A Synthetic Live Bacterial Therapeutic Organism for the Treatment of the Human Metabolic Disease Phenylketonuria (PKU)

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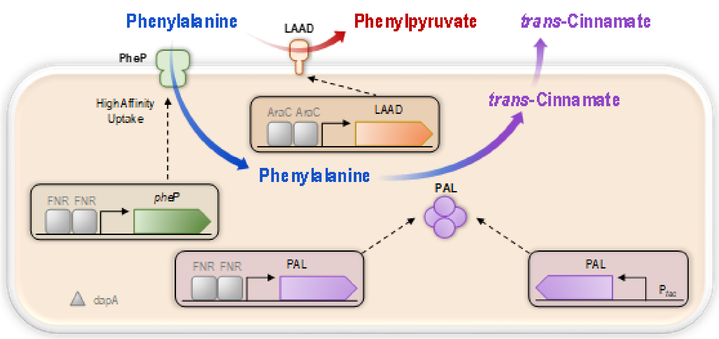
ABSTRACT

Phenylketonuria (PKU) is a human metabolic disease characterized by the inability to metabolize phenylalanine (Phe), resulting in significant neurotoxicity. As a novel therapeutic treatment, we engineered Escherichia coli Nissle (EcN) to express the Phe-metabolizing enzyme phenylalanine ammonia lyase (PAL) in response to anoxic conditions within the mammalian gut. Co-expression of a high affinity Phe uptake system, pheP, resulted in a 7-fold increase in the rate of Phe degradation by PAL in vitro. In addition, expression of the membrane-localized enzyme L-amino acid deaminase (LAAD) was shown to degrade Phe in an oxygen-dependent manner; its inclusion in the therapeutic strain was intended to capitalize on oxygen available in the stomach and proximal GI tract. In a mouse model of PKU, administration of our synthetic strain, SYNB1618, reduced Phe concentration in the blood by 38% compared with the unengineered EcN control, independent of dietary protein intake. Additionally, we established that the breakdown product of Phe by PAL, trans-cinnamate (TCA), was quantitatively converted to hippurate and excreted in urine in vivo, and could act as a non-invasive biomarker of SYNB1618 activity. In healthy Cynomolgus monkeys, we found that SYNB1618 significantly blunted an increase in serum Phe after an oral dietary challenge. Additionally, SYNB1618 was detectable in murine and primate feces after a single oral dose, permitting the evaluation of pharmacodynamic properties. Finally, in a phase I dose escalation trial in healthy human volunteers, SYNB1618 administration resulted in a dose-dependent recovery of urinary hippurate excretion. Our results define a strategy for the translation of live synthetic bacterial therapeutics for the treatment of metabolic disease.

METHODS

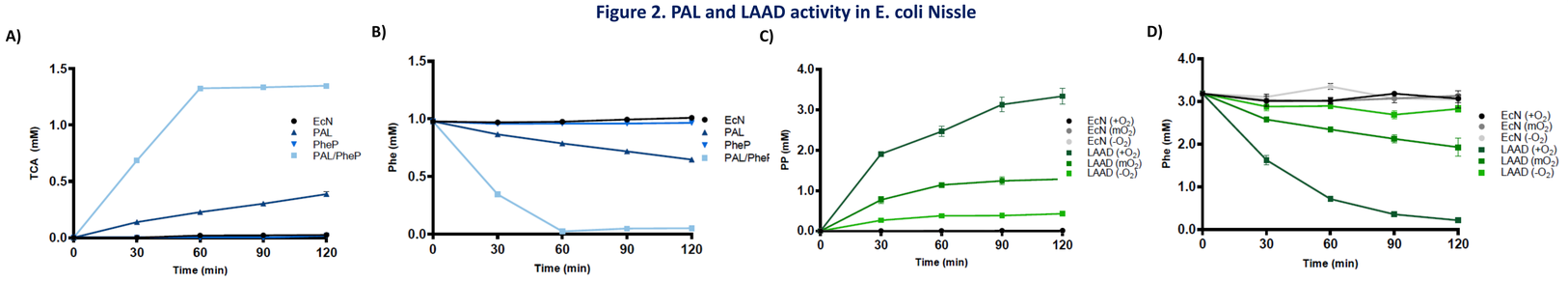
Figure 1: Schematic diagram of SYNB1618



SYNB1618 contains chromosomally integrated genes encoding PheP, a high affinity transporter that can bring Phe into the cell, PAL *(stlA),* which converts Phe to TCA, and LAAD *(pma)* which converts Phe to PP. Regulation of these components is carried out by anaerobic-, IPTG-, and L-arabinose-inducible promoters to enable activation in the mammalian gut or *in vitro.*

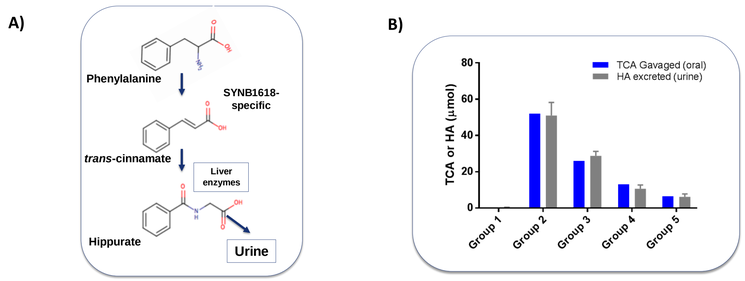
RESULTS

Figure 2. PAL and LAAD activity in E. coli Nissle



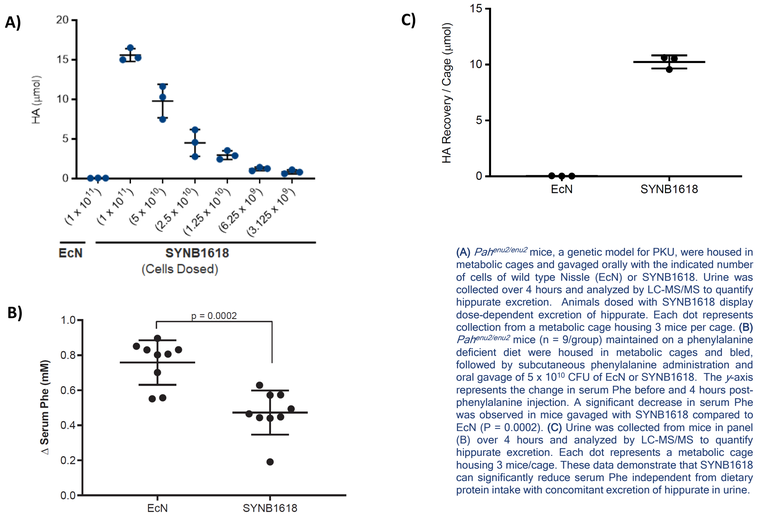
(A and B) EcN expressing PAL and/or PheP were analyzed for production of *trans*-cinnamate (TCA) and degradation of Phe. 10^9 pre-activated cells were suspended in M9 minimal media containing 1mM Phe and TCA (A) and Phe (B) were measured over time using LC-MS/MS. These data demonstrate that PAL-expressing EcN are capable of producing TCA concomitant with Phe degradation, and that Phe degradation is enhanced when PAL expression is coupled with PheP. **(C and D)** EcN expressing LAAD were analyzed for production of phenylpyruvate (PP) and degradation of Phe. 2×10^8 pre-activated cells were suspended in M9 minimal media containing 4mM Phe and incubated under differing oxygenation. PP (C) and Phe (D) were measured over time using LC-MS/MS. These data demonstrate that LAAD can degrade Phe concomitant with PP production, and that the activity of this enzyme is highly dependent on oxygen for function.

Figure 3: Establishment of Hippurate as a quantitative biomarker for trans-cinnamate



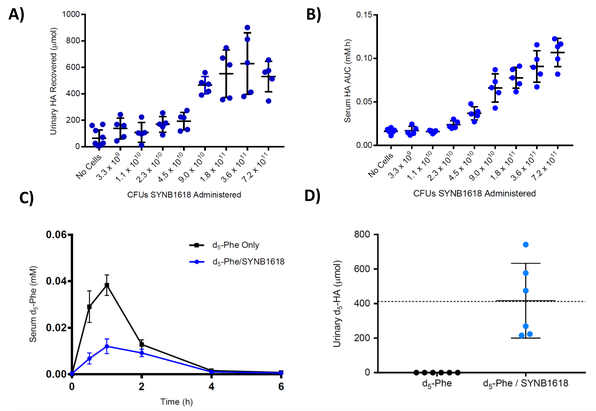
(A) PAL converts Phenylalanine to TCA, a SYNB1618-specific reaction. TCA is further converted to hippurate (HA) in the liver and excreted in urine. **(B)** Groups of mice housed in metabolic cages were gavaged orally with TCA, and urine was collected over 4 hours . Urinary hippurate concentration was measured by LC-MS/MS and multiplied by urinary volume to calculate the total quantity of HA excreted. TCA was converted to HA in a 1:1 ratio across all dose groups, thus allowing its use as a quantitative biomarker of strain activity.

Figure 4: *In vivo* activity and efficacy of SYNB1618 in *Pah*$^{enu2/enu2}$ mice



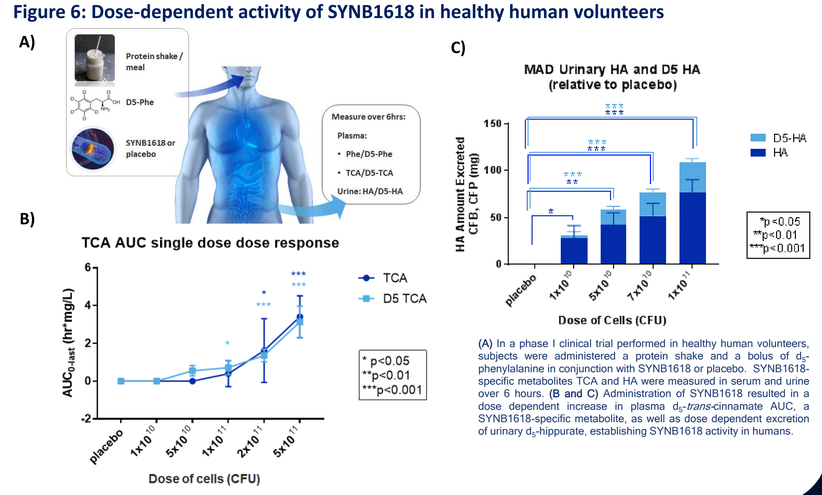
(A) *Pah*$^{enu2/enu2}$ mice, a genetic model for PKU, were housed in metabolic cages and gavaged orally with the indicated number of cells of wild type Nissle (EcN) or SYNB1618. Urine was collected over 4 hours and analyzed by LC-MS/MS to quantify hippurate excretion. Animals dosed with SYNB1618 display dose-dependent excretion of hippurate. Each dot represents collection from a metabolic cage housing 3 mice per cage. **(B)** *Pah*$^{enu2/enu2}$ mice (n = 9/group) maintained on a phenylalanine deficient diet were housed in metabolic cages and bled, followed by subcutaneous phenylalanine administration and oral gavage of 5×10^{10} CFU of EcN or SYNB1618. The *y*-axis represents the change in serum Phe before and 4 hours post-phenylalanine injection. A significant decrease in serum Phe was observed in mice gavaged with SYNB1618 compared to EcN (P = 0.0002). **(C)** Urine was collected from mice in panel (B) over 4 hours and analyzed by LC-MS/MS to quantify hippurate excretion. Each dot represents a metabolic cage housing 3 mice/cage. These data demonstrate that SYNB1618 can significantly reduce serum Phe independent from dietary protein intake with concomitant excretion of hippurate in urine.

Figure 5: Dose response and efficacy of SYNB1618 in healthy non-human primates



(A and B) Fasted non-human primates (n = 5 per group except for no cells control, which was n = 10) were administered a 5 g peptide bolus with the indicated dose of SYNB1618. Urine was collected over 6 hours and serum at 0, 0.5, 1, 2, 4, and 6 hours. Oral SYNB1618 administration was shown to result in a dose-dependent recovery of urinary HA as well an increase in serum HA area under the curve (AUC). **(C and D)** Non-human primates (n = 6 per group) were administered a 70 mg challenge of deuterated (d_5) Phe followed by SYNB1618 or mock (d_5-Phe only). Animals that received SYNB1618 displayed significantly reduced levels of serum d_5-Phe AUC compared to mock administration (P = 0.0149) with concomitant recovery of d_5-hippurate in urine. No d_5-hippurate was recovered in animals that did not receive SYNB1618.

Figure 6: Dose-dependent activity of SYNB1618 in healthy human volunteers



(A) In a phase I clinical trial performed in healthy human volunteers, subjects were administered a protein shake and a bolus of d_5-phenylalanine in conjunction with SYNB1618 or placebo. SYNB1618-specific metabolites TCA and HA were measured in serum and urine over 6 hours. **(B and C)** Administration of SYNB1618 resulted in a dose dependent increase in plasma d_5-*trans*-cinnamate AUC, a SYNB1618-specific metabolite, as well as dose dependent excretion of urinary d_5-hippurate, establishing SYNB1618 activity in humans.

CONCLUSIONS

- SYNB1618 consumes phenylalanine and produces TCA and PP in vitro
- TCA is converted to hippurate in vivo and excreted in urine, allowing its use as a quantitative biomarker of SYNB1618 activity
- Oral SYNB1618 administration in PKU mice led to a significant decrease in serum Phe levels, independent of dietary protein intake
- In healthy non-human primates, SYNB1618 demonstrates dose responsive pharmacokinetics and significant blunting of serum Phe elevation post administration of a Phe challenge.
- Finally, in a Phase I clinical trial, SYNB1618 led to dose-dependent increases in SYNB1618-specific biomarkers TCA and HA in serum and urine, demonstrating its pharmacological activity in healthy humans