**Escherichia coli** cultures maintain stable subpopulation structure during long-term evolution

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Contributed by Michael Lynch, April 4, 2018 (sent for review May 22, 2017; reviewed by Jeffrey E. Barrick and Gregory I. Lang)

How genetic variation is generated and maintained remains a central question in evolutionary biology. When presented with a complex environment, microbes can take advantage of genetic variation to exploit new niches. Here we present a massively parallel experiment where WT and repair-deficient (ΔmutL) *Escherichia coli* populations have evolved over 3 y in a spatially heterogeneous and nutritionally complex environment. Metagenomic sequencing revealed that these initially isogenic populations evolved and maintained stable subpopulation structure in just 10 mL of medium for up to 10,000 generations, consisting of up to five major haplotypes with many minor haplotypes. We characterized the genomic, transcriptomic, exometabolomic, and phenotypic differences between clonal isolates, revealing subpopulation structure driven primarily by spatial segregation followed by differential utilization of nutrients. In addition to genes regulating the import and catabolism of nutrients, major polymorphisms of note included insertion elements transposing into *fimE* (regulator of the type I fimbriae) and upstream of *hns* (global regulator of environmental-change and stress-response genes), both known to regulate biofilm formation. Interestingly, these genes have also been identified as critical to colonization in uropathogenic *E. coli* infections. Our findings illustrate the complexity that can arise and persist even in small cultures, raising the possibility that infections may often be promoted by an evolving and complex pathogen population.

C omprising most of the tree of life and constituting a major determinant of human health and disease, microbes are the most diverse organisms on earth, but how this diversity and genetic variation is generated and maintained remains a central question in evolutionary and population biology. For over 30 y, long-term evolution experiments have examined how prokaryotes and single-celled eukaryotes adapt to novel environments ranging from nutrient-poor environments where *Escherichia coli* are presented with simple carbohydrates as carbon sources (1–3); spatially structured environments where bacteria and yeast evolve strategies to survive oxygen limitation, migration, and cell crowding (4–6); and engineered communities, where microbes evolve under cooperative scenarios (7, 8). Through these experiments microbes have been shown to rapidly adapt to laboratory settings (4, 5, 9–11) by evolving key innovations, such as the ability to utilize a previously unusable carbon source (12), development of cross-feeding behaviors (13), changes in cellular morphology (4), and extreme levels of polyploidization (14, 15).

In nature, microbes are more often faced with ecologically complex environments, and the combination of nutritional and spatial heterogeneity can introduce many novel niches in which microbes may adapt. Nutritional heterogeneity can allow for the evolution of generalist/specialist strategies (16–19), and nutrient-rich environments offer many potential, evolutionary trajectories (20). Further, in spatially structured environments, the two segregated populations may independently evolve and create additional genetic diversity (4, 21, 22).

Here, we report the results of a long-term evolution experiment consisting of 100 *E. coli* populations. These populations have been propagated from either WT K-12 or mismatch-repair deficient genetic backgrounds, at five different transfer densities, for over 3 y (up to 10,000 generations), in growth conditions allowing for both spatial and nutritional specialization. We applied a systems approach for assessing both the evolving populations and derived clones, using genomic, transcriptomic, and exometabolomic data, and growth phenotypes associated with lag time, maximum growth rate, competitive growth (relative fitness when cocultured with the progenitor strain), auxotrophy (inability to create essential nutrients such as amino acids), and spatial variation (location of haplotypes within the culture tube). Long-term population substructure rapidly and stably evolves in these populations, with subpopulation haplotypes being characterized by spatial affinity, differential regulation of metabolism genes, and differential utilization of amino acids.

### Results

**Long-Term Populations Harbor Multiple Dominant Haplotypes.** For over 3 y we have maintained 100 lines of WT (MMR+) and repair-deficient (MMR−) *E. coli* populations by serial transfer in 16×100-mm culture tubes incubated in alternating conditions.

#### Significance

Understanding how microbes adapt to novel environments is essential to understanding acute bacterial infection and long-term disease, as genetic architecture underlying the production and maintenance of genetic variation influences a population’s potential for adaptation. In this in-depth analysis of a highly replicated *Escherichia coli* long-term evolution experiment, we observe rapid diversification into stable subpopulations in response to several environmental variables. This niche separation creates novel genetic backgrounds upon which new traits, such as differential nutrient utilization or antimicrobial resistance, can arise. The observed genetic changes, in a simple and tractable experimental system, mimic events known to occur during bacterial infections.

Author contributions: M.G.B. and M.L. designed research; M.G.B., B.I.C., S.F.M., J.A.K., and W.G. performed research; M.G.B., T.G.D., and J.A.K. analyzed data; and M.G.B., T.G.D., and M.L. wrote the paper.

Reviewers: J.E.B., The University of Texas at Austin; and G.I.L., Lehigh University.

The authors declare no conflict of interest.

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Data deposition: Sequences for all clones and populations analyzed in this paper have been deposited at the NCBI SRA database as BioProject PRJNA448766.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1708371115/-/DCSupplemental.

Published online April 30, 2018.
of 37 °C for 24 h and 25 °C for 48 h while shaking at 175 rpm (Fig. 1). Due to the dimensions of the culture tubes, shaking is inefficient at environmental homogenization, resulting in an oxygen gradient that creates the opportunity for niche-heterogeneity development. In an attempt to manipulate effective population size, five different daily transfer volumes (1 mL, 100 μL, 10 μL, 1 μL, and 10 nL) were used to inoculate 10 mL of LB each transfer. These differences in transfer volume also affected the number of generations experienced by the populations before carrying capacity is reached after each transfer. As such, an approximately fivefold difference in cell division number per transfer exists between the largest and smallest transfer densities. Early in the experiment, we observed widespread development of biofilm at the liquid-air interface. Although cultures are thoroughly vortexed before each transfer, the biofilm rapidly and consistently reforms within 24 h.

Every 6 mo, a fully homogenized sample of each population was collected and sequenced at 100× coverage to identify and track new and segregating polymorphisms. Whole-community sequencing of 100 populations after 1 y of evolution (800–3,400 generations) identified polymorphisms expected to encourage (fimE, a repressor of the type 1 fimbriae; hns, histone-like global regulator which regulates fimB and fimE (23, 24)) and inhibit (acrB; a subunit of the MDR family efflux pump) biofilm formation, as well as many other polymorphisms that accounted for significant portions of the populations.

To gain more insight into the potential linkage of these polymorphisms within distinct haplotypes, we focused on 10 populations shown from population sequencing to contain a combination of polymorphisms in fimE, hns, and/or acrB (Table 1 and Datasets S1 and S2). At the 2.5-y collection, we isolated eight randomly selected clones from each of these 10 populations for whole-genome sequencing and phenotype assessment. Initial plating of the clones on Congo red agar confirms that all clones are capable of forming biofilms, whereas the WT progenitor is not (26, 27) (SI Appendix, Fig. S1). Further, when grown as monocultures, all of these clones formed visible biofilms at the liquid-air interface.

Genomic sequence of the clones revealed multiple haplotypes coexisting in all assayed populations, from two major clades in the WT populations to five clades in repair-deficient populations (SI Appendix, Fig. S2). Further, these haplotypes contribute to a stable population structure. Specifically populations 410 and 417, our populations evolving with the smallest transfer sizes, have experienced a population-wide fixation event (Fig. 2; red: haplotype A, blue: haplotype B, green: haplotype C). Sequenced clones were additionally surveyed for cooccurrence of biofilm-promoting (fimE and hns) and biofilm-inhibiting (acrB) mutations.

Table 1. Summary of genetic background, daily transfer population size, and number of cumulative generations for each population analyzed in this study

<table>
<thead>
<tr>
<th>Population*</th>
<th>Initial genotype</th>
<th>Biofilm-related polymorphisms†</th>
<th>Transfer volume</th>
<th>Generations‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>113</td>
<td>ΔmutL</td>
<td>acrB; fimE</td>
<td>1 mL</td>
<td>2,000</td>
</tr>
<tr>
<td>125</td>
<td>WT</td>
<td>hns; fimE</td>
<td>1 mL</td>
<td>2,000</td>
</tr>
<tr>
<td>126</td>
<td>ΔmutL</td>
<td>acrB; fimE</td>
<td>1 mL</td>
<td>2,000</td>
</tr>
<tr>
<td>129</td>
<td>WT</td>
<td>acrB; fimE; hns</td>
<td>1 mL</td>
<td>2,000</td>
</tr>
<tr>
<td>210</td>
<td>ΔmutL</td>
<td>acrB; fimE; hns</td>
<td>100 μL</td>
<td>4,150</td>
</tr>
<tr>
<td>221</td>
<td>WT</td>
<td>hns</td>
<td>100 μL</td>
<td>4,150</td>
</tr>
<tr>
<td>233</td>
<td>ΔmutL</td>
<td>acrB; fimE; hns</td>
<td>100 μL</td>
<td>4,150</td>
</tr>
<tr>
<td>326</td>
<td>ΔmutL</td>
<td>hns</td>
<td>10 μL</td>
<td>6,330</td>
</tr>
<tr>
<td>410</td>
<td>ΔmutL</td>
<td>acrB; fimE</td>
<td>1 μL</td>
<td>8,500</td>
</tr>
<tr>
<td>417</td>
<td>ΔmutL</td>
<td>acrB; fimE</td>
<td>1 μL</td>
<td>8,500</td>
</tr>
</tbody>
</table>

*The first digit of each population encodes the transfer volume treatment.
†After 1 y.
‡After 2.5 y.
and only one population, 417 (10^6 transfer density, MMR−), harbored clones containing null mutations (frameshift, IS-element insertion, or large deletion) in both fimE and acrB.

Parallel Mutation Is Extensive Across Populations. Across all 10 focus populations, extensive parallel mutation, beyond biofilm-associated genes, is observed. We identified 66 genes that acquired either an SNP or indel mutation in at least 5 of 10 populations (expectation based on 10,000 random permutations = 33, P value < 10^-4) (SI Appendix, Table S1). Interestingly, the order in which several shared mutations arose and spread is similar across populations. For example, a nonsynonymous change in trkH (potassium transporter) commonly occurred early and increased to high frequencies, followed by reversion of a known laboratory-associated mutation in prfB (peptide chain release factor RF-2) to its WT form (28) and nonsynonymous changes in dcuS (sensor kinase for dicarboxylates), ompF (outer membrane porin), and treB (trehalose importer), with mutations in adhE (acetaldehyde-CoA dehydrogenase) and fimH (minor component of type 1 fimbriae) reliably occurring later in evolutionary time (SI Appendix, Table S2). We additionally identified 23 loci that acquired an IS-element insertion or a long deletion in at least two populations (SI Appendix, Table S3).

We additionally compared the parallel mutations identified in our populations to the mutations identified across two different publicly available datasets (SI Appendix, Table S4): (i) Lenski’s most-characterized population, Ara-1 (9), contained only one gene, yghJ (since renamed sslE), a putative secreted and surface-associated lipoprotein mucinase, that also exhibited mutations in our 10 focus populations (Jaccard index = 0.011, P = 0.396) and (ii) Helling’s population characterized by Kinnersley et al. (32) shared 11 of the 66 genes containing parallel mutations within our populations (Jaccard index = 0.016, P = 0.26). Although the Lenski and Helling experiments also focus on long-term evolution of E. coli strains, differences exist in both culture conditions and E. coli genetic background with respect to our study. Specifically, Lenski’s populations involve E. coli B and are batch-cultured in a minimal glucose medium, and the Helling lines were evolved in a chemostat containing a minimal glucose medium, despite the shared usage of K-12–derived E. coli. Given the different experimental conditions, the difference in the parallel mutations observed in these populations was due to selection and not an artifact of target size, we conducted a log-likelihood ratio test (G-test) for both the WT and repair-deficient populations (31). We then compared the resulting G-scores to an expectation calculated by bootstrapping 1,000 random G-score distributions based on the total number of nonsynonymous mutations observed in each genetic background (55 mutations in WT; 5,371 mutations in repair-deficient) and significance was determined with a z-test (31). For both genetic backgrounds, calculated G-scores were significantly different from expected (WT: G_{obs} = 472, G_{exp} = 522.53, P = 0.00147; repair-deficient: G_{obs} = 5,736.32, G_{exp} = 7,756.81, P = 1.39 \times 10^{-35}) (Dataset S3).

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Evolved isolates and populations show increased fitness in heterogeneous environments but not in homogeneous environments. To associate the evolved genotypes within our populations to phenotypes, we focused further on eight clones isolated from population 125, which experienced the fewest SNPs throughout the experiment (26 SNPs) and has a WT (MMR+) background. Through whole-genome sequencing of the individual clones, we identified two major haplotype groups from population 125, with clones 125-1, 125-3, and 125-6 (haplotype A) characterized by an early IS-element insertion upstream of *lns* and clones 125-2, 125-4, 125-5, 125-7, and 125-8 (haplotype B) characterized by an IS-mediated deletion of *thiDM*, rendering the latter clones auxotrophic for the B vitamin thiamine. Individual haplotypes are defined as members of a clade and are not identical to each other, although they share defining and presumably early mutations.

All individuals isolated from population 125 had significantly slower maximum growth rates than the WT progenitor strain when assayed in a 96-well plate (Welch’s *t* = -14.236, *P* = 2.11 × 10^-5^) (Fig. 3A and B). Additionally, a revived sample of population 125 collected at the same time the clones were isolated (after 2.5 y of evolution or 2,000 generations) had an even slower maximum growth rate than each of the individual clones (Welch’s *t* = 10.792, *P* = 8.52 × 10^-7^). Further, when we examined lag time, all clones except 125-3 experienced longer lag times than the WT progenitor strain when grown in isolation (Fig. 3C).

Surprisingly, these slower growth rates did not negatively impact the fitness of the individual clones in competition with the progenitor, as they all outperformed the progenitor when cocultured in growth tubes (with paired *t* test significance ranging from *P* = 0.078 for clone 125-2 to *P* = 0.006 for clone 125-4) (Fig. 3D, dark bars) under conditions similar to the evolution experiment. However, when the individual isolates were cocultured with the progenitor in a well-mixed, homogeneous environment, all performed either the same or worse than the progenitor strain (with paired *t* test significance ranging from *P* = 0.792 for clone 125-1 to *P* = 0.006 for clone 125-2) (Fig. 3D, light bars).

In light of the extreme fitness differences between the individual derived clones and the WT progenitor, we asked if the presence of haplotype A and B within a population is truly stable. To this end, we reconstructed the population by coculturing each of the haplotype A clones (125-1, 125-3, and 125-6) with each of the haplotype B clones (125-2, 125-4, 125-5, 125-7, and 125-8) and transferring 1 mL of culture to a new culture tube containing fresh media every day for 7 d (~22 generations). Comparison of the frequencies of the two haplotypes after 1 and 7 d revealed no difference in the abundance of each haplotype between these two time points (paired *t* test: *t* = 1.6787; *P* = 0.0961), implying mutual stability (*SI Appendix*, Fig. S3). Further, we calculated the minimum expected change between the two haplotypes in the absence of population stability using the fitness data shown in Fig. 3D. For example, assuming fitness is transitive, the fitness of haplotype A’s clone 125-1 relative to haplotype B’s clone 125-4 is ~1.45/1.42, or ~1.02, ignoring sampling error. If we let the starting proportion of clone 125-1 (*p*=) equal 0.402, as it is in *SI Appendix*, Fig. S3A, and the strength of selection (*s*) equal 0.02, as determined by the relative fitness of clone 125-1, then the expected proportion of clone 125-1 after 22 generations (*p*~22~) can be solved for with the equation *p*~22~/(*1−*p*) = *p*~0~/*(1−*p*)e^*s* *t* (33), or ~0.510. However, the proportion...
of clone 125-1 found in the experimental data is 0.410, or essentially unchanged, suggesting that the presence of the two sub-populations is, in fact, stable.

**Individual Clones Show Differential Utilization of Key Nutrients.**
Stable subpopulation structure is often engineered or evolved on the basis of syntrophy—cross-feeding of nutrients (34, 35) or specialization on a specific novel nutrient (36). As LB is a complex growth medium with many potential nutrients, specifically amino acids that clones could potentially specialize on, we looked for evidence for syntrophy in the study population. First, we plated all characterized individual clones from population 125 on M9 minimal media to determine if any auxotrophy had evolved within the population. If a particular clone is auxotrophic, then growth is observed on LB media but not on M9, as the latter forces the bacteria to biosynthesize their own amino acids and vitamins. While no amino acid auxotrophy is observed, we observed auxotrophy to the B vitamin thiamin in haplotype B. This auxotrophy was confirmed as haplotype B clones have an observed deletion of the thiamin biosynthesis genes (thiDM) and are able to grow on M9 media supplemented with thiamin.

Next, to further investigate if differential amino acid usage could be a source of syntrophy, we used an exometabolomic approach to survey the spent media of four individual clones representing each haplotype in population 125 as well as the WT progenitor (SI Appendix, Table S5). For 14 of the 17 amino acids surveyed, no distinguishable difference was detected in utilization compared with the WT progenitor (Fig. 4A–C). However, there are extreme differences within and among haplotypes in the utilization of threonine, lysine, and cysteine (Fig. 4D–F and Dataset S4).

**Individual Clones Show Major Transcriptional Changes Suggestive of Their Potential Niche.** While parallel mutation can highlight candidate genes that may be important in establishing sub-population structure, genomic data alone are unable to confirm if nonsynonymous mutations result in gain or loss of function, or if there are downstream effects on metabolic or regulatory pathways. Additionally, any epigenetic changes that occur, such as adenine methylation (which can be associated with phenotypes regulated by phase variation), would go undetected by genomic analysis alone. Thus, we performed transcriptome analysis of mRNA extracted at midlog phase from clones isolated from population 125 and the WT progenitor.

Analysis of the transcriptomes revealed that the similarity of clones based on differential gene expression resembles their phylogenetic relationship (SI Appendix, Fig. S4 and Dataset S5). Major differences between the two haplotypes include up-regulation of the nupG and the deo operon (both associated with nucleotide salvage) in haplotype A and down-regulation of two cold-shock proteins, cspG (haplotype A) and cspC (haplotype B). Within haplotype A, there appears to be further distinction between clone 125-1 and clones 125-3 and -4 with an additional 38 differentially regulated genes, suggesting further structure within this particular haplotype. Specifically, up-regulation of the cad operon (involved in lysine import and catabolism) is detected in clone 125-1, a possible cause for the increased utilization of lysine in this clone as observed in the exometabolomic analysis. Up-regulation of hydrogenase-2 (hybACO) and spermidine acetyltransferase (speG) and down-regulation of cytochrome bd-II (cbsdAB) are also observed in clone 125-1 but not in the other clones belonging to haplotype A.

In addition to haplotype-specific regulation, some loci experienced parallel differential expression in both haplotypes A and B. Clones 125-3 and -6 (haplotype A) and clones 125-2, -4, -5, -7, and -8 (haplotype B) have increased expression of the tde operon (threonine import and catabolism) consistent with the increase in threonine uptake, while clones 125-3 and -6 (haplotype A) and clone 125-2 (haplotype B) have up-regulation in flu, an epigenetically regulated phase-variable gene encoding a biofilm-promoting autotransporter. In addition to influencing biofilm formation, flu also appears to play a role in flocculation, potentially

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**Fig. 4.** Uptake/release of free amino acids from single clones in LB broth through an initial 6 h of growth. Amino acid availability is demonstrated as a ratio of the concentration of a particular amino acid at a particular time point (T) and that for the same amino acid at 0 h (T0) as detected by GC-MS. (A) phenylalanine, (B) proline, and (C) valine show no significant difference in net uptake across samples, while (D) threonine, (E) lysine, and (F) cysteine show significant differences in net uptake and net excretion between the evolved clonal isolates and the WT progenitor.

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E6464 | www.pnas.org/cgi/doi/10.1073/pnas.1708371115

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lending insight into the spatial segregation of haplotypes within the evolving population (37, 38).

Haplotypes Show Affinity for Different Spatial Niches. Finally, we evaluated whether the different haplotypes display an affinity for different spatial niches within the growth environment. As previously mentioned, haplotype B clones are auxotrophic for this amine, which allows simple quantification of the two haplotypes by replica plating from solid LB media to M9. Thus, we aspirated 500 μL of cells from liquid-culture replicates of population 125 in two assays, one from the top half of the liquid culture and one from the bottom. In both assays, despite being the less-common haplotype (accounting for only ~30% of the entire population according to whole-population sequencing), haplotype B comprised 75% of the cells isolated from the planktonic, more anaerobic, niche, while haplotype A accounted for the other 25%. Further, when the biofilm itself was surveyed, only individuals belonging to haplotype B were present, contrary to expectations based on the genomically surveyed genotypes. Two possibilities for the underrepresentation of haplotype A in our sampling are that (i) haplotype A is concentrated in flocs, making aspiration of those individuals more difficult, or (ii) some members of haplotype A have become so sensitive to traditional plating methods that we are unable to recover them.

To determine whether aerobic growth could impede our ability to sample clones without bias, we again aspirated 500 μL of cells from both the top half and bottom half of six liquid-culture replicates of population 125. From each aspirate, 100 μL of cells were spread on two LB plates before incubating one replicate aerobically and the other replicate in an anaerobic jar. While the difference in cell counts between the two treatments was not statistically significant (paired \( t \) test, \( t_{18} = 1.80, P = 0.146 \)), there was a trend of higher cell counts on the anaerobic plates, suggesting the existence of a subset of individuals not captured with aerobic plating (SI Appendix, Fig. S5).

Discussion

Parallel Mutation Provides Evidence for Possible Adaptive Targets. Within this study, we have identified a substantial number of parallel mutations among replicate populations. While some mutations, such as IS-element insertions upstream of \( \text{nhs} \), have such broad downstream effects that it is difficult to elucidate the specific adaptive effects, others such as deletion of the \( \text{gat} \) operon [which is notably highly expressed in ancestral K-12 (39)] have the adaptive potential to reduce bioenergetic costs by reducing expression. This is further supported through our transcriptional analysis, where \( \text{gatB} \) and \( \text{gatF} \) were observed to be within the top 1.5% of all expressed genes in the progenitor line. A previous study also observed the \( \text{gat} \) operon as a deletion hotspot during long-term evolution of \( \text{E. coli} \) K-12 in chemostats and concluded that the benefits of this deletion could be attributed to conservation of resources otherwise wasted by constitutive expression of \( \text{gat} \) (40).

In addition to this shared evolutionary behavior of the \( \text{gat} \) operon across studies, we also identified similarities between mutations in our population and another earlier long-term evolution experiment (13, 32, 41), even though the structural and nutritional environments differed: Helling’s population as characterized by Kinnesler et al. (2) originated from a clone (JA122) harboring the same IS-element insertion in \( \text{fimE} \) acquired across our experimental populations. It is possible that impairing the function of \( \text{fimE} \) creates an adaptive constraint, as 4 of the 11 genes experiencing parallel mutation between our populations and the Helling populations (\( \text{cytR}, \text{dcuS}, \text{fimH}, \) and \( \text{rpsG} \)) are mutationally overrepresented as indicated by their high G-scores (SI Appendix, Table S4).

Potential Drivers of Subpopulation Structure. The stable coexistence of multiple dominant haplotypes over thousands of generations may have a number of causes. However, our analyses suggest the evolution of subpopulation structure in population 125 was a two-step process: spatial differentiation, followed by metabolic differentiation. Early mutations affecting biofilm-related genes along with the absence of haplotype A from the biofilm at the liquid–air interface support this hypothesis. While both haplotypes within population 125 experience some sympatric interaction as evidenced by their cooccurrence within the planktonic niche, it is possible that the two haplotypes hardly compete at all within the experimental culture environment, with the majority of haplotype A existing within flocs in the bottom of the culture tube. As such, the coexistence of the two major haplotypes may not involve frequency-dependent selection (42, 43) or clonal reinforcement (32). If a frequency-dependent relationship is operating, then it is most likely occurring within haplotype A, where clone 125-1 appears quite different both genotypically and transcriptionally from the other haplotype A clones, 125-3 and 125-6. We observe that haplotype A clone 125-6 overproduces lysine, whereas haplotype A clone 125-1 overutilizes lysine; the overproduction of lysine has been previously shown to support cooperation in synthetic communities (34).

Earlier studies have suggested that inhabiting the liquid–air interface is advantageous owing to the availability of oxygen within this niche (4). However, few explanations, if any, have been offered for the potential advantage of occupying the oxygen-poor bottom-dwelling niche. Is haplotype B simply the fitter haplotype and rewarded with the oxygen-rich niche, or does haplotype A have a distinct advantage in the oxygen-poor niche? One potential benefit of the bottom-dwelling niche that is unavailable at the surface–air interface involves nutrients scavenged from dead cells. Transcriptome data revealed that the major regulatory difference between haplotypes A and B is the overexpression of \( \text{nupG} \) and the \( \text{deo} \) operon, both responsible for nucleotide salvage in haplotype A. Free nucleotides are not an abundant component of LB media (44), which is composed mainly of amino acids. However, the main byproduct of amino acid metabolism is ammonia, which causes a rapid increase in pH within the culture environment. While \( \text{E. coli} \) is well-equipped to handle acid stress, the common result of growth in glucose (44), handle acid stress, the common result of growth in glucose (44), the pH increases to 8.7 within 8 h after transfer. The effect of this increase was observed in our initial exometabolomic assays, where results become complicated after 6 h, as cell lysis caused a substantial release of amino acids into the culture medium. This resulting cellular debris would introduce a source of nutrients primarily accessible to clones specializing in the bottom-dwelling niche.

Another example of a transcriptional difference affecting niche specialization is the differential expression of \( \text{speG} \) (spermidine acetyltransferase). Haplotype A clone 125-1 overexpresses \( \text{speG} \) while clones 125-2, -3, -4, -5, -7, and -8 (belonging to both haplotypes A and B) all have \( \text{speG} \) activity knocked down due to an IS-element insertion in \( \text{yhfB} \), which is located upstream in the same operon as \( \text{speG} \). Overexpression of \( \text{speG} \) may be beneficial in a basic environment as a means of increasing catabolism of free spermidine, as higher pH exacerbates polyamine stress (45). This stress is less of an issue for clones located in flocs (125-3 and -6; haplotype A) or in the surface biofilm (125-2, -4, -5, -7, and -8; haplotype B), as these complex structures insulate cells from environmental stressors (46, 47). The presence of these seven clones within complex structures (flocs and biofilms) is further supported by their increased utilization of threonine (Fig. 4). Recently, threonine has been shown to be an important nutrient in biofilms and other hypoxic, amino acid-rich environments, as threonine can be fermented into 1-propanol (48). This fermentation via the \( \text{tdc} \)
operon, which is also overexpressed in these seven clones, preserves cellular redox balance and is expected to have a fitness advantage during anaerobic and biofilm growth.

Clinical Relevance

The long-term evolution experiment described here provides an excellent model for examining adaptation, specialization, and diversification within microbial populations experiencing environmental spatial heterogeneity. The usefulness of this model extends from questions of microbial speciation to those with clinical implications. For example, the products of both fimE and hms regulation are genes known to be important to uropathogenic *E. coli* for adherence to the epithelial wall, allowing colonization of the urinary tract (49–51). Further, as in LB medium, adaptation to a high environmental pH is advantageous for long-term colonization of the urinary tract, as nitrogen-based compounds (which metabolize to ammonia) are also the major nutrient source in urine (52).

Particularly relevant results to the diagnosis and treatment of infection are (i) the rapidity with which subpopulation structure develops, with many of the characteristic mutations appearing before our first sampling point, and (ii) the possibility that traditional plating methods may exclude some subpopulations of cells. These observations suggest that the diagnostic practice of characterizing a single infective isolate may be misleading in understanding and combating chronic urinary tract infections. With the power of whole-population sequencing, it is now possible to investigate clinical samples to determine if similar population structure arises during infection as in our experimental system, potentially changing the way we think about infection from an isolate view to a population view.

Materials and Methods

Strain Information and Culture of Populations. The WT progenitor strain PFM2 is a prototrophic derivative of the *E. coli* K-12 reference strain MG1655, while the repair-deficient strain PFM5 is a derivative of PFM2 with the mutL gene replaced by an in-frame scar sequence (53). Populations were established by inoculating 10 μL of LB-Miller broth (BD Difco) with a single-isolated progenitor colony cultivated overnight at 37 °C on LB agar plates. Resulting cultures were maintained in 16 × 100-mm glass culture tubes containing 10 mL of LB broth shaking at 175 rpm, alternating transfers after 24 h, growth at 37 °C for 48 h, and periodic, thorough vortexing immediately before transfer to ensure equal likelihood of plating and duplicate removal and performance. These colonies were then selected from the five plates and prepared for long-term storage. To determine which isolate acquired resistance to rifampicin while actively evolving for 2.5 y were streaked onto LB agar plates and cultured overnight. RNA was isolated from mid-log-phase LB broth cultures using the FastRNA Pro Blue Kit (MP Biomedicals) and purified with the RNeasy Kit (Qiagen) to remove DNases before submission to the Center for Genomics and Bioinformatics at Indiana University for library preparation and sequencing. Paired-end sequencing libraries were constructed using the Nextera DNA Library Preparation Kit (Illumina) following an augmented protocol for optimization of reagent use (54) and sequenced on an Illumina NextSeq500 300-cycle, midoutput run. RNA isolation was performed with TRIzol (54) and submitted to the Hubbard Center for Genome Studies at the University of New Hampshire for sequencing on an Illumina HiSeq2500 75-cycle, high-output run. Sequencing data are available on the SRA database at NCBI, BioProject PRJNA484766. For more information on RNA library preparation see SI Appendix, Extended Materials and Methods.

Assembly and Analysis of Genomic Sequences. To identify mutation variation within clonal isolates, paired-end sequencing reads were quality-controlled using the cutadapt software (55) and mapped to the *E. coli* K-12 MG1655 reference sequence (NC_000913.3) (56) with bowtie-mem version 0.7.10 (57). The entire workflow can be found at https://github.com/behrimg/Murlams. Further, we applied GATK version 3.4-0 (58) for base quality score recalibration, indel realignment, and duplicate removal and performed SNP and INDEL discovery across all eight clones per population simultaneously using standard hard filtering parameters according to GATK Best Practices (59, 60). Variants identified with GATK were then annotated with Snpeff version 4.1 (61). To detect structural variants in our clonal sequences, we used Trimmomatic version 0.32 (62) to crop sequencing reads to 90 bp to infer structural variation with GRASPER (63, 64). To identify mutational variation within populations of individual clones during the course of long-term evolution, isolates were plated on solid M9 minimal media and cultured for 96 h at 37 °C, checking every 24 h for growth. To screen for the nutrient requirement for which the clones were auxotrophic, supplementation tests were performed with crossed pools of amino acids, nucleotides, and vitamins (68).

Determination of Relative Growth Phenotypes. To conduct the growth assays, individual clones were grown overnight in culture tubes containing 10 mL of LB broth at 37 °C. We then inoculated 100 μL of fresh liquid LB-Miller with 1 μL of the overnight culture in an octuplicate in 96-well culture plates. On every growth assay culture plate we included eight wells of inoculum-free LB media, followed by alternating rows of eight wells inoculated with evolved clones and eight wells inoculated with the MG1655 progenitor strain. Culture plates were loaded into a SynergyMx Microplate Reader (BioTek) and incubated at 37 °C with shaking for 24 h, recording OD readings at 600 nm, every 15 min. Maximum growth rate was calculated by fitting a modified Gompertz equation to OD readings (https://github.com/LennonLab/Growth_Curves).

Determination of Competitive Growth Phenotypes. A selectable competition strain was created by culturing 10 μL of cells from the MG1655 progenitor strain on 5 LB agar + 0.01% rifampicin plates at 37 °C for 24 h. Eight random colonies were then selected from the five plates and prepared for long-term storage. To determine which isolate acquired resistance to rifampicin while maintaining the fitness phenotype of the WT progenitor, the eight rifampicin-resistant isolates and the MG1655 progenitor were each grown overnight in culture tubes containing 10 mL of LB broth at 37 °C. A fresh culture tube of 10 mL LB broth was then inoculated with 100 μL of a single rifampicin-resistant isolate and 100 μL of the MG1655 progenitor. Immediately after inoculation 100 μL of well-vortexed culture was serial diluted in PBS and spread onto LB agar plates plated for initial colony counting. Following an overnight incubation at 37 °C, 100 μL of well-vortexed culture was serially diluted in PBS and spread onto LB agar plates and incubated at 37 °C overnight for final plate counting. To separate the rifampicin-resistant
colonies from the MG1655 progenitor colonies, a replica plating technique was used. Initial and final countable LB agar plates were stamped onto velvet felts and transferred to LB agar plates + 0.01% rifampicin. The LB–rifampicin plates were also incubated at 37 °C overnight for plate counting. The following equation was then used to determine fitness, where ω represents relative fitness, A represents the number of MG1655 CFUs after subtracting E, the number of rifampicin-resistant CFUs counted on the LB–rifampicin plates from the total number of CFUs on the LB agar:

\[ \omega = \ln \left( \frac{A}{E} \right) / \ln \left( \frac{E}{L} \right) \]

We then selected the isolate with the ω value closest to 1 to represent the ancestor. For other competitive assays, to determine the relative fitness of population 125, the coculture fitness assessment experiment was repeated with triplicate cultures of population 125 as well as clonal isolates from 125 competed against the rifampicin-resistant progenitor strain in both glass culture tubes as well as 10 mL of culture in 100-mL flasks.

Determination of Growth Behavior. To confirm the potential for biofilm-forming behavior, an isolated colony of the rifampicin clone and the WT progenitor were each streaked on Congo red BHI agar and incubated overnight at 37 °C. Presence of bright-red colonies, opposed to pale-pink, confirm the ability to form biofilms (27). We then aimed to identify the localization of the different haplotypes of population 125 within the long-term culture environment. Using the evolved thiamine auxothrophy of haplotype B in population 125 we are able to separate and count colonies of haplotype A and haplotype B using a replica plating technique. Triplicate cultures of population 125 were grown overnight in 16 × 100-mm polyurethane culture tubes containing LB broth. We concluded that the subpopulation of polyurethane for glass did not disrupt the subpopulation structure to the persistence of the biofilm at the surface-air interface. By inserting a 20-gauge needle through the side of the tube, we aspirated 0.50 mL of culture from both the bottom quarter and the upper quarter of the culture tube. After aspiration, cells belonging to the biofilm were then collected from the side of the tube where the air–liquid interface once resided. Once collected, cells were cultured at 10° dilutions, 37 °C overnight on LB to obtain accurate colony counts. To identify colonies of auxotrophic clones aspired from the different regions, colonies were replica-plated on M9 minimal media using velvet felts and incubated at 37 °C for 24 h. To confirm the presence of oxygen-sensitive clones this experiment was repeated by culturing six replicates of population 125 at 10° dilutions on LB and incubating both aerobically and in anaerobic jars (Alcore International) containing an anaerobic gas pack (AnaeroGen; Thermo Scientific) at 37 °C overnight.

Finally, we used replica plating to investigate the stability of the subpopulation structure by reconstructing the population through coculture of a single clone from each haplotype. Cocultures were established by inoculating culture tubes containing 10 mL of LB with 100 μL of an overnight culture containing a clone from haplotype A and 100 μL of an overnight culture containing a clone from haplotype B in triplicate. Cocultures were then shaker-incubated at 37 °C for 24 h before fully vortexing and transferring 1 mL of coculture into a fresh culture tube containing 10 mL of LB broth. After 24 h, 72 h, and 168 h, fully vortexed samples were plated in triplicate on LB and incubated at 37 °C before colony counting and replica plating onto M9.

Determination of Amino Acid Uptake. A single colony from the WT progenitor and clones 125-1, 125-2, 125-3, and 125-5 were used to inoculate culture tubes containing 10 mL of LB-Miller and incubated overnight at 37 °C. We transferred 100 μL from each culture tube into four 100-ML flasks containing fresh LB broth. Each flask thus represented a time point in which the spent media was to be surveyed (0, 2, 4, and 6 h). The sets of 2-, 4-, and 6-h treatments were incubated at 37 °C shaking at 200 rpm, while the 0-h treatments were collected immediately. Spent media from all treatments was collected via filter centrifugation and immediately refrigerated before submission for GC-MS analysis (for more information about GC-MS sample preparation and analysis see SI Appendix, Extended Materials and Methods).

ACKNOWLEDGMENTS. We thank D. A. Drummond, P. Foster, W.-C. Ho, J.-F. Gout, J. Lennon, H. Long, J. McKinlay, and the reviewers for their helpful suggestions. Library construction and sequencing was conducted by Center for Genomics and Bioinformatics at Indiana University. The High Performance Computing Cluster was maintained by the National Center for Genome Analysis Support at Indiana University. This work was supported by Army Research Office Grants WRO65308-LS-MU and W911NF-14-1-0411 and National Institutes of Health Grant F32GM123703.