Precise Genome Editing via CRISPR/Cas9 for *in vivo* Production of Sialyllactose

Freya Körtje [1,2], Miriam Aßmann [1], Paul Bubenheim [2], Andreas Liese [2], Jürgen Kuballa [1]

[1] GALAB Laboratories GmbH, Am Schleusengraben 7, 21029 Hamburg, Germany
[2] Institute of Technical Biocatalysis, Hamburg University of Technology, Denickestr. 15, 21073 Hamburg, Germany
Corresponding author: Freya.Koertje@galab.de

Motivation

- Human milk oligosaccharides (HMOs) such as sialyllactose (SL) play a crucial role in human health and promote the growth of naturally occurring human gut bacteria like bifidobacteria. In addition, HMOs prevent the adsorption of pathogens by acting as acceptor mimics for pathogen binding [1].
- For the development of a SL-producing strain for use in whole-cell biocatalysis, *E. coli* W3110 wild-type (WT) strain is modified (Fig. 1) using CRISPR/Cas9.
- To overcome challenges such as need for cofactor regeneration, substrate uptake, product release, and prevention of intermediate degradation in competing pathways, gene mutations were introduced (*). Additionally, specific genes were overexpressed (orange), knocked-out (X) and knocked-in (green).
- As a result, two substrates are required for the multi-enzyme SL production: glucose for product synthesis and glycerol for cell growth.

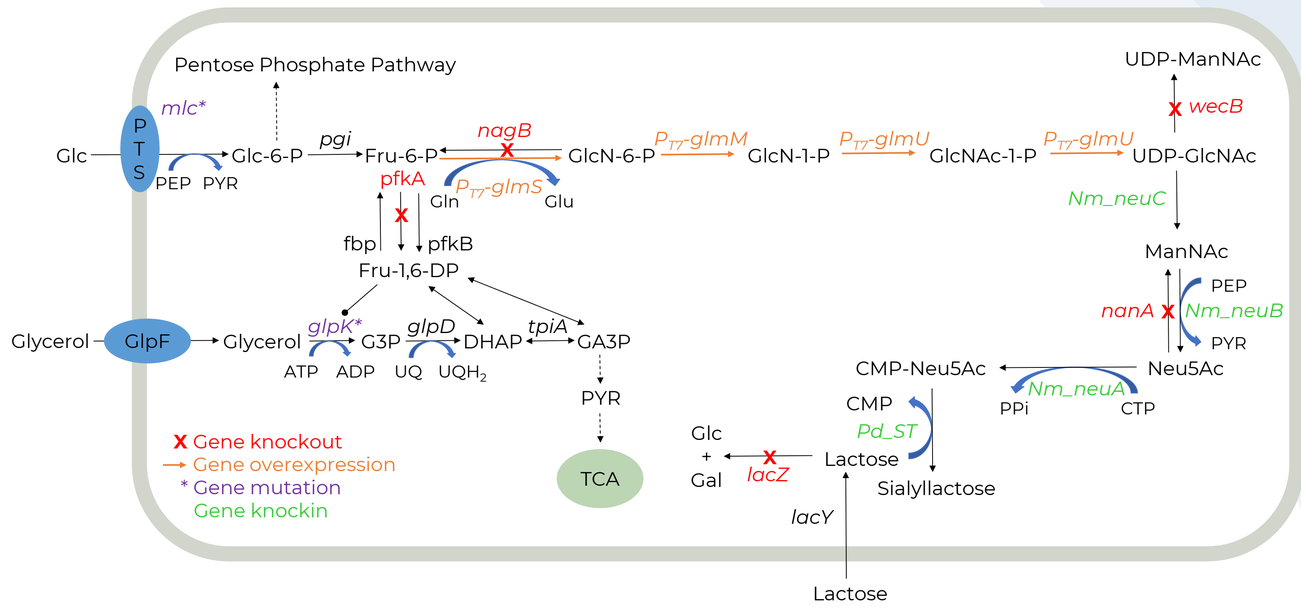

Fig. 1: Reaction scheme of the SL-producing *E. coli* cell for the use in whole-cell biocatalysis starting from glucose.

Approach

Theoretical strain construction and genome editing using CRISPR/Cas9:

- Design of guideRNA-plasmids (gRNA) for specific targeting of the genomic *E. coli* DNA (Fig. 2) via PCR, restriction digest, and ligation.
- Synthesis of double-stranded donor DNA (dsDonorDNA) via overlap extension PCR.
  - ➢ knock-out: gene sequence deletion except of the start codon and the last 7 amino acids.
  - ➢ knock-in: introduction of gene of interest (GOI) sequences.
- Transformation of *E. coli* with pEcCas-plasmid, pgRNA-Plasmid, and dsDonorDNA via electroporation.
- Check-PCR of potential edited colonies, sequencing and growth analysis.

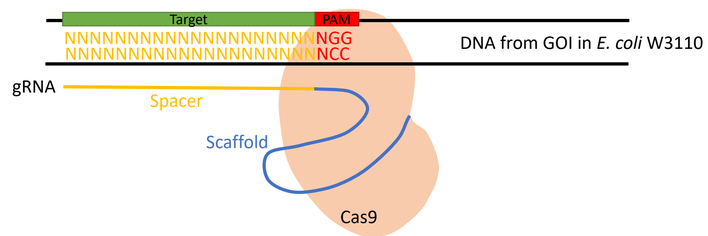

Fig. 2: gRNA-Cas9 complex binding the genomic DNA of *E. coli*.

Experimental results

- Modification of the *E. coli* W3110 genome: Δ*pfkA*, Δ*nanA*, Δ*wecB*, Δ*nagABC*.
- Comparative growth analysis in triplicates in LB- and M9-minimal-medium (Fig. 3 and Fig. 4) and determination of max. specific growth rate μ_{max} (Table 1).

Comparative growth analysis in LB-medium:

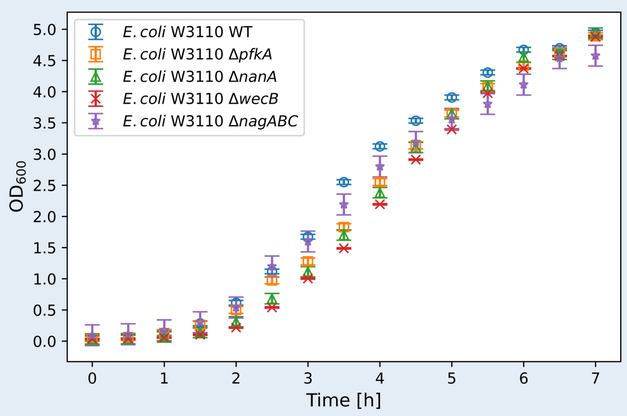

Fig. 3: Batch growth analysis of CRISPR/Cas9-modified *E. coli* strains in comparison to wild-type *E. coli* W3110, performed as triplicates in 100 ml LB-medium at 37 °C and 200 rpm in 500 ml shake flasks .

Comparative growth analysis in M9-medium:

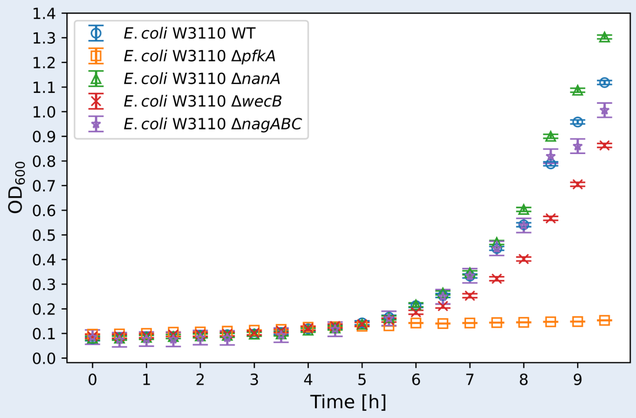

Fig. 4: Batch growth analysis of CRISPR/Cas9-modified *E. coli* strains in comparison to wild-type *E. coli* W3110, performed as triplicates in 100 ml M9-medium with 2 g/L glucose at 37 °C and 200 rpm in 500 ml shake flasks .

Table 1: Comparison of max. specific growth rate μ_{max} for *E. coli* W3110 strains in LB- and M9-media.

Strain name	μ_{max} in LB	μ_{max} in M9 + 2 g_{Glc}/L
E. coli W3110 WT	1.49	0.54
E. coli W3110 Δ*pfkA*	1.35	-
E. coli W3110 Δ*nanA*	1.40	0.55
E. coli W3110 Δ*wecB*	1.49	0.49
E. coli W3110 Δ*nagABC*	1.27	0.52

μ_{max}: max. specific growth rate in 1/hour, Glc: glucose

E. coli W3110 Δ*pfkA*	• Reduced, wildtype-comparable cell growth in LB-medium • No cell growth in M9-minimal-medium	→ Damage in glycolysis
E. coli W3110 Δ*nanA*	• Reduced, wildtype-comparable cell growth in LB-medium • Slightly increased cell growth in M9-medium	→ Decreased pyruvate production, minor ATP consumption for non-essential pathways
E. coli W3110 Δ*wecB*	• Reduced, wildtype-comparable cell growth, no differences in max. specific growth rate μ_{max} in LB-medium • Reduced cell growth in M9-minimal-medium	→ Disorder in cell wall biosynthesis
E. coli W3110 Δ*nagABC*	• Reduced, wildtype-comparable cell growth, lower max. specific growth rate μ_{max} in LB- and M9-minimal-medium	→ Disorder in glycolysis and pentose phosphate pathway

Conclusion & Outlook

The results of the comparative growth analysis in LB- and M9-minimal-medium show that the elimination of competing pathways for SL-production does not significantly affect the strain's growth. However, glucose metabolism is affected when deleting the *pfkA* gene. Therefore, in further growth studies the M9-minimal-medium will be supplemented with glycerol.

To ensure the cofactor synthesis and regeneration as well as the prevention of unwanted degradation of substrates and intermediates, final genome-editing will be performed. The edited strain will be characterized and optimized in regard of a high titer of sialyllactose.

References:
[1] C. Li et al., 'Metabolic Engineering of Escherichia coli for High-Titer Biosynthesis of 3'-Sialyllactose', Journal of Agricultural and Food Chemistry 2024, 72(10), 5379-5390

11th International Congress on Biocatalysis 2024