Final report for OTKA PD 105654

In this proposal we have undertaken to apply high throughput methodologies, particularly *in vitro* compartmentalization, in order to develop protein assays that are able to detect enzyme activities and antimicrobial peptides with weak functions that can be improved with directed evolution methodologies. The project consists of three parts: establishment of the infrastructure (1), development of assays to screen for novel enzyme activities, especially weak promiscuous ones (2) and antimicrobial peptides with improved functional efficiency (3). These subprojects have been carried out simultaneously and are presented separately in this report.

1. Establishment of the infrastructure for high throughput screening

In the proposed projects the more efficient protein and peptide variants are selected based on fluorescent signals. For high throughput analysis of fluorescent particles the most convenient way is with conventional cell sorters. Thus, we have adapted the *in vitro compartmentalization* methodology to conventional cell sorters with my previous lab at the University of Cambridge (Anal Chem. 2014 Mar 4;86(5):2526-33). To make this methodology available within the host institute we have carried out the purchase of a high performance cell sorter instrument (Beckman Dickinson FACS Jazz) that is suitable to sort not only cells, but also water-in-oil droplets with high accuracy and made the instrument available for the whole institute.

2. Development of sensor assays to detect weak promiscuous enzyme reactions

As a source for enzyme reactions that can be improved with directed evolution we have chosen the bacteria *Escherichia coli*. The advantage of this organism is that all of its genes are available on an expression plasmid

collection. Using this library of plasmids I have designed a high throughput genome-wide screen using the methodology, where weak promiscuous activities were mapped systematically in the metabolic network of Escherichia coli. The data from the experimental screen then were used to validate a computational model, which integrates the weak promiscuous functions into the metabolic network of the same organism: The significant overlap between the results of the experimental screen and the computational model shows that the prediction of metabolic innovations that forms the basis of evolutionary adaption to new environments is possible. The results were published in the prestigious journal Proceedings of the National Academy of Sciences last year (*PNAS,* 2014, 12;111(32):11762-7). We have selected some of the reactions that we have described here (D-lyxose isomerization, dephosphorilation of D-2-deoxyribose) and currently carry out directed evolution experiments to improve the weak promiscuous functions of these enzymes. Additionally, we apply and develop further a state-of-the-art genome-engineering tool (Multiplexed Automated Genome-Engineering) to carry out the directed evolution procedure not only on plasmid, but also in the genome.

Recently I have also devoted my time to finalize a publication where weak promiscuous enzyme activities were detected from metagenomic libraries using the microfluidic droplet screening procedure (Nat Commun. 2015 Dec 7;6:10008). The observation of these evolutionary start points in environmental samples may predict if adaptation to the presence of mane-made xenobiotics by enzymatic degradation can undergo or not. The plausibility of this hypothesis was demonstrated by detecting many enzymatic potentials in microbial consortiums that cleave phophotriester pesticides a class of xenobiotics against resistance was developed very quickly after its introduction into the environment.

3. Directed evolution of antimicrobial peptides in water-in-oil droplets

The aim of these experiments is to establish a droplet-based directed evolution methodology to improve the function of antimicrobial peptides.

Therefore, we have collected antimicrobial peptides with clinical or industrial relevance and tested their killing capacity in water-in-oil droplets and synthetized them. In droplet the assay the antimicrobial peptides were co-compartmentalized with *Escherichia coli* cells, expressing a GFP signal that could be followed optically upon cell lysis. The test reactions in the droplets showed that the oil phase decreases the efficacy of the peptides, probably by absorbing them with hydrophobic interactions. We hypothesized that instead of the water-in-oil droplets a similar *in vitro compartmentalization* procedure, alginate beads can be used for this experiment as an alternative reaction compartment, where no oil is used for the micro-compartment. As we did not have experience with manufacturing and screening of such reaction compartments we have started to build collaboration with Prof. Sven Panke (ETH Zürich, Switzerland), who is one of the leaders of the field and they have recently demonstrated evolution of antimicrobial peptides with their facility. Together with them, we have designed an experiment where the procedure of the co-evolution of antimicrobial peptide and bacterial resistance can be investigated. To this aim we have produced antimicrobial peptide resistant *Micrococcus flavus* strains and started to screen antimicrobial peptide libraries to see if there are peptides in the sequence space that are still effective in spite of the developed resistance. Using next generation sequencing techniques we are going to explore the distribution of the fitness effects of all possible mutations of the antimicrobial peptide. This part of the proposal is unfortunately running behind, but significant progress was demonstrated last year. Still, foreseeable the experimental part of the proposed subproject will be finished in the beginning of 2016, followed by the preparation of the manuscript.

In summary, in the frame of the OTKA PD fellowship we have (1) established the infrastructure that is necessary for the high throughput screening procedures; (2) carried out sub-project 1 and already published part of the results (*PNAS,* 2014, 12;111(32):11762-7); (3) we made significant progress in subproject 2, first screens of antimicrobial peptides have been carried out, but the experiments still needs to be finalized at the beginning of 2016.

The publications describing our results that were produced in the frame of this and my previous fellowships, but published during the OTKA PD fellowship in the last three years are the following:

Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics
Colin PY, **Kintses B**, Gielen F, Miton CM, Fischer G, Mohamed MF, Hyvönen M, Morgavi DP, Janssen DB, Hollfelder F.
Nat Commun. 2015 Dec 7;6:10008.

Network-level architecture and the evolutionary potential of underground metabolism
Notebaart RA, Szappanos B, **Kintses B**, Pál F, Györkei Á, Bogos B, Lázár V, Spohn R, Csörgő B, Wagner A, Ruppin E, Pál C, Papp B.
Proc Natl Acad Sci U S A. 2014 Aug 12;111(32):11762-7.

One in a million: flow cytometric sorting of single cell-lysate assays in monodisperse picolitre double emulsion droplets for directed evolution
Zinchenko A, Devenish SR, **Kintses B**, Colin PY, Fischlechner M, Hollfelder F.
Anal Chem. 2014 Mar 4;86(5):2526-33.