See Table S5.2 for ANOVA tables). IS elements were observed within all subsequent time points, but only in population T4 was the same IS element observed consistently throughout the experiment. Clones isolated from population AT2 at transfer 16 had IS*5* within *ompF* and a SNP in *gfcD* (encoded within an inactive operon in MG1655-K12 (Peleg et al., 2005)), which were lost from the population, displaced by a lineage which inactivated *ompF* through the insertion of IS*1*. Populations AT3 and AT5 had one of three different IS elements; IS*1*, IS*2* and IS*5* within *ompF.* These results suggest that the beneficial loss of function of *ompF* evolved independently multiple times within a single population (Figure 5.3). However, the variation in MIC among clones from these populations at any one time point was low (Figure S5.5-5.7), showing that independent lineages with the same functional mutations had similar resistance profiles.

Across all populations the next beneficial mutations to occur were SNPs within the plasmid bourn *tetA* or *tetR* genes*,* encoding a tetracycline specific efflux pump and its negative regulator, respectively (Schnappinger and Hillen, 1996). These mutations reduced the level of resistance to tetracycline when present in a wild-type background, but also reduced the cost of the plasmid (Bottery et al., 2017). This order of mutations is consistent with the hypothesis that mutations within *tetA* and *tetR* are reliant upon the prior acquisition of *ompF* mutations. Interestingly, there was no significant difference in the MIC between clones from transfer 16 and 40 (Post-hoc Tukey Tests: Transfer 16:Transfer 40 $p > 0.05$), suggesting that *ompF* mutations alone mask the decreased efficacy of the mutated plasmid-encoded efflux pump.
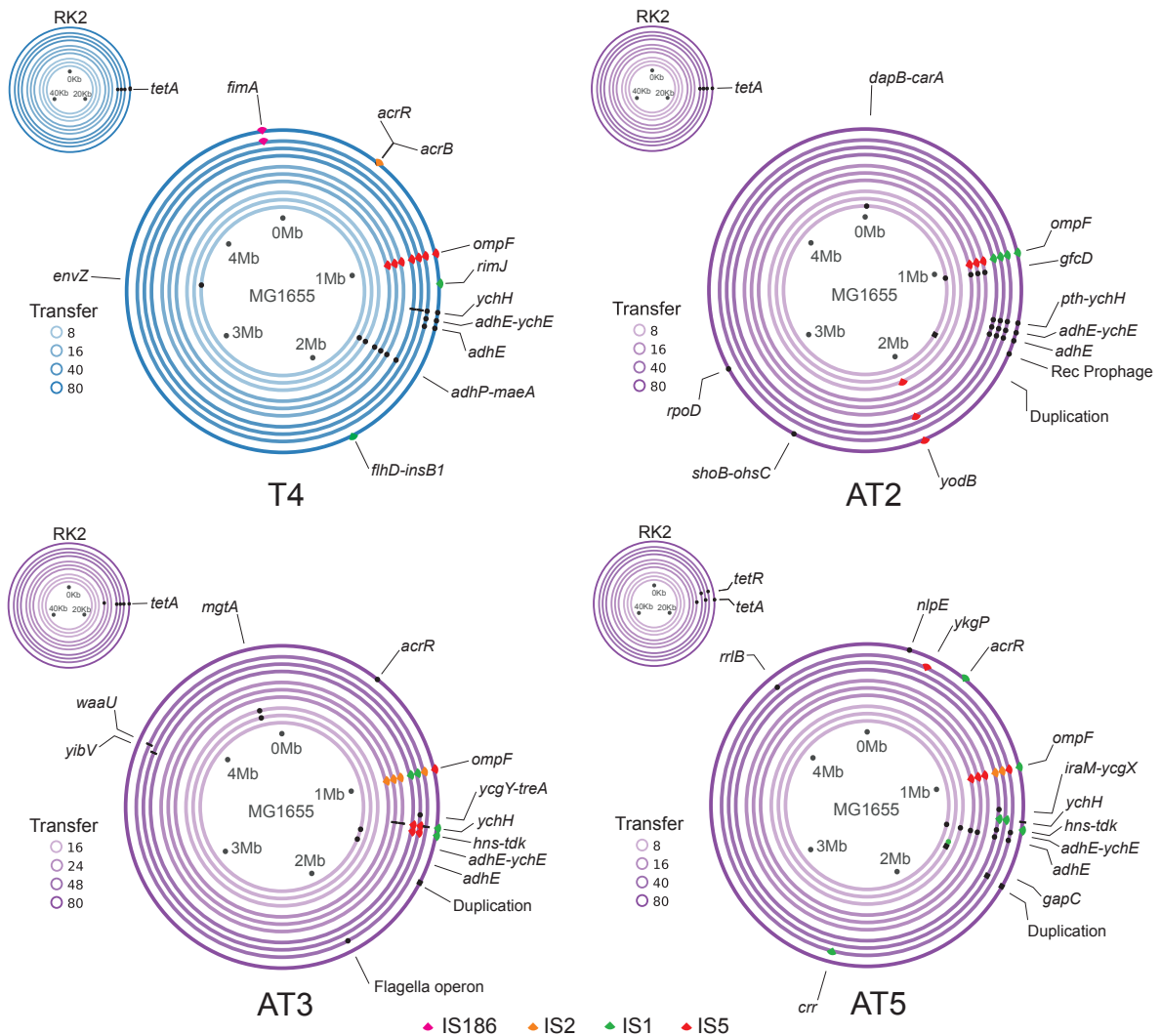
Figure 5.3 | Each panel shows the mutations observed within each population during constant selection under tetracycline treatment. Concentric rings represent *E. coli* chromosomes or RK2 plasmids, ring colour represent the time point the clones were isolated (see keys); inner lighter rings to outer darker rings represent the progression through time. Sets of three concentric rings are represent three independent clones isolated from the same time point within that population. Points on the plot represent mutations at specific loci, circles = non-synonymous mutations, bars = frame shift inducing deletions, squares = duplications, and triangles = IS elements (colours show type of IS element, see key).
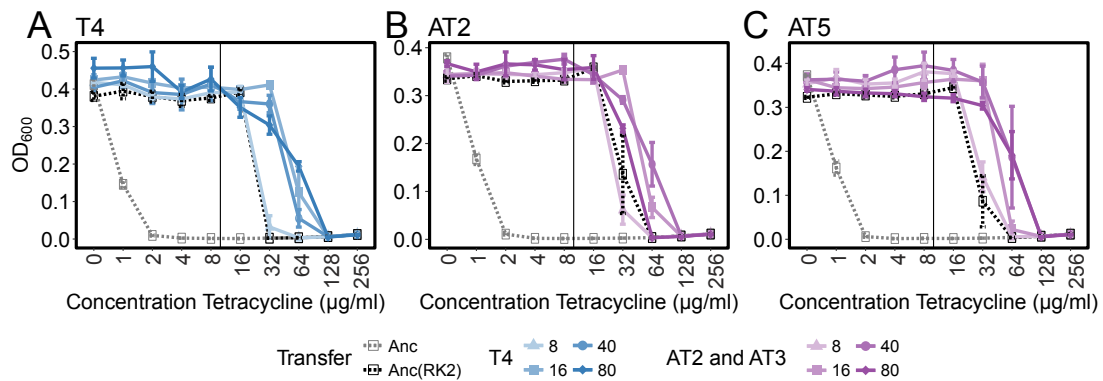
Figure 5.4 | The change in tetracycline resistance profile through time in population **A** T4, **B** AT2, and **C** AT5. The vertical line represents the concentration of tetracycline selected for during the evolution experiment. Points represent the collective mean of the three sequenced independent clones from each time point with error bars showing SEM. For individual MIC curves for each clone see Figure S5.5-5.7.

Transfer 40 is the first time point that we see the loss of function of *ychH,* a hypothetical gene encoding a general stress response (Lee et al., 2010), potentially not required under tetracycline selection. Loss of function was either through acquisition of IS elements or mutations generating frameshifts early within the gene, with both methods of inactivation present within populations AT3 and AT5. The final mutation to occur across all populations was within *acrR*, which negatively regulates the *acrAB* genes (Su et al., 2007), being only observed within clones isolated from the end of the evolution experiment (transfer 80). The loss of function of *acrR* is associated with the overexpression of AcrAB-TolC multidrug efflux pump, leading to MDR phenotypes (Okusu et al., 1996; Wang et al., 2001) and increased growth in the presence of tetracycline (Bottery et al., 2017). Genetically different mutations within *tetA*, *ychH* and *acrR* occurred within multiple independent lineages containing different IS elements within *ompF* (Figure 5.3). The acquisition of these secondary mutations within *ychH* and *acrR* in populations T4 and AT5 did not significantly increase the resistance to tetracycline (Figure 5.4, Post-hoc Tukey Tests, populations T4, AT5: 16:40:80 all $p > 0.05$). Interestingly, population AT2 did not acquire mutations within either *ychH* or *acrR* and unlike populations T4 and AT5, the

MIC decreased in clones isolated from population AT2 at transfer 80, reduced back down to a level which was not significantly different from the ancestral strain (Figure 5.4, Anc-RK2:AT2-80 p = 0.481). Potentially, the acquisition of subsequent parallel mutations is required to maintain high levels of resistance observed within populations T4 and AT5. End-point clones from populations T4 and AT5, also showed increased growth rates when compared to the ancestral strains (although these gains were not significant, Figure S5.8), whereas, end-point clones from population AT2 did not show this effect, potentially due to the lack of mutation of *acrR* and *ychH*. Thus, the early chromosomal mutations had the large benefit of allowing cells to grow significantly faster in the presence of tetracycline, while subsequent mutations had smaller effects on growth that are difficult to distinguish through analysis of growth curves alone.

The order of beneficial mutations was consistent across all four populations, with mutations of *ompF* followed by *tetA/R*, then *ychH* and finally *acrR* (Figure 5.3). The only parallel mutations not to show temporal consistency between populations was a highly parallel SNP within *adhE*, encoding an alcohol dehydrogenase which is involved in multiple metabolic pathways (Kessler et al., 1991) and known to exhibit binding activity to the 30S ribosome under aerobic respiration (Shasmal et al., 2016), the primary target of tetracycline. These mutations fluctuate in frequency both within and between populations, being observed only early on in population AT3, consistently in all transfers in population AT5 and only in the later half of the experiment in populations T4 and AT2.

5.5 Discussion

Our results demonstrate that plasmids not only act as facilitators of HGT, but due to evolutionary conflict with the host chromosome, also selects for intragenomic coevolution. The acquisition of the RK2 MDR plasmid provides the benefit of resistance in the short term despite costs associated with carriage of the plasmid. However, over longer periods of antibiotic selection with tetracycline, a highly parallel set of mutations occurs both on the plasmid and host's chromosome, reducing the plasmids cost (Bottery et al., 2017). Here we show that the observed coevolution between plasmid and host follows the same

order of mutations between independently evolving populations. This was despite the coexistence of independently competing lineages within some of the populations. We found that across all lineages, mutations within a chromosomally encoded outer membrane pore were followed by the mutation of the plasmid encoded tetracycline resistance genes. Subsequent mutation of chromosomal stress response and multidrug efflux systems followed. Their phenotypic effects upon resistance and growth of the bacteria potentially govern the common order of mutations.

The expression of the RK2 encoded tetracycline specific efflux pump is costly (Modi et al., 1991; Nguyen et al., 1989); mutations in the pump were able to reduce both the cost and efficiency of the pump (Bottery et al., 2017). But, in the presence of tetracycline, these mutations reduce the growth of the ancestral bacteria, as there are no additional resistance mechanisms to compensate for the reduction in efflux efficacy. Therefore the reduction in resistance caused by the plasmid mutations was hypothesised to rely upon the prior acquisition of additional chromosomal resistance mutations. Supporting this hypothesis, the mutational loss of function of *ompF* always occurred first across all of the sequenced populations. This mutation was likely selected for due to the significantly decreased length of the lag phase it conferred to cells in the presence of tetracycline. But, importantly the mutational loss of function of *ompF* also increased the level of resistance to tetracycline, providing a buffer to allow the mutational reduction in plasmid bourn resistance to sweep into the population. Following the early acquisition of *ompF* and *tetA/R* mutations, secondary resistance associated mutations in *ychH* and then *acrR* arose within the populations. Again the order of these mutations was highly parallel both within and between populations. End-point clones that had gained these mutations maintained high levels of resistance and showed an increase in growth rate, whereas a population that never acquired mutations within *ychH* and *acrR* did not, suggesting the potential importance of continual innovation when under tetracycline selection.

Here we measured the phenotypic effects the incremental acquisition of mutations, these help to explain the parallelism of the order of mutations between lineages. However, the highly repeatable order of mutations may also, in part, be due to diminishing returns epistasis (Chou et al., 2011), where by the extent of the benefit provided by mutations governs when they are selected

within a population. It would be expected that mutations with larger fitness effects will be selected early, with less beneficial mutations appearing in the population later. This pattern is implicit in the phenotypic data, with the early mutation of *ompF* having the largest phenotypic effect. However, in order to elucidate the direct fitness benefits of the incremental mutations described here, the direct competition between evolved lineages is required.

There is a growing body of evidence that the gaining of a plasmid can promote reciprocal coevolution of both plasmid and bacteria, reducing the cost imposed by the plasmid and helping to stabilise their existence within lineages (Bottery et al., 2017; Loftie-Eaton et al., 2016). The footprints of coevolution between host and plasmid are also present in bacterial genomes, with the rapid expansion and diversification of *E. coli* sequence types following the acquisition of MGEs by multiple independent lineages (McNally et al., 2016; von Mentzer et al., 2014). Our data suggests that this coevolution between plasmid and host can be highly structured, following a specific path governed by the phenotypic effects of mutations upon the resistance and growth of the bacteria. Further work will have to be conducted to determine if the dynamics of other examples of reciprocal coevolution between plasmid and host are similarly highly repeatable.

6. Discussion

Plasmids play a fundamental role in the evolution of antimicrobial resistance (AMR). The ability of plasmids to transmit between often distantly related bacterial lineages and accumulate multiple resistance genes means that they pose one of the greatest challenges in dealing with the emergence of AMR. Yet, our understanding of the selection and evolution of multidrug resistance (MDR) plasmids is limited. The data presented in this thesis demonstrates that the dynamics of both selection and evolution of MDR plasmids can vary dependent upon the plasmid encoded antimicrobial genes (ARGs) under selection. I show that the sociality of the contrasting antibiotic resistance mechanisms encoded by a MDR plasmid determined the selective conditions for the plasmid (Chapter 2). Furthermore, the mechanistic details of antibiotic resistance, along with mode of action of the antibiotic, were shown to be essential to produce mathematical models that accurately predict selection for resistance (Chapter 3). These two chapters address how short-term antibiotic selection can overcome the costs of plasmid carriage, but in the longer-term, compensatory evolution can stabilise MDR plasmids within bacterial populations by reducing the costs associated with plasmid carriage. I went on to examine how the mode of compensatory evolution varies with antibiotic treatment. I found that tetracycline treatment, but not ampicillin treatment led to intragenomic coevolution between the RK2 plasmid and it's host *Escherichia coli*. Coevolution ameliorated the cost of plasmid borne tetracycline resistance, but also created a co-dependency between the plasmid and its host (Chapter 4). The coevolutionary dynamics between *E. coli* and RK2 were strikingly repeatable across the independently evolving populations despite the emergence of multiple competing lineages within populations (Chapter 5). The data presented in the thesis demonstrates that a combination of positive selection and compensatory evolution can help to explain why MDR plasmids persist within bacterial populations despite their inherent fitness cost.

6.1 Selection for Multidrug Resistance Plasmids

The thesis highlights the importance of plasmid cost in the selection and maintenance of MDR plasmids. Although the underlying molecular bases of

plasmid costs are poorly understood (Millan and MacLean, 2017), the fitness burden imposed by plasmids is a fundamental driver of plasmid and host evolution (Harrison et al., 2015; Loftie-Eaton et al., 2017; San Millan et al., 2015). The cost of plasmid carriage generates purifying selection against plasmid-carrying strains, the thesis first explores how positive selection for plasmid-encoded traits can overcome this purifying selection. Recent studies have shown that it can take only a very small concentration of antibiotic, far below the minimum inhibitory concentration (MIC) of sensitive plasmid-free strain, to select for costly plasmids (Andersson and Hughes, 2014; Gullberg et al., 2014). However, MDR plasmids can encode qualitatively different mechanisms of resistance, ranging from cooperative to selfish. In Chapter 2 I show that the sociality of resistance can alter the selective conditions for a MDR plasmid. Plasmid encoded cooperative inactivation of ampicillin was selected for at concentrations of antibiotic higher than the MIC of the plasmid-free strain. The enzymatic inactivation of ampicillin by resistant cells permitted a sensitive sub-population to survive past their MIC by reducing the environmental ampicillin concentration. In contrast, selection for a tetracycline efflux pump, encoded on the same MDR plasmid, occurred at just 1/100th of the MIC of the sensitive plasmid-free strain. Furthermore, selection for a cooperative resistance mechanism, but not a selfish resistance mechanism, was negatively frequency dependent, resulting in a higher fitness of plasmid containing strain when they were rare. Mass action modelling helped confirm that the inactivation of antibiotic did provide a shared benefit of resistance, but importantly showed that the inclusion of both mode of action and mechanism of resistance are required to accurately predict the selection for resistance. Interestingly, the inactivation of bacteriostatic antibiotics permitted cooperation across a wider range of parameters than the inactivation of bactericidal antibiotics.

The levels of antibiotic contaminating natural environments can vary across orders of magnitude, ranging from very high concentrations – mg ml^{-1} in industrial sewage (Larsson et al., 2007) – to very low concentrations – ng or pg ml^{-1} in soil and water (Kümmerer, 2009; Zhang and Li, 2011). Our results show that even at these very low sub-MIC concentrations of antibiotic, selfish resistances such as efflux pumps will be selected for, driving the competitive exclusion of sensitive cells. Selfish resistances therefore seem to be especially important for the selection of MDR plasmids in environments with very low concentrations of antibiotic.

Multiple different antibiotics are often present at low levels within a single environment (Kim et al., 2011; Valcárcel et al., 2011). This is of concern due to the combined effects of multiple antibiotics demonstrated by the results presented in chapter 2 and 3. Combining two antibiotics at very low concentrations, each of which individually was insufficient to select for the resistance plasmid, together selected for the plasmid when in combination. Environmental pollution with multiple antibiotics may therefore be a contributing factor for the persistence of environmental MDR plasmids, even when each antibiotic is present at concentrations below the level required to select for resistance.

Coexistence of sensitive and resistant bacteria can occur when the benefit of resistance is shared in well-mixed populations, via the inactivation of the antibiotic. However, bacteria often live within complex spatially structured communities such as biofilms (Nadell et al., 2016). Limited diffusion and local interactions mediated by structured environments, such as biofilms, could vastly change population level effects of resistance by disproportionally directing the benefits of social resistances to the resistant sub-population. Spatial segregation and reduced diffusion within biofilms has been observed to promote cooperative behaviours in bacteria, such as the production and secretion of iron scavenging siderophores where the public good is preferentially utilised by neighbouring co-operators (Kümmerli et al., 2009). Whether biofilms promote or suppress cooperative antibiotic resistance is unknown. However, the modelling results presented in chapter 3 suggest that the survival of a sensitive sub-population may be more likely when in co-culture with a resistant population that inactivates bacteriostatic antibiotics as an initially high concentration of bacteriostatic does not kill sensitive cells. This may be of particular importance within structured populations where the public good of reduced antibiotic concentration is not immediately available to the sensitive sub-population. In support of this hypothesis, one of the few studies that has examined cooperative antibiotic resistance within spatially structured environments showed that the inactivation of chloramphenicol (a bacteriostatic antibiotic) allowed the survival of sensitive cells both on agar plates and within *in vivo* mouse models (Sorg et al., 2016).

Understanding how antibiotic resistance is selected for is a central goal of studying the spread of MDR plasmids. There are many forms of both selfish (Li

et al., 2015) and cooperative (Wright, 2005) resistances that can be encoded upon MDR plasmids. The social implications of these plasmid-encoded resistances are often overlooked; the thesis highlights the importance of quantifying the social interactions imparted by resistances in order to gain an understanding of the selection and maintenance of MDR plasmids within bacterial populations.

6.2 Evolution of Plasmid Borne Resistance

Antibiotic treatment can lead to the competitive exclusion of antibiotic sensitive cells from a population and positively select for MDR plasmids. However, positive selection alone does not resolve the intragenomic conflicts between plasmid and host; the underlying costs of plasmid carriage are still present even when the plasmid is acting as a mutualist (in the presence of positive selection). Studies have shown that compensatory evolution can act to ameliorate the cost of plasmid carriage when a plasmid is acting as a parasite (in the absence of positive selection) but also when it is in a mutualistic relationship with the bacterial host (Harrison et al., 2015). An outstanding question was how the mode of compensatory evolution following the acquisition of a MDR plasmid varies with antibiotic treatment. We addressed this question through experimental evolution of *E. coli* carrying the MDR plasmid RK2 under a range of antibiotic treatments. We found that populations treated with tetracycline had an evolutionary trajectory distinct from populations that were not treated with tetracycline, which led to mutations both on the chromosome and plasmid. *De novo* chromosomal resistance mutations that arose under tetracycline treatment, despite pre-existing plasmid resistance, provided cross-resistance to other antibiotics and a shortened lag phase in the presence of tetracycline. These chromosomal mutations enabled the acquisition of mutations within the plasmid borne tetracycline efflux pump that reduced its level of resistance and cost. Further examination of populations evolving under constant tetracycline treatment revealed that the coevolutionary dynamics were highly repeatable. Despite clonal interference between multiple competing lineages within populations, the order of beneficial mutations was always the same.

A worrying consequence of the intragenomic coevolution observed under tetracycline treatment was that the coevolved plasmid and host together acted multiplicatively (Bottery et al. 2017), providing an increased level of resistance to tetracycline and cross-resistance to chloramphenicol at a reduced cost. However, neither the *de novo* chromosomal resistance mutations nor the evolved plasmid efflux pump provided a sufficient level of resistance for the *E. coli* to survive at the level of tetracycline it had been selected for. Thus, following intragenomic coevolution the two replicons became co-dependent, this potentially limited the ability of the plasmid to be transmitted and stability maintained in other lineages due to the weaker resistance it encoded.

It is clear from our results that the evolutionary trajectory following the acquisition of a MDR plasmid does vary with antibiotic treatment. An outstanding question arising from this result is what caused the differences in the evolutionary trajectories following treatment with ampicillin or tetracycline. Our results suggest the main cause of the differences in evolutionary trajectories were due to the costs of resistance. Although the *bla* gene encoding β-lactamase on the RK2 plasmid is constitutively expressed (Wilson et al., 1997), the β-lactamase does not require any other substrates in order to catalyse the hydrolysis of ampicillin (Shaikh et al., 2015). Therefore, it is likely that this resistance mechanism is not particularly costly, as it does not consume cellular resources once the β-lactamase proteins have been produced. In contrast, although the expression of the TetA efflux pump is strongly negatively repressed by TetR in the absence of tetracycline, high levels of expression of *tetA* can be triggered with very little tetracycline (Nguyen et al., 1989). Once expressed, the efflux pump, unlike β-lactamase, is energy dependent – potentially limiting cellular resources (Levy, 2002) – but it also disrupts the cell physiology through the insertion into the membrane (Nguyen et al., 1989). This difference in the cost of expression between the two resistance mechanisms may have been the cause of two differing evolutionary trajectories, allowing the acquisition of immediate effect chromosomal mutations and modulation of the plasmid-borne resistance when under tetracycline selection.

The mechanism of action of the two antibiotic resistances may have also contributed to the differences between the evolutionary trajectories adopted under ampicillin and tetracycline selection. The RK2 encoded β-lactamase is predicted to inactivate all ampicillin within the microcosm within just five hours of

growth (see chapter 3). This will have resulted in the bacteria to be under periodic ampicillin selection, with bacteria exposed to very little antibiotic for the majority of the experiment. In contrast, the efflux of tetracycline from the cell is a continual process required throughout the growth cycle of the cells. The more constant selection pressure imposed by tetracycline treatment and not ampicillin treatment may have also contributed to the difference in evolution observed between the two treatments.

To gain a clearer picture of how antibiotic treatment alters evolution following the bacterial acquisition of a MDR plasmid a greater range of resistance plasmids must be examined. A future direction may be to examine the positive selection of plasmids harbouring potentially less costly resistance mechanisms which remain under constant selection, such as *tetM,* which acts to block the binding of tetracycline to the ribosome (Dönhöfer et al., 2012). An alternative is to explore the selection of a potentially more costly form of antibiotic inactivation such as group transfer – kanamycin phosphotransferase or chloramphenicol acetyltransferase for example – which requires energy in the form of ATP and acetyl-CoA respectively (Leslie, 1990; Wright and Thompson, 1999).

The horizontal acquisition of ARGs encoded on MDR plasmids accelerates the evolution of antibiotic resistance by introducing new resistance genes into bacteria in a single step, by-passing the need for rare *de novo* resistance mutations (Bennett, 2008; Jain et al., 2003). However, bacterial genomes show the footprints of coevolution between horizontally acquired genetic elements and host chromosomes. The pathogenic *E. coli* lineage ST131 was observed to have multiple co-existing subtypes; chromosomal mutations within each were associated with the maintenance of different horizontally acquired genetic elements, including plasmids (McNally et al., 2016). The data presented in chapters 4 and 5, together with these recent findings, is suggestive of a more dynamic evolutionary process, whereby the acquisition of a conjugative MDR plasmid can act as a catalyst for the adaptation of both chromosomal and plasmid encoded genes.

6.3 Conclusions

Antibiotic resistance in bacteria is a global threat to public health, and the horizontal transfer of MDR plasmids is a key vector for the acquisition and spread of antibiotic resistance in commensals and pathogens (Bennett, 2008). Although MDR plasmids benefit bacteria under antibiotic treatment, the carriage of plasmids can also impose a significant fitness cost upon novel bacterial hosts. Research into the maintenance of MDR plasmids has begun to identify the role of fitness costs, positive selection and compensatory evolution in explaining plasmid-host dynamics. My research demonstrates the interaction between these factors: both positive selection and compensatory evolution can help to explain the persistence of MDR plasmids in the clinic and environment.

The results of the thesis paint a worrying picture, even when MDR plasmids are costly only a very small concentration of antibiotic is required to overcome this cost and select for resistance. When resistance is cooperative it may take a higher relative concentration to select for resistance; however, resistance will likely be maintained within bacterial populations exposed to sub-MIC levels of antibiotic through negative frequency dependent selection. Importantly, the point at which resistance will be selected for is dependent upon the cost of resistance. Therefore, cost free resistance mechanisms could be maintained within populations without any exposure to antibiotics. I have shown that it only takes a short period for compensatory mutations to arise and reduce the cost of an MDR plasmid. Moreover, positive selection for resistance can lead to not only a reduction in the cost of resistance, but also an increased level of resistance and cross-resistance. It is critical therefore that we reduce the levels of antibiotic exposure in the environment, and optimise current drug regimens to limit long-term exposure to sub-MIC levels of antibiotics. Despite the advances presented here and elsewhere (Gullberg et al., 2014; Harrison et al., 2015; Loftie-Eaton et al., 2017; Porse et al., 2016; San Millan et al., 2015), our ability to predict the evolution of plasmid-mediated antibiotic resistance remains limited, particularly within clinically relevant settings. Future work needs to explore how the evolution of antibiotic resistance proceeds in clinically relevant pathogen-plasmid combinations within more complex microbial environments.

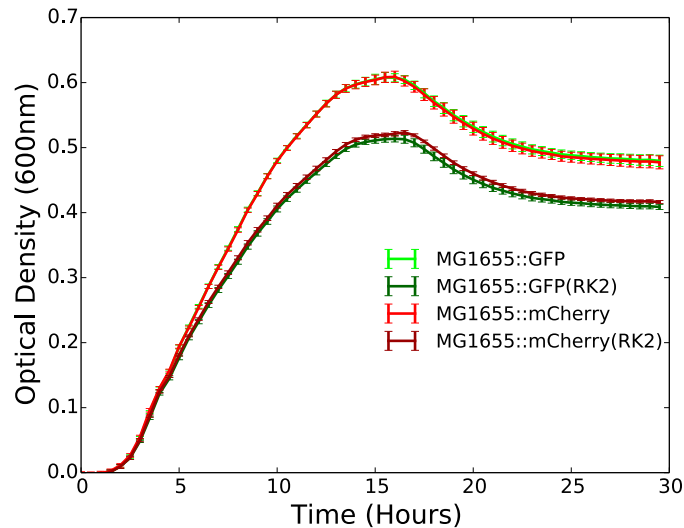# Appendices

Appendix A: Chapter 2

Figure S2.1 | Growth curves of marked strains show no difference in growth dynamics between marked strains, with or without RK2 plasmid. Bacteria were grown in 96-well plates at 37°C with periodic shaking in a Tecan Infinite 200 Pro plate reader, with OD_{600} measured every 30 mins. Error bars represent SEM (n=6).
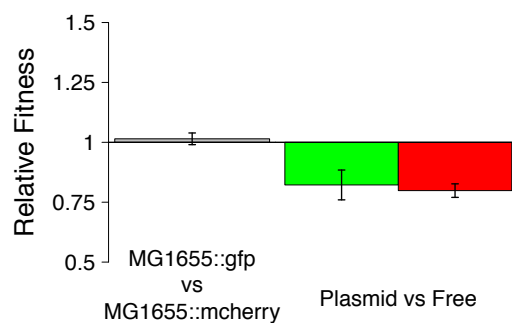

Figure S2.2 | No significant difference in fitness of MG1655::gfp strain and MG1655::mcherry strain (t test t = 0.6001, df = 11, p-value = 0.5606) or between MG1655::gfp(RK2) and MG1655::mcherry(RK2) (two sample t test t = 0.5186, df = 16, p-value = 0.6944) when competed in antibiotic free media. Green bar represents RK2 plasmid in MG1655::gfp background competed against MG1655::mcherry and red bars are RK2 plasmid in MG1655::mcherry background competed against MG1655::gfp. Error bars represent SEM (n=6).
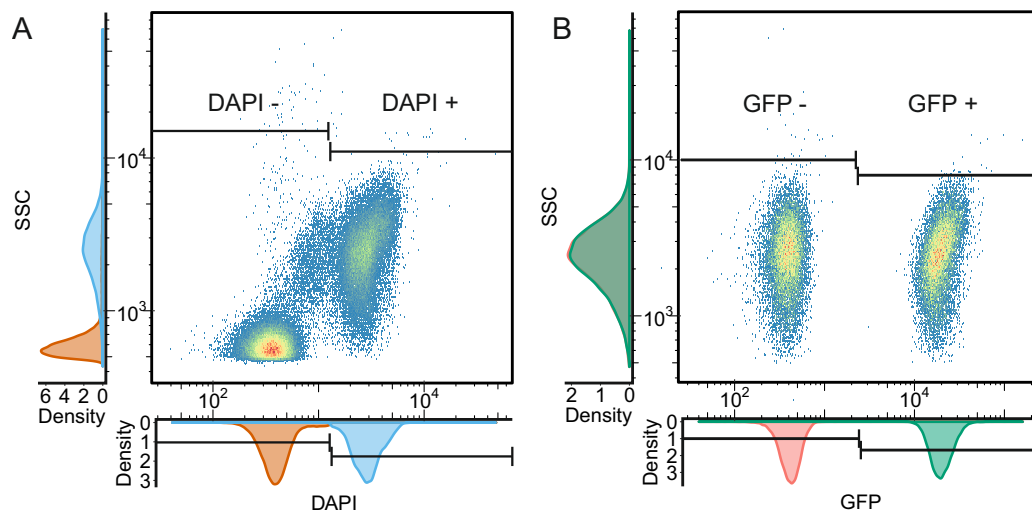
Figure S2.3 | Quantification of GFP and mcherry marked MG1655 strains in co-culture using flow cytometry. **A** Density plots showing SSC vs DAPI signals, bacterial cells were differentiated from noise based on a positive DAPI signal as SSC alone does not accurately split the two. **B** Density plots showing SSC vs GFP signal, GFP expressing cells are easily distinguishable from mcherry expressing cells, ratios of the two strains were used to calculate selection rates.
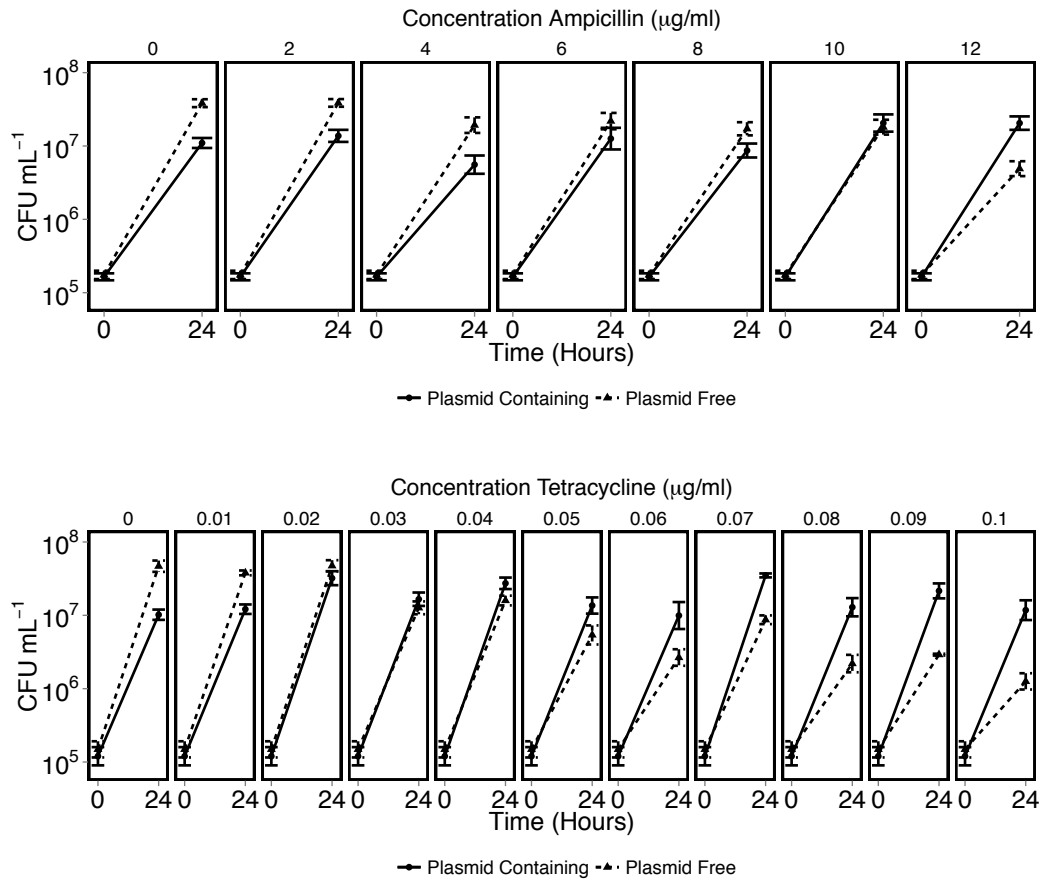
Figure S2.4 | Plots of cell densities of plasmid free and RK2 plasmid containing populations in competition, these data were used to calculate the relative fitness of plasmid bearing strain (figure 2.3A/B in text). Note that relative fitness (*W*) relies on positive growth of both competing strains. Error bars represent SEM (n=6).
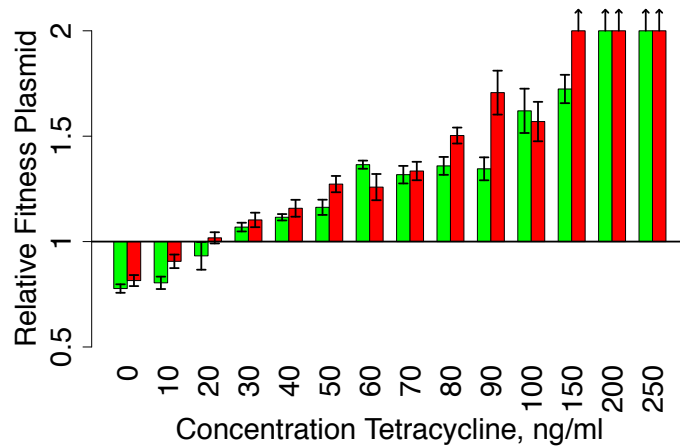
Figure S2.5 | High concentrations of tetracycline cause extinction of sensitive population when in competition. Green bars are the RK2 plasmid in MG1655::gfp background competed against MG1655::mcherry and red bars are the RK2 plasmid in MG1655::mcherry background competed against MG1655::gfp. Upward arrows represent a relative fitness above 2 i.e. no observable sensitive bacteria within the population. Error bars represent SEM (n=3).
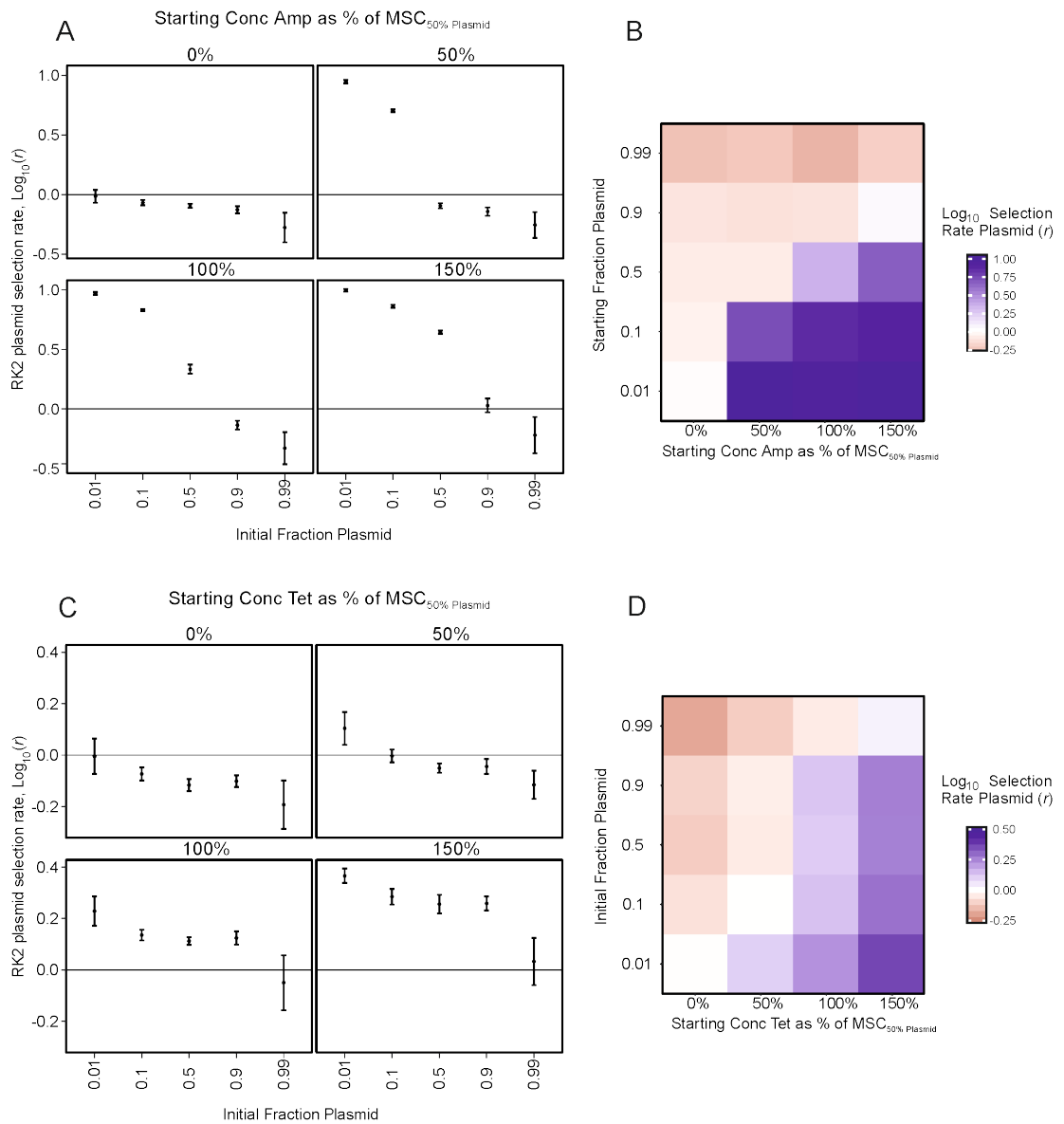
Figure S2.6 | Log_{10} selection rates (*r*) of plasmid bearing *E. coli* vs plasmid free *E.coli* as a function initial plasmid frequency under (A,B) ampicillin selection and (C,D) tetracycline selection. Points show means of six independent replicates; with error bars showing SEM. **B** and **D** show the same data as panels plotted as heat maps.
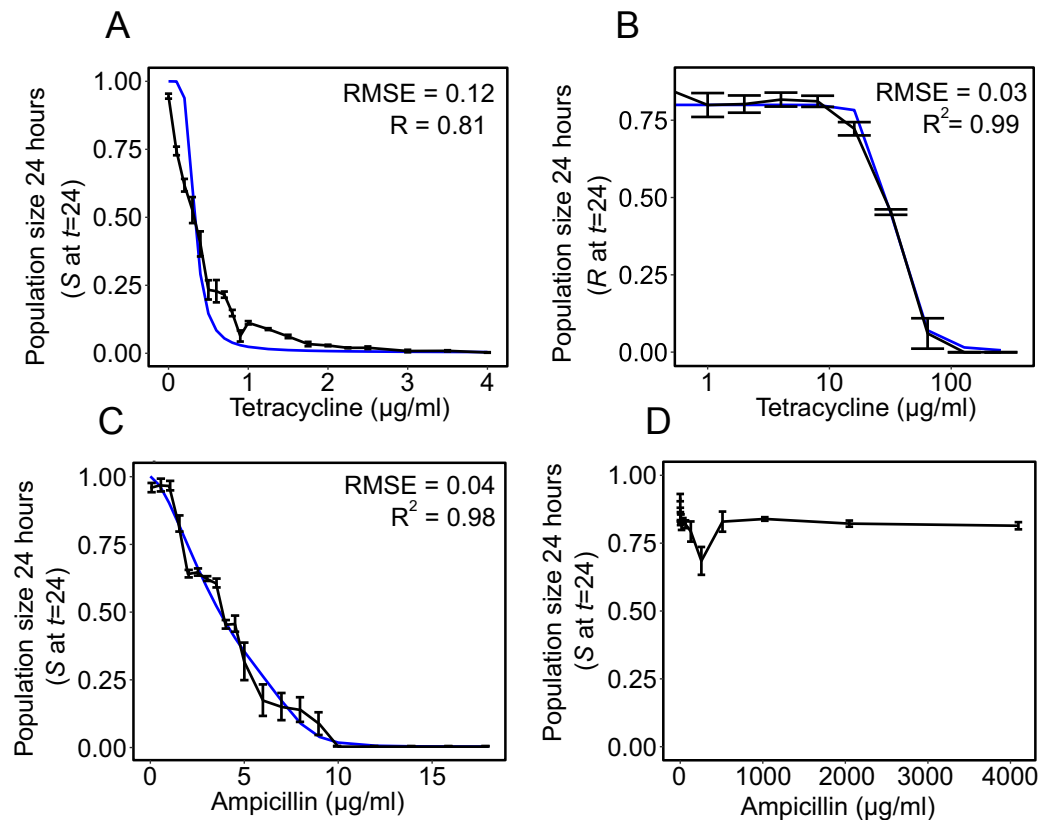
Appendix B: Chapter 3

Figure S3.1 | Fitting of simulated monocultures to empirical MIC data. The black line shows *E. coli* density after 24 hours growth at increasing concentrations of tetracycline or ampicillin, with error bars representing SEM (n=6). The blue lines show the population density at t=24 hr of the best parameter fit of numerical solution to models (1)-(3) when simulated in monoculture. **A** Plasmid free MIC in tetracycline, 2 µg ml^{-1}, $Kmax_{tS}$ = 1.25, KA_{50tS} = 0.08 **B** Plasmid containing MIC in tetracycline, 64 µg ml^{-1}, $Kmax_{tR}$ = 1, KA_{50tR} = 10.6 **C** Plasmid free MIC in ampicillin, 9 µg ml^{-1}, $Kmax_{cS}$ = 1.5, KA_{50cS} = 4.5 **D** Plasmid containing MIC in ampicillin, >4096 µg ml^{-1}, as the full curve was not available, KA_{50cR} was selected to provide growth at 4096 µg ml^{-1} ampicillin, $Kmax_{cR}$ = 1.5, KA_{50Cr} = 3200.
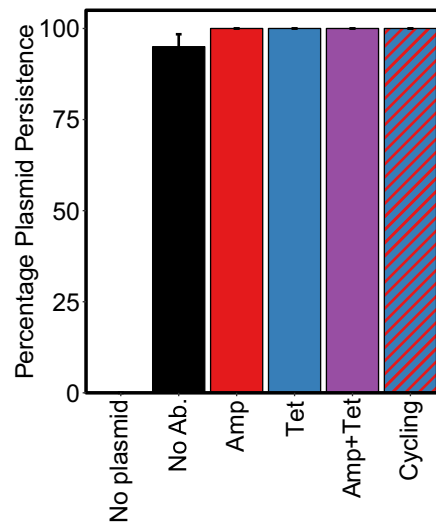
Figure S4.1 | Persistence of plasmid at transfer 80, generation ~530. Bars show mean percentage of 20 evolved colonies from transfer 80 positive for RK2 plasmid by PCR, 6 independent populations per treatment, error bars show SEM.
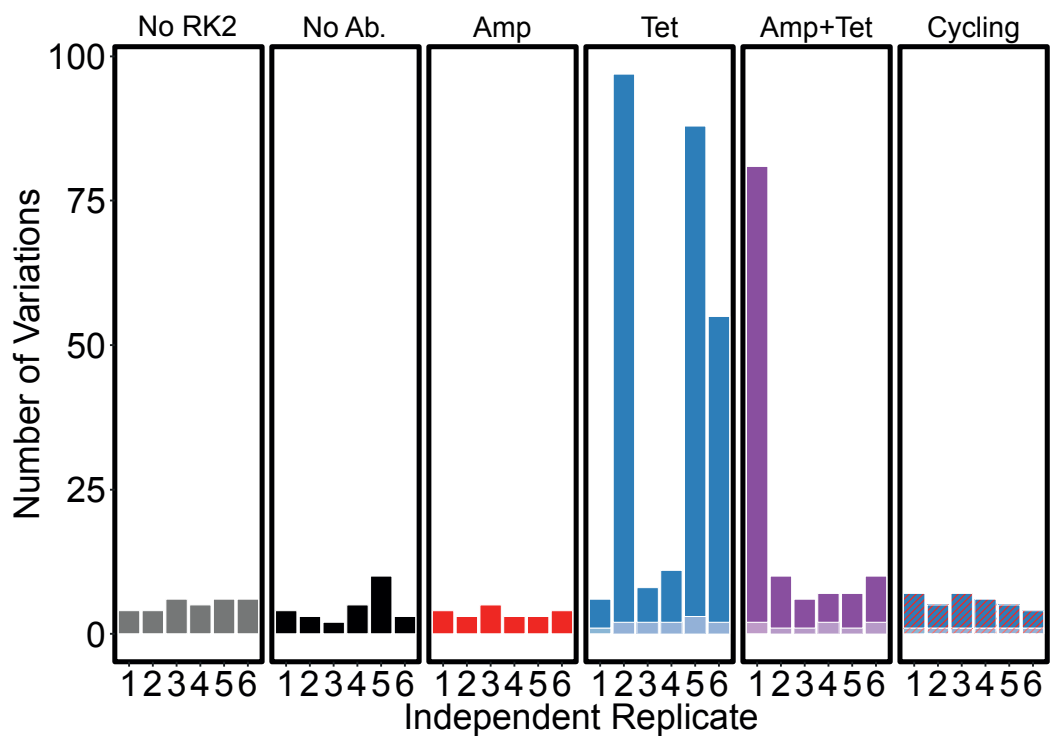

Figure S4.2 | Number of mutations per genome. Bar charts show number of mutations per evolved clone in each treatment. The dark area of bar represent chromosomal mutations and the light area represent plasmid mutations.

Figure S4.3 | Mutations observed in each evolved independent population. Each ring represents the chromosome or RK2 from a single clone from each independent population, populations 1 to 6 from inner ring to outer ring. Dots (●) represent SNPS and sort indels, triangles (▲) represent insertion sequences, squares (■) represent large duplications and bars (Ι) represent large deletions. Mutations which are present within the same loci in two or more clones from the same treatment are labelled.
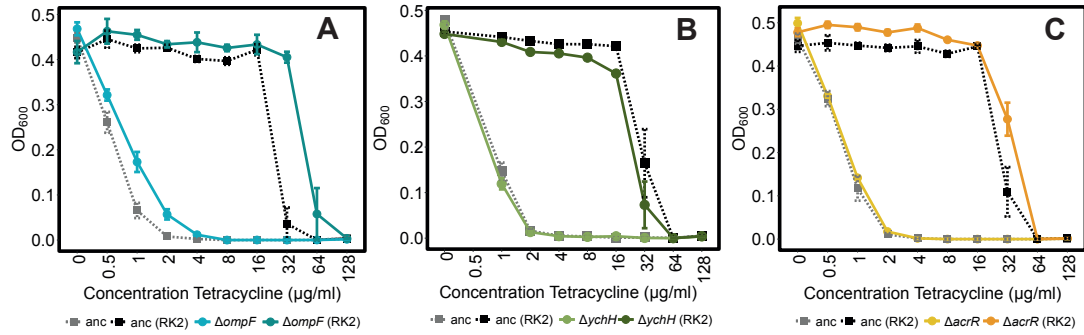
Figure S4.4 | Loss of function of OmpF multiplicatively increases tetracycline resistance when in combination with plasmid based efflux pump. Growth of keio knockout strains in the presence tetracycline in comparision to ancestral MG1655. Dashed lines represent ancestral strains, solid lines represent knockout strains, with light lines showing plasmid free growth and dark lines showing growth when harbouring RK2 **a**, *E. coli* K-12 Δ*ompF* (JW0912) **b**, *E. coli* K-12 Δ*ychH* (JW1196) **c**, *E. coli* K-12 Δ*acrR* (JW0453). Error bars represent SEM of six replicates.

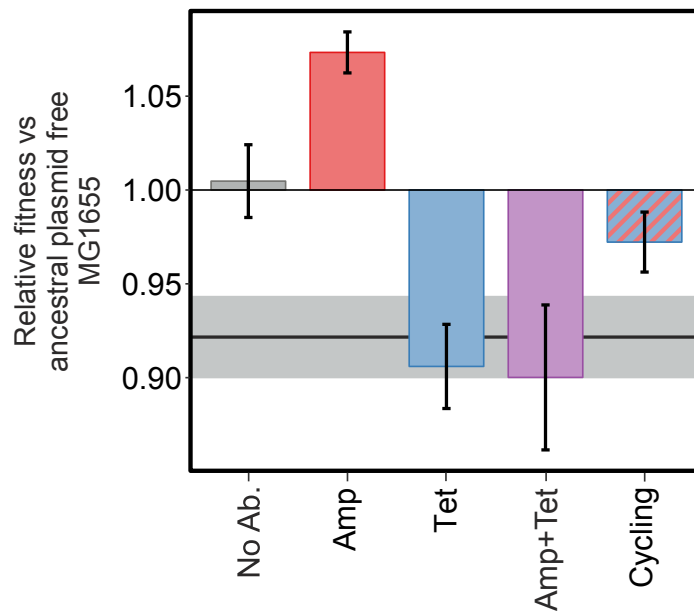**Figure S4.5 |** Relative fitness of evolved plasmid bearing populations. The relative fitness of evolved plasmid containing strains verses ancestral plasmid free strains when under no antibiotic selection. Bars represent mean of one randomly selected clone from each of the six replicate populations, error shows SEM. The black line represents the fitness of the ancestral plasmid carrying strains verses ancestral plasmid free, with the shaded grey area showing SEM (n=6). Evolved clones selected without antibiotics or with AMP increased in fitness (one-sample *t* test from 1 N: mean = 1.0047, s.e. = 0.019, t_5 = 0.2445, p = 0.8166, A: mean = 1.0733, s.e. = 0.0109, t5 = 6.709, p < 0.05). Evolved clones constantly exposed to TET (T and AT treatments) remained significantly less fit than the ancestral plasmid free MG1655 in antibiotic-free media (one-sample *t* test from 1 T: mean = 0.9059, s.e. = 0.0224, t_5 = -4.192, p < 0.05, AT: mean = 0.8938, s.e. = 0.0.0321, t_5 = -3.300, p < 0.05).
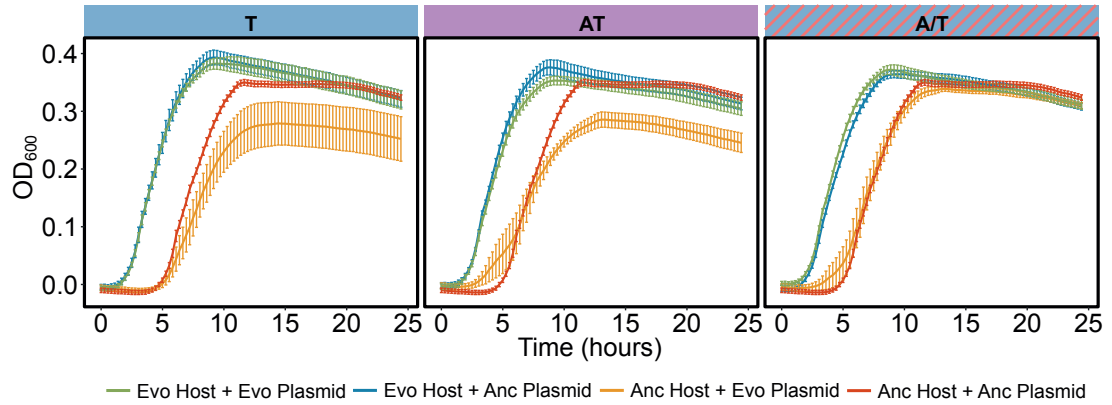
Figure S4.6 | Chromosomal resistances reduce lag time. Growth curves of: evolved host with evolved plasmid, evolved host with ancestral plasmid, ancestral host with evolved plasmid and ancestral host with ancestral plasmid, in TET 10μg/ml (the TET concentration used in the selection experiment), from either T, AT or A/T treatments. Mean of one randomly selected strain from each of the six replicate populations per treatment, error bars represent SEM.
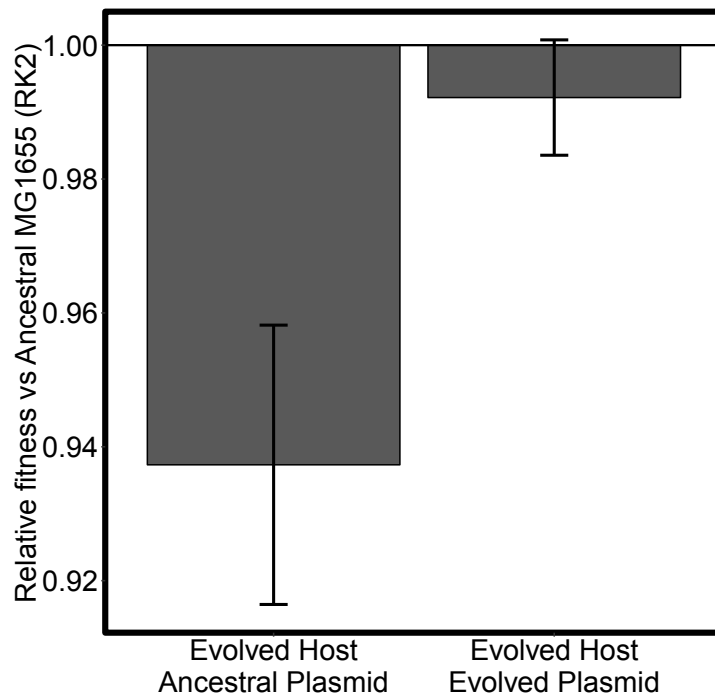
Figure S4.7 | Plasmid mutations ameliorate the cost of chromosomal resistances. The relative fitness of evolved strains from T treatment harbouring either their evolved plasmid or ancestral plasmid competed against ancestral MG1655 with ancestral RK2 plasmid, bars show the mean of 18 replicates, with error bars showing SEM.
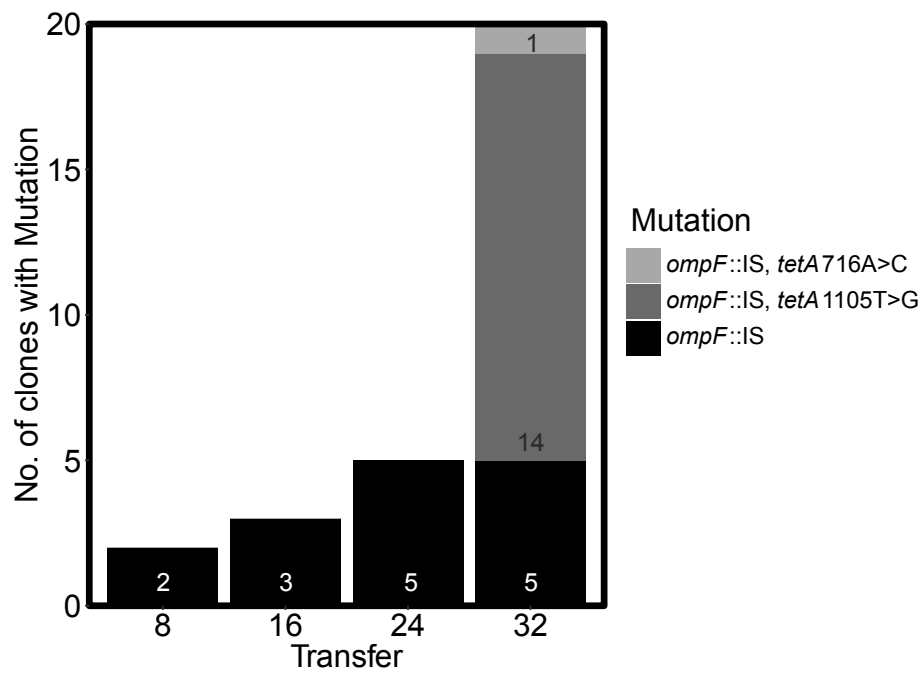
Figure S4.8 | Plasmid *tetA/tetR* mutations only occur after acquiring insertion sequences in *ompF*. Bars represent the number of independent clones from population AT2 that have *ompF*::IS mutations, detected by PCR, and the number of clones within this background with *tetA* or *tetR* mutations, detected by Sanger sequencing.
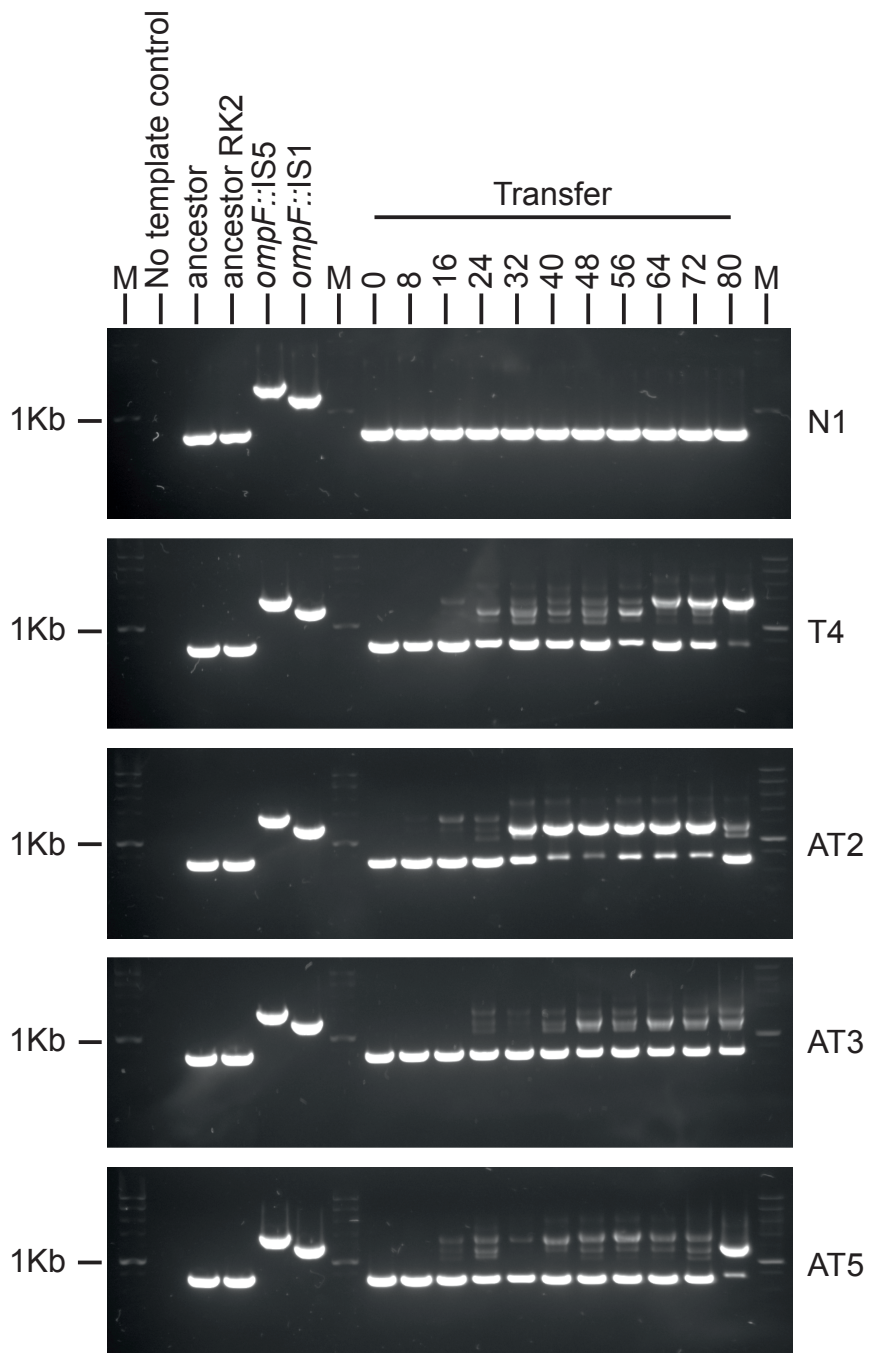
Figure S5.1 | Whole population PCR of *ompF* within populations N1, T4, AT2, AT3 and AT5. Short bands of 716 bp indicate on aquisiiton of IS elements within *ompF*. Bands of 1484 bp show the acquisition of IS1, and bands of 1911 bp show the acquisition of IS5 within *ompF*. The insertion of IS elements was never ombserved within the control population evolving with RK2 under no antibiotic selection. Lanes marked with M contain size markers.
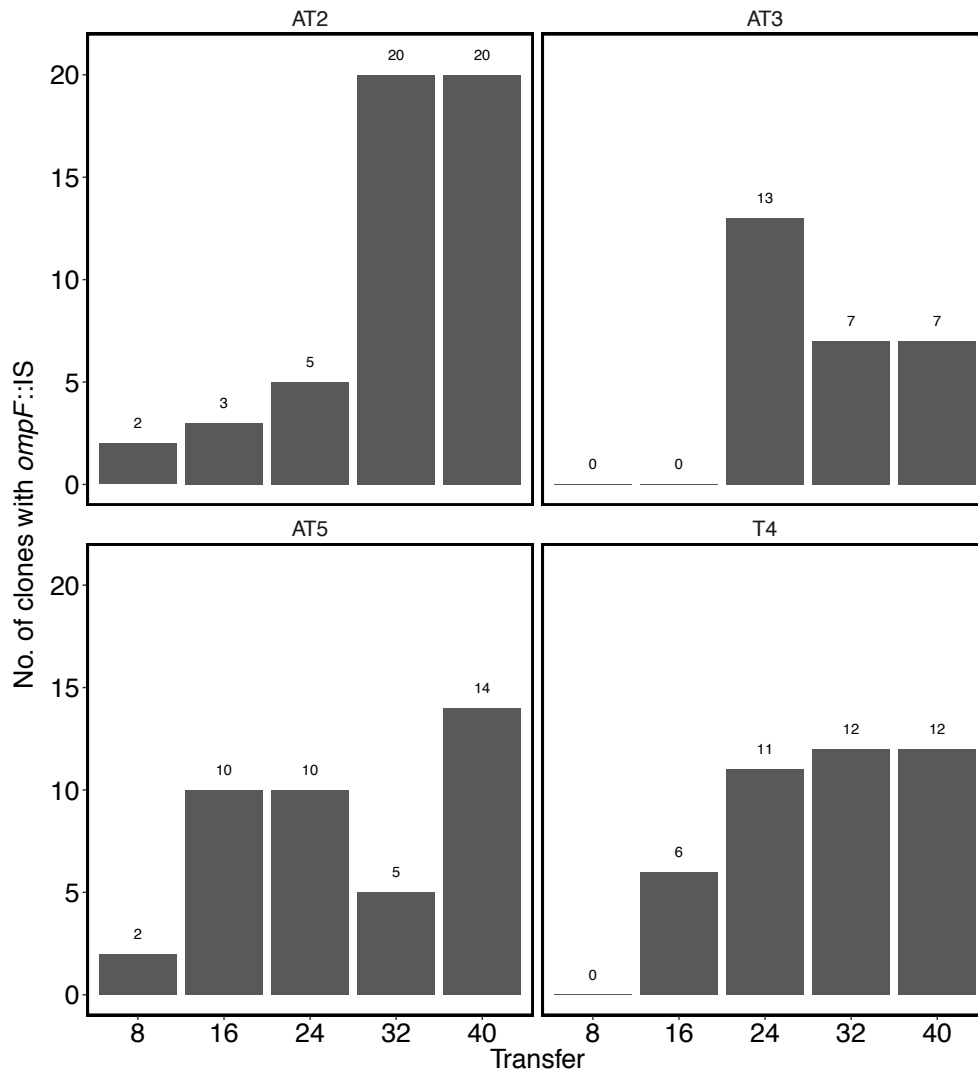
Figure S5.2 | Frequency of IS elements witin *ompF* between transfers 8 and 40 in population T4, AT2, AT3 and AT5. Bars represent the number of independent clones from population AT2 that have *ompF*::IS mutations, detected by PCR.
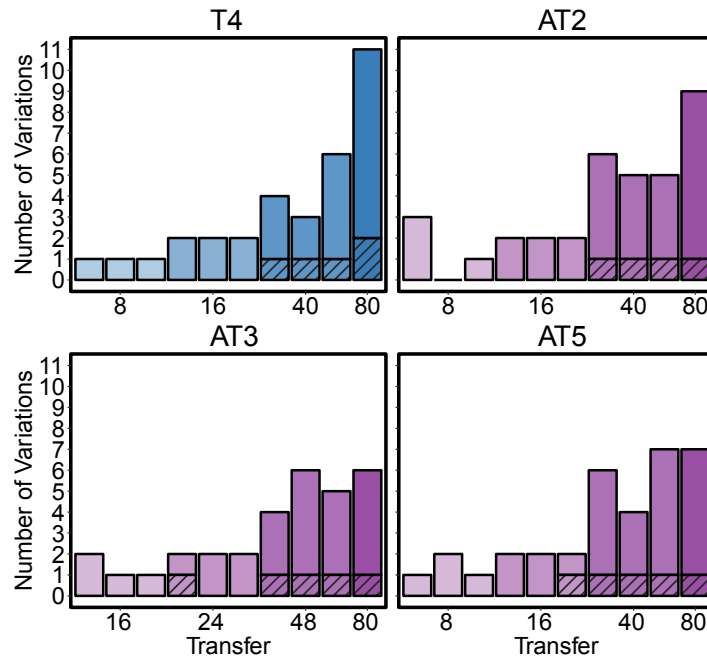
Figure S5.3 | Number of mutations within each clone through time in populations T4, AT2, AT3 and AT5. Solid fill shows chromosomal mutilations, dashed fill shows plasmid mutations.
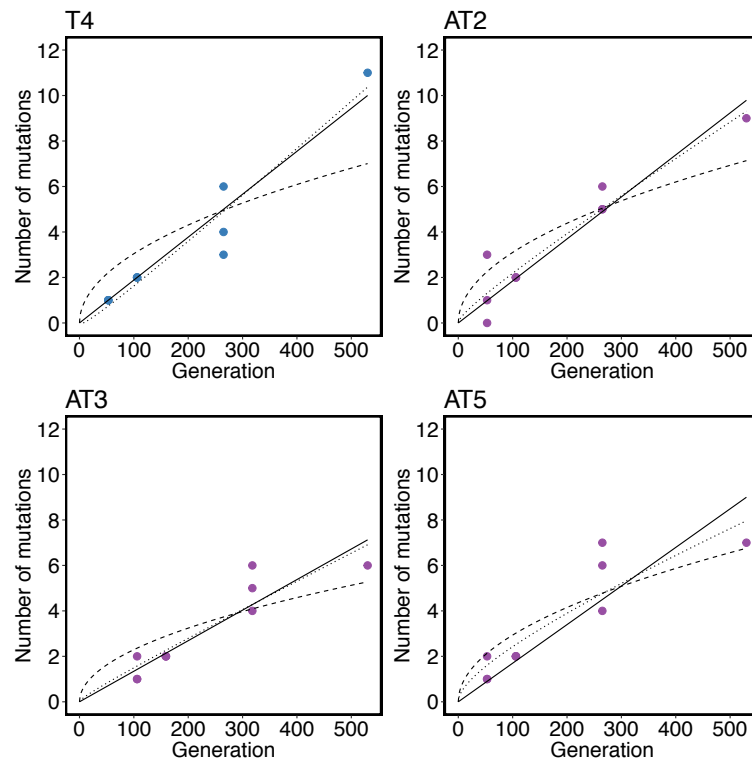
Figure S5.4 | Fits of models to the acquisition of mutations within populations T4, AT2, AT3 and AT5. The solid line shows the best fit to a linear model, the dashed line shows the fit to a root-squared model, and the dotted line shows the fit to the two parameter model, incorporating both the linear and root-squared terms.
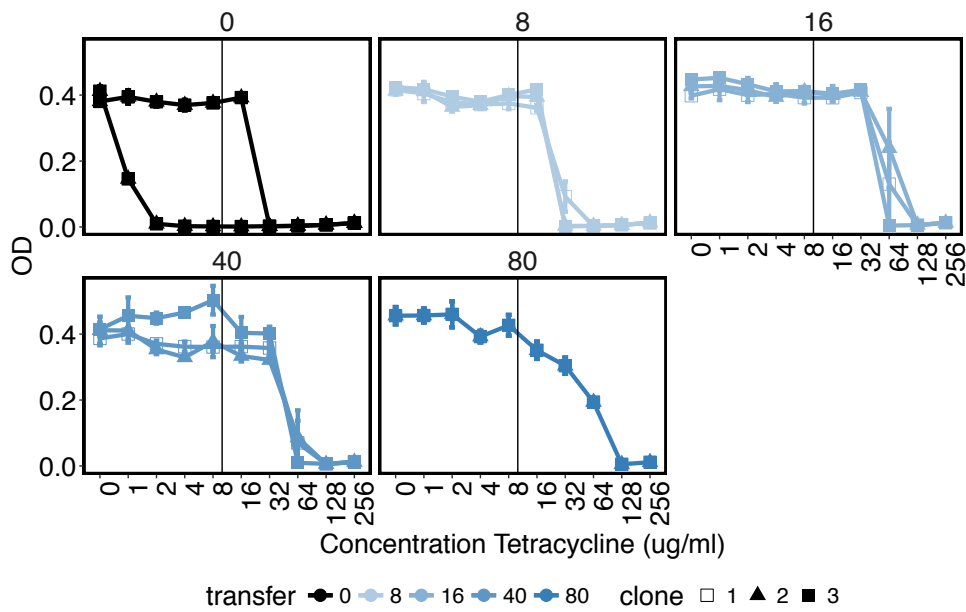
Figure S5.5 | The change in resistance profile through time of independent clones within population T4. Faceted by transfer clones were isolated from. The vertical line represents the concentration of tetracycline selected for during the evolution experiment. Points represent the mean of the three technical replicates with error bars showing SEM.
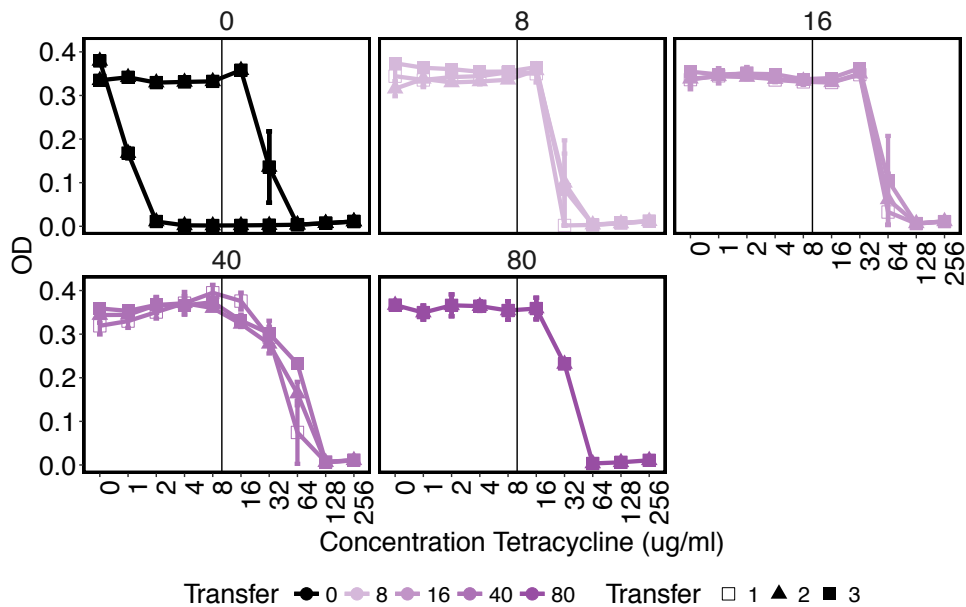
Figure S5.6 | The change in resistance profile through time of independent clones within population AT2. Faceted by transfer clones were isolated from. The vertical line represents the concentration of tetracycline selected for during the evolution experiment. Points represent the mean of the three technical replicates with error bars showing SEM.
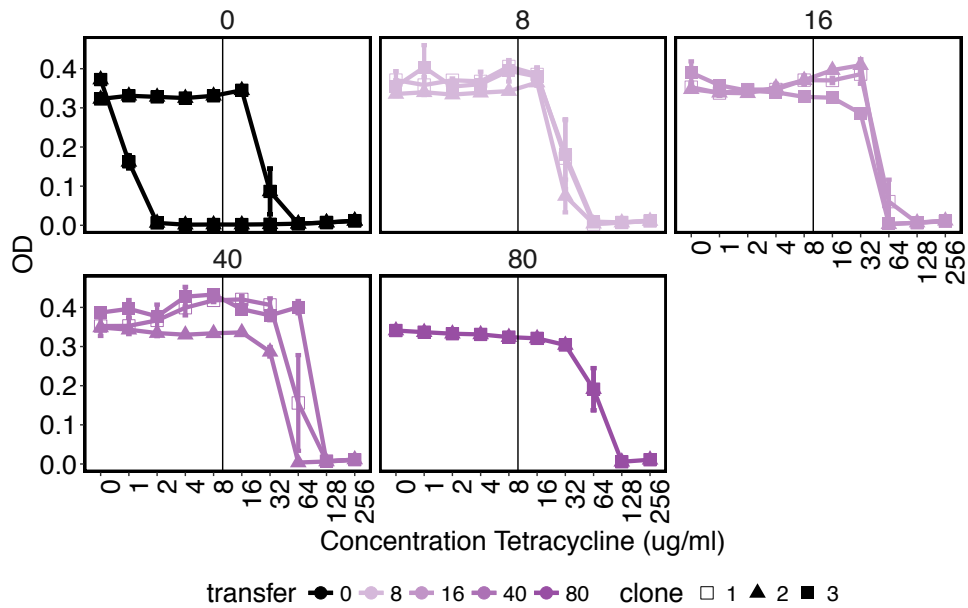
Figure S5.7 | The change in resistance profile through time of independent clones within population AT5. Faceted by transfer clones were isolated from. The vertical line represents the concentration of tetracycline selected for during the evolution experiment. Points represent the mean of the three technical replicates with error bars showing SEM.
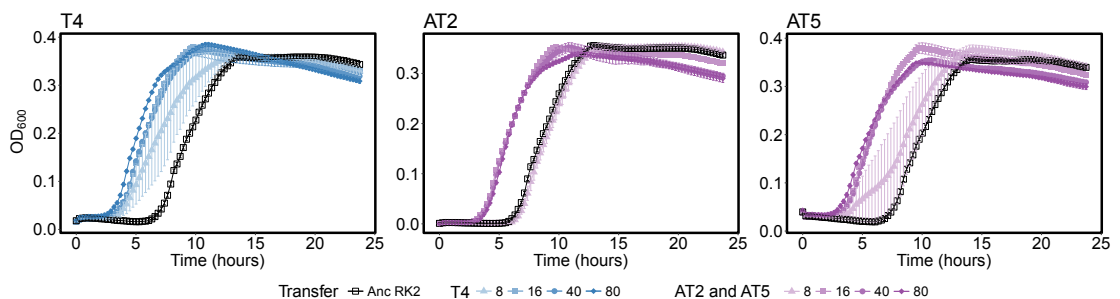
Figure S5.8 | Growth curves of the ancestral MG1655(RK2) and clones from transfers 8, 16, 40 and sequenced clones from transfer 80 from populations T4, AT2 and AT5 in the presence of tetracycline 10 µg/ml, the same concentration used in the selection experiment. Points are the mean of three replicates with error bars representing SEM.

Table S5.1 | ANOVA comparison of tetracycline resistance integrals with Tukey multiple comparison tests of **A** population T4, **B** population AT2, and **C** population AT5.

A Population T4

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	2.438	0.609	23.56	6.84e-09
Residuals	30	0.776	0.026		

Comparison	Difference	P adjusted
Anc – 8	-0.075	0.916
Anc – 16	-0.647	0.0001
Anc – 40	-0.464	0.0001
Anc – 80	-0.631	0.0001
8 – 16	-0.572	0.000
8 – 40	-0.389	0.0001
80 – 8	0.556	0.0001
40 – 16	-0.183	0.138
80 – 16	-0.017	0.999
80 – 40	0.167	0.535

B Population AT2

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	0.982	0.245	11.1	1.21e-05
Residuals	30	0.663	0.022		

Comparison	Difference	P adjusted
Anc – 8	0.033	0.994
Anc – 16	-0.274	0.019
Anc – 40	-0.372	0.001
Anc – 80	-0.179	0.482
8 – 16	-0.308	0.001
8 – 40	-0.405	0.000
80 – 8	0.211	0.231
40 – 16	0.097	0.639
80 – 16	-0.096	0.868
80 – 40	-0.193	0.315

C Population AT5

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	1.337	0.334	7.864	0.0002
Residuals	30	1.274	0.042		

Comparison	Difference	P adjusted
Anc – 8	-0.217	0.346
Anc – 16	-0.379	0.020
Anc – 40	-0.599	0.0001
Anc – 80	-0.380	0.111
8 – 16	-0.163	0.465
8 – 40	-0.382	0.004
80 – 8	0.164	0.756
40 – 16	0.219	0.186
80 – 16	0.001	1.000
80 – 40	-0.218	0.515

Table S5.2 | ANOVA comparison of length of lag phase when growing in tetracyclin 10 µg/ml with Tukey multiple comparison tests of **A** population T4, **B** population AT2, and **C** population AT5.

A Population T4

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	59.53	14.882	18.23	2.71e-09
Residuals	50	40.81	0.816		

Comparison	Difference	P adjusted
Anc – 8	2.179	0.0002
Anc – 16	3.339	0.000
Anc – 40	3.392	0.000
Anc – 80	3.796	0.000
8 – 16	1.160	0.008
8 – 40	1.213	0.005
80 – 8	-1.617	0.009
40 – 16	-0.053	0.999
80 – 16	-0.457	0.863
80 – 40	-0.404	0.908

B Population AT2

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	109.33	27.331	558.7	<2e-16
Residuals	50	2.45	0.049		

Comparison	Difference	P adjusted
Anc – 8	-0.246	0.214
Anc – 16	2.776	0.000
Anc – 40	2.759	0.000
Anc – 80	2.583	0.000
8 – 16	3.022	0.000
8 – 40	3.005	0.000
80 – 8	-2.829	0.000
40 – 16	0.018	0.999
80 – 16	0.193	0.448
80 – 40	0.176	0.543

C Population AT5

Factor	d.f.	Sum Squares	Mean Squares	F Value	P
Transfer	4	110.03	27.507	25.17	1.86e-11
Residuals	50	54.64	1.093		

Comparison	Difference	P adjusted
Anc – 8	1.336	0.113
Anc – 16	3.849	0.000
Anc – 40	3.726	0.000
Anc – 80	4.175	0.000
8 – 16	2.513	0.000
8 – 40	2.390	0.000
80 – 8	-2.829	0.000
40 – 16	0.123	0.998
80 – 16	-0.316	0.977
80 – 40	-0.439	0.925

Abbreviations

AIC	Akaike information criterion
AMP	Ampicillin
AMR	Antimicrobial Resistance
ARG	Antibiotic Resistance Gene
bp	Base Pair
CFU	Colony Forming Units
CML	Chloramphenicol
DNA	Deoxyribonucleic Acid
ESBL	Extending Spectrum β-lactamse
GFP	Green Fluorescent Protein
HGT	Horizontal Gene Transfer
ICE	Integrative and Conjugative Elements
IS	Insertion Sequence
kb	Kilobase
Mb	Mega-base
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSC	Minimum Selectable Concentration
NB	Nutrient Broth
NDM	New Delhi metallo-β-lactamase
OD	Optical Density
PCR	Polymerase Chain Reaction
PK-PD	Pharmacokinetic-Pharmacodynamic
SEM	Standard Error [of the] Mean
SNP	Single Nucleotide Polymorphism
ssDNA	Single Stranded Deoxyribonucleic Acid
TA	Toxin-Antitoxin
TET	Tetracycline

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