Thesis of the PhD dissertation

Link between iron homeostasis, oxidative mutagenesis and antibiotic resistance evolution

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Szeged

2014

Introduction

Antibiotic resistance is an increasingly urgent, present-day medical issue. The emergence of resistant strains threatens not only the treatability of contagious diseases, but other medical treatments as well for which the use of antibiotics is indispensable. Besides its medical implications studying the mechanisms governing antibiotic resistance evolution represents an important evolutionary biology issue as well. It is one of the few evolutionary processes which can be studied in real time.

In the case of microorganisms the two main mechanisms contributing to the evolution of antibiotic resistance are the horizontal gene transfer and the accumulation of chromosomal resistance mutations. Dominance of either mechanism is antibiotic- and bacterial species-dependent, but in many cases both contribute to the development of a very high level of resistance.

Resistance to a number of antibiotics (e.g. fluoroquinolones, rifampicines) develops predominantly by acquisition of *de novo* mutations during antimicrobial therapy. *De novo* mutation based resistance mechanisms can be classified into three main categories: 1) modification of the antibiotic target, 2) alterations in antibiotic transport (decreased uptake or increased efflux) or 3) increased expression of enzymes degrading or neutralizing the antibiotic.

Our work focused on *de novo* evolution of antibiotic resistance during a lethal ciprofloxacin exposure. The fluoroquinolone antibiotic ciprofloxacin is a widely used bactericid antibiotic, and its molecular mechanism has been well studied. Laboratory evolutionary experiments performed on the *Escherichia coli* Gram-negative bacterium represented the main methodology of our investigations.

Aims of the study

We investigated the mechanisms underlying the evolution of ciprofloxacin resistance from two aspects, while searching for the answers for the following questions:

1) Are there non-essential genes in *E. coli* whose inactivation boosts the evolution of resistance against a lethal dose of ciprofloxacin? If yes, which are the underlying mechanisms?

2) What role does iron homeostasis play in the evolution of ciprofloxacin resistance?

Methods and techniques

- Culturing *E. coli* wild type and mutant strains
- Preparation of electrocompetent cells and transformation
- Measuring minimum inhibitory concentration (MIC) of different antibiotics
- Measuring *de novo* antibiotic resistance development by short-term, high-throughput evolutionary experiments
- Cell viability assay in the presence of antibiotics
- Rifampicin and Lac reversion based mutation rate measurements
- Fluorescence based promoter activity measurements
- Measuring antibiotic induced ROS production with redox sensitive fluorescent dye
- Molecular biology methods and technics: genomic DNA-, RNA-, and plasmid isolation, polymerase chain reaction (PCR), agarose gel electrophoresis, basic DNA cloning technics, gene deletion by P1 phage-transduction method.
- Membrane permeability measurement by Hoechst dye accumulation assay
- Microarray based gene expression measurements

Summary of the results

In order to investigate the impact of gene inactivation on *de novo* evolution of antibiotic resistance, we performed a genome wide screen by using the *E. coli* KEIO collection, containing ~ 4000 single-gene knockout strains. We identified five genotypes with a massive increase in the frequency of resistant populations in comparison with the wild type strain. Based on the function of the encoded protein the identified genes could be classified into three main categories: 1) methyl-directed mismatch repair (*mutS*, *mutH*, *mutL*), 2) translation fidelity (*miaA*) and 3) iron homeostasis regulation (*fur*). As a common characteristic of these genes, their inactivation elevates the spontaneous mutation rate of the cell, providing a mutator phenotype, which significantly increases the chance of resistance development. Our results emphasize the significant role of mutator phenotypes in promoting the evolution of antibiotic resistance. In case of extreme antibiotic stress, bacteria benefit from an enhanced mutation rate, as this can provide mutations that protect the population from death.

Fishing out the *fur* gene, coding for a central regulator of iron-homeostasis, drew our attention to a potential link between intracellular iron-homeostasis and antibiotic resistance. We demonstrated that perturbation of iron homeostasis promotes the evolution of resistance against a lethal dose (6.25 times the minimal inhibitory concentration of wild-type *E. coli*) of ciprofloxacin.

Transcriptomics data have shown that ciprofloxacin caused a major reprogramming of gene expression across the genome of Δ*fur* strain. Genes involved in iron uptake comprised a significant part of those with changed expression.

We proved that regulation of intracellular free iron concentration plays a crucial role in the evolution of resistance against ciprofloxacin. Increased level

of intracellular free iron leads to oxidative stress via the hydroxyl radical-generating Fenton reaction. Oxidative stress may lead to the appearance of mutations and hence to the development of antibiotic resistance. Minimizing the intracellular free iron concentration either by inhibition of siderophore mediated iron uptake, enhancement of iron storage or chelation of intracellular free iron using a cell permeable iron chelator caused a significant decrease in the frequency of ciprofloxacin resistant populations in the case of Δ*fur* strain.

Next to the intracellular free iron concentration the level of superoxide also represents a key factor in the evolution of resistance. In case of Δ*sodAB* strain, deficient in superoxide detoxification, the frequency of resistant populations, similarly to Δ*fur* strain, is elevated. The mutagenic effect of enhanced superoxide formation was contingent upon iron uptake and probably relies on Fenton chemistry, similar to what was observed in Δ*fur*. It seems that increased iron and superoxide levels enhance each other's effect, contributing to oxidative mutagenesis and consequent resistance development. Moreover, anaerobic conditions drastically diminished both strains' (Δ*fur* and Δ*sodAB*) resistance promoting "capacity", which supports the important role of oxidative mutagenesis in antibiotic resistance development.

Prior studies have shown that bactericidal antibiotics induce perturbations in cell metabolism, including iron homeostasis, that stimulate intracellular accumulation of reactive oxygen species (ROS). We demonstrated that ciprofloxacin treatment induces a slight, but significant increase in the level of ROS. We hypothesize that increased intracellular iron level and ciprofloxacin induced oxidative stress contribute to the increased evolvability of Δ*fur* strain by strengthening each other's effect.

Based on our results the mutation generating activity of Pol IV and Pol V error-prone DNA polymerases has only a minor contribution to the appearance of resistance mutations in Δ*fur*. We hypothesize that a significant part of the oxidized nucleotides is incorporated into the DNA strand by the Pol III

replicative polymerase. By overexpressing the MutS protein, we have shown that appearance of the resistance mutations in Δ*fur* is independent of the activity of the methyl-directed mismatch repair system.

In our experimental setup ciprofloxacin-induced oxidative stress together with an intracellular iron overload promotes oxidative mutagenesis, thus resistance development, rather than lethality. Based on this, the enhancement of antibiotics induced oxidative stress, suggested by other works does not seem to be a proper strategy to improve the efficiency of antibiotics, because the presence of ROS promotes mutagenesis instead of eradication.

Publications related to this thesis

Méhi O, Bogos B, Csörgő B, Pál C. 2013. **Genomewide Screen for Modulators of Evolvability under Toxic Antibiotic Exposure.** Antimicrob. Agents Chemother. **57**:3453–3456.
I.F.: 4.606

Méhi, O., Bogos, B., Csörgő, B., Pál, F., Nyerges, Á., Papp, B., Pál, C. 2014. **Perturbation of Iron Homeostasis Promotes the Evolution of Antibiotic Resistance.** Mol Biol Evol **31**: 2793–2804.
I.F.: 14.308

Other publications

Fehér T, Bogos B, Méhi O, Fekete G, Csörgő B, Kovács K, Pósfai G, Papp B, Hurst LD, Pál C. 2012. **Competition between Transposable Elements and Mutator Genes in Bacteria.** Mol Biol Evol **29**:3153–3159.
I.F.: 10.353

Lázár V, Pal Singh G, Spohn R, Nagy I, Horváth B, Hrtyan M, Busa-Fekete R, Bogos B, Méhi O, Csörgő B, Pósfai G, Fekete G, Szappanos B, Kégl B, Papp B, Pál C. 2013. **Bacterial evolution of antibiotic hypersensitivity.** Molecular Systems Biology **9**.
I.F.: 11.340

Lázár V, Nagy I, Spohn R, Csörgő B, Györkei Á, Nyerges Á, Horváth B, Vörös A, Busa-Fekete R, Hrtyan M, Bogos B, Méhi O, Fekete G, Szappanos B, Kégl B, Papp B, Pál C. 2014. **Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network.** Nat Commun **5**.
I.F.: 10.015