Final report (2013.09.01.-2016.08.31.) on „Development of genome engineering approaches to study the evolution of antibiotic resistance" (OTKA grant PD BIOIN 109572).

The original goals of my research proposal were twofold, first, the development and optimization of high throughput genome editing methods in bacteria, and second, using these advances to study molecular mechanisms underlying multidrug resistance in *E. coli*. Specifically, my proposal focused on the improvement of the single-strand oligonucleotide-based allelic replacement method and its high-throughput derivative, multiplex automated genome engineering (MAGE) (1). MAGE is a powerful tool that is unique among genome editing techniques in that it enables rapid, automated and high-throughput genome editing in a highly multiplexable fashion. From accelerated optimization of biosynthetic pathways (2) to the construction of a so-called "genomically recoded organism" by genome-wide codon replacement engineered to depend on synthetic amino acids allowing for genetic isolation (3, 4), MAGE has allowed genome engineering endeavors of unparalleled complexity. However, as with any technique, MAGE has its drawbacks. The most significant of these have been the following: it has been optimized for a few laboratory model strains only, it demands extensive prior modification of the host strain, and finally, it leads to the accumulation of numerous off-target modifications, sometimes outnumbering the desired ones.

Beginning with work started in 2013, we started to tackle these limitations of MAGE and started perfecting the method for broader and more precise applications. By using temperature-sensitive variants of methyl-directed mismatch (MMR) proteins, we were able to achieve high-efficiency MAGE with a markedly reduced off-target mutagenesis. By implementing MMR protein variants with temperature-sensitive defects, it allowed for the rapid switching between the mutator and nonmutator states and was able to control MMR activity throughout the course of numerous genome editing cycles. We published our results in *Nucleic Acids Research* in 2014 (5), this work has since received 17 citations. Additionally, multiple labs have asked for and are now working with our strains, which are available through the Addgene strain and plasmid repository. However, when it comes to the first two issues raised about MAGE (rapid use and portability), these temperature-sensitive alleles still have to be generated within the chromosome of the host bacteria prior to use, greatly increasing the preparation time, and have been optimized only for *Escherichia coli*.

In subsequent work, we developed the so-called pORTMAGE (portable, plasmid-based MAGE) method to combat the remaining limitations of the MAGE technique. The method is based on a set of vectors (named pORTMAGE-1, -2, -3, and -4) we constructed that are specifically optimized for the MAGE process. pORTMAGE is a widely applicable method for large-scale, high-precision, multiplex bacterial genome-engineering. Briefly, the technique is based on temperature-controlled expression of a dominant negative mutator allele of the key *E. coli* MMR protein MutL, which allows a transient switch from a nonmutator to mutator phenotype of the host cell. Importantly, this particular mutator allele cannot be complemented by the native MutL protein, therefore no disruption of the genomic copy is needed. Additionally, because of the highly conserved nature of MMR proteins, this dominant negative mutator allele of MutL produced the same phenotypic effect in a wide range of Enterobacteria. The pORTMAGE plasmids express the λ Red recombinase enzymes as well as the dominant negative mutator allele of MutL, all under the control of the cI857 temperature-sensitive repressor. The temperature-

dependent nature of expression means that at non-permissive temperatures (30-34 °C), the mutant protein is not expressed, therefore allowing the native MMR system to function properly, limiting the number of background mutations to wild-type level. During each genome engineering cycle, expression of the synthetic operon containing the λ Red recombinases and the dominant negative MutL mutant is induced by a simple temporal shift inactivating the native MMR system and allowing for highly efficient allelic replacement to take place. Importantly, when using pORTMAGE, no modifications need to be made to the standard MAGE protocol, as it already incorporates a shift in temperature when activating the expression of the λ Red recombinase enzymes. Using pORTMAGE, we were able to achieve unbiased, highly efficient multiplex recombineering in a wide range of gram-negative bacteria, all without any prior genomic modification of the host organism. This also allowed us to compare the effects of specific antibiotic resistance mutations in different bacterial species, opening a new door for the investigation of differences between antibiotic resistance mechanisms of different bacterial organisms. Our work was published in *Proceedings of the National Academy of Sciences of the USA* earlier this year of which I am co-first author and also co-corresponding author (6). We have deposited the pORTMAGE plasmid set also in the Addgene plasmid repository, with our work also highlighted in the official Addgene newsletter which reaches over 20 000 scientists worldwide. Our plasmid has since been purchased through Addgene by over 15 labs worldwide and requested from me personally by another 15 labs, from such institutions and companies as the Pasteur Institute and New England Biolabs. Building on this work, we are currently developing a technique aimed at highly efficient protein engineering in a genomic context. We are preparing the manuscript describing this method and hope to be able to publish the details of this work sometime in 2017.

To address the second key aim of my research proposal, that is the implementation of the optimized genome engineering methods that we develop into the study of molecular mechanisms behind antibiotic resistance in *E. coli*, several projects have been initiated and are now at an advanced stage. Because one of the major focal points of our lab, headed by Csaba Pál, is the study of bacterial evolution towards antibiotics, it is only natural that the tools we develop be put towards helping other projects currently running in the lab (7, 8). With the development of pORTMAGE, the process of generating specific mutations previously identified by whole-genome sequencing has become much easier and faster. For an earlier project, I used our temperature-controlled MAGE protocol to generate various genome modifications in cells where, for instance, a verification of a resistance conferring mutation was needed. For work published in *Nature Communications*, I generated over 30 different strains carrying various combinations of mutations which my colleagues previously identified through laboratory evolution experiments (8). These verifications were vital in supporting the conclusions made in this work, namely that convergent molecular evolution is prevalent across antibiotic treatments, resistance conferring mutations simultaneously enhance sensitivity to many other drugs, and 27% of the accumulated mutations generate proteins with compromised activities, suggesting that antibiotic adaptation can partly be achieved without gain of novel function.

In an ongoing project, we have generated all possible combinations of antibiotic resistance mutations we have identified in two different antibiotic-adapted strains back into wild-type strains. This set of strains allows us to measure antibiotic resistance fitness landscapes providing key insights into the molecular

mechanisms and evolutionary processes behind drug resistance. This work involves measuring the antibiotic susceptibility of over 40 different strains against 12 different antibiotics. In close collaboration with my colleagues, we have developed a high-throughput protocol using a robotic liquid handling system to measure drug resistance levels of strains in a rapid fashion. I am now co-supervising a fifth year undergraduate student (Gábor Apjok) from the University of Szeged who is helping me with this part of the work. Also, as stated previously, pORTMAGE allows the comparison of resistance mechanisms simultaneously in different organisms, not just laboratory model organisms. We have started a project where we generate the same resistance-conferring mutations in species other than *E. coli* (such as *Salmonella enterica* and *Citrobacter freundii*). Using these sets of mutant variants, we will be able to compare the epistatic effects influencing certain resistance mechanisms. A manuscript describing these two works can realistically be completed in 2017.

During the duration of my research project, I have also taken part in several projects that were not originally part of my research proposal, but nonetheless tied into my work on the development of genome engineering methods. Through these side-projects, I have been able to put the techniques I have developed to good use, showing the advantages of these techniques in a variety of research applications. Recently, I used the various genome editing tools that I have worked with and developed to generate experimental evidence for a bioinformatics research project from the group of Balázs Papp, a close collaborator of the Pál lab. Specifically, I used genome editing and experimental lab evolution to show that *E. coli* could metabolically adapt to the utilization of a complex carbon source (ethylene-glycol) by first adapting to utilize a different carbon source (propylene-glycol). This supports a so-called "dynamic environment" hypothesis for complex evolutionary adaptations, meaning that an adaptation to one environment can serve as a stepping stone for adapting to a seemingly unrelated different environment. A manuscript (of which I am a joint first-author) describing this work was recently published in *Nature Communications* (9).

In another project collaborating with the group of Balázs Papp, I generated specific mutant variants of enzymes that were later shown to have increased side-activities. One of these mutants was used as an example to show that enzymes have the evolutionary potential to increase their underground activities leading to potential metabolic innovations. This work was published in *Proceedings of the National Academy of Sciences of the USA* in 2014 (10).

For another work published recently in *Molecular Biology and Evolution*, I used our optimized MAGE method, in conjunction with other genome engineering methods such as homologous recombination based suicide-vector modifications and P1 phage transduction to generate over 40 different strains that were needed to help in the study of the role of intracellular iron level regulation in the evolution of antibiotic resistance (11). The strains I generated using these various genetic engineering techniques allowed us to show the important, and previously overlooked effects of elevated intracellular iron levels on the evolution of antibiotic resistance.

Recently, my expertise in genome engineering methods was made to good use in another project from the lab, where I implemented the CRISPR-Cas9 genome editing method (12) to generate specific mutations in yeast. This study aims at understanding the role that phenotypic heterogeneity plays during

adaptive evolution. Depending on the so-called noisiness of the expression of certain genes, the organism shows different capabilities in adapting to stress. It is during this adaptation process that specific mutations arise depending on the expression noise. Generation of these mutants in different backgrounds gave further support for the role of heterogeneous expression during adaptation. A manuscript describing this work has currently been submitted.

In another project, I used the pORTMAGE protocol to inactivate all of the so-called error-prone polymerase enzymes of the BL21(DE3) strain of *E. coli*. This was done in collaboration with my former group, headed by György Pósfai and a post-doctoral fellow in his lab, Kinga Umenhoffer. The aim of this specific work was to stabilize the genome of the biotechnologically important BL21(DE3) strain of *E. coli* through the removal of all prophage sequences and the inactivation of all Insertion Sequence (IS) elements. To further stabilize this strain, I used pMAGE to inactivate the SOS-inducible polymerase enzymes. During my PhD work in the Pósfai lab, I showed that inactivation of these enzymes increases genome stability in a different strain of *E. coli* (13). This is advantageous when the goal is to use a strain to maintain different synthetic constructs or when using a strain to overexpress proteins. Indeed, the absence of these enzymes increased the stability of a toxic, synthetic construct maintained in the modified BL21(DE3) strain. Preparation of the manuscript describing the whole, larger project is currently under way.

Finally, I was recently presented the opportunity to take part in the writing of a review article describing the enormous progress that has recently been made in the area of microbial genome engineering and its prospects heading forward. Thanks to the several works and publications that have recently been authored from our lab, we were in the fortunate position to take part in a special issue focusing on microbial systems biology in the journal *Current Opinion in Microbiology* (14). This review, of which I am the first author, is now available online and will appear in the October issue of the journal.

The many different projects I was fortunate enough to be involved in show the versatility and great usefulness of genome engineering methods and specifically our pORTMAGE technique. It is now apparent that the pORTMAGE protocol developed over the past years in our lab greatly increases the speed and precision of genome engineering in bacteria. I have been able to capitalize these advantages in my own main work and also the work of others in our lab.

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Phenotypic heterogeneity guides adaptive evolution.

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