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BOOK OF ABSTRACTS

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Keynote Lectures

Toward A World of ElectroGenetics

With the advent of the internet of things, interconnected electronic devices are starting to dominate our daily lives and are reaching the control complexity of living systems, and yet work radically different: While human metabolism uses ion gradients across insulated membranes to simultaneously process slow analog chemical reactions and communicate information in multicellular systems via soluble or volatile molecular signals, electronic devices use multicore central processing units to control the flow of electrons through insulated metal wires with gigahertz frequency and communicate information across networks via wired or wireless connections. While analog biological systems and digital electronic devices efficiently work in their respective worlds there are no efficient interfaces between electronics and genetics. We will report our first attempts to design direct electro-genetic interfaces and our progress toward a world of ElectroGenetics and the internet of the body.

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RNA origami robots: Genetically expressible nanodevices that can sense, compute, and actuate

Ebbe Sloth Andersen

Abstract:

As biomolecular designers we strive to develop advanced molecular devices to eventually rival the molecular machines and regulatory systems found in Nature. Due to the relative ease of programming base-pairing patterns, nucleic acid nanotechnology has provided a playground for developing nanomechanical devices that, when being able to sense, compute and act, are often referred to as nanorobots. In this talk I will describe our efforts in improving our RNA origami method [1] towards the development of genetically encodable RNA robotic devices. We initially developed a new software package, that facilitates the design of RNA origami structures. We then characterized a large panel of RNA origamis using atomic force microscopy (AFM) [2] and cryo-electron microscopy (cryo-EM) [3] to explore architectural principles and optimize yield. Finally, we have demonstrated a set of RNA robotic devices that sense a variety of input signals, compute simple Boolean logic, and provide fluorescent signals as output [4]. We suggest that this type of devices can be used as components for molecular circuit in synthetic biology or for controlled release of RNA medicine.

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Synthetic biology tools for yeast cell factories

Verena Siewers, Chalmers University of Technology, Gothenburg/Sweden

Saccharomyces cerevisiae has been engineered to produce a plethora of industrially relevant compounds including biofuels, chemicals, nutraceuticals and pharmaceuticals. These approaches are accompanied by various challenges that can be addressed using synthetic biology tools.

Introduced heterologous pathways can suffer from low enzyme activity or specificity. In such cases, directed evolution is often employed to improve enzyme properties. Traditionally, this comprises iterative rounds of gene diversification *in vitro*, transformation, selection or screening and isolation of improved variants, which can be time consuming. In the past years, several methods that allow for targeted mutagenesis *in vivo* - and thus continuous directed evolution - have been developed. Here, two systems based on Cas9 variants will be presented: yEvolvR, where a Cas9 nickase directs an error-prone DNA polymerase to its designated target site, and the employment of nucleobase deaminases coupled to a deactivated Cas9. The latter was employed to improve the performance of a heterologous transporter in *S. cerevisiae*.

A second challenge is the fine-tuning of endogenous pathways to improve product formation. In a recent study, we predicted target genes for improved recombinant protein production using a proteome-constrained genome-scale protein secretory model. The target genes were evaluated using CRISPR interference/activation (CRISPRi/a) libraries combined with droplet microfluidics screening. We were able to demonstrate that the simultaneous fine-tuning of three genes within central carbon metabolism can increase recombinant protein production 3-fold.

Reservoir Computing with Bacteria

Paul Ahavi, Thi-Ngoc-An Hoang, Jean-Loup Faulon, Micalis, INRAE, University of Paris Saclay, Jouy-en-Josas, France

In contrast to the traditional bottom-up approach of building devices to perform computations within organisms, this presentation explores a top-down strategy utilizing bacterial strains in reservoir computing (RC) to solve regression and classification tasks.

We employ an *E. coli* K-12 MG1655 strain as the reservoir, train it on M9 minimal media supplemented with 28 metabolites, and measure growth rates across various media compositions. Our RC system outperforms traditional methods such as multi-linear regression (MLR) and multi-layer perceptron (MLP) in several regression tasks, particularly when using a generative model to search out the optimum media. Additionally, we find that the performance of RC systems based on genome-scale metabolic models (GEMs) for several bacterial species correlates with the diversity and complexity of phenotypes they produce.

As a practical application for classification, we train our *E. coli* reservoir on a set of gene knock-out *E. coli* mutants grown on samples from moderate and severe Covid-19 patients. Our RC system successfully identifies several mutants capable of accurately classifying the samples.

These findings highlight the potential of top-down synthetic biology for biocomputing, particularly in solving complex computational tasks typically reserved for digital systems. They also suggest future research directions for medical diagnostics and for the integration of the bacterial RC approach with emerging technologies, such as neuromorphic computing and engineered living materials.

Sub topic: AI AND SYNTHETIC BIOLOGY

Engineering *Pseudomonas* as a universal platform for detection of chemicals

Prof. Dr. Pablo Ivan Nikel

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Traditional chemical sensing techniques used to test the performance of cell factories, while effective, often lack the specificity, sensitivity, and portability that biosensors can provide. Most biosensors, however, require engineered transcription factors tailored to detect specific molecules, limiting their versatility. In this talk, I will present a whole-cell biosensor platform based on engineered *Pseudomonas putida* strains to detect a wide range of chemicals. *P. putida* is a versatile bacterium widely used owing to its robust metabolic capabilities and adaptability across environmental niches. By integrating synthetic auxotrophies within its central metabolism with fluorescent reporters in growth-coupled setups, we have created a biosensor platform that overcomes multiple limitations of traditional sensing systems. This whole-cell platform enabled the detection of diverse chemicals (e.g., D-lactate and protocatechuate) under various conditions, including co-cultures of producer and sensing strains. The application of this biosensor platform in monitoring complex biochemical processes is illustrated by adopting the degradation of plastics by both purified hydrolytic enzymes and metabolically engineered bacteria as a test case. This versatile system provides a rapid, sensitive, and adaptable tool for monitoring microbial factory performance, biotechnological processes, and environmental analyses—both online and offline.

Cell-free synthetic biology of parts, molecular assemblies and networks

Henrike Niederholtmeyer, Technical University of Munich, Straubing/Germany

Cell-free expression (CFE) systems contain the molecular machinery for RNA and protein synthesis from DNA templates. CFE systems accelerate design-build-test-learn cycles and prototyping of DNA-encoded functions by allowing assays in miniaturized reaction volumes. Additionally, as open reactions, CFE enables tight control over reaction conditions and direct assays for functionality without a need for protein purification. I will present our recent work on developing rapid prototyping systems, exploring self-assembly of bacteriophages and on regulatory communication networks in two dimensions.

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Lectures

Engineering Genetic Tools to Control Individual Microbes and Microbiota without Antibiotic Resistance Genes at a Single Strain Level

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Matthew Amrofell, Washington University in St. Louis, St. Louis, MO, United States
Steven Vo, Washington University in St. Louis, St. Louis, MO, United States
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Subtopic: synthetic genomics and gene circuits

Key Words: kill switch; biocontainment; microbiome; antibiotic resistance spread; machine learning

Microbial biocontainment is essential for engineering safe living therapeutics [1, 2]. However, the genetic stability of biocontainment circuits is a challenge. Kill switches are among the most difficult circuits to maintain due to the evolution of escape mutants. We engineered two CRISPR-based, chemical- or temperature-inducible kill switches in the probiotic *Escherichia coli* Nissle and demonstrated mutationally robust biocontainment [3]. In this presentation, we will discuss our machine learning-based microbiota engineering tools that are useful to manipulate microbiota and kill pathogens at a single strain level [4]. Specifically, we will discuss the development and validation of a novel computational program, ssCRISPR, which designs strain-specific CRISPR guide RNAs (gRNAs) that can be utilized to modify complex consortia. As a proof of concept, we applied the program to two novel applications: the isolation of specific microbes from consortia through plasmid transformations and the removal of specific microbes from consortia through liposome-packaged CRISPR antimicrobials. Additionally, we will discuss antibiotic resistance gene-free plasmid systems that prevent antibiotic resistance spread via horizontal gene transfer [5-8]. This new technology has vast implications in designing strain-specific antimicrobials and combating the growing concern of antibiotic- and bacteriocide-resistant microbes.

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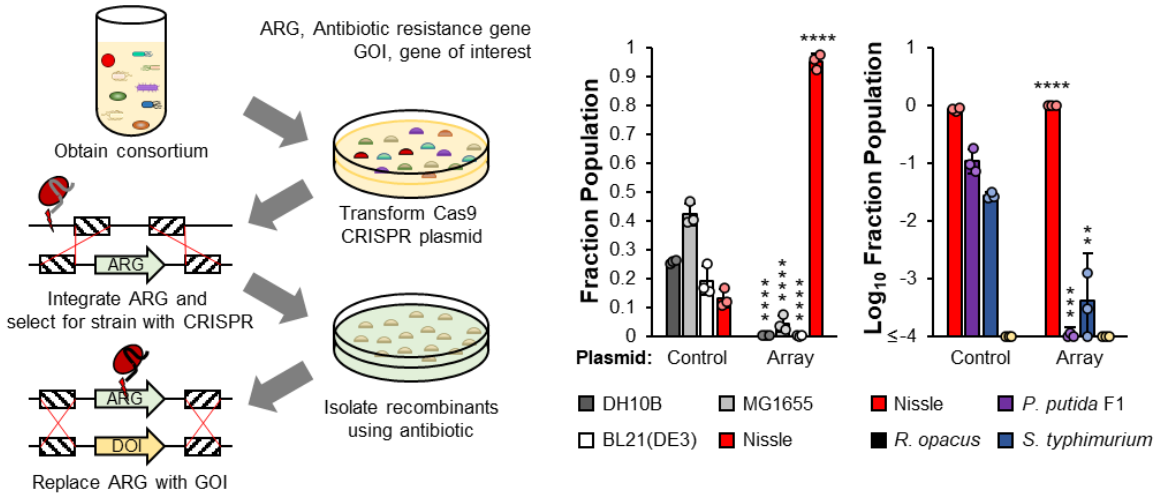

Figure. Strain-specific isolation of microbes from environments. Machine learning-based algorithm guides gRNA design to specifically target only one microbe in the consortium. Nissle was isolated from both synthetic consortia.

A combinatorial approach to improve laccase activity through yeast surface display

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Eva Garcia-Ruiz, Instituto de Catálisis y Petroleoquímica, ICP-CSIC, Madrid, Spain.

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The current demand of petroleum-derived compounds and the limitation of oil highlights the necessity of seeking greener and more sustainable carbon sources. Lignocellulose is a major component in the plant cell wall, accounting for 60% of total biomass on Earth. It is therefore a promising source of organic material for industry. The valorization of lignin remains underutilized compared to cellulose due to its recalcitrant nature.

In nature, secreted enzymes from white-rot fungi are responsible for the degradation of lignin into its basic monomers, which are further metabolized by the associated bacterial community [1]. Among ligninolytic enzymes, laccases play a major role in the degradation of lignin. These enzymes are of interest due to their ability to oxidize a wide range of substrates from phenolic, amines and non-phenolic compounds, releasing only H_2O as by-product [2].

Synthetic biology and synthetic genomics have become prominent research fields in recent years allowing now for the redesign and synthesis of whole designer genomes. We use synthetic yeast strains of the Synthetic Yeast Project (Sc2.0) and synthetic biology tools to improve lignin valorization. We combine modular cloning strategies, directed evolution and yeast surface display to improve the functionality of laccases. At the same time we use the Sc2.0 SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution) to combine protein engineering and chassis engineering for optimal lignin valorization [3].

References

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Orchestrating artificial operons using an ECFσ-based synthetic master regulator in *Alphaproteobacteria*

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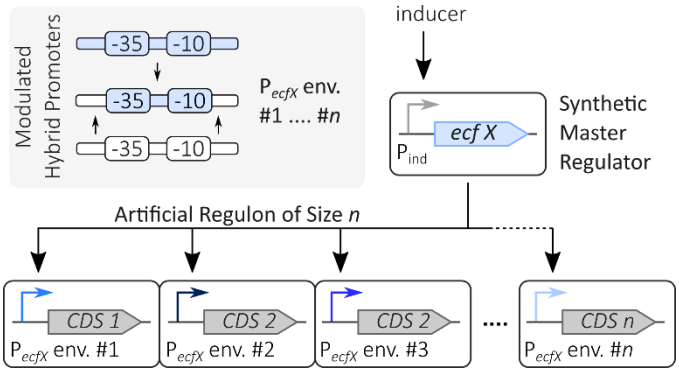
Bacteria constantly face environmental stresses, and their survival hinges on finely tuned genetic responses. Sigma factors, key components of the RNA polymerase, direct this response by guiding the transcription machinery to stress-responsive genes. Extracytoplasmic function sigma factors (ECFσs), the most diverse group of alternative sigma factors, enable transcription from specific promoters for genes coding for stress-response proteins or virulence factors. Groups of co-regulated genes, so-called regulons, can range from single stress-mitigating genes to complex networks. In large regulons, fine-tuning individual gene expression within the promoter region is essential for a balanced stress response.

ECFσs as Synthetic Regulators



Beyond their natural role in bacterial resilience, ECFσs have emerged as versatile tools in synthetic biology. Recently, we showed that ECFσ-based switches designed for the γ-proteobacterium *E. coli* could be transferred to the plant-symbiotic α-proteobacterium *Sinorhizobium meliloti* while maintaining consistent properties between the distantly related organisms (Meier et al., 2023, doi: 10.34133/bdr.0025). Next to autonomous genetic timers, synthetic regulons are an interesting application of heterologous expressed ECFσs, thus reconstructing their natural function in an artificial circuit.

(Semi-)Synthetic ECFσ-dependent Promoters

To recreate such tunable response network, we designed synthetic hybrid promoters based on environments of well-known promoters from *S. meliloti* and the core motifs (-35 motif, spacer, -10 motif) of ECFσ-dependent promoters belonging to phylogenetic

classes 02 and class 11 (abbreviated P_{ecf02} and P_{ecf11}, respectively). We designed and characterized a library of 20 promoter environments that modulate a given ECFσ-dependent promoter in terms of dynamic range and both ON and OFF state activities which are consistent between ECF02 and ECF11. These environmental hybrid promoters can be specifically activated by their cognate ECFσ and maintain orthogonality towards other heterologous ECFσs. Furthermore, we describe a critical UP element-like feature, as short as 3 bp, located upstream of the -35 region, that is essential for highly active hybrid promoters in *S. meliloti* and related *Alphaproteobacteria*.

Towards Artificial Regulons

We harvested the library of synthetic ECF-dependent promoters to construct an artificial regulon consisting of a total of 6 reporter genes controlled by different hybrid promoters of varying strengths and dynamic ranges which are activated by the same ECFσ. With that, we show that a single heterologous ECFσ can activate expression of multiple genes to predictably tunable levels. This result enables the design of more complex pathways, e.g., the controlled expression of natural product biosynthesis gene clusters or induced reprogramming of cellular states such as exopolysaccharide composition.

Using synthetic yeast chromosomes to map and probe large scale-deletions in high-throughput

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Daniel Schindler, Max Planck institute for Terrestrial Microbiology, Marburg, Germany

The Synthetic Yeast Genome Project (Sc2.0) aims to produce the first synthetic eukaryotic genome.[1] All synthetic chromosomes have been synthesized and characterised in individual strains. The sixteen synthetic chromosomes and the tRNA neochromosome are being consolidated in a single cell. All chromosomes have been redesigned according to strict principles.[1] One notable feature is the insertion of symmetrical *loxP* sites downstream of almost every non-essential gene. Upon activation of Cre recombinase, this leads to highly complex structural variations, turning a single genotype into a population. Here we present our strategy and technology for using synthetic yeast to systematically study the effects of large-scale deletions, complementing our knowledge of single gene deletion libraries.

The *loxP* sites are 34 nucleotides in length. We use this as a landing pad to randomly integrate a modified marker by homologous recombination (HR). The resulting population is transformed with a plasmid overexpressing a system to introduce a double-strand break in the marker. The DNA is degraded *in vivo* until HR occurs at *loxP* sites. Counter selection ensures that only candidates that have lost the marker are obtained. We use laboratory automation to select and characterise large numbers of candidates. Based on our high-throughput, low-cost end-point genotyping, we can rapidly identify the deletion size for each strain.[2,3] This is followed by phenotyping of the relevant strains.

We have developed and established a strategy for targeted introduction of large-scale deletions into synthetic yeast chromosomes. The large deletions will be characterised

using molecular and phenotyping techniques to understand their impact on cell viability.

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Laying the Foundations for Plant Synthetic Genomics: Establishing a Plant Artificial Chromosome in *Physcomitrium patens*

Uriel Urquiza-Garcia, Heinrich Heine Universität, Düsseldorf

We are currently engineering the structure of a Neo-chromosome for Physcomitrium patens. This endeavor involves constructing approximately 100Kb of neutral synthetic DNA, meticulously designed to be targeted by an inactive Cas9 protein, facilitating the initiation of a neo-centromere. Our research delves into the potential of various centromeric-associated proteins to catalyze neo-centromere formation. By leveraging of the natural chromosome of P. patens as a foundation, we are building the body of the synthetic neo-chromosomes thanks to P. patens' inherent capability for homologous recombination. Subsequently, the neo-chromosome body will be excised through Cre/lox recombination, producing an episome. This episome will then be precisely edited with a secondary cut by Cas12a (Cpf1) at a site characterized by telomeric repeats. Potentially resulting in a linear neo-chromosome. The genome's stability will be rigorously evaluated, integrating developmental and physiological metrics. This innovative approach has the potential to significantly advance the field of genome biology and evolution. For instance, it could facilitate paleogenomic studies by offering fully synthetic scaffolds for the recreation of ancestral genomic structures. Additionally, it opens the prospect of developing neo-chromosomes for implementation in current crop species, heralding new possibilities in agricultural biotechnology.

Synthetic biology optimization methodology for manufacturing natural products in yeast

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Secondary natural products (NPs) show promise for drug discovery, but their limited availability in nature and the complexity of chemical synthesis pose challenges. Yeasts like *Saccharomyces cerevisiae* and *Pichia pastoris* offer efficient platforms for NP production. Our research aims to create a reliable method for sustainably producing rae NPs using these yeasts. We focus on using synthetic regulators and high-throughput optimization techniques. Integrating automated bioprocessing and single-cell analysis into our synthetic biology platform allows us to identify the best conditions for producing target NPs in microbial cell factories.

Biochemical and metabolic engineering of *Saccharomyces cerevisiae* for the production of olivetolic acid, a key precursor in cannabinoid biosynthesis

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Cannabinoids comprise a large class of bioactive compounds found primarily in the plant species *Cannabis sativa* and are of interest due to their pharmacological and therapeutic potential. The aromatic polyketide, olivetolic acid (OA), is a major precursor in the biosynthesis of cannabinoids and is derived from hexanoyl-CoA and malonyl-CoA by the action of olivetol synthase (OLS) and olivetolic acid cyclase (OAC) from *C. sativa*. To date, most microbial cannabinoid production systems rely on the external supplementation of hexanoic acid together with the overexpression of *AAE1* from *C. sativa* to form hexanoyl-CoA. Here, we describe various metabolic engineering strategies to overcome the need for precursor supplementation and address further limitations within the OA biosynthetic pathway using the yeast *Saccharomyces cerevisiae*. Through the overexpression of a multi-species derived heterologous reverse β-oxidation pathway and the knocking-out of competing pathways, we ensured an endogenous supply of hexanoyl-CoA. Next, we introduced multiple copies of ^{Cs}*OLS* and ^{Cs}*OAC* into the genome and we present a mutant phenylacetate-CoA ligase from *Penicillium chrysogenum* (^{Pc}*PCL-K*) with superior hexanoyl-CoA ligase activity to ^{Cs}*AAE1*. We subsequently mutated known phosphorylation sites within acetyl-CoA carboxylase 1 (*ACC1*$^{S659A/S686A/S1157A}$) to increase the cytosolic pool of malonyl-CoA and overexpressed a feedback resistant pantothenate kinase from *E. coli* (^{Ec}*coaA*^{R106A}) to increase coenzyme A biosynthesis. Finally, we combined these modifications to develop a single recombinant strain capable of the *de novo* production of up to 200 mg L^{-1} OA. Our results provide details of the metabolic engineering steps critical for the biosynthetic production of cannabinoids and their precursors in *S. cerevisiae*.

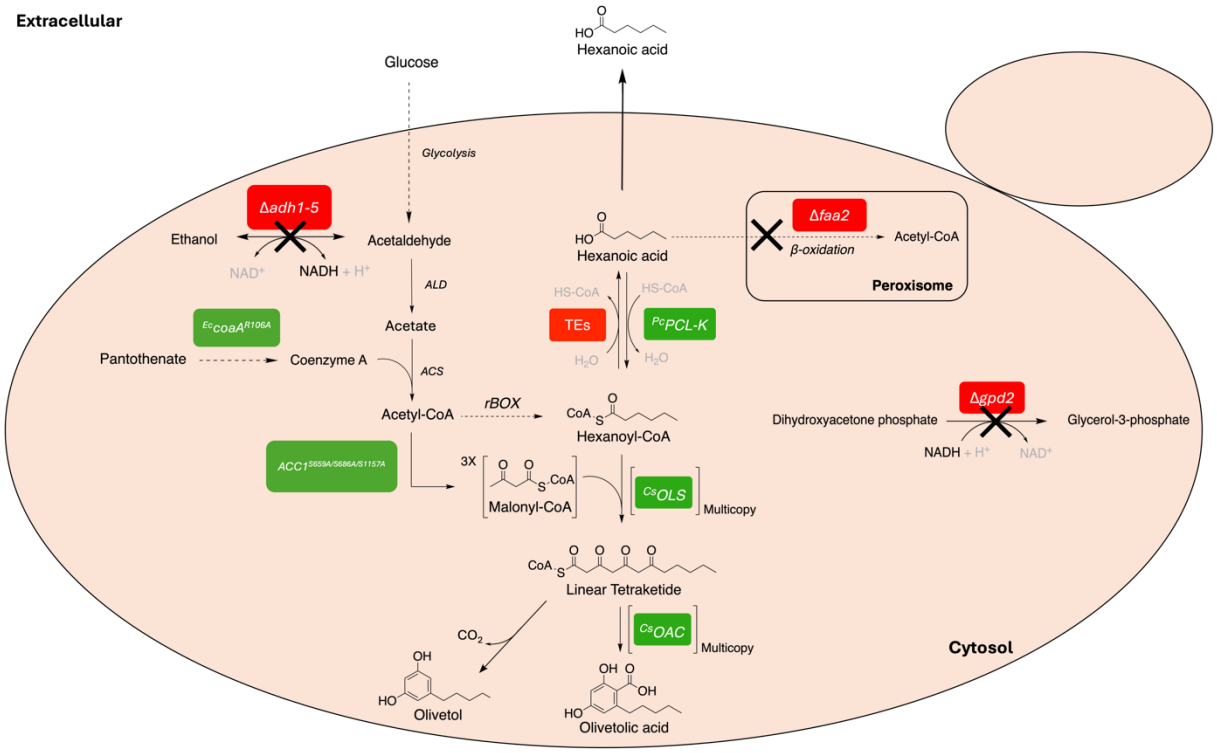

Figure 1 | Overview of the olivetolic acid biosynthesis pathway in *S. cerevisiae*.

Green boxes represent heterologous or modified genes involved in the olivetolic acid (OA) biosynthetic pathway. Red boxes represent genes from competing pathways. Knocked out pathways are indicated with a black cross. *ADH1-5* and *GPD2* were knocked out to provide NADH for the rBOX pathway. *FAA2* was knocked out to prevent the degradation of hexanoic acid via peroxisomal β-oxidation. Olivetol is a side product formed through the spontaneous decarboxylative cyclization of the linear tetraketide intermediate. *ADH1-5* – Alcohol Dehydrogenase 1-5; *GPD2* – Glycerol-3-Phosphate Dehydrogenase 2; *FAA2* – Medium Chain Fatty Acyl-CoA Synthetase; rBOX – Reverse β-Oxidation Pathway; TEs – Thioesterases; *OLS* – Olivetol Synthase; *OAC* – Olivetolic Acid Cyclase; *ACC1* – Acetyl-CoA Carboxylase 1; *PCL* – Phenylacetate-CoA Ligase; *coaA* – Pantothenate Kinase; *Pc* – *Penicillium chrysogenum*; *Cs* – *Cannabis sativa*; *Ec* – *Escherichia coli*; *ALD* – Aldehyde Dehydrogenase; *ACS* – Acetyl-CoA Synthetase.

Engineering yeast towards vitamin B12 dependency and B12 production

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For many years, the industrial production of vitamin B12 (cobalamin) has relied on bacterial strains as the primary producers. However, due to limited methods for optimizing strains and the challenging handling of such strains, there is a growing interest in identifying alternative hosts capable of producing vitamin B12. *Saccharomyces cerevisiae* emerges as a promising candidate due to its inherent independence from vitamin B12, its potential for genetic engineering, and its straightforward cultivation conditions. Nevertheless, the complexity of the B12 synthesis pathway presents a significant challenge in engineering and evolving recombinant yeast cells for B12 production. To address this challenge, we have first developed an *S. cerevisiae* strain that depends on vitamin B12 for its growth (Lehner and Boles 2023). This was accomplished by replacing yeast's B12-independent methionine synthase Met6 with a B12-dependent methionine synthase MetH obtained from *Escherichia coli* (see Fig. 1). Adapted laboratory evolution has proven helpful for gradual adaptation to the utilization of vitamin B12 for methionine production. Our experiments demonstrate that additional high-level expression of a bacterial flavodoxin/ferredoxin-NADP+ reductase (Fpr-FldA) system is crucial for *in vivo* reactivation of MetH activity and subsequent growth. The growth of MetH/Fpr/FldA-containing yeast cells on methionine-free media is only possible with the supplementation of adenosylcobalamin or methylcobalamin (Fig. 2 A and B). Interestingly, the incorporation of a heterologous vitamin B12 transport system is unnecessary for cobalamin uptake. This strain offers a robust platform for engineering B12-producing yeast cells, which could potentially be utilized to fortify food and beverages with this essential nutrient in the future. So far, we have implemented the whole heterologous vitamin B12 production pathway in the B12-dependent strain and are now moving towards *de novo* vitamin B12 production.

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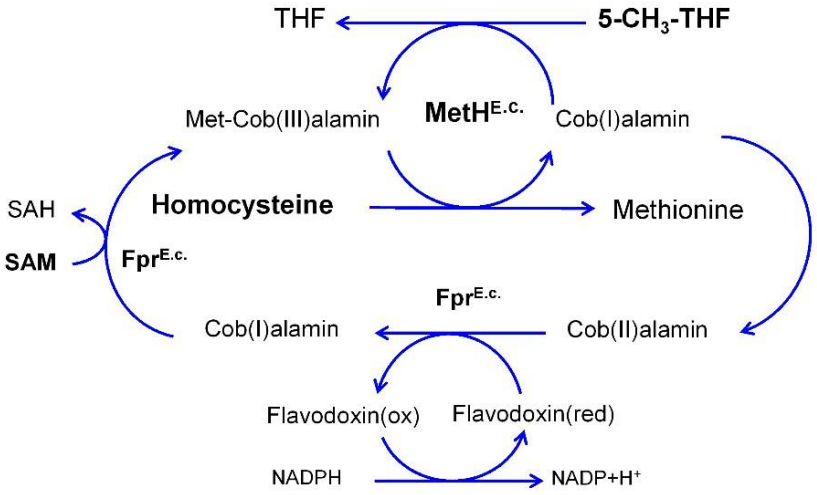

Figure 1: **The vitamin B12-dependent methionine synthase MetH from *E. coli***. The turnover reaction of MetH is supported by a methionine synthase reductase (Fpr) and a flavodoxin (FldA). MetH promotes the transfer of a methyl group from 5-methyl-tetrahydrofolate (5-CH_3-THF) to homocysteine, resulting in tetrahydrofolate (THF) and methionine. Cob(I)alamin is occasionally oxidized to Cob(II)alamin, blocking MetH for further reactions. Fpr is using reduced flavodoxin and S-adenosylmethionine (SAM) to restore MetH activity.

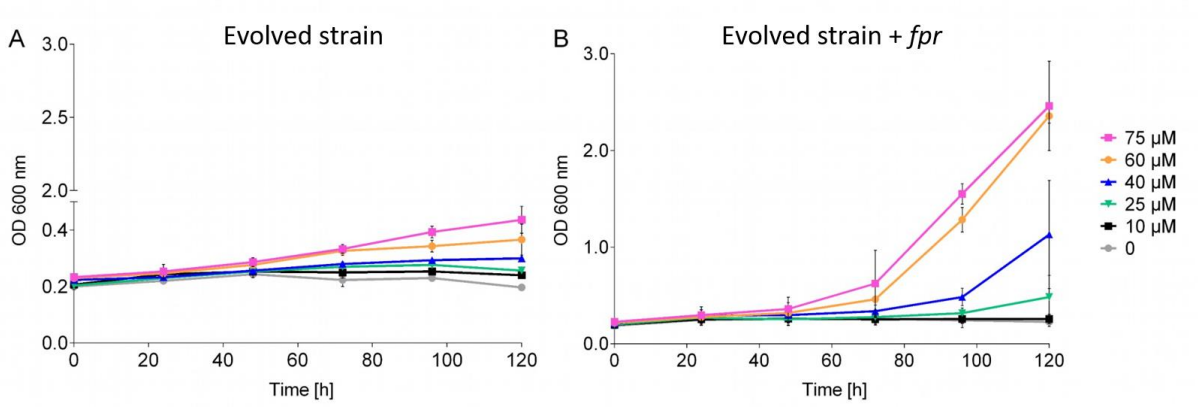

Figure 2: **Growth of the Meth/Fpr/FldA evolved yeast strain in media containing different B12 levels.** The evolved strain alone (A) or expressing additional copies of fpr on a high copy plasmid SARV062 (B) were cultivated on SCD-Met medium supplemented with different amounts of MetB12 (0-75 µM).

Physiological response of *Vibrio natriegens* to low intracellular ATP levels

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Productivity is a key performance indicator of bio-production processes which is crucially limited by the biomass-specific substrate uptake rate of the employed strain. For the model organism *Escherichia coli*, the engineering of the energy metabolism, particularly the ATP pool, has for long been recognized as a promising strategy to increase substrate uptake and productivity by limiting the intracellular availability of ATP [1]. Different methods were applied, such as introducing “ATP wasting” strategies (e.g. ATP futile cycling, implementation of pathways with low ATP yields [1, 2], overexpression of the ATP hydrolysing subunits of F_0F_1-ATPase [3, 4], etc.) or inactivating the ATP synthase complex to abolish ATP formation via oxidative phosphorylation [5, 6]. These works indicated that the *E. coli* glycolytic flux is controlled by the ATP demand and that limiting the intracellular ATP availability leads to increased substrate uptake rates under various cultivation conditions [4]. In recent years, the marine bacterium *Vibrio natriegens* has been attracting wide attention as a host strain for microbial production processes, due to its exceptionally high growth and substrate uptake rates (μ and q_S, respectively). In aerobic bioreactor cultivations with defined mineral medium containing glucose as sole source of carbon and energy, *V. natriegens* can grow at μ = 1.48–1.70 h^{-1} with q_S values of 21.4-21.7 $mmol_{Glc}\ g_{CDW}^{-1}\ h^{-1}$, the latter being about three times higher when compared to *E. coli* [7, 8].

To gain insight into the mechanisms which control substrate uptake in *V. natriegens*, we investigated its physiological response to low intracellular ATP levels and compared its behaviour to that of the model organism *E. coli*. First, the method of plasmid-borne ATPase expression was applied. For both *E. coli* and *V. natriegens* ATPase-overexpressing strains, a similar relative increase of ATPase activity (1.6-1.9 fold) was observed when compared to the respective control strains. In contrast to *E. coli*, in *V. natriegens* the introduction of ATP wasting did not lead to a significant increase of glucose uptake rate during exponential phase. However, under non-

growing conditions, up to 360 % improved glucose uptake rates were observed for non-growing *V. natriegens* ATPase cells compared to the respective control strains. Despite these observations, the highest glucose uptake rates observed for *V. natriegens* ATPase-overexpressing strains (4.43-4.62 mmol g^{-1} h^{-1}) did not reach the maximal rates observed for the *E. coli* ATPase strain (8.78 mmol g^{-1} h^{-1}) in stationary phase.

As a second strategy to ATPase overexpression, the ATP synthase complex was inactivated in *V. natriegens*. Since two ATP synthase operons have previously been bioinformatically annotated in the genome of *V. natriegens* (*atp1, atp2*), we constructed strains deleted for one and two ATP synthase complexes. Differently than observed for ATPase overexpression, an increase of q_S of up to 118-123 % was reached for the *V. natriegens Δatp1* and *Δatp1 Δatp2* strains compared to the wild type under growing conditions indicating that the glycolytic flux in *V. natriegens* is indeed controlled by ATP demand. However, the extent of this control in *V. natriegens* seems to be limited compared to *E. coli* as the relative increases of q_S caused by reducing the intracellular ATP availability in *V. natriegens* were overall lower than observed for the model organism *E. coli* under all tested cultivation conditions.

Overall, our work highlights the importance to further investigate the regulation mechanisms which govern substrate uptake and glycolytic flux in *V. natriegens* to develop this organism into a high-performing industrial producer strain.

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Genome reduction in *Paenibacillus polymyxa* DSM 365 for chassis development

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The demand for highly robust and metabolically versatile microbes is of utmost importance for replacing fossil-based processes with biotechnological ones. Such an example is the implementation of *Paenibacillus polymyxa* DSM 365 as a novel platform organism for producing value-added products such as 2,3-butanediol or exopolysaccharides. For this, a complete genome sequence is the first requirement towards further developing this host towards a microbial chassis. Herein, we report the first complete genome sequence of *P. polymyxa* DSM 365, which consists of 5,889,536 bp with 45 RNAs, 106 tRNAs, 5370 coding sequences and an average GC content of 45.6 %. The additional nucleotide data revealed a novel NRPS synthetase that may contribute to the production of tridecaptin. Building on these findings, we initiated the top-down construction of a chassis variant of *P. polymyxa* by utilising a CRISPR-Cas9 based system. In the first stage, single knock-out mutants of non-essential genomic regions were created and evaluated for their biological fitness. As a result, two out of 18 variants showed impaired growth. The remaining deletion mutants were combined in two genome-reduced *P. polymyxa* variants which either lack the production of endogenous biosynthetic gene clusters or non-essential genomic regions including the

insertion sequence IS*Pap1*, with a decrease of the native genome of 3.0 % and 0.6 %, respectively. Both variants showed identical growth characteristics to the wild-type. Endpoint titers of 2,3-butanediol and EPS production were also unaffected, validating these genome-reduced strains as suitable for further genetic engineering.

Design of biosynthetic pathways in amoebae for production of a cannabinoid precursor

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The rise of synthetic biology is directly linked to the progressive improvement of sequencing technologies, continuously disclosing a plethora of genomic data. Only due to this profound research and the linkage between DNA sequence and function we can access and utilize databases to construct novel biological entities. One possibility is the generation of novel metabolic pathways using enzymes from different biological origins.

Many plant secondary metabolites such as aromatic polyketides show a range of biological activities and are nowadays applied directly as drugs or raw materials for semi-synthetic modifications. A common approach for the production of those phytochemicals is the reconstitution of biosynthetic pathways from plants into microbial host organisms. Thereby, a single molecule can be formed in contrast to the rich bouquet of substances produced by plant donors. The high compound purity and reproducibility of microbial processes are especially crucial for pharmaceutical production. Many representatives of aromatic polyketides have important medical applications as antibacterial, antiparasitic, cholesterol-lowering or pain-relieving drugs. Especially the microbial production of plant substances is still limited due to low yields. This is probably attributed to the use of common model organisms such as *Escherichia coli* or *Saccharomyces cerevisiae* and the associated excessive genetic engineering efforts for sufficient precursor supply. In contrast, the amoeba *Dictyostelium*

discoideum is a native polyketide producer and hence exhibits the genetic repertoire for the biosynthesis of secondary metabolites.

Here we present a novel approach combining viral 2A sequences with the utilization of bacterial and plant-derived enzymes to enable the production of the cannabinoid precursor olivetolic acid (OA) in amoebae starting from hexanoic acid. To facilitate OA biosynthesis from primary metabolites, we further engineered an amoeba/plant inter-kingdom hybrid enzyme based on the StlB polyketide synthase from *D. discoideum* and the olivetol synthase from the plant *Cannabis sativa.* Hence, we developed a shortcut in the cannabinoid biosynthetic pathway and provide evidence, that *D. discoideum* is a suitable chassis for the construction of biosynthetic pathways and biotechnological production of aromatic polyketides.

A new Zymomonas mobilis platform strain with tunable PDC expression

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Objective:

Bio-based production of platform chemicals is an important step away from fossil fuels and towards renewable resources. Efficient processes are needed to produce the large quantities of platform chemicals required. The bacterium *Zymomonas mobilis* is well known for its outstanding ability to produce ethanol with both high specific productivity and with a yield close to the theoretical maximum. In order to exploit the favourable properties of *Z. mobilis* for the production of substances other than ethanol, it is necessary to redirect the fluxes of the central metabolism away from ethanol towards alternative products. The key enzyme in the ethanol production pathway is the pyruvate decarboxylase (PDC), which converts pyruvate to acetaldehyde. Since its gene, *pdc,* is widely considered to be essential, metabolic engineering strategies aiming to produce other compounds derived from pyruvate must find ways to reduce PDC activity.

Results:

We have constructed a new platform strain of *Z. mobilis* (sGB027) in which the native promoter of *pdc* has been replaced by the IPTG-inducible P_{T7A1}. This allows the expression of *pdc* to be controlled by the addition of the inducer IPTG. In parallel to the construction of the strain, a modular cloning toolbox Zymo-Parts was developed that greatly facilitated the construction of this strain as well as genetic engineering of Z. mobilis in general [1,2].

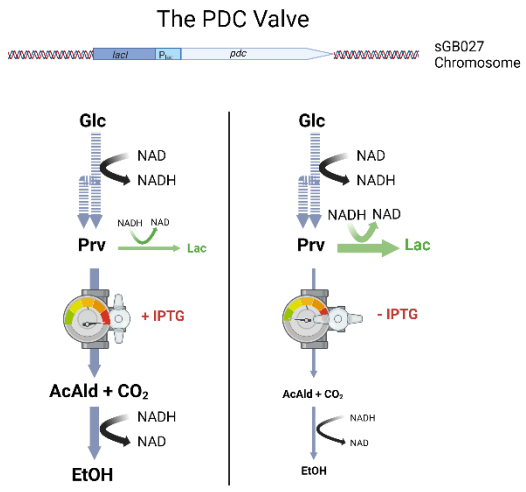

Fig.1: Control of PDC expression and activity in the platform strain *Z. mobilis* sGB027 (created with BioRender.com)

We were able to show that the growth rate of sGB027 can be controlled by the amount of IPTG added to the medium. To achieve redox balance of metabolism in the mutant, alternative NADH-consuming reactions must be provided. To this end, we introduced a plasmid expressing the lactate dehydrogenase from *E. coli* into sGB027 and demonstrated that the resulting strain produced high amounts of lactate. Similarly, we introduced a plasmid expressing the alanine dehydrogenase from *Geobacillus stearothermophilus.* The resulting strain produced significant amounts of alanine but its growth was impaired [3].

For both strains, the yields and production rates could probably be improved further by optimising the process conditions. However, ethanol production is still present in both strains and reduces the yield of the respective product. We are therefore developing the strain further by additionally reducing the activity of the alcohol dehydrogenases. This could also increase the stability of the strain.

Conclusion: We present a new platform strain that enhances the production of compounds derived from pyruvate with *Z. mobilis.* The strain, but also the extensive toolbox developed for genetic modification of Z. mobilis, is a step towards establishing this organism as a workhorse for biotechnological production processes.

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CoNoS – Synthetic Communities of Niche-optimized Strains for biotechnological production and basic research

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Background

For biotechnological production processes, the efficient use of the available resources is a key factor for their success. Current processes are mostly based on pure cultures of engineered strains with versatile metabolic capacities. In the comparably well-defined environment of a bioreactor, metabolic flexibility is much less required and results in suboptimal use of carbon and energy sources due to e.g. enzymes that are formed but not used. In nature, microorganisms have frequently evolved in communities where genome-reduced, auxotrophic strains cross-feed each other, suggesting that there must be a significant advantage compared to growth without cooperation.

Objectives

Our aim was to generate synthetic Communities of Niche-optimized Strains (CoNoS), composed of complementary auxotrophic strains of *Corynebacterium glutamicum,* which produce an amino acid more efficiently than a monoculture. Furthermore, we wanted to understand the critical factors influencing community-based production processes.

New Results

We established several CoNoS by combining engineered auxotrophic strains that i), lacked full amino acid synthesis pathways and ii), slightly overproduced the amino acid required by the partner strain (Fig. 1). Those CoNoS had a growth rate close to a wild type monoculture[1]. By combining CoNoS with adaptive laboratory evolution (Fig. 1), we identified critical factors for community growth and also new amino acid production traits. One example was the identification of the new arginine uptake system ArgTUV, whose deletion led to a faster and 24 % higher arginine production in comparison to the parental strain[2].

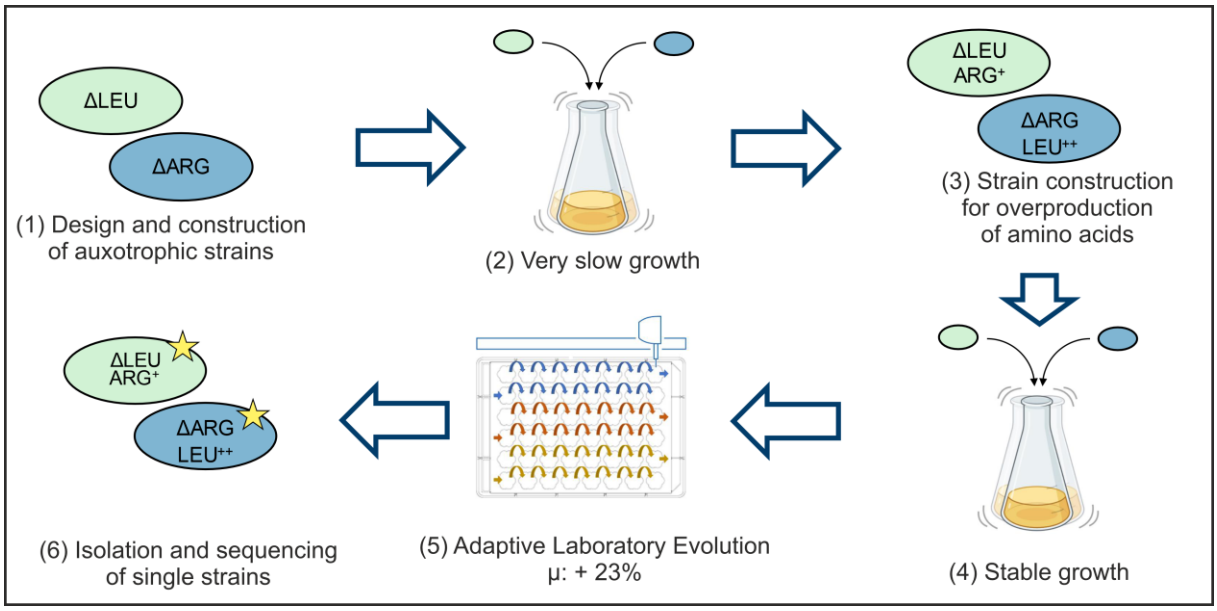

Fig. 1: CoNoS workflow consisting of (1) construction of complementary auxotrophic strains, (2) initial co-cultivation, (3) further strain construction, (4) second co-cultivation, (5) growth enhancement using ALE and (6) isolation and sequencing of individual strains. Parts created with BioRender.com.

Conclusion

Our work demonstrates the power of the CoNoS-approach for evolution-guided identification of non-obvious production traits, which can also advance amino acid production in monocultures. The approach can easily be extended to all kinds of metabolite cross-feeding pairings of different organisms or different strains of the same organism, thereby enabling the identification of relevant transport systems and other favorable mutations.

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Artificial Enzymes Meet Artificial Intelligence – Engineering Novel Biocatalysts by Active Learning

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Biocatalysis and metabolic engineering play a central role in the transition to a sustainable bioeconomy. Currently, these disciplines are restricted to the natural repertoire of enzymes, which catalyze a limited set of reactions compared to the wide range of transformations known in organic chemistry. Artificial metalloenzymes (ArMs) could substantially increase the scope of enzymatic catalysis by incorporating synthetic metal cofactors into proteins[1]. However, most ArMs display a modest activity and are not biocompatible. To overcome these limitations, we have developed protein engineering pipelines that allow for the efficient engineering of ArMs towards higher activity in a cellular environment. These pipelines rely on expression and assembly of the ArM in the periplasm of *Escherichia coli*[2] (Figure 1A). Previously, we have used this approach to establish a screening platform that allows for the rapid discovery of active ArMs for various reactions[3]. More recently, we have augmented the pipeline in order to efficiently navigate very large sequences spaces[4]. To this end, we made use of lab automation and a cost-efficient sequencing strategy based on next-generation sequencing. This allowed us to acquire large and informative sequence-activity data sets. In addition, we employed machine learning to direct screening rounds to regions of sequence space that are of particular interest. By applying these techniques in an iterative fashion (Figure 1B), we were able to substantially increase the hit rate of our screening. Our results hold important insights for machine learning-directed protein engineering. For example, we demonstrate that model-guided exploration is an efficient strategy for improving models and illustrate the importance of considering experimental noise during model development. We expect that automation and machine learning-guided engineering will substantially boost the performance of ArMs and thus enable exciting applications in biocatalysis and synthetic biology.

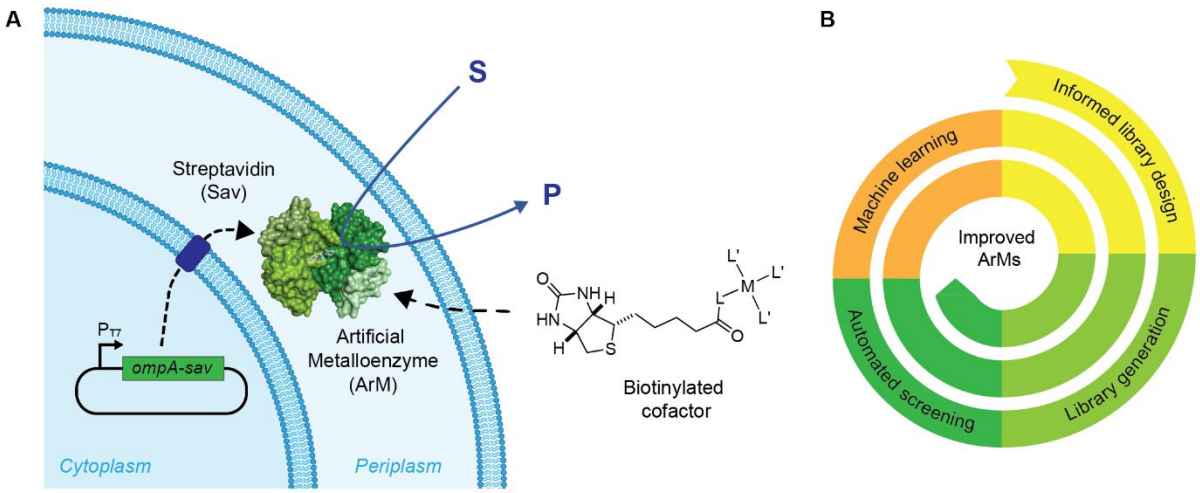

Figure 1: Engineering artificial metalloenzymes for in vivo applications. A) *Periplasmic compartmentalization strategy for ArMs based on streptavidin. Streptavidin is exported to the periplasm of E. coli, where it binds a biotinylated metal cofactor. The resulting ArM can catalyze new-to.nature reactions in a whole-cell setup.* ***B)*** *Active learning strategy for ArM engineering. Iterative cycles of library design, library generation, automated screening, and machine learning allow for the efficient engineering of ArMs towards higher activity.*

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Capabilities of KIWI-Biolab's robotic ecosystem by orchestration of model-mased DoEs and fast in-depth analytics for synthetic biology bioprocesses

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The selection of possible production strains under process-relevant conditions is a key task in bioprocess development. In our applications, we focus on the development of high cell density processes for difficult-to-express recombinant proteins in *Escherichia coli*, such as hydrogenases and elastin-like proteins.

To test the big amount of possible parameters with a reasonable effort, model based DoEs are applied. These use mathematical models of different complexity that describe the dynamic properties of the cell factory. The aim of the experiments is then to identify the cell-specific parameters that depend on the strain and the respective environmental conditions. With the parameterized cell model, further process development can benefit greatly from computational simulations.

Efficient parameterization of parallel cell factories during a running experiment is a major concern in the KIWI-biolab. Here, well-controlled fed-batch fermentations are performed in parallel 48x10mL or 8x100ml bioreactor systems. Fully automated and integrated process analytics enable quick access to the data for extensive parallel simulations and real-time process control. This allows to pursue multiple objectives, such as the characterization of cell factories (1), the maximization of cell or product yields, and even to run the cultivations with a very high reproducibility within predefined constraints by model-based control (2).

In different projects we have proven the efficiency of this approach. Relevant actual examples include the functional expression of a recombinant oxygen-tolerant regulatory [NiFe]-hydrogenase from *Cupriavidus necator* in *E. coli* and the combination of this expression with an orthogonal translation system for the incorporation of a non-canonical amino acid.

One key technology to perform a robust fed-batch fermentation with well-controlled physiological conditions over a long period in a small-scale screening system is the

enzyme supported substrate delivery technique (Enbase®). While this technology is established in many laboratories as a standard for expression optimization of recombinant proteins, only a constant feeding rate could be used so far. For robotic applications, we have further developed this technology to allow control of the specific growth rate, similar to exponential feeding in bench-top bioreactor fed-batch processes (3).

Acknowledgement

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Analysis of the combination suitability between different dandelion species for rubber yield enhancement by evaluation of NMR metabolite profiles using artificial intelligence methods

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Objectives

The objective of the project is to find a connection between certain properties of dandelion species (e.g., high rubber content) and their metabolite profiles using multivariate and machine learning methods. The calculated models shall help to gain insights into relevant individual metabolites and metabolite networks and, thus, to understand the underlying biochemical mechanism. The metabolite profiles required for the analysis are calculated automatically from one-dimensional 1H NMR spectra of the dandelion plants with the help of a self-written computer program.

Keywords: Dandelion, natural rubber, NMR, machine learning, metabolomics

Significance of the work

Today, natural rubber is mainly produced in Asia, Africa, and Latin America, and it is likely that these sources will no longer be able to meet global demand within this decade. Domestic production of natural rubber with the help of dandelions would not only create an alternative source of supply, while reducing the destruction of climate-protecting and biodiverse ecosystems in other parts of the world, but also lead to the development of significant regional value chains. So far, only molecular approaches have been pursued to identify relevant genetic markers in dandelions as selection tools for rubber content and root morphology. The metabolome has not been considered although it is the level of information that best reflects the actual phenotype.

Methods

The data was acquired as follows. For plant material, leaves and roots were chosen. Optimized sample preparation processes (lyophilization, grinding, etc.) allow

quantitative, fast, and reproduceable one-dimensional 1H NMR measurements, with a 600 MHz Bruker AV NEO NMR spectrometer used. The metabolites are identified and quantified automatically from 1D 1H NMR spectra of dandelion plants using a self-written identification algorithm, non-linear optimization methods and an extensive database. No precise details can be given about the further data analysis, as these studies had just begun at the time of submission.

Results

As part of this project, 142 metabolites have been measured and manually identified in a dandelion matrix. Based on these measurements, 34 metabolites already known in the literature were confirmed. In addition, 21 metabolites not yet detected by NMR were identified. The total of these metabolites almost completely account for the signals in dandelion spectra, while the remaining surveyed metabolites are present in smaller concentrations and thus intensity. Most of the 142 manually identified metabolites can already be automatically identified and quantified with high accuracy and can be used as a basis for subsequent analyses. Initial results of statistical evaluations of the metabolite list are also presented and their significance for a deeper understanding of the underlying biochemical processes is discussed.

Conclusion

To conclude, this project represents a crucial step towards understanding the biochemical mechanisms underlying rubber yield enhancement in dandelion species, facilitated by advanced methods of data analysis. The identification of key metabolites and networks offers promising opportunities for the development of sustainable domestic rubber production. These findings emphasize the importance of continuing research into the use of artificial intelligence for metabolome analysis to pave the way for further advances in biotechnology and sustainable agriculture.

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Improving carbon efficiency in lignin valorisation through C1 assimilation and integration of green hydrogen

Abstract:

Current strategies for biotechnological lignin valorisation predominantly employ co-feeding methods using refined sugars or organic acids as auxiliary carbon sources. While this practice supports microbial growth, it inadvertently compromises the carbon efficiency of biotechnological processes and contributes to competition within food supply chains. In particular, lignin valorisation processes typically generate C1 and C2 by-products. While the C2 fraction, mainly acetyl-CoA, is efficiently utilised as a carbon source by microbial hosts, the C1 fraction, formaldehyde, is typically oxidised to carbon dioxide (CO_2) to mitigate intracellular toxicity, inadvertently contributing to greenhouse gas emissions. This study proposes the integration of C1 assimilation pathways to increase the carbon pool available for microbial growth, thereby eliminating the need for additional carbon sources in lignin valorisation efforts. Preliminary metabolic modelling suggests that energy constraints significantly inhibit C1 assimilation pathways, such as the reductive glycine pathway and the RuMP cycle, leading to oxidation of assimilated carbon and subsequent CO_2 emission. To circumvent the loss of carbon as CO_2, this research proposes the use of hydrogen derived from renewable energy sources, coupled with heterologous expression of hydrogenase, to efficiently energise C1 assimilation pathways. By exclusively using lignin-derived by-product fractions as carbon sources and harnessing electrons from green hydrogen, the proposed next-generation lignin valorisation strains promise to achieve 100% molar efficiency, thereby eliminating the need for co-feeding additional carbon sources.

Enhanced mineral weathering by engineered marine bacteria for CO_2 sequestration

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Harvard Medical School, Boston, USA;

According to the IPCC report, humans must remove about 1000 Gt of CO_2 from the atmosphere by 2100 to prevent the worst effects of climate change. Enhanced rock weathering technologies are a promising negative emission technology. Natural weathering of silicate minerals results in an increase in ocean alkalinity, which promotes the uptake and storage of CO_2 [Eq. 1].

$$Mg_{1.8}Fe_{0.2}SiO_4 + 4CO_2 + 4H_2O \rightarrow 1.8Mg^{2+} + 0.2Fe^{2+} + 4HCO_3^- + H_4SiO_4 \qquad \text{[Eq. 1]}$$

Natural weathering is slow, however, such that technologies for accelerated dissolution are required. Bacteria have been shown to accelerate the weathering of the widely available mineral olivine using siderophores, which remove iron from the mineral surface. We sought to engineer a marine bacterium, *Alteromonas macleodii*, for enhanced siderophore secretion and mineral dissolution.

The siderophore production in *A. macleodii* is strictly regulated through the ferric uptake regulator (fur) and repressed in the presence of iron. We substituted the native promoter of the putative siderophore synthesis genes with a constitutive promoter. We showed that the overproduction of siderophores results in a higher dissolution rate of olivine (Fig. 1a).

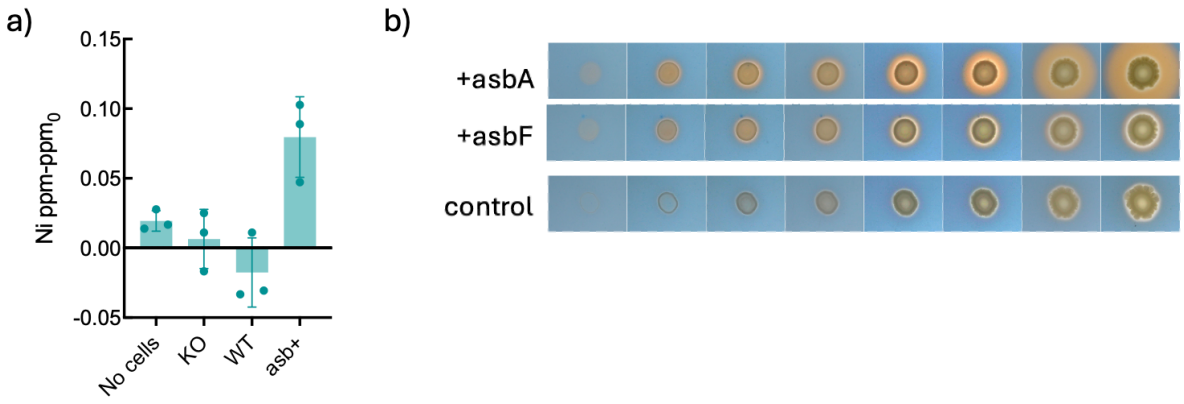

Figure 1: a) Dissolution rates of olivine in the presence of different A. macleodii strains. b) CAS Assay for siderophore production and secretion of different A. macleodii strains.

Furthermore, we have identified the enzyme AsbA (spermidine-citrate ligase) as the key bottleneck in the siderophore production pathway (Fig. 1b). These results will be implemented in the development of new strains. Overall, we envision using the marine model organism *A. macleodii,* engineered for overproduction of siderophores and for the enhanced dissolution of olivine and the alkalinization of ocean water in a bioreactor.

Self-decorating cells via surface-initiated enzymatic controlled radical polymerization

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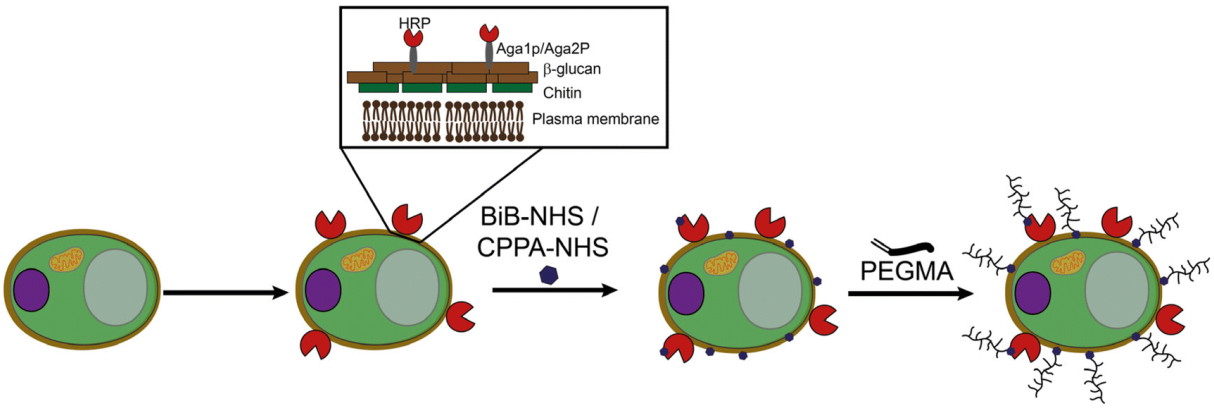

Objective: This study aims to use surface-displayed horseradish peroxidase (HRP) to catalyse enzymatic controlled radical polymerizations (bioRAFT and bioATRP) on *Saccharomyces cerevisiae* cells, creating surface polymers that alter cellular phenotype.

Methods and Results: Using grafting-from polymerization techniques, we synthesised hydrophilic, biocompatible polymer brushes from poly(ethylene glycol) methyl ether methacrylate (PEGMA), azide-functionalized PEGMA (PEGMA-N_3), and N-isopropyl acrylamide (NIPAM). The polymerizations were controlled by attaching either a chain transfer agent for RAFT or an alkyl halide ATRP initiator to the cell surface and allowing the surface HRP to perform the reaction. The findings indicate that bioATRP is more effective than bioRAFT in these polymerizations, creating PPEGMA and PNIPAM brushes extending from the cell wall, as evidenced by the different growth profiles,

resistance to digestion, aggregation and the conjugation of non-native enzymes. This polymerization could be repeated across generations of yeast, allowing the continuous propagation of the modified phenotype.

Conclusions and Significance: The self-produced polymer coatings permit the encapsulation of yeast cells within synthetic polymers and functionalization with biomolecules, markedly influencing several aspects of their behaviour. The generational carryover of surface polymers stabilises the desired traits and also enhances the cells' utility in successive biotechnological applications. These modifications pave the way for advances in bioorthogonal cell-surface engineering and the creation of engineered living materials.

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Synthesis of valued ferulate-based natural products via biocatalytic cascades

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Coenzyme A (CoA) conjugates represent the base for the biosynthesis of many phenolic natural products. In plants, they are the starting materials for structurally diverse and industrially important metabolite classes such as cinnamic amides, benzaldehydes, chalcones, or aromatic 2-pyrones. In the presented study, we used biorthogonal, multistep enzyme catalysis to access these natural products. First, suitable enzymes were developed by means of screening and subsequent engineering in order to synthesize different phenolics in coupled in vitro reactions. As a second step, the developed enzyme cascades were integrated into microbial production strains in order to provide a sustainable and scalable access to the natural products and derivatives.

Examples for this strategy comprise the formation of aroma compounds (i.e. benzaldehydes and 2-pyrones) or antimicrobial amides from cinnamates. In these cascades, the carboxylates are activated by promiscuous CoA ligases, which provides access not only to natural but also to new-to-nature CoA conjugates. This library was used to screen for enzymes which subsequently accept these substrates for a transformation into valued products. First, the aroma benzaldehyde vanillin and its more potent derivative ethylvanillin were formed by retro-aldol-type conversion catalyzed by a hydratase/lyase enzyme in a two-step *in vitro* reaction. Second, the application in a cascade containing a polyketide synthase yielded a set of novel alkylpyrones. In a third reaction, the ligase was coupled to an amide synthase which gave rise to a library of aromatic and aliphatic amides with different substitution pattern and bioactivity. Advantageously, the latter transformation was also functional in *E. coli* as a heterologous host, which produced selected amides from fed carboxylates. In summary, biocatalytic cascades represent a simple strategy to create structural diversity from carboxylates via conjugation to CoA.

From Toolkit to Application: Optimizing modular Golden Gate Assembly and plastic degradation with the fast-growing chassis *Vibrio natriegens*

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With its remarkable doubling time of less than 10 minutes, *Vibrio natriegens* presents an opportunity to significantly accelerate the cycle of design-build-test-learn in synthetic biology. However, the full potential of *V. natriegens* as a synthetic biology platform and protein production host is highly dependent on the availability of specialized genetic tools tailored to its unique biology. Therefore, the development of synthetic biology toolboxes, such as the Marburg Collection,[1] is crucial for unlocking the capabilities of *V. natriegens*. This Golden Gate cloning toolbox comprises standardized genetic parts designed for modularity and ease of assembly. Our recent collaborative work has focused on creating a significant expansion of this collection, enhancing the efficiency, number, and quality of available parts. For example, we tested an improved design for dropout parts, built a selection software for easier part management, and characterized additional inducible promoters, novel operon connectors, as well as homology flanks for NT-CRISPR. Moreover, our work focuses on establishing *V. natriegens* as a protein production host, particularly for plastic degrading enzymes. As a proof of concept, we focus on the degradation of polyethylene terephthalate (PET) with *V. natriegens* by building Golden Gate constructs with relevant genes from different soil bacteria. Taken together, this work contributes to the ongoing advancement of genetic tools, their cross compatibility, and protein production in *V. natriegens* to increase the applicability of this promising chassis.

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Selective photocontrol of deleterious but chemotherapeutically essential glutaminase activity in *Escherichia coli* asparaginase type-II

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Light is an efficient tool for the artificial, spatio-temporal control of enzymes, which are an important class of macromolecules and in particular of interest for therapeutic end-applications. The homotetrameric asparaginase type-II from *Escherichia coli* (EcAII) is a well-suited target for photocontrol owing to its role as chemotherapeutic drug. It has been used for the treatment of acute lymphoblastic leukemia since the 1970s but has especially encountered difficulties for the treatment of solid cancers partly owing to its mode of action. Certain tumor cells, including leukemic cells, rely on the intake of asparagine and glutamine from the extracellular environment and starve to death when supplied EcAII depletes both amino acids. While the promiscuous glutaminase activity of EcAII is required for its high efficacy, for example, in pancreatic adenocarcinoma, the entailed reduced glutamine levels can cause detrimental side effects due to a depression of the central nervous system.

We envision the selective photocontrol of the EcAII glutaminase activity to be a viable solution to ultimately reduce the toxicity in chemotherapy of especially solid tumors such as pancreatic cancer. To this end, we set out to regulate enzyme activity in EcAII by incorporation of the photoswitchable unnatural amino acid phenylalanine-4'-azobenzene (AzoF) in initial investigative studies. We selected ten positions for incorporation either in the catalytically essential active site flexible loop or within 6 Å distance thereof. By screening the enzyme activity before and after irradiation we identified two light-sensitive EcAII variants. While their asparaginase activity was unaffected by light, the glutaminase activity could be reversibly photocontrolled by 2-fold. Moreover, both variants showed a high asparaginase activity in the range of 57–79% of wild type EcAII, and a glutaminase activity in the more active state of 15–65% of wild type EcAII as determined in steady-state kinetics. By testing various reaction conditions we could increase the photocontrol efficiency to 3- and 7-fold. In summary, our findings provide the first step towards photocontrolled chemotherapy and allow us to further optimize the approach *in vitro* and *in vivo*.

Cell-free reaction systems for ATP regeneration from low cost sugar and alcohol substrates

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Adenosine triphosphate (ATP) plays a crucial role in various biological processes, particularly as a cofactor for group transfer in enzymatic reactions. ATP-dependent enzymatic syntheses have great industrial potential, with applications ranging from cell-free protein synthesis to the production of fine chemicals. Unfortunately, the economic viability of such *in vitro* bioprocesses is limited by the high cost of the ATP cofactor. Different cell-free reaction systems for ATP-regeneration have been established using high-energy compounds such as acetyl-phosphate or phosphoenolpyruvate. However, these substrates are either expensive or unstable and release phosphate in large quantities, which may inhibit product synthesis. A pyruvate-based ATP regeneration system previously proposed by Kim and Swartz overcomes these obstacles and was applied for the synthesis of proteins or lacto-N-biose [1–3]. However, this process requires aeration, which results in higher energy costs and foam formation associated with complicated reaction control.

Therefore, we developed *in vitro* reaction systems which rely on a non-natural phosphoketolase (PKT) activity to synthesize acetyl phosphate from non-phosphorylated sugars or the basic chemical ethylene glycol. Subsequently, acetyl phosphate is used to regenerate ATP from ADP by a highly active acetate kinase (Figure 1). These PKT-based reaction systems have great potential for cost-efficient ATP regeneration as they use

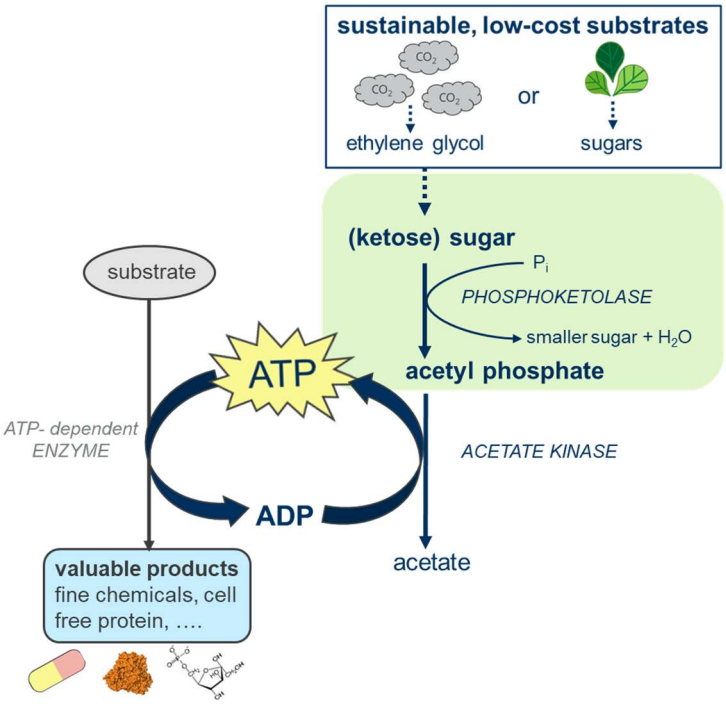

Figure 1 In vitro reaction systems for ATP regeneration from low-cost substrates based on the enzyme phosphoketolase.

low-cost substrates with a theoretical yield of up to 3 $mol_{ATP}/mol_{Substrate}$. In our work, these enzyme cascades were implemented to provide ATP for the conversion of glycerol to *sn*-glycerol-3-phosphate (G3P) via glycerol kinase. G3P is a precursor for synthetic phospholipids, which are widely used in various pharmaceutical applications [4].

To achieve a high ATP recycling rate, it is necessary to optimize the key enzyme PKT. Here, the phosphoketolase from *Bifidobacterium adolescentis*, which is naturally active on fructose 6-phosphate and xylulose 5-phosphate, was engineered by rational design to improve its catalytic performance on smaller, non-phosphorylated sugars. Our cell-free ATP regeneration systems were shown to enable higher G3P productivity and yield with the improved PKT variants compared to the wild type enzyme.

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Giant vesicles get energized: correlating membrane potentials to ion transport through fluorescence analysis

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Electrochemical potentials are essential for cellular life. For instance, cells generate and harness electrochemical gradients to drive a myriad of fundamental processes from nutrient uptake and ATP synthesis to neuronal transduction. To generate and maintain these gradients, all cellular membranes carefully regulate ionic fluxes using a broad array of transport proteins. For that reason, it is also extremely difficult to untangle specific ion transport pathways and link them to membrane potential variations in live cell studies. Conversely, synthetic membrane models, such as black lipid membranes and liposomes, are free of the structural complexity of cells and thus enable to isolate particular ion transport mechanisms and study them under tightly controlled conditions. Still, there is a lack of quantitative methods for correlating ionic fluxes to electrochemical gradient buildup in membrane models. Consequently, the use of these models as a tool for unravelling the coupling between ion transport and electrochemical gradients is limited. We developed a fluorescence-based approach for resolving the dynamic variation of membrane potential in response to ionic flux across giant unilamellar vesicles (GUVs). To gain maximal control over the size and membrane composition of these micron-sized liposomes, we developed an integrated microfluidic platform that is capable of high-throughput production and purification of monodispersed GUVs[1]. By combining our microfluidic platform with quantitative fluorescence analysis, we determined the permeation rate of two biologically important electrolytes – protons (H^+) and potassium ions (K^+) – and were able to correlate their flux with electrochemical gradient accumulation across the lipid bilayer of single GUVs[2,3]. Through applying similar analysis principles, we also determined the permeation rate of K^+ across two archetypal ion channels, gramicidin

A and outer membrane porin F (OmpF). We then showed that the translocation rate of H^+ across gramicidin A is four orders of magnitude higher than that of K^+ unlike in the case of OmpF where similar transport rates were evaluated for both ions[3].

Subtopic – Cell-free and Bottom-up Systems (Protocells)

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Poster

Genetically-stable optogenetic gene switches modulate spatial cell morphogenesis in two- and three-dimensional tissue cultures

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Genomically engineered optogenetic gene switches represent an approach for precisely regulating cellular behavior in 2D and 3D mammalian tissue models. Here, we implement blue and red light-responsive gene switches into model tissue cultures and achieve precise control of cell death and morphogen-directed patterning in 2D and 3D tissues by optogenetically regulating cell functions including necroptosis, apoptosis, and synthetic WNT3A signaling at high spatial resolution (see Figure 1). This is accomplished using custom-built patterned illumination systems, including a digital mirror device (DMD) and photomasks, as well as laser techniques. We developed and make available a modular and affordable hardware/software framework utilizing open DMD technology and software solutions for high-resolution pattern projection and imaging that enables 'cybergenetic' feedback control of optogenetic tissue regulation. The biology-hardware-software interface opens new avenues for designing programmable 3D tissue and organ models and may advance the precision of optogenetic tissue engineering research in the future.

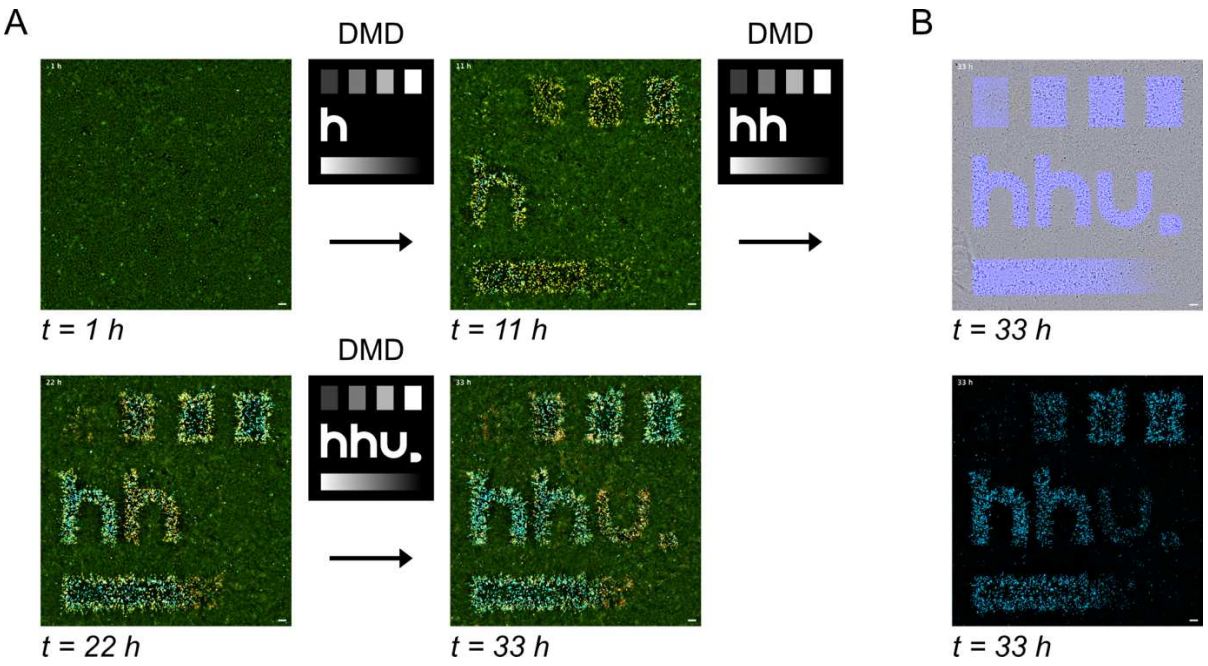

Figure 1. Spatial induction of necroptosis in an engineered optogenetic mammalian cell line. **A)** Time series of dynamic and quantitative control of optogenetic necroptosis induction using the digital mirror device for patterning and variation of light intensities across the pattern. An overlay of EGFP, mCherry, and SYTOX Blue (cell death stain) signals is shown. **B)** Brightfield and blue light pattern projection (top) and SYTOX Blue signal (bottom) of the experiment in (A) at 33 h. Scale bar, 100 µm.

Peptide Barcodes for Label-Free Enzyme Screening at High-Throughput

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Enzymes operate optimally under natural conditions for natural reactions and often fail to work effectively when applied in technical settings for practically important syntheses. This limitation is often addressed by directed evolution. But despite numerous advances in the field [1] the screening step remains a major bottleneck, with each distinct reaction requiring its own tailored, often optical, readout. Alternatively, mass spectrometry (MS) can be used to establish rather generic readouts. However, MS readouts are slow due to their confinement to microtiter plates and are not suitable for integration with microfluidics systems. To overcome these limitations, we aim to develop a new screening method that allows for reading out a library address and the reaction in one MS measurement (Fig.1). For this, we want to use diverse short

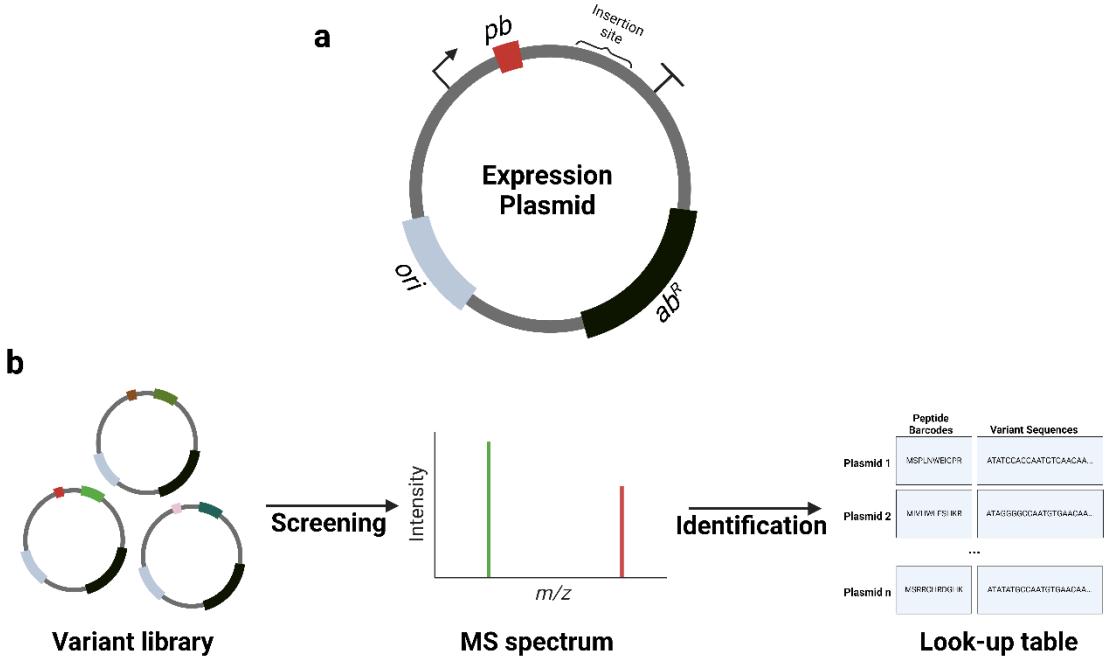

Figure 1: Screening of an enzyme variant library based on a peptide address. **a)** Expression plasmids contain genes for a peptide “barcode” and an insertion site for the gene for a protein of interest (POI). **b)** Next generation sequencing is performed on a pooled library of barcoded plasmids with unique peptide- and POI combinations. The association between peptide- and DNA barcode is stored in a look-up table. Mass spectra are obtained in the screening step. The mass signal of a peptide barcode links to the POI on the respective plasmids and thus allows for the retrieval of the DNA sequence of specific samples. Created with BioRender.com.

peptides as an “address” that could be specific to a library member. However, as there are few examples of successful production of short peptides in model bacteria such as *Escherichia coli*, we are first establishing the boundary conditions for successful peptide production.

Designing stable and MS-detectable peptide barcodes

So far, we developed a machine learning-based genetic algorithm [2] that outputs peptide sequences with high MS-detectability scores. Furthermore, we employ an aggregation tag [3], aiming to protect the peptide barcodes from degradation by keeping them insoluble. In fact, we were successful in detecting a variety of peptides in the cytoplasm of *E. coli* when applying whole cells to MALDI-MS analysis (Fig. 2).

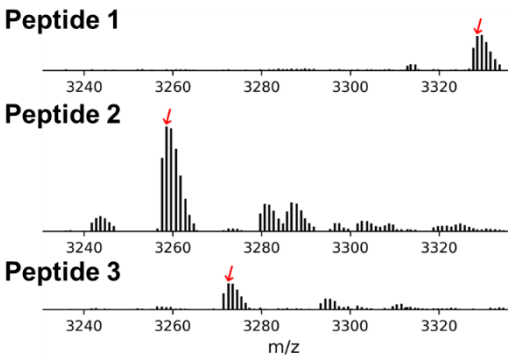

Figure 2: MALDI-MS spectra of three peptides produced *in vivo* (red arrows).

Outlook

With detectable peptide barcodes at hand, the next step involves further refinement to allow for a proof-of-principle screening experiment, and later the design of highly diverse peptide libraries. This requires a robust expression system for peptide barcodes and a POI library as well as a suitable screening setup.

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Tandem photobiocatalysis in artificial cells enables communication with natural cells

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Abstract

Photobiocatalysis provides more simplified and versatile strategies for discovering new-to-nature synthetic routes[1, 2]. However, photocatalytically generated reactive oxygen intermediates can denature biomolecules, posing challenges in combining the merits of photocatalysis and biocatalysis[3, 4]. Inspired by the evolution of subcellular multi-compartment structure in natural cells, we developed artificial biomimetic systems for spatiotemporal control over photobiocatalytic cascade reactions to deal incompatible restrictions. In this study, we design artificial cells that are characterized by physically confined segregation of a photocatalytic cofactor regeneration system and a biocatalytic cascade system within distinct silica nanocapsules (SiNCs). This subcellular selective compartmentalization strategy permits the integration of both incompatible catalytic modules within the same artificial cell while shielding enzymes from direct exposure to reactive oxygen intermediates. A hydrophilic photocatalytic polymer (PC) is synthesized and confined in SiNCs to create the photocatalytic cofactor regeneration system (SiNC@PC). Concurrently, oxidized cofactor-dependent enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), are spatially coupled in SiNCs at their optimized ratio, forming the biocatalytic nanoreactor (SiNC@ADH/ALDH). Finally, SiNC@PC and SiNC@ADH/ALDH are encapsulated within polymeric giant unilamellar vesicles through a bottom-up approach, producing functional artificial cells. Upon coincubation with living cells, these artificial cells exhibit excellent biocompatibility and significantly reduce the oxidative stress of hepatocytes exposed to alcohol and acetaldehyde *in vitro*. This precision-designed strategy that integrates incompatible photobiocatalytic modules in spatial control not only breaks the

shackles of photobiocatalytic bio-applications but also opens a new avenue for artificial cells transformations.

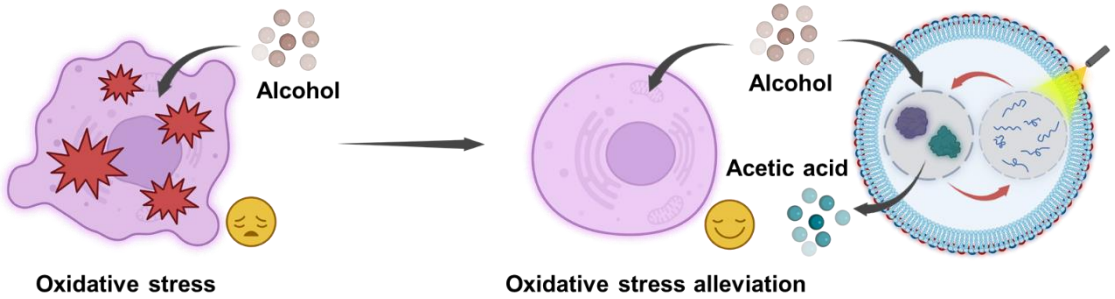

Figure 1. Schematic representation of artificial cells alleviating the oxidative stress caused by alcohol in living cells.

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Ligand-specific biosensing for aromatic compounds and neurochemicals in engineered probiotics

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Subtopic: biomedical applications

Key Words: ligand specificity; biosensor; aromatic amino acid; neurochemical; disease

Microbial biosensors have diverse applications in metabolic engineering and medicine [1-3]. Specific and accurate quantification of chemical concentrations allows for adaptive regulation of enzymatic pathways and temporally precise expression of diagnostic reporters [1]. Although ideal biosensors should differentiate structurally similar ligands with distinct biological functions, such specific sensors are rarely found in nature and are very challenging to create. Using *E. coli* Nissle 1917, a 'generally regarded as safe' microbe, we developed and characterized several biosensor systems that promiscuously recognize aromatic amino acids or neurochemicals [4]. To improve the sensors' selectivity and sensitivity, we combined rational protein engineering with directed evolution techniques, applicable to both transcription repressors and activators. The generalizable approach involved searching for optimal evolutionary starting points, identifying critical residues in ligand binding, and screening mutagenesis libraries. Our method also provided insights into the previously uncharacterized structures of transcription regulators and elucidated the corresponding specificity control strategies.

Specifically, we successfully demonstrated the ligand-specific biosensors for phenylalanine, tyrosine, indole-3-acetic acid, phenylethylamine, tyramine, and tryptamine. Each of these structurally similar compounds serves as a distinct biomarker or regulator that influences intestinal functionality and human health. These results lay the groundwork for developing kinetically adaptive microbes for potential applications, ranging from monitoring food quality and detecting diseases to facilitating therapy administration. In this presentation, we will discuss our ongoing progress toward specific biosensor development [5].

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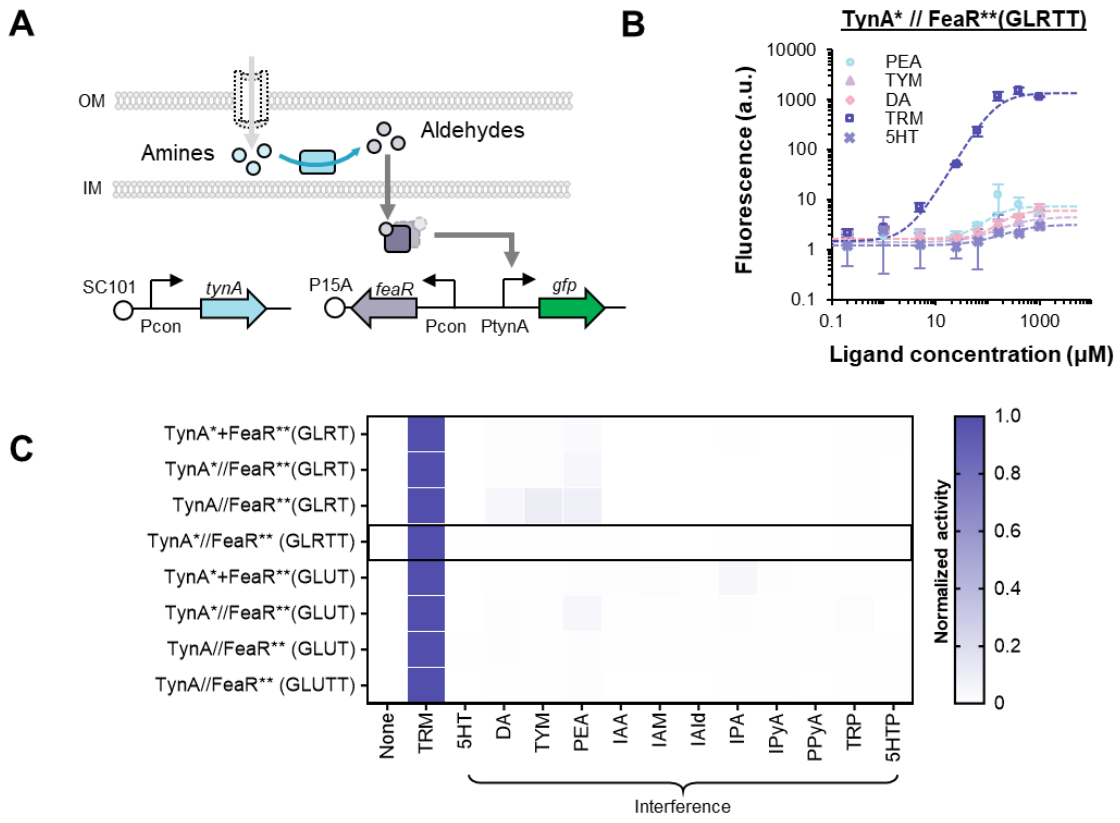

Figure. Engineering tryptamine (TRM) biosensor specificity. (A) Schematic of TynA-FeaR-based TRM-specific biosensor with elevated expression of a FeaR variant (FeaR**). (B) Enhanced specificity profiles of the TRM-specific sensor with increased expression of FeaR** in glycerol medium supplemented with thiamine and trace elements (GLRTT). (C) Cross-reactivity profiles of TynA-FeaR-based TRM-specific biosensors varying TynA activity, media, and source (* or **). GLR = glycerol, GLU = glucose, T = thiamine, TT = thiamine + trace element. The negative control (None) and 13 chemicals were tested.

Synthetic Notch Receptor-Based Virus Sensing and Neutralization: A Versatile Tool for Virological Research

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Background

The synthetic Notch (SynNotch; SN) technology can be used to yield a customized output in response to a specific input signal in mammalian cells. Herein, we adopted the SN technology to set up a versatile and broadly applicable virological assay, e.g. to assess neutralizing antibodies.

Objective

Live virus or pseudoviral neutralization assays provide the virological golden standard to assess protective antibodies against SARS-CoV-2, e.g. in immunosurveillance programs and for vaccine development. However, those assays tend to be sluggish in adapting to novel variants of concern (VOC) or zoonotic strains and require infectious virus. The objective of this study was to explore SN virus sensing and establish a virus-free neutralization assay using a SN core fused to a viral entry receptor.

Method

The Notch core was fused to an ACE2 extracellular domain and an intracellular transcription factor (Gal4 linked to VP64). SARS-CoV-2 Spike binding to ACE2-SN engineered cells activates reporter transcription and detection marker (mCherry) expression. ACE2-SN constructs were transiently transfected or stably expressed in HEK-293T cells. Triggering of ACE2-SN sensor cells was achieved via several formats

displaying multiple Coronavirus Spike proteins and neutralization was quantified using patient antisera.

Results

SN-ACE2 engineered cells sensed a variety of Spike-displaying particles such as live virus, lentiviral and vesicular stomatitis virus derived infectious and non-infectious particles and Spike-transfected cells. Gold particles presenting chimeric Spike-RBD moieties to sensor cells revealed that multimerization is a necessary requirement for ACE2-SN activation and that both the level of RBD multimerization as well as the particle size critically influence the signal output. Neutralizing antibodies could be detected using Spike-transfected cells and IC50 values for human sera correlated highly significantly with an established lentiviral neutralization assay. Adaptability was shown by using a CD4-SN receptor to dose dependently detect HIV-envelope protein on cells and HIV virus-like particles.

Conclusion

A reliable, easily adaptable, broad and high throughput compatible neutralization assay was established using a SN receptor. Versatility was shown by activation with various particles and quick adaption to other viruses. This newly adapted tool provides evidence that synthetic biology principles can be applied effectively in virological assays.

Characterizing and Engineering Homologs of a Template-Independent DNA Polymerase for Signal Recording Applications

Teresa Rojas Rodríguez, Marija Milisavljevic, and Keith Tyo, Northwestern University, Evanston/United States of America

Measuring cellular signals is essential for studying complex biological systems and understanding and curing diseases. DNA-based recording offers the potential for less invasive and higher-throughput methods, as DNA is a high-density information storage medium that can be deployed in cells. However, existing DNA recorders rely on signal-induced expression of DNA-editing enzymes for signal transduction, which occurs at slower timescales and prevents recording of sub-hour signals. Recently, temporal recording of environmental signals at minute resolution was demonstrated in vitro using terminal deoxynucleotidyl transferase (TdT), a template-independent polymerase. TdT adds deoxyribonucleotides (dNTPs) to the 3' end of single stranded DNA (ssDNA) and changes dNTP preference depending on environmental conditions; signals are therefore encoded in the composition of TdT-synthesized ssDNA. However, TdT currently has limitations as a recording tool. It is inhibited by ribonucleotide presence (abundant in cellular environments), shows minimal response to some signals of interest, and exhibits slower incorporation rates compared to replicative polymerases. To overcome these limitations through protein engineering, understanding TdT sequence-structure-function relationships is necessary. To explore these relationships, we characterized a diverse set of natural TdT homologs, assessing catalytic activity, dNTP selectivity, responsiveness to cation signals, and activity in the presence of ribonucleotides. Certain homologs exhibit higher responses to cations *in vitro*, and multiple sequence alignment of TdT sequences revealed shared amino acid identity at specific residues common to more responsive homologs. We introduced equivalent point mutations in a low-response homolog to understand the effects of altering these non-conserved TdT regions. We found regions of TdT that play a role in dNTP selectivity, responsiveness to cations, and activity in the presence of ribonucleotides. This new knowledge is important to understand which regions can be modulated to improve TdT's molecular recording properties, thereby guiding targeted protein engineering efforts to optimize TdT as a recording tool.

Expansion of the genetic code by a reverse engineered protein ligase

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Nature employs a limited and conservative set of amino acids to synthesize proteins. The ability to genetically encode an extended set of building blocks can be used in diverse applications, including approaches to study and control protein function as well as to design novel therapeutics. Non-natural amino acids (NAA) are co-translationally incorporated into proteins by orthogonal pairs consisting of aminoacyl-tRNA synthetase and cognate tRNA.

However, the current repertoire can neither display the full natural diversity of NAAs and is especially limited for backbone modifications. Excitingly, we now succeeded in reverse engineering a protein ligase into new-to-nature tRNA synthetases that can load tRNA either with β-amino acids (βaa) or expand the genetic code by novel non-natural α-amino acids including advanced glycation endproducts.

Synthetic dual-input hybrid riboswitches – optimized genetic regulators for complex circuit design

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Abstract

Riboswitches are genetic elements composed entirely of RNA that are capable of stabilizing their structure after binding a target molecule (ligand) with high affinity and specificity. The compact size (50-100 nucleotides), no need for auxiliary factors (e.g. repressor proteins) and the resulting low metabolic burden on the host cell make riboswitches perfect candidates for the design of complex genetic circuits. In yeast, insertion of a riboswitch into the 5' UTR of an mRNA allows control of translation initiation by creating a physical "roadblock" for the scanning ribosome. A side effect of riboswitch insertion is reduced expression of the controlled gene even in the absence of a ligand (basal expression) due to the steric hindrance introduced by the pre-structured state of the riboswitch. We report here that the impact on basal expression can be reduced and switching efficiency simultaneously increased by creating constructs containing two different ligand binding pockets in one continuous structure. Our hybrid riboswitches outperform single-input riboswitches and, due to their dual-input nature, are able to emulate Boolean logic gate switching patterns. Both rational design and screening methods were tested for hybrid riboswitch optimization, and a Boolean NAND gate was created using a combined Sort-Seq and machine learning approach.

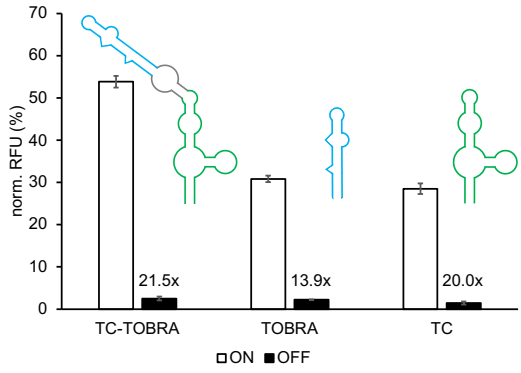

Figure 1: hybrid vs. parental single-input riboswitches

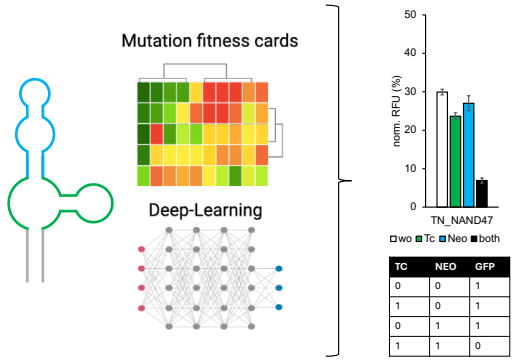

Figure 2: NAND gate hybrid riboswitch

Using Diversity Generating Retroelements for Cas9 protein engineering

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For over a decade, CRISPR-Cas9-based tools have emerged as one of the leading methods in genetic engineering. The range of their applications includes genome engineering, base editing, RNA-guided transcriptional repression, and activation, as well as chromatin remodeling. Tools utilizing this system leverage the ability of Cas9 to recognize specific DNA sequences, which depends on two key features: the customizable guide RNA and the invariant protospacer adjacent motif (PAM), a fixed short sequence required for correct target recognition, which varies among different Cas9 proteins. Consequently, there is significant interest in creating Cas9 variants that relax this requirement to allow the targeting of diverse sequences. This effort involves generating and screening libraries of Cas9 and dCas9 variants through various methods.

In this work we adapted a specific method called DGRec to generate dCas9 libraries *in vivo*. This strategy is based on diversity-generating retroelements (DGR) natural systems capable of accelerating evolution by rapidly mutagenizing protein-encoding target gene. During DGR mutagenesis variable repeat (VR) within a target gene is modified by mutated cDNA from a similar template repeat (TR). This process involves the synthesis of dgrRNA containing the TR, followed by error-prone reverse transcription of the TR, and recombination of resulting cDNA with the VR. Two proteins are essential for this event the reverse transcriptase (RT) and an accessory subunit

(Avd), which together with the dgrRNA form the active complex. Notably, DGR RT shows a unique mutation bias predominantly adding random nucleotides instead of adenines. This feature enables the application of this system for modifying various genes of interest. Our DGRec strategy combines DGR system mutagenesis and ssDNA oligonucleotide recombineering.

To adapt the DGRec method for producing dCas9 libraries with PAM-modified variants, we developed a two-plasmid diversification system complemented by selection techniques that facilitate the isolation of dCas9 variants generated by DGRec with modified amino acid sequences and novel properties.

This new Cas9 protein engineering technique has great potential for generating Cas9 variants *in vivo*. It can be utilized not only to alter PAM recognition but also to reduce off-target binding activity and enhance Cas9 binding and cleavage efficiency.

The ART of RNAylation: Harnessing RNAylated proteins as molecular probes

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ABSTRACT

Protein-oligonucleotide conjugates have emerged as a promising class of molecular probes in biosensing applications. As they leverage the properties of both nucleic acids and proteins, these conjugates possess expanded applicability and detection capabilities compared to conventional fluorophore- or radio-labeled probes. Continued growth in this area relies on developing conjugation approaches that can be more site-specific and robust. Our research group has recently reported a novel post-translational protein modification termed 'RNAylation' of proteins. In this reaction, the ADPribsolytransferase (ART) ModB generates RNAylated proteins by conjugating NAD-capped RNAs (NAD-RNA) to target proteins. This conjugation is highly site-selective, occurs under mild conditions and requires few reagents. Although RNAylation offers promising potential as a bio-conjugation approach, the possibility to implement RNAylated proteins in biosensing applications remains completely unexplored.

The project here presented aims at exploiting RNAylation to generate highly sensitive and modular probes made of RNAylated proteins for the detection of nucleic acids and proteins. As a proof of principle, we aim to apply our probes for the detection of target transcripts and proteins in blotting techniques such as northern and western blots. Additionally, to demonstrate the flexibility and applicability of our approach, we seek to apply RNAylated probes on fixed cells *in situ* to detect target transcripts using microscopy. RNAylated proteins applied as probes could constitute a modular and highly sensitive technology for the detection of biomolecules in cells and could stand as an alternative to fluorophore or radioactivity-based probes.

Unlocking Therapeutic Success: Vector Design and Plasmid Quality are the Keys in Cell and Gene Therapy

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Plasmids are the backbone of cell and gene therapy (CGT), serving as carriers of therapeutic genes essential for treating various diseases. Thus, the success of these therapies hinges on strategic vector design and high-quality plasmids to enhance construct performance, stability, and safety.
Conventional methods of plasmid production are time-consuming and limit the achievable design space. Here, we show the importance of specific elements in plasmids for various CGT approaches and that their design should be considered from the earliest stages of development. We argue that optimized plasmid design and working with high-quality plasmids is a necessity for efficient development and optimal therapeutic outcomes. This can be achieved most efficiently by *de novo* synthesis which allows maximum freedom of design as well as sequence optimization using GeneArt™ GeneOptimizer™ technology.
Leveraging over 20 years of experience in complex plasmid synthesis, we present a streamlined approach to plasmid production for therapeutic development. From initial design to pre-clinical development, we offer custom, optimized plasmid solutions. Coupled with robust quality control measures, our techniques address common challenges in therapeutic plasmid production, enabling faster progression of cell and gene therapies.

Investigation of the unusual PE-III phycobiliprotein of *Prochlorococcus marinus* SS120, using *E. coli* and *Synechocystis* sp. PCC 6803 as a biosynthetic platform

Jacqueline Hackh and Nicole Frankenberg-Dinkel, RPTU Kaiserslautern-Landau

The marine cyanobacterium *Prochlorococcus* is known to be the smallest photosynthetic organism on this planet, nonetheless, its sheer abundance makes it ecological one of the most significant cyanobacteria in the ocean. Unlike other cyanobacteria, *Prochlorococcus* has abandoned the effective light harvesting complexes, called phycobilisomes. Instead, this organism relies on divinyl-chlorophyll-antenna, harvesting blue light very efficiently. Interestingly, *Prochlorococcus* kept small amounts of a remnant of phycobilisomes (PBS) in form of a single phycobiliprotein, phycoerythrin III (PE-III). This PE-III of low-light adapted *P. marinus* SS120 (CCMP1375) is composed of an α- and β-subunit (SU) and likely carrying covalently attached phycoerythrobilin (PEB) and phycourobilin (PUB) chromophores in a 1:3 ratio. Genes required for the assembly of PE-III are encoded in a ~10 kb gene cluster, encoding the SUs and five putative phycobiliprotein lyases for the proper stereochemical attachment of chromophores to apo-PE-III. Apart from the cluster, genes are found encoding biosynthetic enzymes for PEB and phycocyanobilin (PCB). The function of lyases and their role in the assembly of apo-PE-III were investigated by a heterologous *E. coli* expression system. To date only the function of lyase CpeS was confirmed by this approach, ligating (3Z)-PEB to Cys82 on the β-SU CpeB. Although the remaining lyases can be expressed in *E. coli* as verified by Western blot, none of them displayed activity, pointing to a problem related to protein folding in the foreign host, possibly due to a bias in codon usage. *P. marinus* SS120 has a global GC content as low as 36.82 % shifted towards AT at the 3rd position, compared to *E. coli* with 51.7 % of GC. Synonymous codon substitution might be “silent” in the amino acid sequence but can impact gene expression levels and protein folding. The codon usage is known to regulate the translational elongation speed, efficiency and accuracy. The speed in turn affects the time available for the co-translational folding process, hence influencing protein structure and

function. Just optimizing the genes of lyases for the expression in *E. coli* might not necessarily solve the problem. Since translation kinetics would still differ from those in *Prochlorococcus*. Therefore, the tool MinMax% was used to evaluate the relative usage frequencies of the synonymous codons (Rodriguez *et al.*, 2017). While the algorithm CHARMING allowed to replicate the codon usage pattern of genes of *P. marinus* SS120 in the expression host *E. coli* (Wright *et al.*, 2022). With this approach it was possible to show the function of lyase isomerase MpeX, covalently attaching PUB to the double linkage site of CpeB at Cys50/61, resulting in an additional absorption peak at 494 nm and fluorescence at 567 nm (Fig.1). MpeX activity requires that CpeB is first chromophorylated by the lyase CpeS and needs the assistance of chaperone-like lyase CpeZ.

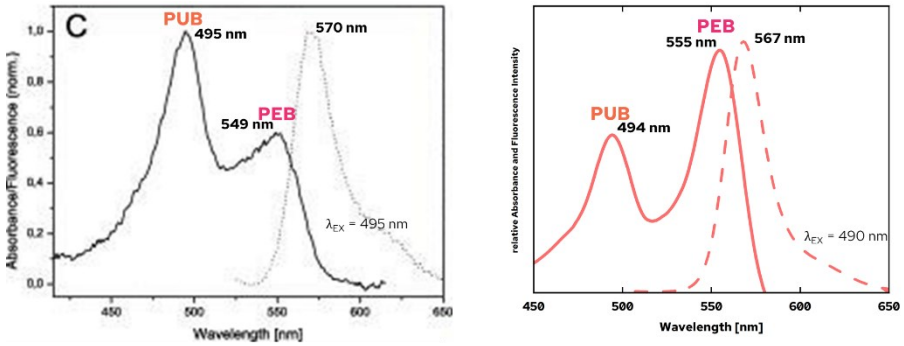

Figure 1 Absorption (solid line) and fluorescence emission spectra (dotted line). (**Left**) Native PE-III isolated from *P. marinus* SS120 showing an emission maximum at 570 nm. The absorption spectrum of PE shows two maxima at 495 nm (PUB) and at 549 nm (PEB) (Steglich *et al.*, 2002). (**Right**) Purified CpeB after co-production with the lyases CpeS, MpeX and CpeZ shows two absorption maxima at 494 nm (PUB) and 555nm (PEB) and emission at 567 nm, resembling already the spectrum of native PE-III.

With a second approach, we will introduce the components of the PE-III gene cluster and different chromophore biosynthesis enzymes into the cyanobacterium *Synechocystis* sp. PCC 6803. The modular cloning system CyanoGate will allow a quick exchange of different combinations of lyases as well as the generation of a synthetic polycistronic operon, resembling the gene cluster of *P. marinus* SS120 (Vasudevan *et al.*, 2019). If PE-III can be assembled, it might interact with the present PBS and/or the thylakoid membrane of PCC 6803 and thus give hints towards a possible function of PE-III.

Overall, this project aims at understanding the assembly and function of the unusual phycobiliproteins in low-light adapted *P. marinus* strains.

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Vasudevan R, Grant A.R. Gale, Alejandra A. Schiavon, Anton Puzorjov, John Malin, Michael D. Gillespie, Konstantinos Vavitsas, Valentin Zulkower, Baojun Wang, Christopher J. Howe, David J. Lea-Smith, Alistair J. McCormick, CyanoGate: A Modular Cloning Suite for Engineering Cyanobacteria Based on the Plant MoClo Syntax, *Plant Physiology*, Volume 180, Issue 1, May 2019, Pages 39–55.

Metabolic burden and resource allocation in engineered *Pseudomonas putida* strains: the fate of amino acids

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Technical University of Munich, Germany

The biotechnological production of heterologous proteins is often limited by metabolic burden as it interferes with the host's cellular capacity to allocate resources. Draining cellular resources toward heterologous gene expression creates additional stress, often leading to low productivity and decreased growth rates. In this study, we aimed to investigate whether resupplying cellular resources (here amino acids) could help to alleviate the metabolic burden and boost heterologous protein production in engineered *Pseudomonas putida*. To this intent, four different synthetic fusion-proteins were designed, consisting of eGFP fused to proteins with varying sizes and amino acid composition but with the same ribosome binding site upstream of the respective genes. The resulting strains were grown in minimal medium, with and without amino acid supply and bacterial growth, protein production rates and amino acid uptake were analysed. Amino acids can have different fates once taken up; they can serve as building blocks for protein synthesis, as energy source, as nitrogen source, and/or as carbon source. We could show that the size of the fusion-protein did not directly impact the metabolic burden in *P. putida.* The supplementation of amino acids shortened the lag phase and stimulated growth but also, counterintuitively, led to a decrease in the protein production rate. To exclude that this phenomenon originates from the promoter system employed for gene expression, we exchanged the XylS/*Pm* promoter of the burden plasmid with a promoter system not native to *P. putida.* Still, the effect of decreased protein production upon amino acid supplementation was observed. By increasing the translation rate of the fusion-protein by employing a stronger RBS, the decrease in protein production rate became less apparent. This suggests an uneven allocation of the additional resources between growth-related processes and those involved in heterologous protein production. As we observed this phenomenon in the presence of abundant potential carbon sources, carbon catabolite repression (CCR) could be one of the factors involved.

Boosting unnatural amino acid facilitated photocontrol of enzyme activity by directed evolution

Sabrina Mandl, Reinhard Sterner, Andrea Kneuttinger
University of Regensburg and Regensburg Center for Biochemistry, Germany

Light is an excellent tool for the regulation of biochemical processes as it offers high resolution in time, space, and intensity. Moreover, it is environmentally friendly, cost-effective, and easy to apply. Therefore, the artificial, light-induced control of especially enzymes, which are implemented as sustainable biocatalysts in the chemical industry or as biotherapeutics in the medical sector, is of growing interest. Particularly reversible on and off switching of enzyme activity is highly desirable for various research fields. This can be tackled by incorporating photoswitchable unnatural amino acids (UAA) into enzymes. The integrated photoswitches change their shape upon irradiation with light of one wavelength, and reverse back upon irradiation with light of another wavelength. We have previously shown that we can photocontrol enzyme allostery and activity in the bi-enzyme complex imidazole glycerol phosphate synthase (ImGPS), in which substrate binding to the synthase subunit HisF stimulates the glutaminase subunit HisH. Incorporation of the photoswitchable UAA phenylalanine-4'-azobenzene (AzoF) at position 123 in HisH (hW123AzoF) [1] resulted in a ~10-fold regulation of HisH activity. Regarding the applicability of this strategy in the biocatalytic industry or biotherapy, we were wondering whether we can further increase the photocontrol efficiency by directed evolution (Figure 1).

To this end, we aimed to optimize the protein environment of AzoF incorporated at position 123 in HisH using a semi-rational design approach. We generated focused DNA libraries targeting positions in the vicinity of AzoF, translated them into enzyme libraries, and screened for an increased photocontrol efficiency of these enzyme libraries by using a light-responsive activity screening. In this way, we identified variants that facilitated to control HisH activity up to ~40-fold. In-depth characterization of these variants provided additional insights into the principles and mechanisms of photocontrol in ImGPS. Our findings reveal that photoswitchable UAAs can be used as a powerful tool for the spatiotemporal control of enzymes by light and that directed evolution is a promising approach to further extend this regulation potential.

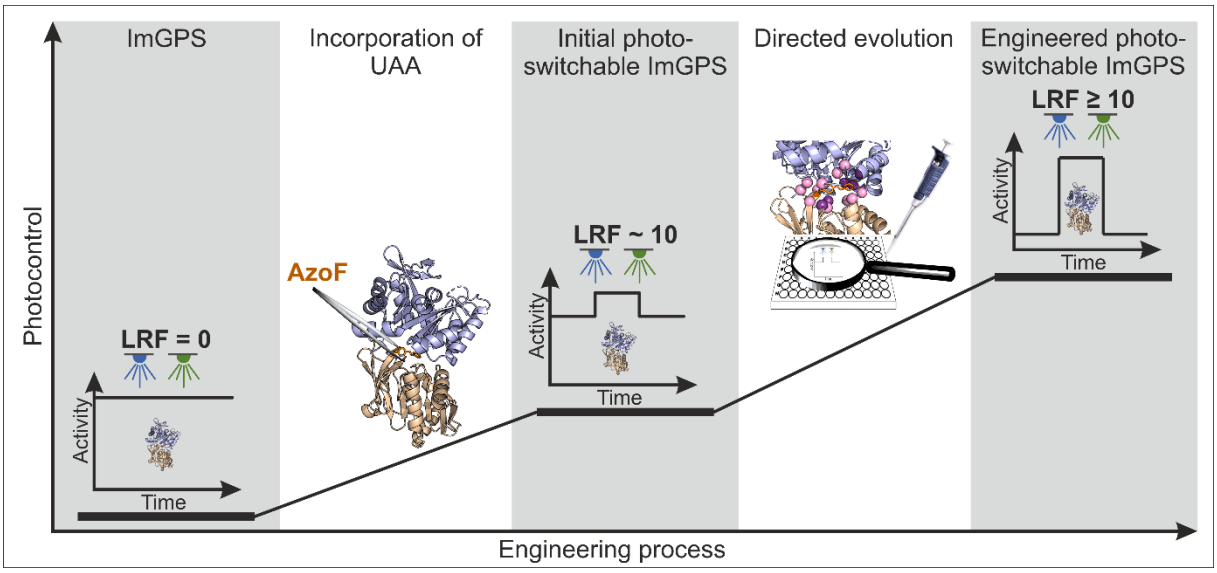

Figure 1. AzoF and directed evolution facilitated optimization of photocontrol in ImGPS. The light-regulation factor (LRF) is a measure of photocontrol efficiency.

[1] A.C., Kneuttinger; C., Rajendran; N.A., Simeth; A., Bruckmann; B., König; R., Sterner *Biochemistry*, **2020**, 59, 2729–2742.

Expanding the repertoire of photoswitchable unnatural amino acids for the versatile photocontrol of enzymes

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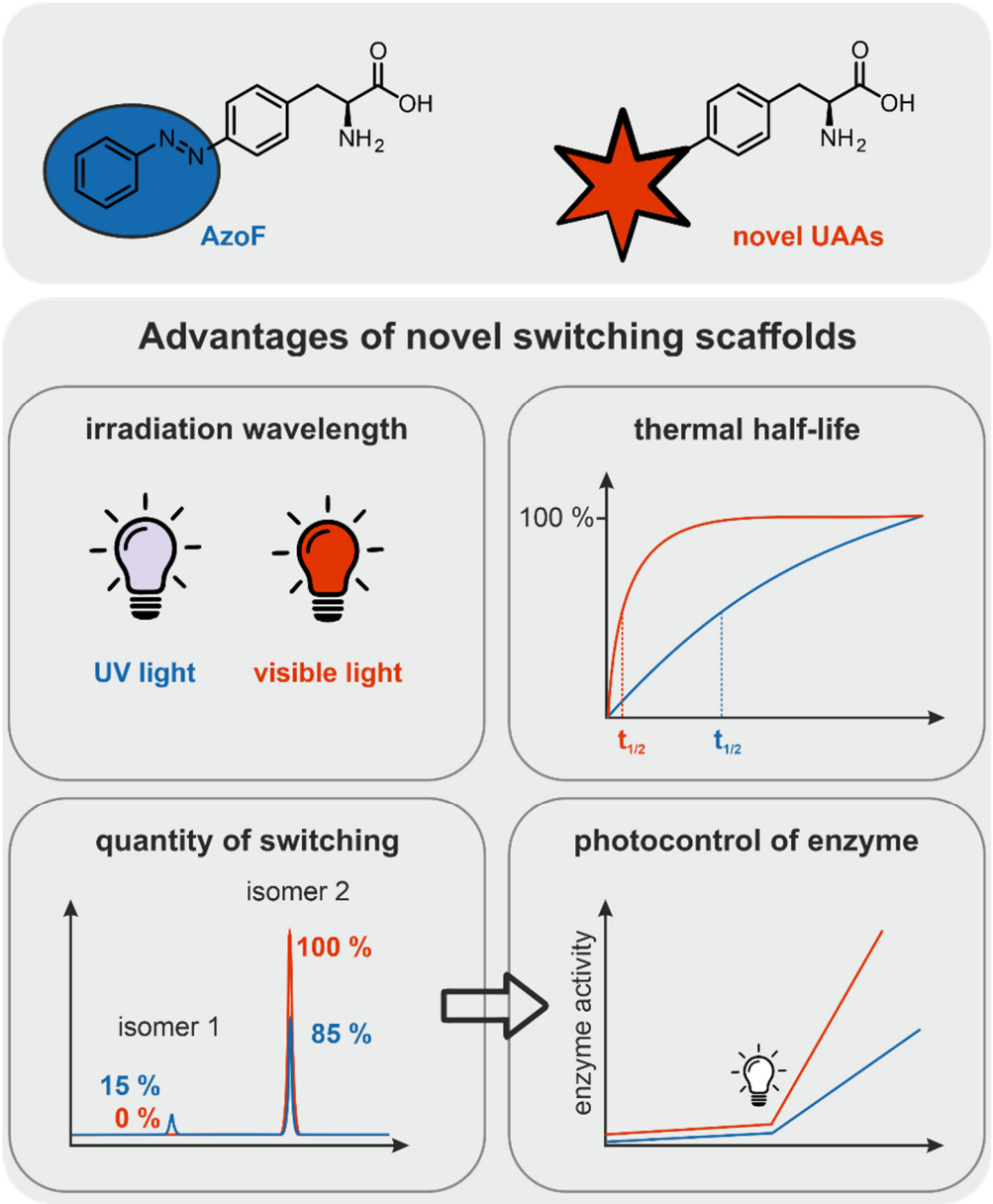
University of Regensburg and Regensburg Center for Biochemistry, Germany

In the last decades, the design of photocontrolled enzymes has been of growing interest in various research fields, including *in vivo* studies, therapeutic approaches, and industrial biocatalysis. One commonly used method involves the incorporation of light-sensitive unnatural amino acids (UAAs) by means of genetic code expansion generating so-called photoxenoproteins. The photoswitchable UAA phenylalanine-4'-azobenzene (AzoF) interconverts between an *E* and *Z* isomer with UV and visible light, respectively, thereby offering the advantage of reversibility. However, some of its properties limit the engineering of photoxenoproteins: i) *E*↔*Z* switching is not quantitative and has caused problems with the recovery of the initial activity, and ii) *E*↔*Z* interconversion relies on irradiation with short wavelengths, which is toxic and has only a short penetration depth.

For this reason, we decided to expand the repertoire of photoswitchable UAAs. To this end, we chose several other photoswitches that have previously demonstrated excellent photochemical properties. Besides further AzoF derivatives, we synthesized UAAs based on arylazopyrazole, arylazothiazole, hemithioindigo, and spiropyrane scaffolds. Analysis of their photochemical properties revealed several advantages compared to AzoF such as a more quantitative *E*↔*Z* switch, differences in thermal stability, as well as switching wavelengths in the visible range. To achieve the site selective incorporation of these UAAs into proteins, screening of various aminoacyl-tRNA synthetase variants identified suitable synthetases for the incorporation of four of the seven synthesized UAAs. We further tested the photophysical properties of these UAAs within an enzyme and determined differences in the photocontrol efficiency of enzyme activity. We anticipate that this set of photoswitchable UAAs will facilitate the fine-tuning and improvement of photoxenoproptein engineering, thereby significantly expanding the range of applications.


AzoF
novel UAAs
Advantages of novel switching scaffolds
irradiation wavelength
UV light
visible light
thermal half-life
100 %
quantity of switching
isomer 2
100 %
isomer 1
85 %
15 %
0 %
photocontrol of enzyme
enzyme activity

Optimizing the Reaction Conditions for a mammalian Cell-free Expression System using Design of Experiments

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Sartorius Stedim Cellca GmbH, Ulm/Germany

A Cell-free expression (CFE) platform utilizes the translational machinery derived from either prokaryotic or eukaryotic cells to express a protein of interest (POI) in vitro. Cell lysates with an eukaryotic origin commonly suffer from low protein yield in comparison to prokaryotic-based lysates. Therefore, there is a need to develop a mammalian CFE system that can match up to the bacterial and plant-based systems in terms of protein yields while being able to perform the post-translational modifications relevant for the recombinant expression of therapeutic proteins. The performance relies on the correct function of numerous enzymes involved in the molecular pathways of protein synthesis. The crude lysate is supplemented with various components that enable transcription and energy regeneration in addition to the DNA template carrying the POI sequence. We describe the development and optimization of a Chinese hamster ovary cell (CHO) derived CFE system.

Due to the open nature of CFE, it is well suited for sequential design-build-test cycles utilizing automated liquid handling and design of experiments (DoE) to optimize the reaction conditions. The DoE-based approach allows for the identification of interaction as well as synergistic effects between individual factors, in contrast to only investigating one factor at a time. Central composite designs were used to optimize the concentration of HEPES, potassium glutamate, magnesium glutamate, ATP, GTP, CTP, UTP, Creatine Phosphate, Creatine Kinase, T7 Polymerase and DNA Plasmid. Overall, we achieved more than a two-fold increase in protein yield after an 8-hour reaction, using the turboGFP variant as a model protein. The optimized CHO-derived CFE system surpasses the yields of commercially available kits, representing a considerable advancement in mammalian cell-based lysate systems.

Characterisation and engineering of biomolecular condensates

Emma Crean, Aukse Gaizauskaite, Henrike Niederholtmeyer, Technical University of Munich, Campus Straubing for Biotechnology and Sustainability, Straubing/Germany

Overview

Compartmentalisation is a key spatio-temporal organisation principle that is tightly linked to the evolution of life. Compartments formed by membranless organelles, termed 'biomolecular condensates', can spontaneously assemble and disassemble via phase separation. The dynamic formation, combined with the benefits of compartmentalisation make condensates a highly attractive avenue to explore for researchers, especially in the area of synthetic biology.

However, currently the unique and diverse properties of condensates, are poorly characterised. Of particular interest is the interplay between protein sequence, physical properties and functionality. Untangling part of this mystery would allow for tailored manipulation of condensates and their use in synthetic biology, and other fields. Synthetic, membraneless compartments could enable researchers to endow them with functional specificity, enrich targeted molecules to boost reaction kinetics or sequester molecules for protection.

Objectives

The overall objective of this project is to characterise and engineer biomolecular condensates. Investigating the formation of condensates and understanding the intricate connections between their sequence, material properties, and functionality represents a novel and crucial research area, especially in the context of cell free transcription-translation (TXTL). This consists of three primary objectives.

1. Engineering and screening of a wide variety of candidates to expand the library of known condensate-forming proteins in TXTL reactions.
2. Characterisation of the material properties of condensates formed by a variety of condensate-forming proteins to enhance understanding of the interrelation between material properties and external influences upon them.
3. Characterisation of how the material properties of condensates influence their functioning. Engineering the sequestration of molecules into condensates and subsequent characterization of enzymatic reactions within condensates will

expand knowledge about the internal biochemical environments of condensates, and how it is influenced by material properties.

Significance

Investigation into condensates, especially their properties and functional capabilities, is an exciting area of research. Expanding this knowledge base could enable the eventual engineering of condensates with specific properties and functionality. Tailored condensates would have enormous applicability in synthetic biology and many other research fields.

Development of cell-free cascades to generate multi-antennary, complex glycans on insect-cell derived glycoproteins

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Almost all viruses that have caused epidemic are enveloped viruses with glycoproteins as part of their envelope. These glycoproteins are typically a major antigen target for vaccine development. It is well-known that the glycans attached to the backbone of these proteins fulfil multiple roles [1, 2]. As an example, influenza A virus hemagglutinin (HA) possesses several *N*-glycosylation sites which can carry glycans that shield the epitope from the host's immune system. Thereby, the glycans can help to evade the host's immune response [3, 4]. Yet, the impact of specific homogeneous glycoforms on viral membrane glycoproteins is only poorly characterized due to native microheterogeneity. The use of recombinant glycosyltransferases to homogenize the glycoprofile of these vaccine candidates would be an important step towards the study of the influence of specific glycans on immunogenicity.

For this purpose, we have developed a platform for *in-vitro* glycoengineering of glycoproteins. The platform comprises seven enzymes (MGAT1 [5], MGAT2 [5], GalT [5], MGAT4A, cMGAT4C, hMGAT4C and MGAT5) that were recombinantly expressed in *Escherichia coli* strains. The enzymes are applied in cascade reactions to generate glycan structures of interest.

In a case study, HA from Sf9 cells was chosen as a target glycoprotein. Recombinant proteins expressed in insect cells typically carry paucimannose-type *N*-glycans that are a suitable starting point to explore the range of complex glycans that can be generated by our newly established platform. For example, complex-type *N*-glycans such as A3G3 and A4G4 were converted from paucimannose-type *N*-glycans with high turnover. In the future, the generated samples can be used for detailed structure-function studies in animal models.

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Compartmentalisation-driven Feedback Dynamics in Cell-free Systems

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Through compartmentalisation, cells separate incompatible reactions, co-localise and concentrate molecules, establish concentration gradients, and regulate reactions by controlling reactant availability. Synthetic compartments have been engineered to achieve spatial control. Although generated through different methods, most share two main characteristics: They are separated through a physical barrier, and their assembly dynamics cannot be easily controlled.

Recently, biomolecular condensates have been discovered and studied as a new type of genetically encodable, membrane-less compartments. These condensates can be formed by RNA-RNA, Protein-Protein, and RNA-proteins interactions. When a threshold concentration is reached, weak interactions between self-assembling binding partners cause phase separation into a condensed and dilute phase. Subsequently, cargo can be sequestered into the condensate, and reagents can be removed from the dilute phase or concentrated. Biomolecular condensates offer a genetically encodable platform for engineering defined membrane-less reaction compartments with temporal control over assembly and disassembly. Engineered biomolecular condensates have been reported and are typically characterised in cells or using purified proteins.

By employing continuous flow microfluidics and cell-free synthesis, I aim to engineer spatiotemporal control over membrane-less compartmentalisation. To achieve this goal I plan utilise oscillating gene networks.

In this work I aim to engineer condensate-forming proteins that allow for defined sequestration of target molecules into the synthetic compartment. Thereby, local enrichment of biomolecules will induce a feedback loop, which is followed by dynamic assembly and disassembly of the engineered compartment. Dynamic feedback control over condensate assembly will allow synthetic biologists to engineer autonomously adapting cellular compartments.

Synthetic cell-based artificial tumor immune microenvironments in 3D cancer cultures

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Over the past decades, great progress has been made in the field of cancer therapy. In particular, the various immunotherapeutic approaches, which involve using components of the immune system for treatment, have had a major impact on the survival rate and regression of the disease. However, a variety of cancers are able to evade detection by the immune system by developing a suppressive tumor immune microenvironment (TIME), a dense structure of extracellular matrix proteins and anti-inflammatory immune cells that interact with both tumor and immune cells. Research into the influence of the various components of TIME on treatment efficacy is hampered by both its inherent complexity and the lack of suitable *in vitro* models.

To meet this challenge, we developed a bottom-up assembled synthetic cell system, referred to as droplet-supported lipid bilayers (dsLBs). DsLBs are soft mimetic structures designed to imitate the surface properties of immune cells by displaying immune cell-associated proteins on their surface. We are able to decorate dsLBs with defined quantities of surface receptors and regulatory proteins, allowing us to reproduce the cell-cell interfaces that naturally occur between tumor and TIME in a highly controlled manner. We show that cancer cells adhere closely to these dispersed synthetic cells and incorporate them into 3D spheroids during their self-assembly formation process, creating a hybrid material that we call the artificial tumor-immuno-microenvironment (ART-TIME). Using this approach, we demonstrated that incorporation of dsLBs decorated with different immune cell-derived receptors results in distinct ART-TIMEs that affect gene expression of pancreatic ductal adenocarcinomas (PDAC) cancer cell lines, including several proteins involved in the outcome of cancer therapy and relevant for immune evasion. This included transcriptome analysis and phosphoprotein signaling pathway assays to explore the functional mechanisms behind evasive behavior in bispecific T cell engager therapies.

We envision ART-TIMEs to be applied as controllable *in vitro* models for research into cancers-immune evasion and for systematic analysis of receptor co-signalling during phenotypic cancer.

Workflow for extract-based cell-free synthesis of heterologous enzymes followed by an *in situ* assay

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Introduction

The speed of enzyme discovery is currently limited by traditional protein expression methods. Cell-free protein synthesis (CFPS) systems are valuable tools to accelerate biotechnological workflows. Especially top-down extract-based CFPS systems are easily accessible, relatively cheap and higher yielding compared to more defined CFPS systems such as PURE. In this work, we developed an extract-based CFPS workflow for the expression and *in situ* assay of heterologous proteins. The assay can be performed in a plate-reader format. The workflow can be completed within a working day, and delivers qualitative information about protein solubility and activity.

New results

Enzyme assays are frequently done through detection of the absorbance at 340 nm, where the reaction can be followed by the consumption of NAD(P)H/NAD(P)$^+$ electron carriers in the reaction. An NAD(P)H based assay is very straightforward to apply for a purified enzyme, however, that is not the case when the enzyme had been expressed using an extract based cell-free expression platform. This is because within the extract proteome there are many other enzymes which also utilize these ubiquitous biological molecules, resulting in a very high background signal in the experiments. This background can be overcome by a implementing a single additional step into the workflow – a purification of the CFPS reaction prior to its use in the enzyme assay. The purification of the completed CFPS reaction was done with a 0.5 mL Zeba Spin size exchange chromatography column (7K MWCO). This resulted in the removal of all small metabolites from the reaction while all the proteins were transferred to the buffer of choice. Enzymatic assays before and after SEC purification are shown in Figure 1. Enzymes investigated so far were oxidoreductases and dehydratases (EC classes 1 and 4 respectively).

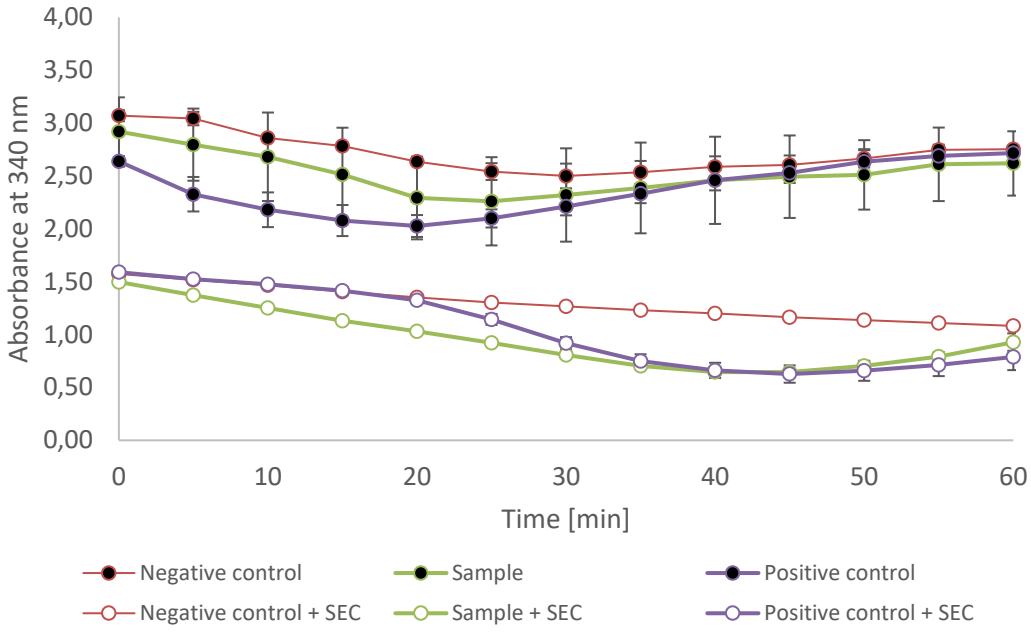

Figure 1. Assay of enzymes A (dehydratase) and B (oxidoreductase) after production through extract-based CFPS, with and without processing by size exchange chromatography (SEC). Without SEC (closed circles), the signal from the negative control, positive control and sample is indistinguishable. With SEC (open circles), there is a clear difference between the negative and positive controls, and it can be determined that the enzymes A and B produced through CFPS were active. Assay conditions - 37 °C for 60 min, absorbance at 340 nm, n = 3. Negative control – enzyme X produced through CFPS; Positive control – purified enzymes A and B added to the CFPS-assay mixture; Sample – enzyme A and B produced through CFPS.

Outlook

Further work on this topic will involve addressing open questions and developing best practices for reliable prototyping results. The protocol will also be expanded to test other enzyme classes and assay formats. Development of such protocols would bring us closer to routinely using faster CFPS-based workflows for enzyme discovery on a medium-throughput scale.

Microfluidic Platform for Engineering Biomolecular Condensates in Cell-Free Systems

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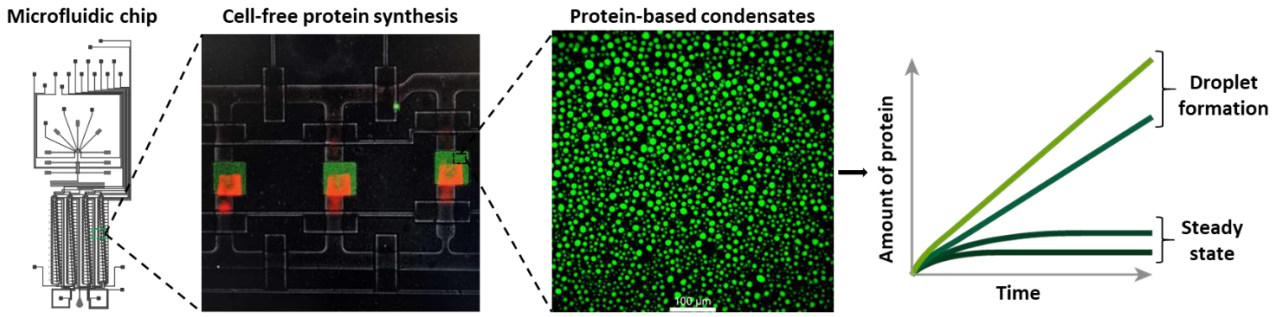
Intrinsically disordered regions (IDRs) and high multivalency give some proteins the ability to self-associate into liquid-like droplets, called condensates. While living cells employ condensates as membranelles organelles for spatial organisation, it is challenging to recreate such compartmentalisation *in vitro* with a complexity matching living cells. In order to facilitate this challenge, we designed a microfluidic platform, which helps to characterise protein-based condensates in cell-free transcription and translation (TX-TL) systems. Micrometre-size environments in the chip with the diffusive supply of TX-TL reagents ensure long-term continuous protein synthesis with a constant supply of fresh reaction components. Taking advantage of microfluidic techniques, this device allows the screening of multiple condensing proteins to be carried out simultaneously and efficiently with minimal usage of reaction components. Easier characterisation of protein variants opens an opportunity to design synthetic condensates as programmable and dynamic assemblies. This not only expands the complexity of artificial life-like systems but also lets the creation of biochemically active compartments that are able to carry or sequestrate particular molecules *in vivo* and *in vitro*.



Cell-mimics continuously communicate in 2D, supported by 3D microfluidic perfusion

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Cell free transcription and translation provides a well-controlled environment to test gene regulatory networks and their products *ex vivo*. Gene regulatory networks distributed in communities of cell-mimics could lead to the formation of self-organized patterns in gene expression.

To achieve this goal, it is necessary to increase the life-likeness of the cell free *in vitro* system using a continuous perfusion setup maintaining the reaction far from equilibrium. However, perturbations, from the feeding of fresh reagents, mix establishing concentration gradients in an unprotected reaction space.

We present a multilayer, microfluidic chip separating the feeding flow from the protected, 2D reaction chamber. As cell-mimics, we use porous capsules, with immobilized DNA cargo in an internal, primary hydrogel, to distribute gene regulatory networks in a discrete 2D array. An external hydrogel immobilizes the capsules after arrangement and constitutes the reaction space. Overflowing the gel pad with reagents creates a two-layer system with separated feeding and reaction spaces.

This setup allows us to model communication between synthetic cells in two dimensions, a prerequisite for multicellular organization. Using this device, we aim to characterize increasingly complex gene regulatory networks, leading to dynamic and static self-organizing patterns.

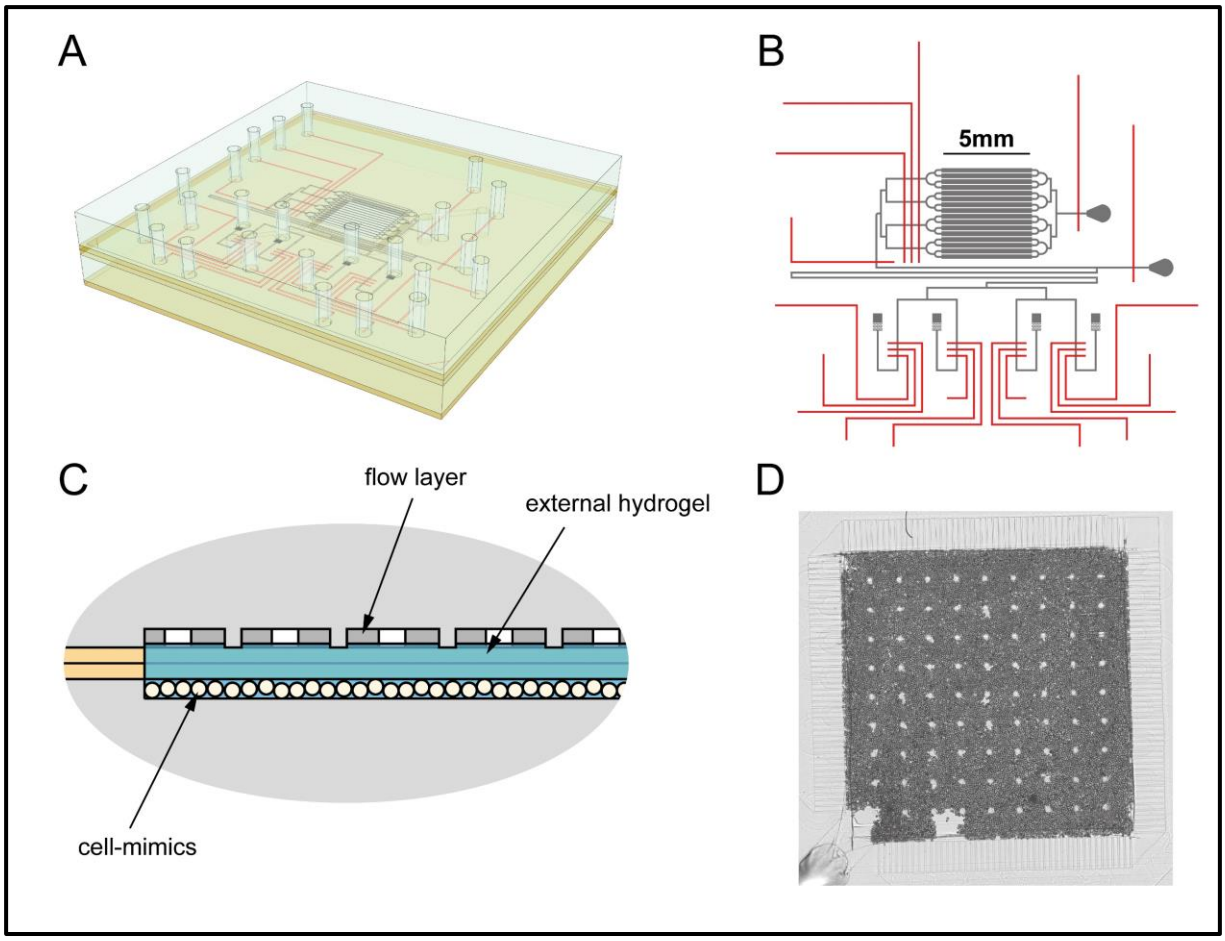

Figure 1: A) 3D rendering of microfluidic chip. Membrane-thickness (yellow) is exaggerated for visualization. B) Double layer chip design with flow layer (grey) and pneumatic valves (red) for on chip flow control. C) Cross-section of device layers at relative scale. D) Microscopy image of cell-mimic array in fully assembled chip.

Microfluidic assisted fabrication of stimuli-responsive artificial cell for pH modulation

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Synthetic cells are multi-compartmentalized systems that mimic the structure and function of a biological cell. In the recent years, they have gained enormous attention due to their hierarchal structure which resemble the cytosolic organization in eukaryotic cells. Due to their compartmentalized structure, they can perform biological tasks efficiently and therefore they find applications in fields such as biomimetic synthesis, molecular sensing and biomedicine.[1]

In this work, we present the fabrication of polymersomes based synthetic cells as a mechanically robust and chemically tunable system. We use droplet microfluidics as a high-throughput technique to create these polymer based artificial cells with controlled membrane permeability using pH as a stimuli.[2,3] Membrane permeability allows fine controlling important reactions inside the artificial cells. Furthermore, we aim to create a fully functioning reactor system inside the polymersome to further mimic the presence of artificial organelles inside polymersome. The artificial organelle is designed to be responsive to external stimuli (i.e. feeding of reaction component) through enzymatic reaction. The reaction is then further cycled through cascade reaction. Finally, we aim to use these functional cell-like reactors for pH modulation.

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Optimization of a protocol for the production of a bottom-up cell free system for high-throughput enzymatic screening applications

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Protein Synthesis Using Recombinant Elements (PURE), a cell free protein synthesis platform derived from purified proteins, offers a precisely defined milieu with significant potential for enzymatic high throughput screening applications. However, the high price associated with commercial PURE systems remains a great hurdle to overcome. This makes commercial Bottom-Up synthetic biology systems unattractive for many groups. The individual purification of the 36 protein components of the PURE system is a resource intensive task. In the OnePot PURE protocol, 36 *E. coli* clones, each overexpressing a component of the expression machinery, are co-cultured and purified. Following the original protocol, this cell free expression platform yielded low expression levels compared to a commercial PURE system. Additionally, OnePot PURE, as well as many other cell free systems, are heavily reliant on the availability of high quality commercial tRNA preparations. However, the only sufficiently pure product has been discontinued. We thus set out to solve two problems: First, we adopted and optimized the OnePot PURE protocol, developed by Lavickova et al., for the low-cost preparation of the PURE cell free protein synthesis platform in-house. Second, we developed a reliable protocol for the high yielding production of tRNA that can be used in the energy solution of the PURE system.

In our OnePot PURE preparations (see batch A & B in figure 1) the level of functionally expressed protein at most corresponds to 2.5 % of the commercial system. We have identified partial promoter deletions as a major problem with the reproducibility of the protocol. The OnePot PURE system not only relies on the co-culture, but also utilizes two different expression systems. While 20 proteins are stably encoded on pET derived vectors, the other 16 are encoded on either pQE30 or pQE60 vectors. Due to a repetitive operator sequence and the lack of tight expression control, the pQE vectors are highly prone to promoter deletions throughout the OnePot PURE workflow. We have fixed this issue by transferring 15 of the 16 genes into T7 based expression vectors and producing all proteins in the *E. coli* BL21 (DE3) pLysS strain. Further improvement in the protein yield was achieved by separately purifying the T7 RNA Polymerase (see figure 1 PURE Batch C). Through careful adjustment of its concentration in the cell free reaction, competition with the energy demand of translation was minimized without significantly added production effort. Another major task for in-house PURE production was the high yielding purification of tRNA. For this purpose, a phenol extraction based protocol was developed and optimized. Through multiple precipitation steps (see figure 2) and size exclusion chromatography, tRNAs can be selectively enriched largely free of other nucleic acid and small molecule contaminants that could interfere with enzymatic assay reactions in a cell free screening workflow.

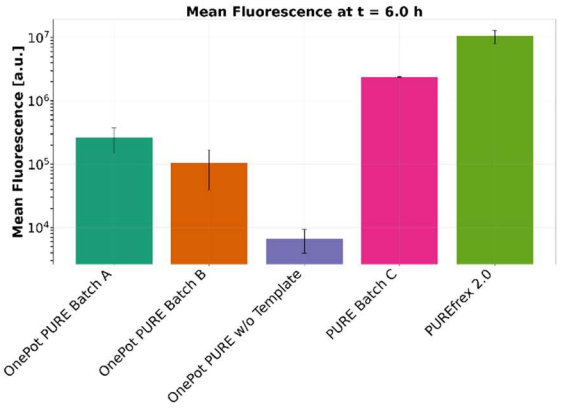

Figure 1: Expression of deGFP in two different batches of the OnePot PURE system compared to the commercial PURExpress 2.0 system and PURE batch C, produced using our improved protocol; Data displayed at a logarithmic scale

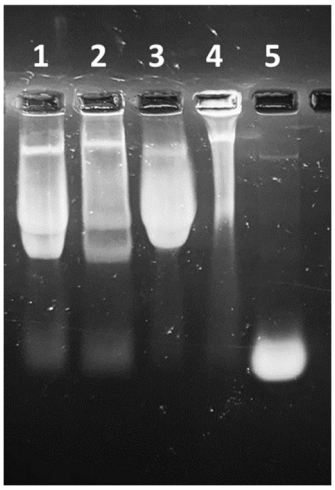

Figure 2: Agarose gel of successive tRNA purification steps. 1: total nucleic acids after phenol extraction, 2: bulk after precipitation of large RNAs, 3: precipitate of large RNA species, 4: residual DNA precipitate, 5: pure tRNA fraction

The present project has successfully developed a methodology for the economical production of the PURE cell-free protein synthesis platform, making it amenable to high throughput screening applications. We developed a protocol for the high-yielding purification of *E. coli* tRNA that constitutes a major bottleneck in the preparation of the PURE energy solution component.

Synthetic cell-based 3D artificial lymph nodes to study the activation dynamics of T-cells

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Ex vivo T-cell activation and expansion is crucial for effective immunotherapy. In this context, synthetic tissue-mimetics of lymphoid organs, containing artificial antigen presenting cells (aAPCs), are a promising approach. However, mimicking the biochemical and biophysical cues provided by natural APCs and required for reliable expansion of therapy relevant T-cell phenotypes is a formidable engineering challenge. For strong T-cell activation, established and commercially available T-cell activation beads rely on biochemical triggers in the form of immune-stimulatory antibodies present on their non-mobile surface. This results in T cell phenotypes of reduced quality.

In contrast to this, we demonstrate that lateral ligand mobility as a key factor involved in T-cell activation and expansion. For this, we developed a bottom-up synthetic cell assembly strategy to form dispersed droplet-supported lipid bilayers (dsLBs) able to emulate the natural lipid membrane characteristic of APCs. To take the system one step closer to *in vivo* conditions, we engineered the dispersed synthetic cells to self-assemble into 3D reaction rooms to form artificial lymphoid bottom-up tissue (lymphBUTs) structures. LymphBUTs provide T-cells with controllable biochemical and biophysical cues for a reliable therapeutically relevant T-cell expansion. Co-culture of T-cells within lymphBUTs result in infiltration of the T-cells into the synthetic tissue and subsequent expansion of a $CD8^+$ phenotype with regulatory- and memory-like function.

In a future perspective, the focus will be on engineering heterogeneous lymphBUTs comprising functional reaction zones. This compartmentalization approach within the lymphBUTs leads to increased control of T cell activation based on the biomimetic tissue structure. In a broader point of view, self-assembled BUT structures with therapeutic potential could open new avenues in biomedicine such as organ replacement, immuno-therapy and vaccination.

Production of recombinant human insulin from a novel bacterial expression system

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The number of diabetes patients and the price of the drug used to treat the condition, i.e., insulin, have considerably increased in recent decades. Despite impressive advancements over the years, multiple bottlenecks still need to be addressed to provide inexpensive and effective insulin therapy, given the increased demand for the drug. The shortcomings of existing expression methods include inclusion body formation, hazardous inducers and high maintenance costs. Here, we report a novel *Pseudomonas fluorescens*-based expression system created via vector engineering and fusion protein strategy (Figures 1 and 2) for recombinant human insulin production. Soluble and high-titer proinsulin fusion protein expression is achieved by tuning the co-expression of the chaperones. The fusion of native signal peptides further enhanced proinsulin secretion and soluble expression. Synergistic effects of multiple signal peptides and chaperones are examined for soluble fusion protein production. Enzyme-linked immunosorbent assay and western blot are performed to assess the validity of expressed proinsulin and insulin. Machine learning-aided RSM-based culture media optimization led to ~368 mg/l of human proinsulin fusion protein production in the metabolically engineered expression system (Figure 3). The scalability of the bioprocess for recombinant human insulin production was checked in a batch bioreactor with a 2-l working volume. Genome-scale metabolic model-based pathway optimization involving specific gene knock-in and gene knock-out via CRISPR-Cas9 can further enhance titer.

Keywords: Insulin, Expression system, Fusion protein, ELISA

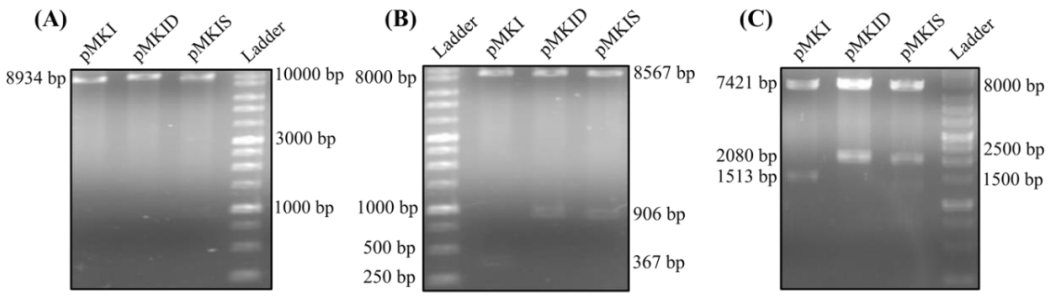

Figure 1. Clone confirmation of pMKI, pMKID, and pMKIS using restriction digestion **(A)** *Hin*dIII; **(B)** *Eco*RI and *Hin*dIII; **(C)** *Mlu*I and *Xho*I.

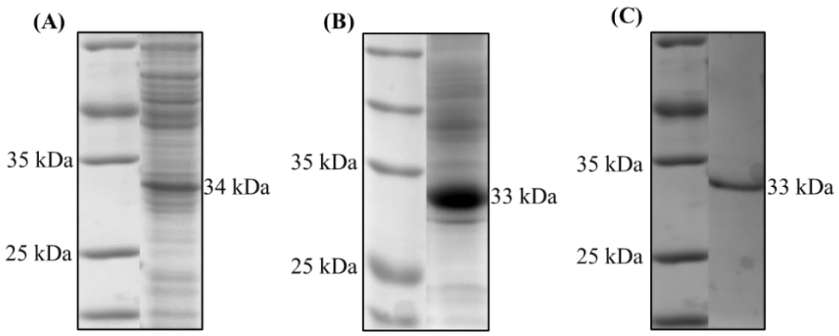

Figure 2. Expression profile of DsbA-Proinsulin fusion protein **(A)**; GST-Proinsulin fusion protein in *P. fluorescens* **(B)**; Purified GST-Proinsulin **(C)**.

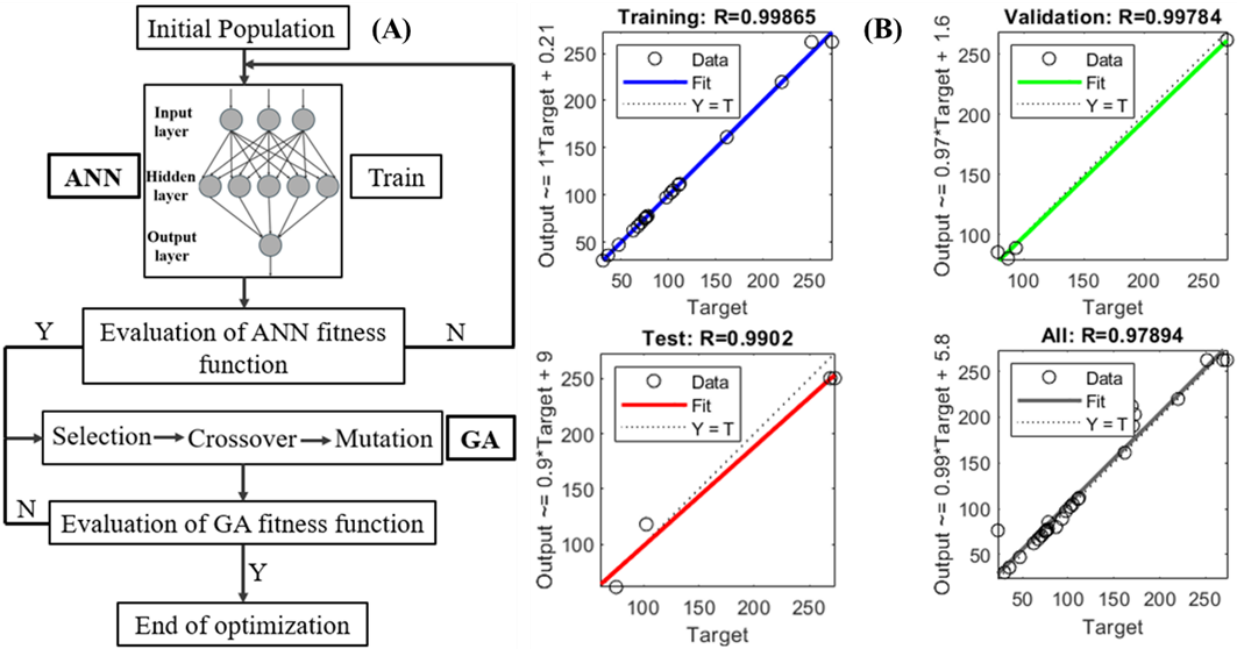

Figure 3. Schematic representation of a (3-5-1) ANN-GA network **(A)**; The prediction performance of ANN-GA models **(B)**.

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CRISPR/Cas-mediated activation in the social amoeba *D. discoideum* for natural product discovery

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Discovery of novel natural products is crucial for modern pharmaceutical industry and the development of potent drugs. The social amoeba *Dictyostelium discoideum* bears a high potential to find new bioactive molecules of therapeutic relevance. *D. discoideum* lives ubiquitously in soil and leaf litter, and has a unique developmental cycle in which it transforms from a unicellular organism to a multicellular fruiting body with various cell types. Due to its interesting lifecycle, the amoeba is used as a research model for numerous biological processes and discovery of elusive secondary metabolites. The haploid genome encodes a wide spectrum of biosynthetic genes, including up to 45 potential type I polyketide synthase and 11 terpene synthase genes. However, most of these genes are not expressed in the vegetative cell state, limiting the natural product discovery in wild-type strains so far.

Therefore, novel technologies to activate these transcriptionally silent genes are essential to elucidate the corresponding secondary metabolites. Recent advances in CRISPR/Cas-mediated genome editing facilitate the generation of *D. discoideum* mutant strains with specific alterations, including knock-out, knock-in and knock-down mutations. In this study, we aim to develop a CRISPR/Cas-based activation (CRISPRa) system for *D. discoideum* to transcriptionally activate biosynthetic genes. In general, CRISPR/Cas systems consist of two components, a Cas nuclease and programmable RNAs guiding the Cas to specific genomic loci. Here, a nuclease-deficient Cas9 mutant is fused to the highly potent transactivation domain VP16, previously used for inducible Tet^{ON} expression in amoeba. This construct is encoded on the same vector as the target-specific guide RNAs, providing

an easy-to-use all-in-one vector system. As proof of concept, the newly designed CRISPRa system will be used to activate the native promoters of the terpene synthase 5 (*tps5*) and polyketide synthase 5 (*pks5*) in *D. discoideum* wild-type. These genes were selected as their respective secondary metabolites are already disclosed and can be easily detected by mass spectrometry-based analytics. This CRISPRa system would enable a rapid, convenient and simultaneous activation of multiple silent biosynthetic genes in *D. discoideum* to unravel their biological function and thus their potential for biotechnology and pharmaceutical industry.

Metabolic engineering of *Corynebacterium glutamicum* for the production of the low-caloric natural sweetener D-allulose

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Introduction

High-calorie sugars have a bad record in the field of nutrition, since their consumption has been linked with an increased risk of obesity, type 2 diabetes, and cancer. Therefore, low-caloric natural sweeteners that provide a sweet taste without adding much calories gained public and industrial interest, since they represent an attractive alternative for sweetening processed foods and beverages. D-Allulose is a promising candidate, which is currently produced in industry from fructose in fixed-bed reactors at high temperatures with immobilized allulose or tagatose 3-epimerases. This process requires purification and immobilization of large quantities of protein and an elaborate separation of the product from the substrate.

Objectives

Microbial processes for allulose production should be established, which could offer advantages compared to the current process with immobilized enzymes.

Materials and methods

A *C. glutamicum* strain unable to grow with fructose as sole carbon source was constructed by gene deletion via homologous recombination. The genes required for the production of allulose were cloned into the pPREx2 plasmid, in which target gene expression is regulated by the tac promotor via IPTG induction. Transformed strains were cultivated in minimal medium with 2 % (w/v) glucose and 2 % (w/v) fructose in baffled shake flasks. Fructose consumption and allulose formation were detected via HPLC.

Results

For microbial production of allulose from fructose, the industrially established cell factory *C*. *glutamicum* was used as host. To inhibit metabolization of fructose in central metabolism, a fructose-negative strain was constructed. Two pathways were tested for

allulose production: one based on the conversion of fructose to allulose by the allulose 3-epimerase, and a second one based on the conversion of fructose 6-phosphate to allulose 6-phosphate followed by dephosphorylation to allulose. Both pathways led to the production of allulose from fructose. The strain with allulose 3-epimerase enabled 21.5 % conversion within the first 48 h of cultivation. For the strain with allulose 6-phosphate epimerase, a conversion of 13.2 % was obtained.

Conclusion

Two microbial production pathways for allulose were established in *C. glutamicum*, of which the direct epimerization of fructose to allulose is currently more efficient. Future studies aim at further improvement of the strain using the dephosphorylation method for the production of D-allulose.

Engineering new-to-nature biochemical conversions by combining fermentative metabolism with respiratory modules

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Anaerobic microbial fermentations provide high product yields and are a cornerstone of industrially established bio-based processes. However, the need for redox balancing limits the array of fermentable substrate-product combinations. To overcome this limitation, we designed an aerobic fermentative metabolism that allows the introduction of selected respiratory modules. Through these modules, oxygen can be used to re-balance otherwise unbalanced fermentations, hence allowing controlled respiro-fermentative growth. Following this design, we engineered and characterized an obligate fermentative Escherichia coli strain that aerobically ferments glucose to stoichiometric amounts of lactate. We then re-integrated the quinone-dependent glycerol 3-phosphate dehydrogenase and demonstrated glycerol fermentation to lactate while selectively transferring the surplus of electrons to the respiratory chain. To showcase the platform potential of this novel fermentation mode, we replaced

lactate with isobutanol production and demonstrated its growth-coupled synthesis from glycerol. In summary, our design permits the use of oxygen with unprecedented selectivity for the re-balancing of fermentations. This concept is a groundbreaking advance for freeing highly efficient microbial fermentation from the limitations imposed by redox balancing.

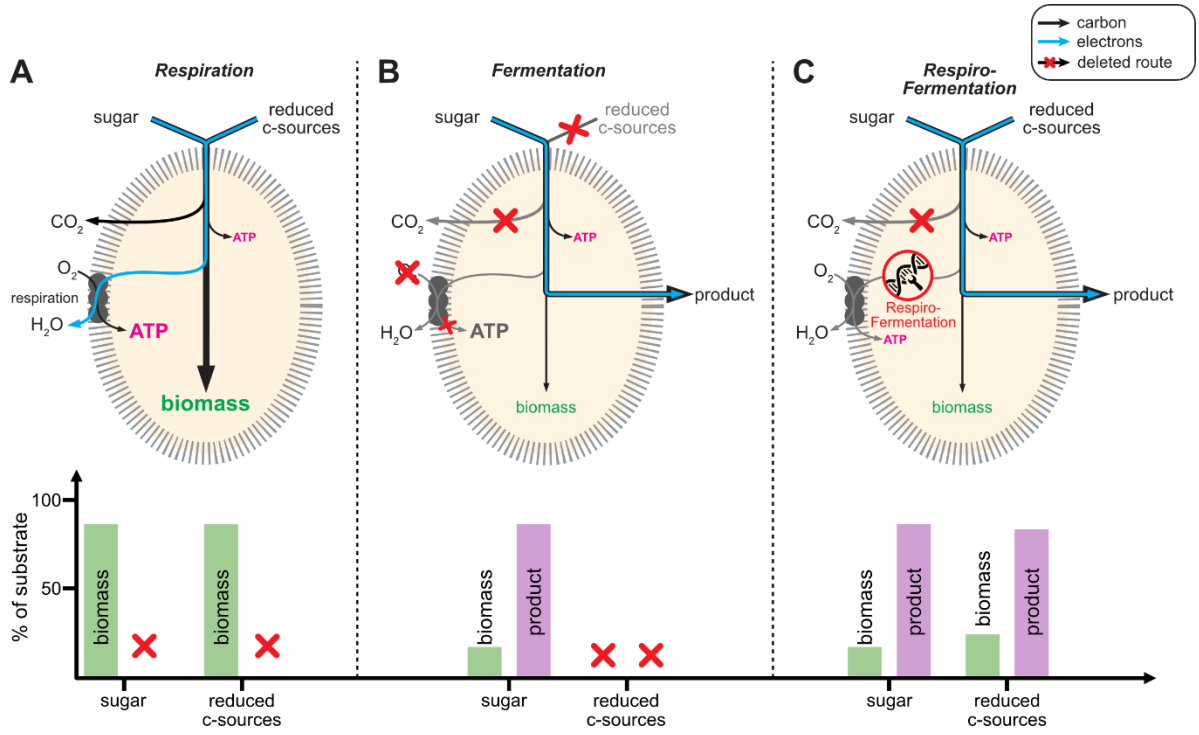

Figure 1 *Comparison of different growth modes.* ***A****: Under aerobic conditions, biomass production (growth) is prioritized. No fermentation products are formed, but a variety of carbon sources can serve as growth substrates.* ***B****: Under anaerobic conditions, biomass production decreases. Instead of biomass, a large portion of the carbon is converted into fermentation products. This is only possible with sugars as a carbon source, as substrate and product must have the same degree of reduction.* ***C****: The novel metabolic mode of controlled Respiro-Fermentation allows the aerobic production of fermentation products from various carbon sources, regardless of their degree of reduction.*

Engineering a pyruvate auxotrophic *Escherichia coli* strain to use growth coupling approaches for enzyme engineering, bioproduction, and metabolite sensing

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Pyruvate, the final product of glycolysis, plays an essential role in central metabolism. It is involved in various metabolic pathways and synthesis routes and furthermore is a byproduct of numerous pathways. Here, we engineer and characterize a pyruvate auxotrophic *E. coli*. This strain possesses 19 genetic modifications in total, including the replacement of the phosphotransferase system with ATP-dependent glucokinase, the replacement of anaplerosis from phosphoenolpyruvate to pyruvate and deletion of all pyruvate generating reactions. The resulting strain lacks any biosynthesis pathway for pyruvate production. Therefore, the strain can only grow in the presence of pyruvate. Strikingly, the strain's design allows to tune its pyruvate requirement, e.g. by supplementing carbon sources entering glycolysis or the TCA cycle. Growth can also be restored by introducing a pathway or enzymatic reaction that produces pyruvate. This strategy allows optimization of enzyme or pathway activity through adaptive laboratory evolution.

The pyruvate auxotrophic strain offers broad application potential (**Figure 1**). First, it can be utilized for growth coupled enzyme selection and evolution. By introducing a pathway or reaction that results in the production of pyruvate, superior candidates from enzyme libraries can be screened effectively. In this work we showcase the use of the pyruvate auxotroph by selecting for the activity of the DAHMS pathway. Second, it serves as a platform strain for the growth coupled bioproduction that ensures the stoichiometric production of a high minimal yield. Here, we demonstrate the feasibility of producing anthranilate, an industrially significant compound. The anthranilate synthase produces pyruvate as a byproduct, making the pyruvate auxotrophic platform strain suitable for overproduction. The third application is to use the strain for pyruvate sensing. Maximum optical density directly correlates with on pyruvate availability. By labeling the strain with a fluorescent protein, we show that

the signal intensity readout can be used to accurately correlate with the pyruvate concentration in the growth medium.

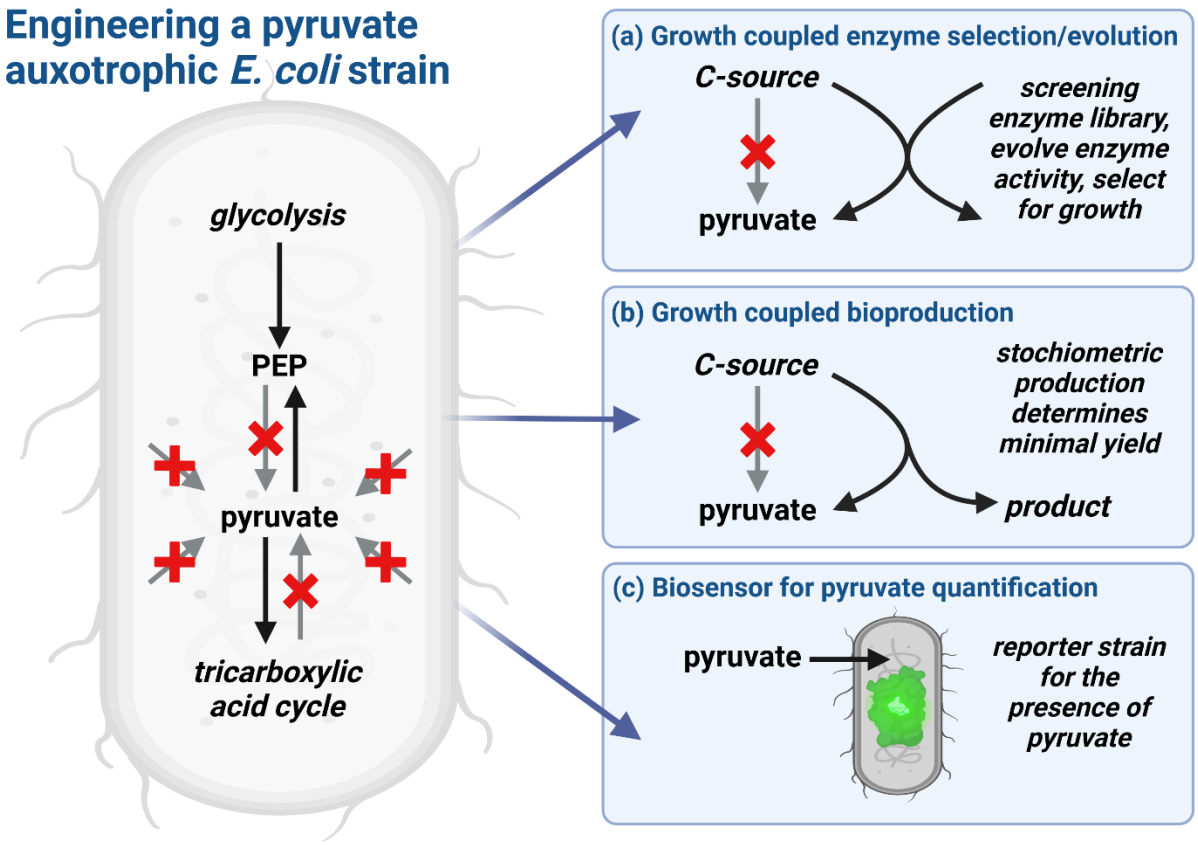

Figure 1: The application potential of the engineered pyruvate auxotrophic *E. coli* can be divided into three categories. (a) The strain serves as platform to screen libraries of enzymes which produce pyruvate as product or byproduct whereas the readout is growth. Moreover, it can be used for growth coupled enzyme optimization through adaptive laboratory evolution. (b) The strain serves as a platform for growth coupled bioproduction. (c) The fluorescently labelled strain can be used as a pyruvate biosensor strain, where growth parameters are dependent on the pyruvate availability. Figure was created with Biorender.

Blue Petal Coloration: Genomic and Transcriptomic Analysis of Flower Color for Pigmentation Engineering

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The engineering of flower coloration through anthocyanin synthesis has long been a topic of interest in horticultural research due to its commercial and aesthetic value. However, the induction of blue flower coloration and its potential applications, particularly in disease detection, remain underexplored.

The SynBio2024 team at TU Braunschweig aims to investigate the genetic induction of blue coloration in plants, using the cornflower (Centaurea cyanus) as a model. Specifically, we focus on transferring the genes responsible for the synthesis of protocyanin, a blue pigment, into the model organism Arabidopsis thaliana. Protocyanin is formed through the metal-ion complexation of cyanidin, an anthocyanin pathway product, and apigenin from flavone biosynthesis.
Our goal is to develop a method for inducing color changes in plants that not only enhance aesthetic appeal but also serve practical purposes. By increasing the presence of the blue metal complex in targeted plants, we aim to engineer a visible biomarker that can indicate various conditions, such as nutrient deficiencies, early signs of plant disease, or environmental stress.

Biotin independence – engineering of a robust *E. coli* strain to produce highly pure streptavidin

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Biotin is an essential co-factor required for carboxylation reactions in all forms of life. In addition to its role in metabolism, biotin is known for its exceptionally tight, non-covalent binding to proteins such as avidin and streptavidin, with dissociation constants in the range of 10^{-14} M. This unique property has led to numerous biotechnological applications of the biotin-(strept-) avidin interaction, in which the high affinity is exploited for the specific capture or labelling of a variety of different target molecules [1].

On the downside, this high affinity binding to biotin makes the biosynthesis of streptavidin difficult and inefficient resulting in high product prices (> 100 €/mg). In particular, the overexpression of streptavidin leads to biotin depletion in the microbial host cell resulting in a growth deficit. Furthermore, the presence of biotin in the cytoplasm hampers the purity of the product due to partial saturation of the binding sites with biotin. In efforts to overcome these limitations, we have previously reported on a metabolic bypass of acetyl-CoA carboxylase which represents the only essential biotin-dependent enzyme in *E. coli,* and which catalyzes the synthesis of malonyl-CoA [2, 3]. To this end, we installed the two heterologous genes, *matB* and *matC*, under the control of a lac promoter, which facilitate the uptake of malonate and its subsequent ligation to CoA. While this solved the toxicity problem associated with streptavidin production, the residual biotin in the cytoplasm still compromised product quality. In addition, the engineered strains exhibited a pronounced sensitivity to the extracellular malonate concentration and only showed growth within a narrow concentration range.

Therefore, we aim to engineer a next-generation of improved biotin-independent *E. coli* strains, which combine robust growth behavior across different growth phases with the possibility to work under entirely biotin-free conditions without the necessity of practically complicated schemes for the feeding of malonate.

For this, we followed two conceptual strategies in knock-out strains lacking native biotin biosynthesis. First, we rewired the regulation of the *matBC* operon to a set of growth phase-dependent promoters, which mitigated the aforementioned toxicity and

improved the robustness against different malonate concentrations thus simplifying production-scale cultivations. Preliminary data also suggest that these strains are well suited for the production of highly pure streptavidin. In the second approach, we aim to directly derive malonate from central metabolism through the implementation of heterologous synthetic pathways. This would circumvent the need to supplement the dicarboxylic acid in the medium and thus further simplify the process and increase practical utility of the strains. To this end, initial data demonstrate principal feasibility of this approach while slow growth rates indicate the need for further optimization of the metabolic flux through synthetic biology techniques.

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Engineering the amoeba *Dictyostelium discoideum* for biosynthesis of a cannabinoid precursor and other polyketides

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Polyketides are structurally and functionally diverse secondary metabolites with a broad spectrum of bioactivities and relevance as therapeutics. Advances in genome sequencing technologies are continuously increasing the number of new polyketide synthase (PKS) genes with an untapped potential to identify novel natural products among all major taxonomic groups. The genomes of social amoebae like *Dictyostelium discoideum* comprise a wide, but largely unexplored, repertoire of mostly silent PKS genes representing a hidden source for novel biosynthetic routes. Among those genes, there are two exceptions that encode for unique hybrid enzymes, having a type I fatty acid synthase (FAS) fused to a plant-like type III PKS. Due to its biosynthetic potential, we aimed to exploit the amoeba *D. discoideum* as a new microbial chassis to produce native polyketides but also pharmaceutically relevant compounds of plant origin.

By expressing its native and cognate plant PKS genes in amoeba, we show *in vivo* production of phlorocaprophenone, methyl-olivetol, resveratrol, and olivetolic acid (OA) as the key intermediate in the cannabinoid biosynthetic pathway. Cannabinoids are known for their psychoactive, calming and pain-relieving properties, and are extensively studied for their use as alternative pain medication. Production of such phytochemicals in model hosts such as *Escherichia coli* and *Saccharomyces*

cerevisiae has been limited by extensive metabolic engineering for a proper supply of required precursors. As *D. discoideum* is a native producer of polyketides and terpenoids, we aimed to realize OA biosynthesis in amoeba without additional accessory pathways. To address this, we engineered an amoeba/plant inter-kingdom hybrid enzyme, where a type III PKS from *Cannabis sativa* occurs as a C-terminal fusion to the amoeba FAS domain. This unique hybrid enzyme enabled OA production exclusively from primary metabolites and provides a shortcut in a synthetic cannabinoid pathway. As alternative to chemical syntheses and laborious extractions from native plant material, an amoeba-based bioprocess may offer an innovative and environmentally friendly approach for cannabinoid production from renewable resources.

Optimization of a synthetic 2,4-dihydroxybutyric acid pathway by engineering enzyme cofactor dependency

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(L)-2,4-dihydroxybutyrate (DHB) is a versatile compound which can serve as a precursor for the chemical production of the methionine analogon HMTB or as a building block for new advanced biopolymers. However, DHB is not a natural metabolite in cells and, thus, no natural metabolic pathway exists for its biosynthesis. Therefore, we and others recently engineered a new-to-nature biosynthetic pathway for the aerobic production of DHB from glucose [1], which proceeds through the characteristic intermediate homoserine. The pathway relies on the deamination of homoserine followed by the reduction of 2-keto-4-hydroxybutyrate (OHB) yielding DHB by an enzyme bearing OHB reductase activity.

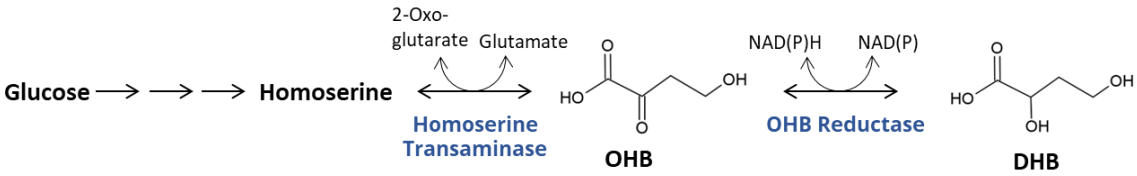

Figure 1 | Synthesis of *(L)*-2,4-dihydroxybutyric acid (DHB) from glucose via homoserine.

The pathway was implemented in *E. coli* and employs a NADH-dependent OHB reductase. However, the typical intracellular ratios of [NADH]/[NAD^+] and [NADPH]/[$NADP^+$] in *E. coli* cells cultivated under aerobic conditions are 0.03 and 30, respectively [2], thus indicating that the use of a NADPH-dependent OHB reductase provides a strong thermodynamic advantage. Therefore, we have engineered a NADPH-dependent OHB reductase using the previously developed NADH-dependent OHB reductase $Ec.Mdh^{5Q}$ [3] (that is, the *E.coli* malate dehydrogenase mutant I12V:R81A:M85Q:D86S:G179D) as template.

Based on multiple sequence alignments supported by the use of the a structure-guided web tool [4], key cofactor discriminating positions have been identified and point mutations were introduced at these sites in $Ec.Mdh^{5Q}$. Following this approach, we identified two mutations which strongly increased the NADPH-dependent OHB-

reductase activity. The resulting Ec.Mdh^{7Q} variant had a 1500-fold higher specificity for NADPH than for NADH compared to the Ec.Mdh^{5Q} reference enzyme. Furthermore, we show that replacing Ec.Mdh^{5Q} by the NADPH-dependent Ec.Mdh^{7Q} variant in an *E. coli* producer strain increases the DHB yield on glucose by 30 %.

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Metabolic burden and resource allocation in *Pseudomonas putida* KT2440 during the expression of synthetic pathways for terpenoid and carotenoid production

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Technical University of Munich, Germany

Incorporating heterologous metabolic pathways into a bacterial host increases the demand for energy and essential cellular resources such as amino acids, ribosomes, and polymerases. This situation demands a shift in the cell's allocation of resources, diverting them from their regular functions to support the new, introduced processes. Often, a scarcity in any of these crucial resources leads to reduced cell growth and cessation of heterologous production, a scenario referred to as metabolic burden.

In this study, we investigate the metabolic burden derived from the integration of a synthetic pathway for the production of a valuable product, such as the carotenoid lycopene (Hernandez-Arranz et al., 2019), the terpenoids perillyl alcohol (Van Beilen et al., 2015), or geranic acid (Mi et al., 2014) on *Pseudomonas putida* KT2440.

To this end, we employed *P. putida* CAP, a derivative of *P. putida* KT2440, to track cellular capacity during the expression of heterologous proteins. This strain carries the mCherry fluorescent protein gene under the control of a constitutive promoter integrated into the chromosome. The mCherry signal serves as a proxy for the cellular capacity by mirroring the allocation of cellular resources to unregulated gene expression, and together with the growth rate, this capacity monitor aids in delineating the metabolic burden. Equipped with this tool, our objective was to meticulously assess how the production of heterologous proteins impacts the cell, with the ultimate goal of applying these insights to the expression of complete metabolic pathways.

We used a plasmid-based approach to introduce the synthesis pathways for geraniol, perillyl alcohol and lycopene, respectively, into *P. putida* CAP. Genes that encode these metabolic pathways were introduced via plasmids. Additionally, further plasmids were constructed to augment the precursor availability for enhanced product formation. To quantify the metabolic burden of the engineered strains induced by the expression of the heterologous pathway, the production rate, yield, growth rate, and capacity approximated by the mCherry production rate is measured in different conditions.

In the strains that harbor the plasmids for the synthetic geraniol pathway, we observed that with increasing size of the plasmid, the strains experienced a higher metabolic burden. This was evidenced by reduced growth rates, diminished mCherry fluorescence, and extended lag phases. As conventional methods such as HPLC and GC failed to provide clear results for geraniol production, an alternative detection strategy had to be developed. We have, therefore, established an enzyme-based test based on GeoA, a geraniol dehydrogenase from *Castellaniella defragrans* to detect the geraniol concentration (Lüddeke et al., 2012). Enzymatic assays are known for their exceptional sensitivity and specificity, which facilitate the detection of even the smallest amounts of target substances.

In summary, the data reflect the extensive metabolic burden caused by the introduction of a synthetic pathway for heterologous production of valuable compounds on *P. putida*.

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Engineering *Escherichia coli* Nissle 1917 for enhanced tartaric acid metabolization

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Tartaric acid (TTA) is found in various plants, most prominently grapes[1,2]. TTA is generally recognized as safe (GRAS) and commonly used as a food additive[3,4]. Upon consumption, TTA is metabolized by the gut microbiome into beneficial compounds like short-chain fatty acids[2]. However, dysbiosis of the gut microbiome or excessive TTA consumption can lead to toxic effects through high body levels of TTA, including metabolic acidosis[5]. To avoid these toxic effects microbes that are capable of degrading TTA within the gut environment gain relevance. The probiotic *Escherichia coli* Nissle 1917 is natively able to metabolize TTA under anaerobic conditions, provided that a co-substrate is supplied for the reduction equivalents[6].

To further investigate the TTA metabolism, we performed adaptive laboratory evolution (ALE) of *E. coli* Nissle 1917 aiming at accelerating anaerobic growth on TTA with glycerol as the co-substrate. Additionally, TTA concentrations were gradually increased, resulting in an ALE strain with an increased growth rate and a higher TTA tolerance than the wild type. Genome resequencing of the ALE strain identified multiple mutations, that potentially lead to the phenotypic changes. Out of these, mutations in transporter and oxidoreductase genes were deemed most promising.

Based on these findings, reverse engineering the most promising mutations found in the ALE strain will be performed to explore the impact on TTA metabolization. Additionally, metabolic engineering will be applied to potentially avoid the need for a co-substrate.

This work marks the first step for the utilization of engineered *E. coli* Nissle 1917 strains to counteract high body levels of TTA and the associated toxic effects.

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Expanding the synthetic biology molecular toolbox by modulation of protein homeostasis

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Synthetic biology has revolutionized the field of metabolic engineering by enabling precise design, construction, and manipulation of biological systems. Central to this paradigm shift is the development of a versatile molecular toolbox. While significant progress has been made in standardizing genetic components, devising modular assembly strategies, and establishing genome editing tools, the application window of these tools remains limited. In particular, the cumbersome adaptation of essential genes presents a major bottleneck for the available molecular tools.

To address this limitation, we showcase a novel methodology that leverages direct modulation of protein levels via the native protein homeostasis network to broaden the scope of the yeast molecular toolbox. To assess the potential of protein homeostasis as an application, we incorporated a protein degradation signal (degron) that was encountered to promote triterpenoid production [1, 2], to enhance the degradation of common reporters including fluorescent proteins and enzymes. In analogy with previous studies characterizing protein degrons, functional read outs ranging between a complete knockout and wild type were observed, underscoring the transferability of our engineered degron, an essential trait for molecular tools. Next, we demonstrate the potential of protein degradation signals to modulate essential genes by fusing the degron to metabolic enzymes involved in early ergosterol biosynthesis. An accumulation of 2,3-oxidosqualene was observed that was successfully steered towards heterologous terpenoids, including ginsenosides [1, 2].

In conclusion, protein homeostasis forms a critical application asset that circumvents gene essentiality, thereby broadening the scope of the ever-expanding synthetic biology toolbox. This approach not only enhances the versatility of molecular tools but also opens new avenues for metabolic engineering and biotechnological applications.

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Development of *Saccharomyces cerevisiae* for potential production of high purity cellulose.

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Biotechnological produced cellulose harbours great advantages for modern industrial solutions towards high precision solutions like synthetic organs and tissues. Especially the purity allows produced bacterial cellulose resistance towards physical and chemical stresses. However, optimizing cellulose producing bacteria for cellulose production remains difficult because they are genetically difficult to modify and hardly suitable for industrial production. Several catalytic subunits of catalytic active cellulose synthases (CesA/ BcsA), producing high purity cellulose have been described recently. Cellulose synthases use UDP−glucose to produce β−1−4 glucan chains, which are extruded. These fibrils spontaneously align to each, called microfibrils, which form a network known as cellulose.

This project aims to express catalytic subunits of cellulose synthases and investigate the yeast *Saccharomyces cerevisiae*, a biotechnologically well established microorganism, for possible cellulose production. For this an UDP−glucose accumulating and glucose sensitive yeast strain was developed, expressing various cellulose synthase subunits. Furthermore, the cellulose synthase expressing yeast cells were cultivated and analysed by an enzymatic digestion assay and physiological analyses.

Our experiments demonstrate a simple and effective analysis workflow for the investigation of additional glucan synthesis in *S. cerevisiae*. The expression of the reported catalytic active subunits of cellulose synthase do not allow glucose sensitive yeast strains to overcome the visible growth deficits (Figure 1). Interestingly the single cell weight determination of the yeast cells allows predicting a possible endoplasmic stress response and excludes additional β−1−4 glucan accumulation (Figure 2). Subcellular localisation studies indicate an intracellular localisation of the heterologous cellulose synthases. Our work demonstrates a clear and precise workflow towards the analysis of possible cellulose synthase activity in *Saccharomyces cerevisiae*.

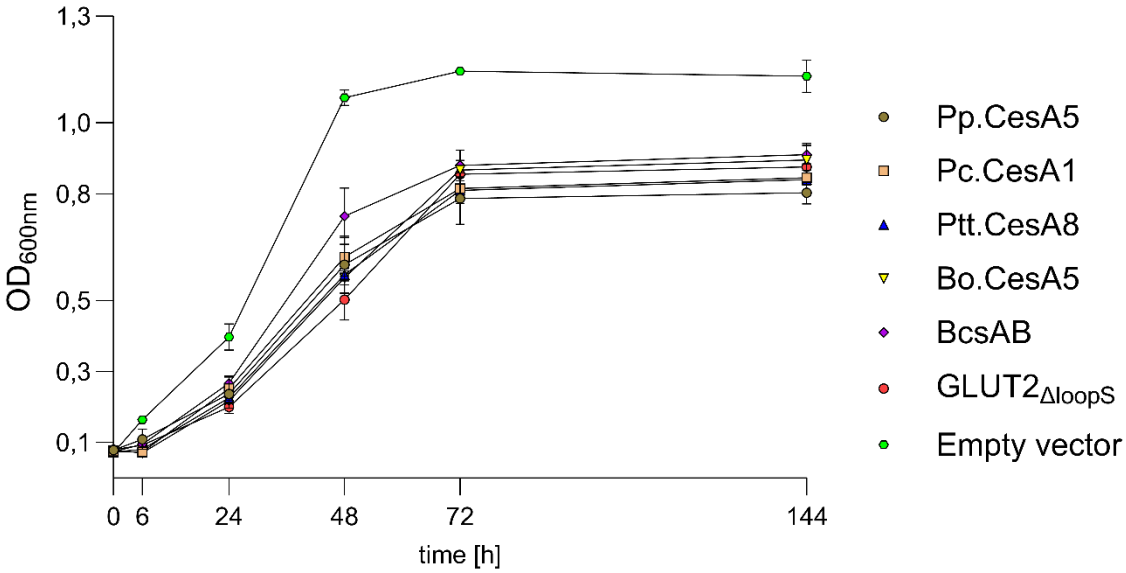

Figure 1: **Detoxification assay of CEN.PK2-1C *pgi1*Δ *fks1*Δ expressing various cellulose synthases.** Yeast cells expressing CesA5 from *Physcomitrella patens*, CesA1 from *Phytophora capsici*, CesA8 from *Populus tremula* × *tremuloides*, Bo.CesA5 from *Bambusa oldhamii*, BcsA & B from *Rhodobacter sphaeroides* and an endoplasmic stress control GLUT2ΔloopS and an empty vector control, were cultivated in SC medium containing 2% fructose and 0.1% glucose.

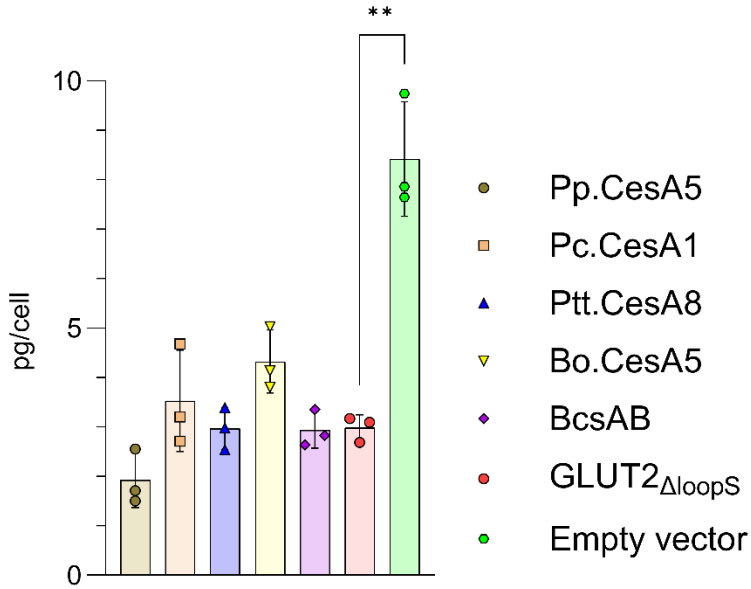

Figure 2: **Dryweight determination of CEN.PK2-1C *pgi1*Δ *fks1*Δ expressing various cellulose synthases.** Yeast cells expressing CesA5 from *Physcomitrella patens*, CesA1 from *Phytophora capsici*, CesA8 from *Populus tremula* × *tremuloides*, Bo.CesA5 from *Bambusa oldhamii*, BcsA & B from *Rhodobacter sphaeroides* and an endoplasmic stress control GLUT2ΔloopS and an empty vector control, were cultivated in SC medium containing 2% fructose and 0.1% glucose for 48 hours.

Upcycling waste polyethylene terephthalate and other plastics into high-value bioproducts using novel synthetic biology tools

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Subtopic: metabolic engineering

Key Words: polyethylene terephthalate; plastic upcycling; carotenoid; lipid; synthetic biology tools

Polyethylene terephthalate (PET), a polyester with a high ratio of aromatic components, is one of the most abundant plastics with its global annual production reaching almost 70 million tons. The accumulation of recalcitrant PET in the environment has led to the current global plastic pollution crisis. Currently, hydrolysis has been commonly utilized to depolymerize PET into monomers terephthalic acid (TPA) and ethylene glycol (EG), and these monomers can be repolymerized into virgin PET for further processing without any value added. To realize the valorization of PET waste, synthetic biology can be applied.

Our previous study has identified a non-pathogenic bacterium *Rhodococcus jostii* PET (RPET) that can directly utilize TPA and EG from alkaline hydrolysis of PET as the sole carbon source [1, 2]. Despite its robust capability to catabolize a wide range of aromatic compounds and accumulate diverse storage compounds, few genetic tools have been identified in *R. jostii*. To overcome this limitation, we have developed and characterized a series of genetic tools to control gene expression, construct genetic circuits, and integrate large heterologous pathways into the genome of RPET. These genetic tools have been utilized to improve the productivity of either lipids or lycopene in RPET using TPA and EG from alkaline hydrolysis of PET as the sole carbon source. Our results demonstrate that those genetic tools can facilitate our efforts to valorize waste PET and other plastics into sustainable biochemicals.

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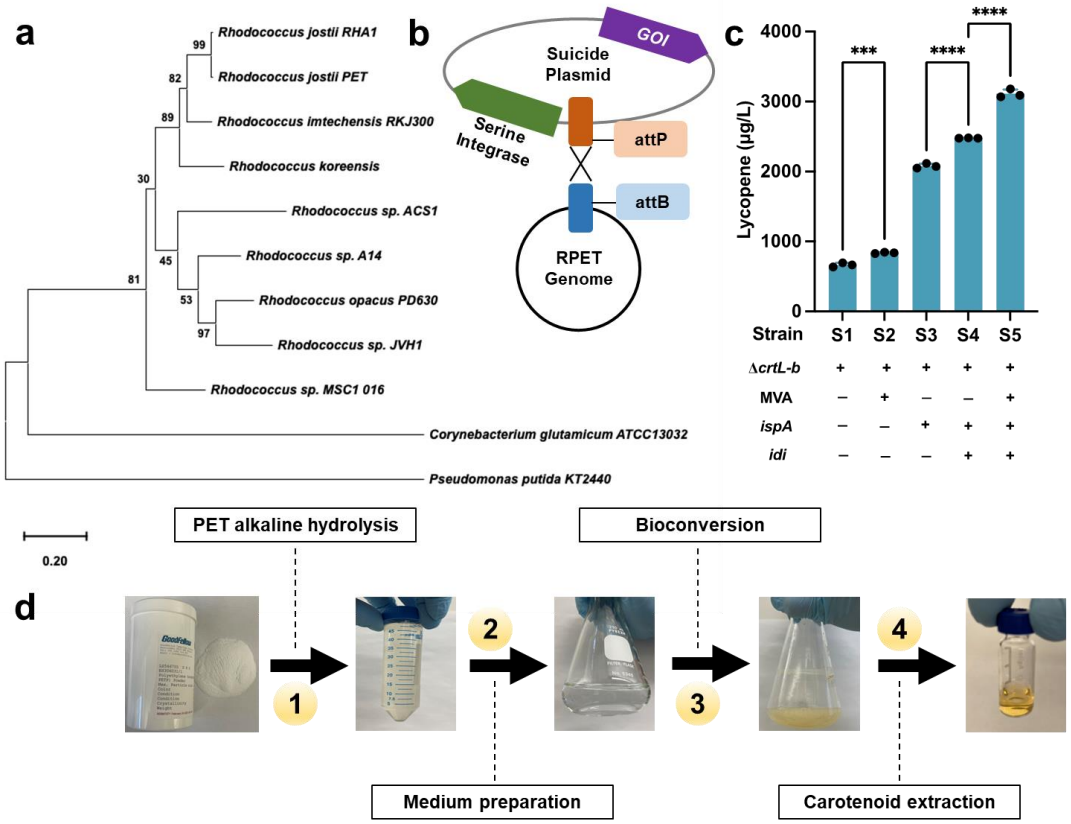

Figure. Waste PET plastic upcycling. **a**. Bioprospecting using synthetic metagenomics and >2,000 harsh-condition-tolerant, engineered strains stored in the Moon lab to discover a plastic-utilizing microbe. **b**. Tool development in RPET. **c**. Metabolic engineering to produce high-valued lycopene from plastic waste. **d**. Developing and optimizing a chemical-biological hybrid process to upcycle real-world plastic waste into carotenoids such as lycopene.

Understanding the evolution of the Geminiviridae family of plant viruses through recombination and mutation: a bioinformatics analysis

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Introduction

Geminiviridae is the family of the largest group of plant viruses, currently consisting of 15 genera and over 500 species. It has single-stranded circular genome DNA that is either monopartite or dipartite. It causes devastating losses to susceptible host crops like beans, tomatoes, and cassava. Mutation and recombination are the driving forces behind the emergence of new species and genera at a very high rate. This poses a challenge in rendering established surveillance, understanding of disease epidemiology, and management strategies ineffective, thus threatening global food security. Previous studies on recombination in Geminiviridae were more centered on the genus Begomovirus, whereas the possibilities of recombination exist in other destructive genera. Hence, this study investigated possible intra- and inter-genera recombination, mutation, and diversity in the entire genera within the family.

Methods

The complete genomes of the virus sequences belonging to each monopartite and DNA-A of the dipartite genus of the family Geminiviridae were retrieved from GeneBank. Downloaded sequences were exported to the sequence demarcation tools (SDT) version 1.3 for classification and partitioning. Similarly, DNA-B of the dipartite was also sampled. The metadata of all the downloaded virus sequences was also retrieved from the Genebank and served as the main data for the geographical map illustration. Explicitly, the species of the virus were collapsed into their respective genera and used with the country where the species were reported to

construct the map. The sampled virus sequences for each genus were aligned with multiple alignments using the Fast Fourier transform (MAFFT) algorithm. The alignment was performed with and without outgroup sequence for the diversity and recombination analyses, respectively.

Results

We found ≥ 95% pairwise similarity in viral sequences within each of the Topocuvirus, Topilevirus, Maldovirus, and Eragrovirus. We also found ≥ 92% pairwise similarity for Capulavirus, Citlodavirus, Curtovirus, Becurtovirus, Grablovirus, Mulcrilevirus, Opunvirus, Turncurtovirus, and Welwivirus. In addition, we found ≥ 80% pairwise similarity in Mastrevirus and Begomovirus. We visualized the evolutionary relationship among the entire genera of the Geminiviridae using a phylogenetic tree, while RDP5 was used to detect possible recombination.

Conclusion

Our results show significant evidence of intra-genus and inter-genera mutation among various genera of the Geminiviridae plant viruses. This indicates evidence of evolutionary means for the creation of new species and genus of such a destructive plant virus family. This understanding informs novel approaches for mitigating the spread and reducing the impact of the Geminiviridae plant viruses. This also informs efforts in strengthening and ensuring global food security.

Process modelling with dynamic flux balance analysis and enzyme cost minimization

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In the past years, the use of resource allocation to improve constraint-based model performance has become widespread. For example, models can be constrained by considering that the total protein in a cell is limited, and thus, the total metabolic enzymes in a cell are limited. This typically takes the form of additional enzyme capacity constraints in flux balance analysis (GECKO, MOMENT, or ecGEM)[1]; that is, setting maximal bounds on metabolic fluxes proportional to the enzyme turnover numbers. The turnover numbers (k_{cat}) can be obtained from databases or estimated via machine-learning models to parametrize even genome-scale models. These enzyme-constrained models improve FBA by, for example, accurately predicting phenomena like overflow metabolism. However, in this approach only the enzyme maximal velocity is considered, with only a very broad correction for enzyme saturation levels across the proteome.

Another optimization approach to metabolic modelling, enzyme cost minimization[2], explicitly includes enzyme saturation and thermodynamics to more accurately estimate enzyme levels for a given flux distribution. As such, it has to be used in a separate step after FBA. Besides turnover numbers, it needs the Michaelis and equilibrium constants of the reactions. Here, we successfully incorporate the enzyme cost minimization algorithm into a flux balance analysis scheme. We use dynamic-FBA simulations based on the static optimization approach to model the anaerobic production of succinate with different *Escherichia coli* mutants. We show that the new scheme leads to improved model accuracy, especially when simulating production after an aerobic-anaerobic shift. While we limit ourselves to a model of the core metabolism of *E. coli*, the continued improvement of machine-learning models for

parameter estimation should also allow this scheme to be used for genome-scale models in further work.

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Optimization of an L-phenylalanine production process using a coarse-grained model to describe overflow metabolism

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One of the biggest limitations to productivity in the cultivation of *Escherichia coli* on an industrial scale is overflow metabolism and the resulting accumulation of acetate in the medium. Not only is the carbon source converted into the mostly unwanted acetate, but the metabolism itself changes, resulting in slower cell growth, for example (Bernal, Castaño-Cerezo and Cánovas, 2016). In recent years, various models have been used to explain overflow metabolism, including coarse-grained models (Basan *et al.*, 2015). These models aggregate individual cellular processes into higher-level groups, leading to a considerable reduction in the number of state variables compared to whole-cell models, while still maintaining sufficient complexity for an adequate description of the system.

The aim of this work is to minimize overflow metabolism in a biotechnological fed-batch process using a coarse-grained model, thereby increasing productivity. Recently, it has already been shown that the dynamic profile of an L-phenylalanine production process can be modeled using a coarse-grained model (Doan *et al.*, 2022). In this model, an external substrate S is taken up and metabolized to a central metabolite M. Starting from this metabolite M, different cell reactions such as protein and residual biomass production, along with respiration, overflow, and product formation, occur. The reaction rates of these cellular processes depend on proteome sectors, which are, in turn, formed with a protein production rate.

In this fed-batch process, the amino acid L-phenylalanine is produced from glycerol using the strain *E. coli* FUS4 ($pF81_{kan}$) (Weiner *et al.*, 2017). The process is divided into three phases, an initial batch phase, a two-stage biomass production phase followed by the product formation phase after induction with IPTG. Due to amino acid auxotrophy, growth can be controlled precisely, resulting in minimal growth during the product formation phase. However, 24 hours after induction, a halt in product formation at approximately 20 g L^{-1} and accumulation of acetate can be observed. Product inhibition can be excluded since a product concentration of approximately 33 g L^{-1} L-phenylalanine has already been reached with glucose (Rüffer *et al.*, 2004).

As a possible cause for the cessation of product formation and transition to overflow metabolism, the approach of Basan *et al.* (2015) will be utilized. This concept relies, in this case, on the idea that cells are forced to transition to a more proteome-efficient energy production (overflow metabolism) because the formation of new non-essential proteins leaves insufficient capacity for endogenous proteins. By incorporating this approach, the aim is to predict the optimal amount of target proteins necessary for product formation without affecting productivity due to overflow metabolism or similar factors. Reducing the inducer concentration is a method to decrease the non-essential proteins in the cells, yet if the inducer concentration is too low, it also leads to decreased productivity due to not enough production proteins.

In the laboratory, a significantly improved yield and reduced formation of by-products have already been observed for the L-phenylalanine process at a lower inducer concentration. In the next step, additional inducer concentrations will be tested. Subsequently, the dependency of product formation on the inducer concentration will be integrated into the coarse-grained model, allowing for a better estimation of the protein fraction for product formation. Then, using the model, a process with the optimal inducer concentration with minimal by-product formation will be predicted and subsequently verified in the laboratory.

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A Modular Approach for Comparison and Evaluation of Metabolic Models with Bioreactor Simulations

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Succinate, a valuable platform chemical, has gained significant interest in the biotechnology industry. Extensive research efforts have focused on improving succinate production in *Escherichia coli* through metabolic engineering strategies. Strategies such as knockout of genes involved in acetate, lactate, and ethanol formation and inactivating the glucose phosphotransferase transport system (PtsG) have shown significant improvements in succinate yields.

Through genome-scale metabolic network reconstructions, the metabolism of *E. coli* has been described in various mathematical metabolic models. In this study, two commonly used models, namely the core model and genome-scale model *i*JO1366, were compared and evaluated using the COBRA toolbox in MATLAB to investigate succinate production behavior. Furthermore, the *i*JO1366 model was curated to explore the feasibility of modular coarse-grained (coarse-grained^{M}) modelling. Additionally, dynamic flux balance analysis (dFBA) algorithms were applied to simulate the succinate production process under various scenarios and achieve process optimization.

Based on the results, the *i*JO1366 genome-scale model was deemed more useful for predicting microbial metabolism and simulating mutant behavior. The exploration of coarse-grained^{M} modeling is still ongoing and requires further in-depth study. Through dFBA simulation, the wild-type strain of *E. coli* showed higher volumetric productivity in fed-batch mode compared to the other two mutant strains studies: pointing to the need for further strain improvement. This computational work has shown promise in optimizing succinate production. However, further validation and comparison with experimental data is necessary to ensure their accuracy and reliability.

Metabolic Modelling of Synthetic CO_2-Fixation Pathways to Improve Carbon Use Efficiency in Plants at Night

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Plant growth in agriculture is constrained by limited carbon availability. This is partly due to nocturnal carbon losses from cellular respiration, which increase under stress conditions. The objective of this work was to use constraint-based metabolic modelling to evaluate whether implementing synthetic CO_2-fixation pathways at night would benefit plant growth by improving carbon use efficiency.

For this purpose, we first created a carbon-limited plant metabolic model with separate but connected day and night phases. We then added reactions representing the previously published crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle[1] in the night phase only. The activity of the cycle and the rest of the plant metabolic network was assessed, in terms of the feasibility of operating the CETCH cycle at night and any additional metabolic engineering efforts that may be required.

While the CETCH cycle was used in the model to refix most nocturnal, respiratory CO_2, this came at a substantial energetic cost. Respiration increased to generate the additional ATP and NADPH required to run the cycle. This resulted in further losses of CO_2, thereby opposing the very purpose of adding this synthetic CO_2-fixation cycle.

Particularly NADPH regeneration at night limited metabolic efficiency. To further validate this finding, we replaced NADPH with NADH consumption in the CETCH cycle reactions. In this scenario, the CETCH cycle refixed 100% of nocturnally produced CO_2 without substantially increasing fluxes throughout energy and carbon metabolism.

In conclusion, the efficiency of a nocturnal CO_2-refixation pathway depends crucially not just on its ATP usage, but also on which redox cofactors are used and how they are regenerated. However, we acknowledge that different cofactor use would be difficult to achieve in enzymes and could detrimentally affect the thermodynamics of the pathway within the cellular environment.

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Analysis of the combination suitability between different dandelion species for rubber yield enhancement by evaluation of NMR metabolite profiles using artificial intelligence methods

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Objectives

The objective of the project is to find a connection between certain properties of dandelion species (e.g., high rubber content) and their metabolite profiles using multivariate and machine learning methods. The calculated models shall help to gain insights into relevant individual metabolites and metabolite networks and, thus, to understand the underlying biochemical mechanism. The metabolite profiles required for the analysis are calculated automatically from one-dimensional 1H NMR spectra of the dandelion plants with the help of a self-written computer program.

Keywords: Dandelion, natural rubber, NMR, machine learning, metabolomics

Significance of the work

Today, natural rubber is mainly produced in Asia, Africa, and Latin America, and it is likely that these sources will no longer be able to meet global demand within this decade. Domestic production of natural rubber with the help of dandelions would not only create an alternative source of supply, while reducing the destruction of climate-protecting and biodiverse ecosystems in other parts of the world, but also lead to the development of significant regional value chains. So far, only molecular approaches have been pursued to identify relevant genetic markers in dandelions as selection tools for rubber content and root morphology. The metabolome has not been considered although it is the level of information that best reflects the actual phenotype.

Methods

The data was acquired as follows. For plant material, leaves and roots were chosen. Optimized sample preparation processes (lyophilization, grinding, etc.) allow

quantitative, fast, and reproduceable one-dimensional 1H NMR measurements, with a 600 MHz Bruker AV NEO NMR spectrometer used. The metabolites are identified and quantified automatically from 1D 1H NMR spectra of dandelion plants using a self-written identification algorithm, non-linear optimization methods and an extensive database. No precise details can be given about the further data analysis, as these studies had just begun at the time of submission.

Results

As part of this project, 142 metabolites have been measured and manually identified in a dandelion matrix. Based on these measurements, 34 metabolites already known in the literature were confirmed. In addition, 21 metabolites not yet detected by NMR were identified. The total of these metabolites almost completely account for the signals in dandelion spectra, while the remaining surveyed metabolites are present in smaller concentrations and thus intensity. Most of the 142 manually identified metabolites can already be automatically identified and quantified with high accuracy and can be used as a basis for subsequent analyses. Initial results of statistical evaluations of the metabolite list are also presented and their significance for a deeper understanding of the underlying biochemical processes is discussed.

Conclusion

To conclude, this project represents a crucial step towards understanding the biochemical mechanisms underlying rubber yield enhancement in dandelion species, facilitated by advanced methods of data analysis. The identification of key metabolites and networks offers promising opportunities for the development of sustainable domestic rubber production. These findings emphasize the importance of continuing research into the use of artificial intelligence for metabolome analysis to pave the way for further advances in biotechnology and sustainable agriculture.

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Evaluating the functional dynamics and interoperability of three programmable lytic systems in *Pseudomonas putida* chassis strains

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In recent years, *Pseudomonas putida* has emerged as a favoured platform for applied synthetic biology endeavours thanks to its physiological robustness and metabolic versatility. However, the presence of a double-membrane cell wall makes it challenging to efficiently secrete certain proteins and products of cellular metabolism (e.g. biopolymers) out of the cell. The implementation of highly functional, programmable "killing"/lytic systems could not only streamline the release of such intracellular bioproducts from cells either in downstream (*1*) or upstream processing (*2*) but also pave the way for strategies that utilise temporal cell growth control, such as in synthetic microbial consortia (*3*).

In this work, we aimed to construct three programmable lytic systems with distinct modes of action – *i)* esterase autotransporter derived from *P. putida* disrupting the integrity of outer cell membrane (EstPbeta), *ii)* novel prophage endolysin with amidase activity, identified by *in silico* analysis in *P. putida* genome (Enlys), and *iii)* colicin E3 (ColE3) inhibiting the proteosynthesis as 16S RNase (**Figure 1**). The performance of these three lytic systems, in the so-called digitalizer module (*4*), was compared and critically evaluated in terms of functionality and efficiency to release a model reporter green fluorescent protein (GFP) in the wild-type *P. putida* KT2440 and its genome-reduced variants – the surface-naked EM371 strain and prophage-free EM42 strain.

We demonstrated that all three lytic systems can lyse all three *P. putida* strains "from within" with various lysis dynamics depending on the system type, the timing of induction and the genomic context of surrogate microbial host. The EstPbeta system showed the best lysis performance in the "naked" EM371 strain, likely due to its weakened outer membrane. In contrast, the Enlys system was the most effective at breaking down the cells of other two strains (KT2440 and EM42), achieving over 3-fold higher release of GFP into the culture medium compared to EstPbeta and ColE3.

Regarding the ColE3, our results suggest that this system, compared to cell wall-targeting molecules, seemed to by the least suitable for programmed cell lysis strategies with potential use in biotechnology.

On the other hand, we nonetheless showed that at least *P. putida* EM42 can inactivate the lethal activity of all tested systems over time. In two out of three analysed mutants, the driving force behind this escape was a mutational alteration of the transcriptional regulator in the inducible expression system.

Our in-depth comparative analysis of selected lytic systems in *P. putida* strains demonstrate the critical importance of considering the bacterial genomic context when selecting an appropriate lytic system. Additionally, our findings highlight the need to develop strategies to enhance the evolutionary stability of genetic components in lytic devices under less favourable conditions.

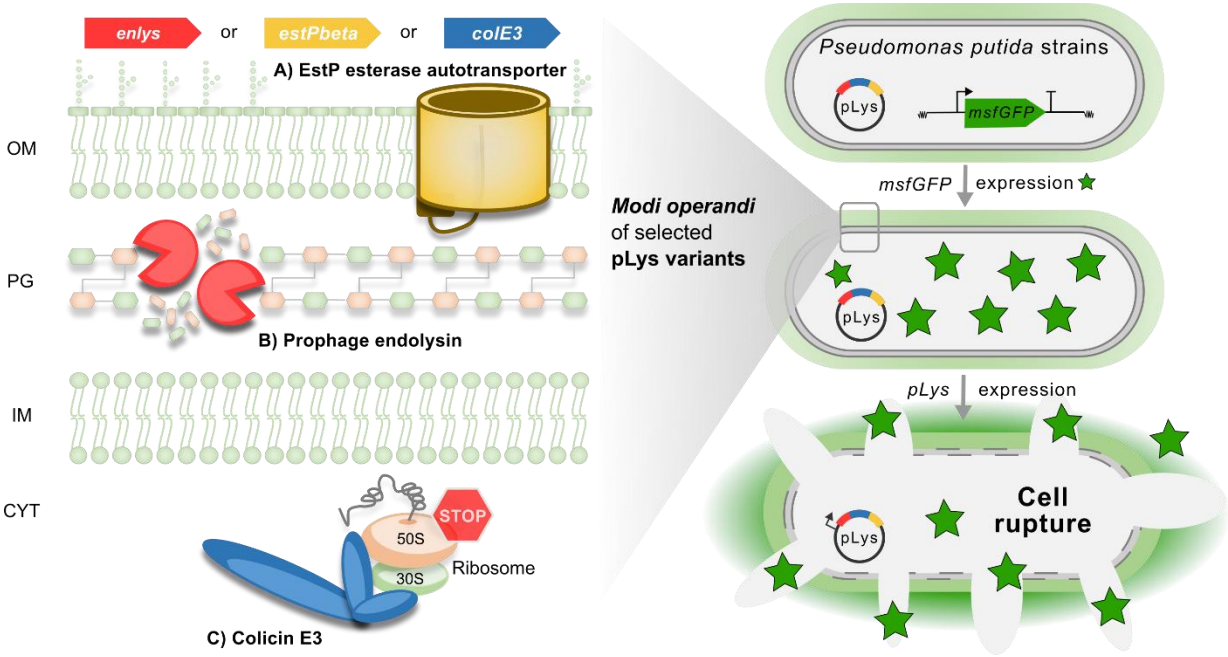

***Figure 1*: Mechanisms of action of the lytic systems used in this work.**

OM – outer membrane, PG – peptidoglycan, IM – inner membrane, CYT – cytoplasm

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Exploring the properties of cohesin-dockerin interactions under application-relevant conditions

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Cohesins (Coh) and dockerins (Doc) (Fig. 1) are binding modules that facilitate the assembly of large protein complexes (cellulosomes) on the surface of certain cellulolytic bacteria. The extremely high affinity of their interaction renders the Coh-Doc pairs highly promising candidates for the construction of synthetic protein complexes, both on the cell surface and in locations remote from it. However, a comprehensive systematic study that explores and enhances the binding efficiency of diverse Coh-Doc pairs in a biotechnologically relevant model system is lacking.

The remarkable strength and stability of Coh-Doc interactions are typically determined for purified proteins through biophysical assays and incubation at elevated temperatures followed by non-denaturing PAGE. These methods are useful for elucidating the differences between Coh-Doc pairs from different species under the specific experimental conditions. However, these conditions do not necessarily reflect those in potential future applications, making the transfer of this knowledge into practice a challenge.

In this project, we aim to describe the Coh-Doc interaction efficiency under biotechnologically relevant conditions – displayed on the surface of a model Gram-negative bacterium of industrial significance, *Pseudomonas putida*. The presented work includes an *in vivo* comparison of wild-type Coh-Doc pairs originating from different bacterial species, which revealed a statistically significant difference in binding efficiency in favor of the *Acetivibrio thermocellus* Coh-Doc pair when compared to Coh-Doc pairs from *Acetivibrio cellulolyticus* and *Acetivibrio clariflavus*.

Moreover, we endeavor to engineer superior Coh-Doc pairs with enhanced stability and/or binding energy. Diverse engineering designs compare some previously published computational simulations and analyses with novel approaches carried out as part of this project. Additionally, we present a comparison of the results obtained from *in silico* modelling and *in vivo* assays.

In conclusion, our work contributes to a better understanding of the Coh-Doc interaction in conditions that are applicable in numerous conceptual synthetic biology studies as well as in prospective industrial applications. In addition, it compares various targeted engineering approaches to obtain superior Coh-Doc pairs.

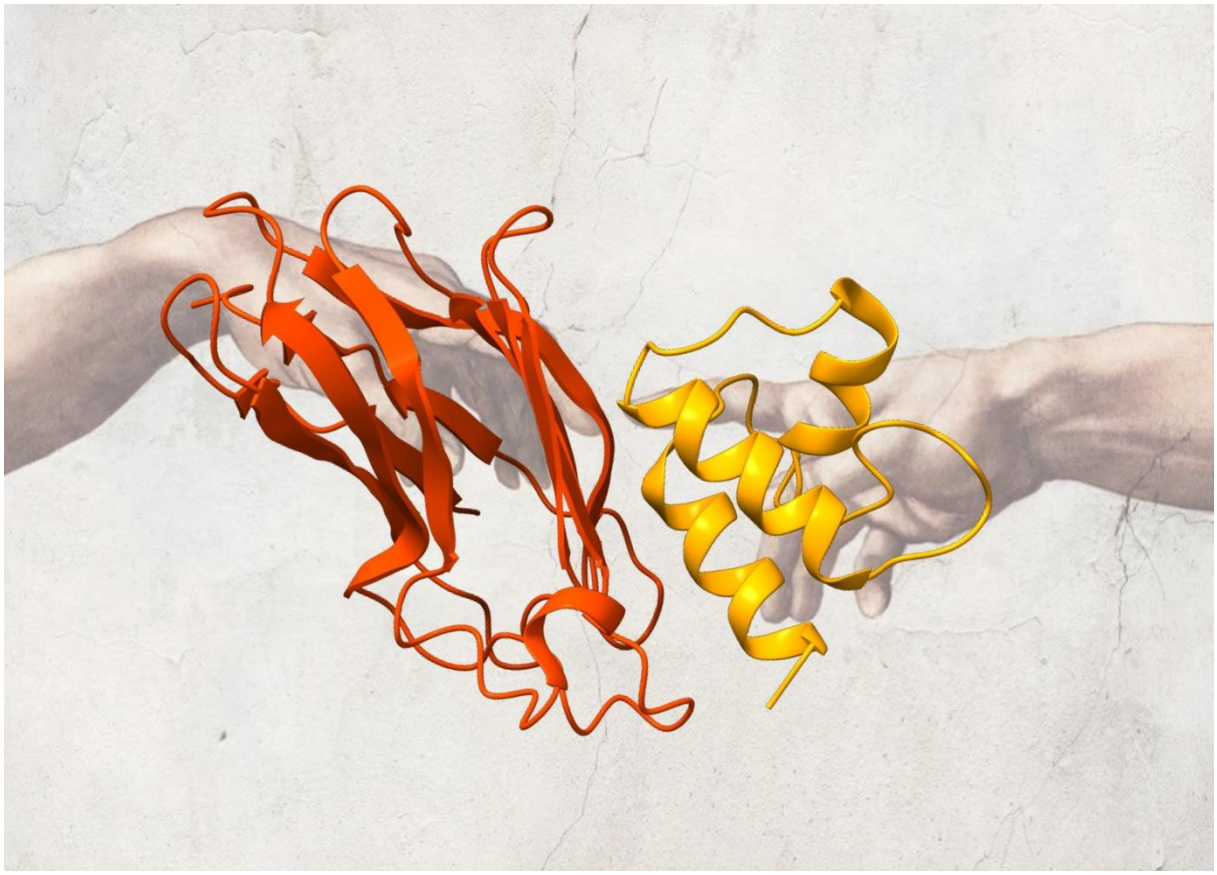

Fig. 1. A dockerin (yellow) attaches to a cohesin (orange) with one of the strongest noncovalent interactions found in nature. These binding modules are promising candidates for construction of synthetic protein complexes that could be used in a wide range of applications, including degradation of recalcitrant polymeric substrates and immobilization of complex enzymatic pathways.

Testing components of the CRISPR/Cas system for genetic manipulation of thermophilic bacteria of the genus *Caldimonas*

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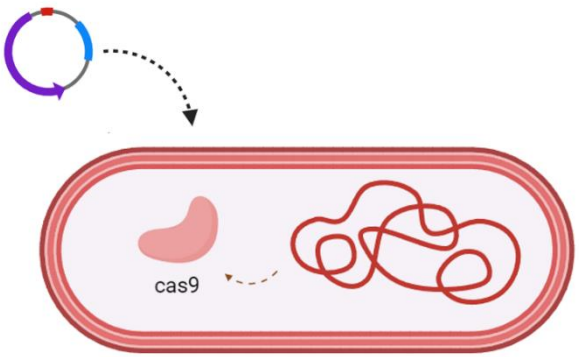
The life of thermophilic bacteria is tightly connected with high temperature. This feature brings many advantages that could be utilized in biotechnological processes, such as low contamination risk, good solubility of the substrate and reduced energy requirements associated with cooling the culture in bioreactors. Moreover, these organisms appear to be promising biofuel and biopolymer producers. [1] However, the lack of thermostable genome editing tools stands in the way of unlocking full biological and industrial potential of these organisms.

Caldimonas thermodepolymerans is a gram-negative producer of biodegradable polymers, specifically polyhydroxyalkanoates (PHA). Its unique capability lies in the highly efficient production of PHA from xylose, the second most common sugar in waste plant biomass. [2] The aim of our research is to expand the genome editing toolbox of this bacterium with a new CRISPR/Cas system to increase PHA yield and to bring it closer to industrial application. The obstacle in genome editing of the thermophilic bacteria is that most of the characterized thermostable Cas9 enzymes are cytotoxic to non-host organisms. [3]



To overcome this problem, we decided to use native Cas9 enzyme present in *Caldimonas.* This approach benefits from the small size of vector used, which contains only guide RNA and template for homologous recombination, and low cytotoxicity risk. [4]

To prove the functionality of the CRISPR system in *Caldimonas*, three guide RNAs were designed to target the *pyrF* gene. Successful editing is being confirmed by looking for auxotrophic mutants resistant to 5-floroorotic acid.

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Design of more thermostable DuraPETase variants by alchemical free energy calculations

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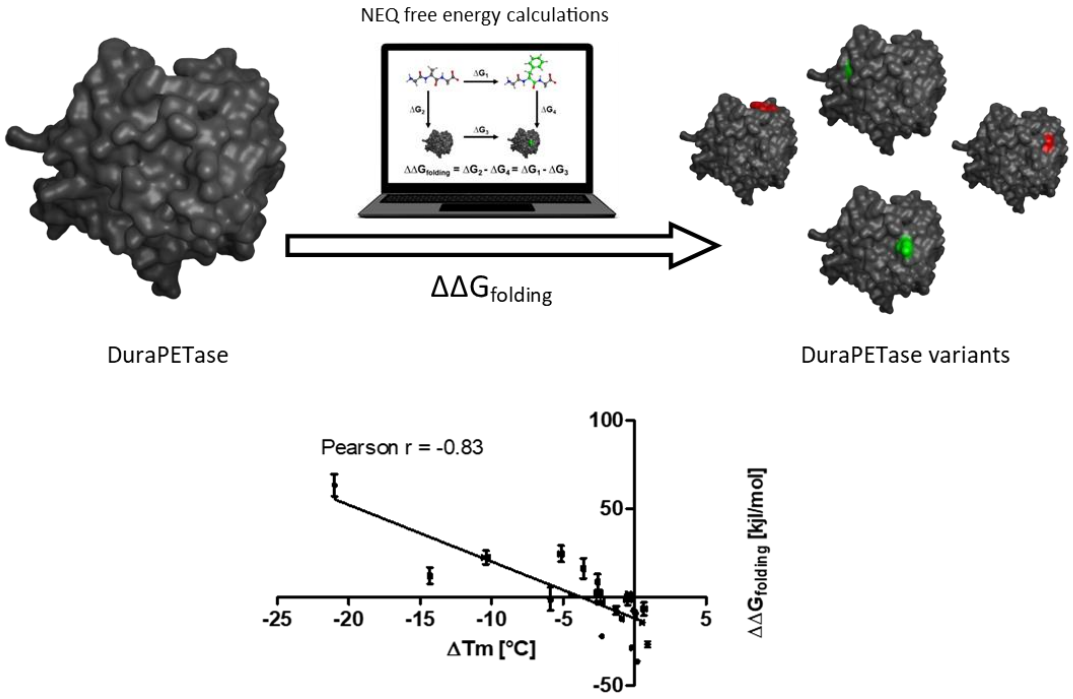
Non-equilibrium (NEQ) alchemical free energy calculations are an emerging tool for accurately predicting changes in protein folding free energy resulting from amino acid mutations. Here, we systematically evaluated the performance of this method in combination with the rosetta *ddg monomer* tool for the prediction of more thermostable variants of the polyethylene terephthalate (PET) degrading enzyme DuraPETase. The relative change in folding free energy of 96 single amino acid mutations was calculated by NEQ alchemical free energy calculation. 23 of these variants were purified and melting temperature (T_m) were determined experimentally. The calculated relative change in folding free energy showed an excellent correlation with these experimentally determined melting temperatures resulting in a Pearson's correlation coefficient of r = - 0.83. We could also show, that the rosetta *ddg monomer* tool efficiently enriched promising mutations prior to more accurate prediction by NEQ alchemical free energy calculations. Among 10 of the highest scoring variants, two mutations (DuraPETase^{S61M} and DuraPETase^{S223Y}) were identified that increased T_m of the enzyme by up to 1 °C. Based on these results, a two-step process is proposed, that leverages the efficiency of the rosetta *ddg monomer* calculations and the high predication accuracy of the NEQ alchemical free energy calculations. Limitations in the prediction of strongly stabilizing mutations were, however, encountered and are discussed. Despite these challenges, this study demonstrates the practical applicability of NEQ alchemical free energy calculations in enzyme engineering projects.



Graphical Abstract

Autodisplay-Toolbox: a plasmid library for simple surface display of recombinant proteins and its optimization.

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**These authors contributed equally to this work.*

The display of proteins at the bacterial cell surface has appealing benefits for the analysis of binding partner, screening applications and whole cell biocatalysis. Here, a plasmid library is presented for the surface display of proteins, to be used by experts and non-experts, including a simple, but powerful option for optimization. Based on a classical (EhaA) autotransporter protein, variants were constructed with four different signal peptides (including adjacent region) and six different linkers. The variants were set under control of a *rhaBAD* promoter, resulting in a library of 24 plasmids named **A**utodisplay-**T**ool**B**ox (ATB). For proof of concept, a β-glucosidase (β-Gluc) as model passenger was subjected to combinatorial permutation using the plasmids of the ATB (pATB) with subsequent pATB library screening. The best combination based on enzymatic activity was pATB_45-β-Gluc with an OprF signal peptide, an adjacent C-terminal spacer (GGGDDNAAPA), and a comparably short linker (ΔepitopeΔβ1). Its activity was 4.9-fold increased compared to the initial construct. The ATB can be extended by two promoters (P_{Rox306}, P_{araBAD}) and by variants of an inverse AT (YeeJ), to optimally control the expression strength and to enable inversed protein display, respectively. Besides using the whole pATB library, individual plasmids can be picked for custom-made surface display.

Enhanced mineral weathering by engineered marine bacteria for CO_2 sequestration

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According to the IPCC report, humans must remove about 1000 Gt of CO_2 from the atmosphere by 2100 to prevent the worst effects of climate change. Enhanced rock weathering technologies are a promising negative emission technology. Natural weathering of silicate minerals results in an increase in ocean alkalinity, which promotes the uptake and storage of CO_2 [Eq. 1].

$$Mg_{1.8}Fe_{0.2}SiO_4 + 4CO_2 + 4H_2O \rightarrow 1.8Mg^{2+} + 0.2Fe^{2+} + 4HCO_3^- + H_4SiO_4 \qquad \text{[Eq. 1]}$$

Natural weathering is slow, however, such that technologies for accelerated dissolution are required. Bacteria have been shown to accelerate the weathering of the widely available mineral olivine using siderophores, which remove iron from the mineral surface. We sought to engineer a marine bacterium, *Alteromonas macleodii*, for enhanced siderophore secretion and mineral dissolution.

The siderophore production in *A. macleodii* is strictly regulated through the ferric uptake regulator (fur) and repressed in the presence of iron. We substituted the native promoter of the putative siderophore synthesis genes with a constitutive promoter. We showed that the overproduction of siderophores results in a higher dissolution rate of olivine (Fig. 1a).

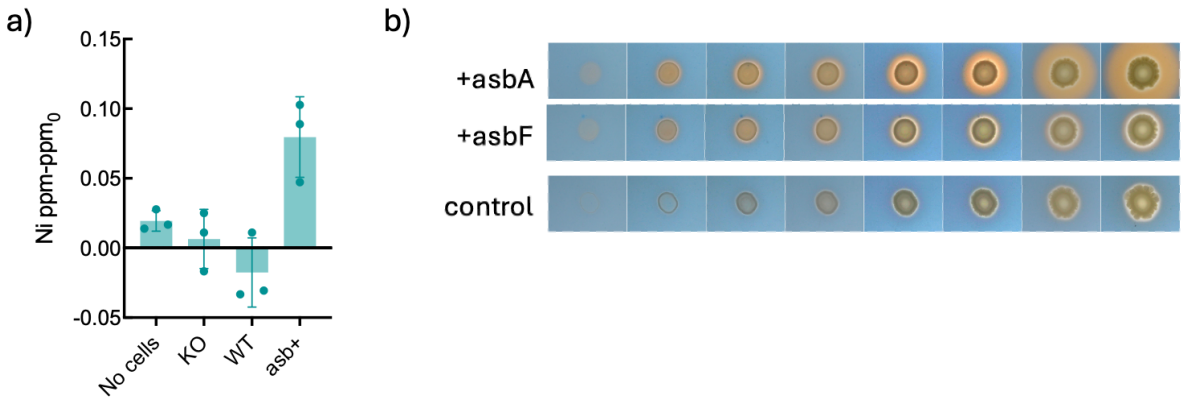

Figure 1: a) Dissolution rates of olivine in the presence of different A. macleodii strains. b) CAS Assay for siderophore production and secretion of different A. macleodii strains.

Furthermore, we have identified the enzyme AsbA (spermidine-citrate ligase) as the key bottleneck in the siderophore production pathway (Fig. 1b). These results will be implemented in the development of new strains. Overall, we envision using the marine model organism *A. macleodii,* engineered for overproduction of siderophores and for the enhanced dissolution of olivine and the alkalinization of ocean water in a bioreactor.

Metabolic pathway for biosynthesis of (L)-2,4-Dihydroxybutyric acid for the sustainable production of the methionine analogon HMTB

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The industrial production of the sulfur-containing amino acid methionine and its analogue 2-hydroxy-4-(methylthio)butyrate (HMTB) is still dominated by petroleum-based synthesis. With its annual production of over 1 million tons and the growing shortage of fossil raw materials, the development of a sustainable production of methionine and HMTB are of significant importance. Due to the high metabolic cost of incorporating sulfur, the fermentative production of methionine results in low product yields and productivities [1]. A promising alternative could be a two-stage process, in which a precursor molecule is produced by fermentation in the first step, followed by the chemical incorporation of sulfur with a nearly 100 % carbon yield [2].

For the production of HMTB, (L)-2,4-dihydrohybutyric acid (DHB) is used as the precursor molecule. For the microbial synthesis of DHB no natural metabolic pathway has been described so far. Therefore, a synthetic metabolic pathway was constructed and implemented into the well-known organism *Escherichia coli*. The presented pathway is built like the synthesis pathway of the natural occurring amino acid homoserine, which is structurally similar to DHB [3].

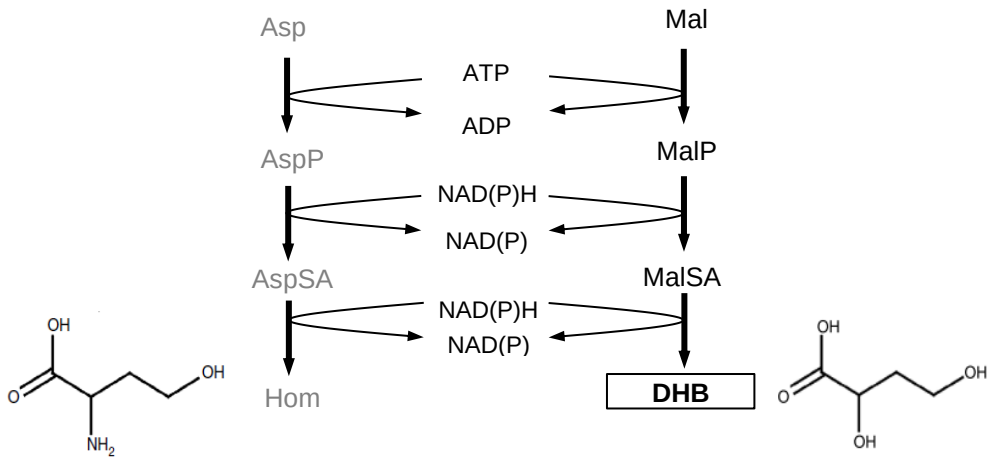

Figure 1 Synthetic pathway for the production of (L)-2,4-dihydrohybutyric, which is constructed in analogy to the natural homoserine synthesis pathway.

For the synthetic pathway, enzymes with malate kinase as well as malate semialdehyde dehydrogenase and malate semialdehyde reductase activity were

introduced into *E. coli*. Because DHB is derived from the Krebs cycle intermediate malate, a malate-insensitive phosphoenolpyruvate carboxylase (Ppc_{K620S}) was over-expressed to increase the carbon flux through the Krebs cycle. Additionally, chassis strains that over-produce malic acid were used as the host strains to optimize the carbon flux distribution [4]. With this approach the DHB yield could be improved from 0.06 mol mol^{-1} to 0.2 mol mol^{-1}.

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Data-driven Engineering and Design of Novel Transcription Factor-based Biosensors for Biotechnological Applications

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In pursuit of a sustainable bio-economy, the use of microorganisms to produce industrially relevant compounds has emerged as a viable alternative to environmentally damaging petrochemical production methods. Consequently, avenues to engineer microorganisms and create highly desired microbial factories have been extensively explored. In that regard, directed evolution approaches based on iterative cycles of diversification and screening are typically undertaken to identify microbial variants with superior production performance. Unfortunately, conventional screening methods impose a major bottleneck on this process due to the limited number of variants that can be simultaneously studied. Transcription Factor Biosensors (TFBs) have a transformative potential to that end, enabling the screening of up to 10^9 variants per campaign. Despite considerable cases of success involving the use of TFBs as a screening tool, the natural repertoire of transcription factors is limited, hindering their wider application in that context. Consequently, there is a strong interest in engineering TFBs with tailored sensory features (fold change, operational range, etc.) towards their native inducer and, beyond that, to new-to-nature inducers, therefore expanding the existing toolkit of TFBs and, concomitantly, enabling directed evolution campaigns for new targets.

In my PhD, I aim to develop and apply methods to rationally design robust and customized TFBs without the need for time-consuming trial-and-error optimization campaigns for each case at hand. To achieve this, I have established a fungible genetic architecture based on DNA recorders to enable the acquisition of large datasets that couple millions of TFB sequence variants with their corresponding quantitative sensory function. The generated data will enable us to better understand the underlying sequence-function relationships that rule over TFBs design. Further, I will exploit these newly acquired datasets to train machine learning models that are able to predict and forward design the sensory function of untested TFB variants in silico. Finally, I will use these TFB design capabilities to develop screening tools *à la carte* for their use in directed evolution campaigns towards enzymatic carbon fixation, as well as in

metabolic pathways for the production of flavor and fragrance molecules such as vanillin.

Catalytic Bio-hybrid Coating-based Degradation of Haloalkanes in the Gas-phase

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Haloalkanes are a group of halogenated hydrocarbons that can be harmful to the environment and human health. Several of these compounds are persistent and can be found in air, water, sediment, and biota globally and are considered as emerging contaminants. While emission of haloalkanes has been severely limited under the Montreal Protocol of 1988, anthropogenic sources are still the main contributor to their occurrence in the environment. Haloalkanes contribute to the depletion of the ozone layer, which has negative impacts on crop production, regional climate, and human health. Exposure to haloalkanes can cause a variety of health issues, including respiratory, dermal, and neurological effects and can contribute the occurrence of cancer.

To address this global threat we have employed engineered, transgene expressing microbial cells embedded in state-of-the-art coating components to generate a bio-hybrid coating able to detoxify an array of these toxic and environmentally harmful chemicals. This work thus represents a meaningful step in the direction of using genetically engineered, bio-hybrid materials to move towards a more sustainable and healthy future for both the environment and its inhabitants.

Efficient Production of n-Butanol from CO_2 via the Synthetic Acetyl-CoA (SACA) Pathway

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The global demand for sustainable alternatives to traditional fossil-based fuels and chemicals has spurred considerable interest in developing novel biotechnological approaches for renewable production. Here, we present our research focused on the production of n-butanol from CO_2 using the recently constructed Synthetic Acetyl-CoA (SACA) pathway.

The production of n-butanol from CO_2 via formaldehyde holds significant promise for various applications. Firstly, n-butanol serves as a valuable industrial chemical with diverse uses, including as a solvent, intermediate in chemical synthesis, and additive in the manufacturing of plastics, coatings, and textiles. Furthermore, n-butanol has garnered attention as a biofuel due to its high energy density and compatibility with existing infrastructure. By harnessing formaldehyde as a precursor for n-butanol production, we not only expand the range of renewable feedstocks but also mitigate reliance on fossil resources, aligning with the global shift towards greener and more sustainable energy and chemical production pathways. Acetyl-CoA, a pivotal metabolite in cellular metabolism, is conventionally derived from complex carbon sources. However, the SACA pathway offers an innovative route to generate acetyl-CoA from formaldehyde, a simple and abundant feedstock. In order to improve the formation of acetyl-CoA, we want to engineer glycolaldehyde synthase (GALS) and acetyl-phosphate synthase (ACPS). For this, we want to use a combined approach with (semi-) rational design and high-throughput methods such as microfluidics. Furthermore, we integrate this pathway with an enzyme cascade aimed at the conversion of acetyl-CoA to n-butanol. This work could contribute to the bioprocess development for renewable production of valuable chemicals and biofuels as well as to the transition towards a more sustainable and environmentally friendly bioeconomy.

The Synthetic Microbiome Platform for The Living Cell Medicine

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Abstract

Modern synthetic biology often focuses on single-cell designs, which can present challenges such as high design complexity, implementation difficulties, and adverse effects on cellular mechanisms. The development of whole-cell bacterial consortium models offers a more robust solution for implementing complex genetic circuits composed of multiple elements. In these adaptations, the tuning and optimization of each individual cell within the consortium are tailored to specific tasks, providing a more reliable, and feasible method with fewer risks of affecting the internal structure and behavior of the cells. In our study, we demonstrate the advantages of using a consortium to address a real-world problem, namely inflammatory bowel disease (IBD). We present a synthetic microbiome model designed for the diagnosis and therapy of IBD. Our system allows some cells to detect inflammatory biomarkers and localize inflammation regions in the gut using quorum sensing, while other cells can locally produce and release anti-inflammatory drugs. This dual functionality shows efficacy not only in vitro but also in DSS-induced murine models that mimic IBD. Our model effectively reduces inflammation and enables the regulated release of therapeutics. We employ various biomarkers whose efficacy has also been demonstrated in vivo in DSS-induced murine models. Our system is highly flexible and can be easily expanded by incorporating new cells with additional functions into the consortium. This work highlights the promising potential of synthetic microbiomes for developing real-world applications in biomedicine and biotechnology.

Cas9-guided ADP-ribosylation enables distinct editing in bacterial and mammalian cells

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[1]Helmholtz Institute for RNA based Infection Research, Würzburg, [2]North Carolina State University, Raleigh, [3]Medical faculty, University of Würzburg.

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The discovery of Cas-nucleases as easy-to-program DNA scissors was a major leap in the field of genome editing. However, creation of unwanted edits through double stranded breaks changed the landscape of editing applications for this protein. Currently, it assumes a dominant role as an RNA-guided DNA targeting entity, enabling expanded functionalities through recruitment of other proteins. Within this realm, proteins that can remove either an amino group (deamination) or the entire nucleobase (glycosylation) are recruited with Cas-nucleases to generate base edits. Given the diversity of DNA modifying proteins, addition of chemical groups to the targeted base is yet, an unexplored avenue. We therefore reasoned that, adding chemical groups to bases could potentially trigger distinct cellular repair pathways, further expanding gene-editing abilities. To investigate our hypothesis, we harnessed the DarT2 protein from an anti-phage defense system. DarT2 naturally adds ADP-ribosyl groups to targeted Thymine residues on single stranded DNA in a sequence specific manner. Fusing an attenuated DarT2 to a nicking Cas9 nuclease enables template-mediated recombination in bacteria, offering efficient, flexible and scar-free edits without counter-selection. Interestingly, the same approach preferentially drives base editing of the targeted thymine in mammalian cells. The edits reveal a biased replacement of Thymine with cytosine and adenine, and outcomes dependent on neighboring sequence. The editor also exhibited minimal indels and by-stander mutations with editing efficiencies dependent on Cas9 targeting and position of the DarT2-specific motif. This approach also enabled Thymine base editing in alternate cell lines, yeast and plants. Our work probes a new avenue in genome editing through programmable addition of chemical groups, enabling distinct repair outcomes across organisms.

One kit fits all: Expansion of the Yeast Toolkit for increased modularity and cross chassis uses

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Centre for Synthetic Biology, Graduate School Life Science Engineering, TU Darmstadt, Germany

Abstract

Synthetic biology has a need for improvements in standardization and automation to achieve the goal of completely forward-engineered designs. Numerous standardized cloning systems for different chassis already exist, but they often lack transferability. This is partly due to different requirements in the control elements of the transcriptional units, as most clearly demonstrated by the differences between eukaryotes and prokaryotes.

In this work, we present an extension of the widely used Yeast Toolkit (YTK) [1], which enables construction of plasmids for both eukaryotes (*S. cerevisiae*, HEK293) and prokaryotes (*E. coli*, *V. natriegens*). To increase the flexibility and usefulness in both prokaryotes and eukaryotes, the promoter part (type 2) was split into three subparts, named 2a, 2b, and 2c (as shown in Figure 1). These are the promoter, the ribozyme insulator, and the RBS/Kozak sequence, respectively. This allows for a higher degree of customizability in the choice of designs, as promoters, insulators, and translation initiation elements can be chosen independently. Furthermore, it is possible to split the promoter further into 2a1 and 2a2 to enable the use of tandem promoters in prokaryotes and promoters and enhancers in eukaryotes.

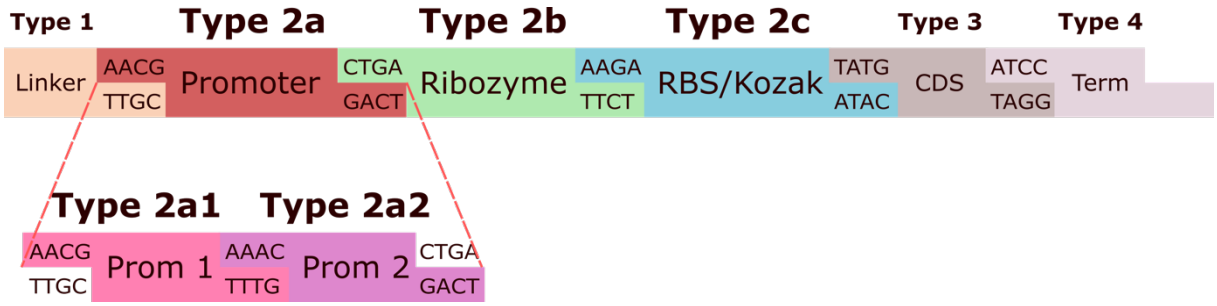

Figure 1: Graphic showing the newly adapted overhangs of the subdivisions of the type 2 parts of the YTK.

To demonstrate the usefulness of this extended cloning toolkit, we are building genetic circuits composed of protein repressors in eukaryotes and protein repressors and regulatory RNAs in prokaryotes. Newly constructed backbones with additional insulators are used across all chassis, thus highlighting the transferability inherent in this system. These constructs are based both on well characterized circuits [2] and on the genetic circuit design tool ARCTIC [3].

Lastly, we present an automated plasmid cloning pipeline. This open-source software will allow the user to easily design the protocol, based on the available parts and the desired output plasmid(s), for an acoustic liquid handler. Overall, this extension and improvements will enable accelerated construction of plasmids for all who use them.

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Engineering RNA-based Feedforward Loops in TXTL

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Background incl. objective

RNA-based circuits offer significant potential due to their rapid response times and lower metabolic costs compared to protein-based systems. One of the strategies to regulate gene expression at the RNA level is using small transcriptional activators (STARs) and toehold switches to construct genetic circuits. Feedforward loops are essential motifs in biological networks that offer dynamic regulation of gene expression. In this study, coherent and incoherent feedforward loops (C-FFL and I-FFL) were based on the STARs and toehold switches. Additionally, RNA sponges (antiSTAR and antiTrigger), which are RNA molecules designed to sequester and inhibit the function of specific RNAs, were utilised. The aim was to implement and characterise synthetic C-FFL and I-FFL in an *E. coli* lysate-based cell-free transcription-translation system (TXTL) to study parameter changes and optimal construct combinations.

Methods

The study measures the fluorescence of sfGFP and the Pepper aptamer to analyse dose-response relationships in C-FFLs and I-FFL in the TXTL system. The concentration of the circuit activating STAR plasmid ranged from 0 to 30 nM, while other circuit components remained constant. Given multiple RNA interactions, we analysed these complexes with Nupack, a thermodynamic model suite. Additionally, we developed a package that generates rule-based models of RNA circuits, enabling rapid prototyping and analysis of complex systems from simulations. We implemented a parallel tempering Markov Chain Monte Carlo algorithm to fit models to experimental data, capturing the behaviour of RNA components in circuits.

Results

This study measures fluorescence signals generated by C-FFL and I-FFL. In the combined C-FFLs example (Figure 1. D), the circuit operates with two STARs. One

STAR is constantly present, regulating the expression of antiSTAR (aSTAR), while the concentration of the other STAR varies from low to high to activate the circuit. The data shows that at low concentrations of the activating STAR, aSTAR effectively acts as a sponge RNA, preventing the initiation of sfGFP production and resulting in low fluorescence. However, at high concentrations of the activating STAR, aSTAR's capacity to sequester STAR is exceeded, leading to high sfGFP expression.

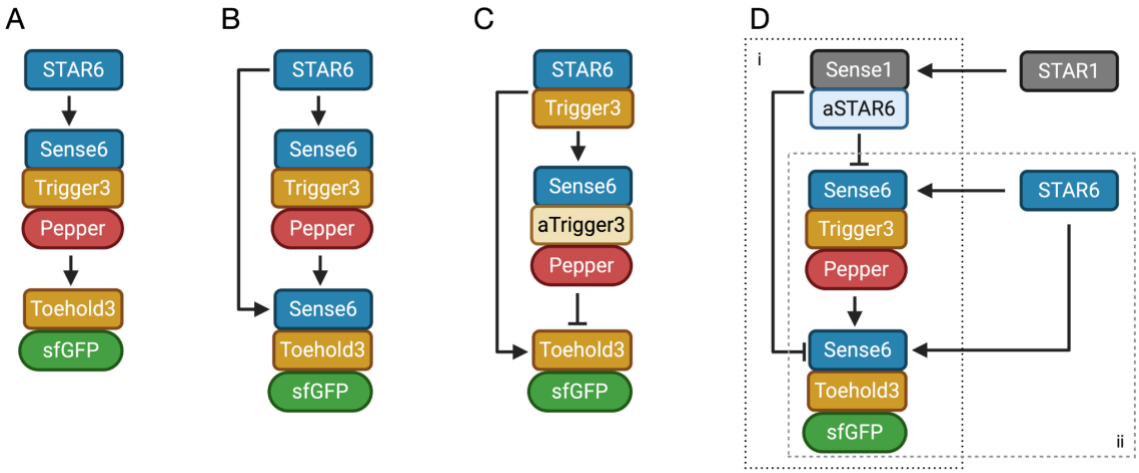

Figure 1. Mechanism of the circuits. A) Scheme of the cascade. B) Scheme of the coherent feedforward loop - type 1. C) Scheme of the incoherent feedforward loop - type 1. D) Scheme of the circuit with a combined coherent feedforward loop of type 1 (ii) and type 2 (i).

Conclusion

Complex RNA-based feedforward loops can be constructed by employing STARs, Toehold switches, and RNA sponges. Combining experimental data with *in silico* simulations offers valuable insights into design principles and effectiveness of these circuits, enhancing our understanding of synthetic circuit design.

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Engineering of single-spanning transmembrane receptors activated with red light

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Single-spanning transmembrane receptors include such proteins as receptor tyrosine kinases (RTKs), toll-like receptors (TLRs) and a number of cytokine receptors, such as EPOR. We developed a generalized approach, which allows engineering single-spanning transmembrane receptors activated with red light, instead of their ligands. In this approach, the ligand-binding extracellular domain of the single-spanning transmembrane receptor is swapped with the light-sensitive bacterial phytochrome of *Deinococcus radiodurance* (DrBphP) and transmembrane helical domain of RTK HER2 serves as a linker, connecting DrBphP and intracellular signalling domains of RTKs, TLRs and EPOR. This strategy allowed us to engineer a set of single-spanning transmembrane receptors (opto-RTKs, opto-TLRs, opto-EPOR), activated with red light (λ=660nm). Systematic optimization of the helical linker, connecting DrBphP and signalling domains of single-spanning transmembrane receptors allowed us to improve performance of opto-RTKs, opto-TLRs and opto-EPOR, so that they strongly activated downstream ERK, NFkB and JNK/STAT-dependent gene expression in mammalian cells in tens of seconds upon action of red light and stayed inactive in darkness. Additionally, we demonstrated that minimally invasive stimulation of the opto-TrkB via skull of mice with red light induces neural activity, and cFos gene expression in neocortex. Such red-light activated RTKs, TLRs and EPOR can be used for non-invasive and orthogonal of cell signalling in mammalian cells and behaving animals.

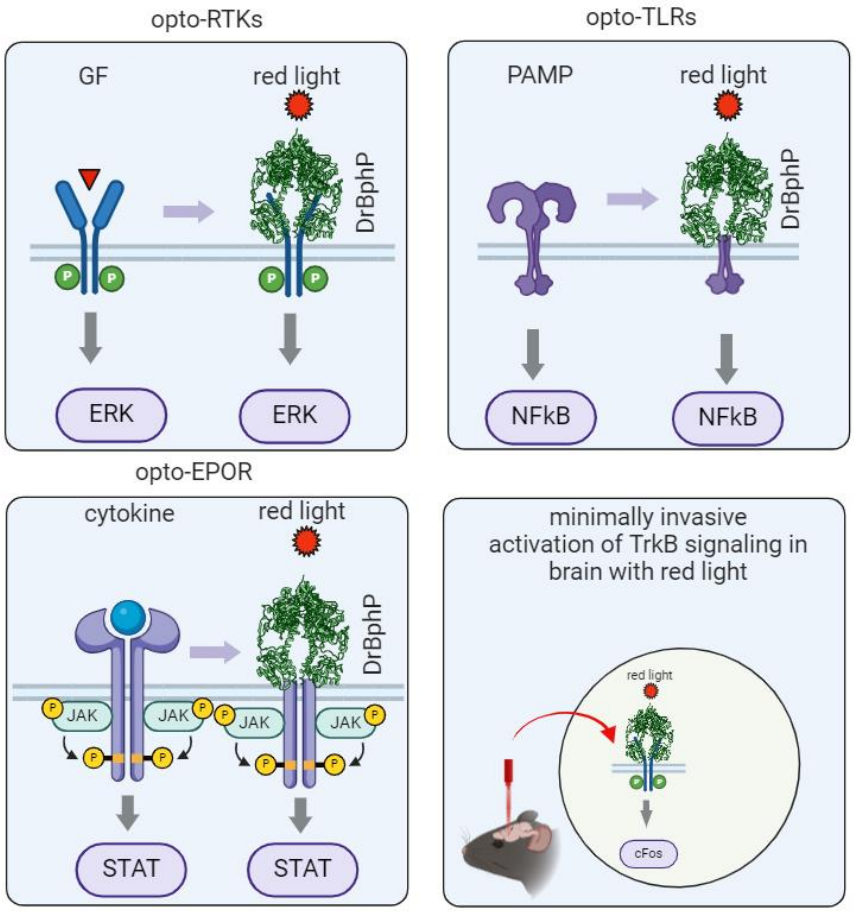

Figure 1. Engineering of single-spanning transmembrane receptors activated with red light. *opto-RTKs, opto-TLRs, opto-EPOR:* Extracellular ligand-binding domains of RTKs and TLRs are swapped to the photosensory core module of *Deinococcus radiodurance* bacterial phytochrome (DrBphP). Illumination of DrBphP with red light leads to conformational changes, which lead to the reorganization and activation of the intracellular signaling domains within the opto-RTKs, opto-TLRs and opto-EPOR. Activated opto-RTKs, opto-TLRs and opto-EPOR activate, correspondingly, ERK, NFkB and JAK/STAT signaling. Minimally invasive activation of TrkB signaling in brain with red light: red-light activated TrkB (opto-TrkB) is expressed in neocortex of mice. Activation of opto-TrkB with red light via the skull activates cFos in freely behaving mice.

Tuning Ultrasensitivity in Logic Gates Using Feedback Mechanisms

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In this work we will investigate and discuss potential feedback mechanisms in order to tune the steepness of the gate's transition region in inverting logic gates. Additionally, those mechanisms could potentially reduce leakiness and be used to adjust the transition location.

One of the approaches is to use antisense RNAs (asRNAs), which are expressed together with the output protein in a single transcript. Upon cutting of the transcript via Csy4, the output is split in a coding sequence, which is stabilized by hairpin structures at the 3'-end, and an asRNA which has the residue of one Csy4 hairpin on the 3' end. The asRNA is complementary to the transcript of the input repressor, inducing a sequestration reaction by blocking and/or degrading the target transcript. This leads to reduced input protein levels if the output promoter is in on-state.

The second approach is similar, with the difference that the sequestration reaction is performed on protein level rather than RNA level. The output protein is expressed together with a coiled-coil peptide (SynZip) [1][2] fused to a degron. This is done by self-cleaving 2A peptides. Those coiled-coil peptides form strong heterodimers, resulting in the peptide to bind to the input repressor, which is fused to another coiled-coil peptide. Therefore, comparable to the first mechanism, expression of the output protein results in downregulation of the input protein. With a strong enough degradation, this reaction is also a sequestration, in which one molecule is "subtracting" the other molecule.

Using these sequestration reactions for feedback within inverting logic gates, we can achieve a very steep transition region, suggested by stochastic and numerical analysis.

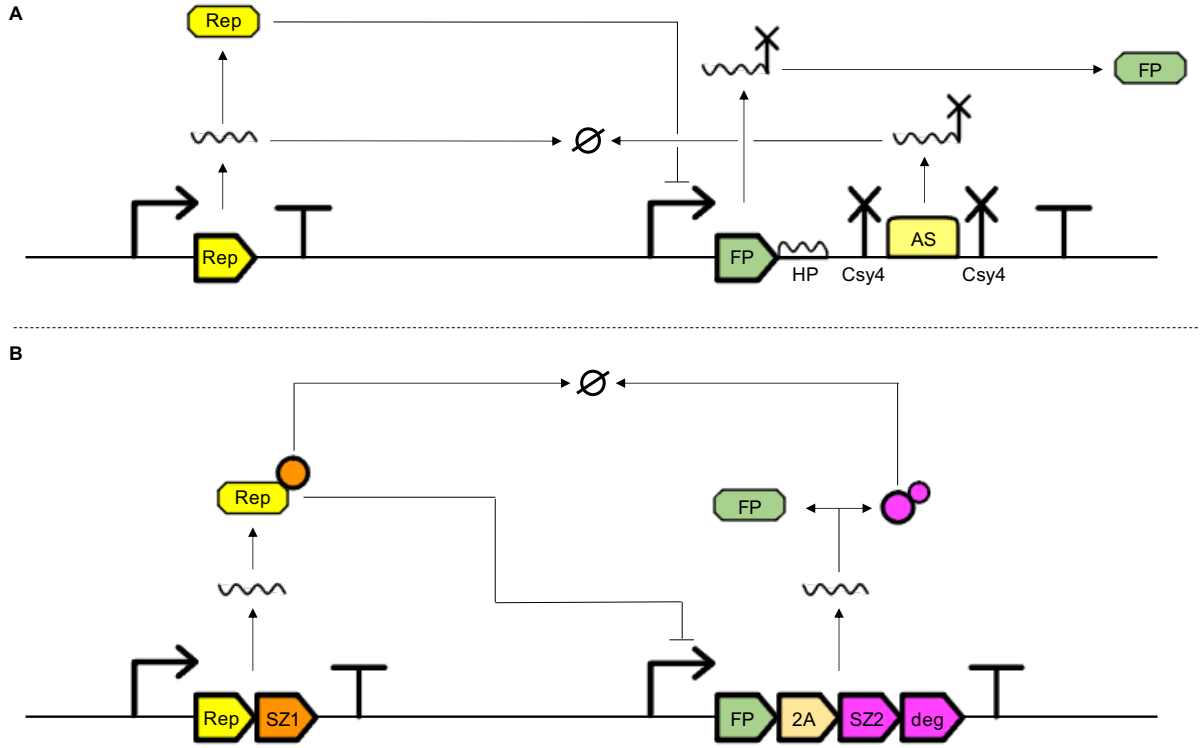

Figure 1: (A) NOT-Gate with feedback mechanism based on antisense-RNA (AS). The repressor (Rep) of the input promoter is inhibiting the output promoter. This promoter is driving the expression of a transcript consisting of a fluorescent protein (FP) and antisense-RNA. The antisense is cut out of the original transcript by Csy4 sites. Therefore, the coding sequence can be translated, while the antisense-RNA is sequestering the RNA of the input repressor. (B) NOT-Gate with feedback mechanism based on heterodimerization. The repressor of the input promoter is fused to a coiled-coil peptide (SynZip [SZ]). The output promoter, which can be repressed by the input repressor, is expressing a fluorescent protein and another SZ fused to a degradation tag (deg). During translation, the self-cleaving 2A peptide in between the FP and the SZ is cleaved, releasing the fluorescent protein, as well as the SZ fused to a degron. Upon binding to the input repressor, the protein-peptide complex is degraded by the host's endogenous degradation machinery.

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Design of Synthetic Riboswitches that modulate mRNA Stability.

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Structured elements in the viral RNA genome play an important role in the regulation of the replication and infection cycle of a virus. An interesting example are subgenomic RNA fragments that modulate the pathogenicity and cytopathicity of flaviviruses like Dengue, Yellow fever, or Zika viruses. In a defense reaction of the host, the single-stranded viral (+) genome is degraded by the 5'-3'-exonuclease XRN1. In the 3'-UTR, highly structured elements block the progression of this exonuclease, resulting in the release of the subgenomic RNA fragments. These XRN1-resistant structures are called xrRNA and are characterized by the formation of a ring-like structure that stalls XRN1, preventing the degradation of the downstream RNA region. This property can be utilized to generate synthetic regulatory RNA elements that modulate the half-life of individual RNA molecules.

Here, we are constructing riboswitches that use xrRNA as an expression platform. The figure below depicts the fundamental pattern for such a design process. An aptamer (red) and expression platform (blue) are positioned with a significant nucleotide overlap between the two elements. In the presence of a ligand, the aptamer conformation is energetically stabilized and incorporates nucleotides that are required for the functional fold of the expression platform (Figure 1, top right). Conversely, a functional xrRNA can only be formed by utilizing nucleotides that are required for formation of the aptamer. This scenario describes an OFF-switch, where the xrRNA is only active when the aptamer structure is destabilized due to the

absence of the ligand. Such a construct requires synthetic RNA with two alternative base pairings, as shown in the left part of Figure 1. Since a candidate sequence must not only provide complementary bases for both a degradation-competent structure (top) and a protective xrRNA (bottom), but also balance thermodynamic and kinetic factors, this proves to be a computational challenge.

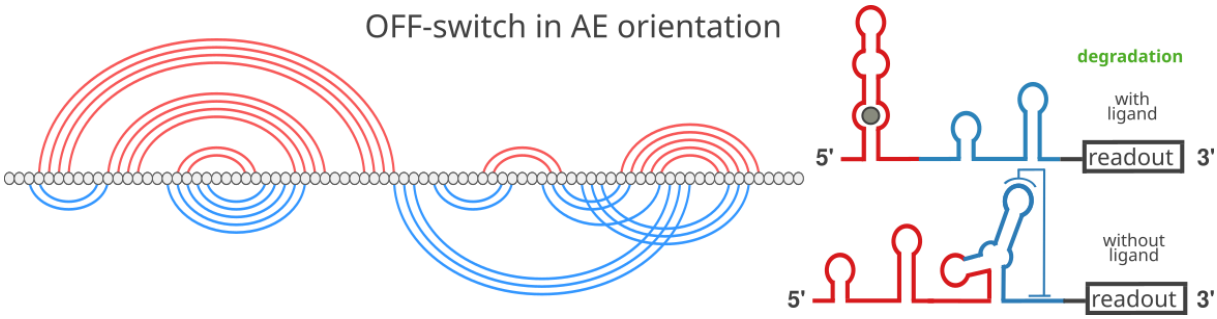

Figure 1: Left: RNA sequence having a possible base pairing with an inactive (top) or active (bottom) xrRNA. Right: Secondary structure of a xrRNA OFF-switch with (top) and without (bottom) a ligand present.

Besides providing a deeper insight into the intricate details of xrRNA structure-function associations, such riboswitches represent a novel tool to regulate RNA stability. Applications range from fine-tuning mRNA vaccines to regulating gene expression in synthetic biology or gene therapy. In our pilot experiments, we could demonstrate that the xrRNA from the mosquito-borne Aroa virus is stable against XRN1 and can be used as an expression platform in a theophylline-dependent riboswitch. Further experiments are currently being conducted to understand the function of individual xrRNA motifs in the switching process of these constructs and the resistance to degradation by XRN1.

Phage-assisted synthetic evolution of cytosine deaminases for genome editing

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Introduction: Base editing is a genome editing technique that allows targeted changes of single DNA bases. Fusions of TALEs (transcription activator-like effectors) and the cytosine deaminase DddA can be used for C-to-T base editing on dsDNA in chromosomes, mitochondria and chloroplasts. The efficiency of the native DddA is strongly restricted to cytosines in a 5'-TC context, but the enzyme has previously been evolved to also recognize 5'-AC and 5'-CC, but not 5'-GC targets. In contrast, SsdA is a cytosine deaminase with broad specificity for the target C context, but it prefers ssDNA over dsDNA. Both, DddA and SsdA are toxins and their activities need to be carefully controlled.

Objectives: We use phage-assisted evolution to develop DddA variants for efficient cytosine base editing in all contexts. In addition, we aim to evolve SsdA as a novel cytosine deaminase for genome editing on single- and double-stranded DNA.

Results: We set up the selection systems for the evolution of DddA to gain higher activity in different target DNA contexts. Coiled-coil tags were successfully established to recruit DddA to the TALE protein at the target site, to evolve DddA within the M13 phage independently of the other components. DddA is evolved via phage-assisted evolution in non-continuous and continuous *E. coli* cultures. Further progress on DddA evolution will be reported. SsdA was tested first as a CRISPR/Cas9 fusion in a plant reporter system and we could thereby establish this enzyme as a novel cytosine deaminase for base editing. Further steps to evolve this enzyme for use in a TALE base editor will be reported.

Conclusions: Phage-assisted evolution is a powerful technique to domesticate enzymes and optimize the activity of genome editing tools.

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