



Antibiotic Resistance

Adaptive Evolution & Dissemination of Resistance Genes

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Technical University
of Denmark

Antibiotic Resistance

Adaptive Evolution
&
Dissemination of Resistance Genes

PhD thesis
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January 2014

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Abstract

Bacteria can avoid extinction during antimicrobial exposure by becoming resistant. They achieve this either via adaptive mutations or horizontally acquired resistance genes. If resistance emerges in clinically relevant species, it can lead to treatment failure and ultimately result in increasing morbidity and mortality as well as an increase in the cost of treatment. Understanding how bacteria respond to antibiotic exposure gives the foundations for a rational approach to counteract antimicrobial resistance.

In the work presented in this thesis, I explore the two fundamental sources of antimicrobial resistance: (1) adaptive mutations and (2) horizontal acquisition of resistance genes from antibiotic gene reservoirs.

By studying the geno- and phenotypic changes of *E. coli* in response to single and drug-pair exposures, I uncover the evolutionary trajectories leading to adaptive resistance. I find, in contrast to a general assumption of independent responses, that there is a high degree of interactions between the evolutionary responses to the individual drugs, which is manifested in collateral changes in drug susceptibility. Specifically, I show that collateral

sensitivity can be exploited to rationally design drug combinations that limit the evolution of antibiotic resistance due to counteracting evolutionary trajectories. My results highlight that an in-depth knowledge about the genetic responses to the individual antimicrobial compounds enables the prediction of responses to drug combinations.

In the second study I focus on horizontal gene transfer as a way of achieving resistance. More specifically, I focus on gene acquisition from environmental reservoirs. The study investigates the resistance gene reservoirs in several wastewater treatment plants (WWTPs) sampled over a two years period. I find, that although the resistance gene reservoir is highly shared across different WWTPs, there is only a small overlap with resistance genes from other environments. This finding suggests, that there is a dissemination barrier preventing the spread of functional resistance genes across environmental niches.

Dansk resume

Bakterier som bliver udsat for antibiotika kan overleve ved at blive resistente. Det kan de enten opnå via chromosomale mutationer eller horisontal erhvervelse af resistensgener. Resistens i sygdomsfremkaldende bakterier er et omfattende problem som kan resultere i en uvirksom behandling med en øget morbiditet og mortalitet som konsekvens. For effektivt at kunne imødegå en stigende udfordring fra resistente bakterier, er det nødvendigt at opnå en detaljeret forståelse af de mekanismer der fører til resistens. I mit PhD-forløb har jeg studeret to centrale aspekter ved resistensudvikling: (1) adaptiv resistens og (2) horisontalt erhvervet resistens.

Ved at udsætte *E. coli* for enkel- og flerstof antibiotika og efterfølgende studere de geno- og fænotypiske responser, har jeg afdækket de evolutionære veje der fører til resistens i denne organisme. I modsætning til en generel forestilling om uafhængige responser, fandt jeg, at der er et stort overlap i de evolutionære responser som bl.a. manifesterer sig ved kollaterale ændringer i antibiotika overfølsomheden. Specifikt har jeg vist, at resistensudviklinger er stærkt nedsat overfor antibiotikakombinationer hvor responserne til komponentstofferne medfører kollateral sensitivitet. Mine resultater viser, at en detaljeret forståelse af resistensudvikling kan benyttes til rationelt at sammensætte antibiotikakombinationer, som er særligt velegnet til at forebygge resistensudvikling.

I mit andet studium har jeg fokuseret på resistensudvikling via horisontal erhvervelse af resistensgener. Mere specifikt, har jeg fokuseret på generhvervelse fra ikke-

humane miljøer. Studiet har undersøgt resistensgen-reservoirs i en række forskellige spildevandsrensingsanlæg samlet over en toårig periode. Jeg fandt, at selvom der var et omfattende og stabilt resistensgen-reservoir, var resistensgenerne fra spildevandsrensingsanlæg ikke delte med andre miljøer. Dette antyder, at der er en barriere som forhindrer omfattende genudveksling mellem bakterier fra forskellige miljøer.

Introduction

Entering the antibiotic era

In 1909 the German physician Paul Ehrlich discovered the first antibacterial compound¹. This marked the beginning of a new era where bacterial infections could be treated for the first time and cured with great success. Ehrlich had only a year earlier received the Nobel Prize for his work on immunology and differential cyto-staining, and using knowledge gained from this research he hypothesized that if he could develop dyes that selectively stained different cell types, he should also be able to develop compounds that selectively killed bacterial cells. This theory later got popularized as the “magic bullet” theory. Using a synthesis and screening approach he started looking for an anti-syphilis drug. He reasoned, that using a toxic arsenic compound as starting molecule he would be able to chemically “tune” the compound to become selectively toxic against *Treponema pallidum*, the cause of syphilis. Using a *T. pallidum* rabbit model the staff in his lab screened for active derivatives that successfully cured the rabbit. In 1909 this led to the discovery of the active compound 606, marketed only a year later under the name Salvarsan².

Inspired by this successful discovery, chemical companies and scientists began screening compounds for antibacterial activity, and in 1932 the German consortium of dye manufactures IG Farben discovered the first sulfa drug later to be marketed in 1935 under the name Prontosil^{2,3}.

Prior to these first successful developments of antibacterial compounds, the field of infectious medicine had undergone period of major discoveries. In 1861 Louis Pasteur proved that microorganisms do not arise spontaneously, a theory held by many of his colleagues². Using swan neck bottles he showed that preventing airborne microorganisms from settling directly into sterilized broth kept the broth sterile. As a direct consequence of Pasteur’s work, the British surgeon Joseph Lister started to experiment with the use of antiseptic coated linens to shield surgical sites during surgery. Lister’s work quickly proved effective at reducing surgical wound infections and marked the beginning of antiseptic surgery. Then, in 1884, following Roberts Koch’s discovery of the tubercle bacilli as the cause of Tuberculosis, he together with another German physician, Friedrich Loeffler, formulated their famous postulates for establishing a causal relationship between the presence of bacteria and a disease. Taken together, these discoveries that bacteria are the cause of disease and that they can spread via air (or other vehicles) paved the way for modern day clinical bacteriology².

Natural compounds

In 1928, while the chemical industry was busy screening synthetic libraries, the Scottish biologist Alexander Fleming made his famous discovery of a penicillium mold producing an antibacterial compound. Fleming named the compound penicillin⁴. This discovery inaugurated the period of natural compound discovery, later proving to be the most successful source of antibacterial agents (Figure 1)⁵. Despite realizing the medical potential of the compound, Fleming was unable to purify the active substance. It was not until the early 1940s when Ernest Chain, a biochemist working in the lab of Howard Florey at Oxford, managed to produce penicillin in sufficient quantities to test it, that the enormous potential of penicillin was realized^{2,4}. Today, penicillin and its derivatives still make up the backbone of the antibacterial armory. Following the discovery of penicillin, other scientists began to search for producer organisms of antibacterial agents. The first major breakthrough in this process happened in 1943, in the lab of Selman Waksman, where the graduate student Albert Schatz discovered streptomycin. Like many successful antibiotics later to be discovered, this drug was produced by bacteria belonging to the genus *Streptomyces*, hence its name⁵. Streptomycin was the first drug with activity against *Mycobacterium tuberculosis*, the cause of tuberculosis (TB), making it an extremely important antibiotic. Furthermore, streptomycin was also the first pharmaceutical drug to be evaluated in a randomized controlled double-blinded trial. In this

Timeline of antibiotics



Figure 1. Timeline of antibiotics
a) Time of production
b) Commonly used in combination with sulfamethoxazole

historical trial, the British Medical Research Council pioneered clinical trials and showed that streptomycin was more effective against tuberculosis than the standard treatment of bed rest⁶.

In the following 40 years many new antibacterial agents were discovered. Most of these were identified via massive screening projects in which thousands of microorganisms were screened for the production of antibacterial molecules, and while chemical modification of natural compounds has led to numerous successful semi-synthetic derivatives, remarkably few completely synthetic compounds have been developed (Figure 1).

The decline of the antibiotic era

In response to the increased use of antibacterial compounds throughout the 20th century, bacteria have evolved ways to overcome the effect of antibiotics and thereby become resistant. Consequently, previously effective drugs no longer cure infections, resulting in increasing morbidity and mortality^{7,8}. The prevalence of antibiotic resistance varies greatly from species to species and across geographical location. However, for certain species previously effective drugs have almost completely lost their clinical applicability. For instance, *Staphylococcus aureus*, which was initially sensitive to penicillin and ampicillin, is now almost completely resistant to these drugs. In many parts of the world the high prevalence of methicillin resistant *S. aureus* (MRSA) also makes beta-lactamase resistant derivatives such as dicloxacillin or nafcillin obsolete^{9,10}. Likewise with common the uropathogen, *Escherichia coli*, that was previously sensitive to ampicillin and sulfa drugs, close to half of the isolates are now resistant⁹. Fortunately there are still alternative antibiotics that remain effective against these bacteria. However, resistance against these “last resort” drugs is regularly being reported^{11,12}.

Further complicating the situation is the gap in the time between the initiation of empirical treatment and the lab result from resistance tests. Consequently, as resistance becomes more prevalent, more patients will receive an ineffective empirical treatment and therefore experience a worsening of their illness. An example is ciprofloxacin resistance in *E. coli*, which is now greater than 10 % in many countries. This broad spectrum orally available drug is a popular alternative to beta-lactam drugs, especially with penicillin allergic patients, but its usefulness is being undermined by an increasing prevalence of resistance. This case exemplifies how the extensive use (and misuse) of an antibiotic can quickly reduce its applicability.

Although most infections can still be treated successfully with antibiotics, there is a need for the development of new and safe antibiotics. Such development takes many years and should therefore be initiated while resistance is still manageable. Over the years, the scientific communities have increasingly been trying to make politicians and other decision makers aware of the lack of the development of new antibiotics^{13,14}. At the same time, physicians at hospitals around the world are beginning to encounter pan-resistant bacteria such as multiple resistant enterobacteriaceae and extensively drug resistant tuberculosis (XDR-TB). Resultantly, drugs that were abandoned long ago due to high toxicity are now being reintroduced as “last resort” drugs¹⁵⁻¹⁷.

However, resistance is not always emerging as fast as in the cases highlighted above. For more than 60 years, *Streptococcus pneumoniae*, the most common cause of pneumonia, has been successfully treated with penicillin, and still penicillin resistance in *S. pneumoniae* is a relatively rare phenomena^{9,12}. These differences in resistance development emphasizes, that understanding the factors that drive the evolution of resistance in different organisms, is key to developing strategies to counteract emergence of resistance.

Antibiotic classes and their targets

The term antibiotic broadly refers to chemical entities that selectively inhibit microorganisms. Yet in everyday jargon, it is commonly used to denote antibacterial agents. However, even when considering this narrowing of the definition, the term antibiotic still covers a wide range of different chemical compounds. These compounds can be organized in many different ways depending on the relevance of the different properties that characterize them. The properties most commonly used to characterize antibiotics include their structure, molecular mechanism of action, pharmacodynamics, pharmacokinetics, target spectrum, potency, bioavailability, toxicity and price. In this section, the main focus will be on giving an overview of the chemical classes and the cellular target of selected antibiotics. However, it is important to emphasize that knowledge about the other properties is essential to understand the utility of a given antibiotic in a clinical setting.

The chemical structure and class of antibiotics are closely connected parameters, and most new structures are the result of modifications of known and well-tested drugs scaffolds. At the fundamental level, the different classes of antibiotics can be separated into synthetic, natural and semi-synthetic classes, where the latter is the result of modifying naturally occurring structures. Hence semi-synthetic antibiotics often represent derivatives of existing

drugs normally with improved properties such as increased spectrum, potency, half-life or bioavailability. Table 1 lists some of the most important antibiotic classes used in human medicine along with representative drugs for each class.

Antibiotic class	Example	Target
Beta-lactam	Penicillins (ampicillin), Cephalosporins (cefotaxime), Carbapenems (meropenem)	Cell wall synthesis
Quinolone	Nalidixic acid, Ciprofloxacin	Gyrase / topoisomerase IV
Aminoglycoside	Streptomycin, Gentamicin, Amikacin	30S ribosomal subunit / cell membrane
Macrolide	Erythromycin, Azithromycin	Peptide exit tunnel in 50S ribosomal subunit
Tetracycline	Tetracyclin, Tigecycline	tRNA binding in 30S ribosomal subunit
Oxazolidinones	Linezolid	Peptidyl transferase center in 50S ribosomal subunit
Phenicol	Chloramphenicol	Peptidyl transferase center in 50S ribosomal subunit
Licosamide	Clindamycin, Lincomycin	Peptide exit tunnel in 50S ribosomal subunit
Sulfonamides	Sulfamethoxazole	Tetrahydrofolate synthesis
Benzylopyrimidine	Trimethoprim	Tetrahydrofolate synthesis
Rifamycin	Rifampicin	RNA polymerase
Nitroimidazoles	Metronidazole	General DNA damage
Nitrofurans	Nitrofurantoin	General DNA damage
Lipopeptide	Daptomycin	Cell membrane
Glycopeptide	Vancomycin	Cell wall synthesis

Table 1. Overview of selected antibiotics
The table presents selected antibiotic classes and their target

Aside from the different classes of anti-TB drugs, the most important drug class is the beta-lactam class. This class can be divided into the penicillin, cephalosporin, carbapenem and mono-bactam groups with each group encompassing many different compounds (Table 1)¹⁸. Common for all antibiotics belonging to the beta-lactam class is their beta-lactam core consisting of a four-membered cyclic amide (lactam). All beta-lactam drugs exert their antibacterial effect by inhibiting cell wall synthesis. Specifically, they bind to penicillin binding proteins (PBPs) and inhibit the trans-peptidase activity that normally cross-link penta-peptides in the bacterial cell wall peptidoglycan. Beta-lactam drugs are in most cases bactericidal, but require the bacterial cells to be actively dividing to exert their antibacterial effect. Due to the lack of peptidoglycan in human cells, they generally display low toxicity making them a one of the most preferred drug classes¹⁸.

Another important drug class is the quinolones. This class of synthetic antibiotics consists of four generations of drugs including ciprofloxacin (2. gen.), levofloxacin (3. gen) and

moxifloxacin (4. gen.). The quinolones have a high orally bioavailability, good tissue penetration and a broad spectrum of activity. The latter is especially true in the third and fourth generations which are also active against *Streptococci* spp.. Quinolones exert their antibacterial effect by inhibiting the gyrase enzyme, which is essential for replication of the genome. Like the beta-lactam drugs, they are bactericidal but require active cell division to kill¹⁸.

The aminoglycoside represent another bactericidal class of antibiotics, however, their clinical applicability is limited due to adverse side effects. All though their binding site in the 30S ribosomal subunit is well studied, this class of drugs has a complicated mechanism of bactericidal action involving induction of protein mistranslation and reduced membrane integrity¹⁹.

The bacteriostatic drugs include a broad range of drug classes that reversibly inhibit mRNA translation by interfering with essential sites on the ribosome. Of particular importance are the tetracyclines that interfere with the tRNA docking in the 30S subunit, the phenicols, lincosamides and oxazolidinones that inhibit the peptidyl transferase reaction in the 50S subunit, and the macrolides that block the protein exit tunnel also in the 50S subunit²⁰. Especially the latter class has clinical relevance, as it is useful in the treatment of *Mycoplasma pneumonia* and to treat intracellular pathogens such as *Chlamydomphila*¹⁸.

The last important antibiotic classes are the two classes of tetrahydrofolate (THF) inhibitors: the sulfonamides and trimethoprim. Both are widely used, especially in countries outside Denmark, where mecillinam is not available to treat uncomplicated urinary tract infections. The THF-inhibitors also have great historical importance, as it was some of the first safe and effective antibiotics to be developed. They are often used in a combination, as their step-wise inhibition of the THF pathway is very synergistic¹⁸.

Antibiotic resistance

Traditionally microbiologists divide antibiotics resistance into two categories: intrinsic resistance and acquired resistance^{21,22}. The former refers to the situation where a bacterial species is unaffected by an antibiotic due to its fundamental physiological properties e.g. beta-lactam resistance in *Mycoplasma* spp. due to this genus' lack of cell wall, or vancomycin resistance in enterobacteriaceae due to the outer membrane of Gram-negative

species. In contrast, acquired resistance refers to the situation where a bacterium that *used* to be susceptible to an antibiotic at a given concentration is no longer inhibited at that concentration. For all practical purposes, only the relative form of antibiotic resistance, i.e. acquired resistance, has important clinical implications. This is because the emergence of resistance renders previously effective treatments useless, resulting in increased morbidity and mortality, especially in the transition phase where resistance is too low to motivate a change in the empirical treatment.

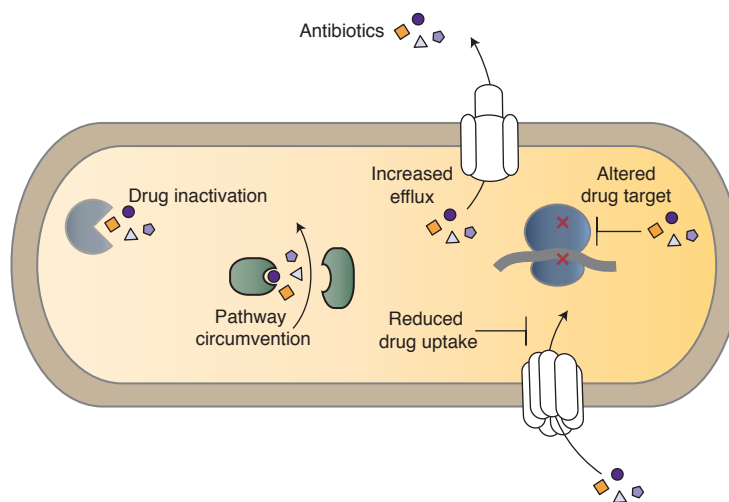


Figure 2. Mechanisms of antibiotic resistance
Schematic presentation of the common mechanisms behind antibiotic resistance.

Acquired resistance can be further divided into horizontally acquired resistance and mutational acquired resistance. Horizontally acquired resistance refers to the situation where resistance emerges as a result of horizontal gene transfer (HGT), commonly in the form of plasmid conjugation, phage transduction or non-specific DNA uptake. In contrast, mutational acquired resistance occurs when the bacterial genome mutates to overcome the effect of an antibiotic and normally only involves the change of one or a few nucleotides. Both horizontally and mutational acquired resistance play a major role in clinical relevant resistance, however, an in depth understanding of mutational resistance has until recently been unfeasible due to limited sequencing capabilities. With the advent of high-throughput sequencing platforms this situation is now changing. It is now possible to sequence bacterial

genomes at a large scale and identify single nucleotide polymorphisms (SNPs) across sensitive and resistant isolates²³⁻²⁵. All though this technique is not yet applied routinely, future projects studying the evolution of resistance by whole genome sequencing will without doubt give important insight into the dynamics of resistance evolution.

Antibiotic resistance mechanisms

From a mechanistic point-of-view, resistance can be divided into five categories: drug-target alterations, reduced drug uptake, increased drug efflux, circumvention of drug target essentiality and drug inactivation (Figure 2)²².

Drug target alterations can either occur at the gene-level, i.e. as mutational acquired resistance, or post-transcriptionally/post-translationally via different modification enzymes such as methyltransferases. Target modifications usually impose structural changes in RNA (commonly rRNA or tRNA) or proteins, an exception being the changes in the terminal amino acid in the peptidoglycan structure leading to vancomycin resistance.

Reduced drug uptake is primarily observed in Gram-negative species and is commonly caused by reduced permeability of the outer membrane. This is usually the result of reduced expression of the outer membrane porins (OMPs), caused either by mutations in the OMP genes or in regulatory genes that control the OMP expression²⁶. Reduced uptake has been reported to augment the effect of ESBL resistance enzymes, thereby leading to carbapenem resistance^{27,28}. This highlights how multiple resistance mechanisms can act jointly to increase the spectrum of resistance. Furthermore, the combination of multiple resistance mechanisms makes it very difficult to uncover the individual resistance contributors, especially in routine diagnostics.

Like target alterations, increased efflux can be the result of both mutational and horizontal acquired resistance. In the case of mutational resistance, the mutations are usually located in regulatory genes resulting in up-regulation of housekeeping efflux pumps. Alternatively, mutations within the efflux pumps can change its substrate spectrum making it more promiscuous and thereby enabling the pump to expel antibiotics²⁹. Bacteria can also horizontally acquire novel resistance efflux pumps, such as the well studied TetA tetracycline efflux pump^{30,31}.

Circumvention of the drug target essentiality leaves the cell unaffected by the inhibitory

effect of the antibiotic. This resistance mechanism is by nature always horizontally acquired and probably the best example is the acquisition of penicillin binding protein 2a (PBP2a) in *S. aureus* that results in the MRSA phenotype. This PBP has a very low affinity for beta-lactam antibiotics and while the beta-lactam antibiotics still inhibit housekeeping PBPs, this inhibition does not have an effect as the PBP2a complements their function²¹. Other examples of target circumvention include circumvention of THF inhibition via acquisition of enzymes insensitive to THF inhibitors. In this case, the acquired enzyme is often a mutated version of a housekeeping enzyme that is spreading via HGT³².

Drug inactivation is a common resistance mechanism, involving either degradation or modification of the antibiotic. It is most commonly the result of HGT. However, it can also occur via up-regulation of housekeeping enzymes, as for instance in the case of up-regulation of *ampC* leading to beta-lactam resistance. Currently one of the biggest resistance problems challenging the successful treatment of infections is the spread of extended spectrum beta-lactamases (ESBL)³³. These enzymes inactivate most beta-lactam drugs by hydrolyzing the beta-lactam core³⁴. Another resistance challenge is the aminoglycoside modifying enzymes. These enzymes modify the aminoglycoside structure by add phospho, acetyl or nucleotidyl groups to the aminoglycoside drugs, thereby reducing the drug affinity for the 30S ribosomal target³⁵. These examples highlight the breadth of resistance mechanisms that function via inactivation of an antibiotic.

In any cases of resistance, whether it is mutationally or horizontally acquired, the clinical relevance of the phenotype depends highly on the degree of dissemination. If a specific resistance mechanisms is only observed in a few cases, it might be interesting from a molecular point-of-view, however, its clinical importance is negligible. Therefore, it is important to realize that surveillance of antibiotic resistance is key to prioritizing research and improving efforts of overcoming the challenges of antibiotic resistance. Currently such surveillance is based on phenotypic screening, but as technologies advance, this will hopefully be augmented with whole genome sequencing, enabling scientists to study the genetics of resistance dissemination.

Thesis investigations

When a bacterial population is exposed to an antibiotic, the resistant members will keep dividing and ultimately take over the population. However, this view on the emergence of antibiotic resistance assumes that the population contains resistant members. Resultantly, the question of how these resistant members emerge is highly relevant.

In the case of mutational resistance, it is fair to assume that the probability of a population containing a resistant member is proportional to the number of mutations required for resistance and the size of the population. If it is further assumed that resistance against a specific class of antibiotics requires a specific set of mutations, then more mutations are required to achieve multi-drug resistance. This reasoning has led to the simultaneous use of multiple antibiotics, to reduce the risk of resistance emerging.

When the emergence of antibiotic resistance occurs via HGT, it is fair to assume that the probability of acquiring a resistance gene is proportional to concentration of resistance genes and the microbial density of the environment.

In my thesis work I have investigated both of these sources of antibiotic resistance in an attempt to get a more detailed picture of the underlying mechanisms affecting the emergence of antibiotic resistant bacteria. The results are presented in two separate sections. In section one I investigate the relationship between the use of drug-combinations and the emergence of resistance, and in section two I investigate the overlap in resistance gene reservoir between different environments.

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Section 1

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Evolutionary responses to antibiotic combinations

Introduction to section 1

Drug combinations

Drug combinations are routinely used in the treatment of infectious diseases, as for instance in the treatment of HIV or TB¹⁻³. In both cases, the argument for using multiple drugs is to reduce the emergence of resistance. In a similar fashion, multidrug treatment is also used to reduce the emergence of resistance during treatment of certain types of cancer⁴. Common for these otherwise very different diseases is the fact that they require long term or, as in the case of HIV, life long treatment with an antibiotic or chemotherapeutic agent. The long treatment duration greatly increases the risk of resistance development, as it allows for longer periods of sub minimal inhibitor concentration (MIC) selection, which effectively selects for resistant mutants. Therefore, assuming that there is no overlap in the resistance mutations, the simultaneous use of multiple drugs will reduce the development of resistance⁵.

While this approach to reducing resistance has proven very successful in reducing resistance during long-term treatment regimes, it is generally not used for shorter regimes.

The two other main arguments for using combinations of antibiotics are increased spectrum of target organisms and increased potency⁶. In the case of the combination of an aminoglycoside and a beta-lactam, which is routinely used to treat bloodstream infections, both increased spectrum and in vitro synergy are used as arguments for the use of this combination. However, clinical trials have failed to show superiority of this combination treatment over mono-therapy with a beta-lactam antibiotic^{7,8}. These findings highlight the importance of randomized controlled trials to bridge laboratory findings and clinical practice. Another widely used combination is the combination of a penicillin drug plus a beta-lactamase-inhibitor. This combination re-sensitizes bacteria that produce beta-lactamase-inhibitor sensitive beta-lactamases, which include the common CTX-M ESBL enzymes⁹. Also commonly used, is the combination of trimethoprim plus sulfamethoxazole. This combination exhibits great in vitro synergy, however, clinical trials have again not convincingly shown an increased efficacy of the combination over mono therapy with trimethoprim¹⁰.

Studies of drug combinations

With the increase in multidrug and broad-spectrum resistant bacteria there has been a renewed interest in the possibilities of overcoming resistance by using combinations of antibiotics, often as higher-order combinations¹¹⁻¹³. This has resulted in much laboratory-

based research being published, with very little clinical follow-up research to sustain in vitro findings¹⁴⁻¹⁶. However, the interest in drug combinations has also initiated more research into the fundamental phenotypic and genotypic responses to antibiotics¹⁷⁻¹⁹. Particularly the study of epistatic drug interactions has been the focus of many studies²⁰⁻²³.

Epistatic interactions basically describe the phenomena where one component in a system is modulated by another component in the system. In the case of antibiotics, synergy and antagonism between drugs are examples of epistasis^{13,24}. Synergy describes the situation where the effect of a drug-combination is greater than the combined effect of the individual drugs. In other words, the presence of one drug potentiates the effect of the other drug. In a similar fashion, antagonism describes the situation where the effect of a drug-combination is less than the combined effect of the individual drugs. Depending on the prior assumptions about drug interactions, the non-epistatic interaction can either be additive or non-interacting. Assuming additivity, the non-epistatic interaction is the situation where the effect of a drug combination is equal to the combined effect of the individual drugs. In contrast, if no interaction is assumed, the non-epistatic state is the situation where the effect of a drug combination is equal to the effect of the most potent single agent¹³.

Measuring drug interactions

Different models are used to assess the degree of drug epistasis, which makes comparison across studies difficult. However, what is common to all models is that they assess epistasis by comparing the inhibitory capacity of a drug-combination to the inhibitory capacity of the individual drugs²⁴. Normally, the inhibitory capacity is expressed as the amount of drug required to inhibit the growth of a target organism relative to a no-drug control, although sometimes it may also be quantified by the relative reduction in growth rate. A commonly used measure of drug activity is the amount of drug required to inhibit bacterial growth by 90 % relative to a no-drug control after a fixed incubation time. This measure is normally referred to as the IC90. Experimentally, the IC90 can be derived from dose-response curves obtained by inoculating the target organism into a drug gradient.

Two models are commonly used to assess the interactions between antibiotics: the Bliss independence model and the Loewe additivity model²⁴⁻²⁷. They differ fundamentally in their prior assumptions about drug interactions and it is therefore important to consider the nature of the interactions before choosing a model.

The Bliss independence model assumes that drugs act independently of each other.

Therefore, in a combination of two truly independent acting drugs, a and b, the effect of drug b will be exerted on the fraction that drug a is not affecting, more generally this can be described as:

(1): $E(a, b) = E(a) + (1 - E(a)) * E(b)$, where E is the inhibitory effect of a drug relative to a no-drug control

In other words for the non-epistatic case, the effect of the combination of drug a and b is equal to the effect of drug a plus the effect of drug b on the fraction unaffected by drug a.

Often (1) is rewritten as:

(2): $E(a, b) = E(a) + E(b) - E(a)*E(b)$

Deviations from this equation indicate drug epistasis, which can be characterized as either synergistic $E(a, b) > E(a) + E(b) - E(a)*E(b)$ or antagonistic $E(a, b) < E(a) + E(b) - E(a)*E(b)$.

Another model, generally considered to be more applicable to the study of antibiotic interactions, is the Loewe additivity model^{24,25}. In contrast to the Bliss independence model, this model assumes that drugs acts in an additive fashion such that the effect of a combination of drugs, a and b, can be described as the sum of the fractional effect of the individual drugs:

(3): $E(a, b) = ICx(a)ab / ICx(a)a + ICx(b)ab / ICx(b)b$, where ICx is the drug concentration required for x % inhibition relative to a no-drug control.

That is to say, if the drugs a and b have no epistatic effect on each other, then the effect of a combination of drug a and b will be the sum of the fractional inhibition of each drug in the combination relative to the single drug. The effect of the drug-combination and the single drugs is reported at a given effect level (ICx) e.g. IC90. Commonly, the result of using the Loewe additivity model is termed the fractional concentration inhibitory index (FICI). Consequently, for additive drugs $FICI = 1$, while for synergistic drugs $FICI < 1$ and for antagonistic drugs $FICI > 1$.

An important difference between the two models is that the Loewe additivity model assesses epistasis at a given effect level e.g. IC₉₀, whereas the Bliss independence model assesses epistasis at given drug concentrations. Consequently, the Loewe model requires a dose-response curve while the Bliss model only requires single concentration points.

It is important to emphasize, that if the drugs do not fulfill the independence assumption, estimations of epistasis using the Bliss independence model will not be valid as it overestimates the degree of synergy between drugs. This point is best illustrated by considering the scam experiment in which drug a and b are identical. In this situation a combination of drug a and b will be highly synergistic according to the Bliss independence model, which is clearly wrong. Conversely, according to the Loewe additivity model, such scam combination would be correctly identified additive.

Resultantly, assuming independent actions is erroneous when assessing combination of drugs that belong to the same drug class, e.g. different beta-lactam drugs. Likewise, it seems difficult to uphold an argument of independent action for drugs that have the same target, e.g. drugs that target the ribosome. Therefore, Loewe additivity is generally accepted as a better model for assessing antibiotic drug interactions.

A new view on synergistic combinations

Traditionally, research into interactions between antibiotic drugs has focused on identifying synergistic combinations^{16,24}. The reason for this being, that increased potency, conferred by the synergy, would improve treatment outcome and, in the case of resistance, might serve as a way to overcome resistance.

Recently, the search for synergy has been questioned by a number of studies showing that synergistic combinations accelerate evolution of resistance while antagonistic combinations reduces evolution of resistance^{20,21}. The rationale behind this observation hinges on the idea that as resistance develops against one drug in a drug combination, the epistatic drug interactions are lost. Consequently, for a synergistic combination there will be a strong evolutionary pressure selecting for resistance, as this would ameliorate the drug synergy. Conversely, for antagonistic combinations, selection for resistance is reduced, as it would lead to a loss of drug interactions, resulting in increased drug potency. These findings leads to the paradoxical situation in which increased potency, due to synergy between antibiotics, comes at the price of increased resistance development. Hence, these studies highlight that antagonistic combinations should be preferred over synergistic due to their ability to reduce

resistance.

A key weakness of the studies is that they are based on sub-MIC measurements. From a clinical perspective, resistance is referred to an increase in the MIC relative to the wild-type MIC. Hence, the translation of sub-MIC experiments to a clinical scenario may be difficult. Resultantly, more in-depth studies of the impact of epistatic drug interactions on the evolution of resistance increase above the wild-type MIC resistance is warranted. In addition, potential collateral impacts of the resistance development are not accounted for in these studies.

Collateral impact of resistance

Collateral impact of resistance is manifested as either collateral resistance or collateral sensitivity, the former being the situation where resistance to one drug also confers resistance to other drugs, while the latter describes the situation where resistance to one drug increases sensitivity to other drugs. Szybalski and Bryson pioneered the study of these phenomena in the early 1950s, of which particularly collateral resistance has been described in many studies of adaptive resistance²⁸⁻³⁰. More recently, there has been an increased interest in collateral sensitivity as a mean to overcome or reduce resistance development. Large scale adaptive evolution studies have shown that the phenomena is common and that collateral sensitivity can be exploited to re-sensitize resistant populations by applying a drug cycling regime^{17,19}. Moreover, collateral sensitivity has been demonstrated in both bacteria, virus and cancer cell lines, suggesting that general principles can be developed for the exploitation of the phenomena to overcome resistance³¹⁻³⁴.

Thesis work

In my work I have studied the adaptive responses of *E. coli* to five antimicrobial drugs and all possible pairwise combinations of these. In contrast to previous studies of responses to drug combinations, which are primarily based on sub-MIC adaptations, all adaptation experiments in the present work are conducted in antibiotic gradients with the specific aim to measure how combinations affected increase the MIC relative to single drug exposure (Figure 3). Both the genotypic and phenotypic responses were studied using full genome sequencing and comprehensive MIC testing. This resulted in a detailed analysis of the evolutionary responses during drug exposure.

I find that during adaptive resistance evolution against drug-combinations, the collateral impact has a major influence on the rate of resistance development. Specifically I find that the responses to the single-drug adapted lineages can be used to predict the responses to the drug-pairs.

Furthermore I find no correlation between the epistatic drug interactions and the evolution of resistance as measured by increasing MIC (Figure 4). These results highlight that during adaptive evolution of resistance to drug-combinations, the collateral MIC changes have a major impact on the resistance development, while the effect of the epistatic drug

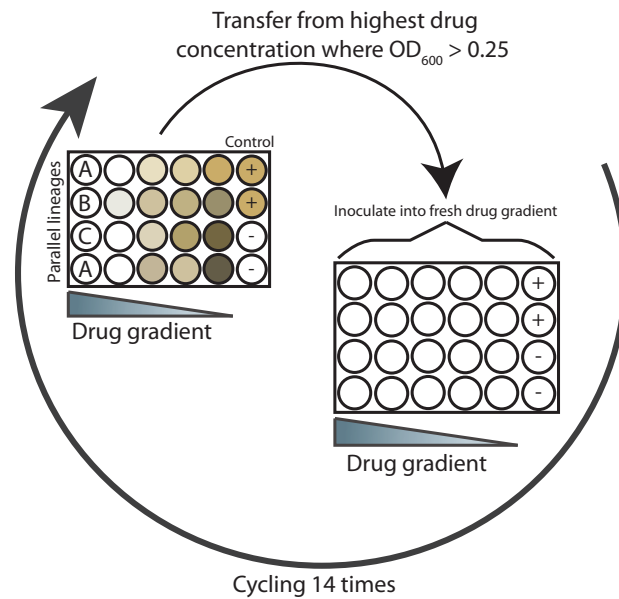


Figure 3. Overview of the experimental setup. The in vitro evolution experiment was conducted in 24 well plates with drug gradients across the columns. The last column contained positive and negative controls. Every 20 h cells were diluted 1:40 from the highest drug concentration where the OD₆₀₀ was greater than 0.25 into a freshly prepared drug gradient. The rows contained the parallel-evolved lineages.

interactions on the evolution of resistance is limited. My findings show that the ability of drug combinations to prevent evolution of resistance is different for sub- and increasing MIC adaptations. In the case of evolution of clinical relevant resistance, which by definition is above the wild-type MIC, my results suggests that resistance preventing drug combinations can be designed by studying the collateral impact of single drug evolved strains.

In a parallel experiment, my colleague Mari Evgrafov headed an investigation of MIC adaptation in *Staphylococcus aureus*. She exposed *S. aureus* to three different drug combinations; ciprofloxacin + ampicillin, amikacin + fusidic acid and erythromycin + fusidic acid, as well as their component drugs. These drug-combinations represent additive,

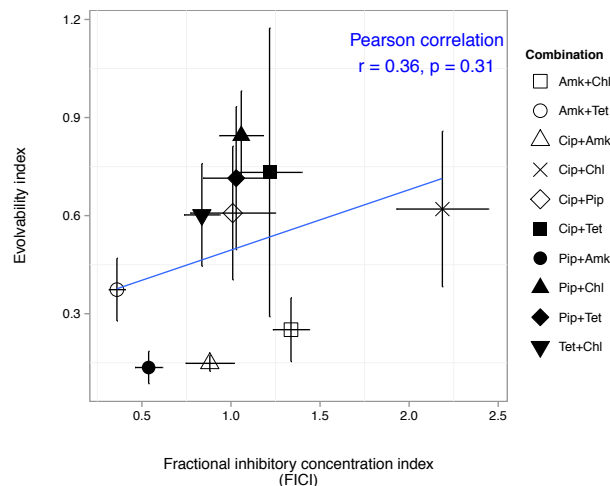


Figure 4. Correlation between epistatic drug interactions and the evolution of resistance. Epistatic drug interactions are measured using the Loewe additivity model, resulting in a FICI. Evolution of resistance is measured as the increase in MIC of the individual drugs in the drug-pair exposed lineages relative to the MIC increase of the single-drug exposed lineages (mean \pm s.e.m, n = 3 biological replicates).

antagonistic and synergistic interactions, respectively²².

The study confirmed that epistatic drug interactions do not predict evolution of increased MICs to drug combinations. Furthermore, it corroborated that collateral changes in MIC in response to single drug adaptation predicted evolution of resistance during drug combination adaptation. Importantly, this study used a different organism and different drug combinations yet found the same overall principles dictating the evolution of increased MIC during drug-combination adaptation.

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Manuscripts
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Section 1

Evolutionary interactions between environmental selection pressures drive phenotypic evolution

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For a population to avoid extinction during environmental change it must be able to adapt or evolve phenotypes that allow survival under the new condition. The success of this process is closely connected with the magnitude and the complexity of the environmental perturbation as survival rates decline with increasing selection pressure and selection complexity^{1,2}. This paradigm provides the basis for the use of drug combinations to reduce resistance development during treatment of for instance tuberculosis, HIV and cancer³⁻⁵. Traditionally, the ability of multi-component environmental perturbations to reduce fixation of mutations has been viewed as the result of having to acquire multiple independent mutations². Yet, we propose that evolutionary interactions between environmental perturbations play a key role in determining the rate of phenotypic evolution. Here we show, that the ability of a multi-component perturbation to prevent fixation of mutations can be predicted from the evolutionary responses to the constituent perturbations. By studying the phenotypic and genotypic evolution of *E. coli* during single- and dual-component perturbations we found, that combinations of perturbations for which the responses to the individual perturbations conferred collateral sensitivity, significantly reduced the evolution of otherwise beneficial genotypes. Notably we found, that the dual-component perturbation with the highest degree of collateral sensitivity almost completely prevented fixation of the canonical mutations found in the single-component evolved lineages. These findings highlight, that the ability of a multi-component perturbation to prevent fixation of mutations is not only the result of a lowered probability of simultaneously evolving along multiple individual trajectories, but instead a complex process where evolutionary interactions play a major role in determining the rate of

adaptation. The results provide a framework that could contribute to rational design of drug combination treatments that reduce evolution of drug resistance.

In order to investigate how multi-component perturbations affect evolutionary trajectories relative to single-component perturbations we used an *E. coli* based model system. We characterized the genotypic and phenotypic responses of *E. coli* to 5 different antibiotics (amikacin (Amk), ciprofloxacin (Cip), piperacillin (Pip), tetracycline (Tet) and chloramphenicol (Chl)) and all possible pairwise combinations, in total 15 different perturbations. Antibiotics were chosen as the environment perturbation since it facilitated a quantitative assessment of the degree of phenotypic evolution by measuring the relative increase in the 90 % inhibition concentration (IC₉₀) (Supplementary Data 1). Additionally, the genotypic responses underlying the resistance phenotype could be readily identified due to the significant knowledge on antibiotic resistance mutations in *E. coli*. Triplicate evolution experiments were performed for each condition by daily passaging the 45 separate lineages into increasing antibiotic concentrations selecting for increasing resistance. To investigate how dual environmental perturbations affect the phenotypic evolution relative to single perturbations, we measured the IC₉₀ of all the evolved lineages and calculated the increase in IC₉₀ relative to the ancestral wild type IC₉₀ (Fig. 1a-e). In contrast to the general expectation, many dual-perturbations did not significantly reduce the relative phenotypic evolution compared to single-perturbations (Fig. 1c-e). However, for combinations containing amikacin or ciprofloxacin, the presence of any of the other four drugs significantly reduced the relative phenotypic evolution (Fig. 1a and b). This effect could either be caused by one of the components acting as a ceiling factor in the dual-perturbation scenario or by a genuine repression of the phenotypic evolution of both

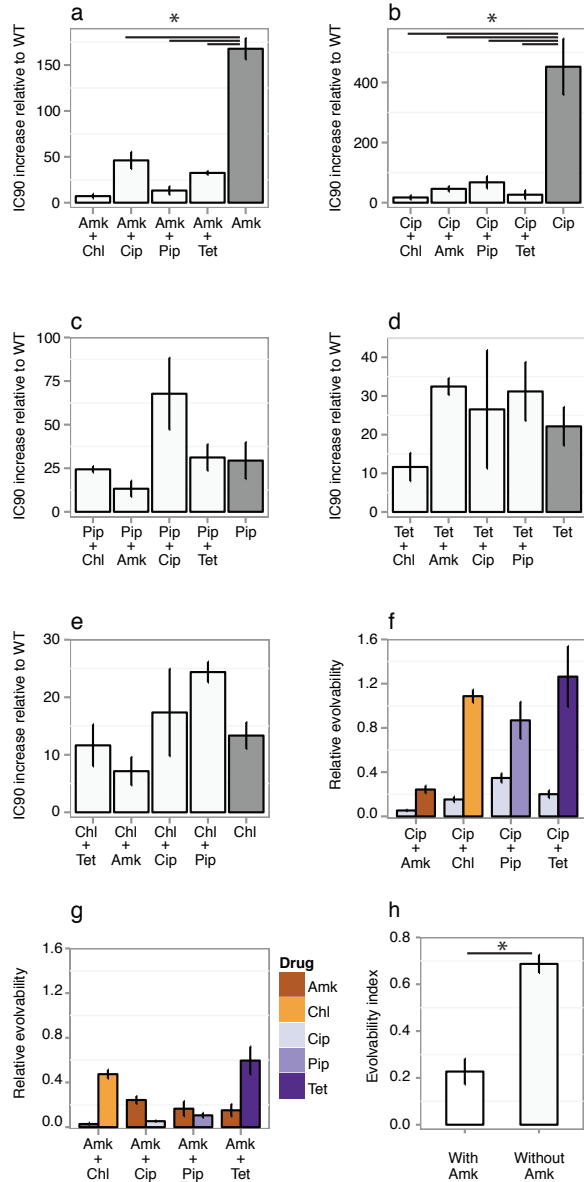


Figure 1. Amikacin containing combinations reduce the phenotypic evolution.

Increase in IC90 (mean \pm s.e.m, $n = 3$ biological replicates) of the evolved lineages relative to IC90 of the ancestral *E. coli* MG1655. Panel a-e depict data for lineages evolved to single or dual perturbations by Amk, Cip, Pip, Tet and Chl, respectively. Only combinations containing either Amk (a) or Cip (b) significantly reduce the relative increase in IC90 compared to a perturbation by the single component (* $P < 0.05$, ANOVA followed by Tukey's test). Panel f and g; evolvability of the individual components in Cip containing combinations (f) and Amk containing combinations (g) measured as increase in IC90 (mean \pm s.e.m, $n = 3$ biological replicates) of the individual components relative to the IC90 increase in single-perturbed lineages, only Amk containing combinations reduces the evolvability of both components. Panel h; evolvability index of combinations with and without amikacin (mean \pm s.e.m, $n = 12$ (with amikacin) $n = 18$ (without amikacin), biological replicates) calculated as the mean evolvability of the individual components in each combination. Amk containing combinations reduce the phenotypic evolutionary response significantly more than the other combinations (* $P < 0.05$, Student's t-test).

components.

To distinguish between these two phenomena we determined how the evolution of resistance to one individual component is affected by the presence of the other component in a dual-component selection. For each lineage evolved to a dual-perturbation containing either amikacin or ciprofloxacin, we quantified the relative evolvability of the individual components. This describes the increase in IC90 for each component in a dual-perturbation relative to the increase in IC90 in response to the mono-perturbation (see Supplementary Methods). If a component has a relative evolvability of 1, the presence of the other component does not influence the phenotypic evolutionary response. Conversely, if the relative evolvability is less than 1 it implies that the presence of the other component reduces the phenotypic evolutionary response, while a value greater than 1 implies that the other component accelerates the phenotypic evolutionary response. In the ciprofloxacin containing combinations, the presence of the other component only affects the phenotypic evolutionary response to ciprofloxacin, whereas the response to the other component is unaffected (except when it is amikacin) (Fig. 1f). Hence, any reduction in the phenotypic evolutionary response against ciprofloxacin containing combinations is merely the result of a ceiling effect exerted by the other component (Fig. 1f). In contrast, for combinations containing amikacin the phenotypic evolutionary response to both components is reduced, providing an example of genuine repression of phenotypic evolution (Fig. 1g).

To further examine the capacity of amikacin-containing combinations to reduce the phenotypic response, we calculated the evolvability index for each combination-perturbed lineage and compared the amikacin-containing combinations to the other combinations (Fig. 1h). The evolvability index is calculated as the average of the relative evolvability for each component in a combination (see Supplementary Methods). It describes the overall ability of a given combination to affect the phenotypic evolution. The comparison revealed, that amikacin-containing combinations reduce the overall phenotypic evolution to a significantly higher degree compared to the other combinations (Fig. 1h). Moreover, the high evolvability index of the combinations that did not contain amikacin revealed that the common assumption of independent responses to each component in a multi-component perturbation is overly simplified. Instead, these results suggest that there is a high degree of interactions between the evolutionary trajectories, and that this plays a large role in the phenotypic evolution to multi-component perturbations.

To uncover the underlying genotypic changes of the

evolved lineages, we genome sequenced all the evolved lineages along with the ancestral *E. coli* MG1655 wild type strain. We identified SNPs and INDELs (Supplementary Table 1) (Figure 2). Aside from the canonical primary target mutations in the ciprofloxacin and piperacillin perturbed lineages^{6,7}, we found that the lineages perturbed by ciprofloxacin, piperacillin, tetracycline and chloramphenicol had overlapping evolutionary trajectories, while those perturbed by amikacin evolved along a distinct trajectory (Fig. 2b). The ciprofloxacin, piperacillin, tetracycline and chloramphenicol perturbed lineages carried mutations in the regulatory genes *acrR*, *marR*, *soxR* and *rob* which all induce the well characterized multiple antibiotic resistant phenotype (mar

phenotype)^{8,9}(Fig. 2d). These regulatory genes control a common regulon known to confer resistance to all the tested antibiotics, except amikacin¹⁰, via increased efflux pump expression and down-regulation of porin expression (Fig. 2d)¹¹. In contrast, the amikacin-perturbed lineages all carried mutations in the *fusA*, *cpxA* and *sbmA* genes, known to be involved in aminoglycoside resistance¹²⁻¹⁵. Interestingly, none of the amikacin-perturbed lineages had mutations known to induce the mar-phenotype, highlighting that the amikacin-perturbed lineages follow a unique evolutionary trajectory (Fig 2b).

The differences between the genotypic responses to single perturbations by amikacin and by the other compounds correspond to the differences seen in the evolvability index of the dual perturbed lineages. This suggests that the amount of overlap in the responses to the single perturbed lineages predicts the responses to the combinations of these perturbations. This was generally confirmed by the genotypes of the dual perturbed lineages (Fig. 2a and c). As expected, we found that lineages perturbed by combinations of ciprofloxacin, piperacillin, tetracycline and chloramphenicol all evolved towards the mar-phenotype with an average of 1.5 mar related mutations per lineage (Fig 2c). In contrast, even though all the amikacin containing combinations also contained a compound that selected for the mar-phenotype, these lineages did not accumulate the mar mutations to the same extent having an average of only 0.6 mar related mutations per lineage (Fig. 2a). Furthermore, the accumulation of the canonical amikacin mutations was also reduced with none of the lineages containing the *sbmA* mutations and many of the lineages only having either the *fusA* or the *cpxA* mutations (Fig 2a). In particular the amikacin+chloramphenicol combination was impaired in its accumulation of the canonical mutations found in the component-perturbed lineages (Fig. 2a). Hence, it appears that there is a high degree of incompatibility between the evolutionary responses to amikacin and chloramphenicol.

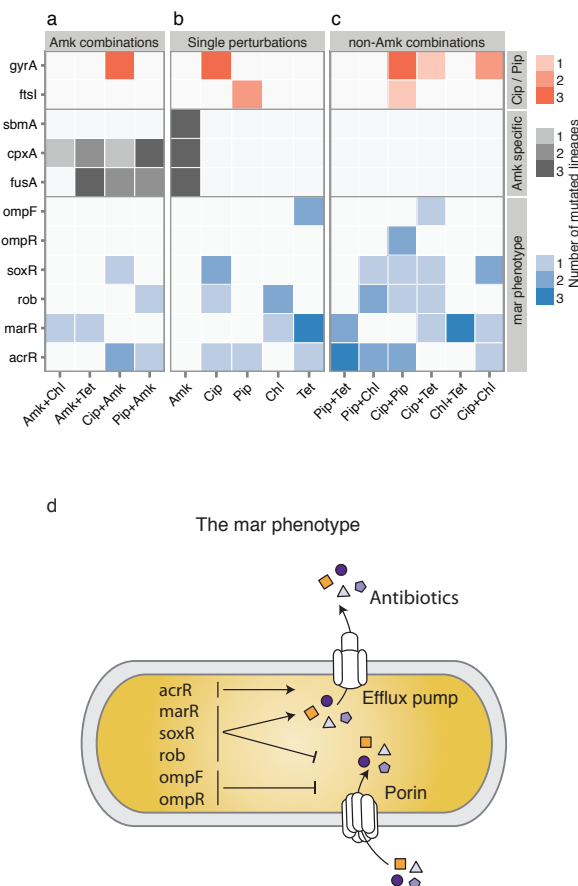


Figure 2. Compatible and incompatible genotypic evolution.

Panel a; heatmap depicting mutations (SNPs and INDELs) in genes known to be involved in resistance. The three parallel evolved lineages are collapsed by perturbation condition. The gene targets are grouped by the phenotypic characteristics (Red: Cip and Pip specific mutations. Grey: Amk specific mutations. Blue: mutations known to confer the mar phenotype). The legend indicates the number of parallel lineages containing the mutation. Panel b: illustration of the genes conferring the mar phenotype, arrows indicate up-regulation of the AcrAB-TolC multidrug efflux pump, bars indicate down-regulation of the outer membrane porins.

We hypothesized, that such evolutionary incompatibility could be the result of a collateral impact on the phenotype resulting from the genotypic evolutionary response to the individual components. Collateral sensitivity and resistance in response to antibiotic treatment was originally studied in the early 1950s by Szybalski and Bryson, however, recent studies have brought renewed attention to the phenomena and its potential relation to evolution of resistance^{12,16,17}. To quantify the collateral impacts of evolution to single component perturbations we measured the changes in antibiotic sensitivity (IC90) of the single perturbed lineages against the four antibiotics they had not been exposed to (Fig. 3a). This revealed a high degree of collateral effects resulting from evolution to each of the

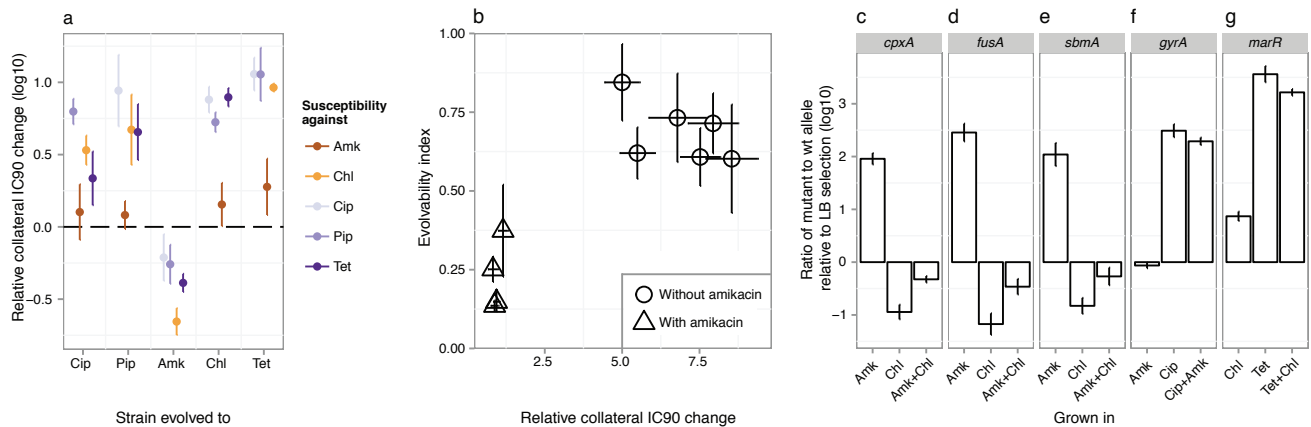


Figure 3. Collateral IC90 changes affect phenotypic evolution.

Panel a; quantification of the collateral impact in response to single-component perturbations. IC90 of each of the single-perturbed lineages was determined for the compounds they had not been exposed to and the collateral changes in IC90 are reported relative to the ancestral wt *E. coli* (mean \pm s.e.m, $n = 3$ biological replicates). Panel b; evolvability index of the dual-perturbed lineages as a function of the mean of the collateral IC90 change for the corresponding single-perturbed lineages (see Supplementary Methods) (mean \pm s.e.m, $n = 3$ biological replicates). Triangles represent combinations containing amikacin, circles represent combinations without amikacin. Panel c-g; competition assay between mutant and wt alleles. For each competition experiment the ratios of mutant to wt allele after competitive growth in sub-inhibitory concentrations of either single- or dual-perturbations (listed below each panel) are reported relative to the ratio in an unperturbed experiment (mean \pm s.e.m, $n = 3$ biological replicates). Alleles are; *cpxA*, *fusA*, *sbmA*, *gyrA*, *marR*, for panel c-g respectively.

single-component perturbations (Fig. 3a). In agreement with the overlapping evolutionary trajectories found in the genome sequences, we observed a high degree of collateral resistance between the lineages perturbed by ciprofloxacin, piperacillin tetracycline and chloramphenicol. In contrast, we found that the amikacin-perturbed lineages all displayed a consistent pattern of collateral sensitivity to the other drugs with chloramphenicol as the most sensitized drug (Fig. 3a). These findings confirm recent studies demonstrating significant collateral sensitivity in aminoglycoside exposed lineages^{12,17}.

Based on these results we hypothesized that the reduced evolutionary response to the amikacin-containing combinations is due to an evolutionary “tension” between the evolutionary responses to the individual components. To explore this idea, we used the measurements of collateral IC90 changes to calculate the average collateral impact for every pair of single-perturbed lineages and compared that to the evolvability index of the dual-perturbed lineages (Fig. 3b)(see Supplementary Methods). In this comparison the combinations divide into two populations, one with the amikacin containing combinations and one with the remaining combinations. It shows that the extent of the collateral impact within the single-component perturbed lineages predicts the capacity of a given combination to reduce the evolutionary response.

To investigate how the collateral impact between evolutionary trajectories affect fixation of mutations we performed a competition experiment using engineered single mutants with either a *cpxA*, *fusA*, *sbmA*, *gyrA* or *marR* mutation¹⁸ (see Supplementary Methods and

Extended Data Table 1). These mutations represent examples of evolutionary trajectories with collateral sensitivity (*cpxA*, *fusA*, *sbmA*), no collateral impact (*gyrA*) and collateral resistance (*marR*). Each mutant was mixed 1:1 with an ancestral wild type strain and grown overnight in sub-inhibitory concentrations of relevant antibiotics. Subsequently, the ratio between WT and mutant alleles was measured using qPCR and the changes were reported relative to growth without antibiotics (Fig. 3c and Extended Data Fig. 1). As predicted from the phenotypic and genotypic data, the results show that each of the three mutations originally found in the amikacin perturbed lineages (*cpxA*, *fusA* and *sbmA*) were enriched in the populations exposed to amikacin (Fig 3c-e). However, the experiment also revealed that the *cpxA*, *fusA* and *sbmA* mutations were counter selected in chloramphenicol and that the counter selection even occurred when the engineered strains were grown in chloramphenicol plus amikacin, highlighting that evolutionary tension can prevent fixation of otherwise beneficial mutations (Fig. 3c-e). In contrast, the *gyrA* mutation was strongly enriched by ciprofloxacin alone and ciprofloxacin plus amikacin, yet growth in amikacin alone did not affect the frequency of the mutant relative to growth without antibiotics (Fig. 3f). Accordingly, the *gyrA* mutation can be categorized as compatible with amikacin selection. Finally, the *marR* mutation was positively selected both in tetracycline alone and chloramphenicol alone as well as the combination of the two drugs (Fig. 3g). The *marR* mutation represents an example of how overlapping evolutionary trajectories, where a mutation is beneficial under multiple conditions, can reduce the ability of a multi-component perturbation to reduce an

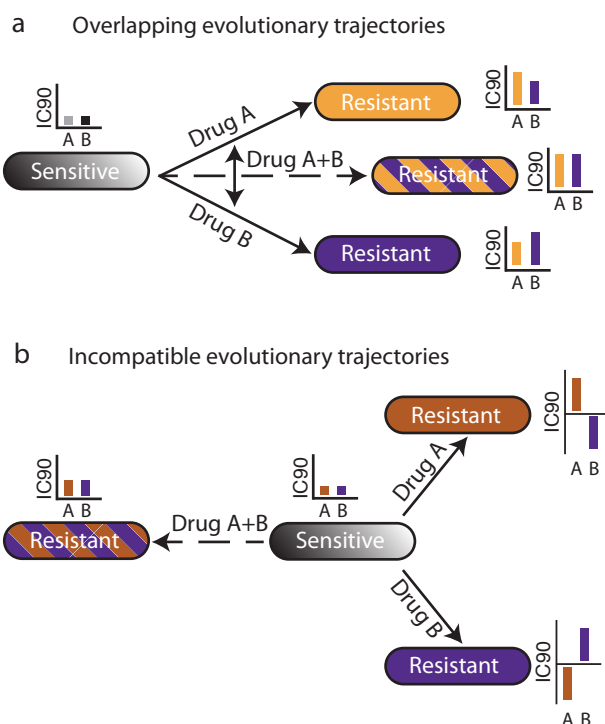


Figure 4. Collateral impact of single-perturbation can guide effective combinations.

Panel a; if two single-perturbations have overlapping evolutionary trajectories (e.g. Tet and Chl), a dual-perturbations by a combination of the two will not effectively reduce the phenotypic evolution. In contrast (panel b), if the two single-perturbations have incompatible evolutionary trajectories (e.g. Amk and Chl) a combination of the two will be effective at reducing the phenotypic evolution.

evolutionary response (Fig 1c-e).

These results show that the collateral impact of the individual evolutionary trajectories greatly affects the ability of a dual-perturbation to prevent fixation of the mutations commonly found in response to the component perturbations. If a population is exposed to a combination of perturbations where the responses to the individual components are compatible or even overlapping (e.g. tetracycline+chloramphenicol), fixation of the component mutations will not be effectively reduced (Fig 4a). In contrast, if the combined perturbation consists of individually incompatible evolutionary trajectories (e.g. amikacin+chloramphenicol) the evolutionary response to the combination will be greatly reduced relative to the response to the components (Fig. 4b).

Our findings highlight, that interactions between evolutionary trajectories as a result of distinct perturbations are common. Furthermore, these evolutionary interactions play a key role in determining the rate of phenotypic evolution to multi-component

perturbations. Indeed, evolutionary interactions between environmental perturbations can both prevent the fixation of canonical genotypes or lead to rapid adaptation through compatible or overlapping evolutionary trajectories. Notably, we find no correlation between epistatic interactions, e.g. synergism or antagonism, of the components and the phenotypic evolution¹⁹ (Extended Data Fig. 2). Instead, collateral resistance and sensitivity, a hallmark of evolutionary interactions, plays a key role in determining the rate of phenotypic evolution in response to combined perturbations. Collateral resistance and sensitivity has been described in bacteria, viruses and cancer cell lines^{17,20-22}. Accordingly, we expect that development of resistance-reducing drug combinations, on the basis of evolutionary interactions, should be applicable to a broad range of therapeutic areas, including bacterial infections, cancer and HIV management.

Method Summary

E. coli MG1655 was exposed to increasing concentrations of five antibiotics: ciprofloxacin hydrochloride (AppliChem), tetracycline hydrochloride (Sigma), amikacin sulfate (Sigma), chloramphenicol (Sigma) and piperacillin sulfate (Sigma) as well as all pairwise combinations thereof. The evolution experiments were performed in triplicates for each drug condition using LB medium as the growth medium. Selection was carried out by daily passaging of the lineages into two-fold dilution series of the antibiotics in 24 well plates using 1 ml total volume (Extended Data Fig. 3). All lineages were evolved for the same period of time and the experiment ended when all strains exposed to single-components had reached their respective clinical break point (see Supplementary Methods). For each single-component perturbed lineage, the IC90 of a representative clone was determined against all single components as well as dual-components containing the perturbation component. For the dual-perturbed lineages the IC90 against the combination and its components determined. In addition the IC90 of the ancestral strain was determined against all conditions (raw data in Supplementary Table 2). The genome of a representative clone from each lineage was sequenced using the SOLiD platform. For the competition experiment mutant strains were created using Multiplexed Automated Genome Engineering (MAGE)¹⁸ each mutant was mixed with a $\Delta lacZ$ wt strain and allele frequencies were determined with qPCR and confirmed by plating on Xgal+IPTG (see Supplementary Methods, Extended Data Fig. 1 and Extended Data Table 1 and 2).

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Evolution of high-level resistance to drug combination treatment is not predicted by epistatic drug interactions

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ABSTRACT

As drug resistant pathogens continue to emerge, combination therapy will increasingly be relied on to treat infections and to help combat the further development of multidrug resistance. At present a dichotomy exists between clinical practice, which favors therapeutically synergistic combinations, and the established scientific model, which maintains that this interaction provides a greater selective advantage toward resistance development than other interaction types. We explore the role that drug interactions play in the evolution of clinically relevant resistance through a series of adaptive evolution experiments using *Staphylococcus aureus*. Interestingly, no relationship between drug interaction type and resistance evolution was found as resistance increased significantly beyond wild type levels. All drug combinations, irrespective of interaction type, effectively limited resistance evolution compared to mono-treatment. Comparative genomic analyses reveal overlap in the mutations causing resistance between the drug combination evolved lineages and the individual drug evolved strains highlighting the importance of the component drugs in determining the rate of resistance evolution. Results of this work suggest that the emphasis placed on drug interactions as the driving force behind resistance evolution should be reconsidered.

SIGNIFICANCE STATEMENT

Combination therapy has been instrumental in treating illness where resistance potential is high. Presently a paradox exists between clinical practice, which favors therapeutically synergistic combinations, and

the established scientific mode, which maintains that this interaction favors resistance evolution. The role of drug interactions in the evolution of clinically relevant resistance was investigated through a series of adaptive evolution experiments using *Staphylococcus aureus*. All drug combinations slowed resistance evolution with the synergistic combination limiting it best. Combination and single drug evolved lineages shared mutations in primary targets highlighting the importance of constituent drugs in predicting rates of resistance evolution. Results of this work challenge the emphasis placed on drug interactions as the driving force behind resistance evolution.

INTRODUCTION

The discovery and deployment of antibiotics has been credited with revolutionizing medicine (1, 2). Unfortunately, microbes very quickly demonstrated a deft ability to adapt and evolve to these new wonder drugs rendering them less effective. Indeed, evolution of resistance in human pathogens has closely followed the deployment of every new antibiotic (3). Furthermore, the evolution of antibiotic resistance has been exacerbated by the misuse of antibiotics, particularly in the livestock industry (4), which has contributed to the spread and emergence of resistant strains (5-7). The ramifications of this situation are dire. Left unresolved antibiotic resistance will increase the cost of health care as a result of prolonged illness and the need for more expensive treatment, threaten medical advancements such as chemotherapy and organ transplants, scale back progress already made against certain infectious diseases, and ultimately

result in increased morbidity and mortality (8).

One means of reducing resistance development during treatment is to use drug “cocktails” instead of individual drugs to treat infections. A multidrug approach relies upon the notion that spontaneous resistance is rare and multiplicative and so the likelihood of an organism suddenly gaining resistance to a cocktail in a single step will be much less than the prospect of resistance to any one of the individual drugs that make up the mixture acting alone (9). This reasoning assumes that resistance acquisition occurs independently for each component of the cocktail.

Drug combinations are categorized according to their interactions, which can be described using the Loewe additivity model (7). The ideal case, referred to as synergistic, occurs when the treatment outcome of the mixture is significantly better than what would be expected from summing the effect of the component drugs acting alone. A mixture where the drugs interfere with each other and the overall therapeutic effect is worse than expected from summing the effect of the components acting alone is referred to as antagonistic. Finally, a combination where the treatment outcome of the mixture is the same as that of the individual components summed together is referred to as additive (10).

Combination therapy has been instrumental in improving the lifespan of individuals infected with HIV (11-13) and in treating tuberculosis (14, 15). The success of combination therapy coupled with the increasing frequency of resistant organisms has spawned interest in understanding how drug interactions can affect resistance development (16-22). Recent studies have suggested that the epistatic interactions of the drug combination are correlated with the development of resistance (18, 19, 22). Moreover, of the three aforementioned drug interaction types, combinations of a synergistic nature have been hypothesized to favor the emergence of resistance (18, 19, 21, 22). The rationale behind this phenomenon is that the mutations conferring resistance to a single drug will have a more pronounced effect on the fitness of the organism in the presence of a synergistic combination because of the cooperative interaction of the components in the mixture (18, 19).

The current body of in vitro experimental evidence for this hypothesis conflicts with the practice of combination therapy in clinical settings, where therapeutically synergistic combinations are favored over additive or antagonistic combinations as a means for treating resistant pathogens and to clear infections (7). Thus a paradox exists between the need for effective treatment and the desire to limit resistance evolution. Resolution of this contradiction is important as it has the potential to affect the treatment of a wide variety of infectious diseases, including tuberculosis (14, 15), HIV (11-13, 23, 24), as well as cancer (25).

The current paradigm is built upon experimental work at or near the wild type (WT) minimum inhibitory concentration (MIC) level (16-18, 22). The caveat of this approach is that clinically relevant resistance usually occurs at concentrations substantially greater than the WT MIC levels (26) used to develop the existing model and hypothesis. It is the evolution of resistance in the range substantially above the WT MIC, which has not yet been properly explored, that we aimed to investigate. We hypothesize that beyond WT MIC levels, resistance evolution is largely driven by the response to the individual component drugs rather than the epistatic interactions between the drugs in a combination.

To explore our hypothesis, we performed a series of resistance evolution experiments using a medically relevant Gram-positive species, *Staphylococcus aureus* strain Newman (27), and five clinically relevant antibiotics - ampicillin (beta lactam), ciprofloxacin (fluoroquinolone), amikacin (aminoglycoside), fusidic acid, erythromycin (macrolide) – which target a broad range of processes, including cell wall construction, the structure and function of DNA, and protein synthesis, respectively. The selected antibiotics had previously been used to develop the current resistance paradigm (18) and were employed here to determine if the paradigm extended substantially beyond WT MIC levels. Erythromycin(28), fusidic acid (29, 30), and ciprofloxacin (28) are important agents for treating methicillin sensitive *Staphylococcus aureus* infections. We considered evolution to each individual drug as well as three combinations, ciprofloxacin-ampicillin (cpr-amp), amikacin-fusidic acid (fus-amik), and erythromycin-fusidic acid (fus-ery), which had previously been characterized as having additive, antagonistic and synergistic interactions, respectively (18). It is important to note that the fusidic acid-amikacin combination is not considered to be an antagonistic drug combination for the treatment of methicillin resistant *Staphylococcus aureus* (31). For simplicity we will refer to these combinations by their interaction type when commenting on them.

Resistance evolution experiments were conducted according to standardized methods (32) following determination of the WT MIC (Table S1). Briefly, we challenged our WT organism with increasing concentrations of the aforementioned individual drugs or drug combinations. All evolution experiments were performed in liquid culture and each condition was investigated with three parallel experiments, designated as A, B, and C, in order to ensure experimental robustness. Growth inhibition was assessed after 18 hours of incubation using optical density measurements. The most resistant culture for each condition and experiment was selected for cultivation in fresh liquid media at the appropriate drug(s) concentration(s) overnight and then used

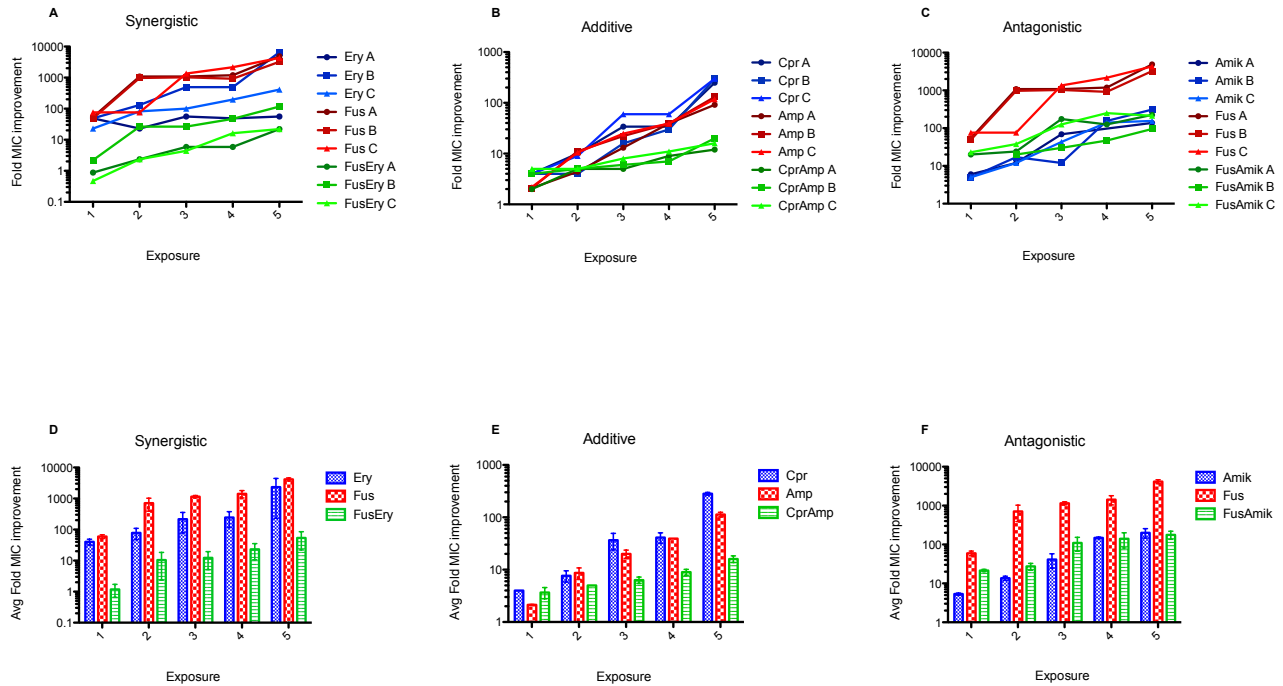


Figure 1. Individual and averaged resistance evolution for each of the conditions investigated. Individual drugs are shown in blue and red, while drug combinations are displayed in green. First figure row (A, B, C) shows fold MIC improvement for individual lineages. Fold MIC improvement is defined as the IC90 of the resistant strain divided by the WT IC90. Second figure row (D, E, F) shows average fold MIC improvement for each condition investigated. Values represented here are an average of all replicate IC90 values for each condition (n=3, \pm SEM).

to inoculate the next experiment. A total of five exposure improvement experiments were conducted for individual drug and drug combination. This corresponded to an average cumulative number of cell divisions of 8.6×10^{12} (33).

RESULTS

Resistance evolution experiments

Results of the evolution experiments show that all lineages exhibited a substantial increase in resistance, defined as the fold increase in the 90% inhibition value (IC90) relative to the WT, following five rounds of selection (Fig. 1A,B,C). The exception to this trend was erythromycin-evolved lineage A, which experienced almost no additional resistance gain following the first exposure (Fig 1A). The average resistance improvements of the combination evolved (CE) lineages were different from their corresponding single drug evolved (SDE) lineages (Kruskal-Wallis nonparametric ANOVA, all $p < 0.0001$) (Fig 1D,E,F). The SDE strains achieved average fold MIC improvements that ranged from approximately one hundred (ampicillin) to greater than four thousand (fusidic acid) (Fig 1D,E,F). This corresponded to drug concentrations ranging between 20-500 $\mu\text{g/mL}$ (Fig S1). These concentrations were six to several hundred times greater than the reported clinical breakpoints for these drugs for *S. aureus* (Table S2) (26). The

average fold resistance increase of the erythromycin evolved lineages was 2346, however variance in the evolution among the replicate strains translated to an equally large standard error of the mean (2115). The CE lineages had less average fold resistance increase compared to their corresponding SDE strains. Of the three CE lineages only the fus-amik strains had an average fold MIC improvement of greater than 100.

Resistance evolution limited by all three combinations

Previous work has suggested that synergistic drug combinations favor resistance evolution more than antagonistic combinations (18, 19, 22). To examine the influence of epistatic interactions on resistance evolution in our experiments we determined the evolvability index (EI) of each combination. The EI is a measure of the evolution of the CE lineages relative to the SDE lineages at a given effect level and is determined by taking the average of the sum of the evolvability fractions for each drug in a mixture (for more detail see Methods). An EI value of one signifies that the combination lineage evolved to same extent as its component lineages. A value greater than one indicates that the CE strains evolved to be more resistant than their corresponding SDE lineages, while a value less than one means that CE lineages evolved less than their SDE strains.

The evolvability fraction reflects how resistance

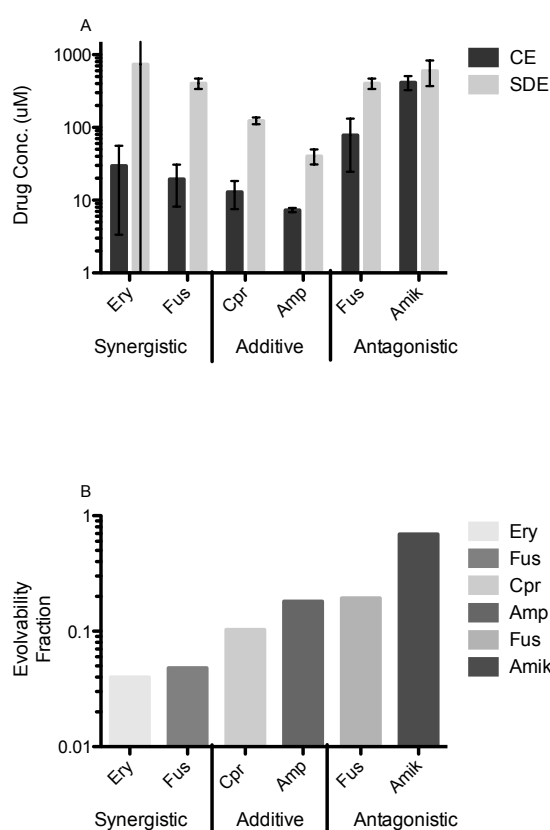


Figure 2. Concentrations and ratios used to determine evolvability indices (EI) for each drug pair examined. (A) Resistance concentration for combination evolved (CE) strains tested against individual component drugs ($n=12$, \pm SD). Single drug evolved (SDE) concentrations are shown alongside for comparison ($n=12$, \pm SD). (B) Fraction of resistance to an individual component attained by CE strain relative to resistance attained by SDE strain.

evolution toward an individual drug is impacted as a result of being used in a combination compared to being used alone. To make this assessment IC₉₀ values for individual component drugs were obtained for the CE strains (Fig. 2A) and subsequently divided by the corresponding SDE values (Fig. 2B). Overall, the evolvabilities of all CE lineages were limited with all fractions being less than one (Fig. 2B). Resistance development towards amikacin by the antagonistic combination was exceptionally high with the CE strains having IC₉₀ values of close to 70% of the SDE strains (Fig. 2B). This outcome is surprising because despite being in a combination, particularly an antagonistic one, resistance appears to have developed quickly. Pairwise comparisons of each drug's CE and SDE IC₉₀ values determined that the concentrations were statistically different (Mann-Whitney, $p < 0.05$). Summation of the appropriate evolvability fractions yielded the EI values for each of the CE strains. EI values for all three combinations were less than one meaning that the CE lineages were less evolved

than their corresponding SDE strains (Table S3). Interestingly, the evolvability was highest for the antagonistic drug combination (fus-amik EI = 0.44) and lowest for the synergistic drug combination (fus-ery EI = 0.04). This result is in direct contrast to previous reports based on sub MIC adaptations (18, 19, 22).

Drug combination interactions change with resistance adaptation

In the current scientific model, drug interactions are considered to be static properties of the agents involved with no consideration given to the biological response provoked by the combination. To ascertain the stability of epistatic interactions post resistance adaptation, we calculated a fractional inhibitory concentration (FIC) index for each interaction type using our WT and drug combination adapted lineages. The FICI is based on the Loewe additivity zero interaction theory and describes, for a given effect level, drug interactions as the sum of the fractional inhibition of each drug in a combination relative to the drug acting alone (34). A FICI of one denotes an additive interaction while values greater than one indicate an antagonistic interaction and those less than one point to a synergistic interaction.

The interactions of each drug combination were tested with the WT strain prior to beginning the evolution experiments. The interaction types were as expected: fus-ery was synergistic ($FICI=0.86\pm0.13$), cpr-amp was additive ($FICI=1.05\pm0.13$) and fus-amik was antagonistic ($FICI=1.45\pm0.1$) (Fig. 3 A, B, C). After five rounds of resistance improvement, the FICI values of the CE strains were re-evaluated by decoupling the final combination resistance concentrations into individual components and obtaining IC₉₀ values for individual component drugs. (Fig. 3 A, B, C). The additive combination underwent the greatest shift in FICI value with all replicate lineages becoming antagonistic (average $FICI = 2.71\pm0.32$). Strains adapted to the synergistic and antagonistic combinations each had one replicate lineage, whose interaction type changed completely (Fig. 3 A, B). Results of our analyses show that drug interactions will change in response to resistance adaptation and that this change may be dramatic. This effect has previously been observed (22)

Mutations are shared between combination evolved and single drug evolved lineages

To explore the molecular basis of drug resistance evolution in our experiment, we sequenced the genomes of our most evolved strains and our wild type. Comparative genomic analyses revealed that the CE and SDE lineages had mutations in several of the same primary targets (Fig 4, complete listing available in Data S1). For example, strains evolved to fus-ery and erythromycin had mutations in genes coding for several key ribosomal proteins including L4 and L22.

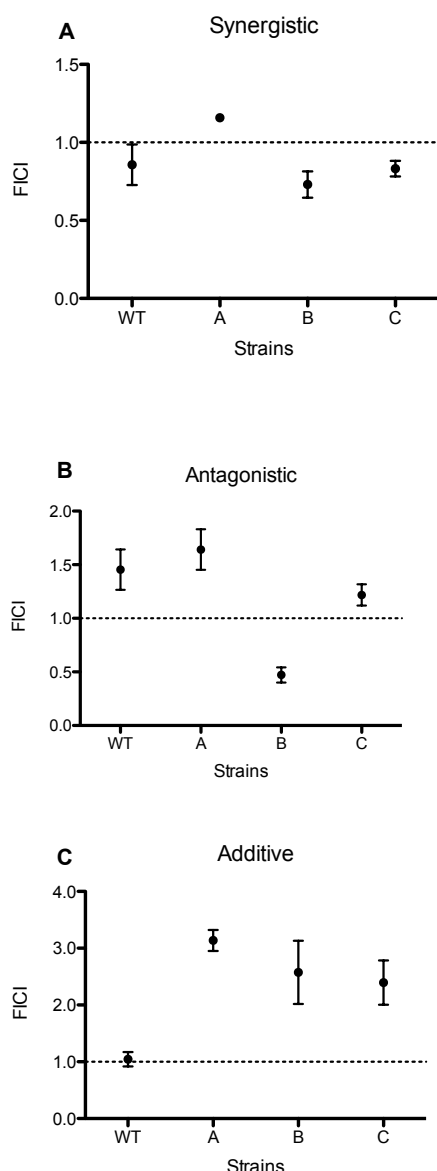


Figure 3. Fractional inhibitory concentration index (FICI) values for each interaction type investigated.

Following resistance adaptation, the most evolved combination adapted strains had their FICI values re-evaluated (n=4, +SD). The wild-type value is shown alongside as a reference (n=8, +SD). Lineages adapted to the additive combination (C) had the greatest change in FICI value. Notable shifts in FICI value are also observed in the synergistic (A) and antagonistic (B) evolved strains.

Mutations in these proteins have been correlated with macrolide resistance in several bacterial species (35, 36), including *S. aureus* (37). Mutations in the *fus* gene, known to confer fusidic acid resistance (38), were observed in both the *fus-ery* and fusidic acid evolved lineages. Likewise, strains evolved to *cpr-amp* and ciprofloxacin had mutations in the *parC* gene, which has been well documented in conjunction with ciprofloxacin resistance (39). Beta-lactam resistance

observed in the *cpr-amp* and ampicillin evolved lineages was the result of mutations in the *pbpA* gene. Some instances of expected mutations missing were also noted. For example, *gyrA* mutations were observed in the ciprofloxacin strains, but not in the *cpr-amp* lineages. Mutations in the *parC* gene grant only low-levels of resistance (40), while mutations in the *gyrA* gene have been identified as being responsible for higher levels of quinolone resistance (41). When present together, an organism has high level quinolone resistance (40, 42). The presence or absence of *gyrA* mutations in the ciprofloxacin and *cpr-amp* lineages is reflected in their ciprofloxacin tolerance (Fig. 2A).

In addition to mutations in primary targets, a wide variety of auxiliary mutations were also observed in both the CE and SDE strains. These supplementary mutations were assessed and grouped according to function (Data S1). Several of the auxiliary mutations were part of a larger stress response network, which likely conferred resistance. For example, all three *cpr-amp* evolved strains had mutations in the *relA* gene. Under environmental stress, RelA initiates the stringent response, which controls the production of the alarmone ppGpp, which in turn serves as a regulator of a variety of metabolic pathways and processes and has been shown to play an essential role in decreased sensitivity to penicillin (43-46) and quinolones (47). *relA* mutations were also observed in the *fus-amik* evolved strains. Shared auxiliary mutations between SDE and CE lineages were limited. However, the numerical distribution of these mutations was approximately equal among all strains. The apparent absence of new primary resistance mutations in response to combination treatment suggests that CE strains became resistant by acquiring mutations in the same genes as the SDE lineages, chiefly those in primary targets, as well as auxiliary mutations in stress response genes.

Collateral resistance between component drugs is correlated to resistance evolution towards drug combinations.

The evolvability indices revealed that all three of the CE strains evolved less than their corresponding SDE lineages and that the extent of evolution among these strains differed. One explanation for the difference in resistance levels among the CE strains may have been the extent of collateral resistance between the component drugs. Component drugs selecting for resistance mediated by compatible trajectories can result in collateral resistance, which means that the development of resistance to one drug results in resistance to other drugs as well (48). We explored the role of this phenomenon in the resistance development of our CE strains by testing our SDE strains to the other drug in their respective drug pair (Fig. 5).

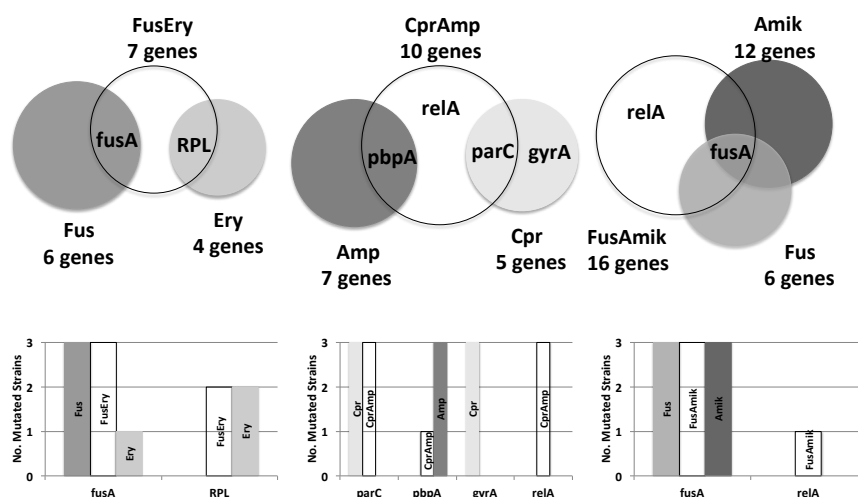


Figure 4. Mutations most likely responsible for causing resistance in the evolved strains. Lineages are shown as colored circles and genes containing mutations are shown as text. Circle size corresponds with the number of mutated genes. Overlap among the lineages represents shared genes with mutations. It is observed that the combination-evolved lineages share most of the primary resistance mutations with the single drug evolved. Bar graphs show the number of mutated strains (y axis) with a mutation in a particular gene (x axis). RPL stands for ribosomal proteins.

Results of these experiments were used to calculate a resistance ratio (RR) for each of the SDE lineages. The RR is determined by dividing the IC₉₀ towards drug A of an SDE strain evolved to drug B by the IC₉₀ towards drug A of the WT. We employed the conservative resistance ratio cut off value of greater than or equal to two to define collateral resistance (49). The majority of RR values were between 0.5 and 2 indicating that resistance to one drug does not affect resistance to other drugs (Fig. 5). Only the amikacin

evolved strains demonstrated any significant shift from this range. These lineages showed a high degree of collateral resistance to fusidic acid (Fig. 5), which can likely be attributed to mutations in the fusA gene (Fig. 4), the most characterized mechanism of fusidic acid resistance, and the only primary target mutations found in the amikacin evolved lineages. Indeed, mutations in fusA have previously been shown to confer resistance to aminoglycosides in *Staphylococcus aureus* (30). Interestingly, the fusidic acid evolved strains did not exhibit a corresponding collateral cross-resistance towards amikacin, highlighting that the collateral resistance in this case is uni-directional. Additionally collateral resistance to ampicillin was observed for the ciprofloxacin evolved strains. It is interesting to note that there is a quantitative correlation between the degree of cross-resistance between the component drugs (Fig 5) and the rate by which resistance evolved towards to the drug combination (Fig 2). This suggests that evolutionary interactions resulting from collateral impacts of resistance evolution could be important in predicting how fast resistance evolves towards drug combinations.

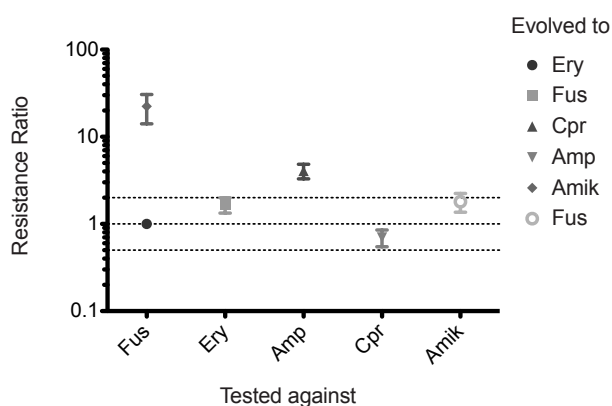


Figure 5. Assessing collateral resistance as a result of resistance evolution to one drug. Single drug evolved (SDE) lineages were MIC tested to their corresponding partner drug (n=4, +SD). These values were then compared to the WT MICs (Resistance Ratio). Values less than 0.5 indicate collateral sensitivity, while those greater than 2 suggest collateral resistance. Values between 0.5 and 2 are considered to be insensitive. SDE strains are listed in the legend, while drugs they were tested against are on the x-axis. We observe a strong collateral resistance towards fusidic acid of the amikacin-evolved strain, and a weaker collateral resistance of the ampicillin evolved strain towards ciprofloxacin.

DISCUSSION

Combination therapy is used as a means for treating resistant pathogens and for combating the development of multidrug resistance. The current paradigm, which stems from a limited number of experiments, asserts that the interaction between drugs in a combination is the primary factor affecting the rate by which resistance develops. In particular, combinations acting in a therapeutically synergistic fashion are believed to favor resistance evolution. This model conflicts with clinical practice where such combinations are preferred for their ability to stop bacterial growth.

The objective of this work was to assess the role of individual components in driving resistance evolution of a drug combination at antibiotic concentrations relevant to clinical resistance. Our results suggest that drug combinations, whatever their theoretical interaction, effectively curb resistance evolution. Moreover, these interactions were found to be modulated during resistance adaptation to the drug combinations. A finding that has previously been observed (22). Therefore, the current canons, which depend upon a static model of drug interactions, did not predict our results. Rather, the types of mutations arising from adaptation towards the component drugs appear to be an influential factor in resistance evolution. Absence of a key primary target mutation directly translated to a reduction in resistance.

Additional aspects, such as pleiotropic effects, variation in mutational rate, variation in drug concentration, and pharmacodynamics, etc., have also been hypothesized (18, 19) to influence the rate of adaptation; however, the impact of these factors was not explored in earlier work. In investigating our hypothesis, the role of pleiotropic effects in resistance evolution became clearer. Mutations that performed double duty (i.e. conferring resistance to two drugs) resulted in the greatest increase in resistance evolution. Likewise, the degree of collateral resistance, arising from these pleiotropic effects, in our SDE lineages could be correlated with the rate of resistance evolution in our CE strains. Results of our work suggest that this factor is more important than drug pair interactions for evolution of clinically relevant resistance. The role of the remaining factors in influencing the rate of adaptation still need to be explored and serve as a caveat to any resistance evolution paradigm.

In conclusion, the results of our work present a challenge to the current paradigm regarding the driving force behind multidrug resistance evolution. When evaluating resistance evolution over a wider spectrum of concentrations, which better reflects what is observed in clinical resistance, the current theories regarding drug interactions influencing resistance evolution could not be substantiated. Instead our data bolster the idea that resistance development in combination therapy has more to do with mutation acquisition and the individual drugs of the combination. While the breadth of our study is limited, we expect that the findings uncovered here are not unique to our tested organism, antibiotics and drug pairings, and would hold true if extended to a broader range of organisms and drugs.

Materials and Methods

Bacteria and Reagents

A drug sensitive *Staphylococcus aureus* strain Newman was adapted to five antibiotics: amikacin sulfate

(Sigma), ampicillin sodium salt (Sigma), ciprofloxacin hydrochloride (AppliChem), erythromycin (Sigma), and fusidic acid sodium salt (Sigma) and the following drug pair combinations: fusidic acid-amikacin, fusidic acid-erythromycin, and ampicillin-ciprofloxacin. Drug stock solutions were prepared weekly. All evolution and MIC experiments were performed using a modified Luria broth (LB) media. Briefly, the salt content was reduced to 4g/l instead of 5g/l.

Evolution of antibiotic resistance

A wild type IC90 was established for each antibiotic. Drug pair combinations were a 1:1 IC90 mixture of the component drugs. Wild type IC90s were also established for each drug pair. All evolution experiments began one dilution step below their respective IC90 concentration. Evolution experiments involved challenging a wild-type organism with increasing concentrations, in steps of the square root of two, of individual drugs or drug combinations. All evolution experiments were performed in triplicate in a modified Luria-Bertani (LB) broth in microtiter plates. Each experiment included both negative and positive control wells. The positive control was the inoculating strain in LB media only. Following a 18-hour growth period at 37 °C, the microtiter plates were measured for optical density (OD) at wavelength 600nm. The value of the experimental positive control was used to normalize the evolution data. A cut off of 60% inhibition was used to determine the starting concentration of the next experiment. This concentration was referred to as the experimental MIC. The 60% inhibition value was chosen based on pre-experimental work that found that this value consistently ensured a resistant population was used in subsequent exposure experiments. The replicate with the best growth at the experimental MIC concentration was used as seed material for the next experiment. The selected seed was added to fresh LB media containing the appropriate drug(s) concentration and allowed to grow over night. The overnight culture was then used to inoculate the next challenge experiment. A portion of this culture was saved. The challenge process was repeated a total of five times for each individual drug and drug combination.

IC90 determination

Strains from all evolution steps were tested to determine their IC90 fold improvement. Lineages from the final evolution experiment received additional testing -single drug evolved strains were MIC tested against the corresponding combination and other component drug, while combination drug evolved lineages were tested against their component drugs. All IC90 experiments were performed in 96 well micro titer plates in quadruplicate using two fold dilution steps. Positive, tested straining LB media only, and negative controls were included in each test.

Innoculated plates were placed on an orbital shaker and incubated at 37°C for at least 16 hours. After the allotted growth period, OD600 was read on a BioTek Epoc plate reader.

Calculation of CCD

Using the equation set forth by Lee et al (33), n is the number of generations for each growth step. In our case there are two growth steps - the resistance experiment and the test tube pre growth period prior to each resistance experiment. n values were calculated for each evolved lineage and the two growth steps. We performed growth kinetic experiments that allowed us to calculate a generation time (G in min⁻¹) for each strain. These values were then used to determine the number of generations for each strain in an eight-hour period (assumed log growth phase) or n . In the Lee equation CCD is:

$$\sum_{i=1}^n N_0 (2^N - 1)$$

where N_0 is the initial number of cells in each well or test tube during evolution. We used representative values of N_0 , reflecting each growth condition, for each strain to calculate the CCD for the test tube and resistance experiment periods. The subsequent CCD values were multiplied by five to reflect the number of evolution periods for each growth condition. A mean CCD value and associated standard error mean was calculated for each drug or drug combination and each growth condition. These values are listed in a table in the supplementary data section. The CCD range given in the text comes from adding the two growth conditions together.

Data Analysis

The OD600 data were analyzed using Excel and Prism (GraphPad Software). Briefly, negative control values were subtracted from all growth wells yielding dose response values. These data were then normalized by the positive control data and then used to determine the fraction of inhibition, calculated as: 1- normalized dose response of strain X. Inhibition data was plotted in Prism and IC90 read from graph.

Calculation of Evolvability Index

The evolvability index assesses how resistance evolution toward a combination compares to individual drug resistance evolution. The index is determined by summing a combination evolved strain's resistance to each of its component drugs relative to the resistance development of the corresponding single drug evolved lineages and then taking an average. Each term individual fraction can be used to assess how

resistance evolution to an individual component is impacted as a result of being used in a combination. The evolvability index is calculated as:

$$\text{Evolvability Index} = \frac{1}{n} \left(\frac{IC90[A]_{AB}}{IC90[A]_A} + \frac{IC90[B]_{AB}}{IC90[B]_B} \right)$$

where the n is the number of components in a mixture and is used to determine an average value. IC90[A]_{AB} refers to the IC90 of the AB evolved lineage tested against drug A.

Sequencing

Genomic DNA from our most evolved strains and wild type was isolated using either an UltraClean® Microbial DNA Isolation Kit (Mobio Laboratories, Inc.) or a modified chloroform/phenol extraction method. Briefly, lysostaphin in conjunction with proteinase K were used to disrupt the cell wall. The extracted DNA was sheared into 200bp fragments using a Covaris E210 and barcoded libraries were constructed for illumina or IonTorrent sequencing. Illumina sequencing was performed by Partners HealthCare Center for Personalized Genetic Medicine (Cambridge, Massachusetts). IonTorrent sequencing was performed by DTU Multi-Assay Core (Kongens Lyngby, Denmark). All reads were aligned to *Staphylococcus aureus* subsp. *aureus* str. Newman (NC_009641.1) using Bowtie2 version 2.0.0-b6 with the default options (50). An average of 99.6% (minimum 97.5%) of the genome was covered with three times read coverage or greater, as determined using bedtools (51). Variant calling for SNPs and INDELs was done using SAMTools version 0.1.17 with the -B,-L 1000 options (52). SNPs were filtered leaving only those with a phred score of at least 30 and at least 80% of the reads aligned at the site having the variant. INDELs were verified by aligning constructed contigs around INDEL sites to the reference genome (53, 54). The BioCyc database collection (55) was used to identify and annotate mutation sites.

Author Contributions

M.C.R.D.E. designed and performed research, analyzed data and wrote paper, H.G. helped analyze data, C.M. helped analyze data, and T.T.T. helped perform research, M.O.A.S. designed research, analyzed data and wrote paper.

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Conflicts of Interest

None

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Supplementary pages

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Section 1

Supplementary Methods

Evolutionary interactions between environmental selection pressures drive phenotypic evolution

Adaptive evolution experiment

E. coli MG1655 was evolved to the five antibiotics: ciprofloxacin hydrochloride (AppliChem), tetracycline hydrochloride (Sigma), amikacin sulfate (Sigma), chloramphenicol (Sigma) and piperacillin sulfate (Sigma) as well as all pairwise combinations thereof. The evolution experiments were performed in triplicates for each drug condition using LB medium as the growth medium. Selection was carried out in two-fold dilution steps of antibiotics in 24 well plates using 1 ml total volume. Each plate contained two medium control wells, none of these were showed growth during the experiment. After 20 hours of incubation at 37°C with shaking, OD600 was read and 25 µl from the lowest antibiotic concentration with OD600 greater than 0.25 was diluted into a new antibiotic gradient. In total 14 passages were performed. The drug-pairs were evolved to a 1:1 IC₉₀ mixture of the component drugs, the resulting molar ratios were: Cip:Pip 0.012; Cip:Amk 0.015; Cip:Chl 0.004; Cip:Tet 0.021; Pip:Amk 0.559; Pip:Chl 0.230; Pip:Tet 0.641; Amk:Chl 0.191; Amk:Tet 0.585; Tet:Chl 0.144. The evolution experiment was ended when the single-drug evolved strains had reached the clinical break-point for the antibiotic. Clinical breakpoints were defined according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST): Ciprofloxacin 1 µg/ml, Piperacillin 16 µg/ml, Amikacin 16 µg/ml, Chloramphenicol 8 µg/ml and Tetracycline 16 µg/ml. On the last day all strains were streaked on LB agar plates to be used for IC₉₀ determination.

IC₉₀ determination

IC₉₀ determination was performed in 96 well micro-titer plates prepared using a Hamilton Star pipetting robot. Each drug gradient consisted of 11 points in a two fold dilution series prepared in MHBII (Sigma) medium with a total of 150 µl in each well. For the single drug evolved strains, the IC₉₀ was determined for all single drugs as well as all combinations containing the evolved-to drug, and the experiments were carried out in triplicates and quadruplicates, respectively. For the drug-pair evolved strains, the IC₉₀ was determined for the two drugs in the combination as well as the drug combination, and experiments were done in five replicates. For every IC₉₀ test, the wt strain was included to determine the IC₉₀ reference point. The IC₉₀ plates were inoculated with approximately 10⁵ cells per well using a 96 pin replicator. The plates were incubated at 37°C with shaking for 18 – 20 hours and OD600 was read on a BioTek H1 plate reader.

Data analysis

The OD600 data files were analyzed using R²⁵. In brief, control wells were analyzed (one contamination out of 616 blanks and growth in all positive control). To obtain inhibition curves the OD600 values for the dose-response series were converted into values of percent inhibition calculated as $1 - (\text{OD}_{600}[\text{x}] - \text{OD}_{600}[\text{negative control}] / (\text{OD}_{600}[\text{positive control}] - \text{OD}_{600}[\text{negative control}]))$ and plotted against the molar concentration of the antibiotic and a dose-response curve was fitted using the drc() package with the default four variable logistic model: $f(x, (b, c, d, e)) = c + (d - c) / (1 + \exp\{b(\log(x) - \log(e))\})$ ²⁶. IC₉₀ was calculated via the inverse function of the fit. Graphs were made in R with the packages plotrix and ggplot2^{27, 28}.

FICI was calculated as $\text{IC}_{90}[\text{AB}] \times \omega / \text{IC}_{90}[\text{A}] + \text{IC}_{90}[\text{AB}] \times (1 - \omega) / \text{IC}_{90}[\text{B}]$
Where ω signifies the molar fraction of drug A in the drug combination AB.

Calculation of evolvability

The evolvability is used to assess how the development of resistance against a drug is affected by the presence of another drug. By summing the effect from each component drug in a drug-pair, it gives an overall value to describe the degree of drug resistance development in drug-pair evolved lineages relative to the resistance development in single-drug evolved lineages. Specifically, evolvability is

calculated the average of the sum of the change in resistance development to the component drugs in a drug-pair divided by the change in resistance development in the single drug evolved strains:

$$\text{Evolvability} = \left\{ \left(\frac{IC_{90}[A]_{AB}}{IC_{90}[A]_{wt}} \right) / \left(\frac{IC_{90}[A]_A}{IC_{90}[A]_{wt}} \right) + \left(\frac{IC_{90}[B]_{AB}}{IC_{90}[B]_{wt}} \right) / \left(\frac{IC_{90}[B]_B}{IC_{90}[B]_{wt}} \right) \right\} / 2 = \{ IC_{90}[A]_{AB} / IC_{90}[A]_A + IC_{90}[B]_{AB} / IC_{90}[B]_B \} / 2$$

Where $IC_{90}[A]_{AB}$ signifies the IC_{90} of the AB evolved strain tested against drug A.

Collateral IC_{90} change is the average collateral IC_{90} change between two single-drug evolved strains calculated as $(IC_{90}[A]_B + IC_{90}[B]_A) / 2$

Where $IC_{90}[A]_B$ signifies the IC_{90} of the B evolved strain tested against drug A.

SOLiD sequencing

A single colony from each evolution experiment was grown up in LB and DNA was extracted using the DNeasy kit (Qiagen, Germany). The DNA was sheared into 200 bp fragments using Covaris E210 and barcoded libraries were made for SOLiD sequencing. SOLiD reads were aligned to *E. coli* MG1655 reference genome (NC_000913) using Bowtie2²⁹. Each sample had at least 98 % of the genome covered with 3 times coverage or greater, and the mean percentage with at least 3 times coverage was 99.71 %³⁰. The alignments were further tuned by GATK³¹ by re-aligning identified possible INDEL sites to discriminate between SNP and INDEL sites³². Variant calling for SNPs and INDELs was done using SAMtools³³, with INDELs verified by aligning constructed contigs around INDEL sites to the reference genome^{34,35}. Further analysis was done by custom written scripts using Biopython³⁶.

Competitive growth selection.

The five single mutants *cpxA*, *fusA*, *sbmA* *gyrA*, and *marR* were engineered using the MAGE technique¹⁸ (Extended Data Table 1). A wt *lacZ* mutant was engineered in a similar fashion. Each of the five single-mutants were mixed 1:1 with the wt *lacZ* mutant at OD600 = 0.1. 1 µl of this mixture was inoculated into sub-inhibitory concentrations of antibiotics in the following order: *cpxA*, *fusA* and *sbmA* was inoculated into Amk, Chl Amk+Chl and LB; *gyrA* was inoculated into Cip, Amk, Cip+Amk and LB; *marR* was inoculated into Tet, Chl, Tet+Chl and LB. All were grown overnight at 30°C. To confirm that the antibiotic concentration was indeed sub-inhibitory, the wt *lacZ* was inoculated into all antibiotic solutions and the growth rate was measured (Data not shown). The competitive growth selection experiments were performed in triplicates.

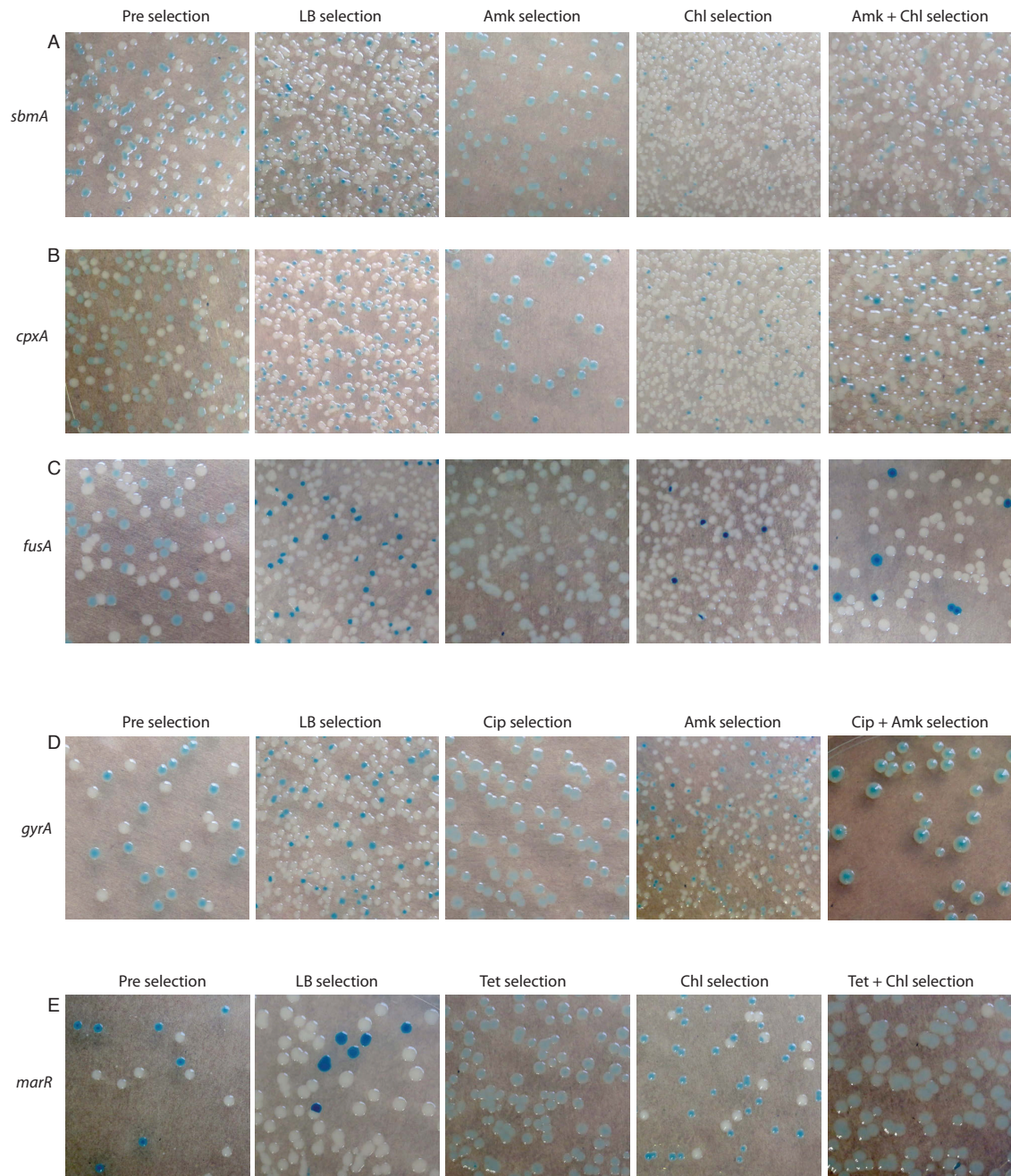
qPCR assay.

For each of the five mutant alleles in the competitive growth selection experiment a wt primer pair and a mutant primer pair was designed. The optimal annealing temperature was identified in a temperature gradient, and for each growth condition a separate qPCR reaction was performed with the wt primer pair (Extended Data Table 2) and the mutant primer pair using 1 µl of a 100-fold dilution of the overnight competitive selection culture as template in a SYBR green qPCR (SSO BioRad). The ΔCT for the wt and mutant primer pair was calculated and normalized to the ΔCT for the no antibiotic growth resulting in a $\Delta\Delta CT$ value.

Plating validation of the competitive growth selection.

After overnight competitive growth selection cultures were diluted and plated on LB with IPTG and Xgal.

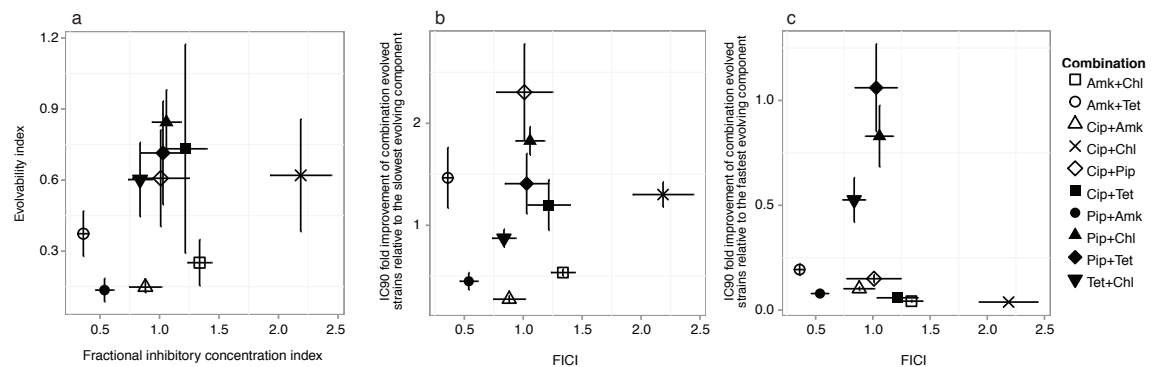
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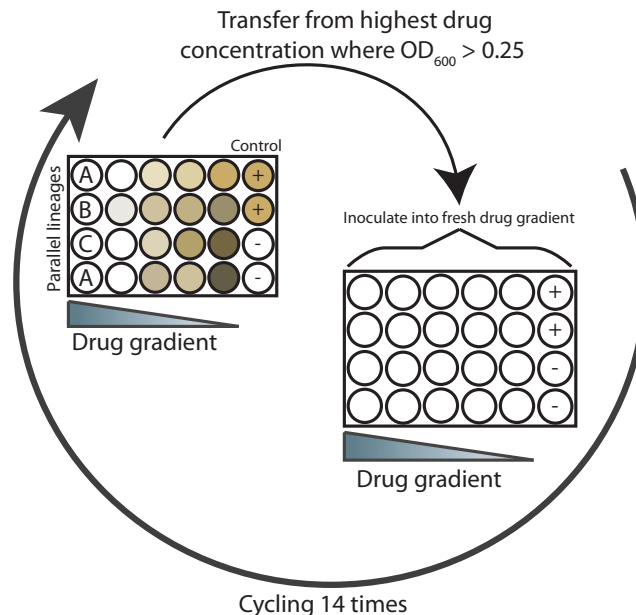
Extended data figure 1. Competition between WT Δ lacZ and mutant.

WT and mutant was mixed 1:1 and grown overnight in sub-inhibitory concentrations of AB and plated on LB+IPTG+Xgal. The presence of chloramphenicol selects against the three mutations that confers amikacin resistance (*sbmA*, *cpxA* and *fusA*). In contrast the *gyrA* mutation is always selected when ciprofloxacin is present and it is not counter selected by amikacin.

marR confers resistance to both tetracycline and chloramphenicol and is hence selected by both drugs.



Extended Data Figure 2. Epistatic interactions between the perturbations do not correlate with phenotypic evolution. Panel a; for each dual-perturbation the epistatic interaction was quantified by calculating the fractional inhibitory concentration index (FICI) (Loewe additivity) ²⁴(mean \pm s.e.m, $n = 3$ biological replicates). An index of 1 indicates no interaction between the perturbations whereas an index less than one indicates a synergistic interaction while an index greater than one indicated an antagonistic interaction. We found no correlation between the nature of the epistatic interaction and phenotypic evolution (measured as the evolvability index (mean \pm s.e.m, $n = 3$ biological replicates)) (Panel a; $r = 0.36$, $p > 0.05$, Pearson correlation). To investigate if different measures of the phenotypic evolutionary response correlated better with the drug interactions we also quantified the phenotypic evolution as the relative increase in IC90 divided by the relative increase of the slowest component (panel b) and the fastest component (panel c). However, we found no strong when comparing these different measures of phenotypic evolution to the FICI. (Panel b; $r = 0.11$, $p > 0.05$, Pearson correlation) and (Panel c; $r = -0.15$, $p > 0.05$, Pearson correlation).



Extended Data Figure 3. Overview of the experimental setup. The in vitro evolution experiment was conducted in 24 well plates with drug gradients across the columns. The last column contained positive and negative controls. Every 20 h cells were diluted 1:40 from the highest drug concentration where the OD600 was greater than 0.25 into a freshly prepared drug gradient. The rows contained the parallel-evolved lineages.

Loci	MAGE-Oligo
cpxA_G4102763A-	TTAATGTGGTGGCGCGCTCTGTCCGGGCGATTGATAAGTGGGTACCGCCAGGACAGCGTTTGTATTGGTGACCACCGAAGGCCGCGTG
fusA_G3469714A+	GAGTTTCTACTTCAACCTTCATGATCGGCTCAAGCAGAAGTGGTTTCACTTTCTTAAAGCCTTCTTTAAAGGCGATAGAAGCAGCCAGTT
sbmA_C396368A-	ATATGACGCAGTTGTTGCCAGTTGCGCCATGTAATATTCGTTTCATCTCTGTACGCCAGCGGAACACGTAGTGACTGACAAAGAAGTTGTTC
gyrA_T2337183C+	ACCGAAGTTACCCTGACCGTCTACCAGCATATAACGCAGCGAGAATGGCTGCGCCATGCGGACGATCGTGCCATAGACCGCCGAGTCACC
marR_C1617480T+	GTAACAACTTACCACCGGCGCGCGCAATATGTGAACAATGCCATTAATTAGTTGGCCAGGACCTGCACCAAGAATTAACAAAAACCTG

Extended Data Table 1. MAGE oligos.

The MAGE Oligos were designed according to (Wang and Church, 2011)²³. The oligo name indicates the SNP change at the position number according to E. coli MG1655 (Accession no. NC_000913). The + and – denote whether the oligo is on the plus or the minus strand.

Loci	WT forward primer	Mutant forward primer	Reverse primer
cpxA	ACGCTGTCCTGGCGGTG	ACGCTGTCCTGGCGGTA	CACGCCAGATGACCGAGC
fusA	GCTCAAGCAGAACTGGTTTCG	GCTCAAGCAGAACTGGTTTCA	TAATCCCTGGCGAATACATCC
sbmA	GTTCCGCTGGCGTACAGC	GTTCCGCTGGCGTACAGA	GCGGAGAGCGTTACCAGC
gyrA	CCATGCGGACGATCGTGT	CCATGCGGACGATCGTGC	GCGATGTCGGTCATTGTTGG
marR	GCAATATGTGAACAATGCCATC	GCAATATGTGAACAATGCCATT	TGGCGATTCCAGGTTGTCC

Extended Data Table 2. qPCR primers.

qPCR primers used to quantify the ration of mutant to wt allele. The forward qPCR primers are identical except for the 3' nucleotide being altered according to the introduced SNP. The reverse primer is common for both forward primers.

Extended Data Figure 1. Competition between mutant and a Δ lacZ wt MG1655.

Panel a-e, mutant and wt Δ lacZ were mixed 1:1 and grown overnight in sub-inhibitory concentrations of single- and dual-perturbations and plated on LB+IPTG+Xgal, here the mutant will appear blue while the wt will be white. The presence of chloramphenicol selects against the three mutations that confers amikacin resistance (sbmA, cpxA and fusA) even during growth in a combination of Amk+Chl (panel a-c). In contrast, the gyrA mutation is always selected when ciprofloxacin is present and it is not counter selected by amikacin (panel d). marR confers resistance to both tetracycline and chloramphenicol and is hence selected by both drugs (panel e).

Supplementary Table 1. Individual SNPs and INDELs.

A) SNPs that were identified in the WT non-evolved strain of MG1655 when compared to the deposited Genbank sequence of MG1655 (NC_000913). Annotations were obtained from the deposited Genbank record.

B) INDELs that were identified in the WT non-evolved strain of MG1655 when compared to the deposited Genbank sequence of MG1655 (NC_000913). Annotations were also obtained from the deposited Genbank record.

C) SNPs that were identified in the evolved strains based on comparison to the deposited Genbank sequence of MG1655 (NC_000913) with SNPs found in the non-evolved strains.

D) INDELs that were identified in the evolved strains based on comparison to the deposited Genbank sequence of MG1655 with INDELs found in the non-evolved strains.

A. SNPs Identified in the starting *E. coli* MG1655 Strain

Position	Mutation	Genbank ID	Gene	Product	Conservative
547694	A->G		ylbE		Yes
802885	C->A	NP_415292.2	ybhJ	predicted hydratase	No
1903785	G->A	NP_416335.4	yebN	conserved inner membrane protein	No
3957957	C->T				

B. INDELs Identified in the starting *E. coli* MG1655 Strain

From Position	Type	INDEL	Genbank ID	Gene	Product
547832	insertion	G->GG			

C. SNPs Identified in the evolved MG1655 Strain

Strain/AB_selection	Position	Mutation	Genbank ID	Gene	Product	Conservative
Amk_A	396442	G->A	NP_414911.1	sbmA	microcin B17 transporter	No
Amk_A	1363139	G->C	NP_415817.1	puuB	gamma-Glu-putrescine oxidase; FAD/NAD(P)-binding	No
Amk_A	1976527	G->T				
Amk_A	1976528	G->A				
Amk_A	3469498	T->C	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
Amk_A	3469708	G->T	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
Amk_A	3875481	A->C	NP_418153.4	yidB	conserved protein; DUF937 family	No
Amk_A	4102908	C->A	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
Amk_B	396368	C->A	NP_414911.1	sbmA	microcin B17 transporter	No
Amk_B	2021141	G->A	NP_416460.1	fliR	flagellar export pore protein	Yes
Amk_B	3469714	G->A	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
Amk_B	4102763	G->A	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
Amk_C	396611	G->A	NP_414911.1	sbmA	microcin B17 transporter	No

Amk_C	1976527	G->T				
Amk_C	1976528	G->A				
Amk_C	1990891	A->T				
Amk_C	2690454	C->T	YP_026170.1	purL	phosphoribosylformyl-glycineamide synthetase	No
Amk_C	3469709	G->T	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
Amk_C	4025094	T->A	NP_418286.1	fre	NAD(P)H-flavin reductase	No
Amk_C	4102449	A->T	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
AmkChl_A	4184421	C->G	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
AmkChl_B	1617423	C->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
AmkChl_B	1976527	G->T				
AmkTet_A	810513	G->T	NP_415297.1	bioF	8-amino-7-oxononanoate synthase	No
AmkTet_A	4101770	C->T	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
AmkTet_B	3469759	A->C	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
AmkTet_C	1285929	G->A				
AmkTet_C	3451124	C->G	NP_417780.1	rpsJ	30S ribosomal subunit protein S10	No
AmkTet_C	3470824	A->T	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
AmkTet_C	4092723	G->A				
AmkTet_C	4092726	C->T				
AmkTet_C	4092728	C->T				
AmkTet_C	4102680	T->A	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
AmlChl_C	1150959	T->C	NP_415612.1	acpP	acyl carrier protein (ACP)	No
AmlChl_C	1977294	C->A				
AmlChl_C	1998241	G->T	NP_416430.1	fliY	cystine transporter subunit	No
AmlChl_C	4102440	C->T	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
Chl_A	1976527	G->T				
Chl_A	1976528	G->A				
Chl_A	2089530	C->T	NP_416524.1	hisD	bifunctional histidinal dehydrogenase/histidinol dehydrogenase	No
Chl_A	4632601	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
Chl_B	1500383	G->T				
Chl_B	1617399	A->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
Chl_C	848200	G->A				
Chl_C	882884	C->A				
Chl_C	1977294	C->A				
Chl_C	3370896	G->A	NP_417692.1	nanA	N-acetylneuraminate lyase	Yes
Chl_C	4632867	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
ChlTet_A	1977294	C->A				
ChlTet_B	1617492	C->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No

ChlTet_B	1977291	C->T				
ChlTet_B	1977294	C->A				
ChlTet_B	3212876	G->A	NP_417539.1	rpoD	RNA polymerase; sigma 70 (sigma D) factor	No
ChlTet_B	4184369	G->T	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
ChlTet_C	1617492	C->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
ChlTet_C	3350464	A->C	YP_026207.1	arcB	aerobic respiration control sensor histidine protein kinase; cognate to two-component response regulators ArcA and RssB	No
Cip_A	1976527	G->T				
Cip_A	2337184	C->A	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Cip_A	2337220	C->T	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Cip_A	4187448	C->G	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
Cip_A	4187449	A->T	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	Yes
Cip_A	4187450	G->C	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
Cip_B	1195443	C->T	NP_415654.1	icd	e14 prophage	Yes
Cip_B	1195455	C->T	NP_415654.1	icd	e14 prophage	Yes
Cip_B	1195468	T->C	NP_415654.1	icd	e14 prophage	Yes
Cip_B	1195470	A->G	NP_415654.1	icd	e14 prophage	Yes
Cip_B	1210633	C->A				
Cip_B	1210635	A->G				
Cip_B	1976527	G->T				
Cip_B	1976528	G->A				
Cip_B	2337183	T->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Cip_B	3876753	A->C	YP_026241.1	gyrB	DNA gyrase; subunit B	No
Cip_B	4632867	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
Cip_C	485058	C->A	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
Cip_C	2337183	T->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Cip_C	2337202	C->A	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Cip_C	3256446	A->C	YP_026204.1	tdcG	L-serine dehydratase 3	No
CipAmk_A	442247	T->C				
CipAmk_A	1990685	C->T	NP_416422.1	pgsA	phosphatidylglycerophosphate synthetase	No
CipAmk_A	4092726	C->T				
CipAmk_A	4092728	C->T				
CipAmk_A	4102470	G->A	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
CipAmk_B	485144	G->T	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
CipAmk_B	1976527	G->T				
CipAmk_B	1976528	G->A				
CipAmk_B	3469714	G->T	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
CipAmk_C	1977294	C->A				
CipAmk_C	2069371	T->A		isrC		No

CipAmk_C	2337183	T->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
CipAmk_C	2398061	C->T	NP_416786.4	nuoG	NADH:ubiquinone oxidoreductase; chain G	No
CipAmk_C	3469714	G->T	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
CipAmk_C	4031406	T->A	YP_026273.1	trkH	potassium transporter	No
CipAmk_C	4275937	T->A	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing	No
CipChl_A	1195443	C->T	NP_415654.1	icd	e14 prophage	Yes
CipChl_A	1195455	C->T	NP_415654.1	icd	e14 prophage	Yes
CipChl_A	1210633	C->A				
CipChl_A	1210635	A->G				
CipChl_A	1617394	T->A	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
CipChl_A	1718801	C->T	NP_416159.2	slyA	DNA-binding transcriptional activator	No
CipChl_A	1976528	G->A				
CipChl_A	2040948	A->G				
CipChl_B	4187354	A->C	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
CipChl_B	4275549	C->T	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing	No
CipChl_C	111493	G->T				
CipChl_C	882884	C->A				
CipPip_A	156256	T->G				
CipPip_A	2337183	T->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
CipPip_A	4183254	G->T	NP_418414.1	rpoB	RNA polymerase; beta subunit	No
CipPip_A	4632867	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
CipPip_B	1622859	A->C	NP_416055.1	ydeJ	conserved protein	Yes
CipPip_B	1976527	G->T				
CipPip_B	1976528	G->A				
CipPip_B	3534356	G->A	NP_417864.1	ompR	DNA-binding response regulator in two-component regulatory system with EnvZ	No
CipPip_B	4187442	T->G	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
CipPip_B	4275914	A->G	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing	Yes
CipPip_C	92851	G->T	NP_414626.1	ftsI	transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)	No
CipPip_C	92852	C->T	NP_414626.1	ftsI	transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)	Yes
CipPip_C	2337202	C->A	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
CipPip_C	3534290	G->T	NP_417864.1	ompR	DNA-binding response regulator in two-component regulatory system with EnvZ	No
CipTet_B	1976527	G->T				
CipTet_B	1976528	G->A				
CipTet_B	3048873	T->C				
CipTet_B	3533133	C->T	NP_417863.1	envZ	sensory histidine kinase in two-component regulatory system with OmpR	No

CipTet_C	1363138	C->G	NP_415817.1	puuB	gamma-Glu-putrescine oxidase; FAD/NAD(P)-binding	No
CipTet_C	1363139	G->A	NP_415817.1	puuB	gamma-Glu-putrescine oxidase; FAD/NAD(P)-binding	No
CipTet_C	1617483	T->G	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
CipTet_C	2337201	C->T	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A	No
Pip_A	92851	G->T	NP_414626.1	ftsI	transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)	No
Pip_A	485070	T->G	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
Pip_A	1363140	C->G	NP_415817.1	puuB	gamma-Glu-putrescine oxidase; FAD/NAD(P)-binding	No
Pip_A	1617132	A->C				
Pip_A	1617133	G->C				
Pip_A	1617134	G->T				
Pip_B	1617133	G->C				
Pip_B	1617134	G->T				
Pip_B	4183082	A->G	NP_418414.1	rpoB	RNA polymerase; beta subunit	No
Pip_C	92326	C->T	NP_414626.1	ftsI	transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)	No
Pip_C	99209	G->T	NP_414631.1	ftsW	Lipid II flippase	No
Pip_C	1976527	G->T				
Pip_C	1977294	C->A				
Pip_C	3533169	A->C	NP_417863.1	envZ	sensory histidine kinase in two-component regulatory system with OmpR	No
PipAmk_A	485598	G->A	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
PipAmk_A	4102886	A->G	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
PipAmk_B	3470123	G->A	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
PipAmk_C	2623803	G->C	NP_416997.1	ppx	exopolyphosphatase	No
PipAmk_C	3471188	C->A	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding	No
PipAmk_C	4102452	C->T	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR	No
PipAmk_C	4632747	A->C	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
PipChl_A	485024	C->T	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
PipChl_A	1976527	G->T				
PipChl_A	1976528	G->A				
PipChl_A	4187340	C->T	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
PipChl_A	4275549	C->T	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing	No
PipChl_B	1976527	G->T				
PipChl_B	1976528	G->A				
PipChl_B	4184379	G->C	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
PipChl_B	4632867	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
PipChl_C	484997	A->G	NP_414997.1	acrR	DNA-binding transcriptional repressor	No

PipChl_C	1976527	G->T				
PipChl_C	3214645	T->C				
PipChl_C	4632867	C->T	NP_418813.1	rob	right oriC-binding transcriptional activator; AraC family	No
PipTet_A	485076	C->A	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
PipTet_A	1906727	G->A	NP_416340.1	mgrB	regulatory peptide for PhoPQ; feedback inhibition	No
PipTet_A	1976527	G->T				
PipTet_A	1976528	G->A				
PipTet_A	3533586	G->A	NP_417863.1	envZ	sensory histidine kinase in two-component regulatory system with OmpR	No
PipTet_B	484998	C->T	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
PipTet_B	2729754	T->A	NP_417083.1	clpB	protein disaggregation chaperone	Yes
PipTet_B	4187339	G->A	NP_418415.1	rpoC	RNA polymerase; beta prime subunit	No
PipTet_C	484997	A->G	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
PipTet_C	1976527	G->T				
PipTet_C	1976528	G->A				
PipTet_C	3533368	C->T	NP_417863.1	envZ	sensory histidine kinase in two-component regulatory system with OmpR	No
PipTet_C	4183090	G->T	NP_418414.1	rpoB	RNA polymerase; beta subunit	No
Tet_A	484987	G->A	NP_414997.1	acrR	DNA-binding transcriptional repressor	No
Tet_A	1617480	C->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance	No
Tet_A	3438262	C->T	NP_417754.1	rpoA	RNA polymerase; alpha subunit	No
Tet_B	483223	G->T	NP_414995.1	acrB	multidrug efflux system protein	No
Tet_B	985121	A->C	NP_415449.1	ompF	outer membrane porin 1a (Ia)	No
Tet_C	985350	G->A	NP_415449.1	ompF	outer membrane porin 1a (Ia)	No

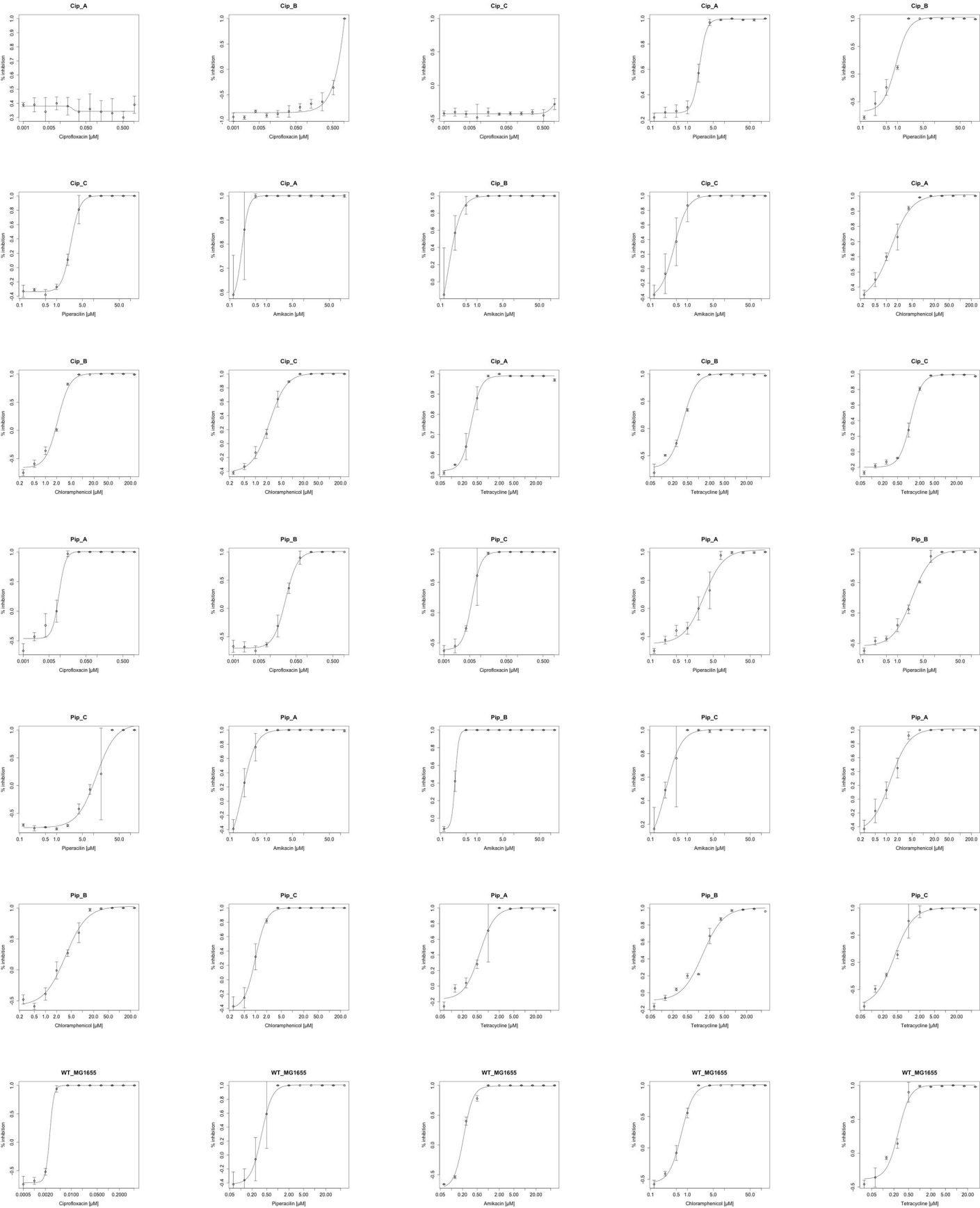
D. INDELs Identified in the evolved MG1655 Strain

Strain/AB_selection	From Position	Type	INDEL	Genbank ID	Gene	Product
Amk_B	753063	insertion	C->CTACGGTTCGCAC	NP_415248.1	gltA	citrate synthase
Amk_B	2925947	insertion	T->TTATAT			
Amk_B	4081530	deletion	GCCATGC->G	NP_418330.1	fdoG	formate dehydrogenase-O; large subunit
AmkTet_A	1617207	insertion	T->TAATC	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance
AmkTet_A	3469704	insertion	C->CAAC	NP_417799.1	fusA	protein chain elongation factor EF-G; GTP-binding
AmkTet_A	4468534	deletion	CCG->C			
AmkTet_B	484953	deletion	AT->A			
Chl_A	882788	insertion	A->AGTAATAAT			
Chl_B	4254115	deletion	ACACTGG->A	NP_418465.4	plsB	glycerol-3-phosphate O-acyltransferase
ChITet_A	1617515	deletion	CA->C	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance

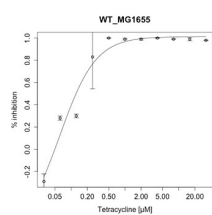
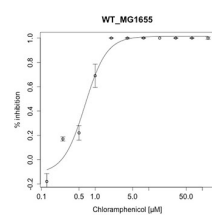
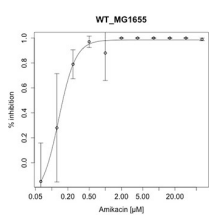
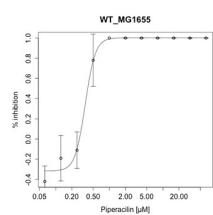
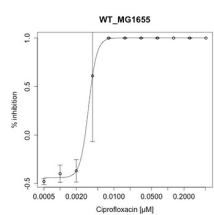
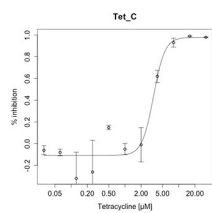
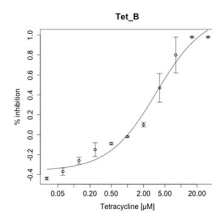
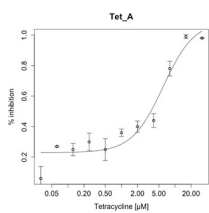
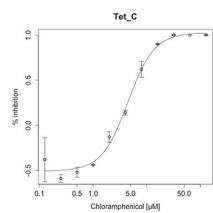
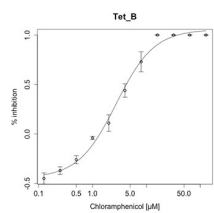
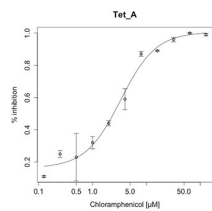
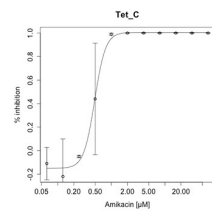
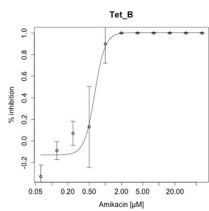
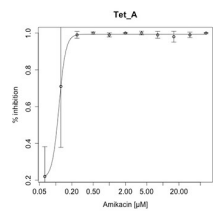
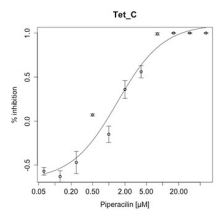
Cip_A	4275869	deletion	CGCG->C	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing
Cip_C	987835	insertion	G->GGCG	NP_415450.1	asnS	asparaginyl tRNA synthetase
Cip_C	1142060	insertion	A->ACCATCGC	NP_415602.1	rne	fused ribonucleaseE: endoribonuclease/RNA-binding protein/RNA degradosome binding protein
Cip_C	3445977	deletion	TGG->T	NP_417769.1	rplN	50S ribosomal subunit protein L14
Cip_C	4275871	deletion	CGCA->C	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing
CipAmk_A	485481	deletion	AT->A	NP_414997.1	acrR	DNA-binding transcriptional repressor
CipAmk_A	2337194	deletion	CCGA->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A
CipAmk_B	2337194	deletion	CCGA->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A
CipChl_A	458212	deletion	GA->G	NP_414973.1	lon	DNA-binding ATP-dependent protease La
CipChl_A	2337194	deletion	CCGA->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A
CipChl_B	485598	insertion	C->CGCC	NP_414997.1	acrR	DNA-binding transcriptional repressor
CipChl_B	986403	deletion	TAAC->T			
CipChl_C	2337194	deletion	CCGA->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A
CipChl_C	4275869	deletion	CGCG->C	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing
CipPip_B	485036	insertion	T->TG	NP_414997.1	acrR	DNA-binding transcriptional repressor
CipPip_B	2337194	deletion	CCGA->C	NP_416734.1	gyrA	DNA gyrase (type II topoisomerase); subunit A
CipPip_C	485136	deletion	CA->C	NP_414997.1	acrR	DNA-binding transcriptional repressor
CipPip_C	4275889	insertion	A->AGTA	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing
CipTet_A	986138	deletion	TGCAG->T	NP_415449.1	ompF	outer membrane porin 1a (Ia)
CipTet_A	4184028	insertion	C->CAGC	NP_418415.1	rpoC	RNA polymerase; beta prime subunit
CipTet_A	4275869	deletion	CGCG->C	NP_418487.1	soxR	DNA-binding transcriptional dual regulator; Fe-S center for redox-sensing
CipTet_B	2105	deletion	CATCA->C	NP_414543.1	thrA	fused aspartokinase I and homoserine dehydrogenase I
CipTet_B	484937	deletion	AT->A			

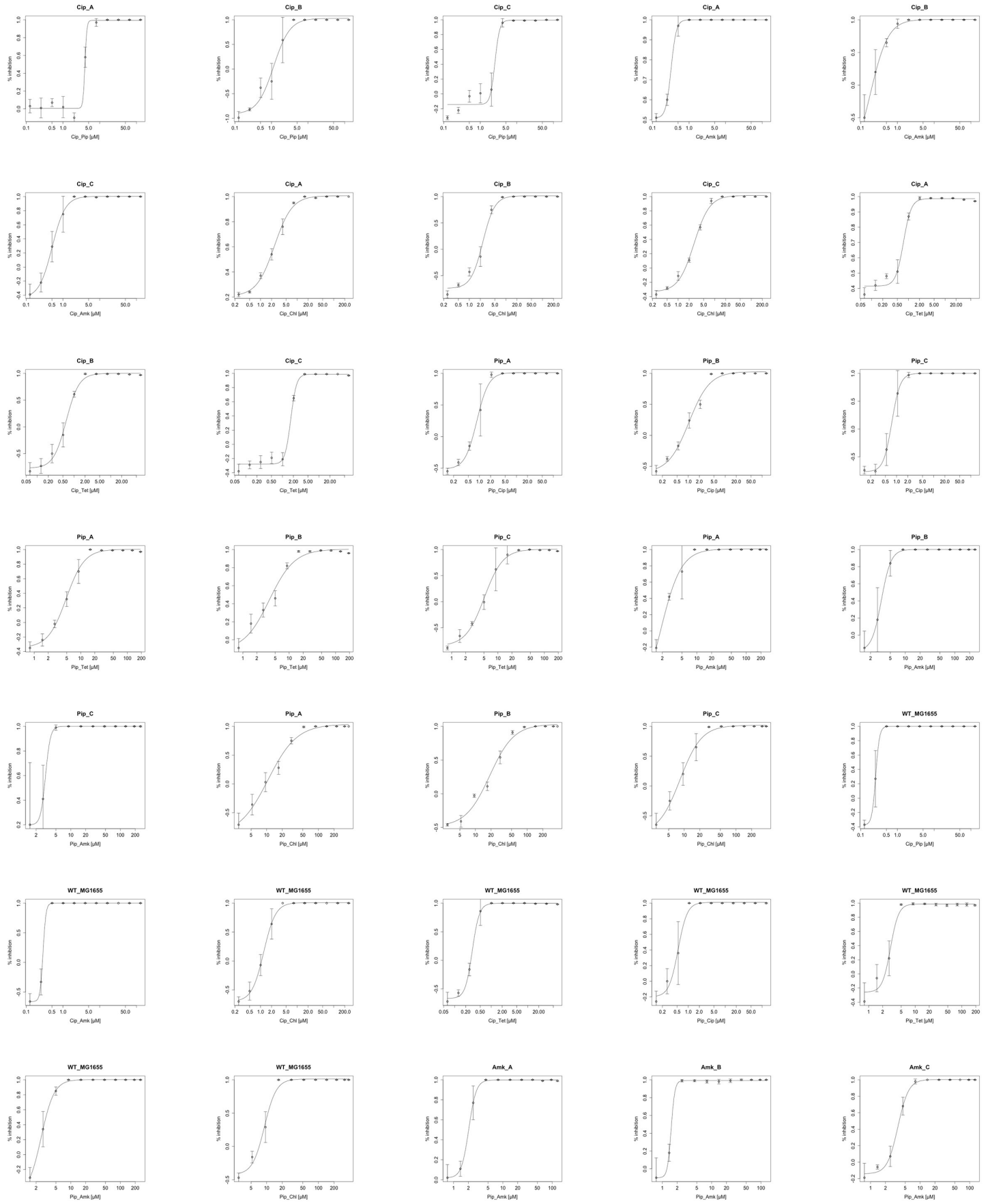
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Pip_A	1617115	deletion	TGCAACTAATTACTTGCCAGG->T			
Pip_B	1712342	deletion	CT->C			
PipAmk_B	4102928	insertion	C->CACCAACATCAAC	NP_418347.1	cpxA	sensory histidine kinase in two-component regulatory system with CpxR
PipTet_B	1617324	insertion	A->AA	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance
PipTet_B	3533825	deletion	GCAAGGTGACGAT->G	NP_417863.1	envZ	sensory histidine kinase in two-component regulatory system with OmpR
PipTet_C	1617318	deletion	TG->T	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance
Tet_B	1617384	insertion	A->AAAAGGCTGGGTGG	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance
Tet_C	1617384	insertion	A->AAAAGGCTGGGTGG	NP_416047.4	marR	DNA-binding transcriptional repressor of multiple antibiotic resistance

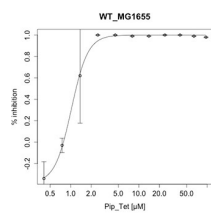
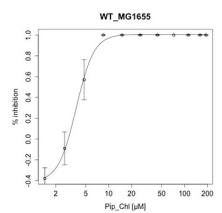
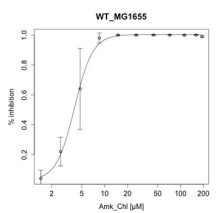
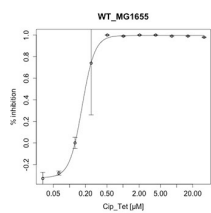
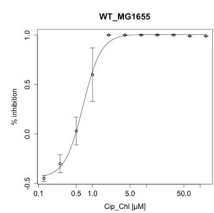
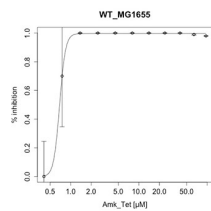
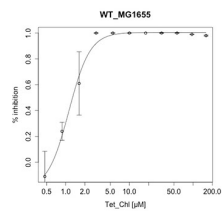
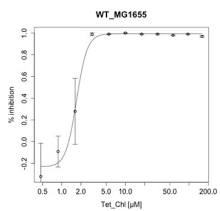
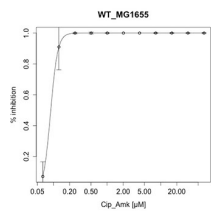
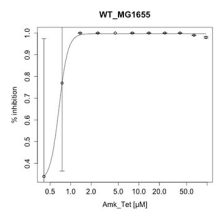
Supplementary Data 1: Dose-response curves
Dose-response curves for the evolved and the wt strains. The header denotes the evolved-to condition. The X-axis is the antibiotic concentration in μM , the Y-axis is the % inhibition.

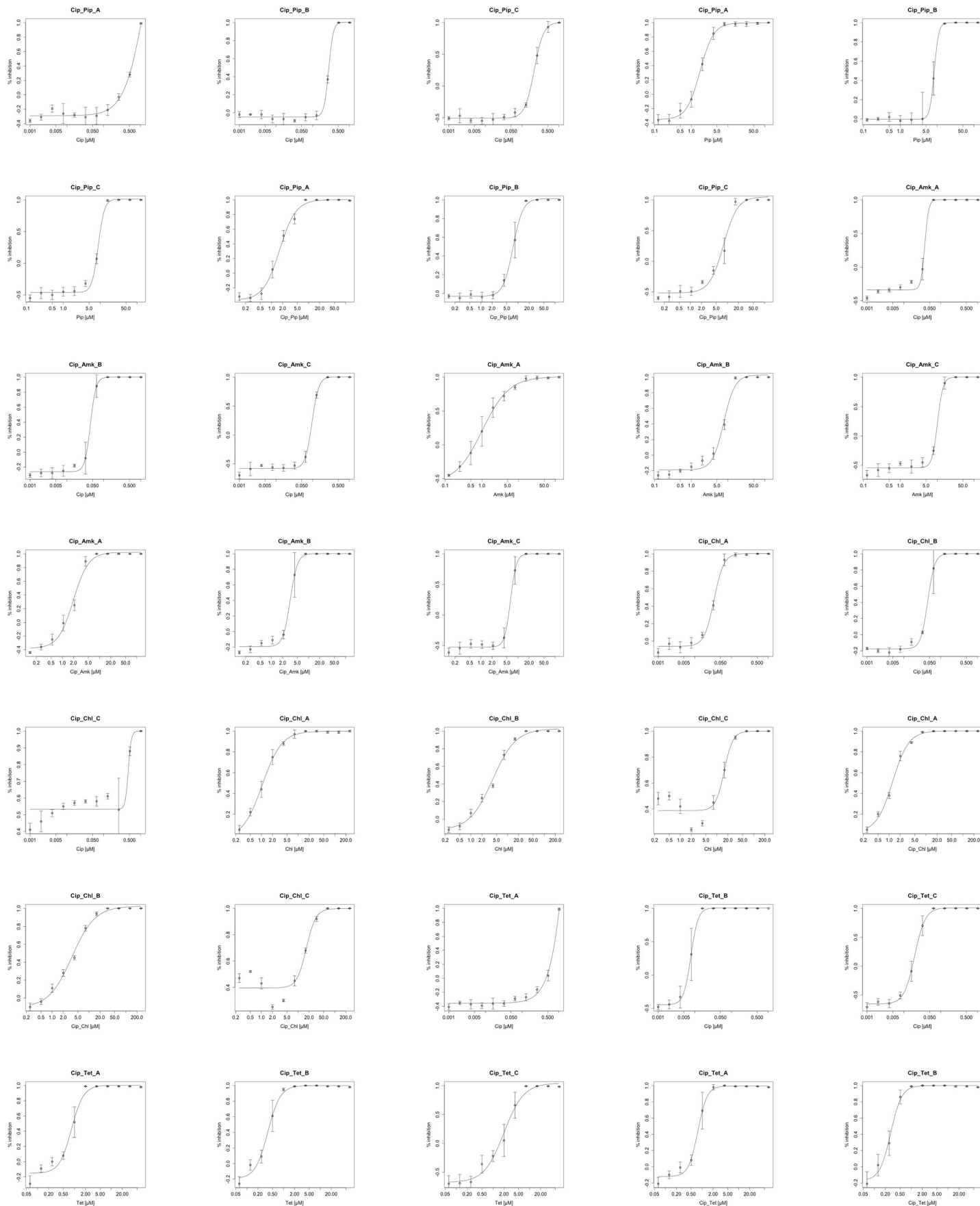


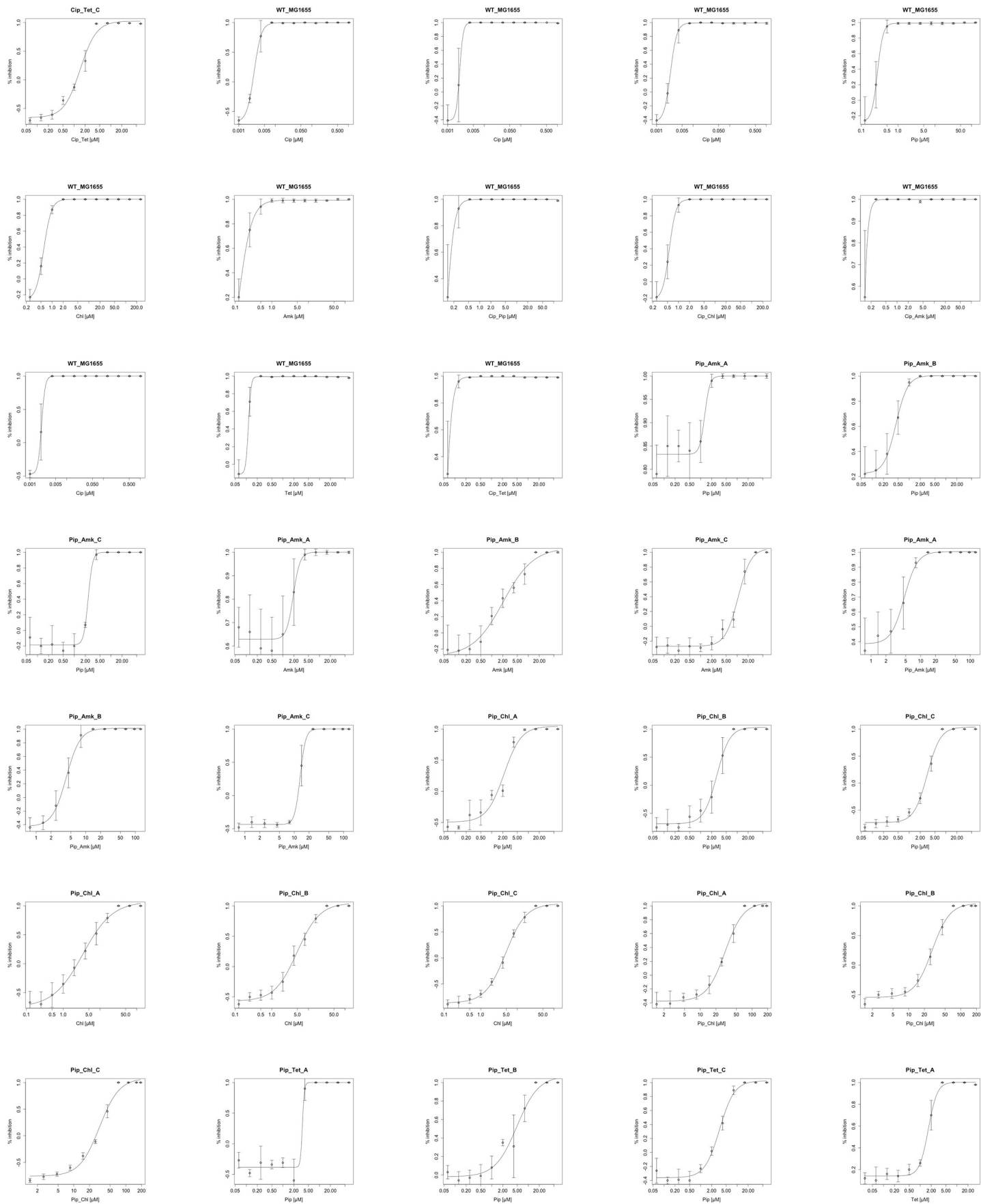


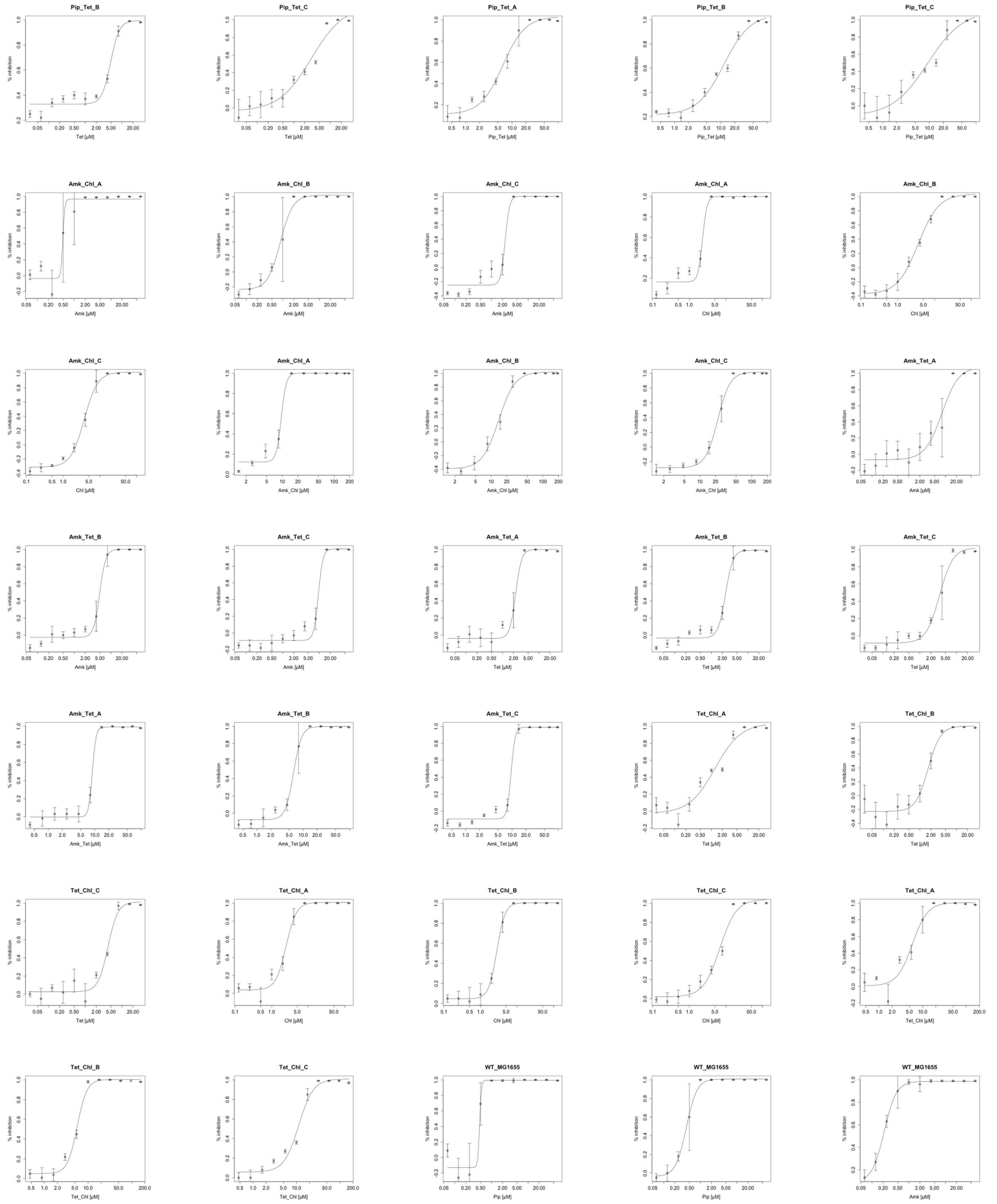


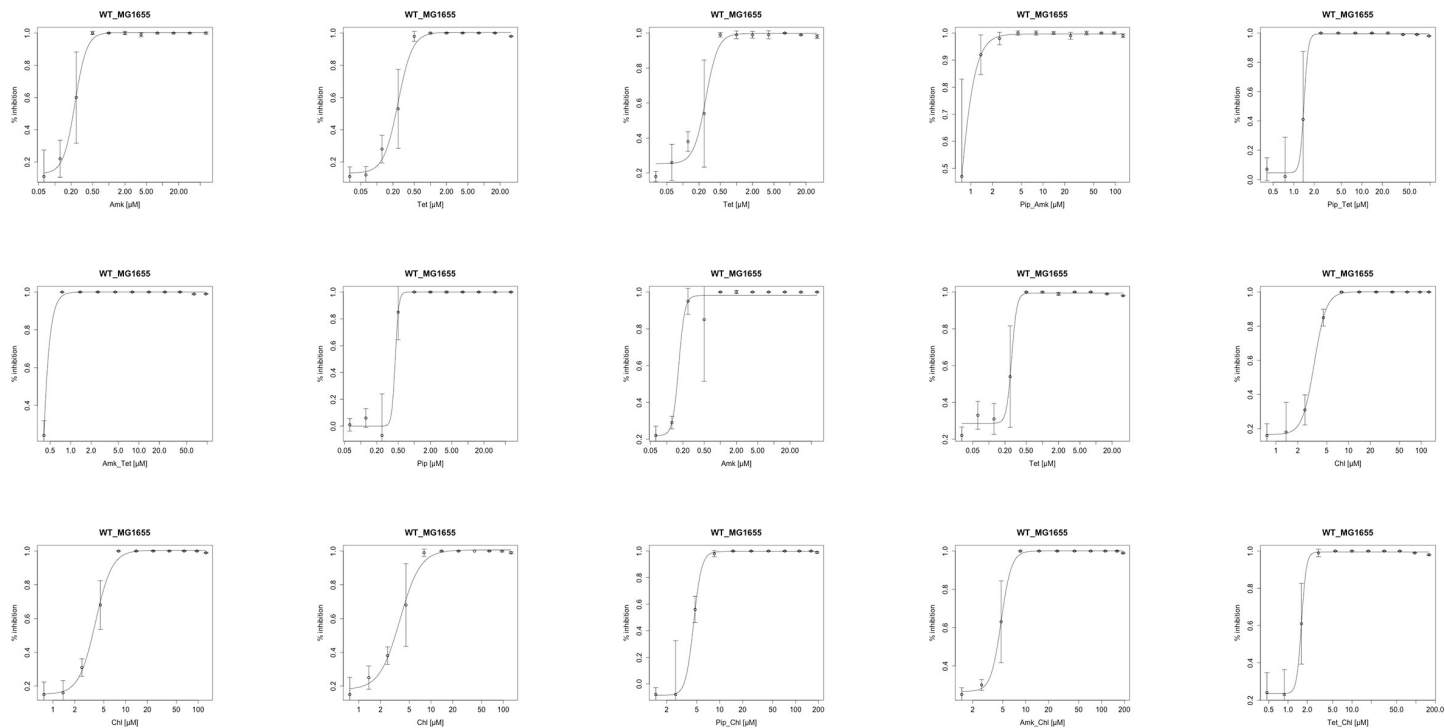












Supplementary information

Evolution of high-level resistance to drug combination treatment is not predicted by epistatic drug interactions

1. Detailed Analysis of Observed Mutations
2. Supplementary Figure S1

1. Detailed analysis of observed mutations

Erythromycin.

The target of macrolides is the protein synthesis process where they disrupt peptide chain elongation by binding near the peptide chain exit located on the 50S subunit and blocking the exit tunnel (1-3). Large macrolides can directly affect peptide bond formation in the peptidyl transferase center by extending up into the exit tunnel (4, 5). Macrolides are also known to prevent the assembly of new ribosomal units (6-9).

Mutations at or near nucleotide site 2058 (*Escherichia coli* reference) alter the macrolide binding point in the 50S ribosome providing resistance against macrolide drugs. Modifications at this site are generally the result of erm associated methyltransferase (10, 11), which is usually achieved via a resistance gene not present in *Staphylococcus aureus* Newman (12). In the absence of this gene, alteration of the macrolide binding site can be achieved by mutations to rplD and rplV genes (13), which respectively code for L4 and L22 ribosomal proteins. Both proteins are in proximity to the binding site, which is located near the exit tunnel at the back of the 50S ribosome (14, 15). Mutations in these proteins near the binding site can lead to changes in the exit tunnel shape thereby affecting rRNA access. Two out of three erythromycin evolved strains and two out of three combination evolved strains had mutations the rplD (L4) gene. Mutations in the gene coding for this ribosomal protein have been associated with macrolide resistance in several bacterial species (16-18) including *Staphylococcus aureus* (19) and are increasingly being observed in clinical isolates (20, 21). The most characterized erythromycin associated L4 mutations are those involving insertions or deletions (17), however neither phenomenon was observed here. Instead several different missense mutations were observed. Recent work has shown that over 200 different amino acid substitutions in the L4 and L22 proteins can result in macrolide resistance (13, 17) and that these substitution mutations do no adversely impact growth rate (13).

An six nucleotide duplication was observed in the extended loop region of the L22 ribosomal protein (coded by gene rplV) of erythromycin-fusidic acid evolved lineage A. Duplication mutations in L22 have been associated with erythromycin resistance in different organisms (18, 22-25), including *Staphylococcus aureus* (26). A duplication mutation of this size at a different residue position in this protein has previously been observed to slow the growth of an *E. coli* mutant (18), however growth experiments performed on our strain found no negative impact on growth rate.

In addition to primary target changes, auxiliary mutations were found in the erythromycin evolved strains and included modifications to ribosomal proteins L3 and L27 and the ribosomal RNA large subunit methyltransferase N (NWMN1128). Three missense mutations in ribosomal protein L3 were found in erythromycin evolved lineage B. Ribosomal protein L3 is a secondary binding protein in 12S region of the 50S ribosome (27). Mutations to this particular ribosomal protein have been associated with linezolid (28-30) and tiamulin (31) resistance. Subsequent experiments to test for linezolid cross resistance in this particular lineage did not find additional resistance beyond established MIC values. Lineage B also had a single missense mutation in NWMN 1128, which is responsible for the methylation of the adenine at position 2503 in the 23S rRNA (32). The adenosine of 2503 is part of a group of adenosines located at the narrow entrance to the peptide exit tunnel (33). This group is thought to play a “quality control role” in monitoring peptides leaving the peptide tunnel (34). The position of 2503 is close to the binding sites of several peptidyl transferase center antibiotics (1, 35)

and modification of the adenosine at 2503 is known to confer resistance to drugs targeting this region (32, 36). Together the supplementary ribosomal mutations observed erythromycin lineage B most likely serve in a complementary capacity to the primary target mutation in ribosomal protein L4. The single mutation found in ribosomal protein L27 in lineage A may also serve in a complimentary role to the mutation in L4. Ribosomal protein L27 plays a critical role in the proper functioning of the 50S ribosome (37, 38). Organisms without this protein have hindered growth, impacted ribosome assembly and reduced peptidyl transferase activity (37). The role of ribosomal protein L27 in resistance is unknown.

Fusidic acid and amikacin.

Mutations to the *fusA* gene were observed in the *fus*-ery, *fus*-amik CE lineages as well as in the *fus*, *amik* and *ery* strains. Subsequent cross-resistance tests determined that the erythromycin-evolved strain was not resistant to fusidic acid above the WT MIC. *fusA* gene mutations in the amikacin evolved strains were conferred resistance to both amikacin and fusidic acid. No aminoglycoside primary target mutations were observed in the amikacin-evolved lineages.

The mechanisms of action for fusidic acid and amikacin are distinctly different; however, the targets of these two drugs are similar, namely the translocation step of protein synthesis. Fusidic acid targets the final step of the protein synthesis process by preventing the release of elongation factor G from the ribosome. These two agents work together to catalyze the movement of the mRNA-tRNA complex in the final step of peptide chain elongation (39). When fused together elongation G and the ribosome block the next stage of protein synthesis (40, 41).

Amikacin, an aminoglycoside, has a two-pronged bactericidal effect. First, it disrupts the protein synthesis process by binding to a region on the 30S ribosome, which is critical for translational fidelity (42). Second, it can disrupt the integrity of the cell envelope if mistranslated proteins are inserted into the cell membrane thereby affecting permeability and leading to an increased uptake of amikacin (43, 44). Mutations in the *fusA* gene resulting in aminoglycoside resistance have previously been observed in *Staphylococcus aureus* (45, 46).

Amino acid substitutions in the *fusA* gene (codes for EF-G) are the most characterized mechanism of fusidic acid resistance. To date there are more than 30 substitutions in *fusA*, which are known to confer varying extents of resistance (47-49). With the exception of just two mutations, all missense mutations found in our evolved fusidic acid, amikacin, erythromycin and fusidic acid combinations strains have previously been described (46, 48, 50-55). The two novel mutational changes observed were amino acid substitutions E444L in the fusidic acid-amikacin combination evolved lineage C and I619F in the amikacin-evolved lineage B. The fusidic acid-amikacin combination strain C had substitutions in two amino acids- 444 and 457. Amino acid 444 is situated in a known resistance region (47) and is therefore unsurprising. Substitutions to amino acid 457 are frequently part of a mutation combination (49). This may suggest that E444L/H457Q is a new resistance mutation combination. The I619F mutation is particularly interesting as it is outside the known resistance regions and is the only *fusA* mutation observed in amikacin evolved lineage B. Moreover, this particular strain did not possess any additional mutations associated with fusidic acid or amikacin resistance.

Mutations to the *fusA* gene are known to have fitness costs. The extent of these costs varies from severe to negligible depending on which amino acid is affected. Earlier work by Nagaev (47) demonstrated that the fitness costs of many of the mutations observed in this study may be considered moderate (average relative growth rate 80% of wild type) to negligible (no difference between mutant and wild type growth rate). A severe fitness cost was observed for only one substitution (T436I) present in fusidic acid-amikacin combination evolved lineage A. The cost of this particular substitution has previously been shown to reduce growth rate of *Staphylococcus aureus* in LB to 58% of the wild type (47). Strains containing moderate fitness cost mutations were found to have similar to wild type growth rates, perhaps indicating that under conditions where competition is absent, the presence of *fusA* mutations makes little difference to a strain's fitness (Table S4). Fusidic acid evolved lineage B is notable because it is the only isolate to have mutations in an alternative fusidic acid resistance mechanism, gene *rplF*, which codes for the ribosomal protein L6. Resistance solely due to mutations in this gene is rare and is known to have a large fitness cost (46). Growth experiments demonstrated that the additional mutation did not adversely impact the growth rate of this particular lineage, as the doubling time of this strain was similar to the wild type. This finding coupled with the presence of a

mutation in the primary fusidic acid resistance mechanism suggests that the rplF mutation is most plays a supplementary role at best.

Ciprofloxacin and ampicillin.

Sequencing results of the ciprofloxacin-ampicillin group of strains showed an interesting cascade of mutations. Ciprofloxacin-evolved strains were observed to have mutations in the parC and gyr A genes, both of which have been well documented in conjunction with ciprofloxacin resistance (56), while ampicillin-evolved lineages had mutations in the pbpA gene. In addition to their respective primary target mutations, both sets of evolved strains had mutations in the SaeS gene, part of a two component regulatory system directly involved in the expression of several pathogenicity gene and virulence factors. The SaeR/S system is known to be activated in response to environmental conditions (57), including sub inhibitory concentrations of beta lactams (58, 59), which may explain the mutations observed in the ampicillin evolved strains. The SaeR/S system does not appear to be responsible for resistance to beta lactams (59).

The ciprofloxacin-ampicillin combination evolved strains also had mutations in the parC and pbpA genes and shared an unusual mutation with the ampicillin-evolved strains. Both evolved groups had mutations in the NWMN13 gene, a hypothetical signaling protein with strong associations to rplI (50S ribosomal protein L9) and cca (a cca adding enzyme), as predicted by STRING 9.0 (60). Mutations in NWMN13 may be in response to environmental stress brought on by the presence of ampicillin. The link between stress response genes and resistance remains unknown for many strains (61).

It is no surprise that supplementary mutations may arise as a result of the stressful environment created by two drugs. Mutations in the relA, NWMN600, and NWMN1950 genes were unique to the combination-evolved strains and probably serve as added support mechanism to primary target mutations. The relA gene controls the production of the alarmone ppGpp, which serves as global transcriptional regulator and signal that affects a variety of metabolic pathways and bacterial physiological processes, including cell wall synthesis, ribosome synthesis, virulence and nucleic acid metabolism, and is triggered by environmental stress. The relationship between production of ppGpp and antibiotic resistance is well established (62, 63). Increased ppGpp production results in a slowing or shut down of cell wall synthesis, leading to a loss of target for beta lactam drugs (64, 65). This phenomena was recently observed in *Staphylococcus* isolates, where a single nucleotide substitution in relA resulted in a prolonged stringent response activity and reduced growth of clinical *Staphylococcus* (66). Results of the growth kinetics tests did not reveal an adverse effect in the growth rate of combination-evolved strains with relA mutations (Table S4). Elevated ppGpp concentrations have also been linked to fluoroquinolone survival in *Pseudomonas aeruginosa* (67, 68). Perhaps a similar protective benefit may be yielded under the experimental conditions here. Finally, it has been suggested that ppGpp may also play a role in drug efflux pump regulation (69). This proposed function provides an additional resistance mechanism, which may help to explain the mutations in NWMN600, a Na⁺/H⁺ exchange protein, and NWMN1950, an ammonium transporter, which were unique to the combination-evolved strains.

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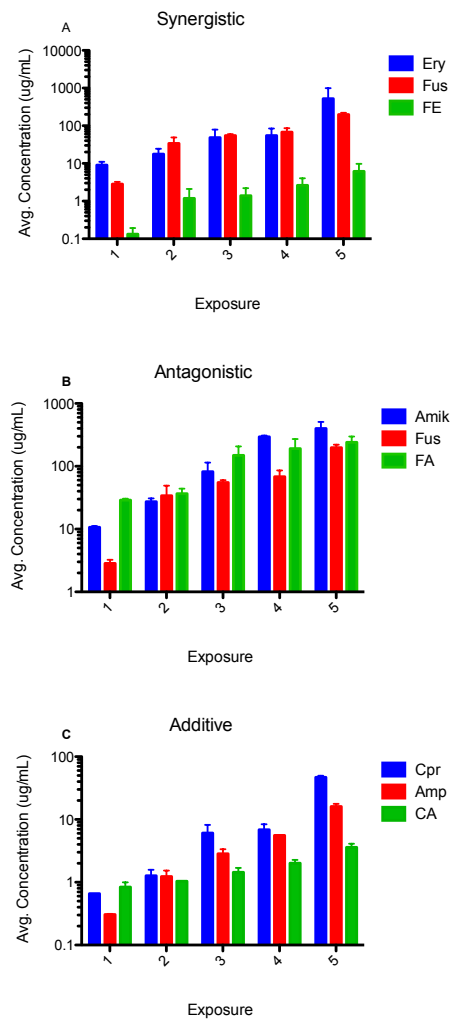


Figure S1. Averaged resistance evolution concentrations for each of the conditions investigated. Individual drugs are shown in blue and red, while drug combinations are displayed in green. Values shown were determined by taking an average of all the replicate concentrations for each lineage.

Table S2. Clinical breakpoints as published by EUCAST and the final resistance concentrations for each antibiotic used in this work.

Drug	Resistance breakpoint (ug/mL)	Final Experimental Conc (ug/mL)		
		Replicate A	Replicate B	Replicate C
Amikacin	16	273	615	308
Ampicillin	NR	13	19	17
Ciprofloxacin	1	41	50	50
Erythromycin	2	12	1468	92
Fusidic Acid	1	232	155	207

NR = not reported

Resistant values taken from EUCAST Clinical breakpoints tables published online 2013-02-11
http://www.eucast.org/clinical_breakpoints/

Table S3. Calculated evolvability index (EI) for each drug pair investigated.

Combination	EI
Fus-Ery	0.04
Cpr-Amp	0.14
Fus-Amik	0.44

Section 2

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Horizontal dissemination of antibiotic resistance genes

Introduction to section 2

Horizontal transfer of antibiotic resistance genes

Resistance genes predate anthropogenic production and dissemination of antimicrobial agents, highlighting that they belong to the core repertoire of bacterial functionality^{1,2}. Furthermore, genes conferring resistance to penicillin have been predicted to be located on plasmids before the wide use of penicillin showing that even mobilization of resistance genes predates the period of high antibiotic consumption³. However, since the mid 1900s the rise in antibiotic consumption has selected strongly for mobilization and dissemination of antibiotic resistance genes, a process mainly facilitated through horizontal gene transfer (HGT)⁴. HGT short-circuits traditional vertical evolution and allows bacterial to share favorable genes even across diverging phylogeny⁵. In contrast to mutational responses, it offers a rapid response to changes in the environment. Consequently, HGT play a key role in the emergence and dissemination of antibiotics resistance genes.

Commonly, the resistance gene reservoir within bacterial communities is referred to as the “resistome”⁶. As such it represents all genes that have the potential to become a resistance gene. Transition of genes from the resistome to the pool of clinical relevant resistance genes is believed to be an important source of novel resistance genes^{4,7,8}. This process involves horizontal transfer of antibiotic resistance genes from environmental species to human pathogens, which can occur via conjugation, transduction or natural competence. In the case of plasmid-mediated transfer, the resistance gene needs to be mobilized onto the plasmids⁹. This process is facilitated by transposons or integrons that can hijack chromosomal encoded genes and move them to other genetic locations. Such mobilization processes is the first step in bridging environmental resistance gene reservoirs with clinical relevant strains.

Studies of the current pandemic extended spectrum beta-lactamase (ESBL) family, CTX-M, indicate that this enzyme originate from the chromosome of *Kluyvera* spp.¹⁰. The CTX-M enzyme family has in fewer than 10 years become the dominating ESBL gene family and exemplifies the process of mobilization and dissemination of novel resistance genes from environmental gene reservoirs to human pathogens¹¹.

As a consequence, there has been an increased interest in studying the resistome of different

environments¹². Traditionally this has been done using PCR or qPCR to look for specific resistance genes in total DNA from environmental samples. However, an increasing number of studies have also used a more unbiased functional selection approach to uncover resistance gene reservoirs^{13,14}.

Even though many studies have suggested that environmental reservoirs are key sources of clinically relevant antibiotic resistance genes, more recent studies indicate that HGT is confined by environmental barriers^{15,16}.

In a study by Forsberg et al. functional metagenomic screening was applied to identify resistance genes in different soil samples¹⁵. The identified genes were compared to resistance genes in clinically relevant species and they found that only 7 out of 110 antibiotic resistance genes were shared with clinical relevant species. This shows that even though antibiotic resistance genes are ubiquitous to soil environments, only a small subset are shared with human pathogens.

It is believed that studying resistance reservoirs of different environments gives insight into possible future antibiotic resistance genes that can become major threats to human health¹². An environment of particular concern is wastewater treatment plants (WWTPs). In this environment bacteria from many different origins are mixed, providing the perfect conditions for extensive gene exchange. The majority of bacteria that enter these facilities come from either humans or animals and many have been exposed to antibiotics with consequent selection of antibiotic resistance. In addition, pathogenic bacteria also enter the WWTPs especially from wastewater from hospital sources. Resultantly, WWTP are conceived as the ideal site for human pathogens to acquire novel resistance genes^{8,12,17,18}.

Thesis work

Functional metagenomic selection

A short perspective article that gives an in-depth description of the advantages and disadvantages of functional metagenomic selection.

Resistance gene reservoir of wastewater treatment plants

We wanted to comprehensively map the resistance gene reservoir in wastewater treatment plants (WWTPs) and identify the overlap with clinically relevant bacteria. We did this by combining functional metagenomic selection with metagenomic sequencing. We found that the core antibiotic resistome of WWTPs is highly stable and shared across different WWTPs. Furthermore, we found that only 6 of the 80 identified WWTP resistance genes had an identity of >95 % to the GenBank database, highlighting that there is very little overlap between WWTPs' resistance gene reservoir and other environments, including human pathogens.

This conclusion challenges the common belief that WWTPs play a key role in dissemination of antibiotic resistance genes. Instead, it suggests that mobilization and dissemination across environmental niches is a significant barrier preventing the spread of resistance genes.

Horizontal gene transfer in the human gut

Via a collaboration with the Clinical Microbiology Department at the Sahlgrenska University Hospital in Sweden, we had the opportunity to study horizontal gene transfer (HGT) events occurring *in situ* in the human gut. As a part of the ALLERGYFLORA study, clinicians at Sahlgrenska sampled the infant gut-microbiota to study its relations to the development of allergy¹⁹. Fecal samples were collected from the enrolled infants over their first year of life and were cultivated on different media selective for relevant species. This resulted in an *E. coli* collection that was MIC tested against several antibiotics^{19,20}. In one of the infants that had not received antibiotics, an *E. coli* isolate's resistance profile changed at the 2-month sampling point. Initially, isolates from this child were sensitive to ampicillin, piperacillin, streptomycin and sulfamethoxazole. However, after the two-months sampling point, they were resistant to these drugs.

In a study headed by my colleague Heidi Gumpert, we investigated, at the genomic level, the changes in these *E. coli* strains to uncover the genetic events that lead to the change

in resistance profile. We found that horizontal transfer of a large multi-drug resistance plasmid was the cause of the change in resistance profile. This is to our knowledge, the first observation of HGT of antibiotic resistance genes *in situ* of the human gut without any antibiotic selection. In addition to the plasmid transfer, we also observed several remodeling events of the *E. coli* genomes, including large deletions and phage insertions. The results of this study show that dissemination of genetic material is inherent to bacterial communities, and that transfer of antibiotic resistance genes does not require a selective pressure. Furthermore, the study highlights the power of single isolate genome sequencing to uncover strain dynamics in a complex bacterial community.

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Manuscripts
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Section 2



Functional metagenomic investigations of the human intestinal microbiota

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The human intestinal microbiota encode multiple critical functions impacting human health, including metabolism of dietary substrate, prevention of pathogen invasion, immune system modulation, and provision of a reservoir of antibiotic resistance genes accessible to pathogens. The complexity of this microbial community, its recalcitrance to standard cultivation, and the immense diversity of its encoded genes has necessitated the development of novel molecular, microbiological, and genomic tools. Functional metagenomics is one such culture-independent technique, used for decades to study environmental microorganisms, but relatively recently applied to the study of the human commensal microbiota. Metagenomic functional screens characterize the functional capacity of a microbial community, independent of identity to known genes, by subjecting the metagenome to functional assays in a genetically tractable host. Here we highlight recent work applying this technique to study the functional diversity of the intestinal microbiota, and discuss how an approach combining high-throughput sequencing, cultivation, and metagenomic functional screens can improve our understanding of interactions between this complex community and its human host.

Keywords: functional metagenomics, human intestinal microbiota, antibiotic resistome

INTRODUCTION

A growing body of evidence indicates that human microbial communities play a role in the pathogenesis of diseases as diverse as neonatal necrotizing enterocolitis, asthma, eczema, inflammatory bowel disease, obesity, atherosclerosis, insulin resistance, and neoplasia. Because the composition of the intestinal microbiota is highly variable in early infancy and largely stabilizes by the end of the first year of life, understanding the determinants of the composition of the infant enteric microbial community is of particular interest (Vael and Desager, 2009). The decreased rates of early childhood infections, atopic disease, diabetes, and obesity in breastfed infants have been well-documented (Oddy, 2004; Bartok and Ventura, 2009; Duijts et al., 2009; Le Huerou-Luron et al., 2010; Gouveri et al., 2011), as have the differences in the composition of the intestinal microbiota between breast- and formula-fed infants. In breastfed infants, *Bifidobacterium* spp. rapidly become the predominant group of organisms (Harmsen et al., 2000), while formula-fed infants develop a different microbial community comprised of some *Bifidobacteria* and large proportions of other potentially pathogenic organisms, including *Bacteroides*, *Staphylococcus*, *Enterobacteria*, *Clostridia*, and *Enterococcus* spp. (Yoshioka et al., 1983; Rubaltelli et al., 1998). Fermentative metabolites generated by *Bifidobacterium* and other

saccharolytic species decrease stool pH, inhibiting the growth of potential pathogens in breastfed infants (Bullen et al., 1976). Relative decreases in the proportion of *Bifidobacteria* and concomitant increases in other enteric flora in infancy have been linked to disease states later in life: increased numbers of *Escherichia coli* and *Clostridium difficile* are associated with the development of atopic disease such as asthma and eczema (Penders et al., 2007), while lower *Bifidobacterial* counts and greater numbers of *Staphylococcus aureus* are associated both with overweight mothers (Collado et al., 2010) and an increased risk of the infant becoming overweight in early childhood (Kalliomaki et al., 2008). *Bifidobacteria* may also enhance intestinal barrier function, decreasing the likelihood of bacterial translocation during periods of metabolic stress (Wang et al., 2006; Ruan et al., 2007). The gastrointestinal microbiota appear essential to the development of the immune system (Round and Mazmanian, 2009), can act as a reservoir for antibiotic resistance genes (van der Waaij and Nord, 2000), and may contribute to chronic inflammatory states (Erridge et al., 2007; Ghanim et al., 2009). Together, these data suggest that understanding the interactions between microbial communities and their human hosts may illuminate the pathogenesis of complex human diseases such as obesity and the metabolic syndrome, atopic disease, and autoimmune disorders,

and thereby provide a rich source for mining novel therapeutic approaches.

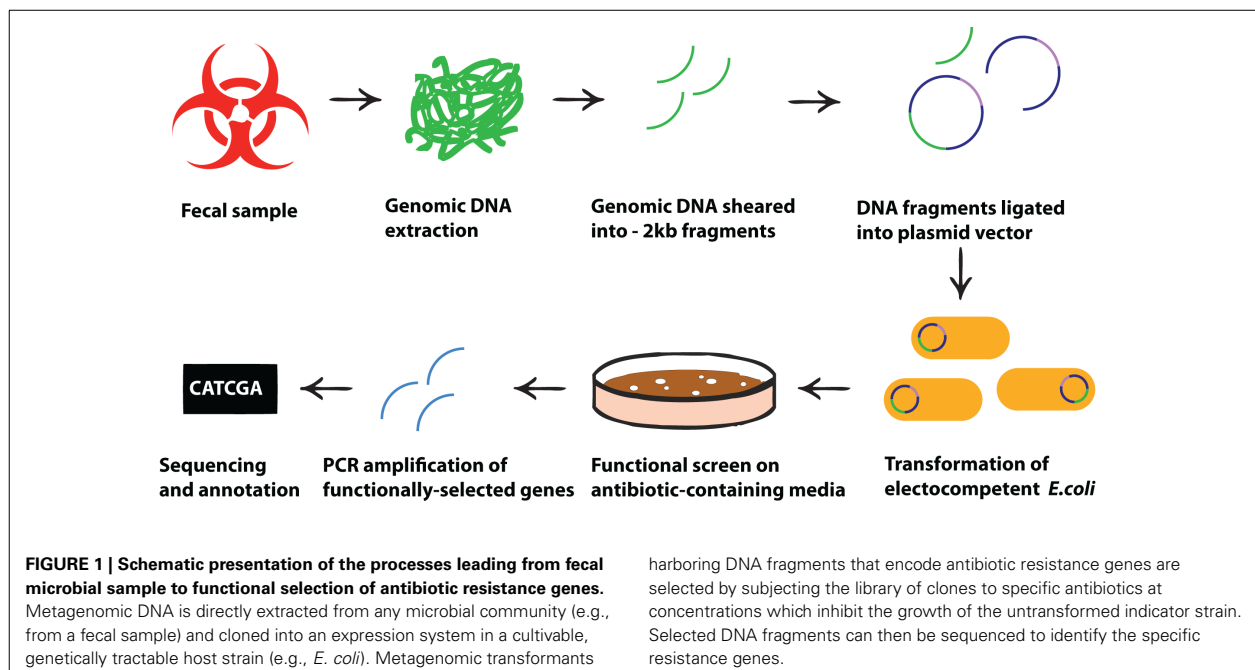
To understand microbial community effects on human health, both the phylogenetic profile of human microbial communities and the functional capacity of their members must be characterized. Much progress has been made toward these ends using direct bacterial culture, 16S sequencing, shotgun metagenomic sequencing, PCR probing for specific genes, and chemical profiling of microbial metabolites. These approaches have yielded incredible insights ranging from shifts in prevalent bacterial phylotypes and altered metabolic profiles in human subjects with inflammatory bowel disease, variations in the composition of the intestinal microbiota with human diet and functional differences in the gut microbiota related to host body habitus, developmental changes in the composition of the gastrointestinal microbiota during infancy and childhood, and the genetic epidemiology of antibiotic resistance in the intestinal microbiota. (Rimbara et al., 2005; Qin et al., 2006; Turnbaugh et al., 2006; Bezabeh et al., 2009; Jansson et al., 2009; Paliy et al., 2009; Gillevet et al., 2010; Kang et al., 2010; Koenig et al., 2011; Rigsbee et al., 2011). In this perspective, we will focus on the emerging application of functional metagenomic screens, a technique developed for investigating unculturable environmental microbes that neatly complements the aforementioned techniques currently used to characterize the human microbiota.

Direct culture, historically the *sine qua non* of microbiology, readily provides information on the functional characteristics of the species being investigated. The majority of gastrointestinal microbiota, however, are obligate anaerobes recalcitrant to culture. Traditional estimates are that only 15–20% of the gastrointestinal microbiota are culturable, precluding direct characterization of the majority of bacterial species (Langendijk et al., 1995; Eckburg et al., 2005; Gill et al., 2006). A recent report by Goodman et al. (2011) showed, using high-throughput 16S sequencing in combination

with extensive anaerobic culturing, that up to 56% of gastrointestinal microbial species are culturable. Although this represents a dramatic improvement over standard culturing techniques, there remains a significant proportion of unculturable organisms that must be characterized using complementary techniques. Different approaches have been employed to overcome this problem ranging from simple PCR-based screens to large metagenomic sequencing analyses and functional metagenomic screens. Together, these methods have expanded our knowledge about the fraction of the GI tract microbiota that cannot be characterized by culture-based approaches.

FUNCTIONAL METAGENOMICS: AN EMERGING TECHNIQUE FOR CHARACTERIZING UNCULTURABLE ORGANISMS

Functional metagenomic screens, originally proposed as a method to characterize the unculturable fraction of soil microbiota (Handelsman et al., 1998; Rondon et al., 2000) and successfully used for years to characterize the functional diversity of microbes in a variety of environments (Warnecke et al., 2007; Allen et al., 2009b; Berlemont et al., 2009; Torres-Cortes et al., 2011), has relatively recently been adapted to characterize the functions of human microbial communities, representing an interesting cross-pollination between environmental microbiology and biomedical science. The functional metagenomic screening method, based on clone libraries containing genomic DNA from a microbial community, does not require direct culture of fastidious organisms. Instead, clone libraries are constructed by first extracting and shearing DNA from a sample of a microbial community, then cloning the fragmented DNA into a relevant vector, and subsequently transforming this vector into a suitable host strain (Figure 1). Once a library is constructed, it can be functionally screened by cultivation on selective media or by employing a reporter system. Using this approach, it is possible to identify



harboring DNA fragments that encode antibiotic resistance genes are selected by subjecting the library of clones to specific antibiotics at concentrations which inhibit the growth of the untransformed indicator strain. Selected DNA fragments can then be sequenced to identify the specific resistance genes.

genes encoding a variety of functions such as antibiotic resistance, metabolism of complex compounds, and modulation of eukaryotic cells. Subsequent sequencing and *in silico* analysis of the DNA inserts from isolated clones provides information about the source of the genes and the putative mechanisms of action of their products.

INTERACTIONS WITHIN MEMBERS OF THE INTESTINAL MICROBIOTA: ANTIBIOTIC RESISTANCE

One area of early success for functional metagenomic screens is in the discovery of new antibiotic resistance genes in the human gastrointestinal microbiota. Multidrug resistant bacteria are increasingly prevalent in both hospitals and the community, and pose a growing threat to human health (Boucher et al., 2009; Högberg et al., 2010). Infections with antibiotic resistant organisms are associated with increased mortality and cost of treatment (Maragakis et al., 2008), and novel antibiotic discovery has not kept pace with the emergence of microbial resistance to existing agents (Högberg et al., 2010). In order to develop a rational approach to curtail the emergence of antibiotic resistance in human pathogens, a deeper understanding of the flow of resistance genes within microbial communities is required. Pathogenic organisms present in the environment may acquire resistance genes from soil or water microbes, while commensal gastrointestinal organisms that are continuously exposed to the outside environment via host ingestion of food, may also come in contact with pathogenic bacteria during the course of an infection. Although great strides have been made in recent years documenting genetic resistance reservoirs and patterns of gene flow within and between environmental and human commensal microbiota, fully characterizing the diversity and mobility of the environmental resistome will be crucial to control the emergence of ever more resistant organisms (Aminov and Mackie, 2007; Martinez, 2008; Aminov, 2009; Allen et al., 2010).

Multiple studies demonstrate the efficacy of simple PCR screens in the detection and quantification of antibiotic resistance genes present in the gastrointestinal microbiota. PCR assays have been used to detect the presence of known tetracycline resistance genes (*tet*) in fecal samples from antibiotic-naïve infants (Gueimonde et al., 2006). Similarly, qPCR has been used to quantify the levels of *tet* and *erm* genes, which confer resistance to tetracycline and macrolide, lincosamide, and streptogramin B antibiotics respectively, in animal and human waste water (Smith et al., 2004; Auerbach et al., 2007; Chen et al., 2010). The extraordinary specificity of PCR-based studies is also an important limitation of the technique: because PCR can only be used to interrogate a sample for known genes, it is an ineffective method for identifying novel resistance genes.

Functional metagenomic screens obviate this problem by identifying genes by their function in an expression vector rather than by a specific sequence used for PCR probing. Using this approach, novel antibiotic resistance genes have been identified in different environments including oral microbiota, soil microbiota, and moth gut flora (Diaz-Torres et al., 2003; Riesenfeld et al., 2004; Allen et al., 2009a). Sommer et al. (2009) demonstrated the power of metagenomic functional screens to identify novel antibiotic resistance genes in fecal samples from two healthy adults. Metagenomic libraries with a total size of 9.3 Gb

(gigabases) and an average insert size of 1.8 kb (kilobases) were screened for resistance against 13 different antibiotics, revealing 95 unique inserts representing a variety of known resistance genes as well as 10 novel beta-lactamase gene families (Sommer et al., 2009). Genes identified using metagenomic functional screens were, on average, 61% identical to known resistance genes from pathogenic organisms, while genes identified via aerobic culturing of isolates from the same individuals had greater than 90% sequence identity to previously described resistance genes. One of the novel resistance genes identified with the functional metagenomic screen had 100% sequence identity to a protein of unknown function, demonstrating the power of metagenomic functional screens to identify novel resistance genes even in fully sequenced and apparently well-annotated organisms. Antibiotic resistance with high sequence identity to known genes were more likely than novel genes to be flanked by mobile genetic elements such as transposases, possibly indicating that the novel genes represent a potential resistance reservoir that has not yet become widely disseminated. Recent work by Goodman et al. (2011) demonstrated that interindividual differences in gastrointestinal antibiotic resistance genes can be detected by subjecting both uncultured fecal samples as well as pools of phylogenetically representative fecal culture collections to metagenomic functional screens. Notably, the presence or absence of specific resistance genes (e.g., those encoding amikacin resistance) in uncultured samples, as determined by functional metagenomics, correlated with the fraction of cultured isolates phenotypically resistant to those compounds, and the presence of the exact genes identified by functional metagenomic screens was reconfirmed by PCR assay in phenotypically resistant cultured strains. These authors also found that the nearest genome-sequenced phylogenetic neighbors of the resistant strains isolated from the gastrointestinal microbiota of the sampled individuals lacked similar resistance genes, further highlighting the diversity and individualized nature of antibiotic resistomes. Together, these studies indicate that the gastrointestinal microbiota are likely to harbor many more resistance genes that will continue to be revealed by further investigation.

Functional metagenomic screens have also been used to mine the resistance reservoir in the intestinal microbiota of farm animals. Livestock are frequently dosed with antibiotics to treat infections and promote growth, and mounting evidence indicates that these practices lead to increased antibiotic resistance not only in the microbiota of the treated animals but also in their human caregivers (Sorum et al., 2006). The scope of this problem is highlighted by the findings of Kazimierczak et al. (2009), who employed metagenomic functional screens to identify both known and novel tetracycline resistance genes in fecal samples from organically farmed pigs that had not been exposed to antibiotics. Most of these genes were associated with mobile genetic elements, possibly explaining their persistence in an environment without any obvious selection pressure. The clinical and epidemiologic relevance of resistance genes present in the intestinal microbiota must be further defined by examining secondary effects of these genes, such as fitness costs or benefits associated with their expression, as well as by demonstrating the potential for direct transfer of the resistance gene to pathogenic organisms.

FUNCTIONAL METAGENOMICS FOR UNDERSTANDING THE GENETIC DETERMINANTS OF METABOLIC FUNCTION IN THE GASTROINTESTINAL MICROBIOTA

As previously noted, specific variations in the composition of the gastrointestinal microbial community have been linked to important states of human health and disease. Recent advances in understanding the interactions between bacterial metabolites and the host cellular machinery have begun to illuminate the physiologic basis of microbial contributions to human pathology. Metabolites generated either directly or indirectly by saccharolytic species may provide a mechanistic explanation for the observed human health outcomes associated with different compositions of the enteric microbial community. Conjugated linoleic acids, which are generated by some *Bifidobacterial* species (Rosberg-Cody et al., 2004), modulate tumorigenesis in animal models (Kelley et al., 2007), and are being investigated for a role in modulating inflammation and risk for neoplasia in humans (Bhattacharya et al., 2006; Coakley et al., 2009). Short-chain fatty acids (SCFAs) are bacterial metabolites that have wide-ranging effects on human physiology. In animal models of prematurity, some SCFAs (acetic and butyric acid) directly injure colonic mucosa in a dose-dependent fashion in the most immature age groups (Lin et al., 2002), an effect that disappears with increasing postnatal age (Nafday et al., 2005). This suggests a possible role for bacterial metabolites in the complex pathogenesis of necrotizing enterocolitis, a necroinflammatory disease commonly seen in preterm infants but non-existent in older age groups. Butyrate, a SCFA that is produced by fermentation of dietary fiber, has a variety of effects modulating inflammation and risk for neoplasia (Hamer et al., 2008). Butyrate is taken up by colonocytes via the MCT1 and SMCT1 transporters (downregulated in cancer cells), and is protective against colon cancer in animal models. It also inhibits histone deacetylase and inhibits TNF- κ B activation, which may explain its role in modulating inflammation. Acetate and propionate, two other SCFAs, have opposing effects on cholesterol biosynthesis (Wong et al., 2006). Microbe-generated SCFAs also may contribute to host obesity via interaction with fasting-induced adipocyte factor (Fiaf), AMPK, and Gpr41, which modulate pathways regulating fatty acid storage in adipocytes, fatty acid oxidation, gastrointestinal motility, and nutrient absorption (Backhed et al., 2007; Samuel et al., 2008).

Functional metagenomic screens offer a powerful means for detecting the genetic determinants of microbial metabolism. Jones et al. (2008) employed a functional screen using a large-insert metagenomic library to identify bile salt hydrolases within the human gastrointestinal microbiota. End-sequencing of clones displaying bile salt hydrolase activity revealed a broad phylogenetic distribution of bile salt hydrolase enzymes suggesting that this metabolic capacity is a conserved trait among bacteria adapted to life in the human gastrointestinal tract. Since bile salts play important roles in the processing and uptake of dietary fats in the intestines, microbial catabolism of these compounds may affect the amount of energy extracted from the diet.

Catabolism of fibers indigestible by the human host, another significant activity of the human intestinal microbiota, has been investigated using successive rounds of functional screens to enrich the metagenomic library with carbohydrate-metabolizing

enzymes followed by high-throughput sequencing to identify genetic determinants of carbohydrate metabolism within the human gastrointestinal microbiota (Tasse et al., 2010). They identified 73 carbohydrate-metabolizing enzymes from the enriched library, representing a fivefold increase in active genes identified compared to metagenomic sequencing without enrichment. This highlights the strong potential of serial functional screens combined with high-throughput sequencing to identify novel genes and yield increasingly comprehensive information on the metabolic potential of a given microbial community.

INTEGRATING FUNCTIONAL SCREENS WITH SHOT-GUN METAGENOMIC SEQUENCING ANALYSIS

The advent of convenient applications for metagenomic data analysis such as MG-RAST and MEGAN have simplified annotation and comparative analysis of functionally selected genes, which together with the declining cost of high-throughput sequencing, offer an efficient complement to functional screens (Huson et al., 2007; Meyer et al., 2008). Several studies have used this approach to connect functional genes with metabolic capacities and to identify pathways, such as metabolism of sugars, amino acids, and nucleotides, that are enriched in the gastrointestinal microbiota relative to representative genome-sequenced strains (Gill et al., 2006; Kurokawa et al., 2007; Turnbaugh et al., 2009; Arumugam et al., 2011). Moreover, by ranking functional gene clusters according to their frequencies, a minimal gut genome and a minimal gut metagenome have been described (Qin et al., 2010). The former reflects the minimal set of genes required by a single member of the gastrointestinal microbiota, while the latter indicates the minimal set of genes required to sustain the aggregate gastrointestinal microbiota. The minimal gut genome includes genes essential to all bacteria (e.g., replication, transcription, translation) as well as gut-specific genes such as those facilitating adhesion to epithelium. In contrast, the minimal gut metagenome includes genes necessary for metabolism of complex sugars, underscoring the importance of coupled metabolism in sustaining the GI tract microbiota. The importance of confirming gene function *in vitro* and *in vivo* to ensure reliable annotation is illustrated by Hess et al. (2011), who used metagenomic sequencing to identify >20,000 carbohydrate active genes from the cow rumen microbiota. From this gene set, they selected 90 *in silico* predicted carbohydrate-metabolizing genes, expressed them, subjected them to functional assays, and found that 51 genes were enzymatically active *in vitro* (Hess et al., 2011). These studies exemplify how metagenomic sequencing, automated annotation of large data sets, and functional screening comprise a powerful toolkit capable of characterizing functional networks in highly complex environments such as the GI tract microbiota.

FUNCTIONAL MAPPING OF INTERACTIONS BETWEEN HUMANS AND THEIR INTESTINAL MICROBIOTA

Functional metagenomic screens may also illuminate the genetic determinants of microbial interactions with host cells. The intestinal microbiota have long been known to modulate intestinal epithelia, for instance, by stimulating intestinal cell differentiation (Bry et al., 1996). In order to identify specific bacterial gene products that directly influence the fate of human cells, Gloux et al.

(2007) used cell lysate from individual clones in a gastrointestinal metagenomic library to screen for modulation of cell growth in CV-1 kidney fibroblast and HT-29 human colonic tumor cells. Using this approach, they identified 30 growth-stimulating and 20 growth-inhibiting clones, with Bacteroidetes as the dominant phylum among both sets. Using transposon mutagenesis on these sets of clones, they identified seven candidate genes with putative growth modulation effects.

Functional metagenomic screens have also been designed to investigate the immune-modulatory capacity of the gastrointestinal microbiota. To identify clones modifying the host immune response, Lakhdari et al. (2010) constructed an NF- κ B activated reporter system from a human colorectal carcinoma cell line. By screening metagenomic libraries of GI tract microbiota from patients with Crohn's disease, in which NF- κ B activity is frequently elevated (Ellis et al., 1998), they identified several clones either inducing or inhibiting NF- κ B activity. Together, these studies demonstrate the potential for functional metagenomic screens to illuminate the genetic mechanisms for microbial community contribution to the development of the human immune system and the pathogenesis of atopic, autoimmune, and neoplastic disease, which may provide novel therapeutic targets for these conditions.

In addition to interacting with human cells, commensal bacteria can also use quorum-sensing to convey signals over distances and thereby coordinate community gene expression. Guan et al. (2007) used a metabolite regulated expression (METREX) screen based on a quorum-sensing inducible promoter fused to *gfp* to identify genes encoding a new class of quorum-sensing inducing molecules in moth gut microbiota, demonstrating the power of functional metagenomics for characterizing the determinants of community behavior in uncultured organisms.

FUNCTIONAL METAGENOMICS FOR REFINING PRE- AND PRO-BIOTIC INTERVENTIONS

Increased understanding of the effects of gastrointestinal microbiota on human health has generated interest in targeting these communities for therapeutic intervention (Cani and Delzenne, 2011). Short-chain carbohydrates that are indigestible by humans but are fermentable by some microbes have demonstrable efficacy in increasing the populations of *Lactobacilli* and *Bifidobacteria* in the human gastrointestinal tract (Wang and Gibson, 1993). Investigations of galactose oligosaccharides (GOS) and fructose oligosaccharides (FOS) as additives to infant formula have demonstrated increased *Bifidobacterial* populations, decreased stool pH, generation of a stool fatty acid profile more similar to that found in breastfed infants, and reduced populations of potential pathogens such as *Clostridia* spp., *Bacteroides* spp., and *E. coli* (Fanaro et al., 2005; Knol et al., 2005; Costalos et al., 2008; Magne et al., 2008; Rao et al., 2009). Prebiotic supplementation with oligosaccharides

may promote blooms of beneficial bacteria more effectively than direct administration of pro-biotic organisms: a study directly comparing infant formula containing *Bifidobacterium animalis* with GOS/FOS-supplemented formula revealed a significantly greater proportion of *Bifidobacterial* species in the infants fed oligosaccharide-containing formula but no difference between the *Bifidobacterial* supplemented formula and control formula groups (Bakker-Zierikzee et al., 2005). Administration of prebiotics such as inulin and oligosaccharides in adult humans have shown some effect on hunger and satiety mechanisms (Whelan et al., 2006; Cani et al., 2009) but inconsistent results when applied to pathologies such as atopy and inflammatory bowel disease (Guarner, 2005; Roberfroid et al., 2010). Functional metagenomics has the potential to refine current prebiotic therapies by more completely defining the genetic determinants of metabolism for given constituents of a microbial community, providing a rational basis for more precise design of prebiotic agents intended to promote blooming of a specific subset of organisms.

TOWARD A COMPLETE FUNCTIONAL REPRESENTATION OF THE GASTROINTESTINAL MICROBIOTA

Functional metagenomic screens have been successful in elucidating novel genes encoding microbial antibiotic resistance, metabolic machinery, and immune-modulatory elements. Despite their demonstrable utility, functional metagenomic screens have several important limitations. First, the DNA insert must be compatible with the host's expression machinery and the gene product must be non-toxic and functional in the host (for an in-depth review, see Uchiyama and Miyazaki, 2009). Second, the host must be suited for the screen: when screening for antibiotic resistance genes, a host sensitive to the antibiotic of interest must be chosen. Third, the insert size may restrict the diversity of functions portrayed in a screen; a small insert library cannot reveal the function of genes organized in large operons such as many metabolic pathways or some efflux pumps associated with antibiotic resistance. Finally, the expression level of the insert can significantly affect the result of a functional screen. Using a high-copy plasmid as vector or a strong synthetic promoter can result in an overestimation of functionality. Conversely, overexpression of potentially lethal proteins may cause underestimation of functional genes, (e.g., cell lysis due to overexpression of efflux pumps). Despite these limitations, multiple studies demonstrate the potential for functional metagenomic screens to powerfully complement direct culture, 16S sequencing, shotgun metagenomic sequencing, and metabolomic analysis to offer new insight into the complex interactions between microbial communities and their human hosts. Used in concert, these techniques promise to expand our understanding of microbial community function, its impact on human health, and to provide novel targets for therapeutic development in the coming years.

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Limited exchange of antibiotic resistance genes between wastewater treatment plants and human pathogens

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Antibiotic resistance genes are ubiquitous in bacterial communities throughout all environments¹⁻³. When present in non-pathogenic bacteria these genes can be considered as a resistance gene reservoir, commonly referred to as the resistome⁴. It has been shown that horizontal acquisition of resistance genes from such reservoirs represent an important contributor to novel antibiotic resistance determinants in human pathogens⁵. One particular environment believed to play a central role in the transfer of antibiotic resistance genes is wastewater treatment plants (WWTPs)^{6,7}. In these facilities a mixture of resistant and pathogenic bacteria, originating from many diverse sources such as hospitals, households and animal production farms, can interact and exchange genetic material. However, the overall impact of the resistance reservoir in WWTPs on resistance in human pathogens is poorly understood. Using a combination of metagenomic functional selections and comprehensive sequencing we show that WWTPs contain a highly stable resistance gene reservoir that is shared among members of the WWTP communities and across different WWTP facilities. Surprisingly, we find that only 6 of the 80 genes in this reservoir are shared with bacteria found outside the WWTPs. This suggests that there exists a dissemination barrier limiting the spread of resistance

genes and that only a few genes are capable to crossing this barrier.

Horizontal gene transfer is a major contributor to the emergence and dissemination of antibiotic resistance genes⁸. Investigations of environmental sources of antibiotic resistance genes have shown that many clinical relevant resistance genes such as the cephalosporin resistance gene family *ctx-m* and the *van* vancomycin resistance genes, have close homologous on the chromosomes of environmental non-pathogenic species^{5,6,9}. This highlights that mobilization of environmental gene-reservoirs represents an important source of novel resistance genes.

Of particular concern is the dissemination of antibiotic resistance genes in wastewater treatment plants^{7,10,11}. These facilities daily receive hundreds of tons of wastewater from different sources, including hospital and animal farms where antibiotic consumption is high. Wastewater from these locations often contains antibiotics in concentrations high enough to select for resistance¹²⁻¹⁴. Accordingly, the microbe-dense WWTPs are viewed as the ideal hub for horizontal exchange of resistance genes between a wide range of bacterial species.

Several PCR based studies have shown that clinical relevant resistance genes, including *tem1*, *ampC*, *ndm-1*, *vanA* and *ermB*, can be found in WWTPs¹⁵⁻¹⁸. In addition, cultivation

Antibiotic	Concentration (µg/ml)	Code	Class	Number of colonies
Ampicillin	16	AMP	Beta-lactam / Penicillin	660
Amoxicillin	16	AMX	Beta-lactam / Penicillin	500
Carbenicillin	64	CAR	Beta-lactam / Penicillin	1100
Piperacillin	16	PIP	Beta-lactam / Penicillin	800
Ceftazidime	1	CAZ	Beta-lactam / Cephalosporin	350
Amikacin	16	AMK	Aminoglycoside	220
Gentamicin	8	GEN	Aminoglycoside	40
Spectinomycin	32	SPC	Aminoglycoside	330
Azithromycin	16	AZI	Macrolide	120
Erythromycin	100	ERY	Macrolide	200
Tetracycline	4	TET	Tetracycline	100
Chloramphenicol	6	CHL	Phenicol	30
Rifampicin	16	RIF	Rifamycin	210
Trimethoprim	4	TMP	Benzylpyrimidine	2000
Sulfamethoxazole / Trimethoprim	32:4	SXT	Sulfonamide / Benzylpyrimidine	1500
Total				8160

Table 1. Antibiotics used for functional metagenomic selection. In total 15 different antibiotics were used, resulting in 8160 resistant colonies.

based studies have shown that important resistant pathogens such as vancomycin-resistant enterococci, methicillin-resistant staphylococci and cephalosporin-resistant enterobacteriaceae can be isolated from WWTPs^{17,19}. Due to these findings, it is generally believed that WWTPs are a hot-spot for emergence and transfer of novel resistance genes^{7,10}. However, because this conclusion is based on studies relying on PCR based screening and cultivation, these findings are expected to be biased by sequence specificity and cultivation conditions, respectively. Thus, the magnitude of resistance gene exchange between the WWTP resistome and clinically relevant bacteria remains poorly understood. We combined functional metagenomic selection and metagenomic sequencing to fully investigate the WWTP antibiotic resistome. Compared to PCR and cultivation, functional metagenomic selection represents an unbiased approach to identifying genes with specific functions, and it has been used to identify resistance genes in several different environments^{2,20,21}. By coupling this screening technique with high-throughput metagenomic sequencing, we are able to comprehensively investigate the overlap in resistome across different environments.

We used functional metagenomic selection to screen an *Escherichia coli* expression library containing more than 0.8 Gb of DNA isolated from a large modern WWTP that receive both hospital and household wastewater. The library was screened on 15 different antibiotics

representing seven chemical classes; beta-lactam, aminoglycoside, macrolide, tetracycline, phenicol, rifamycin, sulphonamide and benzylpyrimidine (Table 1). Inserts conferring resistance were found for every antibiotic tested. In total 8540 resistant clones were identified with an average of 534 colonies per antibiotic ranging from 30 colonies for chloramphenicol to 2000 clones for trimethoprim (Table 1). Of the 8540 clones identified, the inserts of 749 clones, selected proportionally among the different classes of antibiotics, were sequenced resulting in 80 unique resistance genes (Extended Data Table 1). Rarefaction curve analysis indicated that the functional screening approach recovered most of the antibiotic resistome within the cloned metagenome (Figure 1a).

Inserts were annotated and we found that clones isolated on the same class of antibiotic displayed a high degree of conservation in their functional classification (Extended Data Table 1). Clones isolated on beta-lactam antibiotics were primarily classified as beta-lactamases (86 % (21/24)), clones isolated on aminoglycosides were classified as aminoglycoside-transferases (phospho-, acetyl- or adenylyltransferases) (100 % (9/9)), clones isolated on macrolides were primarily classified as rRNA methyltransferases (63 % (5/8)), while clones isolated on rifampin were classified as ADP ribosyl transferases (83 % (5/6)) and clones selected on trimethoprim or sulfamethoxazole were classified as dihydrofolate reductases or thymidylate synthases (90 % (28/31)). For

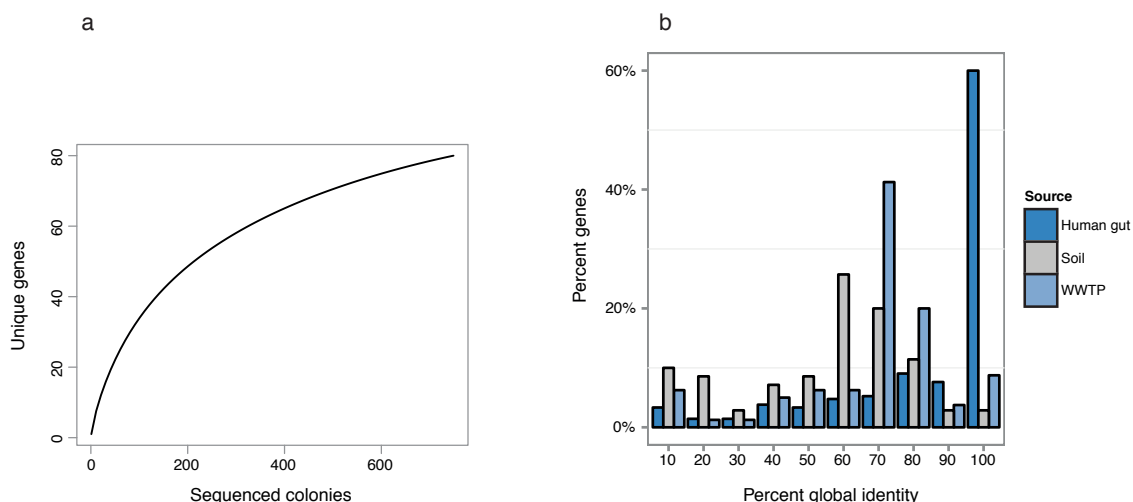


Figure 1. Functionally identified resistance genes

a) Assessment of sampling effort. In total 749 colonies was sequenced, resulting in 80 unique resistance genes comprising most of the cloned resistance genes.

b) Nucleotide identity distribution. The functional selected resistance genes were compared to the genbank database. On average the WWTP resistance genes had 62 % to genbank, in comparison functional selected resistance genes from the Human gut and Soil has an average identity of 82 % and 50 %, respectively.

tetracycline and chloramphenicol only one unique gene was identified, a tetracycline efflux-transporter protein and a chloramphenicol acetyltransferase, respectively. In all cases the expected resistance mechanism is well understood and except for rifampicin, the most abundant resistance functions identified represent the commonly found resistance mechanisms in clinical isolates^{12,22,23}.

Knowing that the functionally selected resistance genes from the WWTP encode resistance proteins that are functionally equivalent to well-known resistance genes we wanted to investigate if they also shared high sequence identity with known sequences. Comparing the identified resistance genes against the genbank nucleotide database revealed that the identified ORFs had an average sequence similarity of 62 % and that just 6 ORFs had a sequence identity greater than 95 % indicating that the vast majority of the functional selected resistance genes represent novel sequences (Figure 1b). In order to put this finding into perspective, we compared the nucleotide identity distribution of the WWTP resistome with functional selected resistance genes from other environments (Figure 1b). We reanalyzed online available functional selected resistance genes from the human gut and from soil^{2,20,21,24,25}. We found, using the current genbank database, that the 210 functionally selected resistance genes

from the human gut had an average of 82 % identity to genes in genbank while the 70 genes from soil had 50 % average identity to genbank (Figure 1b).

The low identity of functionally selected resistance genes from the WWTP and soil to genbank, compared to genes found in more established reservoirs such as the human gut, suggests that there is a limited flow of resistance genes between environmental gene reservoirs and the human gut. This observation is in line with a recent study of the shared antibiotic resistome of soil and human pathogens²⁶.

To assess how geographical and temporal variations affected recovery of the WWTP resistance gene reservoir we sequenced 15 metagenomes from five full scale WWTPs all located in Denmark (AAV, EGA, EJB, HJO and AAE). Two of the plants were sampled repeatedly over a two-year period and all plants, except AAE, received domestic as well as hospital wastewater (Extended Data Table 2). Mapping of the WWTP metagenomic reads to the functionally selected resistance genes revealed that on average each sample contained 35 (44 %) of the functionally selected resistance genes (Figure 2a). From AAV, the facility corresponding to the functional selected genes, we could map 64 (80 %) of the selected genes and 33 (52 %) of these were present in four or more of the seven longitudinal samples

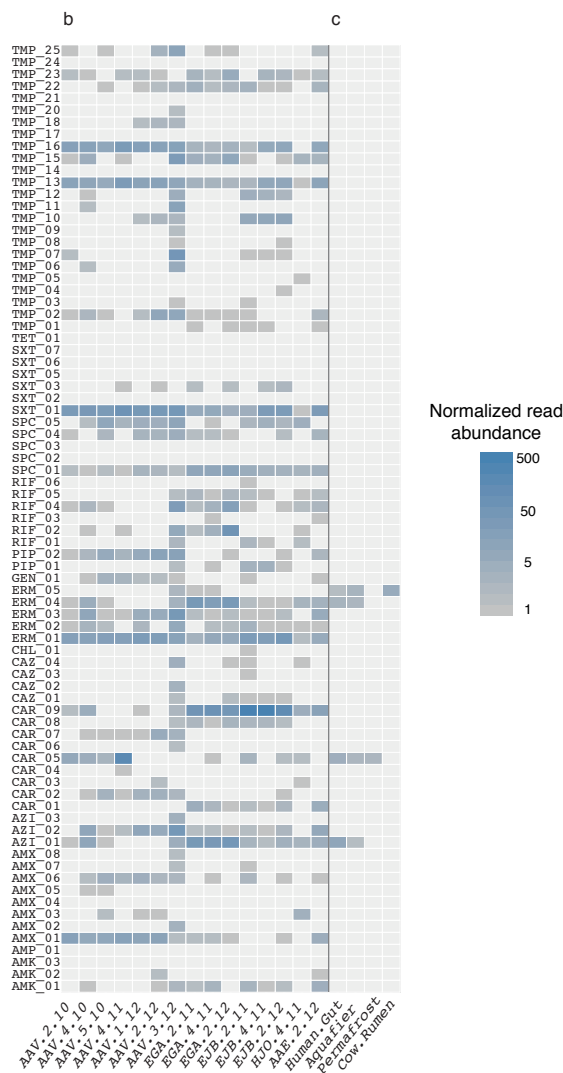
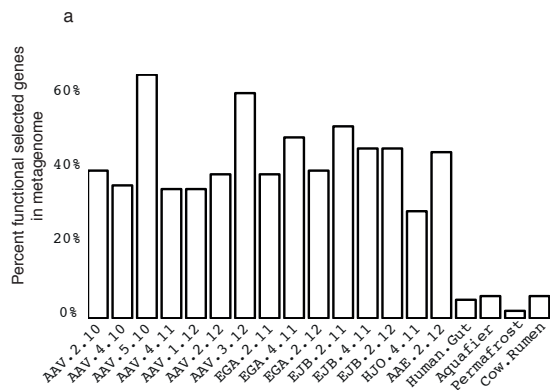


Figure 2. Mapping metagenomic reads to functional selected genes.

The metagenomes from 19 environments; 15 WWTP sample and 4 auxiliary environments were mapped to the functional selected resistance genes. a) On average each WWTP sample contained 35 of the 80 selected resistance genes while the auxiliary metagenomes on average only contained 4.

Normalized abundance of reads from WWTPs (a) and auxiliary (b) metagenomes that mapped with > 95 % identity to the functional selected resistance genes. The WWTP samples represent five different WWTPs, 58 (73 %) of the functional selected genes were present in more than one WWTP. In contrast only 6 (8 %) of the resistance genes were also found in auxiliary metagenomes.

from this location (Figure 2a and b). The common resistance reservoir shared between three or more of the five WWTPs contained 40 (50 %) of the originally identified resistance genes, and 17 (21 %) of the genes were present in all WWTPs (Figure 2a and b). This shows, that the WWTP resistome is shared and highly stable across different treatment facilities and sampling times.

In order to investigate if the resistance genes found in the WWTP core resistome were shared with metagenomes from different environments we mapped reads from online available metagenomes to the functionally selected resistance genes from the WWTPs. These auxiliary metagenomes contained sequences from the human gut, cow rumen, permafrost and aquifer. Interestingly, only 6 (7.5 %) of the WWTP genes were sampled within these other metagenomes, confirming that the WWTP resistome is distinct from previously characterized environments (Figure 2a and c). All six genes found in these other metagenomes had an identity greater than 95% to clinically relevant resistance genes, highlighting that a PCR based approach would successfully identify these genes. Rarefaction curve analysis showed that the small overlap between the environmental metagenomes and the WWTP resistome did not result from differences in sampling depth of the environments (Figure 3a).

We wanted to investigate whether the functionally selected resistance genes were present in only one genetic background (i.e. a specific bacterial strain) or were disseminated between different genetic backgrounds in the WWTPs. By aligning metagenomic reads that

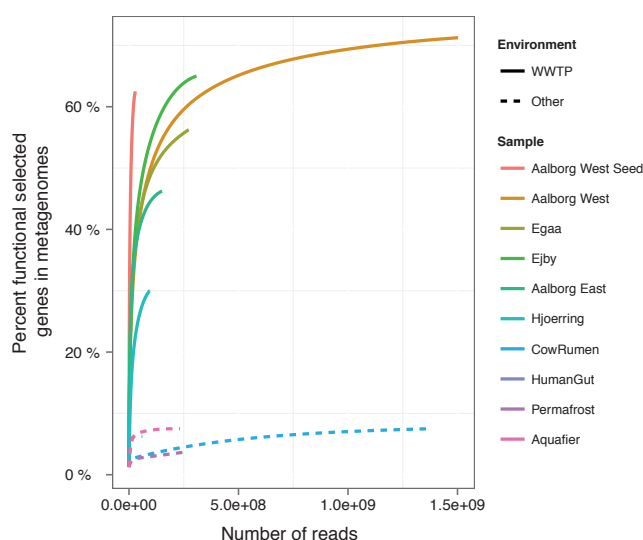


Figure 3. Metagenomic sampling effort and gene recovery.

To investigate the effect of sampling depth on the recovery of the functional selected resistance genes from the metagenomes we conducted a rarefaction analysis of each metagenome. The WWTP corresponding to the functional selected sample (seed) was clearly under sampled. Generally the WWTP metagenomes generally seem to saturate at 5×10^8 reads. In contrast the auxiliary metagenomes saturate much earlier, highlighting that sampling effort cannot account for the low abundance of functional selected genes in these metagenomes.

spanned the resistance gene region and the flanking regions we could identify variability in the flanking regions. Interestingly we found that the part of the reads that mapped to the resistance gene was highly conserved while the part that extended outside of the resistance gene were very diverse (Figure 4a This figure is still under construction). This reveals that the functionally selected resistance genes are widely shared among members of the bacterial WWTP communities .

Integrating functional metagenomic selection with metagenomic sequencing represents a powerful approach to comprehensively map the shared functionality across different environments.

Using this new approach we found that even though WWTPs contain many functional resistance genes, only few of these are also found outside the WWTP environment. This conclusion contrasts the general belief that WWTPs are a key source of resistance genes. Our findings suggest that mobilization, rather than functionality, is the main barrier preventing spread of resistance genes from environmental reservoirs into human pathogens. This conclusion is in line with a general theory of ecological niches as drivers of HGT²⁷. However, whether certain microbial environments contribute more than others to the emergence of clinical relevant resistance genes still remains an open question.

Materials and methods

Sampling

A total of 15 activated sludge samples were collected over a period of two years from five different Danish WWTPs; Aalborg Vest (AAV), Aalborg East (AAE), Hjoerring (HJO), Ejby (EJB) and Egaa (EGA), with enhanced biological phosphorus removal (Extended Data Table 2).

Metagenomic DNA extraction

DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions except an initial phenol incubation step for 3 min at 90°C. Briefly, 1 ml sample aliquots were centrifuged at 13.000 rpm for 5 min and the supernatant discarded. The pellets were dissolved in 250 µl phosphate buffer and transferred to the FastDNA bead beating tubes. Next, 750 µl preheated phenol was added to each tube (Biological grade, pH 8, 0.1M EDTA, Sigma-Aldrich) and the samples were incubated for 3 min at 90°C with occasional shaking. Subsequently, the samples were homogenized using the FastPrep instrument according to the manufacturer's instructions and centrifuged at 13.000 rpm for 10 min to separate the phenol phase from aqueous phase. The DNA was extracted and further purified using the FastDNA kit according to the manufacturer's instructions. DNA concentrations were measured using a Qubit (Life technologies) and DNA integrity was evaluated using gel electrophoresis.

Library construction

DNA used for functional selection was sheared by Covaris E220 instrument (USA). In brief, 200 µl DNA was added to a Covaris mini-tube and sheared according to Covaris shearing protocol for 2 kbp DNA fragments. Sheared DNA was end repaired using the End-It end repair kit (Epicentre) according to the manufacture's instructions. End repaired DNA was size selected by low melting point agarose gel electrophoresis in 0.5X TBE buffer and fragments between 1-3 kbp DNA were extracted using the QIAquick Gel Extraction Kit (Qiagen). The sheared DNA was blunt ligated into pZE21 MCS 1 vector at the HincII site using the Fast Link ligation kit (Epicenter) according to the manufactory's instruction. Next, 3 µL of the fresh ligation mixture was electroporated into 50 µl electrocompetent *E. coli* TOP10 cells (Invitrogen). Subsequently, the cells were recovered in 1 ml SOC medium for 1 h at 37 °C. Libraries were titrated by plating out 1 µL, 0.1 µL and 0.01 µL of recovered cells onto LB agar plates containing 50 µg/mL kanamycin. For each library the insert size distribution was estimated by colony PCR products using primers flanking the HincII site of the multiple cloning site of the pZE21 MCS1 vector. The average insert size was found to be 2.07 kbp. The total metagenomic library size was estimated to 828 Mbp by multiplying average PCR based insert size with the number of colony forming units (cfu). The remaining recovered cells were grown overnight in 10 ml LB containing 50 µg/ml kanamycin. The overnight culture was stored in 15 % glycerol at -80 °C.

Functional selections of antibiotic resistant clones

For each metagenomic and genomic library tested, 100 µL of library freezer stock corresponding to 0.5×10^7 cfu were plated out on LB agar plates containing binary combinations of kanamycin (50 µg/mL) and one of 15 different antibiotics (Table 1) and incubated at 37 °C for 16 hrs.

To enable functional selections for resistance to multiple compounds, the transformed cells were allowed to grow up overnight before freezing stocks, allowing each clone harboring a unique DNA insert to grow, resulting in an amplification of the particular clone in the library. Based on the original library titers as well as titers of the freezer stocks, the average amplification of

a given library can be estimated. On average each unique clone in the libraries screened was plated out in 10-100 copies depending on library size, corresponding to 10-100 fold amplification. To minimize the redundancy of clones in subsequent analysis, on average the number of colonies picked for sequencing corresponded to approximately the number of resistant clones on an agar plate divided by the estimated average library amplification (Sommer et al., 2009).

Each of the clones picked was inoculated into 96 deep well plates containing liquid LB medium supplemented with kanamycin (50 µg/mL) and the relevant antibiotic to which resistance had been selected (Table 1) and grown overnight to verify resistance phenotype.

Sequencing and analysis of functional metagenomic inserts

Functional selected metagenomic inserts harbored by clones were sequenced using Sanger sequencing. A total of 749 clones were sequenced unidirectional using the reverse primer. In 153 of these the resistance gene could not be annotated, consequently these were sequenced bi-directionally using the following primers:

>Forward primer_pZE21_81_104_57C

5'-GAA TTC ATT AAA GAG GAG AAA GGT-3'

>Reverse primer_pZE21_151_174rc_58C

5'- TTT CGT TTT ATT TGA TGC CTC TAG -3'

All reads (≥ 500 base pairs) obtained after reverse primer Sanger sequencing was assembled into contigs using CLC Main Workbench 6. The sequence from each contig corresponds to single clone containing unique insert. To get a full sequence of unique insert each single clone corresponds to respective contigs were sequenced with forward primer Sanger sequencing. The full insert sequence for each unique insert was obtained by merging the forward primer sequence reads and reverse primer sequence reads using EMBOSS:Merger server (bioinfo.nhri.org.tw/cgi-bin/emboss/merger, used at MARCH 20, 2013). Open reading frames (ORFs) were identified and annotated using ORFfinder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>, used at April 20, 2013).

ORFs were annotated using tblastx, which computes local sequence alignment between the nucleotide query translated in all 6 frames and the GenBank non-redundant nucleotide database translated in all 6 frames (May 2013) (Sommer et al., 2009). For each insert, the

genbank ID and the alignment coordinate for the top scoring tblastx hit was obtained. The top hit were inspected manually and expected resistance gene candidates were identified. Pairwise sequence alignments between the GenBank non-redundant nucleotide hit and the identified ORFs were computed using EMBOSS:Matcher and the percentage global identity was calculated by dividing the number of matches with the total query length plus possible gaps.

Metagenome by Illumina high-throughput sequencing

Samples were prepared for sequencing using the Nextera DNA Sample Preparation Kits (Illumina Inc.) with 50 ng of DNA. The library DNA concentration was measured using the QuantIT kit (Molecular Probes) and paired-end sequenced (2x151 bp) on an Illumina HiSeq2000 using the TruSeq PE Cluster Kit v3-cBot-HS and TruSeq SBS kit v.3-HS sequencing kit (Illumina Inc.). The sample AAV-2012-3 was paired-end sequenced (2x301 bp) on the Illumina MiSeq platform using the MiSeq Reagents kit v2 (Illumina Inc.).

Illumina high-throughput sequencing quality filtering and data analysis

Metagenome reads in fastq format were imported to CLC Genomics Workbench v. 5.5.1 (CLC Bio) and trimmed using a minimum phred score of 20, a minimum length of 50 bp, allowing no ambiguous nucleotides and trimming off Illumina nextera sequencing adapters if found. In addition, metagenome reads from four other environments (Human gut (ERS006497), Permafrost (), Cow Rumen (SRP004875) and an Aquafier () were downloaded from the NCBI SRA and used for comparison.

The trimmed metagenome reads were mapped to all antibiotic contigs using a minimum of 95% similarity and the number of reads hitting within the putative antibiotic gene were counted. All subsequent data analysis was conducted using R (<http://www.r-project.org>).

The trimmed metagenome reads from the AAV-5-10 sample were assembled using CLC's de novo assembly algorithm, using a kmer of 63 and a minimum scaffold length of 2 kbp.

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Transfer of multiple antibiotic resistance genes *in situ* of the infant gut microbiota

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Highlights

- Antibiotic resistance genes exchanged between co-existing *E.coli* lineages in the gut
- Resistance genes spread and remain in gut microbiome without antibiotic selection
- Extensive genome dynamics occur in *E. coli* lineages colonizing the gut microbiota

Summary

The human gut is one of the densest microbial ecosystems and is believed to play an important role in the exchange of antibiotic resistance genes. We study the dynamics of co-existing *Escherichia coli* lineages in a longitudinally followed infant not receiving antibiotics. Using whole genome sequencing, we capture the exchange of multidrug resistance genes and identify a clinically relevant conjugative plasmid mediating the transfer. Quantification of the co-existing lineages reveals that the resistant transconjugant is maintained for months, demonstrating that antibiotic resistance genes disseminate and remain in the gut microbiome even in absence of antibiotic selection. Furthermore, we observe a large genomic deletion, as well as acquisition and loss of phages in these lineages during their colonization of the human gut. Our findings highlight the dynamic nature of the human gut microbiota and provide the first genomic description of antibiotic resistance gene transfer between bacteria in the unperturbed human gut.

Introduction

Resistance to antibiotics is an ever-growing public health concern. Antibiotic resistance in bacteria can either be achieved through mutation in the genome or via horizontal acquisition of foreign genetic material that confers resistance (Davies and Davies, 2010). While antibiotic resistance achieved through mutation is of significant importance during long term treatment of chronic infections (Yang *et al*, 2011), horizontal gene transfer plays a key role in the ever-growing problems

with multidrug resistant human pathogens (Hawkey and Jones, 2009).

The environment is considered a reservoir of antibiotic resistance genes (D'Costa *et al*, 2006), and indirect evidence has shown that antibiotic resistance genes have disseminated from the environment to both commensal intestinal microbes and to clinical pathogens (Forsberg *et al*, 2012; Sommer *et al*, 2009). In addition to the flow of genetic material from environmental reservoirs to humans, antibiotic resistance genes have also been suggested to spread between bacteria within the human microbiome (Sommer and Dantas, 2011). In the human intestine, this transfer would not only be between members of the commensal microbiota, but would also include non-commensal bacteria transiting through the intestine (Salyers *et al*, 2004). While the gut microbiome has been the subject of numerous metagenomic studies (Forslund *et al*, 2013; Project, 2013; Qin *et al*, 2010), including a recent study constructing complete genomes of various species and strains from metagenomic data (Sharon *et al*, 2013), the use of metagenomics is not well-suited to detect horizontal gene transfer events between species or strains in the human gut.

Transfer of antibiotic resistance genes within the gut microbiota has been shown to occur in animals (McConnell *et al*, 1991), as well as in humans (Lester *et al*, 2006; Trobos *et al*, 2008). These studies have directly demonstrated antibiotic resistance transfer between bacteria in the gut. However, the studies set up an artificial scenario where the host is fed a strain that can donate antibiotic resistance genes to

the commensal microbiota. A recipient strain or the commensal bacteria is subsequently monitored to detect if the antibiotic resistance genes have been transferred from the donor strain. Despite employing an artificial experiment set-up, studies have started to elucidate factors known to influence transfer between bacteria. For example, Stecher *et al* reported that pathogen-induced gut inflammation of lab mice gives rise to an environment where lineages of various Enterobacteriaceae species can bloom resulting in unprecedented rates of horizontal gene transfer between these bacteria (Stecher *et al*, 2012).

A few instances of natural transfer of antibiotic resistance genes in the unperturbed human gut microbiota have been published in reports describing changes in the antibiotic resistance profiles of strains collected from infants undergoing antibiotic treatment (Bidet *et al*, 2005; Karami *et al*, 2007). Additionally, one study demonstrates that extensive resistance gene exchange has occurred between species of Bacteroides in a collection of strains assembled over a period of 40 years (Shoemaker *et al*, 2001). However, full genomic data for strains exchanging antibiotic resistance genes in situ of the human gut has yet to be reported. In this study, we characterize the transfer of antibiotic resistance between co-existing Escherichia coli lineages in the infant gut at the genomic level using whole genome sequencing.

Results

E. coli lineage sampling

Our study material was selected from an infant enrolled in the ALLERGYFLORA study, which was designed to examine the link between the infant gut microbial colonization pattern and the development of allergies (Nowrouzian *et al*, 2005). Faecal samples obtained over the course of the first year of life were serially diluted and cultured on a range of non-selective and selective media, including media for Enterobacteriaceae selection. Isolates resembling Enterobacteriaceae were selected from each positive culture based on differing colony morphology and size. The sensitivity threshold of the culture method was 102.5 colony-forming units (CFU) per gram of fecal matter (Nowrouzian *et al*, 2005), which is several orders of magnitude more sensitive than current metagenomic sequencing studies (Sharon *et al*, 2013). Isolates identified as *E. coli* by biotyping were initially assigned to specific lineages based on random amplification of polymorphic DNA (RAPD). The antibiotic resistance profile was determined for each isolate. Interestingly, a change in the antibiotic resistance profile was detected in a set of consecutive isolates from one lineage colonizing one of the enrolled infants. The set of *E. coli* lineages collected from this infant are the subject of this study, chosen to investigate the genome dynamics involved in the acquisition and persistence of antibiotic resistance.

E. coli isolates were obtained from the infant at two

and four weeks, and at two, six and twelve months after birth (Figure 1). In total, three distinct lineages were identified: A, B and C. The sampling at two and four weeks after birth yielded only isolates belonging to lineage A, which were sensitive to all antibiotics tested (Figure 1, Table S1). At the 2-month sampling time, lineage B was observed. Lineage B was resistant to the antibiotics ampicillin, piperacillin, streptomycin and sulfamethoxazole (Figure 1). At the two-month sampling time, the antibiotic resistance profile of lineage A changed. Lineage A was now resistant to ampicillin, piperacillin, streptomycin and sulfamethoxazole. Notably, the change in the resistance profile of lineage A at two months matches that of the incoming lineage B suggesting a transfer of resistance determinants from lineage B to A. Lineages A and B were both present at the six months sampling time with no changes in the antibiotic resistance profile. However, at the 12-month sampling time only lineage B was still present, with the addition of lineage C, which is only resistant to sulfamethoxazole (Figure 1).

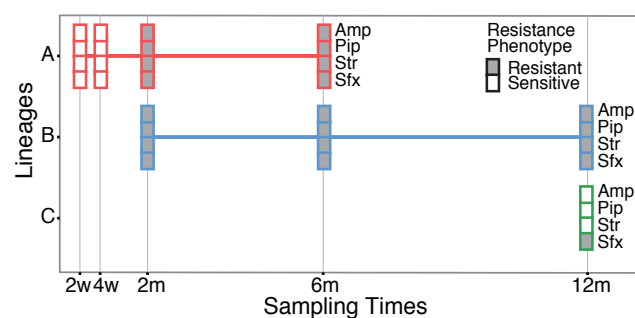


Figure 1. Sampling and antibiotic resistance of the *E. coli* strains. A total of three *E. coli* lineages were sampled from the infant's intestinal microbiota over the course of the first year of life. Circles indicate both the presence of the lineage at the sampling points, in addition to their antibiotic resistance profile to ampicillin (Amp), piperacillin (Pip), streptomycin (Str), and sulfamethoxazole (Sfx) at the time points. Filled colored circles indicate resistant isolates, and non-filled circles indicate sensitive isolates (Table S1 for MICs).

Isolates from the lineages A, B and C were genome sequenced from each of the sampling points. In order to confirm lineage identities of the isolates, Illumina single-end reads were first assembled into contigs for each isolate. Next, reads from all isolates were cross-aligned to the assembled contigs of the other isolates and the number of SNPs between the isolates were counted to establish strain relationships (Table S2). The three lineages were apparent based on the low number of SNPs between isolates in each lineage. Interestingly, isolates from lineages A and C had an order of magnitude fewer SNPs between them than when compared to lineage B (approximately 5,000 SNPs vs. 70,000 SNPs). Furthermore, lineage A and C shared 94.0% of the genomic content. For subsequent analyses, contigs were assembled for each lineage by

pooling reads together from all sampling points. Evolutionary relationships amongst the isolates within a lineage were established based on the number of SNPs identified by aligning reads from each isolate to the pooled lineage contigs (Table S3 for full list of SNPs). SNPs occurring in non-genomic regions or in homologous regions after genomic deletions or acquisitions were filtered, as to not skew the relationships of the isolates in the lineages. SNPs identified in isolates from both lineages A and B produce consistent phylogenetic trees and show a progression in the acquisition of SNPs (Figure 2). In the A lineage, one SNP was identified between the two and four week isolates, and this SNP was again identified in all isolates of lineage A subsequently sampled. Similarly, lineage B had two SNPs propagated to all isolates subsequently obtained after the initial SNP detection. The consistent phylogenetic tree and the progression of SNPs indicate that the isolates sampled are representative clones for both the A and B lineages.

To get further information about the isolated lineages we compared the isolates to previously sequenced *E. coli* genomes found in the NCBI reference sequence database, based on genomic content in common and number of SNPs. Lineage A had 93.2% genomic content in common with the asymptomatic bacteriuria (ABU) strain, ABU 83972 (NC_017631.1) (Zdziarski *et al*, 2010). ABU 83972 was also the closest previously sequenced strain to lineage C as well, sharing 91.5% genomic content. The ABU 83972 strain, which is closely related to the pathogenic CFT073 *E. coli* strain, has been reported to show a tendency to evolve toward commensalism from virulence during asymptomatic colonization of the bladder via the loss of gene function (Zdziarski *et al*, 2010). Lineage B shared 95.4% genomic content with UMN026 (NC_011751.1), an extra-intestinal pathogenic strain (Touchon *et al*, 2009). The UMN026 strain belongs to clonal group A, which is a globally spread uropathogenic clone that frequently carry multiple antibiotic resistance determinants (Lescat *et al*, 2009).

Lineage genome dynamics

By examining the changes in the isolates collected from a lineage, we observed that the lineages were highly dynamic. Genome dynamics included acquisition of antibiotic resistance, a major genomic deletion, and phage infections (Figure 3A).

To identify the genomic changes underlying the acquisition of antibiotic resistance in lineage A, sequence data collected from the sensitive isolates (2 and 4 weeks) was compared to sequence data from the resistant isolates (2 and 6 months). Two non-conservative genomic mutations in the betaine aldehyde dehydrogenase (*betB*) and phosphoenolpyruvate carboxylase (*pckA*) genes were identified, however, these would not be expected to contribute to antibiotic resistance. Instead, additional

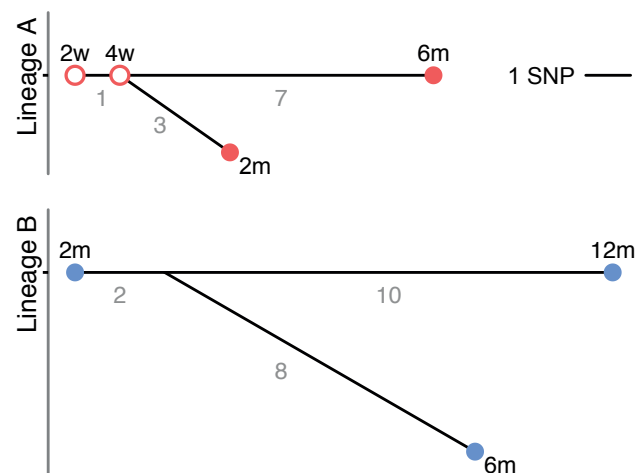


Figure 2. Phylogenetic tree of the isolates.

Phylogenetic trees based on the number of SNPs found in each of the isolates of lineages A and B. The grey value next to each branch indicates the number of SNPs between isolates.

genetic information totaling 90kb was found in the resistant isolates from the A lineage that were collected at the two and six month sampling points compared to the A lineage isolates from the 2 and 4 week time points (Figure 1). The read coverage of the newly acquired genetic information had 2.0 times greater coverage compared to that of the genome, suggesting that a newly acquired low-copy number plasmid harbored the antibiotic resistance determinants. This putative plasmid contained conjugative transfer genes (*trb*, *tra* and *pil* operons). Additionally, the putative plasmid contained a beta-lactamase (*bla*TEM-1c), which has been reported to confer resistance to penicillins, including ampicillin (Livermore, 1995). The TEM-1 beta-lactamases are widely disseminated amongst several different pathogens including *E. coli*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Livermore, 1995). Furthermore, two genes, aminoglycoside 3'-phosphotransferase (*strA*) and streptomycin phosphotransferase (*strB*), known to mediate resistance to streptomycin and other aminoglycosides were identified, the latter being widely disseminated on a broad host range plasmid (Ramirez and Tolmasky, 2010). Lastly, a dihydropteroate synthase gene (*sul2*) was found in the putative plasmid. The *sul2* gene is known to mediate resistance to sulfonamides and is also frequently found in clinical isolates (Sköld, 2000). Searching sequence databases for similar plasmids yielded the clinically important conjugative, IncI1-type pHUSEC41-1 plasmid of 91,942bp (Kunne *et al*, 2012). Aligning contigs from the isolates in this study to pHUSEC41-1 resulted in 99.3% coverage of the plasmid with an average of 99.0% identity. The alignment also showed that there were no insertions in the transferred plasmid compared to pHUSEC41-1. The pHUSEC41-1 plasmid was initially identified in the *E. coli* serotype O104:H4 strain HUSEC41 isolated from a child in Germany with hemolytic-uremic syndrome (HUS) (Kunne *et al*, 2012). The plasmid

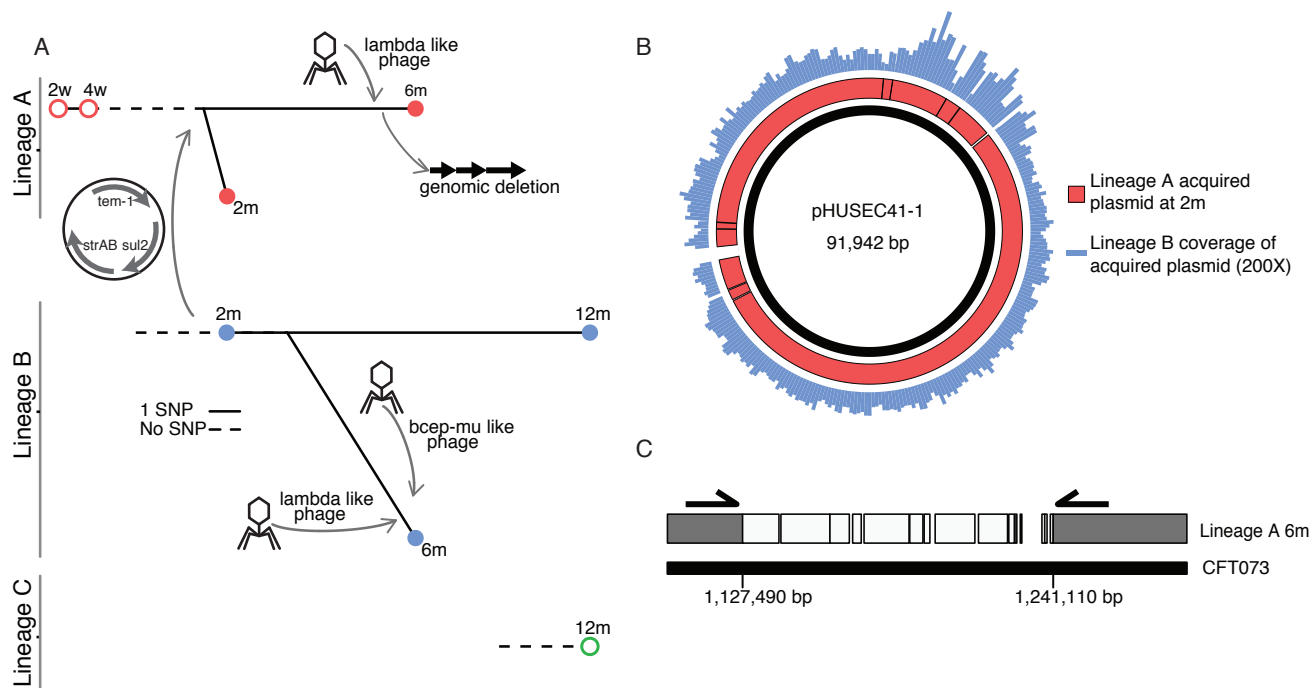


Figure 3. **Lineage genome dynamics.**

A. Overview of lineage genome dynamics. The transfer of a multidrug resistance plasmid from the B lineage to the A lineage occurred before the 2 month sampling time. The transfer occurred before diversification of the A lineage. At the 6 month sampling point, a bcep-mu like phage infecting the B lineage was detected. In addition, both the A and B lineages were infected by lambda-like phages at this time point. A large genomic deletion occurred in the A lineage after the 2 month but before the 6 month sampling point, as detected in the isolate obtained from the 6 month sample. No isolates of lineage A were obtained at the final sampling time at 12 months, but a new isolate from lineage C is sampled along with lineage B.

B. Transfer of a plasmid mediating antibiotic resistance.

Contigs corresponding to the newly acquired plasmid were identified by analyzing differences in the read alignment coverage before and after the change in the resistance profile. Reads from lineage B are mapped to the acquired plasmid contigs of A, displaying coverage depth. High coverage and identity between the strains was observed. The acquired plasmid contigs of A were aligned to the sequence of pHUSEC41-1.

C. Large deletion in the genome of lineage A. Contigs from strain A were aligned to reference genome CFT073. Dark grey colored contigs represent regions flanking the excision. Pale colored contigs represent the region lost due to the deletion. Arrows indicate the position of the primers designed based on the CFT073 genome used to confirm the genomic excision.

has additionally been found in other sequenced *E. coli* isolates of serotype O104:H4 isolated from patients in France with sporadic cases of HUS (Grad *et al*, 2013). Here, the plasmid was found in *E. coli* strains of serotypes O73: K-:H18 and O179: K12:H- isolated from a Swedish infant, highlighting the wide dissemination of this multiple antibiotic resistance plasmid amongst various *E. coli* lineages.

The phenotypic resistance patterns (Figure 1) suggested that the horizontally acquired resistance was transferred from strain B to strain A. To assess this we analyzed strain B to see if it contained the acquired genetic information of strain A. Aligning reads from strain B to the acquired genetic information of strain A resulted in coverage of 100% of the new genetic information with only one identified SNP variant (Figure 3B). The high-degree of identity between the plasmids and the change in the antibiotic resistance profile of strain A to match the profile of strain B is consistent with strain B transferring its antibiotic resistance plasmid to strain A.

Interestingly, a large deletion was detected in the A lineage at the six-month sampling point, i.e. after acquisition of the resistance plasmid. The lost

contigs, totaling 83kb, aligned to a contiguous region in a similar, sequenced strain, *E. coli* CFT 073 (Figure 3C) (NC_004431). The ABU 83972 strain, which has high homology to the CFT 073 strain, was not used due to a documented prophage integrated in this region (Zdziarski *et al*, 2010). Using the alignment information, PCR assays were conducted to establish that the deletion was an excision (Figures 3C, S1).

Annotated genes located in the deleted segment included iron scavenging genes, such as the *iroA* gene cluster and the hemolysin activator protein, peptide antibiotic genes microcin H47 and colicin-E1, which target gram-negative bacteria in general and *E. coli* specifically, respectively, and antigen 43, which may have a role in adhesion (Selkrig *et al*, 2012). Lastly, genes involved in fatty-acid synthesis, carbohydrate and amino acid metabolism were also lost as a result of the deletion (Table S4 for complete list).

Furthermore, phage content varied in the lineages across sampling time points, in addition to the several stably integrated phages. At the 6-month sampling time, a phage bloom had occurred where both strains A and B were infected by phages (Figure 3A). Strain A was infected by a 27.1 kilobase (kb) Lambda-like

phage, while strain B was infected by two phages: a different Lambda-like phage of 22.5kb, and a Bcepμ-like phage of 40.9kb. Notably, the Bcepμ-like phage infecting the B lineage had very high similarity to an integrated phage in the genome of the A lineage (at least 99.9% similarity covering 97.7%), meaning that the B lineage had just acquired an extra 40kb of genetic material on par with the A lineage. However, the phage likely did not originate from the A lineage due to the differences in both sequence and structure in one region. The integration of several phages at this time point comprising more than 90kb highlights how phages play a vital role in horizontal gene transfer. Interestingly, the phages that infected the lineages were not detected in any isolates collected at the later sampling time (12 months), suggesting that they did not fully establish in the population.

E. coli lineage population counts in relation to genome dynamics.

To investigate whether the various genomic events occurring in the *E. coli* lineages possibly affected their fitness in the gut microbiota, we examined the population counts of lineages A and B at the different sampling points (Figure 4). In general, population numbers of *E. coli* decrease in the gut of infants over the first year of life, in parallel with the establishment of a microbiota dominated by anaerobic bacteria (Nowrouzian *et al*, 2003).

Interestingly, the acquisition of the resistance plasmid in lineage A was associated with a very steep drop in population counts, from $10^{10.2}$ CFU per gram of fecal matter in the 4 week sample to $10^{7.8}$ per gram in the 2 month sample. This could possibly relate to a fitness cost imposed on lineage A from carrying the resistance plasmid. This possibility was further supported by pair-wise in vitro growth competition experiments comparing the growth of a lineage A isolate from the 4 week sampling, before acquisition of the plasmid, and a lineage A isolate from the 2 month sampling, after acquisition of the plasmid. In these experiments carriage of the plasmid incurred a cost of $-6.3\% \pm 1.9\%$ per generation on lineage A. However, despite the fitness costs the lineage persisted in the gut for at least another 4 months, and even increased in numbers during this time.

At the 2 month sampling time, when lineage B was first sampled, lineages A and B had roughly the same population counts, $10^{7.8}$ and $10^{7.7}$ CFU/g, respectively. At the 6 month sampling time, the population counts of lineage A were several times higher than the counts of lineage B ($10^{8.7}$ versus $10^{8.0}$ CFU/g,) and had also increased competitive advantage.

Discussion

The human gut, as a hub for horizontal gene exchange, is expected to play an important role in the exchange of antibiotic resistance genes. Yet to our knowledge,

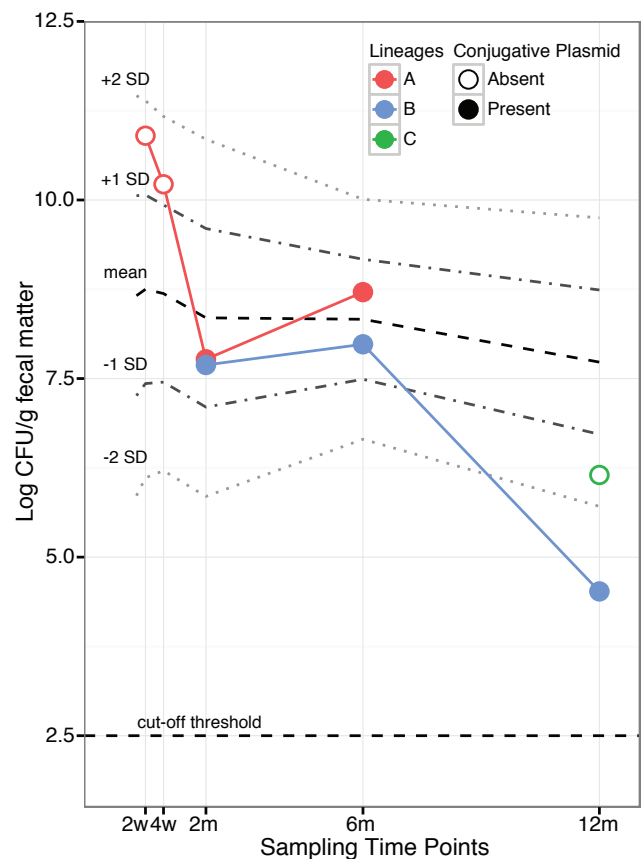


Figure 4. Population counts of co-existing *E. coli* lineages
Faecal population counts of *E. coli* lineages A, B and C at different sampling points during the first year of life of the infant studied. For comparison, the mean population levels and ± 1 and 2 standard deviations (SD) at the same sampling points for 272 *E. coli* strains isolated from 128 infants in the ALLERGYFLORA cohort are indicated in the figure.

there are no prior reports documenting horizontal gene exchange events at the genomic level between bacteria in the unperturbed human gut. Here, by genome sequencing a collection of co-existing *E. coli* strains, we describe several cases of horizontal gene transfer mediated by both integrative phages and conjugative plasmids. The substantial genome dynamics captured in this study highlight the highly dynamic nature of the gut microbiota.

Specifically, we document at the genomic level transfer of a multiple drug resistance plasmid between co-existing bacterial lineages in the human gut. Notably, the transfer occurred in the gut of an infant not treated with antibiotics. This provides compelling evidence to support the idea of the human gut serving as a hotspot for horizontal resistance gene exchange even in absence of selective pressure from antibiotics. Furthermore, the A lineage persisted in the infant gut for months after acquisition of the resistance plasmid. The fact that the transconjugant survived and even increased its population counts highlights that resistance genes might easily disseminate also in healthy individuals never treated with antibiotics.

We also identify the entire mobile genetic element

responsible for this horizontal gene transfer and discover that it is closely related to the clinically relevant multidrug resistance plasmid pHUSEC41-1. This study highlights the advantages of studying the longitudinal dynamics of co-existing bacterial lineages in the gut microbiota as a complement to metagenomic sequencing studies. The power of this approach is expected to increase as cultivation methods for representatively sampling the gut microbiota further improves and we anticipate that further studies augmenting metagenomic sequencing with genomic sequencing will provide a richer and more detailed view of the highly dynamic nature of strains in the human gut microbiota.

Experimental Procedures

Strain Isolation. Fecal samples were placed in airtight containers with an anaerobic sachet (AnaeroGen Compact, Oxoid Ltd, Basingstoke, UK) and refrigerated until brought to the lab, where they were serially diluted and cultivated within 24 hours on Drigalski medium for the isolation of Enterobacteriaceae. Colony types were selected based on size, shape, color or texture, and speciated using API 20E biotyping (API Systems, SA, La Balme les Grottes, Montalieu-Vercieu, France). Each morphotype was enumerated separately, and the limit of detection was 102.5 CFU/g fecal matter. Isolates identified as *E. coli* were initially typed to the strain level using random amplification of polymorphic DNA (RAPD) (Nowrouzian *et al*, 2003) and strain identity was confirmed by performing pulse field gel electrophoresis (PFGE), prior to genomic sequence verification.

Minimum Inhibitory Concentration (MIC) determination.

MIC determination was performed in 96 well micro-titer plates. Each drug gradient consisted of 11 points in a two-fold dilution series prepared in MHBII (Sigma) medium with a total of 150 µl in each well. The MIC plates were inoculated with approximately 10⁵ cells per well using a 96-pin replicator. The plates were incubated at 37°C for 18 – 20 hours and the optical density (OD) at 600 nm was read on a BioTek Epoch plate reader.

Genome Sequencing.

Genomic DNA from each isolate was obtained using an UltraClean® Microbial DNA Isolation Kit (Mobio Laboratories, Inc.). The extracted DNA was sheared into 200bp fragments using a Covaris E210 and barcoded libraries were constructed for Illumina sequencing and performed by Partners HealthCare Center for Personalized Genetic Medicine (Cambridge, Massachusetts). Single-end reads were assembled using Velvet (Zerbino and Birney, 2008), with a k-mer size of 31 and a coverage cut-off of 3. Contigs were corrected by aligning reads using Bowtie2

(Langmead and Salzberg, 2012), calling single-nucleotide polymorphisms (SNPs) using SAMtools (Li *et al*, 2009), and edited using custom biopython scripts (Cock *et al*, 2009). Contigs with less than 500 base pairs were filtered. Genomes were annotated using the RAST server (Aziz *et al*, 2008). Bowtie2 and SAMtools were also used to determine the number of SNPs between isolates. In addition, BEDtools (Quinlan and Hall, 2010) was used to calculate read coverage across genomes and thus identify acquired or deleted genomic information. MUMmer was used to align sequences (Khan *et al*, 2009).

Phage Identification.

The PHAST phage search tool server (Zhou *et al*, 2011) was used to identify possible intact phages. In addition, BLAST was used to identify similar previously described phages.

Genomic Deletion Verification.

Based on the alignment of contigs to the genome of CFT 073 (NC_004431), primers were designed to show that the deletion was an excision, in addition to show contiguity prior to the deletion, as well as controls for showing the occurrence of the deletion only in the lineage A isolate sampled at 6 months.

Competition experiments.

To assess the fitness costs in vitro of carriage of the plasmid closely resembling pHUSEC41-1. To assess fitness cost, pair-wise growth competition in Davis minimal medium with 25 mg/mL glucose (DM25) was performed using isolates of lineage A sampled at 2 weeks and 2 months, respectively, the latter which had acquired the plasmid closely resembling pHUSEC41-1 (Enne *et al*, 2004).

Author Contributions

NK, IA and AW designed the study, made the original finding and analyzed the preliminary data regarding transfer of a resistance phenotype and a conjugative resistance plasmid between *E. coli* lineages in situ. NK performed all experiments in the early stage of the study. HG processed the sequencing data and performed experiments in the late stage of the study. HG, CM and MS analyzed all sequencing data and wrote the manuscript with input from NK, IA and AW.

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Supplementary pages

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Section 2

Supplementary data

Limited exchange of antibiotic resistance genes between wastewater treatment plants and human pathogens

Extended Data Table 1

Resistance genes identified using metagenomic functional selections from WWTP. Gene ID is made up of the 2 or 3-letter code for the antibiotics used for the selections (e.g. CAR denotes Carbenicillin, For each gene identified, the most similar gene from any organism in GenBank was identified using tblastx). Global sequence identities at the nucleotide level were computed using EMBOSS:Stretcher.

Antibiotics	Gene ID	Gene length (bp)	Gene annotations	Top hit [gbID title position]	GPNI
Carbenicillin	CAR_01	507	Beta-lactamase	JN559393.1 Pseudomonas aeruginosa 8651-9454	56.1
	CAR_02	1005	Beta-lactamase	FN640464.1 Uncultured bacterium lpxB gene for putative lipid-A-disaccharide synthase and bla gene for beta-lactamase class A, clone Ap6-8w 1523-2482	55.9
	CAR_03	909	Beta-lactamase	CP002859.1 Runella slithyformis DSM 19594 5147053-5147949	66.2
	CAR_04	798	NDM metallo-beta-lactamase	JN104597.1 E. coli strain EC405 115-927	57.2
	CAR_05	861	Beta-lactamase	JQ624676.1 Mammalian expression vector pSA95 4195-5055	100
	CAR_06	900	Beta-lactamase	GU441460.1 E. coli strain R170 plasmid pRZA92 3220-4125	60
	CAR_07	480	Beta-lactamase	CP000383.1 Cytophaga hutchinsonii ATCC 33406 2205559-2206377	38.1
	CAR_08	825	OXA-2 beta-lactamase	JX846494.1 Pseudomonas aeruginosa strain Pa314 Class I integron OXA-2 like protein 109-909	97.1
	CAR_09	882	Beta-lactamase	CP000450.1 Beta-lactamase	80.7

Piperacillin	PIP_01	771	Beta-lactamase	Nitrosomonas eutropha C91 1096410-1097369 CP002961.1 Emticicia oligotrophica DSM 17448 1889449-1890237	57.7
	PIP_02	816	Beta-lactamase class D	CP000383.1 Cytophaga hutchinsonii ATCC 33406 2205559-2206377	62.6
	CAZ_01	759	Beta-lactamase	CP002961.1 Emticicia oligotrophica DSM 17448 870369-871124	70.5
	CAZ_02	768	Beta-lactamase	CP002961.1 Emticicia oligotrophica DSM 17448 870369-871124	69.7
Ceftazidime	CAZ_03	732	Metallo-beta- lactamase	FP476056.1 Zobellia galactanivorans 970040- 970783	58.1
	CAZ_04	768	Hypothetical protein	CP003787.1 Riemerella anatipestifer RA-CH-1 30846- 31760	100
	AMX_01	651	Beta-lactamase	CP000356.1 Sphingopyxis alaskensis RB2256 2002725- 2003594	80
	AMX_02	915	Beta-lactamase	CP002859.1 Runella slithyformis DSM 19594 5147053-5147949	67.8
Amoxicillin	AMX_03	975	Beta-lactamase	CP001220.1 Comamonas testosteroni CNB-2 2966741- 2967490	57.9
	AMX_04	762	Beta-lactamase	CP000361.1 Arcobacter butzleri RM4018 1485957- 1486718	81.2
	AMX_05	417	Beta-lactamase	CP002859.1 Runella slithyformis DSM 19594 5147053-5147949	32.4
	AMX_06	915	Beta-lactamase like protein	CP000248.1 Novosphingobium aromaticivorans DSM 12444 1837704-1838573	56.2
	AMX_07	858	Penicillin binding protein	CP002961.1 Emticicia oligotrophica DSM	62.9

Ampicillin	AMX_08	846	transpeptidase Beta-lactamase	17448 2038928-2039734 CP000269.1 <i>Janthinobacterium</i> sp. Marseille 647835-648734	46.4
	AMP_01	1020	Beta-lactamase	AP012047.1 <i>Arcobacter</i> butzleri ED-1 DNA 1416170- 1416931	60.3
Spectinomycin	SPC_01	792	Aminoglycoside adenylyltransferase	JN849689.1 Uncultured bacterium plasmid pRSB113 6315-7106	100
	SPC_02	1005	Spectinomycin phosphotransferase	FN650140.1 <i>Legionella</i> longbeachae NSW150 1157668-1158690	56.3
	SPC_03	801	Spectinomycin phosphotransferase	FN650140.1 <i>Legionella</i> longbeachae NSW150 1157668-1158690	26.9
	SPC_04	1014	Aminoglycoside - (3'')(9)- adenylyltransferase	FJ172373.1 Uncultured bacterium clone BF7_C6 class 1 integron qacH and aadA genes 598-1443	56.7
	SPC_05	1092	Aminoglycoside phosphotransferase	CP001824.1 <i>Sphaerobacter</i> thermophilus DSM 20745 940698-941714	50.7
Gentamycin	GEN_01	636	GCN5-like N- acetyltransferase	CP002447.1 <i>Mesorhizobium</i> ciceri biovar biserrulae WSM1271 525402-525917	59.3
Amikacin	AMK_01	339	Aminoglycoside 6'-N- acetyltransferase	AY566824.1 Uncultured soil bacterium clone CR6 putative glucosamine-fructose-6- phosphate aminotransferase gene, partial cds; aminoglycoside 6'-N- acetyltransferase gene 249-797	45.2
	AMK_02	567	Aminoglycoside 6'-N- acetyltransferase	AY566824.1 Uncultured soil bacterium clone CR6 putative glucosamine-fructose-6- phosphate aminotransferase	66.4

Chloramphenicol - Tetracycline Azithromycin Erythromycin	AMK_03	501	Aminoglycoside 6'-N-acetyltransferase	gene, partial cds; aminoglycoside 6'-N-acetyltransferase gene 249-797 AY566820.1 Uncultured soil bacterium clone 85C1 putative cation efflux family protein and aminoglycoside 6'-N-acetyltransferase genes 354-905	65.9
	CHL_01	1428	Chloramphenicol resistant protein	KC176455.1 RNAi silencing vector pCAPD 3580-4239	46.2
	TET_01	1191	Putative tetracycline resistant protein	CP003504.1 Enterococcus hirae ATCC 9790 2237403-2238605	54.1
	AZI_01	759	Ribosomal RNA adenine dimethylase family protein	FR720602.1 Streptococcus oralis Uo5 1839657-1840394	96.8
	M_02	777	rRNA (adenine N-6-)-methyltransferase	CP001778.1 Stackebrandtia nassauensis DSM 44728 2735336-2736172	53.9
	AZI_03	618	MscS mechanosensitive ion channel	CP002084.1 Dehalogenimonas lykanthroporepellens BL-DC-9 1509935-1510822	40.5
	ERM_01	399	Dimethyl adenine transferase	CP001686.1 Kytococcus sedentarius DSM 20547 1860415-1861149	35.3
	ERM_02	636	GTP binding protein Hflx	CP002876.1 Nitrosomonas sp. Is79A3 1604493-1605845	43.6
	ERM_03	438	rRNA(adenine N-6-)-methyltransferase	CP001778.1 Stackebrandtia nassauensis DSM 44728 2735336-2736172	31.8
	ERM_04	405	rRNA (adenine N-6-)-methyltransferase	CP003922.1 Streptococcus suis SC070731	53.1

Trimethoprim	ERM_05	513	Macrolide-efflux protein	715601-716338 EU870852.1 <i>Streptococcus pyogenes</i> strain MB56Spyo005 1-1227	40.8
	TMP_01	306	Dihydrofolate reductase	CP000267.1 <i>Rhodospirillum rubrum</i> strain T118 3291945-3292439	40.7
	TMP_02	696	Thymidylate synthase	CP002419.1 <i>Neisseria meningitidis</i> G2136 1675708-1676502	59.2
	TMP_03	408	Thymidylate synthase	CP001681.1 <i>Pedobacter heparinus</i> DSM 2366 1689195-1689680	52
	TMP_04	489	Dihydrofolate reductase	CP002046.1 <i>Croceibacter atlanticus</i> HTCC2559 2891530-2892012	57.1
	TMP_05	495	Dihydrofolate reductase	CP000449.1 <i>Maricaulis maris</i> MCS10 2207968-2208489	61
	TMP_06	501	Dihydrofolate reductase	CP002691.1 <i>Haliscomenobacter hydrossis</i> DSM 1100 4468410-4468913	58.9
	TMP_07	489	Dihydrofolate reductase	CP003178.1 <i>Niastella koreensis</i> GR20-10 5722263-5722763	61.8
	TMP_08	495	Putative oxidoreductase	AP012337.1 <i>Caldilinea aerophila</i> DSM 14535 4963388-4964050	41.4
	TMP_09	468	Dihydrofolate reductase	CP000089.1 <i>Dechloromonas aromatica</i> RCB 646778-647269	58.9
	TMP_10	537	Dihydrofolate reductase	HE965806.1 <i>Bordetella bronchiseptica</i> 253 2778656-2779153	51.4
	TMP_11	432	Thymidylate synthase	CP003418.1 <i>Ignavibacterium album</i> JCM 16511 2413030-2413917	76.4

TMP_12	510	Dihydrofolate reductase	CP002281.1 Ilyobacter polytropus DSM 2926 545109-545585	48.3
TMP_13	762	dihydrofolate reductase	CP001013.1 Leptothrix cholodnii SP-6 1345906-1346406	43.3
TMP_14	480	Dihydrofolate reductase	CR954246.1 Pseudoalteromonas haloplanktis str. TAC125 2808343-2808810	54.4
TMP_15	816	Thymidylate synthase	CP002542.1 Fluviicola taffensis DSM 16823 960816-961610	70.4
TMP_16	525	Dihydrofolate reductase	CP001339.1 Thioalkalivibrio sulfidophilus HL-EbGr7 2890732-2891220	58.2
TMP_17	483	Dihydrofolate reductase	CP001638.1 Geobacillus sp. WCH70 1654473-1654961	55
TMP_18	492	Dihydrofolate reductase	CP000148.1 Geobacter metallireducens GS-15 3337712-3338197	60.8
TMP_19	483	Dihydrofolate reductase	CU207366.1 Gramella forsetii KT0803 328588-329070	69.2
TMP_20	753	Putative oxidoreductase	AP012337.1 Caldilinea aerophila DSM 14535 4963388-4964050	48.3
TMP_21	663	Bifunctional deaminase/reductase protein	CP002040.1 Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111 3369988-3370797	39.6
TMP_22	516	Dihydrofloate reductase	CP000316.1 Polaromonas sp. JS666 1886763-1887257	62.6
TMP_23	525	Dihydrofloate reductase	CP000747.1 Phenylobacterium zucineum HLK1 902351-902869	60.7
TMP_24	531	Bifunctional dihydrofolate reductase-thymidylate synthase	XM_002512892.1 Ricinus communis 1-1587	23.4
TMP_25	495	Thymidylate synthase	FQ859181.1 Hyphomicrobium sp. MC1 3728475-3729269	38.2

Sulfonamide	SXT_01	795	Thymidylate synthase	FO082820.1 Rhizobium sp. str. NT-26 2040923-2041717	71.9
	SXT_02	687	Thymidylate synthase	CP000284.1 Methylobacillus flagellatus KT 953119-953913	52.2
	SXT_03	444	Thymidylate synthase	JF924881.1 Uncultured bacterium clone tri1 390-794	65.1
	SXT_04	525	Dihydrofolate reductase	CP000747.1 Phenylobacterium zucineum HLK1 902351-902869	60.7
	SXT_05	414	Thymidylate synthase	HE774682.1 Flavobacterium indicum GPTSA100-9 562831-563655	33.5
	SXT_06	831	Thymidylate synthase	CP000082.1 Psychrobacter arcticus 273-4 2477380-2478282	48.7
	SXT_07	483	Dihydrofolate reductase	CU207366.1 Gramella forsetii KT0803 328588-329070	69.2
Rifampicin	RIF_01	429	Rifampin ADP-ribosyl transferase	JX875536.1 Uncultured bacterium clone WGRif3028 3134-3583	72.3
	RIF_02	582	Pentapeptide repeat protein	CP002542.1 Fluviicola taffensis DSM 16823 847426-847995	59.1
	RIF_03	426	Rifampin ADP-ribosyl transferase	HE577629.1 Vibrio splendidus partial integrative and conjugative element ICEVspPor2 7798-8245	62.9
	RIF_04	744	Rifampin ADP-ribosyl transferase	BA000045.2 Gloeobacter violaceus PCC 7421 4141456-4141998	55.7
	RIF_05	750	Rifampin ADP-ribosyl transferase	FJ418586.4 Oscillatoria sp. PCC 6506 cylindrospermopsin	72
	RIF_06	447	Rifampin ADP-ribosyl transferase	CP002859.1 Runella slithyformis DSM 19594 5150381-5150830	74.9

GPNI = Global percent nucleotide identity

Extended Data Table 2

Sampling and sequencing depth for the 15 metagenomes. Aalborg Vest (AAV), Aalborg East (AAE), Hjoerring (HJO), Ejby (EJB) and Egaa (EGA). Quarter “5” refers to December.

WWTPs	Year	Quarter	Sequencing platform	Trimmed reads (millions)
AAV	2012	3	MiSeq, 2x301	29
AAV	2012	2	HiSeq2000, 2x151	66
AAV	2012	1	HiSeq2000, 2x151	59
AAV	2011	4	HiSeq2000, 2x151	130
AAV	2010	5	HiSeq2000, 2x151	1109
AAV	2010	4	HiSeq2000, 2x151	24
AAV	2010	2	HiSeq2000, 2x151	113
AAE	2012	2	HiSeq2000, 2x151	151
HJO	2011	4	HiSeq2000, 2x151	94
EJB	2012	2	HiSeq2000, 2x151	91
EJB	2011	4	HiSeq2000, 2x151	127
EJB	2011	2	HiSeq2000, 2x151	90
EGA	2012	2	HiSeq2000, 2x151	59
EGA	2011	4	HiSeq2000, 2x151	137
EGA	2011	2	HiSeq2000, 2x151	78

Supplementary information

Transfer of multiple antibiotic resistance genes in situ of the infant gut microbiota

Supplemental Information

Inventory:

Table S1: Minimum Inhibitory Concentrations (MICs) of *E. coli* lineage isolates.

Related to main Figure 1, “Sampling and antibiotic resistance of the *E. coli* strains.”

Table S2: Genomic verification of *E. coli* lineage identities.

Related to “*E. coli* lineage sampling” section of paper to support assembling one set of contigs per *E. coli* lineage.

Table S3: Identified SNPs in *E. coli* lineages A and B across time.

Related to main Figure 2, “Phylogenetic tree of the isolates”, as the SNP data is used to generate the phylogenetic trees.

Table S4: List of Annotated Deleted Genes in Lineage A.

Related to main Figure 3C, “Lineage genome dynamics - Large deletion in the genome of lineage A.” This supplementary table lists the gene name annotations that were in the deleted region.

Figure S1: Confirmation of excision deletion lineage A.

Related to main Figure 3C, “Lineage genome dynamics - Large deletion in the genome of lineage A.” The A panel of the figure shows the design of the PCR primers to verify the occurrence of the deletion and appropriate controls. The B panel of the figure shows the corresponding PCR products separated on a gel.

Table S1. Minimum Inhibitory Concentrations of *E. coli* lineage isolates.

Lineage	Lineage A				Lineage B			Lineage C
Sampling Time	2w	4w	2m	6m	2m	6m	12m	12m
Antibiotic (ug/mL)								
Ampicillin	2	2	512	512	512	512	512	2
Piperacillin	1	1	64	64	32	32	32	0.5
Mecillinam	0.125	0.125	1	1	1	1	0.5	0.125
Ceftazidime	0.25	0.25	0.25	0.25	0.0625	0.125	0.125	0.125
Cefuroxime	8	8	8	8	2	4	2	2
Cefoxitin	2	4	4	4	8	4	8	2
Chloramphenicol	1	1	1	1	1	1	1	1
Gentamycin	0.5	0.5	0.5	0.5	1	1	1	1
Tobramycin	0.25	0.25	0.5	0.125	0.5	0.25	0.5	0.5
Streptomycin	2	2	64	128	128	128	128	4
Nalidixic Acid	1	1	1	1	1	0.5	1	0.5
Tetracycline	0.5	0.5	0.5	0.5	1	1	0.5	1
Trimethoprim	4	4	2	4	0.125	0	0.125	0.125
Sulfamethoxazole	8	16	1024	1024	512	1024	1024	512

Table S2. Genomic verification of *E. coli* lineage identities.

			Contigs Used as Reference						
			Lineage A				Lineage B		
			2w	4w	2m	6m	2m	6m	12m
Reads Aligned to Reference Contigs	A	2w	0	1	4	34	78404	77641	78132
		4w	4	0	4	44	78160	77406	77788
		2m	4	3	0	22	78096	77369	77678
		6m	12	9	16	0	77893	77053	77559
	B	2m	78314	68731	77415	76060	0	48	38
		6m	78421	68893	77622	76157	58	0	73
		12m	78359	68773	77455	76147	10	125	0
	C	12m	5714	4728	5575	5450	77836	77044	77446

Contigs were assembled from reads from each isolate (columns). Then reads from all isolates were aligned to the assembled contigs from each isolate, and the number of SNPs enumerated (rows). For example, there was 1 SNP when reads from the 2 week isolate from lineage A were aligned to the contigs from 4 week isolate from lineage A.

Table S3. Identified SNPs in *E. coli* lineages A and B across time.

Lineage A		Base in reference	SNP				Annotation					
Contig	Position		2 w	4 w	2 m	6 m	Start	End	Strand	Gene Name	Original Amino Acid	Alternate Amino Acid
2013A_NODE_149_length_3031_cov_38.721542	188	A				G						
2013A_NODE_149_length_3031_cov_38.721542	410	G				A						
2013A_NODE_154_length_1161_cov_75.563309	409	A				G						
2013A_NODE_15_length_140483_cov_65.503220	113786	T		C	C	C						
2013A_NODE_180_length_75468_cov_64.760590	28271	C				A	27193	28752	+	Putative oxidoreductase subunit	A	E
2013A_NODE_26_length_113048_cov_61.368729	83882	G			A		83529	84140	+	Formate hydrogenlyase subunit 2	P	P
2013A_NODE_33_length_347794_cov_61.142704	219543	G				A	219217	219663	+	Putative inner membrane protein	Q	Q
2013A_NODE_392_length_545_cov_42.056881	331	T				C						
2013A_NODE_78_length_48679_cov_62.558968	28660	C			T		27455	30106	-	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	V	M
2013A_NODE_8_length_41036_cov_59.454357	37903	A			C		37419	38891	+	Betaine aldehyde dehydrogenase (EC 1.2.1.8)	K	T
2013A_NODE_9_length_30547_cov_49.230759	30352	A				G						

Lineage B		SNP			Annotation						
Contig	Position	Base in reference	2 m	6 m	12 m	Start	End	Str and	Gene Name	Orig. Amino Acid	Alt. Amino Acid
2013B_NODE_101_length_90707_cov_87.091919	71886	A			C	71308	72321	+	FIG021952: putative membrane protein	T	T
2013B_NODE_1072_length_36437_cov_89.532646	30658	T		A		30642	31139	+	C-terminal domain of CinA type S	L	Q
2013B_NODE_1080_length_11379_cov_61.034187	10791	T		A		10460	10804	-	FIG00639301: hypothetical protein	K	I
2013B_NODE_117_length_21335_cov_94.341034	14791	C		A	A	14176	15462	+	DamX, an inner membrane protein involved in bile resistance	P	T
2013B_NODE_128_length_60697_cov_86.632339	24352	G			C	23271	25238	-	FUSARIC ACID RESISTANCE PROTEIN FUSB / FUSARIC ACID RESISTANCE PROTEIN FUSC	A	G
2013B_NODE_1435_length_1638_cov_86.649574	842	T			A	416	964	-	Putative transporting ATPase	E	D
2013B_NODE_177_length_21153_cov_93.690826	5021	C			T	4861	5193	+	LSU ribosomal protein L22p (L17e)	A	V
2013B_NODE_182_length_44822_cov_88.078667	7185	A			T	6422	7549	+	N-succinyl-L2CL-diaminopimelate desuccinylase (EC 3.5.1.18)	N	I
2013B_NODE_185_length_42572_cov_81.927017	33197	G			A	32668	33357	-	D-Galactonate repressor DgoR	A	V
2013B_NODE_262_length_46086_cov_82.565529	20849	G		A		20296	21306	-	Alcohol dehydrogenase (EC 1.1.1.1)	A	V
2013B_NODE_282_length_58359_cov_90.223686	37237	T			C	37199	38137	+	LysR family transcriptional regulator IrlhA	L	L
2013B_NODE_343_length_18910_cov_70.166420	7016	A			T	6526	8442	+	Chaperone protein DnaK	E	V
2013B_NODE_350_length_24378_cov_81.994049	125	C			A						
2013B_NODE_391_length_130930_cov_86.418434	2925	C			T						
2013B_NODE_391_length_130930_cov	56602	A		T		56431	57087	-	Putative amidohydrolase	H	Q

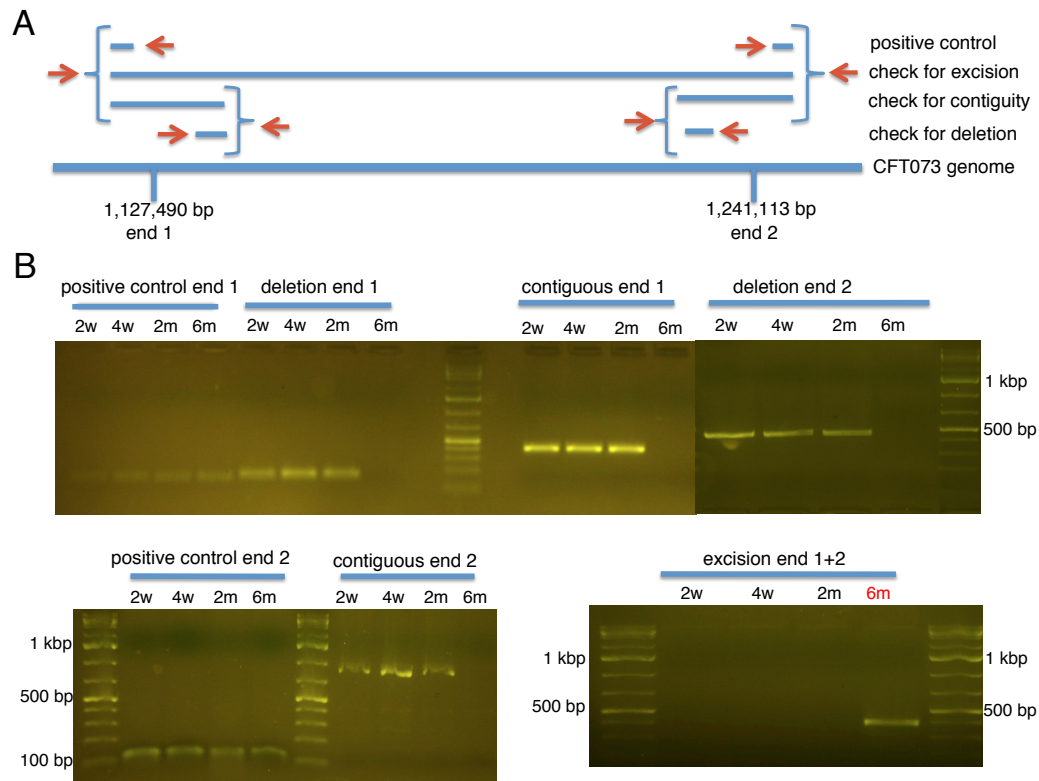
86.418434										
2013B_NODE_399 _length_5672_cov_ 30.594675	691	T		A	155	703	+	Type III secretion inner membrane protein (YscU, SpaS, EscU, HrcU, SsaU, homologous to flagellar export components)	N	K
2013B_NODE_419 _length_45352_cov_ 85.486702	35483	A		T	35020	36114	-	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	I	N
2013B_NODE_45_I _length_26469_cov_ 84.598854	23531	A								
2013B_NODE_472 _length_8834_cov_ 90.308243	943	C		T	894	1847	+	Iron(III) dicitrate transmembrane sensor protein FecR	S	F
2013B_NODE_550 _length_43301_cov_ 83.552345	21521	A		G						
2013B_NODE_963 _length_7960_cov_ 87.411812	2701	A		C	2514	3449	+	Dipeptide transport system permease protein DppB (TC 3.A.1.5.2)	E	A

Table S4. List of Annotated Deleted Genes in Lineage A.

Fatty Acid Synthesis	(3R)-hydroxymyristoyl-[ACP] dehydratase (EC 4.2.1.-) 3-hydroxydecanoyl-[ACP] dehydratase (EC 4.2.1.60) 3-oxoacyl-[ACP] reductase (EC 1.1.1.100) 3-oxoacyl-[ACP] synthase 3-oxoacyl-[ACP] synthase (EC 2.3.1.41) FabV like Acyl carrier protein Acyl carrier protein (ACP1) FIG002571: 4-hydroxybenzoyl-CoA thioesterase domain protein FIG018329: 1-acyl-sn-glycerol-3-phosphate acyltransferase FIG143263: Glycosyl transferase / Lysophospholipid acyltransferase FIGfam138462: Acyl-CoA synthetase, AMP-(fatty) acid ligase
Carbohydrate and Amino Acid Metabolism	D-galactarate dehydratase (EC 4.2.1.42) D-galactarate dehydratase (EC 4.2.1.42) Tagatose-6-phosphate kinase AgaZ (EC 2.7.1.144) Putative O-methyltransferase Aspartate aminotransferase (EC 2.6.1.1) 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha (EC 2.5.1.54)
Iron Scavenging	Glycosyltransferase IroB ABC transporter protein IroC Trilactone hydrolase IroD Periplasmic esterase IroE Outer Membrane Siderophore Receptor IroN Hemolysin activator protein precursor Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family
Bacteriocins and Virulence	antigen 43 precursor Colicin-E1* mannose-specific adhesin FimH Probable microcin H47 secretion/processing ATP-binding protein mchF (EC 3.4.22.-) MchC protein Putative F1C and S fimbrial switch Regulatory protein type 1 fimbriae adaptor subunit FimF type 1 fimbriae anchoring protein FimD type 1 fimbriae major subunit FimA type 1 fimbriae protein FimI2C unknown function YeeV toxin protein
Other	Transposase Transposase Transposase

	Transposase Putative Transposase Mobile element protein Integrase IS2C phage, Tn: Transposon-related functions Putative metal chaperone, involved in Zn homeostasis, GTPase of COG0523 family entry exclusion protein 2 membrane: Transport of small molecules: Cations FIG021862: membrane protein, exporter FIG027190: Putative transmembrane protein putative membrane protein putative membrane protein putative secretion permease putative regulatory protein NgrB tRNA-Arg-TCT
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Figure S1. Confirmation of an excision deletion lineage A.



A. Schematic of PCR reactions that were designed to test whether the deletion that occurred in lineage A was an excision. The schematic shows the positive controls, confirmation of contiguous regions, deletion and excision, for both ends of the deletion.

B. Images of gels of separated PCR reaction products. Reactions for each end of the deletion and the contiguous region produced a product prior to the deletion. A PCR product using primers from the region flanking the deletion confirmed that the deletion was an excision.