Ph.D. thesis summary

The *de novo* evolution of bacterial antibiotic resistance and its collateral consequences

Réka Spohn

Supervisor: Csaba Pál, Ph.D.

Ph.D School in Biology, University of Szeged

Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences

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Introduction

Therapeutic application of antibiotics substantially improved life expectancy. Without antibiotics, humanity would not only loose its ability to treat bacterial infections, but also cancer therapies, transplantations and other immunosuppressive therapies, invasive surgeries and premature care would become impossible. One does not have to go far to face the tragic consequences of the shortage of antibiotics: in those countries that seriously lack resources, bacterial infections cause the second most deaths and are responsible for 60% of the pediatric deaths. Therefore, the public health hazard brought on by the spread of antibiotic resistance cannot be overemphasized.

Despite the fact that antibiotic resistance has long been observed, our knowledge on the evolution of resistance and its collateral consequences is limited. The evolution of antibiotic resistance in the clinic is facilitated by mutations accumulating in the bacterial genome. The accumulation of these mutations could serve as a first step for the development of more specific resistance mechanisms providing clinically relevant resistance level. Additionally, these resistance mutations could affect bacteria's susceptibility to other antibiotics as well. These collateral effects are referred to as evolutionary interactions. Cross-resistance or multidrug-resistance occurs when resistance evolving to a given antibiotic causes increased resistance to one or more other antibiotics as well. Collateral sensitivity or hypersensitivity occurs in the exciting cases when resistance to a given antibiotic causes increased sensitivity to one or more other antibiotics. Collateral sensitivity was first described in a pioneering study 60 years ago. In this study, despite the then current laboratory conditions, the evolutionary interactions of 15 antibiotics were systematically investigated. This experiment was primarily aimed at understanding the differences and similarities in the modes of action of antibiotics.

Despite the obvious clinical relevance, apart from this pioneering phenomenological study 60 years ago, no one investigated the evolutionary interactions of antibiotics until recently. This long hiatus of studies is surprising for two reasons: first, it has been known for long that antibiotic resistance has considerable fitness cost in antibiotic-free environment; and second, hypersensitivity, or in other words collateral sensitivity, has been expansively studied in cancer chemotherapy. Recent technological advances on the fields of laboratory automation and whole-genome sequencing have facilitated the systematic investigation of collateral sensitivity. Thanks to these advances, our first results on collateral sensitivity were published amongst other studies on this topic, and there are also several ongoing investigations on the possible clinical utilization of collateral sensitivity.

Aims

During my work, our aim was to answer one of the most urgent questions of our days, namely to understand, what kind of genetic changes appear during the *de novo* evolution of antibiotic resistance and furthermore, how these genetic changes affect the susceptibility of bacteria towards other antibiotics. In order to systematically investigate these evolutionary interactions, we set up the following goals:

- Investigation of the evolutionary forces driving the evolution of antibiotic resistance by a high-throughput laboratory evolution experiment, followed by whole-genome sequencing, on *Escherichia coli* bacteria.

- Establishment of a high-throughput antibiotic susceptibility screening protocol to map the cross-resistance and collateral sensitivity interactions of 12 clinically relevant, conventional antibiotics that represent a diverse set of modes of action.

- Investigation of the correlation between the physiological and evolutionary interactions of antibiotics.

- Understanding the main patterns behind the cross-resistance interactions.

- Uncovering the molecular mechanisms behind collateral sensitivity by the application of biochemical assays. Understanding how frequent collateral sensitivity is and how can we utilize it in the clinics.

Methods

Laboratory evolutionary experiment. During the evolution we adapted parallel populations of *Escherichia coli* K12 BW25113 towards the gradually increasing concentration of one of 12 antibiotics, in batch cultures transferring 1% of the population into fresh medium every 24 hours. 96 parallel populations were started for each antibiotic and the evolution was continued until at least 10 parallel populations could grow or until we reached the solubility limit of an antibiotic. Depending on the growth and solubility limit, the evolution lasted approximately 240-384 generations.

Whole genome sequencing. In order to identify the mutations behind the acquired resistance, the ancestor (*Escherichia coli* BW25113) and 63 adapted lines were next generation sequenced on SOLiD platform. The whole genome sequencing and the analysis and validation of the acquired raw data was performed by the Sequencing Platform of the Biological Research Centre, under the direction of István Nagy, PhD. The raw results of the sequencing are available in the NCBI BioProject database (Accession: PRJNA248327 ID: 248327).

Measuring the mutation rate. The mutation rate was measured by Luria-Delbrück fluctuation assay that is based on the frequency of the emergence of rifampicin resistance. The mutation rate was measured by my colleague, Orsolya Méhi, PhD.

High-throughput antibiotic susceptibility screening. During the high-throughput screening we measured the susceptibility (cross-resistance and collateral sensitivity) of the 120 parallel adapted lines towards those 11 antibiotics that they have not met before. For the measurement of these 1320 (120*11) data points we developed a high-throughput screening protocol followed by a robust statistical analysis.

Measuring the relative fitness of the adapted lines. The relative fitness of the 120 parallel adapted lines was measured during the antibiotic susceptibility screening by performing the screen on antibiotic-free medium as well.

Calculating the chemical similarity. Chemical similarity was measured by the Tanimoto coefficient that captures the chemical fingerprint similarities of antibiotics. This analysis was performed by my colleague, Ádám Györkei.

Calculating the chemogenomic profiles. Chemogenomic profiles were based on the database of Girgis et al on the genes affecting susceptibility for each antibiotic. Chemogenomic profile similarity was calculated as the Jaccard coefficient for each pairs of antibiotics. The database of Girgis et al includes data for 9 of the 12 antibiotics applied by us. The chemogenomic profile similarities were calculated by my colleague, Viktória Lázár, Ph.D.

Reinsertion of single mutations. In order to understand the molecular mechanisms behind the acquired resistance and evolutionary interactions, we chose 9 mutations that could play a key role in these mechanisms and reinserted each of these mutations separately into the wild type genetic background. Mutations were reinserted by a scarless-markerless method based on a suicide plasmid. The reinsertion was performed by my colleague, Csörgő Bálint, Ph.D.

Measuring the bacterial membrane potential. In order to measure the effect of resistance to different antibiotics on the bacterial membrane potential, we randomly chose 2 adapted lines for each antibiotic and measured their membrane potential by flow cytometer, applying the BacLight Bacterial Membrane Potential Kit.

Hoechst accumulation measurement. 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.

Measuring the minimal inhibitory concentration (MIC) on dilution series. In most cases MIC was measured on standard linear dilution series in liquid medium. To maximize reproducibility and precision, the 11-step dilution series were prepared by a robotized liquid handling system.

Measuring the minimal inhibitory concentration (MIC) by E-test strips. Inoculum preparation and plating on agar, as well as the placing of the E-test strips and reading of MIC values were performed according to the manufacturer's instructions.

Effect of AcrAB efflux pump overexpression and deletion on susceptibility. In order to understand the role of the AcrAB efflux system in the observed evolutionary interactions, we modified 3 different strains as follows: we either deleted the *acrB* gene by P1 transduction, or we transformed a multicopy plasmid (pUCacrAB) encoding the AcrAB efflux pump into these 3 strains. The pUCacrAB plasmid was kindly provided by Kunihiko Nishino, Ph.D. and Akihito Yamaguchi, Ph.D. (Osaka University, Osaka, Japan).

Results

I) High-throughput laboratory evolution to establish lines with high resistance towards one of 12 antibiotics. The evolution was initiated from a common ancestor (*Escherichia coli* BW25113) towards the gradually increasing concentrations of one of 12 antibiotics, with 96 parallel populations per antibiotic in batch culture. Under this strict gradual increasing the experiment was continued until at least 10 parallel populations were growing for the antibiotic or the concentration reached the solubility limit of the given antibiotic. In the end the evolution lasted for around 240-384 generations and despite the short timeframe, bacteria could even grow at as high concentrations as 328-fold over the minimal inhibitory concentration (MIC) of the applied antibiotic.

II) Adaptive mutations dominated in the laboratory evolved antibiotic-resistant lines. Several results suggest that mutations identified in protein coding regions were driven by the selective pressure of the antibiotic. First, 87% of the point mutations were non-synonymous, causing amino acid susbtitution. Second, we identified several mutations that are identical to those identified in environmental or clinical resistant isolates not only on the level of amino acid susbstitution, but also in the exact nucleic acid substitution. This second result is also extremely important from another aspect: it shows that laboratory evolution can predict some of the genomic resistance mutations that appear in the clinic.

III) Loss of function mutations are prevalent. 27% of the observed point mutations, small deletions and insertions (between 1-100 basepairs) generated early, in-frame stop codons, frameshifts or the disruption of the start codon. These mutations most probably lead to the production of inactive proteins or proteins with compromised activities. In several cases, these loss of function mutations provide resistance to multiple antibiotics, like in the case of loss of function mutations of the transcriptional repressores of the antibiotic stress-response (for example the *acrR*, *marR*, *mprA*). These mutations alone could provide multidrog resistance.

IV) Evidences of parallel evolution. Signs of parallel evolution were present on both the level of affected functional modules, genes, amino acids and even nucleotides. First, 35% of the affected genes acquired mutations in at least 2 independent, parallel adapted lines. Moreover, 8% of the identified point mutations appeared in at least 2 independent, parallel adapted lines. Remarkably, 66% of those genes that acquired mutations in independent lines acquired mutations in lines adapted to different antibiotics. This result suggests that despite the different mechanisms of action of various antibiotics, the key elements of the development of resistance are provided by overlapping functional modules.

V) High-throughput screening of the antibiotic susceptibilities of the adapted lines. The high-throughput protocol was validated by 2 independent control experiments. First, while optimizing the protocol colony sizes were measured on agar plates in parallel. Second, results for cross-resistance were validated by measuring the minimal inhibitory concentrations (MIC) with standard E-test stripes for a subset of the adapted lines. Based on this MIC measurement the false positive rate was found to be 5%, while the false negative rate was found to be 16%.

VI) Treatment with a single antibiotic can lead to multidrug resistance. The evolution of multidrug resistance was frequent despite applying the antibiotics alone in monotherapy: 52% of the investigated antibiotic pairs showed cross-resistance interaction in at least one direction. The strength of cross-resistance was very diverse, from as mild as 2-fold increase in the minimal inhibitory concentration (MIC) to as high as 128-fold increase in the MIC. Strong cross-resistance interactions were not limited to antibiotics with similar modes of action or belonging to the same antibiotic class.

VII) Parallel evolution is partially responsible for the observed cross-resistance interactions. Despite the fact that the 12 applied antibiotics cover a wide range of mode of action, we found signs of parallel evolution even between distinct antibiotic classes. While analysing the mutation profile similarity of the independent adapted lines, we found that those antibiotic pairs for which there was only slight similarity in the mutation profile of the adapted lines exhibited fewer cross-resistance interactions than those antibiotic pairs where the mutation profile was highly similar. We further demonstrated the role of individual mutations in the cross-resistance interactions by reinserting 9 of the identified mutations individually into the wild-type genetic background and measuring their effect on susceptibility towards the 12 antibiotics applied.

VIII) The effect of antibiotic properties on cross-resistance interactions. The chemical structure similarity (measured in the Tanimoto coefficient that prepares a chemical fingerprint for each antibiotic based on the presence and absence of specific structural motifs and then compares these motifs) showed almost no correlation with the cross-resistance interactions. Correlation between the similarity of mode of action and cross-resistance was tested by comparing the mutation profile similarity and the chemogenomic profile similarity of each antibiotic pair. In this case we found that those antibiotic pairs that showed high overlap in their chemogenomic profile similarity also led to the acquisition of a similar set of mutations in the laboratory evolution. Thus we can conclude that although the chemical structure has no effect on the cross-resistance interaction, the chemogenomic profile similarity significantly influences the appearance of these interactions.

IX) Collateral sensitivity: the Achilles' heel of multidrug-resistant bacteria. Collateral sensitivity interactions are surprisingly frequent: 35% of all investigated antibiotic pairs showed collateral sensitivity in at least one direction. Most of these collateral sensitivity interactions appeared between antibiotic pairs that show negligible overlap in their chemogenomic profiles. Accordingly, collateral sensitivity is more likely to appear between antibiotics that have distinct modes of action. The observed collateral sensitivity network was dominated by aminoglycosides: 44% of all collateral sensitivity interactions were observed between an aminoglycoside and a non-aminoglycoside. The strength of collateral sensitivity was usually around a 2-10-fold decrease in the minimum inhibitory concentration (MIC). This decrease is proportional to the 2-8-fold increase in MIC provided by multidrug efflux pump mutations.

X) Multiple mechanisms behind aminoglycoside resistance. The complexity of aminoglycoside resistance is at first suggested by the number of mutations: aminoglycoside-adapted lines acquired an average of 11.4 mutations per line, while non-aminoglycoside adapted lines acquired only an average of 5.65 mutations per line. A pathway enrichment analysis revealed that one of the main targets of selection in case of the aminoglycosides was the translation machinery, in agreement with their mode of action. Moreover, mutations were also enriched in genes that play roles membrane transport processes, phospholipid biosynthesis and the maintenance of cell envelope homeostasis. The biosynthesis pathway of polyamines, that can bind intracellular reactive oxygen species, was also affected. Another large group of mutated genes were those genes that can directly or indirectly influence the membrane potential of the bacteria.

XI) Opposing effects of aminoglycoside-resistance mutations on membrane potential. We hypothesized that those mutations in the aminoglycoside-adapted lines that have an effect on the membrane potential of the bacteria play a key role in the development of collateral sensitivity. Our hypothesis is based on two key observations from the literature: first, aminoglycosides uniquely require proton motive force for their bacterial uptake; second, several non-aminoglycoside antibiotics are eliminated by proton motive force dependent multidrug efflux pumps from the bacteria. Based on these two observations we thought that in order to rapidly acquire an initial small resistance to aminoglycosides, *Escherichia coli* drastically decreases its membrane potential. However, these mutations diminish the activity of the major proton motive force dependent multidrug efflux pumps. Indeed, many of the observed mutations in aminoglycoside-adapted lines directly or indirectly influence the membrane potential. On top of these mutations we also performed to biochemical assays to prove our hypothesis. First, we used $DiOC_2(3)$ fluorescent dye to demonstrate that the membrane potential is actually significantly lower in aminoglycoside-adapted lines. Second, we also detected increased intracellular Hoechst dye accumulation in these aminoglycoside-adapted lines, that suggests decreased activity of the major efflux pumps.

XII) A single mutation in the gene *trkH* leads to widespread collateral sensitivity. TrkH supports the bacterial membrane potential by the uptake of potassium ions. *TrkH* acquired mutations in 64% of the aminoglycoside-adapted lines. Reinsertion of one of these mutations, that lies close to the ion channel, into the wild-type genetic background could alone provide resistance to aminoglycosides and collateral sensitivity to many other antibiotics. The trkH* single mutant strain also exhibited lower membrane potential and increased Hoechst accumulation. These findings further supported our hypothesis that this collateral sensitivity is caused by a significant decrease in the proton motive force.

XIII) Collateral sensitivity is partly related to the AcrAB efflux system. The AcrAB multidrug efflux pump works on a proton motive force dependent way, therefore, we assumed that this efflux system plays a key role in the collateral sensitivity pattern observed by us. To investigate the role of AcrAB pump in collateral sensitivity, we transformed the trkH* single mutant strain and an aminoglycoside-adapted line with a multicopy plasmid encoding the *Escherichia coli* AcrAB efflux pump and its native promoters. The sensitivity of these transformed strains was tested towards four antibiotics that are all known substrates of the AcrAB efflux pump. The AcrAB overexpressing plasmid provided significant resistance towards all tested antibiotics in the wild-type background, however, the same plasmid had very little effect in the trkH* strain and the aminoglycoside-adapted line.

Publications related to this thesis

V Lázár*, I Nagy*, **R Spohn***, B Csörgő , Á Györkei, Á Nyerges, B Horváth, A Vörös, R Busa-Fekete, M Hrtyan, B Bogos, O Méhi, G Fekete, B Szappanos, B Kégl, B Papp, Cs Pál **Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network** NATURE COMMUNICATIONS Jul 8;5:4352. (2014) IF: 11.470

* shared first authors

V Lázár, G Pal Singh, **R Spohn**, I Nagy, B Horváth, M Hrtyan, R Busa-Fekete, B Bogos, O Méhi, B Csörgő, Gy Pósfai, G Fekete, B Szappanos, B Kégl, B Papp, Cs Pál **Bacterial evolution of antibiotic hypersensitivity** MOLECULAR SYSTEMS BIOLOGY Oct 29;9:700. (2013) IF: 14.099

Other publications

RA Notebaart, B Szappanos, B Kintses, F Pál, Á Györkei, B Bogos, V Lázár, **R Spohn**, B Csörgő, A Wagner, E Ruppin, Cs Pál, B Papp **Network-level architecture and the evolutionary potential of underground metabolism** PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA Aug 12; 111(32): 11762–11767. (2014) IF: 9.674

V Lázár, A Martins, **R Spohn**, L Daruka, G Grézal, G Fekete, M Számel, PK Jangir, B Kintses, B Csörgő, Á Nyerges, Á Györkei, A Kincses, A Dér, FR Walter, MA Deli, E Urbán, Zs Hegedűs, G Olajos, O Méhi, B Bálint, I Nagy, TA Martinek, B Papp, Cs Pál **Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides** NATURE MICROBIOLOGY May 24; 3(6): 718-731. (2018) IF: NA