The Role of ROS and Genome Plasticity During De Novo Acquisition of Antibiotic Resistance
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Antibiotic resistance is considered one of the major dangers threatening human health. Bacteria initially susceptible to antibiotics can develop resistance through adaptation on a phenotypic level, horizontal gene transfer, or de novo acquisition of resistance. Sublethal concentrations in the environment are an important factor driving both the emergence and spread of antibiotic resistance. Fundamental knowledge about the molecular mechanisms driving acquisition of de novo antibiotic resistance are necessary to design interventions that can interfere with or impair development of resistance. In this thesis, these molecular mechanisms that drive development of resistance are studied, with particular focus on two aspects: the role of ROS, and the relevance of genome plasticity during acquisition of resistance.

Chapter 1 is used to briefly introduce the topic. An overview of the mechanism of action of antibiotics, the effect of antibiotic treatment on bacterial cells, and the mechanisms that drive acquisition of resistance is provided.

In Chapter 2, the effects of stress on development of antibiotic resistance were explored, with focus on pH regulation, oxidative stress, and outer porin function. Acquisition of resistance to antibiotics with different modes of action, such as amoxicillin and enrofloxacin, was differentially affected by knocking out genes carrying these functions. Knocking out the SOS response, in contrast, affected acquisition of resistance to both antibiotics. This indicates that the development of resistance to bactericidal antibiotics might be caused by activation of similar factors, but the cellular response during resistance development might differ for each class of antibiotic.

Chapter 3 focuses on the role of ROS during acquisition of resistance. We showed that cells with acquired resistance to one bactericidal antibiotic can adapt faster to a second bactericidal antibiotic, but not to a bacteriostatic antibiotic, while no cross-resistance could be found when measuring MICs. When cells with acquired resistance to a single bactericidal antibiotic were stimulated with a second bactericidal antibiotic from a different class, significantly lower ROS levels were measured when comparing to wild-type stimulated cells, and bactericidal-resistant cells are temporarily protected against the killing effects of other antibiotics. Overall, the results suggest that common elements involved in development of resistance might be found in cells with acquired resistance to bactericidal antibiotics.
The resistant strains generated in the work we present in Chapter 3 were analyzed using a whole-genome sequencing approach. The results are described in Chapter 4 and 5. In Chapter 4, all SNPs and indels identified in strains with either a single or a double acquired resistance are documented. Adaptation to a single antibiotic resulted in a number of well-known resistance-conferring mutations, as well as mutations not yet before associated with antibiotic resistance. In strains with a second acquired resistance, mutations associated with resistance in wild-type E. coli were not always identified, and a more varied set of mutations was acquired, indicating that more mutation trajectories are available in cells with a previously acquired resistance. When cells were exposed to a second antibiotic, the maintenance of original mutations and resistance was dependent on the combination of antibiotic with no apparent pattern. The number of mutations maintained did not always correspond with the level of residual resistance, suggesting a role for adaptation on a gene expression level during de novo acquisition of resistance.

Chapter 5 describes the larger-scale genomic alterations occurring in the strains with a single or double acquired resistance, many of which have been never reported to occur during acquisition of antibiotic resistance. In all cells with acquired resistance to high concentrations of amoxicillin, a DNA fragment containing ampC, was amplified, with copy numbers ranging from 48-65. Excision of prophage e14, known to occur upon activation of the SOS response, was observed in strains with acquired resistance to any of the four antibiotics, but only upon secondary exposure. In addition to amplifications and deletions, various insertion sequence transpositions were identified. In general, most genome rearrangements could be correlated with exposure to a specific antibiotic and occurred in multiple independent replicates, indicating that these events do not occur randomly. Overall, the observed genome rearrangements indicate the importance of larger-scale genome rearrangements, which are considered to be more disruptive for genome integrity, during acquisition of resistance in E. coli.

In Chapter 6, the effect of oxygen availability on acquisition of resistance is explored. E. coli was adapted to different antibiotics under anaerobic and aerobic conditions. In the absence of oxygen, adaptation to increasing sub-MIC concentrations of antibiotic was very limited and could only be maintained for a few days, in contrast to cells adapted under aerobiosis. As no difference could be observed in minimal medium containing no alternative electron acceptors, and LB medium, containing nitrate, the
absence of antibiotic-induced radical species was not the limiting factor. As the expected mutations did also not accompany development of resistance under anaerobiosis, most likely, the metabolic changes necessary to allow growth under anaerobic conditions limit acquisition of resistance to increasing concentrations of antibiotic in these conditions.

Chapter 7 is used to discuss the results presented in the preceding chapters. First, the role of ROS during antibiotic action and development of resistance is discussed. We attempt to place our results within the current framework, and suggest possible mechanisms that explain our observed results. Next, genome plasticity is discussed and we hypothesize on common mechanisms driving the observed genome rearrangements. This is followed by a discussion on the relationship between metabolism and antibiotic resistance, and how our results add to the information already available on this topic. Last, we discuss our experience with Crispr-Cas9 genome editing and some pitfalls we observed.

Finally, in Chapter 8, the applicability of the obtained results for veterinary use of antibiotics is outlined. We discuss the role of the environment, the role of a previously acquired resistance, the possibility of de novo acquired multidrug resistance, and the dangers of sublethal concentrations of antibiotic.