Ph.D. Thesis summary

Systematic genome engineering approaches to investigate mutational effects and evolutionary processes

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Introduction

Methods of bacterial genome engineering offer an unprecedented opportunity to systematically investigate phenotype-to-genotype relationships and evolutionary processes, i.e., the evolution of bacterial resistance to antibiotics, one of the most pressing healthcare challenge of the 21st century. However, available tools for bacterial genome engineering suffer from limitations. Previous methods have been optimized for a few laboratory model strains only (such as *Escherichia coli* K-12 MG1655, the "work-horse" strain of laboratory and biotechnological research) or demand the extensive modification of the host genome, labor-intensive cloning steps, or DNA synthesis prior to genome editing. Moreover, most methods lead to the accumulation of undesired, off-target modifications which, in extreme cases, may outnumber the desired edits and may mask the effect of intentional engineering. These issues have severe implications on the widespread applicability of genome engineering in both basic and applied research.

In this thesis, we present advancements on these challenging fronts. As our first step, we have improved the currently available, most high-throughput and multiplexable genome engineering method, ssDNA-based recombineering (recombination-based genetic engineering), to achieve broad host range functionality and to reduce off-target effects to negligible levels. This new method, termed pORTMAGE offers an all-in-one tool for enterobacterial genome engineering. Next, we have further advanced genome engineering to allow the systematic mutagenesis of extended genomic segments.

The resulting methods now enable the systematic comparison of mutational effects across different species, as well as the exploration of the phenotypic effects of a vast number of mutations in their native genetic context. Moreover, the application of these developments has contributed to a better understanding of bacterial evolution and resistance-processes against antibiotics. As a step-forward towards the predictability of antibiotic resistance, these methods have allowed us to predict the evolution of resistance against an antibiotic currently in clinical trials.

Aims

Our research has focused on improving bacterial genome engineering towards precision, increased throughput, multiplexability, and a broader host range to facilitate its applications in basic and applied research. Specifically, we first aimed to develop a broad host range, plasmid-based ssDNA-recombineering system which can efficiently operate in a wide range of enteric bacteria, including those frequently used in microbial fermentations or are significant human pathogens. Next, building on this advancement, we aimed to develop an extremely high-throughput genome engineering technique that efficiently generates a vast number of genotypic alterations in bacterial genomes in their native genetic context. Finally, we examined whether ssDNA-recombineering enables an accelerated analysis of microbial mutational processes and the directed evolution of antibiotic resistance in multiple bacterial species.

To achieve these goals, our research included the following steps:

- We characterized a dominant mutator allele of the methyl-directed mismatch repair system of *E. coli*, and analyzed the phenotypic conservancy of its effect across enterobacterial species.
- We designed and constructed a broad host range, plasmid-encoded system for ssDNA-mediated genome engineering to allow simultaneous mismatch-repair control and efficient genome editing.
- We developed a method of DNA synthesis which can introduce large genetic diversity into user-defined oligonucleotide strands without mutational bias in a cost-effective way.
- We developed a method for ssDNA-mediated genome engineering which can introduce randomly distributed, random mutations and their combinations along the entire length of multiple long and continuous genomic segments simultaneously in multiple bacterial species.
- We compared the mutational effects that give rise to antibiotic resistance phenotypes across phylogenetically related bacterial strains.
- We identified evolutionary processes that can lead to antibiotic resistance to new antibiotic candidates that are currently under clinical development.

Methods

I. Synthesis of soft-randomized DIvERGE oligonucleotides

We designed and optimized the synthesis of DIvERGE oligonucleotides on an ABI 3900 DNA synthesizer, according to a customized phosphoramidite chemistry-based protocol. The manufacturing of DIvERGE oligonucleotides was performed in collaboration with Györgyi Ferenc at the DNA Synthesis Laboratory of the Biological Research Centre, Szeged.

II. Construction of pORTMAGE plasmids

pORTMAGE1 (Addgene plasmid ID: 72680) and derivatives were constructed by introducing the gene encoding *E. coli* MutL (*ecmutL*) containing an E32→K mutation (*ecmutL*E32K) into the pSIM8 plasmid by using standard cloning techniques. pSIM8 was donated by Donald L. Court (National Cancer Institute in Frederick, MD, USA). Next, we constructed kanamycin and chloramphenicol resistance marker-based variants of pORTMAGE: pORTMAGE3, and 4, respectively.

III. ssDNA-recombineering and Multiplex Automated Genome Engineering

We performed ssDNA-recombineering and iterative Multiplex Automated Genome Engineering (MAGE) cycles to insert specific point mutations and generate mutant libraries with pORTMAGE and DIvERGE in *E. coli* K-12 MG1655, *E. coli* UPEC CFT073, *Salmonella enterica* LT2, and *Citrobacter freundii* ATCC 8090.

IV. MP6 plasmid-based *in vivo* mutagenesis

MP6 is a potent, inducible, plasmid-based mutagenesis system that enhances mutation rate 322,000-fold over the basal level in *E. coli*, thus surpassing the mutational efficiency of other, widely used *in vivo* mutagenesis methods. We generated MP6-mutagenized *E. coli* K-12 MG1655 cell populations and assessed the mutational composition of the resulted cell libraries.

V. Assessing the allelic replacement efficiency of pORTMAGE and DIvERGE across multiple bacterial species

In order to measure allelic-replacement efficiencies uniformly across species, we integrated an artificial landing pad sequence into the genome of *E. coli* K-12 MG1655, *S. enterica* LT2, and *C. freundii* ATCC 8090. This landing pad sequence was integrated into the endogenous *asnA* in every organism by utilizing dsDNA-recombineering. Next, the efficiency of ssDNA-recombineering from pORTMAGE and the performance of DIvERGE were assayed by

performing oligonucleotide integration at this landing pad sequence, followed by high-throughput amplicon sequencing of the target region on Illumina MiSeq.

VI. Whole genome sequencing to assess off-target effects of ssDNA-recombineering

After 24 iterative ssDNA-recombineering cycles, we selected one independently edited clone from *E. coli* K-12 MG1655 + pORTMAGE, MG1655 Δ*mutS* + pSIM8, and MG1655 + pSIM8. To quantify off-target mutations, next, the genomes of these parental and the MAGE-derived clones were whole-genome sequenced on an Ion Personal Genome Machine System at Seqomics Ltd (Mórahalom, Hungary). Mutations that were not targeted by recombineering and were detected only in the edited clones, besides being absent in the parental strains, were voted as off-target mutations.

VII. High-throughput sequencing of soft-randomized oligos

To analyze the characteristics and mutational spectrum of soft-randomization-based oligo synthesis for DIvERGE, we synthesized 90 nucleotide long soft-randomized oligos. Within these oligos, each nucleotide position was soft-randomized with up to 2% of all three possible mismatching nucleobases. To analyze their nucleotide composition, we developed a modified Illumina MiSeq sequencing library construction and sequencing protocol that allowed us to accurately assess the mutational profile of DIvERGE oligos.

VIII. Mutational analysis of DIvERGE-mutagenized cell libraries

To quantify the distribution and spectrum of mutation at *folA*, at the *asnA*-integrated landing pad, and at *gyrA*, *gyrB*, *parC*, and *parE* in our DIvERGE experiments, we developed a PCR amplicon deep sequencing-based mutational assay and subsequent bioinformatic analysis workflow. This assay, in conjunction with subsequent Illumina and Pacific Biosciences Single Molecule Real-Time sequencing and strict sequencing noise removal during sequence data processing, allowed us to precisely identify mutations at these target sites. To access Pacific Biosciences sequencing service, we built up a fee-for-service collaboration with the Norwegian Sequencing Centre, a national sequencing technology platform hosted by the University of Oslo in Norway, while Illumina sequencing was performed in collaboration with Seqomics Ltd (Mórahalom, Hungary). DNA sequencing and data analysis protocols for Illumina DNA sequencing were developed jointly with Péter Bihari, Balázs Bálint, Bálint Márk Vásárhelyi, and István Nagy from Seqomics Ltd, while Pacific Biosciences Single Molecule Real-Time sequencing projects were performed and evaluated jointly with Ave Tooming-Klunderud, Eszter Ari and Balázs Bálint.

IX. Mutation rate measurements

We measured the phenotypic effect of the plasmid-based expression of *E. coli* MutL E32→K (*ecmutL* E32→K) on the mutation rates of *E. coli* K-12 MG1655, *Salmonella enterica* LT2, *Citrobacter freundii* ATCC 8090, *Edwardsiella tarda* ATCC 15947, and *Escherichia hermannii* HNCMB 35034 in a rifampicin frequency-of-resistance and fluctuation assay.

X. *In vitro* growth rate measurements

We measured bacterial fitness as the growth rate in a rich bacterial medium (ie., LB^{L}) under aerobic conditions. Following inoculation, cells were grown at 30 °C in a Powerwave XS2 (Biotek) automated microplate spectrophotometer under continuous agitation. Growth curves were recorded by measuring the optical density of the cultures at every 7 minutes for 24 hours. Finally, growth rates were calculated from the obtained growth curves.

XI. Antibiotic drug susceptibility measurements

Antibiotic susceptibilities of selected bacterial mutants and their wild-type parental strains were determined according to the corresponding experiment. Minimal Salt + casamino acid agar-surface-based minimum inhibitory concentrations of trimethoprim for *E. coli* K-12 MG1655, *E. coli* CFT073, and *Salmonella enterica* LT2 were assessed by using E-test strips according to the manufacturer's protocol (bioMerieux). Trimethoprim resistance of individually isolated bacterial strains was quantified as the 75% inhibitory concentration of trimethoprim (IC75) in minimal salt + casamino acid broth. Specifically, the IC75 value of the given bacterial isolate was calculated as the trimethoprim concentration at which the area under the growth curve of the given cell population was equal to 25% of a control cell population of the same strain that was grown without antibiotic. Maximal IC75 values within pooled mutant libraries were determined by competition-based antibiotic susceptibility measurements. Ciprofloxacin and gepotidacin minimum inhibitory concentrations were determined by a microdilution-based MIC assay according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Results

I. Mismatch repair control by a dominant mutant allows precise genome editing

As our first step, we extended the most high-throughput microbial genome engineering method (multiplex automated genome engineering, shortly MAGE) towards straightforward applicability in multiple bacterial species. To achieve that, we first characterized a dominant mutation in *Escherichia coli* MutL, a key protein of the methyl-directed mismatch repair system, and utilized its dominant effect to precisely control mismatch-repair processes. We have demonstrated that this MutL E32→K mutant of *E. coli* rapidly abolishes mismatch repair activity even in the presence of the wild-type protein. By integrating this MutL E32→K variant into the MAGE workflow, we have developed a plasmid-based system, termed pORTMAGE, for genome engineering, and we have successfully demonstrated its applicability for high-throughput genome editing at multiple loci. Importantly, whole-genome sequencing has revealed that the pORTMAGE-modified strains lack off-target mutations, which is a significant improvement compared to prior MAGE-based methods.

II. Conserved functionality of pORTMAGE across various bacterial species

Exploiting the highly conserved nature of bacterial methyl-directed mismatch repair, the application of the dominant MutL E32→K provided a solution for the interspecies portability. The dominant mutator phenotype of MutL E32→K was conserved across a diverse set of enterobacteria. Thus, by placing the entire synthetic operon encoding all necessary elements of MAGE and mismatch repair control to a broad-host-range vector, we have successfully adapted ssDNA-recombineering to a wide range of biotechnologically and clinically relevant enterobacteria. In turn, pORTMAGE has allowed of the rapid generation of specific mutations, and thus the development of large, unbiased mutant libraries mutagenized at desired positions in these species.

These results have been published in the *Proceedings of the National Academy of Sciences of the United States of America*, on February 16, 2016, https://doi.org/10.1073/pnas.1520040113.

III. Systematic analysis of phenotype-to-genotype associations

The systematic investigation of genotype-to-phenotype associations for complex traits, however, still remained a significant challenge at that point, partly because the evolution of such evolutionary innovations frequently require the acquisition of multiple, rare mutations at

the same time. In turn, the availability of genome engineering tools which enable the targeted combinatorial mutagenesis of multiple loci is an inherent prerequisite for these goals to be met. To address the shortcomings above, we have advanced pORTMAGE-based recombineering to allow the systematic multiplex mutagenesis of long genomic segments. By building on the efficiency, multiplexability, and throughput of multiplex automated genome engineering (MAGE) and the portability of pORTMAGE, as well as by combining these with a method for mutagenic chemical DNA synthesis, we have developed a novel method for *in vivo* directed evolution. Thus, this new method termed directed evolution with random genomic mutations or DIvERGE for short (I) targets multiple, user-defined genomic regions, (II) has a broad and controllable mutagenesis spectrum for each nucleotide position, (III) allows of up to a million-fold increase in mutation rate at the target sequence, (IV) enables multiple rounds of mutagenesis and selection in a fast and continuous way, (V) is applicable to a wide range of enterobacterial species without the need for prior genomic modification(s), (VI) avoids off-target mutagenesis, and (VII) is also cost-effective, as it relies on soft-randomized oligos which can easily be manufactured at a modest cost. In summary, DIvERGE utilizes soft-randomized ssDNA oligos, coupled to pORTMAGE-based ssDNA-recombineering, and thereby allows for the in-depth exploration of the sequence space.

IV. Soft-randomized oligos randomize extended targets

First, by using DIvERGE, we have demonstrated that it can mutagenize multiple, distinct genomic segments at nucleotide level precision, without affecting non-targeted regions. A unique application of soft-randomized oligos has enabled us to extend the target sequence undergoing mutagenesis up to 87% of the length of an entire oligonucleotide, by using only a single oligo. Next, the partially overlapping design of such oligos, as well as the coverage of entire genes and their regulatory regions permitted rapid protein engineering through random mutagenesis.

V. Precise control of mutagenesis

Second, modifications of the parameters for soft-randomized oligo synthesis have made it possible to execute an unbiased introduction of mutations at each targeted nucleotide position, resulting in a more comprehensive generation of combinatorial mutants. Besides the types of introduced sequence alterations, the rate of mutagenesis can also be precisely adjusted by controlling the parameters and composition of soft-randomization of the oligos during synthesis, as well as by the number of iterative DIvERGE cycles. Thereby, mutation rate can

be upregulated to achieve an increase of up to a million-fold compared to wild-type mutation rates at the targeted loci, a range exceeding that of most *in vivo* methods.

VI. Iterative cycles of recombineering accelerate laboratory evolution

Third, we have demonstrated that DIvERGE can be performed iteratively using the same oligo pools which are designed at the beginning of a given experiment. Thereby it permits multiple rounds of directed evolution (consisting of iterative mutagenesis and selection steps), which has been demonstrated to facilitate the rapid attainment of bacterial variants highly resistant to trimethoprim, a widely used antibiotic drug, in our case. By performing multi-round directed evolution of *folA*, we have successfully demonstrated the rapid generation of variants containing up to 10 mutations. This feature is particularly important, as it facilitates the combination of independently generated mutations whose co-occurrence would normally be highly unlikely under laboratory conditions, and would require time-consuming laboratory evolution protocols. Thereby, DIvERGE accelerates the laboratory evolution of slowly evolving traits.

VII. Broad-host mutagenesis identifies strain-specific mutational effects

Fourth, we have demonstrated the portability of DIvERGE by mutagenizing multiple enterobacterial species, including biotechnologically and clinically relevant organisms. DIvERGE has enabled us to explore the *in vivo* evolution of drug resistance in pathogen bacteria, in a much faster and more comprehensive manner compared to prior techniques. Using DIvERGE, we have identified numerous previously undetected resistance-conferring mutations. Moreover, we have also demonstrated that phenotypic effects of certain trimethoprim resistance-associated mutations vary considerably across phylogenetically related strains. Despite a nearly 99% sequence similarity of *folA* between *E. coli* K-12 MG1655 and the uropathogenic strain *E. coli* CFT073, the relative resistance level induced by the very same mutation differed between the two strains. One of the identified mutations, FolA Ala7→Ser was associated with a decreased trimethoprim susceptibility only in the uropathogenic isolate.

VIII. High-throughput recombineering is well suited to analyze antibiotic resistance evolution

Together, pORTMAGE and DIvERGE are paving the way towards the high-throughput *in vivo* exploration of fitness landscapes of endogenous genes or gene networks in multiple species. In a direct clinically relevant application, these methods have allowed us to explore the evolutionary routes which induce target-specific antibiotic resistance in multiple bacterial species. As the new method executes an exceptionally high mutation rate at the drug's target, and also lacks mutational biases, using DIvERGE we have comprehensively generated resistance-conferring mutations at specific drug targets. We have successfully demonstrated that DIvERGE is capable of rapidly identifying mutations which contribute to the development of resistance against three distinct antibiotic classes. Utilizing DIVERGE we have identified several previously described mutations conferring resistance against trimethoprim and fluoroquinolone antibiotics, and furthermore, we have identified a combination of two specific mutations at *gyrA* and *parC* that might lead to clinically significant resistance against a novel antibiotic that is under clinical development (gepotidacin, GSK2140944) in the near future.

These results have been published in the Proceedings of the National Academy of Sciences of the United States of America, on June 19, 2018, 115(25)E5726-E5735, https://doi.org/10.1073/pnas.1801646115, and a patent application was also filed that covers our invention: Nyerges Akos Jozsef, Pal Csaba, Csorgo Balint, Kintses Balint (2017) Mutagenizing Intracellular Nucleic Acids, PCT/EP2017/082574.

List of Publications and Patents

MTMT identification number: 10043320

First-author publications:

1*. **Nyerges, Á.**, Csörgő, B., Draskovits, G., Kintses, B., Szili, P., Ferenc, G., Révész, T., Ari, E., Nagy, I., Bálint, B., Vásárhelyi, B.M., Bihari, P., Számel, M., Balogh, D., Papp, H., Kalapis, D., Papp, B., Pál, C., 2018. Directed evolution of multiple genomic loci allows the prediction of antibiotic resistance. PNAS 115, E5726–E5735. https://doi.org/10.1073/pnas.1801646115 IF: 9.504

2*. **Nyerges, Á.**, Csörgő, B., Nagy, I., Bálint, B., Bihari, P., Lázár, V., Apjok, G., Umenhoffer, K., Bogos, B., Pósfai, G., Pál, C., 2016. A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. PNAS 201520040. https://doi.org/10.1073/pnas.1520040113 IF: 9.661

* These publications served as the basis of the current Ph.D. dissertation.

3. **Nyerges, Á.**, Csörgő, B., Nagy, I., Latinovics, D., Szamecz, B., Pósfai, G., Pál, C., 2014. Conditional DNA repair mutants enable highly precise genome engineering. Nucleic Acids Res 42, e62–e62. https://doi.org/10.1093/nar/gku105. IF: 9.112

4. **Nyerges, A.**, Balint, B., Cseklye, J., Nagy, I., Pal, C., Feher, T., 2018. CRISPR-interference based modulation of mobile genetic elements in bacteria. bioRxiv 428029. https://doi.org/10.1101/428029 . IF: N.A.

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5. **Nyerges Akos J**, Pal C, Csorgo B, Kintses B (2017) Mutagenizing Intracellular Nucleic Acids, PCT/EP2017/082574 (WO2018108987)

6. Tihomir Tomašič, Lucija Peterlin Mašič, **Akos Nyerges**, Csaba Pal, Danijel Kikelj, et. al. LU100918, Luxembourgian Patent Application, (2018) New class of DNA gyrase B and/or topoisomerase IV inhibitors with activity against Gram-positive and Gram-negative bacteria

Co-authored publications:

7. Szili, P., Draskovits, G., Revesz, T., Bogar, F., Balogh, D., Martinek, T., Daruka, L., Spohn, R., Vasarhelyi, B.M., Czikkely, M., Kintses, B., Grezal, G., Ferenc, G., Pal, C.*, **Nyerges, A***., 2018. Antibiotic usage promotes the evolution of resistance against gepotidacin, a novel multi-targeting drug. bioRxiv 495630. https://doi.org/10.1101/495630 IF: N.A.

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