Keynote 1
Probing gene essentiality in *Pseudomonas putida* using SynBio tools

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Keynote 2
Pseudomonas taiwanensis VLB 120: a versatile biofilm workhorse in biotechnology

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Pseudomonas taiwanensis VLB 120, for a long time also known as *Pseudomonas* sp. VLB120 is a Pseudomonas isolated from the microbiome of an air filter system cleaning styrene contaminated offgas. Not surprising, it turned out to be able to utilize styrene as a sole source of carbon and energy, and exhibited an extraordinary solvent tolerance. Multiple variants of this strain have been designed over the years, among them a knock out strain, which is capable of converting styrene enantioselective to (*S*)-styrene oxide when growing on an alternative carbon source. Amongst others, this variant has been extensively used in biotech-research to learn about solvent tolerance of organisms and two liquid phase biotransformations. Furthermore, it was discovered, that *P. taiwanensis* VLB120 is an excellent biofilm former, readily sticking to surfaces when encountering hydrodynamic stress. We exploited this ability and used *P. taiwanensis* VLB 120 in capillary biofilm reactors as primary producer as well as biofilm supporter strain in multi-trophy biofilms. In my presentation I will focus on biofilm applications using this organism, in axcenic as well as in mixed species biofilms, light-driven biofilms.

Keynote 3
Application of *Pseudomonas putida* in Biotechnology – Strain and process engineering for rhamnolipid production

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Keynote 4
Modelling approaches in systems biology

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Mathematical modeling (MM) is a critical step in understanding complex systems in both technical and non-technical sciences, including life sciences. In systems biology, MM can be seen as a way to formalize our knowledge of a system and gain a better understanding of its inner workings. Systems theory offers a framework for developing models that can be characterized along different dimensions. For example, one dimension is the level of detail of the model, which can range from simple qualitative interaction networks to large, mass-conservative, and quantitative models that describe processes inside cells and their fluctuating environment. Another dimension is whether the model represents an average cell or single cells in an environment. Further dimensions include whether the system is static or dynamic, and/ or whether the model needs structural elements such as an objective function to explore the (possibly infinite) solution space. This talk will briefly summarize the steps involved in developing a mathematical model (before writing down any equation), provides an overview of typical model equation systems used in systems and synthetic biology, and gives short examples of the authors' own work on bacterial systems like *Escherichia coli* and *Pseudomonas putida*.

Keynote 5
From sunlight to bioplastics: the fairy tale of *Synechococcus elongatus* and *Pseudomonas putida*

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Once upon a time, there dwelled a cyanobacterium, known as *Synechococcus elongatus cscB,* endowed with the ability to secrete sucrose, who yearned to find its perfect partner, a companion to turn the boundless powers of light and CO_2 into bioplastics, known as polyhydroxyalkanoates (PHA). An ally named *Pseudomonas putida cscRABY* was encountered, and together, they embarked on a grand adventure that would lead them towards the realisation of their dream…

This defined synthetic co-culture composed of *S. elongatus cscB* providing sucrose to the heterotrophic *P. putida cscRABY*, will be presented from two perspectives: First, we will discuss the streamlining of the co-cultivation process for efficient PHA production from light and CO_2. Critical to PHA accumulation is the molC/molN ratio. By individualising the nitrogen sources to be exclusively available for one partner, we could estimate the molC/molN ratio from experimental data and predict the necessary feeding rates to obtain a specific ratio. This resulted in an increased maximal PHA titer and PHA production rate.
Next, as we had observed a remarkable growth-promoting effect on the cyanobacterium by the presence of *P. putida cscRABY*, we set out to identify potential interactions between both partners. A suitable reference process was established in a parallel operating photobioreactor system, which sets the basis for the co-culture analysis at the transcriptome, proteome, and metabolome level. This comprehensive multi-OMICs approach unveiled several moderate changes, including alterations in the metabolism, transportation, and stress response in both microbes. These findings offer valuable insights into the complex dynamics within the co-culture system, suggesting the exchange of further molecules beyond the unidirectional feeding with sucrose and enriching our understanding of this scientific partnership.

… and they lived happily ever since.

Oral 1
Unraveling the finetuned transcriptional regulation of specialized metabolite production in *Pseudomonas* using ONT-cappable-seq

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Specialized metabolites are complex chemical molecules that enable their natural producer to thrive in certain niches. In case of the *Pseudomonas* genus, their ubiquitous nature is reflected by the versatility of specialized metabolites they produce. The past century, scientists have directed their efforts to repurpose these natural products as antibiotics, herbicides, etc. However, their natural production titers are generally low, and genetic engineering is required to facilitate biotechnological applications. These efforts are often limited by the lack of knowledge concerning regulatory mechanisms that drive specialized metabolite production.

To bridge this gap, we developed a long-read RNA-sequencing approach termed ONT-cappable-seq[1] that allows 5'-to-3' sequencing of primary microbial transcripts. The obtained transcriptional landscape subsequently provides a window in the complex regulatory network involving transcriptional landmarks, sRNAs and riboswitches. In addition, novel insights in the finetuned transcriptional regulation of specialized metabolite production will aid future genetic engineering efforts to improve production titers.

Here, we would like to present mono-rhamnolipid production by *Pseudomonas chlororaphis* as a case-study. This specialized metabolite shows biological activity against carcinogenic cell lines as well as agricultural and foodborne pathogens, which makes mono-rhamnolipids an interesting target for industrial upscaling. We used ONT-cappable-seq to unravel new mechanisms driving rhamnolipid biosynthesis gene expression in *P. chlororaphis*. This technique revealed several regulatory bottlenecks, which can now be addressed to efficiently engineer the host and to further improve mono-rhamnolipid titers.

1. Putzeys, L. *et al.* Development of ONT-cappable-seq to unravel the transcriptional landscape of Pseudomonas phages. *Comput. Struct. Biotechnol. J.* **20**, 2624–2638 (2022).

Oral 2
Engineering of *Pseudomonas taiwanensis* for *de novo* production of aromatics

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Aromatics are a cornerstone of modern society. Their current production is mostly dependent on petrochemical processes and a bio-based production is required for the transition towards a sustainable economy. Microbial biocatalysis is a promising approach to enable *de novo* biosynthesis of value-added chemicals from renewable substrates.
We deeply engineered solvent-tolerant *Pseudomonas taiwanensis* as microbial host for the bioproduction of a wide range of different aromatics[1-5] applying *chassis*, tolerance and metabolic engineering. Initially, a tyrosine overproducing platform strain was generated for the production of phenol by a combination of forward and reverse engineering of leads obtained from previous aromatics-producing *P. putida* strains that were obtained through random mutagenesis and high-throughput screening[1]. Genes were deleted, point mutations introduced, and heterologous genes overexpressed to increase the flux into the shikimate pathway, avoid product or intermediate loss, and enable product formation. The engineering of streamlined genome-reduced *chassis* (GRC) strains with improved bioprocess features further enhanced phenol production regarding titer, rate, and yield[2]. The resulting tyrosine-producing GRC strain served as basis for subsequent studies to diversify the product spectrum to other tyrosine-derived hydroxylated aromatics (including 4-coumarate, 4-hydroxyphenylacetate, and 4-vinylphenol) with low byproduct formation[5] and to enable production of phenylalanine-derived chemicals (such as *trans*-cinnamate[3], benzoate, and *cis,cis*-muconate[4]). More recently, we established and enhanced production of chorismate-derived hydroxybenzoates. In ongoing work, we study the tolerance towards and the production of hydrophobic and highly toxic chemicals such as styrene and aromatic aldehydes (i.e., cinnamaldehyde) and focus on substrate in addition to product diversification.

1. Wynands et al 2018, Metab Eng 47:121-133
2. Wynands et al 2019, ACS Synth Biol 8:2036-2050
3. Otto et al 2019, Front Bioeng Biotechnol 7:312
4. Otto et al 2020, Biotechnol J 15:2000211
5. Wynands et al 2023, Metab Eng 78:115-127

Oral 3
Lord of the Aromatic Rings: Harnessing Native Metabolism in *Pseudomonas umsongensi*s GO16 for the Production of Plastic Alternatives

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2,5-Furandicarboxylic acid (FDCA) is a biobased molecule functionally similar to terephthalic acid (TPA), one of the monomers of the ubiquitous plastic, polyethylene terephthalate (PET). FDCA can substitute TPA in PET, producing polyethylene 2,5-furanoate (PEF). However, the use of FDCA for plastic applications is limited by high production costs. Biotechnology offers an alternative production route by using fewer toxic reagents, milder conditions, and waste stream derived feedstocks for upcycling.

Pseudomonas umsongensis GO16, which can natively metabolise the PET monomers ethylene glycol (EG) and TPA as growth and energy substrates, has been used to upcycle these molecules to polyhydroxyalkanoates and hydroxyalkanoxyl-alkanoates previously. Building on this ability to convert PET monomers to valuable compounds, this bacterium is being explored as a microbial chassis for the synthesis of FDCA.

A precursor of FDCA is 5-hydroxymethylfurfural (HMF). *P. umsongensis* GO16 possesses an *hmf* operon, enabling it to utilise HMF as a sole growth substrate, with FDCA as an intermediate. FDCA decarboxylase (HmfF) has been identified as the enzyme responsible for converting FDCA to further metabolism. A CRISPR/Cas9 system was used to delete this gene and allow FDCA accumulation.

EG-grown GO16 Δ*hmfF* cells in a biotransformation reaction fully converted 50 mM HMF into FDCA after 48 hours. To improve this conversion rate, the enzymes responsible for the oxidations of HMF to FDCA have been identified. Their expression is currently being optimised to create a more potent biocatalyst from this FDCA producing strain.

Oral 4

3rd *Pseudomonas* Grassroots Meeting
Munich 2023

Engineered Pseudomonas putida strains enable combined chemo- and biocatalytic conversion of technical lignin into muconic acid

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Pseudomonas putida is regarded as a promising cell factory for the valorization of lignin in a biorefinery concept based on lignocellulosic biomass due to its ability to catabolize a wide range of monoaromatic compounds into products of industrial interest. However, access to these monoaromatics is challenging due to the recalcitrance and heterogeneity of the original lignin streams.

In the present work, we explored the use of *P. putida* in the conversion of different industrial lignin-streams for the production of muconic acid, a valuable platform chemical that can be used in the synthesis of polyamides and polyethylene terephthalate [1]. Challenges posed by heterogeneity and inhibition of the substrate were addressed by careful choice of strain and optimizing the chemical depolymerization process conditions. This led to a successful conversion of lignin-derived guaiacol and vanillin into muconic acid.

[1] ACS Sustainable Chem. Eng. 2021, 9, 24, 8097–8106

Oral 5
Construction of a synthetic mixotrophic *Pseudomonas putida* strain *via* a Calvin-Benson-Bassham shunt

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Climate change is perhaps the most severe threat that humanity is facing nowadays. Carbon emissions must be reduced to effectively tackle this issue, and efficient routes towards CO_2 capture from the atmosphere and upcycling into useful products should be deployed to this end. Biological carbon upcycling offers such an opportunity. *Pseudomonas putida* presents several phenotypic and metabolic traits that make it an almost ideal host for metabolic engineering and industrial biotechnology—yet C_1 substrates are not part of its biochemical 'palate'. Enabling this host to assimilate CO_2 or soluble derivatives (i.e. formic acid and methanol) would provide a microbial cell factory capable of converting carbon emissions into useful products. Here, we aim at transitioning the lifestyle of *P. putida* from a heterotroph into a chemoautotroph by implementing a synthetic Calvin-Benson-Bassham cycle, i.e. the most important carbon fixation cycle in the biosphere. We implemented growth-coupled selection principles by constructing a strain that is unable to grow on ribose unless the CBB shunt, composed by ribulose 1,5-bisphosphate carboxylase/oxidase (RuBisCO) and phosphoribulokinase, is active. The application of this engineering strategy, coupled with laboratory evolution, yielded a synthetic mixotrophic *P. putida* strains that grow on ribose and CO_2 as the sole substrates via the CBB shunt. These strains and its derivatives will be used for bioproduction of value-added chemicals using CO_2 as a co-substrate and further engineered towards full chemoautotrophy.

Oral 6
CRISPR-base editing unravels the role of *P. putida* porins in carbohydrate metabolism

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Choosing a suitable microbial host is pivotal in biotechnologies and some of the former, well-established go-to organisms are being replaced by more specialized producers. In lignocellulose technologies, *Pseudomonas putida* has emerged as a promising alternative, boasting a range of advantages, including enhanced stress resistance, efficient aromatics metabolism, inherent production of valuable chemicals, and absence of pathogenic determinants. This study focuses on the outer membrane porin complement of *P. putida*

and its impact on the utilization of lignocellulosic sugars, leveraging a powerful CRISPR-base editing tool for precise gene manipulation.

In contrast to *E. coli*, *Pseudomonas* porins exhibit substrate-specificity, contributing to a sophisticated system governing substrate uptake and its regulation. This unique feature also endows *P. putida* with the capability to thrive in the presence of toxins that would inhibit other microorganisms. We comprehensively mapped the porin complement of *P. putida* EM42 and selected candidate genes encoding carbohydrate-selective porins for targeted gene interruption. Next, we systematically inactivated selected porin-encoding genes and subjected the modified strains to various carbohydrate substrates for growth analysis. Our results suggest that the role of carbohydrate-specific porins in *P. putida* is more complex than generally assumed. This study also underscores the versatility of the CRISPR-base editor as a powerful tool for unraveling the complex phenotypes in industrially relevant microbial hosts.

Poster 1
Pseudomonads at (my) work: Novel Applications in Lignin Biotechnology, Plastics degradation, Electrofermentation and Infectious Disease Research

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Among the 250 strains of the genus Pseudomonas known today many have proven highly promising for industrial application as cell factories for microbial production of biodegradable and biobased plastics, biosurfactants, platform chemicals, pharmaceutical enzymes, and natural compounds [1]. Other Pseudomonads however are hazardous human, insect, and plant pathogens that are intensively studied to develop novel drugs and therapies [2].

In this presentation, we give an overview about the different research activities at the Institute of Systems Biotechnology (iSBio) with a special focus on applications and available analytical and modeling tools:

(i) *P. putida* and its genome-reduced derivatives are used as chassis strainsto convert depolymerized lignin aromatics or plastic monomers into *cis,cis*-muconic acid (MA) and PHA [3,4]. MA can be further processed into PA66 (Nylon) and PET [3,5]. Here, we study gene expression, intracellular precursor level and metabolic fluxesin various strain backgrounds and under diverse growth, stress and production conditions using microarrays, GC- and LC-MS, and ^{13}C metabolic flux analysis [6,7] with the aim to create superior cell factories;

(ii) *P. putida* is studied in bioelectrochemical systems under anoxic conditions. We take a systems biological look at the strain under electro-fermentative conditions and inspect mRNA and protein levels, as well as metabolic activities using available ‘omics technologies [1,8];

(iii) we study *P. aeruginosa* growing on lung-specific carbon sources at the metabolic level by integrating transcriptomics and proteomics data with intracellular metabolite pools (e.g. CoA esters) and metabolic fluxes, eventually unraveling links between carbon metabolism and the cells’ infectivity [2,9].

[1] Weimer et al. (2020) Appl Microbiol Biotechnol 104:7745–7766.
[2] Dolan et al. (2020) mBio 11:e02684-19.
[3] Kohlstedt et al. (2018) Metab Eng. 47:279-293.
[4] Borrero-de Acuña et al. (2021) Microb Biotechnol 14:2385-2402.
[5] Kohlstedt et al. (2022) Metab Eng 72:337-352.
[6] Gläser et al. (2020) Microb Cell Fact 19:160.
[7] Kohlstedt et al. (2019) Metab Eng 54:35–53.
[8] Pause et al. (2023) Microb Biotechnol. Under revision.
[9] Dolan et al. (2022) mBio 13:e02541-22.

Poster 2
Metabolic Engineering of *Pseudomonas putida* for Enhanced Malonyl-CoA Availability and Polyhydroxyalkanoate Production

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Malonyl-CoA serves as a crucial precursor for the biosynthesis of various high-value compounds, including polyketides, carboxylic acids, biofuels, and polyhydroxyalkanoates (PHAs). However, its intracellular availability is limited due to competition with essential cellular metabolic pathways. To address this limitation, we undertook metabolic engineering of *Pseudomonas putida* strain SEM11. We strategically knocked out specific combinations of genes involved in glycolysis, TCA cycle, and fatty acid biosynthesis to enhance the malonylCoA pool. To quantify malonyl-CoA accumulation, we employed an enzyme-coupled biosensor based on the rppA gene, encoding type III polyketide synthase RppA, which converts malonyl-CoA into flaviolin, displaying a red color. Strains exhibiting enhanced malonyl-CoA accumulation were identified via a colorimetric screening method, displaying increased red pigmentation. HPLC analysis confirmed that four modified strains exhibited statistically significant increases in flaviolin production compared to the parental strain. To assess PHA production, we utilized Nile Red, a lipophilic fluorescent dye that binds to PHA granules, for microscopy analysis and to compare the PHA production of the strains over time. Our results demonstrated increased PHA production in most engineered strains during the early stages of production.

Poster 3
Mutagenesis of the bifunctional chorismate mutase / prephenate dehydratase PheA and its influence on viability and production in *Pseudomonas taiwanensis* VLB120

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Environmental pollution and decreasing fossil resources require the focus on bio-production of valuable compounds. As chorismate represents one common precursor for various compounds, the generation of a chorismate production chassis and its combination with different production modules might be a convenient step towards a more unified production setup.

Pseudomonas taiwanensis naturally bears a versatile metabolism, enabling the usage of various sustainable carbon sources combined with a high tolerance towards accumulation of aromatic compounds. In this organism, chorismate is generated via the shikimate pathway and used for aromatic amino acid biosynthesis or secondary metabolite production. Previously, genome reduction and metabolic engineering were successfully applied to increase chorismate formation and its flux towards tyrosine or phenylalanine. However, not only its generation but also its availability for heterologous enzymes should be considered. Thus, this work addresses the increased availability of chorismate exemplarily for hydroxybenzoate production. Therefore, targeted mutations were inserted in the bifunctional chorismate mutase / prephenate dehydratase (PheA) that catalyzes the integration of chorismate in aromatic amino acid biosynthesis. To prevent auxotrophy-related increased costs and workload of potential industrial applications, a reduced instead of obliterated PheA activity was aimed.

In total, up to ten different *pheA* variants combined with 11 production modules for five different hydroxybenzoates were tested and characterized regarding growth as well as production in comparison to the performance of respective *pheA* wildtype strains. This way, chorismatederived production of e.g. salicylate could already be increased from 2.7 mM to 3.3 mM equaling yields of 16.0±0.1 %Cmol/Cmol and 19.1±0.3 %Cmol/Cmol respectively

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Poster 4
Generate quantitative mechanistic insights into processes governing intracellular solvent concentrations using fluorescent biosensors

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Today, we still rely on the production of bulk building blocks asthe basis of our petrochemical industry. In contrast, PROSPER targets the bio-based production of hydrophobic aromatics such as styrene, benzene and ethylbenzene, addressing major challenges such as

climate change and environmental pollution. However, the toxicity of these products requires the development of improved solventtolerant biotechnological chassis to ensure the efficient production of hydrophobic aromatics.

In my project within PROSPER, I aim to use fluorescent whole-cell biosensors to gain a deep mechanistic insight into the tolerance to intracellularly produced rather than an externally added chemicals. To this end, fluorescent biosensors will be developed based on transcriptional regulators of genes that encode solvent efflux pumps or catabolic pathways for aromatic solvents. Once the biosensors are successfully characterized, they will be used to study solvent tolerance with regard to the difference between internal production and external addition of a solvent. The results of these studies will be used to rationally develop *Pseudomonas taiwanensis* chassis that exhibit increased solvent production tolerance. These chassis and biosensors will serve as a foundation for enhancing of the microbial production efficiency and achieving the second phase of hydrophobic aromatics.

During the 3rd *Pseudomonas* Grassroots Meeting, I will focus on the construction and characterization of various fluorescent whole-cell biosensors for detecting intracellular aromatic solvents in different *Pseudomonas taiwanensis* chassis. Additionally, I will provide initial insights into the tolerance mechanisms through external addition, and I will illustrate further aspects of my research schematically.

Poster 5
Engineering novel metabolic pathways for the production of aromatic compounds

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Aromatic hydrocarbons are traditionally produced by oil refining, associated with energy-intensive processes and fossil resources. To manage the transformation of the petrochemical industry towards a sustainable bio-based industry it would a key achievement to establish microbially catalyzed syntheses for these aromatic compounds. In this study we used highly solvent-tolerant *Pseudomonas taiwanensis* strains to produce 4-ethylphenol in a *de novo* synthesis from glucose or glycerol as carbon source. This was achieved by the expression of three heterologous enzymes converting tyrosine to the final aromatic product. However, the last enzyme in this cascade is a major bottleneck. Although its activity can be increased by the supplementation of osmolytes we aim to significantly improve the enzyme by coupling the production of 4-ethylphenol to growth, allowing us to perform adaptive laboratory evolution. Furthermore, we are aiming to establish metabolic routes for the production of highly hydrophobic aromatic solvents such as benzene, toluene, and ethylbenzene. We are screening and engineering several enzymes such as aldehyde-deformylating oxygenases to produce alkylbenzenes from their corresponding aldehydes. The issue of low initial enzymatic activity is tackled by random mutagenesis and subsequent biosensor screening or adaptive laboratory evolution employing a strain that is able to catabolize the final product.

Poster 6
Regulate EG and TA metabolism in Pseudomonas umsongensis GO16

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Pseudomonas umsongensis GO16, isolated from the soil of a polyethlyene terephthalate (PET) bottle processing plant, exhibits remarkable versatility in utilising various substrates, including PET monomers, terephthalic acid (TA) and ethylene glycol (EG). Additionally, GO16 can synthesise both medium and short-chain length polyhydroxyalkanoates (PHA), making it a promising candidate for upcycling PET waste into versatile PHA materials.

While the proof-of-concept for the biotechnological strategy to upcycle PET has been demonstrated, enhancing the efficiency of TA and EG metabolism remains a challenge. To address this issue, genetic engineering tools to delete, overexpress and integrate genes were employed. In *P. umsongensis* GO16, TA can be catabolised by the enzymes encoded in *tph* operon into protocatechuate (PCA). PCA is metabolised into TCA cycle intermediates through β-ketoadipate pathway. The deletion of the native *pcaGH* to disable the PCA ortho-cleavage pathway and the strain couldn't grow with TA. The introduction of the PCA meta-cleavage pathway from *Sphingobium* sp. SYK-6 recovered the growth. Regarding EG metabolism, a LysR type transcriptional regulator (LTTRs) *ttdR* is located 5'- of the gene for glyoxylate carboligase (*gcl*) was deleted. While the wild-type GO16 can utilise EG as a sole carbon and energy source, the GO16 Δ*ttdR* lost this capacity, highlighting the critical role of *ttdR* in EG metabolism. Furthermore, overexpressing ttdR resulted in a 10-hour shorter lag phase and a 1.25- fold higher biomass compared to the control strain when cultivated in 30 mM EG.

Poster 7
Mining phage transcription regulatory elements for synbio applications using ONT-cappable-seq

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Bacteriophages are potent 'bio-engineers' that are able to convert their host bacteria within a matter of minutes for a streamlined production of phage progeny. To achieve this, they encode a plethora of effector proteins and tailored mechanisms for their regulation. Since the advent of molecular biology these have been harvested and put to use as molecular tools and incorporated into functional DNA parts such as (orthogonal) promoters and terminators that are widely adopted. However, most of this pioneering work has been oriented towards *Escherichia coli* and its phages. Parts and tools from these are being transferred to other species, often leading to aberrant performance as these are not tailored to these new hosts.

We set out to acquire new tools and DNA parts from phages tailored towards *Pseudomonas* species. To this end, we have developed an RNA sequencing method termed ONT-cappable-seq that enables end-to-end sequencing of primary unprocessed RNAs (1). Applied on pseudomonads and their phages, this resulted in the large scale identification of new (orthogonal) promoters and terminators as well as other RNA features such as introns and sRNAs. The performance of promoters and terminators were characterized in pseudomonads, demonstrating their potential for application in synthetic DNA circuits and orthogonal expression cassettes (2, 3). Our method thus enables straight-forward identification and development of potent DNA parts for non-model hosts. In addition, it provides a better understanding of the operon layout of different phages, enabling better informed phage engineering approaches.

1. Putzeys,L., Boon,M., Lammens,E.-M., Kuznedelov,K., Severinov,K. and Lavigne,R. (2022) Development of ONT-cappable-seq to unravel the transcriptional landscape of Pseudomonas phages. *Comput. Struct. Biotechnol. J.*, **20**, 2624–2638.
2. Lammens,E.-M., Putzeys,L., Boon,M. and Lavigne,R. (2023) Sourcing Phage-Encoded Terminators Using ONT-cappable-seq for SynBio Applications in Pseudomonas. *ACS Synth. Biol.*, **12**, 1415–1423.
3. Lammens,E.-M., Feyaerts,N., Kerremans,A., Boon,M. and Lavigne,R. (2023) Assessing the Orthogonality of Phage-Encoded RNA Polymerases for Tailored Synthetic Biology Applications in Pseudomonas Species. *Int. J. Mol. Sci.*, **24**, 7175.

Poster 8
NxtGenWood: Converting wood-based phenols to value added products

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Lignin, one of the most abundant natural polymers on the planet, is also one of the most underutilized due to its recalcitrant nature. Our project aims to investigate novel ways to valorise lignin into valueadded products utilizing bacterial cultures as biocatalysts. In order to achieve this, different bacterial species were selected from the literature due to their reported ability to use lignin derived phenols. The bacteria were tested for growth on the three main phenolic acids present in lignin (ferulic, sinapic and *p*-coumaric acid). The results from these experiments showed that *Pseudomonas* species showed the most promise for growth on phenolic acids.

As sinapic acid turned out to be an unstable compound that was not suitable as growth substrate, we focused on ferulic and *p*-coumaric

acid. Our next objective was to obtain a candidate that showed high productivity from the mentioned phenolic acids. We compared the different Pseudomonas species for their ability to accumulate the internal polymer PHA, one key indicator of productivity. Out of the selected candidates we decided to continue with *P. putida* KT2440 as it is a promising model for genetic modification and for valorisation of lignin based phenols. Future experiments will be focused on developing a process using the optimal bacterial strains for the biotransformation of lignin derived phenols into value-added products.

Poster 9
Precision method to edit the TOL system encoded by the plasmid pWW0 of *Pseudomonas putida* mt-2

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We have implemented a precise method to edit the TOL system borne by pWW0 of *Pseudomonas putida* mt-2. This soil bacterium has the catabolic capacity to use recalcitrant pollutants such as toluene, xylene and related compounds as sole carbon source. Although intensive biochemical, metabolic and genetic research has been performed over many years, the physical architecture and arrangement of its enzymatic components are yet to be precisely described. To shed light on this matter, genetic editions of the native pWW0 plasmid (i.e. deletions and protein fusions) are necessary. For this purpose, the pEMG/pSW-I system is used for either insertion of heterologous DNA segments (coding for fluorescent proteins), as well as for scarless deletions. The procedure is based on the homologous recombination forced by the appearance of double-strand breaks (DSBs) upon cleavage in vivo by I-SceI. Integration of pEMG + edited DNA into the pWW0 plasmid endows flanking I-SceI target sequences in the region of interest. Intracellular expression of the I-SceI enzyme is brought about *in vivo* by the cognate pSW-I (I-SceI) plasmid. Finally, homologous recombination leads to edited cells. The steps of the insertion/deletion process are: 1) cloning regions homologous to those flanking the desired deletion/replacement into pEMG; 2) cointegrating the resulting plasmid into the target host; 3) introduction of pSW-I into cells bearing the cointegrate; 4) induction of the DSBs; 5) selection of the deleted/ replaced strain and; 5) pSW-I curation.

Poster 10
Unveiling *Pseudomonas*' enzymatic toolbox for 5-(hydroxymethyl)furfural (HMF) detoxification: Towards accelerated 2,5-furandicarboxylic acid (FDCA) production

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Due to its tolerance properties, *Pseudomonas* has gained particular interest as host for oxidative upcycling of the toxic aldehyde HMF into FDCA, a promising biobased alternative to terephthalate in polyesters. However, until now, the native enzymes responsible for aldehyde oxidation are unknown. Here, we report the identification of the primary HMF converting enzymes of *P. taiwanensis* VLB120 and *P. putida* KT2440 by extended gene deletion experiments opening up new opportunities for targeted tolerance engineering of these organisms. The key players in HMF oxidation were found to be a molybdenum-dependent periplasmic oxidoreductase and a cytoplasmic dehydrogenase. Deletion of the corresponding genes almost completely abolished HMF oxidation, leading instead to aldehyde reduction. In this context, two aldehyde-reducing dehydrogenases were also revealed. These discoveries enabled us to specifically improve Pseudomonas' aldehyde oxidation machinery by genomic overexpression of the respective genes via exchange of the native promoter regions for strong and constitutive synthetic promoters. The resulting BOX strains (Boosted OXidation) represent useful hosts for biotechnological synthesis of FDCA from HMF since aldehyde toxicity as drawback of wholecell catalysis is attenuated by increased conversion rates. Furthermore, the non-oxidizing deletion mutants provide a solid foundation for future investigations into secondary aldehyde tolerance mechanisms that are distinct from rapid conversion.

Poster 11
Enhancing production tolerance with growth-coupled evolution

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Aromatic compounds are vital in industry, serving as organic solvents and key materials in plastics, resins, adhesives, and more. Although bio-based production offers solutions to environmental challenges like climate change and pollution, the competitiveness of biotechnological production is hindered by the economic advantage of fossil bulk chemicals and the lack of effective bio-production techniques. One major challenge in this context is the toxicity of these compounds to microorganisms commonly used in biotechnology.

The PROSPER project aims to address this challenge by demonstrating the bio-based synthesis of hydrophobic aromatic chemicals, with a primary focus on overcoming tolerance limitations during production. Within the scope of PROSPER, this project concentrates on enhancing solvent tolerance through engineering and evolution of robust producer strains. To achieve this, different strategies will be employed to link microbial growth with solvent production. This coupling will be established by either integrating the production pathway into the central metabolism or using a solvent biosensor to regulate growth based on solvent levels. Once this coupling is achieved, adaptive laboratory evolution will be conducted to cultivate producer strains and improve their tolerance to the produced solvents. This process aims to identify key mutations responsible for enhanced solvent tolerance. Our research will not only contributes to the development of robust *Pseudomonas taiwanensis* chassis strains but also will advances our understanding of solvent tolerance in *Pseudomonas* species.

At the 3rd *Pseudomonas* Grassroots Meeting, we will present our progress in constructing robust producer strains and provide a schematic overview of the concept of our research.

Poster 12
A SEVA-based, CRISPR-Cas3-assisted genome engineering approach for *Pseudomonas* with efficient vector curing

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The development of CRISPR-Cas-based engineering technologies has revolutionized the microbial biotechnology field. Over the years, the Class 2 Type II CRISPR-Cas9 system has become the gold standard for genome editing in many bacterial hosts. However, the Cas9 system does not allow efficient genomic integration in *Pseudomonas* without the assistance of recombineering enzymes. The proposed CRISPR-Cas3 editing system facilitates the creation of genomic alterations in *Pseudomonas putida* and *Pseudomonas aeruginosa* in a straightforward manner, with efficiencies exceeding 80%. The two-vector system combines a broad host range CRISPR-Cas3 targeting plasmid with a SEVA (Standard European Vector Architecture) plasmid for homologous directed repair, which introduces clean deletions, insertions or substitutions in the bacterial genome within a week. In addition, by applying a universal self-targeting spacer present on a second SEVA plasmid post-modification, the Cas3 system rapidly cures all helper vectors, including itself, in only a few days, with up to 100% curing efficiency. The system has also been successfully applied for *Pseudomonas* bacteriophage genome editing. Moreover, a vector set of the plasmids with multiple antibiotic markers allows for application in various Gram-negative hosts and different designs, making it a broad engineering tool for viral and bacterial engineering.

Poster 13

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Bacterial motility is energy-intensive, and thus more bacterial resources are allocated to protein production at the expense of motility. *Pseudomonas putida* is used for various industrial applications, including the expression of recombinant proteins, because of its inherent properties. In this study, the *fleQ* gene, which primarily regulates flagella synthesis, was deleted in *P. putida* to enhance gene expression capacity. Consequently, the *fleQ*-null strain lacked cellular motility and formed less biofilm. Furthermore,

the expression level of circuit genes increased in the mutant strain cultured in nutrient-rich media when compared with that in the wild-type strain. This was attributed to the reallocation of cellular resources caused by the reduction in the cost of cellular motility, which thereby increased the availability of ribosomes and ATP production in the cell. The overexpression of *fleQ* negatively impacted cellular fitness while restoring cellular mobility and biofilm formation. In addition, the wild-type strain showed higher expression of the reporter gene but a significant decrease in growth when grown in minimal media. This result is caused by a trade-off between reporter protein synthesis and cellular fitness due to limited cellular resources. Interestingly, the mutant strain maintained cellular fitness and activity of reporter proteins when cultured under nutrient-poor conditions. Thus, manipulating resource allocation by inactivating cellular motility contributes to developing a chassis strain with nutrient-independent gene expression capacity.

Poster 14
Developing a straightforward I-sceI mediated one-plasmid recombination system for efficient genome editing in *P. putida* KT2440

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Pseudomonads have become an increasingly important and reliable platform for bioproduction due to its versatile metabolism and high resistance towards oxidative stress. To better exploit the production potential of Pseudomonads, some genome editing methods have been established and implemented, but they are still quite complex and time consuming. Hence, we design and demonstrate a straightforward genome editing method for *Pseudomonas putida* KT2440 that is very fast, robust and user-friendly. It's based on the widely-used I-sceI mediated recombination method and has been obtained after two rounds of optimization. Firstly, a counterselection system was constructed by the use of the levansucrase coding gene sacB from *Bacillus subtilis,* which could effectively skip the repetitive plasmid curing steps; Secondly, all modular components are integrated into a single plasmid, which could significantly minimize the steps required and also enabling stable and efficient operation; Finally, a straightforward genome editing method that takes only three days is perfectly demonstrated. This will give a considerable boost to the development of studies on Pseudomonads.

Poster 15
De novo styrene production and biotransformation from trans-cinnamic acid using *Pseudomonas taiwanensis*

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Organic solvents within the $\log P_{O/W}$ range of 1.5 to 4 are highly toxic for microorganisms [1], but some bacteria such as *Pseudomonads* show a high natural tolerance primarily mediated by solvent efflux pumps. The *Pseudomonas taiwanensis* GRC strains [2] feature improved bioprocess performance and provide a platform for production of aromatics, including the solvent styrene. The styrene biosynthesis pathway starts with deamination of phenylalanine by phenylalanine ammonia lyase (PAL), yielding trans-cinnamic acid (tCA). In a second step, tCA is decarboxylated to styrene catalysed by ferulic acid decarboxylase (FDC).

Here, we report *de novo* bioproduction of styrene from glucose using phenylalanine overproducing – and hence more sensitive – *P. taiwanensis* strains with concentrations up to 1.1 mM dissolved in the medium. In a different approach, we integrated a styrene production module in strains without modifications in precursor synthesis but high solvent tolerance and supplemented tCA to the medium. Cell growth and complete conversion of tCA was achieved for addition of up to 20 mM to the medium using glucose as carbon source. Biotransformation of high amounts of tCA resulted in emulsification of the culture, increasing the solubility of styrene far beyond the solubility limit of 2.8 mM in aqueous solution.

[1] Ramos, J.L., et al. (2002). Annu Rev Microbiol. **56**(1), 743-768.
[2] Wynands, B., et al. (2019). ACS Synth Biol. **8**(9), 2036-2050.

Poster 16
In situ extraction of oleochemicals produced by *Pseudomonas taiwanensis* VLB120

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Biotechnological production of long-chain hydrocarbons is a promising, sustainable alternative to petroleum- or plant-oil-based processes. Due to its high redox cofactor regeneration rate and high tolerance to organic compounds, *Pseudomonas taiwanensis* VLB120 is an excellent production host for such attempts. Regarding product purification, *in situ* product extraction was shown to be beneficial for the removal of oleochemicals from the cultivation broth. By application of a well-suited solvent, product inhibition and product degradation can be circumvented and the number of downstream processing steps can be reduced. However, out of countless potential solvent candidates, one wants to choose the solvent with the best overall performance.

Here, we carried out a comprehensive, reductive solvent screening for *in situ* extraction of oleochemicals produced by genetically engineered *P. taiwanensis* VLB120. In a first pre-screening, 95 solvents were investigated regarding their physicochemical properties such as the density, the flash point, and the log *P* value. The best 20 candidates of the pre-screening were evaluated with regard to the product's partition coefficient, separation of aqueous and organic phase, and solvent safety. In the last step, five candidates were tested for their biocompatibility and biodegradability. Here, the high tolerance of *P. taiwanensis* VLB120 towards potentially growth-inhibiting organic solvents was shown to be beneficial.

The screening resulted in 2-undecanone as a solvent for efficient extraction of oleochemicals. This solvent outperforms the previously applied organic phases regarding biocompatibility, process control, and safety. We believe that this methodology can also benefit other biotechnological applications.

Poster 17
Broad host range genomic modification tool based on fluorescence selection

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Genomic modifications can be a time-consuming work. Even if the method can modify a strain in some days, curing and removing resistance genes requires time. In 2011, Martínez-García et al. developed the pEMG vector, which is widely used in Pseudomonas. Four steps are needed: 1) integration of the pEMG vector; 2) introduction of an inducible I-*SceI* expressing vector; 3) counterselection to find clones who lost the marker from step 1; 4) PCR to verify the modification and curing.

Here, we demonstrate a new suicide vector for targeted genomic modifications based on the pEMG vector. It contains an arabinose inducible I-*SceI* gene, a constitutively expressed *msfGFP*, and a resistance gene. The vector is transferred by electroporation to the strain. Green fluorescent colonies were transferred to LB media and induced with arabinose. Dilution series were plated on LB agar plates, and arising colonies with no green fluorescence were tested by PCR to verify the modification. Using green fluorescence allows for a fast selection of clones with the integration and the subsequent release of the vector from the genome.

The new method shown here enables the generation of genomic modifications in three to four days. It is based on homologous recombination, double-strand breaks, and an optical selection by GFP fluorescence. We were able to circumvent the need for plasmid replication; therefore, the method can be used in many different bacteria. This tool improves strain engineering for many bacterial strains, and we hope to support the synthetic biology community by generating modified organisms.

Poster 18
E-Biotech-Project *ePseudomonas*

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Poster 19

Genetic Manipulation to Expand the Product Spectrum of a Co-Culture consisting of *Synechococcus elongatus* PCC 7942 *cscB* and Pseudomonas putida EM178 *att*Tn7::*cscRABY*

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Facing climate crisis, industries should reduce greenhouse gas emissions, e. g. by consuming them in sustainable production processes. Such a process represents our photoheterotrophic co-culture: From CO_2 and light, the cyanobacterium *Synechococcus elongatus* PCC 7942 *cscB* accumulates sucrose under salt stress. The secreted disaccharide is used by the soil bacterium *Pseudomonas putida* EM178 *att*Tn7::*cscRABY* to synthesize the plastic alternative polyhydroxyalkanoates.

Intending to transform the co-culture into a platform process for bioproduction, a transfer plasmid was designed to expand the heterotrophic´s product spectrum. Plasmid selection was realized *via* the sucrose operon *cscRABY* instead of antibiotics. Subsequent size reduction first eliminated the operon´s repressor CscR, while the final minimal plasmid encoded only CscY, a porin essential for sucrose uptake. As first “product”, eGFP was chosen for simple quantification. Different plasmid variants served to evaluate the effect of plasmid carriage and protein expression on growth.

The growth behavior of the resulting *P. putida* strains was studied in axenic growth experiments, in both microtiter plates and shake flasks. Compared *to P. putida* EM178 *att*Tn7::*cscRABY* with 0.37 ± 0.02 h-1 , their specific growth rate on sucrose was about 0.14 ± 0.02 h-1 . Moreover, all strains produced eGFP. One-week experiments with *S. elongatus* confirmed the suitability of the plasmid-bearing heterotrophs for the co-culture: they grew similarly to their genomic relative and had no adverse effects on the phototrophic partner.

Poster 20

Amino acid auxotrophy in *Pseudomonas putida*: Experimental and *in silico* investigation of amino acid demand in a continuous system

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Amino acids, being the building blocks of proteins, are an essential cellular resource. Amino acid auxotrophy, the inability to synthesize amino acid(s), is a well-studied phenomenon in popular biotechnological hosts like *Escherichia coli*. In contrast, knowledge on amino acid auxotrophy in *Pseudomonas putida* is scarce. In this work, we aimed to quantify the specific demand for single amino acids in *Pseudomonas putida*, using amino acid auxotrophy as a tool.

To this end, strains lacking the ability to synthesize l-leucine (considered the most abundant amino acid in *P. putida*) or l-tryptophan (considered one of the least abundant amino acids) were engineered using CRISPR assisted cytidine base editing [1]. Consecutively, amino acid sensitivity assays were performed and specific amino acid uptake rates were determined in shake flasks. This information assisted in the design of a continuous bioreactor process, where the growth rate is dictated by limiting amino acid supply, rather than by the supply of glucose.

First results of these continuous processes show that different stationary states were reached, in which the biomass, CO_2 production rate and oxygen uptake rate remained constant over time. In parallel, adapted versions of the genome-scale model (*i*JN1462) of *P. putida*, lacking the respective genes for amino acid biosynthesis, were created [2]. Using the COBRA Toolbox, flux balance distributions were found that predict growth rates in dependence of the specific amino acid uptake rate. With this *in silico* analysis and our experimental data, we show that there are quantitative differences for specific amino acid demand in *P. putida*.

[1] Volke, Daniel C., et al. (2022) *Nature Communications* 13.1: 3026.
[2] Nogales, Juan, et al. (2020) *Environmental microbiology* 22.1: 255-269.

Poster 21

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The soil bacterium *Pseudomonas putida* is a robust biomanufacturing host that assimilates a broad range of substrates while efficiently coping with adverse environmental conditions. *P. putida* is equipped with functions related to one-carbon (C1) compounds (e.g. methanol, formaldehyde, and formate) oxidation—yet pathways to assimilate these carbon sources are largely absent. In this work, we adopted a systems-level approach to study the genetic and molecular basis of C1 metabolism in *P. putida*. RNA sequencing identified two oxidoreductases, encoded by *PP_0256* and *PP_4596*, transcriptionally active in the presence of formate. Quantitative physiology of deletion mutants revealed growth defects at high formate concentrations, pointing to an important role of these oxidoreductases in C1 tolerance. Moreover, we describe a concerted detoxification process for methanol and formaldehyde, the C1 intermediates upstream formate. Alcohol oxidation to highly-reactive formaldehyde by PedEH and other broad-substrate-range dehydrogenases underpinned the (apparent) suboptimal methanol tolerance of *P. putida*. Formaldehyde was mostly processed by a glutathione-dependent mechanism encoded in the *frmAC* operon, and thiol-independent FdhAB and AldB-II overtook detoxification at high aldehyde concentrations. Deletion strains were constructed and characterized towards unveiling these biochemical mechanisms, underscoring the worth of *P. putida* for emergent biotechnological applications—e.g. engineering synthetic formatotrophy and methylotrophy.

Poster 22

Model-driven analysis of the metabolic burden of heterologous protein production in *Pseudomonas putida*

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The production of heterologous proteins is a drain of energy and resources for engineered cells. In this work, we used a model-driven approach to quantitatively analyze this metabolic burden in *Pseudomonas putida* KT2440. Experimentally, we used a strain with two fluorescent reporter proteins: mCherry integrated into the genome with a constitutive promoter, and the fusion protein MBP-GFP in a plasmid with an inducible promoter. The strain was cultured in a benchtop bioreactor in batch mode, with minimal media with ^{13}C-glucose. Afterwards, we quantified the intracellular fluxes using a model of the central carbon metabolism of *P. putida* for the strain with increasing levels of metabolic burden, i.e., with increasing inductor concentrations. We then used a constraint-based approach with a genome-scale model to analyze the metabolic burden in terms of the rearrangements of both carbon and energy fluxes.

Poster 23

Engineering *P. putida* as a whole-cell biosensor for secreted metabolites detection and PET-degradation characterization.

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Here, we propose to use *P. putida* as a universal product sensor for a broad range of chemicals. *P. putida* harbors a multitude of degradation pathways that enable growth on many compounds as sole carbon or nitrogen source. This metabolic diversity includes transporters, regulators and certainly the enzymes, which can be exploited for the construction and implementation of new biosensors. Biosensors typically rely on the synthesis of a fluorescent protein mediated by a transcriptional regulator that recognizes the analyte of interest. While simple to implement and useful for the detection of many compounds, such designs also suffer from severe technical limitations. For example, the dynamic and operational ranges are often narrow, meaning that the concentration difference between the limit of detection and saturation of the system is relatively small and, also, the signal intensity difference is normally low. Furthermore, biosensors are often specific for one metabolite and cannot be repurposed easily. Additionally, the output of biosensors typically reflect the concentration inside the cells, while extracellular concentrations (titers) are the key performance indicator for microbial fermentations.

An alternative strategy is the coupling of growth to the presence of the compound of interest. This strategy has not been broadly applied

as it is often difficult to render the analyte essential for the cells. The versatile metabolism of *P. putida* can be exploited to this end. As indicated above, *P. putida* grows on structurally unrelated carbon and nitrogen sources, e.g., vanillate, ferulic acid or serine. Therefore, the metabolic network of *P. putida* can be engineered to make the cell growth dependent on these compounds. *P. putida* SENS was constructed in our lab by deleting all importers and kinases of glucose, gluconate and 2-ketogluconate as well as genes responsible for sugar oxidation. Thereby, P. putida SENS was rendered incapable to metabolize glucose or its 2 derivatives. Upon equipping the SENS strain with a constitutively expressed fluorescent marker, reliable fluorescence signals were detected in cultures supplemented with different chemicals. This method showed a broad operational range and high sensitivity. In the presented poster, I will show the flexibility of the system created to detect both analytes of industrial (lactate) and environmental interest (PET-degradation subproducts); and its adaptation to different screening formats as it could be: co-culture systems, analysis of culture supernatants, or detection of sub-products of an enzymatic in-vitro degradation.

Poster 24
Valorization of the C1-fraction from processed lignin by engineered *Pseudomonas putida*

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Exploration of new sustainable feedstocks for biomanufacturing is mandatory to make the bioeconomy reality. Whereas the first generation feedstocks are derived from biomass, which is also consumed by humans, and therefore compete with food production, second generation feedstocks do not. Lignocellulosic biomass is a promising source of feedstock. Besides the easy to degrade cellulosic fraction, the lignin part is hard to process. However, to achieve economical feasible biorefineries for lignin, all components of lignocellulosic biomass have to be valorized either for fuels, bulk chemicals or fine chemicals. While much focus is put on the valorization of the phenylpropanoids, the C1 molecules, methanol and formaldehyde are often overlooked.

In this project, I will establish *Pseudomonas putida* as a cell factory for utilizing the C1-fraction of processed lignin, using synthetic biology and Adaptive Laboratory Evolution (ALE). There are several possible pathways, which can be implemented to allow *P. putida* growth on methanol and foraldehyde. Here, I will implement the RuMP cycle, as it allows relative high growth rates combined with good energy efficiency. Through the use of artificial auxotrophies, growth of engineered strain with the RuMP implemented will be increasingly tight coupled to the use of methanol and/or formaldehyde as carbon source until these C1 molecules can serve as sole carbon source. During the evolution of the strain, attention will be placed on retaining the general fitness of the strain in the intended application in diverse growth phases. These traits have been neglected in previous studies for generation of synthetic methylotrophs leading to strains, which exhibit lag phase of up to 20 days, are not viable in stationary phase or do not grow in liquid medium.

Poster 25
Metabolic burden in *Pseudomonas putida*: from single proteins to entire pathways

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The introduction of heterologous metabolic pathways in a bacterial host entails an increased demand for energy and cellular resources such as amino acids, ribosomes, or polymerases. This leads to a redistribution from normal cellular functions towards the additionally implemented task. Limitations in any of these resources usually result in a decrease in growth rate and cessation of heterologous production, a condition defined as metabolic burden. We recently constructed *P. putida* CAP, a strain that allows to follow the cellular capacity during heterologous protein expression. This was achieved by placing the fluorescent mCherry gene under the control of a constitutive promotor in the chromosome of P. putida. With this strain at hand, we aimed to analyze in detail the effect of the expression of single heterologous proteins with the aim to expand this towards the expression of an entire pathway. By analyzing the effects of heterologous protein expression on transcriptional and translational level we could conclude that the bottle neck most likely can be found on the post-transcriptional level. Interestingly, the addition of amino acids did not result in more protein production but had a positive effect on growth. As a next step, we shifted our interest towards an entire metabolic pathway and now focus on the metabolic burden during the production of the monoterpenoid geranic acid, which carries great potential for various industrially relevant applications, e.g., as a fragrance or antifungal agent.
