Synthetic Biology Approaches for the Optimization and Improvement of a Live Bacterial Therapeutic for the Treatment of Phenylketonuria (PKU)

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Abstract

Phenylketonuria (PKU) is a human metabolic disease characterized by an inability to degrade phenylalanine (Phe), causing neurotoxicity. A novel therapeutic treatment to consume Phe in the GI tract, *Escherichia coli* Nissle (EcN) SYNB1618 was engineered to express two independent enzymes, PAL and LAAD, which metabolize Phe into the respective metabolites, *trans*-cinnamic acid (TCA) and phenylpyruvate (PP). Uptake of Phe is facilitated by a high efficiency transporter, PheP. In a Phase 1 trial, SYNB1618 led to dose-dependent increases in SYNB1618-specific biomarkers in healthy humans. To further develop our Synthetic Biotic™ platform, a more potent PKU strain was sought through optimization of whole cell PAL activity. *In vitro* analyses suggested the rate-limiting step was feedback inhibition of PAL by TCA. Increasing PAL expression did not increase whole cell activity but did increase activity in lysate, while salicylate, an efflux pump inducer, increased whole-cell PAL activity. Using Zymergen's proprietary sensor engineering platform, an allosteric transcription factor biosensor controlling GFP expression was developed which responded specifically to TCA in a dose-dependent manner. Biosensor intensity correlated with PAL activity as measured by GFP and TCA production. Utilizing a directed evolution approach, PAL variants were designed for improved activity using phylogenetic analysis combined with computational protein design algorithms. Iterative screening of oligo-based combinatorial libraries resulted in progressive activity improvement over multiple rounds of enrichment. Unique PAL variants with increased activity were identified and characterized. SYNB1934 was constructed by integrating four copies of a lead PAL variant into the chromosome of EcN modified with key features of SYNB1618. *In vitro*, SYNB1934 exhibited a 2-fold increase in TCA production rate compared to SYNB1618. In healthy non-human primates, SYNB1934 treatment resulted in increases of strain activity biomarkers *in vivo*. These results define a strategy for optimizing live bacterial therapeutics.

Results

To further develop our Synthetic Biotic™ platform, a more potent PKU strain was sought through optimization of whole cell PAL activity.

Fig. 1. *In vitro* activity suggests feedback inhibition of PAL by TCA is rate limiting

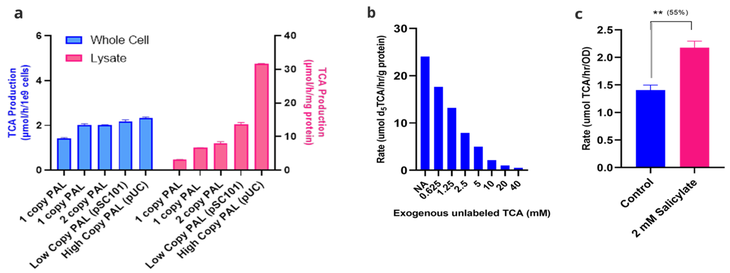

Fig. 1. EcN engineered to express wild-type PAL (a) Cells were incubated as whole cells and lysates for 2 h at 37°C in M9 media containing 40mM Phe and 0.5% glucose. Blue bars represent whole cell PAL activity (normalized to cell number); pink bars represent the corresponding PAL activity from lysates (same cell batches; activity normalized to total soluble protein content from lysate). Increased copy number had little effect on whole-cell TCA production but corresponded to increased activity in lysed cells (b) Cell lysates were assayed for 2 h at 37°C in M9 media containing 30mM d_5Phe, 0.5% glucose, and increasing concentrations of unlabeled TCA. PAL activity decreased in the presence of increasing exogenous TCA, demonstrating feedback inhibition of PAL by TCA (c) Cells were incubated in 5mM Phe, 0.5% glucose, with and without 2 mM salicylate, a general inducer of efflux pumps. Increased rates of PAL activity were shown in the presence of salicylate, demonstrating that potential efflux of TCA aids in increasing PAL activity.

Fig. 2. Zymergen developed TCA-responsive biosensor and HTS campaign

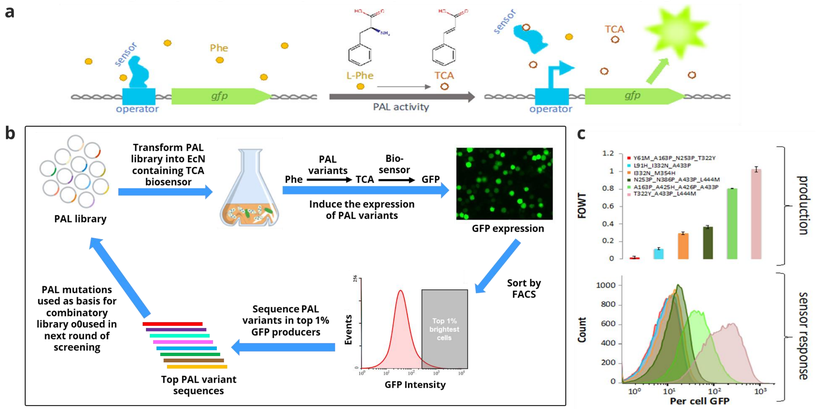

Fig. 2. (a) specific TCA-responsive allosteric transcription factor biosensor represses expression of the fluorescent reporter gene *gfp* in the absence of TCA; when TCA is introduced to the system, the repression is relieved and *gfp* is expressed (**b**) Directed evolution method where library of PAL variants (on low copy plasmids) were transformed into EcN with biosensor (on high copy plasmid), grown as a pool, washed, encapsulated by microfluidics in water-in-oil droplets containing Phe and inducer of PAL expression (aTc) at ~1 cell/droplet, incubated, and sorted by FACS for highest GFP producers. Sequences of top 1% PAL variants used to library design and construction for next round of screening. This ultra-high-throughput screening approach allowed screening of >1M-member combinatorial libraries. **(c)** Demonstration of rank-order GFP to production correlation. PAL variants with different levels production (upper panel) from Round 1 (Fig. 3) correspond to correlated levels of GFP expression (lower panel). Colors are consistent between graphs.

Fig. 3. PAL evolution demonstrated improvement in TCA production over four rounds of enrichment

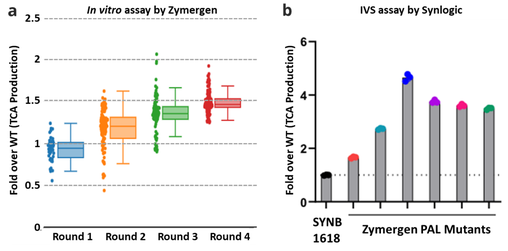

Fig. 3. (**a**) Cells grown and activated in deep well plates were incubated for 4 h at 37°C in M9 media containing 40mM Phe and 0.5% glucose. TCA was quantified by reading OD290 of cell supernatants and calculated based on TCA standard curve and normalized by OD600. PAL variants with increased TCA production were enriched in iterative rounds of evolution. (**b**) Fermenter produced cells were tested in the *in vitro* gastric simulation (IVS) model. Cells (2.5e9) were incubated under microaerobic conditions for 2 h at 37°C in simulated gastric fluid containing 20mM Phe. TCA was quantified by LC-MS/MS.

Conclusions

- A specific TCA-responsive allosteric transcription factor biosensor as well as large library of PAL variants were developed for initial HTS in whole cell format using microfluidics and FACS sorting.
- Using the directed evolution platform to screen >1M-member combinatorial library, PAL variants were identified with increased activity; top PAL variant (mPAL) with mutations: S92G, H133M, I167K, L432I, V470A.
- SYNB1934 was developed by integrating four copies of mPAL in *E. coli* Nissle containing PheP and LAAD.
- SYNB1934 demonstrated 2-fold higher TCA production in vitro compared to SYNB1618 and increased strain specific biomarkers in non-human primates compared to SYNB1618.
- SYNB1934 is currently undergoing IND-enabling studies and warrants further exploration in the clinic as a potential treatment for PKU.

Fig. 4. Homology model of PAL with key residues involved in increased PAL activity

Fig. 4. A homology model of PAL was constructed, and active site residues were identified. To improve PAL activity, positions around the active site were targeted for variation. Phylogenetic analyses were completed to compensate for instabilities introduced by mutations in and around the active site and indicated designed mutations widely dispersed across PAL. **(a)** Active site residues are highlighted in red, residues mutated in the initial library templates are highlighted in green, and additional mutations targeted during combinatorial mutagenesis are shown in pink (**b**) Active site residues are shown in red, residues mutated in SYNB1934 are highlighted in yellow, and residues mutated in other top hits are highlighted in blue.

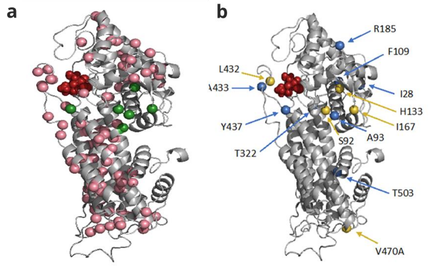

Fig. 5. Key engineered genetic elements of SYNB1934

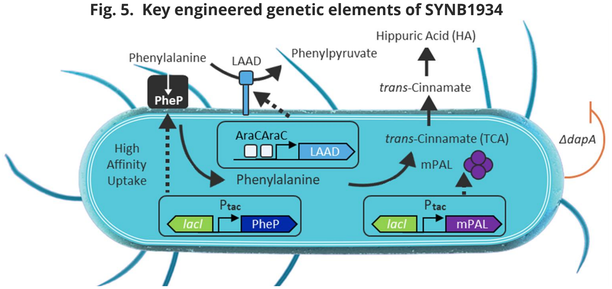

Fig. 5. SYNB1934 contains chromosomally integrated genes encoding PheP, a high affinity Phe transporter, mPAL, phenylalanine ammonia lyase from *Photorhabdus luminescens* with the following engineered mutations: S92G, H133M, I167K, L432I, V470A which converts Phe to TCA, and LAAD, L-amino acid deaminase, which converts Phe to phenylpyruvate (PP). Regulation of PheP and mPAL is carried out by IPTG inducible promoters and LAAD is L-arabinose inducible promoter. For biocontainment, the strain is a diaminopimelate (DAP) auxotroph.

Fig. 6. SYNB1934 demonstrated higher activity *in vitro* and increased biomarker *in vivo*

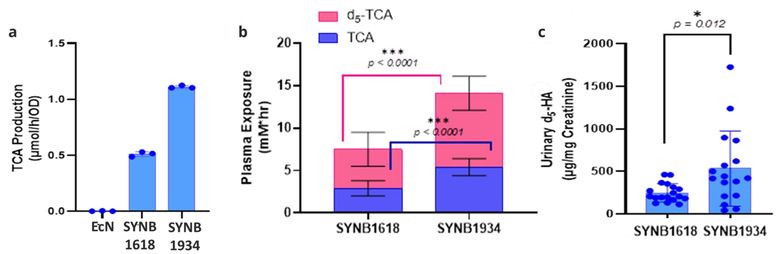

Fig. 6. (**a**) Fermenter produced cells were incubated statically under reduced oxygen conditions for 90 min at 37°C in M9 media containing 4mM Phe. The concentration of TCA was determined by LC-MS/MS. The data demonstrate that SYNB1934 is capable of consuming higher levels of Phe compared to SYNB1618 *in vitro* and the increase is due to expression of the evolved mPAL enzyme. (**b and c**) Non-human primates (NHPs) were dosed orally with a 5 g peptide and 0.25 g d_5-Phe bolus followed by dosing with 1 x 10^{11} resuspended lyophilized SYNB1618 or SYNB1934 cells. Plasma areas under the curve (AUCs) for strain-specific biomarkers TCA and d_5-TCA (**b**) and urinary d_5-HA concentration normalized to creatinine (**c**) are shown. NHPs that received SYNB1934 demonstrated a significant increase in plasma exposure to both TCA and d_5-TCA as well as a urinary d_5-HA concentration 2-fold higher than NHPs that received SYNB1618.