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Adaptive Strategies of the Candidate Probiotic *E. coli* Nissle in the Mammalian Gut

Graphical Abstract



Highlights

- Carbohydrate availability in the gut drives *E. coli* Nissle adaptation *in vivo*
- Gut monocolonization selects for glycosyl hydrolases enabling population cross-feeding
- Mutations that enhance mucin utilization are enriched in lowdiversity guts
- Prior antibiotic exposure in conventional guts can lead to evolved probiotic resistance

Authors

Nathan Crook, Aura Ferreiro, Andrew J. Gasparrini, ..., Stephen Dobrowolski, Daniel Peterson, Gautam Dantas

Correspondence

dantas@wustl.edu

In Brief

E. coli Nissle is a probiotic and chassis for engineered biotherapies, but its adaptive behavior in the gut is unclear. Crook et al. report host-mediated selective pressures modulating carbohydrate utilization and metabolism of *E. coli* Nissle. This in-host evolution also promotes probiotic survival by enabling effective stress responses during colonization.



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Adaptive Strategies of the Candidate Probiotic *E. coli* Nissle in the Mammalian Gut

Nathan Crook,^{1,7,8} Aura Ferreiro,^{1,2,7} Andrew J. Gasparrini,^{1,9} Mitchell W. Pesesky,^{1,10} Molly K. Gibson,^{1,11} Bin Wang,¹ Xiaoqing Sun,¹ Zevin Condiotte,^{1,12} Stephen Dobrowolski,³ Daniel Peterson,^{4,13} and Gautam Dantas^{1,2,5,6,14,*}

¹Edison Family Center for Genome Sciences & Systems Biology, Washington University School of Medicine, St. Louis, MO 63110, USA ²Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO 63130, USA

³Department of Pathology, Children's Hospital of Pittsburgh, Pittsburgh, PA 15224, USA

⁴Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

⁵Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA

⁶Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA

⁷These authors contributed equally

⁸Present address: Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27606, USA ⁹Present address: VL55, 55 Cambridge Pwky, Cambridge, MA 02142, USA

¹⁰Present address: Department of Chemical Engineering, University of Washington, Seattle, WA 98195, USA

¹¹Present address: Flagship Pioneering, 55 Cambridge Pkwy, Cambridge, MA 02142, USA

¹²Present address: Graduate School of Biomedical Sciences, Baylor College of Medicine, Houston, TX 77030, USA

¹³Present address: Eli Lilly & Company, 307 East McCarty Street, Indianapolis, IN 46225, USA

¹⁴Lead Contact

*Correspondence: dantas@wustl.edu

https://doi.org/10.1016/j.chom.2019.02.005

SUMMARY

Probiotics are living microorganisms that are increasingly used as gastrointestinal therapeutics by virtue of their innate or engineered genetic function. Unlike abiotic therapeutics, probiotics can replicate in their intended site, subjecting their genomes and therapeutic properties to natural selection. We exposed the candidate probiotic E. coli Nissle (EcN) to the mouse gastrointestinal tract over several weeks, systematically altering the diet and background microbiota complexity. In-transit EcN accumulates genetic mutations that modulate carbohydrate utilization, stress response, and adhesion to gain competitive fitness, while previous exposure to antibiotics reveals an acquisition of resistance. We then leveraged these insights to generate an EcN strain that shows therapeutic efficacy in a mouse model of phenylketonuria and found that it was genetically stable over 1 week, thereby validating EcN's utility as a chassis for engineering. Collectively, we demonstrate a generalizable pipeline that can be applied to other probiotics to better understand their safety and engineering potential.

INTRODUCTION

Probiotics are live microorganisms that provide health benefits when consumed, including enhancement of gut epithelial barrier function, blocking of pathogen binding, and vitamin synthesis (Hill et al., 2014). There has been increasing success in expanding the therapeutic scope and efficacy of probiotics through genetic engineering, with prior work demonstrating preclinical efficacy against infectious and metabolic diseases (Durrer et al., 2017; Hwang et al., 2017; Isabella et al., 2018; Palmer et al., 2018). Engineered probiotics are exciting platforms for in situ drug synthesis and delivery, provided they maintain appropriate abundance and activity at their target site. Unlike conventional (abiotic) therapeutics, wild-type (WT) or engineered probiotics replicate in the gut and are therefore subject to natural selection, potentially to the detriment of their intended therapeutic effect and safety profile. Indeed, both commensal and pathogenic microbes adapt in a host-specific manner during gut passage (Lieberman et al., 2014; Zhao et al., 2018). In addition, probiotic efficacy and colonization of the gut is highly variable between subjects, and some of this variability can be attributed to person-specific gut microbiome features prior to treatment, such as alpha diversity and diet (Rothschild et al., 2018; Suez et al., 2018; Zmora et al., 2018). Clinical use of probiotics, especially genetically engineered probiotics, therefore, will benefit from a thorough understanding of their in vivo evolutionary trajectories under diverse schemes of microbiome complexity and host diet. In this study, we use mouse models to determine the selective forces acting on probiotics in vivo.

We focus on the probiotic *Escherichia coli* Nissle 1917 (EcN), an *E. coli* clade B2 commensal (Figure S1). EcN has a long history of use in humans (Westendorf et al., 2005), has demonstrated efficacy against inflammatory bowel disease (Scaldaferri et al., 2016), and has been gaining increased attention as a chassis for engineered function (Hwang et al., 2017; Isabella et al., 2018; Kurtz et al., 2019; Palmer et al., 2018). We expected several factors to drive adaptation of EcN in the mouse gut. First, EcN cannot natively degrade many of the complex polysaccharides present in the intestine (Fabich et al., 2008; Hoskins et al., 1985) and is dependent on other species for production of metabolizable sugars (Conway and Cohen, 2015), which may lead to adaptations in carbohydrate utilization, especially in low-diversity microbiomes. Second, the high prevalence of

Table 1. Summary of Functional Metagenomic Selection Experiments

Microbiome	Diet	# Mice
Germ-free	mouse	6
Germ-free	human	5
Germ-free	human + dextrin	5
Germ-free	human + inulin	5
Germ-free	human	3
13-member	human	3
13-member	human + dextrin	3
13-member	human + inulin	3
See also Figure S2 a	nd Tables S1, S2, and S4 for de	scriptions of the se

See also Figure 52 and Tables 51, 52, and 54 for descriptions of the selection experiments, diet compositions, and the 13-member community.

antagonistic interspecies interactions in the gut (Wexler et al., 2016) may drive adaptation in EcN's encoded colicins (Patzer et al., 2003). Third, since EcN normally resides in multi-species biofilms on the outer mucin layer (Conway and Cohen, 2015), introduction to the murine gut may drive adaptation in adhesins, increasing their specificity to mouse mucins. Finally, expression of a phenylalanine (Phe)-degrading enzyme (modeling an engineered probiotic therapy) (Isabella et al., 2018) may impose a metabolic burden (Fletcher et al., 2016; Lechner et al., 2016).

We tested these hypotheses using both whole-genome sequencing of in vivo-adapted isolates and in vivo functional metagenomic selections. Isolates were adapted and metagenomic libraries (derived from healthy human fecal samples) were selected in mice subject to a spectrum of diets and microbiome complexities. In addition, some mice were the genetic model (Pah^{enu2-/enu2-}) for phenylketonuria (PKU). This combination of whole-genome adaptive evolution and functional metagenomics enabled us to investigate the endogenous adaptations and human-gut-metagenome-encoded genes that can confer a selective advantage to EcN. The functional metagenomic selections served as an approximate model for adaptation via horizontal gene transfer, which is a major mechanism driving bacterial evolution (Ferreiro et al., 2018; Soucy et al., 2015) and frequently associated with key functional determinants such as antibiotic resistance (Smillie et al., 2011). In total, we analyzed 225 longitudinal samples from functional metagenomic selections in 33 mice (Table 1) and 401 EcN isolates from 20 mice (Figure S2; Table S3).

RESULTS

Rationale for Functional Metagenomic Selections in the Low-Diversity Gut

The source of our metagenomic DNA was stool from two healthy human infants and six healthy human mothers to approximate the diversity of microbial taxa present in the gut through different life stages (Moore et al., 2015). Our library comprised $\sim 1.5 \times 10^7$ unique 2–5 kb fragments of metagenomic DNA. We confirmed in a longitudinal *in vivo* experiment that the empty vector remained stable in EcN over 5 weeks in the mouse gut (Figure S3). By competing millions of recombinant EcN strains against each other *in vivo*, we expected to observe significant enrichment of a subpopulation of strains with DNA inserts conferring a fitness

advantage to EcN. Specifically, we hypothesized that functional metagenomic selections in germ-free mice would select for enzymes capable of degrading complex polysaccharides, a function that *E. coli* typically depends on gut anaerobes to carry out (Conway and Cohen, 2015; Fabich et al., 2008).

Selection for Carbohydrate Utilization in the Germ-Free Gut

Metagenomic libraries were delivered to six germ-free C57BL/6 mice. These mice consumed a mouse diet (MD) composed primarily of plant-based carbohydrates and protein (Figure 1A; Tables S1 and S2). Over 5 weeks, we observed a striking reduction in the diversity of metagenomic inserts-the same 5 sequences were selected in all 6 mice (Figure 1B). These inserts rose from a low starting abundance (0.187% for the most abundant fragment on day 1 of selection) to over 75% combined abundance in each mouse. Four out of these five inserts contained genes encoding a predicted glycosyl hydrolase family 32 (GH32) enzyme, and several of these inserts also contained genes encoding predicted transporters and other enzymes involved in sugar utilization (Figure 2B). To test our hypothesis that these inserts enhance carbohydrate utilization, we cloned these metagenomic fragments into WT EcN and looked for conferred carbon source utilization using phenotypic microarrays (Data S1).

Each GH32-containing fragment enabled growth on sucrose, which commensal E. coli, including EcN, are unable to consume (Conway and Cohen, 2015) (Figure 2A). Several GH32-containing fragments enabled growth on longer-chain oligosaccharides containing sucrose as a terminal moiety, including raffinose and stachyose (Figure 2A). The growth rate of EcN strains containing two different GH32-encoding fragments (MD02 and MD05) was higher on these longer-chain oligosaccharides than sucrose (MD02, 0.04 h^{-1} on sucrose versus 0.208 h^{-1} on raffinose [p = 0.0017, t test, two tailed, unequal variances] and 0.144 h^{-1} on stachyose [p = 0.011]; MD05, 0.028 h^{-1} on sucrose versus $0.252 h^{-1}$ on raffinose [p = 8.8 × 10⁻⁵] and 0.153 h⁻¹ [p = 0.00073] on stachyose), indicating specialization toward these molecules. We hypothesized that GH32 activity by an EcN subpopulation would release sugars upon which the rest of the EcN population can cross feed. To test this, we grew WT EcN on raffinose minimal media pre-conditioned by EcN strains expressing recovered GH32 enzymes or by WT EcN. We found that WT EcN could only grow on media pre-conditioned by the EcN strains expressing GH32-encoding inserts (Figure 2D), indicating that these strains can support cross-feeding within the EcN population.

The remaining fragment (MD04), which did not encode GH32, encoded a transcription factor that was identical (100% nucleotide identity) to the *gadX* gene in EcN and also contained the native *gadX* promoter. GadX plays an important role in acid tolerance by regulating the expression of the *gad* operon, whose combined activity generates a cycle that reduces cytosolic H+ (Tramonti et al., 2002). The presence of MD04 enabled EcN to recover more rapidly than WT from a 30-min exposure to acidic conditions (pH 2.5) (Figure 2C). Also, expression of this fragment in EcN uniquely increased cell density at 24 h after switching from lysogeny broth (LB) to minimal media with galactose, a mucin component, as the sole carbon source (Tailford et al.,



Figure 1. Functional Metagenomic Selections in EcN Mono-Colonized Mice

(A) Experimental design and sampling timeline. Mice were gavaged on day 0. N, number of replicate mice.

(B–E) Relative abundance of metagenomic fragments in replicate mice fed standard mouse diet and water (B), a high-fat human diet and water (C), a high-fat human diet and dextrin (D), or a high-fat human diet and inulin (E). MD, fragments enriched in mice fed the mouse diet; HD, fragments enriched in mice fed human diet.

See also Figures S1-S4 and Table S6.

2015) (Figure 2A). At high extracellular concentrations, galactose is imported into *E. coli* by a low-affinity galactose permease (GalP), which is a proton symporter (Weickert and Adhya, 1993). Overexpression of *gadX* may therefore also mitigate an increase in cytosolic pH in galactose minimal media. Together with Gene Ontology (GO) annotation of the genes contained within recovered fragments (Figure 3A), these results indicated that the dominant selective pressure on EcN in the mono-colonized mouse gut is limited carbon source availability.

Diet-Dependent Selection

Because we found strong selection for expanded carbohydrate utilization in the previous experiment, we hypothesized that polysaccharide-specific enrichment of DNA inserts would occur in response to dietary prebiotics. To test this hypothesis, we delivered the EcN library to 3 groups of 5 germ-free mice. All mice were maintained on a high-fat, high-carbohydrate diet that mimics the diet of Western humans, referred to here as human diet (HD) (Plump et al., 1992). One group of mice consumed HD alone, while the other two were supplemented with the prebiotics inulin or dextrin in the drinking water. Inulin is not degradable by WT EcN, while dextrin can support limited EcN growth (Fabich et al., 2008) (Figure S4). Over 5 weeks, we again observed convergence toward \leq 6 metagenomic fragments in each dietary condition (Figures 1C-1E). Supporting our diet-dependency hypothesis, the collection of enriched fragments in each condition was different. However, one fragment was enriched in all three groups (HD04). Among enriched fragments, HD04 alone contained a gene encoding a GH32 enzyme, replicating our prior observations. Indeed, this fragment enabled EcN to consume sucrose and a water-soluble extract of HD, possibly because of the high amount of sucrose (341 g/kg) it contains (Figure 2A).

We classified open reading frames (ORFs) in each enriched fragment into GO pathways (Figure 3A). We observed strong clustering of ORF annotation based on prebiotic supplementation. Reflecting the ubiquity of fragment HD04, "carbohydrate metabolic process" was abundant (>23%) in all samples. In the presence of dextrin, we also observed "pyrimidine nucleotide biosynthetic process," reflecting a predicted glutamate synthase. In the presence of inulin, the major fragment (reaching 32%-67% relative abundance in all mice at the end of the experiment) contained a gene encoding a predicted "ATPase-coupled sulfate transmembrane transporter." In the absence of prebiotic, the dominant fragment was HD04. No fragment besides HD04 enabled increased growth on dextrin or inulin or on HD extract broth. We conclude that in these mice, the dominant selective pressure remained the ability to degrade long-chain polysaccharides, but once this function was fulfilled by a subpopulation of GH32-carrying EcN, secondary functions were enriched in a diet-specific manner.

Advantageous Functions in a Simplified Microbial Community

Based on these results, we hypothesized that in the presence of polysaccharide-degrading taxa, non-polysaccharide-degrading functions would be advantageous to EcN. To test this hypothesis, 8 germ-free C57BL/6 mice were colonized with 13 human-gut-derived bacteria that model the phylogenetic makeup of the human microbiome (Goodman et al., 2011) (Table S4), after which the EcN library was delivered and allowed to colonize for 5 weeks. 16S rRNA sequencing revealed that EcN colonized these mice at an average relative abundance of 15.3% (Figure S5). Sequencing revealed a high diversity of enriched functions relative to the mono-colonization experiments. While no individual fragment



Figure 2. Selection for Carbohydrate Utilization and Acid Tolerance in EcN Mono-Colonized Mice

(A) Growth curves of strains expressing fragments enriched in the functional selections on minimal media supplemented with different carbon sources. $*p < 6 \times 10^{-3}$, Student's t test comparing time taken to reach OD₆₀₀ = 0.25 between WT and MD04. The shaded regions represent one standard deviation above and below the mean of three experimental replicates.

(B) Annotations of select metagenomic fragments enriched in the functional selections.

(C) Growth curves of 3 biologically replicate EcN strains expressing the *gadX* DNA insert (MD04) or EcN carrying empty vector (WT) after a 30-min pulse in acidic (pH 2.5) or neutral (pH 7.0) conditions. *p < 0.001, Student's t test for the time needed to return to the maximum optical density prior to the pulse between MD04 fragments and WT, Bonferroni correction for multiple comparisons. The shaded regions represent one standard deviation above and below the mean of four experimental replicates.

(D) Growth of WT EcN on 1% raffinose minimal media pre-conditioned by EcN strains carrying MD01 or MD02, or by WT EcN, or on unconditioned 1% raffinose minimal M9 media (left). No growth was observed in uninoculated conditioned media (right). *p < 0.001, Student's t test on the *K* parameter of logistic models fit to the data using the Growthcurver R package and the Bonferroni correction for multiple comparisons. The shaded regions represent the standard deviation of 8 experimental replicates. N = 6 experimental replicates.

See also Figures S2 and S4 and Table S6.

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Figure 3. Comparison of Functions Enriched in Functional Selections in Mice with Different Gut Microbiome Complexities

(A) Heatmap of the relative abundances of GO classifications present in EcN mono-colonized mice and mice pre-colonized with a 13-member defined community at the end of the selection period. Dendrograms represent the clustering applied to group similar rows or columns of the heatmap.

(B and C) Growth curves of EcN strains harboring metagenomic inserts shown in (D) on gluconate minimal media (B), or glucose (C). n = 6 experimental replicates, error bars represent one standard deviation above and below the mean.

(D) Annotations of the metagenomic fragments characterized in (B) and (C).

See also Figures S2, S4, and S5 and Table S6.

reached a high abundance (>20%) in all mice in the same experimental treatment, we did observe functional convergence. As in prior experiments, the GO term "carbohydrate metabolic process" was consistently recovered across the majority of replicates. We also observed condition-specific enrichment for "gluconate metabolic process," reflecting an *E. coli* transcriptional regulator (*gntR*), which achieved >7.6% abundance in mice containing the 13-member defined community (Figures 3A and 3D).



We hypothesized that these fragments altered the ability of EcN to metabolize gluconate. When these fragments were introduced to un-adapted EcN, the recombinant strain was no longer able to grow on minimal media containing gluconate as the sole carbon source, yet it maintained its growth on glucose (Figures 3B and 3C), consistent with strong overexpression of *gntR*. Gluconate utilization has been shown to be necessary for *E. coli* to colonize the conventional or streptomycin-treated mouse gut (Chang et al., 2004; Sweeney et al., 1996a), so the recovery of fragments abolishing gluconate utilization could indicate an environment-specific benefit to gluconate use.

Together, these data indicated that the primary selective pressures faced by EcN in the low-diversity mammalian gut are related to carbon source limitation and that once other taxa were introduced, this selective pressure was removed. The presence of a strong constitutive promoter in our cloning vector did not bias ORF orientation. Rather, across all experiments and all recovered fragments, full-length ORFs in the same orientation as the promoter accounted for only 47.0% of full-length ORFs. We were intrigued by the recovery of *E. coli* genes in our selections (*gadX* and *gntR*). Based on this observation, we asked what genomic changes EcN undergoes after *in vivo* passage.

Within-Genome Evolutionary Change

We next performed whole-genome sequencing on 401 EcN isolates collected from mice with four levels of microbiota complexity: mono-colonized with EcN, colonized with a defined

Figure 4. Summary of Mutations Detected in *In Vivo*-Adapted EcN Isolates

The EcN chromosome, its 2 native plasmids pMut1 and pMut2, and the expression vector pZE21 are depicted in a Circos plot. Light gray regions are scaled 100 × that of the rest of the chromosome for visibility. Genes of interest (orange) are labeled around the plot. The outer track depicts all detected intergenic (orange) and nonsynonymous (green) SNPs. The next track depicts all small insertions (blue) and deletions (red). The third track depicts all large deletions >1 kb (shaded purple). The inner track depicts the number of isolates that contained each mutation. Mutations found in only one isolate are excluded from the inner track for visibility. Isolate counts for each mutation are capped at 25, which only affects the rsmG mutants (167).

See also Figure S6 and Tables S3 and S5-S7.

community, streptomycin treated, or conventional. Mice from each of these conditions were fed MD, HD, or HD+dextrin (Figure S2; Tables S1–S3). We observed 456 total (336 unique) genomic changes across 171 genes (Figures 4 and S6B) relative to a high-quality closed assembly for WT EcN we generated. The most highly mutated strain had 5 SNPs, while each strain on average had 0.86 SNPs per genome. From longitudinal sampling of 2 mice fed a human diet and treated with

streptomycin prior to EcN gavage, we estimate the mutation rate of EcN in the mouse gut to be 0.007 (\pm 0.002) SNPs per genome per generation (Figure S6A), assuming an *in vivo* generation time of 100 min (Rang et al., 1999), which is in broad agreement with that obtained for *E. coli* grown *in vitro* in the absence of antibiotic exposure, 0.001 SNPs/genome/generation (Lee et al., 2012). As evidence for consistent *in vivo* selective pressures, we observed multiple instances of SNP accumulation in the same gene in isolates from separately caged mice.

Adaptations Involving Nutrient Utilization

We found 10 different mutations to nagC in 19 isolates (Figure 5A). These isolates were from 5 monocolonized mice used in the functional metagenomic selections. All mutations resulted in either a premature stop codon, elimination of the start codon, or a frameshift. NagC is a repressor of utilization of the mucin component N-acetylglucosamine (GlcNAc) (Tailford et al., 2015). We hypothesized that NagC inactivation would enhance the ability of EcN to consume GlcNAc. To test this hypothesis, three isolates with mutations to nagC were cured of their metagenomic vector (which encoded either gadX or GH32). We performed in vitro diauxic growth experiments and found that while these plasmid-cured nagC mutants grew similarly to WT EcN on glucose or GlcNAc minimal media, they were able to grow better on GlcNAc under glucose-limiting conditions (Figure 5B). In porcine mucin minimal media under glucose-limiting conditions, the mutants had diauxic growth profiles that differed

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Figure 5. Phylogenies and Phenotypic Effects of nagC and gntT Mutations

(A and C) Maximum parsimony gene phylogenies for adapted isolates based on their nucleotide sequences for (A) *nagC* and (C) *gntT*. The length of the branches corresponds to the number of SNPs separating the isolate gene sequence from the reference sequence. Color tracks depict the treatment conditions and the mouse IDs from which isolates were isolated. The prefix of each mouse ID indicates the cage in which the mouse was housed.

from WT, but these differences were not statistically significant (Figure S7A). *NagC* mutants were exclusively isolated from mice mono-colonized with EcN populations expressing GH32 enzymes; mutations enhancing growth on a less preferred carbon source may be due to a low diversity of polysaccharide degradation functions present and a concomitant carbon source limitation in the gut. It has also been found that *nagC* is mutated during adaptation to osmotic stress (Winkler et al., 2014), which may provide EcN an advantage during transit through the gastro-intestinal (GI) tract.

GntT was found to be mutated in 6 different ways across 21 strains isolated from five different mice, including 2 isolates with >10 kb deletions containing this gene (Figures 4 and 5C). The other 4 mutation types included 3 early stop codons and a D395N mutation. GntT encodes a high-affinity gluconate transporter (Porco et al., 1997). GntT mutants were significantly enriched in mice that had been pre-colonized with a 13-member defined community (p = 1.06×10^{-6} , hypergeometric test), although gntT mutants were also observed in EcN-monocolonized mice (Figure 5C). We hypothesized that *qntT* mutants would have an impaired ability to utilize gluconate, and so, we tested the growth of two representative isolates in gluconate minimal media: Isolate 21 (gntT D395N) and Isolate 3 (gntT/ nagC double mutant). Isolate 3 was unable to grow before 20 h, and Isolate 21 had significantly impaired growth (Figure 5D). Although this was the same phenotype conferred by the gntR-encoding fragments we recovered, we did not observe any mutations in gntR or in its surrounding intergenic regions in our isolate genomes.

Since we recovered inactivation of gluconate metabolism in independent experiments that spanned mouse litters, cages, and years, we investigated further. Surprisingly, we discovered that Isolates 3 and 21 grew better than WT on GlcNAc minimal media. Further, both grew better on porcine gastric mucin minimal media (Figure 5D). These results were unexpected, as there is no apparent regulatory or metabolic link between gluconate and GlcNAc utilization. Interestingly, we found that WT EcN transformed with *gntR*-containing fragments were able to grow better than WT on porcine gastric mucin minimal media (Figure 5E), yet they were not able to grow better than WT on GlcNAc minimal media. GntR is a negative repressor of GntT; the data suggest that repression of the entire Gnt-I gluconate utilization system, but not inactivation or inhibition of GntT alone, precludes enhanced consumption of GlcNAc. Both adaptations, however, enhance growth on mucin.

A metabolite in the Entner-Duodoroff pathway responsible for gluconate metabolism, 2-keto-3-deoxy-6-phosphogluconate, is toxic to E. coli (Sweeney et al., 1996b). We hypothesized that in the low-diversity, carbon-limited mouse gut where all gluconate utilization fragments or mutants were identified, increased degradation of the mucin layer increased local gluconate concentration, leading to toxicity. We found that in glucose minimal media, growth profiles of gntT mutants remained the same across gluconate concentrations of 0.5-8 mM while growth of WT EcN progressively declined (Figures 5F and 5G). However, in GlcNAc minimal media, these dynamics changed. The growth profiles of Isolate 3 (gntT/nagC) and WT were similar, whereas Isolate 21 (gntT) had significantly improved growth at 1 mM gluconate but approached the other strains at higher gluconate concentrations (Figures 5F and 5G). The D395N mutation (present in Isolate 21) was common among gntT mutants, with 11 of 21 isolates from 3 separately caged mice hosting this mutation, suggesting this mutation offered a greater fitness advantage. Indeed, when grown on porcine gastric mucin minimal media, Isolate 21 consistently outperformed Isolate 3 and WT, although all strains responded similarly to increasing gluconate concentrations (Figure S7B). Notably, both WT and Isolate 3 exhibited two growth stages (suggesting diauxic growth) on porcine gastric mucin minimal media while Isolate 21 did not. Together, these data suggest that there is a link between gluconate and GlcNAc utilization in EcN and that inhibited gluconate utilization is advantageous when mucin components are the primary carbon source.

Adaptations Involved in Stress Response

sgrR was mutated 4 different ways in 7 isolates from 2 mice (Figure 6C). These mutations were nonsynonymous and did not result in a stop codon. All strains were isolated in the context of a very low diversity microbiome. Under conditions of excess sugar phosphates, SgrR is necessary to protect the cell from stress (Vanderpool and Gottesman, 2007). It is possible that in the low-diversity gut, lack of the preferred substrate glucose resulted in cytosolic accumulation of phosphates of less efficiently metabolized sugars, such as GlcNAc-6-P or gluconate-6-P. Additionally, *rssB* was mutated 4 different ways in 9 strains. Strikingly, all were frameshift mutations. RssB regulates RpoS expression by directing it to the ClpX protease. RssB mutants

(F) gntT mutants were grown in 20 mM glucose (top) or 20 mM GlcNAc (bottom) minimal media supplemented with indicated gluconate.

⁽B) Growth curves of *nagC* mutants on 0.4% glucose minimal media (left), 0.4% GlcNAc minimal media (middle), and 0.2% GlcNAc and 0.2% glucose minimal media (right). N = 3 experimental replicates per strain, *p < 0.05, Welch's t tests comparing the *K* parameter of logistic growth models fit to the data for WT or the mutant isolates, with Benjamini-Hochberg correction for multiple comparisons.

⁽D) Growth curves of *gntT* or *gntT/nagC* double mutants on gluconate (left), GlcNAc (middle), or porcine gastric mucin minimal media (right). fs, frameshift mutation S197T. *p < 1 \times 10⁻⁵, Welch's t tests comparing the empirical areas under the curves for WT and the mutant isolates, Bonferroni correction for multiple comparisons.

⁽E) Growth curves of EcN transformed with plasmid-borne *gntR*-containing inserts from the functional metagenomic selections in 1.5% porcine gastric mucin (left, middle, 2 independent experiments), or in 20 mM GlcNAc minimal media (right). n = 4-6 experimental replicates per strain, *p < 0.05, Welch's t tests comparing the empirical areas under the curves for WT and the mutant isolates, Benjamini-Hochberg correction for multiple comparisons.

⁽G) Summary of F, showing the areas under the growth curves (AUC) and the K parameter of logistic growth models fit to the data. n = 2-4 experimental replicates per strain, *p < 0.05, Welch's t tests, Benjamini-Hochberg correction for multiple comparisons. Black stars indicate both Isolates 3 and 21 had a significantly greater value than WT at a given gluconate concentration. Blue, only Isolate 3; red, only Isolate 21.

For all panels, K and AUC values were calculated using the growthcurver R package. Error bars and shaded regions represent one standard deviation above and below the mean. See also Figure S7.

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Figure 6. Phylogenies and Phenotypic Effects of kfiB, sgrR, and rsmG Mutations

Maximum parsimony gene phylogenies for adapted isolates based on their nucleotide sequences for (A) *kfiB*, (C) *sgrR*, and (D) *rsmG*, as in Figure 5. (B) Percentages of *kfiB* mutant cells adherent to Caco-2 (left) or HT29-MTX (right) monolayers after 3 washes. Shown are the means of ratios of adherent CFU to total CFU counts (n = 3 experimental replicates). Error bars are one standard deviation above and below the mean. *p < 0.05, **p < 0.001, Welch's t test with Benjamini-Hochberg correction for multiple comparisons.

display elevated RpoS levels (Battesti et al., 2011). RpoS is a master regulator of the stress response in *E. coli*, and *rpoS* mutants show enhanced resistance to stress (Fontaine et al., 2008).

Adaptation in Membrane-Associated Functions

kfiB was mutated in 24 strains from 4 mice, each with the same loss of an early stop codon at amino acid 366, resulting in a full-length protein of 562 amino acids (Figure 6A). This potential gain-of-function variant was significantly enriched in mice that were pre-colonized with a defined community ($p = 3.01 \times 10^{-15}$, hypergeometric test). *kfiB* is a member of the K5 capsule polysaccharide (heparosan) biosynthesis cluster, which has been implicated in mediating the interaction between EcN and epithelial cells (Hafez et al., 2010; Leroux and Priem, 2016). We hypothesized that our *kfiB* variants would exhibit altered adhesion to human epithelial cells. To test this hypothesis, we carried out an adhesion assay with 3 representative *kfiB* mutants and either

Caco-2 or HT29-MTX cell monolayers. HT29-MTX cells produce MUC3, a membrane-bound mucin found throughout the gastrointestinal tract (Tailford et al., 2015). We found that the *kfiB* mutation provided significantly enhanced adhesion to Caco-2 and HT29-MTX cells (Figure 6B). This loss of truncation may reflect readaptation of our reference EcN strain, which likely has been passaged often under laboratory conditions, to *in vivo* conditions.

Antibiotic Resistance Mutations

In streptomycin-treated mice, we observed that the majority of isolates (167 of 204) contained mutations in *rsmG*, which encodes a 16S rRNA methyltransferase (Figure 6D). The three positions we identified with mutations (53, 71, and 79) were also mutated in prior work to give low-level streptomycin resistance (Benítez-Páez et al., 2012). We also observed 2 mutations to *rpsL* in 11 strains. All mutations occurred at residue 86 and converted it to a cysteine or serine. These mutations only occurred



Figure 7. In Vitro and In Vivo Degradation of Phenylalanine by EcN:PAL2

(A) Phenylalanine concentration over time in minimal media supplemented with glucose and 10 μ M Phe and inoculated with EcN expressing PAL2 under promoters of indicated strength. Shown are the mean of three experimental replicates. Error bars represent one standard deviation above and below the mean. *p < 0.01, Welch's t test with Bonferroni correction for multiple comparisons.

(B and C) Paired serum phe concentrations for male (B) and female (C) homozygous mutant Pah^{enu2} mice before and 24 h after gavage with WT EcN or EcN-expressing PAL2 under a high- or low-strength promoter. Shown are means and standard deviations for three replicate mice. ***p < 0.0001, **p < 0.01; p values were calculated using the generalized linear hypothesis test with Bonferroni correction (glht function from the multcomp R package) on the coefficient estimates from a linear mixed-effects model generated by regressing Phe measurements on an interaction factor comprised of mouse gender, measurement timepoint (before or after treatment), and treatment (Ime function from the nIme R package). Random intercepts by mouse were specified in the model. The model accounts for repeat measures variability.

in streptomycin-treated mice, and 10 out of 11 times occurred in conjunction with a mutation in *rsmG*. Residue 86 is adjacent to residues that have been mutated previously to provide streptomycin resistance to EcN (Timms et al., 1992). These streptomycin-resistant genotypes were preserved 5 weeks after streptomycin exposure, suggesting that this resistance did not carry a fitness trade-off. These results could indicate residual streptomycin present in the mouse gut and are highly relevant considering the common practice of administering probiotics and fecal microbiota transplants after antibiotic administration (Hempel et al., 2012; Koenigsknecht and Young, 2013).

No In Vivo Plasmid Acquisition

We searched our genome assemblies for sequences that did not map to the EcN genome. Interestingly, we did not observe acquisition of new sequences. The cause for this may be that EcN contains two endogenous plasmids (Grozdanov et al., 2004) in addition to the cloning vector we used for this study, which may pose a barrier due to plasmid incompatibility groups or other defense mechanisms. In agreement with these results, EcN has been shown to be a poor recipient of horizontally transferred DNA (Sonnenborn and Schulze, 2009).

In Vivo Excision of Large Genomic Sections

In total, we observed 8 deletions larger than 1 kb in size in 7 isolates. Two of these large deletions (24.3 kb and 15.5 kb in size) encompassed *gntT*. While EcN contains 5 putative phage regions (Arndt et al., 2016) (Table S5), only one (11.5 kb) was observed to be excised. 3 deletions (23.3 kb, 25 kb, and 93 kb in size) contained inverted repeat sequences, transposase, or integrase sequences. Copy number variation analysis indicated that multiple isolates from the same mouse exhibited copy number increases in two hypothetical proteins; a BLAST search revealed that these hypothetical proteins align significantly to an integrase and an inovirus Gp2 family protein. Together, these results indicate that large deletions or duplications occur in the EcN genome at low but significant rates during gut colonization, partially mediated by phage and transposon activity.

PKU Treatment by Engineered EcN

PAH-deficient PKU creates a blockage in the tyrosine synthetic pathway leading to Phe accumulation in tissues, which can be neurotoxic and impair cognitive development. As a model for an engineered probiotic therapy, we expressed PAL2 from *Arabidopsis thaliana* in EcN (McKenna and Nielsen, 2011) under the control of a weak, medium, or strong synthetic promoter (Anderson, 2006). The rationale for using different promoters is the sexual dimorphism exhibited in the Pah^{enu2} mouse model, where females show higher serum Phe than males (Sarkissian et al., 2008). These expression cassettes were placed on a high copy plasmid and transformed into EcN. As expected, the weak promoter drove slower Phe consumption *in vitro* than the medium or strong promoters (Figure 7A).

In vivo, male PKU mice treated with EcN expressing PAL2 with a strong promoter, and female mice treated with strains harboring either the strong or weak promoters, saw significant reductions in serum Phe concentrations 24 h after probiotic gavage (Figures 7B and 7C). In male mice, serum Phe levels were reduced to half of their baseline levels by the strongly expressing probiotic (Figure 7B). As expected, we observed differences in efficacy among male and female mice (Figure 7C) and between the weak and strong promoters. No serum Phe reductions were observed after 1 week. We recovered isolates containing the PAL gene from feces 1 week after delivery and did

not observe any mutations to PAL2. Only 1 of 16 strains accrued genomic changes, corresponding with an estimated mutation rate of 0.001 SNPs/genome/generation. The two SNPs we observed in this strain were (1) a nonsynonymous mutation in the endoribonuclease ybeY and (2) a nonsense mutation in 4-alpha-glucanotransferase malQ. No other isolate sequenced during this study had mutations in these genes. Given the low frequency of the recovered mutations, our data did not support the hypothesis that PAL2 expression confers a significant selective pressure to EcN in the PKU gut.

DISCUSSION

Unlike conventional abiotic therapeutics, the performance characteristics of probiotics can vary during treatment in response to natural selection. While probiotics have been broadly used in humans for decades, engineering probiotics for therapeutic applications in humans will only be acceptable if it is demonstrated that probiotics exhibit long-term safety. This study represents a necessary first step towards this aim and takes advantage of strictly controlled mouse models. We have demonstrated that in the context of low microbiome diversity, the greatest selective pressure on EcN is carbon source limitation. In this context, we also observed consistent inactivation of gluconate metabolism and report evidence of a possible link between metabolism of gluconate and GlcNAc. Our observation of mutations in stress response pathways in low-diversity guts suggest that low gut diversity, known to be pathologic to the host (Lane et al., 2017), can also subject the present bacteria to stress, which might exacerbate genomic instability. As a gut commensal, we found the lowest evidence of selection in a high diversity microbiome, but as a probiotic and a chassis for engineered therapies, we can expect future clinical use of EcN to occur in the context of dysbiosis. Future work should therefore include a systematic assessment of probiotic efficacy and adverse effects in relationship to personalized microbiome features prior to treatment. These results collectively indicate that EcN defines its gut niche through metabolic competition, painting EcN as not an opportunistically offensive player but instead as a scavenger and survivalist.

In the context of engineered PAL activity in PKU mice, we achieved up to 50% reduction in serum Phe levels, depending on strain design and the extent of hyperphenylalaninemia, indicating the opportunity for personalized engineered probiotic therapies. We were unable to observe significant changes to the genome or plasmids of our engineered probiotic over 1 week of treatment, supporting EcN's suitability as a chassis for engineered therapies. Coupled with continued development of biocontainment approaches (Chan et al., 2016), we envision the experiments described herein as a general pipeline to be applied to other probiotic strains in development in the context of diverse healthy and dysbiotic microbiomes and other in vivo habitats to better understand their safety and engineering potential. The major selective pressures operating on any engineered probiotic may be substantially different from their WT counterparts and would warrant further study of their in vivo evolutionary trajectories. As such, this work presents a generalizable framework for developing and regulating living therapeutics.

STAR***METHODS**

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j. chom.2019.02.005.

ACKNOWLEDGMENTS

We would like to acknowledge Chyi Hsieh, Andrew Kau, and members of their labs for shared setup and maintenance of our germ-free mouse facility; Jessica Hoisington-Lopez, Eric Martin, and Brian Koebbe for next-generation sequencing and high-throughput computing support at the Edison Family Center for Genome Sciences and Systems Biology at Washington University in St. Louis School of Medicine; and Thaddeus Stappenbeck, Tae Seok Moon, Phillip Tarr, and members of the Dantas lab for helpful discussions. This work was supported by the NIH (DP2DK098089, R01GM099538, R01Al123394, R01HD092414, and R01AT009741 to G.D.; T32DK077653 to N.C. and A.J.G.; T32GM007067 to A.J.G. and M.W.P.), the NSF (DGE-1143954 to M.K.G.), the Kenneth Rainin Foundation (13H5 to G.D.), the Chancellor's Graduate Research Fellowship Program at Washington University in St. Louis (to A.F.), and the Mr. and Mrs. Spencer T. Olin Fellowship at Washington University in St. Louis (to M.K.G.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

AUTHOR CONTRIBUTIONS

Conceptualization, N.C., A.F., A.J.G., M.W.P., M.K.G., and G.D.; Data Analysis, N.C., A.F., A.J.G., M.W.P., M.K.G., and G.D.; *In Vivo* Functional Metagenomic Selections, N.C., A.F., A.J.G., M.W.P., M.K.G., X.S., and D.P.; *In Vitro* Experiments, N.C. and A.F.; PKU Experiments, N.C. and A.F.;

Manuscript Preparation, N.C., A.F., and G.D.; Library Creation and Engineering of GFP-Tagged EcN, B.W.; Engineering of EcN:PAL2 Strains, Z.C.; Creation, Sharing, and Guidance on Pah^{enu2}-Mouse Model, S.D.

DECLARATION OF INTERESTS

The authors declare no competing interests. The following authors have the current affiliations: A.J.G., VL55; M.K.G., Flagship Pioneering; D.P., Eli Lilly & Company. The authors assert that VL55, Flagship Pioneering, and Eli Lilly & Company were not involved in and did not influence this research.

Received: January 7, 2019 Revised: February 6, 2019 Accepted: February 19, 2019 Published: March 26, 2019

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STAR***METHODS**

KEY RESOURCES TABLE

	SOURCE	
Readenin of Nesource		IDENTIFIEN
	Dr. Dhil Tarr	N/A
Escherichia coli Nissie 1917	bi. Fini Tan	Catt C640002
12 member defined microbial community		
Observice les Particles and Parametricent Particip	Dr. Andrew Goodman	See Table 34
Chemicals, Peptides, and Recombinant Proteins	S	0-111 1075 4
inulin Decteir	Sigma	
Dextrin	Sigma	
Glutamax	ThermoFisher	
Taq Reddymix	Fisher Scientific	
14 Polymerase	NEB	Cat# M0203S
14 PNK	NEB	Cat# M0201S
Taq Polymerase	NEB	Cat# M0273S
T4 DNA Ligase	NEB	Cat# M0202S
Phusion HF Mastermix	ThermoFisher	Cat# F531S
Taq Hot-Start DNA Polymerase	Takara	Cat# R028A
Nextera XT Tagmentation reagents	Illumina	Cat# FC-131-1096
Genomic DNA Buffer Set	Qiagen	Cat# 19060
NEBNext FFPE Repair Mix	NEB	Cat# M6630
NEBNext End repair / dA-tailing Module	NEB	Cat# E7546
MinION Ligation Sequencing Kit 1D	Oxford Nanopore	Cat# SQK-LSK108
MinION Adapter Mix	Oxford Nanopore	Cat# AMX1D
NEB Blunt/TA Ligase Master Mix	NEB	Cat# M0367
Minion Adapter Bead Binding Buffer	Oxford Nanopore	Cat# ABB
Running Buffer with Fuel Mix	Oxford Nanopore	Cat# RBF
Library Loading Bead Kit	Oxford Nanopore	Cat# EXP-LLB001
Critical Commercial Assays		
Phenylalanine Assay Kit	Sigma	Cat# MAK005
PowerSoil DNA Extraction Kit	Qiagen	Cat# 12955-4
MinElute PCR Purification Kit	Qiagen	Cat# 28006
MinElute Gel Extraction Kit	Qiagen	Cat# 28606
PicoGreen kit	Invitrogen	Cat# P11496
DNeasy UltraClean 96 Microbial kit	Qiagen	Cat# 10196-4
BIOLOG Phenotypic Microarrays	BIOLOG, Inc	Cat#s PM1 and PM2A
Deposited Data		
Raw reads for functional metagenomic selections	This paper	BioProject: PRJNA517544
Raw reads for 16S sequencing	This paper	BioProject: PRJNA517716
Raw reads for EcN isolates	This paper	BioProject: PRJNA517347
Assembled wild-type EcN genome	This paper	Genbank: CP035486-CP035489
Assembled functional metagenomic contigs	This paper	ID# BioSample: SAMN10936612
Experimental Models: Cell Lines		
Caco-2	ATCC	Cat# ATCC HTB-37
HT29-MTX	Sigma	Cat# 12040401

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Mus musculus C57BL/6 (Germ-free)	University of Michigan	N/A
Mus musculus C57BL/6 (Germ-free)	UPenn	N/A
Mus musculus C57BL/6J (Conventional)	Jackson Labs	Cat# JAX:000664
Mus musculus C57BL/6J Pah ^{enu2}	Dobrowolski Lab	N/A
Oligonucleotides		
Primers	IDT	Table S6
Recombinant DNA		
pSPAL2At	Addgene	Cat# 78286
Other plasmids	This paper	Data S2
pZE21	Expressys	pZE21MCS
Software and Algorithms		
PARFuMS	Boolchandani et al., (2017)	N/A
CD-HIT	Fu et al., (2012)	http://weizhongli-lab.org/cd-hit/
Bowtie2	Langmead and Salzberg, (2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
SAMtools	Li, (2011)	http://www.htslib.org/
BLASTp	Boratyn et al., (2012)	ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/
Interproscan	Jones et al., (2014)	https://www.ebi.ac.uk/interpro/download.html
BLAST2GO	BioBam	https://www.blast2go.com/
R	R Core Team, 2018	https://www.r-project.org/
heatmaply	Galili et al., (2018)	https://cran.r-project.org/web/packages/heatmaply/vignettes/ heatmaply.html
USEARCH7	Edgar, (2010)	https://drive5.com/usearch/
QIIME 1.9.0	Caporaso et al., (2010)	http://qiime.org/
Trimmomatic	Bolger et al., (2014)	http://www.usadellab.org/cms/?page=trimmomatic
SPAdes	Bankevich et al., (2012)	http://cab.spbu.ru/software/spades/
LAST	Frith and Noé, (2014)	http://last.cbrc.jp/
IGV	Thorvaldsdóttir et al., (2013)	http://software.broadinstitute.org/software/igv/
Prokka	Seemann, (2014)	https://github.com/tseemann/prokka
Breseq	Deatherage and Barrick, (2014)	http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenome Resequencing
VarScan2	Koboldt et al., (2012)	http://varscan.sourceforge.net/
Annovar	Wang et al., (2010)	http://annovar.openbioinformatics.org/en/latest/
Phaster	Arndt et al., (2016)	http://phaster.ca/
CNVkit Pipeline	Talevich et al., (2016)	https://cnvkit.readthedocs.io/en/stable/
PHYLIP 3.695	Felsenstein, (2005)	http://evolution.genetics.washington.edu/phylip.html
ITOL	Letunic and Bork, (2016)	https://itol.embl.de/
Roary	Page et al., (2015)	https://sanger-pathogens.github.io/Roary/
RaxML	Stamatakis, (2014)	https://cme.h-its.org/exelixis/web/software/raxml/index.html
HyPhy	Delport et al. (2010)	http://hyphy.org/
Other		
Human Diet	Envigo	Cat# TD.88137
Mouse Diet	Purina	Cat# 5K67

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gautam Dantas (dantas@wustl.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbial Strains

E. coli Nissle was obtained as a kind gift from Dr. Phillip I. Tarr (Washington University in St. Louis School of Medicine) and authenticated via whole-genome sequencing. Unless otherwise indicated, EcN was grown in LB media under aerobic conditions at 37° C. Agar was added at a concentration of 15 g/L for growth on solid media. For maintenance of the pZE21 plasmid (Lutz and Bujard, 1997) and its derivatives, kanamycin was added to a final concentration of 50 μ g/mL after autoclaving.

Thirteen strains that were selected to mimic a healthy human microbiota composition were obtained as a kind gift from Dr. Andrew Goodman (Yale University) (Table S4) and authenticated via sequencing of the 16S region. Strains were cultured in TYG medium supplemented with D-(+)-Cellobiose (0.1% w/v; Sigma), D-(+)-Maltose (0.1% w/v; Sigma), D-(-)-Fructose (0.1% w/v; Sigma), Tween 80 (0.05% v/v; Sigma), Meat Extract (0.5% w/v; Sigma), ATCC Trace Mineral Supplement (1% v/v), ATCC Vitamin Supplement (1% v/v), N-butyric acid (4mM), propionic acid (8mM), isovaleric acid (1mM), and acetic acid (30mM) as in Goodman et. al. (Goodman et al., 2009), referred to as Mega Medium. Culturing occurred under anaerobic conditions in a soft-sided plastic anaerobic chamber (Coy Laboratory Products). Stocks of strains were stored in E-Z crimp top vials (Wheaton) at -80°C in Mega Medium with 20% glycerol. Stocks were titered by plating on BHI blood agar plates. Strains were pooled immediately prior to gavage such that each member was equally represented, and washed 3x in PBS. *Eubacterium rectale* and *Ruminococcus torques* displayed poor recovery rates from freezer stocks, so overnight cultures at early stationary phase were included in the final pool *in* lieu of stocks. Mice were gavaged with a total dose of 1×10^8 CFU in a volume of 200 µL.

Mice

All mouse experiments were approved by the Washington University in Saint Louis School of Medicine Division of Comparative Medicine.

E. coli Nissle monocolonization experiments were performed in germ-free C57BL/6 mice (University of Michigan). Upon arrival, mice were provided food and water *ad libitum* for one week prior to colonization. Mouse experiments were not blinded. For the mouse chow experiment, mice were provided autoclaved feed (Purina Conventional Mouse Diet (JL Rat/Mouse 6F Auto) #5K67) and autoclaved water. Human diet consisted of Envigo TD.88137, irradiated and vacuum-sealed. Inulin or Dextrin (Sigma) was provided in the drinking water at a concentration of 20 g/L, and autoclaved. 6 (for the mouse chow experiment) or 5 (for the Human Diet experiment) co-housed male mice were exposed to 10^8 colony forming units of *E. coli* Nissle via oral gavage of 100 µL cell suspension in phosphate buffered saline on day 1 of the experiment. Feces were collected at weekly intervals and immediately frozen at -80 °C. At the end of the experiment, mice were sacrificed through carbon dioxide asphyxiation and cecal contents were collected.

Experiments involving the 13-member synthetic microbiota were also performed in 3-5 week old germ-free C57BL/6 mice (University of Pennsylvania Gnotobiotic Mouse Facility). Upon arrival, 3 germ-free mice (1-2 male, 1-2 female) per condition (monocolonized + human diet, 13-member + human diet, 13-member + human diet + dextrin, 13-member + human diet + inulin) were housed separately in germ-free isolators. Mice were acclimated to their diet for one week prior to colonization with the synthetic microbiota. The synthetic microbiota was allowed to stabilize for one week prior to delivery of 10^8 colony forming units of *E. coli* Nissle via oral gavage of 100 µL cell suspension in phosphate buffered saline on day 1 of the experiment. Fecal samples were subsequently collected weekly for 5 weeks, and immediately frozen at -80 °C. At the end of the experiment, mice were sacrificed through carbon dioxide asphyxiation and cecal contents were collected.

Experiments involving the conventional mouse microbiota were performed in a specific-pathogen-free facility with mice 3-5 weeks of age. 2 cages of 5 mice (Jackson Labs C57BL/6J (RRID:IMSR_JAX:000664)) for each condition were maintained on the Human Diet with or without prebiotic for one week after arrival. After one week, mice were deprived of food and water for 4h, and given either 20mg streptomycin in 100 μ L water, or 100 μ L water alone via oral gavage. After this treatment, food and water was immediately returned. Daily over the next three days, 10⁸ CFU of EcN was delivered in 100 μ L PBS via oral gavage. Fecal samples and intestinal contents were collected as above.

Experiments involving PKU mice (C57BL/6 mice homozygous for the Pah^{enu2} mutation) were performed in a specific-pathogenfree mouse facility. For each condition (High PAL expression, Low PAL expression, or No PAL expression), 3 Male and 3 Female PKU mice (10-12 weeks of age, Washington University in St. Louis) were co-housed by gender and condition and maintained on Phe-containing mouse chow (18 mice total from 5 different litters). 100 µL blood was sampled from the femoral artery one week before the experiment to serve as a baseline for each mouse. Then, 10⁹ CFU of the appropriate EcN strain was delivered via oral gavage. One day after gavage, 100 µL blood was again sampled from the femoral artery. Fecal samples were collected 1 week after gavage. After collection, all blood samples were allowed to coagulate then centrifuged in BD Microtainer ® SST[™] tubes for 2 minutes at 10,000g to collect serum. Serum samples were then passed through 10 kDA molecular weight cut-off filters by centrifugation. Phe levels of the filtrates were determined using a Phenylalanine Assay Kit (MAK005, Sigma-Aldrich).

For assessment of *in vivo* plasmid loss, 10 male C57BL/6J (RRID:IMSR_JAX:000664) mice 3-5 weeks of age housed in two separate cages were deprived of food and water for 4h and given 20 mg streptomycin in 100 µL water as above. Daily over the next three days, 10⁸ CFU of EcN containing the pZE21 vector was delivered in 100 µL PBS via oral gavage. Fecal pellets were collected on the final day of gavage and weekly for 5 weeks. Pellets were weighed and then homogenized in 300 µL PBS on a bench-top vortexer. Dilutions were plated in parallel on MacConkey agar and LB agar with 50 ug/mL kanamycin and incubated overnight at 37 C, with the prior selecting for aerobic lactose fermenters and the latter selecting for EcN containing the pZE21 vector. CFUs/mg feces

were estimated for each sample after colony enumeration. The colonies enumerated on the MacConkey agar plates were confirmed to be EcN by colony PCR of the chromosomally integrated GFP locus. A LOESS regression was applied to the longitudinal data for each selective agar.

Cell Lines

Caco-2 or HT29-MTX cells were seeded overnight in a 24-well plate at $50,000 \text{ cells/cm}^2$ in DMEM-HEPES media supplemented with Glutamax (10564-011, ThermoFisher) and 10% Fetal Bovine Serum. They were incubated at 37 °C, 5.0% CO₂ and 90% relative humidity. Caco-2 cells (ATCC HTB-37) were derived from a human male, and HT29-MTX cells (Sigma Aldrich 12040401) were derived from a human female. The cell lines were authenticated by the commercial vendors.

METHOD DETAILS

Plasmids Used in This Study

All plasmids used in this study were constructed using a Golden Gate Assembly Mastermix from NEB, according to the manufacturer's instructions. Plasmid pSPAL2At containing the PAL2 enzyme was obtained from Addgene (cat #78286), as a kind gift from David Nielsen (McKenna and Nielsen, 2011). Genbank files of all plasmids are provided in [Data S2].

Metagenomic Library Construction

Metagenomic libraries were sourced from a prior study examining the antibiotic resistome of the healthy maternal and infant microbiomes (Moore et al., 2015). Frozen glycerol stocks containing these libraries were thawed on ice, and plasmid DNA was extracted using a Qiagen Spin Miniprep kit. 100 ng of DNA was transformed into electrocompetent *E. coli* Nissle, and allowed to recover in SOC media for 1h at 37 °C. After 1h, 1 μ L of SOC media was plated on LB+kan, to count transformants, and the remainder was placed in 50mL LB media containing kanamycin at room temperature overnight. After overnight growth, the library was centrifuged at 4000 rpm for 7 m, resuspended in 10mL LB+15% w/v glycerol, and frozen in 1mL aliquots at -80 °C. After freezing, one aliquot was thawed on ice, and serial dilutions were plated on LB+kan media to count viable cells. This procedure yielded a library containing 32.77 Gb of metagenomic DNA.

Functional Metagenomic Library Sequencing

DNA was extracted from fecal samples and intestinal contents using the PowerSoil DNA extraction kit (MoBio/Qiagen). 10 ng of this DNA was used as a template for a multiplex PCR with primers 1-6 [Table S6] at equimolar concentrations using Taq Reddymix (Fisher Scientific). PCR recipe per sample was as follows: 12.5 μ L Taq Reddymix, 3 μ L primer mix (10 μ M total concentration), 10 ng template, nuclease free water to 25 μ L. PCR protocol was as follows: 94 °C for 10 m, 94 °C for 45 s, 55 °C for 45 s, 72 °C for 5.5 m, go to step 2.24 times, 72 °C for 10 m, 4 °C forever. PCR products were purified using a Qiagen Spin PCR Purification Kit. 500 ng PCR products in 200 μ L elution buffer (from purification kit) were then placed in an AB1900 half-skirted plate (Fisher Scientific) and sonicated using a Covaris E220 sonicator. The settings on the sonicator were as follows: Peak Incident Power: 140, Duty Cycle: 10%, Cycles per Burst: 200, Treatment Time: 600 s, Temp: 7 °C. Sonicated products were cleaned using a Qiagen MinElute PCR purification kit according to the manufacturer's instructions, and eluted in 22 μ L elution buffer. 20 μ L sonicated products were end-repaired through addition of 2.5 μ L DNA ligase buffer with 10 mM ATP (NEB), 1 μ L 1 mM dNTP, 0.5 μ L T4 Polymerase (NEB), 0.5 μ L T4 PNK (NEB), and 0.5 μ L Taq Polymerase (NEB), and incubating for 30 m at 25 °C, followed by 20 m at 75 °C. Then, 0.8 μ L T4 DNA ligase (NEB) and 5 μ L pre-annealed sequencing barcodes were added to the full end-repair reaction, followed by 40 m at 16 °C, and 10 m at 65 °C.

Sequencing barcodes were designed according to the template described in Primers 7-8 (Table S6), and synthesized by IDT in 2 96-well plates to a concentration of 500 μ M. Each barcode was designed to be at least 2 mismatches away from every other barcode. 2 μ L of each primer pair were added to 96 μ L of TES buffer (10mM Tris, 1mM EDTA, 50mM NaCl, pH 8.0), and this mixture was diluted 1:10 in TES buffer, to a final concentration of 1 μ M. Adapters were annealed by incubating at 95 °C for 1 m, followed by slowly cooling (0.1 °C per second) to 4 °C. Annealed oligos were kept cold and transferred to a -20 °C freezer until use.

Next, ligation products were size-selected to 300-400bp in size through gel electrophoresis. Briefly, the total ligation volume was loaded on a 2% (w/v) agarose gel in 0.5x TBE buffer using thin combs. This gel was run at 120V for 2h, or until the loading dye front reached \sim 2/3 of the way to the end of the gel. DNA fragments between 300 and 400bp were excised, and purified using the Qiagen MinElute Gel Extraction Kit, eluting in 15 μ L elution buffer.

Sequencing adaptors were then added to the size-selected ligation products through PCR. PCR recipe was as follows: 12.5 μ L Phusion HF Mastermix (ThermoFisher), 9.5 μ L nuclease-free water, 1 μ L 10 μ M primer mix (Primers 9-10) and 2 μ L gel-purified DNA. PCR conditions were as follows: 98 °C for 30 s, 98 °C for 10 s, 65 °C for 30 s, 72 °C for 30 s, return to step 2 17 times, 72 °C for 5 min, 4 °C forever. PCR products were then size-selected on a 2% agarose, 0.5x TBE gel as above. Sequencing libraries were quantified on a Qubit fluorimeter, and pooled at equimolar concentrations for sequencing. 100,000 2x150 paired-end sequencing reads were obtained per sample.

Functional Metagenomic Sequencing Analysis

Functional metagenomic contigs were assembled from sequencing reads and annotated using PARFuMS (Boolchandani et al., 2017; Forsberg et al., 2012). For each experiment, contigs were clustered at 95% nucleotide identity using CD-HIT (Fu et al., 2012). Then,

vector-trimmed sequencing reads (from PARFuMS) were uniquely mapped to each contig using bowtie2 (Langmead and Salzberg, 2012), and mapping reads were counted using SAMtools (Li, 2011). Contig abundance was estimated as the fraction of reads mapping to each contig. To perform Gene Ontology mapping, open reading frames (from PARFuMS) were mapped against all nonredundant protein sequences using BLASTp (Boratyn et al., 2012), and annotated using InterProScan (Jones et al., 2014). Outputs of these two operations were then used as input to Blast2GO, according to manufacturer's instructions. Each contig was associated with a GO annotation if at least one of its constituent open reading frames was. The abundance of each GO annotation was then estimated as the sum of contig abundances associated with that GO annotation. Heatmaps of GO annotations were plotted using the heatmaply package in R.

16S rRNA Sequencing and Analysis

We performed sequencing of the variable region (V4) of the 16S rRNA gene on 7 longitudinal fecal samples from each of 17 mice (102 samples total). We used 515F/806R PCR primers including Illumina flowcell adapter sequences to amplify the V4 region using the Earth Microbiome Protocols (Caporaso et al., 2012) (described in more detail here: http://www.earthmicrobiome.org/emp-standard-protocols/16s/).

The following 25 µL reaction was prepared in 96-well plates: 10 µL H20, 12.5 µL Taq Hot-Start DNA Polymerase (Takara R028A), 1 µL forward primer (10 uM), 1 µL reverse primer (10 uM), 0.5 µL template DNA (1 ng/uL). PCR cycle temperatures were as follows: 98 C for 30 s, then 35 cycles of [98 C for 10 s, 50 C for 30 s, 72 C for 30 s], then 72 C for 2 min. PCR reactions were carried out in triplicate for each reaction. In addition, a template-less control reaction was carried out for each primer pair. PCR products were assessed via gel electrophoresis to verify the amplicon size and absence of contamination, then were quantified using the PicoGreen kit (Invitrogen P11496), following the manufacturer's protocols. 16S rRNA gene amplicons were sequenced by 2x250 bp paired-end sequencing on the Illumina MiSeq platform using custom primers (read 1: 5'-TAT GGT AAT TGT GTG CCA GCM GCC GCG GTA A-3'; read 2: 5'-AGT CAG TCA GCC GGA CTA CHV GGG TWT CTA AT-3'; and index: 5'-ATT AGA WAC CCB DGT AGT CCG GCT GAC TGA CT-3') at a loading concentration of 8pM with 25% PhiX spike-in.

OTUs were generated using USEARCH7 (Edgar, 2010). Reads were demultiplexed by sample and read pairs merged (usearch -fastq_mergepairs -fastq_maxdiffs 0 -fastq_truncqual 3 -fastq_maxmergelen 258 -fastq_minmergelen 248). Merged reads were quality filtered (usearch -fastq_filter -fastq_maxee 0.5), dereplicated (usearch -derep_fulllength -sizeout), sorted and singletons removed (usearch -sortbysize -minsize 2), clustered (usearch -cluster_otus), and checked for chimeric sequences using the Gold database (usearch -uchime_ref -db gold.fa -strandplus -nonchimeras). OTUs were renamed (uparse/fasta_number.py), reads were mapped back to OTUs at 97% identity (usearch -usearch_global -strand both -id 0.97), and converted to the final OTU table (uparse/uc2otutab.py).

Taxonomy was assigned using the QIIME 1.9.0 (Caporaso et al., 2010) pipeline with the GreenGenes database. The OTU table was subsampled to 10,000 reads per sample (*single_rarefaction.py*).

Isolate Sequencing

To isolate *in vivo*-adapted EcN isolates, intestinal contents or fecal samples were vortexed in phosphate-buffered saline and streaked on LB plates containing kanamycin. Single colonies were placed in liquid media and grown at 37 °C overnight. Un-adapted EcN strains were also grown overnight. Total DNA was extracted from each culture using the DNeasy UltraClean 96 Microbial kit (10196-4, Qiagen), and prepared for whole-genome sequencing using Nextera Tagmentation (Baym et al., 2015). Briefly, gDNA was brought to a concentration of 0.5 ng/µL in 1 µL volume. To each sample, 1.25 µL TD buffer, 0.125 µL TDE1 enzyme, and 0.125 µL nuclease-free water was added, and incubated at 55 °C for 15 m. Then, 11.2 µL KAPA HiFi master mix was added to each tagmented sample. Indexed sequencing adaptors (5 µM each) were thawed, and 8.8 µL was added to each sample. Then, PCR was performed using the following protocol: 72 °C for 3 m, 98 °C for 5 m, 98 °C for 10 s, 63 °C for 30 s, 72 °C for 30s, return to step 2 13 times, 72 °C for 5 m, 4 °C forever. PCR reactions were purified using AMPure XP beads according to the manufacturer's protocol, and eluted in 60 µL resuspension buffer. Sequencing libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Fisher Scientific), and pooled at equimolar concentrations for sequencing. 2 million 2x150bp sequencing reads were obtained per sample.

MinION Sequencing

Genomic DNA from 30 mL of overnight culture of control EcN was extracted using a Genomic DNA Buffer Set (19060, Qiagen) with the Qiagen protocol for Gram-negative bacteria (Qiagen Genomic DNA Handbook 06/2015) and the Genomic-tip 500/G (10262, Qiagen). Purified genomic DNA was sheared to a target fragment size of 10 kilobases using the Covaris g-TUBE[™]. All following steps were carried out in Eppendorf DNA LoBind tubes. Sheared DNA was repaired using the NEBNext FFPE Repair Mix (M6630), and repaired DNA purified from the reaction using Agencourt AMPure XP beads. DNA was then end-repaired and dA-tailed using the NEBNext End repair / dA-tailing Module (E7546) and again purified with AMPure XP beads. DNA was then prepared for MinION sequencing using the MinION Ligation Sequencing Kit 1D (SQK-LSK108). Briefly, adapters were ligated to the DNA using the MinION Adapter Mix (AMX1D) and NEB Blunt/TA Ligase Master Mix (M0367). Then the DNA product was purified using AMPure XP beads and the Minion Adapter Bead Binding Buffer (ABB). 350 ng of DNA product was then sequenced on a MinION R9.4 flow cell for 48 hours using the Running Buffer with Fuel Mix (RBF) and the Library Loading Bead Kit (EXP-LLB001). Average read lengths were 7 kilobases and the genome was sequenced with an average coverage of 50 reads.

Isolate Sequencing Data Analysis

Illumina sequencing reads were quality-filtered and adaptor-trimmed using Trimmomatic (Bolger et al., 2014). Isolate genomes were assembled with Illumina data using SPAdes (Bankevich et al., 2012). The control EcN genome (which had both Illumina and MinION data) was assembled using SPAdes in hybrid mode. Illumina reads were mapped to the assembled genome using bowtie2 and MinION reads were mapped using LAST (Frith and Noé, 2014). Mapping results were converted to indexed, sorted bam files using SAMtools and visualized using IGV (Thorvaldsdóttir et al., 2013). The control EcN genome was then corrected manually by inspecting read alignments for dips in coverage, and correcting these regions using MinION data as well as homology to a published EcN genome (Reister et al., 2014) using an iterative process. After each iteration, Illumina and MinION reads were re-mapped to the corrected genome, and read alignments were inspected to verify an improved assembly. Coding sequences were annotated using Prokka (Seemann, 2014). One region of spurious assembly was due to imperfect tandem repeats present in the UpaH gene; this region was resolved by PCR amplification from the genome followed by Sanger sequencing. The result of this finishing process became the control genome to which all *in vivo*-adapted isolates were compared.

Mutation Calling

Single-nucleotide polymorphisms and indels were annotated using two complementary approaches. The first method was Breseq, using default parameters (Deatherage and Barrick, 2014). The second method was VarScan2, where SNPs and indels were called if supported by a minimum read coverage of 30 and 80% abundance in those reads (Koboldt et al., 2012). VarScan2 variant call format output was then annotated with ANNOVAR (Wang et al., 2010). Mutations called by each method were manually verified through visualization of Illumina read alignments using IGV. Anecdotally, both methods did recover some false positive mutation calls, primarily due to the presence of a homologous metagenomic DNA insert causing spurious read alignments to the EcN genome. Phage sequences were identified using the Phaster web server (Arndt et al., 2016). To look for potential plasmid sequences, assembled contigs for each isolate were aligned to 1) all nonredundant nucleotide sequences, and 2) the control EcN genome using BLASTn. Our criteria for plasmid sequences was the following: >500bp in length, <90% of bases mapping to the EcN genome, no homology to the functional metagenomic insert present in the strain (if any), and no obvious genomic contamination from other species. No contig satisfied these criteria, and therefore we concluded that no acquired plasmid sequences were obviously present in our *in vivo* adapted strains.

Annotation

To perform Gene Ontology mapping, mutated open reading frames were mapped against all nonredundant protein sequences using BLASTp, and annotated using InterProScan. Outputs of these two operations were then used as input to Blast2GO, according to manufacturer's instructions. The abundance of each GO annotation was then estimated as the number of open mutated open reading frames with that annotation among the strains within a certain condition. Heatmaps of GO annotations were plotted using the heatmaply package in R.

Copy Number Variation

Copy number variation was assessed using the CNVkit pipeline (Talevich et al., 2016). Briefly, short reads for each isolate were mapped to an indexed reference assembly using bowtie2 with the -very-senstitive-local option to generate SAM files. SAM files were then converted to sorted BAM files using Samtools. Coverage was calculated in bins using the CNVkit coverage function and compared to the coverage of the reference strain using the CNVkit reference function with the -no-edge option. Biases were adjusted using the CNVkit fix and segment functions. CNV hits were called if the bias-adjusted log₂ copy ratio was greater than 1.3 or less than -1.3.

Growth Assays on Functional Metagenomic Hits

Three biological replicates of EcN containing the appropriate plasmid were grown overnight in LB media containing kanamycin at 37 °C under anaerobic conditions. These were the overnight culture conditions for all growth assays unless otherwise indicated. The cell densities of these overnight cultures were measured, and EcN was transferred to M9 MOPS minimal media containing the carbon source of interest at a starting optical density of 0.01 in a 96-well plate. BIOLOG plates were prepared with M9-MOPS minimal media (Liu et al., 2015) per the manufacturer's recommendations. A Breathe-Easy Sealing Membrane (Sigma) was placed over the plate. This plate was placed in a plate reader set to maintain 37 °C and read optical density (absorbance at 600 nm) every minute for 72h.

To test the acid tolerance of EcN strain MD04, overnight cultures of MD04 and WT EcN harboring the empty pzE21 plasmid were subcultured in LB broth pH 7.0 to an OD600 of approximately 0.4, then were washed with PBS and resuspended in either LB pH 7.0 or LB pH 2.5 (adjusted with HCl). The cultures were incubated at 37 °C for 30 minutes, then were washed again and resuspended in LB pH 7.0 and grown for 15 hours at 37 °C in a plate reader with optical density measured at 20 minute intervals.

To test whether EcN expressing glycosyl-hydrolase encoding fragments from the functional metagenomic selection can support growth of WT EcN, overnight cultures of EcN strains MD01, MD02, or WT EcN carrying empty pZE21 vector were subcultured in LB to an OD600 of 0.5, then washed three times with PBS and resuspended in 1% raffinose minimal M9-MOPS media. The cultures were incubated at 37 °C for 3 hours, then centrifuged at 6000g for 5 minutes. The supernatants were filter-sterilized using 0.2 µm filters. These pre-conditioned media, as well as unconditioned 1% raffinose minimal M9 media were inoculated with WT EcN and grown for 15 hours at 37 °C with optical density measured at 20 minute intervals.

To assess the ability of HD15 inserts to grow on mucin or GlcNAc, overnight cultures were normalized to an optical density of 0.1 in 1.5% porcine gastric mucin M9-MOPs minimal media or 20 mM GlcNAc M9-MOPs minimal media and incubated in a plate reader at 37 °C for 20 hours with optical density measured at 20 minute intervals. The assay in porcine gastric mucin M9-MOPs minimal media was repeated on separate days.

Growth Assays on Mutant Isolates

NagC mutants were grown in standard or diauxic growth experiments. As before, overnight cultures were normalized to an optical density of 0.1 in 0.4% glucose, 0.4% GlcNAc, or 0.2% glucose / 0.2% GlcNAc M9-MOPS minimal media. Alternatively, overnight cultures were transferred to 0.2% glucose / 0.75% porcine gastric mucin M9-MOPS minimal media. The plates were set in a plate reader at 37 °C for 20 hours with optical density measured at 20 minute intervals.

Two representative *gntT* mutants were grown on 0.4% gluconate, 0.4% GlcNAc, or 1.5% porcine gastric mucin M9-MOPS minimal media. Overnight cultures were normalized in M9-MOPS minimal media supplemented with either of the three carbon sources at a starting optical density of 0.1 in a 96-well plate as before and incubated in a plate reader at 37 °C for 24 hours with optical density measured at 20 minute intervals.

For the gluconate sensitivity assays overnight cultures of the same *gntT* mutants were normalized as before in 20 mM glucose or 20 mM GlcNAc M9-MOPS minimal media supplemented with either 0.5, 1, 2, 4, or 8 mM gluconate and incubated in a plate reader at 37 °C for 20 hours with optical density measured at 20 minute intervals.

Epithelial Cell Binding Assay

Overnight cultures of representative *kfiB* mutants or WT EcN were incubated in LB at 37 °C. The next day, wells with Caco-2 or HT29-MTX cells were inoculated with mutant or WT EcN that had been washed and resuspended in PBS. The multiplicity of infection was 1:100. The human and bacterial cells were co-incubated 37 °C, 5.0% CO₂ and 90% relative humidity for 3 hours. Media was aspirated from each well. Three replicate wells for each strain were gently washed 3x with PBS, while three replicate wells for each strain were left untreated. All wells were then treated with 100 μ L of 1% Triton-X for 10 minutes at room temperature to lyse the human cells. 900 μ L LB was added to each well and the cells resuspended by pipetting. These cultures were diluted and plated on LB agar plates and incubated overnight at 37 °C. The next day CFUs were enumerated, and the percentage of adherent cells was calculated as the mean ratio of CFUs/mL from the washed and unwashed wells for each strain.

In Vitro Phenylalanine Degradation Assay

EcN strains expressing PAL2 in the pZE21 plasmid under promoters of low (p08), medium (p01), or high (p19) strength, or harboring only empty vector, were grown overnight in LB at 37 °C. The next day the optical densities (absorbance at 600nm) of these cultures were measured and the strains were transferred to M9 minimal media supplemented with 0.4% glucose and 20 μM L-phenylalanine with a starting optical density of 1.0. Fractions of these cultures were removed at 0, 30, 60, and 90 minutes and immediately centrifuged. Supernatants were passaged through 10 kDa molecular weight cut-off filters and the phenylalanine in the filtrates was measured using a Phenylalanine Assay Kit (MAK005, Sigma-Aldrich). Phenylalanine levels were reported as average absolute nanomoles of phenylalanine per well. Three technical replicates were carried out for each strain.

Gene Phylogenies and Enrichment Testing

We searched for genes with variants significantly enriched in specific diet conditions or in specific microbiome complexities using the hypergeometric test for enrichment and Bonferroni correction for multiple hypothesis testing. The significant genes included *gntT*, *nagC*, *sgrR*, *rsmG*, and *kfiB*. Gene phylogenies were created for these genes by aligning the gene sequence for all isolates involved in the comparison in which the gene variants were found enriched in a condition (that is, all isolates along the axis of different diets, or the axis of microbiome complexity). Alignments were generated using Clustal Omega. Maximum parsimony phylogenies for each gene were generated using the Dnapars program included in PHYLIP 3.695 (Felsenstein, 2005), and visualized using iTOL (Letunic and Bork, 2016).

E. coli Phylogeny Construction

Representative *E. coli* genomes were downloaded from NCBI. PROKKA was used to generate general feature format (gff) files for each *E. coli* genome, including our assembled reference EcN genome (Seemann, 2014). Roary was used to generate a core gene alignment, using these gff files and the corresponding genome sequences (Page et al., 2015). The strain phylogeny was inferred with RaxML using the GTRGAMMA model and 1000 bootstraps (Stamatakis, 2014). The best tree was visualized using iTOL.

Analysis of Selection

SgrR was analyzed for evidence of positive or negative selection. The previously generated gene alignment and phylogeny were provided as input to the aBSREL, MEME, and FUBAR modules included in the HyPhy application hosted by the Datamonkey webserver (Delport et al., 2010). Neither of the three modules reported evidence of selection.

QUANTIFICATION AND STATISTICAL ANALYSIS

All of the statistical details of experiments, including significance criteria, sample size, definition of center, and dispersion measures can be found in the figure legends and in the Results section. For all *in vitro* experiments involving synthetic DNA, n refers to the number of biological replicates of each strain (i.e. separate colonies) assayed per condition. For all *in vitro* experiments involving *in vivo*-adapted probiotic isolates, n refers to the number of technical replicates of each strain (i.e. same master stock). For mouse experiments, n refers to the number of mice in each group. All significance values were FDR-corrected as described. No sample size calculations were performed during the design of experiments. No samples were excluded. No blinding was performed.

DATA AND SOFTWARE AVAILABILITY

The raw reads for functional metagenomic selections have been deposited in NCBI under ID code PRJNA51754. Assembled contigs for functional metagenomic selections have been deposited in DDBJ/ENA/GenBank under the accession SHCT00000000. The version described in this paper is version SHCT01000000. The raw reads for all EcN isolates have been deposited in NCBI under ID code PRJNA517347. The assembled wild-type EcN genome has been deposited in NCBI under ID codes CP035486-CP035489. 16S rRNA sequencing for experiments involving the 13-member community have been deposited in NCBI under ID PRJNA517716.

Cell Host & Microbe, Volume 25

Supplemental Information

Adaptive Strategies of the Candidate Probiotic

E. coli Nissle in the Mammalian Gut

Nathan Crook, Aura Ferreiro, Andrew J. Gasparrini, Mitchell W. Pesesky, Molly K. Gibson, Bin Wang, Xiaoqing Sun, Zevin Condiotte, Stephen Dobrowolski, Daniel Peterson, and Gautam Dantas

Supplementary Information

Supplementary Figures



Figure S1. Phylogeny of *Escherichia* strains. Related to Figure 1. Maximum likelihood
phylogeny of representative *Escherichia* strains annotated by clade (inner track, branches) and by
strain type (outer track).



Figure S2. Schematic of *in vivo* functional metagenomic selections and adaptation 9 experiments. Related to Table 1, Figures 1-3, and Figures 5-6. Experimental design and 10 sampling timelines for A) functional metagenomic selections in germ-free mice B) functional 11 metagenomic selections in mice pre-colonized with a defined community, C) genomic adaptation 12 experiments in mice fed different diets, D) genomic adaptation experiments in mice with different 13 14 gut microbiota complexities, E) longitudinal analysis of genomic adaptation in conventional, streptomycin treated mice, F) analysis of adaptations specific to gut sites, and G) genomic 15 adaptation experiments in Pahenu2 mutant mice (PKU model) that were treated with EcN 16 17 harboring either empty vector or the Pal2 gene. Mice were gavaged with EcN on day 0. N: number of replicate mice, n: total number of sequenced isolates. Pink bars: sampling time point from which 18 EcN isolates were harvested. Mice with a defined community were pre-colonized 7 days before 19 EcN Gavage. 20



Figure S3. Assessment of *in vivo* plasmid loss. Related to Figure 1. CFUs/mg of EcN recovered from mouse fecal samples over 5 weeks when plated either on MacConkey agar (selects for all EcN) or LB agar with Kanamycin (selects for EcN containing pZE21). Shaded regions represent 95% confidence intervals of LOESS regressions on the data. 10 mice from two different cages were sampled.





29 Figure S4. Growth of wild type EcN on mono- and polysaccharides. Related to Figures 1-3.

In vitro growth of EcN on minimal media supplemented with the monosaccharides glucose or lactulose, or with various polysaccharides under aerobic (**A**) and anaerobic (**B**) conditions. Shown are means of 3 replicates. Error bars are one standard deviation above and below the mean.

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Figure S5. Relative taxonomic abundances from gnotobiotic mice humanized with a defined 13-member community. Related to Figure 3. gDNA from longitudinal fecal samples was analyzed by 16S rRNA sequencing. Mice were fed a Human Diet, and in some arms were supplemented with prebiotic in the drinking water. One week after humanization with the 13member community, mice were gavaged with EcN containing either empty pZE21 vector (-Library) or the functional metagenomic library (+Library).











Α

after gavage from two streptomycin treated mice are plotted against number of generations, where 48 the *in vivo* generation time is assumed to be 100 minutes (Rang et al., 1999), and the number of 49 SNPs at time 0 is assumed to be 0. 10 isolates per mouse per timepoint were whole-genome 50 sequenced for SNP detection. The shaded area represents the 95% confidence interval of a linear 51 regression of new unique SNPs/genome on generation. The estimated mutation rate of 0.007 (\pm 52 53 (0.002) mutations per genome per generation is on the same order of magnitude as the previously published rate of 0.001 new SNPs per genome per generation (Lee et al., 2012). B) Heatmap of 54 the cumulative number of nonsynonymous SNPs detected in genes grouped by their GO terms. 55 56 Inclusion criteria for the heatmap was any function with at least two cumulative mutations identified across isolates from at least two mice. Dendrograms represent the clustering applied to 57 group similar rows/columns of the heatmap. With 168 mutations, the GO pathway for Cell 58 Division was the most commonly-mutated, but it should be noted that this GO pathway was almost 59 entirely (165/168 mutations) composed of the ribosomal genes mutated during streptomycin 60 treatment. The next most abundant pathways were transcription (57 mutations), regulation of 61 transcription (23 genes), and gluconate transmembrane transport (22 mutations). 62



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Figure S7. Growth of NagC and gntT mutants on mucin-containing minimal medias. Related 64 to Figure 5. A) Diauxic growth curves of *NagC* mutants on glucose and porcine gastric mucin 65 minimal media. B) Growth curves of gntT mutants on porcine gastric mucin minimal media 66 supplemented with either 0.5, 1, 2, 4, or 8 mM gluconate. The gray panel summarizes the growth 67 via the areas under the curves (AUC) for each gluconate concentration. N = 3, * P < 0.05, Welch's 68 t-tests, Benjamini-Hochberg correction for multiple comparisons. Black stars indicate that both 69 70 Isolate 3 and Isolate 21 had a significantly greater AUC at a given gluconate concentration; red only Isolate 21. Areas under the curves were calculated using the growthcurver R package. 71

72 Supplementary Tables

Table S1. Comparison of nutrient compositions of the standard Mouse Chow diet
(Laboratory Rodent Diet 5001*, LabDiet) and the High-fat ('Human') Diet (TD.88137,
Teklad). Related to Table 1.

	Mouse Chow		High-fat ('Human') Diet	
	% By Weight	% kcal from	% By Weight	% kcal from
Protein	24.1	28.7	17.3	15.2
Fat	11.4	13.4	21.2	42
Carbohydrates	48.7	57.9	48.5	42.7
kcal/gm	2.8'	9	4 50	
Koul/gill	2.0		4.50	

- 77 Table S2. Comparison of formulaic compositions of the standard Mouse Chow diet
- 78 (Laboratory Rodent Diet 5001*, LabDiet) and the High-fat ('Human') Diet (TD.88137,
- 79 **Teklad**). **Related to Table 1.** Ingredients are listed in order of most to least abundant by weight.

Mouse Chow	High-fat ('Human') Diet
Ground Corn	Sucrose
Dehulled Soybean Meal	Anhydrous Milk Fat
Dried Plain Beet Pulp	Casein
Fish Meal	Corn Starch
Ground Oats	Cellulose
Dehydrated Alfalfa Meal	Mineral Mix
Brewers Dried Yeast	Vitamin Mix
Cane Molasses	Calcium Carbonate
Wheat Germ	DL-Methionine
Dried Whey	Cholesterol
Porcine Animal Fat	Ethoxyquin
Porcine Meat and Bone Meal	
Wheat Middlings	
Salt	

Calcium Carbonate	
Vitamin Mix	
Mineral Mix	

81 Table S3. Summary of *in vivo* adapted EcN isolates. Related to Figure 4.

					#	#
Microbiome	pZE21 Plasmid Insert	Diet	Organ	Day	Isolates	Mice
None	Metagenomic	Human	Cecum	35	20	2
None	Metagenomic	Human	Cecum	35	20	2
None	Metagenomic	Human+Dextrin	Cecum	35	20	2
None	Metagenomic	Mouse	Cecum	21	20	2
None	No insert	Human	Feces	35	20	2
13-member	No insert	Human+Dextrin	Feces	35	20	2
Mouse+strep	No insert	Human	Prox. SI	35	2	2
Mouse+strep	No insert	Human	Med. SI	35	22	3
Mouse+strep	No insert	Human	Dist. SI	35	25	3
Mouse+strep	No insert	Human	Cecum	35	20	2
Mouse+strep	No insert	Human	Colon	35	30	3
Mouse+strep	No insert	Human	Feces	5	5	1
Mouse+strep	No insert	Human	Feces	12	20	2
Mouse+strep	No insert	Human	Feces	19	20	2
Mouse+strep	No insert	Human	Feces	26	20	2
Mouse+strep	No insert	Human	Feces	33	20	2
Mouse+strep	No insert	Human+Dextrin	Feces	35	20	2
Mouse	No insert	Human	Feces	35	20	2
Mouse	No insert	Human+Dextrin	Feces	35	20	2
PKU Mouse	No insert	Mouse	Feces	35	17	2
PKU Mouse	PAL2	Mouse	Feces	35	20	2

82

83 Table S4. Taxonomic composition of the 13-member defined bacterial community (Goodman

et al., 2011). Related to Table 1.

Species	ATCC/DSMZ
Bacteroides caccae	ATCC 43185T

Parabacteroides distasonis	ATCC 8503
Bacteroides novel WH2	A. Salyers strain collection
Bacteroides ovatus	ATCC 8483T
Bacteroides uniformis	ATCC 8492
Bacteroides vulgatus	ATCC 8482
Clostridium scindens	ATCC 35704
Clostridium symbiosum	ATCC 14940
Dorea longicatena	DSM 13814
Eubacterium rectale	ATCC 33656
Ruminococcus obeum	ATCC 29174
Collinsella aerofaciens	ATCC 25986
Ruminococcus torques	ATCC 27756

86 Table S5: Putative phage regions in the EcN genome. Related to Figure 4.

REGION_LENGTH	SPECIFIC_KEYWORD	REGION_POSITION
18.8Kb	transposase, terminase	276890-295730
45.5Kb	transposase,lysis,head,capsid,tail	1325775-1371307
59Kb	tail,head,portal,terminase,lysin,integrase	2011601-2070656
12.5Kb	integrase, capsid, head	2535374-2547895
38.8Kb	integrase, transposase, tail	3318001-3356837

87

88 Table S6: Primers. Related to Figures 1-3 and Figure 4.

Primer	Primer Name	Primer Sequence
#		
1	pZE21_79_102For	CCGAATTCATTAAAGAGGAGAAAG
2	pZE21_80_103For	CGAATTCATTAAAGAGGAGAAAGG
3	pZE21_81_106For	GAATTCATTAAAGAGGAGAAAGGTAC
4	pZE21_126_149rc	GATATCAAGCTTATCGATACCGTC
	Rev	
5	pZE21_128_150rc	CGATATCAAGCTTATCGATACCG
	Rev	
6	pZE21_129_151rc	TCGATATCAAGCTTATCGATACC
	Rev	

7	Barcode1	/5Phos/ANNNNNNGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
8	Barcode2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN*T
9	IlluminaPEAmpF	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC
		GATCT
10	IlluminaPEAmpR	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTC
		TTCCGATCT

90 Table S7: Mutations in pZE21. Related to Figure 4

Position	Type of mutation	Mutation	# isolates	# mice	# cages
74	intergenic (in ori)	G→T	1	1	1
295	intergenic (between kanR and ori)	G→T	1	1	1
313	intergenic (between kanR and ori)	G→T	3	2	1
386	intergenic (between kanR and ori)	G→T	1	1	1
480	intergenic (between kanR and ori)	C→A	1	1	1
532	intragenic (in kanR)	P249P (CCC→CCA)	7	3	2
648	intragenic (in kanR)	R211R (CGG→AGG)	1	1	1
742	intragenic (in kanR)	P179P (CCC→CCA)	3	1	1
802	intragenic (in kanR)	D159E (GAC→GAA)	1	1	1
805	intragenic (in kanR)	L158L (CTG→CTT)	1	1	1
890	intragenic (in kanR)	T130N (ACC→AAC)	3	2	1
963	intragenic (in kanR)	H106N (CAC→AAC)	1	1	1
1,019	intragenic (in kanR)	A87E (GCG→GAG)	1	1	1
1,085	intragenic (in kanR)	A65V (GCG→GTG)	1	1	1
1,372	intergenic (upstream of promoter)	G→T	1	1	1
1,440	intergenic (upstream of promoter)	T→C	1	1	1
1,482	intergenic (upstream of promoter)	G→T	1	1	1
1,558	intergenic (in promoter)	G→T	1	1	1
1,681	intergenic (in terminator)	G→T	1	1	1
1,717	intergenic (in terminator)	G→T	1	1	1

The kanR mutations could have been accumulated during growth in selective media prior to delivery. As all kanamycin was removed from the gavage bolus by 3 washes in PBS, no antibiotic selection was present during growth *in vivo*. Further, these isolates were from mice never treated with streptomycin, eliminating cross-resistance as a possible explanation. No inactivating mutations in kanR were observed, potentially limited by the fact that kanamycin was used to recover EcN from fecal and intestinal contents.