The role of the gut microbiota in the subsistence of antibiotic resistance

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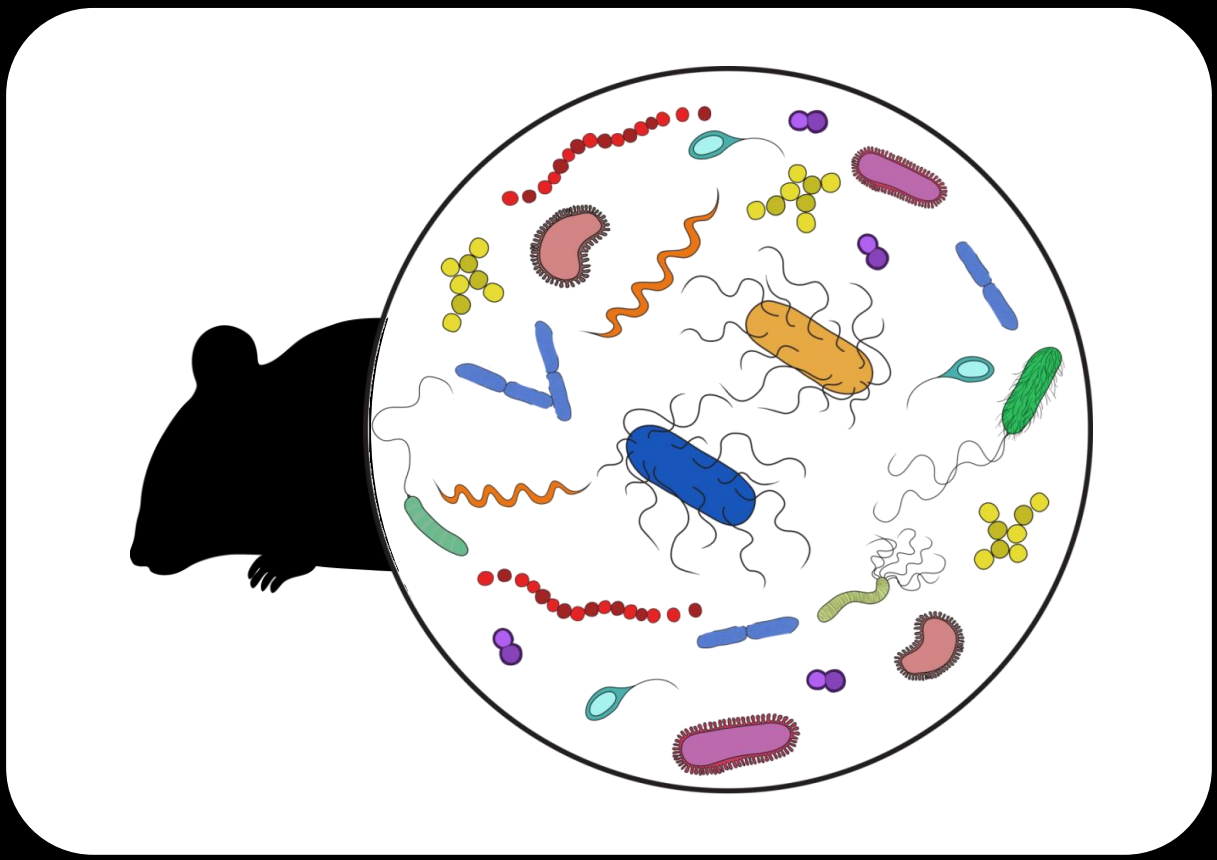

Dissertation presented to obtain the Ph.D degree in Integrative Biology and Biomedicine

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
March, 2020

UNIVERSIDADE NOVA DE LISBOA

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Research work coordinated by: INSTITUTO GULBENKIAN DE CIÊNCIA

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Cover image:

A depiction of *Escherichia coli* in the mammalian gut environment, in which it has to co-exist and compete with several microbial species. Credits go to Joana Carvalho for the original illustration.

The work described in this PhD thesis received financial support from Fundação para a Ciência e Tecnologia, through the grant PD/BD/106003/2014, awarded to Luís Leónidas Cardoso.

This work was conducted at Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal, under the supervision of Dr. Isabel Gordo.

Acknowledgements

This thesis is a product of a long journey- 5 years of scientific life, full of exciting and joyful moments, and also some hardships. If I made it out, it is thanks to the people who cherished and supported me throughout this quest, and whom I would like to acknowledge for our shared deed.

I would first like to thank my family, and in particular my parents, for their constant and unconditional support. They have been exceedingly comprehensive in these 5 years and their wisdom was essential in helping me handle adversity. I would like to thank my paternal grandparents, who have been present throughout my life have been role-models for me in many aspects. To my cousins, which have been true brothers and sisters to me in many occasions. To my maternal grandparents, to uncle Casimiro, to Têtê and other loved ones which are not with us anymore, but have had a great impact in shaping the person that I am today.

I would like to thank Isabel for betting on me and giving me the opportunity to perform this work, for the supervision and for all that she taught me along the thesis. Also, for letting me be a part of a group full of wonderful people. I learned a lot with everyone, from bacterial genetics with Roberto, to mice handling and gavages with Nelson and Catarina, statistics and sequencing tips by Ricardo and Hugo, together with many helpful know-hows passed by our mommy Dani. Paulo's judgment and experience were really important in shaping our story, and his great sense of humor helped a great deal to cope with tough moments. Massimo's contribute was the perfect add-on, the theoretical component that allowed us to go one step forward. It has been a pleasure working with both of you, and I am looking forward to share with the world our endeavors. I would also like to extend my thanks to the former and current members of the BE and BAS groups, for their helpful nature and strong companionship.

I would like to thank the IGC Community. Everyone was extremely helpful and good natured, from the ladies at the cantine to the admin office, making the life at the

Institute more enjoyable and cozier. Of course, this work was only possible thanks to the support and resourcefulness of the members of the Institute facilities, and I am particularly thankful to them. I would also like to acknowledge the PhD Programme personnel, namely Élio, Manuela and Ana for all the support. I would also like to acknowledge my thesis committee, Karina Xavier and Joana Sá, thank you for your insight and helpful discussions.

I would like to thank a core group of friends that I had the pleasure to meet, some since high school, others during my stay at the IGC - Gonçalo Matos, Filipe Vieira, Henrique Colaço, Yash Pandya, André Barros e André Carvalho. Thank you for the profound bro-hood and for all the moments of laughter.

I would like thank my fellow colleagues from IBB2015. In them I have found true and lasting friendship. Although we don't meet as frequently as before, every time we do is a moment to cherish and remember.

I would also like to do a special mention to my "outside of IGC" friends. I thank João F., Ricardo C., Manuel S. and Miguel L. for the unwinding moments of board and roleplay gaming - I hope we can have more of those together one day.

I would like to thank *Dibengos*, who have reached for me even when I did not know I needed it. For the solidarity and for the simple, genuine, happy moments.

To those mentioned, and to those that I did not mention directly but were an important part of this journey, my thanks for making this achievement possible.

Summary

Antibiotic resistance is one of the major contemporary threats to global health. Studies on evolutionary biology, molecular biology and genetics have revealed that many phenomena contribute for the subsistence of resistant bacteria. The environment has been shown to be a key factor, capable of altering fitness costs and the epistasis patterns between resistance determinants. Still, few studies have ventured to assess the costs of antibiotic resistance in natural environments, and such studies are centered on pathogens. It is now known that commensal bacteria can act as reservoirs of resistance, and that resistant commensals can evolve to express pathogenicity and share resistance genes with pathogens. Here, we explore how selection acts on resistant, commensal *E. coli* in the mouse gut.

We observe that the fitness effects of resistance mutations in the gut are not predictable by experiments in standard laboratory media, and that after an antibiotic perturbation, the presence of microbiota affects the outcome of competitions with the sensitive strain. The costs become host-specific and lead to situations in which the resistant strain bears no cost, suggesting a role of the microbiota in the maintenance of resistance. We then report that when resistant bacteria are evolving in this system, they acquire a multitude of mutations that do not correspond to classic compensatory mutations, and that the latter appear at a different pace in mice carrying different microbiota. For the studied mutants, adapting to the gut environment seems to prevail over compensation. We also present a study in which we look for frequency-dependent selection in two environments with different complexity. We find an association between frequency-dependent selection and secretome-related functions in a minimal medium environment, and we observe Darwinian selection in the mouse gut, with a single mutant dominating the competition against the wild-type and other mutants in genes related to the secretome.

The research presented on this thesis highlights the gut microbial community as a factor that influences the survival of resistant and multi-resistant bacteria, and as a whole, stresses the importance of studying bacteria in environments that reflect

their place in nature. Following studies taking into account the biotic environment in which bacteria are inserted may help to prevent and reverse resistance.

Resumo

A resistência a antibióticos é uma das maiores ameaças contemporâneas à saúde a nível global. Estudos de biologia evolutiva, biologia molecular e genética revelaram vários fenómenos que contribuem para a subsistência das bactérias resistentes. O ambiente é um factor-chave, capaz de alterar os custos de *fitness* e os padrões de epistasia entre determinantes de resistência. Ainda assim, os estudos focados no custo da resistência em ambientes naturais são escassos e centrados em agentes patogénicos. Hoje, as bactérias comensais são reconhecidas como reservatórios de resistência, e sabe-se que comensais resistentes podem evoluir no sentido de expressar patogenicidade e partilhar genes de resistência com bactérias patogénicas. Aqui, exploramos como é que a seleção actua sobre *E. coli* comensais resistentes, no intestino de ratinho.

Reportamos que no intestino, os efeitos no fitness das mutações de resistência, não correspondem ao previsto através de meios de laboratório convencionais, e que após uma perturbação através de um antibiótico, a presença da microbiota afecta o desenlace de competições com a estirpe sensível. Os custos tornam-se específicos consoante o hospedeiro, levando a situações nas quais as estirpes resistentes não têm custo, o que sugere um papel da microbiota na manutenção da resistência. Reportamos de seguida que quando bactérias resistentes evoluem neste sistema, adquirem uma variedade de mutações que não correspondem a mutações compensatórias clássicas. Estas aparecem a um ritmo diferente em ratinhos com microbiotas diferentes. Para os mutantes estudados, a adaptação ao ambiente do intestino parece prevalecer em relação à compensação. Por último, apresentamos um estudo no qual procuramos por selecção dependente da frequência em dois ambientes de complexidade distinta. Encontramos uma associação entre selecção dependente da frequência e funções relacionadas ao secretoma num ambiente de meio mínimo, mas observamos selecção Darwiniana no intestino, no qual um mutante domina a competição contra o *wild-type* e contra outros mutantes para genes ligados ao secretoma.

O trabalho de investigação presente nesta tese realça a comunidade microbiana do intestino como um factor capaz de influenciar a sobrevivência de bactérias resistentes e multirresistentes e dá ênfase à importância de estudar as bactérias em ambientes que reflictam o seu lugar na natureza. Estudos subsequentes que tenham em consideração o ambiente biótico que rodeia as bactérias poderão contribuir para a prevenção e a reversão da resistência.

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Thesis Outline

When a bacterium acquires a mutation, its survivability will depend on the fitness effect of the genetic modification. The fitness effect can vary across environments, reflecting different selective pressures, which may stem from the activity of other microbes.

This study was designed to evaluate the contribution of microbiota to the fitness effect of chromosomal mutations in a natural environment – the mouse gut - focusing on how the gut microbiota changes the fitness effect of mutations conferring antibiotic resistance, how it conditions the evolution of antibiotic resistant strains, and to assess the occurrence of stable polymorphisms for public traits in simple and complex environments.

Chapter I presents a general introduction on antibiotic resistance, going through the cell targets of the main classes of antibiotics, how resistance is acquired, and the mechanisms of resistance. Afterwards, the chapter addresses the fitness cost of resistance and the known mechanisms by which bacteria reduce or circumvent this cost. The role of commensal strains as a reservoir and as a source of resistance determinants is then addressed. The major aims of this thesis are stated at the end of this chapter.

In **Chapter II** the fitness of resistant *Escherichia coli* strains was measured through *in vivo* competitions, showing that the cost of chromosomal resistance mutations is affected by the presence of microbiota, in a host-specific manner.

Chapter III follows the evolution of resistant *E. coli* in the gut environment, identifying mutations through sequencing and revealing a prominent role of adaptation relative to compensation.

Chapter IV is a stand-alone chapter, which looks at frequency-dependent fitness effects on single gene deletion polymorphisms in *E. coli*, finding frequency-dependence for secretome genes in a simple environment, and strong, positive selection in the mouse gut.

Chapter V highlights the main findings of this study, briefly contextualizes them within the current literature and proposes approaches to be addressed in future research.

CHAPTER I

Introduction

The incorporation of antibiotics into medical practice was one of the major landmarks in the history of medicine. Their utilization enhanced the combat against diseases of bacterial origin, many of which fatal by the time of their discovery [1], and allowed the prevention of opportunistic infections in delicate medical practices [2], resulting in an effective reduction in human morbidity and mortality caused by bacterial infections [3]. However, over time, the intensive use of antibiotics has led to the dissemination of resistant bacteria, making the treatments ineffective [1].

The prospect got worse with the early reports of multi-resistance [4,5], which allows pathogenic bacteria to accumulate means of survival to treatments [6]. These worrisome news led to a response of the pharmaceutical industry, with the design of synthetic antibiotics, some of which directed to new cell targets. However, resistance to these new agents was developed [7], indicating that bacteria can evolve and acquire resistance to synthetic compounds as well. As a consequence of the unrestrained, extensive and excessive use of multiple antibiotics for decades [8], antibiotic resistance has turned into one of the major public health problems [1,2], and might lead to the failure of quimioterapy based treatments [9] due to the spread at epidemic levels of infections carried out by resistant bacteria [10]. A post-antibiotic era, in which common infections can once again kill, is indeed a real possibility [11].

Main targets of antibiotics

Antibiotics are grouped in several classes based on their composition, target and mechanism of action. Antibiotics target fundamental processes of the cell, such as cell wall biosynthesis, nucleic acid synthesis, both at the level of folate synthesis, DNA replication and transcription into RNA, and at the level of protein synthesis by targeting subunits of the ribosome [12]. Following is a brief description of the mechanism of action of the most commonly used antibiotics, grouped by the targeted cellular process.

Cell wall biosynthesis

Bacterial cell walls are made of peptidoglycans, long sugar polymers. The peptidoglycan undergoes cross-linking of glycan strands through cross links between peptides. In this process, penicillin binding proteins (PBP) are essential for the cross linking of the D-alanyl-alanine portion of the peptide chains by glycine residues. There are two main classes of antibiotics targeting cell wall synthesis, β-lactams, including extended spectrum β-lactams and large spectrum carbapenems, and glycopeptides. β-lactams compete with alanine for PBP by binding to it, preventing transpeptidation and leading to synthesis disruption [13]. Glycopeptides such as vancomycin also act on the transpeptidation step by binding to the D-alanyl D-alanine portion of the peptides, also preventing the action of PBP on these residues [14]. The prevention of the synthesis of peptidoglycan leads to lysis of the bacterial cell [13].

Replication

Within the bacterial cell cycle, the chromosomal DNA is replicated in order to pass genetic information to both daughter cells. Topoisomerases, such as DNA gyrase and topoisomerase IV are essential in this process, as they control and maintain the topological state of DNA molecules [15]. Gyrase acts while DNA is unwinded by helicase during replication and during the elongation by RNA polymerase in transcription by nicking double-stranded DNA, introducing negative supercoils and resealing the nicked ends. The functional gyrase is a topoisomerase made by joining two A subunits, responsible by the nicking and sealing, and two B subunits, that introduce negative supercoils. In Gram-negative bacteria, quinolones such as nalidixic acid and fluoroquinolones such as ciprofloxacin target DNA replication by binding to the gyrase A subunit with high affinity and blocking its strand cut and reseal ability, although certain analogues such as norafloxacin seem to inhibit gyrase activity while binding directly to DNA [16]. In Gram-positive bacteria, quinolones

primary target is topoisomerase IV [17], which nicks and separates DNA strands after replication without introducing negative supercoils [18]. As with gyrase binding in Gram-negative bacteria, quinolone action leads to the impairment of DNA replication. In both gram positive and gram-negative bacteria, topoisomerase inhibitors lead to the generation of single and double strand breaks and culminate in apoptosis of proliferating cells [19]. While quinolones are typically bactericidal, nalidixic acid was shown to turn bacteriostatic in very high drug concentrations, a paradox that may be related with a secondary effect of the drug blockading RNA synthesis [20].

RNA and protein synthesis

Through the action of the DNA-dependent RNA polymerase, the information coded in DNA molecules is used to synthesize messenger RNA or functional non-coding RNA [21], in a process known as transcription.

RNA polymerase is a complex enzyme composed by five subunits, two α, one β, one β' and an ω subunit [22]. Rifamycins, including rifampicin, inhibit DNA-dependent RNA synthesis by strongly binding in a pocket of the β subunit of the RNA polymerase, deep within the DNA/RNA channel. The binding does not occur in the RNA polymerase active site but sterically blocks the extension of the nascent RNA chain after the RNA transcript becomes 2 or 3 nucleotides in length [23], effectively blocking RNA synthesis (**Figure 1**).

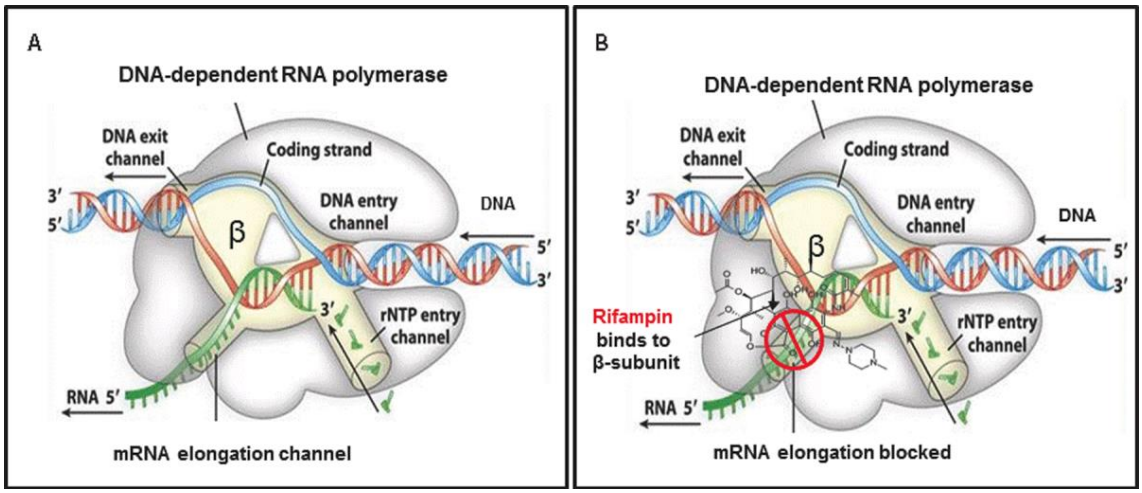


Figure 1 - Mechanism of action of rifampicin. A) Using a DNA strand as a template, RNA polymerase forms an elongated mRNA molecule that corresponds to the coded gene. **B)** By binding to the β-subunit of the DNA-dependent RNA polymerase, rifampicin, also known as rifampin, blocks the elongation of messenger RNA and impairs transcription. Adapted from [24].

The messenger RNA sequence is then processed by the multimeric structure known as ribosome to generate peptides and proteins, with each RNA triplet, named as codon, corresponding to a specific amino-acid residue. This process is called translation. The bacterial ribosome is composed of two subunits, 30S and 50S, made by RNA enveloped by proteins. 30S is composed of 16S rRNA and 21 proteins (S1–S21) whereas 50S is composed of 5S and 23S rRNAs and 36 proteins (L1–L36) [25]. As the two units work together to perform translation, different antibiotics affect protein biosynthesis by targeting either one or the other subunit.

Aminoglycoside antibiotics, such as streptomycin, kanamycin, gentamicin and kanamycin are positively charged molecules, and their uptake depends on their interaction with the negatively charged components of the outer membrane which

lead to an increase in permeability. This process allows the entrance to the cytoplasm through energy-dependent, electro-transport-mediated process [26]. Once in the cytoplasm, aminoglycosides interact with the 16S rRNA of the 30S ribosome subunit through hydrogen bonds in the first ribosome binding site for tRNA, the aminoacyl binding site (A site). Some aminoglycosides, including streptomycin, cause mistranslation of proteins and premature termination of translation [27,28]. The mistranslated proteins can cause damage to the cytoplasmic membrane and facilitate aminoglycoside entry, leading to an increased inhibition of protein synthesis and mistranslation, culminating in cell death [29]. Other aminoglycosides, such as kasugamycin, act by blocking peptide chain initiation [27]. (**Figure 2**). Tetracyclines also interfere with the binding of the t-RNA to the ribosomal A site by acting upon the conserved sequences of 16S rRNA, inhibiting the elongation phase of protein synthesis with a bacteriostatic effect [30,31].

Commonly used drugs such as chloramphenicol, macrolides and oxazolidinones act on the 50S subunit of the ribosome. Chloramphenicol interacts with the peptidyl transferase cavity of the 23S rRNA, also preventing the binding of the tRNA to the A site. Macrolides affect translocation in the early stage of protein synthesis, by targeting the peptidyl transferase center of the 23S rRNA, leading to the premature detachment of incomplete peptide chains. [12]. Oxazolidinones, including the synthetic linezolid, bind to the peptidyl tRNA binding site of the ribosome (P site). Besides affecting the initiation of protein synthesis by inhibiting the formation of the initiation complex, oxazolidinones affect the formation of 70S (the joining of the

two subunits to initiate protein synthesis), and the translocation of the peptide chain if the two ribosomal subunits are already performing translation [32].

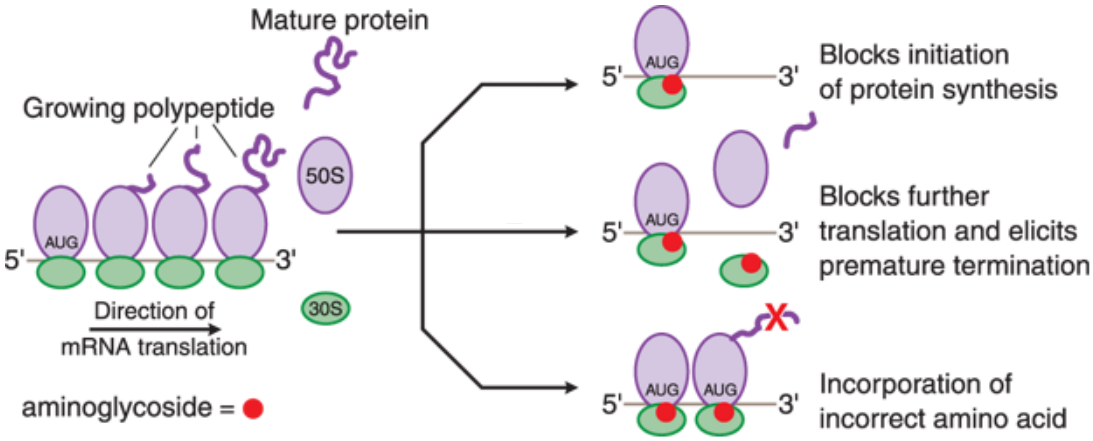


Figure 2 – Mechanisms of action of aminoglycoside antibiotics. All aminoglycosides bind to the 30S subunit of the ribosome. Depending on their structure, they may act by blocking the initiation of protein synthesis or by causing mRNA misreading, which can lead to the the block of ongoing translation and cause translation errors. Streptomycin in particular causes mRNA misreading, promoting mistranslation and translation termination. Adapted and modified from [33].

Metabolism

Some antibiotics have as targets central metabolic pathways of the cell. For instance, sulfonamides and trimethoprim target the essential folic acid pathway. Folate is a critical precursor for the synthesis of glycine, methionine, thymidine triphosphate and purines [34]. Sulfonamides inhibit dihydropteroate synthase through competition with the natural substrate, while trimethoprim acts at a later stage, inhibiting dihydrofolate reductase. Both drugs are bacteriostatic. However, their combination has a synergistic effect, killing the cell and leading to a reduced

probability of evolving resistance [35]. Besides allowing for the expansion of the spectrum of targets of current antibiotics, the development of these drugs is a promising approach to eliminate persistent bacteria - subpopulations of sensitive bacteria that are slow-growing but metabolically active, surviving exposure to antibiotics that act on the dividing clones [36].

Despite the use of a multitude of diverse compounds with the purpose of leading to their death or inhibition, bacteria tend to gain resistance recurrently. To understand how bacteria become able to respond to these harmful agents, it is essential to consider the biologic processes by which resistance is obtained.

The acquisition of resistance

Antibiotic resistance occurs in nature and is found in non-agricultural, non-clinical environments [37]. Furthermore, relatively recent studies indicate that antibiotic resistance is ancient, with targeted metagenomic studies showing the presence of diverse antibiotic resistance determinants in thousands-of-years-old permafrost samples [38,39], and with multi-resistance being reported in environments that were isolated for millions of years [40].

As many antimicrobial compounds are produced by living organisms, bacteria in constant contact with them have evolved to survive in their presence. These bacteria are considered to be intrinsically resistant to one or more antibiotics [41,42]. However, the public health threat of antibiotic resistance does not only come from the expansion of intrinsically resistant bacteria, but also from the acquisition of resistance by previously susceptible strains, including life-threatening pathogens in clinical settings. Resistance can be acquired by spontaneous chromosomal mutations or through the acquisition of genes carrying resistance determinants, obtained from resistant organisms through horizontal gene transfer of mobile genetic elements.

Resistance through horizontal gene transfer

Horizontal gene transfer (HGT) of antibiotic resistance genes has been shown to occur through 3 main mechanisms: transformation [43,44], which involves the incorporation of external DNA [45]; conjugation [46], a contact-dependent exchange of genetic material [47] through the transfer of conjugative plasmids [48] or transposons [49]; transduction [50,51], in which the foreign DNA is transferred through bacteria viruses called phages [52] (**Figure 3**).

HGT is now considered to be a key player in the evolution of bacteria [53]. While it typically ensues between different strains and species, the transfer of homologous regions can also occur between higher taxa [54,55]. As an example, bacteria can acquire antibiotic resistance by transformation with genes of plant origin [56], evidentiating how “promiscuous” bacteria can be. Horizontal gene transfer can occur in various natural habitats. Gene transfer through conjugative plasmids alone has been observed in distinct environments such as soil and rhizosphere [57,58], plant surfaces [59], water [60] and in the mammalian gut [61,62]. Furthermore, environmental conditions can boost the occurrence of HGT. As an example in the context of the mammalian gut, HGT through conjugation [63] and transduction [64] is greatly increased when the intestine is inflamed.

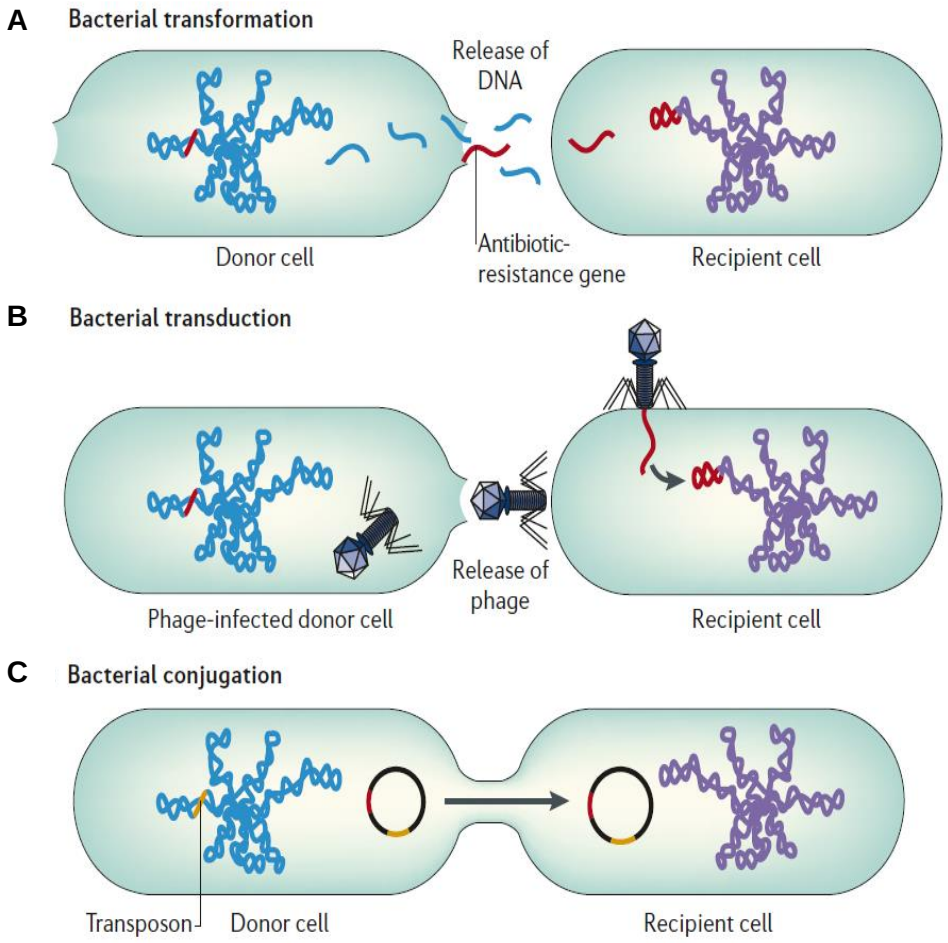


Figure 3 – Horizontal gene transfer between bacteria. A) Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism. The antibiotic-resistance gene can be integrated into the chromosome or plasmid of the recipient cell. **B)** In transduction, antibiotic resistance genes are transferred from one bacterium to another by means of bacteriophages and can be integrated into the chromosome of the recipient cell. **C**) Conjugation occurs by direct contact between two bacteria: plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another. As other mobile genetic elements, transposons can carry antibiotic-resistance genes. Adapted from [65].

Additionally, horizontal gene transfer processes can lead to a very fast spread of new genes in nature. For instance, resident *E. coli* in the mouse gut can transfer prophage genes at an epidemic level to an invading strain in a matter of days, a process that precedes the occurrence of adaptive point mutations [66].

Antibiotic resistance genes coded on mobile genetic elements are frequently acquired in integrons [67]. These DNA elements can be found in conjugative plasmids, phages and transposons, and act as assembly platforms that incorporate exogenous open reading frames through site-specific recombination and convert them to functional genes by securing their expression [68]. Integrons typically encode an integrase, a primary recombination site and an outward-orientated promoter, providing all of the tools for the transcription of the captured gene. Multiple genes can be sequentially integrated as gene cassettes, allowing for the accumulation of functional resistance determinants [67]. In fact, integron-bearing mobile genetic elements are thought to have been major agents in the fast spread of multi-resistance in Gram-negative bacteria through horizontal gene transfer [69]. Very large, integron-like structures can also be found in the chromosome – the super-integrons. Although not mobile, these sequences can contain hundreds of accessory genes, including cassettes related to antibiotic resistance [70], and are present in many bacterial species, being considered to have an important role in genome evolution [68].

Resistance acquisition through mutations

Despite the important role of HGT in its spread, antibiotic resistance can originate *de novo* in sensitive strains. Some bacteria, such as the intracellular parasite *Mycobacterium tuberculosis*, are mostly clonal, and typically acquire resistance in this way [71]. Antibiotic resistance through *de novo* mutation often occurs through single nucleotide substitutions that modify the drug target, but resistance can also be acquired through other classes of spontaneous mutations. For instance, insertions and deletions generate frameshifts and premature stop codons in genes

involved in antibiotic susceptibility [72], while gene duplication can lead to an increased dosage of antibiotic hydrolytic enzymes and efflux gene pumps [73]. While gene duplications are often unstable, they can facilitate the development of a stable resistance phenotype, by allowing survival and population expansion until the occurrence of point mutations conferring higher levels of resistance [74]. Point mutations can confer resistance without modification of the primary antibiotic target. One such example regards point mutations in regulatory regions of porin coding genes that reduce porin expression [75], decreasing susceptibility. Point mutations and deletions on such genes can lead to porin impermeability [76,77] culminating in an increased resistance to carbapanems in Enterobacteraceae. Another example regards multi-drug (MDR) efflux pumps. Point mutations on gene repressors of MDR efflux pump genes, or in the gene regulator to which they bind can lead to overexpression of the efflux pump. On the other hand, mutations of the coding region may change the substrate binding, both at the range and affinity level [41], resulting in an elevated level of resistance.

Mutation rates and effects

Mutations can occur through errors in DNA replication or through DNA damage. As cell division ensues, mutations with fitness effects spanning from fitness benefits to lethality can occur. In haploid microbes, the mean genome mutation rate is estimated to be of 0.003 mutations per DNA replication [78]. In *E. coli*, the mutation rate is estimated to be close to this value [78], and mutation accumulation studies in this species estimate that roughly 1 in every 15 mutations is deleterious (rate of 0.0002; [79]), while 1 in every 150 newly arising mutations is beneficial (rate of 2×10^{-5}, [80]), in line with theoretical and molecular evolution studies suggesting that most mutations are neutral [81,82] or nearly neutral [83–85]. Across haploid species, the variation in mutation rate per base pair is high (≈16000-fold). However, the variation in mutation rate per genome is quite low (≈2.5-fold), suggesting a selective pressure towards a balance between deleterious effects of mutations and the maintenance of a minimal mutation rate in haploid organisms [78]. This hypothesis is supported by data on thermophile microbes. For instance, the estimated mutation rate per genome for the

crenarchaeon *Sulfolobus acidocaldarius* and the bacterium *Thermus thermophilus*, which have evolved in extreme heat conditions, is about 5 times lower than in their mesophile counterparts, possibly reflecting an adjustment of the mutation rate to strong purifying selection [86].In bacteria, mutation frequencies are generally found to be between 10^{-10} and 10^{-9} per replicated base pair [87]. While this mutation rate per nucleotide reflects a low probability of a specific mutation to occur, bacterial populations in nature can reach high population size. Furthermore, mutations conferring resistance can occur in multiple positions in the same target gene. As an example, a classical study mapped 17 mutational distinct alterations able to confer rifampicin resistance [88]. The disposal of multiple mutational options also promotes the recurrent appearance of resistance mutations in natural populations. Additionally, the rate of emergence of antibiotic resistance mutants is affected by cell physiology, genetics, and by aspects of the environment, such as temperature [89] and physical structure [90].

Environmental effects on mutation rate

The mutation rate towards resistance can also change with the presence and dose of antibiotics themselves. In fact, sublethal concentrations of antibiotics can increase the rate and frequency of HGT, recombination and mutagenesis [91], while the nature of the selected resistance mutations can vary with the dose of antibiotic [92]. Furthermore, drug exposition can increase the mutation rate towards resistance to the antibiotic - as an example, the exposition to ciprofloxacin can increase 10000-fold the rate at which ciprofloxacin resistance mutations occur [93,94], through DNA damage and the activation of the SOS response [94]. A mutagenic effect has also been described for other fluoroquinolones [95] and for streptomycin [96].

Successive selective pressures, such as the use of different antibiotics can lead to the emergence of “mutator” bacteria. These strains originate through certain mutations in core genes related with DNA repair, which vastly increase genome mutation rate, sometimes up to 1000-fold [97]. *In vitro* studies indicate that resistance to ampicillin, streptomycin and ofloxacin [98], as well as low level resistance to

rifampicin and ciprofloxacin [99] emerge more frequently in mutator lines of *E. coli*. Mutator phenotypes were reported to occur in natural populations of pathogenic [100–102] and commensal bacteria [103,104] and allow for greater resistance levels than non-mutator populations, particularly when full resistance requires more than one mutational step [99,105]. Furthermore, some mutations conferring mutator phenotype also increase the recombination rate and are under frequent horizontal gene transfer themselves [104], promoting the sharing of potentially beneficial traits such as virulence factors and antibiotic resistance determinants.

Resistance through chromosomal mutations can be easily produced in a laboratory with culturable bacteria through a fluctuation test, by letting bacteria grow into very high population size while accumulating mutations, followed by a strong selection with an antibiotic - a test adapted from classic studies on the origin of resistance phenotypes to phages [106] and to antibiotics themselves [107,108]. This test is often used to estimate the mutation rate of bacteria in a given environment [109]. Due to the ease of production and the immediate acquisition of a selective phenotype, chromosomal resistance is one of the most studied types of genetic alteration. As many spontaneous resistance mutations occur in essential genes, the study of the different types of resistance has led to important findings in molecular and cell biology of bacteria, often coupling molecular and evolutionary mechanisms together.

Mechanisms of resistance

There are several mechanisms by which bacteria can avoid antibiotic mediated killing or inhibition (**Figure 4**). These fall in 3 main categories: mechanisms that decrease target access and minimize the intracellular concentration of the antibiotic; mechanisms that modify the antibiotic target by genetic mutation or post-translational modification; and mechanisms that lead to the inactivation of the antibiotic through hydrolysis or modification [41].

Decreased target access

One way through which bacteria limit the access of the antibiotic target is by reducing permeability. As the outer membrane of Gram-negative bacteria forms a permeability barrier [110,111], hydrophilic antibiotics have to enter the cell through outer membrane porin proteins, which are thought to function as non-specific channels in Proteobacteria [111]. Through the down-regulation of porin expression [112], and through the replacement of porins with selective channels [113], bacteria can block the entry of hydrophilic antibiotics inside the cell.

Bacteria can also limit the access to intra-cellular targets through the expression or overexpression of efflux pumps. Bacterial pumps are a means of active transport and are a major contributor for the intrinsic resistance of Gram-negative bacteria [110]. Some pumps have a narrow substrate specificity [e. g. tetracycline pumps) [114]], but many are able to transport a wide range of structurally dissimilar compounds. Such transporters can confer resistance to a multitude of antibiotics, and hence are known as multidrug resistance (MDR) efflux pumps. While efflux pumps are ubiquitous in the chromosome of bacteria, some are also coded in mobile genetic elements [115–117] and can be shared through horizontal gene transfer.

Another important mechanism of reduction of target access has to do with population level phenotypes such as biofilm formation. Bacteria can encase themselves in a self-produced polymer matrix made of polysaccharide and protein and DNA. These structures can harbor a single or more bacterial species living in a socio-microbiological way [118], and frequently confer increased tolerance to antibiotics and disinfectant chemicals [119], by physically reducing the exposition to the antibiotic through slow penetration [120]. Additionally, biofilms typically generate a gradient of nutrients and oxygen that lead to concentration driven changes in division rate and metabolic activity, with some cells developing a level of tolerance by fine tuning of the expression of genes involved in the additional resistance mechanisms such as efflux pumps and degrading enzymes, leading to further reduced target access [119]. Furthermore, biofilm-growing bacteria can exhibit a higher mutation frequency when

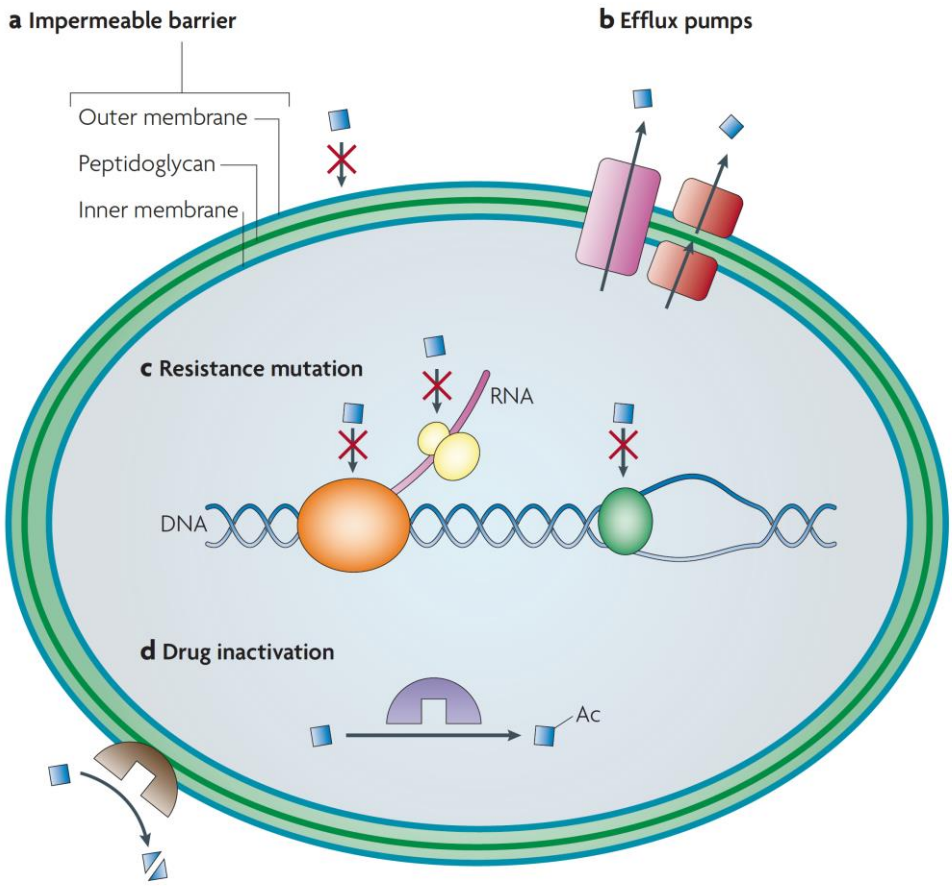


Figure 4 – Main mechanisms of resistance in gram-negative bacteria. a) Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics (blue squares) due to membrane impermeability, while others can limit drug entry through changes in gene expression that reduce drug permeability. **b)** Efflux pumps. Bacteria can also limit target access through the activity of these pumps, which secrete antibiotics to the outside of the cell. **c)** Resistance mutations. These mutations modify the target protein, and reduce target affinity, for example, by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. **d)** Inactivation of the antibiotic. Inactivation can occur by modification covalent modification of the antibiotic, such as that catalyzed by acetylases (purple) acting on aminoglycosides, or by degradation of the antibiotic, such as that catalyzed by β-lactamases acting on β-lactams. Adapted from [37].

growing in a biofilm when compared to planktonic growth [e. g. 105-fold increase in mutability for *Pseudomonas aeruginosa* PA01, [121]], and horizontal gene transfer is also known to be increased in biofilms [122], which in turn can increase the chance of acquiring antibiotic resistance mutations.

Reduced target affinity and target protection

Most antibiotics bind specifically to their targets with high affinity and impair the target's function. However, certain naturally occurring mutations and gene recombination in the target structure can prevent antibiotic binding while still allowing for the target to carry its function, even if not optimally. Such alteration in the target site is a common, ubiquitous mechanism of resistance, as examples of clinical strains with this type of resistance can be found for every single class of antibiotic, regardless of the mechanism of action [123].

Perhaps the most classic examples of resistance through reduced target affinity refer to beta-lactams. The acquisition of spontaneous mutations in penicillin binding proteins conferring resistance has been reported for several genera of bacteria, including *Haemophilus influenzae*, *Helicobacter pylori*, *Proteus mirabilis*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Listeria monocytogenes* [123]. Strikingly, these altered targets with reduced affinity can be shared by horizontal gene transfer. One the best examples refer to β-lactam resistance derived in bacteria such as *Streptococcus pneumoniae* [124] and pathogens such as *Neisseria gonorrhoeae* [125] and *Neisseria meningitidis* [126] through the formation of "mosaic" penicillin binding proteins. These proteins are generated through transformation and recombination with acquired DNA coding beta-lactam insensitive variants of the proteins, usually originated from closely related species, including bacteria of commensal nature [126]. Another reported situation refers to the acquisition of the *mecA* gene by methicillin resistant *Staphylococcus aureus*. The gene encodes an alternative penicillin-binding protein 2 (PBP2a), and is carried on a

large genetic element, the staphylococcal cassette chromosome *mec*, which is presumed to have been acquired by horizontal gene transfer from other *Staphylococcus* species [127].

Mutations conferring resistance to rifampicin and streptomycin are also classic examples of reduced target affinity. Rifampicin is a relevant drug in the treatment of pathogenic bacteria, being particularly important to treat tuberculosis [128]. Resistance to rifampicin typically occurs through chromosomal mutations in the *rpoB* gene, which codes for the target of rifampicin, the β subunit of the DNA-dependent RNA polymerase. The genome alterations include point mutations, with certain single nucleotide modifications being sufficient to grant high levels of resistance, but can also occur through small insertions and deletions [129,130], most of which occurring in a 81 base pair restricted region of the *rpoB* gene called cluster I [129]. Additional resistance mutations can occur in other *rpoB* regions, such as cluster N, cluster II and cluster III [131], but only a fraction of the resistance mutation spectrum is responsible for the majority of clinical rifampicin resistance in *Mycobacterium tuberculosis* [129]. Most resistance mutations map directly in a fork domain of the RNA polymerase, proximal to the catalytic site, or in adjacent regions [88,132]. Amino-acid substitutions in these sites are expected to affect the conformation of the binding pocket and lower its affinity for rifampicin [132], not allowing the drug to bind and block transcription elongation.

Mutations in the ribosomal protein S12, coded by the *rpsL* gene [133] and in the 16S rRNA 530 loop, coded by the *rrn* operons [134,135] can confer high levels of streptomycin resistance. The ribosome 30S subunit contains a conformational switch that is important for the optimization of translation. The H27 switches from an error-prone, ribosomal ambiguity form (*ram*), and an alternative hyperaccurate, “restrictive” form [136]. Streptomycin in particular acts by stabilizing the error prone state and increasing the binding of non-cognate tRNA. Most S12 resistance mutations and 16S mutations in the 530 loop lead to changes in ribosome accuracy. In the respective mutants, the *ram* state is very destabilized, and the stabilization induced by streptomycin does not trap the ribosome in such error prone state [136]. Due to the

balancing equilibrium between ribosomal states, in a fraction of these mutants, streptomycin presence can even become essential [137,138].

Target protection by modification

The antibiotic target can be protected by modifications that do not require mutational change. Protection of targets has been found to be a clinically relevant mechanism of resistance to several antibiotics. One example is the action of erythromycin ribosome methylase on the 16S rRNA, protecting the target from being bound by macrolides and lincosamines [139]. Another example is the chloramphenicol-florfenicol resistance methyltransferase. This enzyme methylates the position A2503 of the 23SrRNA, conferring resistance to a wide range of drugs with nearby targets, such as phenicols, pleuromutilins, streptogramins, lincosamides and oxazolidonones [140]. Resistance to aminoglycosides can also be granted through 16S rRNA methylation [141,142]. Protective agents can also associate with the antibiotic target. Such examples are the quinolone resistance *qnr* genes, which encode pentapeptide repeat proteins. These agents bind to DNA gyrase and topoisomerase IV and protect the enzymes of inhibition by quinolones. While the mechanism of action of these resistance determinants is not fully understood, a model of the mechanism of action based on structural data of QnrN1 suggests that these agents might interact with topoisomerase-quinolone complexes, effectively rescuing the enzyme and allowing it to re-ligate DNA, thus preventing the formation of double-strand DNA breaks that typically occur with the antibiotic action [143]. Some of the antibiotic resistance genes conferring target protection can be encoded by cryptic genes, and a recent study in *Salmonella enterica* shows that such genes can be activated and confer resistance through mutations that affect their expression – a chromosomal mutation induced the expression of an aminoglycoside adenyl transferase in stringent conditions, leading to resistance to streptomycin and spectinomycin [144]. On a similar note, mutations inactivating the 16S rRNA methyltransferase *gidB* were shown to confer low-levels

of streptomycin resistance in *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Escherichia coli* through methylation of the 16S rRNA 530 loop [145], effectively protecting the target from the antibiotic action.

Antibiotic inactivation

Antibiotic inactivation is a major mechanism of antibiotic resistance. Naturally occurring antibiotic resistance through antibiotic modification was first reported in 1940, with the discovery of penicillinase [146], roughly a decade after the discovery of penicillium [147]. Since then, thousands of enzymes degrading or modifying antibiotics of different classes have been identified, including β-lactams, aminoglycosides, phenicols and macrolides [41]. Some of these enzymes are able to degrade different antibiotics of the same class [148], inclusively to modified β-lactams that were designed to be effective against strains producing natural β-lactamases [149,150]. The carriage of these extended-spectrum β-lactamases and carbapenemases has led to a fast, world-wide spread of strains that are resistant to clinically used β-lactam antibiotics [151–153].

Many antibiotics have hydrolytically susceptible chemical bonds which are essential to their biological activity. β-lactamases, conferring resistance to β-lactams, esterases, conferring macrolide resistance and fosfomycin resistance epoxidases cleave these vulnerable bonds, destroying antibiotic activity. As hydrolytic enzymes require only water as a co-substrate, they can be excreted by bacteria and intercept antibiotics before they reach the cell [154].

Another common type of antibiotic modification is group transfer, which is carried out by the large and diverse family of group transferases. These enzymes modify antibiotics covalently, resulting in the prevention of target binding by steric hindrance. Various chemical groups can be transferred, including acyl, thiol, phosphate, nucleotidyl and ribitoyl groups [41,154]. These strategies require molecular co-substrates such as ATP, acetyl-CoA or NAD^+, UDP glucose or gluthatione to react

with the antibiotic, and hence are active only in the cytosol [154]. Aminoglycoside antibiotics are particularly susceptible to modification, since these large molecules can be deactivated by the action of different classes of enzymes [41]. Group transfer mediated antibiotic inactivation is also known to act on other antibiotics such as chloramphenicol [155] and streptogramin [156] through acetyltransferases, and rifamycins through a phosphotransferase enzyme [157]. A bioinformatic analysis of GenBank sequences coupled with heterologous expression experiments suggests that cryptic orthologues of the latter are present across environmental and pathogenic Gram-positive bacteria [157], further supporting that cryptic embedded genes may be a significant fraction of the antibiotic resistome [158]. Besides hydrolysis and group transfer, antibiotics can also be inactivated by oxidation [159,160], and by the action of lyases [161], although these routes seem to be much less common in nature [154].

The fitness effects of antibiotic resistance

A critical aspect for the maintenance of an antibiotic resistant bacteria lies in its ability to compete with antibiotic sensitive ones. In order to predict which strains are maintained in a population, evolutionary biologists estimate and compare their fitness — a quantitative measure of a genotype's competitive ability. Fitness is derived from all phenotypes affecting the ability to survive and reproduce in a given environment. In the absence of strong random genetic drift, fitness will determine the frequency change of a population's genotypes over time [162].

Fitness can be measured in one of two ways: as absolute fitness and as relative fitness. Absolute fitness refers to the variation in absolute numbers of a genotype, while relative fitness refers to the frequency change of the genotype in the population [163]. As every environment has a limit to the number of individuals that it can carry — a carrying capacity — relative fitness is commonly used to predict the fate of a given genotype — maintenance, fixation or extinction. In bacteria, relative fitness is typically measured through a direct competitive fitness assay, in which competing strains are

co-cultured in the same set of growth conditions, or estimated by measuring and comparing quantifiable growth traits, such as maximum growth rate, in single culture growth [164]. When a change in the genetic information affecting one or more phenotypes occurs, it will have a fitness effect in the individuals carrying it. This fitness effect can lead to a higher fitness by rendering a fitness benefit or lead to a lower fitness by imposing a fitness cost.

The cost of antibiotic resistance

As previously discussed, antibiotic targets are often core components of the cell. These components are typically involved in essential cellular functions, such as replication [165], transcription [166], translation [167], and cell wall biogenesis [168]. Mutations conferring resistance by target gene modification can structurally change these elements into a suboptimal state [169] and lead to pleiotropic effects [170–172]. On the other hand, genes obtained by horizontal gene transfer, as well as genes with an amplified expression imply a metabolic cost to the cell due to increased transcription and translation of genes conferring antibiotic resistance [173]. Furthermore, the acquisition of mobile genetic elements can alter the transcription of profile of chromosomal genes [174] and cause chromosomal perturbations if integrated into the host's genome [175]. As a consequence, the acquisition of antibiotic resistance is expected to inflict a fitness cost in the absence of antibiotics [164,176,177]. Several laboratory studies have shown that resistance is effectively associated with deleterious effects, and the fitness cost of resistance has become a well-established concept [178–180].

Selection favoring the maintenance of resistance

As resistant bacteria tend to be inferior competitors than their sensitive counterparts, an intuitive strategy for containing the spread of antibiotic resistance is to suspend the use of ineffective antibiotics until resistant phenotypes decline to low

frequency [176]. This strategy has been adopted by different countries and for different antibiotics, but its effectiveness has been inconsistent [164,181]. In some cases, resistance has decreased as predicted [182–186]. However, in other situations, resistant determinants were not eliminated [185,187,188], and there is evidence of spread of resistance after antibiotic reduction campaigns [189], indicating that in nature, bacteria have ways to persist despite the expected cost of acquiring resistance.

Over time, several biological phenomena allowing the reduction or circumvention of a fitness cost of resistance were reported. These include resistance mutations with no detectable cost, selection for resistance at residual antibiotic concentrations, co-selection, environment influence on the fitness effects, compensatory mutations and epistasis.

Costless mutations

Some resistance mutations have been reported to confer high levels of resistance while imposing reduced costs or even no cost at all [9]. This absence of cost is thought to be related with the nature of the mutations. For instance, certain streptomycin resistance mutations leading to an amino acid change from a lysine to an arginine have an insignificant effect on fitness in *Salmonella enterica* [190,191] and *E. coli* [192], and in the latter study, some nalidixic acid resistance mutations seem to have no cost as well in standard laboratory conditions [192]. The costless mutations in streptomycin happen to match with non-restrictive resistance phenotypes, which are similar to the wild-type's regarding translation speed and fidelity [193]. Furthermore, in *Salmonella enterica* these mutations do not impair virulence [190], suggesting that the corresponding mutants may be able to compete with the wild-type in clinical settings and establish stable populations of resistant bacteria even in the absence of antibiotics.

Selection and mutagenesis at residual antibiotic concentrations

The minimum inhibitory concentration (MIC) is the lowest concentration of a compound that prevents visible growth of a bacterium [194]. Resistant strains have a higher MIC than their sensitive counterparts. MIC measurements allows the detection of resistant strains and the determination of the level of resistance.

It should be noted that the level of resistance can vary extensively depending on the resistance mechanism. Certain mechanisms confer such low-level resistance that it can only be detected by time-kill experiments [195], while others may provide extremely high resistance levels, which may even surpass the solubility limit of the antibiotic [196,197]. Mechanisms involved in drug efflux tend to confer a lower level of resistance than those that modify the antibiotic target or inactivate the drug [198]. Furthermore, bacterial growth may be unaffected by increasing antibiotic concentration until the MIC is reached, such as for amdinocillin resistant mutants in *E. coli* [199], or monotonically decrease with the increasing levels of antibiotic [200,201]. Thus, the fitness of resistant bacteria might be constant or vary extensively as a function of antibiotic concentration [198].

Selection for resistance is classically thought to be driven by high, supra-MIC concentrations of antibiotics used in therapy, animal husbandry and agriculture. However, nowadays, antibiotics are widespread throughout the environment in low concentrations due to contamination from human activities [202], with many interactions between antibiotics and microbial populations occurring in such conditions. It has thus been hypothesized that these low concentrations have a relevant role in the development of resistance [203] and it has been experimentally demonstrated that concentrations below the wild-type's MIC can affect selection and favor resistance [91]. A fitness advantage of resistant strains at sub-MIC concentrations of tetracycline and ciprofloxacin was shown for *E. coli* mutants [204], while in *S. enterica*, such advantage was shown for the same antibiotics and streptomycin [201]. The latter study further shows that resistant mutants occur under a sub-MIC regime, sustaining the idea that residual levels of antibiotics can give rise to resistant phenotypes on their own.

A recent work focused in *S. enterica*'s evolution at sub-MIC levels of streptomycin shows that the acquired resistance mutations can have a different nature than the ones obtained in high drug concentration regimes. Low antibiotic levels are shown to lead to high levels of resistance through the accumulation of different small-effect resistance mutations that either alter the ribosome target, reduce the aminoglycoside uptake or induce a cryptic aminoglycoside modifying enzyme [205]. These observations indicate that low levels of antibiotics foster a greater mutational space for the selection of resistant phenotypes and provide additional evolutionary paths towards high-level resistance. Unlike supra-MIC concentrations of antibiotics, which typically kill non-resistant bacteria, below MIC concentrations still affect and act as a stress to sensitive bacteria. The stress leads to a physiological response of the cell, often leading to the activation of conserved stress response systems and leading to an increase in the rate and frequency of genetic processes that promote resistance acquisition, such as horizontal gene transfer [206–208], recombination [209–211] and mutagenesis [212–214].

One of the main stress responses to sub-MIC concentrations of antibiotics is the SOS response – a systemic reaction to DNA damage in which cell growth is arrested and DNA repair is promoted. This response implies the recruitment of RecA, which is involved in recombination, and of translesion DNA polymerases, which introduce base substitutions at a high frequency [215], increasing mutagenesis. Another major stress response activated by antibiotic stress is the general stress response, mediated by RpoS induction [216]. As with other sigma factors, RpoS it interacts with the core RNA polymerase and controls the expression of a large, yet specific collection of genes [217]. Within such a set, RpoS positively regulates the expression of *sdsR*, a small RNA molecule that represses the messenger RNA of MutS. The latter is a protein involved in the DNA mismatch repair, being pivotal in mending replication errors. Furthermore, the error prone DNA polymerase IV is also a part of the RpoS regulon. Together with the depletion of MutS, the action of DNA polymerase IV leads to a RpoS-mediated induction of mutagenesis in the presence of beta-lactam antibiotics [216], which in turn can generate mutations conferring resistance.

Co-selection and cross resistance

When adapting to a specific environment, bacteria can acquire mutations or genetic elements that are beneficial in other environmental settings. Such acquisitions, which prepare bacteria for environments to which they are not exposed, are broadly called as co-selection. Antibiotic resistance is frequently co-selected with multiple resistance phenotypes, such as resistance to a different antibiotic, to heavy metals or other biocide agents [218,219].

Resistance to an antibiotic can be acquired after exposure to another agent if the two attack the same target, initiate a common pathway to cell death or share a common route of access to their targets, through the evolution or activation of a shared resistance mechanism. This type of co-selection is called cross-resistance, and allows the development of resistance to multiple antibacterial agents through a single selective pressure [220]. Cross-resistance is commonly associated to multidrug resistance elements, especially those involved in efflux mechanisms [221]. Multidrug pumps in particular have often a wide substrate specificity and can lead to elevated levels of resistance to multiple agents [222]. In addition to antibiotics, multidrug pumps can simultaneously confer resistance to metals[223–225], and to the natural substances produced by the bacteria's host such as bile salts, hormones and defense molecules [226]. There is accumulated evidence that multidrug pumps can be involved in bacterial pathogenicity, indicating that in certain situations, the use of an antibiotic may help to select for increased virulence [226].

Co-selection can be driven by co-resistance, which occurs when a resistance determinant is coded in the same genetic element as another, such as plasmids, transposons or integrons [220]. Antibiotic and toxic metal resistance genes are frequently linked in plasmids [227–230]. A key example of co-resistance regards Tn21-like transposons, in which a mercury resistance and multiple antibiotic resistance genes are contained [231–233]. Tn21 is thought to have accumulated multiple antibiotic resistances in its integron while associating to the mercury resistance *mer* operon,

resulting in Tn21 dissemination at a time when clinical, agricultural and industrial practices provided both types of selective agents [233].

There is evidence that permafrost-derived *Pseudomonas* contains transposons that are closely related with ones found in present-day bacteria, but do not carry resistant determinants [234]. Furthermore, the characterization of ancestral R-plasmids from pre-antibiotic era culture collections of Enterobacteriaceae indicate that while a significant fraction of pre-existing R-plasmids had the ability to be transferred, they did not yet carry transferable antibiotic resistance determinants [235]. These studies suggest that co-resistance may have been selected and amplified by the anthropogenic use of antibiotics.

Co-selection can also occur due to a mechanism of co-regulation. Resistance genes can have a linked transcriptional and translation response with other stress response genes. For instance, in *E. coli* MG1655, excess zinc has been shown to cause the upregulation of the *mdtABC* operon [236], which codes for an efflux system that confers resistance to antimicrobial agents such as novobiocin and deoxycholate [237]. Zinc exposition was also shown to induce and select for resistance to the carbapenem antibiotic imipenem in *Pseudomonas aeruginosa*. The heavy metal efflux pump CzcCBA, involved in zinc resistance, was shown to have increased expression, while the expression of the OprD porin, involved in carbapenem transport, was reduced. This was due to a single amino-acid substitution in the CzcS sensor protein, shown to be involved in their regulation [238]. Such co-regulated stress responses can lead to the expression of antibiotic resistance determinants prior to the exposition to the antibiotic and render it ineffective. Unless co-regulation is lost, selection favoring one of the phenotypes can drive the maintenance of the other.

Compensation

Although antibiotic resistance determinants are often deleterious, resistant bacteria can lower their cost through the acquisition of compensatory mutations. These mutations confer a genetic background-specific fitness benefit by

counteracting the phenotype of deleterious mutations, partially or sometimes fully restoring the strain's competitive ability [169]. As such, they are extremely important to secure the competitive ability of resistant strains against sensitive bacteria.

Compensation for single resistance mutations has been widely described in laboratory conditions [239–241], including through *in vivo* studies involving pathogens [190,191,242], and compensatory mutations are often found in clinic isolates [241,243,244].

Bacteria can overcome the deleterious effect of resistance through an extensive array of compensatory mutations [169], including mutations in different gene targets [190,196,240,245,246], making compensation through the acquisition of mutations far more common than genotypic reversion [191,239,240,247,248]. The latter is a specific case of compensation in which the mutant loses the original resistance mutation, fully recovering fitness and antibiotic sensitivity. In this work, I will refer to genotypic reversions in separate from the remaining compensatory mutations.

Compensation can occur via several mechanisms. One of the most common is the restoration of the structure and function of the protein or RNA molecule responsible for the altered phenotype, by the acquisition of additional intragenic mutations [169]. Intragenic compensation has been shown to occur for a wide array of antibiotics, such as fusidic acid [242,247], streptomycin [190,191,240,249], beta lactams [250–252], rifampicin [190,239,245], sulfonamids [253], coumarins [254] and mupirocins [255].

Regarding streptomycin resistance, a fraction of the mutations in the *rpsL* gene increase translation accuracy and decrease the protein elongation rate, a restrictive phenotype that results in an impaired bacterial growth [240,256]. In *Salmonella enterica*, the fitness cost of one such restrictive mutation (*rpsL K42N*) could be compensated by an intragenic mutation (*rpsL H93R*) which restored the translation accuracy and elongation rate by destabilizing the mutant ribosome's restrictive conformation [240].

A similar situation occurs when considering rifampicin resistance mutations. Resistance mutations in the *rpoB* gene have often been reported to impose a cost [177,190,239]. The *rpoB R529C* mutation in particular was shown to be defective in the transition from transcription initiation to the elongation stage, leading to increased

aborted transcription initiation [257]. For other *rpoB* resistance mutations, the increased cost was correlated with a lower transcription efficiency [239]. In the latter study, during the evolution of a resistant strain in the absence of antibiotic, intragenic compensatory mutations were acquired in roughly half (11/20) of the evolved lines. While the compensatory mutations improved growth rate without altering the level of resistance, in 5 out of 7 tested lines, the transcription efficiency was rescued.

Compensatory mutations also occur frequently in other genes besides the one conferring resistance. These targets are intimately related with the resistance gene, with compensation allowing the restoration of the structure and function of multi-subunit complex proteins or organelles, such as the RNA polymerase and the ribosome [169].

In *E. coli* and *S. enterica*, the aforementioned restrictive phenotype of streptomycin resistance mutations, which leads to ribosomal hyper accuracy, could be counter balanced by mutations in other ribosome proteins that decrease the accuracy of the translational process. In most mutants, fitness was partially restored, but in some cases, the compensated strains could grow at a pace similar to the sensitive wild-type's [190,240,249]. As with intragenic compensation, these mutations are thought to destabilize the restrictive conformation of the ribosome, counteracting the phenotype of the restrictive antibiotic resistance mutations [169].

For rifampicin resistance mutations, extragenic *rpoA* and *rpoC* mutations are commonly found in compensation experiments [196,245,258,259]. Furthermore, in a study comparing the genomes of clinical rifampicin resistant and rifampicin susceptible *M. tuberculosis* isolates, mutations in these genes were associated with resistance, with 27.1% of resistant strains carrying non-synonymous mutations in one of the two genes [243]. In a study in *S. enterica*, involving genetic reconstructions through phage transduction, single compensatory mutations in *rpoA* and *rpoC* (and also in *rpoB*) that occurred during the evolution of a resistant strain were shown to be necessary and sufficient for growth compensation [258], effectively proving that that these genes are compensatory targets. In a subsequent, similar study, mutations in the same targets were shown to compensate for the clinically relevant *rpoB S531L* mutation [245].

Compensation can also occur by a bypass mechanism, through which the resistant bacteria evolves to reduce the need of an altered function or by exploring an alternative protein or metabolic pathway to the ones affected by the antibiotic. One such example is compensation of isoniazid resistance in *Mycobacterium tuberculosis*. Resistance mutations impairing KatG catalase peroxidase activity confer resistance but are extremely costly. Such cost can be compensated by mutations increasing the expression of the AhpC alkyl hydroperoxidase, bypassing the need of KatG for protection against organic peroxidases [260]. Another exquisite example of compensation regards resistance to actinonin in *Salmonella enterica*. Resistant mutants have an impaired formylation of methionyl initiator tRNA, and a fraction of the mutants compensated this defect by amplifying the tRNAi encoding genes *metZ* and *metW*. The high tRNAi levels allowed translation initiation to proceed without formylation, effectively heightening fitness by allowing the bypass of the reaction [261].

Compensation studies are usually performed in the absence of antibiotics. Still, some studies have compared the evolution of resistant strains in the presence and in the absence of the drug [239,255]. Both studies suggest that the two regimes imply different loci in the compensation process. Curiously, for mupirocin-resistant mutants, compensatory mutations acquired in the absence of the drug do not confer fitness rescue in its presence [255].

The appearance and spread of compensatory mutations is conditioned by various factors, such as population size, bottlenecks [240] and mutation rate [262], as well as the fitness effect of compensatory mutations, which depend on the genetic background in which they occur [196,259]. Compensation (and adaptation in general) tends to be faster in backgrounds with a higher fitness cost (Couce & Tenaillon, 2015). A study involving antibiotic resistant *E. coli* strains with different costs shows that mutants with a higher cost can compensate rapidly and compete with mutants with a lower initial cost, leading to the subsistence of both compensated strains for hundreds of generations [259]. This observation illustrates how instrumental

compensation can be in the rescue of low fitness resistant mutants from their predicted extinction.

Importantly, certain compensatory mutations acquired in the absence of antibiotics were found to decline resistance. While in most situations the compensated strains still had a higher level of resistance that the sensitive strain, there are reports of full recovery of sensitivity through second site mutations, a process of phenotypic reversion [196,263–265]. In a recent study with 23 strains carrying at least 2 mutations conferring multiple antibiotic resistance, phenotypic reversion was found to be common, with the extent of reversions depending on the considered antibiotic [265]. These observations suggest that the varying success of banishment policies in reducing resistance is related with the antibiotic nature. Further studies on the likelihood of phenotypic reversions could help identify the most suitable antibiotics for the application of banishment policies. Also recently, three studies have focused on the induction of phenotypic reversions. One of the studies applies the treatment of resistant bacteria with a short antisense oligomer that inhibits the expression of *acrA*, a gene involved in efflux system AcrAB-TolC, one of the major intrinsic resistance determinants in *E. coli* [266]. In the second study, the authors developed drug-like molecules that activate a cryptic bioactivation pathway of the prodrug ethionamide in *Mycobacterium tuberculosis*, bypassing the classic pathway in which resistance mutations occur [267]. One such molecule named SMARt-420 (Small Molecule Aborting Resistance) was shown to fully reverse resistance, to clear an ethionamide-resistant infection in mice and to increase basal sensitivity of bacteria to ethionamide. In the third study, the authors perturb the assembly of functional membrane microdomains (FMM) in methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA's β-lactam resistance determinant PBP2a typically accumulates in these domains. Through the use of the staphyloxanthin biosynthesis inhibitor zaragozic acid, the authors were able to disrupt FMM and inhibit resistance *in vitro* and *in vivo* [268].

It should be noted that both resistance and compensatory mutations can have pleiotropic effects in bacteria, affecting multiple relevant phenotypes. In *Salmonella*

enterica, nalidixic acid, rifampicin and streptomycin resistance mutations were shown to impair virulence in mice. While evolving through successive mouse infections, the mutants acquired compensatory mutations that not only heightened their relative fitness, but also and restored virulence [190], with compensation leading to simultaneously fit, resistant and virulent genotypes.

Environment dependence of fitness effects

Different organisms are more or less adapted to specific environments. A change in environment can lead to a shift in selective pressures and favor a previously unfavored genotype. Antibiotic resistance mutations, in particular those which are pleiotropic, can confer environment-specific fitness effects. Many examples of unexpected benefits have been reported. For instance, rifampicin resistance mutations we able to spread into high frequencies in populations under regimes of resource limitation [89,269]. In another study, transposon insertions in the *oprD* gene leading to the loss of the coded carbapenem entry channel, also instigate transcriptional changes in numerous genes and confer a fitness advantage to *Pseudomonas aeruginosa*, both during colonization of the mouse gut and in the dissemination to the spleen [270].

Various studies have addressed the environmental contribution to the growth and fitness effects of resistance mutations by comparing them in multiple environments with the same resistant genotypes [271–274]. In one of these studies, both the fitness of *E. coli* double mutants resistant to rifampicin and streptomycin and the epistasis between resistance mutations were shown to drastically vary between two of the most commonly used laboratory media, LB and M9 minimal medium with glucose [274]. Particularly important are studies comparing the fitness effects on laboratory media compared to bacteria's natural environments [191,242]. In one such study, Bjorkman *et al*. have shown that in *Salmonella enterica*, mutations conferring resistance to fusidic acid and to streptomycin have a different fitness in LB and mice. The authors proceeded to the evolution of the strains through mice intra-peritoneal infection and through growth in LB medium and have observed different

compensatory mutations occurring in the two regimes. Importantly, the compensated mutants also had different fitness in the two environments [191], implying a strong environmental effect in the fitness cost of compensated strains as well.

Epistasis

Epistasis is a phenomenon of genetic interaction, in which the phenotype of an allele in a particular locus is affected by the presence of other alleles on different loci [275]. In other words, the effect of a mutation depends on the genetic background in which it occurs [276]. This type of interaction can involve phenotypes that contribute to fitness and influence evolution.

Considering two mutations under epistasis, mutations with a shared beneficial or detrimental effect on fitness can either act synergistically, amplifying their effect, or antagonistically, with a lower combined effect of the two. When considering deleterious mutations, a synergistic interaction leads to a combined cost that is higher than expected, and consequently, to a lower fitness. This genetic interaction is classified as negative epistasis. Alternatively, epistasis is considered positive if the two deleterious mutations have an antagonistic effect, leading to a lower cost, and a higher fitness than the expected for independent mutations [277,278].

According to its effects, different classes of epistasis can be considered. Epistasis can act maintaining the beneficial or deleterious effects of the mutations, being classified as magnitude epistasis. Alternatively, the effect of a mutation might vary in sign with the genetic background, being beneficial or deleterious depending on the presence or absence of the other mutation. Such scenario is classified as sign epistasis [279]. Considering two deleterious mutations, sign epistasis can be partial, in with the double mutant being fitter than one of the single mutant genotypes, or reciprocal, through which the double mutant is fitter than both single mutants [280]. For this particular class of epistasis, both paths towards the fitness optimum involve a step that lowers it, so the double mutant may get stuck in a sub-optimal fitness peak [280].

In the particular case of antibiotic resistance, genetic interactions between different antibiotic resistance determinants can occur. Positive epistasis between resistance mutations has been reported to occur in bacteria such as *E.coli* [192,274], *Mycobacterium tuberculosis* [281] and *Pseudomonas aeruginosa* [282,283], with multi-resistance being less costly than expected. These studies indicate that epistasis between resistance mutations may promote the maintenance of multi-resistant strains by mitigating their fitness costs. It should also be noted that positive epistasis can occur between a costly and a costless mutation as well [274]. Furthermore, positive epistasis is also observed between plasmids carrying resistance [284], and between conjugative plasmids and chromosomal mutations [285].

In a study demonstrating positive epistasis between chromosomal single nucleotide polymorphisms (SNPs) conferring resistance to nalidixic acid, rifampicin and streptomycin, some double mutants manifested partial sign epistasis [192]. Sign epistasis between mutations conferring resistance to the clinically relevant antibiotics rifampicin and ofloxacin was also described in *Mycobacterium smegmatis* [281]. Furthermore, sign epistasis was shown to occur between chromosomal mutations and conjugative plasmids carrying antibiotic resistance determinants, including a case of reciprocal sign epistasis [285]. These observations are particularly troubling because they imply that for certain combinations of resistance determinants, the multi-resistant strain may be more fit than its single counterparts. In this circumstance, evolution through natural selection will not promote the loss of resistance. In fact, it may select against reversions in the multi-resistant strain.

It has been observed that the beneficial effect of a mutation tends to be smaller as the overall fitness increases [286–288], a phenomenon called diminishing returns of beneficial mutations. A study with rifampicin resistant *Pseudomonas aeruginosa* evolving in a constant environment in the presence of the drug corroborates these classic observations and further discloses that the same mutation can have a different fitness effect across genetic backgrounds, effectively showing that epistasis is driving these diminishing returns in fitness [289]. Two key studies evaluating the fitness effect of every combination of sets of beneficial mutations fully demonstrate

such diminishing returns epistasis [290,291], further evidentiating the fundamental role of the genetic background in the fitness effect of a mutation.

Fortunately, resistance determinants can lead to an increased susceptibility to a different drug [292,293]. This phenomenon of negative cross-resistance, also known as collateral sensitivity, can be particularly useful to determine which drugs to use sequentially in chemotherapy [294].

As epistasis affects fitness, it is expectable that the occurrence of compensatory mutations in multi-resistant bacteria can also be affected by these genetic interactions. In the presence of positive epistasis, compensatory effects are expected to be weaker. In the particular case of sign epistasis, in which the double mutant has an advantage over single resistant clones, compensatory targets are expected to be less due to genetic constraints. In the case of negative epistasis however, a stronger compensatory effect is expectable, as mutations compensating specifically for the deleterious genetic interaction can occur. In a recent study [196], an *E. coli* double mutant for rifampicin and streptomycin resistance bearing negative epistasis between the two chromosomal resistance mutations, as well as the respective single mutants were evolved in the absence of antibiotics. By comparing the genome sequences of the evolved lines, the authors discovered mutations occurring exclusively in the double mutant background. Through genetic reconstruction and fitness assays, the authors proved that a specific amino-acid change in the RNA polymerase subunit β', RpoC, and a mutation causing increased expression of the ribosome-RNA polymerase coupling protein, NusG, were advantageous in the double resistant background, but neutral or deleterious in the respective singles. Furthermore, the authors showed that the compensatory effects were present no more in an environment in which the two mutations did not have negative epistasis, effectively showing that these mutations were specifically compensating for the deleterious genetic interaction [196]. The discovery of compensatory targets for epistasis allows the identification of new genes involved in multiple key cellular mechanisms. As such, the corresponding proteins are potential targets to the development of new antibiotics [295].

Gut commensal bacteria

Host-bacteria interactions can be classified as symbiosis, commensalism and pathogenesis. Symbiosis occurs when at least one of the two species has a clear benefit of the association, without harming the other, and typically involves the exploitation of a nutritional niche that would not be available without such association. Commensalism typically refers to when bacteria coexist with their host, without detriment but also no obvious benefit for any of the partners. Pathogenicity implies damage to the host [296].

Although it is generally believed that the composition of the gut flora is associated with health and disease, the specific roles and potential benefits of most non-pathogenic gut bacteria are still undisclosed. Having this into account, throughout this work, I will be using the term commensal to broadly describe non-pathogenic bacteria.

Commensal bacteria as a reservoir of resistance

Pathogens are agents that directly cause disease, and resistant pathogens are an immediate threat to humans. As such, it is understandable that many of the early resistance and epidemiological studies were focused on them. However, pathogens are only a tiny fraction of the microbes interacting with humans [297]. The realization that commensal bacteria could act as a reservoir for resistance has gradually shifted the focus of resistance studies to natural bacterial communities.

Antibiotic resistance in commensal bacteria is currently recognized as a threat. The recurrent use of antibiotics in the clinics to treat disease and perform prophylaxis, in crops and in animal husbandry selects for resistance not only in disease causing agents, but also on the much vaster commensal and environmental communities [298].

Consecutive rounds of selection lead to the enrichment in resistant bacteria that are not the primary target of antibiotic use [299]. These bacteria can then transfer antibiotic resistance determinants between themselves and potential pathogens, providing an alternative route to generate resistant disease-causing agents [300].

Transfer of multiple drug resistance from commensal to pathogenic enterobacteria was described over half a century ago, depicting the transference of resistance from commensal *E. coli* to pathogen enterobacteria such as *Shigella* sp. [301] and *Salmonella enterica* serovar Typhimurium [302]. Meanwhile, several studies have further contributed to the notion, such as the report of recent dissemination of streptomycin and sulfonamide resistance genes among commensal and pathogenic bacteria [303] and the dissemination of tetracycline resistance gene *tetQ* and of the erythromycin resistance genes *ermF* and *ermG* in *Bacteroides* species [304]. This last report is particularly impressive, as *tetQ* has spread its representation from 30% to more than 80% of *Bacteroides* strains in just 3 decades, while the *erm* genes rose from below 2% to 23% frequency in the isolates. The carriage of resistance was as high in isolates from people without recent history of antibiotic use as in isolates from patients with *Bacteroides* infections, indicating that resistance transfer is occurring outside the clinic environment [304].

Additional studies support the origin of resistance determinants in environmental bacteria. One example relates to the β-lactamase resistance $bla_{\text{CTX-M}}$ genes, which have spread worldwide [305]. Through homology inferences of the genes and their flanking regions, the origin of these genes was pinpointed to the chromosomal DNA from environmental *Kluyvera* species, from where the genes have spread to other bacterial species [306]. Another example relates to quinolone resistance *qnrA* genes, which have disseminated globally in enterobacteria [307]. *Shewanella algae*, a marine and fresh water bacteria, was found to be a reservoir and the likely origin of these resistance determinants [308]. Evidence supporting horizontal gene transfer from commensal to pathogen bacteria extends to Gram-positive bacteria as well. Studies focused on sequence diversity of several commensal and pathogenic streptococci strains have found extensive sequence variation in transpeptidase

genes, conferring β -lactam resistance in both groups, with homologous variants found in between the two [309–312]. These observations strongly suggest that β-lactam resistant variants of *Streptococcus pneumoniae* originated in horizontal gene transfer events from commensal related species [309,310], such as *Streptococcus mitis* and *Streptococcus oralis* [311,312].

Armed with the knowledge that bacteria often exchange genes laterally [53] and evidence of horizontal gene transfer of antibiotic resistance determinants, researchers have focused on studying the whole resistance repertoire of bacterial communities. This repertoire, called resistome [158] refers to all antibiotic resistance determinants and precursors in a given environment or group of environments [313,314].

Many studies have sampled the resistome of environments such as the soil [313], wastewater [315] and human [316] and animal microbiota [317], and confirmed the widespread of antibiotic known resistance determinants, including clinically relevant resistance genes [318]. The application of metagenomic approaches in particular allowed the direct recovery of DNA from all micro-organisms in a sample, avoiding culturability limitations and selective biases [319,320]. Mining of metagenomic data can lead to the discovery of antibiotic resistance genes, inclusively in databases that were not designed to study resistance [321].

On their own, sequence-based studies are limited to the identification of genes that are already known and to the prediction of novel functions of genes with high sequence homology of novel gene functions. Researchers have addressed this limitation by coupling the discovery of candidate genes with phenotypic tests of their function in surrogate hosts [322,323]. Through functional metagenomics, numerous studies have revealed several unknown and unnoticed antibiotic resistance determinants in soil [39,313,324,325], wastewater treatment facilities [326–328], in the microbiota of farm animals [166,329] and of humans [330–332]. Thus, resistomes are more extensive than initially thought, with commensal bacteria indeed acting as large genetic reservoirs of antibiotic resistance determinants.

It should be noted that under certain conditions, bacteria that are typically commensal can evolve to cause disease. Often, pathogenic bacteria are members of a genus in which non-pathogenic or marginally pathogenic bacteria are the norm. In fact, many pathogenic bacteria inhabit the same primary habitat as their commensal relatives, causing disease only after spreading into other areas of the body – their "virulence niche" [333]. Some of these lineages are carried by humans as permanent or transient commensals, with high virulence resulting from the acquisition and expression of traits that promote adaptation to secondary or novel niches. These traits consist in mechanisms that allow adhesion and/or damage to the host tissues, consumption of the secondary niche's nutrients and the ability to evade or overcome antibacterial defenses [333].

Nonpathogenic bacteria can acquire virulence traits through the horizontal gene transfer of virulence factors of foreign nature into the "commensal" genome, through the acquisition of plasmids, phages and pathogenicity islands [334]. The latter are a particular class of genomic island that is present on the genomes of pathogenic strains but absent in nonpathogenic members of the same species and typically encode multiple virulence factors, including adhesins, secretion systems, toxins, invasins, modulins, effectors, superantigens and iron uptake systems, besides carrying other virulence associated genes [335].

Alternatively, commensals can become more pathogenic through the acquisition of patho-adaptive mutations - adaptive mutations that lead to loss or modification of function of pre-existing genes, benefitting bacteria in the virulence niche [333]. These pathoadaptive mutations can confer phenotypes such as increased evasion of phagocytosis [336,337], resistance to phagocytosis [338] increase in resistance to host clearance [339,340], increased capsule production [341], prolonged intracellular persistence [337,342], endotoxin activity [343], increased hemolysin production [344] and increased tropism to uroepithelium [345].

Pathoadaptive changes can act by affecting the host's ability to recognize bacteria. As an example, *E. coli* evolving in the presence of macrophages was shown

to recurrently acquire transposable element insertions that induce altered gene expression profiles both in the bacteria and in the macrophage, resulting in a delay on phagosome maturation by the latter [337].

As with other genetic changes, pathoadaptive mutations can imply trade-offs in fitness and generate a cost in nonpathological habitats. One such example regards mutations in genes encoding the fimH adhesin can change the tissue tropism of commensal *E. coli* and confer a urovirulent phenotype [345]. The pathoadapted variants gained a selective advantage in the colonization of the urinary tract, but at the price of a lowered ability to bind to buccal cells in the presence of the mannose-containing inhibitors bathing mucosal surfaces.

Importantly, pathoadaptive traits, such as growth rate and survival inside macrophages, can be enhanced directly by antibiotic resistance mutations [346,347]. Furthermore, pathoadaptation can impact antibiotic resistance on its own, by increasing or decreasing antibiotic sensitivity. As an example, a recent study shows that besides having a fitness advantage in the colonization of the mouse gut, pathoadapted *E. coli* small colony variants have increased resistance to kanamycin and increased sensitivity to tetracycline, featuring collateral sensitivity *in vitro* and *in vivo* [348].

The gut microbiota

The gut microbiota is one of the most densely populated microbial ecosystems on earth [316]. Recent reports estimate that the number of bacterial cells in the human body is in the same order of magnitude of human cells, with the vast majority (≈99%) present in the colon [349]. A landmark study catalogued the human gut microbial genes by performing metagenomic sequencing from fecal samples of 124 European individuals. A total of 3.3 million non-redundant microbial genes was found, a gene set that is ≈150 times larger the human genome's [350]. With an elevated population

size and an enormous genetic repertoire that has been co-evolving with its hosts' [351–353], the gut microbiota is a core element of our body.

Mammals have become dependent on the microbiota in a wide array of functions[354,355]. These range from metabolization of complex nutrients and production of metabolites, such as fatty acids, bile acids, choline and vitamins, that are essential for host health [356], to organ development and morphogenesis [355] and the maturation and regulation of the immune system [354,357,358]. Microbiota also plays a role in the protection against pathogens by confering colonization resistance to the host [359], either through competitive exclusion or by maintenance of immune homeostasis [360].

Strong microbiota perturbations, such as the ones imposed by antibiotic treatments and diet changes can lead to a reduction of microbiota diversity and affect its functions. This imbalance, known as dysbiosis, is associated health problems such as obesity [361,362], inflammatory bowel disease [363,364] and gastrointestinal cancer [365], functional bowel disorders [366], and allergies [367].

Due to the intimate host-microbe interactions and the critical role in the contact with the external environment, some authors conceptualize the gut microbiota as an effective organ [368,369]. However, the gut microbiota is much less characterized than the host organs - we don't know with precision its composition (it is not fixed), and its roles are still far from being fully explored [370].

As mentioned, recent studies have addressed the resistome of the gut communities and shown that the gut microbiome is indeed a large reservoir for antibiotic resistance genes [330–332]. However, given the expected cost of carrying antibiotic resistance determinants, it is still not clear how this antibiotic resistance repertoire is maintained. Studying how selection acts on resistant commensal bacteria will help us understand how to contain this vast reservoir of antibiotic resistance determinants.

Escherichia coli as a model and as a gut commensal

E. coli is a gram-negative, asporogenous, facultative anaerobe and a common member of the normal gut microbiota community in warm-blooded animals and reptiles [371,372]. Despite being vastly outnumbered by obligate anaerobes in the large intestine of adult individuals [354], *E. coli* is the predominant aerobic organism in the gastrointestinal tract of humans [373]. In the gut, *E. coli* typically resides in the caecum and the colon, namely in the mucus layer covering the epithelial cells and is shed into the intestinal lumen, being excreted in the feces [374]. *E. coli* has adapted to acquire nutrients from mucus, and the ability to harvest mucus-derived sugars has a prominent role in the initiation and maintenance of *E. coli*'s gut colonization [375]. While having the gut as a primary habitat, *E. coli* is able to subsist in secondary habitats such as soil, water and sediments [376]. These nutritionally poor environments act mainly as a transitive habitat until a new host is found [376].

E. coli is one of the first bacteria to colonize the intestine, reaching high cell density shortly after its host's birth [377]. *E. coli* is considered pivotal in the ecological succession of the gut microbiota by modifying the habitat into a reduced state, allowing the gut colonization by strictly anaerobic bacteria such as *Bacteroides*, *Bifidobacterium* and *Clostridium* [378,379]. After the expansion of anaerobes, *E. coli* cell density stabilizes at around 10^8 cfu per gram of feces [373], although its numbers may rise in aged humans [380].

Most *E. coli* strains act as commensals, benefiting from their hosts while causing no harm. *E. coli* is granted access to a limited supply of nutrients for which it competes [381], a relatively stable environment and protection against stresses, as well as transport and dissemination [373]. On the other hand, commensal *E. coli* can contribute to the host's health by producing vitamins [382], by activating the immune system [383,384] and by providing colonization resistance against antibiotic resistant Gram negative bacteria [385] and pathogens such as *Shigella flexneri* [386], *Salmonella enterica* serovar Typhimurium [387] and enterohemorrhagic *E. coli* strain O157:H7 [388].

E. coli was first identified by the physician Theodor Escherich at the end of the 19th century [389]. The K-12 strain in particular was originally isolated by Blair in 1922 from the stool of a convalescent diphtheria patient [390]. Later on, the K-12 strain was selected as a model organism by Edward Tatum for his studies due to its prototrophism, ease of cultivation, short generation time and for permitting the study of very large populations, allowing the detection of rare events, such as mutations. Through these early studies, K-12 allowed for the isolation of auxotrophic mutants [391] that were instrumental on the discovery of recombination [47,392]. Since then, hundreds of strains were derived from the original K-12 either by spontaneous mutations or by treatment with mutation inducing agents such as X rays and UV irradiation [390]. These derivative strains have been key in the advancement of genetics, molecular biology, physiology and biochemistry [373].

With the dawn of sequencing technologies, *E. coli* K-12 MG1655 was the first *E. coli* having its whole genome sequenced. This K-12 sub-strain was chosen due to its minimal genetic manipulation, having only been cured of the lambda phage by means of UV light and of the F plasmid through the use of acridine orange [393]. While other *E. coli* strains were classically used for landmark studies, the proximity of MG1655 with the original, basal *E. coli* K-12 strain and the availability of its sequenced genome promoted the use of MG1655 as a main model for genome and genetic engineering studies and turned this strain into one of the best-studied organisms in biology. The accumulated knowledge on MG1655, its commensal nature and the ability to sequence, detect and identify mutations led to its adoption for evolution studies on the genetic basis of adaptation to the mammalian gut [66,394–397] [e. g., [66,395–398]].

Aims

This work had as a main objective to study the fitness and evolution of gut commensal antibiotic resistant bacteria in their natural environment.

Specifically, we aimed to:

1) Estimate the cost of chromosomal mutations conferring antibiotic resistance in commensal *E. coli*, in its natural environment, the mammalian gut.

2) Assess the presence of epistasis and compare fitness costs and the epistasis pattern with the ones observed in competitions in standard laboratory environments.

3) Determine the genetic basis of the compensatory evolution of resistant strains within the mouse gut.

4) Study the commonality of frequency-dependent selection in single gene polymorphisms, in simple and complex environments.

Our predictions regarding a strain's survival depends on the conditions in which fitness is inferred, and in the way we measure fitness. Studying commensal resistant bacteria in their natural environments will help us comprehend how they subsist in the current era.

CHAPTER II

Personalized fitness costs of antibiotic resistance in the mouse gut

Parts of this chapter are included in a manuscript submitted for publication.

Cardoso, LL, Durão, P, Amicone, M, Gordo, I. *Dysbiosis personalizes fitness effect of antibiotic resistance in the mammalian gut.*

Pre-print available at bioRxiv:

https://doi.org/10.1101/748897

Author contributions for this Chapter:

Isabel Gordo and I designed the initial study. Paulo Durão designed the streptomycin detection test. For the co-housing and resident *E. coli* data, Isabel Gordo, Paulo Durão and myself designed the experiments. I have performed *in vivo* competitions in germ-free and SPF mice, the *in vitro* fitness effects of the resistant mutants on LB and metagenomic analysis of the individually caged mice experiments. Paulo Durão has performed SPF mice competitions, *in vitro* competitions of the resistant strains in minimal medium with glucose and the streptomycin detection test on stool samples. Massimo Amicone has performed the PCoA analysis of the co-housing experiment. Results were edited by Paulo Durão, Massimo Amicone and I. The execution was supervised by Isabel Gordo.

Abstract

Antibiotic resistance mutations typically decrease the competitive ability of bacteria in the absence of antibiotics. This has justified a public strategy of suspending antibiotic use to lower levels of resistance. However, this strategy has met mixed results and the reasons for these different outcomes are still unclear. It has been shown that fitness costs can vary with the environment, but they have been mostly studied *in* vitro. Thus, it became relevant to study the cost of resistance in environments closer to natural conditions. Here we show that following an antibiotic treatment, the cost of resistance for *Escherichia coli* in the mouse gut is highly host-specific. Using mice co-colonized with sensitive and resistant *E. coli*, but lacking microbiota, we find a measurable cost of single and double resistance. In mice with microbiota, the same resistance mutations exhibit a varying fitness effect, being deleterious in some of the hosts, while having no cost or even being beneficial in others. Through competitions in co-housed mice, with normalized microbiota, we observe a reduction of the variance of the fitness effects. Our results suggest that microbiota imbalances following antibiotic treatment can generate ecological conditions where resistance can be neutral or beneficial even in the absence of antibiotics, contributing to subsistence of resistant strains.

Introduction

The discovery and usage of antibiotics was a major medical event in the 20th century, greatly reducing human mortality following bacterial infections [1]. Antibiotics are nowadays central in modern medicine, both to treat and prevent disease [2], but their effectiveness is threatened by antibiotic resistance. Resistance occurs in nature and is present in different ecological settings [38,40,314]. However, the extensive use of antibiotics has led to the spread of resistance, reducing the effectiveness of antibiotics in clinical settings over time [1,8], being a contemporary threat to the effective prevention and treatment of infections [11].

Resistance can be acquired through spontaneous chromosomal mutations affecting core metabolic pathways and physiological processes of the cell, such as transcription [399], translation [167], replication [165] and cell wall biogenesis [168]. As a consequence, it often implies a functional or energetic fitness cost [9], which turns into a lowered ability to survive and reproduce in the absence of the antibiotic [164,176,177].

The existence of a cost predicts that the susceptible strain should out-compete the resistant over time, through natural selection. Having this into account, a commonly adapted procedure is to banish the use of antibiotics that have lost their effectiveness until resistance recedes. However, campaigns promoting the reduction or banishment of antibiotics do not always lead to a decrease in resistance [181]. In some cases, resistance decreased as expected [182–186]. Still, in others it was maintained [185,188,400], and there is even evidence for the spread of resistance after antibiotic reduction campaigns [189]. As the efficacy of this strategy ought to depend on the cost of antibiotic resistance, these observations suggest that in nature, resistant strains may not always pay a fitness cost, even in the absence of the antibiotic that allowed it to rise.

In vitro studies have shown that the fitness effects of resistance are under a strong influence of environmental factors, such as temperature [272] nutrition [272,274] and

the presence of immune cells [346,347]. Additionally, epistasis between resistance mutations in functionally related genes appears to be pervasive [192] and itself environment-dependent [274].

Since resistance mutations can be highly pleiotropic [172], and their fitness effect depends on the environment, a relevant question is whether the fitness measured in standard laboratory settings can be extrapolated to natural systems. Some studies on pathogens have tackled this issue and found fitness costs *in vivo* during the infection of mice hosts [190,191,401] which were different when measured in laboratory media [191,401], reinforcing the need to measure fitness costs in more natural settings.

It is known that commensal bacteria can be a reservoir of antimicrobial resistance genes [297,330,402]. However, the fitness costs of resistance in commensal bacteria in their natural environments is difficult to measure. Furthermore, the role of complex microbial communities such as the gut microbiota in the maintenance of resistance is still poorly explored. To address this gap in knowledge, we have performed *in vivo* competitions to measure the fitness costs of antibiotic resistance when commensal *E. coli* colonize the mouse gut, in the presence and absence of native gut bacterial species.

Methods

Escherichia coli and mice strains

For our experiments, we used fluorescence-labeled, *E. coli* K-12 MG1655-derived strains, with different antibiotic resistances, bearing either a point mutation conferring streptomycin resistance, a point mutation conferring rifampicin resistance, both mutations, or none of the two. We also used a natural streptomycin resistant strain from the mouse gut, and a double resistant strain, derived from the latter.

The point mutations conferring resistance in our K-12 strains, *rpsL K43T* and *rpoB H526Y*, were generated by Trindade *et al.* in a K-12 MG1655 genetic background, and confer streptomycin and rifampicin resistance, respectively [192]. These mutations were moved into isogenic, YFP and CFP fluorescent-labeled strains by Moura de Sousa *et al.* [196]. Since the *gat* operon was found to be a mutation hotspot under strong selection in the mouse gut for K-12 MG1655 derived strains [395,398], we modified our strains to a galactitol negative phenotype via a knock-out of the *gatZ* gene. P1 transductions [403] were performed to substitute the whole *gatZ* gene from our strains with a kanamycin resistance cassete, using *E. coli* JW2082-1 from the KEIO collection [404] as a donor for the transduction. The new strains, RB929 and LC88 (*ΔlacIZYA::scar galK::cat-YFP/CFP* Δ*gatZ::FRT-aph-FRT*), were used as wild-type strains for the competitions. P1 transductions were also used to insert the point mutation *rpoB H526Y* in the wild-type background and to pass the *gatZ* deletion from the wild-type strains to the isogenic antibiotic resistant strains which carried either the point mutation *rpsL K43T* or both *rpsL K43T* and *rpoB H526Y* mutations ($Str^R Rif^R$). The resulting streptomycin resistant (Str^R) strains LC81 and LC82 (YFP/CFP, respectively), the rifampicin resistant (Rif^R) strains RB933 and LC84b (YFP/CFP, respectively), and the double resistant ($Str^R Rif^R$) strains LC85, LC86 (YFP/CFP, respectively) were used to colonize mice in competitions with the sensitive strain. All of these strains possess a chloramphenicol resistance cassete associated with their

fluorescent protein, as well as the kanamycin resistance cassete. As this study is focused on streptomycin and rifampicin resistance, I will be designating our *rpsL rpoB* wild-type strains as sensitive, and the resistant strains as single or double mutants.

For the streptomycin detection test, the strain JB77 *(ΔlacIZYA::scar gatZ::IS1 galK::cat-YFP rpsL K43R*), was used to perform the competitions. For the competitions with resident *E. coli*, we used two streptomycin resistant strains, NF1 and PJD5. The strain NF1 is an *E. coli* strain isolated from the mouse gut, and carries the streptomycin resistance mutation *rpsL K88R*, which emerged when the mice received streptomycin treatment. The strain PJD5 was generated by growing NF1 in Luria-Bertani liquid medium and plating in LB agar medium supplemented with 100 µg/ml of rifampicin. Through Sanger sequencing directed to the *rpoB* gene, PJD5 was found to carry the resistance-conferring *rpoB H526Y* point mutation – the same as in our K-12 isolates.

6-to-13 week-old female C57BL/6J germ-free (GF) mice were used as hosts for the *in vivo* competitions in the absence of microbiota, while 6-to-8 week-old female C57BL/6J specific pathogen free (SPF) mice were used for the *in vivo* competitions in the presence of microbiota. GF mice were bred and raised at the IGC gnotobiology facility in dedicated axenic isolators (La Calhene/ORM). Young adults were transferred into sterile ISOcages (Tecniplast) before the competition experiments.

In vitro competitions

To measure fitness effects in two nutritionally distinct environments *in vitro*, the strains were streaked from the frozen stocks in LB agar with antibiotics corresponding to their resistances and incubated at 37°C for 24 hours, followed by acclimatization for 24h in 150 µl of LB and in minimal medium with glucose [M9 salts, $MgSO_4$ 1 mM, $CaCl_2$ 0.1 mM, glucose 0.4% (w/v)], in 96-well plates, at 37°C, with shaking (700 rpm). Each resistant strain was then mixed with the sensitive wild-type in a 1:1 ratio,

and competitions were performed for 24h in the same conditions as the acclimatization. To determine the initial and final ratios of resistant and susceptible strains in the competition assays, bacteria numbers were quantified with an LSR Fortessa flow cytometer using a 96-well plate autosampler. Samples were always run in the presence of SPHERO (AccuCount 2.0-µm blank particles) in order to accurately quantify bacterial numbers in the cultures. Briefly, flow cytometry samples consisted of 180 µl of PBS, 10 µl of SPHERO beads, and 10 µl of a 100-fold dilution of the bacterial culture in PBS. The bacterial concentration was calculated based on the known number of beads added. Cyan fluorescent protein (CFP) was excited with a 442-nm laser and measured with a 470/20-nm pass filter. Yellow fluorescent protein (YFP) was excited using a 488-nm laser and measured using a 530/30-nm pass filter.

In vivo competitions

To measure the fitness effects of each resistant strain in SPF mice, we used an antibiotic treatment in order to break the colonization resistance and allow colonization[405]. Mice were transferred into individual cages and given autoclaved drinking water containing streptomycin sulfate (5g/L) for seven days, and then were given regular autoclaved drinking water for 2 days, in order to wash out the antibiotic from the gut. Within experimental treatments, each mouse was bred in a unique litter. After 4 hours of starvation for food and water, the mice were gavaged with 100 µl of a $\approx 10^9$ cells/ml suspension with a 1:1 ratio of the two competing strains.

To make the suspension, the strains were streaked from frozen stocks in LB agar plates supplemented with the corresponding antibiotics (concentrations of 100µg/ml) two days before gavage and incubated for 24 hours. Afterwards, an overnight culture of a single colony for each biological replicate in BHI (brain heart infusion) medium with the corresponding antibiotics was grown. The cultures were then diluted 100-fold and grown in BHI medium until an $OD_{600nm} \approx 2$. For the K12 derived strains, fluorescence-associated flow cytometry was used to assess the

number of cells per growth and further adjust the initial number of cells while preparing a suspension in PBS for the gavage. Mice fecal pellets were collected 4 hours and every 24 after gavage, for 5 days, suspended and homogenized, diluted in PBS and plated in LB agar plates. For the K-12 strains, the stool samples plates were diluted in PBS and plated in LB agar plates supplemented with chloramphenicol (30µg/ml). The plates were incubated overnight and the frequencies of CFP and YFP-labeled bacteria were assessed by counting the fluorescent colony forming units (CFU) with the help of a fluorescent stereoscope (SteREO Lumar, Carl Zeiss). For the resident bacteria, the stool samples were diluted in PBS and plated in LB agar plates supplemented with streptomycin (100µg/ml) and with both streptomycin and rifampicin (100µg/ml), to count the total number of *E. coli* CFU and the fraction of double mutants. The samples were also stored in 15% glycerol at -80°C for future experiments. Apart from the streptomycin treatment, the same protocol was used in the competitions with GF mice. In the co-housing experiments, for each of the competing pair, five to six mice originated from 2 different litters were co-housed in the same cage 2 weeks prior to antibiotic treatment, and kept together until colonization with the competing strains (co-housed for a total period of 23 days, including the treatment and the period of antibiotic washout). Apart from the co-housing, the protocol was identical to the one used for individually caged mice.

Selection coefficient and epistasis calculations

In the *in vivo* competitions, the selection coefficient per day (S_{day}) of each mutant strain was estimated through the slope of the linear regression of the logarithm of the ratio of the resistant strain to the reference strain, from day 1 to day 5 after gavage.

In the *in vitro* competitions, the selection coefficient (S) was estimated as the difference in the resistant / wild-type ratio per generation:

$$S = \frac{log\left(\frac{Nm(t)}{Nwt(t)} / \frac{Nm(0)}{Nwt(0)}\right)}{log\left(\frac{Nwt(t)}{Nwt(0)}\right)}$$

In this equation, Nm(0) corresponds to the initial number of mutant cells in the competition, Nm(t) to the final number of mutant cells, Nwt(0) to the initial number of wild-type cells and Nwt(t) to the final number of wild-type cells.

Epistasis was calculated using the selection coefficients of the single and double mutants:

$$\varepsilon = S_{Str^R Rif^R} - (S_{Str^R} + S_{Rif^R})$$

The epistasis error was then estimated through the error propagation method:

$$\sigma\varepsilon = \sqrt[2]{(\sigma S_{Str^R Rif^R})^2 + (\sigma S_{Str^R Rif^R})^2 + (\sigma S_{Str^R Rif^R})^2}$$

Epistasis was considered positive if its value was superior to the error, and negative if it was inferior. Epistasis was considered absent otherwise.

Microbiota analysis

To assess the effect of streptomycin in the gut microbiota composition, we extracted DNA from fecal samples obtained from a subset of individually caged SPF mice belonging to independent litters, and to all mice involved in the co-housing experiments, right before antibiotic treatment and 24 hours after gavage —

simultaneously with the first samples used for selection coefficient calculation. For the analysis of the microbiota regarding the cost in individually caged mice, we divided the samples extracted at 24 hours after gavage in two categories: A) a group where a fitness cost of resistance was estimated when competing against a susceptible strain and B) a group where no fitness cost could be detected when competing against a susceptible strain. With this design, we aimed to test if a specific microbiota composition was associated with the presence or absence of fitness costs independently of the resistance genotype.

Fecal DNA was extracted with a QIAamp DNA Stool MiniKit (Qiagen), according to the manufacturer's instructions and with an additional step of mechanical disruption [406]. 16S rRNA gene amplification and sequencing was carried out at the Gene Expression Unit from Instituto Gulbenkian de Ciência, following the service protocol. For each sample, the V4 region of the 16 S rRNA gene was amplified in triplicate, using the primer pair F515/R806, under the following PCR cycling conditions: 94 ºC for 3 min, 35 cycles of 94 ºC for 60 s, 50 ºC for 60 s, and 72 ºC for 105 s, with an extension step of 72 ºC for 10 min [407]**.** Samples were then pair-end sequenced on an Illumina MiSeq Benchtop Sequencer, following Illumina recommendations.

QIIME2 [408] was used to analyze the 16S rRNA sequences by following the authors' online tutorial (https://docs.qiime2.org/2018.11/tutorials/moving-pictures/). Briefly, the demultiplexed sequences were filtered using the "denoise-single" command of DADA2 [409], and forward and reverse sequences were trimmed in the position in which the 25th percentile's quality score got below 20. Alpha diversity and phylogenetic diversity analyses were also performed as in the QIIME2 tutorial. A sample depth of 543 and 1271 counts was chosen for the individually caged and co-housed mice fitness experiments, respectively, in order to exclude as few samples as possible without lowering too much the number of retained sequences per sample. For taxonomic analysis, OTU were picked by assigning operational taxonomic units at 97% similarity against the Greengenes database [410].

Statistical analysis

The selection coefficient of the *in vivo* competitions was tested for significance using R software, through an F-statistic on a predictive linear model of the mutant/sensitive or double mutant/ single mutant ratio over time, generated through the observed ratio on sampled time-points from 24, 48, 72, 96 and 120 hours after gavage. The null hypothesis was that the slope was equal to 0. When the null hypothesis was rejected (p-value < 0,05), the mutant was considered to have a cost if the slope of the model was negative and to have a fitness benefit if the slope was positive. F tests were performed using the FTEST function on Microsoft Excel. Normality of each treatment was tested through the Shapiro Wilk test and normality of the treatments involving competitions in the presence of microbiota was further tested through Kolmogorov-Smirnov test on GraphPad.

Bacterial load constancy was tested in R, through an F-statistic on a predictive linear model of the total load over time, generated through the loads on sampled time-points from 24, 48, 72, 96 and 120 hours after gavage. Null hypothesis is that the slope is equal to 0, and would be rejected when the p-value < 0.05. If the null hypothesis was rejected, the bacterial load would be considered to change consistently over time. For the competitions involving the resident *E. coli*, bacterial loads in germ-free and SPF mice were compared in GraphPad, through a Mann Whitney U test on sampled time-points from 24, 48, 72, 96 and 120 hours after gavage.

Streptomycin detection test

Fecal pellets originating from mice not treated with antibiotics were collected. The pellets were suspended in PBS, homogenized and stored in 15% glycerol (v/v) at -80ºC. The suspension was thawed and further filtered to remove both the fecal content and bacteria. To facilitate *E. coli* growth, the filtrate was mixed in a 4:1 ratio with LB, generating what we refer in here as fecal medium. By adding streptomycin, we have generated fecal medium with increasing concentrations of the antibiotic (two-fold increases, from 0.5 to 16 µg/ml, capturing the wild-type MIC of 2 µg/ml when grown in LB). We used these media to compete, in a 1:1 ratio, the mutants carrying either *rpsL K43R*, *rpsL K43T* and *rpsL K43T rpoB H526Y* against the sensitive strain, for 24h, at 37ºC. We then calculated the selection coefficients, as described for the other *in vitro* competitions. The values between 1 and 8 were used to build calibration curves.

We then generated fecal medium using our experimental samples, collected 4 hours after gavage. We competed the mutant carrying *rpsL K43R* against our wild-type strain and calculated the selection coefficients. By fitting the selection coefficient values in the corresponding calibration curve, we were able to estimate an "in-sample" estimation of streptomycin concentration. Fecal medium originated from mice under continuous streptomycin was used as a positive control.

In the 3 calibration curves, for concentrations below 1 µg/ml, the selection coefficients were similar to the ones in the absence of streptomycin. As both our calibration and experimental samples were diluted by 50% (v/v) when their frozen stocks were made, our effective limit of detection with this method is of 2 µg/ml.

Results

Environment-dependence of resistance mutations and epistasis

The mutations under study were previously shown to have environment-dependent fitness effects and epistasis in a similar genetic background [274]. To make sure that our *gat* negative strains bearing these mutations kept the environment-dependence, we measured the fitness effect of the resistance conferring mutations in two nutritionally distinct media – LB and minimal medium with glucose - by competing the strain bearing the mutation *rpsL K43T*, which confers resistance to streptomycin, the one bearing the mutation *rpoB H526Y*, conferring resistance to rifampicin, and the double mutant *rpsL K43T rpoBH526Y* which has both resistances, with their isogenic wild-type.

We have found distinct costs for each of our resistant strains in the two media (**Table 1**), confirming that the costs of these mutations are environment-dependent in the *E.coli* K-12 MG1655 *gatZ* knock-out. Moreover, as observed before in a *gat* positive background, epistasis is still negative in LB medium and positive in minimum medium with glucose, confirming the environment-dependence of the fitness effect of mutations and of their genetic interaction in this genetic background.

Table 1 – Fitness effects and epistasis between resistance mutations *in vitro*.
Fitness effects of antibiotic resistance mutations - Str^R (*rpsL K43T*), Rif^R (*rpoB H526Y*), Str^RRif^R (*rpsL K43T rpoB H526Y*) - in the Δ*gatZ* pre-adapted background when competing against the isogenic susceptible strain (also Δ*gatZ* background) in Luria Broth (LB) and in M9 salts minimal medium supplemented with 0.4% glucose (MM). The average selection coefficient per generation and 2 times the standard error are shown. Epistasis was measured using the additive model. The error for epistasis was calculated through the error propagation of the standard errors.

Strain	$S_{/gen}$ LB	$S_{/gen}$ MM	Epistasis in LB	Epistasis in MM
Str^R	-0.11 ± 0.02	-0.16 ± 0.02		
Rif^R	+0.03 ± 0.03	+0.14 ± 0.03		
Str^RRif^R	-0.19 ± 0.03	+0.05 ± 0.03	**-0.11 ± 0.03**	**0.07 ± 0.02**
			Negative	**Positive**

Costs of antibiotic resistance in the absence of microbiota

To evaluate competitive ability in a mammalian host, we decided to measure the fitness effects of the resistance mutations when *E. coli* is colonizing the mouse gut in the absence and presence of other bacterial species (**Figure 1**). We rationalized that the presence and activity of other species would likely change the environment, and as such, be an additional factor affecting fitness.

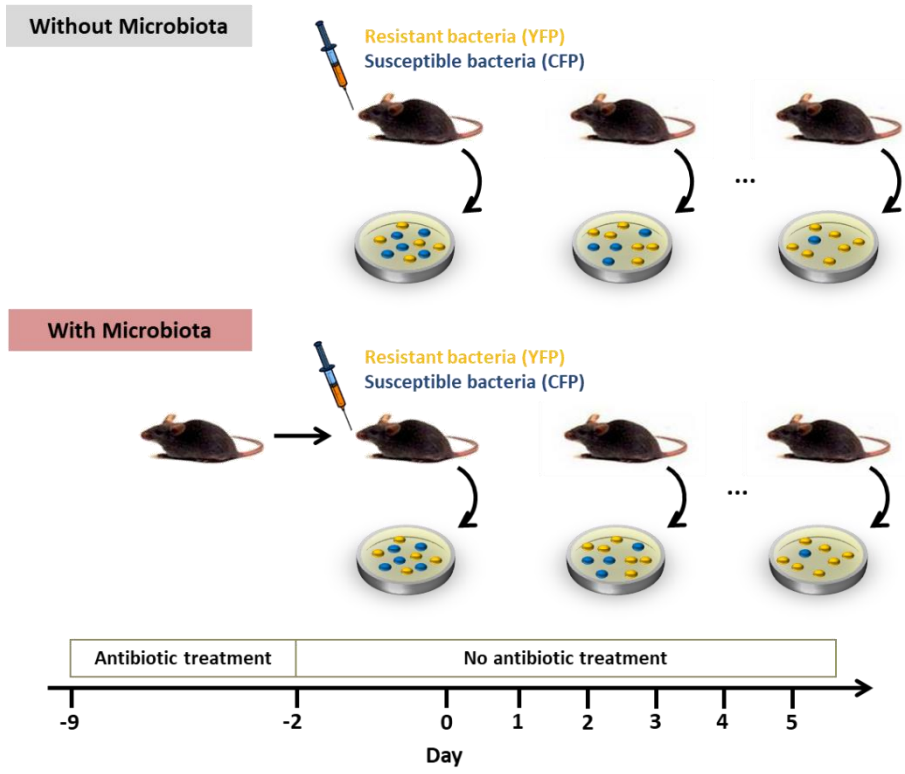


Figure 1- Scheme of the *in vivo* experimental design. This design was used to determine the fitness costs of resistance in mice mono-colonized or with a complex microbiota. For mice with microbiota, a one-week course of streptomycin treatment had to be given to break colonization resistance. The gavage with the experimental *E. coli* was performed two days later in order to give mice time to excrete streptomycin.

We assayed the competitive ability of each K-12 resistant strain by competing the mutants with their isogenic wild-type in mono-colonized germ-free mice (**Figure 2**, left panels). A fitness cost per day was estimated for all of the resistance mutations, with costs of $S_{Str^R/day}$ = -0.20 [± 0.09, 2x standard error (SE)], $S_{Rif^R/day}$ = -0.25 (±0.08) and $S_{Str^R Rif^R/day}$= -0.44 (± 0.10). These are average values, and the fitness effect is similar across biological replicas for each competition, with the slope of the selection coefficient being significantly negative for every mouse (**Table S1**). Assuming 26.2 generations per day in germ-free mice, as observed by Barroso-Batista *et al.* [411], the

estimated costs per generation are S_{Str}^{R} = -0.008 (± 0.003), S_{Rif}^{R} = -0.009 (±0.003) and $S_{Str}^{R}{}_{Rif}^{R}$= -0.017 (± 0.004). These fitness effects are lower in magnitude when compared with the fitness effects *in vitro* (**Table 1**), suggesting that for the K-12 genetic background, negative selection on traits affected by these resistance mutations could be weaker in germ-free mice than *in vitro*. As none of the costs correspond to the values observed in LB nor in minimal medium with glucose, neither of the two media was able to predict the fitness effects of resistance in the mammalian gut. Additionally, the cost of the double mutant is not significantly different than the sum of the costs of the single resistant mutants, indicating the absence of epistasis between these mutations in the germ-free mouse gut. This observation diverges with our results in rich medium, in which epistasis is negative, and in minimal medium with glucose, in which it is positive (**Table 1**), showing yet again that epistasis is environment-dependent [196,274] and that our *in vitro* competitions also failed to predict the epistasis pattern between these two mutations *in vivo*.

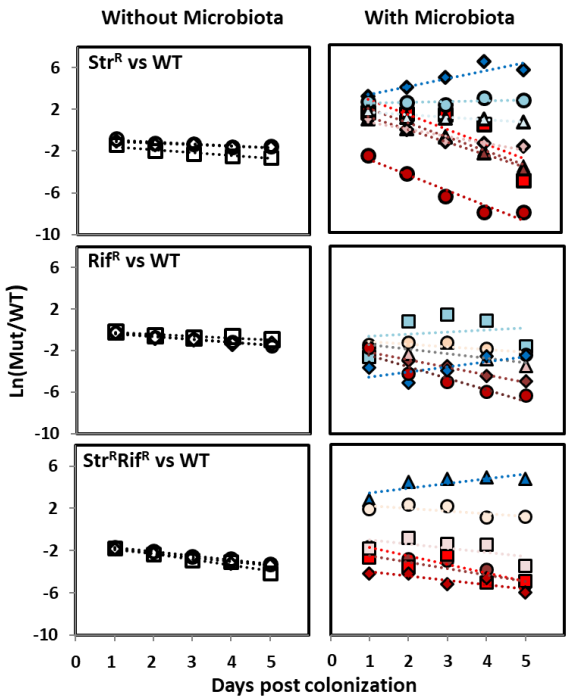


Figure 2- Early fitness effects of resistance after antibiotic treatment are host-specific in the presence of microbiota. Logarithm of the resistant mutant : wild-type ratio over time for *rpsL K43T* (Str^R), *rpoB H526Y* (Rif^R), and *rpsL K43T rpoB H526Y* (Str^RRif^R) mutants, both in mono-colonized mice and in mice with microbiota. The slope of the regression is an estimate of the selection coefficient per day.

Our results suggest than in the absence of other bacteria strains, the double mutant is the less fit genotype. However, there have been reports of non-transitivity in evolving bacteria populations [412], in which, for instance, a genotype would outcompete its recent predecessor but not their ancestral [413]. To control the possibility of non-transitive interactions in the mammalian gut, we performed competitions between the double resistance against its single counterparts (**Figure S1A,** left pannels). We have measured a cost of $S_{DM\ vs\ Str^R/day}$ = -0.26 (± 0.07) and $S_{DM\ vs\ Rif^R/day}$ = -0.21 (± 0.05), with the costs of the rifampicin and of the streptomycin mutations

being very similar to the ones observed previously in the competitions involving the wild-type (**Figure S1B**). The costs of these mutations are therefore transitive in the germ-free gut. Furthermore, our results suggest that the fitness effects of resistance are measurable, which allows us to predict the fate of resistance in this system. Still, strong beneficial mutations could occur before the resistant strains' extinction and change the outcome predicted by the competitions.

The effect of microbiota in the cost of resistance

A key aspect regarding the mammalian gut is the presence of many bacterial species. To understand if inter-species interactions affect the costs of antibiotic resistance, we performed competitive fitness assays in specific pathogen free (SPF) mice, which have a complex microbiota.

We have observed a clear influence of the microbiota in the fitness effects of antibiotic resistance mutations. Strikingly, the fitness effects show wide variation across hosts for all treatments, suggesting host-specificity. For instance, we have observed strong deleterious effects of resistance in some mice while in others no significant cost was found, with the resistant strain maintaining or even rising in frequency (**Figure 2,** right panels). On average, the fitness effects brought a cost in all treatments ($S_{Str}{}^{R}{}_{/day}$ = -0.68 (± 0.58, 2xSE), $S_{Rif}{}^{R}{}_{/day}$= -0.30 (± 0.48) and $S_{Str}{}^{R}{}_{Rif}{}^{R}{}_{/day}$= -0.34 (± 0.35), but the clear disparity between the selection coefficients across mice show us that in this experimental system, individual resistance costs are hard to predict. The observed variation is unlikely to be driven by frequency dependent selection, as the initial frequency of the resistant strain is not predictive of the resistance fate (e.g.: two streptomycin resistant mutants that were at similar frequency at the beginning had different fates; one declined in frequency while the other increased in frequency).

The variation is also unlikely to be due to the occurrence of compensatory mutations, as such events would have to be quite common and have strong effects

to have an early influence in the estimated fitness effects. Moreover, the majority of the mouse competitions can be fairly explained by a linear regression (**Table S2**), suggesting that in the majority of cases, selection remains constant throughout the 5 days. This would not be expected if the resistant strains acquired beneficial mutations. Still, in a few cases, the log (resistant/sensitive) deviated from a linear line during this period (**Figure 2**), revealing that selection can change during this early colonization. However, it is not clear if this is due to beneficial mutations, or actually due to an effective change in the environment, which could be driven by a changing microbiota.

During SPF mouse colonization, we gave a streptomycin treatment in order to break colonization resistance. To exclude an effect of streptomycin in our competitions, we have designed a protocol to test its presence through a competition method (**Figure S2**). We have competed a resistant strain carrying the mutation *rpsL K43R* against the wild-type in LB fecal medium made with our filtered samples, taken 4 hours after colonization (see **Methods**) and compared the selection coefficient values with the respective calibration curve, generated through competitions in different streptomycin concentrations (**Figure S2A**). We have observed that streptomycin was below our threshold level of detection (2ug/ml), and as such, unlikely to be involved in the outcome of the competitions (**Figure S2B**).

Antibiotic perturbation increases variation in microbiota composition

We followed up with a 16S rRNA analysis of the microbiota of a sub-sample of the SPF mice, for which we were able to collect stool samples before streptomycin treatment and 24 hours after the gavage. These last samples correspond to the first time-points that were used to measure selection coefficients, and as such, are representative of the microbiota that our strains encountered during the fitness assay. Our goal was to confirm that the treatment was inducing dysbiosis and leading to distinct microbiota profiles across hosts.

We have found a range of different microbiota compositions both before and after the antibiotic treatment (**Figure 3A**), setting them as a varying factor. Furthermore, the treatment reduced alpha diversity ($p<0.001$, Kruskal-Wallis test) and increased the variation of the host microbiota (**Figure 3B**). These results suggest that the microbiota profiles in our competitions are a combination of pre-existing differences in microbiota composition with antibiotic-driven dysbiosis. We did not observe an association between a specific taxonomic group and the absence of cost (ANCOM analysis), nor did we see a significant relationship between alpha diversity and the presence of a cost ($p=0.70$, Kruskal-Wallis test).

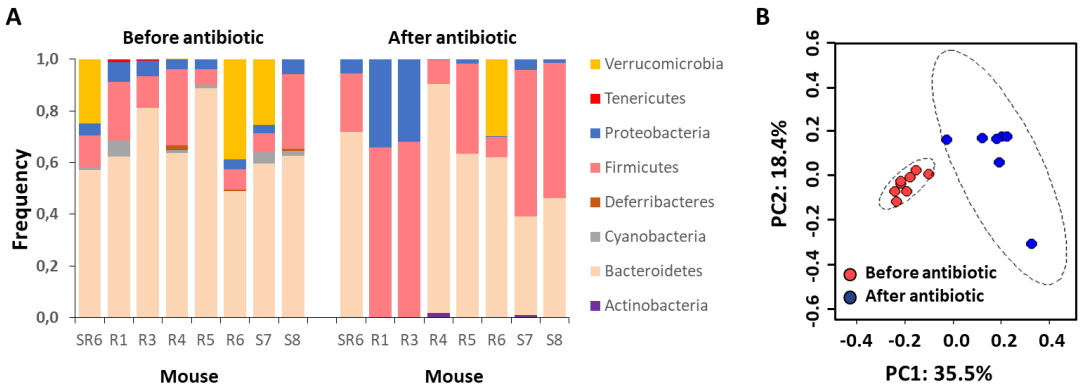


Figure 3 – The effect of antibiotic treatment in microbiota composition. A) Microbiota composition at the phylum level of mutant versus wild-type competitions, before and after antibiotic treatment, estimated through 16S-based metagenomic analysis. **B)** PCoA of an unweighted unifrac (qualitative beta-diversity) of the samples. Dashed lines represent 95% confidence intervals.

Normalization of the microbiota reduces variance of fitness effects

At this point, it was still not clear if a shared microbiota composition could lead to a similar fitness phenotype. Mice are coprophagic [414] sharing microbiota while living together [415], so to test if the microbiota composition could account for the personalized fitness effects, we performed competitions in mice co-housed until *E. coli* colonization.

We observed a reduced variance for the fitness effects in the co-housed mice when compared with individually caged ones (**Figure 4A-B**), with a significantly lower variance for the single streptomycin and the double mutant competitions ($pStr^R$ = 0.005, $pStr^RRif^R$ = 0.04, F-test; **Figure 4B**). Furthermore, the intermediate variance in co-housed mice was not significantly different from the low variance of the germ-free competitions, with the outcome of the competitions becoming more similar across hosts within the first 5 days of competition for all of the resistant strains, although without a significant cost for the double mutant competitions (**Table S3**).

Besides displaying an effect of antibiotic perturbation (**Figure 4C**), 16S metagenomic analysis of the microbiota composition shows a clear co-house identity for all of the mice 24 hours after colonization (**Figure 4D**), confirming a successful normalization of the microbiota.

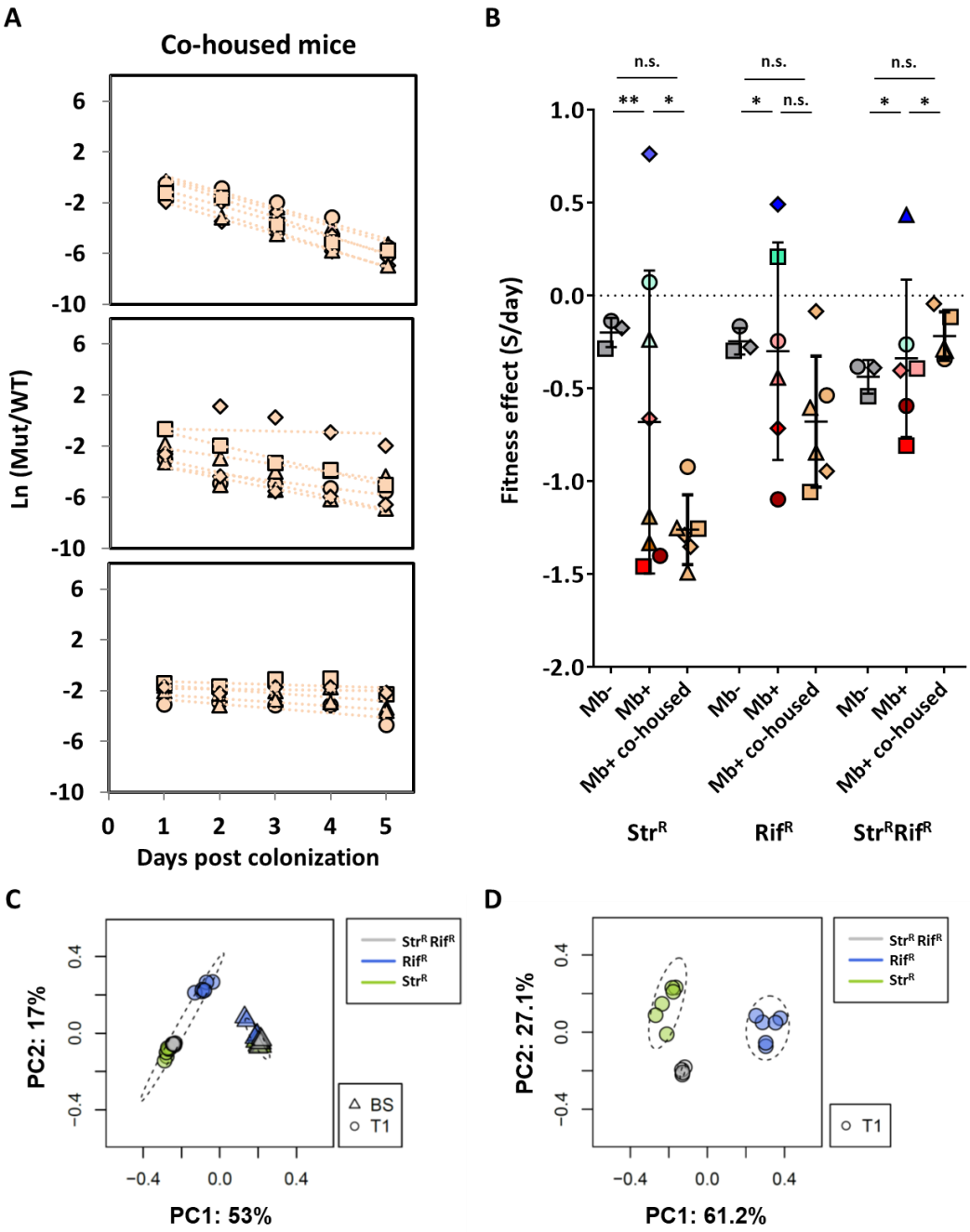


Figure 4 - Co-housing homogenizes the fitness effect of resistance. A) Logarithm of the resistant : wild-type ratio overtime for *rpsL K43T* (Str^R), *rpoB H526Y* (Rif^R), and *rpsL K43T rpoB H526Y* (Str^RRif^R) mutants, in mice that were co-housed for two weeks before and during

antibiotic treatment. **B)** Boxplot of the mean and the standard deviation of the fitness effect of resistance measured in mono-colonized, individually caged and co-housed mice. F-tests to detect differences in variance were performed (*p<0.05;**p<0.01). **C)** PCoA of an unweighted unifrac of the co-housed samples, before antibiotic treatment and 24 hours after colonization, showcasing the effects of antibiotic perturbation. **D)** PCoA of an unweighted unifrac of the samples at 24 hours after colonization, depicting co-house identity. For both PCoA, dashed lines correspond to 95% confidence intervals.

Extended competitions reveal a late disadvantage of multi-resistance

Despite the early fitness effects of resistance after a microbiota perturbation being so variable, the long-term effects could be more predictable, namely if the microbiota would recover to a state in which its competitive ability is similar across mice. On the other hand, adaptive and compensatory evolution could affect the competitive ability of the strains. To query about the long term of multi-resistance, we have prolonged the fitness assay for a subset of 8 mice (2 in which the double mutant was in higher frequency than the wild-type 5 days after gavage, and all of the double mutant competitions with the single resistants), and checked for the strain frequency 3 weeks after the previous sampling event, around 1 month after the gavage. In all of them, the frequency of the double mutant decreased (**Figure S3A-B**), even in one mouse in which it was previously close to fixation (frequency of 99%). While we have not sequenced the strains nor the microbiota, these results suggest that the lack of a cost in some mice might be a temporary consequence of the antibiotic perturbation.

The effect of microbiota in the cost of resistance in a mouse adapted strain

It is known that the cost of antibiotic resistance mutations is determined in part by the strain's genetic background [177]. To check if microbiota could affect the cost of resistance in a natural strain, we have designed a competition between a streptomycin resistant resident *E. coli* (*rpsL K88R*), isolated from fecal samples of a streptomycin treated mouse [66], and an isogenic double resistant strain (*rpsL K88R rpoB H526Y*). The double mutant bears the same rifampicin resistance mutation as the previously tested *E. coli* K12. We have chosen to use two streptomycin resistant strains to immediately exclude a direct effect of streptomycin in the competing strains, without need of further testing. Once again, we performed competitions both in germ-free and in SPF mice (**Figure 5A**). In the absence of microbiota, we have observed a strong deleterious fitness effect of rifampicin resistance in the resident *E. coli*'s *rpsL K88R* background [$S_{Rif}^{R}/_{day}$ germ-free = -0.86 (± 0.15, 2xSE)], suggesting that in the mouse gut, in the absence of other bacteria, the *rpoB H526Y* mutation causes maladaptation. All of the slopes were significantly negative ($p<0.05$, F test), although in a single mouse, one of the slopes lost steepness after 2 days. As this was a single occurrence in all of the germ-free mice tested in this project (1 in 21 mice), it could be the result of a rare event, such as early adaptation or compensation. Although all of the mice were treated with utmost care to keep the monocolonization status, a contamination during the experiment can also not be excluded.

Regarding the SPF mice, we have observed variation of the fitness effects on the resident *E. coli* background, with 2 of the 6 mice having significantly negative slopes, while the other mice had a non-significant selection coefficient (**Table S4**). Curiously, the average cost of resistance was more than 3 times lower than in germ-free mice [$S_{Rif}^{R}/_{day}$ SPF = -0.26 (±0,22) vs $S_{Rif}^{R}/_{day}$ GF = -0.86 (± 0.15); p=0.002, Mann-Whitney U test; **Figure 5B**], suggesting that in an environment with strong inter-species competition, resistance is less disadvantageous. As with the competitions involving *E. coli* K-12, in 2 mice we observe fluctuations in the strain vs strain ratio, indicating that in some mice, the environment could be changing during these first 5

days of competition. Altogether, these observations indicate that the fitness effect of resistance mutations in natural strains can also be changed by microbiota. However, the magnitude of the variance is not significantly higher in SPF mice for the resident competitions (σ^2SPF = 0.07 vs σ^2GF = 0.03; p=0.77, F-test), which might nonetheless be related with the lower average cost of rifampicin resistance in SPF mice.

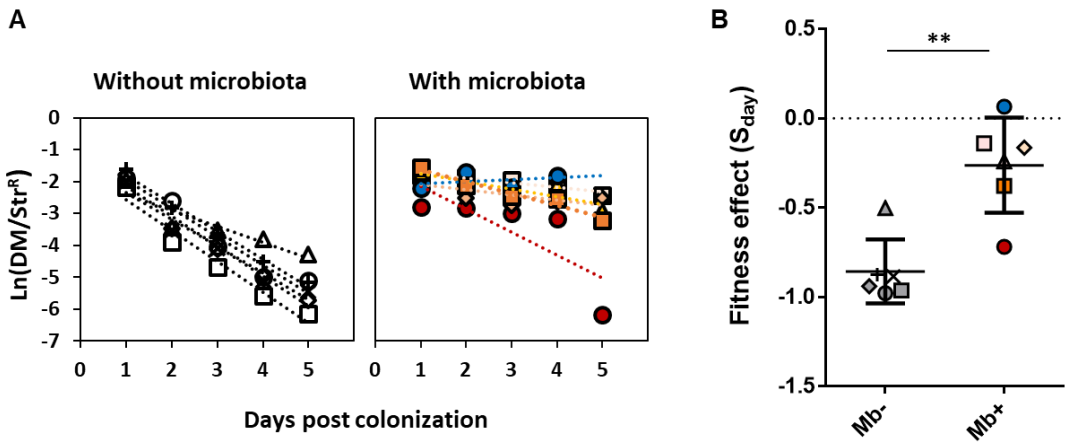


Figure 5 – Distinct fitness effects of a rifampicin resistance mutation in the streptomycin resistant, resident background in the absence and presence of microbiota. (A) Logarithm of the double mutant : single mutant ratio over time in mono-colonized mice and in mice with microbiota. **(B)** Boxplot of the mean and the standard deviation of the fitness effect of resistance measured in mice mono-colonized and with microbiota. The distribution of the fitness effects between the two treatments is significantly different (**p<0.01, Mann-Whithney U test).

Discussion

Causes for variation, nutrition and cross-feeding

Our experiments had as an objective to determine of the fitness effect of resistance mutations in the mammalian gut. As the interactions between bacterial species can have a great impact on their competitive ability and diverse microbial communities are prevalent in nature [416], we also aimed to determine the contribution of the gut microbial community in the cost of these mutations. While colonization resistance against our *E. coli* strains did not allow to study how an unperturbed community affects the fitness of resistant strains, we are assessing the costs of resistance in an increasingly important environment to understand its maintenance: the mammalian gut after an antibiotic treatment.

In individually caged SPF mice, we have observed a variable fitness effect of resistance mutations across hosts. As we do not see such variation of the fitness effects in germ-free mice and as we observe a clear reduction of the differences through co-housing, our observations suggest that selection is acting unequally across mice due to the activity of microbiota. In accord with what has been reported in the literature [417,418], we see a dramatic decrease in microbiota diversity following antibiotic treatment, leading to different microbiota compositions across mice at the phylum level. These differences in microbiota composition are a change in the environment and are likely to be an important source of the variance between hosts.

It is known that the gut microbiota can modulate the host immune system [419,420], which in turn has a prominent role in the colonization by and in the control of microbiota members [421,422]. However, a simpler explanation for the observed variation is related with nutritional niches. The gut is an environment with strong competition for resources, and microbiotas with disparate species can differ in their metabolic abilities, leading to distinct levels of competition for the different gut resources. It has been observed that the gene expression and metabolic activity of a gut strain can

change greatly in the presence of other community members [420,423,424], so the elimination of bacteria with specific functions can cause adjustments on the metabolic activity of other species and further differentiate individual microbiotas. In particular, an altered microbiota can offer different cross-feeding opportunities. Pair-wise cross-feeding interactions between gut bacteria were shown to occur, both *in vitro* [425] and in germ-free mice [423], and higher order cross-feeding interactions, are thought to be involved in complex microbial communities [426,427], with a recent empirical work showing them to be an emerging property of large microbial communities [428]. Furthermore, the disruption of cross-feeding has functional consequences for the microbiota, inclusively at the level of host-microbe interactions [420]. It is thus intuitive that the alterations in terms of microbiota composition, with consequences at the level of nutrient availability, gene expression and on cross-feeding interactions, together with the gut spatial structure and the consequent local competition, can generate singular environments in each host. This in turn can make the fitness effects of resistance personalized.

In order to colonize the mice that had resident microbiota, we had to break colonization resistance by giving a streptomycin treatment for a week. A two-day period of recovery followed, to clear the streptomycin from the mouse before the gavage. We have tested its presence through a competition method and we did not detect an effect of residual streptomycin in our samples. We are aware that our test is not highly sensitive to trace amounts of streptomycin (it cannot detect concentrations lower that 2 µg/ml in our experimental samples). However, it has been observed that streptomycin is quickly removed from mice following oral administration [429]. On another note, a recent study on pig fecal medium shows that the presence of the fecal community increases the minimum selective concentration of gentamicin and kanamycin on sensitive strains of *E. coli* by more than one order of magnitude [430]. Such a protective effect, if applicable for other antibiotics, would further reduce the risk of an effect of residual streptomycin. Furthermore, we see different outcomes in the competitions between two streptomycin resistant strains ($Str^R Rif^R$ vs Str^R, **Figure S1A**) and between two susceptible strains (Rif^R vs WT,

Figure 2) in individually caged mice and lower selection coefficient variation in the co-housed competitions (**Figure 4**), reinforcing that the observed disparities in the former are due to the action of microbiota following the antibiotic treatment, and not due to a direct action of the drug on the competing strains. The dysbiotic state caused by the antibiotic treatment is likely to be the main reason for the observed costs. However, the data in **Chapter III** hints that pre-existing differences in the microbiota might be partially responsible for the outcome of the antibiotic treatment, both at the level of microbial composition and on its consequential fitness costs.

For some of the competitions involving the double mutant, we have extended the sample collection for roughly 1 month after gavage. In these, we could observe that the double mutant would lower in frequency regardless of the dynamics from the early colonization. It has been observed in humans that, after the challenge with an antibiotic, microbiota begins to return to their initial state. However, this recovery is partial [431,432], with some members being lost from the community [417,418], and thus antibiotic treatment may cause a shift to alternative stable states [432]. It is possible that the microbiota in the different mice are converging to a state in which a cost for resistance is expressed. Another more farfetched, but still plausible hypothesis has to do with constraints in the evolution of the multi-resistant strain. The high pleiotropy of the resistance mutations could cause epistasis between them and mutations that would be adaptive in a wild-type background. As genetic interactions generate rugged fitness landscapes [433], the double mutant could get more easily stuck on a sub-optimal fitness peak, which would limit the access to adaptive mutations when compared to a less epistasis-restricted single mutant or wild-type strain. Our observations are clearly insufficient to understand the long-term fate of resistant strains. Besides, we do not know the proximate cause for the late cost of resistance. However, the results suggest that the fitness effects converge across hosts in the long term.

Mutation nature and pleiotropy

The mutations under focus have previously been shown to have disparate fitness effects in two distinct nutritional environments [274], which still holds for the mutants used in this study (**Table 1**). The double mutant, for instance, has a fitness advantage in minimal medium with glucose when competing with the wild-type strain, and a disadvantage in LB. It is reasonable to assume that in an environment in which glucose is the main carbon source, this mutant is more likely to have an advantage than in an environment in which glucose is depleted by other microbes. Following this logic, any gut nutrient for which the consumption efficiency is different in the resistant and wild-type bacteria could be an environmental factor driving selection. While we are currently not aware of the fitness effect of these mutations in the presence of other gut nutrients, it is expected that they exist. Point mutations on the *rpoB* and *rpsL* genes, such as the ones involved in this study, can be highly pleiotropic [170,172,434]. This might be related with the fact that some streptomycin resistance mutations can affect translation speed and accuracy [435], while certain *rpoB* mutations can affect transcription speed [436] and fidelity [437,438]. Cellular processes that depend of the effectiveness of transcription and translation, such as the activation or repression of operons linked to nutrient uptake and consumption, are likely to be affected. Although catabolite-repression is known to occur in bacteria, including *E. coli* [439], bacteria can find a multitude of niches when colonizing the mammalian gut. Successful species must have been selected to readily change their expression according to the niche opportunities or to simultaneously utilize multiple substrates [440]. *E. coli* MG1655 grown in mucus (mimicking the gut nutritionally) has been shown to express genes involved in catabolism of different sugars, such as N-acetylglucosamine, sialic acid, fucose, glucosamine, ribose, glucuronate, galacturonate, gluconate and maltose [441]. Additionally, we are measuring fitness at the population level. It is very likely that in the gut, individual *E. coli* cells are experiencing different niches, while the population as a whole is consuming different carbon sources simultaneously [397]. Therefore, the fitness burden of the resistance

mutation will be a net effect of all affected pathways, whether they are simultaneously active or not.

We suspect that our observations are also true for other mutations affecting traits under selection. Consistent with this hypothesis, Barroso-Batista *et al*. have observed a higher variation for the fitness effect of a mutation in the *gat* operon in SPF mice, when compared with germ-free [411]. As the *gat* operon is involved in the consumption of galactitol, this observation suggests that mutations that affect the consumption of a gut nutrient can produce a different fitness effect across hosts, and that pleiotropy is not required.

In vitro predictions and future approaches to measure fitness costs

We have observed that the costs of resistance of the mutations under study in germ-free mice are different from the *in vitro* competitions in LB and minimal medium with glucose, and as such, not predictable by these results. Both media are commonly used in microbiology, and this observation raises the question of how meaningful are the resistance costs measured using standard laboratory media. Although these media are an asset for growing model bacteria, questions related with fitness effects of mutations in natural environments should be addressed either in *in vitro* media with a similar composition to those environments, or in those environments themselves. Additionally, our *in vivo* results suggest that microbial communities are an essential environmental factor determining the fitness effects of resistance. Alternative *ex vivo* models, such as microfluidics intestine chips are currently being developed [442], and using these environments in future studies to evaluate the cost of resistance, both in the presence and absence of a microbiome, could prove to be a valid alternative in predicting the cost of resistance.

We were not able to determine which microbiota members were affecting the fitness of our resistant strains. This might have had to do with the fact that we had an overall small sample size, with few cases in which the resistance was beneficial,

reducing our statistical power to discriminate specific taxa. On the other hand, as the streptomycin and rifampicin single mutants have different fitness in both LB and minimal medium with glucose, it is entirely possible that the two are not favored by the same microbiota composition. Since the fitness effects of resistance are allele specific [192], a favorable microbiota might be so in a resistance-specific or even in an allele-specific way. Following our observations, studying if and how specific microbiota members modulate the cost of resistance is a promising research topic, which might bring further insights concerning how resistance is maintained in nature. In any regard, future studies on antibiotic resistance in natural and clinical settings should take the metagenome into account as an important factor.

Natural strains and generalizations

While competing a single and a double resistant resident *E. coli*, we have observed a much higher average cost per day of rifampicin resistance in the germ-free gut compared with the SPF mouse gut. At a first glance, this suggests that the maladaptation caused by the *rpoB H526Y* mutation is higher in the absence of microbiota.

In the germ-free gut, in which selection is acting at the intra-species level, *E. coli* has a faster doubling time [411], and the resident *E. coli* reaches a higher bacterial load when compared with the SPF mouse gut (averages of 1.3×10^{10} vs 2×10^{8} CFU per gram of feces, for germ-free and SPF, respectively; $p<0.0001$, Mann Whitney test). It is possible that the *rpoB H526Y* mutation is more disadvantageous in an environment in which more nutrients are available, and in which the resident has to divide faster. *E. coli* is one of the first colonizers in the gut, reaching very high loads before the expansion of anaerobes [373]. To a certain extent, the germ-free gut mimics the environment in which natural *E. coli* colonizes the gut – an habitat that allows a great expansion of *E.coli*, and in which strong intra-species selection will favor fast cell division. In an environment in which there is strong inter-species

competition and the resources are scarcer, both strains are affected by the activity of the other microbes, which could lead to a smaller fitness difference in SPF mice.

Curiously, we do not see such a difference in the average cost of this mutation in the *E. coli* K-12 - the germ-free cost is smaller than in the resident, and similar to the average cost in SPF mice. It is known that the fitness effect of a mutation can vary with the genetic background [443]. It is possible that the most relevant phenotypic aspects for the germ-free colonization are less affected by the *rpoB* mutation in the K-12 background. On the other hand, the resident strain has been evolving in the gut ecosystem and should be more well adapted to readily colonize the mouse intestine than the K-12 strains. As a genotype that is closer to a fitness peak, mutations in the resident background are more likely to have a deleterious effect, particularly when they can affect multiple phenotypes.

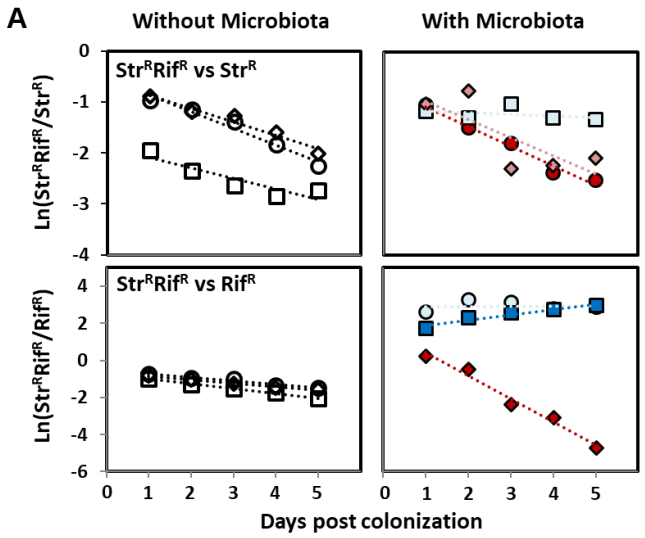
Although we have observed variation in the fitness effect of rifampicin resistance in the presence of microbiota, including dynamic shifts during the competition, it was less wide than in the K-12 background. Assuming that lower bacterial loads reflect a lower rate of cell division, the resident double mutant might be matching closely the wild-type concerning growth-related traits in SPF mice, leading to a small magnitude of costs and benefits. We did not perform metagenomic analysis for these competitions, but as the hosts are from distinct litters, the microbiota composition should still differ. Thus, the lower variation is more easily explainable through similar growth phenotypes between these two strains in the presence of other competitors.

These results alone do not clearly support or disprove the variation of the fitness effects of resistance in natural strains, and further research should address the generality of our observations at different levels: at the level of natural strains, at the level of taxa - asking if the costs of resistant bacteria from other phyla is also variable after an antibiotic treatment, at the level of the resistance profile - if it is specific for rifampicin and streptomycin resistance - or antibiotic target - if variation only occurs for pleiotropic resistance mutations like ours, which affect transcription

and translation - and how does microbiota affect the cost of resistances with a different genetic nature, such as the ones encoded through resistance cassettes. As these can code for a multitude of resistance mechanisms and can be shared through horizontal gene transfer, they had a significant contribution in the spread of antibiotic resistance following the golden age of antibiotics and are seen as an epidemiological threat [444]. Studies regarding microbiota composition and how it affects the selection on mobile genetic elements carrying resistance genes could indeed bring important insights in the fight against resistance.

Supplementary Material

A



B

Competition	S_{day} (± SE) Germ-Free	Epistasis
Str^R vs Wt	-0,20 ±0,05	
Rif^R vs Wt	-0,25 ±0,04	
Str^RRif^R vs Str^R	-0,26 ±0,04	
Str^RRif^R vs Rif^R	-0,21 ±0,03	
Str^RRif^R vs Wt	-0,44 ±0,05	0,01 (0,08)
		Absent

Figure S1 – Transitivity and absence of epistasis *in vivo*, in the absence of microbiota. **(A)** Logarithm of the resistant mutant : wild-type ratio over time for the double mutant while competing with the single mutants. As in the competitions with the wild-type, the selection coefficient can vary between animals in the presence of microbiota. **(B)** Selection coefficients for the resistance mutations in the absence of microbiota (germ-free). Notice that the cost of a mutation in the wild-type background is similar to the cost in a resistant background. In the absence of microbiota, no epistasis between the mutations was found.

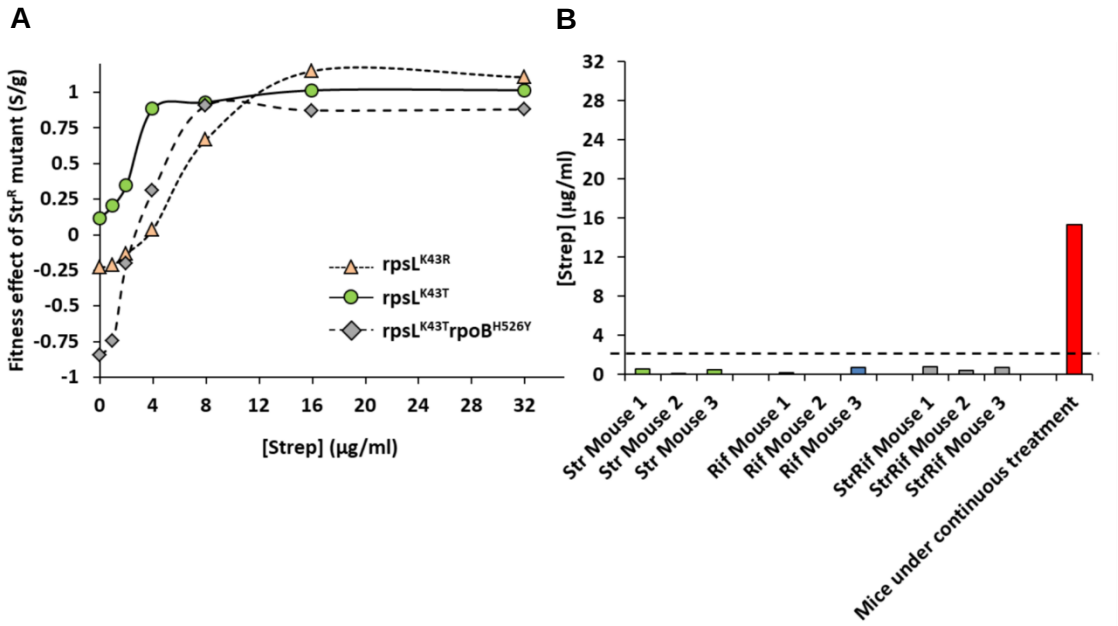
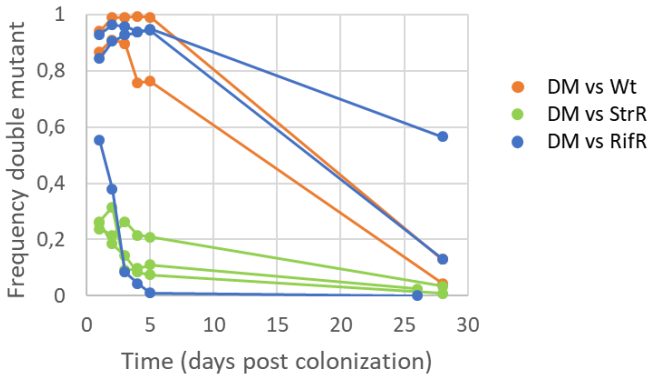


Figure S2 - Indirect estimation of streptomycin concentration in fecal samples.

(A) Effect of different concentrations of streptomycin in pairwise competitions between *E. coli* MG1655 resistant to streptomycin (*rpsL K43T* or *rpsL K43R* single mutants or double mutant *rpsL K43T rpoB H526Y*) against a susceptible strain in fecal medium supplemented with LB, in the presence of known streptomycin concentrations. The effects with *rpsL K43R* were used to generate a calibration curve. **(B)** Estimated streptomycin concentration in fecal samples collected 4 hours after gavage of *E. coli*, through competitions in the respective fecal medium. The method does not allow for the detection of streptomycin below 2µg/ml of streptomycin, corresponding to the dashed line. No antibiotic pressure was detected in the experimental samples.

A



B

	Frequency double mutant	
Mouse	**Day 5**	**Week 4**
SR4	0.99	0.13
SR5	0.76	0.04
DS1	0.07	0.01
DS2	0.21	0.03
DS3	0.11	0.02
DR1	0.94	0.13
DR2	0.95	0.57
DR3	0.01	0.00

Figure S3 - Extended competitions suggest convergence of the double mutant frequency. (A) Dynamics of the double mutant frequency in a subset of mice, extended up to 4 weeks after colonization. **(B)** Frequency of the double mutant in each of the sampled mouse after 5 days and 4 weeks of colonization. For mice DS3 and DR3, the late time-point corresponds to day 26, while for the other mice it corresponds to day 28 after colonization. Although we have observed a variable fitness effect of multi-resistance in the first 5 days, the frequency of the double mutant dropped between day 5 and week 4 in all mice.

Table S1 – Outcome of the competitions for the *E.coli* K-12 MG1655 background in germ-free mice. The selection coefficient per day for each mouse is presented, and within brackets is shown the standard error. Significant values were obtained through an F-statistic for the slope of the linear model of the selection coefficient (ln(mutant/wild-type) or ln (double mutant/single mutant) over time). Fitness effects with a significant score and a positive slope were considered a benefit, while fitness effects with significant scores and a negative slope were considered a cost.

Mouse	Competitors	S_{day}	R^2	Fitness effect
S1	Str^R vs Wt	-0.17 (0.04 SE)	0.84	**Cost**
S2	Str^R vs Wt	-0.29 (0.05 SE)	0.92	**Cost**
S3	Str^R vs Wt	-0.14 (0.03 SE)	0.87	**Cost**
R1	Str^R Rif^R vs Wt	-0.28 (0.04 SE)	0.94	**Cost**
R2	Str^R Rif^R vs Wt	-0.17 (0.05 SE)	0.78	**Cost**
R3	Str^R Rif^R vs Wt	-0.30 (0.03 SE)	0.98	**Cost**
SR1	Str^R Rif^R vs Str^R	-0.39 (0.02 SE)	0.99	**Cost**
SR2	Str^R Rif^R vs Str^R	-0.54 (0.07 SE)	0.95	**Cost**
SR3	Str^R Rif^R vs Str^R	-0.38 (0.03 SE)	0.98	**Cost**
DS1	Str^R Rif^R vs Str^R	-0.32 (0.04 SE)	0.97	**Cost**
DS2	Str^R Rif^R vs Str^R	-0.21 (0.06 SE)	0.83	**Cost**
DS3	Str^R Rif^R vs Str^R	-0.26 (0.03 SE)	0.95	**Cost**
DR1	Str^R Rif^R vs Rif^R	-0.18 (0.02 SE)	0.96	**Cost**
DR2	Str^R Rif^R vs Rif^R	-0.26 (0.01 SE)	0.99	**Cost**
DR3	Str^R Rif^R vs Rif^R	-0.19 (0.02 SE)	0.98	**Cost**

Table S2 – Outcome of the competitions for the *E.coli* K-12 MG1655 background in all single-housed specific pathogen free mice. The selection coefficient per day for each mouse is presented, and within brackets is shown the standard error. Significant values were obtained through an F-statistic for the slope of the linear model of the selection coefficient (ln(mutant/wild-type) or ln ((double mutant/single mutant) over time). Fitness effects with a significant score and a positive slope were considered a benefit, while fitness effects with significant scores and a negative slope were considered a cost.

Mouse	Competitors	S_{day}	R^2	Fitness effect
S1	Str^R vs Wt	-1.4 (0.2 SE)	0.93	**Cost**
S2	Str^R vs Wt	-1.4 (0.6 SE)	0.37	**Neutral**
S3	Str^R vs Wt	0.762 (0.2 SE)	0.25	**Benefit**
S4	Str^R vs Wt	-1.3 (0.1 SE)	0.77	**Cost**
S5	Str^R vs Wt	0.07 (0.08 SE)	0.24	**Neutral**
S6	Str^R vs Wt	-1.2 (0.1 SE)	0.27	**Cost**
S7	Str^R vs Wt	-0.663 (0.1 SE)	0.79	**Cost**
S8	Str^R vs Wt	-0.24 (0.06 SE)	0.90	**Cost**
R1	Rif^R vs Wt	-0.71 (0.04 SE)	0.99	**Cost**
R2	Rif^R vs Wt	0.2 (0.6 SE)	0.03	**Neutral**
R3	Rif^R vs Wt	-0.4 (0.2 SE)	0.58	**Neutral**
R4	Rif^R vs Wt	-0.2 (0.1 SE)	0.62	**Neutral**
R5	Rif^R vs Wt	0.5 (0.3 SE)	0.51	**Neutral**
R6	Rif^R vs Wt	-1.1 (0.2 SE)	0.90	**Cost**
SR1	Str^R Rif^R vs Wt	-0.8 (0.1 SE)	0.94	**Cost**
SR2	Str^R Rif^R vs Wt	-0.6 (0.3 SE)	0.58	**Neutral**
SR3	Str^R Rif^R vs Wt	-0.4 (0.2 SE)	0.70	**Neutral**
SR4	Str^R Rif^R vs Wt	0.4 (0.2 SE)	0.60	**Neutral**
SR5	Str^R Rif^R vs Wt	-0.3 (0.1 SE)	0.55	**Neutral**
SR6	Str^R Rif^R vs Wt	-0.4 (0.3 SE)	0.39	**Neutral**
DS1	Str^R Rif^R vs Str^R	-0.39 (0.035 SE)	0.98	**Cost**
DS2	Str^R Rif^R vs Str^R	-0.03 (0.04 SE)	0.17	**Neutral**
DS3	Str^R Rif^R vs Str^R	-0.4 (0.2 SE)	0.61	**Neutral**
DR1	Str^R Rif^R vs Rif^R	0 (0.1 SE)	0.00	**Neutral**
DR2	Str^R Rif^R vs Rif^R	0.29 (0.05 SE)	0.93	**Benefit**
DR3	Str^R Rif^R vs Rif^R	-1.3 (0.1 SE)	0.98	**Cost**

Table S3 –– Outcome of the competitions for the co-housed mice. The selection coefficient per day for each mouse is presented, and within brackets is shown the standard error. Significant values were obtained through an F-statistic for the slope of the linear model of the selection coefficient (ln(mutant/wild-type) over time). Fitness effects with a significant score and a positive slope were considered a benefit, while fitness effects with significant scores and a negative slope were considered a cost.

Mouse	Competitors	S_{day}	R^2	Fitness effect
S1-CH	Str^R vs Wt	-1.25 (0.04 SE)	0.99	**Cost**
S2-CH	Str^R vs Wt	-1.25 (0.07 SE)	0.99	**Cost**
S3-CH	Str^R vs Wt	-1.47 (0.11 SE)	0.98	**Cost**
S4-CH	Str^R vs Wt	-1.41 (0.08 SE)	0.99	**Cost**
S5-CH	Str^R vs Wt	-1.2 (0.2 SE)	0.93	**Cost**
S6-CH	Str^R vs Wt	-1.25 (0.16 SE)	0.96	**Cost**
R1-CH	Rif^R vs Wt	-0.5 (0.2 SE)	0.74	**Neutral**
R2-CH	Rif^R vs Wt	-0.84 (0.1 SE)	0.94	**Cost**
R3-CH	Rif^R vs Wt	-0.1 (0.5 SE)	0.01	**Neutral**
R4-CH	Rif^R vs Wt	-0.6 (0.1 SE)	0.86	**Cost**
R5-CH	Rif^R vs Wt	-0.9 (0.1 SE)	0.93	**Cost**
R6-CH	Rif^R vs Wt	-1.06 (0.08 SE)	0.98	**Cost**
DM1-CH	Str^R Rif^R vs Wt	-0.3 (0.2 SE)	0.56	**Neutral**
DM2-CH	Str^R Rif^R vs Wt	-0.3 (0.2 SE)	0.47	**Neutral**
DM3-CH	Str^R Rif^R vs Wt	-0.1 (0.1 SE)	0.13	**Neutral**
DM4-CH	Str^R Rif^R vs Wt	-0.3 (0.1 SE)	0.64	**Neutral**
DM5-CH	Str^R Rif^R vs Wt	-0.04 (0.09 SE)	0.07	**Neutral**

Table S4 – Outcome of the competitions between a rifampicin resistant and a rifampicin sensitive strain, both with a gut-adapted genetic background. The two strains bear the K88R mutation, which confers streptomycin resistance. The selection coefficient per day for each mouse is presented, and within brackets is shown the standard error. Significant values were obtained through an F-statistic for the slope of the linear model of the selection coefficient (ln (double mutant/single mutant) over time). Fitness effects with a significant score and a positive slope were considered a benefit, while fitness effects with significant scores and a negative slope were considered a cost.

Mouse	Host Status	S_{day}	R^2	Fitness effect
GF1	Germ-Free	-0.9 (0.1 SE)	0.95	**Cost**
GF2	Germ-Free	-1.0 (0.1 SE)	0.96	**Cost**
GF3	Germ-Free	-0.9 (0.1 SE)	0.96	**Cost**
GF4	Germ-Free	-0.5 (0.1 SE)	0.83	**Cost**
GF5	Germ-Free	-1.0 (0.1 SE)	0.95	**Cost**
GF6	Germ-Free	-0.87 (0.06 SE)	0.99	**Cost**
SPF1	SPF	-0.7 (0.3 SE)	0.60	**Neutral**
SPF2	SPF	-0.14 (0.04 SE)	0.80	**Cost**
SPF3	SPF	-0.2 (0.1 SE)	0.43	**Neutral**
SPF4	SPF	-0.24 (0.09 SE)	0.69	**Neutral**
SPF5	SPF	0.1 (0.1 SE)	0.11	**Neutral**
SPF6	SPF	-0.38 (0.05 SE)	0.95	**Cost**

CHAPTER III

Adaptation prevails over compensation in the mouse gut

Parts of this chapter are included in a manuscript submitted for publication.

Cardoso, LL, Durão, P, Amicone, M, Gordo, I. *Dysbiosis personalizes fitness effect of antibiotic resistance in the mammalian gut.*

Pre-print available at bioRxiv:

https://doi.org/10.1101/748897

Author contributions for this Chapter:

Isabel Gordo and I designed this study. I have performed the propagations and whole genome sequencing analysis. Results were edited by Paulo Durão and I. The execution was supervised by Isabel Gordo.

Abstract

The acquisition of antibiotic resistance typically implies a fitness cost in the absence of antibiotics. Despite this fact, antibiotic resistance has been spreading over time, and resistant bacteria persist in bacterial communities. The maintenance of resistant bacteria can be explained by many factors, including biotic interactions and the evolution through compensatory mutations. Still, the adaptation of commensal strains harboring single or multiple costly chromosomal resistance mutations remains unexplored. We have evolved mutants harboring streptomycin and rifampicin resistance mutations and the respective double mutant in the mammalian gut, in the presence of distinct bacterial communities. We have found that adaptative mutations are ubiquitous during the evolution of these strains, while classical compensatory mutations are not. Furthermore, the occurrence and pace of compensation was markedly different across mouse litters which had a distinct microbiota composition, suggesting host microbiota specificity for the evolution of resistant strains.

Introduction

Bacteria can acquire antibiotic resistance through horizontal gene transfer of accessory elements encoding resistance genes and through chromosomal mutations that modify the target of resistance [9]. While horizontal gene transfer has been found to be common [53] and is recognized as the main driver of the early spread of resistance across pathogens [46,55], resistance mutations occur spontaneously, inclusively in bacteria that do not have the ability to acquire and retain foreign genetic material. One key example is the human pathogen *Mycobacterium tuberculosis*, capable of accumulating mutations that alter cellular antibiotic targets [445] and display extensive drug resistance to multiple antibiotics [446]. Resistance through target modification is effectively a ubiquitous mechanism of resistance, as examples of clinical strains carrying antibiotic resistance mutations are found for every class of antibiotics [123]. Hence, studying how resistant mutations subsist and disseminate in bacterial populations is of crucial importance to contain the antibiotic resistance threat.

Most resistance-conferring chromosomal mutations change cell machinery components into a non-optimal state, affecting metabolic and physiological processes of the cell [169] and leading to deleterious effects in the absence of the drug [8,9,176]. Various biological phenomena allow bacteria carrying antibiotic resistance mutations to reduce or bypass these effects, such as mutations with background-specific beneficial effects [447], environment-dependent fitness effects [272,274] and positive epistasis, either between chromosomal resistance mutations [192] or between chromosomal mutations and plasmids encoding for resistance [285]. However, the maintenance of resistance can also be a product of the evolution of resistant strains towards a higher fitness, namely through the acquisition of compensatory mutations [e.g. [177,191,196,249]].

While adaptive mutations in general can confer a benefit to bacteria irrespectively of their resistance profile, compensatory mutations have background-specific effects, and are selected because they ameliorate the fitness of resistant

strains, either by counter-acting the metabolic impairments caused by the resistance mutation [169] or the negative epistatic effects between resistance mutations [196]. These mutations are particularly troublesome clinically, as they are frequently found in clinic isolates [241,243,244,448], most often allow fitness restoration without the loss of resistance [8] and further hinder reversions due to their epistatic nature [449]. Besides mitigating fitness costs, compensatory mutations can also promote the recovery of relevant phenotypes affected by the resistance mutations, such as virulence in *Salmonella enterica* serovar Typhimurium during mouse infection [190].

Compensation can occur through intragenic mutations, on the gene conferring the resistance, or through mutations on other genes with a closely associated function [169]. Additionally, mutations outside coding regions may also compensate for the cost of resistance through changes in gene expression [196]. As resistant bacteria can ameliorate fitness through a broad range of gene targets, compensation is much more common than genotypic reversions — back mutations on the resistance locus that rescue both fitness and drug sensitivity [191,239,240,247,248]. Second site mutations can also cause resistance decline in the absence of antibiotics. In some situations, phenotypic reversions leading to full recovery of sensitivity have been reported — [196,263–265].

The compensatory evolution of bacteria carrying resistance mutations has been followed in several *in vitro* studies e. g. [196,205,239,240,242,262,450] and also in animal experiments [190,191,242]. One of the latter studies compares the evolution of *Salmonella enterica* serovar Typhimurium carrying either streptomycin or fusidic acid resistance mutations in LB medium and during mouse infection. By sequencing the genes with the resistance mutations and previously known extragenic targets, the authors have observed selection for different compensatory mutations in the two environments, with a different proportion of intragenic mutations, extragenic mutations and genetic reversions [191], highlighting the importance of studying the evolution of resistant bacteria in experimental hosts.

As shown in **Chapter II**, in the antibiotic-treated mammalian gut, the cost of resistance is host-specific in the presence of microbiota. As the strength of selective pressures favoring compensation depends on the fitness effect of the resistance mutation, the occurrence and spread of compensatory mutations could also be host-specific. In fact, a simple ecological model of resource competition based on metabolic trade-offs [451] corroborates the observed microbiota-dependent fitness costs when applied to our data and predicts a varying onset of selective pressure towards compensation (Amicone & Gordo, personal communication). Furthermore, strong selective pressures towards metabolic adaptation to the mouse gut have been observed in commensal *E. coli* [398,452] and it is still not clear if in the mouse gut, a strain bearing costly, resistance mutations is more likely to evolve through compensation or prioritize mutations that promote environment adaptation. To address these questions, we have evolved and sequenced single and double resistant *E. coli* in the mouse gut in two distinct cohorts of mice.

Methods

Eschericha coli and mice strains

For our experiments, we used fluorescence-labeled, *E. coli* K-12 MG1655-derived strains, with different antibiotic resistance profiles, including strains bearing either a point mutation conferring streptomycin resistance, a point mutation conferring rifampicin resistance or both mutations.

The point mutations conferring resistance in our K-12 strains, *rpsL K43T* and *rpoB H526Y*, were generated by Trindade *et al.* in a K-12 MG1655 genetic background, and confer streptomycin and rifampicin resistance, respectively [192]. These mutations were moved into isogenic, YFP and CFP fluorescent-labeled strains by Moura de Sousa *et al.* (2017). Since the *gat* operon was found to be a mutation

hotspot under strong selection in the mouse gut for K-12 MG1655 derived strains [395,398], we modified our strains to a galactitol negative phenotype via a knock-out of the *gatZ* gene. P1 transductions [403] were performed to substitute the whole *gatZ* gene from our strains with a kanamycin resistance cassete, using *E. coli* JW2082-1 from the KEIO collection [404] as a donor for the transduction. P1 transductions were also used to insert the point mutation *rpoB H526Y* in the wild-type background and to pass the *gatZ* deletion from the wild-type strains to the isogenic antibiotic resistant strains which carried either the point mutation *rpsL K43T* or both *rpsL K43T* and *rpoB H526Y* mutations ($Str^R Rif^R$). The resulting streptomycin resistant (Str^R) strains LC81 and LC82 (YFP/CFP, respectively), the rifampicin resistant (Rif^R) strains RB933 and LC84b (YFP/CFP, respectively), and the double resistant ($Str^R Rif^R$) strains LC85, LC86 (YFP/CFP, respectively) were used to colonize mice to perform the evolution experiment. All of these strains possess a chloramphenicol resistance cassete associated with their fluorescent protein, as well as the kanamycin resistance cassete. As this study is focused on streptomycin and rifampicin resistance, I will be designating our strains as single or double mutants. 6-to-8 week-old female C57BL/6J specific pathogen free (SPF) mice were used as hosts for the *in vivo* evolution experiment.

In vivo evolution

To evolve the resistant strains in SPF mice, we used a streptomycin treatment in order to break the colonization resistance [405]. Mice from the same litter were separated into individual cages and given autoclaved drinking water containing streptomycin sulfate (5g/L) for seven days, and then were given regular autoclaved drinking water for 2 days, in order to wash out the antibiotic from the gut. After 4 hours of starvation for food and water, the mice were gavaged with 100 µl of a $\approx 10^9$ cells/ml suspension with a 1:1 ratio of the two isogenic strains. This protocol was applied to two distinct cohorts of mice.

To make the suspension, the strains were streaked from stocks in LB agar with antibiotics corresponding to their resistance two days before gavage and incubated for 24 hours, followed by an overnight culture of a single colony for each biological replicate in BHI (brain heart infusion) medium with the corresponding antibiotics (concentrations of 100µg/ml). The cultures were then diluted 100-fold and grown in BHI medium until an $OD_{600nm} \approx 2$. An equal volume of the suspensions was mixed. Mice fecal pellets were collected 24 hours and every 48 after gavage, for 29 days, and collected once more, at the 39^{th} day of colonization. Upon collection, the samples were suspended, diluted in PBS and plated in LB agar plates with chloramphenicol (30µg/ml). Plates were incubated overnight and the frequencies of CFP- or YFP-labeled bacteria were assessed by counting the fluorescent colonies with the help of a fluorescent stereoscope (SteREOLumar, Carl Zeiss). The samples were also stored in 15% glycerol at -80°C for future experiments.

Reversion test

To test for antibiotic resistance reversion, following the plating of the diluted samples in LB medium plates supplemented with chloramphenicol (30µg/ml), up to 153 random colonies per tested sample were grown in solid LB with no antibiotic, and passed with a replicator into LB agar with streptomycin (100 µg/ml), with rifampicin (100 µg/ml), with both antibiotics and with no antibiotic (control). If an evolved clone would be sensitive to an antibiotic for which the ancestral was resistant, we would classify it as a phenotypic revertant.

DNA extraction for population sequencing

In order to perform population sequencing, PBS suspended dilutions of the fecal samples were plated in LB plates supplemented with chloramphenicol (30µg/ml). The dilutions were done in order to obtain between 500 and 5000 cells of *E. coli*. We use a high number of colonies to allow for a good resolution regarding the detection of low frequency mutants, while the growth in a structured environment can diminish the over-representation of putative fast-growing mutants. The colonies were then scrapped and resuspended in PBS, and genomic DNA was extracted using a standard phenol / chlorophorm method [453].

DNA extractions and whole-genome sequencing analysis

Concentration and purity of DNA were quantified using Qubit and NanoDrop, respectively. The DNA library construction and sequencing were carried out by the IGC genomics facility. Each sample was pair-end sequenced on an Illumina MiSeq Benchtop Sequencer. Standard procedures produced data sets of Illumina paired-end 250 bp read pairs. The reads were filtered using SeqTk version 1.0-r63. For whole genome sequencing, the mean coverage after filtering for the different samples was as follows: 168x and 175x for Str^R1 day 19 and day 39, respectively; 238x and 194x for Str^R2 day 19 and day 39, respectively; 164x and 159x for Rif^R1 day 19 and day 39, respectively; 226x and 202x for Rif^R2 day 19 and day 39, respectively; 148x and 156x for Str^R Rif^R1 day 19 and day 39, respectively; 213x and 220 for Str^R Rif^R2 day 19 and day 39, respectively. Sequences were analyzed using Breseq version 0.31.1, using *E. coli* K12 genome NC_000913.3 as a reference, with the polymorphism option selected, and the following parameters: (a) rejection of polymorphisms in homopolymers of a length greater than 3, (b) rejection of polymorphisms that are not present in at least 3 reads in each strand, and (c) rejection of polymorphisms that do not have a p-value for quality greater than 0.05,

(d) rejection of polymorphisms with less than 3 of coverage in each strand and (e) rejection of polymorphisms with less than 1% frequency. All other Breseq parameters were used as default. Hits that were present in all of our ancestral mutants, as well as homopolymers were discarded. Hits that were likely to be due to misalignment of repetitive regions were also discarded. Regarding the downstream analysis, target genes that appeared only in one sample and had a frequency lower than 5% were not considered.

In order to perform amplicon sequencing, samples were processed as for population sequencing, for every time-point of the double mutant evolution. Specific primers containing overhang adaptor regions were designed and used to amplify ≈400 base pair genome regions harboring the *rpsD Q54L* and *rpoC A784V* mutations, detected on the double mutant background by whole genome sequencing. For each sample, PCRs were performed using the Phusion high fidelity polymerase following the Phusion protocol (https://international.neb.com/Protocols/0001/01/01/pcr-protocol-m0530). The annealing temperatures were adjusted, corresponding to the primer sequences without the adaptor overhangs. PCRs for inserting indices and Illumina sequencing adapters were performed by the IGC Genomics Facility, following an online library preparation guide (https://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf). Samples were then pair-end sequenced by the Facility on an Illumina MiSeq Benchtop Sequencer, following Illumina recommendations.

The DADA2 R package [409] was used to filter and trim the amplicons, remove the adaptors, merge forward and reverse sequences and exclude chimeras. After these operations, the mean coverage of the *rpsD* amplicon ranged from 14899x to 62315x, while the mean coverage for the *rpoC* amplicon ranged from 8868x to 34653x.

Microbiota analysis

To assess the gut microbiota composition of mice, we extracted DNA from fecal samples from the evolution experiment 17 days after gavage, corresponding to the last time-point before the first sequencing event. Fecal DNA was extracted with a QIAamp DNA Stool MiniKit (Qiagen), according to the manufacturer's instructions and with an additional step of mechanical disruption [406]. 16S rRNA gene amplification and sequencing was carried out at the Gene Expression Unit from Instituto Gulbenkian de Ciência, following the service protocol. For each sample, the V4 region of the 16S rRNA gene was amplified in triplicate, using the primer pair F515/R806, under the following PCR cycling conditions: 94 ºC for 3 min, 35 cycles of 94 ºC for 60 s, 50 ºC for 60 s, and 72 ºC for 105 s, with an extension step of 72 ºC for 10 min [407]**.** Samples were then pair-end sequenced on an Illumina MiSeq Benchtop Sequencer, following Illumina recommendations.

QIIME2 [408] was used to analyze the 16S rRNA sequences by following the authors' online tutorials (https://docs.qiime2.org/2018.11/tutorials/). Briefly, the demultiplexed sequences were filtered using the "denoise-single" command of DADA2 [409], and forward and reverse sequences were trimmed in the position in which the 25th percentile's quality score got below 20. Alpha diversity, beta-diversity, and phylogenetic diversity analyses were also performed as in the QIIME2 tutorial. The sample depth was adjusted to 789 – the number of sequences of the sample with the lowest number, excluding the control.

Alpha-diversity was estimated through the Shannon vector and significance was assessed through the Kruskal-Wallis test. An ANCOM [454] was performed in order to assess if specific microbiota members were associated with each litter. For taxonomic analysis, OTU were picked by assigning operational taxonomic units at 97% similarity against the Greengenes database [410].

Results

Litter-specific occurrence of compensatory mutations

To study how resistant commensal bacteria evolve in the mammalian gut, we have followed the evolution of *E. coli* strains resistant to streptomycin, rifampicin or both drugs combined for up to 6 weeks of evolution. Since we have previously observed a microbiota-related, variable fitness cost, and are aware that microbiota composition is more similar in mice from the same litter than in mice from different litters [361,455], we have colonized two separate cohorts of mice, a design in which the same resistant background colonizes two mice from different parents (**Figure 1A**). The two cohorts were expected to have different microbiota composition, and such difference between litters was confirmed through 16S rDNA analysis 17 days after gavage — the closest time-point before the first sequencing event — with a significant over-representation of the Verrucomicrobia phylum in microbiota from litter 1, namely from the *Akkermansia* genus (W=10, ANCOM analysis). Remarkably, despite going through perturbations such as antibiotic treatment and the colonization with our *E. coli* strains, after 26 days, the microbiota of the 3 mice in cohort 1 are very similar regarding their composition (**Figure 1B**). The microbiota in cohort 2 however varied between the hosts. Noticeably, the phylum Bacteroidetes was not present at all in the mouse from cohort 2 hosting the single streptomycin resistant mutant, Str^R2 (**Figure 1C**). No significant variation in alpha diversity between the two cohorts was found (p=0.827, Kruskal-Wallis test).

To detect mutations in the evolved strains, we proceeded by performing whole genome population sequencing at two time-points, around week 3 and 6 after gavage. To identify *bona fide* compensatory mutations in particular, we relied on the fact that these mutations have been studied extensively *in vitro*, in different medium and bacterial species [e.g. [190,196,239,240,258]]. The remaining mutations were classified here as adaptive.

We have observed a wide array of mutation targets, with a constant presence of adaptive mutations in all sequenced samples. Overall, 17 genes and 10 intergenic regions were targeted for global adaptation, including mutations shown to be adaptive during *E. coli* colonization of streptomycin treated mouse gut (**Table S1**). Not a single mutation was found in all mice, and only 12 out of 27 targets (44%) were found in 2 or more genetic backgrounds, suggesting that our evolution experiment did not saturate the loci in which beneficial mutations can occur. Within these adaptive targets, mutations in the coding sequence of the genes *frlR*, *spoT* and *kdgR*, as well as in the intergenic regions of *dcuB-dcuR* and in *ymfE-lit* occurred only in one of the litters. A single adaptive mutation managed to reach fixation – the *tdcA D44G* mutation in the rifampicin resistant background.

Classical compensatory mutations, however, were found to vary between hosts, with a temporal signal of compensatory evolution (**Figure 1D**). In the first cohort of mice, at least one compensatory mutation was detected in all resistance backgrounds, by the 3rd week of evolution, while in the second cohort, compensation was only detected for the streptomycin resistant strain (**Figure 1D**), and even so, at low frequency (≈8% at day 19; **Figure 1E-F**). Strikingly, no compensatory mutations were detected for the double mutant in mice from the second cohort in the 6th week of evolution, while adaptive mutations were already present at week 3, suggesting a prolonged lack of cost of multi-resistance. No compensatory mutation reached fixation.

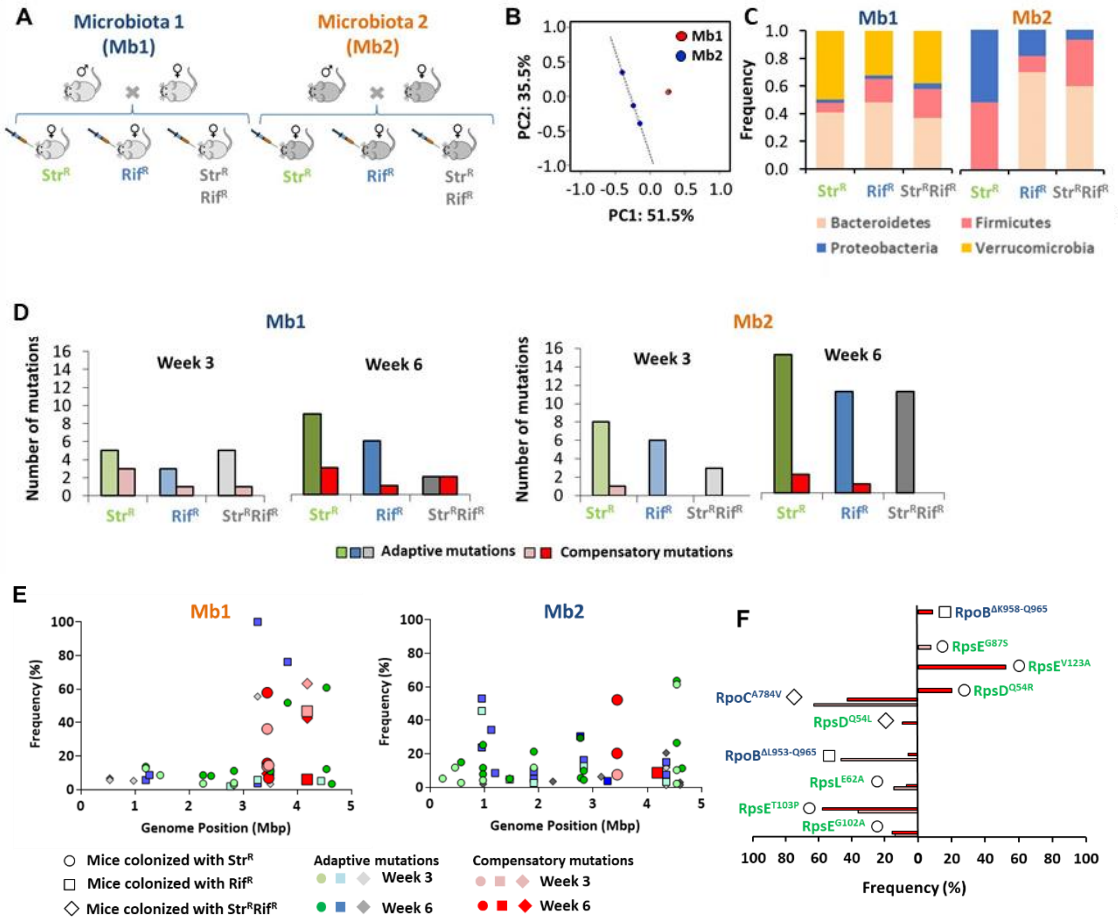


Figure 1 - Dynamics and genetic basis of compensatory evolution of AR strains across hosts. (A) Experimental set up to study the adaption pattern of resistant strains (Str^R, Rif^R and Str^RRif^R). Mice from the same sex and litter were co-housed since birth to homogenize the microbiota across litters. Afterwards, one mouse from each litter was colonized with one of the resistant backgrounds. **(B)** PCoA of an unweighted unifrac of the mice at week 3. Dashed lines correspond to 95% confidence intervals. Mice from the same litter cluster together and have a more similar microbiota. **(C)** Microbiota composition at the phylum level of the mice from the two different litters 3 weeks after colonization. **(D)** Comparison of the number of putative adaptive and compensatory mutations present in the adapted resistant populations after 3 and 6 weeks in the mice gut with different microbiotas. **(E)** Frequency of the detected adaptive and compensatory mutations at week 3 and week 6. **(F)** Genetic basis of the bona fide compensatory mutations detected after 3 or 6 weeks of adaptation in the gut.

Nature of classic compensatory mutations

For the single streptomycin resistant mutants, we detected as compensatory targets a mutation on *rpsL* itself, and extragenic mutations on genes coding for other ribosome subunits, *rpsE* and *rpsD* (**Figure 1E**). These genes have been shown to be compensatory targets for streptomycin resistance [190]. In fact, the mutations *rpsD Q54R* and *rpsE V123A* and the locus *rpsE T103* have been previously described as compensatory for the equivalent allele *rpsL K42N* in *Salmonella enterica* ser. Typhimurium [240].

In single rifampicin resistant mutants, we have observed two small deletions within the *rpoB* gene itself, one in each mouse. The two mutations are located in the same locus, as the deletions overlap (both delete amino acids 957 to 965), and both maintain the gene in-frame, as the two deletions englobe a number of base pairs multiple of 3. Mutations on *rpoB* compensating for fitness costs have been previously observed [239,245,258]. Still, to the best of my knowledge, a *rpoB* deletion was reported as a putative compensatory target only once [196], during the evolution of a double

mutant for rifampicin and streptomycin resistance. The mutation was also an in-frame deletion and was located close to ours (deleting RNA polymerase amino acids 1010-1012), so it could be compensating for rifampicin resistance through a similar mechanism. The frequency of this mutation in the first litter, however, lowers drastically between day 19 and 39 (from 46.7% to 6% - **Figure 1E**), suggesting that other mutations conferring a stronger fitness benefit have emerged in the population.

Focusing on the double mutant, we have detected mutations on the genes *rpsD* and *rpoC*, which have been described as compensatory targets for streptomycin and rifampicin respectively, and were hit during the evolution of the isogenic double mutant in LB medium, in the absence of antibiotics [196]. Curiously, the *rpsD Q54L* mutation affects the exact same codon as one detected in the single streptomycin resistant mutant, further implying this locus as a target for streptomycin resistance

compensation. The *rpoC A784V* mutation occurred only in the double resistant background, both in our and in the aforementioned study [196].

Amplicon sequencing suggests clonal interference between the double mutant's evolving lineages

Focusing on the study of the double mutant, we wanted to pinpoint the occurrence of compensatory mutations in the first cohort and further confirm that none occurred in the second cohort. To do so, we have performed amplicon sequencing throughout every sample of the double-mutant evolution of two ≈400 bp genome regions, centered on the observed compensatory mutations. In the first cohort, we have observed the *rpoC A784V* mutation appearing as early as 7 days after gavage and the mutation *rpsD Q54L* around 27 days post colonization (**Figure 2**). The appearance of the *rpoC* mutation coincides with the fluorescent marker dynamics favoring the YFP background, strongly suggesting that the *rpoC A784V* was under positive selection shortly after its appearance. This mutation rose in frequency until day 17, after which it suffered small fluctuations until around day 29, suggesting clonal interference between the lineage bearing this mutation and others, possibly carrying adaptive mutations. Between day 29 and 39, the frequency of *rpoC A784V* declined while *rpsD Q54L* rose in frequency. As the frequency of the first mutation decayed after the appearance of the second, clonal interference between the two compensated lineages might have ensued. For the second cohort, we did not detect any mutation on the selected regions. While we did not extend our analysis to other classical compensatory targets, the absence of compensatory mutations in these loci together with the whole genome sequencing data support the idea that compensatory mutations were not favored by selection in this specific mouse.

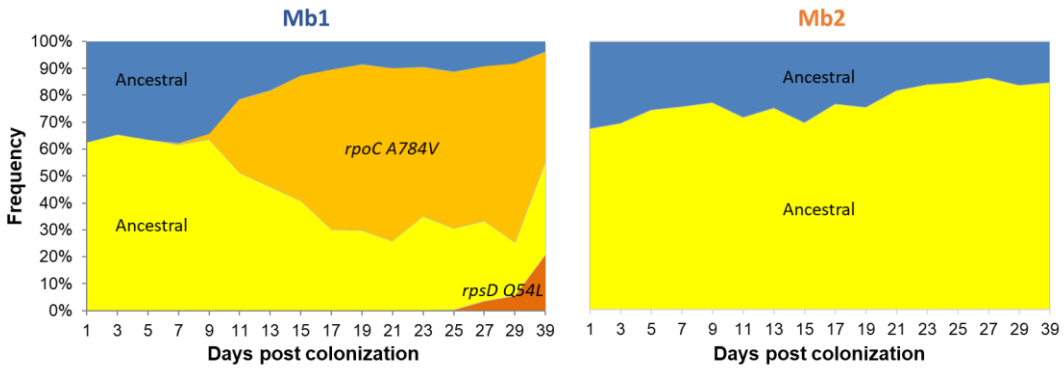


Figure 2 – Frequency over time of candidate compensatory mutations for the double mutant in litter 1 and litter 2. Dynamics suggest clonal interference between clones carrying distinct compensatory mutations in litter 1. No compensatory mutations in these loci were detected in litter 2.

Within-host evolution did not lead to reversion of resistance

Unlike other studies regarding *E. coli*'s *in vivo* adaptation [395,398,411,452], ours does not involve a continuous antibiotic treatment, and evolution is occurring in a streptomycin and rifampicin cleared environment. As such, lethal selective pressures favoring resistance phenotypes are absent, and phenotypic reversions are allowed to occur. To look out for these rare phenomena during the evolution of resistant strains, we have performed a phenotypic reversion test by selecting single clones from within the evolved samples and test them for antibiotic resistance to streptomycin, rifampicin and both drugs together. All of the sampled colonies maintained their resistance profiles – no phenotypic reversions were found and, as expected, none of the single mutants acquired resistance to the other antibiotic (**Table S2**).

Discussion

Microbiota effects on the onset of compensatory mutations

Compensatory mutations are background-specific and ameliorate the fitness of a resistant background by counteracting the phenotype of the resistance mutation. Therefore, the onset of compensatory mutations should be intimately dependent on the presence or absence of a fitness cost of resistance. While performing an evolution experiment in commensal *E. coli* strains carrying resistance mutations, we have observed an earlier onset of compensation in one cohort of hosts when compared with the other, for all tested resistant backgrounds. Both cohorts belong to an inbred, isogenic line of mice, and as such, the only known biological difference between the cohorts lies in their gut microbiota composition, which in turn determines the niches under inter-species competition. The cohort in which compensatory mutations occurred early had a similar microbiota composition across mice. The inter-species competition driven by those microbiota communities may have led to the expression of a fitness cost of our selected antibiotic resistance mutations, prompting a selective pressure towards compensation.

The cohort with earlier compensation had an over-representation of the *Akkermansia* genus. The single species of this genus known to be present in the mammalian intestine, *Akkermansia muciniphila*, is a Gram-negative, anaerobic intestinal mucin-degrading bacteria [456,457]. *A. muciniphila* has a relevant relationship with the mammalian host, triggering host immune and metabolic responses and leading to an increased mucus thickness and gut barrier function [458]. Additionally, it releases nutrients from mucin and produces short-chain fatty acids, beneficial to the host and the other microbiota members [458]. Hence, *Akkermansia* modifies the intestinal niche, impacting the microbiota ecology. The levels of *A. muciniphila* are negatively correlated with different diseases, such as IBD [459], ulcerative colitis [460], obesity [461–463], and diabetes [464]. While the role of *A. muciniphila* in the prevention or

mitigation of these diseases is not fully understood, *A. muciniphila* could act as a reporter of the host's gut health. Under this assumption, the shared microbiota composition in first cohort might reflect a "healthier" microbiota state, in which the typical functions of gut commensals were less affected (resistance) by or recovered faster (resilience) from the antibiotic perturbation [465].

It is tempting to wonder if *Akkermansia* on its own could be interacting with resistant *E. coli* in such a way that resistance would bring a cost. However, our resistant strains express a cost on their own in the absence of microbiota (**Chapter II**) — in the presence of a naïve immune system and without interspecies competition. As in SPF mice, the cost varies in dysbiosis (**Chapter II**), the absence of cost in particular hosts might derive from imbalanced interspecies competition together with the ablation of cross-feeding, which on its own leads to the loss of nutritional niches and affects host-microbe interactions [420]. A potential role of *Akkermansia* is likely to be related with its modulation of the immune system and with its ability to support other bacteria, leading to the restoration of alternative nutritional niches and providing competitive challenges for which wild-type and compensated strains are more fit to face.

Regarding other taxa, we have observed differences in composition between cohorts within the Firmicutes phyla, and these could also have contributed to the low level of parallelism and to the variable onset of compensatory mutations. Furthermore, we are not taking into account variation at lower taxonomic levels nor polymorphisms within the resident bacteria. As a single gene loss or gain of function can change drastically a strain's competitive ability in the mouse gut [397,398], intra-species variation might underlie phenotypes that interfere with the cost of antibiotic resistance mutations and influence which adaptive and compensatory mutations are favored by selection.

Predictability of antibiotic perturbation

Our experiment involves a microbiota perturbation which typically decreases diversity [417,418,432,466,467]. Even so, the two cohorts could be grouped and distinguished through 16S metagenomic analysis, with one of the cohorts presenting strikingly similar microbiota composition between the different mice after antibiotic treatment and prolonged colonization with our resistant strains. This mark of identity suggests that some microbiota communities might react to antibiotic perturbation in a predictable way. Accordingly, a recent study following the microbiota composition in healthy human volunteers shows a consistent association between the presence of a *Bacteroides* enterotype and lower initial microbiome diversity before treatment with the bloom of the opportunistic pathogen *Enterobacter cloacae* after exposure to the antibiotic [468]. These findings highlight a link between the microbial community before and after perturbation and also suggest a degree of predictability. Success at predicting individual consequences of harsh microbiota perturbations such as antibiotic medication would be an extraordinary asset to select appropriate, personalized treatment and to prevent microbiota related diseases. As the cost of antibiotic resistance and the evolution of resistant strains seem to be associated with microbiota, these predictions could also be useful to minimize the rise of antibiotic resistant bacteria as well.

Nature of compensatory mutations

In the both single and double streptomycin resistant background, we have observed the occurrence of mutations in classic compensatory targets, namely, in *rpsL*, the gene containing the antibiotic resistance mutation, and in other genes encoding for ribosomal subunits, *rpsD* and *rpsE*. Streptomycin resistance mutations can confer a restrictive phenotype, which is characterized by an increased rate of proofreading, leading to a higher translation accuracy and a lowered peptide

elongation rate [240,256]. Effectively, the equivalent to our *rpsL K43T* mutation has been shown to decrease peptide elongation rate in a different strain of *E. coli* [256], besides conferring a high cost in minimal medium - a similar fitness phenotype as ours. While we did not test for peptide elongation rate in our single streptomycin resistant mutant, it is very likely that it corresponds to a restrictive phenotype. Compensatory mutations targeting the ribosome can restore the elongation speed and error rate of streptomycin resistant mutants [190,240], and the restoration of this phenotype is typically associated with a fitness recovery towards the wild-type's [240,256]. Our mutations in classic compensatory targets could, therefore, be restoring the ribosome translation speed and accuracy in order to abolish the pleiotropic effects of the resistance mutation.

We have observed rare putative compensatory mutations in the form of intra-gene deletions, in a region of the *rpoB* gene that is not usually associated with compensation. The locus is located in a eubacteria-specific, non-essential region of the gene [469,470], which is thought to be an exposed loop, allowing the flanking domains to interact and perform the enzyme's catalytic function [469]. The deletions allow the maintenance of essential regions of the *rpoB* gene in frame, presumably to not hinder RNA polymerase's essential functions. A potential compensatory role of the deletion could be adjusting the distance between the flanking RNA polymerase domains. Curiously, resistance-conferring indels can have their fitness cost mitigated by compensatory amino acid substitutions [246]. The process of using a SNP to compensate a length defect is seemingly the evolutionary inverse process of what we have observed. Both findings suggest genome versatility of resistant bacteria, and an extended mutational repertoire by which the fitness cost of rifampicin resistance might be mitigated.

The putative compensatory target *rpsD Q54* has emerged in both single and double streptomycin resistant backgrounds, but *rpoC A784V* was restricted to the double resistant background in our study, as well as in another regarding *in vitro* evolution in LB medium [196]. While *rpoC* mutations are known to compensate the cost of rifampicin resistance [243,258,448], we cannot exclude the possibility of certain alleles

conferring benefits for the double mutant only. In fact, another *rpoC* mutation (*rpoC Q1126K*) was shown to give a fitness benefit to the double mutant, but not to the single rifampicin resistant background, compensating specifically for the genetic interaction between the *rpsL K43T* and *rpoB H526Y* alleles, possibly by being mechanistically linked to transcription-translation coupling [196]. Moving our *rpoC* mutation to the single rifampicin and double mutant backgrounds and measuring their fitness effects would help us understand if it is compensating the cost of the *rpoB H526Y* mutation or of the epistatic effect between the two resistance mutations.

Nature of adaptive mutations

Roughly half of the mutations that we are addressing as adaptive were present in a single resistant background, reflecting a low level of parallelism at the gene level. However, most of the mutation targets were described before in *in vivo* adaptation studies. In the mouse gut, an environment with a degree of structure and a variety of nutritional niches, the existence of multiple paths to a higher fitness is to be expected. Most likely, the adaptive targets were not exhausted in our experiment even after around 40 days of evolution. On the other hand, some of the metabolic targets might be under negative epistasis with each other [395], which would also hinder parallelism for adaptive mutations between populations.

Focusing on metabolic genes, their nature combined with their previous observation suggests an adaptive role. Still, the specific mutations that we observe may be a result of shifted fitness landscapes in the resistant mutants due to the pleiotropy of the resistance genes. As with other rifampicin [239] and streptomycin [435] resistance mutations, *rpoB H526Y* and *rpsL K43T* are likely to alter transcription and translation efficiency (respectively). In such a scenario, the strains can recover their fitness either by compensatory mutations acting on these phenotypes, or by correcting downstream effects of the resistance mutations. The latter hypothesis is particularly plausible for the *tdc* operon and the *spoT* gene.

Our rifampicin resistant mutants have acquired mutations in the *tdcA* gene and in the *tdcA-tdcR* intergenic regions, with the *tdcA D44G* point mutation reaching fixation in one of the mice. *tdcA* is required for the autoregulation of the *tdc* promoter [471], while its expression requires a polypeptide coded by *tdcR* [472]. Assuming that these mutations did not raise in frequency by hitchhiking on others, selection must have acted on the expression levels of the operon. *tdc* encodes for the transport and anaerobic degradation of L-threonine and serine [473,474], allowing their use as a source of energy and cell metabolites, and it is the single operon in *E. coli* MG1655 whose transcription is not blocked in the presence of rifampicin – it is actually upregulated in the presence of the drug, suggesting a distinct regulatory mechanism of transcription [475]. Having this into account, it is possible that our rifampicin resistant mutant has an altered *tdc* expression, or that the expression of the operon relative to the rest of the genome is changed when compared with the wild-type's. The lack of an optimal expression of *tdc* genes, or of genes downstream of *tdc* for *rpoB H526Y* mutants could lead to metabolic imbalances and a cost in the *in vivo* context.

Intriguingly, *spoT* was targeted in both the single streptomycin and single rifampicin resistant backgrounds through distinct point mutations. We are not aware of the effect of these mutations – they may be affecting the two strains' phenotypes in a different way. SpoT regulates the "alarmone" ppGpp, a nucleotide that mediates the stringent response - an adjustment of cellular activity affecting the rate of RNA accumulation, the synthesis and breakdown of metabolites and macromolecules upon amino-acid starvation [476]. The nucletide ppGpp binds directly to RNA polymerase to elicit the response [477], but certain *rpoB* mutants have been shown to have a stringent phenotype even in the absence of ppGpp [477–479]. The effect of ppGpp could be affected in our rifampicin resistant mutant, and in such a situation, adjusting the levels of ppGpp through a *spoT* mutation could be beneficial.

On the other hand, a molecular study shows that ppGpp can affect the accuracy of translation by managing the ratio of different tRNA [480]. The mutation in *spoT* could therefore be indirectly compensating phenotypic effects of the *rpsL K43T* mutation on translation accuracy. Furthermore, spoT is necessary to induce the transcription

of iraP, a stabilizer of rpoS [481], the master regulator of the general stress response in *E. coli* [482]. Strikingly, in *Salmonella enterica* var. Typhimurium, a streptomycin resistance mutation on an equivalent locus (*rpsL K42N*) leads to a lower production of the rpoS protein on its own [271]. It is possible that the regulation of starvation or other stress responses is also affected in our resistant strains, requiring an adjusted spoT activity in stressful or resource-depleted environments to "compensate" a lower level of rpoS. Notably, *spoT* mutations occurred in litter 1 only, despite occurring in two distinct genetic backgrounds. As the microbiota composition is remarkably similar within this cohort, a need to adjust the response to stress and/or starvation might have emerged due to inter-species competition. Curiously, in the rifampicin mutant, the *spoT L103F* point mutation has appeared and risen sharply in frequency between day 19 and 39 (Table 1), while the putative compensatory *rpoB* deletion has lowered in frequency drastically, suggesting a stronger beneficial effect of the *spoT* mutation. Clonal interference between lineages may have led to these inverted dynamics, but a change in the gut's selective pressures between the two time-points cannot be discarded.

Some of the "adaptive" targets are related by nature with our resistance mutations. The *rrfH* gene codes for 5S ribosomal RNA, an RNA component of the large subunit of the ribosome [483]; clpX is a molecular chaperone and part of the clpXP and clpAXP protease complexes [484], which are related with viability in stationary phase [485]. rimJ is an acetyltransferase acting on the 30S ribosomal protein S5, which is itself coded by the compensation target gene *rpsE* [486], and *mukF*, found at low frequencies in the double mutant background, codes for bacterial condensin, which is involved in chromosome partition during cell division [487]. Each of the targets was found in a single resistant background, and while these genes have not been previously reported as compensatory mutations, we cannot fully exclude the possibility of them conferring background-specific fitness benefits in the gut environment. Growth condition-specific compensation targets were previously observed in the pathogen *Salmonella enterica* var. Typhimurium [191], so it would not be surprising to uncover new targets in our commensal *E. coli*. Placing these

mutations in clean backgrounds, both sensitive and resistant, and assessing their fitness *in vivo* could help exclude or reveal them as additional compensatory targets of resistance.

The rarity of fixation

In our study, a single adaptive mutation reached fixation during our evolution experiment. Furthermore, we have not observed fixation of any classical compensatory mutation. Our observations suggest an overall pattern of clonal interference, not only between putative compensatory mutations, but also between these and adaptive mutations. In other gut evolution studies involving *E. coli* K12, numerous adaptive mutations are detected, and as in here, most of the acquired mutations do not fix [395,398]. Hence, the low rate of fixation seems to be a common feature in the *in vivo* adaptation of this *E. coli* K12 background.

Bacteria can find a multitude of nutritional niches in the gut [488], and these niches are thought to be under intense resource competition [489]. Cross-feeding interactions are a feature of the gut environment [490], contributing to a higher complexity of its ecology. Thus, it is not surprising that multiple paths towards a higher fitness are available in such an environment. As multiple selective pressures are involved, some of them implying trade-offs between adaptive traits and leading inclusively to sympatric diversification [491], unconditionally beneficial mutations might be rare, even in genetic backgrounds crippled by one or more costly resistance mutations.

Absence of reversions

Reversion of the *rpoB H526Y* mutation has been observed *in vitro*, in a single mutant isogenic to ours [196], and *in vivo* reversion of antibiotic resistance to streptomycin [190] and fusidic acid [191] have been previously observed on *Salmonella* infection models. Armed with this knowledge, we queried if a phenotypic reversion in our rifampicin and streptomycin resistant mutants could have occurred during our gut evolution experiment. However, the resistance phenotypes of our strains remained untouched during *in vivo* adaptation. One possibility is that reversions did not occur at all in our strains. Back mutations have a reduced mutational target size, as they imply the occurrence of a SNP in a specific locus, and as a consequence, are expected to be rare. In fact, several studies show that compensatory mutations are far more frequent than reversions [169]. Regarding phenotypic reversions without a back mutation, they typically occur through compensatory mutations that restore the wild-type's phenotype, leading to restored sensitivity [263]. Still, this phenomenon is highly specific. While streptomycin resistance mutations leading to ribosome hyper-accuracy can be reverted by mutations that lead to lowered accuracy in translation, the recovery of sensitivity is restricted to specific alleles [264]. Supporting the rarity of these events, in a study in which genotypic revertants are found during successive infections by streptomycin resistant *Salmonella typhimurium*, none of the second-site compensatory mutations reverted resistance [190].

Alternative explanations for the absence of genotypic and phenotypic reversions imply that they are not favored by natural selection. One possibility is that the reversion itself does not confer a fitness advantage. In Chapter II, we have observed a host-specific cost of resistance, inclusively hosts in which a cost was not found - reversion would not be favored in such scenario. The presence of compensatory mutations in litter 1 suggests that resistance had a cost in litter 1. However, in litter 2, classical compensatory mutations had a late onset, and did not occur at all in the double mutant, suggesting a low or non-existent cost of resistance. In this particular scenario, reverting to the wild-type background would not confer a

fitness benefit. Additionally, compensatory mutations interact epistatically with the resistance locus. If these mutations would confer a fitness benefit only in the presence of the resistance mutation, a negative genetic interaction with the wild-type allele could also impair reversions by selecting against sensitivity in the evolved strains. In fact, compensatory mutations with a deleterious effect in a sensitive background have been described [196], and such mutations would impair reversions.

It should also be noted that we have a limited number of sampled mice. We cannot exclude the possibility that with a higher number of hosts, covering for different microbiota compositions, we could have observed phenotypic reversions of resistance. Still, our results suggest that reversions are rare in the gut environment.

Future directions

Besides reproducing our observation of a variable onset of compensatory mutations, further experiments with a similar experimental design could help to better discern the genetic background-specificity of mutations, which could reveal new targets for compensation [196], as well as litter-specific adaptive mutations, which in turn could hint into host-specific adaptation or even expose epistatic relationships between adaptive mutations [395]. A very recent work has performed the evolution of *E. coli* in germ-free mice [492] and has shown that a specific genetic adaptation in the gene *lrp*, encoding a global regulator of amino-acid metabolism, was predictably selected within two weeks of mono-colonization, and the addition of a single member of the microbiota (*Blautia coccoides*) was enough to alter the gut metabolic profile and *E. coli*'s evolutionary path, further showing reproducibility on *in vivo* systems in which microbiota is absent or controlled. Performing the evolution of each of the resistant backgrounds in germ-free mice, and in conventional mice from the same litter while warranting a similar microbiota composition before and during antibiotic treatment, would let us test the limits of parallel evolution across hosts.

In the previous chapter, we have performed competitions between a double resistant strain and its ancestral, a streptomycin resistant resident *E. coli*. It could be interesting to evolve these strains in the mouse gut as well. The single streptomycin resistant strain is presumably adapted to the gut environment, so further adaptation should reflect either host-specific or microenvironment-specific fitness determinants. The evolution of the double mutant strain however could allow us to evaluate the maladaptive impact of the acquired *rpoB H526Y* mutation in the resident background. Namely, it would be interesting to note if the mutant would evolve mainly through compensation due to the adapted resident background, or if this mutation would expand the adaptive targets of the double mutant, reflecting a shifted fitness landscape due to the mutation's pleiotropic effects.

Supplementary material

Table S1 – *De novo* mutations on AR *E. coli* genetic backgrounds during gut colonization. Genome position, mutation nature and frequency after 3 and 6 weeks of evolution are shown. Mutations in bold occurred in genes described in the literature as targets for compensation. In the gene column, references on mutational gene targets previously described in the literature are indicated after the mutation target.

				Frequency	
Population	**Genome Position**	**Gene**	**Mutation**	**Week 3**	**Week 6**
Str^R 1	1198436	ymfE / lit	+AATGAAAT	12.6%	13.6%
	1466201	ydbA[493]	T→C	8.5%	
	2259422	psuK / fruA[66]	A→C	3.5%	8.5%
	2406600	lrhA	+TCGAGG		8.1%
	2829125	srlR[394]	C→T	2.9%	
	2829468	srlR[394]	C→A	3.9%	11.1%
	3444923	**rpsE**[190,240]	**T→G**	**13.7%**	**15.6%**
	3444925	**rpsE**[190,240]	**C→G**	**36.1%**	**57.8%**
	3474368	**rpsL**[190,240]	**T→G**	**14.4%**	**6.8%**
	3504197	frlR[394]	G→A		10.9%
	3823019	spoT[493]	G→A		51.8%
	4542161	fimE[66]	IS5 +4 bp		60.8%
	4542457	fimE[66]	IS1 +9 bp		12.2%
	4640748	yjjY → / → yjtD[395]	IS5 +4 bp		3.3%
Str^R 2	228767	rrfH	C→A	5.2%	

	457912	clpX	IS1 +9 bp	11.7%	
	570603	ybcK → / → ybcL	IS2 +5 bp	2.6%	14.9%
	972965	elyC	G→T		11.5%
	973071	elyC	IS1 +9 bp		25.2%
	973103	elyC	IS5 +4 bp	4.1%	25.1%
	973108	elyC	G→T		7.7%
	1466210	ydbA[493]	G→A		5.0%
	1466438	ydbA[493]	T→G		5.0%
	1909535	kdgR[394]	G→A	11.7%	21.3%
	2765463	yfjL ← / ← yfjM	+TATGGCAC		29.4%
	2773761	yfjW	T→C		5.5%
	2829065	srlR[394]	C→T		9.8%
	2829207	srlR[394]	G→A		4.4%
	3441515	**rpsD**[190,240]	**T→C**		**20.2%**
	3444862	**rpsE**[190,240]	**A→G**		**52.1%**
	3444971	**rpsE**[190,240]	**C→T**	**7.5%**	
	4542308	fimE[66]	IS5 +4 bp	2.1%	26.5%
	4542308	fimE[66]	IS5 +4 bp	10.2%	
	4542577	fimE[66]	IS1 +10 bp	61.3%	63.5%
	4640605	yjjY → / → yjtD[395]	IS2 +5 bp		11.3%
Rif^{R} 1	1198437	ymfE ← / → lit	Δ8 bp		5.6%
	1264717	hemA	G→T		8.6%
	2765412	yfjL ← / ← yfjM	Δ8 bp	1.8%	
	3266935	tdcA	T→C	5.7%	
	3266993	tdcA	C→T		100.0%
	3267147	tdcA ← / → tdcR[394]	IS5 +4 bp		3.8%

	3504139	frlR[394]	C→T		10.4%
	3822706	spoT[493]	C→T		76.1%
	4184101	**rpoB**[196,450]	**Δ39 bp**	**46.7%**	**6.0%**
	4439596	ytfK	T→A	5.0%	
Rif^{R} 2	954669	focA ← / ← ycaO[394]	IS5 +4 bp	45.5%	52.9%
	954678	focA ← / ← ycaO[394]	IS5 +4 bp		23.7%
	1125778	rimJ[394]	IS1 +9 bp		34.3%
	1198460	ymfE ← / → lit	+AATGAAAT		8.5%
	1466432	ydbA[493]	A→G	4.6%	
	1466438	ydbA[493]	T→G	4.7%	
	1909925	kdgR[394]	IS5 +4 bp		5.0%
	1910031	kdgR[394]	IS2 +5 bp	2.5%	9.4%
	2765411	yfjL ← / ← yfjM	+GCACTATG		30.4%
	2829687	srlR[394]	A→C	12.4%	16.2%
	3267147	tdcA ← / → tdcR[394]	IS5 +4 bp		3.8%
	4184117	**rpoB**[196,450]	**Δ24 bp**		**8.6%**
	4348862	dcuB ← / ← dcuR[394]	IS5 +4 bp	3.2%	7.4%
	4349082	dcuB ← / ← dcuR[394]	IS2 +5 bp		15.0%
Str^{R} Rif^{R} 1	533245	allR	T→G	5.6%	6.7%
	974859	mukF	Δ3 bp	5.2%	
	3267149	tdcA ← / → tdcR[394]	Δ2 bp	55.5%	
	3441515	**rpsD**[190,240]	**T→A**		**9.4%**
	3504101	frlR[394]	G→T	15.2%	8.2%
	3504565	frlR[394]	C→T	3.2%	
	4187700	**rpoC**[196,450]	**C→T**	**63.2%**	**42.6%**
Str^{R} Rif^{R} 2	974859	mukF	Δ3 bp		1.9%

	1466438	ydbA[493]	T→G		5.1%
	2259416	psuK ← / ← fruA[66]	+AA		3.4%
	3150816	yghA → / ← exbD	C→T		6.2%
	4348862	dcuB ← / ← dcuR[394]	IS5 +4 bp	12.2%	14.2%
	4348971	dcuB ← / ← dcuR[394]	Δ98 bp	1.3%	4.2%
	4348988	dcuB ← / ← dcuR[394]	IS2 +5 bp	11.6%	7.5%
	4349082	dcuB ← / ← dcuR[394]	IS2 +5 bp		1.4%
	4349103	dcuB ← / ← dcuR[394]	IS2 +5 bp		20.5%
	4603111	yjjP ← / → yjjQ	IS5 +4 bp		2.9%
	4603111	yjjP ← / → yjjQ	Δ2 bp		1.4%

Table S2 – Time-point sampling to detect phenotypic reversions during the evolution experiment. Stool samples were diluted and grown in LB with chloramphenicol to select *E. coli* strains. Random colonies were replicated into LB plates with streptomycin, with rifampicin, with both drugs and with no antibiotics. No reversions or gain of double resistance was observed, meaning that all strains maintained their resistance profile in the mammalian gut.

Population/litter	**Day 11**	**Day 17**	**Day 19**	**Day 25**	**Day 29**	**Day 39**	**Total**	**Reversions**
Str^R 1	102	153		152	113	115	**635**	**0**
Rif^R 1	110	100		96	96	96	**498**	**0**
$Str^R Rif^R$ 1	78	135		132	114	102	**561**	**0**
Str^R 2			144				**144**	**0**
Rif^R 2			96				**96**	**0**
$Str^R Rif^R$ 2			144				**144**	**0**

CHAPTER IV

Frequency-dependent selection acting on secretome polymorphisms

Author contributions for this Chapter:

Isabel Gordo and I designed this study. I have performed the competitions, the growth-curves and the polymorphism stability assay. The execution was supervised by Isabel Gordo.

Abstract

The fitness effect of a mutation is a key factor in its spread. In most studies, fitness effects are measured either by comparing the growth rate of the mutant with a reference strain or by direct competition between the two in a 1 to 1 ratio. However, most ecological interactions are dynamic, changing selection according to the density and frequency of the genotypes. Still, most studies do not test for frequency dependence, overlooking potential bacterial interactions with an impact on fitness. Despite being associated with cross-feeding and leaky functions, it is not clear how common frequency dependent selection is. Here, we query about the existence of frequency dependent selection for polymorphisms in distinct cellular functions, and we do find a relationship between secretome functions and magnitude frequency-dependent selection. Furthermore, to enquire for polymorphism stability in a natural environment, we have competed single gene deletion mutants lacking these functions and the respective wild-type strain in the mouse gut. A single mutant strain dominated the competition hinting that in the gut, strong Darwinian selection prevails over polymorphisms maintenance for leaky functions.

Introduction

When a new mutation occurs in a population, it does so at low frequency. The frequency of the new strain will then change, leading either to loss of the mutant, to its fixation or to its maintenance in a polymorphic population. While stochastic fluctuations, termed as “genetic drift”, can have a powerful role in the fate of new mutations, selection acts as an effective modifier of the frequency, benefiting the spread of mutations that confer a fitness advantage and hindering deleterious ones [162]. The fitness effect of a mutation is thus a key factor in its spread.

In most studies with bacteria, the fitness effect of a mutation is typically measured in one of two ways. The first consists in measuring absolute fitness through the growth of the mutant strain, the estimation of its growth parameters, and the comparison of its growth-rate with a reference strain [e. g. [261,271,494,495]]. While this method provides a proxy of fitness, other growth parameters contribute to it [496], and even when taking life-history parameters like the lag phase and the carrying capacity into account, the isolated growth does not integrate the parameters into a single competitive index, nor does take into account particular phenotypes that could foster interactions between strains competing directly. Addressing these limitations, the second widely used approach consists in 1:1 ratio, direct competitions between the mutant and a reference strain [e. g.[192,497–499]], typically the isogenic wild-type background. These competition assays require a way to distinguish the strains (typically through auxotrophy, resistance or fluorescence markers) but allow integration of the differences across the full growth cycle [500], as well as putative interactions between the mutant and the reference strain. The 1:1 ratio is used because it allows the maximization of differences between the competing genotypes under the assumption of constant selection. However, most ecological interactions are dynamic, depending on the number of individuals and the proportion of each strain in the population and affecting fitness according to the density and frequency

of the genotypes [501,502]. Still, in the absence of *a priori* expectations, most fitness studies do not take into account putative frequency-dependent events.

Frequency-dependent selection is thought to be one of the main selective mechanisms through which diversity can be maintained [503,504]. Negative-frequency dependent selection (NFDS) in particular, also known as stabilizing frequency dependent selection, is a form of selection in which the selective value of a variant is a function of its frequency in the population, in such a way that a variant has a fitness advantage when rare and a fitness disadvantage when common, and can lead to a stable coexistence between variants [501,504].

Frequency-dependent polymorphisms can be generated in clonal bacterial populations through evolution, even in simple, constant laboratory environments through cross-feeding [505] or adaptive radiation for the consumption of resources [506], but they have also been shown to occur in complex environments such as the mammalian gut, through metabolic adaptation [395]. It is expected to occur for genes within the accessory genome [507], and there is evidence that leaky, public good-related functions in particular can lead to frequency dependence [508,509].

Despite the importance attributed to frequency dependent selection in the maintenance of polymorphisms, it is not well known how common it is, and how prone are distinct cellular functions, to be under this type of selection. To address these questions, we performed pair-wise competitions between a set of mutants, each carrying a single gene deletion for different functions, including core genes encoding for inner cell mechanisms to accessory genes implied in functions outside of the cell, against their isogenic wild-type at different frequencies, to look out for frequency-dependent effects. We have also competed a subgroup of mutants, lacking leak-prone genes, and the wild-type strain in the mammalian gut to enquire for polymorphism stability in a natural environment.

Methods

Escherichia coli and mice strains

For our experiments, we used fluorescence-labeled, *E. coli* K-12 MG1655-derived strains, bearing single gene deletions of genes covering for different functions, ranging from core cellular processes to secretion. The list of genes can be found in the following table.

Table 1 – *E. coli* strains used in this study and the corresponding genotype.

Strain	Genotype
JB 77	*ΔlacIZYA::scar gatZ::IS1 galK::cat-YFP rpsL K43R*
JB 78	*ΔlacIZYA::scar gatZ::IS1 galK::cat-CFP rpsL K43R*
LC6	JB78 *ΔsrlR::FRT-kan-FRT*
LC7	JB78 *ΔtnaA::FRT-kan-FRT*
LC27	JB78 *ΔyliH::FRT-kan-FRT*
LC29	JB78 *ΔtalB::FRT-kan-FRT*
LC31	JB78 *ΔrpsT::FRT-kan-FRT*
LC32	JB78 *ΔfkpB::FRT-kan-FRT*
LC34	JB78 *ΔhepA::FRT-kan-FRT*
LC39	JB78 *ΔcadA::FRT-kan-FRT*
LC40	JB78 *ΔcadB::FRT-kan-FRT*
LC41	JB78 *ΔcadC::FRT-kan-FRT*
LC46	JB78 *ΔkefC::FRT-kan-FRT*
LC52	JB78 *ΔtbpA::FRT-kan-FRT*
LC60	JB78 *ΔpqqL::FRT-kan-FRT*
RB370	JB78 *ΔhlyE::FRT-kan-FRT*
RB372	JB78 *ΔhtrE::FRT-kan-FRT*
RB374	JB78 *ΔlamB::FRT-kan-FRT*
RB376	JB78 *ΔcbrA::FRT-kan-FRT*
RB378	JB78 *ΔgspK::FRT-kan-FRT*
RB380	JB78 *ΔompG::FRT-kan-FRT*
RB382	JB78 *ΔyebF::FRT-kan-FRT*
RB384	JB78 *ΔkatG::FRT-kan-FRT*
RB414	JB78 *Δagp::FRT-kan-FRT*
RB416	JB78 *ΔcitT::FRT-kan-FRT*
RB418	JB78 *ΔdcuB::FRT-kan-FRT*
RB422	JB78 *ΔidnD::FRT-kan-FRT*
RB424	JB78 *ΔkatE::FRT-kan-FRT*
RB426	JB78 *Δdps::FRT-kan-FRT*
RB428	JB78 *ΔentE::FRT-kan-FRT*

The mutants were constructed through P1 transduction [403], having as donors strains from the KEIO collection [404], which includes precisely defined, single gene knock-outs of all non-essential genes in *E. coli* K12. The genes are replaced by a kanamycin resistance cassete, which we used as a selective marker for the

transduction. Clean, single gene deletions were chosen as mutations because they allow the full abolishment of a gene's function. On this chapter, JB77 will be considered as the wild-type, while individual mutant strains for a matter of convenience, will henceforth be called by their deleted gene.

We have purposely included genes encoding for periplasm and outside of the cell functions (referred as "secretome" in this chapter), which are more likely to be involved in interactions, namely the genes *agp*, *entE*, *gspK*, *hlyE*, *pqqL*, *tbpA* and *yebF* , as well as the ones encoding *E. coli* peroxidases *katE* and *katG*, as it was previously shown that in a dependency context, a peroxidase producer and a beneficiary could show frequency dependent selection [509] We have also included 3 genes from the same operon: *cadA*, coding a lysine decarboxilase, *cadB*, a lysine transporter and *cadC*, the *cadBA* transcription regulator, as the dynamics of a long term evolution experiment in the mouse gut suggest that this operon is under frequency-dependent selection [396]. Some of the remaining genes are involved in nutrient metabolism and host adaptation, and while they could lead to frequency dependent selection via differential resource competition in complex environments such as the mammalian gut, they are not expected do so in a single carbon source, well mixed, *in vitro* environment. Our selection comprises both core and accessory genes, with a wide range of representation within the sequenced *E. coli* genomes (**Table 2**). Although some of these genes went through duplication events, none is duplicated in *E. coli* K12 MG1655 (checked through megablast in https://blast.ncbi.nlm.nih.gov/Blast.cgi).

6-to-8 week-old C57BL/6J specific pathogen free (SPF) mice (2 male and 2 female) were used for the *in vivo* competitions.

Table 2 - Genes under study, their frequency in sequenced *E. coli* genomes and their described function. In bold are core genes, which are present in all strains and for which phylogenetic data does not suggest deletion or duplication events. Frequency and phylogenetic data were obtained through the PanX pipeline [510], and the gene annotation was collected through the EcoCyc database [511].

Gene	Frequency	Annotation
agp	0.98	Periplasmic acid glucose phosphatase
cadA	0.94	Lysine decarboxylase CadA
cadB	0.92	Cadaverine/lysine antiporter
cadC	0.92	CadBA transcriptional regulator
cbrA	0.99	Flavin adenine dinucleotide protein, colicin M resistance
citT	1.00	Citrate carrier
dcuB	1.00	Anaerobic C4-dicarboxylate transporter
dps	1.00	DNA starvation/stationary phase protection protein
entE	1.00	Enterochelin synthase subunit
fkpB	1.00	Peptidyl-prolyl cis-trans isomerase
gspK	0.54	Pseudo pillin of the type II secretion system
hepA	0.99	RNA polymerase-associated protein
hlyE	0.67	Hemolysin E
htrE	0.79	Outer membrane usher protein
idnD	0.42	L-idonate 5-dehydrogenase
katE	0.99	Catalase-peroxidase HPII
katG	1.00	Catalase-peroxidase HPI
kefC	0.99	Potassium-efflux system protein
lamB	1.00	Maltoporin
ompG	0.95	Outer membrane protein G
pqqL	0.96	Zinc protease
rpsT	1.00	30S ribosomal protein S20
srlR	0.99	Sorbitol operon repressor
talB	1.00	Transaldolase 1
tbpA	0.99	Thiamine binding periplasmic protein
tnaA	0.99	Tryptophanase
yebF	1.00	Protein carrier to the outside of the cell
yliH	1.00	Biofilm regulator BssR

In vitro competitions and selection coefficient calculation

In order to access the extent of frequency dependent selection in a simple environment, we performed pairwise competitions between the mutants and the wild-type strain with 3 different mixing frequencies: 1:9, 1:1 and 9:1 mutant to wild type ratio. For future reference, I will be calling these mixing treatments as low, average and high frequency, respectively.

The strains were grown in minimal medium with glucose [M9 salts, $MgSO_4$ 1 mM, $CaCl_2$ 0.1 mM, glucose 0.4% (w/v)] as an acclimatization step. This medium composition was used in all *in vitro* experiments on this chapter. The cell number for each strain was then assessed using fluorescent associated flow cytometry. The strains were then diluted and mixed together in fresh medium order to have a total of 10^6 cells in 200 µl of culture medium, with the mutant either in low, average or high frequency. The mix was sampled through flow cytometry and further incubated at 37ºC with agitation. After 24 hours, the mix was sampled again, propagated into fresh media (5 µl suspension in 200 µl fresh medium) and grown again in the same conditions. This procedure was repeated every 24 hours until 4 days after mixing, in which the propagation was stopped. To determine the ratios of mutant and wild-type strains, bacteria numbers were quantified with an LSR Fortessa flow cytometer using a 96-well plate autosampler. Samples were always run in the presence of SPHERO (AccuCount 2.0-µm blank particles) in order to accurately quantify bacterial numbers in the cultures. Briefly, flow cytometry samples consisted of 180 µl of PBS, 10 µl of SPHERO beads, and 10 µl of a 100-fold dilution of the bacterial culture in PBS. The bacterial concentration was calculated based on the known number of beads added. Cyan fluorescent protein (CFP) was excited with a 442-nm laser and measured with a 470/20-nm pass filter. Yellow fluorescent protein (YFP) was excited using a 488-nm laser and measured using a 530/30-nm pass filter. Yellow fluorescent protein (YFP) was excited using a 488-nm laser and measured using a 530/30-nm pass filter.

As in most competitions the dynamics were shifted in the first day compared with the following days, we considered the first 24 hours as an additional

acclimatization step, namely to the new environment with two strains, and used the day 1 to day 4 measurements to calculate the selection coefficients. The selection coefficients per day were estimated through the slope of the linear regression of the logarithm of the ratio of the mutant to the reference strain. For all mutants, the coefficients in high and low frequency were compared through a T test assuming homogenous variance. Mutants for which the mean difference was statistically significant were considered to be under frequency-dependent selection. In each treatment, mutations for which the average fitness effect surpassed 2 times the standard error were considered to have an effect. If not considered as frequency dependent, mutations that had a fitness effect in only one or two frequency treatments were considered as beneficial if all of the individual slopes in all treatments were positive, deleterious if all slopes in all treatments were negative, otherwise they were considered neutral. A minimum of 3 biological replicates were used to measure fitness for each mutant in each treatment. For each biological replicate, the experiment was performed separately. Wild-type YFP / CFP competitions did not disclose significant differences in fitness between the two fluorescent backgrounds [$S_{cfp,\ low}$= 0.03 (±0.04 2x standard error), $S_{cfp,\ avr}$=0.03 (±0.06) $S_{cfp,\ high}$=-0.01(±0.02); $p_{cfp,\ low}$ =0.27, $p_{cfp,\ avr}$=0.1, $p_{cfp,\ high}$=0.96, One sample T-test].

Growth curves

The strains were grown in minimal medium with glucose as an acclimatization step. The cell numbers were then assessed using fluorescent associated flow cytometry. For each strain, approximately 10^6 cells were transferred to 200 ul of fresh medium, in a honeycomb well plate. The plate was then taken into the Bioscreen, and the OD at 600 nm wavelength was measured every 30 minutes, for 24 hours. After subtracting the OD value for the blank wells, the data was analyzed using the GrowthRates R package (https://cran.r-project.org/web/packages/growthrates/index.html) to estimate the maximum growth rate (μ) and

lag phase (λ). The carrying capacity (K) was estimated directly by asserting the highest OD in each sample. The parameters were each analyzed through a one-way ANOVA with Dunnett's test for multiple comparisons, using the wild-type as a reference treatment for comparison. It was not possible to run all the samples in a single honeycomb plate. To minimize circumstantial differences between plates, the wild-type was grown in each plate, and the statistics were performed using the respective wild-type growth. 3 biological replicates per strain were used to estimate the growth parameters.

In vivo competition

Prior to the experiment, we used an antibiotic treatment in order to break the colonization resistance [405]. Mice were transferred into individual cages and given autoclaved drinking water containing streptomycin sulfate (5g/L) for seven days, and then were given regular autoclaved drinking water for 2 days. After 4 hours of starvation for food and water, the mice were gavaged with 100 µl of a ≈10^9 cells/ml suspension with a 1:7 ratio of the 8 competing strains (each with ≈12.5% frequency).

To make the suspension, the strains were streaked from stocks in LB agar with chloramphenicol (30µg/ml) two days before gavage and incubated for 24 hours, followed by an overnight culture of a single colony for each biological replicate in BHI (brain heart infusion) medium supplemented with chloramphenicol (30µg/ml). The cultures were then diluted 100-fold and grown in BHI medium until an OD_{600nm} ≈ 2. The cells were then resuspended in PBS. Following the gavage, mice fecal pellets were collected twice per day, starting at 6 and 24 hours after gavage for a week, keeping the daily schedule (30 and 48 hours post gavage, and so on). Each time, the cells were suspended and homogenized, diluted in PBS and plated in LB agar plates supplemented with chloramphenicol (30µg/ml). The plates were incubated overnight and the frequencies of CFP and YFP-labeled bacteria were assessed by counting the fluorescent colony forming units (CFU) with the help of a fluorescent stereoscope

(SteREO Lumar, Carl Zeiss). The samples were also stored in 15% glycerol at -80°C for future experiments. For the last time-point (174 hours after gavage), random colonies were picked to perform colony PCR against mutant-specific primer pairs (**Table 3**), in order to identify the mutants which were present at the end of the competition.

Table 3 – **Primers used in this study.**

Primer name	Sequence	Primer homology
LC9	ATGCCTGCTTGCCGAATATCATG	KanR end forward
LC 10	AAGCCACACTGAACAAGATCGC	upstream *hlyE*
LC11	GTAAGTCAGCGATGAATCAGCAACG	upstream *gspK*
LC12	AGGTTGTCGTCATCGAACGG	downstream *entE*
LC14	TGTGGCAGAATAACGGCAGAAG	upstream *yebF*
LC 16	AGATGCCAGAAGCACACCAC	upstream *tbpA*
LC17	GATTGTCTGTTGTGCCCAGTCATAG	KanR start reverse
LC 19	CCGTACTTTCTGGCTTGATGTCAG	upstream *pqqL*
LC 21	TCCTGCAACAGCTTCTTAGCG	downstream *agp*

LC9 amplifies with LC12 in the presence of *ΔentE*, LC 19 in the presence of *ΔpqqL* and LC21 in the presence of *Δagp*; LC17 with LC10 in the presence of *ΔhlyE*, LC11 in the presence of *ΔgspK*, LC14 in the presence of *ΔyebF* and LC16 in the presence of *ΔtbpA*. These primers can produce non-specific, faint bands in the context of a multiplex, so we had to perform regular PCR to identify the mutants.

A fraction of each colony was suspended in 20 µl of MilliQ water. PCR reactions were run with the DreamTaq polymerase, following the standard protocol (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012037_DreamTaq DNAPolymerase_5x500U_UG.pdf), using a 1µl of the colony suspension as template and a 3 minutes long initial denaturation step.

Results

Secretome deletion polymorphisms show magnitude frequency dependent selection

To enquire how likely it is for frequency dependent selection to arise through a single gene polymorphism, we competed *E. coli* strains carrying single gene deletions for different cellular functions against the wild-type strain, in 3 different initial frequencies. We performed these competitions in a simple environment with a single carbon source, in which phenotypic differences should lead to competitive exclusion in the absence of strain interactions.

We have observed magnitude frequency-dependent selection for 6 out of 28 mutations (21%) (**Figure 1A**). For these mutations, the selection coefficient per day at low frequency was significantly higher than in high frequency. The mutations *Δagp*, *ΔhlyE* and *ΔgspK* conferred a fitness benefit throughout the tested frequencies, while the mutant *ΔsrlR* had a slight fitness benefit in low frequency only. The mutations *ΔentE* and *ΔtbpA* brought a fitness cost at high frequency. Both seemingly had no cost at low frequency. Despite these results, we did not observe a change in the sign of the selection coefficient, which would denounce negative frequency-dependent selection. It is worth noticing that 5 out of the 6 genes with frequency-dependent selection code proteins that are either secreted or involved in secretion, with the incidence of frequency-dependent selection being significantly higher in this subset of genes than in the remaining genes (p=0.0003, Fisher's two-sided exact test). Furthermore, although the two remaining mutants in the secretome class within our selected mutants, *ΔyebF* and *ΔpqqL*, did not show a significant fitness difference between high and low frequency, their average fitness does decrease with the frequency as well (table 2), further suggesting that the leakiness of these functions leads to frequency dependence. Although we have observed a deleterious effect due to the absence of the peroxidases KatE and KatG, we did not observe a significant

difference in the fitness effects of these mutations between the competitions in low vs high frequency (p_{katE}= 0.55; p_{katG}=0.68, T test for homoscedastic variances).

Regarding the mutants that do not show frequency dependent selection, 6 out of 22 mutations (27%) confer a constant fitness advantage in direct competition with the wild-type. 6 (27%) bring a constant cost, while 10 (45%) seem to not affect the competitive fitness (**Figure 1B-C)**. The strains are competing in a simple environment, which was not expected to provide mutant-specific benefits. Therefore, the commonness of beneficial effects of the single gene deletions is unexpected. Focusing on the cadaverine cluster, removing the promoter had a beneficial effect on fitness, while the removal of the downstream transporter and lysine decarboxylase genes had milder effects.

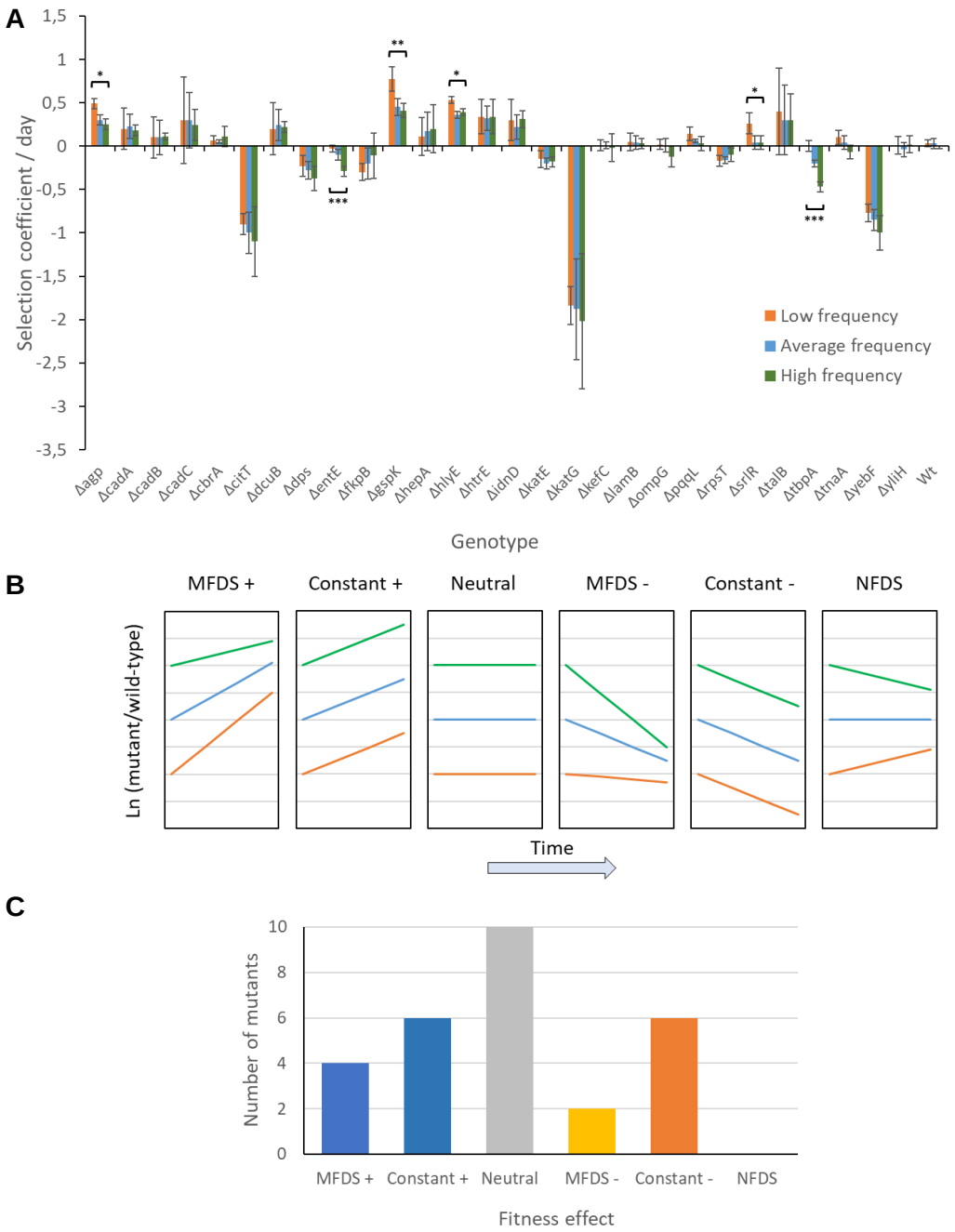


Figure 1 – Frequency-dependent selection acting on single gene deletion polymorphisms. (A) Selection coefficients for the single gene deletions, obtained by pair-wise competition with wild-type strain at low, average and high frequency. Error bars correspond to 2 times the standard error. Significant differences between competitions in low

and high frequencies are highlighted: * p<0.05; **p<0.01; ***p<0.001 (Two tailed T test with homoscedasticity). **(B)** Expectable patterns of selection. **(C)** Number of mutants with each type of selection.

Isolated growth does not fully predict competitive fitness

We have observed a surprising number of mutations with an effect in minimal medium with glucose, inclusively frequency dependent events. These observations could be a product of strain interactions between the wild-type and the mutants, or just be a reflection of a better or worse ability to grow on minimal medium. In order to see to what extent the fitness effects could be predicted by an altered ability to grow, we have performed growth curves of the mutants and the wild-type strain, and assessed the relative growth dynamics between each mutant compared to the wild-type (**Table 4**).

Table 4 - Average growth curve parameters of the mutant strains relative to the wild-type. The parameter μ_m/μ_{wt} stands for the relative maximum growth rate, $1/(\lambda_m/\lambda_{wt})$ for the inverse relative lag phase, and K_m/K_{wt} for the relative carrying capacity. Values below 1 correspond to a worse performance relative to the wild-type, while values above 1 correspond to a better performance. The parameter values that differ significantly from the wild-type's are highlighted in color ($p<0.05$, ANOVA with Dunnett's multiple comparison correction). In orange are highlighted the parameters for which the mutant performed worse, while in green are highlighted the parameters for which the mutant performed better.

Genotype	μ_m/μ_{wt}	$1/(\lambda_m/\lambda_{wt})$	K_m/K_{wt}
Δagp	1.06	3.85	1.04
ΔcadA	1.28	1.01	1.06
ΔcadB	1.04	1.19	1.02
ΔcadC	1.11	2.33	1.10
ΔcbrA	1.56	1.83	1.19
ΔcitT	0.96	0.73	0.96
ΔdcuB	1.07	2.50	1.01
Δdps	0.99	1.63	1.23
ΔentE	1.07	0.37	0.99
ΔfkpB	1.05	0.50	0.99
ΔgspK	1.09	5.00	1
ΔhepA	1.38	1.04	0.96
ΔhlyE	1.00	11.11	1.1
ΔhtrE	1.08	1.35	1.07
ΔidnD	1.17	1.72	1.18
ΔkatE	0.96	0.79	1.12
ΔkatG	0.6	0.13	0.59
ΔkefC	1.05	1.92	1.07
ΔlamB	1.18	0.74	1.05
ΔompG	1.06	1.59	0.55
ΔpqqL	1.08	5.56	1.06
ΔrpsT	1.09	1.05	1.15
ΔsrlR	1.05	0.96	0.96
ΔtalB	1.10	1.75	1.11
ΔtbpA	1.24	3.57	0.98
ΔtnaA	1.10	1.27	1.05
ΔyebF	0.68	0.40	0.77
ΔyliH	0.97	0.93	1.06

+	Beneficial
	Neutral
-	Deleterious

While comparing the relative growth curve parameters with the observed fitness (**Table 5** vs **Table 4**), it becomes clear that the extent by which fitness was predictable by isolated growth was dependent on the considered mutant. The length of the lag phase was negatively correlated with a better performance in the presence of the wild-type strain (R^2 =0.71 when compared with fitness at average frequency). In fact, the lag phase was more correlated with fitness than the relative growth rate and the relative carrying capacity irrespectively of the mutant frequency (**Figure 2**).

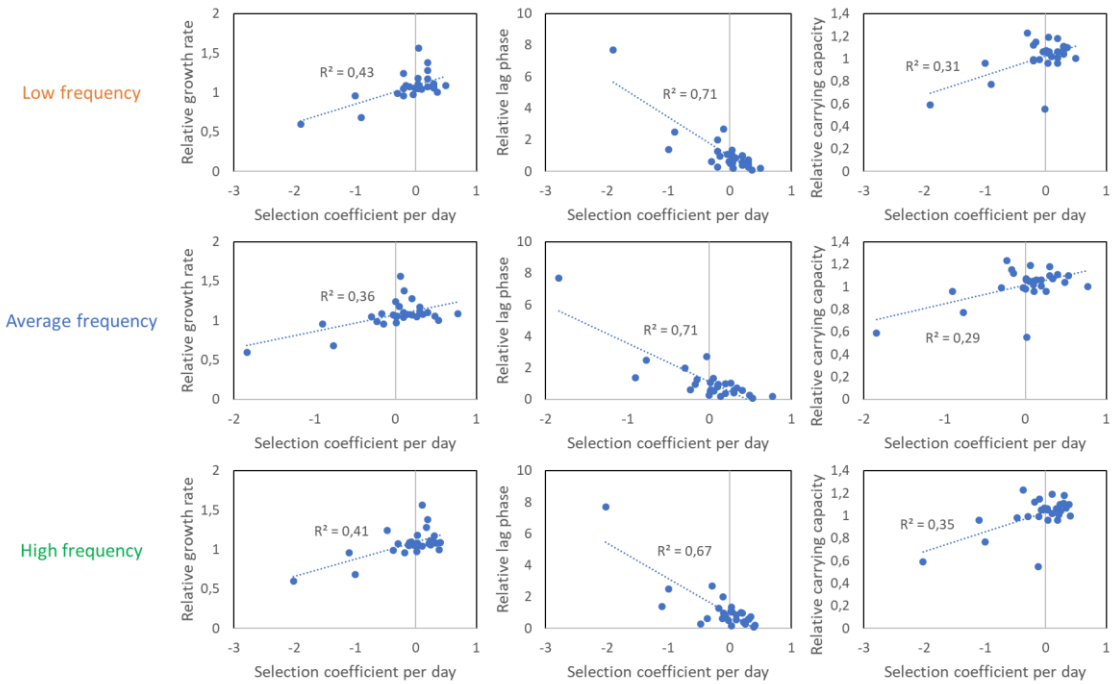


Figure 2 – Correlations between selection in pairwise competitions and relative growth curve parameters. Shown are the correlations between the growth rate, the lag phase and the carrying capacity with the selection coefficient per day of the mutant strains in all tested frequencies. A strong negative correlation between lag phase duration and the selection coefficient was observed.

Our competitions allowed for a small number of generations per transfer (5-6 gens), favoring a swift exit from lag phase, and this could help explain this correlation. For most mutants (22 out of 28), no significant differences in growth rate and carrying capacity were detected. These include three of the most successful mutants in the pairwise competitions – *Δagp*, *ΔgspK* and *ΔhlyE*. However, *ΔhlyE* has a significantly shorter lag phase than the wild-type, which could contribute to the observed fitness advantage. (*Δagp* and *ΔgspK* also have a shorter estimated lag phase, but significance was lost after applying multiple comparison correction).

The mutants *Δcbr* and *Δdps*, which also had a fitness advantage over the wild-type strain, do have a higher relative maximum growth rate and relative carrying capacity, respectively. *ΔkatG* and *ΔyebF*, two costly mutants, have a significantly worse performance than the wild-type in all three growth parameters. For these mutants, the outcome of the competitions could be predicted by the isolated growth. On the other hand, the mutants *ΔhtrE*, which had a fitness advantage, and *ΔcitT*, which had a clear fitness disadvantage had close performances relative to the wild-type when alone, despite their effects in direct competition.

Regarding the mutants that have a frequency-dependent, negative competitive ability in high frequency, *ΔentE* has a longer lag phase, while showing no other growth defects. This phenotype reflects the cost when in high frequency, and might derive from an impaired ability to scavenge iron in the absence of the wild-type strain. *ΔtbpA* did not perform worse than the wild-type in any parameter. In fact, it has a higher maximum growth rate and a shorter lag phase when growing alone, which could support an advantage against the wild-type while in low density. It seems however that the presence of the wild-type in high densities imposes a cost on this mutant (**Table 5**).

Table 5 – Effects on competitive fitness of single gene deletions. Average fitness effects of gene deletions when the mutant is in low, average or high initial frequency when competing with the wild type. Within brackets is shown 2 times the standard error. Absolute values exceeding it were considered significant. In orange are highlighted the treatments for which the mutation was deleterious, while in green are highlighted the treatments for which the mutation was beneficial.

	Frequency		
Genotype	**Low**	**Average**	**High**
Δagp	0.49 (± 0.07)	0.3 (± 0.06)	0.25 (± 0.07)
ΔcadA	0.2 (± 0.2)	0.2 (± 0.1)	0.18 (± 0.07)
ΔcadB	0.1 (± 0.2)	0.1 (± 0.1)	0.11 (± 0.05)
ΔcadC	0.3 (± 0.5)	0.3 (± 0.3)	0.2 (± 0.2)
ΔcbrA	0.06 (± 0.05)	0.05 (± 0.02)	0.1 (± 0.1)
ΔcitT	-0.9 (± 0.2)	-1.0 (± 0.3)	-1.1 (± 0.4)
ΔdcuB	0.2 (± 0.3)	0.2 (± 0.2)	0.22 (± 0.05)
Δdps	-0.2 (± 0.1)	-0.3 (± 0.1)	-0.4 (± 0.1)
ΔentE	-0.03 (± 0.05)	-0.10 (± 0.06)	-0.29 (± 0.06)
ΔfkpB	-0.3 (± 0.2)	-0.2 (± 0.1)	-0.1 (± 0.2)
ΔgspK	0.8 (± 0.1)	0.5 (± 0.1)	0.41 (± 0.08)
ΔhepA	0.1 (± 0.2)	0.2 (± 0.2)	0.2 (± 0.2)
ΔhlyE	0.53 (± 0.03)	0.36 (± 0.04)	0.39 (± 0.08)
ΔhtrE	0.3 (± 0.2)	0.3 (± 0.1)	0.3 (± 0.2)
ΔidnD	0.3 (± 0.2)	0.2 (± 0.1)	0.3 (± 0.1)
ΔkatE	-0.2 (± 0.1)	-0.20 (± 0.07)	-0.18 (± 0.06)
ΔkatG	-1.8 (± 0.2)	-1.9 (± 0.6)	-2.0 (± 0.8)
ΔkefC	0.01 (± 0.05)	0.01 (± 0.04)	0 (± 0.2)
ΔlamB	0.05 (± 0.02)	0.04 (± 0.02)	0.03 (± 0.09)
ΔompG	-0.02 (± 0.06)	-0.01 (± 0.08)	-0.1 (± 0.1)
ΔpqqL	0.14 (± 0.08)	0.06 (± 0.01)	0.03 (± 0.09)
ΔrpsT	-0.17 (± 0.06)	-0.16 (± 0.04)	-0.10 (± 0.08)
ΔsrlR	0.3 (± 0.1)	0.04 (± 0.08)	0.04 (± 0.09)
ΔtalB	0.3 (± 0.5)	0.3 (± 0.4)	0.3 (± 0.3)
ΔtbpA	0 (± 0.07)	-0.20 (± 0.03)	-0.47 (± 0.06)
ΔtnaA	0.10 (±0.09)	0.04 (± 0.08)	-0.07 (± 0.09)
ΔyebF	0.77 (± 0.10)	-0.9 (± 0.1)	-1.0 (± 0.2)
ΔyliH	-0.01 (± 0.09)	-0.04 (± 0.08)	0 (± 0.1)

+	Beneficial
	Neutral
-	Deleterious

Surprisingly, *ΔompG* has a low relative carrying capacity in minimum medium while alone, despite the mutation having a neutral effect in the pair-wise competitions with the wild-type. It is possible that in higher frequencies than the ones tested here, which would impose a starting low density of the wild-type strain, *ΔompG* would actually have a fitness disadvantage and allow the wild-type's invasion. These unexpected disparities between growth parameters and competitive fitness, together with the previously observed frequency-dependent fitness effects support a role for bacterial interactions in our competitions, despite the simple environment.

Darwinian selection prevails over secretome polymorphism stability in the mammalian gut

We have observed frequency-dependent effects in the course of the pairwise competitions, the majority of which involving secretome mutants. While we did not observe negative frequency dependent selection, we wondered if in a complex and natural environment, these particular frequency dependent effects, which do suggest strain interactions, would turn into negative frequency dependent selection and allow polymorphism stability, or into Darwinian selection, in which the most fit genotype would dominate the intra-specific competition. To assess this, we performed competitions involving the wild-type strain and the 7 secretome mutants from the previous experiments, altogether in the mouse gut (**Figure 3A)**. We have decided to exclude *ΔsrlR* from these competitions because its nature is different from the other mutants, being a metabolic mutation that does not require interactions to obtain a fitness advantage. Still, it has previously been shown to be under frequency dependent selection in the gut, through resource specialization [395].

Albeit with a different pace, we have observed the loss of the wild-type strain in all mice within the course of a week after colonization (**figure 3B**). *E. coli* loads were high and constant throughout the competition, supporting the loss of the wild-type strain as an end result of selection, not drift (**Figure 3C**). Within a host and while

in the presence of other microbes, the wild-type was not the most competitive strain, losing to the secretome consortia. To evaluate which mutants were present at the end of the competition, we have performed colony PCR with mutant-specific primer pairs on the last time-point samples. We found out that the competitions were dominated by the *ΔgspK* mutant, as the sampled colonies (10 per mouse) were identified as such (**figure 3D**). The *ΔgspK* mutant had previously performed better than all the other mutants in the *in vitro* competitions, even though its fitness in high frequency was not superior to the fitness of *ΔhlyE* and *Δagp* in low frequency (**Table 4**). Our results indicate that Darwinian selection favored *ΔgspK*, and while we cannot exclude the presence of the other strains at low frequency, we found no evidence of balancing selection for secretome traits in the gut ecosystem.

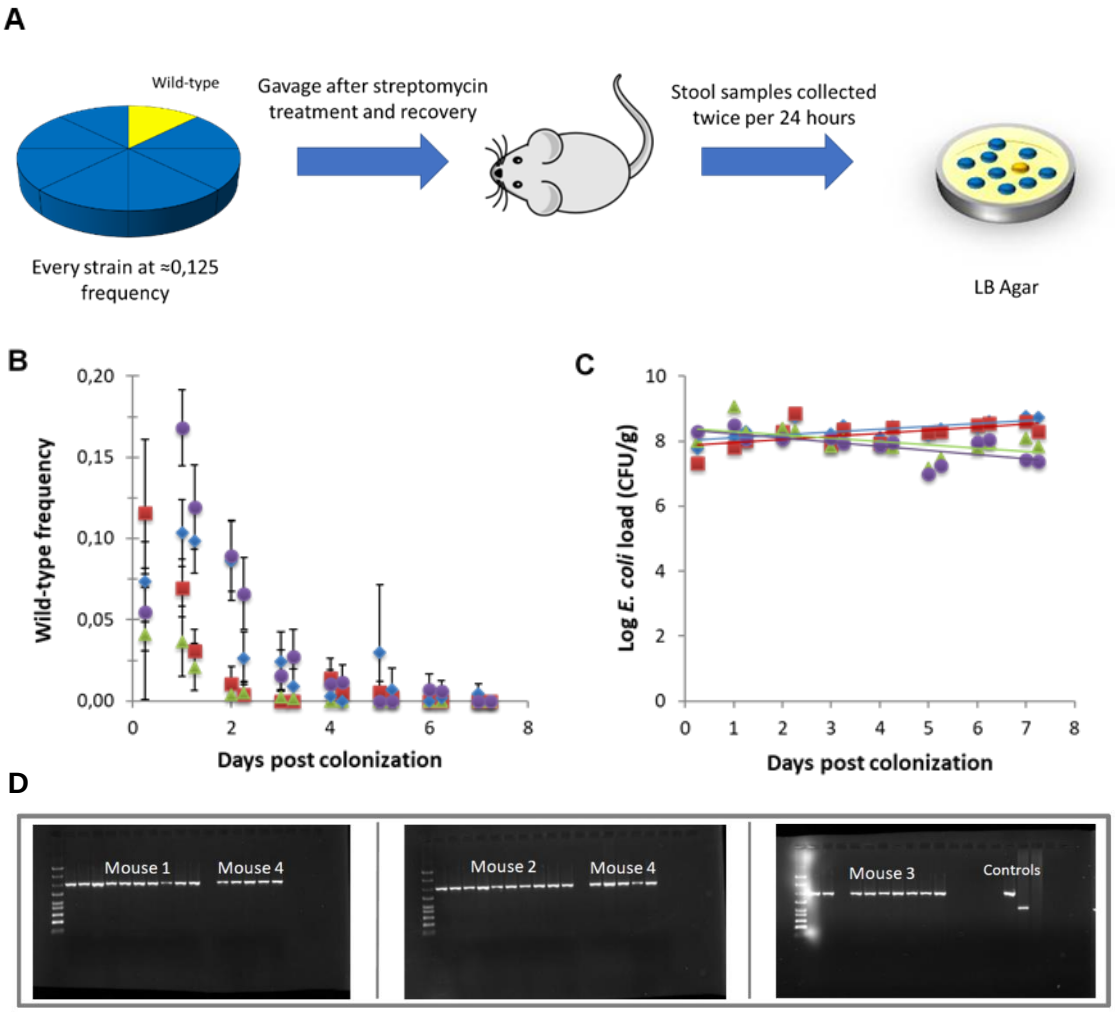


Figure 3 – Multi-strain competition *in vivo*. (A) Design of the multi-strain competition. The initial strain frequency was adjusted to have the strains in equal frequency at the beginning of the competition. (**B)** Exclusion of the wild-type strain. Each color and symbol represent the wild-type frequency in a singular mouse host. The frequency of the wild-type over the course of the competitions is reduced below the limit of detection. (**C)** Total *E. coli* loads (CFUs/g feces). The bacterial load of the competing strains did not suffer significant variation. (**D)** Colony PCR with specific primers for the *gspK* deletion, for stool samples from the last time-point. 3 controls are shown: one with the *ΔgspK* mutant with the respective primers, one with the *ΔentE* mutant with its specific primers, and one with the *ΔentE* mutant with *ΔgspK* primers (negative control). The colony PCR for the empty well in mouse 3 was re-run and confirmed to be *ΔgspK*.

Discussion

Frequency dependent fitness effects and secretome mutants

To assess the existence of frequency dependence in single mutants for a variety of genes involved in distinct functions, we have performed competitions with the wild-type strain in different frequencies. We have observed situations of frequency dependence, in which the performance of the mutants was better in low frequency than in high frequency. Our results suggest that magnitude frequency-dependent selection can occur and should be taken into consideration. However, as expected, its presence is likely to be associated with specific classes of genes. Traits that concern the outside of the cell, secretome traits, are particularly enriched for frequency dependence in our simple environment.

Here we are considering that a polymorphism is under negative frequency-dependent selection only when the strains can mutually invade each other, as without an advantage in rarity, genetic drift is more likely to eliminate rare mutants and lead to a single morph population [162]. Under our definition, we do not observe situations of negative frequency-dependent selection. We did, however, observe 2 cases of frequency-dependent selection in which the mutants, *ΔentE* and *ΔtbpA*, have no cost when rare and a cost when in high frequency, and 1 in which the mutant *ΔsrlR* has an advantage in low frequency only. These fitness effects allow for the maintenance of the mutant and wild-type polymorphism when drift effects are weak, and some authors still consider them as negative-frequency dependent selection [512].

The gene *entE* codes for a subunit of the enterochelin synthase [513]. Looking at the growth dynamics, the *ΔentE* mutant performs worse than the wild-type when growing alone, namely through an extended lag phase. Their maximum growth rate and carrying capacity however, are similar. Therefore, the presence of the wild-type confers a benefit that is not related with the ability to grow fast, but with having physiological conditions to initiate exponential growth. As enterochelin is a conserved

siderophore in *E.coli* [514], the extended lag phase is most likely derived from an impaired ability to scavenge iron. Our results for the *ΔentE* mutant were expectable, considering that the deletion of another component of the enterochelin synthase, entF, has been shown to provide a fitness cost in low iron conditions, but virtually no cost when the mutant is within a numerous wild-type population [512]. The authors did not observe a fitness advantage with mutant/wild-type frequencies as low as 1/1000, possibly due to a degree of privatization at low cell density by the wild-type cells.

Regarding *ΔtbpA*, it is possible that our range of frequencies throughout the competitions did not capture the equilibrium value [515]. Therefore, we cannot exclude the possibility of this mutant being under negative frequency-dependent selection in extremely low frequencies. Alternatively, it is possible that in this simple environment, there is not a frequency in which the sign of selection changes for this polymorphism. Concerning the individual growth, the mutant does not perform worse than the wild-type (it actually achieves a slightly superior maximum growth rate), so one would think that the presence of the wild-type is detrimental. However, while in competition, *ΔtbpA* performs better when surrounded by wild-type cells. *tbpA* encodes the periplasmic subunit of an ABC transporter, that is involved in the import of thiamine and thiamine pyrophosphate (TPP) into the cell [516,517]. Mutants with an impaired transporter system have to rely solely in *de novo* synthesis of thiamine and TPP. Alike our observations for the *ΔtbpA* mutant, *Salmonella* mutants with impaired thiamine transport have no observable growth defects in minimal medium when growing alone [516], further supporting that a lower relative fitness of the *ΔtbpA* mutant is due to an interaction with the wild-type strain. While the wild-type can uptake thiamine, our minimal medium does not have thiamine in its composition, so the wild-type would have to access produced thiamine to gain a frequency-dependent advantage. Some bacteria release thiamine while growing. *E. coli* K12 itself releases a fraction of the accumulated thiamine when exposed to fresh medium [518]. One possibility is that the wild-type has access to the leaked thiamine through the thiamine transporter, but as its frequency increases, the clonal competition for thiamine reduces the benefit. This hypothesis remains to be tested, so for now, the mechanism

behind this mutant's cost in pair-wise competitions and its frequency dependence remain undisclosed.

Polymorphisms in *srlR* were shown to be under frequency dependent selection in the mouse gut [395]. Still, our observations of frequency dependent selection acting on *ΔsrlR*, particularly in minimal medium with glucose, are puzzling. *srlR* codes for a repressor, and as such, its deletion leads to an enhanced expression of the sorbitol operon [519]. In the absence of sorbitol, this represents a waste of resources, and can lead to a deleterious effect. However, the sorbitol operon is subjected to catabolite repression [520] - high glucose levels typically lead to a lowered cAMP-CRP concentration [521], a complex that is essential for the expression of the operon even in mutants lacking a functional srlR repressor [519]. The operon is, therefore, expected to be inactive until late exponential phase and have a neutral effect in the competitions, as well as in the individual growth, which in fact, is not significantly different from the wild-type's (table 3). Our observations require *ΔsrlR* to have an advantage during competition, and to either that advantage or a late cost of the *srl* operon activity to be different in high and low frequency. Given that *srlR* codes a metabolic regulator, I would expect the frequency dependence to result from a pure density-dependent effect and not from a strain interaction. Further experimentation would be required to understand this result.

Absence of dependency and NFDS

A fairly recent theory, the Black Queen hypothesis states that negative frequency dependent selection can arise from the loss of essential genes involved in leaky functions [522]. The underlying idea is that if a communal function entails a fitness cost, there will be a selective pressure towards losing this function, as long as there is another strain or species performing it. After gene loss, the fitness of these "beneficiary" members will be a balance between the benefit of not producing the good and the cost of having a limited access to it, leading to frequency-dependent

balancing selection. Evolution through gene loss and dependency has been experimentally shown to lead to long lasting, stable polymorphisms [509], further corroborating this theory for diversity maintenance.

We used single gene deletions to generate the mutants in our experiments, fully disabling their function. However, our selected genes code non-essential functions of *E. coli* [404]. As such, in commonly used media, while frequency dependence can occur, metabolic dependencies are not expected. The single exception within our sampled genes is the previously mentioned *ΔentE* mutant. As our minimal medium is not supplemented with iron, the inability to scavenge residual iron brought a high, frequency-dependent cost.

A typical case that can result in stabilizing frequency dependent selection is detoxification [523,524], and we have chosen to test *E. coli* peroxidases, KatE and KatG, for frequency dependence because the latter has been associated with a Black Queen function in a natural [525] and in an experimental [509] system. In both cases however, the beneficiary strain was extremely sensitive to hydrogen peroxide, with no alternative catalases to degrade it, while in our case, one catalase is still present. In our hands, both peroxidase single mutants have shown a clear fitness defect, but no frequency dependence. Although the two peroxidases are induced independently [526] and locate differently in the cell [527], they seem to partially compensate each other and buffer putative frequency-dependent effects. Taking these studies into account, our results suggest that negative frequency dependent selection through gene loss of leaky functions might require essentiality of these functions.

Positive directional selection and frequency dependent effects

We have observed 4 cases of magnitude FDS for genes that confer a fitness benefit in low frequency. For the mutants *Δagp*, *ΔgspK* and *ΔhlyE*, selection was

positive even in high frequency. While the frequency dependent effects are not negligible, the fact that these mutants have an advantage irrespective of their frequency suggests that a cost component is always present in the wild-type cell, possibly due to the absence of need for these functions in minimal medium with glucose. The mutant's fitness advantage is particularly surprising for *ΔgspK* and *ΔhlyE*, as these genes were shown to be poorly expressed or not at all by commensal *E. coli* in rich laboratory media [528,529], due to repression by the small chromatin associated protein H-NS [530,531].

The gene *hlyE* codes for haemolysin E, a pore forming toxin that targets mammalian cells [532]. *hlyE* has been shown not to be induced in laboratory strains derived from *E. coli* K12, when grown in LB medium [528,531]. Experiments on *E. coli* K12 BW 3110 as well on other commensal *E. coli* grown on rich media associate the *hlyE* locus to repression through H-NS binding [531,533], although chromosome immuno-precipitation experiments failed to detect H-NS binding to this locus on the *E. coli* K12 MG1655 strain, isogenic to ours, grown in minimal medium [534]. While it is known that gene expression varies with the environment, it is unlikely that the simple conditions in which the competitions were performed, lacking host-derived environmental cues, would lead to the de-repression or to the activation of *hlyE*. On the other hand, the wild-type genetic background of our strains is still quite close to the BW 3110 strain. It is thus unlikely that the *hlyE* gene is fully derepressed in our strains, although a leaky expression scenario is possible. It is known that the secretion of haemolysin E involves a transient leakage of periplasmic contents to the extracellular medium [535], and as such, it would allow sharing of the wild-type periplasmic contents with the mutant cells. While the periplasmic leak could generate a cost, the shared compounds could generate a frequency-dependent fitness effect.

Regarding *ΔgspK*, the respective gene codes a pseudo pilin of the type II secretion system [536]. Experiments on *E. coli* MC4100 have shown a reduced expression by the operon's promoter in LB medium when compared with unrepressed, H-NS knock-out mutants [530]. As with *hlyE*, the *gspCO* operon's repression was shown with other *E. coli* backgrounds than our own and in different

culture media than the one used in this study. Once again, it is unlikely, but still possible that the expression profile of *gspK* is different in our wild-type strain under our laboratory conditions. Additionally, even if we assume that the differences in the environment and genetic background are not having an effect in *gspK* expression, the low level of expression could still generate a phenotype affecting fitness. While the full repertoire of proteins that can be transported by *E. coli*'s type II secretion system is not known, putative secretion products could change the surrounding, shared environment, and such change would lead to frequency dependent effects. Type II secretion systems are widespread among gram-negative proteobacteria and feature a broad range of functions [537], inclusively during host infections by pathogenic bacteria [538]. As our *E. coli* is commensal, it is possible that the usefulness of having *gspK*, as well as other repressed secretome genes, has been lost due to the absence of formerly associated virulence traits. Despite these considerations, the fitness benefit and the frequency dependence of these mutations could still be related with undisclosed effects of the substitution of these genes by the kanamycin resistance cassette.

As ecological interactions between populations are typically density dependent [524], it is thought that density dependence underlies most mechanisms of frequency dependent selection [502]. We performed our competitions in a relatively high cellular density, with relatively small bottlenecks (1:40 dilutions), and we did not test for frequency dependent selection considering different densities. However, it is likely that higher shifts in cell density would change the observed patterns of selection. Starting at a lower density would reduce interactions on the beginning of growth and change their net benefit or cost. One such example is given by the aforementioned work of Scholz and Greenberg [512] on enterochelin, in which the frequency-dependent fitness effect of a mutation is the result of a density-dependent interaction.

Beneficial effects of whole gene deletions

As expected, the lack of core genes had a deleterious impact on fitness. The exception was the deletion of the transaldolase encoding gene *talB*. For this mutant, the variance in the fitness effect of the mutation between biological samples was high (reflected by the high standard error for all treatments, table 4), but still, we have observed, on average, a beneficial effect of the mutation. A *talB* – deficient mutant has been previously generated by other authors (by partial deletion and concomitant insertion of a kanamycin-cassette into the *talB* locus), and its growth in minimal medium with glucose was unaffected [539]. Transaldolase activity takes part in the pentose phosphate pathway non-oxidative branch [540], which in turn is essential for the production of NADPH and nucleotide synthesis percursor metabolites [541]. However, *E. coli* genome possesses another transaldolase encoding gene, *talA*, which acts on the same pathway. One possibility for the lack of cost of *ΔtalB* is therefore, compensation by talA. Furthermore, *talAB* knockout mutants have been shown to growth at rates comparable to the growth of the wild-type cells in single sugar growth, by completing the pentose pathway cycle through recently described, alternative reactions [542]. The lack of transaldolase can, therefore, be bypassed by *E. coli*, and this could also be the reason why we do not observe a cost. Regarding the accessory genes, their deletion had mixed effects. It should be noted however that the deletion of the 4 least represented genes (*ΔidnD*, *ΔgspK*, *ΔhlyE* and *ΔhtrE*) has led to a fitness advantage, in accord with the idea that the accessory genome codes for functions conferring context-specific fitness benefits and will tend to have a cost in the remaining contexts [507].

We have observed in total 9 deletions with a positive effect on fitness on all frequencies (3 with frequency dependent effects) in our simple selective environment. The benefits of gene deletions have also been shown in *Salmonella enterica*, where 25% of 55 random deletion mutants conferred a higher growth rate when compared with the ancestor strain in at least one of 3 laboratory environments [543]. The observed growth benefit could be explained partially (but not totally) by a

decreased energy/mass expenditure on DNA, RNA and protein production. Additionally, a study on the distribution of pseudogenes among the sequenced strains of *Salmonella* suggests that the full deletion of a gene confers a higher fitness benefit than its function loss by inactivation [544]. Having into account that practically all sequences in the genome, including pseudogenes and antisense sequences, are transcribed in *E. coli* [545,546], the beneficial effects of our mutations might come from the full stop of transcription of genes that are not useful in our experimental setting, with the clean, whole gene deletions allowing the avoidance of leaky expression or harm to the genome architecture.

Through comparison of different ways of measuring fitness and the integration of frequency-dependence testing, we have observed discrepancies between the measurements for some of the mutants, hinting into undescribed bacterial interactions. Molecular and ecological studies stemming from these observations are necessary, and could elucidate new ecological relationships within bacterial populations. Our results stress out that studying the fitness effects of a mutation taking into account competitors and the mutant's frequency in the population can reveal otherwise cryptic fitness effects which can have an impact on survival.

Darwinian selection acting on secretome genes

In order to probe for negative frequency dependent selection in *E. coli*'s natural environment, the mammalian gut, we have competed together a pool of 7 mutants plus the wild-type strain in SPF mice. As we observed an enrichment for frequency dependence in traits involved with the outside of the cell, we chose to compete single mutants for genes encoding these traits.

We have observed the dominance of a single mutant in all tested mice. Through what seemed like a process of Darwinian selection, *ΔgspK* has risen in frequency, while the wild-type has fallen under our limit of detection. Besides having a shorter lag phase than the wild-type strain and a beneficial effect with all tested frequencies

in *in vitro* pair-wise competitions, *ΔgspK* has the highest average fitness value within our mutant pool in all frequencies. On the other hand, it is likely that inter-species competition, as well as the gut's structured environment reduce the effect of intra-strain interactions on fitness. Having this into account, the *in vivo* dominance of *ΔgspK* might just reflect the superior competitive ability of this mutant relative to the other tested strains.

It seems clear that this single gene deletion confers a fitness advantage *in vivo*. However, as far as I am aware, mutations on *gspK* were not detected during *E. coli* K12 MG1655 evolution experiments in the mouse gut [[66,395,398,411]; chapter III]. This might have to do with its location in this strain's genome: the *gspCO* operon is located between core genes, including the *rps* ribosomal encoding genes and the transcription elongation factor *tufA* (Ecocyc database). An extensive deletion in this region could affect the cell's inner machinery, or even its viability, and consequently, mutations of this nature would be highly deleterious. Curiously, the gene is not conserved in sequenced *E. coli* (Table 2), further suggesting that this gene is costly to have per se and that the advantages that if confers are context-specific.

The gut is a complex environment that contains a wide range of potential substrates for bacterial growth, derived from host-harvested nutrients, host produced substances, their breakdown products and additional substances synthetized by microflora itself [488]. This multitude of nutrients generates a multitude of niches, with different bacteria having distinct niche specializations, which in turn can allow prolonged coexistence. Accordingly, a data-supported theoretical model suggests that microbiome stability in the gut is achieved through resource competition [489]. Under this scenario, mutations in metabolic regulators can generate metabolic trade-offs between the strains, which in turn are predicted to maintain diversity in environments with multiple species and resources [451]. As mentioned before, *srlR* was shown to be a target for negative frequency-dependent selection in the gut [395], and spontaneous mutations in this gene appear, in several *E. coli* adaptation experiments without reaching fixation [[395,398,411], chapter III, table 1], an empirical example of a metabolism-based balanced polymorphism.

Our results and these observations suggest that for *E. coli*, in a complex environment with a multitude of nutritional niches and strong inter-species competition like the mammalian gut, resource competition is more likely to lead to negative frequency-dependent selection between polymorphisms than intra-species interactions.

CHAPTER V

General Discussion

Studying bacteria in their natural environment is crucial to understand their ecology and evolution. As in other studies [191,401], the research presented in this thesis shows a discrepancy between measuring the fitness effects of mutations in standard laboratory medium and within a mouse host.

We discovered that in the context of the dysbiotic gut, the costs of antibiotic resistance mutations are personalized, and identified the gut microbiota as a factor contributing decisively to the fate of antibiotic resistance in this system. Hence, our results indicate that, besides acting as a reservoir for antibiotic resistance, microbiota can promote the subsistence of resistant strains in the gut on its own. Our novel results add to an increasing evidence regarding the importance of microbial communities. As with our own research, the microbiota has been shown to be a source of variation in the expected outcome of various murine-based studies [547], including some in which it was not initially thought to be involved [548]. For instance, two studies have shown that the gender of mice alters autoimmune phenotypes partially through the action of microbiota. Through the employment of germ-free colonization with defined microbiota [549] and gut microbiota transfer [550], both studies show that, while gender influences microbiota composition, microbiota is partially responsible for the lower incidence of type I diabetes in male nonobese diabetic mice. Another example regards a study on the effect of the mouse microbiota in the gut IgA levels[551]. After observing a binary phenotype of fecal IgA levels between cages, the authors perform a series of co-housing and fecal transplantation experiments and show that the “low IgA level” phenotype is dominant and transmitted horizontally, being driven by bacteria that degrade both IgA and the protective, secretory component SIgA. Taking microbiota-driven variation into account, future studies involving the gut ecosystem should have a mixed approach, in which an effect of microbiota composition is tested through a treatment that allows variation and a treatment that tightly controls it. The latter can be obtained through co-housing, as performed by us, and also through littermate methods, which are more time-consuming but more effective [415]. This mixed approach will allow to assess the

reproducibility of a phenotype across hosts and to simultaneously control for the effects of the microbiome in the phenotype [547].

We have observed variance in competitive fitness, including situations in which a resistant strain had a fitness benefit. While during treatment, selection by an antibiotic agent favors directly the resistant strain, our results suggest that microbiota imbalances can generate conditions for resistance to be neutral or beneficial after the treatment. This state may be temporary, but it will promote the expansion and maintenance of the resistant strain. A recent study corroborates this expectation and further extends it to a community level, by showing that a short-term cefuroxime treatment can increase the general level of resistance in the human gut microbiota [552]. On the other hand, this dysbiotic period is typically a window of opportunity for pathogens [553–555]. If this lack of cost would happen in a resistant pathogen, its elimination would be extremely difficult due to the lack of a method to specifically select against it.

Our experimental system does not allow us to test the fitness effect of antibiotic resistance mutations in an unperturbed microbiota. Assuming that the variance in fitness costs widens due to the microbiome perturbation and that resistant strains do express a cost in equilibrium conditions, an effective way to impair them is to promote microbiota recovery. In a study using the streptomycin treated, dysbiotic mouse gut as a model, the overproduction of the inter-species quorum sensing molecule, AI-2, by a genetically engineered *E. coli* led to a recovery in Firmicutes abundance after the perturbation [406]. This type of approach could shorten the window of opportunity in which resistant strains and pathogens are able to proliferate.

For resistance mutations that affect nutritional metabolism, an alternative way to address these situations could be through diet manipulation, which has been shown to be able to effectively change selective pressure on *E. coli* polymorphic for galactitol consumption [397]. Screening and profiling a resistant strain's nutritional preferences and adjusting the host's diet accordingly could thwart its subsistence in the gut ecosystem. However, dietary patterns affect microbiota composition [556,557],

and diet manipulation readily causes expansion of specific bacterial groups [557], which in turn will affect inter-species competition. Furthermore, inter-species competition itself might limit the effectiveness of diet-based manipulation [558]. Hence, we cannot fully predict the helpful and harmful effects of this approach.

A study with a simplified model microbiota has shown that the presence of a single gut bacterial species can change the outcome of an intra-species competition [559]. Assuming that a such principle is applicable within the gut ecosystem, another plausible strategy is to find competitors that will reliably and specifically outcompete resistant bacteria, or at least force them to express a cost. Once identified, these interfering strains could be administered to the hosts carrying resistant bacteria. Furthermore, once present in the community, these competitors could be expanded and manipulated through the use of prebiotics [560,561] to prime the treatment. Although laborious, studying the fitness effects of resistance mutations in the presence of specific gut microbes or defined collections of microbiota members, focusing on the "molecular-to-ecological" mechanisms through which fitness is being affected and further testing the efficiency of these strains in host models could lead to more optimized approaches for selection against resistance.

The human gut includes an enormous variety of microbes [562]. A key study on the microbiota of healthy humans, the Human Microbiome Project, has revealed that the diversity and the abundance of microbes varies widely among subjects [563]. A subsequent study performed the tracking of individuals based on the project's data and was able to pinpoint over 80% of the subjects [564]. These studies show that even when considering healthy individuals, microbiota composition is personal. As the microbiota can affect the cost of resistance, it is likely that the subsistence of resistant bacteria in humans is also host-specific. Under this scenario, individual microbiomes and resistomes should be taken into account when an antibiotic is prescribed. As with contemporary health problems that depend on genetics, lifestyle and diet [565,566], the fight against antibiotic resistance in the current era might require personalized medicine.

Microbiome research is currently thriving [567], and over time we have gained access to techniques and technologies that allow us to perform studies in these complex communities: The employment of metagenomics [563,568,569], metatranscriptomics [570,571] and metabolomics [572] allows for a detailed characterization of the environment; The adaptation of classical ecological models to the study of microbial communities [573], and the coupling of their predictions with empirical experimentation [428,574] can help us determine properties of bacterial communities; Reliable and reproducible approaches such the as the colonization of germ-free mice with defined culture collections [575] and the use of synthetic microbial communities [576] may let us to go over correlative studies and test specific hypothesis in experimental systems with intermediate complexity, which nonetheless share properties with the natural ones. Coupling these tools with experimental evolution will help us learn more about the selective forces that act in microbial communities, how they are generated and how they shape the evolution of their members.

Evolutionary biology has been instrumental to medicine, in understanding the origin and devising strategies to fight concerning health problems, such as genetic conflicts, aging, cancer, infectious diseases and of course, antibiotic resistance [577,578]. Through the use of an increasingly integrative approach, evolutionary biology might let us learn much more about the ecology and evolution of bacteria (including the ones living within us) and hopefully lead to relevant clinical implementations towards the prevention and reversion of resistance.

References

1. Cohen, M. L. Epidemiology of drug resistance: implications for a post-antimicrobial era. *Science* **257**, 1050–1055 (1992).

2. Martinez, J. L. *et al.* A global view of antibiotic resistance. *FEMS Microbiol. Rev.* **33**, 44–65 (2008).

3. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **8**, 260–271 (2010).

4. Tanaka, T., Hashimoto, H., Nagai, Y. & Mitsuhashi, S. Drug Resistance of Enteric Bacteria. *Jpn. J. Microbiol.* **11**, 155–162 (1967).

5. Arai, T. THE MECHANISMS OF TRANSFER OF R FACTORS. *Keio J. Med.* **16**, 55–82 (1967).

6. Levy, S. B. & Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* **10**, S122–S129 (2004).

7. Neu, H. C. The Crisis in Antibiotic Resistance. *Science (80-.).* **257**, 1064 LP – 1073 (1992).

8. Andersson, D. I. & Hughes, D. Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiol. Rev.* **35**, 901–911 (2011).

9. Andersson, D. I. & Levin, B. R. The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* **2**, 489–493 (1999).

10. Bonhoeffer, S. *et al.* Evaluating treatment protocols to preventent‰antibiotic€ resistance. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12106–12111 (1997).

11. World Health Organization. Antimicrobial resistance: global report on surveillance 2014. (2014).

12. Kapoor, G., Saigal, S. & Elongavan, A. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J. Anaesthesiol. Clin. Pharmacol.* **33**, 300–305 (2017).

13. Tomasz, A. The Mechanism of the Irreversible Antimicrobial Effects of Penicillins: How the Beta-Lactam Antibiotics Kill and Lyse Bacteria. *Annu. Rev. Microbiol.* **33**, 113–137 (1979).

14. Reynolds, P. E. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis.* **8**, 943–950 (1989).

15. Khan, T. *et al.* DNA gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents. *Biomed. Pharmacother.* **103**, 923–938 (2018).

16. Shen, L. L. & Pernet, A. G. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proc. Natl. Acad. Sci.* **82**, 307 LP – 311 (1985).

17. Higgins, P. G. & Schmitz, A. C. F. and F.-J. Fluoroquinolones: Structure and Target Sites. *Current Drug Targets* **4**, 181–190 (2003).

18. Kato, J., Suzuki, H. & Ikeda, H. Purification and characterization of DNA topoisomerase IV in Escherichia coli. *J. Biol. Chem.* **267**, 25676–25684 (1992).

19. Kathiravan, M. K., Khilare, M. M., Nikoomanesh, K., Chothe, A. S. & Jain, K. S. Topoisomerase as target for antibacterial and anticancer drug discovery. *J. Enzyme Inhib. Med. Chem.* **28**, 419–435 (2013).

20. Crumplin, G. C. & Smith, J. T. Nalidixic Acid: an Antibacterial Paradox. *Antimicrob. Agents Chemother.* **8**, 251 LP – 261 (1975).

21. Repoila, F. & Darfeuille, F. Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. *Biol. Cell* **101**, 117–131 (2009).

22. Vassylyev, D. G. *et al.* Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature* **417**, 712–719 (2002).

23. Campbell, E. A. *et al.* Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. *Cell* **104**, 901–912 (2001).

24. Cambau, E. & Williams, D. L. Anti-leprosy drugs: Modes of action and mechanisms of resistance in Mycobacterium leprae. *International Textbook of Leprosy* (2019). Available at: https://internationaltextbookofleprosy.org/chapter/anti-leprosy-drugs-modes-action-and-mechanisms-resistance-mycobacterium-leprae.

25. Schuwirth, B. S. *et al.* Structures of the Bacterial Ribosome at 3.5 Å Resolution. *Science (80-.).* **310**, 827 LP – 834 (2005).

26. Taber, H. W., Mueller, J. P., Miller, P. F. & Arrow, A. S. Bacterial uptake of aminoglycoside antibiotics. *Microbiol. Rev.* **51**, 439–457 (1987).

27. Wilson, D. N. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nat. Rev. Microbiol.* **12**, 35 (2013).

28. Krause, K. M., Serio, A. W., Kane, T. R. & Connolly, L. E. Aminoglycosides: An Overview. *Cold Spring Harb. Perspect. Med.* **6**, (2016).

29. Davis, B. D., Chen, L. L. & Tai, P. C. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. *Proc. Natl. Acad. Sci.* **83**, 6164 LP – 6168 (1986).

30. Schnappinger, D. & Hillen, W. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Arch. Microbiol.* **165**, 359–369 (1996).

31. Connell, S. R., Tracz, D. M., Nierhaus, K. H. & Taylor, D. E. Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrob. Agents Chemother.* **47**, 3675–3681 (2003).

32. Bozdogan, B. & Appelbaum, P. C. Oxazolidinones: activity, mode of action, and mechanism of resistance. *Int. J. Antimicrob. Agents* **23**, 113–119 (2004).

33. Brunton, L. L., Chabner, B. A. & Knollmann, B. C. Aminoglycosides. *Goodman & Gilman's: The Pharmacological Basis of Therapeutics* (2011). Available at: https://basicmedicalkey.com/aminoglycosides-2/.

34. Murima, P., McKinney, J. D. & Pethe, K. Targeting Bacterial Central Metabolism for Drug Development. *Chem. Biol.* **21**, 1423–1432 (2014).

35. Yoneyama, H. & Katsumata, R. Antibiotic Resistance in Bacteria and Its Future for Novel Antibiotic Development. *Biosci. Biotechnol. Biochem.* **70**, 1060–1075 (2006).

36. Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial Persistence as a Phenotypic Switch. *Science (80-.).* **305**, 1622 LP – 1625 (2004).

37. Allen, H. K. *et al.* Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **8**, 251–259 (2010).

38. D'Costa, V. M. *et al.* Antibiotic resistance is ancient. *Nature* **477**, 457–461 (2011).

39. Perron, G. G. *et al.* Functional Characterization of Bacteria Isolated from Ancient Arctic Soil Exposes Diverse Resistance Mechanisms to Modern Antibiotics. *PLoS One* **10**, e0069533 (2015).

40. Bhullar, K. *et al.* Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. *PLoS One* **7**, e34953 (2012).

41. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42–51 (2015).

42. Munita, J. M. & Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol. Spectr.* **4**, (2016).

43. Cohen, S. N., Chang, A. C. Y. & Hsu, L. Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of Escherichia coli by R-Factor DNA. *Proc. Natl. Acad. Sci.* **69**, 2110 LP – 2114 (1972).

44. Harford, N. & Mergeay, M. Interspecific transformation of rifampicin resistance in the genus Bacillus. *Mol. Gen. Genet. MGG* **120**, 151–155 (1973).

45. Avery, O. T., MacLeod, C. M. & McCarty, M. STUDIES ON THE CHEMICAL

NATURE OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES. *J. Exp. Med.* **79**, 137 LP – 158 (1944).

46. Davies, J. Vicious circles: looking back on resistance plasmids. *Genetics* **139**, 1465–1468 (1995).

47. Lederberg, J. & Tatum, E. L. Gene Recombination in Escherichia Coli. *Nature* **158**, 558 (1946).

48. Norman, A., Hansen, L. H. & Sørensen, S. J. Conjugative plasmids: vessels of the communal gene pool. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 2275–2289 (2009).

49. Salyers, A. A., Whittle, G. & Shoemaker, N. B. Conjugative and Mobilizable Transposons. in *Microbial Evolution* 125–143 (American Society of Microbiology, 2004).

50. Schmieger, H. & Schicklmaier, P. Transduction of multiple drug resistance of Salmonella enterica serovar typhimurium DT104. *FEMS Microbiol. Lett.* **170**, 251–256 (1999).

51. Muniesa, M., Colomer-Lluch, M. & Jofre, J. Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. *Future Microbiol.* **8**, 739–751 (2013).

52. Zinder, N. D. & Lederberg, J. Genetic exchange in Salmonella. *J. Bacteriol.* **64**, 679–699 (1952).

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

54. Amábile-Cuevas, C. F. & Chicurel, M. E. Bacterial plasmids and gene flux. *Cell* **70**, 189–199 (1992).

55. de la Cruz, F. & Davies, J. Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol.* **8**, 128–133 (2000).

56. Kay, E., Vogel, T. M., Bertolla, F., Nalin, R. & Simonet, P. In Situ Transfer of Antibiotic Resistance Genes from Transgenic (Transplastomic) Tobacco Plants to Bacteria. *Appl. Environ. Microbiol.* **68**, 3345 LP – 3351 (2002).

57. Lilley, A. K. & Bailey, M. J. The acquisition of indigenous plasmids by a genetically marked pseudomonad population colonizing the sugar beet phytosphere is related to local environmental conditions. *Appl. Environ. Microbiol.* **63**, 1577–1583 (1997).

58. Troxler, J., Azelvandre, P., Zala, M., Defago, G. & Haas, D. Conjugative Transfer of Chromosomal Genes between Fluorescent Pseudomonads in the Rhizosphere of Wheat. *Appl. Environ. Microbiol.* **63**, 213–219 (1997).

59. Björklöf, K., Suoniemi, A., Haahtela, K. & Romantschuk, M. High frequency of

conjugation versus plasmid segregation of RP1 in epiphytic Pseudomonas syringae populations. *Microbiology* **141**, 2719–2727 (1995).

60. Bale, M. J., Fry, J. C. & Day, M. J. Transfer and Occurrence of Large Mercury Resistance Plasmids in River Epilithon. *Appl. Environ. Microbiol.* **54**, 972–978 (1988).

61. Balis, E. *et al.* Indications of in vivo transfer of an epidemic R plasmid from Salmonella enteritidis to Escherichia coli of the normal human gut flora. *J. Clin. Microbiol.* **34**, 977–979 (1996).

62. Maisonneuve, S., Ouriet, M.-F. & Duval-Iflah, Y. Effects of yoghurt intake on plasmid transfer and colonisation with transconjugants in the digestive tract of mice associated with human faecal flora. *FEMS Microbiol. Ecol.* **31**, 241–248 (2000).

63. Stecher, B. *et al.* Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proc. Natl. Acad. Sci.* **109**, 1269–1274 (2012).

64. Diard, M. *et al.* Inflammation boosts bacteriophage transfer between Salmonella spp. *Science (80-.).* **355**, 1211–1215 (2017).

65. Furuya, E. Y. & Lowy, F. D. Antimicrobial-resistant bacteria in the community setting. *Nat. Rev. Microbiol.* **4**, 36–45 (2006).

66. Frazão, N., Sousa, A., Lässig, M. & Gordo, I. Horizontal gene transfer overrides mutation in Escherichia coli colonizing the mammalian gut. *Proc. Natl. Acad. Sci.* **116**, 17906 LP – 17915 (2019).

67. Rowe-Magnus, D. A., Guérout, A.-M. & Mazel, D. Super-integrons. *Res. Microbiol.* **150**, 641–651 (1999).

68. Mazel, D. Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* **4**, 608–620 (2006).

69. Stalder, T., Barraud, O., Casellas, M., Dagot, C. & Ploy, M.-C. Integron Involvement in Environmental Spread of Antibiotic Resistance. *Front. Microbiol.* **3**, 119 (2012).

70. Rowe-Magnus, D. A., Guerout, A.-M. & Mazel, D. Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Mol. Microbiol.* **43**, 1657–1669 (2002).

71. Koch, A. & Wilkinson, R. J. The road to drug resistance in Mycobacterium tuberculosis. *Genome Biol.* **15**, 520 (2014).

72. Wang, G., Wilson, T. J. M., Jiang, Q. & Taylor, D. E. Spontaneous Mutations That Confer Antibiotic Resistance inHelicobacter pylori. *Antimicrob. Agents Chemother.* **45**, 727–733 (2001).

73. Sandegren, L. & Andersson, D. I. Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nat. Rev. Microbiol.* **7**, 578–588 (2009).

74. Sun, S., Berg, O. G., Roth, J. R. & Andersson, D. I. Contribution of Gene Amplification to Evolution of Increased Antibiotic Resistance in Salmonella typhimurium. *Genetics* **182**, 1183–1195 (2009).

75. Tängdén, T., Adler, M., Cars, O., Sandegren, L. & Löwdin, E. Frequent emergence of porin-deficient subpopulations with reduced carbapenem susceptibility in ESBL-producing Escherichia coli during exposure to ertapenem in an in vitro pharmacokinetic model. *J. Antimicrob. Chemother.* **68**, 1319–1326 (2013).

76. Wozniak, A. *et al.* Porin alterations present in non-carbapenemase-producing Enterobacteriaceae with high and intermediate levels of carbapenem resistance in Chile. *J. Med. Microbiol.* **61**, 1270–1279 (2012).

77. Baroud, M. *et al.* Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. *Int. J. Antimicrob. Agents* **41**, 75–79 (2013).

78. Drake, J. W. A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci.* **88**, 7160–7164 (1991).

79. Kibota, T. T. & Lynch, M. Estimate of the genomic mutation rate deleterious to overall fitness in E. coll. *Nature* **381**, 694–696 (1996).

80. Perfeito, L., Fernandes, L., Mota, C. & Gordo, I. Adaptive Mutations in Bacteria: High Rate and Small Effects. *Science (80-.).* **317**, 813–815 (2007).

81. Kimura, M. Evolutionary Rate at the Molecular Level. *Nature* **217**, 624–626 (1968).

82. Kimura, M. The neutral theory of molecular evolution: A review of recent evidence. *Japanese J. Genet.* **66**, 367–386 (1991).

83. Ohta, T. Slightly Deleterious Mutant Substitutions in Evolution. *Nature* **246**, 96–98 (1973).

84. Ohta, T. & Gillespie, J. H. Development of Neutral and Nearly Neutral Theories. *Theor. Popul. Biol.* **49**, 128–142 (1996).

85. Kreitman, M. The neutral theory is dead. Long live the neutral theory. *BioEssays* **18**, 678–683 (1996).

86. Drake, J. W. Avoiding Dangerous Missense: Thermophiles Display Especially Low Mutation Rates. *PLOS Genet.* **5**, e1000520 (2009).

87. Wood, R. D., Gearhart, P. J., Neuberger, M. S. & Bridges, B. A. Hypermutation in bacteria and other cellular systems. *Philos. Trans. R. Soc. London. Ser. B*

Biol. Sci. **356**, 29–39 (2001).

88. Jin, D. J. & Gross, C. A. Mapping and sequencing of mutations in the Escherichia colirpoB gene that lead to rifampicin resistance. *J. Mol. Biol.* **202**, 45–58 (1988).

89. Rodríguez-Verdugo, A., Gaut, B. S. & Tenaillon, O. Evolution of Escherichia coli rifampicin resistance in an antibiotic-free environment during thermal stress. *BMC Evol. Biol.* **13**, 50 (2013).

90. Martinez, J. L. & Baquero, F. Mutation Frequencies and Antibiotic Resistance. *Antimicrob. Agents Chemother.* **44**, 1771–1777 (2000).

91. Andersson, D. I. & Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **12**, 465 (2014).

92. Harmand, N., Gallet, R., Jabbour-Zahab, R., Martin, G. & Lenormand, T. Fisher's geometrical model and the mutational patterns of antibiotic resistance across dose gradients. *Evolution (N. Y).* **71**, 23–37 (2017).

93. Riesenfeld, C., Everett, M., Piddock, L. J. & Hall, B. G. Adaptive mutations produce resistance to ciprofloxacin. *Antimicrob. Agents Chemother.* **41**, 2059–2060 (1997).

94. Cirz, R. T. *et al.* Inhibition of Mutation and Combating the Evolution of Antibiotic Resistance. *PLOS Biol.* **3**, e176 (2005).

95. Ysern, P. *et al.* Induction of SOS genes in Escherichia coli and mutagenesis in Salmonella typhimurium by fluoroquinolones. *Mutagenesis* **5**, 63–66 (1990).

96. Ren, L., Rahman, M. S. & Humayun, M. Z. Escherichia coli Cells Exposed to Streptomycin Display a Mutator Phenotype. *J. Bacteriol.* **181**, 1043–1044 (1999).

97. Chopra, I., O'Neill, A. J. & Miller, K. The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resist. Updat.* **6**, 137–145 (2003).

98. Tanabe, K., Kondo, T., Onodera, Y. & Furusawa, M. A conspicuous adaptability to antibiotics in the Escherichia coli mutator strain, dnaQ49. *FEMS Microbiol. Lett.* **176**, 191–196 (1999).

99. Miller, K., O'Neill, A. J. & Chopra, I. Response of Escherichia coli hypermutators to selection pressure with antimicrobial agents from different classes. *J. Antimicrob. Chemother.* **49**, 925–934 (2002).

100. LeClerc, J. E., Li, B., Payne, W. L. & Cebula, T. A. High Mutation Frequencies Among Escherichia coli and Salmonella Pathogens. *Science (80-.).* **274**, 1208–1211 (1996).

101. Oliver, A., Cantón, R., Campo, P., Baquero, F. & Blázquez, J. High Frequency of Hypermutable Pseudomonas aeruginosa in Cystic Fibrosis Lung Infection.

Science (80-.). **288**, 1251–1253 (2000).

102. Denamur, E. *et al.* High Frequency of Mutator Strains among Human Uropathogenic Escherichia coli Isolates. *J. Bacteriol.* **184**, 605–609 (2002).

103. Matic, I. *et al.* Highly Variable Mutation Rates in Commensal and Pathogenic Escherichia coli. *Science (80-.).* **277**, 1833–1834 (1997).

104. Denamur, E. *et al.* Evolutionary Implications of the Frequent Horizontal Transfer of Mismatch Repair Genes. *Cell* **103**, 711–721 (2000).

105. Macià, M. D., Pérez, J. L., Molin, S. & Oliver, A. Dynamics of Mutator and Antibiotic-Resistant Populations in a Pharmacokinetic/Pharmacodynamic Model of Pseudomonas aeruginosa Biofilm Treatment. *Antimicrob. Agents Chemother.* **55**, 5230 LP – 5237 (2011).

106. Luria, S. E. & Delbrück, M. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics* **28**, 491–511 (1943).

107. Demerec, M. Production of Staphylococcus Strains Resistant to Various Concentrations of Penicillin. *Proc. Natl. Acad. Sci. U. S. A.* **31**, 16–24 (1945).

108. Demerec, M. Origin of bacterial resistance to antibiotics. *J. Bacteriol.* **56**, 63–74 (1948).

109. Foster, P. L. B. T.-M. in E. Methods for Determining Spontaneous Mutation Rates. in *DNA Repair, Part B* **409**, 195–213 (Academic Press, 2006).

110. Randall, C. P., Mariner, K. R., Chopra, I. & O\textquoterightNeill, A. J. The Target of Daptomycin Is Absent from Escherichia coli and Other Gram-Negative Pathogens. *Antimicrob. Agents Chemother.* **57**, 637–639 (2013).

111. Kojima, S. & Nikaido, H. Permeation rates of penicillins indicate that Escherichia coli porins function principally as nonspecific channels. *Proc. Natl. Acad. Sci.* **110**, E2629--E2634 (2013).

112. Lavigne, J.-P. *et al.* An adaptive response of Enterobacter aerogenes to imipenem: regulation of porin balance in clinical isolates. *Int. J. Antimicrob. Agents* **41**, 130–136 (2013).

113. Tamber, S. & Hancock, R. E. W. On the mechanism of solute uptake in Pseudomonas. *Front. Biosci.* **8**, s472-83 (2003).

114. Grossman, T. H. Tetracycline Antibiotics and Resistance. *Cold Spring Harb. Perspect. Med.* **6**, (2016).

115. Dolejska, M., Villa, L., Poirel, L., Nordmann, P. & Carattoli, A. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance–nodulation–cell division/multidrug efflux pump. *J. Antimicrob. Chemother.* **68**, 34–39 (2012).

116. Hansen, L. H., Johannesen, E., Burmølle, M., Sørensen, A. H. & Sørensen, S. J. Plasmid-Encoded Multidrug Efflux Pump Conferring Resistance to Olaquindox in Escherichia coli. *Antimicrob. Agents Chemother.* **48**, 3332–3337 (2004).

117. Yamane, K. *et al.* New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an Escherichia coli Clinical Isolate. *Antimicrob. Agents Chemother.* **51**, 3354–3360 (2007).

118. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. MICROBIAL BIOFILMS. *Annu. Rev. Microbiol.* **49**, 711–745 (1995).

119. Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* **35**, 322–332 (2010).

120. Stewart, P. S. & William Costerton, J. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138 (2001).

121. Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J. & Chopra, I. Increased mutability of Pseudomonas aeruginosa in biofilms. *J. Antimicrob. Chemother.* **61**, 1053–1056 (2008).

122. Molin, S. & Tolker-Nielsen, T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr. Opin. Biotechnol.* **14**, 255–261 (2003).

123. Lambert, P. A. Bacterial resistance to antibiotics: Modified target sites. *Adv. Drug Deliv. Rev.* **57**, 1471–1485 (2005).

124. Dowson, C. G., Coffey, T. J. & Spratt, B. G. Origin and molecular epidemiologY of penicillin-binding-protein-mediated resistance to β-lactam antibiotics. *Trends Microbiol.* **2**, 361–366 (1994).

125. Spratt, B. G. Hybrid penicillin-binding proteins in penicillin-resistant strains of Neisseria gonorrhoeae. *Nature* **332**, 173–176 (1988).

126. Bowler, L. D., Zhang, Q. Y., Riou, J. Y. & Spratt, B. G. Interspecies recombination between the penA genes of Neisseria meningitidis and commensal Neisseria species during the emergence of penicillin resistance in N. meningitidis: natural events and laboratory simulation. *J. Bacteriol.* **176**, 333–337 (1994).

127. Enright, M. C. The evolution of a resistant pathogen – the case of MRSA. *Curr. Opin. Pharmacol.* **3**, 474–479 (2003).

128. Vesely, J. J. K., Pien, F. D. & Pien, B. C. T. Rifampin, a Useful Drug for Nonmycobacterial Infections. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **18**, 345–357 (1998).

129. Ramaswamy, S. & Musser, J. M. Molecular genetic basis of antimicrobial agent resistance inMycobacterium tuberculosis: 1998 update. *Tuber. Lung Dis.* **79**, 3–29 (1998).

130. Brandis, G., Pietsch, F., Alemayehu, R. & Hughes, D. Comprehensive phenotypic characterization of rifampicin resistance mutations in Salmonella provides insight into the evolution of resistance in Mycobacterium tuberculosis. *J. Antimicrob. Chemother.* **70**, 680–685 (2014).

131. Campbell, E. A. *et al.* Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. *Cell* **104**, 901–912 (2001).

132. Alifano, P., Palumbo, C., Pasanisi, D. & Talà, A. Rifampicin-resistance, rpoB polymorphism and RNA polymerase genetic engineering. *J. Biotechnol.* **202**, 60–77 (2015).

133. Ozaki, M., Mizushima, S. & Nomura, M. Identification and Functional Characterization of the Protein controlled by the Streptomycin-resistant Locus in E. coli. *Nature* **222**, 333–339 (1969).

134. Melancon, P., Lemieux, C. & Brakier-Gingras, L. A mutation in the 530 loop of Escherichia coli 16S ribosomal RNA causes resistance to streptomycin . *Nucleic Acids Res.* **16**, 9631–9639 (1988).

135. Springer, B. *et al.* Mechanisms of Streptomycin Resistance: Selection of Mutations in the 16S rRNA Gene Conferring Resistance. *Antimicrob. Agents Chemother.* **45**, 2877–2884 (2001).

136. Carter, A. P. *et al.* Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* **407**, 340 (2000).

137. Newcombe, H. B. & Hawirko, R. SPONTANEOUS MUTATION TO STREPTOMYCIN RESISTANCE AND DEPENDENCE IN ESCHERICHIA COLI. *J. Bacteriol.* **57**, 565–572 (1949).

138. von Ahsen, U. Translational fidelity: error-prone versus hyper-accurate ribosomes. *Chem. Biol.* **5**, R3–R6 (1998).

139. Leclercq, R. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clin. Infect. Dis.* **34**, 482–492 (2002).

140. Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S. & Vester, B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrob. Agents Chemother.* **50**, 2500–2505 (2006).

141. Galimand, M., Courvalin, P. & Lambert, T. Plasmid-Mediated High-Level Resistance to Aminoglycosides in Enterobacteriaceae Due to 16S rRNA Methylation. *Antimicrob. Agents Chemother.* **47**, 2565–2571 (2003).

142. Doi, Y. *et al.* Plasmid-mediated 16S rRNA methylase in Serratia marcescens conferring high-level resistance to aminoglycosides. *Antimicrob. Agents Chemother.* **48**, 491–496 (2004).

143. Vetting, M. W. *et al.* Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor. *J. Biol. Chem.* **286**, 25265–25273 (2011).

144. Koskiniemi, S., Pränting, M., Gullberg, E., Näsvall, J. & Andersson, D. I. Activation of cryptic aminoglycoside resistance in Salmonella enterica. *Mol. Microbiol.* **80**, 1464–1478 (2011).

145. Okamoto, S. *et al.* Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol. Microbiol.* **63**, 1096–1106 (2007).

146. Abraham, E. P. & Chain, E. An Enzyme from Bacteria able to Destroy Penicillin. *Nature* **146**, 837 (1940).

147. Fleming, A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. *Br. J. Exp. Pathol.* **10**, 226–236 (1929).

148. Queenan, A. M. & Bush, K. Carbapenemases: the Versatile β-Lactamases. *Clin. Microbiol. Rev.* **20**, 440–458 (2007).

149. Philippon, A., Labia, R. & Jacoby, G. Extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* **33**, 1131–1136 (1989).

150. Jacoby, G. A. & Medeiros, A. A. More extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* **35**, 1697–1704 (1991).

151. Nordmann, P., Poirel, L. & Dortet, L. Rapid detection of carbapenemase-producing Enterobacteriaceae. *Emerg. Infect. Dis.* **18**, 1503–1507 (2012).

152. Voulgari, E., Poulou, A., Koumaki, V. & Tsakris, A. Carbapenemase-producing Enterobacteriaceae: now that the storm is finally here, how will timely detection help us fight back? *Future Microbiol.* **8**, 27–39 (2013).

153. Johnson, A. P. & Woodford, N. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *J. Med. Microbiol.* **62**, 499–513 (2013).

154. Wright, G. D. Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Adv. Drug Deliv. Rev.* **57**, 1451–1470 (2005).

155. Schwarz, S., Kehrenberg, C., Doublet, B. & Cloeckaert, A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol. Rev.* **28**, 519–542 (2004).

156. Allignet, J. & el Solh, N. Diversity among the gram-positive acetyltransferases inactivating streptogramin A and structurally related compounds and

characterization of a new staphylococcal determinant, vatB. *Antimicrob. Agents Chemother.* **39**, 2027–2036 (1995).

157. Spanogiannopoulos, P., Waglechner, N., Koteva, K. & Wright, G. D. A rifamycin inactivating phosphotransferase family shared by environmental and pathogenic bacteria. *Proc. Natl. Acad. Sci.* **111**, 7102–7107 (2014).

158. Wright, G. D. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* **5**, 175–186 (2007).

159. Speer, B. S. & Salyers, A. A. Characterization of a novel tetracycline resistance that functions only in aerobically grown Escherichia coli. *J. Bacteriol.* **170**, 1423–1429 (1988).

160. Yang, W. *et al.* TetX Is a Flavin-dependent Monooxygenase Conferring Resistance to Tetracycline Antibiotics. *J. Biol. Chem.* **279**, 52346–52352 (2004).

161. Mukhtar, T. A., Koteva, K. P., Hughes, D. W. & Wright, G. D. Vgb from Staphylococcus aureus Inactivates Streptogramin B Antibiotics by an Elimination Mechanism Not Hydrolysis. *Biochemistry* **40**, 8877–8886 (2001).

162. Patwa, Z. & Wahl, L. M. The fixation probability of beneficial mutations. *J. R. Soc. Interface* **5**, 1279–1289 (2008).

163. Orr, H. A. Fitness and its role in evolutionary genetics. *Nat. Rev. Genet.* **10**, 531–539 (2009).

164. Melnyk, A. H., Wong, A. & Kassen, R. The fitness costs of antibiotic resistance mutations. *Evol. Appl.* **8**, 273–283 (2015).

165. Nöllmann, M., Crisona, N. J. & Arimondo, P. B. Thirty years of Escherichia coli DNA gyrase: From in vivo function to single-molecule mechanism. *Biochimie* **89**, 490–499 (2007).

166. Wichmann, F., Udikovic-Kolic, N., Andrew, S. & Handelsman, J. Diverse Antibiotic Resistance Genes in Dairy Cow Manure. *MBio* **5**, (2014).

167. Toivonen, J. M., Boocock, M. R. & Jacobs, H. T. Modelling in Escherichia coli of mutations in mitoribosomal protein S12: novel mutant phenotypes of rpsL. *Mol. Microbiol.* **31**, 1735–1746 (1999).

168. Zapun, A., Contreras-Martel, C. & Vernet, T. Penicillin-binding proteins and β-lactam resistance. *FEMS Microbiol. Rev.* **32**, 361–385 (2008).

169. Maisnier-Patin, S. & Andersson, D. I. Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res. Microbiol.* **155**, 360–369 (2004).

170. Barnard, A. M. L., Simpson, N. J. L., Lilley, K. S. & Salmond, G. P. C. Mutations in rpsL that confer streptomycin resistance show pleiotropic effects on

virulence and the production of a carbapenem antibiotic in Erwinia carotovora. *Microbiology* **156**, 1030–1039 (2010).

171. Qi, Q., Preston, G. M. & MacLean, R. C. Linking System-Wide Impacts of RNA Polymerase Mutations to the Fitness Cost of Rifampin Resistance in Pseudomonas aeruginosa. *MBio* **5**, (2014).

172. Robinson, L. J., Cameron, A. D. S. & Stavrinides, J. Spontaneous and on point: Do spontaneous mutations used for laboratory experiments cause pleiotropic effects that might confound bacterial infection and evolution assays? *FEMS Microbiol. Lett.* **362**, (2015).

173. Zünd, P. & Lebek, G. Generation time-prolonging R plasmids: Correlation between increases in the generation time of Escherichia coli caused by R plasmids and their molecular size. *Plasmid* **3**, 65–69 (1980).

174. San Millan, A., Toll-Riera, M., Qi, Q. & MacLean, R. C. Interactions between horizontally acquired genes create a fitness cost in Pseudomonas aeruginosa. *Nat. Commun.* **6**, 6845 (2015).

175. Touchon, M., Bobay, L.-M. & Rocha, E. P. C. The chromosomal accommodation and domestication of mobile genetic elements. *Curr. Opin. Microbiol.* **22**, 22–29 (2014).

176. Lenski, R. E. Bacterial evolution and the cost of antibiotic resistance. *Int. Microbiol.* **1**, 265–270 (1998).

177. Gagneux, S. *et al.* The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. *Science* **312**, 1944–1946 (2006).

178. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **8**, 260–271 (2010).

179. Vogwill, T. & MacLean, R. C. The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol. Appl.* **8**, 284–295 (2015).

180. Hernando-Amado, S., Sanz-García, F., Blanco, P. & Martínez, J. L. Fitness costs associated with the acquisition of antibiotic resistance. *Essays Biochem.* **61**, 37–48 (2017).

181. Enne, V. I. Reducing antimicrobial resistance in the community by restricting prescribing: can it be done? *J. Antimicrob. Chemother.* **65**, 179–182 (2009).

182. Seppälä, H. *et al.* The Effect of Changes in the Consumption of Macrolide Antibiotics on Erythromycin Resistance in Group A Streptococci in Finland. *N. Engl. J. Med.* **337**, 441–446 (1997).

183. Austin, D. J., Kristinsson, K. G. & Anderson, R. M. The relationship between the volume of antimicrobial consumption in human communities and the

frequency of resistance. *Proc. Natl. Acad. Sci.* **96**, 1152–1156 (1999).

184. Bergman, M. *et al.* Effect of Macrolide Consumption on Erythromycin Resistance in Streptococcus pyogenes in Finland in 1997–2001. *Clin. Infect. Dis.* **38**, 1251–1256 (2004).

185. Bean, D. C., Livermore, D. M., Papa, I. & Hall, L. M. C. Resistance among Escherichia coli to sulphonamides and other antimicrobials now little used in man. *J. Antimicrob. Chemother.* **56**, 962–964 (2005).

186. Gottesman, B. S., Carmeli, Y., Shitrit, P. & Chowers, M. Impact of Quinolone Restriction on Resistance Patterns of Escherichia coli Isolated from Urine by Culture in a Community Setting. *Clin. Infect. Dis.* **49**, 869–875 (2009).

187. Enne, V. I., Livermore, D. M., Stephens, P. & Hall, L. M. C. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. *Lancet* **357**, 1325–1328 (2001).

188. Sundqvist, M. *et al.* Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J. Antimicrob. Chemother.* **65**, 350–360 (2009).

189. Arason, V. A. *et al.* Clonal Spread of Resistant Pneumococci Despite Diminished Antimicrobial Use. *Microb. Drug Resist.* **8**, 187–192 (2002).

190. Björkman, J., Hughes, D. & Andersson, D. I. Virulence of antibiotic-resistant Salmonella typhimurium *Proc. Natl. Acad. Sci.* **95**, 3949 LP – 3953 (1998).

191. Björkman, J., Nagaev, I., Berg, O. G., Hughes, D. & Andersson, D. I. Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* **287**, 1479–1482 (2000).

192. Trindade, S. *et al.* Positive Epistasis Drives the Acquisition of Multidrug Resistance. *PLOS Genet.* **5**, e1000578 (2009).

193. Bohman, K., Ruusala, T., Jelenc, P. C. & Kurland, C. G. Kinetic impairment of restrictive streptomycin-resistant ribosomes. *Mol. Gen. Genet. MGG* **198**, 90–99 (1984).

194. Wiegand, I., Hilpert, K. & Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3**, 163–175 (2008).

195. Lofton, H., Pränting, M., Thulin, E. & Andersson, D. I. Mechanisms and Fitness Costs of Resistance to Antimicrobial Peptides LL-37, CNY100HL and Wheat Germ Histones. *PLoS One* **8**, e68875 (2013).

196. Moura de Sousa, J., Balbontín, R., Durão, P. & Gordo, I. Multidrug-resistant bacteria compensate for the epistasis between resistances. *PLOS Biol.* **15**,

e2001741 (2017).

197. Zhang, F. *et al.* Whole-genome sequencing reveals the mechanisms for evolution of streptomycin resistance in Lactobacillus plantarum. *J. Dairy Sci.* **101**, 2867–2874 (2018).

198. Hughes, D. & Andersson, D. I. Evolutionary Trajectories to Antibiotic Resistance. *Annu. Rev. Microbiol.* **71**, 579–596 (2017).

199. Thulin, E., Sundqvist, M. & Andersson, D. I. Amdinocillin (Mecillinam) resistance mutations in clinical isolates and laboratory-selected mutants of Escherichia coli. *Antimicrob. Agents Chemother.* **59**, 1718–1727 (2015).

200. Nilsson, A. I., Berg, O. G., Aspevall, O., Kahlmeter, G. & Andersson, D. I. Biological costs and mechanisms of fosfomycin resistance in Escherichia coli. *Antimicrob. Agents Chemother.* **47**, 2850–2858 (2003).

201. Gullberg, E. *et al.* Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. *PLOS Pathog.* **7**, e1002158 (2011).

202. Andersson, D. I. & Hughes, D. Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resist. Updat.* **15**, 162–172 (2012).

203. Baquero, F. Low-level antibacterial resistance: a gateway to clinical resistance. *Drug Resist. Updat.* **4**, 93–105 (2001).

204. Liu, A. *et al.* Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: a Simple and Ultrasensitive Color Test for Detection of Antibiotics and Genotoxic Agents. *Antimicrob. Agents Chemother.* **55**, 1204–1210 (2011).

205. Wistrand-Yuen, E. *et al.* Evolution of high-level resistance during low-level antibiotic exposure. *Nat. Commun.* **9**, 1599 (2018).

206. Beaber, J. W., Hochhut, B. & Waldor, M. K. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**, 72–74 (2004).

207. Úbeda, C. *et al.* Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol. Microbiol.* **56**, 836–844 (2005).

208. Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. & Claverys, J.-P. Antibiotic Stress Induces Genetic Transformability in the Human Pathogen Streptococcus pneumoniae. *Science (80-.).* **313**, 89–92 (2006).

209. Hocquet, D. *et al.* Evidence for Induction of Integron-Based Antibiotic Resistance by the SOS Response in a Clinical Setting. *PLOS Pathog.* **8**, e1002778 (2012).

210. López, E., Elez, M., Matic, I. & Blázquez, J. Antibiotic-mediated recombination: ciprofloxacin stimulates SOS-independent recombination of divergent sequences in Escherichia coli. *Mol. Microbiol.* **64**, 83–93 (2007).

211. López, E. & Blázquez, J. Effect of Subinhibitory Concentrations of Antibiotics on Intrachromosomal Homologous Recombination in Escherichia coli *Antimicrob. Agents Chemother.* **53**, 3411 LP – 3415 (2009).

212. Wu, Y. L., Scott, E. M., Po, A. L. I. W. A. N. & Tariq, V. N. Development of resistance and cross-resistance in Pseudomonas aeruginosa exposed to subinhibitory antibiotic concentrations. *APMIS* **107**, 585–592 (1999).

213. Kohanski, M. A., DePristo, M. A. & Collins, J. J. Sublethal Antibiotic Treatment Leads to Multidrug Resistance via Radical-Induced Mutagenesis. *Mol. Cell* **37**, 311–320 (2010).

214. Baharoglu, Z. & Mazel, D. Vibrio cholerae Triggers SOS and Mutagenesis in Response to a Wide Range of Antibiotics: a Route towards Multiresistance. *Antimicrob. Agents Chemother.* **55**, 2438–2441 (2011).

215. Schlacher, K. & Goodman, M. F. Lessons from 50 years of SOS DNA-damage-induced mutagenesis. *Nat. Rev. Mol. Cell Biol.* **8**, 587–594 (2007).

216. Gutierrez, A. *et al.* β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. *Nat. Commun.* **4**, 1610 (2013).

217. Battesti, A., Majdalani, N. & Gottesman, S. The RpoS-Mediated General Stress Response in Escherichia coli. *Annu. Rev. Microbiol.* **65**, 189–213 (2011).

218. Baker-Austin, C., Wright, M. S., Stepanauskas, R. & McArthur, J. V. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* **14**, 176–182 (2006).

219. Wales, A. D. & Davies, R. H. Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. *Antibiotics* **4**, 567–604 (2015).

220. Chapman, J. S. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegradation* **51**, 271–276 (2003).

221. Levy, S. B. Active efflux mechanisms for antimicrobial resistance. *Antimicrob. Agents Chemother.* **36**, 695–703 (1992).

222. Li, X.-Z. & Nikaido, H. Efflux-Mediated Drug Resistance in Bacteria. *Drugs* **64**, 159–204 (2004).

223. Mata, M. T., Baquero, F. & Pérez-Díaz, J. C. A multidrug efflux transporter in Listeria monocytogenes. *FEMS Microbiol. Lett.* **187**, 185–188 (2000).

224. Hayashi, S., Abe, M., Kimoto, M., Furukawa, S. & Nakazawa, T. The DsbA-DsbB Disulfide Bond Formation System of *Burkholderia cepacia* Is Involved in the Production of Protease and Alkaline Phosphatase, Motility, Metal Resistance, and Multi-Drug Resistance. *Microbiol. Immunol.* **44**, 41–50 (2000).

225. Aendekerk, S., Ghysels, B., Cornelis, P. & Baysse, C. Characterization of a new efflux pump, MexGHI-OpmD, from Pseudomonas aeruginosa that confers resistance to vanadium. *Microbiology* **148**, 2371–2381 (2002).

226. Piddock, L. J. V. Multidrug-resistance efflux pumps ? not just for resistance. *Nat. Rev. Microbiol.* **4**, 629–636 (2006).

227. Novick, R. P. & Roth, C. Plasmid-linked Resistance to Inorganic Salts in Staphylococcus aureus. *J. Bacteriol.* **95**, 1335–1342 (1968).

228. Foster, T. J. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.* **47**, 361–409 (1983).

229. Summers, A. O. *et al.* Mercury released from dental 'silver' fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. *Antimicrob. Agents Chemother.* **37**, 825–834 (1993).

230. Ghosh, A., Singh, A., Ramteke, P. W. & Singh, V. P. Characterization of Large Plasmids Encoding Resistance to Toxic Heavy Metals in Salmonella abortus equi. *Biochem. Biophys. Res. Commun.* **272**, 6–11 (2000).

231. Wireman, J., Liebert, C. A., Smith, T. & Summers, A. O. Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates. *Appl. Environ. Microbiol.* **63**, 4494–4503 (1997).

232. Bass, L. *et al.* Incidence and Characterization of Integrons, Genetic Elements Mediating Multiple-Drug Resistance, in AvianEscherichia coli. *Antimicrob. Agents Chemother.* **43**, 2925–2929 (1999).

233. Liebert, C. A., Hall, R. M. & Summers, A. O. Transposon Tn21, Flagship of the Floating Genome. *Microbiol. Mol. Biol. Rev.* **63**, 507–522 (1999).

234. Mindlin, S. *et al.* Present-day mercury resistance transposons are common in bacteria preserved in permafrost grounds since the Upper Pleistocene. *Res. Microbiol.* **156**, 994–1004 (2005).

235. Hughes, V. M. & Datta, N. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**, 725–726 (1983).

236. Lee, L. J., Barrett, J. A. & Poole, R. K. Genome-Wide Transcriptional Response of Chemostat-Cultured Escherichia coli to Zinc. *J. Bacteriol.* **187**, 1124–1134 (2005).

237. Baranova, N. & Nikaido, H. The BaeSR Two-Component Regulatory System Activates Transcription of the yegMNOB (mdtABCD) Transporter Gene Cluster in Escherichia coli and Increases Its Resistance to Novobiocin and Deoxycholate. *J. Bacteriol.* **184**, 4168–4176 (2002).

238. Perron, K. *et al.* CzcR-CzcS, a Two-component System Involved in Heavy Metal and Carbapenem Resistance in Pseudomonas aeruginosa. *J. Biol.*

Chem. **279**, 8761–8768 (2004).

239. Reynolds, M. G. Compensatory Evolution in Rifampin-Resistant Escherichia coli. *Genetics* **156**, 1471–1481 (2000).

240. Maisnier-Patin, S., Berg, O. G., Liljas, L. & Andersson, D. I. Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium. *Mol. Microbiol.* **46**, 355–366 (2002).

241. Besier, S., Ludwig, A., Brade, V. & Wichelhaus, T. A. Compensatory Adaptation to the Loss of Biological Fitness Associated with Acquisition of Fusidic Acid Resistance in Staphylococcus aureus. *Antimicrob. Agents Chemother.* **49**, 1426–1431 (2005).

242. Nagaev, I., Björkman, J., Andersson, D. I. & Hughes, D. Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. *Mol. Microbiol.* **40**, 433–439 (2001).

243. Comas, I. *et al.* Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* **44**, 106–110 (2012).

244. Zhang, H. *et al.* Genome sequencing of 161 Mycobacterium tuberculosis isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat. Genet.* **45**, 1255–1260 (2013).

245. Brandis, G. & Hughes, D. Genetic characterization of compensatory evolution in strains carrying rpoB Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. *J. Antimicrob. Chemother.* **68**, 2493–2497 (2013).

246. Brandis, G. & Hughes, D. Mechanisms of fitness cost reduction for rifampicin-resistant strains with deletion or duplication mutations in rpoB. *Sci. Rep.* **8**, 17488 (2018).

247. Johanson, U., Ævarsson, A., Liljas, A. & Hughes, D. The Dynamic Structure of EF-G Studied by Fusidic Acid Resistance and Internal Revertants. *J. Mol. Biol.* **258**, 420–432 (1996).

248. Moore, F. B.-G., Rozen, D. E. & Lenski, R. E. Pervasive compensatory adaptation in *Escherichia coli*. *Proc. R. Soc. London. Ser. B Biol. Sci.* **267**, 515–522 (2000).

249. Schrag, S. J., Perrot, V. & Levin, B. R. Adaptation to the fitness costs of antibiotic resistance in Escherichia coli. *Proceedings. Biol. Sci.* **264**, 1287–1291 (1997).

250. Orencia, M. C., Yoon, J. S., Ness, J. E., Stemmer, W. P. C. & Stevens, R. C. Predicting the emergence of antibiotic resistance by directed evolution and structural analysis. *Nat. Struct. Biol.* **8**, 238–242 (2001).

251. Huang, W. & Palzkill, T. A natural polymorphism in β-lactamase is a global suppressor. *Proc. Natl. Acad. Sci.* **94**, 8801–8806 (1997).

252. Sideraki, V., Huang, W., Palzkill, T. & Gilbert, H. F. A secondary drug resistance mutation of TEM-1 β-lactamase that suppresses misfolding and aggregation. *Proc. Natl. Acad. Sci.* **98**, 283–288 (2001).

253. Fermér, C. & Swedberg, G. Adaptation to sulfonamide resistance in Neisseria meningitidis may have required compensatory changes to retain enzyme function: kinetic analysis of dihydropteroate synthases from N. meningitidis expressed in a knockout mutant of Escherichia coli. *J. Bacteriol.* **179**, 831 LP – 837 (1997).

254. Blance, S. J. *et al.* Temperature-sensitive suppressor mutations of the Escherichia coli DNA gyrase B protein. *Protein Sci.* **9**, 1035–1037 (2000).

255. Paulander, W., Maisnier-Patin, S. & Andersson, D. I. Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in Salmonella typhimurium. *Mol. Microbiol.* **64**, 1038–1048 (2007).

256. Schrag, S. J. & Perrot, V. Reducing antibiotic resistance. *Nature* **381**, 120–121 (1996).

257. Jin, D. J. & Turnbough, C. L. An Escherichia coli RNA Polymerase Defective in Transcription due to its Overproduction of Abortive Initiation Products. *J. Mol. Biol.* **236**, 72–80 (1994).

258. Brandis, G., Wrande, M., Liljas, L. & Hughes, D. Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. *Mol. Microbiol.* **85**, 142–151 (2012).

259. de Sousa, J., Sousa, A., Bourgard, C. & Gordo, I. Potential for adaptation overrides cost of resistance. *Future Microbiol.* **10**, 1415–1431 (2015).

260. Sherman, D. R. *et al.* Compensatory ahpC Gene Expression in Isoniazid-Resistant Mycobacterium tuberculosis. *Science (80-.).* **272**, 1641–1643 (1996).

261. Nilsson, A. I. *et al.* Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proc. Natl. Acad. Sci.* **103**, 6976–6981 (2006).

262. Levin, B. R., Perrot, V. & Walker, N. Compensatory Mutations, Antibiotic Resistance and the Population Genetics of Adaptive Evolution in Bacteria. *Genetics* **154**, 985–997 (2000).

263. Böck, A., Petzet, A. & Piepersberg, W. Ribosomal ambiguity (ram) mutations facilitate dihydrostreptomycin binding to ribosomes. *FEBS Lett.* **104**, 317–321 (1979).

264. Kraal, B. *et al.* Antibiotic resistance mechanisms of mutant EF-Tu species in Escherichia coli. *Biochem. Cell Biol.* **73**, 1167–1177 (1995).

265. Dunai, A. *et al.* Rapid decline of bacterial drug-resistance in an antibiotic-free environment through phenotypic reversion. *Elife* **8**, e47088 (2019).

266. Ayhan, D. H. *et al.* Sequence-Specific Targeting of Bacterial Resistance Genes Increases Antibiotic Efficacy. *PLOS Biol.* **14**, 1–18 (2016).

267. Blondiaux, N. *et al.* Reversion of antibiotic resistance in Mycobacterium tuberculosis by spiroisoxazoline SMARt-420. *Science (80-.).* **355**, 1206–1211 (2017).

268. García-Fernández, E. *et al.* Membrane Microdomain Disassembly Inhibits MRSA Antibiotic Resistance. *Cell* **171**, 1354-1367.e20 (2017).

269. Hershberg, R. Antibiotic-Independent Adaptive Effects of Antibiotic Resistance Mutations. *Trends Genet.* **33**, 521–528 (2017).

270. Skurnik, D. *et al.* Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing. *Proc. Natl. Acad. Sci.* **110**, 20747–20752 (2013).

271. Paulander, W., Maisnier-Patin, S. & Andersson, D. I. The Fitness Cost of Streptomycin Resistance Depends on rpsL Mutation, Carbon Source and RpoS (σS). *Genetics* **183**, 539–546 (2009).

272. Trindade, S., Sousa, A. & Gordo, I. ANTIBIOTIC RESISTANCE AND STRESS IN THE LIGHT OF FISHER'S MODEL. *Evolution (N. Y).* **66**, 3815–3824 (2012).

273. Hall, A. R., Angst, D. C., Schiessl, K. T. & Ackermann, M. Costs of antibiotic resistance - separating trait effects and selective effects. *Evol. Appl.* **8**, 261–272 (2015).

274. Durão, P., Trindade, S., Sousa, A. & Gordo, I. Multiple Resistance at No Cost: Rifampicin and Streptomycin a Dangerous Liaison in the Spread of Antibiotic Resistance. *Mol. Biol. Evol.* **32**, 2675–2680 (2015).

275. Zeyl, C. How missing genes interact. *Nat. Genet.* **39**, 440–442 (2007).

276. Wong, A. Epistasis and the Evolution of Antimicrobial Resistance. *Front. Microbiol.* **8**, 246 (2017).

277. Segrè, D., DeLuna, A., Church, G. M. & Kishony, R. Modular epistasis in yeast metabolism. *Nat. Genet.* **37**, 77–83 (2005).

278. Jasnos, L. & Korona, R. Epistatic buffering of fitness loss in yeast double deletion strains. *Nat. Genet.* **39**, 550–554 (2007).

279. Weinreich, D. M., Watson, R. A. & Chao, L. Perspective: Sign epistasis and

genetic constraint on evolutionary trajectories. *Evolution* **59**, 1165–1174 (2005).

280. Poelwijk, F. J., Kiviet, D. J., Weinreich, D. M. & Tans, S. J. Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* **445**, 383–386 (2007).

281. Borrell, S. *et al.* Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol. Med. Public Heal.* **2013**, 65–74 (2013).

282. Ward, H., Perron, G. G. & Maclean, R. C. The cost of multiple drug resistance in Pseudomonas aeruginosa. *J. Evol. Biol.* **22**, 997–1003 (2009).

283. Angst, D. C. & Hall, A. R. The cost of antibiotic resistance depends on evolutionary history in Escherichia coli. *BMC Evol. Biol.* **13**, 163 (2013).

284. San Millan, A., Heilbron, K. & MacLean, R. C. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME J.* **8**, 601–612 (2014).

285. Silva, R. F. *et al.* Pervasive sign epistasis between conjugative plasmids and drug-resistance chromosomal mutations. *PLoS Genet.* **7**, e1002181 (2011).

286. Lenski, R. E. & Travisano, M. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6808–6814 (1994).

287. Elena, S. F. *et al.* EVOLUTIONARY DYNAMICS OF FITNESS RECOVERY FROM THE DEBILITATING EFFECTS OF MULLER'S RATCHET. *Evolution (N. Y).* **52**, 309–314 (1998).

288. Schoustra, S. E., Bataillon, T., Gifford, D. R. & Kassen, R. The properties of adaptive walks in evolving populations of fungus. *PLoS Biol.* **7**, e1000250 (2009).

289. MacLean, R. C., Perron, G. G. & Gardner, A. Diminishing returns from beneficial mutations and pervasive epistasis shape the fitness landscape for rifampicin resistance in Pseudomonas aeruginosa. *Genetics* **186**, 1345–1354 (2010).

290. Chou, H.-H., Chiu, H.-C., Delaney, N. F., Segrè, D. & Marx, C. J. Diminishing Returns Epistasis Among Beneficial Mutations Decelerates Adaptation. *Science (80-.).* **332**, 1190–1192 (2011).

291. Khan, A. I., Dinh, D. M., Schneider, D., Lenski, R. E. & Cooper, T. F. Negative Epistasis Between Beneficial Mutations in an Evolving Bacterial Population. *Science (80-.).* **332**, 1193–1196 (2011).

292. Palmer, A. C. & Kishony, R. Understanding, predicting and manipulating the

genotypic evolution of antibiotic resistance. *Nat. Rev. Genet.* **14**, 243–248 (2013).

293. Lázár, V. *et al.* Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat. Commun.* **5**, 4352 (2014).

294. Imamovic, L. & Sommer, M. O. A. Use of Collateral Sensitivity Networks to Design Drug Cycling Protocols That Avoid Resistance Development. *Sci. Transl. Med.* **5**, 204ra132--204ra132 (2013).

295. Durão, P., Balbontín, R. & Gordo, I. Evolutionary Mechanisms Shaping the Maintenance of Antibiotic Resistance. *Trends Microbiol.* **26**, 677–691 (2018).

296. Hooper, L. V & Gordon, J. I. Commensal Host-Bacterial Relationships in the Gut. *Science (80-.).* **292**, 1115–1118 (2001).

297. Marshall B, O. D. L. S. Commensals: Underappreciated Reservoir of Antibiotic Resistance. *Microbe Mag.* **4**, 231–238 (2009).

298. von Wintersdorff, C. J. H. *et al.* Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* **7**, 173 (2016).

299. Francino, M. P. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Front. Microbiol.* **6**, 1543 (2016).

300. Andremont, A. Commensal flora may play key role in spreading antibiotic resistance. *ASM News* **63**, 601–607 (2003).

301. Akiba, T., Koyama, K., Ishiki, Y., Kimura, S. & Fukushima, T. ON THE MECHANISM OF THE DEVELOPMENT OF MULTIPLE-DRUG-RESISTANT CLONES OF SHIGELLA. *Jpn. J. Microbiol.* **4**, 219–227 (1960).

302. Guinee, P. A. Transfer of multiple drug resistance from Escherichia coli to Salmonella typhi murium in the mouse intestine. *Antonie Van Leeuwenhoek* **31**, 314–322 (1965).

303. Sundin, G. W. & Bender, C. L. Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol. Ecol.* **5**, 133–143 (1996).

304. Shoemaker, N. B., Vlamakis, H., Hayes, K. & Salyers, A. A. Evidence for Extensive Resistance Gene Transfer amongBacteroides spp. and among Bacteroides and Other Genera in the Human Colon. *Appl. Environ. Microbiol.* **67**, 561–568 (2001).

305. Hawkey, P. M. & Jones, A. M. The changing epidemiology of resistance. *J. Antimicrob. Chemother.* **64**, i3–i10 (2009).

306. Cantón, R. & Coque, T. M. The CTX-M β-lactamase pandemic. *Curr. Opin. Microbiol.* **9**, 466–475 (2006).

307. Robicsek, A., Jacoby, G. A. & Hooper, D. C. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect. Dis.* **6**, 629–640 (2006).

308. Poirel, L., Rodriguez-Martinez, J.-M., Mammeri, H., Liard, A. & Nordmann, P. Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* **49**, 3523–3525 (2005).

309. Laible, G., Spratt, B. G. & Hakenbeck, R. Interspecies recombinational events during the evolution of altered PBP 2x genes in penicillin-resistant clinical isolates of Streptococcus pneumoniae. *Mol. Microbiol.* **5**, 1993–2002 (1991).

310. Dowson, C. G., Hutchison, A. & Spratt, B. G. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of Streptococcus pneumoniae. *Mol. Microbiol.* **3**, 95–102 (1989).

311. Sibold, C. *et al.* Mosaic pbpX genes of major clones of penicillin-resistant Streptococcus pneumoniae have evolved from pbpX genes of a penicillin-sensitive Streptococcus oralis. *Mol. Microbiol.* **12**, 1013–1023 (1994).

312. Jensen, A., Valdórsson, O., Frimodt-Møller, N., Hollingshead, S. & Kilian, M. Commensal Streptococci Serve as a Reservoir for β-Lactam Resistance Genes in Streptococcus pneumoniae. *Antimicrob. Agents Chemother.* **59**, 3529–3540 (2015).

313. D'Costa, V. M., McGrann, K. M., Hughes, D. W. & Wright, G. D. Sampling the Antibiotic Resistome. *Science (80-.).* **311**, 374 LP – 377 (2006).

314. Forsberg, K. J. *et al.* The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**, 1107–1111 (2012).

315. Rizzo, L. *et al.* Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci. Total Environ.* **447**, 345–360 (2013).

316. Penders, J., Stobberingh, E., Savelkoul, P. & Wolffs, P. The human microbiome as a reservoir of antimicrobial resistance. *Front. Microbiol.* **4**, 87 (2013).

317. Marshall, B. M. & Levy, S. B. Food Animals and Antimicrobials: Impacts on Human Health. *Clin. Microbiol. Rev.* **24**, 718–733 (2011).

318. Wright, G. D. The antibiotic resistome. *Expert Opin. Drug Discov.* **5**, 779–788 (2010).

319. Eckburg, P. B. *et al.* Diversity of the Human Intestinal Microbial Flora. *Science (80-.).* **308**, 1635–1638 (2005).

320. von Wintersdorff, C. J. H. *et al.* High rates of antimicrobial drug resistance gene acquisition after international travel, The Netherlands. *Emerg. Infect. Dis.*

20, 649–657 (2014).

321. Liu, Y.-Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* **16**, 161–168 (2016).

322. Lam, K. N., Cheng, J., Engel, K., Neufeld, J. D. & Charles, T. C. Current and future resources for functional metagenomics. *Front. Microbiol.* **6**, 1196 (2015).

323. Johnson, J., Jain, K. & Madamwar, D. 2 - Functional Metagenomics: Exploring Nature's Gold Mine. in (eds. Gunasekaran, P., Noronha, S. & Pandey, A. B. T.-C. D. in B. and B.) 27–43 (Elsevier, 2017). doi:https://doi.org/10.1016/B978-0-444-63667-6.00002-X

324. Riesenfeld, C. S., Goodman, R. M. & Handelsman, J. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ. Microbiol.* **6**, 981–989 (2004).

325. Torres-Cortés, G. *et al.* Characterization of novel antibiotic resistance genes identified by functional metagenomics on soil samples. *Environ. Microbiol.* **13**, 1101–1114 (2011).

326. Mori, T., Mizuta, S., Suenaga, H. & Miyazaki, K. Metagenomic Screening for Bleomycin Resistance Genes. *Appl. Environ. Microbiol.* **74**, 6803–6805 (2008).

327. Parsley, L. C. *et al.* Identification of Diverse Antimicrobial Resistance Determinants Carried on Bacterial, Plasmid, or Viral Metagenomes from an Activated Sludge Microbial Assemblage. *Appl. Environ. Microbiol.* **76**, 3753–3757 (2010).

328. Munck, C. *et al.* Limited dissemination of the wastewater treatment plant core resistome. *Nat. Commun.* **6**, 8452 (2015).

329. Kazimierczak, K. A., Scott, K. P., Kelly, D. & Aminov, R. I. Tetracycline Resistome of the Organic Pig Gut. *Appl. Environ. Microbiol.* **75**, 1717–1722 (2009).

330. Sommer, M. O. A., Dantas, G. & Church, G. M. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **325**, 1128–1131 (2009).

331. Cheng, G. *et al.* Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS Microbiol. Lett.* **336**, 11–16 (2012).

332. Moore, A. M. *et al.* Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. *PLoS One* **8**, e78822–e78822 (2013).

333. Sokurenko, E. V, Hasty, D. L. & Dykhuizen, D. E. Pathoadaptive mutations:

gene loss and variation in bacterial pathogens. *Trends Microbiol.* **7**, 191–195 (1999).

334. Hacker, J., Blum-Oehler, G., Mühldorfer, I. & Tschäpe, H. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol. Microbiol.* **23**, 1089–1097 (1997).

335. Hacker, J. & Kaper, J. B. Pathogenicity Islands and the Evolution of Microbes. *Annu. Rev. Microbiol.* **54**, 641–679 (2000).

336. Miskinyte, M. *et al.* The Genetic Basis of Escherichia coli Pathoadaptation to Macrophages. *PLOS Pathog.* **9**, e1003802 (2013).

337. Proença, J. T., Barral, D. C. & Gordo, I. Commensal-to-pathogen transition: One-single transposon insertion results in two pathoadaptive traits in Escherichia coli -macrophage interaction. *Sci. Rep.* **7**, 4504 (2017).

338. Mahenthiralingam, E., Campbell, M. E. & Speert, D. P. Nonmotility and phagocytic resistance of Pseudomonas aeruginosa isolates from chronically colonized patients with cystic fibrosis. *Infect. Immun.* **62**, 596–605 (1994).

339. Boucher, J. C., Yu, H., Mudd, M. H. & Deretic, V. Mucoid Pseudomonas aeruginosa in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect. Immun.* **65**, 3838–3846 (1997).

340. Yu, H., Hanes, M., Chrisp, C. E., Boucher, J. C. & Deretic, V. Microbial Pathogenesis in Cystic Fibrosis: Pulmonary Clearance of Mucoid Pseudomonas aeruginosa and Inflammation in a Mouse Model of Repeated Respiratory Challenge. *Infect. Immun.* **66**, 280–288 (1998).

341. Kroll, J. S., Moxon, E. R. & Loynds, B. M. An Ancestral Mutation Enhancing the Fitness and Increasing the Virulence of Haemophilus influenzae Type b. *J. Infect. Dis.* **168**, 172–176 (1993).

342. von Eiff, C. *et al.* A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. *J. Bacteriol.* **179**, 4706 LP – 4712 (1997).

343. Maurelli, A. T., Fernández, R. E., Bloch, C. A., Rode, C. K. & Fasano, A. {\textquotedblleft}Black holes{\textquotedblright} and bacterial pathogenicity: A large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive Escherichia coli. *Proc. Natl. Acad. Sci.* **95**, 3943–3948 (1998).

344. Okuda, J. & Nishibuchi, M. Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of Vibrio parahaemolyticus depends on a particular single base change in the promoter of the thermostable direct haemolysin gene. *Mol. Microbiol.* **30**, 499–511 (1998).

345. Sokurenko, E. V *et al.* Pathogenic adaptation of Escherichia coli by natural

variation of the FimH adhesin. *Proc. Natl. Acad. Sci.* **95**, 8922–8926 (1998).

346. Miskinyte, M. & Gordo, I. Increased survival of antibiotic-resistant Escherichia coli inside macrophages. *Antimicrob. Agents Chemother.* **57**, 189–195 (2013).

347. Durão, P., Gülereşi, D., Proença, J. & Gordo, I. Enhanced Survival of Rifampin- and Streptomycin-Resistant Escherichia coli Inside Macrophages. *Antimicrob. Agents Chemother.* **60**, 4324–4332 (2016).

348. Ramiro, R. S., Costa, H. & Gordo, I. Macrophage adaptation leads to parallel evolution of genetically diverse Escherichia coli small-colony variants with increased fitness in vivo and antibiotic collateral sensitivity. *Evol. Appl.* **9**, 994–1004 (2016).

349. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biol.* **14**, e1002533 (2016).

350. Qin, J. *et al.* A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59–65 (2010).

351. Ley, R. E. *et al.* Evolution of mammals and their gut microbes. *Science* **320**, 1647–1651 (2008).

352. Dominguez-Bello, M. G., Godoy-Vitorino, F., Knight, R. & Blaser, M. J. Role of the microbiome in human development. *Gut* **68**, 1108–1114 (2019).

353. Nishida, A. H. & Ochman, H. A great-ape view of the gut microbiome. *Nat. Rev. Genet.* **20**, 195–206 (2019).

354. Berg, R. D. The indigenous gastrointestinal microflora. *Trends Microbiol.* **4**, 430–435 (1996).

355. Sommer, F. & Bäckhed, F. The gut microbiota — masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238 (2013).

356. Nicholson, J. K. *et al.* Host-Gut Microbiota Metabolic Interactions. *Science (80-.).* **336**, 1262–1267 (2012).

357. Hooper, L. V., Littman, D. R. & Macpherson, a. J. Interactions Between the Microbiota and the Immune System. *Science (80-.).* **336**, 1268–1273 (2012).

358. Arpaia, N. *et al.* Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451–5 (2013).

359. Lawley, T. D. & Walker, A. W. Intestinal colonization resistance. *Immunology* **138**, 1–11 (2013).

360. Spees, A. M., Lopez, C. A., Kingsbury, D. D., Winter, S. E. & Bäumler, A. J. Colonization Resistance: Battle of the Bugs or Ménage à Trois with the Host? *PLOS Pathog.* **9**, e1003730 (2013).

361. Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U.*

S. A. **102**, 11070–11075 (2005).

362. Turnbaugh, P. J., Bäckhed, F., Fulton, L. & Gordon, J. I. Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host Microbe* **3**, 213–223 (2008).

363. Sartor, R. B. Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology* **134**, 577–594 (2008).

364. Mukhopadhya, I., Hansen, R., El-Omar, E. M. & Hold, G. L. IBD—what role do Proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* **9**, 219–230 (2012).

365. Wang, T. *et al.* Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J.* **6**, 320–329 (2012).

366. Simrén, M. *et al.* Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* **62**, 159–176 (2013).

367. Penders, J., Stobberingh, E. E., Brandt, P. A. van den & Thijs, C. The role of the intestinal microbiota in the development of atopic disorders. *Allergy* **62**, 1223–1236 (2007).

368. Baquero, F. & Nombela, C. The microbiome as a human organ. *Clin. Microbiol. Infect.* **18**, 2–4 (2012).

369. Guinane, C. M. & Cotter, P. D. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therap. Adv. Gastroenterol.* **6**, 295–308 (2013).

370. Cani, P. D. Gut microbiota — at the intersection of everything? *Nat. Rev. Gastroenterol. Hepatol.* **14**, 321–322 (2017).

371. Hartl, D. L. & Dykhuizen, D. E. THE POPULATION GENETICS OF ESCHERICHIA COLI. *Annu. Rev. Genet.* **18**, 31–68 (1984).

372. Gordon, D. M. & Cowling, A. The distribution and genetic structure of Escherichia coli in Australian vertebrates: host and geographic effects. *Microbiology* **149**, 3575–3586 (2003).

373. Tenaillon, O., Skurnik, D., Picard, B. & Denamur, E. The population genetics of commensal Escherichia coli. *Nat. Rev. Microbiol.* **8**, 207–217 (2010).

374. Poulsen, L. K. *et al.* Spatial distribution of Escherichia coli in the mouse large intestine inferred from rRNA in situ hybridization. *Infect. Immun.* **62**, 5191–5194 (1994).

375. Chang, D.-E. *et al.* Carbon nutrition of Escherichia coli in the mouse intestine. *Proc. Natl. Acad. Sci.* **101**, 7427–7432 (2004).

376. Savageau, M. A. Escherichia coli Habitats, Cell Types, and Molecular Mechanisms of Gene Control. *Am. Nat.* **122**, 732–744 (1983).

377. Penders, J. *et al.* Factors Influencing the Composition of the Intestinal Microbiota in Early Infancy. *Pediatrics* **118**, 511–521 (2006).

378. Mackie, R. I., Sghir, A. & Gaskins, H. R. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.* **69**, 1035s-1045s (1999).

379. Jones, S. A. *et al.* Respiration of Escherichia coli in the Mouse Intestine. *Infect. Immun.* **75**, 4891–4899 (2007).

380. Mariat, D. *et al.* The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* **9**, 123 (2009).

381. Conway, T., Krogfelt, K. & Cohen, P. The Life of Commensal Escherichia coli in the Mammalian Intestine. *EcoSal Plus* (2004).

382. Bentley, R. & Meganathan, R. Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol. Rev.* **46**, 241–280 (1982).

383. Bambou, J.-C. *et al.* In Vitro and ex Vivo Activation of the TLR5 Signaling Pathway in Intestinal Epithelial Cells by a Commensal Escherichia coli Strain. *J. Biol. Chem.* **279**, 42984–42992 (2004).

384. Fábrega, M. J. *et al.* Activation of Immune and Defense Responses in the Intestinal Mucosa by Outer Membrane Vesicles of Commensal and Probiotic Escherichia coli Strains. *Front. Microbiol.* **7**, 705 (2016).

385. Rastegar Lari, A., Gold, F., Borderon, J. C., Laugier, J. & Lafont, J.-P. Implantation and in vivo Antagonistic Effects of Antibiotic-Susceptible *Escherichia Coli* Strains Administered to Premature Newborns. *Neonatology* **58**, 73–78 (1990).

386. Freter, R. & Abrams, G. D. Function of Various Intestinal Bacteria in Converting Germfree Mice to the Normal State. *Infect. Immun.* **6**, 119–126 (1972).

387. Hudault, S., Guignot, J. & Servin, A. L. Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut* **49**, 47–55 (2001).

388. Schamberger, G. P., Phillips, R. L., Jacobs, J. L. & Diez-Gonzalez, F. Reduction of Escherichia coli O157:H7 Populations in Cattle by Addition of Colicin E7-Producing E. coli to Feed. *Appl. Environ. Microbiol.* **70**, 6053–6060 (2004).

389. Shulman, S., Friedmann, H. & Sims, R. Theodor Escherich: The First Pediatric Infectious Diseases Physician? *Clin. Infect. Dis.* **45**, 1025–1029 (2007).

390. Bachmann, B. Derivations and genotypes of some mutant derivatives of Escherichia coli K-12. in *Escherichia coli and Salmonella typhymurium Cellular and Molecular Biology* 2460–2488 (ASM Press, 1996).

391. Gray, C. H. & Tatum, E. L. X-Ray Induced Growth Factor Requirements in Bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **30**, 404–410 (1944).

392. Tatum, E. L. & Lederberg, J. Gene Recombination in the Bacterium Escherichia coli. *J. Bacteriol.* **53**, 673–684 (1947).

393. Blattner, F. R. *et al.* The Complete Genome Sequence of Escherichia coli K-12. *Science (80-.).* **277**, 1453 LP – 1462 (1997).

394. Barroso-Batista, J. *et al.* The First Steps of Adaptation of Escherichia coli to the Gut Are Dominated by Soft Sweeps. *PLOS Genet.* **10**, e1004182 (2014).

395. Lourenço, M. *et al.* A Mutational Hotspot and Strong Selection Contribute to the Order of Mutations Selected for during Escherichia coli Adaptation to the Gut. *PLoS Genet.* **12**, e1006420–e1006420 (2016).

396. Ramiro, R. S., Durão, P., Bank, C. & Gordo, I. Low Mutational Load Allows for High Mutation Rate Variation in Gut Commensal Bacteria. *bioRxiv* (2019). doi:10.1101/568709

397. Sousa, A. *et al.* Recurrent Reverse Evolution Maintains Polymorphism after Strong Bottlenecks in Commensal Gut Bacteria. *Mol. Biol. Evol.* **34**, 2879–2892 (2017).

398. Barroso-Batista, J. *et al.* The First Steps of Adaptation of Escherichia coli to the Gut Are Dominated by Soft Sweeps. *PLoS Genet.* **10**, (2014).

399. Zhou, Y. N. *et al.* Isolation and Characterization of RNA Polymerase rpoB Mutations That Alter Transcription Slippage during Elongation in Escherichia coli. *J. Biol. Chem.* **288**, 2700–2710 (2013).

400. Enne, V. I., Livermore, D. M., Stephens, P. & Hall, L. M. C. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. *Lancet* **357**, 1325–1328 (2001).

401. Björkholm, B. *et al.* Mutation frequency and biological cost of antibiotic resistance in Helicobacter pylori. *Proc. Natl. Acad. Sci.* **98**, 14607 LP – 14612 (2001).

402. Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. Commensal Escherichia coli of healthy humans: a reservoir for antibiotic-resistance determinants. *J. Med. Microbiol.* **59**, 1331–1339 (2010).

403. Lennox, E. S. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206 (1955).

404. Baba, T. *et al.* Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 (2006).

405. van der Waaij, D., Berghuis-de Vries, J. M. & Lekkerkerk-van der Wees, L.

Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J. Hyg. (Lond).* **69**, 405–411 (1971).

406. Thompson, J. A., Oliveira, R. A., Djukovic, A., Ubeda, C. & Xavier, K. B. Manipulation of the Quorum Sensing Signal AI-2 Affects the Antibiotic-Treated Gut Microbiota. *Cell Rep.* **10**, 1861–1871 (2015).

407. Walters, W. *et al.* Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *mSystems* **1**, (2016).

408. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335–336 (2010).

409. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).

410. DeSantis, T. Z. *et al.* Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069–5072 (2006).

411. Barroso-Batista, J., Demengeot, J. & Gordo, I. Adaptive immunity increases the pace and predictability of evolutionary change in commensal gut bacteria. *Nat. Commun.* **6**, 8945 (2015).

412. de Visser, J. A. G. M. & Lenski, R. E. Long-term experimental evolution in Escherichia coli. XI. Rejection of non-transitive interactions as cause of declining rate of adaptation. *BMC Evol. Biol.* **2**, 19 (2002).

413. Paquin, C. E. & Adams, J. Relative fitness can decrease in evolving asexual populations of S. cerevisiae. *Nature* **306**, 368–371 (1983).

414. Hugenholtz, F. & de Vos, W. M. Mouse models for human intestinal microbiota research: a critical evaluation. *Cell. Mol. Life Sci.* **75**, 149–160 (2018).

415. Robertson, S. J. *et al.* Comparison of Co-housing and Littermate Methods for Microbiota Standardization in Mouse Models. *Cell Rep.* **27**, 1910-1919.e2 (2019).

416. Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* **8**, 15–25 (2010).

417. Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J.* **1**, 56–66 (2007).

418. Jakobsson, H. E. *et al.* Short-Term Antibiotic Treatment Has Differing Long-Term Impacts on the Human Throat and Gut Microbiome. *PLoS One* **5**, e9836 (2010).

419. Belkaid, Y. & Hand, T. W. Role of the Microbiota in Immunity and Inflammation. *Cell* **157**, 121–141 (2014).

420. Sonnenburg, J. L., Chen, C. T. L. & Gordon, J. I. Genomic and Metabolic Studies of the Impact of Probiotics on a Model Gut Symbiont and Host. *PLOS Biol.* **4**, e413 (2006).

421. Hooper, L. V, Littman, D. R. & Macpherson, A. J. Interactions Between the Microbiota and the Immune System. *Science (80-.).* **336**, 1268–1273 (2012).

422. Stecher, B. & Hardt, W.-D. Mechanisms controlling pathogen colonization of the gut. *Curr. Opin. Microbiol.* **14**, 82–91 (2011).

423. Samuel, B. S. & Gordon, J. I. A humanized gnotobiotic mouse model of host{\textendash}archaeal{\textendash}bacterial mutualism. *Proc. Natl. Acad. Sci.* **103**, 10011–10016 (2006).

424. Mahowald, M. A. *et al.* Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. *Proc. Natl. Acad. Sci.* **106**, 5859–5864 (2009).

425. Belenguer, A. *et al.* Two Routes of Metabolic Cross-Feeding between Bifidobacterium adolescentis and Butyrate-Producing Anaerobes from the Human Gut. *Appl. Environ. Microbiol.* **72**, 3593–3599 (2006).

426. Bairey, E., Kelsic, E. D. & Kishony, R. High-order species interactions shape ecosystem diversity. *Nat. Commun.* **7**, 12285 (2016).

427. Levine, J. M., Bascompte, J., Adler, P. B. & Allesina, S. Beyond pairwise mechanisms of species coexistence in complex communities. *Nature* **546**, 56–64 (2017).

428. Goldford, J. E. *et al.* Emergent simplicity in microbial community assembly. *Science (80-.).* **361**, 469–474 (2018).

429. Stebbins, R. B., Graessle, O. E. & Robinson, H. J. Studies on the absorption and excretion of streptomycin in animals. *Proc. Soc. Exp. Biol. Med.* **60**, 68–73 (1945).

430. Klümper, U. *et al.* Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. *ISME J.* **13**, 2927–2937 (2019).

431. De La Cochetière, M. F. *et al.* Resilience of the Dominant Human Fecal Microbiota upon Short-Course Antibiotic Challenge. *J. Clin. Microbiol.* **43**, 5588 LP – 5592 (2005).

432. Dethlefsen, L. & Relman, D. A. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci.* **108**, 4554–4561 (2011).

433. Østman, B. Effects of Epistasis and Pleiotropy on Fitness Landscapes BT -

Evolutionary Biology: Exobiology and Evolutionary Mechanisms. in (ed. Pontarotti, P.) 37–46 (Springer Berlin Heidelberg, 2013). doi:10.1007/978-3-642-38212-3_3

434. Qi, Q., Preston, G. M. & MacLean, R. C. Linking System-Wide Impacts of RNA Polymerase Mutations to the Fitness Cost of Rifampin Resistance in Pseudomonas aeruginosa *MBio* **5**, e01562-14 (2014).

435. Ruusala, T., Andersson, D., Ehrenberg, M. & Kurland, C. G. Hyper-accurate ribosomes inhibit growth. *EMBO J.* **3**, 2575–2580 (1984).

436. Libby, R. T., Nelson, J. L., Calvo, J. M. & Gallant, J. A. Transcriptional proofreading in Escherichia coli. *EMBO J.* **8**, 3153–3158 (1989).

437. Blank, A., Gallant, J. A., Burgess, R. R. & Loeb, L. A. An RNA polymerase mutant with reduced accuracy of chain elongation. *Biochemistry* **25**, 5920–5928 (1986).

438. Strathern, J. N., Jin, D. J., Court, D. L. & Kashlev, M. Isolation and characterization of transcription fidelity mutants. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1819**, 694–699 (2012).

439. Görke, B. & Stülke, J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**, 613–624 (2008).

440. Kovárová-Kovar, K. & Egli, T. Growth Kinetics of Suspended Microbial Cells: From Single-Substrate-Controlled Growth to Mixed-Substrate Kinetics. *Microbiol. Mol. Biol. Rev.* **62**, 646 LP – 666 (1998).

441. Chang, D.-E. *et al.* Carbon nutrition of Escherichia coli in the mouse intestine. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 7427–7432 (2004).

442. Bein, A. *et al.* Microfluidic Organ-on-a-Chip Models of Human Intestine. *Cell. Mol. Gastroenterol. Hepatol.* **5**, 659–668 (2018).

443. Elena, S. F. & Lenski, R. E. Epistasis between new mutations and genetic background and a test of genetic canalization. *Evolution* **55**, 1746–1752 (2001).

444. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **74**, 417 LP – 433 (2010).

445. Nguyen, Q. H., Contamin, L., Nguyen, T. V. A. & Bañuls, A.-L. Insights into the processes that drive the evolution of drug resistance in Mycobacterium tuberculosis. *Evol. Appl.* **11**, 1498–1511 (2018).

446. Velayati, A. A. *et al.* Emergence of New Forms of Totally Drug-Resistant Tuberculosis Bacilli: Super Extensively Drug-Resistant Tuberculosis or Totally

Drug-Resistant Strains in Iran. *Chest* **136**, 420–425 (2009).

447. Luo, N. *et al.* Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. *Proc. Natl. Acad. Sci.* **102**, 541–546 (2005).

448. de Vos, M. *et al.* Putative Compensatory Mutations in the rpoC Gene of Rifampin-Resistant Mycobacterium tuberculosis Are Associated with Ongoing Transmission. *Antimicrob. Agents Chemother.* **57**, (2012).

449. Davis, B. H., Poon, A. F. Y. & Whitlock, M. C. Compensatory mutations are repeatable and clustered within proteins. *Proc. R. Soc. B Biol. Sci.* **276**, 1823–1827 (2009).

450. Moura de Sousa, J., Sousa, A., Bourgard, C. & Gordo, I. Potential for adaptation overrides cost of resistance. *Future Microbiol.* **10**, 1415–1431 (2015).

451. Posfai, A., Taillefumier, T. & Wingreen, N. S. Metabolic Trade-Offs Promote Diversity in a Model Ecosystem. *Phys. Rev. Lett.* **118**, 28103 (2017).

452. Lescat, M. *et al.* Using long-term experimental evolution to uncover the patterns and determinants of molecular evolution of an Escherichia coli natural isolate in the streptomycin-treated mouse gut. *Mol. Ecol.* **26**, 1802–1817 (2017).

453. Kirby, K. S. A new method for the isolation of deoxyribonucleic acids; evidence on the nature of bonds between deoxyribonucleic acid and protein. *Biochem. J.* **66**, 495–504 (1957).

454. Mandal, S. *et al.* Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* **26**, 27663 (2015).

455. Ubeda, C. *et al.* Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J. Exp. Med.* **209**, 1445–1456 (2012).

456. Derrien, M., Vaughan, E. E., Plugge, C. M. & de Vos, W. M. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int. J. Syst. Evol. Microbiol.* **54**, 1469–1476 (2004).

457. Hiippala, K. *et al.* The Potential of Gut Commensals in Reinforcing Intestinal Barrier Function and Alleviating Inflammation. *Nutrients* **10**, (2018).

458. Ottman, N., Geerlings, S. Y., Aalvink, S., de Vos, W. M. & Belzer, C. Action and function of Akkermansia muciniphila in microbiome ecology, health and disease. *Best Pract. Res. Clin. Gastroenterol.* **31**, 637–642 (2017).

459. Png, C. W. *et al.* Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am. J. Gastroenterol.*

105, 2420–2428 (2010).

460. Rajilic-Stojanovic, M., Shanahan, F., Guarner, F. & de Vos, W. M. Phylogenetic analysis of dysbiosis in ulcerative colitis during remission. *Inflamm. Bowel Dis.* **19**, 481–488 (2013).

461. Karlsson, C. L. J. *et al.* The Microbiota of the Gut in Preschool Children With Normal and Excessive Body Weight. *Obesity* **20**, 2257–2261 (2012).

462. Dao, M. C. *et al.* Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut* **65**, 426–436 (2016).

463. Everard, A. *et al.* Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci.* **110**, 9066–9071 (2013).

464. Zhang, X. *et al.* Human Gut Microbiota Changes Reveal the Progression of Glucose Intolerance. *PLoS One* **8**, e71108 (2013).

465. Sommer, F., Anderson, J. M., Bharti, R., Raes, J. & Rosenstiel, P. The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* **15**, 630–638 (2017).

466. Dethlefsen, L., Huse, S., Sogin, M. L. & Relman, D. A. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLOS Biol.* **6**, e280 (2008).

467. Pallav, K. *et al.* Effects of polysaccharopeptide from Trametes Versicolor and amoxicillin on the gut microbiome of healthy volunteers. *Gut Microbes* **5**, 458–467 (2014).

468. Raymond, F. *et al.* The initial state of the human gut microbiome determines its reshaping by antibiotics. *ISME J.* **10**, 707–720 (2016).

469. Borukhov, S. *et al.* Mapping of trypsin cleavage and antibody-binding sites and delineation of a dispensable domain in the beta subunit of Escherichia coli RNA polymerase. *J. Biol. Chem.* **266**, 23921–23926 (1991).

470. Severinov, K. *et al.* A non-essential domain of Escherichia coli RNA polymerase required for the action of the termination factor Alc. *J. Biol. Chem.* **269**, 14254–14259 (1994).

471. Ganduri, Y. L., Sadda, S. R., Datta, M. W., Jambukeswaran, R. K. & Datta, P. TdcA, a transcriptional activator of the tdcABC operon of Escherichia coli, is a member of the LysR family of proteins. *Mol. Gen. Genet. MGG* **240**, 395–402 (1993).

472. Schweizer, H. P. & Datta, P. Identification and DNA sequence of tdcR, a positive regulatory gene of the tdc operon of Escherichia coli. *Mol. Gen. Genet.*

MGG **218**, 516–522 (1989).

473. Sawers, G. The anaerobic degradation of l-serine and l-threonine in enterobacteria: networks of pathways and regulatory signals. *Arch. Microbiol.* **171**, 1–5 (1998).

474. Wu, Y. & Datta, P. Influence of DNA topology on expression of the tdc operon in Escherichia coli K-12. *Mol. Gen. Genet. MGG* **247**, 764–767 (1995).

475. Selinger, D. W., Saxena, R. M., Cheung, K. J., Church, G. M. & Rosenow, C. Global RNA Half-Life Analysis in Escherichia coli Reveals Positional Patterns of Transcript Degradation. *Genome Res.* **13**, 216–223 (2003).

476. Laffler, T. & Gallant, J. spoT, a new genetic locus involved in the stringent response in E. coli. *Cell* **1**, 27–30 (1974).

477. Ross, W., Vrentas, C. E., Sanchez-Vazquez, P., Gaal, T. & Gourse, R. L. The magic spot: a ppGpp binding site on E. coli RNA polymerase responsible for regulation of transcription initiation. *Mol. Cell* **50**, 420–429 (2013).

478. Zhou, Y. N. & Jin, D. J. The rpoB mutants destabilizing initiation complexes at stringently controlled promoters behave like {\textquotedblleft}stringent{\textquotedblright} RNA polymerases in Escherichia coli. *Proc. Natl. Acad. Sci.* **95**, 2908–2913 (1998).

479. Xu, J., Tozawa, Y., Lai, C., Hayashi, H. & Ochi, K. A rifampicin resistance mutation in the rpoB gene confers ppGpp-independent antibiotic production in Streptomyces coelicolor A3(2). *Mol. Genet. Genomics* **268**, 179–189 (2002).

480. Wagner, E. G. & Kurland, C. G. Translational accuracy enhanced in vitro by (p)ppGpp. *Mol. Gen. Genet.* **180**, 139–145 (1980).

481. Bougdour, A. & Gottesman, S. ppGpp regulation of RpoS degradation via anti-adaptor protein IraP. *Proc. Natl. Acad. Sci.* **104**, 12896–12901 (2007).

482. Hengge-Aronis, R. Signal Transduction and Regulatory Mechanisms Involved in Control of the σS (RpoS) Subunit of RNA Polymerase. *Microbiol. Mol. Biol. Rev.* **66**, 373–395 (2002).

483. Brownlee, G. G., Sanger, F. & Barrell, B. G. Nucleotide sequence of 5S-ribosomal RNA from Escherichia coli. *Nature* **215**, 735–736 (1967).

484. Wawrzynow, A. *et al.* The ClpX heat-shock protein of Escherichia coli, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. *EMBO J.* **14**, 1867–1877 (1995).

485. Weichart, D., Querfurth, N., Dreger, M. & Hengge-Aronis, R. Global Role for ClpP-Containing Proteases in Stationary-Phase Adaptation of Escherichia coli. *J. Bacteriol.* **185**, 115–125 (2003).

486. Cumberlidge, A. G. & Isono, K. Ribosomal protein modification in Escherichia

coli: I. A mutant lacking the N-terminal acetylation of protein S5 exhibits thermosensitivity. *J. Mol. Biol.* **131**, 169–189 (1979).

487. Yamanaka, K., Ogura, T., Niki, H. & Hiraga, S. Identification of two new genes,mukE andmukF, involved in chromosome partitioning inEscherichia coli. *Mol. Gen. Genet. MGG* **250**, 241–251 (1996).

488. Freter, R. Mechanisms that control the microflora in the large intestine. *Hum. Intest. microflora Heal. Dis.* 33–54 (1983).

489. Coyte, K. Z., Schluter, J. & Foster, K. R. The ecology of the microbiome: Networks, competition, and stability. *Science (80-.).* **350**, 663–666 (2015).

490. Rakoff-Nahoum, S., Foster, K. R. & Comstock, L. E. The evolution of cooperation within the gut microbiota. *Nature* **533**, 255–259 (2016).

491. De Paepe, M. *et al.* Trade-off between bile resistance and nutritional competence drives Escherichia coli diversification in the mouse gut. *PLoS Genet.* **7**, e1002107–e1002107 (2011).

492. Barroso-Batista, J. *et al.* Specific Eco-Evolutionary Contexts in the Mouse Gut Reveal Escherichia coli Metabolic Versatility. *Curr. Biol.* (2019).

493. Ghalayini, M. *et al.* Evolution of a Dominant Natural Isolate of Escherichia coli in the Human Gut over the Course of a Year Suggests a Neutral Evolution with Reduced Effectiv. *Appl. Environ. Microbiol.* **84**, e02377-17 (2018).

494. Maisnier-Patin, S. *et al.* Genomic buffering mitigates the effects of deleterious mutations in bacteria. *Nat. Genet.* **37**, 1376–1379 (2005).

495. Walkiewicz, K. *et al.* Small changes in enzyme function can lead to surprisingly large fitness effects during adaptive evolution of antibiotic resistance. *Proc. Natl. Acad. Sci.* **109**, 21408–21413 (2012).

496. Vasi, F., Travisano, M. & Lenski, R. E. Long-Term Experimental Evolution in Escherichia coli. II. Changes in Life-History Traits During Adaptation to a Seasonal Environment. *Am. Nat.* **144**, 432–456 (1994).

497. Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *Am. Nat.* **138**, 1315–1341 (1991).

498. Hegreness, M., Shoresh, N., Hartl, D. & Kishony, R. An Equivalence Principle for the Incorporation of Favorable Mutations in Asexual Populations. *Science (80-.).* **311**, 1615–1617 (2006).

499. Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L. & Andersson, D. I. Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics

and Heavy Metals. *MBio* **5**, (2014).

500. Wiser, M. J. & Lenski, R. E. A Comparison of Methods to Measure Fitness in Escherichia coli. *PLoS One* **10**, e0126210 (2015).

501. Levin, B. R., Antonovics, J. & Sharma, H. Frequency-Dependent Selection in Bacterial Populations [and Discussion]. *Philos. Trans. R. Soc. B Biol. Sci.* **319**, 459–472 (1988).

502. Turner, P. E., Souza, V. & Lenski, R. E. Tests of Ecological Mechanisms Promoting the Stable Coexistence of Two Bacterial Genotypes. *Ecology* **77**, 2119–2129 (1996).

503. Kojima, K. & Yarbrough, K. M. FREQUENCY-DEPENDENT SELECTION AT THE ESTERASE 6 LOCUS IN Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **57**, 645–649 (1967).

504. Brisson, D. Negative Frequency-Dependent Selection Is Frequently Confounding. *Front. Ecol. Evol.* **6**, 10 (2018).

505. Helling, R. B., Vargas, C. N. & Adams, J. Evolution of Escherichia coli during growth in a constant environment. *Genetics* **116**, 349–358 (1987).

506. Maharjan, R., Seeto, S., Notley-McRobb, L. & Ferenci, T. Clonal adaptive radiation in a constant environment. *Science (80-.).* **313**, 514–517 (2006).

507. Rankin, D. J., Rocha, E. P. C. & Brown, S. P. What traits are carried on mobile genetic elements, and why? *Heredity (Edinb).* **106**, 1–10 (2011).

508. Ross-Gillespie, A., Gardner, A., West, S. A. & Griffin, A. S. Frequency Dependence and Cooperation: Theory and a Test with Bacteria. *Am. Nat.* **170**, 331–342 (2007).

509. Morris, J. J., Papoulis, S. E. & Lenski, R. E. Coexistence of evolving bacteria stabilized by a shared black queen function. *Evolution (N. Y).* 2960–2971 (2014). doi:10.1111/evo.12485

510. Ding, W., Baumdicker, F. & Neher, R. A. panX: pan-genome analysis and exploration. *Nucleic Acids Res.* **46**, e5–e5 (2017).

511. Keseler, I. M. *et al.* The EcoCyc database: reflecting new knowledge about Escherichia coli K-12. *Nucleic Acids Res.* **45**, D543–D550 (2017).

512. Scholz, R. L. & Greenberg, E. P. Sociality in Escherichia coli: Enterochelin Is a Private Good at Low Cell Density and Can Be Shared at High Cell Density. *J. Bacteriol.* **197**, 2122–2128 (2015).

513. Gehring, A. M., Mori, I. & Walsh, C. T. Reconstitution and Characterization of the Escherichia coli Enterobactin Synthetase from EntB, EntE, and EntF. *Biochemistry* **37**, 2648–2659 (1998).

514. Garénaux, A., Caza, M. & Dozois, C. M. The Ins and Outs of siderophore mediated iron uptake by extra-intestinal pathogenic Escherichia coli. *Vet. Microbiol.* **153**, 89–98 (2011).

515. Gromko, M. H. WHAT IS FREQUENCY-DEPENDENT SELECTION? *Evolution* **31**, 438–442 (1977).

516. Webb, E., Claas, K. & Downs, D. thiBPQ Encodes an ABC Transporter Required for Transport of Thiamine and Thiamine Pyrophosphate inSalmonella typhimurium . *J. Biol. Chem.* **273**, 8946–8950 (1998).

517. Hollenbach, A. D., Dickson, K. A. & Washabaugh, M. W. Overexpression, purification, and characterization of the periplasmic space thiamin-binding protein of the thiamin traffic ATPase in Escherichia coli. *Protein Expr. Purif.* **25**, 508–518 (2002).

518. Kawasaki, T. & Esaki, K. Thiamine uptake in Escherichia coli: III. Regulation of thiamine uptake in Escherichia coli. *Arch. Biochem. Biophys.* **142**, 163–169 (1971).

519. Yamada, M. & Saier, M. H. Positive and negative regulators for glucitol (gut) operon expression in Escherichia coli. *J. Mol. Biol.* **203**, 569–583 (1988).

520. Magasanik, B. Catabolite Repression. *Cold Spring Harb. Symp. Quant. Biol.* **26**, 249–256 (1961).

521. Postma, P. W. & Lengeler, J. W. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. *Microbiol. Rev.* **49**, 232–269 (1985).

522. Morris, J. J., Lenski, R. E. & Zinser, E. R. The Black Queen Hypothesis: Evolution of Dependencies through Adaptive Gene Loss. *MBio* **3**, (2012).

523. Lenski, R. E. & Hattingh, S. E. Coexistence of two competitors on one resource and one inhibitor: A chemostat model based on bacteria and antibiotics. *J. Theor. Biol.* **122**, 83–93 (1986).

524. Levin, B. R. *et al.* Frequency-dependent selection in bacterial populations. *Philos. Trans. R. Soc. London. B, Biol. Sci.* **319**, 459–472 (1988).

525. Morris, J. J., Johnson, Z. I., Szul, M. J., Keller, M. & Zinser, E. R. Dependence of the Cyanobacterium Prochlorococcus on Hydrogen Peroxide Scavenging Microbes for Growth at the Ocean's Surface. *PLoS One* **6**, e16805 (2011).

526. Loewen, P. C., Switala, J. & Triggs-Raine, B. L. Catalases HPI and HPII in Escherichia coli are induced independently. *Arch. Biochem. Biophys.* **243**, 144–149 (1985).

527. Heimberger, A. & Eisenstark, A. Compartmentalization of catalases in Escherichia coli. *Biochem. Biophys. Res. Commun.* **154**, 392–397 (1988).

528. Oscarsson, J., Mizunoe, Y., Uhlin, B. E. & Haydon, D. J. Induction of

haemolytic activity in Escherichia coli by the slyA gene product. *Mol. Microbiol.* **20**, 191–199 (1996).

529. Francetic, O. & Pugsley, A. P. The cryptic general secretory pathway (gsp) operon of Escherichia coli K-12 encodes functional proteins. *J. Bacteriol.* **178**, 3544–3549 (1996).

530. Francetic, O., Belin, D., Badaut, C. & Pugsley, A. P. Expression of the endogenous type II secretion pathway in Escherichia coli leads to chitinase secretion. *EMBO J.* **19**, 6697–6703 (2000).

531. Westermark, M., Oscarsson, J., Mizunoe, Y., Urbonaviciene, J. & Uhlin, B. E. Silencing and Activation of ClyA Cytotoxin Expression in Escherichia coli. *J. Bacteriol.* **182**, 6347–6357 (2000).

532. Lai, X.-H. *et al.* Cytocidal and Apoptotic Effects of the ClyA Protein from Escherichia coli on Primary and Cultured Monocytes and Macrophages. *Infect. Immun.* **68**, 4363–4367 (2000).

533. Higashi, K. *et al.* H-NS Facilitates Sequence Diversification of Horizontally Transferred DNAs during Their Integration in Host Chromosomes. *PLOS Genet.* **12**, e1005796 (2016).

534. Grainger, D. C., Hurd, D., Goldberg, M. D. & Busby, S. J. W. Association of nucleoid proteins with coding and non-coding segments of the Escherichia coli genome. *Nucleic Acids Res.* **34**, 4642–4652 (2006).

535. del Castillo, F. J., Moreno, F. & del Castillo, I. Secretion of the Escherichia coli K-12 SheA hemolysin is independent of its cytolytic activity. *FEMS Microbiol. Lett.* **204**, 281–285 (2001).

536. Forest, K. T. The type II secretion arrowhead: the structure of GspI–GspJ–GspK. *Nat. Struct. Mol. Biol.* **15**, 428–430 (2008).

537. von Tils, D., Blädel, I., Schmidt, M. & Heusipp, G. Type II secretion in Yersinia—a secretion system for pathogenicity and environmental fitness. *Front. Cell. Infect. Microbiol.* **2**, 160 (2012).

538. Cianciotto, N. P. Type II secretion: a protein secretion system for all seasons. *Trends Microbiol.* **13**, 581–588 (2005).

539. Schörken, U. *et al.* Identification of catalytically important residues in the active site of Escherichia coli transaldolase. *Eur. J. Biochem.* **268**, 2408–2415 (2001).

540. Katz, J. & Rognstad, R. The labeling of pentose phosphate from glucose-14C and estimation of the rates of transaldolase, transketolase, the contribution of the pentose cycle, and ribose phosphate synthesis. *Biochemistry* **6**, 2227–2247 (1967).

541. Wamelink, M. M. C., Struys, E. A. & Jakobs, C. The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: A review. *J. Inherit. Metab. Dis.* **31**, 703–717 (2008).

542. Nakahigashi, K. *et al.* Systematic phenome analysis of Escherichia coli multiple-knockout mutants reveals hidden reactions in central carbon metabolism. *Mol. Syst. Biol.* **5**, 306 (2009).

543. Koskiniemi, S., Sun, S., Berg, O. G. & Andersson, D. I. Selection-Driven Gene Loss in Bacteria. *PLOS Genet.* **8**, e1002787 (2012).

544. Kuo, C.-H. & Ochman, H. The Extinction Dynamics of Bacterial Pseudogenes. *PLOS Genet.* **6**, e1001050 (2010).

545. Selinger, D. W. *et al.* RNA expression analysis using a 30 base pair resolution Escherichia coli genome array. *Nat. Biotechnol.* **18**, 1262–1268 (2000).

546. Dornenburg, J. E., DeVita, A. M., Palumbo, M. J. & Wade, J. T. Widespread Antisense Transcription in Escherichia coli. *MBio* **1**, (2010).

547. Stappenbeck, T. S. & Virgin, H. W. Accounting for reciprocal host–microbiome interactions in experimental science. *Nature* **534**, 191–199 (2016).

548. Servick, K. Of mice and microbes. *Science (80-.).* **353**, 741–743 (2016).

549. Yurkovetskiy, L. *et al.* Gender Bias in Autoimmunity Is Influenced by Microbiota. *Immunity* **39**, 400–412 (2013).

550. Markle, J. G. M. *et al.* Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity. *Science (80-.).* **339**, 1084 LP – 1088 (2013).

551. Moon, C. *et al.* Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. *Nature* **521**, 90–93 (2015).

552. Li, J. *et al.* Antibiotic Treatment Drives the Diversification of the Human Gut Resistome. *Genomics. Proteomics Bioinformatics* **17**, 39–51 (2019).

553. Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat. Rev. Immunol.* **13**, 790–801 (2013).

554. Mullineaux-Sanders, C., Suez, J., Elinav, E. & Frankel, G. Sieving through gut models of colonization resistance. *Nat. Microbiol.* **3**, 132–140 (2018).

555. Sorbara, M. T. & Pamer, E. G. Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunol.* **12**, 1–9 (2019).

556. Wu, G. D. *et al.* Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. *Science (80-.).* **334**, 105–108 (2011).

557. Walker, A. W. *et al.* Dominant and diet-responsive groups of bacteria within

the human colonic microbiota. *ISME J.* **5**, 220–230 (2011).

558. Patnode, M. L. *et al.* Interspecies Competition Impacts Targeted Manipulation of Human Gut Bacteria by Fiber-Derived Glycans. *Cell* **179**, 59-73.e13 (2019).

559. de Muinck, E. J. *et al.* Context-Dependent Competition in a Model Gut Bacterial Community. *PLoS One* **8**, (2013).

560. Roberfroid, M. *et al.* Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* **104**, S1–S63 (2010).

561. Gibson, G. R. *et al.* Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **14**, 491–502 (2017).

562. Hugon, P. *et al.* A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect. Dis.* **15**, 1211–1219 (2015).

563. Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).

564. Franzosa, E. A. *et al.* Identifying personal microbiomes using metagenomic codes. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E2930-8 (2015).

565. Ng, P. C., Murray, S. S., Levy, S. & Venter, J. C. An agenda for personalized medicine. *Nature* **461**, 724–726 (2009).

566. Schork, N. J. Personalized medicine: Time for one-person trials. *Nature* **520**, 609–611 (2015).

567. Crow, D. Microbiome Research in a Social World. *Cell* **172**, 1143–1145 (2018).

568. Franzosa, E. A. *et al.* Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* **15**, 962–968 (2018).

569. Lloyd-Price, J. *et al.* Strains, functions and dynamics in the expanded Human Microbiome Project. *Nature* **550**, 61–66 (2017).

570. Bashiardes, S., Zilberman-Schapira, G. & Elinav, E. Use of Metatranscriptomics in Microbiome Research. *Bioinform. Biol. Insights* **10**, 19–25 (2016).

571. Moran, M. Metatranscriptomics: Eavesdropping on Complex Microbial Communities. *Microbe Mag.* **4**, 329–334 (2009).

572. Ursell, L. K. *et al.* The Intestinal Metabolome: An Intersection Between Microbiota and Host. *Gastroenterology* **146**, 1470–1476 (2014).

573. Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M. & Relman, D. A. The Application of Ecological Theory Toward an Understanding of the Human Microbiome. *Science (80-.).* **336**, 1255–1262 (2012).

574. Kelsic, E. D., Zhao, J., Vetsigian, K. & Kishony, R. Counteraction of antibiotic production and degradation stabilizes microbial communities. *Nature* **521**, 516–519 (2015).

575. Goodman, A. L. *et al.* Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6252–6257 (2011).

576. Vrancken, G., Gregory, A. C., Huys, G. R. B., Faust, K. & Raes, J. Synthetic ecology of the human gut microbiota. *Nat. Rev. Microbiol.* **17**, 754–763 (2019).

577. Mertens, G. EVOLUTION, HEALTH AND DISEASE – DARWINIAN APPROACHES TO MEDICINE. *Acta Clin. Belg.* **64**, 2–6 (2009).

578. Nesse, R. M. & Stearns, S. C. The great opportunity: Evolutionary applications to medicine and public health. *Evol. Appl.* **1**, 28–48 (2008).

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

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