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Conquering homocystinuria with engineered probiotics

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https://doi.org/10.1016/j.chom.2024.02.008

Pyridoxine-unresponsive homocystinuria has lifelong implications for health. In this issue, Perreault and colleagues present evidence that orally delivered engineered probiotic *Escherichia Coli* Nissle SYNB1353 is a promising candidate in reducing homocysteine, with successful trials in mice, monkeys, and humans. However, further probiotic optimization and safety assessments are required.

Inborn errors of metabolism are estimated to collectively affect 1 in 800 births in the United States[1,2] and require lifelong treatment. Homocystinuria (HCU), recently estimated to affect ~1 in 10,000 newborns in the US,[3] is caused by disrupted methionine metabolism where a patient presents with increased levels of methionine and increased levels of a toxic methionine byproduct, homocysteine, in plasma and urine.[4] Aberrant levels of homocysteine can lead to oxidative damage and protein misfolding, clinically manifesting across multiple organ systems as joint pain, intellectual impairment, and increased risk of thromboembolism, among other symptoms.[3] Classic HCU, the most common form of HCU, is caused by genetic mutations in the gene-encoding cystathionine β-synthase (CBS), an enzyme involved in the conversion of methionine to cysteine.[5] Diseases of inborn metabolism are currently treated with dietary modifications to avoid metabolic buildup of the toxic intermediates, and additionally, in the case of HCU, vitamin B6 (pyridoxine) therapy. While CBS has pyridoxine-dependent activity, only 50% of patients are responsive to oral pyridoxine therapy.[2] In addition, HCU is life-altering for patients, associated with increased hospital admissions, and therapeutic adherence is low. Thus, new interventions are needed to ensure long-term quality of life for patients with HCU.

Probiotic microorganisms engineered to synthesize therapeutic enzymes, antibodies, or other proteins hold great promise for the targeted delivery of therapies within the gastrointestinal tract. In this issue of *Cell Host & Microbe*, Perreault et al. utilized *Escherichia Coli* Nissle (EcN), a well-studied and widely used probiotic,[6] that has previously been engineered to deliver proteinaceous cargo to treat colitis, cancer, and phenylketonuria.[7] To target HCU, they engineered a candidate EcN probiotic strain, SYNB1353, to digest gut methionine by identifying a suitable bacterial metabolic enzyme, optimizing the enzyme's activity, recombinantly expressing the optimized enzyme, and utilizing several genetic modifications as a biocontainment strategy, or mechanism for preventing unintended probiotic proliferation.

Perreault et al. selected the bacterial enzyme methionine decarboxylase (MetDC), which converts methionine into 3-methylthiopropylamine (3-MTP) as the initial enzyme to perform the therapeutic function. To identify candidate with optimal enzyme activity *in vitro*, they performed a metagenomic screen to assay diverse variants of MetDC.[8] Additionally, they engineered the EcN strain to be a "methionine sink" by deleting the native methionine exporter (*yjeH*) and augmenting the strain with a methionine import system (MetP). Importantly, chromosomal integration of these genetic constructs, initially examined in strains expressing MetDC from a plasmid, did not change the degradation capacity of the probiotic. This enabled animal and human trials to be conducted without antibiotics, which would have

been necessary to maintain plasmid carriage.

Perreault et al. demonstrated the efficacy and safety of the optimized SYNB1353 strain using three mammalian systems: a genetically modified mouse model of HCU (AAV-CBS mice), cynomolgus monkeys, and healthy human volunteers. They utilized 3-MTP production as a biomarker for SYNB1353 activity and measured serum homocysteine and methionine levels in all 3 models.[8]

They established a new mouse model of classic HCU using short hairpin RNA (shRNA)-silencing of CBS gene expression. Correspondingly, mice had elevated serum levels of homocysteine that approximated those in HCU patients. When the mice were treated with SYNB1353 for 28 days, Perreault et al. observed no significant reduction in plasma homocysteine levels despite increased levels of urinary 3-MTP.[8] They speculated this lack of an effect was due to an inability to control dietary methionine intake in the mice. While methionine-free diets may be difficult to manufacture, this would be an important future experiment to verify probiotic-specific activity on methionine levels.

For efficacy studies in cynomolgus monkeys, the authors orally administered methionine immediately followed by SYNB1353 to animals that were been fasted overnight. They observed a significant reduction in serum homocysteine and methionine levels 6 h after SYNB1353 treatment and a marked increase in urinary 3-MTP and 3-MTP glycine.[8] These results were the best observed of all three mammalian models tested. Importantly, these data support evidence that restricting methionine intake via an overnight fast was beneficial in assessing SYNB1353 activity.

Finally, in a phase 1 clinical study, healthy human volunteers received SYNB1353 or placebo over seven days. At the highest dose of SYNB1353, Perreault et al. observed a slight reduction in plasma methionine level and an even smaller reduction in homocysteine levels, a contrast to the results observed from testing in cynomolgus monkeys.[8] They attributed these findings to variations in oral methionine dosage between monkeys and humans. No safety concerns were observed in the volunteers who received SYNB1353, suggesting that humans can tolerate the product at the tested dosage and frequency. However, the presence of 3-MTP glycine in the placebo group raises questions about 3-MTP/3-MTP-glycine suitability as a marker of SYNB1353 activity in humans.

This examination of an engineered probiotic bacterial "methionine sink" across three different mammalian systems indicates a potential novel avenue for therapy for HCU patients. The limitations of this study highlight future avenues for probiotic optimization. Given the modest activity in some *in vivo* settings, it is possible that further probiotic methionine enzyme or transport optimizations could substantially increase the *in vivo* activity of SYNB1353. Further, biocontainment strategies must strike a delicate balance between probiotic fitness, efficacy, therapeutic output, and safety. Future work is warranted to examine the effect of multiple different "safety features" in these strains on therapeutic activity to balance efficacy with safety.

Interestingly, the AAV-CBS model developed by Perreault et al. does not delete the CBS gene as in the classic mouse HCU model but blunts its expression in the liver. In the classic model, 90% of the murine neonates die due to liver dysfunction, which had been addressed by complementing the mice with the human CBS gene.[9] The use of AAV-directed therapy is novel and may speed up therapy development for pre-clinical HCU studies in mice; however, extensive characterization of the model that answers questions about potential liver and cardiovascular effects is crucial to further assess its suitability. While SYNB1353 was demonstrated to be safe in healthy volunteers in the phase 1 trial, other probiotics have been found to restructure gut microbiomes during passage or colonization,[10] and little is understood about gastrointestinal-related risks in the context of patients with inborn errors of metabolism. It will be critical to examine gastrointestinal inflammation and microbiome perturbation in future animal models employing SYNB1353, and in future clinical trials to ensure that putative off-target consequences of this therapy are understood and minimized. Finally, despite the robust measurement of 3-MTP/3-MTP-glycine in murine and cynomolgus monkeys, this marker was less useful in human trials. To robustly claim *in vivo* activity in volunteers or HCU patients, a more precise measurement of probiotic function would need to be identified.

From these studies, EcN has demonstrated significant potential in its ability to modulate methionine levels in the mammalian GI tract, and it will be exciting to uncover novel approaches to increase this probiotic's efficacy. Moreover, the genetic screening strategy and novel animal model described here could be powerful tools for therapeutic development in the context of other inborn errors of metabolism.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Invade to evade: *E. coli*'s gutsy survival strategies

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https://doi.org/10.1016/j.chom.2024.02.006

Antibiotic resistance is often studied *in vitro*, limiting the understanding of *in vivo* mechanisms that affect antibiotic treatment. In this issue of *Cell Host & Microbe*, Rodrigues et al. show that specific mutations allow bacteria to invade intestinal cells in a mouse model, thereby evading antibiotic treatment.

Antibiotic resistance is intensively studied in evolution experiments, typically performed with isolated bacteria growing in artificial culture media.[1] While these laboratory conditions provide precise control, they cannot detect any resistance mechanisms that rely on the niches provided by the infected body. To fill this gap, Rodrigues and co-workers used a new mouse infection model, revealing an intriguing bacterial survival mechanism that exploits niches in the gut environment to evade antibiotic treatment.[2]

The authors studied the evolution of resistance to cefepime, a cell-wall targeting antibiotic commonly used to treat bacterial infections in cancer and stem cell transplant patients. Previous research has shown that interval dosing of antibiotics is problematic in mice, because these drugs are typically cleared much more rapidly than in humans[3]: Antibiotic concentrations in blood plasma drop to sub-inhibitory levels in mice within 4 h. Therefore, the authors implanted a special pump in the mice that continuously releases antibiotics, thereby maintaining inhibitory drug levels and more closely mimicking the situation in humans.

Mice in which all intestinal bacteria had been artificially removed were then colonized with a pathogenic *Escherichia coli* isolate from a pediatric stem cell transplant patient and treated with cefepime via the implanted pump to study its resistance evolution. This clinical isolate was genetically barcoded to allow for lineage tracing, which has previously been used primarily in laboratory strains.[4,5] The authors found that despite an inoculum size of 10^7 bacteria, only about 30 lineages dominated an infection, suggesting a strong founder effect. Furthermore, barcode diversity was reduced by the cefepime treatment compared to the no-antibiotic control, supporting strong selection caused by the antibiotic. Indeed, the strain was undetectable in feces after six days of cefepime treatment, whereas untreated mice showed clear colonization. This observation suggests that the (initially susceptible) strain was simply eliminated by the antibiotic. However, the authors noticed that viable bacteria could be isolated from the intestinal tissue. Remarkably, these bacteria showed no signs of increased cefepime resistance, raising the question of how they survived the continuous antibiotic treatment.

A first clue was the observation that some colonies of isolated bacteria were more translucent than wild type. The authors sequenced two isolates with this phenotype recovered from different mice and identified loss-of-function mutations in the *wbaP* gene in both cases. This gene is involved in the synthesis of the O-antigen, a key virulence factor that is part of the lipopolysaccharide (LPS) layer of gram-negative bacteria and is involved in recognition by the immune system.[6] They hypothesized that alterations in the polysaccharide capsule and/or LPS O-antigen allow increased invasion of colon cells, and that this may explain the survival during the antibiotic treatment.

Consistent with this idea, an *E. coli wbaP* deletion mutant was five times more likely to be recovered from human colon cells in a cell invasion assay than the wild type, confirming that *wbaP* mutations increase colon cell invasion. But how does colon cell invasion affect survival under antibiotic treatment? The authors found that the antibiotic concentration inside the colon cells is about an order of magnitude lower than outside. In fact, when the extracellular drug concentration in the cell invasion assay was comparable to that found in the intestinal tissue of treated mice, the drug level inside the colon cells was below the level