Ph.D. Thesis

Systematic investigation of the compensatory evolution of multidrug-resistant bacteria

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Introduction

The intensive and inappropriate use of antibiotics has led to the increased frequency of multidrug resistance in both hospital- and community acquired infections. Fortunately, antibiotic resistance is one of the few examples of evolution that can be studied in real time. Understanding the evolutionary forces behind the clinical dynamics of antibiotic resistance (acquisition, maintenance and reversion) can guide the development of future strategies for controlling resistance in clinical pathogens.

It is general knowledge that the massive, irresponsible use of antibiotics causes extensive antibiotic resistance, but whether it is working the other way around, namely that decreased antibiotic usage leads to less prevalent resistance, is yet unclear. In the presence of high levels of antibiotics, the acquisition of antibiotic resistance provides a fitness advantage over the susceptible competitors, whereas, in the absence of antibiotics, resistance mechanisms generally decrease the fitness. Resistance does not come for free. In the absence of antibiotics, resistance mechanisms generally confer deleterious effects, typically observed as an increased generation time and reduced survival inside the host. As an example, reduction of porin expression causes resistance to several antibiotics by reducing the membrane permeability, but it comes at a significant cost because not only antibiotics, but also important nutrients are simultaneously excluded from the periplasm. Nevertheless, bacteria can adapt to this new environment (that is the absence of the antibiotic) and increase their fitness. There are two possible ways bacteria can increase fitness, either by decreasing resistance or by keeping their resistance through specific secondary mutations.

Earlier studies on compensation showed an important difference between the results of clinical studies and laboratory experiments. Clinical studies show that resistance is usually lost in pathogenic populations once the application of an antibiotic is reduced or stopped. In contrast, laboratory experiments have almost always found that resistance is maintained in the absence of antibiotics. A reason for this contradiction could be that laboratory experiments have focused on model systems involving a single chromosomal resistance mutation, whereas antibiotic-resistant clinical isolates usually carry multiple resistance mutations. Moreover, those mutations investigated in laboratory experiments are usually target mutations, while mutations in clinical isolates are diverse, including efflux pump mutations, membrane permeability related mutations, etc.

Aims

Antibiotic resistance typically induces a fitness cost that shapes the fate of antibiotic-resistant bacterial populations. However, the cost of resistance can be mitigated by compensatory mutations elsewhere in the genome, and therefore the loss of resistance may proceed too slowly to be of practical importance. We applied a high-throughput experimental approach to study the efficacy and phenotypic impact of compensatory evolution in *Escherichia coli* strains carrying multiple resistance mutations, in order to answer the following questions:

- How costly is antibiotic resistance?

- Is resistance sustainable in the absence of antibiotics?

- What are the differences in compensation between bacteria carrying a single resistance mutation and antibiotic-resistant clinical isolates that carry multiple resistance mutations?

- What kind of mutations emerge during evolution in the absence of antibiotics? Is there a general pattern?

Methods

Antibiotic-resistant strains

The 60 multidrug-resistant strains used in this study were derived from our previous work, where parallely evolving populations of *E. coli* K12 BW25113 were adapted to increasing dosages of one of 12 antibiotics. The evolutionary experiment was continued for ~240–384 generations, at which point the evolving populations reached an up to 328-fold increase in resistance compared to the wild-type ancestor. The 60 antibiotic-resistant strains (4–6 strains per antibiotic) were previously subjected to whole-genome sequencing. The identified resistance mutations affected drug targets, cell permeability or efflux pumps.

Laboratory evolutionary experiment

We started with 23 antibiotic-resistant *E. coli* strains that displayed a significant fitness cost. Six parallel lines were initiated from each antibiotic-resistant strain, and were propagated in 96-well microtiter plates in antibiotic-free medium for 60 days.

High-throughput fitness measurements and determination of growth rate

The starter plates were grown for 24 hr under conditions identical to the evolutionary experiment. Plates were transferred by robotic arm to plate readers every 20 min and cell growth was followed by recording the optical density at 600 nm.

Fitness was approximated by calculating the area under the growth curve (AUGC). For each line and each evolutionary time point, relative fitness was calculated as the median of the normalized AUGC of the technical replicates divided by the median fitness of the wild-type controls. This analysis was performed by my colleague, Gábor Boross, PhD.

Determination of the minimal inhibitory concentration (MIC)

Minimal inhibitory concentrations (MICs) were determined using standard E-test strips. Based on the MIC results, we categorized each strain as being resistant (R), intermediate (IM) or susceptible (S) to each investigated antibiotic according to the Clinical and Laboratory Standards Institute (CLSI Approved Standard M100, 29th Edition) guidelines.

Hoechst 33342 (Bisbenzimide H 33342) accumulation assay

In order to measure the membrane permeability of the adapted lines, we measured the intracellular accumulation of the Hoechst fluorescent dye (H33342 bisbenzimide) by a high-throughput, microplate-based protocol. Data curves analysis was performed by my colleague, Balázs Szappanos, PhD.

Reconstruction of compensatory mutations with the help of pORTMAGE

Utilizing multiplex automated genome engineering (pORTMAGE) we reconstructed two candidate compensatory mutations and the corresponding resistance mutations in the wild-type genetic background (separately and in combination as well), as well as the compensatory mutations in the corresponding initial antibiotic-resistant strains.

Whole-genome sequencing

To identify potential compensatory mechanisms, 15 adapted lines derived from a total of 10 antibiotic-resistant T0 strains were chosen for whole-genome sequencing. The whole genome sequencing and the analysis and validation of the acquired raw data was performed by the Sequencing Platform of the Institute of Biochemistry, University of Szeged, under the direction of László Bodai, PhD. The raw results of the sequencing are available in the NCBI BioProject database (URL: https://www.ncbi.nlm.nih.gov/sra/PRJNA529335).

Results

Initial fitness cost

We estimated fitness by measuring individual fitness of each of the 60 antibiotic-resistant strains and the corresponding wild-type strain in an antibiotic-free medium. In total, 23 antibiotic-resistant strains showed significantly reduced growth compared to the ancestral wild-type strain. We detected strains with especially low fitness values in the antibiotic-free medium which were found to carry more resistance mutations and display especially high levels of resistance.

Laboratory evolution of antibiotic-resistant strains in an antibiotic-free medium

To investigate potential changes in resistance phenotypes upon evolution in an antibiotic-free environment, we initiated parallel laboratory evolutionary experiments with 23 antibiotic-resistant (T0) strains for 60 transfers. Fifty-one percent of these lines were found to exhibit significantly improved fitness (T60), and the analysis has revealed major differences in relative fitness across strains adapted to different antibiotics, indicating that adaptation is mainly driven by the set of resistance mutations present in the initial resistant strains. Generally, the resistant mutations and compensatory mutations influenced the same regulation system or the same gene.

Changes in resistance in antibiotic-free medium

We investigated how laboratory evolution in an antibiotic-free medium shapes antibiotic resistance. For this purpose, we first measured the minimum inhibitory concentrations (MIC) in 71 T60 lines showing significant fitness improvement, as well as in the corresponding 20 T0 strains, against a set of 11 antibiotics. Using the CLSI (CLSI Approved Standard M100, 29th Edition), resistance break-point cut-offs, we categorized each strain as being resistant, intermediate or susceptible to each investigated antibiotic. A decline in resistance was defined by transitions between these categories. We focused on antibiotics to which the corresponding T0 strain exhibited resistance, leading to a total of 195 antibiotic-T60 line combinations.

We have found that resistance declined in as high as 54.8% of the antibiotic-T60 line combinations following evolution under antibiotic stress-free conditions. However, the extent of resistance decline depended on the antibiotic considered. For example, doxycycline and tetracycline resistance was frequently lost, while aminoglycoside resistance was generally maintained in the T60 lines. Overall, 64.7% of the T60 lines displayed significant decline in resistance to at least one antibiotic, and many displayed loss of resistance to multiple drugs. Generally, for multidrug resistant strains with increased fitness, we found a decrease in cross-resistance against several other antibiotics. However, resistant strains with only trifling fitness increase could maintain their resistance.

Phenotypic reversion via compensatory mutations dominates

To gain insights into the underlying molecular mechanisms of resistance loss, 15 independently evolved T60 strains displaying increased fitness were subjected to whole-genome sequencing. Using the Illumina platform and established bioinformatics protocols, we aimed to identify mutations relative to the genome of the corresponding T0 strains. Altogether, 45 independent mutational events were identified, including 16 single nucleotide polymorphisms (SNPs), 16 deletions and 13 insertions. We screened the full bacterial genome to identify resistance-conferring SNPs in the T0 population that revert back to the wild-type sequence in the corresponding T60 strains, but found no such cases. Therefore, the fitness gain in the T60 strains is not due to the molecular reversion of the antibiotic-resistance mutations. Rather, compensatory mutations elsewhere in the genome contribute to the rapid fitness improvement in the evolved strains. A rigorous statistical analysis to test functional relationship between the mutations detected in T0 and T60 was not feasible due to the low number of mutations that have accumulated during the course of laboratory evolution. Nevertheless, we noted several examples on functional relatedness between resistance genes mutated in T0 and genes mutated during lab evolution.

Pleiotropic side effects of a compensatory mutation

Based on whole-genome sequencing we found that several putative compensatory mutations accumulated in functionally-related transcriptional regulatory proteins involved in anti-drug

defense. To gain insights into how the compensatory mutations effect fitness and antibiotic resistance level, we first focused on a multidrug-resistant laboratory evolved doxycycline resistant strain that carries mutations in the *acrR* and *marR* efflux pump system. Following 60 days of evolution in antibiotic free medium, a new mutation appeared in the promoter region of *marR*. We examined functional relationship between initial resistance and compensatory mutations with the help of pORTMAGE, which we used to reconstruct mutant strains. First we measured the growth rates of the wild-type and the antibiotic-resistant T0 strain with and without *marR**. In the absence of antibiotic, the phenotypic effects of this mutation depended on the genetic background. This epistatic effect suggests that *marR** reduces the deleterious side effects of antibiotic-resistance mutations, while it has a fitness cost in the wild-type strain. Next, we studied the impact of the *marR** compensatory mutation on resistance level using standard E-test assays. The corresponding T0 strain was found to display detectable resistance to multiple antibiotics, including doxycycline, ampicillin, chloramphenicol, nalidixic acid and tetracycline, while the corresponding T60 strain lost resistance to all studied antibiotics. This can mainly result from the presence of *marR** in the T60 genome, as the introduction of *marR** to the T0 strain recapitulated the same pattern: the engineered strain lost resistance. Finally, we hypothesized that *marR** shapes resistance and cellular fitness through antagonistic effects on drug uptake. This hypothesis was tested by measuring the intracellular accumulation of a fluorescent probe (Hoechst 33342) as a proxy for membrane permeability in the resistant T0 strain, in the T60 line and in the wild-type strain with and without the *marR** compensatory mutation. Decreased intracellular level of the probe indicates either decreased porin activity or enhanced efflux-pump activity. This is exactly what we found in the resistant T0 strains compared to the wild-type. Importantly, *marR** restored membrane permeability of T0 to the wild-type level.

Similar patterns held for a cefoxitin resistant strain carrying mutation in *ompR* and *ompC*, that change outer membrane diffusion. Reduction of porin expression causes resistance to antibiotics by reducing the membrane permeability, but it comes at a significant cost because not only antibiotics, but also important nutrients are simultaneously excluded from the periplasm. However, compensatory mutation in *envZ* restore the original state of porin expression and simultaneously increase the fitness and decrease ampicillin resistance.

Summary

1) Multidrug-resistance declines rapidly when bacteria were exposed to an antibiotic-free medium.
2) The extent of resistance loss was antibiotic-specific.
3) This process is driven by compensatory mutations that reduce both the resistance level and the pleiotropic side effects of antibiotic-resistance mutations.

Agreement with clinical observations

Our findings appear to be consistent with clinical data. For instance, a Finnish retrospective study assessed the proportion of quinolone-susceptible *E. coli* urine isolates before and after a nationwide restriction of ciprofloxacin use was implemented in Finland. The research revealed that a reduced consumption of quinolone antibiotics resulted in a significant decrease in quinolone-resistance of *E. coli*. Our laboratory study also shows that ciprofloxacin-resistance has declined in 66% of initially resistant populations, following a long-term exposure to antibiotic-free medium. Another study examined the impact of a 24-month voluntary restriction on the use of trimethoprim-containing drugs in Sweden on the prevalence of trimethoprim-resistant *E. coli* isolated from urinary-tract infections. All clinical isolates were found to retain their resistance levels and carried mutation in *folA*, the target gene of trimethoprim, even after 24 months of trimethoprim restriction. In agreement with this clinical study, we have found that all five trimethoprim resistant *E. coli* strains with a *folA* resistance mutation have maintained their resistance following laboratory evolution in antibiotic-free medium.

Publication related to this thesis

A Dunai *, R Spohn*, Z Farkas*, V Lázár*, Á Györkei, G Apjok, G Boross, B Szappanos, G Grézal, A Faragó, L Bodai, B Papp, Cs Pál **Rapid decline of bacterial drug-resistance in an antibiotic-free environment through phenotypic reversion** eLife 2019;8:e47088

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Other publication

K Bhaumik, A Hetényi, G Olajos, A Martins, R Spohn, L Németh, B Jojart, P Szili, A Dunai, P Jangir, L Daruka, I Földesi,D Kata, Cs Pál, T Martinek **Rationally designed foldameric adjuvants enhance antibiotic efficacy via promoting membrane hyperpolarization** Molecular Systems Design & Engineering , 21–33 (2022)