BACK TO THE FUTURE: A BACK AND FORTH MANUFACTURING PROCESS JOURNEY FROM MONOCLONAL ANTIBODIES TO VIRAL VECTORS FOR CELL AND GENE THERAPY

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Key Words: Monoclonal antibody, viral vectors, gene therapy, scalable process platform, continuous bioprocessing

The advent of new gene and cell therapies brings high promises to meet unmet medical needs. But, this also raises questions about how to produce these therapies cost effectively at scale. More specifically, producing enough high quality viral vector is key. Many early production and purification processes relied on techniques that are challenging to scale up, or are not commercially available at larger scales and sometimes even not compliant with cGMP. Scalable production and purification techniques from process development to cGMP compliant commercial manufacturing are therefore required. This feels like travelling back in time when the same challenges arose for the development of monoclonal antibodies. So instead of re-inventing the wheel, can we leverage lessons learnt from this past experience? Considering that processes for both mAbs and viral vectors include similar steps in term of cell culture, harvest, purification and formulation, the technologies developed and optimized for mAb manufacturing should therefore be applicable to viral vector processes. Here we will discuss the process similarities and differences for mAbs on one hand and adeno-associated viruses and lentiviruses on the other hand, focusing on gaps identified in developing process platforms for the production and purification of viral vectors. We will show how even the most recent advances in continuous bioprocessing for mAbs can be implemented quickly for viral vectors and the subsequent benefits generated in term of process productivity and economics.

LIVING BACTERIAL HYDROGELS AS THERAPEUTIC BIOMATERIALS

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Key Words: Living materials, hydrogels, biofilms, curli fibers.

Engineered living materials (ELMs) are growing in interest as they offer the potential to combine the biosynthetic potential of living organisms with the properties of functional materials. ELM research requires the control of a cellular chassis, which can draw resources from the environment and use them to grow macroscopic materials. ELMs are of particular interest to treat and diagnose diseases in the gastrointestinal (GI) tract. In the gut, probiotic bacteria can be used as living factories to produce a living extracellular matrix with tunable mechanical, chemical and biological properties. Specifically, here we report the fabrication of living bacterial hydrogels that can be delivered to the gut and that display mucoadhesive properties. Living bacterial hydrogels are simply produced by concentrating a biofilm-producing bacterial culture on a membrane via vacuum filtration, to collect a viscous product. Extracellular matrix proteins confer the hydrogel its mechanical properties, which can be tuned by genetically modifying the composition and structure of the proteins. In this work, we have engineered self-assembling CsgA proteins, the main subunit of extracellular curli fibers, to tune the adhesive and viscoelastic properties of the gels. We have fused trefoil factors, small mucoadhesive proteins, to the CsgA subunits to produce the engineered therapeutic hydrogels. We have shown that the living hydrogels can adhere to the inner layer of the gut and self-regenerate, both *in vitro* and *in vivo*. Since the curli fibers these hydrogels are highly versatile and can be genetically modified to display a variety of fusion proteins, customizing the properties of the bacterial hydrogels opens up many potential diagnostic and therapeutic applications. Such living gels and biofilms could be used to fill lesions in the GI musoca layer, to track microorganisms in the gut, or to locally deliver small molecules or drugs.

OPTIMIZATION OF E. COLI SOLUPRO™ USING SYNTHETIC BIOLOGY TO GENERATE A HIGH PERFORMANCE CHASSIS MICROBE FOR SCALABLE PRODUCTION OF PROTEIN THERAPEUTICS

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Key Words: E. coli, synthetic biology, antibody, therapeutic

E. coli is a historically important research tool for early phase discovery and development of protein therapeutics. Nevertheless, Chinese Hamster Ovary (CHO) and other mammalian cell lines are the predominant production hosts for current generation antibody and antibody fragment production. Microbial hosts such as *E. coli* are used to produce a minority of approved biologic drugs relative to mammalian cell lines, due in part to perceived limitations in protein solubility and quality related to the complexities of protein folding, maturation, host specific post-translational modifications, as well as regulatory considerations. However, recent advances in our understanding of microbial biology and synthetic biology have enabled rapid progress to be made in the development of microbial cell lines that exceed the performance of best-in-class mammalian cell lines. AbSci has developed a functional reconstruction of the protein production environment of the eukaryotic endoplasmic reticulum in *E. coli*, which includes a semi-oxidized cytoplasm that facilitates appropriate protein folding and disulfide bond formation. Within the physiological context of *E. coli* SoluPro™, a best-in-class synthetic biology strategy that modulates rates of gene expression, protein expression, and protein folding using a plasmid-based design architecture have been validated as a strategy to produce soluble, high titer and quality protein biologics. Following construction of millions of plasmid variants using a pooled DNA construction library approach, plasmids are screened *in vivo* for improvements in protein titer and quality using a fluorescence activated cell sorting (FACS)-mediated antigen binding assay. Next generating sequencing (NGS) is used to identify genotypes enriched within populations of cells with enhanced antigen-binding properties. Secondary assays are performed to validate strain improvements identified by flow cytometry, including an orthogonal screening of antibody-mediated antigen-binding in cell lysates, as well as advanced Mass Spectrometry methods to quantify disulfide bond formation and other protein quality attributes. This strategy has enabled rapid identification of plasmid designs for soluble production of full-length antibodies and antibody fragments that can be scaled to multigram quantities of product in bioreactor fermentations of 48 hours or less. Additional optimization of the *E. coli* SoluPro™ chassis is being tailored to further improve folding and maturation of additional classes of complex therapeutic proteins. The ease of use of *E. coli* and technical robustness of our high-throughput discovery and optimization workflow enables AbSci to rapidly identify key conditions for heterologous protein production and identify protein folding solutions conditions that can exceed Gram level quantities of soluble protein with less than three months of strain optimization effort.

DEVELOPING TOOLBOXES TO ACHIEVE RAPID OPTIMIZATION OF LATE STAGE CELL CULTURE PROCESSES

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THE SEPARATION OF RED BLOOD CELLS BASED SOLELY ON INTRINSIC MAGNETIZATION: CLINICAL AND COMMERCIAL IMPLICATIONS

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Key words: Label free separation, magnetic separation, red blood cells

A rough estimate puts the cell isolation market at approximately $ 6 billion a year worldwide. One of the key commercial technologies uses antibodies conjugated to magnetic micro and nanoparticles (i.e Dynal beads or Miltenyi MACS systems). While clearly effective, whenever antibodies are used, whether conjugated to magnetic particles, or fluorescent molecules (such as used in FACS systems), there is always the issue of the sensitivity and specificity of the antibody for the targeted cell(s). This “issue”, amongst others, is the motivator for “label free” identification and separation technology.

Removal of human red blood cells, hRBCs, from a blood or bone marrow sample for diagnostic, or therapeutic applications is a fundamental laboratory practice/procedure. While difficult to obtain precise numbers, it has been suggested that greater than a billion blood draws are conducted in the US each year. While for a majority of these blood draws an evaluation of the RBCs is an important part, there is still a very large number of tests that focus on the remaining blood components after the RBCs have been removed. While not nearly as common as a blood draw, more than 18,000 bone marrow or umbilical cord blood transplants were performed in the US in 2013. In the case of bone marrow transplants, the RBCs need to be removed prior to transfusion or cryopreservation, regardless of whether the donor and patient’s tissues match.

Viewed from a mechanistic perspective, there are three primary methodologies to remove human RBCs, hRBCs, from a blood draw: 1) RBC lysis, 2) immunological based separation in which a RBC is bound with an affinity ligand which facilitates RBC removal, or 3) separation of the RBC from the nucleated cells based on density differences. The two most commonly used methods are the density difference methods with or without hydrophilic polysaccharide addition (e.g Ficoll density gradient based centrifugation, DGC,). When blood samples are only used for further analysis, the condition and the content of the sample after the RBC removal is only important with respect to how it affects the subsequent analysis; however, when the RBC depleted sample is destined for transfusion into a patient, significantly higher standards are required.

We previously compared RBC removal using the Ficoll-based DGC to lysis protocols. Using either method would remove more than 99% of RBCs; however the average recovery of the spiked cancer cells was 73 and 89% for the Ficoll and RBC lysis, respectively. Poor recovery of targeted cells, such as hematopoietic stem cells, in the initial RBC depletion step is a problem in the bone marrow transplant/regenerative medicine community. In fact, several reports indicate that the recovery of nucleated cells from bone marrow, BMNCs, using Ficoll-based DGC, can be as low as 15-30%. Complementary to these reports, two recent papers suggest that cells with high regenerative potential, such as very small embryonic-like stem cells, VSELs and mesenchymal stromal cells are depleted with DGC. Finally, there are suggestions that Ficoll DGC can impair receptor function of the recovered cells.

It is well established that deoxygenated RBCs are weakly paramagnetic; initially reported by Linus Pauling and coworkers in 1936. Melville and co-workers demonstrated in the mid 1970’s that RBCs can be captured using a ferromagnetic wire mesh when the cells are reduced (chemical turned into a state equivalent to the deoxy-state). More recently, we have demonstrated that RBCs can be captured in HGMS systems (i.e. Miltenyi Biotec MACS columns), magnetically deposited on slides, deposited on the wall of a channel, and continuous removed using a flow through separation system. While these studies demonstrate theoretically, and experimentally, that it is possible to separate RBCs based on intrinsic magnetization, the throughputs in these studies are orders of magnitude lower than needed to practically remove RBCs from a typical blood draw.
In this presentation we will present our latest systems which we suggest can increase the throughputs by orders of magnitude which presents the potential for magnetic separation of RBCs to become a practical alternative to the currently used approaches.

ENCAPSULATION BIOPROCESSES FOR DIABETES CELLULAR THERAPY

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Key Words: Cellular therapy, diabetes, 3D culture, hydrogel, vascularization

The number of cell and gene therapy products approved in North America now exceeds 15, creating a need for bioprocesses tailored to this type of biologics. The Stem Cell Bioprocessing Laboratory at McGill, led by Dr. Hoesli, Canada Research Chair in Cellular Therapy Bioprocess Engineering, is developing new materials and methods to produce and deliver promising cell-based products – in particular for the treatment of diabetes and cardiovascular disease. Type 1 diabetes is an autoimmune disease leading to the loss of the insulin-producing beta cells of the pancreas. Islet transplantation aims to replace these insulin producing cells rather than replacing the missing insulin hormone. Over 85% of islet transplant recipients can live without insulin injections for at least 1 year [1], with some patients remaining insulin-independent for over 5 years. Access to this promising long-term treatment to diabetes is limited by donor islet supply as well as the risks associated with lifelong immunosuppression following this intervention. We and others are developing scalable bioprocesses to generate pancreatic cells from pluripotent stem cells, to purify the cells of interest, and to transplant them while circumventing the need for immunosuppression. This presentation will review upstream processing, downstream processing and formulation/delivery methods related to this challenging cell-based product. Examples of novel bioprocesses for pluripotent stem cell differentiation and pancreatic cell encapsulation in immunoprotective devices will be presented [2-4]. Avenues for future research and development include engineering devices that maximize cell oxygenation and survival while also allowing graft retrieval or replacement. One of these approaches is the engineering of vascularized tissue constructs using 3D printing [5]. As the cell therapy field continues to evolve and mature, complex combination products including therapeutic cells, biomaterials as well as drugs are expected to emerge on the market.

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DEVELOPMENT OF PHOSPHO-TAU SPECIFIC ANTIBODIES: VALIDATION AND ENGINEERING OF SPECIFICITY

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Key Words: post-translational modification, Tau protein, neurodegeneration, protein phosphorylation, phospho-specific, affinity, specificity, antibody

Neurodegenerative disease is a widespread and growing burden with no disease-modifying treatment option. As we gain more knowledge on the source of neurotoxicity, monoclonal antibody-based drugs are actively being pursued as a treatment for neurodegeneration. Moreover, antibodies for detecting trace amounts of biomarker proteins in the cerebrospinal fluid and blood have shown promise in early detection and monitoring of neurodegeneration. However, as evidenced by recent failures in clinical trials of amyloid β targeting antibodies, development of a translatable drug for neurodegeneration is extremely challenging. A major hurdle to overcome is the validation and improvement of specificity towards targets that play a causal role in neurotoxicity. In this regard, the microtubule-associated protein tau and its disease-specific post-translational modification (PTM) sites have emerged as a promising target. Disease-specific PTMs appear prior to and drive the formation of tau oligomers, which have been identified as a key source of toxicity. A major clinical trial is underway with a tau monoclonal antibody targeting phosphorylated tau. However, efforts to validate the specificity of these antibodies has been scarce. An alarming and widespread lack of specificity in PTM-targeting antibodies has only recently been noticed. More importantly, we have lacked the capability to improve antibody specificity even when the source of non-specific binding has been uncovered. This presentation will address our recent efforts to meet this urgent need by applying protein engineering approaches to identify high specificity antibodies targeting disease-specific PTM sites in the tau protein. Starting from a structure-based understanding of specificity in PTM-specific antibodies, I will describe our recent work on developing a quantitative parameter for measuring antibody specificity, *in vitro* directed evolution strategies to identify high specificity antibody variants, and new antibody library screening strategies to discover PTM-specific antibodies. We find that antibody screening approaches that focus solely on affinity improvements likely result in antibody clones that have cross-reactivity towards the non-phosphorylated target. Multi-parameter screens that can measure specificity and affinity were essential in obtaining high quality antibodies that greatly improved phospho-tau detection in human patient samples [1]. These antibodies are invaluable tools for early detection of neurodegeneration, and highlight the importance of specificity validation and engineering in PTM-targeting antibodies.

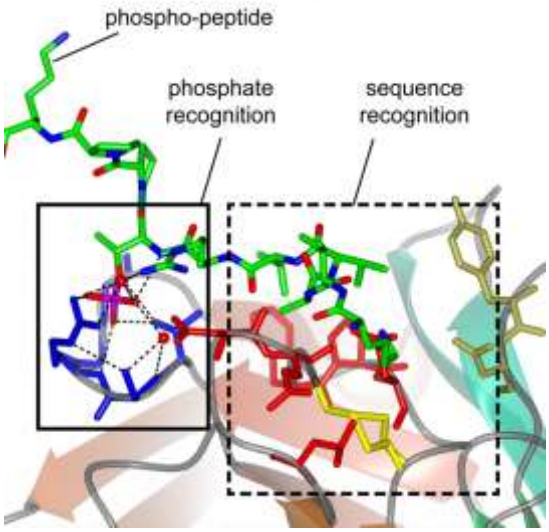

Figure 1. Phospho-specific antibody binding. The interaction is mediated primarily by recognition of the phosphate group (solid box) and the target amino acid sequence (dotted box). Based on Protein Data Bank structure 4GLR.

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LESSONS FROM THE IRON RING

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ENGINEERING NEXT GENERATION THERAPEUTICS TO COMBAT INFECTIOUS DISEASES

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Key Words: antibody, T cell receptor, cytomegalovirus, pertussis, protein engineering

Of the 56 therapeutic monoclonal antibody products currently marketed in the US, four now target infectious disease indications. An additional 40 recombinant antibodies are in clinical trials for infectious indications, with 29 in phase II or III trials. The evolution of antibiotic-resistant bacteria, the emergence of new pathogens, and a growing population of immunocompromised individuals means that in many cases antibodies are an increasingly attractive therapeutic option. Next-generation antibody formats, including antibody-drug conjugates and single-domain antibodies as well as antibody mixtures and bispecific antibodies provide access to novel therapeutic mechanisms and allow for targeting a wider range of epitopes. This talk will provide an overview of recent advances in the field and highlight two on-going projects in my lab. First, to address a resurgence in pertussis in high resource countries and continued high rates of morbidity and mortality in low resource countries, we have developed and antibody therapeutic neutralizing the toxin primarily responsible for symptoms. This antibody has been engineered for high affinity binding, reduced immunogenicity and extended serum half-life. We have also characterized its mechanism of action, using biochemical, structural and cellular assays. We have shown hu1B7 is protective against disease in mouse and adolescent baboon models of disease. Moreover, a single dose can prevent disease symptoms in a neonatal baboon model when administered five weeks before experimental challenge. Second, to address issues with recurrent cytomegalovirus infection in immuno-suppressed individuals, we envision a bispecific antibody able to redirect any passing T cells toward CMV suppression. In our first iteration of this therapeutic, we aim to target infected cells via a T cell receptor (TCR) binding the immuno-dominant peptide-HLA complex. Since TCRs are typically low affinity and express poorly as soluble molecules, we have used a novel eukaryotic-based cell display system that allowed us to rapidly identify variants with higher affinity and enhanced stability. We have generated TCR variants with up to 100-fold improved affinity that retain exquisite peptide selectivity. We will report our initial efforts to use this modified TCR to target CMV-infected cells.

DESIGNING AN ARTIFICIAL GOLGI REACTOR FOR CELL-FREE GLYCOSYLATION

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Key Words: Golgi reactor, cell-free glycosylation, glycosyltransferases immobilization

Glycosylation of therapeutically relevant proteins such as monoclonal antibodies (mAbs), is critical as it can offer increased drug efficiency, efficacy and half-life. Therefore, the production of modern biotherapeutics focuses on controlling the protein glycosylation profile using various methods. Currently, the dominating method is the traditional cell-line engineering of host cells such as mammalian cells. The main goal is to produce mAbs with a human-like glycosylation pattern. However, this approach often struggles due to high sensitivity to the fermentation environment making it difficult to scale up and control. The latter can lead to structural heterogeneity amongst the products which can be immunogenic. In addition to the in vivo methods, there are many in vitro techniques such as chemoselective or enzymatic glycosylation. However, they are often limited by the difficult implementation and, as before, product heterogeneity due to lack of control over the enzymatic reactions.

In line with the need to control glycosylation in the production of therapeutic proteins, we propose an artificial Golgi reactor for in vitro glycosylation. By expressing selected glycosyltransferases and immobilizing them on solid supports we can achieve sequential enzymatic reactions required for protein glycosylation. The spatial separation will allow strict control over the reaction conditions while addressing enzyme promiscuity. Both should enhance product quality. Furthermore, we aim to perform a single-step glycosyltransferases purification/immobilization. Thanks to that, as well as the modularity of our design, the proposed system would be more sustainable and easily tailored for each application, thus producing any desired glycoform to homogeneity. A detailed mathematical approach to design and optimisation of the proposed artificial Golgi reactor focusing on mAb therapeutics has been published [1]. The authors report an optimisation of the reactor design and operational parameters that directs the whole process towards the desired glycan structure.
In this research project, we have achieved expression and in vivo biotinylation of Nicotiana Tabacum GnTI (NtGnTI) and human GalT in E. coli. The biotinylated enzymes were successfully bound to streptavidin beads and used for artificial glycan synthesis. NtGnTI and GalT reacted in a sequential fashion to produce the glycan GalGlcNAcMan5GlcNAc2, as confirmed with MALDI/TOF MS analysis. In the future, we aim in extending the pathway of immobilized enzymes thus demonstrating the importance of this novel platform for in vitro glycosylation.

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AUTOMATED HIGH-THROUGHPUT AND MINIATURISED SEMI-CONTINUOUS CHROMATOGRAPHY

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Key Words: Microscale, Automation, High-Throughput, Continuous Chromatography

The major process limitations of current antibody purification processes are posed by affinity chromatography, although purification platforms based on affinity chromatography are very effective. Typically, protein A-based chromatography can account for more than 70% of downstream processing costs due to resin throughput, cost and complexity of scale up. Thus, there has been increased focus by the industry on developing and implementing continuous chromatography technology to increase resin capacity, reduce buffer consumption and increase productivity of packed bed steps.

At UCB we have publicly presented a novel semi-continuous operation that can be operated on an unmodified chromatography skid named SCRAM (Sequential Chromatography Recycling with Asynchronous Multiplexing), which replicates the functionality and capacity gain of traditional continuous systems without the complexity.

However, increasingly new innovative antibody formats have resulted in significant process platform adaptations to be performed prior to manufacture, and therefore the screening of many conditions to find a suitable window of operation may not be economically feasible at laboratory scale due to the amount of feedstream and resources required for each experiment. To overcome this issue, techniques that can generate data with minimal resource expenditure can be invaluable in early bioprocess development. Automated microscale platforms offer a change in bioprocess development by accelerating process development due to the flexibility for parallel experimentation and automation while requiring microscale quantities of material.

In an industry first, we will demonstrate the application of SCRAM using 600 uL microscale columns on an automated robotic platform performed in parallel to explore large experimental design spaces with minimal resource expenditure. This has resulted in critical bioprocess information to be obtained earlier in development providing a better opportunity to understand process parameters and robustness understanding of this application. Therefore, this approach can be a viable and valuable alternative route for identifying sweet spots during screening studies in bioprocess development.

Within the sector, automated high-throughput and miniaturised chromatographic process development relying on microscale columns is widespread, however, we believe this to be the first report of successful miniaturization of semi-continuous chromatography using microscale columns.

DEVELOPING THE CALCIUM-DEPENDENT CONFORMATIONAL BEHAVIOR OF THE RTX PEPTIDE DOMAIN FOR NOVEL PROTEIN CAPTURE AND RECOVERY APPLICATIONS

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Key Words: Beta Roll Domain, Non-chromatographic Protein Purification, Affinity Purification, Protein Hydrogels

The β-roll domain is a unique, conformationally dynamic peptide secondary structure motif[1]. This peptide is expressed from the repeats-in-toxin (RTX) domains found in some secreted pathogenic proteins. The peptide is intrinsically disordered in the absence of calcium. In calcium rich environments, the peptide binds Ca++ ions and folds into a β-roll secondary structure that resembles a flattened corkscrew. It is composed of two parallel β-sheet faces with a conserved aspartic acid at each turn that is responsible for the Ca++ binding. We have extensively characterized this calcium-responsive RTX domain and evaluated its potential as a new bioseparations platform in both non-chromatographic and affinity chromatography applications, as well as a novel component for the development of advanced protein hydrogels.

We have developed a synthetic peptide, based on RTX domains, which undergoes calcium-responsive, reversible precipitation. This synthetic tag was appended to green fluorescent protein, β-lactamase and alcohol dehydrogenase. After protease cleavage of the precipitating tag, pure and active target proteins were obtained by cycling precipitation steps before and after cleavage. This work demonstrates a new stimulus-responsive precipitating tag that can be used for efficient bioseparations using gentler conditions than existing alternatives, enabling purification of recombinant proteins from microbial lysate in only a few minutes[1].

More recently, we have also shown that this β-roll peptide domain can serve as a new scaffold for engineering controllable biomolecular recognition. Mutant peptide libraries were selected against lysozyme via ribosome display and we were able to identify peptides with mid-nanomolar (63μM) dissociation constants. We showed that the mutant RTX peptides are capable of capturing the lysozyme target in affinity chromatography experiments in the presence of calcium and the bound target is easily eluted upon removal of the calcium ions, in a Catch and Release mechanism[2].

We have previously mutated the amino acids on the faces of the β-roll peptide domain to hydrophobic leucine side chains to enable calcium-induced self-assembly. This provides cross-links that enables calcium-dependent protein hydrogel formation[4,5]. We have introduced our lysozyme-binding β-roll peptide into this platform so that the designed proteins are a viscous liquid in the absence of calcium. Upon calcium addition, a robust hydrogel is formed that specifically binds the target protein, lysozyme[6]. Thus the engineering of the peptide domains has led to new biotechnology applications, where environmentally responsive protein hydrogels are capable of selective and reversible protein capture and immobilization.

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CRISPR-GUIDED DNA POLYMERASE ENABLING DIVERSIFICATION OF ALL NUCLEOTIDES IN A TUNABLE WINDOW

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Key Words: *in vivo* diversifier, directed evolution

The capacity to diversify genetic codes advances our understanding and engineering of biological systems. A method to continuously diversify user-defined regions of a genome without requiring the integration of nucleic acid libraries would enable forward genetic approaches in systems not amenable to high efficiency homology-directed integration, rapid evolution of biotechnologically useful activity through accelerated and parallelized rounds of mutagenesis and selection, and cell lineage tracking. Here we developed EvolvR, the first system that can continuously diversify all nucleotides within a tunable window length at user-defined loci. Our results demonstrate that EvolvR enables multiplexed and continuous diversification of user-defined genomic loci that will be useful for a broad range of basic and biotechnological applications.

SMALL-MOLECULE BIOSENSORS FOR HIGH-THROUGHPUT METABOLIC ENGINEERING

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Key Words: Biosensors, evolution, small-molecule, yeast, metabolic engineering

Allosteric transcription factors (aTFs) have proven widely applicable for biotechnology and synthetic biology as ligand-specific biosensors enabling real-time monitoring, selection and regulation of cellular metabolism. However, both the biosensor specificity and the correlation between ligand concentration and biosensor output signal, also known as the transfer function, often needs to be optimized before meeting application needs. In this presentation we outline a versatile and high-throughput method to evolve and functionalize prokaryotic aTF ligand specificity and transfer functions in a eukaryote chassis, namely baker's yeast *Saccharomyces cerevisiae*. From a single round of directed evolution of the aTF ligand-binding domain coupled with various toggled selection regimes, we robustly select aTF variants evolved for change in ligand specificity, increased dynamic output range, shifts in operational range, and a complete inversion of function from activation to repression. Importantly, by targeting only the ligand-binding domain, the evolved biosensors display DNA-binding affinities similar to parental aTFs and are functional when ported back into a non-native prokaryote chassis. The developed platform technology thus leverages aTF evolvability for the development of new biosensors with user-defined small-molecule specificities and transfer functions. Finally, the presentation will highlight examples on biosensor applications for high-throughput metabolic engineering.

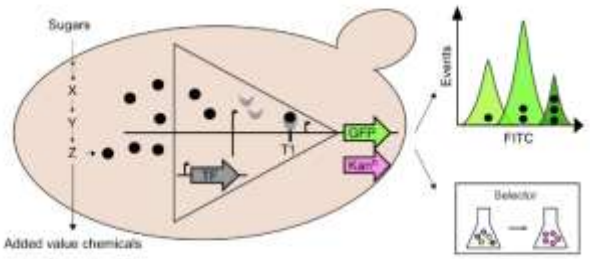

Figure 1 – Schematic outline of a small-molecule biosensor applicable for high-throughput screening and selection of optimal metabolic engineering towards microbial production of added value chemicals

HIGH-THROUGHPUT ENZYME ENGINEERING FOR COMMERCIAL-SCALE PRODUCTION OF NATURAL PRODUCTS

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Key Words: high-throughput, automation, enzyme engineering, bioinformatics, pipeline

The natural products world is unparalleled in its molecular diversity and wide application space. There are however numerous challenges associated with realizing the full potential of these molecules. Amyris has fermentatively produced eight different molecules at commercial scale. This track record is due to investment in advanced tools for strain engineering, high throughput screening, analytics, and bioinformatics. An integrated pipeline encompassing these tools has enabled Amyris to rapidly accelerate the engineering cycle and reduce the number of design-build-test iterations needed for microbial production of any natural product. In this presentation, we will discuss how this infrastructure is now being leveraged for high-throughput enzyme screening and mutagenesis, enabling greater access to natural products and their derivatives. Further, the application of our massive screening infrastructure to enzyme libraries would not be possible without equally sophisticated statistical models and data analysis tools. Scientists at Amyris are accessing ever greater portions of the enzyme sequence space to improve specificity and activity – ultimately enabling sustainable industrial-scale production of natural products. This talk will describe how each aspect of the enzyme engineering pipeline has led to rapid and high-quality screening of hundreds of thousands of mutants for multiple enzymes.

ENGINEERING *CORYNEBACTERIUM GLUTAMICUM* TO PRODUCE THE BIOGASOLINE ISOPENTENOL FROM PLANT BIOMASS HYDROLYSATES.

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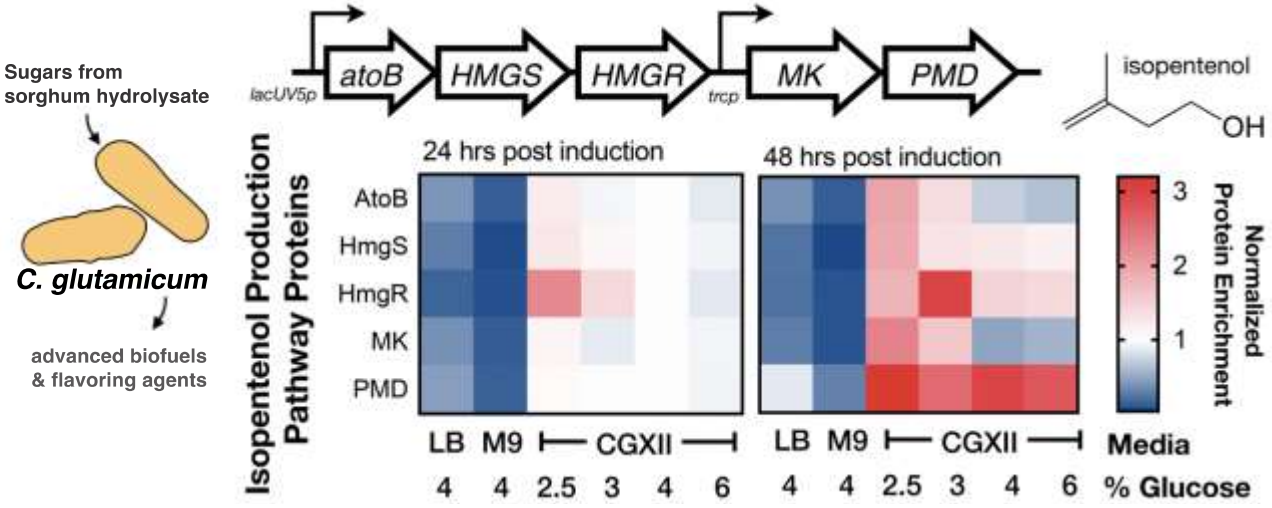
Key Words: Corynebacterium glutamicum, sorghum, hydrolysate, ionic liquid pretreatment, isopentenol.

Many microbes used for the rapid discovery and development of metabolic pathways have sensitivities to final products and process reagents. Isopentenol, a biogasoline candidate, has an established heterologous gene pathway but is toxic to several microbial hosts. Reagents used in the pretreatment of plant biomass, such as ionic liquids, also inhibit growth of many host strains. We explored the use of *Corynebacterium glutamicum* as an alternative host to address these constraints. We found *C. glutamicum* ATCC 13032 to be tolerant to both the final product, isopentenol, as well to three classes of ionic liquids. A heterologous mevalonate-based isopentenol pathway was engineered in *C. glutamicum*. Targeted proteomics for the heterologous pathway proteins indicated that the 3-hydroxy-3-methylglutaryl-coenzyme A reductase protein, HmgR, is a potential rate-limiting enzyme in this synthetic pathway. Isopentenol titers were improved via three routes: media optimization; substitution of an NADH-dependent HmgR homolog from *Silicibacter pomeroyi*; and development of a *C. glutamicum* Δ*poxB* Δ*ldhA* host chassis. We describe the successful expression of a heterologous pathway in the gram-positive industrial microorganism, C. glutamicum, for the production of the biogasoline candidate, isopentenol. We identified and optimized critical genetic and media parameters required to produce 1.25 g/L isopentenol in defined minimal media with D-glucose as the carbon source and similar titers (1 g/L) using sorghum biomass hydrolysates as a carbon source.



YARROWIA LIPOLYTICA: A VERSATILE MICROBIAL WORKHORSE FOR EXPANDING NATURE'S BIOSYNTHETIC CAPACITY

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Key Words: Oleaginous yeast, genetic tools, polyketides, mevalonate pathway, metabolic engineering

Yarrowia lipolytica is an oleaginous yeast that have been substantially engineered for production of oleochemcials and drop-in transportation fuels. It has been considered as a 'generally recognized as safe' (GRAS) organism for the production of organic acids in the food and nutraceutical industry. The high precursor acetyl-CoA and malonyl-CoA flux along with the versatile carbon-utilization capability makes this yeast as a superior host to upgrade low-value carbons into high-value pharmaceuticals and plant natural products (PNPs). Bacteria system in general is less efficient to express the complex gene cluster of plant natural product pathway. Unlike bacteria, yeast has developed spatially separated organelles to partition specialized metabolic functions into distinct cellular compartments. In this talk, we will present strategies to harness the endogenous acetyl-CoA/malonyl-CoA/HMG-CoA metabolism toward engineering efficient yeast cell factories to produce complex oleochemicals, terpenes, polyketides and aromatic commodity chemicals. We identified pathway limitations and assessed genetic engineering strategies to elevate the level of acetyl-CoA, malonyl-CoA, HMG-CoA and NADPH. This work will provide a testbed for engineering *Y. lipolytica* and expanding nature's biosynthetic capacity to produce complex fuels and chemicals from renewable feedstocks.

SOLUTION OF THE MULTI-STEP ASSEMBLY OF *CATHARANTHUS ROSEUS* ANTICANCER ALKALOIDS.

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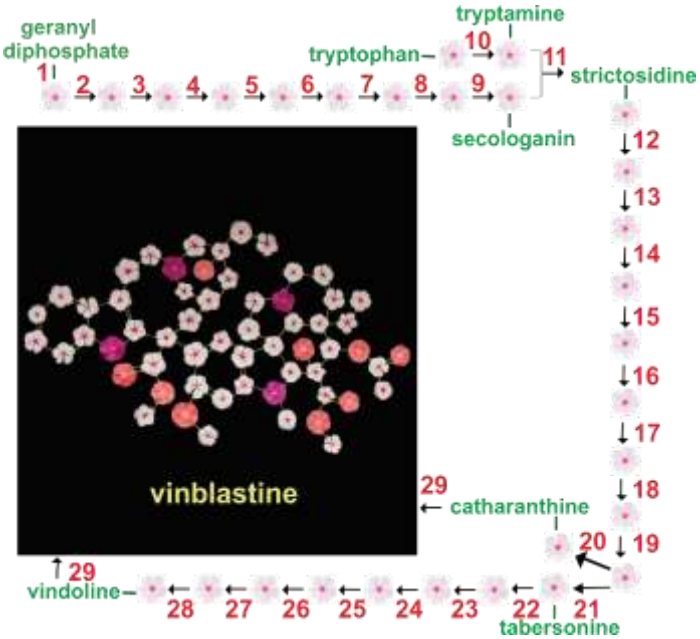
Key Words: Monoterpenoid indole alkaloids, pathway identification and elucidation, metabolic engineering.

Catharanthus roseus, also known as the Madagascar periwinkle, produces low levels of unique dimeric monoterpenoid indole alkaloids (MIAs) that are harvested and used as anticancer drugs to treat Hodgkin's disease and other cancers. The trace levels of dimers occurring in *C. roseus* have made them very expensive to isolate. It is well known that *C. roseus* leaves are the main sites of biosynthesis of the precursors, catharanthine and vindoline, and that each MIA is localized in different leaf cell types, making it difficult to understand how dimer formation takes place. Research efforts in the past 6 years on the assembly of these monoterpenoid indole alkaloids (MIAs) in *C. roseus* has led to the molecular and biochemical characterization of the remaining genes involved in the 29-step pathway required for their biosynthesis from geraniol diphosphate. The formation of strictosidine from geraniol and tryptophan involves 9-steps, most of which have recently been solved and have led to prototype strictosidine expressing pathways in yeast. Enzyme-mediated hydrolysis of strictosidine leads to formation of precursors used in the biosynthesis of several thousand MIAs, including these well-known anticancer drugs. We recently reported the discovery and functional characterization of 10 remaining genes to complete the description of enzymes for tabersonine and catharanthine assembly [*PNAS* (2018) 115: 3180-3185; *Planta* (2018) 247:625-634; *Plant J.*, (2108) 97: 257-266]. These discoveries with our recent successful assembly of vindoline from tabersonine [*PNAS* (2015) 112: 6224-6229) completed the description of the vindoline and catharanthine pathways from geraniol and tryptophan and has set the stage for developing biological systems for synthesis of many different biologically active MIAs. The basic tools and developments leading to these discoveries will be discussed and analyzed. The impacts of elucidation of the vinblastine/vincristine pathways will also be discussed in relation to future discoveries of MIA pathways of biological and biomedical importance.



The *Catharathus roseus* MIA, vinblastine (depicted as a floral diagram), is a dimer of catharanthine and vindoline. The formation of secologanin involves 1) geraniol synthase, 2) geraniol-8-hydroxylase, 3) 8- hydroxygeraniol oxidase), 4) iridoid synthase, 5) 7- deoxyloganetic acid synthase), 6) 7-deoxyloganetic acid glucosyltransferase, 7) loganic acid synthase, 8) loganic acid O-methyltransferase and 9) secologanin synthase. The 10) tryptophan decarboxylase-mediated formation of tryptamine is condensed with secologanin to generate the central intermediate strictosidine via 11) strictosidine synthase. Hydrolysis of thus MIA by 12) strictosidine-β-glucosidase generates the equilibrium mixture of cathenamine/4,21-dehydrogeissoschizine and 13) geissoschizine synthase generates19*E*-geissoschizine. The formation of stemmadenine involves 14) geissoschizine oxidase, 15) Redox1 and 16) Redox 2. Formation of *O*-acetylstemmadenine by 17) stemmadenine-*O*-acetyl-transferase stabilizes the molecule for subsequent transformations involving 18) geissoschizine synthase, 19) *O*-acetyl-stemmadenine oxidase. A reactive intermediate is acted upon by 20) hydrolase 1 to form catharanthine, and by 21) hydrolase 2 to form tabersonine. Tabersonine if converted to vindoline by the action of 22) tabersonine-16-hydroxylase, 23) 16-O-methytransferase, 24) 3-oxidase, 25) 3-reductase, 26) N-methyltransferase, 27) 4-hydroxylase and 28) O-acetyltransferase. 29) peroxidase may then catalyzed the coupling reaction to form vinblastine

PLANT CELL CULTURE PLATFORMS FOR PRODUCTION OF BIOSCAVENGERS FOR BIODEFENSE

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Key Words: transgenic plant cell culture, bioreactors, butyrylcholinesterase, anthrax decoy protein

There is a critical need for flexible, rapid, cost effective biomanufacturing platforms for medical countermeasures. Our team has developed plant cell culture-based manufacturing platforms for production of recombinant protein bioscavengers against organophosphate (OP) nerve agents and anthrax toxins using both stable transgenic cell cultures for known chemical and biological threats, as well as transient production for rapid response to new and/or unanticipated threats. Plant cells offer several advantages over other hosts for production of medical countermeasures, particularly their ability to produce complex biologics and perform post-translational modification, inherent biosafety since they don't harbor or propagate mammalian viruses thereby simplifying and/or eliminating viral clearance steps required for mammalian production systems. Plant cells are robust, have minimal nutrient requirements (grow in simple, chemically defined media containing sucrose, salts and plant hormones), and are relatively insensitive to changes in environmental conditions. These characteristics, robustness of upstream cultivation/use and reduced downstream purification requirements, make plant cells an ideal choice for field-deployable production of medical countermeasures. Here we present results for the production of functional recombinant butyrylcholinesterase (BChE), an OP nerve agent bioscavenger, in transgenic rice cell suspension cultures in different bioreactor configurations, and transient production of a bioscavenger against an anthrax toxin in *N. benthamiana* cell cultures. Techno-economic models for scaled-up versions of these plant cell culture production systems will also be presented.

METABOLIC ENGINEERING OF *SACCHAROMYCES CEREVISIAE* FOR HIGH LEVEL PRODUCTION OF AROMATIC CHEMICALS

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Key Words: Aromatic amino acid | Saccharomyces cerevisiae | Erythrose 4-phosphate | Coumaric acid | Metabolic engineering

The synthesis of functional plant natural products using microbial hosts is considered a safe, cost-competitive and sustainable approach to their production. In particular, the budding yeast has attracted much attention due to its superior ability to express the cytochrome P450s common in downstream of plant pathways for production of these high-value compounds. However, aromatic amino acid (AAA)-based production in yeast is an outstanding challenge. Here we present the construction of a *Saccharomyces cerevisiae* platform strain able to produce *p*-coumaric acid, a common precursor for many commercially valuable chemicals. We demonstrate that carbon flux can be rewired from glycolysis directly to erythrose 4-phosphate formation, enabling an increase in production of AAAs and their derivates. Through systematic removal of bottlenecks in the AAAs biosynthesis pathway and further optimizing carbon distribution between glycolysis and AAAs biosynthesis pathway by using a promoter library screening approach, *p*-coumaric acid production increased to about 3 g l^{-1} under shake flask conditions, with ~ 15% conversion yield on glucose. Furthermore, the engineered strain produced up to 12.5 g l^{-1} of *p*-coumaric acid under fed-batch fermentation conditions, the highest reported titre for aromatic chemicals produced in yeast. The strategies presented here can also be applicable to other microorganisms for production of aromatic chemicals.

ENGINEERED METABOLIC PATHWAYS FOR THE SYNTHESIS OF PLANT NATURAL PRODUCTS

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Key Words: plant natural products, prenylated aromatics, isoprenoids, polyketides, *Escherichia coli*

The structural and chemical diversity of plant natural products (PNPs) offers an enormous chemical space from which molecules with beneficial characteristics can be discovered and produced. To date, thousands of PNPs have been exploited for human benefit with applications ranging from drugs and nutraceuticals to industrial chemicals. However, agricultural and geographic variations and extraction of the desired compound(s) poses a significant challenge for cost-effective production due to the high cost associated with downstream processing and purification. Furthermore, native metabolic pathways for product synthesis often limit the attainable product titers, rates, and yields due to intrinsic inefficiencies and negative cross-talk between product-forming and growth-sustaining reactions. To address these limitations, we are working on engineering microbial-based platforms for the efficient synthesis of PNPs. This includes utilizing native metabolic pathways and plant enzymes for product synthesis in industrially amenable hosts as well as designing and implementing non-natural/synthetic pathways.

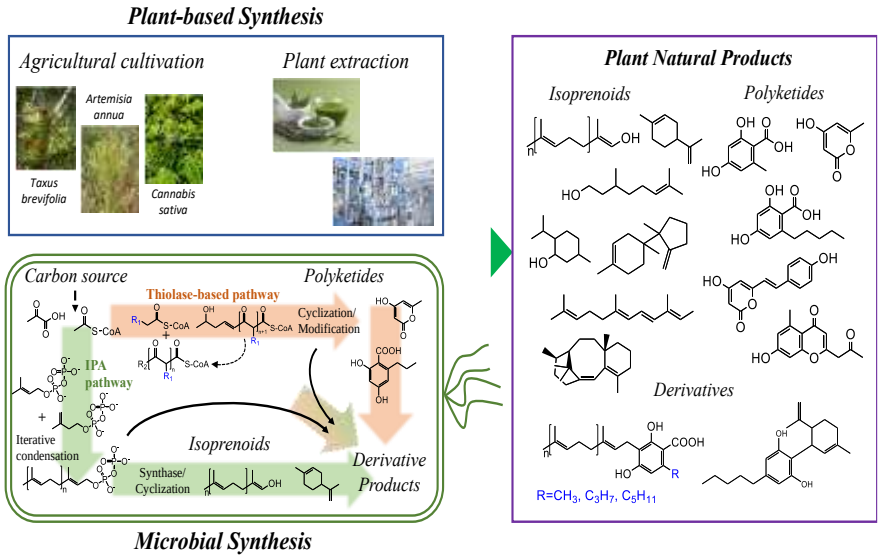

Figure. Engineered microbial platforms for the synthesis of products traditionally sourced from plants

In this talk, I will discuss our recent efforts to develop microbial platforms for the synthesis of isoprenoid and polyketide products as well as their derivatives, including compounds like prenylated aromatics (PAs) that are currently sourced from plants. We have used plant enzymes for product synthesis in *Escherichia coli*, such as the functional expression of a polyketide synthase and olivetolic acid cyclase from *Cannabis sativ*a, which when combined with engineering pathways for precursor generation enabled olivetolic acid production (*ACS Synthetic Biology 7: 1886, 2018*). However, in many cases, such as the integral membrane aromatic prenyltransferases (aPTases) involved in the production of PAs, functional heterologous expression of the plant enzymes in microbial systems can prove a major challenge. We have demonstrated how the use of soluble, bacterial-derived enzymes catalyzing the condensation reaction between a prenyl chain and an aromatic ring can be used in place of plant aPTases to enable the synthesis of PAs in bacteria. Key to our approach was the engineering of soluble aPTases through protein modeling and rational design, resulting in significant improvement to their catalytic efficiency. Expression of engineered aPTases coupled with exogenous addition of aromatic substrates and pyrophosphate supply through an engineered mevalonate pathway enabled the synthesis of an array of PA compounds, including medicinally important cannabigerovarinic, cannabigerolic, and grifolic acids (*Biotechnol. Bioeng. doi:10.1002/bit.26932, 2019*). Finally, I will discuss the engineering of non-natural routes for the generation of the prenyl and polyketide moieties used in the synthesis of PAs. This includes a synthetic pathway for the production of isoprenoids, which we have termed the isoprenoid alcohol or IPA pathway, and a novel platform for the synthesis of polyketide backbones that uses thiolases as opposed to polyketide synthases (*Manuscripts under Review*). These new systems enable the efficient synthesis of isoprenoids, polyketides, and their derivatives, including PAs, in microorganisms.

TAILORING *CORYNEBACTERIUM GLUTAMICUM* TOWARDS EFFICIENT PRODUCTION OF PLANT POLYPHENOLS

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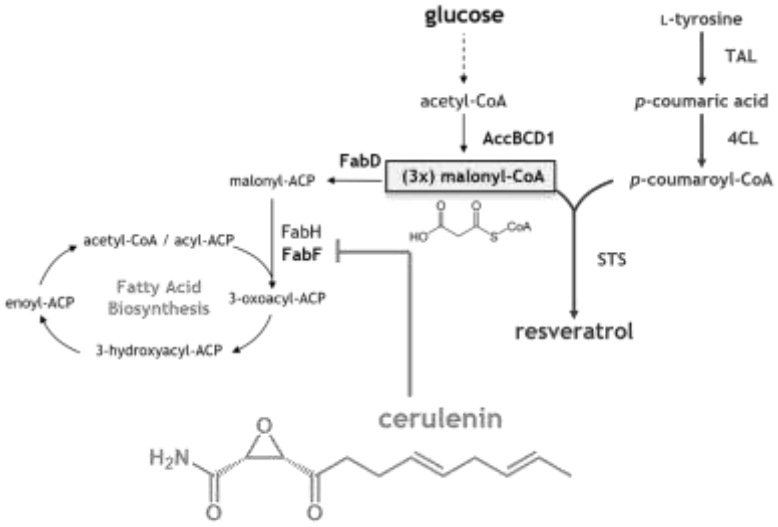
Key Words: *Corynebacterium glutamicum*, polyphenols, malonyl-CoA, resveratrol, biosensors

Corynebacterium glutamicum is an important organism in industrial biotechnology for the microbial production of bulk chemicals, in particular amino acids. Functional integration of plant-derived biosynthetic pathways also allows for the microbial synthesis of various plant polyphenols such as flavonoids or stilbenes either from supplemented phenylpropanoid precursor molecules or directly from glucose.

However, similar to other microorganisms engineered for plant polyphenol synthesis, insufficient malonyl-CoA supply is also limiting polyphenol production with *C. glutamicum*. To date, the antibiotic cerulenin inhibiting fatty acid synthesis is added during microbial cultivations to improve malonyl-CoA availability for product formation at lab-scale. Unfortunately, supplementation of cerulenin is very costly, which prohibits large-scale microbial polyphenol production.



We extensively engineered the central carbon metabolism of *C. glutamicum* with a focus on the TCA-cycle and the fatty acid metabolism to increase malonyl-CoA availability for polyphenol synthesis. In the context of this work, rational metabolic engineering strategies and FACS-based high-throughput screenings using transcriptional biosensors were successfully combined, yielding strain variants accumulating high polyphenol concentrations without supplementation of cerulenin.

We believe that, availability of these new *C. glutamicum* platform strains opens the door towards microbial production of plant polyphenols as well as other high-value aromatic compounds from cheap carbon sources at larger scale.

References:

Kallscheuer N., Vogt M., Stenzel A., Gätgens J., Bott M., Marienhagen J. (2016). Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones. *Metab. Eng.* 38: 47-55.

Milke L., Aschenbrenner J., Marienhagen J. and Kallscheuer N. (2018). Production of plant-derived polyphenols in microorganisms: current state and perspectives. *Appl. Microbiol. Biotechnol*, 102: 1575–1585.

A NOVEL ANTI-DIABETIC METABOLITE FROM PLANTS:
BIOSYNTHESIS, GENE DISCOVERY, AND METABOLIC ENGINEERING OF MONTBRETIN A

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Key Words: Secondary metabolism, bioproducts, flavonoids, glucosyltransferase

Plant specialized metabolites (i.e. secondary metabolites) have been employed by humans for centuries in traditional and modern medicine. They remain an important source for the discovery of new pharmaceuticals and nutraceuticals. Montbretin A (MbA) is a complex acylated flavonoid glycoside discovered in the below-ground storage organs (corms) of the ornamental plant montbretia (*Crocosmia x crocosmiiflora*). MbA a highly potent and selective inhibitor of the human pancreatic α-amylase (HPA), a key enzyme in starch degradation. MbA is being tested for the treatment of type-2 diabetes. However, due to low abundance of MbA in montbretia plants and due the complex chemical structure of MbA, natural product extraction and chemical synthesis are insufficient for MbA production. Our goal is to develop a heterologous plant production system or a microbial production system for MbA. This requires knowledge of the genes, enzymes and regulating factors of the MbA biosynthetic system in montbretia. We achieved the discovery of the complete biosynthetic pathway of MbA using an approach that combined knowledge of montbretia biology, metabolite profiling, differential transcriptome analysis, cDNA cloning, heterologous gene expression in *E. coli*, yeast and tobacco, and enzyme biochemistry. This includes the discovery of five new UDP-sugar dependent glycosyltransferases (UGTs) and a BAHD-acyltransferases (AT) which together catalyze the complete assembly of MbA from its different building blocks. To reconstruct MbA production in tobacco (*Nicotiana benthamiana*) we enhanced the biosynthesis of flavonol precursors using genes for myricetin biosynthesis and transcription factors from montbtretia, which were stacked with genes of the MbA assembly pathway. We will highlight both challenges and opportunities of exploring novel biosynthetic systems of plant specialized metabolites for the development of new drugs, and bioproducts in general.

EVOLUTION OF A MODULAR, MULTI-FUNCTIONAL TARGETED DELIVERY NANOPARTICLE

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Rinchu Mathew, Stanford University, USA

In spite of hundreds of attempts over the last century, Paul Ehrlich's dream of the "magic bullet" targeted delivery system still has not been realized. Yet these studies have clearly identified the many technological barriers that prevent success. This presentation will describe a step-by-step progression that converted an unstable, non-functional viral capsid (a virus-like particle, VLP) into a sophisticated nanoparticle for targeted delivery of drugs, nucleic acids, and proteins.

Initial mutations reduced immunogenicity and antigenicity and provided a new conditional stability that still allows the VLP to disassembly inside the targeted cell to release its cargo. A hexa-histidine tag enables purification of cell-free produced VLP subunits while potentially also serving to trigger endosomal escape by the "proton sponge" effect. The subunits are also extended with a cargo adsorption domain so that simultaneous cargo loading and VLP assembly can be triggered by increasing ionic strength. Finally the VLP subunit protein was further mutated to incorporate non-natural amino acids which then enable precise surface modification by attaching scFv antibody fragments as targeting agents as well as the extracellular domain of the CD47 receptor to avoid immune system interception. To provide an authentic CD47 interactive surface, the ECD has a pyroglutamate N-terminus, two point attachment (mostly) to the VLP surface, and improved solubility. Functional evaluations using cultured cells are promising and results from initial animal studies will be described.

A PLATFORM TECHNOLOGY FOR DYNAMIC CONTROL OF CELL BEHAVIOR

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Key Words: signal dynamics, mammalian genetic circuits, NanoDeg, nanobody, proteasomal degradation

Mammalian cells rely on complex and highly dynamic gene networks to maintain cellular homeostasis in response to environmental stimuli and intracellular signals. Efficient cellular reprogramming thus requires integration of exogenous components for cell engineering with endogenous cellular networks through feedback control systems. We explored the use of post-translational tools for superior feedback regulation of dynamic behaviors. Specifically, we demonstrated efficient detection and manipulation of the main cellular stress response system – the Unfolded Protein Response (UPR) – for the design of high producing cell lines for protein manufacturing and for the development of cell therapies for sustained protein production.

Our approach is based on the use of the NanoDeg[1] – a bifunctional system that mediates proteasomal degradation of a cellular target with high specificity and exquisite control over rate of decay. To achieve input-dependent post-translational control of the output through the NanoDeg, feedforward loop topologies were explored and compared to conventional strategies for circuit design. We defined the ideal circuit architecture for placing both the reporter output and a reporter-specific NanoDeg under control of a common input and regulate the reporter levels through input-dependent transcriptional and post-translational tools. Transcriptional and post-translational modulation of the output results in lowered basal expression *and* rapid decay of the output upon removal of the input, which, in turn, leads to *enhanced output dynamic range and resolution of the input dynamics*. We deployed this approach and, through iterations of mathematical modeling and experimental tests, built a reporter system for sensitive detection of gene expression. A master cell line containing the components for transcriptional and post-translational control was generated and used to build a comprehensive set of derivative cell lines in which these control elements are linked to the expression of different target genes. Experimental measurements were used to refine the model and generate a predictive tool for establishing experimental conditions for maximal signal amplification and a transfer function correlating the measured signal output with expression of the corresponding target gene (Fig. 1).

This work generated a platform technology for quantitative, multiplexed profiling of gene expression signatures of the UPR with high sensitivity and dynamic resolution of the stimulus causing proteotoxic stress. The relative kinetics of activation of the UPR branches determines cellular fate upon UPR induction (i.e., stress attenuation or apoptosis). This technology will be thus useful for monitoring and manipulating the temporal pattern of activation of the different UPR signaling responses, linking the nature of stimulus causing proteotoxic stress with the outcome of UPR induction, and controlling cellular fate.

This study also provides the design rules of a novel cell engineering technology for building complex genetic networks that govern highly dynamic cellular behaviors. Current work is focused on deploying this technology for manipulating the UPR in response to feedback signals generated at different stages of production of recombinant proteins, thereby enhancing the stress attenuation response and delaying induction of apoptosis. Specifically, we will discuss methods to tune recombinant protein expression with the UPR capacity to cope with proteotoxic stress with the ultimate goal to enhance protein production.

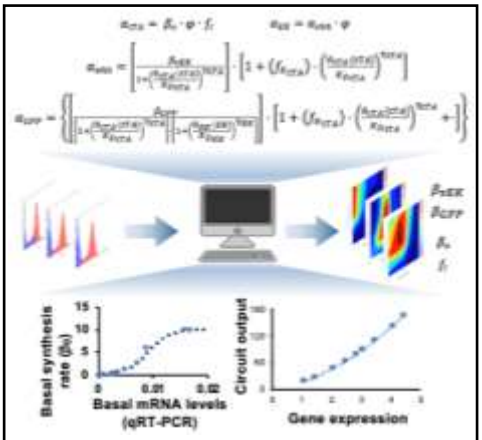

Fig. 1. A platform technology for sensitive detection of gene expression

1. ACS Synth Biol. 2018 Feb 16;7(2):540-552.

UPGRADING THE SYNTHETIC BIOLOGY TOOLKIT FOR DYNAMIC MODULATION OF CELLULAR PHENOTYPES

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Inspired by the remarkable ability of natural protein switches to sense and respond to a wide range of environmental queues, here we report a strategy to engineer synthetic protein switches by using a generalizable strand displacement format to dynamically organize proteins with highly diverse and complex architectures. We showed that both DNA and coiled-coil peptide strand displacements can be used to dynamically control the spatial proximity and the corresponding functions of different proteins for both in vitro and in vivo applications. The general utility of the strategy was used to performing Boolean logic operations to provide explicit control of protein function using multi-input, reversible, and amplification architectures suitable for a wide range of applications.

RECORDING TEMPORAL DATA WITH MINUTES RESOLUTION INTO DNA

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Key Words: Synthetic Biology, Biorecorders, DNA data storage

Recording complex biological signals is a crucial application of synthetic biology and essential for a deeper understanding of biological processes. An ideal “biorecorder” would have the ability to record biological signals over a wide spatial distribution of cells with high temporal resolution. However, the genetically encoded biorecording tools available have very good spatial resolution (cellular level), but currently rely on turning on and off transcription and translation of a protein (e.g., Cas9 or a recombinase) to record the biological signal, making their temporal resolution on the order of hours. Here we introduce a DNA polymerase based biorecorder that can record cationic concentration fluctuations into DNA sequence with a resolution of ~1 minute. We use a template independent DNA polymerase; terminal deoxynucleotidyl transferase (TdT) that randomly incorporates bases onto a single strand of DNA. The preference of base incorporated by TdT changes with the concentration of cations in TdT’s environment. Therefore, by analyzing a strand of DNA that was extended in fluctuating cation concentrations, we can determine the temporal profile of cation concentration from the bases added. Using this method, we can measure a change in Co^{2+} concentration during a one hour period with an accuracy of 1 min. We also show the approach works for Zn^{2+} and Ca^{2+}. We will present our methods for optimizing this biorecorder and characterize its performance *in vitro*. Recording data onto DNA with minutes time resolution could solve many challenging data acquisition problems in neuroscience and developmental biology, and could aid in the use of DNA as a data storage medium.

DEVELOPING AND APPLYING A MICRODROPLET CO-CULTIVATION AND OMICS TOOLBOX FOR ELUCIDATING COMPLEX MICROBIOMES

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Key Words: Microbial community, microdroplet, co-cultivation, microbial interactions, metagenomics

We have been developing, expanding, and applying a technological pipeline, based on nanoliter-scale microfluidic droplets, to co-cultivate and dissect subsets of complex microbial communities in order to elucidate molecular mechanisms underlying their structures and functions. The pipeline consists of droplet generation, co-cultivation, isolation, and analysis such as metagenomic sequencing (Figure 1). We apply this technological framework to the study of a range of microbial communities closely related to human health or the environment. For instance, we demonstrate the anaerobic co-cultivation of diverse sub-communities from a human fecal sample and the *de novo* reconstruction of genomes from a microdroplet. We observe that many of the cultivated bacteria in these encapsulated sub-communities represent the microbial "dark matter". In particular, we have constructed the draft genome of a novel *Neisseriaceae*, representing a new candidate genus with potential contributions to fatty acid utilization and biosynthesis of proatherogenic intermediates.

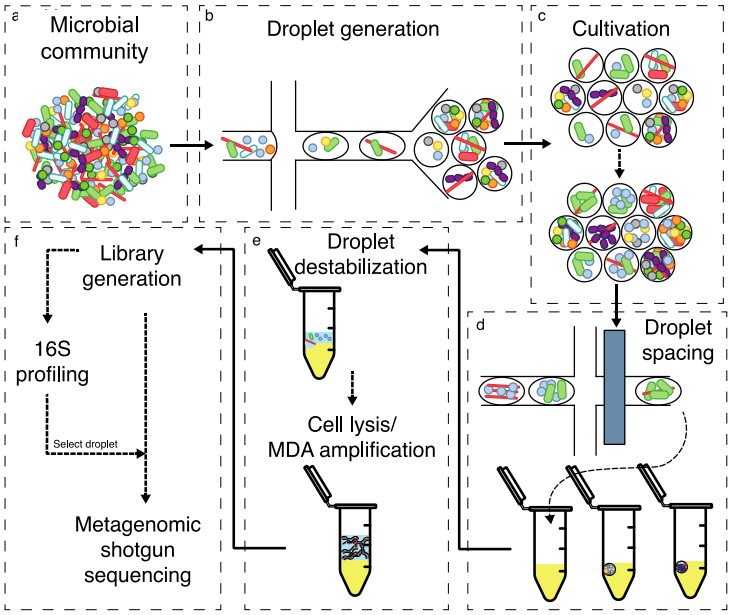

Figure 2 – Experimental workflow of microdroplet encapsulation of subsets of microbial communities (a,b), co-cultivation (c), spacing/isolation (d), metagenome generation (e), and sequencing (f).

EXPLOITING ANAEROBIC CONSORTIA AS NEW TOOLS FOR BIOMASS BREAKDOWN AND SUSTAINABLE CHEMISTRY

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Key Words: Microbiome, metagenomics, synthetic consortia, sequencing, metabolomics

Anaerobic microbes work together in complex communities that decompose and recycle carbon biomass throughout the Earth. Compared to microbes that thrive in the presence of oxygen, anaerobic consortia remain understudied and recalcitrant to culture. However, they are a vast, untapped resource for novel enzymes and strains that degrade woody biomass into sugars for value-added chemical production. Here, we performed several enrichment experiments to isolate biomass-degrading consortia from goat feces, and identify microbes that drive the activity and stability of these cultures. Fecal samples were challenged by four types of biomass (alfalfa, bagasse, xylan, and reed canary grass) and two types of antibiotic treatments (chloramphenicol, penicillin-streptomycin) during cultivation to identify important cross-domain partnerships; 10 billion metagenomic reads spread across 402 enrichment samples tracked biological diversity as the cultures converged to a minimal set of ~20 microorganisms that were stable after more than ten culture generations. Nearly 200,000 carbohydrate active enzymes (CAZyme) domains were identified from the fecal samples alone, constituting nearly 25% of the known CAZymes in existence. 724 genomes were assembled for previously uncultured novel microbes within the herbivore rumen. Surprisingly, consortia dominated by anaerobic fungi generated more than twice the amount of methane compared to prokaryotic consortia, suggesting that fungi play a key role in methane release in ruminant herbivores. The most active microbial consortia comprise cross-domain partnerships between anaerobic fungi from the genus *Neocallimastix* and *Piromyces*, methanogenic archaea from the genus *Methanobrevibacter*, and bacteria from the phylum *Firmicutes*, some of which were enriched nearly 20-fold from the fecal microbiome, produce high yields of methane off-gas, and are capable of cryopreservation and revival. New routes for metabolic cooperation between enriched consortia were also identified, suggesting that an array of bacteria support biomass-degrading microbes by providing essential amino acids while consuming deleterious byproducts. Overall, our analysis points to natural compartmentalization between anaerobes as a means to degrade crude biomass, which can be exploited to harness nature's microbes for sustainable chemical production.

DIRECT CELL-TO-CELL EXCHANGE OF MATTER IN SYNTHETIC *CLOSTRIDIUM* SYNTROPHIES ENABLING CO_2 FIXATION AND AN EXPANDED METABOLIC SPACE

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Key Words: Clostridium, syntrophic co-culture, metabolic expansion, chemicals, exchange of macromolecules

In microbial fermentations to produce metabolites, at least 33% of the sugar-substrate carbon is lost as CO_2 during pyruvate decarboxylation to acetyl-CoA. Previous attempts to reduce this carbon loss focused on engineering a single organism. In nature, microorganisms live in complex communities where syntrophic interactions result in superior resource utilization. Microbial communities are ubiquitous in nature and have a wide range of applications, including production of biofuels and chemicals. Syntrophic and other microbial co-cultures/consortia carry out efficient bio-transformations that are the result of multiple complementary metabolic systems working together. It is now well appreciated that the capabilities of multi-microorganism systems cannot be predicted by the sum of their parts. Rather, synergistic interactions at different levels often result in better overall performance of these systems. Importantly, integration of diverse metabolic systems through syntrophic dependencies make co-culture systems robust to environmental fluctuations.
Clostridium organisms are of major importance for developing new technologies to produce biofuels and chemicals. Three major types of *Clostridium* organisms have been the focus of studies for the sustainable production of fuels and chemicals. Solventogenic clostridia utilize a large variety of biomass-derived carbohydrates (hexoses, pentoses, disaccharides, and hemicellulose), and can produce C2-C4 chemicals. Acetogenic clostridia can fix inorganic H_2, CO_2, and CO to generate C2 acids and alcohols. Other specialized clostridia possess diverse biosynthetic capabilities for production of a wide variety of metabolites including C4 – C8 carboxylic acids and alcohols, which could serve as commodity chemicals, biofuels, or biofuel precursors. Here, we first examined a synthetic syntrophy consisting of the solventogen *Clostridium acetobutylicum*, which converts simple and complex carbohydrates into a variety of chemicals, and the acetogen *C. ljungdahlii*, which fixes CO_2. This synthetic co-culture achieved carbon recoveries into C2-C4 alcohols almost to the limit of substrate-electron availability, with minimal H_2 and CO_2 release. The syntrophic co-culture produced robust metabolic outcomes over a broad range of starting population ratios of the two organisms. Significantly, the co-culture exhibited unique direct cell-to-cell interactions and material exchange among the two microbes, which enabled unforeseen rearrangements in the metabolism of the individual species that resulted in the production of non-native metabolites, namely isopropanol and 2,3-butanediol. Next, we expanded this co-culture system to include *C. kluyveri*, which can metabolite ethanol and acetate to produce C6 and C8 carboxylic acids. Both *C. acetobutylicum* and *C. ljungdahlii* produce ethanol and acetate, which makes *C. kluyveri* and ideal partner for a triple synthetic co-culture system capable to converting biomass-derived carbohydrates to C6 and C8 chemicals.

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DESIGN, CONSTRUCTION AND APPLICATION OF *E. COLI* / *C. GLUTAMICUM* SYNTHETIC CONSORTIA

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Key Words: synthetic consortia, lysine production, alternative carbon sources, mutualism, commensalism.

In the biorefinery concept renewable feedstocks are converted to a multitude of value-added compounds irrespective of seasonal or other variations of the complex biomass substrates. On the one hand, this can be realized by specialized single microbial strains. Alternatively, consortia of several microorganisms or strains can be used. The latter approach allows for modularity, e.g. as various strains for substrate conversion can be combined with various strains for product formation.

We have used *E. coli* for access to carbon sources (Wendisch et al.) and *C. glutamicum* for production of value-added compounds () in order to address the construction of binary microbial consortia based on starch- and sucrose-based production of L-lysine and derived value-added compounds (Sgobba *et al.* 2018). A commensalism-based synthetic consortium for L-lysine production from sucrose was developed combining an L-lysine auxotrophic, naturally sucrose-negative *E. coli* strain with a *C. glutamicum* strain able to produce L-lysine that secretes fructose when grown with sucrose due to deletion of the fructose importer gene *ptsF*. Mutualistic synthetic consortia with an L-lysine auxotrophic, α-amylase secreting *E. coli* strain and naturally amylase-negative *C. glutamicum* strains was implemented for production of valuable fine chemicals from starch.

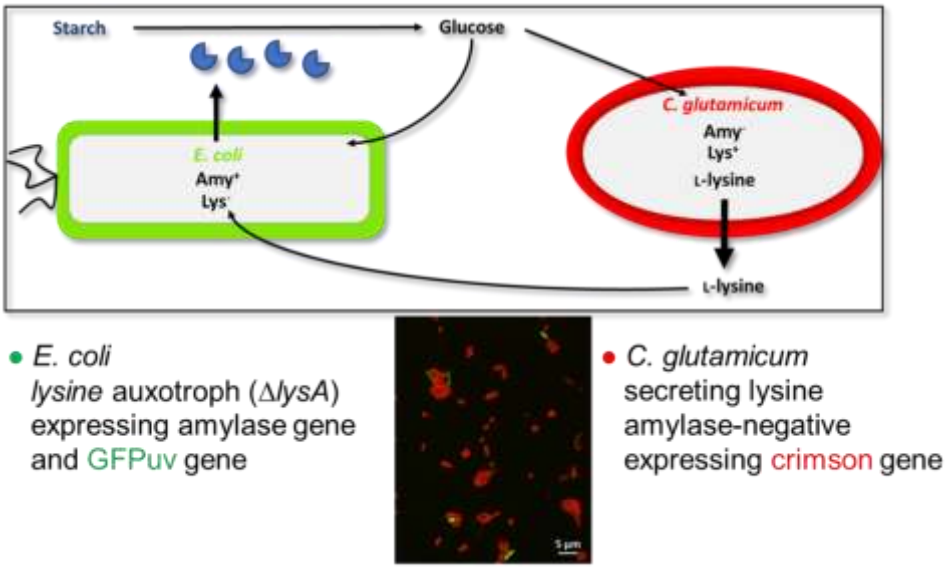

Figure 3 – Design of a mutualistic E. coli/C. glutamicum consortium for starch-based lysine production.

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GENOME EDITING AND SYNTHESIS PLATFORMS WHICH FACILITATE THE CONSTRUCTION OF CELL FACTORIES

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Key Words: Genome editing, Base editor, Genome synthesis, B. subtilis

We have developed the platform technologies such as genome editing and a large gene cluster synthesis systems and are going to integrate to set up the automated systems for efficient construction of microbial cell factories.

By tethering the DNA deaminase activity to nuclease-deficient CRISPR/Cas9 system, we have developed a genome editing tool that enables targeted point mutagenesis. An AID orthologue PmCDA1 was attached to nuclease-deficient mutant of Cas9 (D10A and H840A) to perform highly efficient and target-specific nucleotide editing. This hybrid system, termed Target-AID, induced cytosine point mutation in 3-5 bases range at the distal site within target sequence. Use of nickase Cas9 (D10A), which retains single-strand cleaving activity, greatly increase the efficiency, although it also occasionally induces insertion/deletion (indel) in higher eukaryotes. Uracil-DNA glycosylase inhibitor further increase the efficiency and reduced the indel formation. The toxicity associated with Cas9 has been greatly diminished, enabling application of this technique to wider range of organisms including yeast, bacteria, animals and plants. In addition, by tethering Glycosilase activity to nuclease-deficient CRISPR/Cas9 system, we have developed a genome editing tool that enables targeted randam mutagenesis.

We have also developed an efficient DNA assembly method, namely, Ordered Gene Assembly in *B. subtilis* (OGAB) method. OGAB method can assemble more than 50 DNA fragments in one-step using *B. subtilis*. Thanks to this high processability, even in construction of long DNA (~100 kb), material DNA fragments can be kept in chemical DNA synthesis-friendly and sequencing-friendly small size (< 2 kb). Since there is no *in vitro* DNA synthesis step that may cause unexpected mutation(s), long DNA by OGAB method using sequence-confirmed material DNA thus contains essentially no mutation. We are now constructing user friendly DNA synthesis system by integrating new automation system, such like a liquid handling robot that is specifically developed for OGAB method

These technologies might lead to new pipelines through which functional genomes are cleated with much faster speed to construct microbial cell factories to produce variety of biofuels and chemicals.

METABOLOMICS PROCESS MODELING: A SYSTEMS BIOLOGY APPROACH TO UNDERSTAND VARIABILITY IN COMMERCIAL BIOLOGICS CELL CULTURE PROCESSES

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Key Words: metabolomics

The biopharmaceutical industry strives to develop and operate efficient, robust, reproducible commercial biologics processes. A major challenge of industrial biologics processes is optimization of cell culture conditions to increase productivity while maintaining consistent product quality. The cell culture operations, which involve the use of live cell hosts, have historically introduced significant variability to the overall process. Technological improvements which include the implementation of advanced cell line engineering, chemically defined media, quality by design (QbD) development approaches, and in-line and at-line monitoring, have significantly reduced process variability. Nonetheless, performance variability remains a challenge for many commercial programs. This variability in turn can impact both product yields and product quality. Even small performance differences can become significant in low-yield processes with large campaign sizes, or processes manufactured at multiple sites. The ability to understand and eliminate sources of variability is greatly enhanced by augmenting the quality and quantity of data available from commercial campaigns.

Metabolomics Process Monitoring (MPM) is a data-driven approach to understand sources of manufacturing variability on a cellular level. Here we present a case study of MPM implementation in a legacy commercial biologics program. First, we describe how the MPM workflow was successfully integrated into a commercial manufacturing process. Second, we discuss novel data normalization techniques developed to enable long term trending. Third, we describe the selection of an orthogonal projections to latent structures (OPLS) model to link systems biology and process data. Finally, we share key mechanistic insights obtained from the case study, and provide a vision for how MPM can enhance commercial biologics capabilities going forward.

APPLYING METABOLIC MODELS FOR CONTROL IN ORDER TO ENHANCE ALGAL GROWTH AND LIPID PRODUCTION

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Key Words: *C. vulgaris*, Metabolic models, Control, Fatty acid methyl ester (FAME)

A number of metabolic models have been developed in different algae species in the past five years. In this study, a metabolic model of *C. vulgaris* was applied for controlling algal growth and lipid production. This method optimized nutrient supply by characterizing algal metabolic pathways under different conditions. The approach was validated for autotrophic growth under nitrogen replete condition, in which nitrate requirement was lowered while retaining robust algal growth. Furthermore, this approach was also applied for nitrogen limited environments. Previous studies have found *C. vulgaris* can accumulate high lipid content in biomass, which can be hydrotreated to biodiesel, while decreasing algal growth under nitrogen limitation [1]. Our method optimized the nitrate supply to sustain algal growth while still producing fatty acids efficiently under nitrogen limited conditions. Compared with *C. vulgaris* cultures with complete nitrogen withdrawal, fatty acid methyl esters (FAME) increased substantially. This metabolic model-based approach will have applicability for optimizing nutrient inputs and biomanufacturing across a wide spectrum of organisms ranging from prokaryotes to eukaryotes used to produce a variety of biotechnology products in coming decades.

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AN ADAPTIVE LABORATORY EVOLUTION PLATFORM FOR STRAIN CONSTRUCTION AND ENGINEERING PARTS

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Adaptive Laboratory Evolution (ALE) can be used to obtain useful strains for biotechnology applications. A complete platform containing automation, bioinformatics, and biological selection systems can be used to replace the design and build steps in the rational strain engineering process. In this talk, I will describe the different parts of this platform and current use cases applying the platform to strain construction goals. Examples of advanced biological selection systems which have been developed will be highlighted to show how ALE can be used to obtain production strains for a wider set of products and for focusing selective pressures on targeted enzymatic steps. An additional specific focus will be to highlight what a quality-controlled mutation identification pipeline, a mutation database, and higher order interpretations of mutations using biological network analyses provides for such engineering goals. Finally, I will review what is learned with increasing scale from gathering mutation and strain data from ALE experimentation and what it means for synthetic biology.

VALIDATION AND STABILIZATION OF A PROPHAGE LYSIN OF *CLOSTRIDIUM PERFRINGENS* BY YEAST SURFACE DISPLAY AND CO-EVOLUTIONARY MODELS

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Key Words: antimicrobial proteins, protein engineering, co-evolutionary models, homolog

Bacteriophage lysins exhibit high specificity and activity towards host bacteria with which the phage co-evolved. These properties of lysins make them attractive for use as antimicrobials. Though there has been significant effort to develop platforms for rapid lysin engineering, there have been numerous shortcomings when pursuing the ultra-high throughput necessary for discovery of rare combinations of mutations to improve performance. In particular, the biotechnological utility and evolvability of lysins would be aided by elevated stability. Lysin catalytic domains, which evolved as modular entities distinct from cell wall binding domains, can be classified into one of several families with highly conserved structure and function, many of which contain thousands of annotated homologous sequences. Motivated by the quality of this evolutionary data, the performance of generative protein models incorporating co-evolutionary information was analyzed to predict the stability of variants in a collection of 9,749 multi-mutants across 10 libraries diversified at different regions of a putative lysin from a prophage region of a *Clostridium perfringens* genome. Protein stability was assessed via a yeast surface display assay with accompanying high-throughput sequencing. Statistical fitness of mutant sequences, derived from second-order Potts models inferred with different levels of sequence homolog information, was predictive of experimental stability with AUCs ranging from 0.78 to 0.85. To extract an experimentally derived model of stability, a logistic model with site-wise score contributions was regressed on the collection of multi-mutants. This achieved a cross-validated classification performance of 0.95. Using this experimentally derived model, 5 designs incorporating 5 or 6 mutations from multiple libraries were constructed. All designs retained enzymatic activity with 4 of 5 increasing melting temperature, with the highest performing design achieving an improvement of +4 °C. In addition to validation of a putative lysin and stabilization thereof, the experimental and computational methods presented herein offer a new avenue for improving protein stability and is easily scalable to analysis of tens of millions of mutations in single experiments.

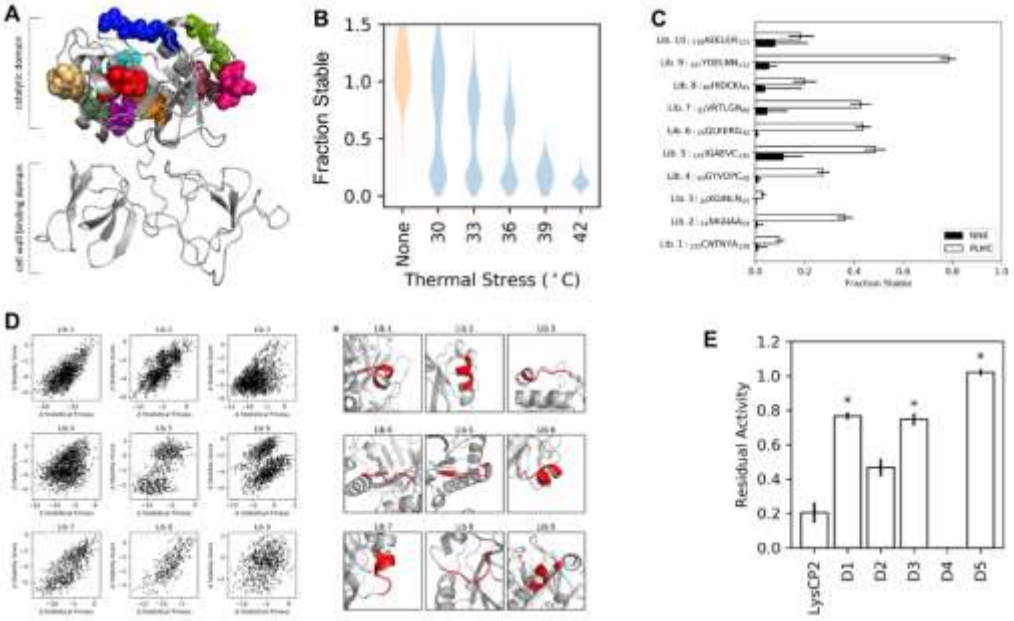

Figure 4. (A) Bacteriophage lysin LysCP2 homology model with diversified sites highlighted. (B) LysCP2 libraries exhibit a broad distribution of stability; high and low stability variants were selected by flow cytometry. (C) The co-evolutionary model yielded more stable lysin variants than a random control library. (D) The predictive quality of the experimentally-derived mutant model was strong at select locations (sub-libraries). (E) Second-generation designed mutants exhibit substantially improved activity after thermal stress relative to wild-type.

UNRAVELING THE METABOLIC AND MACHINERY CONSTRAINTS ON PROTEIN SECRETION THROUGH A NOVEL SYSTEMS BIOLOGY FRAMEWORK

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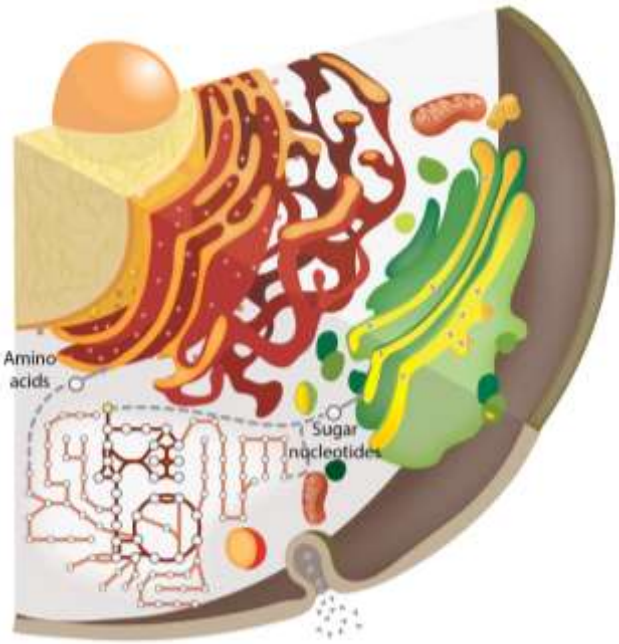
Key Words: metabolism, protein secretion, biotherapeutics, systems biology



In mammalian cells, metabolism is a core process driving homeostasis, but variations in other cell processes largely define cell type identity and cell-type specific functions. The profile of secreted and membrane proteins show substantial cell-type specificity and drive many tissue specific functions. These proteins, encoded by up to 1/3 of mammalian protein-coding genes, include hormones, membrane proteins, and extracellular enzymes, and these are synthesized and trafficked through the secretory pathway. The pathway complexity, however, obfuscates its impact on the secretion of different proteins. Unraveling its impact on diverse proteins is particularly important since the pathway is implicated in many diseases and harnessed for biopharmaceutical production. Through the use of network reconstruction approaches and protein interaction assays, we have mapped out the core secretory pathway and integrated it with our genome-scale metabolic models of human[1], mouse, and Chinese hamster ovary cells[2]. We first deploy graph-based approaches to evaluate the dependency of protein secretion on diverse human secreted proteins[3]. We then deployed constraint-based modeling to quantify the bioenergetic demands for the synthesis and secretion of these proteins[4]. Finally, we deployed these models to engineer mammalian cells for enhanced secretion of high-value biologic drugs. Thus, we present a platform that enables the study and engineering of the mammalian secretory pathway and metabolism for systems biotechnology.

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OPTOGENETICS FOR INTRACELLULAR CODEBREAKING: HOW ERK DYNAMICS CONTROL GENE EXPRESSION AND CELL FATE

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Key Words: Optogenetics, cell signaling, Erk MAP kinase, embryogenesis

Every cell exists in a complex and changing environment. To deal with their complex surroundings, cells have evolved diverse systems to sense external cues and create an internal representation of this information. However, we are still largely in the dark about how external information is stored in patterns of protein activity, and how this information is decoded into specific cell fate decisions. I will talk about our efforts to overcome these challenges using cellular optogenetics: the delivery of precise spatial and temporal activity patterns to a signaling protein of interest. We have developed a suite of optogenetic tools to precisely control MAP kinase (MAPK) signaling. Combining optogenetics and live-cell biosensors enables us to dissect how signaling dynamics are "read out" into target mRNA and protein levels. Applying these tools in the Drosophila embryo further revealed how a model cell fate choice - differentiation into posterior midgut endoderm - is controlled by specific patterns of MAPK activity.

MANIPULATING PHENOTYPES BY EPIGENETIC MECHANISM

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Key Words: epigenetic control, transcriptomes, genome variation, phenotype control

For many years, the plasticity and variation of phenotypes observed in CHO cell lines was attributed to genomic variation. However, while individual mutations of single genes may certainly contribute to a defined phenotype, it typically is the adaptation of the expression pattern of multiple genes which together then modulate and define cellular behavior. Such changes in the transcription pattern are defined by several layers of epigenetic regulation that act on short term and long term, serving both as rapid response mechanisms and as cellular "memory"[1, 2]. These include differential DNA-methylation, predominantly in promoter regions, but also in regulatory regions of the genome. These co-operate and are co-regulated with modifications of histones which change the state of chromatin and thus the accessibility for the transcriptional machinery. On top of these, there are interactions between specific genomic regions and triplex-forming long-non-coding RNAs that can both up- or downregulate transcription by attracting or blocking off transcription factors. The later can serve as very rapid and very strong regulators of transcription.

Such detailed understanding of the underlying mechanisms can be used to advantage to enhance our control over phenotypes both by specifically altering the expression level of individual genes (to the degree of turning them ON or OFF[3]) and by altering the global transcriptome to achieve enhanced cellular performance. Likewise, directed evolution and adaptation protocols also result in a new transcriptome defined by epigenic memory that lays down altered cellular behavior[1]. Ultimately, these tools offer new possibilities for metabolic or cellular engineering, which have the advantage of being fully reversible and dosable, as no changes in the genome sequence are required. Such epigenetic control mechanisms could be used in two directions: i) to increase the phenotypic diversity within a population, for instance during cell line development, to enable isolation of rare variants with superior properties; and ii) to stabilize an already selected phenotype such that more reproducible process outcomes are achieved.

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HIGH-THROUGHPUT MULTICOLOR OPTOGENETICS FOR THE SYSTEMATIC MANIPULATION OF CELL BEHAVIOR

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Key Words: optogenetics, high-throughput, cell signaling, signal dynamics, signal integration

Cellular optogenetics present a powerful approach to control biochemical reactions within living cells and tissues. In principle, optogenetic control offers data-rich experiments with precise perturbations and multiple channels of control (multiple stimulation colors). Further, because light patterns are programmable and light can be applied remotely, optogenetic experiments could be readily scaled up for high-throughput manipulation without the need for expensive robotics, bespoke microfluidics platforms, and perturbative media exchanges. However, there is a lack of methods to perform optogenetic stimulation in a high-throughput microwell format. Here, we describe the optoPlate-96, an LED-based device for independent control of 96 3-color optogenetic experiments in 96- or 384-well plates. The optoPlate-96 is compatible with long-term illumination both in cell culture incubators, as well as in live-cell microscopy. We will first demonstrate how the optoPlates enable more rapid, robust, and reproducible optogenetic experiments. Then, we will briefly detail two studies that highlight the utility of optoPlate experiments. In the first, we used the optoPlate to systematically assess how cancer cells process Ras-Erk signal dynamics differently from normal cells. In the second study, we demonstrated how 3-color (blue, red, far-red) optoPlate-96 illumination enables orthogonal control of red- and blue-responsive optogenetic proteins within the same cell. We used multicolor probing to study signal integration between Ras- and PI3K signaling and uncovered new synergies between these two well-studied pathways. We anticipate that the optoPlate-96 will find broad utility and will help realize the potential of optogenetics for the quantitative dissection and manipulation of living systems.

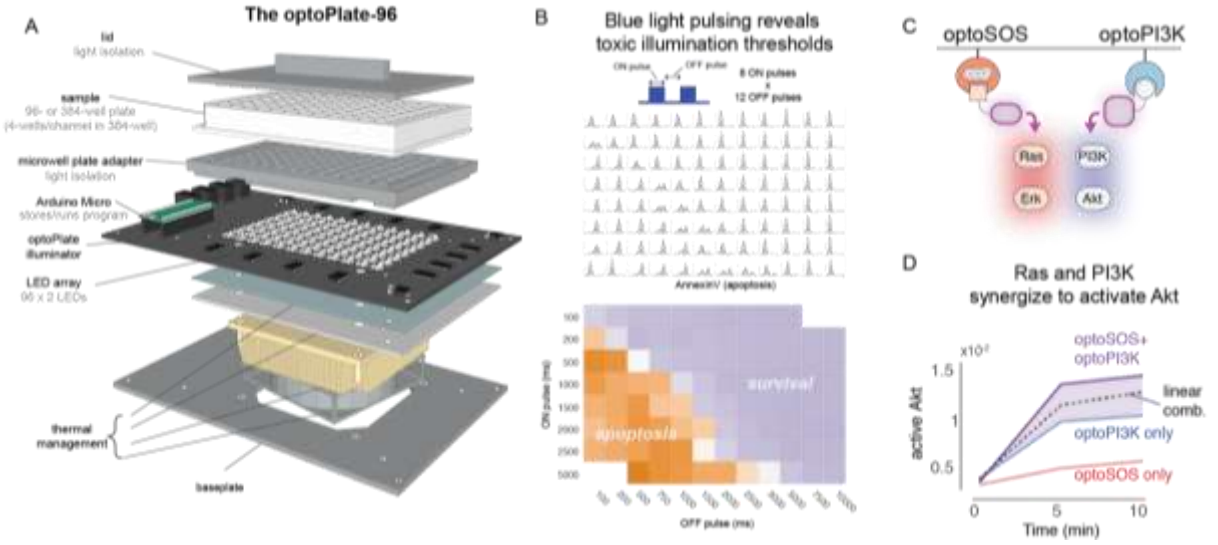

Figure 5 – A) The optoPlate-96 (exploded schematic shown) is an LED-based device for high-throughput microwell optogenetic experiments. B) Rapid scanning of blue light illumination patterns reveals toxic and non-toxic stimuli. C) Multiplexing red-and blue-responsive optogenetic probes uncovers synergy between Ras and PI3K activation of Akt.

OPTOGENETIC MODULATION OF INSULIN FUNCTION IN PANCREATIC BETA-CELLS

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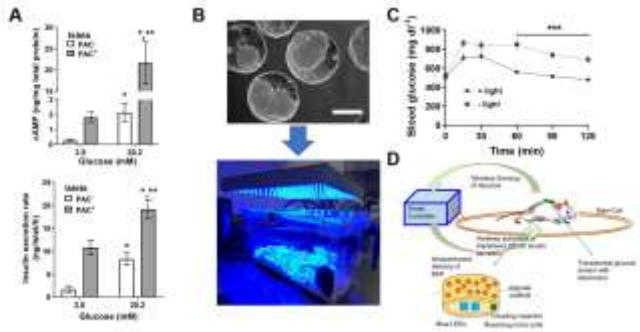
Key Words: Diabetes, optogenetics, pancreas, insulin, β-cells

Diabetes is characterized by elevated blood glucose (BG) due to autoimmune destruction of insulin-producing β-cells (type 1; T1D) or extensive β-cell apoptosis owed to insulin resistance (type 2; T2D). Over 29 million people in the US suffer from diabetes and its complications including blindness, kidney failure and stroke making this the single most expensive disease with total costs of $327 billion in 2017. Pharmacological interventions are linked to serious side effects, are incompatible with abnormal kidney or liver function and their molecular targets have low tissue specificity (e.g. sulfonylurea-targeted ATP-dependent K^+ channels). Daily administration of insulin is essential for the survival of T1D and advanced T2D patients but imposes significant lifestyle restrictions, may cause hypoglycemia, and does not reverse the long-term crippling effects of the disease. Additionally, agents promoting pancreatic endocrine cell regeneration remain elusive. Therefore, novel strategies to achieve BG homeostasis in a glucose-dependent fashion without side effects are highly desirable.

Optogenetic technologies allow the drug-free manipulation of diverse cellular functions through modulation of molecular moieties with unique spatiotemporal precision. One such moiety is cyclic AMP (cAMP), which is an amplifier of glucose-stimulated insulin secretion (GSIS) in β-cells. To this end, we engineered pancreatic β-cells and primary islets to express a photoactivatable adenylyl cyclase (PAC)[1]. PAC-expressing cells exhibited a greater than 5 times rise in cAMP within 5 min of photoactivation and a rapid drop (~12 min) upon termination of illumination. This led to a 2- to 3-fold increase of GSIS in PAC-positive β-cells or islets exposed to blue light vs. those kept in dark (Fig. 1A). Cells exhibited consistently enhanced GSIS over multiple rounds of photoactivation without changes in viability. The pronounced response was comparable to that by β-cells treated with known secretagogues including adenylyl cyclase activators or phosphodiesterase inhibitors[1]. No difference was observed in insulin release with or without illumination in the absence of glucose, further justifying the optogenetic targeting of cAMP, which augments but does not induce hormone secretion. The dependence of GSIS on Ca^{2+} signaling remained intact in engineered β-cells. Light-stimulated secretion of insulin was an order of magnitude greater when PAC-expressing β-cells formed clusters (termed 'pseudoislets'; PIs). The function of PIs carrying PAC was tested *in vivo*. Encapsulated PIs were delivered subcutaneously to mice, which were rendered diabetic upon treatment with streptozotocin (STZ; Fig. 1B). The animals were subjected to a glucose tolerance test (GTT) following a 6-h fasting and intraperitoneal injection of glucose. Blood was sampled before (0 min) and after glucose injection (30, 60, 90 and 120 min) and BG was determined using a glucometer. The mice were exposed to blue light for 3 h starting 1 h before the GTT (Fig. 1C). Animals receiving PAC-expressing β-cells exhibited impaired glucose tolerance in the absence of illumination while the BG returned to baseline in mice with engineered cells undergoing photostimulation.



*(A) Insulin secretion and cAMP of murine islets with (white) or without PAC (gray). *p<0.01 PAC^+ vs. PAC^- islets in matching glucose levels. ANOVA and Tukey post hoc test were performed. **p<0.01, PAC^+ islets at 20.2 vs. 2.8 mM glucose[1]. (B) Encapsulated PIs of PAC^+ β-cells (bar: 100 μm) delivered to STZ-treated mice. Blue LED array (1 ft^2) is shown. (C) GTT data (mean±SEM, 5 mice/group) for STZ-treated mice exposed to light (lower curve) or not (upper curve). Student's 2-tailed unpaired t-test was applied with the Holm-Šídák post hoc test. ***p<0.005 for +light vs. -light. (D) Optogenetic bioartificial pancreas device that is currently being tested.*

Currently, a bioartificial pancreas device (Fig. 1D) is developed integrating β-cells amenable to optogenetic regulation of GSIS, glucose biosensing and remotely powered and controlled microLEDs for GSIS enhancement.

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TOWARDS ELECTROGENETICS: INTEGRATING BIOFABRICATION, SYNTHETIC BIOLOGY, AND MICROELECTRONICS

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Key Words: Synthetic Biology, Biofabrication, Microelectronics

We are developing tools of “biofabrication” that enable facile assembly of biological components within devices, including microelectronic devices, that preserve their native biological function. By recognizing that biological redox active molecules are a biological equivalent of an electron-carrying wire, we have developed biological surrogates for electronic devices, including a biological redox capacitor that enable bi-directional “electron” flow. We have also turned to synthetic biology to provide a means to sample, interpret and report on biological information contained in molecular communications circuitry. Finally, we have developed synthetic genetic circuits that enable electronic actuation of gene expression. That is, using simple reconstructions, one can apply voltage on an electrode and directly actuate genetic responses and associated phenotypes. Cells are stimulated to swim, make other signal molecules, and fluoresce. When stimulated to generate signal molecules, programmed devices are effective agents for modulating biological systems via native molecular means. We have further developed an electronically actuated CRISPR a/i system for simultaneous activation and inhibition of transgenes and host genes, respectively. By integrating engineered cells within hydrogel membranes, these methodologies enable “programmed” biological function. This presentation will introduce the concepts of molecular communication that are enabled by integrating relatively simple concepts in synthetic biology with biofabrication. Our presentation will show how engineered cells and “smart” films represent a versatile means for mediating the molecular “signatures” commonly found in complex environments, or in other words, they are conveyors of molecular communication. Importantly, these methodologies are suggested to enable first ever electronic control of biological production processes in a gene specific manner. Potential applications are abundant for the biopharmaceuticals industry and several specific demonstrations will be presented.

ESTABLISHING A NOVEL CELL THERAPY PLATFORM: SYNTHETIC BIOLOGY AND BIOPROCESS CONSIDERATIONS FOR RATIONAL THERAPEUTIC DEVELOPMENT

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Cell therapies offer exciting, novel strategies for treating acute, chronic, and incurable diseases. This technology represents a “eureka moment,” according to the former FDA head, Scott Gottlieb. This field has undergone several generations of development, from stem cell transplantation, to dendritic cell modification, and developing most recently into autologous CAR-T therapies that have been highly effective against some blood cancers. However, these recent autologous therapies have been limited by daunting costs, highly variable process outcomes, a lack of reliable assays, and supply chains that are both immature and risky. Call this Cell Therapy 1.0. Allogeneic therapies, either from primary donors or induced pluripotent lines, or Cell Therapy 2.0, are being currently developed. As these are nucleated cells with the potential for significant expansion in vivo, they still carry substantial safety concerns. Rubius Therapeutics is leapfrogging the allogeneic adoptive transfer approaches by developing Red Cell Therapeutics™, in which therapeutic proteins are expressed in or on genetically engineered, enucleated red blood cells. This versatile, innovative 3.0 platform enables us to efficiently target immune-oncology, inborn metabolic disorders, and autoimmune indications. It also makes cell therapy more akin to a routine monoclonal antibody business model with greater scale, flexibility, reliability, and cost competitiveness relative to current autologous and allogeneic technologies. However, the traditional process metrics used in manufacturing monoclonal antibodies must be adjusted to account for the unique considerations of red cell manufacture. Rubius monitors a variety of phenotypical and product-specific biomarkers that track the progression of cells through erythroid differentiation in conjunction with seed train steps in the process. Methods to understand which attributes are predictive of process outcome and final product quality are at the forefront of Rubius’ analytical strategy. By understanding allogeneic cell manufacturing – specific process development challenges and developing analytical methods capable of reliably predicting process performance and product quality, Rubius expects to develop the next generation of cellular therapies that are cost effective, consistent, and efficacious against incurable diseases.

BIOCHEMICAL ENGINEERING UNDER STRESS!

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The formation of oxygen on earth, which is a relatively recent phenomenon, resulted in a new and highly toxic environment that nature had to overcome to survive. Plants and bacteria evolved unique secondary natural products through exquisitely constructed metabolic pathways to protect against such oxidative stress and against flourishing microbial threats at higher O2 levels. One common mechanistic thread involved a simple chemical motif – the ubiquitous phenolic – that serves as a “reservoir” of protection against oxidative stress. In this talk, I will highlight two examples where we have exploited phenolic compounds for the generation of new biologically active molecules and in uncovering mechanisms of new therapeutic platforms. The first example involves earlier work in our lab where we developed high-throughput, in vitro metabolic pathway engineering to yield unique type III polyketides with exceptional biological activities. The second example involves the development of a new platform technology – magnetogenetics – where alternating and static magnetic fields can be used to control gene expression leading to potential therapeutic interventions, and where control of biological outcomes can be mediated by phenolic compounds.

CO2 AS CARBON SOURCE FOR MICROBIAL PRODUCTION OF BIOBASED CHEMICALS

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Key Words: CO_2, acetogens, microalgae, bioreactor, photobioreactor

Reducing waste and emissions of greenhouse gases like CO_2 is a major demand for industry. In this context great interest has emerged in biological CO_2-fixing processes which are supposed to be very effective in reducing CO_2 emissions.

Acetogenic bacteria are able to use hydrogen gas for the reduction CO_2. The reductive acetyl-CoA pathway enables the autotrophic production of biobased chemicals like acetate, ethanol, butyrate, butanol, 2,3-butanediol, hexanoate and hexanol. Metabolic engineering of acetogens is a promising approach to enlarge the natural product portfolio. Low product yields and selectivities as well as low biomass densities and inefficient utilization of gaseous substrates are some of the challenges that slow down commercialization so far. The reaction engineering analyses of acetogens and the application of bioreactor designs providing high gas-liquid mass transfer efficiencies will enable new gas fermentation processes overcoming the challenges for further commercialization in the near future.

Microalgae consuming the greenhouse gas CO_2 and using sunlight as energy source could become an important renewable source for biobased chemicals. Since the production cost of most microalgae products from current mass cultivation systems is still prohibitively high, further development is required. To advance economic microalgae mass production new open thin-layer cascade photobioreactors were designed recently up to a pilot scale (50 m^2) for high-cell density cultivation of saline microalgae achieving up to 50 g L^{-1} dry cell mass, which was shown applying physically simulated climate conditions of a Mediterranean summer in Spain in the TUM-AlgaeTec-Center near by Munich, Germany.

Finally, state-of-the-art of phototrophic CO_2-fixation by microalgae and of autotrophic CO_2-fixation by acetogens will be compared with respect to kinetics, process engineering aspects and productivities.

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INCREASED YIELD AND PRODUCTIVITY FOR THE CONVERSION OF ALGAL BIOMASS CARBOHYDRATES

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Key Words: Fermentation, Bioproducts, Yield, Immobilized cells, Biomass

Microbial conversion of bio-based fuels and chemicals requires innovative strategies to achieve the best possible economic and sustainability metrics. While most studies have focused on the conversion of sugars from lignocellulosic hydrolysates, algal biomass is an appealing source of fermentable carbohydrates because of the high growth rates of algae relative to plants. The conversion of carbohydrates in algal biomass via fermentation is an important component of an overall strategy to maximize the areal productivity of fuels and chemicals from algal cultivations. There are two classic challenges to improving the cost and sustainability metrics of chemical production via fermentation: increasing product yield from substrate and increasing productivity. The goal of this project is to achieve these goals for ethanol production from algal hydrolysates.

The strategies relied on the use of immobilized-cell technology in a continuous cultivation system, which has the potential to achieve significantly higher productivities than those from standard batch fermentation using free cells. Importantly, cell immobilization can also facilitate strategies for increasing the yield (carbon conversion efficiency) of sugar-to-product conversion. One such strategy, restricting biomass production, was shown to significantly improve yields. Proteome profiling was used to characterize the effects of this treatment.

Hydrolysates of the alga *Desmodesmus armatus* were prepared using a dilute acid treatment at elevated temperature and pressure. A mock hydrolysate medium containing glucose, mannose, and galactose was also used. *Saccharomyces cerevisae* JAY 270 cells were immobilized in alginate to produce ethanol from algal or mock hydrolysates of *D. armatus.* The immobilized cells were packed in a column that was used in a system that operated either in chemostat or single-pass plug-flow mode. The rates of immobilized-cell production of ethanol were determined, along with the effects of pH, temperature, and residence time in the continuous immobilized-cell bioreactor system.

No added nutrients are required for ethanol production using the algal hydrolysate of *D. armatus* in the continuous immobilized-cell bioreactor system. pH 4 and 35 °C are the optimum conditions for the immobilized yeast fermentation. The productivity of the chemostat-like continuous immobilized-cell bioreactor system could be more than ten times that of free-cell bioreactors. Furthermore, shorter residence times led to higher ethanol productivities but lower glucose conversion rates. A sequence of a continuous well-mixed bioreactor and a plug-flow bioreactor is shown to achieve both goals. The increase in productivity can benefit the economics and sustainability of the overall process for production of algal biofuels, and the strategies described here are applicable to any extracellular metabolite production process.

BIOSWITCHES AND ROBOTICS FOR SYSTEMS METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY OF HYPER MICROBIAL PRODUCTION STRAINS

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Despite impressive progresses in systems metabolic engineering and synthetic biology, there are still several unsolved major challenges in their practical applications for developing effective metabolic pathways and microorganisms for biosynthesis: 1. identification of targets for advanced pathway engineering of productive strains, especially under industrially relevant and *in vivo* conditions; 2. effective means with proper dynamic range and sensitivity for dynamic and concerted control of metabolic pathways; 3. designed parts from synthetic biology often not work well within the host cells, especially for highly productive strains; 4. mathematical models of cellular processes often miss regulatory details inside cells and thus fail to guide biomolecular and cellular design. In this talk, I will illustrate our recent efforts to address some of these questions, especially for developing industrially relevant production strains with high yield and productivity (Ref. 1-5). After a short introduction, I will first present results on rational design of bioswitches (riboswitch and allosteric regulation) for dynamic control of metabolic pathways. Then, I will address the question how we can use host cells as parallel "robots" to identify targets and evaluate designed parts. Also recent work in developing a robotic system for algorithm-guided biomolecular design and optimization will be presented. The methods and techniques are successfully applied to develop highly productive strains for the biosynthesis of L-lysine, tryptophan and 5-aminolevulinic acid.

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SCALING UP *E. COLI* FROM THE LAB TO INDUSTRIAL CONDITIONS: LESSONS LEARNED TO ENGINEER ROBUST PROCESSES AND PRODUCTION HOSTS

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For commercialization, strain and bioprocess developments need to be successfully transferred from the lab to industrial scale. Often, this step crucially decides about economic feasibility and survival of the approach. Accordingly, profound understanding of impact factors that hamper the successful scale-up is key, either to create novel microbial production platforms with enhanced robustness or to improve bioreactor design targeting minimized impact on cellular performance.

Using an experimental scale-up simulator consisting of a stirred tank reactor (STR) and a plug flow reactor (PFR) *Escherichia coli* was exposed to typical large-scale mixing conditions in continuous experiments. Installing mixing times of about 110 seconds and simulating fluctuating availability of carbon and nitrogen sources, short-term responses revealed the repeated on/off switching of about 600 genes (Löffler et al.,*Metab Eng* 2016; Simen et al. *Microbial Biotechnol* 2017). Dynamics of gene expression and protein formation were modelled using an agent-based approach and simulating large-scale conditions (Nieß et al. *Frontiers Microbiol*., 2017). ATP balancing of gene expression and protein formation showed that maintenance demands increased by ~50%. Thereof, strategies for genome reduction were deduced.

Large-scale simulation revealed the dominating role of the alarmone ppGpp which triggers the on/off-switching of the stringent response. Accordingly, a novel chassis was engineered such that intracellular ppGpp levels were no more affected thereby disconnecting the extracellular stimulus from the intracellular response, even under nitrogen or carbon limitation. Experimental studies outline the energetic advantages of stringent response deficient production hosts. Additionally, changes were implemented in central metabolism finally yielding *E. coli* HGT (high glucose throughput, Michalowski et al., *Metab Eng* 2017). The patent-filed strain offers about 10 fold risen glucose uptake rates (relative to maintenance demands under glucose limitation) under resting conditions which is beneficial for large-scale production processes.

CHALLENGES AND SUCCESSES IN TECHNOLOGY SCALE UP

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Key Words: Carbon recycling, Gas fermentation, Scale up.

LanzaTech has commercialized its gas fermentation platform, after a decade of rigorous scale up and multiple plants in the field. With this experience in industrial waste gases, municipal solid waste (MSW)- and biomass-derived syngas, LanzaTech is now poised to deploy its carbon recycling platform across an array of waste feedstocks for production of fuels and everyday chemicals. This talk will expand on this experience, the lessons learned and successes along the way.

ENGINEERING YEAST FOR THE HIGH-LEVEL SYNTHESIS OF POLYKETIDE BIOBASED CHEMICALS

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Polyketides are an important class of biobased chemicals, both as products and as precursors for subsequent conversion to a wide range of compounds. They are synthesized via complex polyketide synthases, and many using acetyl-CoA and malonyl-CoA as starter and extender units. We have combined engineering of the pathway and synthase enzymes, metabolic pathway engineering, and improved cultivation strategies to substantially increase titers and yields in two important yeast species, *Saccharomyces cerevisiae* and the thermotolerant *Kluyveromyces marxianus*. Our work has focused on the polyketide triacetic acid lactone (TAL) as it is can be converted into a wide range of high-value and commodity products. TAL is also a simple polyketide that requires expression of only one synthase enzyme and is easily assayed; it can thus be used as an effective and rapid indicator of strains with the high acetyl-CoA and malonyl-CoA pools needed for polyketide production. We have then extended our successful strategies to the synthesis of 6-methylsalicylic acid and a novel biosurfactant. For high-level TAL production in *S. cerevisiae*, our work has focused on overexpression of native and variant *Gerbera hybrida* 2-pyrone synthases, extensive engineering of the yeast metabolic pathways for increased cofactor and precursor pools, and implementation of fed-batch cultivation strategies. These interventions increased TAL titer from 0.07 g/L to 10.5 g/L and yield from <1% to 44% of theoretical. Recent work has included engineering of native regulatory systems to increase synthesis of polyketides and implementation of CRISPR-based combinatorial methods. In the presentation, we will highlight the critical pathways engineered, and examine the synergy between successful strategies for the polyketide products. We have also demonstrated the promise of the alternate yeast species *K. marxianus* for the high-level synthesis of polyketides from low cost substrates. *K. marxianus* has excellent growth characteristics for biobased chemical production, including rapid growth rate, ability to metabolize an array of substrates, high TCA cycle flux, and tolerance to low pH and high temperatures (in excess of 45^{o}C). Unlike *S. cerevisiae, K. marxianus* is able to grow on low-cost substrates to high cell densities that equal or surpass that in glucose. We have evaluated the effects of temperature, carbon source, 2-PS enzyme variant, and expression method on the synthesis of TAL. Xylose and glycerol were the best carbon sources for TAL production. While the highest TAL titers were observed at 37^{o}C, significant levels were also obtained at 41^{o}C and 43^{o}C. Utilization of a multi-copy pKD1-based plasmid resulted in TAL titers of >1 g/L in testtube culture at 37^{o}C prior to any metabolic engineering of the yeast. Yield on carbon is also the highest reported to date and demonstrates the promise of this rapidly growing and thermotolerant yeast species for polyketide production from a variety of carbon sources. Using our new CRISPR system, we are modifying native and heterologous pathways in *K. marxianus* to increase levels of the acetyl-CoA and malonyl-CoA precursors. Continuing work also includes the synthesis of alternate products, and cultivation in controlled bioreactors to increase titer and yield. We will present our successful strategies to increase the synthesis of this class of biobased chemicals from lower-cost feedstocks in this rapidly growing thermotolerant yeast.

ENGINEERING OF AN ENVIRONMENTAL ISOLATE OF BACILLUS MEGATERIUM FOR BIOCHEMICAL PRODUCTION UNDER SUPERCRITICAL CO_2

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Key Words: *Bacillus megaterium*, supercritical carbon dioxide, continuous bioprocess

Continuous processing is a mainstay for chemical production but is far less common for biochemical processes. The increase in productivity and corresponding decrease in costs make continuous processing an intriguing option for bulk chemicals where price is a major consideration. Among the various challenges of continuous bioprocessing are the risks of contamination and the toxicity of the target products. Supercritical carbon dioxide ($scCO_2$) may provide a means to address both of these issues. $scCO_2$ is an attractive substitute for conventional organic solvents due to its unique transport and thermodynamic properties, its renewability and labile nature, and its high solubility for compounds such as alcohols, ketones and aldehydes. $scCO_2$ is also known for its broad microbial lethality. The isolation and engineering of a microbe that is capable of growth and production in the presence of $scCO_2$ thus represents an opportunity to create a production environment that is both resist to contamination and capable of sequestering toxic products through phase separation. Using a targeted bioprospecting approach by sampling fluid from a natural, deep-subsurface $scCO_2$ well, a strain of *Bacillus megaterium* was isolated that is able to germinate and grow in the presence of $scCO_2$. Transformation is possible using a protoplast-based method, which permitted the identification of promoters capable of inducible heterologous protein expression in both aerobic and anaerobic conditions. A xylose-inducible promoter was evaluated under $scCO_2$ and found to have similar expression under both conditions. We engineered the *B. megaterium* strain to produce isobutanol from 2-ketoisovalerate by introducing a two-enzyme pathway (2-ketoisovalerate decarboxylase (KivD) and alcohol dehydrogenase (Adh)). Due to the strong partition of the aldehyde to the $scCO_2$ phase, we tested five homologous Adh enzymes and found that YqhD from *E. coli* resulted in greater than 85% conversion when grown aerobically. Isobutanol production was also observed when our recombinant strain was cultured under $scCO_2$. Finally, we have developed a process model for an integrated bioprocess and have found conditions that are comparable if not better than existing *in situ* extraction techniques such as gas stripping.

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OPTOGENETICS AS A NEW PARADIGM FOR DYNAMIC CONTROL IN METABOLIC ENGINEERING

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Key Words: Optogenetics, Metabolic Engineering, Dynamic Control, Synthetic Organelles.

We recently developed optogenetic circuits to control engineered metabolic pathways for microbial chemical production with light[1]. Light offers unique capabilities for dynamic control of fermentation processes. It is highly tunable and can be applied or removed instantly, and in any desired schedule, to elicit reversible metabolic responses without chemical inducers or complex media changes. In this talk, I will present our recent progress in the development of this new technological platform. I will describe new optogenetic circuit designs that enhance the transcriptional activation kinetics of inverter (OptoINVRT) circuits upon exposure to darkness, which improve the robustness of light-controlled cell growth and chemical production. In addition, I will introduce a new class of optogenetic circuits for metabolic engineering (OptoAMP), which amplify the transcriptional response to light, enabling strong light-induced gene expression in high cell-density fermentations in lab-scale bioreactors of up to five liters. Furthermore, I will present new optogenetic post-translational controls based on light-dependent assembly of synthetic organelles, which we use to control flux through branched metabolic pathways. I will demonstrate how each of these technologies can be applied to dynamically control engineered metabolisms to boost yields, titers, productivities, and product specificities of fuels and chemicals in microbial fermentations. Finally, I will provide a perspective on how optogenetics may emerge as a new paradigm for dynamic control in metabolic engineering.

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MICROBIAL SYNTHETIC BIOLOGY

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Key Words: Protein Quality Control, Synthetic Biology, Fast-growing Bacterium, Metabolic Engineering

In this talk, I will introduce two topics related with microbial engineering; a novel synthetic biology tool for efficient protein synthesis and a novel microbial platform for biorefinery. Firstly, we developed a synthetic Protein Quality Control (ProQC) system to improve the yield of the synthesis of full-length proteins in E. coli. Tightly coupled transcription and translation system in prokaryotes can cause the production of non-functional polypeptides which are translated from early-terminated or degraded transcripts. We used toehold switches to decouple transcription and translation processes. By placing corresponding trigger sequences at 3'-end of mRNA, intact full-length mRNAs could only be translated. When fluorescence tags were attached at both N- and C-terminal ends, we found that equivalent fluorescent intensities from both ends were observed in our system. This result shows that decoupling of transcription and translation processes can be applicable to elevate the quality of gene expression, especially longer proteins. Secondly, we isolated a novel fast-growing bacterium which shows efficient alginate utilization and whose growth rates and sugar uptake rates with most biomass-derivable sugars are substantially higher than those of E. coli. We systematically characterized its genome as well as transcriptome to elucidate its metabolism and gene expression architecture. Based on this, we were able to develop genetic toolboxes for its engineering and successfully demonstrated rapid production of a broad spectrum of chemicals (ethanol, 2,3-butanediol, and lycopene) from alginate and mannitol mixtures with high productivities and yields. Collectively, this strain is a powerful platform for conversion of brown macroalgae sugars; moreover, its usage will dramatically accelerate production of value-added biochemicals.

BIOCONVERSION OF LEVULINIC ACID TO METHYL-ETHYL KETONE VIA A NOVEL CATABOLIC PATHWAY

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Key Words: Bioprocessing, metabolic engineering, lignocellulose, levulinic acid, *Escherichia coli*.

Levulinic acid (LA) is a common degradation by-product of acid-catalyzed hydrolysis of lignocellulosic biomass. Under the right conditions, LA can instead be made as the dominant hydrolysis product. For this reason, LA was identified as a key renewable building block and motivated development of chemical upgrading strategies for producing value-added chemicals from a renewable feedstock. Biological upgrading of LA has been limited to natural microbial products, mainly polyhydroxyalkanoates (PHA), due to a lack of understanding about the LA catabolic pathway. Recently, we discovered and characterized an enzymatic pathway from *Pseudomonas putida* KT2440 that enables growth on LA in both *P. putida* and when heterologously expressed *Escherichia coli* LS5218. The pathway, described in Rand *et al.*, *Nature Microbiology* (2017), activates LA as a CoA-thioester, reduces 4-keto-moiety, and isomerizes the resulting hydroxyl via a unique ATP-dependent mechanism. The 3-hydroxypentanoyl-CoA generated by the pathway can be incorporated into PHA or further oxidized by the native enzymes of beta-oxidation to enable growth and energy generation. In this talk, we will describe the transposon (traditional and TN-seq) experiments used to identify essential genes in the pathway and the genetic and biochemical studies used to associate each enzyme in a five gene operon (*lvaABCDE*) to roles in the pathway.

We will also describe our efforts to evolve *E. coli* for utilization of LA as a sole carbon source. The evolved strains were derived from *E. coli* LS5218, which contains specific mutations for overexpression of β-oxidation (*fadR*) and short chain fatty acid degradation genes [*atoC*(Con)]. Genome sequencing of the evolved mutants and parent led to the isolation of two key function deletions required for robust growth on LA as well as several other differences away from its presumed *E. coli* parent. Reconstitution of the isolated mutations in wild type LS5218 revealed one to be necessary, *fadE*, and one to confer a beneficial growth phenotype, *atoC*. In this talk, we will describe the genome of LS5218 (Rand *et al*., *Metabolic Engineering Communications* (2017), and how it may be an advantageous strain for metabolic engineering of organic acid biosynthesis.

Lastly, we will describe how we engineered a strain of *E. coli* to perform a bioconversion of LA to the common industrial solvent methyl-ethyl ketone (MEK). Here, we overexpressed an acetoacetyl-CoA transferase (AtoDA) and heterologously expressed an acetoacetate decarboxylase (ADC) to enable flux to MEK. Through genome scale metabolic modeling of the pathway, we identified growth coupling strategies to force cells to perform the bioconversion in order to generate energy (and grow). The resulting strains convert LA to MEK at unit conversion with appreciable rates. In this talk, we will present the unpublished metabolic engineering, modeling, and fed-batch optimization of the process.