CRISPR/Cas9-Mediated Engineering of a Sialyllactose-Producing Strain for Whole-Cell Biocatalysis

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Motivation

Human milk oligosaccharides (HMOs) such as Sialyllactose are important for human health and have beneficial effects on the growth of naturally occurring human gut bacteria, such as bifidobacteria. In addition, HMOs prevent the adsorption of pathogens by acting as acceptor mimics for pathogen binding [1].

SL, present in human breast milk as 3'-SL and 6'-SL, are important components in neutralizing toxins and preventing bacteria and viruses from adhering to the epithelial surface of breast-feed infants [2, 3].

For the synthesis of SL starting from Glucose, an *E. coli* W3110 strain is modified to overcome challenges such as the need for cofactor regeneration, substrate uptake and product release, and the prevention of intermediate degradation (Fig. 1).

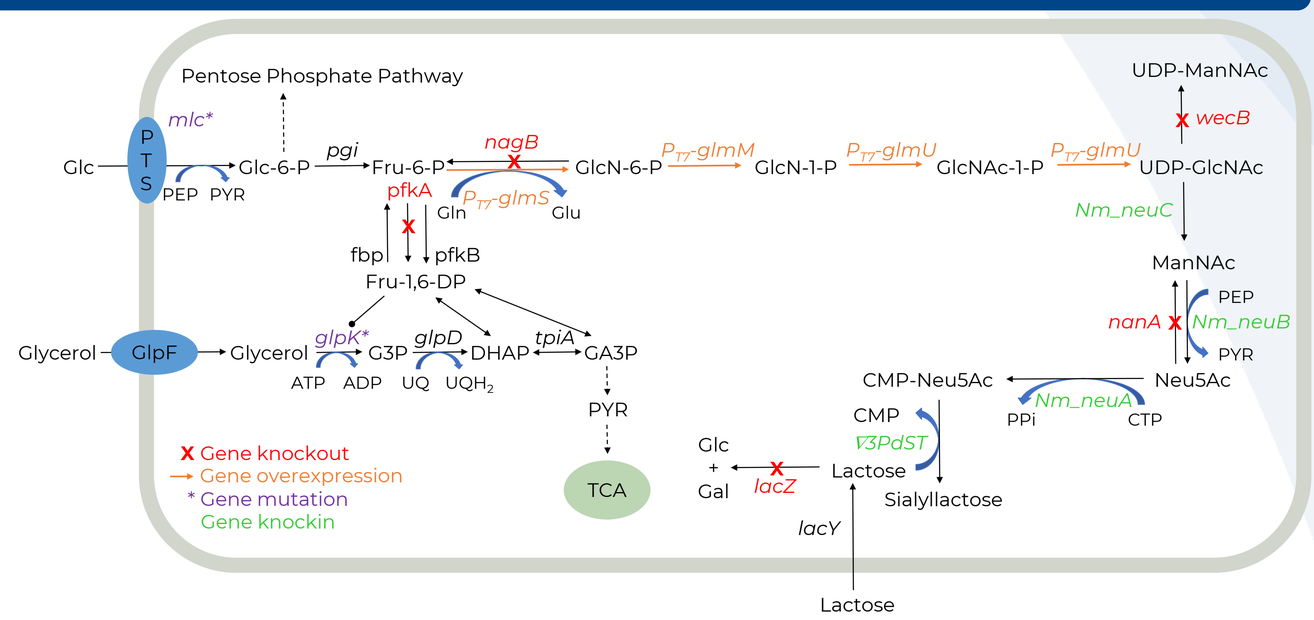

Fig. 1: Reaction scheme of a SL-producing *E. coli* cell for the use in whole-cell biocatalysis starting from glucose.

Approach

After theoretical strain construction, genome-editing is performed using CRISPR/Cas9 to knock-in/out selected genes:

- Isolation of pEcCas-plasmid containing Cas9 enzyme
- Production of guideRNA-plasmids (gRNA) for specific targeting of the genomic *E. coli* DNA (Fig. 2) via PCR, restriction digest, and ligation
- Production of double-stranded donor DNA (dsDonorDNA) via extension PCR
  - Leads to the deletion of the gene sequence except of the start codon and the last 7 amino acids or to the introduction of the sequence of the gene of interest (GOI), respectively
- Transformation of *E. coli* with pEcCas-plasmid, pgRNA-Plasmid, and dsDonorDNA via electroporation
- Check-PCR of potential edited colonies, sequencing and growth analysis

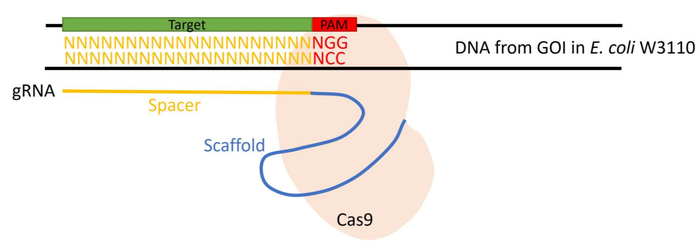

Fig. 2: gRNA-Cas9 complex binding the genomic DNA of *E. coli*.

Experimental results

- Successful modification of the *E. coli* W3110 genome: ΔpfkA, ΔnanA, ΔwecB, ΔnagABC
- Comparative growth analysis in LB-medium and M9-minimal-medium at 37 °C and 200 rpm in 500 ml shake flasks

Comparative growth analysis in LB-medium:

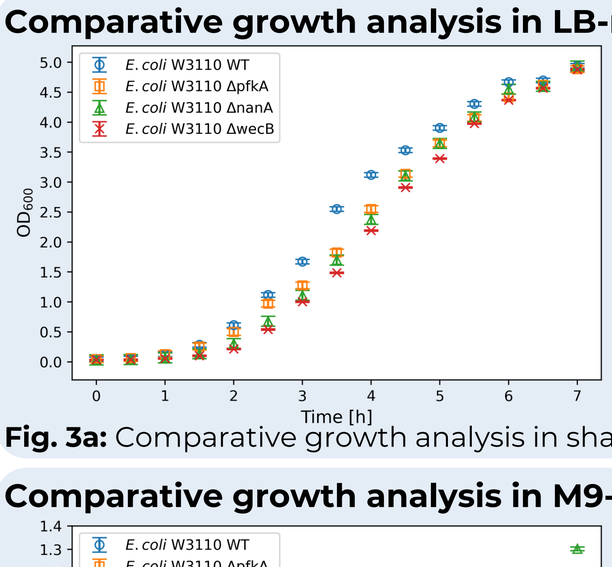

Fig. 3a: Comparative growth analysis in shake flasks for *E. coli* W3110 in 100 ml LB-medium.

No significant differences in cell growth and in specific growth rate μ compared to wild-type (WT) strain.

➢ Slightly reduced cell growth (Fig. 3a) and lower specific growth rate μ for *E. coli* W3110 ΔpfkA and *E. coli* W3110 ΔwecB (Table 1)

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

Strain name	μ in LB	μ in M9 + Glc 2 g/L
E. coli W3110 WT	0.68	0.23
E. coli W3110 ΔpfkA	0.59	-
E. coli W3110 ΔnanA	0.66	0.25
E. coli W3110 ΔwecB	0.59	0.22

μ: specific growth rate in 1/hour, Glc: Glucose

Comparative growth analysis in M9-minimal-medium:

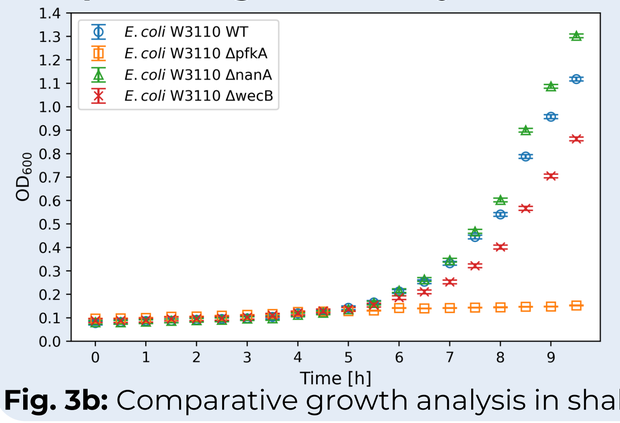

Fig. 3b: Comparative growth analysis in shake flasks for *E. coli* W3110 in 100 ml M9-medium + Glucose (2 g/L).

Differences in cell growth and in specific growth rate μ compared to wild-type (WT) strain.

➢ *E. coli* W3110 ΔpfkA: no cell growth → strain cannot efficiently metabolize glucose in M9 minimal media

➢ *E. coli* W3110 ΔnanA: no significant differences in cell growth and μ (Table 1) → absence of nanA pathway does not hinder growth

➢ *E. coli* W3110 ΔwecB: slightly reduced cell growth (Table 1) → perturbations in cell wall biosynthesis

Conclusion & Outlook

Strain development: the main challenges for HMO production were the strain development of a whole cell biocatalyst, the requirement of multiple enzymes, cofactor regeneration, substrate uptake and product release, as well as the prevention of intermediate synthesis or degradation.

Strain construction: genome-editing was used to ensure that the necessary substrates and cofactors were synthesized and regenerated in the cell to prevent unwanted degradation of substrates/products.
The newly constructed strain will be characterized and optimized to produce a high titer of Sialyllactose.

References:
[1] L. Guo et al., Applied and Environmental Microbiology 2018, Vol. 84 (13).
[2] Z. Li et al., Molecules 2020, Vol. 25 (16).
[3] L. E. Horsfall et al., FEBS J. 2010, Vol. 277 (13).

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