



Adaptation of commensal proliferating *Escherichia coli* to the intestinal tract of young children with cystic fibrosis

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Edited by Lora V. Hooper, The University of Texas Southwestern, Dallas, TX, and approved December 19, 2017 (received for review August 14, 2017)

The mature human gut microbiota is established during the first years of life, and altered intestinal microbiomes have been associated with several human health disorders. *Escherichia coli* usually represents less than 1% of the human intestinal microbiome, whereas in cystic fibrosis (CF), greater than 50% relative abundance is common and correlates with intestinal inflammation and fecal fat malabsorption. Despite the proliferation of *E. coli* and other Proteobacteria in conditions involving chronic gastrointestinal tract inflammation, little is known about adaptation of specific characteristics associated with microbiota clonal expansion. We show that *E. coli* isolated from fecal samples of young children with CF has adapted to growth on glycerol, a major component of fecal fat. *E. coli* isolates from different CF patients demonstrate an increased growth rate in the presence of glycerol compared with *E. coli* from healthy controls, and unrelated CF *E. coli* strains have independently acquired this growth trait. Furthermore, CF and control *E. coli* isolates have differential gene expression when grown in minimal media with glycerol as the sole carbon source. While CF isolates display a growth-promoting transcriptional profile, control isolates engage stress and stationary-phase programs, which likely results in slower growth rates. Our results indicate that there is selection of unique characteristics within the microbiome of individuals with CF, which could contribute to individual disease outcomes.

gastrointestinal microbiome | *Escherichia coli* | cystic fibrosis

The human gut microbiota develops in early life as a result of environmental exposure, dietary habits, and host genetic factors, and it contributes to nutritional status, immune function, metabolism, and physiology (1–5). Altered intestinal microbiomes have been associated with several human health disorders (6–9), including cystic fibrosis (CF) (10), a genetic disease that affects the normal function of multiple organs, most prominently the airway and gastrointestinal (GI) tract. Approximately 85% of people with CF are unable to adequately digest and absorb proteins and lipids, a condition referred to as exocrine pancreatic insufficiency (11). Despite replacement pancreatic enzyme therapy, many individuals have significant malabsorption, excreting high levels of fat and other nutrients in the stool (12). This likely contributes to the significant growth stunting of many individuals with CF, and growth at 1 y correlates with patient life span (13–16). Thus, the GI tract of individuals with CF can contain high levels of fat, composed primarily of glycerol and fatty acids, as well as other nutrients that could select for an altered microbiota.

Escherichia coli usually represents less than 1% of the human intestinal microbiome (17); however, a previous study using a metagenomics approach showed that *E. coli* is significantly more abundant (up to 80–90%) in the fecal microbiota of young children with CF compared with age-matched healthy controls (10), an amount often exceeding that found in patients with other inflammatory intestinal diseases (18–21). Although clonal expansion of a single *E. coli* lineage was common in individual children with CF, different

E. coli lineages with distinct gene repertoires were found across patients (10). This suggested that clonal expansion of *E. coli* in the CF intestine arose independently in different patients and could reflect the propensity of *E. coli* to adapt to the unique growth conditions found in the CF GI tract, such as the ability to survive the inflammatory response and/or metabolize excess nutrients resulting from malabsorption and mucus accumulation. A functional metagenomics analysis of the metabolic capacity of fecal microbiomes of young children with and without CF revealed an overall decreased ability in fatty acid biosynthesis contrasted with an increased capacity for degrading antiinflammatory short-chain fatty acids (SCFAs) (22). This suggested that the initial microbiota composition in the CF gut is selected by and/or adapts to the presence of high levels of fat, which, in turn, creates a proinflammatory environment where bacteria such as *E. coli* can thrive. It seems likely that clonal expansion contributes to pathogenic outcomes; that is, a clone adapts

Significance

Escherichia coli isolated from fecal samples of young children with cystic fibrosis demonstrated an increased growth rate in the presence of glycerol as the sole carbon source, likely as a result of selection pressure from increased intestinal glycerol phospholipids from dietary fat. Therefore, clonal proliferation of microbiota species can occur with specific environmental adaptations, selected as a result of chronic intestinal inflammation and increased fecal fat, and may contribute to human diseases. Understanding the normal and abnormal development, evolution, and adaptation of important microbiota components and what leads to their clonal expansion should give rise to new therapeutic approaches that can help ameliorate chronic disorders.

Author contributions: S.M., H.S.H., L.R.H., and S.I.M. designed research; S.M., H.S.H., K.R.H., K.L., C.E.P., A.-F.I., C.P.M., and S.I.M. performed research; S.M., H.S.H., M.J.B., E.J.W., C.E.P., C.P.M., E.B., L.R.H., and S.I.M. analyzed data; and S.M., H.S.H., and S.I.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The genomic DNA sequence and RNA-seq data reported in this paper have been deposited at the National Center for Biotechnology Information under BioProject ID PRJNA417507 and in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE108846), respectively. *E. coli* isolates are available from the Cystic Fibrosis Research Translation Center (CFRTC) Microbiology Core at the University of Washington (depts.washington.edu/cfrtc/microbiology/).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1714373115/-DCSupplemental.

to intestinal inflammation and proliferates in the altered inflammatory milieu, and this proliferation results in subsequent increased inflammation resulting from stimulation of innate immune responses at the mucosal surface. This feed-forward loop with bacterial adaptation and expansion could be similar to that of *Pseudomonas aeruginosa* within the environment of the CF airway, although in the intestine, endogenous organisms within the complex microbiota would be selected rather than colonization with an environmental organism of the normally sterile areas of the respiratory tract. Therefore, our aim was to decipher the characteristics that make *E. coli* isolated from fecal samples of young children with CF so successful.

Results and Discussion

***E. coli* Isolated from Young Children with CF Exhibits Accelerated Growth Under Aerobic Conditions Compared with Control *E. coli* in Minimal Media with Glycerol as the Sole Carbon Source.** To characterize the *E. coli* present in the GI tract of young children with and without CF, we isolated *E. coli* from the fecal samples of six young children with CF and two healthy controls from a previous study (10) (Fig. 1A). The *E. coli* isolates were tested for their ability to grow in different media supplemented with different carbon sources. Similar growth rates for CF and control isolates were found in nutrient-rich brain heart infusion (BHI) and Luria broth (LB) media and in M9 minimal media supplemented with glucose (GluMM) (Fig. S1). However, when glycerol was used as the sole carbon source in M9 media [minimal media supplemented with glycerol (GlyMM)], CF *E. coli* exhibited accelerated growth rates and produced a large colony phenotype on GlyMM agar plates (Fig. 1B–D). Microscopic examination confirmed that the observed growth rate increase and large colony size were not the result of differences in bacterial cell size or morphology (SI Methods and Fig. S2).

Interestingly, the accelerated growth phenotype of CF isolates on GlyMM in comparison to controls was not observed under anaerobic growth conditions (SI Methods and Table S1). All isolates grew very slowly, and although minor differences in growth were observed across isolates, CF *E. coli* that grew better aerobically on glycerol did not demonstrate a similar phenotype when grown anaerobically. Although the GI tract is commonly thought of as an anaerobic environment, it has been shown that even in the normal gut, there is a zone of relative oxygenation near the mucosal surface (23). Oxygen diffusion from the intestinal tissues creates a GI luminal oxygenation gradient important in shaping the gut microbiota composition (24). In the normal gut, the abundance of *E. coli* and other facultative anaerobes such as Enterobacteriaceae is thought to be kept low by the scarce availability of oxygen and preferred carbon sources in conjunction with the presence of antimicrobial SCFAs (25, 26). SCFAs are not only important for the normal development and maintenance of a healthy intestinal epithelium but also possess antiinflammatory and antimicrobial properties and are known to effectively inhibit *E. coli*'s growth (27). The fecal microbiomes of patients with CF exhibit an increased capacity for degrading SCFAs (22). Depletion of the SCFA butyrate results in increased oxygenation of surface colonocytes, with the consequent aerobic expansion of Enterobacteriaceae (25, 26). In fact, significant expansion of Enterobacteriaceae communities is a common marker of dysbiosis not only in CF but also in other pathologies that lead to increased gut inflammation (28). These observations suggest that dysbiosis in these cases is at least in part due to the oxidative nature of the host inflammatory response. Given this and the fact that the accelerated growth phenotype of the CF isolates is only observed under aerobic conditions, we hypothesize that oxygen was likely an important factor in the evolution and successful gut colonization of the CF isolates. Moreover, the exacerbated inflammation results in the release of luminal neutrophils, which may further reduce the levels of butyrate-producing species and are also an important source of electron acceptors for *E. coli*'s anaerobic respiration in luminal areas where oxygen is not available (25, 26).

Glycerol, a readily metabolized carbon source for *E. coli*, although present in the mammalian gut, is usually rapidly consumed by the gut microbiota (29). However, in Crohn's disease, where fat absorption is compromised similar to CF, elevated levels of glycerol and depletion of antimicrobial SCFAs are two prominent features of these patients' fecal samples (30). Therefore, the observed expansion of *E. coli* in the CF gut may be due to the decreased presence of antimicrobial SCFAs, the increased oxygenation of GI mucosal surfaces as result of inflammation, and the higher availability of glycerol from fat malabsorption, and these factors could be present in other *E. coli* intestinal microbiota expansions apart from those observed in patients who have CF.

CF *E. coli* Isolated from Different Patients Is Genetically Distinct and Has Independently Acquired the Glycerol Growth Phenotype. Multi-locus sequence typing (MLST) and phylogenomic analysis indicate that each of the CF isolates is genetically and evolutionarily distinct (Fig. 2). In the tree shown in this figure, CF and control isolates are found throughout previously described *E. coli* phylogenetic lineages (31, 32), and some isolates exhibiting the glycerol growth phenotype are only distantly related. A genome-wide variant analysis of the eight isolates compared with reference strain MG1655 identified more than 11,000 nonsynonymous single-nucleotide polymorphisms (nsSNPs) present in one or more CF isolate but absent from controls. Between just two CF isolates (CF104.3 and CF108.4) and the two controls, there are still more than 4,500 nsSNPs exclusive to CF; of these, 146 nsSNPs in 116 genes (plus 87 noncoding SNPs) are present in both CF isolates (Dataset S1).

E. coli adaptive mutations to growth on glycerol as the sole carbon source have been shown to be readily isolated in targeted adaptive laboratory evolution (ALE) experiments (33). These experiments indicated that mutations in the genes encoding glycerol kinase (*glpK*) and RNA polymerase β and β' subunits (*rpoB* and *rpoC*, respectively), as well as in genes involved in peptidoglycan biosynthesis (*dapF* and *murE*), pyrimidine starvation (*rph*), and vitamin B6 salvage (*pdxK*), increased bacterial fitness in the presence of glycerol as the sole carbon source (33). Strains carrying specific mutations in *glpK* and *rpoC* were shown to improve glycerol utilization and increase metabolic efficiency, respectively, and to increase growth rate individually and in combination (34). None of the nsSNPs, insertions, or deletions previously described by Herring et al. (33) were found in any of the *E. coli* isolates used in this study. Different nsSNPs in the seven genes (*glpK*, *rpoB*, *rpoC*, *dap*, *murE*, *rph*, and *pdxK*) were found in some isolates (Table S2); however, none segregate as independent of *E. coli* lineage and as specific to CF isolates. The dispersed phylogenetic position of *E. coli* isolates with accelerated growth on glycerol supports the possibility of multiple independent mechanisms conferring an improved growth phenotype on GlyMM. Hence, given the substantial genetic diversity among isolates, a nontrivial set of candidate variants could contribute to the GlyMM phenotype. As there is some evidence that young children with and without CF can carry more than one *E. coli* lineage (10), future studies analyzing SNPs in coevolved strains may provide clues to genotypes underlying the GlyMM phenotype.

Genes in Glycerol Metabolic Pathways Are Similarly Regulated in CF and Control *E. coli* Grown in GlyMM. Since strains from ALE experiments have large transcriptional changes as a result of the RNA polymerase mutations, we wished to examine gene expression in CF isolates to determine if they had dramatically different transcriptional profiles on different carbon sources. Therefore, RNA-sequencing (RNA-seq) analysis was performed to analyze the transcriptomes of CF and control *E. coli* when grown on glycerol or glucose as the sole carbon source. Since the different isolates are not isogenic mutants, transcriptome analysis on GluMM served to establish baseline gene expression for each of these genetically distinct strains. Total mRNA from midexponential growth-phase cultures in GlyMM or GluMM was extracted and sequenced from two CF

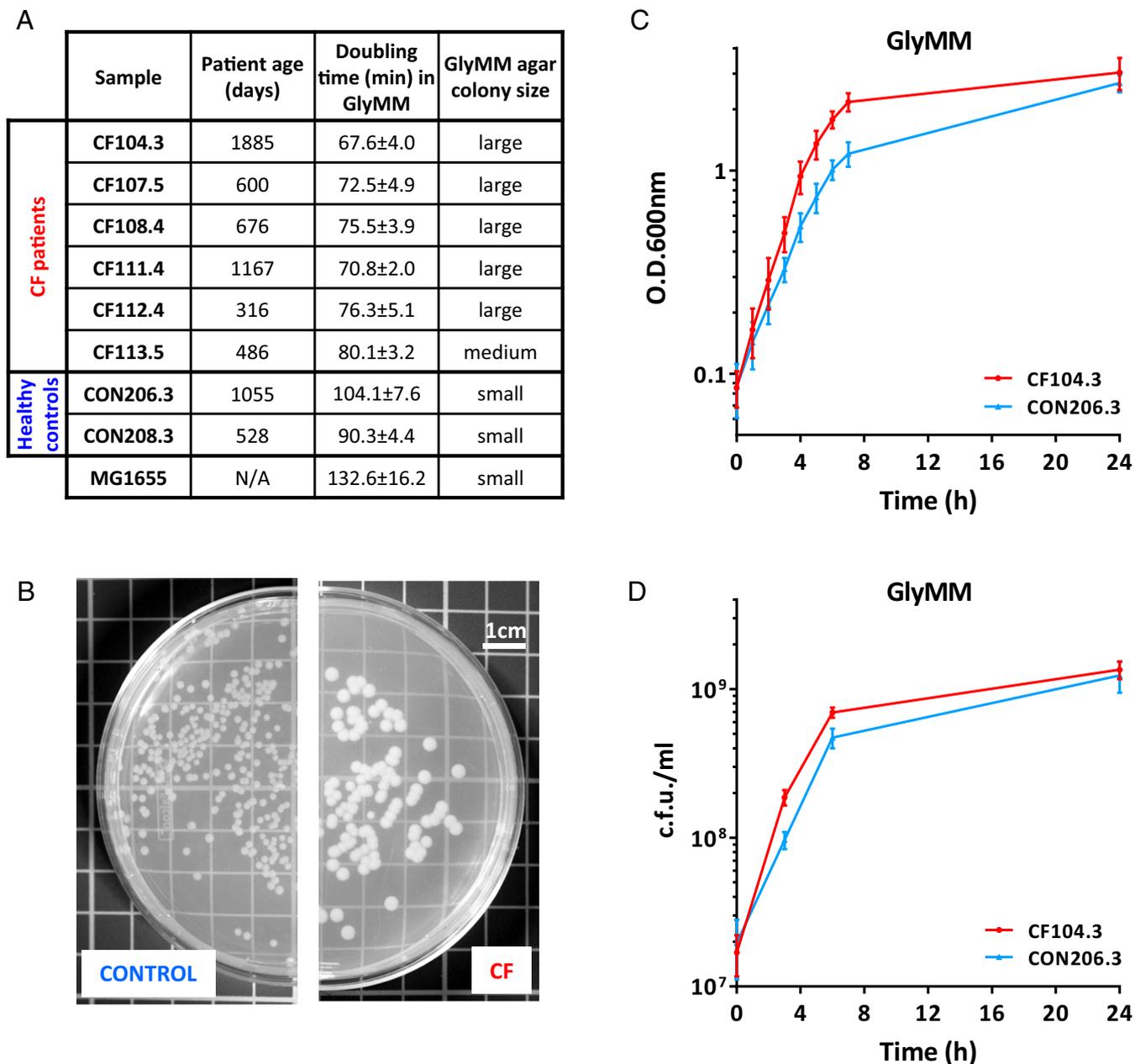


Fig. 1. CF *E. coli* grows faster on GlyMM. (A) *E. coli* isolated from stool samples of patients with CF and healthy controls. Doubling time is given in minutes in comparison to MG1655 in GlyMM. Values represent the mean and SD of at least three independent experiments. N/A, nonapplicable. (B) Example of a control and a CF colony phenotype as observed on GlyMM agar plates. Large colonies averaged >2.0 mm in diameter, and small colonies averaged <2.0 mm in diameter. (C) Representative growth curve in GlyMM. CON, control. (D) Colony-forming units of a control and a CF isolate from GlyMM cultures. In C and D, the mean and SD of at least three independent growth experiments are shown.

isolates (CF104.3 and CF108.4) and two controls (CON206.3 and CON208.3). These isolates were chosen given their growth phenotype in GlyMM, as well as their placement in the phylogenetic tree, and represent the diversity of *E. coli* recovered from patients with CF and healthy controls. Additionally, two of these isolates, CF108.4 and control CON208.3, belong to a single phylogroup (D) and are likely to be genetically more similar to each other than either is to the other CF and control isolates (Fig. 2). Regardless of disease or health status, 213 differentially expressed genes (DEGs) were up-regulated and six DEGs were down-regulated in GlyMM in both the CF and control isolates (Dataset S2). As expected, most of the top up-regulated genes are involved in the glycerol uptake and metabolism pathways (*glpF*, *glpT*, *glpD*, *glpK*, *glpQ*, *glpB*, *glpC*, and

glpA), and the most down-regulated gene is *ptsG*, which is responsible for the phosphotransferase system-dependent transport of glucose into the cell. Furthermore, the magnitude of the regulation level for these genes is very similar between CF and control isolates. Therefore, the accelerated growth rate phenotype observed in the CF isolates on glycerol as the sole carbon source is likely due to differences in other pathways.

CF *E. coli* Down-Regulates Genes Normally Induced on Exposure to Glycerol That Are Associated with Stress and Stationary-Phase Programs and Display a Growth-Promoting Transcriptional Profile in GlyMM. Comparison of CF versus control isolates grown on GluMM and GlyMM revealed an order of magnitude difference in the

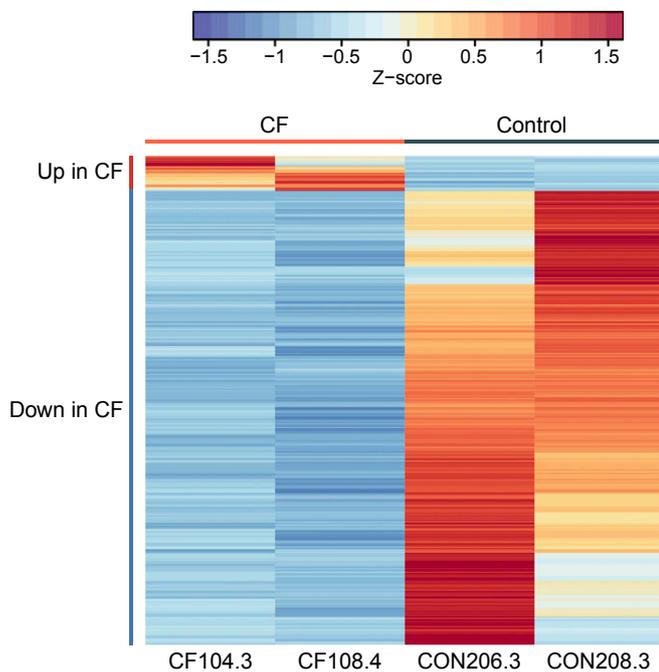


Fig. 3. DEGs in GlyMM in CF *E. coli* isolates relative to controls are predominantly down-regulated. Normalized absolute expression z-scores for each gene and isolate were calculated from gene-wise variance estimates using DESeq2. Pearson correlation was used to cluster genes (rows). Genes up in CF include translation factors, TCA, amino acid synthesis, and energy production (*tufB*, *tufA*, *atpCD*, *rspEG*, *rpmD*, *aceE*, *metE*, *sucBC*, *hisA*, and *ilvE*). Genes down in CF are involved in biofilm, stress, and acid resistance (*azuC*, *ymgC*, *cadB*, *gadBCX*, *hdeABD*, *sra*, *arfA*, *ecpCDR*, *rcaA*, *csgBDG*, *ariR*, *psiE*, *ymgA*, *cspGH*, *bdm*, *adiCY*, *ycgZ*, *rclAC*, *ynhA*, *yjbJ*, *yhcLN*, and *ygiW*). Only genes that were differentially expressed between the CF and control (CON) *E. coli* isolates are shown.

be a consequence of selection of specific phenotypes of individual species. Furthermore, the clonal proliferation of these specific organisms may be detrimental to the delicate symbiotic balance between the microbiota and host. This study is likely the first of many to determine specific unique adaptations of the microbiota to host conditions that could contribute to diseases. Understanding such adaptations should lead to new therapeutic approaches that can avert proliferation of potentially deleterious organisms and improve patient outcomes.

Methods

***E. coli* Isolation and Cultivation.** Fecal samples stored at -80°C were grown aerobically in BHI broth (BD Life Sciences) overnight at 37°C . Cultures were plated onto MacConkey agar and incubated overnight at 37°C to select for lactose-fermenting gram-negative bacteria. Lactose-positive bacteria were grown on Spectra UTI plates (ThermoFisher Scientific) containing a chromogen, which is cleaved by the *E. coli* β -galactosidase enzyme to produce pink colonies. Bacteria producing pink colonies were plated once more on MacConkey agar to eliminate contaminants. Colonies picked from MacConkey agar plates were grown overnight in LB (BD Biosciences) for glycerol stocks. All media were prepared according to the manufacturers' guidelines.

Aerobic Growth on Minimal Media Plates. *E. coli* strains were grown on LB overnight at 37°C , washed, and serially diluted in 1x PBS before plating on Difco M9 (BD Life Sciences) MM plates containing a 0.4% carbon source. Colony size was scored after incubation at 37°C for 72 h.

Minimal Media Growth Curves. Cell growth experiments were always performed at 37°C and followed three steps: seed culture, preculture, and experimental culture. In the seed culture, the cells were grown overnight in LB and then diluted 1:100 in either GlyMM or GluMM for the precultures. Finally, the experimental cultures were started from the overnight-grown

precultures containing the same carbon source at a normalized optical density at 600 nm (OD_{600}) of 0.05. Growth was followed by OD_{600} measurements or by enumeration of colony-forming units on LB agar plates at the specified time points. The specific growth rate (μ) for each isolate was calculated using a linear regression fit ($R^2 > 0.99$) through at least three data points during the exponential growth phase. The doubling time (DT) was extrapolated from $\text{DT} = \ln 2/\mu$ and converted to minutes.

Genomic DNA Extraction and Sequencing. To isolate genomic DNA for sequencing, strains were grown overnight in LB at 37°C . Genomic DNA was isolated using a Genra Puregene Yeast/Bact Kit (Qiagen) according to the manufacturer's directions. For each genome, a random-fragment library was constructed using standard Illumina Nextera libraries (Illumina, Inc.). Libraries from each strain were sequenced according to manufacturer's standards on an Illumina MiSeq system, and 300- or 600-bp paired-end reads were generated at a minimum of 35-fold genome coverage.

MLST. *E. coli* sequence types (STs) were generated from whole-genome shotgun (WGS) sequence reads using the MLST scheme developed by Wirth et al. (37), which uses fragments from seven housekeeping genes: 536 bp of adenylate kinase (*adk*), 469 bp of fumarate hydratase (*fumC*), 460 bp of DNA gyrase (*gyrB*), 518 bp of isocitrate/isopropylmalate dehydrogenase (*icd*), 452 bp of malate dehydrogenase (*mdh*), 478 bp of adenylosuccinate dehydrogenase (*purA*), and 510 bp of ATP/GTP binding motif (*recA*). We created a single MLST reference sequence composed of the scheme's seven-allele template. The Burrows-Wheeler alignment (BWA) algorithm (38) was used to align sequence reads from each strain to the MLST reference sequence using an edit distance of 10, which allowed alignment of reads with up to 10 mismatches. Custom scripts were used to parse alignments to produce consensus sequences for each housekeeping gene, compare consensus sequences with the MLST allele database, and generate STs based on the MLST database of seven allele combinations.

Phylogenetic Analysis. The *E. coli* phylogeny was reconstructed using the kSNP 3.0 software package, in which SNP discovery was based on k-mer analysis (39). The maximum likelihood tree was constructed using 31-mers that were identified in WGS sequence reads for at least 50% of all strains. The 50% requirement provided phylogenetic resolution, while excluding the SNPs present in only one or a small number of genomes, which are more likely to be the result of sequencing or assembly errors. Support for branch nodes was computed by FastTree 2 (40), which is provided in the kSNP package. Tree branches are expressed in terms of changes per total number of SNPs, and not changes per site, as SNP-based trees do not include invariant sites. Local support values are based on the Shimodaira-Hasegawa test on the three alternate topologies at each split in the tree. The trees were drawn using Dendroscope version 3 (41).

Variant Analyses. To identify SNPs and small insertions and deletions in the eight isolates, sequence reads for each were aligned independently to the *E. coli* MG1655 complete genome sequence (GenBank accession no. U00096.3) using the short-read aligner BWA (38) at a minimum mean coverage depth of 43.69 (Dataset S1). The Genome Analysis Toolkit (42, 43) was used to improve the initial alignment, recalibrate base qualities, and call genetic variants. Variants supported by greater than 10 reads and with a frequency of $>80\%$ in at least one isolate were retained and were filtered by custom Python scripts to identify those exclusive to CF isolates. To determine whether variants in seven genes (*glpK*, *rpoB*, *rpoC*, *dap*, *murE*, *rph*, and *pdxK*) segregated as independent of *E. coli* lineage and as specific to CF, the nucleotide sequence of each gene was obtained from high-quality genome assemblies ($n50 > 50,000$ number of contigs < 350 , assembly length > 4.8 Mb) of 212 *E. coli* clinical isolates (44) using BLAST (45). A multiple sequence alignment for each gene was created using MUSCLE (46), and SNPs were called using a custom Python script. Variants identified in CF isolates were manually compared with those in the 212-strain collection while considering MLST and phylogenetic groups.

RNA Isolation and Sequencing. Strains were grown in GluMM or GlyMM, as described. Cultures were harvested at midexponential phase, cells were immediately spun down, and cell pellets were stored at -80°C until processed. RNA was extracted using TRIzol and chloroform in conjunction with a Qiagen RNeasy Mini Kit following the protocol of A. Untergasser (www.molbi.de/protocols/rna_prep_comb_trizol_v1_0.htm) with one modification: Phase Lock Gel tubes (Eppendorf) were used to better separate organic and aqueous phases after the addition of chloroform. DNA was digested with Ambion rDNase I (Life Technologies) for 30 min at 37°C . Total RNA was further cleaned using the RNeasy Mini Kit, and quality was assessed by an

RNA ScreenTape Assay (Agilent Technologies) on a TapeStation 2200 system (Agilent Technologies). Samples with RNA integrity number values greater than 8.0 were treated with an Ambion MICROBExpress Bacterial mRNA Enrichment Kit (Life Technologies) and were used for library construction. The cDNA libraries were barcoded with a TruSeq RNA Sample Prep Kit v2 (Illumina, Inc.). Libraries were sequenced on an Illumina MiSeq system to produce 50-bp single reads. All steps in library construction and sequencing were performed according to the manufacturers' standards.

RNA-Seq Expression Analysis. RNA sequence reads were mapped to the *E. coli* MG1655 complete genome sequence using BWA (38). Multiple mapped reads, gapped reads, and reads with low quality control scores were removed before obtaining feature counts using custom scripts (uwgenomics.org/utilities). Differential gene expression analysis was performed using the Bioconductor package DESeq2 (47) with the default independent filtering disabled. Correction for multiple testing to control the false discovery rate (FDR) was applied with a threshold of FDR < 0.05. Genes were declared differentially expressed for absolute log₂ ratio >1.5. Differential expression analysis was restricted to the 3,564 genes common to all four *E. coli* strains. To increase the power of detection of DEGs across the phylogenetically distinct strains, normalization "size" factors that account for the variable sequencing depth across samples were estimated with DESeq2 using

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235 single-copy genes predicted to be essential in *E. coli* in the Integrated Fitness Information for Microbial genes (IFIM) database (48). Fewer than 3% of the essential genes used for normalization were declared differentially expressed in any contrast. Genes identified as differentially expressed but whose normalized read depth was less than 10 counts per kilobase (equivalent to a 1× average sequence read depth) in expressed strains or conditions were disregarded as having insufficient evidence of expression.

Materials Availability. *E. coli* was isolated from stool samples collected as part of a previous study (10) in which informed consent was obtained following a protocol approved by the Institutional Review Board at the University of Washington (UW). *E. coli* isolates are available from the Cystic Fibrosis Research Translation Center (CFRTC) Microbiology Core at the University of Washington (depts.washington.edu/cfrtc/microbiology/). Genomic DNA sequence and RNA-seq data that support the findings of this study have been deposited at the National Center for Biotechnology Information under BioProject ID PRJNA417507 and GEO (accession no. GSE108846), respectively.

ACKNOWLEDGMENTS. The research was supported by the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health (Grants DK089507 and 1R01DK095869-01A1).

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