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Evolution of *de novo* multidrug resistance in experimental bacterial populations: insights from pharmacodynamic fitness landscapes, recombination, and compensatory mutations

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Abstract

Antibiotic resistance is an emerging global medical crisis. Resistant pathogens can evolve and spread rapidly in response to selection pressures exerted by antibiotics. A major concern in particular is the evolution of resistance to multiple drugs in many clinical pathogenic bacteria. Specifically, multidrug resistance (MDR) can appear either by *de novo* mutations or by acquiring resistance determinants from exogenous sources via recombination. However, the relative influence of mutation and recombination on bacterial adaptation to multiple drugs remains largely unknown. Therefore, I studied the evolution of *de novo* multidrug resistance in *Acinetobacter baylyi*, a Gram-negative environmental bacterium has also recently been found to be associated with clinical infection, by characterizing two-locus pharmacodynamic fitness landscapes, by assessing the role of recombination through natural transformation, and studying the spread of compensatory mutations.

In chapter 2, I characterized two-locus pharmacodynamics fitness landscapes (PDFLs) by constructing three sets of four different genotypes comprising streptomycin, rifampicin and kanamycin resistant mutants. I analysed the resulting PDFLs by characterising epistasis, collateral sensitivity or cross-resistance, drug interactions and mutation selection windows to understand the trajectories and rate of evolutionary adaptation of resistant pathogens at multiple loci under each of the corresponding antibiotic pairs with several hundreds of antibiotics concentrations. My results showed that fitness landscapes were largely non-epistatic between each pair of resistance conferring mutations in all three pairs of drug environments. However, a wider mutant selection window (MSW) was apparent for some drug pairs. I also found that a wider MSW can also occur in presence of cross-resistance (associated with a mutation conferring simultaneous resistance to several drugs belonging to the same class), which may have important implications in resistance evolution. In addition to this, I found a minor sign of an Eagle effect (non-monotonic effect of drug concentration on growth rate). Overall, the results we present here underline the importance of choosing the right drug for both combination and cyclic treatment to exclude the prolonged exposure of sub-MIC level of antibiotics.

In chapter 3, I investigated the impact of recombination via natural transformation on the MDR evolution under static two-drug (rifampicin and streptomycin) antimicrobial combinations by employing an experimental evolution experiment. Here, I characterized the ancestor and the evolved populations with respect to *de novo* multidrug resistance by using different phenotypic assays and by next generation sequencing. Growth rate and competition assays demonstrated higher fitness of the populations propagated under drug pressures, but there was no difference in fitness gain between recombination-proficient and -deficient populations. Moreover, antibiotic

susceptibility assays showed that clones that evolved in presence of drugs had become strongly resistant to rifampicin but resistance to streptomycin was only weak. Additionally, *de novo* resistance to other antibiotic classes including ciprofloxacin was also observed in these populations. Consistent with these findings, whole genome sequencing revealed an abundance of drug specific resistance mutations, including *rpoB* mutation indicating target alteration for rifampicin as a resistance mechanism as well as mutations associated with multidrug efflux system encoding genes representing phenotypic resistance to multiple drugs. In conclusion, adaptive benefit of recombination via natural transformation under sub-lethal concentration of rifampicin and streptomycin is likely constrained by the low number of mutations that were spreading, which prevented clonal interference.

In chapter 4, I investigated the adaptive role of compensatory mutations in a set of resistant populations comprising both single and multidrug resistant *A. baylyi* carrying both rifampicin and streptomycin resistance mutations by employing an evolution experiment in absence of drug pressure. I characterised the evolved endpoint and ancestral populations through phenotypic assays and by next generation sequencing to explore the adaptive mechanisms that have taken place during the course of evolution. My results revealed that the deleterious effects of resistance mutations are compensated for by varying degrees among different sets of resistant genotypes. Specifically, adaptation to the cost of resistance in a set of multidrug resistant and rifampicin resistant populations was higher compared to the subset of streptomycin resistant populations. This greater fitness improvement in multidrug resistant genotype was influenced by some of the fittest lineages following a bimodal fitness distribution without altering the MIC of the primary antibiotics. Whole genome sequencing data revealed that both costly rifampicin resistant and double resistant lineages adapted by compensatory mutations in the RNA polymerase core enzyme. However, only minor fitness compensation to the low cost streptomycin resistance mutation was observed, and without evolving any putative compensatory mutations. We observed a different parallel adaptive evolution in the double resistant genotype, which harboured an unexpected additional deleterious mutation. Surprisingly, half of the lineages originating from this double resistant genotype were better compensated for through reversion mutations, which were also accompanied by distinct compensatory mutations in RNA polymerase core enzyme, and those mutations were not shared by any rifampicin resistant genotypes. Finally our data suggest that adaptation to the cost of multidrug resistance is independent of the genetic background of mutations that appeared in single resistant genotypes.

Declaration by author

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Contributions by others to the thesis

Chapter	Activities	Contribution						
		HCM	JE	DOB	JH	NH	HNNV	SUM
Chapter 1	Writing	100%	0%	0%	0%	0%	0%	100%
Chapter 2	Concept and experimental design	80%	20%	0%	0%	0%	0%	100%
	Resistant genotype construction	95%	0%	0%	0%	5%	0%	100%
	Performed experiments	100%	0%	0%	0%	0%	0%	100%
	Data analysis	95%	5%	0%	0%	0%	0%	100%
	Interpretation of data	95%	5%	0%	0%	0%	0%	100%
	Writing	100%	0%	0%	0%	0%	0%	100%
Chapter 3	Concept and experimental design	80%	20%	0%	0%	0%	0%	100%
	Performed experiments	98%	0%	0%	0%	0%	2%	100%
	Data analysis	80%	10%	10%	0%	0%	0%	100%
	Interpretation of data	95%	5%	0%	0%	0%	0%	100%
	Writing	100%	0%	0%	0%	0%	0%	100%
Chapter 4	Concept and experimental design	90%	10%	0%	0%	0%	0%	100%
	Performed experiments	98%	0%	0%	2%	0%	0%	100%
	Data analysis	95%	5%	0%	0%	0%	0%	100%
	Interpretation of data	95%	5%	0%	0%	0%	0%	100%
	Writing	100%	0%	0%	0%	0%	0%	100%
Chapter 5	Writing	100%	0%	0%	0%	0%	0%	100%

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Keywords

infectious diseases, adaptive evolution, multidrug resistance, pharmacodynamics fitness landscapes, epistasis, drug-interaction and cross-resistance, collateral sensitivity, mutant selection window, clonal interference and recombination, compensatory mutation

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ANZSRC code: 060411 Population, Ecological and Evolutionary Genetics 45%

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FoR code: 0605 Microbiology 50%

FoR code: 0604, Genetics, 40%

FoR code: 1103 Clinical Sciences 10%

Table of contents

LIST OF FIGURES

List of Abbreviations

MDR	Multidrug resistance
PDR	Pandrug resistance
MRSA	Methicillin resistance *Staphylococcus aureus*
VRE	Vancomycin resistant *Enterococci*
VRSA	Vancomycin Resistance *Staphylococcus aureus*
XDR	Extensively Drug Resistance
ND	Not Detected/Determined
Mar	Multiple antibitoc resistance
SA	*Staphylococcus aureus*
TCA	Tricarboxilic Acid Cycle
PBP	Penecillin Binding Protein
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
OMP	Outer Membrane Porin
MGE	Mobile Genetic Element
ESBL	Extended Spectrum β-lactamase
RpsL	Ribosomal protein sub-unit L
RpoB	RNA-polymerase bete-subunit
QRDR	Quinolone Resistance Determining Region
PDFL	Pharmacodynamic Fitness Landscape
CM	Compensatory Mutation
Gyr	Gyrase
NDM-1	New-Delhi Metallo-β-lactamase 1
RNAP	RNA polymerase
MIC	Minimum Inhibitory Concentration
SE	Sign epistasis
nptII	neomycin-phosphotransferase-II
Cfp	Cyan fluoresence protein
Yfp	Yellow fluoresence protein
PCR	Polymerase Chain Reaction
E-test	Epsilometer test
HGT	Horizontal Gene Transfer
ND	No Drug
LD	Low Drug
HD	High Drug

Chapter 1: General introduction

Drug resistance evolution: a global threat

The introduction of antimicrobials to treat against infectious diseases was one of the greatest medical accomplishments in history. The objective was to save millions of lives facing severe infectious diseases. These antimicrobial drugs were used against a wide array of infectious diseases caused by bacteria, fungus, viruses and parasites [2]. However, the global emergence of resistance mechanisms among these pathogens has seriously undermined our current treatment options in many ways. Therefore, treatment failure due to the emergence of antimicrobial resistance has now become a global public health threat and a great economical problem [3]. Among these pathogens, bacteria are the most striking example – in terms of morbidity and mortality. Bacteria exploit many efficient strategies to deactivate the action of antibiotics, often leaving no effective antimicrobial compounds. Such resistant pathogens evolve very rapidly whenever antimicrobials are applied. Successful use of these therapeutic agents is mainly compromised due to the development of tolerance or resistance against them. Pathogens use diverse physiological and biochemical mechanisms in order to develop tolerance or resistance against antimicrobials. Studies concerning the population biology and evolutionary principle of such resistance are largely lacking, which is probably paving the way for these pathogens to develop resistance against diverse classes of antimicrobials.

The first antibiotic to treat against infectious diseases – the sulphonamide – was in clinical practice in 1937, but the development of resistance against this compound was severely compromised by distinct mechanisms of resistance which are still found to operate in current ages. The first discovered naturally occurring antibiotic, namely penicillin was discovered by Alexander Fleming from *Penicillium notatum* in 1928 and introduced in the 1940s; however, the penicillin degrading enzyme penicillinase severely compromised the usefulness of this drug soon after its introduction [4]. The first antibiotic belonging to the aminoglycoside class called streptomycin was introduced in 1944 to treat against *Mycobacterium tuberculosis* – the causative agent of tuberculosis. The usefulness of this antibiotic was also thwarted by as soon as resistance was evolved. In almost every cases, resistance mechanisms against antimicrobials were reported before their introduction, which raises important but yet unsolved questions regarding the origin of resistance and antibiotics [2]. Many studies have highlighted the source of these resistance genes, where most resistance genes were found in natural environments [5].

The bulk of the antibiotics that are used today were discovered and introduced into the market during the 1950s, sometimes referred to as the golden years in the history of the antibiotics era. Before the 1960s, resistance plasmids were found to be responsible for resistance against those antibiotics; therefore many pharmacological studies were carried out during 1960s and those studies mainly concentrated on the understanding of the mode of action of antibiotics as well as their administration. Later on, sixty-one different antibiotics were approved by the FDA (Food and Drug Administration, U.S.A.) between the year 1980 and 2009, but nearly half of them were withdrawn. Some drugs were removed for the safety and effectiveness issues while other were (apparently most of the drugs) removed owing to marketing policy related issues [6], such that it could be due to (1) infrequent prescription of those drugs, (2) the raw materials were very expensive, (3) the production process required multistep complicated process. Together these had made those drugs less profitable for the company; therefore those drugs were withdrawn from the market.

However, the dearth of new antimicrobial classes was apparent between 1943 and 2000; during this period, only a few (~9 different classes) new antimicrobial classes were discovered and launched into the markets (**Figure 1**). Overall, in the last 50 years, only seven new classes of antibiotics, namely linezolid (2000), tigecycline (2005), daptomycin (2006), retaparmulin (2007), telavancin (2008), fidaxomicin (2011), and bedaquiline (2012) have come into clinical application with a single variant of each class [7, 8] . All these new classes of antibiotics were introduced after 2000, and resistance emergence against three of them, namely linezolid, daptomycin and tigecycline were also documented in clinical bacterial strains – for example drug resistance to more than one antibiotic was reported during this time in *Escherichia coli*, *Salmonella*, *Shigella*, MRSA (methicillin-resistant *S. aureus*), VRE (vancomycin-resistant *enterococci*), and others [9]. The rest of the four new classes, including retaparmulin, telavancin, fidaxomicin, and bedaquiline fall into narrow spectrum antibiotics targeting only a limited number of bacterial species belonging mostly to gram positive; no resistance emergence has been reported against them. This situation reflects the fact that the emergence and spread of resistant strains has been faster than antimicrobial drug development [10]. Further, this scenario suggests that antibiotics are gradually decreasing their efficacy against infections due to the evolution of extremely multi-resistant bacteria – mostly deadly Gram negative bacteria via enormous genetic flexibility. Most of these resistant bacteria are found in hospital settings where bacteria encounter high selective pressure exerted by antibiotics [11].

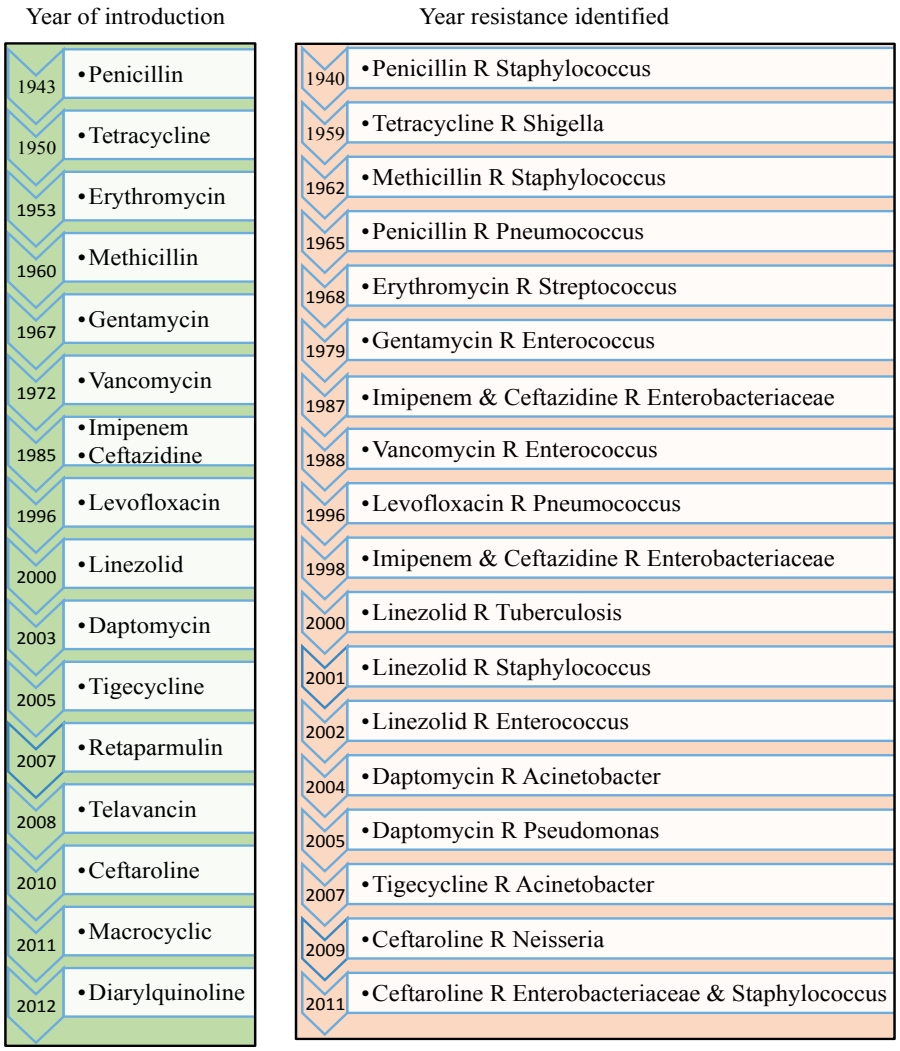

Figure 1 Sequential drug discovery and resistance evolution. Left panel shows year of drug discovery, and the right panel shows year of resistance emergence. No reported case of resistance evolution documented for Retaparmulin, Telavancin, Macrocyclic and Diarylquinolone. R – resistant

Based on the extent of resistance to multiple drugs, bacteria have been classified into three groups. According to this definition, multi-drug resistant (MDR) bacteria are those that are resistant to one agent of three or more antimicrobial classes. Extensively drug-resistant (XDR) bacteria remain non-susceptible to at least one antimicrobial agent of 2 or fewer categories, whereas pandrug-resistant (PDR) bacteria are non-susceptible to all agents of all antimicrobial categories. This definition is applicable to both Gram positive and Gram negative bacteria such as *S. aureus*, *Enterococcus* spp., *Enterobacteriaceae* (other than *Salmonella* and *Shigella*), *Pseudomonas aeruginosa* and *Acinetobacter* spp. [12]. In this study, we defined multidrug

resistance bacteria are being resistant to at least two antimicrobial compounds belonging to either the same chemical class or two different classes.

Antibiotics and their functional targets

Antibiotics belong to different classes of chemicals, are of biological, synthetic or semi-synthetic origin and have selective modes of actions. Based on their mechanism of actions, antimicrobial compounds are classified into two groups, namely bactericidal and bacteriostatic. Drugs are called bactericidal when exposure to this particular group of antibacterial compounds leads to the death of bacteria, whereas bacteriostatic drugs are only able to inhibit or hinder growth of bacteria but cannot kill bacteria. Both of these drug actions are principally targeted to the interference of bacterial cell-wall biosynthesis, DNA or RNA synthesis or repair [4]. Further, antibiotics have been classified based on the cellular component or system they affect [13]. Some of these antimicrobial agents target the synthesis of important cellular components, whereas some other classes target bacterial nucleic acid synthesis or repair [14, 15]. For example, fluoroquinolones target and inhibit important bacterial cellular system, such as topoisomerase II (also known as DNA gyrase), and lead to the cellular death by formation of double-strand DNA breaks [16]. Another class of bactericidal antibiotics is the β-lactam antibiotics (i.e., penicillins, cephalosporins, capbapenems and monobactams), which act by binding to and inhibiting the penicillin binding proteins (PBP) leading to stop in cross-linking or transpeptidations within the bacterial cell wall, thus undergo cellular death [17]. Furthermore, some antimicrobial induced cell death is mediated by common detrimental response to drug-induced stresses. For example, bacteria response to most of the bactericidal antibiotics in a unfavourable way, such that bacterial important metabolic system, including central metabolic system called tricarboxilic acid (TCA) cycle as well as iron metabolism is inhibited by reactive oxygen radicals in response to lethal bactericidal antibiotics, resulting in cellular death [18, 19].

In what follows, I will briefly outline the mechanism of cellular killing by the bactericidal antibiotics namely rifampicin (belongs to ansamycin) as well as streptomycin and kanamycin (both belong to the aminoglycoside), since I have used these antibiotics in all three individual research projects of this PhD thesis.

Rifampicin is a semi-synthetic bactericidal antibiotic that can induce cell death by inhibiting bacterial RNA synthesis. Rifampicin triggers catastrophic effect on prokaryotic nucleic acid metabolism by interfering with β-subunit of RNA polymerase [20]. During execution of normal cellular function, β-subunit forms a stable channel between RNA–polymerase and DNA complex from which newly synthesized RNA strand arises [21-23]. Rifampicin binds stably and with high affinity to the β-subunit of DNA dependent RNA–polymerase (encoded by *rpoB* gene), thus inhibiting the high fidelity transcription and causing cellular death.

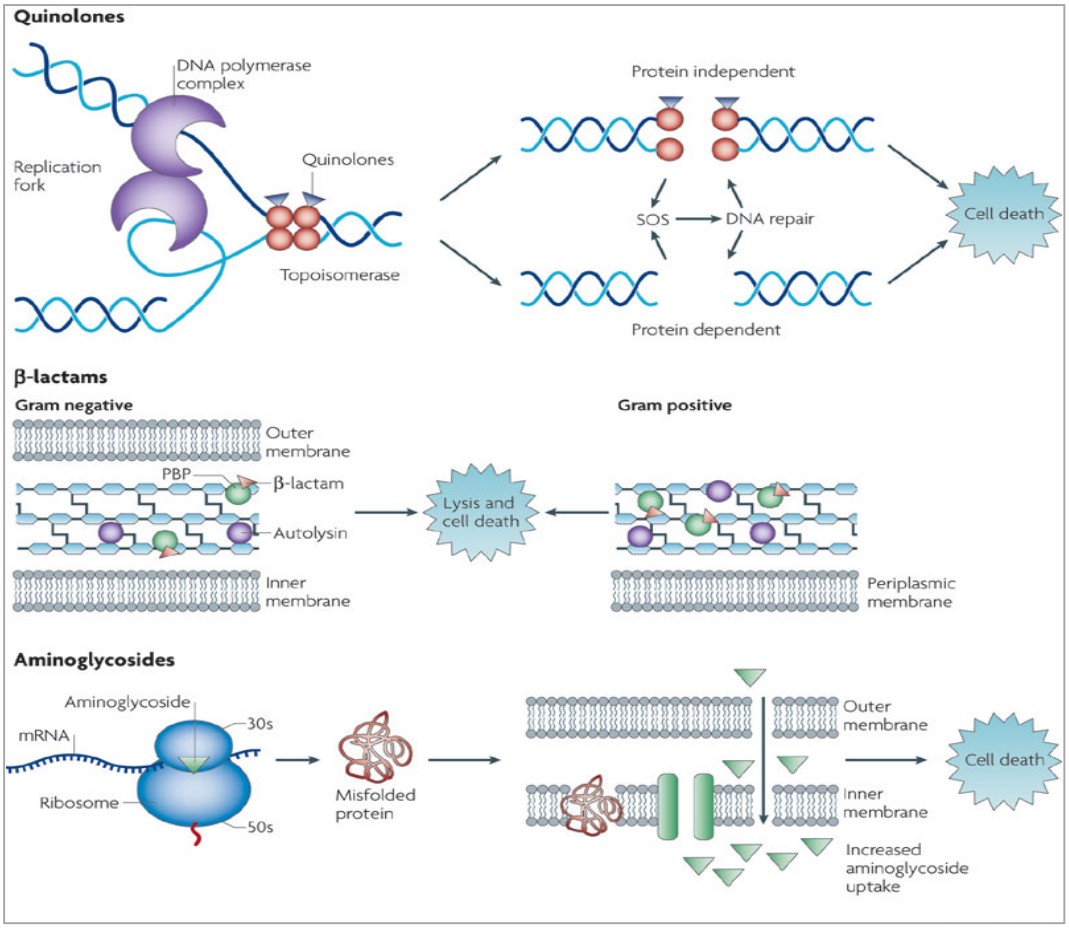

Figure 2 Drugs, their cellular targets and the mechanisms cellular death. This figure shows different drugs and their different targets. Figure 2 reproduced from [1].

Aminoglycosides cause cellular death by interfering with cellular energetics, ribosome binding and protein synthesis [24]. Bacterial protein synthesis through translation of mRNA occurs in a sequential fashion involving the phases including initiation, elongation and termination. This process is operated in the cytoplasmic space involving ribosome as factory and many other

important accessory translation factors available in the cytoplasm [25]. The ribosome is composed of two ribonucleoprotein subunits called 30S (encoded by rpsL gene) and 50S (**Figure 2**). Following formation of a complex between mRNA-transcript, N-formylmethionine-charged aminoacyl tRNA, several initiation factors and a free 30S subunit (this process is called initiation step of translation), ribosome is assembled for the next translational step [25, 26]. Since this translation is a complex process requiring many cellular component parts and translation factors, drugs can interfere with protein synthesis in many different ways. Protein synthesis inhibitors are classified into 50S inhibitor and 30S inhibitor. 50S inhibitors (i.e. erythromycin, clindamycin, streptogramin, chloramphenicol, and linezolid) interfere with protein synthesis by blocking initiation of protein translation (i.e., oxazolidinones), or translocation of peptidyl tRNAs [27, 28]. Inhibition of 30S ribosome involves blocking of the access of aminoacyl tRNAs to the ribosome. 30S ribosome inhibitor comprises tetracyclines and aminoclyclitols. Both spectinomycin and aminoglycosides, including streptomycin, kanamycin and gentamycin bind to the 16S rRNA component of the 30S ribosomal subunit. Aminoglycosides binds to 16S rRNA which in turn alter the conformation of the complex formed by an mRNA codon and its cognate charged aminoacyl tRNA at the ribosome. This altered complex molecule results in defective protein [14, 29, 30].

Types and mechanisms of antibiotic resistance

Antibiotic resistance can be defined as the property inherent in bacteria by which successful uses of therapeutic agents are compromised by the evolution of tolerance or resistance against it [2, 31]. Generally, antibiotic resistance is associated with prolonged exposure to antibiotics. More specifically, bacterial population remains susceptible to antibiotics at the beginning of a treatment, but can sustain and evolve resistance against antibiotics gradually. Therefore, the continuous selective pressure exerted by antibiotics help bacteria evolve resistance to one or more drugs simultaneously [32]. Bacteria can subvert the action of antibiotics through many different ways, and this resistance fall into two major types, namely innate resistance and acquired resistance. In innate resistance, genes encoding resistance to antibiotics are inherently present in bacteria. On the other hand, bacteria can both survive and acquire resistance in presence of lethal effect exerted by antibiotics [33], and this phenomenon of resistance is called acquired or adaptive resistance. Now I will give a brief over view of these two mechanisms:

Intrinsic resistance

With intrinsic resistance, naturally occurring genes in the host cell chromosome impart resistance. All bacterial species exhibit intrinsic resistance to a certain array of antimicrobial

classes. The biology of these resistance mechanisms varies from bacteria to bacteria. Many bacteria carry genes or enzymes giving intrinsically resistance to particular antibiotics. For example, Streptomyces possesses some genes, which provide resistance to streptomycin antibiotic produced by itself. Enzymatic modifications of antibiotics have been well reported in many clinical bacterial populations. Such resistance mechanisms have commonly been documented for natural antibiotics, including aminoglycosides (i.e., kanamycin, amikacin and tobramycin) and b-lactam antibiotics. In each case, certain enzymes can modify the chemical component of the antibiotics, which in turn result in altered drug-target interactions [60]. Commonly observed aminoglycosides modifying enzymes are aminoglycoside acetyltransferase (AAC-3-II), aminoglycoside phosphorylase (APH-3'-I), and adenylate (nucleotidyltransferases). For example, AAC-3-II can modify a number of different aminoglycosides including amikacin, gentamycin and tobramycin. These enzymes are mostly carried by mobile genetic elements, and responsible for resistance to multiple antibiotics. It has been reported that multiple acetyltransferase encoding genes were carried on class-1 integron in clinical P. aeruginosa, and this bacterium was non-susceptible to many other classes of antibiotics including carbapenems and sulfonamides [61]. Aminoglycoside acetyltransferases also exhibits broad-spectrum activity. For example, in E. coli, a variant of aminoglycoside acetyltransferases (i.e. AAC(6')-Ib) has been found to be associated with reduce susceptibility to ciprofloxacin – a synthetic antibiotic compound belongs to the fluoroquinolone class [170]. Enzymatic inactivation of b-lactam antibiotics is also common in many multidrug resistant clinical bacteria. Genes located on plasmids mostly encode these b-lactam degrading enzyme, but chromosomal genes can also encode this enzyme. Most clinical relevant b-lactam hydrolyzing enzymes are b-lactamase (first reported in S. aureus against penicillin), TEM b-lactamase (gives resistance to multiple drugs and commonly found in gram negative bacteria containing also multidrug resistant R plasmids), CTX-M b-lactamase (this enzyme is encoded by chromosome of gram negative bacteria, subsequently transferred to the R plasmid). All these enzymes are belonging to ESBL (extended spectrum b-lactamase) enzyme [62, 63].

Impermeability is another form of intrinsic resistance inherent in bacteria, where antimicrobial compounds cannot pass through the bacterial outer membrane. For example, glycopeptide antibiotics such as vancomycin can only target peptidoglycan cross-linking by binding to peptide chain of D-ala-D-ala of gram-positive bacteria, but it cannot pass through the outer membrane and reach to the peptides in the periplasm in Gram negative bacteria [35]. Another important resistance predominantly found in Gram negative bacilli is intrinsic mechanism of

resistance via efflux pump system. Efflux pumps are the major contributor of intrinsic resistance in Gram negative bacteria, which can actively transport multiple antibiotics out of the cell [59]. Even if an antibiotic can pass through to the periplasmic space by a membrane spanning porin protein (i.e. outer membrane porin (OMP)), it is removed or pumped out from the periplasm by active efflux system. Overexpression of efflux systems has been found to be associated with high-level resistance to many clinically important antibiotics. Efflux systems in bacteria are classified into two major groups based on their substrate specificity: substrate specific efflux pump can only transport a certain antibiotics (e.g., Tet efflux system which can only pump tetracycline out), whereas a broad-spectrum substrate specific efflux system can pump out many different antibiotics (MDR efflux pumps). Chromosomal genes encode these MDR efflux pumps, and sometimes these genes are transferred onto plasmids, which in turn disseminate to many other bacteria. There have been many of such MDR efflux systems reported in MDR bacterial populations [44]. Recently, it has been reported that genes encoding RND (resistance nodulation division) efflux pumps have been mobilized onto plasmid IncH1 in *Citrobacter freundii*, which also carried an NDM-1 metallo-β-lactamase 1[76]. An example of intrinsic resistance mechanisms have been provided by Figure 3 which shows that β-lactam antibiotic (A) targeting a penicillin-binding protein (PBP) first channel through the periplasm via membrane spanning porin protein and binds to the target site of penicillin binding protein (PBP), and thus interfere with bacterial cell wall synthesis – a natural mechanism of action of β-lactam antibiotic. However, antibiotic B can pass through the porin channel but is effectively pumped out from the periplasm via efflux pump, whereas antibiotic C cannot cross bacterial outer membrane. Therefore, such mechanism of resistance to both antibiotics (antibiotic B and C) called intrinsic resistance [36].

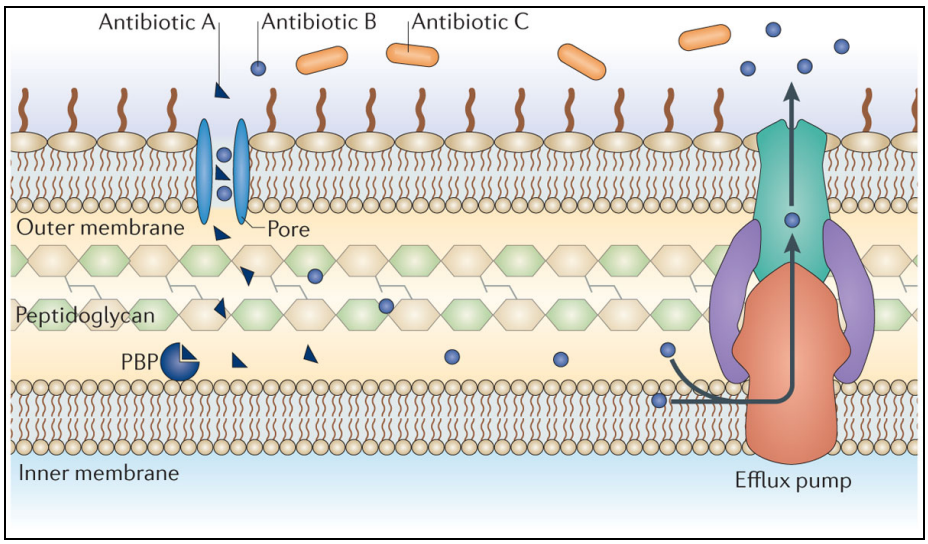

Figure 3 Intrinsic mechanism of antibiotic resistance. Figure 3 reproduced from [36].

Acquired resistance

Acquired mechanisms are associated with mutations in different chromosomal genes targeted by antibiotics, or the acquisition of many different resistant genes from other bacterial species mediated by mobile genetic elements (MGEs) such as plasmids, transposons and integrons [11, 34, 37, 38]. In the case of mutational resistance, bacteria often alter the binding site of the proteins targeted by antibiotics. Bacteria often alter the binding site of the proteins targeted by antibiotics. Mutation in the drug target also promotes over expression of targets during transcription step, these targets otherwise are naturally expressed at very low level. Or it could be another mechanism by which certain mutation can modify the drug target to withstand the lethal effect of an antibiotic. Consequently, the minimum inhibitory concentration (MIC) of a particular antibiotics rises beyond the therapeutic value which in turn preclude the clinical use of the drug [39]. Such mechanisms are not induced by antibiotic exposure, rather such resistance mutations appear during chromosomal replication. In Gram negative bacilli, such as in *Enterobacter* sp. and *Pseudomonas aeruginosa*, mutation in regulatory gene can give overexpression of the *bla*$A_{mpC,}$ together with AmpC cephalosporinases, this overexpressed gene undergoes imbalance between enzyme and substrate ratio, and both of them thus give resistance to both penicillin and extended-spectrum cephalosporin [40].

There have been plenty of such mutational resistances documented in bacterial populations; for example, mutational changes in the penicillin binding protein 2b can result in penicillin resistance in *Pneumococci.* Mutations in *M. tuberculosis, S. aureus, E. coli, P. aeruginosa*, and *Acinetobacter baumannii* give altered ribosomal binding sites and confer high-level resistance

to aminoglycosides. Certain mutations can cause up-regulation of enzyme production or alteration of outer membrane porin (OMP), and this in turn impedes antibiotic penetration inside the bacterial cell, for example mutational change in the outer membrane porin (OMP) in *E. coli* and *P. aeruginosa* gives resistance to many antibiotics. Up-regulation of efflux pumps by mutation can also expel antibiotics out of the bacterial cells, and this phenomenon has been observed in fluoroquinolone resistant *S. aureus and P. aeruginosa* [34, 40-43]. These acquired mechanisms conferred by mutations in different chromosomal genes are collectively called vertical evolution. More example of mutational resistances such as clinically important fluoroquinolone resistance is conferred by mutations within the targets such as DNA gyrase (comprised of GyrA and GyrB protein) and topoisomerase IV (comprised of ParC/GrlA and ParE/GrlB protein) [64]. Most of these mutations conferring resistance to fluoroquinolone are located in a region called quinolone resistance determining region (QRDR) of GyrA and ParC/GrlA protein. Interestingly, mutations occured first in DNA gyrase in gram–negative bacteria, whereas mutations in topoisomerase IV first reported in gram-positive bacteria [53]. Another prominent example of resistance mediated by mutational target alteration is resistance to rifampicin antibiotic. Rifampicin in combination of another drug including isoniazid, pyrazinamide, ethambutol, or streptomycin remains the first-line therapy against tuberculosis infection [65]. However, mutation in *rpoB* gene causes conformational change in RNA polymerase β-subunit, thus inhibiting rifampicin from binding to its target site of the RNA-polymerase. Mutation-conferring resistance to aminoglycosides are also common in many resistant bacterial populations including *M. tuberculosis*, whereas mutations in *rrs* gene conferring resistance to amikacin and kanamycin are also well documented in many other bacterial populations. Mutational alteration in small ribosomal protein (S12) encoded by *rpsL gene,* and also mutation in *rrs* gene conferring resistance to streptomycin or other aminoglycosides in *M. tuberculosis* are well documented [66]. Therefore, target alteration by mutation in many different genes can give rise to multiple drug resistance in clinically important pathogens including *M. tuberculosis.* From a population genetics perspective, these mutational alterations at multiple loci often produce extensive patterns of genetic interactions across the loci in antibiotic resistant bacteria, called epistasis [67-70]. This interaction also determines the evolutionary responses to a variety of environmental conditions, including in presence of multiple antibiotics [71-75]. Thus, even if the drug exposure is halted, resistant mutants may outcompete the susceptible counterparts and adapt in the drug free environment by this evolutionary responses through extensive multilocus genetic interactions, as has been reported in a multidrug resistant bacteria [69].

Certain mutations in bacterial cell wall components can cause altered membrane permeability. For example, in Gram negative bacteria, cells are covered by an additional layer, which restricts the entry of both hydrophobic and hydrophilic compounds into the cell. Under diverse physiological circumstances, bacteria transform this permeability barrier mediated by porin proteins, which helps bacteria transport different compounds in and out of the cell. These evolved porin proteins have been associated with multidrug resistance. For example, OmpF and OprD protein are commonly occurring porin proteins found in *E. coli* and *P. aeruginosa,* respectively. Both of them are involved in non-specific entry and exit points for different antibiotics and small chemical molecules. These porin proteins are involved in both acquired and adaptive resistance to multiple drugs. For example, imipenem and meropenem are passed through this entry, and mutations can cause reduced levels of OprD expression that confers resistance to these drugs. Moreover, mutations in genes involving an altered cell envelope can confer polymixin B resistance in *P. aeruginosa.* There have been numerous bacteria where multidrug resistance through altered membrane permeability have been reported [53].

In addition to the occurrence of mutational resistance, bacteria can acquire resistance elements from outside sources by a process called horizontal gene transfer (HGT). There are three main mechanisms of HGT by which bacteria acquire resistance genes or genetic elements from their exogenous sources (**Figure 4**). For example, a recipient cell can acquire a resistant gene from the donor either by transduction, transformation or conjugation [44]. During transduction process, gene transfer is mediated by bacteriophage where virus infects bacteria on a species-specific mode. Although this process was thought to be relatively rare, bacteria acquire resistance genes via transduction whereby bacteriophages can infect and transfer resistance genes to a new bacterium. One such example is methicillin resistant *Staphylococcus aureus* (MRSA), which was thought to develop resistance through acquisition of *mecA* resistance gene from other bacteria by transduction [45]. Bacterial natural transformation is accomplished by taking up dead or degraded DNA (called donor DNA) by recipient bacterium from its exogenous sources and incorporates this DNA into the recipient's genome by homologous recombination. Such transformation-mediated acquired resistance is thought to have occurred in *Streptococcus pneumoniae,* which acquired genes for altered penicillin binding proteins (PBP2Bs) from degraded *Streptococcus mitis* and conferred low affinity binding with penicillin antibiotics [46]. This mode of resistance acquisition has also been reported in the case of ceftriaxone resistance conferred by *penA* gene in *Neisseria gonorrhoeae* [47].

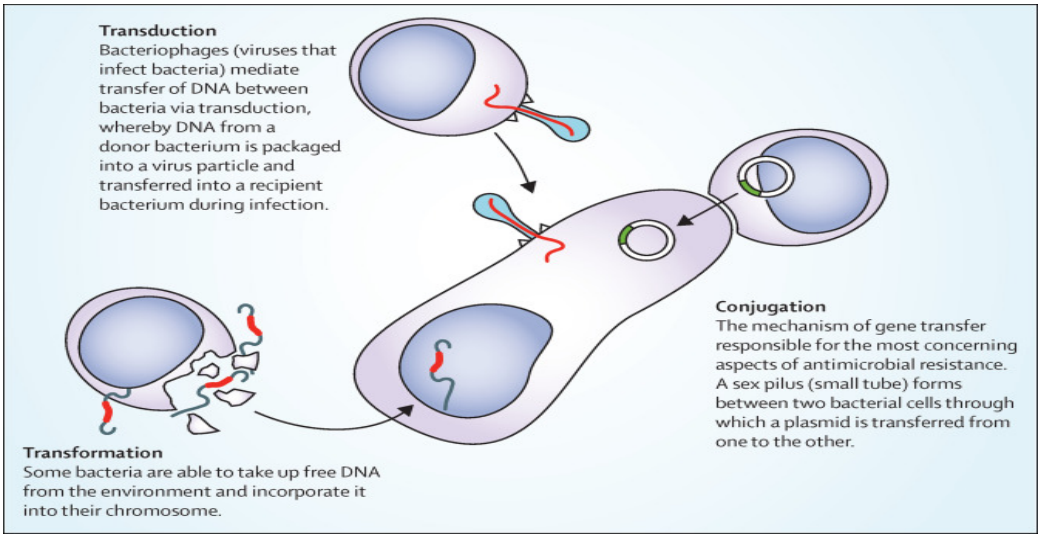

Figure 4 Acquired resistance mechanism in bacteria. Transmission of genetic material by horizontal genetic transfer, which is accomplished by three different mechanisms. Figure 4 reproduced from [44].

Mobile genetic elements (MGEs) are defined as DNA molecules that facilitate both inter– and –intra cellular movement of DNA by encoding the necessary proteins and enzymes in their own DNA [77]. The widespread prevalence of MGEs in bacteria has facilitated the evolution of antibiotic resistance, multidrug resistance in particular. Examples of such MGEs include plasmids, insertion sequences, and integrons. [53]. The general mechanism of resistance transfer through MGEs is as follows: multiple resistant determinants are borne on the MGEs and later mobilize or exchange their genetic elements conferring resistant to multiple antibiotics by the HGT. The majority of resistance genes harbouring plasmids are thought to disseminate to other bacteria by conjugation – the third mechanism of HGT. Some of the most notorious resistance enzymes, including carbapenemases were encoded by plasmids found in Gram negative *Enterobacteriaceae* [48]. Specifically, serine β-lactamases (KPC carbapenemase) and metallo-β-lactamases (e.g., NDM-1: New Delhi Metallo-β-lactamase) belong to carbapenemase enzyme, and both of them are capable of hydrolysing most of the β-lactams currently available in the markets [49-51]. Another striking feature of conjugation is the dissemination of resistance genes between plasmid and bacterial chromosome via integrative chromosomal elements (ICEs) – these are also mobile genetic element (MGE). This type of resistance propagation has found to be prevalent in most of the gram negative bacteria and has been reported in *streptococci* [52].

It has also been reported that some bacteria can swap genes from evolutionarily distant bacteria via HGT (from Gram negative to Gram positive bacteria) [53, 78], this has probably enabled bacteria to evolve resistance to multiple drugs [79]. Therefore, the MGEs are regarded as an open source of bacterial multiple resistance evolution [80], as they can move from one cell to another or from one genetic location to another. To date, various plasmids (such as R factors/R plasmids with varying sizes ranging from 1 to 100 kb), transposons (i.e., Tn1, Tn4, Tn1545, Tn1691) and integrons (i.e., class 1 to 4) have been documented to capture and disseminate multidrug resistance [53]. Multidrug resistance can be transferred into susceptible bacteria by a single-event conjugation of a mobile R plasmid.

Transposons are also mobile genetic elements and are found on the R plasmids, (**Figure 5a**). Transposons can integrate other transposons or can be integrated into host chromosome by transposase or recombinase encoded by its terminal region, thus mobilize resistance genes from plasmid to chromosome. Integron contains many different genes, called gene cassettes. A large integron contains more than one hundred gene cassettes. It has been reported that ~3% of *Vibrio cholerae* genome comprised of these large integrons [81]. Integron with gene cassettes can also be transferred and integrated into new DNA through a single event mediated by integrase enzyme. Integrons can transfer and integrate multiple genes particularly resistance genes. More specifically, integron mediated resistance is accomplished by a site-specific recombination gene sequence called *int*. This gene encodes an integrase enzyme and integrates gene cassette (which may contain different resistance genes) to a specific site [82]. Mechanism of bacterial integron mediated resistance gene captures system is shown in **Figure 5b**.

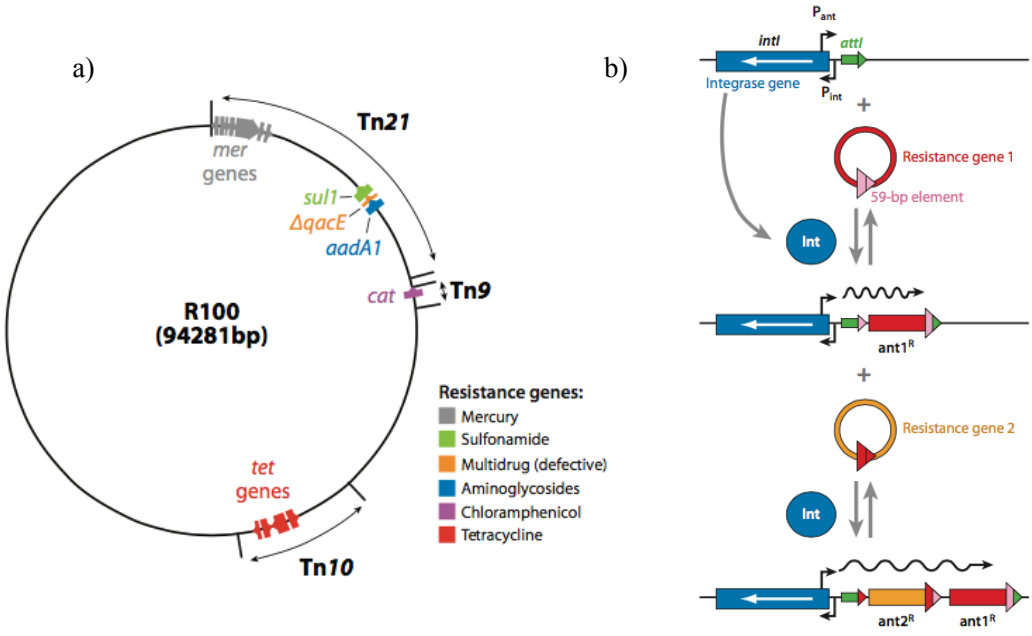

Figure 5 Multidrug resistance via acquisition of mobile genetic element (MGE). Resistant plasmid harbouring many different transposons can confer multidrug resistance by a single conjugation event (a). Integron mediated resistance gene capture system is shown in Figure b. Integrase (transcribed under a downstream promoter (P_{int}) catalyse the insertion of an integron (blue). Resistance gene cassette 1 (red) is integrated into the attI site, which is under the influence of an upstream promoter (P_{ant}). This way, many different resistance genes can be captured repeatedly, where all the resistance genes remain under the influence of the same promoter, thus become a resistance operon. Figure 5 reproduced from [60].

The MDR bacteria and their clinical impact

Simultaneous resistance to several antimicrobial compounds in diverse pathogenic bacterial populations has become the major impediment in treating infectious diseases globally. Since bacteria develop such resistance very rapidly by newly arising resistance mechanisms, it is now very challenging to treat even common infectious diseases [53]. More specifically, our current standard treatment protocols fail to produce any significant therapeutic response against multidrug resistant pathogenic bacteria. Treatment failure due to the evolution of such pathogens also leads to the extended hospital stay with severe illness where patients are mostly exposed to higher risk of morbidity and mortality. The occurrence of such resistance mechanism has been reported in many clinical bacterial populations belonging to both Gram positive and Gram negative bacteria. These populations have been defined as multidrug resistant organisms (MDROs), and these MDROs show *in vitro* resistance to at least three or

more antimicrobial classes [12]. The most problematic MDR bacteria that clinicians frequently encounter are pan-resistant Gram negative bacteria, including *P. aeruginosa*, *E. coli*, *A. baumannii*, extended spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs, including KPC, VIM and NDM-1) producing *K. pneumoniae*, methicillin resistant *S. aureus* (MRSA), vancomycin resistant *enterococci* (VRE), and extensively drug resistant *M. tuberculosis* (XDR) [11, 15, 34, 49, 53-58].

Antibiotics used in this study: action and resistance mechanisms

Aminoglycosides

Streptomycin and kanamycin, both are belonging to the aminoglycoside class, have been used in this PhD work as selective environments. Aminoglycosides are historically important for their role in the treatment of severe bacterial infections, especially infections caused by *M. tuberculosis* and *S. aureus*. Most antimicrobial drugs belonging to the aminoglycosides are bactericidal in nature, exhibit predictable pharmacokinetics, and often produce synergistic interactions when combined with other antibiotics [83-85]. These antibiotics mainly inhibit bacterial protein synthesis by interfering with small ribosomal subunit comprising 16S rRNA encoded by the *rrs* gene and many polypeptide units such as S12 encoded by the *rpsL* gene. 16S rRNA plays a central role in protein synthesis, especially a highly evolutionarily conserved 530 loop region is part of the A site of the 30S ribosome (**Figure 6**). Aminoacyl-tRNA partially binds to this A site and initiates the decoding process. Aminoglycoside molecules bind to the 16S rRNA and S12 protein in the 30S subunit of ribosome. Thus interfere with the decoding site in the vicinity of 16S rRNA and this disrupts the formation of the initiation complex, leading to mistranslation of mRNA and consequently cell death [24, 83, 86-89]. Resistance to aminoglycosides is widespread in many bacterial pathogens, including *M. tuberculosis*, *A. baumannii*, *E. coli,* and *P. aeruginosa*; therefore, the usefulness of aminoglycosides has been greatly compromised. This resistance is mostly attributed to target alteration, enzymatic inactivation and reduced uptake or decreased cell permeability.

First, alteration of the 30S subunit of the bacterial ribosome by commonly occurring single-step chromosomal mutations confers high-level aminoglycoside resistance. This phenomenon has frequently been observed in both clinical and experimental bacterial populations when exposed to streptomycin. Mutations mainly occur in the *rrs* and *rpsL* genes which result in an altered ribosomal binding site for the antibiotics. Mutations in *rpsL* gene, specifically at residues 42 and 87, interact with 18, 27 and 44 helixes of 16S rRNA and prevent drug binding.

It has also been evident that fitness costs caused by *rpsL* mutations can be compensated for by mutations in the rRNA or other ribosomal proteins. A conformational change in 530 loop region of the 16S rRNA due to mutations in *rrs* gene also contributes to the high-level resistance to streptomycin, kanamycin and other aminoglycosides, and this mechanism has been observed both in gram-positive and gram-negative bacteria [89-93].

Significant resistance to streptomycin conferred by mutations in *rrs* and *rpsL* was reported in clinical isolates of *M. tuberculosis*. Most of these mutations were characterized by A → C or A → T transversion at position 513, C → T at position 516 in *rrs* gene; in *rpsL* gene, resistance to streptomycin was due to mutations at residues 42 and 87, and these are associated with Lys → Thr and Lys → Arg substitution respectively. Mutations at multiple sites of these genes conferring resistance to streptomycin and kanamycin have been reported in a wide range of other bacteria including *E. coli, S. typhimurium, Pseudomonas putida* and *Borrelia burgdorferi* [86, 87, 92, 94-98]. Therefore, it is anticipated that in my experimental system using *Acinetobacter baylyi*, similar resistance mutations to streptomycin will arise.

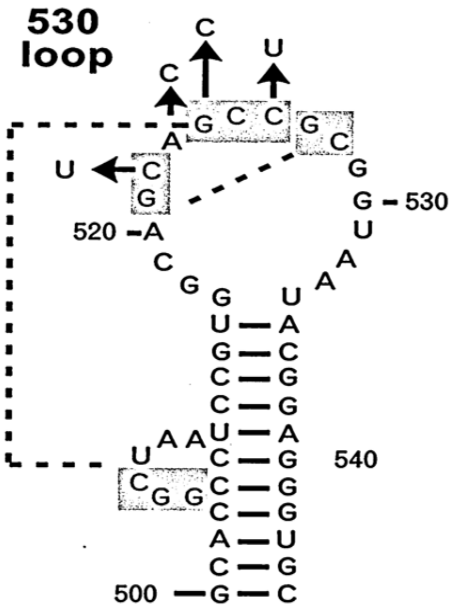

Figure 6 Partial structure of 16S rRNA showing the 530 bps loop. Resistance to streptomycin is conferred by different point mutations in the 530 bps loop region shown by the solid arrows. This figure is based on *E. coli* 16S rRNA nucleotide numbering system. For *M. tuberculosis*, position of the mutations are estimated by subtracting 10 from the 530 bps loop region numbers, and 8 from the 915 region numbers. Figure 6 reproduced from [**99**].

Second, enzymatic inactivation mediated by the aminoglycosides nucleotidyltransferases (ANTs), the aminoglycosides acetyltransferases (AACs) and the aminoglycosides phosphotransferases (APHs). These enzymes are by far the most commonly found enzymes conferring resistance to aminoglycosides and observed in clinically significant bacterial species. Structural modifications catalysed by these enzymes reduce the binding affinity by inducing unfavourable electrostatic or static interactions between aminoglycosides and the target rRNA. This phenomenon has been reported in *P. aeruginosa, K. pneumoniae, E. coli* and other bacteria. To date a vast array of these enzymes has been detected in both Gram positive and Gram negative bacteria of clinical origin, and most of these enzymes are harboured on plasmids and transposons [79, 83, 86, 87, 100]. In the first part of my project, I have used a strain carrying the *nptII* gene inserted in the chromosome. This gene encodes the neomycin phosphotransferase-II enzyme belonging to the aminoglycosides phosphotransferase (APH) family. Enzymes belonging to the APH family can inactivate a number other aminoglycoside antibiotics, including kanamycin. Therefore, strain harbouring *nptII* gene will enable me to ascertain to what extent collateral resistance to another antibiotic from the same class (streptomycin) occurs.

Finally, mutations in the respiratory chain or ATP-synthatase and the regulatory genes encoding the efflux systems (*acrB, mexZ, opmG, opmI*) have also been reported to be associated with aminoglycoside resistance in a diverse bacterial species, especially in *E. coli*, *A. baumannii, S. aureus* and *P. aeruginosa* of clinical and laboratory origin. Aminoglycoside resistance can also be conferred by mutation at the *ksgA* gene encoding S-adenosylmethionine (SAM)-dependent RNA methylase which interferes with post-transcriptional methylation of A1518 and A1519 of 16S rRNA nucleotides leading to inhibition of the initiation step of translation in *E. coli* and *B. stearothermophilus*. However, most clinically relevant 16S rRNA methyltransferase encoding genes are carried by plasmids, for example *rmtA* and *armA* were carried and disseminated by plasmid of *P. aeruginosa* and *K. pneumoniae* species, respectively [34, 57, 86, 101-105].

Ansamycins

Rifampicin, a semisynthetic bactericidal antimicrobial drug. This antibiotic is derived from the ansamycin family, and has been used as a selective environment in this study. This antibiotic has a broad-spectrum antibacterial activity and is particularly effective at low concentration. Rifampicin has long been used as a first-line of drug against *M. tuberculosis*, the causative

agent of tuberculosis. Due to its broad-spectrum action, this antibiotic has also been used for the treatment of various other infections caused by Gram negative bacteria such as *P. aeruginosa, E. coli, A. baumannii* and many more. This antibiotic inhibits DNA-dependent RNA polymerase (RNAP), which has five subunits ($\alpha_2\beta\beta'\sigma$). The RNAP is an important transcriptional component, which catalyses the transcription of DNA to RNA through polymerization of the ribonucleoside triphosphate on a DNA template. Rifampicin binds to and forms a stable complex with the β-subunit of the RNAP, thus suppress the initiation of protein synthesis in susceptible bacterial populations [106-109]. Bacteria acquire resistance to rifampicin at high rate. The most observed form of resistance to rifampicin is by spontaneous mutations in the *rpoB* gene. Specifically, a point mutation in the *rpoB* gene encoding the β-subunit of RNAP is the main mechanism of acquired resistance. Mutation alters the binding site of the β-subunit structure of RNAP enzyme, as a consequent rifampicin loses its binding affinity and cannot produce any inhibitory effect on bacteria. This mechanism of resistance has frequently been observed in clinically significant bacteria, most notably in *M. tuberculosis, P. aeruginosa*, *E. coli* and many more. Commonly occurring rifampicin resistance mutations in *E. coli* are located in the central region of the polypeptide within cluster I, II and III. In *M. tuberculosis*, 40% of resistance is acquired by mutations at codon 432 to 458 within cluster I (**Figure 7**). In clinical *A. baumannii* strains, mutations are observed at multiple nucleotides located within a range of 500 to 580, whereas in laboratory strains mutations are located within a nucleotide region spanning from 1565 to 1741 nucleotide regions [106, 108, 109].

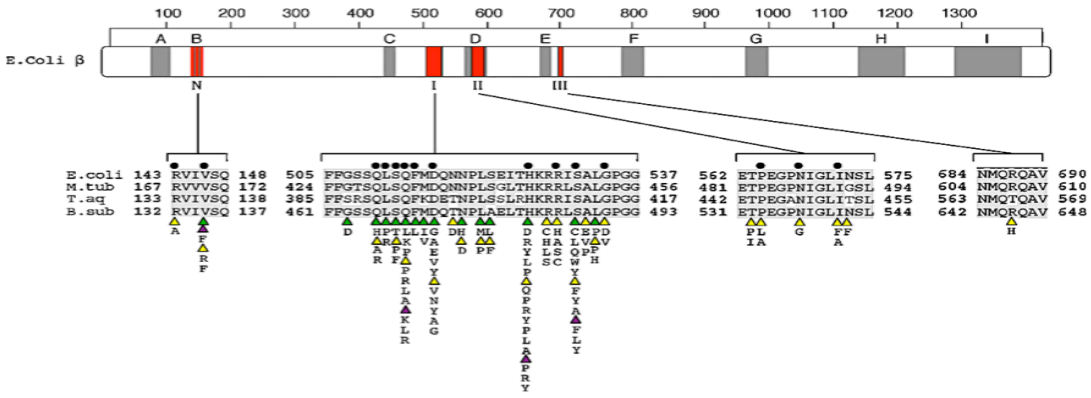

Figure 7 Map of the RNAP β-subunit rifampicin resistance mutations in bacteria. Gray shaded zones correspond to highly conserved regions (labeled A to I) and red shaded zones correspond to rifampicin resistance region of the RNAP β-subunit (labelled I to III). Green, yellow and purple triangles correspond to resistance mutations in *M. tuberculosis*, *E. coli*, and *B. subtilis* respectively. Figure 7 reproduced from [106].

Additionally, many studies have attempted to investigate the potential impact of different

mutations in *rpoB* gene from other species by selecting spontaneous rifampicin mutants [70, 110-113]. Therefore, we also expect to observe mutation in the *rpoB* gene conferring resistance to rifampicin antibiotic. Apart from this, other resistance mechanisms against rifampicin have been reported, including modification of cell permeability or membrane-associated efflux pumps, target duplication (presence of two different RNAP β-subunit genes gives high-level resistance to rifampicin), as well as resistance mediated by enzymatic modification. However, the occurrence of these resistance mechanisms has been reported only in a few instances [22, 106, 113-115].

Evolutionary aspects of drug resistance

Bacterial resistance to antibiotics is thought to be one of the best-known examples of biological evolution. The continued selection pressure exerted by different antimicrobial compounds is the main driving force contributing to this process. For example, an indiscriminate use of different types of antibiotics inhibiting the growth of bacteria in the host environment has resulted in the emergence and spread of resistant determinants in different bacterial populations, thus producing the resistant populations. There are two main processes involving bacterial evolution towards antimicrobial compounds. First, there are processes generating genetic variability, thus providing the raw material in this process of resistance evolution. This variability is attributed to a number of factors, including spontaneous resistance mutation, recombination and horizontal gene transfer. Second, natural selection acts on this genetic variation and thus driving the spread of resistant determinants in presence of a given drug.

Antibiotics on the mutation frequency and mutation rate

Exposure to antibiotics results in hyper mutator or mutator resistant genotypes in the populations. This means that antibiotics exert inhibitory effect on bacterial physiology, which in turn selects for the mutator or hypermutator genotypes. There are several types of antibiotics from different chemical classes can induce the mutagenesis effect in bacteria in many different ways, including ROS (reactive oxygen species) oxidative damage induced by sub-inhibitory concentration of antibiotics in *E. coli* [18], SOS response [116], and general stress response [117]. Among these, SOS response is widespread in bacteria and is activated when DNA damage occurs. The SOS system promotes cell survival by repairing the damaged genome. This system has been extensively studied in *E. coli* involving more than 40 different genes, which are under the control of a repressor protein encoded by *lexA* gene. In response to DNA damage, multiple RecA protein binds at damaged DNA. Following binding to the damaged site, RecA induce proteolysis of the LexA, thereby de-represses the SOS regulon. An

intriguing feature of this system is inducible mutation at an elevated frequency. Many antibiotics activate SOS response in bacteria, including aminoglycosides (translational-stress induced mutagenesis), fluoroquinolone (DNA damaging antibiotics are clearly an SOS inducer since they can directly damage the DNA), and β-lactam classes of antibiotics [118]. The SOS response produces mutator phenotypes by inducing error prone DNA polymerase II, IV and polVI.

The second way of generating mutator phenotype in response to stress (i.e., when nutrients become limited at stationary phase – this is called general stress) is down regulation of the enzymes involved in the correction of post-replicated DNA errors or mismatches. Enzymes and proteins involved in this process called mismatch repair system (MMR). Proteins involved in the MMR system play significant role in maintaining normal cellular function. Defective MMR system results in high mutation rate in the population. For example, in *E. coli* MutS and MutH are important MMR proteins, but both of them are down regulated by general stress (regulated by RpoS) due to the depletion of nutrients at the stationary phase, and thus increased mutation rates in this organism. Hypermutator *P. aeruginosa* have been reported in cystic fibrosis patients with altered MMR system with *mutS* is the most affected gene. These hypermutable genotypes are associated with antibiotic resistance in those patients [119-121]. In *E. coli*, *mutS* gene acquired an increased frequency of A:T→G:C transversion mutation and thus affected the distribution of fitness effect when challenged with rifampicin antibiotic. More specifically, when *mutS*– and *mutS*+ populations of *E. coli* were exposed to rifampicin, greater part of the *mutS*– populations were fixed by one of the two-transversion mutations, but the *mutS*+ strain fixed a wide array of mutations [123].

ROS oxidative damage occurs when aerobic respiration system is distorted by the production of superoxide ($O^{\cdot -}_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Many bactericidal antibiotics and also immune responses produce these ROS species. ROS species have detrimental effect on normal cellular functions of bacteria, for example these molecules damage DNA and membrane proteins. In *E. coli,* drug-target interactions induce oxidation of NADH via respiratory electron transport chain (ETC) system. Hyper activation of the ETC results in ROS species. In experimental *P. aeruginosa*, H_2O_2 was reported to be associated with direct selection of elevated mutation rate. However, mechanism of antibiotic induced-ROS killing in bacteria and the increased mutation frequency has become an active debate since in many bacterial species, including *L. monocytogenes, S. pneumoniae,* bactericidal antibiotics were unable to produce ROS since those bacteria were devoid of cyclic TCA cycle and ETC

system, respectively.

Therefore, it is apparent that resistance evolution is based on the generation of genetic variation (by mutation) in response to antibiotics followed by natural selection acting on this genetic variation [124, 125]. This mutation rate varies in bacterial species due to the MMR system [126].

Role of HGT and recombination

In addition to mutation, there are some other mechanisms in bacteria that produce genetic variability in response to antibiotics. For example, intragenomic reorganization of genomic sequences mediated by intra-chromosomal recombination can produce genetic variability in bacteria [124, 127]. Intragenomic recombination enables transfer of repeated homologous sequences, where a non-reciprocal transfer of information occurs between homologous sequences. Therefore, this process was thought to be less costly in the acquisition of a new mutation, it can maximize the benefits of having a weak mutation by increasing copy number [128]. Bacteria can take up foreign DNA from exogenous sources (i.e., from other organisms) by means of HGT. Both mechanisms play crucial role in bacterial evolution and adaptation to multiple antibiotics [129], as well as play role in immune evasion [130] and increased virulence through acquisition of new genes [131]. In particular, many different resistant determinants borne on MGEs (i.e., plasmids, transposons and integrons) are transferred and disseminated between different bacterial cells and species by means of HGT [125]. Gene recombination produces adaptive response through replacing the deleterious phenotype driven by mutation or by bringing beneficial mutation in diverse population [132]. It is thought that multidrug resistance through recombination; natural transformation in particular, brings more evolutionary benefits to the recombining bacterial population since natural transformation entirely takes place at the chromosomal level [133-135]. It has been reported that evolution of multidrug resistance in clinical strain of *Acinetobacter baumannii* was due to transformation, and later on it was suggested that more than 45 resistant genes had been acquired by this bacterium from other genera including *E. coli*, *Pseudomonas* and *Salmonella* by transformation [136].

Evolutionary dynamics of multidrug resistance

Mutation supply rate determines the genetic variability in the infecting clonal populations under antibiotic selective pressure, as mentioned in preceding sections. Mutation supply rate is determined by population size and rates of mutation and HGT. However, adaptive evolution of

drug resistance, for example the rate at which antibiotic resistance will evolve and spread in the population is determined by several other factors, including relative fitness of the resistant genotypes as the function of drug concentration, strength of selection pressure, clonal interference, compensatory mutation, presence of epistasis, and drug-drug interaction [137].

Relative fitness in absence or presence of drug is a key component in determining how fast fixation and spread of a resistant population occurs at a given mutation supply rate [54]. When selective pressure is reduced, the frequency of resistant populations or reversibility is determined by the relative fitness cost. In many clinical conditions, fitness has been shown to be a key factor in shaping the evolutionary adaptation of pathogen populations of clinical and laboratory origin [138-140]. Both natural and clinical bacterial population confront a wide array of selective pressures in their surroundings. At high drug concentration, the rate of resistance emergence is determined by the pre-existing mutations in the population, whereas at low drug concentration populations are enriched with small-effect resistance mutations [141, 142]. It has been shown in one recent study [143] that strong selection pressure favours high level of cross-resistance (negative collateral sensitivity) to many other antimicrobial classes, whereas under low selection pressure populations enrich with weaker cross-resistance Together, this suggests that the emergence and spread of resistant population is attributed to the strength of selection pressure, which further complicates the resistance evolution.

Number of mutants and their rates of emergence are two important factors, which help predict the simultaneous presence of different resistant mutants in a given population. For example, diverse mutants can appear and simultaneously present in the same population of bacteria, as has been reported in clonal *M. tuberculosis* population during long-term antibiotic treatment. Specifically, this bacterium experienced gradual increase in resistance mutations with apparent clonal sweeps and co-existence of different resistance mutations [144, 145, 171]. Therefore, in this particular situation, clonal interference is thought to influence the evolutionary dynamics [172]. More specifically, when different beneficial mutations (i.e., beneficial in the context of resistance evolution such that a particular resistance mutation appears in response to an antibiotic) arise independently in different lineages, they compete against each other, leading to the loss of most clones from the population and the appearance of dominant clones [146]. This phenomenon has been termed as clonal interference which has been demonstrated in the cost of resistance when experimental resistant bacterial population evolved in absence of particular antibiotic induced selective pressure [147, 148]. Thus, clonal interference is likely potentiated

by the large population size with increased mutation frequency. This phenomenon has already been confirmed experimentally during adaptation of resistance plasmids to their bacterial hosts and adaptation to the fitness cost conferred by resistance mutations [172]. Similarly, multidrug resistance can be the result of concomitant presence of multiple resistance conferring mutations in individual clonal lineage.

Epistatic interactions – where the fitness effects of mutations depends on the genetic background – plays an important role in the evolution of multidrug resistance [75]. Epistasis can occur between genes [149], within a single gene encoding a single resistance protein [68, 150], or between a chromosomal gene and a gene encoded on a plasmid [151]. A large body of studies has identified pervasive epistasis in bacterial adaptive evolution under a variety of conditions. For example, in two studies, positive epistasis (when a double mutant has a higher fitness than expected from the sum of the costs of individual mutations) was reported to be associated with the evolution of multidrug resistance in the cost of resistance [69, 152]. Although reduced use or withdrawing of antibiotic use has been suggested to reverse antibiotic resistance [54], epistasis plays a major role in determining the adaptive potential of resistant populations. For example, in some form of epistasis, called reciprocal sign epistasis, the fitness of multidrug resistant genotypes in the absence of drugs is greater than either of the singly resistant genotypes. This means that acquisition of additional new resistance determinants (new resistance mutation or new resistance plasmid) can further accelerate fitness of the initial resistant genotype. Therapeutic options become limited when this particular form of epistasis arises in clinical pathogens.

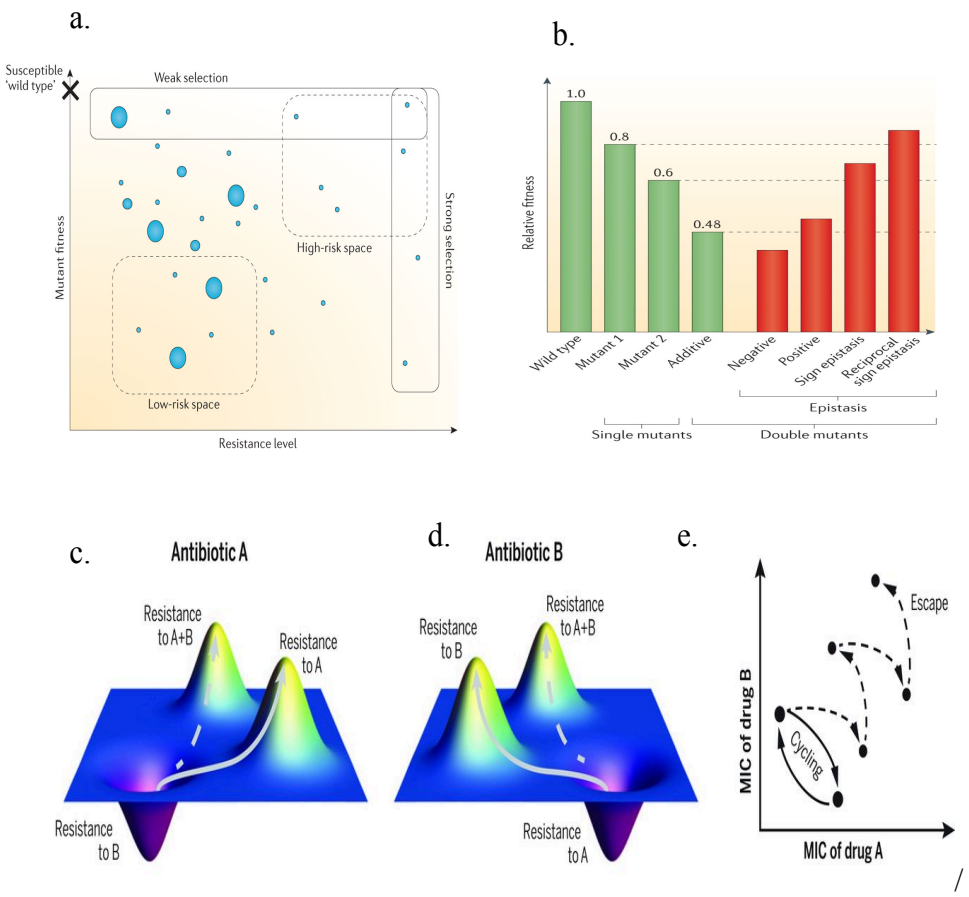

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Figure 8: The relationship between relative fitness, resistance and rate of formation is shown by considering hypothetical sets of all possible drug-resistant mutant variants shown by panel a. Each circle represents one specific resistant mutant, and the size of each circle corresponds its rate of formation. The probability of fixation of a resistant mutant is determined by the rate of formation or mutation rate, extent and level of drug selection and relative fitness. Interaction between two resistant genotypes and the resultant epistasis are shown by panel b. Different mechanisms such as the efficacy and potential failure of cycling collaterally sensitive antibiotics shown by panel c-e. Fitness landscapes with collaterally sensitive antibiotics are shown by panel c and d, which show that genotypes that are resistant to drug A or drug B appear as fitness peaks when the environment contains the drug to which they are resistant but as fitness valleys in alternative drug treatment. Such rotation of the drugs can lead to a cycle of evolution switching between these genotypes (solid arrows). However, doubly resistant mutants can evade this trap (dashed arrows). Panel e shows two possible evolutionary routes in the MICs of component drugs during antibiotic cycling. During cycling treatment bacteria gain resistance to multiple drugs when resistance swaps between two states (shown by solid arrows) even in the case where each individual mutation induces collateral sensitivity (dashed arrow).

In this figure, panel a and b are reproduced from [137], while panel c and d are reproduced from) [153].

Compensatory evolution is another important means of adaptive evolution of antibiotic resistance , which also involves epistasis. Antibiotic resistance is deleterious (on bacterial fitness) in absence of drug pressure. In absence of drug pressure, resistance determinants often impose fitness costs in the form of reduced growth, reduced transmission or reduced virulence [154]. However, secondary mutations may arise in the resistant population that compensate for these costs of resistance. This phenomenon of adaptive evolution has been reported both *in vivo* and *in vitro* studies [155, 156]. Compensatory mutations can also be resistance mutations themselves, which can both compensate and confer resistance to other antibiotics. Here, the initial resistance mutation (conferring resistance to drug A) in absence of drug pressure is deleterious, but is compensated by another mutation conferring resistance to drug B. This phenomenon has recently been observed between a streptomycin resistance mutation and a rifampicin resistance mutation in *E. coli* [152].

Drug interaction is an important factor in determining bacterial evolutionary adaptation to multiple drugs. Drug interactions are classified into two types: physiological interactions and evolutionary interactions. During physiological interaction, two antibiotics are used in combination and they can produce synergistic interaction, antagonistic interaction, or they can suppress each other's effect – called suppressive drug interaction.[157]. Such drug interactions arise when the combined inhibitory effect of two drugs is larger (called synergistic interaction which is more inhibitory) or smaller (called antagonistic interaction, where higher MIC is needed to obtain the same level of inhibition of synergistic drug pair) than expected based on an additive model. During suppressive drug interaction, two drugs in combination produce weaker effect than the null additive expectation and also weaker than the effect of each of the drugs alone. It has been reported that synergistic drug pairs, at a certain concentration threshold, potentiate the evolution of resistance by extending the traditional mutant selection window towards the sub-inhibitory concentration [158, 159]. These studies have shown that certain drug specific resistance mutation arise first (resistance mutation to drug A) in a combination of two drug (drug A + drug B), which diminish the synergistic action of that pair owing to that drug specific resistance mutation. Subsequently, this mutation confers enhanced growth advantage against that drug pair and drives the acquisition of resistance mutation for

another drug (drug B). Thus multiple resistance mutation appears in presence of combination of a synergistic drug pair. Therefore synergistic drug pairs used at concentrations below the minimal inhibitory concentration (MIC) accelerate the resistance emergence. On the contrary, with drugs that interact antagonistically certain mutation or mutation to drug A breaks and convert antagonistic interactions into synergistic. Thus antagonistic drug pairs decelerate the resistance evolution to the second drug. On the other hand, evolutionary interactions are classified into two types: cross-resistance and collateral sensitivity. Resistance mutations or genes – arising through either spontaneous mutation or horizontal gene transfer (HGT) – can simultaneously confer resistance to another drug (called cross-resistance) or become more sensitive to another drug (called collaterally sensitive) [160, 161]. Cross-resistance is the function of the evolutionary response to a single antibiotic; therefore cross-resistance is different to the physiological interactions, which require drugs to be administered in combination.

Study organism

Throughout my PhD work I used the gram-negative bacterium *Acinetobacter baylyi* as my experimental system. This bacterium possesses a highly efficient DNA-uptake system, which makes it an ideal model organism for studying natural transformation [162]. Originally isolated from the environment, in a few instances this bacterium has also been found to be associated with hospital-acquired infections with multiple MBL (metallo-beta-lactamase) carbapenemase enzymes, including $bla_{\text{SIM-1}}$ and $bla_{\text{OXA-23}}$ [163, 164]. This bacterium is also important in terms of drug discovery since systematic chemical-genetic screens have been performed in this bacterium [165] along with other important bacterial pathogens including *M. tuberculosis* [166], *P. aeruginosa* [167] by using a transposon insertion library. This screening has revealed that a large numbers of genes influence drug susceptibility; hence they are regarded as candidate antibiotic potentiation targets. However, the fold-MIC changes in antibiotic concentration ranged from a two-fold to eight-fold between wild type and mutant strains, and this observed hypersensitivity was thought to be typically rather modest. Another important feature is that the whole-genome sequence is available for this bacterium [168, 169], which has made it easier to monitor any genetic changes in the evolved population that has occurred during the course of evolution experiment or in strains associated with chapter 2.

Scope and significance of this thesis

Multidrug resistance (MDR) remains one of the major reasons for therapeutic failure in treating infectious diseases in recent days. The MDR pathogen population can emerge and spread in the

face of selective pressure exerted by drugs or they revert to the original susceptible state when the selective forces are discontinued. This means that evolutionary adaptation to multiple drugs follows a complex evolutionary path determined by many factors. Previously, many studies have made different conclusions on factors contributing to the rate of this resistance evolution under variety of contexts, including the occurrence and molecular characteristics of the resistance mutations, the fitness effects of drug resistance mutations in the presence and absence of drug induced selective pressure and how these factors impact the evolutionary trajectories of the resistant population. Nevertheless, evolution of multidrug resistance is a multifaceted phenomenon involving more factors, which were less studied in earlier studies. For example, among many others, it is yet unclear how a completely susceptible bacterium evolves *de novo* multidrug resistance in an environment enriched with a combination of drugs. Factors important in this context include pharmacodynamics (bacterial fitness as a function of drug concentrations, including drug-drug interactions), epistasis, and compensatory mutations. A complete understanding of these features will be important in predicting and preventing future multidrug resistance evolution in pathogen populations. Therefore, this PhD thesis attempts to expand our understanding further by investigating more factors that influence the development of *de novo* multidrug resistance under laboratory conditions.

From a public health perspective, this study is of importance since antibiotic resistance has become a serious public health problem due to high level of resistance against the treatment of infectious diseases globally. To combat this resistance problem, combination therapy has become an effective therapeutic choice. However, even in combination therapy, we need to better understand to what extent resistance can be selected for and what type of two-drug concentrations affect the potential for this resistance evolution, as resistance to multiple drugs have already threatened our antibiotic arsenal. Therefore, understanding the population genetic processes involving multidrug resistance is essential for proposing different but prudent treatment strategies, including combination therapy or drug-cycling, and this may ultimately help reduce the emergence of multidrug resistance pathogen.

Thesis outline

The first part of this thesis (Chapter 1, literature review) provides a general background on the history, importance, and molecular and evolutionary aspects of antimicrobial resistance, in particular multidrug resistance in bacterial pathogens. In Chapter 2 I report characterisations of pharmacodynamics fitness landscapes, seeking to better understand how antibiotic

concentrations and fitness interactions contribute to the evolution of drug resistance. From an evolutionary perspective, this study will increase our understanding of how populations may adapt to complex environment containing multiple antibiotics. In Chapter 3 I investigate the impact of recombination via natural transformation on the de novo multidrug resistance evolution in two-drug environments in experimental bacterial populations. This perhaps reflects on the actual situation when pathogen population are confronted with different selective pressures posed by antibiotics inside the host body compartments. Chapter 4 investigates the mechanisms of compensation to the cost of multiple drug resistance mutations harboured by bacteria in the absence of selection pressure. This chapter is of particular importance since we do not know how multiple drug resistance bacteria adapt to their fitness costs. Then in Chapter 5 (general discussion) in which I reflect on the main results, their implications, limitations of the research, and future avenues of investigation.

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Chapter 2: Experimental estimation of pharmacodynamics fitness landscapes in the evolution of multidrug resistance

Summary

Combination of multiple drugs has been an effective therapeutic treatment protocol over monotherapy since the success of a single drug therapy is increasingly being threatened by the evolution and spread of antibiotic resistance mutations. The sustained effectiveness of combination treatments depends crucially on the speed at which these resistance mutations arise, spread and are combined to form multidrug resistance. Until now we only know a little about how cross-resistance and collateral sensitivity, interactions between drugs and epistasis determine the evolution of multiple drug resistance. By constructing three pairs of resistance mutations, we studied the resulting fitness landscapes where we measured growth rates as a proxy for fitness in concentration gradients of the corresponding drugs. This was done using *Acinetobacter baylyi*, which has recently been found to be associated with multiple drug resistance, including carbapenem resistance in hospitalised patients.

Overall, the data we obtained here show that fitness landscapes were largely non-epistatic between each pair of resistance conferring mutations under all three pairs of drug environments. A wider mutant selection window was apparent for two antibiotics pairs where the sub-MIC concentration space was many fold lower than the MIC of the susceptible genotype. Thus, these results suggest that the sub-MIC antibiotic concentration is also generated in certain body compartment during combination therapy which may potentiate the evolution of *de novo* multidrug resistance. Our results also point to cross-resistance – a phenomenon where a mutation confers simultaneous resistance to several drugs belonging to the same class. We find that a wider mutation-selection window can also occur in presence of cross-resistance, which may have important implications in resistance evolution. We also observes a minor sign of Eagle effect for a singly resistant genotype treated with an antibiotic, which was non-specific to that particular drug. Overall, our results underlie the importance of selecting the right drug during cyclic treatment and also motivate using optimal treatment dosing regimes during combination therapy that exclude the prolonged exposure of sub-MIC level of antibiotics. More extended analyses of such fitness landscapes considering more drug pairs that target different bacterial physiological pathways is essential to obtain a solid understanding of *de novo* multidrug resistance evolution.

Introduction

The broad occurrence of antibiotic resistance has become a major cause of concern in treating infectious diseases. Specifically, the emergence of resistant bacteria to several antimicrobial compounds – of both synthetic and semi-synthetic origins – over the last decades poses serious threats, which eventually portrays greater lack of understanding of the factors contributing to the evolution of multidrug resistance [1-4]. Despite substantial knowledge of the molecular basis of resistance, the population biological principles determining the evolutionary routes towards drug resistance is largely lacking – for example, complete understanding of selection pressures, environmental variations and evolutionary constraints are important for predicting evolutionary paths towards resistance [5-8].

The fitness landscape [9] – a fundamental concept in evolutionary biology – captures the relationship between genotype and evolutionary fitness. This widespread metaphor portrays the possible evolutionary trajectories of adaptations of a set of genotypes where the genotypes are mapped onto phenotypes (*i.e.* finesses determined by the growth rate of genotypes) by mutational network, which in turn determine the trajectories [10-15], mode and tempo of an adaptive process, or adaptive protein evolution, including evolutionary adaptation of bacterial pathogen to multiple antibiotics [16] and affinity maturation [17, 18]. Fitness landscapes also play important role in many theories, including theories concerning the evolution of sex, speciation, genetic robustness and evolvability [19-22].

An important feature of fitness landscapes is epistasis (**Figure 1**), where fitness effects of alleles at different loci deviate from their individual allelic fitness effects. Positive epistasis predominates when two beneficial mutations (beneficial in the context of a given environment) in a genotype jointly increase the net fitness more than the sum of their individual fitness effects. On the contrary, negative epistasis implies that combined fitness effect of the two beneficial mutations is less than the sum of their individual fitness effect [23, 24]. Furthermore, other patterns of epistasis can also be observed, for example sign or reciprocal sign epistasis. In the case of sign epistasis, the selective effect brought by mutation to a locus depends on the selective effect incurred by mutation to other locus, whereas in reciprocal sign epistasis, selective effect depends on both loci [25-27].

Multidrug resistance usually evolves through adaptation at multiple loci in a genome of a pathogen. Each of these loci harbouring drug specific resistance mutation can also confer resistance to several other drugs. This phenomenon of resistance to multiple drugs is widespread in many clinical bacteria; most notable of such multidrug resistant bacteria, in terms of global morbidity and mortality, are clinical *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [28-30]. In such cases, where drug specific resistance mutations carried by different genotypes, including single drug resistant and double resistant mutant, the rate at which these genotypes will spread in the population is determined by relative fitness. Therefore, the concept of fitness landscape is important for studying the evolutionary dynamics of multidrug resistance driven by mutations at several loci. More specifically, when resistance to several drugs are present in the populations – considering resistance to several drugs conferred by mutations at several drug specific loci – fitness landscape can assign fitness values of all genotypes and determine the rate of adaptation or long-term success in the population under antibiotics environment. Thus, defining different features of this fitness landscape is crucial and could explore new avenue on understanding the protein evolution of multidrug drug resistance.

Epistasis plays an important role in the spread of resistance mutations in the population. Epistasis is measured by determining relative fitness among genotypes comprising both susceptible and resistant genotypes. Therefore, epistasis in presence or absence of drug pressure takes different forms. Theoretical and empirical observations have characterized the fitness landscape by determining epistasis for antibiotic resistance conferred by mutations at several loci under different selective environment [31-34]. From those studies, it is apparent that in presence of drug-selection pressure, fitness of a resistant genotype depends on and further increased by additional mutation, as genotype carrying the first resistance mutation may not be the fittest in the population. Therefore, additional mutation in an additive way forms the fittest genotype sustaining drug-selection pressure and spread in the population. However, in case of epistatic interactions, fitness may increases or decreases when both mutations interact positively or negatively (**Figure 1**, right panel). In absence of antibiotics selection pressure, synergistic cost/substantial decrease in fitness can be observed in singly resistant genotypes or increased in fitness incurred in a double resistant genotype when two costly mutations positively interact each other and increased fitness more than any of the single resistant genotypes (illustrated in **Figure 1**, left panel), a form of epistasis called sign epistasis (SE) [8]. Therefore, it is possible to observe many different types and degree of interactions between

resistance genes or mutations in absence or presence of drug pressure. In addition to this, more different forms of epistasis are presented in **Figure 1**.

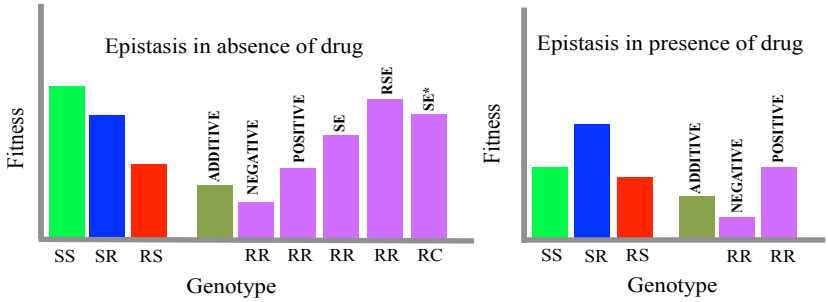

Figure 1 Illustration of the simplest two-locus fitness landscape. Considering a two-locus fitness landscape in presence and absence of drugs, several types of epistasis are possible. 'S and R' denote susceptible and resistant alleles respectively. 'SS' shows fitness for the wild type genotype with green column. Two different single resistant genotypes denoted by 'SR' and 'RS' with their corresponding fitness are shown by blue and red columns, whereas purple colour with 'RR' represents fitness for the genotype carrying both resistant mutations. Here, epistasis is defined as a deviation from the additive effect (shown by brown columns; here, fitness for the double mutant can be predicted from the fitness of the two single resistant genotypes). Different purple columns show different types of epistasis. In absence of drug selection pressure (left panel), different types of epistasis can be observed. In addition to positive and negative epistasis, sign epistasis (SE) can be observed when two resistance mutations interact positively and increase fitness at least more than one of the single resistant genotype. An extreme form of sign epistasis called 'reciprocal sign epistasis (RSE) occurs when two resistance mutations interact in a way that gives rise to a higher fitness than both of the single resistant genotypes. A special type of sign epistasis (denoted by SE^*) can be seen when one resistance mutation compensates for fitness costs imposed by the other. This type of sign epistasis is particularly relevant in the evolution of multidrug resistance. In presence of drugs, both positive and negative epistasis can be observed between resistance mutations (right hand panel).

In experimental population, epistasis between different mutations has been reported. In the case of TEM β-lactam resistance, five point mutations gave strong resistance where extensive reciprocal sign epistasis documented in presence of cefotaxime [11]. However, this type of epistasis was absent in mutations conferring resistance to rifampicin in *P. aeruginosa* but wide spectrum of negative epistasis was prevalent [35]. In absence of drug pressure, studies revealed an abundance of epistatic interactions between mutations conferring drug resistance, where positive epistasis was found to be predominant in the population [16, 36]. Another

study reported a multitude of sign epistasis between chromosomal resistance mutations and resistance mutations harboured by plasmid [27]. In the above-mentioned studies, an important factor was not considered while characterizing fitness landscape of drug resistance: different antibiotic concentrations and their impact on the bacterial growth or death rate. In case of multidrug resistance evolution, evolving bacterial population confront differential drug pressures throughout the environment, and the fitness landscapes underlying drug resistance will vary along with these different concentrations and combinations of drugs. More specifically, the effectiveness of an antibiotic depends on its absorptions, distribution, and decay (which we call pharmacokinetics or PK) as well as the specific functional relationship between the concentration of drugs and the growth or kill rate induced by that drug (which we call pharmacodynamics or PD) [37-41]. The PD functions vary when mutations are acquired across loci; therefore, it is particularly important to investigate how pharmacodynamics and the multilocus population genetics together impact the evolutionary adaptation to multiple drugs. The PD approach has widely been employed for a single drug and for a single (susceptible) genotype. Here, the net growth rate (positive or negative) of a bacterial population is measured in presence of different concentrations of a single drug. Mathematically, this net growth rate as a function of antibiotic concentration is often described by Hill-function [37, 42]. Two previous studies described the pharmacodynamics or growth/death rates of a susceptible genotype at different concentrations of two drugs by employing a two-drug PD approach; these studies revealed that Hill functions give a good fit when considering drug-drug interaction parameter as well as drug concentrations [43, 44]. However, these studies were limited by factors such as drug specific resistant mutants including single and double resistant genotypes were not considered. Therefore, the evolutionary interactions between resistance mutations as well as drug interactions were largely ignorant in those studies.

Drug interactions are an important factor inherent in two-drug pharmacodynamics. Analogous to genetic interactions, drug-drug interactions are classified into three main types namely additive, synergistic and antagonistic, where antagonistic and synergistic interactions are defined on the basis of deviation from an additive effect of a pair of drugs. Additivity can be defined by two main methods namely, Bliss independence and Loewe additivity. According to Bliss independence, the relative effect of a drug at a particular concentration is independent of the presence of the other drug. By contrast, Loewe's definition is premised on the idea that a drug is non-interacting with itself – if two drugs are in fact the same or similar – then their

combined effect would be identical to the effect of one of those drugs used alone in double dose [45-48]. In synergistic drugs pairs, maximum therapeutic outcomes are expected, whereas antagonistic drug pairs debilitate therapeutic success and negatively correlate with bactericidal activity [43]. Studies suggest that antagonistic interactions narrow the drug concentrations and slow down and/or reverse the rate of resistance evolution [48-50]. However, these studies were limited in that either fitness was measured for all genotypes but only in absence vs. presence of drug pressure, or that fitness was measured over a wide range of drug concentrations but only susceptible bacteria were considered.

One recent theoretical study has characterized pharmacodynamics fitness landscapes for multidrug resistance based on Hill pharmacodynamic functions that describe fitness of all genotypes with continuously varying concentrations of two drugs. This theoretical investigation suggested that pharmacodynamic fitness landscapes would be characterised by pervasive epistasis. This epistasis can result from 1) fitness costs of resistance (epistasis is expected to arise at drug concentrations around the MIC of the wild type and resistant genotypes even if the costs are additive; 2) cross-resistance or non-specificity of resistance mutations (such that one particular resistance mutation can also confer resistance to the other drug); and 3) drug interactions (synergistic drug interactions should lead to negative epistasis and antagonistic interactions should lead to positive epistasis). In this chapter I will test this hypothesis experimentally. Specifically, I will characterise two-locus pharmacodynamic fitness landscapes comprising both susceptible and resistant genotypes (both single and double resistant) in absence and presence of the two corresponding drugs at varying concentrations. I then analysed the key properties of these pharmacodynamics fitness landscapes such as epistasis, collateral sensitivity or cross-resistance, and drug-interactions in order to better understand the role of these factors in multidrug resistance evolution.

In the present study all genotypes were constructed by a combination of traditional mutation selection assays (i.e. single resistant genotypes were constructed in presence of rifampicin and streptomycin antibiotics; only kanamycin resistant genotypes were created by insertion of *nptII* gene) and natural transformation assay (all three double resistant genotypes). For further confirmation for additional mutation, we screened for additional mutation by whole genome sequencing. Three different antibiotics we have used belong to two different chemical classes; two of these antibiotics belong to the same chemical classes (i.e. kanamycin and streptomycin belongs to aminoglycoside, both of them typically interfere with important bacterial cellular

processes, including protein synthesis inhibition), and rifampicin belonging to rifamycin class interfere with RNA-polymerase-β-subunit (RNAP). Growth rates - proxy for fitness - were measured for all different representative genotypes (a susceptible, two singly resistant and a double resistant genotype) under all possible antibiotic environments - for example - fitness is measured for each of the four different genotypes in absence of drug pressure, in presence of a single drug and in presence of a combination of both drugs covering a wide range of antibiotic concentrations.

Materials and methods

Bacterial strains, media and growth conditions used in this study

We used Gram negative *Acinetobacter baylyi* derived from ADP1 strain (NC_005966) [53]. These strains were devoid of plasmids as well as bacteriophages, and were recombination efficient with fully functional DNA-uptake machinery for natural transformation assay. One strain we used for rifampicin and streptomycin mutant screening was a tryptophan auxotroph, and also had a insert of cyan fluorescence marker (*ecfp*) (trpE27 ACIAD0921::*ecfp*). The third one called kanamycin resistant strain was also a tryptophan auxotroph harboured an *nptII* (neomycin-phosphotransferase-II) gene conferring resistance to kanamycin and a yellow fluorescence marker (trpE27 ACIAD0921::*eyfp* ACIAD3309::*nptII*).

We used LB medium for all experiments and for most assays (i.e. for bacterial culture preparation, growth rate assays, broth MIC-assay, and for the amplicon and whole genome sequencing). The list of different antibiotics that we used is given in table 1. According to the manufacturer recommendation, liquid LB (lysogeny broth) was prepared at a concentration of 25 g/L, whereas, LB agar was prepared at 32 g/L final concentration. Cultures were grown at 30°C with shaking at 180 r.p.m. Rifampicin and streptomycin antibiotics were purchased from Sigma-Aldrich, and kanamycin was purchased from A.G. Scientific, Inc. Kanamycin and streptomycin solutions were prepared from powder stocks dissolved in sterilized H_2O and rifampicin was prepared from liquid stocks into CH_3OH. Drug gradients were prepared in LB medium by serial dilution method.

Table 1 List of different antibiotics and their targets

Antibiotic name	*Drug class*	*Target*
Streptomycin*	Aminoglycoside	30S ribosome
Kanamycin*	Aminoglycoside	30S ribosome
Gentamycin	Aminoglycoside	30S ribosome
Rifampicin*	Rifamycin	DNA dependent RNA polymerase (RNAP-β-subunit)
Ciprofloxacin	Fluoroquinolone	DNA topoisomerase II (DNA gyrase), IV
Nalidixic acid	Fluoroquinolone	DNA topoisomerase II (DNA gyrase), IV
Trimethoprim	FSI[1]	DFHR
Penicillin	Penicillin	Cell wall synthesis

* Antibiotics used for single drug resistant genotypes and subsequent double resistant genotypes construction for the PDFLs; [1]Folate synthesis inhibitor. DHFR: Dihydro folate reductase

Mutants screening from ancestral sensitive strain and Sanger sequencing

To construct rifampicin and streptomycin mutant, ancestral sensitive strain was streaked on LB agar plate from -80° freezer and incubated overnight at 30°C. From this plate, overnight culture was setup from a single clone in fresh 20mL LB broth in a 50mL falcon tube at 30° C with shaking (180 r.p.m). Mutant screening for a single drug was carried out by plating 100uL of overnight culture on LB agar plates supplemented with either 10mg/mL of rifampicin or 10mg/mL of streptomycin and incubated at 30°C for maximum of 48 hours. The concentrations we used were 10-fold and 5-fold higher than the MIC of rifampicin [~1ug/mL] and streptomycin [~2 ug/mL], respectively. After a maximum of 48 hours of incubation, individual mutants were isolated from both the rifampicin and streptomycin supplemented plates and overnight culture with these individual clones were set up in LB broth supplemented with the respective antibiotics and concentrations. Individual mutants were then frozen in 15% (v/v) glycerol at -80°C for further assays.

Sanger sequencing was performed to sequence targeted genomic locations of the individual mutant. We sequenced two regions of *rpoB* gene (*rpoB*I primer pairs which covered nucleotides ranging from 1–1342, including resistance cluster I, and *rpoB*II which covered the second spanning nucleotides 1240–2226 nucleotides, including resistance cluster II) by using two pairs of primers to detect the mutation responsible for rifampicin resistance (Rif^r). These primer pairs were designed based on a literature search to detect the common regions covering

the occurrence of common rifampicin resistance pathway conferred by mutation in the *rpoB* gene [54]. Similarly, to detect the streptomycin resistance (Stp^r) mutation, two pairs of primers were constructed targeting *rrs* and a single pair of primers for the *rpsL* gene, because most of the mutations conferring resistance to streptomycin appear in these two genetic loci [55, 56]. Detailed primer information, PCR master mix preparation and the PCR program are provided in supplementary **Table S1-S3**. DNA extraction was carried out from these individual resistant clones by using Promega genomic DNA extraction kits (Promega, California), followed by PCR amplification of *rpoB,* and *rrs* gene fragments. Sanger sequencing was carried out on these amplified PCR gene products, and mutations were determined by DNA Sangers sequence analysis on Geneious version 9.1.4 (BioMatters Inc.).

Construction of double resistant strain and Sanger sequencing

In the following step, we constructed three different double resistant genotypes by a natural transformation assay by following a protocol developed by *de* Vries and Wackernagel [57] with slight modification. In short, individual competent cells of rifampicin and streptomycin resistant mutant strains obtained earlier were prepared by adding 1mL overnight culture into 100 mL of LB broth in Erlenmeyer flask for a period of 6-8 hours growth at 30°C with constant shaking at 180 r.p.m., followed by centrifugation at 8500 rpm for 2 minutes and collection of pellets. In the next step, approximately 300uL LB broth supplemented with glycerol (20% v/v) was used to re-suspend the pellets by pipette tips very gently. This suspension of pellet contained competent cells and was stored in a -80°C freezer. The following day, transformation of competent cells with DNA (obtained from rifampicin and streptomycin mutants as donor strain in either way) was carried out by taking 20mL of LB broth supplemented with $MgCI_2$ (0.25mM) and $CaCI_2$ (0.25mM) in 300mL Erlenmeyer flasks. After adding DNA, the flask containing both competent cells and DNA was placed on the shaker (at 30°C with 170 rpm as shaker conditions) for aeration for a period of 90 minutes. This allowed competent cells to take up DNA from its surrounding. After appropriate aeration, the cultures were centrifuged at 6000xg for 5 minutes and re-suspended the pellet by adding 300uL fresh LB into to tube and mix the pellet by pipette tips very gently. From this tube, appropriate volume of this LB broth containing competent cells were streaked on LB agar plate supplemented with both antibiotics, for example, plates were supplemented with 4 ug/uL rifampicin and 20 ug/uL streptomycin in combination. The cultures were then diluted and 100 µL of these cultures were plated and left for adequate dry up for a maximum of 5 minutes. After this, all plates were incubated for a maximum of 40 hours at 30°C and visible individual

colonies were picked up and overnight cultures were setup in LB broth supplemented with 4 ug/uL rifampicin and 20 ug/uL streptomycin in combination. Individual culture of these double resistant mutants (StpRRifR) were frozen in 15% (v/v) glycerol at -80°C freezer. Sanger sequencing was carried out on this StpRRifR to confirm the location of the mutations in *rpoB* and genetic loci.

Structure of two-locus fitness landscape

According to the scheme, this study first aims at determining fitness of all genotypes by constructing different genotypes either by a combination of traditional mutation selection assays (single resistant genotypes) and natural transformation (double resistant genotypes) assay. Background and construction scheme of different genotypes for characterizing the PDFLs is outlined in Figure 2 used three different types of antibiotics belonging to two different classes in my experiments. These antibiotics are typically targeted to perturb bacterial important cellular processes, including inhibition of nucleic acid synthesis as well as protein synthesis inhibition (**Table 1**).

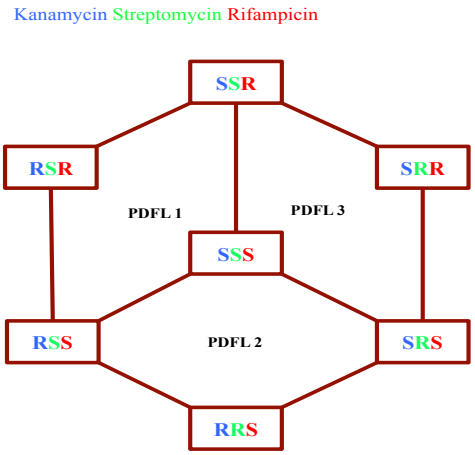

Figure 2 Three resulting pharmacodynamic fitness landscapes (PDFL) characterized in this study. The above figure shows our empirical fitness landscapes comprising three loci and seven different genotypes. Starting from a completely susceptible genotype SSS (with colors indicating the three drugs and corresponding loci), we constructed three single resistant (R) and three doubly resistant genotypes. Each node connected by the line indicating the evolutionary accessibility towards the multiple drug resistant genotypes. The three resulting pharmacodynamic fitness landscapes were studied.

Growth rate assays

Growth rates for seven different genotypes comprising three fitness landscapes were measured by spectrometry (Synergy^{TM} HT microplate reader, Biotech, USA). In doing so, individual

resistant genotypes (i.e., susceptible, single resistant and double resistant genotypes) were first streaked on fresh LB agar plate from the -80°C freezer, and then a single colony from each plate was further grown overnight in LB medium on an orbital shaker (180 rpm) at 30°C. From this overnight culture, the growth rates of each clone were measured by obtaining the growth curve (OD_{600}) in LB broth with or without antibiotics (we used kanamycin, streptomycin and rifampicin in different combinations). Statistical software R was used for finding the best curve fitting a line for data obtained from spectrometer. In short, raw OD values were normalized to a blank OD and log-transformed before analysis. The slope for the exponential growth was determined over a 32 data points correspond to growth over 160 minutes.

Determination of Minimal Inhibitory Concentration (MIC)

We determined the MIC of each of the constructed genotypes to check the level of resistance conferred by the evolved spontaneous drug specific resistant mutations.. Exponential cultures in LB broth were prepared, and samples containing around 5×10^5 colony forming units were transferred to polypropylene microtiter plates containing 180uL LB supplemented with known amounts of antibiotic. After 12 hours of incubation at 37 °C, growth was visually monitored. We carried out this to determine the evolutionary interactions of cross-resistance and negative cross-resistance to other drugs.

Statistical analysis

Various statistical methods were employed at the population level for data analysis. We first employed an analysis of variance (ANOVA) model to compare growth rate of the ancestral genotype with all the mutant genotypes in absence of drug environment to measure the fitness cost of antibiotic resistance. Similar analysis was employed for broth MIC and e-Test assay for the ancestral and mutant genotypes. All statistical analysis was carried out using JMP version 12.

Results

Determination of resistance mutations involved in the two-locus empirical fitness landscapes

In this study, we constructed our three two-locus empirical fitness landscapes from three individual mutations conferring resistance to kanamycin, rifampicin and streptomycin antibiotics respectively. At first we constructed single resistant genotypes from a completely susceptible strain either by mutant screening (i.e. we selected rifampicin and streptomycin resistant genotype) or by site directed mutagenesis (kanamycin resistant genotype was

constructed by insertion of *nptIII* gene through Tn5 transposon insertion). It is worth mentioning here that the frequency of rifampicin resistant mutation was much higher than the frequency of streptomycin resistant mutation (based on the appearance of the number of visible colonies on LB plate supplemented with many fold higher than the MIC of respective antibiotic; data not shown here). This result led us to carry out a targeted amplicon sequencing in the targeted genes (we designed primer by targeting specific allelic position to cover the commonly found mutation in both *rpoB* locus (mutation in this locus confer resistance to rifampicin) and the *rpsL* locus (mutations at this locus cause streptomycin resistance). Our targeted sequencing revealed that indeed the streptomycin resistant genotype carried a point mutation (K43T) in *rpsL* (encoding 30S ribosomal subunit) and rifampicin resistant genotype harboured a point mutation (P571L) at the *rpoB* locus (encoding RNAP, RNA polymerase β-subunit). We then constructed our three double resistant genotypes by introducing two of these unique resistance mutations conferring resistance to two distinct antibiotics respectively by natural transformation assay. To know the level of resistance, all single and double resistant genotypes were tested for the MIC (tested by E-test and broth micro-dilution in LB medium in presence of respective antimicrobial compounds). This antimicrobial susceptibility testing revealed high level of resistance in all different genotypes when we compared them with the susceptible genotype. For example, the MIC for the rifampicin resistant genotype was >32 mg/L (indicated as the highest concentration in the gradient strip) and for the streptomycin resistant genotype the MIC was >1024 mg/L (indicated as the highest concentration in the gradient strip). For the kanamycin resistant genotype the MIC tested with broth micro-dilution method was >1024 mg/L. Similar resistance profile was observed for all the double resistant genotypes.

Table 2 List of genotypes of *A. baylyi* used in this study.

Letter ID	*Genotype*	*AA change*	*Gene affected*	*Fold-MIC change*
SSS	WT	NA	NA	1
RSS	Kan	*nptIII**	*nptIII**	>1032
SRS	Stp	K43T	*rpsL*	>1032
SSR	Rif	P571L	*rpoB*	>32
RSR	KanRif	P571L	*nptIII**; rpoB	>1032
RRS	KanStp	K43T	*nptIII**; rpsL	>1032
SRR	RifStp	P571L; K43T; S325P	*rpsL*; *rpoB*; *cyoA*	>1032

Seven genotypes of *A. baylyi* along with their genetic background and resistance profile used in this study comprising three two-locus fitness landscapes. Experimental ID indicated by three capital letters indicating different genotypes with corresponding resistant loci. Genetic characteristics such as amino acid changes are also provided. Information for an additional mutation in one double resistant genotype indicated by SRR*. The susceptible genotype (trpE27 ACIAD0921::*ecfp*) indicated by SSS where each of these three letters stands for a specific locus giving resistance to three antimicrobial compounds: the first 'S' for kanamycin resistant gene; the second 'S' is for streptomycin resistant gene ; the third 'S' for rifampicin resistant gene *rpoB*. The *nptIII* gene was inserted by Tn5-mutagenesis (ACIAD3309::*nptIII*).

To quantify each of these fitness landscapes, we further examined mutational background for each of the genotypes by carrying out whole genome sequencing to confirm that these mutations were the same and no other additional mutations were present in any of the seven different genotypes. Whole genome sequencing revealed exactly the same resistance mutation in all three double resistant genotypes, and these double resistant strains were constructed through natural transformation assay. However, one additional mutation in the *cyoA* locus was also detected in one double resistant genotype harbouring the K43R *rpsL* and P571L *rpoB* mutations (table 2; see Discussion). We assigned these three fitness landscapes as Kan-Rif, Kan-Stp and Rif-Stp.

Costs associated with single and double resistance genotypes

Antibiotic resistance is often associated with fitness cost under non-selective conditions [58]. We aimed to characterize our two-locus PDFLs in the evolution of multidrug resistance both in presence and absence of drug pressure. Therefore, we first determined the cost of resistance by measuring growth rates in LB medium without antibiotics for each of the six constructed resistant strains and compared them with the ancestral susceptible genotype. Our growth data revealed a significant fitness costs for all the resistant genotypes except for the kanamycin resistance genotype (Figure 3(a): ANOVA, pairwise-comparison given by connecting letters using TK-HSD test). The rifampicin resistant genotype suffered a higher fitness cost compared to the streptomycin resistant genotype. Pairwise comparisons also revealed that all three double resistant genotypes were significantly different from each other. Among them, strikingly, the SRR genotype (dually resistance to rifampicin and streptomycin) experienced the highest fitness cost. Sign epistasis, especially reciprocal sign epistasis through mutually exclusive mutations can make evolutionary trajectories inaccessible and is associated with multi-peak fitness landscapes [59, 60]. We, therefore, further compared fitness between single vs. double resistant genotypes to discern any potential epistatic effects in the cost of resistance. The

fitness of the RRS genotype (resistant to both kanamycin and streptomycin) was greater than that of both corresponding single resistant genotypes, but the difference to SRS was not significant. Similarly, we observed no significant differences in fitness costs between the RSR and SSR genotypes, so that there was also no evidence of sign epistasis in this comparison. With the fitness landscape between rifampicin and streptomycin resistance mutations, we observed an additive fitness cost for the double resistant genotype (SRR) such that the combined costs of the two single resistant genotypes (SRS and SSR) equals the cost of the double resistant genotype (SRR).

Following I analysed the three-factor ANOVA distinguishing between individual mutations and interactions between mutations. This result suggests that both rifampicin and streptomycin resistance mutations incurred significant fitness cost but the kanamycin resistant genotype incurred marginally significant fitness cost. However, we did not observe any significant interactions between any of the resistance pairs suggesting no epistasis in the cost of resistance among them. Parameter estimates for the three-factor ANOVA is given by **Table 3.**

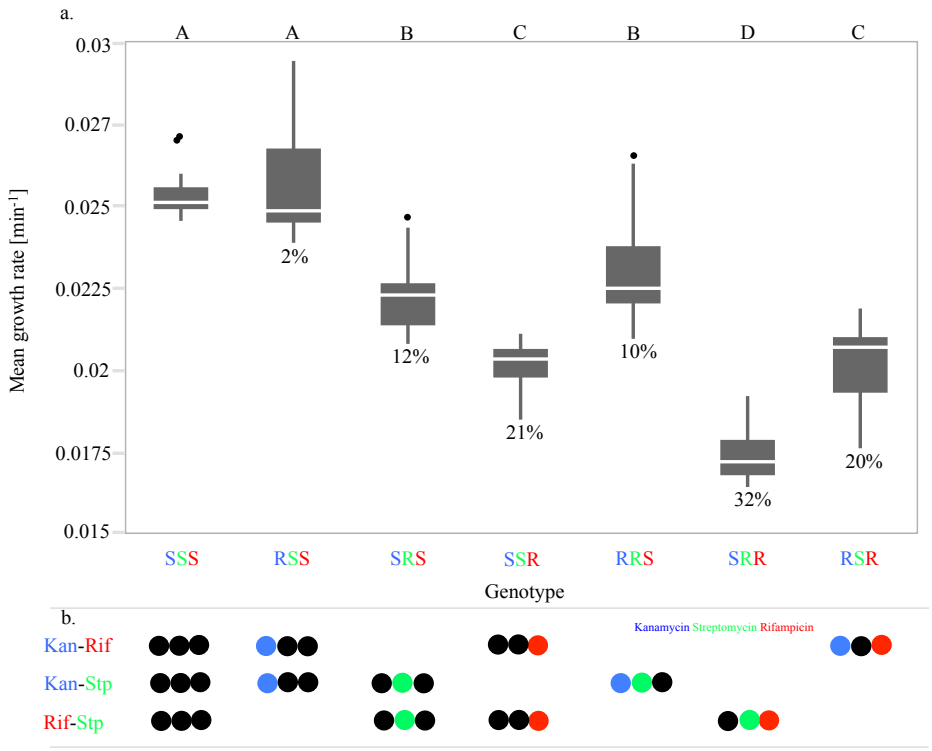

Figure 3 Fitness of different genotypes in absence of drug pressure and the corresponding two-locus fitness landscapes. Panel a shows boxplots of growth rates in absence of antibiotics for the susceptible and the six resistant genotypes, indicating fitness costs of resistance. Relative fitness costs are also given under each box. Thick white horizontal lines indicate the median, the box represents the upper and lower quartiles, vertical lines represent the range between minimum and maximum of growth measurements, and the black circles represent outliers. Growth rates of genotypes connected by the same letter shown on top of the plot are not significantly different (ANOVA: pairwise comparison using TK-HSD test). Panel b illustrates which genotype belongs to which of the three two-locus fitness landscapes (Kan-Rif, Kan-Stp, Rif-Stp). Here, black dots represent the susceptible allele and coloured dots the resistant allele (blue=Kan, red=Rif, green=Stp).

Table 3: Parameter estimates for the three-way ANOVA with different resistance mutation

Source	*Nparm*	*DF*	*SS*	*F Ratio*	*Prob>F*
KanR	1	1	0.00000091	0.6361	0.0426
StpR	1	1	0.00008643	60.2113	<0.0001*
RifR	1	1	0.00024705	172.1098	<0.0001*
KanR*StpR	1	1	0.00000108	0.7533	0.3873
KanR*RifR	1	1	0.00000012	0.0833	0.7734
StpR*RifR	1	1	0.00000048	0.3323	0.5654

Growth rates for the two-locus pharmacodynamics fitness landscapes

We next measured the growth rates for all seven genotypes comprising our three fitness landscapes in presence of combinations of two antibiotics. Thus we obtained growth rate estimates for our three resultant pharmacodynamics fitness landscapes. First, let us consider the Kan-Rif fitness landscape. In the Kan-Rif fitness landscape, the data we obtained here are in accord with general expectations. For example, in presence of antibiotics, an elevated MIC level was observed in genotypes carrying drug specific resistance mutations (**Figure 4**).

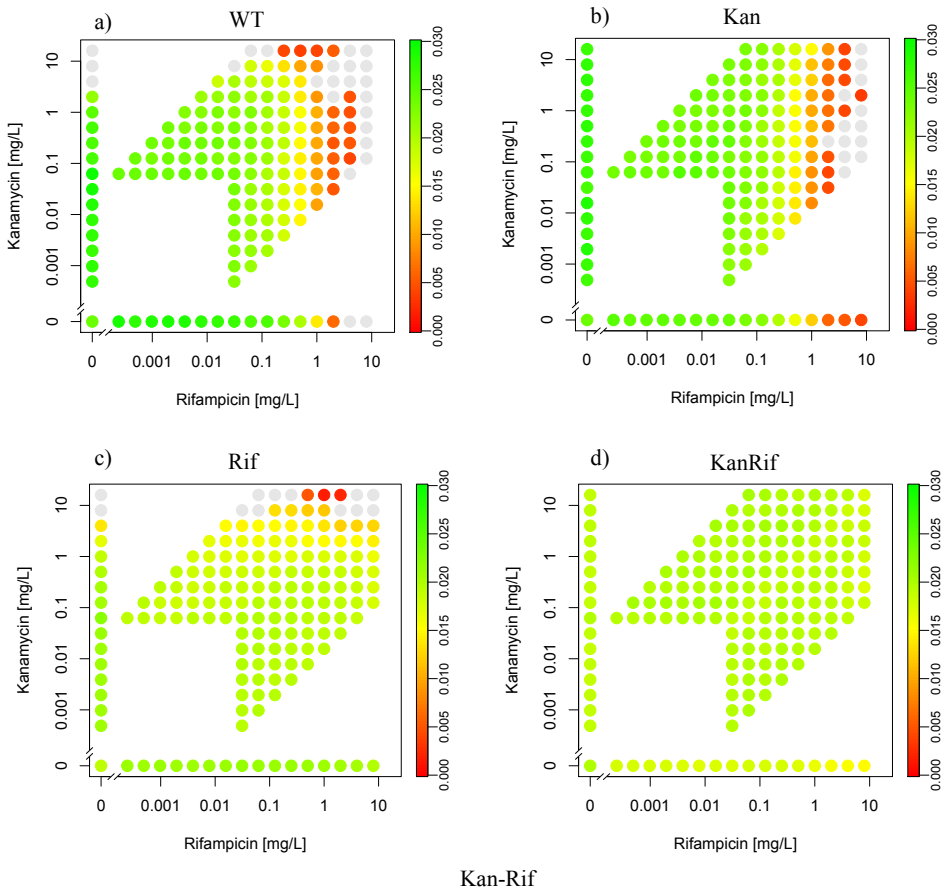

Figure 4 Pharmacodynamic fitness landscape of Kan-Rif. Plots show maximum growth rates [min^{-1}] of four different genotypes in presence of combinations of kanamycin and rifampicin: a fully susceptible genotype (SSS), a genotype resistant to Kan (kanamycin) only (RSS), a genotype resistant to Rif (rifampicin) only (SSR), and a genotype resistant to both antibiotics (RSR). Growth rate is given by gradient scale. Grey dot indicates no growth.

The fully susceptible genotype suffered an abrupt decline in fitness in presence of kanamycin antibiotic but a gradual decrease in fitness was observed for rifampicin antibiotic (**Figure 4a**). A similar pattern of growth reduction was observed for the kanamycin resistant genotype under rifampicin antibiotic, but the growth rate was higher in response to high levels of kanamycin (**Figure 4b**) owing to the *nptIII* gene, which conferred high-level resistance to kanamycin. For the rifampicin resistant genotype, reduction in growth was influenced by kanamycin as has been observed for the susceptible genotype, but no apparent change in reduced growth was observed against rifampicin because the mutation P571L in *rpoB* gene was responsible to withstand high level of rifampicin. In contrast, we observed that the double resistant genotype can grow better by sustaining the high level of inhibitory effects exerted by both drugs, shown by **Figure 4d**.

For the Kan-Stp fitness landscape (**Figure S1**), the susceptible genotype experienced a rapid decline in fitness for kanamycin (as described above) but a steady drop of fitness was observed against streptomycin antibiotic. Maximum reduction in growth rate against streptomycin was observed for the kanamycin resistant genotype, but drug also belongs to the same chemical class, and this phenotypic plasticity towards improved growth advantage has been defined as collateral resistance [61]. Thus, we assume that this improved growth rate would be due to a phenotypic resistance inherent in streptomycin resistant genotype. Subsequently, we observed remarkably a greater growth advantage for the double resistant genotype when streptomycin was used alone suggesting that this higher fitness advantage was due to the high fitness cost associated with K43R mutation at the *rpsL* locus, which helped bacteria grow better in parallel environment (**Figure S1**).

In Rif-Stp fitness landscape, a gradual drop in fitness for both drugs was observed for the susceptible genotype when both rifampicin and streptomycin drugs were used alone or in combination (**Figure S2 panel a**). Between the two single resistant genotypes, a strong growth advantage was provided by the streptomycin resistance mutation in presence of streptomycin, but we observed a gradual growth decline for the streptomycin resistant genotype when tested in presence of rifampicin (which is non-specific to the resistance mutation). However, we observed that this genotype grew slowly at very high concentration of rifampicin, suggesting that a partial cross-resistance was conferred by the streptomycin resistance mutation. On the other hand, a gradual decline in growth was observed for the rifampicin resistant genotype when tested in both specific and non-specific drug environments (Figure S2 panel c). By non-specific we mean that when a single resistant genotype, here rifampicin resistant genotype is tested against a new antibiotic, here we tested growth rate of the rifampicin resistant genotype in presence of streptomycin antibiotic to which this rifampicin resistant genotype is expected to be susceptible against streptomycin and the MIC is expected to be similar to the wild-type MIC. This non-specific slow growth rate of a rifampicin resistant genotype in presence of non-specific drug environment suggests the presence of a possible Eagle effect – a phenomenon where an increased antibiotic concentration promotes bacterial survival such that the dose response curve or kill-curve of an antibiotic is not always monotonic and this effect was previously proposed based on a single antibiotic [62]. This Eagle effect was previously reported in response to antibiotics inhibiting bacterial DNA-synthesis, most strikingly ciprofloxacin antibiotic [Lewin, C. S. et al. 1991 EJCMID]. The role of the Eagle effect in the

evolution of multidrug resistance has not been investigated; therefore it is also important to explore more on how the Eagle effect operates in combination therapy and potentiate the evolution of multidrug resistance such as whether any drug combination against a resistant pathogen provide any selective advantage to the new drug of that combination of drug pair. In this study, we observed a slower growth rate for the double resistant genotype, which grew across the entire concentration gradient of rifampicin and streptomycin antibiotics used in combination (**Figure S2 panel d**).

Drug interactions potentiate the evolution of multidrug resistance

Pairwise drug prescription has been an effective therapeutic option, and being applied in many clinical conditions and infectious diseases, of most notable example is in tuberculosis treatment [48, 63]. However, bacteria show a diverse response to antibiotic combination, for example it has been shown that synergistic drug interaction accelerates the evolutionary adaptations to multiple drugs [49].

In our study, drug interaction was defined as a deviation from a form Bliss independence [60], which assumes that fitness reductions caused by two drugs are additive. Thus, deviation from Bliss independence results in either positive or negative drug interactions arising from a particular drug pair. We used the following formula [51] to calculate the degree of drug interactions from the maximum growth rate and the corresponding reduction in growth caused by two drugs when used in combination:

$$I(A1,A2) = w(0,0) + w(A1,A2) – w(A1,0) - w(0,A2)$$

Here, *I(A1,A2)* stands for drug interaction which is the function of two drugs (*A1* and *A2*) when administered in combination,,*w(0,0)* is for growth rate of a genotype when the concentration of each drug is zero. On the contrary, *w(A1,A2)* stands for growth rate which is caused by the presence of any given concentration of both drugs., *w(A1,0)* and *w(0,A2)* represent growth rates in presence of a single drug only, for example in presence of either drug *A1* or *A2* with a given concentration.

For the kanamycin-rifampicin drug pair (**Figure 5**), a varying degree of drug interactions was apparent. We observed a similar pattern of interactions between susceptible and kanamycin resistant genotypes, especially in concentration gradients where the concentration of kanamycin was higher. In both cases, negative drug interaction was predominant. However,

there were apparently no interactions when the concentration of rifampicin was higher when it combines with low kanamycin drug concentrations. It is worth mentioning here that the fitness between susceptible and kanamycin resistance genotype was indistinguishable.

For the rifampicin resistant mutant, synergistic drug interaction was apparent in presence of this drug pair where the concentration of rifampicin antibiotic was higher. It should be noted here that the fitness cost was significantly higher in rifampicin resistant genotype. For the double resistant genotype, synergistic interaction was predominant in all concentrations ranging from sub-MIC to supra-MIC level. However, one previous study under two-drug treatment environment found a robust negative interaction at sub-MIC drug concentration, but no apparent interactions were identified at supra-MIC of this drug pair [44]. Interestingly, with this drug pair, we observed synergistic drug interaction across the entire range of antibiotic concentrations space predominated by kanamycin tested for the double resistant genotype harbouring drug specific resistance mutations.

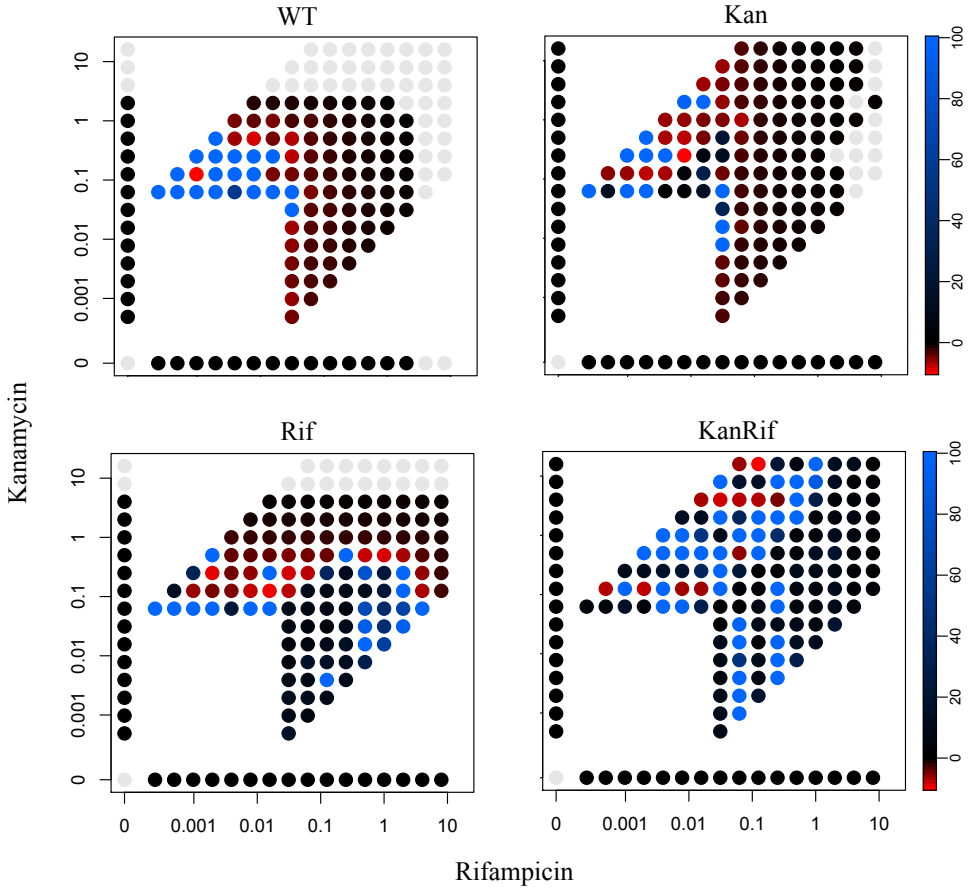

Figure 5 Drug interactions between kanamycin and rifampicin antibiotic. Drug interaction is a function of two antibiotics in combination given by a gradient scale from negative to positive interactions across all genotypes. Concentration of rifampicin is given on the x axis,

concentration of kanamycin is given on the y axis. Grey dots represent concentration where no growth was detected, and black dots represent no interaction rifampicin and kanamycin antibiotics. Red dots are for negative drug interactions. An earlier study reported that synergistic drug interaction between erythromycin and doxycycline for a wide range of concentrations was associated with the emergence of increased frequency of resistance population of *S. aureus* [48]. So, the result we show here supports the fact that synergistic drug combination enables bacteria to evolve with multiple drug resistance with low fitness cost (Figure 4) associated with the resistance mutations.

For the kanamycin and streptomycin, no clear pattern of interactions were observed for the susceptible and two of the drug specific single resistant genotypes, but a wide range of negative drug interaction was pervasive in the double resistant genotype harbouring two drug-specific resistance mutations (**Figure S3**). It is also worth mentioning that these two antibiotics belong to the same chemical class and perturb the same cellular process. The resulting fitness (shown by **Figure S1**) of this genotype for each concentration of this drug pair was lower compared to the kanamycin-rifampicin genotype (**Figure 4**).

We also determined the interaction between rifampicin and streptomycin (**Figure S4**). We observed apparently no drug interaction between these two drugs across entire drug-drug concentration space (compared to other two drug pairs). One previous study on susceptible *M. marinum* predominantly found negative drug interactions at sub-MIC concentration for all different combinations of drugs including rifampicin and streptomycin – this interaction was defined as 'pharmacodynamic antagonism' [44]. In our case, we observed this antagonism for a limited number of sub-MIC concentration in susceptible genotypes, but for the two single resistant genotypes we did not observe any interaction. For the double resistant genotype, we observed both synergistic and antagonistic interactions on a limited number of drug concentrations.

Mutation-selection window (MSW)

We further examined the MSW in presence or absence of a single drug and a combination of drugs. Specifically, we identified which genotype has the highest fitness in absence, presence of a single drug and a combination of both drugs (**Figure 6**). Understanding the MSW is particularly important since resistance evolution takes place in a drug space spanning from the MIC (the concentration at which the frequency of resistant genotype arise at a low number

which means that wild type sensitive genotype is selected for at low frequency at the MIC concentration of administered antibiotics) to the MPC (the concentration at which the frequency of resistant genotype decline to undetectable value). This is a traditional consequence of the MSW [64]. Since the drug concentrations are not static, but always fluctuate in time and space, thus the MSW is also affected, especially concentrations around the MIC. This means that differential drug concentrations around the MIC potentiate the evolution of resistance [65, 66]. The traditional MSW is affected by the sub-MIC drug concentration because selection of resistance has been documented for a single drug at very low concentration (i.e., this concentration is many folds below the MIC) [67], but – though in a limited occasions – this concept of MSW was extended for a multidrug environment by employing a combination of theoretical and experimental approach. Those studies suggested that a narrower MSW can be achieved when two drugs interact antagonistically and also there is a small cross-resistance [48]. It is worth mentioning that this previous study was conducted in presence of combination of two drugs by employing completely a susceptible genotype. In our study we determined the size of the MSW to understand the impact of three different drug combinations and the interactions among them, for example we determined the fittest genotype out of the four belonging to a particular fitness landscape (**Figure 6**).

At first, let us consider kanamycin-rifampicin drug pair (**Figure 6a**). In the absence of antibiotics, susceptible bacteria were predicted to outcompete all three resistant genotypes owing to the fact that the resistance mutations imposed fitness costs in absence of selection pressure. As expected, we found that susceptible bacteria outcompeted all other three resistant genotypes in absence of antibiotics (shown by green stripe). However, both drug specific resistant bacteria outcompeted the susceptible bacteria when the concentration of both drugs extended beyond the MIC point. This point also suggesting that concentration above the MIC is optimal for spontaneous resistance selection, and this also supports the view of a traditional MSW. However, we also observed that both kanamycin and rifampicin resistant genotypes experienced superior growth advantages at the sub-MIC concentrations (which is even below the MIC) of the susceptible strain. In addition to this, we also observe that the kanamycin resistant genotype outcompeted the susceptible genotype at very low concentrations of kanamycin; this could be due to the fact that the kanamycin resistant genotype we used here incurred very low fitness cost.

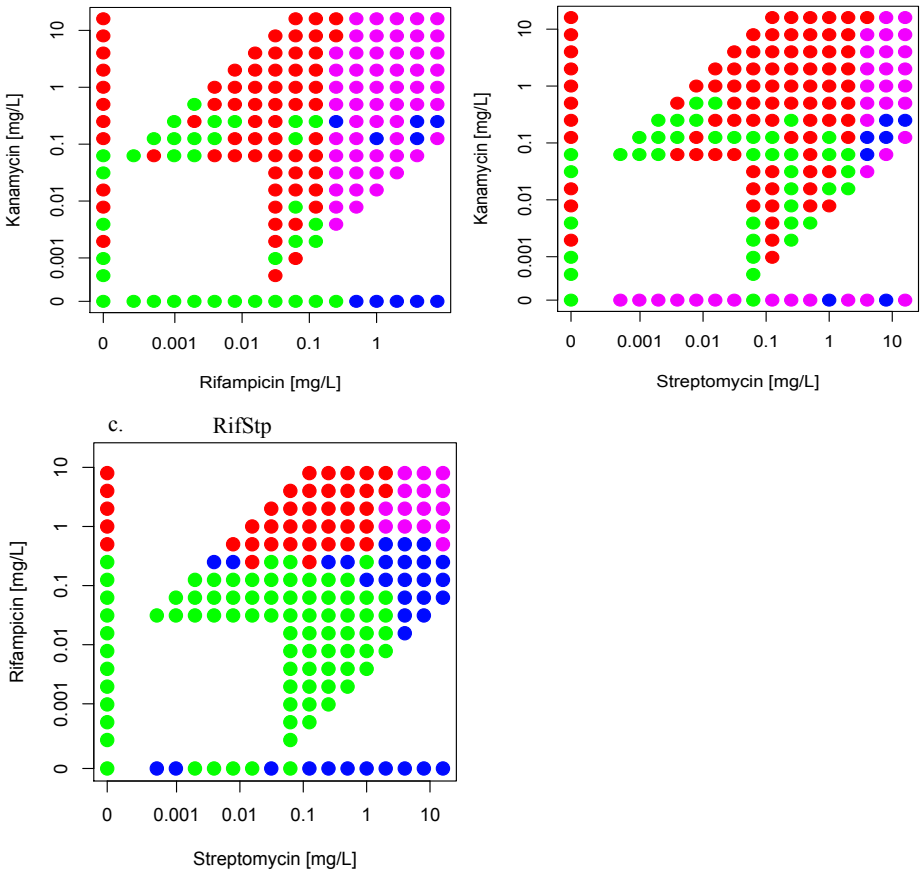

Figure 6 Drug interactions and drug concentrations affect the mutant selection window (MSW) of different genotypes. The MSW indicates at which antibiotic concentration a particular genotype can grow and outcompete other genotypes. The MSWs of each concentration of drugs and drug pairs are plotted (plot a-c for three different drug combinations), with the three double resistant genotypes indicated by purple circles in all there cases, the susceptible genotype indicated by green circle in all there cases, and three single resistant genotypes indicated by red for kanamycin and blue for rifampicin in kanamycin-rifampicin combination; red for kanamycin and blue for streptomycin for kanamycin-streptomycin combination; and red for rifampicin and blue for streptomycin for rifampicin-kanamycin combinations.

But, considering a concentration of a drug pair of kanamycin and rifampicin, ,we observed that sub-MIC concentrations space for susceptible bacteria was narrowed down mostly by the kanamycin resistant genotype, and also by the multidrug resistant genotype. One plausible explanation is that kanamycin resistant genotype perhaps acquired selective advantage which disrupted the synergistic interactions between these two drugs (Figure 6a). Secondly, we did not observe any superiority of the rifampicin resistant genotype at any particular concentrations below the MIC of this drug pair, but we observed this trend sporadically above the MIC concentrations; this could be due to the fact that the rifampicin resistant genotype incurred

highest fitness cost and also required relatively a high concentration of selective environment to regain its optimal growth ability. We also found that rifampicin resistance genotype was fitter in some combination of drug where the concentration of kanamycin was below the MIC. Finally, as expected, the double resistant genotype occupied the entire window ranging from sub-MIC to the MPC (though we did not determine the fitness under such high concentration, but we considered it with respect to the MIC of susceptible genotype as well as the concentration of drug that we used for the mutant screening) as a result of the selective advantage provided by both resistance genes that help occupy the highest drug-drug concentration space.

A similar trend was observed for the kanamycin-streptomycin drug pair, where the only exception was for the double resistant genotype. In a single drug environment of streptomycin, almost the entire single drug space was occupied by the double resistant genotype. This is probably due to the fact that the cost associated with streptomycin resistance was higher than the kanamycin resistance in absence of drug pressure. Therefore, the double resistant genotype grew better in presence of streptomycin to compensate for the costly streptomycin resistance mutation (Figure 6b).

With rifampicin-streptomycin combination, we observed a traditional MSW, where the two single resistant genotypes and a double resistant genotype captured their local concentrations space through drug specific resistant mutations. However, we also observed that the streptomycin resistant genotype had a higher growth rate than the rifampicin and the double resistant genotype (Figure 6c) suggesting that there are some regions below the MIC in a two drug space of the MSW where a less costly resistance mutant can grow better presumably to capture future resistance potential against incoming drug perturbation.

Epistasis between resistance mutations

Finally, we measured epistasis from growth rates for each of the double resistant genotypes belonging to three different fitness landscapes (**Figure 7**). Specifically, we calculated epistasis as deviation from independent effects of alleles at the two loci in presence of two antibiotics with varying concentrations [51]. The formula is given below:

$$E(A1,A2) = w^{SS}(A1,A2) + w^{RR}(A1,A2) - w^{RS}(A1,A2) - w^{SR}(A1,A2)$$

Here, E stands for epistasis in presence of combination of two antibiotics with varying concentrations, w^{SS} is for fitness of the susceptible genotype in presence of the same drug environment, w^{RR} is the fitness for the double resistant genotype in presence of the same drug environment, w^{RS} and w^{SR} represent fitness for the two single resistant genotypes.

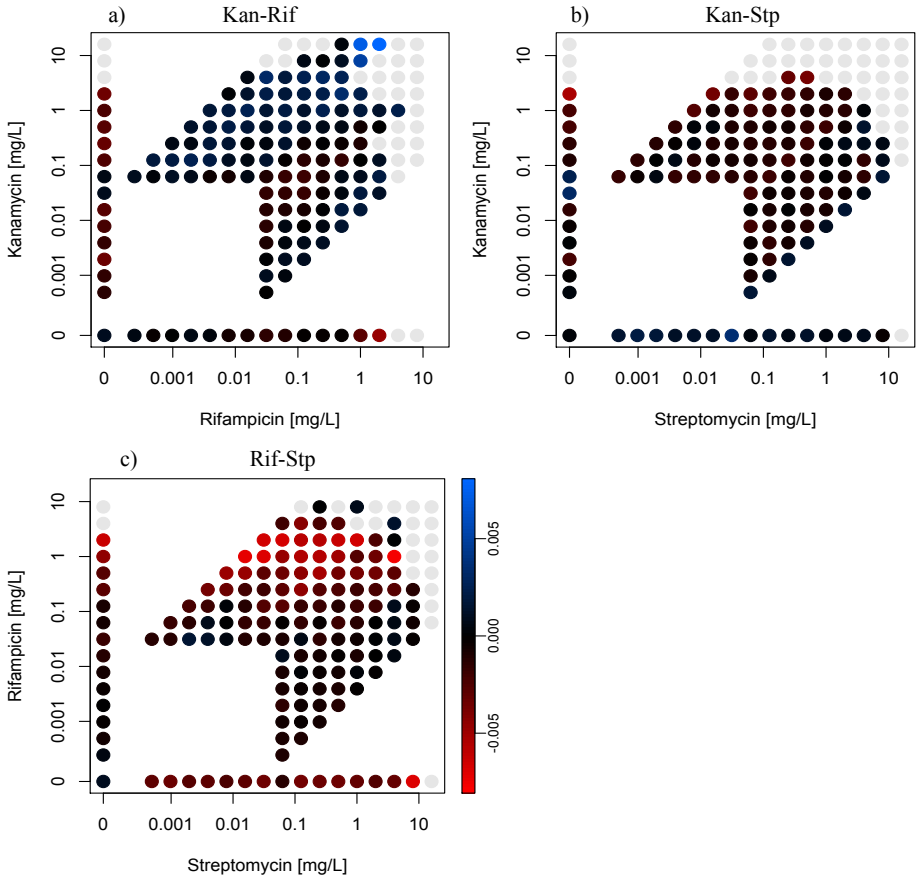

Figure 7 Pairwise epistasis between resistance genes of the three different fitness landscapes. Plots show epistasis in absence and presence of three different drug pairs. Epistasis is given by a gradient scale spanning from negative to positive epistasis. Blue and red dots represent positive and negative epistasis respectively. Grey dots represent no epistasis was detected in particular drug concentrations. Panel (a) shows epistasis between kanamycin and rifampicin resistance mutation, panel (b) shows epistasis between kanamycin and streptomycin resistance mutation, and panel (c) shows epistasis between rifampicin and streptomycin resistance mutation.

Overall, we generally observed that our fitness landscapes are non-epistatic, both in presence of a single drug or both drugs (**Figure 7**). In few instances we observed minor positive epistasis in presence of high concentrations of kanamycin and rifampicin drug pair, which means that the double resistant genotype experiences higher replication rates than expected

based on the growth rate of the susceptible and the single resistant genotypes. On the other hand, in presence of only a single antibiotic, weak negative epistasis was pervasive between kanamycin and rifampicin resistant mutations. We also observed a strong negative epistasis in presence of rifampicin at supra-MIC of the susceptible genotype. This result in presence of rifampicin suggests that negative epistasis beyond the MIC was attributed to the high cost rifampicin resistance mutation which was unable to improve its fitness burden even when it combines with a genetic background having a very low cost kanamycin resistance mutation. Rarely, we also observed positive epistasis at high concentrations of rifampicin and streptomycin antibiotics combination, especially at supra-MIC of kanamycin but around the MIC of rifampicin.

Between kanamycin and streptomycin resistance mutation, we observed a strong positive epistasis at the MIC of kanamycin antibiotic (here, MIC of kanamycin is meaning the MIC of the wild type). But very weak negative epistasis was apparent below the MIC, whereas epistasis progressed towards zero or no epistasis was discernible above the MIC. In presence of only streptomycin, we observed seemingly no epistasis or very weak positive epistasis in presence sub-MIC concentration of streptomycin. Further, this positive and negative epistasis in presence of only a single antibiotic could be explained by the fact that the kanamycin resistance gene imposed less fitness cost compared to the streptomycin resistance mutation. On the contrary, the streptomycin resistance mutation itself was costly at concentrations above the MIC of the susceptible counterparts; therefore growth rate was lower than expected. In line with this, in presence of both drugs, epistasis takes alternative form, for example no epistasis in presence of high streptomycin antibiotic space but negative epistasis was observed in presence of kanamycin antibiotic with concentrations ranging from sub-MIC to the MIC. In general, there was apparently no epistasis observed in presence of both drugs (this drug pair belongs to the same chemical class and acts upon the same target i.e. 30S ribosome) suggesting that evolutionary adaptation to multiple drugs from the same chemical class is independent of epistatic interactions in presence of both drugs which antagonistically inhibit each other (**Figure S3**).

In the third fitness landscape comprising rifampicin and streptomycin resistance mutations, we observed a varying degree of negative epistasis between rifampicin and streptomycin resistance mutations. In combination of rifampicin and streptomycin antibiotics, strong negative epistasis was prevalent when the concentration of rifampicin was higher in that drug pair. This could be

explained by the fact that the high cost of the rifampicin resistance mutation requires higher concentration of rifampicin antibiotic in order to release its high fitness burden. It should be noted here that this double mutant genotype experienced highest fitness cost also carried an additional deleterious mutation at *cyoA* locus (**Figure 3a**; indicated by RSR). We confirmed this by measuring growth rate of this genotype and then compared it with a double resistant genotype that only harboured rifampicin and streptomycin mutations but not the additional *cyoA* mutation. This result revealed that the double mutant genotype harbouring the additional point mutation in *cyoA* locus was less fit than the double mutant genotype without the *cyoA* mutation. Therefore, we consider that the fitness landscape comprising this genotype would presumably be enriched with positive epistatic without this additional mutation at *cyoA* gene. However, in presence of only a single antibiotic, most notably when rifampicin antibiotic was used alone, a steady increase in negative epistasis was observable. In this case, strong negative epistasis was apparent around the MIC of rifampicin (here we are referring to the MIC of the wild type susceptible genotype). A similar observation was also made for the fitness landscape consisting of kanamycin and rifampicin resistance mutations. Whereas, in presence of streptomycin antibiotic alone we observed mostly weak negative epistasis at sub-MIC concentration of streptomycin but strong negative epistasis at the MIC concentrations. Therefore, observed epistasis between streptomycin and rifampicin resistance mutations we observed here was different than the epistasis between streptomycin and kanamycin resistance mutations antibiotics in presence of a single streptomycin antibiotic.

Discussion

Evolution of pronounced resistance to a single drug is mediated by a sequential accumulation of multiple resistance conferring mutations in the same genetic locus. For example, empirical data suggest that a single mutation is not adequate to cause clinically important levels of resistance against antibiotics of the fluoroquinolone class [68-70]. This evolutionary process of the acquisition of multiple resistance mutations has been extensively studied for a single drug [11, 33, 71-73]. Resistance to multiple drugs also involves mutations at multiple loci, where each mutation can give resistance to a specific drug or may confer resistance to other drugs belonging to the same class or a new class by a mechanism called cross-resistance or negative collateral sensitivity.[74-76]. Despite the rapid emergence of multidrug resistance in recent days in many clinical bacterial populations [2, 29, 30, 77-80], only a handful of studies have been attempted to explore the precise evolutionary mechanisms underlying multidrug resistance in bacteria [16, 81]. Such studies characterised fitness landscapes of multidrug

resistance by determining epistasis in the cost of resistance, where positive epistasis was predominant. However, epistasis not only depends on the genetic background at which they arise [82], but the pharmacodynamics of drugs (i.e. fitness in presence of drugs and the interactions between drugs) may also influence the epistasis between multiple resistance conferring genes, as has recently been investigated at theoretical level [51].

Here, we characterised fitness landscapes comprising different resistant genotypes including the susceptible and resistant genotypes under a variety of environmental conditions such as drug free environment, in presence of a single drug and combination of drugs to understand the evolutionary dynamics of mutations at multiple loci conferring resistance to multiple drugs.
Although the pattern of epistasis varied in all three different fitness landscapes, our results indicate that all three fitness landscapes produce weakly negative epistasis or no epistasis. This non-epistatic fitness landscapes are attributed to the types (i.e. antibiotic of specific class and its cellular target), and the concentrations of drugs (i.e. fitness varies with in absence or presence of a single drug or in a combination of both drugs, as well as the types of drugs that have been used). This means that epistasis, though it was weak but on limited occasions, was apparent for a given set of genotypes, which were influenced by the drug specific resistance mutation as well as pharmacodynamics (i.e. relationship between the concentration of drug and fitness) of the administered drugs. In all cases, diminished fitness advantage for all single resistant genotype was apparent in absence of drug pressure. This slower growth rates in absence of drug pressure were mostly due to the fitness cost incurred by drug specific resistant mutations, which may have given rise to non-epistatic fitness landscapes. It is worth mentioning that in two cases, one mutation (i.e. kanamycin resistance genotype) doesn't entail a fitness cost, and in the third case, in addition to the rifampicin and streptomycin resistance mutation, Although the genetic context is different, such non-epistatic fitness landscapes was previously reported in *E. coli* [26], but at the same time our result in absence of drug pressure differed by earlier studies where positive epistasis was predominant [16, 86].

Importantly, whole genome sequencing detected the presence of an additional mutation in the *cyoA* gene in the streptomycin-rifampicin double resistant genotype. In *E. coli*, this gene encodes subunit II of the cytochrome bo_3 terminal oxidase complex. Gene expression analysis of *cyoA* revealed an inter-population variability under tetracycline induced stressful environment, which is associated with adaptive resistance to tetracycline [83-85, 101]. A system level investigation has confirmed that mutations in other components of the cytochrome

oxidase transfer system, such as mutations in *cyoB,* conferred reduced susceptibility to antibiotics belonging to the aminoglycoside class [102]. In our case, we speculate that this *cyoA* mutation arose during strain construction through natural transformation assay, perhaps when the competent cells were treated with salt. Although we did not measure the MIC of this genotype for tetracycline or any other drugs, this mutation might have conferred heterogeneous resistance to other drugs. Our growth rate estimates suggest that this mutation incurred significant fitness cost (**Figure S5**). Therefore, this additional mutation might have affected epistasis of this double resistant genotype.

We also show that our fitness landscapes were influenced by fitness cost such that presence of only a single drug would yet be a non-specific environment for other resistance conferring mutation commonly harboured by a single resistant and a double resistant genotype. In this particular case, for example, a mutation conferring resistance to drug A will affect the growth in presence of drug B to which the MIC is unaffected. This means that growth is further exacerbated by the presence of drug B plus the cost of resistance incurred by the original mutation. Such situation has not been previously investigated experimentally, but here we observed a pervasive negative epistasis (i.e., both weak and strong negative epistasis).

In one case (i.e. between kanamycin and rifampicin resistance mutation) both weak and strong negative epistasis arose in presence of a single drug at concentration spanning from very low to the concentration around the MIC. Most notably, weak negative epistasis was apparent in presence of varying concentration of kanamycin, whereas strong negative epistasis was observed only in presence of the MIC concentration of the rifampicin antibiotic (**Figure 7**). This particular negative epistasis was observed in the case of costly resistant mutants. For example, in presence of kanamycin antibiotic only, the double resistant genotype (harbouring no-cost kanamycin resistance mutation and high-cost rifampicin resistance mutation) can grow slower. Weak epistasis means that decreased fitness advantage of this double mutant genotype is achieved by the integration of a costly rifampicin resistant genotype on a genetic background harbouring less costly resistant mutation. In other word, multidrug resistance evolution presumably takes place when less costly resistant genotype release the fitness burden by integrating a costly resistance mutation.

We also show strong negative epistasis between kanamycin (no-cost resistant genotype) and streptomycin resistance genotype (costly resistant genotype compared to the kanamycin

resistant genotype) in presence of only kanamycin antibiotic at concentration around the MIC. This observation also suggests that strong negative epistasis may decrease the adaptive potential for multidrug resistance evolution given that a low cost resistance mutation acquires a costly resistant mutation.

We then measured epistasis between rifampicin and streptomycin resistance mutation. Earlier studies documented positive epistasis between these two drug resistance mutations in absence of drug pressure [16, 81]. However, we observed a pervasive negative epistasis or no epistasis between rifampicin and streptomycin resistance mutation both in presence of single drug and multidrug environment (**Figure 7**). A strong negative epistasis was present when the concentration of rifampicin was higher or when this drug was combined with streptomycin antibiotics. This could be explained by the fact that the high cost rifampicin resistance mutation requires higher concentration of rifampicin antibiotic in order to release its high fitness cost. However, an additional point mutation (i.e. mutation in *cyoA* gene we identified by whole genome sequencing) was present in this genotype,. This additional mutation further increased the fitness cost in this genotypes (**Figure 7**). We suggest that without this additional mutation, the fitness landscape would presumably be less or non-epistatic. Therefore, we suspect that this type of deleterious mutation may have occurred and confounded previous studies [81, 86, 87], where epistasis was measured without sequencing of bacterial whole genome.

We investigated epistasis on fitness landscapes when there was a cross-resistance or negative collateral sensitivity. Cross-resistance has been defined as a function of the evolutionary response to a single antibiotic. So, unlike physiological interaction, cross-resistance does not require which drugs to be applied in combination [52]. Cross-resistance occurs in situation when evolution of resistance to a drug can simultaneously select for resistance or decreased sensitivity to multiple drugs. This phenomenon of resistance evolution has been reported in many clinical situations, especially in the case of treatment failure due to multidrug resistant bacteria harbouring a single point mutation or a resistant enzyme capable of neutralizing many different antibiotics, including NDM-1-metallo-β-lactamase (New-Delhi metallo-β-lactamase carried by the plasmid) producing *K. pneumoniae* is capable of hydrolysing many other different antibiotics [88], or altered efflux–pump mediated cross-resistance has also been found to be associated with resistance to multiple drugs in many bacterial species, including *P. aeruginosa* and many other gram negative bacilli [89, 90]. Theoretical observation suggests that epistasis can emerge in presence of cross-resistance. For example, in a situation when two

resistance mutations slightly increase the MIC for both drugs individually, but more increase in MIC to both drugs occurs when two mutations act in combination as a result of positive epistasis, or negative epistasis arise in presence or absence of even low level of cross-resistance [51]. Therefore, we considered analysing the concept of cross-resistance to understand the evolutionary dynamics of multiple resistance conferring genes giving rise to epistasis on the pharmacodynamics fitness landscapes. In our study, we observed such cross-resistance conferred by streptomycin resistant genotype, which underwent improved growth advantage in presence of kanamycin and rifampicin antibiotics by increasing its MIC to both drugs. Similar cross-resistance was observed in an earlier systematic study between kanamycin and streptomycin. For example, cross-resistance that we observed here – between rifampicin and streptomycin – was found to be collaterally sensitive to each other in previous study [74]. This difference could be due to the fact that the genetic background and the fitness cost incurred by streptomycin resistance could be different than what was found in previous study. This means that our low-cost streptomycin resistance mutation could be a hyper-accurate phenotype, which is streptomycin dependent [56, 91]. Our growth rate data also support the notion that streptomycin resistance mutation that we observed here resulted in higher fitness compared to the growth rate in absence of drug pressure (**Figure S2**). Therefore, we assume that this hyper-accurate phenotype gives higher growth advantage in presence of streptomycin and, at the same time, it gives improved growth advantage when it is exposed to rifampicin antibiotic whose target also belongs to the same flow of a fundamental cellular process (i.e., **transcription→translation**). Such prediction was made by an earlier study where positive epistasis was pervasive [16]. Another distinct case of cross-resistance we observed in this study was kanamycin resistant genotype gave decreased susceptibility to rifampicin. This result also contradicts the result observed in an earlier study [74].The result we obtained here would be due to the fact that both kanamycin and rifampicin belong to two distinct classes of antibiotics with two different cellular targets, for example rifampicin targets RNA-polymerase and streptomycin targets 30S ribosome. The gene *nptII* we used here encoded neomycin phosphotransferase II enzyme not only conferred high level of kanamycin resistance, it also probably affected the binding affinity of rifampicin antibiotic to the RNA-polymerase core enzyme, which in turn resulted in decreased susceptibility to rifampicin. Therefore, our study highlights the need for more detailed studies for further understanding of cross-resistance,which might be helpful in designing effective treatment strategies in many clinical situations, especially in the case of drug cycling.

We also considered factor such as contribution of drug-drug interactions on bacterial fitness associated with resistance evolutions on the pharmacodynamic fitness landscapes. Antibiotics when used in combination can have synergistic, antagonistic or suppressive effect [46, 95]. In synergistic drug combination, reduction in fitness is more than the expected from the sum of the two individual single drugs, while antagonistic drug interaction is associated with less reduction in fitness. For the suppressive drug interaction (a special form of antagonistic interaction), combined effect of two drugs is weaker than the effect of the individual drugs [45]. Several previous studies examined the effect of drug combinations with varying degree of drug interactions to explore the potential spontaneous resistance evolution against those drugs combinations. For example, both theoretical and empirical studies suggest that synergistic and antagonistic drug interaction leading to negative and positive epistasis respectively can also slow down or accelerate the evolution of multidrug resistance, respectively [50, 51]. Therefore, we attempted to characterise our fitness landscapes when there is drug interaction (deviation from Bliss independence) and drug specific resistance mutations are in actions. We observed a varying degree of synergistic drug interaction between kanamycin and rifampicin, for which we also identified negative epistasis. Our result indicates a good concordance with previous studies [16, 50-51], but differs from the expected positive epistasis in the absence of fitness costs [51]. In absence of fitness cost, we also observed a varying degree of antagonistic interactions between kanamycin and streptomycin. In presence of this drug pair, there was no detectable epistasis observed between those drug specific resistant mutations. This could be explained by the fact that both antibiotics belong to the same chemical class, target the same cellular function, and also undergo a negative collateral sensitivity to each other. In the case of rifampicin and streptomycin drug pair, no apparent interaction were observed nor any epistasis between these two-specific resistant mutations. However, in the cost of resistance, positive epistasis was reported between these two drug specific resistance mutations [16].

The mutant selection window (MSW) is defined as the concentration space between the MIC for the susceptible bacteria and the MPC for the resistant bacteria [96]. We characterise MSWs in presence of multidrug environment. Previous studies have reported that the MSW can vary (i.e. narrower or wider MSW) or remain static under two-drug antimicrobial environment [48, 97] . Therefore, to understand the impact of drug combinations and the interaction between drugs on the size of the MSW, we aimed at determining selective advantage of each genotype under each concentrations of drug that we used for the fitness measurement. Our results indicate that the concentration space for drug pairs that we used (i.e., kanamycin-rifampicin,

kanamycin-streptomycin, and rifampicin-streptomycin) were not confined to the traditional MSWs, rather extended further below the MIC – called the minimal selective concentration (MSC) [98]. This means that selection of resistance occurs at concentration ranging from very low to very high drug concentrations. This wider space for these MSWs was attributed to a number of factors and also implicated with resistance emergence: 1) some drug concentrations allowed wild-type susceptible bacteria to grow at very low concentrations of kanamycin and rifampicin combination. This clearly suggests that selection of resistance, multidrug resistance in particular, may occurs at the sub-MIC concentration of this drug pair. Analogous to this finding, such observation was made by an earlier study in experimental bacterial population in presence of a single drug [99]. We observed similar result for other drug pair for both single and double resistant genotypes, for example in presence of kanamycin and rifampicin antibiotics (when used alone or in combination), we observed a wider MSW for the kanamycin resistant genotype as well as for the double resistant genotype harbouring both kanamycin and rifampicin resistant mutations at sub-MIC combination of this drug pairs 2) similarly, a wider MSW was also observed between kanamycin and streptomycin antibiotic, 3) but a narrower MSW was observed for the rifampicin and streptomycin drug pair which follows a traditional MSW. Overall, our results suggest that selection of resistance against multiple drugs may also occur at sub-MIC combination of two-drugs and/or in a situation when there is a cross-resistance imparted by a singly resistant genotype. The result we presented here also supports the concept of minimal selective concentration (MSC) – biologically relevant sub-MIC selective concentration where antibiotic concentration is several hundred below the MIC of susceptible genotype – which was suggested by an earlier study where selection of resistance at very low antibiotic concentrations was reported [99]. Thus, the results we have presented here may enhance our understanding of the multidrug resistance evolution under variety of drug environments. Our results together with others may implicate the need for the revision of the traditional concept of mutant selection window [96], especially in the case of evolution of multidrug resistance.

In this present study, we characterised our pharmacodynamics fitness landscape by measuring growth rates as a proxy for fitness. The results we have presented here warrant the need of integration of kill rates (negative growth rates) for fitting the Hill-pharmacodynamics function as well as determining the epistasis at concentration beyond the MIC. For further exploration of the consequences of multidrug resistance evolution on the pharmacodynamics fitness landscapes, we should determine other fitness components such as the carrying capacity and

the duration of the lag phase, since a varying competitive ability in both cases was reported earlier [81]. These differential trait effects has been revealed by a recent study with both in single and double resistant genotypes, for example some of the rifampicin resistance mutations were shown to be beneficial in growth rate but all were found to be deleterious on their carrying capacity, despite their competitive superiority [100]. Therefore, both growth rate and carrying capacity are important determinants of the maintenance of resistance. We also found that a double resistant genotype in addition to drugs specific resistant mutations (i.e. resistance mutations in *rpoB* and *rpsL* loci) harboured an additional deleterious mutation in *cyoA* locus, which was detected by sequencing of the whole genome. This additional mutation in *cyoA* locus possibly affected the epistatic interactions between two drug specific resistant mutations in our study. We also suspect that unexpected additional mutations like the one in *cyoA* locus we detected perhaps occurred but not been detected in earlier studies due to the lack of whole genome information. Fitness estimates may therefore was misleading in those studies. Additionally, we only studied two-locus fitness landscape by constructing two specific mutations conferring resistance to two antibiotics. Despite many known resistance mutations in those drug specific resistance loci, several combinations of those drug specific resistant mutations may cause problem in determining the trait effects (i.e. growth rate, kill rate, fitness in carrying capacity). We also determine fitness under static drug environment, but the dynamics nature of multidrug resistance evolution remains to be explored. We have measured the growth rates at constant drug conditions from a master antibiotic solution on everyday basis where possible drug decay may have occurred, which might have affected the constant drug conditions during the growth assay.

In conclusion, epistasis play important role in the adaptive evolution; of special relevance is the recent observations that pharmacodynamics fitness landscapes are characterised under wide range of drug concentrations, including single drug and combination of drugs. Our results indicate non-epistatic fitness landscapes between resistance conferring mutations in absence or presence of drugs, but resistance mutations were previously shown to produce epistasis in presence of fitness cost [103]. However, we observed a wider mutant selection window for two antibiotics pair where the sub-MIC concentration space is many fold lower than the MIC of the susceptible genotype. Thus, these results suggest that the sub-MIC antibiotic concentrations, can also be generated in certain body compartment during combination therapy, may potentiate the evolution of *de novo* multidrug resistance, as has previously been reported by an empirical study for a single drug [99]. Another important aspect of this study was cross-resistance – a

phenomenon where a mutation conferring simultaneous resistance to many other drugs belonging to the same class. We also show that a wider MSW also occurred in presence of cross-resistance, which may be an important implication in resistance evolution. Overall, the results we have presented here underlie the importance of choosing the right drug during cyclic treatment as well as using optimal treatment dosing regimes during combination therapy that exclude the prolonged exposure of sub-MIC level of antibiotics. Therefore, more extended analysis of such fitness landscape is required in determining epistasis in the evolution of multidrug resistance evolution.

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Supplementary materials

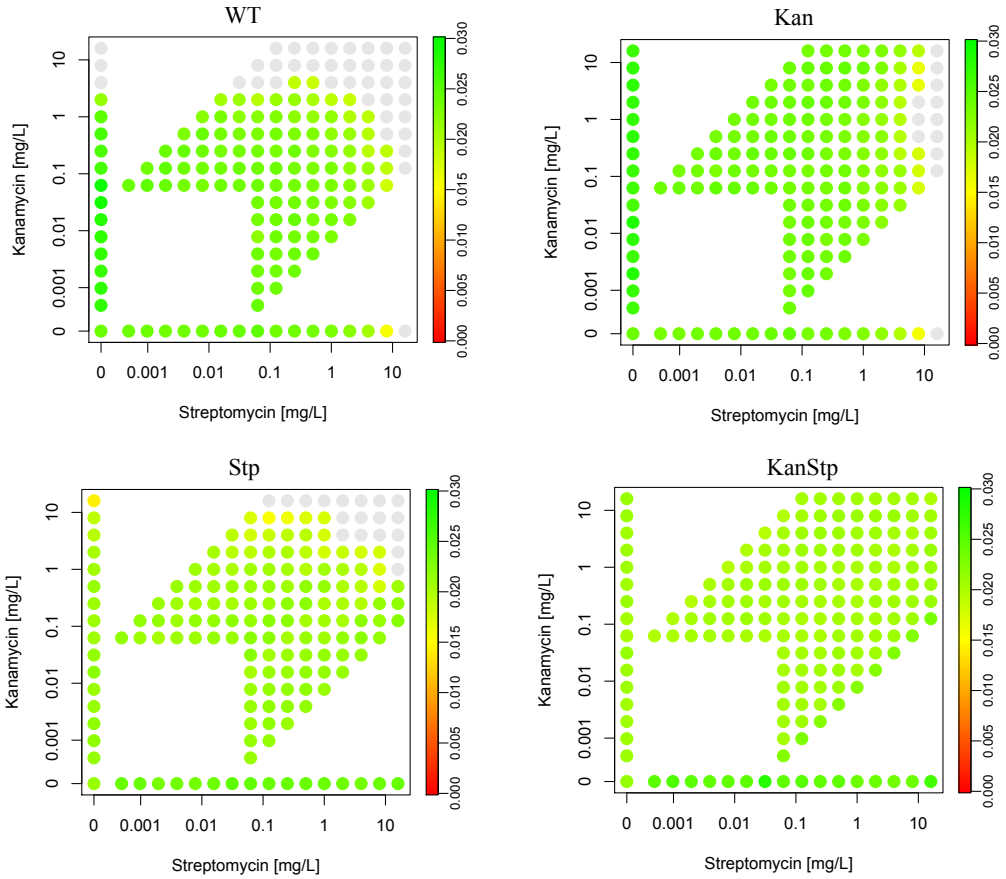

Figure S1 Pharmacodynamic fitness landscape for the Kan-Stp. Plots show fitness of different genotypes in presence of two antibiotics kanamycin and streptomycin combination. Growth rate as a function of two antibiotics is given in gradient scale from 0.0 to 0.03.

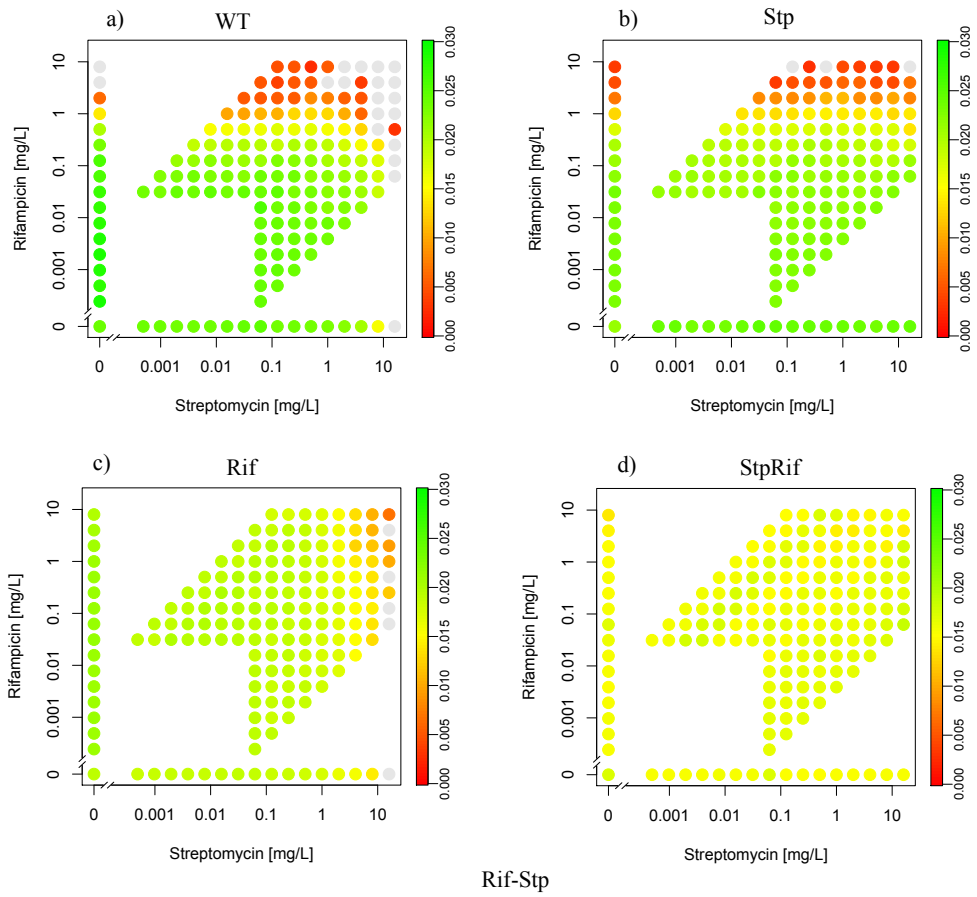

Figure S2 Pharmacodynamic fitness landscape for the Stp-Rif. Plots show fitness of different genotypes in presence of two antibiotics streptomycin and rifampicin combination. Growth rate as a function of two antibiotics is given in gradient scale from 0.0 to 0.03. Gray stripe represents no growth was determined for certain drug concentrations.

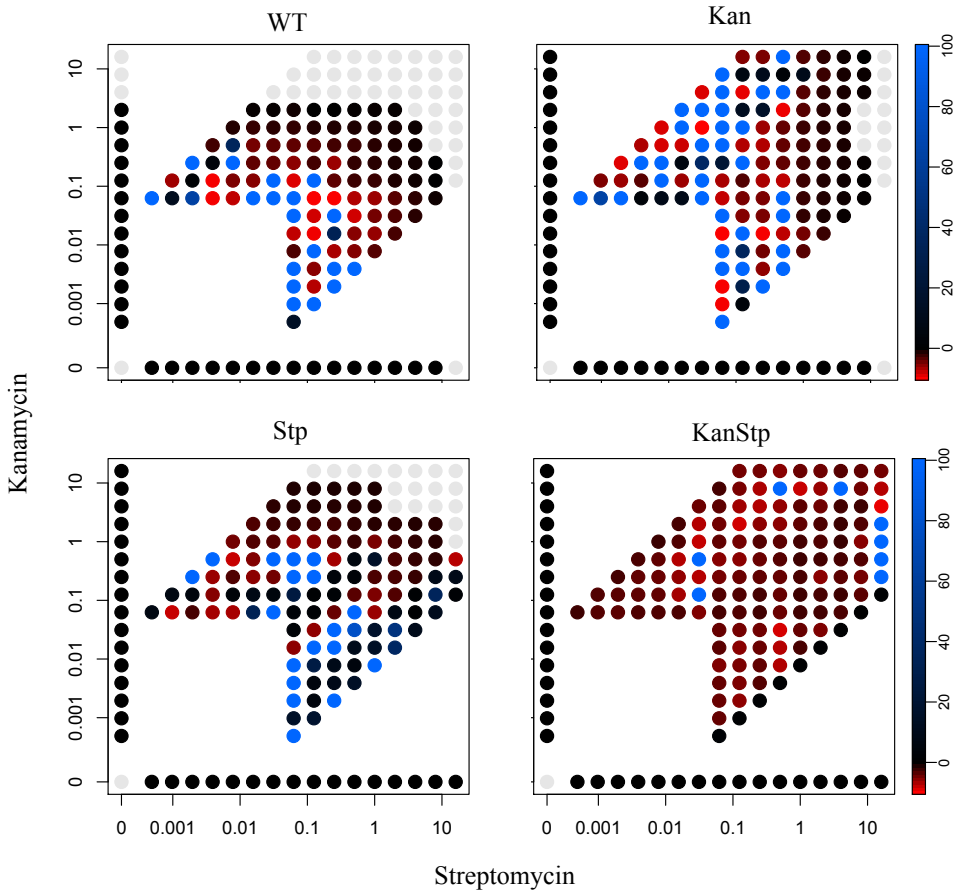

Figure S3 Drug interactions between kanamycin and streptomycin antibiotic. Drug interaction is a function of two antibiotics in combination given by a gradient scale from negative to positive interactions across all genotypes. Grey dots represent concentration where no growth was detected. WT indicates the wild type, Kan indicates kanamycin resistant genotype, Stp indicates streptomycin resistant genotype, and KanRif indicates double mutant genotype harbouring both resistance mutations.

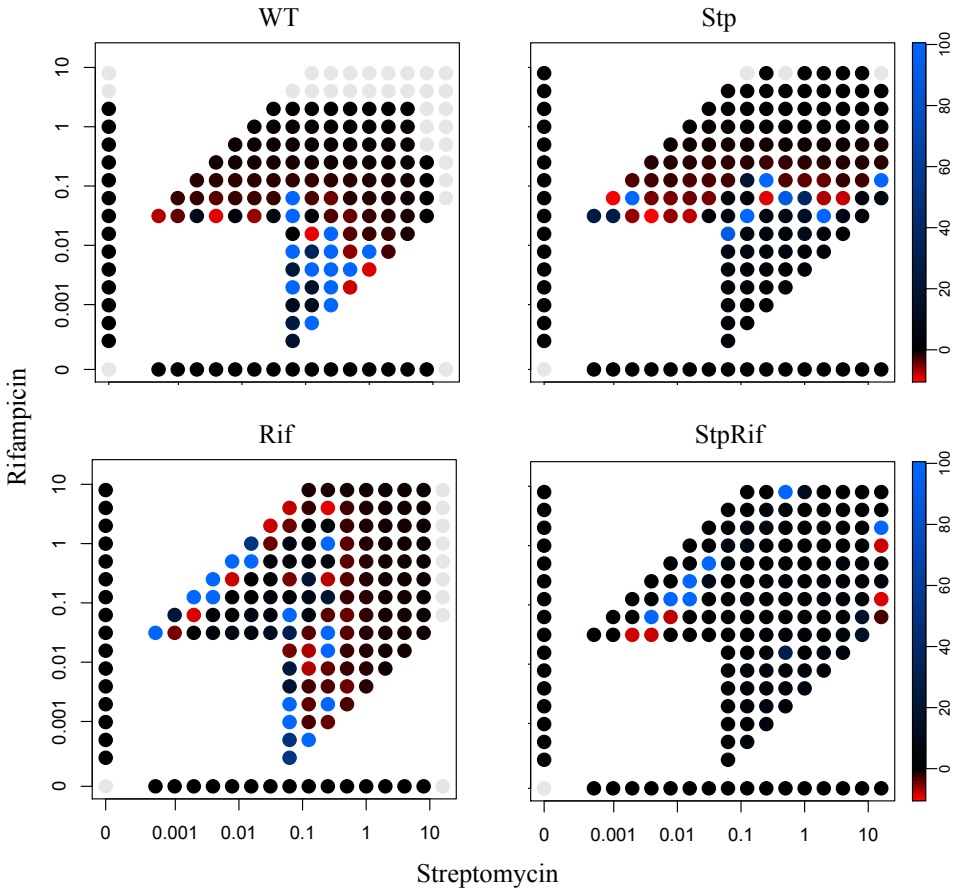

Figure S4 Drug interactions between rifampicin and streptomycin antibiotic. Drug interaction is a function of two antibiotics in combination given by a gradient scale from negative to positive interactions across all genotypes. Grey dots represent concentration where no growth was detected. WT indicates the wild type, Rif indicates rifampicin resistant genotype, Stp indicates streptomycin resistant genotype, and KanRif indicates double mutant genotype harbouring both resistance mutations.

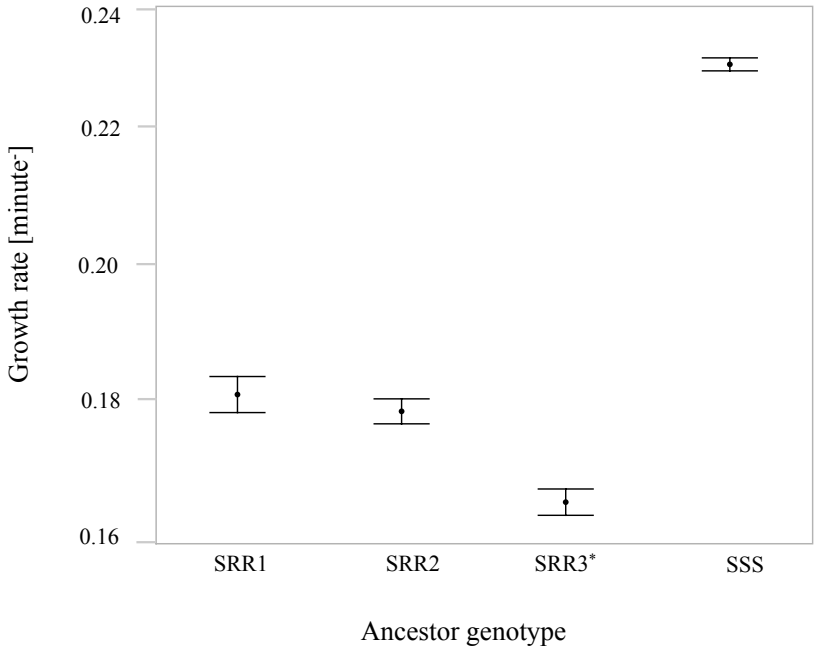

Figure S5 Mean growth rates for three different genotypes. Each error bar was constructed using 1 standard error from the mean of 5 independent population replicates measurements. All genotypes are significantly different from each other, except SRR1 and SRR2 (ANOVA: pairwise comparisons wising TUKEY-HSD post-hoc test: *p*-value = 0.8051). SRR1 and SRR2 were the same strain only differed by construction through natural transformation method such that SRR1 was constructed using StpR as a donor, while SRR2 was constructed using RifR as a donor. SSS = Susceptible/wild-type genotype; both SRR1 and SRR2 are double mutant harbouring both *rpsL* and *rpoB* mutations; SRR* is also a double mutant genotype carrying both *rpoB* and *rpsL* mutation plus an additional mutation at *cyoA* locus, and this genotype was used for characterizing pharmacodynamic fitness landscape.

Table S1 List of primers used in amplifying targeted allele sequences.

Gene name	**Primer sequence**
rpoB 1 (forward)	TTCGATTCAGGTCGACTCGT
rpoB 1 (reverse)	CAGGCGTTCTGGAACAAGAT
rpoB 2 (forward)	TGGATCAAAACAACCCATTG
rpoB 2 (reverse)	ATCGCCACGACCCACTTTAT
rrs 1 (forward)	GGCAGGCTTAACACATGCAA
rrs 1 (reverse)	CTACGCATTTCACCGCTACA
rrs 2 (forward)	CTGGAGGAATACCGATGGCG
rrs 2 (reverse)	TAACCGCCCTCTTTGCAGTT
rpsL (forward)	ATGGCAACAACAAATCAGTT
rpsL (reverse)	TTATTTCTTAGGACGTTTAG

Table S2 PCR master mix preparation per reaction

Reagents	**Amount [uL/reaction tube]**
PCR buffer 10x	2.5
MgCl2 25 mM	2.5
dNTP 10mM	0.5
TaqGold 5U /µL	0.1
Primer mix (conc. 0.5µM) (F+R)	1.5
Sigma H2O	17.9
Template DNA	5
Tolal volume	30

Table S3 PCR program used to amplify the targeted allele sequences

PCR program:		
Step	**Temperature**	**Time**
1 (hold)	95°C	6 minutes
2 (denaturation)	94°C	45 seconds
3 (annealing)	58°C	45 seconds
4 (elongation)	72°C	2 minutes
5 (repetition)	Step 2 to 4 for 35 cycles	
6 (final elongation)	72°C	10 minutes

Chapter 3: Emergence of de novo multidrug resistance in experimental bacteria populations evolving under sub-lethal drug combination: the role of natural transformation

Summary

The emergence of multidrug resistant bacteria has become a major cause of therapeutic failure in treating infectious diseases. Multidrug resistance is frequently acquired by horizontal gene transfers, but can also arise *de novo* through mutations. In the latter case, recombination may still be important in reducing clonal interference between selected resistance mutations that spread simultaneously within the population. Many bacteria, including important pathogens, regularly undergo recombination via natural transformation (uptake of free DNA from the environment), but the role of natural transformation in the evolution of *de novo* multidrug resistance evolution is unclear. Our study aims at characterizing the evolutionary dynamics of *de novo* multidrug resistance through evolution experiments in which the emergence and spread of resistance mutations is monitored and the impact of recombination is assessed. We initiated our evolution experiment with populations comprising either naturally competent or non-competent genotypes of the Gram-negative bacterium *Acinetobacter baylyi*. These populations were then propagated by serial transfer for ~650 generations under sub-lethal doses of rifampicin and streptomycin antibiotics used in combinations. We then characterized our evolved populations by employing different phenotypic assays and by whole genome sequencing. Both growth rate and competition assays demonstrated that the populations propagated under drug pressure had evolved higher fitness when tested in same environment, but there was no difference in fitness gain between competent and recombination-deficient populations. Moreover, our antibiotic susceptibility assays showed that all clones that evolved in presence of drugs had become strongly resistant to rifampicin, whereas resistance to streptomycin was much weaker or absent. Consistent with these findings, whole genome sequencing revealed an abundance of different *rpoB* mutations (indicating target alteration as a resistance mechanism). We have also identified a number of other mutations that have been reported to be associated with resistance to other antibiotics. In conclusion, we saw no evidence that recombination by transformation facilitates adaptation to antibiotics, possibly because the limited number of mutations that were spreading simultaneously prevented clonal interference.

Introduction

Recombination is a complex evolutionary process. This process is ubiquitously present in the nature, including both in prokaryotic and eukaryotic populations. Nevertheless, the benefit and the cost of recombination yet remain a paradoxical question in evolutionary biology [1-3]. It is assumed that allelic associations – called linkage disequilibria (LD) – between different loci that are broken up by recombination determine the adaptive benefit of recombination. For example, recombination can help purge deleterious mutations harboured by particular population lineage [4-6], or accelerate adaptation in situations where beneficial mutations arise at different loci of diverse population lineages and compete against each other – a phenomenon called clonal interference [7, 8].

In many bacterial species, recombination plays a key role in the adaptive process by generating genetic variations by acquisition of genes and genetic elements from their surrounding sources via horizontal gene transfer (HGT) [9-11]. For example, recipient bacteria incorporate DNA from dead cells or other inter bacterial genes and genetic elements such as different gene clusters, plasmids, transposons, prophages [12]. Subsequently these genes or extra chromosomal genetic determinants are integrated into bacterial genomes by single HGT event via recombination.

Recombination helps bacteria to adapt to many environments by evolving new functions necessary for their existence, including adaptation to antibiotics, colonization of new habitats or hosts by increasing virulence or pathogenicity, or metabolization or synthesis of new energy sources [13-16]. This process is accomplished by three main mechanisms: conjugation (mediated by extra chromosomal DNA, such as plasmid), transduction (DNA transfer mediated by phages), and natural transformations (where free DNA from the environment is integrated into the bacterial chromosome) [17].

During transformation process, DNA molecules are released in the environment from degraded bacteria and taken up by recipient bacteria through homologous recombination, which results in transformant bacteria [18]. For example, recipient bacteria capture antibiotic resistance genes from the biosphere by natural transformation [18, 19]. The source of DNA for transformation includes the genomes and extra chromosomal elements of dead cells of the same species or of unrelated organisms, and living cells that actively release DNA [20]. A number of different bacterial species have been reported to be competent for natural

transformation, including *Bacillus subtilis*, *Neisseria gonorrhoeae*, and *Helicobacter pylori* [21-23].

Genetic exchange via natural transformation in bacteria is different from eukaryotic sex. Therefore, a varying degree of benefits and costs is associated with bacterial recombination via this mechanism [24]. For example, apart from bringing beneficial mutations in the same genome, transformation is thought to be an active means of nutrient provider through DNA uptake from surrounding environments to help repair the replicative DNA lesions or to reduce mutational load in the population [25, 26]. However, with regard to reducing the mutational load of a population theoretical studies suggest that transformation decreases fitness when the source of DNA is from closely related dead cells which are originated from low-fitness mutants compared to their living counterparts, or benefit of recombination achieved via natural transformation which can reduce the mutational load in a non-competent genetic background [27, 28]. Furthermore, transformation is regarded as a complex and costly process because it requires many metabolic proteins for the uptake of DNA molecules from the surroundings [25].

Two previous experimental studies provided contrasting views on the benefit and cost of recombination via natural transformation. For example, one study in support of the benefit of natural transformation reported the accelerated adaptation by recombination via natural transformation in *Helicobacter pylori* [29]. Conversely, another study documented conditions where recombination via natural transformation did not play a role in the adaptive process [25]. However, one recent study [30] reported growth phase specific benefit of recombination via natural transformation in *A. baylyi,* while, benefit of recombination via natural transformation was found to be absent in the same species by another recent study [31].

Multidrug resistance is a global medical problem, and it is thought that resistance to multiple antibiotics is an evolutionary process and operated by *de novo* mutations (i.e. point mutation, insertion, deletion or duplication) or by recombination (transfer of resistant genes or genetic determinant from other bacteria) [32-35]. In presence of antibiotics selection pressure, bacteria can adapt and evolve by forming resistance mutations. Adaptation to multiple drugs has been observed in many bacterial pathogens such as *M. tuberculosis* [36], *A. baumannii* [37], and many more.

Analogous to clonal interference, when multiple resistance mutations spread simultaneously in a population, they may compete against each other. In this particular situation, beneficial mutations can be integrated into the same genome by recombination which breaks down this clonal interference [16]. Therefore, we assume that recombination could be an adaptive process in presence of multiple antibiotics. To date, only a single experimental study [13] has investigated the impact of recombination via natural transformation on bacterial adaptation under multiple antibiotics. This study revealed that recombination via natural transformation can accelerate adaption by bringing resistance genes together [13]. More specifically, this study showed that in the absence of recombination, initial single resistant strains inhibited the evolution of multidrug resistance, but adaptation was accelerated by recombination when two resistance genes were incorporated into the same genome via natural transformation. However, this study has some potential shortcomings in terms of de novo multidrug resistance via natural transformation. More specifically, this study was carried out starting with two strains already harboured resistance mutations. This means that bacteria acquired multidrug resistance via new mutations were not considered in this study. Additionally, all the evolved bacteria were exposed to a single drug concentration, but many de novo resistance mutations in presence of that particular drug pressure could also be selected for. This study also did not characterize the evolved strains at genomic level, so the genetic changes that occurred in the population remained unexplored. Typically, this approach only can detect a single adaptive step but does not decipher how multiple resistances are acquired sequentially, or the *recA*-deletion strain used in that study probably encountered a number of pleiotropic effects that were not investigated.

Here, we investigate the impact of recombination via natural transformation in the evolution of de novo multidrug resistance in clonal susceptible bacterial populations under sub-lethal (sub-MIC) concentrations of two-drug antimicrobial combinations by employing an experimental evolution approach. We assume that under two-drug antimicrobial treatment, initial clonal susceptible bacterial populations will diversify by acquiring drug specific resistance mutations and compete against each other over the course of evolution. Consequently, de novo multidrug resistance evolution will take place where recombination via natural transformation will reduce competition between drug-specific multiple beneficial mutations that arise within separate lineages during the course of evolution.

We used *A. baylyi* as our experimental system, which is a soil dwelling gram-negative bacterium possessing an extensive metabolic versatility, and is highly competent for natural transformation [38, 39]. Moreover, this bacterium has recently been found to be associated with hospital-acquired infections and is related to *A. baumannii*, an important nosocomial pathogen [39]. Additionally, whole-genome sequence of this bacterium is available [40]. Together, these features have made *A. baylyi* an attractive model organism in microbiology and evolutionary biology. We used different genetic constructs of competent (Rec+) and non-competent (Rec-) strains to investigate the effect of genetic exchange on laboratory evolution of bacterial de novo multidrug resistance by integrating experimental evolution with whole genome sequencing of the evolved populations to monitor the actual evolutionary steps that have taken place.

Materials and methods

Strains, media and growth conditions used in this study

We used *A. baylyi* derived from ADP1 strain (NC_005966) [40] - details are listed in Table 1. All strains used were devoid of plasmids as well as bacteriophages. Therefore, natural transformation is the only means of recombination among recombination-efficient (Rec+) populations in this study. We used two different Rec+ strains that were fully susceptible to antibiotics and employed in the evolution experiment to investigate how a fully susceptible bacterium adapts to a multidrug environment. The genetic architecture of these two Rec+ strains was identical with the only exception of a fluorescent marker. These fluorescent markers were used to detect possible cross contamination events during the course of experiments. These two strains also possessed fully functional DNA uptake systems making ..

Table 1 Description of different populations of *A. baylyi* used in this study.

Strain	***Genotype***	***Description***	***Reference***
ADP1Rec+3	trpE27 ACIAD0921::*ecfp*	Transformation-efficient ancestor strain; tryptophan auxotroph; cyan fluorescence marker (*ecfp*)	Genome sequenced in this study
ADP1Rec+4	trpE27 ACIAD0921::*eyfp*	Transformation-efficient ancestor strain; tryptophan auxotroph; yellow fluorescence marker (*eyfp*)	Genome sequenced in this study
ADP1Rec-13	trpE27 ΔcomFEBC::*dhfr1*	Transformation-deficient control strain; tryptophan auxotroph; trimethoprim resistance marker (*dfhr1*)	Genome sequenced in this study

these bacterial strains capable of transformation with high efficiency. A Rec- strain was used as a negative control, and this strain was devoid of a functional DNA-uptake system due to the disruption of *comFEBC* gene cluster. All strains were provided by Nils Huelter and Pal Johnsen from the Arctic University of Norway.

We used lysogeny broth (LB) for all experiments and most assays (i.e. for the growth rate, serial passage, competition assays, and bacterial culture preparation for the whole genome sequencing); for antibiotic susceptibility assays, we used Muller Hinton-II (MH-II) medium supplemented with 0.05 tryptophan because all the strains we used in our experiment were tryptophan auxotroph (information provided in table 1).

Experimental evolution experiment

A single colony of each strain of *A. baylyi* (Rec+3, Rec+4, 2xRec-) was grown overnight in LB media. Thirty six population in total founded from each of these four progenitor strains by adding 10uL of overnight culture in 990uL LB medium. The experiment was carried out for three different antibiotic treatments (see below) and three replicate lineages per treatment. Populations were grown in 24-well plates on an orbital shaker (280 rpm) at 30°C, and these growth conditions were maintained throughout the experiment. After 24 hours, 10uL of each population was serially passaged to 990uL of fresh LD medium and propagated for another 24 hours. In this way, one hundred serial transfers were made which resulted in approximately 650 generations of bacterial growth. Samples were stored and frozen at 15 different time points at -80°C in 15% (v/v) glycerol.

Three different selection environments were used in this study: no-drug treatment (ND: LB medium without supplements), low-drug (LD: LB medium supplemented with 0.125 mg/mL of rifampicin and 0.25 mg/mL of streptomycin), and high-drug (HD: LB medium supplemented with 0.25 mg/mL of rifampicin and 0.50 mg/mL of streptomycin). We used these concentrations of two-drug antimicrobial combinations because these combinations were strong enough to exert a significant level of selective pressure on the bacteria whilst still permitting sufficient growth (as ascertained in pilot experiments, see Figure S1).

Growth rate assays

Growth rates for the evolved populations at different time points were measured by spectrometry (SynergyTM HT microplate reader, Biotech, USA). In doing so, individual clones from different time points (transfer 0, 10, 20, 48, 100) were first streaked on fresh LB agar plates from the -80°C freezer, and then a single colony from each plate was further grown

overnight in LB medium on an orbital shaker (180 rpm) at 30°C. From this overnight culture, the growth rate of each clone was measured by obtaining the growth curve (OD600) in LB broth with or without antibiotics in different combinations, including those that were used in the initial evolution experiments. We measured growth rates in 96-well plate (i.e., we used 5uL of culture into 175uL of LB) by spectrometry (SynergyTM HT microplate reader, BioTek, USA). The reader was set at 30°C with continuous shaking for 12 hours. Read (based on absorbance) was collected every 5 minutes interval at wavelengths of 600nm. The individual clones were also stored in -80°C freezer in 15% glycerol (v/v) for further analysis. Statistical software R was used for finding the best curve fitting a line for data obtained from the spectrometer. In short, raw OD values were normalized to a blank OD and Ln (natural log) transformed before analysis. Then the steepest slope over a 32 data points correspond to growth over 155 minutes (data obtained through every 5 minutes reading interval, including the initial read obtained at the beginning of the assay) period was determined.

Antibiotic susceptibility assay by disk diffusion

Antibiotic susceptibility testing was carried out for the same clones for which growth rates were measured. We employed disk diffusion testing as a proxy for determining antimicrobial resistance profiles among clones at different time points of the evolved populations. Individual clones were streaked on LB agar plates followed by overnight growth (18 hours) of a single colony in 15mL fresh LB medium. This overnight culture was diluted to 1:10 and inoculated on fresh LB agar with a sterile cotton swab. Prior to applying the antimicrobial disks, plates were left ajar for five minutes to absorb any excessive moisture on the agar surface. Two types of antimicrobial disks were used for the susceptibility testing which were obtained from Oxoid (Basingstoke, UK). These disks possessed the following concentrations: rifampicin 30ug and streptomycin 25ug representing approximately 10-fold and 5-fold higher MIC than the ancestor strain for the respective antimicrobial compounds (based on earlier growth rate and MIC assays in presence various concentrations of individual drug). Three disks per antibiotic were dispensed to the agar surface using sterile forceps and left for five minutes to make sure that the disk has made thorough contact on the surface of the agar. The disks were placed by maintaining a maximal distance between two antibiotics in order to avoid any potential interactions that may give rise to distortion of inhibition zones. All plates were then inverted and placed inside a plastic bag and incubated at 30°C for 18 hours followed by measurement of diameter of inhibition zones (mm) to determine growth around the disk. Since there is no specific susceptibility breakpoints set by the EUCAST (European Committee for Antimicrobial

Susceptibility Test) nor the CLSI (Clinical and Laboratory Standards Institute, USA) for the tested antimicrobial disks for the strains (*A. baylyi*) used in this study, the antimicrobial susceptibility profile of our evolved strains were interpreted based on earlier growth rate assay.

Antibiotic susceptibility assay by E-test

Additionally, we characterized the MIC profile of our evolved endpoint populations and ancestral populations with E-test assays. Individual clones were streaked on Mueller-Hinton (MH-II; Becton, Dickinson, and company, N.J, U.S.A) plates from frozen stock, and incubated for 18 hours. Suspensions of the organisms were prepared by picking appropriate number of colonies and suspending in 1.5 ml of saline under aseptic condition, and the turbidity was adjusted by measuring the optical density (OD600) of this bacterial saline suspension by spectrometry (SynergyTM HT microplate reader, BioTek, USA). Turbidity of this bacterial saline suspension was adjusted to that of a McFarland 0.06 to 1.0 standard. A total of approximately 0.1 ml of the 1.0 McFarland suspension was plated on a MH-II agar plate by using a sterile cotton swab. In doing this, sterile cotton swab was dipped into the adjusted suspension, and rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excessive inoculum from the swab. Then MH-II agar plate was inoculated by streaking the cotton swab over the whole sterile agar surface. This procedure was repeated at least two more times by rotating the plate approximately 60° each time to ensure an appropriate distribution of inoculum on the whole surface of the plate. In the final step, the rim of the MH-II plate was swabbed at least twice. Then the lid of the plate was kept open for approximately 5 minutes to allow for any excess surface moisture to be absorbed before applying the E-test strip. After adequate drying, a maximum of three E-strips (rifampicin, streptomycin and ciprofloxacin) were placed in one plate. The antibiotic strips were placed by maintaining a maximal distance between two antibiotics in order to avoid any potential interactions (i.e. antagonism, synergism, inhibition or induction) that may give rise to distortion of inhibition zones. All plates were inverted with the lid side up and placed inside a plastic bag and incubated at 30°C for 18 hours. MICs were read from the test strip where the elliptical zone of inhibition intersected with the MIC scale on the strip. Although there is no specific susceptibility breakpoints set by the EUCAST nor the CLSI for the tested antimicrobial stripes for the strain (*A. baylyi*) used in this study, we also carried out E-test on E. coli ATCC 25552 strain as a control strain to interpret the MIC of the experimental strains by comparing with this control strain.

Competitive fitness assay

Competition experiments for each of the evolved clones from the end point population (~650 generations) were carried out in the same culture conditions used to propagate the evolving populations. In this assay, we used a reference tryptophan auxotroph strain harboring *nptII* (neomycin-phosphotransferase-II) (trpE27 ACIAD0921::eyfp ACIAD3309::*nptII*), gene conferring resistance to kanamycin antibiotic. Each of the evolved clones and marker strains were taken from the freezer, acclimatized by plating on LB agar, and incubated overnight at 30°C. Afterwards, a single colony from individual plate was transferred into 1 mL of LB broth contained in individual wells of 24-well plates. These cultures were then incubated for 24 hours on an orbital shaker (250 rpm) at 30°C, which represent one complete growth cycle. Then, to start with the actual competition assay, each acclimated culture (5uL from each of the well representing each of the evolved clones) and reference strain culture (5uL) was inoculated into 9.9mL of LB with or without antibiotics contained in individual wells of 24-well plates, and mixed together. Individual samples (10uL) were taken immediately after mixing followed by serial dilution in PBS saline solution and plated on LB agar plates (at least 3 replicates of 100uL) supplemented with or without kanamycin (50mg/mL). Similarly, samples were taken after 24 hours, and serially diluted and plated on LB supplemented with or without kanamycin (50 mg/mL). LB plates were incubated for ~16 to 18 hours at 30°C, and subsequently colony forming units (CFUs) were counted visually.

Finally, relative fitness (w) was calculated as the ratio of the realized Malthusian parameters of the two competitors over the course of the 24-hours of competition as follows:

$$w = ln(E_f/E_i)/ln(M_f/M_i)$$

where E and M correspond to densities of the evolved population and reference competitor, and subscripts i and f designate initial and final densities, respectively. However, this calculation is highly sensitive to sampling error when the difference between two Malthusian parameters of the two competitor strains arise in different nutrients medium (as in our case is the medium was supplemented with two different antibiotics in combination); therefore, we also calculated the selection rate constant as a measure of relative performance in relative fitness as has previously been done by Travisano and Lenski, 1996 [41].

Bacterial whole genome sequencing and library preparation

Initially, individual clones (the same ones as for the previous fitness assays) were inoculated on LB agar plates from the cryotube followed by overnight incubation at 30°C. A single colony

was then inoculated in 10mL LB broth and incubated overnight at 30°C on orbital shaker (180 rpm). Then DNA extraction from pure culture of individual clone was performed using PureLink® Genomic DNA Kits (Invitrogen). After extraction, DNA quantification was carried out using spectrometer based microplate reader on Take-3 plate (SynergyTM HT, Bio Tek, USA). Then samples were diluted in TRIS buffer to 8-10 ng/uL and stored in -20°C.

Library was prepared using a Nextera XT kit (Illumina, San Diego, CA, USA) according to manufacturer's instructions but with slight modifications. Overall, library preparation was carried out in 5 steps. In step 1, gDNA concentrations were standardized across samples (for example, 8 ng/mL for each strain). Step 2 involved tagmentation of input DNA by the Nextera XT transposome. The Nextera XT transposome simultaneously fragments the input DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps. In step 3, tagmented DNA was amplified for a short cycle with the PCR. In step 4, PCR amplified fragments or library DNA were purified using AMPure XP beads (Agentcourt Ampure XP: Beckman Coulter AMPURE XP, U.S.A), which removed short library fragments from the samples. In the final step, each library was normalized which ensures that equal library representation has been achieved in pooled sample. In doing so, we run our purified samples further on the Shimadzu bioanlyzer for the selection of expected fragment lengths between 300 and 800. Then we pooled the library, and further purified with AMPure XP beads followed by Pippenprip size selection. Finally, the pooled library was sequenced on the Illumina MiSeq platform (300bp paired-end).

Analysis of genome data

The reads (forward and reverse) generated from the Illumina MiSeq run were first quality trimmed with 0.05% error probability, paired and then mapped to our ancestral genomes of *A. baylyi*. Ancestral strains were assembled by mapping their reads against the reference genome of *A. baylyi*, which is available on the NCBI GenBank (NC_005966). All steps were carried out using Geneious version 9.1.4 (BioMatters Inc.). In short, we used 'Find variations/SNPs' tool implemented in Geneious to identify SNPs and indels with minimum reads coverage of 5 and a variant frequency of at least 95%. A subset of SNPs identified in the *rpoB* gene was further verified by Sanger's sequencing of polymerase chain reaction (PCR) amplicons (detailed primer information and PCR program are provided in supplementary table S3-S5). For each SNP in this *rpoB* gene, a subset of clones harbouring this mutations (9 out of 18 mutations identified in the 36 evolved strains) were selected, and were amplified a 500–700 bp

PCR product covering the SNP initially identified during initial SNPs analysis, and directly sequenced the PCR products. All 9 mutations that we cross-examined were successfully confirmed.

Statistical analysis

For data analysis, various statistical methods were employed at the population level. We first used an analysis of variance (ANOVA) model to compare growth rates between ancestral genotypes and evolved endpoint populations with genetic background (i.e. Rec+ and Rec-) and testing environments (ND, LD, and HD) as fixed effects, and replicate evolved populations as random effect. We also employed a fully factorial combined ANOVA model for the relative comparison of competitive fitnesses of the evolved populations from transfer 100 (T100) which represents about ~650 generations of growth (both Rec+ and Rec-) and transfer 0 (T0) which represents ancestral populations (both Rec+ and Rec-). We considered replicate populations as random effect, and treatment and genetic background (Rec+ and Rec-) as fixed factors implemented within the model. We used LS means contrasts in the factorial ANOVA model to compare the Rec+ and Rec- populations at transfer 0 and 100 days. Similar analysis was employed for both disk diffusion and E-test MIC assay for the ancestral and evolved populations. All statistical analyses were carried out using JMP version 12 and R version 3.1.2.

Results

Phenotypic investigation on the evolutionary benefit of natural transformation in de novo multidrug resistance

To investigate the contribution of recombination in the *de novo* multidrug resistance, we evolved replicate populations of competent (Rec+) and non-competent (Rec-) *A. baylyi* under three different treatment environments for ~650 generations. We introduced static antibiotic concentrations comprising no drug (ND), low drug (LD) and high drug (HD) treatment. We confirmed that these concentrations of drugs exerted sufficient growth inhibitions (LD: ~15% growth reduction, HD: ~20% growth reduction, see **Figure S3**). Therefore, we expected that the selection pressures we used were sufficient to produce genetic variability among populations over time, and we assumed that natural transformation would play an important role in adaptation to these environments.

We investigated the level of adaptation by measuring growth rates of the evolved populations from T100 (endpoint population) and T0 (initial population) by exposing them to the same

media and selective pressure that we used earlier in the evolution experiment. In addition to this, we also quantified growth rates for the initial and the endpoint evolved populations in all other treatment environments. This investigation allowed us to directly observe to what extent adaptation had taken place in Rec+ and Rec- evolved populations after ~650 generations.

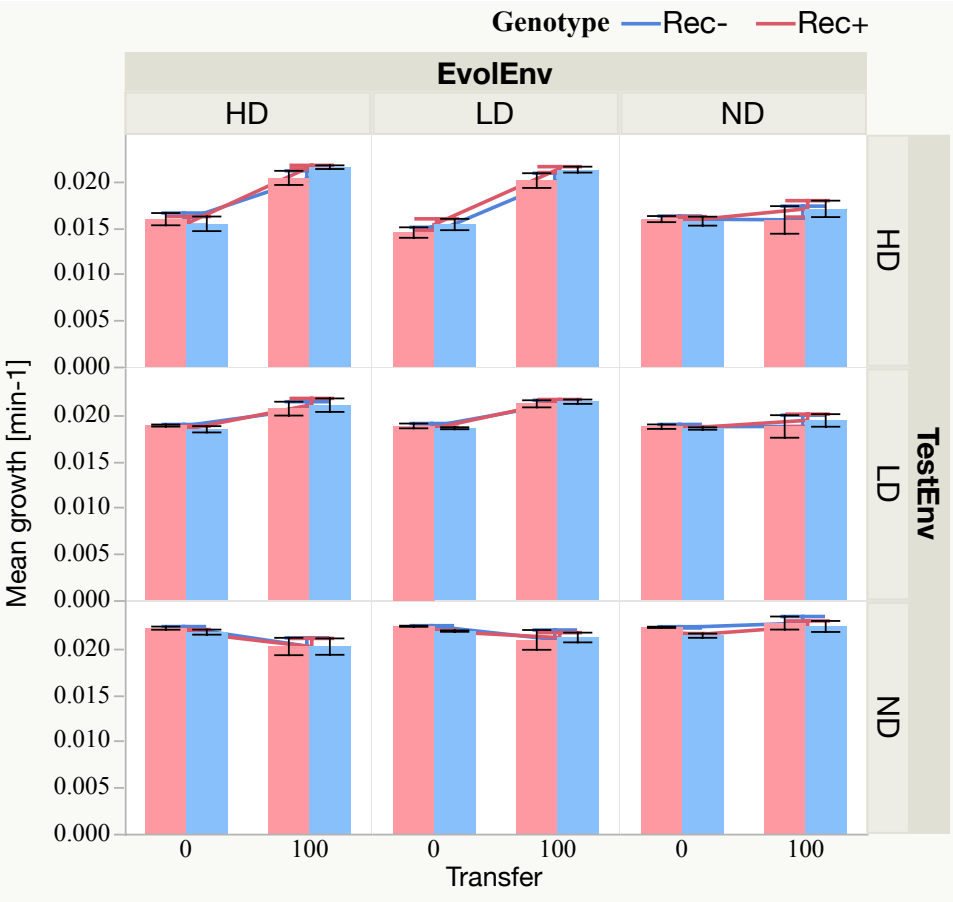

Figure 1 Growth rate for the ancestral and endpoint population. Growth rates were measured for ancestral (T0) and evolved endpoint (T100) populations. Plot shows mean growth rates for the ancestral (T0) and evolved endpoint (T100) populations. Error bar = SEM (±1).

Following ~650 generations, growth rate assays indicate that populations propagated under selective environments had evolved higher growth rates ($F_{1,\ 226}$ = 198.96, *p*-value = <0.0001, see **Figure 1**). This was apparent for all the populations when tested in the same selective environments. For example, populations with higher growth rates were observed when evolved and tested under LD and HD environments. Another important observation was that populations evolved under the LD and HD treatments showed lower growth rates when tested in ND treatment environment. This indicates possible fitness cost incurred by resistance mutations in the evolved populations when tested in absence of drug pressure, as has been suggested by previous studies [19, 42]. Therefore, our results support the fact that populations

evolved in the presence of combination treatments adapted better than those evolved under no antibiotics. However, no differences in growth rates were observed between Rec+ and Rec- populations indicating a uniform adaptive response across Rec+ and Rec- populations (**Figure 1**). We further investigated the statistical basis of fitness improvement in the endpoint populations. More specifically, we were interested to see to what extent populations acquired higher growth advantage when tested in the same selective environments. Therefore, we investigated growth rates of the evolved endpoint populations by employing a linear mixed effect model. In this model, growth rates were explained with the main effects of genotype (Rec+ and Rec-), evolved environment, and testing environment, and all their two-way and three-way interactions. Plates and strains in which genotypes were nested were used as a random factor as one replicate of each treatment was found on each of three plates.

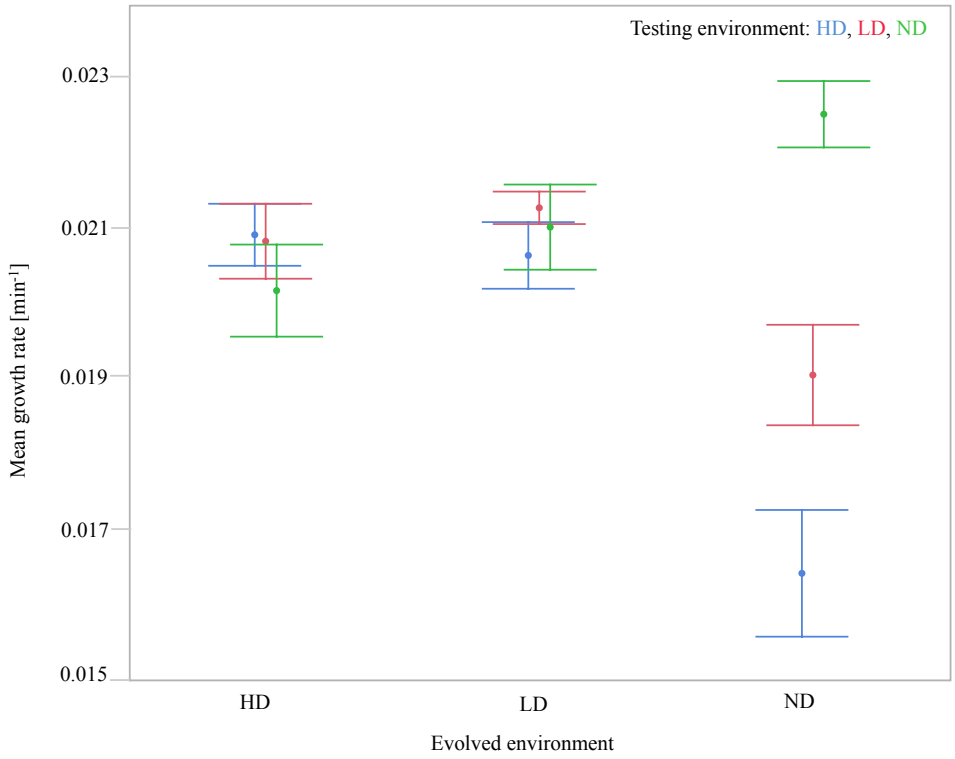

Figure 2 Mean growth rates for the endpoint populations. This plot is based on evolved environment vs. testing environment as a measure of adaptation. Each experimental replicate was measured at least five times. These are repeated measurements of the final outcome so they have been averaged. Error bar = SEM (±1).

Our results indicate that populations evolved better when they were tested under the same environment (**Figure 2**). We observed a significant association between evolved and testing environment (*p*-value = <.0001*). However, this analysis did not reveal any significant

association between genotypes and evolved environments (*p*-value = 0.9966), nor any association between genotypes and testing environments (*p*-value = 0.3413). All the parameter estimates are given in the following **Table 2**.

Table 2 Growth rates of the endpoint populations. Growth rates explained with evolved environments (EvolEnv) and testing environments (TestEnv). Genotype has two biological replicates (Rec+ and Rec-) for each level and three technical replicates (i.e., three plates) for each of the two levels. Testing environment (TestEnv) has three levels (i.e., ND, LD and HD), and it is replicated across the two Rec+ and two Rec- strains for three times (i.e., three plates).

Source	**Nparm**	**DF**	**DFDen**	**F-ratio**	**Prob > F**
Genotype	1	1	86	2.3227	0.1312
Strain [Genotype]	2	2	86	1.0899	0.3409
EvolEnv	2	2	86	8.8130	0.0003*
EvolEnv*Genotype	2	2	86	0.0034	0.9966
TestEnv	2	2	86	10.6571	<0.0001*
TestEnv*Genotype	2	2	86	1.0885	0.3413
TestEnv*Genotype*EvoEnv	4	4	86	0.0678	0.9914
TestEnv*EvolEnv	4	4	86	13.3149	<0.0001*

The above results indicate an elevated growth rates for the populations evolved and tested under antibiotics supplemented selective environment. However, based on these results we cannot infer any potential contribution of recombination in the elevated growth rates among these populations. Therefore, we then tested the adaptive potential of recombination via natural transformation (the main hypothesis of this chapter) by employing a mixed effect model using Rec and adaptation: growth rate ~ genotype * evolved environments + plate + strain [genotype], where plate and strain [genotype] are random effects. This analysis revealed that populations evolved in the face of selective environments adapted better but no significant differences were observed in growth rates between Rec+ and Rec- genotypes evolved under three different treatment environments.

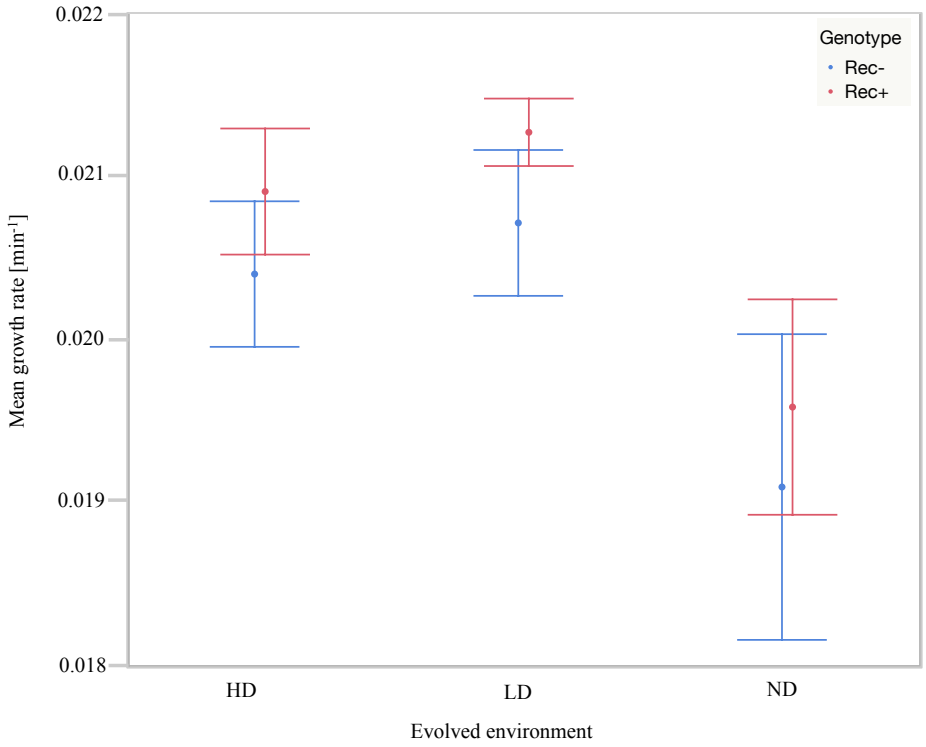

Figure 3 Mean growth rates for the evolved endpoint populations as a measure of adaptation. This plot is based on genotype vs. testing environment as a measure of adaptation. Each experimental replicate was measured at least five times. These are repeated measurements of the final outcome so they have been averaged. Standard error = SEM (±1).

Table 3 Parameter estimates for the fixed effect test for the growth rates of the endpoint populations. Growth rates explained with evolved environments (EvolEnv) and genotypes with two biological replicates (Rec+ and Rec-) for each level and three technical replicates (i.e., three plates) for each of the two levels. Evolved environment (EvolEnv) has three levels (i.e., ND, LD and HD), and each time it is replicated across the two Rec+ and the two REC- strains three times in three plates.

Source	Nparm	DF	DFDen	F-ratio	Prob > F
Genotype	1	1	98	1.3963	0.2402
Strain [Genotype]	2	2	98	0.6552	0.5216
EvolEnv	2	2	98	5.2979	0.0065*
EvolEnv*Genotype	2	2	98	0.0020	0.9980

To summarize the above findings, I presented the following Figure (**Figure 4**) for the evolved endpoint populations. From this figure, it is clear that population grew better in their respective environments; however a substantial fitness cost was also incurred when the evolved

populations were tested in alternative environments meaning that drug specific resistance mutations incurred fitness cost in the absence of selective environments.

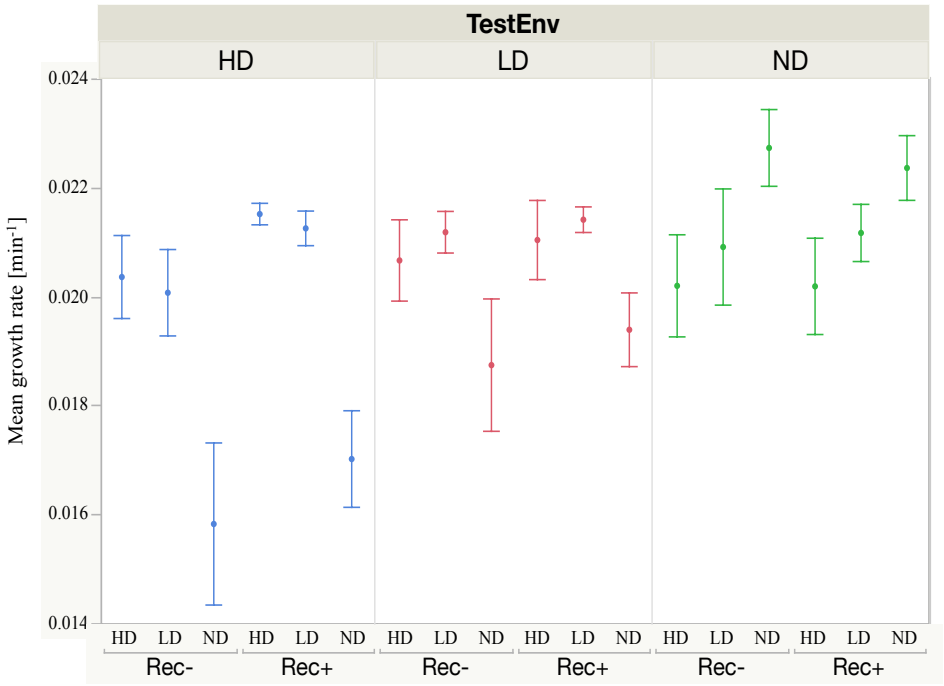

Figure 4 Mean growth rates for the evolved endpoint populations after ~650 generations. In this Figure, X-axis represents genotype by evolved environment by testing environments. Error bar = SEM (±1).

Growth rate measurement was not sufficient to determine the observed fitness differences between Rec+ and Rec- populations as one recent study reported differences in fitness trade-off between adaptation to active growth phase and survival in stationary/death phase caused by pleiotropic antagonism [30]. This finding may mirror the similar pattern of growth phase specific fitness differences between Rec+ and Rec-, which means that that both Rec+ and Rec- populations may experience fitness differences in active growth phase and stationary phase. We did not determine death rate or growth rate during active growth phase in our evolved strains, however we further explored these differential adaptive traits by measuring competitive fitness. To this end, we competed terminal and ancestral populations (i.e. Rec+ and Rec-) against a reference strain by employing pair-wise competition assays Then we compared the level of adaptation relative to the ancestral strains. We then estimated relative changes in competitive fitness by measuring the selection rate constant (Ds) by calculating the natural log difference in population in CFU/mL after 24 hours of competitions for each experimental and ancestral lines [41]. We observed that the relative fitness of competent and non-competent populations evolved in the presence of static stress induced by rifampicin and streptomycin

was significantly distinguishable from no drug treatment environment indicating that stressful conditions significantly favoured adaptations compared to benign environment (F-test = 115.58, DF = 2, 175, p-value = <0.0001) (**Figure 5**).

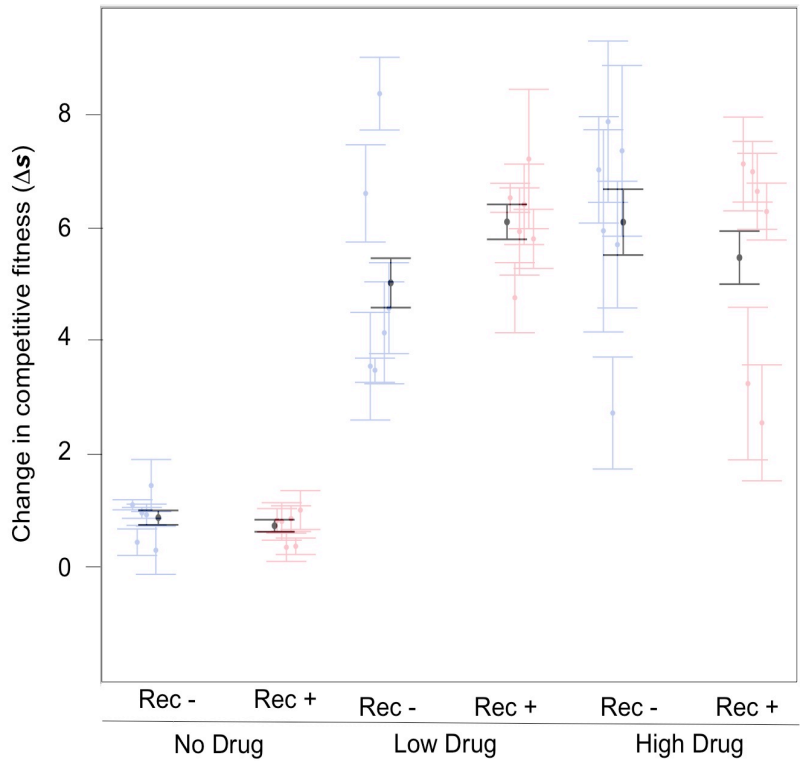

Figure 5 Competitive fitness for the evolved endpoint populations. The plot shows relative change in competitive fitness (Δs) for the evolved thirty-six endpoint populations. Rec+ and Rec- populations are indicated by coloured dots representing the mean of at least five independent competitive fitness measurements for a single population from each of the Rec- and Rec+ population that tested under different environments. Each black bar overlaid represents the mean of the six representative strains. Error bar = SEM (±1)

However, we observed that the level of adaptation is independent of the strength of selection indicating that there were no differences in fitnesses observed between low drug and high drug treatment across all populations (**Figure 5**). Contrary to the expectation that competence (Rec+) accelerates adaptation, we found instead that there were no fitness differences between Rec+ and Rec- evolved terminal populations (F-test = 0.0762, DF = 1, 175, p-value = 0.7829) (**Figure 5**).

This indistinguishable competitive fitness between Rec+ and Rec- led us into further investigation to know whether fitness among genotypes derived from the endpoint populations also varied across three different replicate plates. For example, evolved environment we used

has three levels (ND, LD, HD) and it is replicated across two Rec+ and two Rec- genotypes three times in three plates (i.e., P1, P2, P3). To investigate this we performed a linear mixed effect model using evolved environment and genotype as fixed effects, and plate and strain [genotype] as random effects. Our analysis did not reveal significant effect of recombination on the competitive fitness nor any interaction between evolved environment and genotype. For the random effect tests, the test results revealed no significant effect of recombination for each the plates (**Figure 6**). We only observed a significant effect of the evolved environment on competitive fitness. Detailed parameter estimates for this test is given in **Table 4**.

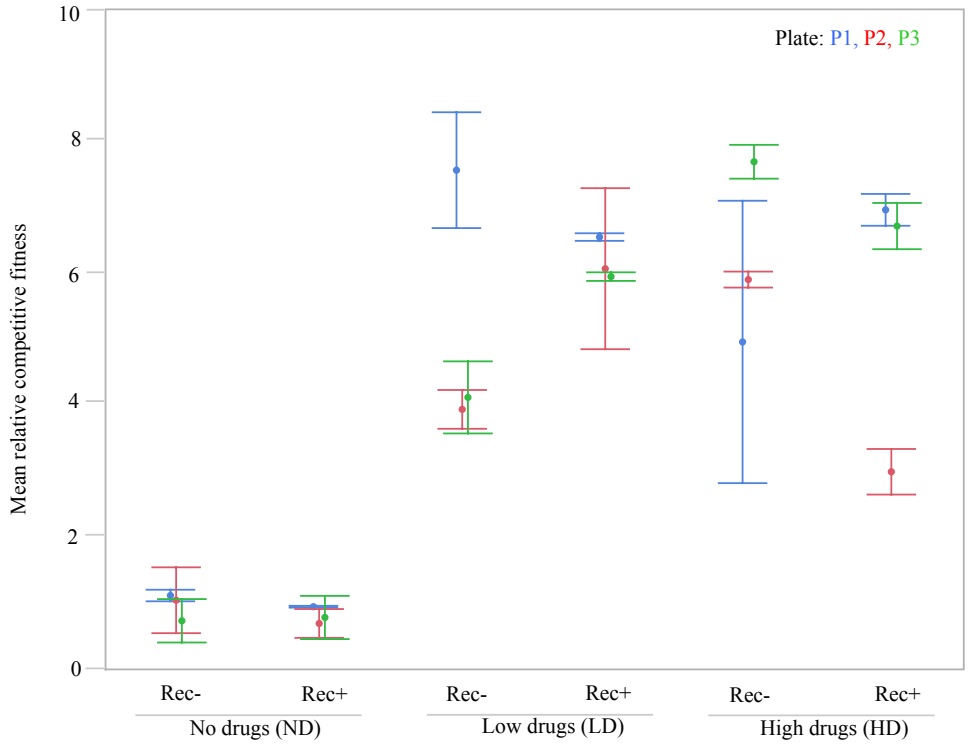

Figure 6 Mean competitive fitness vs. evolved environment across replicate plates. Each of the plates represented by coloured bar with the mean of two distinct genotypes. Each bar represents each plate with two genotypes. Error bar = SEM (±1).

Table 4 Parameter estimates for the fixed effect test for the mean relative competitive fitness of the endpoint populations.

Source	Nparm	DF	DFDen	F-ratio	Prob > F
Genotype	1	1	26	0.0198	0.8891
Strain [Genotype]	2	2	26	0.0337	0.9669
EvolEnv	2	2	26	48.8780	<0.0001*
EvolEnv*Genotype	2	2	26	1.0483	0.3649

We further investigated the diversity of susceptibility profile across population where we tested both ancestral and evolved endpoint populations. We explored this by carrying out an antimicrobial susceptibility test by using rifampicin and streptomycin antibiotic disks. We used these disks since our evolved environments (i.e., LD and HD) were supplemented with these two antimicrobial compounds in combination (i.e. LD and HD treatment) throughout our evolution experiment. We interpreted the zone of inhibition as measure for the adaptive response to the stress. We observed a significant effect on the observed inhibition zone (F-test = 4.7596; DF= 2; p-value = 0.01145). This observation indicates that zone of inhibition was significantly affected by the evolved environments. But, no significant differences were observed between Rec+ and Rec- population (F-test = 0.5808; DF = 2; p-value = 0.56203).

		No drug		Low drug		High drug	
		Strp	Rif	Strp	Rif	Strp	Rif
Com+	P1	21	20	21	21	18	20
	P2	21	23	20	21	21	0
	P3	18	20	17	12	20	16
	P4	20	23	18	16	15	0
	P5	24	23	21	10	21	21
	P6	20	22	22	14	18	21
	WT	21	24	21	24	21	24
Com-	P1	19	23	20	21	20	19
	P2	22	24	20	0	20	11
	P3	18	20	17	20	19	20
	P4	19	22	20	21	19	14
	P5	23	22	21	11	20	18
	P6	18	22	20	20	20	20
	WT	21	24	21	24	21	24

Figure 7 Disk diffusion test as a measure of resistance evolution. Each number represents the zone of inhibition [mm] and is the mean of three independent measurements. In this

experiment, the concentration of the disk for streptomycin was 25 μg/mL, and 30 μg/mL for rifampicin. Com+ and Com- indicates recombination proficient and deficient populations respectively. Genotypes indicated with P1 to P6 representing the total replicate populations in three different plates where each plate has two Rec+ and Rec- for each of the three different evolved environments. WT represents the original wild-type susceptible genotypes. The gradient colour corresponds to zone of inhibition, and represents the extent of resistance, for example inhibition zone of 0 [mm] represents highly resistance (indicated by red colour, and blue indicates low level of resistance. We observed a significant interaction between evolved environments and tested drugs (F-test = 2.9908; DF = 2; p-value = 0.05653); this result also supports that resistance was more pronounced to rifampicin antibiotic (Figure 7), which inhibits bacterial transcription by binding to the β-subunit of RNA-polymerase encoded by *rpoB* gene. However, very low or no resistance was observed to streptomycin, which inhibit protein synthesis by interfering with 30S ribosomal subunit; this might reflect the fact that translation machinery are evolutionarily conserved (relatively low mutation rate) and costly (relatively high fitness cost) [43, 44].

However, the disk diffusion test that we performed was not sufficient enough to determine the level of resistance against the selective environments after ~650 generations. Therefore, we performed E-test assay on the endpoint population to determine the effect of these drug specific resistance mutations. This approach allowed us to further determine change in the MIC profile by using not only the antibiotics we used in our evolution experiment, but we also tested the MIC for an additional class of antibiotic (ciprofloxacin) as a proxy to determine whether the evolution of multidrug resistance was taken place under sub-lethal selective environments. We further investigated into the statistical basis of the elevated level of MIC (we consider this as an adaptive response and we used fold MIC change), and this high MIC is also the result of recombination. Therefore, we employed a mixed effect model such that: **MIC ~ Rec * antibiotic + Plate + Strain [Rec]**, where plate and strain [Rec] are used random effects. The test result revealed no significant effect of recombination on the MIC or any interaction between antibiotic and Rec. Rather we observed that antibiotic had significant effect on the level of the MIC irrespective of Rec type (Rec is referring to the recombination proficient (Rec+) and deficient (Rec-) genotype). Parameter estimates for this model is given in **Table 4**.

Table 5 Parameter estimates for the fixed effect test for the mean fold-MIC change of the endpoint populations.

Source	NParm	DF	DFDen	F-ratio	Prob > F
Genotype	1	1	26	0.2498	0.6214
Antibiotic	2	2	26	19.7554	<0.0001*
Genotype*Antibiotic	2	2	26	0.1666	0.8475
Strain [Genotype]	2	2	26	0.5744	0.5700

Therefore, the E-test results revealed a significant increase in fold-MIC change to both rifampicin and ciprofloxacin but very low to the streptomycin, which is indistinguishable between Rec+ and Rec- (**Figure 8**). Similar results also observed for the disk diffusion test suggesting drug specific resistant mutations occurred during the course of evolution.

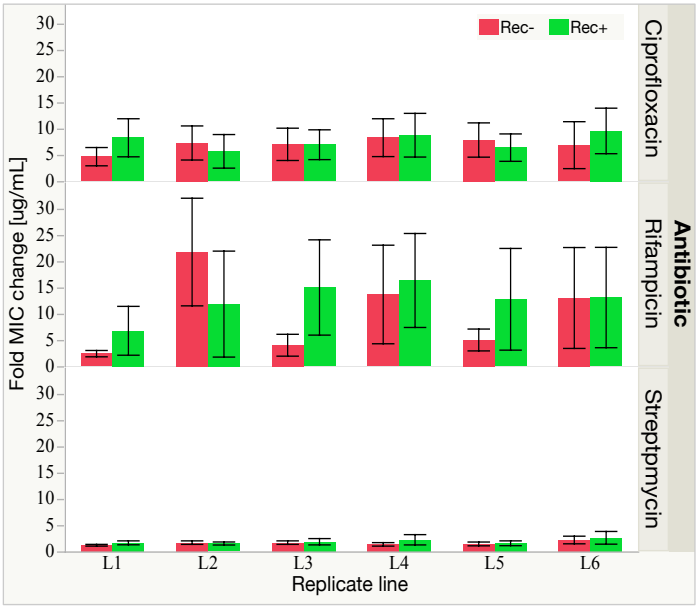

Figure 8 MIC distribution for three different antibiotics. The MIC was determined by using E-test assays. *De novo* resistance to ciprofloxacin (Cip) was observed in all populations (irrespective of Rec+ and Rec-) evolved under LD and HD environments suggesting an adaptive resistance through mutational alteration in the efflux pump system.

Our results are also in line with the observation that the frequency of streptomycin resistance mutations was very low as seen in the previous mutant screening where we selected for spontaneous streptomycin resistant mutants on plates with even only 5-fold higher concentration than the wild type MIC (data not shown here). Based on this data, we speculated that other phenotypic changes might contribute to the mild but indistinguishable adaptive

response to streptomycin across Rec+ and Rec- population. Taken together, these results suggest two important conclusions about different genotypes and their adaptation to LD and HD environment: (1) adaptation does occur, and it is driven primarily by selection of resistance to streptomycin and rifampicin antibiotics; and (2) disk diffusion test revealed no significant difference in zone of inhibition between Rec+ and Rec- (**Figure S1**). This could be explained by the underlying genetic architecture of the endpoint populations; for example, drug specific evolved environments helped both Rec+ and Rec- to acquire resistance against those drugs.

Genomic investigation of natural transformation in the evolution of multidrug resistance

We anticipated that Rec+ populations could potentially alter the evolutionary dynamics by bringing beneficial mutations (resistant mutations) from separate cells into a single genetic background, and would thereby increase the fixation rate of beneficial mutations (Fisher-Muller effect). Therefore, we expected an increased frequency of multidrug resistant genotypes. As noted earlier that we did not find any fitness differences between Rec+ and Rec- lines under sub-inhibitory stressful conditions. However, there is a possibility that this selection pressure in combination might have mutagenic effects on bacterial competence. This is because stress induced by antibiotics greatly increases the competence in many bacterial species which are lacking of SOS-system [45, 46], thus SOS-induced mutagenesis could also result in elevated drug specific resistance mutations conferring higher fitness in Rec- populations.

Therefore, we investigated the influence of competence (Rec+) and non-competence (Rec-) by sequencing the genomes of the evolved clones that were also assayed phenotypically. More precisely, we further wanted to investigate into the indistinguishable benefit of recombination by looking at the genomic level to know whether this indistinct phenotypic property between Rec- and Rec+ population is also mirrored by the low number of overall mutation in the evolved endpoint population. Therefore, we sampled one genotype from each of the 36 evolved endpoint populations along with their ancestral genotypes. We obtained a median coverage of ~38-fold per genotype (mean = 49.5; range 25–50) on the Illumina MiSeq platform, using 300bp paired-end reads. One previous study suggested that 15/20-fold coverage is adequate for identifying a modest number of mutations in laboratory selected microbial strains [47]. In our study, the depth of coverage we achieved should, therefore, allow us to detect all SNPs and small indels throughout the genome that have occurred during the course of evolution over a hundred day of transfer. In addition, the sequenced genomes were surveyed for the large

insertion/deletion events, such as mobile genetic elements insertions or excisions were manually investigated by looking at the specific deleted regions identified initially. We were unable to inspect some portion of the genome for some strains due to low coverage (<5). We selected this coverage threshold arbitrarily with a notion to detect all the nucleotide changes in the entire populations and then to further validate them by traditional amplicons sequencing of a subset of SNPs that were identified with higher frequency in the populations.

Across all evolved lines, we identified 168 mutations comprising 129 nucleotide changes (22 of which were intergenic), and 25 large deletions. Overall, these mutations were affecting a total of 146 genes (some genes were affected in high number). In some instances, large deletion events were found (insertion are not identified yet). We then classified these mutations into two groups based on the regions of the genomes that were affected by them: structural and regulatory mutations (**Figure 9**). This classification revealed an abundance of mutations that affected structural

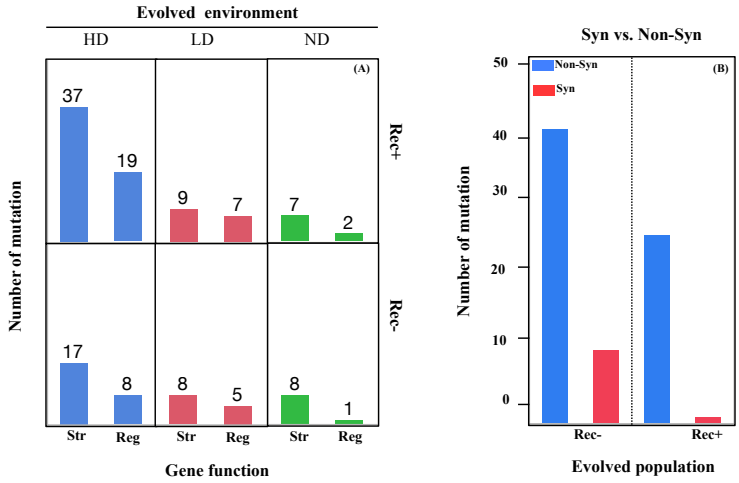

Figure 9 Different mutational events identified in the evolved endpoint population. In panel A, all the mutations based on their functions are classified into structural (genes that encode functional enzymes) and regulatory gene (regulates important cellular processes) mutation. Panel B shows the number of synonymous and non-synonymous mutations observed in Rec- and Rec+ populations after ~650 generations.

genes of the genomes evolved in HD environment, and was higher in Rec+ population (37 vs. 17, shown by Figure 9(A). Next, we identified the genomic substitutions that were not uniformly distributed across treatments and populations (Figure 9(B)). We saw that the frequency of the non-synonymous mutations relative to synonymous mutations were higher in Rec- than Rec+ (i.e. 74/97 vs. 19/97), and the majority of these mutations were detected in

clones that had evolved in stressful conditions. However, the relative abundance of overall mutations in Rec+ and Rec- in stressful environments (LD and HD) was higher compared to unstressed (ND) environment. In ND environment, we observed very few mutations as has previously been observed [48, 49].

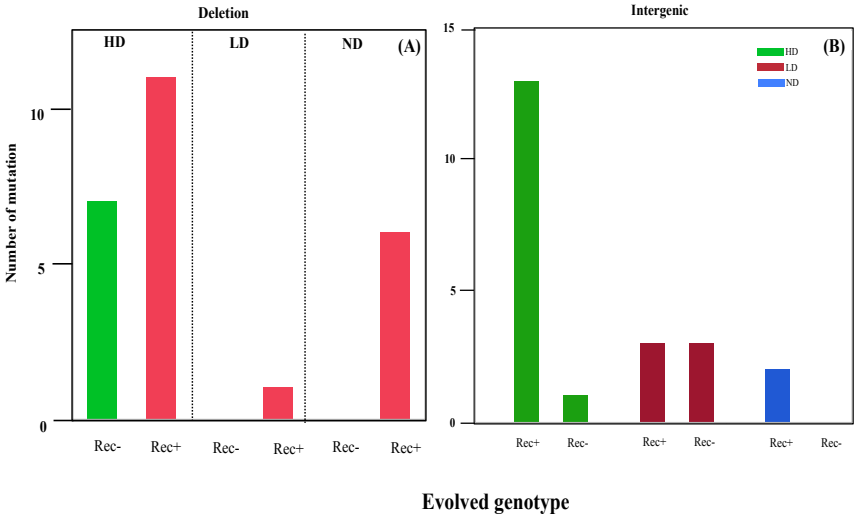

Figure 10 Number of deletions and intergenic mutations occurred in the evolved endpoint population. Panel A shows number of large deletions (from 100 to 49K bp long) events observed in both Rec+ and Rec- populations. Panel B shows the number of intergenic mutations observed in Rec- and Rec+ populations after ~650 generations.

Some earlier studies provided evidence of the genomic basis of adaptation in experimentally evolved population, where mutation rates were ranging from 1.07/100 to 4/100 generation [50, 51]. In the later case, this difference was predicted as a signature of strong selection imposed by antibiotic treatment [52]. In line with this, we compared the number of mutations in the populations that were evolved with or without antibiotics. We observed a high number of mutations in stressed populations suggesting that the selection pressure we used was strong enough to produce an antibiotic-mediated benefit among these genotypes. However, the difference between Rec+ and Rec- suggests a possibility that natural selection in some populations evolved under HD environment could have played role in shaping the spectrum of mutations. We found that non-synonymous and synonymous mutations (as mentioned earlier) in both Rec+ and Rec- populations were different (**Figure 9B**). Large deletions and intergenic mutations were also different in both Rec+ and Rec- (**Figure 10**). In line with the above explanations, we observed an increased abundance of *rpoB* mutations both in Rec+ and Rec- clones evolved under LD and HD environment (**Figure 11**). Detailed mutational background is given in supplementary **Table S1**.

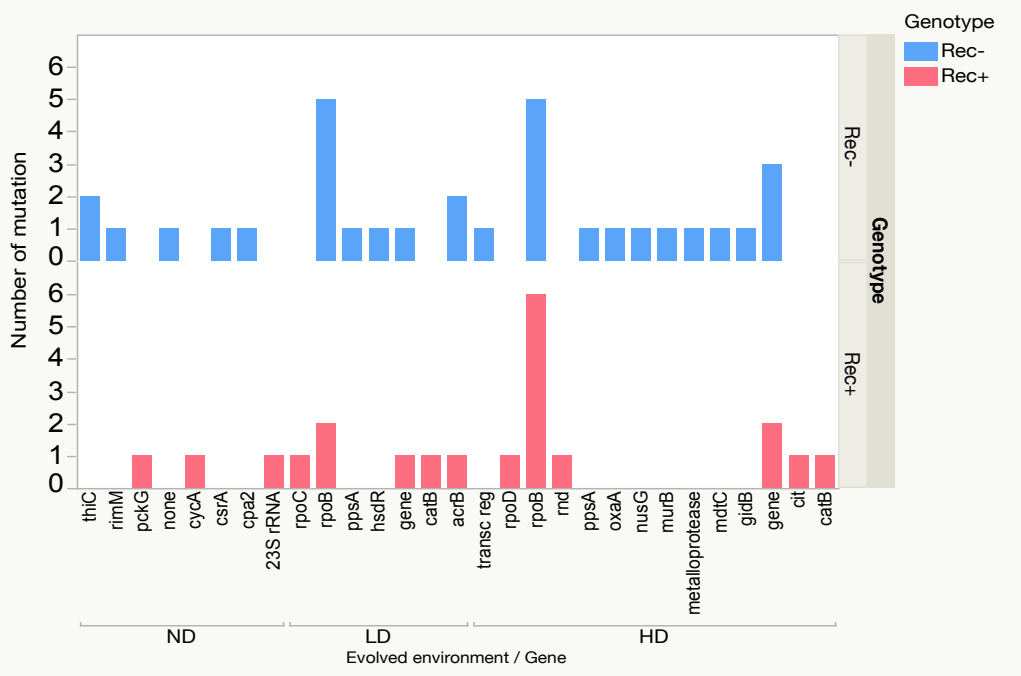

Figure 11 Major mutations identified in the endpoint populations. The plot shows major SNPs found across Rec+ and Rec- genotypes, a majority of them involved in drug resistance. High abundance of drug resistance mutations was found in the *rpoB* gene in both Rec+ and Rec- genotypes, responsible for rifampicin resistance. No observed mutations were detected in *rpsL* gene known for streptomycin resistance. Mutations were also detected in the efflux system encoding genes such as *mdtC*, *rnd* and *acrB*, which are well known for phenotypic multidrug resistance.

Mutation in *rpoB* gene encoding RNA-polymerase β-subunit is widely responsible for resistance to rifampicin antibiotics [44, 53]. In addition to *rpoB* mutation, we observed mutations in *rpoC*, *rpoD*, *acrB*, *ndtc* and *rnd*. It is well known that secondary mutation in *rpoC* and *rpoD* are associated with adaptation in resistant population by compensating the fitness cost associated with resistance mutations [43, 44], whereas mutations in efflux pump system including *acrB*, *rnd* and *ndtc* contribute to the high level of phenotypic resistance to multiple drugs, including common fluoroquinolones resistance [54-56]. Thus, we speculated that this high number of mutations possibly played a role in the adaptive process in populations evolved under LD and HD environment. However, both Rec+ and Rec- lineages carried *rpoB* mutations indicating a substantial parallel evolution, which means that natural selection acted upon *rpoB* mutation in LD and HD environment. This genotypic parallelism probably appeared in the earlier generations of the population during the course of evolution. This parallel evolution can further be supported by looking at the fitness or growth rates of different lineages from different generations, as has been documented in many experimentally evolved bacteria,

including evolution of *P. aeruginosa* under sub-lethal concentration of ciprofloxacin antibiotic [50]. Together with this, *rpoB* and other lineage-specific mutations arose in our evolved populations are beneficial. We observed the same mutation in each replicate population line suggesting that the majority of parallel evolution was indeed due to the selection of drug specific resistance mutations.

To determine the effect of these drug specific resistance mutations in the evolved population, we carried out E-test phenotypic assay by which we further determined the MIC by using not only the antibiotics we used in our evolution experiment, but also an additional antibiotic (ciprofloxacin) as a proxy to determine the putative role of additional mutations associated with the efflux system as well as other unknown mutations. We observed an elevated level of MIC for ciprofloxacin (Cip) (**Figure 8**) for the strains evolved under LD and HD environment, but no changes were observed in populations evolved under ND environment. This indicates possible adaptive evolution attributed to these drug specific genomic mutations or other mutations.

Among others, we also observed a mutation in the *hsdR* regulatory gene. This gene encodes the conserved HsdR protein and belongs to type 1 restriction-modification (RM) system. This system protects bacterial DNA from restriction (degradation) by modification (methylation) of specific sequences that are recognised by restriction enzymes of the RM system [57]. The RM system in *E. coli* protects bacteria from invading foreign DNA such as bacteriophage genomes. Inactivation of *hsdR* mediated type 1 RM systems was also found to be associated with the transformation process in *S. aureus* [58]. In a previous study by Waldron and Lindsay, it was reported that the conserved type I RM system was solely responsible for the inability to transform *S. aureus* isolates with *E. coli* derived plasmid DNA and a premature stop codon in the type I restriction gene (*hsdR*) was identified [59]. Furthermore, disruption of this restriction barrier can reduce the degree of virulence in clinical isolates of MRSA lineages CC22, CC30, and CC45 [60]. Although we observed a synonymous substitution in this gene in one of the Rec- strains, this mutation might have entailed profound evolutionary implications for adaptation in that particular environment by elevated resistance to many antibiotics without altering this restriction modification system.

Overall, we identified a low number of functional mutations in the Rec+ populations. For the low mutations rate, we further searched for the putative known mutator lineages in the evolved

populations because mutator phenotypes increase the mutation rate through error prone DNA replications in presence of antimicrobial stress [61, 62]. Although we did not observe any putative mutation in the Rec+ population for the SOS inducer, Rec- constituted majority of the mutations including mutation in *nusG* gene encodes transcription elongation factors. Some earlier studies reported that transcription anti-termination gene *nusA* as well as rifampicin resistant *rpoB* mutation are required for stress-induced mutagenesis in *E. coli* [63, 64]. Rifampicin resistance mutation affects the cellular anti-termination system involved in the synthesis of stable RNA by interacting with *nusG*, and also acts as a possible compensatory mutation in *P. aeruginosa* genes such as *gyrA*, *gyrB* and *nfxB* under ciprofloxacin antibiotic pressure [50]. Therefore, Rec- populations indicate mutator phenotypes in our experiment. However, based on the mutations we observed, we did not determine the mutation rate differences in each population from Rec+ and Rec- group. It is evident that stress induces both competence and SOS-pathway in bacteria [45]. In order to distinguish mutator strains from non-mutators and infer the role of competence and non-competence in the adaptive process, we first categorised all the populations by giving a rank based on the highest number of mutations (nucleotide substitutions) occurred in a single genome, where strain with highest mutation is categorised as Rank1 (>5 mutation plus a putative gene responsible for stressed-associated mutagenesis) to Rank3 (<3 mutation with no putative SOS-associated gene). According to this, all the Rec+ populations belonged to Rank3. In the Rec- populations, mutation in the *nusG* gene (functions in transcription anti-termination) was found in one strain with other mutations including mutation in *rpoB* gene. In some other Rec- strains, mutations were detected in some other genes including *murB* (peptidoglycan biosynthesis, stressed and recombination inhibition) and *rimM* (16S rRNA-processing protein RimM; essential for efficient processing of 16S rRNA and for the SOS response). Taken together, this rank based classification suggest that Rec- populations were more genetically diverse compared to Rec+ which helped them adapt in the HD environment, probably by inducing SOS systems in some of the strains, for example interactions between *rpoB* and *nusG* genes. By contrast, in Rec+ strains, we observed less broad patterns of genetic diversity where *rpoB* mutation was predominant (*rpoB* mutations 8/12 strains); however, we did not observe any evidence for antibiotics mediated genetic divergence in Rec+ population suggesting no recombination via natural transformation.

Intergenic mutation between two functional genes could be an indication of possible recombination event such that population acquiring non-synonymous intergenic mutations can be acquired with other mutations in functional genes by recombination, and this can be tracked

by targeted sequencing as has been shown by one recent study in *A. baylyi.* [30]. We did not sequence our populations from different time points, but intergenic mutations in the endpoint populations could provide some evidence of recombination. We identified 25 intergenic mutations, and most of these mutations were detected in Rec- population (19/25). This result suggests that Rec- population could be mutator phenotypes. One previous study reported intergenic mutation mostly occurred in mutator phenotypes [50]. In the present study, we identified intergenic mutations between *glcB* and ATPase, and between *fimU* (encoding the type 4 fimbrial biogenesis protein FimU, which modulates virulence in many different bacteria) and *ispH* (encoding IspH and functioning in the MEP pathway). Overall, this high number of intergenic mutation indicates that some of these mutations were possibly driven by selection. However, we only detected 6 intergenic mutations in Rec+ populations, of which 2 were found in populations that evolved under ND treatment, three in LD environment and a single intergenic mutation in the HD environment. Therefore, this low number of intergenic mutations in Rec+ populations suggests that these mutations were not driven by recombination.

We further focused specifically on the genes that were affected by non-synonymous substitutions, because non-synonymous mutations cause functional effect on the gene level. We detected 64 non-synonymous changes in protein coding genes where Rec+ populations bore 25 substitutions (ND = 4, LD = 8, HD = 13), and Rec- population bore 41 substitutions (ND = 3, LD = 11, HD = 27). Furthermore, we identified two clones from Rec- bore a substitution that caused a stop codon in the *mdtc* gene. This *mdtc* belongs to heteromultimeric RND superfamily of transporter, which is well reported for multidrug resistance including resistance to β−lactams, novobiocin, and many more [65].

Genome reduction by large deletions has been reported to be an efficient means of adaptive processes in many different environments, including during adaptation of *P. aeruginosa* to cystic fibrosis patients [66]. We also observed many such reductive events through various deletions; among these, most notable were 15 large deletions (we considered these large when they exceeded 100bp). We only found 3 such deletion events in two of the Rec- populations, whereas the Rec+ populations harboured most of these deletions. The largest deletion event was ~49kb, observed in three replicate population of AB3_Rec+ and two AB4_Rec+. Interestingly, all these deletion events were detected in Rec+ populations that evolved in the HD environment. This represents a strong signature of parallel evolution, as has also been observed in *Methylobacterium extorquens* [67]. The large deletion mainly involved many

functional domains of the genome including *comF*, integrase, and many unknown hypothetical proteins in our evolved strains. These large deletions of crucial genes together with other deletions possibly contributed to the accelerated adaptation under HD treatment. Thus, this large deletion could be an effective mean of sustaining multiple drug pressure and involves a significant genome size reduction. Based on these results, these large deletion events in Rec+ population probably open up a new path towards a limited niche leading to further genome changes to cope up with upcoming perturbation.

Based on the above genetic and phenotypic results, our data show that Rec+ population under LD environment adapted better through reduction of mutation fixations and also the mutator phenotypes. However, Rec+ population under HD environment faces larger deletions of important regulatory genomic portions which encompasses competence associated genes as well as other important accessory and essential gene pools suggest that this reduced genetic architecture overcome competences by generation of effective population size through genome reduction, or possibly deletions may have epistatically induced fitness by producing deletion vs. mutation, or deletion vs. deletion interactions, perhaps because these were exploited among these strains as less costly but efficient strategies for optimal adaptation under strong selection pressure.

Discussion

Recombination via natural transformation is argued to be an important adaptive evolutionary process in many bacterial species, ranging from soil dwelling bacteria to important clinical pathogens [68-73]. Specifically, this adaptive process enables bacteria to evolve multiple drug resistance as well as enrich many pathogenic bacteria with novel modes of virulence mechanisms [71, 74]. However, this adaptive benefit of recombination via natural transformation in the evolution of de novo multidrug resistance is not well studied in experimental bacterial population under two-drug environment with varying concentrations. In previous work, the role of transformation was assessed by employing either naturally competent or non-competent bacterial populations already harboured antibiotic resistance genes, where the effect of competence was investigated by employing only a specific drug targeting bacterial important cellular processes whose disruption created heterogeneous population, and thus facilitated transformation process, or the effect of recombination was not investigated at genomic level [13, 29, 49]. Therefore, the actual benefit of recombination was not fully understood in all those studies. Here, we employed an experimental approach

consisting of both naturally competent Rec+ and non-competent Rec- *A. baylyi*, and systemically investigated how a fully susceptible bacterial population exposed to two-drug combinations evolves multiple drug resistance via mutation, selection and – potentially – natural transformation. To the best of our knowledge, this is the first time that the impact of recombination via natural transformation in the evolution of de novo multidrug resistance has been assessed.

We found that the strength of selection pressure we used affected the benefit of recombination via natural transformation. In particular, in the LD environment, the Rec+ population evolved higher fitness compared to the Rec- counterparts (Figure 5 & 6). This LD environment decreased bacterial growth significantly (*p*-value = <0.0001) before we started the evolution experiment (**Figure S2**). Therefore, we speculated that the greater fitness under this stress was the consequence of recombination. On the contrary, in the HD environment, there was no fitness difference between Rec+ and Rec- populations. This HD treatment also caused a significant growth disadvantage (*p*-value = <0.0001) in the ancestral genotypes (**Figure S2**).

Therefore, we predicted that selection of de novo resistance mutations occurred during serial transfer over one hundred days in these selection regimes, irrespective of Rec+ and Rec-. Our phenotypic data based on the fold MIC change (**Figure 8**) revealed that adaptation to both drugs that we used in LD and HD treatments was strong enough to produce a selection-based benefit in all the population, irrespective of competence or non-competence, as we observed a varying degree of adaptation to rifampicin. On the other hand, for streptomycin resistance, we did only observe mild selection after ~650 generations. Moreover, we did not detect any known mutations such as in rpsL and rrs genes that are commonly associated with streptomycin resistance. However, in two cases, we observed a mutation in *gidB* (16S rRNA methyltransferase GidB; SAM-dependent methyltransferase; glucose-inhibited division protein B; methylates the N7 position of guanosine in position 527 of 16S rRNA). This gene has been reported to be associated with low-level of resistance to streptomycin; in one report a *gidB* mutation was found together with an *rpoB* mutation in *M. tuberculosis* [75, 76]. We considered some plausible alternative explanations for low number of mutations or no known streptomycin resistance mutations spread in our evolved populations such that: either known resistance mutations had not yet arisen, or other unknown mutations were selected for in these populations. Alternatively, for the streptomycin resistance, mutants were present but they were only slowly increasing in number and eventually lost from the population (Figure 6; table S1) due to limited benefits at the streptomycin concentrations they faced after ~650 generations. Or

no resistance or low resistance to streptomycin could be due to adaptive resistance to streptomycin either by mutation in efflux pumps system [77]. Furthermore, based on our previous mutation-screening assay (data not shown here) by plating appropriate dilution of bacterial cultures (plate streptomycin concentration was 10 fold higher than the MIC) indicated a lower mutation frequency for streptomycin compared to rifampicin supports this idea. This has probably affected to follow the benefit of recombination via natural transformation, and thus the benefit of recombination in HD environment is counterpoised by the observed untraceable differences in relative competitive fitness (DS) (**Figure 2**) among Rec+ and Rec- population after ~650 generations.

Another possibility is that the fitness costs induced by sub-MIC could be low for both LD- and HD-evolved population when tested in the absence of drug pressure compared to the initial ancestral strains (**Figure 1**). This could be due the fact that only particular but strong resistant clones, for example, it could be *rpoB* mutation, competed weakly against susceptible clones (which were not killed yet but their growth was arrested by adaptive genes involved in growth bi-stability [78]) at these sub-MIC drug combinations (here we consider situation in both LD and HD). This weak competition (low cost resistance, less than 20%, in absence of drug) between resistance and arrested clones may be associated with or perhaps delayed the emergence of new strong resistant genotypes to another drug (in this case, streptomycin resistant genotypes). Or the newly emerged resistant clones could have carried resistance mutations imposing very high costs, and thus those clones were lost from the population. Overall, if this prediction was correct, the competition between low cost and high cost resistant clones would prevent clonal interference (assumed in the traditional Fisher-Muller model) through the loss of costly mutants from the Rec+ populations, which would result in no recombination but other mechanism to adapt better in the HD environment for both Rec+ and Rec- population. One recent study reported the evolution of low-cost but strong resistance at sub-MIC of streptomycin in *S. coelicolor* while concurrently escaping the cost associated with this phenotype [79]. But in our case of a two-dug HD environment, we did not observe any significant fitness costs in the evolved strains, perhaps because there were other mechanisms that alleviate the cost of resistance. We expected such other mechanisms because the HD treatment would increase the overall mutation rates in the populations through mutagenic effects, including SOS-response or other yet unidentified genomic mutations – as has been observed in response to kanamycin, streptomycin and ciprofloxacin [80-82]. Therefore, we aimed at determining whether this drug pressure accelerate mutations frequency by inducing

natural transformation through the SOS-system. Because previous studies indicate that antibiotics targeting DNA-replication system induce SOS system or induce competence, which has no repressor of the SOS-system, leading to the hypothesis that DNA-damage inducing competence provide genomic plasticity and stress resistance [45]. In our evolution experiment, we evolved the populations under static streptomycin and rifampicin antibiotics in combination that target two different important cellular pathways. Therefore, our observed indistinguishable fitness characteristics between Rec+ and Rec- population could be due to other complex mechanisms, and thus we further pursued to investigate fitness at the population level. However, we also observed increased MIC to ciprofloxacin, which is a different antibiotic than what we had not used in our evolution experiment. This suggests that sub-MIC concentrations of some particular classes of antibiotics can give rise to multidrug resistance evolution with elevated MIC to a new class of drug (**Figure 9**).

We obtained the whole genome sequences of clones from the evolved endpoint populations, and compared them with clones from the ancestral populations to determine the impact of recombination via natural transformation in the evolution of multidrug resistance at the genomic level. We expected that under sub-lethal concentrations of rifampicin and streptomycin, the populations would be enriched with drug specific resistance mutations at first, for example, mutations in *rpsL* and *rpoB* genes, and that these two drug specific resistance mutations compete against each other. Thus, recombination via natural transformations will reduce these competitions (clonal interference) by bringing these competing mutations into a single genome, which will in turn results in multidrug resistance. Our whole genome sequences revealed that both Rec+ and Rec- were enriched with different mutations that were evolved under LD and HD treatment environments. We only observed an abundance of *rpoB* mutations both in Rec+ and Rec- populations across LD and HD treatments. These *rpoB* mutations confer resistance to rifampicin in many bacteria. In a few cases, we only found *gidB* mutation, which is conditionally responsible for the low level of streptomycin resistance (Table SI). In some instances, Rec+ population evolved under LD and HD environments often carried additional mutations, and most notably, in two cases, we observed that population carrying *rpoB* mutation also carried *rpoC* and *rpoD*. Mutations in *rpoD* are responsible for increased resistance or compensate the cost of resistance in *S. typhi*, whereas *rpoC* mutations are responsible for high-level resistance to cefuroxime (CEF). Furthermore, we observed some mutations including a mutation in *nusG* gene in Rec- population, which was previously reported to be associated with stress induced by rifampicin

and streptomycin. This mutation also contributes to the elevated level of resistance to ciprofloxacin. From phenotypic MIC data, we observed an elevated level of resistance (8-fold) to ciprofloxacin but low-level resistance to rifampicin (3 fold) and streptomycin (2-fold) in these strains (**Figure 9**). The remaining non–synonymous changes that occurred in many genes – irrespective of competent and non-competent populations – were previously reported to confer resistance to multiple drugs in many clinical bacterial isolates, including *M. tuberculosis, E. coli, A. baumanii*, *P. aeruginosa* and other important pathogens. [54, 55, 83-88]. However, we did not observe any such mutations in the Rec+ populations, providing no support for the possibility that accelerated competitive fitness under HD environment was driven by recombination.

Genome reduction by inducing many deletion mutations was previously reported as an active means of adaptive process in many bacteria [89, 90]. We also observed such genome reductions via deletions mutations, most notably larger deletions were detected in Rec+ population from the HD environment (**Figure 9A**). In some instances, these were involved in the deletions of many regulatory proteins as well as transformation related proteins; therefore, we expected that deletion mutations could be a strategy of creating an increased growth competitive ability in this stressful environment by counterbalancing the costly transformation process. However, these deletion events cannot explain the contribution of recombination in the adaptive potential (i.e. whether deletion was accelerated by recombination process) under stressed environment for at least two reasons: 1) homogenous competitive fitness between Rec+ and Rec- evolved under LD and HD; 2) deletions were also observed in the LD and ND environments.

After one hundred days of the evolution under sub-lethal concentrations of rifampicin and streptomycin combinations, our phenotypic data revealed no significant differences in fitness gain between recombination proficient and deficient populations. Whole genome sequencing data also revealed a low number of substitution mutations in the evolved endpoint populations. Togther these results disqualify the possible effect of recombination in the adaptive process under multidrug environments. Although most of the mutations that we found here have many different effects on a variety of genes and on their functions, strikingly these genomic changes in the majority of our evolved populations (**table S1**) under these selection regimes were previously shown to be associated with resistance to multiple antimicrobial compounds through targeted and off-targeted mechanisms [91, 92]. This has been reflected in the elevated

level of ciprofloxacin resistance (**Figure 11**). Finally, we observed no significant evidence that recombination by natural transformation facilitates adaptation to multiple antibiotics, presumably because the limited number of mutations that were spreading simultaneously prevented clonal interference.

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Supplementary materials

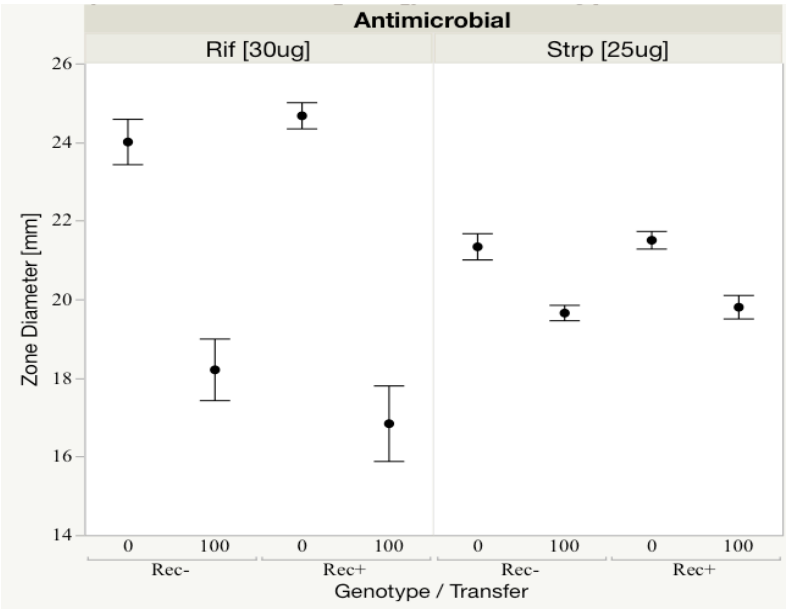

Figure S1 Plot shows disk diffusion test assay between genotype vs. transfer (indicated by 0 for ancestral and 100 for the evolved endpoint population). **Each bar from the evolved endpoint populations represents the mean of the six population replicates of each genotype from three different plates. Each of these six populations was measured with three independent measurements. SEM = ±1**

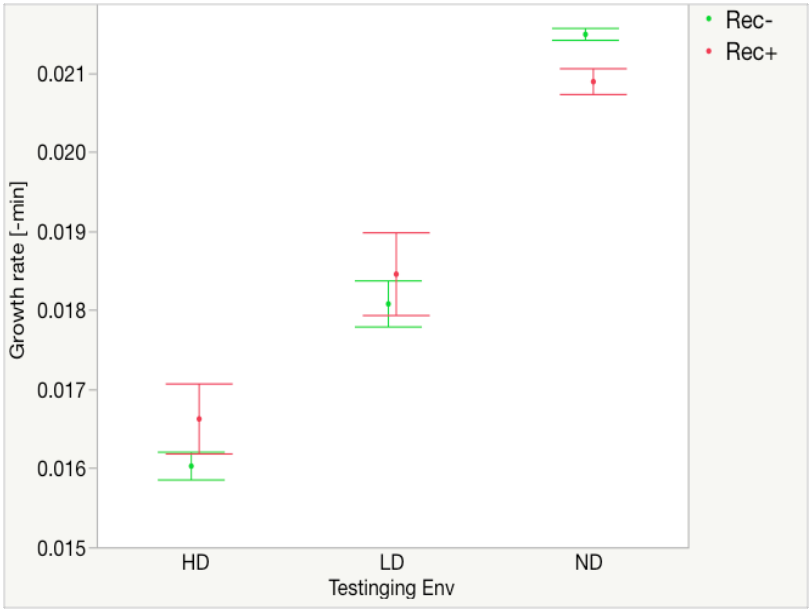

Figure S2 Growth rates for the ancestral genotypes. Each bar represents the mean of more than 3 independent measurements under three different treatment environments. Error bar was calculated using 1 standard error from the mean. ANOVA: F-test ratio = 56.5423; DF = 2; p-value = <0.0001*).

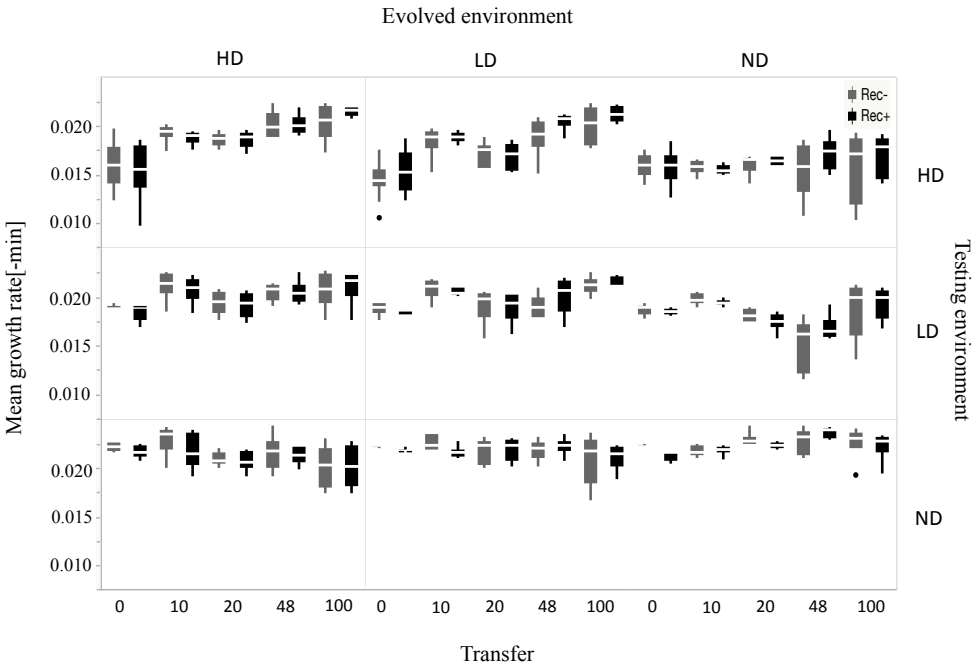

Figure S3: Box plot of growth rates for the Rec+ and Rec- populations at different time points. The horizontal line in each box plot represents the mean, and the black circles present the outliers. ND: no drug; LD: low drug; HD: high drug

Table S1 List of mutations observed in the endpoint populations previously reported for antibiotic resistance.

Population	RecType	AA change	Nucleotide change	Gene	Antibiotic resistance
ADP1Rec+4	Rec+	Ala ->Val	C -> T	*catB*	Chloramphenicol
ADP1Rec+4	Rec+	Asp->Tyr	G -> T	*rpoB*	Rifampicin
ADP1Rec+4	Rec+	Gly->Ser	G -> A	*rpoB*	Rifampicin
ADP1Rec+4	Rec+	Glu->Arg	A->G	*rpoB*	Rifampicin
ADP1Rec+4	Rec+	Glu->His	A->C	*rpoB*	Rifampicin
ADP1Rec+4	Rec+	Arg->His	G -> A	*cycA*	Cycloserine
ADP1Rec+3	Rec+	Ser->Leu	C->T	*rpoB*	Rifampicin
ADP1Rec+3	Rec+	Arg->His	G->A	*rpoD*	Rifampicin
ADP1Rec+3	Rec+	Asp->Glu	T->G	*rpoB*	Rifampicin
ADP1Rec+3	Rec+	Leu->Pro	G -> A	*rpoC*	Cephalosporine
ADP1Rec+3	Rec+	Ser->Arg	A->C	*catB*	Chloramphenicol
ADP1Rec+3	Rec+	Gly->Asp	G->A	*acrB*	Mar
ADP1Rec-132	Rec-	Iso->Thr	T->C	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Ser->Leu	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Gly->Asp	G -> A	*gidB*	Streptomycin
ADP1Rec-132	Rec-	Arg->Cys	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Arg->Cys	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Pro->Leu	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Glu->Lys)	G -> A	*ppsA*	Pyrazinamide
ADP1Rec-132	Rec-	Pro->Ser	C -> T	*salA*	Antibiotic resistance
ADP1Rec-132	Rec-	Glu->Lys	C -> T	*mdtc*	Mar
ADP1Rec-132	Rec-	Met -> Arg	T -> G	*fstK*	Ampicillin, cefoxitin, and piperacillin
ADP1Rec-132	Rec-	Met->Val	A -> G	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Iso->Val	A -> G	*ABC*	Antibiotic resistance
ADP1Rec-132	Rec-	Pro->Ser	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Pro->Ser	C -> T	*salA*	Lincosamides, Streptogramin
ADP1Rec-132	Rec-	Val->Ala	T -> C	*ppsA*	Pyrazinamide

ADP1Rec-132	Rec-	Ser->Leu	C -> T	*rpoB*	Rifampicin
ADP1Rec-132	Rec-	Leu->Leu	G -> A	*gidB*	Streptomycin

Reference for these resistance genes has been provided in the result sections. In this study, we tested resistance to rifampicin, streptomycin and ciprofloxacin. RecType indicates Rec+ and Rec- population; Mar (multiple antibiotic resistances) = Tetracycline, Ampicillin, Puromycin, Nalidixic acid, Rifampicin, Chloramphenicol, and Ciprofloxacin.

Table S2 List of different intergenic mutations. Different intergenic mutations arose in the evolved populations. RecType indicates recombination proficient/deficient, population evolved under different treatment environments indicated by EvolEnv, whereas Rec+ and Rec- indicating recombination efficient and deficient evolved genotypes, RepPlate for replicate plates.

Strain	**RecType**	**EvolEnv**	**RepPlate**	**Change**	**SNP Type**
ADP1Rec+4	Rec+	HD	P2	C -> T	SNP (transition)
ADP1Rec+4	Rec+	LD	P1	C->A	SNP (transition)
ADP1Rec+4	Rec+	LD	P2	C->A	SNP (transition)
ADP1Rec+4	Rec+	ND	P1	G -> T	SNP (transition)
ADP1Rec+4	Rec+	ND	P2	G -> T	SNP (transition)
ADP1Rec+3	Rec+	LD	P3	C->T	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	C->T	SNP (transition)
ADP1Rec-131	Rec-	HD	P3	T -> C	SNP (transition)
ADP1Rec-131	Rec-	LD	P1	T -> C	SNP (transversion)
ADP1Rec-131	Rec-	LD	P2	G -> A	SNP (transition)
ADP1Rec-131	Rec-	LD	P3	T -> C	SNP (transition)
ADP1Rec-131	Rec-	ND	P1	T -> C	SNP (transition)
ADP1Rec-131	Rec-	ND	P2	G->A	SNP (transition)
ADP1Rec-131	Rec-	ND	P2	A -> C	SNP (transversion)
ADP1Rec-131	Rec-	HD	P2	A -> G	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	C -> T	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	A -> G	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	C -> T	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	C -> T	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	G -> A	SNP (transition)

ADP1Rec-131	Rec-	HD	P2	T -> A	SNP (transversion)
ADP1Rec-131	Rec-	HD	P2	T -> C	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	T -> C	SNP (transition)
ADP1Rec-131	Rec-	HD	P2	T -> C	SNP (transition)
ADP1Rec-131	Rec-	HD	P3	T -> C	SNP (transition)

Table S3 List of primers used in amplifying targeted *rpoB* allele sequences.

Gene name	**Primer sequence**
rpoB 1 (forward)	TTCGATTCAGGTCGACTCGT
rpoB 1 (reverse)	CAGGCGTTCTGGAACAAGAT
rpoB 2 (forward)	TGGATCAAAACAACCCATTG
rpoB 2 (reverse)	ATCGCCACGACCCACTTTAT

Table S4 PCR master mix preparation per reaction

Reagents	**Amount** [uL/reaction tube]
PCR buffer 10x	2.5
MgCl2 25 mM	2.5
dNTP 10mM	0.5
TaqGold 5U /µL	0.1
Primer mix (conc. 0.5µM) (F+R)	1.5
Sigma H2O	17.9
Template DNA	5
Tolal volume	30

Table S5 Temperature profile for the PCR for amplifying *rpoB* allele sequences

PCR program:		
Step	**Temperature**	**Time**
1 (hold)	95°C	6 minutes
2 (denaturation)	94°C	45 seconds
3 (annealing)	58°C	45 seconds
4 (elongation)	72°C	2 minutes
5 (repetition)	Step 2 to 4 for 35 cycles	
6 (final elongation)	72°C	10 minutes

Table S6: Parameter estimates of the fixed effect test using populations from different time points.

Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Genotype	1	1	0.00000146	0.2210	0.6385
Transfer	4	4	0.00051062	19.3081	<.0001*
Treatment	2	2	0.00000389	0.2945	0.7450
Genotype*Transfer	4	4	0.00002968	1.1222	0.3451
Genotype*Treatment	2	2	0.00000622	0.4704	0.6250
Transfer*Treatment	8	8	0.00009798	1.8524	0.0652

Chapter 4: Compensatory evolution of the costs of single versus multidrug resistance in *Acinetobacter baylyi*

Summary

The phenomenon of drug resistance is pervasive in pathogen populations whenever they face any selective pressure exerted by antibiotics. Often, resistant bacteria suffer fitness costs once the drug usage is discontinued. However, this deleterious effect of resistant mutations can be ameliorated by compensatory mutations (CM), thus preventing the rapid elimination of the resistant bacteria from the population. Although this mechanism of compensation has been extensively studied in bacterial populations resistant to only a single drug, adaptation to the costs associated with multiple drug resistance mutations remains unexplored. We therefore tested the mechanism of compensation in multidrug resistant bacteria by employing an evolution experiment. We constructed a set of genotypes comprising a completely susceptible genotype, a streptomycin resistant genotype carrying the K43T mutation at *rpsL* locus, a rifampicin resistant genotype carrying the P573L mutation at *rpoB* locus, and a doubly resistant genotype carrying both of these mutations. Sixteen populations from each of these genotypes were subjected to daily serial transfer for ~325 generations. To follow the dynamics of fitness compensation and track the mutational spectrum, the resultant endpoint populations with their ancestral counterparts were phenotyped and their genomes sequenced. Our phenotypic results show that the deleterious effects of resistance mutations can be compensated for by a varying degree of fitness improvement. Specifically, we found that adaptation to the cost of resistance in a set of multidrug resistant populations was higher compared to the subset of single resistant populations. The MIC data also suggest that fitness was improved without altering their resistance level. Moreover, this greater fitness improvement was influenced by some of the fittest lineages following a bimodal fitness distribution. Our whole genome sequencing data revealed that both costly rifampicin resistant and costly double resistant lineages adapted by compensatory mutations in RNA polymerase core enzyme. We also observed minor fitness compensation to the low cost streptomycin resistant mutation where we detected no evidence of putative compensatory mutations but parallel secondary mutations in hypothetical genes. However, we observed a different parallel adaptive evolution in double resistant genotype, which harboured an unexpected additional deleterious mutation. Surprisingly, half of the lineages originating from this double resistant genotype was better compensated through reversion mutation in the same nucleotide position of the same genetic locus, which were also accompanied by distinct compensatory mutations in the RNA

polymerase core enzyme, and those mutations were not shared by any rifampicin resistant genotypes. Our data suggest that adaptation to the cost of multidrug resistance is independent of the genetic background of mutations that appeared in single resistant genotypes. Furthermore, our study indicates how selection at other locations in the genome can influence the dynamics of resistance alleles in multidrug resistant clinical populations.

Introduction

The majority of mutations – even synonymous mutation [1, 2] and mutations in intergenic regions [3, 4] – have been found to be associated with organism's fitness in an unfavourable way. Some of these deleterious mutations may disappear from the population or revert back to the original wild type state, but in some cases such deleterious effect is reduced by so called second-site compensatory/suppressor mutations (CMs) at the same or a different locus in the genome. By definition, CMs are deleterious or at best neutral when on their own but beneficial when co-occurring with the original deleterious mutation, thus representing a form of sign-epistasis (see also Chapter 2). This poorly understood biological phenomenon has important implications, not only for the evolutionary consequences of mutations, but also for the genetic complexity of adaptation. In instances of resistance evolution such as bacterial resistance to antibiotics, pesticide resistance and resistance of HIV-1 to antiretroviral therapy, this phenomenon has contributed an adaptive advantage to pathogen populations [1]. In order to gain a better understanding of the biological relevance of CMs in the organism's fitness – both in terms of evolutionary significances of mutation and also for the genetic consequences of adaptation – both theoretical and empirical studies have been conducted. For example, theories suggest that the CMs appear in the population to mask the deleterious effect of another mutation or they arise independently as deleterious mutations in the same population but are neutral when they combine with other mutations [2, 3]. In evolutionary biology and genetics, the role of CMs have further been assessed under a variety of contexts including the evolution of sex, the structure of fitness landscapes and epistasis, mutational load, the extinction of populations, and the mechanism of suppressions in determining various regulatory or functional interactions between protein or RNAs [4-9].

It is well known that bacteria can evolve resistance to antibiotics by acquiring drug specific new genomic mutations or by acquiring horizontally transferred genetic material carrying resistance determinants [10-12]. During acquisition of resistance mutations, these genetic changes often involve deleterious effects as they weaken or interfere with important cellular

functions including cell wall synthesis, regulation of DNA supercoiling, transcription and protein synthesis, or undergo many other metabolic disruptions. Not surprisingly, these interruptions of essential cellular functions by antibiotic resistance mutations are often associated with fitness costs. This means that the resultant resistant bacterial population experiences decreased fitness in the absence of drugs, reflected in, for example, decreased survival, reduced growth rate, reduced transmission, and/or reduced virulence in pathogenic bacteria [13-15]. The cost associated with this resistance mutation has been empirically studied both in vitro and in vivo in many bacterial pathogens in many different settings [9, 16-22]. These studies have provided evidence that when the drug-selective pressure is removed, the resistance mutations become a disadvantage for the organisms with reduced fitness compared to the parental susceptible ones. This means that the resistant subpopulation may go extinct because of their low fitness compared to their susceptible counterparts.

In some cases the fitness cost of this genomic single mutation conferring resistance to a particular drug in bacteria can be alleviated temporarily by increasing the activity of the mutated enzymes. However, compensation may also arise on a permanent basis by additional point mutations throuh which the focal mutated-target-proteins become more active, or strengthen the flow of this protein through biochemical pathways [5]. Alternatively, it has been shown that drug resistance mutations can incur high levels of fitness cost by increasing the degree of catalytic activity of the target antibiotics, and this high affinity biochemical-specificity-associated fitness cost is compensated by additional point mutations in the same gene through the thermodynamic activity of that mutated enzyme [23, 24]. One earlier study by Schrag and Perrot [25] provided direct evidence of the mechanism of compensation by evolving streptomycin resistant *E. coli* populations in the absence of streptomycin. This study found that resistance was stably maintained by evolving a second-site compensatory mutation that reduced the fitness cost by 6%. Subsequently, many other observations have been made by studying both clinical and laboratory populations, including in *Staphylococcus aureus*, *Salmonella typhimurium*, *M. tuberculosis* and *P. fluorescens* [26-28].

In all previous studies, the genetic and molecular mechanisms of compensation were investigated only for single chromosomal resistance mutations. Nevertheless, it is important to also ascertain the role of additional mutations that have been frequently associated with multiple drug resistance, but to date this has not been investigated systematically. From a clinical perspective, compensation is of special interest since resistant organisms may still

maintain their resistance while adapting genetically to its costs, resulting in the stabilization of resistant organisms in patients. Thus, it may make it difficult to eradicate the pathogen population from infection sites. At the same time, there is a possibility that some fraction of resistant populations may acquire additional mutations or compensatory mutations, which may perhaps carry higher fitness costs during the course of compensation (**Figure 1**). One empirical study reported that epistatic interactions drive the acquisition of multiple drug resistance and also compensate the cost of initial resistance mutation by evolving the second mutation [29].

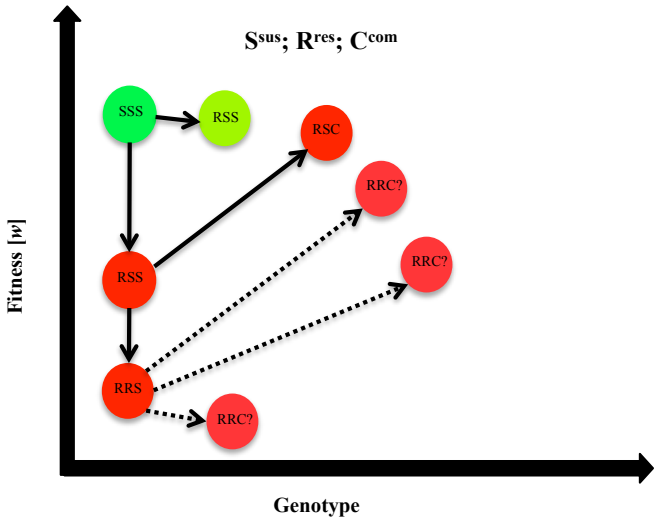

Figure 1 Genetics of compensation to the cost of resistance. Horizontal axis shows genotypes and vertical axis represents fitness. Here, 'S' indicates a susceptible locus, 'R' indicates a resistant locus, 'C' indicates compensatory locus. Strain carrying 'SSS' indicates a susceptible genotype with higher fitness in absence of antibiotics, where the first two 'S' indicating drug resistant loci that can acquire two distinct resistant mutations, and with the third 'S' indicating a locus that acquire a compensatory mutation (indicated by green circle). Two single resistant genotypes can be achieved by acquiring a single drug specific resistant mutation indicated by circle with 'RSS'. Here, for the simplicity I only consider the first locus, but another single resistant genotype conferring resistance to a new drug can be selected for by another point mutation in the second locus. The green 'RSS' genotype can also be achieved by negligible or low cost resistance mutations, whereas red 'RSS' resistant genotype has lower fitness because of a costly resistant point mutation. For the double resistant genotype indicated by red 'RRS' fitness is greatly reduced because of the two distinct costly resistant mutations. The solid arrow indicates the known mechanism of compensation for a single resistant mutation. The mechanism is unknown in terms of fitness and mutational spectrum for multidrug resistant bacteria indicated by broken arrow – here two plausible outcomes can be expected: the double mutant may acquire a fitness compensatory mutation (i.e., this mutation could be similar to the

single drug or a distinct new compensatory mutation) or may acquire a deleterious mutation that can further exacerbate the cost of resistance.

Since this work concerns the possible differences between compensation of single vs. multidrug resistance, both in terms of fitness and the spectrum of mutations, we investigated the adaptive role of compensatory mutations by employing a set of genotypes comprising a completely susceptible genotype, two single drug resistant genotypes, and a multidrug resistant bacteria derived from those two singly resistant genotypes through an evolution experiment for ~325 generations. We then followed the dynamics of fitness compensation and the mutational spectrum of these evolved populations by characterizing them both at the phenotypic (growth rate, competitive fitness, MIC-assay) and genomic level (whole genome sequencing). We attempted to identify de novo mutations that accumulated in these genotypes during the course of evolution to uncover the mutational spectrum as well as to what extent these mutations are involved in the dynamics of fitness compensations in both single vs. double resistant genotypes Thus, we will be able to determine whether evolutionary adaptation to the cost of multiple drug resistance by compensatory mutations generates substantial genetic variations which differ from compensation to the cost of single-drug resistant mutations. This should shed light on our understanding of new evolutionary processes that may also be present in multidrug resistant bacteria of clinical origin.

Materials and methods

Genetic background of strains and growth conditions

To construct mutant genotypes, a completely susceptible, tryptophan auxotroph *A. baylyi* ADP1 strain containing a cyan fluorescence marker (trpE27 ACIAD0921::ecfp) was used. Before initiating the experiments, these strains were preserved in 15% glycerol (v/v) and stored in -80° freezer.

Genotypes constructions

To construct rifampicin and streptomycin resistant mutants, the ancestral sensitive strain was streaked on LB agar plate and incubated at 30°C overnight. From this plate, an overnight culture was initiated from a single clone in 20mL LB broth in a 50mL tube at 30° C with constant shaking at 180 r.p.m. Mutant screening for a single drug was carried out by plating this overnight culture on LB agar plates supplemented with appropriate antibiotics. Specifically, 100uL of overnight culture was inoculated on LB agar plates supplemented with either 10ug/mL of rifampicin or 10ug/mL of streptomycin antibiotics and incubated at 30°C for

a maximum of 48 hours. The concentrations we used were 10-fold and 5-fold higher than the MIC of rifampicin (1ug/mL) and streptomycin (2ug/mL), respectively. After a maximum of 48 hours of incubation, individual mutants were isolated from both the rifampicin and streptomycin supplemented plates and overnight cultures with these individual clones were established in LB broth supplemented with the respective antibiotics and concentrations. Individual mutants were then frozen in 15% (v/v) glycerol at -80°C for further assays.

Sanger sequencing was employed to sequence the targeted genomic locations of the individual mutants. We sequenced two regions of the *rpoB* gene (*rpoBI* primer pair which covered nucleotides 1–1342, including resistance cluster I, and *rpoBII* primer pair which covered a second region spanning nucleotides 1240–2226, including resistance cluster II) [30-32] to detect the mutation responsible for rifampicin resistance (Rif). These primers pairs were designed based on an extensive literature search to detect the common regions covering the occurrence of common rifampicin resistance pathway conferred by mutation in the rpoB gene [31]. Similarly, to detect the streptomycin resistance (Stp) mutation, two pairs of primers were constructed targeting rrs and a single pair of primers for the rpsL gene, because most of the mutations conferring resistance to streptomycin appear in these two genetic loci [27, 32]. Detailed primer information can be found in the supplementary Table S1. DNA extraction was carried out from these individual resistant clones using Promega genomic DNA extraction kits (Promega, California), followed by PCR amplification of rpoB, rpsL and rrs gene fragments. Detailed PCR protocols can be found in supplementary Tables S2 and S3. Sanger sequencing was carried out on these amplified PCR gene products at the Australian Genome Research Facility, and mutations were determined by using CodonCode Aligner version 5.0 (CodonCode, Deadham, MA).

In the following step, we constructed a double resistant genotype (resistant to both rifampicin and streptomycin) by a natural transformation assay according to de Vries and Wackernagel [33] with slight modification. In short, individual competent cells of rifampicin and streptomycin resistant mutant strains obtained earlier were prepared by adding 1mL overnight culture into 100 mL of LB broth in Erlenmeyer flask for a period of 6-8 hours growth at 30°C with constant shaking at 180rpm, followed by centrifugation at 8500rpm for 2 minutes and collection of pellets. In the next step, approximately 300uL LB broth supplemented with glycerol (20% v/v) was used to re-suspend the pellets by pipette tips very gently. This suspension of pellet contained competent cells and was stored in a -80°C freezer. The

following day, transformation of competent cells with genomic DNA (obtained from rifampicin and streptomycin mutants as the donor strain in either way) was carried out by taking 20mL of LB broth supplemented with MgCI2 (0.25mM) and CaCI2 (0.25mM) in 300mL Erlenmeyer flasks. After adding DNA, the flask containing both competent cells and DNA was placed on the shaker (at 30°C with 170 rpm as shaker conditions) for aeration for a period of 90 minutes. This allowed competent cells to take up DNA from their surroundings. After appropriate aeration, the cultures were centrifuged at 6000g for 5 minutes and the pellet re-suspended by adding 300uL fresh LB and gently mixing the pellet with pipette tips. From this tube, 100 uL of culture was streaked on an LB agar plate supplemented with both antibiotics, for example, plates were supplemented with 4 ug/uL rifampicin and 20 ug/uL streptomycin in combination. The cultures were then diluted and 100 μL of these cultures was plated and left for adequate time to dry (maximum of 5 minutes). After this, all the plates were incubated for a maximum of 40 hours at 30°C, visible individual colonies were picked and overnight cultures were setup in LB broth supplemented with 4ug/uL rifampicin and 20ug/uL streptomycin in combination. Cultures of these double resistant mutants (StprRifr) were frozen in 15% (v/v) glycerol at -80°C. Sanger sequencing was carried out on this StprRifr strain to confirm the location of the mutations in rpoB and rpsL genetic loci.

Measuring cost of resistance

To determine the cost of resistance, we measured the growth rate of the Rifr, Stpr, and StprRifr mutants in 96-well plates in 180uL LB broth where optical density (OD600) was estimated in triplicate by using a Tekan Synergy HT plate reader (BioTek., Synergy, USA). Each strain taken from the -80°C freezer was grown on an LB agar plate followed by transfer of a single colony into 20mL LB broth for overnight growth at 30°C with continuous shaking at 180rpm for a period of 18 hours. Then 5uL of this overnight culture was transferred into a 96-well microplate where each well contained 175uL of LB broth (without any antibiotics). The spectrometer was run for 12 hours, which gave 145 reads in total with a five-minute read interval. Specifications for the run protocol for the spectrometer were: set point temperature 30°C, wavelength 600nm, continuous shaking at medium speed.

Evolution experiment

A single colony of each mutant strain of *A. baylyi* – a rifampicin resistant genotype (Rifr), a streptomycin resistant genotype (Stpr) and a double mutant genotype (RifrStpr), and the ancestral genotype AB3 (SS) – were grown independently overnight in LB media in absence of any antibiotics. Sixteen lineages were founded from each of these four progenitor strains by

adding 10uL overnight culture in 990uL of LB medium without antibiotics. All sixteen lineages per genotype were grown in 24-well plates on an orbital shaker (280 rpm) at 30°C for 24 hours, and these growth conditions were maintained throughout the experiment. After 24 hours, 10uL of each population was serially passaged to 990uL of fresh LB medium and propagated for another 24 hours. In this way, fifty serial transfers were made which resulted in approximately 325 generations of bacterial growth. Samples were stored and frozen at 8 different time points at -80°C in 15% (v/v) glycerol solution.

Growth rate measurement

Since our aim was to determine the fitness trajectories of all lineages founded from a single colony of each of the four initial genotypes, growth rates for the evolved populations at different time points were first measured by spectrometry (SynergyTM HT microplate reader, BioTek, USA). In doing so, for each linage, 5uL of mixed culture from 24 hours culture contained in 24-well plate were directly transferred into 175 uL of LB broth containing 96-well plates of different generations at different time (day) points (0, 7, 14, 21, 28, 35, 42, 50). For each lineage, we measured this growth rate in duplicates by using two plate readers. We also measured the growth rates of all of our evolved endpoint populations and the initial population from time 0 (day 1) by taking individual clones; for example, we measured growth rates for all sixteen population lineages derived from each of the four different initial genotypes. In this case, we first streaked individual lineages of mixed samples from the frozen stock on fresh LB agar plates from a -80°C freezer, and then a single colony was picked and further grown overnight in LB medium without adding any antibiotics at 30°C with 180 r.p.m on an orbital shaker. From this overnight culture, the growth rate of each clone was measured by obtaining the growth curve (OD600) in LB broth without adding any antibiotics. The individual clones were also stored in a -80°C freezer in 15% glycerol (v/v) for further analysis. The statistical software R version 3.3.0 was used for estimating the maximum exponential growth rate from the OD data. Here, raw OD values were normalized to a blank well and Log-transformed before analysis. Then the steepest slope over a 32 data point range, corresponding to growth over 160 minutes, was determined.

Antibiotic susceptibility assay by E-test

To characterize the antibiotic susceptibility profile of the ancestral and evolved endpoint populations, we carried out E-test assays. Individual clones were streaked on Mueller-Hinton (MH-II; Becton, Dickinson, and company, N.J, U.S.A) plates from frozen stock, and incubated for 18 hours. Suspensions of the organisms were prepared by picking the appropriate number

of colonies and suspending in 1.5 ml of saline under aseptic conditions, and the turbidity was adjusted by measuring the optical density (OD_{600}) of this bacterial saline suspension by spectrometry (Synergy™ HT microplate reader, BioTek, USA). Turbidity of this bacterial saline suspension was adjusted to that of a 0.06 to 1.0 McFarland standard. A total of approximately 0.1ml of the 1.0 McFarland and suspension was plated on a MH-II agar plate by using a sterile cotton swab. In doing this, the sterile cotton swab was dipped into the adjusted suspension, and rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excessive inoculum from the swab. Then the MH-II agar plate was inoculated by streaking the cotton swab over the whole sterile agar surface. This procedure was repeated at least two more times by rotating the plate approximately 60° each time to ensure an appropriate distribution of inoculum on the whole surface of the plate. In the final step, the rim of the MH-II plate was swabbed at least twice. Then the lid of the plate was kept open for approximately 5 minutes to allow for any excess surface moisture to be absorbed before applying the E-test strip. After adequate drying, a maximum of three E-strips (rifampicin, streptomycin and ciprofloxacin) were placed in one plate. The antibiotic strips were placed by maintaining a minimal distance between two antibiotics in order to avoid any potential drug interactions (i.e. antagonism, synergism, inhibition or induction) that may give rise to distortion of inhibition zones. All plates were inverted with the lid side up and placed inside a plastic bag and incubated at 30°C for 18 hours. MICs were read from the test strip where the elliptical zone of inhibition intersected with the MIC scale on the strip. Although there are no specific susceptibility breakpoints set by either the EUCAST (European Committee for Antimicrobial Susceptibility Testing) nor the CLSI (Clinical and Laboratory Standards Institute, USA) for the tested antimicrobial stripes for the strain (*A. baylyi*) used in this study, we also carried out E-tests on *E. coli* strain ATCC 25552 as a control to better interpret the MIC of our experimental strains.

Fitness estimation by competition experiment

For the competition assay, a kanamycin resistant *A. baylyi* ADP1 strain (trpE27 ACIAD0921::ecfp ACIAD3309::nptIII) was used as a reference marker strain [30]. Pairwise competition experiments for each of the evolved clones from the end point (~325 generations) populations and the clones from ancestral genotypes were carried out in the same culture conditions used to propagate the evolving populations. Specifically, each of the evolved clones, ancestral clones and the marker strain were taken from the freezer, plated on LB agar plates and incubated overnight at 30°C. Then, a single colony was transferred into 1mL of LB broth

in 24-well plates. These cultures were then incubated for 24 hours on an orbital shaker at 30°C with continuous shaking at 250 rpm, representing one complete growth cycle as has been done for the actual evolution experiment. Then, to start with the actual competition assay, equal densities of tested clone and reference marker clone were mixed in 990uL LB broth without antibiotics (i.e. 5uL of competitor and 5uL of reference culture was added into a well containing 990 uL of LB broth which represents the conditions of the actual evolution experiment). Individual samples (10uL) were taken immediately after mixing from the individual wells of 24-well plate into 990uL PBS containing 2mL tubes, returning the 24-well plate to the incubator, followed by dilution and plating of 100 uL on LB agar plates supplemented with or without kanamycin (at least 3 replicate plates per dilution per plate type). The rationale of this sampling at the beginning of the competition experiment was to determine the number of cells of both evolved clone and reference clone, then calculate the number of cells after 24 hours of competition, which gives an indication of the competitive ability of the evolved and reference strain. Thus, similarly, samples were taken after 24 hours, and serially diluted and plated on LB agar supplemented with or without kanamycin antibiotics. All plates were incubated for ~16 to 18 hours at 30°C, and subsequently colony forming units (CFUs) were counted visually. Then the relative fitness of the ancestral and evolved strains was determined according to Travisano et al. [34].

Genome sequencing

Based on initial growth rates, we selected eight replicate lineages from each of the sixteen evolved end-point resistant genotypes for whole genome sequencing. We also selected a completely susceptible and three different initial resistant genotypes (i.e. rifampicin resistant genotype, streptomycin resistant genotype and a double resistant genotype harbouring both rifampicin and streptomycin resistance mutations) for whole genome sequencing. All these populations were also assessed with competitive fitness assays. Initially, individual clones were inoculated on LB agar plates from the cryotube followed by overnight incubation at 30°C. A single colony was then inoculated in 10mL LB broth and incubated overnight at 30°C on an orbital shaker (180 rpm). Then DNA extractions from pure culture of the individual clones were performed using PureLink® Genomic DNA Kits (Invitrogen). After extraction, DNA quantification was carried out using a spectrometer based microplate reader on Take3 plate (SynergyTM HT, Bio Tek, USA). Then samples were diluted in TRIS buffer to 8-10 ng/uL and stored at -20°C. Libraries were prepared using a Nextera XT kit (Illumina, San Diego, CA, USA) according to manufacturer's instructions but with slight modifications. Overall, library

preparation comprises five different modules. In short, module 1 involves standardization of gDNA concentrations across samples. In module 2, input DNA is tagged and fragmented by the Nextera XT transposome. The Nextera XT transposome simultaneously fragments the input DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps. In module 3, tagmented DNA is amplified via a limited-cycle PCR program. In module 4, PCR-amplified DNA fragments library is subject to purify using AMPure XP beads to remove short library fragments from the samples. In the final module, each library is normalized which ensures that equal library representation has been achieved in pooled sample. In doing so, we run our purified samples further on the Shimadzu bioanlyzer for the selection of expected fragment lengths between 300 and 800bp. Then we pooled the library and further purified with AMPure XP beads followed by Pippin-Prep size selection (250-800 bps). Finally, the pooled library was sequenced using 300bp paired-end reads on the Illumina MiSeq platform.

Analysis of sequencing data

The reads including both forward and reverse reads generated from Illumina MiSeq were first quality trimmed and paired into consensus sequences, and then aligned with our original wildtype *A. baylyi* which we had previously assembled against the reference genome of *A. baylyi*, from the NCBI GenBank database (Accession NC_005966) [30]. All these steps were carried out using the program Geneious version 9.1.4 (Biomatters, Inc.). We used the 'Find variations/SNPs' tool implemented in Geneious to identify SNPs and indels with a minimum sequencing coverage of 5 and a variant frequency of at least 95% (see Table S2 for different parameters used in bioinformatics analysis). Unique mutations were identified by direct comparison of the ancestral strains with the evolved resistant strains. We also classified other spontaneous mutations that could potentially emerge in the populations propagated in medium without supplementing any antibiotics during the course of the evolution experiment [35] for example, we speculated that some mutations will emerge in the populations to adapt to LB medium only. A subset of SNPs identified in different locations of the genome will be further verified by Sanger sequencing of polymerase chain reaction (PCR) amplicons as has been done in chapter 3.

Statistical analysis

Various statistical methods were employed at the population level for data analysis. We first employed an analysis of variance (ANOVA) to compare growth rate of the ancestral genotypes with evolved endpoint populations with the genetic background and replicate lines as fixed

factor. Differences in growth rates among genotypes were compared between ancestral and evolved lines, and were measured using an ANOVA model where genetic background and transfer were taken as fixed factors and replicate lines were used as random factor. We also employed a similar model for the relative comparison of finesses for evolved population between transfer 50 (T50) representing about ~325 generations of evolved populations, and transfer 0 (T0) representing the ancestral populations. We used least square (LS) means contrasts in the factorial ANOVA model. Similar analysis was employed for both competitive fitness and e-Test MIC assay for the ancestral and evolved populations. All statistical analyses were carried out using JMP version 12 (SAS Institute Inc., Cary, NC, 1989-2007).

Results

Determining mutants genotypes

From a susceptible ancestral genotype, we obtained a streptomycin resistant and a rifampicin resistant genotype through mutant screening. Sanger sequencing indicated a K43T substitution at the rpsL locus to be responsible for resistance to streptomycin (MIC >128 ug/mL) and a P573L mutation at the rpoB locus responsible for rifampicin resistance (MIC >32 ug/mL). We constructed a double resistant genotype carrying both of these mutations through natural transformation. The presence of both mutations was confirmed by Sanger sequencing and the strain was indeed found to be resistant to both streptomycin and rifampicin (MIC for rifampicin: >32ug/mL; MIC for streptomycin: >128ug/mL). This double resistant genotype forms the main focus of this study.

Costs of resistance

Growth rate and competitive fitness assays revealed that significant fitness costs are associated with the two single and the double resistant genotype (**Figure 2**). Fitness costs in growth rate were significantly higher in the double resistant genotype ($p < 0.0001$) compared to single resistant genotypes, and the cost associated with the rifampicin genotype was higher than that of the streptomycin genotype ($p < 0.0001$), and the streptomycin resistant genotype was also significantly different from the ancestral wild-type genotype (**Table1**).

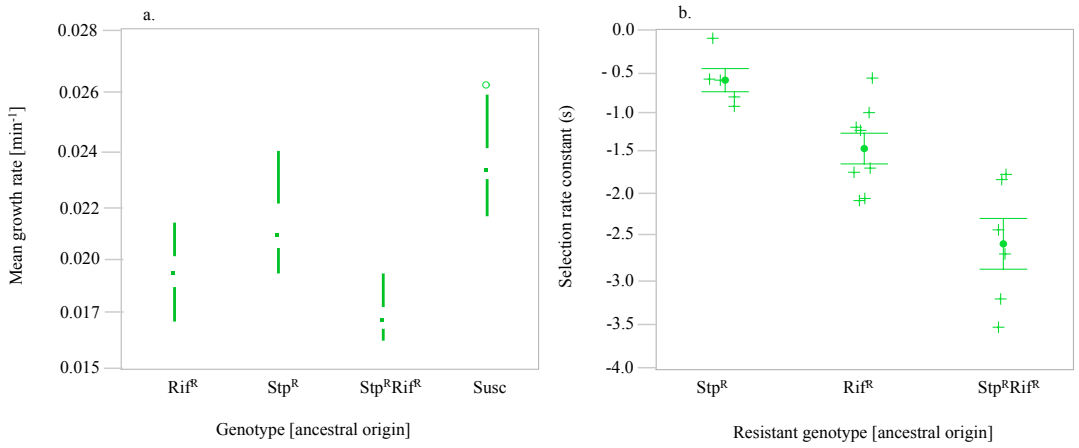

Figure 2 Fitness cost among ancestral resistant genotypes. Results are based on growth rate are shown panel (a) in box plot. Here, green lines indicate the lower and upper quartile with an open circle represents an outlier. In panel (b), fitness cost is shown in negative competitive ability among three different resistant genotypes. Filled circle represent the mean negative competitive fitness connected. Error bar represents ±1 standard error from the mean.

Table 1 ANOVA with mean growth rate vs. strain. Ordered differences report for comparisons for all pairs using Tukey-Kramer HSD test. Positive difference in a pair indicates significantly fitter genotype.

Genotype	-Genotype	Differences	SE of Diff.	Lower CL	Upper CL	*p*-value
Susc	StpRRifR	0.0067385	0.000286	0.0061752	0.0073019	<0.0001*
Susc	RifR	0.0047685	0.0001811	0.0044119	0.0051252	<0.0001*
StpR	StpRRifR	0.004050	0.0002995	0.003460	0.0046399	<0.0001*
Susc	StpR	0.0026886	0.0001774	0.0023391	0.0030380	<0.0001*
StpR	RifR	0.002080	0.0002017	0.0016826	0.0024773	<0.0001*
RifR	StpRRifR	0.001970	0.0003017	0.0013758	0.0025643	<0.0001*

Similarly, significant fitness cost in competitive ability was also found in all the resistant strains, where streptomycin and rifampicin mutant genotypes were significantly fitter than the double resistant genotype. However, no significant difference was between streptomycin and rifampicin resistant genotypes (see **Table 2** for parameter estimates).

Table 2 LS means differences in competitive fitness cost between different genotypes. Comparisons for all three pairs using Tukey HSD. Positive difference in a pair indicates significantly fitter genotype.

Genotype	-Genotype	Differnece	Std Err Di	Lower CL	Upper CL	*p*-value
Stp	StpRif	1.924304	0.3337942	1.06300	2.785603	<0.0001*
Rif	StpRif	1.120122	0.2977052	0.35194	1.888300	0.0046
Stp	Rif	0.0804182	0.3142568	-0.00670	1.615068	0.0521

Growth rate trajectories over time

We measured the growth rates of mixed populations for all sixty-four replicate populations evolving from the four different genotypes at eight different time points during the course of the evolution experiment. First, we observed that the mean growth rate trajectories for all different genotypes were stably maintained throughout the evolution experiments except fir all lines that originated from rifampicin and the double resistant genotype. Overall, mean fitness varied most in double resistant genotypes, and two distinct groups are apparent: one group comprising seven lineages exhibited markedly increased growth rates whereas the other nine lineages did not. Rifampicin resistant genotypes also showed varying degrees of fitness compensation. For the rest of the genotypes, no apparent trajectories were classifiable, except for one lineage (L13) from the streptomycin genotype, which showed a series of adaptive walks but this lineage converged with others in the late phase of the evolution experiment (**Figure 3**).

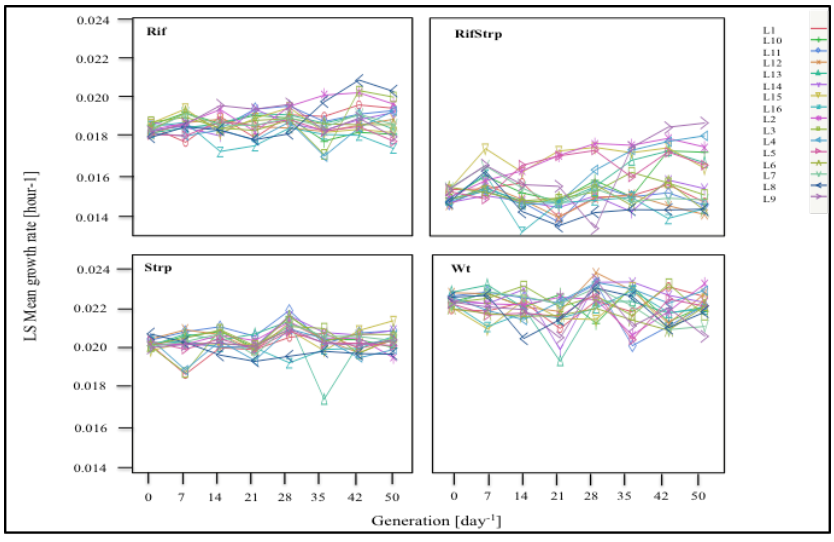

Figure 3 Growth rate trajectories over time. The x-axis shows different sampling time points in day, which also corresponds to generation time. Mean growth rates over different time points

for all 16 mixed populations (indicated by L1-L16) evolved from each of the ancestral genotypes.

Further, we investigated whether the dynamics of fitness for all the genotypes over different sampling points imply any statistical significance by employing a linear mixed effect model. We find that all the genotypes were significantly different from each other (p = <0.0001*), and also they significantly differ during the course of evolution (p = <0.0025*). All statistical parameter estimates are given in **Table 3.**

Table 3 Results of a general mixed effect model. Table shows the result on the dynamics of fitness for different genotype and transfer.

Source	**No. parm**	**DF**	**SSs**	**F-ratio**	***p*-value**
Genotype	3	3	0.00045397	196.9585	<0.0001*
Transfer	7	7	0.00002989	5.5577	<0.0001*
Gen*Tra	21	21	00.00003450	2.1385	<0.002*

The level of adaptation was further assessed by comparing growth rates of the evolved strains (i.e. after transfer 50, corresponding to ~325 generations) with their corresponding ancestral strains. We observed significant fitness differences between the T0 and T50 lineages originated from the double resistant genotype (StpRRifR) (ANOVA: F1, 30 = 4.3250, p = 0.0462). However, no significant differences were observed for the rifampicin and streptomycin resistant lineages (F1, 30 = 3.6629, p = 0.0652 and F1, 30 = 1.2779, p = 0.2672).

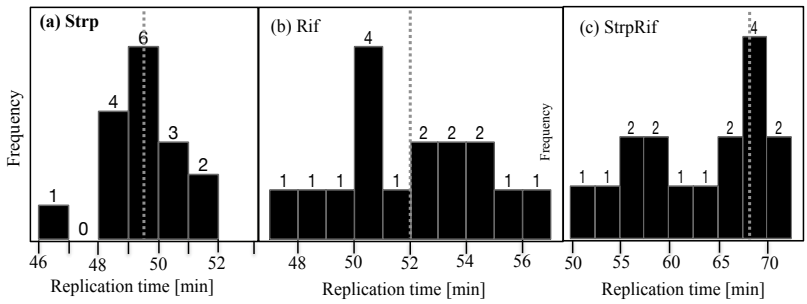

Figure 4 Histogram of minimum replication times. Histogram of replication time after ~325 generation representing distribution of fitness improvement in populations (founded on each of the resistant genotypes) in the absence of drug. Replication time for each of the ancestral

resistant genotypes is indicated by dashed vertical line. Numbers indicated above the bars represent the number of evolved lines.

In addition to this, we determined the distribution of minimum replication times for all different genotypes derived from endpoint population. We observed a higher number of double resistant lineages with improved fitness (10/16) than single mutant genotypes (**Figure 4**).

Growth compensation as a measure of mean competitive ability

Fitness compensation for each of the resistant genotypes from endpoint populations was further assessed among a subset of lineages by carrying out a head-to-head competition assays.

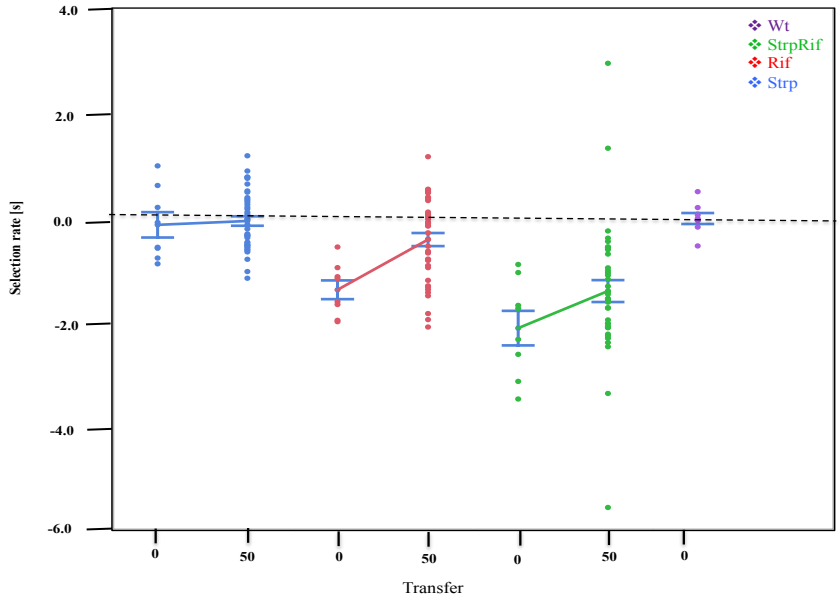

Figure 5 Mean selection rate (competitive fitness) vs. transfer (generation) of a subset of evolved resistant genotypes after ~325 generation. For each resistant genotype, competitive fitness was measured over a subset of 8 different endpoint populations at transfer 50 (8 populations per genotype were selected based on earlier growth rate trajectories). For example, 8 total populations for each resistant endpoint populations were tested for competitive fitness; At least 5 replicate measurements were made on each population and the resultant fitnesses (selection rates) were averaged, and finally grand completive fitness was calculated from these 8 populations. Competitive fitness for each ancestral genotype was carried out for at least 5 replicate measurements including the original wild type ancestor genotype. Error bar = ±1 SEM.

The mean relative improvement of fitness compensation was higher in rifampicin and double mutant genotypes than their ancestral resistant counterparts indicated by transfer 0 (**Figure 5**).

Further, pairwise mean comparisons using Student's t-tests among these genotypes revealed that the double resistant genotypes were significantly different from single resistant genotypes. This test also revealed significant differences between the two of singly resistant genotypes (see parameter estimates in **Table 4**).

Table 4 LS means differences. Comparison of each pair using Student's t-test.

Genotype	-(Genotype)	Differences	SE of Diffe.	Lower CL	Upper CL	*p*-value
StpR	StpRRifR	1.410458	0.1886498	1.037186	1.783731	<0.0001*
RifR	StpRRifR	1.010824	0.2009188	0.613228	1.40842	<0.0001*
StpR	RifR	0.399634	0.1998093	0.004238	0.79503	0.04760

MIC-determination as a means of tracking the evolvability

The stability of the resistance profile was determined with the end point populations. This was done because the change in MIC in absence of drug pressure may be linked to adaptation in a number of ways: a decrease in MIC to a particular drug could indicate reversion of resistance, whereas an increase in MIC may indicate acquisition of compensatory mutations that at the same time are also new resistance mutation. More specifically, if the fitness improvement occurs through reversion, it can be expected that antibiotic resistance level will be reduced to the level of wild-type ancestor strain. Alternatively, fitness improvement can be effected through compensatory mutations without altering or even increasing the MIC. Thus, the MIC of rifampicin and streptomycin was determined using E-test for all genotypes previously assessed with the competitive fitness assay.

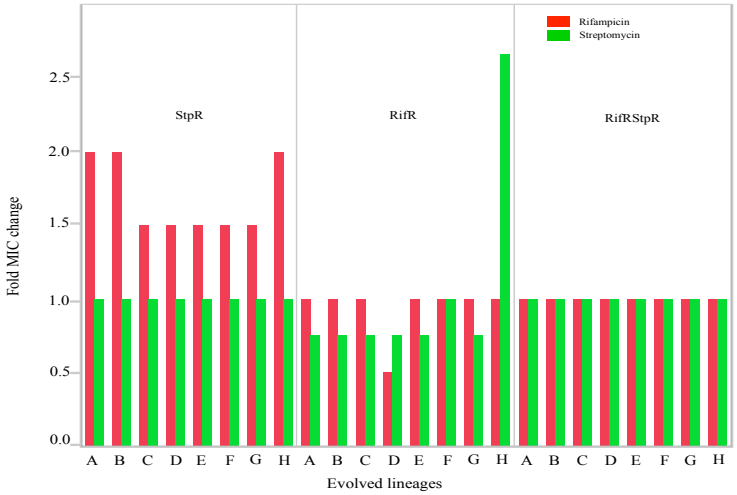

Figure 6 Stability of the antibiotic-resistant phenotype in the subsets of resistance populations after ~325 generations. MICs of ancestral rifampicin resistant population (Rif) was 32 mg/mL, ancestral streptomycin resistant genotype (Stp) was 128 mg/mL, and ancestral double mutant genotype ($Stp^{R}Rif^{R}$) was 32 mg/mL for rifampicin and 128 for streptomycin (this is based on the maximum E-stripe concentration for each antibiotic).

This MIC profile among different resistant genotypes (**Figure 6**) revealed that most lineages did not exhibit any change in MIC, and that there was no an incident of reversion. For streptomycin resistant genotypes, marginal increases in MIC to rifampicin were observed without shifting the level of resistance to the original streptomycin antibiotic. For the rifampicin resistant genotype, in most of the cases, we observed no apparent changes in MIC to streptomycin, except in one case where resistance to streptomycin was increased by more than 2.5 fold. This higher MIC to streptomycin in this lineage could be the result of either acquisition of new drug specific resistance mutation (i.e. mutation in *rpsL* or *rrs* gene) or this lineage could have acquired adaptive resistance through phenotypic heterogeneity. In one evolved lineage of rifampicin resistant genotype, we observed a two-fold reduction in MIC of rifampicin antibiotics; and this could be due to either the original resistance mutation was swapped by a newly arising but weaker mutation or by other mutation during the course of evolution. Interestingly, all double resistant populations kept their MIC level unchanged, indicating that double mutant genotypes improved their average fitness through other mechanisms.

Genomic basis of compensation

We further explored the genomic basis of compensatory adaptations by carrying out whole genome sequencing on the evolved populations. Our objective was to investigate the actual mechanism of compensation at the genomic level after ~325 generations to primary resistance

mutations carried by both single and double resistant genotypes. We attempted to identify de novo mutations that accumulated in these genotypes during the course of evolution to uncover: 1) how pervasive these adaptive mutations are, and where they appear in the genome, 2) to what extent compensatory mutations are influenced by already existing drug specific resistance mutations, 3) which adaptive routes are more accessible, for example, adaptation to the cost of single vs. double resistance mutation, 4) to what extent de novo gene disruption can affect the evolvability in both single and double resistant genotypes, 5) to determine what genetic changes further exacerbate the cost of resistance (this is of particular importance for infection control), 6) to determine the mutations that improve fitness to an optimal level (for example, by rare mutation suggested by Fisher geometric model), 7) to determine mutations that are associated with pleotropic effects, for example, a genotype with a newly arisen mutation in a costly background is further away from the fitness peak, 8) or whether newly arising mutations that can both compensate and give rise to resistance to a new drug through epistatic interactions between resistance and compensatory mutations [29, 36], 9) whether compensation achieved by inactivation of other genes, for example through truncation or premature termination in the costly genome. Thus, we will be able to determine whether evolutionary adaptation to the cost of multiple drug resistance by compensatory mutations generates any substantial genetic variations, which differ from compensation to the cost of single drug resistant mutations. Therefore, we sequenced clones from eight independently evolved lines from each of the three evolved resistant genotypes, all of which we previously assessed for growth rate, competitive fitness, and antimicrobial susceptibility. Additionally, we sequenced the three ancestral resistant genotypes to compare the genomic changes underlying compensatory adaptations.

Whole genome sequencing investigation on the ancestral genotypes

Whole genome sequencing of ancestral origin has revealed an additional mutation in a less characterised *cyoA* locus in a double resistant genotype. This mutation unexpectedly occurred during construction of this genotype through natural transformation and this mutation also produced significant fitness cost (ANOVA: F-test: 319.87; DF: 2; p-value = <0.001). Although we did not construct any genotypes with identical *cyoA* mutation alone or in combination with other resistance mutations to determine the actual effect on bacterial physiology we assume that this additional mutation may also cause additional fitness cost through epistatic interaction (**Figure S1**). However, it has been reported that disruption in *cyoA* can give adaptive resistance to multiple antibiotics [37, 38]. We included this double resistant genotypes in our evolution

experiment and evolved for ~325 generations along with other three different genotypes since natural populations always undergo simultaneous acquisition of genetic determinants along with resistant elements from their exogenous sources via recombination. Therefore, we assumed that such additional deleterious mutations might occur in both natural populations as well as in the laboratory populations but the true role of such additional mutations were unexplored in absence of whole genomic information.

Whole genome sequencing investigation on the evolved endpoint populations

Whole genome sequencing detected 27 secondary mutations in each subset of the evolved endpoint populations founded on three different genotypes during the course of evolution (**Table 5**). Among these, we identified seven different secondary mutations in five of the eight different StpR evolved populations. However, none of these mutations occurred in putative compensatory loci such as *rpsL*, *rpsD*, *rpsE*, and *rrs* (detailed information on this mutation can be found in supplementary **table S4**). Three out of those five StpR evolved lineages each carried a single point mutation; two lineages each carried two different mutations. One lineage carried a secondary mutation in gene encoding FSR protein, which greatly benefitted growth improvement (by ~6%). Minor growth improvement (~2%) was also observed in a single lineage carrying an intergenic mutation between a transcriptional regulatory protein and citrate transporter protein. We also identified 4 different secondary mutations in three different genes of unknown function encoding hypothetical proteins (HPs). We observed these 4 different mutation in three different lineages. Two lineages (C and E) harboured the identical substitution mutation in the same genetic location suggesting parallel evolution. Lineage C improved its fitness cost by ~3%, which was accompanied by an additional point mutation (synonymous) in a gene encoding an ABC-transporter protein.

	Protein/locus affected										
Genotype	**FSR**	**Int**	**ABC**	**HP**	**RNAP**	**S4**	**HRP**	**TCS**	**Lrp**	**CyoA**	**Lon**
Rif^R	0/8	0/8	0/8	0/8	6/8	0/8	1/8	1/8	1/8	0/8	0/8
Stp^R	1/8	1/8	1/8	4/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Rif^RStp^R	0/8	0/8	0/8	1/8	3/8	1/8	0/8	1/8	0/8	4/8	1/8

FSR-follic siderophore receptor protein; Int-intergenic mutation; ABC- abc tranporter protein; RNAP-RNA-polymerase; S4-ribosomal subunit protein 4; TCS-two component sensory kinase; Lrp- Leucine responsive protein; Lon – Lon protease

Mutations in these hypothetical loci suggest that all these mutations are alone not sufficient to improve the fitness burden, but the cost reduction is accelerated by a mutation in another genetic locus through an epistatic interaction. However, lineage E carried two secondary mutations (one non-synonymous and another one synonymous mutation) in a hypothetical gene that caused about 5% increase in fitness cost, and another lineage (line F) carried a single mutation in a hypothetical gene that caused about 1.4% increased fitness cost. However, three lineages (3/8) did not carry any secondary mutations where fitness was improved by ~2% among two of these lineages (lineage G and H), but the cost was further increased in one lineage (lineage D). The MIC of streptomycin was unchanged in all the evolved lineages, but interestingly increased resistance to rifampicin was observed among all these replicate populations evolved from streptomycin resistance genotypes (**Figure 6**) suggesting that less sensitive resistant genotypes (i.e. streptomycin resistance mutation with low fitness cost) helped these populations develop resistance to a new antibiotic (i.e. rifampicin) by avoiding both extreme beneficial or deleterious secondary mutations in the absence of selection pressure.

In seven out of eight RifR evolved populations, we identified a total of nine secondary mutations, of which six are intragenic mutations in *rpoB* (the same locus that carries the primary mutation conferring resistance to rifampicin), and four extragenic mutations (secondary mutations in loci other than the primary resistance locus). In one lineage we did not identify any secondary mutation. Among these six secondary mutations found in *rpoB*, four were newly arisen putative compensatory mutations of which two lineages carried the same mutation (lineage A and B), and thus suggesting a parallel compensatory evolution in this resistance locus. Surprisingly, in one lineage (lineage D), the primary resistance mutation (*rpoB*P571L) was replaced by another mutation (*rpoB*L571H). This lineage experienced greater fitness improvement (by more than 23%) than any other rifampicin resistant evolved lineages. In this clone, we also observed a reduced MIC to rifampicin (**Figure 6**) suggesting that this mutation represents an incomplete reversion mutation at the phenotypic level. This lineage also carried an additional truncation mutation (Q->Stop) in a gene encoding a multifunctional two-component sensory (TCS) kinase. A similar truncation mutation such as a premature stop codon in glutamine amino acid (Q->Stop) was also observed in a gene encoding two-component sensory kinase (lineage E) whose fitness cost was further intensified by more than 5% (detailed information for the observed mutation can be found in supplementary **Table S5**). In lineage H, a new extragenic secondary mutation was also

observed in *lrp* gene encoding a global regulatory protein (leucine responsive protein) where fitness was improved by 2%.

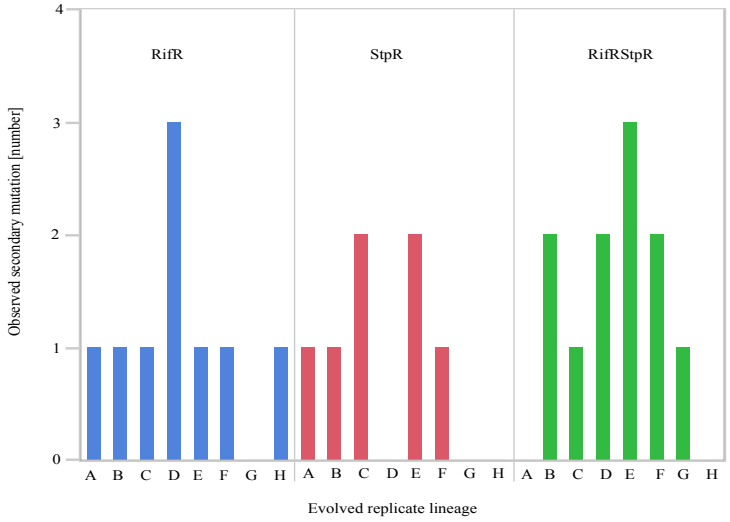

Figure 7 Observed secondary mutations in different resistance lineages after ~325 generations in absence of drug pressure. Empty bar represents no secondary mutations observed after ~325 generations for particular lineages.

Disruption in this gene has been associated with reduced virulence in *E. coli* [39] suggesting a putative new mechanism of compensatory adaptation to the cost of rifampicin resistance. Overall, all the secondary mutations observed in the evolved RifR populations were sufficient to ameliorate the fitness cost incurred by *rpoB* P571L rifampicin resistance mutation (fitness cost improvement ranging from 3 to 23 %), these secondary mutations observed in rifampicin resistance evolved lineages are thus regarded as putative compensatory mutations. Such intragenic compensation has previously been identified in experimental bacterial population [17]. No other known secondary mutations (i.e. putative compensatory mutations in *rpoA* and *rpoC*) were observed in any of the eight evolved Rifr lineages (**Table S5**).

Following the evolution experiment, the double resistant lineages had acquired 11 different secondary mutations distributed in five out of eight lineages (**Figure 9**). Among these, three lineages of each carried a single intragenic secondary mutation where a putative compensatory mutation appeared in the same *rpoB* locus. Four lineages (B, C, E, F) carried a reversion mutation in the *cyoA* gene and, surprisingly, all these four lineages improved their growth rates ranging from 5% to 17%. Among these 4 lineages, we also identified a mutation in *rpsD* gene encoding small subunit of ribosome called S4 together with a secondary mutation in *rpoB* and a *cyoA* reversion mutation, and the highest fitness improvement was observed in this lineage (E).

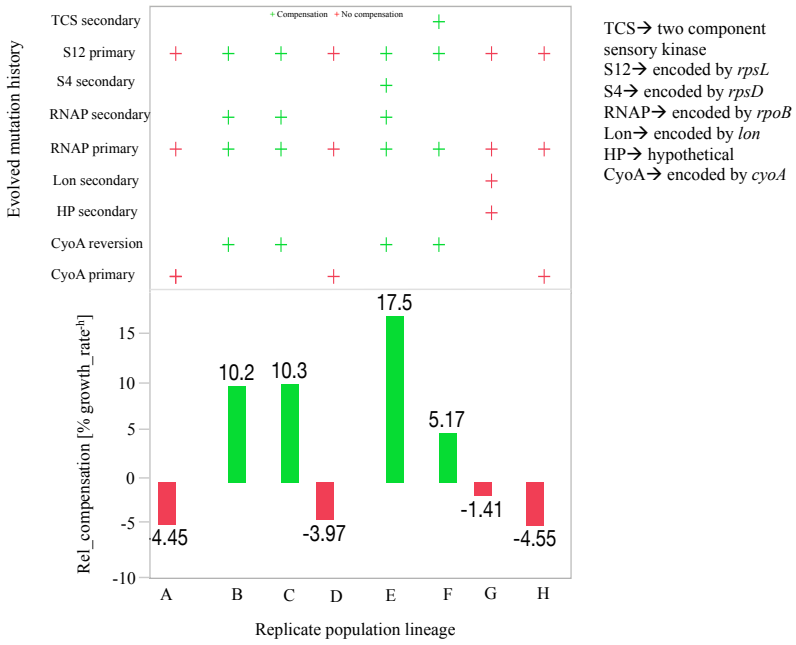

Figure 8 Observed mutations and corresponding fitness compensation among Rif^{R}Stp^R double resistant evolved lineages. Top panel shows the secondary mutations observed in the evolved lineages, and the bottom panel shows relative fitness compensation [% relative growth rate]. All the green bars and red bars represent positive and negative fitness respectively. Lineage G (indicated by the red bar) compensated cost compared to other three (A, D and H) by harbouring a secondary mutation in *lon* the gene.

However, one lineage (F) improved fitness by 5% without harbouring any secondary mutations in *rpoB* or *rpsL* locus suggesting that the greater fitness compensation in this lineage was mediated by *cyoA* reversion together with a secondary mutation in gene encoding TCS kinase (**Figure 8**). Interestingly, all the *rpoB* mutations found in the double resistance genotypes were distinct, and none of them were found to be common in any lineages initialised with the RifR resistant genotype (**Figure 9**).

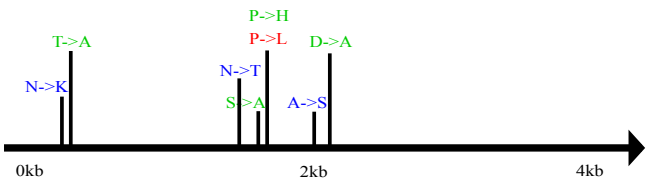

Figure 9 A map of *rpoB* gene showing the distribution of secondary mutations observed in the populations evolved from both Rif^R and Rif^{R}Stp^R after ~325 generations. The amino acid change highlighted in red is the primary rifampicin resistance mutation. Green and blue colour indicates the secondary mutations observed in the Rif^R and Rif^{R}Stp^R population, respectively.

By contrast, in four lineages (A, D, G and H), we did not identify any secondary mutation in *rpoB*, *rpsD* or *cyoA*, and there was no compensation of the cost of resistance among these three lineages. However, we observed a secondary mutation in a gene encoding Lon protease in one lineage (G). This lineage experienced improved fitness compared to other three lineages (A, D, and H) suggesting a putative compensatory mutation. Spontaneous mutation in the lon gene has been found to be associated with tetracycline or chloramphenicol resistant *E. coli* [40]. However, in a recent study, it has been shown that mutations in lon genes confer low level of multidrug resistance via the activation of efflux pump systems [41]. For example, mutated Lon protease stabilizes MarA and SoxA transcriptional activators and induces the AcrAB-TolC efflux-pump, which in turn confers multidrug resistance. However, the co-occurrence of this mutation along with other resistance mutations may suggest that this mutation may be involved in simultaneously improving fitness as well as conferring strong resistance to other drugs through epistatic interaction.

Discussion

The frequency of resistant bacteria and their subsequent adaptation in the absence of antimicrobial compounds are associated with the amount of antibiotics used, the cost of resistance mutations (i.e., reduced competitive ability compared to the susceptible counterparts), and the ability of resistant genotypes to compensate this cost [14, 36-39]. This phenomenon of compensation in bacterial populations has been well characterised by employing both in vivo and in vitro experiments in many studies [17, 18, 40-42]. It is assumed that compensatory adaptation to the cost of resistance is critical to these evolutionary dynamics because it can slow or prevent the extinction of resistant genotypes in the absence of selection pressure. More specifically, the mechanism of compensation is thought to depend on many factors such as mutation in a particular genetic background, the degree of cost associated with the resistance mutation as well as adaptation to the infected hosts [13, 14, 22, 42]. In previous studies [16-18, 43, 44], the mechanism of compensation was mostly investigated by employing a bacterial population resistant to a single drug. For example, the mechanism of compensation was studied in strains carrying known rifampicin (*rpoB* resistance) and streptomycin (*rpsL* resistance) mutations. However, no comparative studies at the genomic level have been conducted to uncover the genetics of compensation in strains carrying multiple drug resistance mutations.

In this study, whole genome sequencing of a double resistant genotype of ancestral origin revealed an additional mutation in the *cyoA* gene (which encodes cytochrome bo terminal oxidase A associated with respiratory chain system) with other two distinct drug-specific resistance mutations in the *rpoB* and *rpsL* loci. This mutation in *cyoA* locus unexpectedly occurred during construction of this genotype by natural transformation assay. The mutation may have arisen for a number of reasons, for example: 1) natural transformation requires competent cells which are more vulnerable to the environmental perturbation, 2) natural transformation itself a costly metabolic process which involves many genes to take up the foreign DNA pieces into cytoplasm, and 3) this process also requires sets of other genes in the final step of homologous recombination. In our case, the additional cost of this transformation process incurred by a costly *rpoB* mutation may have given rise to this additional mutation. This mutant has previously been found to be associated with reduced rates of cellular respiration [45]. Recent studies also suggested that altered cellular respiration could be the result of the altered translation process, or translation inhibition may have other non-metabolic effects on the cell which in turn could give rise to decreased susceptibility to bactericidal antibiotics, as has recently been shown by a study carried out with mutant *cyoA* harbouring *E. coli*, which conferred high a level of resistance against bactericidal antibiotics such as norfloxacin, ampicillin, and gentamicin [37, 38]. Therefore, motivated by these findings, we also assume that this additional *cyoA* mutation was responsible for reduced growth rate through epistatic interaction, and also may have given rise to high-level resistance to other bactericidal antibiotics in our study organism that we have not tested in our yet. In addition to this, additional mutations in other genetic loci may occur in both single and multidrug resistant bacterial population of laboratory and natural origin. This additional mutation may have profound effects on the rate of bacterial adaptation in the absence of drug pressure, especially in cases where resistance to multiple drugs conferred by mutations at multiple loci. One recent study [44] has opined the importance of general beneficial mutations in overcoming the fitness cost of the compensatory mutation. This study has suggested to include antibiotic-resistant mutants that carry other types of resistance mutations and assess compensatory adaptation in those genotypes evolved from experimental evolution, since the mechanism of antibiotic resistance is diverse and many other parts of the genome are also affected by antibiotics, for example resistant genotypes may enriched with other beneficial or deleterious mutations in the genome.

One of the main findings in our study is that 50% of evolved multidrug resistant lineages (4/8 lineages) carried reversion mutations in the *cyoA* gene, suggesting parallel evolution [46, 47]. Thus, the *cyoA* gene in these populations under strong positive selection also played a central role in the adaptive process. Surprisingly, three of these lineages (B, C, E) experienced higher fitness compensation and also harboured a secondary mutation in the *rpoB* locus, but one lineage (F) was less fit because this lineage evolved with a mutation in a gene encoding TCS kinase protein with no secondary mutation in the *rpoB* locus. This result suggests that all the *rpoB* secondary mutations that occurred in these populations were indeed involved in the compensatory adaptation. Most notably one lineage (E) that experienced greater fitness improvement (17.5%) harboured a secondary mutation in the *rpsD* gene. Mutations in the *rpsD* gene are well known for fitness compensation of *rpsL* mutations conferring resistance to streptomycin [48]. It is also worth mentioning that *rpsD* mutations are associated with fitness compensation of streptomycin resistance mutations in the *rpsL* gene, but in the absence of *rpsL* mutations *rpsD* mutations alone can confer resistance to streptomycin [43]. Thus, these results suggest that, even in presence of an additional mutation in the *cyoA* locus, both *rpoB* and *rpsD* are indeed common loci, where most of the secondary mutations involved in fitness compensation for rifampicin and streptomycin resistant genotypes occurred. In one case, we have also found a minor fitness compensation induced by a secondary mutation in a gene encoding two-component sensory kinase (TCS). This result suggests that adaptation to the cost of multiple resistance mutations can also be achieved by acquiring secondary mutation in off-targeted region (different from commonly found compensatory regions, such as *rpoB* and *rpoD*) of the genome. However, we have not observed any fitness compensation among four other lineages owing to the fact that none of these lineages harboured any secondary mutations like what we have observed in four different fitness-compensatory double resistant lineages. Interestingly, in one lineage, we have observed that fitness cost was further exacerbated by a mutation in the *lon* gene previously reported to be associated with low level of multidrug resistance [41, 49-51]. Competitive fitness estimate for all the double resistant genotypes also revealed that all the lineages harbouring secondary mutations together with *cyoA* reversion greatly improved fitness cost after ~325 generation. Together with this, our results suggest that fitness compensation among multidrug resistant population is caused mainly by secondary mutations commonly occurring at compensatory loci (targeted regions such as *rpoB* and *rpsL*) and also follow a parallel evolutionary trajectory (i.e. reversion mutation) for costly mutations in other loci in the same genome. Furthermore, our results suggest that in the absence of compensation multidrug resistant populations can intensify their fitness cost further by

acquiring additional drug resistance mutations in other locations of the genome. Reversion in the *cyoA* locus may also suggest that some genomic loci may accidentally acquire deleterious mutations, which can then revert back to the original state at high rate owing to the cost associated with it.

We have observed seven different secondary mutations in five of the eight different StpR evolved populations, but none of them were found in the expected *rpsD* or rpsE loci [16, 48]. Most of the observed secondary mutations in StpR resistant evolved lineages were either neutral or deleterious given that some mutations were either involved in negligible fitness compensation or were found to be associated with further increase in fitness cost. This could be due to the fact that mutation conferring resistance to streptomycin antibiotic incurred low fitness cost in absence of drug pressure; thus this low cost resistance mutation has given rise to secondary mutations in less-known genetic loci which are involved in minor fitness compensation or slight increases in fitness cost, as has been found in earlier studies [52]. Among these, in two lineages, we have observed identical transversion mutation in genes encoding hypothetical proteins, which were not involved in significant fitness compensation. This observation, though found in a limited number of lineages, may also suggest parallel evolution in uncharacterised hypothetical genes of strains carrying streptomycin resistance mutation in *rpsL* locus. Among these, only a single lineage carried a secondary mutation in a gene encoding ferric siderophore receptor protein greatly benefitted from growth improvement (by ~6%). However, one lineage that carried a secondary mutation in a hypothetical gene improved its fitness by about 3%, which was accompanied by an additional point mutation (synonymous) in a gene encoding the ABC-transporter protein. Mutations in these hypothetical loci suggest that all these mutations are alone not sufficient to improve the fitness burden, but the cost reduction could be accelerated by a mutation in other genetic locus through an epistatic interaction [16, 53]. The MIC of streptomycin was unchanged in all the evolved lineages, but interestingly increased resistance to rifampicin was observed among all these replicate populations evolved from streptomycin resistance genotypes (**Figure 6**) suggesting that less sensitive resistant genotypes (i.e. a streptomycin resistance mutation with low fitness cost) helped these populations develop resistance to a new antibiotic (i.e. rifampicin) by avoiding both extremely beneficial or deleterious secondary mutations in the absence of selection pressure. However, none of these secondary mutations were commonly detected in any of the double resistant genotypes. This could be explained by the fact that selection for the low-cost resistance mutation (conferred by streptomycin resistance mutation) in the double resistant

background is less effective since the selection of compensatory adaptation for the costly rifampicin resistant mutation in *rpoB* locus in the genome is stronger.

We observed secondary mutations in six different RifR resistant evolved lineages, whereas no secondary mutation was observed for two lineages. Interestingly, two of these lineages have evolved with an identical secondary mutation in the *rpoB* gene suggesting parallel evolution and thus this mutation was fixed in these populations by positive selection. Interestingly, this mutation has also helped these lineages recover maximum fitness. We have also identified identical transition mutations in two other lineages but in two different loci; in one lineage this mutation was accompanied by a secondary mutation in the *rpoB* gene and improved fitness, whereas this transition mutation was found in another locus but not accompanied by other secondary mutations and fitness cost was further accelerated in this lineage. These results support the proposition that this secondary mutation in *rpoB* locus is indeed a fitness compensatory mutation which can improve fitness to a greater extent in presence of a transition mutation in other loci, but fitness is deteriorated further by a secondary transition mutation in the absence of a secondary mutation in the *rpoB* locus. In addition to this, our results support the notion that owing to the costly resistance mutation, rifampicin resistant genotypes evolved with more compensatory mutations rather than other beneficial mutation.

Furthermore, we were interested to see whether the secondary mutations that occurred in RifR lineages were also observed in the RifRStpR lineages. Therefore, we mapped the location of all *rpoB* secondary mutations (**Figure 9**). The map shows that none of these mutations were commonly shared by any RifR and RifRStpR evolved populations suggesting that the genetic basis of compensation of multidrug resistant bacteria is independent of the genetic backgrounds associated with the single drug resistant bacteria. In three other lineages of RifRStpR, fitness costs were further magnified where no secondary mutations were observed. In all these double mutant lineages, the MIC to both rifampicin and streptomycin remained unchanged.

Our experimental evolution experiment using single and double resistant genotypes with an additional mutation has revealed the genetic basis of adaptation to the cost of resistance. In most cases, we have observed minor improvements in fitness due to off-targeted beneficial mutations, and this has constrained the compensatory evolution which is thought to occur in the same protein or pathway where antibiotic resistance mutations also arise [1, 54] . In our

case, we have observed pathway-specific secondary mutations, which improved highest fitness among genotypes belonging to both rifampicin resistant genotype and the double resistant genotype, highlighting the dependency of compensatory adaptation on costly resistance mutation.

In conclusion, our data supports the empirical observation that genetic basis of compensation in both single and double resistant genotype depends on both intragenic and extragenic mutations. In double resistant genotypes, this compensation is independent of the genetic background of mutations occurred in single resistant genotypes. Furthermore, we have identified some regions in the *rpoB* locus from costly RifR and RifRStpR lineages where most of the secondary mutations were observed. Overall, the results we have presented here further increase our understanding of the adaptive molecular evolution, and partially explain the increasing clinical problem of multidrug resistant bacteria.

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Supplementary materials

Table S 4 List of primers used in amplifying target genes.

Gene name	Primer sequence
rpoB 1 (forward)	TTCGATTCAGGTCGACTCGT
rpoB 1 (reverse)	CAGGCGTTCTGGAACAAGAT
rpoB 2 (forward)	TGGATCAAAACAACCCATTG
rpoB 2 (reverse)	ATCGCCACGACCCACTTTAT
rrs 1 (forward)	GGCAGGCTTAACACATGCAA
rrs 1 (reverse)	CTACGCATTTCACCGCTACA
rrs 1 (forward)	CTGGAGGAATACCGATGGCG
rrs 1 (reverse)	TAACCGCCCTCTTTGCAGTT
rpsL (forward)	ATGGCAACAACAAATCAGTT
rpsL (reverse)	TTATTTCTTAGGACGTTTAG

Table S5 PCR master mix preparation per reaction

Reagents	Amount [uL/reaction tube]
PCR buffer 10x	2.5
MgCl2 25 mM	2.5
dNTP 10mM	0.5
TaqGold 5U /µL	0.1
Primer mix (conc. 0.5µM) (F+R)	1.5
Sigma H2O	17.9
Template DNA	5
Tolal volume	30

Table S6 Setup for PCR program

PCR program:		
Step	**Temperature**	**Time**
1 (hold)	95°C	6 minutes
2 (denaturation)	94°C	45 seconds
3 (annealing)	58°C	45 seconds
4 (elongation)	72°C	2 minutes
5 (repetition)	Step 2 to 4 for 35 cycles	
6 (final elongation)	72°C	10 minutes

Table S7 Observed mutations in the evolved streptomycin resistant lineages.

Original lineage	Technical lineage	Position	Amino Acid Change	Protein Effect	Mutaion history
3	A	732,196	V -> M	Substitution	FSR
3	A	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
5	B	2,755,427	Intergenic	Intergenic	Int (between Trp and CitN)
5	B	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
6	C	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
6	C	943,763	I -> R	Substitution	HP
6	C	1,487,504	L -> L	Synonymous	ABC transporter
7	D	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
8	E	943,763	I -> R	Substitution	HP
8	E	865,048	K -> T	Substitution	Original *rpsL*

					resistance mutation
8	E	2,747,986	T-> T	Synonymous	HP
9	F	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
9	F	294,112	M -> T	Substitution	HP
10	G	865,048	K -> T	Substitution	Original *rpsL* resistance mutation
11	H	865,048	K -> T	Substitution	Original *rpsL* resistance mutation

Table S8 Observed mutation in the evolved rifampicin resistant lineages.

Lineage	**Technical lineage**	**Position**	**Amino acid change**	**Protein ffect**	**Mutation history**
1	A	304,167	P -> L	Substitution	*rpoB*, original mutaion
1	A	302,864	T -> A	Substitution	*rpoB*, compensatory
2	B	302,864	T -> A	Substitution	*rpoB*, compensatory
2	B	304,167	P -> L	Substitution	*rpoB*, original mutaion
3	C	304,167	P -> L	Substitution	*rpoB*, original mutaion
3	C	304,608	D -> A	Substitution	*rpoB*, compensatory
4	D	304,167	P -> H	Substitution	New mutation in *rpoB*
4	D	931,154	Q -> Stop	Truncation	hemagglutinin/hemolysin-related protein
5	E	304,167	P -> L	Substitution	*rpoB*, original mutaion
5	E	3,392,952	Q -> Stop	Truncation	Two component sensor kinase
8	F	304,167	P -> L	Substitution	*rpoB*, original mutaion
8	F	304,196	S -> A	Substitution	*rpoB*, compensatory
10	G	304,167	P -> L	Substitution	*rpoB*, original mutaion
15	H	115,202	Y -> C	Substitution	*Lrp*
15	H	304,167	P -> L	Substitution	*rpoB* original mutation

Table S9 Observed mutation in the evolved double resistant evolved lineages

Lineage	Technical lineage	Position	Amino acid change	Type of mutaion	Mutation history
1	A	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
1	A	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
1	A	2,391,960	S -> P	Nonsynonymous	Original *cyoA* mutation
2	B	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
2	B	304,466	A -> S	Nonsynonymous	Compensatory
2	B	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
2	B	2,392,267	S -> L	Nonsynonymous	Compensatory
4	C	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
4	C	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
6	D	302,860	N -> K	Nonsynonymous	Compensatory
6	D	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
6	D	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
9	E	304,029	N -> T	Nonsynonymous	Compensatory
9	E	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
9	E	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
9	E	3,123,145	S -> F	Nonsynonymous	Compensatory
10	F	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
10	F	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
10	F	3,392,837	S -> R	Nonsynonymous	Compensatory
11	G	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
11	G	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
11	G	1,102,100	G -> D	Nonsynonymous	Compensatory
11	G	2,391,960	S -> P	Nonsynonymous	Original *cyoA* mutation
16	H	304,167	P -> L	Nonsynonymous	*rpoB* original resistance mutation
16	H	865,048	K -> T	Nonsynonymous	Original *rpsL* resistance mutation
16	H	2,391,960	S -> P	Nonsynonymous	Original *cyoA* mutation

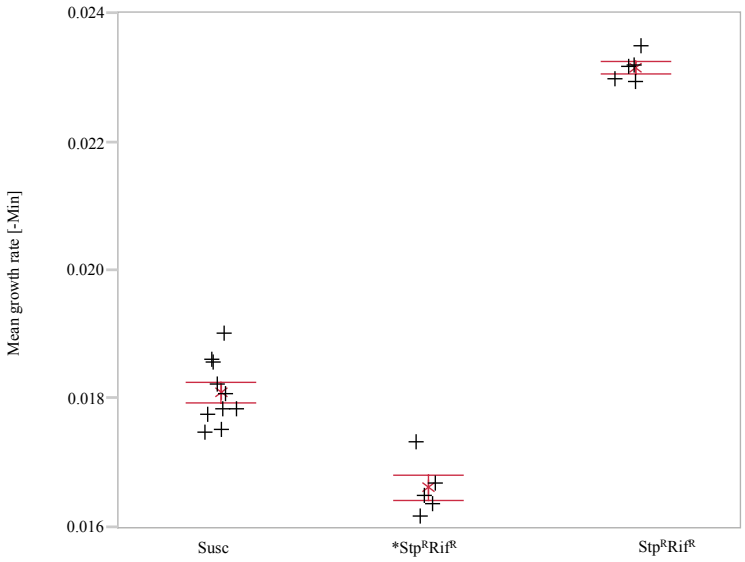

Figure S5 Fitness of double resistant genotype. Plot shows two different double mutant genotypes ($Stp^{R}Rif^{R}$) with a star indicating an additional mutation in *cyoA* harboured by one double resistant ($Stp^{R}Rif^{R}$) genotype. locus and a completely susceptible genotype. Error bar = ±1 S.E.M. Susc: completely susceptible genotype; $Stp^{R}Rif^{R}$: double mutant genotype harbouring both K43T in *rpsL* gene and P571L mutation in *rpoB* gene.

Chapter 5: General discussion

Multidrug therapy has been regarded as an effective treatment strategy over traditional monotherapy because of the rapid evolution of resistant pathogen against single drug. For example, combination therapy produces effective therapeutic response by producing synergistic interactions with different cellular targets of the pathogen simultaneously [1]. Therefore, drug combination yet regarded as a standard-of-care treatment. One prominent example of such treatment protocol widely being used is against *M. tuberculosis.* In recent days this therapy is also extensively being prescribed against many other important clinical pathogens, such as Gram negative bacilli [2]. However, the widespread prevalence of bacterial resistance to multiple antibiotics has restricted our current therapeutic arsenal to a greater extent. This is due to the fact that multidrug resistance remains a poorly understood biological phenomenon. Therefore, evolution of *de novo* resistance and subsequent adaptation to multiple drugs present both practical and theoretical challenges for proposing successful therapeutic interventions and control of such problematic infectious organisms.

Many earlier studies have drawn different conclusions concerning factors contributing to the rate of resistance evolution under varying contexts, including the molecular characteristics of the resistance mutations, the fitness effects of drug resistance mutations in the presence and absence of drug induced selective pressure, and how these factors impact the evolutionary trajectories of the resistant population [3-7]. However, it is not fully understood how *de novo* multidrug resistance evolution takes place in an environment enriched with drug combinations. In relation to this, we remain largely ignorant of many factors, for example to what extent two-drug pharmacodynamics, drug-drug interaction, cross-resistance, epistasis, recombination and compensatory mutations contribute to the emergence, spread and adaptation of *de novo* multidrug resistance in bacteria. These factors are important in predicting and preventing future multidrug resistance evolution [8]; therefore these factors deserve to be assessed by taking proper experimental approaches. This PhD thesis attempted to expand our understanding further by investigating these factors involving the de novo multidrug resistance evolution and subsequent adaptation under laboratory conditions.

In chapter 2, fitness landscapes comprising both susceptible and resistant genotypes were characterised under two-drug antimicrobial therapy to understand the evolutionary dynamics of

mutations at multiple loci conferring resistance to multiple drugs. Specifically, motivated by a recent theoretical work [9], I investigated to what extent two-drug pharmacodynamics, drug-drug interaction, and cross-resistance are associated with epistasis – an important property of the pharmacodynamic fitness landscapes and a determinant of the evolution of antibiotic resistance [10, 11]. Epistasis can occur between genes [12], within a single gene encoding a single resistance protein [13, 14], or between chromosomal gene and plasmid [15]. Many studies have identified a pervasive epistasis in bacterial adaptive evolution of multidrug resistance under diverse conditions. For example, in two studies positive epistasis between resistance mutations was reported in the cost of resistance [16, 17].

Here, our results show mostly weak or no epistasis between different sets of resistance conferring mutations in absence or presence of varying concentrations of two-drug combinations. In all but except one cases (i.e., epistasis between kanamycin-rifampicin and kanamycin-streptomycin resistance mutation) diminished fitness for all double resistant genotype was apparent in absence of drug pressure. These slower growth rates in absence of drug pressure were mostly due to the fitness cost incurred by drug specific resistant mutations occurred in the chromosomes, which has given rise to non-epistatic fitness landscapes. Although the genetic context is different, such non-epistatic fitness landscape was previously reported in *E. coli* [18], but at the same time our result in absence of drug pressure differs from earlier empirical studies where positive epistasis was pervasive [17, 19]. Therefore, from our data it is apparent that in order to have an epistatic effect on the fitness landscape, genetic background or mutational history with degree of fitness cost incurred by different resistance conferring mutations is important component. We observed negative epistasis between rifampicin and streptomycin resistance mutation when the concentration of rifampicin was higher and combined with streptomycin antibiotics. This could be explained by the fact that the high cost rifampicin resistance mutation requires higher concentration of rifampicin antibiotic in order to release its high fitness cost. However, it is also important to mention here that an additional point mutation (i.e. mutation in *cyoA* gene we identified by whole genome sequencing) was present in this genotype; *cyoA* was previously reported to be involved in bacterial intrinsic physiological constraint [20-22] and also incurred additional fitness cost. We speculate that without this additional mutation, the fitness landscape comprising this genotype might also be less or non-epistatic. Therefore, we suspect that this type of deleterious mutation may be also have occurred and confounded previous studies where epistasis was measured without conducting whole genome sequencing [16, 17, 23, 24].

We further investigated the occurrence of cross-resistance and its association with epistasis. Theoretical observation suggests that epistasis can emerge when cross-resistance is pervasive. For example, in presence of cross-resistance (i.e., when both drugs confer resistance to each other with the same degree) positive epistasis will ensue when two resistance mutations jointly increase the MIC of both drugs but negative epistasis will follow when combined effect of both mutations only entails slightly increase or does not increase in MIC compared to individual mutational effect [51]. Multidrug resistance through cross-resistance has already been reported in many clinical situations. For example, bacteria harbouring a single point mutation or a resistance enzyme capable of neutralizing many different antibiotics, including NDM-1 (New-Delhi Metallo-β-lactamase carried by the plasmid) producing *K. pneumoniae* capable of hydrolysing many other different antibiotics [25], or altered efflux–pump mediated cross-resistance has also been found to be associated with resistance to multiple drugs in many bacterial species, including *P. aeruginosa* and many other Gram negative bacilli [26, 27]. We also observed cross-resistance in single resistant genotype; for example streptomycin resistant genotype can grow slowly in presence of high concentration of rifampicin or kanamycin. The observed cross-resistance between streptomycin and kanamycin would be due to the fact that both antibiotics belong to the same chemical class and also target the same cellular process. Therefore, only a single resistance gene can provide accelerated growth advantage. Our result is also well aligned with a previous systematic study, which also identified cross-resistance between these two drugs [28]. However, in another case we observed that the same resistant genotype exhibited cross-resistance when exposed to rifampicin antibiotics and this finding differ by previous study [28]. One plausible explanation is such that both antibiotics target the same flow of the cellular process of protein synthesis, involving bacterial transcription and translation inhibition. This means that a genotype with an altered protein synthesis machinery may be capable of conferring resistance to other antibiotics when the target of both antibiotics are belonging to a fundamental cellular process. Therefore, our study highlights the need for more detailed studies for further understanding of cross-resistance, which might be helpful in designing effective treatment strategies in many clinical situations, especially in the case of drug cycling.

Theoretical and empirical studies suggest that synergistic and antagonistic drug interaction leading to positive and negative epistasis can also accelerate or slow down the evolution of multidrug resistance, respectively [9, 29]. We observed a varying degree of synergistic drug

interaction between kanamycin and rifampicin. This result indicates a good concordance with previous studies [17, 29], but differs from the expected epistasis [9], where we observed no epistasis. This result signifies the fact that evolution of multidrug resistance can be accelerated by synergistic drug interaction with non-epistatic fitness landscapes.

Although our fitness landscapes were largely non-epistatic, we further explored how the mutant selection window (MSW) was affected by different genotypes in the presence of multidrug environment. Previous studies have reported that the MSW can vary or remain static under two-drug antimicrobial environment [30, 31]. In our case, we observed extended MSWs ranging from minimal selective concentration (MSC) to the minimum inhibitory concentration (MIC). This wider MSW is implicated with resistance emergence such that some drug concentrations allowed wild-type susceptible bacteria to grow at very low concentrations of kanamycin and rifampicin combination. This clearly suggests that selection of resistance, multidrug resistance in particular, may occur at the MSC concentration of this drug pair. Similar observations was made by an earlier study in experimental bacterial population in presence of a single drug [32]. Wider MSW was observed for the kanamycin-streptomycin antibiotic combination, but a narrower MSW was observed for the rifampicin and streptomycin drug pair followed a traditional MSW. Overall, our results suggest that selection of resistance against multiple drugs may also occur at sub-MIC combination of two-drugs and/or in a situation when there is a cross-resistance imparted by a singly resistant genotype. The result we have presented here also supports the concept of minimal selective concentration (MSC) as has been suggested by an earlier study [32]. Overall, the results we have presented here may enhance our understanding of the multidrug resistance evolution under variety of drug environments. Our results together with previous study [33] suggest that resistance selection occurs at very low antibiotic concentration. Therefore, our study warrants the need for the revision of the traditional concept of mutant selection window, especially in the case of evolution of multidrug resistance.

Our results may be constrained by some factors such that we use only growth rate as the proxy for fitness, but we have not measured kill rates, which may be associated with epistasis beyond the MIC. Besides this, additional fitness components such as carrying capacity and growth rate at lag phase were not assessed which are also determinants of the maintenance of resistance. In one previous study, it has been shown that rifampicin resistant genotypes were beneficial with respect to growth rate but deleterious with respect to their carrying capacity despite their competitive superiority [20]. Our results also highlight the importance of studying epistasis

between multiple resistance mutations in various organisms to explore the pattern of epistasis across species belonging to both Gram positive and Gram negative, since previous studies, including ours were predominantly carried out in a limited number of bacterial systems of Gram negative origin.

In chapter 3, an experimental evolution experiment was employed where replicate populations of both naturally competent (Rec+) and non-competent (Rec-) *A. baylyi* was propagated by daily serial transfer in presence of two antibiotics used in combinations for ~650 generation. Following, evolved populations were systemically investigated to understand how a fully susceptible bacterial population gives rise to multiple drug resistance by recombination via natural transformation under sub-lethal concentration of rifampicin and streptomycin combination. While our phenotypic observation revealed a minor effect of adaptive potential of recombination via natural transformation under low selection pressure, but indistinguishable fitness between Rec+ and Rec- populations invalidated the benefit of recombination. We hypothesized that such uniform fitness between Rec+ and Rec- populations might be due to static selection pressure maintained throughout the serial transfer, which only affected selectively important regulatory genes of both Rec+ and Rec- populations such that resistance mutations arose and selected during the early phase of evolution and this has resulted in elevated fitness improvement irrespective of genetic competence. This idea was supported by one previous study, which reports selection of resistance at very low MIC [32]. In our study, this prediction was supported by the fold-MIC changes in antimicrobial susceptibility, which revealed that adaptation to both drugs that we used for the low and high drug treatments was strong enough to produce selection based benefit for all the populations, irrespective of competence or non-competence. Taken together, these results further suggest that under antibiotic combination, adaptation did occur, and it was primarily driven by (1) selection of resistance mutations against drug pairs that we applied, and (2) limited genetic diversity in the evolving population in response to these selection pressures.

It is assumed that recombination via natural transformation can accelerate bacterial adaptation with higher mutation rates or by decreasing mutational loads [34]. The mechanistic basis of high mutational load in the population is accomplished by bringing beneficial mutations (i.e., here different drug resistance mutations) from separate cells into a single genetic background. This phenomenon is called Fisher and Muller effect), this would increase the fixation rate of the recombinant cells [35]. Conversely it was suggested that competence could reduce the

fixation rate of the recombinant cells and eliminate them from the experimental population given that deleterious mutations would be naturally selected and integrated into a single genome alone or in combination by epistatic interaction, or the new mutations arising in the recipient strain would be replaced or corrected by wild type allele in the population. In the latter case, recombination event would be undetectable [36]. In our case, under antibiotic combination, we anticipated that Rec+ populations would potentially alter mutation fixation rate by bringing drug specific resistant mutations from separate cells into a single genetic background and increase the fixation rate of the recombinant cells conferring resistance to multiple drugs. However, after one hundred days of evolution our genome data revealed a lower abundance of substitution mutations compared to previous naturally transformable opportunistic pathogen *S. pneumoniae* [36], *H. pylori* [37], *Haemophilus influenza* [38], and *P. aeruginosa* [39]. Even in the presence of such low number of mutations, Rec- populations were enriched with high number of mutations. Large deletions and intergenic mutations were also different in both Rec+ and Rec- population. One plausible explanation for the high number of mutations in Rec- would be due to the disruption of competence associated genes, and this has likely help them acquire more mutations than Rec+ populations. On the contrary, Rec+ populations were enriched with high number of intergenic and deletion mutations than Rec- populations. Together these results also suggest that some of these mutations were naturally selected irrespective of recombination efficient and deficient populations, as has been the case in *P. aeruginosa* [52].

Mutation in *rpoB* locus was predominantly observed in both Rec+ and Rec- populations. Mutation in *rpoB* gene encoding RNA-polymerase β-subunit is widely responsible for resistance to rifampicin antibiotics [46, 55]. This high abundance of *rpoB* mutation correlates with elevated fitness gain during early phase of evolution – irrespective of Rec+ and Rec- populations. These results also support that *rpoB* mutation was fixed by natural selection at the early phase of evolution, and also suggest that the frequency of rifampicin resistance is higher than the streptomycin resistance. In addition to the *rpoB* mutation, we observed mutations in other genetic loci as has been found in clinically significant bacterial pathogens. Among these, some mutations have been found to be associated with fitness compensation of rifampicin resistance mutation [45, 46], while others contribute to the high level phenotypic resistance to multiple drugs, including common fluoroquinolone resistance [56-58]. Thus, we suggest that this high number of mutations in the populations evolved under stressful environments possibly played an adaptive process. Furthermore, high number of *rpoB* mutation indicates a

substantial parallel evolution, which means that natural selection acted upon this mutation in antibiotics induced selective environments. Genotypic parallelism has been documented in many experimentally evolved bacterial populations, including evolution of *P. aeruginosa* under ciprofloxacin antibiotic drug pressure [52]. Similar *rpoB* mutation in each replicate population line suggesting that the majority of parallel evolution was indeed due to the selection of drug specific resistance mutations.

We further attempted to determine to what extent low number of genomic mutations that we have observed is associated with the evolution and maintenance of natural transformation under sub-lethal concentration of rifampicin and streptomycin antimicrobials. Therefore, we explored transformation frequency (data not presented here) among Rec+ ancestral genotype of *A. baylyi* in identical selective environments, as has been used for the evolution experiment. Our result on the low frequency of tranformants cells in the populations across all three environments suggesting that the selective environment that we have used is independent of transformation frequency. Additionally, low number of transformants may also reflect the fact that different pathways involving DNA recombination process basically corrected the altered DNA strand, which occurs during taking up DNA under sub-lethal selection environments. Thus, we observed a low number of mutations or we were unable to detect the benefit of natural transformations in the endpoint populations. Such diminished transformation efficiency in *A. baylyi* was previously reported under variety of environments [40, 41]. In general, the genomic changes that we have identified were previously shown to potentiate resistance to multiple other antimicrobial compounds through targeted and off-targeted mechanisms [42, 43]. This has been reflected by the elevated level ciprofloxacin resistance after ~650 generations. High substitution rates were previously thought to be involved in simultaneous fixation of several mutations in presence of an antibiotic environment [44], our results here suggest that several drug specific resistance mutations can be fixed under sub-lethal combination of two drugs even in presence of low substitution rates. Overall, we observed no significant evidence that recombination by natural transformation facilitates adaptation to multiple antibiotics, presumably because the limited number of mutations that were spreading in the populations prevented clonal interference.

Under antibiotics induced selective environments, bacteria evolve resistance to particular antibiotics by acquiring drug specific genomic mutations (as has been shown in chapter 2 and 3) or by horizontally transferred genetic material carrying resistance determinants [27, 45, 46].

These mutational alterations or acquisition of new genomic determinants weaken or interfere with bacterial important physiological functions. And not surprisingly these interruptions are often associated with fitness cost, which has resulted in decreased survival, reduced growth rate, reduced transmission, and/or reduced virulence in pathogenic bacteria [3, 4, 47]. In absence of antibiotics, some of these resistance mutations disappear from the population or revert back to the original wild type state, or in some cases the deleterious effect of resistance mutations is reduced by so called second-site compensatory/suppressor mutations (CMs) at the same or different locus in the genome [48]. CMs are thought to be deleterious when they appear alone but beneficial when co-occurring with the original deleterious mutation thus representing a form of sign-epistasis. This is yet a poorly understood biological phenomenon, and has important implications not only for the evolutionary consequences of mutations but also for the genetic complexity of adaptation in many biological systems, including bacterial resistance to antibiotics [48]. The study conducted by Schrag and Perrot [49] provided direct evidence of the mechanism of compensation by evolving streptomycin resistant *E. coli* population in the absence of streptomycin which found 6% of fitness improvement by evolving second-site compensatory mutation. Since then, many other observations have been made by studying both clinical and laboratory populations, including in *Salmonella typhimurium* [50], *M. tuberculosis* [51], *Pseudomonas* species [52-54] and many more. In the mentioned studies, mechanisms of compensation were investigated only for single chromosomal resistance mutations. However, the mechanism of compensation of the additional mutations that have been frequently associated with multiple drug resistance is not explored properly. From a clinical viewpoint understanding of compensatory adaptation in multidrug resistant populations is crucial since resistant pathogens can stabilize inside patients by simultaneous adaptation to the cost followed by conservation of the resistance phenotype to the drugs. This particular scenario probably makes treatment very difficult to eradicate the pathogen from the patients by traditional antimicrobial therapy.

In chapter 4, we investigated this adaptive role of compensatory mutations by employing an experimental evolution experiment to explore the potential phenotypic and genomic basis of compensatory adaptation in resistant genotypes of both single and multidrug resistance origin. Specifically, we intended to explore some of the yet unknown questions in terms of the dynamics of mutational spectra, effect of additional deleterious mutations on compensation, or other genetic changes that arise during adaptive evolution. Uncovering these factors is important to determine whether evolutionary adaptation to the cost of multiple drug resistance

mutations generate any substantial genetic variations which differ from compensation to the cost of single drug resistance mutation.

Although the genetic locations of the evolved mutations were different, whole genome sequence data revealed an abundance of pathway specific secondary mutation, namely in *rpoB* locus in both rifampicin resistant genotype and the doubly resistant genotype suggesting a putative compensatory mutation. Secondary mutations in other non-specific loci, intergenic locations or truncation mutations in both single and double resistant genotypes also provided genetic basis of compensation since none of these mutations were detected in the susceptible genotype. Surprisingly whole genome sequencing revealed an identical reversion mutation in *cyoA* locus, which was an additional mutation harboured by multidrug resistance populations. This additional mutation also contributes to the elevated fitness compensation when coupled with putative compensatory or small effect beneficial mutation in costly resistance locus. This finding suggests, in terms of clinical relevance, that natural populations constantly facing different environmental perturbations inside the body host may acquire deleterious mutation. Therefore, the rate of compensation by reversion in these populations is high compared to the traditional laboratory reversion rate. We suspect that such additional mutation may have occurred in previous studies [50, 55-63] where mechanism of compensation was studied for a single drug resistance mutation with unexploited genomic information. Therefore, the true biological role of compensatory mutations in those studies may have been misrepresentative. However, this additional mutation may have profound effects on the rate of bacterial adaptation in absence of drug pressure, especially in the case where resistance to multiple drugs conferred by mutation at multiple loci in clinical populations. Another relevance for the inclusion of this genotype is that this mutation may have occurred during recombination process; therefore it is also possible that this type of mutations may frequently appear in dynamic host environment where bacteria frequently acquire resistant determinants by horizontal gene transfer.

In this study, putative compensatory mutations were not identified for the less costly streptomycin resistant genotype but were present in the costly rifampicin resistant genotype. Such trend also was apparent in double resistant genotype. Two important plausible reasons can be drawn: 1) *rpoB* acts as a global regulator for other genes and thus affect many different important cellular pathways. Therefore, any alteration in this global regulator entails significant fitness burden, 2) the simplest explanation for the absence of compensatory mutations to streptomycin mutation is presumably owing to the low fitness cost, or *rpsL* gene is more

restrictive because of the high fidelity translation process, thus only minor fitness cost conferring single mutation is accessible for streptomycin resistance but cannot accommodate more mutations in any ribosomal proteins but can improve fitness by acquiring small step secondary mutations in other loci, 3) similar explanation could be drawn for the double resistant genotype where we observe compensatory mutation in the *rpoB* locus, except one where *rpsD* compensatory mutation was also observed. This is due to the fact that *rpsL* mutation can cause streptomycin dependency, which means that streptomycin resistant genotype can grow better in presence of elevated concentration of streptomycin antibiotic, and secondary mutation in *rpsD* can release the streptomycin dependency and also can compensate for the fitness cost. Overall, we did not find any overlap across the three mutant backgrounds suggesting that genetic basis of compensation is independent of genetic background between single and multidrug resistant bacteria.

In conclusion, determining evolutionary routes towards antibiotic resistance is essential, and this will extend the successful use of antibiotics in real clinical situation. Among many factors, evolution of resistance can be predicted once we know about factors such as the mutation supply rate, the degree and level of resistance conferred by different resistance mechanisms, the fitness (i.e., growth rate and death rate) of the resistant bacteria in presence and absence of antibiotics, and the strength of selective pressures [3, 5, 64, 65]. Additionally, other factors such as epistatic interactions, compensatory evolution, co-selection of drug resistances, and population bottlenecks with clonal interference can strongly impact the resistance evolution [8]. Determining all these factors is challenging owing to the lack of quantitative data. However, this PhD thesis attempted to explore some of these factors involving the evolution of antibiotic resistance, multidrug resistance in particular. For example, our findings suggest that the sub-MIC antibiotic concentrations thought to be generated in certain body compartment during combination therapy may potentiate the evolution of *de novo* multidrug resistance, as has previously been reported for different single drugs [66, 67]. Another important aspect of this current study is the occurrence of cross-resistance [28]. We suggest that a wider MSW can also occur in presence of cross-resistance. Overall, our results underlies the importance of choosing the right drug during cyclic treatment, as well as using optimal treatment dosing regimes during combination therapy that exclude the prolonged exposure of sub-MIC level of antibiotics. In future more extension of our works would be necessary to pinpoint different factors implicating the evolution of de novo multidrug resistance in bacteria.

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