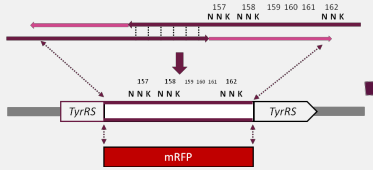
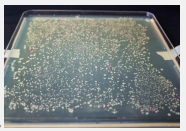
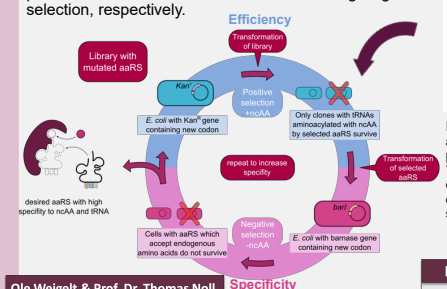
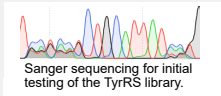
Library and Selection



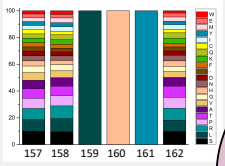


For the translational incorporation of ncAAs, a suitable tRNA/aminoacyl-tRNA synthetase pair (tRNA/aaRS) is required. Our generated tyrosyl-tRNA synthetase (TyrRS) library was adapted for the ncAA 2-nitro-L-phenylalanine. Selection for incorporation efficiency and specificity, resulted in ~ 800 clones after positive and ~ 100 clones after the following negative selection, respectively.





Sanger sequencing for initial testing of the TyrRS library.



Illumina sequencing revealed the amino acid distribution on variable positions of our TyrRS library in 70,000 clones. The final library encodes >> 95 % of all 9,261 different possible protein sequences.

Ole Weigelt & Prof. Dr. Thomas Noll, co-founders of Xell AG

Photolysis has a high innovation potential and could be used as a new purification method, e.g. for the very fragile essential blood-clotting protein factor VIII.

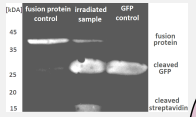
Prof. Dr. Nediljko Budisa (TU Berlin)

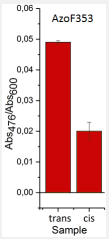
Budisa and his group provided insights into tRNA-synthetase selection and hosted one team member in their lab in Berlin.

Applications

Photolysis

We were able to cleave a streptavidin tag from a GFP fusion protein through the photolabile ncAA 2-NPA. It was incorporated by an aaRS [1]. Cleavage was achieved by irradiation with UV light from our self designed LED panel.



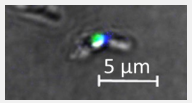


Photoswitching

We confirmed enzyme activity regulation on protein level using our self designed LED panel (shown in the exhibition space) to switch the *cis-trans*-conformation of the ncAA AzoF, incorporated in CrtI of the lycopene pathway.

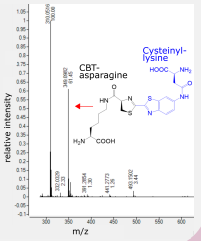
Labeling

We showed colocalization of RuBisCO (blue) [2], labeled with the fluorescent ncAA CouAA, along with the carboxysome [2], labeled with GFP (green).



Fusing

We designed and synthesized a novel ncAA called CBT-asparagine. It enables a highly specific covalent fusing reaction to another ncAA, cysteinyl-lysine. We confirmed the fusion *in vitro* via MS and successfully modeled several aaRS sequences for the translational incorporation of CBT-asparagine into proteins.



Dr. Sandip Jadhav (Bielefeld University)

Sandip Jadhav advised Daniel in synthesizing CBT-asparagine and cysteinyl-lysine and provided us with a chemical background knowledge.

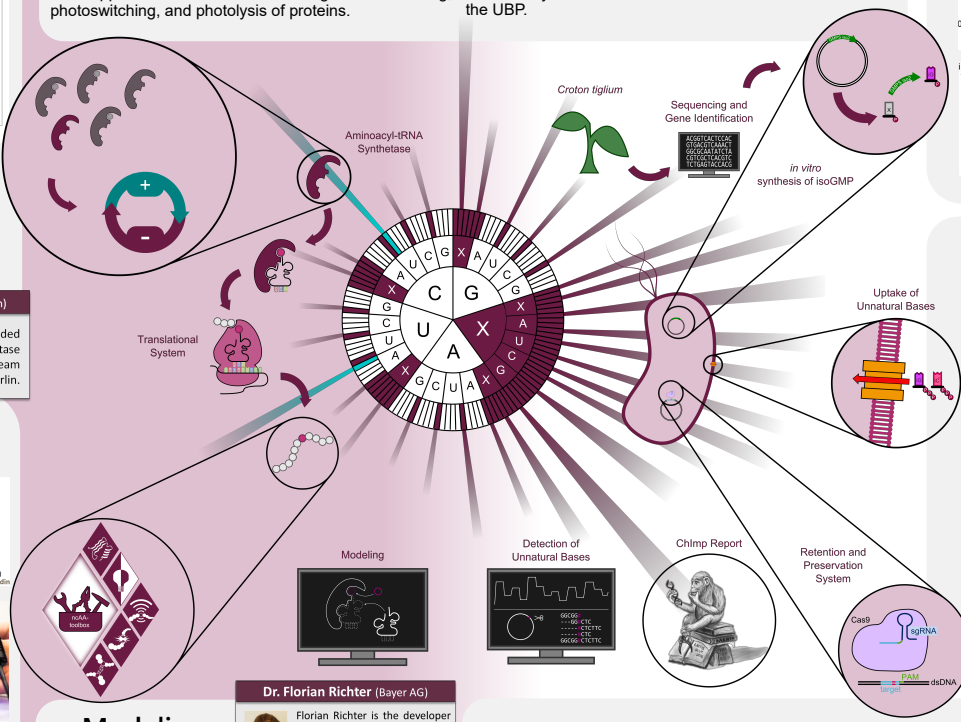
Abstract

Daniel Bergen, Christina Drake, Saskia Dymek, Maximilian Edich, Markus Haak, Lennard Karsten, Denise Kerkhoff, Yannic Kerkhoff, Michelle Liebers, Camilla März, Laura Schlüter, Olga Schmidt, Svenja Vinke, Christopher M. Whitford

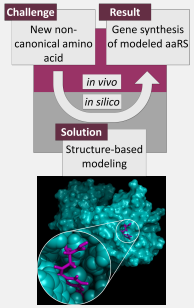
Protein design is usually limited to 20 amino acids. Since the chemical abilities of these amino acids are limited, a lot of interesting functions are not applicable in protein design. We aim to enable the **translational incorporation of non-canonical amino acids** (ncAAs) through an expanded genetic code.

First, we repurposed the rarely used amber stop codon for translational incorporation. Therefore, we adjusted aminoacyl-tRNA synthetases (aaRS) which charge the ncAA to the tRNA. We designed a **library** of aaRS and a suitable **selection** system for the development of customized aaRS which specifically incorporate the ncAA of interest. As a proof of concept, we applied different ncAAs e.g. for labeling, photoswitching, and photolysis of proteins.

To further improve the translational incorporation of ncAAs, we explored a new way to expand the genetic code by development of a semisynthetic organism. An additional **unnatural base pair** (UBP) generates 61 additional codons. Therefore, we improved an **UBP uptake system** and identified and characterized a **biosynthesis** pathway for *in vivo* production of isoG in *Escherichia coli*. We engineered a retention system and and established detection methods for the UBP.



Modeling



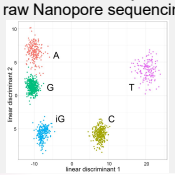
Dr. Florian Richter (Bayer AG)

Florian Richter is the developer of the Enzyme Design protocol ROSETTA. We met him in Cologne, where he gave us advice on using the protocol.

We modeled several tyrosyl-tRNA synthetase 3D structures with our own non-canonical amino acid CBT-asparagine in the active center. The ROSETTA enzyme design protocol [3] was used to predict promising amino acid residues to target in order to change synthetase specificity. Experimental validation *in vivo* was achieved by applying our own selection system.

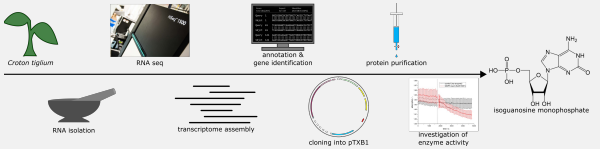
Detection & Software

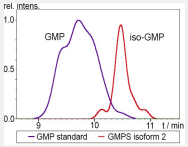
Our software **iCG** detects UBPs in a known sequence context based on linear discriminant analysis of normalized, raw Nanopore sequencing data.



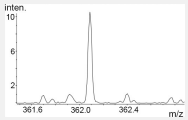
Our software **M.A.X** (Mutational Analysis Xplorer) filters databases to find enzymes with overlapping recognition sites, differing at a single position. Only if a UBP is located at this position, the enzymes cannot cut. We applied M.A.X to demonstrate the presence of an UBP.

Biosynthesis





We investigated the biosynthesis pathway of isoguanosine in *Croton tiglium*. After RNA isolation, RNA-Seq and transcriptome assembly, an isoform of guanosine monophosphate synthetase (GMPS-2) was identified as the most promising enzyme candidate. The functionality of the purified enzyme was confirmed through *in vitro* enzyme activity assays after heterologous expression in *E. coli*.



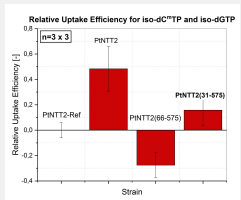
For further validation of the reaction products, we applied high performance liquid chromatographic analysis followed by mass spectrometry analysis.

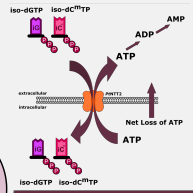
Dr. Vitor B. Pinheiro (UCL)

Pinheiro gave us very valuable advice regarding possible toxicity of UBPs in *E. coli*.

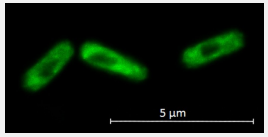
Uptake

Uptake of iso-dC^{m}TP and iso-dGTP in *E. coli* was facilitated by heterologous expression of the nucleotide transporter *Pt*NTT2 from the algae *Phaeodactylum tricornutum* [5]. Different transporter variants were designed and characterized. Since import of iso-dC^{m}TP and iso-dGTP leads to ATP export, the strains growing the weakest compared to the reference take up iso-dC^{m}TP and iso-dGTP.





Subcellular localization of a transporter-GFP fusion protein in the cell envelope was visualized by confocal laser scanning microscopy.



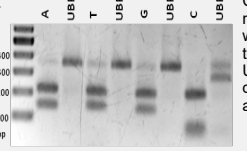
Biologio B.V. (The Netherland)

Biologio

Scientific discussions about base modifications throughout the entire project

Retention and Preservation

For the incorporation of an UBP in a plasmid we annealed two complementary ssDNA oligomers containing the unnatural nucleobases isoG and isoC^{m}, respectively, and performed a sticky end ligation. Different polymerases are able to incorporate isoG and isoC^{m} during *in vitro* replication (PCR).



For *in vivo* replication, a CRISPR/Cas9 system [4] was designed to retain and preserve the UBP. This works using sgRNAs complementary to the DNA sequence without the UBP. Hence, Cas9 induces the degradation of all plasmids without an UBP.

References:

[1] **Peters F.B.,** *et al.,* (2009). Photocleavage of the Polypeptide Backbone by 2-NitrophenylalaninePeters.*Chem. Biol. 16: 148–152.*

[2] **Fust A.,** *et al.,* (2014). The Transformers. From Carbon Dioxide to Biofuel, **DOI: 10.13140/ RG.2.2.30504.98566**

[3] **Richter F.,** *et al.,* (2011). De novo enzyme design using Rosetta3. PloS one, **6(5): e19230.**

[4] **Zhang, Y.,** (2017). A semisynthetic organism engineered for the stable expansion of the genetic alphabet. Proc. Natl. Acad. Sci. **114**: 1317–1322.

[5] **Ast, M.** *et al.,* (2009) Diatom plastids depend on nucleotide import from the cytosol. Proc. Natl. Acad. Sci. U. S.A. 106, 3621–3626.

Acknowledgements: Boas Pucker, Julian Droste, Dr. Christian Rückert, Prof. Dr. Kristian Müller, Prof. Dr. Jörn Kalinowski and the research group Microbial Genomics and Biotechnology and all members of the CeBiTec.

Submitted BioBricks:

Library & Selection: BBa_K2201900, BBa_K2201901, BBa_K2201411, BBa_K2201400

Application: BBa_K2201200, BBa_K2201207, BBa_K2201204, BBa_K2201208, BBa_K2201302

Modeling: BBa_K2201301, BBa_K2201302, BBa_K2201300

Retention & Preservation: BBa_K2201032,

Uptake: BBa_K2201004, BBa_K2201000, BBa_K2201001, BBa_K2201002, BBa_K2201003

Biosynthesis: BBa_K2201060, BBa_K2201061

Partner

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